European Journal of Neuroscience, Vol. 2, pp. 414–429

Synaptic and Nonsynaptic Localization of Benzodiazepine/$GABA_A$ Receptor/Cl^- Channel Complex Using Monoclonal Antibodies in the Dorsal Lateral Geniculate Nucleus of the Cat

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Key words: GABA, receptors, synapse, immunocytochemistry, inhibition, thalamus

Abstract

The two monoclonal antibodies, bd-17 and bd-24, are specific for β- and α-subunits of the $GABA_A$/benzodiazepine receptor/chloride channel complex respectively. An abundance of both subunits has been revealed in the visual thalamus of the cat by light microscopic immunocytochemistry using these antibodies. The α-subunit specific antibody and electron microscopy were used to determine the subcellular distribution of immunoreactivity with respect to specific cell classes in the dorsal lateral geniculate nucleus. Immunoreactivity was always associated with membranes and the degree of immunoreactivity varied greatly between different types of cell as defined by: (i) immunoreactivity for GABA; (ii) soma area; (iii) presence or absence of cytoplasmic laminated bodies (CLB). GABA negative neurons with the smallest soma area showed the strongest immunoreactivity, mainly in the endoplasmic reticulum and also on the somatic plasma membrane. Cytoplasmic laminated bodies could be found in the majority of these neurons. Large GABA negative cells without CLBs were strongly immunoreactive on the plasma membrane of the soma and dendrites, but showed scant if any intracellular immunoreactivity. GABA-positive cells showed weak intracellular immunoreactivity but negligible if any immunoreactivity at the somatic and proximal dendritic plasma membrane. A similar reaction pattern was found in GABA negative cells which contained no CLBs and which constituted a medium sized cell population. It is suggested that the degree of intracellular receptor immunoreactivity is positively correlated with receptor turnover. The dendrites of projection cells, particularly outside the glomeruli, showed strong immunoreactivity on the plasma membrane. The synaptic junctions formed by many boutons (F terminals) establishing symmetrical synapses with dendrites of relay cells were immunopositive, but no immunoreactivity could be detected at the synapses established by the presynaptic dendrites of the local interneurons. Many axo-somatic F1 junctions were also immunoreactive. However, immunoreactivity for the receptor/channel complex was also widely distribution on nonsynaptic plasma membranes of somata and dendrites. Thus GABA may act at both synaptic and non-synaptic sites. Furthermore, the correlation of immunoreactivity for the $GABA_A$ receptor complex with previously published properties of physiologically identified cells suggests that the strongly immunoreactive, small, GABA negative cells with CLBs might correspond to the 'lagged' X-type cells, and the large GABA negative receptor outlined cells without CLBs might correspond to some of the Y-type neurons.

Introduction

Gamma-aminobutyric acid (GABA) mediated inhibition has been shown to play a major role in gating the transmission of visual information from the retina to the visual cortex in the dorsal lateral geniculate nucleus (LGN) of the cat (Singer and Bedworth, 1973; Dubin and Cleland, 1977; Lindstrom, 1982; Sillito and Kemp, 1983; Berardi and Morrone, 1984; Sherman and Koch, 1985; Ahlsen *et al.*, 1985; Pape

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Received 11 July 1989, revised 1 December 1989, accepted 20 December 1989

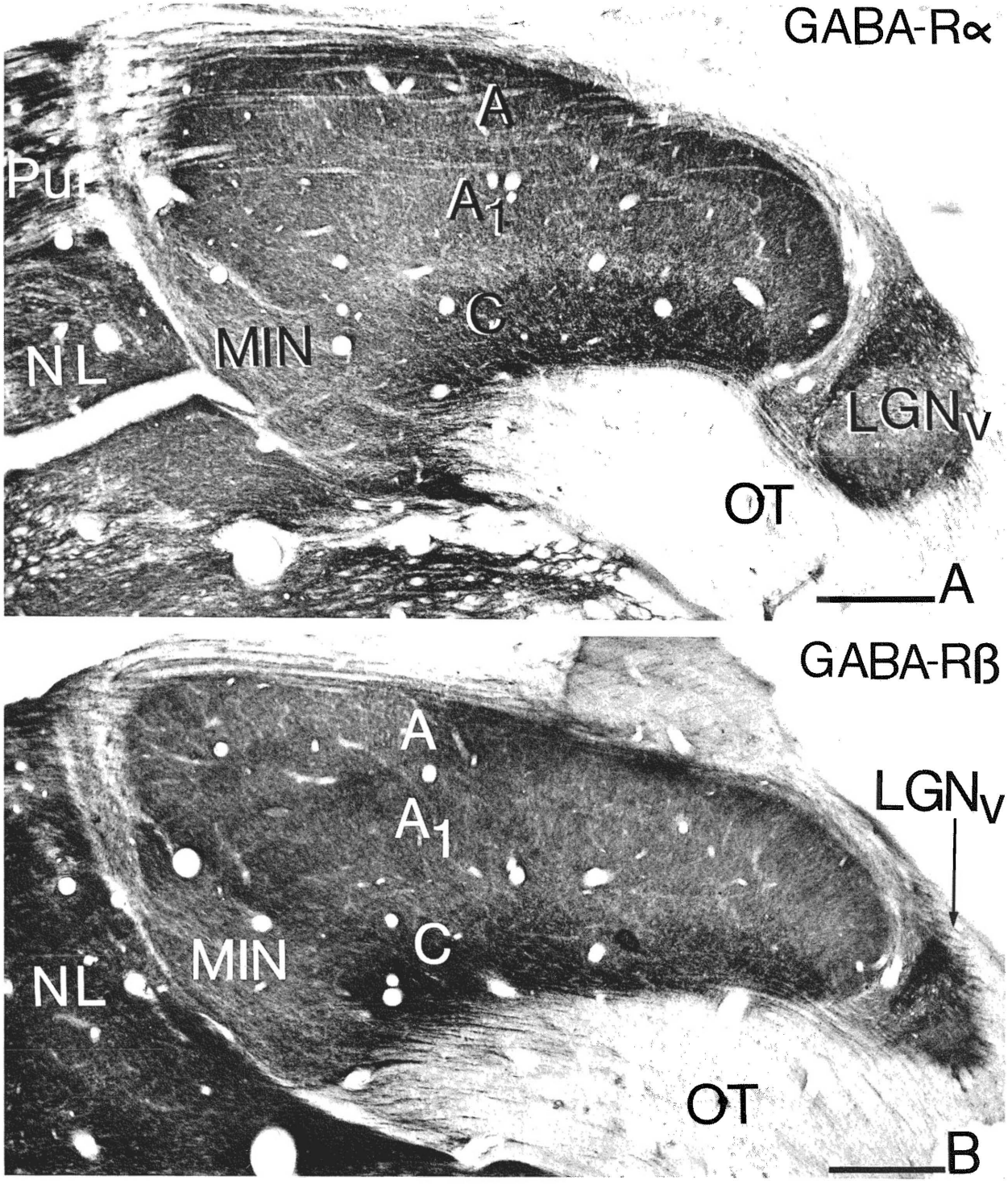

FIG. 1. Distribution of GABA$_A$-R immunoreactivity in the visual thalamus of cat, as shown by monoclonal antibody bd-24 (A), specific for the α-subunit of the receptor, and monoclonal antibody bd-17 (B), specific for the β-subunit of the receptor complex. Sections (coronal plane) were treated with Triton X-100, therefore both antibodies gave strong reaction throughout the neuropil of the thalamus, but not in fibre tracts. The stronger reaction at the periphery of the sections was not observed consistently. A, A$_1$, C, laminae of the dorsal lateral geniculate nucleus; LGN$_v$, ventral LGN; MIN, medial interlaminar nucleus; NL, nucleus lateralis; OT, optic tract; Pul, pulvinar. Scale: 500 μm.

and Eysel, 1986). Neurons in the LGN receive GABAergic input from axons of the perigeniculate nucleus, from axons of intrinsic neurons and from presynaptic dendrites of these same intrinsic neurons (Guillery, 1969; Hamori *et al.*, 1974; Lieberman and Webster, 1974; Sherman and Spear, 1982; Fitzpatrick *et al.*, 1984; Mason *et al.*, 1984; Montero and Singer, 1985; Montero, 1986). *In vivo* microelectrode

studies have revealed the existence of optic tract activated inhibition, and combined electrophysiological and pharmacological studies using the $GABA_A$ antagonist bicuculline have demonstrated that at least a substantial part of this inhibition is mediated by $GABA_A$ receptors (Sillito and Kemp, 1983; Vidyasagar, 1984; Berardi and Morrone, 1984; Pape and Eysel, 1986; Eysel *et al.*, 1987). It is apparent that different types of cells can be defined on the basis of the spatial and temporal properties of the GABA mediated inhibitory influences.

The LGN of the cat offers great advantages not only for physiological studies on inhibition, but also for the localization of the contributing molecular machinery. The LGN contains at least one local circuit cell population (Guillery, 1966; Szentagothai, 1973; Hamori *et al.*, 1974; Hamos *et al.*, 1985), which probably uses GABA as a transmitter (Montero and Singer, 1985; Montero and Zempel, 1985; Gabbott *et al.*, 1985; Madarasz *et al.*, 1985; McCormick and Pape, 1988). Physiologically distinct cell populations can be delineated also by size distribution of their somata (Friedlander *et al.*, 1981; Stanford *et al.*, 1983; Humphrey and Weller, 1988a,b). Furthermore, almost all constituents of the neuropil can be identified by electron microscopy (Peters and Palay, 1966; Guillery, 1969; Famiglietti and Peters, 1972; Szentagothai, 1973; Robson and Mason, 1979; Mason *et al.*, 1984; Wilson *et al.*, 1984; Hamos *et al.*, 1985). The regional distribution of one of the main components of the inhibitory mechanism, the $GABA_A$/benzodiazepine receptor/chloride channel complex has been mapped in the thalamus using radioligand binding (Palacois *et al.*, 1981; Schoch *et al.*, 1985; Bowery *et al.*, 1987) and immunohistochemistry (Schoch *et al.*, 1985; Richards *et al.*, 1987; De Blas *et al.*, 1988; Vitorica *et al.*, 1988). Although these studies show high levels of binding sites and immunoreactivity in the LGN of rodents, they have not provided information on localization of the receptor with regard to particular populations of cells. This study was undertaken to identify the cellular and subcellular distribution of the $GABA_A$/benzodiazepine receptor/chloride channel complex.

We used two monoclonal antibodies specific for the α- and β-subunit of the receptor complex respectively (Schoch *et al.*, 1984, 1985; Haring *et al.*, 1985), and an immuno-electronmicroscopic method (Somogyi *et al.*, 1989a) in order to relate the receptor distribution to different classes of cells. The distribution of GABA-containing neurons was also studied in relation to receptor distribution. Some of the results have been presented in preliminary form (Somogyi, 1989).

Materials and methods

Preparation of animals and tissue

Three adult male cats (2–3 kg) were used. They were deeply anaesthetized with chloral hydrate (400 mg/kg i.p. initially, supplemented as required), and perfused transcardially with saline for 1 min followed by fixative for about 30 min at room temperature. In the first 10 min a fixative containing paraformaldehyde (4%), glutaraldehyde (0.025%) and picric acid (approx 0.2%), made up in 0.1 M phosphate buffer (PB, pH 7.2–7.4) was used (Somogyi and Takagi, 1982), then perfusion was continued with a similar fixative but without glutaraldehyde.

A tissue block containing the LGN was dissected from the fixed brain and 50μm thick sections were cut on a vibratome. The sections were washed in 0.1 M PB and then placed in solutions of the same buffer containing 10% and 20% sucrose for about an hour each. To facilitate the penetration of reagents most of the sections were frozen in liquid N_2 then thawed in 0.1 M PB as described earlier (Somogyi and Takagi, 1982). Thereafter sections were treated with 1% sodium borohydride (Willingham, 1983) dissolved in phosphate buffered saline (PBS, pH 7.4). This treatment enhanced immunoreactivity.

Antibodies

The purification of $GABA_A$/benzodiazepine receptors and the preparation of mouse monoclonal antibodies have been described earlier (Haring *et al.*, 1985; Schoch *et al.*, 1984, 1985). As shown by immunoblotting bd-24 recognizes one major protein (M_r 50 000–53 000), an α-subunit of the receptor containing the benzodiazepine binding site (Schoch *et al.*, 1984, 1985). The other antibody bd-17 recognizes one major protein band (M_r 55 000–56 000; Fuchs *et al.*, 1988), the β-subunits of the receptor containing the GABA binding site. Both antibodies precipitate the GABA, the benzodiazepine and the t-butylbicyclophosphorothionate (TPBS) binding sites in solubilized brain preparations, indicating that the receptor channel complex contains both subunits (Schoch *et al.*, 1985; Mohler *et al.*, 1986). The antibodies are gamma-isotype IgGs. In the present experiment tissue culture supernatant, containing 10% calf serum, was used for immunocytochemistry.

Immunocytochemistry

For the localization of the receptor complex the same methods were used as reported earlier (Somogyi *et al.*, 1989a). Briefly, free floating sections were incubated either overnight or for 2 days at 4°C, first with serum to block nonspecific binding of the antibodies, then with hybridoma supernatant containing either bd-24 or bd-17. Supernatants were undiluted or diluted 2–4 times.

In the ABC method 10% normal sheep serum was used for blocking and then, following the primary antibody, biotinylated sheep IgG (dil. 1:50, Vector) was applied for 1 h. The sections were then washed in PBS containing 1% normal sheep serum, followed by incubation in avidin–biotin–HRP complex (dil. 1:100, Vector) for 1 h. In the indirect antibody method 10% normal rabbit serum was used for blocking, then following the primary antibody the sections were incubated in HRP-conjugated rabbit antimouse IgG (dil. 1:100, Dako) for 3 h. All steps but the primary antibody incubations were done at room temperature with agitation on a shaker. In some cases Triton X-100 (0.05%) was added to the primary antibody solution to increase penetration of the antibodies and to reveal possible masked immunoreactive sites.

For specimens used in light microscopy only (Fig. 1) Triton X-100 (0.2%) was included in all solutions. These sections received contrast enhancement with 0.1% OsO_4 for a few minutes.

We have tried several methods using particulate markers such as colloidal gold, which gives higher resolution localization of antigens than immunoperoxidase. It has not been possible to achieve penetration of these markers into the narrow membrane delineated spaces in tissue sections using pre-embedding incubation. So far we have also failed to achieve immunoreaction in postembedding procedures.

As controls for method specificity, some sections were incubated in tissue culture medium, and other sections were incubated with omission of the primary antibody step from the sequence.

The peroxidase enzyme reaction was carried out in the dark by preincubating the sections for 30 min in 0.05% diaminobenzidine tetrahydrochloride (Sigma), dissolved in 50 mM Tris buffer (pH 7.4), followed by incubation in the same solution containing 0.01% H_2O_2 for 3–10 min. After washing in PB the sections for combined light and electron microscopy were treated with OsO_4 (1% in PB) for 30 min, dehydrated in ethanol and embedded flat on glass slides in

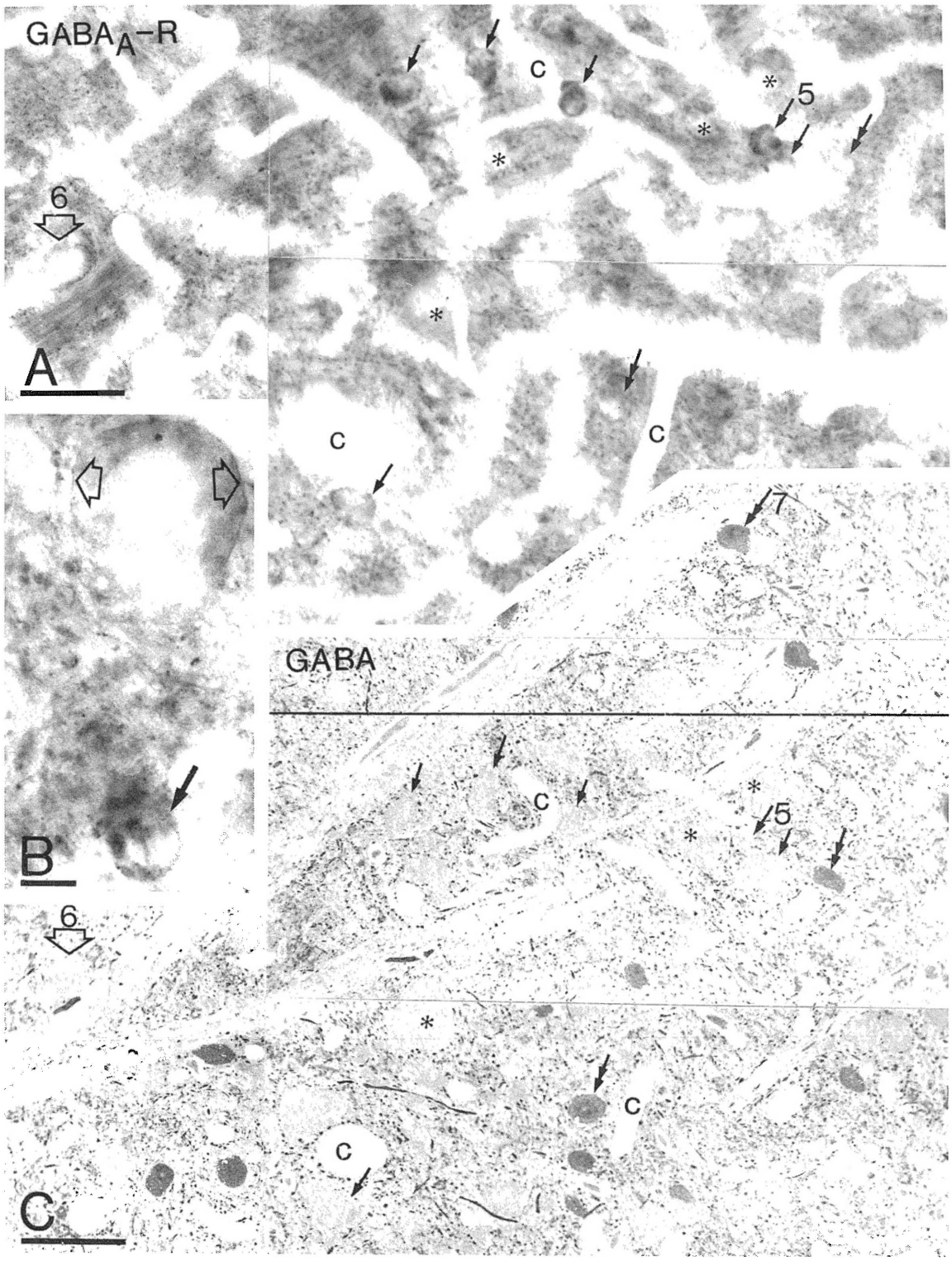

FIG. 2. Immunoreactivity for the $GABA_A$-R in different cell classes of the dorsal LGN using monoclonal antibody bd-24, specific for the α-subunit. (A–B) Light micrographs of 50 μm thick osmium treated section, showing small, strongly immunoreactive (SI in text) cells (arrows) amongst more numerous apparently unstained cells (e.g. asterisks). Immunoreactivity is evident on the plasma membrane of a large cell (open arrow). (C) Immunoreactivity for GABA on a semithin (0.5 μm) section cut from an overlapping region of the thick section shown in A. The comparison of immunoreactivities shows that cells strongly stained (arrows) or outlined (open arrow) by $GABA_A$-R immunoreactivity are negative for GABA. Neurons positive for GABA are negative or very faintly stained for $GABA_A$-R (e.g. double arrows). Neurons 5, 6 and 7 are shown in electron micrographs of subsequent figures. c, capillaries; scales: A, C, 50 μm; B, 10 μm.

epoxy resin (DURCUPAN ACM, Fluka). To increase contrast for electron microscopy the sections were treated with 1% uranyl acetate in 70% ethanol for 40 min during dehydration. Lead staining was not used. Ultrathin sections were cut from the surface layers of the thick vibratome sections because the immunoreactivity was usually limited to the superficial 10–15 μm of the sections. At least two areas from each animal were cut for electron microscopy.

Postembedding immunocytochemistry for GABA was carried out on semithin (0.5 μm thick) sections cut from the resin-embedded, vibratome sections (50 μm thick), which had been immunoreacted for the receptor complex before embedding. Rabbit antiserum to GABA (Code No. 9) and previously described procedures were used (Hodgson *et al.*, 1985; Somogyi *et al.*, 1985). Alternate semithin sections were either stained with toluidine blue/azure II for the identification of cytoplasmic laminated bodies, or immunoreacted for GABA.

Morphometric analysis

The outlines of neuronal somata were drawn along their largest extent, as projected by a drawing tube in the plane of the section, from the osmium-treated vibratome sections immunoreacted for the $GABA_A$ receptor complex. Only neurons with immunoreaction recognizable using a 50x oil immersion objective were drawn. The areas of these two dimensional projections were measured with the aid of a bitpad attached to a computer and evaluated using the package Bioquant IV (R. & M. Biometrics Inc.). Statistical analysis was carried out using the two-tailed Student's *t*-test. Numerical values are expressed in the text as mean ± SD.

Results

Controls

Immunoreactivity could not be detected when the tissue culture supernatant containing the monoclonal antibodies was either omitted from the incubation or replaced by fresh tissue culture medium.

Distribution of immunoreactivity as detected by light microscopy

There were substantial differences between sections incubated with and without Triton X-100. When the detergent was included very strong immunoreactivity was observed throughout the neuropil in all areas of the visual thalamus including the dorsal and ventral lateral geniculate nucleus, the medial interlaminar nucleus, the pulvinar and the lateral posterior complex (Fig. 1). The immunoreactivity was similar with both the α- and the β-subunit specific antibodies. The strong immunoreactivity in the neuropil made the evaluation of individual neurons difficult in detergent treated sections.

The distribution was different in sections which were not treated with detergent. Immunoreactivity could not be detected light microscopically in and around the LGN with undiluted hybridoma supernatant containing antibody bd-17, specific for the β-subunit of the receptor complex. With antibody bd-24 the immunoreactivity was easily detectable at both light and electron microscopic level, but immunoreactivity in the neuropil was substantially weaker than in detergent treated sections. This differential decrease in immunoreactivity allowed better visualization of neuronal somata (Fig. 2A). We therefore used antibody bd-24 for detailed analysis. The most striking feature of the pattern of immunoreactivity as seen in the light microscope was the presence of small, strongly immunoreactive (SI) cells amongst the more numerous nonimmunoreactive somata. These cells often seemed to

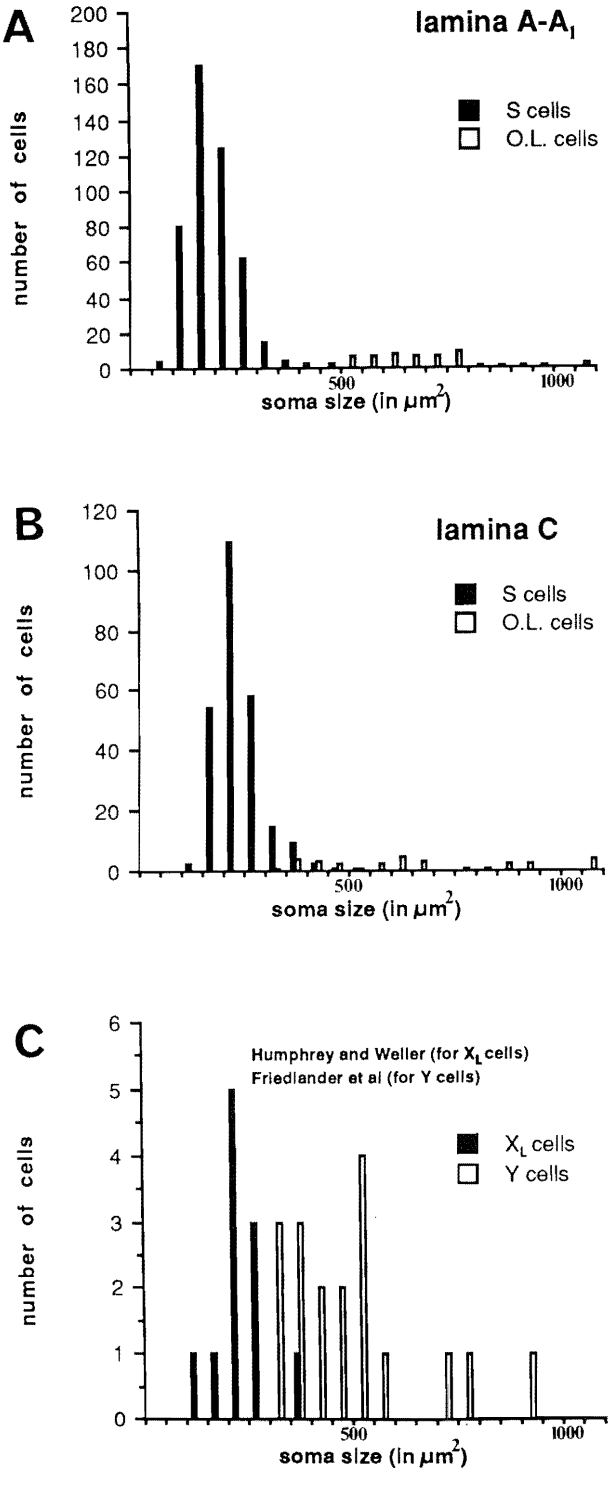

FIG. 3. Distribution of soma sizes (as measured by their light microscopically projected area) of different populations of neurons in the lateral geniculate nucleus of the cat. (A–B) Neurons immunoreactive for the α-subunit of the $GABA_A$-R were classified as strongly immunoreactive throughout the cell body (filled columns in A and B) or outlined by immunoreactivity along the plasma membrane (open columns). Populations are shown separately for the A (A) and C laminae (B). (C) For comparison the physiologically characterized populations of lagged X cells (X_L, Humphrey and Weller, 1988b) and Y cells (Friedlander *et al.*, 1981) are also shown.

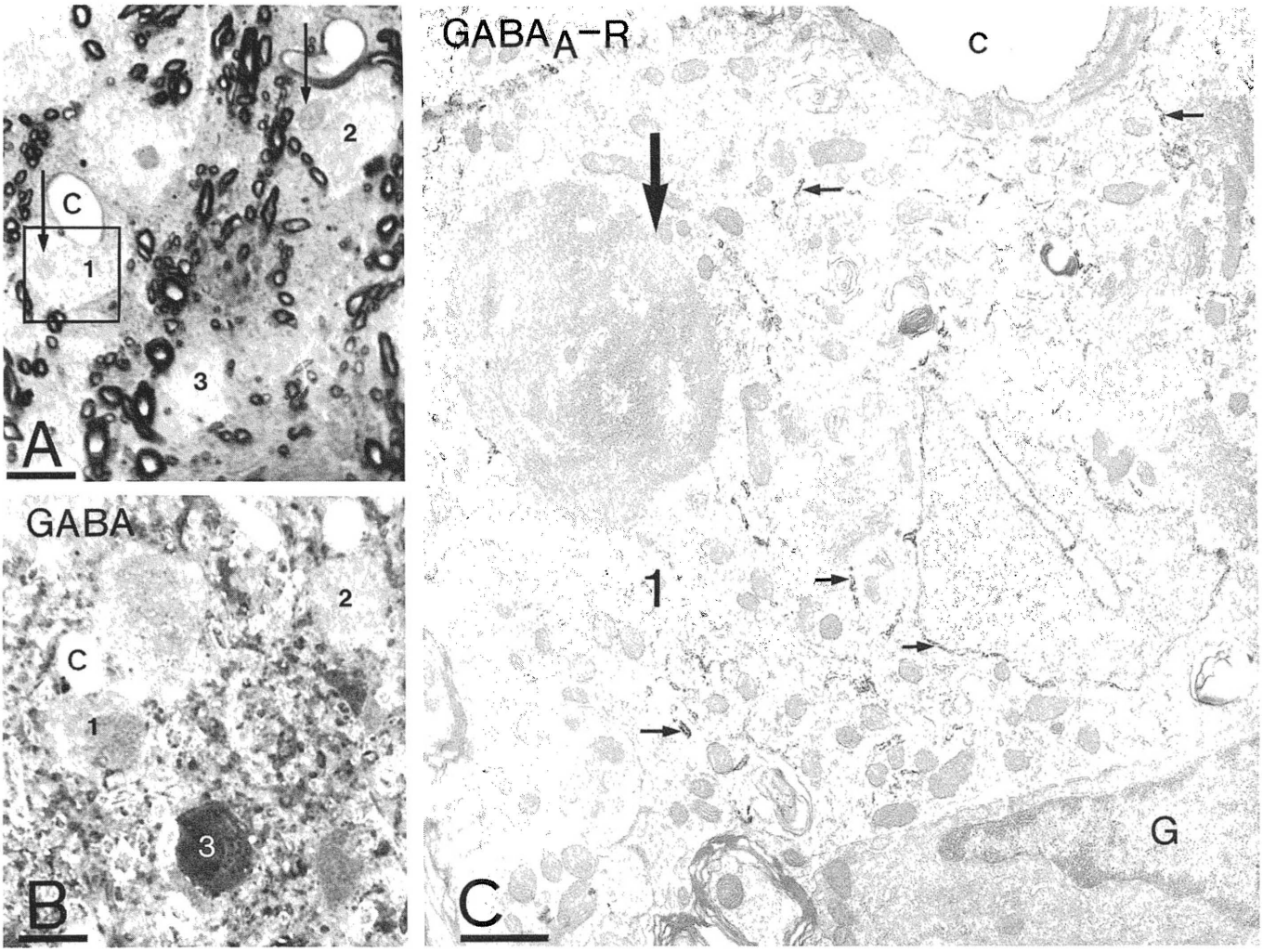

FIG. 4. Cytoplasmic laminated body (vertical arrows) containing neurons show strong immunoreactivity for $GABA_A$-R. (A–B) Light micrographs of semithin sections of the same cells stained with toluidine blue (A) or immunoreacted for GABA (B). Neurons (1,2) containing CLBs (vertical arrows) are GABA negative, while cell 3 is GABA positive. (C) Electron micrograph of the area framed in A, showing strong $GABA_A$-R immunoreactivity in the endoplasmic reticulum (small arrows) of cell 1. The glial cell (G) shows no immunoreactivity. c, capillary. Scales: A and B, 10 μm; C, 1 μm.

occur in groups of three or four (Fig. 2A), and they could be observed in all three laminae of the dorsal lateral geniculate nucleus (dLGN).

Another type of immunoreactive cell was characterized by a larger soma than cells of the previous group and was outlined by immunoreactivity along the presumed plasma membrane. No immunoreactivity could be detected within the somata of these neurons. The immunoreactivity of the plasma membrane was not confined to the somata, but in most cases the proximal dendrites were also outlined (Figs 2A and 2B and 6). We will refer to these neurons as 'outlined' cells. Like the SI cells the outlined cells could be found in all three laminae. In order to characterize the SI and outlined cells further the neurons were tested for the presence of GABA by immunocytochemistry. Out of about 20 cells tested from each category none was immunoreactive for GABA (Fig. 2). Numerous GABA-positive neurons were found in all laminae. In micrographs taken from the thick vibratome sections before sectioning for postembedding GABA reaction, the GABA-positive cells showed negligible immunoreactivity for the receptor/channel complex. Toluidine blue staining of 1 μm thick sections revealed that the SI cells frequently contained CLBs. Due to the limited and variable penetration of antibodies, only cells in the surface 10–15 μm of the section were immunoreactive. Inevitably many strongly immunoreactive (SI) cell

TABLE 1. Comparison of soma sizes (μm^2) of two populations of neuron distinguished on the basis of immunoreactivity for the α-subunit of the $GABA_A$-R in laminae $A-A_1$ and C (student *t*-test, two tailed). One population showed strong immunoreactivity (SI) throughout the cell body, the other one contained cells with light microscopically undetectable immunoreactivity intracellularly, but outlined by the immunoreaction endproduct (OL)

Cell types (laminae)				*P* values
SI($A-A_1$)	201 ± 56	vs. OL($A-A_1$)	691 ± 149	$P < 0.001$
SI($A-A_1$)		vs. SI(C)	190 ± 56	$0.02 > P > 0.01$
SI($A-A_1$)		vs. OL(C)	791 ± 293	$P < 0.001$
OL($A-A_1$)		vs. SI(C)		$P < 0.001$
OL($A-A_1$)		vs. OL(C)		$0.7 > P > 0.6$
SI(C)		vs. OL(C)		$P < 0.001$

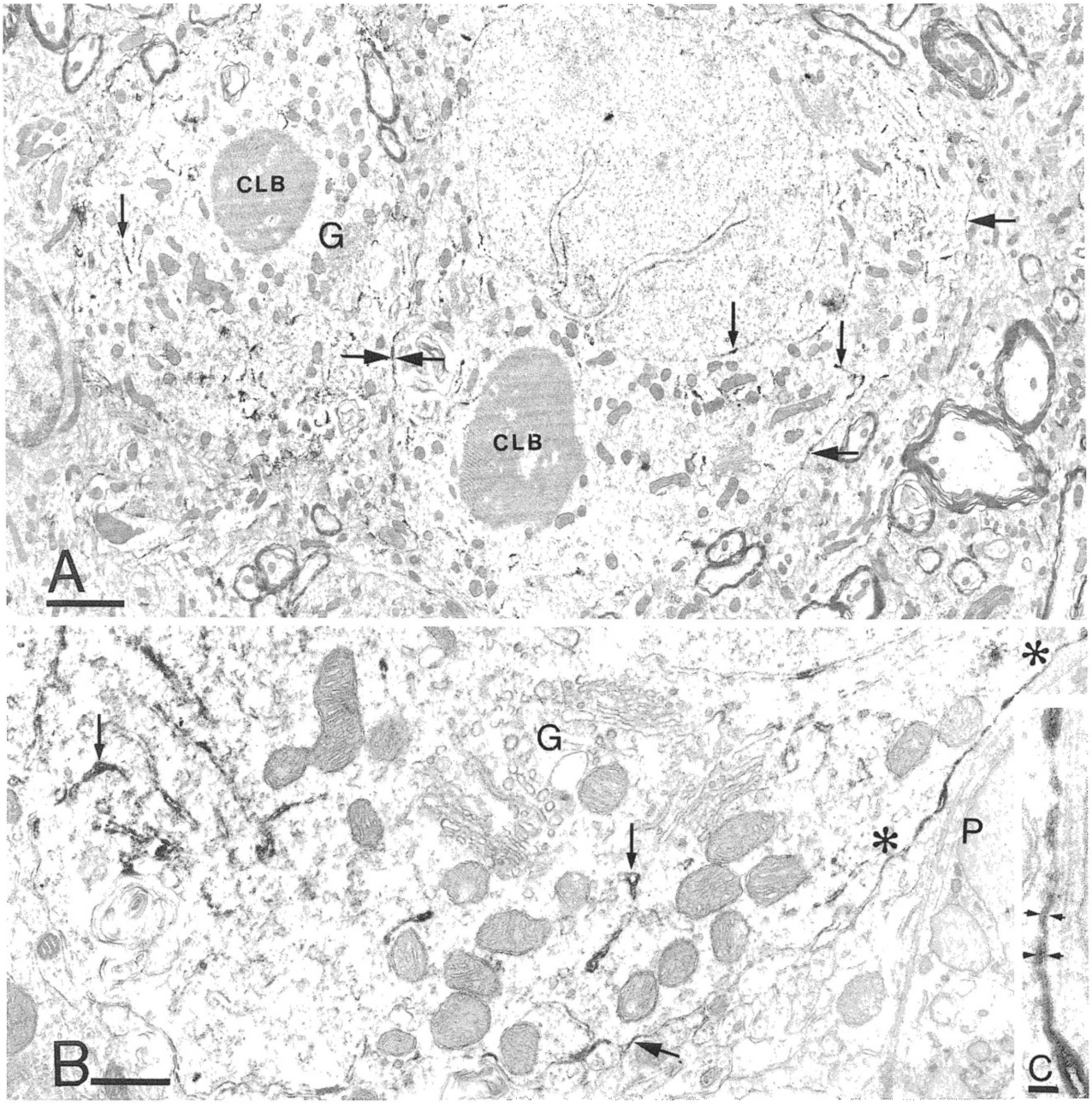

FIG. 5. Intra- and extracellular $GABA_A$-R distribution in strongly immunoreactive (SI in text) cells shown also in Figure 2 (arrows 5). (A) Electron micrograph of two CLB containing cells showing immunoreactivity in the cisternae of the endoplasmic reticulum and nuclear envelope (small vertical arrows). The plasma membrane is also immunoreactive in patches (horizontal arrows) including the area where the two cells are in direct apposition (opposing arrows). (B) Higher magnification view of another cell showing immunoreactivity in the ER, but not in the Golgi apparatus. The plasma membrane is also immunopositive including the area along glial lamellae (between asterisks) next to a pericapillary pericyte (P). (C) Detail of the plasma membrane showing immunoreaction product between the neuronal and the glial membrane in the extracellular space (arrows). Scales: A, 2 μm; B, 0.5 μm; C, 0.05 μm.

bodies were cut and only partially present in the section. This made it impossible to ascertain whether every SI cell contained CLBs. On the other hand, cells having CLBs were almost invariably strongly immunoreactive for the α-subunit of the receptor complex (95%, n = 42, confirmed in the electron microscope) (Fig. 4). The two exceptions were in many respects similar; they were rich in ribosomes, but they had disintegrated endomembranes indicating that these cells might have been poorly preserved for immunocytochemistry. CLBs were never observed in outlined or GABA positive neurons. Each CLB containing cell identified in semithin sections was also double-checked in the electron microscope to exclude the possibility of misidentification of these cytoplasmic organelles. Confirming previous observations

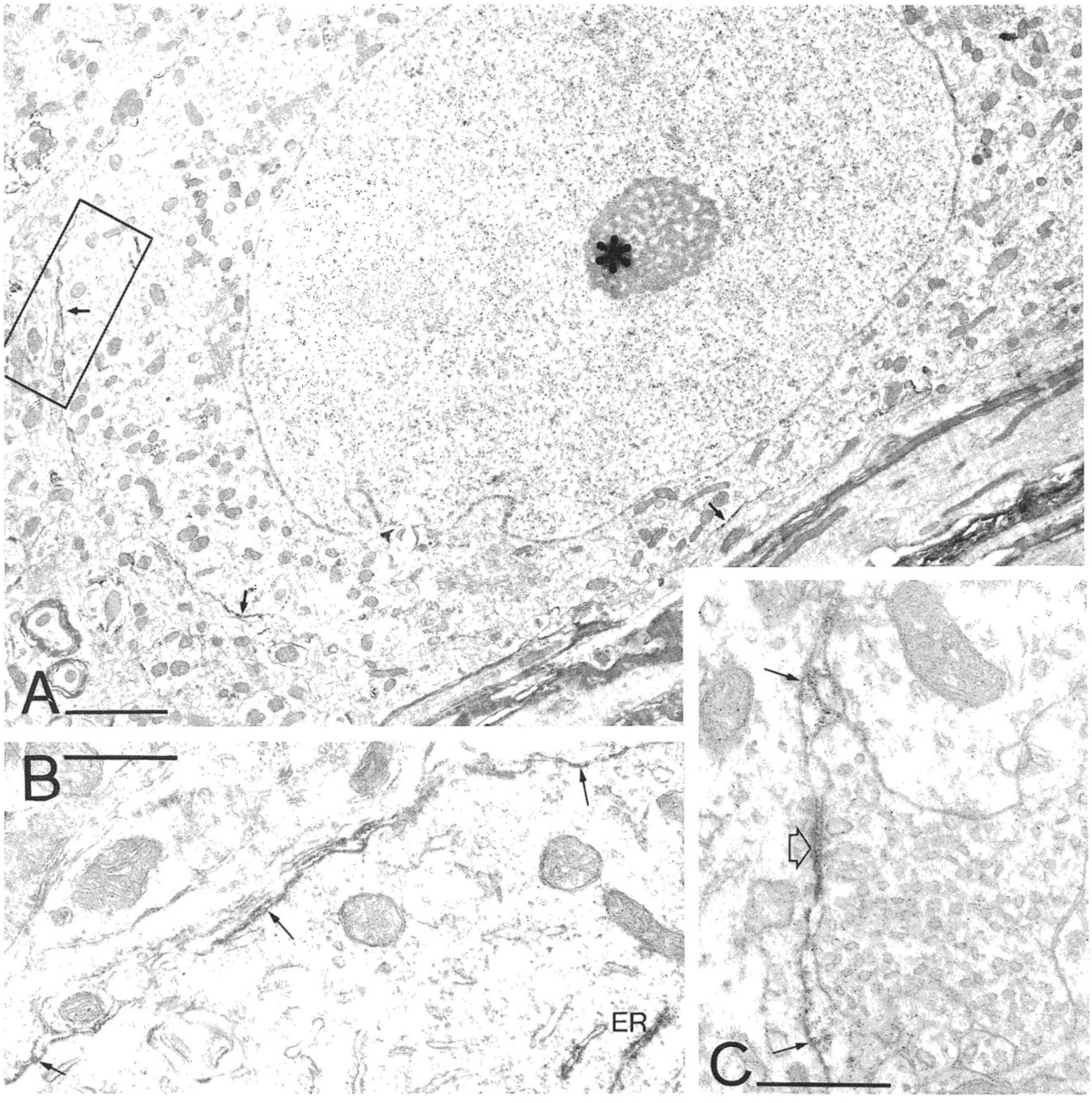

FIG. 6. $GABA_A$-R immunoreactivity associated with large cells outlined by immunoreaction end-product. (A–B) Electron micrographs showing the cell (asterisk) marked by the open arrow 6 in Figure 2A. Framed area in A is shown at higher magnification in B. (B) Immunoreactivity is present on the plasma membrane (arrows) and very rarely in the endoplasmic reticulum (ER). (C) Immunoreactivity is present in the synaptic cleft (open arrow) of an F type bouton and another cell of the same type, as well as along the nonjunctional plasma membrane (small arrows). Scales: A, 2 μm; B and C, 0.5 μm.

(Kalil and Worden, 1978), CLB containing cells appeared clustered in small groups. Two cells were found to have two CLBs (see also Kalil and Worden, 1978), and they were in every respect similar to SI cells having only one CLB.

To measure the sizes of somata immunoreactive for the receptor complex samples of cells were taken from two animals. Soma areas were measured separately for each animal, lamina and immunoreactive cell type (SI or outlined). As no difference was found between the two animals, and between laminae A and A_1, the data were pooled. The pattern of immunoreactivity in the C laminae was similar to the A laminae, but fewer immunoreactive cells could be seen. As shown in Table 1 and Figure 3, in both the A and C laminae, the somatic 'area' of SI cells (201 $\pm$ 56 μm^2, n = 462 in lam.A–A_1, and 190 $\pm$ 56 μm^2, n = 251 in lam.C) was significantly ($P < 0.001$) smaller than those of the outlined neurons (691 $\pm$ 149 μm^2, n = 53 in lam.A–A_1, and 712 $\pm$ 293 μm^2, n = 34 in lam.C).

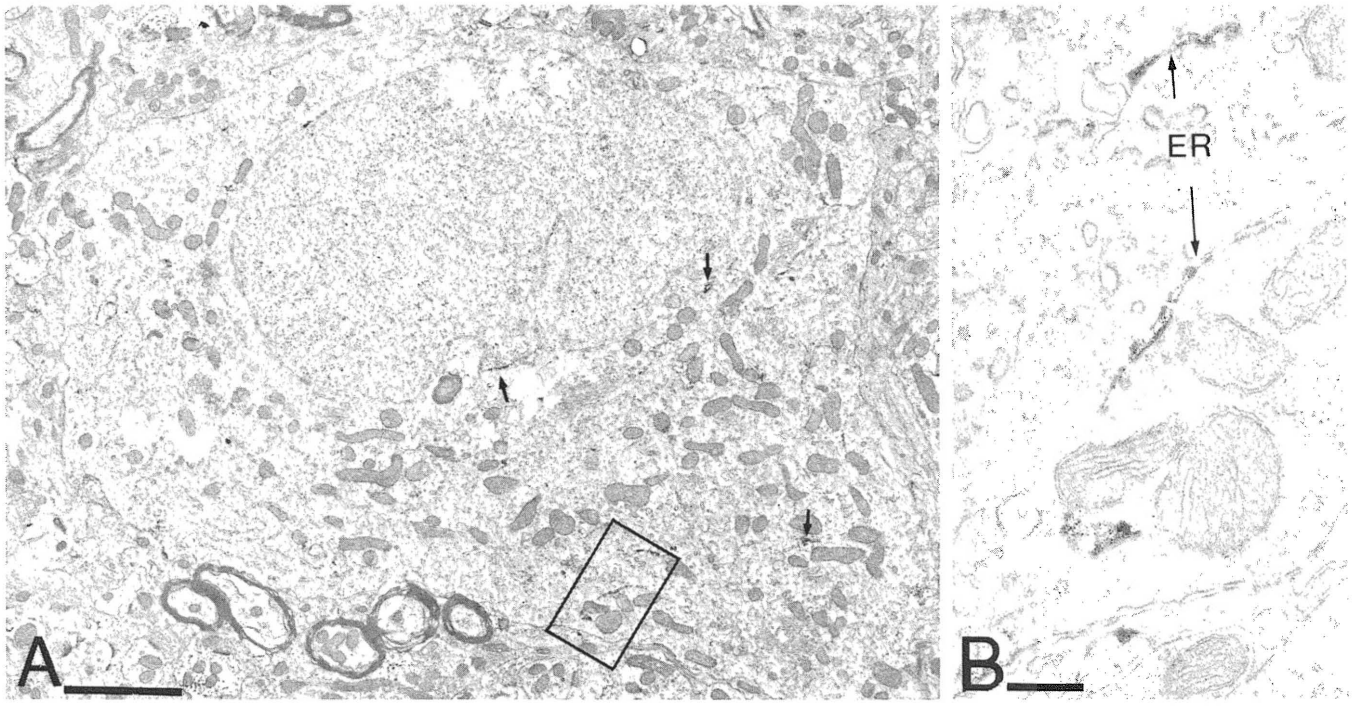

FIG. 7. Electron micrograph of the cell marked by double arrow 7 in Figure 2C showing sparse immunoreactivity in the endoplasmic reticulum (ER and arrows) for the α-subunit of the $GABA_A$-R. Framed area in A is shown at higher magnification in B. This neuron also contains GABA as shown in Figure 2C therefore it is very likely a local circuit neuron. Scales: A, 2 μm; B, 0.25 μm.

Distribution of immunoreactivity as detected by electron microscopy

Tissue that was treated with detergent showed poor localization of the immunoreaction end-production. Nevertheless it could be seen that the reaction was associated with somata and dendrites, and it was usually stronger along the plasma membrane. Since the membranes were disrupted the end-product also spread onto all intracellular organelles. The detergent treated material did not reveal additional immunoreactive sites when compared with material not treated with detergent. Therefore, the subcellular distribution has been described and illustrated on nontreated material where the localization can be clearly defined.

Membrane localization

In material not treated with detergent the immunoreaction endproduct was mainly limited to membrane delineated spaces. This included the extracellular gap between cells and processes, and the endoplasmic reticulum cisternae (Figs. 4–8). The Golgi saccules and vesicles only occasionally showed immunoreactivity, even in cells with heavy intracellular staining. From the deposition of the peroxidase reaction endproduct it could be established that the epitope reacting with the antibody was located on the extracellular surface of the plasma membrane and on the internal surface of both the granular and the agranular endoplasmic reticulum. Using the immunoperoxidase method the reaction endproduct formed in the narrow extracellular space is deposited on both of the closely juxtaposed membranes and often fills the space between them (Figs. 5C, 6 and 9). As a result, it is impossible to determine on which of the two membranes the antigen is located. However, if the plasma membranes of two cells of the same type were directly apposed and immunoreaction endproduct was present between them, this was taken as direct evidence that the membrane of that cell type contains the molecule carrying the epitope. Unfortunately such arrangements were rarely found. The clustered SI cells were an exception; their somata were frequently in direct membrane contact with other, often CLB containing SI cells (Fig. 5A).

Immunoreactive neuronal classes

The plasma membrane of outlined cells showed strong immunoreactivity confirming the light microscopic prediction (Fig. 6). This contrasted with their very sparse intracellular immunoreactivity. Only rarely were short segments of the endoplasmic reticulum and the nuclear envelope found to contain reaction endproduct (Fig. 6) although, like the SI cells, these neurons also had abundant endoplasmic reticulum. The plasma membrane was fairly evenly immunopositive both at somatic and proximal dendritic sites. Immunoreactivity was present in the synaptic cleft of F terminals establishing synaptic junctions with the proximal dendrites or somata (Fig. 6C) of outlined cells. The plasma membrane of outlined cells was also immunoreactive when apposed to glial cells.

SI cells were heavily immunoreactive in their endoplasmic reticulum, including the nuclear envelope, and along the plasma membrane as predicted by light microscopy. Occasionally a few cisternae of the Golgi apparatus were also immunopositive. CLBs never showed any immunoreactivity.

GABA positive cells identified by correlated light and electron microscopy showed only moderate levels of immunoreactivity (Fig. 7). Few immunoreactive endoplasmic reticulum profiles were observed, and these contained reaction endproduct in short segments (Fig. 7B). Immunoreactivity has not been found on the somatic membrane of these cells.

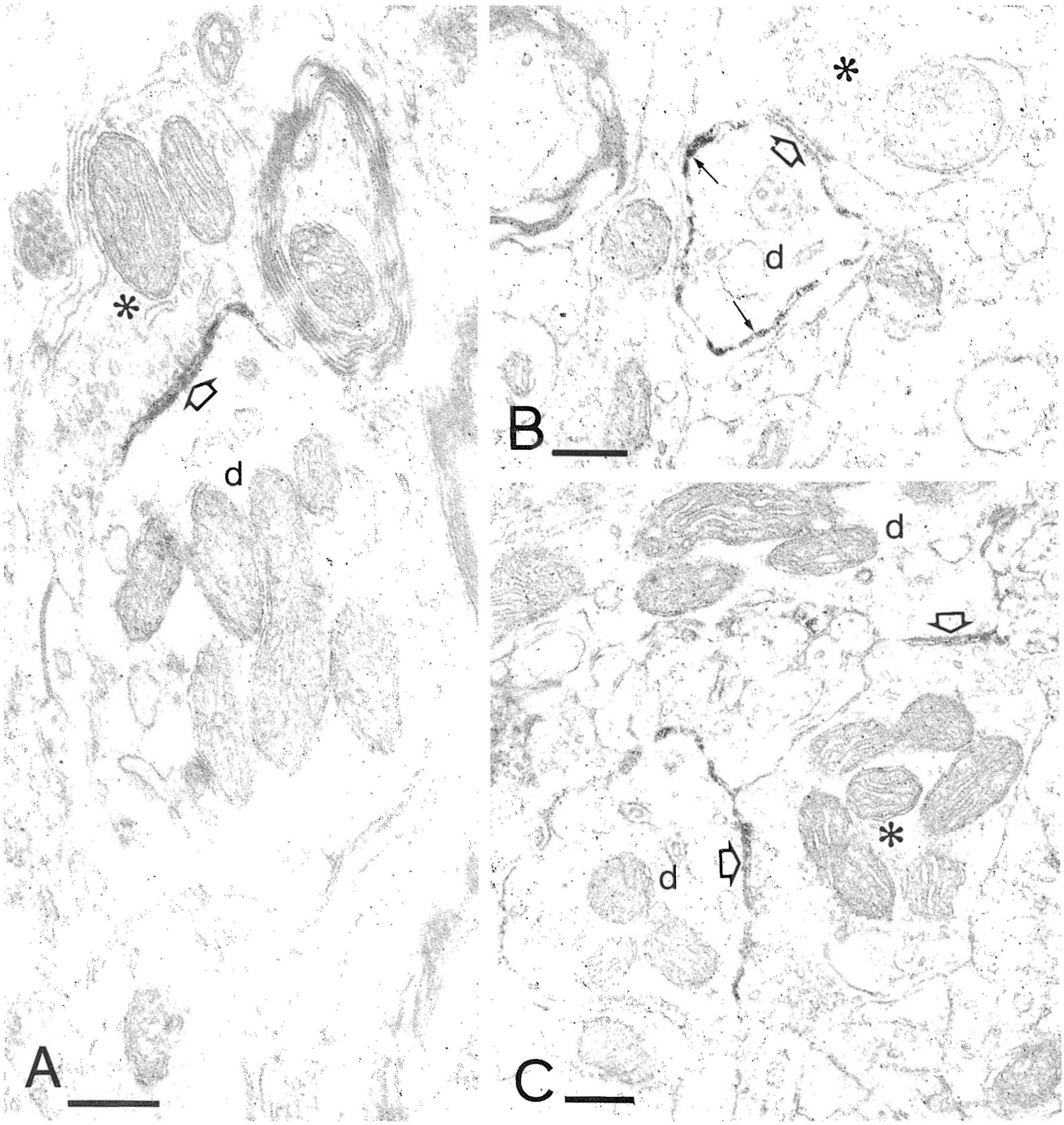

FIG. 8. Immunoreactivity for the $GABA_A$-R α-subunit in the neuropil of the cat LGN. (A–C) Dendrites (d), probably originating from relay cells show immunoreactivity both at nonjunctional sites (small arrows) and at synaptic junctions (open arrows in A and C) formed with F terminals (asterisks). In many cases the synaptic junctions involving F terminals were immunonegative (e.g. open arrow in B). Scales: A, B and C 0.25 μm).

Immunoreactivity in the neuropil

The reaction was similar to that described above for the somata in that the reaction product was localized on the plasma membrane both at junctional and nonjunctional sites (Fig. 8). Projection cell dendrites showed varying degrees of immunoreactivity. We did not attempt to classify these dendrites as belonging to X or Y cell categories given the current debate concerning the morphological classification of physiologically identified neurons (Mastronarde, 1987a,b; Humphrey and Weller, 1988a,b). Immunoreactivity was observed at dendritic membranes apposed to other dendrites, to glial processes, or in synaptic junctions with F terminals (Fig. 8). Synaptic terminals making symmetrical synapses were classified as either F1 or F2 on previous criteria (Guillery, 1969; Hamori *et al.*, 1974; Lieberman and Webster, 1974; Szentagothai, 1973), when one or more of the following characteristics could be seen. Terminals that were postsynaptic to other boutons were considered F2 presynaptic dendrites. These terminals

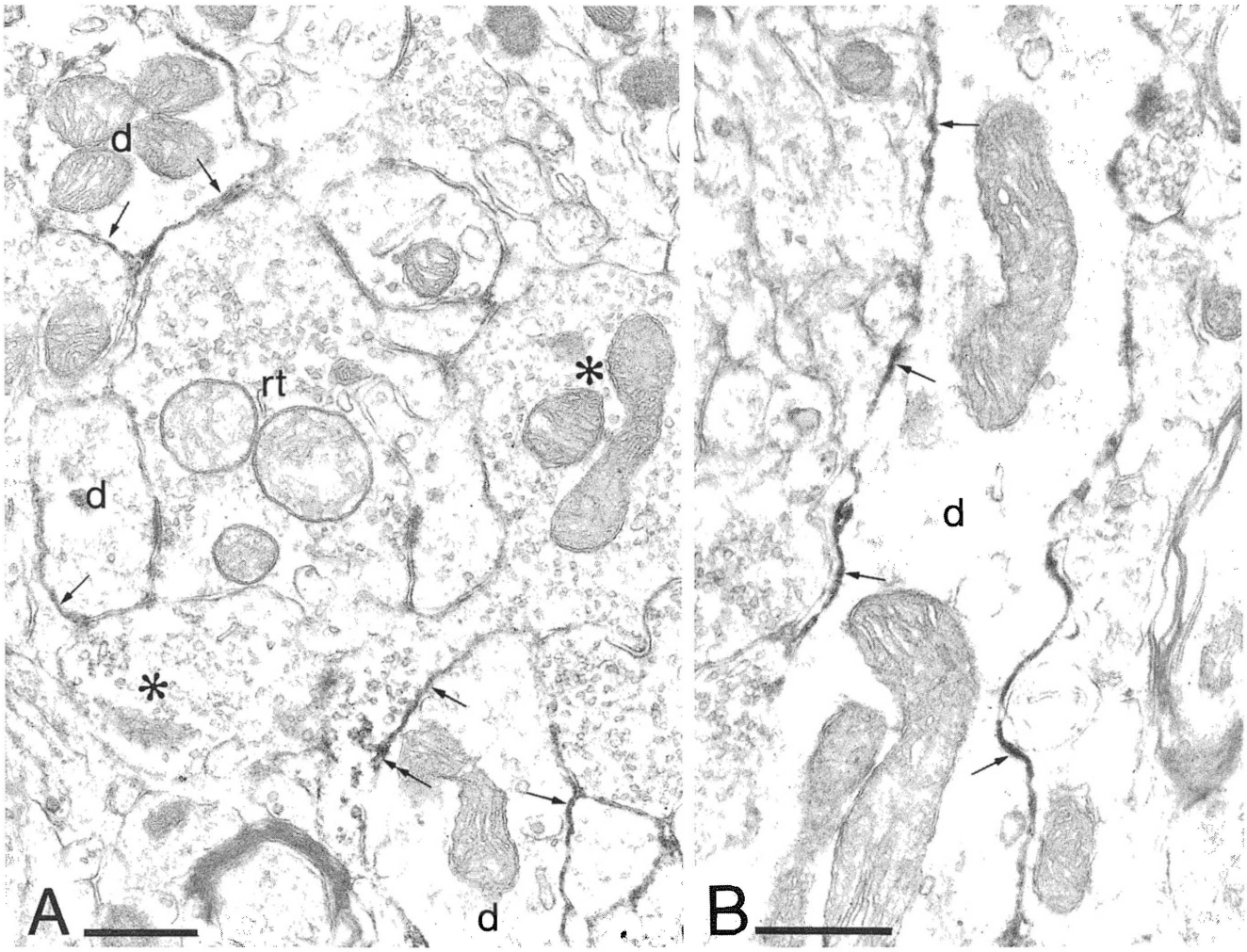

FIG. 9. (A) Immunoreactivity (e.g. small arrows) for the $GABA_A$-R α-subunit around dendrites (d), probably originating from relay cells and situated near a retinal terminal (rt) in a glomerulus. At a site where the plasma membrane is broken (double arrow) the immunoreaction end-product spread into a neighbouring nerve terminal. Asterisks mark probable F terminals. (B) Same material as shown in A, but the dendrite (d) is not in a glomerulus. Receptor immunoreactivity (arrows) is present along nonsynaptic plasma membrane. Scales: 0.5 μm.

usually had light cytoplasm, loosely scattered vesicles and were often associated in groups within glomeruli. In the glomeruli, terminals were classified as F2 even in the absence of boutons presynaptic to them if the other characteristics were present. Other terminals making symmetrical synapses had more densely packed vesicles and more electron-dense cytoplasm and were classified as F1 (axonal) boutons. It was generally easy to find F1 profiles at immunoreactive synaptic junctions, but synapses made by F2 profiles (presynaptic dendrites) were largely immunonegative. F2 profiles postsynaptic to F1 or other F2 terminals were not immunoreactive at the synaptic or at the non-synaptic sites (Figs. 8 and 9). Similarly, F1 profiles showed no immunoreactivity at membrane regions outside the F1 to dendrite/soma contact zone (Figs. 6 and 8). Immunoreactivity seemed to be absent from or very rare in the synaptic clefts established by retinal terminals or by synaptic endings of presumed cortical or projection cell origin identified on the basis of fine structural criteria (Guillery, 1969). Synaptic junctions made by other than F terminals showed no immunoreactivity generally, although in cases of heavy immunoreactivity on adjacent sites some reaction endproduct could be detected in these synaptic clefts. However, as in all pre-embedding immunocytochemistry, technical factors, such as differential penetration of the antibodies, different density or masking of the epitopes, could account for some of the negative findings.

The membranes of glial processes were covered with reaction endproduct only when apposed to projection cell dendrites, indicating that in these cases the epitope recognized by the antibody was localized on the extracellular surface of the projection cell dendrite and not on the glial membrane. Myelinated fibres of the optic tract or axons of different origin were never immunoreactive.

Discussion

The present immunocytochemical study on the dLGN of cat extends the results of previous reports demonstrating the presence of high levels of $GABA_A$ receptors in the dLGN of the rat. Autoradiographic ligand binding studies found a high or very high concentration of binding sites

for the $GABA_A$ receptor (Bowery *et al.*, 1987, Palacois *et al.*, 1981) in the dLGN of the rat. Immunohistochemical studies using monoclonal antibodies also demonstrated a very high concentration of $GABA_A$/benzodiazepine receptor proteins in the dLGN of rodents (Richards *et al.*, 1987; De Blas *et al.*, 1988).

Specificity of the immunoreaction

The recent recognition of a superfamily of ligand gated receptor/channel proteins (Barnard *et al.*, 1987; Grenningloh *et al.*, 1987; Schofield *et al.*, 1987) that includes the $GABA_A$/benzodiazepine, the glycine and the nicotinic acetylcholine receptors, raises the question of antibody crossreactivity with several of these proteins. It has been discussed elsewhere that the recognition of proteins other than the α- and β-subunit of the $GABA_A$/benzodiazepine receptor by bd-24 and bd-17 respectively is highly unlikely (Richards *et al.*, 1987; Somogyi *et al.*, 1989a). In the case of the dLGN, crossreactivity with glycine receptors can be excluded because strychnine-sensitive glycine receptors are undetectable both by autoradiography (Wamsley and Palacois, 1984; Bristow *et al.*, 1986; Probst *et al.*, 1986) or by immunohistochemistry (Araki *et al.*, 1988), and there does not seem to be significant binding of glycine to strychnine-insensitive sites either (Bristow *et al.*, 1986). Nicotinic acetylcholine receptors are present in high concentration in the dLGN as revealed by ligand binding (Clarke *et al.*, 1985) and immunocytochemical (Swanson *et al.*, 1987) studies. However, from the distribution of immunoreactivity patterns for the $GABA_A$/benzodiazepine receptor (Richards *et al.*, 1986, 1987; De Blas *et al.*, 1988) and the nicotinic acetylcholine receptor (Swanson *et al.*, 1987) it is clear that our antibodies do not cross-react with brain nicotinic receptor sites.

Considering the strong immunoreactivity in Triton-treated sections, it was surprising that in the absence of detergent only negligible immunoreactivity could be detected with the β-subunit specific antibody, even with the electron microscope. In the cerebellum of the cat only the strongest sites showed some immunoreactivity with this antibody while in the monkey the two antibodies gave similar levels of immunoreactivity (Somogyi *et al.*, 1989a). Immunoreactivity with both antibodies and in all species is clearly enhanced by detergent, but the epitope recognized by bd-17 in the cat seems to be masked more than in other species.

Recent studies indicate that there are several different α-subunits in the brain (Fuchs *et al.*, 1988) and these may differ in their pharmacological characteristics (Levitan *et al.*, 1988). As our antibody may only recognize some of the α-subunit proteins it cannot be established at present whether the differences between different classes of cells are due to variation in subunit composition or to the degree of receptor expression.

Subcellular distribution of immunoreactive receptors

Immunoreactivity in the endoplasmic reticulum and in the Golgi apparatus presumably reflects the biosynthesis of the protein and the addition of carbohydrate residues respectively. The level of intracellular and plasma membrane immunoreactivity is not well correlated in the dLGN, or in the cerebellum. Thus, some classes of neuron such as cerebellar granule cells (Somogyi *et al.*, 1989a) and large GABA-negative cells in the dLGN can have strong immunoreactivity on their plasma membrane but negligible immunoreactivity intracellularly. The degree of immunoreactivity is probably related to the intensity of receptor synthesis and receptor turnover, while intensity of plasma membrane immunoreactivity is probably related to receptor density (Somogyi *et al.*, 1989a). It has been suggested from *in situ* hybridization studies that the α-, but not the β-subunit of the receptor complex is expressed in cerebellar Purkinje cells (Siegel, 1988; Richards *et al.*, unpublished observation). Our immunocytochemical studies showed that immunoreactivity for both subunits can be detected on Purkinje cells, but the intracellular immunoreactivity was significantly stronger for the α-subunit (Somogyi *et al.*, 1989a). This may explain the stronger signal obtained for this subunit in *in situ* hybridization (Siegel, 1988; Richards *et al.*, unpublished observation). The situation seems to be similar in the dLGN, where *in situ* hybridization studies demonstrated very high hybridization signals with an α-subunit specific probe, but very low signals with a β-subunit probe (Sequier *et al.*, 1988).

With regard to the distribution of receptors in the plasma membrane, it should be noted that the epitope recognized by bd-24 is unknown, although it must be outside the ligand recognition site since neither GABA nor benzodiazepine binding is inhibited by the antibody (Haring *et al.*, 1985). It is surprising that the receptor is widely distributed both at synaptic junctions and at non-synaptic plasma membrane. A previous preliminary electron microscopic study, using antibody bd-17, emphasized the synaptic location of the receptor complex (Richards *et al.*, 1987). The present study, as well as results obtained in the cerebellum (Somogyi *et al.*, 1989a) and in the neocortex (Somogyi *et al.*, 1989b) demonstrated that the junctional and non-junctional distribution of the receptor complex is a general feature of the central nervous system. However, the distribution is not uniform even on the same cell, because synaptic junctions of F terminals, most of which have been shown to contain GABA (Ohara *et al.*, 1983; Fitzpatrick *et al.*, 1984; Montero, 1986, 1987; Montero and Singer, 1985), were frequently immunopositive. In contrast, junctions of retinal and presumed cortical boutons, which are not GABAergic, were almost without exception immunonegative. It was shown in the cerebellum, that the nonjunctional immunoreactivity cannot result from the diffusion of the reaction end-product, but reflects the distribution of the receptor protein (Somogyi *et al.*, 1989a).

The function of the nonjunctional receptors remains to be established. We have proposed that the widespread distribution of the receptor on the plasma membrane means that the topography of GABAergic influence mediated by $GABA_A$ receptors is governed by the precise placement of GABA-releasing terminals, and not by the location of the receptors (Somogyi *et al.*, 1989a).

Differential expression of the receptor complex by different classes of neuron

Immunohistochemical studies have revealed significant differences in $GABA_A$ receptor concentration between different types of neuron located in the same brain region (Richards *et al.*, 1987; De Blas *et al.*, 1988; Somogyi *et al.*, 1989a). These differences have been confirmed by *in situ* hybridization (Sequier *et al.*, 1988; Siegel *et al.*, 1988) demonstrating differences in the concentration of mRNAs. As in other brain regions the α-subunit specific antibody revealed differences between morphologically and functionally different classes of neuron in the dLGN of the cat.

Immunoreactivity of projection cells

The two cell types found by immunostaining may correspond to functionally distinct neural types. The first type is the SI cell, which frequently contained CLBs and was GABA-negative with a soma area distribution almost identical (Fig. 3) to that of the so called 'lagged'

X cells (X_L) identified recently morphologically by Humphrey and Weller (1988b). These cells show an early, short duration inhibition in their response to visual stimulus and this is thought to be produced by intrageniculate inhibitory interneurons (Mastronarde, 1987a,b; Humphrey and Weller, 1988a,b). The different types of X cells form about 76% of the relay cell population (Wilson *et al.*, 1976) and about 57% of the total neuronal population in lamina A. Since approximately every third X cell has X_L characteristics, about 19% of the total neuron population is of X_L cell type. This figure agrees well with that reported by Kalil and Worden (1978) for CLB-containing cells which comprise somewhat less than 20% of neurons in the LGN. In addition, the soma size distribution of CLB-containing cells (Kageyama and Wong-Riley, 1985, 196 ± 21.5 μm^2) is very similar to that of the X_L cells (Humphrey and Weller, 1988b, 236 ± 66 μm^2) and SI cells (present study, 201 ± 56 μm^2). The SI cells in the C laminae might correspond to X-type cells recorded there previously (Wilson *et al.*, 1976; Sherman and Spear, 1982; Stanford *et al.*, 1983; Mize *et al.*, 1986; Sur *et al.*, 1987). Furthermore, Kalil and Worden (1978) reported that though CLBs are present in the C laminae, they are less frequent than in the A laminae. This finding parallels our observation of fewer SI cells in the C laminae. If our SI cells correspond to the X_L cells, the CLBs can be used as a marker for these cells, though it remains to be established if all CLB-containing cells show strong immunoreactivity for the receptor complex.

Our antibody also labelled the plasma membrane of a cell population with a soma area distribution similar to that of the physiologically identified medium and large Y cells (Fig. 3). A probable Y cell population with large somatic areas has been marked by the monoclonal antibody Cat-301 (Hendry *et al.*, 1988), indicating that neuronal diversity might exist in the Y cell population as well (Mastronarde, 1988). The smaller Y cell population and the medium-sized possibly non-lagged X cells seem to be only weakly immunopositive for the α-subunit.

The difference between the staining pattern of the somatic and dendritic membranes is of interest. Neurons in the LGN are thought to receive synapses from F terminals, most of which are GABAergic, mainly on or close to the cell body (Guillery, 1969; Wilson *et al.*, 1984; Hamos *et al.*, 1987). The inhibitory input to X cells is thought to be predominantly of the F2 (presynaptic dendrite) type, forming triadic synaptic arrangements on dendritic appendages. In addition some F1 terminal (axonal) input can be found both on the somata and proximal dendrities (e.g. cell 1 with a CLB of Hamos *et al.*, 1987; Wilson *et al.*, 1984). On the other hand Y cells which do not seem to receive F2 input have comparatively more F1 terminals on or close to the soma (e.g. cell 4 of Hamos *et al.*, 1987; Mason *et al.*, 1984). Thus, the strong immunoreactivity of outlined cells on the soma and proximal dendritic membrane might reflect this larger somatic GABAergic input of Y cells.

Immunoreactivity of local circuit GABAergic neurons

Interneurons in the LGN showed weak immunoreactivity with antibody bd-24 similarly to the cerebellar Golgi cells (Somogyi *et al.*, 1989a), which are also GABAergic. It is interesting that other GABAergic interneurons in the cerebellum (Somogyi *et al.*, 1989a), the basket and stellate cells, and some GABA-positive cells in the cortex and hippocampus (unpublished observation) show the strongest intracellular $GABA_A$ receptor immunoreactivity. These differences may reflect quantitative differences in the degree of receptor expression, and possibly the different subunit structure of the receptor complex.

Inhibitory influences in the dGLN in relation to $GABA_A$ receptor distribution

It has been suggested that $GABA_A$ receptors play a role in several neuronal operations in the dLGN of the cat. Evidence from micro-ionophoretic application of the $GABA_A$ antagonist bicuculline indicates that binocular inhibition (Pape and Eysel, 1986), inhibition responsible for orientation bias (Vidyasagar, 1984), centre-surround inhibition (Sillito and Kemp, 1983), and global lateral inhibition outside the antagonist receptive field (Eysel *et al.*, 1987) all involve $GABA_A$ receptors. Furthermore, physiologically identified cell types in the dLGN of the cat differed mainly in qualities determined by the GABA mediated inhibition. For example in the spatial domain the differences in the spatial frequency tuning of X and Y cells (Maffei and Fiorentini, 1973; Lehmkuhle *et al.*, 1980; Derrington and Fuchs, 1979) are due to a bicuculline-sensitive local GABAergic inhibitory system (Berardi and Morrone, 1984). The temporal frequency tuning curves of X and Y cells are also affected differently by bicuculline, demonstrating that the preference for objects moving with a particular velocity is shaped differentially by GABAergic influences (Bernardi and Morrone, 1984). The present evidence that one of the two receptors for GABA is distributed differently on projection cell types is in line with the physiological differences. However, it should be noted that not all of the characteristics defining X and Y neuronal classes in the dLGN are sensitive to bicuculline (Berardi and Morrone, 1984).

The subclass of lagged X cells is of particular interest. These cells are subjected to a strong, stimulus induced inhibition lasting for 10–40 ms (Mastronarde, 1987a,b; Humphrey and Weller, 1988a), and as we suggested above may correspond to strongly $GABA_A$ receptor immunoreactive neurons. This inhibition seems to originate from the GABAergic (and non-lagged) interneurons, as perigeniculate cells have long (up to 70 ms) latency following visual stimulation (Humphrey and Weller, 1988a). The latency and duration of inhibition is very similar to that of the short latency, short duration, Cl^- dependent and bicuculline-sensitive $GABA_A$ inhibitory postsynaptic potentials described in the dLGN both *in vivo* (Singer and Creutzfeldt, 1970; Singer and Bedworth, 1973; Francesconi *et al.*, 1988; Bloomfield and Sherman, 1988) and *in vitro* in rats (Hirsch and Burnod, 1987; Crunelli *et al.*, 1988) and cats (Soltesz *et al.*, 1989). Therefore it seems reasonable to suggest that the dip in firing of lagged X cells is caused by the activation of $GABA_A$ receptors. The strong immunoreactivity for the $GABA_A$ receptor complex shown here in cells which have similar somatic size to X_L cells, may underline high receptor turnover and the enhanced sensitivity of these cells to GABAergic inhibition.

GABAergic perigeniculate cells and local GABAergic neurons make F1 boutons in the dLGN. As indicated by the results of the present study, the synaptic contacts made by many F1 terminals on projection neurons were positive for $GABA_A$ receptor complex. Thus the perigeniculate contacts probably involve $GABA_A$ receptors. This is consistent with the electrophysiological studies of Thomson (1988) who observed Cl^--dependent IPSPs in projection neurons of the rat ventrobasal thalamus when the nucleus reticularis was stimulated.

In contrast to contacts made by F1 terminals, immunoreactivity at the synaptic junctions of projection cell dendrites postsynaptic to F2 terminals was rare and weak. Since all presynaptic dendrites contain GABA, strong receptor immunoreactivity was expected in the glomeruli. At present we have no explanation for this difference between F1 and F2 terminals. Even after detergent treatment only main relay cell dendrites associated with glomeruli were immunopositive

and dendritic appendages receiving synapses from F2 terminals were largely immunonegative, indicating that lack of antibody penetration is an unlikely explanation for the negative result. Antibodies to other subunits may reveal differences in receptor composition between different parts of the same cell.

Acknowledgements

The authors are grateful to Miss J. Ellis and Mr Frank Kennedy for excellent technical assistance, to Mrs P. Twynam for secretarial help and to Mr Attila Gulyas for his participation in one of the experiments. H. Takagi was supported by a grant from the Wellcome Trust. I. Soltesz was supported by the Herbert von Karajan Neuroscience Research Trust and acknowledges support from the Neuroscience Research Laboratory of the Hungarian Academy of Sciences.

Abbreviations

General abbreviations

bd-17	monoclonal antibody specific to β-subunit(s) of the receptor complex
bd-24	monoclonal antibody specific to α-subunit(s) of the receptor complex
GABA	gamma aminobutyric acid
$GABA_A$-R	gamma aminobutyric acid receptor type A
OL	outlined cell
PB	phosphate buffer
PBS	phosphate buffered saline
SI	strongly immunoreactive
TPBS	butylbicyclophosphorothionate

Anatomical abbreviations

CLB	cytoplasmic laminated body
ER	endoplasmic reticulum
F terminal	synaptic terminals with pleomorphic vesicles making symmetrical synaptic contacts
LGN	lateral geniculate nucleus
dLGN	dorsal lateral geniculate nucleus

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