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THE CEREBELLUM: FROM STRUCTURE TO CONTROL

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CHAPTER 7

Compartmentalised distribution of $GABA_A$ and glutamate receptors in relation to transmitter release sites on the surface of cerebellar neurones

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Introduction

Amino acid neurotransmitter receptors are thought to be present at synaptic junctions close to the transmitter release site. Indeed, the fast onset and rise time of $GABA_A$ receptor mediated inhibitory post-synaptic currents (IPSCs) and AMPA receptor mediated excitatory post-synaptic currents (EPSCs) suggest that ionotropic $GABA_A$ and AMPA receptors are present in the post-synaptic membrane opposite the vesicle fusion site (Edwards et al., 1990; Hestrin et al., 1990; Konnerth et al., 1990; Malinow, 1991; Otis and Mody, 1992; Silver et al., 1992; Jonas et al., 1993; Llano and Gerschenfeld, 1993; Traynelis et al., 1993; de Koninck and Mody, 1994; Puia et al., 1994; Soltesz and Mody, 1994; Kaneda et al., 1994; Wyllie et al., 1994). Furthermore, AMPA-type glutamate receptors have low affinity for glutamate (Patneau and Mayer, 1990) which indicates that these receptors are close to the release site where glutamate reaches concentrations in the millimolar range (Clements et al., 1992). However, it has been shown that outside out patches from extrasynaptic somatic and dendritic membranes of central neurones also contain functional $GABA_A$ and glutamate receptor channels (Cull-Candy and Ogden, 1985; Edwards et al., 1990; Colquhoun et al., 1992; Jonas and Sakmann, 1992; Llano and Gerschenfeld, 1993; Wyllie et al., 1993; Kaneda et al., 1994; Macdonald and Olsen, 1994; Puia et al., 1994; Wyllie and Cull-Candy, 1994; Spruston et al., 1995). In line with these results immunoreactivity for $GABA_A$ and glutamate receptors has been found at extrasynaptic sites (Richards et al., 1987; Somogyi et al., 1989; Yazulla et al., 1989; Soltesz et al., 1990; Waldvogel et al., 1990; Hansen et al., 1991; Greferath et al., 1993; Martin et al., 1993; Molnar et al., 1993; Baude et al., 1994, 1995; Caruncho and Costa, 1994; Gao and Fritschy, 1995; Jaarsma et al., 1995; Nusser et al., 1995a,b, 1996). The quantitative relationship between the synaptic and extrasynaptic receptors has not yet been established using electrophysiological approaches.

In theory, electron microscopic immunocytochemistry should have the resolution to localise receptors at synaptic and non-synaptic sites. Electron-dense, peroxidase reaction end-product has been found on synaptic specialisations for AMPA, NMDA, metabotropic glutamate and $GABA_A$ receptors, and it was generally interpreted as visualising synaptic receptors (Richards et al., 1987; Somogyi et al., 1989; Soltesz et al., 1990; Wald-

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vogel et al., 1990; Martin et al., 1992; Petralia and Wenthold, 1992; Gorcs et al., 1993; Greferath et al., 1993; Martin et al., 1993; Molnar et al., 1993, 1994; Baude et al., 1994; Huntley et al., 1994; Petralia et al., 1994a,b; Siegel et al., 1994; Jaarsma et al., 1995; Sassoe-Pognetto et al., 1995; Takayama et al., 1995). However, the immunoperoxidase method is not reliable for localising synaptic receptors (see next section), nor it is suitable for quantification of receptor immunoreactivity. The most direct support for a possible enrichment of $GABA_A$ and AMPA receptors at synaptic sites came from the results of experiments using immunofluorescence labelling and confocal laser microscopy, showing intense immunoreactive spots on the surface of cultured hippocampal, spinal cord and cerebellar neurones (Craig et al., 1993, 1994; Bohlhalter et al., 1994; Gao and Fritschy, 1995). In order to compare synaptic and extrasynaptic sites on neurones in situ, we investigated to what extent the ionotropic glutamate and $GABA_A$ receptors were enriched in the synaptic junctions compared with the extrasynaptic membrane, and whether the degree of enrichment was similar at different types of synapses. Finally, we also tried to establish what was the precise subsynaptic distribution of receptors in relation to the transmitter release site.

The cerebellar cortex was chosen to study the above questions, because the origin and the position of GABA- and glutamatergic synapses are well known and both the pre- and post-synaptic elements can easily be identified. Electron microscopic immunoperoxidase and pre- and post-embedding immunogold methods were employed. The advantages and the limitations of each technique are summarised briefly in the following section.

Comparison of immunocytochemical methods for electron microscopy

Four immunocytochemical techniques have been applied to obtain a comprehensive picture of the cellular, subcellular and subsynaptic distribution of glutamate and $GABA_A$ receptors in the cerebellum. First, the cellular distribution of certain receptor subtypes was determined using the pre-embedding immunoperoxidase method (ABC method, with diaminobenzidine as chromogen). In order to determine the possible subcellular origin of the immunostaining observed at light microscopic level, correlated electron microscopy was always carried out (Somogyi et al., 1989). Pre-embedding immunoperoxidase method provides reliable information about whether the origin of the immunoreaction is pre- or post-synaptic; whether the epitope(s), recognised by the primary antibody, is(are) located on the intra- or extracellular face of the plasma membrane; and, for certain epitopes, whether any reaction is associated with endomembranes. This method is also suitable for the visualisation of extrasynaptic receptors under certain conditions (Somogyi et al., 1989). However, due to the diffusable nature of the peroxidase reaction end-product, this method is generally not suitable for localising receptors at synaptic site. The possibility cannot be excluded that the presence of the electron-dense reaction end-product on the post-synaptic specialisation did not diffuse there from extrasynaptic sites. For example, when the 1α form of the metabotropic glutamate receptor (mGluR) was localised with the peroxidase method, reaction end-product was found to be deposited on the post-synaptic densities of asymmetrical synapses in the cerebellar molecular layer (Fig. 1A; and also see Martin et al., 1992; Gorcs et al., 1993). However, using either pre-embedding immunogold localisation on 70-μm thick, free-floating sections (Fig. 1B) and on ultrathin frozen sections (Fig. 1D), or post-embedding immunogold localisation on Lowicryl-embedded ultrathin sections (Fig. 1C), it was discovered that immunoparticles were not present in the main body of the same type of synapse.

Antibodies bound to tissue sections under pre-embedding conditions can also be visualised by colloidal gold particles. Gold particles, over 5 nm in diameter, are easily detectable in the electron microscope, but immunoglobulins coupled to these large particles do not penetrate well into

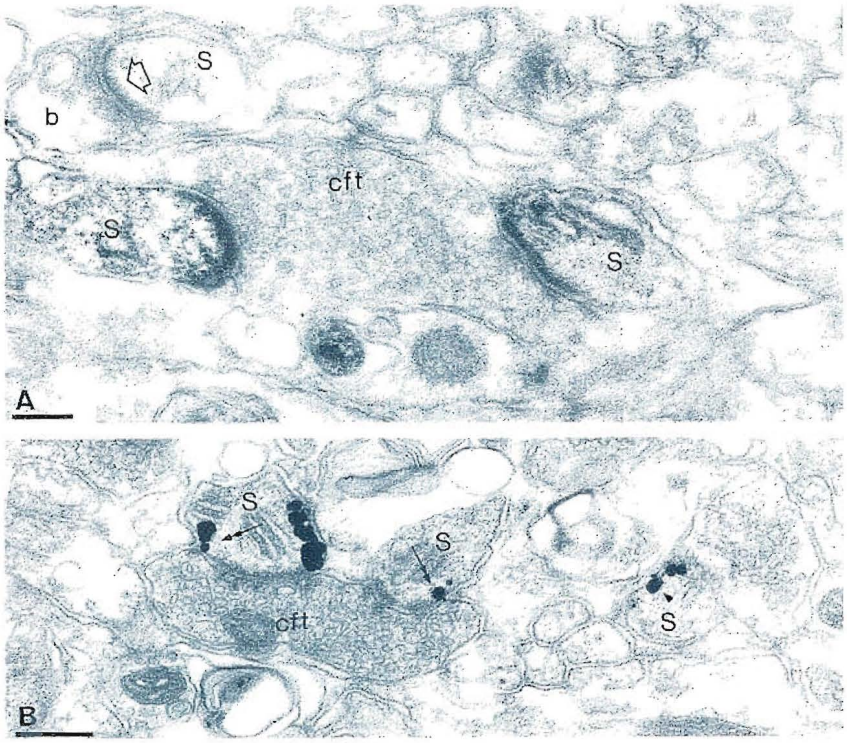
the tissue. Therefore, 1.4 nm gold particles coupled to secondary antibodies were employed in order to facilitate the penetration of antibodies. Silver intensification of the gold particles was carried out to make the particle size detectable in the electron microscope. The pre-embedding immunogold method has several advantages compared with the peroxidase technique. First of all, it produces a non-diffusable label, so the precise site of the reaction can be determined with a resolution of about 20 nm. Secondly, particulate markers are quantifiable. Thirdly, this method can be relied upon to localise receptors at extrasynaptic sites. However, synaptic receptors could not always be revealed with the pre-embedding immunogold method (Baude et al., 1995; Nusser et al., 1995a,b, 1996), therefore another method was developed and applied in order to visualise synaptic receptors.

Cerebellar slices were slam-frozen, freeze-substituted and embedded in Lowicryl HM20 (Baude et al., 1993; Nusser et al., 1995b). Lowicryl-embedded material has different electron microscopic appearance compared with conventional epoxy resin-embedded, osmium-treated tissue. Nevertheless, different cellular profiles such as nerve terminals, dendrites, spines, synapses, etc., are easily identifiable. This method is reliable for the synaptic localisation of receptors and reveals an enrichment of the ionotropic glutamate and $GABA_A$ receptors in the synaptic junctions (Nusser et al., 1994; 1995a,b). Extrasynaptic receptors are also revealed with the post-embedding method. Since the surface of the electron microscopic sections is directly in contact with the antibodies, there is no difference between the exposed tissue elements in their access to antibodies, making quantitative comparison possible. Such a comparison can be made between different subcellular compartments of the same cell (Nusser et al., 1995a), among synapses of the same population (Nusser et al., 1995a; 1996) or between synapses on distinct cell types (Nusser et al., 1994).

In summary, an immunoperoxidase and two immunogold methods have been compared for electron microscopic localisation of receptors on three different kinds of section. Correlated light and electron microscopy can easily be made using the immunoperoxidase method for the cellular localisation of receptors. The pre-embedding immunogold method is suitable for revealing extrasynaptic receptors and in some cases for visualising receptors at synaptic sites (Baude et al., 1995; Fig. 2). Only the post-embedding immunogold method is reliable for the visualisation of synaptic receptors and it is suitable for revealing quantitative differences in receptor density on the surface of the same cell or on distinct cell types.

Synaptic enrichment of ionotropic amino acid neurotransmitter receptors

The relative quantitative relationship in receptor density was studied on the surface of cerebellar neurones using the immunogold localisation of AMPA and $GABA_A$ receptors. We found that AMPA receptor subunits were present at low density on the extrasynaptic somatic and dendritic membranes of granule and Purkinje cells and were concentrated in the main body of asymmetrical synapses (Figs. 2 and 3B). Similar results were also obtained in the hippocampus (Baude et al., 1995) and in the spinal cord (Phend et al., 1995) using post-embedding immunogold localisation of AMPA receptor subunits. Several subunits of the $GABA_A$ receptor were also found to be highly concentrated in symmetrical synapses on Purkinje (Fig. 4) and granule cells (Figs. 5 and 6) in the cerebellar cortex as well as in the hippocampus (Nusser et al., 1995b). The same subunits have a much lower density at extrasynaptic sites (Figs. 4–6). The ionotropic glycine receptors are also enriched in synapses on spinal cord neurones (Triller et al., 1985). Therefore it seems that, as a general rule, ionotropic amino acid receptors are concentrated in the post-synaptic membrane specialisation of synaptic junctions on central neurones opposite to the transmitter release site. They can be present uniformly across the junction or in one or more clusters within the synaptic specialisation.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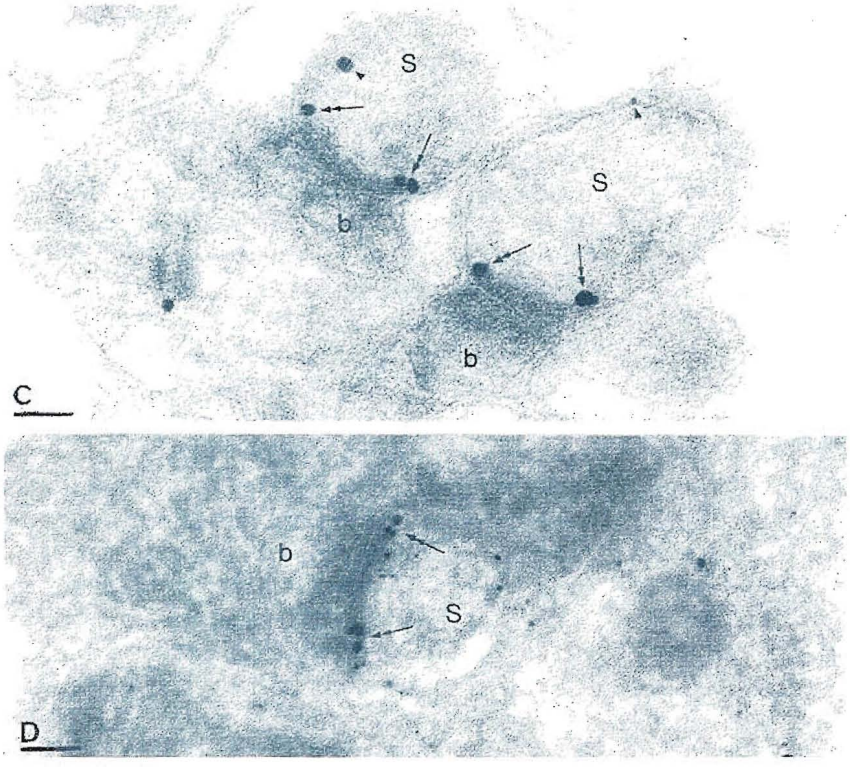
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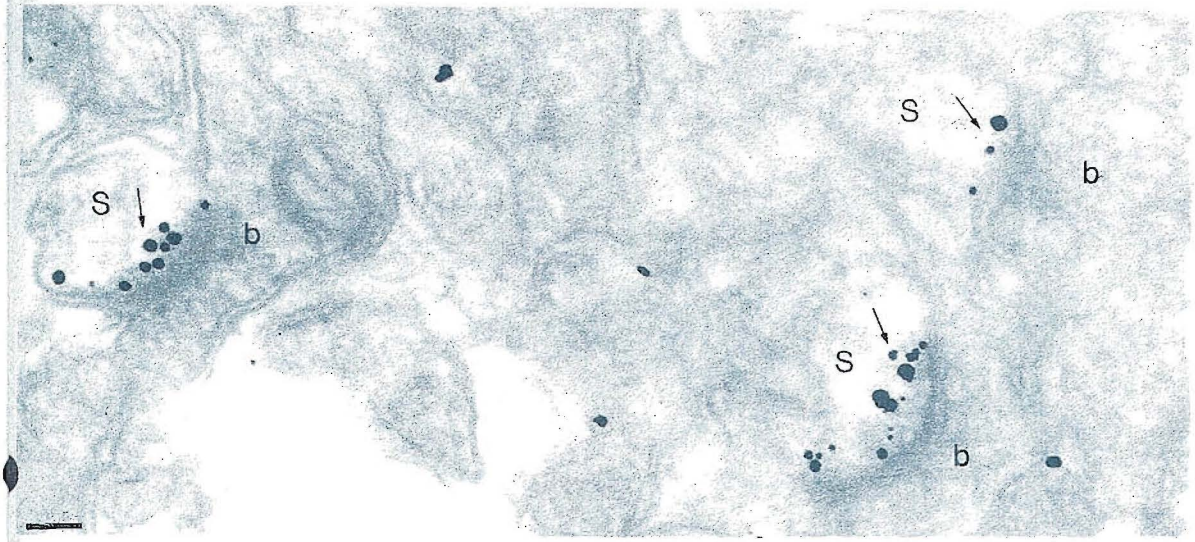


Fig. 2. Electron micrograph showing immunoreactivity for the GluRD subunit of the AMPA-type GluR in the cerebellar molecular layer as demonstrated by a pre-embedding immunogold method. Immunoparticles are enriched at the cytoplasmic side of post-synaptic densities of asymmetrical synapses (arrows) made by parallel fibre terminals (b) with Purkinje cell spines (s). Scale bar: 0.1 μm.

The degree of enrichment of $GABA_A$ receptors in synaptic junctions was studied in cerebellar granule cells. Immunoparticle densities for the $\alpha 1$ and $\beta 2/3$ subunits of the $GABA_A$ receptor were calculated on four different compartments of the surface of the cells (Nusser et al., 1995a), namely, junctional membrane post-synaptic to mossy fibre terminals (MS), extrasynaptic somatic membrane (BG), extrasynaptic dendritic membrane of granule cell (DG), and junctional membrane post-synaptic to Golgi cell terminals (GS). Immunoparticle densities for the $\alpha 1$ and $\beta 2/3$ subunits are approximately 230 and 180 times higher, respectively, in immunopositive GABAergic Golgi synapses than in the extrasynaptic somatic membrane. The concentration of these subunits on non-synaptic somatic membrane is significantly lower than on the extrasynaptic dendritic membrane. Synaptic junctions between glutamatergic mossy fibre terminals and granule cell dendrites are immunonegative for these $GABA_A$ receptor subunits. The ratios of immunoparticle density on the four examined compartments are approximately MS/BG/DG/GS = 0:1:2:230 and

Fig. 1. Electron micrographs showing immunoreactivity for mGluR1α as demonstrated by four different methods in the cerebellar molecular layer. A: Pre-embedding immunoperoxidase method. Reaction end-product covers the post-synaptic specialisations at asymmetrical synapses made by a climbing fibre terminal (cft) with Purkinje cell spines (s). The reaction end-product also diffuses to the cytoplasm of the spines covering the spine apparatus. A neighbouring asymmetrical synapse (open arrow) between an axon terminal (b) and a spine is immunonegative for mGluR1α. B: Pre-embedding immunogold method. Immunoparticles are located at the intracellular face of non-synaptic spine membranes (arrowhead) and at the periphery (double arrows) of asymmetrical synapses made by a climbing fibre terminal (cft) with spines. C: Post-embedding immunogold method on Lowicryl-embedded tissue. Immunoparticles are concentrated at the edge (double arrows) of post-synaptic specialisations established by boutons (b) on spines (s), but they also occur at extrasynaptic sites (arrowheads). D: Pre-embedding immunogold method on ultrathin frozen section. Immunoparticles are concentrated at the periphery of a synaptic junction (double arrows) made by a bouton (b) with a spine (s). Scale bars: A, B: 0.2 μm; C, D: 0.1 μm.

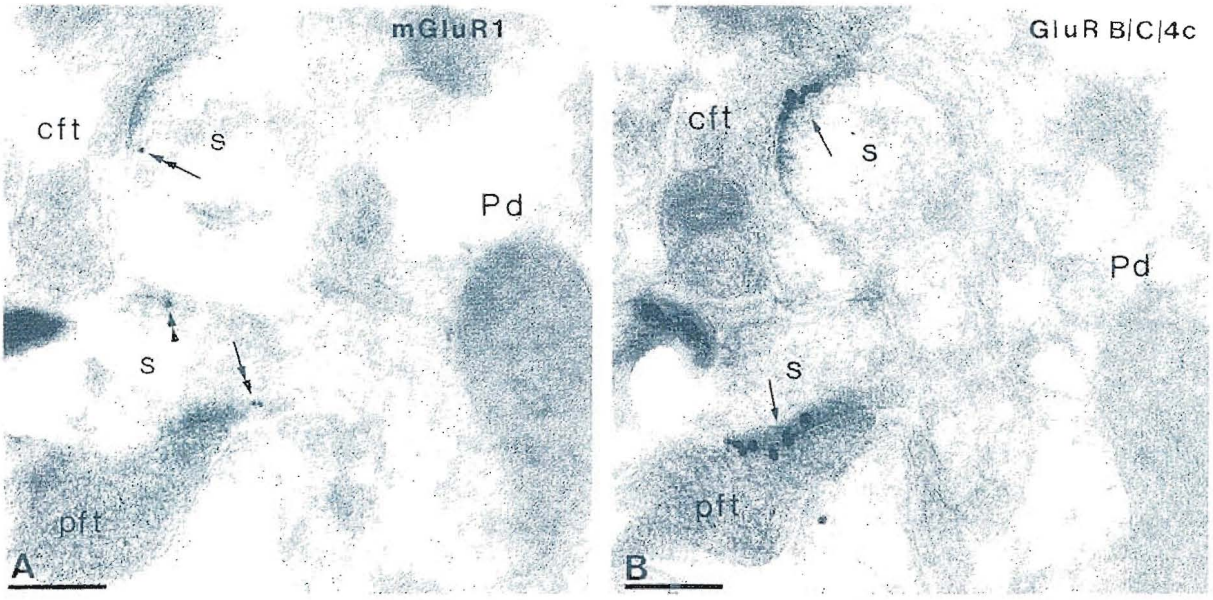


Fig. 3. Electron microscopic demonstration of the subsynaptic segregation of metabotropic (A; mGluR1α) and ionotropic (B; Glu B/C/4c) GluRs. Immunoparticles for mGluR1α are concentrated at the edge of the synaptic specialisation (double arrows in A whereas particles for GluR B/C/4c are concentrated in the middle (arrows in B) of asymmetrical synapses established by paralle (pft) and climbing (cft) fibre terminals with Purkinje cell spines (s). The climbing fibre terminal was identified from its larger size multiple synaptic junctions and several mitochondria outside the field of view. Extrasynaptic mGluR1α are often observed (doubl arrowhead). Pd, Purkinje dendrite. Adopted from Nusser et al. (1994). Scale bars: 0.2 μm.

MS/BG/DG/GS = 0:1:3.3:180 for the α1 and β2/3 subunits, respectively (Fig. 7). Only a small fraction of the granule cell membrane is covered by GABAergic synapses. Taking into account the measured synaptic and non-synaptic dendritic plasma membrane ratio in the glomeruli (although not all extrasynaptic immunoreactivity could be measured, therefore the value is an underestimate), it can be calculated that at least 1.25 and 3 times more α1 and β2/3 subunits exist (respectively) at extrasynaptic glomerular sites than at synaptic sites (Nusser et al., 1995a). These values are further increased by adding the non-glomerular dendritic and somatic extrasynaptic receptors. Although, the functional role of the extrasynaptic receptors is unknown, this result demonstrates that the overall amount of extrasynaptic receptors probably exceeds that of synaptic ones.

It is not yet possible to establish the absolute number of GABA or glutamate channels in a given membrane because the immunogold density has not been calibrated. The degree of synaptic receptor enrichment may depend on the cell and receptor type, because both the synaptic and the extrasynaptic density of receptors vary on different cell types.

Quantitative comparison of glutamate and $GABA_A$ receptor density between synapses on different post-synaptic cells

Spontaneously occurring tetrodotoxin resistant miniature EPSCs (mEPSCs) and IPSCs (mIPSCs) vary in their amplitude within a single cell and between different cell types (Silver et al., 1992; Jonas et al., 1993; de Koninck and Mody, 1994; Soltesz and Mody, 1994; Edwards, 1995). The site of their origin is not revealed using patch clamp recordings, therefore the contribution of den

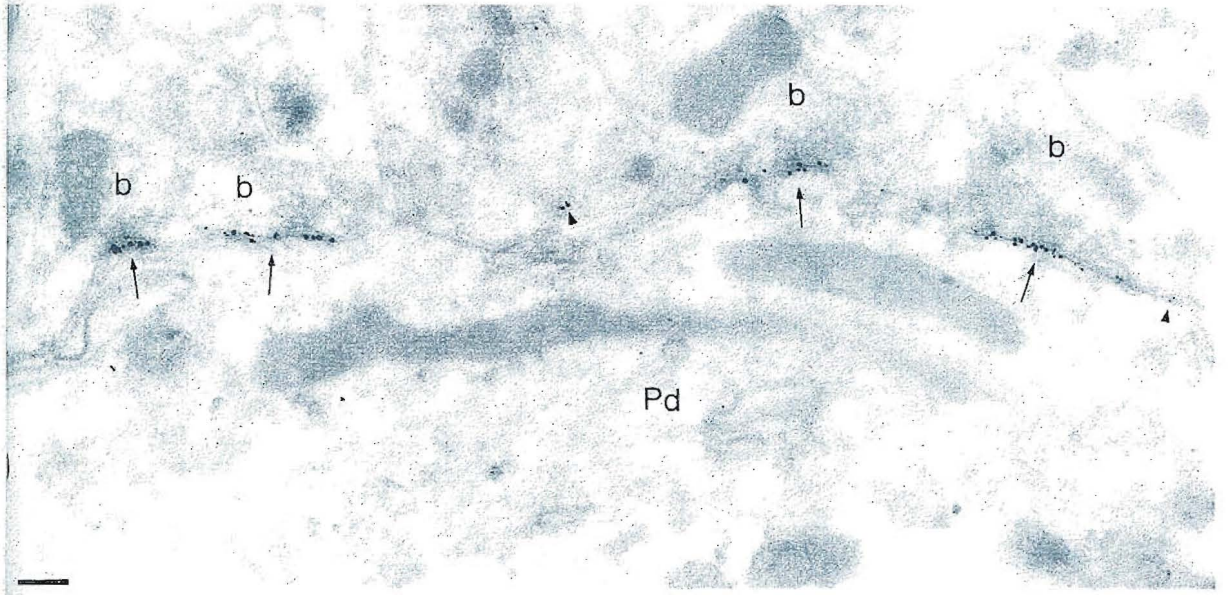


Fig. 4. Post-embedding, silver intensified immunogold demonstration of immunoreactivity for the α_1 subunit of the $GABA_A$ receptor in the molecular layer of rat cerebellum using a rabbit polyclonal antibody (P16). A high density of immunoparticles (arrows) is present at synaptic junctions made by axon terminals (b) with a Purkinje cell dendrite (Pd). Only occasional particles can be found at extrasynaptic sites (arrowheads). Scale bar: 0.2 μm.

dritic filtering and attenuation to the variability is not known. However, the amplitude distribution of the fastest events, which probably originate proximally and are not distorted by dendritic filtering, is still skewed towards larger events (Silver et al., 1992; de Koninck and Mody, 1994; Soltesz and Mody, 1994; Soltesz et al., 1995). It is thought that synaptic AMPA and $GABA_A$ receptors are saturated upon the synaptic release of transmitter (Mody et al., 1994; Tang et al., 1994; Tong and Jahr, 1994). Therefore, the skewed amplitude distribution of the fastest mIPSCs and mEPSCs may be due either to different numbers of channel in individual synapses; or to the differential distribution of channels from synapse to synapse with different single channel conductances; or to the combination of these two factors. The single channel conductance of synaptic channels did not vary considerably among synapses within a cell (Traynelis et al., 1993; Soltesz and Mody, 1994). Therefore, the most likely explanation of the skewed amplitude distribution is that individual synapses on the same cell contain different numbers of channel. This hypothesis is supported by our results showing that immunoparticle density for the GluRB/C/4c subunits varies considerably among asymmetrical synapses on Purkinje cells (Nusser et al., 1994) and also on CA1 pyramidal cells (Baude et al., 1995).

Post-embedding immunocytochemistry with immunogold as label reveals quantitative differences in receptor density between different sites such as distinct types of synapses. The first such comparison was carried out for the GluRB/C/4c subunits content of mossy fibre to granule cell synapse and the parallel fibre to Purkinje cell synapse (Nusser et al., 1994). The number of immunoparticles for GluRB/C/4c subunits is approximately twice as high in the parallel fibre synapses on Purkinje cells as it is in mossy fibre synapses on granule cells (Fig. 8). This difference is not due to a more limited subunit recognition by the antibodies in the mossy synapses, because the majority of AMPA receptor subunits expressed by

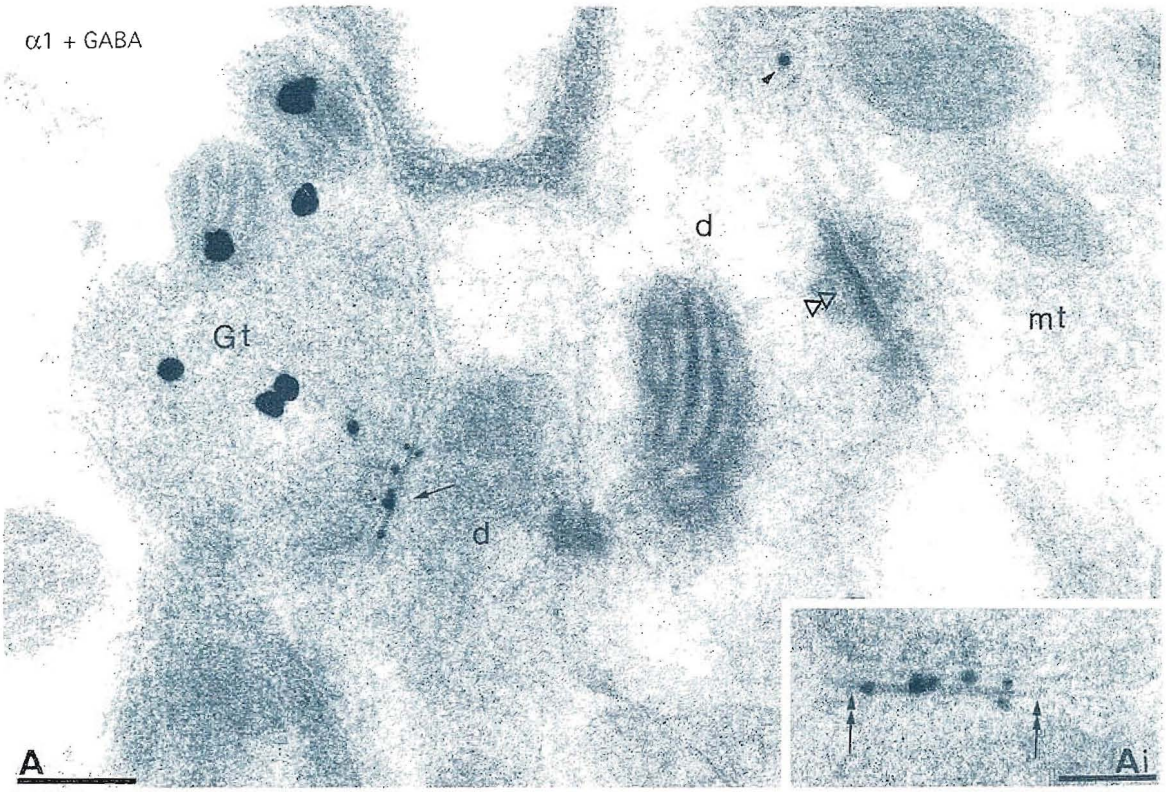
granule cells (Gallo et al., 1992; Sato et al., 1993) are recognised by this antibody. On the contrary, only a subset of the AMPA receptor subunits are recognised by the antibody in parallel fibre synapses since neither the GluRA nor the GluRD subunits expressed by Purkinje cells (Fig. 2; and see Sato et al., 1993; Baude et al., 1994) are recognised by the antibody used for the comparison. Thus, the twofold difference in immunoreactivity is an underestimate of the real difference in channel number.

Another example that a given receptor subunit has different concentration at distinct synapses on the surface of different cell types is evident in the cerebellum. Immunoparticles for the $\alpha 1$ subunit of the $GABA_A$ receptor have a much higher density in symmetrical synapses on Purkinje cell somata and dendrites than in synapses made by Golgi cell terminals with granule cell dendrites (Figs. 4 and 5A).

Although quantitative immunocytochemistry reveals differences in receptor density, it remains uncertain whether the higher number of glutamate or GABA receptors under certain types of synapses will result in a larger synaptic conductance, because single channels may not be the same in all synapses. Electrophysiological recordings of synaptic responses and quantitative immunocytochemistry of receptors can provide complementary information about the kinetic properties, number and molecular composition of synaptic transmitter gated ion-channels.

Heterogeneity of cerebellar Golgi cell to granule cell synapses according to their $GABA_A$ receptor α subunit content

Cerebellar granule cells receive excitatory input from brain stem nuclei and from the spinal cord



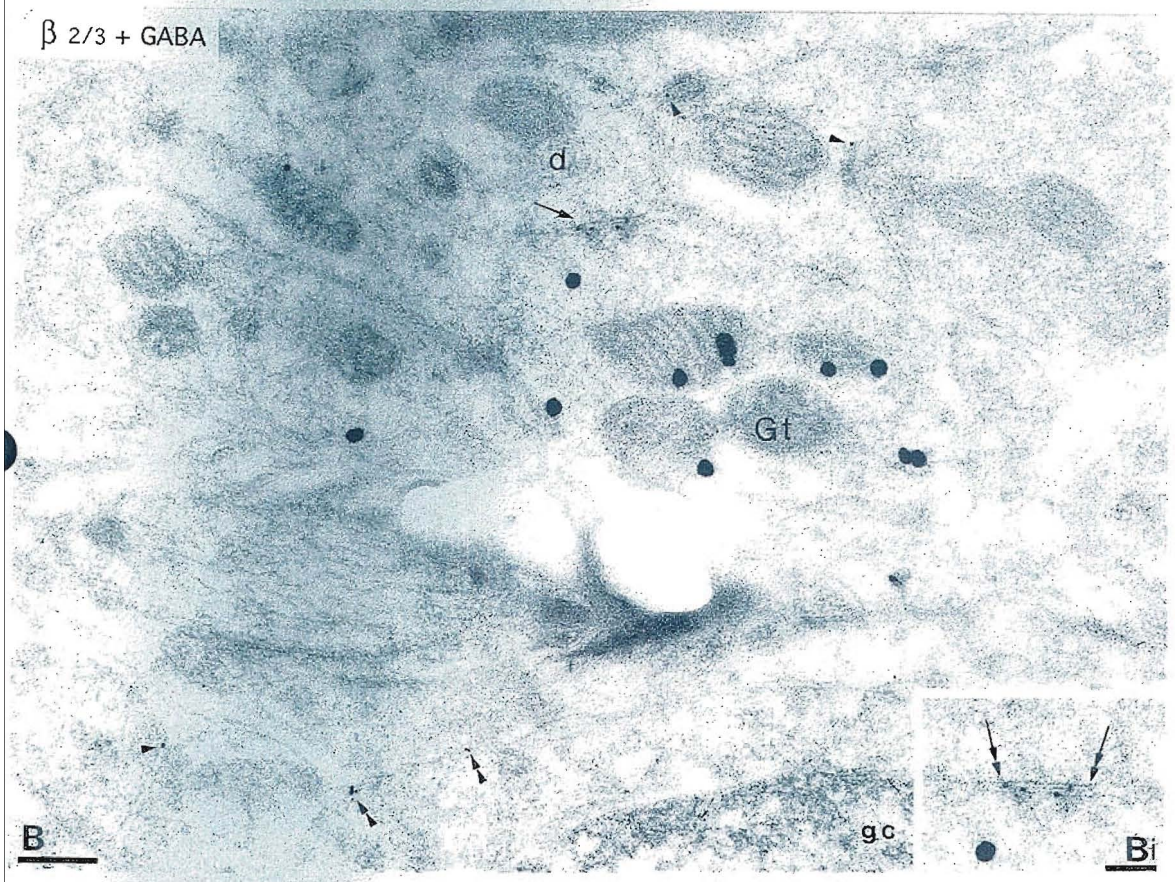


Fig. 5. Electron micrographs of freeze-substituted, Lowicryl-embedded ultrathin sections of cat cerebellum demonstrating immunoreactivity for the α_1 (A, Ai) or $\beta_{2/3}$ (B, Bi) subunits of the $GABA_A$ receptor (small particles, silver intensified 1 nm gold) and for GABA (large particles, silver intensified 15 nm gold). The enrichment of large particles shows GABA immunoreactivity of Golgi cell terminals (Gt). The synaptic junctions (arrow) are covered by small particles showing an enrichment of receptor immunoreactivity. Note the presence of immunoparticles for the receptor on the somatic (B, double arrowheads) and the extrasynaptic dendritic membrane (arrowheads) of granule cells (gc) and the immunonegative synapse (double triangles) established by a mossy terminal (mt). The insets (Ai, Bi) show the demarcation of the edge of synaptic junctions (double arrows) based on the electron dense post-synaptic membrane specialisation. Adopted from Nusser et al. (1995a). Scale bars: A, B, 0.2 μm; Ai, Bi, 0.1 μm.

through mossy fibres. They receive inhibitory input from Golgi cells only. Granule cells express mRNAs for 8 different subunits of the $GABA_A$ receptor (Laurie et al., 1992; Persohn et al., 1992) and immunocytochemical studies have already shown that six out of the eight subunits are expressed on the surface of granule cells (Somogyi et al., 1989; Turner et al., 1993; Gutierrez et al., 1994; Fritschy and Mohler, 1995; Gao and Fritschy, 1995, Nusser at al., 1995a; 1996). These subunits assemble into several types of $GABA_A$ receptor channels (Duggan et al., 1991; McKernan et al., 1991; Endo and Olsen, 1993; Mertens et al., 1993; Pollard et al., 1993; Khan et al., 1994; Quirk et al., 1994; Togel et al., 1994; Pollard et al., 1995) which may be distributed in a similar manner on the surface of granule cells resulting in identical $GABA_A$ receptor channels at synaptic and extrasynaptic sites. Alternatively, distinct subunits may have dissimilar subcellular locations on the granule cell surface, resulting in distinct synaptic and extrasynaptic $GABA_A$ receptors. To decide between these possibilities, immunogold localisation of the $\alpha 1$, $\alpha 6$ and $\beta 2/3$ subunits was

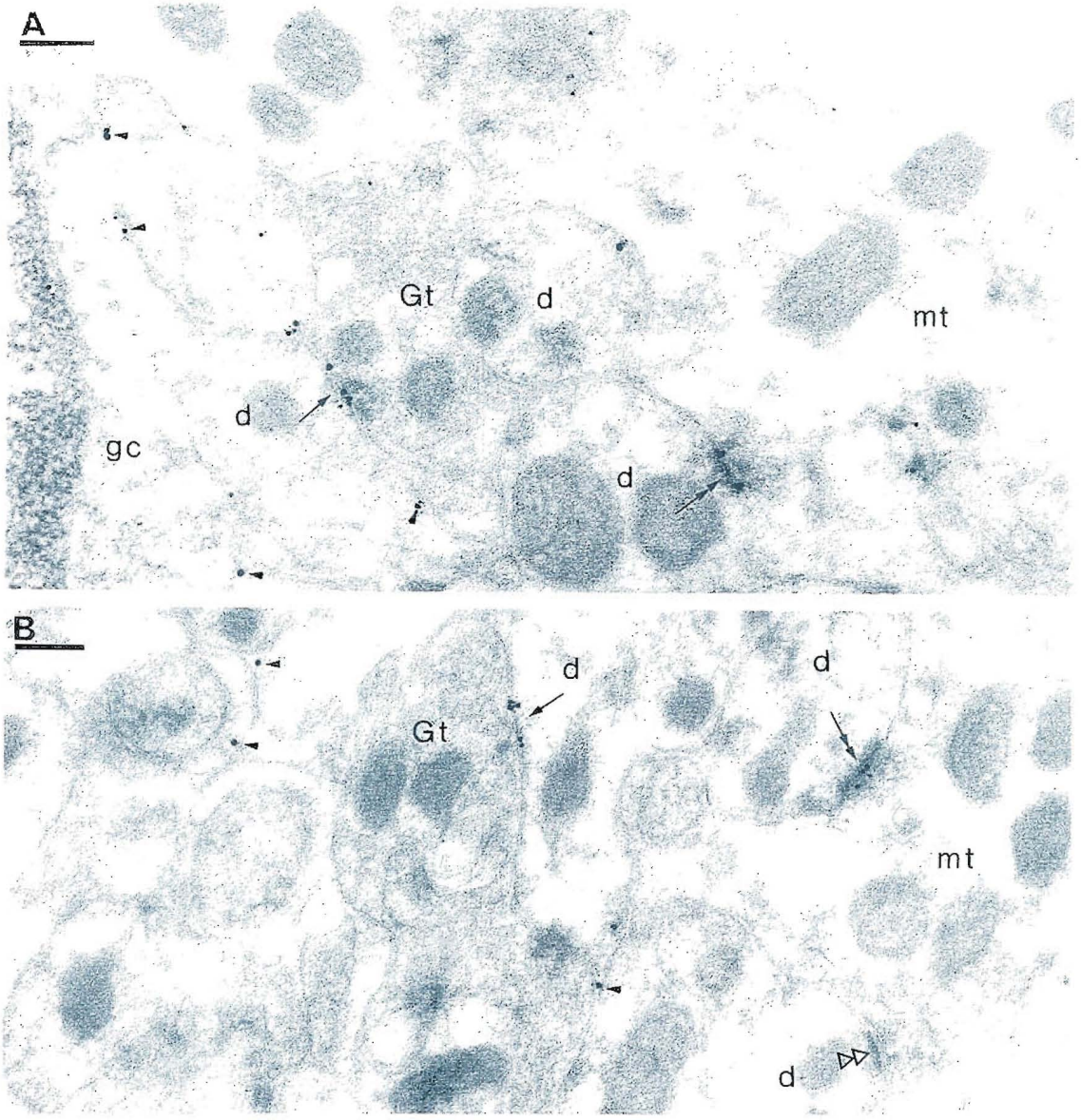


Fig. 6. Electron micrographs showing immunoreactivity for the α_6 subunit in the cerebellar glomeruli as demonstrated by a post-embedding immunogold localisation using polyclonal antibodies to the N-terminal (A) or to the C-terminal (B) end of the protein. A, B: An enrichment of immunoparticles is detected in synapses made by both Golgi cell terminals (Gt; arrows) and some mossy fibre terminals (mt; double arrows) with granule cell dendrites (d). Golgi terminals are usually located at the periphery of glomeruli, close to the somata of granule cells (gc in A). Immunonegative mossy synapses are also found in the same glomeruli (double open triangles in B). Particles are also present on the extrasynaptic somatic and dendritic membranes (arrowheads). Adapted from Nusser et al. (1996). Scale bars: 0.2 μm.

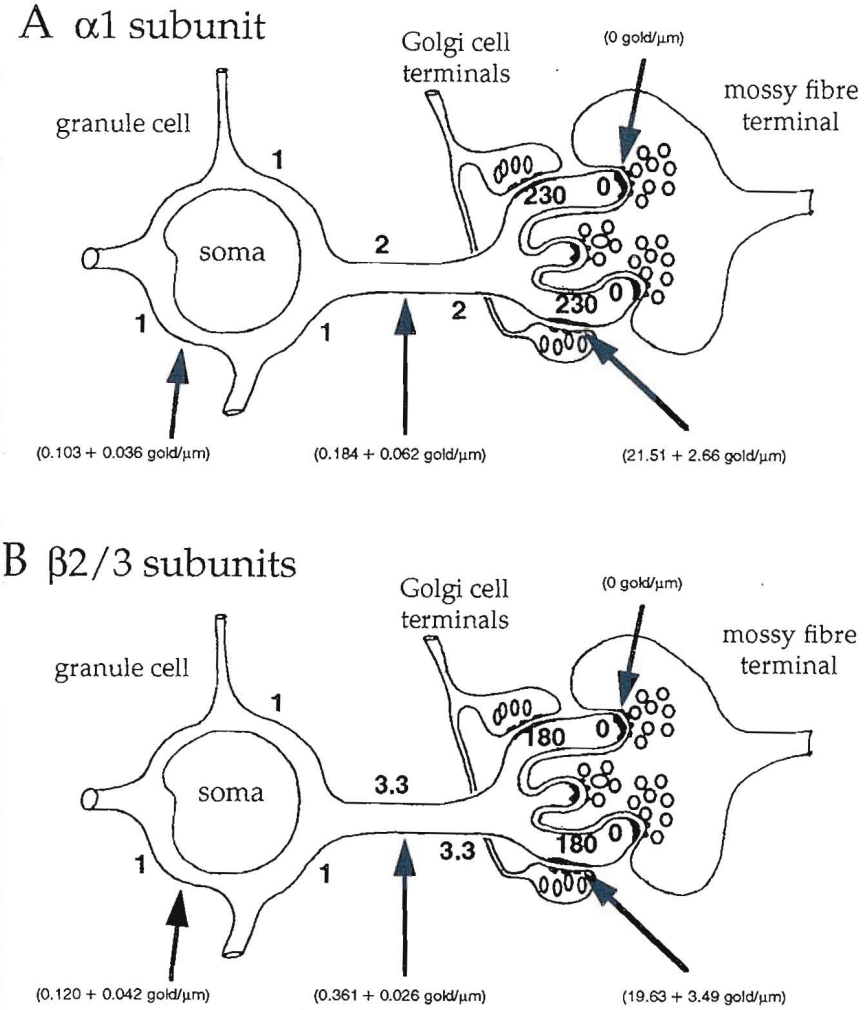


Fig. 7. Schematic drawings of the cerebellar glomerulus showing absolute and relative immunoparticle densities for the α_1 (A) and the $\beta_{2/3}$ (B) subunits of the $GABA_A$ receptor on four different compartments of the granule cell surface in cat cerebellum. Immunoparticle densities on the extrasynaptic somatic membranes are 0.103 (0.036) [mean (SD)] and 0.120 (0.042) particle/μm membrane for the α_1 and the $\beta_{2/3}$ subunits, respectively. Immunoparticle densities on the extrasynaptic dendritic membranes are significantly higher, being 0.184 (0.062) and 0.361 (0.026) particle/μm membrane for the α_1 and the $\beta_{2/3}$ subunits, respectively. Immunoparticle densities on the membranes post-synaptic to immunopositive GABAergic Golgi cell terminals are 21.51 (2.66) and 19.63 (3.49) particle/μm membrane for the α_1 and the $\beta_{2/3}$ subunits, respectively. Post-synaptic membranes to excitatory mossy fibre terminals are immunonegative for both subunits. The ratios between immunoparticle density on membrane post-synaptic to mossy fibre terminals (MS), membrane of granule cell body (BG), extrasynaptic dendritic membrane of granule cell (DG), and membrane post-synaptic to Golgi cell terminals (GS) are approximately MS:BG:DG:GS = 0:1:2:230 and MS:BG:DG:GS = 0:1:3.3:180 for the α_1 and the $\beta_{2/3}$ subunits, respectively. Data from Nusser et al. (1995a).

carried out (Nusser et al., 1995a; 1996). All of these $GABA_A$ receptor subunits are concentrated in GABAergic Golgi synapses and they have a much lower concentration in the extrasynaptic somatic and dendritic membrane (Figs. 5 and 6). Interestingly, the $\alpha 6$ subunit was found to be concentrated in excitatory mossy fibre synapses as well (Nusser et al., 1996; Figs. 6 and 10). The

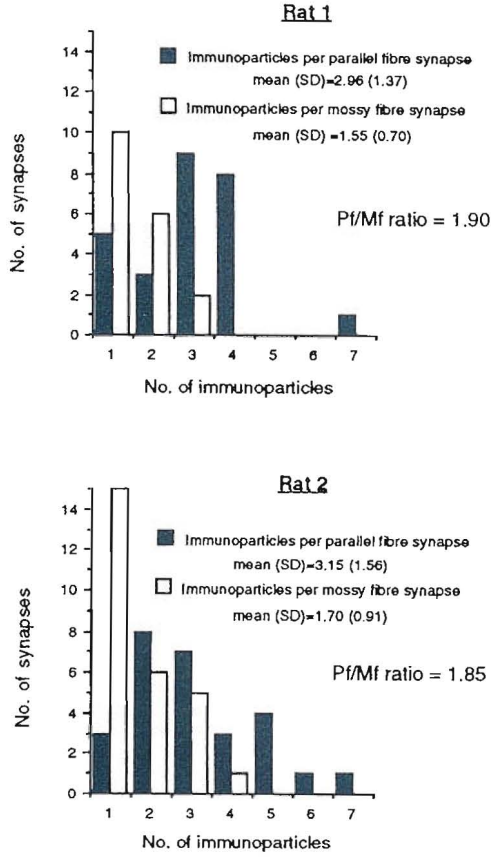


Fig. 8. Quantitative distributions of immunopositive parallel and mossy fibre synapses according to their immunoreactivity for GluR B/6/4c subunits. Immunoreactivity in synapses made by parallel fibre terminals with spines of Purkinje cells is significantly higher ($P < 0.001$, Mann-Whitney test, rat 1: $Z = -3.46$; rat 2: $Z = -3.59$) than in synapses established between mossy fibre terminals and granule cell dendrites. Adopted from Nusser et al. (1994).

possible functional significance of the presence of the $GABA_A$ receptor $\alpha 6$ subunit in mossy fibre synapses is unknown. Since the $\alpha 1$ and the $\beta 2/3$ subunits were not found in glutamatergic mossy synapses, this result shows that there is a partially differential synaptic targeting of distinct $GABA_A$ receptor subunits on the surface of granule cells.

Golgi synapses were found to be homogeneous according to their $\beta 2/3$ subunits content (Fig. 9), but they are heterogeneous with regard to their $\alpha 1$ subunit concentration (Fig. 9). Two populations of synapses were found, one with a high, another with a low or zero $\alpha 1$ subunit immunoreactivity (Fig. 9). A possible explanation for the heterogeneity of Golgi synapses with regard to their $\alpha 1$ subunit content is that synapses with low $\alpha 1$ subunit content may contain high concentrations of the $\alpha 6$ subunit. In order to decide whether the $\alpha 1$ and $\alpha 6$ subunits are located in the same synapses, they are segregated into two populations of synapses, or both possibilities occur, immunogold localisation of the $\alpha 1$ and $\alpha 6$ subunits was carried out on consecutive, electron microscopic sections of the same synapses. Although numerous Golgi synapses are immunopositive for both the $\alpha 1$ and $\alpha 6$ subunits, many synapses were found to be immunopositive for only one of these α subunits (Fig. 10). This result demonstrates the heterogeneity of Golgi synapses. There are synapses with very high $\alpha 1$ to $\alpha 6$ subunit ratio, with ratio approximately one, and with very low $\alpha 1$ to $\alpha 6$ ratio. These results are in agreement with the findings that spontaneous IPSCs in granule cells decayed with both a fast and a slow components. These two components could be differentially modified by diazepam, indicating that they may correspond to the activation of two distinct $GABA_A$ receptors (Puia et al., 1994). The faster, diazepam-sensitive current component may be due to the activation of $GABA_A$ receptors containing $\alpha 1$, βx, $\gamma 2$ subunits, whereas the slower, diazepam-insensitive component may correspond to the activation of the $\alpha 6$ subunit containing $GABA_A$ receptors (Pritchett et al., 1989; Luddens et al., 1990; Khan et al., 1994; Quirk et al., 1994). The proportion of the fast and slow current components varied within single granule cells (Puia et al., 1994), which is in agreement with the variable $\alpha 1$ to $\alpha 6$ subunit ratio.

The ratio of α subunits in Golgi synapses may be determined by the activity of pre- or post-synaptic elements. Such an activity-dependent modification of synaptic receptor composition could lead to a differentiation of post-synaptic responses according to the needs of the system. The use

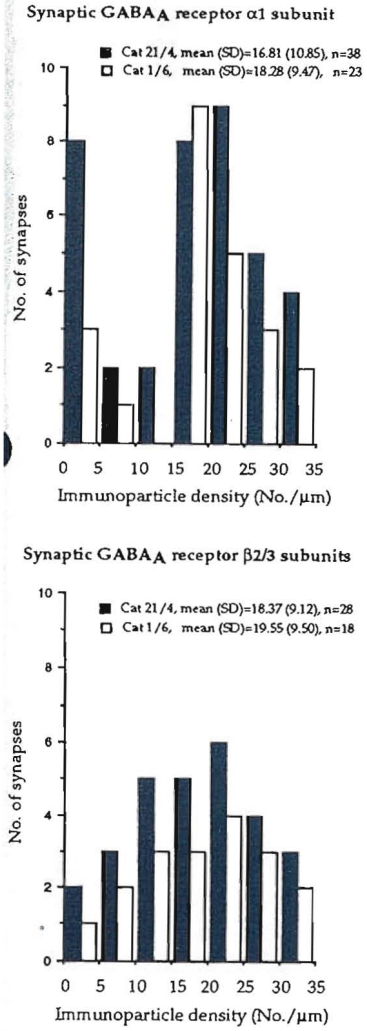


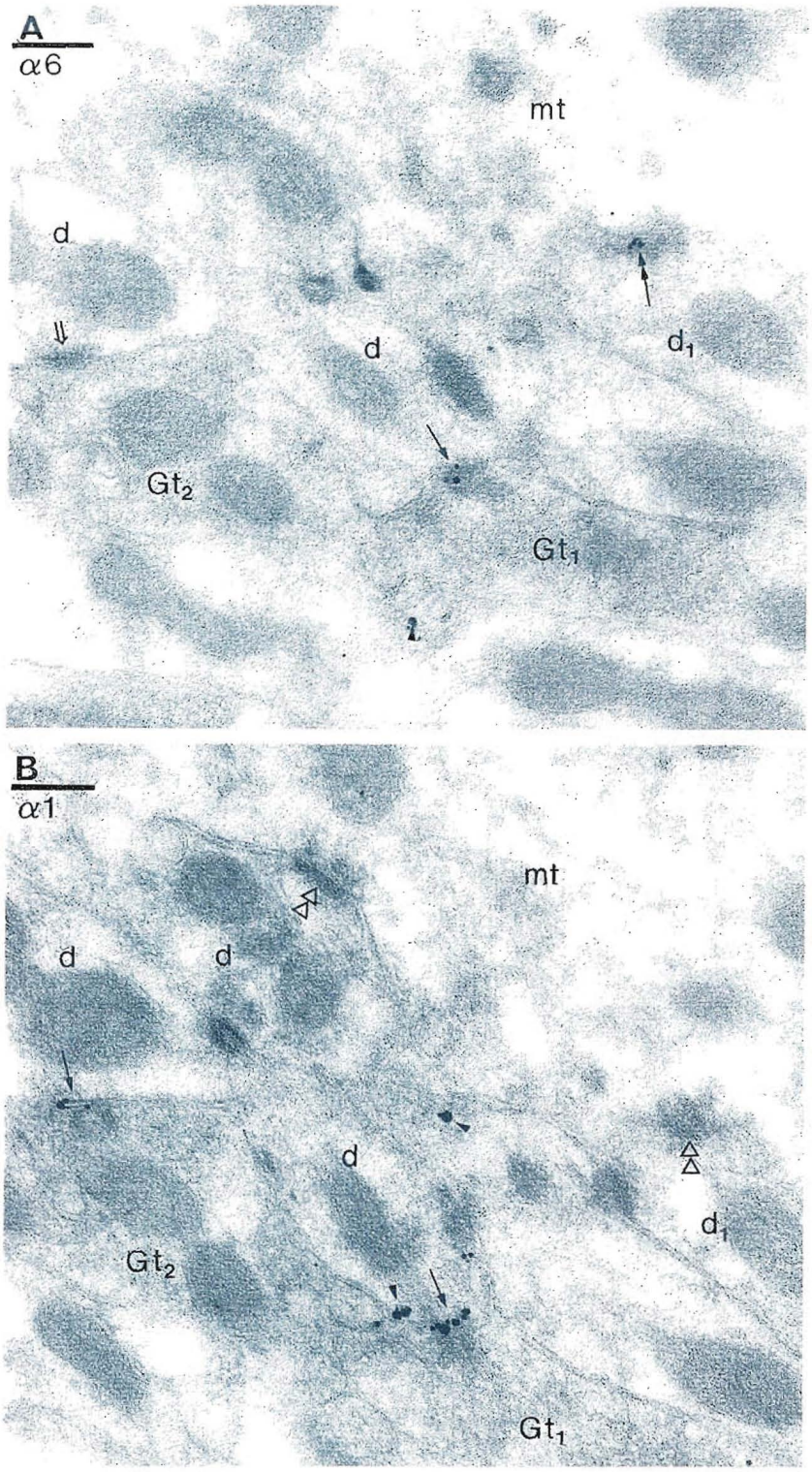
Fig. 9. Quantitative distribution of synapses established by Golgi cell terminals and granule cell dendrites according to their immunoreactivity for the α_1 (A) and $\beta_{2/3}$ (B) subunits in cat cerebellum. A, The distribution of Golgi synapses with regard to α_1 subunit immunoreactivity is different from normal distribution (Chi-square test: $\chi^2 = 17.46$, $P < 0.002$) indicating two populations of synapses. The distribution in the two animals is not different (Mann–Whitney test: $Z = -0.11$, $P = 0.91$). B, Frequency distribution of Golgi synapses immunoreactive for the $\beta 2/3$ subunits is normal in both animals (Chi-square test: cat 21/4, $\chi^2 = 0.115$, $P = 0.862$; cat 1/6, $\chi^2 = 0.096$, $P = 0.997$), indicating one population of Golgi synapses for $\beta_{2/3}$ subunits immunoreactivity. Adopted from Nusser et al. (1995a).

of quantitative electron microscopic immunocytochemistry has particular potential in brain areas where synapses, apparently homogeneous anatomically, may contain different sets of receptors. Creating subpopulations of synapses according to their receptor content could increase the computational capacity of neural circuits.

Subsynaptic segregation of ionotropic and metabotropic glutamate receptors

In addition to the segregation of receptors on the cell's surface between different synapses, the post-synaptic membranes can also be parcellated by distinct receptor types. For example, mGluR1α was found to be concentrated around the post-synaptic specialisation of cerebellar parallel and climbing fibre synapses, but it was absent from the anatomically defined synaptic junctions on Purkinje cells (Fig. 1 and 3A) and also on hippocampal neurones (Baude et al., 1993). Similarly, the mGluR5 subtype is also concentrated around the synaptic specialisation of asymmetrical synapses in hippocampal pyramidal cells (Lujan et al., 1996). Furthermore, the G-protein coupled neurokinine type I receptor (Lujan, Shigemoto and Somogyi, unpublished observation) as well as dopamine receptors (D1, D2; Yung et al., 1995) have been found to be excluded from the main body of synaptic junctions in the neostriatum. By contrast, the ionotropic glutamate, GABA$_A$ and glycine receptors are concentrated in the middle of the synaptic junctions in the CNS (see above). Thus the two types of receptor appear to be at different parts of the post-synaptic disc when localised in separate experiments. We therefore tested whether the same synapse contains both receptor types (Nusser et al., 1994). The co-localisation of the AMPA-type and metabotropic GluRs in the same synaptic junction in the cerebellar cortex confirmed the subsynaptic segregation of these two receptor classes (Fig. 3). It is possible that the perisynaptic location of the G-protein coupled receptors and the enrichment of the ionotropic receptors in the main body of the synaptic junctions are general features of their distribution.

A possible reason for the more peripheral position of the G-protein coupled receptors is that


A
α6
mt
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d1
Gt2
Gt1
B
α1
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they are in precise conjunction with voltage and/or ligand-dependent Na^+, K^+ and Ca^{2+} channels that they modulate (e.g. Charpak et al., 1990; Baskys, 1992; Crepel et al., 1994; Guerineau et al., 1994, 1995). These channels may only be present in the non-junctional membrane of post-synaptic cells. The subsynaptic segregation of ionotropic and metabotropic receptors may also result in their differential operation and/or regulation by intracellular modulators. Finally, a likely consequence of this spatial segregation of receptors is that low frequency presynaptic activity exclusively activates the ionotropic receptors, whereas high frequency presynaptic stimuli leads to the activation of both the ionotropic and metabotropic receptors (Bashir et al., 1993; Batchelor and Garthwaite, 1993; Miles and Poncer, 1993; Batchelor et al., 1994).

Conclusions

We have demonstrated that nerve cells are able to express receptor proteins at different concentrations on their surface, and distinct $GABA_A$ receptor subunits are differentially targeted to synapses on the surface of the same type of neurone. The enrichment of ionotropic receptors in the synaptic junctions with an abrupt decrease in receptor density at the edge of the junction can provide the structural basis for the uniformity of the fast post-synaptic response at a given synapse. The perisynaptic location of post-synaptic mGluRs may have the consequence that only high frequency presynaptic activity results in their activation. The degree of post-synaptic mGluR activation may depend upon the frequency of presynaptic release and the extent of glutamate spill-over. Whether the subsynaptic segregation of transmitter gated ion channels and G-protein coupled receptors activated by the same transmitter applies to other transmitters such as GABA or acetylcholine remains to be tested.

Abbreviations: ABC, avidin-biotinylated horseradish peroxidase complex; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; CNS, central nervous system; EPSC, excitatory post-synaptic current; GABA, γ-aminobutyric acid; GluR, glutamate receptor; GluR B/C/4c, B, C and 4c subunits of the glutamate receptor; GluRD, D subunit of the glutamate receptor; IPSC, inhibitory post-synaptic current; mGluR, metabotropic glutamate receptor; mGluR1α, 1α subtype of the metabotropic glutamate receptor; mGluR5, 5 subtype of the metabotropic glutamate receptor; NMDA, *N*-methyl-D-aspartate;

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Fig. 10. Serial ultrathin sections of rat cerebellum reacted either for the α_6 (A; C-terminal antibody) or the α_1 (B; antibody P16) subunit of the $GABA_A$ receptor. A synapse (arrow) made by a Golgi cell terminal (Gt_1) with a granule cell dendrite (d) shows immunoreactivity for both the α_6 and α_1 subunits. Another synaptic junction between a Golgi cell terminal (Gt_2) and a granule cell dendrite is immunopositive for the α_1 (arrow in B), but immunonegative for the α_6 subunit (open arrow in A). Mossy synapses are immunonegative for the α_1 subunit (double open triangles in B), but one of them is immunopositive for the α_6 subunit (double arrow in A). Immunoparticles are also present at extrasynaptic sites (arrowheads). Adapted from Nusser et al. (1996). Scale bars: 0.2 μm.

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