European Journal of Neuroscience, Vol. 13, pp. 428–442, 2001

Input-dependent synaptic targeting of α_2-subunit-containing $GABA_A$ receptors in synapses of hippocampal pyramidal cells of the rat

Gábor Nyíri,[1,2] Tamás F. Freund[1] and Péter Somogyi[1,2]
[1]Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, PO Box 67, H-1450 Hungary; [2]Medical Research Council, Anatomical Neuropharmacology Unit, Oxford University, Mansfield Road, Oxford OX1 3TH, UK

Keywords: basket cell, benzodiazepine, GABA, inhibition, neurotransmission, Zolpidem

Abstract

Pyramidal cells, expressing at least 14 subunits of the heteropentameric $GABA_A$ receptor, receive GABAergic input on their soma and proximal dendrites from basket cells, activating $GABA_A$ receptors and containing either parvalbumin or cholecystokinin and vasoactive intestinal polypeptide. The properties of $GABA_A$ receptors are determined by the subunit composition, and synaptic receptor content governs the effect of the presynaptic neuron. Using a quantitative electron microscopic immunogold technique, we tested whether the synapses formed by the two types of basket cell show a difference in the subunit composition of $GABA_A$ receptors. Terminals of one of the basket cells were identified by antibodies to parvalbumin. Synapses made by parvalbumin-negative terminals showed five times more immunoreactivity for the α_2 subunit than synapses made by parvalbumin-positive basket cells, whose synapses were frequently immunonegative. This difference is likely to be due to specific $GABA_A$ receptor α subunit composition, because neither synaptic size nor immunoreactivity for the $\beta_{2/3}$ subunits, indicating total receptor content, was different in these two synapse populations. Synapses established by axo-axonic cells on axon initial segments showed an intermediate number of immunoparticles for the α_2 subunit compared to those made by basket cells but, due to their smaller size, the density of the α_2 subunit immunoreactivity was higher in synapses on the axon. Because the two basket cell types innervate the same domain of the pyramidal cell, the results indicate that pyramidal cells have mechanisms to target $GABA_A$ receptors, under presynaptic influence, preferentially to distinct synapses. The two basket cell types act via partially distinct $GABA_A$ receptor populations.

Introduction

Hippocampal pyramidal cells receive GABAergic innervation from several distinct populations of interneurons, which differ in the expression of calcium binding proteins, neuropeptides and ion channels, as well as in their input (Freund & Buzsaki, 1996). However, the most striking distinguishing feature of interneurons is their selective synaptic targeting of distinct domains of the postsynaptic cells (Ramon y Cajal, 1893; Somogyi *et al*., 1983, 1998; Freund & Buzsaki, 1996). Axo-axonic cells innervate only the axon initial segment (AIS), basket cells innervate the soma and the proximal dendrites, and other interneurons innervate only the dendrites, often coaligned with particular glutamatergic inputs. The same postsynaptic part of the pyramidal cell may be targeted by more than one class of interneuron. For example, the somata of pyramidal cells is innervated by basket cells expressing either parvalbumin (PV) or cholecystokinin and vasoactive intestinal polypeptide (CCK/VIP) (see Freund & Buzsaki, 1996). These two neurochemically distinct types of basket cell differ in soma position and local and subcortical innervation (Gulyas *et al*., 1996; Papp *et al*., 1999) as well as in the presynaptic control of transmitter release (Hajos *et al*., 1998; Katona *et al*., 1999), predicting distinct roles in the hippocampal network.

Correspondence: Dr Gábor Nyíri, as [1]above.
E-mail: nyiri@koki.hu

Received 10 August 2000, revised 14 November 2000, accepted 20 November 2000

So far, all unitary connections from identified interneurons evoked γ-aminobutyric acid type A ($GABA_A$) receptor-mediated responses. $GABA_A$ receptors are heteropentamers of $\alpha_{1–6}$, $\beta_{1–3}$, $\gamma_{1–3}$, δ, ε, π, $\rho_{1–3}$ and θ subunits in mammals (see Sieghart, 1995; Stephenson, 1995; McKernan & Whiting, 1996; Barnard *et al*., 1998; Bonnert *et al*., 1999). Most receptors are thought to be assembled from 3–4 different subunits, usually two α and two β subunits in combination with a γ subunit; but the subunit composition of these receptors is highly variable in different brain regions and the same channel may include more than one α or β subunit species (refs above).

Paralleling the diversity of the presynaptic source of GABA, pyramidal cells express at least 14 subunits ($\alpha_{1–5}$, $\beta_{1–3}$, $\gamma_{1–3}$, δ, $\rho_{1,2}$, Wisden *et al*., 1992; Fritschy & Mohler, 1995; Sperk *et al*., 1997; Ogurusu *et al*., 1999). The precise subunit combination of native receptors on the surface of pyramidal cells is not known, and the location of only the α_1, α_2, $\beta_{2/3}$ and γ2 subunits has been studied at the synaptic level (Nusser *et al*., 1996a; Somogyi *et al*., 1996).

The subunit combination of the channel confers specific kinetic and pharmacological profile to the receptor, which has important consequences for synaptic function as well as for the behavioural and therapeutic action of drugs. For instance, the α_2-subunit-containing receptors showed a 10-fold higher affinity for GABA than did α_1-subunit-containing receptors in Xenopus oocytes (Levitan *et al*., 1988). Receptors containing α_1 subunits exhibit 4–20-fold higher affinity (type 1 benzodiazepine receptors) for the ligands 2-

oxoquazepam, Cl 218872 or the widely used hypnotic drug Zolpidem, compared to receptors containing α_2 or α_3 subunits (Pritchett *et al*., 1989; Pritchett & Seeburg, 1990). Furthermore, the α_2 subunit confers faster activation and slower deactivation rates on receptors than does the α_1 subunit (Lavoie *et al*., 1997). Because both the α_1 and the α_2 subunits are expressed at high levels in CA1 pyramidal cells, a potential differential participation of these subunits in domain-specific synapses would result in specific functional and pharmacological characteristics. Indeed, Thomson *et al*. (2000) demonstrated that Zolpidem enhanced the IPSPs of the fast spiking population of visualized basket cells significantly more than the IPSPs evoked by the regular spiking basket cells. Therefore, it is possible that the postsynaptic cell targets receptors of a specific subunit combination specifically to synapses receiving GABA from a particular type of interneuron.

High-resolution immunocytochemical localization of the α_1 and α_2 subunits (Nusser *et al*., 1996a; Fritschy *et al*., 1998a,b; Loup *et al*., 1998) showed that the α_2 subunit was more frequently found in synapses on the AIS innervated by the axo-axonic cell than in synapses on soma and proximal dendrites innervated by basket cells. Both basket and axo-axonic cell synapses were immunoreactive for the α_1 subunit. However, the relative lack of α_2 subunit in synapses on the somata may reflect a selective exclusion from the somatic membrane rather than a preferential targeting of the α_2 subunit to the AIS (Nusser *et al*., 1996a). In other words, the apparently selective distribution of the synaptic α_2-subunit-containing receptors may reflect targeting of receptors to specific cell domains rather than to specific inputs.

In the present study, we investigated whether preferential synaptic targeting of $GABA_A$ receptors takes place within the same postsynaptic domain of a cell. The synapses of two distinct converging basket cell populations were tested for their immunoreactivity for the α_2 and $\beta_{2/3}$ subunits of the $GABA_A$ receptor using immunogold labelling.

Materials and methods

Preparation of animals and tissues

Three adult male Wistar rats (≈ 150 g; animal codes: 2/59/99, 9/61/99, 2/61/99) obtained from Charles River, UK, were anaesthetized with Sagatal (pentobarbitone sodium, 220 mg/kg i.p.) and perfused through the heart with 0.9% NaCl followed by fixative containing 4% paraformaldehyde, 0.05% glutaraldehyde, and ≈0.2% picric acid in 0.1 M phosphate buffer (PB; pH = 7.4) for 20–25 min. After perfusion the brains were left *in situ* for 10–15 min; then they were removed from the skull. Blocks from the dorsal hippocampi were dissected and washed in 0.1 M PB, followed by sectioning on a vibratome at 500 μm thickness. They were postfixed for 15–20 min and washed in 0.1 M PB overnight.

Freeze substitution and low temperature embedding in Lowicryl resin

The same procedure was used as described earlier (Baude *et al*., 1993; Nusser *et al*., 1995). Briefly, after washing in PB overnight, the sections were placed into increasing concentrations of sucrose solutions (0.5, 1 and 2 M sucrose for 0.5, 1 and 2 h, respectively) for cryoprotection. After slamming onto copper blocks cooled in liquid N_2 and following low temperature dehydration and freeze-substitution, the sections were embedded in Lowicryl HM 20 resin (Chemische Werke Lowi, Waldkraiburg, Germany).

Antibodies

All antibodies used were affinity purified and have been characterized previously. A guinea pig polyclonal antiserum was raised against a synthetic peptide corresponding to residues 1–9 of the α_2 subunit of the $GABA_A$ receptor coupled to keyhole limpet haemocyanine through an additional C-terminal cysteine (Marksitzer *et al*., 1993). The antibody reacts with a single band corresponding to a M_r of 51–52 kDa in immunoblots of rat hippocampal membranes (Marksitzer *et al*., 1993; Benke *et al*., 1994). The antibody was used for immunocytochemistry at a final protein concentration of ≈65 μg/mL. The mouse monoclonal antibody bd-17 (Haring *et al*., 1985; Schoch *et al*., 1985) has been shown to react with both the β_2 and β_3 subunits of the $GABA_A$ receptor (Ewert *et al*., 1990). Therefore, the immunoreactivity detected by this antibody is referred to as representing the $\beta_{2/3}$ subunits. The antibody was diluted to 10–20 μg protein/mL. A rabbit polyclonal antiserum (Code No. R301, diluted 1 : 150 or 1 : 100) was raised to rat muscle PV (Calbiochem, Nottingham, UK), and was a gift from Dr K. G. Baimbridge. Immunostaining of the hippocampus was reported to be completely prevented by preincubating the antiserum, diluted 1 : 5000, with 10 ng/mL (Calbiochem) rat muscle PV (Sloviter, 1989). Further characterization of the antiserum, reported by Mithani *et al*. (1987), showed that in SDS-PAGE of rat brain and muscle soluble proteins the antiserum labels a single band, which corresponds to rat muscle PV. The antiserum was reported to have no cross-reactivity with other calcium binding proteins (Mithani *et al*., 1987).

Postembedding immunocytochemistry on ultrathin sections

Postembedding immunocytochemistry was carried out on 70-nm-thick serial sections of slam-frozen, freeze-substituted, Lowicryl-embedded hippocampi from three rats (Baude *et al*., 1993). The sections were picked up on pioloform-coated nickel grids. They were then incubated on drops of blocking solution for one hour, followed by incubation on drops of primary antibodies overnight. The blocking solution, which was also used for diluting the primary and secondary antibodies, consisted of 0.05 M Tris-HCl (pH = 7.4) containing 0.9% NaCl (tris-buffered saline, TBS) and 2% human serum albumin (Sigma-Aldrich, Poole, UK). After incubation overnight in a mixture of primary antibodies to $GABA_A$ receptor subunits and parvalbumin, sections were washed in TBS and incubated for 4 h on drops of a mixture of secondary antibodies (IgG) coupled to 10- or 5-nm gold particles (British BioCell Int., Cardiff, UK). Following several washes, sections were washed in ultra pure water, then contrasted with saturated aqueous uranyl acetate followed by lead citrate.

Measurement of immunoreactivity

Measurements were taken from well-preserved Lowicryl-embedded long electron microscopic serial sections colabelled either for PV and the α_2 subunit, or for PV and the $\beta_{2/3}$ subunits. Parvalbumin primary antibodies were labelled with 5-nm gold particle-conjugated secondary antibodies on all serial sections to differentiate between PV-positive and -negative synaptic boutons on pyramidal cell somata (Fig. 1). $GABA_A$ receptor subunits were labelled with 10-nm gold particles. One block was used from each of the three rats. Following extensive testing and adjusting of reaction conditions, final measurements were carried out from serial sections from each animal reacted on different days. Sections from one animal were reacted on two different occasions and because no difference was observed the results were pooled. The reactions resulted in remarkably consistent results from reaction to reaction and from animal to animal (see Results). Type II

synapses (Gray, 1959) on pyramidal cell somata and axon initial segments were identified in the hippocampal CA1 region. Axon initial segments are easily differentiated from dendrites in Lowicryl-embedded and contrasted material, on the basis of the electron-dense membrane undercoating (Nusser *et al*., 1996a; Somogyi *et al*., 1996). Because PV-negative boutons making synapses on pyramidal somata are relatively infrequent (see below), in order to sample a sufficient number, such boutons were searched for selectively; therefore, somatic synapses were not sampled randomly. However, once a synapse of the required character with respect to parvalbumin immunoreactivity was encountered, it was included without any further selection. Each synaptic junction included in the study for measuring immunoreactivity per synapse was photographed in every section; thus all synapses were fully reconstructed for quantitative analysis. Immunoparticles for α_2 and $\beta_{2/3}$ subunits were counted within the anatomically defined synaptic junction. The synaptic areas were assessed by measuring the length of the synaptic specialization in every section of the series and multiplying the length by 70 nm, assumed to be the thickness of the sections based on the ultramicrotome setting which agreed with a chart of interference colour of the sections.

As controls for the specificity of the method, primary antibodies were either omitted or replaced by 5% normal rabbit, mouse and guinea pig sera. Selective labelling, resembling that obtained with the specific antibodies, could not be detected under these conditions. When a mixture of two antibodies raised in two different species was used in double immunogold labelling experiments, the two different sized gold particles were distributed to different subcellular locations, excluding the possibility that any of the secondary antibodies recognized both primary antibodies.

The concentrations of primary antibodies were chosen such that they resulted in low background labelling, as assessed over synaptic vesicle-containing profiles, which are not thought to contain $GABA_A$ receptors. The density of 10 nm particles used for labelling $GABA_A$ receptors was 0.30 particles/μm^2 over areas occupied by synaptic vesicles. This is assumed to represent background labelling. Theoretically, in the method used, the centre of a 10-nm particle can be to a maximum of $\approx$25 nm from the epitope, i.e. the outer leaflet of the cross-sectioned plasma membrane. However, in most cases the plasma membrane is cut at an angle different from 90°, which means that the surface epitopes available for the antibody can be >25 nm from the image of the plasma membrane, which is formed from the whole thickness of the section. Therefore, a band of two times 35 nm (two times 2 mm on the original prints) was chosen along the synaptic membrane specialization as an area representing synaptic $GABA_A$ receptor labelling. The average length of membrane specialization per somatic synapse, obtained by summing the lengths found in serial sections, was 1144 $\pm$ 304 µm. Therefore the average area on the surface of serial sections over which gold particles were counted was 0.080 μm^2/synapse. The contribution of background labelling, assumed to be represented by the density of particles over synaptic vesicle-containing areas, to the particle number over an average postsynaptic membrane specialization, was calculated as 0.02 gold particles/synapse. The weighted median number of particles per somatic synapse was 3.72, so the potential background contribution could be at most 0.53%. Accordingly, subtraction of background from synaptic immunogold numbers was not carried out and each particle, including labelling by one particle, was considered to represent immunolabelling.

Measurement of the ratio of synapses established by PV-positive or -negative boutons

The results showed that synapses made by PV-positive or -negative boutons on pyramidal cell somata are similar in size (see below); therefore, their frequency, as counted in single sections, was used to establish their ratio. Counts were taken from double-immunogold-labelled sections of two animals (2/59/99, 9/61/99) by scanning the specimens and recording all type II synapses found on somata.

Statistics

We used nonparametric statistics for the analysis of the distribution of gold particles and for the comparison of populations of synapses, because in many cases their distributions were not normal, as shown by the Kolmogorov–Smirnov test. Similarly, synaptic area and immunolabelling density distributions were not always normal; therefore, we also used nonparametric statistics. Some data are also given as mean $\pm$ SD. The Kruskal–Wallis and χ^2 tests were used for comparing data from three groups and, when necessary, were followed by *post hoc* comparisons using the Mann–Whitney test with Bonferroni correction. Two groups were compared using the Mann–Whitney test. Spearman rank correlation analysis was used to test for possible association between two variables. All statistical analyses were carried out using the software package Statistica™ (StatSoft, Tulsa, OK, USA).

Results

In the postembedding immunoreaction, electron microscopic sections were uniformly exposed to the antibody solutions on the whole cut surface of the section; therefore, different parts of the same cell or different cells may be compared for the degree of labelling. In the present study, we used mixtures of antibodies raised in different species for dual immunogold labelling for PV (5-nm particles) and either the α_2 subunit or the $\beta_{2/3}$ subunits of the $GABA_A$ receptor (10-nm particles) in long consecutive series of sections (Fig. 1).

Immunolabelling of nerve terminals for parvalbumin

Boutons of the pyramidal layer were either densely covered by 5-nm gold particles or had no, or only occasional, particles over them, similar to the somata of pyramidal cells, which do not express parvalbumin (Celio, 1986; Kosaka *et al*., 1987; Sloviter, 1989). Therefore, the boutons could be unequivocally classified as immunopositive or immunonegative (Fig. 1), probably corresponding to basket cells expressing parvalbumin, or CCK and VIP, respectively (for review see Freund & Buzsaki, 1996). Axo-axonic cells innervating the axon initial segment also express parvalbumin (Kosaka *et al*., 1987) and most synaptic terminals in the present study were parvalbumin-immunopositive.

For the quantitative interpretation of synaptic receptor labelling, first we estimated the ratio of the synapses formed by PV-positive or PV-negative boutons on the somata of pyramidal cells in two rats, by systematically searching and identifying each terminal forming a type II synapse. In the first animal 19 (70%) of 27 synaptic boutons, and in the second 15 (65%) of 23 synaptic boutons, were PV-positive. Therefore, on average $\approx$68% of the synapses were established by PV-positive and $\approx$32% by PV-negative boutons on pyramidal cell somata.

Immunolabelling of synapses for the subunits of the $GABA_A$ receptor

Synapses on the somata of pyramidal cells have been shown to contain α_1, α_2, $\beta_{2/3}$ and γ_2 subunits by electron microscopic

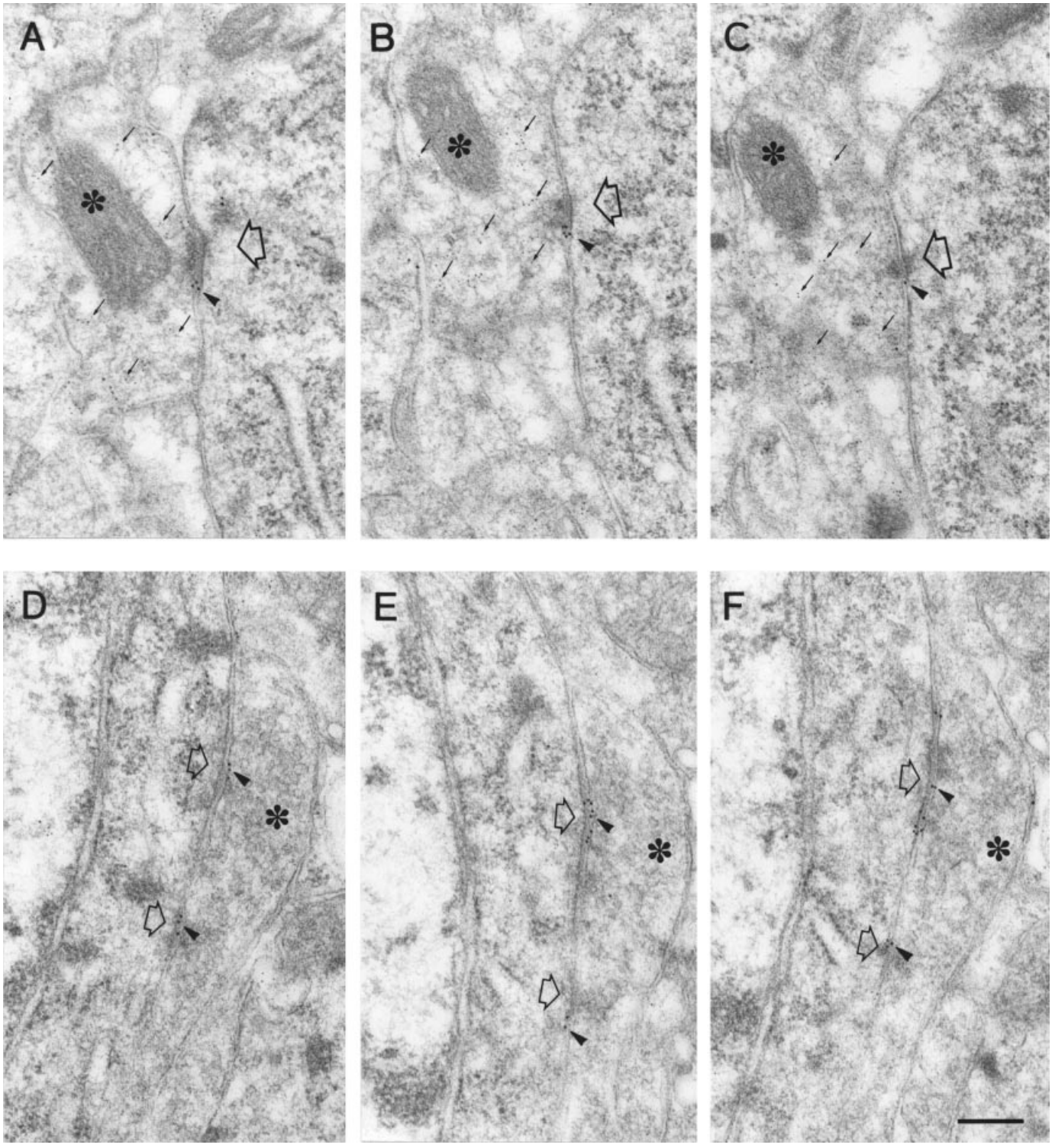

FIG. 1. Comparison of immunolabelling for the $\beta_{2/3}$ subunits of the $GABA_A$ receptor in synapses (open arrows) located on pyramidal cell bodies. Electron micrographs show three serial sections each of two synapses made by (A–C) a PV-positive or (D–F) a PV-negative bouton (asterisks). Large (10-nm) gold particles (e.g. arrowheads) correspond to immunoreactivity for the $\beta_{2/3}$ subunits; small (5-nm) gold particles (small arrows in A–C) indicate PV immunoreactivity. The PV-negative bouton in D–F establishes two $\beta_{2/3}$-subunit-immunoreactive active zones. Scale bar, 0.2 μm (A–F).

immunogold labelling (Nusser *et al*., 1996a; Somogyi *et al*., 1996). In the study of Nusser *et al*. (1996a), immunoreactivity of synapses was assessed from single sections using a silver-intensified immunogold reaction, which results in a higher number of particles per synapse than the method using 10-nm particles without silver intensification. However, it is more difficult to count silver intensified particles because they are of heterogeneous size and the silver shells of closely situated particles may coalesce. The silver intensification may also result in clumps of particles whose origin is difficult to interpret. Therefore, in the present study 10-nm particles were used, and the

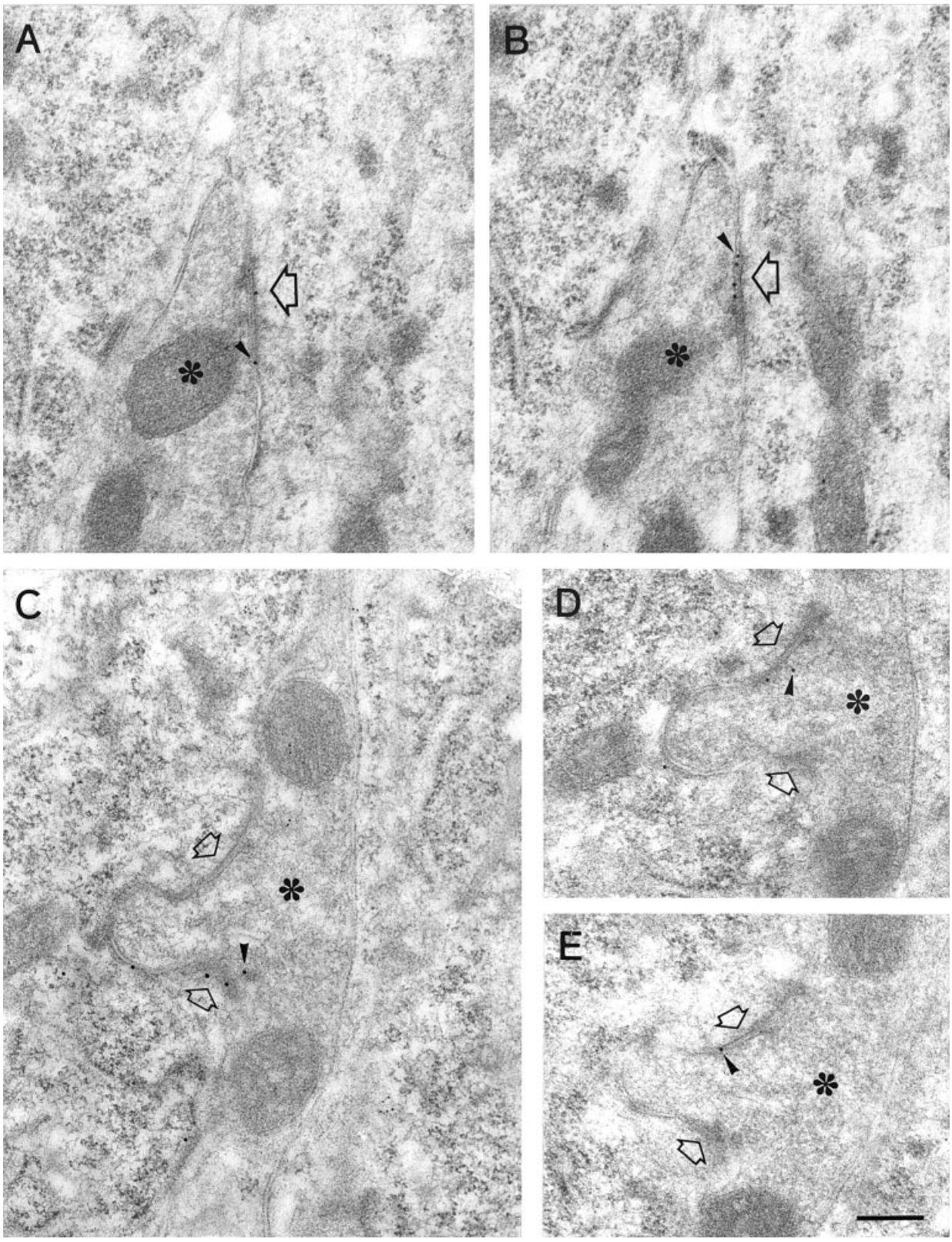

FIG. 2. (A–E) Immunolabelling for the α_2 subunit of the $GABA_A$ receptor in synapses (open arrows) on the soma of pyramidal cells. Electron micrographs of serial sections showing synapses formed by two PV-negative boutons (asterisks: bouton 1, A and B, and bouton 2, C–E). Large (10-nm) gold particles (arrowheads) correspond to α_2 subunit immunoreactivity. (C–E) Intrusions of boutons into the pyramidal cell soma were found only at synapses of PV-negative boutons, and in most of these cases two synaptic active zones were present. The lower active zone is cut tangentially. Although there are no PV-immunopositive structures in these fields, scattered 5-nm gold particles, due to background labelling, can be observed. Scale bar, 0.2 μm (A–E).

concentration of antibodies was chosen to result in a negligible background labelling. Due to the low background labelling (see Materials and methods), synaptic particle counts were not corrected for background. In order to sample each synapse thoroughly and to minimize the false negative cases, each synapse was completely serially sectioned. Immunoparticles for GABA$_A$ receptor subunits appeared over the image of the postsynaptic membrane, the synaptic cleft and the presynaptic membrane as well as above the pre- and postsynaptic cytoplasm. This presumably occurs because the centre of immunoparticles may be up to 25 nm from the epitope, and the synapses are cut at various angles, but the contribution of the presynaptic membrane to the labelling cannot be excluded with this

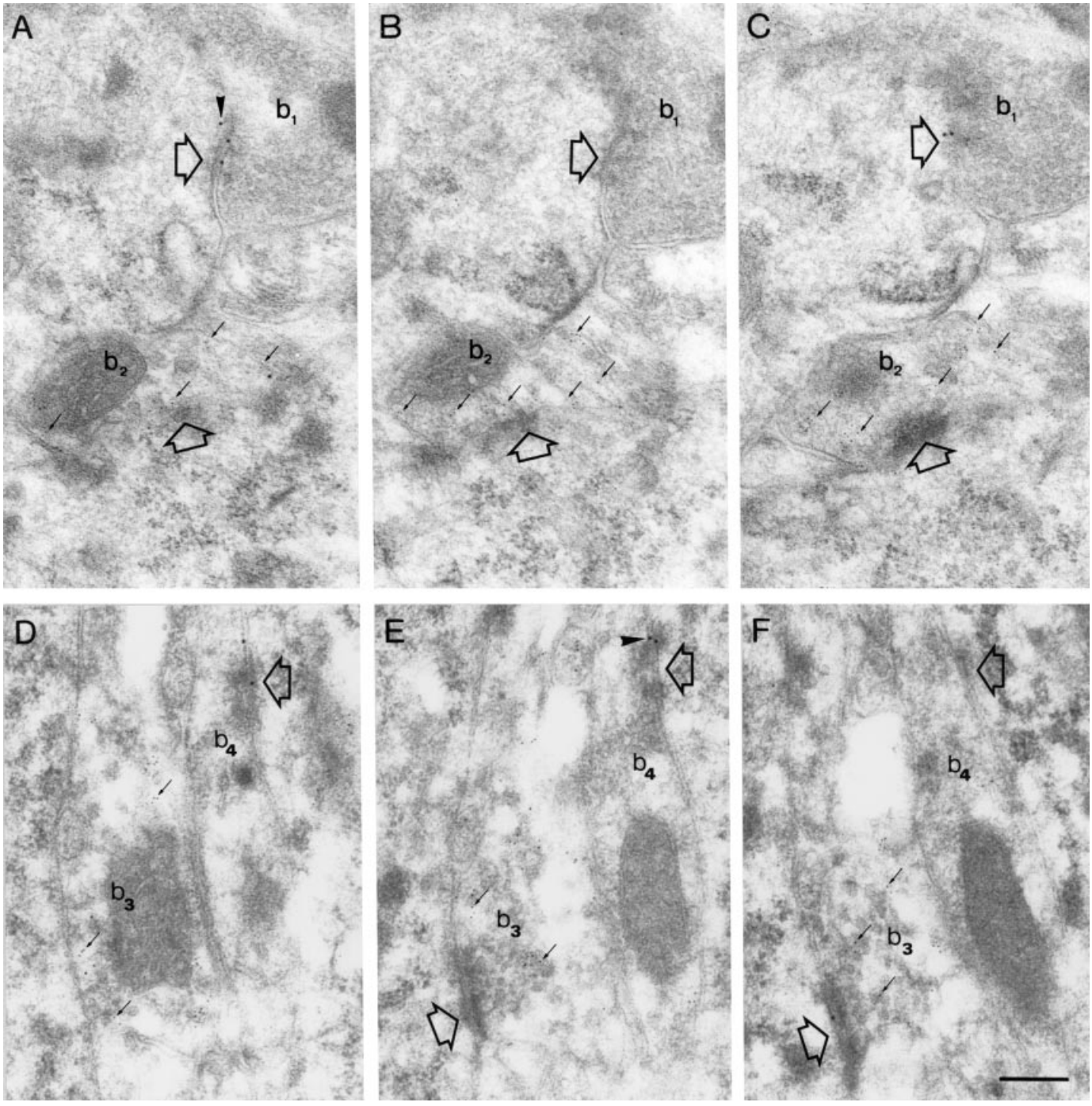

FIG. 3. Comparison of immunolabelling for the α_2 subunit of the GABA$_A$ receptor (10 nm gold particles, arrowheads) in adjacent synapses (open arrows) formed by PV-negative (b_1 and b_4) or PV-positive (b_2 and b_3) boutons on the somata of adjacent pyramidal cells. Electron micrographs show three serial sections of two areas (A–C and D–F), representing a part of the synapses. Of the two synapses established by PV-positive boutons, one is unlabelled for the α_2 subunit (b_2), and the other contains only one particle (b_3). In contrast, one PV-negative bouton (b_1) is labelled by five 10-nm gold particles (arrowheads) for the α_2 subunit, the other one (b_4) by four particles. Small arrows indicate some of the 5-nm gold particles demonstrating PV immunoreactivity. Some of the sections were cut tangentially to the synaptic active zone (e.g. synapse of b_2 in A–C, synapse of b_1 in B and C). Scale bar, 0.2 μm (A–F).

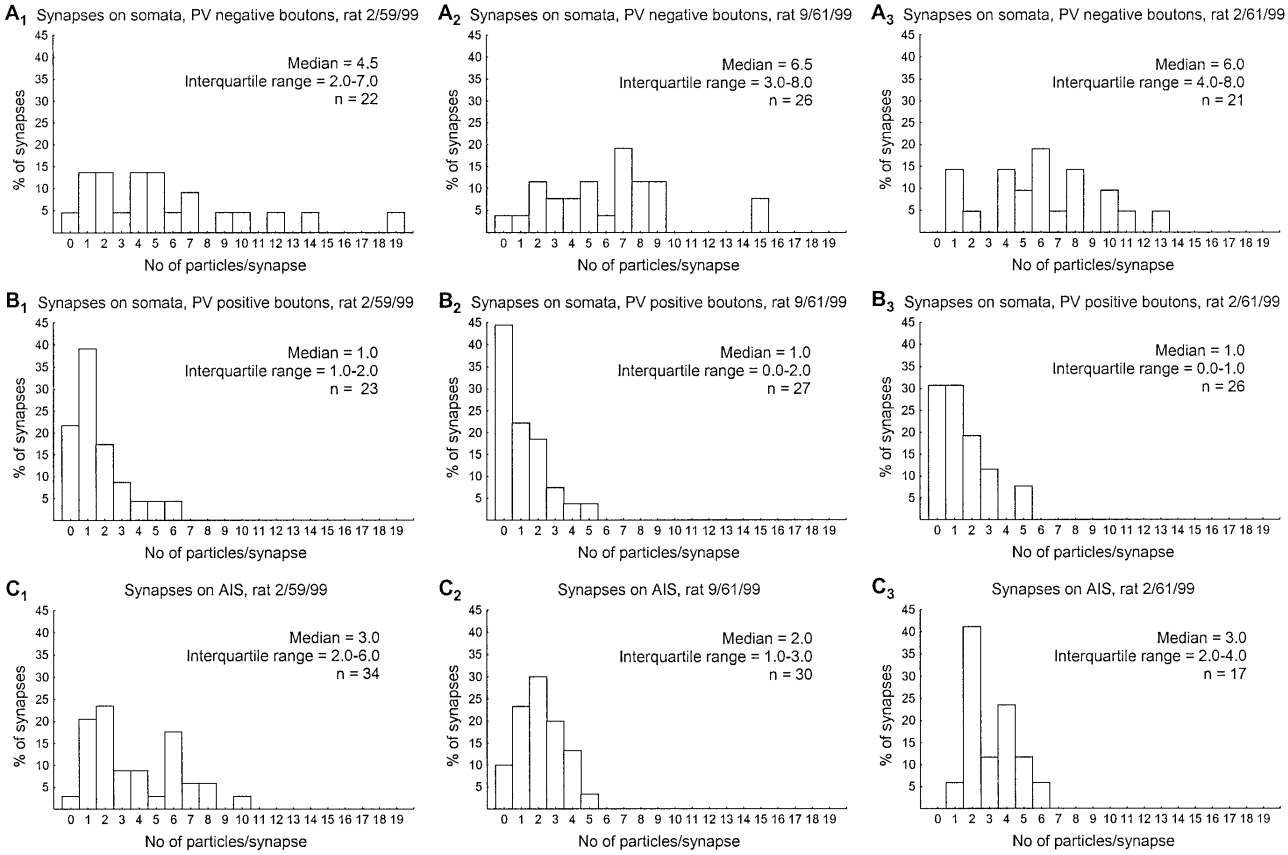

FIG. 4. Distribution of synapses on pyramidal cell somata and axon initial segments according to immunoreactivity for the α_2 subunit of the $GABA_A$ receptor in three adult rats (A_1–C_1, A_2–C_2 and A_3–C_3, respectively). Graphs show synapses established by PV-negative or PV-positive boutons on somata, and boutons on axon initial segments. Differences among synapse populations are consistent in the three animals. The number of particles per synapse for a given synapse population from the three animals was not different (Kruskal–Wallis test, $P > 0.1$ for somatic synapses, $P = 0.041$ for AIS; χ^2 test in each case, $P > 0.1$); therefore, they were pooled and are shown in Fig. 5.

method. Immunolabelling for receptors was also observed over the endoplasmic reticulum and Golgi apparatus, particularly in the somata, but this signal was not analysed in the present study.

Quantitative distribution of immunolabelling for the α_2 subunit of the $GABA_A$ receptor in synapses on pyramidal cell somata and AISs

Analysis of immunoreactivity from three animals revealed striking differences in the distribution of the α_2 subunit in synapses on CA1 pyramidal cells (Figs 2 and 3). Three different populations of synapses have been examined for their α_2 subunit content: synapses from parvalbumin-immunonegative boutons on somata, synapses from parvalbumin-positive boutons on somata, and synapses on axon initial segments (AIS). The number of gold particles per synapse within each synapse population on somata from the three animals was statistically not different (Fig. 4, Kruskal–Wallis test, $P > 0.1$, χ^2 test, $P > 0.1$); therefore, they were pooled for analysis (Fig. 5). For synapses on the AIS the difference was significant ($P = 0.041$) using the Kruskal–Wallis test, but not with the χ^2 test ($P > 0.1$). Nevertheless, for comparability we also pooled the synapses on the AIS from the three animals. Immunoreactivity of PV-negative synapses was five times stronger (median, 5 gold particles/synapse; interquartile range, 3–8 gold particles/synapse; mean $\pm$ SD, 5.90 ± 3.92, $n = 69$) than immunoreactivity of PV-positive synapses (median, 1 gold particle/synapse; interquartile range, 0–2 gold particles/synapse; mean, 1.39 ± 1.47, $n = 76$). Synapses on AISs contained intermediate numbers of gold particles for the α_2 subunit (median, 2 gold particles/synapse; interquartile range, 2–4 gold particles/synapse; mean, 2.99 ± 2.06, $n = 81$). The three synapse populations were significantly different from each other in all combinations (Fig. 6A, Kruskal–Wallis test, $P < 0.01$; χ^2 test, $P < 0.01$; *post hoc* Mann–Whitney test with Bonferroni correction, $P < 0.01$).

The proportions of α_2 subunit-positive synapses, containing at least one gold particle, were also calculated in the three synapse populations. This measure showed that on somata 97.1% and 67.1% of the synapses formed by PV-negative and PV-positive boutons, respectively, were immunopositive for the α_2 subunit, and 95.1% of the synapses were positive on AISs. This observation strongly suggests that on statistical grounds (χ^2 test, $P > 0.1$ in both cases) all synapses formed on somata by PV-negative boutons and all synapses on AISs included α_2 subunit-containing $GABA_A$ receptors.

Comparisons of the size of synapses and the density of α_2 subunit labelling in somatic and axon initial segment synapses

The difference in total α_2 subunit immunoreactivity between the two populations of somatic synapses raised the question of whether it could be caused by a difference in the size of synapses. The area of synaptic specializations was calculated from serial sections by measuring the lengths of the synaptic active zones in each section

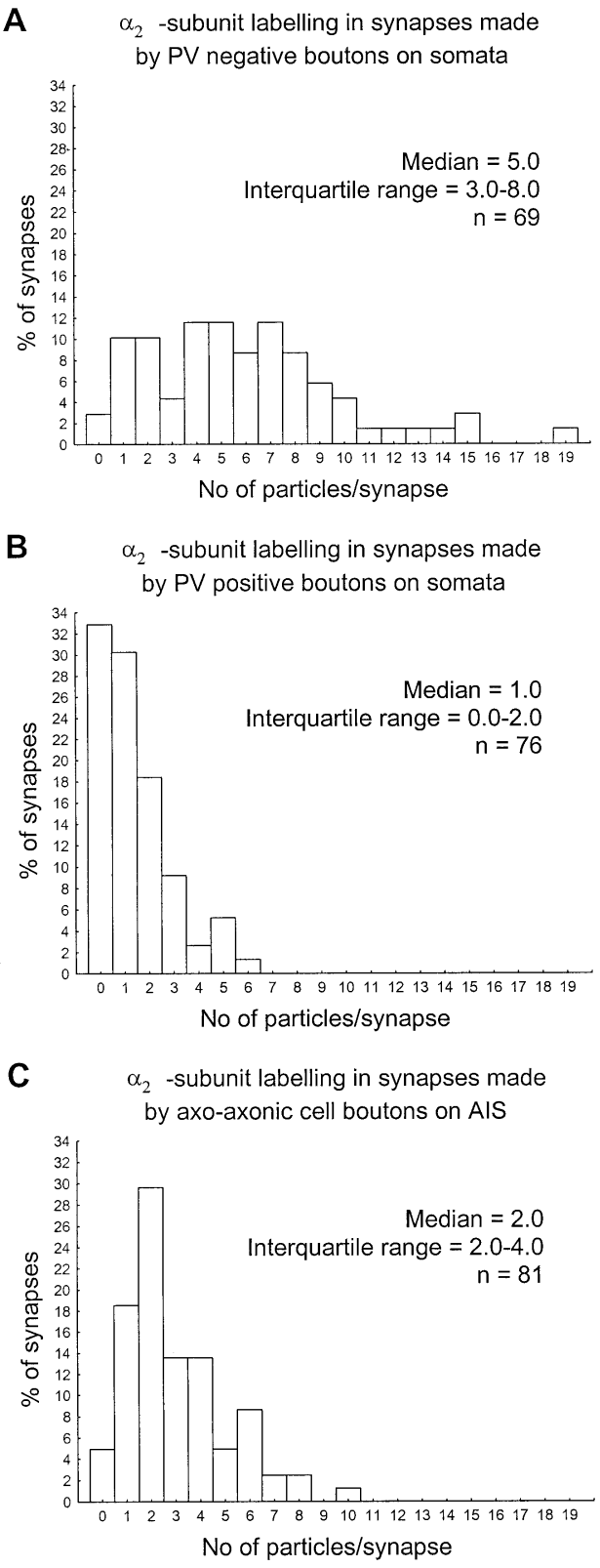

FIG. 5. Pooled distributions of synapses on somata and AIS according to immunoreactivity for the α_2 subunit of the GABA$_A$ receptor from three animals shown individually in Fig. 4. Measurements are from synapses traced and tested fully in serial sections. The degrees of labelling of the three populations of synapses were significantly different (Kruskal–Wallis test, $P < 0.01$; χ^2 test, $P < 0.01$).

and multiplying the sum by the estimated section thickness of 70 nm (Fig. 6B). The medians of synapse sizes established by PV-negative and PV-positive boutons were 0.0753 (interquartile range, 0.0454–0.1222) and 0.0728 (0.0491–0.0932) μm^2, respectively, and they were statistically not different (Mann–Whitney test, $P > 0.1$). However, the median size of the AIS synapses (0.0333 μm^2; interquartile range, 0.0259–0.0419), was less than half of the size of the pooled somatic synapses, and the difference was statistically significant (Mann–Whitney test, $P < 0.01$).

Following from the differences in the sizes of synapses, the density of the immunogold particle labelling was different in synapses on somata and AIS (Kruskal–Wallis test, $P < 0.01$; χ^2 test, $P < 0.01$). The highest median density of α_2 subunit labelling was found in AIS synapses (median, 75.4 gold particles/μm^2; interquartile range, 52.3–121.5), followed by synapses established by PV-negative boutons with a median of 63.7 gold particles/μm^2 (interquartile range, 45.3–95.3). These two populations were not significantly different from each other (*post hoc* Mann–Whitney test with Bonferroni method, $P > 0.1$). Synapses made by PV-positive boutons had a median of 13.7 gold particles/μm^2 (interquartile range: 0–24.7 gold particles/μm^2), significantly lower than the other two synapse populations (*post hoc* Mann–Whitney test with Bonferroni correction, $P < 0.01$). This means that, on average, in synapses made by PV-positive boutons the immunogold particle density was almost five times smaller than in the somatic synapses made by PV-negative boutons (Fig. 6C).

Distribution of immunolabelling for the $\beta_{2/3}$ subunits of the GABA$_A$ receptor in synapses on somata

The high level of α_2 subunit immunoreactivity in somatic synapses made by PV-negative terminals on CA1 pyramidal cells may be due to an overall higher density of GABA$_A$ receptors, which had the same subunit composition as the receptors in synapses made by PV-positive boutons. Most GABA$_A$ receptors probably include the β_2 and/or the β_3 subunits, because the β_1 subunit is expressed at low level by pyramidal cells (Wisden *et al.*, 1992; Sperk *et al.*, 1997). Therefore, the level of $\beta_{2/3}$ subunit immunolabelling was compared in somatic synapse populations (Figs 1 and 7). Practically all synapses were immunopositive for the $\beta_{2/3}$ subunits (97%, χ^2 test, $P > 0.1$). The number of gold particles/synapse was statistically not different in the three animals (Kruskal–Wallis test, $P > 0.1$); therefore, they were pooled for the comparison. The number of gold particles in synapses made by PV-negative boutons (median, 9 gold particles/synapse; interquartile range, 5.5–13.5, mean, 10.36 ± 7.41, $n = 68$) was indistinguishable from that in synapses formed by PV-positive boutons (median, 9 gold particles/synapse, interquartile range, 5.0–13.0; mean, 10.05 ± 6.06, $n = 60$, Mann–Whitney test, $P > 0.1$). These data indicate that synapses made by PV-positive and -negative neurons on somata of pyramidal cells contain similar numbers of GABA$_A$ receptors (Fig. 7).

Correlation of synaptic area and immunogold labelling

It has been reported that the overall amount of synaptic GABA$_A$ receptors containing the α_1 and $\beta_{2/3}$ subunits positively correlates with the size of synapses in the cerebellum (Nusser *et al.*, 1997; Somogyi *et al.*, 1998). Therefore, we have examined whether such a correlation existed in the present material. Because synapses made by PV-negative and PV-positive boutons on somata are similar in size and in the degree of immunoreactivity for $\beta_{2/3}$ subunits, the data from these synapses were pooled to test the relationship of $\beta_{2/3}$ subunit content and synaptic size (Fig. 7C). The correlation between the synaptic area and the number of gold particles representing

immunoreactivity for the $\beta_{2/3}$ subunits was strongly positive (Spearman rank correlation, $r = 0.67$, $P < 0.01$). A positive linear correlation has been also found between the synaptic area and the number of immunogold particles for the α_2 subunit in all three synapse populations (Fig. 8). The correlation for the PV-positive boutons making synapses on somata was weak (Spearman rank correlation, $r = 0.29$, $P < 0.05$), with a shallow slope of the fitted linear regression line, indicating that immunolabelling for the α_2 subunit in these synapses increases very little with increasing size of the synapses. The correlations were higher for synapses formed by PV-negative boutons on somata (Spearman rank correlation, $r = 0.71$, $P < 0.01$) and for synapses on the AISs ($r = 0.44$, $P < 0.01$), and the slopes of linear regression lines were also steeper.

Synaptic size and immunogold labelling density did not correlate for any of the subunits examined. Our observation that the number of $GABA_A$ receptor subunits correlates with synaptic size, but that the density of receptors in a given synapse population is uniform, suggests a regulation of subunit clustering.

Discussion

$GABA_A$ receptor subunits in synapses made by basket cells on pyramidal cell somata

The main finding of the present study is that α_2-subunit-containing $GABA_A$ receptors are present at a much higher level in synapses formed by PV-negative basket cells than in synapses formed by PV-positive basket cells. Because the size of the synapses in the two populations was similar and both synapse populations contained the same level of immunoreactivity for the $\beta_{2/3}$ subunits, it is very likely that the difference in α_2 subunit immunoreactivity is a consequence of an input-specific difference in the subunit composition of the receptors in these synapses. Presumably, most of receptors are located in the postsynaptic membrane, but optimal testing of this would require antibodies to epitopes on the intracellular surface of the plasma membrane and the use of smaller immunogold and immunoglobulins than those in the present study. Immunoreactivity for the α_2 subunit is also expressed throughout the dendritic domain (Zimprich *et al.*, 1991; Fritschy & Mohler, 1995; Sperk *et al.*, 1997), so it appears that synapses of PV-positive basket cells on the soma contain selectively few α_2-subunit-containing receptors. We used immunolabelling for parvalbumin only to identify one presynaptic cell population, and there is no reason to assume that this calcium

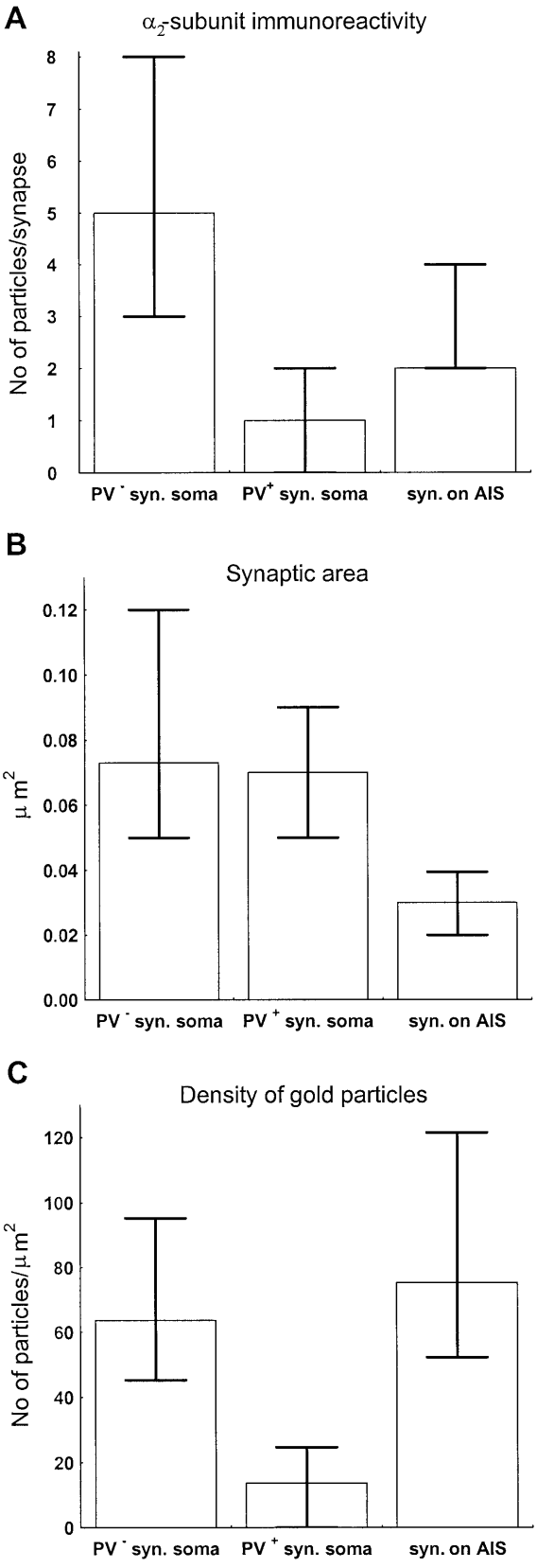

FIG. 6. (A–C) Comparison of PV-positive and PV-negative somatic synapses with those on axon initial segments. The height of the columns represents the median, and line bars show interquartile ranges of a given distribution. In the second columns shown in A and C, the lower limit of the interquartile ranges is 0, and it is 2 in the third column shown in A. (A) Summed α_2 subunit immunoreactivity of the $GABA_A$ receptors ($n = 3$ rats for each population), demonstrating significant differences in the degree of immunolabelling between each pair of populations (Kruskal–Wallis test, $P < 0.01$; χ^2 test, $P < 0.01$; *post hoc* Mann–Whitney with Bonferroni correction, $P < 0.01$). (B) Comparison of synaptic areas, demonstrating that the two somatic synapse populations were not different (Mann–Whitney test, $P > 0.1$), but that synaptic active zones on AISs were significantly smaller than those on the soma (Mann–Whitney test, $P < 0.01$). (C) Comparison of the density of synaptic α_2 subunit labelling, obtained by dividing the number of particles by the estimated synaptic area for each synapse. The synapses made by PV-positive boutons have significantly lower density of α_2 subunit labelling than the other two populations (Kruskal–Wallis test, $P < 0.01$; χ^2 test, $P < 0.01$; *post hoc* Mann–Whitney with Bonferroni correction, $P < 0.01$).

binding protein is directly connected to the difference in receptor expression between basket cells. For example, axo-axonic cells innervating the axon initial segment also express parvalbumin (Kosaka *et al*., 1987), but the α_2 subunit density in their synapses is much higher than that in synapses made by parvalbumin-positive basket cells.

There are two major interneuron populations targeting the somatic surface of pyramidal cells, the PV-positive basket cells and the CCK/VIP-positive but PV-negative basket cells (for review see Acsady *et al*., 1996; Freund & Buzsaki, 1996). The only other source of somatic GABAergic innervation has been reported as originating from the bistratified cells (Buhl *et al*., 1994; Halasy *et al*., 1996); these, however, make very few synapses on somata and probably do not express parvalbumin. Therefore, it is reasonable to assume that perisomatic synapses from PV-positive boutons originate mainly from PV-expressing basket cells, and the PV-negative boutons are established almost exclusively by CCK/VIP-containing basket cells.

The uniform quantitative distribution of the $\beta_{2/3}$ subunits in the synapses of the two basket cell populations suggests similar receptor numbers, making it unlikely that our results are explained by identical receptor composition expressed at different densities. All GABA$_A$ receptors, with the possible exception of the θ-subunit-containing receptor and the homomeric ρ receptors, are thought to contain β subunits. Receptors including the θ subunit are unlikely to be present in the hippocampus (Sinkkonen *et al*., 2000), and the α_2 subunit does not associate with the ρ subunits. Due to the lack of suitable antibodies we could not test the immunoreactivity of synapses for the β_1 subunit, which may also combine with the α_2 subunit. Although it is unlikely that the $\alpha_2\beta_1$-subunit-containing receptors would be expressed at selectively high level in the synapses made by PV-negative boutons, this cannot be excluded at present. Even if this were the case, the results would demonstrate a quantitative, input-specific subunit difference in GABA$_A$ receptors on the same postsynaptic domain and cell type, the soma of pyramidal cells.

Somatic synapses have also been shown to include the α_1 (Nusser *et al*., 1996a; Somogyi *et al*., 1996), $\beta_{2/3}$ and γ_2-subunit-containing receptors (Somogyi *et al*., 1996), and the α_1 and the γ_2 subunits were demonstrated in the same individual synapses (Somogyi *et al*., 1996). In the present study virtually all synapses were shown to contain the $\beta_{2/3}$ subunits, so it is likely that basket cells act through $\alpha_1\beta_{2/3}\gamma_2$-, $\alpha_2\beta_{2/3}\gamma_2$- and/or $\alpha_1\alpha_2\beta_{2/3}\gamma_2$-subunit-containing receptors, but the immunogold method does not provide information on the subunit composition of single receptor channels. The location of other α subunits, expressed by pyramidal cells, such as the α_5 and α_4 subunits, has not been studied at the synaptic level. Fritschy *et al*. (1998a) published a confocal image of immunolocalization of the α_5 subunit, which shows strongly immunolabelled puncta near the somata of cells in stratum pyramidale of the CA3 area. Therefore, it is

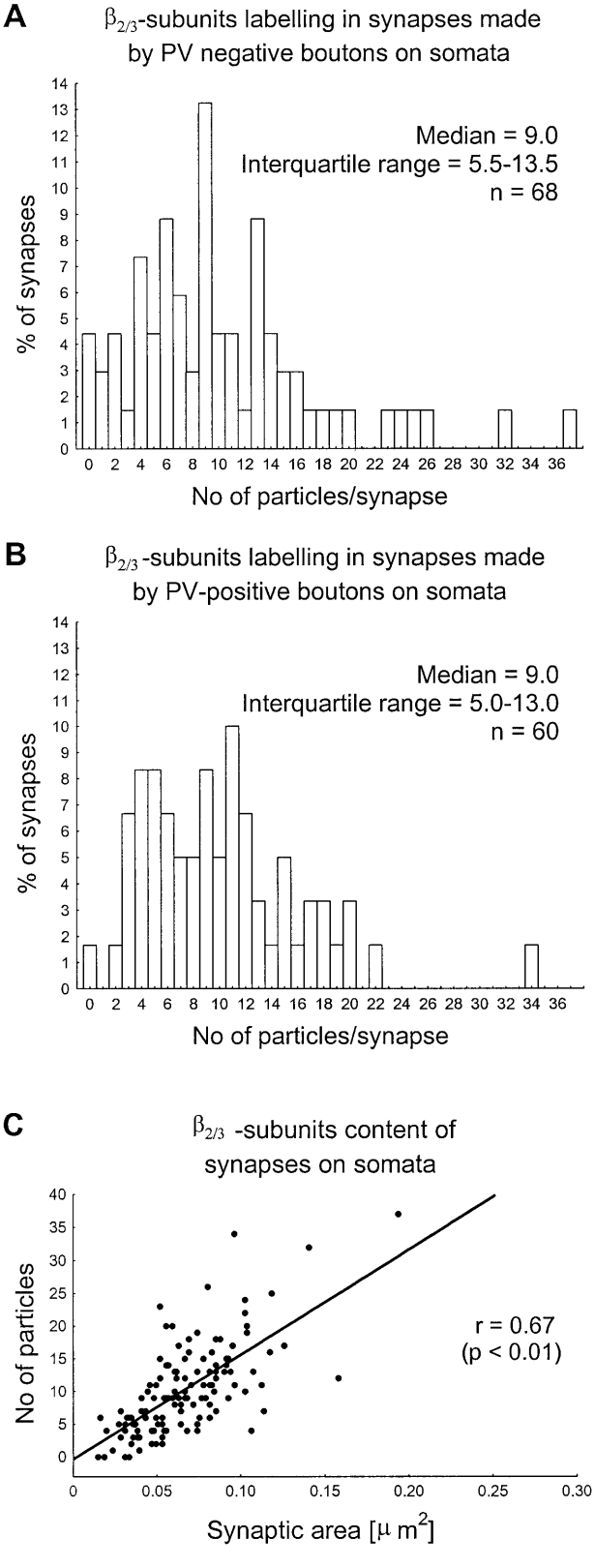

FIG. 7. Comparison of the distributions of synapses on pyramidal cell somata according to immunoreactivity for the $\beta_{2/3}$ subunits of the GABA$_A$ receptor. (A and B) Fully reconstructed synapses were collected from three rats, and data were pooled after confirming that the distributions were statistically not different (Kruskal–Wallis test, $P > 0.1$). The PV-positive and -negative boutons are not different with regard to immunolabelling for the $\beta_{2/3}$ subunits (Mann–Whitney test, $P > 0.1$). (C) Relationship of GABA$_A$ receptor $\beta_{2/3}$ subunit immunolabelling to synaptic area on pyramidal cell soma. There is a strong positive linear correlation between immunoparticle number and synaptic area. Data were pooled from synapses established by PV-positive and PV-negative boutons, because these synapses were not different according to their size (Mann–Whitney test, $P > 0.1$). The regression line intercepts the ordinate close to zero, indicating that even small synapses may be immunolabelled.

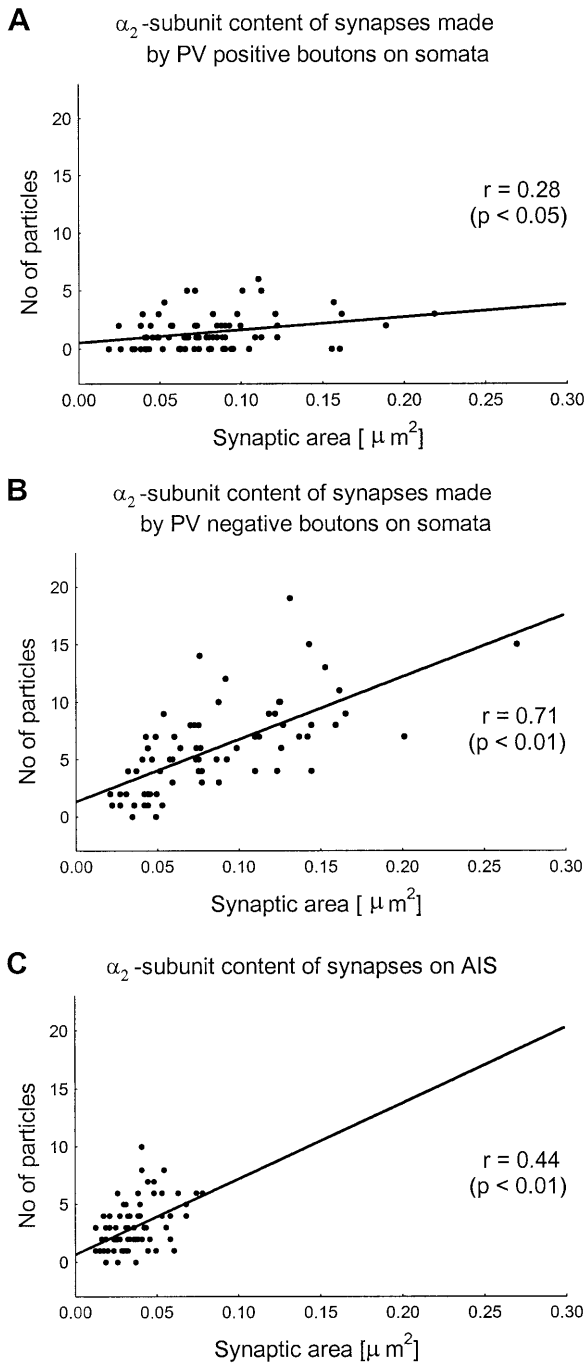

FIG. 8. Comparison of the relationships between immunoreactivity for the α_2 subunit of the $GABA_A$ receptor and synaptic area of the three populations of synapses. In all cases, there are positive linear correlations between immunoparticle number and synaptic area (Spearman rank correlation); the correlation is strongest for synapses made by PV-negative boutons and is weakest for the PV-positive boutons on somata. The regression lines intercept the ordinate close to zero, indicating that even small synapses may be immunolabelled.

possible that the α_5 subunit is also expressed in some basket cell synapses, but the labelled elements and the identity of the cells remain to be established. The α_5 subunit is unlikely to be coassembled with the α_2 subunit in the hippocampus (Fritschy *et al*., 1997).

Comparison of the $GABA_A$ receptor subunit composition of synapses made by basket cells and axo-axonic cells

Virtually all GABAergic synapses on AISs contained the α_2 subunit, and the synaptic density of the α_2 subunits was on average 75.4 gold particles/μm^2, the highest among the three populations of synapses. Taking into account the heterogeneity of receptor content in somatic synapses, and given the estimate that 32% of synapses are established by PV-negative boutons, the average density of immunogold particles for the α_2 subunit is only 22.8 particles/μm^2 (weighted average of median densities of the two populations). This estimate covers a wide range of immunoreactivity of individual synapses, from a significant population of immunonegative synapses to synapses immunolabelled at the same density as those on the AIS. Nevertheless, the higher density of synapses on the AISs, together with their uniformly high density of α_2-subunit-containing receptors, is expected to result in the appearance of much higher immunoreactivity on the AIS, as described in light microscopic studies (Fritschy *et al*., 1998a,b; Loup *et al*., 1998). The quantitative immunogold measurements of Nusser *et al*. (1996a) showed that, in random single sections of synapses, 81.4% of the synapses on AIS and 17.1% on somata were immunopositive for the α_2 subunit. The estimate for the AIS is in agreement with the results of 95% immunopositive synapses in the present study, because at the labelling intensity of Nusser *et al*. (1996a), in serial sections, virtually all synapses would have been immunopositive. The proportion of somatic α_2-subunit-containing synapses appears to have been underestimated, in comparison to the results of the present study, as we found that 76.7% (weighted average of median density values of the two populations) of somatic synapses were labelled by at least one immunoparticle. This difference is due to at least two factors. First, because of the higher background labelling as compared with the present study, Nusser *et al*. (1996a) used a criterion of at least two particles per single section of an active zone to consider a synapse immunopositive. Therefore, synapses containing few α_2 subunits and labelled by one particle, which in the present study are 30% of the synapses made by PV-positive boutons even in serial sections, would have been classified as immunonegative. Second, a synapse considered immunonegative on the basis of one section is often immunopositive in subsequent sections, and the underestimation resulting from this factor affects more synapses of low α_2 subunit density, which are the majority of synapses on the soma, than synapses of high α_2 subunit density, such as the ones on the AIS. Indeed, when the observed labelling frequency of synapses made by PV-negative and PV-positive boutons in single sections is used, and weighted in the ratio of the frequency (32 : 68) of the two somatic synapse populations, the proportion of somatic immunopositive synapses decreases to 24% on the basis of single sections. This proportion, using the criterion of one particle for immunopositivity, is close to the reported 17.1% of Nusser *et al*. (1996a) using a criterion of two particles.

On the basis of the calculations above, the measurements of the two studies are in agreement, but the serial section analysis together with the identification of the two basket cell terminal populations in the present study leads to a modified conclusion. Instead of a preferential targeting of α_2-subunit-containing receptors to axo-axonic cell synapses as opposed to basket cell synapses (Nusser *et al*., 1996a), the synapses of PV-positive basket cells have selectively few α_2-subunit-containing channels. However, α_2-subunit-containing channels may occur in synapses made by PV-negative basket cells at a density similar to that in synapses of axo-axonic cells. Moreover, due to the larger size of synapses made by PV-negative basket cells, their synapses contain, on average, $\approx$2.5 times more α_2-subunit-

containing receptors than synapses made by axo-axonic cells. The results show only a preferential targeting and not an absolute selectivity between the two somatic synapse populations, because 67% of synapses made by PV-positive boutons were labelled by at least one particle and a few of them were relatively strongly labelled. The latter PV-positive boutons may not necessarily originate from basket cells, because a small proportion of the axo-axonic cells, which also express parvalbumin, also innervate the somata of pyramidal cells to a small extent in Wistar rats (P. Somogyi, E. H. Buhl, R. Lujan and J. D. B. Roberts, unpublished observation). If these somatic synapses of axo-axonic cells were supplied with α_2-subunit-containing receptors to the same degree as their synapses on the axon initial segment, then they may account for the few more strongly labelled α_2-positive synapses on the soma. The possibility that axo-axonic cell terminals on the soma may contribute to the presence of α_2 subunit immunoreactive synapses was also suggested by Nusser *et al*. (1996a).

In single sections of synapses on AIS, about 53.5% of active zones were immunopositive for the α_1 subunit (Nusser *et al*., 1996a). Using the distribution of the number of sections per synapse from the current serial section data (not shown; mean 3.09 $\pm$ 1.17 sections per synapse), and assuming that synapses on the AIS are homogenous for GABA$_A$ receptor subunit composition, at least 87% of synapses would contain α_1 subunits as well as the α_2 subunit, the $\beta_{2/3}$ and the γ_2 subunits (Somogyi *et al*., 1996). Indeed, the α_1, $\beta_{2/3}$ and γ_2 subunits were demonstrated in the same individual synapses (Somogyi *et al*., 1996). Whether the α_1 and α_2 subunits are present in the same or in separate receptors cannot be established with the immunogold method. In comparison, in single sections of somatic synapses, at least 65.5% of them were immunopositive for the α_1 subunit (Nusser *et al*., 1996a). Because these synapses are not homogeneous in receptor composition, as shown by the α_2 subunit labelling results, it is not possible to calculate the frequency of α_1 subunit occurrence in the somatic synapse population. Nevertheless, considering the frequency of single-section labelling for the α_1 subunit, and the low labelling density of synapses made by PV-positive basket cells for the α_2 subunit, it is possible that most somatic synapses include α_1-subunit-containing receptors to some extent. For the synapses made by PV-negative boutons, it remains to be tested if their enrichment in α_2-subunit-containing channels leads to a decrease in the amount of α_1-subunit-containing receptors, as suggested by the relatively lower potentiation of synaptic responses of regular spiking basket cells by Zolpidem (Thomson *et al*., 2000). If the density of α_1-subunit-containing receptors were the same in the synapses of the two basket cell populations, then one would expect that immunoreactivity for the ubiquitous $\beta_{2/3}$ subunits was higher in synapses of PV-negative boutons, containing the additional α_2-subunit-containing receptors. This is clearly not the case. Therefore, the results indicate that the enrichment of α_2-subunit-containing receptors in one of the synapse populations is accompanied by a lower number of α_1-subunit-containing receptors.

Consequences of input-specific differences in synaptic GABA$_A$ receptor composition

The calculations above suggest an input-specific synaptic enrichment of the α_2 subunit in somatic synapses on pyramidal cells made by PV-negative basket cells. This is expected to result in pharmacological and kinetic differences in the postsynaptic responses evoked by the two distinct basket cell populations. The benzodiazepine (BZ) binding pocket is formed at the interface of the α and γ subunits (see Sigel & Buhr, 1997). The $\alpha_1\gamma_2$-subunit-containing receptors are expected to show the classical BZ$_1$ type, whereas the $\alpha_2\gamma_2$-subunit- or $\alpha_3\gamma_2$-subunit-containing receptors are expected to show the BZ$_2$ type receptor pharmacology. Receptors including both α_1 and α_2 subunits, which are probably present in the cortex (Duggan *et al*., 1991; Pollard *et al*., 1993), are expected to show intermediate responses, as they probably have one binding pocket of each type (Araujo *et al*., 1996). The most widely prescribed hypnotic drug Zolpidem (Rush, 1998) binds to α_1-subunit-containing receptors with higher affinity than to those containing the α_2 subunit (Pritchett *et al*., 1989; Pritchett & Seeburg, 1990). The recent demonstration that fast spiking basket cells evoked synaptic responses in CA1 pyramidal cells which were potentiated about twice as much by Zolpidem as those evoked by regular spiking basket cells (Thomson *et al*., 2000), taken together with the present results, makes it very likely that the fast spiking basket cells correspond to the PV-positive and the regular spiking ones to the PV-negative basket cells. Diazepam, which acts on both α_1- and α_2-subunit-containing receptors, potentiated responses to both types of basket cells equally (Thomson *et al*., 2000). Further selective pharmacological characteristics have also been reported for the IPSPs evoked by dendritically terminating interneurons (Pawelzik *et al*., 1999; Thomson *et al*., 2000).

Much less is known about the consequences of preferential receptor location on the kinetic properties of postsynaptic responses. From parameters of fast GABA application evoked responses of recombinant receptors (Lavoie *et al*., 1997), the responses to GABA released by PV-negative, probably CCK/VIP-expressing, basket cells, are expected to have faster rise times and slower decay kinetics. In hypothalamic neurons there was a strong correlation between mRNA levels for the α1 and α2 subunits and synaptic response kinetics (Brussaard *et al*., 1997). So far, postsynaptic currents evoked by only one identified CCK-positive and one PV-positive basket cell have been reported (Maccaferri *et al*., 2000); therefore, the two populations cannot be compared. In sharp electrode recordings, no correlation was found between Zolpidem sensitivity and rise or decay times of IPSPs (Thomson *et al*., 2000). However, the assessment of kinetic parameters in unitary connections is complicated by the dispersed location of up to 10 synaptic junctions not only on the soma, but also on the proximal dendrites at different electrotonic distances (Buhl *et al*., 1994; Halasy *et al*., 1996). Therefore, probably a large number of connections will have to be evaluated to test potential kinetic differences resulting from the differences in subunit composition of GABA$_A$ receptors at distinct synapses.

Examples of selective cell surface targeting of distinct GABA$_A$ receptors

Receptors may be targeted selectively to a functionally distinct part of a neuron on the basis of cell-domain-specific mechanisms, which can differentiate, for instance, amongst the axon, soma and dendritic domains. Another possibility is that the incorporation of specific receptor complexes into synapses is governed by the influence of the presynaptic neuron, through as yet unknown signals. The latter mechanism is clearly supported by the present results, which show a preferential enrichment of α_2-subunit-immunopositive receptors in synapses formed by CCK/VIP-expressing basket cells as compared to PV-positive basket cells, even though both basket cell populations target the soma. Similarly, the α_1- or α_6-subunit-containing receptors were found to be differentially targeted on the surface of cerebellar granule cells (Nusser *et al*., 1996b); α_6-subunit-containing receptors were present in both glutamatergic and GABAergic synapses, whereas α_1-subunit-containing receptors were only in the GABAergic synapses. On retinal α ganglion cells, the α_1, α_2 and α_3 subunits were also found mostly at different spots on the cell surface, suggesting innervation possibly from different sources

(Koulen *et al*., 1996). On retinal bipolar cells, bicuculline-sensitive $GABA_A$ receptors were present on both the dendrites and terminals of bipolar cells, but ρ-subunit-containing receptors appear to be present only on the terminals (Shields *et al*., 2000). Differential functional requirements for synaptic and extrasynaptic receptors may also lead to differences in receptor subunit composition and location. The δ-subunit-containing $GABA_A$ receptors were found to be excluded from synaptic junctions, but were found at high density in the extrasynaptic membrane of cerebellar granule cells (Nusser *et al*., 1998). Considering the diversity of $GABA_A$ receptors and the expression of many subunits (Turner *et al*., 1993; Moreno *et al*., 1994; Fritschy & Mohler, 1995), as well as numerous receptor subtypes by a single neuronal type in the CNS (e.g. Jechlinger *et al*., 1998), differences in receptor subunit composition, depending on the source of GABA to the same cell, are expected to be widespread.

Molecular specificity supporting differential functional roles of basket cells

The differences in the roles of the two basket cell types and the axo-axonic neurons in the hippocampal network and throughout the cortex are still unclear. There is convincing evidence that somatic and perisomatic GABAergic innervation regulates the precise timing of principal cell discharge (Buzsaki & Chrobak, 1995; Cobb *et al*., 1995; Miles *et al*., 1996), but so far no differences have been revealed amongst the three types of interneuron. A difference in the roles of the two basket cell populations is suggested by their distinct presynaptic modulation. Katona *et al*. (1999) demonstrated that, unlike PV-positive basket cells, CCK/VIP-containing basket cells express presynaptic type 1 cannabinoid receptors, inhibiting GABA release. In contrast, only the PV-positive basket cells and axo-axonic cells express presynaptic m2 muscarinic receptors, also inhibiting GABA release (Hajos *et al*., 1998). The inputs of these basket cells are also different, at least partly; the VIP/CCK-expressing basket cells, but not the PV-positive cells, are innervated by calretinin-expressing local GABAergic cells (Gulyas *et al*., 1996), and a similar bias was reported in their 5-HT innervation (Papp *et al*., 1999), suggesting divergence in their roles of influencing pyramidal cells. The difference in subunit composition of $GABA_A$ receptors in their synapses, together with recent behavioural studies on transgenic animals (Rudolph *et al*., 1999; Low *et al*., 2000; McKernan *et al*., 2000), indicates a state-dependent functional differentiation, as outlined below.

The α_2-subunit-containing receptors are present at selectively low level in the synapses formed by PV-positive basket cells, whose action therefore is likely to be mainly mediated by α_1-subunit-containing receptors. Transgenic animals, which carry a point mutation in the α_1 subunit, rendering α_1-subunit-containing receptors insensitive to benzodiazepines, are selectively resistant to the sedative and amnesic actions of diazepam (Rudolph *et al*., 1999; McKernan *et al*., 2000). The probable reliance of PV-positive basket cells mainly on α_1-subunit-containing receptors in their output, the high level of α_1 subunit immunoreactivity in their extrasynaptic somato-dendritic membrane (Gao & Fritschy, 1994; Nusser *et al*., 1995) and the role of the hippocampus in memory processes implies that these basket cells participate in activity related to vigilance/exploration and memory formation. In contrast, in the same transgenic animals diazepam was as effective an anxiolytic as in control animals, suggesting that its anxiolytic action is mediated by receptors and synapses that include, amongst others, α_2-subunit-containing receptors. This has been shown recently by rendering α_2-subunit-containing receptors insensitive to diazepam, resulting in the elimination of its anxiolytic effect (Low *et al*., 2000). Furthermore, a reduction of the expression of the γ_2 subunit in $\gamma 2 + / -$ mice led to heightened anxiety and a decreased clustering of the α_2-subunit-containing receptors both in the dendritic and in the somatic layers of the hippocampus (Crestani *et al*., 1999). Therefore, the output synapses of CCK/VIP-expressing basket cells, rich in α_2-subunit-containing synaptic receptors, are candidates for mediating the anxiolytic effects of benzodiazepines. Interestingly, drugs acting on CCK-B receptors change responses in tests of anxiety (see Dauge & Lena, 1998). A further link of the latter basket cell type to activity related to anxiety is the expression of high levels of presynaptic cannabinoid receptors (Katona *et al*., 1999) and the well known anxiolytic effects of cannabinoid agonists (Navarro *et al*., 1997). Mechanistic explanations linking pharmacological sensitivity and specific synaptic activity to behavioural states await the recording of the differential activity of the two types of basket cell in behaving animals. Nevertheless, these findings clearly demonstrate that pre- and postsynaptic molecular differentiations support the differential role of the two populations of basket cell.

Acknowledgements

We thank Drs Yanis Dalezios, Francesco Ferraguti, Jean-Mark Fritschy and Zoltan Nusser for their critical comments on a previous version of the manuscript and Dr Laszlo Marton for calculating the probability of AIS labelling for the α_1 subunit. The authors are grateful to Dr J.-M. Fritschy for the anti-α_2 and anti-$\beta_{2/3}$ subunit antibodies and to Dr K. G. Baimbridge for the antiparvalbumin antiserum. The work was also supported by the Howard Hughes Medical Institute, McDonell Foundation, NINDS (30549), and OTKA (T032251) Hungary and a European Commission Shared Cost RTD Programme Grant (No. BIO4CT96-0585).

Abbreviations

AIS, axon initial segment; BZ, benzodiazepine; CA1, cornus ammonis 1 hippocampal region; CCK, cholecystokinin; $GABA_A$, γ-aminobutyric acid type A; PB, phosphate buffer; PV, parvalbumin; TBS, tris-buffered saline; VIP, vasoactive intestinal polypeptide.

References

Acsady, L., Arabadzisz, D. & Freund, T.F. (1996) Correlated morphological and neurochemical features identify different subsets of vasoactive intestinal polypeptide-immunoreactive interneurons in rat hippocampus. *Neuroscience*, **73**, 299–315.

Araujo, F., Tan, S., Ruano, D., Schoemaker, H., Benavides, J. & Vitorica, J. (1996) Molecular and pharmacological characterization of native cortical γ-aminobutyric $acid_A$ receptors containing both α_1 and α_3 subunits. *J. Biol. Chem.*, **271**, 27902–27911.

Barnard, E.A., Skolnick, P., Olsen, R.W., Mohler, H., Sieghart, W., Biggio, G., Braestrup, C., Bateson, A.N. & Langer, S.Z. (1998) International Union of Pharmacology. XV. Subtypes of γ-aminobutyric $acid_A$ receptors: classification on the basis of subunit structure and receptor function. *Pharmacol. Rev.*, **50**, 291–313.

Baude, A., Nusser, Z., Roberts, J.D.B., Mulvihill, E., McIlhinney, R.A.J. & Somogyi, P. (1993) The metabotropic glutamate receptor (mGluR1α) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. *Neuron*, **11**, 771–787.

Benke, D., Fritschy, J.-M., Trzeciak, A., Bannwarth, W. & Mohler, H. (1994) Distribution, prevalence, and drug binding profile of γ-aminobutyric acid type A receptor subtypes differing in the β-subunit variant. *J. Biol. Chem.*, **269**, 27100–27107.

Bonnert, T.P., McKernan, R.M., Farrar, S., le Bourdelles, B., Heavens, R.P., Smith, D.W., Hewson, L., Rigby, M.R., Sirinathsinghji, D.J.S., Brown, N., Wafford, K.A. & Whiting, P.J. (1999) θ, a novel γ-aminobutyric acid type A receptor subunit. *Proc. Natl Acad. Sci. USA*, **96**, 9891–9896.

Brussaard, A.B., Kits, K.S., Baker, R.E., Willems, W.P.A., Leyting-Vermeulen, J.W., Voorn, P., Smit, A.B., Bicknell, R.J. & Herbison, A.E. (1997) Plasticity in fast synaptic inhibition of adult oxytocin neurons caused by switch in $GABA_A$ receptor subunit expression. *Neuron*, **19**, 1103–1114.

Buhl, E.H., Halasy, K. & Somogyi, P. (1994) Diverse sources of hippocampal unitary inhibitory postsynaptic potentials and the number of synaptic release sites. *Nature*, **368**, 823–828.

Buzsaki, G. & Chrobak, J.J. (1995) Temporal structure in spatially organized neuronal ensembles: a role for interneuronal networks. *Curr. Opin. Neurobiol.*, **5**, 504–510.

Celio, M.R. (1986) Parvalbumin in most γ-aminobutyric acid-containing neurons of the rat cerebral cortex. *Science*, **231**, 995–997.

Cobb, S.R., Buhl, E.H., Halasy, K., Paulsen, O. & Somogyi, P. (1995) Synchronization of neuronal activity in hippocampus by individual GABAergic interneurons. *Nature*, **378**, 75–78.

Crestani, F., Lorez, M., Baer, K., Essrich, C., Benke, D., Laurent, J.P., Belzung, C., Fritschy, J.-M., Luscher, B. & Mohler, H. (1999) Decreased $GABA_A$-receptor clustering results in enhanced anxiety and a bias for threat cues. *Nature Neurosci.*, **2**, 833–839.

Dauge, V. & Lena, I. (1998) CCK in anxiety and cognitive processes. *Neurosci. Biobehav. Rev.*, **22**, 815–825.

Duggan, M.J., Pollard, S. & Stephenson, A. (1991) Immunoaffinity purification of $GABA_A$ receptor α-subunit iso-oligomers. *J. Biol. Chem.*, **266**, 24778–24784.

Ewert, M., Shivers, B.D., Luddens, H., Mohler, H. & Seeburg, P.H. (1990) Subunit selectivity and epitope characterization of mAbs directed against the $GABA_A$/benzodiazepine receptor. *J. Cell Biol.*, **110**, 2043–2048.

Freund, T.F. & Buzsaki, G. (1996) Interneurons of the hippocampus. *Hippocampus*, **6**, 347–470.

Fritschy, J.M., Benke, D., Johnson, D.K., Mohler, H. & Rudolph, U. (1997) $GABA_A$-receptor α-subunit is an essential prerequisite for receptor formation in vivo. *Neuroscience*, **81**, 1043–1053.

Fritschy, J.-M., Johnson, D.K., Mohler, H. & Rudolph, U. (1998a) Independent assembly and subcellular targeting of $GABA_A$-receptor subtypes demonstrated in mouse hippocampal and olfactory neurons in vivo. *Neurosci. Lett.*, **249**, 99–102.

Fritschy, J.-M. & Mohler, H. (1995) $GABA_A$-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. *J. Comp. Neurol.*, **359**, 154–194.

Fritschy, J.M., Weinmann, O., Wenzel, A. & Benke, D. (1998b) Synapse-specific localization of NMDA and $GABA_A$ receptor subunits revealed by antigen-retrieval immunohistochemistry. *J. Comp. Neurol.*, **390**, 194–210.

Gao, B. & Fritschy, J.M. (1994) Selective allocation of $GABA_A$ receptors containing the α_1 subunit to neurochemically distinct subpopulations of rat hippocampal interneurons. *Eur. J. Neurosci.*, **6**, 837–853.

Gray, E.G. (1959) Axo-somatic and axo-dendritic synapses of the cerebral cortex: an electron microscope study. *J. Anat.*, **93**, 420–433.

Gulyas, A.I., Hajos, N. & Freund, T.F. (1996) Interneurons containing calretinin are specialized to control other interneurons in the rat hippocampus. *J. Neurosci.*, **16**, 3397–3411.

Hajos, N., Papp, E.C., Acsady, L., Levey, A.I. & Freund, T.F. (1998) Distinct interneuron types express M2 muscarinic receptor immunoreactivity on their dendrites or axon terminals in the hippocampus. *Neuroscience*, **82**, 355–376.

Halasy, K., Buhl, E.H., Lorinczi, Z., Tamas, G. & Somogyi, P. (1996) Synaptic target selectivity and input of GABAergic basket and bistratified interneurons in the CA1 area of the rat hippocampus. *Hippocampus*, **6**, 306–329.

Haring, P., Stahli, C., Schoch, P., Takacs, B., Staehelin, T. & Mohler, H. (1985) Monoclonal antibodies reveal structural homogeneity of γ-aminobutyric acid/benzodiazepine receptors in different brain areas. *Proc. Natl Acad. Sci. USA*, **82**, 4837–4841.

Jechlinger, M., Pelz, R., Tretter, V., Klausberger, T. & Sieghart, W. (1998) Subunit composition and quantitative importance of hetero-oligomeric receptors: $GABA_A$ receptors containing α_6 subunits. *J. Neurosci.*, **18**, 2449–2457.

Katona, I., Sperlagh, B., Sik, A., Kafalvi, A., Vizi, E.S., Mackie, K. & Freund, T.F. (1999) Presynaptically located CB1 cannabinoid receptors regulate GABA release from axon terminals of specific hippocampal interneurons. *J. Neurosci.*, **19**, 4544–4558.

Kosaka, T., Katsumaru, H., Hama, K., Wu, J.-Y. & Heizmann, C.W. (1987) GABAergic neurons containing the Ca^{2+}-binding protein parvalbumin in the rat hippocampus and dentate gyrus. *Brain Res.*, **419**, 119–130.

Koulen, P., Sassoe-Pognetto, M., Grunert, U. & Wassle, H. (1996) Selective clustering of $GABA_A$ and glycine receptors in the mammalian retina. *J. Neurosci.*, **16**, 2127–2140.

Lavoie, A.M., Tingey, J.J., Harrison, N.L., Pritchett, D.B. & Twyman, R.E. (1997) Activation and deactivation rates of recombinant $GABA_A$ receptor channels are dependent on α-subunit isoform. *Biophys. J.*, **73**, 2518–2526.

Levitan, E.S., Schofield, P.R., Burt, D.R., Rhee, L.M., Wisden, W., Kohler, M., Fujita, N., Rodriquez, H.F., Stephenson, A., Darlinson, M.G., Barnard, E.A. & Seeburg, P.H. (1988) Structural and functional basis for $GABA_A$ receptor heterogeneity. *Nature*, **335**, 76–79.

Loup, F., Weinmann, O., Yonekawa, Y., Aguzzi, A., Wieser, H.-G. & Fritschy, J.-M. (1998) A highly sensitive immunofluorescence procedure for analyzing the subcellular distribution of $GABA_A$ receptor subunits in the human brain. *J. Histochem. Cytochem.*, **46**, 1129–1139.

Löw, K., Crestani, F., Keist, R., Benke, D., Brunig, I., Benson, J.A., Fritschy, J.-M., Rülicke, T., Bluethmann, H., Mohler, H. & Rudolph, U. (2000) Molecular and neuronal substrate for the selective attenuation of anxiety. *Science*, **290**, 131–134.

Maccaferri, G., Roberts, J.D.B., Cottingham, C.A. & Somogyi, P. (2000) Cell surface domain specific postsynaptic currents evoked by identified GABAergic neurones in rat hippocampus in vitro. *J. Physiol. (Lond.)*, **524**, 91–116.

Marksitzer, R., Benke, D., Fritschy, J.-M., Trzeciak, A., Bannwarth, W. & Mohler, H. (1993) $GABA_A$-receptors: drug binding profile and distribution of receptors containing the α2-subunit in situ. *J. Rec. Res.*, **13**, 467–477.

McKernan, R.M., Rosahl, T.W., Reynolds, D.S., Sur, C., Wafford, K.A., Atack, J.R., Farrar, S., Myers, J., Cook, G., Ferris, P., Garrett, L., Bristow, L., Marshall, G., Macaulay, A., Brown, N., Howell, O., Moore, K.W., Carling, R.W., Street, L.J., Castro, J.L., Ragan, C.I., Dawson, G.R. & Whiting, P.J. (2000) Sedative but not anxiolytic properties of benzodiazepines are mediated by the $GABA_A$ receptor α_1 subtype. *Nature Neurosci.*, **3**, 587–592.

McKernan, R.M. & Whiting, P.J. (1996) Which $GABA_A$-receptor subtypes really occur in the brain? *Trends Neurosci.*, **19**, 139–143.

Miles, R., Toth, K., Gulyas, A.I., Hajos, N. & Freund, T.F. (1996) Differences between somatic and dendritic inhibition in the hippocampus. *Neuron*, **16**, 815–823.

Mithani, S., Atmadja, S., Baimbridge, K.G. & Fibiger, H.C. (1987) Neuroleptic-induced oral dyskinesias: effects of progabide and lack of correlation with regional changes in glutamic acid decarboxylase and choline acetyltransferase activities. *Psychopharmacology*, **93**, 94–100.

Moreno, J.I., Piva, M.A., Miralles, C.P. & De Blas, A.L. (1994) Immunocytochemical localization of the β_2 subunit of the gamma-aminobutyric $acid_A$ receptor in the rat brain. *J. Comp. Neurol.*, **350**, 260–271.

Navarro, M., Hernandez, E., Munoz, R.M., del Arco, I., Villanua, M.A., Carrera, M.R.A. & Rodriguez de Fonseca, F. (1997) Acute administration of the CB_1 cannabinoid receptor antagonist SR 141716A induces anxiety-like responses in the rat. *Neuroreport*, **8**, 491–496.

Nusser, Z., Cull-Candy, S. & Farrant, M. (1997) Differences in synaptic $GABA_A$ receptor number underlie variation in GABA mini amplitude. *Neuron*, **19**, 697–709.

Nusser, Z., Roberts, J.D.B., Baude, A., Richards, J.G., Sieghart, W. & Somogyi, P. (1995) Immunocytochemical localization of the α1 and β2/3 subunits of the $GABA_A$ receptor in relation to specific GABAergic synapses in the dentate gyrus. *Eur. J. Neurosci.*, **7**, 630–646.

Nusser, Z., Sieghart, W., Benke, D., Fritschy, J.-M. & Somogyi, P. (1996a) Differential synaptic localization of two major γ-aminobutyric acid type A receptor α subunits on hippocampal pyramidal cells. *Proc. Natl Acad. Sci. USA*, **93**, 11939–11944.

Nusser, Z., Sieghart, W. & Somogyi, P. (1998) Segregation of different $GABA_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. *J. Neurosci.*, **18**, 1693–1703.

Nusser, Z., Sieghart, W., Stephenson, F.A. & Somogyi, P. (1996b) The α6 subunit of the $GABA_A$ receptor is concentrated in both inhibitory and excitatory synapses on cerebellar granule cells. *J. Neurosci.*, **16**, 103–114.

Ogurusu, T., Yanagi, K., Watanabe, M., Fukaya, M. & Shingai, R. (1999) Localization of GABA receptor ρ2 and ρ3 subunits in rat brain and functional expression of homooligomeric ρ3 receptors and heterooligomeric ρ2 ρ3 receptors. *Receptors Channels*, **6**, 463–475.

Papp, E.C., Hajos, N., Acsady, L. & Freund, T.F. (1999) Medial septal and median raphe innervation of vasoactive intestinal polypeptide-containing interneurons in the hippocampus. *Neuroscience*, **90**, 369–382.

Pawelzik, H., Bannister, A.P., Deuchars, J., Ilia, M. & Thomson, A.M. (1999) Modulation of bistratified cell IPSPs and basket cell IPSPs by pentobarbitone sodium, diazepam and Zn^{2+}: dual recordings in slices of adult rat hippocampus. *Eur. J. Neurosci.*, **11**, 3552–3564.

Pollard, S., Duggan, M.J. & Stephenson, F.A. (1993) Further evidence for the existence of α subunit heterogeneity within discrete γ-aminobutric $acid_A$ receptor subpopulations. *J. Biol. Chem.*, **268**, 3753–3757.

Pritchett, D.B., Luddens, H. & Seeburg, P.H. (1989) Type I and type II $GABA_A$-benzodiazepine receptors produced in transfected cells. *Science*, **245**, 1389–1392.

Pritchett, D.B. & Seeburg, P.H. (1990) γ-aminobutyric $acid_A$ receptor α5-subunit creates novel type II benzodiazepine receptor pharmacology. *J. Neurochem.*, **54**, 1802–1804.

Ramon y Cajal, S. (1893) Estructura del asta de ammon y fascia dentata. *Anal. Soc. Espan. Historia Natural*, **22**, 53–114.

Rudolph, U., Crestani, F., Benke, D., Brunig, I., Benson, J.A., Fritschy, J.-M., Martin, J.R., Bluethmann, H. & Mohler, H. (1999) Benzodiazepine actions mediated by specific γ-aminobutyric $acid_A$ receptor subtypes. *Nature*, **401**, 796–800.

Rush, C.R. (1998) Behavioral pharmacology of Zolpidem relative to benzodiazepines: a review. *Pharm. Biochem. Behav.*, **61**, 253–269.

Schoch, P., Richards, J.G., Haring, P., Takacs, B., Stahli, C., Staehelin, T., Haefely, W. & Mohler, H. (1985) Co-localization of $GABA_A$ receptors and benzodiazepine receptors in the brain shown by monoclonal antibodies. *Nature*, **314**, 168–171.

Shields, C.R., Tran, M.N., Wong, R.O.L. & Lukasiewicz, P.B. (2000) Distinct ionotropic GABA receptors mediate presynaptic and postsynaptic inhibition in retinal bipolar cells. *J. Neurosci*, **20**, 2673–2682.

Sieghart, W. (1995) Structure and pharmacology of γ-aminobutyric $acid_A$ receptor subtypes. *Pharm. Rev.*, **47**, 181–234.

Sigel, E. & Buhr, A. (1997) The benzodiazepine binding site of $GABA_A$ receptors. *Trends Pharmacol. Sci.*, **18**, 425–429.

Sinkkonen, S.T., Hanna, M.C., Kirkness, E.F. & Korpi, E.R. (2000) $GABA_A$ receptor ε and θ subunits display unusual structural variation between species and are enriched in the rat locus ceruleus. *J. Neurosci.*, **20**, 3588–3595.

Sloviter, R.S. (1989) Calcium-binding protein (calbindin-D28k) and parvalbumin immunocytochemistry: Localization in the rat hippocampus with specific reference to the selective vulnerability of hippocampal neurons to seizure activity. *J. Comp. Neurol.*, **280**, 183–196.

Somogyi, P., Fritschy, J.-M., Benke, D., Roberts, J.D.B. & Sieghart, W. (1996) The γ2 subunit of the $GABA_A$ receptor is concentrated in synaptic junctions containing the α1 and β2/3 subunits in hippocampus, cerebellum and globus pallidus. *Neuropharmacology*, **35**, 1425–1444.

Somogyi, P., Nunzi, M.G., Gorio, A. & Smith, A.D. (1983) A new type of specific interneuron in the monkey hippocampus forming synapses exclusively with the axon initial segments of pyramidal cells. *Brain Res.*, **259**, 137–142.

Somogyi, P., Tamas, G., Lujan, R. & Buhl, E.H. (1998) Salient features of synaptic organisation in the cerebral cortex. *Brain Res. Rev.*, **26**, 113–135.

Sperk, G., Schwarzer, C., Tsunashima, K., Fuchs, K. & Sieghart, W. (1997) $GABA_A$ receptor subunits in the rat hippocampus. I. Immunocytochemical distribution of 13 subunits. *Neuroscience*, **80**, 987–1000.

Stephenson, F.A. (1995) The $GABA_A$ receptors. *Biochem. J.*, **310**, 1–9.

Thomson, A.M., Bannister, A.P., Hughes, D.I. & Pawelzik, H. (2000) Differential sensitivity to Zolpidem of IPSPs activated by morphologically identified CA1 interneurons in slices of rat hippocampus. *Eur. J. Neurosci.*, **12**, 425–436.

Turner, J.D., Bodewitz, G., Thompson, C.L. & Stephenson, F.A. (1993) Immunohistochemical mapping of gamma-aminobutyric acid type-A receptor alpha subunits in rat central nervous system. In Stephens, D.N. (ed.), *Anxiolitic* B-*Carbolines: from Molecular Biology to the Clinic.* Springer-Verlag, Berlin, pp. 29–49.

Wisden, W., Laurie, D.J., Monyer, H. & Seeburg, P.H. (1992) The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. *J. Neurosci.*, **12**, 1040–1062.

Zimprich, F., Zezula, J., Sieghart, W. & Lassmann, H. (1991) Immunohistochemical localization of the α1, α2 and α3 subunit of the $GABA_A$ receptor in the rat brain. *Neurosci. Lett.*, **127**, 125–128.