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Nitric Oxide/Cyclic Guanosine Monophosphate Pathway in the Peripheral and Central Auditory System of the Rat

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ABSTRACT

The neuronal isoform of nitric oxide synthase (nNOS) and soluble guanylate cyclase (sGC) were localized in the cochlea, the cochlear nucleus (CN), and the superior olivary complex (SOC) of Fisher 344 rats. In the cochlea, nNOS was identified in spiral ganglion cells by using nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase histochemistry and in situ hybridization. NADPH-diaphorase staining also was detected in blood vessels of the modiolus. By using immunohistochemistry against cyclic guanosine monophosphate, cochlear sGC activity was localized to pericytes in the spiral ligament as well as nerve fibers innervating outer hair cells. In the lower auditory brainstem, nNOS was localized to principal cells of the medial nucleus of the trapezoid body (MNTB) with NADPH-diaphorase histochemistry and in situ hybridization. NADPH-diaphorase activity also was observed in the lateral and medial superior olive (LSO and MSO, respectively), the superior periolivary nucleus (SPN), the ventral and lateral nuclei of the trapezoid body (VNTB and LNTB, respectively), and the ventral cochlear nucleus (VCN). Transcripts of the β-subunit of sGC were localized in rat brainstem by using in situ hybridization. mRNA for sGC was expressed in neurons within the SPN, LSO, MSO, LNTB, MNTB, VNTB, and VCN. Highest levels of sGC expression were seen in the SPN. These results suggest that the NO/cGMP pathway is involved in both the ascending and descending pathways of the auditory brainstem. J. Comp. Neurol. 404:52–63, 1999.

Indexing terms: auditory brainstem; cochlea; nicotinamide adenine dinucleotide phosphate-diaphorase histochemistry; nitric oxide synthase; soluble guanylate cyclase

Many neurotransmitters are amino acids or amino acid derivatives stored within neuronal synaptic vesicles. On depolarization of the neuron and subsequent calcium influx, these bioactive molecules are released into the synaptic cleft, where they bind to receptors that usually are present in the postsynaptic cell membrane. Nitric oxide (NO) is a recently discovered neurotransmitter that also is derived from an amino acid (L-arginine) but with a different mode of action. NO is a gas; therefore, it can diffuse across cell membranes. NO does not act upon plasma membrane receptors; instead, it stimulates a cytosolic protein, soluble guanylate cyclase (sGC), to produce cyclic guanosine monophosphate (cGMP). Thus, the NO/cGMP pathway is a unique neurotransmitter/neuromodulator system with a role in the nervous system that is only beginning to be understood.

In the central nervous system (CNS), the NO/cGMP pathway has been best characterized in the cerebellum (Garthwaite et al., 1988; Bredt and Snyder, 1989), where it has been implicated in the induction of long-term depres-

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sion (LTD; Shibuki and Okada, 1991), a form of synaptic plasticity. The neuronal isoform of nitric oxide synthase (nNOS) is expressed in cerebellar granule cells as well as basket cells of the molecular layer (Bredt et al., 1990). In contrast, sGC is restricted to the Purkinje cells (Nakane et al., 1983), which receive input from the nNOS-positive granule cells. Purkinje cells also express downstream targets of the NO/cGMP pathway, such as cGMP-dependent protein kinase (cGK) and a putative cGK substrate (Schlichter et al., 1980; Lohmann et al., 1981).

In the peripheral nervous system (PNS), nNOS is expressed in nonadrenergic, noncholinergic (NANC) nerves (Sheng et al., 1992), which innervate the smooth muscle lining of numerous organs, including intestine, lung, and penis. NO produced from these nerves (Bult et al., 1990; Sanders and Ward, 1992) activates sGC in the smooth muscle, resulting in gut peristalsis, bronchodilation, and penile erection (for review, see Grozdanovic et al., 1994).

In the auditory system, the NO/cGMP pathway has been characterized in the cochlea of the guinea pig (Fessenden et al., 1994; Zdanski et al., 1994; Fessenden and Schacht, 1997) but not in the central auditory pathways, which process sensory information originating in the cochlea. On acoustic stimulation, inner hair cells release glutamate, which stimulates dendritic processes from type I spiral ganglion neurons. The spiral ganglion cells project to the cochlear nucleus (CN), which innervates several different nuclei within the superior olivary complex (SOC), an important relay station for auditory signal processing. Both ascending pathways (eventually leading to the auditory cortex, where hearing is perceived) and descending pathways (leading back to the cochlea) are present in the SOC (for reviews, see Helfert et al., 1991; Spangler and Warr, 1991).

The central auditory system and the NO/cGMP pathway both are activated by the neurotransmitter L-glutamate. This amino acid stimulates the spiral ganglion cells as well as neurons in the CN and the SOC (Helfert et al., 1991; Eybalin, 1993). Glutamate also preferentially activates nNOS, which is physically coupled to N-methyl-D-aspartate (NMDA)-type glutamate receptors (Brenman et al., 1996). NMDA receptors are expressed throughout the neuronal auditory pathways, including the spiral ganglion cells (Puel et al., 1991; Safieddine and Eybalin, 1992; Kuriyama et al., 1993; Niedzielski and Wenthold, 1995), CN (Hunter at al., 1995; Petralia et al., 1996; Sato et al., 1998), and SOC (Sato et al., 1998). Thus, it can be postulated that the NO/cGMP pathway is expressed in the neuronal auditory pathways. In this study, we localized nNOS and sGC in both central and peripheral auditory tissues of the rat.

MATERIALS AND METHODS

Fisher 344 albino rats, 6–8 weeks old, were used in this study. All experimental protocols on animal use were approved by the University of Michigan Committee on Use and Care of Animals. Animal care was under the supervision of the University of Michigan's Unit for Laboratory Animal Medicine in accordance with National Institutes of Health guidelines. Except where noted, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Tissue preparation: Brainstem

Rats were anesthetized by using CO_2 gas and then decapitated. The cerebellum and brainstem were quickly removed and frozen at −30°C. Coronal sections (15 μm) that were obtained by using a cryostat (Bright Instrument Co., Huntingdon, United Kingdom) were thaw-mounted onto slides precoated with an aqueous solution of 5 mg gelatin and 0.5 mg $CrK(SO_4)_2$/ml. Sections were stored at −80°C. Before use, sections were fixed in a solution of 4% paraformaldehyde in phosphate-buffered saline (PBS; 10 mM Na_2HPO_4/NaH_2PO_4, 138 mM NaCl, 2.7 mM KCl, pH 7.4) for 1 hour at room temperature.

Tissue preparation: Cochlea

After decapitation, the bullae were quickly removed, opened, and placed in 4% paraformaldehyde in PBS. The apex of the otic capsule was opened, and fixative was perfused through the round and oval windows. The cochlea was incubated in fixative for 1 hour at room temperature and then washed three times in PBS.

To obtain cochlear sections, the cochlea was decalcified in 5% ethylenediaminetetraacetic acid (EDTA) in PBS for 2–5 days at room temperature. The tissue was then cryoprotected by incubation in 30% sucrose in PBS overnight at 4°C. Cochleae used for in situ hybridization were decalcified and cryoprotected in the same solutions supplemented with 4% paraformaldehyde. The cochleae were then frozen in Tissue-Tek mounting medium (Miles Inc., Elkhart, IN), and 15-μm sections were obtained as described for the brainstem.

For experiments performed on surface preparations, the otic capsule was removed, and the modiolus containing the neurosensory epithelium was isolated. The tectorial membrane and lateral wall tissues were discarded. This modiolar preparation was then processed for immunohistochemistry.

cGMP immunocytochemistry

sGC was localized by using anti-cGMP immunocytochemistry (de Vente et al., 1989; Fessenden and Schacht, 1997). Briefly, rat cochleae were isolated, opened, and placed in Hanks' balanced salt solution (HBSS; Gibco-BRL, Gaithersburg, MD) containing 1 mM isobutylmethylxanthine (IBMX). The cochleae were perfused either with HBSS/IBMX alone (control) or HBSS/IBMX supplemented with 1 mM diethylamine NONOate (DEA-NO; Cayman Chemical, Ann Arbor, MI), an NO donor. Fifteen minutes after the perfusion, the reactions were terminated by fixation with 4% paraformaldehyde for 1 hour. Cochlear tissues were incubated for 1 hour at room temperature in 1 ml of blocking solution [5% normal goat serum (Vector Laboratories; Burlingame, CA) and 0.1% Triton X-100 in PBS]. Tissues were then incubated overnight at 4°C in 100 μl of a 1:300 dilution of anti-cGMP antibody (de Vente et al., 1989) followed by an incubation for 1 hour at room temperature in 500 μl of a 1:200 dilution of anti-rabbit fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Vector Laboratories). The neurosensory epithelium and lateral wall tissues were mounted as surface preparations in Vectashield fluorescence mounting medium (Vector Laboratories).

Immunolabeling was detected by using a Nikon fluorescent microscope (Tokyo, Japan). Sections were photographed by using Kodak Ektachrome 160T film (Eastman-

Kodak, Rochester, NY) at 160 ASA. Alternatively, labeling was detected by using a Bio-RAD 600 laser scanning confocal unit (Bio-RAD, Richmond, CA) attached to a Nikon Diaphot TMD inverted microscope with a ×60 oil-immersion objective. Fluorescent images were processed digitally by using Adobe Photoshop 3.0 (Adobe Systems, Mountain View, CA) for Macintosh (Apple Computers, Cupertino, CA).

Nicotinamide adenine dinucleotide phosphate-diaphorase histochemistry

Cochlear or brainstem sections were prepared as described above. For visualization of nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase staining, 200 µl of a solution containing 1 mM NADPH, 0.5 mM nitro blue tetrazolium, 0.2% Triton X-100, and 50 mM Tris-HCl, pH 8.0, was added to each slide. After a 2-hour incubation at 37°C, slides were washed twice in PBS and mounted with GVA mount (Zymed Laboratories, South San Francisco, CA), and the coverslips were sealed with nail polish. Sections were photographed by using brightfield illumination.

Molecular cloning

The full-length complementary DNA (cDNA) for the neuronal NOS isoform (GenBank accession no. X59949) and the sGC β subunit (GenBank accession no. M22562) from rat were kindly provided by Drs. Michael Marletta and Michael Uhler, respectively. For nNOS, a 350-nucleotide SstI-ApaI fragment corresponding to positions 296–646 from the rat neuronal NOS cDNA was subcloned into pGEM -7Zf vector (Promega, Madison, WI). For sGC, a 489-nucleotide PvuII-BglII fragment corresponding to positions 131–620 from the rat sGC β subunit cDNA was subcloned into pGEM-3Zf vector (Promega). The identity of the subcloned cDNAs was confirmed by DNA sequencing.

Riboprobe generation

Riboprobes were synthesized from linearized templates in a 20 µl reaction containing 1 × transcription buffer (Epicentre Technologies, Madison, WI), 10 mM dithiothreitol, 500 µM adenosine triphosphate (ATP), 500 µM cytidine triphosphate (CTP), 500 µM guanosine triphosphate (GTP), 20 U RNasin (Promega), 0.5–1.0 µg template DNA, 100 µCi ^{35}S-uridine triphosphate (UTP), and 25 U RNA polymerase (Epicentre Technologies). Antisense probes were generated with T7 RNA polymerase for nNOS and SP6 RNA polymerase for sGC. After a 2-hour incubation at either 37°C (T7) or 42°C (SP6), reactions were terminated with 80 µl of G50/50 buffer, which consisted of 100 mM Tris-HCl, 12.5 mM Na_4EDTA, 150 mM NaCl, and 0.2% sodium dodecyl sulfate, pH 7.5. The reaction medium was loaded onto a 1 ml G50 Sephadex column that had been preequilibrated with G50/50 buffer. Eight 100 µl fractions were collected, and peak fractions corresponding to labeled riboprobe were quantified by scintillation counting and stored at −80°C until use.

In situ hybridization

Fixed tissue sections were washed three times in 2 × standard saline citrate (SSC). Next, slides were placed in either 1 µg/ml (brain sections) or 0.1 µg/ml (cochlear sections) proteinase K for either 10 minutes (brain) or 5 minutes (cochlea) at 37°C. Slides were washed once in water for 2 minutes and then incubated in 100 mM triethanolamine with 0.25% acetic anhydride, pH 8.0, for 10 minutes. Slides were washed once in 2 × SSC for 2 minutes and then dehydrated in a stepwise series of graded ethanol solutions (50%, 70%, 80%, 95%, 95%, 100%, and 100% ethanol; 1.5 minutes each). Slides were left to air dry for at least 1 hour.

Riboprobes were diluted to a concentration of between 33,000 CPM/µl and 40,000 CPM/µl by using 50% formamide cocktail (Amresco, Solon, OH) supplemented with 10 mM dithiothrietol. Riboprobes were added to slides, which were then covered with coverslips and sealed with rubber cement. Slides were incubated with riboprobes overnight at 55°C in a humidified chamber.

Coverslips were removed in 2 × SSC, and the slides were washed twice in 2 × SSC. The sections were then incubated in 10 mM Tris-HCl, 0.5 M NaCl, 200 µg/ml RNase A, pH 8.0, for 1 hour at 37°C. Next, sections were washed for 5 minutes in 2 × SSC, for 5 minutes in 1 × SSC, and for 60 minutes in 0.1 × SSC at 65°C. Sections were washed once in 0.5 × SSC for 5 minutes and then dehydrated as described above. Sections were placed under autoradiographic film (Eastman Kodak) for 3–5 days for preliminary determination of labeling. Sections were then dipped in Kodak NTB-2 autoradiographic emulsion (Eastman Kodak) and exposed for 2–4 weeks in the dark. Sections were developed in D-19 developer (Eastman Kodak) for 2 minutes, washed for 20 seconds in water, fixed for 3 minutes in Rapid Fix (Eastman Kodak), and washed in water for 10 minutes. Slides were then counterstained with cresyl violet for 2 minutes, dehydrated, and incubated in xylene (two washes, 10 minutes each), and coverslips were mounted by using Permount (Aldrich, Milwaukee, WI).

Slides were examined and photographed with Leitz (Wetzlar, Germany) or Zeiss (Thornwood, NY) photomicroscopes under brightfield, differential interference or darkfield illumination to visualize silver grains. Identification of cell types was based on size, shape, and position within regions. In the cochlear nucleus, classifications and criteria developed by Cant (1991) were used.

The intensity of label with in situ hybridization was evaluated with densitometric measurements by using the Metamorph image-analysis system (Metamorph; Universal Images Corp., West Chester, PA). A strong signal was defined as labeling at least 6.0 times more intense than background. A moderate signal was defined as labeling between 2.5 times and 6.0 times more intense than background.

RESULTS

Positive controls

The cerebellum was used as a positive control for in situ hybridization experiments. The nNOS antisense probe strongly labeled basket cells in the molecular layer and granule cells of the granular layer (Fig. 1A), whereas the Purkinje cells of the Purkinje cell layer were not labeled above background. The sGC probe, on the other hand, labeled the Purkinje cells strongly, whereas it labeled basket cells only moderately (Fig. 1B). These findings are consistent with previous localizations of nNOS and sGC by in situ hybridization (Bredt et al., 1991; Matsuoka et al., 1992; Furuyama et al., 1993). The sense probes for nNOS and sGC did not label any cells in the cerebellum (Fig. 1C,D). Also, pretreatment of the tissue with RNase A abolished labeling by antisense probes (data not shown).

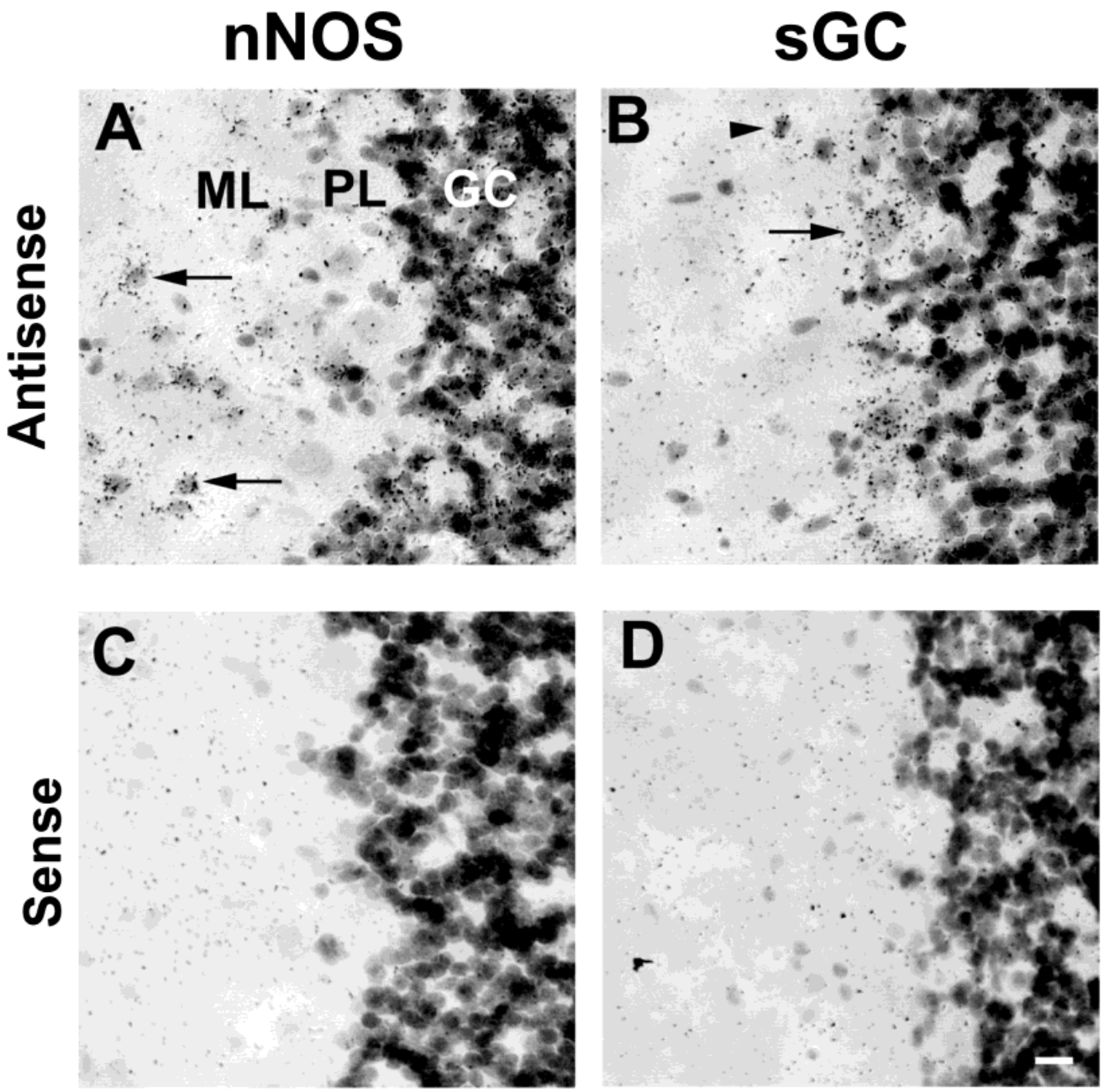

Fig.1 Cerebellar localization of mRNA for the neuronal isoform of nitric oxide synthase (nNOS) and soluble guanylate cyclase (sGC)-β using in situ hybridization. **A:** The nNOS antisense probe labeled the granule cell layer (GL) heavily as well as basket cells (arrows) in the molecular layer (ML). The Purkinje cell layer (PL) was unlabeled. **B:** The sGC antisense probe strongly labeled the Purkinje cell layer (arrow). Basket cells were moderately labeled (arrowhead). **C,D:** nNOS (C) and sGC (D) sense probes did not label any cells in the cerebellum. Scale bar = 20 μm.

Cochlea

NOS was localized in the rat cochlea by using NADPH-diaphorase histochemistry and in situ hybridization. NADPH-diaphorase activity was detected in spiral ganglion cells and blood vessels within the modiolus. Nerve fibers in Rosenthal's canal also were stained (Fig. 2A). By using in situ hybridization, mRNA for nNOS was detected in spiral ganglion cells of the modiolus (Fig. 2B).

In cochlear surface preparations, sGC activity was detected in pericytes lining blood vessels of the lateral wall tissues (Fig. 3A). In the upper cochlear turns, nerve fibers innervating outer hair cells contained sGC activity (Fig. 3B).

Lower auditory brainstem: General

NADPH-diaphorase histochemistry as well as nNOS and sGC β mRNA expression were evaluated in the ventral

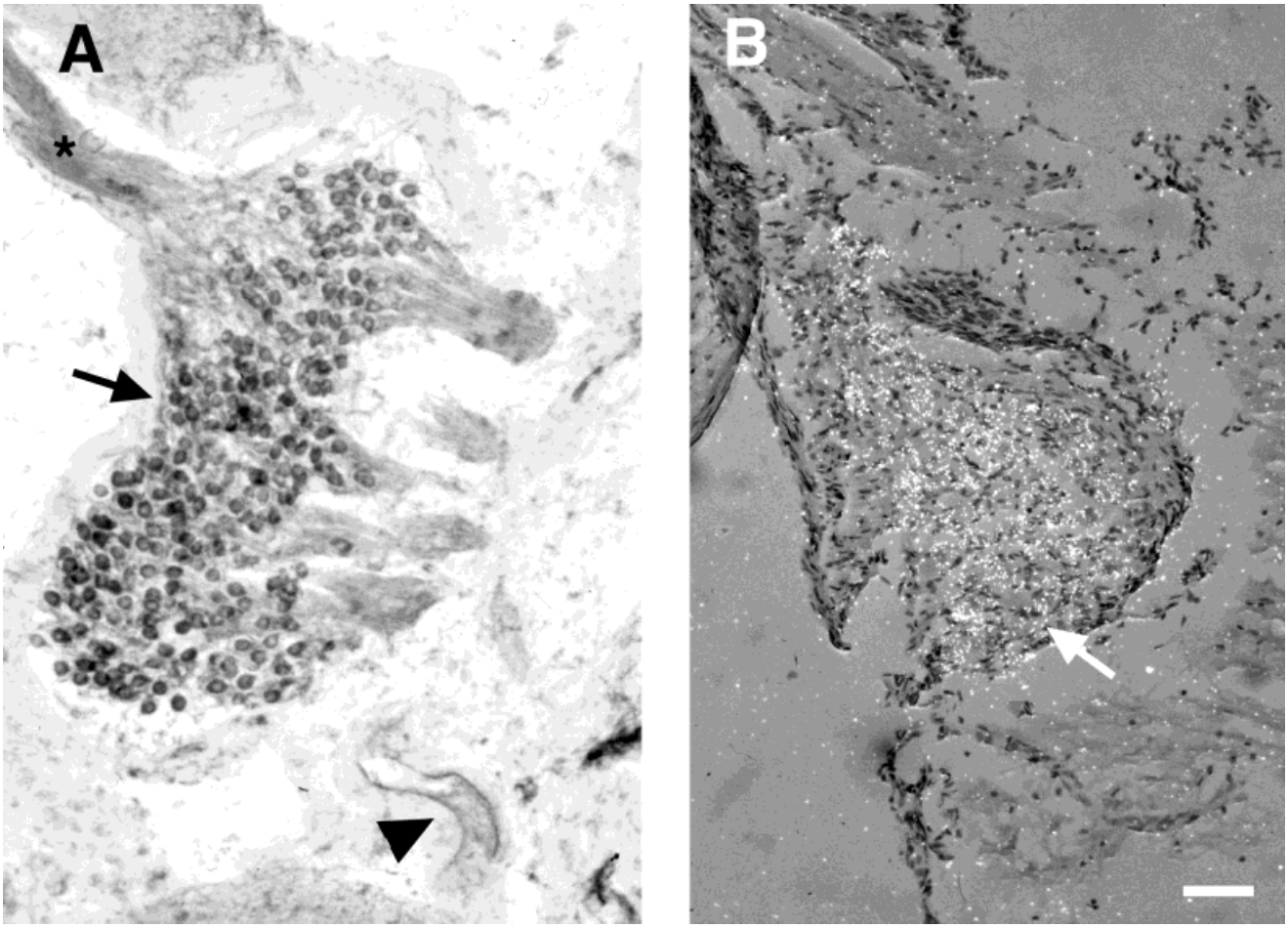

Fig. 2. Localization of NOS in the cochlear modiolus by nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase labeling and in situ hybridization. **A:** NADPH-diaphorase activity was detected in spiral ganglion cells (arrow) and blood vessels (arrowhead). In addition, nerve fibers in Rosenthal's canal were stained (asterisk). **B:** mRNA for nNOS was detected in spiral ganglion cells (arrow). Scale bar = 50 μm.

cochlear nucleus (VCN) and in six regions of the SOC: the medial, ventral, and lateral nuclei of the trapezoid body (MNTB, VNTB, and LNTB, respectively); the medial and lateral superior olivary nuclei (MSO and LSO, respectively); and the superior periolivary nucleus (SPN). Strong expression of mRNA for nNOS was detected in principal cells of the MNTB (Fig. 4A) at a level of expression comparable to that observed in cerebellar basket cells. In contrast, expression of mRNA for the sGC β subunit in neurons was much more widespread (Fig. 4B), a finding consistent with previous localization of this subunit in the brainstem (Furuyama et al., 1993).

VCN

NADPH-diaphorase activity was observed in VCN neuropil (which could reflect labeling of central processes of spiral ganglion cells) and in some, but not all, spherical and globular bushy cells (Fig. 5A,D). Stellate multipolar cells and octopus cells were not labeled. mRNA for nNOS was expressed in the shell region of the VCN. Silver grains were observed over some of the smaller shell neurons, likely to be granule cells, based on the classification of Hutson and Morest (1996). Spherical and globular bushy cells and stellate multipolar cells within the VCN did not show expression over background levels (Fig. 5B,E).

mRNA for the sGC β subunit was also seen in the VCN (Fig. 5C), where some neurons in the shell region moderately expressed this mRNA. Moderate expression of sGC β subunit mRNA was found in most spherical and globular bushy cells (Fig. 5F) as well as in some stellate multipolar cells.

SOC

In the SOC, NADPH-diaphorase activity was present in principal cells of the MNTB and in neuropil of the LSO, SPN, and VNTB (Fig. 6A), whereas mRNA for nNOS was detected only in the principal cells of the MNTB (Fig. 6B). mRNA for the sGC β subunit was present in all SOC regions, with the strongest expression seen in the SPN (Fig. 6C).

NADPH-diaphorase activity also was present in some cells in regions with abundant labeling of neuropil. For example, in the LSO (Fig. 7A), a granular staining pattern of some cells was seen in addition to neuropil labeling (Fig. 7B). This staining pattern contrasted with a more diffuse labeling of MNTB principal cells (Fig. 7C).

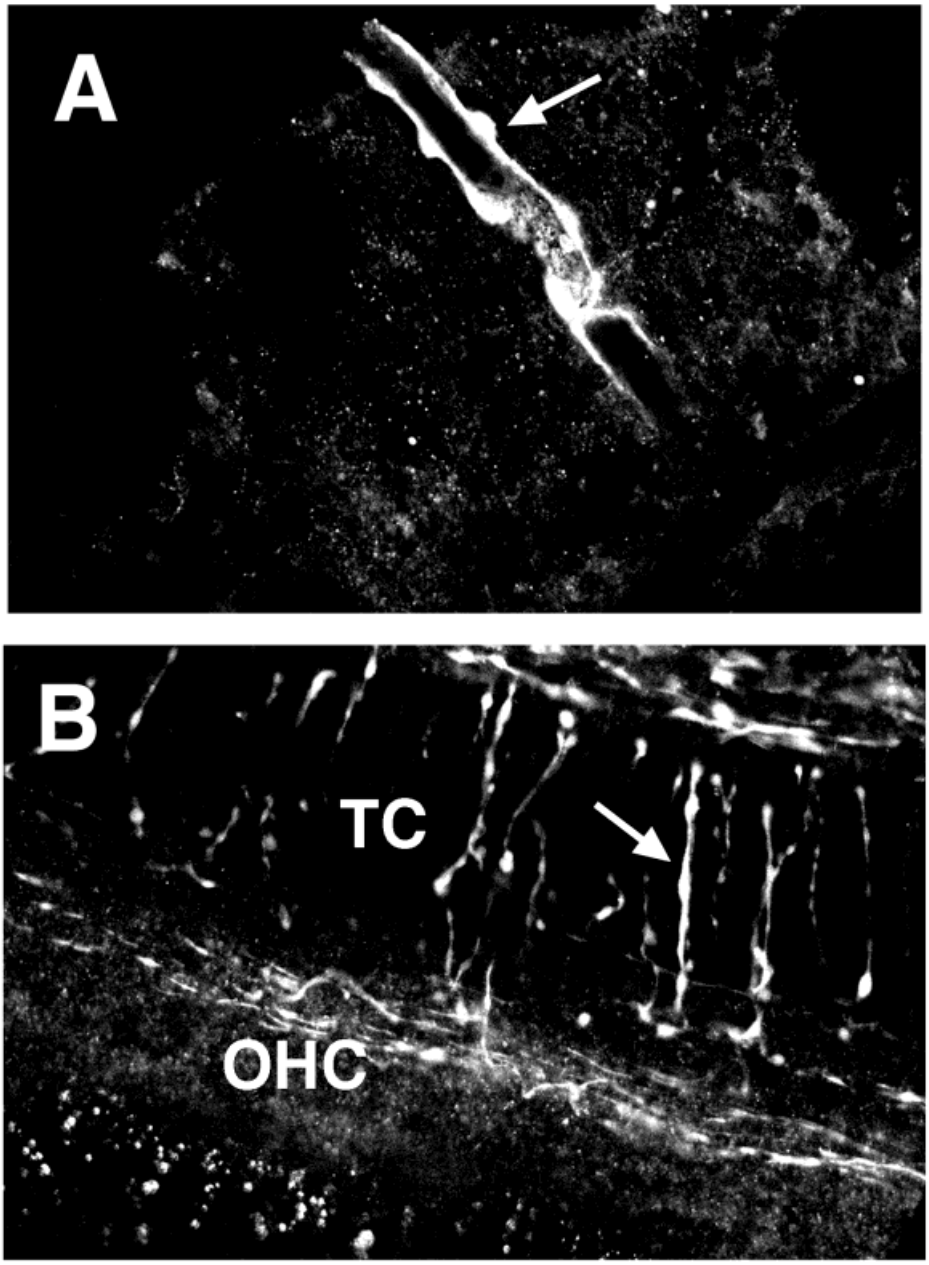

Fig. 3. sGC activity in rat cochlear surface preparations. **A:** sGC activity was evident in pericytes of the spiral ligament (arrow). **B:** In the organ of Corti, sGC activity was found in nerve fibers (arrow) crossing the tunnel of Corti (TC) to the outer hair cell region (OHC).

mRNA for nNOS was found only in principal cells of the MNTB (Fig. 8A). In contrast, mRNA for the sGC β subunit was detected in all brainstem regions surveyed (LSO, MSO, MNTB, VNTB, LNTB, and SPN). Examples of labeled cells in some of these regions (Fig. 8B,D,F,H) indicate that the strongest level of mRNA for sGC-β expression was seen in the SPN.

DISCUSSION

Distribution of the NO/cGMP pathway

The NO/cGMP pathway is distributed widely in the peripheral and central auditory system. NADPH-diaphorase activity and nNOS mRNA are colocalized in cells activated by glutamate (spiral ganglion, MNTB), suggesting that glutamate acts on the NO/cGMP pathway in these areas. The detection of nNOS by in situ hybridization and NADPH-diaphorase histochemistry in spiral ganglion cells confirms and extends observations in guinea pig (Fessenden et al., 1994; Franz et al., 1996) and rat (Zdanski et al., 1994). The characterization of this pathway in the auditory brainstem is a novel contribution to our understanding of neurotransmission and modulation in these structures.

Glutamate released from the inner hair cells activates spiral ganglion cells, which express a variety of glutamate receptor subtypes, including NMDA receptors (Safieddine

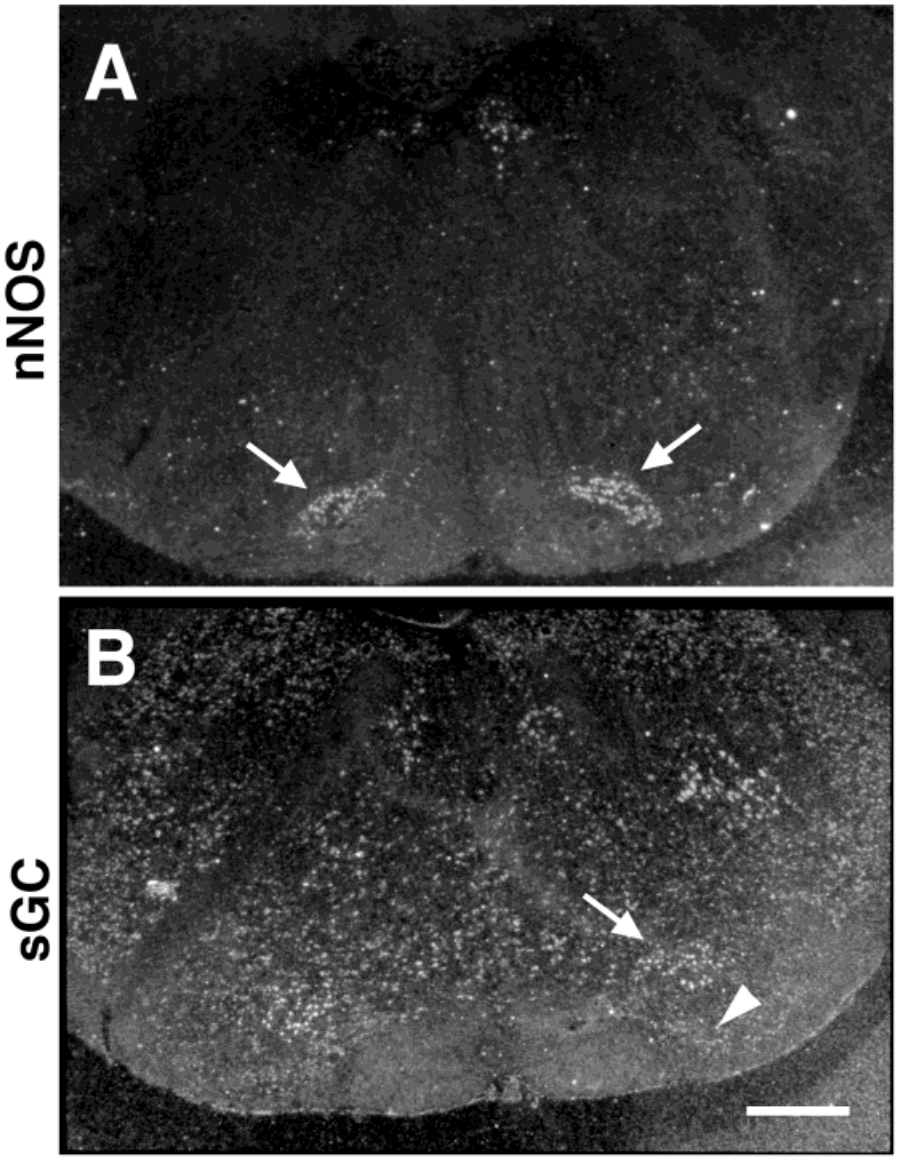

Fig. 4. Brainstem localization of mRNA for nNOS and sGC-β using in situ hybridization. **A:** The nNOS antisense probe labeled relatively few cell groups in the brainstem. The medial nucleus of the trapezoid body (arrows) was labeled. **B:** In contrast, the sGC-β antisense probe labeled many cells throughout the brainstem, such as the neurons of the superior periolivary nucleus (arrow) and the medial nucleus of the trapezoid body (arrowhead). Scale bar = 500 μm.

and Eybalin, 1992; Kuriyama et al., 1993; Niedzielski and Wenthold, 1995). The presence of nNOS in afferent fibers is consistent with the established activation of this enzyme by calcium influx through NMDA receptors. In fact, nNOS may be associated physically with this receptor type (Brenman et al., 1996).

Projections of spiral ganglion cells (i.e., the auditory nerve) synapse on neurons in the cochlear nucleus. The diffuse NADPH-diaphorase activity found in neuropil in the VCN may be derived from such auditory nerve fibers and terminations. In contrast, NADPH-diaphorase labeling of spherical and globular bushy cells often had a granular appearance.

These cell types also contain mRNA for the β subunit of sGC, suggesting that NO produced from NADPH diaphorase-positive auditory nerve terminals may activate sGC in spherical/globular bushy cells. Because these cells project out of the VCN, it is possible that the NO/cGMP pathway also influences these projections to other parts of the brainstem. The β subunit is the more conserved protein of the two subunits of sGC and is used frequently to extrapolate to the localization of sGC (Matsuoka et al., 1992; Giuili et al., 1994). It should be noted, however, that the α subunit has a more limited distribution in some brain regions (Furuyama et al., 1993).

In principal cells of the MNTB, again, nNOS may be linked to NMDA receptors. MNTB principal cells preferentially express the NMDA receptor subunit 2B (Saito et al., 1998). This subunit is coupled to nNOS through the adapter protein PSD-95 (Brenman et al., 1996). Thus, stimulation of principal cells of the MNTB by glutamate may lead to nNOS activation.

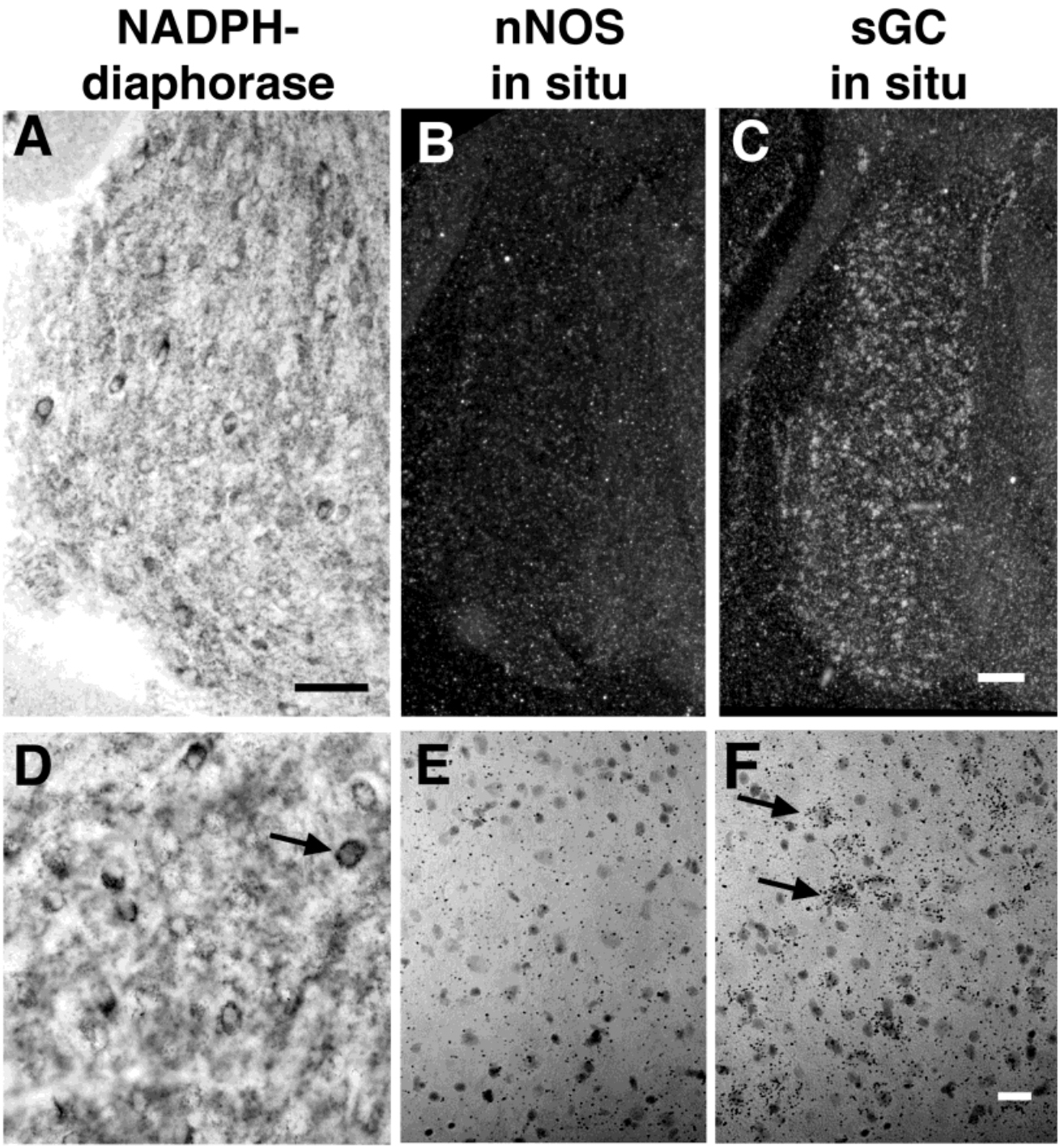

Fig. 5. **A–F:** Localization of NADPH-diaphorase activity and nNOS and sGC-β mRNA in the ventral cochlear nucleus (VCN). A,D: NAPDH-diaphorase activity of VCN neuropil was seen diffusely throughout the entire VCN. In addition, some cells were labeled (arrow in D). B,E: No labeling of neurons within the VCN was detected with the nNOS antisense probe, as visualized by using either darkfield (B) or brightfield (E) microscopy. C,F: Abundant labeling of neurons within the VCN was seen with the sGC-β antisense probe (F, arrows). Scale bars = 100 μm in A and C (C also applies to B), 20 μm in F (also applies to D,E).

The MNTB principal cells project to other SOC regions with large inputs to the LSO and the SPN (Helfert et al., 1991). It is reasonable to assume that nNOS-positive fibers from MNTB principal cells may be responsible for the NADPH-diaphorase activity of neuropil in these regions. The granular labeling of cells in the LSO and the SPN, similar to the appearance in the VCN, suggests NOS-positive nerve terminals on these cells. Because cells that express mRNA for sGC β also were found in these areas, the NO/cGMP pathway may be involved in connections between MNTB principal cells and the neurons in the SPN and the LSO. The NO/cGMP pathway may have a particu-

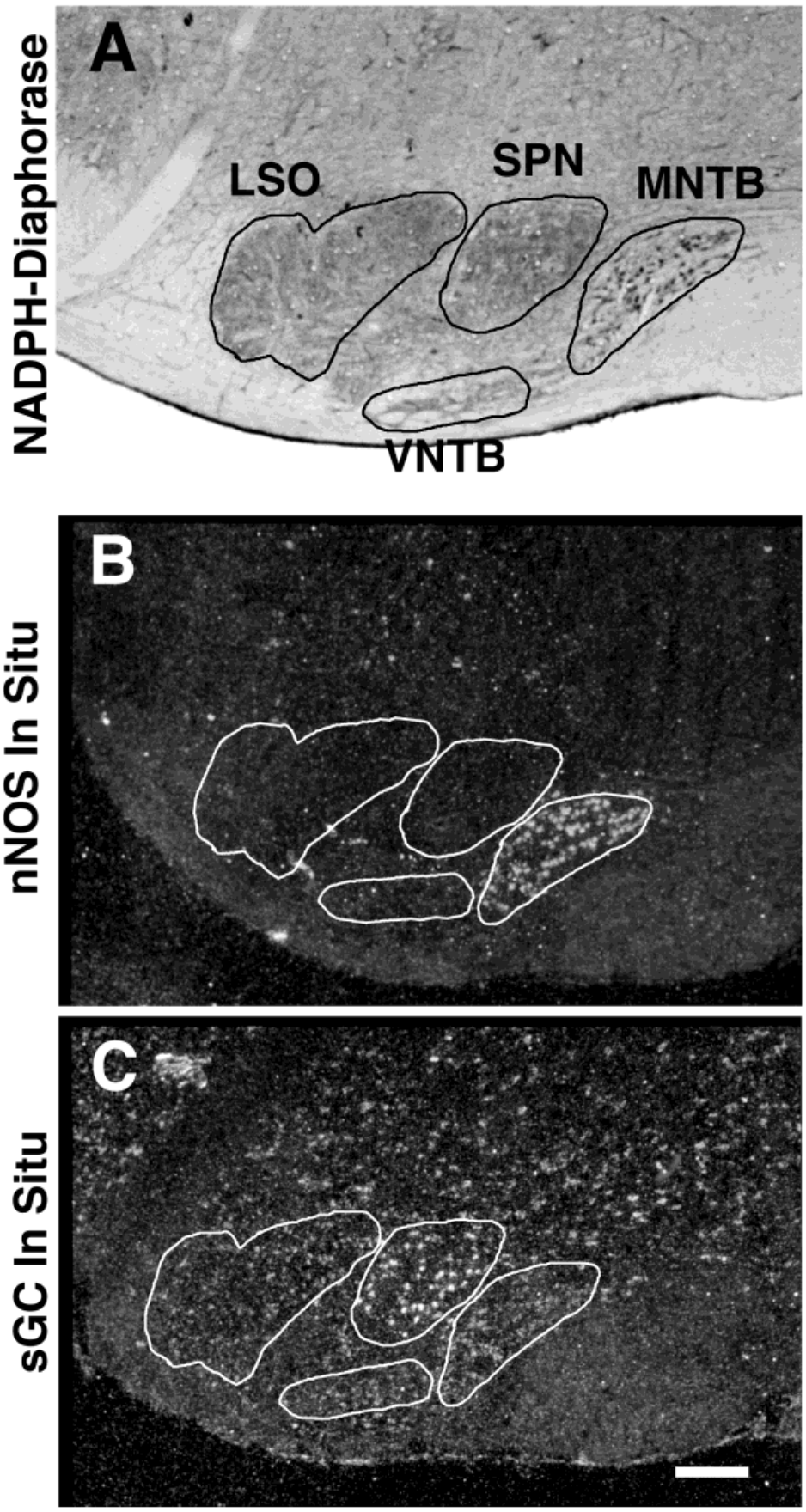

Fig. 6. Localization of NAPDH-diaphorase activity and nNOS and sGC-β mRNA in the superior olivary complex. **A:** NADPH-diaphorase labeling in the superior olivary complex (SOC). Principal cells in the medial nucleus of the trapezoid body (MNTB) are labeled. In addition, labeling of neuropil in the lateral superior olive (LSO), the superior periolivary nucleus (SPN), and the ventral nuclei of the trapezoid body (VNTB) is apparent. **B:** Distribution of mRNA for nNOS in the SOC. Principal cells in the MNTB are labeled. **C:** Distribution of mRNA for sGC in the SOC. Cells within the LSO, VNTB, SPN, and MNTB are labeled, with strong labeling in the SPN. Scale bar = 200 µm.

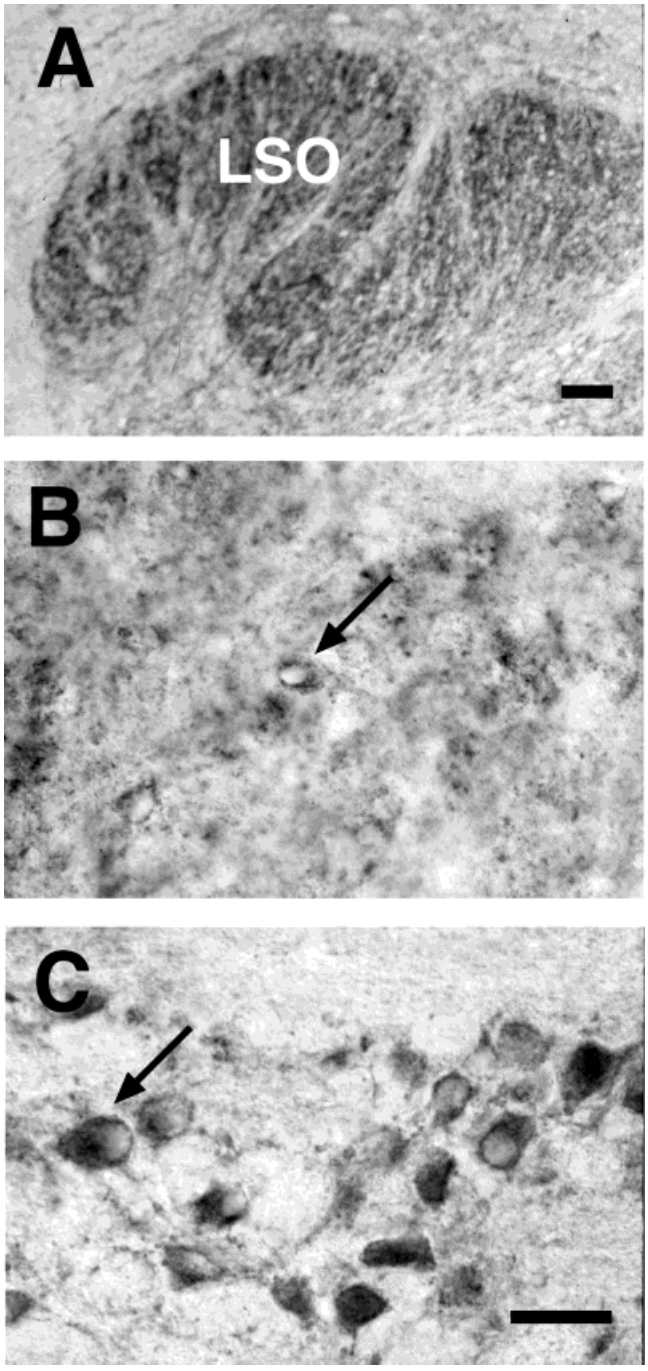

Fig. 7. NADPH-diaphorase labeling in the SOC. **A,B:** Labeling of LSO with granular-type labeling (arrow). **C:** Labeling of MNTB principal cells. NAPDH-diaphorase activity in these cells is more diffuse (arrow). Scale bars = 100 μm in A, 50 μm in C (also applies to B).

larly important role in the SPN, because mRNA for sGC β was present in the highest amounts in this location.

The VNTB contains several different cell types, including olivocochlear neurons that project back to the outer hair cells of the cochlea. The VNTB contained both NADPH-diaphorase activity and sGC-β-positive cells. Because the efferent projections to the outer hair cells show sGC activity, the neurons labeled by in situ hybridization in the VNTB may include medial olivocochlear neurons. This suggests the participation of the NO/cGMP pathway in the descending auditory pathways.

The localization of sGC to efferent fibers in the rat cochlea is intriguing, because, in guinea pig, sGC activity is found in supporting cells rather than in efferent fibers (Fessenden and Schacht, 1997). Species differences in cochlear localization exist for a number of cell components, including proteins involved in regulatory processes. For example, the calcium-binding protein calretinin is present in Deiters' cells in guinea pig and gerbil but is absent from these cells in rat (Dechesne et al., 1991). Similarly, calbindin is found in pillar cells in rat cochlea (Dechesne et al., 1991) but not in guinea pig or gerbil (Slepecky and Ulfendahl, 1993; Pack and Slepecky, 1995). Such differences in biochemical regulatory pathways between species may have important consequences on cochlear function that remain to be explored.

Differences in labeling between NADPH-diaphorase histochemistry and nNOS in situ hybridization

In the VCN and SOC, NADPH-diaphorase activity was detected in some cells that did not express mRNA for nNOS. Because all NOS isoforms exhibit NADPH-diaphorase activity, these cells may express a different NOS isoform, such as endothelial NOS (which also has been localized to neurons of the brain; Dinerman et al., 1994). Another strong possibility is that an alternatively spliced isoform of nNOS exists in these cells. One such isoform, nNOS β, lacks exon 2 but retains enzymatic activity (Brenman et al., 1996). Our nNOS riboprobe was directed against exon 2; therefore, we would not have been able to detect this splice variant by using in situ hybridization. Indeed, recent experiments indicate that nNOS β is present in the VCN (Eliasson et al., 1997), which is compatible with the suggestion that the cellular NADPH-diaphorase activity in this location is due to this isoform.

Functional implications for the NO/cGMP pathway in the auditory system

Consistent with its proposed activity in other brain regions, the NO/cGMP pathway could modulate neurotransmitter release in the auditory brainstem. In cultured neurons and slices from a variety of brain areas (Hanbauer et al., 1992; Montague et al., 1994), exogenous NO can elicit the release of different neurotransmitters, such as acetylcholine, dopamine, glycine, and glutamate. Because all of these neurotransmitters are found in the auditory brainstem, it is conceivable that the NO/cGMP pathway could regulate their release.

In addition, NO in the central auditory pathways may act as a feedback inhibitor of glutamate receptor activity. For example, NO produced by activation of NMDA-type glutamate receptors (Manzoni and Bockaert, 1993) or supplied from exogenous NO donors (Manzoni et al., 1992; Lipton et al., 1993) inhibit NMDA receptor activity. NO-mediated inhibition of glutamate receptors also has been described in the visual system (McMahon and Ponomareva, 1996). Because excess glutamate can cause neurotoxicity in the CNS, this type of inhibition of glutamate receptors by NO itself may protect neurons from toxic damage. In the auditory pathways, NO may inactivate glutamate receptors during periods of excessive neuronal activity, like what would occur during acoustic overstimulation. Such a protective effect would be expected in neurons in

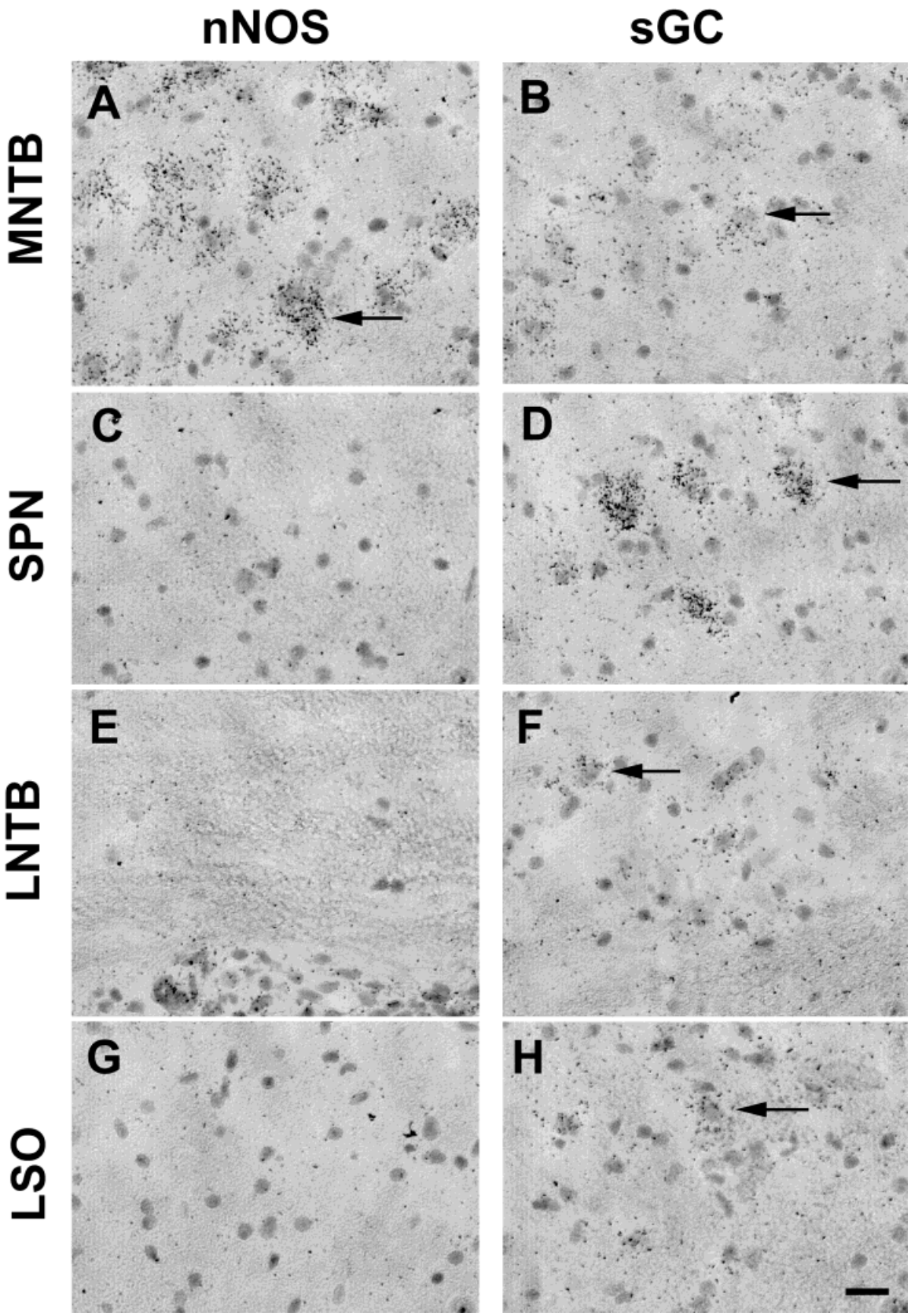

Fig. 8. Brightfield visualization of in situ hybridization labeling in various SOC regions. In situ hybridization experiments using nNOS (A,C,E,G) and sGC-β (B,D,F,H) antisense probes were performed on the MNTB (**A,B**), SPN (**C,D**), VNTB (**E,F**), and LSO (**G,H**). Labeled cells are indicated by arrows. mRNA for nNOS was detected only in the MNTB (A), whereas mRNA for sGC was detected in all regions (B,D,F,H). Labeling was strongest in the SPN (D). Scale bar = 20 μm.

which nNOS and NMDA receptors are coexpressed, such as spiral ganglion cells and MNTB principal cells.

In summary, the NO/cGMP pathway is distributed widely in the ascending and descending auditory pathways of the rat. nNOS is associated largely with structures that contain NMDA receptors, consistent with the notion that NO is a major modulator and mediator in glutamatergic neurons. The localization of both NOS and the NO target, sGC, now allows for rational experimentation to test the role(s) of this fascinating pathway in audition.

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