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Immunolocalization of Metabotropic Glutamate Receptor 1α (mGluR1α) in Distinct Classes of Interneuron in the CA1 Region of the Rat Hippocampus

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ABSTRACT: In the hippocampal CA1 region, metabotropic glutamate subtype 1 (mGluR1) receptors have been implicated in a variety of physiological responses to glutamate, which include modulation of synaptic transmission and plasticity, as well as neuronal excitability and synchronization. The mGluR1α isoform is characteristically expressed only by nonprincipal cells, and it is particularly enriched in somatostatin (SS)-containing interneurons in stratum oriens-alveus. Anatomical and physiological data have indicated the presence of mGluR1α in several distinct classes of interneurons with their somata located also in strata pyramidale, radiatum, and lacunosum moleculare. Each different interneuron subtype, as defined by functionally relevant criteria, including input/output characteristics and expression of selective molecular markers, subserves distinct functions in local hippocampal circuits. We have investigated which of the different CA1 interneuron classes express mGluR1α by immunofluorescent labeling, combining antibodies to mGluR1α, calcium-binding proteins, and neuropeptides, and by intracellular labeling in vitro. Several types of interneuron that are immunopositive for mGluR1α each targeted different domains of pyramidal cells and included (1) O-LM interneurons, found to coexpress both SS and parvalbumin (PV); (2) interneurons with target selectivity for other interneurons, expressing vasoactive intestinal polypeptide (VIP) and/or the calcium-binding protein calretinin; (3) procholecystokinin-immunopositive interneurons probably non-basket and dendrite-targeting; and (4) an as-yet unidentified SS-immunoreactive but PV-immunonegative interneuron class, possibly corresponding to oriens-bistratified cells. Estimation of the relative proportion of mGluR1α-positive interneurons showed 43%, 46%, and 30% co-labeling with SS, VIP, or PV, respectively. The identification of the specific subclasses of CA1 interneurons expressing mGluR1α provides the network basis for assessing the contribution of this receptor to the excitability of the hippocampus. © **2004 Wiley-Liss, Inc.**

KEY WORDS: immunohistochemistry; cholecystokinin; parvalbumin; mGluR7; somatostatin; vasoactive intestinal polypeptide

INTRODUCTION

Local γ-aminobutyric acid (GABA)ergic interneurons play multiple roles in the control of the output and network activity patterns of principal cells in the hippocampus (Traub and Miles, 1991; McBain and Fisahn, 2001). Physiological and anatomical evidence points to the existence of several distinct classes of interneurons, each potentially serving different functions in hippocampal circuits (Ramon y Cajal, 1893; Lorente de No, 1934; Freund and Buzsaki, 1996; Somogyi et al., 1998). Different classes of interneuron selectively innervate distinct membrane domains of pyramidal cells (Somogyi et al., 1998) or are specialized to control other interneurons (Freund and Buzsaki, 1996). The selective expression of neurochemical markers, such as calcium-binding proteins, neuropeptides, and neurotransmitter receptors, by subpopulations of interneurons has facilitated their classification into different populations (Kawaguchi et al., 1987; Nitsch et al., 1990; Freund and Buzsaki, 1996; Somogyi et al., 1998).

In the hippocampus, one of the alternatively spliced isoforms of the metabotropic glutamate receptor subtype 1 (i.e., mGluR1α), is characteristically expressed only by nonprincipal cells. It is particularly enriched in somatostatin (SS)-containing interneurons of the CA1 stratum oriens-alveus (O/A) (Baude et al., 1993; Hampson et al., 1994). Scattered interneurons immunopositive for mGluR1α have also been reported in strata pyramidale, radiatum, and lacunosum moleculare (Martin et al., 1992; Baude et al., 1993; Lujan et al., 1996; Shigemoto et al., 1997). Alternative splicing at the mGluR1 gene generates at least two other receptor proteins: mGluR1β and mGluR1d (Tanabe et al., 1992; Laurie et al., 1996). These splice variants are expressed in the hippocampus in both principal and nonprincipal cells (Shigemoto et al., 1992; Berthele et al., 1998; Ferraguti et al., 1998). Immunoreactivity (IR) for mGluR1β is particularly enriched in interneurons and pyramidal cells of the CA3 area but was undetectable in the CA1 area (Ferraguti et al., 1998). The location of the mGluR1d receptor protein is unknown, because antibodies that selectively identify this variant are not yet available. Nevertheless, some interneurons in strata oriens and pyramidale of the CA1 area were shown to have abundant mRNA expression for mGluR1d (Berthele et al., 1998).

A variety of physiological responses to glutamate appear to implicate mGluR1 receptors in the CA1 area. These responses include the modulation of synaptic transmission and plasticity (McBain et al., 1994;

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Ouardouz and Lacaille, 1995; Woodhall et al., 1999; Perez et al., 2001), as well as the control of neuronal excitability and synchronization (Whittington et al., 1995; van Hooft et al., 2000). Synchronous oscillations in the hippocampus, which have been implicated in several physiological functions (Singer, 1993; Buzsaki, 2002), appear to be generated by networks of interconnected interneurons (Buzsaki and Chrobak, 1995; Freund and Buzsaki, 1996). Oscillations dependent on inhibitory postsynaptic currents (IPSCs), within the range of 40 Hz, have been elicited in hippocampal slices in vitro by a number of manipulations, including the application of muscarinic (Fisahn et al., 1998) and metabotropic glutamate receptor agonists (Whittington et al., 1995; Boddeke et al., 1997). Bath application of the nonselective mGluR agonist 1S,3R-aminocyclopentane dicarboxylic acid (ACPD) evoked oscillatory inward currents with long latency and slow time course in CA1 O/A interneurons (McBain et al., 1994; Woodhall et al., 1999; van Hooft et al., 2000). Recently, it has been suggested that the activation of mGluR1 is involved in evoking rhythmic action potential firing (van Hooft et al., 2000), and that a network composed of several classes of interneurons concurs to generate collective 40-Hz rhythms (Whittington et al., 1995; Jefferys et al., 1996). At least three distinct classes of interneuron have shown ACPD-mediated inward currents, although the size of the current varied among interneuron types (McBain et al., 1994; Woodhall et al., 1999; van Hooft et al., 2000). Two of these interneuron types also showed rhythmic firing and were identified as oriens-lacunosum moleculare (O-LM) and calbindin-immunopositive cells, respectively (van Hooft et al., 2000).

Given that several interneuron classes may express mGluR1α, it is possible that the activation of this receptor may have distinct functional roles according to the specific classes of interneuron involved. Hence, their identification is of importance for the understanding of the contribution of mGluR1α to the excitability of local neuronal networks.

To this end, we have examined the co-localization of mGluR1α with the neuropeptides SS, vasoactive intestinal polypeptide (VIP), and pro-cholecystokinin (CCK), the calcium-binding proteins calretinin and parvalbumin (PV), and the presynaptic glutamate receptor mGluR7a, all of which are known to label well-characterized subpopulations of interneuron. Because several classes of interneuron express SS, IR of O-LM cells for mGluR1α was tested by identifying interneurons on the basis of their axonal projection, using combined whole-cell recording and intracellular biocytin labeling. In addition, we have attempted to characterize interneurons that target other interneurons selectively, by recording and filling cells within the CA1 stratum pyramidale in vivo, followed by labeling and reconstruction of their dendritic and axonal arbors. Some of the results presented in the present report have been published previously in abstract form (Pollard et al., 2000; Ferraguti et al., 2001).

MATERIALS AND METHODS

All procedures involving experimental animals were carried out in accordance with the UK Animals (Scientific Procedure) Act 1986 and associated procedures. All efforts were made to minimize stress to the animals and the number of animals used. For immunocytochemical studies, adult (n = 9) Wistar rats (Charles River, UK) were deeply anesthetized with Sagatal (pentobarbital sodium; 100 mg/kg, i.p.) and transcardially perfused with saline, followed for 20 min by a fixative composed of 4% paraformaldehyde, ~0.2% picric acid in 0.1 M phosphate-buffer (PB, pH 7.2–7.4). Brains were quickly removed, extensively rinsed in PB, and sectioned in the coronal plane at 50-μm thickness on a vibratome.

Double Immunofluorescent Labeling Using Tyramide Signal Amplification

Free-floating sections were treated with a solution of 1% $NaBH_4$ (Sigma) in PB for 20 min and extensively washed in PB. After a further wash in Tris-buffered saline (TBS; 0.9%), nonspecific protein binding was blocked by incubation in 20% normal goat serum (NGS) for 2 h. Sections were then incubated overnight (4°C) in primary antibodies (see Table 1 for complete listing and dilutions), in combination or alone, prepared in 1% NGS, 0.1% Triton X-100, TBS (TBS-T). The following day, after extensive washes in TBS, sections were incubated overnight (4°C) in secondary antibodies (Table 1). After three washes in TBS, the FITC-tyramide reagent (diluted 1:50 in amplification diluent; NEN) was applied to the sections for 10 min. Sections were then mounted onto gelatin-coated slides and coverslipped with Vectashield (Vector Laboratories).

Triple Immunofluorescent Labeling Using Primary Antibodies Raised in Different Species

As in double immunofluorescent experiments, free-floating sections were pre-blocked for 2 h in 20% NGS or 20% normal swine serum (swine serum was always used in combination with the guinea pig anti-mGluR1α) and were subsequently incubated overnight (4°C) in a combination of three different primary antibodies raised in different species (see Table 1 for combinations and dilutions). Sections were then washed in TBS and processed overnight (4°C) with a mixture of appropriate secondary antibodies (Table 1). When biotinylated secondary antibodies were used, sections were subsequently incubated, after extensive washes in TBS, in a solution containing streptavidin conjugated to either AMCA (Vector) or Cy3™ (Amersham) in TBS-T.

Controls

The two mGluR1α antisera used in this study showed an identical staining pattern, although, under our experimental conditions, weak labeling of the nuclei of pyramidal cells could be also observed with the guinea pig antibody. Both antisera were tested on brain slices of mice lacking mGluR1 (kindly provided by Dr. F. Conquet), which were perfused in the same way, and with the same fixative solution (except that the fixative was perfused for 10 min), as carried out for rats. No immunolabeling was detected with either of the antibodies in sections of mice with gene-targeted deletion of mGluR1, with the exception of some labeled cell nuclei with the antibodies raised in guinea pig. Labeling in wild-type mice

TABLE 1.

Antibodies and Conditions Used

Antibody	Species (raised in)	Dilution	Combinations of antibodies in double and triple labeling exp.[a]										Secondary antibodies[b]	Dilution	Source of primary antibodies	Characterization reference
mGluR1α	Rabbit	1:500	*v*	*v*	*v*								Sw anti-Rb-HRP (Dako)-TSA	1:100	DiaSorin	Alvarez et al., 2000
						v			*v*				Gt anti-Rb Alexa 488 (Mol. Probes)	1:1,000		
										v			Gt anti-Rb Cy3™ (Jackson)	1:400		
mGluR1α	Guinea pig	1:500					*v*	*v*			*v*	*v*	Gt anti-GP Biotin (Vector)	1:100	Dr. M. Watanabe (Hokkaido University, Japan)	Tanaka et al., 2000
mGluR7a	Rabbit	1:200									*v*		Dnk anti-Rb Cy3™ (Jackson)	1:400	Dr. R. Shigemoto (Natl. Inst. Physiol. Sci., Okazaki, Japan)	Shigemoto et al., 1997
VIP	Mouse	1:10,000		*v*									Gt anti-Ms Cy3™ (Jackson)	1:400	Biogenesis	Dey et al., 1988
							v	*v*			*v*	*v*	Gt anti-Ms Alexa 488 (Mol. Probes)	1:1,000		
									v				Gt anti-Ms Biotin (Vector)	1:100		
SS	Mouse	1:500	*v*										Gt anti-Ms Cy3™ (Jackson)	1:400	Dr. A. Buchan (MRC Reg. Peptide Group, Canada)	Vincent et al., 1985
										v			Gt anti-Ms Cy5™ (Amersham)	1:500		
SS	Rabbit	1:1,000										*v*	Gt anti-Rb Cy5™ (Amersham)	1:500	Dr. R. Benoit (McGill University, Canada)	Benoit et al., 1985
CCK	Rabbit	1:1,000						*v*					Gt anti-Rb Cy5™ (Amersham)	1:500	Dr. A. Varro (Liverpool University, UK)	Morino et al., 1994
CR	Mouse	1:1,000				*v*							Gt anti-Ms Cy3™ (Jackson)	1:400	SWant	Schwaller et al., 1995
CR	Rabbit	1:800					*v*						Gt anti-Rb Cy5™ (Amersham)	1:500	Chemicon	Jacobowitz and Winsky, 1991
PV	Guinea pig	1:1,000			*v*				*v*				Dnk anti-GP Cy3™ (Jackson)	1:400	Dr. K.G. Baimbridge (University British Columbia, Canada)	Personal communication
										v			Gt anti-GP Alexa 488 (Mol. Probes)	1:1,000		

Biotin, biotinylated; CCK, pro-cholecyslokin; CR, calretinin; Dnk, donkey; GP, guinea pig; Gt, goat; Ms, mouse; PV, parvalbumin; Rb, rabbit; SS, somatostatin; Sw, swine; TSA, tyramide signal amplification; VIP, vasoactive intestinal polypeptide.

[a]Each column represents the mixture of primary antibodies for a reaction.

[b]The position of V identifies the secondary antibody used in a specific reaction in combination with the primary antibodies.

for both antibodies was comparable to that observed in rat sections. For the guinea pig antibody, even low-intensity specific immunosignal could be easily discriminated from the nonspecific nuclear labeling. The labeling pattern observed with the guinea pig anti-PV antibody, when either used alone or in combination with rabbit anti-mGluR1α, was identical to the immunostaining obtained with a well-characterized mouse anti-PV antibody (Swant). Therefore, we report only the results produced with the guinea pig antibody. To control for a possible cross-reactivity between IgGs in double and triple immunolabeling experiments, some sections were processed through the same immunocytochemical sequence, except that only one primary antibody was applied, but the full complement of secondary antibodies was maintained. In addition, the secondary antibodies used were highly pre-adsorbed to the IgGs of numerous species. All anti-rabbit secondary antibodies tested showed some degree of cross-reactivity with the guinea pig anti-mGluR1α. However, under our experimental conditions, including the use of goat anti-rabbit Cy5-conjugated (Amersham) and donkey anti-rabbit Cy3-conjugated (Jackson ImmunoLab) secondary antibodies, the intensity of the labeling due to the cross-reactivity was very low and could be easily distinguished from the specific staining, also on the basis of the distinctive cellular compartments labeled. All other combinations always resulted in a lack of labeling of the species-unrelated secondary antibodies, confirming the specificity of the immunosignals.

Image Acquisition and Analysis

Full penetration of antibodies was tested using a LSM-510 confocal microscope (Zeiss). Immunofluorescence in different cell

populations was studied using a Leitz DMRB microscope with epifluorescence illumination equipped with the following filter blocks: A4 (excitation filter BP 360/40 nm, reflection short pass filter 400 nm, suppression filter BP 470/40 nm), L5 (excitation filter BP 480/40 nm, reflection short pass filter 505 nm, suppression filter BP 527/30 nm), Y3 (excitation filter BP 545/30 nm, reflection short pass filter 565 nm, suppression filter BP 610/75 nm), and Y5 (excitation filter BP 620/60 nm, reflection short pass filter 660 nm, suppression filter BP 700/75 nm). Images, recorded through a CCD camera (Hamamatsu C4742-95), were analyzed and displayed using the Openlab software (version 3.0.2; Improvision). Brightness and contrast were adjusted for the whole frame, and no part of a frame was modified in any way.

Semiquantitative analysis of co-labeled cells was performed in the CA1 area of the dorsal hippocampal sections capturing individual immunopositive cells with a ×40 objective lens (PL Fluotar, NA 0.70). Sections from three adult rats were analyzed for this study. Somatic profiles were counted separately in each hippocampal layer. At first, each cell showing IR for VIP, SS, or PV was captured at the same focal plane with both L5 and Y3 filter blocks. Once this data collection was completed, all neuronal cell bodies immunolabeled for mGluR1α were imaged. This provided the total number of immunopositive cells for each molecular marker as well as of co-labeled neurons. Two and four hemisections per animal were analyzed for the mGluR1α/SS and mGluR1α/PV or mGluR1α/VIP reactions, respectively. Co-localization between mGluR1α and the other markers was independently assessed by two of the investigators. The size of the soma of different types of interneuron can vary significantly, as a consequence the frequency of sampling of distinct cell classes might have been affected.

Whole Cell Recording and Immunofluorescent Labeling of O-LM Cells

Co-immunolabeling for SS and mGluR1α displayed several populations of cells; therefore, it became necessary to test further the identity of the strongly mGluR1α positive neurons, which can only be classified by revealing their axonal arbor. Horizontal slices (300 μm) of hippocampus were prepared from juvenile male Sprague-Dawley rats (postnatal day 13–24) as described in detail elsewhere (Cope et al., 2002; Maccaferri et al., 2000). Slices were incubated for ≥1 h at room temperature before being transferred to a recording chamber and being continuously perfused with a warmed (33 ± 1°C), oxygenated (95% O_2: 5% CO_2) artificial cerebrospinal fluid (CSF) of the following composition (in mM): 130 NaCl, 24 $NaHCO_3$, 3.5 KCl, 1 NaH_2PO_4, 2 $CaCl_2$, 1 $MgSO_4$, and 10 glucose. Neurons in stratum O/A were visualized using a Zeiss Axioskop (Zeiss) equipped with a ×40 immersion differential interference contrast objective coupled to an infrared camera (Hamamatsu). Whole-cell current clamp recordings were made with an Axoclamp 2B or Axopatch 1D preamplifier (Axon Instruments). Patch pipettes (final resistance 4–8 MΩ) contained (in mM): 126 potassium gluconate, 4 KCl, 4 Mg-ATP, 0.3 Na_2-GTP, 10 Na_2-phosphocreatine, 10 HEPES, and 0.5 % biocytin. Data were filtered at 2–5 kHz before being acquired at 10 kHz directly to a computer, using pClamp software via a DigiData 1200 analogue/digital converter (both Axon Instruments).

After termination of recording, slices were fixed for ≤3 h in a solution containing 4% paraformaldehyde, 0.05% glutaraldehyde, and ~2% picric acid in 0.1 M PB (pH 7.4). Fixed slices were then embedded in gelatin and were resectioned at 60-μm thickness. Immunofluorescent labeling was carried out as described above. Sections were incubated in a mixture of the following primary antibodies: rabbit anti-mGluR1α (diluted 1:500) and mouse anti-SS (diluted 1:500). AMCA-conjugated streptavidin (1:1,000; Vector Laboratories) was used to visualize biocytin. Detection of primary antibodies was obtained using goat anti-rabbit IgG conjugated to Alexa 488 (1:1,000; Molecular Probes, Eugene, OR) and goat anti-mouse IgG conjugated to Cy3™ (1:400; Jackson ImmunoLab). Cells were studied using a Leica dichromatic mirror system, as described above. The immunonegativity of a cell for a marker can be due to damage caused by the recording, an undetectable low level of the molecule, or the genuine absence of the molecule; therefore, only the positive detection of IR is informative after extensive whole-cell recording. The recorded cells usually showed significantly lower IR than nearby unrecorded cells.

After immunocytochemical processing, sections were demounted, washed in TBS and 0.1% Triton X-100 and incubated in biotinylated horseradish peroxidase (HRP) (1:100; Vector Laboratories) overnight at 4°C. Slices were then further washed and incubated in avidin-biotinylated HRP (1:100) for ≥6 h. 3,3′-Diaminobenzidine (0.05%; DAB) was used as the chromogen and 0.01% H_2O_2 as the substrate in the peroxidase reaction carried out in 50 mM Tris buffer. Sections were then dehydrated and permanently mounted on slides. The axonal and dendritic patterns were analyzed at high magnification to determine the identity of each recorded neuron.

Juxtacellular Recording

Sprague-Dawley rats (300–350 g) were anesthetized with halothane followed by an intraperitoneal injection of urethane (1.5 g/kg, 33% w/v), and were placed in a stereotactic apparatus on a heated water blanket. The scalp was removed and a bone window (1.5 × 3 mm) was drilled above the hippocampus to allow entry of the recording/iontophoretic electrode (AP = −4.16, ML = +2.5 from bregma) and a field electrode (AP = −4.3, ML = +2.5 from bregma) angled at 30° from the vertical plane. The electrode design consisted of three borosilicate glass capillaries with filament (1.5-mm outer diameter [OD]) glued, under microscopic control, with one glass protruding ~10 μm. The electrode arrangement was reinforced with two to three smaller pieces of glass capillary glued over the gaps and covered with molten wax. The protruding electrode served to record cells juxtacellularly, and the other two electrodes were used for drug application and current balancing with 1 M NaCl, respectively. The juxtacellular electrode contained 1.5% neurobiotin tracer in 0.5 M NaCl. As the recording electrode was farther away from the iontophoretic application, the juxtacellular responses recorded did not result directly from extracellular potential changes due to current application, but were due either to the drug itself or to indirect activation by other cells near the

iontophoretic electrode. In order for the field electrode to reach the stratum LM at the specified coordinates from bregma, the electrode was positioned 3.2 mm posterior to this point. Juxtacellular recordings were separated into two channels at the level of the Axoprobe-1A amplifier. The first channel was amplified (×10, Axoprobe-1A;×100, Neurolog System) and filtered (500-Hz to 3-kHz bandwidth, Neurolog System) for recording action potentials. The second channel was amplified ×10, Axoprobe-1A) and filtered (5-kHz LP, Axoprobe-1A) in order to record any phase reversal of oscillatory activity, which would serve as a reference for the placement of the field electrode. The field electrode was also amplified ×100) and filtered (500-Hz LP) using the Axoprobe-1A amplifier, but via a separate headstage. ECG recording (Neurolog System) served not only to monitor the animal's welfare, but also as a basis for discriminating behaviorally relevant rhythmic activity from that resulting from homeostatic functions. Data were stored on DAT tapes and transferred onto the Spike2 software via the 1401 CED interface (Cambridge Electronic Design) for analysis.

Filling and Visualization of In Vivo Recorded Cells

At the end of the recording, the electrode was moved closer to the cell; positive current pulses (300 ms on and 500 ms off) were applied for 15 min in order to release neurobiotin from the juxtacellular electrode at a magnitude that evoked a train of action potentials (Pinault, 1996). The animal was given additional urethane anesthesia 1 h later, and perfused through the heart with saline followed by fixative consisting of 4% paraformaldehyde, 0.05% glutaraldehyde, and ~0.2% picric acid made up in PB. The brain was removed and left in the fixative consisting of 4% paraformaldehyde, ~0.2% picric acid in PB until sectioning. Coronal sections of 70 μm were cut on a vibratome. Sections were thoroughly rinsed first in PB and then in TBS-T, and were incubated overnight at 4°C with an avidin-biotin HPO complex in TBS (ABC Kit, Vector Laboratories). The following day, sections were washed repeatedly in TBS and were incubated with a buffered DAB solution (0.5 mg/ml in TB) for 15 min; H_2O_2 was then added, for a final concentration of 0.01%. The reaction was stopped after 8 min by repeated washes with TB. Sections were mounted on gelatin-coated microscope slides, treated with 1% OsO_4 in PB for 10 min, dehydrated, and coverslipped with Xam mounting medium (BDH). Because of its relevance to this study, one labeled cell was reconstructed with a drawing tube using a ×100 oil objective.

RESULTS

After immunostaining with the rabbit antiserum to mGluR1α, numerous neuronal processes and perikarya of nonprincipal cells were observed in the hippocampus with a distribution similar to that described earlier (Martin et al., 1992; Baude et al., 1993; Hampson et al., 1994; Shigemoto et al., 1997). However, the procedures used in the current experiments provided greater sensitivity, permitting better visualization of weakly labeled or previously undetected mGluR1α positive-neuronal elements. The alveus and adjoining stratum oriens of the CA1 area contained a population of strongly immunoreactive cells (Fig. 1). In addition, we found numerous weakly labeled and a few strongly labeled nonprincipal cells in stratum pyramidale (Fig. 1) and, less frequently, in strata radiatum and lacunosum-moleculare (LM). When the antiserum was applied to brain sections of mice lacking mGluR1, in order to assess its specificity, no labeling could be detected (data not shown).

In hippocampal interneurons, IR to mGluR1α was reported to coexist largely, if not completely, with SS (Baude et al., 1993; Hampson et al., 1994; Kerner et al., 1997; Yanovsky et al., 1997). However, the large number of weakly mGluR1α-immunopositive cells found in strata pyramidale and radiatum appears to be inconsistent with the known distribution of SS-positive cell bodies (Kohler and Chan-Palay, 1982; Kosaka et al., 1988; Buckmaster et al., 1994). This observation prompted us to reinvestigate the colocalization between mGluR1α and SS, by means of double immunofluorescence labeling. The vast majority of intensely mGluR1α-immunopositive cells, particularly in stratum O/A (~97%, Table 2, Fig. 2A), did indeed coexpress SS (Fig. 3A), but a large proportion of weakly mGluR1α-labeled neurons in strata pyramidale (82.5%) and radiatum (88.7%) were not co-labeled (Table 2, Fig. 2A). In addition, the immunofluorescence signal for mGluR1α was not detected in many SS-immunoreactive neurons (Table 2, Fig. 3B) in stratum O/A (47.6%) and pyramidale (60.5%).

Identification of the Axonal Projection of mGluR1α/SS-Immunopositive Cells

Somatostatin-IR is present in at least two populations of hippocampal interneurons: the O-LM and oriens-bistratified (O-Bi) cells, which share similar somatodendritic organization but have distinct laminar axonal targets (Maccaferri et al., 2000). To elucidate whether one of these two cell populations coexpresses mGluR1α and SS, we have recorded from interneurons with somata located in stratum oriens using whole-cell patch clamp recording in vitro. The cells were immunocytochemically tested for the presence of mGluR1α and SS, and their axonal patterns were displayed by biocytin labeling. The membrane potential of the cells was held close to −60 mV by current injection to analyze the firing properties, which are not reported in the present report because they lie outside the scope of the current study. The membrane time constant and input resistances were 53 ± 22 ms and 427.7 ± 290 MΩ (mean ±SD), respectively, for O-LM cells (n = 12), and 40 ± 20 ms and 265.8 ± 83.6 MΩ, respectively, for O-Bi cells (n = 3); the mean age of the animals was 16.6 ± 2.5 postnatal days. Five O-Bi and 16 O-LM cells were anatomically verified (Fig. 4) and immunocytochemically tested for mGluR1α, of which one O-Bi and 14 O-LM cells were immunopositive (Fig. 4). The overall intensity of the immunolabeling for mGluR1α in the slices was generally lower than in similar regions of perfusion-fixed brains. This decrease of IR might be attributed to holding the brain slices in vitro and to the immersion fixation protocol. All O-LM cells tested for SS (n = 13) were found immunopositive, while two

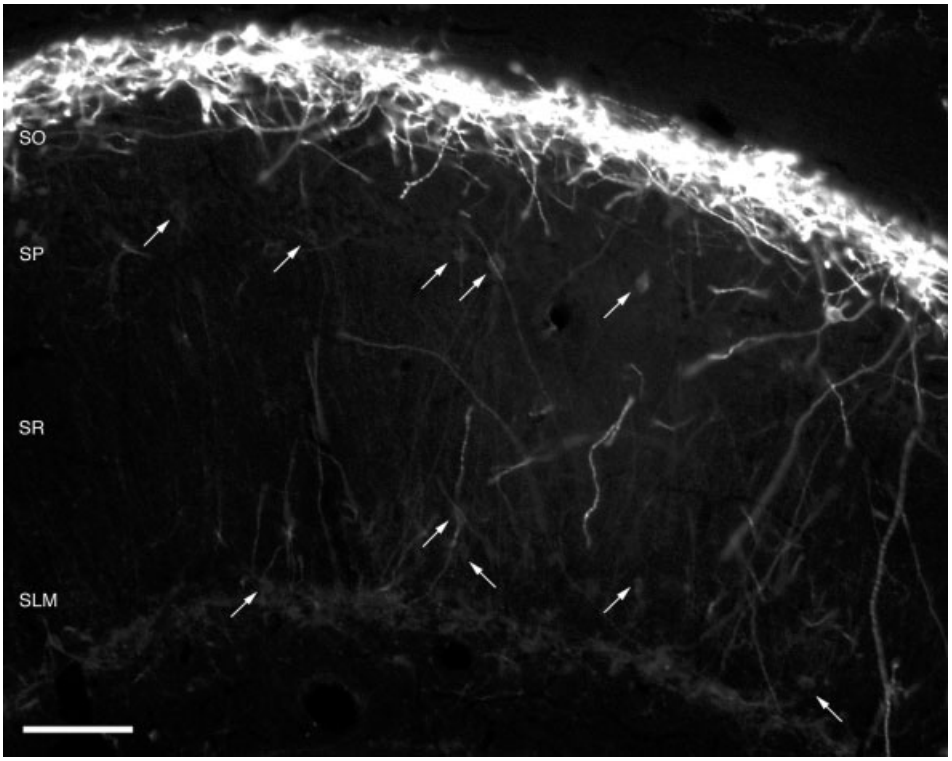


FIGURE 1. Fluorescence image showing the distribution of metabotropic glutamate receptor 1α (mGluR1α)-immunoreactive neurons in the CA1 area of an adult rat hippocampus. Weakly immunoreactive cells located in the strata pyramidale, radiatum, and lacunosum-moleculare are indicated by arrows. SO, stratum oriens/alveus; SP, stratum pyramidale; SR, stratum radiatum; SLM, stratum lacunosum-moleculare. Scale bar = 100 μm.

O-Bi cells, out of the five tested, showed SS-IR. These findings provide conclusive evidence that in the rat hippocampus intensely mGluR1α-immunopositive cells are mainly O-LM cells, and suggest that at least a subpopulation of O-Bi interneurons also expresses this receptor.

Interneurons Selectively Targeting Other Interneurons Display mGluR1α-IR

It appears that a relatively large component of mGluR1α-immunopositive cells does not contain SS (~57%). Most of these interneurons express a distinctively low level of the receptor and their somata are located primarily in strata pyramidale, radiatum, and LM. Interneurons selectively targeting other interneurons (IS) appeared as prime candidates for this group of mGluR1α-immunopositive cells. Such interneurons form a heterogeneous class of cells expressing either VIP or calretinin (CR), or both. Some of the VIP-containing interneurons (IS-3) heavily innervate O-LM cells, and their somata are located primarily in stratum pyramidale and radiatum (Acsady et al., 1996a,b; Freund and Buszaki, 1996).

To investigate whether IS interneurons, in particular the VIP-immunopositive ones, contain mGluR1α, we carried out double immunofluorescence experiments followed by an assessment of the extent of VIP and mGluR1α coexpression. Estimation of mGluR1α- and VIP-immunolabeled neurons showed that, in strata pyramidale, radiatum, and LM, a very high proportion was immunoreactive to both molecules (Table 2, Figs. 2 and 5). Conversely, in stratum O/A, only ~2% of mGluR1α neurons showed VIP-IR (Table 2, Fig. 2A), which represents ~17% of total VIP-immunoreactive cells in this layer (Table 2, Fig. 2B). The mGluR1α/VIP-immunopositive cells were generally fusiform with horizontal dendrites and were located in the portion of the alveus bordering the white matter (Fig. 5E). In stratum LM, ~60% of VIP-containing interneurons also displayed mGluR1α-IR (Table 2, Figs. 2B and 5F). An inverse correlation in the degree of intensity between the two immunofluorescence signals was detected.

In addition to VIP-positive cell bodies, several plexi of varicose axons were seen, as previously described (Acsady et al., 1996a,b; Blasco-Ibanez and Freund, 1995). The termination zone of a plexus of VIP-immunoreactive axon terminals in stratum O/A largely overlapped with the dendrites of mGluR1α-immunopositive neurons (Fig. 5D), whereas cell bodies appeared to be relatively spared by this innervation. Interestingly, because labeling for mGluR1α revealed the dendrites completely, we could follow the VIP-positive boutons to the very tip of dendritic branches (Fig. 5D).

Three classes of IS interneurons have been described in the hippocampus on the basis of their axonal projections and neurochemical features (Acsady et al., 1996a,b). The coexpression of VIP with CR characterizes interneurons projecting to the stratum O/A (Acsady et al., 1996a). Immunofluorescence labeling for

TABLE 2.

Number of mGluR1α-Immunopositive, SS-Immunopositive, and Double-Labeled Cells Counted in the CA1 Area of Rat Dorsal Hippocampus

Layer	Cells mGluR1α-positive only (n)	Cells SS-positive only (n)	Double-labeled cells (n)	Double-labeled cells as % of mGluR1α-positive cells (%)	Double-labeled cells as % of SS-positive cells (%)
Oriens/Alveus	7	201	222	96.9 ± 1.3	52.4 ± 4.7
Pyramidale	246	75	56	17.5 ± 6.5	39.5 ± 11.9
Radiatum	128	6	15	11.3 ± 3.9	76.5 ± 16.1
Total	381	282	293	43.5	51.0

Number of mGluR1α-Immunopositive, VIP-Immunopositive, and Double-Labeled Cells Counted in the CA1 Area of the Rat Dorsal Hippocampus

Layer	Cells mGluR1α-positive only (n)	Cells VIP-positive only (n)	Double-labeled cells (n)	Double-labeled cells as % of mGluR1α-positive cells (%)	Double-labeled cells as % of VIP-positive cells (%)
Oriens/alveus	274	32	6	2.2 ± 0.8	17.2 ± 7.9
Pyramidale	117	25	200	63.0 ± 4.0	89.2 ± 1.9
Radiatum	54	21	104	66.9 ± 4.2	82.4 ± 4.9
Lac.-moleculare	18	50	79	81.7 ± 1.1	60.9 ± 1.1
Total	463	128	389	45.7	75.2

Number of mGluR1α-Immunopositive, PV-Immunopositive, and Double-Labeled Cells Counted in the CA1 Area of the Rat Dorsal Hippocampus

Layer	Cells mGluR1α-positive only (n)	Cells PV-positive only (n)	Double-labeled cells (n)	Double-labeled cells as % of mGluR1α-positive cells (%)	Double-labeled cells as % of PV-positive cells (%)
Oriens/alveus	41	203	142	75.4 ± 8.0	39.7 ± 9.4
Pyramidale	254	419	26	9.5 ± 1.3	5.9 ± 0.6
Radiatum	116	19	4	3.6 ± 1.6	23.2 ± 9.6
Total	411	641	172	29.5	21.2

*Values represent the total number of immunolabeled neurons and means ±SEM of the percentage of double-labeled neurons among animals (n = 3). Data were obtained from four and two hemisections/animal of the dorsal hippocampus for the mGluR1α/VIP and mGluR1α/SOM or mGluR1α/PV, respectively.

mGluR1α and CR confirmed the presence of double-stained interneurons throughout all layers, although they were most frequently observed in strata pyramidale and radiatum (data not shown). In order to investigate various IS interneurons displaying mGluR1α-IR, we have performed triple immunoreactions combining antisera to mGluR1α, VIP and CR raised in different species. For these studies an additional anti-mGluR1α antiserum, raised in guinea pig (Tanaka et al., 2000) has been used. In strata pyramidale and radiatum, neurons exhibiting all possible combinations were found, although the patterns most frequently observed were (1) cells immunopositive for mGluR1α/VIP/CR; (2) cells immunopositive for mGluR1α/VIP, but not CR; (3) cells immunopositive only for VIP and CR; and (4) neurons immunopositive for either mGluR1α or CR only (Fig. 6). In stratum O/A, most neurons displaying mGluR1α-IR did not contain either CR-IR or VIP-IR (Fig. 6). In this layer, most cells immunolabeled for VIP were not immunopositive for CR, and, likewise, most CR-immunofluorescent neurons did not show VIP-IR, although a few VIP/CR co-labeled neurons could be found (data not shown). The relatively rare mGluR1α/VIP-immunoreactive neurons in alveus were immunonegative for CR (data not shown).

Two major groups of mGluR1α-immunopositive cells appear to be present in CA1, namely those expressing SS or VIP. As observed from double immunolabeling experiments, in stratum O/A only a small number (~2%) of mGluR1α-immu-

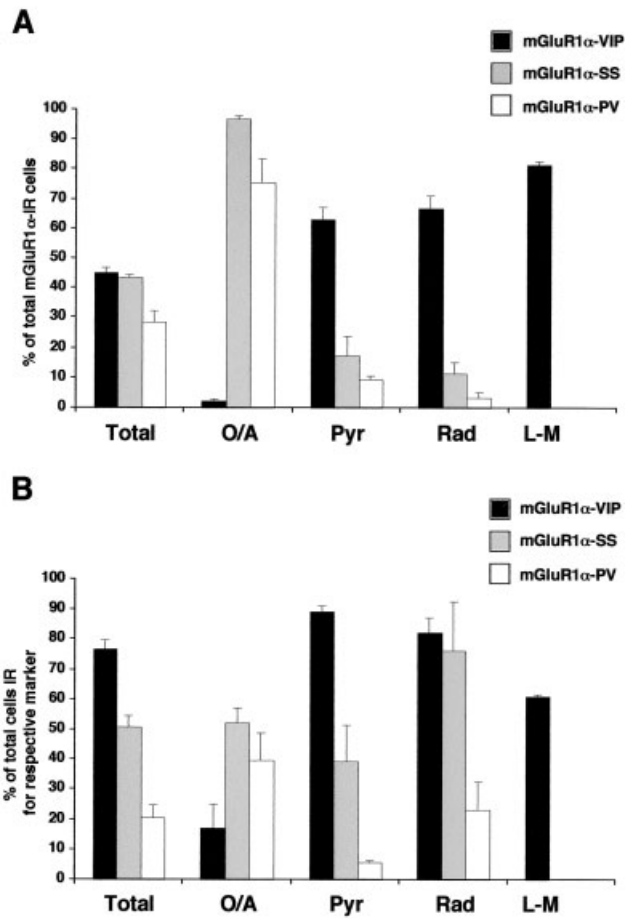


FIGURE 2. Semiquantitative analysis of neurons showing co-localization of metabotropic glutamate receptor 1α (mGluR1α) with either somatostatin (SS)-, vasointestinal peptide (VIP)-, or parvalbumin (PV)-immunoreactivity (IR). A: Histograms show the percentage of double-labeled neurons as a proportion of the total mGluR1α-immunoreactive neuronal population in the respective layers. B: Histograms show the percentage of double-labeled neurons as a proportion of the total immunopositive cells for either VIP, SS, or PV. Data were obtained from four (mGluR1α/VIP) and two (mGluR1α/SS and mGluR1α/PV) hemisections per animal (n = 3). Values represent the mean ± SEM. L-M, stratum lacunosum-moleculare; O/A, stratum oriens/alveus; Pyr, stratum pyramidale; Rad, stratum radiatum.

nopositive neurons were co-labeled with VIP, whereas the vast majority (~97%) coexpressed SS. To investigate whether VIP and SS are mutually exclusive in mGluR1α-immunopositive neurons, we carried out qualitative triple immunolabeling experiments combining antisera for mGluR1α, VIP and SS. All triple immunolabeling experiments were performed omitting the TSA intensification step. Our immunofluorescence experiments revealed that, in all CA1 strata, neurons displaying mGluR1α/VIP-IR did not exhibit detectable levels of SS-IR (Fig. 7). Likewise, cells showing mGluR1α/SS-IR had no detectable levels of VIP-IR (Fig. 7).

From the findings presented above, we propose that the mGluR1α/VIP/CR-immunopositive neurons in strata pyramidale and radiatum correspond to IS-3 interneurons, which innervate other interneurons in stratum O/A, whereas VIP-positive cells that are CR-negative in these strata may correspond to basket cells.

Immunoreactivity for mGluR1α May Be Absent From Basket Cells

To test the mGluR1α-IR of basket cells, we have carried out triple-labeling immunofluorescence studies with antisera against VIP, mGluR1α, and either CCK or PV. Two main classes of basket cells have been described (Pawelzik et al., 2002; for review, see Freund and Buzsaki, 1996) that contain either PV (Kosaka et al., 1987; Sik et al., 1995) or CCK and VIP (Nunzi et al., 1985; Gulyas et al., 1991; Acsady et al., 1996a,b). Immunolabeling for CCK discriminated among three distinct categories of neurons: (1) cells immunoreactive to CCK and VIP, but not to mGluR1α (Fig. 8); (2) cells containing both CCK- and mGluR1α-immunolabeling, but not VIP; and (3) cells displaying only CCK-IR.

In the triple immunolabeling tests for PV, mGluR1α, and VIP, we unexpectedly found that a very large proportion of mGluR1α-immunolabeled and VIP-immunonegative neurons in stratum O/A were also immunopositive for PV (Fig. 9). However, we also found numerous intensely PV-positive cells, which were immunonegative for mGluR1α (Fig. 9). In contrast, in stratum pyramidale, the vast majority of neurons displaying PV-IR did not exhibit mGluR1α-IR (Fig. 9). In all layers, PV-IR and VIP-IR were never found in the same cell. Accordingly, cells double-labeled for mGluR1α and VIP in all cases lacked detectable levels of immunofluorescent signal for PV (Fig. 9). These results, taken together with previous reports establishing the presence of PV or CCK/VIP in two distinct populations of basket cells (Freund and Buzsaki, 1996), suggest that neither of these two basket cell populations is likely to be among mGluR1α-immunopositive interneurons.

O-LM Cells Are Characterized by the Coexpression of mGluR1α/SS/PV and Innervation by Intensely mGluR7a-Positive Boutons

Because most of the mGluR1α-immunopositive interneurons in stratum O/A also contain SS (Table 2, Fig. 3A), which was shown to be expressed by O-LM cells (Maccaferri et al., 2000; Klausberger et al., 2003), we have investigated whether they also contained PV. A triple immunofluorescent reaction to mGluR1α, SS, and PV antisera showed that most mGluR1α/SS-immunoreactive cells also exhibited PV-IR (Fig. 10). The intensity of the IR for PV appeared distinctively different between mGluR1α-immunopositive and -immunonegative cells, with the former ones displaying a weaker immunofluorescent signal (Fig. 10). Consistent with this observation and the findings obtained from the co-localization of mGluR1α, VIP, and PV, strong PV-immunopositive cells in strata pyramidale and O/A were not found co-labeled for mGluR1α. The co-localization between mGluR1α and PV in different layers was further studied by assessing the relative degree of their coexistence. As shown in Table 2, approximately 75% of mGluR1α-immunopositive neurons in stratum O/A also contained PV, whereas only ~9.5% and ~3.6% of neurons

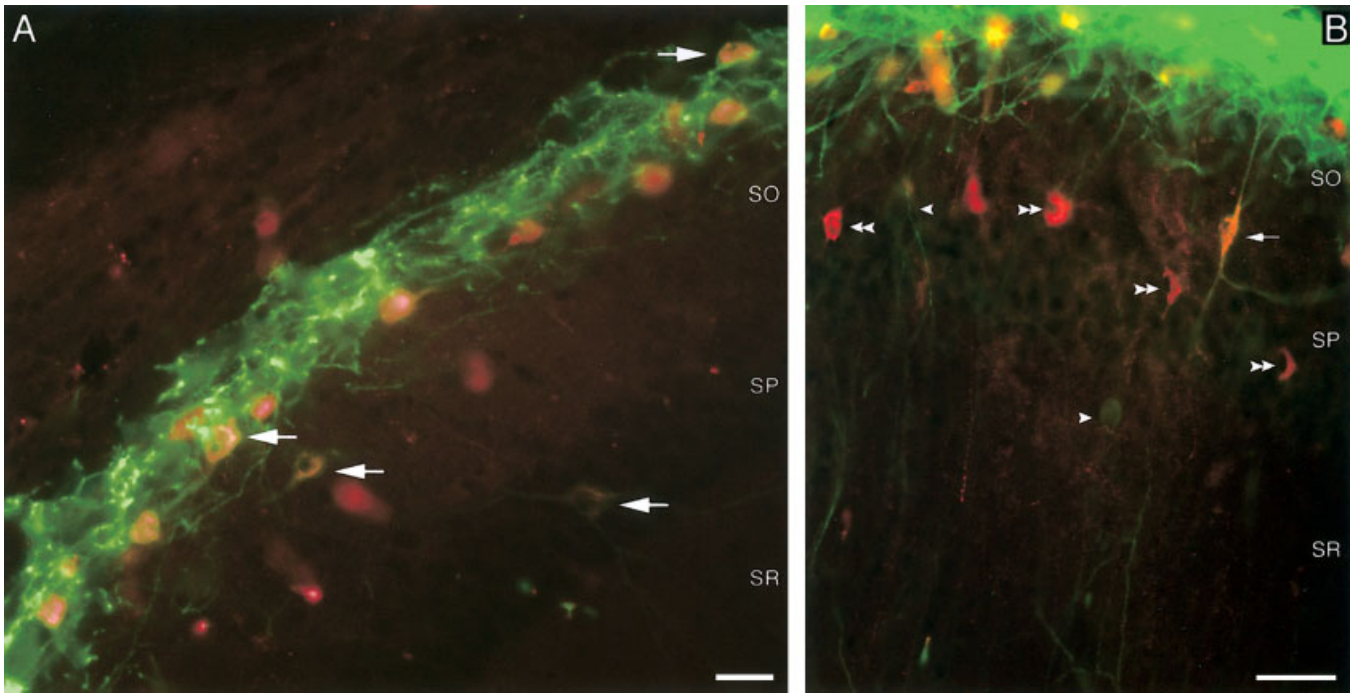


FIGURE 3. Immunofluorescent co-localization of metabotropic glutamate receptor 1α (mGluR1α) and somatostatin (SS) in CA1 hippocampal interneurons. A: The vast majority of mGluR1α-immunopositive (green) interneurons in stratum oriens/alveus are co-labeled for SS (red), as indicated by arrows. B: In strata pyramidale and radiatum, most interneurons are either immunopositive only for mGluR1α (single arrowheads) or SS (double arrowheads), although a few also appear co-labeled (arrow). SO, stratum oriens/alveus; SP, stratum pyramidale; SR, stratum radiatum. Scale bars = 25 μm in A; 50 μm in B.

expressing mGluR1α coexpressed PV in strata pyramidale and radiatum, respectively (Fig. 2A).

At the border between strata radiatum and LM, we occasionally found neurons characterized by intense IR for mGluR1α, but not for VIP or CCK, and by their dendrites being covered with spines (Fig. 11). These neurons also expressed both SS and PV (data not shown).

Immunoreactivity for the presynaptic receptor mGluR7a is particularly intense around strongly mGluR1α-positive interneurons in stratum O/A (Shigemoto et al., 1996, 1997), which we have shown, in this study, to be O-LM cells. This has been confirmed in the present study by triple labeling immunofluorescence for mGluR7a, VIP, and mGluR1α. In addition, we have found that mGluR1α/VIP-immunopositive interneurons are also outlined by mGluR7a-labeled terminals (Fig. 12). However, the intensity of the mGluR7a-immunofluorescent signal around mGluR1α/VIP-positive interneurons, although stronger than the staining in the surrounding neuropil that corresponds to terminals innervating pyramidal cell spines, appeared significantly weaker than the one observed on mGluR1α-immunopositive, but VIP-immunonegative, cells (Fig. 12).

A few perikaryal and dendritic profiles in both strata O/A and radiatum, which appeared to be immunonegative for mGluR1α, were also found delineated by strongly mGluR7a-immunofluorescent puncta (Fig. 12), but these were rare, and their identity could not be established. These findings suggest that O-LM cells are not the only interneurons to be heavily innervated by terminals enriched in mGluR7a.

Dendritic and Axonal Patterns of an Interneuron Targeting Oriens/Alveus Neurons

Among the candidate cell types expressing mGluR1α, there is a wealth of anatomical and physiological information about O-LM cells from both in vitro (Maccaferri et al., 2000; Martina et al., 2000) and in vivo (Sik et al., 1995) recordings. In contrast, the current knowledge about IS interneurons, many of which, as shown in the present study, are likely to express mGluR1α, is limited to immunolabeling studies (Acsady et al., 1996a,b), which provide only a partial visualization of the processes of the cells.

Among a series of interneurons recorded extracellularly in vivo and visualized by juxtacellular labeling with neurobiotin, one cell was identified that resembled interneurons immunolabeled for VIP in the pyramidal cell layer (Acsady et al., 1996a). Because of the lack of information about these cells, we report its features in the present report. Juxtacellular recording was carried out using a glass microelectrode combined with an iontophoretic micropipette assembly. Because most hippocampal neurons are silent in anesthetized as well as in awake animals, quisqualate (5 mM in the pipette), an agonist acting at both group I mGluRs and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors, was applied to induce cell firing while the electrode was stepped into the hippocampus. Ejection of quisqualate evoked a barrage of multiunit neuronal activity, which included spikes of varying amplitude. Insuffi-

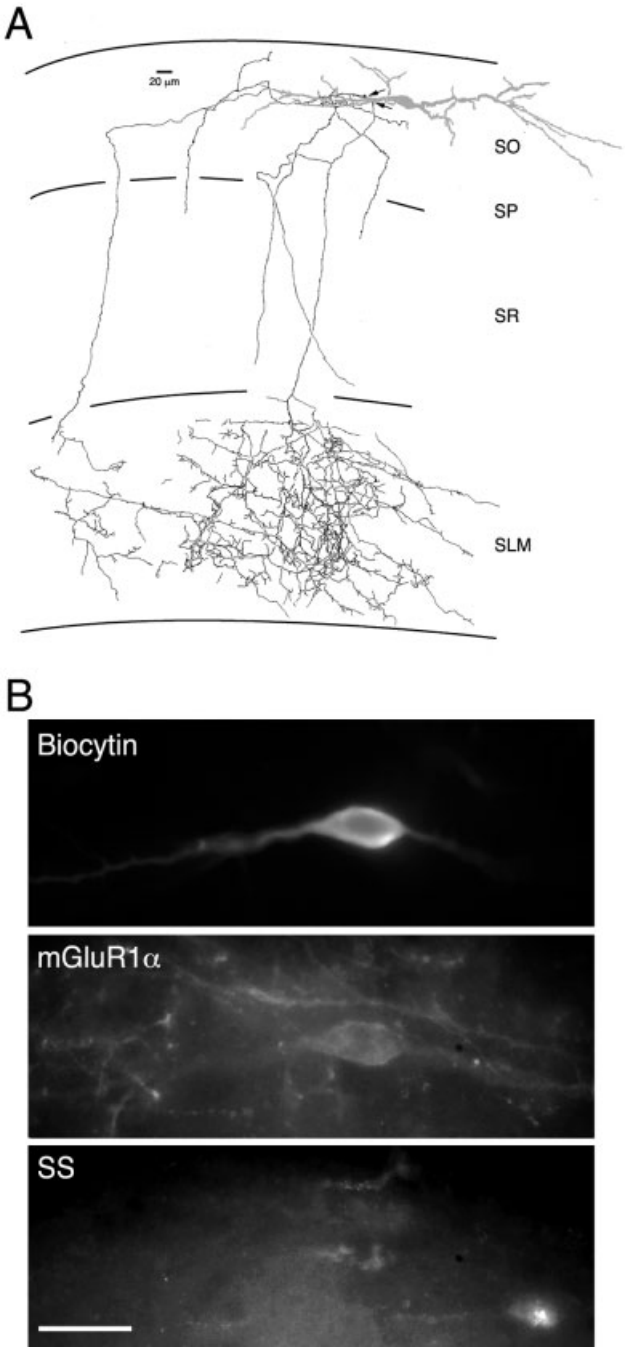


FIGURE 4. Axonal projection pattern of a metabotropic glutamate receptor 1α (mGluR1α)/SS immunoreactive oriens-lacunosum moleculare (O-LM) cell recorded in vitro. A: The soma and dendrites (gray) were located in stratum oriens/alveus. Two main axons (black) branched from a proximal dendrite (arrows) and descended perpendicularly to the stratum radiatum into the stratum lacunosum-moleculare, where they produced a dense tuft of collaterals. B: The cell, visualized by intracellular injection of biocytin shown by 7-amino-4-methylcoumarin-3-acetic acid (AMCA) immunofluorescence (top), was found immunopositive to mGluR1α (middle) and somatostatin (SS) (bottom). Immunoreactivity for SS was restricted to the cytosol, whereas mGluR1α-IR (rabbit antibody) was present on both the somatic and dendritic membranes. SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum; SLM, stratum lacunosum-moleculare.. Scale bar = 30 μm in B.

cient data were collected to attempt physiological characterization. Small positive current steps were applied to label neurons at the recording site. Labeling was carried out for 15 min; although it was not optimal for a complete visualization of all axonal processes (Pinault, 1996), it resulted in good labeling of a single cell, and its anatomical characteristics were analyzed. The soma of the cell was in the pyramidal layer and emitted four primary dendrites, which branched both proximally and distally from the soma forming a tuft in strata LM and oriens (Fig. 13). The dendritic tree was strongly labeled and the dendrites could be followed to their apparent natural ends. The descending main axon originated from the soma and gave rise to recurring collaterals in stratum radiatum and near the border to the LM. These varicose branches formed local clusters and seemed to surround other interneurons. The recurring main collaterals branched frequently in the stratum O/A, providing a dense plexus of varicosities in the neuropil among myelinated axon bundles (Fig. 13). These more distal axonal branches were weakly filled and could not be traced to their natural ends, as the labeling faded with distance.

DISCUSSION

Previous anatomical and physiological studies demonstrated the presence of mGluR1 on structurally and functionally heterogeneous hippocampal interneurons in the CA1 area (Baude et al., 1993; McBain et al., 1994; van Hooft et al., 2000). However, the identity of mGluR1α-expressing interneuron classes, in terms of their input and output characteristics and their relative proportions to the total of mGluR1α-positive interneurons, remains uncertain.

This study has several conclusions:

1. mGluR1α is expressed both by interneurons innervating the pyramidal cell dendritic domains and by interneurons selectively targeting other specific subsets of interneurons.
2. We have found an incomplete overlap between mGluR1α- and SS-immunoreactive interneurons.
3. In vitro labeling of oriens interneurons confirmed that intensely mGluR1α-immunopositive cells coexpressing SS are O-LM interneurons.
4. VIP-containing terminals selectively target the dendritic domain of O/A interneurons intensely labeled for mGluR1α.
5. O-LM cells are characterized by the expression of PV in addition to SS and mGluR1α.
6. We have found an as-yet unidentified mGluR1α/SS-immunoreactive, but PV-immunonegative, interneuron class, possibly corresponding to oriens-bistratified cells.
7. Interneurons intensely immunolabeled for PV do not show IR for mGluR1α.
8. In interneurons coexpressing CCK and VIP, no IR for mGluR1α was detected.
9. A subpopulation of CCK-immunopositive interneurons coexpresses mGluR1α.

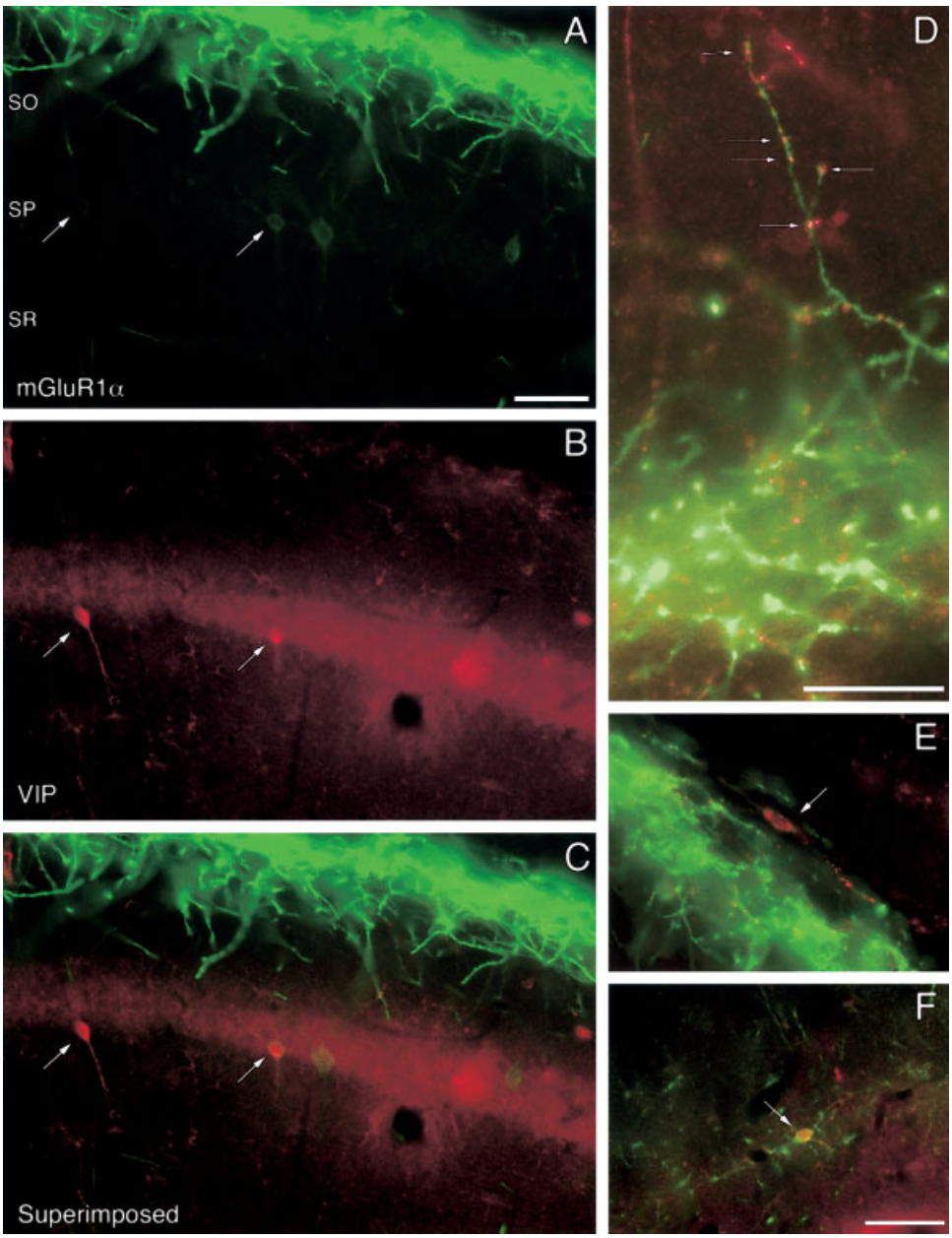


FIGURE 5. Immunofluorescent co-localization of metabotropic glutamate receptor 1α (mGluR1α) and vasointestinal peptide (VIP) in interneurons of the CA1 area of the rat hippocampus. A: The alveus and adjoining stratum oriens contain the most intensely mGluR1α-immunolabeled neurons (green); several weakly labeled interneurons can be observed in all other layers, as shown here in stratum pyramidale. B,C: Many, but not all, of these neurons are also immunopositive (arrows) for VIP (red). D: Superimposed image of strongly mGluR1α-immunolabeled dendrites (green) of interneurons in stratum oriens/alveus innervated by VIP-immunopositive axon terminals (red). The image shows how terminals containing VIP (arrows) follow the distal dendrites of mGluR1α-immunopositive neurons up to their tips. E: Double-labeled neuron (arrow) for mGluR1α (green, mainly on the plasma membrane) and VIP (red, cytoplasmic) in stratum oriens/alveus. F: Double-labeled neuron (arrow) for mGluR1α (green, mainly on the plasma membrane) and VIP (red, cytoplasmic) in stratum lacunosum-moleculare. SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. Scale bars = 50 μm in A–C; 30 μm in D; 50 μm in E,F.

10. Terminals intensely immunopositive for mGluR7a target VIP/mGluR1α-containing interneurons and mGluR1α-immunonegative cells, in addition to mGluR1α-/SS positive cells.
11. A reconstruction of an IS interneuron is reported.

A schematic diagram tentatively summarizing these results and depicting mGluR1α-positive interneurons within the CA1 circuitry is given in Figure 14.

Interneuron Selective (IS) Interneurons Express mGluR1α Receptors

The most reliable identification of hippocampal interneurons with nonoverlapping properties has been provided by the correlation of several functionally relevant criteria, which include axonal targeting, source of synaptic input, laminar position, expression of neurochemical markers (e.g., calcium-binding proteins, neuropeptides, and neu-

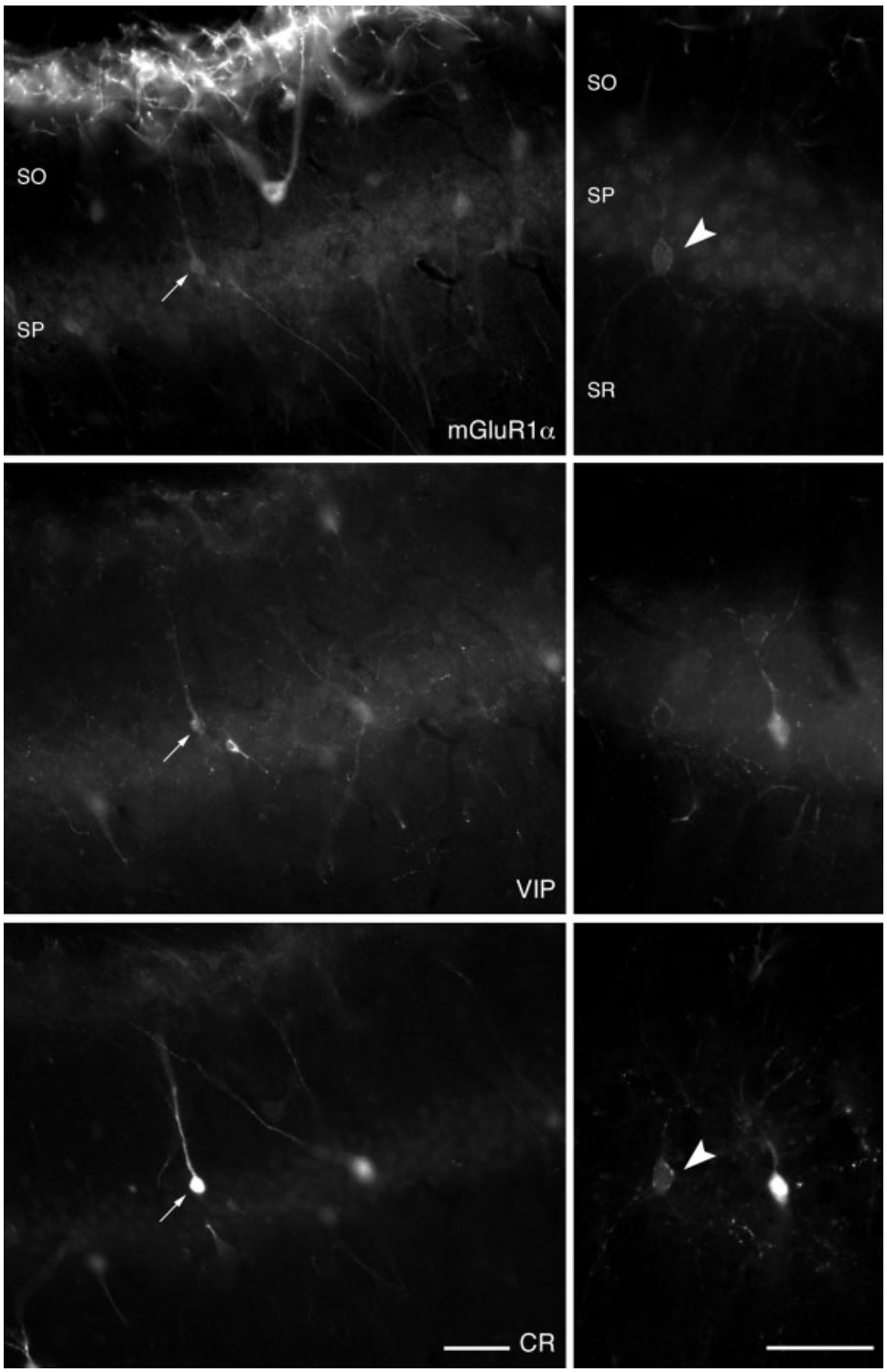


FIGURE 6. Immunofluorescent co-localization of metabotropic glutamate receptor 1α (mGluR1α), vasointestinal peptide (VIP), and CR in interneurons of the CA1 area of the rat hippocampus. The very same hippocampal fields were taken at the same focal plane for mGluR1α (top), VIP (middle), and calretinin (CR) (bottom). In the left column, the arrow shows an interneuron immunopositive for all three proteins. As shown in the right column, an interneuron in stratum pyramidale is immunopositive for both mGluR1α and CR, but not for VIP (arrowhead), although terminals containing VIP and CR IR appear to delineate the soma. A neighboring cell is VIP and CR positive but clearly immunonegative for mGluR1α. SO, stratum oriens/alveus; SP, stratum pyramidale. Scale bar = 50 μm in left and right column panels.

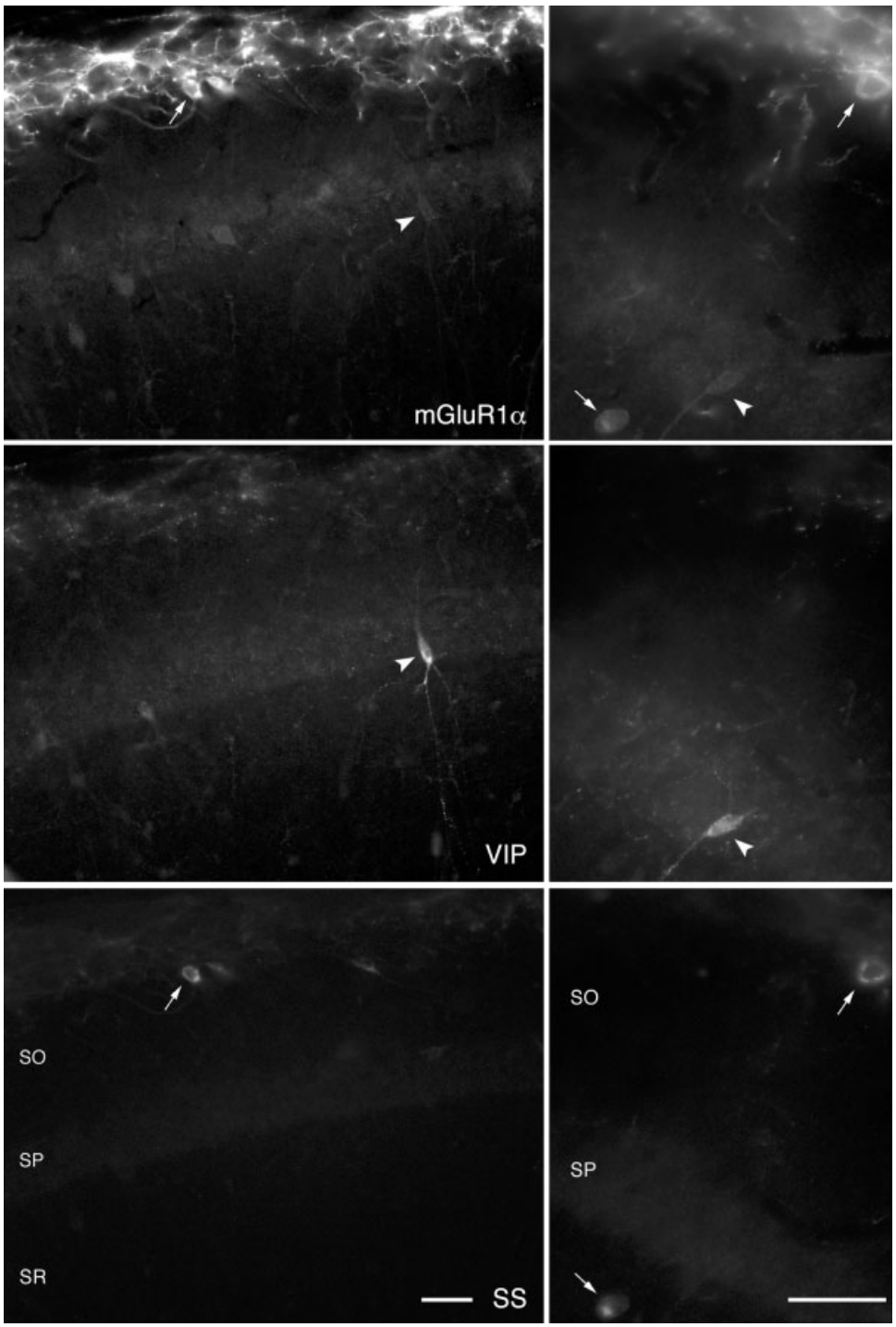


FIGURE 7. Immunofluorescent co-localization of metabotropic glutamate receptor 1α (mGluR1α) (top row), vasointestinal peptide (VIP) (middle row), and somatostatin (SS) (lower row) in interneurons of the rat CA1 hippocampal area. In two different fields (left and right columns), interneurons immunopositive for both VIP and mGluR1α are immunonegative for SS (arrowheads). Likewise, interneurons immunopositive for mGluR1α and SS do not express VIP (arrows). SO, stratum oriens/alveus; SP, stratum pyramidale. Scale bars = 50 μm in left and right column panels.

rotransmitter receptors), and intrinsic biophysical characteristics (for review, see Freund and Buzsaki, 1996; Somogyi et al., 1998, McBain and Fisahn, 2001). A few discrete classes of interneuron have been established; yet several others remain to be unambiguously identified (Parra et al., 1998). Because in this study our data are restricted mainly to the immunocytochemical detection of molecular markers, apart from well-established interneuron types, cellular identification has been limited to correlational evidence.

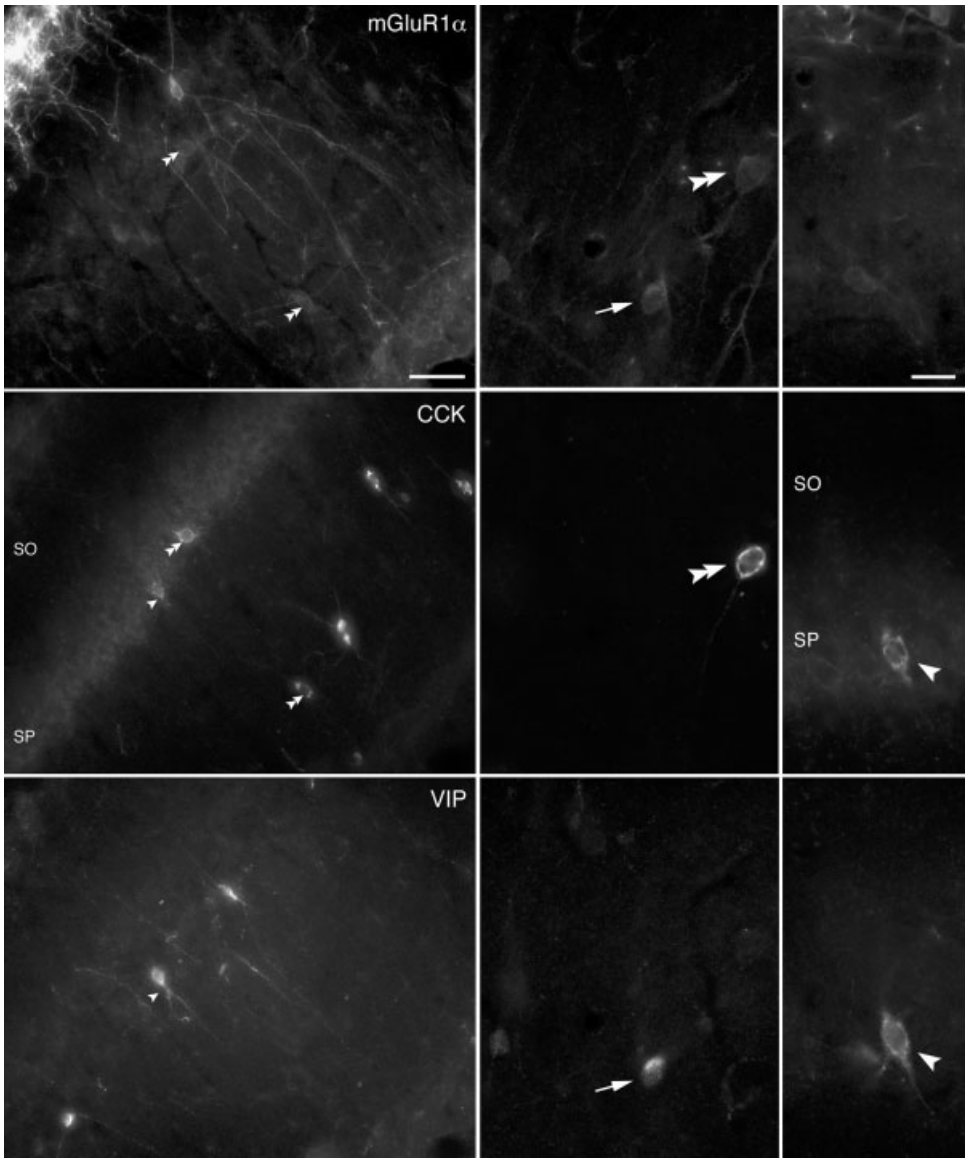


FIGURE 8. Immunofluorescent co-localization of metabotropic glutamate receptor 1α (mGluR1α), vasointestinal peptide (VIP), and cholecystokinin (CCK) in interneurons of the rat CA1 hippocampal area. Three different fields (columns) are shown. Some interneurons (double arrowheads) are immunopositive for mGluR1α (top) and CCK (middle), but not VIP (bottom). Other cells (single arrowheads) are immunoreactive for CCK and VIP, but not mGluR1α. As shown in the middle column panels, a neuron in deep stratum radiatum is immunopositive for both CCK and mGluR1α, but it is clearly VIP-immunonegative, while a neighboring cell (arrow) is mGluR1α- and VIP-positive and CCK-negative. The right column panels show a VIP- and CCK-immunopositive putative basket cell, which is mGluR1α-negative. SO, stratum oriens/alveus; SP, stratum pyramidale; SR, stratum radiatum. Scale bars = for left column, 50 μm; for middle and right column, 20 μm.

Distinct GABAergic interneurons innervate the soma, axon initial segment, or dendritic domains of pyramidal cells, or otherwise selectively target the soma and/or dendrites of other interneurons. The latter interneurons were described as three different groups differing in their connectivity and differential expression of VIP and/or CR (Acsady et al., 1996a,b). This study provides evidence for the coexistence in some CA1 interneurons of mGluR1α with VIP and/or CR. Although the axon of individual neurons co-labeled for mGluR1α, VIP, and/or CR could not be visualized, based on the observed neurochemical features of these neurons, we suggest that all three reported classes of IS interneurons express mGluR1α. Nevertheless, because some CR-immunopositive, but VIP-immunonegative, cells did not display mGluR1α-IR, it is possible that some IS cells either do not express this glutamate receptor or express it at a very low level. These results parallel recent findings in neocortical interneurons showing transcript coexpression of mGluR1 with VIP and/or CR (Cauli et al., 2000).

In stratum O/A, cells intensely immunolabeled for mGluR1α were shown to be innervated by VIP-positive terminals, which may

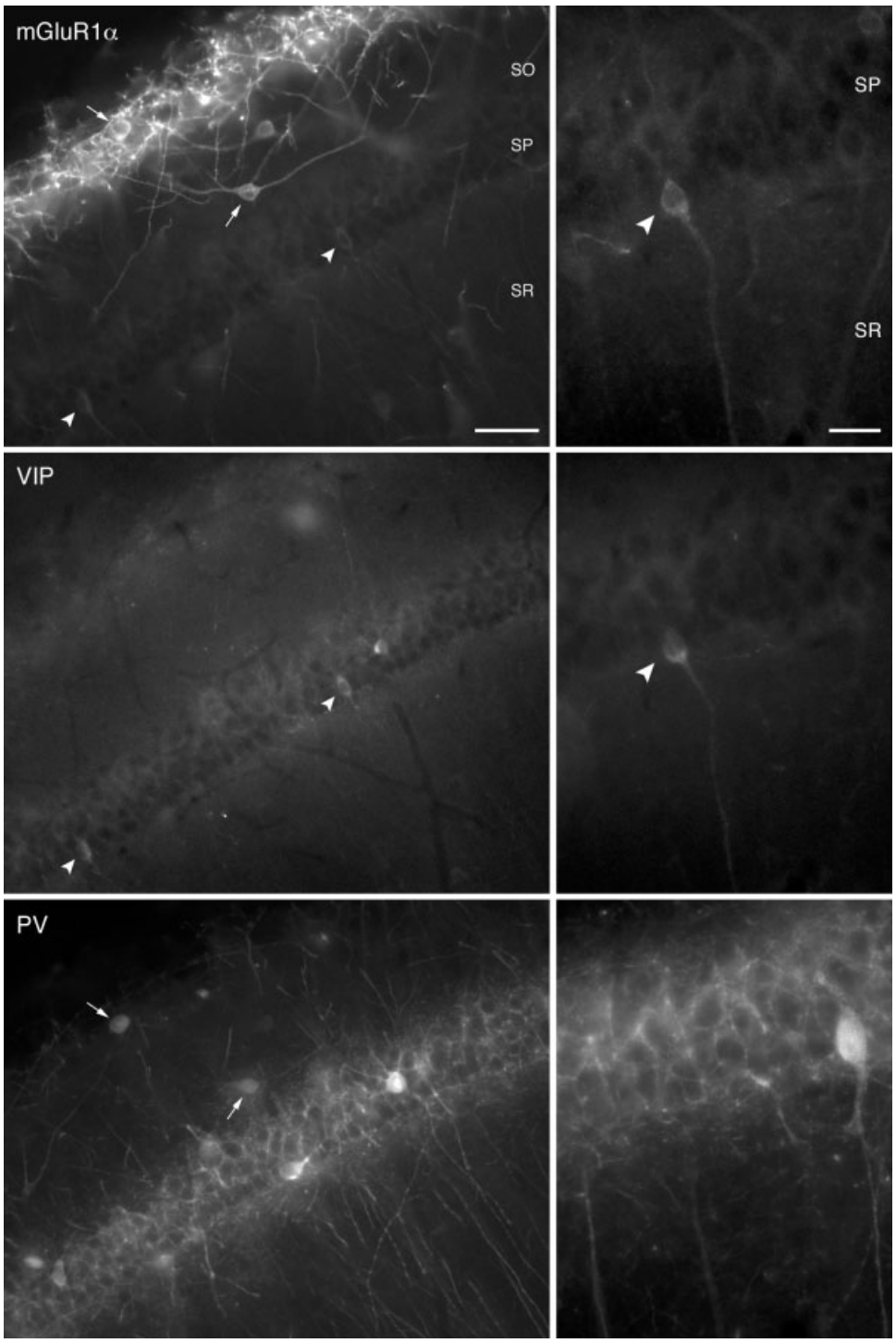


FIGURE 9. Immunofluorescent co-localization of metabotropic glutamate receptor 1α (mGluR1α), vasointestinal peptide (VIP), and parvalbumin (PV) in the CA1 area of the rat hippocampus. Two fields (columns) are shown. Arrows show neurons immunoreactive for both mGluR1α (upper row) and PV (lower row), but immunonegative for VIP (middle row). Arrowheads indicate interneurons immunopositive for mGluR1α and VIP, but immunonegative for PV. Right column panels show that a strongly PV-immunopositive neuron (lower panel) is immunonegative for mGluR1α and VIP. SO, stratum oriens/alveus; SP, stratum pyramidale. Scale bars = for panels on the left, 50 μm, for panels on the right, 20 μm.

contact O-LM cells selectively in this layer (Blasco-Ibanez and Freund, 1995; Freund and Buzsaki, 1996). In the present study, we have reported that VIP-containing axon terminals primarily target the distal dendrites of mGluR1α-immunolabeled neurons, sparing primarily the soma and proximal dendrites. These interneurons are also characteristically associated with high levels of

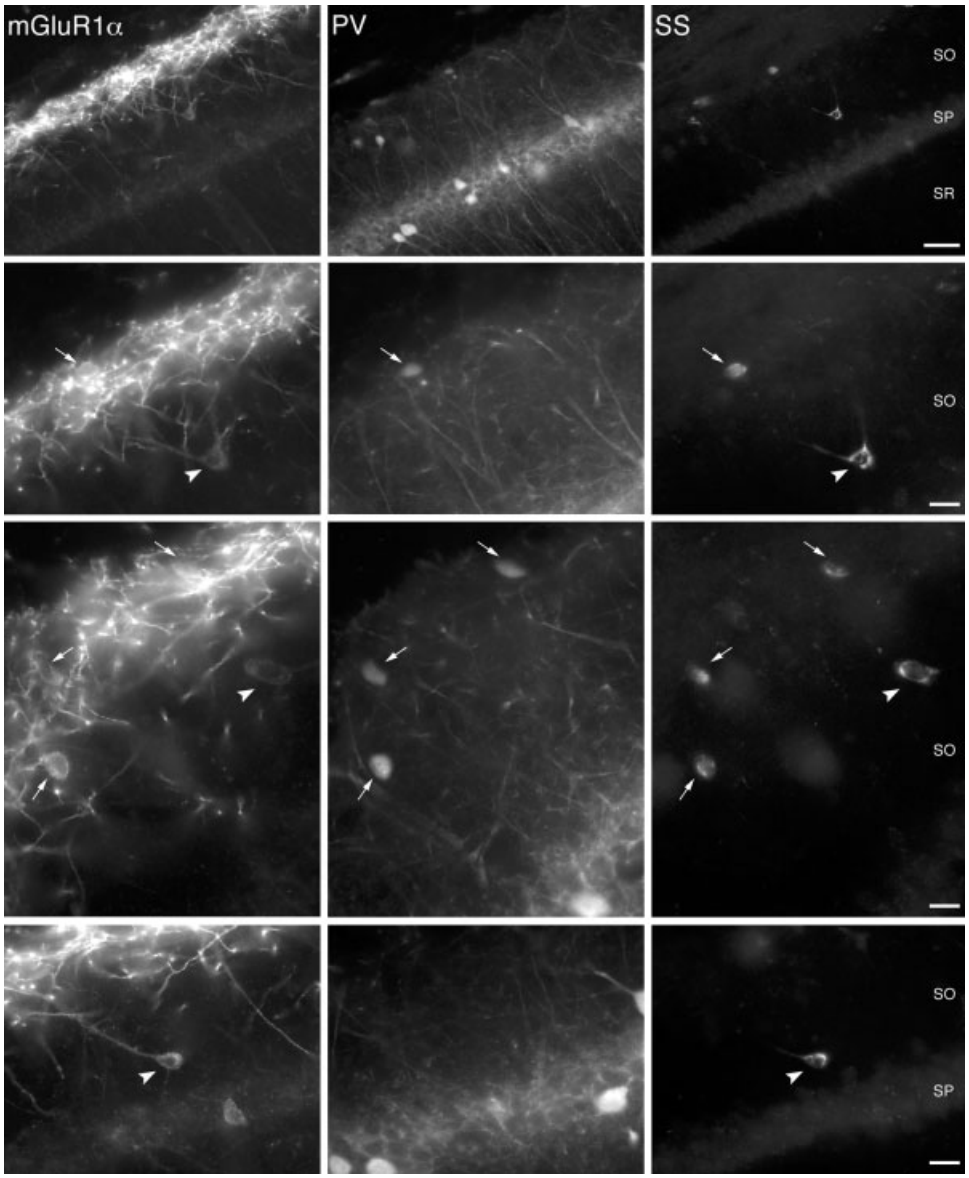


FIGURE 10. Immunofluorescent co-localization of metabotropic glutamate receptor 1α (mGluR1α), parvalbumin (PV), and somatostatin (SS) in interneurons of the CA1 area of the rat hippocampus. The same hippocampal fields (rows) immunoreacted for mGluR1α (left column), PV (middle column) and SS (right column) are shown. Arrows show neurons containing IR for all three proteins. Arrowheads indicate interneurons immunopositive for mGluR1α and SS, but immunonegative for PV. Scale bars = 50 μm in first row; 20 μm in all other rows.

mGluR7a on the terminals of their major excitatory input originated from CA1 pyramidal cells (Shigemoto et al., 1996, 1997; Losonczy et al., 2002). Type II (symmetrical) synapses, most of which are GABAergic in the hippocampus, were also shown to contain mGluR7a (Shigemoto et al., 1997). Therefore, VIP-containing axon terminals of IS3 interneurons, which are presumed to be GABAergic, may possess in their presynaptic active zone mGluR7a acting as a heteroreceptor at these synapses. Consistent with this hypothesis, in preliminary experiments some VIP-positive terminals were found to be immunoreactive for mGluR7a (Somogyi et al., 1999), and in the somatosensory cortex virtually all VIP-positive terminals innervating mGluR7a-decorated neurons were shown to contain mGluR7a (Dalezios et al., 2002).

Interneurons of the IS3 type appear to receive a major excitatory input from the entorhinal cortex, which might activate mGluR1α present on their dendrites. The juxtacellularly recorded cell that resembles IS3 interneurons (Acsady et al., 1996a) had dendrites branching extensively in stratum LM. Interestingly, in addition to the axonal projection to O/A, as reported by Acsady et al. (1996a), the filled axon of the cell shown here also innervated stratum radiatum and appeared to contact primarily other interneurons. The entorhinal input and the receptor array mediating its effect to-

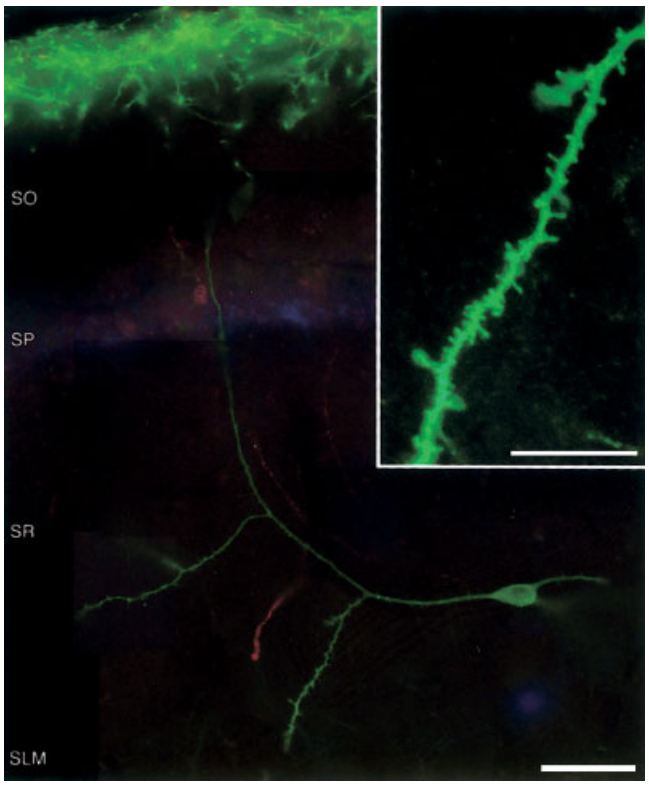


FIGURE 11. Interneurons displaying many of the characteristic features of oriens-lacunosum moleculare (O-LM) cells can be observed occasionally in CA1 stratum radiatum. A: The soma and dendrites of an intensely metabotropic glutamate receptor 1α (mGluR1α) (green) immunolabeled interneuron are shown in stratum radiatum, close to the border with stratum LM. This interneuron shows no detectable IR for either VIP (red) or CCK (blue). The insert shows a portion of a dendrite at high magnification. SO, stratum oriens/alveus; SP, stratum pyramidale; SR, stratum radiatum; SLM, stratum lacunosum-moleculare. Scale bars = A, 50 μm; insert, 20 μm.

gether with the interconnectivity among hippocampal interneurons may facilitate their participation in oscillations (see below, under Functional Implications).

Heterogeneity in SS-Containing Interneurons

In previous studies, it was suggested that all SS-containing interneurons coexpress mGluR1α (Baude et al., 1993; Kerner et al., 1997; Yanovsky et al., 1997). We confirm in the present study that interneurons intensely labeled for mGluR1α in stratum O/A contain SS. However, we also found evidence that the coexistence between the two molecules is not complete because, when mGluR1α-expressing interneurons in all CA1 layers and showing both strong and weak IR were taken into account, only 43% of them appeared immunopositive for SS; in addition, 49% of SS-containing neurons were immunonegative for mGluR1α.

At least two populations of SS-immunopositive interneurons, namely O-LM and O-Bi cells, having a distinct laminar axonal pattern (McBain et al., 1994; Buhl et al., 1994; Sik et al., 1995; Ali et al., 1998; Maccaferri et al., 2000; Losonczy et al., 2002), are present in the CA1 hippocampal region. In the absence of established molecular markers that clearly identify each of these two cell types, we have unequivocally separated them from each other on the basis of their axonal distribution. Subsequently, we have assessed them immunocytochemically to establish whether they expressed mGluR1α. In this study, we provide conclusive evidence that, in the rat, intensely mGluR1α/SS-immunopositive interneurons are mostly O-LM cells, although some O-Bi cells may express mGluR1α at an as-yet undetermined level, as also seen in the mouse hippocampus (Losonczy et al., 2002). Consistent with these findings, interneurons with axonal arborization in strata oriens and radiatum and immunopositive for calbindin were shown to respond with a small inward current to the application of ACPD and to express mGluR1 mRNA (van Hooft et al., 2000).

A relatively large proportion of mGluR1α/SS interneurons (~75%) in stratum O/A also expresses PV, although at a distinctively lower level than other PV-immunopositive cells. This observation is consistent with previous demonstrations that SS and PV are significantly co-localized in interneurons in stratum O/A (Bering et al., 1995; Jinno and Kosaka, 2000). These interneurons most likely are the O-LM cells, which selectively innervate the most distal part of the pyramidal cell dendritic tree in conjunction with entorhinal and thalamic afferents (Naus et al., 1988; Baude et al., 1993; McBain et al., 1994). This conclusion is supported by a previously in vitro recorded and reconstructed O-LM cell that was found immunopositive for SS and PV (Maccaferri et al., 2000) and by the recent identification of 3 O-LM cells labeled in vivo and displaying SS-IR, mGluR1α-IR, and PV-IR (Klausberger et al., 2003). From our analysis, ~40% of SS-containing interneurons in stratum O/A would be O-LM cells. This estimate deviates somewhat from the quantitative assessment of CA1 interneurons coexpressing SS/PV (11.5%), reported by Jinno and Kosaka (2000) in the mouse dorsal hippocampus. Although this inconsistency could be attributed to inter-species variations, differences in fixation protocols may provide an alternative explanation. At present, although unlikely, it cannot be excluded that some O-LM cells lack SS, PV, or both molecules.

If our hypothesis that the coexpression of mGluR1α/SS/PV identifies O-LM cells is correct, the SS-immunopositive neurons that do not coexpress both mGluR1α and PV may constitute a population of bistratified interneurons, thus accounting for ~60% of the population of SS-IR interneurons in stratum O/A. Bistratified interneurons may themselves include several different cell types (Sik et al., 1995; Maccaferri et al., 2000; Pawelzik et al., 2002). Interneurons double-labeled for mGluR1α/SS and immunonegative for PV are likely to correspond to a subpopulation of O-Bi cells. One of these cells has been recorded and reported in this study, and similar ones were observed in the mouse hippocampus (Losonczy et al. (2002).

Sporadically, we have observed intensely labeled mGluR1α interneurons also characterized by spiny dendrites and IR for SS and PV, but not CCK or VIP, at the stratum radiatum/LM border. Recently, transgenic mice were generated that expressed enhanced green fluorescent protein (EGFP) in a subpopulation of GABAergic neurons (Oliva et al., 2000). Interestingly, all EGFP-expressing interneurons were found to be immunoposi-

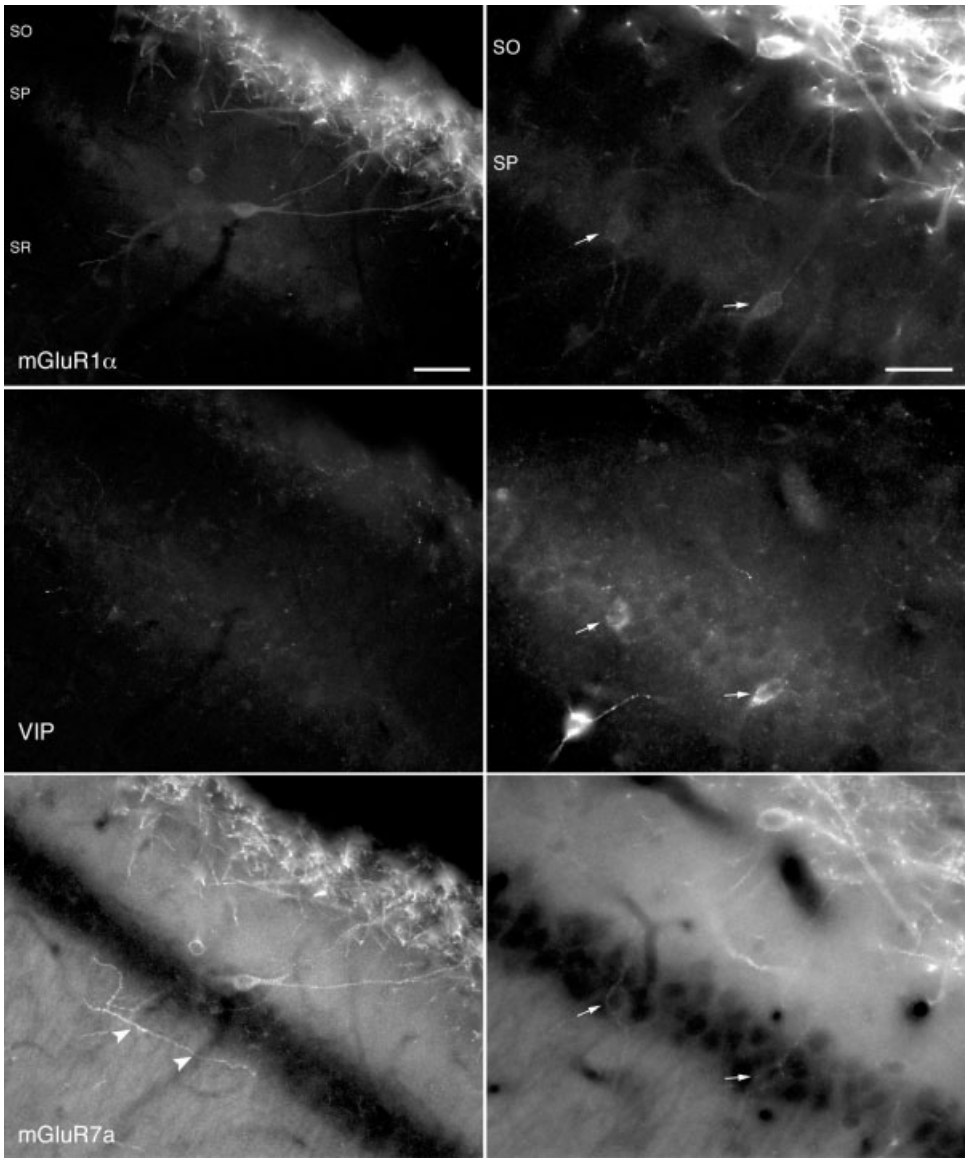


FIGURE 12. Immunofluorescent co-localization of metabotropic glutamate receptor 1α (mGluR1α) (top row), vasointestinal peptide (VIP), and mGluR7a in interneurons of the CA1 area of the rat hippocampus. The very same hippocampal fields taken at the same focal plane are shown for mGluR1α, VIP (middle row), and mGluR7a (bottom row). Arrows show neurons immunoreactive for mGluR1α and VIP outlined by terminals immunopositive for mGluR7a. Arrowheads indicate a dendrite decorated by intensely immunopositive mGluR7a terminals, but immunonegative for mGluR1α. SO, stratum oriens/alveus; SP, stratum pyramidale; SR, stratum radiatum. Scale bars = 50 μm in left column; 30 μm in right column.

tive for mGluR1α and the authors reported the infrequent identification of EGFP-containing interneurons in stratum radiatum with selective axonal innervation of stratum LM and neurophysiological properties very similar, if not identical, to those of O-LM cells. It is likely that the neurons reported by Oliva et al. (2000) in the mouse, as well as those seen in the present study in the rat, are homologous. These neurons display most of the typical morphological, functional, and biochemical characteristics of O-LM cells. We therefore propose that these neurons do not constitute a distinct interneuronal class, but rather are the equivalent of O-LM cells involved in a local circuit in association primarily with pyramidal cells located in stratum radiatum (Maccaferri and McBain, 1996; Gulyas et al., 1998), which have extensive local axon collaterals in stratum radiatum (G. Vida and P. Somogyi, unpublished observation).

Basket Cells Do Not Appear to Express mGluR1α

The absence of detectable IR to mGluR1α on both PV- and CCK/VIP-containing presumed basket cells suggests that interneurons innervating the somatic region of pyramidal cells are activated through partially different mechanisms from those innervating the dendrites. By single-cell reverse transcription-polymerase

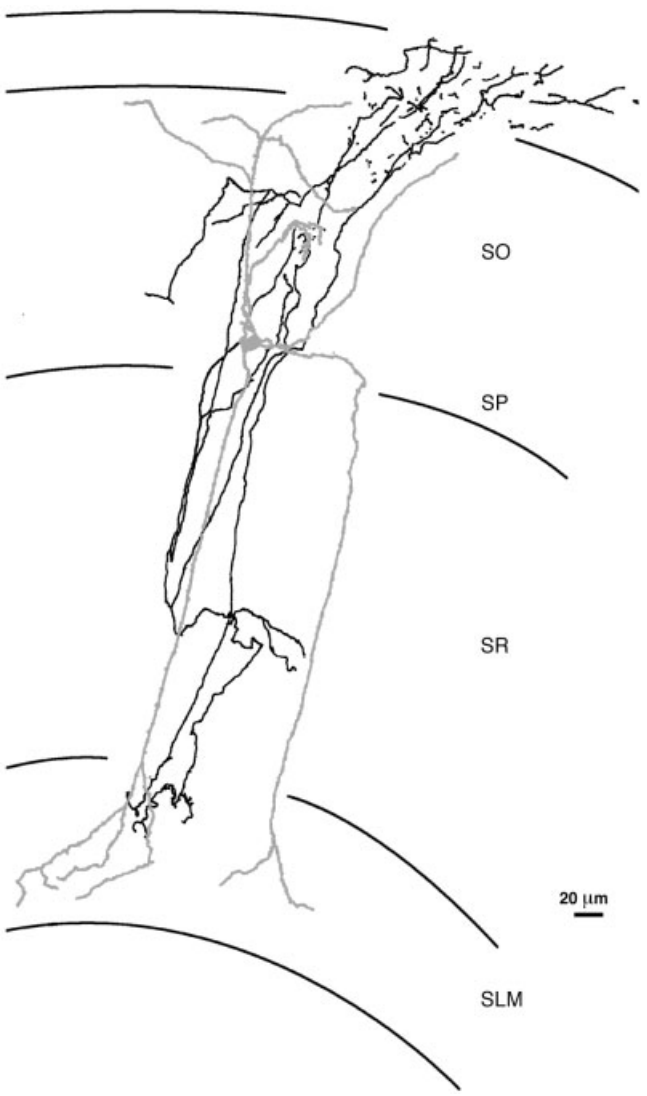


FIGURE 13. Reconstruction of an interneuron recorded in vivo and labeled by juxtacellular application of neurobiotin. The soma was in stratum pyramidale and gave rise to four main dendrites (gray) terminating in tufts in both stratum oriens and lacunosum-moleculare. The main axon (black) descended into the stratum radiatum, branched locally and projected back to stratum oriens densely innervating the alveus. The axon was weakly filled and could only be partially reconstructed in stratum oriens. SO, stratum oriens/alveus; SP, stratum pyramidale; SR, stratum radiatum; SLM, stratum lacunosum-moleculare.

chain reaction (RT-PCR), van Hooft et al. (2000) were able to amplify products corresponding to mGluR1 in ~20% of their type III interneurons, which they considered to be basket cells, partly because the cells expressed PV. However, the primers used in that study were designed in a region common to all mGluR1 splice variants. Hence, some basket cells may express a mGluR1 isoform other than mGluR1α. Alternatively, the expression level of mGluR1α in these cells may be below the threshold of detection of our immunofluorescence techniques, or not all the so-called type III interneurons may have been basket cells.

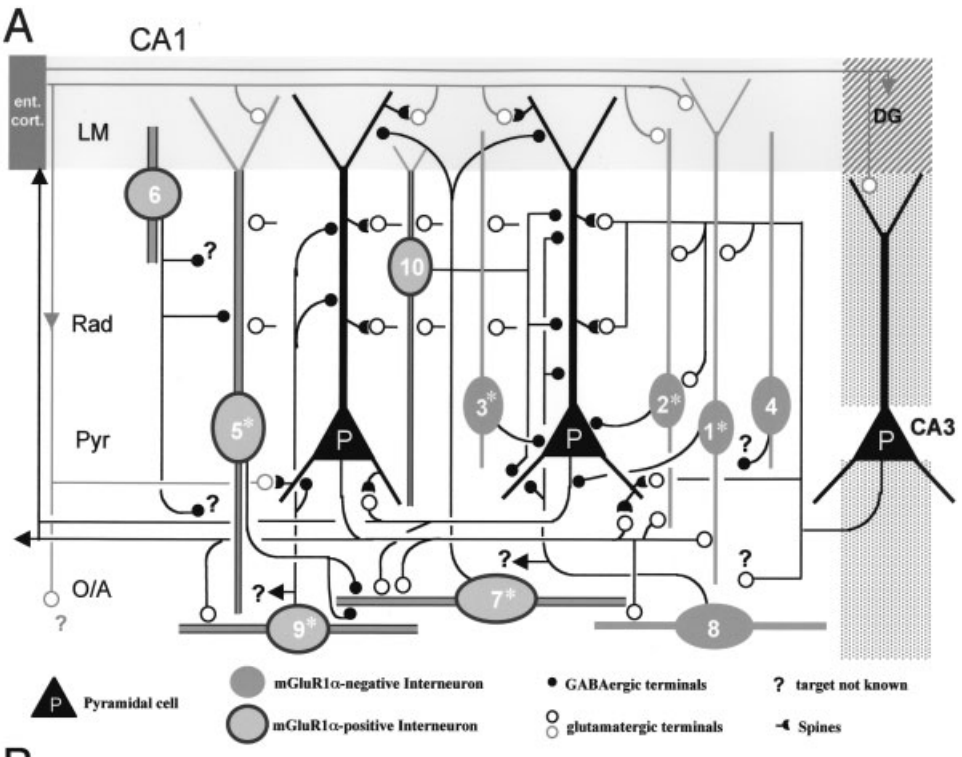
A Subpopulation of CCK-Immunopositive Interneurons Coexpresses mGluR1α

Recent combined physiological and anatomical studies have shown that CCK-immunoreactive interneurons are not exclusively basket cells, but also include a variety of interneurons innervating dendrites and displaying different discharge patterns, i.e., fast-spiking, regular-spiking, and burst firing (Cope et al., 2002; Pawelzik et al., 2002). In the present study, we show that a subpopulation of CCK-immunopositive interneurons also expresses mGluR1α. The perikarya of these neurons were found predominantly in stratum pyramidale or radiatum close to the LM border, and their dendrites, identified by mGluR1α-IR, were mainly localized to strata radiatum and oriens or LM. Some of these characteristics are suggestive of the CCK-positive Schaffer collateral-associated interneurons, whose axons overlap with the Schaffer collateral/commissural pathway in strata radiatum and oriens (Vida et al., 1998; Cope et al., 2002)., A definitive conclusion as to whether Schaffer collateral-associated interneurons possess mGluR1α receptors on their somatodendritic domain awaits combined anatomical and physiological studies able to show the axonal projection of these cells. Likewise, no experimental evidence is available for a complete coexistence of CCK and VIP in basket cells, leaving open the possibility that some of the CCK- and mGluR1α-IR interneurons might be basket cells.

Functional Implications

Little is known about the differences in the glutamatergic activation of specific types of hippocampal interneuron and, in particular, which physiologically relevant conditions produce activation of mGluR1α-positive cells. Several types of interneuron are differentially affected by the in vitro activation of group I mGluRs (McBain et al., 1994; Woodhall et al., 1999; van Hooft et al., 2000), which may thus influence hippocampal function in multiple interneuron-specific ways.

Because of its peri- and extrasynaptic location (Baude et al., 1993; Lujan et al., 1996), mGluR1α is expected to be strongly activated during population bursts of pyramidal cells, when glutamate increases in the extracellular space. The most prominent population burst occurs during sharp waves, a phenomenon that represents synchronization across the hippocampal-cortical loop (Csicsvari et al., 2000). Hippocampal interneurons also participate in both γ and θ frequency rhythmic activity (Lytton and Sejnowski, 1991; Cobb et al., 1995; Ylinen et al., 1995; Buzsaki, 2002). Such rhythmic activity may arise through several different mechanisms, each probably involving distinct subtypes of interneurons (Klausberger et al., 2003). Agonists of group I mGluRs can evoke slow oscillatory inward currents and rises in intracellular Ca^{2+} in subsets of CA1 interneurons (McBain et al., 1994; Carmant et al., 1997; Woodhall et al., 1999; van Hooft et al., 2000). The mGluR-induced inward currents were large in O-LM cells and small in calbindin-positive interneurons as well as in interneurons near stratum LM (McBain et al., 1994; Woodhall et al., 1999; van Hooft et al., 2000). Rhythmic action potential firing was reported only in O-LM cells and in a fraction of calbindin-immunopositive interneurons (van Hooft et al., 2000; but see also



B

Interneuron Class	mGluR1α	VIP	SS	CCK	CR	PV	mGluR7a terminals
1 Axo-axonic	-	-	-	-	-	+++	-
2 Basket-PV	-	-	-	-	-	+++	-
3 Basket-CCK/VIP	-	+++	-	+++	-	-	-
4 Basket(?)-CCK	- (?)	-	-	+++	- (?)	-	(?)
5 IS 3	+	+++	-	-	+++	-	++
6 IS 1/2	+	+/-	-	-	++/-	-	(?)
7 O-LM	+++	-	+++	-	-	+	+++
8 O-Bistratified I	-	-	+++	-	(?)	(?)	(?)
9 O-Bistratified II	++	-	+++	-	(?)	-	(?)
10 Schaff. Coll. Assoc.-CCK	++ (?)	-	-	+++	-	-	(?)

FIGURE 14. Interneuron classes immunopositive and immunonegative for metabotropic glutamate receptor 1α (mGluR1α) in the basic CA1 hippocampal circuit. Only some of the known connections among interneurons are shown for clarity. A: The identified or proposed presence of mGluR1α in some classes of interneuron is shown in a schematic diagram of the basic CA1 hippocampal circuit. The interneuron classes included in the diagram are limited to those that probably were tested by the immunofluorescent and in vitro recording experiments. Asterisks indicate cell types for which definitive experimental evidence for the expression of mGluR1α has been obtained. B: Relationship between interneuron classes and the tested immunochemical markers. Degree of immunoreactivity: +++, intense; ++, moderate; +, weak; −, absent; (?), unknown, or identity of cell has not been directly demonstrated.

McBain et al., 1994), and it has been suggested to be primarily, if not exclusively, mediated by mGluR1 (van Hooft et al., 2000). Thus, it is possible that mGluR1α promotes oscillatory activity also at physiologically relevant frequencies in vivo.

Synchronization may enhance cooperativity in neuronal networks and may generate synaptic potentiation or depression (Singer, 1993; Chapman and Racine, 1997). Perez et al. (2001) elicited long-term potentiation (LTP) of excitatory postsynaptic currents (EPSCs) by θ burst stimulation associated with postsynaptic depolarization in O/A interneurons, but not in interneurons in strata radiatum or LM. Evoking LTP in O/A interneurons, both in those with axonal projections to stratum LM (O-LM cells) and

to strata oriens and radiatum (O-Bi cells), required the activation of mGluR1 and was NMDA receptor-independent (Perez et al., 2001). This is consistent with our identification of two categories of SS and mGluR1α-positive O/A interneurons. The effectiveness of θ burst stimulation suggests that during hippocampal θ activity, the inputs from rhythmically active pyramidal cells, such as place cells, are potentiated and the activated O-LM interneurons may sustain rhythmic hyperpolarization of the distal pyramidal dendrites assisting in the maintenance of the efficacy of the entorhinal input (Losonczy et al., 2002).

The GABAergic innervation provided by IS cells to other hippocampal interneurons gives rise to a complex interaction among GABAergic neurons. Group I mGluR agonists are effective on some interneurons in the strata radiatum and LM, and mGluR1 was identified as the receptor involved (Ouardouz and Lacaille, 1995; Woodhall et al., 1999; Perez et al., 2001). Substantial differences were reported between interneurons of strata O/A and LM in mGluR1 mediated responses (Ouardouz and Lacaille, 1995; Woodhall et al., 1999; Perez et al., 2001). Although IS interneurons express a much lower concentration of mGluR1α than O-LM cells, their highly specific relationship to other interneurons could amplify the effect of mGluR activation as each innervated GABAergic neuron innervates thousands of pyramidal cells.

In conclusion, the identification of at least six different mGluR1α-positive types of interneuron, which project to distinct laminae and cell types, shows a participation of this receptor in multiple synaptic links and circuits, which may form a specific subset. This is suggested by the presence of the receptor in two strongly connected cell types: the O-LM cells and their main GABAergic input neurons, the IS-3 cells. Some of the O-Bi cells are also in a position to receive input from IS-3 neurons, and the CCK-positive Schaffer collateral associated cells probably receive IS-1/2 input, linking them to the network. What is common in all the GABAergic cell types shown to express mGluR1α in this study is that they innervate dendrites. The activation of the receptor on the interneurons and the circuits may be related to some of the distinct network states of the hippocampus, which requires the GABAergic fine tuning of dendritic signal processing in pyramidal cells.

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