

Differential synaptic localization of two major γ -aminobutyric acid type A receptor α subunits on hippocampal pyramidal cells

(neurotransmission/hippocampus/inhibition/targeting/immunocytochemistry)

ZOLTAN NUSSER*, WERNER SIEGHART†, DIETMAR BENKE‡, JEAN-MARC FRITSCHY‡, AND PETER SOMOGYI*

*Medical Research Council, Anatomical Neuropharmacology Unit, University of Oxford, Mansfield Road, Oxford OX1 3TH, United Kingdom;

†Department of Biochemical Psychiatry, Psychiatrische Universitätsklinik, Währinger Gürtel 18-20, A-1090 Vienna, Austria; and

‡Institute of Pharmacology, University of Zurich, Winterthurerstrasse 190, CH-8057, Zurich, Switzerland

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ABSTRACT Hippocampal pyramidal cells, receiving domain specific GABAergic inputs, express up to 10 different subunits of the γ -aminobutyric acid type A (GABA_A) receptor, but only 3 different subunits are needed to form a functional pentameric channel. We have tested the hypothesis that some subunits are selectively located at subsets of GABAergic synapses. The $\alpha 1$ subunit has been found in most GABAergic synapses on all postsynaptic domains of pyramidal cells. In contrast, the $\alpha 2$ subunit was located only in a subset of synapses on the somata and dendrites, but in most synapses on axon initial segments innervated by axo-axonic cells. The results demonstrate that molecular specialization in the composition of postsynaptic GABA_A receptor subunits parallels GABAergic cell specialization in targeting synapses to a specific domain of postsynaptic cortical neurons.

Inhibition in hippocampal pyramidal cells is mediated by distinct sets of GABAergic local-circuit neurons, which govern several integrative properties such as the threshold of activation (1–4), the pattern of action potential firing (5–7), and the modification of the synaptic strength (8). Different types of GABAergic interneurons innervate separate domains of pyramidal cells and at least three types have been shown to act through γ -aminobutyric acid type A (GABA_A) receptors (4, 9–14). Pentameric GABA_A receptors require only three to four different subunits to display the functional properties of native channels. However, hippocampal pyramidal cells express 10 subunits of the GABA_A receptor (15–18), which may be coassembled into GABA_A receptor channels differing in their pharmacological and kinetic properties depending on subunit composition (19–22). The multiple sources of GABA-releasing terminals on the one hand and the potential of assembling a variety of functionally distinct GABA_A receptors on the other raise the possibility that the segregation of inputs coincides with a specialization in postsynaptic receptors resulting from selective targeting of particular receptor subtypes to certain synapses. To test this hypothesis, a postembedding immunogold method was applied on Lowicryl-embedded ultrathin sections to compare the cell surface distribution of the $\alpha 1$ and $\alpha 2$ subunits, which endow GABA_A receptors with distinct pharmacological properties.

MATERIALS AND METHODS

Preparation of Animals and Tissues. Five adult Wistar rats were anaesthetized with Sagatal (pentobarbitone sodium, 220 mg/kg i.p.) and perfused through the heart with saline followed by fixative containing 4% paraformaldehyde, 0.05–0.1% glutaraldehyde, and $\approx 0.2\%$ picric acid for 13–25 min. After perfusion the brains were removed, and blocks from the dorsal

hippocampi were cut out and washed in several changes of 0.1 M phosphate buffer.

Antibodies. Rabbit polyclonal antiserum (code no. P16) was raised to a synthetic peptide corresponding to residues 1–9 of the rat $\alpha 1$ subunit. Antibody specificity has been described (23). Immunoreactions with affinity-purified P16 antibody were carried out at a final protein concentration of 1.4–7 $\mu\text{g/ml}$.

Guinea pig polyclonal antiserum was raised against a synthetic peptide corresponding to residues 1–9 of the $\alpha 2$ subunit of the GABA_A receptor (24). Immunoblotting was carried out as described (25) using SDS/10% PAGE. The affinity-purified antibody recognizes a single protein with a M_r of 51 kDa in immunoblots of rat hippocampal membranes (result not shown). Affinity-purified antibody was used for immunocytochemistry at a final protein concentration of 15–30 $\mu\text{g/ml}$.

Controls. Selective labeling, resembling that obtained with the specific antibodies, could not be detected when the primary antibodies were either omitted or replaced by 5% normal rabbit serum. Using polyclonal antibodies to synapsin (26) no plasma membrane labeling was observed with our methods, demonstrating that the labeling observed on the plasma membrane is due to the antibodies prepared to peptide sequences present in receptor subunits.

Freeze Substitution and Low Temperature Embedding in Lowicryl Resin. The same procedure was used as described in refs. 27 and 28. Briefly, after perfusion, blocks of tissue were washed in 0.1 M phosphate buffer (PB) followed by vibratome sectioning (500- μm thickness) and washing in 0.1 M PB overnight. The sections were placed into 1 M sucrose solution in 0.1 M PB for 2 hr for cryoprotection before slamming, freeze-substitution, and embedding in Lowicryl HM 20 or in Unicryl resin (Chemische Werke Lowi, Waldkraiburg, Germany).

Postembedding Immunocytochemistry on Ultrathin Sections. Postembedding immunocytochemistry was carried out on 70-nm thick sections of slam-frozen, freeze-substituted, Lowicryl-embedded hippocampi from five rats (28). The sections were picked up on pioloform coated nickel grids. They were then incubated on drops of blocking solution for 30 min, followed by incubation on drops of primary antibodies overnight or for 3 days. The blocking solution, which was also used for diluting the primary and secondary antibodies, consisted of 0.1 M PB saline (pH 7.4), containing 0.8% ovalbumin (Sigma), 0.1% cold water fish skin gelatine (Sigma), and 5% fetal calf serum. After incubation in primary antibody, sections were washed and incubated on drops of goat anti-rabbit or goat anti-guinea pig IgG coupled to 1.4 nm gold (diluted 1:100, Nanoprobes, Stony Brook, NY) for 2 hr at room temperature. Following several washes, sections were fixed in a 2% glutaraldehyde solution for 2 min and then transferred to drops of

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Abbreviations: AIS, axon initial segment; GABA, γ -aminobutyric acid.

ultrapure water prior to silver enhancement in the dark with HQ Silver (Nanoprobes) kit for 4–5 min. After further washing in ultrapure water, the sections were contrasted with saturated aqueous uranyl acetate followed by lead citrate.

Measurements of GABA_A Receptor Immunoreactivity. Measurements were taken from a well-preserved strip of Lowicryl-embedded ultrathin sections immunoreacted for the GABA_A receptor subunits. One block from each of five rats were used. Measurements were taken from three blocks for the $\alpha 1$ subunit and from four blocks for the $\alpha 2$ subunit, two blocks being measured for both subunits. Somata in continuity with proximal dendrites were selected in the ultrathin sections and every second cell was analyzed. Every symmetrical synaptic junction on these somata/proximal dendrites was photographed, providing the sample for our quantitative analyses. For axon initial segments (AISs), every synaptic junction was photographed and analyzed. Immunoparticles were counted within the anatomically defined synaptic junctions. An arbitrary criterion of at least two immunoparticles was used for accepting a synapse as immunopositive. This probably leads to an underestimation of synapses that contain a given subunit, but the stochastic nature of particle labeling does not make it possible to interpret a single particle on a given synapse.

For calculating the postsynaptic element distribution of immunopositive synapses for the $\alpha 2$ subunit, well-preserved strips (≈ 50 – $80 \mu\text{m}$) of the ultrathin sections from two blocks were systematically searched from the alveus to the hippocampal fissure. Every symmetrical synapse containing more than one immunoparticle was photographed, and the postsynaptic elements were categorized as being AIS, soma, proximal or distal dendrites, or unidentified profiles. The later group was not included in the calculation of postsynaptic element distribution.

RESULTS

Immunopositive synapses (at least two gold particles per synapse; see *Materials and Methods*) for the $\alpha 1$ subunit were found on somata (Fig. 1A), proximal (Fig. 1B) and distal dendrites, spines (Fig. 1C), and AISs (see Fig. 4C) of pyramidal cells, as well as on some GABAergic interneurons (not shown). The presence of the $\alpha 1$ subunit in somatic and AIS synapses predicts that GABAergic basket and axo-axonic cells (which have been shown to innervate these domains of pyramidal cells) activate GABA_A receptor channels containing this subunit. The presence of immunopositive synapses on dendrites in strata oriens, radiatum, and lacunosum moleculare shows that several types of dendrite targeting GABAergic interneuron (4, 11–14, 29) also exert their influence through $\alpha 1$ subunit-containing receptors. Quantitative analysis of immunoreactivity revealed that approximately 65 and 55% of symmetrical synapses on somata/proximal dendrites and AISs, respectively, are immunopositive for the $\alpha 1$ subunit (Fig. 3A). The absence of immunolabeling in some synapses may be due to a lower level, or the genuine lack of the $\alpha 1$ subunit in some of these synapses.

In contrast to the $\alpha 1$ subunit, the proportion of synapses immunoreactive for the $\alpha 2$ subunit was highest on AISs (Fig. 2A and B). Only a fraction of symmetrical synapses on somata, proximal, and distal dendrites showed immunoreactivity for the $\alpha 2$ subunit (Fig. 2C). Immunopositive synapses were also present on the somato-dendritic domain of a subset of GABAergic interneurons in all layers (not shown). Quantitative evaluation revealed that only 17% of the symmetrical synapses on somata/proximal dendrites were immunopositive for the $\alpha 2$ subunit (Fig. 3B), whereas 81% of synapses on AISs showed immunoreactivity (Fig. 3B). The high proportion of synapses labeled for the $\alpha 2$ subunit on AISs is unlikely to reflect an overall higher synaptic GABA_A receptor density because the number of immunoparticles for the $\alpha 1$ subunit in

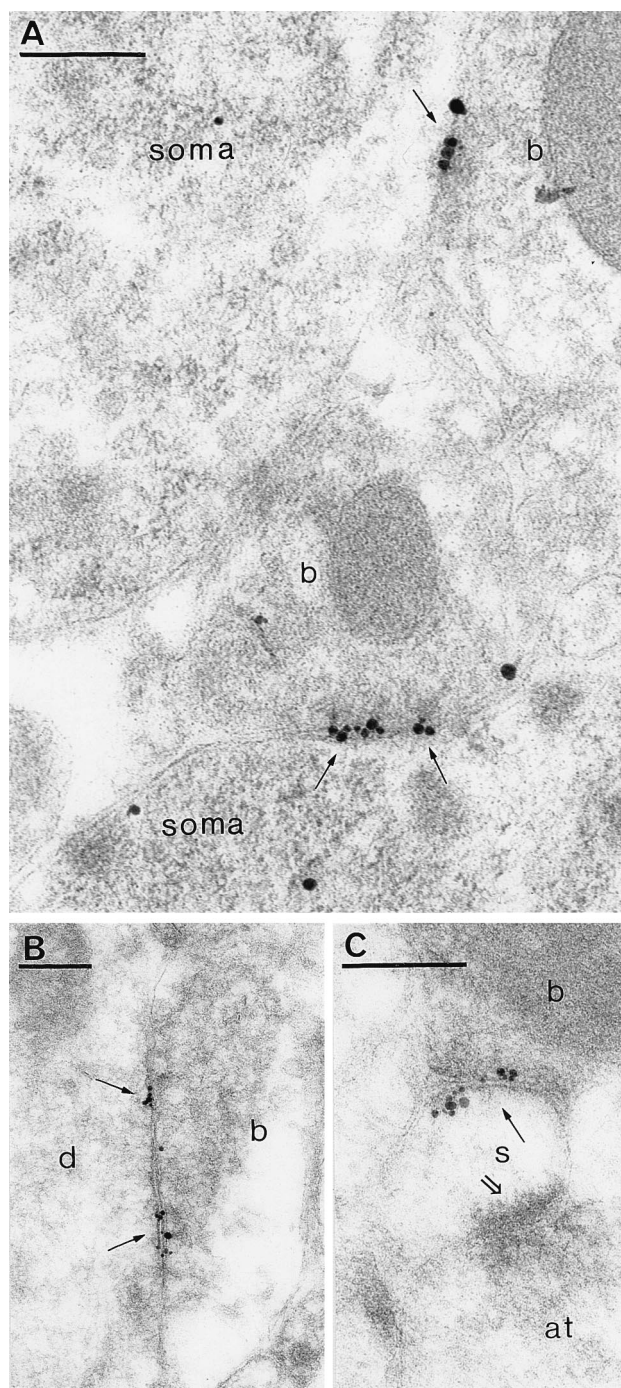


FIG. 1. GABA_A receptors in synaptic junctions on somata (A), a dendrite (B), and a dendritic spine (C) as shown in electron micrographs using a postembedding, silver-intensified immunogold technique on Lowicryl-embedded tissue and an antibody to the $\alpha 1$ subunit. (A) Two axon terminals (b), probably originating from basket cells, establish immunopositive synapses (arrows) with somata of pyramidal cells. (B) A high density of immunoparticles (arrows) is present in a symmetrical synapse made by an axon terminal (b) with a proximal dendrite (d) of a pyramidal cell in the stratum radiatum. (C) A high density of immunoparticles can be seen in a synapse (solid arrow) made by a bouton (b) with a pyramidal cell spine (s). The same spine also receives an immunonegative asymmetrical synapse (open arrow) from an axon terminal (at). Note that immunoparticles can be present in more than one cluster within the synaptic junction. (Scale = $0.2 \mu\text{m}$.)

immunopositive synapses was not significantly different between the somata/dendrites [4.1 ± 1.7 particles per synapse, mean (SD)] and AISs [3.5 ± 1.0 particles per synapse]. Similarly, the number of particles in synapses immunopositive

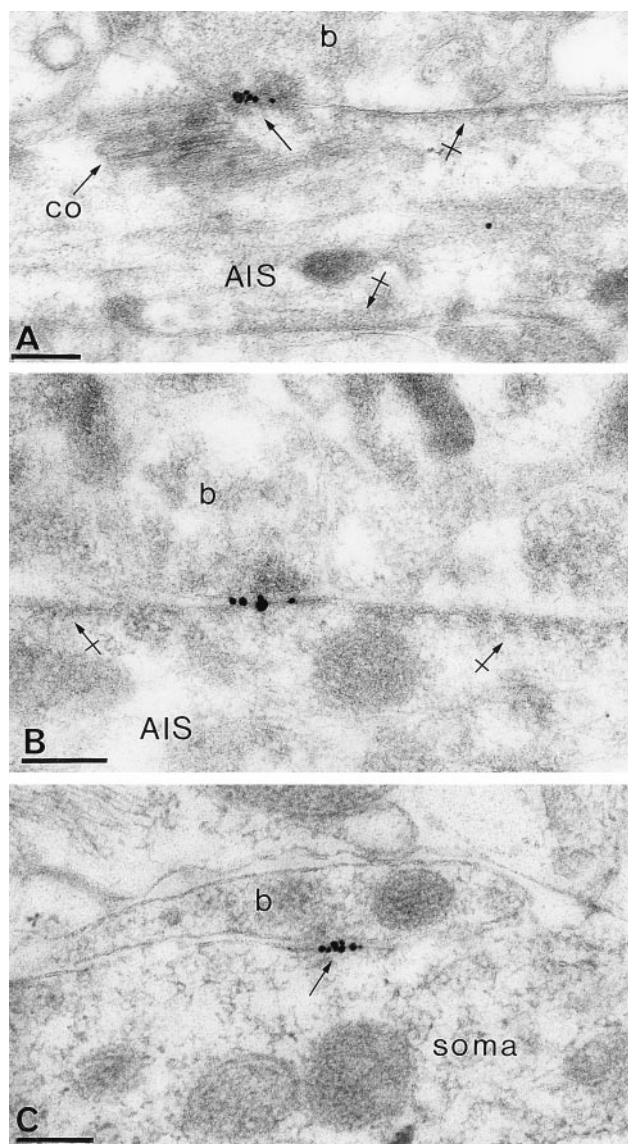


FIG. 2. Immunoreactivity for the $\alpha 2$ subunit of the GABA_A receptor. Postembedding reactions on Lowicryl-embedded ultrathin sections. (A and B) Axon terminals (b) establish immunopositive synaptic junctions (arrows) with AISs, which can be recognized by their fine structural characteristics (e.g., membrane undercoating, crossed arrows; cisternal organelle, co). (C) A rare immunopositive synapse (arrow) is shown on a pyramidal cell soma. The density of immunoparticles at individual synapses on the soma and on the AIS is similar. (Scale = 0.2 μm .)

for the $\alpha 2$ subunit on the somata/dendrites [3.5 ± 2.1 particles per synapse] and AISs [4.3 ± 1.8 particles per synapse] was not significantly different, indicating that immunonegative synapses on somata/dendrites formed a separate population. The distribution of synapses on the AIS was normal with regard to their $\alpha 2$ subunit immunoreactivity (χ^2 test: $\chi^2 = 7.03$, $P = 0.134$, $n = 52$); immunonegative synapses on AISs do not appear to form a separate population, but rather represent the left tail of a single population. The AIS is exclusively innervated by axo-axonic cells, whereas the somato-dendritic domain of pyramidal cells is innervated by several distinct types of GABAergic interneuron. Accordingly, the presynaptic elements of the $\alpha 2$ subunit immunopositive synapses on AISs can be identified as axo-axonic cell terminals. However, the origin of GABAergic terminals, making $\alpha 2$ subunit immunopositive synapses on somata, proximal, and distal dendrites cannot be identified at present, because several types of interneuron innervate these domains.

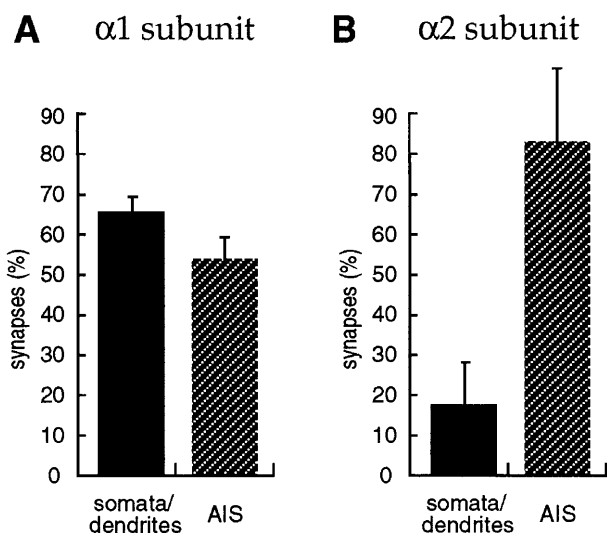


FIG. 3. Quantitative distribution of synapses immunopositive for the $\alpha 1$ (A) and the $\alpha 2$ (B) subunits of the GABA_A receptor on somata/proximal dendrites (solid column) or on axon initial segments (AIS; striped column) of pyramidal cells. (A) Of the symmetrical synapses, $65.5 \pm 4.2\%$ [mean of two animals, SD, between animals] on somata/proximal dendrites ($n = 27$) and $53.5 \pm 5.0\%$ on AISs ($n = 15$ in two animals) are immunopositive for the $\alpha 1$ subunit. (B) The majority [$81.4 \pm 18.2\%$] of the synapses on AIS ($n = 52$ in three animals) are immunopositive for the $\alpha 2$ subunit, whereas only $17.1 \pm 10.7\%$ of the symmetrical synapses ($n = 44$ in three animals) are immunopositive on somata/proximal dendrites.

We also collected a sample of all $\alpha 2$ subunit immunopositive synapses ($n = 45$ in two rats) in a strip through all layers to identify their postsynaptic elements. The results show that the targets are dendritic shafts (68.9%), AISs (24.4%), and somata (6.7%); therefore, the frequency of synapses immunopositive for the $\alpha 2$ subunit on somata/dendrites is only three times higher than on AISs. In the neocortex (30) and the dentate gyrus (31) only 0.1% and 4.8%, of GABAergic terminals innervate AISs; and a similar low proportion (1.4%) has been found in the CA1 area, where the rest of the terminals innervate somata (57.2%), dendrites (40%), and spines (1.4%) (K. Halasy, G. Tamas, and G. Nyiri, personal communication). Thus, the frequency of GABAergic synapses on somata/dendrites is approximately 70 times higher than that on AISs. Accordingly, the proportion of AIS as a target of $\alpha 2$ subunit immunopositive synapses is much higher than expected from the proportion of AIS among the targets of all GABAergic terminals.

The absence of immunolabeling in the majority of symmetrical synapses on the somato-dendritic domain could be due either to the lack (or an undetectable density) of the $\alpha 2$ subunit, or to an uneven preservation of GABA_A receptors in the area studied. To exclude the last possibility, 44 synapses on somata/proximal dendrites were tested for the presence or absence of both the $\alpha 1$ and the $\alpha 2$ subunits. Similar to that obtained in two animals (Fig. 3A), in this additional rat 77.3% of the symmetrical synapses are immunopositive for the $\alpha 1$ subunit, but only 4.5% of the same synapses show immunoreactivity for the $\alpha 2$ subunit (Fig. 4A), confirming the results of the previous experiments on the subunit composition of somatic synapses. This sample provided a somewhat lower proportion of $\alpha 2$ subunit immunopositive synapses on somata/dendrites (compare with Fig. 3B), but it was not due to a poor $\alpha 2$ reaction, because synapses on AISs have been strongly labeled (as shown in Fig. 4D). This result indicates that GABA_A receptor channels are well preserved at synapses on somata, but the $\alpha 2$ subunit is present at detectable density only in a small proportion of synapses. To determine whether the

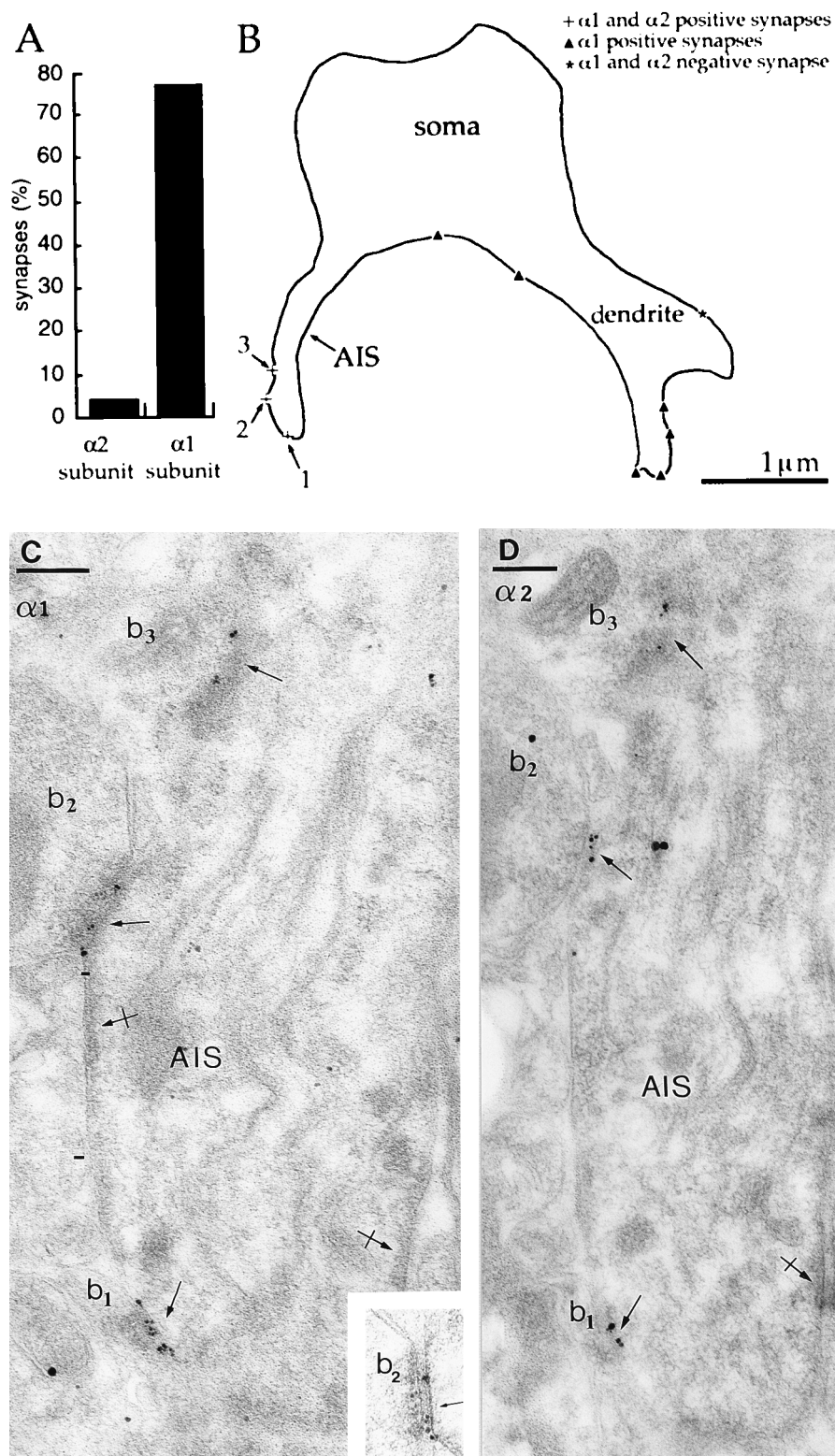


FIG. 4. (A) Receptor immunoreactivity of synapses tested for both the $\alpha 1$ and the $\alpha 2$ subunits on somata/proximal dendrites. Serial ultrathin sections of each synapse ($n = 44$) were reacted for the $\alpha 1$ or $\alpha 2$ subunits. The majority (77.3%) of the symmetrical synapses are immunopositive for the $\alpha 1$ subunit, but only 4.5% of these synapses show immunoreactivity for the $\alpha 2$ subunit. (B) GABA_A receptor subunit distribution on an individual pyramidal cell. The AIS and one of the basal dendrites are emerging from the soma at a level where serial ultrathin sections were immunoreacted for the $\alpha 1$ and $\alpha 2$ subunits. Three synapses on the AIS (+, shown in C and D) are immunopositive for both α subunits. Six of seven symmetrical synapses on soma/basal dendrite are immunopositive for the $\alpha 1$ subunit alone (▲) and only one synapse is immunonegative for both α subunits (asterisk). (C and D) Electron microscopic demonstration on serial sections of the colocalization of the $\alpha 1$ and $\alpha 2$ subunits at synapses on the AIS shown in B. Three boutons (b1, b2, b3) establish immunopositive synaptic junctions (arrows) for both the $\alpha 1$ (C) and $\alpha 2$ (D) subunits with an AIS. Crossed arrows point to the membrane undercoating of the plasma membrane, identifying the process as an AIS. Synapses made by b1 and b3 are cut tangentially, therefore, the synaptic cleft is not visible. However, when tilted in the electron microscope, for example of the synaptic junction made by b2 (C Inset), it is apparent that immunoparticles are present in the synaptic cleft. (Scale = 0.2 μm .)

dominant targeting of the $\alpha 2$ subunit to the AIS is present on a representative *individual* pyramidal cell that expresses the $\alpha 1$ subunit in most of its synapses, seven synapses were tested on the soma and a proximal dendrite and three on the AIS of a pyramidal cell (Fig. 4B). All three synapses on the AIS were immunopositive for both α subunits (Fig. 4C and D), but six of the seven synapses on the soma and basal dendrite were immunopositive for the $\alpha 1$ subunit only (Fig. 4B), demonstrating that these α subunits are differentially targeted on the surface of a single neuron that expresses both subunits.

DISCUSSION

We have demonstrated that the $\alpha 1$ and $\alpha 2$ subunits of the GABA_A receptor are differentially targeted on the surface of hippocampal pyramidal cells. The $\alpha 2$ subunit is only present at a detectable level in a small subset of synapses on somata and dendrites and in most synapses on AISs, whereas the $\alpha 1$ subunit is uniformly distributed in synapses over the axo-somato-dendritic domains.

A neurotransmitter receptor may be targeted selectively to a functionally distinct part of a nerve cell by a postsynaptic mechanism, which differentiates amongst the axon, soma, and dendritic domains. Another possibility is that the clues for the synaptic clustering of a receptor at certain sites are provided by the presynaptic neuron. The latter possibility is supported by the high proportion of $\alpha 2$ subunit positive synapses on the AIS, which is selectively innervated by axo-axonic cells. This distribution raises the possibility that axon terminals making synapses immunopositive for the $\alpha 2$ subunit on somata/dendrites of pyramidal cells also originate from a subset of GABAergic interneurons. Several GABAergic cell types that selectively innervate the somato-dendritic domain of pyramidal cells have been discovered in the CA1 area. For example, it has been shown, that the "trilaminar cell" innervates rather evenly each layer (14). Primarily dendrite targeting cells, like the "bistratified cell," innervate strata radiatum and oriens and act through GABA_A receptors (4). There are further cell types with cell bodies in the alveus/oriens (13) or stratum lacunosum-moleculare (11), which innervate the distal dendrites of pyramidal cells. Furthermore, GABAergic basket cells form at least two populations according to their parvalbumin or cholecystokinin content (32-34). Thus, there is a large variety of GABAergic interneurons, some of which could be responsible for the synaptic clustering of the $\alpha 2$ subunit on the somata/dendrites of pyramidal cells.

Principal cells throughout the cortical mantle are innervated by distinct populations of GABAergic local circuit neuron on their AISs and somata/proximal dendrites. Both axo-axonic cells innervating the AIS and basket cells innervating the soma exert their synaptic action exclusively through GABA_A receptors (4, 35). Here we have shown that the $\alpha 2$ subunit is associated with axo-axonic cell synapses to a much greater extent than with somatic synapses. Other biochemical differences between the somatic and initial segment synapses include the presence of a specialized calcium-storing organelle at the initial segment synapses (36) and the preferential localization of gephyrin-like immunoreactivity in synapses on AISs (D. Lozsadi, M. F. Kritzer, H. Betz and P.S., unpublished observations), with an antibody (code no. 5a) to this glycine receptor associated membrane protein (37, 38).

The $\alpha 2$ subunit, when coexpressed with the $\beta 1$ and $\gamma 2$ subunits, results in receptors with type II benzodiazepine receptor properties which, in contrast to $\alpha 1$ subunit containing receptors, show low affinity for 2-oxoquazepam, zolpidem, the triazolopyridazine CL218872, and β -carbolines (19-22, 39). Therefore, because of the subunit composition of the synapses, both exogenous drugs and possible endogenous modulators of the benzodiazepine site are likely to affect the action of basket and axo-axonic cells differently. The difference in the roles of

these two GABAergic neurons in the network is not known, but both may be involved in the precise timing of principal cell discharge, because they can either suppress or induce action potentials in pyramidal cells in interaction with intrinsic membrane conductances (7). Basket cells discharge rhythmically in phase with the theta rhythm of hippocampal activity in anaesthetized animals (14, 40) and are particularly effective in synchronizing the sub- and suprathreshold activity of large populations of pyramidal cells (7). They also discharge at high frequency during γ oscillations and may be contributing to the timing of action potentials at these higher frequencies (41). However, since the AIS is the site of the generation of action potentials (42), it is quite possible that axo-axonic cells innervating this region of the postsynaptic cell have evolved to govern the timing of action potentials when the demand for precision is increased, i.e., at higher frequencies of discharge. The molecular difference revealed here between basket and axo-axonic cell synapses is probably an adaptation to their distinct roles and will help to establish the function of axo-axonic cells, provided drugs acting preferentially on $\alpha 2$ subunit-containing channels can be developed.

In addition to the present example of differential targeting of two α subunits, the $\alpha 1$ and $\alpha 6$ subunits of the GABA_A receptor have also been shown to be differentially targeted on the surface of cerebellar granule cells (43). Similar conclusions have also been obtained in identified retinal α ganglion cells. Koulen *et al.* (44) have shown that the $\alpha 1$, $\alpha 2$, and $\alpha 3$ subunits are differentially distributed on the surface of individual cells. The molecular determinants of selective subunit assembly and surface expression of the GABA_A receptors are unknown. However, in epithelial cells transfected to express $\alpha 1$ and/or $\beta 1$ subunits, it could be shown that the $\beta 1$ subunit can influence the routing of the $\alpha 1$ subunit to the plasma membrane (45). It is therefore possible, that the $\alpha 2$ subunit assembles with β and/or γ subunits different from those in $\alpha 1$ subunit containing channels, producing GABA_A receptors with distinct subunit composition, which can be differentially targeted on the surface of nerve cells.

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