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$GABA_A$ RECEPTOR SUBUNITS IN THE RAT HIPPOCAMPUS I: IMMUNOCYTOCHEMICAL DISTRIBUTION OF 13 SUBUNITS

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Abstract—The $GABA_A$ receptor is a ligand-operated chloride channel. It has a pentameric structure. In mammalian brain different subunits are recruited from four gene subfamilies. Using immunocytochemistry, we investigated the distribution of the 13 $GABA_A$ receptor subunits in the hippocampus of the rat. $GABA_A$ receptor subunits were heterogeneously distributed within different hippocampal subfields. High concentrations of α_1-, α_2-, α_4-, β_3-, γ_2- and δ-immunoreactivities were observed within the molecular layer of the dentate gyrus, representing the dendritic area of the granule cells. In the hippocampus proper, the predominant $GABA_A$ receptor subunits were α_1, α_2, α_5, β_3 and γ_2 that were located throughout the strata radiatum and oriens of CA1 to CA3. Immunocytochemical staining was there less prominent for α_4-, β_1-, β_2-, γ_3- and δ- subunits. In the hippocampus proper, the β_1 subunit was preferentially located in CA2. The α_4- and δ-subunits were somewhat more abundant in CA1 than in CA3. Numerous local circuit neurons in the hippocampus proper and the hilus of the dentate gyrus contained α_1-, β_2-, γ_2- and/or δ-subunits. α_3 and γ_1 were present only in minute amounts and no α_6-IR was detected in the hippocampal formation.

The distribution of the $GABA_A$ receptor subunits indicates the existence of heterogenously constituted $GABA_A$ receptor complexes within various hippocampal subfields, which may exert different physiological or pharmacological properties upon stimulation by GABA or its agonists. © 1997 IBRO. Published by Elsevier Science Ltd.

Key words: basket cells, benzodiazepines, dentate gyrus, limbic system, local circuit neurons.

GABA is the principal inhibitory neurotransmitter in the vertebrate brain. It is acting through two major classes of receptors: (i) $GABA_A$ receptors, which are ligand-gated chloride channels, and (ii) $GABA_B$ receptors which are G-protein coupled. $GABA_A$ receptors are presumably assembled in a pentameric structure from a number of subunit classes. Four classes of subunits, with multiple isoforms, have been identified by their sequence homology so far. In rodents, they comprise six α-subunits (α_1–α_6), three β-subunits (β_1–β_3), three γ-subunits (γ_1–γ_3) and one δ-subunit. Proteins derived from a fifth gene subfamily encoding ρ-subunits (ρ_1, ρ_2) presumably constitute so-called $GABA_C$ receptors in the retina.[34] Even assuming that a functional $GABA_A$ receptor is assembled in a pentameric structure and that it requires a combination of at least one α-, one β- and one γ- (or δ-) subunit, a rather high number of subunit combinations may be considered. Recent studies on the regional distribution of mRNA species encoding for the different subunits in the brain and first immunocytochemical investigations indeed suggest the existence of different subunit combinations which may constitute molecularly and functionally different $GABA_A$ receptor subtypes.[11,30,45]

The hippocampal formation is important for acquisition of short-term memory.[1] Some of its structures are especially prone to neuronal damage in chronic epilepsy and ischemia.[36] Benzodiazepines and barbiturates are the treatment of choice in severe status epilepticus, although they may induce mild retrograde amnesia.[46] These actions, at least in part, may be mediated by $GABA_A$ receptors located within the hippocampal formation. Only limited information on the subunit composition of $GABA_A$ receptors in individual subfields of the hippocampus is available. Studies performed at the mRNA level suggest a widespread distribution of the 13 known $GABA_A$ receptor subunits within the hippocampus.[30,45] On the protein level, the distribution of eight different subunits (α_1, α_2, α_3, α_5, $\beta_{2,3}$, γ_2 and δ) has been described.[10,14,25,28,50] The $\beta_{2,3}$ antibodies used in these studies, however, did not distinguish between the β_2 and β_3 subunits.[10,25] We now

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Abbreviations: IR, immunoreactivity; KA, kainic acid; PBS, phosphate-buffered saline.

Table 1. Antibodies used for labelling different $GABA_A$ receptor subunits

Subunit	Antigens	Amino acids	Final conc. (μg/ml)	References
α1	QPSQDELKD (KLH-coupled)	1–9	0.5	[28,48–50]
α2	REPVLGVSP (KLH-coupled)	416–424	2	[12,48–50]
α3	AIKGMIRKQ (KLH-coupled)	459–467	3	[12,48–50]
α4	MBP-α4-7His-fusion protein	379–421	5	[8,20,26]
α5	MPTSSVQDE (KLH-coupled)	2–10	5	[35]
α6	VSSTVE (KLH-coupled)	429–434	5	[29]
β1	MBP-β1-7His-fusion protein	350–404	2	(Fuchs *et al.*, unpublished observations)
β2	MBP-β2-7His-fusion protein	351–405	2 or 15	(Fuchs *et al.*, unpublished observations)
β3	MBP-β3-7His-fusion protein	345–408	2	[41]
γ1	MBP-γ1-7His-fusion protein	324–366	2	[26]
γ2	MBP-γ2-7His-fusion protein	316–352	1	[26]
γ3	RSRRVEEDD (KLH-coupled)	1–9	5	[42]
δ	MBP-δ-7His-fusion protein	1–44	5	(Pelz *et al.*, unpublished observations)

investigated by immunocytochemistry the distribution of all 13 $GABA_A$ receptor subunits in the hippocampus including a differential analysis of the β_2 and β_3 subunits. In accompanying papers changes in immunoreactivities of these subunits and their mRNAs in an animal model of temporal lobe epilepsy are described.[33,43]

EXPERIMENTAL PROCEDURES

Animals and tissue preparation

Male Sprague–Dawley rats (250–350 g, Forschungsinstitut für Versuchstierzucht, Himberg, Austria) were injected with a lethal dose of thiopental (150 mg/kg, i.p., Sanabo, Austria) and perfused immediately through the ascending aorta with 50 ml ice-cold phosphate-buffered saline (PBS; 50 mM phosphate buffer, pH 7.4 in 0.9% NaCl) followed by 200 ml chilled 4% paraformaldehyde in PBS. The brains were removed from the skulls and divided by coronal cuts into three parts. They were postfixed in the same fixative for 90 min at 4°C, then transferred to 20% sucrose in PBS and kept there for 24 h at 4°C. Thereafter the brains were rapidly frozen by immersion in −70°C isopentane for 3 min and, after evaporating the isopentane, stored in tightly sealed vials at −70°C. For immunocytochemistry of the α_3-subunit a different protocol was followed without prior perfusion. The brains were immersed into isopentane (−70°C) cut in a cryostat and the mounted sections were fixed in acetone (5 min, room temperature).[48]

Immunocytochemistry

Coronal sections (40 μm) were obtained from the dorsal hippocampus and kept in Tris−HCl-buffered (50 mM, pH 7.2) saline containing 0.1% sodium azide (4–6°C).

Immunocytochemistry for each receptor subunit was performed in separate experiments on adjacent free-floating sections of the same animals at three different levels of the dorsal hippocampus. The indirect peroxidase–antiperoxidase technique of Sternberger[39] was used as described in detail before.[37] Free-floating sections were rinsed in Tris–HCl-buffered saline and the endogenous peroxidase was blocked by incubation (15 min) with 0.6% H_2O_2 in Tris–HCl-buffered saline and 20% methanol. Then the sections were preincubated with 10% normal goat serum (Biomedica, Vienna, Austria) and 10% egg albumin (Bender, Vienna, Austria) in Tris–HCl-buffered saline for 90 min, followed by incubation with the primary antibodies at 4°C for 48–72 h. Subsequently they were incubated with goat anti-rabbit secondary antibodies (1:200; Vector Al-1000, Szabo, Vienna, Austria) for 60 min and with peroxidase-antiperoxidase complex (1:300, Dako Z-113, Vienna, Austria) for 120 min at room temperature. These sections were reacted with 0.4 mM 3,3-diaminobenzidine (Sigma, Munich, Germany) and 0.01% H_2O_2 for 4–6 min. After each incubation step, except the preincubation, three 5-min washes with Tris–HCl-buffered saline were performed. All buffers and antibody dilutions, except those for washing and reacting with diaminobenzidine, contained 0.4% Triton X-100. Normal goat serum and egg albumin (4% each) were included at all antibody dilutions and during the incubation with the peroxidase-antiperoxidase complex. Sodium azide (0.1%) was added to the primary antibody solutions to avoid growth of microorganisms.

In each experiment controls were included using the primary antibodies preadsorbed with 10 μg/ml (24 h, 4°C) of the respective synthetic peptide or fusion protein (except for γ_2 for which 50 μg/ml of the fusion protein were used). Furthermore, slices were incubated without the primary antibody.

Antibodies

The antibodies used and their dilutions are indicated in Table 1. They were raised in rabbits either against synthetic peptides corresponding to specific sequences of the respective $GABA_A$ receptor subunits, coupled to keyhole-limpet haemocyanin (α_1–α_3, α_5, α_6 and γ_3) or against fusion proteins derived from DNA constructs of the maltose-binding protein gene with specific sequences of the receptor subunit genes and transcribed in *Escherichia coli* (α_4, β_1–β_3, γ_1, γ_2 and δ).[26] All antibodies have been characterized extensively before by various methods including immunoprecipitation, Western blotting and immunocytochemistry (for details see references given in Table 1). Antisera against β_1, β_2 and δ were purified with affinity chromatography with the respective GST-subunit fusion proteins.[26] Antibodies were characterized by immunoprecipitation and western bloting. The β_1 and β_2 antibodies did not immunoprecipitate the other β subunit proteins. The δ antibody recognized several isoforms of the δ subunit proteins with an apparent molecular weight of 53–57,000 and did not precipitate a α1β3γ2 receptor complex.[18]

RESULTS

Antibodies

In the present experiments the antibodies appeared highly specific, revealing staining patterns characteristic for each subunit in different brain areas (data not shown). Notably, subunits which were less prominently expressed in the hippocampus were detected at considerable concentrations in other

brain regions: e.g., α_3 in the cortex, striatum and tuberculum olfactorium, α_6 in granule cells of the cerebellum, β_1 in the cerebral cortex, tuberculum olfactorium and cerebellum, γ_1 in the ventral pallidum and γ_3 in the glomerular layer of the olfactory bulb and in Purkinje cells of the cerebellum (data not shown). The general distribution of immunostaining for the different subunits was in agreement with data published so far on the immunocytochemical distribution of $GABA_A$ receptor subunits[10,11,50] and with data on the distribution of mRNAs.[30,43,45] In all instances staining was blocked by pre-incubation of the antibodies with the respective antigens. Staining for the α_3 subunit revealed a pattern slightly different from previously published data when paraformaldehyde-perfused brain sections were used (see below).[11,48] Therefore, in accordance with a previous report on the characterization of this antibody, acetone-fixed sections were used for labelling α_3.[50] Staining intensities for the individual subunits certainly depend on the properties of the individual antibodies. Different antibody dilutions have therefore been used to reveal comparable staining. Thus, it has to be kept in mind that differences in staining intensities obtained with the subunit-specific antibodies do not necessarily reflect real differences between the concentrations of the respective proteins in the membrane.

α-Subunits

Among the six α-subunits investigated only five (α_1–α_5) were present within the hippocampal formation (Fig. 1). The most prominent staining was observed for the α_1-, α_2-, and the α_5-subunits. Less immunoreactivity (IR) was detected for α_4 and only minute amounts for α_3. α_6-IR was not detected in the hippocampus although it was observed at high concentrations within the granular cell layer of the cerebellum (not shown).[29]

α_1-subunit. The α_1-subunit of the $GABA_A$ receptor was found within fibres throughout the hippocampal formation (Fig. 2). It was almost evenly distributed in the dendritic areas of pyramidal cells, in strata oriens and radiatum CA1 to CA3. Within the molecular layer of the dentate gyrus and the stratum lacunosum moleculare, even somewhat higher concentrations of the protein were detected (Fig. 2a,c). There was no staining within the principal cell layers and in the stratum lucidum. Most of the α1-IR appeared to be located in perikarya and fibres of interneurons at the hilar surface of the granule cell layer (pyramidal-shaped basket cells) and throughout the pyramidal cell layer (Fig. 2b,d,e).

α_2-subunit. The α_2-subunit was found in all subfields of the hippocampus (Fig. 3a). The highest concentration of α_2-IR was present in the molecular layer of the dentate gyrus (Fig. 3a,c). Staining for α_2 was more prominent in the strata oriens and radiatum of CA3 than of CA1. Noticeable α_2-IR was seen also in the stratum lacunosum moleculare, especially at the transition zone to CA3. Staining was restricted to fibres and was not observed within perikarya.

α_3-subunit. As shown in Fig. 1, besides α_6, the α_3-subunit was the least expressed α-subunit. In acetone-fixed sections faint staining was detected in the inner molecular layer of the dentate gyrus, in CA3 and in the stratum lacunosum moleculare (Fig. 1). In sections obtained from paraformaldehyde-perfused brains, faint (but specific) staining was also observed within principal cells (not shown).

α_4-subunit. Staining for the α_4-subunit was diffuse and weaker than for the α_1-, α_2- and α_5-subunits (Figs 1, 3b). It was quite strong in the molecular layer of the dentate gyrus. Staining was also observed in the stratum oriens and radiatum of CA1 and the stratum lacunosum moleculare (Fig. 3b,d). In other parts of the hippocampus only marginal staining was detected. α_4-IR appeared to be restricted to fibres and was not seen in perikarya. Its overall distribution paralleled that of the δ-subunit (Figs 1, 7)

α_5-subunit. The α_5-subunit was present in most parts of the hippocampus proper at high concentrations (Fig. 3f). In contrast to the other α-subunits, α_5 was considerably more concentrated in the dendritic areas (strata radiatum and oriens) of CA1 to CA3 and in the stratum lacunosum moleculare than in the molecular layer of the dentate gyrus (Fig. 3e–g). Staining appeared to be restricted to dendrites. α_5-IR was not observed within the hilus of the dentate gyrus and not within the principal cell layers.

β-Subunits

β_1- and β_3-subunits. The β_1-subunit was present throughout the dendritic areas of the hippocampus, the strata oriens and radiatum CA1 to CA3 and the molecular layer of the dentate gyrus (Fig. 4a). It was especially concentrated within the dendrites of the CA2 sector (Fig. 4a,c). No β_1-IR was observed within the pyramidal cell layers and in the hilus of the dentate gyrus.

The distribution of the β_3-subunit largely matched that of β_1. β_3-IR, however, in general was found at higher concentrations than β_1-IR (Fig. 4b,e). β_3-IR was most prominent in the molecular layer of the dentate gyrus and intense in the strata oriens and radiatum of CA1 and CA3. In CA2 considerably less β_3- than β_1-IR was observed (Fig. 4a–c). The layers of principal neurons and the terminal field of mossy fibres were spared for β_1 and β_3 (Fig. 4a,b).

β_2-subunit. Only faint staining was detected when the β_2 antibody was used at a similar concentration as the other antibodies (2 μg/ml) although prominent

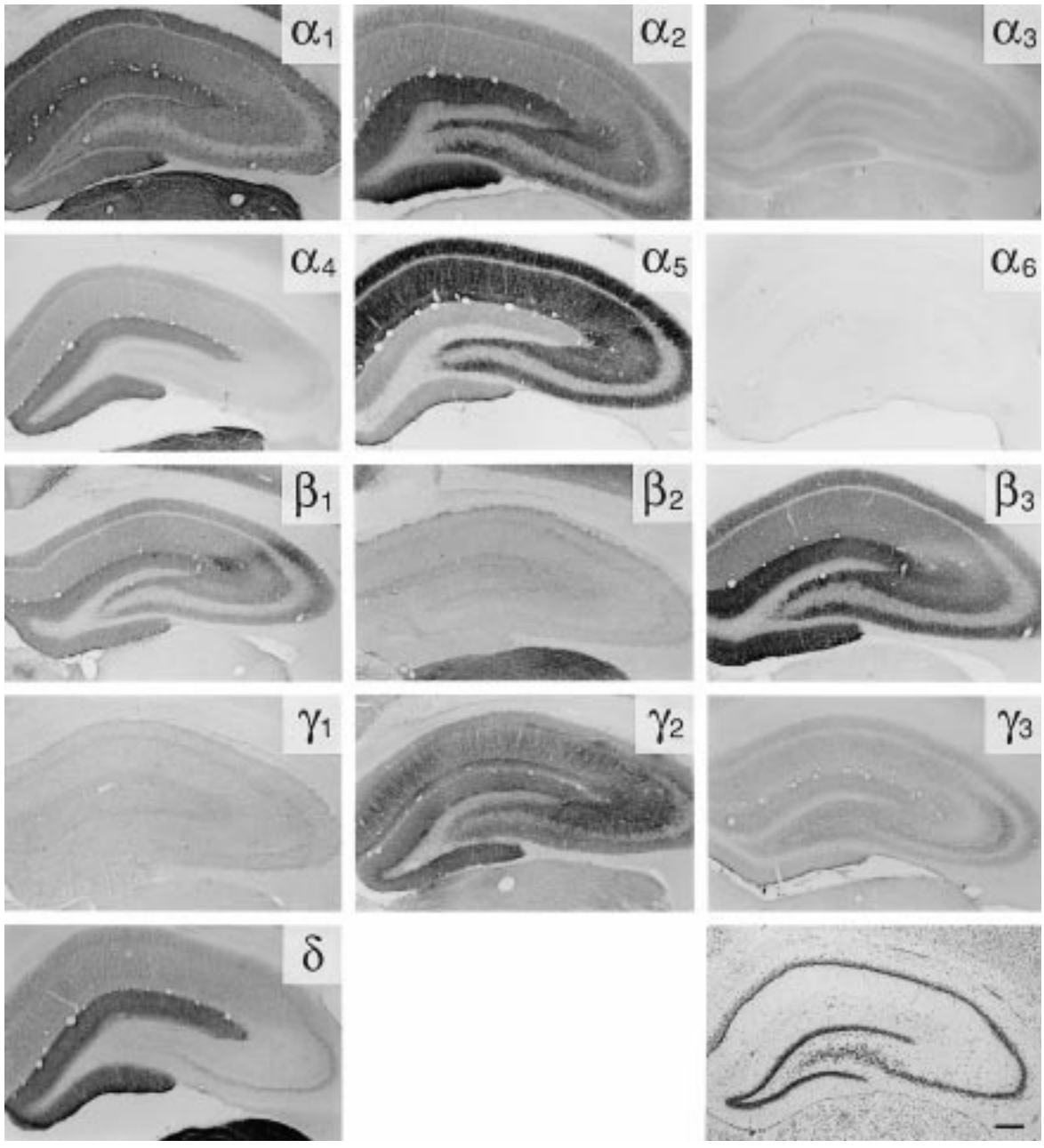

Fig. 1. Differential distribution of 13 $GABA_A$ receptor subunits in the dorsal hippocampus. Note the different subunit expression in the molecular layer of the dentate gyrus (containing preferentially α_1-, α_2-, α_4-, β_1-, β_3-, γ_2-, δ-IR), in CA1 (containing especially α_1-, α_5-, β_3-, γ_2- and some α_2-, α_4-, β_2-, δ-IR), in CA2 (containing α_5-, β_1- and γ_2-IR but little β_3-IR), in CA3 (being especially rich in α_1-, α_2-, α_5-, β_3- and γ_2-IR). Note the rather similar distributions of the α_4- and δ-subunits. For β_2 a similar concentration (2 µg/ml) of the β_2 antibody was used as for the other antibodies, resulting in a faint staining. Compare staining for β_2 in Fig. 5, where a higher antibody concentration (15 µg/ml) was applied. In the lower right corner a Nissl-stained section is shown. Scale bar=250 µm.

staining was observed in brain areas outside the hippo-campus, e.g., in the thalamus (Fig. 1), globus pallidus and substantia nigra. Among the β-subunits, thus β_2 was the least abundant in the hippocampus. In the course of this study we then used comparatively high concentrations of the β_2 antibody (15 µg/ml), obtaining still specific immunostaining. Marked labelling of a great number of interneurons throughout the hippocampal formation was observed (Fig. 5). Only weak staining was seen in dendrites of pyramidal neurons (preferentially in CA1) and of granule cells (molecular layer of the dentate gyrus). The β_2 antibody intensely labelled the perikarya and the dendritic trees of pyramidal-shaped basket cells in

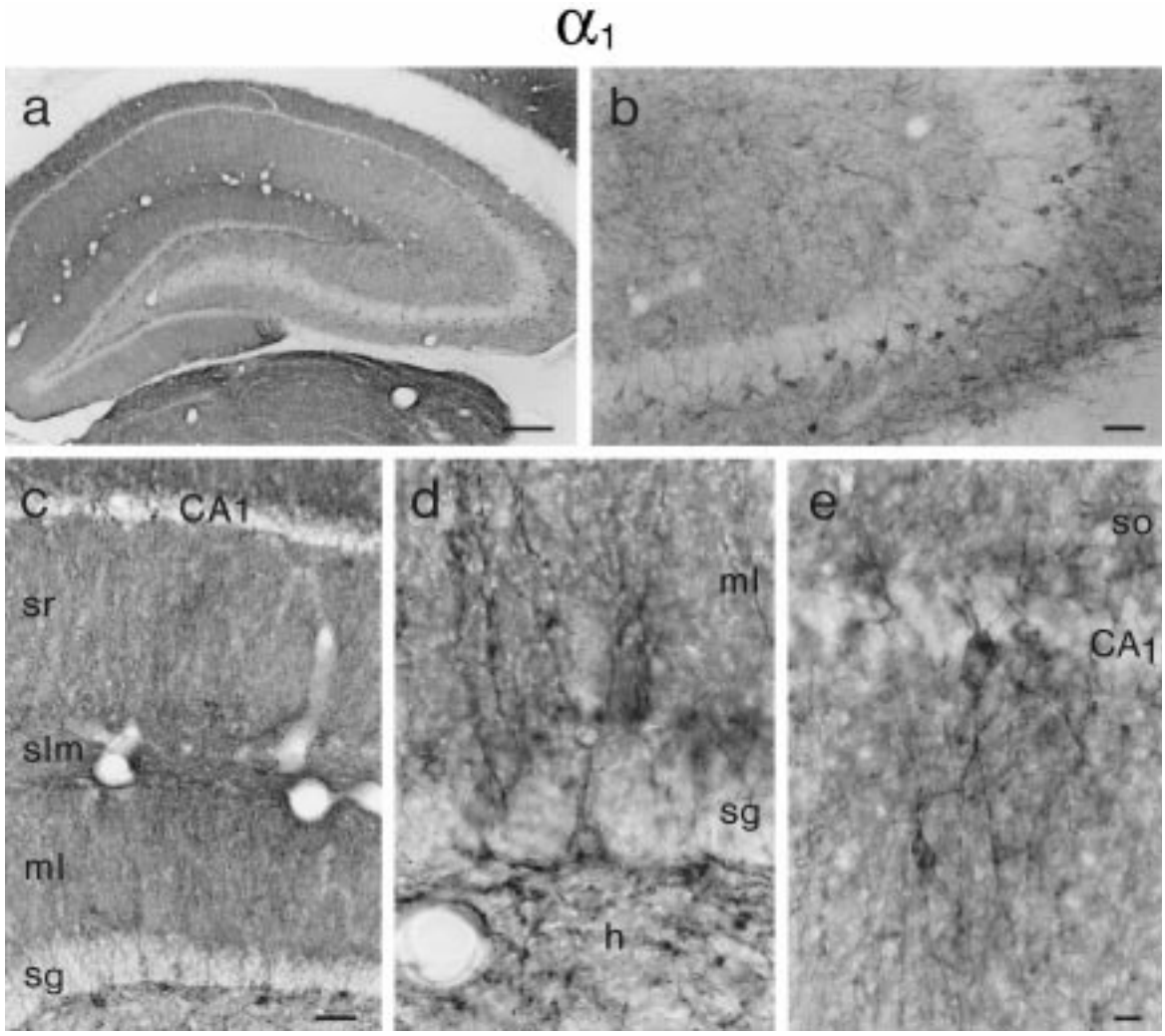

Fig. 2. α_1-Subunit of the $GABA_A$ receptor. Note the preferential localization of α_1-IR in interneurons: overview of the hippocampus (a); note the dense plexus of interneurons in CA3a (b); area between the stratum granulosum and the stratum oriens CA1 (c); pyramidal shaped basket cell projecting with its apical dendrite through the stratum granulosum (d); basket cell in CA1 (e). sg, stratum granulosum; ml, molecular layer; slm, stratum lacunosum moleculare; sr, stratum radiatum; so, stratum oriens; h, hilus. Scale bars: in a=250μm; in b and c=50 μm and in e=10 μm (for d and e).

the hilus of the dentate gyrus (Fig. 5b–d) and basket cells located in the CA1 pyramidal cell layer (Fig. 5e,g) and in CA3 (Fig. 5f). A band of neurons located within the middle and inner molecular layer of the dentate gyrus also exposed β_2-IR (Fig. 5b,c).

γ-Subunits

γ_2-subunit. Among the γ-subunits, only γ_2-IR was found at high concentrations in neuronal structures of the hippocampus. It was concentrated in dendrites of the stratum radiatum of CA1 to CA3 (Fig. 6a,b,d,e), in the stratum lacunosum moleculare and in the molecular layer of the dentate gyrus (especially in its inner part; Fig. 6d). Staining in the stratum oriens was diffuse but strong. Prominent γ_2-IR was present in perikarya located throughout the dentate hilus (Fig. 6c). Numerous presumable type II basket cells, located close to the inner surface of the granule cell layer, were strongly labelled (Fig. 6c). However, only a few faintly stained (type I) pyramidal-shaped basket cells were found. Occasionally staining of pyramidal neurons was seen in CA1 and CA3.

γ_1- and γ_3-subunits. For the γ_1- and γ_3-subunits only faint and diffuse immunoreactivity was detected in all parts of the hippocampus (Fig. 1). The faint γ_1-immunostaining appeared to be associated with astrocyte-like cells and fibres (not shown). γ_3-IR was observed in fibres throughout the hippocampus especially in the terminal field of mossy fibres and, to a lesser extent, in the molecular layer and the hilus of the dentate gyrus (Fig. 1).

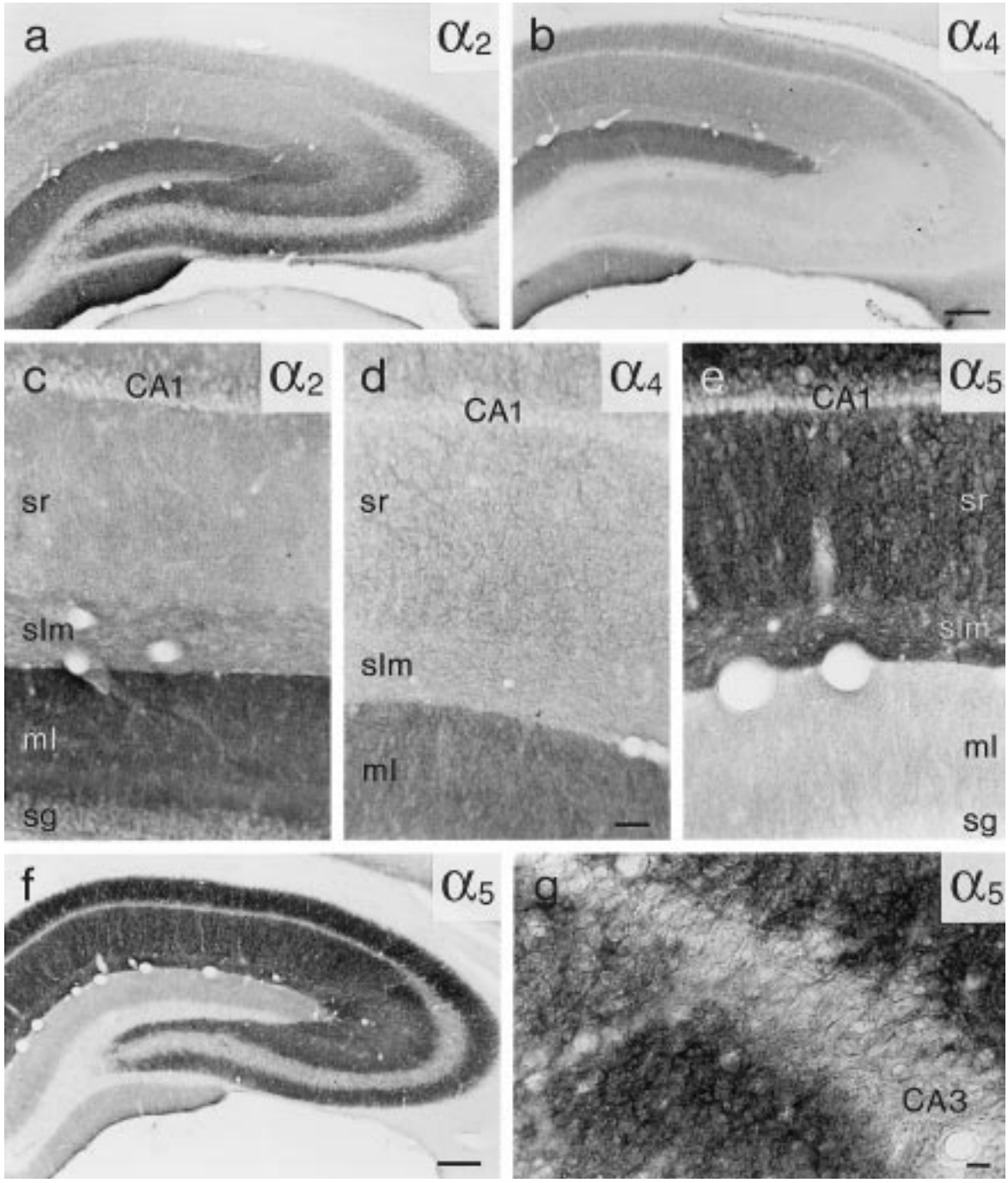

Fig. 3. Comparative distribution of α_2-, α_4- and α_5-subunits. Overviews of the subunits are shown in a, b, f. In c–e the area between the stratum granulosum and the stratum oriens CA1 and in g α_4-IR in an aspect of CA2 is depicted. Note the differential distribution of these three α-subunits in the molecular layer of the dentate gyrus (containing α_2- and α_4-, but almost no α_5-IR), the high concentration of α_5-IR, present throughout the dendritic fields of CA1 to CA3 (e–g), the distribution of α_2-IR (being enriched in CA2 and CA3) and the complementary expression of α_4-IR in CA1. Note also the differential expression of the subunits in the stratum lacunosum moleculare (slm in c–e). For abbreviations see Fig. 2. Scale bars: in d=50 µm (for c–d); in b and f=250 µm (for a, b, f); in g=10 µm.

δ-Subunit

The overall distribution of the δ-subunit largely matched that of α_4. δ-IR was preferentially located in the molecular layer of the dentate gyrus (Fig. 7a–d). It was also observed within the granule cell layer (Fig. 7c,d). Faint staining was present in the strata radiatum and oriens of CA1 and in the pyramidal cell layer of CA1 to CA3 (Fig. 7a,e). Dense δ-IR was also

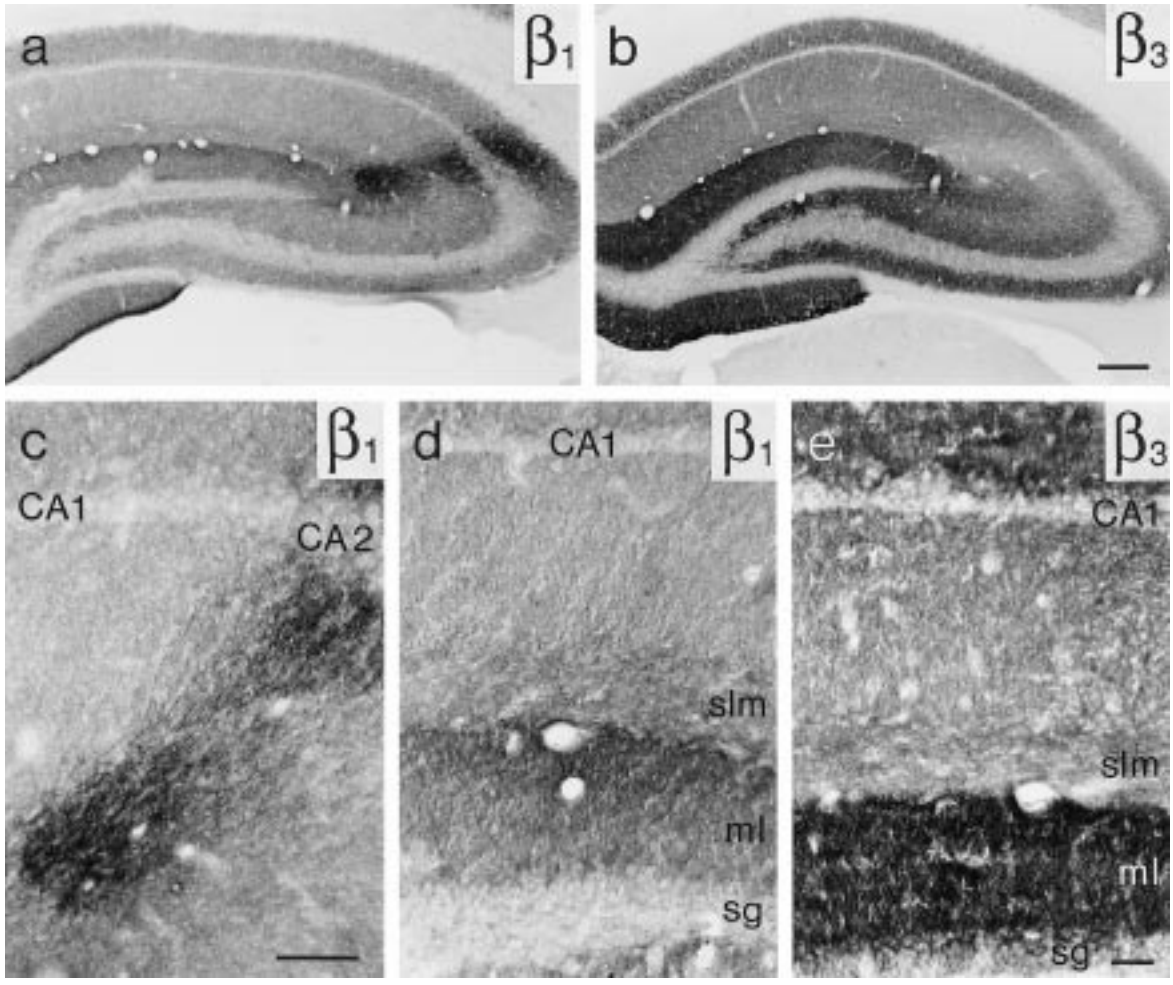

Fig. 4. Distribution of the β_1- and β_3-subunits. Both subunits appear to be contained within the molecular layer of the dentate gyrus and in the dendritic fields of CA1 and CA3 (a,b). Note however their differential distribution in CA2 (a–c). For abbreviations see Fig. 2. Scale bars: in b=250 µm (for a and b); in c=50 µm; in e=50 µm (for d, e).

observed in pyramidal-shaped basket cells in the hilus of the dentate gyrus and in numerous interneurons of CA3 (Fig. 7d,e). A plexus of immunoreactive fibres was observed in the stratum lacunosum moleculare (Fig. 7b).

DISCUSSION

The present data provide a detailed survey of the immunocytochemical distribution of 13 $GABA_A$ receptor subunits in the hippocampal formation in the rat.

Specificity of antibodies and efficacy of the immunocytochemical staining

The specificity of the subunit-directed antibodies is based on their extensive previous characterization (see references in Table 1). In the present study, the validity of the immunocytochemical staining was verified by competition of the primary antibodies with their corresponding peptide antigens and by replacing primary antibodies with a non-immune serum. The regional distribution of the individual subunit proteins within the hippocampal formation and in other brain areas (data not shown) generally is in agreement with the distribution of the respective mRNAs.[30,43,45]

Possible localization of $GABA_A$ receptor subunits in glia

By far the largest portion of immunoreactive $GABA_A$ receptor subunits appears to be located on neuronal structures. Faint β_1-, γ_1- and possibly α_4-IR is found in astrocyte-like structures of the hippocampus (not shown). This staining is blocked by preincubation of the antibodies with the respective antigens. It was rather weak and not unequivocal. We therefore did not include these results in our present report. Following kainic acid-induced seizures, however, transient expression of some α_3- (in

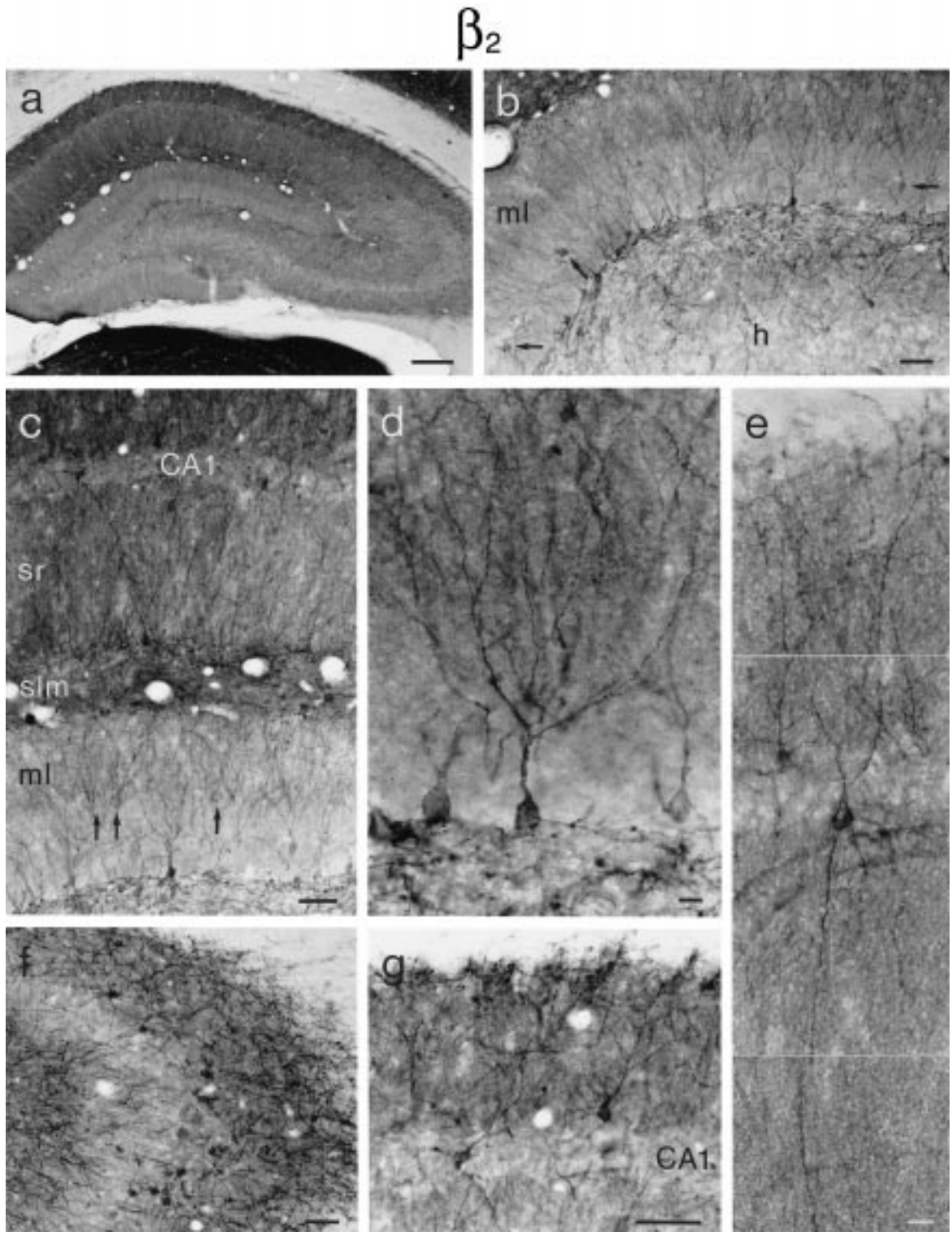

Fig. 5. The β_2-subunit of the $GABA_A$ receptor. In this experiment 15 µg/ml β_2 antibody were used (compare Fig. 1 in which 2 µg/ml β_2 antibody were used). An overview (a); aspect of the dentate gyrus (b); the area extending from the stratum granulosum to the stratum oriens CA1 (c); high magnification of pyramidal-shaped basket cells with their apical dendrites extending to the molecular layer (d); basket cell in CA1 with dendrites extending to the stratum oriens (up) and the stratum lacunosum moleculare (down; e); dense plexus of interneurons with their processes in CA2 (f); interneurons in CA1 with fibres extending into the stratum oriens (g). The β_2 subunit is preferentially located within interneurons of all hippocampal subfields. Note the faint β_2-IR neurons located in the middle molecular layer of the dentate gyrus (arrows in b and c) forming an immunoreactive band (a). Scale bars: in a=250 µm; in b, c, f and g=50 µm; in d and e=10 µm.

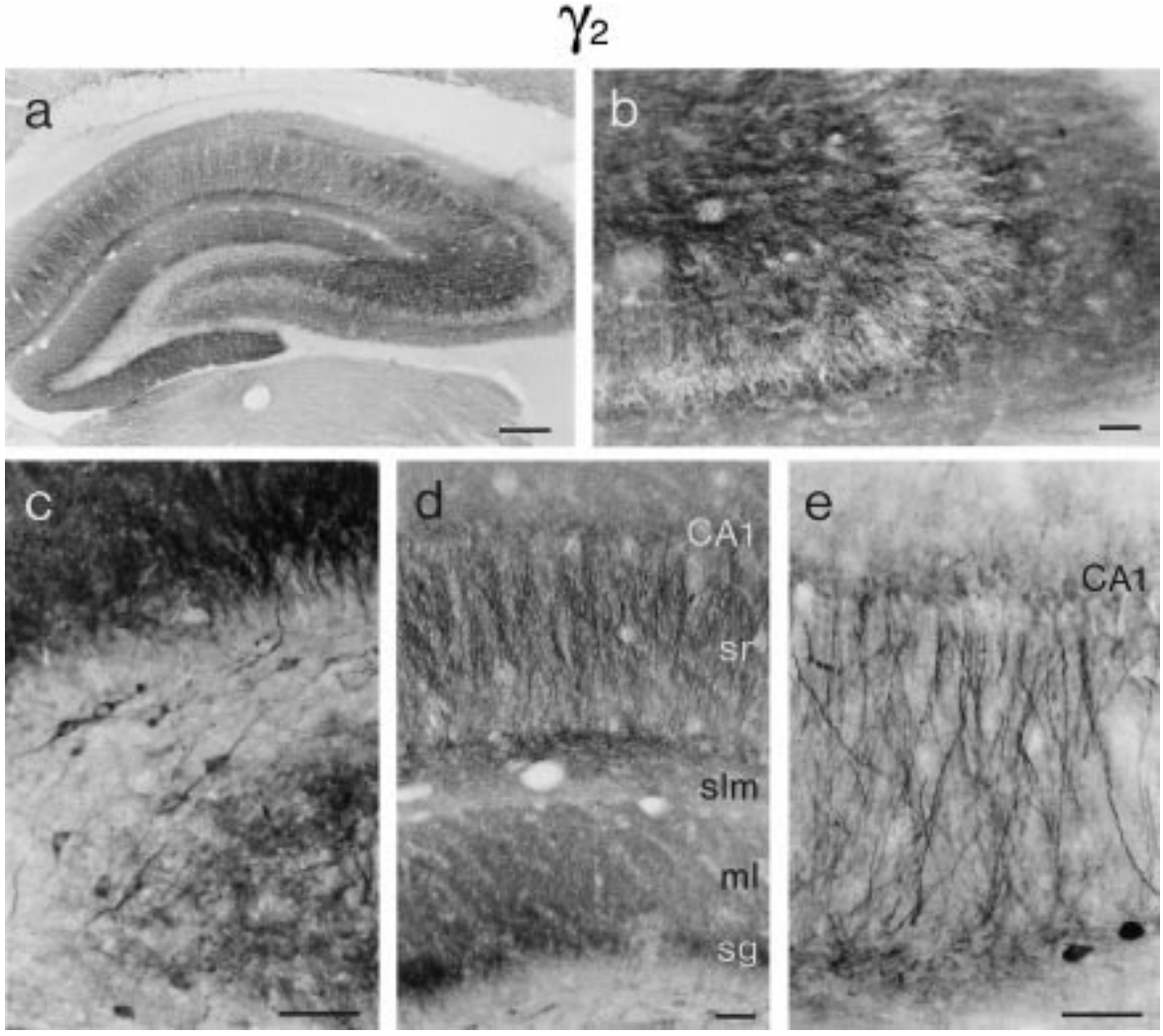

Fig. 6. The γ_2-subunit of the $GABA_A$ receptor. a, Overview; b, CA3a sector; c, interneurons in the dentate hilus; d, the area between the stratum granulosum and the stratum oriens CA1; higher magnification of the stratum radiatum CA1. Note the almost ubiquitous distribution of the γ_2 subunit within the hippocampus (a) and its high concentration in interneurons of the hilus (c). Note the dense immunoreactive fibres in the supergranular layer and in the stratum lacunosum moleculare (a,d) Intense γ_2-immunoreactivity is present in dendrites of the stratum radiatum of CA1 (d) and in CA3 (b). High concentrations of γ_2-IR are present in pyramidal neurons and their dendrites in CA1. For abbreviations see Fig. 2. Scale bars: in a=250 μm, in b–e=50 μm.

paraformaldehyde-fixed sections), α_4-, α_5-, γ_1-, γ_3- and δ-IR is observed in astrocytes of the hippocampus, being most prominent two or 30 days (γ1) after KA injection.[33] Also in epileptic animals such a staining has to be judged with caution. Other proteins expressed at high concentrations in glia after KA-induced seizures easily could interfere.[6,22,27]

Distribution of $GABA_A$ receptor subunits in the hippocampal formation

The distribution of $GABA_A$ receptor subunits in different parts of the hippocampal formation is summarized in Table 2 (see also Fig. 1). Within the hippocampal formation, immunoreactivity of the different subunits appears to be expressed at different levels. In general, extraordinarily strong staining was found for the α_1-, α_2-, α_5-, β_3- and the γ_2-subunits. Within some hippocampal subfields (especially in the molecular layer of the dentate gyrus), rather strong staining is seen for α_4, β_1 and for δ. Weak immunoreactivity is observed for α_3, γ_1 and for γ_3. This coincides with the comparatively low expression levels of mRNAs encoding the latter subunits. β_1 and β_2 mRNAs are expressed in all principal cell layers at a considerably lower level than β_3.[30,43,45] This coincides with the moderate overall staining observed for these two subunits. The observed conspicuous labelling of interneurons for β_2-IR, as for α_1 and γ_2, was in agreement with the mRNA expression.[30,43,45]

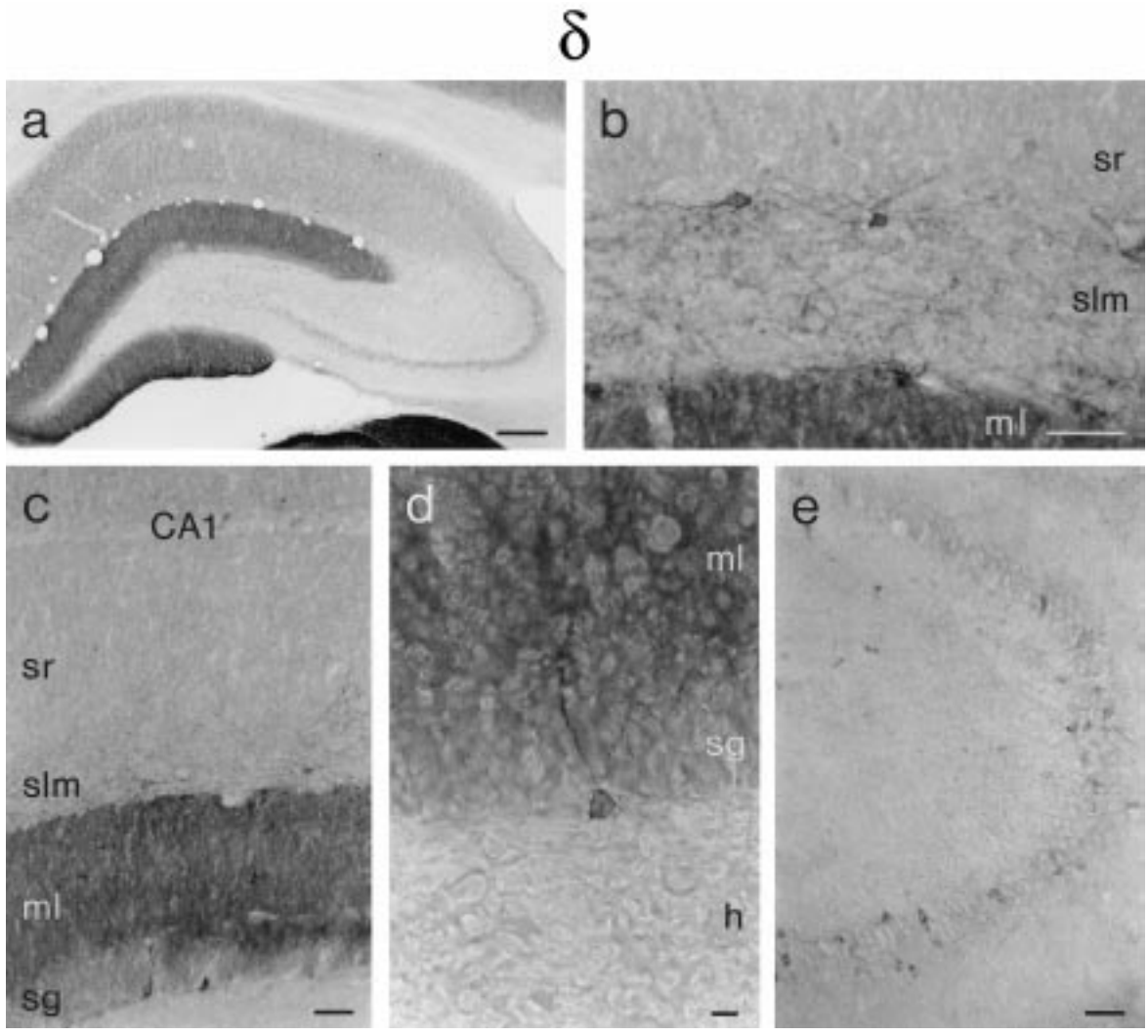

Fig. 7. The δ-subunit of the $GABA_A$ receptor. An overview (a); the stratum lacunosum moleculare (b); the area between the stratum granulosum and the stratum oriens CA1 (c); pyramidal shaped basket cell at the hilar surface of the stratum granulosum (d); CA3a sector (e). Note the intense immunostaining within the molecular layer of the dentate gyrus. The δ-subunit is expressed at moderate concentration within interneurons of CA3 and in fibres of the stratum lacunosum moleculare (b). Moderate concentrations are found in dendrites of CA1 pyramidal neurons (a,c). For abbreviations see Fig. 2. Scale bars: in a=250 μm; in b, c, e=50 μm; d=10 μm.

The monoclonal antibody (62-361), recently developed by Moreno *et al.* and directed against an intracellular loop of the β_2-subunit[25] as well as the one used by Fritschy and Möhler,[11] are less specific and detect simultaneously the β_2- and β_3-subunits. Thus, staining obtained by these groups has to be compared with the almost complementary distributions of β_2 and β_3 as observed in the present study (see below). This fact, however, stresses the importance of using selective antibodies that differentiate between the two subunits. Our data also revealed that in the hippocampus the combination of $\alpha_1\beta_2\gamma_2$, thought to be highly abundant in other brain areas,[24,38] is present preferentially in interneurons.

Molecular layer of the dentate gyrus

The molecular layer of the dentate gyrus contains an extraordinary high variety of immunoreactive $GABA_A$ receptor proteins expressed at high concentrations, α_1, α_2, α_4, β_1, β_3, γ_2 and δ (and of β_2 in interneurons). In general these subunits are almost uniformly distributed within the different segments of the molecular layer. This implies that these $GABA_A$ receptor subunits are predominantly located upon dendrites of granule cells. A slight clustering of α_4- in the outer, of β_2-IR in the middle and inner molecular layer and of γ_2-IR in the inner molecular layer may be supportive of a partial direct association with synapses of GABAergic projections to these parts of

Table 2. Distribution of $GABA_A$ receptor subunits in the hippocampal formation of control rats

	α_1	α_2	α_3	α_4	α_5	β_1	β_2	β_3	γ_1	γ_2	γ_3	δ
Dentate gyrus												
Granule cell layer	–	–	–	±	–	–	–	-	-	-	-	+
Subgranular zone	+	+	+	–	–	–	+					
Molecular layer	+++	+++	+	+++	+	++	+	+++	±	+++	+	+++
Deep hilus (fibres)	++	+	–	–	–	+	+	-	-	++	+	+
Hilar interneurons	+++	–	–	–	–	–	++	-	-	+++	-	+
Hippocampus proper												
CA1												
Pyramidal cell layer	–	–	–	–	–	+	±	-	+	+	-	-
Interneurons	++	-	-	-	-	-	++	-	-	+++	-	+
Stratum lac. molec.	+++	++	–	–	+++	++	+	++	+	+++	-	-
Stratum radiatum	+++	+	+	+	+++	++	+	++	-	+++	+	+
Stratum oriens	+++	+	+	++	+++	++	+	++	-	++	+	+
CA2												
Pyramidal cell layer	–	–	–	–	–	–	±	-	+	+	-	-
Interneurons	+++	–	–	–	–	-	++	-	-	+++	-	+
Stratum radiatum	++	++	+	–	+++	+++	–	+	-	+++	+	-
Stratum oriens	++	++	+	-	+++	+++	-	+	-	++	+	-
CA3												
Pyramidal cell layer	–	–	–	–	±	+	±	-	+	+	-	+
Interneurons	+++	–	–	–	–	-	++	-	-	+++	-	++
Stratum radiatum	+++	+++	+	–	+++	++	–	++	-	+++	++	-
Stratum oriens	+++	+++	+	-	+++	++	-	++	-	++	+	-

+++, very strong; ++, strong; +, weak; –, no immunoreactivity.

the dentate gyrus. Granule cell dendrites are densely innervated by a great variety of GABAergic interneurons located in the hilus of the dentate gyrus and exerting an important role in gating the impulse flow transmitted through the granule cell axons, the mossy fibres, to the CA3 sector of the hippocampus. The co-expression of at least three different α-, two β- and one γ- and one δ-subunit suggests a striking heterogeneity of $GABA_A$ receptors within the same dendritic area. It has been demonstrated before that GABAergic interneurons synapse at different sites of the granule cells and their dendrites.[16,17] Thus, it may be imagined that such a diversity of GABAergic innervation in conjunction with the broad variety of functionally different $GABA_A$ receptors within granule cells may allow a more decisive differentiation of the information processed through these structures. Indeed, experimental evidence for a heterogenous localization of two important α-subunits of the $GABA_A$ receptor at different domains of the same hippocampal cell type has been provided recently by Somogyi's group in an elegant study using immunoelectron microscopy.[2] The authors demonstrated for the α_2-subunit a preferential localization at the axon initial segments of pyramidal cells, whereas the α_1-subunit was found at all postsynaptic domains. A differential distribution of $GABA_A$ receptor subunits within individual regions of the postsynaptic cell may be of high relevance because, e.g., $GABA_A$ receptors containing either α_1- or α_2-subunits exert different pharmacological properties[23,24,34,38,40] and α_4-subunit-containing receptors have a very low affinity for classical benzodiazepines such as diazepam.[34,44] The subunit composition of the $GABA_A$ receptors might also influence the modulation by endogenous ligands (e.g., neurosteroids or Zn^{2+})[5,47] and by second messenger systems (e.g., phosphorylation).[15,19,21,34]

Interneurons of the hilus of the dentate gyrus and the hippocampus proper

Our data demonstrate that at least four subunits of the $GABA_A$ receptor, α_1, β_2, γ_2 and δ are present in GABAergic interneurons of the hilus of the dentate gyrus and in interneurons located in the strata oriens and radiatum of CA1 to CA3. These data are in agreement with previous observations using immunocytochemistry[11,13,25] and *in situ* hybridization.[30,43,45] They suggest that interactions between GABAergic neurons (or possibly presynaptic $GABA_A$ receptor-mediated actions of GABA neurons) within the hippocampus may be preferentially mediated through the major class of type I $GABA_A$ receptors consisting of α_1-, β_2-, and γ_2-subunits. The genes for these subunits are clustered on the human chromosome 5q34–35[31,32] and on the mouse chromosome 11.[2]

It is interesting to note, that type I pyramidal-shaped basket cells in the dentate hilus express (at least in some instances) the δ-subunit but rarely γ_2. The γ_2-subunit was found at high concentrations in other interneurons of the dentate hilus. Thus, a heterogeneous distribution of the γ_2- and the δ-subunits in various types of interneurons has to be considered. On the other hand, the α_1- and the β_2-subunits may be expressed by a great number of local circuit neurons of the hippocampus. Gao and Fritschy,[13] however, have shown in an elegant study using double-labelling immunocytochemistry of

the α_1 subunit with various neuropeptides and calcium-binding proteins, that α_1 is not expressed in calbindin-D_{28}- or cholecystokinin-containing neurons. And, whereas the α_1 subunit is expressed in most somatostatin/GABA neurons of the hippocampus proper it is not contained in somatostatin neurons of the hilus of the dentate gyrus.[7,13] Similar studies are required to establish the exact localization of the different $GABA_A$ receptor subunits on the neurochemically well-defined subpopulations of hippocampal interneurons.[9]

Hippocampus proper

There is also a striking heterogeneity of $GABA_A$ receptor subunits within the dendritic areas of the pyramidal neurons (Table 2). The α_1-, α_2-, α_5-, β_3- and the γ_2-subunits are present at high concentrations throughout the strata oriens and radiatum CA1 to CA3. β_1-, β_2-, α_4- and δ- are expressed at somewhat lower concentrations. Only faint or no staining was detected for α_3-, α_6-, γ_1- and γ_3. The α_2-subunit is less, α_4 somewhat more extensively expressed in the CA1 sector than in other parts of the hippocampus proper. The dendritic areas of CA2 appear to contain more α_5-, and β_1-protein and in reverse somewhat less α_2-, and β_3-IR than the neighbouring areas. The δ-subunit is found preferentially in CA1, however, less concentrated than in the dentate molecular layer. Both, the immunocytochemical data and *in situ* hybridization of the mRNAs[30,43,45] suggest a low level of expression for the α_3-, α_4- (except for CA1), β_2- (besides its expression in interneurons), γ_1- and the γ_3-subunit.

As elegantly shown by Somogyi and co-workers, morphologically different GABA neurons innervate pyramidal cells at different domains and impose distinct physiological signals to these cells.[3,4,28] It is tempting to speculate that distinct subpopulations of $GABA_A$ receptors constituted of different receptor subunits with different properties of the ligand-gated Cl^- channel may mediate such diverse functions. It is, however, noteworthy that on the light microscopic level no clustering of staining for individual subunits is observed dendritic (strata oriens and radiatum, molecular layer of the dentate gyrus) indicating that both synaptic and extrasynaptic receptors are labelled.

CONCLUSION

A heterogeneous distribution of $GABA_A$ receptor subunits has been found within the hippocampus. The predominant subunits within the molecular layer of the dentate gyrus are α_1, α_2, α_4, β_1, β_3, γ_2 and δ. Within the dendritic areas of the hippocampus proper the α_1-, α_2-, α_5-, β_3- and the γ_2- subunits predominate. $GABA_A$ receptors within local circuit neurons of the hippocampus consist mainly of α_1, β_2, and of either γ_2- or δ-subunits (or both γ_2 and δ). These data imply heterogeneously constituted $GABA_A$ receptors with different physiological and pharmacological properties within individual subfields of the hippocampus.

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