

QUANTIFICATION OF IMMUNOGOLD LABELLING REVEALS ENRICHMENT OF GLUTAMATE IN MOSSY AND PARALLEL FIBRE TERMINALS IN CAT CEREBELLUM

P. SOMOGYI,* K. HALASY,† J. SOMOGYI,*‡ J. STORM-MATHISEN§ and O. P. OTTERSEN§

*MRC Anatomical Neuropharmacology Unit, South Parks Road, Oxford, OX1 3QT, U.K.; ‡1st Department of Anatomy, Semmelweis Medical School, Tuzolto u.58, Budapest, H-1450, Hungary; and

§Anatomical Institute, University of Oslo, 0162 Oslo 1, Norway

Abstract—The glutamate immunoreactivity of different cell populations was compared quantitatively in the cerebellar cortex of cat, using an antiserum raised against glutamate coupled to bovine serum albumin by glutaraldehyde. Neuronal and glial processes were identified on serial electron microscopic sections which were processed by a postembedding immunogold procedure. The surface density of colloidal gold particles was used for statistical comparison of the relative levels of glutamate in cell populations, or in different parts of the same population.

The terminals of mossy and parallel fibres had significantly higher levels of glutamate immunoreactivity than Golgi cell terminals, granule cell dendritic digits, Purkinje cell dendrites or dendritic spines. Golgi cell terminals were identified by their position and GABA immunoreactivity as revealed by immunogold in serial sections. The dendritic digits of the putative glutamatergic granule cells had significantly higher glutamate immunoreactivity than did Purkinje cell dendrites and dendritic spines. Glial cell processes in the molecular layer had lower level of glutamate immunoreactivity than any of the neuronal processes.

The results demonstrate that the highest levels of glutamate immunoreactivity occur in mossy and parallel fibre presynaptic terminals that are known to have an excitatory effect. This supports previous suggestions that glutamate may be a transmitter at these synapses. The measurement of the levels of putative amino acid transmitters in identified neuronal populations, or in different parts of the same population, could have wide applications in studies on the chemical neuroanatomy of the nervous system.

The development of antisera recognizing amino acids fixed in tissue sections²⁷ opened the way to revealing the localization of amino acids in specific pathways and neurons that may use them as neurotransmitters.¹⁷ This approach was particularly important for the localization of the acidic amino acid glutamate. Although the majority of neurons probably have receptors for this substance,⁵ before the development of antisera to conjugated glutamate it was not possible to localize glutamate-rich nerve terminals, due to the lack of suitable markers. Glutamate is an essential component of intermediary metabolism and so, together with its metabolic enzymes, it can be expected to be present in all cells of the brain. However, several neurochemical indicators, including the level of glutamate, appear to be enriched in pathways that are thought to use acidic amino acids as transmitter (for reviews see^{7,8,17}). Therefore it was expected that the higher levels of glutamate would make it possible to localize selectively the transmitter pool of glutamate in histological sections.²⁷ In support of this prediction the first immunocytochemical studies certainly revealed an uneven distribution of glutamate in the brain,^{16,17} in agreement with the biochemical results.^{7,8} Because all cells will react with

antiserum to glutamate to some extent, quantitative methods are required for the evaluation of different levels of reactivity. The most probable site where a difference in glutamate levels between neurons that use glutamate as transmitter and neurons using other transmitters would occur is in the nerve terminals that store and release the transmitters.

To test this hypothesis, in the present study we quantified the glutamate immunoreactivity of identified cell populations in the cerebellum, where the different cells and processes can easily be recognized at the fine structural level.^{6,20} We compared the glutamate immunoreactivity of mossy and parallel fibre terminals, two populations of nerve terminals with excitatory action⁶ and which have been suggested to use glutamate as transmitter^{1,3,4,9,10,13,14,21,22,25,28,29} with the reactivity of Golgi cell terminals, a GABAergic terminal population (for review see¹⁷), and with two populations of postsynaptic dendrites, those of granule cells and those of Purkinje cells, as well as with the processes of glial cells.

EXPERIMENTAL PROCEDURES

Animals and specimens

Two male adult cats were sedated with ketamine hydrochloride (Vetalar, 25 mg/kg, i.m., Parke-Davis) and deeply anaesthetized with chloral hydrate (BDH, 35%, 350 mg/kg, i.p.) and perfused through the heart with saline, followed by a fixative at room temperature and containing 1% para-

†Present address: Department of Zoology, Jozsef Attila University, Szeged, Egyetem u-2., Hungary.

Abbreviations: EM, electron microscopic; GLU, glutamate; GABA, γ -aminobutyrate.

Table 1. Statistical comparison of glutamate immunoreactivity of different cellular elements in the cerebellar cortex

Cell process	Mean surface density SEM (<i>n</i>)		Statistical comparison				
Glial c.p.	12.27 ± 0.74 (125)	Glial c.p.					
Mossy f.t.	63.54 ± 2.68 (25)	<i>P</i> < 0.005	Mossy f.t.				
Golgi c.t.	25.35 ± 1.34 (48)	<i>P</i> < 0.005	<i>P</i> < 0.005	Golgi c.t.			
Parallel f.t.	45.96 ± 1.22 (156)	<i>P</i> < 0.005	<i>P</i> < 0.005	<i>P</i> < 0.005	Parallel f.t.		
Purkinje d. and s.	26.58 ± 1.13 (171)	<i>P</i> < 0.005	<i>P</i> < 0.005	<i>P</i> < 0.3	<i>P</i> < 0.005	Purkinje d. and s.	
Granule c.d.	38.85 ± 1.66 (110)	<i>P</i> < 0.005	<i>P</i> < 0.005	<i>P</i> < 0.005	<i>P</i> < 0.005	<i>P</i> < 0.005	<i>P</i> < 0.005

Values represent the surface density of gold particles expressed as the mean number/ $\mu\text{m}^2 \pm$ standard error of the mean (SEM). The number of measured profiles (*n*) is shown in parentheses in each case. The profiles of cellular processes were identified in electron micrographs of ultrathin sections immunoreacted by the postembedding gold method. This measurement was made from an incubation using the antiserum to glutamate at a dilution of 1:500. The means are for the data shown in Fig. 3. The pattern of differences was identical when the antiserum was used at a dilution of 1:1000, or when the measurement was made in another animal, although the absolute values of the surface density of gold were different. Student's *t*-test was used to ascertain the significance or otherwise of the differences between the mean values. Glial c.p. = glial cell processes in molecular layer; Golgi c.t. = Golgi cell terminals; Granule c.d. = granule cell dendritic digits; Mossy f.t. = mossy fibre terminals; Parallel f.t. = parallel fibre terminals; Purkinje d. and s. = Purkinje dendritic shafts and spines.

formaldehyde, 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2). Blocks of tissue from lobulus V of the vermis were dissected, postfixed for 2 h in the same fixative, treated with 1% osmium tetroxide for 1 h, dehydrated routinely and embedded in Durcupan ACM (Fluka) resin. The blocks were stained at the 70% ethanol stage for 40 min in 1% uranyl acetate.

Postembedding immunocytochemistry

Ultrathin sections were mounted on single slot Formvar-coated gold grids and reacted to reveal either glutamate or GABA immunoreactivity using the postembedding immunogold method and procedure I of Somogyi and Hodgson.²³ Following the reaction sections were stained with lead citrate.

The production and characterization of rabbit antisera to conjugated glutamate (serum 13^{16,17,18,19,27}) and GABA (Code No. 9^{11,24}) have been described. The antiserum to glutamate was purified through a sequence of columns (1–2) of albumin-coupled Sepharose reacted with (1) glutaraldehyde and (2) glutaraldehyde-GABA.¹⁸ To reveal the primary antibodies colloidal gold (15 nm) coated with goat antirabbit IgG (Janssen, Beerse, Belgium) was used. One folium was reacted from each animal at primary antiserum dilutions of both 1:500 and 1:1000 for glutamate, and 1:2000 for GABA. Each area was also reacted with antisera that were preincubated with one of the following substances conjugated to polyacrylamide beads with glutaraldehyde as described earlier:¹¹ L-glutamic acid, L-aspartic acid, L-glutamine, GABA and glycine (all AnalaR, BDH). Preincubation of the antiserum to glutamate with conjugated glutamate and preincubation of the antiserum to GABA with conjugated GABA resulted in the almost complete absence of gold particles from the sections, but none of the other substances affected the distribution of gold particles, except a slight attenuation of glutamate immunoreactivity by preincubation with conjugated glutamine.

Measurement of glutamate immunoreactivity

To see whether the differences in glutamate immunoreactivity were statistically significant, the surface density of gold particles was measured over neuronal and glial processes identified on the basis of their fine structural features and their location in the cortex.^{6,20} From each incubation 150 micrographs were taken systematically at a primary magnification of 21,000, along vertical stripes from the pia to the white matter. Areas were selected so that each frame included maximal numbers of profiles of interest. The sample of glial processes was measured in the molecular

layer. Using a Bioquant (R & M Biometrics, Inc., Nashville, U.S.A.) system, the area of 3209 cellular profiles was measured on micrographs at a final magnification of 54,000 and all gold particles, including those over mitochondria, were counted over each profile. Aggregates of two or more gold particles were counted as one. Aggregates could be recognized from the uniform small distance between the particles. Occasionally closely but independently attached particles may have been counted as one. This would occur mainly in densely labelled structures resulting in the underestimation of particle density. Since, at the most, only a few per cent of the particles were aggregates, this underestimation would not affect the differences between cell populations in the present study. Particles over the plasma membrane may represent epitopes inside or outside the membrane. Therefore to prevent overestimation of gold density over the measured profile the circumference of the profiles was divided into two approximately equal lengths by a vertical line drawn by hand. Particles touching the membrane were included only on the left side of the vertical line.

RESULTS AND DISCUSSION

In sections reacted with antiserum to glutamate, gold particles were found over all neuronal and non-neuronal cells, but their surface density differed recognizably even upon visual inspection: the most conspicuous difference was the low surface density over glial cells and the high density over parallel and mossy fibre terminals (Figs 1A, 2). Mitochondria were often the most densely labelled subcellular organelles both for glutamate and for GABA, but only in those processes that also had high density of gold over vesicles and other parts of the terminal. The mitochondrial labelling could result either from genuine differences in the amino acid content of mitochondria in terminals using different transmitters, or from the chemical coupling by glutaraldehyde of transmitter amino acids to mitochondria during fixation.

Statistical comparison of the immunoreactivity of different processes, based on the density of gold particles, gave similar results in both animals and at

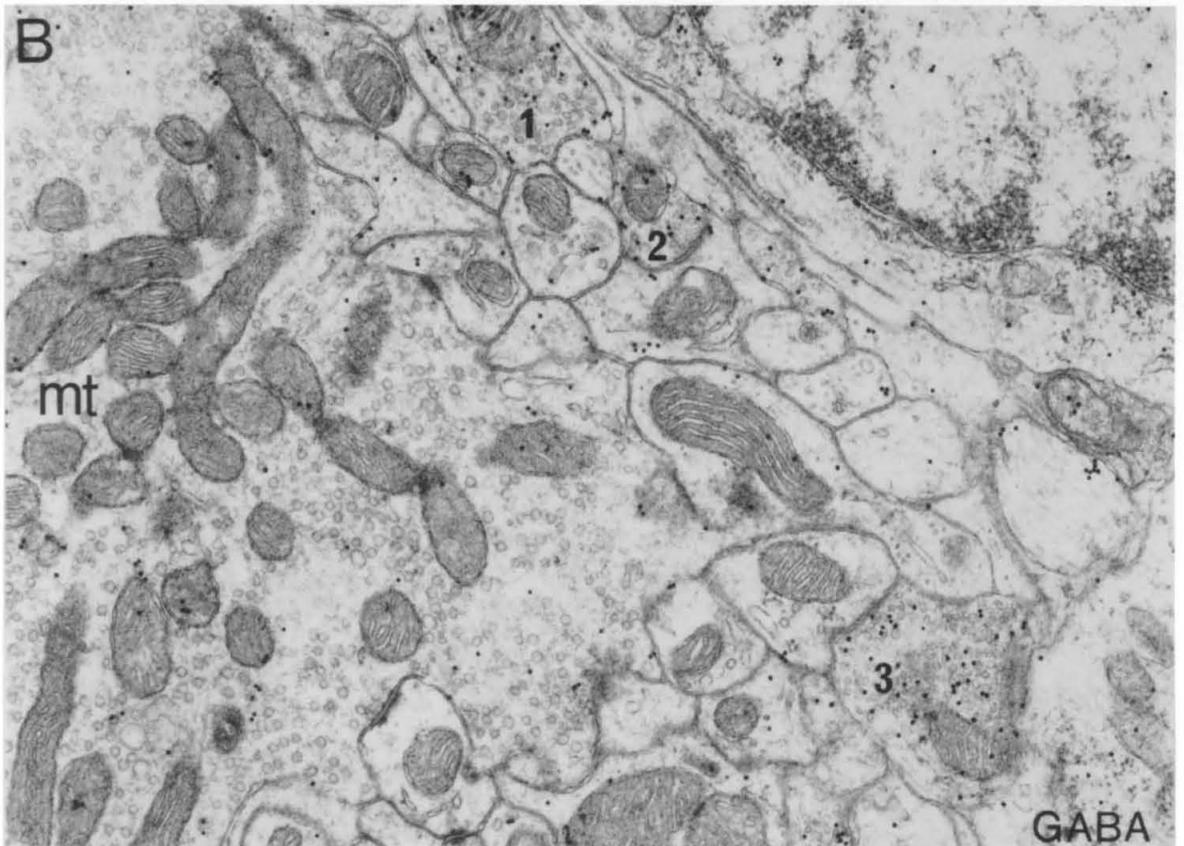
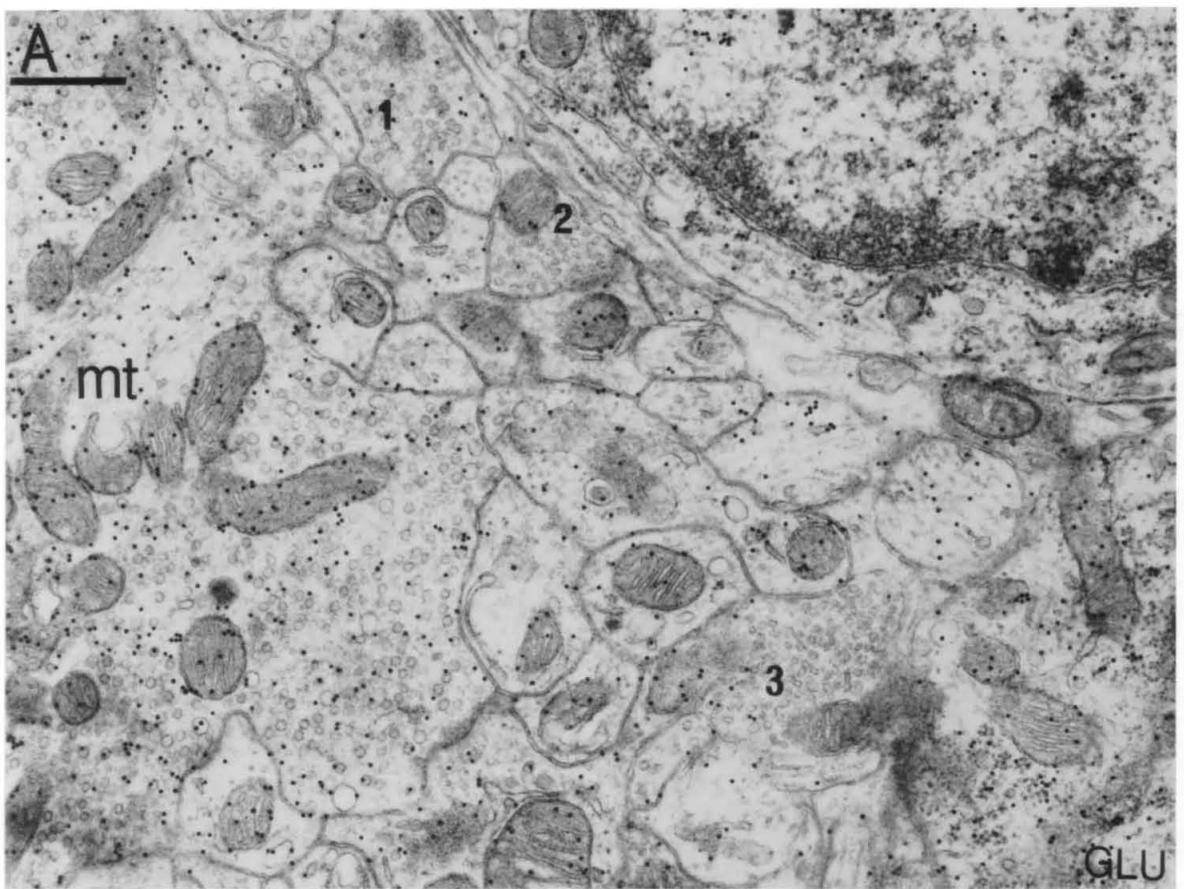


Fig. 1. Electron micrographs of serial sections through a glomerulus in the granular layer of the cat's cerebellar cortex. The section shown in (A) was reacted with antiserum to glutamate (GLU), the section in (B) with antiserum to GABA. The electron-dense gold particles show immunoreactive sites. For GLU the highest density of gold appears to be over the mossy fibre terminal (mt) and the lowest over glial processes and Golgi cell terminals (1-3). This was confirmed by statistical comparison of the populations (Table 1). The same Golgi cell terminals are strongly reacting for GABA, while other processes have only a low surface density of gold. Scale: (A) and (B), same magnification, 0.5 μ m.

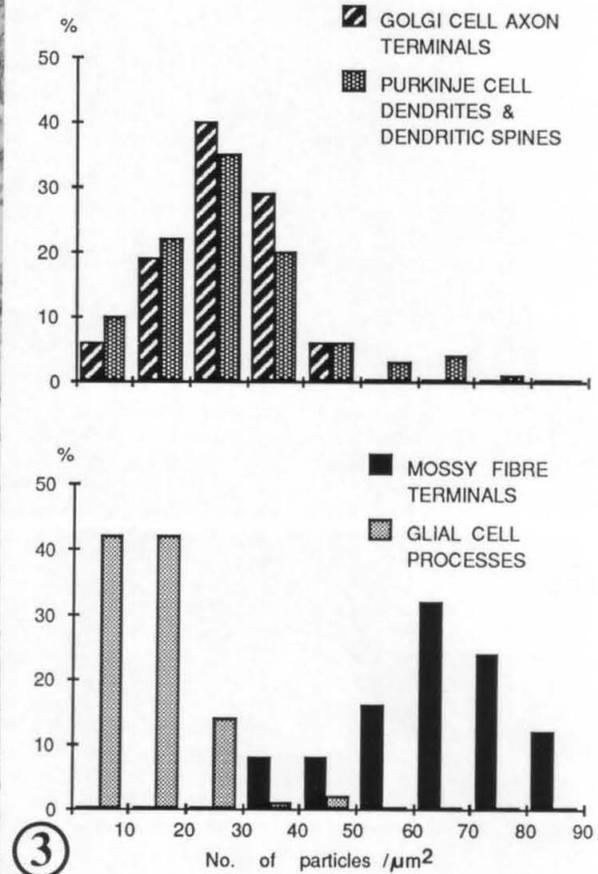
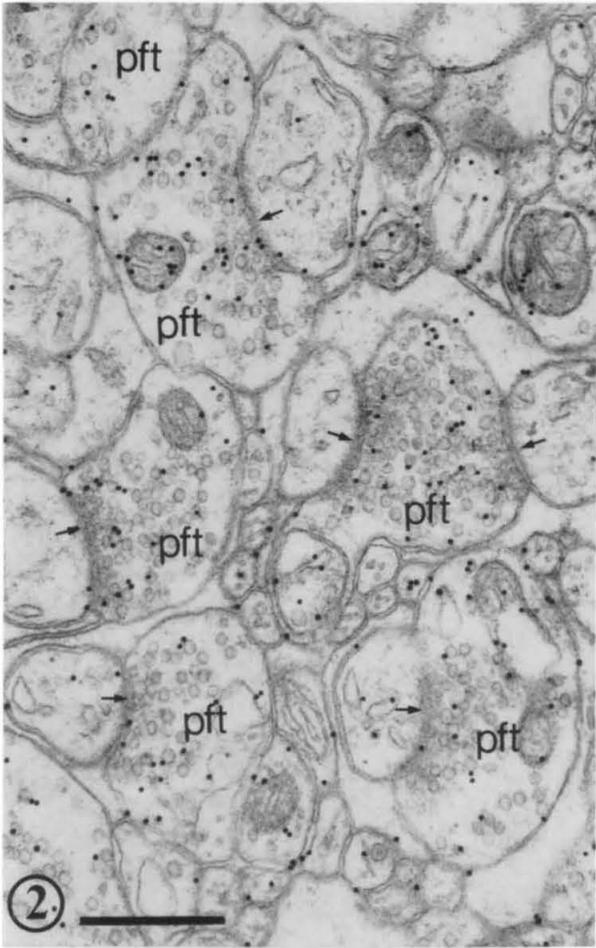


Fig. 2 Electron micrograph of a section through the molecular layer reacted for glutamate. Parallel fibre terminals (pft) react strongly for glutamate as evidenced by the high density of gold, while Purkinje cell dendritic spines that receive synapses from them (arrows) and glial processes react weakly. Scale: 0.5 μ m.

Fig. 3. Distribution of glutamate immunoreactivity as measured by the density of gold particles over various cellular processes of immunoreacted sections of the cat's cerebellar cortex. The ordinate represents the proportion of measured cellular profiles in a bin. Note that glia had the lowest glutamate immunoreactivity, and mossy fibre terminals the highest. Purkinje cell dendrites, spines and the GABAergic Golgi cell terminals had intermediate levels which were significantly higher than that of glia, but significantly lower than that of mossy terminals (Table 1). The glutamate immunoreactivity of parallel fibre terminals (not shown here) was lower than that of the mossy fibres but still significantly higher than the level in any of the other populations of processes measured in this study.

both dilutions of the antiserum to glutamate in each animal. The results of a representative measurement are shown in Table 1. The statistical analysis bore out the most conspicuous differences predicted from the visual inspection, but in addition provided further information.

Nerve terminals that are known to exert an excitatory action (see⁶), i.e. the terminals of mossy and parallel fibres, have significantly higher levels of glutamate-like immunoreactivity than the putative GABAergic Golgi cell terminals, or other neuronal processes. This is strong evidence that glutamate is indeed concentrated in these excitatory terminals, as has previously been inferred from biochemical studies,^{14,21,22,28,29} and strongly supports the suggested transmitter role of glutamate at parallel^{3,4,10,13,22,25} and mossy^{10a,11a} fibre synapses. All mossy terminals encountered in the present study showed strong glutamate immunoreactivity, but it cannot be excluded that some mossy fibres use other transmitters as suggested earlier.^{2,12} The glutamate-like immunoreactivity of mossy terminals in the cat in our study is in good agreement with that found by light microscopy in mossy terminals in rat cerebellar slices fixed by glutaraldehyde *in vitro* (O. P. Ottersen and J. Storm-Mathisen, unpublished) and/or numerous neuronal perikarya in the pontine nuclei in rat and mouse.¹⁶ Pontine neurons are also stained by a monoclonal antibody to γ -glutamylglutamate in material fixed by carbodiimide and glutaraldehyde.¹ Many of the mossy fibres in the cerebellar cortex originate in the pons and our sample probably included some of them.

The strong labelling of parallel fibre boutons compared with that of Purkinje cell dendrites and glial processes confirms light microscopic observations in the rat cerebellar molecular layer, where the latter types of structure stand out as unstained.²⁶ The very low level of immunoreactivity in glia agrees with the notion that in these cells glutamate is converted to glutamine by glutamine synthetase.¹⁵

The results show significantly higher levels of glutamate in the terminals of granule cells than in their dendritic digits, which receive input from glutamate-containing mossy fibres. This strongly suggests that the high level of glutamate in the terminal is related to presynaptic function, i.e. transmitter storage. The results raise the possibility that subcellular differences in transmitter levels within the same population of cells can be evaluated under different experimental conditions.

The quantitative analysis revealed that the dendrites of the excitatory granule cells, which may use glutamate as transmitter, have significantly higher levels of glutamate than the dendrites of the Purkinje cells that probably use GABA as transmitter (for review see¹⁷). This demonstrates that neuronal populations with high glutamate levels in their terminals may contain higher levels throughout than other neuronal types. If this proves to be a general phenomenon, then it may be possible to identify the dendrites and somata of neurons that use glutamate as transmitter. The glutamate immunoreactivities of Purkinje dendrites and Golgi cell terminals were not different and their glutamate levels may represent the general neuronal metabolic pool. Both of these neuron populations are probably GABAergic, and so will use glutamate as precursor for GABA. Consequently their glutamate levels may be lower than those of neurons using other transmitters and not represented in the cerebellum. More measurement on other neurochemically distinct neuron populations, as well as on putative glutamatergic populations, combined with drug treatments that are known to influence transmitter levels, are needed to evaluate further the general applicability of the method.

Acknowledgements—We are grateful to Miss Patricia McChlery and Mr Frank Kennedy for technical assistance, and to Mrs Dorothy Rhodes for secretarial support. Supported in part by the Norwegian Research Council for Science and the Humanities.

REFERENCES

- Beitz A. J., Larson A. A., Monaghan P., Altschuler R. A., Mullett M. M. and Madl J. E. (1986) Immunohistochemical localization of glutamate, glutaminase and aspartate aminotransferase in neurons of the pontine nuclei of the rat. *Neuroscience* **17**, 741–753.
- Chan-Palay V. (1975) Fine structure of labeled axons in the cerebellar cortex and nuclei of rodents and primates after intraventricular infusions with tritiated serotonin. *Anat. Embryol.* **148**, 235–265.
- Chujo T., Yamada Y. and Yamamoto C. (1975) Sensitivity of Purkinje cell dendrites to glutamic acid. *Expl Brain Res.* **23**, 293–300.
- Crepel F., Dhanjal S. S. and Sears T. A. (1982) Effect of glutamate, aspartate and related derivatives on cerebellar Purkinje cell dendrites in the rat: an *in vitro* study. *J. Physiol., Lond.* **329**, 297–317.
- Curtis D. R. and Johnston G. A. R. (1974) Amino acid transmitters in the mammalian central nervous system. *Ergebn. Physiol.* **69**, 97–188.
- Eccles J. C., Ito M. and Szentagothai J. (1967) *The Cerebellum as a Neuronal Machine*. Springer, Berlin.
- Fagg G. E. and Foster A. C. (1983) Amino acid neurotransmitters and their pathways in the mammalian central nervous system. *Neuroscience* **9**, 701–719.
- Fonnum F. (1984) Glutamate: a neurotransmitter in mammalian brain. *J. Neurochem.* **42**, 1–11.
- Freeman M. E., Lane J. D. and Smith J. E. (1983) Turnover rates of amino acid neurotransmitters in regions of the rat cerebellum. *J. Neurochem.* **40**, 1441–1447.
- Gallo V., Ciotti M. T., Coletti A., Aloisi F. and Levi G. (1982) Selective release of glutamate from cerebellar granule cells differentiating in culture. *Proc. natn Acad. Sci. U.S.A.* **79**, 7919–7923.

- 10a. Garthwaite J. (1986) Parallel-fibre- and mossy-fibre-mediated synaptic transmission in rat cerebellar slices monitored using a 'grease-gap' method. *J. Physiol., Lond.* **371**, 16P.
11. Hodgson A. J., Penke B., Erdei A., Chubb I. W. and Somogyi P. (1985) Antiserum to γ -aminobutyric acid. I. Production and characterisation using a new model system. *J. Histochem. Cytochem.* **33**, 229–239.
- 11a. Holm J. A. and Logan S. D. (1986) Release of amino acids and a novel peptide from isolated fragments of rat cerebellar glomerulus. *J. Physiol., Lond.* **373**, 28P.
12. Kan K.-S., Chao L. and Eng L. F. (1978) Immunohistochemical localization of choline acetyltransferase in rabbit spinal cord and cerebellum. *Brain Res.* **146**, 221–229.
13. Levi G., Gordon R. D., Gallo V., Wilkin G. P. and Balazs R. (1982) Putative acidic amino acid transmitters in the cerebellum I. Depolarization-induced release. *Brain Res.* **239**, 425–445.
14. McBride W. J., Nadi N. S., Altman J. and Aprison M. H. (1976) Effects of selective doses of X-irradiation on the levels of several amino acids in the cerebellum of the rat. *Neurochem. Res.* **1**, 141–152.
15. Norenberg M. D. and Martinez-Hernandez A. (1979) Fine structural localization of glutamine synthetase in astrocytes of rat brain. *Brain Res.* **161**, 303–310.
16. Ottersen O. P. and Storm-Mathisen J. (1984) Glutamate- and GABA-containing neurons in the mouse and rat brain, as demonstrated with a new immunocytochemical technique. *J. comp. Neurol.* **229**, 374–392.
17. Ottersen O. P. and Storm-Mathisen J. (1984) Neurons containing or accumulating transmitter amino acids. In Classical transmitters and transmitter receptors in the CNS Part II (eds Bjorklund A., Hokfelt T. and Kuhar M. J.), *Handbook of Chemical Neuroanatomy*, Vol. 3, pp. 141–246. Elsevier, Amsterdam.
18. Ottersen O. P. and Storm-Mathisen J. (1985) Different neuronal localization of aspartate-like and glutamate-like immunoreactivities in the hippocampus of rat, guinea-pig and Senegalese baboon (*Papio papio*), with a note on the distribution of γ -aminobutyrate. *Neuroscience* **16**, 589–606.
19. Ottersen O. P., Storm-Mathisen J., Madsen S., Skumlien S. and Stromhaug J. (1986) Evaluation of the immunocytochemical method for amino acids. *Med. Biol.* **64**, in press.
20. Palay S. L. and Chan-Palay V. (1974) *Cerebellar Cortex. Cytology and Organization*. Springer, Berlin.
21. Roffler-Tarlov S. and Turey M. (1982) The content of amino acids in the developing cerebellar cortex and deep cerebellar nuclei of granule cell deficient mutant mice. *Brain Res.* **247**, 65–73.
22. Sandoval M. E. and Cotman C. W. (1978) Evaluation of glutamate as a neurotransmitter of cerebellar parallel fibers. *Neuroscience* **3**, 199–206.
23. Somogyi P. and Hodgson A. J. (1985) Antiserum to γ -aminobutyric acid. III. Demonstration of GABA in Golgi-impregnated neurons and in conventional electron microscopic sections of cat striate cortex. *J. Histochem. Cytochem.* **33**, 249–257.
24. Somogyi P., Hodgson A. J., Chubb I. W., Penke B. and Erdei A. (1985) Antiserum to γ -aminobutyric acid. II. Immunocytochemical application to the central nervous system. *J. Histochem. Cytochem.* **33**, 240–248.
25. Stone T. W. (1979) Glutamate as the neurotransmitter of cerebellar granule cells in the rat: electrophysiological evidence. *Br. J. Pharmac.* **66**, 291–296.
26. Storm-Mathisen J. and Ottersen O. P. (In press) Antibodies against amino acid neurotransmitters. In *Neurohistochemistry: Modern Methods and Applications* (eds Panula P., Paivarinta H. and Soinila S.), pp. 107–136. Alan R. Liss, New York.
27. Storm-Mathisen J., Leknes A. K., Bore A. T., Vaaland J. L., Edminson P., Haug F.-M. S. and Ottersen O. P. (1983) First visualization of glutamate and GABA in neurones by immunocytochemistry. *Nature* **301**, 517–520.
28. Valcana T., Hudson D. and Timiras P. S. (1972) Effects of the X-irradiation on the content of amino acids in the developing rat cerebellum. *J. Neurochem.* **19**, 2229–2232.
29. Young A. B., Oster-Granite M. L., Herndon R. M. and Snyder S. H. (1974) Glutamic acid: selective depletion by viral induced granule cell loss in hamster cerebellum. *Brain Res.* **73**, 1–13.

(Accepted 18 July 1986)