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Colocalization of glycine-like and GABA-like immunoreactivities in Golgi cell terminals in the rat cerebellum: a postembedding light and electron microscopic study

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Consecutive sections of rat cerebella were incubated with antisera raised against glycine or γ-aminobutyric acid (GABA) conjugated to protein by glutaraldehyde. The sections were subsequently processed according to the peroxidase–antiperoxidase technique (semithin sections) or treated with a secondary antibody coupled to colloidal gold particles (ultrathin sections). Corroborating previous light microscopic observations based on pre-embedding immunocytochemistry[20], a major proportion (about 70%) of the Golgi cell bodies showed immunoreactivity for both glycine and GABA. Analyses of semithin sections further suggested that the two immunoreactivities were colocalized in the same glomeruli and even in the same Golgi cell terminals. This was confirmed by electron microscopy. Quantification of the immunogold labelling for glycine (which is assumed to play metabolic roles in addition to its presumed role as a transmitter) showed that the net gold particle density was an order of magnitude higher over Golgi cell terminals than over the other constituents of the cerebellar glomeruli (mossy fibre terminals and granule cell dendrites). The total particle density over the latter was only slightly higher than the background level (over empty resin), suggesting that the concentration of 'metabolic' glycine is generally low compared to the concentration of glycine in Golgi cells. The stellate and basket cell terminals (which similarly to the Golgi cells are thought to release GABA as transmitter) were immunoreactive for GABA, but (with very few exceptions) virtually unlabelled for glycine, suggesting that our results were not confounded by any crossreactivity of the glycine antiserum with fixed GABA. Direct evidence that the sera reacted selectively with fixed glycine or GABA under the conditions used was obtained by incubating the tissue sections together with test sections containing a series of different amino acid–glutaraldehyde–brain macromolecule conjugates. Adsorption tests with soluble amino acid–glutaraldehyde complexes similarly suggested that the double-labelling of the Golgi terminals indeed reflected a colocalization of glycine and GABA. The results show that two 'classical' transmitters, both being inhibitory and acting on Cl^- channels, may coexist in the same nerve terminals.

INTRODUCTION

Among the inhibitory transmitter candidates so far identified in the mammalian central nervous system (CNS), GABA (γ-aminobutyric acid) and glycine are the most widespread. GABA is thought to act as a transmitter at all levels of the neuraxis, whereas the presumed glycine-using neurons show a more restricted localization with a preference for caudal parts of the CNS (reviews see refs. 2, 8, 17, 21). While the prevailing view has been that GABA and glycine are used by separate neuronal systems, recent evidence indicates that the two amino acids may be more closely related than previously assumed, both functionally and distributionally. According to patch clamp analyses GABA and glycine receptors act on the same or very similar chloride channels (although with different effects on their conductance states)[3,11]. Further, electron microscopic studies (in the spinal cord) have shown that terminals immunoreactive for the GABA synthesizing enzyme, glutamic acid decarboxylase (GAD), can be apposed to glycine receptors identified by means of specific antibodies[37], providing a possible morphological corre-

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late for the finding that GABA is capable of modifying allosterically the glycine receptor in certain systems[40]. Finally, by using antisera directed against fixed amino acids it has been shown at the light microscopical level that GABA- and glycine-like immunoreactivities (GABA-LI and glycine-LI) coexist in certain neuronal cell bodies[20,24,39] including those of the cerebellar Golgi cells[20,24], raising the possibility that these two amino acids could be coreleased from the same neurons. The purpose of the present study is to further investigate this possibility by resolving whether the two amino acids coexist also in the nerve terminals of the cerebellar Golgi cells.

MATERIALS AND METHODS

Experimental material

Male Wistar rats ($n = 8$, 250–300 g) were anesthetized with pentobarbital (50 mg/kg) and perfused through the heart with a mixture of 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, at room temperature (15 min, 50 ml/min), following a brief flush (10–15 s) of 2% dextran (mol. wt. 70,000) in the same buffer (4 °C). All animals developed neck rigidity indicative of fixation within 90 s after thoracotomy. The brains were postfixed for 3 h or overnight in the perfusion liquid and then transferred to the above buffer. Tissue blocks from the cerebellum were osmicated, dehydrated in graded ethanols and propylene oxide, and embedded in Durcupan (ACM, Fluka). Serial semithin (0.5 μm) and ultrathin sections were cut in the transverse or sagittal plane with a Reichert ultramicrotome and mounted on gelatinized glass slides, and nickel mesh grids, respectively.

Sera

GABA antisera 26 (ref. 22) and 09 (ref. 13) were obtained from rabbits after repeated immunizations with GABA–glutaraldehyde–bovine serum albumin (BSA) complexes. GABA antiserum 33 and glycine antiserum 31 were raised according to a slightly different strategy modified from Seguela et al.[29]; instead of using BSA throughout, the carrier was changed for each immunization[7,20,25]. For electron microscopy the crude antisera were preadsorbed with 60 μg/ml glutaraldehyde-treated brain macromolecules (antiserum 26), 1% normal sheep serum (antiserum 09), or glutamate–glutaraldehyde fixation complexes[25] (final concentration 300 μM with respect to glutamate; antiserum 33 and 31). The light microscopical observations were obtained with aliquots of antisera 26 and 31 that had been purified in solid phase (agarose) by sequential adsorptions with glutaraldehyde-treated BSA and amino acid–glutaraldehyde–BSA conjugates[7,20,22]. The amino acids used were glutamate (for antiserum 31) and glutamate followed by taurine (for antiserum 26). Antiserum 31 was subjected to an additional purification step with the individual carrier proteins treated with glutaraldehyde; this step did not, however, further improve the specificity. The antisera 26, 31 and 33 have been tested (concentrations up to 1:50) against a large series of amino acids and other compounds (alanine, β-alanine, arginine, asparagine, aspartate, α-aspartylglycine, cadaverine, carnosine, cysteic acid, cysteine sulfinic acid, cysteine, cystine, epinephrine, GABA, glutamate, glutamine, glycine, histidine, homocarnosine, leucine, leucine-enkephalin, lysine, methionine, methionine-enkephalin, *N*-acetyl aspartate, *N*-methyl aspartate, norepinephrine, ornithine, phosphoethanolamine, proline, putrescine, reduced glutathione, serine, spermidine, spermine, taurine, threonine, tryptophan, tyrosine, valine) conjugated to brain macromolecules by glutaraldehyde and have shown significant reactivity only with the amino acid against which the serum was raised[25]. Antiserum 09 has been similarly characterized[13].

Immunocytochemical procedures

Post-embedding staining of semithin sections. The procedure was essentially similar to that of Somogyi et al.[33]. Briefly, the sections were etched with sodium ethanolate, and treated with 1% sodium periodate (to remove osmium), followed by sequential incubations in: (1) 20% normal sheep serum; (2) glycine antiserum 31 or GABA antiserum 26, diluted 1:50 or 1:100, respectively in Tris-phosphate-buffered saline containing 1% normal sheep serum (18 h in room temperature); (3) sheep anti-rabbit IgG (own produce, 1:10); (4) rabbit peroxidase–antiperoxidase complex (Dakopatts, 1:100); and (5) diaminobenzidine/H_2O_2. The staining was intensified by treating the sections with OsO_4 (2 drops of 1% OsO_4 in 50 ml 0.1 M sodium phosphate buffer, pH 7.4, 5 min). The

sections were thoroughly rinsed between the steps.

Post-embedding immunogold staining of ultrathin sections. The procedure was modified from Somogyi and Soltész[34] and involved the following treatments: (1) 1% HIO_4 in H_2O (7 min) followed by 1% $NaIO_4$ in H_2O (7 min) to remove osmium; (2) 1% human serum albumin (10 min); (3) glycine antiserum 31, or GABA antisera 33, 26, or 09; (4) polyethylenglycol (mol. wt. 20,000; 50 mg/100 ml 0.05 M Tris buffer, pH 7.4, 5 min); (5) goat anti-rabbit IgG coupled to colloidal gold particles with mean diameter 15 nm (Janssen), diluted 1:20 in the solution used in the preceding step (1 h); (6) 1% uranyl acetate (20 min) followed by lead citrate (1–3 min). The primary antisera were diluted in Tris-phosphate-buffered saline (dilutions varying between 1:100 and 1:500 for antisera 31, 33, and 26, and between 1:1000 and 1:3000 for antiserum 09), containing additives as described above, and were applied to the sections for 60 min to 3 h. An essential difference between the present procedure and that of Somogyi and Soltész[34] was the omission of normal serum in the blocking and rinsing solutions, and in the diluting buffer for the primary antisera (except for antiserum 09). For antisera 31, 33, and 26, the addition of normal serum greatly increased the level of unspecific labelling. Another difference between the two procedures was that in the present study the grids were immersed in the different solutions instead of floated on the droplets. The GABA antiserum 33 required special incubation conditions: the concentration of NaCl in the buffer was kept at 0.03 M (instead of 0.12 M for the other antisera) and the incubation was performed at 25 °C in a thermostat (instead of at room temperature). The sections were extensively rinsed between the steps, except after steps 2 and 4, and were dried before staining with uranyl acetate and lead citrate.

Specificity controls

Semithin (0.5 μm) or ultrathin Durcupan sections (Figs. 1, 5, 6) containing a series of different amino acid–glutaraldehyde–brain macromolecule conjugates were mounted on glass slides (in close proximity to the tissue section) or on nickel mesh grids, respectively[18], and immersed in the same drops of sera and buffers as the tissue sections throughout the immunocytochemical procedure. For additional control, aliquots of the different antisera were preadsorbed with glutaraldehyde fixation complexes of glycine or GABA[25]. Some sections were incubated with a preimmune serum instead of an antiserum.

Computer analysis

Gold particle densities over test conjugates and cell profiles were assessed in electron micrographs with a final magnification of 51,800×. Areas were calculated on a Calcomp 9480 digitizer connected to a Cromemco CS300 computer, and the numbers of gold particles over each profile were subsequently counted and entered manually from the keyboard[18, 19]. The computer programme (Morforel 8) was developed by Th.W. Blackstad.

RESULTS

Light microscopy

Glycine-like immunoreactivity (glycine-LI) was concentrated in Golgi cell bodies and in circularly arranged dots interpreted as Golgi cell terminals (Fig. 1A). The molecular layer was unlabelled except for scattered processes and bouton-like dots and an occasional cell body (0–5 per sagittal section compris-

TABLE I

Specificity of antisera assessed on sections of Durcupan-embedded amino acid–glutaraldehyde macromolecule conjugates

The data presented were obtained from the same ultrathin test sections as Figs. 5 and 6. Values represent number of gold particles/μm^2 ± S.E.M. after subtracting the gold particle density over empty resin (12.1 for the left column and 8.3 for the right column). The number of observations is shown in parenthesis (*n*). *None* denotes conjugates made by reacting brain macromolecules with glutaraldehyde without adding amino acids. Asterisks represent values significantly different from 'None': $*P < 0.05$; $**P < 0.001$ (Student's *t*-test). The absolute density values are not comparable to those over tissue profiles (Table II), since the test conjugates were prepared at an amino acid concentration corresponding to as high as 200 mM in the brain and were condensed during embedding[18].

Amino acid	*Mean gold particle density ± S.E.M. (n)*	
	Glycine antiserum 31 (1:100)	*GABA antiserum 33 (1:200)*
GABA	79.5 ± 17.7 (6)	2068.6 ± 151.1 (7)**
Glutamate	81.0 ± 17.8 (6)	43.3 ± 8.0 (7)*
Taurine	96.1 ± 17.1 (6)	61.7 ± 26.1 (8)
Glycine	2144.6 ± 213.0 (7)**	36.1 ± 10.8 (10)
None	78.0 ± 12.6 (6)	19.8 ± 2.7 (6)
Aspartate	60.4 ± 18.1 (5)	29.3 ± 5.8 (8)
Glutamine	75.2 ± 11.8 (7)	37.6 ± 18.4 (6)

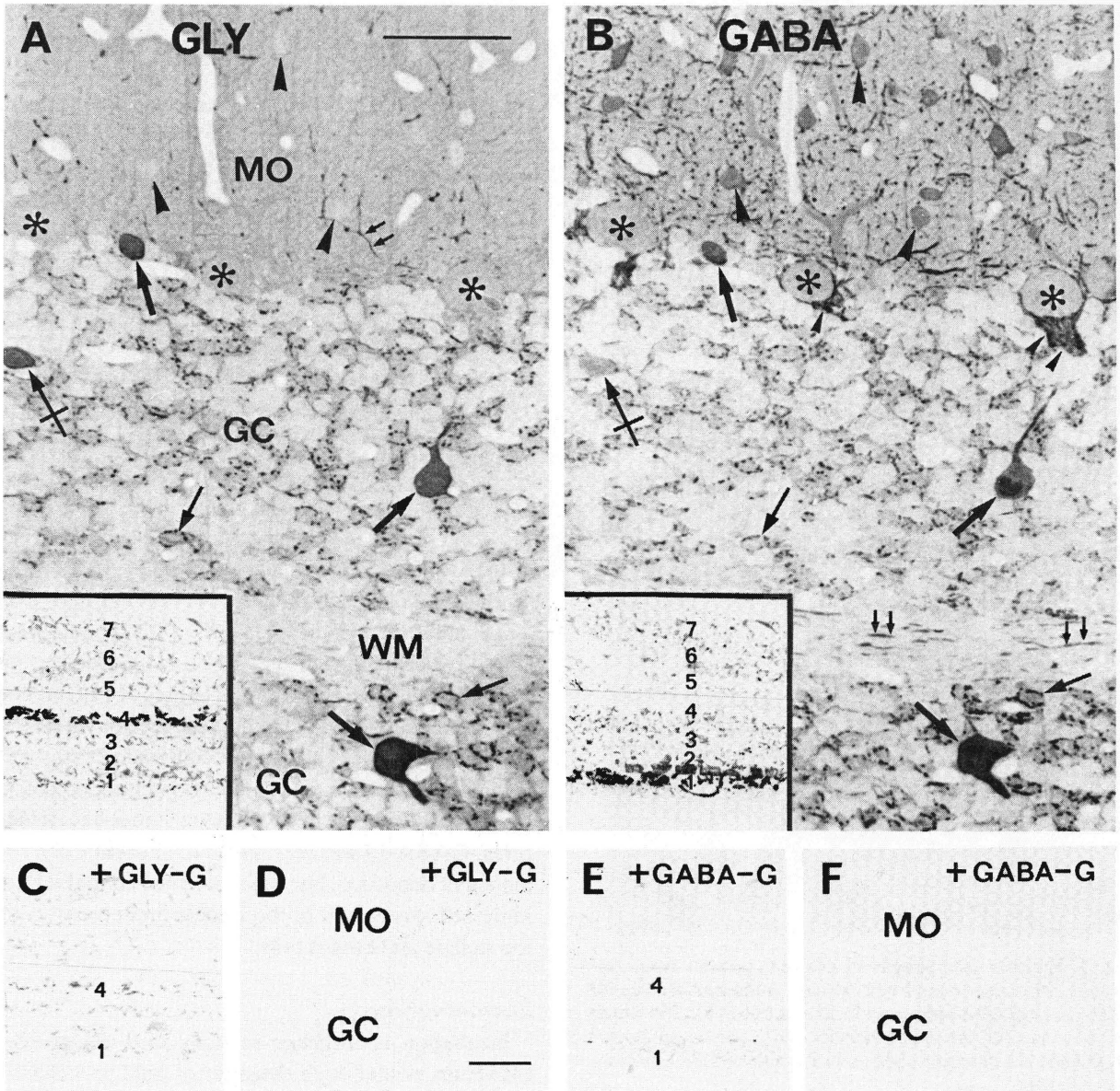

Fig. 1. Photomicrographs of semithin (0.5 μm) tissue and test sections incubated with glycine antiserum 31 diluted 1:50 (A,C.D) or GABA antiserum 26 diluted 1:100 (B,E,F). A and B: 3 of the 4 Golgi neurons that are glycine immunoreactive (thick arrows) are also stained with the GABA antiserum in the adjacent section; the fourth glycine-positive neuron (crossed arrow) is virtually immunonegative for GABA. Most if not all glomeruli (thin arrows) show GABA-LI-positive as well as glycine-LI-positive Golgi cell terminals. The molecular layer contains no glycine-LI positive structures except for a few fibrous processes (small arrows in A). The terminals of the basket (small arrowheads) and stellate cells and their respective cell bodies (large arrowheads) are glycine immunonegative, but GABA immunopositive. Asterisks, Purkinje cell bodies; small arrows in B, GABA immunoreactive fibres (probably of Purkinje cell origin) in the white matter (WM). Other abbreviations: MO and GC, molecular and granule cell layers. Insets show test sections mounted on the same slides as the respective tissue sections and incubated together with these. The test sections are cut perpendicularly through sections containing different amino acid conjugates, alternating with rat brain sections used as spacers[18]. The conjugates were made by reacting a crude brain macromolecular extract (extensively dialyzed to remove all small molecular weight compounds) with glutaraldehyde and GABA (1), glutamate (2), taurine (3), glycine (4), aspartate (6), or glutamine (7), or with glutaraldehyde alone (5). Note selective staining of the respective amino acid conjugates. The dark structures between 1 and 2 in B (inset) represent GABA-LI positive cell bodies in spacer section (taken from rat hippocampus). C–F: adsorption controls. The addition of glutaraldehyde (G) complexes of glycine or GABA (200 μM with respect to the amino acid) to the respective antisera almost abolished staining of test conjugates (C,E) and tissue sections (D,F). Complexes of other amino acids did not have this effect. Bar = 50 μm.

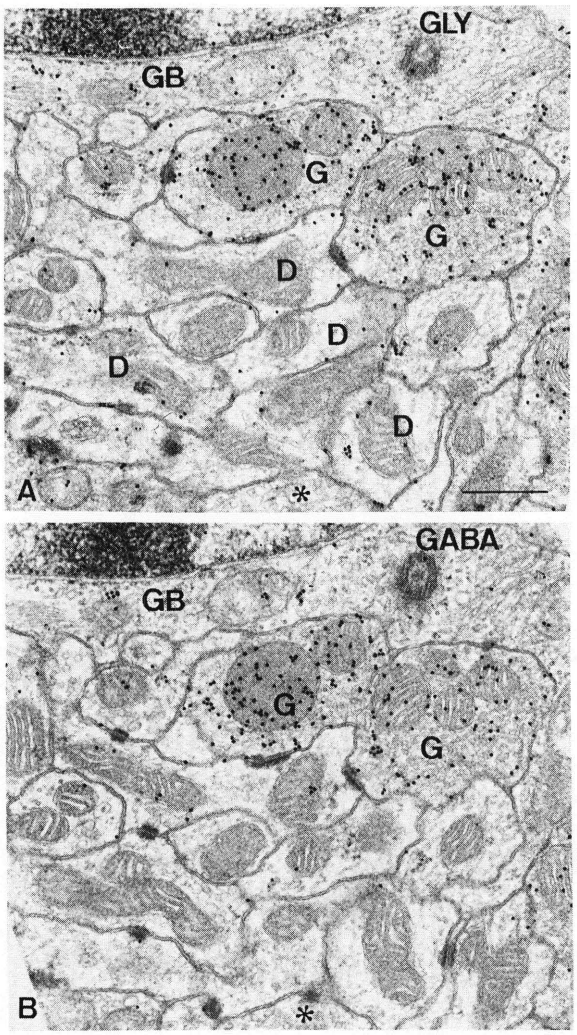

Fig. 2. Electron micrographs of adjacent sections from the granule cell layer treated with glycine antiserum 31 diluted 1:100 (A) or GABA antiserum 33 diluted 1:200 (B). The Golgi cell terminals (G) are double labelled. GB, granule cell body; D, granule cell dendritic digits; asterisk, mossy fibre terminal. Bar = 0.4 μm.

ing all folia). No glycine-LI could be detected in the Purkinje cells or in the subcortical white matter. Adjacent sections (Fig. 1B) incubated with a GABA antiserum (antiserum 26) showed labelling of Golgi cells, most of which were also identified as glycine immunoreactive; countings revealed that about 70%

TABLE II

Glycine-like immunoreactivity in the different components of the cerebellar glomeruli

All data were obtained from the same ultrathin section (also represented in Fig. 2A) incubated with glycine antiserum 31 diluted 1:100. Values represent mean number of gold particles/μm^2 ± S.E.M., after subtracting the particle density over empty resin (7.0 particles/μm^2). The number of observations is shown in parenthesis (*n*).

Cell profile	*Mean gold particle density ± S.E.M. (n)*
Golgi cell terminals	143.1 ± 9.6 (10)**
Mossy fibre terminals	14.4 ± 1.5 (8)*
Granule cell dendritic digits	7.3 ± 2.2 (10)

* Significantly different ($P < 0.05$) from the following value.
** Significantly different ($P < 0.001$, Student's *t*-test) from the two following values.

of the Golgi cells* were double-labelled. In a representative pair of sagittal sections containing a total of 56 stained Golgi neurons, 8 cells showed only glycine-LI, 8 cells showed only GABA-LI, whereas 40 cells showed both immunoreactivities. Most, if not all, of the glomeruli contained both glycine immunoreactive and GABA immunoreactive elements, and in many cases it seemed as if the two immunoreactivities resided in the same nerve terminal-like structures. GABA-LI was very low in the Purkinje cell somata and dendrites, but was concentrated in the Purkinje cell axons, and in the somata and processes of the stellate and basket cells.

Electron microscopy

Incubation of ultrathin sections with the glycine antiserum yielded high densities of gold particles in Golgi cell terminals (Fig. 2) and in Golgi cell bodies (not illustrated). A few Golgi cell terminals showed particle densities similar to background level. Within the immunopositive Golgi terminals, the particles were located over mitochondria as well as over areas containing synaptic vesicles. The remaining cell profiles in the granule cell layer showed labelling close to background level, although the mossy fibre terminals were slightly more intensely labelled than the gran-

* In the present paper, all non-granule cell bodies in the granule cell layer were defined as Golgi cells, including the fusiform neurons situated directly beneath the Purkinje cells. The fusiform neurons may belong to a particular class of neurons (the Lugaro cell[16,26]); however, they shared the staining characteristics of the remaining non-granule cells, the majority containing glycine-LI as well as GABA-LI.

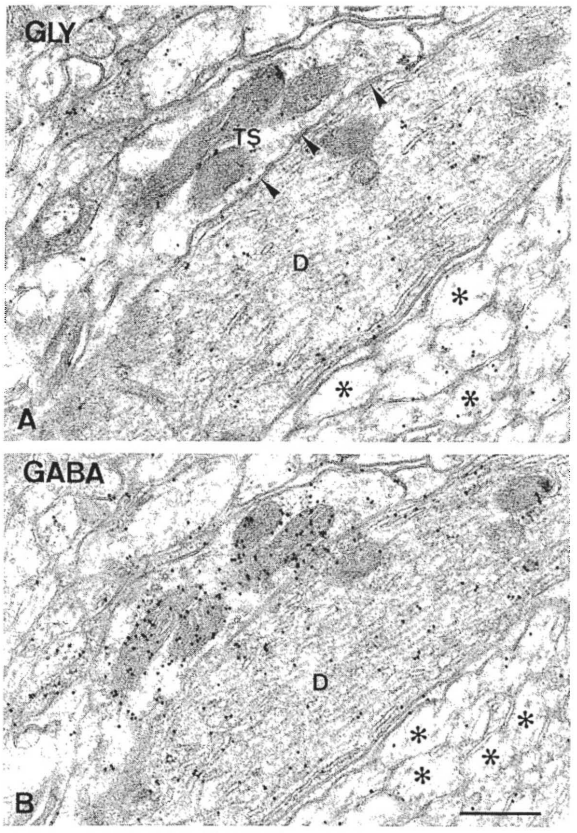

Fig. 3. Electron micrographs of adjacent sections from the molecular layer incubated with glycine antiserum 31 diluted 1:100 (A), or GABA antiserum 33 diluted 1:500 (B). A stellate cell terminal (TS) establishing symmetric contacts (arrowheads) with a Purkinje cell dendrite (D) is GABA immunopositive, but glycine immunonegative. The parallel fibers (asterisks) are unlabelled with both antisera. Bar = 0.4 μm.

ule cell dendrites (Table II). In the molecular layer, the particle density was very low over the parallel fibre terminals (Fig. 4A) and, similarly, over the vast majority of the boutons showing flattened or pleomorphic vesicles and symmetric junctions (Fig. 3A). However, occasional boutons of the latter type did contain glycine-LI; these terminals established synaptic contacts with Purkinje or non-Purkinje dendrites (Fig. 4A) or, very rarely, with Purkinje cell bodies (not illustrated). The origin of the glycine immunoreactive terminals in the molecular layer is unclear. The most likely possibilities are that they derive from the (very few) immunopositive stellate or basket cell bodies, or from Lugaro cells, the axons of which may enter the molecular layer[26]. The Golgi cells proper are not believed to contribute axons to the molecular layer.

Adjacent ultrathin sections treated with a GABA antiserum revealed that most of the glycine immunoreactive Golgi cell terminals also contained GABA-LI (Fig. 2). The distribution of gold particles within these terminals was similar to that observed after incubation with the glycine antiserum. In the molecular and Purkinje cell layers, GABA-LI occurred in the stellate and basket cell bodies and processes (Figs. 3B, and 4B,C); as mentioned above, these profiles were usually immunonegative for glycine. However, GABA-LI was also found in the few terminals in the molecular layer that did contain glycine-LI and could be identified in adjacent sections (Fig. 4C). In agreement with the light microscopic data, the Purkinje cell dendrites (Fig. 3B) and somata (Fig. 4B) were only weakly GABA-immunoreactive, whereas the mossy fibres, and the granule cells and their processes, showed particle concentrations similar to background level (Fig. 2B). The background labelling (assessed by the density of gold particles over empty resin) varied among the different experiments, but could be minimized by careful selection of incubation parameters (Fig. 2B). The 3 different GABA antisera produced essentially the same patterns of labelling.

Control experiments

The test sections incubated together with the tissue sections showed selective labelling of the conjugate against which the serum was raised (Figs. 1, 5, 6). With the glycine antiserum, only the glycine conjugates showed particle densities significantly different from that over glutaraldehyde-treated brain macromolecules (Table I). Corresponding results were obtained with the GABA antiserum, except for a barely significant ($P < 0.05$) reactivity with the glutamate conjugates. Preadsorption of the glycine antiserum with glycine-glutaraldehyde complexes, or the GABA antiserum with GABA-glutaraldehyde complexes (added to the diluted serum at a final concentration of 200 or 600 μM with respect to the amino acid) abolished labelling of test and tissue sections, both at the light microscopic (Fig. 1) and electron microscopic (not illustrated) levels. The specific labelling remained, however, after adsorption of the glycine antiserum with GABA-glutaraldehyde com-

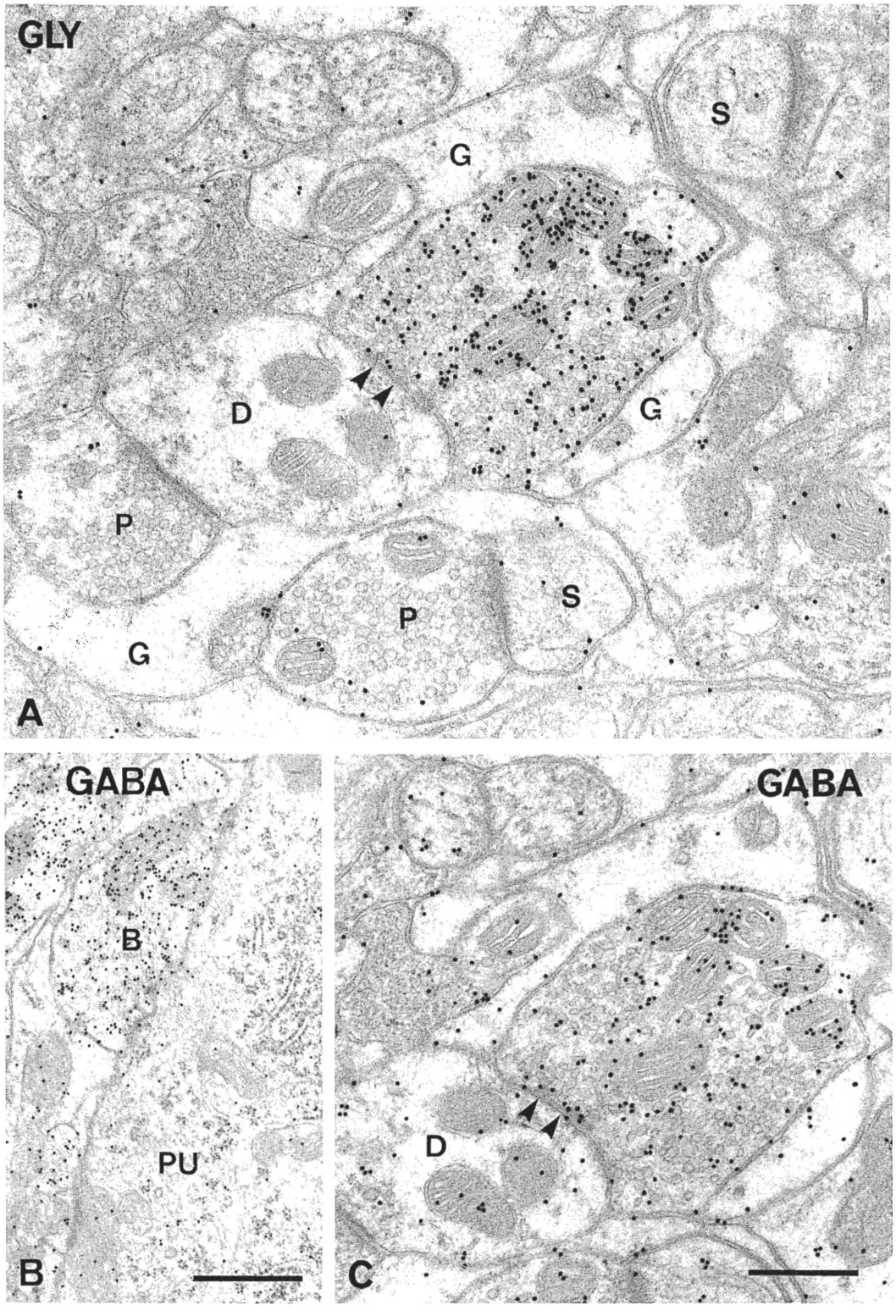

Fig. 4. Electron micrographs of cerebellar sections treated with glycine antiserum 31 diluted 1:150 (A), GABA antiserum 33 diluted 1:200 (B), or GABA antiserum 09 diluted 1:2000 (C). A: a glycine immunopositive nerve terminal with pleomorphic vesicles establishes a symmetric contact (arrowheads) with a non-Purkinje cell dendrite (D) in the molecular layer. (Most terminals of this kind are glycine immunonegative; see Fig. 3.) This terminal is also rich in GABA-like immunoreactivity (C). Note, in A, the very weak labelling of glial profiles (G), parallel fibre terminals (P), and Purkinje cell dendritic spines (S). B: a GABA immunoreactive basket cell terminal (B) contacts a Purkinje cell body (PU). This section was incubated with an antiserum that had been preadsorbed with 600 μM glycine–glutaraldehyde complexes. Bars: B, 0.6 μm; C, 0.4 μm.

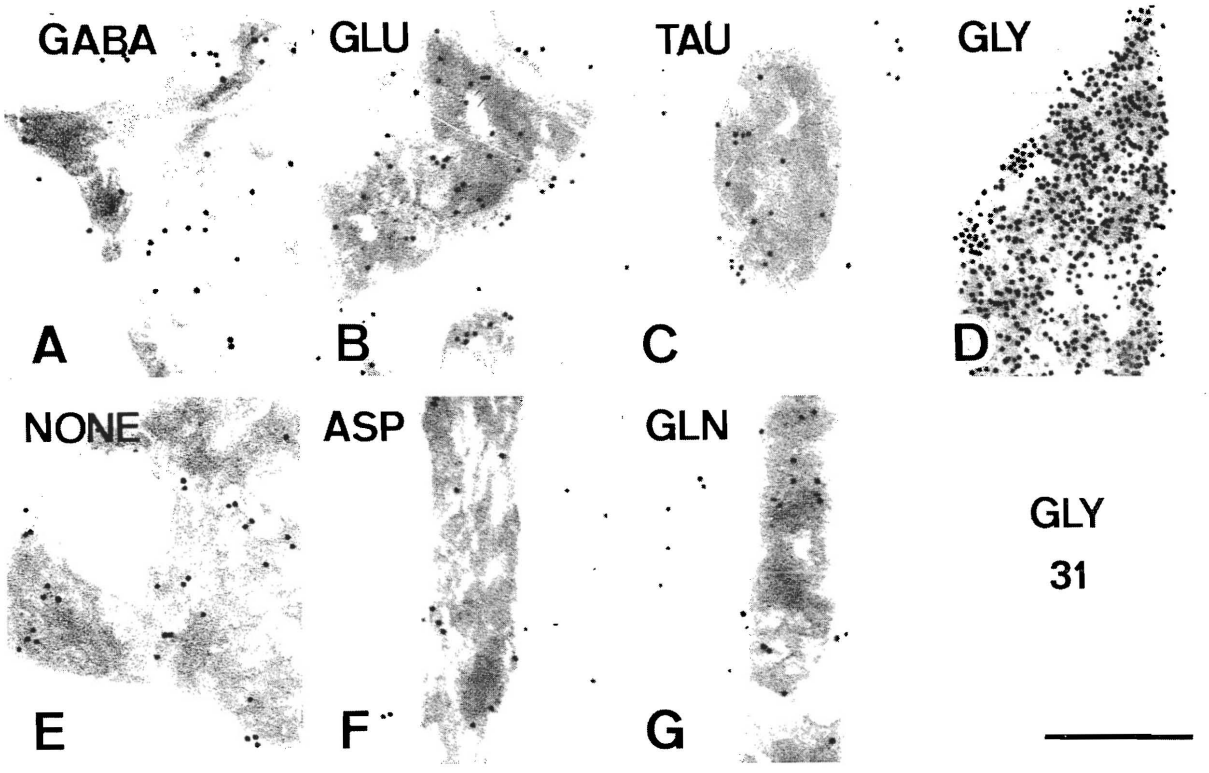

Fig. 5. Electron micrographs of amino acid–glutaraldehyde–brain macromolecule conjugates from a test section similar to those shown in Fig. 1 (but ultrathin), treated with glycine antiserum 31 diluted 1:100. Note the selective labelling of the glycine conjugates (D; also see Table I). Standard abbreviations for amino acids. NONE = glutaraldehyde-treated macromolecules (no amino acid added). The conjugates are condensed in clumps with diameters about 1 μm or less. Bar = 0.4 μm.

plexes** or the GABA antiserum with glycine–glutaraldehyde complexes at the same concentrations as above (Fig. 4B; also see Fig. 2 in ref. 20). Sections treated with a preimmune serum instead of the specific antisera were devoid of labelling.

DISCUSSION

In our previous study[20], based on Vibratome sections processed for light microscopy, we showed that glycine-LI and GABA-LI coexist in a subpopulation of Golgi cell bodies in rat cerebellum. In the present study we have exploited the superior resolution offered by post-embedding stained semithin and ultrathin sections to demonstrate that the two immunoreactivities are also colocalized in the same glomeruli, and even in the same Golgi cell boutons.

The distribution of GABA-LI in the cerebellum has been described in numerous reports[9,21,22,28,29,32], and our results are in good agreement with these. The three GABA antisera used in the present paper appear to be selective for fixed GABA; notably, there is no crossreactivity with glycine.

The glycine antiserum has been tested against a long list of compounds, including GABA, taurine, and β-alanine, and has been found to react selectively with fixed glycine. In immunocytochemical preparations, the antiserum labels neuronal populations for which there is strong evidence that glycine is the actual transmitter, such as the commissural interneurons of the *Xenopus* spinal cord[7], and the Renshaw cells in mammalian spinal cord[23]. That the high selectivity also obtains under the present post-embedding conditions was confirmed by incubating test sections,

** The addition of GABA–glutaraldehyde complexes to the glycine antiserum was followed by a slight decrease in labelling intensity, most notably at the electron microscopic level. This effect is probably due to unspecific interaction between the antibodies and the amino acid complexes, since the test sections revealed no significant crossreactivity with GABA (Figs. 1, 5).

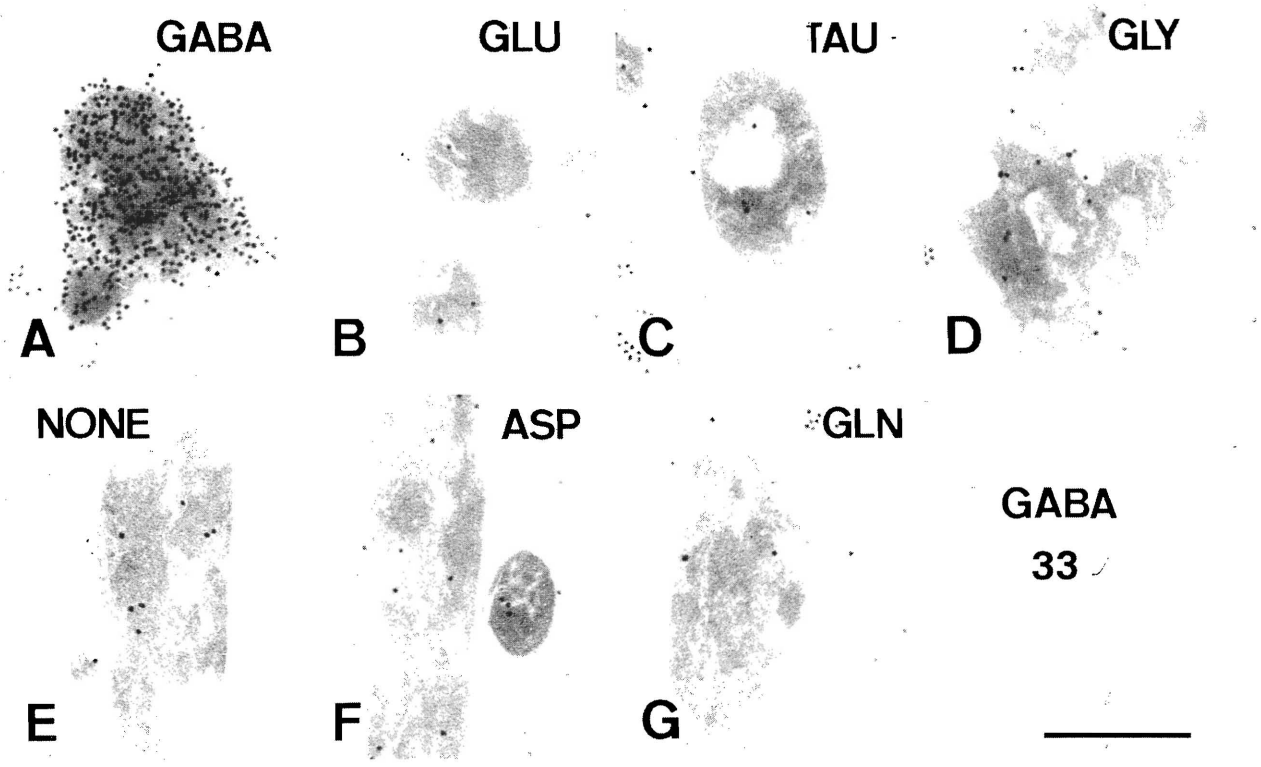

Fig. 6. Test conjugates from section adjacent to that shown in Fig. 5, but treated with GABA antiserum 33 diluted 1:200. The GABA conjugates (A; also see Table I) are selectively labelled. Bar = 0.4 μm.

containing a series of different amino acid conjugates, together with the tissue sections. The possibility that the labelling of Golgi cell terminals should be due to crossreactivity with GABA or other compounds was further minimized by the observation that the glycine serum failed to label the vast majority of stellate and basket cell terminals (which like the Golgi cell terminals are enriched in GABA-LI), and by adsorption tests. However, as is always the case in immunocytochemistry, the possibility cannot be totally excluded that the immunolabelling pattern could have been confounded by unknown substances sharing the antibody binding properties of the assumed antigen. As judged from the proposed structure of amino acid–glutaraldehyde complexes (with the amino acids attached by their amino groups[12]), the most likely interfering substances would be oligopeptides with a C-terminal glycine residue. It is important therefore, that neither glutathione (γ-glutamylcysteinylglycine), nor α-aspartylglycine, show crossreactivity in their fixed state[20].

Our finding of glycine-LI in Golgi cells also receives support from a study with a glycine antiserum raised independently of ours[5] and from an autoradiographic study at the electron microscope level[41]. The latter investigation indicated that a proportion of the Golgi cell terminals selectively accumulate radiolabelled glycine. The results were not entirely conclusive as to whether uptake of GABA and glycine could occur in the same terminals or whether the two amino acids were accumulated by strictly separate populations of terminals, although the authors interpreted their data to support the latter alternative (see ref. 20 for a more detailed discussion on this point). However, further analysis of the material indicated that a substantial proportion of the Golgi cell terminals could accumulate both amino acids[6].

Before the biological significance of a coexistence of glycine and GABA in the same Golgi terminals can be fully appreciated, it must be resolved whether both substances can be subject to synaptic release. In contrast to GABA, which is thought to function exclusively or primarily as a transmitter, glycine subserves several metabolic roles in the CNS in addition

to its transmitter role[2], raising the possibility that the glycine-LI in Golgi terminals merely reflects the presence of a (highly concentrated) metabolic pool. This is unlikely, for several reasons. First, since the Golgi cell terminals appear to be the only type of terminal in the cerebellum containing sizeable amounts of glycine, these are the probable source of the glycine that can be released Ca^{2+}-dependently in cerebellar slices[36]. Second, as noted above, the Golgi cell terminals (or a subpopulation of these) have been shown to sustain selective high-affinity glycine uptake[41]; a property that is assumed to be specific for terminals capable of glycine release. Third, the synthesis of glycine from serine (its major precursor) occurs at a higher rate in the cerebellum than in any other CNS region including the spinal cord[15]. A high turnover rate is typical of transmitter pools of amino acids. It must also be noted that the effects of glycine in the cerebellum are compatible with its presumed role as a transmitter (data reviewed in ref. 20). The transmitter release from Golgi cell terminals will now be studied by applying our amino acid antisera to cerebellar slices subjected to depolarization and other experimental manipulation in vitro. This strategy has previously proved successful for studying the synaptic handling of glutamate in the hippocampus[35].

A comparison of the present results with data on glycine receptor distribution fails to give a coherent picture. Antibodies selective for the glycine receptor[1,4,10,37,38] produced more intense labelling in the molecular layer than in the granule cell layer[37]. In the former layer immunoreactive material was frequently found postsynaptic to glutamic acid decarboxylase (GAD)-positive stellate cell terminals, whereas the granule cell layer was reported to exhibit immunoreactive patches postsynaptic to GAD-negative, but not to GAD-positive Golgi cell terminals. GABA receptor antibodies[27] have so far not given results at a comparable level of resolution. The spatial relationship between GABA and glycine and the respective receptors will have to be reexamined in material where the distributions of the appropriate markers can be directly compared. In the light of recent electrophysiological data[14] it would also be interesting to know whether the glycine-containing terminals are localized in close proximity to *N*-methyl-D-aspartate receptors.

Apart from the Golgi cells and scattered profiles in the molecular layer all structures in the cerebellar cortex showed very modest levels of glycine-LI. This suggests that the concentration of glycine in 'metabolic pools'[2] is low compared to that in 'transmitter pools', at least in the cerebellum. The small differences in gold particle density among the weakly labelled structures (e.g. between the granule cell dendrites and mossy fibre boutons) may reflect variations in the size of the metabolic pool. However, when attempts are made to interpret differences of this small magnitude, even the weak affinity of the antibodies to glutaraldehyde-treated brain macromolecules may become significant.

The high resolution of the post-embedding immunogold technique allows inference to be made about the antigen localization down to the level of organelles. In the Golgi cell terminals, glycine-LI is concentrated over groups of synaptic vesicles, but is also high over mitochondria. A high content of glycine in mitochondria would be consistent with the predominant mitochondrial localization of the enzyme serine hydroxymethyl transferase[2], which converts serine to glycine. However, it deserves emphasis that GABA-LI, glutamate-LI[31], and taurine-LI[19] are similarly accumulated in mitochondria of the labelled structures. This could be evidence of high levels of the amino acids in mitochondria, but also raises the possibility that the mitochondrial labelling could reflect an artifactual redistribution of amino acids during the perfusion procedure. Alternatively it could be due to particularly favourable conditions for the retention and fixation of amino acids in these organelles. The dense mitochondrial labelling is not caused by unspecific antibody binding since it was never observed in structures that were otherwise immunonegative, and was, like all other labelling, except background (over empty resin), competitively and specifically inhibited by addition of glutaraldehyde-treated glycine (200 μM) to the serum. Whether glycine-LI is indeed enriched in synaptic vesicles compared to the surrounding cytosol is an important question which in addition to the problems mentioned for the mitochondrial localization, also faces problems of spatial resolution. Studies are planned to address this issue.

In conclusion, the present study strongly suggests that glycine and GABA are colocalized in a majority of Golgi cell terminals in the cerebellum, pointing to their possible roles as cotransmitters.

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