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DEMONSTRATION OF A RELEASABLE POOL OF GLUTAMATE IN CEREBELLAR MOSSY AND PARALLEL FIBRE TERMINALS BY MEANS OF LIGHT AND ELECTRON MICROSCOPIC IMMUNOCYTOCHEMISTRY

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INTRODUCTION

The mossy fibres constitute the quantitatively most important afferent input to the cerebellum. Whereas the anatomical organization of this fibre system has been elucidated in great detail (5, 8, 9, 10, 47), little information has been available concerning the neurotransmitter(s) involved in this projection. Early reports favouring acetylcholine as a possible transmitter in the mossy fibres (24) have not been corroborated by more recent immunocytochemical studies of the acetylcholine synthesizing enzyme, choline acetyltransferase (28, 29). Several neuroactive peptides, including enkephalins (25, 40, 48), corticotropin-releasing factor (13), and substance P (26), have been localized to mossy fibres, but none of these are likely to mediate the fast excitation (15, 23) provided by these fibres. Moreover, several of the peptides associated with mossy fibres have only been detected in certain species or at certain developmental stages (e.g. ref. 22).

Several lines of evidence suggest that glutamate is responsible for fast signalling in a large proportion of the synapses in the CNS (12, 17). To test whether this could also be the case for the mossy fibre system, we have employed immunocytochemical procedures based on the use of antisera that selectively recognize fixed glutamate in tissue sections (30, 31, 43). In describing the results, the mossy fibre terminals will be compared with the terminals of the parallel fibres, for which glutamate is already accepted as a very strong transmitter candidate (review: ref. 30), and which, like mossy fibres, contain a high concentration of glutamate according to recent immunocytochemical studies in cat (42) and rat (27, 34). Parts of the presented results have been published previously (34, 36, 37, 38, 39).

METHODS

Perfusion fixation

Adult male Wistar rats (n = 10) were perfusion-fixed through the heart with a freshly prepared mixture of 2.5% glutaraldehyde and 1% (para) formaldehyde in 0.1 M sodium

phosphate buffer, pH 7.4. The perfusion procedure has been described in detail in previous reports (35).

Incubation and immersion fixation of tissue slices

After a brief transcardial rinse with ice-cold buffer, rat brains (n = 8) were quickly removed and 400 μm cerebellar slices cut on a Vibratome with the tissue immersed in ice-cold artificial cerebrospinal fluid (ACSF). The slices were incubated in ACSF in vitro. The standard ACSF was composed as follows (numbers indicate concentration in mM). NaCl 124 (in some experiments: NaCl 74 + Tris/HCl 50), KCl 3.8, $NaHCO_3$ 26, KH_2PO_4 1.2, $MgSO_4$ 1.3, glucose 10, $CaCl_2$ 2.4. Slices were depolarized by adding 40 or 55 mM KCl and reducing the concentration of NaCl (or, alternatively, that of Tris/HCl) correspondingly. Calcium dependence was tested by reducing the Ca^{2+} concentration to 0.1 mM and increasing the Mg^{2+} concentration to 10 mM ("calcium free medium"). In most experiments, consecutive slices from the same animal were incubated simultaneously for 20—60 min in four different media (1-4): calcium-containing ACSF with "physiological" (1) or "high" (2) K^+ concentration, or "calcium free" ACSF with "physiological" (3) or "high" (4) K^+ concentration. All slices were stabilized in medum 1 for 40—60 min at the start of the experiment. The media (pH 7.4, 33°C) were continuously gassed with 95% O_2/5% CO_2. After incubation, the slices were immersion-fixed for 1 h in a mixture of glutaraldehyde/paraformaldehyde (same composition as above).

Further processing of the tissue

Tissue specimens from perfusion-fixed or immersion-fixed material were osmicated, dehydrated, and embedded in Durcupan (ACM, Fluka). Semithin (0.5 μm) or ultrathin sections were mounted on glass slides or nickel grids, respectively. The semithin sections were etched with a mixture of ethanol and sodium hydroxyde and thereafter processed according to the peroxidase-antiperoxidase procedure as previously described (34; method based on ref. 41). The ultrathin sections were first treated with HIO_4 and $NaIO_4$, to alleviate the masking effect of osmium, and thereafter exposed to the primary antiserum (glutamate antiserum 03 or 13) followed by a secondary antibody coupled to colloidal gold particles (34, 37, 38, 42). The glutamate antisera, which had been purified in solid phase by absorption with agarose-albumin-glutaraldehyde-GABA (31), were further preadsorbed in liquid phase with glutaraldehyde fixation complexes (33) of glutamine and aspartate (8-16 h; 100 μM of each with respect to the amino acid). The optimum serum dilution was 1:300-1:1500. Electron micrographs were obtained at ×6300 primary magnification in a Philips EM 400 or CM 10. The tissue profiles were recorded on a digitizing tablet and the gold particle densities calculated by means of a computer programme (Morforel) (3, 4).

Specificity testing

The purified glutamate antisera used in the present report have been subjected to extensive specificity tests (31, 32). To ensure that the high degree of specificity also obtains under the conditions of the present immunocytochemical procedures, test conjugates were assembled in a "sandwich" (34), sections of which could be incubated together with the ultrathin and semithin tissue sections (Figs. 1, 5). These test conjugates were prepared from the six most abundant amino acids in the CNS by letting them react with glutaraldehyde and a macromolecular extract from rat brains.

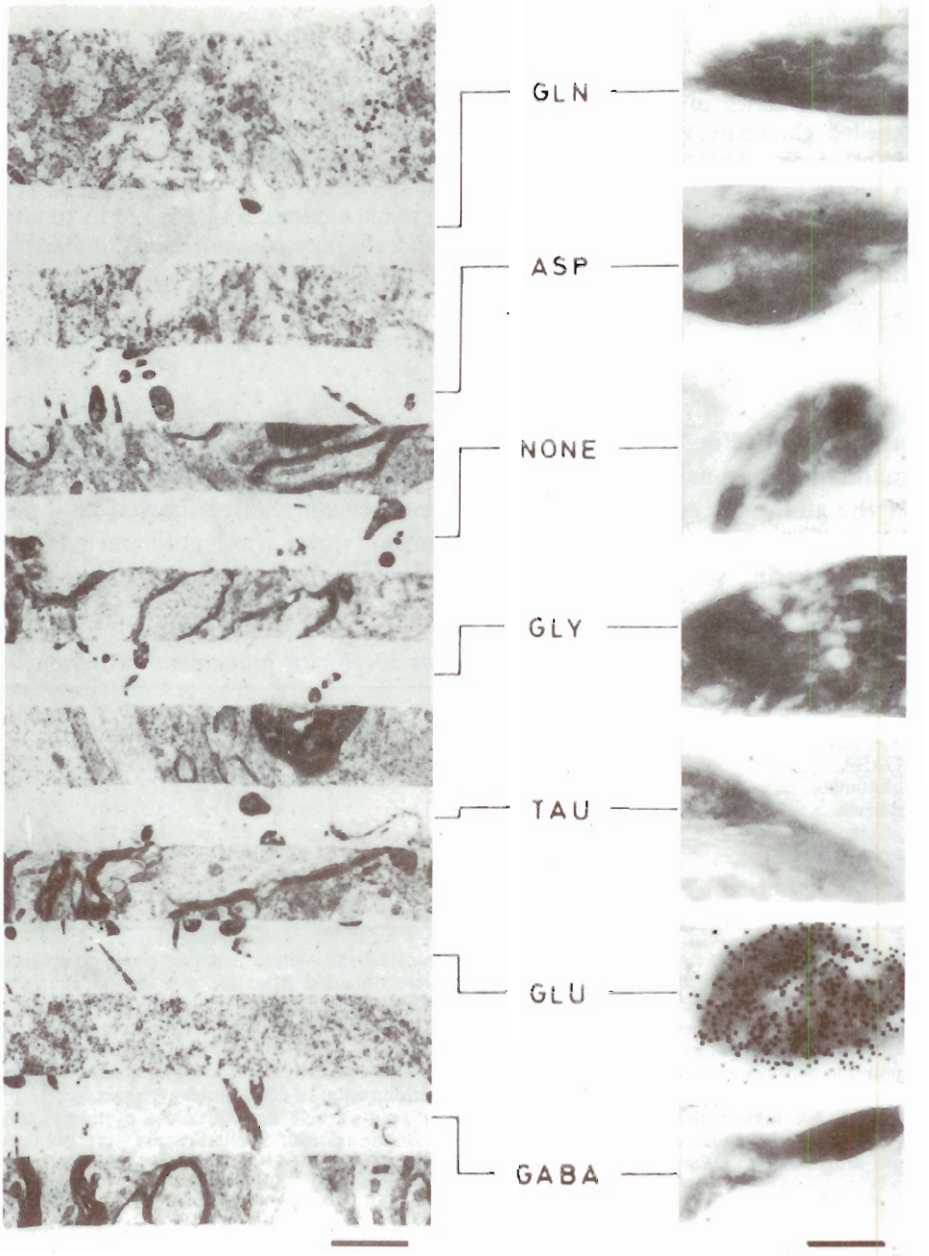

Fig. 1. – *Specificity testing.*

Ultrathin test sections, preparated as described in ref. 34 and incubated in the same drops of serum as the ultrathin tissue sections. The test conjugates appear as discrete clumps. The right panel shows that the glutamate antiserum produces selective labelling of the corresponding amino acid conjugate (see Table 1). Standard abbreviations of amino acids. None: conjugate made by reacting glutaraldehyde with brain macromolecules (i.e., no amino acid added to the reaction mixture). Brain tissue (which in this case was obtained from rat hippocampus) was used to separate the different amino acid conjugates. Bars: left panel, 2 μm; right panel, 0.2 μm. Glutamate antiserum 13 diluted 1:500. Modified from ref. 37.

Graded sections

Glutamate conjugates were prepared in the same way as the test conjugates described above, using a series of different glutamate concentrations (37, 38). Trace amounts of radiolabelled glutamate were added to the reaction mixture to permit calculation of the fixation efficiency. The conjugates were incorporated in a "sandwich" which could subsequently be cut to provide sections resembling that shown in Fig. 1. These "graded" sections (not illustrated) were incubated together with the tissue sections, and allowed us to establish the relationship between gold particle densities and absolute concentrations of fixed amino acid (for methodological details, see ref. 38).

RESULTS

1. *Specificity.* — Fig. 1 shows test conjugates incubated in the same drops of glutamate antiserum as the tissue sections. Gold particles are accumulated selectively over the glutamate conjugate; none of the other conjugates showed gold particle densities significantly different from background level (i.e., gold particle density over empty resin; see Table 1).

Table 1. — *Quantitative analysis of antiserum specificity (glutamate antiserum 13).*

Amino acid conjugate	*Mean gold particle density ± S.E.M. (n)*
GABA	11.5 ± 9.6 (8)
glutamate	1253.6 ± 57.1 (10)*
taurine	3.7 ± 3.5 (10)
glycine	8.9 ± 3.6 (8)
none	4.1 ± 5.2 (8)
aspartate	3.8 ± 3.6 (9)
glutamine	0.2 ± 2.7 (7)

The section containing test conjugates (amino acid - glutaraldehyde - brain macromolecule complexes) were incubated together with the tissue sections. The data were obtained from the test section shown in Fig. 1. Values represent number of gold particles/μm^2 ± S.E.M. over n different conjugate clumps after correction for the gold particle density over tissue-free resin (7.1 particles/μm^2). Asterisk represents the only value that is significantly different ($p < 0.001$; Student's t-test) from "none" (i.e., glutaraldehyde - brain macromolecule conjugates prepared in the absence of any free amino acid). To obtain a sensitive specificity test, all test conjugates were prepared at an amino acid concentration of 2500 μmol/g protein, (corresponding to as high as 250 mM in the brain). Thus the *absolute* gold particle density values over test conjugates are not comparable to those over tissue profiles. Serum dilution 1:500. Similar results were obtained with glutamate antiserum 03. (Data from ref. 37).

2. *Perfusion-fixed material.* — As previously described, our glutamate antisera produced a varying density of labelling over all tissue profiles in the cat (42) as well as in the rat (34, 37, 38) cerebellum: in both species, the highest accumulation of gold particles was found over mossy fibre terminals (also see ref. 27). Relatively strong labelling was also found over parallel fibre terminals (Fig. 5A in ref. 34), whereas glial cell processes and Golgi cell boutons were weakly labelled (Figs. 2, 3). Intermediate labelling intensities were found over granule cell bodies

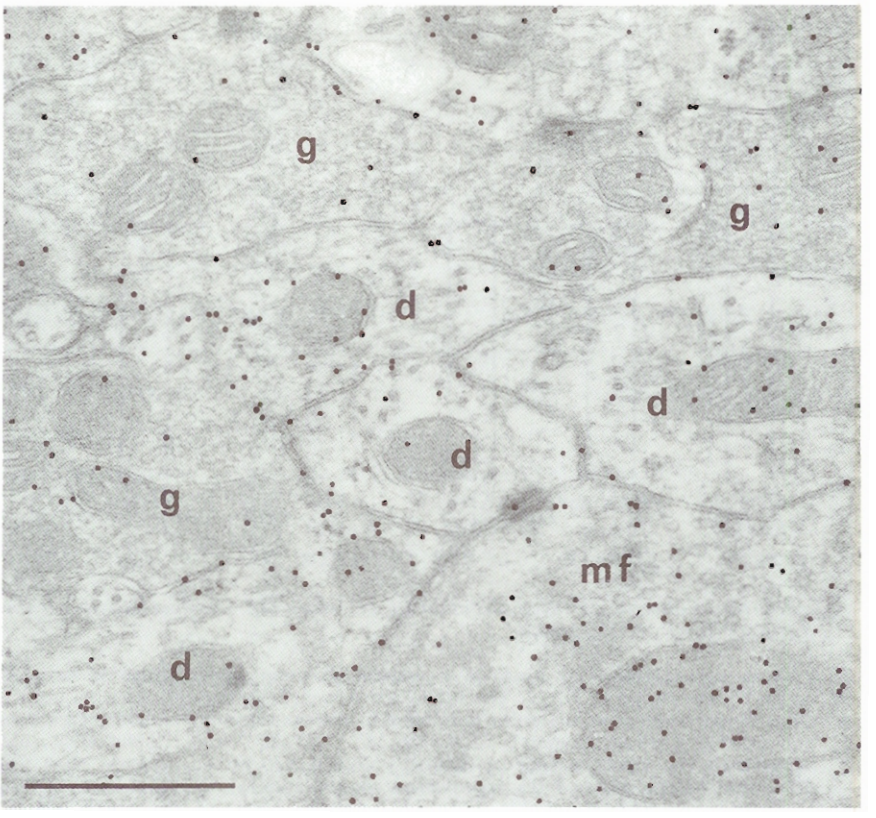

Fig. 2. – *Distribution of glutamate-like immunoreactivity in the granule cell layer of a perfusion-fixed rat cerebellum.*

Gold particles signalling the presence of fixed glutamate are more concentrated over mossy fibre terminals (mf) than over granule cell dendritic digits (d) and Golgi cell terminals (g). See Fig. 3 for a quantitative analysis of a similar preparation. Bar: 0.5 μm. From ref. 36.

and dendrites. Graded sections incubated together with the tissue sections revealed a close to linear relationship between gold particle densities and absolute concentration of fixed glutamate (37, 38). Thus, the relative differences in labelling intensities are likely to reflect quite closely the differences in actual antigen concentrations. This means, for example, that the fourfold difference between the Golgi cell terminals and the mossy fibres with regard to the gold particle densities (Fig. 3) should reflect a similar difference in concentration of fixed glutamate between these structures. Having established the relationship between gold particle density and antigen concentration, we could also calculate the mM concentration of fixed glutamate in the different tissue elements. Although we do not claim accuracy in this respect, our data indicate that the concentration of fixed glutamate in the mossy fibre terminals is approximately 28 mM, compared to about 6 mM in the Golgi cell terminals (Fig. 3). The in vivo concentrations of *free* glutamate in these profiles

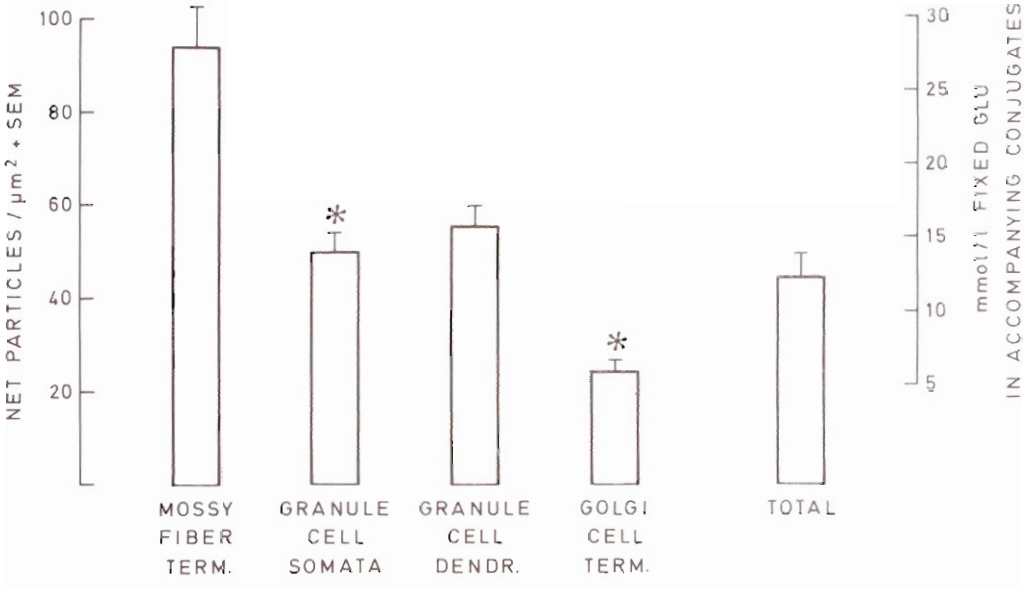

Fig. 3. – *Quantitative assessment of the distribution of glutamate-like immunoreactivity in the granule cell layer of perfusion-fixed rat cerebellum.*

The density values were calculated by means of a computer program (Morforel) on the basis of electron micrographs similar to that shown in Fig. 2. The values are corrected for background (i.e., particle density over empty resin). Standard error of the mean is indicated. Asterisks denote values significantly different ($p < 0.001$) from preceding values (Student's t-test, two tails). "Total" refers to the mean gold particle density as recorded over the whole surface of randomly chosen electron micrographs from the granule cell layer. Scale in right margin indicates the approximate concentration of fixed glutamate in the different tissue profiles, as calculated based on a section containing "graded" conjugates that was incubated together with the tissue section (see text, and ref. 38). From ref. 37.

are likely to exceed the above values, since a proportion of the free glutamate in the brain will inevitably remain unfixed and be lost during the perfusion procedure or subsequent handling of the tissue (see Discussion in refs. 37 and 38).

3. *Immersion-fixed tissue.* — In tissue fixed by immersion after incubation in standard ACSF, the different types of tissue profiles showed the same rank order of labelling intensities as after perfusion fixation. However, the *contrast* in labelling between the different tissue elements was considerably increased. For example, instead of a fourfold difference in gold particle density between mossy fibre terminals and Golgi cell terminals (the ratio found in perfusion-fixed tissue), these profiles now showed a more than tenfold difference in gold particle covering (Fig. 4). As in perfusion fixed tissue the parallel fibre terminals were slightly less intensely labelled than the mossy fibre terminals (Table 2). When sections from perfusion-fixed and immersion-fixed tissue were incubated in the same serum drops to permit direct comparison, it could be seen that the in vitro incubation had led to an absolute increase in the gold particle covering of the mossy and parallel fibre

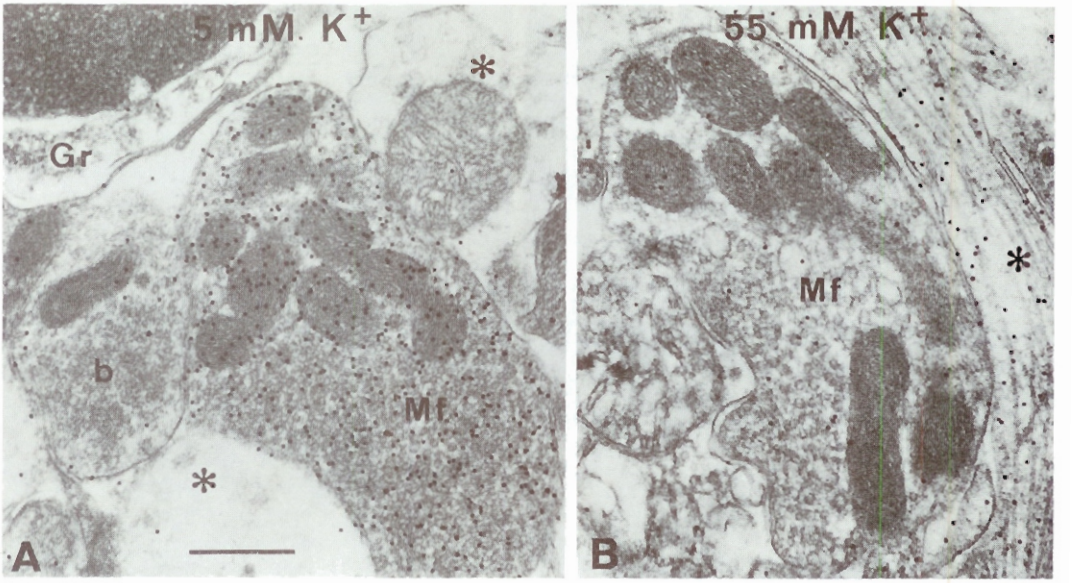

Fig. 4. – *Glutamate-like immunoreactivity in the granule cell layer of immersion-fixed rat cerebellum: Ca^{2+}-dependent K^+-evoked depletion.*

Increasing the K^+ concentration from 5 mM (A) to 55 mM (60 min; B) leads to a dramatic decrease in the level of glutamate-like immunoreactivity in the mossy fibre boutons (Mf). See Table 2 for a quantitative analysis of a similar preparation. b, Golgi cell bouton; Gr, granule cell body; asterisks, granule cell dendrites (dilated in A). Bar: 0.4 μm. Glutamate antiserum 13, diluted 1:500. Modified from ref. 37).

Table 2. — *Effect of K^+-depolarization on the distribution of glutamate-like immunoreactivity in rat cerebellar slices.*

Type of profile	*Mean gold particle density ± S.E.M. (n)*	
	5 mM K^+	*55 mM K^+*
Mossy fibre terminals	178.0 ± 13.1 (11)	20.0 ± 5.0 (13)††
Parallel fibre terminals	142.6 ± 6.5 (39)*	20.0 ± 3.9 (33)††
Glial cell processes	34.2 ± 3.1 (14)**	48.9 ± 3.0 (30)**†

Values represent number of gold particles/μm^2 ± S.E.M. over n different profiles. The values were corrected for background (i.e., 0.65 particles/μm^2 over tissue-free resin). The glial cell processes analysed included only Bergmann processes with distinct glial fibrils. The slices represented in the right column were exposed to high K^+ concentration for 60 min. Asterisks represent values that are significantly different from the previous (*, $p < 0.05$) or the previous two (**, $p < 0.001$) values in the same column. Daggers denote values that are significantly different from corresponding value in left column (†, $p < 0.01$; ††, $p < 0.001$; Student's t-test, two tails). Case 2896; glutamate antiserum 03 diluted 1:300. From experiment similar to that shown in Fig. 4.

terminals, as well as to an absolute decrease in gold particle covering over the remaining tissue elements.

Depolarization of the tissue slices with high concentrations of K^+ led to a decrease in the gold particle density over mossy and parallel fibre terminals (Fig. 4,

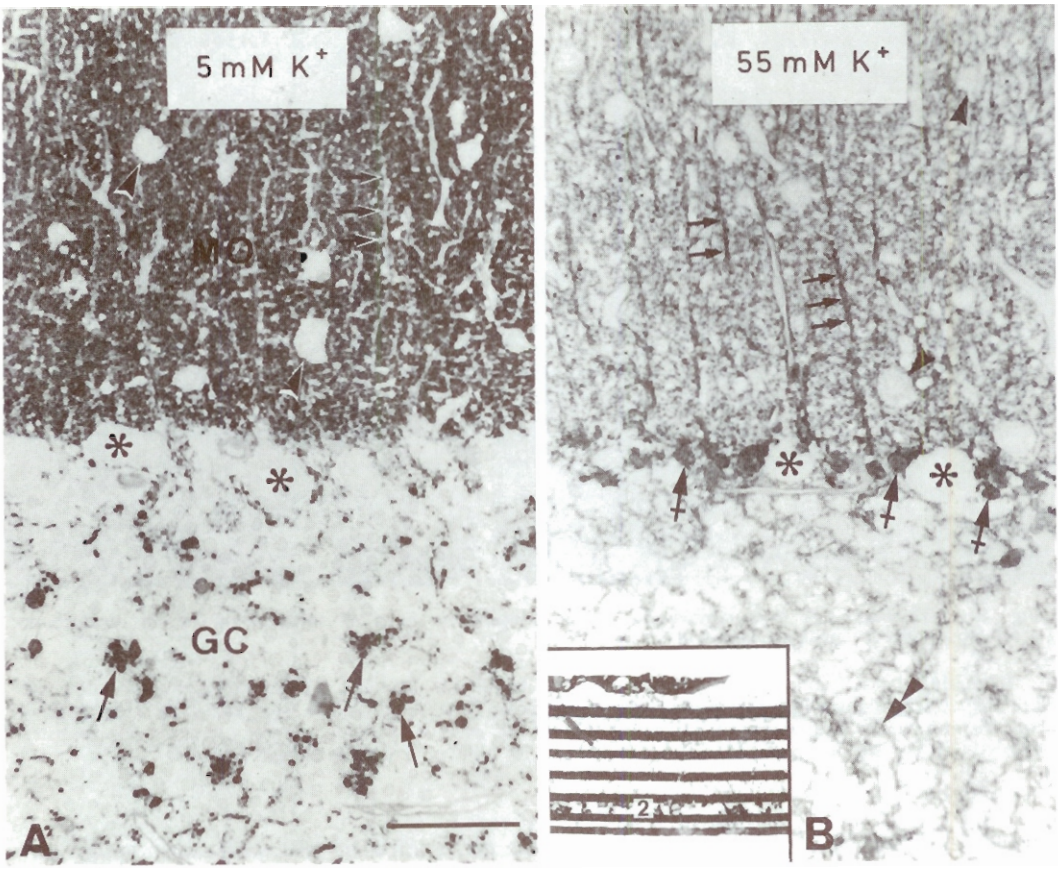

Fig. 5. – *Glutamate-like immunoreactivity in immersion-fixed rat cerebellum: redistribution on K^+-depolarization.*

Increasing the K^+-concentration from 5 mM (A) to 55 mM (60 min, B) leads to a loss of glutamate-like immunoreactivity from structures interpreted as mossy fibre terminals (arrows) and from the interstices between the Purkinje cell dendrites in the molecular layer (corresponding to the location of the parallel fibre terminals). Bergmann glial cell bodies (crossed arrows) and processes (small arrows) show an increased staining intensity after depolarization. The labelled processes in the granule cell layer in B (double arrowhead) probably also represent glial elements. Arrowheads, stellate/basket cell bodies; asterisks, Purkinje cell bodies. MO, molecular layer; GC, granule cell layer. Inset in B: semithin test section (composed as shown in Fig. 1) indicates that the labelling is highly selective for fixed glutamate (lane 2). This test section was mounted on the same slide as the tissue sections in A and B and was thus subjected to exactly the same treatments as the latter two. Bar: 50 μm. Glutamate antiserum 13, diluted 1:1000. Peroxidase-antiperoxidase procedure. From the same experiment as Fig. 4. Modified from ref. 39.

Table 2). (This decrease was more pronounced with 55 mM K^+ than with 40 mM K^+ and more pronounced with 60 min than with 20 min of depolarization.) Glial cell processes showed, in contrast, an enhanced labelling intensity after depolarization (Table 2).

These changes were also evident at the light microscopic level (Figs. 5, 6). Slices incubated at "physiological" K^+ concentration showed a dense punctate labelling in the molecular layer, corresponding to the distribution of the parallel fibre termi-

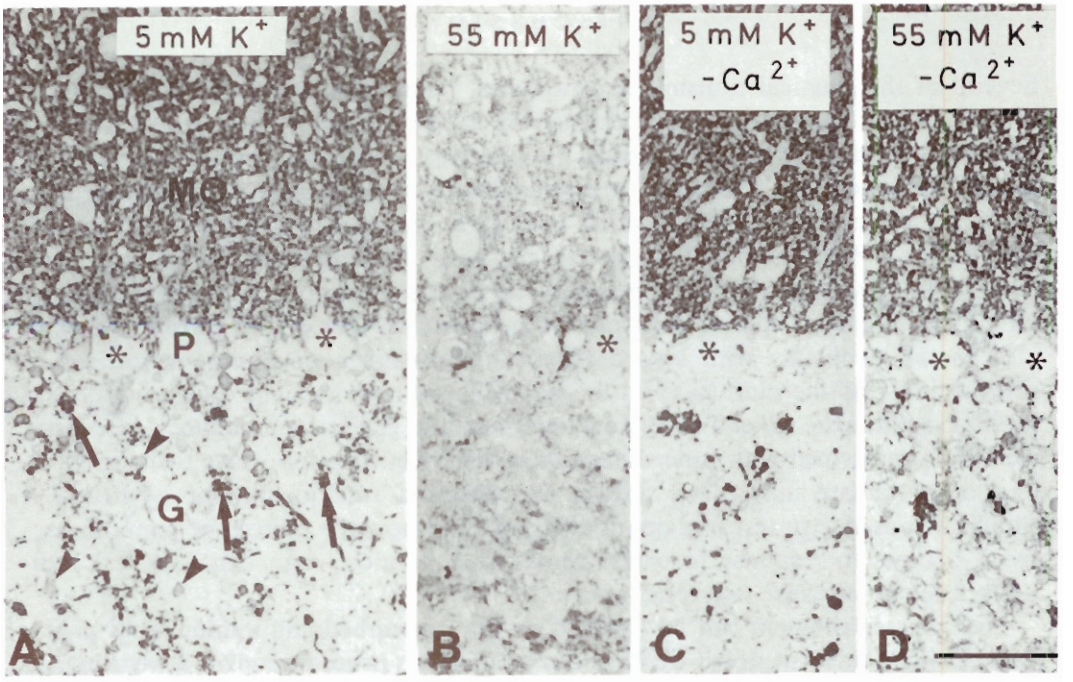

Fig. 6. – *The effects of depolarization with high K^+ concentration are counteracted by reducing the calcium concentration in the medium.*

The patterns of glutamate-like immunoreactivity in A and B (2.4 mM Ca^{2+}, 1.3 mM Mg^{2+}) are similar to those in the previous figure, except that in the present experiment there is a less conspicuous increase in the glial cell labelling after depolarization (60 min). The staining patterns in panels C and D (0.1 mM Ca^{2+}, 10 mM Mg^{2+}) are both very similar to that in A. Mo, molecular layer; P, Purkinje cell layer; G, granule cell layer; arrows, labelled structures probably representing mossy fibre terminals; arrowheads, weakly to intermediately labelled granule cell bodies; asterisks, Purkinje cells. Bar: 60 μm. Same serum dilution and immunocytochemical procedure as in the previous figure.

nals, and also displayed immunoreactive profiles in the granule cell layer with a morphology typical of mossy fibre terminals. Bergmann glial processes, and stellate, basket, Golgi and Purkinje cell bodies, were very weakly labelled, as were most of the granule cell bodies. The remaining granule cell bodies showed an intermediate labelling intensity, i.e., they were much less intensely stained than the mossy and parallel fibre terminals. Depolarization with high K^+ concentration severely depressed the nerve terminal labelling whereas it increased the labelling intensity of glial cell bodies and processes. However, the depolarization-induced changes in glial labelling were not equally prominent in all experiments (compare Fig. 5B with Fig. 6B). The effects of exposure to high K^+ concentration were largely abolished under Ca^{2+}-free conditions (Fig. 6D). Removal of Ca^{2+} at normal K^+ concentration had no effect on the immunostaining (compare Fig. 6A with Fig. 6C). Whether the addition of KCl was adjusted for by a reduction of NaCl or of Tris/HCl did not influence the results obtained.

DISCUSSION

Several of the classical transmitter candidates, including GABA and acetylcholine, are commonly believed to occur exclusively in the terminals from which they are released as transmitters. Glutamate, in contrast, subserves several functions in the CNS, in addition to that of a transmitter (17). Glutamate is an intermediary in important metabolic pathways, it acts as a precursor of GABA, and is also involved as a building block in protein synthesis. Matching the diversity of functional roles, glutamate-like immunoreactivity occurs in varying amounts in all cellular compartments, including terminals of presumed GABA-ergic neurons. This creates special problems with regard to the interpretation of immunocytochemical preparations, as the glutamate antibodies will naturally recognize transmitter and metabolic glutamate alike. To facilitate interpretation, glutamate immunocytochemistry should be performed with procedures that allow quantification, since the concentration of glutamate may differ among the different functional pools. Further, it should be possible to test in a quantitative manner how the pattern of immunoreactivity changes in response to experimental manipulation, e.g., exposure to conditions known to cause synaptic release.

The first requirement can be fulfilled by the postembedding immunogold procedure. Using graded sections, we have shown that this procedure gives a well defined relationship between immunolabelling intensity and antigen concentration (37, 38). The second requirement can be met by applying the postembedding immunogold procedure to tissue slices that have been incubated under different conditions in vitro before immersion fixation. A similar strategy has previously been used to study the synaptic handling of glutamate at the light microscopic level (20, 44, 45, 46).

With regard to the issue studied in the present report, this combination of techniques has given the following major results: mossy fibre terminals are enriched in glutamate-like immunoreactivity relative to other tissue elements (as previously shown in the cat; see ref. 42), and are even more strongly labelled than the parallel fibre terminals, which are some of the most well established glutamatergic terminals in the brain (review: see ref. 30). This enrichment is obvious in perfusion-fixed tissue (in which the distribution of glutamate is likely to reflect quite closely the in vivo distribution of free amino acid) and is even more pronounced in immersion-fixed material. In the latter material, differences in immunolabelling intensities may be accentuated by differences in the rates of glutamate synthesis and uptake and by glutamate "leakage" from neuronal pools (cf. ref. 6; for a full discussion of this issue see refs. 37 and 46). Moreover, the glutamate-like immunoreactivity in the mossy fibre terminals can be strongly depressed in a calcium-dependent manner on prolonged exposure to conditions that are known to induce synaptic release. Indeed, under the latter conditions, part of the glutamate-like immunoreactivity appears to be translocated from nerve terminals to glial elements, a finding that is in agreement with biochemical data suggesting that a proportion of the released glutamate is being taken up by glia (17).

Thus, our results provide strong evidence that mossy fibre terminals use gluta-

mate as a transmitter. This would be in accord with recent immunocytochemical investigations in the cerebellar cortex of cat (42) and rat (27) [1], and with electrophysiological and pharmacological studies (18, 19) indicating that the mossy fibre terminals cause excitation mediated by one or several classes of excitatory amino acid receptors. Whether glutamate is used as a transmitter in all or only in a subpopulation of mossy fibres is not known. It should be emphasized, however, that we have analysed material from most parts of the cerebellum without noticing any regional differences in the pattern of glutamate immunolabelling. To explore this issue further, we are currently combining glutamate immunocytochemistry with anterograde transport of HRP-WGA (cf. ref. 11), to see whether the different contingencies of mossy fibres differ with regard to their level of glutamate-like immunoreactivity.

Our finding that most, if not all, of the mossy fibres contain and release glutamate does not exclude the possibility that these terminals in addition contain and release other neuroactive substances. As pointed out in the Introduction, a considerable number of such substances have been reported to exist in the mossy fibre system. Direct evidence for a colocalization of glutamate and a neuroactive peptide (substance P) has recently been obtained in the spinal cord (14). Efforts should now be made to resolve if and how glutamate interacts at the synaptic level with its presumed co-contained neuroactive substances.

Recently, a small subpopulation of mossy fibres in the cat cerebellum was reported to display GABA-like immunoreactivity (21; also see ref. 1). These terminals did not differ morphologically from the majority of mossy fibre terminals, in as much as they contained round vesicles and established asymmetric junctions with dendritic digits of granule cells. Whether these terminals are also enriched in glutamate-like immunoreactivity has not been established. Evidence from other systems suggests that such a colocalization may indeed occur: Bradford *et al.* (7) have shown that presumed GABAergic terminals (immunoprecipitated by means of antibodies to GAD) sustain a calcium dependent release of glutamate. Studies are now in progress to further explore this issue.

SUMMARY

The chemical substance(s) responsible for the fast signalling in the mossy fibre to granule cell synapses in the cerebellum has not been identified, although recent studies suggest that glutamate is a strong candidate. In the present investigation, this issue was explored by means of a quantitative electron microscopic im-

[1] It has been reported that most of the neurons in the pontine nuclei are stained with antisera to glutaraldehyde-fixed (30) and carbodiimide-fixed (2) glutamate. However, these findings cannot be taken as strong support for a transmitter role of glutamate in the mossy fibers since perikaryal labelling in general may be greatly influenced by "metabolic" pools of glutamate.

munocytochemical procedure. Ultrathin sections of plastic-embedded rat cerebella were treated with an antiserum specific for glutaraldehyde-fixed glutamate, followed by a secondary antibody coupled to colloidal gold particles. The gold particle density over mossy fibre terminals was assessed in tissue that had been rapidly fixed by perfusion, as well as in tissue that had been incubated in artificial cerebrospinal fluid in vitro before immersion fixation. In both preparations the mossy fibres appeared as the most intensely glutamate-immunoreactive profile type in the cerebellar cortex, and the parallel fibre terminals were also strongly labelled. Corresponding resuls were obtained at the light microscopic level. Most of the immunoreactivity in the mossy and parallel fibre terminals could be depleted in a Ca^{2+}-dependent manner by depolarization with a high K^+ concentration. These data suggest that the mossy and parallel fibre terminals contain a glutamate pool that behaves as a transmitter pool.

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