THE JOURNAL OF COMPARATIVE NEUROLOGY 377:149–164 (1997)

Distribution of GABA, Glycine, and Glutamate Immunoreactivities in the Vestibular Nuclear Complex of the Frog

I. REICHENBERGER,[1,*] H. STRAKA,[1] O.P. OTTERSEN,[2] P. STREIT,[3] N.M. GERRITS,[4] AND N. DIERINGER[1]

[1]Department of Physiology, University of Munich, D-80336 Munich
[2]Department of Anatomy, Institute of Basic Medical Sciences, University of Oslo, Blindern, N-0317 Oslo 3, Norway
[3]Brain Research Institute, University of Zürich, CH-8029 Zürich, Switzerland
[4]Department of Anatomy, Erasmus University, 3000 DR Rotterdam, The Netherlands

ABSTRACT

This study describes the localization of γ-aminobutyric acid (GABA), glycine, and glutamate immunoreactive neurons, fibers, and terminal-like structures in the vestibular nuclear complex (VNC) of the frog by using a postembedding procedure with consecutive semithin sections at the light microscopic level. For purposes of this study, the VNC was divided into a medial and a lateral region.

Immunoreactive cells were observed in all parts of the VNC. GABA-positive neurons, generally small in size, were predominantly located in the medial part of the VNC. Glycine-positive cells, more heterogeneous in size than GABA-positive cells, were scattered throughout the VNC. A quantitative analysis of the spatial distribution of GABA or glycine immunoreactive cells revealed a complementatory relation between the density of GABA and glycine immunoreactive neurons along the rostrocaudal extent of the VNC. In about 10% of the immunolabeled neurons, GABA and glycine were colocalized. Almost all vestibular neurons were, to a variable degree, glutamate immunoreactive, and colocalization of glutamate with GABA and/or glycine was typical. GABA, glycine, or glutamate immunoreactive puncta were found in close contact to somata and main dendrites of vestibular neurons. A quantitative analysis revealed a predominance of glutamate-positive terminal-like structures compared to glycine or GABA containing profiles. A small proportion of terminal-like structures expressed colocalization of GABA and glycine or glycine and glutamate.

The results are compared with data from mammals and discussed in relation to vestibulo-ocular and vestibulo-spinal projection neurons, and vestibular interneurons. GABA and glycine are the major inhibitory transmitters of these neurons in frogs as well as in mammals. The differential distribution of GABA and glycine might reflect a compartmentalization of neurons that is preserved to some extent from the early embryogenetic segmentation of the hindbrain. J. Comp. Neurol. 377:149–164, 1997.

Indexing terms: immunocytochemistry; amino acids; colocalization; neurotransmitter; comparative aspects

The termination field of afferent fibers from the vestibular and auditory sense organs of the labyrinth defines the vestibulo-auditory area in the dorsal alar plate of the brainstem of frogs. Several nuclei have been distinguished within this vestibulo-auditory area according to cytoarchitecture and afferent input from individual end organs (Larsell, 1934; Gregory, 1972; Hillman, 1972; Opdam et al., 1976; Matesz, 1979; Kuruvilla et al., 1985; Montgomery, 1988). However, the descriptions of the various authors differ with respect to location, extent, subdivision, and nomenclature. The discrepancies are largely due to the low neuronal density and the small differences in

Contract grant sponsor: Deutsche Forschungsgemeinschaft; Contract grant number: Di 205/7-1

*Correspondence to: Dr. I. Reichenberger, Physiologisches Institut der Universität München, Pettenkoferstrasse 12, D-80336 München, Germany. E-mail: u7224ag@sunmail.LRZ-muenchen.de

Received 22 September 1995; Revised 4 September; Accepted 13 September 1996

neuronal size and shape that make the borders between subnuclei difficult to define. Since *Rana temporaria* has a similar nuclear composition as *Rana catesbeiana*, we favor the nomenclature of Kuruvilla et al. (1985). These authors distinguished six nuclear groups in the vestibulo-auditory area of the bullfrog based on location, cytoarchitectonical features, and afferent innervation pattern: dorsal (acoustic) nucleus, cerebellar nucleus, ventral (lateral) vestibular nucleus (VVN), medial vestibular nucleus (MVN), descending vestibular nucleus (DVN), and superior vestibular nucleus (SVN). Each of the four vestibular nuclei receives information from all vestibular end organs and is assumed to be the equivalent of the vestibular nuclei of mammals (see also Matesz, 1979).

Additional synaptic inputs known to converge upon central vestibular neurons of frogs originate from cerebellar Purkinje cells (Stern and Rubinson, 1971; Magherini et al., 1975), from contralateral vestibular neurons via commissural fibers (Fuller, 1974; Ozawa et al., 1974; Cochran et al., 1987; Montgomery, 1988), from different levels of the spinal cord (Precht et al., 1974; Antal et al., 1980), and from ipsilateral reticulo-vestibular neurons (Straka and Dieringer, 1996). Major vestibular pathways project to the cerebellum, the ocular motor nuclei, and the spinal cord (Fuller, 1974; Magherini et al., 1974; Grover and Grüsser-Cornehls, 1984; Tóth et al., 1985; Montgomery, 1988). These projections of vestibular neurons of frogs are similar to the patterns described for mammals (see Gerrits, 1990).

A comparison of results from pharmacological, biochemical, and immunocytochemical studies in frogs with those obtained in mammals (see de Waele et al., 1995) shows similarities as well as differences in the organization of vestibular reflex pathways. Afferent vestibular nerve input is mediated by glutamate or a related substance via glutamate receptors of the NMDA and non-NMDA subtypes in frogs (Cochran et al., 1987; Reichenberger and Dieringer, 1994; Straka et al., 1996a,b) and rats (Kinney et al., 1994). Abducens motoneurons and internuclear neurons are inhibited by uncrossed, glycinergic vestibular neurons in frog as well as in cat (Straka and Dieringer, 1993; Spencer et al., 1989). Evidence for an inhibitory, GABAergic cerebellar Purkinje cell input to the vestibular nuclear complex as reported in mammals (see Ito, 1984; Ottersen and Storm-Mathisen, 1984a) was also found in frogs (Dieringer and Precht, 1979; Reichenberger et al., 1993). With respect to commissural inhibition between bilateral canal-related vestibular neurons as described in the cat (Shimazu and Precht, 1966), studies in the frog have yielded different results. Whereas Ozawa et al. (1974) report an absence of commissural inhibition, Dieringer and Precht (1979) showed in chronic hemilabyrinthectomized frogs crossed inhibition in approximately 30% of the vestibular neurons recorded on the operated side. Shorter latency inhibitory responses persisted after cerebellectomy but could be blocked by application of picrotoxin, suggesting a GABAergic connection via the brainstem. Therefore, commissural inhibition may be present in frogs but probably in a weaker form than in the cat.

The general lack of information concerning location and distribution of inhibitory vestibular interneurons and projection neurons in frogs prompted us to study the number and distribution of GABA and glycine immunoreactive vestibular neurons. Glutamate immunoreactivity was studied simultaneously, since a previous study has shown a consistent colocalization of this amino acid with glycine in vestibular nerve fibers (Reichenberger and Dieringer, 1994).

Preliminary results of this investigation have been published in abstract form (Reichenberger et al., 1992).

MATERIALS AND METHODS

Tissue preparation

Five adult frogs (*Rana temporaria*) were anaesthetized with 0.1% 3-aminobenzoic acid ethyl ester (MS 222) and perfused transcardially with a modified Ringer solution (75 mM NaCl, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM KCl, 0.5 mM $MgCl_2$, and 11 mM glucose; pH 7.4; 5 ml), followed by a mixture (40 ml) of 2.5% glutaraldehyde and 0.5% formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M phosphate buffer (PB; pH 7.4). The brains were removed, postfixed for 2 hours and kept in PB overnight. The brainstems were osmicated (1% OsO_4), dehydrated in increasing concentrations of ethanol and propylene oxide, and embedded in epoxy resin (Epon 812) at 60°C for 72 hours.

Antibody characterization

Serial semithin sections (0.5–1 μm) were cut transversely with a diamond knife and mounted on gelatinized slides. Three sets of serial sections were stained with antibodies characterized in earlier reports: monoclonal anti-GABA antibodies (mAb 3A12; Matute and Streit, 1986), polyclonal anti-glycine antibodies (No. 290; Kolston et al., 1992), or monoclonal anti-glutamate antibodies (mAb 2D7; Liu et al., 1989). The antibodies had a high selectivity, and only the glycine antiserum exhibited a weak cross-reactivity with conjugated β-alanine. All antibodies have been tested previously in frog material (Reichenberger et al., 1993; Reichenberger and Dieringer, 1994).

Postembedding immunocytochemistry

The immunostaining was carried out according to the procedure described by Liu et al. (1989). Briefly, the sections were etched with a mixture of potassium hydroxide, methanol, and propylene oxide, washed with methanol and methanol/0.1 M potassium phosphate buffered saline (KPBS, pH 7.4), treated with 1% sodium periodate, incubated with 0.1 M sodium borohydride (this step was skipped for glutamate immunocytochemistry), and then washed in KPBS. Subsequently, the sections were preincubated with 0.5% ovalbumine in KPBS and incubated with the primary antibodies (for dilution see below) overnight at 4°C. After rinsing in KPBS, the slides were incubated with goat anti-rabbit IgG (1:100, DAKO) for glycine and goat anti-mouse IgG (1:100, P. Streit) for GABA and glutamate, and rinsed again in KPBS, incubated in rabbit peroxidase-antiperoxidase complex (1:100, DAKO) and mouse peroxidase-antiperoxidase complex (1:750, P. Streit), respectively. Before the last two steps the slides were preincubated with 10% bovine serum albumin (BSA). The peroxidase was detected with 0.05% diaminobenzidine and 0.01% H_2O_2 and the diaminobenzidine reaction product was silver intensified and gold substituted (0.05% gold chloride).

All antibodies were diluted in 10% BSA: anti-GABA antibodies 1:2,000, anti-glycine antibodies 1:1,000, and anti-glutamate antibodies 1:12,000. To ensure a high

selectivity the anti-glycine antiserum was preincubated with glutaraldehyde conjugates of structurally similar amino acids (β-alanine, GABA, glutamate) for 18 hours prior to use.

Controls

As a positive control, sections of the vestibular nuclear complex were immunostained together with cerebellar sections, known to contain neurons that are immunopositive for the tested anti-GABA or anti-glycine antibodies (Reichenberger et al., 1993). As a negative control the staining was blocked by adding to the antiserum glutaraldehyde conjugates of those amino acids against which the antibody had been raised. Preabsorption of the antibodies with glutaraldehyde complexes of other amino acids had no effect, except that preincubation of the glycine antiserum with β-alanine-glutaraldehyde decreased the staining intensity. As an additional control for the specificity tissue sections were incubated together with semithin test sections containing different amino acids, fixed by glutaraldehyde to rat brain macromolecules (Ottersen, 1987). Each antibody reacted specifically with its respective amino acid conjugate. Cell bodies and fibers were considered to be immunonegative if their staining was very weak with respect to known GABA- or glycine-positive structures.

Quantitative analysis

Immunoreactive structures were investigated in transverse sections of the vestibular nuclear complex. Four out of five animals were used for quantification of GABA and glycine immunoreactive neurons, in order to detect regional differences in their distribution. The number of GABA and glycine immunoreactive cell bodies in these animals was determined in 28 different rostro-caudal levels, each separated by 80 µm. At each level 18 consecutive semithin sections were used for GABA, glycine, or glutamate immunocytochemistry, and mean values of GABA and/or glycine immunoreactive cell bodies per section were estimated. Neurons in the medial and lateral parts of the VNC were counted separately.

The colocalization of amino acids in cell bodies, and in terminal-like structures or fibers, was investigated in 1 µm and 0.5 µm thick consecutive sections, respectively.

RESULTS

The distribution of GABA, glycine, and glutamate immunoreactivities in the vestibular nuclear complex (VNC) was investigated over its rostro-caudal extent, stretching from the nucleus cerebelli to the level of exit of N.XI. Since a clear delineation of individual vestibular nuclei is already difficult in frozen sections and practically impossible in the semithin sections used in the present study, the VNC was subdivided into two larger areas that could be distinguished more readily on the basis of their cyto- and myelo-architecture, i.e. a medial (mVNC) and a lateral (lVNC) part of the VNC (Figs. 1, 2). The mVNC borders the fourth ventricle (Fig. 1A,B), contains mainly small neurons (8–15 µm) and comprises the MVN and the medial part of the DVN of Kuruvilla et al. (1985). The mVNC is separated from the lVNC by the medial extent of the internal arcuate fibers leaving or entering the vestibulo-auditory area (Fig. 1C,D). The lVNC was further characterized by the presence of medium-sized (≤ 30 µm) and large (> 30 µm) neurons in addition to numerous small ones (Fig. 1A,B). The lVNC comprises the VVN, the SVN, and the lateral part of the DVN of Kuruvilla et al. (1985). Dorsally, the VNC was delimited by the dorsal (acoustic) nucleus. The ventral border is at the level of the sulcus limitans of His. The dorsal and ventral borders of the VNC are clearly illustrated by the extent of the terminal field of the anterior branch of the N.VIII (Fig. 1E,F).

GABA immunoreactive cells

Brainstem sections were processed for GABA immunoreactivity and analyzed quantitatively. Most GABA immunoreactive (GABA-IR) neurons were found in the rostral and caudal parts of the mVNC (Figs. 2A, 3A, 4A), predominantly in the ventral aspect. Only few GABA-IR neurons were observed in the intermediate part of mVNC, between the caudal aspect of the entry of N.VIII and the entry of N.IX (Fig. 4A). The lVNC also contained few GABA-IR neurons, present throughout its entire extent (Figs. 2A,B, 4B, 5A). GABA-IR neurons were always small (8–15 µm) and homogeneously stained (Fig. 3A).

Glycine immunoreactive cells

Consecutive sections from the same brainstems were processed for glycine immunoreactivity. Glycine immunoreactive (Gly-IR) neurons were numerous in both the mVNC and the lVNC, but their density differed along the rostro-caudal axis of VNC (Figs. 2C,D, 3B, 4C,D, 5B). Gly-IR neurons were almost uniformly distributed in the mVNC (Fig. 4C), whereas in the lVNC the Gly-IR neurons were found predominantly caudal to the entry of N.VIII (Fig. 4D). Gly-IR neurons were more heterogeneous in size than GABA-IR cells (Figs. 3A,B, 5B) with a diameter ranging from 8 to 50 µm. The cytoplasm and the nucleus of Gly-IR somata were homogeneously labeled (although with various intensity), but the nucleoli always remained unlabeled. Some of the large neurons in lVNC (Deiters' neurons) exhibited Gly-IR (Fig. 2D), but the staining intensity was weak compared to that of smaller neurons.

Averaged per level over the four individuals used for quantification, the total number of GABA-IR and Gly-IR neurons did not differ significantly (Fig. 4E) but the spatial distribution of both types showed a complementary distribution over the rostrocaudal extent of the VNC. GABA-IR neurons predominated in the rostral one third of the VNC and Gly-IR neurons in the intermediate one third. The caudal one third of the VNC contained about equal numbers of Gly-IR and GABA-IR neurons.

Glutamate immunoreactive cells

Glutamate immunoreactivity was observed in almost all neurons of the VNC (Fig. 2E,F) with a wide range of staining intensities (Figs. 3C, 5C). Nuclei were often more strongly labeled than the cytoplasm, but the nucleolus remained unstained. Very few neurons contained a low level of glutamate immunoreactivity and were therefore considered as immunonegative. In particular, the immunolabeling revealed a narrow band of more or less strongly glutamate immunoreactive (Glu-IR) neurons at the transition between the dorsal (acoustic) nucleus and the mVNC (Fig. 6A,B). This band was present between the entries of N.IX and N.XI, and consisted of a row of 10–25 neurons with a dorsoventral extent of about 200 µm. A few of these neurons were also Gly-IR (Fig. 6C) or GABA-IR (Fig. 6D).

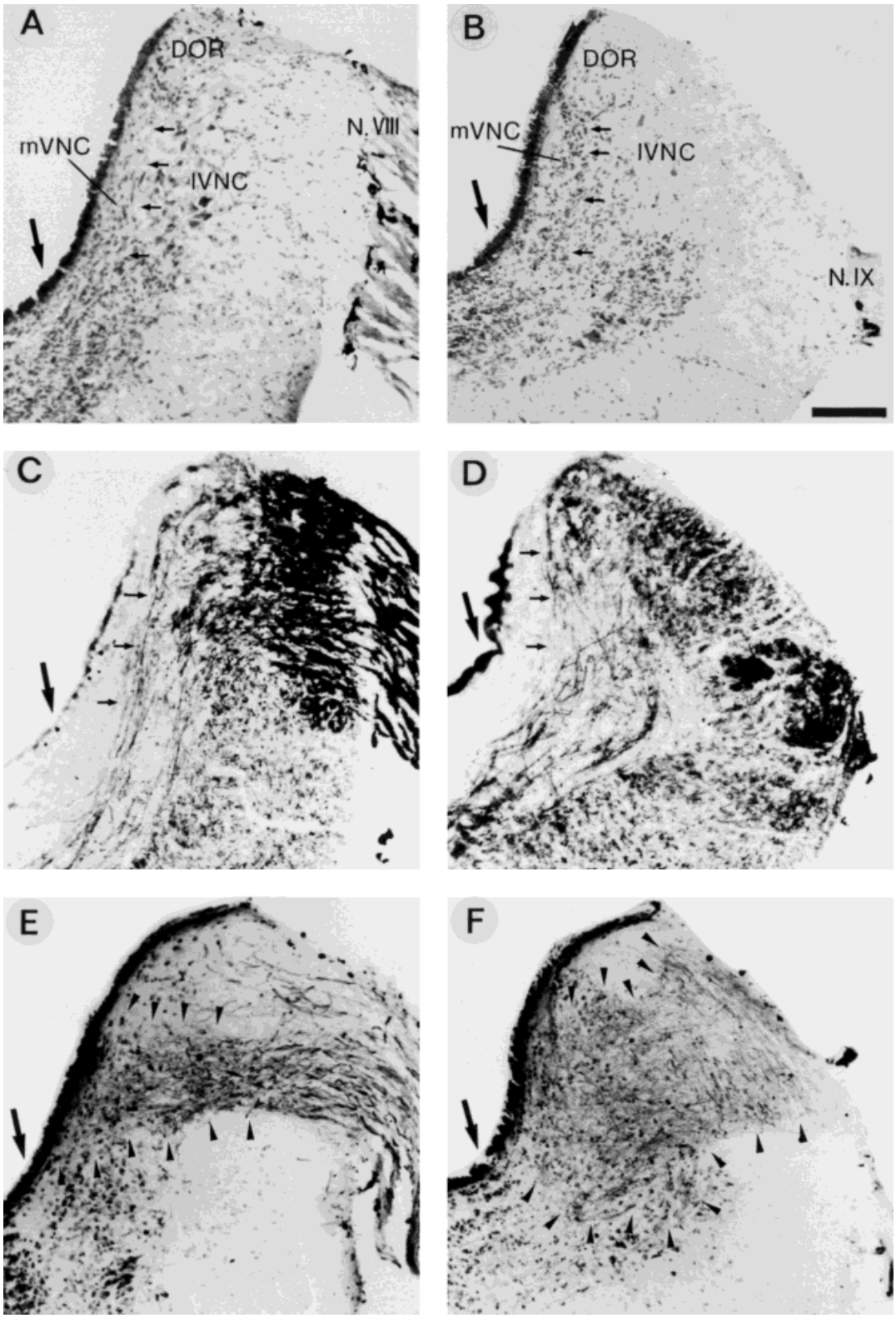

Fig. 1. Identification of the medial (mVNC) and the lateral (lVNC) part of the vestibular nuclear complex on the basis of cyto- and myeloarchitecture, and vestibular nerve labeling. Photomicrographs show transverse sections (50 µm) through the vestibulo-auditory area in the brainstem at the level of N.VIII (A,C,E) and more caudally at the level of N.IX (B,D,F). **A,B:** Distribution of neurons as revealed by Nissl-staining. Note the difference in cell size between mVNC and lVNC. **C,D:** Location of fibers as revealed by myelin-staining. The medial extent of the internal arcuate fibers delineates the mVNC from the lVNC (small arrows, in A–D). **E,F:** Termination field of vestibular nerve fibers labeled by anterograde transport of horseradish peroxidase from the anterior branch of N.VIII and subsequent diaminobenzidine treatment. The termination field defines the dorsal and ventral borders of the vestibular nuclear complex (arrowheads). DOR, dorsal (acoustic) nucleus. Large arrows point at the sulcus limitans of His. Scale bar = 190 µm.

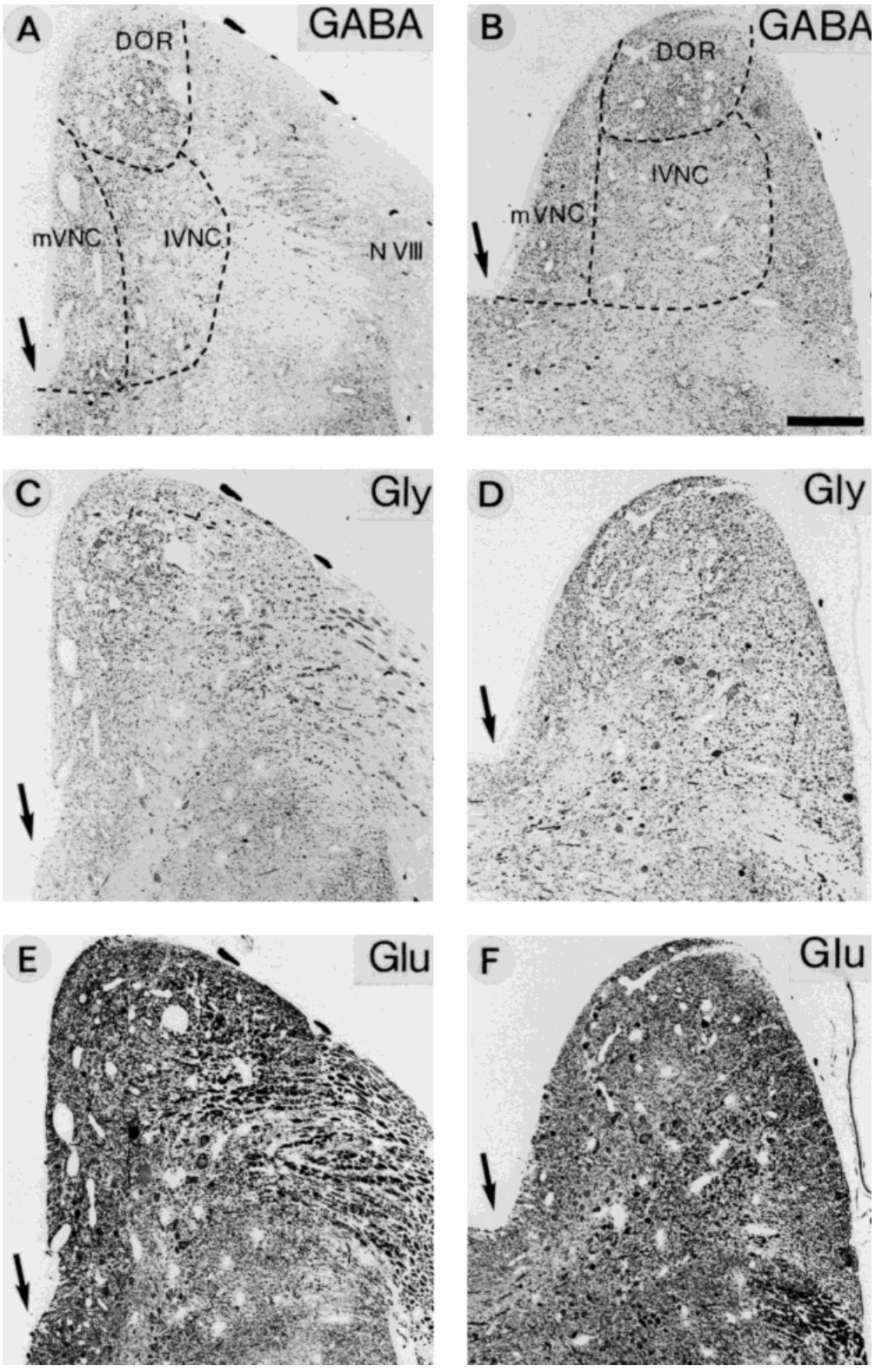

Fig. 2. Photomicrographs of the vestibulo-auditory area in the brainstem. Consecutive, transverse semithin sections through the brainstem at the level of N.VIII (**A,C,E**) and at a level 720 µm more caudally (**B,D,F**) were treated for GABA (A,B), glycine (C,D), or glutamate (E,F). Note glycine- and glutamate-positive fibers in the N.VIII (C,E), and glycine-positive large neurons in the lateral part of the vestibular nuclear complex (lVNC) (D). DOR, dorsal (acoustic) nucleus; mVNC, medial part of the vestibular nuclear complex. Arrows point at the sulcus limitans of His. Scale bar = 150 µm.

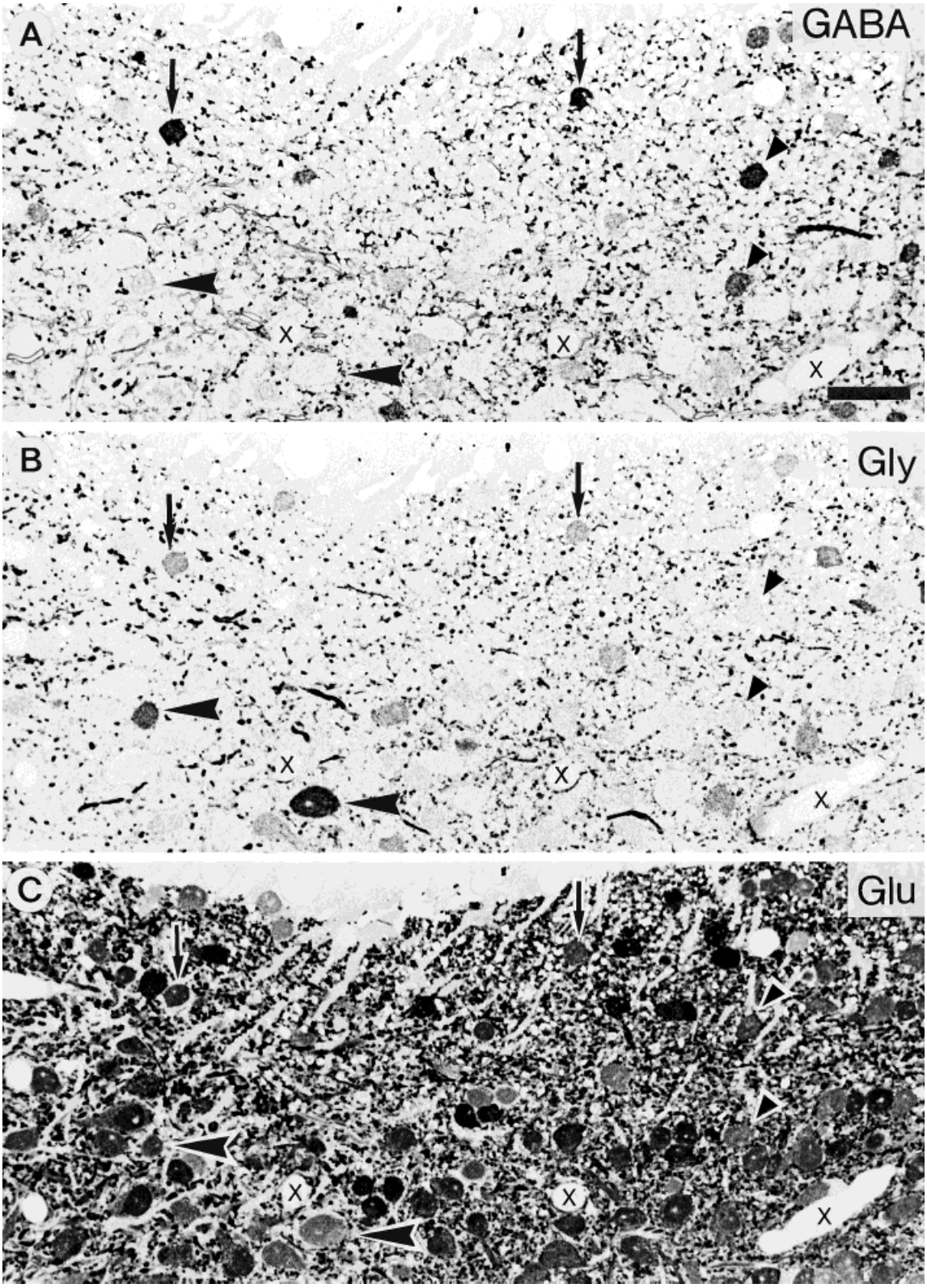

Fig. 3. Photomicrographs of labeling in the medial part of the VNC. Consecutive sections were treated with antibodies against GABA (**A**), glycine (**B**), or glutamate (**C**). Some of the neurons are immunostained either for GABA (small arrowheads), glycine (large arrowheads), or both amino acids (arrows). All neurons are more or less intensely stained for glutamate, but GABA and glycine immunoreactive cells are always among the weaker glutamate immunostained neurons. Ependymal cells (on top of each section) are free of immunolabeling. Some blood vessels are marked by crosses. Medial is up and dorsal is to the left. Scale bar = 30 µm.

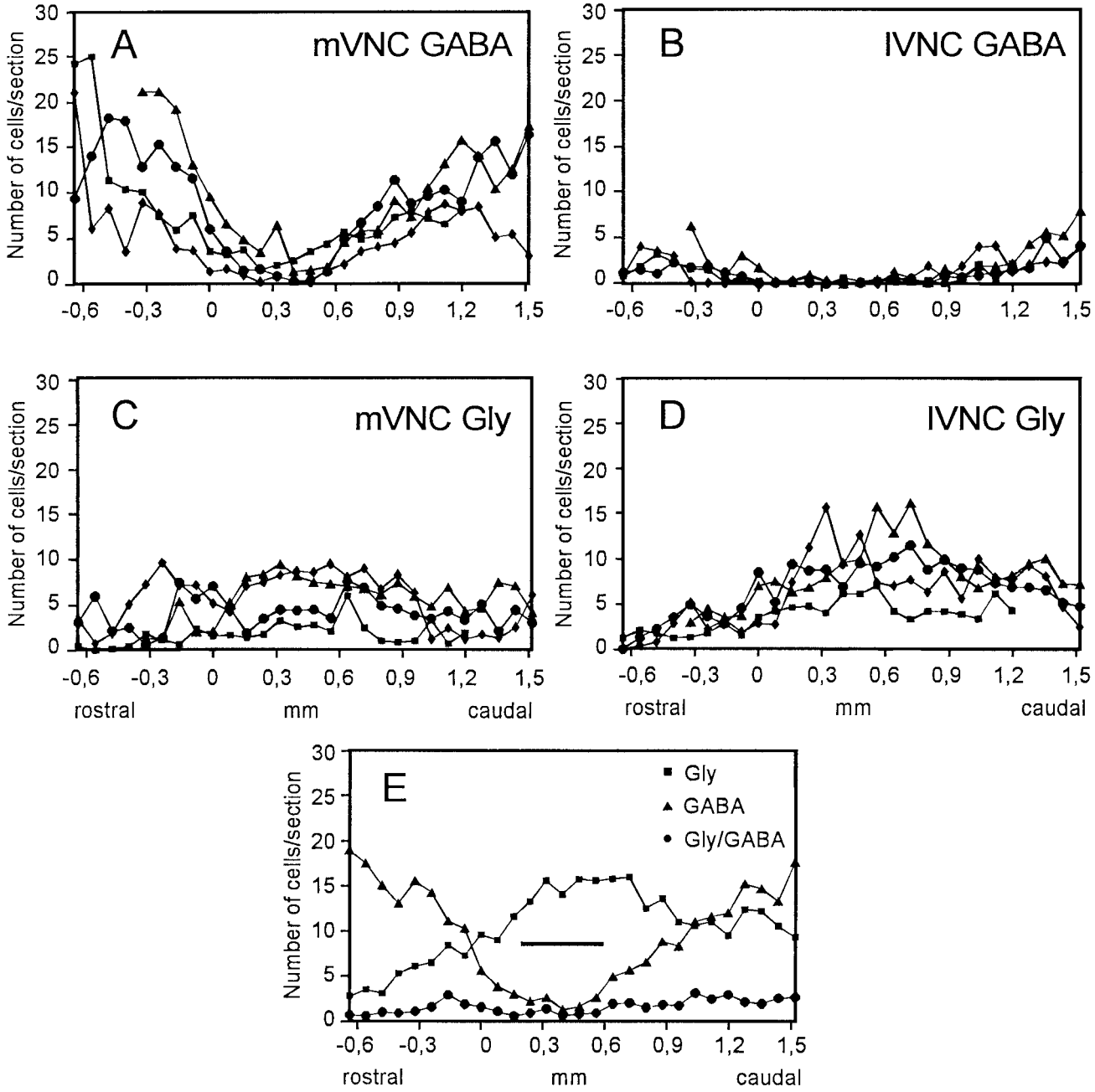

Fig. 4. Rostro-caudal distribution of GABA (**A,B**) and glycine immunoreactive (**C,D**) cells in the medial (mVNC in A,C) and the lateral (lVNC in B,D) part of the VNC. The number of immunoreactive cells per section was counted in 80 μm intervals in four animals (each represented by a different symbol). Diagram in **E** summarizes the distribution of immunoreactive cells as the mean values from the data shown in A–D. Note the inverse distribution of neurons that contain either glycine or GABA. Zero on the ordinate corresponds to the caudal end of the entry of the VIIIth nerve. Bar in E represents the extent of the abducens nucleus.

Colocalization of amino acids

Colocalization of GABA and glycine immunoreactivities was observed in only 2–3 somata per section (Fig. 3A,B, 4E, 7), located almost exclusively in the mVNC. These cells were always small and uniformly distributed over the rostro-caudal extent of the mVNC (Fig. 4E). Since almost all neurons contained a significant amount of glutamate, the GABA-IR or Gly-IR neurons also colocalized glutamate. Nevertheless, the intensity of glutamate immunolabeling in these double or triple stained neurons was lower than in most of the single labeled neurons (Figs. 3, 5).

Terminal-like structures and fibers

Immunopositive puncta were abundant throughout the VNC and may represent crosscut dendrites or axons or may represent axon terminals. Some of the immunoreac-

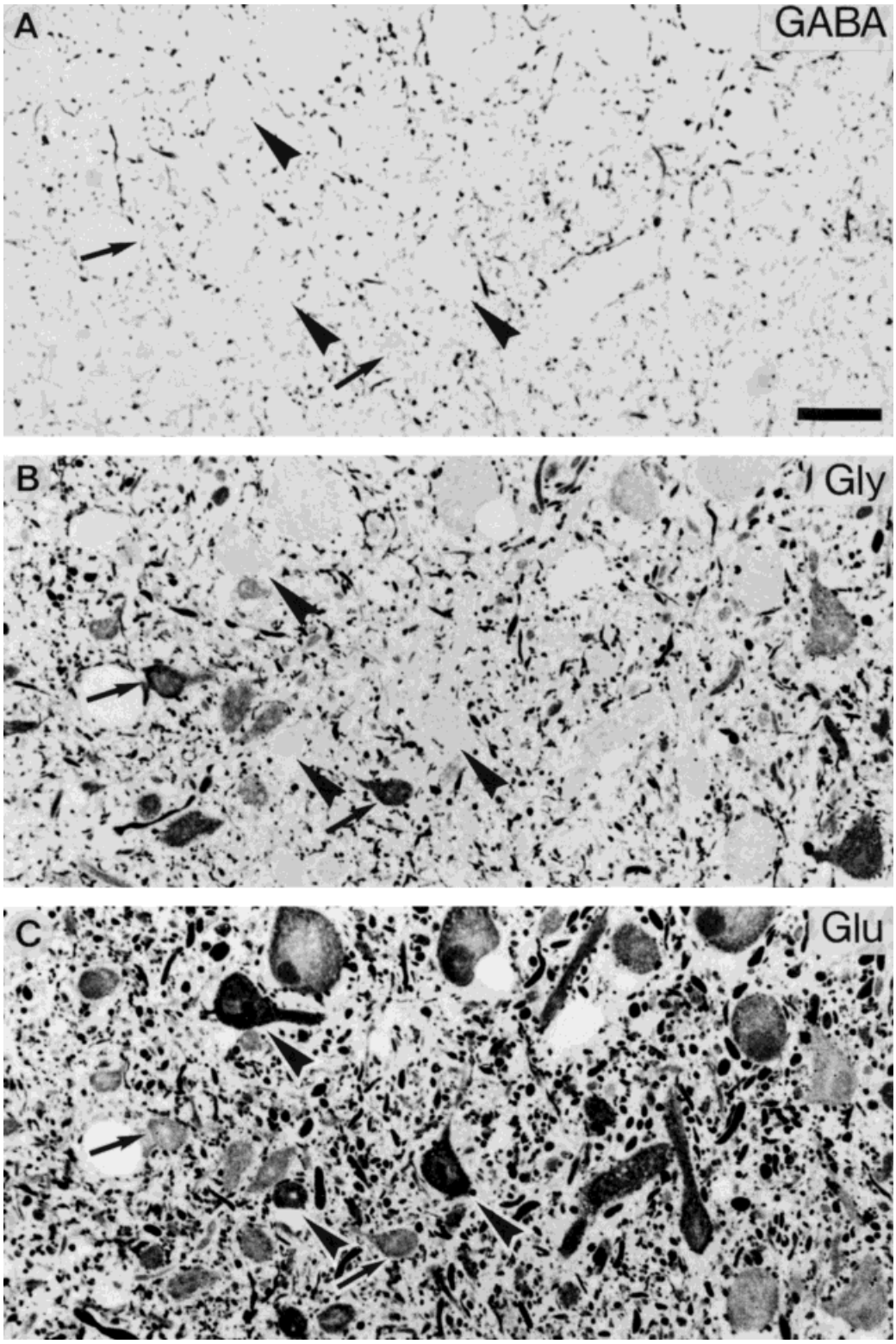

Fig. 5. Photomicrographs of labeling in the lateral part of the VNC. Consecutive semithin sections were treated with antibodies against GABA (**A**), glycine (**B**), or glutamate (C). None of the cells was stained for GABA. Note that strongly glycine immunoreactive cells are weakly glutamate immunoreactive (arrows). Moderate to strongly glutamate immunoreactive neurons do not colocalize glycine (arrowheads). The majority of fibers are glutamate immunoreactive and some of them express colocalization of glycine. Few fibers show colocalization of GABA and glycine. Scale bar = 30 µm.

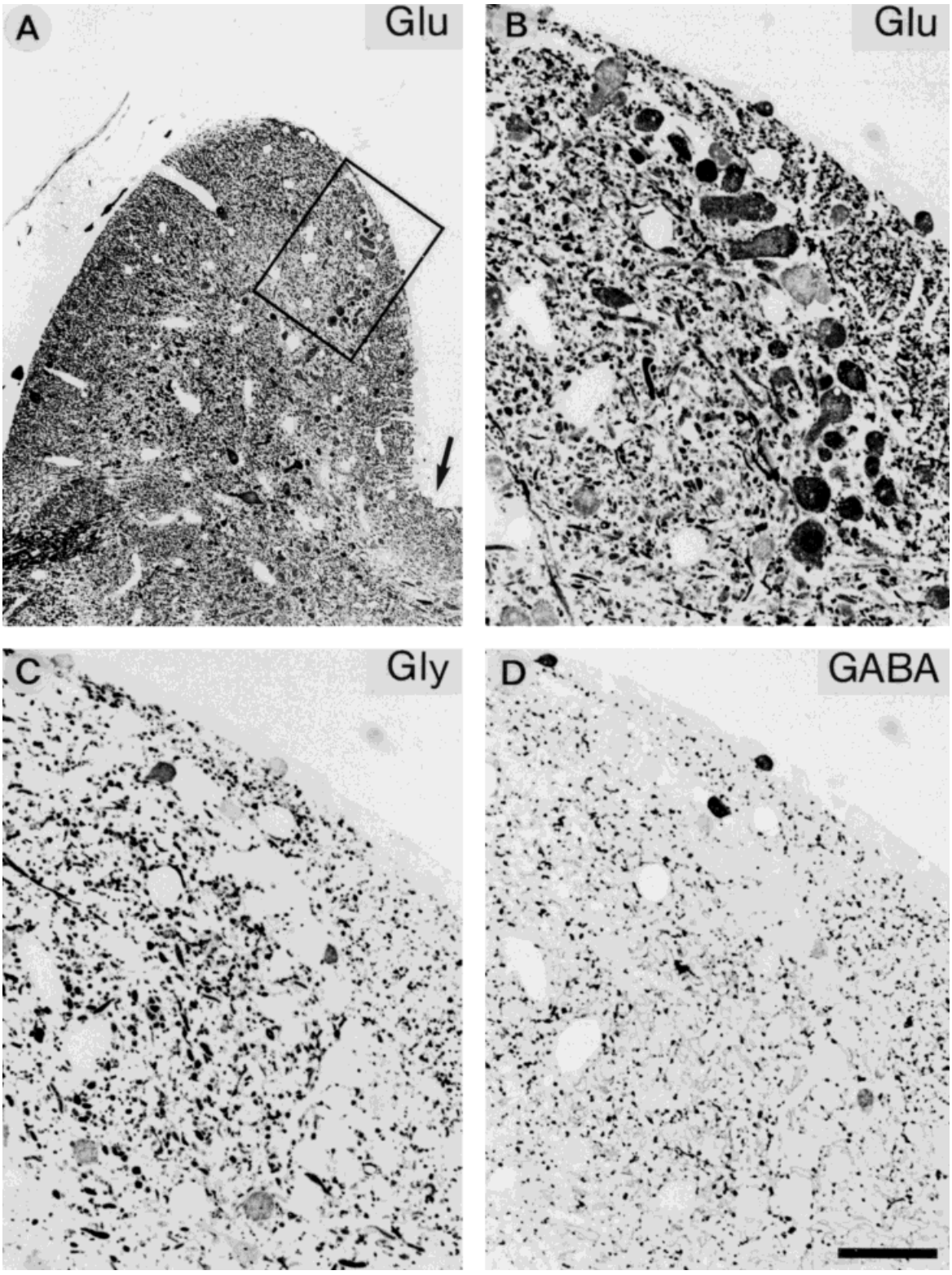

Fig. 6. Photomicrographs of a particular group of neurons in the medial part of the VNC. **A:** Location of the neurons (boxed area) as revealed by treatment with antibodies against glutamate. Arrow indicates the sulcus limitans of His. **B:** Higher magnification of the boxed area. **C,D:** Sections consecutive to B immunostained for glycine (C) and GABA (D). Scale bar = 170 μm for A, 40 μm for B–D.

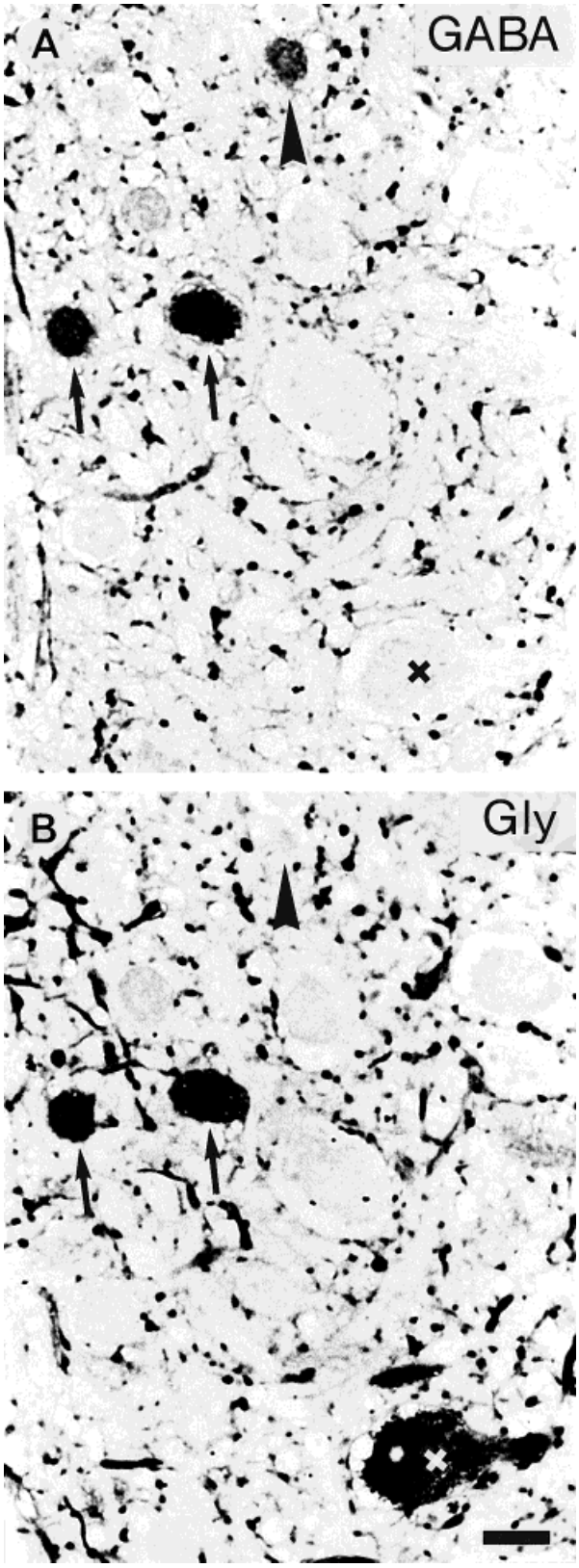

Fig. 7. Colocalization of GABA (**A**) and glycine (**B**) in vestibular neurons. Consecutive sections show one GABA immunoreactive neuron (arrowhead), one glycine immunoreactive neuron (cross), and two neurons immunoreactive for both GABA and glycine (arrows). Scale bar = 10 µm.

tive puncta apposed to cell bodies (Fig. 8) and main dendrites are likely to be synaptic terminals. In absence of an electron microscopic identification of synaptic contacts, these puncta are considered as terminal-like structures, including the possibility that some of them do not establish synaptic contacts.

GABA, glycine, or glutamate immunoreactive terminal-like structures often outlined dendrites but were usually less densely distributed around cell bodies. In 0.5 µm thick serial sections, cell bodies with a visible nucleus were quantitatively investigated for the presence of immunoreactive, terminal-like structures. Cell bodies (N = 218) were more frequently contacted by Glu-IR terminal-like structures (N = 2,417) than by Gly-IR (N = 2,003) or GABA-IR terminal-like structures (N = 1,349). Some of the terminal-like structures appeared to colocalize GABA and glycine (N = 547) or glycine and glutamate (N = 609). Figure 9 shows that the proportional distribution of terminal-like structures on the somata in both vestibular subdivisions is very similar.

The colocalization of GABA and glutamate in terminal-like structures was not tested for technical reasons. Since sections processed for GABA were adjacent to those processed for glycine but not for glutamate, the gap (0.5 µm) between GABA and glutamate sections was too large to allow a reliable comparison of related structures. The total number of terminal-like structures colocalizing two amino acids might be an underestimate since some terminal-like structures were too small to permit a definitive conclusion about colocalization.

Fibers of the N.VIII were Glu-IR (Fig. 2E). In addition, several thick nerve fibers were also Gly-IR (Fig. 2C), while GABA immunoreactivity was not observed (Fig. 2A). In the VNC, immunoreactive obliquely and cross-sectioned fibers were more abundant in the lVNC (Fig. 5) than in the mVNC (Fig. 3). Most of these fibers were Glu-IR and some of them colocalized glycine. GABA-positive fibers were few in number and approximately 10% of them colocalized glycine. Colocalization of GABA and glutamate was rarely observed in fibers.

DISCUSSION

This study describes the differential distribution of GABA, glycine, and glutamate immunoreactive neurons in the vestibular nuclear complex of the frog. As a major result we demonstrate a partial inverse distribution of GABA-IR and Gly-IR cell bodies along the rostrocaudal extent of the VNC. The results of this study will be compared with data obtained in mammals and the significance of the spatial differences will be discussed.

Methodological aspects

The antibodies against GABA, glycine, and glutamate used in this study have already been applied in nervous tissue from several species (frog: Reichenberger et al., 1993; Reichenberger and Dieringer, 1994; chick: Matute and Streit, 1986; pigeon: Domenici et al., 1988; rat: Matute and Streit, 1986; Liu et al., 1989; Kolston et al., 1992; Reichenberger and Dieringer, 1994; Grandes et al., 1994; human: Davanger et al., 1994) and are assumed to label selectively those neurons that contain these amino acids. This is supported by the control experiments and results obtained in this study.

GABA seems to be concentrated exclusively in neurons that use GABA as a neurotransmitter. This was demon-

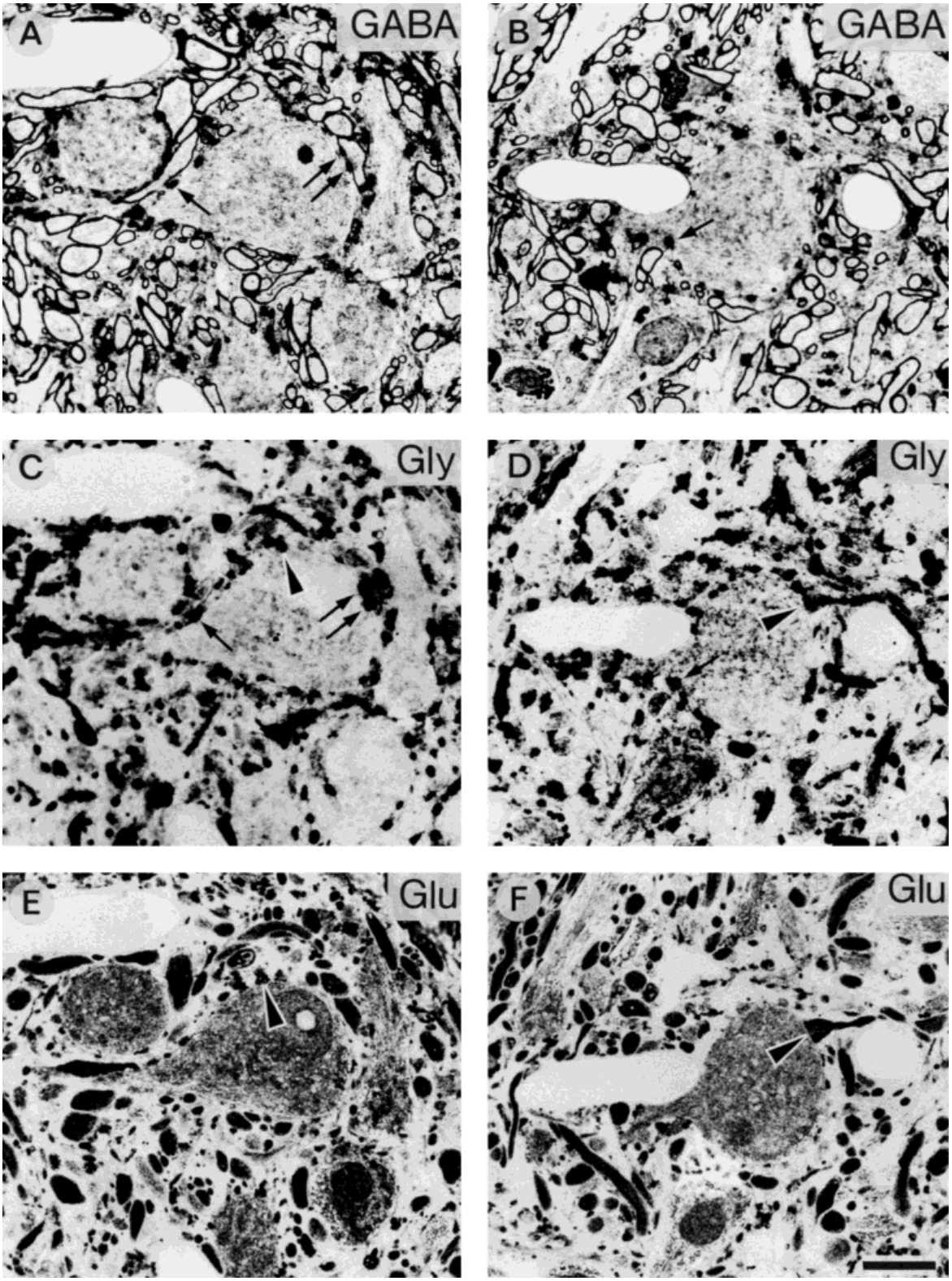

Fig. 8. Terminal-like structures contacting glutamate immunoreactive somata in the lateral part of the VNC. Consecutive semithin sections (0.5 µm) from two different areas (A,C,E and B,D,F) were treated with antibodies against GABA (**A,B**), glycine (**C,D**), or glutamate (**E,F**). Some terminal-like structures colocalized GABA and glycine (arrows in A–D), others colocalized glycine and glutamate (arrowheads in C–F). Scale bar = 15 µm.

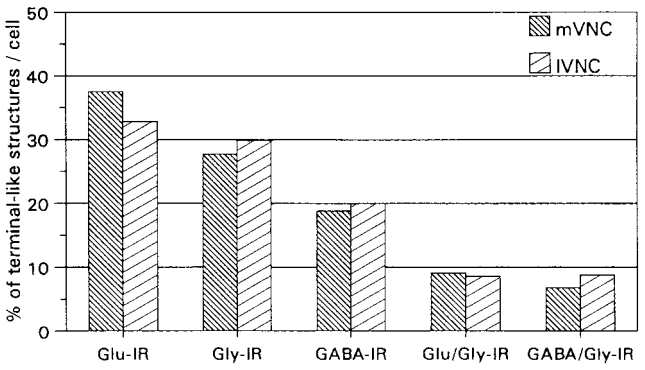

Fig. 9. Proportional distribution of terminal-like structures on somata in the medial (mVNC) and lateral (lVNC) parts of the vestibular nuclear complex immunostained for glutamate (Glu-IR), glycine (Gly-IR), or GABA (GABA-IR). Colocalization was investigated for glycine and glutamate (Gly/Glu-IR), and for GABA and glycine (GABA/Gly-IR). Data are based on N = 3,065 terminal-like structures counted on 123 mVNC cells, and on N = 3,860 terminal-like structures counted on 95 lVNC cells.

strated in the cerebellum, where anti-GABA antibodies only label neurons of undisputed GABAergic nature. Thus, GABA immunoreactivity is generally taken as a reliable marker of GABAergic neurons and we assume that this is also the case in the vestibular nuclei of frogs.

In contrast to GABA, glutamate is involved in protein synthesis and in a series of metabolic reactions (Hertz et al., 1983). The amount of glutamate in cell bodies may primarily reflect its metabolic demand and is likely to be a poor indicator of transmitter identity. This differs from the situation in nerve terminals where a high concentration of glutamate in synaptic vesicles is considered a hallmark of their glutamatergic nature (Burger et al., 1989; Ji et al., 1991; Shupliakov et al., 1992). Therefore, it is possible that the strongly immunostained terminal-like structures in the present material use glutamate as a transmitter, but the same cannot be assumed from the Glu-IR somata.

Like glutamate, glycine is engaged in several metabolic pathways (Daly, 1990). However, immunocytochemical data from numerous species and brain regions suggest that the cellular distribution of glycine is much more differentiated than that of glutamate and that a high level of glycine is a characteristic feature of putative glycinergic neurons. Thus, it is likely that the metabolic pool of glycine is modest compared to the transmitter pool (Ottersen et al., 1990). However, some instances have been reported of an enrichment of glycine in cell bodies (Davanger et al., 1991; Reichenberger and Dieringer, 1994) and terminals (Davanger et al., 1994) that are thought to use glutamate as transmitter. In such cases the question arises whether glutamate and glycine are subject to corelease. This question is particularly relevant when the terminals face NMDA receptors at which glutamate and glycine are known to act as coagonists (Johnson and Ascher, 1987).

Comparison of results from different vertebrates

Immunocytochemical studies on the vestibular nuclear complex in mammals have predominantly dealt with the distribution of GABA or its synthesizing enzyme glutamate decarboxylase (GAD). Fewer studies analyzed the distribution of glycine or glutamate immunoreactive vestibular neurons. Assuming homology between the vestibular nuclei in frogs and mammals (Matesz, 1979; Kuruvilla et al., 1985) our results are quite similar to some of the data obtained in mammals.

GABA immunoreactivity

The distribution of GABA or GAD immunoreactive neurons in the vestibular nuclei was studied in squirrel monkey, cat, rat, guinea pig, and mouse (Nomura et al., 1984; Ottersen and Storm-Mathisen, 1984b; Mugnaini and Oertel, 1985; Kumoi et al., 1987; Carpenter et al., 1990; Walberg et al., 1990; Spencer and Baker, 1992). Most authors found higher densities of GABA-IR neurons in the MVN and DVN than in the LVN and SVN. This observation is quite comparable with the results obtained in the frog. Here, GABA-IR neurons were found throughout the VNC but predominated in the rostral and caudal parts of the mVNC, including the MVN and part of the DVN. As in mammals, the GABA-IR neurons in the frog were small.

Studies in cat, rabbit, and frog indicated that GABA is the transmitter in the inhibitory vertical vestibulo-ocular reflex. Stimulation of the VIIIth nerve evoked disynaptic inhibitory postsynaptic potentials in oculomotor and trochlear motoneurons that could be blocked by application of the GABA antagonist picrotoxin or bicuculline (Ito et al., 1970; Obata and Highstein, 1970; Precht et al., 1973a; Cochran, 1992). Unilateral lesions of the medial longitudinal fasciculus, known to contain the inhibitory axons from the SVN to the trochlear and oculomotor nuclei, led to a reduction of the amount of GABA in these two nuclei on the ipsilateral side (Precht et al., 1973a). The inhibition of oculomotor motoneurons following vestibular nerve stimulation is similar to the inhibition observed after local application of GABA (Obata and Highstein, 1970). More recently an electron microscopic study in the rabbit (Wentzel et al., 1995) confirmed the electrophysiological and pharmacological data. Thus, the inhibitory GABAergic vestibular input to the oculomotor nucleus originates from the ipsilateral SVN.

In frogs, most vestibular neurons that project to the oculomotor nucleus are located in the rostral VNC that contains the SVN (Straka and Dieringer, 1991). It is likely that part of the GABA-positive neurons observed in this area mediate the inhibition of ipsilateral oculomotor and trochlear motoneurons (Cochran, 1992). This is supported by the fact that the somata of identified frog oculomotor and trochlear neurons were more frequently contacted by GABA-positive than by glycine-positive terminal-like structures (unpublished data).

Part of the GABA-IR neurons in the VNC might contribute to descending pathways. GAD immunoreactivity was observed in about half of the rabbit's DVN and MVN neurons that projected to the spinal cord (Blessing et al., 1987). Vestibulo-spinal neurons in the frog appear to be located primarily in the caudal portion of the VNC (Fuller, 1974). Unfortunately, pharmacological data on the vestibulo-spinal projection in frogs are absent.

The vestibular nuclei on either side of the brainstem are connected by commissural fibers (frog: Fuller, 1974; rabbit: Epema et al., 1988; cat: Ladpli and Brodal, 1968; Gacek, 1978). Electrical stimulation of the contralateral semicircular canal nerves of the cat evoked inhibitory postsynaptic potentials in central vestibular neurons that could be excited by stimulation of the coplanar canal nerve from the ipsilateral side (Kasahara and Uchino, 1971). The commis-

sural inhibition is di- and polysynaptic and mediated by inhibitory interneurons or intrinsic neurons (Shimazu and Precht, 1966). Pharmacological studies showed that GABA is the putative transmitter (Precht et al., 1973b; Furuya et al., 1991). Unlike in the cat, commissural inhibition is only rarely observed in intact frogs. Central vestibular neurons in this species are weakly excited following stimulation of the contralateral N.VIII and glutamate seems to be the putative transmitter (Ozawa et al., 1974; Cochran et al., 1987; Knöpfel, 1987). Commissural neurons of frogs are located between the entry of N.VIII and N.IX (Fuller, 1974). The paucity of GABA-IR neurons in this region is consistent with the rarely observed commissural inhibition.

Part of the GABA-IR neurons in the VNC might represent vestibular interneurons that are activated monosynaptically by vestibular nerve fibers and which in turn inhibit ipsilateral second-order vestibular neurons via GABA (Straka and Dieringer, 1996). This disynaptic inhibition expresses itself in the eighth nerve evoked field potentials in the VNC and allowed an analysis of the spatial distribution of the effects of bicuculline on the vestibular field potentials. Interestingly, a significant bicuculline-sensitive component was detected in the rostral and caudal parts but not in the intermediate part of the VNC (Straka and Dieringer, 1996).

GABA-positive fibers and terminal-like structures may in part originate from cerebellar Purkinje cells. These neurons are GABA immunoreactive (Reichenberger et al., 1993), terminate on central vestibular neurons (Stern and Rubinson, 1971), and exert an inhibitory effect (Magherini et al., 1975).

Glycine immunoreactivity

Walberg et al. (1990) observed in the cat small to medium-sized, Gly-IR neurons in the MVN, LVN, and DVN, but not in the SVN. This is slightly different from the results obtained in frog, since here Gly-IR cells were found in all parts of the VNC, including the rostral region which contains the SVN. Another difference with the cat was the observation that some of the large neurons in the lVNC (Deiters' neurons) were Gly-IR.

Ample evidence has been presented in cats and frogs that glycine is the inhibitory transmitter of the horizontal vestibulo-ocular pathway. Abducens motoneurons could be inhibited by stimulation of the ipsilateral vestibular nerve and excited by stimulation of the contralateral nerve (Baker et al., 1969; Highstein, 1973; Magherini et al., 1974; McCrea et al., 1987; Straka and Dieringer, 1993). The uncrossed disynaptic inhibition could be blocked by the glycine antagonist strychnine but not by the GABA antagonist picrotoxin (Spencer et al., 1989; Straka and Dieringer, 1993). Injection of [^{3}H]-glycine in the abducens nucleus of the cat resulted in retrograde labeling of neurons located predominantly in the ipsilateral MVN (Spencer et al., 1989). In addition, immunocytochemistry revealed a higher density of Gly-IR than of GABA-IR terminal-like structures in the abducens nuclei of the frog (unpublished observations) and the cat (Spencer et al., 1989). Therefore, part of the Gly-IR cells found in the present study may be involved in the projection to the abducens nucleus. Other Gly-IR central vestibular neurons may participate in inhibitory projections to the spinal cord (Felpel, 1972).

Another group of Gly-IR neurons in the VNC might represent vestibular interneurons, given that the majority of second-order vestibular neurons recorded by Straka and Dieringer (1996) exhibited a disynaptic, glycinergic inhibitory response upon stimulation of the ipsilateral eighth nerve. Interestingly, the spatial distribution of the effects of strychnine on the evoked field potentials in the VNC was complementary to the spatial distribution of the effects of bicuculline. A significant strychnine-sensitive component was detected in the intermediate and caudal parts but not in the rostral part of the VNC (Straka and Dieringer, 1996). The correspondence of the results obtained by this immunocytochemical study and the above-mentioned pharmacological study in the same species implies a spatial restricted extension of the axonal projections of inhibitory interneurons in the VNC.

Glutamate immunoreactivity

Consistent with reports in the squirrel monkey and the cat (Carpenter et al., 1990; Walberg et al., 1990) most vestibular neurons in the frog were Glu-IR. As discussed before, the presence of glutamate in cell bodies may reflect a metabolic demand and does not necessarily signify its role as a transmitter. Nevertheless, glutamate seems to be the major transmitter of excitatory vestibular pathways to oculomotor, trochlear, and abducens motoneurons (Demêmes and Raymond, 1982; Kevetter and Hoffman, 1991; Straka and Dieringer, 1993). Transmission in excitatory vestibulo-spinal pathways is probably mediated by glutamate as well. Deiters' neurons, which were immunoreactive for glutamate in cat and squirrel monkey (Carpenter et al., 1990; Walberg et al., 1990), exert a facilitatory influence on ipsilateral spinal motoneurons (Grillner et al., 1970).

Although Glu-IR terminal-like structures were very abundant in the VNC of the frog, they never surrounded cell bodies as densely as has been reported for the MVN of the cat (Walberg et al., 1990) or in the dorsal (acoustic) nucleus of frog (unpublished data). Throughout the frog VNC, Glu-IR terminal-like structures were observed more frequently in close proximity of main dendrites than of somata. The high proportion of Glu-IR terminal-like structures is compatible with the presumed role of glutamate as the transmitter of afferent vestibular nerve fibers and excitatory vestibular commissural fibers (Cochran et al., 1987; Straka et al., 1996a, b).

The location of the narrow band of rather strongly Glu-IR neurons in the mVNC coincides with that of the nucleus caudalis nervi octavi as described by Opdam et al. (1976). However, Kuruvilla et al. (1985) considered this cell group as a subdivision of the MVN. At present the afferent and efferent connections of these neurons are unknown.

Colocalization of amino acids

A large number of studies in different species have demonstrated the colocalization of different amino acids in neurons and terminals, but few reports concerned the VNC. The present study has demonstrated the combined presence of GABA, glycine, and glutamate in neurons as well as in terminal-like structures.

Colocalization of GABA and glycine was observed within the entire VNC. Almost all somata containing both amino acids were located in the mVNC, whereas double-labeled fibers and terminal-like structures were more equally

stributed in both mVNC and lVNC. Walberg et al. (1990) analyzed the colocalization of GABA and glycine in the VNC of the cat and concluded that double-labeled structures were most frequent in the LVN where all Gly-IR profiles also contained GABA. In the frog, about 20% of the Gly-IR profiles (somata, fibers, or terminal-like structures) were also GABA-IR. The apparently smaller number of GABA and glycine double-labeled profiles as compared to the cat reflects a difference, similar to the one already described for the cerebellum (Reichenberger et al., 1993). Whether the colocalization of GABA and glycine indicates the presence of two neurotransmitter pools or the presence of a metabolic pool of one amino acid that is unrelated to the neurotransmitter pool of the other remains to be analyzed in the frog at the electron microscopic level. In the cerebellum, however, corelease of GABA and glycine has been shown by Morales and Tapia (1987) and by Ottersen et al. (1990). Although both amino acids act on receptors that are coupled to chloride channels, the inhibitory effect of glycine and GABA could still differ, depending on the subtypes of the receptors activated by these two amino acids.

Since virtually all VNC neurons in the present study were Glu-IR, the observed colocalization of this amino acid with GABA or glycine was not surprising and in accordance with similar observations in the cat (Walberg et al., 1990). Despite the absence of electron microscopic analysis and the uncertainty about the functional role of amino acids colocalized in neuronal profiles, it might be considered that some of the terminal-like structures colocalizing glutamate and glycine represent terminals of thick vestibular nerve fibers. These fibers are known to colocalize glutamate and glycine (Reichenberger and Dieringer, 1994), to exhibit a high specificity uptake for glycine (Straka et al., 1996b) and to activate NMDA receptors (Straka et al., 1996a). Since glutamate and glycine are known to act as co-agonists on NMDA receptors (Johnson and Ascher, 1987), it is possible that glutamate and glycine are subject to corelease from afferent vestibular nerve fibers. An electron microscopic analysis of this issue is in progress.

In the frog, some of the Deiters' neurons colocalized glutamate and glycine, whereas in the cat, Deiters' neurons were immunoreactive for glutamate and aspartate, but not for glycine (Walberg et al., 1990).

Functional and developmental aspects

From the distribution of GABA-IR and Gly-IR neurons along the rostrocaudal extent of the VNC, the conclusion could be drawn that the VNC of the frog contains three subdivisions. The rostral part is characterized by an abundancy of GABA-IR and a small number of Gly-IR neurons, while this relation is inverse in the intermediate part. The caudal subdivision contains a high number of both GABA-IR and Gly-IR neurons. This compartmentalization of the VNC in regions with different inhibitory transmitters could reflect a segregation of inhibitory vertical and horizontal canal-related vestibulo-ocular neurons, and inhibitory vestibulo-spinal neurons. As described before, inhibitory vertical canal-related vestibulo-ocular neurons are GABAergic and located in the SVN of mammals and the rostral VNC compartment of frogs. Inhibitory horizontal canal-related neurons are glycinergic and located in the MVN of mammals and probably in the intermediate compartment of frogs. Inhibitory vestibulospinal neurons are GABA- and glycinergic and predominate in the mammalian caudal MVN and the DVN, and in the caudal compartment of the frog VNC.

The presence of regions with different inhibitory transmitters in adult frogs could have its origin in the early embryogenetic segmentation of the hindbrain (see Gilland and Baker, 1993). Data from chicken embryos suggest that vestibulo-ocular and vestibulo-spinal neurons are derived from clusters of vestibular neurons, each of which has a characteristic spatial domain in the hindbrain (Glover and Petursdottir, 1991). Thus, the complementary distribution of GABA-IR and Gly-IR neurons might have originated during the pattern formation at early embryonic stages and might be still conserved to some extent in the adult frog.

ACKNOWLEDGMENTS

The authors thank Dr. J. Büttner-Ennever and Dr. T. Heinzeller who allowed us to use their laboratories. This research was supported by a grant (Di 205/7-1) from Deutsche Forschungsgemeinschaft.

LITERATURE CITED

Antal, M., I. Tornai, and G. Székely (1980) Longitudinal extent of dorsal root fibres in the spinal cord and brain stem of the frog. Neuroscience *5:*1311–1322.

Baker, R.G., N. Mano, and H. Shimazu (1969) Postsynaptic potentials in abducens motoneurons induced by vestibular stimulation. Brain Res. *15:*577–580.

Blessing, W.W., S.C. Hedger, and W.H. Oertel (1987) Vestibulospinal pathway in rabbit includes GABA-synthesizing neurons. Neurosci. Lett. *80:*158–162.

Burger, P.M., E. Mehl, P.L. Cameron, P.R. Maycox, M. Baumert, F. Lottspeich, P. De Camilli, and R. Jahn (1989) Synaptic vesicles immunoisolated from rat cerebral cortex contain high levels of glutamate. Neuron *3:*715–720.

Carpenter, M.B., Y. Huang, A.B. Pereira, and L.B. Hersh (1990) Immunocytochemical features of the vestibular nuclei in the monkey and cat. J. Hirnforsch. *31:*585–599.

Cochran, S.L. (1992) Synaptic input to oculomotor neurons of the frog. Soc. Neurosci. Abstr. *18:*28.

Cochran, S.L., P. Kasik, and W. Precht (1987) Pharmacological aspects of excitatory synaptic transmission to second-order vestibular neurons in the frog. Synapse *1:*102–123.

Daly, E.C. (1990) The biochemistry of glycinergic neurons. In O.P. Ottersen, and J. Storm-Mathisen (eds): Glycine Neurotransmission. Chichester: John Wiley and Sons Ltd., pp. 25–66.

Davanger, S., O.P Ottersen, and J. Storm-Mathisen (1991) Glutamate, GABA, and glycine in the human retina: An immunocytochemical investigation. J. Comp. Neurol. *311:*483–494.

Davanger, S., J. Storm-Mathisen, and O.P. Ottersen (1994) Colocalization of glutamate and glycine in bipolar cell terminals of the human retina. Exp. Brain Res. *98:*342–354.

Demêmes, D., and J. Raymond (1982) Radioautographic identification of [^{3}H]glutamic acid labeled nerve endings in the cat oculomotor nucleus. Brain Res. *231:*433–437.

Dieringer, N., and W. Precht (1979) Mechanisms of compensation for vestibular deficits in the frog. II. Modification of the inhibitory pathways. Exp. Brain Res. *36:*329–341.

Domenici, L., H.J. Waldvogel, C. Matute, and P. Streit (1988) Distribution of GABA-like immunoreactivity in the pigeon brain. Neuroscience *25:*931–950.

Epema, A.H., N.M. Gerrits, and J. Voogd (1988) Commissural and intrinsic connections of the vestibular nuclei in the rabbit: A retrograde labeling study. Exp. Brain Res. *71:*129–146.

Felpel, L.P. (1972) Effects of strychnine, bicuculline and picrotoxin on labyrinthine-evoked inhibition in neck motoneurons of the cat. Exp. Brain Res. *14:*494–502.

Fuller, P.M. (1974) Projections of the vestibular nuclear complex in the bullfrog (*Rana catesbeiana*). Brain Behav. Evol. *10:*157–169.

Furuya, N., T. Yabe, and T. Koizumi (1991) Neurotransmitters regulating vestibular commissural inhibition in the cat. Acta Otolaryngol. (Stockh.) *481:*205–208.

Gacek, R.R. (1978) Location of commissural neurons in the vestibular nuclei of the cat. Exp. Neurol. *59:*479–491.

Gerrits, N.M. (1990) Vestibular nuclear complex. In G. Paxinos (ed): The Human Nervous System. San Diego: Academic Press, pp. 863–888.

Gilland, E., and R. Baker (1993) Conservation of neuroepithelial and mesodermal segments in the embryonic vertebrate head. Acta Anat. *148:*110–123.

Glover, J.C., and G. Petursdottir (1991) Regional specificity of developing reticulospinal, vestibulospinal, and vestibuloocular projections in the chicken embryo. J. Neurobiol. *22:*253–276.

Grandes, P., F. Ortega, and P. Streit (1994) Glutamate-immunoreactive climbing fibres in the cerebellar cortex of the rat. Histochemistry *101:*427–437.

Gregory, K.M. (1972) Central projections of the eighth nerve of frogs. Brain Behav. Evol. *5:*70–88.

Grillner, S., T. Hongo, and S. Lund (1970) The vestibulospinal tract. Effect on alpha-motoneurones in the lumbosacral spinal cord in the cat. Exp. Brain Res. *10:*94–120.

Grover, B.G., and U. Grüsser-Cornehls (1984) Cerebellar afferents in the frogs, Rana esculenta and Rana temporaria. Cell Tissue Res. *237:*259–267.

Hertz, L., E. Kvamme, E.G. McGeer, and A. Schousboe (1983) Glutamine, Glutamate and GABA in the Central Nervous System. New York: Alan R. Liss.

Highstein, S.M. (1973) Synaptic linkage in the vestibulo-ocular and cerebello-vestibular pathways to the VIth nucleus in the rabbit. Exp. Brain Res. *17:*301–314.

Hillman, D.E. (1972) Vestibulocerebellar input in the frog: Anatomy. Prog. Brain Res. *37:*329–339.

Ito, M. (1984) The Cerebellum and Neural Control. New York: Raven Press.

Ito, M., S.M. Highstein, and T. Tsuchia (1970) The postsynaptic inhibition of rabbit oculomotor neurones by secondary vestibular impulses and its blockage by picrotoxin. Brain Res. *17:*520–523.

Ji, Z.Q., J.E. Aas, J. Laake, F. Walberg, and O.P. Ottersen (1991) An electron microscopic, immunogold analysis of glutamate and glutamine in terminals of rat spinocerebellar fibers. J. Comp. Neurol. *307:*296–310.

Johnson, J.W., and P. Ascher (1987) Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature *325:*529–531.

Kasahara, M., and Y. Uchino (1971) Selective mode of commissural inhibition induced by semicircular canal afferents on secondary vestibular neurons in the cat. Brain Res. *34:*366–369.

Kevetter, G.A., and R.D. Hoffman (1991) Excitatory amino acid immunoreactivity in vestibulo-ocular neurons in gerbils. Brain Res. *554:*348–351.

Kinney, G.A., B.W. Peterson, and N.T. Slater (1994) The synaptic activation of N-methyl-D-aspartate receptors in the rat medial vestibular nucleus. J. Neurophysiol. *72:*1588–1595.

Knöpfel, T. (1987) Evidence for N-methyl-D-aspartic acid receptor-mediated modulation of the commissural input to central vestibular neurons of the frog. Brain Res. *426:*212–224.

Kolston, J., K.K. Osen, C.M. Hackney, O.P. Ottersen, and J. Storm-Mathisen (1992) An atlas of glycine- and GABA-like immunoreactivity and colocalization in the cochlear nuclear complex of the guinea pig. Anat. Embryol. *186:*443–465.

Kumoi, K., N. Saito, and C. Tanaka (1987) Immunocytochemical localization of γ-aminobutyric acid- and aspartate-containing neurons in the guinea pig vestibular nuclei. Brain Res. *416:*22–33.

Kuruvilla, A., S. Sitko, I.R. Schwartz, and V. Honrubia (1985) Central projections of primary vestibular fibers in the bullfrog. I. The vestibular nuclei. Laryngoscope *95:*692–707.

Ladpli, R., and A. Brodal (1968) Experimental studies of commissural and reticular formation projections from the vestibular nuclei in the cat. Brain Res. *8:*65–96.

Larsell, O. (1934) The differentiation of the peripheral and central acoustic apparatus in the frog. J. Comp. Neurol. *60:*473–527.

Liu, C.-j., P. Grandes, C. Matute, M. Cuénod, and P. Streit (1989) Glutamate-like immunoreactivity revealed in rat olfactory bulb, hippocampus and cerebellum by monoclonal antibody and sensitive staining method. Histochemistry *90:*427–445.

Magherini, P.C., M.L. Giretti, and W. Precht (1975) Cerebellar control of vestibular neurons of the frog. Pflügers Arch. *356:*99–109.

Magherini, P.C., W. Precht, and P.C. Schwindt (1974) Functional organization of the vestibular input to ocular motoneurons of the frog. Pflügers Arch. *349:*149–158.

Matesz, C. (1979) Central projection of the VIIIth cranial nerve in the frog. Neuroscience *4:*2061–2071.

Matute, C., and P. Streit (1986) Monoclonal antibodies demonstrating GABA-like immunoreactivity. Histochemistry *86:*147–157.

McCrea, R.A., A. Strassman, E. May, and S.M. Highstein (1987) Anatomical and physiological characteristics of vestibular neurons mediating the horizontal vestibulo-ocular reflex in the squirrel monkey. J. Comp. Neurol. *264:*547–570.

Montgomery, N.M. (1988) Projections of the vestibular and cerebellar nuclei in Rana pipiens. Brain Behav. Evol. *31:*82–95.

Morales, E., and R. Tapia (1987) Neurotransmitters of the cerebellar glomeruli: uptake and release of labelled γ-aminobutyric acid, glycine, serotonin and choline in a purified glomerulus fraction and in granular layer slices. Brain Res. *420:*11–21.

Mugnaini, E., and W.H. Oertel (1985) An atlas of the distribution of GABAergic neurons and terminals in the rat CNS as revealed by GAD immunohistochemistry. In A. Björklund, and T. Hökfelt (eds): Handbook of Chemical Neuroanatomy, Vol 4, GABA and Neuropeptides in the CNS, Part I. Amsterdam: Elsevier, pp. 436–608.

Nomura, I., E. Senba, T. Kubo, T. Shiraishi, T. Matsunaga, M. Tohyama, Y. Shiotani, and J.-Y. Wu (1984) Neuropeptides and γ-aminobutyric acid in the vestibular nuclei of the rat: An immunohistochemical analysis. I. Distribution. Brain Res. *311:*109–118.

Obata, K., and S.M. Highstein (1970) Blocking by picrotoxin of both vestibular inhibition and GABA action on rabbit oculomotor neurones. Brain Res. *18:*538–541.

Opdam, P., M. Kemali, and R. Nieuwenhuys (1976) Topological analysis of the brain stem of the frogs Rana esculenta and Rana catesbeiana. J. Comp. Neurol. *165:*307–332.

Ottersen, O.P. (1987) Postembedding light- and electron microscopic immunocytochemistry of amino acids: Description of a new model system allowing identical conditions for specificity testing and tissue processing. Exp. Brain Res. *69:*167–174.

Ottersen, O.P., and J. Storm-Mathisen (1984a) Glutamate- and GABA-containing neurons in the mouse and rat brain, as demonstrated with a new immunocytochemical technique. J. Comp. Neurol. *229:*374–392.

Ottersen, O.P., and J. Storm-Mathisen (1984b) Neurons containing or accumulating transmitter amino acids. In A. Björklund, T. Hökfelt and M.J. Kuhar (eds): Handbook of Chemical Neuroanatomy, Vol 3, Classical Transmitters and Transmitter Receptors in the CNS, Part II. Amsterdam: Elsevier, pp. 141–246.

Ottersen, O.P., J. Storm-Mathisen, and J. Laake (1990) Cellular and subcellular localization of glycine studied by quantitative electron microscopic immunocytochemistry. In O.P. Ottersen, and J. Storm-Mathisen (eds): Glycine Neurotransmission. Chichester: John Wiley and Sons, Ltd., pp. 303–328.

Ottersen, O.P., J. Storm-Mathisen, and P. Somogy (1988) Colocalization of glycine-like and GABA-like immunoreactivities in Golgi cell terminals in the rat cerebellum: A postembedding light and electron microscopic study. Brain Res. *450:*342–353.

Ozawa, S., W. Precht, and H. Shimazu (1974) Crossed effects on central vestibular neurons in the horizontal canal system of the frog. Exp. Brain Res. *19:*394–405.

Precht, W., R. Baker, and Y. Okada (1973a) Evidence for GABA as the synaptic transmitter of the inhibitory vestibulo-ocular pathway. Exp. Brain Res. *18:*415–428.

Precht, W., P.C. Schwindt, and R. Baker (1973b) Removal of vestibular commissural inhibition by antagonists of GABA and glycine. Brain Res. *62:*222–226.

Precht, W., A. Richter, S. Ozawa, and H. Shimazu (1974) Intracellular study of frog's vestibular neurons in relation to the labyrinth and spinal cord. Exp. Brain Res. *19:*377–393.

Reichenberger, I., and N. Dieringer (1994) Size–related colocalization of glycine and glutamate immunoreactivity in frog and rat vestibular afferents. J. Comp. Neurol. *349:*603–614.

Reichenberger, I., H. Straka, H., and N. Dieringer (1992) Distribution of GABA-and glycine-immunoreactive cells in the cerebellum and the vestibular nuclei of the frog. Eur. J. Neurosci. Suppl. *5:*301.

Reichenberger, I., P. Streit, O.P. Ottersen, and N. Dieringer (1993) GABA- and glycine-like immunoreactivities in the cerebellum of the frog. Neurosci. Lett. *154:*89–92.

Shimazu, H., and W. Precht (1966) Inhibition of central vestibular neurons from the contralateral labyrinth and its mediating pathway. J. Neurophysiol. *29:*467–492.

Shupliakov, O., L. Brodin, S. Cullheim, O.P. Ottersen, and J. Storm-Mathisen (1992) Immunogold quantification of glutamate in two types of excitatory synapse with different firing patterns. J. Neurosci. *12:*3789–3803.

Spencer, R.F., and R. Baker (1992) GABA and glycine as inhibitory neurotransmitters in the vestibuloocular reflex. Ann. NY Acad. Sci. *656:*602–611.

Spencer, R.F., R.J. Wenthold, and R. Baker (1989) Evidence for glycine as an inhibitory neurotransmitter of vestibular, reticular, and prepositus hypoglossi neurons that project to the cat abducens nucleus. J. Neurosci. *9:*2718–2736.

Stern, T.A., and K. Rubinson (1971) Efferent projections of the cerebellar cortex of Rana pipiens. Anat. Rec. *169:*438.

Straka, H., and N. Dieringer (1991) Internuclear neurons in the ocular motor system of frogs. J. Comp. Neurol. *312:*537–548.

Straka, H., and N. Dieringer (1993) Electrophysiological and pharmacological characterization of vestibular inputs to identified frog abducens motoneurons and internuclear neurons in vitro. Eur. J. Neurosci. *5:*251–260.

Straka, H., and N. Dieringer (1996) Uncrossed disynaptic inhibition of second-order vestibular neurons and its interaction with monosynaptic excitation from vestibular nerve afferent fibers in the frog. J. Neurophysiol. *76:* in press.

Straka, H., K. Debler, and N. Dieringer (1996a) Size-related properties of vestibular afferent fibers in the frog: Differential synaptic activation of N-methyl-D-aspartate and non-N-methyl-D-aspartate receptors. Neuroscience *70:*697–707.

Straka, H., I. Reichenberger, and N. Dieringer (1996b) Size-related properties of vestibular afferent fibers in the frog: Uptake of and immunoreactivity for glycine and aspartate/glutamate. Neuroscience *70:*685–696.

Tóth, P., Gy. Csank, and Gy. Lázár (1985) Morphology of the cells of origin of descending pathways to the spinal cord in Rana esculenta. A tracing study using cobaltic-lysine complex. J. Hirnforsch. *26:*365–383.

Waele, C. de, M. Mühlethaler, and P.P. Vidal (1995) Neurochemistry of the central vestibular pathways. Brain Res. Rev. *20:*24–46.

Walberg, F., O.P. Ottersen, and E. Rinvik (1990) GABA, glycine, aspartate, glutamate and taurine in the vestibular nuclei: An immunocytochemical investigation in the cat. Exp. Brain Res. *79:*547–563.

Wentzel, P.R., C.I. De Zeeuw, J.C. Holstege, and N.M. Gerrits (1995) Inhibitory synaptic inputs to the oculomotor nucleus from vestibulo-ocular-reflex-related nuclei in the rabbit. Neuroscience *65:*161–174.