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GABA- and Glycine-Immunoreactive Projections From the Superior Olivary Complex to the Cochlear Nucleus in Guinea Pig

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ABSTRACT

Retrograde transport of horseradish peroxidase was combined with immunocytochemistry to identify the origins of potential γ-aminobutyric acid (GABA) -ergic and glycinergic inputs to different subdivisions of the cochlear nucleus. Projection neurons in the inferior colliculus, superior olivary complex, and contralateral cochlear nucleus were examined, but only those from the superior olivary complex contained significant numbers of GABA- or glycine-immunoreactive neurons. The majority of these were in periolivary nuclei ipsilaterally, with a sizeable contribution from the contralateral ventral nucleus of the trapezoid body. Overall, 80% of olivary neurons projecting to the cochlear nucleus were immunoreactive for GABA, glycine, or both. Most glycine-immunoreactive projection neurons were located ipsilaterally, in the lateral and ventral nuclei of the trapezoid body and the dorsal periolivary nucleus. This suggests that glycine is the predominant neurotransmitter used by ipsilateral olivary projections. Most GABA-immunoreactive cells were located bilaterally in the ventral nuclei of the trapezoid body. The contralateral olivary projection was primarily GABA-immunoreactive and provided almost half the GABA-immunoreactive projections to the cochlear nucleus. This suggests that GABA is the predominant neurotransmitter used by contralateral olivary projections. The present results suggest that the superior olivary complex is the most important extrinsic source of inhibitory inputs to the cochlear nucleus. Individual periolivary nuclei differ in the strength and the transmitter content of their projections to the cochlear nucleus and may perform different roles in acoustic processing in the cochlear nucleus. J. Comp. Neurol. 381:500–512, 1997.

Indexing terms: auditory pathways; inhibitory neurotransmitters; inferior colliculus; horseradish peroxidase

The cochlear nucleus (CN) contains many morphologically different types of neurons receiving seemingly similar inputs from the cochlear nerve. Their responses to excitatory stimuli (both current injection and tonal stimuli) are different, for example, regularly or irregularly timed spike trains, transient or sustained. The role that different membrane properties and excitatory cochlear nerve inputs and synaptology play has received much attention (Cant and Morest, 1984; Oertel et al., 1988; Manis and Marx, 1991; Rhode and Greenberg, 1992; Feng et al., 1994). Interneuronal, feedback, and feedforward circuits could also play a role in shaping the responses of CN neurons. Noncochlear synaptic inputs make up greater than 50% of the synaptic input to some cell types in the CN. Many of these inputs have inhibitorylike morphologies and are strategically located on the somata and axon hillocks of the principal output cell types (Smith and Rhode, 1987, 1989; Ostapoff and Morest, 1991; Ryugo and Sento, 1991). However, either the sources of these inhibitory endings are not known or the relative strengths from the different sources are not known.

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Inhibitory inputs have been shown to exert significant influences on signal processing in the cochlear nucleus (CN), both in the response to tonal stimuli at a neuron's best frequency and in side-band reductions of response rates measured extracellularly (Palmer and Evans, 1982; Smith and Rhode, 1987, 1989). Intracellular recordings have shown inhibitory potentials in response to tones and auditory nerve stimulation (Smith and Rhode 1987, 1989; Wickesberg and Oertel, 1990; Feng et al., 1994). Pharmacological manipulations also reveal ongoing inhibitory effects on the responses of cells in the CN (Palombi and Caspary, 1992; Caspary et al., 1993, 1994). Inhibitory effects on some cell types in the dorsal cochlear nucleus (DCN) are evoked by contralateral acoustic stimulation (Young and Brownell, 1976). Inhibitory inputs elicited from best frequency and off-best frequency sources have been used in models mimicking the responses of different cell types in the CN (Arle and Kim, 1991; Banks and Sachs, 1991).

There is considerable evidence to suggest that γ-aminobutyric acid (GABA) and glycine are major inhibitory neurotransmitters in the CN (Altschuler et al., 1993; Caspary et al., 1993; Evans and Zhao, 1993; Oertel and Wickesberg, 1993; Potashner et al., 1993; Saint Marie et al., 1993). Briefly, both neurotransmitter substances, when applied exogenously, cause a rapid and reversible inhibition of spontaneous and tone-evoked activity in CN neurons, and this inhibition can be manipulated in each case by using the pharmacologically appropriate compounds, indicating that postsynaptic receptors sensitive to both GABA and glycine are present (Caspary et al., 1993, 1994). Elevated levels of glycine, GABA, and related enzymes have been found in all three divisions of the CN (Godfrey et al., 1978, 1988), as have mechanisms for the high-affinity uptake and calcium-dependent release of both neurotransmitters (summarized in Potashner et al., 1993). None of these activities is diminished by cochlear ablation, indicating that they most likely originate from noncochlear sources (Potashner et al., 1985; Staatz-Benson and Potashner, 1988). The neurotransmitters and postsynaptic receptors have been localized cytochemically to synapses with Gray's type 2 morphology (summarized in Altschuler et al., 1993), which are abundant in all three divisions of the CN and have been shown to survive cochlear ablation (summarized in Saint Marie et al., 1993).

Sources of noncochlear projections to the CN, from which the GABAergic or glycinergic synapses in the CN may originate, include some intrinsic connections within the CN itself, commissural projections from the contralateral CN, and bilateral projections from the inferior colliculus (IC) and superior olivary complex (SOC). For example, many of the GABA- and glycine-immunoreactive neurons, which have been described in the CN, are thought to be intrinsic or local interneurons, such as the cartwheel and small stellate neurons of the DCN (Thompson et al., 1985; Peyret et al., 1986, 1987; Wenthold et al., 1986, 1987; Aoki et al., 1988; Osen et al., 1990; Saint Marie et al., 1991; Kolston et al., 1992). Projections from the contralateral CN (Wenthold, 1987; Benson and Potashner, 1990; Saint Marie et al., 1993) and certain intranuclear projections, for example, those from the DCN to the anteroventral cochlear nucleus (AVCN) (Wickesberg and Oertel, 1990; Saint Marie et al., 1991; Kolston et al., 1992), also appear to use glycine as a neurotransmitter. On the other hand, inhibitory projections from the IC to the CN have not been described, even though 20% of the neurons in the central nucleus of the IC contain GABA-like immunoreactivity (Saint Marie et al., 1993; Oliver et al., 1994). By contrast, the periolivary nuclei of the SOC have been shown to contain numerous neurons with high levels of GABA and glycine (Helfert et al., 1989). Previously, we demonstrated that neurons in the SOC that project to the CN (or olivocochlear nucleus neurons [OCN]) form descending projections that have neurotransmitter-selective uptake mechanisms for GABA and glycine (Benson and Potashner, 1990; Ostapoff et al., 1990; summarized in Potashner et al., 1993).

In order to better define the role(s) that these inputs may play in the processing of acoustic information in the CN, we sought to determine which of the descending and commissural projections to the CN from the IC, SOC, and contralateral CN contain GABA or glycine or both and whether the transmitter content of these projections differs depending on their origins and/or targets in the CN.

Abbreviations

ALPO	anterolateral periolivary nucleus
AVCN	anteroventral cochlear nucleus (A in figures)
CN	cochlear nucleus
DCN	dorsal cochlear nucleus (D in figures)
DMPO	dorsomedial periolivary nucleus
DPO	dorsal periolivary nucleus
$GABA^+$	labeled by anti-GABA antibody
$GABA^-$	unlabeled by anti-GABA antibody
GLY^+	labeled by antiglycine antibody
GLY^-	unlabeled by antiglycine antibody
IC	inferior colliculus
LSO	lateral superior olivary nucleus
LTB	lateral nucleus of the trapezoid body
MSO	medial superior olivary nucleus
MTB	medial nucleus of the trapezoid body
nVII	facial nucleus
OCN	olivocochlear nucleus, periolivary neurons projecting to the cochlear nucleus
PPO	posterior periolivary nucleus
PVCN	posteroventral cochlear nucleus (P in figures)
SOC	superior olivary complex
V	spinal trigeminal tract
VTB	ventral nucleus of the trapezoid body
VII	facial nerve root

MATERIALS AND METHODS

Ten albino guinea pigs (Buckberg, NJ) received injections of either 20% horseradish peroxidase (HRP), 2% HRP conjugated to wheat-germ agglutinin (WGA-HRP), or both into the CN (Table 1). All procedures were approved by the University of Connecticut Health Center Animal Care Committee and were in compliance with NIH guidelines. Animals were anesthetized with 28–35 mg/kg pentobarbital, supplemented with 1–2 mg diazepam, as indicated by periodic monitoring of withdrawal and corneal reflexes. A partial craniotomy, followed by aspiration of the cerebellar tissue overlying the CN, allowed visualization of the caudal CN. Our largest injections were achieved by introducing a micropipet (20–40 μm tip diameter) into the CN and injecting the HRP at two locations in the ventral CN and at one location in the DCN. To inject only the DCN, a micropipet was visually placed and advanced approximately 0.75 mm into the tissue. To inject only the AVCN, the micropipet was directed down through the cerebellar peduncles, rostral to the DCN, ensuring that the micropipet did not enter the DCN. These latter cases were also

TABLE 1. Summary of HRP Injections into the CN

Case no.	Injection site[1]	Survival time (h)	Tracer conc. (%)	Volume (nl)
CN injections				
20213	**DPA**	24	2 (WGA-HRP)	200
20216	**DPA**	24	20 + 2 (WGA-HRP)	700
21213[2]	d**PA**	24	20	60
DCN injections				
10213	**D**	24	2 (WGA-HRP)	45
10314	**D**	48	20 + 2 (WGA-HRP)	500
20314[3]	**D**a	48	20 + 2 (WGA-HRP)	750
AVCN injections				
10104	**A**	48	20	20
10227	**A**	24	2 (WGA-HRP)	40
20227[3]	**A**	24	2 (WGA-HRP)	35
PVCN injection				
20410[3]	d**P**a	24	2 (WGA-HRP)	20

A/a, anteroventral cochlear nucleus; D/d, dorsal cochlear nucleus; P, posteroventral cochlear nucleus; WGA-HRP, wheat germ agglutinin-horseradish peroxidase complex.
[1]Center of the injection site is marked in bold, encroachment into other subdivisions is indicated by lower case.
[2]Data plotted in Figures 3 and 4.
[3]Data plotted in Figure 5.

used in a previous study of projections from the DCN to the AVCN (Saint Marie et al., 1991). A single injection was made in the posteroventral cochlear nucleus (PVCN) by directing the pipet ventral and lateral to the visualized DCN. All injections were made with a calibrated air-pressure injection system (Picospritzer II, General Valve Corp.).

After survival times of 24–48 hours (Table 1) the animals were transcardially perfused with a mixture of aldehydes (1% paraformaldehyde and 2.5% glutaraldehyde) in 0.1 M phosphate buffer (pH 7.4). The brainstems were dissected and sectioned in the transverse plane at 60 µm (Vibratome, Sorvall). Adjacent sections from two series (at an interval of 600 µm between pairs) were processed with metal-intensified diaminobenzidine (DAB) (Adams, 1981), which generated a black reaction product. One section from each pair was then reacted by using affinity-purified, polyclonal rabbit antisera to bovine serum albumin-glutaraldehyde conjugates of glycine (1:1.6K primary dilution) and the other section reacted by using a similar antisera to GABA (1:4K primary dilution), followed by avidin-biotin peroxidase complex histochemistry (Vector Labs), which generated a brown reaction product. These antisera have been previously characterized (Wenthold et al., 1986, 1987). Sections were mounted on glass slides, cleared, and coverslipped.

Our search criteria for identifying cells in the superior olivary complex that project to the cochlear nucleus was to locate neuronal cell bodies that were bisected by the cut surface of a section. Even cells unreactive to either of the antibodies had sufficient background labeling (light yellow) to allow their identification. Each cell body was observed while focusing the microscope up and down to ascertain whether it contained HRP granules. The fact that the HRP was visible below the level of the immunocytochemical staining made the unequivocal identification of even darkly stained (with antibody) neurons more certain. Ambiguous cases were not included. The labeled cell was then drawn, along with local landmarks (blood vessels, etc.) and its immunoreactivity assessed. The drawing was then aligned with the adjacent section (stained with the other antibody), the cell relocated, the presence of HRP granules confirmed and the immunoreactivity to the other antibody assessed. Cells were discarded if HRP granules could not be confirmed in both bisected portions. The intensity of the immunostaining was evaluated by visual comparison with the background (nonspecific) staining present in adjacent nuclei in the same sections in which few, if any, neurons were immunoreactive (e.g., facial nucleus, medial superior olivary nucleus). Control sections, in which the primary antiserum was eliminated or replaced by nonimmune rabbit serum or in which the primary antiserum was preadsorbed with antigen, showed no labeling above background (data not shown). Four categories of staining were observed: Cell bodies could be stained by only GABA ($GABA^+/GLY^-$), only glycine($GLY^+/GABA^-$), both antibodies ($GLY^+/GABA^+$), or neither antibody ($GLY^-/GABA^-$).

Proportions of OCN neurons (i.e., SOC neurons projecting to the CN) were based on the number of retrogradely labeled cells within each nucleus, divided by the total number of OCN neurons in each animal. These proportions were used for analyses of variance followed by corrected t tests to determine differences in the distribution and immunoreactivity between different experimental (injection site) groups.

Periolivary nomenclature

Schofield and Cant (1991) have provided a detailed description of the periolivary nuclei in the pigmented guinea pig by using classical Nissl and Golgi methods as well as cytochrome oxidase staining. Since they compared the published nomenclature for several species and drew analogies between the different classification schemes, we will simply indicate here the overlap between the subdivisions used in the present report and those described by them. The nomenclature we use for the periolivary nuclei includes posterior periolivary (PPO) (Schofield and Cant's posteroventral periolivary nucleus); lateral nucleus of the trapezoid body (LTB); anterolateral periolivary nucleus (ALPO) (Schofield and Cant's ALPO plus rostral and dorsolateral periolivary nuclei); dorsal periolivary nucleus (DPO); dorsomedial periolivary nucleus (DMPO) (Schofield and Cant's superior paraolivary nucleus); medial nucleus of the trapezoid body (MTB); and ventral nucleus of the trapezoid body (VTB) (Schofield and Cant's VTB plus the anteroventral and ventromedial periolivary nuclei). In order to retain comparability between the present report and our previously published reports (Benson and Potashner, 1990; Ostapoff et al., 1990; Saint Marie and Baker, 1990; Potashner et al., 1993; Saint Marie et al., 1993) we will retain our own nomenclature, which is based on transverse sections using Nissl and immunocytochemical labeling.

RESULTS

Large injections of tracers in the DCN or the ventral cochlear nuclei produced large numbers of retrogradely labeled neurons bilaterally in the IC and periolivary nuclei of the SOC. A much smaller number of neurons also labeled in the opposite CN. Though small in number, the projection from the opposite CN was almost exclusively glycine-immunoreactive, as reported previously (Wenthold, 1987; Saint Marie et al., 1993). By contrast, we examined 189 retrogradely labeled IC neurons and only 3 were GABA-immunoreactive and none were glycine-immunoreactive. A very large proportion of retrogradely labeled periolivary neurons in the SOC were immunoreactive for GABA or glycine. Details of the topography and

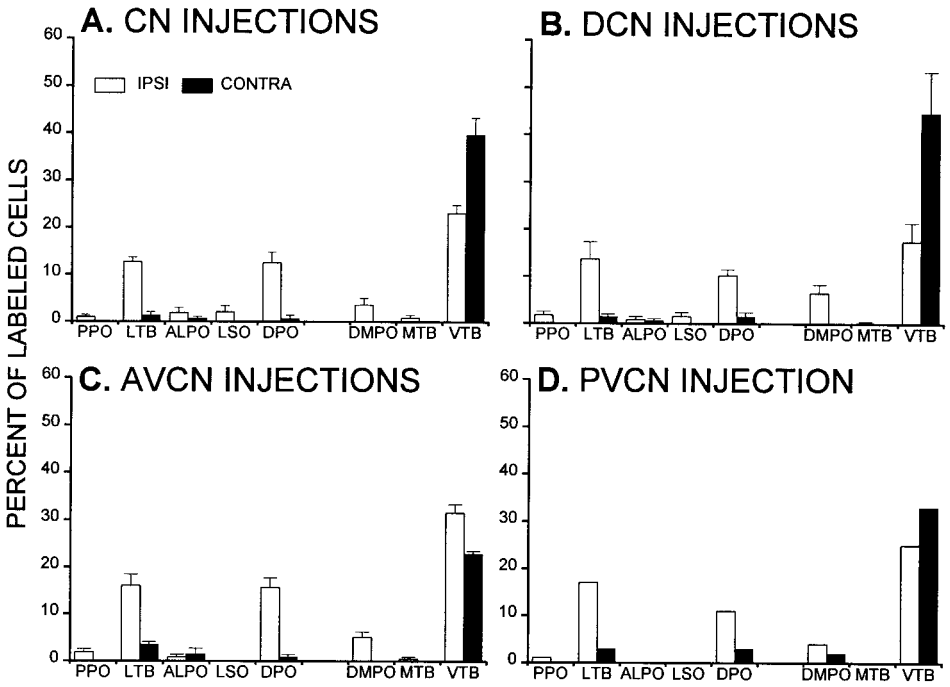

Fig. 1. Distribution of retrogradely labeled cells in the superior olivary complex (SOC) following horseradish peroxidase (HRP) injections that included: (**A**) portions of all three divisions of the cochlear nucleus (CN); (**B**) dorsal cochlear nucleus (DCN); (**C**) anteroventral cochlear nucleus (AVCN; n = 3 for each group), and (**D**) posteroventral cochlear nucleus (PVCN; one case only). The mean percent of all retrogradely labeled cells in the SOC (determined for each individual animal) ± SEM is shown. The ratios of ipsilaterally projecting to contralaterally projecting olivocochlear nucleus (OCN) neurons were 58:42 for the CN group, 55:45 for the DCN group, 70:30 for the AVCN group, and 61:39 for the PVCN case. The total number of retrogradely labeled OCN cells was A = 720, B = 293, C = 137, D = 223.

transmitter immunoreactivity of this important descending projection of the SOC follow.

Distribution of OCN neurons

Figure 1 shows the distribution of retrogradely labeled cells in the SOC after large injections that included all three divisions of the CN (Fig. 1A), and smaller injections centered in the DCN (Fig. 1B), AVCN (Fig. 1C), or PVCN (Fig. 1D). After large injections, approximately 58% of the retrogradely labeled cells were located on the ipsilateral side. These were located mainly in the LTB, DPO, and VTB (with 13%, 13%, and 23% of all OCN neurons, respectively). Most of the retrogradely labeled cells on the contralateral side were located in the VTB (40% of the total).

After injections that were centered in the DCN, 52% of the retrogradely labeled cells were located ipsilaterally and 48% contralaterally. In this group, proportionately more labeled cells were observed in the contralateral VTB (45%) and fewer in the ipsilateral VTB (17%) than were observed after the large injections. The ipsilateral LTB (14%) and DPO (10%) had slightly larger and smaller proportions of labeled cells, respectively, than after the large injections.

After injections into the AVCN, 71% of the retrogradely labeled cells were located in the ipsilateral SOC. In this group, the ipsilateral VTB had a significantly larger proportion of labeled cells (31%), and the contralateral VTB had a smaller proportion (23%), than did the large-injection and DCN groups. The ipsilateral LTB (16%) and DPO (16%) had slightly larger proportions of labeled cells than did the large-injection and DCN groups. In the single PVCN case, 60% of the labeled OCN neurons were located ipsilaterally and 40% contralaterally; 25% of the labeled cells were located in the VTB ipsilaterally and 33% contralaterally. This distribution was similar to the large-injection group and intermediate between the AVCN group and the DCN group. These patterns of labeled cells differed between the injection site groups, with the AVCN group having significantly fewer contralateral and more ipsilateral labeled OCN cells in the VTB ($P < .02$) than the other groups.

Immunocytochemistry of OCN neurons

Examples of OCN neurons that were immunoreactive for either GABA or glycine are presented in Figure 2. In general, the antisera dilutions employed in this study produced a light immunoreaction that facilitated the visualization of the HRP granules. The drawback of this approach was that some of the lightest immunoreactive neurons may have been counted as unstained. Also, HRP granules in some of the darkest immunoreactive neurons may have been obscured and therefore undetected. Because of this propensity to produce some false negatives, the proportions of GABA- and glycine-immunoreactive projection neurons reported here may be underestimates. Both large and small periolivary neurons were immunoreactive in this study. No attempt was made to analyze and compare the size distribution of the immunoreactive and nonimmunoreactive OCN neurons because of the limited penetration of the antibodies (~5 μm) and the uncertainty

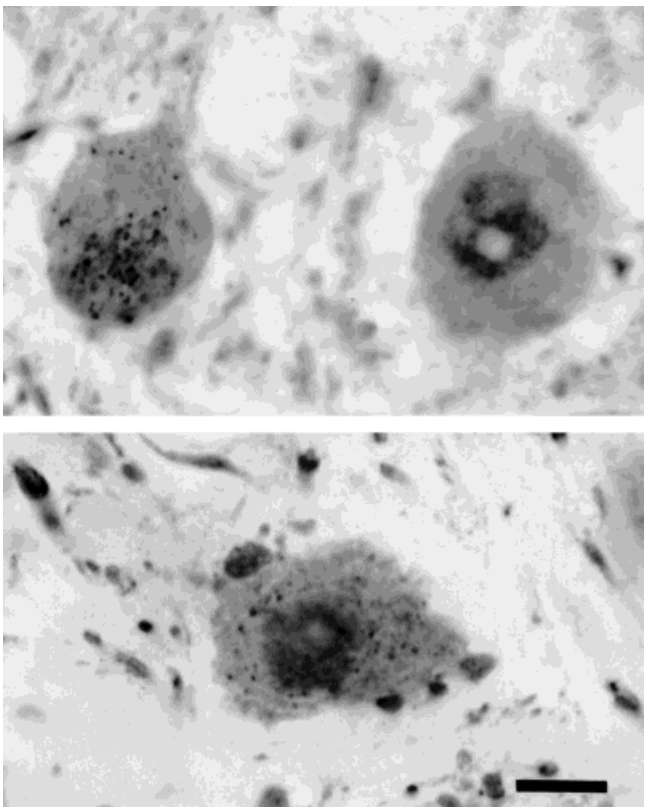

Fig. 2. Photomicrographs of HRP and immunocytochemical staining. **Top:** Two GABA- positive (GABA+) neurons in the dorsal periolivary nucleus (DPO), only the left one contains HRP granules from an injection in the PVCN. **Bottom:** Glycine-positive (GLY^+) OCN neuron in the lateral nucleus of the trapezoid body (LTB). Images were digitized from 35mm slide film (Kodak Ektachrome 64T) by using a 2700 dpi slide scanner (Polaroid SprintScan) digitally enhanced to maximize contrast using Adobe PhotoShop (v 3.0), and printed at 300 dpi with a Kodak XLS 8600 dye-sublimation printer. Scale bar = 10 μm for both.

of delineating the boundaries of the nonimmunoreactive neurons in this tissue.

Data from the three cases with large injections that included parts of all three CN subdivisions are summarized in Figures 3 and 4, along with plots showing the distribution of immunoreactive and nonimmunoreactive OCN neurons for one of those cases. Immunoreactive and nonimmunoreactive OCN neurons were found in nearly all the periolivary nuclei, regardless of the location or size of the injection site.

If one considers each series of sections alone, as if only one antibody had been available, 51% of all the OCN neurons were glycine immunoreactive for the cases with large CN injections (Fig. 3). The proportion of glycine-immunoreactive neurons projecting to the ventral CN (AVCN and PVCN) was larger (64% and 61% of all OCN neurons, respectively) than that projecting to the DCN (51%). In the cases with large CN injections (Fig. 4), 55% of all OCN neurons were GABA-immunoreactive. Again, projections to DCN and AVCN differed, with DCN receiving a smaller GABA projection (48% of total OCN neurons) and AVCN a larger one (62%). Neurons labeled by both antibodies (see below) are included in the above percentages for each antibody, hence the total of more than 100%.

Approximately 70% of the OCN glycine-immunoreactive neurons were located in the ipsilateral nuclei. This proportion was higher for populations projecting to the ventral CN (75%), and lower for those projecting to the DCN (61%). The highest proportions found in the ipsilateral SOC were located in the LTB (average of 19% of the total number of glycine-positive neurons in all cases), DPO (15% of the total number of glycine-positive neurons), and VTB (23% of the total number of glycine-positive neurons). Conversely, OCN neurons that were not stained by the glycine antibody were more evenly distributed on both sides. Of all the unstained OCN neurons in sections treated with the glycine antibody, 51% were found on the ipsilateral side and 49% on the contralateral side.

The distribution of GABA-positive neurons was different from that of the glycine-positive neurons, with nearly equal proportions projecting from ipsilateral (49.9% of the total number of GABA-positive OCN neurons) and contralateral (50.1%) nuclei, for the large CN injection group (Fig. 4). Again, projections to DCN and AVCN differed, with DCN receiving a greater GABA-immunoreactive contralateral projection (56% of the total number of GABA-positive OCN neurons) and AVCN a stronger ipsilateral one (65%). In the large-injection group, nearly 70% of the OCN neurons that did not label with the anti-GABA antibody were found ipsilaterally. Together, the ipsilateral and contralateral VTB's provided 54% of all the glycine-immunoreactive neurons and 72% of all the GABA-immunoreactive neurons projecting to the CN in the large-injection group.

Colocalization of GABA and glycine in OCN neurons

We examined the immunoreactivity of OCN neurons that were bisected by the sectioning process on opposing surfaces of the adjacent sections to determine whether individual OCN neurons contained both GABA and glycine. Figure 5 illustrates the distribution of immunocytochemical staining of OCN neurons after injection of the DCN, AVCN, or PVCN. Although there are differences in the absolute number of OCN neurons depending on the location and size of the injections, the same general trends described above and illustrated in Figures 3 and 4 are evident in these three cases.

Figure 6 shows the cumulative percentage of all OCN neurons for the four nuclei that contribute more than 5% of the total OCN projections (LTB, DPO, and ipsi- and contralateral VTB) and their immunoreactivities. In general, it may be seen that for all of the injection-site groups, the bulk of OCN neurons that are immunoreactive only for glycine were found in the ipsilateral nuclei (19% of total OCN neurons vs. only 6% located contralaterally) Conversely, the majority of the GABA-only-immunoreactive OCN neurons were found in the contralateral VTB (14% vs. 10% of total OCN neurons located ipsilaterally). In addition, it appears that the AVCN group may have proportionately more OCN neurons that are simultaneously immunoreactive to both GABA and glycine (42% of all OCN neurons in that group) than the other groups (range: 25–27%). An analysis of variance followed by a Duncan test ($P < .05$) showed that the VTB on the contralateral side had significantly more GABA-only-immunoreactive OCN neurons than any of the ipsilateral nuclei. Overall, 80% of the OCN projections were immunoreactive for one or both transmitters (Table 2). The propor-

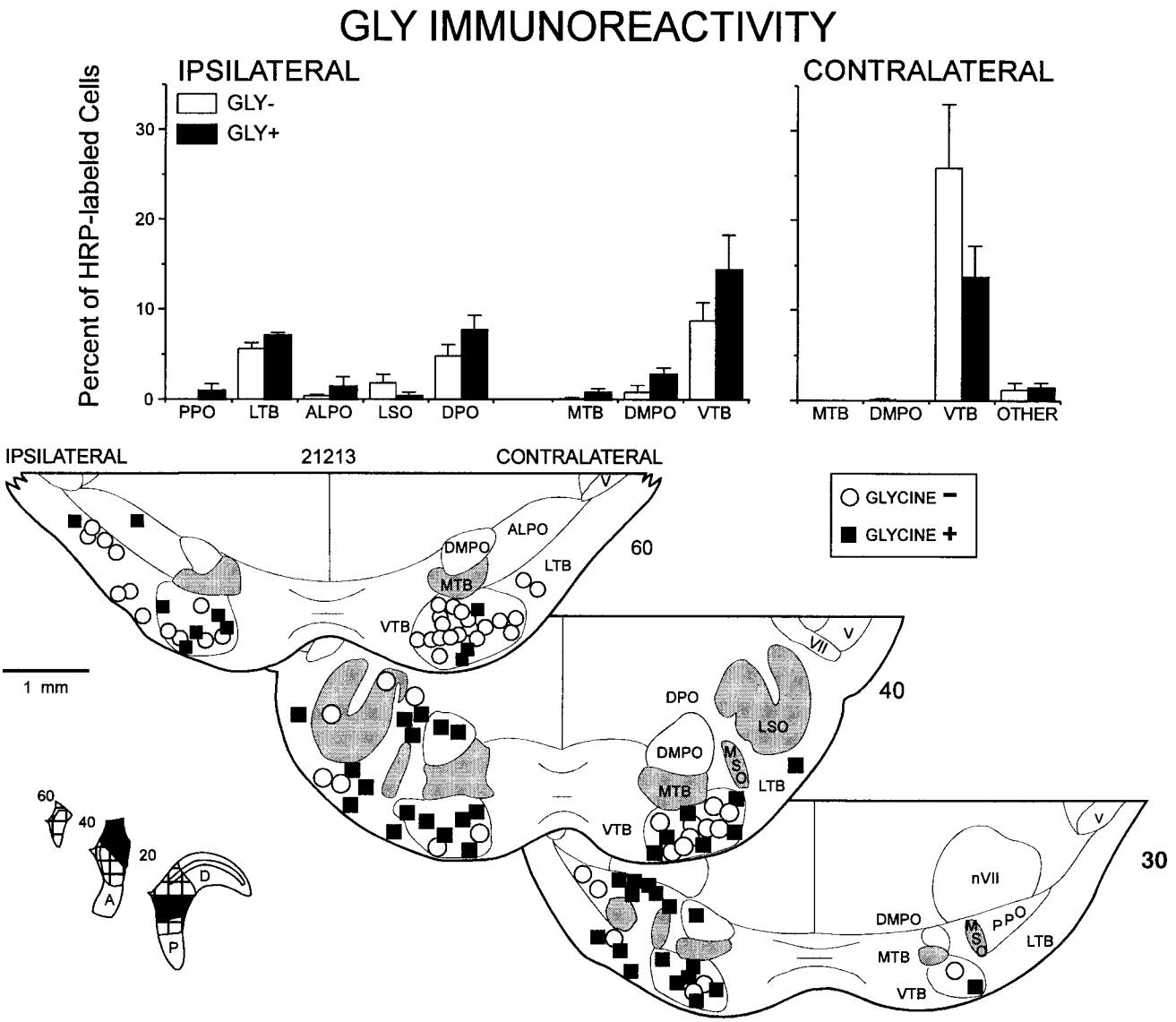

Fig. 3. Distribution of OCN neurons that were labeled or unlabeled with antibodies to glycine. Histograms across the top show the means (±SEM) of the percent OCN GLY^+ and GLY^- neurons in each nucleus from the three animals with large CN injections. The periolivary nuclei are grouped into ipsilateral and contralateral nuclei. "Other" includes the small number of OCN cells lateral to the MSO on the contralateral side. Below these are plots of the distribution and immunoreactivity of OCN neurons in the SOC in case 21213 from this group. The injection site is shown in inset, lower left: the central core of injection is black; lighter, diffuse labeling is shaded. The most rostral section is upper left.

tion was greatest for OCN neurons projecting to the ventral CN (85–92%) and least for those projecting to the DCN (74%).

Figure 7 shows the same data replotted as a percentage of the different immunoreactivities based on the number of OCN neurons found in each nucleus. Calculated this way, the dominance of the OCN neurons located in the ipsilateral nuclei that were immunoreactive to only the glycine antibody is even more obvious. For example, over 53% of the OCN neurons (overall average) in the LTB (range: 39–71%) were labeled only by the glycine antibody. Equally evident is the predominance of OCN neurons in the contralateral VTB that were labeled only by the GABA antibody (overall average: 37.5%; range: 30–52%).

DISCUSSION

The results of this study confirm that major projections to the CN in the guinea pig arise bilaterally from the periolivary regions of the SOC, and from the inferior colliculus and the contralateral CN. The SOC may be the most important source of extrinsic inhibitory inputs to the CN because the overwhelming majority of GABA-positive and glycine-positive neurons that projected to the CN were periolivary. Overall, 80% of the OCN neurons were GABA-positive or glycine-positive, and both types were found in all periolivary nuclei. The principal source of glycine-positive inputs to the DCN and ventral CN was the ipsilateral SOC, while the principal source of GABA-

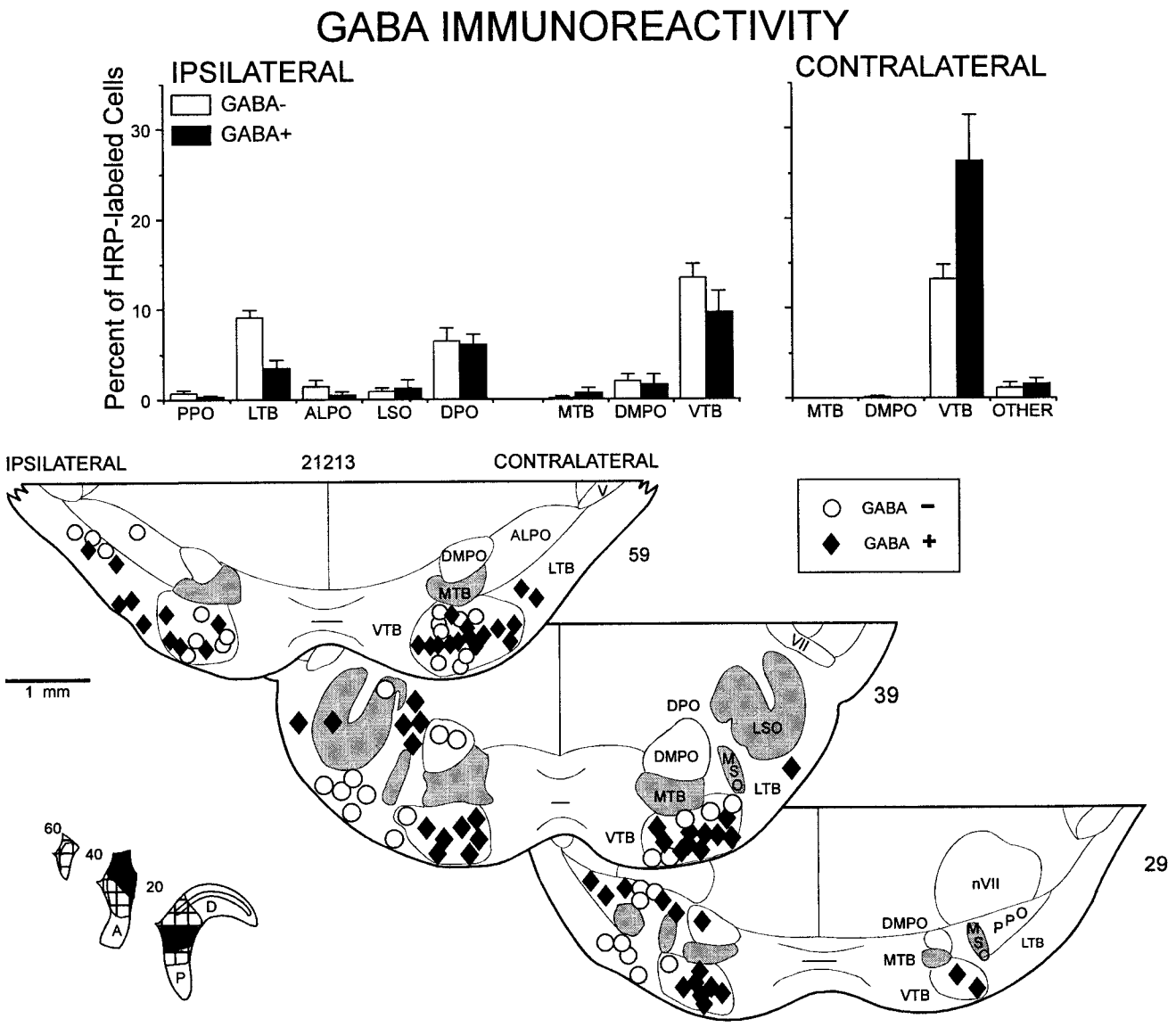

Fig. 4. Distribution of OCN cells in the SOC that are labeled or unlabeled with antibodies to GABA. Layout is similar to Figure 3.

positive inputs was the VTB bilaterally. Some OCN neurons contained both transmitter substances and others contained neither. Both of the latter types were found throughout the SOC but were especially prevalent in the VTB bilaterally. The periolivary inputs to the AVCN were different from those to the DCN. Inputs from the ipsilateral SOC were more pronounced in AVCN, and those from the contralateral VTB were more pronounced in DCN.

Origins of projections to the CN

The sources of noncochlear inputs to the CN in guinea pigs reported here are largely consistent with those reported by others (Winter et al., 1989; Shore et al., 1991), and include the IC and SOC bilaterally and the CN contralaterally. In particular, we observed a similar contralateral bias in the distribution of retrogradely labeled cells in the VTB after injections into the DCN as compared to injections into the ventral CN. By contrast, the OCN projections reported in cats (Adams, 1983; Spangler et al., 1987) appear somewhat dissimilar to those in the guinea pig. It is clear from our data and that of Shore and colleagues (1991) that there is a numerically larger population of OCN neurons in the VTB and a relatively smaller one in the LTB in the guinea pig than in the cat. Also, the preferential projection of the contralateral VTB to the DCN is not so striking in the cat as in the guinea pig. The functional consequences of these differences in projections between species are not presently clear.

SOC may be the main source of extrinsic inhibition in the CN

Studies in several species have shown that the cochlear nucleus receives substantial inputs from other auditory

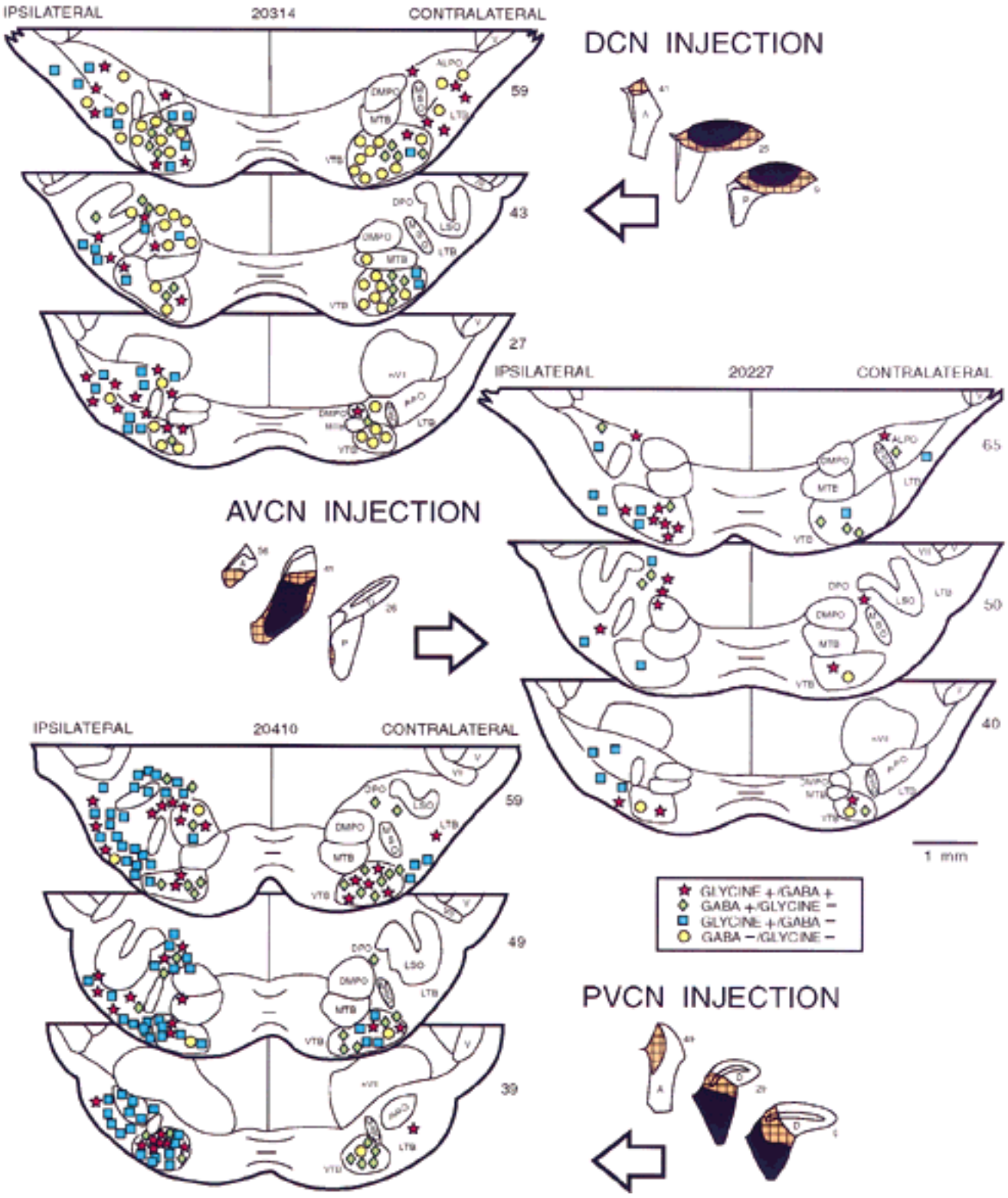

Fig. 5. The distribution of OCN cells in the SOC after injection of HRP into the DCN (**top**), AVCN (**middle**), and PVCN (**bottom**). Only OCN neurons identified on the cut surface between two adjacent sections, one stained with GABA antibody and the other with GLY antibody, are included. Red stars, location of cells that were labeled by both antibodies; green diamonds, cells with only $GABA^+$ staining; blue squares, cells with only GLY^+ staining; yellow circles, location of cells labeled by neither antibody. The injection sites are illustrated by the appropriate insets, similar to Figures 3 and 4.

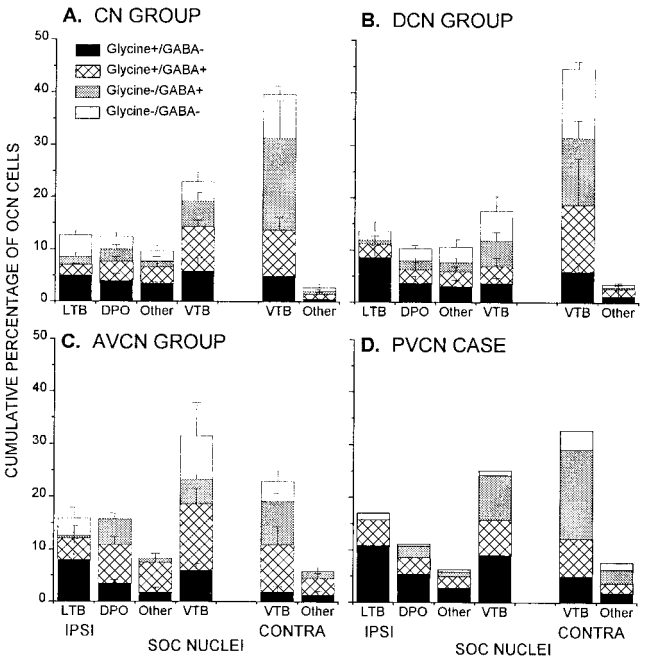

Fig. 6. Cumulative percentages (±SEM) of immunocytochemically labeled and unlabeled OCN cells in the three injection site groups **(A–C)** and the PVCN case **(D).** Shown are the four SOC nuclei that contributed more than 5% of the total OCN projection neurons. Data for the other SOC nuclei are combined into ipsilateral and contralateral "other" bars.

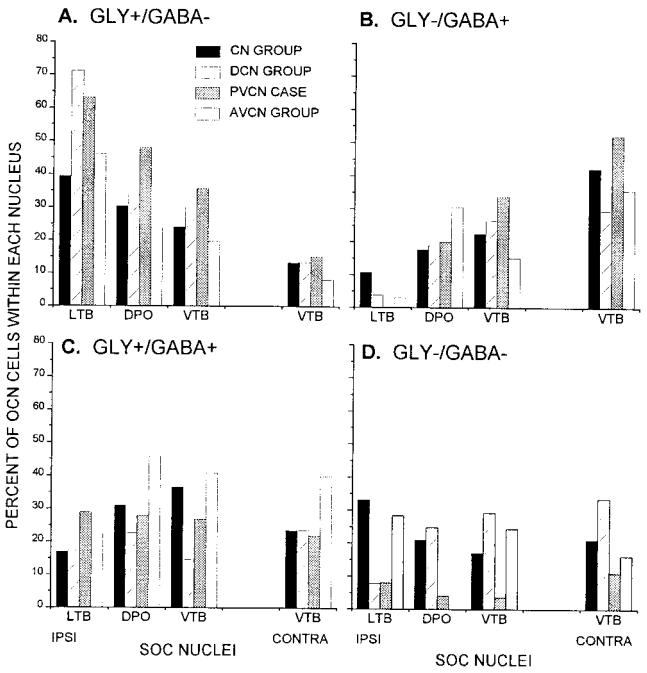

Fig. 7. Immunocytochemistry of OCN neurons. Each panel **(A–D)** shows the number of immunocytochemically labeled neurons of each type in each nucleus divided by the total number of OCN neurons in the same nucleus (expressed as a percentage) for the three groups and the PVCN case. Only those nuclei with more than 5% of the total number of OCN neurons are shown. The general trends of high levels of glycine-positive labeling ipsilaterally and high levels of $GABA^+$ labeling contralaterally are evident. Proportionately more cells projecting to the AVCN tended to be positive to both antibodies. An increased percent of cells projecting to the DCN did not label with either antibody ($GLY^-/GABA^-$).

TABLE 2. Percent[1] of OCN Cells Labeled by anti-GLY and/or anti-GABA Antibodies[1]

	CN	DCN	AVCN	PVCN	All cases
Total OCN neurons	720	293	137	223	1,373
$GLY^+/GABA^-$	24 (5.0)	26 (4.9)	23 (5.2)	35	25 (4.6)
$GABA^+/GLY^-$	28 (8.1)	22 (4.1)	20 (2.4)	31	24 (5.0)
$GLY^+/GABA^+$	27 (3.4)	25 (6.1)	42 (8.3)	26	31 (6.3)
$GLY^-/GABA^-$	21 (5.5)	26 (6.4)	15 (9.6)	8	20 (7.0)
Total inhibitory	79	74	85	92	80

GABA, γ-aminobutyric acid; GLY, glycine.
[1]The number of OCN neurons in each antibody labeling category divided by total OCN neurons per animal, averaged for each injection-site group (±SEM) × 100.

structures, including small inputs from the auditory cortex and the contralateral cochlear nucleus, and larger inputs from the inferior colliculi and the superior olivary complexes on both sides (Elverland, 1977; Cant and Gaston, 1982; Adams, 1983; Covey et al., 1984; Spangler et al., 1987; Winter et al., 1989; Shore et al., 1991; Feliciano et al., 1995). There is evidence from immunocytochemistry as well as measurements of synaptic uptake and release that projections from the contralateral cochlear nucleus may be glycinergic (Wenthold, 1987; Benson and Potashner, 1990; Saint Marie et al., 1993). This projection is diffuse, innervating each of the principal subdivisions of the CN, that is, DCN, PVCN, and AVCN. However, as their numbers are small, these neurons are unlikely to be a major source of GABA and glycine in the CN.

Projections from the inferior colliculi, on the other hand, are primarily to the DCN and granule cell layer of the ventral CN (e.g., Rasmussen, 1964; Adams and Warr, 1976; Conlee and Kane, 1982; Caicedo and Herbert, 1993). Ablation of the IC does not affect the synaptic uptake and release of either GABA or glycine in the CN (Bergman et al., 1989). The present study provides additional evidence that the transmitter used by this pathway is neither GABA nor glycine. This is consistent with recent findings that suggest projections from the IC to the CN may be glutamatergic and excitatory (Saint Marie, 1996).

Studies that used transmitter-specific uptake of [^{3}H]GABA or [^{3}H]glycine (Benson and Potashner, 1990; Ostapoff et al., 1990) have suggested that GABAergic and glycinergic projections from the superior olive to the CN are substantial, but the magnitude of these putative, inhibitory projections, relative to the total olivary projection, could not be determined. In the present study, we find that the olivary projection is not only substantial, but also largely GABA- or glycine-immunoreactive. More than 90% of the projection in some cases (mean: 80%) was immunoreactive for one or both putative inhibitory transmitters. The relative contributions of the different periolivary nuclei in this study followed patterns similar to those transporting [^{3}H]GABA or [^{3}H]glycine, in that the principal contributors to these projections were the LTB, VTB, and DPO, ipsilaterally, and the VTB, contralaterally. Projections transporting glycine arose primarily from ipsilateral nuclei (Benson and Potashner, 1990) and those transporting GABA originated in the VTB bilaterally (Ostapoff et al., 1990). The previously reported GABA contribution of the contralateral VTB was not as large as suggested by the present study, but this may have been related to the

distance of that locus from the injection site, the time required for axonal transport, and the lability of [^{3}H]GABA.

Colocalization of GABA and glycine

OCN neurons that contained both GABA and glycine immunoreactivity comprised 31% of all the labeled OCN projections and 39% of the immunoreactive OCN neurons in this study. These were especially prevalent after AVCN injections. This is consistent with reports of doubly labeled axons and synaptic endings in the AVCN (Wenthold et al., 1987; Kolston et al., 1992; Altschuler et al., 1993). Moreover, immunoreactivity to the glycine postsynaptic receptor has been shown to appose presynaptic endings containing GABA immunoreactivity in AVCN (Oberdorfer et al., 1988). These doubly labeled synapses are unlikely to be of local origin because only occasional colocalization of these transmitters has been reported in small neuronal somata and not in larger cell types in the ventral CN (e.g., Wenthold et al., 1986, 1987; Kolston et al., 1992). In cats, presumed tuberculoventral neurons (those projecting from the DCN to the ventral CN) and their axons stained for both transmitters and may contribute some of these endings (Osen et al., 1990). In guinea pigs, however, tuberculoventral neurons and their axons were glycine-positive but not GABA-positive (Saint Marie et al., 1991; Kolston et al., 1992). In the latter study, GABA and glycine were colocalized in trapezoid body fibers, and this is consistent with the present findings, which suggest that doubly labeled OCN neurons provide many of these endings in the ventral CN.

By contrast, GABA and glycine have been colocalized in large populations of intrinsic neurons in the DCN in both cat and guinea pig (Wenthold et al., 1987; Osen et al., 1990; Saint Marie et al., 1991; Kolston et al., 1992). Additionally, both transmitters have been colocalized in synaptic endings within the terminal fields of cartwheel cell axons (Mugnaini, 1985). These observations, combined with our finding of a proportionally smaller projection of doubly labeled OCN neurons to the DCN, suggest that local connections and not the OCN projections are the major contributor of this type of ending in DCN (Osen et al., 1990).

Colocalization of GABA and glycine in the same neurons and synaptic endings has also been reported in spinal cord (van den Pol, 1985; Taal and Holstege, 1995) and cerebellum (Ottersen et al., 1987, 1988). Several hypotheses have been advanced for why these substances colocalize, but none has yet been established. These include the possibility that one of the substances has a nontransmitter role at the synapse or that one modulates the postsynaptic actions of the other (e.g., GABA modulation of glycine receptors) (Werman, 1980). One of the postsynaptic effects of both substances involves fast-acting chloride channels that differ in their conductance states and duration of channel openings (Hamill et al., 1983; Barker et al., 1986; Bormann, 1988). GABA can also have long-lasting inhibitory effects via GABA-B presynaptic receptors (Thompson and Gähwiler, 1989). Thus, the co-release of both transmitters could result in postsynaptic effects that differ from those produced by either transmitter alone, providing a more refined response. Our finding of substantial numbers of OCN neurons that are immunoreactive to both GABA and glycine suggests it might be interesting to observe the effects of application of combinations of GABA and glycine and their blockers simultaneously on the responses of CN neurons to different kinds of acoustic stimuli.

Possible roles of GABA and glycine in the CN

The responses of neurons in the CN to tonal stimuli show evidence of acoustically driven inhibition (reviewed in Young et al., 1988; Rhode and Greenberg, 1992) in both their driven rate as a function of frequency and intensity (e.g., Evans and Nelson, 1973a, 1973b; Palmer and Evans, 1982) and their pattern of responses over time (e.g., Kiang et al., 1973; Blackburn and Sachs, 1989; Feng et al., 1994). Other possible inhibitory effects on the output of neurons in the ventral CN have been reported in response to more complex stimuli, such as off-CF in combination with CF tones (Blackburn and Sachs, 1992), responses to band-stop masking noise in combination with CF tones (Winter and Palmer, 1990) and forward masking responses in the CN (Shore, 1995), which may be altered by interruption of centrifugal projections to the CN (Shore, 1996). This latter result is particularly intriguing in light of this report. If inhibitory inputs contribute to the effects of forward masking in the ventral CN then the GABA-positive and glycine-positive projections described here would certainly appear to be the prime candidates for the sources of that inhibitory input.

Iontophoretic application of pharmacological agonists or antagonists of inhibitory transmitters has been used to associate specific transmitters with some of these inhibitory phenomena. For example, Evans and Zhao (1993) have shown that side-band inhibition, important for spectral contrasts and dynamic range, is probably mediated by glycine in the DCN. Glycine affects the ongoing discharge rates within the excitatory response areas of many neuronal types in the ventral CN, including PVCN phasic-firing neurons and AVCN neurons with chopper and primarylike response patterns (Palombi and Caspary, 1992; Caspary et al., 1993, 1994). This has been hypothesized to act either to control dynamic range and/or detection of signals in noise. $GABA_A$ receptors are responsible for a tonic inhibition found in the DCN, which alters the spontaneous activity and may be important for adjusting excitatory-inhibitory contrasts (Evans and Zhao, 1993). In contrast, $GABA_A$ receptors have more prominent effects on the responses to off-characteristic frequency stimuli in a majority of the AVCN primarylike neurons tested (Caspary et al., 1994). Some of these inhibitory phenomena are thought to arise from intrinsic connections, especially in the DCN (e.g., Wickesberg and Oertel, 1990; Evans and Zhao, 1993). The GABA-positive and glycine-positive projections from the SOC to the CN described here may account for other of the above inhibitory effects on the responses of CN neurons. In addition, it is possible that these OCN projections may serve a modulatory role on acoustic processing in the CN by either $GABA_B$ receptors or other secondary messenger systems yet to be elucidated.

Subpopulations of OCN neurons may participate in different functional circuits

Axons from different cell types in the CN project to different subsets of periolivary nuclei (Borg, 1973; Warr, 1982, 1992; Tolbert et al., 1982; Covey et al., 1984; Friauf and Ostwald, 1988; Smith et al., 1991, 1993a, 1993b). In addition to their well-known projections to the MTB, LSO, and MSO, axons from both globular and spherical bushy cells project to the ipsilateral LTB and the contralateral

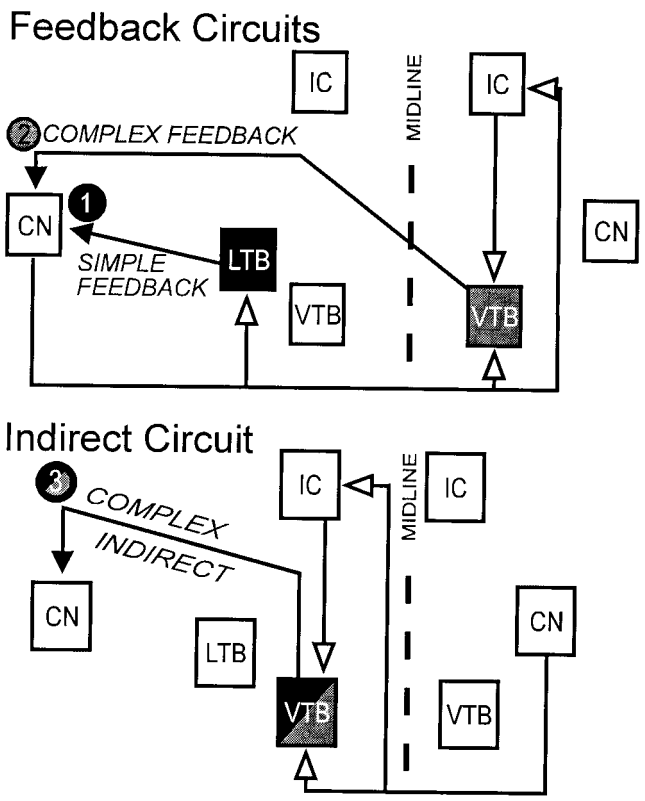

Fig. 8. Three hypothetical OCN circuits differentiated by their possible innervation by the cochlear nucleus and inferior colliculus and the transmitter that they might use. Possible glycinergic projections are filled in black; possible GABAergic projections are shaded gray. See text for additional details.

VTB, and globular bushy cells also project to the ipsilateral PPO and DLPO nuclei and the contralateral DMPO nucleus (Tolbert et al., 1982; Friauf and Ostwald 1988; Smith et al., 1991, 1993a). At least some types of multipolar cells in the CN appear to be a major source of inputs to the contralateral VTB (Smith et al.,1993b) and form distinctive patterns of axosomatic and axodendritic endings on periolivary cells (e.g.,Thompson and Thompson, 1991; reviewed in Warr, 1992). Descending innervation from the IC projects heavily to the ipsilateral VTB and DMPO nuclei, but there is only a minor projection to the LTB (van Noort, 1969; Andersen et al., 1980; Henkel and Spangler, 1983).

These varied, overlapping inputs to the periolivary nuclei may form the anatomical bases for functionally distinct OCN circuits (Fig. 8). One such circuit includes cells in the LTB, which receive most of their inputs from the ipsilateral CN to which they also project. This projection is primarily glycine-immunoreactive and may form a *simple inhibitory feedback circuit*. A more *complex inhibitory feedback circuit* may include OCN neurons in the VTB, which receive a large number of inputs from the ipsilateral IC, in addition to those from the contralateral CN, to which they send primarily GABA-immunoreactive projections. Together, these two circuits receive their afferent, presumably excitatory input from the same CN to which they project. A third *complex indirect inhibitory circuit* may include ipsilaterally projecting OCN neurons in the VTB, which receive inputs from the contralateral CN and ipsilateral IC. These are GABA- and/or glycine-immunoreactive and project to the opposite CN than the one from which they receive their inputs. One or more of the inhibitory effects due to stimulation of the contralateral ear (discussed above) may arise from this latter OCN circuit. Further anatomical studies examining both the afferent inputs to OCN neurons and their targets in the CN are required to establish the linkage between these anatomical pathways and their physiological functions.

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