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Depression of GABAergic input to identified hippocampal neurons by group III metabotropic glutamate receptors in the rat

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Abstract

The release of GABA in synapses is modulated by presynaptic metabotropic glutamate receptors (mGluRs). We tested whether GABA release to identified hippocampal neurons is influenced by group III mGluR activation using the agonist L-(+)-2-amino-4-phosphonobutyric acid (L-AP4) on inhibitory postsynaptic currents (IPSCs) evoked in CA1 interneurons and pyramidal cells. In interneurons, characterized with biocytin and immunolabelling for somatostatin, evoked IPSCs were depressed by 50 μM L-AP4 (activating mGluR4 and 8) to 68 ± 6% of control, but they were rarely depressed in pyramidal cells (96 ± 4% of control). At 300–500 μM concentration (activating mGluR4, 7 and 8), L-AP4 depressed IPSCs in both interneurons (to 70 ± 6%) and pyramidal cells (to 67 ± 4%). The change in trial-to-trial variability and in paired-pulse depression indicated a presynaptic action. In interneurons, the degree of IPSC depression was variable (to 9–87%), and a third of IPSCs were not affected by L-AP4. The L-AP4-evoked IPSC depression was blocked by LY341495. The depression of IPSCs was similar in O-LM cells and other interneurons. The lack of cell-type selectivity and the similar efficacy of different concentrations of L-AP4 suggest that several group III mGluRs are involved in the depression of IPSCs. Electron microscopic immunocytochemistry confirmed that mGluR4, mGluR7a and mGluR8a occur in the presynaptic active zone of GABAergic terminals on interneurons, but not on those innervating pyramidal cells. The high variability of L-AP4-evoked IPSC suppression is in line with the selective expression of presynaptic mGluRs by several distinct types of GABAergic neuron innervating each interneuron type.

Introduction

Metabotropic glutamate receptors (mGluRs) are expressed in a cell type- and subcellular domain-specific manner by hippocampal neurons (Masu *et al*., 1991; Shigemoto *et al*., 1992, 1996, 1997; Tanabe *et al*., 1992, 1993; Baude *et al*., 1993; Ohishi *et al*., 1994, 1995; Lujan *et al*., 1996, 1997). All but one of the eight receptor subtypes, and most of their splice variants, have been localized to pre- and/or postsynaptic domains of some types of neuron (Shigemoto *et al*., 1997). In accordance with the distribution, physiological studies revealed cell type- and/or target cell-specific effects of mGluR activation (Aniksztejn *et al*., 1992; Bashir *et al*., 1993; Otani *et al*., 1993; McBain *et al*., 1994; Ouardouz & Lacaille, 1995; Carmant *et al*., 1997; Scanziani *et al*., 1998; Bortolotto *et al*., 1999). Similar to other presynaptic receptors (Vizi, 1979), the activation of presynaptic group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR7, mGluR8) mGluRs was shown to depress the release of glutamate (Baskys & Malenka, 1991; Desai *et al*., 1994; Gereau & Conn, 1995; Vignes *et al*., 1995; Scanziani *et al*., 1998) and GABA (Gereau & Conn, 1995; Poncer *et al*., 1995, 2000; Morishita *et al*., 1998; Morishita & Alger, 2000; Semyanov & Kullmann, 2000; Cossart *et al*., 2001) in the hippocampus, and in other parts of the brain (Salt & Eaton, 1995; Schrader & Tasker, 1997; Schaffhauser *et al*., 1998; van den Pol *et al*., 1998; Wittmann *et al*., 2001). In the Schaffer collateral–commissural connection, the depression of glutamatergic transmission is cell type-selective; the activation of group III mGluRs depressed glutamate release to GABAergic neurons but not to pyramidal cells from the same stimulated presynaptic axon (Scanziani *et al*., 1998). This selective functional effect appears to parallel a cell type-specific expression of a much higher level of presynaptic group III mGluRs in the terminals received by some GABAergic neurons than in terminals given by the same pathway to pyramidal cells (Shigemoto *et al*., 1996, 1997). However, the receptor subtype(s) involved in the depression of transmitter release in the Schaffer collateral–commissural pathway has not been identified.

A distinct type of interneuron in the CA1 area of the hippocampus, the oriens–lacunosum moleculare (O-LM) cell, expresses somatostatin (Maccaferri *et al*., 2000) and has horizontally extended dendrites in stratum oriens–alveus, which overlap with the area of axon collaterals from pyramidal cells. The somatic and dendritic membrane of O-LM

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cells contains a high level of mGluR1α (Baude *et al*., 1993; Pollard *et al*., 2000; Losonczy *et al*., 2002; Ferraguti *et al*., 2004). The axon of O-LM cells projects to stratum lacunosum moleculare, providing a dense and restricted GABAergic innervation of this layer overlapping with the input from entorhinal cortex (McBain *et al*., 1994; Sik *et al*., 1995). The highly specialized anatomical organization of O-LM cells has generated strong interest in their properties and potential roles (Lacaille *et al*., 1987; Maccaferri & McBain, 1995, 1996a,b; Hajos & Mody, 1997; Somogyi *et al*., 1998; Martina *et al*., 2000). Interestingly, most input terminals to O-LM cells, including those originating from pyramidal cells and therefore releasing glutamate, are highly enriched in presynaptic mGluR7a immunoreactivity as compared to the terminals innervating other types of interneuron or pyramidal cells (Shigemoto *et al*., 1996; Losonczy *et al*., 2002). However, the role of the high level of mGluR7a on these synaptic terminals remains unknown.

Recently we found that, in addition to the glutamatergic terminals, GABAergic terminals also express high levels of mGluR7a selectively on O-LM cells (Somogyi *et al*., 2003). In the CA3 area, other group III mGluRs, mGluR7b, mGluR4 and mGluR8, have been shown to be present in the presynaptic active zone of type 2 synapses (Shigemoto *et al*., 1997), most of which are probably GABAergic. Such selectivity may endow the GABAergic input of these interneurons with specific pharmacological and physiological properties. Indeed, inhibitory postsynaptic currents (IPSCs) were strongly depressed by the group III mGluR agonist L-(+)-2-amino-4-phosphonobutyric acid (L-AP4), in unidentified interneurons of stratum radiatum, but only slightly reduced in pyramidal cells in the guinea pig (Semyanov & Kullmann, 2000) or the rat (Cossart *et al*., 2001). However, the interneuron types affected, the receptor subtypes mediating the effect and the source of innervation of stratum radiatum interneurons and whether this effect applies to all interneurons and to other species, remain unknown. Therefore, we tested the effect of L-AP4 on postsynaptic IPSCs evoked in characterized interneurons of stratum oriens and compared the effect to that on IPSCs evoked in pyramidal cells. The interneurons were identified on the basis of their dendritic and axonal arborization revealed by biocytin labelling and somatostatin immunoreactivity. The identification of cell types was deemed to be important, because the high level of mGluR7a expression in the presynaptic input appeared to be cell type-specific (Shigemoto *et al*., 1996; Somogyi *et al*., 2003), and stratum oriens–alveus of the hippocampus contains at least six distinct types of GABAergic cell (Sik *et al*., 1995; Freund & Buzsaki, 1996; Maccaferri *et al*., 2000).

Preliminary results have been published in abstract form (Kogo *et al*., 1999; Somogyi *et al*., 1999).

Materials and methods

Slice preparation

Hippocampal slices were prepared from young rats (Wistar, age P12–P20) deeply anaesthetized using isoflurane and killed by decapitation according to a procedure approved by the Home Office in line with The Animals (Scientific Procedures) Act 1986. The brain was quickly removed and placed into ice-cold artificial cerebrospinal fluid (ACSF) of the following composition (in mM): NaCl, 130; $NaHCO_3$, 24; KCl, 3.5; NaH_2PO_4, 1.25; $CaCl_2$, 1; $MgSO_4$, 3; and glucose, 10; saturated with 95% O_2 : 5% CO_2, at pH 7.4. The hemisected brain was then glued onto the stage of a vibrating microtome (Leica, Milton Keynes, UK) and sections of 300 μm thickness were cut and stored in an incubation chamber at room temperature for ≈ 1 h before use. During recording, ACSF with the same concentration as above, except containing 3 mM $CaCl_2$ and 1.5 mM $MgSO_4$, was used. The ACSF contained 100 μM D(–)-2-amino-5-phosphonopentanoic acid (D-AP5) and 20 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) (both from Tocris, Bristol, UK) to block ionotropic glutamate receptors during the recording of monosynaptic IPSCs. The temperature of ACSF inside the recording chamber was 32–35 °C and controlled via a Peltier device connected to the chamber (Luigs & Neumann, Ratingen, Germany) and monitored via a thermometer applied to the metal frame supporting the recording chamber.

Electrophysiological recording

Both stratum (str.) oriens–alveus interneurons and pyramidal cells were identified visually as potential targets using a Zeiss Axioskop microscope (Zeiss, Oberkochen, Germany) equipped with a 40× immersion differential interference contrast (DIC) objective coupled to an infrared camera system (Hamamatsu, Hamamatsu City, Japan). Conventional whole-cell recordings were applied. Cells were voltage-clamped at a holding potential of −70 mV using an Axopatch 1-D (Axon Instruments, USA) amplifier and pipettes of 3–7 MΩ direct current resistance. Two different solutions were used to fill the electrodes. One of them consisted of (in mM): KCl, 130; ATP (Mg salt), 4; GTP (sodium salt), 0.3; and HEPES, 10; with biocytin, 0.5%; the pH was adjusted to 7.3. The other solution contained 125 mM CsCl instead of KCl and, in addition, included 5 mM phosphocreatine and 10 mM QX314, and was used to record three interneurons (four IPSCs) and five pyramidal cells (five IPSCs). Results obtained with these two different pipette solutions were similar; therefore, data were pooled for analysis.

Pairs of rectangular constant-current pulses (0.1 or 0.2 ms duration, 100 ms interpulse interval), repeated at 0.1 Hz, were delivered through an isolation unit (Model A360D, World Precision Instruments, USA) to a stimulus electrode. Most often a monopolar extracellular glass electrode, filled with ACSF, was used to evoke IPSCs. The stimulus electrode was placed in str. oriens to evoke IPSCs in str. oriens–alveus neurons. For 25 str. oriens–alveus neurons, a concentric metal electrode or an 8 × 2 matrix metal electrode (FHC, Bowdoinham, USA) was used and placed in str. oriens. The results reported here were not obviously different with these three different stimulus electrodes, and the data were pooled. For IPSCs evoked in pyramidal cells, the monopolar glass pipette stimulating electrode was placed in the vicinity of the soma to increase the likelihood that inputs on or close to the soma would be activated (Davies *et al*., 1990).

Series resistance was not compensated and it was constantly monitored during recording by injecting −10 mV 20 ms command pulses to the cell 750 ms after each stimulation. Only stable recordings with a <30% change in series resistance (Rs) for the duration of the control, drug application and washout periods were included for analysis. Plots of Rs vs. time were made for the recorded cells. In the cells included in the analysis, there was no correlation between the change in IPSC peak amplitude during L-AP4 application and washout and any change in Rs. Likewise, no systematic changes in IPSC kinetics were observed during L-AP4-induced effects. Recordings with series resistance >50 MΩ were discarded. All drugs were delivered by bath application for 10 or 15 min. The washout of drugs was monitored for 20–30 min.

Data analysis

Data were filtered at 5 kHz and acquired directly on a hard disc of an Intel Pentium II-based computer using a Digidata 1200 A/D board (50 μsec sampling rate) controlled by Clampex software (Axon Instruments, Foster City, USA). The data were also recorded in a DAT recorder (Biologic, France) continuously throughout whole cell recording. Analysis of IPSCs was performed using Axograph software packages (Axon Instruments) and IGOR Pro software (WaveMetrics,

Inc., Portland, OR, USA). Thirty consecutive IPSCs from the end of control, drug application and washout periods were collected and averaged for the comparison of IPSC peak amplitudes. Peak amplitudes of a population of 30 individual responses during control and drug application periods were compared for each cell using the nonparametric Mann–Whitney *U*-test. The Wilcoxon rank test was used to compare mean peak amplitudes within groups of cells before and during drug application.

The variability of IPSCs was analysed for periods before and during the application of L-AP4 in order to test for potential pre- and/or postsynaptic effects. The inverse square power of the coefficient of variation (CV) of each IPSC was calculated with the formula: $CV^{-2} = M^2(\sigma_r^2 - \sigma_n^2)^{-1}$, where M is the mean amplitude of the IPSC, σ_r^2 is the variance of the noise-contaminated IPSCs and σ_n^2 is the variance of the noise. To obtain the amplitudes of individual IPSCs, first, 30 consecutive IPSCs in the control period and separately in the period of L-AP4 application were averaged and the time to the peak of the averaged responses was measured. Then, the amplitude of individual IPSCs was measured during a 1-ms period centred at the time to peak of the averaged response, and the mean amplitude (M) and standard deviation (σ_r) of the 30 signals were calculated. Standard deviation of noise (σ_n) was measured in the same way, except at the time preceding IPSC onset, with the same period as between IPSC onset and the peak time. The paired-pulse ratio was calculated by dividing the mean IPSC2 by the mean IPSC1. Data are presented as mean ± SEM unless otherwise indicated.

Stability test and measures for IPSCs

The stability of IPSC amplitude was recorded to test for any time-dependent change in our recordings of the amplitude of evoked IPSCs at 0.1 Hz. Two interneurons and four pyramidal cells were recorded for 32.5 ± 11.6 min (range 22–54 min) without drug application, and running averages of the amplitude of 30 responses were obtained. Stability was acceptable if over the recording period the mean of any 30 responses was within one SD of the first 30 responses. This was fulfilled for the two interneurons and for three of the pyramidal cells. Therefore, in general, once the method to determine the stimulus site and conditions mentioned above were applied, our IPSC recordings were stable enough for the testing of the effect of the drug over at least 30 min including the control period.

In the pharmacological tests, during the washout period the responses did not always return to the predrug control level. This may be due to numerous factors, including long-term change in synaptic efficacy induced by the drug or the rundown of the response due to dialysis, etc. As mentioned above, if the amplitude of the IPSC during the washout did not return to at least 70% of the control level, the data were discarded. In order to characterize the overall difference in IPSC amplitudes between the control and the washout period, the slope of the linear regression line fitted to the two sets of data points was calculated for each recording included in this study. From the average slope it was calculated that, if there was a time-dependent gradual decline in IPSC amplitude, on average, an IPSC would decline by 1.3% over 10 min. Considering that the measured control period was 5 min before drug application, which was typically for 10 min, in the analysed IPSC population, on average, <2% decline in IPSC amplitude was expected to be due to factors other than the effect of the drug.

Immunocytochemistry for light microscopy and visualization of recorded cells

The identity of the cells was verified anatomically. Briefly, slices were sandwiched between two Millipore filters to avoid deformations and fixed for a period of 2 h to 1 day in 2.5% paraformaldehyde, 0.05% glutaraldehyde and 15% (v/v) saturated picric acid in 0.1 M phosphate buffer (pH 7.4). Following gelatin embedding, the slices were re-sectioned at 60 μm thickness and washed in phosphate-buffered saline (PBS). In triple fluorescence labelling experiments (Maccaferri *et al*., 2000) two of three antibodies, either to somatostatin, mGluR1α or parvalbumin, were employed together with visualization of biocytin by 7-amino-4-methylcoumarin-3-acetic acid (AMCA)-labelled streptavidin. Although acceptable labelling of nearby cells for mGluR1α and parvalbumin was often obtained, the recorded cells generally displayed undetectable or very weak immunoreactivity, probably due to the long recording and dialysis of the neurons, so only the results for somatostatin labelling are reported. Non-specific protein binding was blocked by incubation in normal goat serum for 1 h. A monoclonal antibody to somatostatin (Code: SOMA8, ascites fluid diluted 1 : 500, gift from Dr A. Buchan, Department of Physiology, University British Columbia, Canada), characterized by Vincent *et al*. (1985), was used to detect O-LM and O-bistratified cells. The antibody was diluted in PBS containing 0.2% Triton X-100 and applied overnight. The sections were subsequently washed in PBS and incubated for 2 h in a mixture of Alexa Fluor® 488-conjugated goat antimouse IgG (diluted 1 : 1000; Molecular Probes, Leiden, The Netherlands), or Cy™3-conjugated goat antimouse IgG (diluted 1 : 100; Jackson ImmunoResearch Laboratory, West Grove, Pennsylvania, USA) and AMCA-conjugated streptavidin (diluted 1 : 1000, Vector Laboratories, Burlingame, CA, USA). Biocytin-filled cells were studied using a Leica dichromatic mirror system (Maccaferri *et al*., 2000). Cells were recorded on a cooled 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. The immunonegativity of a cell for a marker could be due to damage caused by the recording, an undetectably low level of the molecule or the genuine absence of the molecule; therefore, only the positive detection of immunoreactivity is informative following extensive whole-cell recording. The recorded cells usually showed significantly lower immunoreactivity than nearby unrecorded cells, suggesting that the recording decreased the somatic level of somatostatin.

Following immunocytochemical testing, the sections were removed from the slides, washed in PBS and processed for peroxidase-based visualization of the recorded cells in order to evaluate the axonal and dendritic patterns and for permanent storage. The sections were incubated in biotinylated horseradish peroxidase (diluted 1 : 100; Vector) before further washing and incubation in avidin-biotinylated-horseradish peroxidase complex (diluted 1 : 100; Vector). Peroxidase activity was revealed with 0.05% diaminobenzidine as chromogen and 0.01% H_2O_2 as substrate.

Method specificity was tested by replacing the primary antibodies with 1% normal mouse serum, and for the antibody to somatostatin by preincubating the antibody with 10 mM somatostatin (Sigma, Poole, UK) for 5 h at room temperature before applying the mixture to the sections. This completely prevented immunolabelling. From the control experiments we conclude that the labelling detected by fluorescence was due to the selective binding of the primary antibody.

Immunocytochemistry for electron microscopy

Two adult (350–400 g) male Wistar rats were deeply anaesthetized with Sagatal (pentobarbitone sodium, 100 mg/kg i.p.) and transcardially perfused with 0.9% saline followed for 20 min by a fixative containing 4% paraformaldehyde, 0.05% glutaraldehyde and ≈0.2% picric acid in 0.1 M phosphate buffer (pH ≈7.4). Brains were then

sectioned and treated as described earlier (Dalezios *et al*., 2002). Briefly, free-floating sections were preincubated in 20% normal goat serum (NGS) diluted in TBS for 2 h and then incubated for 48 h at 4 °C in a mixture of two primary antibodies diluted in TBS−1% NGS. Three different combinations of primary antibodies were used in which antibodies to mGluR7a, mGluR8a or mGluR4 were coincubated with human antibodies to GAD. Human antibodies to GAD, purified from the plasma of a patient suffering from Stiff-man syndrome (Oe *et al*., 1996; Dalezios *et al*., 2002), were a gift from Dr K. Tanaka (Niigata University, Japan). They were used at 1 μg/mL protein concentration. The specificity of polyclonal antibodies to mGluR7a (rabbit, 1 μg/mL), mGluR7b (rabbit, 1 μg/mL) and mGluR8a (guinea pig, 1.7 μg/mL) were reported previously (Kinoshita *et al*., 1998; Shigemoto *et al*., 1996, 1997). Affinity-purified polyclonal antibodies to mGluR4 were raised in rabbit against amino acid residues 834–912 of rat mGluR4 (Corti *et al*., 2002) and used at a final protein concentration of 1 μg/mL. Primary antibodies were followed by a mixture of goat antihuman affinity-purified Fab′ fragment coupled to 1.4-nm gold (1 : 100; Nanoprobes Inc., Stony Brook, NY, USA) and biotinylated goat antirabbit (1 : 100, Vector) or goat antiguinea pig IgG (1 : 100, Vector). Immunoreaction for mGluR7b did not include colabelling for GAD. For the other three antibodies, immunolabelling for GAD was always visualized with silver-intensified gold reaction and group III mGluR immunolabelling with peroxidase reaction. Gold particles were enhanced by silver amplification for 8–12 min using the HQ Silver kit (Nanoprobes Inc.). Subsequently, sections were incubated overnight in avidin–biotin–horseradish peroxidase complex (ABC, 1 : 100; Vector), which was visualized with diaminobenzidine (0.5 mg/mL) and 0.01% H_2O_2 as substrate. Sections were treated with 2% OsO_4 and contrasted in 1% uranyl acetate before embedding in epoxy resin (Durcupan ACM, Fluca, Sigma-Aldrich, Gillingham, UK). Serial electron microscopic sections (70–80 nm) were collected on pioloform-coated copper slot grids. To test for cross-reactivity of secondary antibodies, some sections were always incubated with either none or only one primary antibody and the full complement of secondary antibodies. No cross-reactivity was detected, and the low level of nonspecific signal allowed unequivocal localization of the respective molecules.

Results

Identification of recorded neuron populations

In total, data were included from 52 interneurons with 59 IPSCs and 24 pyramidal cells with 28 IPSCs in the CA1 area. From these, five cells (two interneurons and three pyramidal cells) were used only to test the stability of recordings, and 13 cells (nine interneurons, four pyramidal cells) were used only to assess the effect of bicuculline on IPSCs. Five of the interneurons were tested from two different stimulation sites; therefore, data on interneurons are presented from 46 extracellular stimulation sites. Two sites on two cells were used for testing two different doses of L-AP4 and for each dose an independent control IPSC was obtained; therefore, they have been treated as independent IPSCs. Consequently, 41 interneurons with 48 IPSCs and 17 pyramidal cells with 19 IPSCs were used to test the effects of L-AP4 and the antagonist LY341495 (Table 1). Interneurons were selected under DIC optics and infrared illumination on the basis of their spindle-like, elongated cell body parallel with the hippocampal layers in stratum oriens bordering the alveus. Their nonpyramidal nature was confirmed in all but the three CsCl-recorded cases by their characteristic firing pattern, and in 39 cases by subsequent recovery and anatomical analysis of the biocytin-filled cells (Table 1). In two cases the cells were not filled or the tissue was lost during processing. Immunocytochemical labelling was also used to assist the identification of the neurons. Somatostatin immunoreactivity was detected in 18 (51%) of 35 tested neurons.

In the pharmacologically tested interneuron population of 41 cells (Table 1), 17 interneurons were characterized by the laminar pattern of their dendritic and axonal arborizations, and an additional 22 only by their dendritic arborization. Briefly, O-LM cells ($n = 13$) were defined as cells having a dendritic tree largely restricted to str. oriens–alveus and an axon mostly innervating str. lacunosum moleculare (Fig. 1). Six O-LM cells were somatostatin-immunopositive (Fig. 3). The oriens–bistratified (O-Bi) cells ($n = 3$) were recognized on the basis of a dendritic tree largely restricted to str. oriens–alveus and an axonal arborization mostly innervating str. radiatum and oriens (Fig. 2, Maccaferri *et al*., 2000). Two O-Bi cells were somatostatin-immunopositive. In 22 cases the main axon of the cell was cut during the

TABLE 1. Identification of neurons tested with L-AP4 and number of IPSCs tested with L-AP4*

	Cells				IPSCs								
					50 μM L-AP4			300–500 μM L-AP4			Total		
		Somatostatin immunoreactivity			Depressed			Depressed			Depressed		
Cell type	Number recorded	Tested	Immuno-positive	Immuno-negative	No.	%	No. tested	No.	%	No. tested	No.	%	No. tested
O-LM	13	11	6	–	5	50	10	5	71	7	10	59	17
O-Bistratified	3	3	2	–	0	–	0	2	–	3	2	–	3
Horizontal, not enough axon recovered	22	20	10	–	9	56	16	7	78	9	16	64	25
Total (O-LM, O-Bi and horizontal)	38	34	18	–	14	54	26	14	74	19	28	62	45
Basket	1	1	0	–	1	–	1	0	–	0	1	–	1
Not recovered interneuron	2	–	–	–	2	–	2	0	–	0	2	–	2
Total (interneuron)	41	35	18	–	18	62	29	14	74	19	32	67	48
Somatostatin-immunopositive			18	–	7	58	12	9	90	10	16	73	22
Somatostatin-immunonegative			–	17	6	50	12	4	50	8	10	50	20
Pyramidal recovered	13	0	–	–	1	13	8	5	83	6	6	43	14
Pyramidal not recovered	4	–	–	–	1	20	5	–	–	–	1	20	5
Total (pyramidal)	17	0	–	–	2	15	13	5	83	6	7	37	19

*Some of the cells were tested from more than one stimulation site or for both doses of L-AP4, and the resulting IPSCs are counted separately.

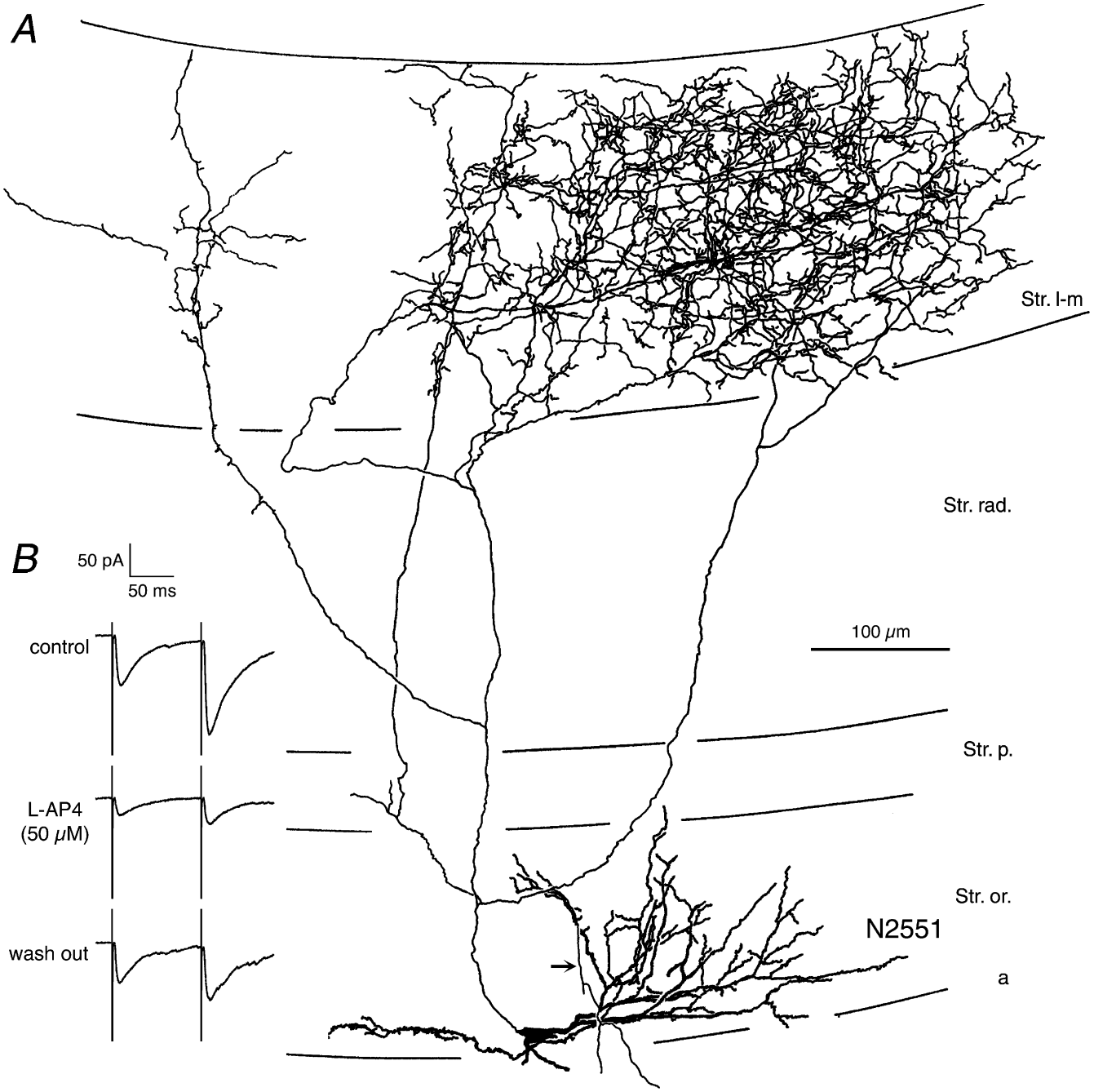

FIG. 1. Depression of IPSCs by L-AP4 in a somatostatin-immunopositive identified O-LM cell (N2551) of the hippocampus (age, P14). (A) Reconstruction of the cell showing a dendritic arbor restricted to stratum oriens (Str. Or.) and alveus (a), and an axon innervating str. lacunosum moleculare (Str. l-m). One axon collateral (arrow), displaying varicosities, returned to str. oriens–alveus. (B) Extracellular paired-pulse stimulation at 100 ms interpulse interval evoked inward currents, in the presence of 100 μM D-AP-5 and 20 μM DNQX, when the recording pipette contained 130 mM KCl. In other cells such currents were completely blocked by bicuculline; therefore, they are considered to be $GABA_A$ receptor-mediated IPSCs. The IPSCs showed paired-pulse facilitation in this cell, were depressed by L-AP4, and the effect was washed out. Scale bar for A, 100 μm.

preparation of the slice; therefore, it could not be decided which of them were O-LM, O-Bi or other interneurons. All these cells had a dendritic arbor orientated parallel with the hippocampal layers; therefore, they are called here 'horizontal cells' (Table 1). Most of the dendritic arbors had the characteristics of O-LM cells in that they were emitting numerous spines, thin filopodia and appendages. Of the horizontal cells, 10 of 20 tested were somatostatin-immunopositive, supporting the notion that most of them were O-LM or O-Bi cells. One of the identified cells was a basket cell innervating str. pyramidale and the adjacent regions.

Pyramidal cells were selected on the basis of their shape as seen in infrared DIC microscopy. The identity of some of them was confirmed by light microscopy following visualization by biocytin. Of the 17 recorded cells, eight out of 12 cells tested with 50 μM of L-AP4 and six out of six cells tested with 400 μM L-AP4 were recovered and anatomically confirmed to be pyramidal cells.

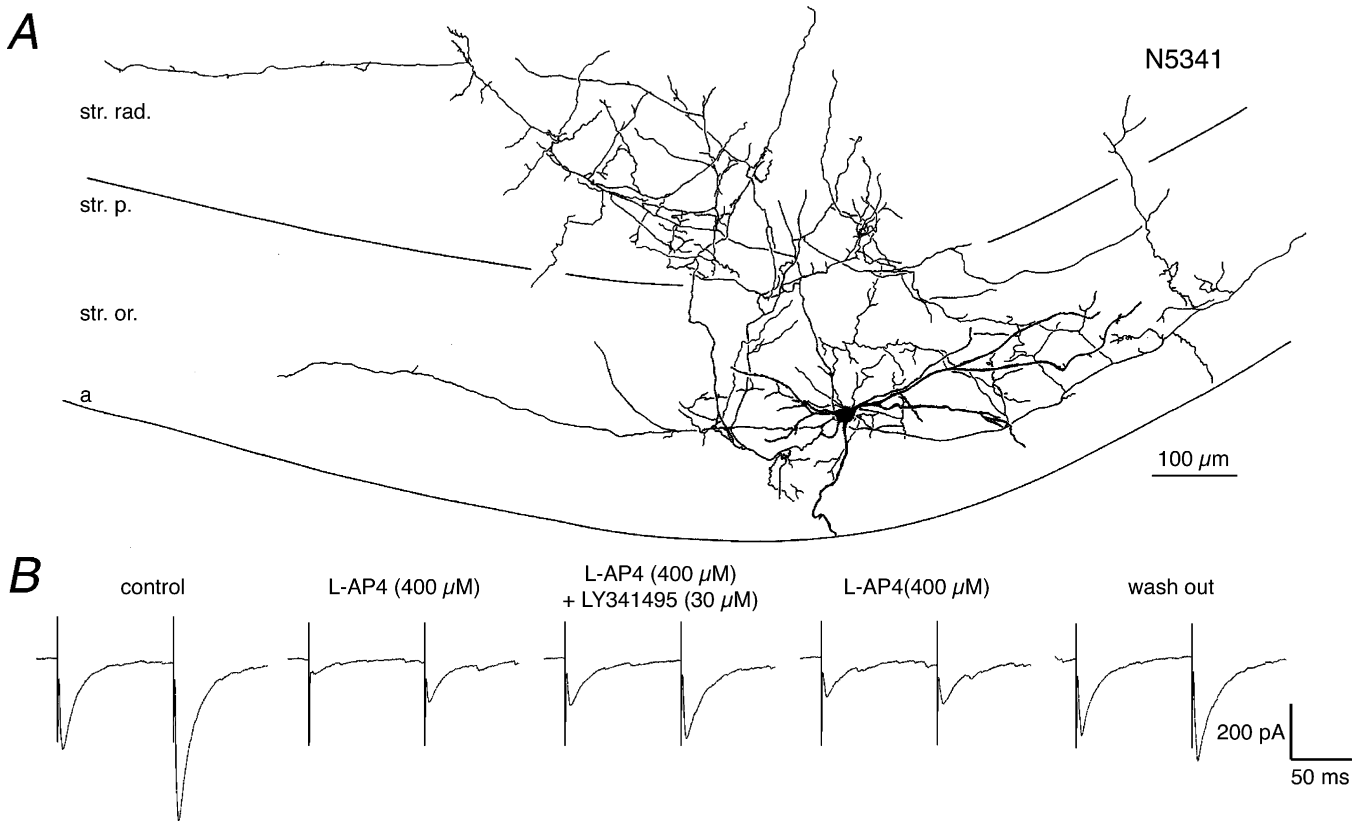

FIG. 2. Depression of IPSCs by L-AP4 in a somatostatin-immunopositive identified O-Bi cell (N5341, P16). (A) Reconstruction of the cell showing dendritic arbor restricted to the str. oriens–alveus and an axon mostly terminating in str. radiatum and oriens. The cell was reconstructed from four 60-μm-thick sections which were cut slightly tangentially to the layers, resulting in a drift of laminar boundaries from section to section; therefore, only the approximate boundaries of str. radiatum and str. pyramidale are indicated. (B) Extracellular paired-pulse stimulation at 100-ms interpulse interval evoked IPSCs showing pronounced facilitation in this cell. Both IPSCs were depressed by L-AP4. The depression was antagonized by LY341495 showing the involvement of mGluRs, and the effect was partially washed out. Scale bar for A, 100 μm.

Evoked IPSCs in stratum oriens interneurons and pyramidal cells

Stimulation in str. oriens evoked inward currents in interneurons ($n = 41$) held at −70 mV membrane potential (Figs 1 and 2). The inward currents were mediated by $GABA_A$ receptors, as 10 μM bicuculline ($n = 9$ cells) either completely abolished the current or, in one case, reduced it to <10% (data not shown). Paired-pulse stimulation at 100 ms interpulse interval evoked IPSCs with an average first IPSC amplitude of 235.3 ± 37.7 pA (mean ± SEM, $n = 48$, range 24.9–1556.4 pA). Only one IPSC exceeded 1 nA. All amplitude values below represent the first of the pair of evoked IPSCs. Paired-pulse ratios (IPSC2/IPSC1) showed wide variation (range 0.46–1.80), but the average was close to one (0.92 ± 0.04).

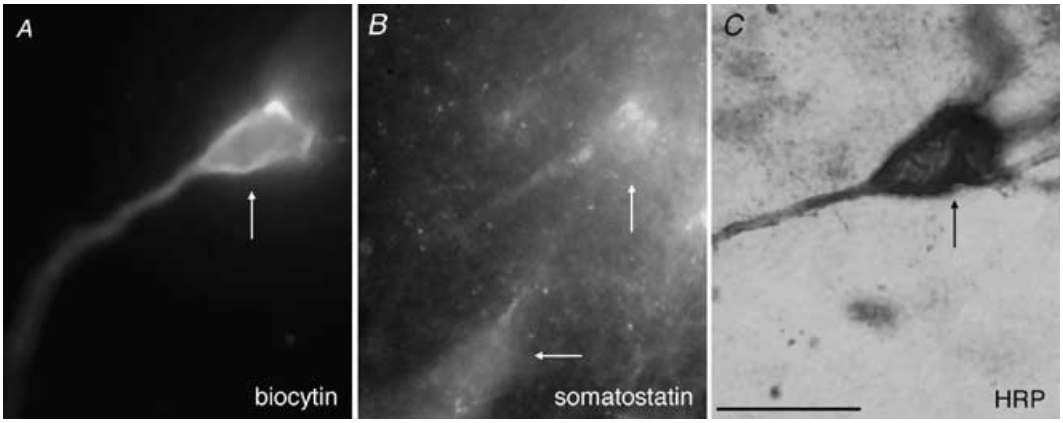

FIG. 3. Somatostatin immunoreactivity in an identified O-LM cell (P13, case N5331), which had an evoked IPSC depressed by 400 μM L-AP4 (not shown). The O-LM cell (vertical arrow) displays fluorescence for (A) AMCA, demonstrating the presence of biocytin, and for (B) Cy3™, demonstrating the presence of somatostatin immunoreactivity. Another somatostatin-immunolabelled cell, which was not recorded, having a soma out of focus and proximal dendrites showing granular labelling, is also seen (horizontal arrow). (C) For the identification of the cell by its axonal arborization, biocytin was subsequently visualized with the peroxidase reaction. Scale bar, 20 μm.

Pyramidal cells ($n = 17$), held at −70 mV membrane potential, were recorded in two separate series of experiments with two different pipette solutions. In the first series, using KCl as the main solute, the average amplitude of evoked IPSCs was 151.1 ± 28.6 pA ($n = 13$ stimulation sites for 12 cells, range 38.9–323.1 pA). Paired-pulse stimulation at 100 ms interpulse interval evoked IPSCs showing a significant ($P < 0.005$, Wilcoxon test) paired-pulse depression (IPSC2/IPSC1, 0.77 ± 0.02; range 0.61–0.89, $n = 13$). In the second series of recordings, using CsCl as the main solute and QX314 to block some voltage-sensitive channels, significantly larger IPSCs were evoked, but only one IPSC exceeded 1.5 nA. The average amplitude of IPSCs was 1126.2 ± 329.1 pA ($n = 5$; range, 257.1–2157.0 pA) and a paired-pulse depression (IPSC2/IPSC1, 0.78 ± 0.04; $P < 0.05$, range 0.68–0.90) similar to that found in the first series was observed.

Effect of L-AP4 on IPSCs recorded in interneurons

Bath-applied L-AP4, a selective group III mGluR agonist, at a concentration of 50 μM depressed IPSCs to $68 \pm 6\%$ ($n = 29$, $P < 0.001$) of control (Figs 1B, 4 and 5). The depression of IPSCs in interneurons was highly variable. Interneurons receive GABAergic input from several sources, and this input in some cases is highly specific for a given cell type (Freund & Buzsaki, 1996). It is possible that the variability in the response is a result of stimulating different types of presynaptic GABAergic axons, some of which may not contain mGluRs, or may differ in the expression of mGluR subtypes. A statistical comparison of the population of control IPSCs with IPSCs in the presence of L-AP4 showed that, at a significance level of $P < 0.05$ (Mann–Whitney *U*-test, Table 1), evoked IPSCs were depressed in 18 of 29 cases (62%) to an average of $51 \pm 6\%$ of control (range, 12–87%, $P < 0.001$).

The agonist L-AP4 was also tested at higher doses, because it has been reported that at human group III mGluRs expressed in a heterologous system, L-AP4 is highly potent at mGluR4 and mGluR8 subtypes with an EC_{50} of <1 μM, whereas the EC_{50} at mGluR7 is 175 μM (Wu *et al*., 1998). At a concentration of 300 μM of L-AP4, IPSCs were significantly reduced in three of four interneurons; at a concentration of 400 μM IPSCs were significantly reduced in 11 of 14 interneurons, and at a concentration of 500 μM the IPSC was significantly reduced in one tested interneuron. The higher doses of L-AP4 depressed the IPSCs to $70 \pm 6\%$ ($n = 19$) of control (Fig. 5). The depression of IPSCs in interneurons was as variable as at the lower dose (Fig. 5). Evoked IPSCs were significantly depressed in 14 cells to $62 \pm 7\%$ of control ($P < 0.001$, range 9–87%). Overall, the proportion of IPSCs (74%) depressed by 300–500 μM L-AP4 was not significantly higher ($P > 0.1$, χ^2 test) than the proportion (62%) obtained with 50 μM of the agonist. The mean depression of IPSCs elicited by the low

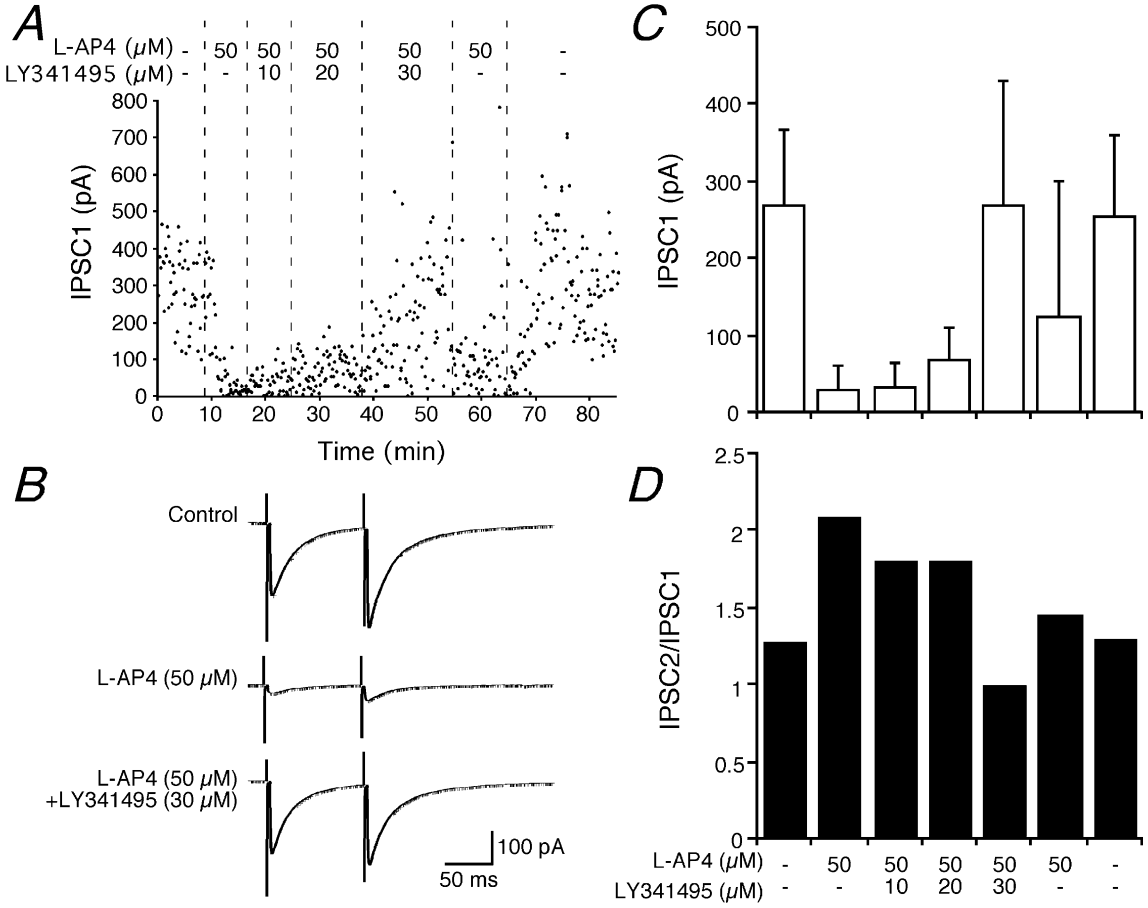

FIG. 4. Time course of the depression of IPSCs in an interneuron by L-AP4 (P15, case N3661). Only the soma and dendrites were recovered following histological processing and the cell was classified as a horizontal cell. (A) The amplitude of individual IPSCs, evoked at 0.1 Hz, is shown and demonstrates a strong depression by L-AP4, which is antagonized by the mGluR antagonist LY341495 in a dose-dependent manner. Upon discontinuing LY341495, the depressive effect of L-AP4 returned. Washing out L-AP4 led to the restoration of IPSCs to predrug level. (B) Average traces of the last 30 IPSCs before a change to 50 μM L-AP4 (Control), in 50 μM L-AP4 (middle) and 50 μM L-AP4 plus 30 μM LY341495 30 μM (bottom). (C) Average IPSC amplitudes (± SD) of the last 30 IPSCs before a change in the drugs in each period. (D) Average paired-pulse ratios of the last 30 IPSCs before a change in the drugs in each period. Note the increased paired-pulse ratio during suppression of the IPSC and decrease upon return of the response to control level.

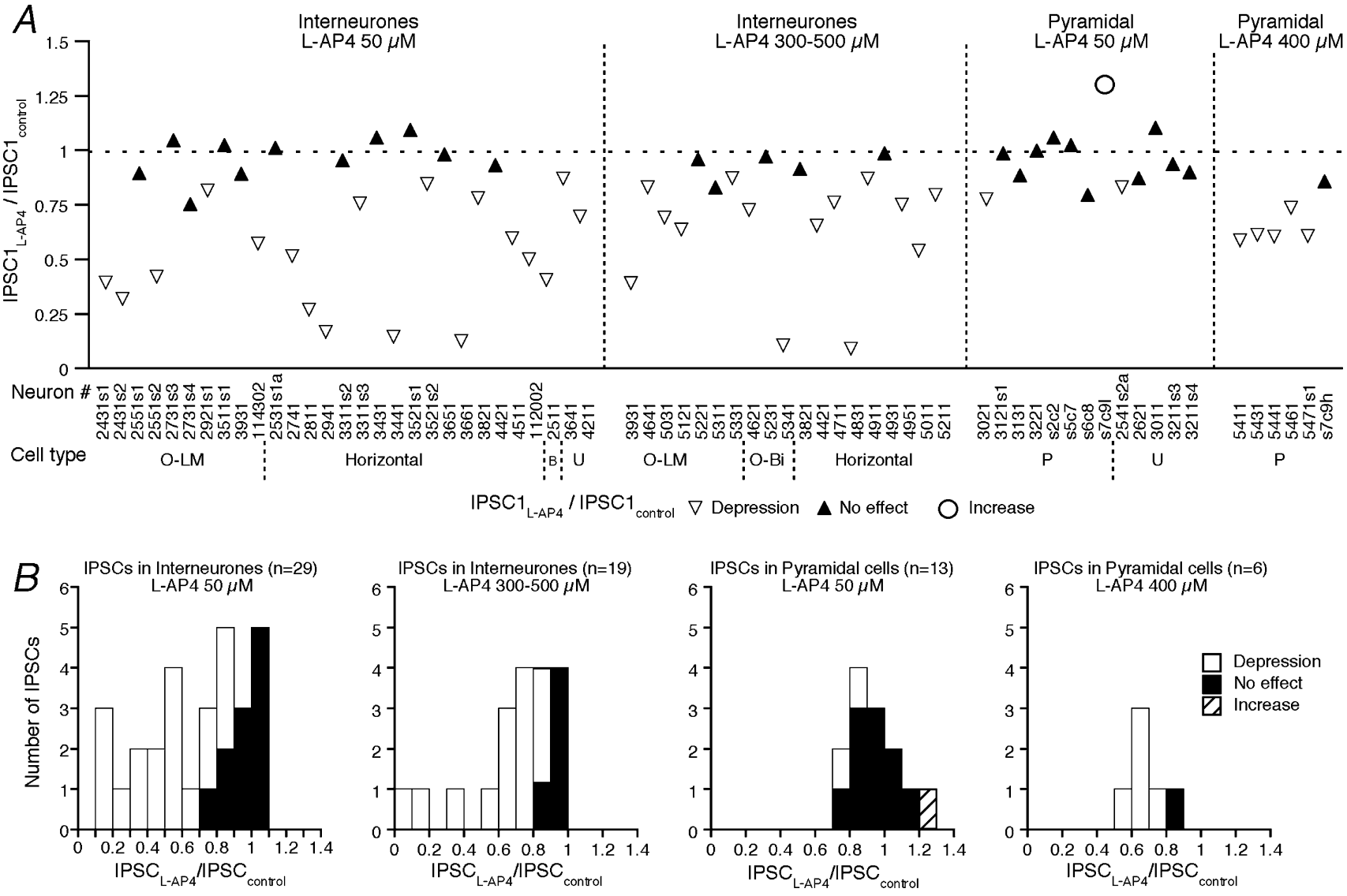

FIG. 5. Variability in the effect of low or high doses of L-AP4 on IPSCs in interneurons of stratum oriens–alveus and in pyramidal cells. (A) The effect of the drug on the first IPSCs of a pair evoked by extracellular stimulation is shown for each neuron. IPSCs were normalized to the respective control IPSCs. Above the code number of each neuron, the triangle represents the first of a pair of IPSCs. Statistically significant depression is shown by open triangles. The cells are grouped as O-LM, O-Bi, basket (B), horizontal (the axon not recovered), pyramidal (P), or unidentified (U). (B) The histograms show the distribution of the degree of depression of the IPSCs by L-AP4. In interneurons, about the same proportion of IPSCs were affected (open columns) by either the low or the high dose of L-AP4. However, in pyramidal cells a high dose of L-AP4 depressed most IPSCs, whereas the low dose affected relatively few.

and high doses of L-AP4 was also not different ($P > 0.1$, Mann–Whitney *U*-test).

We also applied 50 μM L-AP4 twice with an interval of 40 ± 9 min (range 35–50 min, $n = 3$) to test whether the first application of the drug produced desensitization. Clearly, this was not the case because both the first and second applications of L-AP4 reduced significantly ($P < 0.05$, Mann–Whitney test) the IPSCs in all three cells tested, to $40 \pm 24\%$ (first application, range 12–57%) and $59 \pm 11\%$ (second application, range 47–68%) of the control value.

There was no significant difference (χ^2 test, $P > 0.05$) in the proportion of IPSCs depressed by the low or high doses of L-AP4 amongst interneurons. In the most frequent cell type, the O-LM cell, there appeared to be a higher probability of depression of IPSCs by the higher doses of L-AP4 than by the 50 μM concentration (71 vs. 50%), but this difference did not reach statistical significance. Similarly, the proportion of IPSCs depressed (90%) by 300–500 μM L-AP4 in somatostatin-positive cells was not significantly higher (χ^2 test, $P > 0.1$) than that in somatostatin-immunonegative cells (50%). At 50 μM L-AP4 concentration, significant depression of IPSCs was observed in a basket cell.

Effect of L-AP4 on IPSCs recorded in pyramidal cells

In contrast to the results obtained in interneurons, in the majority of the pyramidal cells tested 50 μM L-AP4 did not reduce IPSCs ($96 \pm 4\%$ of control, $n = 13$; Fig. 5). L-AP4 (50 μM) depressed only two out of 13 IPSCs (15%) to 78 and 83%, respectively, and enhanced one by 30% (cf. Evans *et al*., 2000). In contrast, evoked IPSCs were significantly depressed in five out of six cases by 400 μM L-AP4 to a mean of $67 \pm 4\%$ ($n = 6$) of control. The degree of depression and the frequency of depressed IPSCs were significantly higher than that caused by 50 μM L-AP4 (for both, $P < 0.01$; Mann–Whitney U and χ^2 tests, respectively). Paired-pulse depression at 100 ms interpulse interval was not significantly reduced in pyramidal cells by 400 μM L-AP4 (IPSC2/IPSC1, before 0.84 ± 0.07, after 0.96 ± 0.09; $114 \pm 11\%$, $n = 6$).

The average depression of IPSCs caused by 50 μM L-AP4 ($P < 0.05$, Mann–Whitney *U*-test) and the frequency of a significant depression was higher ($P < 0.01$, χ^2 test) in interneurons than in pyramidal cells. There was no difference between interneurons and pyramidal cells in the frequency ($P > 0.5$) or the average degree ($P > 0.1$) of depression of IPSCs by 300–500 μM L-AP4.

The effect of the mGluR antagonist LY341495

The mGluR antagonist LY341495 (10–30 μM; Kingston *et al*., 1998) reduced L-AP4-evoked depression of IPSCs in a dose-dependent manner (Figs 2 and 4). Following the depression of IPSCs by 50 μM L-AP4–$33 \pm 1\%$ of control ($n = 4$ interneurons), the application of 30 μM LY341495, in the continuing presence of L-AP4, returned IPSCs to $97 \pm 1\%$ of control. Likewise, LY341495 at 30 μM either completely (to 114%) or partially (to 75%) restored the IPSCs previously reduced by 400 μM L-AP4 ($n = 2$). In both cases the sensitivity of the IPSC to L-AP4 was reconfirmed following the washout of LY341495. None of the IPSCs tested in four interneurons was changed significantly by 30 μM LY341495 alone ($96 \pm 4\%$ of control; $n = 4$), ruling out the possibility that a tonic activation of mGluRs affected IPSCs in interneurons. There was no change in the paired-pulse ratio either.

L-AP4 depressed IPSCs, probably through a presynaptic mechanism

Next we investigated the site of action of mGluRs activated by L-AP4. First, we analysed the change in PPR relative to control conditions. If a compound acts presynaptically by affecting transmitter release it modulates the paired-pulse ratio, whereas a postsynaptic action produces a similar change in both the first and second responses to a pair of stimuli (Manabe *et al*., 1993). Paired-pulse ratio (IPSC2/IPSC1) was increased by 50 μM L-AP4 from 0.90 ± 0.05 to 1.10 ± 0.08 ($n = 29$, Wilcoxon test, $P < 0.05$) but no significant change was caused by 300–500 μM L-AP4 (from 0.96 ± 0.05 to 1.05 ± 0.10, $n = 19$) for the overall population of IPSCs in interneurons (Fig. 6). However, there was a significant increase in PPR of those IPSCs that showed significant depression of the IPSC by 50 μM ($46 \pm 17\%$, from 0.92 ± 0.07 to 1.24 ± 0.11, $n = 18$, $P < 0.05$), and also by 300–500 μM L-AP4 ($15 \pm 6\%$, from 0.94 ± 0.07 to 1.10 ± 0.13, $n = 14$, $P < 0.05$). The paired-pulse ratio of the IPSCs that were not depressed did not change significantly (Fig. 6)

Second, we plotted CV^{-2} against the mean amplitude of IPSCs significantly depressed by L-AP4 ($n = 16$), both normalized to the individual control IPSC. In all but one case CV^{-2} decreased together with the IPSC amplitudes (Fig. 7). The points representing each IPSC fell close to the line of unitary slope, with the majority of shifts having slopes close to 1, hence indicating a presynaptic action of the drug. The group average for the effect of 50 μM L-AP4 on the first IPSC of a pair resulted in a slope of 1.47 ± 0.27 (n = 16) and the group average for the effect of 300–500 μM L-AP4 was 1.22 ± 0.37 ($n = 14$). The same trend was caused by 400 μM L-AP4 for the IPSCs in six pyramidal cells, with a mean slope of 0.75 ± 0.95, which was not significantly different from 1 ($P > 0.5$, one-sample sign test, Fig. 7). Overall, the analysis indicated a presynaptic effect of L-AP4.

Next, to establish the location of group III mGluRs directly in the synapses tested above by physiological methods, we carried out an

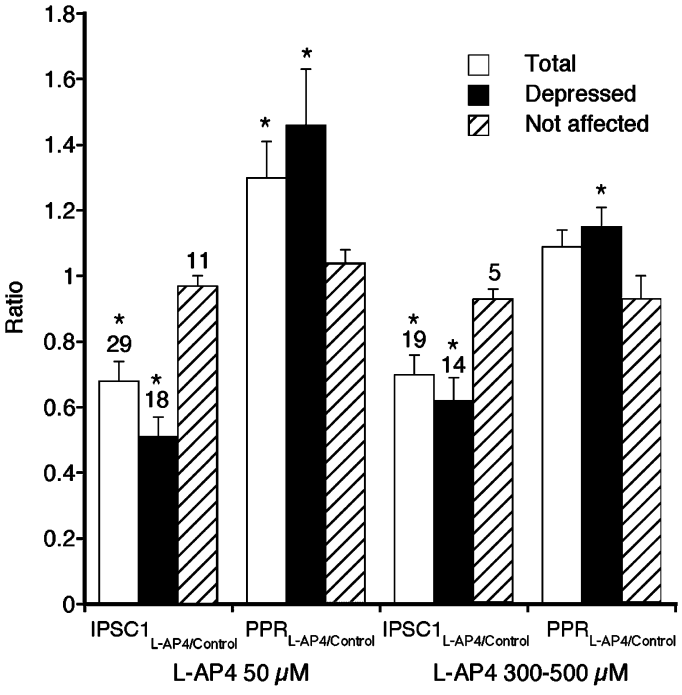

FIG. 6. Normalized data showing the effect of low and high doses of L-AP4 on IPSCs and paired-pulse ratios (PPR) in interneurons. The first column (open) in each case shows the mean (± SEM) depression of IPSCs in all interneurons; the second column (filled) shows the mean depression of those IPSCs which individually showed a significant depression, and the third column (stippled) shows the mean of those IPSCs which individually were not significantly affected by L-AP4. The low and high doses of L-AP4 affected IPSCs similarly. Numbers above the columns indicate the number of IPSCs. $^*P < 0.05$ vs. 1.0.

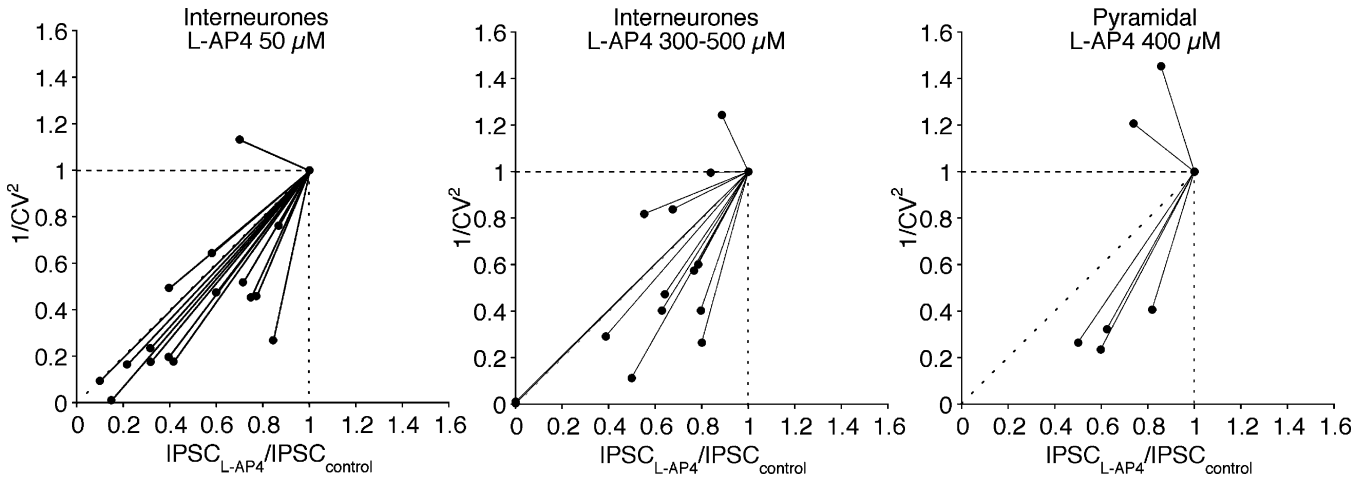

FIG. 7. Effect of L-AP4 on the relationship between mean IPSC amplitude and CV^{-2} (inverse square of the coefficient of variation) of the first IPSC of a pair evoked by extracellular stimulation. All points show averages of 30 IPSCs normalized to the control IPSC. In most cases, L-AP4 caused a reduction in CV^{-2} along with a depression of the IPSC. Most points fall close to the line of unitary slope (dotted), consistent with a decrease in the amount of transmitter released.

electron microscopic immunocytochemical double-labelling analysis for glutamic acid decarboxylase (GAD), the enzyme responsible for the synthesis of GABA, and group III mGluRs (Fig. 8). The presence of mGluR7a in some hippocampal GABAergic terminals has been established in a recent study (Somogyi *et al*., 2003), and we compared its location to the location of mGluR4 and mGluR8a. In order to differentiate the immunoreactivity for mGluRs and GAD in the same sections, GAD was demonstrated with the silver-intensified immunogold reaction, producing electron-opaque particles as a signal, whereas mGluRs were demonstrated with an immunoperoxidase reaction giving a homogeneous electron-opaque deposit. Being an enzyme-based reaction the immunoperoxidase reaction is more sensitive than the immunogold reaction; therefore it was used for the visualization of the receptors. Immunoreactivity for mGluR4, mGluR7a and mGluR8a

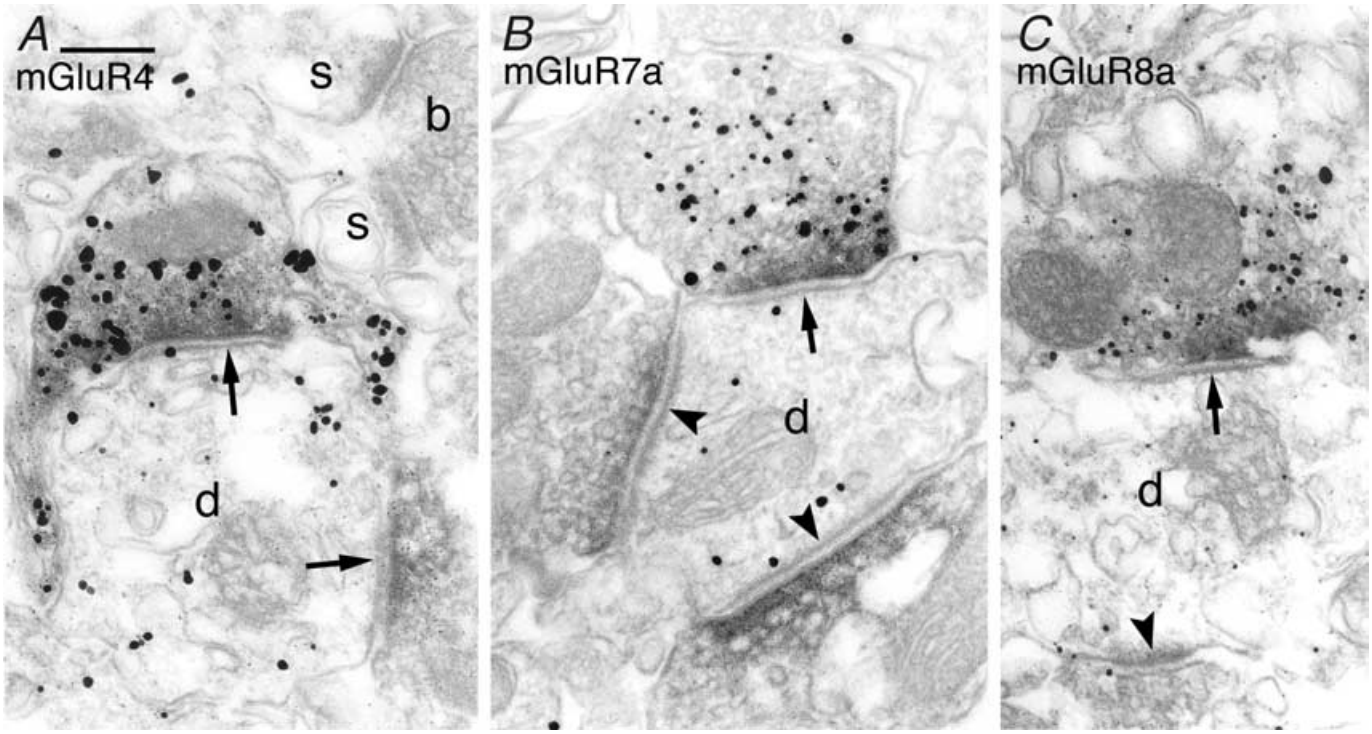

FIG. 8. Electron microscopic demonstration of immunoreactivity for group III mGluRs in the presynaptic active zones of GABAergic and non-GABAergic synaptic terminals innervating interneuron dendritic shafts (d) in str. oriens. Immunoreactivity for GAD is demonstrated by silver-intensified immunogold reaction (particles); mGluRs are demonstrated by immunoperoxidase product, a homogeneous electron-opaque deposit in the active zone. The GAD-immunopositive boutons form type II synapses (arrows), whereas the GAD-immunonegative boutons form type I synapses (arrowheads), except the one in A, which forms a type II synapse. (A) A GAD-immunopositive (top) and a GAD-immunonegative (right) bouton forming synapses (arrows) with a GAD-immunopositive dendrite are both immunoreactive for mGluR4. A bouton (b) forming synapses with two spines (s) is immunonegative. (B) A GAD-immunopositive (top) and two GAD-immunonegative (left and bottom) boutons forming synapses with a GAD-immunopositive dendrite are immunoreactive for mGluR7a. (C) A GAD-immunopositive (top) and a GAD-immunonegative (bottom) bouton forming synapses with a dendrite are seen. Only the GAD-positive bouton is immunoreactive for mGluR8a. Scale bar, 0.2 μm (A–C).

was selectively located in the presynaptic active zone of both GAD-immunopositive and -immunonegative boutons (Fig. 8). Terminals immunopositive for GAD formed type II (frequently called symmetrical) synapses, whereas the vast majority of GAD-immunonegative boutons formed type I (frequently called asymmetrical) synapses. MgluR7b was also present in boutons forming either type I or type II synapses; it has not been tested together with GAD (data not shown). The postsynaptic targets of mGluR4-, mGluR7b- and mGluR8a-positive boutons were dendritic shafts; mGluR7a-positive boutons innervated both dendritic shafts and dendritic spines. The vast majority of dendritic shafts could be identified as originating from interneurons, because they were either immunopositive for GAD (Fig. 8A and B) or they received type I synapses (Fig. 8C) which are not present on the dendritic shafts of pyramidal cells (Megias *et al*., 2001). Interestingly, if a dendritic shaft received a group III mGluR-immunopositive synapse, usually most of the other synapses were also immunopositive on that particular dendrite whereas on neighbouring dendrites they were immunonegative, as reported previously for these three mGluRs (Shigemoto *et al*., 1996, 1997; Corti *et al*., 2002; Dalezios *et al*., 2002; Somogyi *et al*., 2003). However, some of the GAD-positive terminals making synapses on dendrites receiving innervation from group III mGluR-enriched terminals were mGluR-immunonegative. Although false-negative reaction due to the lack of antibody penetration into the tissue cannot be excluded with this method, the consistent presence of mGluR-negative and GAD-positive boutons in well-reacting areas of the section suggests a heterogeneous expression of group III mGluRs in GABAergic boutons innervating interneurons. The overlap in the postsynaptic cell populations innervated by the three mGluRs could not be established in the current study. No group III mGluR immunoreactivity could be detected in synapses on pyramidal cell somata, proximal dendrites or axon initial segments.

Discussion

The comparison of the effect of a group III mGluR agonist, L-AP4 (50 μM), on $GABA_A$ receptor-mediated synaptic transmission in identified stratum oriens GABAergic interneurons and pyramidal cells has revealed a depression of IPSCs in interneurons, but only occasional and small effects in pyramidal cells. A higher concentration of L-AP4, however, also depressed IPSCs in pyramidal cells, as reported earlier (Morishita *et al*., 1998). The degree of depression of IPSCs in interneurons was highly variable, and the increase in paired-pulse ratio under the influence of L-AP4 suggested a presynaptic action of the drug. This was further supported by variance analysis of the response, which showed a proportional decrease in the mean amplitude and CV^{-2}, as also shown previously for str. radiatum interneurons (Semyanov & Kullmann, 2000). Direct evidence for the presynaptic location of mGluR4, mGluR7a and mGluR8a in GABAergic terminals on str. oriens interneurons was obtained by electron microscopic immunocytochemistry. However, these results do not exclude an additional postsynaptic effect of L-AP4, and further work on miniature IPSCs (mIPSCs) is required to clarify the mechanism of presynaptic depression. These observations demonstrate that GABAergic inputs to different cell types in the same brain area may express different presynaptic receptors, as found here for pyramidal cells and interneurons.

Differential distribution and effect of presynaptic mGluRs in hippocampus

Cells expressing somatostatin and a high density of mGluR1α receive synaptic input which is highly enriched in presynaptic mGluR7a as compared to that of pyramidal cells (Shigemoto *et al*., 1996). A similar interneuron target-specific enrichment was also found in glutamatergic terminals for mGluR4 and mGluR8 (Shigemoto *et al*., 1997; Corti *et al*., 2002). Both mGluR7 splice variants and, less frequently, the other group III mGluRs, mGluR4 and mGluR8a, were found in the presynaptic active zone of type 2 synapses (Shigemoto *et al*., 1997; Corti *et al*., 2002; Dalezios *et al*., 2002; Somogyi *et al*., 2003), most of which are GABAergic in the hippocampus. Here we have demonstrated that in the CA1 area some GABAergic terminals contain high levels of at least one of the three group III mGluRs known to be present in the hippocampus; therefore, these receptors are candidates for mediating the depression of IPSCs in interneurons. The antibodies to mGluR4, mGluR8 and two of the splice variants of mGluR7 did not reveal receptor immunoreactivity in the proximal GABAergic input to pyramidal cells, but high doses of L-AP4, which are known to activate mGluR7, depressed IPSCs. It is possible that other splice variants of mGluR7 reported recently (Schulz *et al*., 2002) and not recognized by our antibodies are responsible for this effect.

The GABAergic input to interneurons as well as pyramidal cells was differentially affected by L-AP4 as shown here in rat, and previously in the guinea pig (Semyanov & Kullmann, 2000). However, Semyanov & Kullmann (2000) studied unidentified interneurons, at room temperature, in stratum radiatum, whereas we have investigated identified types of interneuron at 32–35 °C in stratum oriens. Despite differences in the species, temperature and populations of interneurons, the two studies are in general agreement. Similarly to stratum oriens, stratum radiatum also contains a heterogeneous population of interneurons (Freund & Buzsaki, 1996).

Variability of L-AP4-mediated depression of IPSCs, diversity of cell types and sources of GABAergic terminals

The variability of IPSC depression may be due to heterogeneity of postsynaptic cells or presynaptic inputs differing in presynaptic receptors. Interneuron populations can be subdivided into broad groups using molecular markers, but can only be identified on the basis of their postsynaptic targets as reflected in their axonal pattern (Somogyi *et al*., 1998). The somatostatin-expressing cells in stratum oriens include the O-LM and O-Bi cells (Maccaferri *et al*., 2000) and the hippocampo-septal projection neuron (Gulyas *et al*., 2003). We had a reasonably large sample of axonally identified O-LM cells showing high variability in the effect of L-AP4 on IPSCs between individual cells, which did not appear to differ from the IPSCs in other less frequently encountered cell types. Many of the cells without axonal recovery were probably also O-LM cells, because they showed very similar dendritic trees to identified O-LM cells. Therefore, diversity of interneuron types in the sample is probably not the major contributor to the variability of the effect of L-AP4.

Each type of neuron is likely to be innervated by multiple, functionally distinct GABAergic cells (Freund & Buzsaki, 1996; Hajos & Mody, 1997; Maccaferri *et al*., 2000), which may differ in presynaptic mGluR expression. This is well documented for pyramidal cells (Gulyas *et al*., 1993; Buhl *et al*., 1994; Freund & Buzsaki, 1996; Vida *et al*., 1998), but it is less clear for many interneuron types. The most frequent cell in our sample, the O-LM cell, is densely innervated by a specific class of GABAergic cell, called the IS-III (interneuron-specific type III) cell, expressing vasoactive intestinal polypeptide (Blasco-Ibanez & Freund, 1995; Acsady *et al*., 1996). The septo-hippocampal GABAergic projection also innervates somatostatin-positive cells (Gulyas *et al*., 1990). Furthermore, O-LM cells also innervate each other (N. Kogo & P. Somogyi, unpublished observation). Therefore, GABAergic innervation of O-LM cells originates from at least three sources. Some of these different presynaptic GABAergic neurons may either lack or express distinct subtypes of presynaptic group III mGluRs. Other factors, such as the variation of

presynaptic receptor level in individual terminals, or the state of activation or desensitization of receptors by endogenous agonists in the control condition (Awatramani & Slaughter, 2001), may have also contributed to the variability in L-AP4 effect.

Identity of group III mGluR(s) mediating the effect of L-AP4

Our results show that three presynaptic group III mGluRs are present in GABAergic terminals, and the results do not exclude their coexistence in the same terminal. Although the effective concentration of L-AP4 on mGluR7a *in situ* at the synapses is not known, at human mGluRs expressed in a mammalian cell line, the EC_{50}s of L-AP4, for the inhibition of forskolin-stimulated cAMP production, were 0.32, 175 and 0.061 μM for mGluR4, 7 and 8, respectively (Wu *et al*., 1998). If these values were applicable to presynaptic mGluRs, the involvement of subtypes other then mGluR7 would be expected from the depression obtained by 50 μM L-AP4. A similar conclusion was reached for the mGluR-mediated depression of IPSCs to stratum radiatum interneurons on the basis of both agonist concentration dependence and the blocking of the response by α-methylserine-O-phosphate (Semyanov & Kullmann, 2000), an antagonist that was described as ineffective on mGluR7. In our study, increasing the dose of L-AP4 to a level expected to activate mGluR7 did not significantly enhance the effect in interneurons. Therefore, either a maximal activation of the effector mechanism is already achieved by the low dose of L-AP4 having high efficacy on another receptor, or the potency of L-AP4 is higher on synaptic mGluR7 than in expression systems. Unfortunately, there are no agonists or antagonists selective to mGluR7, which has the lowest affinity among the group III mGluR subtypes not only to L-AP4 but also to glutamate (Wu *et al*., 1998; Cartmell & Schoepp, 2000). The reduction in the increase of PPR at the high doses of L-AP4, as compared to 50 μM, could be due to an additional postsynaptic effect, although no consistent postsynaptic immunolabelling was seen with the specific antibodies and method used here, in agreement with previous studies (Shigemoto *et al*., 1997; Corti *et al*., 2002; Somogyi *et al*., 2003).

In pyramidal cells IPSCs were depressed only by a high dose of L-AP4, but not by a low dose, suggesting the involvement of mGluR7. Immunoreactivity for mGluR7a or mGluR7b has not been found on GABAergic terminals innervating the somatic region of pyramidal cells in the present study. Therefore, the identity of the receptor involved in the depression of evoked IPSCs in pyramidal cells remains to be established. All in all, the results indicate that receptor subtypes other than mGluR7 contribute to the depression of IPSCs in interneurons, but the involvement of mGluR7 is not excluded.

Functional roles of presynaptic mGluRs

The functional roles of presynaptic mGluRs on the GABAergic terminals depend on the source of the endogenous agonist for their activation. It is possible, but at present least likely, that an agonist, such as L-serine-O-phosphate, which is present in the brain at high levels, is released by the GABAergic terminals and acts as a feedback modulator through autoreceptors. Glutamate is unlikely to be released by most GABAergic terminals, because they contain a low level of this amino acid (Somogyi *et al*., 1986), which serves as a precursor for GABA synthesis by GAD. However, in rare cases, both GABA and glutamate might be stored (Sandler & Smith, 1991; Somogyi, 2002) and released (Walker *et al*., 2001) by the same nerve terminals. In other synapses, there is evidence for two other possible mechanisms of receptor activation: (i) glutamate, released from nearby excitatory terminals, may diffuse through the extracellular space (Vizi & Kiss, 1998) and reach high enough concentration to activate presynaptic mGluRs (spillover; Mitchell & Silver, 2000; Semyanov & Kullmann, 2000); (ii) postsynaptic dendrites may release glutamate or other transmitters specifically to the presynaptic terminals (Zilberter, 2000).

The depolarization-induced suppression of inhibition in the hippocampus can also be enhanced by the activation of postsynaptically located group I mGluRs, leading to the suppression of presynaptic transmitter release via the retrograde activation of CB1 cannabinoid receptors on GABAergic terminals (Daniel & Crepel, 2001; Maejima *et al*., 2001; Ohno-Shosaku *et al*., 2002; Yoshida *et al*., 2002). Group III mGluRs do not seem to be involved in this effect. In contrast, long-term depression of excitatory input to some stratum radiatum interneurons in the CA3 region requires postsynaptic Ca^{2+} influx through Ca^{2+}-permeable AMPA receptors as well as activation of a presynaptic group III mGluR with properties similar to mGluR7 (Laezza *et al*., 1999).

The selectively high level of presynaptic mGluR7 on the mGluR1α–somatostatin-expressing hippocampal interneurons suggests that these cells govern presynaptic receptor level via a retrograde signal in most of their synaptic input irrespective of the transmitter mechanism, i.e. the target cell-specific rather than origin-specific property of synapses. These cells show strong excitatory postsynaptic potential (EPSP) frequency facilitation in both the hippocampus and the isocortex (Ali & Thomson, 1998; Reyes *et al*., 1998; Losonczy *et al*., 2002, 2003). Intraterminal calcium dynamics play a key role in this frequency facilitation (Rozov *et al*., 2001), and group III mGluR activation can depress the calcium current in terminals at the calyx of Held (Takahashi *et al*., 1996). We found that most IPSCs on the hippocampal cells lack paired-pulse depression, as it has been found also in the isocortex (Reyes *et al*., 1998). Similarly to other synapses (Scanziani *et al*., 1998; Semyanov & Kullmann, 2000), the activation of group III mGluRs increased paired-pulse facilitation of IPSCs. This suggests a possible role for presynaptic mGluRs, i.e. modifying the pattern of frequency response of postsynaptic potentials depending on the rate of presynaptic activity and the resulting extracellular glutamate level (Awatramani & Slaughter, 2001; Losonczy *et al*., 2003). However, if such a mechanism were operating at GABAergic synapses it may require specific conditions, because in our preparation the application of an mGluR antagonist alone did not cause any modification of the synaptic response. It is possible that the differences in the affinities of group III mGluR subtypes to glutamate support distinct roles when they coexist in the same synapses (Wada *et al*., 1998). Some may act as extracellular glutamate sensors, and other ones for the detection of possible retrograde messengers.

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Abbreviations

ACSF, artificial cerebrospinal fluid; AMCA, 7-amino-4-methylcoumarin-3-acetic acid; CV, coefficient of variation; D-AP5, D(–)-2-amino-5-phosphonopentanoic acid; DIC, differential interference contrast; DNQX, 6,7-dinitroquinoxaline-2,3-dione; GAD, glutamic acid decarboxylase; IPSC, inhibitory postsynaptic current; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; mGluR, metabotropic glutamate receptor; mGluR1α, mGluR subtype 1, 'α' splice variant; MGluR4, mGluR subtype 4; mGluR7a, mGluR subtype 7, 'a' splice variant; mGluR8, mGluR subtype 8; O-Bi, oriens–bistratified; O-LM, oriens–lacunosum moleculare; PBS, phosphate-buffered saline; Rs, series resistance; Str., str., stratum.

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