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Subcellular Localization of Benzodiazepine/$GABA_A$ Receptors in the Cerebellum of Rat, Cat, and Monkey Using Monoclonal Antibodies

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Two monoclonal antibodies, bd-17 and bd-24, specific for the β- and α-subunit of the $GABA_A$/benzodiazepine receptor/chloride channel complex, respectively, were used to determine the subcellular distribution of immunoreactivity in the cerebellum by electron microscopy. The 2 antibodies showed similar antigen distribution on the plasma membrane (except in the rat; bd-24 does not recognize the rat antigen), but intracellular immunoreactivity was more prevalent for the α-subunit.

The plasma membrane of all neuronal types was immunopositive. The degree of immunoreactivity varied greatly between different types of cell, but it was stereotyped among individual cells of the same type. Granule cells showed the strongest immunoreactivity, not only on their dendrites which receive synapses from GABA-containing Golgi cell terminals, but also on their somata which do not receive synapses. Stellate and basket cells were somewhat weaker in immunoreactivity. Purkinje cells were only weakly positive on their somatic membrane but stronger on their dendritic shafts and spines. Golgi cells showed negligible if any immunoreactivity. Neurons of the deep cerebellar nuclei were strongly immunopositive along their plasma membrane. Immunoreactivity was strong in cisternae of the endoplasmic reticulum and in the Golgi saccules of stellate and basket cells, variable in Purkinje cells, while granule cells were rarely immunoreactive intracellularly. It is suggested that these differences reflect differences in the turnover of the receptor complex in the different cell types.

The synaptic clefts established by boutons of the GABAergic stellate, basket, and Golgi cells were immunopositive, as were many synapses in the deep cerebellar nuclei. However, immunoreactivity was also present along the nonjunctional plasma membrane, and it was concluded that this reflected the distribution of the antigen. The synaptic clefts at the presumed glutamate-releasing parallel and mossy fiber terminals were almost always immunonegative. No immunoreactivity was detected on axons, nerve terminals, or glial cells.

The results demonstrate that different neuronal types express the $GABA_A$/benzodiazepine receptor/chloride channel complex to different degrees. The distribution of the receptor complex suggests that the cellular topography of GABAergic influence is not governed by the precise spatial arrangement of the receptors but by the precise placement of the GABA-releasing terminals, a characteristic of the cerebellar circuit.

The $GABA_A$ receptor belongs to the family of ligand-gated receptor–channel complexes. The activation of this receptor leads to opening of chloride channels and inhibition of the neurons that possess it (for review, see Alger, 1985). The receptor is also the site of action for such clinically important drugs as benzodiazepines and barbiturates. The structure and localization of the receptor is therefore of considerable scientific interest. The structure of the receptor is relatively well known. The receptor proteins have been purified (Schoch and Mohler, 1983; Sigel et al., 1983; Schoch et al., 1984; Taguchi and Kuriyama, 1984; Kirkness and Turner, 1986), and the α- and β-subunits have been cloned (Schofield et al., 1987). Pharmacological studies have revealed complex interactions between the various ligand binding sites of the receptor complex (Mohler and Okada, 1977; Squires and Braestrup, 1977; for review, see Haefely et al., 1985).

The regional distribution of $GABA_A$-specific binding sites has also been mapped in the CNS (Young and Kuhar, 1979, 1980; Palacios et al., 1981; for review, see Wamsley and Palacios, 1984) through the use of radioactive ligands in autoradiographic studies. More recently, monoclonal antibodies to the receptor complex (Haring et al., 1985; Schoch et al., 1985; Vitorica et al., 1988) have allowed the mapping of the regional distribution of the receptor proteins in the brain (Richards et al., 1987; De Blas et al., 1988; Houser et al., 1988). The antibodies have been used to reveal also the localization of receptors at some synaptic junctions (Richards et al., 1986, 1987).

However, most areas of the brain contain a diverse population of neurons supplied by an equally diverse population of GABA-releasing synaptic boutons. These boutons differ in their origin, and in their postsynaptic targets, i.e., in the position of their parent cells in the neuronal networks, as well as in their biochemical characteristics (e.g., Baimbridge and Miller, 1982; Hendry et al., 1984; Somogyi et al., 1984; Nakagawa et al., 1986; Kosaka et al., 1987). It would help the understanding of the action of GABA and the drugs that influence GABAergic transmission if the localization of the receptors could be achieved with a resolution that permitted their identification on particular cell membranes and at synapses where the identity of the pre- and postsynaptic elements could be established. An area where

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the position, origin, and role of GABAergic synapses is best known is the cerebellar cortex, in which both the pre- and the postsynaptic elements can easily be identified on anatomical grounds. Therefore, the cerebellum lends itself to the clarification of several questions concerning the cellular and subcellular distribution of the receptors.

This study was undertaken to determine if the presence of the $GABA_A$/benzodiazepine receptor complex is restricted to synaptic junctions formed by GABAergic boutons of known origin or if it could be found at other, non-GABAergic synapses, as well as at extrasynaptic sites. Since the cerebellar cortex contains 5 major neuronal types identifiable with both light and electron microscopy, the distribution of receptors can be compared on neurons receiving different GABAergic input on different parts of their somatic and dendritic membranes. Furthermore, there is evidence from biochemical studies on brain cell cultures that, in addition to the receptors which are accessible to ligands that do not cross the cell membrane, there is a pool of presumed intracellular receptors (Czajkowski and Farb, 1986). Since no such receptors have been localized by ligand binding or immunocytochemical studies, we searched for intracellular sites that contained immunoreactive receptors.

To clarify the above questions, immunoelectron microscopy and 2 well-characterized monoclonal antibodies specific to the α- and β-subunits of the receptor, respectively (Schoch et al., 1984, 1985; Haring et al., 1985), were used.

Materials and Methods

Preparation of animals and tissue. One Wistar and 6 Sprague-Dawley female rats (160–200 gm), 2 adult male cats, and 1 adult male cynomolgus monkey (*Macaca fascicularis*) were used. The cats and rats were deeply anesthetized with chloral hydrate (400 mg/kg, i.p.), and the monkey was first sedated with ketamine hydrochloride (intramuscular, Ketalar, Parke Davis) and then deeply anesthetized with sodium thiopentone (i.v., Penthotal, May and Baker). Fixation of the brains was carried out by transcardial perfusion with saline for 1 min, followed by fixative (200 ml/rat; 1.5—1/cat; 2—1/monkey) for about 30 min at room temperature.

The following fixatives were used:

1. Paraformaldehyde (4%), glutaraldehyde (0.05%), and picric acid (~0.2%), made up in 0.1 M (pH 7.2–7.4) phosphate buffer (Somogyi and Takagi, 1982), was used for 3 rats (including the Wistar one) and 1 cat.
2. The same fixative as above, perfused for 10 min, followed by a similar fixative but without glutaraldehyde for 20 min was used for 1 rat and 1 cat. Tissue from these 2 animals had stronger immunoreactivity than that obtained with fixative 1 and it was therefore used for the illustrations.
3. Paraformaldehyde (4%) made up in 0.1 M phosphate buffer (PB, pH 7.2–7.4) was used for the monkey. Strong immunoreactivity was obtained with this fixative, but the fine structural preservation was worse than with the 2 previous fixatives.
4. Periodate–lysine–paraformaldehyde fixative (McLean and Nakane, 1974) was used for 1 rat. Immunoreactivity was inferior to that obtained with fixative 2.

Following perfusion the cerebellum was removed and sliced sagitally in buffer. Postfixation was not used. Sections (50 μm thick) 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 and then thawed in 0.1 M PB as described earlier (Somogyi and Takagi, 1982). Some sections were also treated with 1% sodium borohydrate (Willingham, 1983) dissolved in PBS, pH 7.4. This treatment enhanced immunoreactivity.

Immunocytochemistry. The purification of $GABA_A$/benzodiazepine receptors from bovine cerebral cortex and the preparation of mouse monoclonal antibodies bd-24 and bd-17 have been described earlier (Schoch et al., 1984, 1985; Haring et al., 1985). Both antibodies are gamma-isotype IgGs. Immunoblots show that antibody bd-24 recognizes an α-subunit (M_r 50,000); antibody bd-17 recognizes a β-subunit (M_r 55,000) of the receptor complex (Haring et al., 1985). Incubation of receptor preparations with hybridoma supernatants containing either of the 2 antibodies led to the immunoprecipitation of both low- and high-affinity GABA binding sites, flunitrazepam binding sites (Schoch et al., 1985), as well as *t*-butylbicyclophosphorothionate (TBPS) binding sites (Mohler et al., 1986). This indicates that the antibodies interact with the GABA/benzodiazepine receptor–chloride channel complex.

Three different immunocytochemical procedures were used to visualize the immunoreactive sites. In all 3 methods, free-floating sections were incubated either overnight or for 2 d at 4°C, first with a serum to block nonspecific binding of the antibodies and then with either undiluted hybridoma supernatant containing bd-24 or with undiluted or 1.5 times diluted supernatant containing bd-17.

(1) In the unlabeled antibody peroxidase–antiperoxidase (PAP) method, 10% normal rabbit serum (Nordic) was used as blocking solution for 30 min; then, following the primary antibody, rabbit anti-mouse IgG (dilution, 1:40, Dako) was applied for 2 hr. Washing was done in PBS containing 1% normal rabbit serum, followed by mouse PAP (1:80, Nordic) for 2 hr.

(2) Using the ABC method, 10% normal sheep serum was used for blocking and then, following the primary antibody, biotinylated sheep IgG (1:50, Vector) was applied for 1 hr. After washing in PBS containing 1% normal sheep serum, the sections were incubated in avidin–biotin–HRP complex (1:100, Vector) for 1 hr.

(3) 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 anti-mouse IgG (1:100, Dako) for 3 hr. All steps but the primary antibody incubations were done at room temperature with agitation on a shaker. Triton X-100 (0.1%) in the primary antibody step substantially increased the intensity of immunoreactivity. However, because of its disruptive effect on fine structural detail, specimens produced with Triton were not included in the electron microscopic analysis.

Particulate markers such as colloidal gold or ferritin may give higher-resolution localization of antigens than immunoperoxidase. We have tried several methods for the localization of the $GABA_A$ receptor complex, but so far it has not been possible to achieve penetration of these markers into the narrow membrane delineated spaces. 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 were treated with OsO_4 (1% in PB) for 30 min, dehydrated in ethanol, and embedded flat on glass slides in 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. No lead staining was 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 2, but usually 3–5, areas were cut for electron microscopy from each animal.

Results

Controls

Immunoreactivity was absent in all 3 species when the hybridoma supernatant containing the monoclonal antibodies was omitted from the incubation series or when it was replaced by culture medium, containing 10% fetal calf serum, that was not used for growing myeloma cells.

Distribution of immunoreactivity as detected by light microscopy

Hybridoma supernatant bd-17, specific for the β-subunit of the receptor, was used in all 3 species. In addition, supernatant containing the antibody bd-24, which is specific for the α-subunit but does not recognize the rat antigen, was also used in the cat and monkey. The pattern of immunoreactivity was very

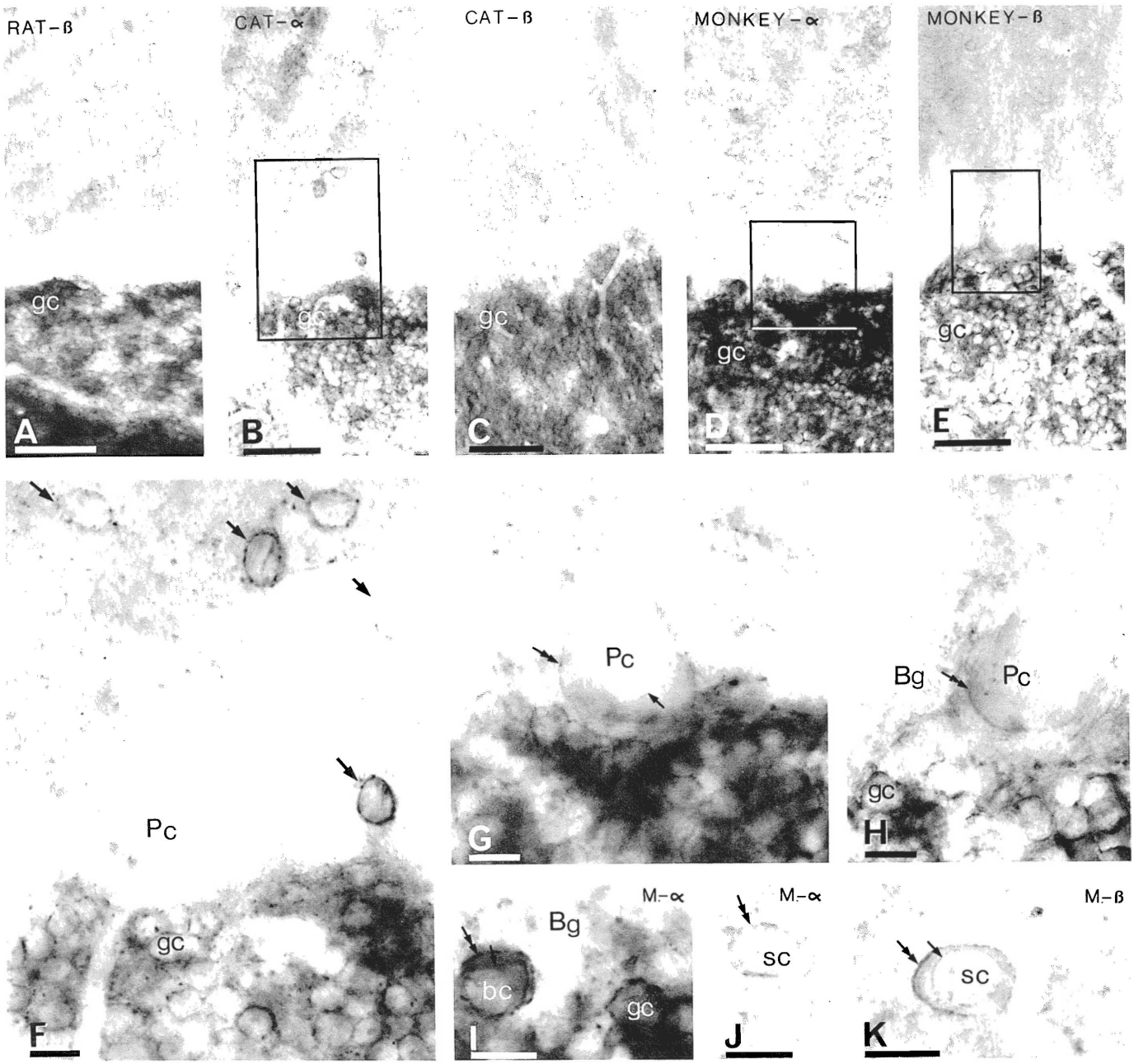

Figure 1. Immunoreactivity for the $GABA_A$/benzodiazepine receptor in the cerebellar cortex, using monoclonal antibodies to the α- (bd-24) and β-subunit (bd-17) proteins. Light micrographs of 50-μm-thick osmium-treated sections; *boxed areas* are shown at higher magnification in *F–H*. In all species, the layer of granule cells (*gc*) shows the strongest immunoreactivity. Only weak immunoreactivity can be seen around some Purkinje cells (*Pc, double arrows* in *G* and *H*) and occasionally along their nuclear envelope (*arrow* in *G*). Strong staining is evident in stellate (*sc*) and basket (*bc*) cells (*arrows* in *F*) with antibody to the α-subunit. At higher magnification, it is evident that the immunoreactivity in these cells is present along the cell membrane (*double arrow* in *I–K*), as well as intracellularly, as best seen along the nuclear envelope (*arrow* in *K*). No immunoreactivity is detected in association with Bergman glial cells (*Bg*). Scale bars: *A–E,* 50 μm; *F–K,* 10 μm.

similar in the 3 species (Fig. 1). Confirming earlier results, obtained with the same (Richards et al., 1986, 1987) or with different (De Blas et al., 1988) antibodies, the strongest immunoreactivity was found in the granule cell layer outlining the somata of granule cells (Fig. 1, *A–H*). Golgi cells showed no immunoreactivity at the light microscopic level. The Purkinje cells were surrounded by much weaker immunoreactivity than the granule cells (Fig. 1, *F–H*); in some cases, none at all could be detected. Using the antibody specific to the α-subunit, lightly stained patches were observed in the somata of Purkinje cells (Fig. 1*G*). The molecular layer contained punctate immunoreactivity, relatively sparse compared with the granular layer. The main difference detectable between sections reacted with antibodies bd-17 and bd-24 was the much more prevalent immunoreactivity obtained with bd-24 in the somata of stellate and basket cells (Fig. 1, *B, F, I, J*). These cells were distinctly outlined, and the nuclear membrane was sharply delineated, suggesting intracellular reaction in the cat and monkey (Fig. 1,

Table 1. Distribution of GABA$_A$ receptor immunoreactivity in different neuronal cell types of the cerebellum

Cell type	Structure	Species				
		Rat	Cat		Monkey	
Granule cell	Somatic membrane	β	α	β	α	β
	Dendritic membrane	β	α	β	α	β
	Golgi apparatus	0	0	0	0	0
	ER cisternae	0	(α)	—	—	—
	Golgi cell synapse	β	α	—	α	β
	Mossy terminal nonsynaptic membrane	β	α	—	α	β
	Mossy terminal synapse	—	—	—	—	—
Purkinje cell	Somatic membrane	β	α	—	α	β
	Dendritic membrane	β	α	—	—	β
	Spine membrane	β	α	—	—	β
	ER cisternae	(β)	α	—	α	(β)
	Golgi apparatus	β	α	—	—	β
	Basket cell synapse	—	α	—	—	β
	Stellate cell synapse	β	α	—	—	β
	Assym. spine synapse	0	0	—	—	0
Stellate and basket cells	Somatic membrane	β	α	—	α	β
	Dendritic membrane	β	α	—	α	β
	ER cisternae	(β)	α	—	α	—
	Golgi apparatus	β	α	—	—	—
	Synapses received	β	α	—	α	β
Golgi cell	Plasma membrane	0	0	0	0	(β)
	Intracellular membrane	0	0	0	0	(β)
Large neurons in dentate nucleus	Plasma membrane	β	α	—	—	—
	Synaptic clefts (some)	β	α	—	—	—

The table provides a summary of the observations, and the lack of positive data does not imply the absence of the antigen. For example, incubations for the β-subunit in the cat and for the α-subunit in the monkey repeatedly produced only weak immunoreactivity, some sites probably remaining below detectability. Code: α, immunoreactivity with antibody (bd-24) to the 50,000 Da subunit (this antibody does not recognize the rat antigen); β, immunoreactivity with antibody (bd-17) to the 55,000 Da subunit; ( ), very rare and weak; 0, no immunoreactivity detected under our conditions in sections where immediately adjacent sites were immunopositive; dash, not examined or no conclusion can be drawn for technical reasons.

F, I, K). The Bergman glial cells that can be recognized at the border of the granular and molecular layers showed no immunoreactivity (Fig. 1, *F, H*).

In the deep cerebellar nuclei, the majority of the cell bodies and also the dendrites were outlined by immunoreactivity resulting from the application of either of the 2 antibodies.

Distribution of immunoreactivity as detected by electron microscopy

The reaction end product was almost exclusively limited to membrane-delineated spaces, including the extracellular gap between cells and processes (Figs. 2, 4, 5), the endoplasmic reticulum cisternae and the Golgi saccules and vesicles (Fig. 3). From the deposition of the peroxidase reaction end product, it could be established that the epitopes reacting with both the α- and β-subunit specific antibodies were located on the extracellular surface of the plasma membrane (e.g., Figs. 2*E*, 4*B*) and on the internal surface of the endoplasmic reticulum and Golgi membranes (Fig. 3, *B–D*). The subcellular distribution of the immunoreactivity is summarized in Table 1.

Using the immunoperoxidase method, the reaction end product formed in the narrow extracellular space is deposited on both of the closely juxtaposed membranes and often fills the space between them (e.g., Fig. 2*E*). As a result, it is impossible to determine on which of the 2 membranes the antigen is located. Thus, conclusions regarding the presence of the receptor in one or the other of the apposed plasma membranes have to be drawn by comparing the same membrane apposed to the membranes of different cell types. If the plasma membranes of 2 cells of the same type are directly apposed and immunoreaction end product 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 tightly packed granule cells were an exception; both their dendrites and somata were frequently in direct membrane contact with other granule cells (Fig. 2, *A, B, D, E*).

No evidence could be detected for the presence of the antigenic sites within or on the surface of astroglia, oligodendroglia, or Bergman glia. The different types of neuronal cells differed greatly in the intensity of both their intracellular and plasma membrane immunoreactivities. Confirming the light microscopic results, the plasma membrane of granule cells showed the strongest immunoreactivity (Fig. 2). This contrasted with their almost total lack of intracellular immunoreactivity; only rarely were short segments of the ER and the nuclear envelope found to contain reaction end product (Fig. 2*C*). The plasma membrane was evenly immunoreactive both at somatic and dendritic sites. Since soma-to-soma and dendrite-to-dendrite appositions are frequent, it could be unequivocally demonstrated that the granule cell membrane carries the epitope (Fig. 2, *A, B, E*). Immunoreactivity was present in the synaptic cleft of the Golgi cell terminals establishing junctions with the dendrites of gran-

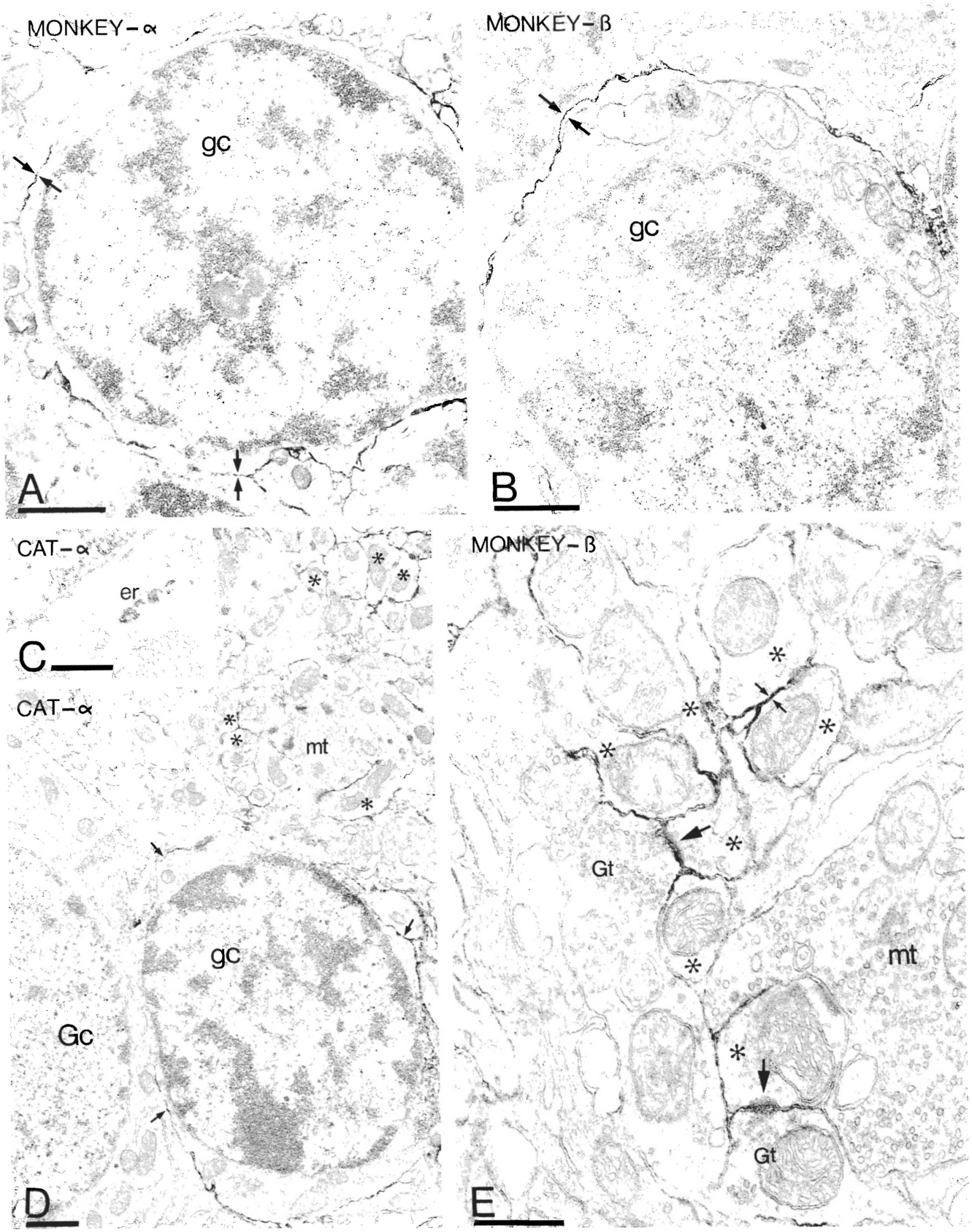

Figure 2. Electron micrographs of the granule cell layer. Immunoreactivity is always present along the somatic plasma membrane of granule cells (*gc*), including regions where 2 cells are directly apposed (*opposing arrows* in *A* and *B*). The endoplasmic reticulum (*er* in *C*) very rarely showed immunoreactivity. The membrane of dendrites (*asterisks*) also shows immunoreactivity at the synaptic junctions with Golgi cell terminals (*Gt, large arrows* in *E*), at nonjunctional sites, including some areas facing mossy terminals (*mt*), and at dendrite-to-dendrite contacts (*opposing arrows* in *E*). Immunoreactivity was largely absent from Golgi cells (*Gc*), their membrane had reaction end product only when apposed (*arrows* in *D*) to immunopositive granule cells. Scale bars: *A, B,* and *D,* 1 μm; *C,* 0.2 μm; *E,* 0.5 μm.

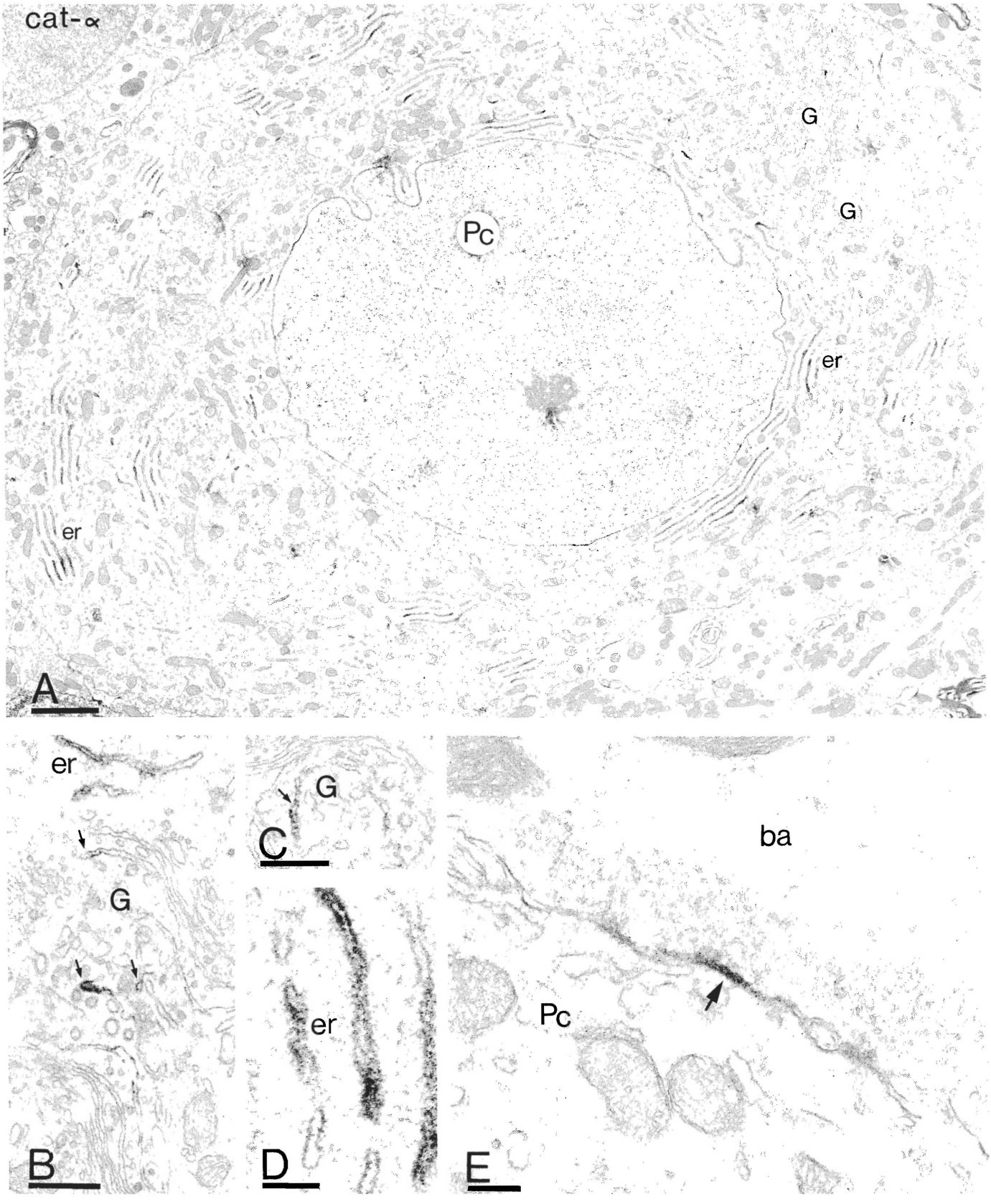

Figure 3. Immunoreactivity to the α-subunit associated with Purkinje (*Pc*) cells of the cat. The reaction end product is present mainly in the endoplasmic reticulum (*er*) and in some of the Golgi (*G*) saccules (*arrows*), as shown at higher magnification in *B–D*. The immunoreactivity is associated with the inner surface of the endoplasmic reticulum (*er*) membrane. *E*, Only weak immunoreactivity was found in patches on the somatic plasma membrane; immunoreactivity in the synaptic junction (*arrow*) between a basket cell axon (*ba*) and a Purkinje cell (*Pc*). Scale bars: *A*, 2 μm; *B* and *C*, 0.5 μm; *D* and *E*, 0.2 μm.

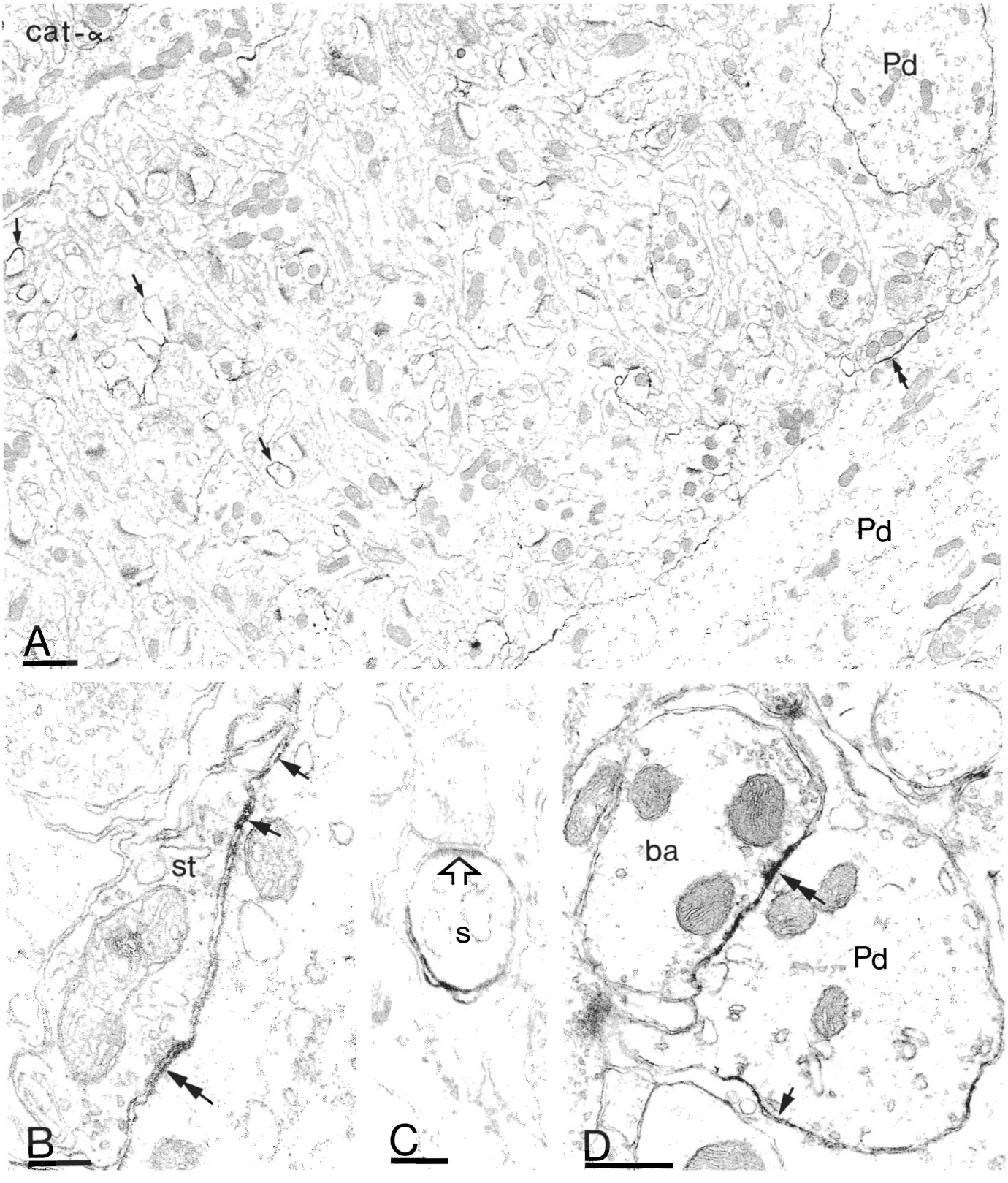

Figure 4. Immunoreactivity to the α-subunit in the molecular layer of the cat. The plasma membranes of Purkinje cell dendrites (*Pd*) as well as dendritic spines (*arrows*) are immunopositive in patches. The contact (*double arrows*) made by a probable stellate cell bouton (*st*) is shown in *B* from a serial section. Immunoreactivity is present in the synaptic cleft (*double arrow*), as well as at the nonjunctional membrane (*arrows*) of the Purkinje cell dendrite. *C,* Immunoreactivity along the nonjunctional membrane of a dendritic spine (*s*) receiving a synapse (*open arrow*). *D,* Immunoreactivity along the plasma membrane (*arrow*) of a Purkinje cell dendrite (*Pd*) including the cleft at a synapse received from a basket cell axon (*ba*). The latter can be identified from the numerous neurofilaments. Scale bars: *A,* 1 μm; *B* and *C,* 0.2 μm; *D,* 0.5 μm.

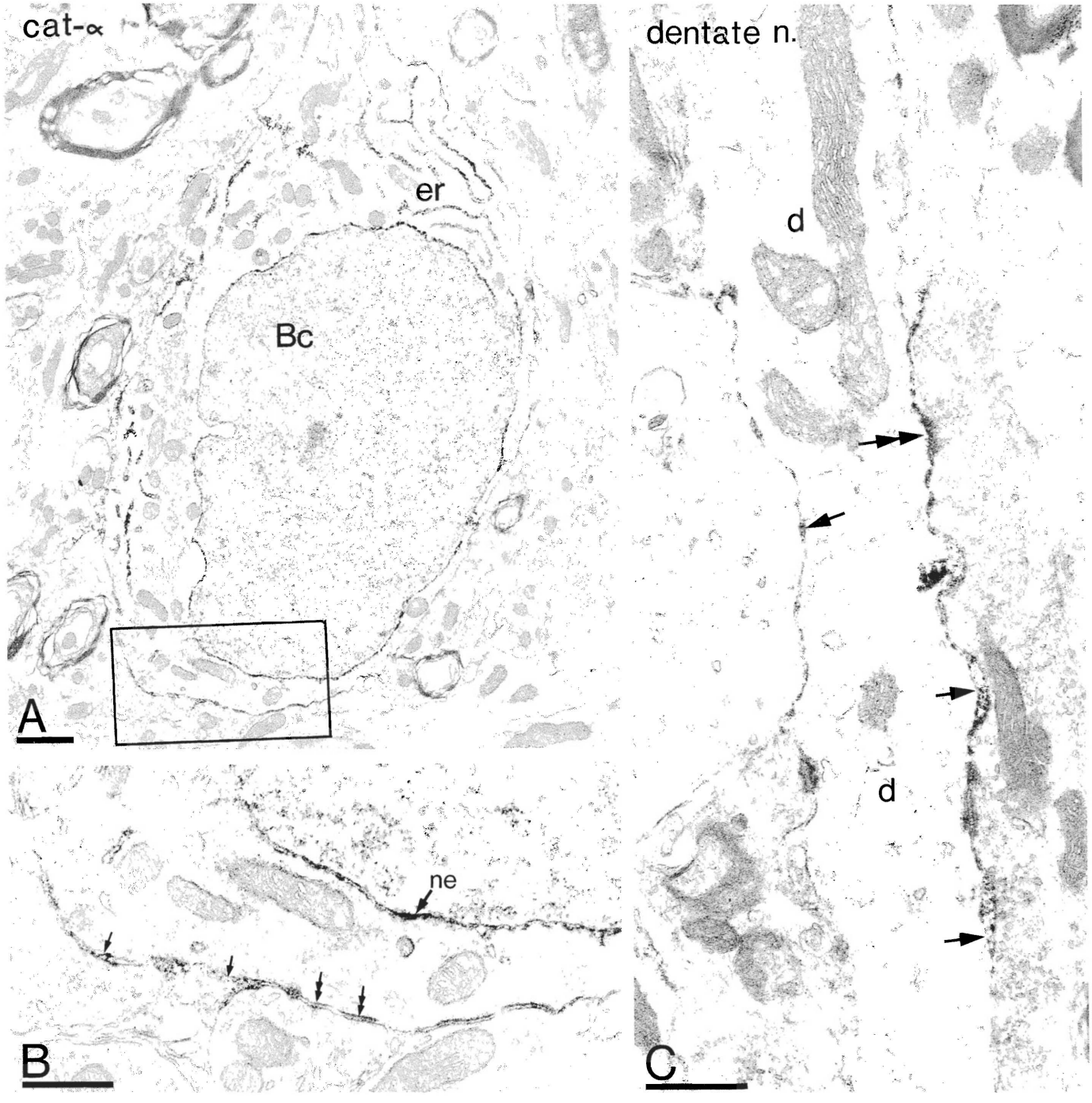

Figure 5. Immunoreactivity to the α-subunit in the cat. *A,* Basket cell (*Bc*) showing strong immunopositivity in the endoplasmic reticulum (*er*) and along its plasma membrane. *B,* Boxed area from *A* demonstrating immunoreactivity along most of the plasma membrane (arrows), including synaptic junctions (*double arrows*). The nuclear envelope (*ne*) is also immunopositive. *C,* Immunoreactivity along synaptic (*double arrow*) and nonjunctional (*arrows*) plasma membranes of a dendrite (*d*) in the dentate cerebellar nucleus. Scale bars: *A,* 1 μm; *B* and *C,* 0.5 μm.

ule cells (Fig. 2*E*). The dendrites of granule cells were also immunoreactive when apposed to glial or Golgi cells and less frequently when next to mossy terminal membranes. The synaptic junctions between mossy fibers and granule cell dendritic digits showed no immunoreactivity in general; only in 2 out of several hundred junctions was reaction end product found in the synaptic cleft, and in both cases there was heavy immunoreactivity at the neighboring nonjunctional membranes. Thus, in these cases the reaction end product could have diffused into the cleft from neighboring sites.

Purkinje cells consistently showed weaker immunoreactivity on their plasma membrane than did granule cells and the immunoreactivity was patchy. The synaptic junctions formed by basket cells were often immunopositive (Fig. 3*E*). Using the antibody to the α-subunit, immunoreactivity was readily seen in the endoplasmic reticulum and in the Golgi saccules, vesicles,

and in multivesicular bodies (Fig. 3, *A–D*). The dendritic membrane was also immunopositive at the synaptic junctions received by the dendrites and also at nonjunctional sites (Fig. 4, *A, B, D*). Many of the dendritic spines showed immunoreactivity along most of their surface, the junctional cleft with the granule cell terminals being an exception (Fig. 4*C*).

Stellate and basket cells were strongly immunopositive in their endoplasmic reticulum, including the nuclear envelope, in the Golgi apparatus, and also occasionally in multivesicular bodies. Their plasma membrane was also strongly immunopositive, as predicted by light microscopy (Fig. 5, *A, B*). In contrast, Golgi cells showed no immunoreactivity, even in areas where neighboring granule cells were strongly immunopositive. Only in the monkey was weak immunoreactivity for the β-subunit associated with Golgi cells.

In the dentate nucleus the subcellular distribution of immunoreactivity was very similar to that of the cortex in that the reaction end product was localized along most of the plasma membrane both at junctional and nonjunctional sites (Fig. 5*C*). Most of the dendrites and somata were immunopositive.

Discussion

Cerebellar distribution of $GABA_A$/benzodiazepine receptors

The present immunocytochemical study of the cerebellum of 3 species has confirmed the regional distribution of the $GABA_A$ receptors as described previously by ligand-binding autoradiographic (Young and Kuhar, 1979; Palacios et al., 1981; for review, see Wamsley and Palacios, 1984; Herkenham and McLean, 1986; Kuhar et al., 1986) and immunocytochemical (Richards et al., 1986, 1987; De Blas et al., 1988) studies. Immunoreactive receptors were found on all cerebellar neuronal classes with the possible exception of Golgi cells. While the presence of receptors was clearly demonstrated on the somatic and dendritic membrane, no evidence was obtained for immunoreactivity on axons and nerve terminals, though the results do not exclude the presence of presynaptic receptors on terminals. It is possible that under our conditions sites with lower concentrations of the antigen were not revealed.

It is not known which domain of the receptor molecule represents the epitope for the antibodies. Since neither GABA nor benzodiazepine binding is inhibited by the antibodies (Haring et al., 1985), sequences outside the ligand recognition sites in the extracellular domain must be responsible for antibody recognition. The good agreement (Richards et al., 1986) of ^{3}H-*t*-butylbicyclophosphorothionate binding sites, thought to demonstrate Cl^- channels (Squires et al., 1983), and receptor immunoreactivity suggests that the epitope distribution reflects the distribution of Cl^- channels. The intense staining of the granule cell layer for both the α- and β-subunits (Richards et al., 1986, 1987) also matches well with the great density of high-affinity GABA binding sites demonstrated with ^{3}H-muscimol and the immunoreactivity obtained with other antibodies (De Blas et al., 1988). However, the distribution of immunoreactivity, while overlaps, does not fully agree with the distribution of either GABA binding sites (high-affinity ones revealed by ^{3}H-muscimol or low-affinity ones revealed by ^{3}H-bicuculline) or benzodiazepine binding sites in the cerebellum (Herkenham and McLean, 1986; Kuhar et al., 1986; Richards et al., 1988; Zezula et al., 1988; for comparison, see Richards et al., 1986). It remains to be established whether all receptor/chloride channel complexes recognized by the antibodies have the complete set of ligand binding sites. For example, $GABA_A$ receptors exist without the benzodiazepine binding site (Unnerstall et al., 1981; Olsen et al., 1984).

The different cell types of the cerebellar cortex exhibited different degrees of immunoreactivity, but the staining was stereotyped among individual members of the same class of cell, as in the hippocampus (Houser et al., 1988). The subcellular distribution, as detected by high-resolution immunoelectron microscopy, revealed unexpected results. In contrast to previous light microscopic studies (Richards et al., 1986, 1987; De Blas et al., 1988), immunoreactivity could be shown both in the cell body and on the somatic membrane of Purkinje cells. The results also demonstrate that the plasma membrane of granule cells is uniformly immunoreactive, including the somatic membrane which never receives GABAergic synapses. Although the perisomatic immunoreactivity has been noted previously (Richards et al., 1986, 1987; De Blas et al., 1988), the presence of immunoreactivity in the glomeruli has been emphasized (De Blas et al., 1988). The glomerular regions of the granule cell layer could appear more strongly stained in light microscopic specimens probably because they contain a higher volume density of immunopositive granule cell dendritic plasma membrane.

The presence of immunoreactive receptors on cells in the deep cerebellar nuclei in cat agrees with the results of Richards et al. (1986, 1987) in the rat. Apart from local GABAergic neurons, the main GABA-containing input comes from the cortical Purkinje cells, which exert an inhibitory action (Obata et al., 1967; Ito et al., 1970).

Synaptic versus nonsynaptic receptors

At the subcellular level, the most striking finding of the present study is that the receptor proteins are present not only at the synaptic junctions but also at nonjunctional sites. However, the distribution of receptors is not completely uniform over the plasma membrane. On the one hand, when the sections were taken from the zone of tissue that was penetrated by the antibodies, the synaptic junctions formed by the boutons of Golgi, basket, and stellate cells that have been shown to contain GABA (for review, see Ottersen and Storm-Mathisen, 1984; Mugnaini and Oertel, 1985) were invariably immunopositive for the $GABA_A$ receptor complex. On the other hand, the synaptic junctions formed by the mossy terminals and parallel fiber boutons, which are rich in glutamate (Somogyi et al., 1986) and probably use it as transmitter (Garthwaite, 1986; for review, see Ottersen and Storm-Mathisen, 1984; Levi and Gallo,1986), were immunonegative for the receptor.

The GABA receptor complex may be unusual among neurotransmitter receptors because of its broad distribution at both synaptic and nonsynaptic sites. Electron microscopic studies of a closely related amino acid-gated receptor–channel complex, the glycine receptor, show that it is mainly associated with synaptic junctions (Triller et al., 1985, 1987). This was found not only for the intracellular epitopes localized in the postsynaptic membrane specialization but also for an extracellular epitope that was localized with an immunoperoxidase method similar to the one used in our study (Triller et al., 1985). Thus, the difference in the membrane distribution of GABA and glycine receptors cannot be attributed to technical factors but probably represents genuine differences in the distribution of the receptor proteins.

There are a number of possible explanations for the localization of the GABA/benzodiazepine receptor chloride channel complex at sites distant from the GABA-releasing terminals.

1. The immunoreactive sites outside the synaptic junction may represent cross-reacting proteins, possibly other receptors, such as the nicotinic ACh receptor or the glycine receptor, which have been shown to have sequence homologies with the $GABA_A$ receptor complex (Barnard et al., 1987; Greeningloh et al., 1987; Schofield et al., 1987). The possibility of cross-reactivity with the glycine receptor proteins is easily excluded because the glycine receptors are distinctly localized only at synaptic junctions (see above). The possibility of cross-reactivity with the nicotinic ACh receptor is also unlikely to result in the immunoreactivity pattern observed in the present study because the regional distribution of the immunoreactivity in the cerebellum as well as in the brain in general (Richards et al., 1986, 1987) is different from the distribution of nicotinic receptor sites (Clarke et al., 1985; Swanson et al., 1987). The nicotinic ACh receptor also seems to be localized preferentially at synaptic junctions, at least on peripheral neurons (Jacobs et al., 1986). While cross-reactivity with as yet unknown components cannot be excluded, it is at present an unlikely explanation for the observed distribution of immunoreactivity.

2. It could be argued that the receptors distant from the synaptic sites do not represent active receptors capable of opening ion channels; the immunoreactivity merely shows the presence of receptor proteins that will become functional only when moved into the synaptic membrane. This argument clearly awaits patch-clamp recording of somatic membranes of granule cells, which do not receive GABAergic synapses on their somata but exhibit a high density of immunoreactive receptors. That the receptors at extrasynaptic sites are capable of binding the appropriate ligands is indicated by the similarly high density of high-affinity $GABA_A$ sites, as demonstrated by ^{3}H-muscimol, and immunoreactive sites in the granule cell layer (Richards et al., 1986). The binding would be patchy, localized to glomeruli in the granule cell layer, if only receptors at synaptic junctions were labeled. The good agreement of the 2 patterns strongly supports the view that the immunoreactive sites correspond to ^{3}H-muscimol binding sites. Further work on cerebellar cells can reveal whether the extrasynaptic channels are also active with regard to Cl^- conductance.

The membrane mobility of benzodiazepine receptors has been studied on chick neurons *in vitro* (Thompson et al., 1988). The results indicated that 36% of the receptors on the soma and 75% on the processes of the cells were immobile. This suggests that a substantial proportion of the extrasynaptic receptors demonstrated in the present study could not easily move to synaptic junctions, if receptor mobility was similar in the presence of functional GABAergic circuits *in vivo*.

3. The explanation for the widespread extrasynaptic distribution of the receptor complex may lie in the general role of the GABA-gated chloride channel (Alger, 1985). The synaptic and nonsynaptic receptors may be used under different levels of neuronal activity, providing a mechanism for adaptation. It is possible that at low levels of neuronal activity GABA acting at the synaptic junctions provides adequate inhibitory control of the neurons. The moderate amounts of GABA released can be removed by reuptake or glial uptake (Martin, 1976; Nicklas, 1983; Krnjevic, 1984) without reaching the extrasynaptic sites. However, at increased excitatory input, the number of channels operating at the junctions may not be adequate to keep the activity of the cell in the range of optimal sensitivity, in which case channels at extrasynaptic sites would be opened by GABA diffusing from the release sites (see Alger and Nicoll, 1982). Consequently, the topography of GABAergic effects would primarily be regulated not by the precise placement of receptors but by the precise placement of GABA-releasing synaptic terminals and by the amount of GABA released. This could explain the great degree of differentiation in GABAergic synaptic circuits in the cerebellum (for review, see Eccles et al., 1967; Palay and Chan-Palay, 1974) and elsewhere in the brain (Freund et al., 1983; Somogyi, 1986).

The operation of the mechanism outlined above would require the presence of GABA in the extracellular space away from synaptic sites. Indeed, GABA can be detected in micromolar concentration in the cerebrospinal fluid (Enna et al., 1977; Manyam and Hare, 1983), and the increased level of GABA in the cerebrospinal fluid following increased neuronal activity, e.g., epileptic discharge (Lloyd et al., 1986), suggests that neuronally released GABA may reach receptors distant from synaptic junctions. Extrasynaptic receptors may also be influenced by GABA released from glial cells under certain conditions (Brown, 1979; Jaffe and Cuello, 1981).

It is noteworthy that the granule cells, which are the recipients of the main activating system of the cerebellum, the mossy fiber input, carry the highest density of immunoreactive receptors broadly distributed on their dendritic and somatic membrane, while their GABA input is well defined to the distal dendrites. Thus, the GABAergic system through the synaptic, and at increased excitatory input through the nonjunctional, receptors could set the activity of cortical cells by influencing the gain of the first synapse, the mossy fiber synapse, through the $GABA_A$ receptors on the granule cells. Since these receptors receive GABA from Golgi cells that are activated by the same granule cells that they innervate, the release of GABA is governed by the overall activity in the granule cell population which is under feedback control by Golgi cells.

Comparison with glycine receptors

Glycine receptors have been localized immunocytochemically at synapses in the cerebellum formed by glutamate decarboxylase-containing boutons, presumably originating from stellate cells (Triller et al., 1987). They have also been shown to be present on some of the granule cell dendrites receiving Golgi cell terminals (Triller et al., 1987). It has been suggested that these Golgi cell terminals were not GABAergic because they did not contain glutamate decarboxylase. However, in view of the coexistence of GABA and glycine in a very large proportion of Golgi cell terminals, and in some stellate cell terminals (Ottersen et al., 1988), it is possible that the absence of detectable GAD immunoreactivity was due to technical reasons. The synaptic contacts made by stellate and Golgi cells were positive for the $GABA_A$ receptors in the present study; thus, the appropriate receptors are present for both GABA and glycine at the same synapses. This raises the question of whether GABA and glycine activate the same Cl^- channels. Physiological evidence suggests that in the spinal cord at least, the 2 receptors activate separate populations of channels with similar conductance characteristics (Hamill et al., 1983).

Intracellular localization of receptors

The demonstration of intracellular receptors is in agreement with biochemical studies (Czajkowski and Farb, 1986) and is very similar to the nicotinic ACh receptor localization in neurons of the chicken ciliary ganglion (Jacob et al., 1986). The stronger immunoreactivity obtained for the α-subunit intracel-

lularly probably reflects the differential maturation of the epitopes for the 2 antibodies. Immunoreactivity in the endoplasmic reticulum and in the Golgi apparatus is explained by the synthesis of the protein in the ER and by the addition of carbohydrate residues (Sweetnam and Tallman, 1986; Kuriyama and Taguchi, 1987) in the Golgi system. It is interesting that the different cell types differed in the intensity of the intracellular immunoreactivity. Possibly this reflects differences between the classes of cells in the turnover of the receptor. Surprisingly, the granule cells that react most strongly on their plasma membrane showed the least immunoreactivity intracellularly. This may suggest that the turnover of their receptor population is low compared with that of the stellate and basket cells, which showed the strongest intracellular staining. The latter 2 types of cell also synthesize GABA, and it could be argued that GABAergic cells synthesize more receptor. Indeed, in other parts of the brain such as the hippocampus and cortex, the strongest intracellular $GABA_A$ receptor immunoreactivity is also found in some of the GABA-synthesizing cells (unpublished observation). However, this is not a general rule since the cerebellar Golgi cells that are also GABAergic showed negligible receptor immunoreactivity.

Functional significance of $GABA_A$ receptors in the cerebellar cortex

It has been suggested that some of the behavioral effects of benzodiazepines may be explained by their influence on GABAergic neurotransmission in the cerebellum (Richards et al., 1986, 1987). The cerebellar cortical neurons, with the exception of granule cells, exert inhibitory influence on their postsynaptic cells (Andersen et al., 1964; Ito and Yoshida, 1966; Eccles et al., 1967; Ito et al., 1970; Obata et al., 1970; Woodward et al., 1971). The presence of GABA and its synthetic enzyme in the synaptic boutons of Purkinje basket, stellate, and Golgi cells (for review, see Ottersen and Storm-Mathisen, 1984; Mugnaini and Oertel, 1985), together with pharmacological evidence (Obata et al., 1967, 1970; Curtis and Felix, 1971; Woodward et al., 1971), makes it very likely that they use GABA as an inhibitory transmitter (but see also Okamoto et al., 1983; Ottersen et al., 1987). Since the present study has demonstrated that $GABA_A$ receptors are present at the postsynaptic membrane of the synapses established by these major GABAergic cells, their inhibitory effect could be mediated by $GABA_A$ receptors. Indeed, the inhibition of Purkinje cells via the parallel fiber to basket cell pathway is antagonized by bicuculline (Curtis and Felix, 1971). Furthermore, the iontophoretic application of the $GABA_A$ antagonist bicuculline shifted the modulation of simple spike discharge of Purkinje cells involved in the vestibulo-ocular reflex (Miyashita and Nagao, 1984), presumably by antagonizing the effects of the GABAergic interneurons influencing the Purkinje cells. The different roles of the 3 types of GABAergic interneurons are not yet clear, but the above study indicates that local inhibition mediated by $GABA_A$ receptors is essential for the normal function of the cerebellar cortex.

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