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Morphology of GABAergic Neurons in the Inferior Colliculus of the Cat

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ABSTRACT

The goal of the present study was to provide a comprehensive and quantitative description of neurons immunoreactive for γ-aminobutyric acid (GABA) in the inferior colliculus (IC) of the cat. Neurons were investigated with two different antisera and two different incubation methods. Free-floating frozen or vibratome-cut sections were incubated either with an antiserum to glutamic acid decarboxylase (GAD) or to GABA conjugated to protein with glutaraldehyde. Additional 1.5-μm-thick sections were incubated with the GABA antiserum after embedding and removal of the plastic. Quantitative data were obtained from much of this material. Despite the use of these different antisera and reaction methods, the results obtained were remarkably similar.

The results show that GAD- or GABA-positive neurons represent a significant population of cells in the central nucleus of the IC, up to 20% of the neurons. Most of these neurons have large or medium-sized perikarya. In contrast, immunonegative neurons are medium-sized or small. Many GABA-positive neurons had proximal dendrites or somata oriented in parallel to the fibrodendritic laminae of the central nucleus and are presumed to be disc-shaped neurons. Others have an orthogonal orientation and are presumed to be stellate cells. Large GABA-positive neurons form two groups, those with many axosomatic endings and those with few. Collectively, these observations suggest that there are several types of GABAergic neuron in the central nucleus and, by extension, that these may participate in many types of inhibitory circuits.

Key words: auditory pathways, inhibitory synapses, axosomatic endings, neuropil

Neural responses in the inferior colliculus (IC) may depend upon local circuits. When IC neurons are injected intracellularly in vivo with horseradish peroxidase (HRP), almost every type of neuron elaborates a local axon collateral (Oliver et al., '91). Local axons terminate near the cell of origin and in a symmetric position in the opposite IC (Saldaña and Merchán, '92). Some local axons are surprisingly extensive and could provide a structural substrate for feed forward excitation/inhibition, sharpened tuning, or amplification/suppression of lateral interactions. If these axons are excitatory, their intrinsic connections could supplement the effects of ascending afferent systems. However, if local axons suppress other IC neurons, then the intrinsic collaterals might have an entirely different set of physiologic implications. An essential first step toward understanding the function of the local connections within the IC is to establish which neurons use inhibitory neurotransmitters.

Despite the potential importance of local inhibitory transmission, there have been few studies to identify inhibitory neurons within the IC. Surveys of neurons immunopositive for γ-aminobutyric acid (GABA) or its synthetic enzyme, glutamic acid decarboxylase (GAD), in the gerbil (Roberts and Ribak, '87a), rat (Roberts and Ribak, '87b), guinea pig (Thompson et al., '85), and bat (Winer et al., '94) describe many such neurons. However, only brief reports have related GABA immunoreactivity and neuron type in the cat (Oliver et al., '88; Oliver and Beckius, '89; Paloff et al., '91). GABA immunoreactivity reveals that the transmitter is present in axonal endings (Oliver and Beckius, '92). However, the GABAergic synapses remain to be associated with

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specific types of local neurons or to extrinsic sources that project upon the auditory midbrain (e.g., DNLL). Identification of GABAergic neurons would provide a framework for considering more critically the specific actions of GABAergic circuits.

The goal of the present study was to provide a comprehensive and quantitative description of intrinsic GABAergic neurons and to relate them to cell types distinguished in Golgi studies. To meet this goal, we have pooled the results from three laboratories that have used different methodologies. Neurons were investigated with different types of antisera to GABA and GAD and with different reaction methods. Despite the differences, the results obtained are remarkably similar. We conclude that several types of IC neurons may use GABA as a neurotransmitter and that, together, these neurons represent a substantial proportion of the total neuronal population.

MATERIALS AND METHODS

Neurons in the IC were examined for their ability to bind antibodies to GAD or GABA with either preembedding or postembedding techniques. Material from 11 adult cats was examined in transverse, horizontal, or parasagittal sections.

GAD immunocytochemistry

To show GAD-immunoreactive neurons and axonal terminals, five animals were deeply anesthetized with sodium pentobarbital (35–40 mg/kg, i.p.). Depth of anesthesia was assessed by careful examination of cutaneous nociceptive and corneal reflexes. The protocols for anesthesia and animal care were approved by an institutional animal care committee, and appropriate veterinary supervision was provided in an accredited facility. Animals were perfused through the heart with 500 ml of normal saline followed by 2,500–3,000 ml of 0.5% zinc salicylate in 10% unbuffered formalin at pH 6.5 at 20°C (Mugnaini and Dal, '83). These solutions were delivered at constant pressure via a peristaltic pump or, less often, by gravity. Cold 10% sucrose in normal saline, pH 6.5, was pumped through to displace the fixative 1–2 hours later. The tissue was stored overnight in 30% sucrose dissolved in normal saline, pH 6.5, at 4°C, then sectioned either on a freezing microtome or Vibratome at 25–50 μm. Pairs of adjacent sections were either immunostained or Nissl stained. The procedures for the immunoreaction are similar to those used to study the auditory thalamus of the cat and rat (Winer and Larue, '88, '89; Winer, '91, '92). Sections were incubated in the primary antiserum, GAD-1440 (Oertel et al., '81), at a 1:2,000 dilution and immunostained with either the peroxidase-antiperoxidase (Sternberger and Joseph, '79) or the avidin-biotin (Hsu et al., '81) method. The latter, using a double-bridge technique (Roberts et al., '85), produced superior immunostaining, and the contrast of these sections was further enhanced by the osmium-thiocarbohydrazide-osmium procedure (Willingham and Rutherford, '84).

GABA immunocytochemistry

To show GABA-immunoreactive neurons and axonal terminals, six cats were anesthetized as noted above and perfused with phosphate buffer (50–100 ml, 0.12 M, pH 7.2–7.4, 0.004% $CaCl_2$, 2% sucrose, 0.06% lidocaine) followed by two successively more concentrated mixtures of buffered paraformaldehyde and glutaraldehyde (Oliver and Beckius, '92). Solutions were delivered by gravity at 37°C. Brains remained in the final perfusate (1% paraformaldehyde, 3% glutaraldehyde, and 0.004% $CaCl_2$ in 0.12 M phosphate buffer; pH 7.2–7.4) overnight at 4°C. The next day, the brainstem was sectioned on a vibratome while submerged in chilled phosphate buffer (0.12 M, pH 7.4; 0.004% $CaCl_2$). Sometimes 6% sucrose was added.

For preembedding immunocytochemistry, 50–100-μm-thick tissue slices were placed in 5% normal goat serum in phosphate buffer (1 hour) and then incubated overnight with affinity-purified GABA antiserum diluted 1:1,000–1:16,000 in buffer (Wenthold et al., '86). Binding of the GABA antibodies was detected by biotinylated goat antirabbit IgG and an avidin-biotin-peroxidase complex (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) with diaminobenzidine as the chromagen (Hsu et al., '81). All procedures preceding the peroxidase reaction were performed at 4°C with continuous agitation. Treated sections were sometimes postfixed with buffered 0.5–2.0% osmium tetroxide for 15–120 minutes, stained with 2% uranyl acetate overnight, and flat embedded in Durcupan ACM or Polybed 812 (Polysciences, Inc., Warrington, PA).

For postembedding immunocytochemistry, thick untreated vibratome slices were postfixed in 0.5% osmium tetroxide and flat embedded in Polybed as described above. From these slices, semithin (1.5-μm-thick) sections were cut from the plastic-embedded material and mounted on glass slides. After removal of the plastic and osmium with 10% sodium hydroxide and 0.3% hydrogen peroxide in methanol (Saint Marie et al., '89), the sections were exposed to a blocking solution for 1 hour and then incubated

Abbreviations

I, II, III, IV	layers of the dorsal cortex
B	brachium of the inferior colliculus
C	pars centralis of the central nucleus
CC	caudal cortex of the inferior colliculus
CG	central gray
CN	central nucleus of the inferior colliculus
CL	lateral nucleus of the commissure
CP	cerebral peduncle
DC	dorsal cortex of the inferior colliculus
DM	dorsomedial nucleus
DNLL	dorsal nucleus of lateral lemniscus
HYP	hypothalamus
IcT	intercollicular tegmentum
L	pars lateralis of the central nucleus
LGB	lateral geniculate body
LN	lateral nucleus of the inferior colliculus
M	pars medialis of the central nucleus
MCP	middle cerebellar peduncle
MGB	medial geniculate body
MLF	medial longitudinal fasiculus
MRF	midbrain reticular formation
OR	optic radiation
RP, RPN	rostral pole nucleus of the inferior colliculus
SA	sagulum
SU	subcollicular zone
TM	mesencephalic trigeminal nucleus
V	pars ventralis of the central nucleus
VB	ventrobasal complex
VL	ventrolateral nucleus of the inferior colliculus
VNLL	ventral nucleus of the lateral lemniscus
VT	third ventricle

overnight with the affinity-purified GABA antiserum (diluted 1:500 to 1:2,000 with the blocking solution). Antibody binding was detected by sheep antirabbit IgG and peroxidase antiperoxidase complex (Cappel, Organon Teknika, Rockville, MD). The chromogen for the postembedding immunoreaction was nickel- and cobalt-intensified diaminobenzidine (Adams, '81). In control reactions, the primary antiserum was eliminated, replaced with nonimmune serum, or preadsorbed with antigen; the details have been reported elsewhere (Saint Marie et al., '89). Labeling above background was absent in all control conditions.

All of the cases used for postembedding immunocytochemistry were from previous experiments in which HRP was injected in either an auditory (three cases) or a nonauditory structure (one case). Two of the cases (C1020287 and C1062987) were injected in the contralateral IC and were described previously (Saint Marie et al., '89).

Analysis

Neurons labeled with GAD or GABA antisera were readily distinguished from unlabeled cells. While some differences in the intensity of labeling were seen, these were, in our hands, never related to the type of neuron nor to any particular collicular locus. Therefore, we did not make subjective classifications of cells based on the intensity of immunolabeling. Since the signal-to-noise ratio in our material was quite good (see, e.g., Fig. 6), immunopositive cells were easily distinguished from immunonegative cells. Previous studies (Oertel et al., '81; Wenthold et al., '87) have shown the cross reactivity for these antibodies to below.

Immunoreacted material was examined at high magnification and drawn with a camera lucida. Plots depicting the locations of the labeled cells in the section were drawn at low-power (×20) with a camera lucida. To analyze semithin sections, low-power (×100) drawings were made of all neurons with a visible nucleolus in unreacted, toluidine blue-stained sections of the central nucleus of the IC. An adjacent, immunoreacted, semithin section was used to learn whether each neuron was immunopositive (Saint Marie et al., '89). Cell size and shape were measured by tracing ×500 drawings with a Numonics 2200 digitizer interfaced to an LSI-11 computer equipped with custom software (Oliver, '85; Shneiderman et al., '88; Saint Marie et al., '89). Statistical comparisons of cell size were made with Student's t test.

RESULTS

Distribution of GABAergic Cells in the IC

GABAergic neurons were found in every subdivision of the IC. The distribution of GAD- and GABA-positive neurons is shown in Figure 1. With a few exceptions, the immunopositive neurons were uniformly distributed in the IC and the adjoining subcollicular tegmentum. In the central nucleus, GAD-positive neurons were found in each subdivision (Fig. 1: M, C, L, V). In the dorsal cortex (Fig. 1: I–IV) the most superficial layer (I) had few GAD-positive cells, compared to the deeper cortical layers. The caudal cortex had immunostained cells that were larger and more plentiful (Fig. 1C,D: CC). Outside the central nucleus and cortex, the other subdivisions contained fewer GAD-positive cells (Fig. 1: DM, LN, VL, CL). Some of these differences may be related to the high density of fibers, e.g., layer I of the cortex, the lateral zone (LN),and the commissural nucleus (CL).

GABAergic neurons varied in their concentrations among the different subdivisions. The incidence of immunoreactive neurons was sometimes correlated with the general cell packing density. This is shown in Figure 2, where GABA-positive (solid squares) and GABA-negative neurons (open circles) are shown in a 1.5-μm-thick section. In the central nucleus, GABA-positive neurons represented about 20% of all the neurons (medial subdivision 22%, central subdivision 21%). Only the lateral part of the central nucleus (Fig. 2: L) had a smaller proportion (11%). In the dorsal cortex, the frequency of GABA-positive cells was similar to that in the central nucleus, although the total number of neurons was greater, suggesting that the relative proportion of GABAergic cells was lower than in the central nucleus.

GABA-positive cells formed clusters consisting of four or five somata within 500-μm-wide areas in the central nucleus and in the superficial part of the dorsal cortex. Such clusters also were apparent in both GAD- (Fig. 1A–C) and GABA-labeled (Fig. 1D) preembedded material. Areas as large as 1.0 mm^2 could be devoid of GABAergic cells, even in semithin sections (Fig. 2: below IV).

The remaining observations are limited to the central nucleus. Qualitative and quantitative observations on neuronal size, dendritic orientation, and the patterns of perisomatic endings show that several types of central nucleus neurons are GABAergic.

Somatic size

GABAergic neurons had large, medium-sized, or small perikarya (Table 1), and these corresponded to different neuronal classes recognized in Golgi preparations from mature specimens (Oliver and Morest, '84). This diversity was readily evident in a representative sample of GAD-immunoreactive neurons (Fig. 3) in the central nucleus and was confirmed by measurements of 516 GABA-labeled cell bodies (Fig. 4). Most GABA-labeled neurons had somatic cross-sectional areas between 150 and 650 μm^2. The range of sizes included small (Fig. 3: 13, 26), medium-sized (Fig. 3: 17–19), or large (Fig. 3: 31, 79) cells. Occasionally, neurons up to 900 μm^2 were encountered. In the medial part of the central nucleus, GABA-positive cells averaged 300 μm^2 (Table 2), while, in the central and lateral subdivisions, they were usually larger, approximately 370 μm^2 on average (Table 2). Large neurons also were apparent in GAD-labeled material from the central nucleus, as illustrated in Figure 5, where the largest GAD-positive neurons are shown (Fig. 5: 10, 43, 52, 53). Some smaller GAD-positive neurons were included for comparison (Fig. 5: 6, 24, 55, 71).

When the relative sizes of the GABA-positive neurons from samples reacted with different concentrations of antisera were compared, the sections reacted with the most concentrated antiserum contained the most immunopositive neurons (Fig. 4, 1:1,000). Nevertheless, cell size and distribution were similar within the subdivisions at all dilutions (Fig. 4, Table 2).

A more critical comparison of the GABA-positive and GABA-negative neurons was available in the material prepared for postembedding immunocytochemistry. Both types of cells were readily visualized in such material (Fig. 6). Furthermore, portions of the same neurons were visible in serial, adjacent, 1.5-μm-thick sections stained with tolu-

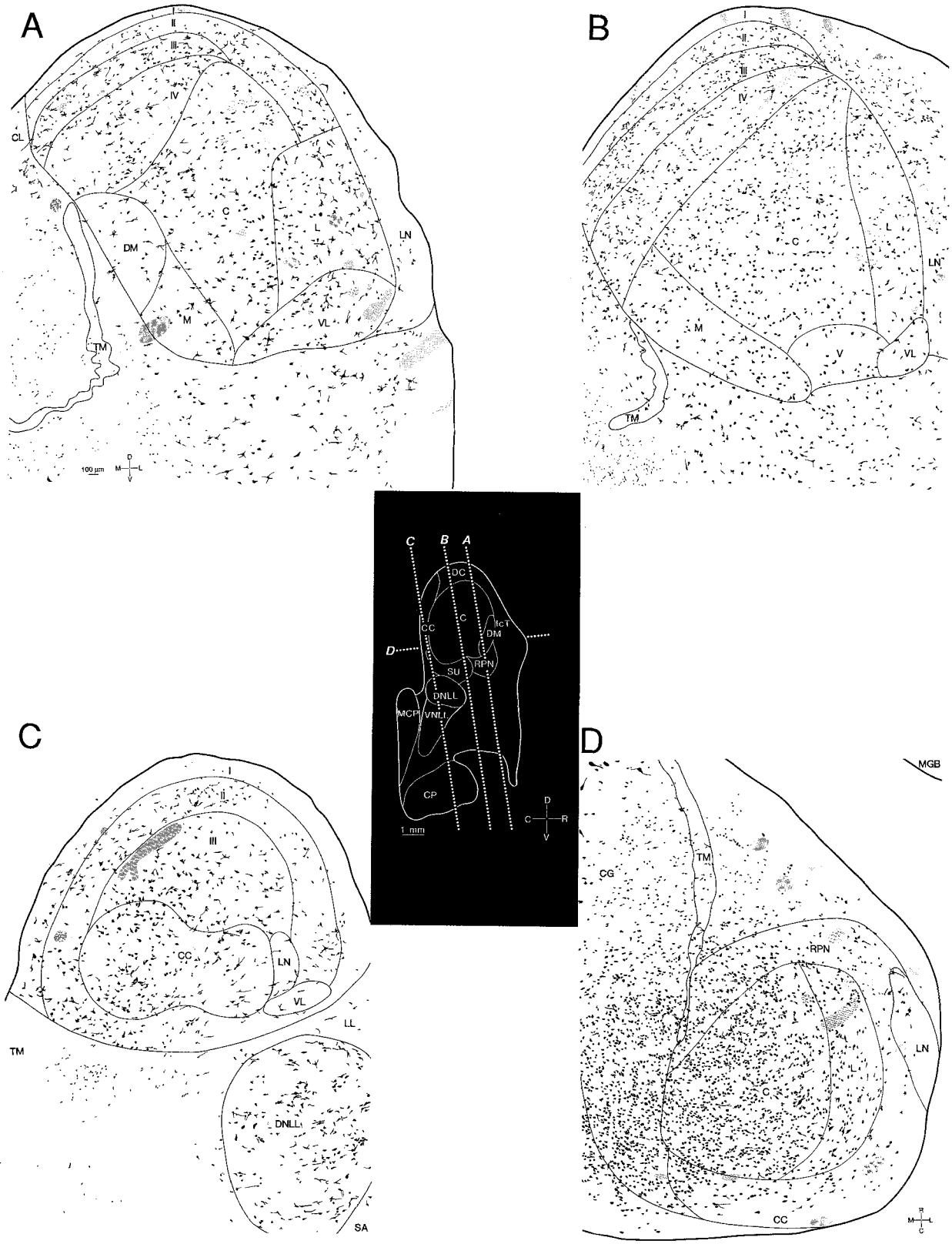

Fig. 1. Location of GABAergic cells in thick slices of the auditory midbrain. Tissue was treated with GAD (**A–C**) or GABA (**D**) antiserum with preembedding techniques. A–C: Each composite drawing shows GAD-positive cells from two consecutive 25-μm-thick sections in the transverse plane. A: Rostral IC. B: Middle IC. C: Caudal IC including the dorsal nucleus of lateral lemniscus. D: Composite drawing of GABA-positive cells from four consecutive 50-μm-thick sections in the horizontal plane. **Inset** shows the approximate locations of A–D projected onto a parasagittal section. Planapochromat, N.A. 0.32. ×200.

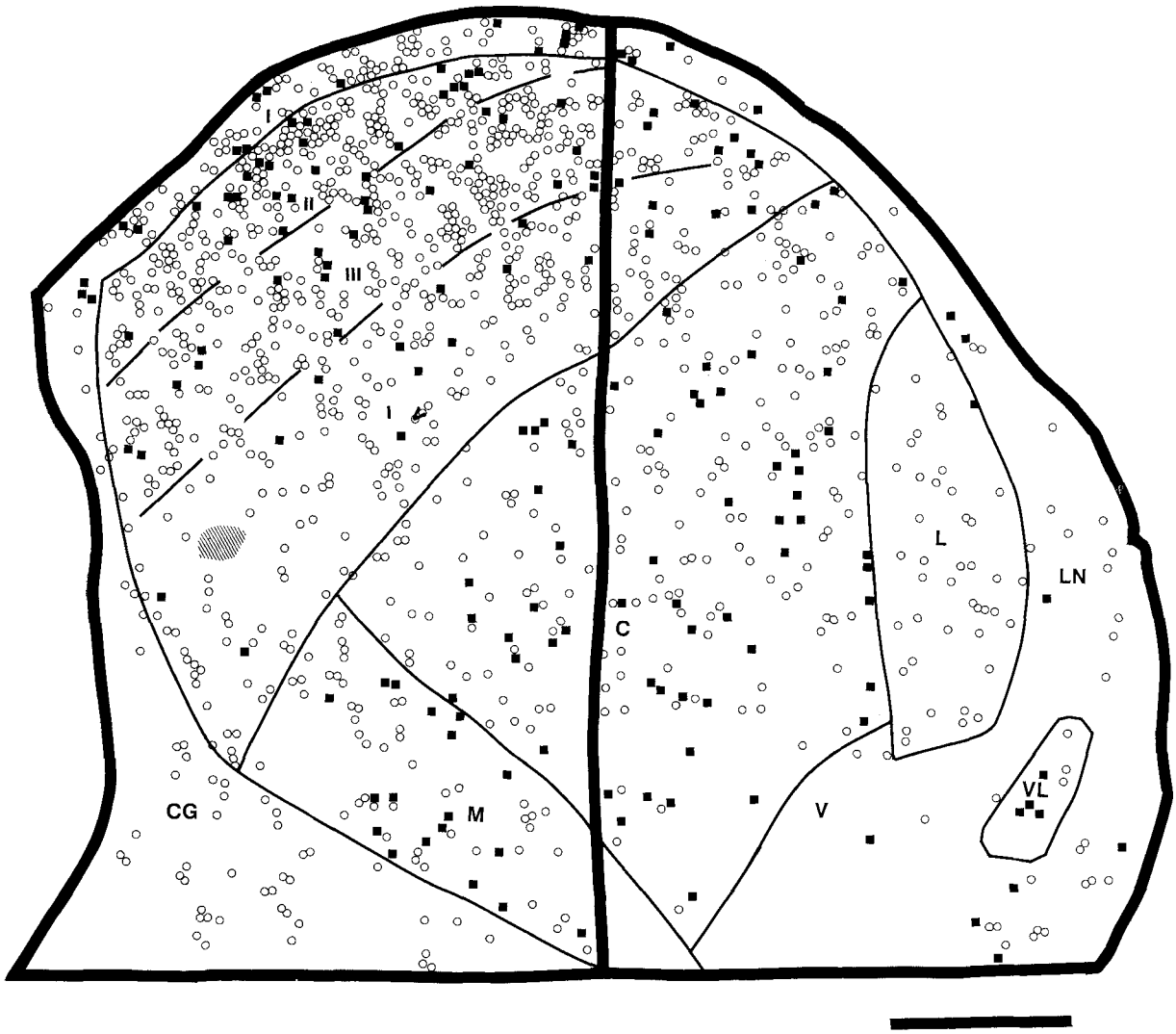

Fig. 2. Location and density of GABA-positive and GABA-negative cells in 1.5-μm-thick plastic sections after postembedding immunoreactions. Two side-by-side sections were made from the surface of the same vibratome slice and, together, represent a complete transverse plane through the inferior colliculus. GABA-positive cells (solid boxes); GABA-negative cells (open circles). Scale bar = 1 mm.

idine blue. Small (Fig. 6A,B), medium-sized (Fig. 6E), and large (Fig. 6G,I) GABA-positive neurons were found, and GABA-negative neurons were often next to the GABA-positive cells (Fig. 6E–H). These populations were easily distinguished from one another even when GABA-positive neurons were lightly stained (Fig. 6G).

A quantitative comparison of the size range of GABA-positive and GABA-negative cells in the central nucleus was made in one complete cross section of the IC in four different animals (Fig. 7, Table 3). Each of the three main subdivisions of the central nucleus was considered (shown in columns: medial, central, and lateral). GABAergic neurons were significantly larger than non-GABAergic cells collectively within the central nucleus and within each of its subdivisions (Table 3). The average cross-sectional areas of immunonegative cells for all subdivisions ranged from 151 to 255 μm^2 for the four animals (Table 3). In contrast, GABA-positive cells were larger and ranged from 392 to 477 μm^2. While medium-sized neurons in these populations overlap, most of the small cells (< 200 μm^2) were GABA-negative and most of the largest cells (> 450 μm^2) were GABA-positive. The same was true in each of the three subdivisions of the central nucleus (Fig. 7).

When data across all subdivisions and all four animals were pooled (Fig. 8), measurements of cross-sectional area (Fig. 8, top) and average diameter (Fig. 8, bottom) showed a similar result. Despite considerable overlap, these populations differed in size. Most GABA-positive neurons ranged from 200 to 800 μm^2 in area, whereas the GABA-negative neurons ranged from 40 to 440 μm^2. GABA-positive cells ranged from 16 to 38 μm in diameter, while the GABA-negative cells ranged from 8 to 27 μm in diameter. That the histograms are unimodal suggests that somatic size alone cannot distinguish between different types of GABAergic cells. We turn now to a consideration of dendritic arrangements as a collateral criterion.

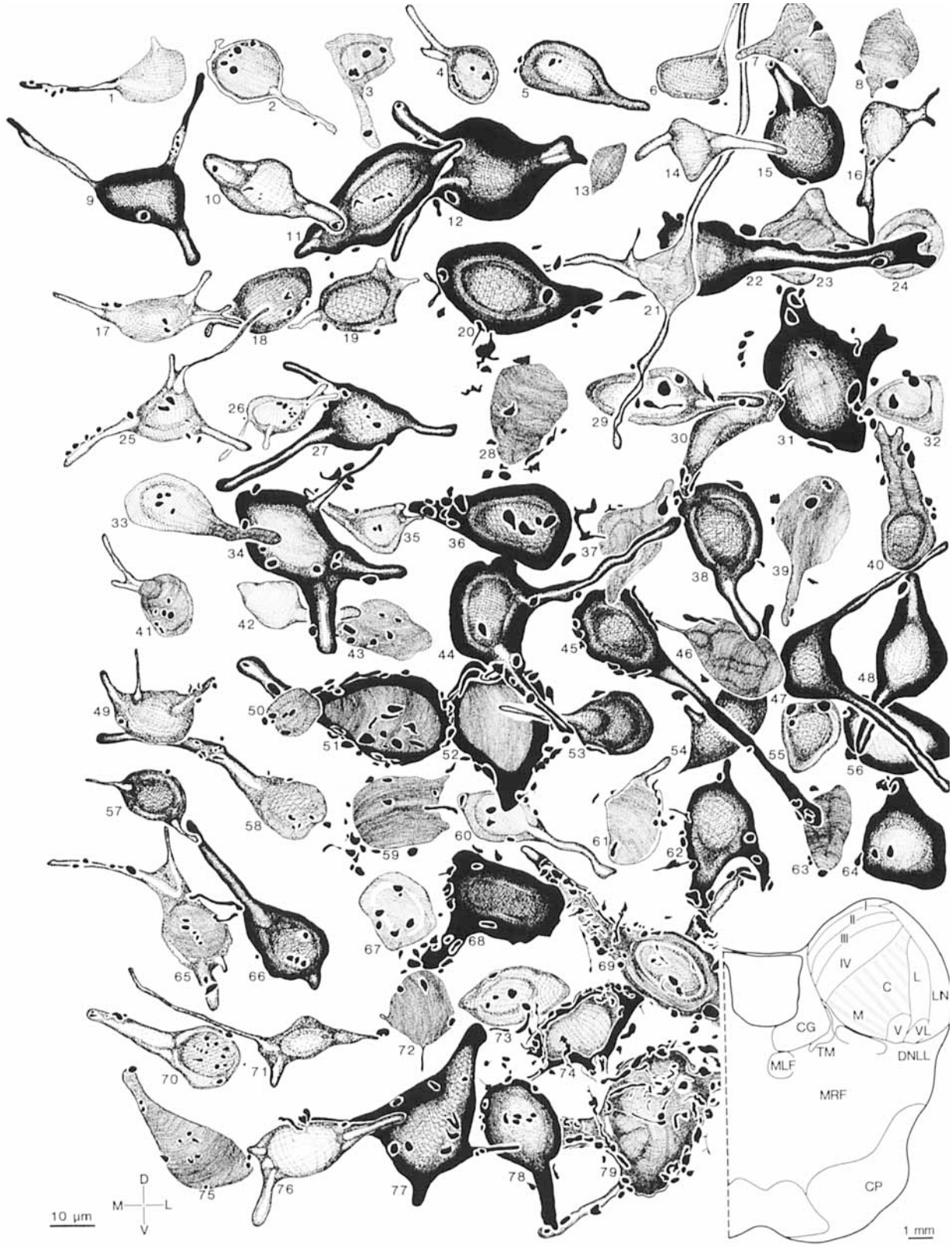

Fig. 3. GAD-positive neurons from pars centralis (C) that show variations in size, somatic and dendritic orientation, and relative number of axosomatic puncta. Large, medium-sized, and small neurons were stained. Some proximal dendrites of the GAD-positive cells are parallel to the dorsomedial to ventrolateral orientation of these laminae, while other dendrites are perpendicular. **Inset** shows the level of inferior colliculus from which these cells were drawn and the orientation of the fibrodendritic laminae in pars centralis (gray bars). Axosomatic puncta are most common on larger cell bodies. Planapochromat, N.A. 1.32, ×2,000.

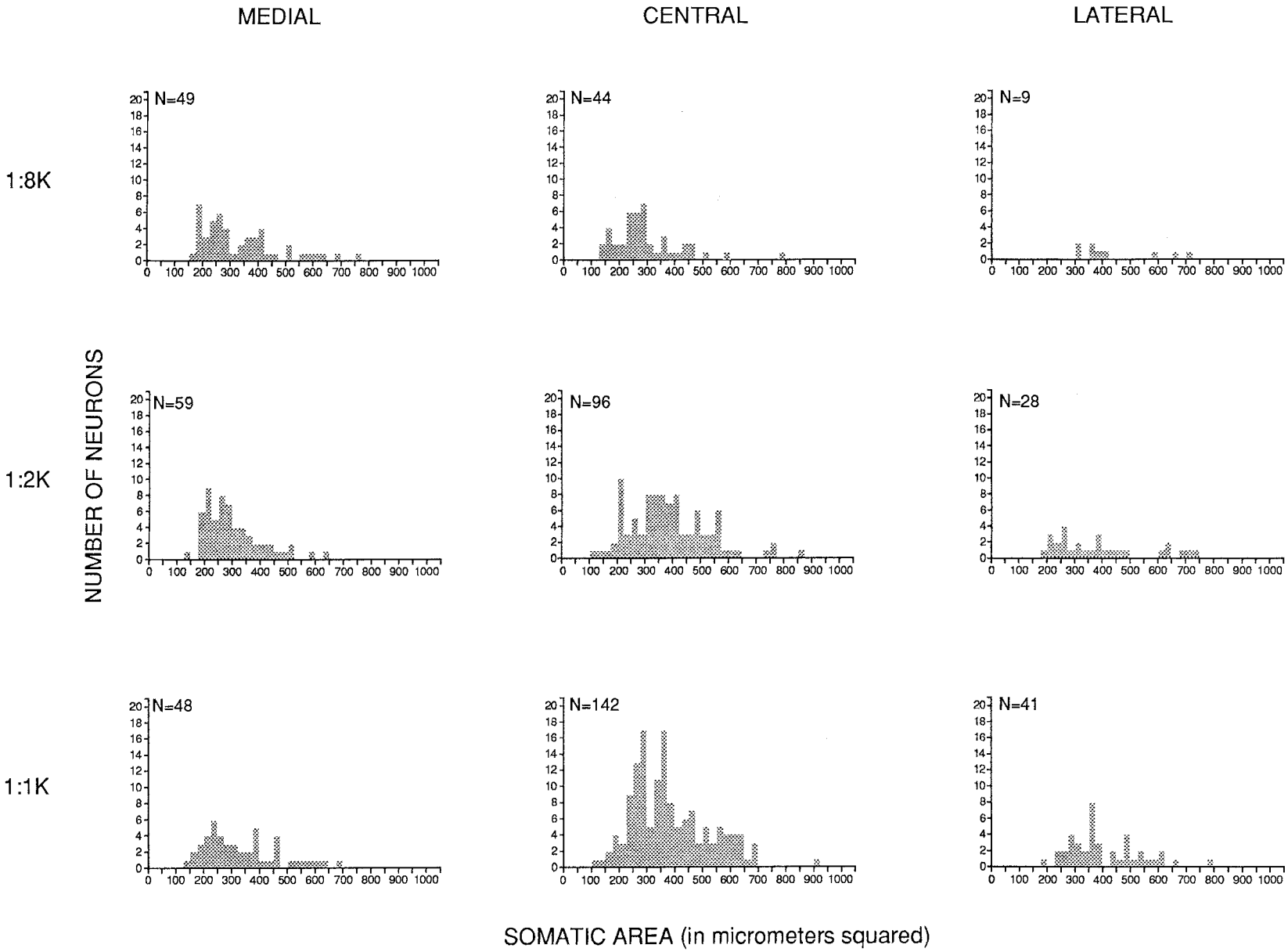

Fig. 4. Sizes of GABA-positive cell bodies from thick vibratome sections prepared with preembedding methods. Cells from each of three subdivisions from the central nucleus were measured in sections prepared with primary antisera at one of three dilutions (1:8,000, **upper row**; 1:2,000, **middle row**, 1:1,000, **lower row**). Average areas of these cells are shown in Table 2.

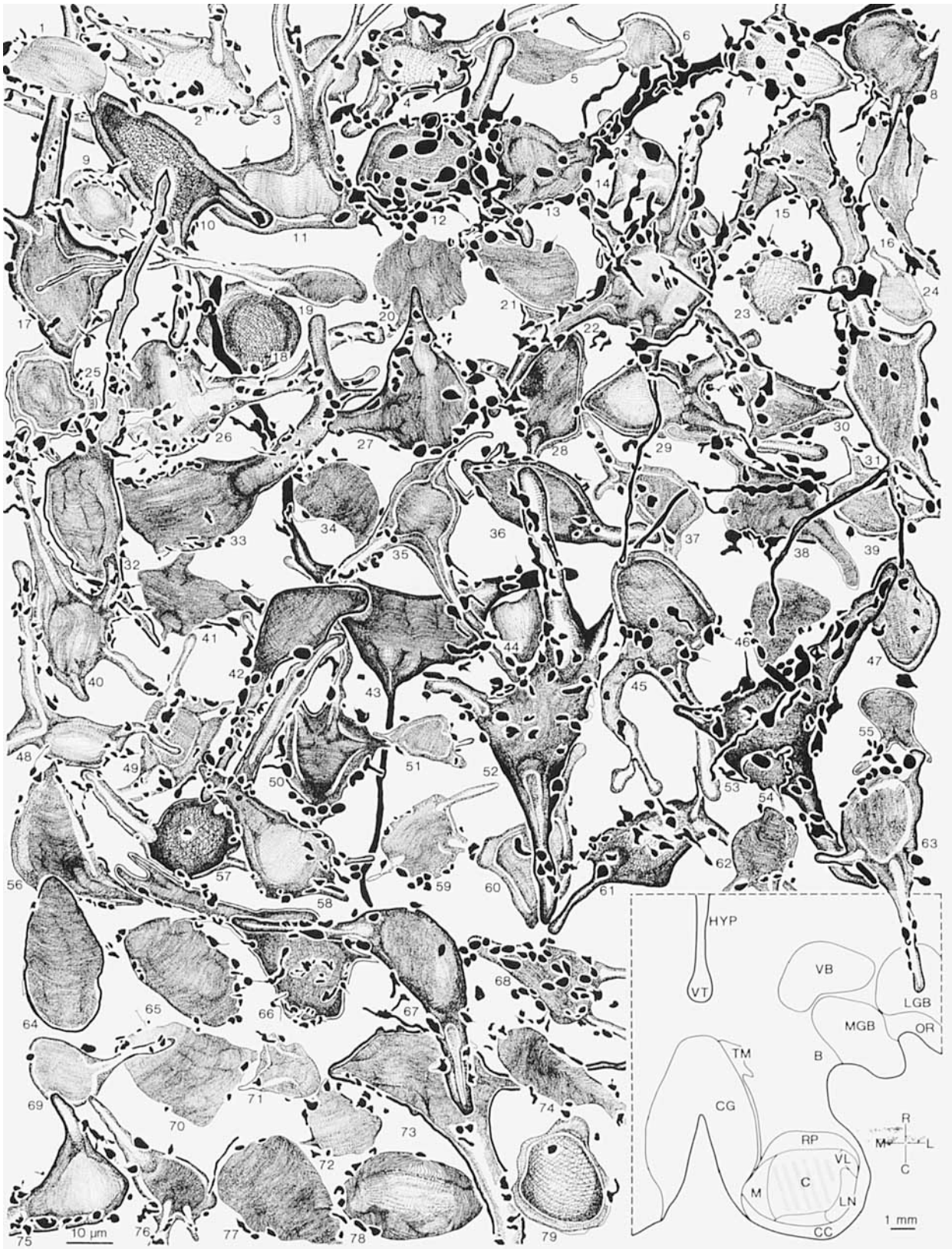

Fig. 5. Dendritic orientations and axosomatic puncta on GAD-labeled somata in pars centralis (C) in the horizontal plane of section. The orientations of the proximal dendrites can be compared to the orientation of the fibrodendritic laminae (gray bars) in the **inset.** Many cells show rostrocaudally oriented dendrites parallel to the laminae and may be disc-shaped neurons. Roughly one-half the larger neurons have numerous GAD-labeled puncta on the somata. Planapochromat, N.A. 1.32. ×2,000.

TABLE 1. Neuron Sizes in the Central Nucleus of the IC

Somatic sizes	Average diameters in Golgi study[1] (μm)	Corresponding areas[1] (μm²)	Range of diameters[2] (μm)	Range of areas[2] (μm²)
Large	29	660	26–35	531–962
Medium	21	415	19–25	284–491
Small	18	255	10–18	79–255

[1]Adult cat measured in Golgi preparations (Oliver and Morest, '84).
[2]Ranges used in the present study.

TABLE 2. Average Areas of Cells Labeled With Preembedded GABA Immunohistochemistry in Three Subdivisions of the Central Nucleus

Antisera dilutions	Medial	Central	Lateral
1:8,000			
N	49	44	9
Average area (μm²)	328	289	442
Standard error	21.03	19.50	51.57
1:2,000			
N	59	96	28
Average area (μm²)	291	373	377
Standard error	13.75	14.77	31.97
1:1,000			
N	48	142	41
Average area (μm²)	327	369	396
Standard error	19.95	11.73	20.16

Dendritic orientation of immunostained neurons

The extensive immunostaining of the dendrites was used to estimate the orientation of the dendritic fields (Figs. 3, 5). Such staining helped to establish the correspondence between cells in this study and those seen in Golgi impregnations (Oliver and Morest, '84), after intracellular injection (Oliver et al., '91), or those marked by retrograde labeling from the thalamus (Oliver, '84b). In transverse sections, the dendrites of GAD-positive cells were often oriented from dorsomedial to ventrolateral (Fig. 3: 2, 10, 45, 58, 66, 69). This illustrates the fibrodendritic laminae in the central part of the central nucleus (Morest and Oliver, '84; Oliver and Morest, '84) and is congruent with the prevailing orientation of the disc-shaped cells that are the principal cell type. Other neurons had their perikarya and dendrites arranged along a medial to lateral axis (Fig. 3: 11, 12, 14, 22, 71). This latter form was characteristic of stellate cells. Still other neurons had a much less regular dendritic arrangement, with both lamina specific and translaminar components (Fig. 3: 21, 44, 47, 62, 77).

An analogous picture was evident in GAD-immunostained horizontal sections (Fig. 5). Many cells had proximal dendrites and somata oriented rostrocaudally, a pattern reminiscent of that for disc-shaped cells (Fig. 5: 17, 30, 32, 52, 67, 73). In contrast, other cells had perikarya and proximal dendrites oriented parallel to the mediolateral axis (Fig. 5: 4, 5, 13, 22, 33). This result suggested that some GAD positive neurons were stellate cells.

To estimate of the proportions of different GABAergic cell types, the principal orientation of 547 GABA positive cells was measured. Both the somatic and the proximal dendritic arrangements were compared with the laminar orientation in the central nucleus as seen in Golgi preparations (Morest and Oliver, '84; Oliver and Morest, '84). Some 39–57% of the immunopositive cells in each subdivision conformed to the fibrodendritic laminae (Table 4), suggesting that they correspond to disc-shaped cells. This pattern was most evident in the medial and lateral parts of the central nucleus. Most neurons parallel to the laminae had medium-sized or large somata (Table 5). In contrast, only 10–15% of the GABA-positive cells were oriented orthogonal to the laminae (Table 4), and 50–60% of these cells were medium sized (Table 5). Those neurons with orthogonally oriented dendrites probably were stellate cells. Since 33–46% of the neurons had dendrites that were either oblique to the laminae or had sparse dendritic immunostaining, their classification as disc-shaped or stellate could not be determined.

Morphology of GABAergic axonal endings

These terminals were so consistent in form and density that they alone would have been sufficient to identify the subdivisions of the IC. Since a complete consideration of the endings is beyond the scope of the present study, only a brief comment is warranted. In the central part of the central nucleus, these endings were numerous (Fig. 9). The puncta were diverse in size, 0.5–2.5 μm in diameter, with most about 1 μm. Their shapes were equally varied and included small, granular endings (Fig. 9: beside cell number 12), oval puncta (Fig. 9: above cell number 9), coarse globular profiles (Fig. 9: to the right of cell number 25), and elongated terminals (Fig. 9: near cell 18). Sometimes, thread-like preterminal segments were visible (Fig. 9: between cell numbers 12 and 26), though for the most part only puncta themselves were evident. Other subdivisions had fewer puncta than the central part of the central nucleus. By comparison, some subdivisions contained puncta that appeared larger (e.g., in DNLL and LN) or smaller (e.g., in the lateral part of central nucleus) than those in the central part of the central nucleus.

Differences in the number of axosomatic endings on GABA- and GAD-positive cells also suggested that there may be several populations of GABAergic cell types in the central nucleus. Many GAD-positive cells had just a few immunopositive axosomatic puncta (Fig. 3: 18, 50, 71, 76; Fig. 9: 2, 13). This pattern was most common for the small and medium-sized neurons and was observed in the GABA-positive postembedded material (Fig. 6A,B). On the other hand, some larger cells displayed many more immunoreactive puncta (see, e.g., Fig. 3: 36, 69, 74, 79; Fig. 5: 12, 26, 27, 52, 53, 66, 68; Fig. 6E,G,I; Fig. 9: 1, 6, 12). Not every large perikaryon shared this characteristic. For example, when large cells were preferentially selected, many were covered with immunopositive puncta (see, e.g., Fig. 5: 12, 22, 52, 53; Fig. 9: 17, 26), but others were not (Fig. 5: 10, 32, 64, 79; Fig. 9: 22–27). This suggests that the largest GABAergic cells include at least two subtypes, those with many GABAergic axosomatic endings and those with fewer. Thus the number and spatial distribution of axosomatic endings may be specific to particular types of neurons.

DISCUSSION

The results show that GAD- or GABA-immunopositive neurons represent a significant population IC cells, approximately 20% of the neurons, and include the largest cells in the central nucleus. In contrast, the smallest neurons are immunonegative. Medium-sized neurons are either immunopositive or negative. Many GABAergic neurons have proximal dendrites or somata oriented in parallel to the fibrodendritic laminae of the central nucleus, while others have an orthogonal orientation or are not oriented. Large GABA-positive neurons make up two groups, those with

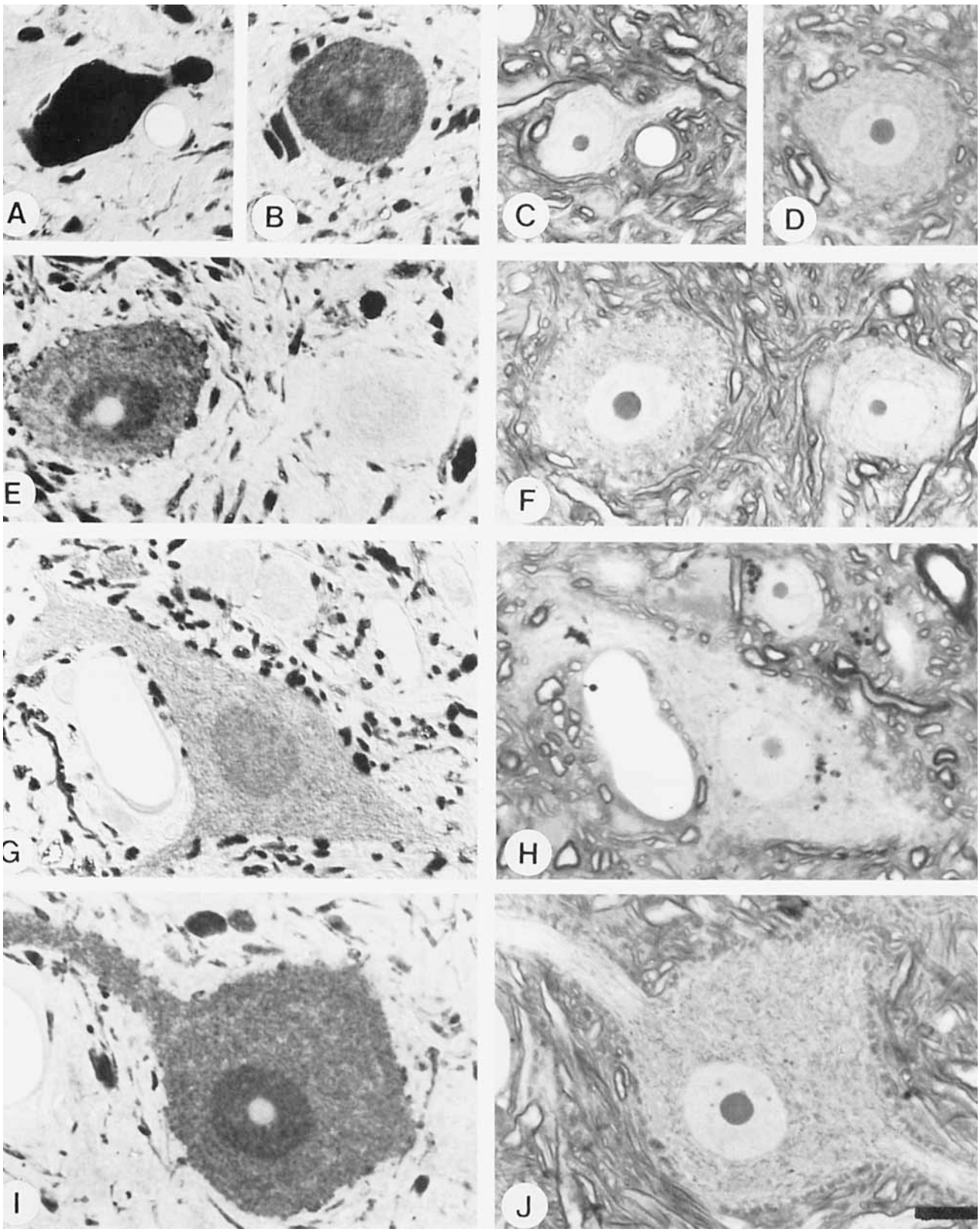

Fig. 6. Photomicrographs of neurons in 1.5-μm-thick immunostained and nonstained sections. **A,B,E,G,I:** GABA-positive cells. **C,D, F,H,J:** the same cells in adjacent toluidine blue-stained sections. **E–H:** immunonegative cells near GABA-positive cells in the same panels. **F, H, J:** Larger neurons with axosomatic endings; **E, G, I:** some of these endings are GABA positive. Scale bar = 10 μm.

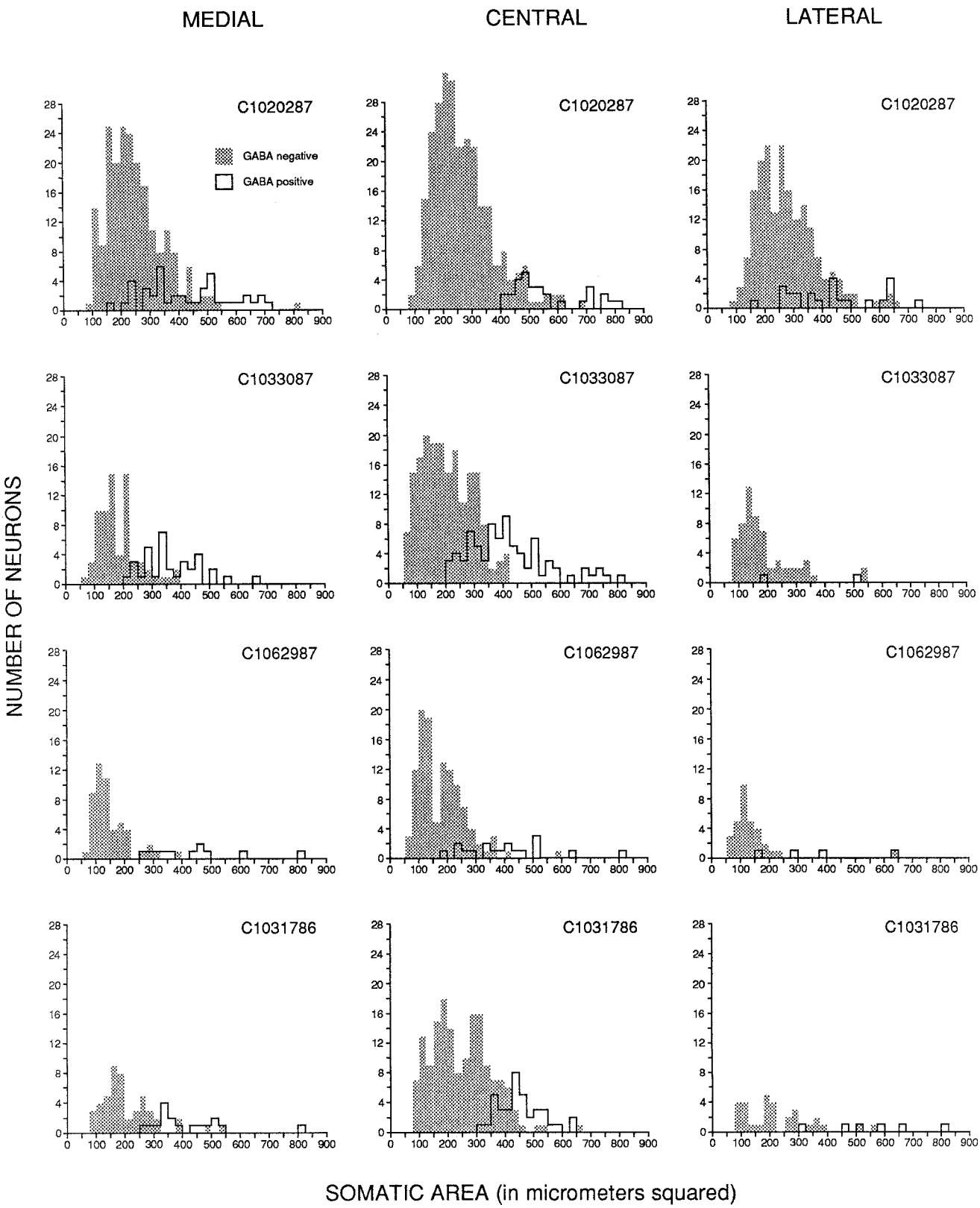

Fig. 7. Sizes of GABA-positive vs. negative somata from 1.5-μm-thick sections. In each of four animals, neurons were measured in the medial, central, and lateral subdivisions of the central nucleus. In all samples, GABA-positive cell bodies (open bars) tend to be larger than most GABA-negative cells (gray bars). Numerical data from these cells are shown in Table 3.

TABLE 3. Proportions and Average Areas of Neurons in Postembedded Material[1]

	Medial		Central		Lateral		Total	
	+[2]	−	+	−	+	−	+	−
C1020287	44	208	60	272	26	186	130	666
Proportion (%)	17	83	18	82	12	88	16	84
Area (μm^2)	443	239	506	259	469	266	477	255
Standard error	28.9	7.0	33.4	4.8	46.3	4.6	20.5	4.2
C1033087	34	71	79	188	2	60	115	319
Proportion (%)	32	68	30	70	3	97	26	74
Area (μm^2)	359	171	408	193	334*	174*	392	185
Standard error	17.5	8.2	15.5	6.3	154.8	12.4	12.1	4.8
C1062987	11	52	18	113	4	31	33	196
Proportion (%)	17	83	14	86	11	89	14	86
Area (μm^2)	427	138	401	168	352	112	404	151
Standard error	48.9	9.2	36.9	7.8	102.5	7.5	6.8	5.5
C1031786	20	48	41	162	6	30	67	240
Proportion (%)	29	71	20	80	17	83	22	78
Area (μm^2)	376	196	394	237	550	215	403	226
Standard error	20.5	6.8	18.8	8.2	71.9	22.1	15.3	6.9
Total	109	379	198	735	38	307	345	1421
Proportion (%)	22	78	21	79	11	89	20	80

[1]In each group, the areas of immunopositive (+) and immunonegative (−) neurons were compared with a t test and the t distribution for two-tailed tests. All tests were significant at $P < 0.001$, except for the lateral subdivision of C1033087 (*), which was significant at $P < 0.05$.
[2]+, GABA positive; −, GABA negative.

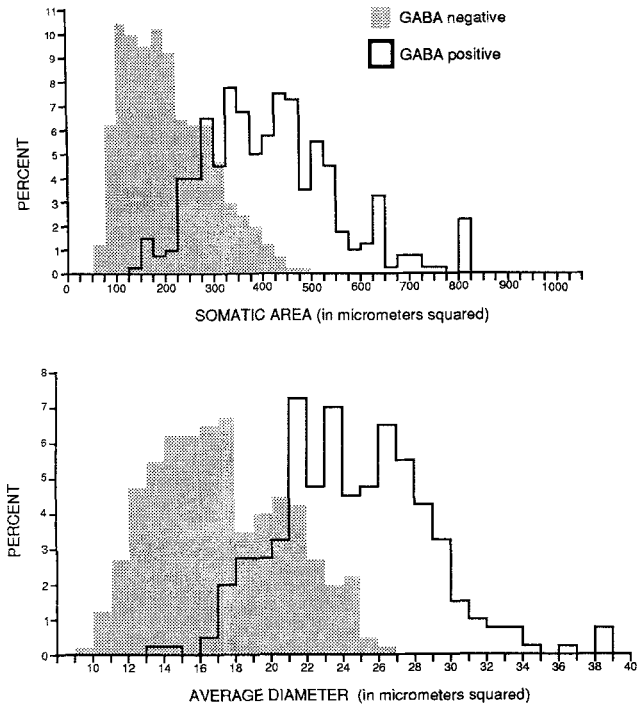

Fig. 8. Cumulative histograms of area and average diameters of GABA-labeled and unlabeled cells. To compare the average diameter and area of GABA-stained vs. GABA-negative cells in 1.5-μm-thick sections, the data from Figure 8 were normalized and summed across all samples.

many axosomatic endings and those with few. These observations suggest that there are several types of GABAergic neurons in the central nucleus of the IC, and, by extension, that there are several types of inhibitory circuits in which they participate.

TABLE 4. Orientation of GABA-Labeled Somata and Dendrites Relative to the Fibrodendritic Laminae of the Central Nucleus

	Parallel[1] (%)	Oblique[2] (%)	Orthogonal[3] (%)	No orientation (%)
Pars medialis (N = 165)	57	18	10	15
Pars centralis (N = 291)	39	22	15	24
Pars lateralis (N = 91)	43	19	15	23

[1]Orientations within 45° (±22°) to the midline were considered parallel to the fibrodendritic laminae.
[2]Orientations parallel to the sagittal or horizontal planes of cut were considered oblique.
[3]Orthogonal cells were oriented 90° (±22°) to the parallel category.

TABLE 5. Percentage of GABA-Positive Neurons by Size and Orientation With Respect to the Fibrodendritic Laminae

Orientation	Small (%)	Medium (%)	Large (%)
Parallel	21	55	24
Oblique	26	51	23
Orthogonal	30	60	10
Nonoriented	41	50	9

Comparison of different immunocytochemical methods

In this study, complementary methods were employed to characterize the GABAergic neurons in sufficient detail to identify them as specific morphological types. GABA was localized with postembedding immunocytochemistry in semithin (1.5 μm), plastic-embedded sections and also with preembedding immunocytochemistry on thick (50 μm), hydrated material. Moreover, glutamate decarboxylase (GAD), the synthetic enzyme of GABA, is localized immunocytochemically in 25–50 μm sections. The quality of the staining (signal vs. background, overall specificity of immunoreactivity, and resolution of cellular details) achieved with all three approaches is similar.

The preembedding methods provide abundant details about the dendritic arrangement of the immunostained cells, since the sections are thicker. This is most noteworthy in GAD material in which the penetration of the antisera is greatly enhanced by the frozen sectioning and by the absence of glutaraldehyde as a fixative. The requirement that tissue immunostained for GABA be fixed to protein with glutaraldehyde stands as one principal drawback to using preembedding methods. Consequently, the modest antibody penetration obscures small, reactive preterminal axons and endings except superficially, where the tissue is most abraded by sectioning.

One advantage of postembedding immunocytochemistry is that labeled cells could be characterized further by examining them in adjacent, toluidine blue-stained, semithin sections. Furthermore, since immunonegative neurons also appear in these Nissl-stained preparations, one can learn their relative proportions with some precision and compare immunoreactive to nonimmunoreactive populations. The uniform thickness and complete penetration of the antibodies and reagents are signal advantages. One constraint is that far less of the cell is present, and the continuity of dendrites with the perikaryon is reduced consequently. Since each method has limitations, we chose to use all three to maximize our ability to distinguish between GABAergic and non-GABAergic neurons and to distinguish different classes of the former.

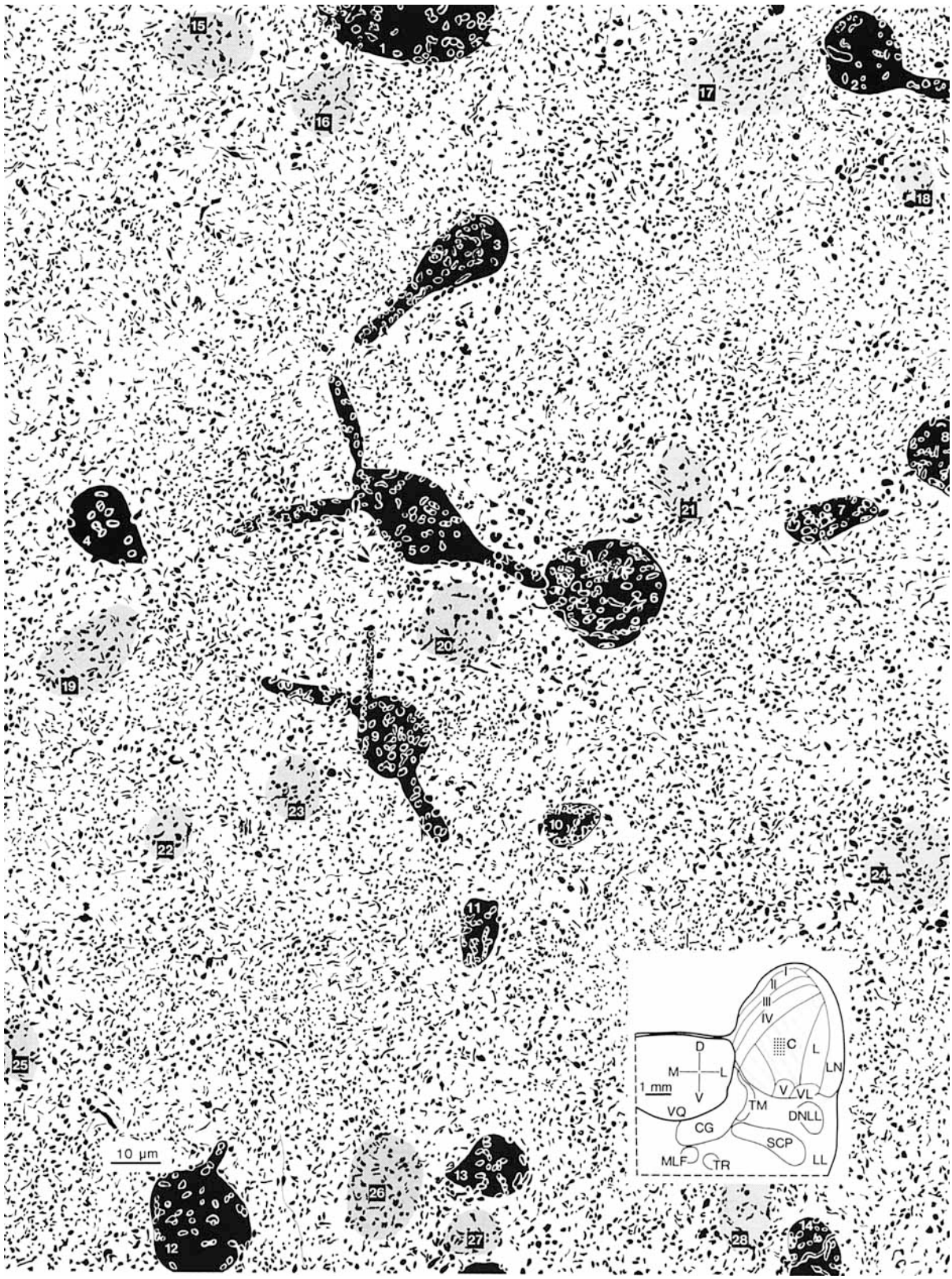

Fig. 9. Uniform density of GAD-immunoreactive puncta in the central nucleus. Besides the obvious axosomatic endings, some GABA-positive cells received appreciable numbers of peridendritic terminals (cell numbers 3, 5, 9). **Inset** shows the approximate disposition of central nucleus (C) laminae (gray bars) and the location from which this panel was drawn (box). Planapochromat, N.A. 1.32. ×2,000.

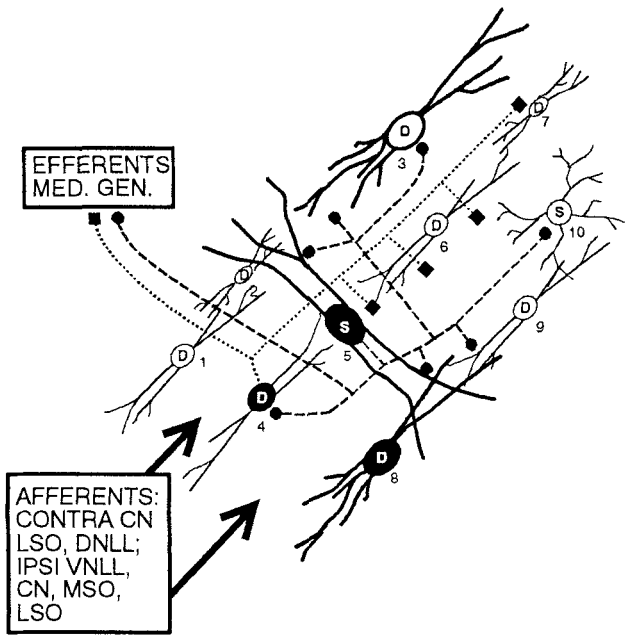

Fig. 10. Schematic drawing illustrating possible patterns of synaptic organization of GABAergic (black cells 4, 5, 8) and non-GABAergic (white cells 1–3, 6, 7, 9–11) neurons in the central nucleus of the IC. Disc-shaped neurons (D) form parallel fibrodendritic laminae with their axons (dashed line and solid squares) confined to the laminae, while stellate cell (S) dendrites cross the laminae and their axons (dotted line and solid circles) interconnect many layers. Both types of axons are thought to form efferent projections. The ascending afferent axons (large arrows) run parallel to the disc-shaped neurons but are not illlustrated otherwise.

Which neurons in the IC are GABAergic?

Golgi studies of the IC in many species identify two main cell types in the central nucleus: disc-shaped and stellate neurons (Geniec and Morest, '71; Rockel and Jones, '73; FitzPatrick, '75; Oliver and Morest, '84; Faye-Lund and Osen, '85; Meininger et al., '86; Herrera et al., '88, '89). These are shown schematically in Figure 10. The first type, the disc-shaped cell (Fig. 10: cells 1–4, 6–9), has a dendritic field that is flattened and is parallel to the afferent axons from the lateral lemniscus (Fig. 10, arrows). Together, the disc-shaped neurons and these ascending fibers form the fibrodendritic laminae characteristic of the central nucleus. Disc-shaped neurons make up about 80% of the cells in the central nucleus and are a diverse group among which subvarieties are distinguished by size (Oliver and Morest, '84). As is shown in Figure 10 and Table 1, the range of disc-shaped neurons includes large (26–35 μm; Fig. 10: cells 3, 8)-, medium-sized (19–25 μm; Fig. 10: cells 1, 6, 9)-, and small (10–18 μm; Fig. 10: cells 2, 7)-diameter perikarya. Some disc-shaped cells are distinguished by the presence or absence of dendritic appendages (Gonzalez-Hernandez et al., '89). At the electron microscopic level, neurons with many axosomatic endings include some large disc-shaped cells (Oliver, '84b).

Stellate cells are the second major type of neuron identified in Golgi impregnations (Fig. 10: cells 5, 10). These are multipolar cells with spherical or elliptical dendritic fields that often cross the fibrodendritic laminae. Stellate cells also are a heterogeneous group (Oliver and Morest, '84; Gonzalez-Hernandez et al., '89) and include large or giant neurons with "simple" dendritic branching patterns and few spines (Fig. 10: cell 5). These also appear to receive many axosomatic endings (Oliver, '84b). "Complex" stellate cells (Fig. 10: cell 10) are medium-sized or large cells with many more higher order dendritic branches and a variety of dendritic appendages. "Small" stellate cells are rarely seen (Oliver and Morest, '84).

Many GABAergic neurons in this study appear to correspond to large or medium-sized disc-shaped cells (Fig. 10: cells 4, 8). These have large or medium-sized perikarya with proximal dendrites oriented parallel to the fibrodendritic laminae. The finding of large GABA-positive somata with many GABAergic axosomatic endings closely matches previous descriptions of large disc-shaped cells (Oliver, '84b). Since few small cells are immunopositive in this study, it is unlikely that the smallest disc-shaped cells are GABAergic.

Large stellate cells also are likely to be GABAergic (Fig. 10: cell 5), since most of the large neuronal perikarya in the IC are GABAergic. Moreover, 15–20% of the immunopositive neurons have dendrites oriented oblique or orthogonal to the fibrodendritic laminae, a characteristic of stellate cells. While the number of stellate cells may be small compared with the number of disc-shaped cells, we suspect that the majority, but not all (see, e.g., Fig. 10: cell 10), of the stellate cells may be GABAergic.

GABAergic neurons in different species

GABAergic cells have been identified in the IC of the rat, gerbil, guinea pig, and bat. In the rat (Roberts and Ribak, '87b), several types of GABAergic neurons are found, but some of these types are different from those reported here in the cat. For example, small stellate (multipolar, < 15 μm) cells are the most common GABAergic neurons in the rat, yet these rarely stain in the cat. Many medium-sized stellate cells (15–25 μm in diameter) are GABAergic in the rat; however, most of the medium-sized GABAergic cells in the cat are probably disc-shaped neurons. Only a few GABAergic disc-shaped (bipolar) cells are observed in the rat. Finally, few large GABAergic neurons with radiate dendrites are reported in the rat, while, in the cat, the large disc-shaped and large stellate cells represent many of the GABAergic neurons.

The GABAergic organization of the central nucleus in the mustached bat's IC shares many features in common with the cat (Vater et al., '92; Winer et al., '92, '94). All sizes of immunopositive neurons and a wide range of shapes are evident. The finding that many of the smallest neurons are GABAergic in the bat's IC is a striking departure from the case in the cat, where such cells are consistently immunonegative. As in the cat, immunopositive neurons have a diverse axis of orientation, sometimes conforming closely to the fibrodendritic laminae. At other times, they are arranged orthogonally, or they are situated obliquely. Finally, the pattern of GAD-positive puncta is very similar to that seen in the cat. Fine, granular endings are especially prominent on immunonegative cells and in the neuropil and are less evident on the perikarya of most immunoreactive neurons.

Are GABAergic cells interneurons and projection neurons?

The present study raises, but cannot resolve, the issue of what constitutes a local circuit neuron within the brain. For example, classic Golgi type II neurons in the thalamus are usually regarded as small cells (with soma < 10 μm in diameter) with a drumstick-shaped perikaryon; thin, sparsely branched dendrites with a few long appendages;

and a fine, unmyelinated axon with extensive local projections (Morest, '75). Many such cells have proved to be GABAergic in the thalamus (Winer and Larue, '88) and cortex (Prieto et al., '94a,b).

While many neurons share the attributes of the classical interneuron, others depart from it significantly in their large size and long axonal projections. For example, cerebellar Purkinje neurons, cortical basket cells, and projection neurons in the DNLL each represent an exception to one or more of these features. Purkinje cells are GABAergic (McLaughlin et al., '74a,b; Oertel et al., '81), and their axons project a comparatively long distance to the deep cerebellar nuclei (Cajal, '09). The GABAergic basket or multipolar cells in the neocortex are among the largest nonpyramidal cells (Prieto et al., '94a), and some varieties have an axon that projects several millimeters (DeFelipe et al., '86). The projection neurons of the DNLL are medium-sized to large neurons and are among the GABAergic afferents to the IC (Adams and Mugnaini, '84; Shneiderman et al., '88, '93; Shneiderman and Oliver, '89).

The present results suggest that many GABAergic neurons in the IC do not conform to the idea of classical interneurons. GABAergic cells in the IC are predominately large or medium in size, not small, and a single distinct class of interneuron seems to be absent. Regarding whether GABAergic cells participate in local circuits in the IC, previous studies have shown that most neurons contribute local axonal collaterals (Morest, '66; Geniec and Morest, '71; Rockel and Jones, '73; Oliver and Morest, '84; Oliver et al., '91). Thus the GABAergic neurons in the IC can be expected to contribute to local inhibitory synapses. Simultaneously, GABAergic neurons in the IC may be projection neurons. Most neuronal types in the IC have ascending projections, especially large and medium-sized neurons (Oliver, '84b), and intracellular injections show axons that branch locally and enter the brachium of the IC (Oliver et al., '91). Although GABAergic cells may function as both local circuit and projection neurons, this remains to be proved experimentally.

Possible roles of GABAergic synapses in the IC

There are nearly as many type II, putative inhibitory, synapses in the central nucleus of the IC as there are synapses with presumed excitatory function (Oliver, '85, '87; Shneiderman and Oliver, '89; Oliver and Beckius, '92). The most common GABAergic synapse has a presynaptic axonal ending that contains pleomorphic synaptic vesicles and makes symmetrical membrane contacts on postsynaptic dendrites (Oliver and Beckius, '92). However, more than one type of ending is immunopositive. These data are consistent with the notion that GABAergic synapses may arise from multiple sources, that is, from both local GABAergic neurons and from extrinsic sources.

Organization of extrinsic GABAergic axons. GABAergic afferent to the IC arise primarily from the DNLL (Adams and Mugnaini, '84; Thompson et al., '85; Moore and Moore, '87; Roberts and Ribak, '87a; Shneiderman et al., '88; Hutson et al., '91). Additional GABAergic projections from the periolivary nuclei cannot be excluded. Axons from DNLL terminate in the central nucleus as topographically restricted bands of axons that parallel the fibrodendritic laminae. This banded, topographic organization of the DNLL input is similar to other ascending projection systems to the central nucleus, except that DNLL afferents probably have inhibitory effects on their postsynaptic targets (see, e.g., Henkel and Spangler, '83; Oliver, '84a, '87; Shneiderman and Henkel, '87).

Organization of intrinsic axons. These may have an entirely different arrangement. Intracellular filling experiments suggest two patterns of local axonal branching: One is a widespread pattern and the second is restricted to a single lamina (Oliver et al., '91). Such patterns could produce several functionally distinct inhibitory intrinsic circuits.

Intrinsic axons with the widespread pattern (Fig. 10: cell 5; dotted axon with round terminal boutons) extend across the fibrodendritic laminae to innervate neurons on either flank. These axons could influence many neurons. This pattern was characteristic of stellate cells of several sizes and of oriented cells in the dorsal cortex (Oliver et al., '91). Thousands of terminal boutons were sometimes observed on a single axon. The accumulated evidence suggests that the largest stellate cells are probably GABAergic and emit local axons with a broad translaminar distribution (Fig. 10: cell 5). If such a pattern is confirmed experimentally, it would suggest that GABAergic neurons could contribute to a remote suppression of neural activity across frequencies. Other axonal collaterals arisng from these cells could project in either ascending or descending pathways.

In contrast, the laminar distribution pattern of intrinsic axons (Fig. 10: cell 4, dashed axon with square terminal boutons) is an arrangement whose primary influence appears to be focused on neurons in a single fibrodendritic lamina. Disc-shaped cells are the source of these axons (Oliver et al., '91). Many GABAergic cells have processes oriented in parallel with the fibrodendritic laminae; therefore, they may be disc-shaped neurons whose axons are confined to the lamina of origin. If such a pattern is confirmed experimentally, it would suggest that GABAergic disc-shaped neurons could produce a temporally delayed suppression of other neurons with similar best frequencies.

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