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Immunocytochemical Localization of the α1 and β2/3 Subunits of the $GABA_A$ Receptor in Relation to Specific GABAergic Synapses in the Dentate Gyrus

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Abstract

Dentate granule cells receive spatially segregated GABAergic innervation from at least five types of local circuit neurons, and express mRNA for at least 11 subunits of the $GABA_A$ receptor. At most two to four different subunits are required to make a functional pentamer, raising the possibility that cells have on their surface several types of $GABA_A$ receptor channel, which may not be uniformly distributed. In order to establish the subcellular location of $GABA_A$ receptors on different parts of dentate neurons, the distribution of immunoreactivity for the α1 and β2/3 subunits of the receptor was studied using high-resolution immunocytochemistry. Light microscopic immunoperoxidase reactions revealed strong $GABA_A$ receptor immunoreactivity in the molecular layer of the dentate gyrus. Pre-embedding immunogold localization of the α1 and β2/3 subunits consistently showed extrasynaptic location of the $GABA_A$ receptor on the somatic, dendritic and axon initial segment membrane of granule cells, but failed to show receptors in synaptic junctions. Using a postembedding immunogold technique on freeze-substituted, Lowicryl-embedded tissue, synaptic enrichment of immunoreactivity for these subunits was found on both granule and non-principal cells. Only the postembedding immunogold method is suitable for revealing relative differences in receptor density at the subcellular level, giving ~20 nm resolution. The immunolabelling for $GABA_A$ receptor occupied the whole width of synaptic junctions, with a sharp decrease in labelling at the edge of the synaptic membrane specialization. Both subunits have been localized in the synaptic junctions between basket cell terminals and somata, and between axo-axonic cell terminals and axon initial segments of granule cells, with no qualitative difference in labelling. Receptor-immunopositive synapses were found at all depths of the molecular layer. Some of the boutons forming these dendritic synapses have been shown to contain GABA, providing evidence that some of the GABAergic cells that terminate only on the dendrites of granule cells also act through $GABA_A$ receptors. Double immunolabelling experiments demonstrated that a population of GABA-immunopositive neurons expresses a higher density of immunoreactive $GABA_A$ receptor on their surface than principal cells. Interneurons were found to receive $GABA_A$ receptor-positive synapses on their dendrites in the hilus, molecular and granule cell layers. Receptor-immunopositive synapses were also present throughout the hilus on presumed mossy cells. The results demonstrate that both granule cells and interneurons exhibit a compartmentalized distribution of the $GABA_A$ receptor on their surface, the postjunctional membrane to GABAergic terminals having the highest concentration of receptor. The α1 and β2/3 subunits have a similar distribution in synapses on the axon initial segment, soma, proximal and distal dendrites of granule cells. The very strong immunoreactivity of a subpopulation of GABAergic interneurons for $GABA_A$ receptors containing the α1 and β2/3 subunits predicts their high sensitivity to GABA and modulators of the receptor complex.

Introduction

Granule cells in the hippocampal dentate gyrus respond to the activation of their synaptic input by both $GABA_A$ and $GABA_B$ receptor-mediated mechanisms (Andersen *et al.*, 1966; Thalmann and Ayala, 1982; Misgeld *et al.*, 1986; Rausche *et al.*, 1989; Edwards *et al.*, 1990; Muller and Misgeld, 1990; Scharfman *et al.*, 1990; Otis and Mody, 1992). GABAergic innervation of granule cells is mainly provided by specific types of hippocampal interneurons (Ribak *et al.*, 1978; Lubbers and Frotscher, 1987; Soriano and Frotscher, 1989,

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1993; Seay-Lowe and Claiborne, 1992; Han *et al.*, 1993). The great degree of target selectivity of these interneurons makes it possible to predict the origin of GABAergic synapses from their location on the surface of the granule cells. Thus, basket cell axons terminate mainly on the soma and proximal dendrites of granule cells (Lubbers and Frotscher, 1987; Ribak, 1992; Halasy and Somogyi, 1993b). Axo-axonic cell terminals specifically innervate the axon initial segment of granule cells (Kosaka, 1983; Soriano and Frotscher, 1989; Soriano *et al.*, 1990; Halasy and Somogyi, 1993b; Buhl *et al.*, 1994a, b). These two types of interneurons are immunoreactive for GABAergic biochemical markers (Ribak *et al.*, 1978; Somogyi *et al.*, 1985a; Soriano and Frotscher, 1989) and elicit short-latency, $GABA_A$ receptor-mediated inhibitory postsynaptic potentials (IPSPs; Buhl *et al.*, 1994a). At least four further types of local circuit interneurons, terminating in the molecular layer, have been described and classified according to the spatial distribution of their dendrites and the highly selective distribution of their axonal arbours (Han *et al.*, 1993; Soriano and Frotscher, 1993): (i) hilar cells, forming a dense axonal plexus in the inner one-third of the molecular layer (HICAP cells), the termination zone for commissural and association pathways; (ii) hilar cells with dense axonal fields associated with the termination of the perforant pathway in the outer two-thirds of the molecular layer (HIPP cells); (iii) molecular layer cells having an axonal arbour restricted to the perforant path terminal field (MOPP cells); and (iv) interneurons with cell bodies in the granule cell layer and having axons apparently ramifying in the whole depth of the molecular layer (Soriano and Frotscher, 1993; MOLAX cells). The terminals of the HICAP and MOPP cells and the somata of MOLAX cells have been shown to contain GABA. The HIPP cells are thought to be the somatostatin-containing GABAergic interneurons (Baude *et al.*, 1993; Halasy and Somogyi, 1993b). The multiple and spatially segregated GABAergic innervation of granule cells raises several questions about possible differences in the effect of GABA on these cells at particular synapses. Do the interneurons selectively innervating the dendrites exert their influence through $GABA_A$ and/or $GABA_B$ receptors? Does the diverse and spatially segregated GABAergic input coincide with selective expression of different subunits of the $GABA_A$ receptor on the surface of granule cells?

The distribution of GABA receptor binding sites has been studied in the dentate gyrus by autoradiography (Young and Kuhar, 1979; Palacios *et al.*, 1981; Wamsley *et al.*, 1984; Richards *et al.*, 1986; Bowery *et al.*, 1987; Sieghart *et al.*, 1987) and shows high concentrations of $GABA_A$ (muscimol, benzodiazepines) and $GABA_B$ (baclofen) binding sites in all layers of the dentate gyrus. Thus both $GABA_A$ and $GABA_B$ receptor binding sites could be present on the dendrites of granule cells (Bowery *et al.*, 1987), but their precise location in the membrane in relation to synaptic inputs is not known. The pharmacological and physiological properties of $GABA_A$/benzodiazepine receptors depend on the subunit composition of the channels (Sigel *et al.*, 1990; Verdoorn *et al.*, 1990; Angelotti and Macdonald, 1993), each probably including five subunits. Although the subunit composition of native channels in dentate cells is not known, it is thought that not all the subunits are different in an individual channel (Barnard *et al.*, 1989; Amin and Weiss, 1993). Granule cells express mRNA for at least 11 subunits of the $GABA_A$ receptor (Persohn *et al.*, 1992; Wisden *et al.*, 1992). If only three or four different subunits are needed to form a functional channel, potentially many pharmacologically and/or functionally different channels might be present on the surface of granule cells. These receptors might not all be expressed at synapses; physiological (Cull-Candy and Ogden, 1985; Edwards *et al.*, 1990; Llano and Gerschenfeld, 1993; Puia *et al.*, 1994) and immunocytochemical (Richards *et al.*, 1987; Somogyi 1989; Somogyi *et al.*, 1989; Yazulla *et al.*, 1989; Soltesz *et al.*, 1990; Waldvogel *et al.*, 1990; Hansen *et al.*, 1991; Greferath *et al.*, 1993; Spreafico *et al.*, 1993) studies on other neurons have demonstrated an abundance of extrasynaptic receptors.

The best resolution for receptor distribution is provided by immunocytochemical methods. Light microscopic localization of the α1 (Schoch *et al.*, 1985; Richards *et al.*, 1987; Houser *et al.*, 1988; Zimprich *et al.*, 1991; Turner *et al.*, 1993; Gao and Fritschy, 1994), α2 (Zimprich *et al.*, 1991; Marksitzer *et al.*, 1993; Turner *et al.*, 1994), α3 (Zimprich *et al.*, 1991; Turner *et al.*, 1993), α5 (Turner *et al.*, 1993) and β2/3 (Schoch *et al.*, 1985; Richards *et al.*, 1987; Houser *et al.*, 1988) subunits of the $GABA_A$ receptor have shown homogenous immunostaining of both the somatic and dendritic layers and more distinct, strong staining of interneurons (Richards *et al.*, 1987; Houser *et al.*, 1988; Turner *et al.*, 1993; Gao and Fritschy, 1994). However, the receptor protein distribution has not been analysed in relation to the synaptic inputs, and neither has the subcellular and subsynaptic distribution of different subunits been established. Therefore, in the present study high-resolution pre- and postembedding immunogold techniques were used in order to define the membrane distribution of the most abundant α1 and β2/β3 subunits of the $GABA_A$ receptor in the hippocampus.

In addition to granule cells, GABAergic non-principal neurons in the hippocampus also receive GABAergic innervation either from the medial septum (Bilkey and Goddard, 1985; Freund, 1992) or from other GABAergic local circuit interneurons (Nunzi *et al.*, 1985; Misgeld and Frotscher, 1986; Lacaille and Schwartzkroin, 1988; Scharfman *et al.*, 1990; Buhl *et al.*, 1994a). Input from either source may provide inhibition of inhibitory cells producing disinhibition of granule cells (Scharfman *et al.*, 1990; Misgeld *et al.*, 1992), but a $GABA_A$ receptor-mediated excitatory effect has also been proposed (Michelson and Wong, 1991). Therefore, we have examined the pattern of $GABA_A$ receptor distribution on GABAergic interneurons with particular reference to their synaptic inputs. An immunoperoxidase method and two immunogold methods have been compared for the electron microscopic localization of receptors. The limitations of each technique have been critically evaluated, and it is shown that the three methods provide complementary information and are best used in combination in order to define the precise distribution of receptors.

Materials and methods

Preparation of animals and tissue

Four adult male cats were used; two of them were deeply anaesthetized with chloral hydrate (400 mg/kg i.p.) and the other two with 0.7 ml of a mixture of one part Rompun (20 mg/ml xylazine) and two parts Vetalar (100 mg/ml ketamine). The first cat was perfused through the heart, first with 0.9% saline for 1 min, then with a fixative containing 3% paraformaldehyde, 0.025% glutaraldehyde and ~0.2% picric acid dissolved in 0.1 M phosphate buffer (PB), pH 7.4 for 10 min, followed by a similar fixative without glutaraldehyde for 20 min. The other three cats were perfused in a similar way (7–30 min), but the fixative contained 4% paraformaldehyde and 0.05% glutaraldehyde and approximately 0.2% picric acid (Somogyi and Takagi, 1982). Four female Wistar rats (120–150 g) were anaesthetized with Sagatal (pentobarbitone sodium, 220 mg/kg i.p.) and perfused through the heart with saline followed by the fixative (for 7–13 min) used for the three cats. After perfusion the brains were removed and blocks from

the dorsal hippocampus were cut out and washed in several changes of 0.1 M PB.

Antibodies and controls

Two monoclonal antibodies, bd-17 and bd-24, were raised to purified $GABA_A$/benzodiazepine receptor complex from bovine brain; their characterization has been described by Haring *et al.* (1985) and Schoch *et al.* (1985). Antibody bd-17 recognizes both β2 and β3 subunits of the human receptor (Ewert *et al.*, 1990) and reacts with GABA receptors of the cat and rat. Antibody bd-24 reacts with the human α1 subunit; it does not recognize the α1 subunit in rat (Ewert *et al.*, 1990) but reacts with the receptor complex of the cat. In our experiments either tissue culture supernatant (1:1, 1:2 dilution) or affinity-purified monoclonal antibodies were used. In addition, affinity-purified rabbit polyclonal antibodies to the α1 subunit were used (Zezula *et al.*, 1991; Zimprich *et al.*, 1991). The antiserum (code no. P16) was raised to a synthetic peptide corresponding to residues 1–9 of the rat α1 subunit. The peptide was conjugated to keyhole limpet haemocyanin for immunization (Zezula *et al.*, 1991). In immunoblots, this antiserum recognizes a single protein with 51 kDa in rat brain membranes or affinity-purified $GABA_A$ receptors (Zezula *et al.*, 1991; Zezula and Sieghart, 1991). A band with identical mobility was photolabelled by [^{3}H]flunitrazepam. The purified antibodies used in immunoaffinity purification retained between 25–40% of specific [^{3}H]flunitrazepam binding from rat brain (Zezula *et al.*, 1991). Immunoreactions with affinity-purified P16 antibodies were carried out at a final concentration of 2.1 μg/ml under pre-embedding conditions and 6.3 μg/ml under postembedding conditions. Rabbit polyclonal antiserum to GABA (code no. 9; Hodgson *et al.*, 1985) was used at a dilution of 1:1000 on semithin epoxy resin-embedded sections, and at a 1:3000 dilution on Lowicryl-embedded ultrathin sections. This antiserum has been tested for cross-reactivity to numerous tissue components; it reveals GABA in fixed brain sections (Hodgson *et al.*, 1985; Somogyi *et al.*, 1985b).

Selective labelling, resembling that obtained with the specific antibodies, could not be detected when the primary antibodies were either omitted or replaced by 5% normal mouse serum, tissue culture supernatant or 5% normal rabbit serum. Using monoclonal antibodies to somatostatin (Vincent *et al.*, 1985) or polyclonal antibodies to synapsin (Naito and Ueda, 1981), no plasma membrane labelling was observed with our methods, demonstrating that the labelling observed on the plasma membrane is due to the anti-receptor antibodies bd-24, bd-17 and P16.

Pre-embedding immunohistochemistry

Immunoperoxidase reactions

These were carried out as described earlier (Somogyi *et al.*, 1989; Molnar *et al.*, 1993). Briefly, following cryoprotection in sucrose the blocks were frozen in liquid nitrogen and thawed in PB, then 70 μm thick Vibratome sections were cut. Floating sections were incubated in normal serum diluted in 50 mM Tris–HCl, pH 7.4, containing 0.9% NaCl (TBS) to reduce non-specific binding of antibodies to the tissue. They were then treated either with the hybridoma supernatant, affinity-purified monoclonal antibodies (final concentration 10 μg protein/ml for bd-17 and 0.07 μg protein/ml for bd-24), or polyclonal antibodies P16 (2.1 μg protein/ml) overnight at 4°C. After several washes in TBS, the sections were incubated in either biotinylated goat anti-rabbit or horse anti-mouse IgG (diluted 1:50; Vector Laboratories) in TBS for 2 h, followed by incubation in avidin-biotinylated horseradish peroxidase complex (diluted 1:100) for 2 h. Peroxidase enzyme reaction was carried out with 3,3′-diaminobenzidine tetrahydrochloride as chromogen and H_2O_2 as oxidant. In some cases Triton X-100 (0.3%) was added to the TBS throughout the experiment and the sections were processed only for light microscopic examination.

Immunogold method

Up to the addition of secondary antibodies the method was identical to that described above. The sections were then incubated in 1:100 goat anti-rabbit or 1:100 goat anti-mouse IgG coupled to 1.4 nm gold (Nanoprobes, Stony Brook, NY) in TBS overnight at 4°C. After several washes the sections were postfixed in 1% glutaraldehyde (in 0.1 M PB) for 10 min. Washing with ultra-pure water was followed by silver enhancement of the 1.4 nm gold particles with an HQ Silver kit (Nanoprobes) for 16–22 min as described by the manufacturer. The peroxidase-reacted and gold-reacted sections were then routinely processed for electron microscopy, including OsO_4 treatment, as reported earlier (Somogyi *et al.*, 1989; Molnar *et al.*, 1993).

Freeze-substitution and Lowicryl embedding

The same procedure was used as described earlier (Baude *et al.*, 1993; Nusser *et al.*, 1994). Briefly, after perfusion, blocks of tissue were washed in 0.1 M PB followed by Vibratome sectioning (500 μm thickness) and washing with 0.1 M PB overnight. The sections were placed into 1 M sucrose solution in 0.1 M PB for 2 h for cryoprotection before they were slammed to a polished copper block cooled with liquid N_2 (Reichert MM80 E). The sections were then transferred to a freeze-substitution apparatus (Leica CS auto) for dehydration in methanol at −80°C and embedding in Lowicryl HM 20 at −50°C (Chemische Werke Lowi GmBH, Germany).

Postembedding immunocytochemistry

Semithin sections

Postembedding immunoreactions for GABA were carried out on 0.5 μm thick sections, cut from 70 μm thick sections embedded in epoxy resin (Durcupan ACM, Fluka), which had been immunostained for the β2/3 subunits of the $GABA_A$ receptor. Rabbit polyclonal antiserum to GABA (code no. 9) and a previously described procedure was used (Somogyi *et al.*, 1985b).

Electron microscopic sections

Postembedding immunocytochemistry on slam-frozen, freeze-substituted, Lowicryl-embedded tissue was carried out on sections of ~90 nm thickness of the dentate gyrus of both cat and rat. They were picked up on pioliform-coated nickel grids (100 mesh). The sections were incubated on drops of blocking solution for 30 min, followed by incubation on drops of primary antibodies overnight at room temperature. The blocking solution, which was also used for diluting the primary and secondary antibodies, consisted of 0.1 M PB containing 0.8% ovalbumin (Sigma), 0.1% cold-water fish skin gelatin (Sigma) and 5% fetal calf serum. The following two protocols were used.

(i) Single immunostaining for the α1 or β2/3 subunits of the $GABA_A$ receptor using affinity-purified monoclonal antibodies at a final concentration of 20 μg protein/ml for bd-17 and 0.25 μg protein/ml for bd-24, or affinity-purified polyclonal P16 antibodies (6.3 μg protein/ml). After incubation in primary antibody, sections were washed and incubated on drops of goat anti-mouse or goat anti-rabbit IgG coupled to 1.4 nm gold (diluted 1:100, Nanoprobes Inc) for 2 h at room temperature.

(ii) Double immunostaining for β2/3 subunits (monoclonal antibody

bd-17) and for GABA on the same ultrathin sections. After incubation in primary antibody, sections were washed and incubated with a mixture of goat anti-mouse IgG coupled to 1.4 nm gold (diluted 1:100; Nanoprobes) for visualizing the receptors and goat anti-rabbit IgG coupled to 15 nm gold (diluted 1:25; BioClinical Services, Cardiff, UK) for visualizing GABA.

Following several washes, sections were fixed in a 2% glutaraldehyde solution (dissolved in 0.1 M PB) for 2 min, and then transferred to drops of ultra-pure water prior to silver enhancement in the dark with HQ Silver kit (Nanoprobes) for 4 min. After further washing in ultra-pure water, the sections were contrasted with saturated aqueous uranyl acetate followed by lead citrate.

Results

Postsynaptic components of the dentate layers

The distribution of $GABA_A$ receptors in the dentate gyrus of rats and cats was determined. This receptor type is mainly postsynaptic. Since none of the hippocampal layers is homogenous in its neuronal composition, it is necessary to summarize briefly the terminology used in this paper, and the GABA-recipient postsynaptic elements of the different laminae. Throughout this paper the term *principal cell* will be used for granule, mossy and pyramidal cells; the term *non-principal cell* or *interneuron* will be used to include all other cell types, some of which may have hippocampofugal axons. In the dentate *molecular layer* the main postsynaptic elements are the dendrites of granule cells, though the somata and dendrites of scattered interneurons, and the dendrites of interneurons having cell bodies in the granule cell layer, the hilus and the CA3c region, are also present as postsynaptic targets. Occasionally, hilar mossy cells may also send dendrites to the molecular layer (Frotscher *et al.*, 1991; Scharfman, 1991). The *granule cell layer* is densely packed with the cell bodies of granule cells, their proximal dendrites and axon initial segments. Cell bodies of interneurons are present throughout the granule cell layer, but they are especially prominent at the interface with the hilus. Interneuron dendrites originating from cell bodies in the granule cell layer, the hilus and the CA3c region are also present. The *hilus* proper contains many non-principal cell bodies and dendrites, the latter originating from cell bodies in the granule cell layer, the hilus itself and the CA3c region. The principal cells of the hilus are the mossy cells receiving GABAergic synapses on their cell bodies, dendrites and axon initial segments. The border region of the hilus with the granule cell layer also contains the axon initial segments of granule cells. The *CA3c region* was also present in our sections, and $GABA_A$ receptor immunoreactivity is described at the light microscope level in comparison with the hilus.

The monoclonal antibody bd-24, specific for the α1 subunit of the $GABA_A$ receptor, could only be used in the cat. The polyclonal antibody P16, also specific for the α1 subunit of the $GABA_A$ receptor, was obtained at a late stage of this study, and was used only in rats. Both cat and rat tissues were studied with monoclonal antibody bd-17, specific for β2/3 subunits.

Distribution of $GABA_A$ receptor immunoreactivity as detected by light microscopy

In the molecular and granule cell layers, as well as in the hilus, the pattern of immunoreactivity for the α1 and β2/3 subunits was similar in the two species. The strongest regional immunoreactivity was detected in the molecular layer (Fig. 1A, B), which is in agreement with previous results for the β2/3 subunits in rats (Richards *et al.*, 1987) and for the α1 subunit in rats (Gao and Fritschy, 1994) and man (Schoch *et al.*, 1985; Houser *et al.*, 1988). Most of the immunoreactivity in the molecular layer appeared homogenous and could not be allocated to particular cellular elements at the light microscope level of investigation. In some cats, the inner one-third of the molecular layer, the terminal field of the commissural/associational pathways and the GABAergic HICAP cells, was more strongly immunopositive for the α1 subunit than the outer two-thirds of the molecular layer (Fig. 1A). This pattern was in contrast to that obtained with antibody for the β2/3 subunits, which in some rats labelled the inner one-third of the molecular layer less than the outer two-thirds (Fig. 1D).

The somata and dendrites of many non-principal cells were more strongly immunopositive than the surrounding neuropil of the molecular layer (Figs 1D and 2B). The somatic and dendritic membranes were sharply delineated by the peroxidase reaction end-product in detergent-free, osmium-treated sections (Figs 1C, D and 2A–C). Strongly immunopositive dendrites originating from interneurons in other laminae also entered the molecular layer.

In contrast to the more strongly immunopositive non-principal neurons in the cell body layer, the somata of granule cells showed moderate immunostaining (Figs 1A, B and 2A–C). The density of the immunopositive non-principal cells in the granule cell layer was higher than in the hilus. The strongly immunopositive hilar interneurons were often located in the infragranular layer (Fig. 1C). The overwhelming majority of the strongly immunostained dendrites of hilar cells were free of spines, although there were also some spiny dendrites. In rats a well defined border could be seen at the boundary of the hilus and the strata oriens and radiatum of the CA3c area of the hippocampus (Fig. 1B), the latter being more strongly and homogeneously immunopositive, similar to the dentate molecular layer. The CA3c area also contained interneurons delineated along their membrane by immunoreactivity for the α1 and β2/3 subunits.

In order to characterize further the strongly immunopositive non-principal neurons in the rat, 0.5 μm semithin sections were cut from thick sections immunoreacted for the β2/3 subunits and immunostained for GABA. In all areas studied, most of the strongly receptor-immunopositive non-principal cells examined were immunopositive for GABA in their cell bodies (Fig. 2C, D). Similar results have been obtained recently for strongly α1 subunit-positive cells in the rat (Gao and Fritschy, 1994).

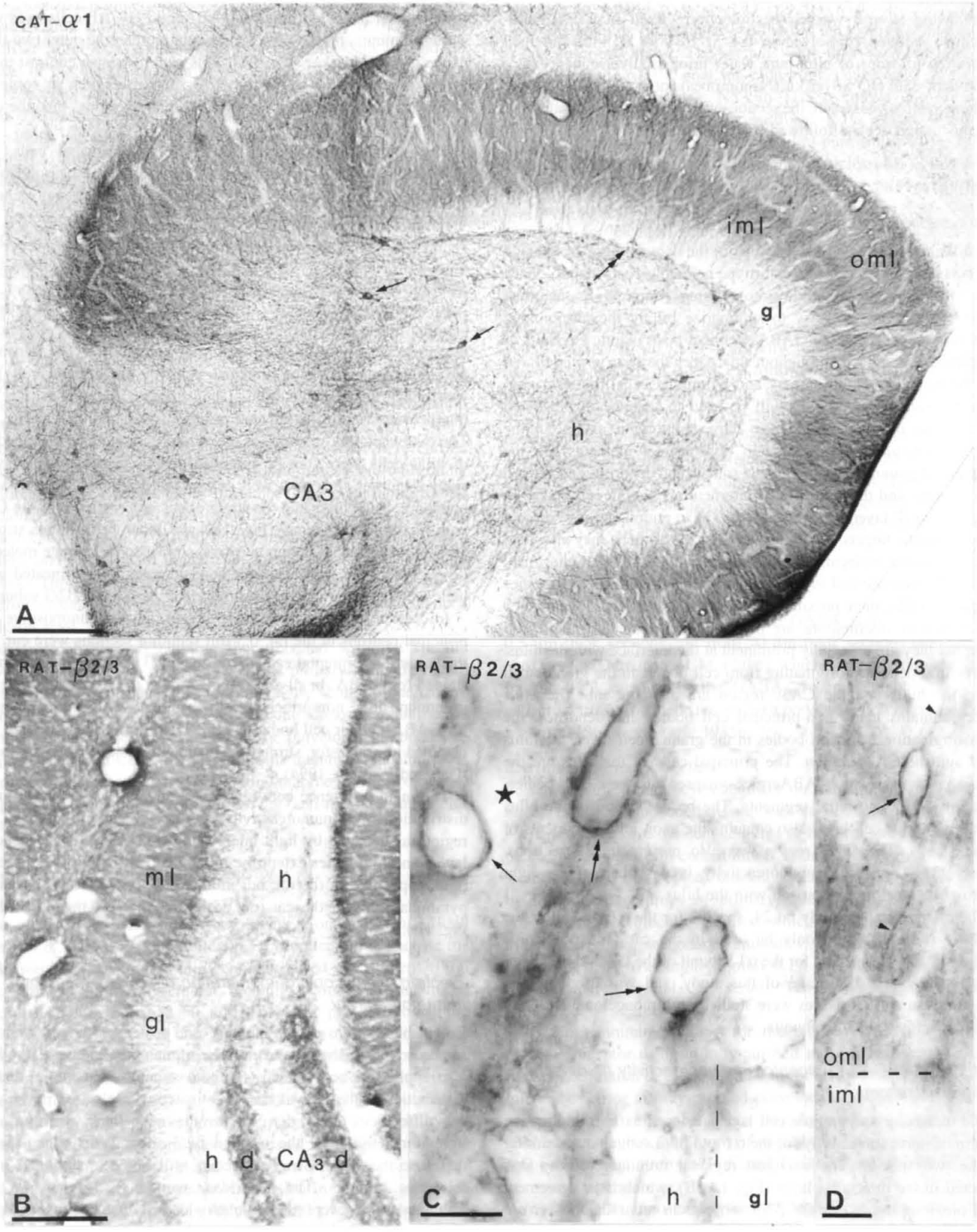
The main difference between the rat and cat was observed in the distribution of immunoreactivity for the α1 subunit in the CA3c region, as detected by light microscopy (Fig. 1A, B). The dendritic layers and to a lesser extent the pyramidal cell bodies were strongly immunopositive in the rat, but immunoreactivity was very weak on pyramidal cells in the cat (not visible at the low magnification in Fig. 1B).

Electron microscopic demonstration of $GABA_A$ receptor immunoreactivity

Both subunits were studied with pre- and postembedding immunogold methods in both the rat and cat. The immunoperoxidase method was also applied to both species for both subunits, but the α1 subunit distribution in the rat was not investigated with electron microscopy. No differences were detected in the subcellular distribution of immunoreactivity for the α1 and β2/3 subunits in either species; therefore the subunit or the species will not be mentioned in the following sections. The peroxidase reaction end-product and the immunoparticles were predominantly located on the extracellular face

of the synaptic membranes, the extrasynaptic membrane of somata and dendrites as well as on the intracisternal surface of the endoplasmic reticulum membrane, corresponding to the predicted location of the epitopes recognized by antibodies bd-24, bd-17 and P16.

The cell bodies of granule cells, most interneurons and mossy cells are identifiable in electron micrographs using previously published criteria (Lubbers and Frotscher, 1987). However, dendrites which are not in continuity with a cell body are not always identifiable. The



following criteria were used to predict the origin of $GABA_A$ receptor-immunopositive dendrites. In the molecular layer, dendritic shafts receiving few or no asymmetrical synaptic junctions and having irregular contours and/or emitting spines were classified as originating from granule cells. Similar dendrites in the hilus were considered as originating from mossy cells. Occasional mossy cell dendrites in the molecular layer may be indistinguishable from granule cell dendrites. However, their overall proportion is negligible in comparison with granule cell dendrites, and our description is based on the overall pattern. The dendritic shafts of many interneurons have numerous synapses, mostly of the asymmetrical type, and these dendrites rarely emit spines. However, there are also interneurons having numerous spines on part or most of their dendrites (Seay-Lowe and Claiborne, 1992; Halasy and Somogyi, 1993b; Han *et al.*, 1993; Soriano and Frotscher, 1993). In electron micrographs probably not all interneuron dendrites can be recognized, thus our description is based on those which could be positively identified on the basis of their input, consisting of many axodendritic asymmetrical and fewer symmetrical synapses. It should also be noted that different types of interneurons differ in their $GABA_A$ receptor immunoreactivity (Gao and Fritschy, 1994), but for technical reasons we can only comment on the immunopositive cases.

Pre-embedding peroxidase technique

Sparse intracellular immunoreactivity associated with intracellular membranes and faint immunostaining of the plasma membrane of granule cell bodies was observed. Immunolabelling of the extrasynaptic dendritic and synaptic membranes was also found. As predicted from the light microscopic observations, more prominent immunostaining of non-principal cells was observed (Fig. 3). The electron-dense reaction end-product filled the cisternae of the endoplasmic reticulum and outlined the nuclear envelope (Fig. 3A). The extrasynaptic somatic and dendritic membranes of interneurons also showed stronger immunoreactivity than that of granule cells (Fig. 3A, B). Peroxidase end-product deposition in the cleft of symmetrical synapses was found in all layers (Fig. 3C). The relative densities of immunoreactivity on the membrane of the endoplasmic reticulum cisternae, on the extrasynaptic somatic and dendritic membrane and in the synaptic junctions of the same cells were indistinguishable using the immunoperoxidase method (Fig. 3). This result may indicate that the receptor protein is present in the membranes everywhere in similar concentration, or that the density of peroxidase reaction end-product is a poor indicator of differences in receptor density at the various subcellular sites. Furthermore, the precise location of the receptors is not possible to determine because the peroxidase reaction end-product diffuses along the membranes. Therefore a particulate, non-diffusible marker was also used in an attempt to overcome the technical limitations of the peroxidase method.

Pre-embedding immunogold technique

This reaction results in irregularly shaped silver particles produced by the silver enhancement of the small gold particles coupled to the secondary antibodies. The size of the particles depends on the duration of silver intensification. The density of particles decreases gradually in the depth of the thick sections due to the limited penetration of the immunoreagents; therefore quantitative comparisons of immunoparticle density on different populations of cells is not possible using the pre-embedding immunogold technique. Immunoparticles were always associated with cell membranes, mostly the plasma membrane and rarely with endomembranes in dendrites and cell bodies (Fig. 4). The presence of immunoparticles along the extrasynaptic somatic and dendritic membrane of granule cells demonstrates that the $\alpha 1$ and $\beta 2/3$ subunits of the $GABA_A$ receptor are present in the extrasynaptic membrane of granule cells (Fig. 4). Dendritic shafts surrounded by immunolabel were most conspicuous, but many spines were also outlined by immunoparticles (Fig. 4). The extrasynaptic membrane of axon initial segments was also labelled (Fig. 5). Similar subcellular labelling patterns were observed on the extrasynaptic somatic and dendritic membranes of the non-principal cells throughout all layers (Fig. 4). However, a higher density of immunoparticles was detected on the immunopositive non-principal cells compared to neighbouring granule cells (Fig. 4).

Immunoparticles were never found in asymmetrical synaptic junctions in the dentate gyrus (Fig. 4B–D). Surprisingly, the symmetrical synapses on dendrites, somata and axon initial segments of granule cells (Figs 4E, F and 5A) and on the somata and dendrites of interneurons (Fig. 4B) were immunonegative for $GABA_A$ receptor using this method. The lack of synaptic reaction was not due to a failure of epitope recognition, since the antibodies were able to label the tissue, as demonstrated by the specific labelling of the extrasynaptic membranes. To investigate whether the lack of immunoreactivity in the presumed GABAergic symmetrical synaptic junctions was genuine or due to a technical limitation of the pre-embedding method, postembedding immunogold reactions were carried out on Lowicryl-embedded dentate gyrus.

Postembedding immunogold technique

The immunoreagents in this reaction have direct access to the surface of the electron microscopic sections, including the whole sectioned length of the plasma membrane exposed on the surface. Immunoparticles for the $\alpha 1$ and $\beta 2/3$ subunits of the $GABA_A$ receptor were enriched in the symmetrical synapses made by axon terminals of axo-axonic cells on the axon initial segment of granule cells (Fig. 5C, D). The axon initial segment of granule cells was recognized by characteristics of its fine structure, the membrane undercoating and the microtubule fascicles (Fig. 5). Immunoparticles were also concentrated in some axo-dendritic synaptic junctions in both the inner and outer molecular layers (Fig. 6A–E). Symmetrical synapses made by the terminals of basket cells on the somata of granule cells were also immunopositive for the tested subunits of the $GABA_A$ receptor (Fig. 7A). In Lowicryl-embedded material the fine structural characteristics of synapses are not always easily defined. Therefore, double immunolabelling experiments, using two sizes of gold par-

FIG. 1. Light microscopic immunoperoxidase demonstration of the $\alpha 1$ (A) and $\beta 2/3$ (B–D) subunits of the $GABA_A$ receptor in the dentate gyrus of the cat (A) and rat (B–D), using monoclonal antibodies. (A, B) Triton-treated sections. (C, D) Osmium-treated sections used for subsequent electron microscopy. Immunoreactivity for both subunits is prominent in the molecular layer (ml), but faint in the granule cell layer (gl). Sometimes the inner molecular layer (iml in panel A) was more strongly immunopositive for the $\alpha 1$ subunit than the outer molecular layer (oml in panel A). Immunoreactivity for the $\beta 2/3$ subunits in the rat, but not in the cat, is weaker in the inner than in the outer molecular layer (D). Strongly labelled non-principal neurons (arrow in D) and their dendrites (arrowheads in D) are conspicuous in the molecular layer. Pyramidal shaped non-principal neurons in the granule cell layer are stained more intensely than granule cells (e.g. double arrows in A). The reticular staining in the hilus (h) results from the dendritic labelling of the strongly immunopositive hilar cells (arrows in A and C and double arrows in C). Many of the hilar cells are immunonegative for the $\alpha 1$ and/or $\beta 2/3$ subunits (e.g. star in C). Note the plasma membrane localization of the peroxidase end-product (arrows and double arrows in C and D). In contrast to the reticular staining of the hilus, the strata oriens and radiatum of the CA3c area are intensely immunoreactive for the $\beta 2/3$ subunits in the rat (d in panel B). Scale bars: A, B, 200 μm; C, D, 15 μm

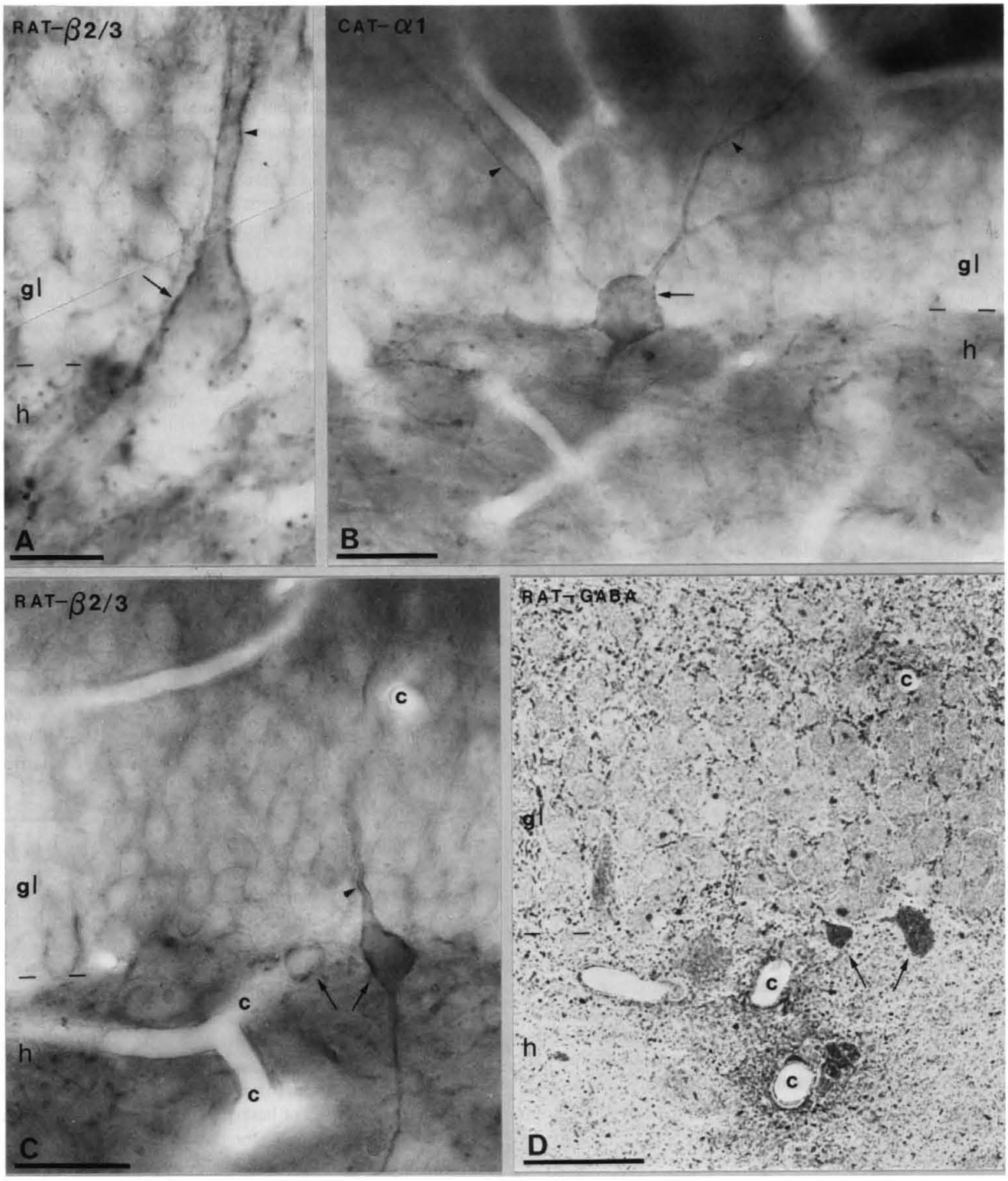


FIG. 2. Immunoreactivity for the α1 (B) and β2/3 (A and C) subunits in the granule cell layer (gl) and hilus (h) of the cat (B) and rat (A, C, D) using monoclonal antibodies and immunoperoxidase reaction. These osmium-treated sections were used for subsequent electron microscopy. Pyramid-shaped (arrows in A and C) and multipolar (arrow in B) non-principal neurons are prominently outlined by the peroxidase reaction end-product, which is associated mainly with the somatic (arrows in A–C) and dendritic membranes (arrowheads in A–C). The two strongly receptor-positive neurons in C (arrows) are shown to be immunoreactive for GABA in D (arrows). GABA immunostaining was performed on a 0.5 μm semithin section cut from the same region of the receptor-reacted thick section shown in C. c, capillary. Scale bars: A, 15 μm; B–D, 30 μm

ticles—large for GABA and small for the $GABA_A$ receptor subunits (Fig. 6E)—were carried out in rats. The high immunoreactive GABA content of axon terminals establishing receptor-immunopositive synapses with granule cell dendrites showed that the receptor immunoreactivity was associated with GABAergic synapses. Similar synaptic enrichment of immunoreactivity for the $GABA_A$ receptor subunits was found on the dendritic shafts of interneurons throughout all layers (Fig. 7B, D, E). Receptor-immunonegative synapses formed by GABA-positive boutons were encountered in all layers.

Immunoparticles for the $GABA_A$ receptor were present along the

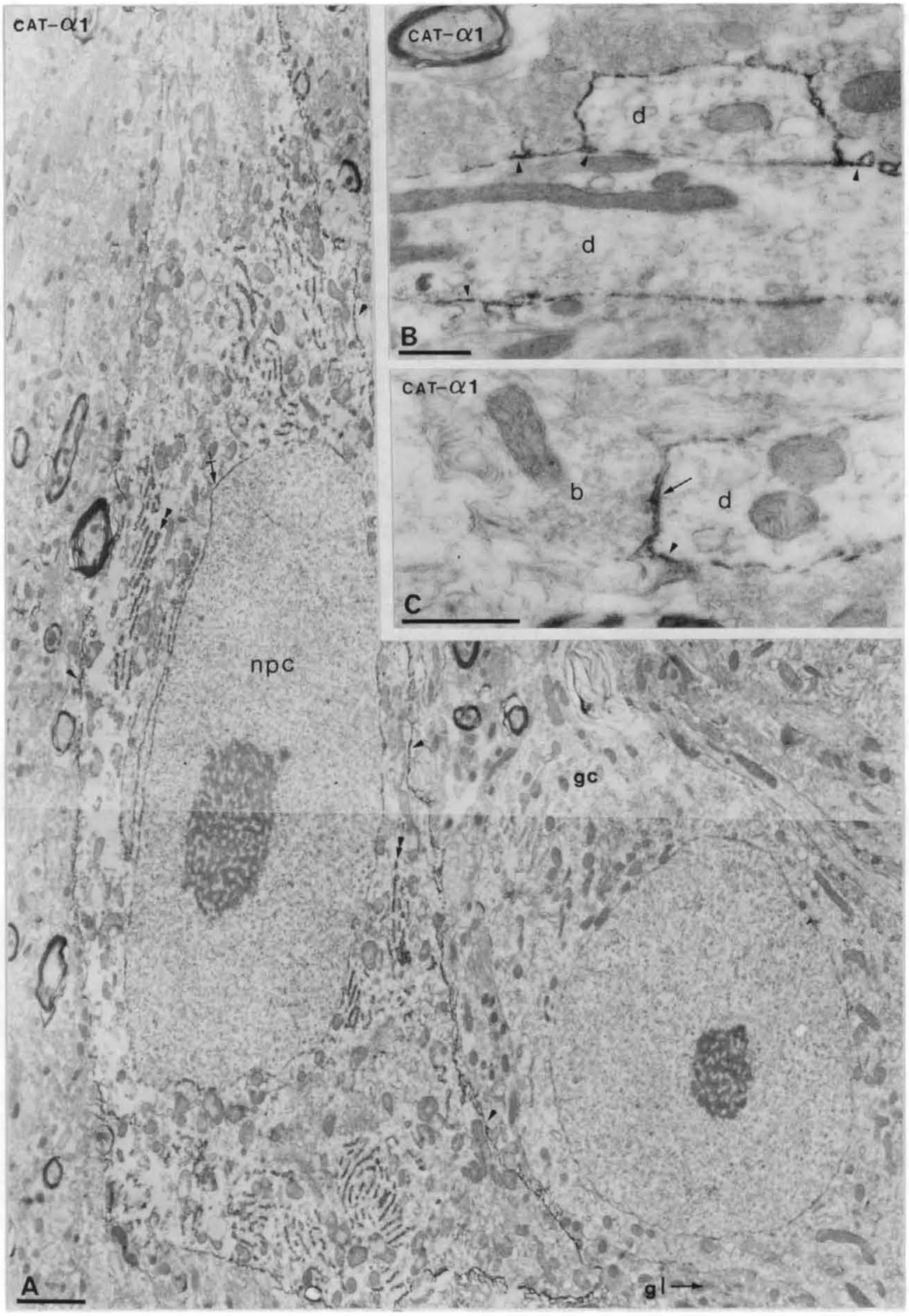


FIG. 3. Electron microscopic immunoperoxidase demonstration of immunoreactivity for the α1 subunit in cat. (A) The plasma membrane (arrowheads), endoplasmic reticulum (double arrowheads) and the nuclear envelope (crossed arrow) of a non-principal cell (npc) at the hilar/granular layer (gl) border are strongly labelled. (B) The extracellular face of non-synaptic dendritic (d) membranes is covered by the end-product (arrowheads) in the hilus. (C) Peroxidase end-product can be seen in the synaptic cleft (arrow) of a symmetrical synapse made by an axon terminal (b) with a hilar dendrite (d), as well as at adjacent extrasynaptic membrane (arrowhead). Note that the intensities of immunoreactivity in the endoplasmic reticulum, the extrasynaptic somatic, dendritic membranes and in the synaptic junction are indistinguishable (A–C). gc, granule cell. Scale bars: A, 2 μm; B, C, 0.5 μm

whole length of the synaptic membrane specialization, either in a continuous row of particles or in small groups (Figs 5–7). Sometimes the particles only covered a part of the synaptic specialization. It is possible that in these cases not the whole length of the synaptic specialization is cut at the surface of the section. The electron image of the junction is formed from the whole depth of the thin section,

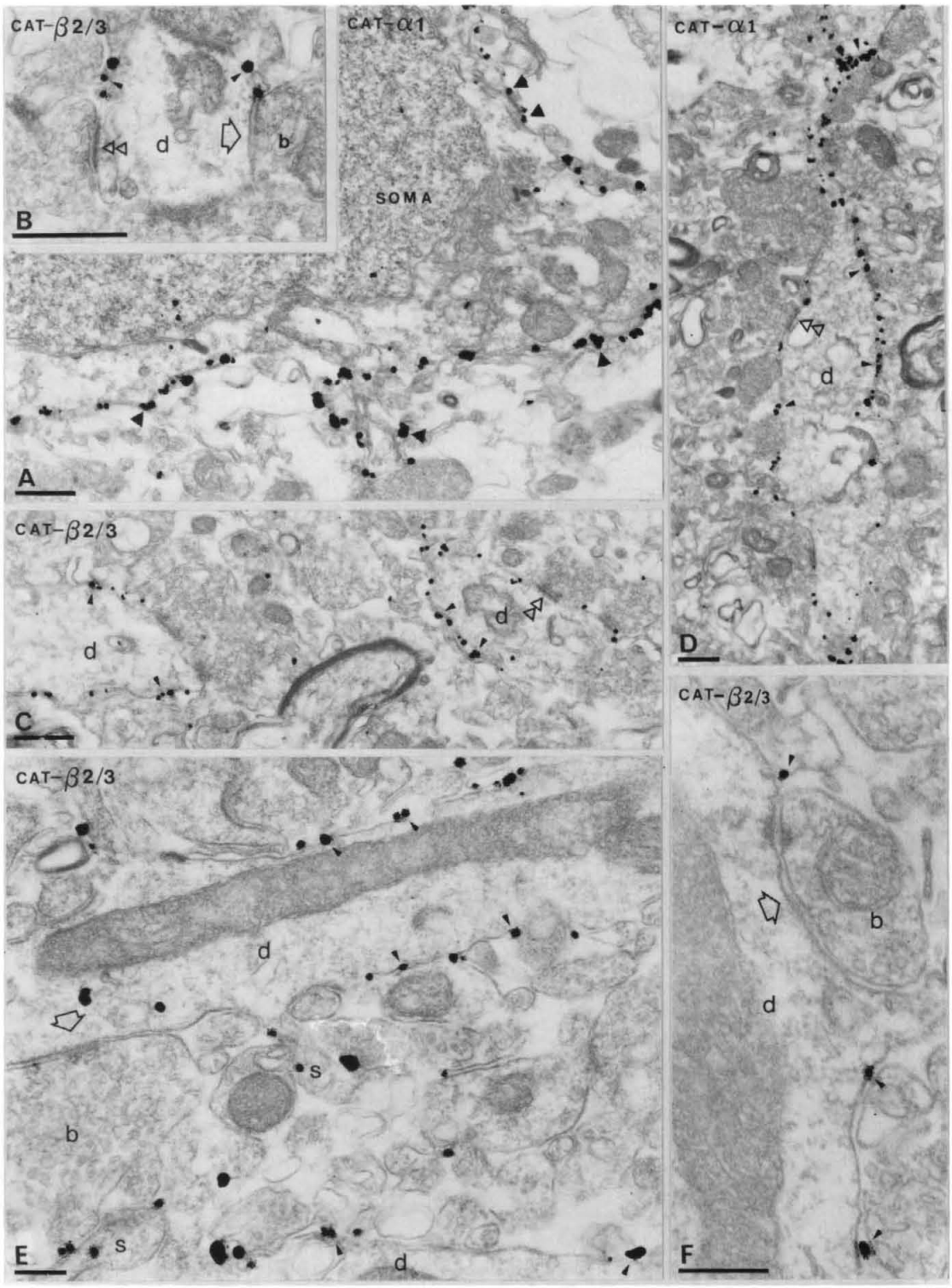


FIG 4. Distribution of the immunoreactivity for the α1 (A, D; same ultrathin section) and β2/3 (B, C, E, F; the latter three pictures from the same ultrathin section) subunits on the somatic (A) and dendritic (B–D; hilus) membranes of interneurons and on the dendritic membrane of granule cells (E, F; molecular layer). Pre-embedding immunogold reactions. Immunoparticles are located at the extracellular surface of non-synaptic dendritic and somatic membranes (arrowheads) corresponding to the location of epitopes, recognized by monoclonal antibodies bd-24 and bd-17 on the extracellular face of the plasma membrane. Immunoparticles (arrowheads) are more densely packed on the extrasynaptic somatic and dendritic membrane of interneurons than on granule cells (compare A–D with E, F). Immunoreactive receptors are also present on spines (s in panel E). Immunoparticles were not found with the pre-embedding method in asymmetrical (e.g. double triangles in B–D) or symmetrical synapses (open arrow) of interneurons (e.g. B) or granule cells (E, F) for either subunit in the two species. Scale bars: A–D, 0.5 μm; E, F, 0.2 μm.

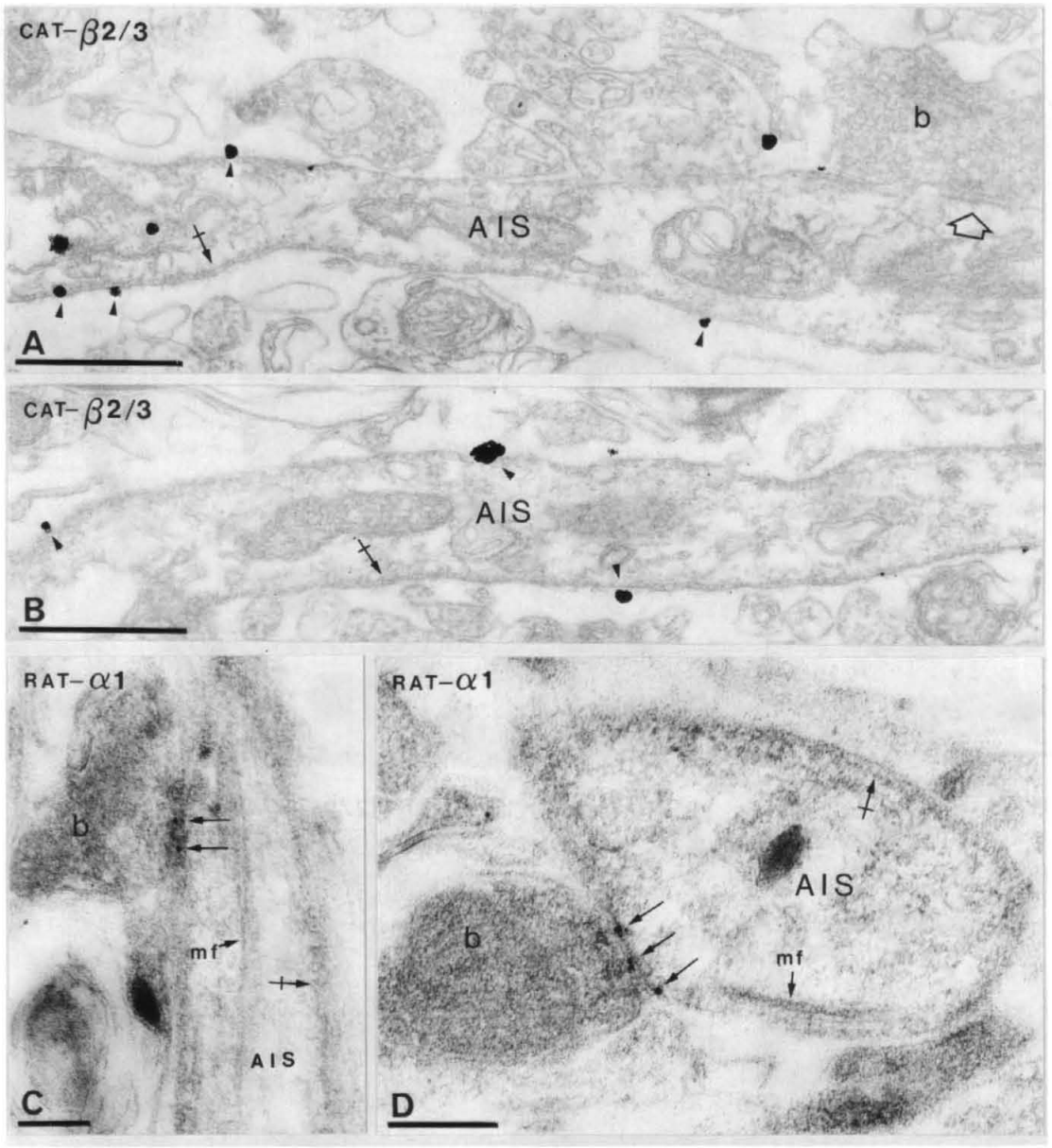


FIG. 5. Pre-embedding (A, B; cat) and postembedding (C, D; rat) immunogold demonstration of immunoreactivity for the α1 (C, D; rabbit polyclonal antibodies) and β2/3 (A, B; monoclonal antibody) subunits on the axon initial segment of granule cells. (A, B) Immunoparticles (arrowheads) are located on the extrasynaptic membrane of the axon initial segment, which can be recognized by its fine structural characteristics (e.g. membrane undercoating, crossed arrows in A–D; microtubule fascicles, mf). Using the pre-embedding technique, immunoparticles were never found in the symmetrical synapses made by boutons of axo-axonic cells (e.g. open arrow in A). However, immunoparticles (arrows in C and D) are frequent in the same type of synapse using the postembedding technique. The synaptic immunoparticles are distributed over the whole length of the synaptic junction. Scale bars: A, B, 0.5 μm; C, D, 0.2 μm

but the immunoreagents only react with one surface of the section. Therefore in some cases, particularly at the edge of a cross-sectioned synaptic disc, a substantial part of the junction may not be present at the surface of the section that had been exposed to the antiserum. There was a sharp decrease in immunoparticle density at the edge of the synaptic specialization. The extrasynaptic plasma membrane was labelled at a much lower density than the synaptic junction. The labelling was also much weaker on equivalent extrasynaptic membranes than that obtained with the pre-embedding method. Extrasynaptic immunoparticles were found on the somata, dendrites and axon initial segments of granule cells and on the somata and dendrites of interneurons (Figs 6 A, C, E and 7A–D). No immunoreaction could be allocated to presynaptic axon terminals.

Discussion

Immunocytochemical localization of receptors in comparison with other methods

The presence of both the α1 and β2/3 subunits was expected in dentate granule cells and non-principal cells from the expression of mRNA for these subunits (Persohn *et al.*, 1992; Wisden *et al.*, 1992). Previous data on the regional distribution of $GABA_A$/benzodiazepine

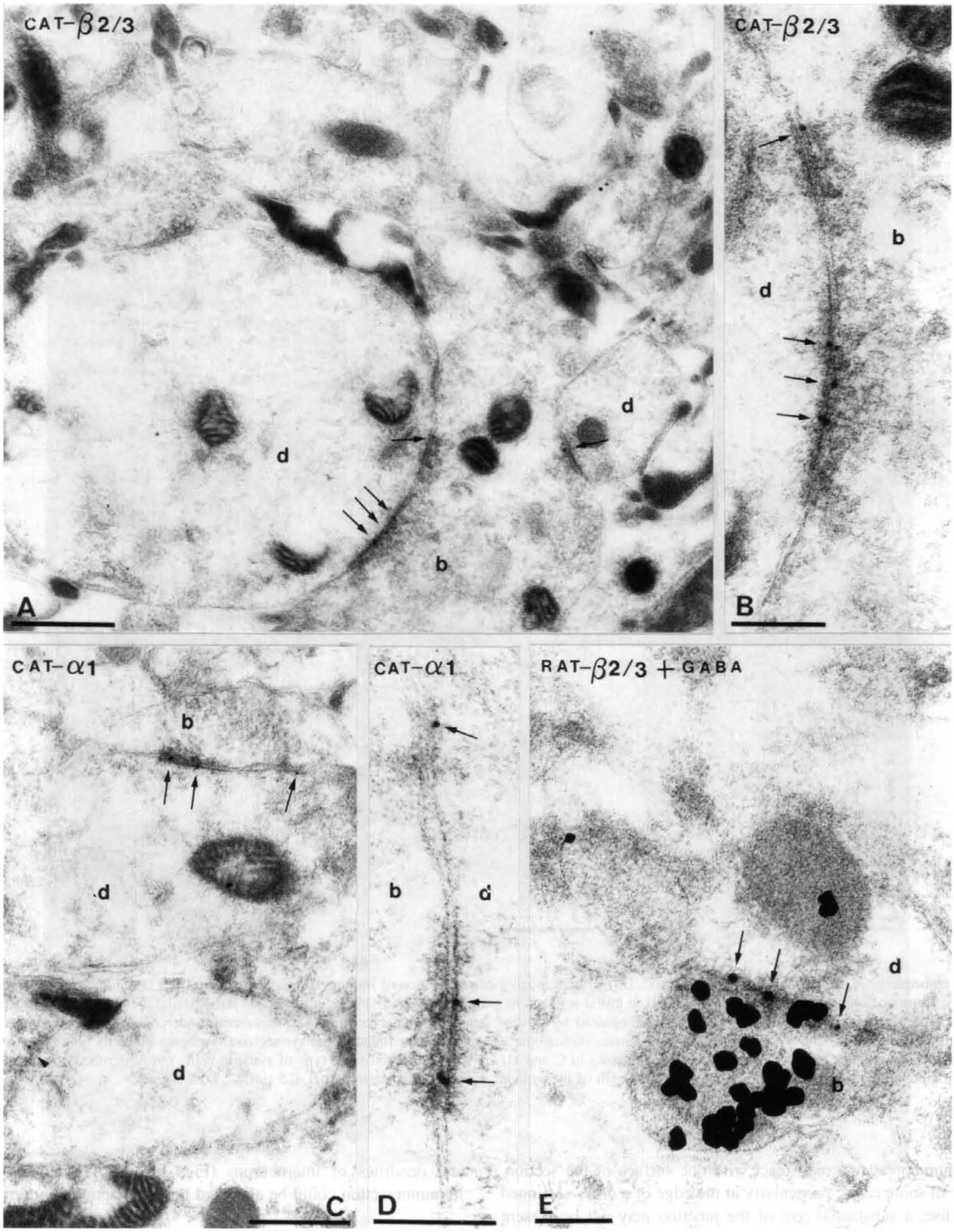


FIG. 6. Electron micrographs from postembedding-immunoreacted (freeze-substituted, Lowicryl-embedded) molecular layer showing immunoreactivity for the α1 or the β2/3 subunits alone (C, D and A, B respectively) or for the β2/3 subunits (small particles; silver-intensified 1 nm gold) and for GABA (E, large particles, silver-enhanced 15 nm gold). (A) An axon terminal (b) establishes three immunopositive symmetrical synaptic junctions (arrows) on dendritic shafts (d) in the inner molecular layer. (B) Higher magnification view of the immunopositive synaptic junctions shown in A. (C, D) Extrasynaptic (arrowhead) and synaptic (arrows) presence of immunoparticles for the α1 subunit on presumed granule cell dendrites (d). The same synapse is shown at higher magnification in D. (E) A GABA-immunopositive bouton (large particles, b) establishes a symmetrical synapse with a presumed granule cell dendrite (d) in the outer molecular layer. The postsynaptic membrane is immunopositive for the β2/3 subunits of the $GABA_A$ receptor (arrows, small particles). Scale bars: A, C, 0.5 μm; B, D, E, 0.2 μm

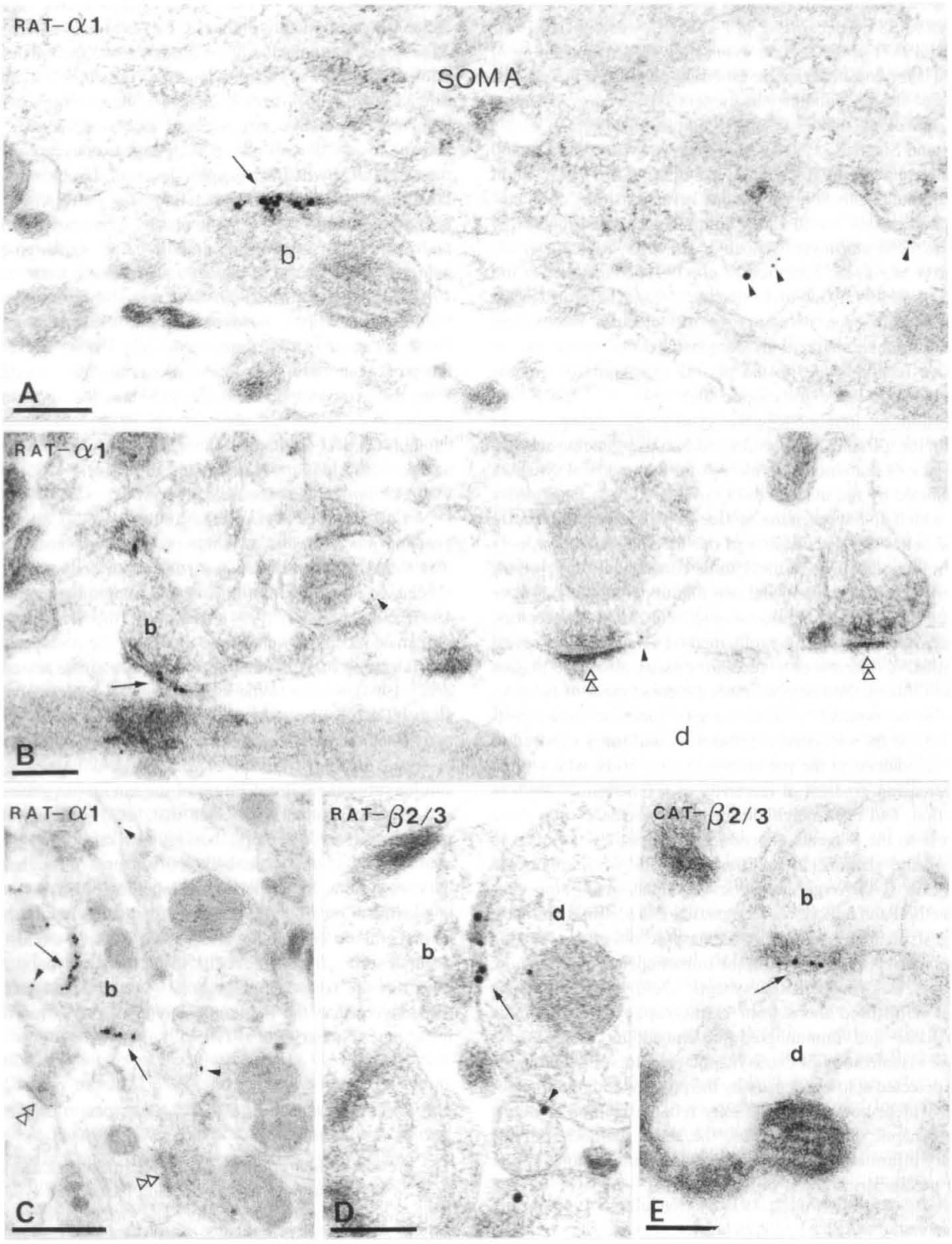


FIG. 7. Precise distribution of the α1 (A, B, C; rabbit polyclonal antibodies) and β2/3 (D, E; monoclonal antibody) subunits of the $GABA_A$ receptor on granule cell soma (A) and in the hilus (B–D), as demonstrated by a postembedding technique on Lowicryl-embedded tissue. (A) A high density of immunoparticles is present in the synaptic junction (arrow) made by a probable basket cell terminal (b). The extrasynaptic somatic membrane is labelled at lower density (arrowheads). Dendritic shafts (d in B, D, E) of hilar cells receive immunonegative asymmetrical synapses (double triangles in B, C) and symmetrical synapses (arrows in B, D, E) immunopositive for either the α1 or β2/3 subunits. A very high density of immunoparticles was found in some GABAergic synaptic junctions in the hilus (e.g. arrows in C). A few extrasynaptic immunoparticles (arrowheads in B, C, D) are also seen. Scale bars: A, B, D, E, 0.2 μm; C, 0.5 μm

receptor ligands (Young and Kuhar, 1979; Palacios *et al.*, 1981; Wamsley *et al.*, 1984; Richards *et al.*, 1986; Bowery *et al.*, 1987; Sieghart *et al.*, 1987) in the dentate gyrus showed dense labelling of the molecular layer and more sparse labelling of the cell body layer. The presence of the α1 subunit that confers type I benzodiazepine receptor pharmacology on the channel complex (McKernan *et al.*, 1991; Zezula and Sieghart, 1991) is in agreement with type I ligand binding in the dentate gyrus (for review see Olsen *et al.*, 1990). Light microscopic immunohistochemical studies revealed strong immunostaining of interneurons and a rather homogeneous distribution of labelling in the molecular layer (Schoch *et al.*, 1985; Richards *et al.*, 1987; Houser *et al.*, 1988; Zimprich *et al.*, 1991; Marksitzer *et al.*, 1993; Turner *et al.*, 1993; Gao and Fritschy, 1994). Therefore, in the present study it has been necessary to use electron microscopy and particulate immunomarkers to demonstrate that some of the immunoreactive receptors are on the plasma membrane of granule cells, at both synaptic and extrasynaptic sites.

The proportion of immunonegative symmetrical synapses was variable within the ultrathin sections. In addition to a genuine absence of receptors, lack of immunoreactivity in some symmetrical synapses can also be caused by the inaccessibility of receptors in tangentially cut synapses which do not reach the surface of the section, or receptor levels may be below the detectability of our method. Therefore, only the positive immunolabelling results can be conclusively interpreted.

An immunoperoxidase method and two immunogold methods have been compared for the electron microscopic localization of receptors. Only the postembedding immunogold method is suitable to reveal relative quantitative differences in receptor density along the plasma membrane, and this method demonstrates the enrichment of $GABA_A$ receptors in the anatomically defined synaptic junction. In the postembedding method the sectioned membrane is uniformly exposed to the reagents, in contrast to the pre-embedding methods, which often produce a decreasing gradient of reactivity as a function of depth in the thick section, and even neighbouring tissue elements may have different access to the reagents depending whether they are open to the surface of the section. In addition, the lack of immunogold reactivity in the GABAergic synaptic junctions, using the pre-embedding method and antibodies recognizing extracellular epitopes, strongly suggests that antibodies may not have access to the synaptic cleft due to its matrix composition. In the immunoperoxidase method, reaction product was found in the synaptic cleft, but it cannot be excluded that it diffused there from extrasynaptic sites. Both the immunoperoxidase and immunogold pre-embedding methods are reliable for the visualization of extrasynaptic receptors. Extrasynaptic receptors are detected at lower density by the postembedding immunogold method, which, however, is the only reliable method to detect receptors in synaptic junctions. Thus, the three methods provide complementary information and are best used in combination in order to define the precise distribution of receptors.

Enrichment of $GABA_A$ receptor in the synaptic junction

The fast onset and rise times of $GABA_A$ receptor-mediated inhibitory postsynaptic currents suggest that the receptors are close to the release site of GABA (Edwards *et al.*, 1990; Konnerth *et al.*, 1990; Llano and Gerschenfeld, 1993; Soltesz and Mody, 1994; Puia *et al.*, 1994). Since relatively few GABA channels (30–60) are thought to be activated on dentate granule cells by a single quantum of GABA (Edwards *et al.*, 1990; de Koninck and Mody, 1994; Soltesz and Mody, 1994), the question arises whether the density of channels is higher near the release site than in the non-synaptic plasma membrane. It is generally assumed that synaptically activated receptor channels are in the anatomically defined synaptic junctions, but the resolution of previous anatomical studies did not make the unequivocal localization of receptors in synaptic junctions possible. Only the postembedding immunogold method provides the resolution necessary to compare the relative synaptic and extrasynaptic receptor densities, and the results demonstrate a high degree of receptor enrichment in the anatomically defined synaptic junction. Using immunoperoxidase labelling, abundant immunoreactivity for the $GABA_A$ receptor has been found on the extrasynaptic plasma membrane of soma, dendrites and axon initial segment of granule cells, similar to that found on neurons in other parts of the central nervous system (Richards *et al.*, 1987; Somogyi *et al.*, 1989; Somogyi, 1989; Yazulla *et al.*, 1989; Soltesz *et al.*, 1990; Waldvogel *et al.*, 1990; Hansen *et al.*, 1991; Greferath *et al.*, 1993; Spreafico *et al.*, 1993). These extrasynaptic receptors seem to be fully functional, as outside-out patches, obtained from the extrasynaptic dendritic and somatic membranes of dentate granule cells as well as other neurons in the central nervous system, contain $GABA_A$ receptor channels which are activated by GABA and pass chloride ions (Cull-Candy and Ogden, 1985; Edwards *et al.*, 1990; Llano and Gerschenfeld, 1993; Puia *et al.*, 1994).

Not all amino acid neurotransmitter receptors are enriched in the synaptic junction. The metabotropic glutamate receptor (mGluR1α) was found to be excluded from the main body of the postsynaptic specialization using non-diffusible immunomarkers (Baude *et al.*, 1993; Nusser *et al.*, 1994). However, the ionotropic AMPA-type glutamate receptors were found to occupy the postsynaptic membrane specialization of synapses opposite the glutamate release site (Nusser *et al.*, 1994), similar to the ionotropic $GABA_A$ receptor in the present study. The glycine receptor was also found to be concentrated in patches on the surface of central neurons using confocal immunofluorescence mapping (Becker *et al.*, 1991; Triller *et al.*, 1991). The similar subsynaptic localization of the ionotropic glutamate, $GABA_A$ and glycine receptors indicates that, as a general rule, ionotropic neurotransmitter receptors, having fast onset and rise times, are concentrated in the main body of synapses throughout the central nervous system. Nevertheless, the labelling of extrasynaptic receptors is significant on all cell types in the dentate gyrus. In the cerebellum, where granule cells receive GABAergic input exclusively on their distal dendrites from Golgi cells, the density of synaptic immunoreactivity for the α1 and β2/3 subunits was 230 and 180 times higher respectively than the immunoreactivity of the extrasynaptic somatic membrane (Nusser *et al.*, 1995).

Subunit composition of $GABA_A$ receptors in basket and axo-axonic cell synapses

The present results confirm recent pharmacological evidence (Buhl *et al.*, 1994a) that both axo-axonic and basket cell synaptic transmission involves $GABA_A$ receptors. The strict segregation of the axo-axonic- and basket cell-innervated synapses, on the axon initial segment and the soma/proximal dendrite region of cortical principal cells respectively, suggests different functions for these synapses (Somogyi *et al.*, 1982; Douglas *et al.*, 1990; Lytton and Sejnowski, 1991). Although it has been clear for a long time that under normal conditions hyperpolarizing $GABA_A$ receptor-mediated responses are generated in or near to the cell body of hippocampal pyramidal and granule cells (Andersen *et al.*, 1964, 1966, 1980; Newberry and Nicoll, 1985), the close proximity of axo-axonic and basket cell synapses precluded their pharmacological separation. A recent study of identified synaptic junctions demonstrated short-latency IPSPs in

pyramidal and granule cells in response to the action potentials of single axo-axonic and basket cells, and both responses were blocked by bicuculline (Buhl *et al.*, 1994a). The pharmacological similarity, together with the presence of the α1 and β2/3 subunits in both synapses, suggests an overall similarity rather than a difference in the mechanism of synaptic transmission at axo-axonic and basket cell synapses. However, differences may still arise in the action of synaptically released GABA in several ways: (i) the synaptic responses may have different kinetics, (ii) receptor activation and inactivation may be under different regulation, and (iii) the synapses may be active at different times and for different durations. There is as yet no information on the differential activation of basket and axo-axonic cells, therefore only the first two points are considered below.

(i) Synaptic responses evoked by axo-axonic cells appear to be faster than those evoked by basket cells when compared in sharp electrode recordings from the soma (Buhl *et al.*, 1994a). Differences in the kinetics and pharmacology of $GABA_A$ receptors may arise from the subunit composition of the channels (Sigel *et al.*, 1990; Verdoorn *et al.*, 1990; Angelotti and Macdonald, 1993). Although the α1 and β2/3 subunits of the $GABA_A$ receptor are present at both synapses, further studies are necessary to compare quantitatively their contribution to the respective synapses. The presence of the α1 subunit at these synapses indicates that the action of these two major types of GABAergic interneurons can be influenced by benzodiazepines of the type I pharmacological group (Pritchett *et al.*, 1989; Zezula and Sieghart, 1991). Both granule and pyramidal cells express several other subunits (Persohn *et al.*, 1992; Wisden *et al.*, 1992) which are thought to be associated with type II benzodiazepine pharmacology (Pritchett *et al.*, 1989; Zezula and Sieghart, 1991; Marksitzer *et al.*, 1993); their distribution remains to be established.

In the CA1 region some IPSPs evoked by single basket cells were followed by a postinhibitory depolarization, which could lead to action potential firing in the pyramidal cell and it was suggested that this may provide a clue for the function of basket cells in synchronizing the activity of principal cells (Buhl *et al.*, 1994a). Such depolarizing responses, which may be due to voltage-dependent conductances, have not been reported for axo-axonic cell IPSPs. Although more data are necessary to confirm a possible difference, the same subunit composition of synaptic receptors could lead to different synaptic responses if the synapses were closely associated with different sets of voltage dependent channels in the somatic or axon initial segment membrane. The different 'channel environment' in the membrane may explain the strict segregation of GABAergic synapses.

(ii) In addition to subunit-specific allosteric sites, $GABA_A$ receptors are also regulated by protein kinases and phosphatases in a calcium- or cAMP-dependent manner (for review see Raymond *et al.*, 1993). The basket and axo-axonic cell synapses may differ in their calcium-dependent regulation, since the latter are specifically associated with the calcium-storing cisternal organelles, which are not present at basket cell synapses (Benedeczky *et al.*, 1994). Thus the same subunit composition may lead to different synaptic response kinetics depending on the regulation of the channels. Further differences may arise from a possible differential presynaptic regulation of GABA release from axo-axonic and basket cell terminals (Lambert and Wilson, 1993; Buhl *et al.*, 1994a).

Possible roles of dendritic $GABA_A$ receptors on granule cells

In the dentate gyrus $GABA_A$ and $GABA_B$ receptor-mediated responses seem to be evoked by different presynaptic neurons (Rausche *et al.*, 1989; Muller and Misgeld, 1990). Since basket and axo-axonic cell synapses definitely contain $GABA_A$ receptors, neurons that activate $GABA_B$ receptors probably terminate on the dendrites of granule cells. We found $GABA_A$ receptor-immunonegative synapses made by GABA positive boutons in the molecular layer, but from immunocytochemical experiments alone the absence of $GABA_A$ receptors at a particular site cannot be established for technical reasons. Unfortunately, a marker for the high-resolution localization of $GABA_B$ receptors is not yet available.

The dendrites are a major site for the action of GABA since 75% of all GABAergic synapses on granule cells were found in the molecular layer (Halasy and Somogyi, 1993a). The role of this prominent dendritic GABAergic innervation, originating from MOPP, HIPP, HICAP (Han *et al.*, 1993) and MOLAX cells (Soriano and Frotscher, 1993), is not well understood. Physiological recordings of spontaneous or stimulus-evoked $GABA_A$ receptor-mediated IPSPs and inhibitory postsynaptic currents (Edwards *et al.*, 1990; Scharfman *et al.*, 1990; Misgeld *et al.*, 1992; Otis and Mody, 1992; de Koninck and Mody, 1994; Soltesz and Mody, 1994) did not reveal the location of the GABAergic synapses on the surface of granule cells. The synapses responsible for these $GABA_A$ receptor-mediated responses may have been located exclusively on the soma or axon initial segment of granule cells, where basket and axo-axonic cells elicit $GABA_A$ receptor-mediated IPSPs (Buhl *et al.*, 1994a). Interneurons in the stratum lacunosum moleculare in the CA1 region of the hippocampus, having their axonal arbour mainly localized to the stratum lacunosum moleculare, have been shown to elicit IPSPs with slow onset and decay, indicating that they inhibit CA1 pyramidal cells through $GABA_B$ receptors in a feed-forward manner (Lacaille and Schwartzkroin, 1988). Based on the anatomical similarity of stratum lacunosum–moleculare interneurons and MOPP cells, it is likely that the latter also act through $GABA_B$ receptors.

The synaptic $GABA_A$ receptors demonstrated in the present study in the outer two-thirds of the molecular layer could be at synapses provided by other types of neurons, namely the HIPP cell (Han *et al.*, 1993) and the MOLAX cell (Soriano and Frotscher, 1993). In addition to the α1 and β2/3 subunits, the α2 and α3 subunits are also distributed in the molecular layer (Zimprich *et al.*, 1991; Marksitzer *et al.*, 1993; Turner *et al.*, 1993). The dendritic $GABA_A$ receptors are distributed in the termination zones of two major glutamatergic afferents to granule cells, suggesting a role in the downward rescaling of either the entorhinal or the associational pathway excitatory postsynaptic potential (Halasy and Somogyi, 1993b). Stimulation of the molecular layer produced $GABA_A$ receptor-mediated shunting of excitatory currents on the dendrites, particularly those mediated by *N*-methyl-D-aspartate receptors (Blanpied and Berger, 1992; Staley and Mody, 1992). Further selectivity in the effects of dendritic $GABA_A$ receptor activation may arise from local interaction with voltage-sensitive cation channels. For example, it has been shown in Purkinje cells that dendritic inhibition is particularly effective in preventing the activation of calcium channels in distal dendrites, and thus inhibition influences local excitability (Midtgaard, 1992). Dentate granule cells express several types of calcium channel, some high-threshold channels apparently being located preferentially in the dendrites (Blaxter *et al.*, 1989), where $GABA_A$ receptor-mediated hyperpolarization may selectively prevent their activation.

The sublaminar differences in light microscopic labelling intensity between the inner one-third and the outer two-thirds of the molecular layer, which receive partially separate GABAergic innervation, are not present in all animals in our material. Similar difference have been noted for the α3 subunit as well (Turner *et al.*, 1993). Quantitative immunogold studies will be necessary to determine the subcellular sites giving rise to the observed labelling differences.

GABAergic interneurons express $GABA_A$ receptors in high concentration

Hippocampal GABAergic interneurons receive synapses from glutamic acid decarboxylase and GABA-positive axon terminals (Freund and Antal, 1988; Misgeld and Frotscher, 1986; Danos *et al.*, 1991; Leranth *et al.*, 1992), but not at an appreciably higher density than principal cells. Therefore, the very strong immunoreactivity for $GABA_A$ receptors (Richards *et al.*, 1987; Houser *et al.*, 1988; Zimprich *et al.*, 1991; Turner *et al.*, 1993; Gao and Fritschy, 1994) is a consequence not of a higher density of GABAergic synapses compared to principal cells, but of the higher density of receptor in the membrane, which has also been demonstrated for extrasynaptic sites in the present study. Several types of interneuron express a high density of $GABA_A$ receptor, including cells with aspiny but also spiny dendrites in the hilus. In double labelling experiments, calretinin-, somatostatin- and neuropeptide-Y-positive GABAergic interneurons were strongly stained for the $\alpha 1$ subunit, but calbindin-, cholecystokinin- and vasoactive intestinal polypeptide-positive interneurons did not show strong labelling (Gao and Fritschy, 1994). Our results extend the finding to the cat and in addition demonstrate that both the $\alpha 1$ and $\beta 2/3$ subunits are present at GABAergic synapses; in this respect interneurons do not differ from principal cells. The main difference between the two populations is the higher density of receptors on interneurons. The high density of $GABA_A$ receptors on certain types of GABAergic neurons is not unique to the hippocampal cortex as it has also been demonstrated in the neocortex (Somogyi, 1989; Hendry *et al.*, 1994), suggesting that it is a fundamental feature of cortical circuits.

One well defined source of GABAergic innervation of interneurons is from the medial septum, which has been shown to innervate predominantly GABAergic interneurons in the hippocampal formation (Bilkey and Goddard, 1985; Freund and Antal, 1988). In addition, interneurons receive synapses from GABA- and/or cholecystokinin-immunopositive axon terminals of local circuit interneurons in the hippocampus (Nunzi *et al.*, 1985; Buhl *et al.*, 1994a). Intrahippocampal GABAergic input to interneurons has also been demonstrated by intracellular recording following perforant path stimulation (Misgeld and Frotscher, 1986; Lacaille and Schwartzkroin, 1988; Buhl *et al.*, 1994b). It is very unlikely that these $GABA_A$ receptor-mediated IPSPs originated from the stimulation of the septohippocampal fibres. Furthermore, paired intracellular recording from interneurons revealed reciprocal hyperpolarizing IPSPs (Scharfman *et al.*, 1990), supporting the hypothesis (Bilkey and Goddard, 1985; Freund and Antal, 1988) that GABAergic input to interneurons leads to disinhibition of principal cells. Further support for this idea comes from the demonstration of a paradoxical increase in inhibition of dentate granule cells following bath application of bicuculline *in vitro* (Scharfman, 1994). This observation could be explained by the higher sensitivity of inhibitory interneurons to $GABA_A$ receptor blockers. Similar preferential action of drugs that modulate $GABA_A$ receptor function may also occur on interneurons due to their higher $GABA_A$ receptor density.

Acknowledgements

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Abbreviations

AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid
GABA	γ-aminobutyric acid
HICAP cell	hilar cell with axon in the commissural and association pathway termination field
HIPP cell	hilar cell having an axon associated with the perforant path projection
IPSP	inhibitory postsynaptic potential
mGluR1α	metabotropic glutamate receptor type 1α
MOLAX cell	dentate interneuron with axon mainly in the molecular layer
MOPP cell	molecular layer neuron having an axon associated with perforant path input
PB	phosphate buffer
TBS	Tris-buffered saline

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