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The Metabotropic Glutamate Receptor (mGluR1α) Is Concentrated at Perisynaptic Membrane of Neuronal Subpopulations as Detected by Immunogold Reaction

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Summary

An antiserum to mGluR1α labeled a 160 kd protein in immunoblots of membranes derived from rat brain or cells transfected with mGluR1α. Immunoreactivity for mGluR1α was present in discrete subpopulations of neurons. The GABAergic neurons of the cerebellar cortex were strongly immunoreactive; only some Golgi cells were immunonegative. Somatostatin/GABA-immunopositive cells in the neocortex and hippocampus were enriched in mGluR1α. The hippocampal cells had spiny dendrites that were precisely codistributed with the local axon collaterals of pyramidal and granule cells. Electron microscopic immunometal detection of mGluR1α showed a preferential localization at the periphery of the extensive postsynaptic densities of type 1 synapses in both the cerebellum and the hippocampus. The receptor was also present at sites in the dendritic and somatic membrane where synapses were not located.

Introduction

The neurotransmitter effects of glutamate are mediated by two classes of receptors, the ionotropic receptors, which are ligand-gated cationic channels, and the metabotropic receptors. Sladeczek and colleagues (1985) demonstrated that glutamate and quisqualate induced the production of inositol-1,4,5-trisphosphate (IP_3), and this increase in IP_3 was subsequently shown to be mediated through a receptor coupled to a G protein (Sugiyama et al., 1987). The development and use of specific antagonist and agonist drugs influencing metabotropic glutamate receptors (see Baskys, 1992) have revealed that these receptors have many roles in the brain (e.g., Anwyl, 1991; Bashir et al., 1993; Miles and Poncer, 1993; Miller, 1991; Zorumski and Thio, 1992), and it has been suggested that they may also be involved in some cerebral disorders (Schoepp and Conn, 1993).

At least six different cDNAs encoding metabotropic glutamate receptors have been cloned (mGluR1–6) (Houamed et al., 1991; Masu et al., 1991, Tanabe et al., 1992; Nakanishi, 1992). The sequenced cDNA for mGluR1 codes for a protein with an estimated molecular mass of 133 kd. Two alternatively spliced forms of this receptor with shorter C-termini have now been isolated and designated as mGluR1β and c (small forms) to differentiate them from mGluR1α (long form), originally described (Pin et al., 1992; Tanabe et al., 1992). In vitro expression experiments have shown that metabotropic glutamate receptors differ not only in their sequence, but also in their signal transduction mechanisms. In transfected cells, the activation of mGluR1 and mGluR5 stimulates IP_3 formation and intracellular calcium mobilization, as well as induces arachidonic acid release (Abe et al., 1992; Aramori and Nakanishi, 1992; Masu et al., 1991; Nakanishi, 1992). In contrast, mGluR2, 3, 4, and 6 appear to inhibit forskolin-stimulated cAMP formation in transfected cells (Tanabe et al., 1992; Nakanishi, 1992). Furthermore, metabotropic glutamate receptors have been subdivided according to their pharmacological characteristics (Barnes and Henley, 1992) into three groups: group 1 includes mGluR1 and 5, group 2 includes mGluR2 and 3, and group 3 includes mGluR4 and 6 (Nakanishi, 1992). All these data suggest that the action of glutamate and the effect of drugs on glutamatergic neurotransmission through intracellular signal transduction will vary depending on the presence of specific metabotropic glutamate receptor subtypes at specific sites in the brain.

In situ hybridization of mRNAs coding for mGluR2 (Tanabe et al., 1992; Ohishi et al., 1993) and for mGLuR1 (Masu et al., 1991; Shigemoto et al., 1992) has suggested that metabotropic glutamate receptors are differentially expressed throughout the brain, as described for the ionotropic glutamate receptor subunits (Nakanishi, 1992). Moreover, the same type of neuron may contain mRNAs coding for more than one metabotropic glutamate receptor subtype. For example, granule cells in the hippocampal dentate gyrus and Golgi cells in the cerebellar cortex contain mRNAs coding for mGluR1, 2, and 5 (Abe et al., 1992; Ohishi et al., 1993; Shigemoto et al., 1992; Tanabe et al., 1992).

One of the major questions raised by the heterogeneity of the metabotropic glutamate receptors is how these receptors are distributed on the surface of cells in the brain, particularly in relation to specific excitatory amino acid-releasing afferents. Anti-peptide antibodies directed to different subunits of the γ-aminobutyric acid (GABA) and glutamate ionotropic receptors (e.g., Baude et al., 1992; Benke et al., 1991; Blackstone et al., 1992; Molnar et al., 1993; Petralia and Wenthold, 1992; Zimprich et al., 1991) as well as to mGluR1α (Martin et al., 1992; Gorcs et al., 1993) have proved to be useful tools for such studies. Martin et al. (1992) showed, using the immunoperoxidase procedure, that immunoreactivity for mGluR1α was present at high levels in particular brain areas and sometimes only in a discrete subpopulation of neurons in a given area. They also found that the immuno-

peroxidase reaction end product could be found intracellularly near synapses. Using a similar strategy, we have used an antiserum raised against the C-terminus of mGluR1α together with immunoperoxidase and immunometal methods to establish the precise location of immunoreactivity for this metabotropic glutamate receptor subtype in relation to afferent synaptic terminals. Different ionotropic amino acid transmitter receptors have been found at extrasynaptic sites (e.g., Molnar et al., 1993); therefore, for comparison we have also studied the distribution of mGluR1α at nonsynaptic areas of the plasma membrane.

Since metabotropic receptors are thought to be involved in plastic changes in several areas of the brain, including the hippocampus (Bashir et al., 1993; Miles and Poncer, 1993; Zheng and Gallagher, 1992), the latter area was analyzed in more detail. The distribution of strongly mGluR1α-immunoreactive cells in the hippocampus (Martin et al., 1992) is very similar to that observed for the somatostatin-immunoreactive cells (Bakst et al., 1985; Kosaka et al., 1988; Sloviter and Nilaver, 1987; Somogyi et al., 1984). Somatostatin cells are the topic of intensive research, since some of them have been shown to be highly vulnerable to excitotoxicity following ischemia or epilepsy (Sloviter, 1987; Freund et al., 1992). Therefore, double labeling experiments were performed to test for the presence of metabotropic glutamate receptor in somatostatin-immunopositive cells of the hippocampus.

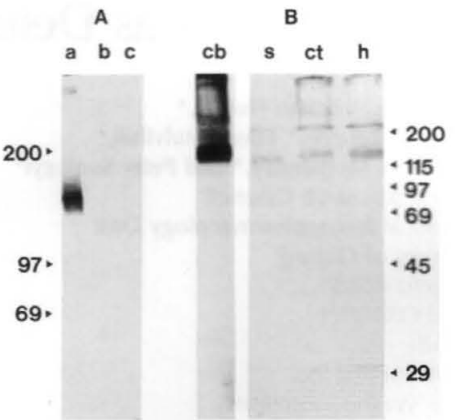


Figure 1. Immunolabeling of Membrane Proteins with Antibodies (A4) Directed to the C-Terminal Part of mGluR1α

Proteins were blotted to nitrocellulose from SDS-polyacrylamide gels following electrophoresis of solubilized membrane from transfected cultured cells ([A] gradient gels) and rat brain ([B] linear gels). The serum reacts with a band corresponding to a 160 kd protein only in the cells transfected with the mGluR1α gene (lane a) and not with cells transfected with the mGluR1β gene (lane b) or with the plasmid only (lane c). The antiserum stains a protein of identical molecular weight in cell membranes prepared from spinal cord (lane s), neocortex (lane ct), hippocampus (lane h), and cerebellum (lane cb); the highest immunoreactivity is observed from the cerebellum. The higher molecular weight proteins probably correspond to aggregates. The positions of the molecular weight standards are indicated on the left for the linear gels and on the right for the gradient gels. The marker proteins used were myosin (200 kd), β-galactosidase (115 kd), phosphorylase b (97 kd), bovine serum albumin (69 kd), ovalbumin (45 kd), and carbonic anhydrase (29 kd).

Results

Immunoblotting of Cell and Brain Membranes

The specificity of the antiserum was assessed by immunoblotting of membranes derived from cells transfected with mGluR1α or mGluR1β (Figure 1A) and rat brain membranes prepared from cortex, spinal cord, cerebellum, or hippocampus (Figure 1B).

Membranes from cells transfected with the gene for mGluR1α (Figure 1A, lane a), with the gene for mGluR1β (lane b), and with the plasmid only (lane c) were analyzed on 4%–15% SDS-polyacrylamide gradient gels. The immunoblot, developed using the crude antiserum directed to mGluR1α (code number A4), clearly showed that the A4 antiserum reacted only with a broadly stained band of protein, (approximate molecular weight of 160,000) in the cells transfected with mGluR1α and showed no reaction with the mGluR1β or vector only transfected cell lines. Separate experiments (data not shown) have demonstrated that the cells transfected with mGluR1β do express this receptor subtype at comparable levels to mGluR1α.

Rat brain membranes (50 μg per lane) from spinal cord, neocortex, hippocampus, and cerebellum were analyzed on 9% SDS-polyacrylamide linear gels (Figure 1B). The immunoblot was developed with affinity-purified antibodies directed to mGluR1α at a concentration of 5 μg per ml of protein. In all brain areas, the major reactive band was a protein of 160 kd, with fainter components of higher molecular weight most clearly seen in the noncerebellar areas. The protein band stained by antibodies to mGluR1α was broad, as seen with the transfected cells, suggesting that the receptor is heavily glycosylated. This result is predicted by the presence of multiple (five) N-linked glycosylation signals in the cDNAs (Houamed et al., 1991; Masu et al., 1991). The molecular weight estimates obtained are consistent with the values predicted by analysis of the cloned mGluR1α (Masu et al., 1991) and also obtained in a previous study using immunoblots of cerebellar membranes (Martin et al., 1992). These results confirm that antiserum A4 reacts with mGluR1α.

The reaction with the cerebellum was much stronger than with other brain areas, and consequently, this color reaction was stopped after 5 min; other brain areas were allowed to react with the chromogen for an additional 15 min. This longer reaction brought up several lower molecular weight bands, which demonstrated inconsistent staining intensity from experiment to experiment. Furthermore, their staining was not blocked by prior treatment of the affinity-purified antibodies directed to mGluR1α with the peptide used as immunogen (EFVYEREGNTEEDEL, 5 μg/ml), unlike the staining of the higher molecular weight proteins. This staining would therefore be due

Table 1. Distribution of Immunoreactivity for mGluR1α in Selected Brain Areas

Brain Area	Immuno-reactivity	Frequency	Brain Area	Immuno-reactivity	Frequency
Olfactory bulb			Basal ganglia		
Glomeruli	****	4	Caudate-putamen	**	1
External plexiform layer	***	4	Accumbens	**	1
Granular layer	(*)	2	Globus pallidus	***	4
Mitral cell	***		Entopeduncular nucleus	***	4
Tufted cell	***		Ventral pallidum	***	4
			Islands of Calleja	***	4
Anterior olfactory nucleus	**	3	Subthalamic nucleus	**	3
Neocortical nonprincipal cells	*	2	Thalamus		
Piriform cortex	**	3	Reticular nucleus	ND	
Cingulate cortex	**	3	Habenula	*	1
			Other nuclei	***	4
Hippocampal nonprincipal cells					
CA1 area	****		Zona incerta	**	3
Alveus		4			
Stratum oriens		2	Hypothalamus		
Stratum pyramidale		1	Arcuate nucleus	ND	
Stratum radiatum		1	Dorsomedial nucleus	(*)	2
Stratum lacunosum moleculare		1	Lateral hypothalamic area	**	3
			Preoptic area	***	2
CA3 area	**		Ventromedial nucleus	ND	
Alveus		3	Supraoptic nucleus	***	3
Stratum oriens		3	Bed nucleus of the stria terminalis	*	1
Stratum pyramidale		1	Other hypothalamic areas	**	2
Stratum lucidum		2			
Stratum radiatum		3	Midbrain		
Stratum lacunosum moleculare		1	Central gray	*	1
			Inferior collicus	(*)	1
Dentate gyrus	**		Interpeduncular nucleus	**	3
Hilus		4	Oculomotor nucleus	*	4
Granule cell layer		1	Substantia nigra	***	4
Stratum moleculare		1	Ventral tegmental area	**	3
			Superior colliculus	***	4
Subiculum	***	3	Red nucleus	(*)	1
Amygdala			Cerebellum		
Cortical nuclei	**	3	Molecular layer	****	4
Basolateral nucleus	**	2	Purkinje cells	****	
Basomedial nucleus	**	1	Stellate cells	***	
Central nucleus	ND		Basket cells	***	
Intramedullary grey	***	3	Bergmann glial cells	ND	
Lateral nucleus	**	1	Granule cells	ND/**	1
Medial nucleus	ND		Golgi cells	**	2
			Lugaro cells	**	
Lateral septum	*	1	Deep nuclei	*	3
Medial septum	*	2			
Bed nucleus of the anterior commissure	***	3	Inferior olive	***	4

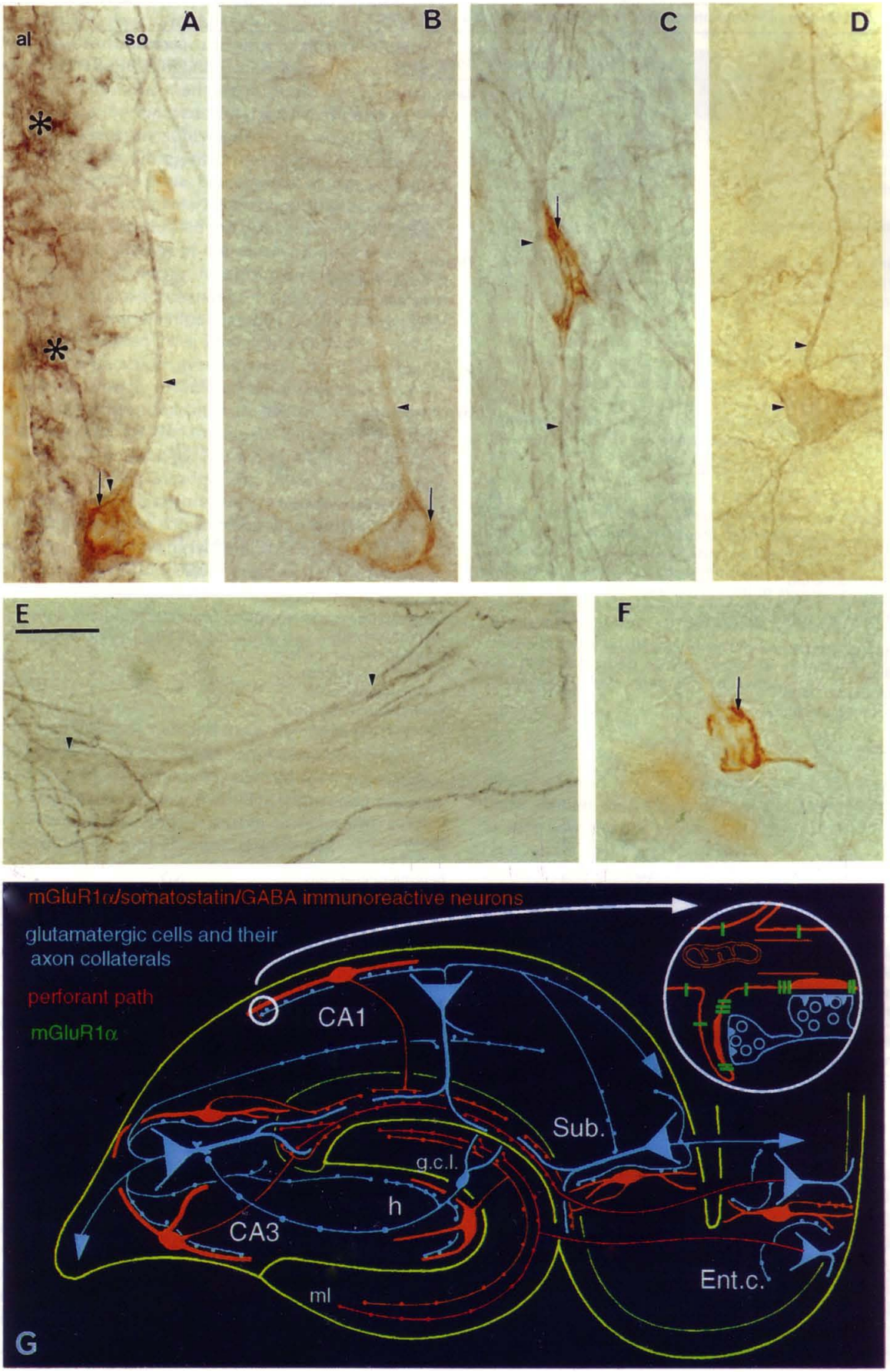
Immunoreactivity: ****, very intense; ***, intense; **, strongly positive; *, positive; (*), weakly positive; ND, not detectable. Frequency of immunopositive somata and dendrites: 1, rare; 2, sparse; 3, high; 4, very high.

to nonspecific binding of the antibody. The higher molecular weight components could represent polymeric aggregates of the receptor and were observed also in the transfected cells (Figure 1A, lane a). The staining of these was blocked by the peptide used as immunogen.

Distribution of the Immunoreactivity for mGluR1α in Rat Brain

Both omission of primary antiserum and preadsorption of purified A4 antibodies with the corresponding peptide resulted in the lack of specific labeling in immunoperoxidase as well as immunogold experiments, confirming the specificity of the immunocytochemical methods.

Immunoreactivity for mGluR1α was widely and selectively distributed in neurons throughout the rat brain (Table 1). The results confirm and extend, particularly in the cerebellum and hippocampus, previous immunocytochemical studies using other antibodies (Martin et al., 1992; Gorcs et al., 1993). Different intensities of immunolabeling were found consistently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both in different brain areas and in the various cell types present in these areas (Table 1).

Distribution of mGluR1α in the Hippocampus and Its Localization within Somatostatin-Immunopositive Neurons

In the hippocampus, no immunoreactivity was detectable in the pyramidal cells of the CA1 and CA3 areas, or in granule and mossy cells of the dentate gyrus. Numerous nonprincipal neurons were strongly immunoreactive in all areas of the hippocampus (Table 1; Figure 2; Figure 3A). The alveus and adjoining stratum oriens of the CA1 area were the strongest immunoreactive areas (Figure 3A). The CA3 area exhibited a more uniform reactivity than the CA1 area owing to scattered nonprincipal cells in all layers. In both CA1 and CA3 areas, the immunopositive cells were strongly immunoreactive throughout their plasma membrane. In the hilus, the density of labeled cells was high, but the intensity of the immunostaining of both cell bodies and dendrites was consistently weaker than that observed in cells of the CA1 and CA3 areas.

The cell bodies and dendrites of immunoreactive nonprincipal cells could be found mainly in the alveus and adjoining stratum oriens in the CA1 area, with sparsely dispersed cells and dendrites in the other layers. On the contrary, in the CA3 area, strongly immunopositive cell bodies and dendrites were distributed in all layers. The dentate hilus contained a high density of immunopositive neurons and their dendrites. A few immunopositive dendrites from hilar neurons entered the molecular layer. Very few positive somata were present among principal cells in the stratum pyramidale or the granule cell layer. Occasionally, weakly immunolabeled somata were located in the dentate molecular layer. The immunoreactive dendritic processes travelled widely through several strata (Figure 3B), or were confined to more restricted areas (Figures 3C and 3F). The most conspicuous characteristic of the cells immunoreactive for mGluR1α was the high density of spines on their dendrites (Figures 3B, 3C, and 3F). Some of the spines had a very long neck (Figure 3D). Immunoreactive spines were distributed all along the dendrites (Figures 3C and 3F; see also Figure 4), or were exclusively confined to the more distal parts of the dendrites (Figure 3B).

The above distribution of mGluR1α-immunoreactive neurons resembled that of somatostatin/GABA-positive cells described previously (Kosaka et al., 1988; Somogyi et al., 1984), some of which are among the most vulnerable neurons in the hippocampus. Therefore, double immunolabeling experiments were carried out to establish the degree of overlap between the populations of cells labeled for the metabotropic glutamate receptor and those labeled for somatostatin. Because of the different peroxidase reactions, immunoreactivity for mGluR1α appeared as a blue-gray reaction end product and immunoreactivity for somatostatin appeared as a brown reaction end product (Figures 2A–2F). Moreover, immunoreactivity obtained with the antibodies directed to mGluR1α was present at the somatic and entire dendritic membrane of immunoreactive cells (Figures 2D and 2E; Figure 3), whereas that obtained with the monoclonal antibody to somatostatin was restricted to the Golgi apparatus in the soma and proximal part of the primary dendrites (Figure 2F).

In these experiments, all the cells of the hippocampus immunoreactive for somatostatin were strongly immunoreactive for mGluR1α, and likewise the cells immunoreactive for mGluR1α were also immunoreactive for somatostatin (Figures 2A and 2B). Numerous double labeled cells were also observed in the anterior olfactory nucleus (Figure 2C), and the majority of mGluR1α-positive cells were also somatostatin reactive in the neocortex. However, in the same double-labeled sections, cells reacted only for mGluR1α in the globus pallidus (Figure 2D) and cortex (Figure 2E), or only for somatostatin in the caudate putamen (Figure 2F), showing that the colabeling of neurons in the cortex and anterior olfactory nucleus was specific.

Cerebellum

In the cerebellar cortex, virtually all the basket, stellate, and Purkinje cells were strongly immunoreactive, resulting in a very intense labeling of the neuropil

Figure 2. Characterization of mGluR1α-Positive Neurons

(A–F) Light micrographs showing immunoreactivity for mGluR1α (DAB–nickel as chromogen, blue-gray reaction end product) in neurons, some of which are also immunoreactive for somatostatin (DAB alone as chromogen, brown reaction end product). Immunoreactivity for somatostatin is visible as a meshwork restricted to the Golgi apparatus (arrows) in the cell body and proximal dendrites. The somatic and dendritic membranes exhibit a blue-gray reaction for mGluR1α (arrowheads). Somatostatin-positive neurons in the alveus ([A] al) and stratum oriens (so) as well as in the hilus (B) of the hippocampus and in the anterior olfactory nucleus (C) are also delineated by immunoreactivity for mGluR1α. Note the high density of strongly mGluR1α-immunoreactive fine dendrites in the neuropil of the alveus (asterisks). (D–F) In the same sections treated for double labeling, some populations of cells are labeled only for mGluR1α, e.g., in the neocortex (D) and globus pallidus (E), or only for somatostatin, e.g., in the caudate putamen (F). (G) Summary of the localization of strongly mGluR1α-immunopositive cells in the network of the hippocampal formation. The dendrites of neurons strongly immunoreactive for mGluR1α and somatostatin are precisely codistributed with the local recurrent axon collaterals of granule and pyramidal cells, which presumably provide their major glutamatergic input. In the plasma membrane the receptor is concentrated at the edge of the postsynaptic density and is also present at extrasynaptic sites (electron microscopic results are shown in the white circle). The somatostatin-positive cells have been shown previously to contain GABA, and their axons terminate mainly in conjunction with the perforant path originating from the entorhinal cortex (Ent.c.). g.c.l., granule cell layer; h, hilus; ml, molecular layer; Sub., subiculum.

Bar, 15 μm (A–F).

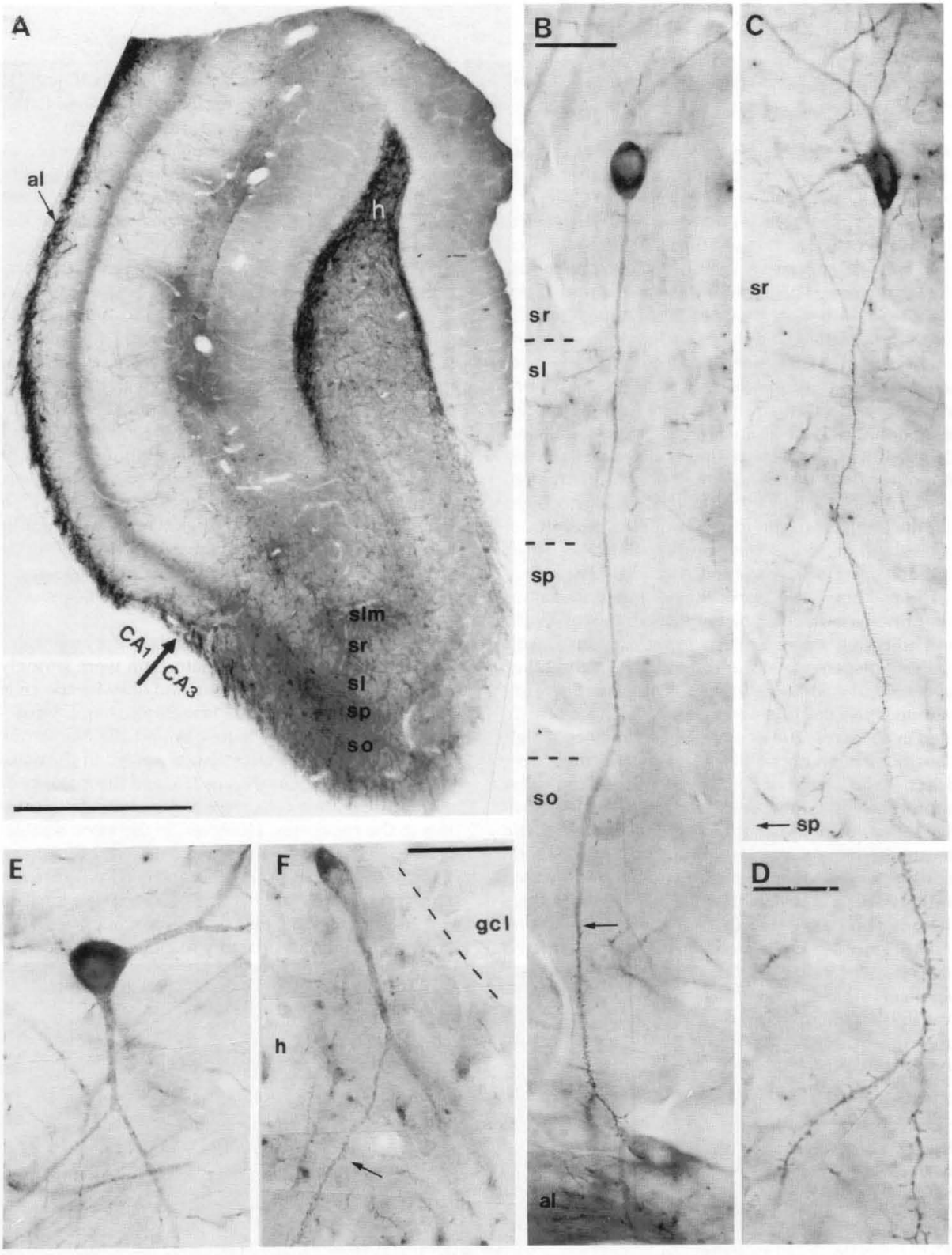


Figure 3. Light Micrographs Showing Immunoreactivity for mGluR1α in the Dorsal Hippocampus

(A) Some nonprincipal neurons and their dendrites are strongly immunoreactive in the hilus (h), throughout the CA3 region, and in the alveus (al) of the CA1 area. (B–E) Strongly immunoreactive nonprincipal neurons in the CA3 area have spiny and/or smooth dendrites. (B) The radially descending dendrite of a cell in the stratum radiatum becomes densely spiny (arrow) from the lower stratum oriens and particularly in the alveus. (C) An immunoreactive cell in the stratum radiatum has dendrites parallel to the stratum pyramidale (direction as arrow, sp) and restricted to the strata radiatum and lucidum. (D) The same dendrite as in (C) emits many spines with long necks. (E) Another immunoreactive cell in the stratum radiatum has thick and aspiny main dendrites. (F) A nonprincipal neuron emitting thick and spiny dendrites (arrow) in the hilus. gcl, granule cell layer; so, stratum oriens; sp, stratum pyramidale; sl, stratum lucidum; sr, stratum radiatum; slm, stratum lacunosum moleculare.

Bars, 0.5 mm (A); 30 μm (B, C, and E); 10 μm (D and F).

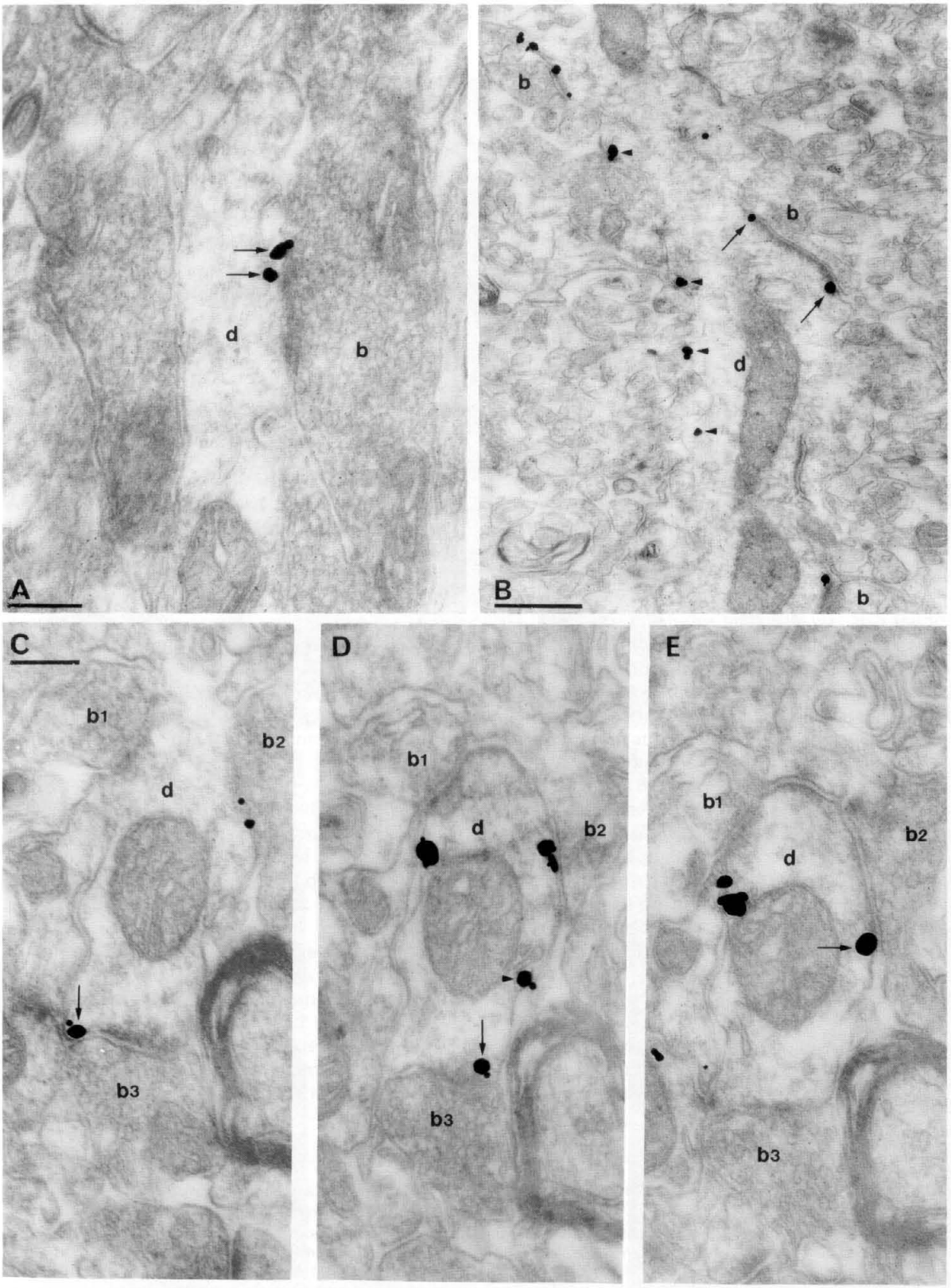
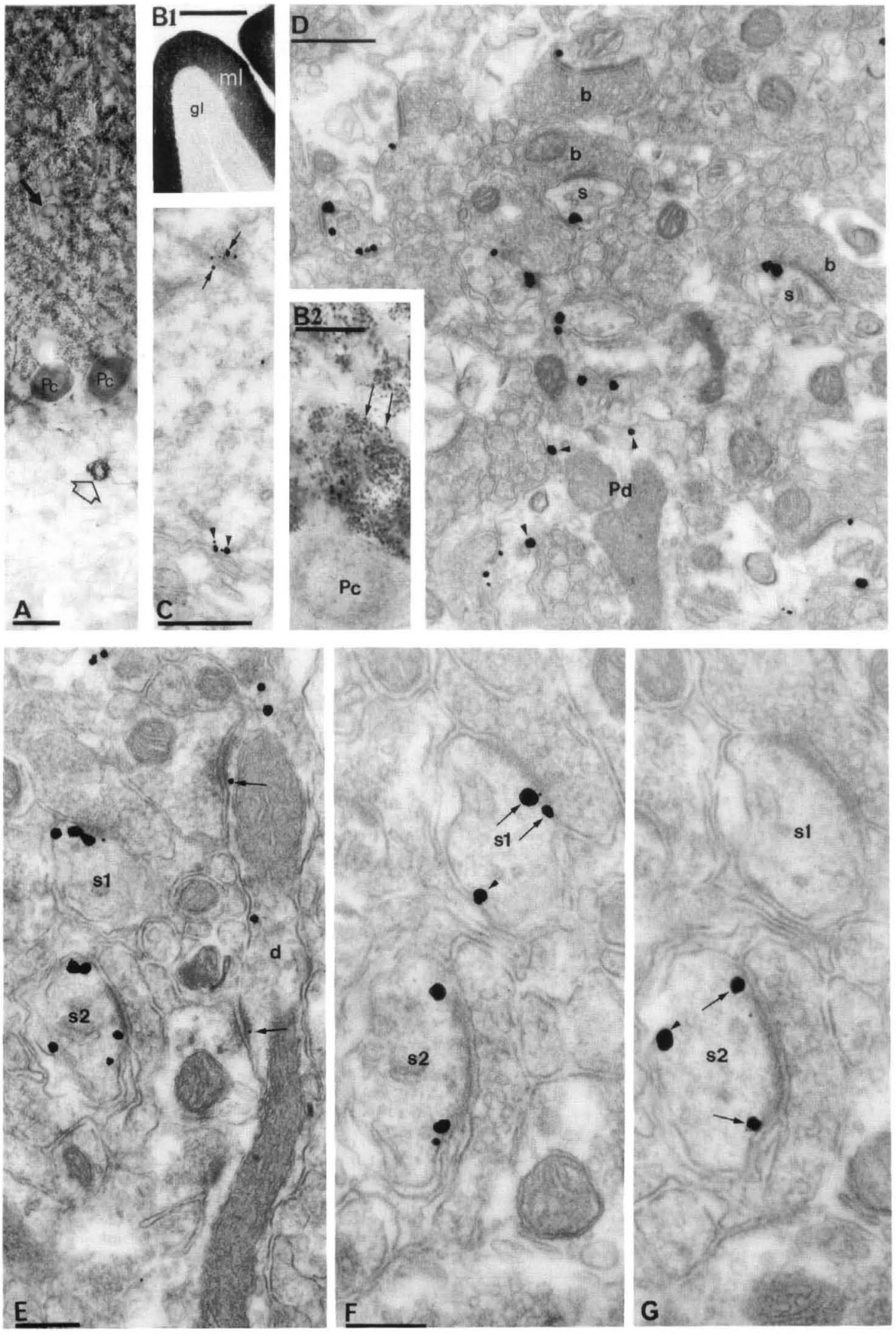


Figure 4. Electron Micrographs of Immunoreactivity for mGluR1α in the Hippocampus as Demonstrated with a Preembedding Silver-Intensified Immunogold Technique

Immunometal particles are located at the edge of synapses (e.g., arrows) in the postsynaptic dendrites, or at some distance from synaptic junctions (e.g., arrowheads), but they are not associated with the main body of postsynaptic densities. (A) An immunoreactive synapse between a dendritic shaft (d) and a bouton (b) probably originating from a granule cell local collateral in the hilus. (B) An immunoreactive dendritic shaft (d) receives type 1 synapses from axon terminals (b) in the alveus of the CA1 area. (C–E) An immunoreactive dendritic shaft (d) is shown in three consecutive serial sections in the CA1 area. Three axon terminals (b1–b3) are seen making type 1 synapses.

Bars, 0.2 μm (A); 0.5 μm (B); 0.2 μm (C–E).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of the molecular layer (Figures 5A and 5B). In the granular layer many Lugaro cells (a subpopulation of cells lying just below the Purkinje cell layer), but only some of the Golgi cells and their dendrites, were stained with antibodies to mGluR1α. Immunoreactive somata of granule cells and strongly immunopositive glomeruli (Figure 5A) were scattered in the folia of the cerebellar cortex unevenly. In some folia no immunopositive granule cells were seen, whereas in others a high density of labeled cells and glomeruli was detected.

Subcellular Distribution of mGluR1α in the Hippocampus and Cerebellum

To establish the subcellular distribution of mGluR1α, we used a high resolution immunometal method that produces silver particles on a gold core, in three different immunocytochemical techniques. These included preembedding experiments on thick sections, reaction on frozen ultrathin sections, and postembedding reaction on ultrathin sections obtained from Lowicryl-embedded tissue. The use of these combined techniques allowed the precise localization of mGluR1α at the membrane of immunoreactive cells. Results were obtained from the hippocampus (Figure 4) and cerebellum (Figure 5; Figure 6; Figure 7) and can be summarized as follows.

– Immunometal particles were found in somata along the cytoplasmic face of the endoplasmic reticulum cisternae (Figure 5C), or at the internal face of the plasma membrane (Figure 5C) of cell bodies, dendrites (Figure 4; Figures 5D and 5E), and spines (Figures 5D–5G). This location correlates with the intracellular location of the C-terminal part of mGluR1α to which antibodies were raised, as expected by the transmembrane topography of the receptor deduced from the amino acid sequence (Houamed et al., 1991; Masu et al., 1991).

– At synaptic junctions, immunometal particles were located most frequently at a "perisynaptic" position at the edge of the synaptic junction surrounding the postsynaptic density of so called type 1 synapses, which are characterized by an extensive postsynaptic density (Gray, 1959). The examination of serial ultrathin sections showed that the immunolabel surrounds the synapses in a ring formation (Figures 4C–4E; Figures 5E–5G) at both axo-spinous and axo-dendritic synaptic junctions. Using the preembedding method, immunolabel was never seen in the main body of the postsynaptic density. Since it could be argued that the immunoreagents were not able to penetrate the dense material present in the postsynaptic densities, which may mask receptors, postembedding immunocytochemistry on Lowicryl-embedded, freeze-substituted tissue was carried out. This technique overcomes the problem of penetration of immunoreagents in tissue, since antibodies have access to molecules present at the very surface of the section along the entire cut length of membranes. The results were similar to those obtained using preembedding techniques; most particles were located around the edge of synaptic densities (Figure 6). Furthermore, an identical localization (Figure 7) was observed after reacting ultrathin frozen sections. The results of these three different immunocytochemical techniques demonstrate that the perisynaptic location of the gold particles does reflect the high concentration mGluR1α at a "perisynaptic" location.

– Immunoreactivity for mGluR1α was also found at dendritic and somatic membranes not associated with synaptic junctions at "extrasynaptic" locations in both the hippocampus (Figure 4B) and the cerebellum (Figures 5C–5G). In particular, immunometal particles were present at the Purkinje cell somatic membrane (Figure 5C), which does not receive excitatory amino acid synapses but is known to be almost entirely covered by Bergmann glial processes and GABAergic basket cell axons. Extrasynaptic immunoreactivity was also observed after postembedding reactions and on ultrathin cryosections at somatic, dendritic, and spine membranes (Figure 6; Figure 7).

None of the techniques used in this study revealed immunoreactivity in axon terminals.

Discussion

Peri- and Extrasynaptic Location of mGluR1α as Detected by a Particulate Immunomarker

As revealed by a high resolution technique using a

Figure 5. Light and Electron Micrographs Showing Immunoreactivity for mGluR1α in the Cerebellar Cortex, as Demonstrated by Immunoperoxidase or Preembedding Silver-Intensified Immunogold Techniques

(A) Immunoperoxidase technique; (B–G) preembedding silver-intensified immunogold technique. (A and B) The strong immunoreactivity in the molecular layer (ml) appears as small dots at higher magnification (thin arrows); occasional glomeruli (open arrow) are also strongly immunopositive in the granular layer (gl). Purkinje cells (Pc) and stellate cells (e.g., thick arrow in [A]) are strongly stained with the peroxidase method. (C) In the soma of a Purkinje cell, immunometal particles are located along the cytoplasmic face of endoplasmic reticulum cisternae (arrows) and at nonsynaptic areas along the internal face of the somatic membrane (arrowheads). (D–G) Presumed parallel fiber boutons (e.g., b) establish type 1 synapses with immunoreactive spines (e.g., s). Immunometal particles are deposited along the internal face of the plasma membrane of the spines preferentially at the periphery of the synaptic disk and at a distance from the synaptic area (arrowheads). (D) Metal particles are also distributed at nonsynaptic areas (arrowheads) along the membrane of Purkinje cell dendrites (Pd). (E–G) Two spines (s1 and s2) are shown in three serial sections. They show perisynaptic and extrasynaptic immunoreactivity for mGluR1α. In (E) the synapse of spine s1 is just beginning to appear so the particles appear to face the junction, but as shown in the consecutive serial section (F), the metal particles are actually located at the edge of the synaptic disk (arrow in s1). (E) A dendritic shaft (d), probably originating from an interneuron, is also immunoreactive (arrows).
Bars, 20 μm (A and B2) 1 mm (B1); 0.5 μm (C and D); 0.25 μm (E); 0.2 μm (F and G).

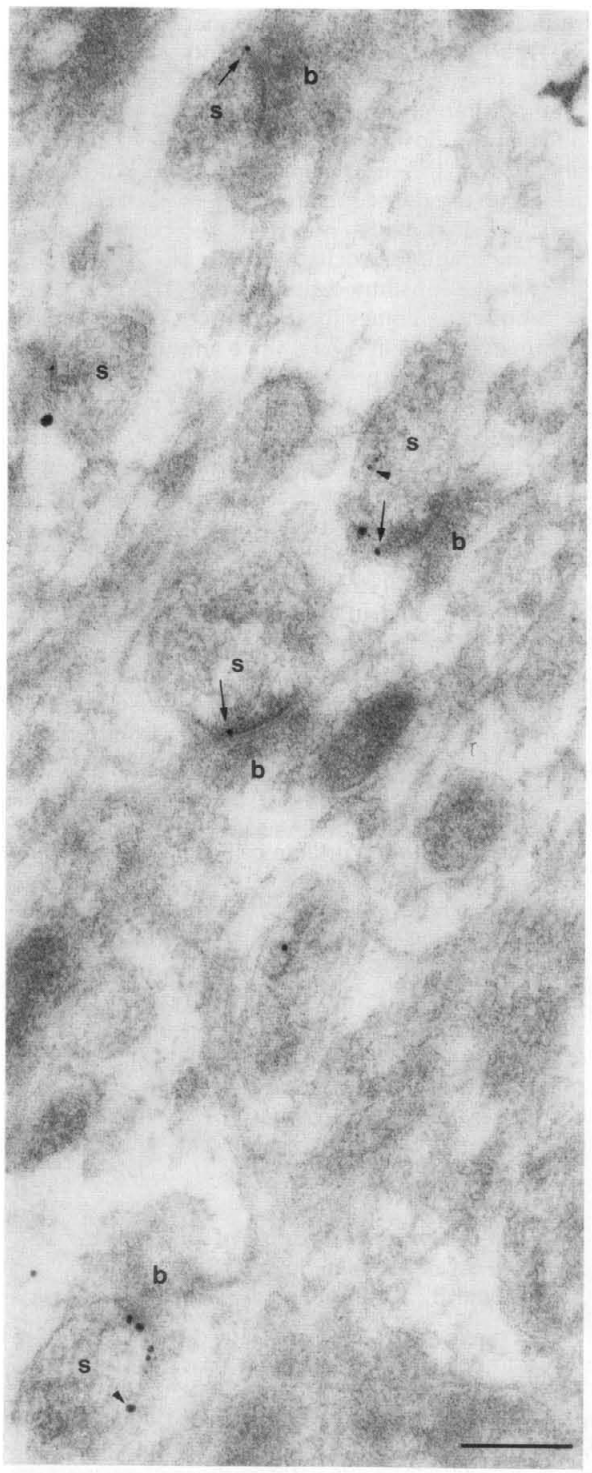

Figure 6. Electron Micrograph of Immunoreactivity for mGluR1α in the Molecular Layer of the Cerebellar Cortex, as Demonstrated by a Postembedding Silver-Intensified Immunogold Reaction on Chemically Fixed, Freeze-Substituted, Lowicryl-Embedded Tissue

Presumed parallel fiber boutons (b) establish synapses with immunoreactive spines (s). The gold particles are located at the edge of the postsynaptic densities (arrows) or at some distance from the synapses (arrowheads). Under postembedding conditions, antibodies have access to the whole length of the cut synaptic plasma membranes; nevertheless, immunoreactivity is not detected in the main body of synaptic disks, similar to data shown in Figures 4 and 5.
Bar, 0.2 μm.

nondiffusible marker, the most striking result of the present study is that immunoreactivity for mGluR1α is not found in the main body of the postsynaptic density, but is concentrated in the postsynaptic membrane *at the periphery of synaptic junctions*. It is possible that, given the sensitivity of available techniques, low amounts of receptor were not visualized, and mGluR1α may be present at lower concentration in the central part of synapses. Although this possibility can never be excluded, our results demonstrate a high concentration of receptor close to the transmitter release site, but not in the main body of the synaptic junction. It is very unlikely that the exclusively peri- and extrasynaptic location of immunoreactivity was due to technical limitations, as an identical localization of the receptor was found with three different techniques. Therefore, allowing for the limitations in sensitivity, it can be concluded that the perisynaptic and extrasynaptic location of immunometal particles at the surface of the plasma membrane reflects the genuine selective distribution of mGluR1α. It is likely, but remains to be shown, that the ionotropic glutamate receptors occupy the main body of the synaptic junction. The results of the localization of mGluR1α are at variance with previous immunoperoxidase localization of the same receptor, also confirmed using our antibodies (data not shown), revealing peroxidase reaction end product on the postsynaptic densities and in the cytoplasm of spines (Gorcs et al., 1993; Martin et al., 1992). It is likely that staining of postsynaptic densities results from secondary deposition of the diffusible peroxidase reaction end product formed at peri- and extrasynaptic sites.

Whenever located at perisynaptic location, immunometal particles were always associated with type 1 synapses, which have extensive postsynaptic densities, established between dendritic spines, shafts, and axon terminals. In the hippocampus, immunoreactive synapses were found between dendrites and presynaptic boutons that had characteristics similar to those known to be enriched in glutamate and thought to release it as a neurotransmitter (Bramham et al., 1990; Cotman et al., 1987; Grandes and Streit, 1991; Liu et al., 1989). In the cerebellar cortex, immunolabeled synapses were found between dendritic spines of Purkinje cells and the other GABAergic cells, and parallel fiber terminals, which are known to contain high concentrations of glutamate (Somogyi et al., 1986). These results suggest that glutamate released from these axon terminals is the endogenous ligand of the perisynaptic mGluR1α. The extrasynaptic location of immunometal particles corresponds to receptor protein located at somatic or dendritic surface membranes that are not associated with axon terminals. It remains

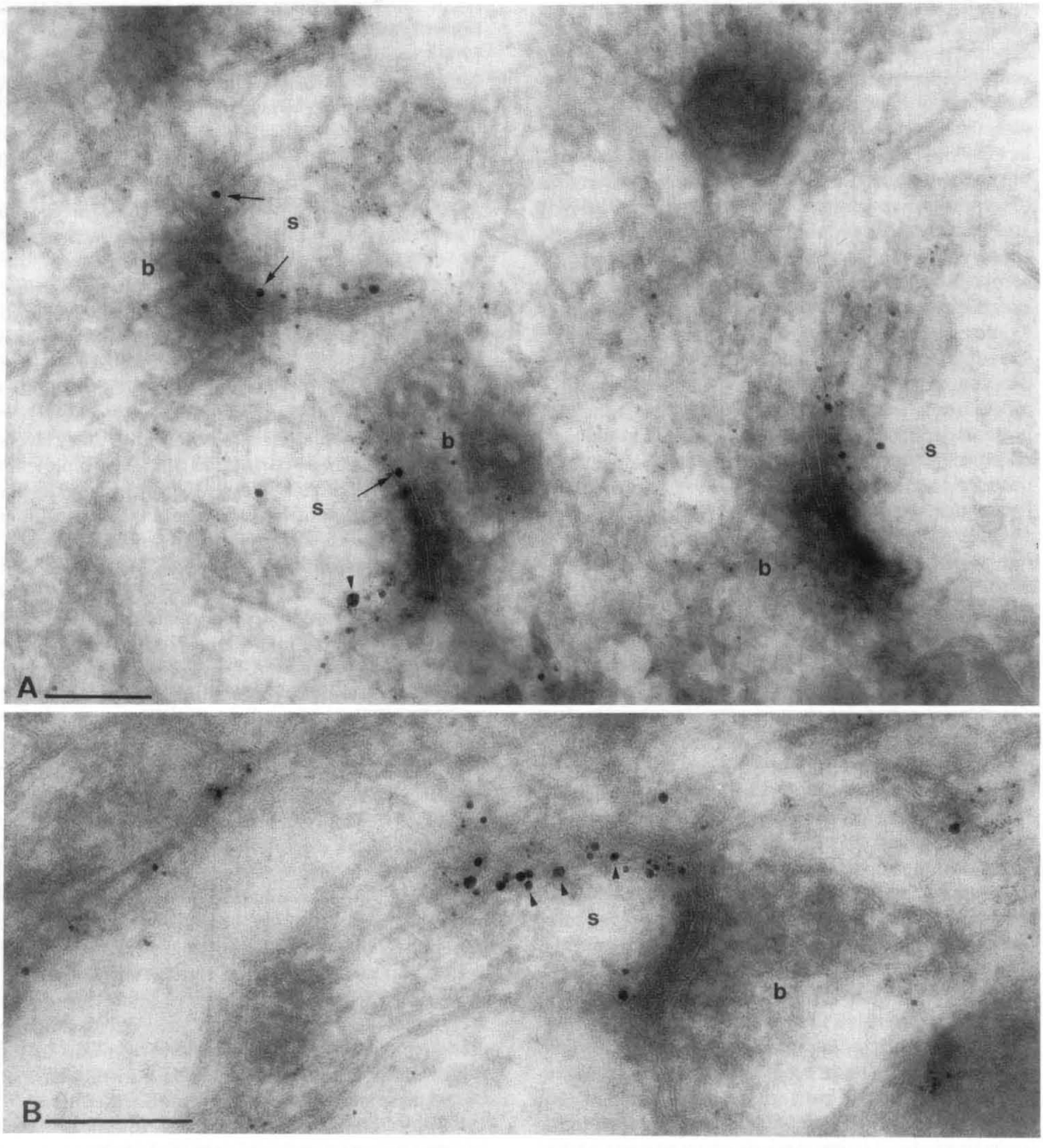

Figure 7. Electron Micrographs of Ultrathin Frozen Sections of the Molecular Layer of the Cerebellar Cortex Demonstrating Immunoreactivity for mGluR1α Using a Silver-Intensified Immunogold Technique

Presumed parallel fiber boutons (b) are shown making synapses with immunoreactive dendritic spine(s). Immunolabeling is strong at the edge of synaptic disks (e.g., arrows) and at sites at some distance from synapses (e.g., arrowheads).
Bars, 0.2 μm (A and B).

to be established whether the extrasynaptic receptor protein found at large distances from glutamate release sites can participate in the full sequence of G protein–coupled transduction mechanisms.

The peculiar peri- and extrasynaptic distrubution of mGluR1α may contribute to the physiological roles mediated by the G protein–coupled mechanisms. Metabotropic glutamate receptors seem to contribute little to the excitatory responses evoked by low frequency stimulation of synaptic input, but are activated by high frequency stimuli (Bashir et al., 1993; Miles and Poncer, 1993). Low frequency stimuli may not release enough glutamate to reach the perisynaptic mGluRs at a significant concentration and would activate mainly the ionotropic receptors presumably located in the main body of the synaptic junction.

High frequency stimuli-evoked transmitter release would also activate the perisynaptic metabotropic receptors, leading to long-term changes in synaptic efficacy demonstrated to depend on metabotropic glutamate receptor activation both in the hippocampus (Behnisch and Reymann, 1993; Bashir et al., 1993) and in Purkinje cells in vitro (Linden et al., 1991).

Another explanation for the compartmentalization of the postsynaptic membrane may lie in the different and incompatible requirements for biochemical machinery necessary for metabotropic and ionotropic receptor operation and regulation. At glutamatergic synapses, ionotropic receptors mediating fast signals would be expected to be as close as possible to release sites, e.g., at the membrane of the area facing the presynaptic release sites. Ionotropic receptors have mechanisms for their regulation (e.g., phosphorylation and dephosphorylation) that require large amounts of proteins such as the major postsynaptic density protein, calcium/calmodulin-dependent protein kinase II (for review see Colbran, 1992; Raymond et al., 1993). In contrast, metabotropic receptors require the movement of G proteins in the membrane and the diffusion of second messengers into the cytoplasm (Hille, 1992), which may be obstructed by the high protein concentration in the postsynaptic density. Therefore, there may be a partitioning of available postsynaptic membrane, with the slower and longer duration metabotropic machinery placed at the periphery. However, an extrasynaptic as well as synaptic location has also been demonstrated for $GABA_A$ receptors subunits (Richards et al., 1987; Soltesz et al., 1990; Somogyi et al., 1989) and for the GluR1 subunit of the AMPA-type glutamate receptor (Molnar et al., 1993), using immunoperoxidase methods. It remains to be established using particulate markers whether the latter two ionotropic receptors are present at different densities at the synapses and extrasynaptic sites.

Distribution of mGluR1α in the Rat Brain

The comparison of our immunocytochemical data with in situ hybridization experiments should take into account that the antiserum we used recognized only the α form of mGluR1, whereas the probes used for in situ hybridization were directed to parts of the mRNA identical for the α, β, and c forms of mGluR1 (Masu et al., 1991; Shigemoto et al., 1992). Nevertheless, with some exceptions, the regional pattern of the immunoreactivity for mGluR1α correlates well with the distribution described for the mRNA coding for mGluR1 (Shigemoto et al., 1992). This suggests that either the α form is the dominant mGluR1 throughout the rat brain, or the isoforms are generally coexpressed in the same cells.

One discrepancy between in situ hybridization mapping of mGluR1 and immunocytochemistry for the α form of mGluR1 is in the hippocampus, where pyramidal cells of the CA3 area and granule cells of the dentate gyrus contain high levels of mRNA coding for mGluR1, but these cells were immunonegative in our study. These results suggest that the β and/or c forms are expressed in the principal cells of the hippocampus and that mGluR1α is present predominantly, if not exclusively, in nonprincipal cells. A previous immunocytochemical study using a similar antiserum to the α form of mGluR1 also described strong immunostaining of the nonprincipal cells (Martin et al., 1992) in the hippocampus and reported, but did not illustrate, a few immunoreactive pyramidal and granule cells. This apparent discrepancy with our data could result from the use of colchicin by Martin et al. (1992), which may increase the somatic receptor content. Another possibility is that immunoreactive cells observed in the stratum pyramidale of CA1 and CA3 areas and in the granule cell layer of the dentate gyrus were not in fact principal cells, but were nonprincipal cells, which we also observed in those layers. In any case, it can be concluded that some hippocampal nonprincipal cells are preferentially enriched in mGluR1α. This indicates that their activation by glutamate may have a unique mechanism and kinetics.

There may be differences in the transduction mechanisms of the α and β forms of the receptor, as the stimulation of phosphoinositide hydrolysis by mGluR1α had both pertussis toxin-sensitive and -insensitive components, whereas mGluR1β showed only a toxin-insensitive stimulatory effect (Pickering et al., 1993). In addition, the calcium transients, as measured by calcium imaging or voltage clamp, are strikingly different (Simoncini et al., 1993; Biophys. J., abstract; Pin et al., 1992). Exposure of cells expressing mGluR1α to constant 100 μM glutamate causes a single rapid intracellular calcium transient, lasting 50–100 s. This contrasts with cells expressing mGluR1β or 1c, in which the same stimulation results in a delayed response appearing on average 1 min postexposure and generating repeated calcium spikes with a constant period that varies from 1.5 to 4 min in individual cells. Since, apart from the extension of the α form at the C-terminal part, the two forms have identical amino acid sequence, this difference must be due to the C-terminal segment located at the intracellular face of the plasma membrane, as demonstrated here.

In the cerebellum, in addition to Purkinje and basket cells reported earlier (Gorcs et al., 1993; Martin et al., 1992), other GABAergic cell types, such as stellate and Lugaro cells, were also strongly immunoreactive for mGluR1α using our antibodies. These additional cell types were probably revealed by the higher sensitivity of our reactions. Immunopositive Golgi cells were reported (Martin et al., 1992) in the granule cell layer, and we also observed that some but not all of them were immunoreactive. In addition, we observed that a subpopulation of granule cell bodies were immunoreactive for mGluR1α as well as some glomeruli, reflecting receptors in the granule cell dendrites. In situ hybridization described a homogeneous autoradiographic signal over the granule cell layer, reflecting the expression of mGluR1 in virtually all the

granule cells (Pin et al., 1992; Shigemoto et al., 1992). The rarity of mGluR1α-immunoreactive granule cells suggests that mGluR1β and/or mGluR1c are more evenly expressed among the granule cell population than mGluR1α. It will be interesting to see whether the mGluR1α-immunoreactive glomeruli are supplied by a specific mossy fiber population.

Somatostatin-Immunoreactive Neurons Are Selectively Enriched in mGluR1α

In the hippocampus all the strongly mGluR1α-positive nonprincipal cells seem to contain somatostatin and vice versa. Most of these cells are thought to be local circuit neurons, and they also contain GABA (Kosaka et al., 1988; Somogyi et al., 1984). Some of them are enriched in other receptors; for example, a subpopulation of somatostatin cells has been shown to exhibit a high level of muscarinic acetylcholine receptors (van der Zee et al., 1991). Furthermore, some of these neurons also contain neuropeptide Y, but not all the neuropeptide Y cells contain somatostatin (Kohler et al., 1987). Antibodies directed to somatostatin reveal a relatively limited portion of the dendritic arborization of cells in the hippocampus and dentate gyrus even when colchicine is used (Bakst et al., 1985; Leranth et al., 1990; Sloviter and Nilaver, 1987). In the present study, we have demonstrated that mGluR1α immunoreactivity is a marker that specifically and strongly labels the entire surface of the somatostatin-positive cells, leading to three main conclusions.

–The codistribution of the recurrent axon collaterals of principal cells and dendrites from mGluR1α/somatostatin-immunoreactive neurons in the hippocampus suggests that glutamatergic transmission at the synapses between them is partially mediated through mGluR1α (see Figure 2G). A similar conclusion probably also applies to the neocortex, where most of the strongly mGluR1α-positive neurons were also immunoreactive for somatostatin.

This conclusion follows from the observations that most of the immunoreactive dendrites are distributed in areas and sublaminae where the recurrent axon collaterals arising from granule and pyramidal cells are located (Claiborne et al., 1986; Li et al., 1993; Tamamaki et al., 1987). The local collaterals exert their influence through the release of excitatory amino acids (Miles, 1990; Thomson and Radpour, 1991). Furthermore, the mGluR1α-positive cells have spiny dendrites, and the density of spines seems to reflect the location of recurrent synaptic input. In the CA1 area, pyramidal cells distribute axon collaterals mainly in the alveus and bordering stratum oriens and only occasionally to the other layers (Knowles and Schwartzkroin, 1981; Tamamaki et al., 1987); this is matched by the distribution of the mGluR1α-positive dendrites. In the CA3 area, pyramidal cells have a much more extensive local axon collateral system, which also richly innervates the stratum radiatum (Ishizuka et al., 1990; Li et al., 1993). This pattern of recurrent axon distribution is also reflected in the even distribution of mGluR1α/somatostatin-immunoreactive cells over the laminae of the CA3 area, with a sharp change at the CA1/CA3 boundary. In the dentate gyrus, granule cells have recurrent collaterals mainly in the hilus (Ramon y Cajal, 1893; Frotscher, 1991; Scharfman, 1992), which again is rich in mGluR1α/ somatostatin-immunoreactive cells and their dendrites. Our electron microscopic examination confirmed that boutons making type 1 synapses with dendrites immunoreactive for mGluR1α possess the anatomical features of the granule cell terminals, in agreement with a previous report that the dendrites of the hilar somatostatin cell are innervated by mossy fibers (Leranth et al., 1990).

–The glutamatergic activation of one of the recurrent inhibitory pathways is uniquely enriched in a receptor mechanism mediated by mGluR1α (see Figure 2G). The axons of the somatostatin/GABA-immunoreactive hilar cells (Kosaka et al., 1988; Somogyi et al., 1984), which we have shown here to have a high density of mGluR1α, terminate in the outer two-thirds of the dentate molecular layer (Leranth et al., 1990; Sloviter and Nilaver, 1987). Intracellular filling of hilar neurons revealed a cell type with spiny dendrites, whose axon was distributed widely in the other two-thirds of the dentate molecular layer and also penetrated the stratum lacunosum moleculare of the CA1 region, in precise conjunction with the glutamatergic perforant path originating in the entorhinal cortex (Han et al., 1993). The name HIPP cell (hilar cell having an axon associated with the perforant path) was suggested for these neurons to differentiate them from other hilar GABAergic cells (Han et al., 1993). Electron microscopic analysis of HIPP cell synapses showed that the majority of the synaptic targets are granule cell dendrites (Halasy and Somogyi, 1993). A similar conclusion was drawn for the somatostatin-immunopositive boutons (Leranth et al., 1990). Since the mGluR1α/somatostatin/GABA-containing cells have spiny dendrites largely restricted to the hilar area, it is suggested that they are the HIPP cells and provide recurrent inhibitory synapses influencing the gain of the perforant path input to the outer dendrites of granule cells. A similar mechanism may also operate in the CA1/CA3 areas and in the neocortex, where the axon patterns have not yet been clarified. It has been shown that in the CA3 area, tetanic activation of inhibitory interneurons through metabotropic glutamate receptors leads to prolonged enhancement of inhibition of principal cells (Miles and Poncer, 1993). Although the types of interneuron responsible for this action were not identified, and distinct types of interneurons probably express specific subtypes of metabotropic glutamate receptors, the mGluR1α-immunopositive cells may have contributed to the increased inhibition. The selective enrichment of mGluR1α at the synapses that activate a type of recurrent inhibitory cell is an example of the great biochemical differentiation of cortical circuits.

–The somatostatin-positive cells show different in-

tensities of mGluR1α immunoreactivity according to hippocampal areas. Hilar cells appeared to be less immunoreactive for mGluR1α than the cells in areas CA1–CA3, demonstrating a biochemical difference between the two populations. The hilar somatostatin cells are highly vulnerable to ischemia and epileptic activity, both insults probably mediated by excitotoxicity (Freund et al., 1992; Sloviter, 1987). At present it is difficult to interpret this vulnerability in terms of receptor subspecies, since contradictory effects of metabotropic glutamate receptor agonists have been reported (Lipartiti et al., 1993). In some studies, agonists have been described as neuroprotectors (e.g., Chiamulera et al., 1992; Silipandri et al., 1992), whereas in other reports they have been shown to potentiate N-methyl-D-aspartate-induced toxicity (Sacaan and Schoepp, 1992; McDonald and Schoepp 1992). Nevertheless, a differential distribution of receptors should be taken into account when interpreting selective neurotoxicity, and our results contribute to the characterization of receptors on this most vulnerable hilar cell population. Clearly, additional studies are needed to understand the precise role of mGluR1α in the normal activation as well as in the pathological degeneration of hilar somatostatin cells.

Experimental Procedures

Generation of Affinity-Purified Antibodies Directed to mGluR1α

The peptide EFVYEREGNTEEDEL was synthesized as a linear peptide derived from a cytoplasmic region (residues 1117–1130; Houamed et al., 1991; Masu et al., 1991) of mGluR1α. Purification of the peptides was carried out by reverse phase high performance liquid chromatography. The purity of the final product was assessed by mass spectrometry. Peptide was conjugated to keyhole limpet hemocyanin by cross-linking with glutaraldehyde. Rabbits were immunized with 100–200 μg of conjugated peptide injected subcutaneously at three different sites. Injections continued every 3 weeks, and sera were collected 10 days following the third injection. Antibodies (code number A4) were purified using the ProtOn Kit #1 (Multiple Peptide Systems) as recommended by the manufacturer.

Cell Cultures

Baby hamster kidney cells (BHK-ts 13) tranfected with the genes (Busby et al., 1991; Pickering et al., 1993) coding for the metabotropic receptor subtypes were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 2 mM L-glutamine, and 1 mM sodium pyruvate. Membranes were prepared from the cells by washing the monolayer cultures with phosphate-buffered saline and lysing the cells in 10 mM $NaHCO_3$ (2 ml/25 cm^2 flask). During lysis, the cells were triturated with a Pasteur pipette to ensure their removal from the flask before being transferred to microfuge tubes on ice. The cells were sheared using at least ten passes of the lysate through a 23 gauge needle attached to a hypodermic syringe. The crude membranes were recovered by pelleting at 3000 rpm in an Eppendorf microfuge for 15 min at 4°C and either used immediately or stored at −70°C.

Immunoblotting of Cell and Brain Membranes

The specificity of antiserum A4 was assessed by imunoblotting of membranes derived from cells transfected with the different metabotropic glutamate receptors and rat brain membranes prepared from cortex, spinal cord, cerebellum, or hippocampus. Cell membranes were prepared as described above, and rat brain membranes were prepared as described by Molnar et al. (1993). Membranes from transfected cells were subjected to SDS-polyacrylamide gel electrophoresis on 4%–15% polyacrylamide gradient gels, and immunoblotting was performed using crude antiserum. Rat brain membranes were analyzed on 9% SDS-polyacrylamide gels, and immunoblotting was carried out using affinity-purified antibody as described by Molnar et al. (1993).

Immunocytochemistry

Preparation of Animals and Tissue

Ten adult female or male rats (100–400 g) were deeply anesthetized with sodium pentobarbital (150 mg/kg, i.p.). They were perfused through the aorta with NaCl solution (0.9%, 1 min) followed by ice-cold fixative containing 4% paraformaldehyde, 0.025%–0.1% glutaraldehyde, and approximately 0.2% picric acid (Somogyi and Takagi, 1982) made up in 0.1 M phosphate buffer (PB, pH 7.2), for 10–30 min.

Preembedding Immunocytochemistry

Immunocytochemical treatments were as described earlier (Molnar et al., 1993). Primary antiserum A4 to mGluR1α was used at a final dilution of 1:1000, corresponding to 1 μg per ml of protein. In control incubations the primary antiserum was replaced by normal rabbit serum at the same dilution, or by purified antiserum A4 at the final dilution after preincubation with the corresponding peptide (75 μg/ml) for 4 hr. Immunoperoxidase reaction was carried out using the avidin biotinylated horseradish peroxidase complex method (ABC, Vector). Preembedding immunogold reaction was performed using anti-rabbit IgG coupled to 1.4 nm gold particles at 1:100 (Nanogold, Nanoprobes Inc.). Gold particles were silver enhanced for 10–20 min using the HQ Silver kit as described by the manufacturer (Nanoprobes Inc.). Sections were then routinely processed for either light or electron microscopic examination.

For the double labeling experiments, sections were incubated for 2 hr in a mixture containing mouse monoclonal antibody to somatostatin (final dilution, 1:2000) and rabbit antiserum to mGluR1α (final dilution, 1:1000). The generation and characterization of the monoclonal antibody to somatostatin (SOM8) were described earlier by Vincent et al. (1985). Sections were then incubated for 1 hr in a mixture of biotinylated goat anti-rabbit IgG (1:50; Vector) and goat anti-mouse IgG (1:50; Sigma), followed by washing and addition of ABC solution. Peroxidase was revealed using 3, 3'-diaminobenzidine tetrahydrochloride (DAB, 0.015%) dissolved in 50 mM Tris-HCl buffer (pH 8) containing 0.6% ammonium nickel sulphate and 0.005% H_2O_2. The enzymatic reaction resulted in a blue-gray reaction end product. After extensive washing, sections were incubated in mouse peroxidase anti-peroxidase complex (1:100, Dako) overnight. Peroxidase was revealed using DAB (0.05%) in 50 mM Tris-HCl buffer (pH 7.6) and 0.01% H_2O_2, resulting in a brown reaction end product.

Freeze Substitution Lowicryl Embedding

After perfusion, blocks of cerebellum were extensively washed in PB. Vibratome sections (300 μm) were cut and placed in 30% glycerol in PB for 15 min. Sections were cryofixed on a cooled copper block with a Leica MM80E cryofixation apparatus. Small blocks were trimmed from the cryofixed tissue and transferred to a Leica CS Auto at −80°C, where freeze substitution proceeded as follows: methanol at −80°C for 36 hr; the temperature was increased at 10°C/hr to −50°C (all the following steps were conducted at this temperature); methanol, Lowicryl HM20 (Chemische Werke Lowi GMBH & Co) 1:1 for 90 min, 1:2 for 90 min; neat Lowicryl for 90 min; fresh Lowicryl overnight; after embedding, polymerization in fresh Lowicryl under UV light for 48 hr. Ultrathin sections were cut and picked up on nickel grids coated with pioloform (Agar Scientific Ltd.).

Ultracryotomy

Animals were deeply anesthetized and perfused as described before, except perfusion with 10% sucrose dissolved in PB followed the perfusion with fixative. Vibratome sections (300 μm thick) were cut and washed in 10% sucrose in PB and infiltrated with 25% polyvinylpyrrolidone (molecular weight 10,000; Sigma) sucrose overnight at 4°C (Tokuyasu, 1989). After freezing

in liquid nitrogen, ultrathin sections were cut with a diamond knife on a Reichert Ultracut E fitted with a Leica FC4E cryoattachment. Sections were collected on 2.3 M sucrose in PB and transferred to 100 mesh pioloform-coated nickel grids that had been carbon coated and floated on 1% human serum in phosphate buffered saline (PBS).

Immunocytochemistry on Ultrathin Sections

A similar immunocytochemical sequence was used to detect mGluR1α in ultrathin Lowicryl-embedded sections and ultrathin frozen sections. Grids were incubated for 30 min in blocking serum consisting of 5% fetal calf serum (Sigma) in PBS for frozen sections and with an additional 0.8% bovine serum albumin (Sigma) and 0.1% cold water fish skin gelatin (Sigma) for Lowicryl sections. Sections were then transferred to primary antiserum A4 1:10 to 1:20 in blocking serum overnight at room temperature. After washing in the respective blocking sera, sections were incubated for 90 min in goat anti-rabbit IgG coupled to 1.4 nm gold particles in blocking sera, with an additional 0.1% cold water fish skin gelatin for the frozen section. Sections were washed in PB for 30 min, then put on 2% glutaraldehyde in PB for 5 min, washed in PB, and washed several times in ultrapure water (18 MΩ:UHQ.PS, Elga) prior to silver enhancement in the dark with HQ Silver kit. Silver enhancement times were 4 min for ultrathin Lowicryl-embedded sections and 8–12 min for ultrathin cryosections, followed by ultrapure water washing. Ultrathin Lowicryl-embedded sections were then contrasted in saturated aqueous uranyl acetate and lead citrate before air drying. Ultrathin cryosections were treated with 2% OsO_4 in PB for 30 min, washed in distilled water, and then floated on saturated aqueous uranyl acetate for 30 min. The grids were transferred directly to drops of 3% polyvinyl alcohol (molecular weight 10,000; Sigma) for 5 min, picked up with a wire loop, and drained before air drying (Tokuyasu, 1989).

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