European Journal of Neuroscience, Vol. 17, pp. 2503–2520, 2003

High level of mGluR7 in the presynaptic active zones of select populations of GABAergic terminals innervating interneurons in the rat hippocampus

Peter Somogyi[1,2], Yannis Dalezios[1,*], Rafael Luján[1,†], J. David B. Roberts[1], Masahiko Watanabe[3] and Ryuichi Shigemoto[2,4]

[1]MRC Anatomical Neuropharmacology Unit, Department of Pharmacology, Mansfield Road,, Oxford University, Oxford OX1 3TH, UK

[2]Division of Cerebral Structure, National Institute for Physiological Sciences, Okazaki, Japan

[3]Department of Anatomy, Hokkaido University School of Medicine, Sapporo 060–8638, Japan

[4]CREST, Japan Science and Technology Corporation, Kawaguchi, Japan

Keywords: GABA, immunogold labelling, inhibition, metabotropic glutamate receptor, neuropeptide, synapse

Abstract

The release of neurotransmitters is modulated by presynaptic metabotropic glutamate receptors (mGluRs), which show a highly selective expression and subcellular location in glutamatergic terminals in the hippocampus. Using immunocytochemistry, we investigated whether one of the receptors, mGluR7, whose level of expression is governed by the postsynaptic target, was present in GABAergic terminals and whether such terminals targeted particular cells. A total of 165 interneuron dendritic profiles receiving 466 synapses (82% mGluR7a-positive) were analysed. The presynaptic active zones of most GAD-(77%) or GABA-positive (94%) synaptic boutons on interneurons innervated by mGluR7a-enriched glutamatergic terminals (mGluR7a-decorated) were immunopositive for mGluR7a. GABAergic terminals on pyramidal cells and most other interneurons in str. oriens were mGluR7a-immunonegative. The mGluR7a-decorated cells were mostly somatostatin- and mGluR1α-immunopositive neurons in str. oriens and the alveus. Their GABAergic input mainly originated from VIP-positive terminals, 90% of which expressed high levels of mGluR7a in the presynaptic active zone. Parvalbumin-positive synaptic terminals were rare on mGluR7a-decorated cells, but on these neurons 73% of them were mGluR7a-immunopositive. Some type II synapses innervating interneurons were immunopositive for mGluR7b, as were some type I synapses. Because not all target cells of VIP-positive neurons are known it has not been possible to determine whether mGluR7 is expressed in a target-cell-specific manner in the terminals of single GABAergic cells. The activation of mGluR7 may decrease GABA release to mGluR7-decorated cells at times of high pyramidal cell activity, which elevates extracellular glutamate levels. Alternatively, the presynaptic receptor may be activated by as yet unidentified endogenous ligands released by the GABAergic terminals or the postsynaptic dendrites.

Introduction

Synaptic interactions mediated by GABA are essential for the precise timing and selectivity of neuronal responses in the brain. In the neuronal network of the cerebral cortex, including the hippocampal formation, many distinct types of interneuron release GABA, often in a target-cell-type- and domain-specific manner (Ramon y Cajal, 1893; Szentagothai & Arbib, 1974; Szentagothai, 1975; Freund & Buzsaki, 1996; Somogyi *et al*., 1998). Many of the rules governing interneuronal organization in the cortex are seen most clearly in the hippocampal formation due to the alignment of functionally equivalent domains of the pyramidal cells. For example, it was revealed that neurons releasing GABA also differ in their synaptic relationships to each other (Freund & Antal, 1988; Acsady *et al*., 1996; Gulyas *et al*., 1996; Cobb *et al*., 1997; Toth *et al*., 1997). The laminar selectivity of the distribution of cell bodies, dendrites, local and afferent axonal arbours makes the identification of cells and their synaptic relationships easier than in other cortical areas (Lorente de No, 1934; Kawaguchi & Hama, 1987; Frotscher, 1991; Ceranik *et al*., 1997; Ali *et al*., 1999; Miles, 2000; Thomson *et al*., 2000; Vida & Frotscher, 2000; McBain & Fisahn, 2001; Pawelzik *et al*., 2002). One recent example was the discovery that a distinct type of GABAergic hippocampal neuron, expressing both somatostatin and the postsynaptic metabotropic glutamate receptor mGluR1α, receives synaptic input which is highly enriched in the presynaptic metabotropic glutamate receptor subtype 7, 'a' splice variant (mGluR7a) (Shigemoto *et al*., 1996; Shigemoto *et al*., 1997). In the hippocampus most of these synaptic terminals originate from local pyramidal cells (Blasco-Ibañez & Freund, 1995; Shigemoto *et al*., 1996) and release glutamate which is expected to activate mGluRs. Pyramidal cell axons express the receptor in a target-cell-specific manner, allocating much higher levels to terminals that form synapses with the mGluR1α-expressing cells

Correspondence: Professor P. Somogyi, [1]MRC Anatomical Pharmacology Unit, as above. E-mail: peter.somogyi@pharm.ox.ac.uk

[*]*Present address*: Department of Basic Sciences, Faculty of Medicine, University of Crete, Heraklion, Greece.

[†]*Present address*: Centro Regional de Investigaciones Biomédicas, Facultad de Medicina, Universidad de Castilla–La Mancha, 02071 Albacete, Spain.

Received 12 December 2002, revised 23 March 2003, accepted 26 March 2003

doi:10.1046/j.1460-9568.2003.02697.x

than to terminals that make synapses with pyramidal or other types of GABAergic cells (Shigemoto *et al*., 1996; Shigemoto *et al*., 1997). Similar interneuron-target-specific enriched receptor expression also was shown in glutamatergic terminals for other group III mGluRs in the hippocampus (Shigemoto *et al*., 1997; Corti *et al*., 2002).

Electron microscopic analysis showed that the vast majority of terminals on cells which received mGluR7a-enriched input expressed high levels of mGluR7a in the presynaptic active zone (Shigemoto *et al*., 1996; Shigemoto *et al*., 1997). Because each cortical neuron receives GABAergic as well as glutamatergic terminals, it is possible that some of the terminals that showed high levels of mGluR7a were in fact not excitatory amino-acid-releasing terminals but originated from GABAergic neurons. Indeed, another group III mGluR, mGluR7b, has been demonstrated in the presynaptic active zone of type II ('symmetrical') synapses, most of which are GABAergic in the hippocampus. Further, mGluR7a has been demonstrated in the presynaptic active zones of GABAergic terminals in the islands of Calleja (Kinoshita *et al*., 1998). More recently, the presynaptic location of mGluR7a was demonstrated in GABAergic terminals forming type II synapses innervating interneurons, which received mGluR7a-enriched innervation in the isocortex (Dalezios *et al*., 2002). Many of the mGluR7a-positive terminals that formed type II synapses were also immunoreactive for vasoactive intestinal polypeptide (VIP). The latter neuropeptide is expressed in specific interneuron types, which innervate mainly other interneurons in the hippocampus, such as the strongly mGluR1α-positive somatostatin-expressing interneurons (Acsady *et al*., 1996).

The aim of the present investigation was to establish whether mGluR7a also was located in the presynaptic active zone of GABAergic inputs to hippocampal neurons. Of particular interest were the somatostatin–mGluR1α-expressing cells, because the high levels of mGluR7a expression in their presynaptic input appeared to be interneuron-type-specific (Shigemoto *et al*., 1996). However, the str. oriens and alveus of the CA1 area, where they mostly are located, contains at least six distinct types of GABAergic cell (Sik *et al*., 1995; Freund & Buzsaki, 1996; Maccaferri *et al*., 2000; Pawelzik *et al*., 2002). Dual- or triple-labelling electron microscopic immunocytochemistry and antibodies to GABAergic interneuronal molecular markers and mGluR7a were used. Some results have been published in preliminary form (Lujan *et al*., 1998; Kogo *et al*., 1999; Somogyi *et al*., 1999; Dalezios *et al*., 2001).

Materials and methods

Tissue preparation

Fifteen adult (150–250 g), two 15–17-day-old and one 20-day-old-male Wistar rats were deeply anaesthetized with Sagatal (pentobarbitone sodium; 60 mg/mL i.p.) and perfused transcardially first with 0.9% saline for ≈1 min. This was followed by perfusion with one of three fixatives for ≈15–30 min: (A) a solution containing 4% paraformaldehyde, 0.05% glutaraldehyde and 15% (v/v) saturated picric acid made up in 0.1 M phosphate buffer (PB; pH ≈7.4) for 16 rats; (B) solution A but containing 0.1% glutaraldehyde for one adult rat; and (C) solution A but containing 0.2% glutaraldehyde for the 20-day-old-rat. The treatment of animals was carried out in accordance with the UK Animals (Scientific Procedures) Act of 1986 and associated procedures. All efforts were made to minimize the number of the animals used, and the project was approved by the Ethical Review Committee of Oxford University. Processing of the tissue fixed with solution A was carried out as described earlier (Dalezios *et al*., 2002). Briefly, for electron microscopic immunocytochemistry, blocks of the brain were incubated for cryoprotection then freeze-thawed in order to increase the penetration of the reagents. For pre-embedding immunocytochemistry the blocks were sectioned at 70 μm thickness. For Lowicryl embedding 500-μm-thick sections were cut from blocks freeze–thawed twice. For light microscopy the cryoprotection and freeze–thawing steps were omitted and 60-μm-thick sections were cut.

Antibodies

The specific primary antibodies and references describing their characterization are summarized in Table 1. Further references are found in a previous paper (Dalezios *et al*., 2002). It is not known whether the human antiserum to glutamic acid decarboxylase (GAD) recognizes GAD-65 and/or GAD-67.

Immunocytochemistry for light microscopy

The immunocytochemical procedure was identical to that reported earlier (Dalezios *et al*., 2002), and was carried out on two adult and two P15–17-day-old-animals for mGluR7a and two adult animals for mGluR7b. Briefly, floating sections were incubated in 20% normal goat serum (NGS) diluted in 50 mM Tris buffer (pH 7.4) containing 0.9% NaCl and 0.1% Triton X-100 (TBST) for 1 h, followed by a mixture of three primary antibodies (Table 1). The secondary antibody solution contained a mixture of donkey antimouse 7-amino-4-methylcoumarin-3-acetic acid (AMCA; diluted 1 : 100; Jackson ImmunoResearch, West Grove, PA, USA), antirabbit Alexa®-488 (diluted 1 : 1000; Molecular Probes, Leiden, The Netherlands) and donkey antiguinea pig Cy-3® (diluted 1 : 400; Jackson ImmunoResearch, West Grove, PA, USA). Immunofluorescence was studied using a Leica dichromatic mirror system and the A4, L5 and Y3 filter blocks. Images were recorded on a CCD camera, analysed and displayed using the Openlab software (Improvision, Coventry, UK). Brightness and contrast were adjusted for the whole frame; no part of a frame was enhanced or modified in any way.

Pre-embedding immunocytochemistry for electron microscopy

Floating sections were first incubated for 1 h in 20% NGS diluted in 0.9% NaCl buffered with 50 mM Tris (pH 7.4; TBS). Sections were then incubated in a solution of a primary antibody or in a mixture of up to three antibodies diluted in TBS containing 1% NGS, at least overnight or for several days. The following reactions were carried out:

(i) one primary antibody and immunoperoxidase reaction,

(ii) one primary antibody and silver-intensified immunogold reaction,

(iii) two primary antibodies, one of which was visualized by an immunoperoxidase reaction carried out first followed by silver-intensified immunogold reaction,

(iv) two primary antibodies one of which was visualized by a silver-intensified immunogold reaction carried out first, followed by an immunoperoxidase reaction,

(v) three primary antibodies, two of which were visualized together by a silver-intensified immunogold reaction but differentially localized on the basis of subcellular location, followed by an immunoperoxidase reaction for the third antibody.

After primary antibody incubation, the sections were incubated overnight at 4 °C in one, or in a mixture, of the following antibodies: goat antihuman, goat antirabbit and/or antiguinea pig IgG (Fab' fragment, diluted 1 : 100) coupled to 1.4-nm gold (Nanoprobes Inc., Stony Brook, NY, USA) and made up in TBS containing 1% NGS, and biotinylated goat antimouse, or goat antirabbit, or goat antihuman second antibodies (diluted 1 : 100, Vector Laboratories, Burlingame, CA, USA). When silver intensification was carried out first, the sections were washed several times in TBS, then in double-distilled water, followed by silver enhancement of the gold particles with an HQ Silver kit (Nanoprobes) for 8–12 min. Subsequently, in some cases the

TABLE 1. Summary of the sources, concentrations and combinations of antibodies

Antibody against	Species (raised in)	Dilution	Primary antibodies and combinations										Secondary antibody	Dilution	Detection signal	Source of primary antibody	References for characterization
			1	2	3	4	5	6	7	8	9	10					
GABA	Rabbit *Oryctolagus cuniculus*	1 : 1000	+	–	–	–	–	–	–	–	–	–	Goat anti-rabbit IgG coupled to 10-nm gold		EM gold	P. Somogyi; MRC Anatomical Neuropharm Unit, Oxford, UK	Hodgson *et al.* (1985)
GAD	Human *Homo sapiens*	1 μg/mL	–	+	–	–	–	–	–	–	–	–	Goat antihuman nanogold (Fab′ fragment; Nanoprobes)	1 : 100	EM Au–Ag	K.Tanaka; Fac. Medicine, Niigata University, Japan	Oe *et al.* (1996)
			–	–	+	–	–	–	–	–	–	–	Biotinylated goat antihuman (Vector Laboratories)	1 : 100	EM HRP		
GAD	Mouse *Mus musculus*	1 μg/mL	–	–	–	+	–	–	–	–	–	–	Biotinylated goat antimouse	1 : 100	EM HRP	Chemicon, Temecula, USA	Kinoshita *et al.* (1988)
mGluR7a	Rabbit *Oryctolagus cuniculus*	1 μg/mL	–	+	–	–	–	–	–	–	–	–	Biotinylated goat antirabbit	1 : 100	EM HRP	R. Shigemoto; National Inst. of Physiology, Okazaki, Japan	Shigemoto *et al.* (1996, 1997) Sansig *et al.* (2001)
			+	–	+	+	+	+	+	–	–	–	Goat antirabbit nanogold	1 : 100	EM Au–Ag		
			–	–	–	–	–	–	–	+	–	–	Goat antirabbit Alexa 488® (Molecular Probes)	1 : 1000	LM Fluor.		
Parvalbumin	Mouse *Mus musculus*	1 : 2000	–	–	–	–	–	+	–	–	–	–	Biotinylated goat antimouse	1 : 100	EM HRP	P-3171, Sigma	
VIP	Mouse *Mus musculus*	1 : 5000	–	–	–	–	–	+	+	–	–	–	Biotinylated goat antimouse	1 : 100	EM HRP	East Acres Biologicals and Biogenesis	Dey *et al.* (1988)
mGluR1α	Guinea pig *Cavia porcella*	1 : 700	–	–	–	–	–	–	+	–	–	–	Goat antiguinea pig nanogold	1 : 100	EM Au–Ag	M. Watanabe; Dept of Anatomy, Hokkaido University, Japan	Tanaka *et al.* (2000)
			–	–	–	–	–	–	–	+	+	–	Goat antiguinea pig Cy3™ (Jackson ImmunoResearch)	1 : 400	LM Fluor.		
Somatostatin	Mouse *Mus musculus*	1 : 500	–	–	–	–	–	–	–	+	+	–	Donkey antimouse AMCA (Jackson ImmunoResearch)	1 : 100	LM Fluor.	A. Buchan; Dept of Physiology, University of British Columbia, Canada	Vincent *et al.* (1985)
mGluR7b	Rabbit *Oryctolagus cuniculus*	1 μg/mL	–	–	–	–	–	–	–	–	–	+	Biotinylated goat antirabbit	1 : 100	EM HRP	R. Shigemoto; National Inst. of Physiology, Okazaki, Japan	Kinoshita *et al.* (1998)
			–	–	–	–	–	–	–	–	+	–	Goat antirabbit Alexa 488® (Molecular Probes)	1 : 1000	LM Fluor.		

sections were incubated in avidin-biotinylated horseradish peroxidase complex (ABC; diluted 1 : 100; Vector Laboratories, Burlingame, CA, USA) in TBS overnight at 4 °C and washed in Tris-buffer (TB, pH ≈7.4). Peroxidase was visualized with DAB (0.05%) using 0.01% H_2O_2 as substrate for 5–10 min. The same reagents were used for peroxidase reaction alone, or when the reaction was carried out first in double labelling, followed by the silver intensification sequence after several washes in double-distilled water. Following the final washes, the sections were treated with 2% OsO_4 in PB for 30–50 min, washed in PB followed by double-distilled water and then contrasted in freshly prepared 1% uranyl acetate for 40–50 min. The sections were then dehydrated in a series of ethanol and propylene oxide and embedded flat in epoxy resin (Durcupan ACM; Fluka, Sigma-Aldrich, Gillingham, UK) on slides. After polymerization of the resin, selected areas of the hippocampal sections were re-embedded in Durcupan blocks for sectioning. Serial 70–80-nm-thick sections were collected on pioloform-coated copper grids. Electron microscopic sections from the immunoperoxidase-stained material were not contrasted by lead; those containing only silver-intensified immunogold labelling were contrasted with lead citrate. Unless otherwise stated, electron microscopic samples were obtained from two different rat brains and two blocks of each animal were cut for electron microscopy.

The immunoreaction using rabbit polyclonal antibodies to mGluR7b was reported earlier (Shigemoto *et al*., 1997). In the present study, further analysis was carried out on electron microscopic samples of the CA1 area.

Lowicryl embedding and postembedding immunocytochemistry for electron microscopy

This procedure was used for testing GABA immunoreactivity in material that was reacted for mGluR7a using the pre-embedding silver-intensified immunogold reaction as described above, except that 500-μm-thick floating sections were reacted. The tissue was obtained from rats perfused with fixative containing 0.1% or 0.2% glutaraldehyde. After the silver intensification the sections were washed in distilled water, cryoprotected, frozen by slam-freezing and embedded in Lowicryl HM-20 resin following dehydration at low temperature as described earlier (Baude *et al*., 1993). Serial sections were cut at 70–90 nm thickness, perpendicular to the plane of the 500-μm-thick section, and picked up on pioloform-coated single-slot nickel grids and reacted with antibodies to GABA for the routine postembedding reaction.

The combined pre- and postembedding procedure was developed because we did not obtain a sufficiently strong signal for mGluR7a detection using the postembedding immunogold method even after extended trials. Although mGluR7a immunoreactivity was observed in synaptic junctions, the frequency of such synapses was much lower than that obtained using the pre-embedding method, so the level of signal detection was not suitable for a quantitative study. On the other hand, the postembedding GABA reaction has the advantage of revealing the majority of GABAergic terminals, because the method does not suffer from limitations of antibody penetration into the tissue encountered in the pre-embedding procedure. Ideally, GABA is best fixed by a high concentration of glutaraldehyde which, however, turned out to be detrimental for detecting mGluR7a. Therefore, after trying various concentrations, acceptable reactions eventually were obtained in two rats fixed with 0.1% and 0.2% glutaraldehyde, respectively. Because mGluR7a detection required a double freeze–thaw in glutaraldehyde-fixed tissue the fine structural preservation of the tissue following Lowicryl embedding was relatively poor. Nevertheless, the method proved to be suitable for quantitative evaluation of the GABA immunoreactivity of mGluR7a-positive terminals.

Controls

To test method-specificity of the procedures for light or electron microscopy, the primary antibody was omitted or replaced with 1% (v/v) normal serum of the species of the primary antibody. No selective labelling was observed. When double or triple labelling was used some sections were always incubated with only one primary antibody and the full complement of secondary antibodies to test for any cross-reactivity of secondary antibodies. Other sections were incubated with two or three primary antibodies and one secondary antibody, followed by the full sequence of signal detection. In general (see below), no cross-labelling was detected which would influence the results. Specifically, the combination of two or three primary antibodies and both gold-labelled or biotinylated secondary antibodies, followed by the ABC reagent and peroxidase reaction but no silver intensification, resulted only in amorphous HRP end product, and no metal particles were detected. Using the same sequence but only silver intensification without HRP reaction produced silver granules but no amorphous HRP end-product. In other control experiments, the silver-intensified gold reaction was used to detect a single primary antibody, but the full ABC sequence was also applied using a biotinylated secondary antibody that was raised to IgG from a species different from that of the applied primary antibody, followed by DAB reaction. Under these conditions, only infrequent small patches of nonspecific HRP end product were detected, and the patches were not associated selectively with any particular cellular profile. In addition, the selective location of the signals in structures labelled with only one or the other of the signalling products within the same section, as well as having side by side double-labelled structures, showed that our procedures did not produce false-positive double-labelling results. We found one case of potential cross interference of signals, namely when carrying out the immunoperoxidase reaction first followed by silver intensification to reveal the 1.4-nm gold-labelled antibodies. Some of the peroxidase-labelled cellular profiles were decorated at the edge of the peroxidase reaction product by highly electron-opaque irregular (presumably silver) precipitate. This precipitate could be differentiated from the intensified immunogold particles because the latter had a much more regular round shape. Nevertheless, due to this and other (see Results) limitations, this labelling sequence was not used for quantification of nerve terminals.

Statistical analysis

Statistical differences between the frequencies of synaptic terminals labelled with a specific antibody were evaluated using the χ^2 test or Fisher's exact test when appropriate (Zar, 1999), setting significant differences at $P < 0.01$.

Results

Distribution of mGluR7a in relation to mGluR1α- and/or somatostatin-expressing interneurons in the developing and adult CA1 area

Several previous studies have reported the codistribution of somatostatin and mGluR1α (Baude *et al*., 1993; Hampson *et al*., 1994) and, separately, of postsynaptic mGluR1α and presynaptic mGluR7a (Shigemoto *et al*., 1996, 1997; Ferraguti *et al*., 2001) in the CA1 area of the rat hippocampus. However, direct evidence for the cellular co association of the three molecules has not been reported. It is important to test this possibility because some mGluR1α-positive cells do not express somatostatin (Ferraguti *et al*., 2001). In the present study, coimmunolabelling with three antibodies showed that the vast majority of strongly mGluR1α-immunopositive neurons were also somatostatin-positive and were decorated by mGluR7a-immunopositive terminals in

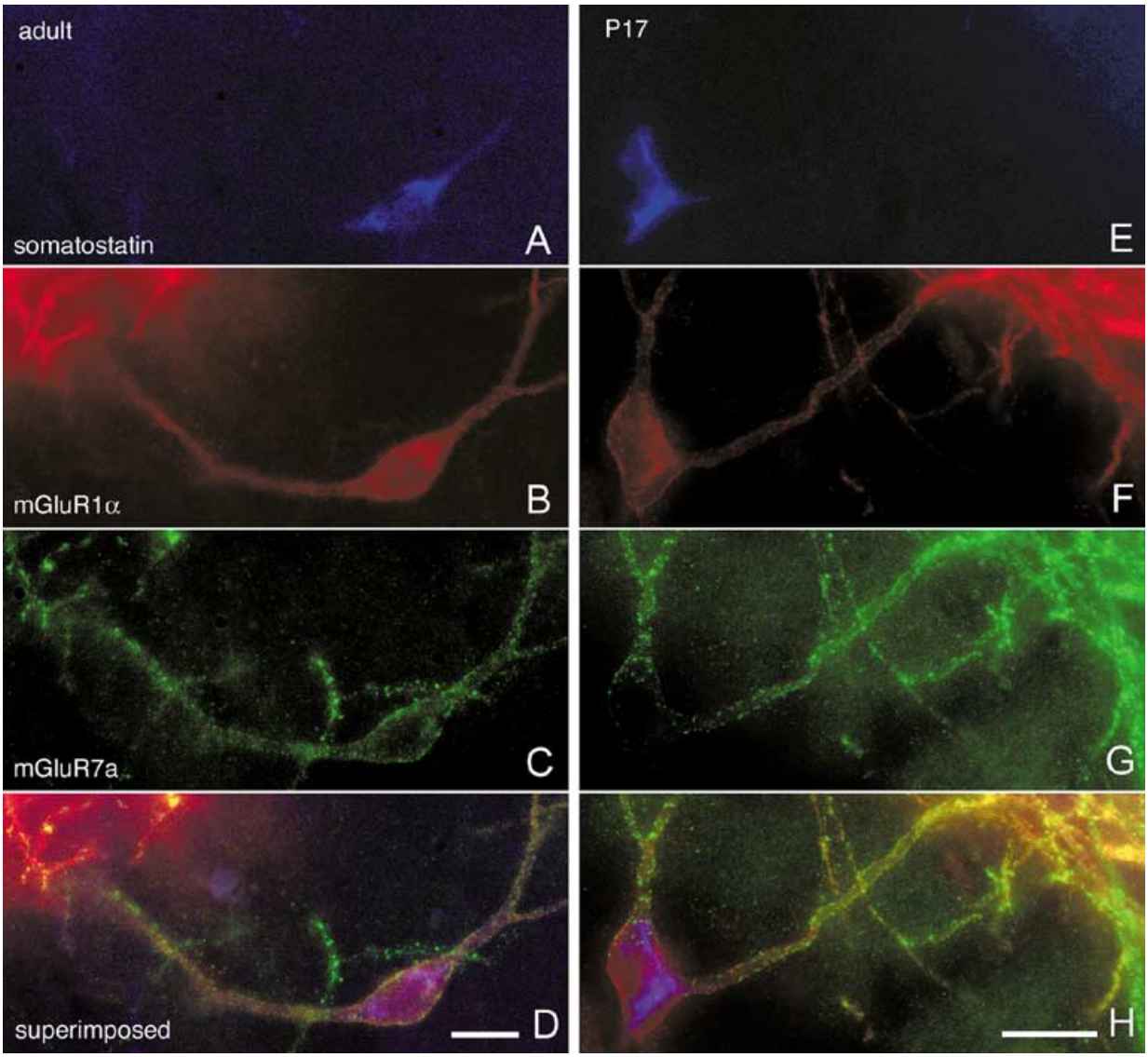
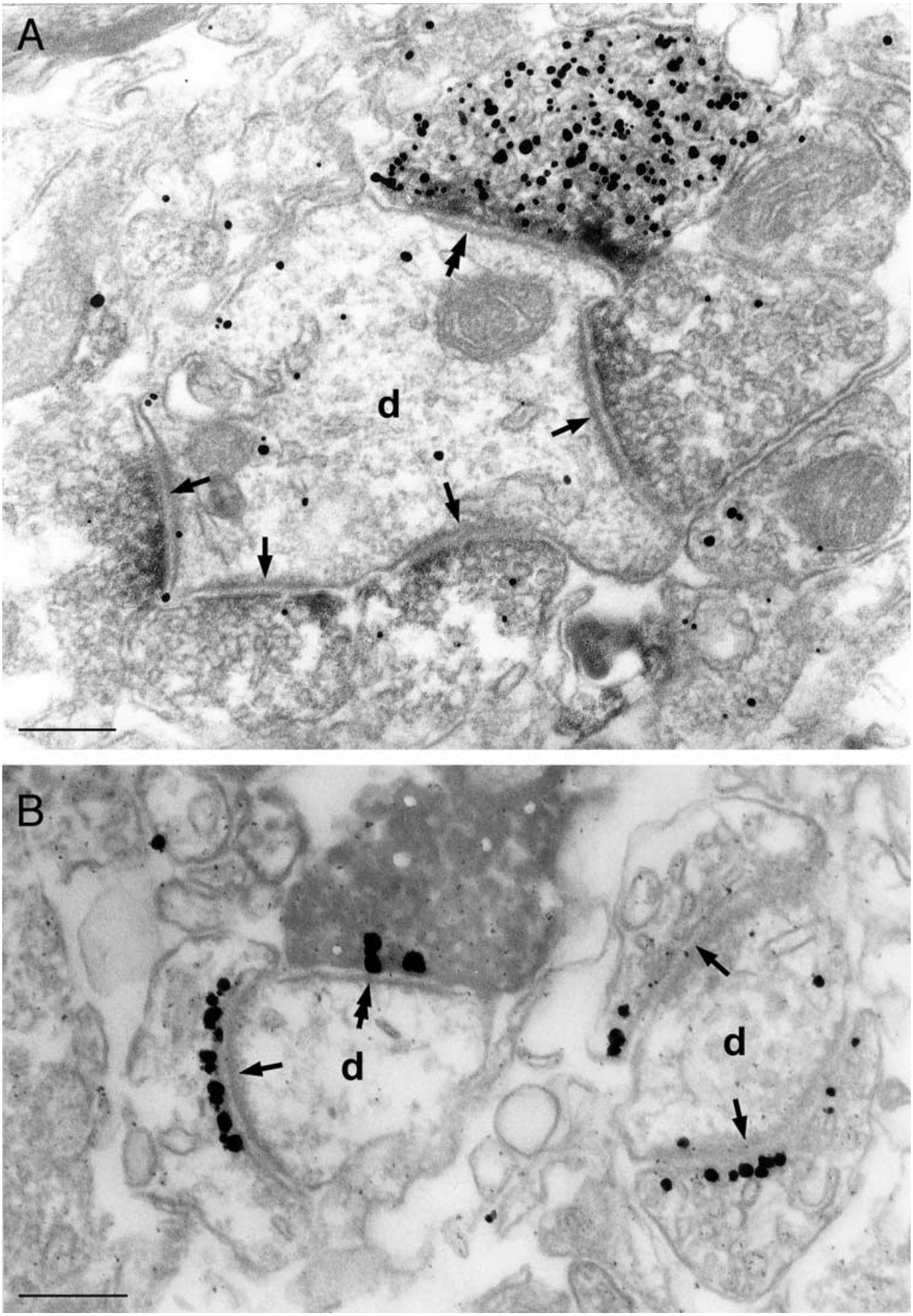


FIG. 1. Many somatostatin-immunopositive neurons express high levels of mGluR1α and their input is enriched in mGluR7a in str. oriens of (A–D) the adult and (E–H) developing (P17) hippocampus. Triple-immunofluorescence labelling for somatostatin, mGluR1α and mGluR7a. There is a good correspondence between the strongly mGluR1α-positive dendrites, mostly originating from somatostatin-positive cells, and the boutons expressing high levels of mGluR7a. Scale bars, 20 μm.

both adult and P15–17-day-old-rats (Fig. 1). Young rats were included in this study because animals at this age are frequently used in electrophysiological experiments to test the function of glutamate receptors on these cells *in vitro* (Pollard *et al*., 2000; Losonczy *et al*., 2002), and because the expression of some receptors may be developmentally regulated. However, no differences in immunostaining patterns were observed between animals of different ages. In addition to the presence of strongly mGluR1α-immunopositive dendrites, at both ages, a few dendrites that displayed weak mGluR1α reactivity were also decorated by mGluR7a-positive terminals.

Distribution of mGluR7a in nerve terminals

Strata pyramidale, oriens and the alveus were systematically studied in sections containing labelling for mGluR7a by either immunoperoxidase or silver-intensified immunogold methods. Electron microscopic examination confirmed that nerve terminals innervating spines had a highly variable immunoreactivity. Terminals innervating dendritic shafts of interneurons also had highly variable immunoreactivity. Some dendritic shafts were innervated mostly by immunonegative boutons, whereas on some other dendritic shafts the majority of boutons making type I and type II synapses in str. oriens and the alveus exhibited a high concentration of labelling for mGluR7a in the presynaptic active zone in both adult (Shigemoto *et al*., 1996) and developing hippocampus. However, such mGluR7a-decorated dendritic shafts were the minority of interneuron dendrites even in str. oriens and the alveus region, where they were most numerous. No mGluR7a immunoreactivity was observed in terminals innervating the axon initial segment or the soma and proximal dendritic shafts of pyramidal cells, after studying at least 50 synapses at each of the three different domains; therefore they were not evaluated further quantitatively. Furthermore, the type II synapses on the somata and dendrites of large, very densely innervated interneurons in and around the pyramidal cell layer, which later were confirmed to be parvalbumin-positive cells (see also Gulyas *et al*., 1999), were also devoid of presynaptic mGluR7a immunoreactivity.


A
d
B
d
d

The amorphous electron-opaque peroxidase reaction product was concentrated at the presynaptic active zone and, depending on the strength of the reaction, diffused amongst the vesicles into the terminal (Fig. 2A). The silver-intensified immunogold particles were lined up at the inner leaflet of the presynaptic membrane, usually along the whole extent of the active zone in well-labelled terminals (Fig. 2B). The two signals were easily differentiated on all the reactions used in the present study. A total of 165 interneuron dendritic profiles receiving 466 type I and type II synapses were analysed. On the mGluR7a-decorated population of dendrites (see definition below), 82% of presynaptic boutons were mGluR7a-immunopositive and 83% of dendrites received more than one (2–16) synapse. Because most type II synapses are made by GABAergic boutons in the hippocampus, next we tested whether those showing mGluR7a immunoreactivity contained immunoreactivity for the enzyme GAD.

Distribution of mGluR7a in GAD-immunopositive terminals

Double immunolabelling for mGluR7a and GAD revealed that the majority of boutons forming type II synapses on mGluR7a-decorated dendrites were indeed GAD-positive as expected (Fig. 2). However, the frequency of mGluR7a immunoreactivity in GAD-positive terminals depended on the method and the sequence of the antibody visualization.

Following immunoperoxidase labelling for GAD as the first reaction, subsequent silver intensification to reveal mGluR7a by immunometal labelling resulted usually only in weak labelling (Fig. 2B) in terminals, which were densely filled with peroxidase product. It is likely that the high density of peroxidase product masked the gold particles and prevented their silver intensification, thereby resulting in a false negative reaction for mGluR7a in some GABAergic terminals. Therefore, silver intensification was carried out as a first reaction for quantitative analysis, followed by the peroxidase enzyme reaction. In this sequence, double-labelled GAD-positive terminals on average had somewhat weaker mGluR7a labelling than the neighbouring GAD-negative terminals. To reduce the possibility of false negative labelling for mGluR7a, GAD was detected with the immunogold method (first reaction) and the sensitive immunoperoxidase reaction was used for visualizing mGluR7a (second reaction) for the quantitative assessment of double-labelled material (Fig. 2A).

Each interneuron probably receives GABAergic innervation from several distinct presynaptic sources and these may not all express the same presynaptic receptors. The majority of synaptic boutons on interneuron dendrites are not GABAergic, and our initial attempt to collect a sample of synapses randomly in str. oriens indicated that the data collections would be prohibitively time consuming. Therefore, we introduced a sampling strategy for the quantitative assessment of only the mGluR7a-decorated dendrites. Dendrites which were innervated by at least one GAD-positive and one mGluR7a-positive bouton within 2–5 serial sections were selected, irrespective of whether the two molecules were in the same terminal or not. This strategy increased the frequency of GABAergic terminals relative to the non-GABAergic terminals in the sample and therefore was not representative for the proportion of GABAergic synapses on the mGluR7a-decorated cells. It also should be pointed out that in the pre-embedding procedure differential penetration of antibodies, even in neighbouring neuronal profiles, cannot be excluded and this might produce false-negative labelling. Nevertheless, at present there is no practical alternative method to provide synaptic data. All selected dendrites were followed in up to 26 serial sections. The synaptic terminals were placed in four categories and this applies to the other molecular markers as well, which are described below: (i) only GAD-positive; (ii) only mGluR7a-positive; (iii) both GAD- and mGluR7a-positive; (iv) immunonegative (Fig. 3).

A total of 56 dendritic shafts receiving 194 synapses in tissue from two adult animals was analysed. The dendrites received on average 3.5 ± 2.9 (SD; range 1–16) synapses in the short series examined. The majority (77%) of GAD-positive boutons ($n = 73$), as well as the majority (87%) of GAD-negative boutons, were mGluR7a-positive (Figs 2–4) giving an overall proportion of 83% mGluR7a-positive terminals on these dendrites. The proportion of mGluR7a-negative boutons was not statistically different between the GAD-positive and GAD-negative ($n = 121$) terminal populations ($P > 0.05$, Fischer's exact test). This could be explained either by the even sampling from a tissue zone, which was uniform with respect to the limitation of penetration of antibodies to mGluR7a, if all terminals in reality contained the receptor, or by a similar proportion of terminals not expressing the receptor in the GAD-positive and -negative populations. To test whether GABAergic terminals detected by a different method and antibody can be characterized better, the postembedding immunogold procedure was used to reveal immunoreactivity for GABA.

Distribution of mGluR7a in GABA-immunopositive terminals

The detection of mGluR7a was carried out using the pre-embedding silver-intensified immunogold reaction alone, and the tissue was embedded in Lowicryl resin to provide high sensitivity for detecting GABA using postembedding immunolabelling. The advantage of the postembedding immunoreaction is that there are no antibody access limitations in the thin electron microscopic section. However, because the detection of mGluR7a proved to be sensitive to high concentrations of glutaraldehyde fixation, which was optimal for GABA, the concentration of glutaraldehyde had to be lowered to 0.1% or 0.2%. Nevertheless, terminals could clearly be divided into GABA-positive and GABA-negative ones, which correlated with the extent of the postsynaptic membrane specialization, being thinner in synapses made by GABA-positive boutons (Fig. 5). In addition to the cytoplasm and vesicle-covered areas, mitochondria also showed high levels of 10-nm immunogold labelling in GABA-positive terminals in comparison with terminals making type I synapses (Fig. 5B). Serial section analysis showed consistent labelling which made the evaluation of boutons unequivocal (Fig. 5). The silver-intensified immunogold particles signalling mGluR7a were much larger and more irregular in shape than the 10-nm immunogold particles signalling GABA (Fig. 5).

The sample included 24 postsynaptic dendrites (73 synapses) from an adult and 21 dendrites (43 synapses) from a P20 rat. Dendrites were selected on the basis of receiving at least one synapse from a GABA-positive bouton and one synapse from an mGluR7a-positive bouton, irrespective of whether the two molecules were in the same terminal.

FIG. 2. Electron micrographs demonstrating mGluR7a immunoreactivity in the presynaptic active zone of GAD-positive boutons. (A) Five mGluR7a-immunolabelled (electron-opaque peroxidase product) boutons make synapses with a dendrite (d). Four boutons make type I synapses (arrows) and are immunonegative for GAD; the fifth bouton making a type II synapse (double arrow) is GAD-positive (gold–silver particles). The peroxidase reaction product is most dense at the presynaptic membrane, but it also spreads amongst the vesicles. Scattered particles over the dendrite indicate that it originates from a GABAergic neuron. (B) mGluR7a-immunolabelled (gold–silver particles) boutons make synapses with dendrites (d). Three boutons making type I synapses (arrows) are immunonegative for GAD; the fourth bouton making a type II synapse (double arrow) is GAD-positive (peroxidase labelling). The gold–silver particles (mGluR7a) are concentrated at the presynaptic membranes. Scale bars, 0.2 μm.

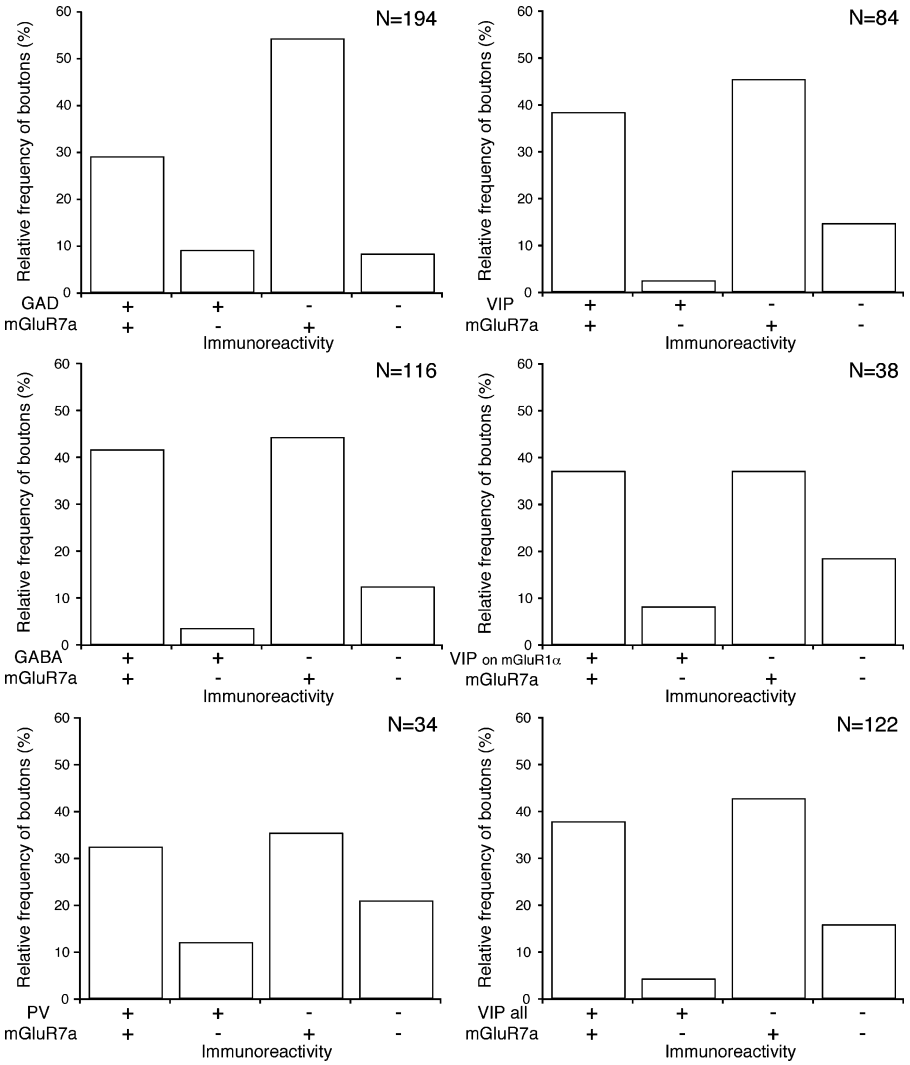


FIG. 3. Relative proportions of synaptic nerve terminals innervating dendrites in str. oriens which received at least one synapse from a bouton immunopositive for mGluR7a, and at least one synapse immunopositive for one of the other tested molecules. The distribution of boutons shows that the majority of synapses were immunopositive for mGluR7a on these dendrites, including the majority of boutons shown or presumed to originate from GABAergic neurons. Note that the selection criterion of having one synapse from a putative GABAergic bouton strongly increased their proportion and is not representative for the total population of boutons on these cells.

The dendrites received on average 2.5 ± 1.6 (SD; range 1–10) synapses in the short series examined. Boutons were placed in four categories as for GAD-reacted terminals. There was no statistical difference between the two animals in the relative proportions of the four categories of boutons ($P > 0.05$, χ^2 test); therefore the two samples were pooled. The majority (94%) of GABA-positive boutons as well as the majority (80%) of GABA-negative boutons were mGluR7a-positive (Figs 3 and 4) giving an overall proportion of 86% mGluR7a-positive terminals on these dendrites, a value very similar to the GAD-tested sample. Terminals positive or negative for GABA did not differ statistically in the fraction of mGluR7a-positive synapses ($P > 0.05$, Fischer's exact test).

There are multiple sources of GABA in the hippocampus and some of the GABA-releasing neurons can be differentiated based on the expression of molecular markers such as calcium-binding proteins and neuropeptides. We attempted to identify the source of mGluR7a-expressing synaptic boutons using antibodies to parvalbumin and VIP, two molecules which are known to be expressed by distinct sets of presynaptic neurons (Freund & Buzsaki, 1996).

Frequency of mGluR7a immunoreactivity in parvalbumin-positive terminals

In the alveus and the bordering str. oriens, where mGluR7a-positive terminals were most frequent, parvalbumin-immunoreactive terminals were rare. To ensure that as many parvalbumin-positive terminals as possible were sampled, the highly sensitive immunoperoxidase method was used, and mGluR7a was detected by the silver-intensified immunogold reaction in two adult animals. Fifteen dendrites (34 synapses) were found that received at least one synapse from a parvalbumin-immunopositive and one synapse from an mGluR7a-positive terminal (Fig. 6A and B). The dendrites received, on average, 2.3 ± 0.8 (SD; range 1–4) synapses in the short series examined. The parvalbumin-positive terminals expressed a noticeably lower level of mGluR7a than the parvalbumin-negative terminals making synapses on the same dendrites. Both the mGluR7a-positive and -negative parvalbumin-expressing terminals made type II synapses. The majority (73%) of parvalbumin-positive boutons as well as the majority (63%) of parvalbumin-negative boutons were mGluR7a-positive

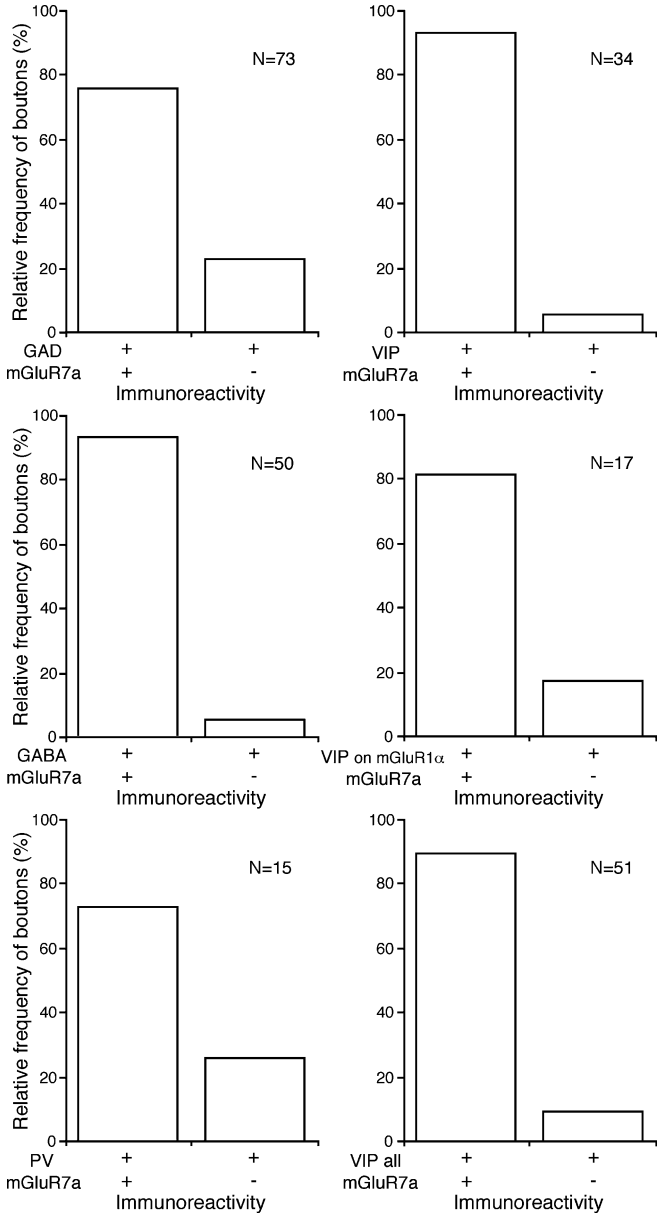


FIG. 4. Proportions of mGluR7a-immunopositive synaptic nerve terminals as a fraction of boutons positive for one of the putative GABAergic markers. Part of the same sample shown in Fig. 3, as innervating dendrites, which received at least one synapse from a bouton immunopositive for mGluR7a and at least one synapse immunopositive for one of the other tested markers. The data show that the majority of putative GABAergic synapses were immunopositive for mGluR7a on these dendrites.

(Figs 3 and 4) giving an overall proportion of 68% mGluR7a-positive terminals on these dendrites, a value somewhat lower than in the GAD- and GABA-tested samples. This indicated that either a somewhat different dendrite population which receives input with less presynaptic mGluR7a-expressing boutons was sampled, or that the mGluR7a reaction strength was lower in the parvalbumin-reacted specimens. The latter is less likely, because the parvalbumin and VIP reactions described below were conducted together.

Some of the postsynaptic dendrites decorated by mGluR7a-immunoreactive terminals were weakly parvalbumin-positive. There were also strongly parvalbumin-positive dendrites in str. oriens and pyramidale, which originated from strongly parvalbumin-positive somata in and around the pyramidal cell layer. The type II synapses on these somata and dendrites lacked presynaptic mGluR7a immunoreactivity.

Frequency of mGluR7a immunoreactivity in VIP-immunoreactive terminals

Interneurons expressing mGluR1α in str. oriens are innervated by local VIP-positive interneuron-specific (IS) neurons (Blasco-Ibañez & Freund, 1995; Acsady *et al*., 1996), which are thought to be GABAergic. Although the proportion of VIP-positive terminals in the GABAergic innervation of mGluR1α-expressing cells is not known, in view of the above data showing that 77–94% of GABAergic terminals are mGluR7a-positive, it was predicted that VIP-positive boutons were in the mGluR7a-positive population. This hypothesis has been tested. Immunoreactivity for VIP was visualized by peroxidase reaction, because the immunogold method gave a low detection rate, and mGluR7a was visualized by silver-intensified immunogold reaction in three adult animals. Quantitative evaluation of a total of 32 dendrites receiving at least one synapse from a VIP-positive bouton and one synapse from an mGluR7a-postive bouton (Fig. 6C) showed that virtually all (94%) of the VIP-positive boutons as well as the majority (76%) of VIP-negative boutons were mGluR7a-positive (Figs 3 and 4). The two VIP-positive boutons that were mGluR7a-negative made type II synapses. The dendrites received, on average, 2.6 ± 1.0 (SD; range 1–5) synapses in the short series examined. The overall proportion of mGluR7a-positive terminals on these dendrites was 83%, very similar to the GAD- and GABA-tested populations.

Frequency of VIP and mGluR7a-immunoreactive terminals innervating mGluR1α-positive dendrites, and overall distribution

Although mGluR1α-expressing cells are a major target of VIP-positive IS cells, it is not yet known whether all VIP-positive terminals target these cells, or whether some of the other interneurons in str. oriens and alveus, which do not express mGluR1α, are also amongst the recipients. This is an issue because, due to our selection criteria, some dendrites in the above sample received only one synapse from a VIP and mGluR7a double-labelled terminal. We tested the mGluR1α-expressing dendrites by a triple immunoreaction relying on the exclusively postsynaptic membrane location of mGluR1α (silver–gold signal) outside the postsynaptic density, and the location of mGluR7a (silver–gold signal) in the presynaptic active zone, as well as using the peroxidase reaction product to detect VIP-positive terminals (Fig. 6D and E). In sections perpendicular to the membrane, the silver–gold particles were at the cytoplasmic face of the plasma membrane, so their allocation to pre- or postsynaptic cells was straightforward. In tangential sections of the plasma membrane, it is not always possible to allocate the particles to one of the plasma membranes; therefore the sections were tilted in the electron microscope. When this failed to reveal the location of the particles unequivocally, the dendrite was not included in the sample. A total of 17 dendrites (38 synapses) was analysed from one 15–17-day-old and one adult animal. The dendrites received, on average, 2.2 ± 1.1 (SD; range 1–5) synapses. The majority (82%) of VIP-positive boutons as well as the majority (67%) of VIP-negative boutons were mGluR7a-positive (Figs 3 and 4) on the mGluR1α-positive dendrites. The overall proportion of mGluR7a-positive terminals on these dendrites was 74%, a somewhat lower proportion than that in the VIP-, GAD- and GABA-tested populations. This probably is due to the overall weaker mGluR7a reactivity in these samples.

However, comparison of the frequency of the four categories of terminals in this sample with that obtained in the reaction without including the antibody to mGluR1α showed no statistical difference ($P > 0.1$, χ^2 test). Therefore, the two samples were pooled. Overall, 49

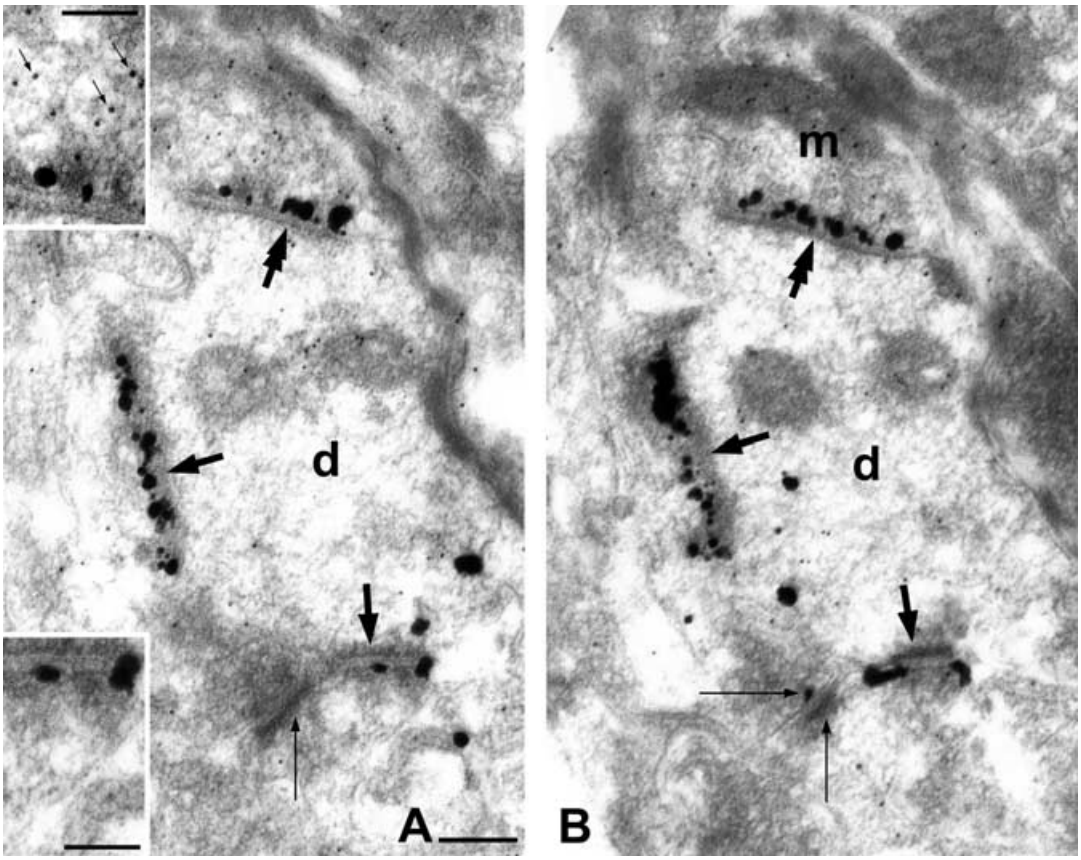


FIG. 5. Immunoreactivity for mGluR7a in the presynaptic active zones of a GABA-positive and GABA-immunonegative boutons. (A and B) Electron micrographs of serial sections reacted for mGluR7a (pre-embedding reaction, large gold–silver particles) followed by Lowicryl embedding and postembedding reaction for GABA (10-nm particles). A dendrite (d) receives synapses from two GABA-negative (arrows) and one GABA-positive (double arrow) bouton, the latter making a type II synapse. The bouton is judged GABA-positive because the cytoplasm and vesicle covered area (small oblique arrows in upper left inset) as well as the mitochondrion (m, in B) contain higher density of 10 nm gold, than the boutons making type I synapses (bottom left inset). The insets show equivalent areas. One of the GABA-negative boutons (bottom) makes a type I synapse; the other one is cut tangentially and cannot be assessed. A nearby small bouton is only labelled with one particle in B (horizontal small arrow) and makes a type I synapse (vertical small arrow) with a presumed spine. Note the lack of 10-nm gold particles over the boutons making type I synapses. Scale bars, 0.2 μm (A and B), 0.1 μm (insets).

dendrites receiving 122 synaptic boutons were evaluated. The majority (90%) of VIP-positive boutons as well as the majority (73%) of VIP-negative boutons were mGluR7a-positive (Figs 3 and 4) on the sampled dendrites. The overall proportion of mGluR7a-positive terminals on these dendrites was 80%.

Comparison of mGluR7a immunoreactivity in neurochemically identified terminals

The electron microscopic tests for the coexistence of mGluR7a with GAD, GABA, parvalbumin and VIP were conducted separately and the postsynaptic dendrite populations may not necessarily be the same. The selection criteria of interneuron dendrites were designed to ensure that, as far as possible, similar dendrite and terminal populations were tested. Unfortunately, due to the low frequency of GABAergic terminals on dendrites in random electron microscopic sections, a complete statistical comparison of all postsynaptic profiles in str. oriens proved prohibitively time consuming, so we restricted the sample to mGluR7a-decorated dendrites. In order to minimize the probability of type I error (incorrect rejection of null hypothesis) we accepted a significant $P \leq 0.01$ for the subsequent χ^2 tests. There was no difference in the mean number of synapses per dendritic profile analysed in the four samples ($P > 0.05$, Kruskal–Wallis test) and overall the dendrites received, on average, 2.8 ± 2.0 (SD; range 1–16) synapses.

The frequency of the four categories of synaptic terminals in the four samples [reacted for GAD, GABA, parvalbumin, VIP (pooled)] was not statistically different ($P > 0.01$, χ^2 test). However, subsequent analysis by subdividing the statistical contingency table (Zar, 1999) showed that the GAD-tested population was significantly different from the pooled population of remaining immunoreactions ($0.001 < P < 0.005$, χ^2 test), whereas exclusion of the GAD population from the analysis supported the null hypothesis that there were no differences in the terminal frequencies reacted for GABA, parvalbumin and VIP (pooled). Comparisons between GABA vs. parvalbumin, GABA vs. VIP (pooled) and parvalbumin vs. VIP (pooled) showed no statistically significant differences. Further analysis showed that the GAD-reacted population was not significantly different from the GABA-($P > 0.01$, χ^2 test), the pooled VIP- ($P > 0.01$) or the parvalbumin-reacted ($P > 0.05$) populations. The fraction of mGluR7a-positive boutons in the subpopulations of terminals immunopositive for GAD, GABA, parvalbumin or VIP (pooled) was not different ($P > 0.05$). Therefore, it is likely that we tested a similar dendritic population in the above reactions.

Presence of mGluR7b immunoreactivity in the CA1 area

The above data indicate that there is a strong expression of mGluR7a in GABAergic terminals on a subpopulation of interneurons, but

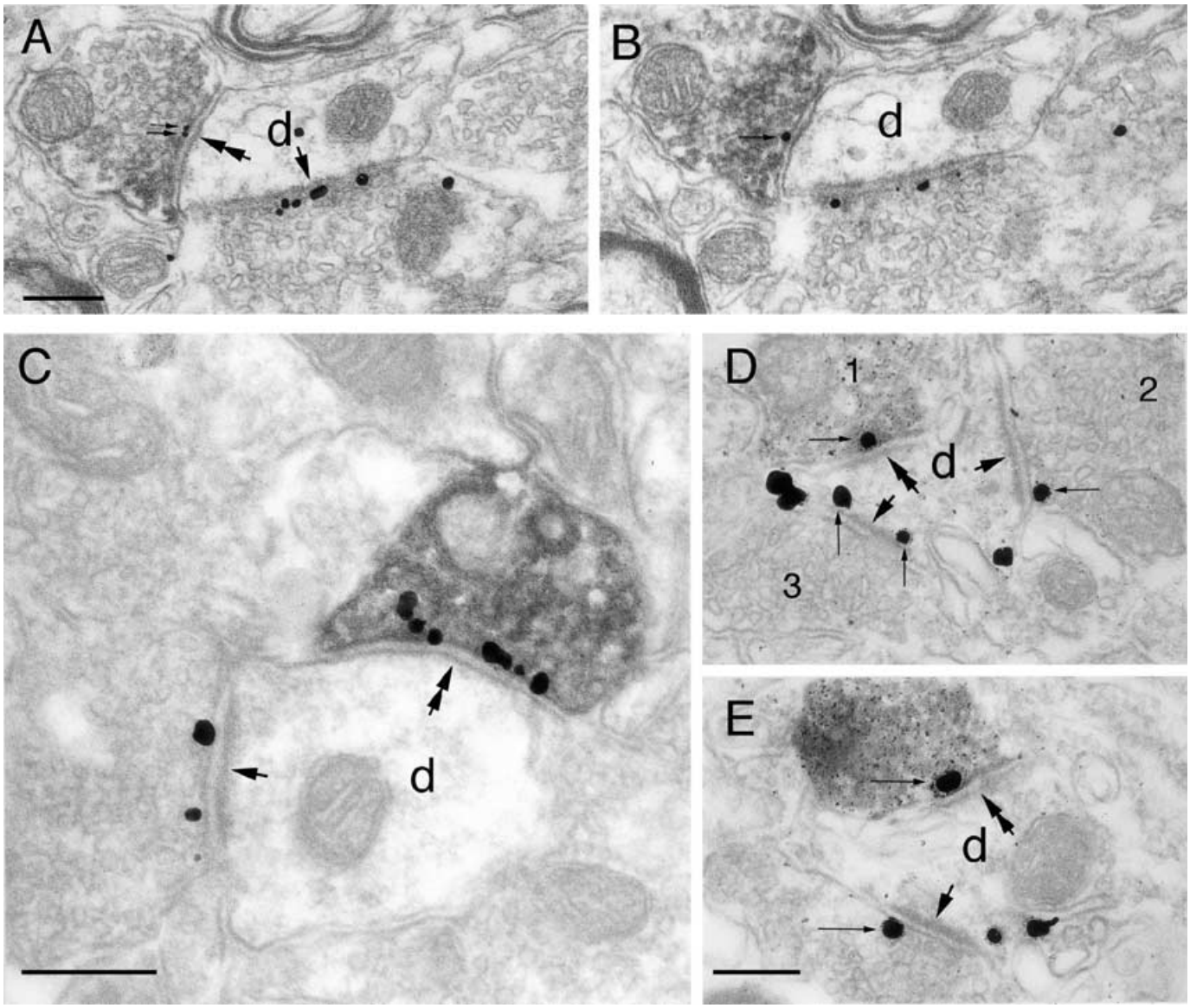


FIG. 6. Electron micrographs showing (A and B) parvalbumin- or (C–E) VIP-immunopositive boutons expressing mGluR7a (gold–silver particles), in the presynaptic active zone. (A and B) Serial sections of a dendrite (d) receiving a type II synapse (double arrow) from a parvalbumin-positive bouton (peroxidase labelling), which is also labelled for mGluR7a (small horizontal arrows). A parvalbumin-negative bouton making a type I synapse is more heavily labelled for mGluR7a. (C) A VIP-positive bouton (peroxidase labelling) making a type II synapse (double arrow) is strongly labelled for mGluR7a. A nearby VIP-immunonegative bouton making a type I synapse (arrow) is also mGluR7a-immunopositive. (D and E) Two nonconsecutive sections from a series cut of a dendrite (d), which is immunopositive for mGluR1α (gold–silver particles) in perisynaptic (small vertical arrows) and in extrasynaptic (unlabelled) positions. It receives synapses from three boutons (1–3) labelled for mGluR7a (gold–silver particles, small horizontal arrows) in the active zones. Bouton 1, also labelled for VIP (peroxidase product), makes a type II synapse (double arrow); boutons 2 and 3 make type I synapses. Note that both in the dendrite and in the terminals the metal particles are associated with the cytoplasmic face of the plasma membrane corresponding to intracellular epitopes of the receptors. Scale bars, 0.2 μm.

not in the somatic and perisomatic GABAergic terminals innervating pyramidal cells. However, high concentrations of the group III mGluR agonist L-AP4 also suppress IPSCs in pyramidal cells (Kogo *et al*., 1999). Therefore, we re-examined the possible expression of the other splice variant, mGluR7b, in the CA1 area. The strongest expression of mGluR7b is in the terminals of dentate granule cells innervating interneurons, but in the CA3 area it was also reported in type II synapses, which may be GABAergic (Shigemoto *et al*., 1997). In the alveus and the neighbouring str. oriens of the CA1 area, weak but consistent immunoreactivity for mGluR7b was observed by triple-labelling immunofluorescence reaction. The soma and dendrites of somatostatin–mGluR1α immunolabelled cells were decorated (not shown), just as for mGluR7a. In the immunoperoxidase labelling, the pattern in the alveus and str. oriens also resembled the patterns obtained with antibodies to mGluR1α and mGluR7a. In addition, rarely, interneurons were also decorated by mGluR7b-immunopositive terminals in str. radiatum. No immunoreactivity was apparent in str. pyramidale. Electron microscopic examination of str. oriens and alveus revealed mGluR7b immunoperoxidase reaction in the presynaptic active zone of terminals making either type I or type II synapses (Fig. 7). The colocalization of mGluR7b with the other molecules examined in this study was not pursued due to the relatively weak reaction obtained.

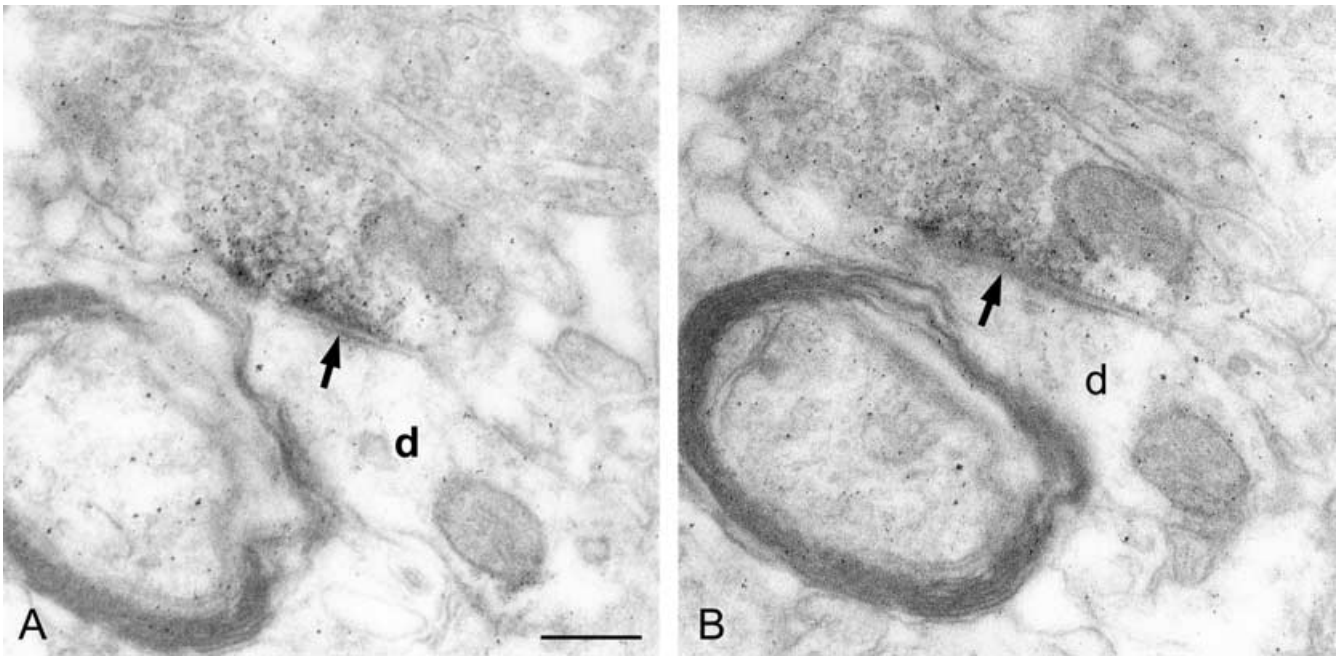


FIG. 7. Electron micrographs of serial sections of a dendrite (d) in str. oriens receiving a type II synapse (arrow) from a bouton labelled by electron-opaque peroxidase reaction product for mGluR7b in the presynaptic active zone. Immunoperoxidase reaction; the small particles are due to inadequate washing before osmium treatement. Scale bar, 0.2 μm.

Discussion

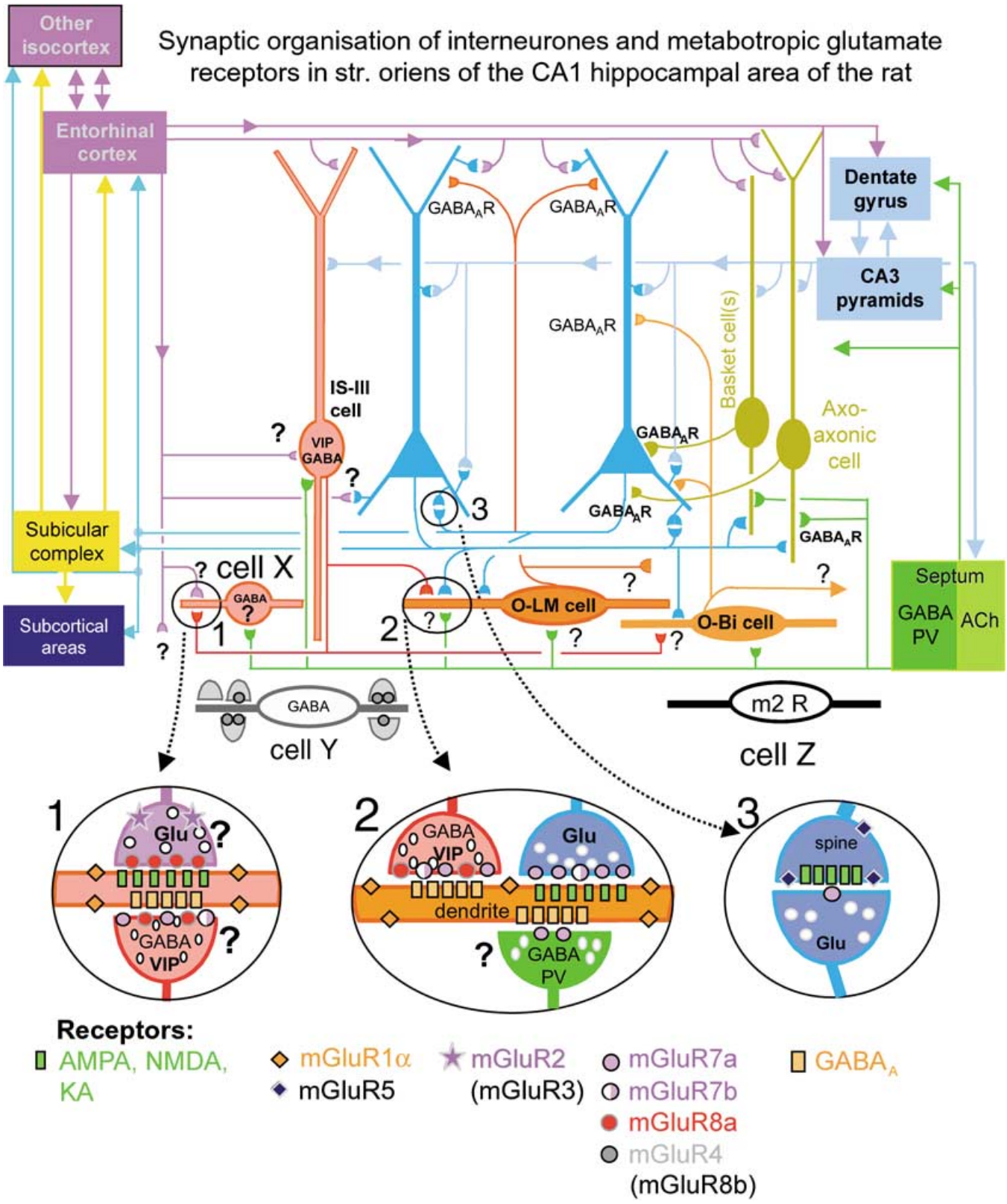
The results show that the GABAergic input of interneurons that receive innervation from mGluR7a-enriched glutamatergic terminals is also enriched in mGluR7a compared to GABAergic terminals on pyramidal cells or most other interneurons in str. oriens. The mGluR7a-decorated interneurons are mostly oriens–lacunosum moleculare (O-LM) cells. They were named so in the CA1 area (McBain *et al*., 1994), because their soma and dendrites are mostly located in str. oriens and their axon projects to str. lacunosum-moleculare. A major GABAergic input to these cells originates from VIP-positive terminals, which have been shown directly to express high levels of mGluR7a in the presynaptic active zone. The selectively high level of presynaptic mGluR7 on both glutamatergic and GABAergic terminals on the mGluR1α–somatostatin-expressing hippocampal interneurons suggests that these cells govern presynaptic receptor levels via a retrograde signal in most of their synaptic input, irrespective of the transmitter released. A homologous circuit and receptor distribution was reported in the isocortex (Dalezios *et al*., 2002), indicating that such an organization is fundamental to cortical function. For the interpretation of these results one must consider the identity and the position of the postsynaptic interneuron(s) in the hippocampal network, which is shown schematically in Fig. 8 (see below).

Is the expression of mGluR7 in GABAergic terminals target-cell-specific along the same axon?

The demonstration of mGluR7a in GABAergic terminals and mGluR7b in type II synapses confirms and extends a previous demonstration of group III mGluRs in type II synapses of the hippocampus (Shigemoto *et al*., 1997). Another study demonstrated the high level of mGluR7a expression in type I synapses on some interneurons (Bradley *et al*., 1996), but did not report the presence of mGluR7a in type II hippocampal synapses.

A target-cell-specific enrichment of presynaptic mGluR7a was directly proven for glutamatergic terminals along the same pyramidal cell axon (Shigemoto *et al*., 1996). In addition, a group III mGluR-mediated target-cell-specific EPSP suppression has also been reported (Scanziani *et al*., 1998). We were not able to test this principle for GABAergic terminals. Some GABAergic neurons innervate both pyramidal cells and several types of interneuron (Cobb *et al*., 1997; Katona *et al*., 1999; Pawelzik *et al*., 2002), whereas others, such as the axo-axonic cell, innervate either only pyramidal cells (Somogyi *et al*.,

FIG. 8. Summary of the known or assumed location of mGluRs in str. oriens of the hippocampal CA1 area. Many details remain to be clarified and several types of interneuron published previously (e.g. X, Y and Z), mainly on the basis of immunocytochemical labelling, remain to be rigorously defined, but the scheme may provide a framework for discussion and further analysis. Pyramidal cells receive GABAergic innervation from domain-specific presynaptic interneurons acting through $GABA_A$ receptors and express mGluR5, particularly at a perisynaptic location in spines (3). The glutamatergic terminals innervating spines express a variable level of presynaptic mGluR7a. The O-LM cells, expressing mGluR1α and somatostatin, are decorated by strongly mGluR7a-expressing GABAergic and glutamatergic terminals (2). Many terminals also express mGluR7b to a small extent. The GABAergic terminals originate mainly from VIP-expressing IS-III interneurons, which are also mGluR1α-positive. Some parvalbumin-positive GABAergic boutons expressing mGluR7a may originate in the septum or locally. The origin of mGluR8a-expressing terminals is not known; a potential source is the entorhinal cortex, which sends a small projection to the str. oriens and is known to express mGluR2 (1). Some local GABAergic neurons may also express mGluR8a, which may be colocalized with mGluR7a in the input to some cells. No presynaptic mGluRs have been found on basket or axo-axonic cell terminals so far. The relationship of the mGluR8a-decorated cells (X), the cells richly decorated by mGluR4-immunopositive terminals (Y) and the strongly muscarinic (m2) receptor-expressing cells (Z) remains to be defined. The possible presence and location of mGluR3 and mGluR8b on neurons has not been established. Presynaptic kainate receptors and group II mGluRs on GABAergic terminals are not included. Question marks indicate assumptions or unknown identity.


Synaptic organisation of interneurones and metabotropic glutamate receptors in str. oriens of the CA1 hippocampal area of the rat
Other isocortex
Entorhinal cortex
Dentate gyrus
CA3 pyramids
Subicular complex
Subcortical areas
Septum
GABA PV
ACh
GABA$_A$R
Basket cell(s)
Axo-axonic cell
IS-III cell
VIP GABA
cell X
GABA
O-LM cell
O-Bi cell
cell Y
cell Z
m2 R
Glu
dendrite
spine
Receptors:
AMPA, NMDA, KA
mGluR1α
mGluR5
mGluR2
(mGluR3)
mGluR7a
mGluR7b
mGluR8a
mGluR4
(mGluR8b)
GABA$_A$

1983), or only other interneurons (Acsady *et al*., 1996; Gulyas *et al*., 1996). Most GABAergic terminals on mGluR7a-decorated dendrites were labelled for mGluR7a whereas those on pyramidal cell somata and axon initial segment were not. This cannot be taken as evidence for a target-cell-specific expression of the receptor along the same axon, as the GABAergic terminals to the somatic–perisomatic region of pyramidal cells and to the mGluR7a-decorated dendrites very probably originate from different presynaptic interneurons (Freund & Buzsaki, 1996). Due to our sampling criteria we obtained data only for mGluR7a-decorated interneuron dendrites, predominantly originating from O-LM cells (Losonczy *et al*., 2002), which may receive GABAergic innervation mainly from the VIP containing interneuron-specific (IS)-III type cells (Acsady *et al*., 1996). It is not known whether IS-III cells only innervate O-LM cells. There are many types of neuron with horizontal dendrites in str. oriens and alveus, where the IS-III cells distribute their axons. Nevertheless, there is a strong association of VIP terminals with mGluR1α-labelled dendrites suggesting a preferential innervation. In the current work, mGluR7a-immunopositive type II, or identified GABAergic synapses, which were not associated with mGluR7a-decorated dendrites, were extremely rare in str. oriens and alveus. At present the most parsimonious explanation for the selective mGluR7a labelling of GABAergic terminals is that the IS-III VIP-containing interneurons express mGluR7 throughout their terminal arbor, which preferentially targets the O-LM cells. However, if some terminals of IS-III cells innervate other cell types, it would be interesting to examine whether those terminals also expressed mGluR7a.

The effect of group III mGluR activation on IPSPs and IPSCs in cortical neurons

Several presynaptic glutamate receptor types regulate GABA release. The most widely studied of these are kainate receptors (Min *et al*., 1999; for review see Kullmann, 2001; Lerma *et al*., 2001) and mGluRs. Both group II and group III mGluRs were shown to depress glutamate as well as GABA release from hippocampal neurons (for review see Anwyl, 1999; Schoepp, 2001). Activation of group III mGluR1 probably reduces transmitter release through reducing of calcium channel activity and/or cAMP production (Takahashi *et al*., 1996; Perroy *et al*., 2000; Millán *et al*., 2002), or by activating a potassium conductance, as in the lamprey reticulo-spinal axon (Cochilla & Alford, 1998).

In hippocampal pyramidal cells, the group III mGluR agonist L-AP4 (1 mM, rat, Gereau & Conn, 1995; 50 μM, guinea pig, Semyanov & Kullmann, 2000) was without effect on monosynaptic IPSCs, but 50 μM L-AP4, or extracellular stimulation-evoked transmitter release, depressed IPSCs in unidentified interneurons of str. radiatum in the hippocampus of the guinea pig (Semyanov & Kullmann, 2000). This effect was blocked by the group III mGluR antagonist alpha-methyl-serine-O-phosphate. The effectiveness of a relatively low concentration of L-AP4 and the antagonist suggests that receptors other than mGluR7 were involved. The same concentration of L-AP4 also depressed IPSCs in str. oriens interneurons, some of which were identified as O-LM and oriens–bistratified (O-Bi) cells in the rat (Kogo *et al*., 1999). A higher concentration of L-AP4 (500 μM) did not significantly increase the degree of suppression suggesting either, (i) that both low and high affinity group III mGluRs are present on the same GABAergic terminals, or (ii) that the *in situ* affinity of mGluR7 for L-AP4 in the active zone of nerve terminals is different from that measured in cellular test systems (Okamoto *et al*., 1994; Wu *et al*., 1998). In support of the involvement of other group III mGluRs in the suppression of IPSCs, both mGluR4 (Corti *et al*., 2002) and mGluR8 (Dalezios *et al*., 2001) have been found in boutons making type II synapses and innervating interneurons in str. oriens.

Another interesting feature of the input of O-LM and the homologous bitufted cells of the isocortex is the strong frequency facilitation of their glutamatergic inputs (Ali & Thomson, 1998; Markram *et al*., 1998; Reyes *et al*., 1998), and the unchanged amplitude of IPSCs evoked by paired-pulse stimulation (Reyes *et al*., 1998; Kogo *et al*., 1999), which is in contrast to most other interneurons. These dynamic properties are due to a relatively low initial transmitter release probability of the input synapses (Reyes *et al*., 1998; Rozov *et al*., 2001). Because mGluR7 activation probably depresses transmitter release, it is possible that glutamate and GABA release to O-LM cells are regulated similarly and may be related to the expression of mGluR7. The molecular mechanisms and time scale of such regulation of transmitter release remain to be tested. However, it is noteworthy that an mGluR7-like receptor was implicated in long-term depression of glutamatergic inputs to some hippocampal interneurons (Laezza *et al*., 1999), and in the mGluR7-deficient mouse the recovery to the initial low release probability from frequency facilitation is delayed in isocortical bitufted cells (Sansig *et al*., 2001).

What endogenous agonist(s) activate mGluR7?

Disruption of the mGluR7 gene leads to a number of behavioural changes in mice (Masugi *et al*., 1999; Sansig *et al*., 2001), but the circuits that are disrupted have not been identified. The presence of the receptor in terminals which release GABA and probably not glutamate raises the question of the identity of the endogenous agonist for mGluR7a. The most frequent scenario is that heteroreceptors are activated by glutamate spilling over from neighbouring glutamatergic synapses (Mitchell & Silver, 2000; Semyanov & Kullmann, 2000; Woodhall *et al*., 2001; for review see Vizi & Kiss, 1998). However, glutamate has a low efficacy at mGluR7 (Okamoto *et al*., 1994; Wu *et al*., 1998) which, therefore, may not be activated this way. Furthermore, the release of an agonist for presynaptic group III mGluRs from the GABAergic terminal cannot be excluded. High concentration of both GABA and glutamate have been reported in the terminals of hippocampal granule cells (Sandler & Smith, 1991) and in some terminals innervating spinal cord motoneurons (Somogyi, 2002). The activation of hippocampal granule cells has been reported to evoke postsynaptic responses mediated by both glutamate and $GABA_A$ receptors (Walker *et al*., 2001), indicating the release of both transmitters. The endogenous amino acid content of GABAergic terminals innervating O-LM cells and expressing group III mGluRs is not known. As they very probably originate from a specialized subclass of interneurons, they may have specific neurochemical properties not shared by the more widely studied interneurons innervating pyramidal cells. In addition, a vesicular glutamate transporter, VGLUT3, is expressed by some hippocampal interneurons making type II synapses in addition to neurons using ACh or 5-HT as transmitters (Fremeau *et al*., 2002; Gras *et al*., 2002). It remains to be clarified whether VGLUT3 transports glutamate in GABAergic terminals, and whether these particular terminals express group III mGluRs. A potential further source of endogenous agonist for presynaptic mGluRs is the postsynaptic dendrite, which may release substances such as L-serine-O-phosphate.

The two splice variants mGluR7a and mGluR7b may be coexpressed in synapses on O-LM cells

Immunolabelling for mGluR7b was much weaker than for mGluR7a in the CA1 area, but both splice variants were enriched in the input of mGluR1a–somatostatin-expressing cells, indicating coexpression in the same terminals, although this was not directly tested in the present study. The same pattern was also described for mossy fibre terminals and the axon collaterals of granule cells innervating mGluR1α-positive

interneurons in the hilus and CA3 area (Shigemoto *et al*., 1997). However, in contrast to the mossy fibre synapses, which express stronger immunoreactivity for mGluR7b than for mGluR7a, the terminals innervating str. oriens interneurons have much higher immunoreactivity for mGluR7a than that for mGluR7b. This comparison of relative immunoreactivities is valid, because it is made in the same sections. Most of the terminals innervating the CA1 str. oriens cells originate from CA1 pyramidal cells (Blasco-Ibañez & Freund, 1995). Therefore it is likely that these terminals when making synapses with O-LM cells (Ali & Thomson, 1998), express both splice variants in the presynaptic active zone (Fig. 8). A comparison with the mossy terminals shows that the relative proportion of the two splice variants is presynaptic cell-type-specific. The same rule may apply to GABAergic terminals on these cells as we found type II synapses immunoreactive for mGluR7b (Fig. 8), but their origin and neurochemical nature remains to be tested directly.

Sources of GABAergic innervation of interneurons in str. oriens

The GAD–mGluR7a-immunopositive terminals originate from several sources, as indicated by their parvalbumin and VIP content, which are unlikely to be colocalized to a significant extent. Most of the postsynaptic interneurons in the present sample belong to the somatostatin–mGluR1α-expressing cells, which include O-LM cells and possibly a class of projection neuron (Ferraguti *et al*., 2003). The vast majority of them are probably O-LM cells (Pollard *et al*., 2000; Maccaferri *et al*., 2000; Losonczy *et al*., 2002). These neurons are innervated by the GABAergic IS-III cells (Acsady *et al*., 1996), some of which have cell bodies in str. pyramidale and radiatum and express VIP and calretinin (Acsady *et al*., 1996; Gulyas *et al*., 1996). Therefore, it is very likely that the mGluR7a–VIP-immunolabelled terminals originate from IS-III cells. This is supported by the high proportion of mGluR7 expression in VIP-expressing GABAergic cells in the isocortex (Cauli *et al*., 2000). Because these neurons do not appear to express parvalbumin, the mGluR7a–PV-immunopositive terminals originate either from another type of hippocampal interneuron or from the medial septum, which sends a GABAergic projection to the hippocampus, and these axons are immunopositive for parvalbumin (Freund & Buzsaki, 1996). However, whether the terminals express detectable levels of parvalbumin remains to be demonstrated; the scheme in Fig. 8 should therefore be considered tentative. At least one type of parvalbumin-expressing interneuron, the O-LM cell (Maccaferri *et al*., 2000; Ferraguti *et al*., 2001; Klausberger *et al*., 2003), has a limited axonal arborization selectively in str. oriens (Ali & Thomson, 1998; Maccaferri *et al*., 2000). Furthermore, there are several other parvalbumin-expressing cell types in both str. oriens and pyramidale which might contribute to the innervation of interneurons (Freund & Buzsaki, 1996; Fukuda & Kosaka, 2000). Therefore, the origin of the parvalbumin-positive boutons studied here remains to be established.

Selective expression of presynaptic mGluRs in relation to specific cell types and circuits

The highly concentrated expression of mGluR7 in the input of specific interneuron classes also applies to mGluR4 (Shigemoto *et al*., 1997; Corti *et al*., 2002) and mGluR8a (Shigemoto *et al*., 1997; Dalezios *et al*., 2001), which have been found at elevated levels in the input of some, as yet undefined, interneurons. We have summarized the most likely distribution of presynaptic mGluRs in relation to some str. oriens interneurons in the CA1 area (Fig. 8). Many details remain to be clarified (indicated by '?' in Fig. 8), and several types of interneuron published previously, mainly on the basis of immunocytochemical labelling, remain to be rigorously defined. Nevertheless, the scheme may provide a framework for discussion and further analysis.

Pyramidal cells receive GABAergic innervation from domain-specific presynaptic interneurons (only four shown in Fig. 8) acting via $GABA_A$ receptors (Miles *et al*., 1996; Ali & Thomson, 1998; Ali *et al*., 1999; Maccaferri *et al*., 2000; Martina *et al*., 2000; Thomson *et al*., 2000; Pawelzik *et al*., 2002) and express mGluR5, particularly at a perisynaptic location in spines (Lujan *et al*., 1996). The glutamatergic terminals innervating spines express a variable level of presynaptic mGluR7a (Shigemoto *et al*., 1996); some terminals can have the same high density of immunolabelling for mGluR7a as those innervating O-LM cells. The mGluR1α–somatostatin-expressing O-LM cells are decorated by strongly mGluR7a-expressing glutamatergic (Shigemoto *et al*., 1996; Losonczy *et al*., 2002) and GABAergic terminals. Many of the terminals also express mGluR7b to a small extent. Here, we have found mGluR7b in terminals making type II synapses, but we have not identified the postsynaptic cell or the source of the terminals; therefore, the suggestion of mGluR7b in terminals expressing mGluR7a, as depicted in Fig. 8, remains hypothetical. The GABAergic terminals innervating O-LM cells originate mainly from VIP-expressing interneurons (Acsady *et al*., 1996), which are also mGluR1α-positive (Ferraguti *et al*., 2001, 2003). It is not yet known whether these VIP-containing interneurons exclusively innervate the O-LM cells and probably each other, or innervate other interneurons such as the O-Bi cells. Some parvalbumin-positive GABAergic boutons expressing mGluR7a may also innervate O-LM cells, as suggested by the present results, but their origin is not known. Some or all of them may originate in the septum, which sends a GABAergic projection innervating somatostatin-expressing cells (Freund & Buzsaki, 1996). However, there are several classes of somatostatin-expressing cells (Freund & Buzsaki, 1996; Katona *et al*., 1999; Maccaferri *et al*., 2000; Ferraguti *et al*., 2001) so the innervation of O-LM cells remains to be tested directly.

In the simplest scenario, the depression of GABA release by mGluR7 activation could lead to a disinhibition of O-LM cells and increased GABA release at their terminals in the entorhinal input zone to pyramidal cells (Fig. 8). However, if glutamate released by CA1 and CA3 pyramidal terminals was the endogenous agonist activating mGluR7, it would also suppress glutamate release as these terminals are also endowed with a high level of mGluR7. The O-LM cells are able to fire repetitive action potentials at beta frequency over long periods *in vivo*, rhythmically, phase-locked to the theta rhythm (Klausberger *et al*., 2003). They are inhibited during high frequency pyramidal cell population bursts called sharp waves (Klausberger *et al*., 2003), lasting for ≈100 ms, when extracellular glutamate levels are likely to be at their highest. However, such an event may be too rapid and transient for significant mGluR7 activation. Therefore, a more likely state for mGluR7 activation *in vivo* is theta activity, which can last for many minutes and involves rhythmic pyramidal cell firing and consequent glutamate release. The suppression of both glutamate and GABA release during prolonged pyramidal cell firing may be a mechanism to preserve presynaptic stores of transmitter to enable prolonged rhythmic transmitter release to O-LM cells from both glutamatergic and GABAergic terminals.

Some interneurons are decorated by mGluR8a-expressing terminals (Dalezios *et al*., 2001) of unknown origin. A potential source is the entorhinal cortex, which is known to express mGluR8 (Saugstad *et al*., 1997) and which sends a small projection to str. oriens, and in addition, is known to be immunoreactive also for mGluR2 (Shigemoto *et al*., 1997). Pyramidal cells may also express mGluR8 (Saugstad *et al*., 1997; not included in scheme). A large proportion (up to 60%) of the entorhinal input may innervate interneurons in str. oriens (T. Sanz, E.

Buhl & P. Somogyi, unpublished observation), but the identity of the postsynaptic cells is not know, as indicated by the question marks. An other potential source would be the amygdala (Pikkarainen *et al*., 1999). The presence of mGluR8a immunoreactivity in terminals making type II synapses (Dalezios *et al*., 2001) suggests that some local GABAergic neurons may also express mGluR8a, which may be colocalized with mGluR7a in the input to some cells. No presynaptic mGluRs have been found on basket or axo-axonic cell terminals so far, but potential novel splice variants of mGluR7 (Schulz *et al*., 2002) have not been tested. Group II mGluRs, which suppress GABA release (Poncer *et al*., 2000), are not indicated on GABAergic cells because the cell types remain to be identified.

The relationship of several cell types, such as the mGluR8a-decorated cells (marked X in Fig. 8), the cells richly decorated by mGluR4-immunopositive terminals (marked Y in Fig. 8) (Corti *et al*., 2002) and the strongly muscarinic M2 receptor-expressing cells (marked Z in Fig. 8) (Hajos *et al*., 1998), to the circuits remains to be defined. There may be a partial or full overlap in the interneuron types innervated by group III mGluR-enriched input, as the immunoreactions were conducted in separate studies and the degree of coexistence has not been tested directly. However, both mGluR8a- and mGluR4-decorated interneurons are much less frequent than those decorated by mGluR7. Therefore, O-LM cells are unlikely to contribute to these populations, which does not exclude that they receive occasional boutons from the inputs expressing mGluR8a and mGluR4. The possible presence and location of mGluR3 and mGluR8b on neurons remains to be established. Immunoreactivity for mGluR3 has been reported in the CA1 area (Tamaru *et al*., 2001). The highly specific distribution of presynaptic mGluRs in the inputs of distinct interneuron classes is probably involved in fine-tuning the efficacy of their inputs in an activity-dependent manner.

Acknowledgements

We are grateful to Drs Ayae Kinoshita, Constantinos Paspalas and Akos Kulik, and Mr Philip Cobden for help with some of the pre-embedding immunoreactions and electron microscopy. We also thank Dr A. Buchan (Department of Physiology, University British Columbia, Vancouver, Canada) for antibodies to somatostatin and Dr K. Tanaka (Niigata University, Faculty of Medicine, Department of Neurology, Japan) for human anti-GAD antibodies. We thank Dr F. Ferraguti for his comments on a previous version of the manuscript. Miss Zahida Ahmad provided excellent technical assistance and Mr Paul Jays and Mr Akira Uesugi helped with photography. Y.D. was supported by a Wellcome Trust Advanced Training Fellowship.

Abbreviations

GAD, glutamic acid decarboxylase; IS, interneuron-specific; IS-III cells, interneuron-specific type III cells; mGluR7a, metabotropic glutamate receptor subtype 7, 'a' splice variant; NGS, normal goat serum; O-Bi, oriens–bistratified; O-LM, oriens–lacunosum moleculare; str., stratum; TBS, Tris-buffered saline; VIP, vasoactive intestinal polypeptide.

References

Acsady, L., Gorcs, T.J. & Freund, T.F. (1996) Different populations of vasoactive intestinal polypeptide-immunoreactive interneurons are specialized to control pyramidal cells or interneurons in the hippocampus. *Neuroscience*, **73**, 317–334.

Ali, A.B., Bannister, A.P. & Thomson, A.M. (1999) IPSPs elicited in CA1 pyramidal cells by putative basket cells in slices of adult rat hippocampus. *Eur. J. Neurosci.*, **11**, 1741–1753.

Ali, A.B. & Thomson, A.M. (1998) Facilitating pyramid to horizontal oriens-alveus interneurone inputs: dual intracellular recordings in slices of rat hippocampus. *J. Physiol. (Lond.)*, **507**, 185–199.

Anwyl, R. (1999) Metabotropic glutamate receptors: electrophysiological properties and role in plasticity. *Brain Res. Rev.*, **29**, 83–120.

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.

Blasco-Ibañez, J.M. & Freund, T.F. (1995) Synaptic input of horizontal interneurons in stratum oriens of the hippocampal CA1 subfield: structural basis of feed-back activation. *Eur. J. Neurosci.*, **7**, 2170–2180.

Bradley, S.R., Levey, A.I., Hersch, S.M. & Conn, P.J. (1996) Immunocytochemical localization of group III metabotropic glutamate receptors in the hippocampus with subtype-specific antibodies. *J. Neurosci.*, **16**, 2044–2056.

Cauli, B., Porter, J.T., Tsuzuki, K., Lambolez, B., Rossier, J., Quenet, B. & Audinat, E. (2000) Classification of fusiform neocortical interneurons based on unsupervised clustering. *Proc. Natl Acad. Sci. USA*, **97**, 6144–6149.

Ceranik, K., Bender, R., Geiger, J.R.P., Monyer, H., Jonas, P., Frotscher, M. & Lubke, J. (1997) A novel type of GABAergic interneuron connecting the input and the output regions of the hippocampus. *J. Neurosci.*, **17**, 5380–5394.

Cobb, S.R., Halasy, K., Vida, I., Nyiri, G., Tamas, G., Buhl, E.H. & Somogyi, P. (1997) Synaptic effects of identified interneurons innervating both interneurons and pyramidal cells in the rat hippocampus. *Neuroscience*, **79**, 629–648.

Cochilla, A.J. & Alford, S. (1998) Metabotropic glutamate receptor-mediated control of neurotransmitter release. *Neuron*, **20**, 1007–1016.

Corti, C., Aldegheri, L., Somogyi, P. & Ferraguti, F. (2002) Distribution and synaptic localisation of the metabotropic glutamate receptor 4 (mGluR4) in the rodent CNS. *Neuroscience*, **110**, 403–420.

Dalezios, Y., Lujan, R., Shigemoto, R., Roberts, J.D.B. & Somogyi, P. (2001) Group III metabotropic glutamate receptors (mGluRs) on GABAergic terminals of hippocampal and neocortical interneurons. *Soc. Neurosci. Abstr.*, **27**, progr. no. 259.5.

Dalezios, Y., Lujan, R., Shigemoto, R., Roberts, J.D.B. & Somogyi, P. (2002) Enrichment of mGluR7a in the presynaptic active zones of GABAergic and non-GABAergic terminals on interneurons in the rat somatosensory cortex. *Cereb. Cortex*, **12**, 961–974.

Dey, R.D., Hoffpauir, J. & Said, S.I. (1988) Co-localization of vasoactive intestinal peptide- and substance P-containing nerves in cat bronchi. *Neuroscience*, **24**, 275–281.

Ferraguti, F., Cobden, P., Pollard, M., Cope, D., Shigemoto, R., Watanabe, M. & Somogyi, P. (2003) Immunolocalization of metabotropic glutamate receptor 1α (mGluR1α) in distinct classes of interneuron in the CA1 region of the rat hippocampus. *Hippocampus*, **13**, in press.

Ferraguti, F., Cobden, P., Pollard, M., Watanabe, M. & Somogyi, P. (2001) Distinct populations of mGluR1α-immunoreactive interneurones in the hippocampal CA1 area. *Soc. Neurosci. Abstr.*, **27**, progr. no. 913.21.

Fremeau, R.T., Jr Burman J., Qureshi, T., Tran, C.H., Proctor, J., Johnson, J., Zhang, H., Sulzer, D., Copenhagen, D.R., Storm-Mathisen, J., Reimer, R.J., Chaudhry, F.A. & Edwards, R.H. (2002) The identification of vesicular glutamate transporter 3 suggests novel modes of signaling by glutamate. *Proc. Natl Acad. Sci. USA*, **99**, 14488–14493.

Freund, T.F. & Antal, M. (1988) GABA-containing neurons in the septum control inhibitory interneurons in the hippocampus. *Nature*, **336**, 170–173.

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

Frotscher, M. (1991) Target cell specificity of synaptic connections in the hippocampus. *Hippocampus*, **1**, 123–130.

Fukuda, T. & Kosaka, T. (2000) Gap junctions linking the dendritic network of GABAergic interneurons in the hippocampus. *J. Neurosci.*, **20**, 1519–1528.

Gereau, R.W. & Conn, P.J. (1995) Multiple presynaptic metabotropic glutamate receptors modulate excitatory and inhibitory synaptic transmission in hippocampal area CA1. *J. Neurosci.*, **15**, 6879–6889.

Gras, C., Herzog, E., Bellenchi, G.C., Bernard, V., Ravassard, P., Pohl, M., Gasnier, B., Giros, B. & Mestikawy, S. (2002) A third vesicular glutamate transporter expressed by cholinergic and serotoninergic neurons. *J. Neurosci.*, **22**, 5442–5451.

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.

Gulyas, A.I., Megias, M., Emri, Z. & Freund, T.F. (1999) Total number and ratio of excitatory and inhibitory synapses converging onto single interneurons of different types in the CA1 area of the rat hippocampus. *J. Neurosci.*, **19**, 10082–10097.

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.

Hampson, D.R., Theriault, E., Huang, X.-P., Kristensen, P., Pickering, D.S., Franck, J.E. & Mulvihill, E.R. (1994) Characterization of two alternatively spliced forms of a metabotropic glutamate receptor in the central nervous system of the rat. *Neuroscience*, **60**, 325–336.

Hodgson, A.J., Penke, B., Erdei, A., Chubb, I.W. & Somogyi, P. (1985) Antisera to γ-aminobutyric acid. I. Production and characterization using a new model system. *J. Histochem. Cytochem.*, **33**, 229–239.

Katona, I., Acsady, L. & Freund, T.F. (1999) Postsynaptic targets of somatostatin-immunoreactive interneurons in the rat hippocampus. *Neuroscience*, **88**, 37–55.

Kawaguchi, Y. & Hama, K. (1987) Two subtypes of non-pyramidal cells in rat hippocampal formation identified by intracellular recording and HRP injection. *Brain Res.*, **411**, 190–195.

Kinoshita, A., Shigemoto, R., Ohishi, H., van der Putten, H. & Mizuno, N. (1998) Immunohistochemical localization of metabotropic glutamate receptors, mGluR7a and mGluR7b, in the central nervous system of the adult rat and mouse: a light and electron microscopic study. *J. Comp. Neurol.*, **393**, 332–352.

Klausberger, T., Magill, P.J., Márton, L.F., Roberts, J.D.B., Cobden, P.M., Buzsáki, G. & Somogyi, P. (2003) Brain state- and cell type-specific firing of hippocampal interneurons *in vivo*. *Nature*, **421**, 844–848.

Kogo, N., Shigemoto, R., Roberts, J.D.B. & Somogyi, P. (1999) Suppression of GABAergic inputs to CA1 O-LM interneurons by group III metabotropic glutamate receptors. *Soc. Neurosci. Abstr.*, **25**, 1259.

Kullmann, D.M. (2001) Presynaptic kainate receptors in the hippocampus. Slowly emerging from obscurity. *Neuron*, **32**, 561–564.

Laezza, F., Doherty, J.J. & Dingledine, R. (1999) Long-term depression in hippocampal interneurons: Joint requirement for pre- and postsynaptic events. *Science*, **285**, 1411–1414.

Lerma, J., Paternain, A.V., Rodriguez Moreno, A. & Lopez Garcia, J.C. (2001) Molecular physiology of kainate receptors. *Physiol. Rev.*, **81**, 971–998.

Lorente de No, R. (1934) Studies on the structure of the cerebral cortex. II. Continuation of the study of the ammonic system. *J. Psychol. Neurol.*, **46**, 113–177.

Losonczy, A., Zhang, L., Shigemoto, R., Somogyi, P. & Nusser, Z. (2002) Cell type dependence and variability in the short-term plasticity of EPSCs in identified mouse hippocampal interneurones. *J. Physiol. (Lond.)*, **542**, 193–210.

Lujan, R., Nusser, Z., Roberts, J.D.B., Shigemoto, R. & Somogyi, P. (1996) Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the rat hippocampus. *Eur. J. Neurosci.*, **8**, 1488–1500.

Lujan, R., Shigemoto, R. & Somogyi, P. (1998) A presynaptic metabotropic glutamate receptor, (mGluR7) is located at the vesicle release site on GABAergic terminals in the rat hippocampus. *Eur. J. Neurosci. Suppl.*, **10**, 128.

Maccaferri, G., Roberts, J.D.B., Szucs, P., 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.

Markram, H., Wang, Y. & Tsodyks, M. (1998) Differential signaling via the same axon of neocortical pyramidal neurons. *Proc. Natl Acad. Sci. USA*, **95**, 5323–5328.

Martina, M., Vida, I. & Jonas, P. (2000) Distal initiation and active propagation of action potentials in interneuron dendrites. *Science*, **287**, 295–300.

Masugi, M., Yokoi, M., Shigemoto, R., Muguruma, K., Watanabe, Y., Sansig, G., van der Putten, H. & Nakanishi, S. (1999) Metabotropic glutamate receptor subtype 7 ablation causes deficit in fear response and conditioned taste aversion. *J. Neurosci.*, **19**, 955–963.

McBain, C.J., DiChiara, T.J. & Kauer, J.A. (1994) Activation of metabotropic glutamate receptors differentially affects two classes of hippocampal interneurons and potentiates excitatory synaptic transmission. *J. Neurosci.*, **14**, 4433–4445.

McBain, C.J. & Fisahn, A. (2001) Interneurons unbound. *Nature Revs.*, **2**, 11–24.

Miles, R. (2000) Diversity in inhibition. *Science*, **287**, 244–246.

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.

Millán, C., Luj,·N.R., Shigemoto, R. & Sánchez-Prieto, J. (2002) The inhibition of glutamate release by metabotropic glutamate receptor 7 affects both $[Ca^{2+}]_c$ and cAMP. Evidence for a strong reduction of Ca^{2+} entry in single nerve terminals. *J. Biol. Chem.*, **277**, 14092–14101.

Min, M.-Y., Melyan, Z. & Kullmann, D.M. (1999) Synaptically released glutamate reduces γ-aminobutyric acid (GABA)ergic inhibition in the hippocampus via kainate receptors. *Proc. Natl Acad. Sci. USA*, **96**, 9932–9937.

Mitchell, S.J. & Silver, R.A. (2000) Glutamate spillover suppresses inhibition by activating presynaptic mGluRs. *Nature*, **404**, 498–501.

Oe, H., Miyashita, K., Tanaka, K., Naritomi, H., Kinugawa, H. & Sawada, T. (1996) [A case of progressive continuous muscular rigidity and painless and rhythmic muscle spasm associated with autoantibody against glutamic acid decarboxylase (article in Japanese)]. *Rinsho Shinkeigaku*, **36**, 1166–1171.

Okamoto, N., Hori, S., Akazawa, C., Hayashi, Y., Shigemoto, R., Mizuno, N. & Nakanishi, S. (1994) Molecular characterization of a new metabotropic glutamate receptor mGluR7 coupled to inhibitory cyclic AMP signal transduction. *J. Biol. Chem.*, **269**, 1231–1236.

Pawelzik, H., Hughes, D.I. & Thomson, A.M. (2002) Physiological and morphological diversity of immunocytochemically defined parvalbumin- and cholecystokinin-positive interneurones in CA1 of the adult rat hippocampus. *J. Comp. Neurol.*, **443**, 346–367.

Perroy, J., Prezeau, L., De Waard, M., Shigemoto, R., Bockaert, J. & Fagni, L. (2000) Selective blockade of P/Q-type calcium channels by the metabotropic glutamate receptor type 7 involves a phospholipase C pathway in neurons. *J. Neurosci.*, **20**, 7896–7904.

Pikkarainen, M., Ronkko, S., Savander, V., Insausti, R. & Pitkanen, A. (1999) Projections from the lateral, basal, and accessory basal nuclei of the amygdala to the hippocampal formation in rat. *J. Comp. Neurol.*, **403**, 229–260.

Pollard, M., Kogo, N., Roberts, J.D.B., Ferraguti, F. & Somogyi, P. (2000) Cell types expressing somatostatin and/or mGluR1α in the rat hippocampal CA1 area. *Soc. Neurosci. Abstr.*, **26**, 427.10.

Poncer, J.C., Mckinney, R.A., Gahwiler, B.H. & Thompson, S.M. (2000) Differential control of GABA release at synapses from distinct interneurons in rat hippocampus. *J. Physiol. (Lond.)*, **528**, 123–130.

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

Reyes, A., Lujan, R., Rozov, A., Burnashev, N., Somogyi, P. & Sakmann, B. (1998) Target-cell-specific facilitation and depression in neocortical circuits. *Nature Neurosci.*, **1**, 279–285.

Rozov, A., Burnashev, N., Sakmann, B. & Neher, E. (2001) Transmitter release modulation by intracellular Ca^{2+} buffers in facilitating and depressing nerve terminals of pyramidal cells in layer 2/3 of the rat neocortex indicates a target cell-specific difference in presynaptic calcium dynamics. *J. Physiol. (Lond.)*, **531**, 807–826.

Sandler, R. & Smith, A.D. (1991) Coexistence of GABA and glutamate in mossy fiber terminals of the primate hippocampus: an ultrastructural study. *J. Comp. Neurol.*, **303**, 177–192.

Sansig, G., Bushell, T.J., Clarke, V.R.J., Rozov, A., Burnashev, N., Portet, C., Gasparini, F., Schmutz, M., Klebs, K., Shigemoto, R., Flor, P.J., Kuhn, R., Knoepfel, T., Schroeder, M., Hampson, D.R., Collett, V.J., Zhang, C., Duvoisin, R.M., Collingridge, G.L. & van der Putten, H. (2001) Increased seizure susceptibility in mice lacking metabotropic glutamate receptor 7. *J. Neurosci.*, **21**, 8734–8745.

Saugstad, J.A., Kinzie, J.M., Shinohara, M.M., Segerson, T.P. & Westbrook, G.L. (1997) Cloning and expression of rat metabotropic glutamate receptor 8 reveals a distinct pharmacological profile. *Mol. Pharmacol.*, **51**, 119–125.

Scanziani, M., Gahwiler, B.H. & Charpak, S. (1998) Target cell-specific modulation of transmitter release at terminals from a single axon. *Proc. Natl Acad. Sci. USA*, **95**, 12004–12009.

Schoepp, D.D. (2001) Unveiling the functions of presynaptic metabotropic glutamate receptors in the central nervous system. *J. Pharmacol. Exp. Ther.*, **299**, 12–20.

Schulz, H.L., Stohr, H. & Weber, B.H.F. (2002) Characterization of three novel isoforms of the metabotrobic glutamate receptor 7 (GRM7). *Neurosci. Lett.*, **326**, 37–40.

Semyanov, A. & Kullmann, D.M. (2000) Modulation of GABAergic signaling among interneurons by metabotropic glutamate receptors. *Neuron*, **25**, 663–672.

Shigemoto, R., Kinoshita, A., Wada, E., Nomura, S., Ohishi, H., Takada, M., Flor, P.J., Neki, A., Abe, T., Nakanishi, S. & Mizuno, N. (1997) Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. *J. Neurosci.*, **17**, 7503–7522.

Shigemoto, R., Kulik, A., Roberts, J.D.B., Ohishi, H., Nusser, Z., Kaneko, T. & Somogyi, P. (1996) Target-cell-specific concentration of a metabotropic glutamate receptor in the presynaptic active zone. *Nature*, **381**, 523–525.

Sik, A., Penttonen, M., Ylinen, A. & Buzsaki, G. (1995) Hippocampal CA1 interneurons: an in vivo intracellular labeling study. *J. Neurosci.*, **15**, 6651–6665.

Somogyi, J. (2002) Differences in ratios of GABA, glycine and glutamate immunoreactivities in nerve terminals on rat hindlimb motoneurons: a possible source of post-synaptic variability. *Brain Res. Bull.*, **59**, 151–161.

Somogyi, P., Ganter, P., Kogo, N., Maccaferri, G., Paspalas, C., Paulsen, O., Roberts, J.D.B., Shigemoto, R. & Szucs, P. (1999) Compartmentalization and properties of synapses and receptors in a feedback circuit of the cerebral cortex. *J. Physiol. (Lond.)*, **518P**, 22S.

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.

Szentagothai, J. (1975) The 'module-concept' in cerebral cortex architecture. *Brain Res.*, **95**, 475–496.

Szentagothai, J. & Arbib, M.A. (1974) Conceptual models of neural organization. *Neurosci. Res. Prog. Bull.*, **12**, 305–510.

Takahashi, T., Forsythe, I.D., Tsujimoto, T., Barnes-Davies, M. & Onodera, K. (1996) Presynaptic calcium current modulation by a metabotropic glutamate receptor. *Science*, **274**, 594–597.

Tamaru, Y., Nomura, S., Mizuno, N. & Shigemoto, R. (2001) Distribution of metabotropic glutamate receptor mGluR3 in the mouse CNS: Differential location relative to pre- and postsynaptic sites. *Neuroscience*, **106**, 481–503.

Tanaka, J., Nakagawa, S., Kushiya, E., Yamasaki, M., Fukaya, M., Iwanaga, T., Simon, M.I., Sakimura, K., Kano, M. & Watanabe, M. (2000) Gq protein α subunits Gαq and Gα11 are localized at postsynaptic extra-junctional membrane of cerebellar Purkinje cells and hippocampal pyramidal cells. *Eur. J. Neurosci.*, **12**, 781–792.

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.

Toth, K., Freund, T.F. & Miles, R. (1997) Disinhibition of rat hippocampal pyramidal cells by GABAergic afferents from the septum. *J. Physiol. (Lond.)*, **500**, 463–474.

Vida, I. & Frotscher, M. (2000) A hippocampal interneuron associated with the mossy fiber system. *Proc. Natl Acad. Sci. USA*, **97**, 1275–1280.

Vincent, S.R., McIntosh, C.H.S., Buchan, A.M.J. & Brown, J.C. (1985) Central somatostatin systems revealed with monoclonal antibodies. *J. Comp. Neurol.*, **238**, 169–186.

Vizi, E.S. & Kiss, J.P. (1998) Neurochemistry and pharmacology of the major hippocampal transmitter systems: Synaptic and nonsynaptic interactions. *Hippocampus*, **8**, 566–607.

Walker, M.C., Ruiz, A. & Kullmann, D.M. (2001) Monosynaptic GABAergic signaling from dentate to CA3 with a pharmacological and physiological profile typical of mossy fiber synapses. *Neuron*, **29**, 703–715.

Woodhall, G., Evans, D.I.P. & Jones, R.S.G. (2001) Activation of presynaptic group III metabotropic glutamate receptors depresses spontaneous inhibition in layer V of the rat entorhinal cortex. *Neuroscience*, **105**, 71–78.

Wu, S., Wright, R.A., Rockey, P.K., Burgett, S.G., Arnold, J.S., Rosteck, P.R., Johnson, B.G., Schoepp, D.D. & Belagaje, R.M. (1998) Group III human metabotropic glutamate receptors 4, 7 and 8: molecular cloning, functional expression, and comparison of pharmacological properties in RGT cells. *Mol. Brain. Res.*, **53**, 88–97.

Zar, J.H. (1999) *Biostatistical Analysis*. Prentice Hall Inc, New Jersey.