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Morphological Identification of Nitric Oxide Sources and Targets in the Cat Oculomotor System

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ABSTRACT

Nitric oxide (NO) production by specific neurons in the prepositus hypoglossi (PH) nucleus is necessary for the correct performance of eye movements in alert cats. In an attempt to characterize the morphological substrate of this NO function, the distribution of nitrergic neurons and NO-responding neurons has been investigated in different brainstem structures related to eye movements. Nitrergic neurons were stained by either immunohistochemistry for NO synthase I or histochemistry for reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase. The NO targets were identified by cyclic guanosine monophosphate (cGMP) immunohistochemistry in animals treated with a NO donor immediately before fixation of the brain. Connectivity between cells of the NO-cGMP pathway was analyzed by injections of the retrograde tracers horseradish peroxidase or fast blue in different structures. The motor nuclei commanding extraocular muscles did not contain elements of the NO-cGMP pathway, except for some scattered nitrergic neurons in the most caudal part of the abducens nucleus. The PH nucleus contained the largest number of nitrergic cell bodies and a rich neuropil, distributed in two groups in medial and lateral positions in the caudal part, and one central group in the rostral part of the nucleus. An abundant cGMP positive neuropil was the only NO-sensitive element in the PH nucleus, where no cGMP-producing neuronal cell bodies were observed. The opposite disposition was found in the marginal zone between the PH and the medial vestibular nuclei, with a large number of NO-sensitive cGMP-producing neurons and almost no nitrergic cells. Both nitrergic and NO-sensitive cell bodies were found in the medial and inferior vestibular nuclei and in the superior colliculus, whereas the lateral geniculate nucleus contained nitrergic neuropil and a large number of NO-sensitive cell bodies. Some of the cGMP-positive neurons in the marginal zone and medial vestibular nucleus projected to the PH nucleus, predominantly to the ipsilateral side. These morphological findings may help to explain the mechanism of action of NO in the oculomotor system. J. Comp. Neurol. 435:311–324, 2001.

Indexing terms: NADPH diaphorase; cGMP; soluble guanylyl cyclase; prepositus hypoglossi nucleus; marginal zone; eye movements

Nitric oxide (NO) is an intercellular messenger released by specific neurons expressing NO synthase I (NOS I) in the central and peripheral nervous system. Due to its chemical properties as a diffusible gas molecule, NO allows intercommunication of adjacent neurons in both anterograde (from terminals to postsynaptic cells) and retrograde (from postsynaptic structures to nerve terminals) directions; in addition, it can affect any possible target in the neighborhood of the cells containing activated NOS (Gally et al., 1990; Schuman and Madison, 1994). The ability of NO to behave as a retrograde neurotransmitter provides nitrergic neurons with the unique capacity to modulate their own synaptic input. Over the past 10 years, the

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role of NO as a cerebral messenger has been well established, and NO has been implicated in several brain functions (Garthwaite and Boulton, 1995) including the tight coupling between local blood flow and synaptic activity (Iadecola, 1993), functional synaptic plasticity (Schuman and Madison, 1994; Fedele and Raiteri, 1999), synapse formation and stabilization (Roskams et al., 1994), and modulation of sensory information (Cudeiro and Rivadulla, 1999).

The physiological participation of NO in a sensorymotor function has been demonstrated in the eye movement control system. Functional experiments have shown that a balanced production of NO by neurons of the preoculomotor nucleus prepositus hypoglossi (PH) is necessary for the correct performance of eye movements in alert cats (Moreno-López et al., 1996). Unilateral injections of NOS inhibitors within this nucleus produced velocity imbalances in a direction contralateral to the injected side, resulting in a dramatic and long-lasting nystagmus, which was more accentuated in darkness. Furthermore, local injections of NO donors produced a nystagmus in a direction opposite to that of NOS inhibitors, together with an alteration in eye position generation (Moreno-López et al., 1998). These functional data indicate that NO is tonically produced in the PH nucleus in normal conditions and suggest that NO acts by a retrograde action on nerve terminals from medial vestibular neurons projecting to the PH nucleus.

The most common cellular mechanism of action for NO is the activation of soluble guanylyl cyclase on the target cells, resulting in a transient increase in intracellular cyclic guanosine monophosphate (cGMP) concentration (Murad et al., 1978; Bredt and Snyder, 1989), which, by regulating certain ionic channels (Ahmad et al., 1994), may control neurotransmitter release (Montague et al., 1994). The actions of NO in the PH nucleus are most probably due to cGMP synthesis, because local administration of the permeant cGMP analog 8-Br-cGMP in this nucleus produced an effect identical to that of NO donors (Moreno-López et al., 1996, 1998). Identification in the oculomotor structures of neuronal cell bodies, nerve fibers, or terminals able to increase their cGMP concentration in response to NO might provide valuable information about the possible targets of NO in the control of eye movements. Labeling of these targets can be achieved by applying cGMP immunohistochemistry to brain sections obtained from animals that have been perfused with the NO donor sodium nitroprusside immediately before fixation (Southam and Garthwaite, 1993).

To gain a better understanding of the mechanism of action of NO in the oculomotor system, we have now analyzed the distribution of nitrergic neurons and their possible targets, the neural elements containing NO-sensitive cGMP immunoreactivity (cGMP-ir), in combination with the use of retrograde markers, in the brainstem structures related to eye movement control in the cat. We have found regions where cell bodies of both types existed in close proximity, allowing cell-cell interactions. Other areas presented either nitrergic cell bodies and cGMP-containing neuropil or nitrergic neuropil in combination with NO-sensitive somas, suggesting that NO may act as a retrograde or an anterograde messenger in different nuclei of the oculomotor system. These results may help to elucidate the cellular bases of the functional influence of NO in the control of eye movements.

MATERIALS AND METHODS

Nine adult cats of either sex, obtained from an authorized supplier (Illa-Credo L'Arbesle, France) were used as experimental subjects. Experiments were performed in accordance with the European Union directive 609/86/CEE and with Spanish legislation (RD 233/89) on the use of laboratory animals. For retrograde tracer injections, the cats were anesthetized with 35 mg/kg sodium pentobarbital, i.p.; before transcardial perfusion, 50 mg/kg of the same drug were used, except for animals whose brain sections were processed for cGMP immunohistochemistry, which were anesthetized with 35 mg/kg ketamine and 1 mg/kg xylidine-dihydrothiazine, i.m., because barbiturates have been shown to interfere with this technique (de Vente et al., 1990).

Identification of structures containing NO-sensitive soluble guanylyl cyclase

To identify cellular structures in which cGMP was produced in response to NO stimulation, the technique described by Southam and Garthwaite (1993) was used with some modifications. Four cats were anesthetized and perfused (200 ml/min, 5 minutes) through the ascending aorta with a physiological solution containing the NO donor sodium nitroprusside (SNP; 10 mM), to activate soluble guanylyl cyclase, and the phosphodiesterase inhibitor isobutyl-3-methylxantine (IBMX, 1 mM). The perfusion fluid (with the following composition, in mM: 120 NaCl, 2 KCl, 2 $CaCl_2$, 26 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, and 11 glucose) was bubbled with 95% O_2 and 5% CO_2 and maintained at 37°C. Two additional animals were perfused under identical conditions, but without SNP, and were used as control for NO-independent cGMP. Immediately after the physiological solution, the animals were perfused with freshly prepared 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.4, at 4°C. The brainstems were removed, postfixed for 2 hours, and cryoprotected by incubation for 2 days with 30% sucrose in PB, at 4°C. Coronal 40-μm-thick sections were cut with a freezing microtome and collected in phosphate-buffered saline solution (PBS), pH 7.4.

Brain sections from SNP-treated and control animals were incubated for 30 minutes in a Tris-buffered saline solution (TS), pH 7.4, containing 0.1% Triton X-100, 2.5% bovine serum albumin, and 0.25% sodium azyde. The tissue was then incubated overnight at 4°C with an antibody raised in sheep against a cGMP-paraformaldehyde-bovine thyroglobulin complex (1:20,000 in TS; Tanaka et al., 1997). After incubation with biotinylated anti-sheep IgG (Chemicon, Temecula, CA), the tissue was processed according to the avidin-biotin peroxidase complex procedure, by using an ABC kit (Vector Laboratories, Burlingame, CA). Horseradish peroxidase (HRP) was made visible by incubation with a solution containing 0.05% 3-3′-diaminobenzidine and 0.003% hydrogen peroxide in 0.05 M Tris, pH 7.6, for 10 minutes. After staining, the brain sections were mounted on gelatin-coated slides, dehydrated, cleared in xylene, and coverslipped. No immunostaining was observed when the primary antibody was omitted. Only structures in which a significantly different staining for cGMP was observed between animals perfused with and without SNP were considered as possible targets for NO.

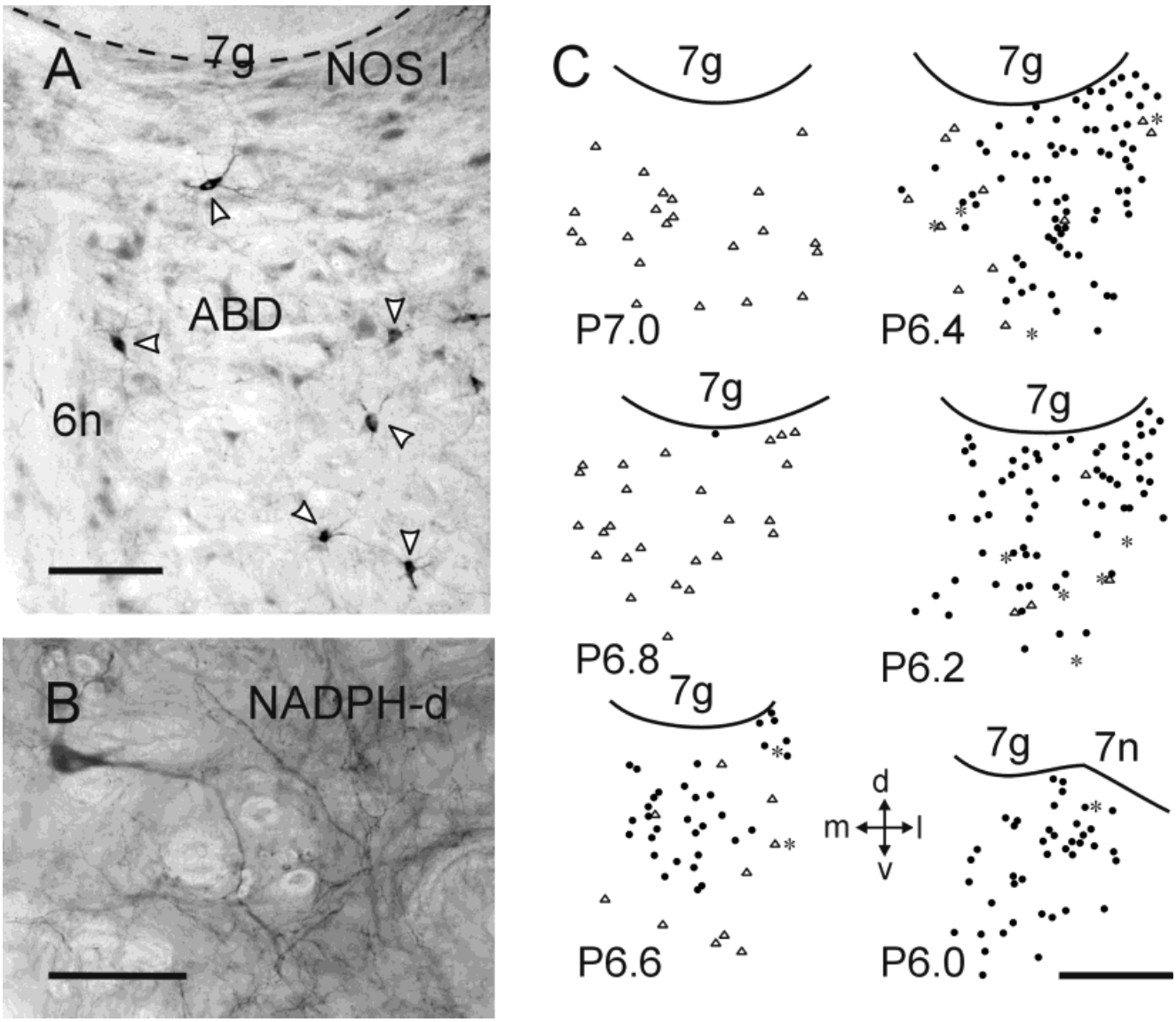

Fig. 1. Distribution of nitrergic neurons in the abducens nucleus of the adult cat. **A:** Photomicrograph showing a coronal brainstem section through the most caudal part of the abducens nucleus processed for nitric oxide synthase I (NOS I) immunohistochemistry. Scattered neurons (arrowheads) are observed all over the nucleus area. **B:** Higher magnification of a coronal brainstem section through the abducens nucleus stained for reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemistry revealing the nitrergic neuropil. **C:** Camera lucida drawings representing a series of abducens nucleus coronal sections, 200 μm apart, showing the distribution of nitrergic neurons (triangles), motoneurons (dots), and motoneurons stained for NADPH diaphorase (asterisks). The planes, according to the atlas of Berman (1968), are indicated. The orientation of the photographs and the drawings is the same and is indicated in C. ABD, abducens nucleus; 6n, abducens nerve; 7g, facial genu; 7n, facial nerve; d, dorsal; l, lateral; m, medial; v, ventral. Scale bars = 150 μm in A; 50 μm in B; 300 μm in C.

Detection of nitrergic structures

Nitrergic neurons were identified by either reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemistry (Dawson et al., 1991; Hope et al., 1991) or NOS I immunohistochemistry. NADPH diaphorase activity was made visible by incubation of the tissue sections in a mixture containing 1 mM β-NADPH, 1 mM nitro blue tetrazolium, and 0.1% Triton-X-100, in 0.1 M Tris buffer, pH 8.0, for 30 minutes at 37°C. For NOS I immunohistochemistry, free-floating sections were incubated with an antibody raised in rabbit against a 22.3-kDa protein fragment corresponding to amino acids 1095–1289 of human neuronal NOS (1:500; Transduction Laboratories, Lexington, KY), followed by a 1-hour incubation at room temperature with a biotinylated anti-rabbit IgG (1:200; Sigma) and processing with the ABC kit as indicated before.

For distribution analysis, consecutive series of sections were stained with cresyl violet and NOS I or cGMP immunohistochemistry. When NOS I and cGMP were immunostained in the same brainstem section, the tissue was incubated overnight at 4°C with a solution containing

anti-NOS I and anti-cGMP (1:4000), followed by a 1-hour incubation at room temperature with biotinylated anti-rabbit IgG (1:200; Vector) and Cy3-bound anti-sheep IgG (1:400; Jackson ImmunoResearch, West Grove, PA). The tissue was mounted in Vectashield, images were captured under fluorescence microscopy, and the brain sections were further processed for biotin visualization with the ABC method.

Retrograde tracing studies

HRP was injected in the abducens nucleus of two cats, to identify the PH neurons that project to this motor nucleus. The animals were anesthetized as described, and a stimulating electrode was implanted in one lateral rectus muscle to stimulate the motoneurons antidromically in the ipsilateral abducens nucleus. A 4 × 4-mm hole was drilled through the occipital bone to allow access to the posterior brainstem via the cerebellum. A glass micropipette (12.5-μm tip diameter) was advanced through the cerebellum toward the abducens nucleus, which was identified by the recording of the antidromic field potential induced by electrical stimulation (<0.1 mA, 50 μsec) of the muscle. A 20% solution of HRP (Boehringer Mannheim, Germany) in 0.05 M NaCl and 0.05 M Tris-HCl buffer, pH 7.6, was electrophoretically applied to the abducens nucleus by using positive current pulses (4 μA, 500 msec, 1 Hz), for 10 minutes. Two injections were performed within the nucleus, 500 μm apart.

In one additional animal, HRP was injected in both lateral rectus muscles, to identify motoneurons in the abducens nuclei. The animal was anesthetized as described, the lateral rectus muscle was exposed under a dissecting microscope, and 10 μl of 20% HRP solution in 2% dimethylsulfoxide was injected by using a Hamilton syringe.

After a survival period of 48 hours, the three animals injected with HRP were deeply anesthetized and transcardially perfused with PBS, followed by 4% paraformaldehyde in PB at 4°C. The brainstems were removed, and coronal sections were obtained as described through the abducens and PH regions. The sections were processed for HRP histochemical localization by using diaminobenzidine as the chromogen and subsequently for NADPH diaphorase histochemistry as described above.

The retrograde tracer fast blue was injected in the rostral third of the PH nucleus in two cats, to identify neurons projecting to the PH nucleus in different structures. The animals were prepared as described for HRP injections in the abducens nucleus. The PH nucleus was localized in the same parasagittal plane and 1–1.5 mm posterior to the abducens nucleus, just below the floor of the fourth ventricle. The correct position of the micropipette was confirmed by recording and characterizing the firing discharge of PH neurons in relation to eye position and velocity (Escudero et al., 1992). Injections were performed by means of glass micropipettes with tip diameters of 50 μm, filled with 1% fast blue in PB. Two air pulses (1 kg/cm^2, 1 second) were applied with an air pressure device connected to the injection micropipette to deliver a total of 300–400 nl. Five days later the animals were perfused as indicated for cGMP immunohistochemistry. Brain sections were stained for cGMP to obtain double immunofluorescent labeling of those neurons projecting to the PH nucleus that were possible targets for the NO produced by PH nitrergic neurons.

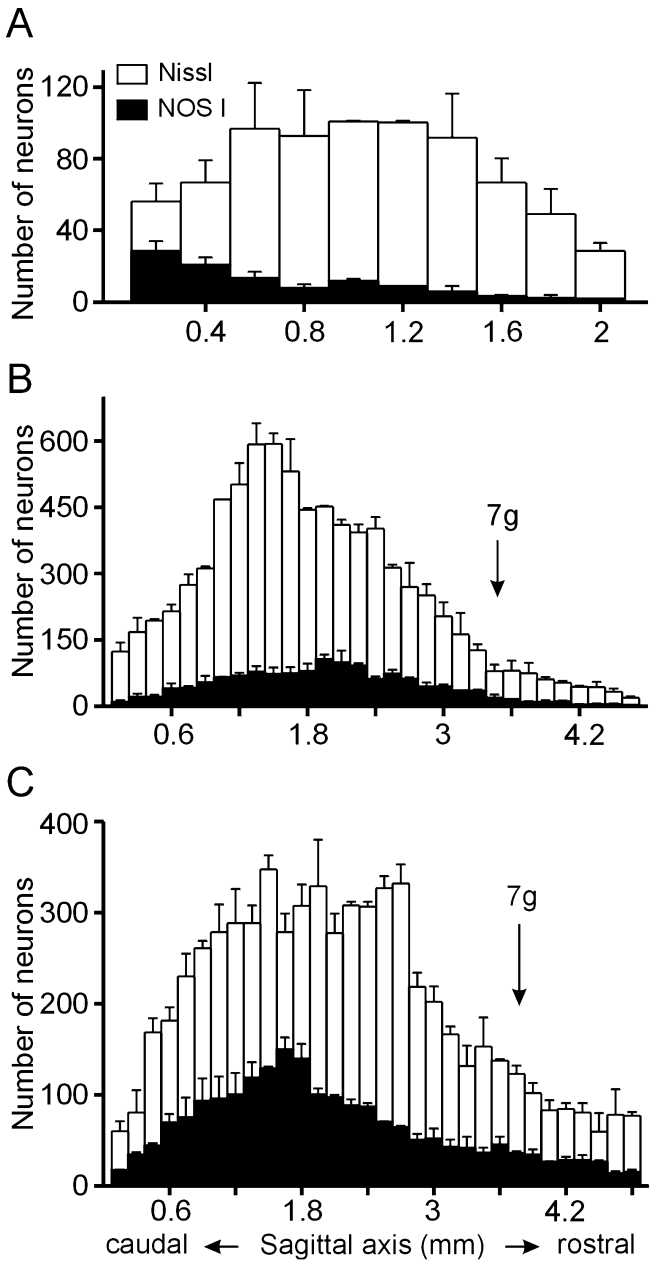

Fig. 2. Histograms showing the rostrocaudal distribution of neurons stained with NOS I immunohistochemistry (solid bars) and Nissl staining (open bars) in a series of consecutive sections in the abducens (**A**), prepositus hypoglossi (**B**), and medial vestibular (**C**) nuclei. Each pair of sections was separated by 120 μm in A and by 160 μm in B and C. In the abscissa, 0 represents the most caudal section quantified in each case, corresponding to P7.2 in A, P11.6 in B, and P11.2 in C, according to Berman (1968). The caudal limit of the facial genu (7g) is indicated in B and C as an anatomical reference. Data are presented as means ± SD of the results obtained in two animals.

Analysis of the data

Sections were analyzed by using an epifluorescence BX60 Olympus microscope fitted with the appropriate filters for independent visualization of Cy3 and fast blue. To investigate whether NOS I and cGMP immunoreactivities were present in the same neurons, images of cGMP im-

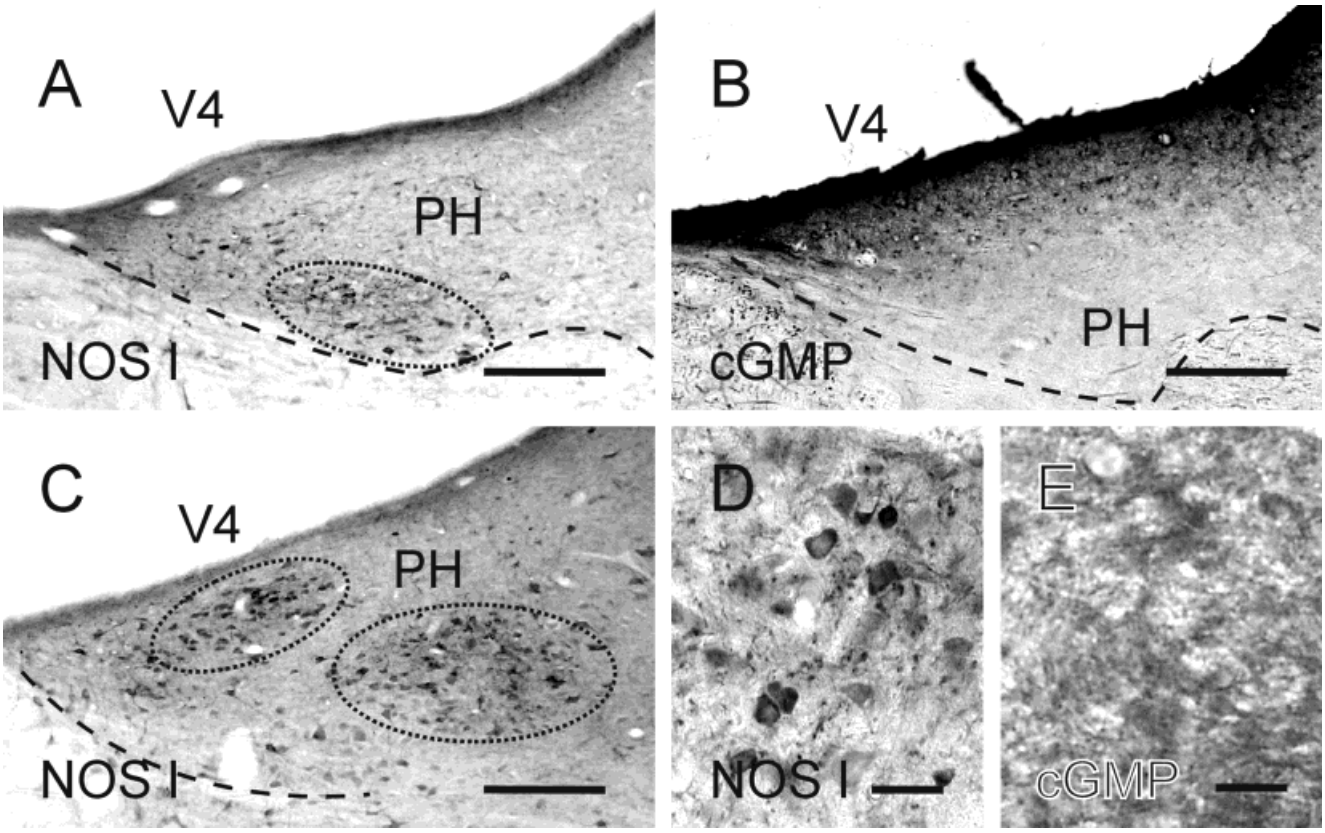

Fig. 3. Distribution of nitric oxide synthase I (NOS I) and cyclic guanosine monophosphate (cGMP) immunoreactivity in the prepositus hypoglossi nucleus of the adult cat. **A,C:** Low-magnification photomicrographs of coronal brainstem sections through the prepositus hypoglossi (PH) nucleus processed for NOS I immunohistochemistry. In the rostral part of the PH nucleus (A; coronal plane P8, according to Berman, 1968), a well-defined group of neurons (dotted lines) is observed, whereas, in a more caudal section (C; coronal plane P10.1, according to Berman, 1968), the stained neurons are organized in two groups (dotted lines). Additional scattered neurons expressing NOS I can be observed in both sections. **B:** Low-magnification photomicrograph of a coronal brainstem section similar to that shown in A, processed for cGMP immunohistochemistry in an animal perfused with SNP. No cGMP neuronal cell bodies were found. The immunostaining was present in the neuropil, in the dorsal limit of the PH, close to the fourth ventricle (V4). The dashed line represents the limits of the PH nucleus. **D,E:** Higher magnification photomicrographs of the PH nucleus stained for NOS I and cGMP immunohistochemistry, respectively. Neuronal cell bodies, neuropil, and bouton-like structures containing NOS I are observed, whereas only a dense neuropil stained for cGMP. Scale bars = 150 μm in A–C; 25 μm in D,E.

munofluorescence were first captured, and the same fields were studied under light microscopy after visualization of NOS I with the avidin-biotin-HRP technique. Images were captured with a DP10 digital camera and analyzed by using the MicroImage software, both from Olympus. The number of nitrergic neurons, or of neurons retrogradely labeled with HRP, was counted in the different structures and compared with the total number of cells revealed with Nissl staining in consecutive sections. The average size of the neurons is indicated by their maximum and minimum diameter; unless otherwise indicated, at least 100 neurons were measured for each datum provided. Unless otherwise indicated, data are expressed as means ± SD.

RESULTS

Distribution of nitrergic neurons and NO-sensitive cGMP-producing structures in the oculomotor nuclei

Immunohistochemical detection of NOS I or NADPH diaphorase histochemistry revealed the presence of some positive neurons and neuropil within the abducens nucleus (Fig. 1), whereas no stained cells were observed in the oculomotor or the trochlear nuclei (not shown). In the abducens nucleus, nitrergic neurons represented 13.6 ± 1.2% of the total number of Nissl-stained neurons, although they were unevenly distributed, with the majority of them located in the periphery and in the most caudal part of the nucleus (Figs. 1C, 2A). To analyze whether motoneurons were able to synthesize NO, the retrograde tracer HRP was injected in both lateral rectus muscles of one cat, and brain sections were double stained for HRP and NADPH diaphorase. Motoneurons, containing HRP, constituted 57.8 ± 5.4% (mean ± SD of the left and right abducens nuclei) of the Nissl-stained cells in the nucleus, and only 5.1 ± 0.2% of the HRP-positive cells were labeled for NADPH diaphorase (not shown). These double-stained neurons, in turn, constituted 15 ± 2.4% of the nitrergic cells. Among the neurons projecting to the lateral rectus muscle, the double-labeled cells constituted a subgroup, which was characterized by their smaller size (21.8 ± 4.9 μm and 12.0 ± 2.9 μm, maximum and minimum diame-

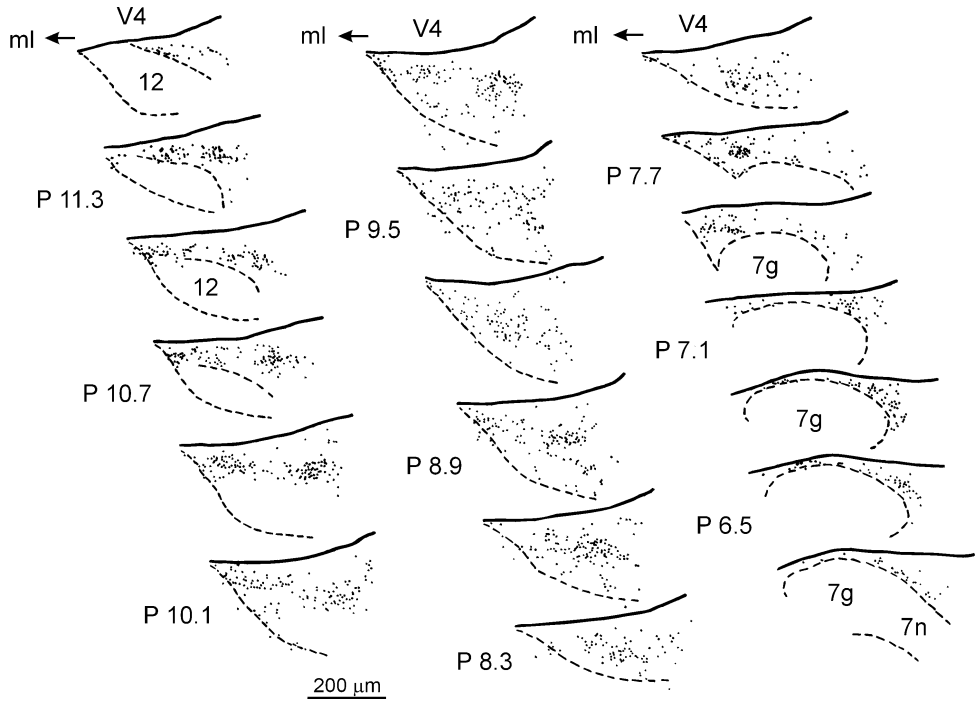

Fig. 4. Distribution of nitrergic neurons along the prepositus hypoglossi (PH) nucleus. Camera lucida drawings of 40μm-thick sections, 260 μm apart, processed for NOS I immunohistochemistry. Coronal sections are arranged in caudal to rostral sequence from top to bottom and from left to right. Nitrergic neurons are represented by black dots. Coordinates according to Berman (1968) are shown for alternate sections. The midline (ml) direction is indicated by arrows. The dashed lines represent the medial and ventral limits of the PH nucleus and, when indicated, the ventral limit of the hypoglossal nucleus (12) or of the facial nerve (7n). 7g, facial genu.

ters, respectively; n = 50) compared with the average size of NADPH diaphorase-negative motoneurons (33.6 ± 7.1 μm and 18.8 ± 4.9 μm, maximum and minimum diameters, respectively).

None of the oculomotor nuclei contained NO-sensitive cGMP-producing structures, except for some large fibers running across the nuclei. Some cell bodies slightly stained for cGMP were also visible, but no differences were observed between animals perfused with or without SNP (not shown).

Distribution of nitrergic neurons and NO-sensitive cGMP-producing structures in preoculomotor nuclei

The PH nucleus was highly enriched in neurons positive for NOS I immunoreactivity (Fig. 3A,C,D), which constituted 32.5 ± 2.2% of the Nissl-stained neurons. The percentage of nitrergic somas was relatively constant in the different coronal sections (Fig. 2B), although the distribution varied significantly along the rostrocaudal axis. In the caudal portion of the nucleus (Figs. 3C, 4), two distinct columns appeared, with a medial group containing oval medium-sized neurons (16.8 ± 3.1 μm and 7.4 ± 1.3 μm, maximum and minimum diameters, respectively) and a lateral group containing smaller multipolar cells (11.6 ± 2.2 μm and 7.1 ± 1.5 μm, maximum and minimum diameters, respectively), in a slightly more ventral position. These two columns fused, in the rostral part of the nucleus, into one central group (Figs. 3A, 4), which contained cells similar in size and shape to those of the medial group. In spite of this preferential distribution, scattered nitrergic neurons were also found all over the nucleus. Abundant neuropil and bouton-like structures were also widely observed (Fig. 3D).

In cats perfused with SNP, cGMP-ir appeared in a dense neuropil in the PH nucleus, with a preferential distribution in the dorsal part of the nucleus (Fig. 3B,E). No neuronal somas positive for cGMP-ir were found. No significant staining was evident in animals perfused without SNP.

The medial vestibular nucleus (MVN) contained nitrergic neurons (Figs. 5A,C, 6), sparsely distributed all over the nucleus. The percentage of nitrergic neurons compared with the total number of neurons visualized with Nissl staining was relatively constant all along the nucleus (Fig. 2C), with an average of 17.2 ± 0.6%. Nitrergic neurons were larger than those found in the PH nucleus (24.4 ± 6.4 μm and 12.9 ± 4.3 μm, maximum and minimum diameters, respectively). Neuronal cell bodies containing NO-sensitive cGMP were also observed in this nucleus (Figs. 5B,D, 6). These cells (21.4 ± 5 μm and 13.0 ± 3 μm, maximum and minimum diameters, respectively) were predominantly found in the central and rostral parts of the nucleus. Double immunofluorescence studies indicated that these markers were never colocalized in the same neuron. Figure 6 shows the distribution of nitrergic and NO-sensitive cGMP-producing neurons in the MVN, in coronal sections 500 μm apart.

The inferior vestibular nucleus (IVN) contained a small number of NOS I-immunoreactive neurons but was highly enriched in cGMP-ir cell bodies, especially in the most dorsal and caudal part of the nucleus (Figs. 5F,G, 6).

A dense group of neurons and neuropil heavily labeled with cGMP-ir in SNP-treated animals was present in the

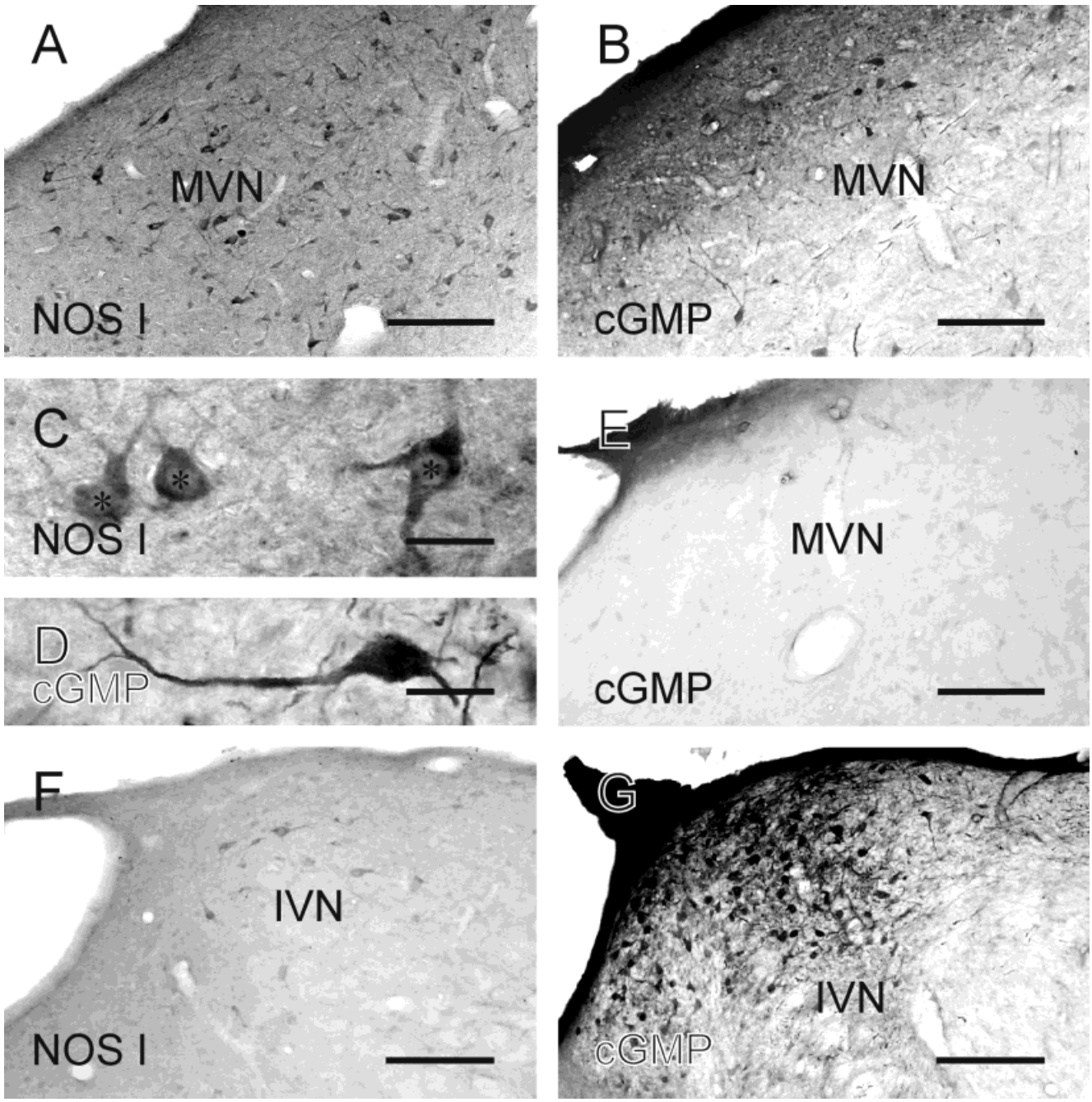

Fig. 5. Distribution of nitric oxide synthase I (NOS I) and cyclic guanosine monophosphate (cGMP) immunoreactivity in the medial and inferior vestibular nuclei of the adult cat. **A:** Low-magnification photomicrograph of a coronal brainstem section through the medial vestibular nucleus (MVN) processed for NOS I immunohistochemistry. Nitrergic neurons are observed all over the nucleus. **B:** Low-magnification photomicrograph of a similar coronal brainstem section processed for cGMP immunohistochemistry. Both neuronal cell bodies and neuropil are present. **C:** High-magnification photomicrograph showing several NOS I-positive neurons with clear nuclei (asterisks) and large dendritic branches. **D:** High-magnification photomicrograph of a cGMP-positive medial vestibular neuron with a prominent dendritic branch. **E:** Control brainstem section through the MVN, obtained from one cat perfused without SNP and processed for cGMP immunohistochemistry as in B. No immunolabeling was observed in the absence of NO stimulation. **F:** Low-magnification photomicrograph of a coronal brainstem section through the inferior vestibular nucleus (IVN) processed for NOS I immunohistochemistry. Small numbers of nitrergic neurons were observed all over the nucleus. **G:** Low-magnification photomicrographs of a similar coronal brainstem section through the IVN processed for cGMP immunohistochemistry. A large number of heavily stained neuronal cell bodies as well as a rich neuropil was present in this nucleus. Scale bars = 150 μm in A,B,E–G; 25 μm in C,D.

ventromedial limit of the MVN, in an intermediate region between this nucleus and the PH nucleus (Fig. 7A,C). The cGMP-ir cell bodies were similar in size (21.7 ± 5 μm and 13.0 ± 3 μm, maximum and minimum diameters, respectively) and shape to those found in the MVN and the IVN. No nitrergic neurons were observed in this particular re-

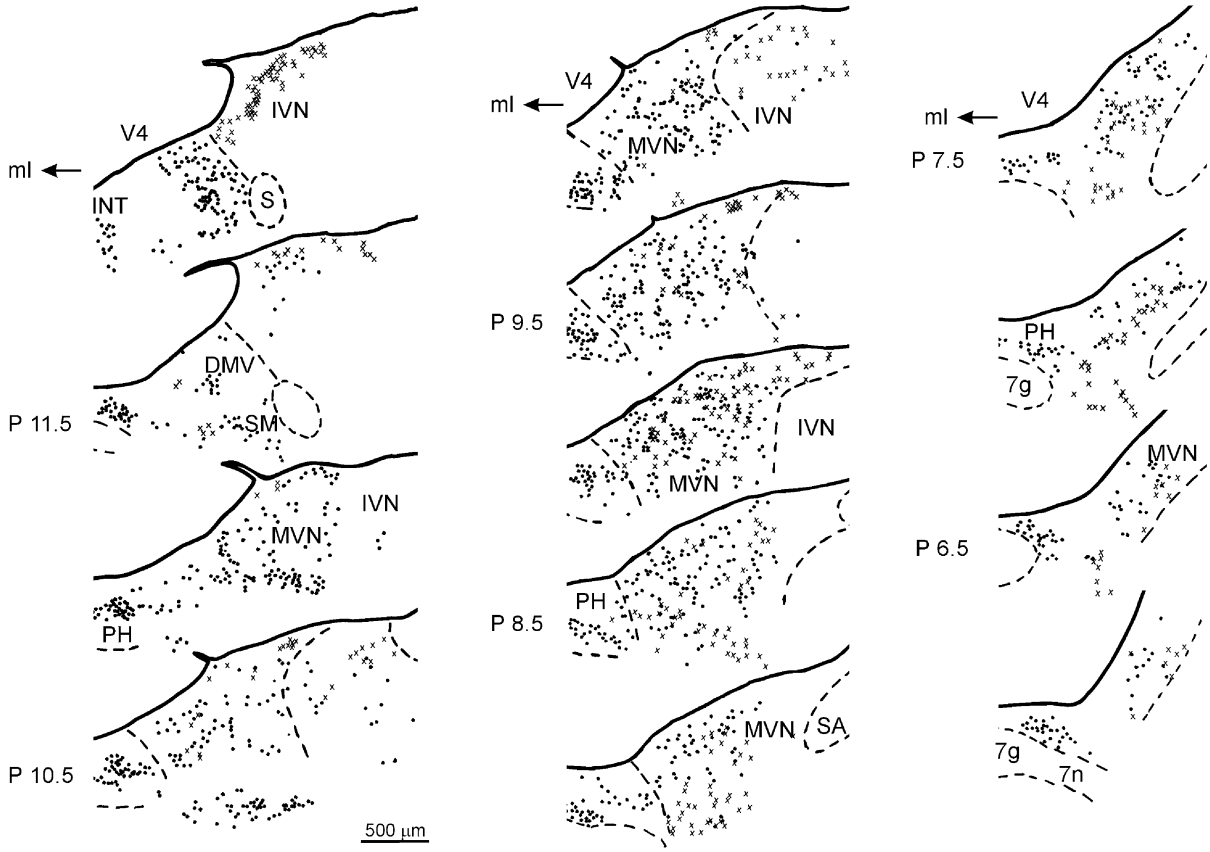

Fig. 6. Distribution of NOS I- and cGMP-immunoreactive neurons along the inferior and medial vestibular nuclei. Camera lucida drawings of two consecutive 40-μm sections, one stained for NOS I and the other for cGMP, were superimposed. Each pair of sections shown was 420 μm apart. Coordinates according to Berman (1968) are shown in alternate sections. Nitrergic neurons (dots) and cGMP-positive neurons (x) are indicated in each section. The midline (ml) direction is indicated by arrows. Note the large number of cGMP neurons and the absence of nitrergic neurons in the inferior vestibular nucleus (P11.5–12.0) and in the marginal zone (P6.5–8.0), in a ventral position, between the prepositus hypoglossi and the medial vestibular nuclei. In contrast, only nitrergic neurons appear in the most caudal region of the medial vestibular nucleus (P10.0–11.0). Both cell types were abundant in the intermediate and rostral parts of the medial vestibular nucleus (P7.0–9.0). The dashed lines indicate the borders of the different structures. 7g, facial genu; 7n, facial nerve; DMV, dorsal motor nucleus of the vagus; INT, nucleus intercalatus; IVN, inferior vestibular nucleus; MVN, medial vestibular nucleus; PH, prepositus hypoglossi nucleus; S, solitary tract; SA, stria acustica; SM, medial nucleus of the solitary tract; V4, fourth ventricle.

gion (Fig. 7B). In the rostrocaudal direction, this cGMP-ir enriched area was visible in sections between P6.5 and P8, according to Berman (1968) (Fig. 6) and could not be differentiated from the surrounding structures by cytoarchitectonic criteria, as observed with Nissl staining (Fig. 7D). Topographically, this area is equivalent to the marginal zone described in primates.

The superior colliculus presented both NOS I and cGMP-ir neurons with a clear differential distribution. Nitrergic neurons and neuropil were preferentially found in the intermediate and deep layers and cGMP-ir cell bodies and neuropil in the most superficial layer (Fig. 8A,B). The lateral geniculate nucleus contained a large number of NO-sensitive GMP-producing neuronal cell bodies and neuropil, whereas NOS I was only found in the neuropil (Fig. 8C,D). In the reticular formation, nitrergic neurons sparsely distributed all along the medulla and pontine regions were found; however, no evident groups of labeled cells were detected at the locations that have been described to project to the oculomotor nuclei (Langer et al., 1986). Some cGMP-ir cell bodies were observed, but no differences were found in animals perfused with or without SNP. Neither NOS I nor cGMP-ir was detected in the interstitial nucleus of Cajal.

Characterization of the nitrergic neurons in the PH nucleus

Injections of HRP were performed in the abducens nucleus (Fig. 9A), which is one of the projection sites of the PH nucleus (Escudero and Delgado-García, 1988), to investigate whether nitrergic PH neurons directly innervated that motor nucleus. The quantitative results appear in Table 1. Retrogradely labeled neurons were found in both PH nuclei, although they were more abundant in the contralateral side (140–180% of the ipsilateral). Neurons projecting to the ipsilateral abducens nucleus increased in number progressively from caudal to rostral sections, with most of the neurons (≈75% of the total) found in the rostral third of the PH nucleus. Contralateral projections, however, were more homogeneously distributed and predominated in the medial third of the nucleus (≈47% of the total). Double labeling with retrogradely transported HRP and NADPH diaphorase (Fig. 10A–C) showed a colocalization of both markers in 20–28% of the HRP-

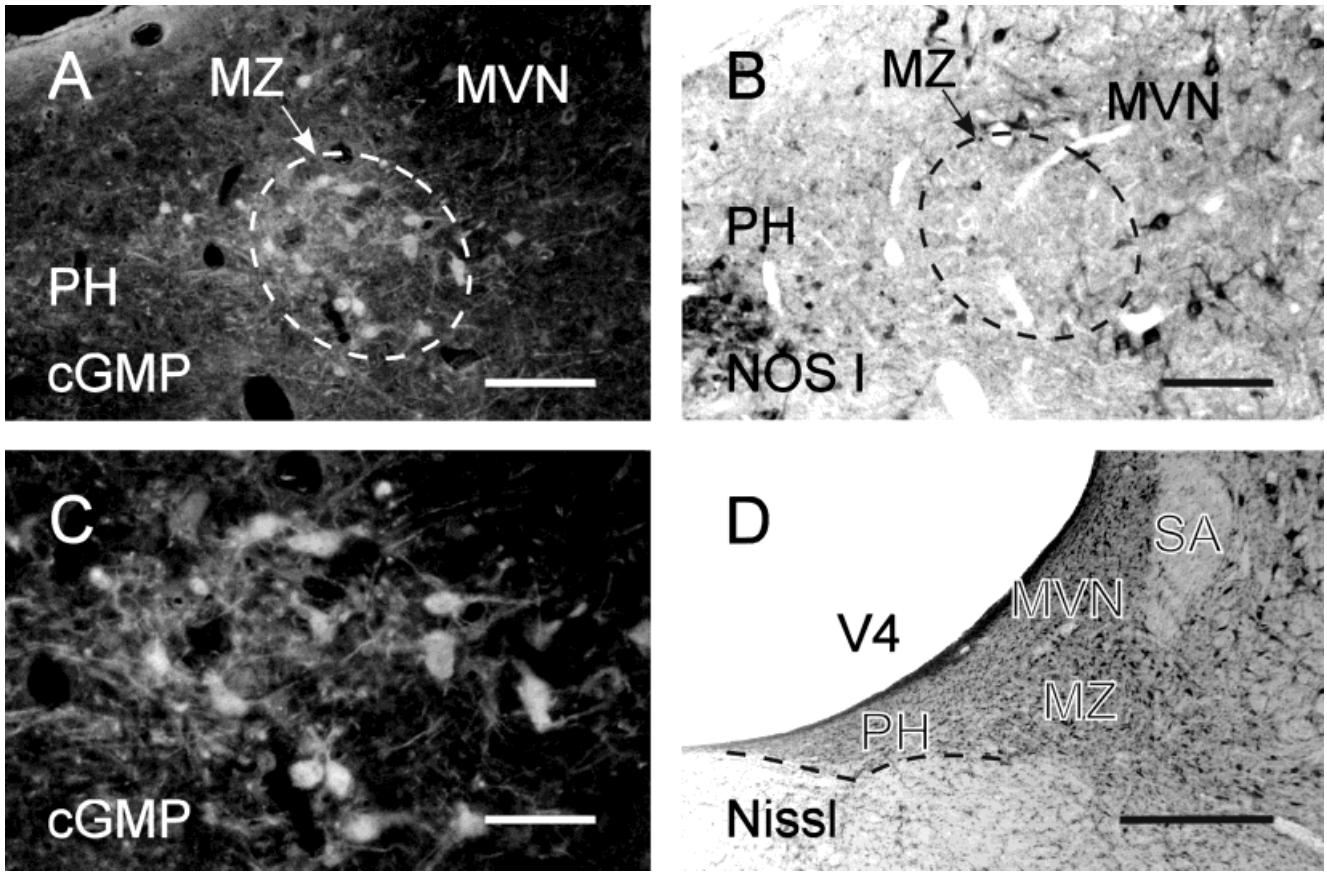

Fig. 7. Distribution of nitrergic and NO-sensitive cyclic guanosine monophosphate (cGMP)-producing structures in the marginal zone. **A,B:** Photomicrographs of the same coronal brainstem section, obtained from one cat perfused with SNP, and double labeled for cGMP; (A) and nitric oxide synthase I (NOS I; B). The marginal zone (MZ) corresponds to the intermediate region between the medial vestibular nucleus (MVN) and the prepositus hypoglossi (PH) nucleus. Note the enrichment in cGMP labeling and the absence of nitrergic neurons within this region. **C:** Higher magnification of the MZ shown in A. Neuronal cell bodies and neuropil stained for NO-sensitive cGMP are observed. **D:** Low-magnification photomicrograph showing the region of the marginal zone in a brainstem section with Nissl staining. No specific cytoarchitectonic features can be observed in this area. SA, stria acustica; V4, fourth ventricle. Scale bars = 100 μm in A,B; 50 μm in C; 500 μm in D.

containing neurons in both sides, without significant differences between the three rostrocaudal regions analyzed (Table 1). The NADPH diaphorase-positive cells projecting to the abducens nucleus represented less than 2% of the nitrergic neurons found in the PH nucleus.

Identification of possible targets for NO produced in the PH nucleus

Injections of the retrograde tracer fast blue in the PH nucleus (Fig. 9B) resulted in the appearance of labeled neuronal cell bodies in different structures including the ipsi- and contralateral PH nucleus, MVN, IVN, marginal zone, and reticular formation. The vestibular nuclei and the marginal zone were preferentially labeled in the ipsilateral side, whereas the reticular formation presented similar numbers of retrogradely labeled cell bodies in both sides. NO-sensitive cGMP-producing cells were identified among the retrogradely labeled neurons in the vestibular nuclei and the marginal zone (Fig. 10D,E). In the reticular formation a very small percentage of neurons were double labeled only in one of the injected animals. The distribution of neurons labeled with fast blue and double labeled with fast blue and cGMP-ir in each of the two injected cats is shown in Table 2. The largest number of double-labeled cells was found in the ipsilateral MVN, followed by the ipsilateral marginal zone.

DISCUSSION

The results described in this study show the anatomical distribution of NO-producing and NO-sensitive sites in the cat oculomotor system, providing a morphological substrate that may explain the functional alterations observed when NO synthesis is impaired in the PH nucleus of alert animals. In addition, we have identified for the first time the marginal zone in cats, a discrete structure that contains a high number of NO-sensitive cell bodies and that may be involved in oculomotor integration.

Two general features of the possible NO influence in the control of eye movements can be deduced from the general distribution of the NO-cGMP pathway in the oculomotor structures analyzed. First, the absence of NO-responsive structures in the motor nuclei suggests that NO does not directly modulate the activity of motoneurons, but rather acts on premotor structures, in which cGMP-ir neurons and/or neuropil were detected. Second, neurons that may

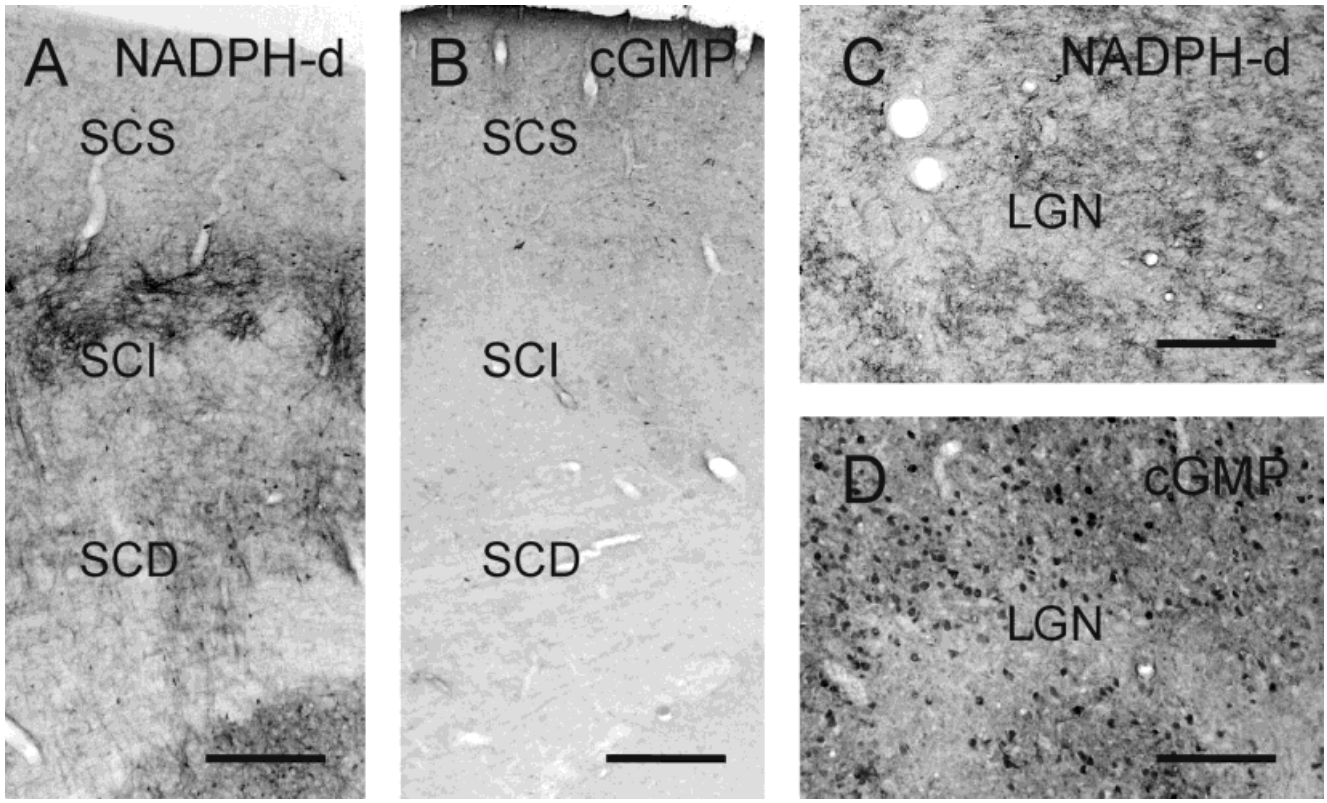

Fig. 8. Distribution of nitrergic and NO-sensitive cyclic guanosine monophosphate (cGMP)-producing structures in the superior colliculus and lateral geniculate nucleus. **A,B:** Photomicrographs of equivalent coronal brainstem sections through the superior colliculus, stained for reduced nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase histochemistry (A) and cGMP immunohistochemistry (B). Nitrergic cell bodies can be observed throughout the colliculus, whereas cGMP-containing neurons are predominantly located in the superficial layer. A heavily stained nitrergic neuropil is apparent in the intermediate layer. **C,D:** Photomicrographs of equivalent coronal brainstem sections through the lateral geniculate nucleus, stained for NADPH-diaphorase histochemistry (C) and cGMP immunohistochemistry (D). In this nucleus, an abundant nitrergic neuropil co-exists with a large number of cGMP-ir cell bodies. LGN, lateral geniculate nucleus; SCD, superior colliculus deep layer; SCI, superior colliculus intermediate layer; SCS, superior colliculus superficial layer. Scale bars = 300 μm in A,B; 150 μm in C,D.

be sources or targets of NO were preferentially found in those structures related to horizontal, rather than vertical, eye movements. Thus, nitrergic cells and neuropil were observed exclusively in the abducens, among the motor nuclei. Furthermore, the largest numbers of nitrergic neurons, together with NO-responsive neuropil, were present in the PH nucleus, which is involved in eye position generation for horizontal eye movements (Fukushima et al., 1992), whereas neither NOS I nor cGMP-ir were detected in the interstitial nucleus of Cajal, which accomplishes an equivalent function for eye movements in the vertical plane (Fukushima et al., 1992).

The functional significance of the nitrergic neurons present in the abducens nucleus is unclear. A local action of NO can be ruled out, due to the absence of cGMP-ir cells and the lack of functional alterations in eye movements when NOS inhibitors were injected within this nucleus (Moreno-López et al., 1996). Alternatively, the abducens neurons expressing NOS I might release NO in their terminals outside the nucleus. In this context, 15% of the nitrergic neurons projected to the lateral rectus muscle, because they were retrogradely labeled when HRP was injected in this muscle. The double-labeled cells constituted a small subgroup, which could be differentiated by their smaller size, compared with the rest of the HRP-labeled motoneurons. Because cGMP is produced by vascular smooth muscle cells in response to NO stimulation (Rapoport and Murad, 1983), the possibility exists that a small number of nitrergic fibers travel together with the motor nerve fibers to release NO in the proximity of the vascular smooth muscle, therefore contributing to the increased blood flow that occurs during muscle contraction. The remaining NADPH-positive neurons that did not project to the muscle may be internuclear neurons, which project to the medial rectus subdivision of the oculomotor nucleus (Delgado-García et al., 1977; McCrea et al., 1986). Although the oculomotor nucleus is devoid of nitrergic neuropil or NO-sensitive cGMP-producing structures, putative nitrergic internuclear interneurons may contribute to the nitrergic neuropil present in the PH nucleus, because part of the internuclear neurons send collaterals to the PH nucleus (Highstein et al., 1982; McCrea et al., 1986). However, because the caudal limits of the abducens nucleus are difficult to establish, we cannot rule out the possibility that the nitrergic neurons found in planes posterior to P6.8 may correspond to the pontine reticular formation.

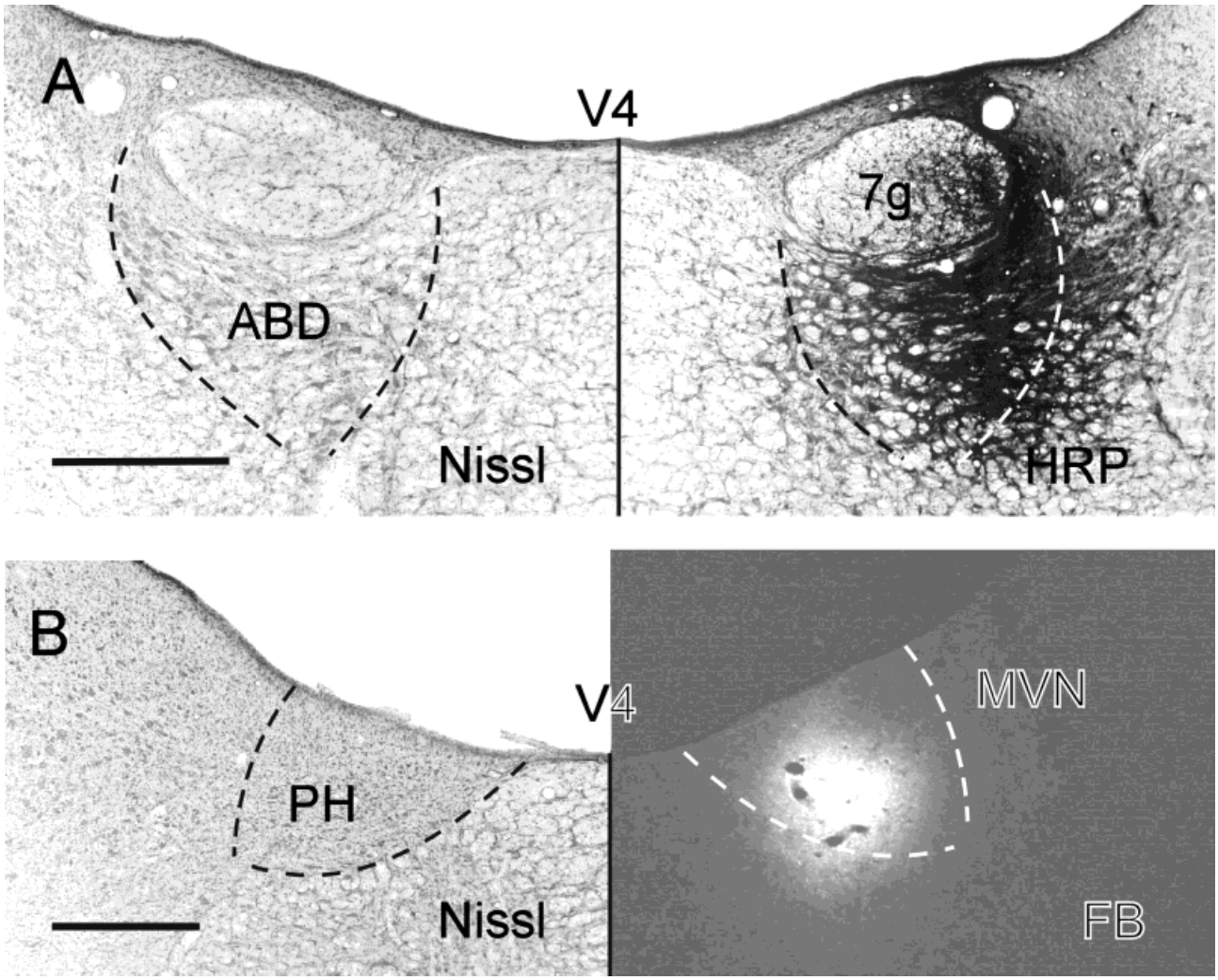

Fig. 9. Injection sites of the retrograde tracers horseradish peroxidase (HRP) and fast blue (FB). **A:** Nissl staining of a brainstem coronal section through the abducens nucleus (ABD), showing the HRP injection site in the right side and the lateral and medial limits of the nuclei (dashed lines). The animal was injected with HRP 48 hours before perfusion, as indicated in Materials and Methods. **B:** Composition of two photomicrographs of the same brainstem plane showing the medial and lateral limits of the prepositus hypoglossi (PH) nucleus (dashed lines) in a Nissl-stained section (left), and the site where FB was injected 5 days before perfusion (right). MVN, medial vestibular nucleus; 7g, facial genu; V4, fourth ventricle. Scale bars = 500 μm.

TABLE 1. Rostrocaudal Distribution of PH Neurons and Nitrergic PH Neurons, Projecting to the Abducens Nucleus in the Cat[1]

		HRP				HRP+NADPH-d			
		No. of neurons		% of total		No. of neurons		% of total	
Region	Cat no.	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral
Caudal	9	4	70	2.1	21.1	1	12	1.7	15.4
	10	5	81	1.8	21.3	1	20	1.8	22.9
Central	9	38	161	20.3	48.6	10	44	16.7	56.4
	10	65	177	24.0	46.5	17	45	32.0	51.7
Rostral	9	145	100	77.5	30.2	49	22	81.7	28.2
	10	200	122	74.0	32.1	45	22	85.0	25.3
Total	9	187	331			60	78		
	10	270	380	100		63	87	100	

[1]Two animals were injected with HRP in one abducens nucleus. Two days later the animals were perfused, the brainstem sections were stained for HRP, and nitrergic neurons were identified by NADPH-diaphorase histochemistry. One of three 40-μm brainstem sections were used for the analysis. PH, prepositus hypoglossi; HRP, horseradish peroxidase; NADPH-d, reduced nicotinamide adenine dinucleotide phosphate-diaphorase.

The largest density of nitrergic neuronal cell bodies appeared in the PH nucleus, together with an abundant neuropil. The presence of a large number of nitrergic neurons in the PH nucleus from rats and cats has been previously reported (Mizukawa et al., 1989; Vincent and Kimura, 1992; Rodrigo et al., 1994; Moreno-López et al.,

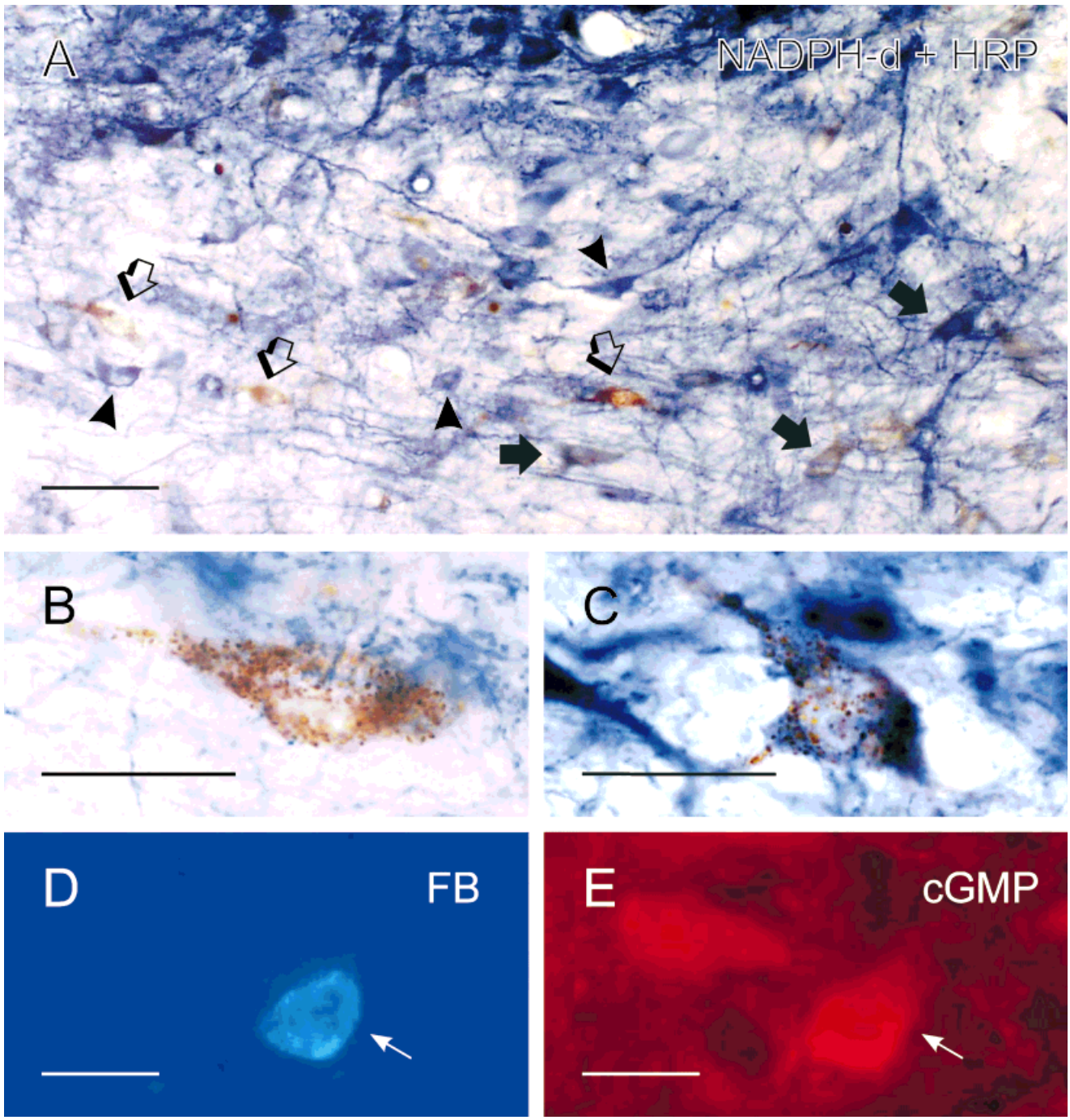

Fig. 10. Photomicrographs of retrogradely labeled neurons in the prepositus hypoglossi and medial vestibular nuclei. **A–C:** Reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase staining of coronal sections of the prepositus hypoglossi nucleus from animals that were injected with horseradish peroxidase (HRP) in the abducens nucleus 2 days before perfusion. Nitrergic neurons (arrowheads), HRP-positive neurons (open arrows), and double-labeled neurons (solid arrows) are observed in A. Examples of a non-nitrergic (B) and a nitrergic (C) neuron, both containing HRP, are shown at higher magnification. **D,E:** High-magnification photomicrographs of a coronal section through the medial vestibular nucleus of an adult cat that was perfused 5 days after injection of fast blue (FB) in the ipsilateral PH nucleus. A neuron containing fast blue (D) and positive for cGMP immunohistochemistry (E) is indicated by the arrow. Scale bars = 150 μm in A; 15 μm in B–E.

1996). Nitrergic neurons in the PH nucleus constituted an heterogeneous group that, according to their morphology, with oval or multipolar shapes, share some characteristics with the "principal cells" described by McCrea and Baker (1985). However, nitrergic neurons were smaller (<20 μm) than the average size of PH neurons reported by these authors. Also, the distribution pattern of the nitrergic neurons in the PH nucleus does not correlate well

TABLE 2. Neurons Projecting to the PH Nucleus and Producing cGMP in Response to NO Stimulation in Different Brainstem Structures Related to Eye Movements

		FB		FB + CGMP-ir			
		Neurons counted		No. of neurons		% FB	
Structure	Cat no.	Ipsilateral	Contralateral	Ipsilateral	Contralateral	Ipsilateral	Contralateral
Reticular formation	36	308	330	10	7	3.2	2.1
	37	185	192	2	0	1.1	0
PH nucleus	36	140	203	0	0	0	0
	37	60	213	0	0	0	0
MVN	36	209	88	15	6	7.2	6.8
	37	248	174	32	5	12.9	2.9
MZ	36	115	11	7	4	6.1	36.3
	37	91	21	9	1	9.9	4.7
IVN	36	33	22	4	5	12.1	22.7
	37	32	20	4	2	12.5	10.0

Animals were injected with the retrograde tracer fast blue (FB) in one prepositus hypoglossi (PH) nucleus. Five days later, brainstem sections were stained by cyclic guanosine monophosphate (cGMP) immunohistochemistry (cGMP-ir), as described in Materials and Methods. Five series of 40-μm brainstem sections were obtained from each cat. One series from each cat was counted for the reticular formation and PH nucleus. Two series from each cat were counted for the rest of the structures. Injection in cat #37 was small and circumscribed to the rostral part of the PH nucleus, whereas injection in cat #36 was slightly larger and affected the most medial border of the medial vestibular nucleus. IVN, inferior vestibular nucleus; MVN, medial vestibular nucleus; MZ, marginal zone.

with the regions identified by cytoarchitectonic criteria. According to McCrea and Baker (1985), neurons in the caudal PH are distributed in a dorsolateral group of small cells and a ventromedial group of large cells. In this region, nitrergic neurons were also organized in two groups of slightly different sizes, but the medial group was in a dorsal position in relation to the lateral one.

The severe nystagmic eye movements observed upon NOS blockade in the PH nucleus of alert cats can be explained by a local action of NO produced by PH neurons. The absence of neuronal cell bodies able to increase their cGMP concentration in response to NO rules out a cell-cell interaction between PH neurons. Hence the unique target for NO within the PH nucleus is the cGMP-ir neuropil observed in the most dorsal part, which necessarily originates outside the nucleus. The neurons giving rise to this neuropil were investigated by injecting the retrograde tracer fast blue in the PH nucleus and by identifying cGMP-ir neuronal cell bodies among those retrogradely labeled. Following this strategy, the possible NO targets were found in the vestibular nuclei and the marginal zone, predominantly in the ipsilateral side. The double-labeled cells found in only one cat in the reticular formation and in the contralateral marginal zone and vestibular nuclei were probably due to some penetration of the FB injection into the medial border of the adjacent MVN in this animal.

In addition to the local action of NO in the PH nucleus, a possible release of NO in the projection sites of PH nitrergic neurons cannot be discounted. The presence of NOS I in approximately 25% of the neurons projecting to the abducens nucleus probably has little functional significance because, as was mentioned above, the extraocular motor nuclei are devoid of NO-sensitive neural elements, and no functional changes were detected upon NOS blockade in the abducens nucleus (Moreno-López et al., 1996). Furthermore, the neurons expressing NOS I and projecting to the abducens nucleus constituted a very small percentage of the nitrergic neurons in the PH nucleus. Release of NO by PH neurons in some of their other main projection areas, such as the vestibular nuclei and/or the cerebellum (McCrea and Baker, 1985; Escudero et al., 1996) may also occur, because these regions contain NO-responsive cells (de Vente et al., 1989; Southam et al., 1992; and present results). Although chemical lesions (Godaux et al., 1993) or electrical stimulation (Yokota et al., 1992) of the medial vestibular nuclei produced nystagmus, the functional effects of NOS blockade in these structures has not been investigated.

A condition opposite to that observed in the PH nucleus, this is, the presence of a dense group of NO-sensitive cGMP containing cells and the absence of neurons expressing NOS I, was found in an intermediate area between the PH and the medial vestibular nucleus. This area, which cannot be identified by cytoarchitectonic criteria in the absence of cGMP staining, has a location equivalent to the marginal zone described in primates (Langer et al., 1986; Belknap and McCrea, 1988). Functional experiments have identified neurons within this area that fire with eye position (McFarland and Fuchs, 1992), and therefore the primate marginal zone has been proposed as part of the integrator controlling eye movements (Kaneko, 1997). We have recently reported that this NO-responsive region may also have a role in the generation of the eye position after saccadic eye movements in the cat, based on the alterations in eye position observed when NO donors were injected in the proximity of these neurons (Moreno-López et al., 1998). However, the physiological significance of these results is not clear, because the marginal zone is devoid of nitrergic neurons, and the closest source of NO, the nitrergic PH neurons, is relatively distant for NO to reach the marginal zone cell bodies at adequate concentration. A NO-cGMP interaction may occur in the projection areas of the marginal zone neurons, if they innervate nitrergic cells able to activate soluble guanylyl cyclase retrogradely. According to our retrograde tracer data, one of these interaction sites might be the PH nucleus, which has been shown to receive inputs from the marginal zone in primates (Belknap and McCrea, 1988).

Other possible sites of action of the NO-cGMP pathway in the oculomotor system are the vestibular nuclei and the superior colliculus, where both NOS and cGMP-ir neuronal cell bodies were observed. In the MVN, a moderate number of both types of neurons was found, with a slightly different distribution along the rostrocaudal axis, whereas in the inferior vestibular nucleus, NO-responsive cells predominated. Finally, the disposition of NO-producing and NO-responsive elements in the LGN suggests that in this structure NO may act as an anterograde messenger on the neuronal cell bodies containing cGMP.

Knowledge of the anatomical distribution of the possible NO sources and targets in the oculomotor system may

help to elucidate the role of NO in eye movement control. The finding that the MVN and the marginal zone contain NO-responsive neurons projecting to the ipsilateral PH nucleus suggests the hypothesis that NO produced by PH neurons acts as a retrograde messenger on vestibular afferents. This mechanism of action explains the physiological effect of NO in the control of eye movements observed in alert animals.

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