Target-cell-specific concentration of a metabotropic glutamate receptor in the presynaptic active zone

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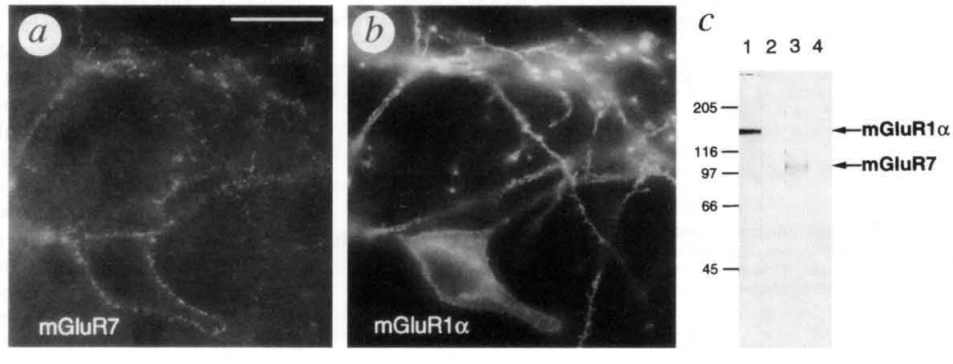
The probability of synaptic neurotransmitter release from nerve terminals is regulated by presynaptic receptors responding to transmitters released from the same nerve terminal or from terminals of other neurons. The release of glutamate, the major excitatory neurotransmitter, is suppressed by presynaptic autoreceptors[1–3]. Here we show that a metabotropic glutamate receptor (mGluR7) in the rat hippocampus is restricted to the presynaptic grid, the site of synaptic vesicle fusion. Pyramidal cell terminals presynaptic to mGluR1α-expressing interneurons have at least a ten-fold higher level of presynaptic mGluR7 than terminals making synapses with pyramidal cells and other types of interneuron. Distinct levels of mGluR7 are found at different synapses made by individual pyramidal axons or even single boutons. These results raise the possibility that presynaptic neurons could regulate the probability of transmitter release at individual synapses according to the postsynaptic target.

The release of glutamate is suppressed by L-2-amino-4-phosphonobutyrate (AP4)-sensitive, presynaptic metabotropic glutamate receptors[2,4–6], which have been postulated to serve as autoreceptors[1,3]. We used an antibody specific to mGluR7 (Fig. 1*c*), the only known AP4-sensitive mGluR expressed abundantly in the hippocampus[7–9], to establish its location by means of a high-resolution immunodetection method[10]. Light microscopy revealed extensive distribution of mGluR7 immunoreactivity throughout the hippocampal dendritic fields[11], some neuronal profiles having strongly immunoreactive puncta, prominent over the weaker neuropile labelling (Fig. 1*a*). Double immunolabelling with an antibody specific to mGluR1α (Fig. 1*c*) showed that these profiles corresponded to an interneuron population[10] selectively expressing mGluR1α in stratum oriens of the CA1 area (Fig. 1*b*), the CA3 area and the hilus (not shown). Electron-microscopic analysis demonstrated that immunoparticle labelling for mGluR7 was highly restricted to the presynaptic membrane specialization of axon terminals making type I (asymmetrical) synapses (Fig. 2*a*), which are known to contain glutamate. All terminals that make type I synapses with dendrites that express mGluR1α had a much higher density of the mGluR7 labelling (Fig. 2*a*, *b*) than terminals making synapses with mGluR1α-immunonegative dendritic shafts (Fig. 2*b*) or pyramidal-cell spines (not shown). Measurement of the immunoparticle density at individual synapses (Fig. 2*c*) indicated a highly significant difference between synapses on mGluR1α-positive ($76 \pm 16\%$, mean ± s.d., $n = 16$) and negative ($7.1 \pm 7.2\%$, $n = 44$) neuronal elements (Mann–Whitney test, $Z = -5.9$, $P < 0.0001$). It is unlikely that the differential immunolabelling is caused by a selective lack of access of antibodies to the presynaptic grid in synapses on spines and some dendritic shafts because the differential pattern was observed in the surface of the tissue and in detergent-treated material for fluorescence microscopy. The expression of a selectively high level of mGluR7 does not depend on the presence of mGluR1α *per se*, as the pattern was the same in genetically altered mice lacking mGluR1α expression (R. Shigemoto, unpublished observation).

The presence of nerve-terminal populations with two distinct levels of presynaptic mGluR7 raises the question of whether the terminals originate from two different populations of presynaptic neuron, or whether both types of terminal occur along a single axon with the difference depending on the identity of the postsynaptic neuron. This was investigated by identifying individual axons of pyramidal cells using *Phaseolus vulgaris* leucoagglutinin (PHAL) injected in the CA3 (Fig. 3) and CA1 areas and double labelling the material for mGluR7 with immunoparticles. Single, PHAL-labelled associational axons of CA3 pyramidal cells occasionally contacted mGluR7-decorated dendritic shafts (Fig. 3*a*), which originate from mGluR1α-positive interneurons (Figs 1 and 2). Electron-microscopic analysis showed that in 9 of 18 tested cases these PHAL-labelled boutons formed synapses with the dendrites, and the presynaptic grid was densely labelled with immunoparticles for mGluR7 (Fig. 3*c*). In 7 cases in the CA3

FIG. 1 Correlated distribution of strong immunoreactivity for metabotropic glutamate receptors mGluR7 and mGluR1α in rat hippocampus. Punctate immunofluorescence for mGluR7 (*a*) decorates mGluR1α-immunopositive soma and dendrites (*b*) of interneurons in stratum oriens/alveus of the CA1 area. Double immunolabelling for mGluR7 and mGluR1α was photographed with different filters for Texas Red (mGluR7) or fluorescein (mGluR1α). Scale bar, 20 μm. *c*, Immunoblots of a membrane fraction from the whole brain with antibodies to mGluR1α (lane 1) and mGluR7 (lane 3), showing major immunoreactive bands of relative molecular mass 145,000 (M_r 145K) and 102,000 (M102K), respectively. For the immunoblots in lane 2 and 4, antibodies were preabsorbed with the corresponding fusion proteins before immunoreaction. M_r markers are indicated on the left.
METHODS. Guinea-pig antibodies to mGluR1α and rabbit antibodies to mGluR7 were raised using *trpE*–mGluR fusion proteins[30]. cDNA fragments encoding C-terminal amino-acid residues of rat mGluR1α[1] (859–1,199) and mGluR7 (ref. 8) (874–915) were inserted into pATH3 vectors and the *trpE*–mGluR proteins were overexpressed, purified and used for immunization. After removing antibodies to the *trpE* protein, antibodies to mGluR sequences were purified with antigen columns. For double-immunofluorescence histochemistry, hippocampal sections 20 μm thick from rat, fixed by perfusion with 4% paraformaldehyde[13], were reacted with antibodies to mGluRs (0.5–1.0 μg ml^{-1}), then with fluorescein isothiocyanate-conjugated anti-guinea-pig-IgG antibody and biotinylated anti-rabbit-IgG antibody combined with Texas-Red-conjugated avidin. When either of the primary antibodies was omitted, no fluorescence signal of the omitted primary antibody was observed (not shown). For immunoblots, a crude membrane fraction prepared from the rat brain was separated on 7% SDS–PAGE and transferred to polyvinylidenofluoride membrane[13]. The membranes were reacted with antibodies to mGluRs (1.0 μg ml^{-1}), and the bands were visualized with alkaline phosphatase-labelled secondary antibodies.



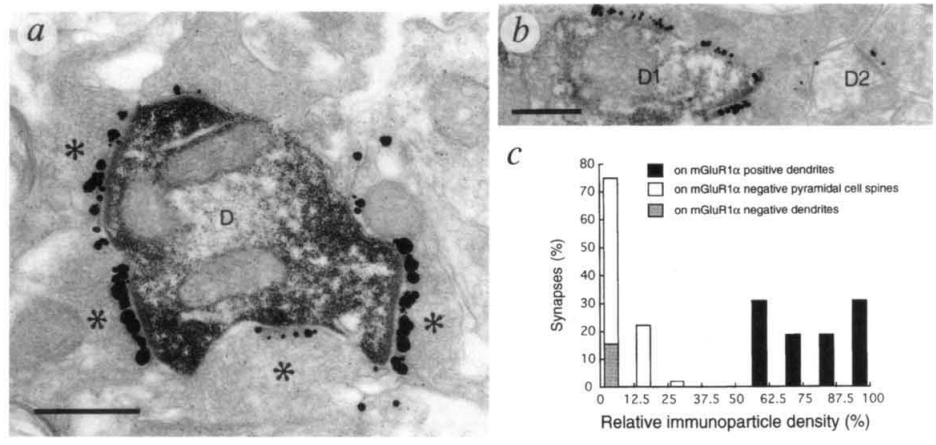


FIG. 2 Expression of mGluR7 in the presynaptic grid of terminals on mGluR1α immunopositive neurons. *a*, *b*, Electron micrographs of terminals labelled for mGluR7 (particles) and dendrites (D, D1) labelled for mGluR1α (peroxidase product) in the CA3 area. The transmitter release site of terminals (asterisks) making synaptic junctions with mGluR1α-immunoreactive dendrites (D, D1) are heavily labelled for mGluR7 with silver-enhanced immunogold particles. Terminals forming synapses on a mGluR1α-negative dendrite (D2) or spines (not shown) are labelled only weakly, if at all. Scale bars, 0.4 μm. *c*, Measurement of presynaptic mGluR7 immunoreactivity in the CA3 area showed that asymmetrical synapses on mGluR1α-positive dendrites (black column) have much higher density of receptor ($P < 0.0001$) than those on dendrites of other interneurons (grey column) and on pyramidal cell spines (white column).

METHODS. Wistar rats were anaesthetized (sodium pentobarbital, 150 mg per kg, intraperitoneal) and perfused with a fixative containing 4% paraformaldehyde, 0.05% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer. Vibratome sections (50 μm thick) were incubated with antibodies (0.5–1.0 μg ml^{-1}) to mGluR1α (guinea-pig) and mGluR7 (rabbit), then with biotinylated anti-guinea-pig-IgG antibody and 1.4 nm gold-coupled anti-rabbit-IgG antibody. After silver enhancement, sections were reacted with the avidin-biotinylated peroxidase complex (Vector), and mGluR1α immunoreactivity was visualized with diaminobenzidine tetrahydrochloride. When either of the primary antibodies was omitted, no signal for the omitted antibody was observed (not shown). Measurements of immunoparticle density were taken from electron micrographs having mGluR1α-positive dendrites in the centre and covering an area of 26 μm^2. Every synaptic profile that had a well-defined synaptic cleft and thick postsynaptic density was measured. Particle density was determined at presynaptic grids by dividing the total area of particles (measured with OptiLab image-processing software) by the length of the synaptic specialization. Density values from two consecutive sections were averaged and normalized by taking the highest value within each sample as 100%. The numbers of synapses are normalized within categories according to the presence or absence of postsynaptic mGluR1α immunoreactivity.

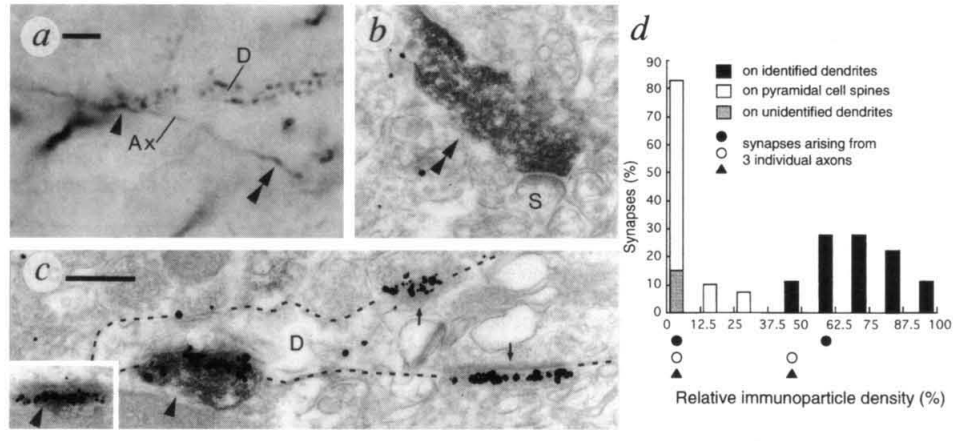


FIG. 3 Individual presynaptic terminals along the same pyramidal cell axon have different levels of presynaptic mGluR7, depending on the identity of the postsynaptic neuron. Corresponding light (*a*) and electron (*b*, *c*) micrographs showing a pyramidal cell axon collateral (Ax) and mGluR7 immunogold labelling along dendrites (one is labelled D) in the CA3 area. The axon is likely to originate form a pyramidal cell, as identified by anterograde labelling with PHAL (visualized with immunoperoxidase) injected in the ipsilateral CA3 stratum radiatum. One PHAL-labelled bouton (single arrowhead) makes a synapse that is heavily labelled by immunoparticles for mGluR7 (*c*; and inset from a tilted consecutive section), whereas the other PHAL-labelled bouton (double arrowheads) makes an immunonegative synapse (*b*) with a dendritic spine (S) of a pyramidal cell. Other synapses (arrows in *c*) on the same dendrite (D, broken lines) are also heavily labelled for mGluR7. Scale bars: *a*, 3 μm; *b*, *c*, same magnification, 0.3 μm. *d*, Distinct levels of presynaptic mGluR7 immunoreactivity in individual boutons along 3 identified pyramidal cell axons (symbols) according to the identity of postsynaptic targets. The level of mGluR7 in PHAL-labelled terminals was compared to that of other asymmetrical synapses around the identified boutons in the CA3 area. Asymmetrical synapses on dendrites (black columns), which received mostly heavily labelled terminals, have a much higher density of receptor ($P < 0.0001$) than those on dendrites of unidentified interneurons (grey column) and spines of pyramidal cells (white columns). Different boutons of the same axon contribute to both populations of synapses.

METHODS. Double labelling for mGluR7 (immunogold) and PHAL (immunoperoxidase) was performed as described in Fig. 2, but biotinylated goat anti-PHAL antibody (Vector) was used instead of the mGluR1α and secondary antibodies. Relative immunoparticle densities were measured from three blocks of two animals. For quantification see Fig. 2.

area the same axons could be followed in serial sections to a consecutive synaptic bouton (Fig. 3, double arrowheads), making a synapse with a pyramidal-cell dendritic spine. The presynaptic grid of the latter boutons was only weakly labelled, or not labelled at all, for mGluR7 (Fig. 3*b*). Measurements of three pairs of synapses (Fig. 3*d*), together with all other synapses found around the PHAL-labelled boutons, demonstrate that a single axon contributes individual boutons to two populations of synapses (Mann–Whitney test, $Z = -6.1$, $P < 0.0001$), having either high ($69 \pm 17\%$, mean ± s.d., $n = 18$) or low ($7.3 \pm 10.0\%$, $n = 41$) levels of presynaptic mGluR7. Similar results were obtained in the CA1 area, where three different axons of CA1 pyramidal cells (2–4 boutons measured from each), identified by PHAL labelling, were found to contribute to populations of synapses with either high ($54 \pm 21\%$, $n = 30$) or low ($7.1 \pm 8.5\%$, $n = 59$) mGluR7 density (Mann–Whitney test, $Z = -7.5$, $P < 0.0001$). Individual synapses with high or low mGluR7 density were found even in single boutons (Fig. 4*a*) when the postsynaptic targets were a dendrite of a presumed mGluR1α-expressing interneuron and a pyramidal-cell dendritic spine. These results demonstrate that the distinct levels of presynaptic mGluR7 density depend on the identity of the postsynaptic target.

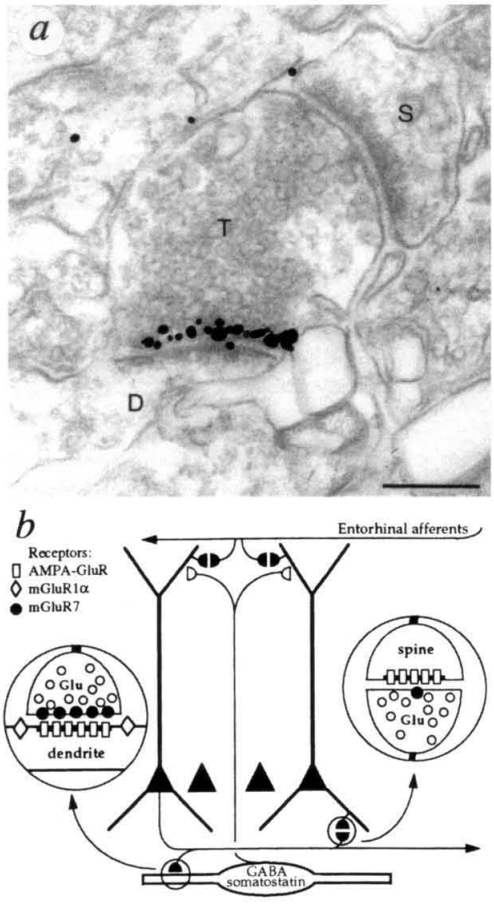


FIG. 4 Postsynaptic target determines presynaptic receptor density. *a*, The presynaptic receptor mGluR7 is differentially expressed at two synapses of a single nerve terminal (T), as demonstrated by immunogold labelling in the CA3 area. The heavily receptor immunolabelled presynaptic grid (particles) faces a dendritic shaft (D) characteristic of mGluR1α-expressing interneurons, whereas the unlabelled synapse is on a pyramidal cell dendritic spine (S). Scale bar, 0.2 μm. *b*, Summary of the molecular architecture of pyramidal-cell synapses demonstrating the position of some of the glutamate receptors in relation to the mGluR1α/somatostatin/GABA-containing interneuron. Ionotropic α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-type receptors are enriched in the postsynaptic membrane specialization on both the dendritic spines of pyramidal cells and on the dendritic shafts of interneurons[31]. Postsynaptic mGluR1α is concentrated in a perisynaptic annulus outside the synaptic junction[10]. The somatostatin/GABA-containing interneurons, which receive mainly recurrent axon collateral input[10,24,25], terminate in conjunction with the entorhinal cortical input on distal dendrites[27]. There is a high density of the presynaptic autoreceptor mGluR7 in the presynaptic grid of pyramidal terminals which target mGluR1α-positive cells, but only a low density in those terminals which innervate pyramidal-cell spines. This selective distribution may result in a low-pass frequency filter for glutamate release (see text).

The results provide evidence of a presynaptic receptor restricted to the site of transmitter release. Taken together with the low affinity of mGluR7 to glutamate (EC_{50} of 1 mM)[8] compared with those of other mGluRs ($EC_{50} \leqslant 56\ \mu M$)[3], it is possible that mGluR7 functions as an autoreceptor activated only by glutamate released at the site where the receptor is located. Such a synapse-specific autoregulation of transmitter release would be in contrast to the postulated heterosynaptic regulation mediated by mGluR2 (refs 12,13), which has much higher affinity to glutamate (EC_{50} of 12 μM)[3] than does mGluR7, and is located distant from the transmitter release site on presynaptic boutons and axons in the hippocampus[11]. Voltage-sensitive calcium channels that trigger synaptic vesicle fusion[14] are also thought to be concentrated at the presynaptic active zones[15], and are inhibited in a membrane-delimited manner by neurotransmitters through G-protein-coupled receptors[14,16,17]. The mechanism of signal transduction for mGluR7 in the hippocampus is not known, but AP4-sensitive receptors pharmacologically similar to mGluR7 decrease the probability of glutamate release in the CA1 area[5,6,18]. The apparently complete segregation of mGluR7 between two synapses within single boutons (Fig. 4*a*) suggests that coupling of the receptor with its effector is likely to be spatially restricted, and probably membrane delimited.

The clustering of postsynaptic receptors, regulated by trophic factors derived from the presynaptic nerve ending, has been extensively studied for nicotinic acetylcholine receptors at the neuromuscular junction[19]. In retinal bipolar cells, targeting of mGluR6 to postsynaptic sites is also dependent on the presynaptic neuronal element[20]. The clustering of mGluR7 demonstrates a correlation between levels of presynaptic receptor expression and postsynaptic element identity. The phenomenon may underlie target-dependent variation in probability of transmitter release[21,22], and raises the possiblity that postsynaptic neurons influence presynaptic receptor density in a retrograde manner.

That the input[23,24] to mGluR1α-expressing GABAergic[10,25] cells is endowed by autoregulation stronger than that to pyramidal cells and other interneurons[26] might be due to their place in the hippocampal network (Fig. 4*b*). They make synapses in conjunction with the entorhinal input to pyramidal cells[27]. The high level of presynaptic mGluR7 in the input terminals may suppress the release of glutamate when action potentials arrive at high frequency, allowing glutamate release to follow only relatively low-frequency presynaptic firing, that is, it could act as a low-pass filter. The activity of hippocampal principal cells and their entorhinal input shows gamma-frequency (30–60 Hz) oscillations modulated at theta (4–12 Hz) frequency[28,29]. The time course of the recovery of glutamate release could be tuned to one of these frequencies, allowing the activation of the cells and recurrent GABA release to distal dendrites of pyramidal cells preferentially at one of the above frequencies. Thus the specifically high level of mGluR7 expression may provide pyramidal cells with a means to assist GABA-mediated timing of entorhinal input. □

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