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SUBSYNAPTIC SEGREGATION OF METABOTROPIC AND IONOTROPIC GLUTAMATE RECEPTORS AS REVEALED BY IMMUNOGOLD LOCALIZATION

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Glutamate is a major neurotransmitter in the brain that acts both through fast ionotropic receptors and through slower metabotropic receptors coupled to G proteins. Both receptors are present throughout the somatodendritic domain of neurons as shown by immunohistochemical[5,6,19,20,24] and patch clamp recording studies.[8,9,16,28,37] Immunogold labelling revealed a concentration of metabotropic receptors at the edge, but not within the main body of anatomically defined synapses,[6] raising the possibility that ionotropic and metabotropic receptors are segregated. We applied double immunogold labelling to study glutamatergic parallel and climbing fibre synapses in the cerebellar cortex. The ionotropic AMPA type receptors occupy the membrane opposite the release site in the main body of the synaptic junction, whereas the metabotropic receptors are located at the periphery of the same synapses. Furthermore, immunoreactivity for AMPA receptors is at least twice as high in the parallel fibre synapses as in glutamatergic mossy fibre synapses. We suggest that the spatial segregation of ionotropic and metabotropic glutamate receptors permits the differential activation of these receptors according to the amount of glutamate released presynaptically, whereas the different densities of the ionotropic receptor at distinct synapses could allow the same amount of glutamate to evoke fast responses of different magnitude.

Glutamatergic synapses throughout the central nervous system display a wide range of pharmacologically and kinetically distinct responses[14,28,29,31,37] which are a consequence of glutamate receptor (GluR) subtypes, their regulation and possibly also their location relative to glutamate release sites. By means of immunofluorescence or immunoperoxidase methods the cellular and subcellular[5,6,12,19,20,24,26] distribution of GluRs has been described at synaptic and extrasynaptic sites.[5,6,19,24] However, these methods do not have the resolution to determine the localisation at the subsynaptic level or reveal quantitative differences in receptor densities. A non-diffusable particulate marker, immunogold, revealed an apparent exclusion of the metabotropic receptor mGluR1α from the main body of the postsynaptic specialization and its enrichment at the periphery of synapses.[6] Thus GluRs are either (i) generally located at the periphery of synaptic junctions, or (ii) receptor subtypes are segregated in the postsynaptic membrane specialization. To decide between these two possibilities, double labelling immunogold procedures were developed for the α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) type ionotropic receptor and for a metabotropic receptor at the same synapses in the cerebellar cortex. The cerebellar cortex was chosen because it contains three well characterized excitatory synapses receiving glutamate from mossy fibres terminating on granule cells and the climbing and parallel fibres converging onto Purkinje cells.[25,33] Thus comparisons can be made both between GluR-bearing postsynaptic cells and distinct glutamatergic synapses on the same cell type.

Large bulbous varicosities containing several mitochondrial profiles and making multiple asymmetric synapses with spines were considered to originate from climbing fibres (Fig. 1A,B). The rest of the presynaptic boutons making single asymmetric axospinous synapses were considered to originate from parallel fibres. In single immunoreaction for mGluR1α an enrichment of immunoreactive receptors was found in a perisynaptic position at both parallel and climbing fibre synapses established on Purkinje cells (Fig. 1A). In contrast, immunoparticles for the ionotropic AMPA receptors were found

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; GluR, glutamate receptor; GluR-B/C/4c, B, C and 4c subunits of the glutamate receptor; LTD, long-term depression; mGluR1α, 1α form of the metabotropic glutamate receptor; PB, phosphate buffer; PBS, phosphate-buffered saline.

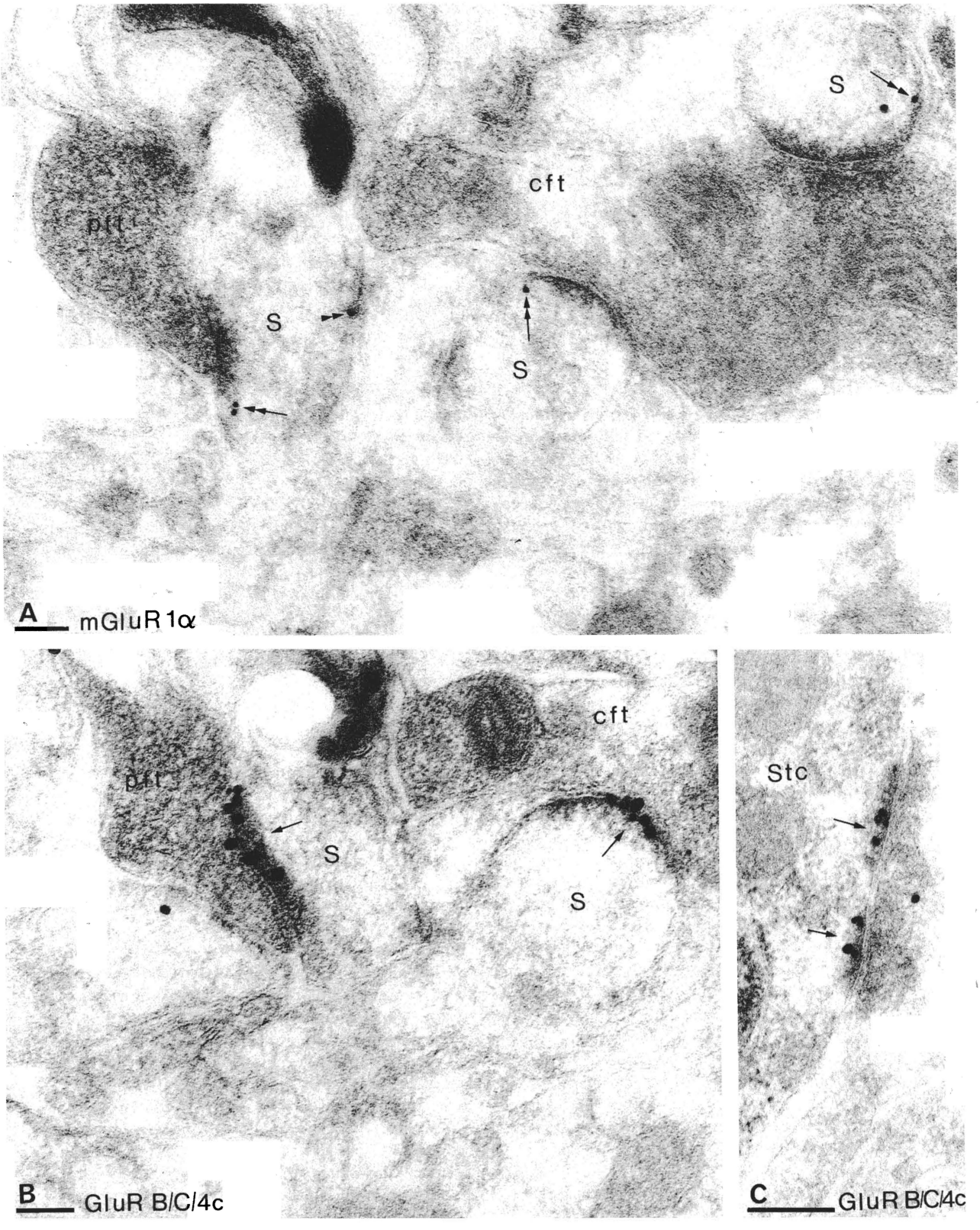

Fig. 1.

across the whole asymmetrical postsynaptic specialization using either poly- or monoclonal antibodies that recognize the GluR-B, C and 4c subunits of the receptor (Figs 1B,C; 2A–C). In the molecular layer, postsynaptic elements were identified as being spines of Purkinje cells (Figs 1A,B; 2A,B), dendrites and cell bodies of GABAergic interneurons (Fig. 1C). Immunolabelling was also found for both ionotropic and metabotropic receptors at non-synaptic sites along the surface of Purkinje cells as described earlier.[5,6,19] Immunoreactivity for the mGluR1α or the GluR-B/C/4c subunits could not be found on the surface of Bergmann glia in agreement with previous studies.[6,19,20,26] In the granule cell layer, enrichment of immunoparticles was found in the synapses between mossy fibre terminals and granule cell dendrites (Fig. 2C). This predominantly synaptic localization is in agreement with the results of electrophysiological experiments showing fast, AMPA receptor-mediated components in the excitatory postsynpatic currents of the Purkinje[18] and granule cells.[35]

As the two receptor types appeared to be at different parts of the synaptic disc when localized separately, we tested whether the same individual synapses contained both receptors. Reactions either on consecutive sections of the same synapse with antibodies to GluR-B/C/4c or mGluR1α (Fig. 1A,B), or by visualising the two types of receptors on the same section with different sizes of particles (Fig. 2A,B) confirmed the subsynaptic segregation of the metabotropic and ionotropic GluRs. Indeed, quantification of immunoparticles revealed that more than 90% of the labelling for mGluR was located no further from the edge of the postsynaptic specialization than 1/6th of its length, whereas particles for the ionotropic GluRs were evenly distributed along the postsynaptic specialization (Fig. 3).

The fast rise time of synaptic currents mediated by ionotropic GluRs[8,16,18,32,35,37] suggests that the activated receptors are close to the glutamate release site. The high concentration of ionotropic receptor immunolabelling in the main body of the postjunctional membrane, and the abrupt decrease in labelling outside the junction provides a structural basis for the fast activation of receptors.[7,10] Metabotropic receptor responses have a slower rise time, therefore the additional time required for the diffusion of glutamate to the periphery of a synaptic junction, where the receptor is located, is negligible compared to the onset of the mGluR-mediated response, most of which is taken up by the transduction mechanism mediated by G-proteins. A more likely explanation

Fig. 1. Electron microscopic demonstration of segregated subsynaptic localization of immunoreactive metabotropic (mGluR1α) and iontropic (B/C/4c) GluRs. Immunoparticles for mGluR1α are concentrated at the edge (double arrows in A), whereas immunoparticles for GluR-B/C/4c are concentrated in the main body (arrows in B) of asymmetric synaptic junctions established by parallel (pft) and climbing (cft) fibre terminals with spines (s) of Purkinje cells. The two receptors are shown in consecutive sections of the same synaptic junctions. Extrasynaptic mGluR1α receptors were often observed (double arrowhead in A). C, Immunoreactivity for the GluR-B/C/4c subunits (arrows in C) was always very strong on the GABAergic interneurons in the molecular layer, such as a stellate cell soma (Stc). Scales: A, B, 0.1 μm; C, 0.2 μm. Adult female Wistar rats (150 g) were deeply anaesthetized with sodium pentobarbital (150 mg/kg, i.p.), and intracardially perfused with fixative.[24,34] Cerebellar sections were placed into 1 M sucrose solution in 0.1 M phosphate buffer (PB) for 2 h before slamming, freeze substitution and embedding in Lowicryl HM 20.[6] Ultrathin sections were incubated in blocking solution (0.1 M phosphate-buffered saline (PBS) containing 0.8% ovalbumin, 0.1% cold water fish skin gelatine (Sigma, Pool, U.K.) and 5% fetal calf serum) for 30 min. Antibodies were also made up in this solution. The monoclonal antibody, mAb 1F1, used in the double labelling experiments was raised to a synthetic peptide (Kem-En-Tec, Copenhagen, Denmark) containing the 13 C-terminal amino acids of rat GluR-B with an added cysteine residue (EGYNVYGIESVKIC). The 13 amino acids are conserved in GluR-B/C/4c, GluR-C and 4c differ from GluR-B by a single and the same residue. The peptide was coupled to albumin by glutaraldehyde and monoclonal antibodies were developed (H.-P. Ottiger and P. Streit, unpublished observations). In immunoblot analysis of membrane proteins from rat brain, mAb 1F1 labelled a single band migrating with a $W_r = 105{,}000$, the band being broader for cerebral cortex than for cerebellum, with no trace of it in liver. The same patterns were obtained in immunostaining of rat brain sections as those described for polyclonal rabbit antibodies recognizing GluR-B/C/4c.[19,26] For single receptor labelling on serial sections, affinity purified rabbit polyclonal antibodies[26] (Chemicon Int. Inc., London, U.K.) to the same peptide sequence in the GluR-B/C/4c subunits were used. Metabotropic GluRs were visualized by rabbit polyclonal antibodies to mGluR1α.[6] Immunoreactivity could not be detected when the primary antibodies were either omitted or replaced by 5% normal rabbit serum, or tissue culture medium for the monoclonal antibody. Two methods were used: (1) Double immunoreaction experiments on the same ultrathin section using a mixture of rabbit anti-mGluR1α and monoclonal anti-GluR-B/C/4c antibodies; (2) Alternating serial sections incubated for mGluR1α and GluR-B/C/4c on separate grids using rabbit antibodies. The sections were incubated in primary antibodies overnight followed by washing and incubation either in goat anti-mouse or goat anti-rabbit IgG coupled to 1.4 nm gold (Nanogold, Nanoprobes Inc. Stony Brook, U.S.A.), or in a mixture of goat anti-mouse IgG coupled to 1.4 nm gold for GluR-B/C-4c and goat anti-rabbit IgG coupled to 5 nm gold (BioClinical Services Ltd. Cardiff, U.K.) for mGluR1α. In some double labelling experiments Triton X-100 (0.05%) was present in all antibody solutions resulting in a higher specific as well as higher background labelling (e.g. Fig. 2B). After the reaction, particle size was increased by silver intensification, followed by uranyl and lead staining as described earlier.[6]

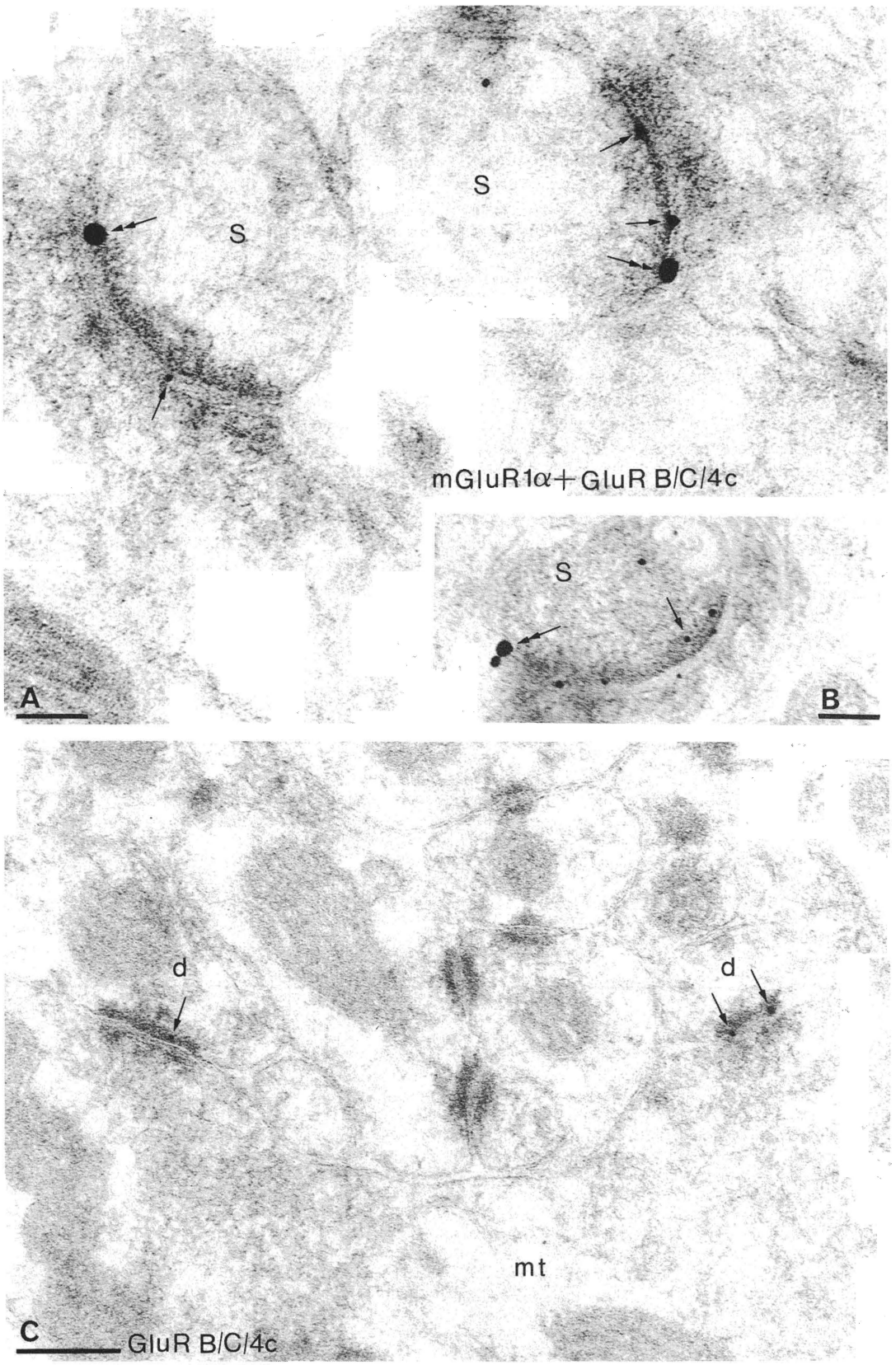

Fig. 2.

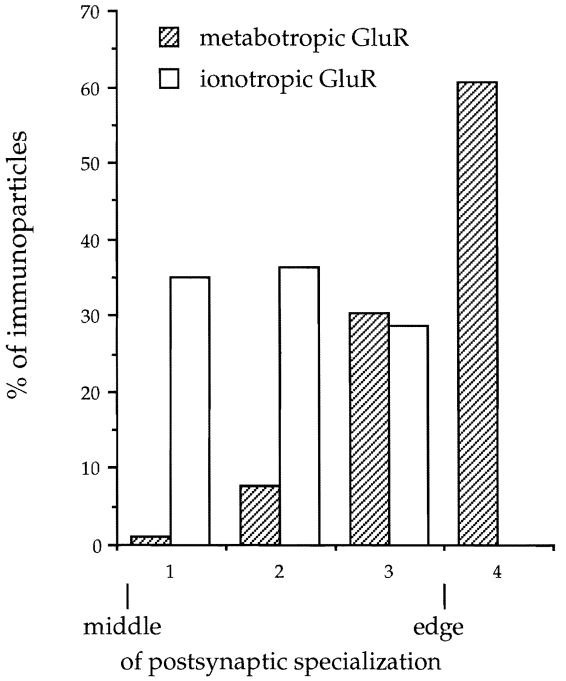

Fig. 3. Spatial segregation of the AMPA type ionotropic and metabotropic (mGluR1α) GluRs in the synaptic junction of parallel fibre terminals. The majority of immunoparticles (61% of 92 particles at 47 synapses) for mGluR1α were outside the postsynaptic specialization and only 9% were located inside of the edge, further than 1/6th of the length of the specialization. In contrast, immunoparticles for the ionotropic GluRs were evenly distributed on the specialization (77 particles, 27 synapses). Each bin represents 1/6th of the length of the postsynaptic specialization.

for the segregation of the two receptor types lies in the different biochemical mechanisms necessary for their operation and regulation. The postsynaptic density contains a high concentration of calcium/calmodulin-dependent protein kinase II[36] known to regulate ionotropic GluRs.[21,27] The high local protein concentration may be incompatible with the free movement of G-proteins in the membrane and diffusion of second messenger in the cytoplasm necessary for mGluRs.[13] Voltage gated ion channels that are regulated by mGluRs (e.g. potassium channels)[3,23] may only be present in the non-junctional postsynaptic plasma membrane, therefore the placement of mGluRs close to the glutamate release site, but outside the junction could place them in precise conjunction with their molecular targets. In addition the distinct localization of the ionotropic and metabotropic GluRs may also be reflected in their differential affinity for activation by glutamate.

A consequence of, and perhaps a reason for, the more peripheral position of metabotropic receptors is that individual presynaptic action potentials may not release enough glutamate to reach a concentration sufficient to activate the perisynaptic mGluRs. According, a high frequency of action potentials may be necessary to evoke the mGluR-mediated part of postsynaptic responses as has been suggested for long-term changes in synaptic efficacy.[2,4,22] One well studied form of long term change is the calcium-mediated long-term depression (LTD) of parallel fibre synaptic responses of Purkinje cells by climbing fibre activation.[15] We have found no difference in the position of mGluR1α, which mediates Ca^{2+} release from intracellular stores, at climbing and parallel fibre synapses. Therefore, the segregation of GluRs may play a general role in excitatory neurotransmission rather than just being involved in LTD.

Purkinje and granule cells have different synaptic responses upon activation of their glutamatergic inputs.[18,32,35] A major difference between these two cells with respect to their AMPA receptors is the apparent absence of GluR-A and C subunits from granule cells and their presence at parallel and climbing fibre synapses.[5,17,19,26,30] We found no qualitative differences in the subsynaptic localization of the GluR-B/C/4c subunits between the climbing and parallel fibre synapses on Purkinje cells and mossy fibre synapses on granule cells. However, using the immunogold method, we were able to look for quantitative differences in the levels of immunoreactivity at different synapses. Comparison of the mossy and parallel fibre synapses for reactivity to the GluR-B/C/4c subunits of the AMPA receptor demonstrated labelling about twice as strong (1.9 and 1.85 times in two animals) at parallel fibre synapses on Purkinje cells (Fig. 4). This quantitative difference is not due to a more limited subunit recognition of the total AMPA receptor subunit content by the antibodies in the mossy fibre synapses, because the majority of AMPA receptor subunits known to be expressed by granule cells[11,30] are recognized by these antibodies. On the contrary, immunoreactivity for an additional subunit, the GluR-A (not recognized by our antibodies), has already been shown in the parallel fibre synapses,[5,19] and so the twofold difference in immunoreactivity is an underestimate (see also Fig. 4) of the real differences in channel numbers. Physiological studies have demonstrated approximately 10

Fig. 2.A,B. Electron micrographs showing subsynaptic segregation of the ionotropic and metabotropic GluRs as revealed by double immunolabelling. Large immunoparticles (double arrows) demonstrate immunoreactivity for mGluR1α (using rabbit antibody), whereas small particles (arrows) represent immunoreactivity for GluR-B/C/4c (using mAb 1F1) in the synapses on spine (s) of Purkinje cells. The synapse in B is the only one shown from Triton treated material. C, generally a lower density of immunoparticles (arrows) for GluR-B/C/4c (using rabbit antibody) has been found in synapses between mossy fibre terminals (mt) and granule cell dendrites (d) than in the parallel fibre synapses (compare to Fig. 1B). Scale bars = 0.1 μm; (A,B) 0.2 μm (C).

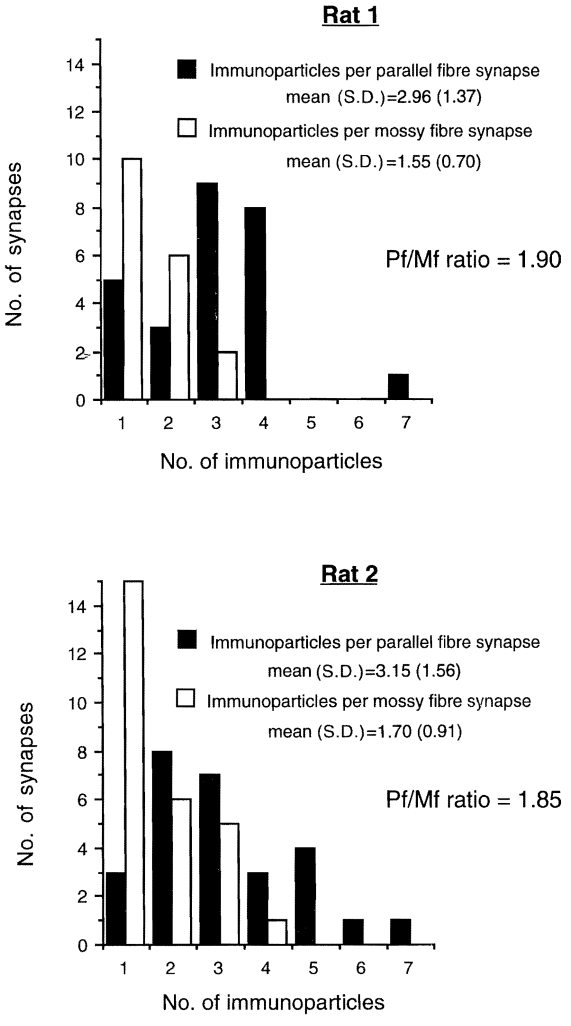

Fig. 4. Distributions of parallel and mossy fibre synapses according to their immunoreactivity for GluR-B/C/4c subunits. Immunoreactivity in the synapses made by parallel fibre terminals with spines of Purkinje cells is significantly higher ($P < 0.001$, Mann–Whitney test, rat 1: $Z = -3.46$; rat 2: $Z = -3.59$) than in the synapses established between mossy fibre terminals and granule cell dendrites. Quantification of GluR-B/C/4c receptor immunoreactivity was carried out using the rabbit polyclonal antibodies[26] described in Fig. 1. Preservation of cellular integrity and immunoreactivity were correlated and uneven in slammed tissue, therefore we defined a reproducible method of sampling. The specimen was scanned randomly until the first immunopositive mossy or parallel fibre synapse was found. An arbitrary criterion of at least one immunoparticle in the synaptic junction was used for accepting the area as immunoreactive. This synapse was placed in the middle of the photograph and a $4 \times 3\,\mu m$ area was analysed at a final magnification of $\times 46{,}600$. Immunoparticles were counted on every synapse within this rectangle. Immunonegative synapses are not included in the evaluation because it is uncertain whether the lack of immunoreaction is due to the absence of receptor protein, or to technical limitations such as sensitivity, or steric hindrance of antibody access to tangentially cut synapses which do not reach the surface of the section. The calculated immunoreactivity ratios may be underestimates of the real differences in channel numbers for two reasons: (i) the frequency of immunonegative synapses (not shown) was higher for mossy fibre synapses; (ii) differential subunit expression by granule and Purkinje cells, i.e. granule cells express mRNA for the GluR-B subunit,[30] but not for the A or C subunits, and a significant subset of the GluR-D transcript is the GluR-4c subtype,[11] therefore the majority of the AMPA receptor subunits are recognized by our antibody in the mossy fibre synapses. In contrast Purkinje cells express mRNAs for the GluR-A, B and C subunits of the AMPA receptors,[11,17,30] thus only a subset of these subunits (GluR-B and C but not GluR-A) are recognized by the antibodies.

non-NMDA channels activated by a single packet of transmitter at the mossy fibre synapse.[35] It remains to be established whether the higher number of AMPA channels in the parallel fibre synapses can be correlated with a synaptic conductance similar to that in the mossy synapses but provided by individual channels having lower conductances as found for extrasynaptic glutamate channels on Purkinje cell.[10a] Alternatively, a higher number of channels in the parallel fibre synapse may lead to a larger synaptic conductance in the parallel as compared to the mossy fibre synapse. The latter possibility seems to be supported by a recent report demonstrating that the activation of as few as 50 parallel fibre synapses, comprising 0.03% of parallel fibre input to a Purkinje cell, may be sufficient to bring the cell to threshold.[1]

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