Symposium

Immunocytochemistry of Glutamate at the Synaptic Level

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Introduction

There is an obvious need to study the microscopic localization of small biological molecules such as amino acids. There is particular interest in the amino acids connected with neurotransmission. Among the excitatory amino acids, glutamate seems the most important but has proved quite difficult to study, in spite of the fact that it is one of the most abundant amino acids in the brain. One of the difficulties is due to the multiple roles of this amino acid, which make it hard to distinguish "metabolic" and "transmitter" glutamate pools.

High concentrations of glutaraldehyde (2-5%) were found optimal for fixation of glutamate. In the absence of glutaraldehyde, (para)formaldehyde does not permanently retain L-[3H]-glutamate or D-[3H]-aspartate previously taken up into brain slices. Rats were fixed by rapid transcardial perfusion with 2.5% glutaraldehyde/1% (para)formaldehyde, and brain samples osmicated, embedded in epoxy resin, sectioned, and exposed to specific antisera to glutamate (conjugated to carrier protein by glutaraldehyde), followed by colloidal gold-labeled second antibody. The gold particle density was higher over putative glutamatergic nerve terminals than over any other tissue elements (two to three tissue average in cerebellum and hippocampus). Calibration by test conjugates containing known concentrations of fixed glutamate processed in the same fluid drops as the tissue sections indicated that the concentration of fixed glutamate in putative glutamatergic terminals in hippocampus CA1 was c. 20 nmol/liter. The grain density over the parent cell bodies was only slightly higher than the tissue average. (Grain densities over test conjugates of other amino acids, aldehyde-fixed to brain macromolecules, were similar to that over empty resin. Labeling was blocked by glutamate-glutaraldehyde but not by other glutaraldehyde-treated amino acids.)

In other experiments, brain slices were incubated in oxygenated artificial cerebrospinal fluid (CSF) and then immersion-fixed and processed as above. Here, the ratio of grain densities in putative glutamatergic terminals vs other tissue elements was greater than in perfusion-fixed material. Comparison of intra-terminal areas poor and rich in synaptic vesicles suggested that in this preparation vesicles contained at least three times the glutamate concentration of cytosol. In the glutamatergic synapses of the giant reticulospinal axons in lamprey the ratio was over 30. Prolonged K+ depolarization of hippocampal and cerebellar slices reduced the nerve terminal glutamate immunoreactivity in a Ca2+-dependent manner. The results suggest that glutamate is released by exocytosis at excitatory synapses and show that immunocytochemistry can be used to study the cellular processing of small molecules. (J Histochem Cytochem 38: 1733-1743, 1990)

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Materials and Methods

Fixation Efficiency. Rat hippocampal slices (300 μm) were cut and incubated with radiolabeled amino acids as previously described (38). The slices were distributed consecutively among the groups to be fixed differently (Figure 1) in such a way that each group consisted of a similar series of seven (or eight) slices. The incubation medium contained 1.8 μmol/liter of L-[U-14C]-glutamate (285 Ci/mol: Amersham, Poole, UK) and 0.14 μmol/liter of D-[2,3-3H]-aspartate (15,000 Ci/mol; NEN/DuPont, Boston,

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Incubation was for 15 min at 21°C in 250 µl oxygenated phosphate-buffered Krebs' solution. After two rinses (5 min each) in fresh Krebs' the slices were fixed for 3 hr in the fixatives indicated, and subsequently fixed four times in 0.1 mol/liter sodium phosphate buffer, pH 7.4: rinse I and III, 15 min 20°C; rinse II and IV overnight at 4°C. Formaldehyde was freshly prepared by depolymerizing paraformaldehyde. Glutaraldehyde (EM grade; TAAB) was added just before use. All fixatives were made up in the buffer mentioned. The volumes of fixatives and rinses were 2 ml. The slices were digested in 500 µl of Soluene-150. Slices and 200-µl aliquots of fixatives and rinses were counted with 10 ml Picofluor in a Packard Tri-Carb 460C liquid scintillation spectrometer giving dpm of 14C and 3H. (Tests confirmed that the isotopes were adequately separated.) Previous experiments (32) have indicated that in the case of glutamate over 80% of the radioactivity present in the slices before fixation represents the authentic amino acid; D-aspartate is poorly metabolized.

Results
Fixation Efficiency
The best retention of tissue glutamate was obtained by a fixative containing a relatively low concentration of formaldehyde in addition to a high concentration of glutaraldehyde (Figure 1). The fixative containing 2.5% glutaraldehyde + 1% formaldehyde has been adopted as standard (the molar concentrations of the two aldehydes are 0.25 and 0.33 in this mixture, i.e., 50% excess of aldehyde groups from the dialdehyde). Increasing the ratio of formaldehyde to glutaraldehyde reduced the efficiency of fixation. In addition, the amount of radioactivity in the second rinse increased. Whereas pure glutaraldehyde was nearly as efficient as the standard fixative, pure formaldehyde was only about one tenth as efficient. Interestingly, the amount of radioactivity in the latter fixative was not higher than in the glutaraldehyde-containing fixatives, but that in the rinses was much higher.

Depolarization-induced Depletion of Glutamate. Slices of rat cerebellar cortex and hippocampus were cut at 400 µm on a vibratome and further processed as described (22). Briefly, the slices were pre-incubated in oxygenated bicarbonate-buffered Krebs' solution at 35°C for 1 hr and then for a further 20 or 60 min in Krebs' solution with either physiological (5 mmol/liter) or elevated (40 or 55 mmol/liter) concentration of K+. The osmolality was kept constant by correspondingly reducing the concentration of either NaCl or Tris-HCl (normally 74 and 30 mmol/liter). After incubation the slices were fixed by immersion and treated as described above.

Specificity Control and Calibration
To estimate labeling specificity under conditions resembling as closely as possible the immunocytochemical situation, amino acids were fixed (conjugated) to rat brain macromolecules by glutaraldehyde and test sections made (see Materials and Methods) that contained conjugates of several amino acids. Only the conjugate containing glutamate showed appreciable labeling with the glutamate antisera (Figure 2a). Conjugates prepared with glutaraldehyde and formaldehyde in the proportion used to fix tissue specimens showed equally good specificity. The series of conjugates shown was incubated together with the tissue sections to be investigated in all our experiments. These are the amino acids that occur at the highest concentrations in brain about (2 mmol/liter and above) and are most closely related to glutamate. Quantitative analysis showed the glutamate conjugate to have a net labeling intensity generally on the order of 1000 particles/µm² and the net particle densities of non-glutamate conjugates to be less than 1% of this value and similar to the density over empty resin, which is 0.5–7 particles/µm² (depending on the experiment). The concentration of fixed amino acid in the embedded conjugates used for specificity testing was about 150 mmol/liter. This high concentration was chosen in order to obtain a high sensitivity towards low levels of crossreactivities.

The same type of conjugates, prepared to contain fixed glutamate at a series of concentrations in a biologically more relevant range, gave a curvilinear relationship between concentration and particle density in the lower part of the range, but approached linearity above 6 mmol/liter (Figure 2b). Even at the lowest concentration of fixed glutamate (0.09 mmol/liter) the net particle density
Figure 1. Effect of fixative on retention of L-[^14]C-glutamate and D[^3]H-aspartate in fixed tissue. Slices of rat hippocampus were incubated with a mixture of the radiolabeled amino acids in Krebs' solution, fixed, and processed as described in Materials and Methods. The results are given as percent of the total amounts of ^14C and ^3H in slices, fixative, and rinses (expressed per slice). The amounts recovered in the fluids are means of two experiments in close agreement. F, formaldehyde; G, glutaraldehyde. Figures in front of F and G, percent (w%) of the fixing agent; rinse I, 15 min 20°C; rinse II, overnight 4°C. The values for rinses III and IV (not shown) ranged from 0.4–2.2%. Note progressive decrease in fixation efficiency on reduction of glutaraldehyde below 25% and rapid reversibility of fixation by formaldehyde. Asterisks denote statistically significant difference from fixative on the left (*, p<0.01; **, p<0.001; ns, p>0.05; Wilcoxon-Van Eiteren, two tails). Fixation of glutamate was marginally better than that of aspartate (p<0.005, except for 2.5G+1F; Wilcoxon matched-pairs signed-ranks test, one tail).

was significantly higher than that over the "none" conjugate (8.5 vs 3.5 particles/µm^2). If such a series of conjugates is incubated in the same drops of fluids as the tissue sections to be examined, it is possible to estimate the approximate concentrations of fixed glutamate in the tissue (see below).

Because the amino acid conjugates were prepared without exposure to OsO_4, and because OsO_4 treatment is necessary to preserve ultrastructure during tissue embedding, it was necessary to check the effect of osmication on labeling intensity. An osmium-treated conjugate was therefore included in the sandwich of calibration conjugates (Figure 2b). The particle density over this type of conjugate was 70–100% (depending on the experiment) of that over the corresponding non-osmicated one. The OsO_4-treated conjugate is useful for monitoring the efficiency of "de-osmication" by periodate. Unfortunately, osmication led to large variations in the texture densities of the embedded conjugate clumps and increased variability in the gold particle densities, which made osmicated conjugates unsuitable for general use in calibration.

Localization in Tissue

In slices of cerebellar (Figure 3) or hippocampal cortex (not shown)
Figure 2. System for specificity testing (20) and calibration (17,18) of the electron microscopic post-embedding immunogold method. Test conjugates of amino acids were prepared by reacting the latter with glutaraldehyde (or the mixture of aldehydes routinely used for tissue fixation) and macromolecules from rat brain, freeze-dried, embedded in epoxy resin, sandwiched (with spacer sections of rat brain), and resected into grids. The grids were immersed in drops of diluted purified antiserum together with grids carrying tissue to be examined. (a) Electron micrographs of a series of conjugates prepared with glutaraldehyde. The concentration of amino acid (usual three-letter symbols) in the embedded conjugate clumps was about 150 mmol/liter. Note high densities of 15-nm gold particles over glutamate conjugate (and spacer sections of glutamate-containing tissue) and low particle densities over empty resin and non-glutamate conjugates. None, macromolecules reacted with glutaraldehyde in the absence of amino acid. (b) Relationship between glutamate concentration in the embedded conjugates (estimated by radioactive tracer; see ref. 17) and gold particle density. This series was prepared with the glutaraldehyde/formaldehyde mixture. Background labeling over empty resin (1.5 particles/μm²) is subtracted. o, mean net particle densities ± SEM over non-osmicated glutamate conjugates; •, result for osmicated conjugate. An aliquot of the conjugate with the highest glutamate concentration (32 mmol/liter) was treated with OsO₄ followed by dialysis and freeze-drying before resin embedding and incorporation into the model system. The 20% reduction in density suggests that there was some residual masking effect of OsO₄ after the HIO₄/NalO₄ treatment. This model section was incubated together with the tissue sections represented in Figure 4, II-IV. Bars: a (left column) = 2 μm; a (right column) = 0.2 μm. (Results from ref. 4)
fixed by immersion in vitro, glutamate-like immunoreactivity was very conspicuously concentrated over putative glutamatergic nerve endings relative to all other tissue elements. Thus, the terminals of the cerebellar mossy fibers and parallel fibers were heavily labeled, and so were the terminals of hippocampal mossy fibers, of perforant path fibers and of fibers derived from the CA3 pyramids. (These terminals are characteristic enough to be recognized by their morphologies and locations; identification by anterograde axon tracing was done for the spino-cerebellar mossy fibers). On the other hand, terminals of inhibitory neurons, such as cerebellar Golgi and basket cells and hippocampal axo-somatic and axo-dendritic terminals with symmetric synaptic thickenings, showed very low grain densities, as did dendrites, neuron perikarya, and glia.

In perfusion-fixed material from the same regions the differences in particle densities were smaller, but still the various types of alleged glutamatergic nerve endings had the highest immunoreactivities. In the cerebellar granular layer the particle density over mossy fiber endings was more than twice the total tissue average, whereas the density over Golgi cell terminals was about half this value. In the hippocampal formation corresponding results were obtained (Figure 4). In all cases the various categories of putative glutamatergic nerve endings had the highest particle densities, varying somewhat between the categories. Glial profiles were the least immunoreactive. Axo-somatic and axo-dendritic terminals with symmetric contacts (not shown in Figure 4) showed particle densities similar to those in glial processes.

Perikarya and dendritic profiles, mostly belonging to putative glutamatergic cells, showed glutamate-like immunoreactivities considerably lower than those in the terminals forming asymmetric contacts. The value for dentate granule cell bodies was about half of that for their axon terminals (mossy fiber boutons), and a corresponding relation was found between perikarya and terminals of the CA3 pyramidal cells, and between cerebellar granular cell bodies and parallel fiber terminals (Figure 4, and additional data not shown). Dendrites were usually somewhat lower in immunoreactivity than the corresponding cell bodies, and in hippocampus and cerebellum had particle densities similar to the tissue average (not shown). Nuclei and perikaryal cytoplasm showed about equal levels of immunoreactivity. In contrast to the situation in conventional light microscopic preparations, the nucleolus was as reactive as the nucleoplasm.

The compilation of data shown in Figure 4 demonstrates that it is possible to achieve good reproducibility of the method between animals and experiments and over time. This contrasts with
Figure 4. Distribution of glutamatelike immunoreactivity in the hippocampal formation; interexperimen
tal reproducibility in nine different rats (I-IX) fixed by transcardial perfusion (2.5% glutaraldehyde + 1% formaldehyde). Columns show mean net gold particles/μm² ± SEM, corrected for background over tissue-free resin (0.5–3 particles/μm² depending on the experiment). Numbers of profiles examined are given over columns. Numbers on abscissas indicate profile types (see lower left corner; missing numbers correspond to profiles recorded infrequently and omitted for clarity). Not all profile types are represented for each case, because the ultra-thin sections usually comprised either the area dentata or the CA1/CA3 regions. In case I the exposed blade of fascia dentata (open columns) as well as the hidden blade (hatched columns) was examined. The sections were processed in different experiments, except those from cases II–IV, which were incubated together. Note that the rank order of labeling densities over the different profile types is consistent from case to case, and that the actual densities agree reasonably well. (Modified from ref. 4)

an apparent variability in the relative immunoreactivity of nerve terminals and neuron perikarya observed in semi-thin sections of the same material processed by the post-embedding, peroxidase–antiperoxidase method (not illustrated).

Combining the data of Figure 4 II–IV with the corresponding calibration curve (preparations incubated in the same drops; Figure 2b) suggests a fixed glutamate concentration on the order of 12 mmol/liter in the terminals forming asymmetric contacts on spines in stratum radiatum of CA1. Taking account of the incomplete de-osmication (Figure 2b) and retention of tissue glutamate by perfusion-fixation (some 70%; see Discussion), this translates to 21 mmol/liter. Another series of data, in which more complete de-osmication was attained, gave a similar figure. For the hippocampal mossy fiber terminals the correspondingly corrected value would be on the order of 13 mmol/liter. The concentration in gial processes, which display one sixth to one third the particle density of the CA1 terminals (Figure 6), is only about 2 mmol/liter, according to Figures 2b and 4 and the same corrections as above. The
Figure 5. (a) Selective concentration of glutamate-like immunoreactivity over synaptic vesicles in a probably glutamatergic synapse, the giant reticulo-spinal axon excitatory synapse in lamprey, *Ichthyomyzon unicuspis* (see ref. 6). C, axoplasm (note that gold particles are spread over cytosol); D, postsynaptic dendrite; V, cluster of synaptic vesicles apposed to membrane specialization. Quantitative evaluation showed the following net labeling intensities (mean net particles/μm² ± SEM): V, 51.9 ± 8.0; C, 1.4 ± 0.3; D, 9.9 ± 0.6; total of micrographs examined, 5.6 ± 0.8 (background over empty resin 2.5 was subtracted). The differences between V, C, and D were all significant at the 0.001 level (Student’s t-test as well as Wilcoxon rank-sum test). Lamprey tissue provided by L. Brodin and S. Grillner. (b) Samples (glutamate, taurine, glycine) of test conjugates from model section (see Figure 2b) processed together with the lamprey sections in the same drips of fluids. Note selectivity of labeling and low background. Bars = 0.5 μm.

**tissue average for hippocampus translates to an estimated in vivo glutamate concentration of roughly 7 mmol/liter, which is consistent with biochemically measured values (1,2).**

**Compartmentation of Glutamate at the Synapse**

This question was studied in the giant reticulo-spinal axons of lamprey as well as in rat hippocampal mossy fibers, both of which are likely to terminate with glutamatergic synapses. In the giant reticulo-spinax axons, synaptic vesicles form distinct clusters surrounded by vast areas of organelle-poor axoplasm, consisting of cytosol with scattered neurofilaments and neurotubules. Glutamate-like immunoreactivity was highly concentrated over the vesicular clusters (Figure 5). The net particle density here was 37 times that in the surrounding axoplasm (which was barely above background) and five times that in the postsynaptic dendrites.

In the hippocampal mossy fiber boutons, the particle density over clusters of synaptic vesicles was about twice that over organelle-free cytosol and 50% higher than the terminal average (97 ± 6, 44 ± 6, and 64 ± 8 particles/μm², means ± SEM, measurements in immersion-fixed slices), both of these differences being statistically significant (p<0.002: Student’s t-test, two tails). Assuming the vesicular volume to be about half of the cluster volume (which would be the case if the vesicles were densely packed, spherical, and equally sized) and the glutamate concentration in the intervening space to be equal to that in the surrounding cytosol, the
vesicular glutamate concentration is well in excess of calculated, to be about the lowest part of the range (Figure 2b), the results indicate that the particle density rises most steeply with concentration in the cells. In the hippocampus similar results were obtained. The changes in mitochondria (129 ± 7 particles/μm², mean ± SEM) are presented for the number of profiles indicated over the columns, correcting for background (0.85 particles/μm² over empty resin). MF, mossy fiber terminals; PF, parallel fiber terminals; GP, glial processes (only Bergman glial processes with distinct filaments are included). Asterisks denote statistically significant difference(s) from column(s) on the left (*, p<0.05; **, p<0.001). Open stars denote significant difference between results at 5 and 55 mmol/liter of K⁺ (*, p<0.01; ***, p<0.001; Student’s t-test, two tails). (Data from ref. 22)

Depolarization-induced Depletion of Glutamate

In cerebellar slices exposed to high concentrations of K⁺ for extended periods, glutamate-like immunoreactivity was drastically redistributed (Figure 6). Putative glutamatergic nerve terminals lost most of their labeling, whereas there was a significant increase in glial cells. In the hippocampal similar results were obtained. The changes were not seen when the Ca²⁺ in the medium was lowered to 0.1 mmol/liter and the Mg²⁺ was raised to 10 mmol/liter, conditions known to block synaptic transmitter release. The extent to which the nerve terminals were depleted of glutamate-like immunoreactivity increased with the K⁺ (40 and 55 mmol/liter) and time (20 and 60 min) and varied among the different types of putative glutamatergic terminals. Thus, the terminals making asymmetric synapses on spines in CA1 were more resistant to glutamate depletion than the mossy fiber terminals in hippocampus or cerebellum.

Discussion

Fixation

For the immunocytochemical investigation of small molecules it is essential to fix the molecule in situ as quickly as possible to prevent redistribution. Therefore, for perfusion-fixation we have chosen the transcortical approach, which allows introduction of the fixative within a few seconds of opening the thorax, a high flow rate (50 ml/min), and neck stiffness within 2 min (19).

The efficiency of the fixative is as important as the rate at which it is introduced. (Para)formaldehyde alone cannot be recommended as a fixative for amino acids. This was shown already in 1967 (29) and confirmed in the present study on immersion-fixation of brain slices, which further suggests that amino acids initially retained by formaldehyde are lost during subsequent processing. On the other hand, the smaller molecule formaldehyde may penetrate quicker than glutaraldehyde and, as the present results suggest, may assist in the permanent fixation by the latter. However, increasing the concentration of formaldehyde while reducing that of glutaraldehyde reduces the proportion of retained amino acid. It is reasonable to assume that a high proportion of amino acid retained is associated with a low level of artifactual redistribution. The presence of a moderate amount of (para)formaldehyde in a high-glutaraldehyde fixative gives the highest retention of acidic amino acids and has been adopted as standard fixative. A statistically significant augmentation by low formaldehyde has been shown in a longer series of experiments (34). For further discussion on the fixation of amino acids by glutaraldehyde and formaldehyde, see ref. 35.

The maximum retention of glutamate in immobilized slices is on the order of 50%. In perfusion-fixed tissue the possibilities for loss of constituents to the medium can be assumed to be less, since the tissue is intact and the distance from the fixation medium in the vasculature to each site in the tissue is shorter and more uniform than in the slices. The proportion fixed on perfusion with our standard fixative has been estimated (14) in the case of taurine by removing pieces of cerebellar cortex under anesthesia before and after perfusion of rats pre-loaded with the radiolabeled amino acid, and was found to be 73 ± 9% (mean ± SD, four animals). The slow turnover of taurine and its relatively good penetrability into brain make this type of investigation possible. Such experiments cannot easily be done for glutamate or aspartate.

Specificity

From the outset (33), our attitude has been that antibody specificities should be tested in conditions mimicking as closely as possible the conditions of the immunocytochemical situation. Therefore, we have made macromolecular conjugates (i.e., chiefly protein bound to endogenous lipopolipoprotein compounds) of rat brain, or in special cases of the tissue to be examined (9), and coupled different amino acids to this by glutaraldehyde (33) or the aldehyde mixture used for tissue fixation. Such conjugates were used...
to produce a spot-test system (26) mimicking the situation in the
light microscopic tissue sections stained by the peroxidase-antiper-
oxidase method. The specificities of the sera used have been exam-
ined in this system. No appreciable crossreactivity was detected
against a large series of low molecular weight tissue constituents
(28). In addition, thin-layer chromatograms of acid ethanol extract
of rat brain displayed only a single band that migrated like authentic
glutamate (23).

The approach has been extended to the post-embedding im-
munogold method (Figure 2) (18,20). The conjugates used for spec-
ficity testing were prepared at high concentrations of amino acids
(2500 μmol/g protein) and the concentrations of fixed amino acids
in the embedded conjugates were about 150 mmol/liter. This is
appreciably higher than the concentrations likely to be encoun-
tered in the tissue specimens (average in brain tissue about 10
mmol/liter) and ensures a high sensitivity for detection of cross-
reactivities. It should be noted that the density of gold particles
over the "none" conjugate is close to background, suggesting that
glutamate residues of proteins are non-reactive. The importance
of including such conjugates in the experiments cannot be over-
emphasized, particularly in the case of the post-embedding elec-
tron microscopic immunogold method, which depends on so many
factors that are difficult to control.

In addition to this "positive control," it is useful to perform "nega-
tive controls" by absorbing the antibody preparation with aldehy-
d-treated amino acids, either in solid phase attached to a resin or
in liquid phase. The aldehyde-treated amino acids are soluble and
easy to prepare (9,28). They are used for checking that the product
of the amino acid against which the antibody preparation was raised
specifically absorbs the labeling of tissue sections and test conjugates.
In addition, they are convenient for removing slight crossreactivi-
ties from the antisera.

Calibration

The first experiments (17,18) suggested a linear relationship be-
tween concentration of fixed glutamate and gold particle density
up to at least 150 mmol/liter. In the biologically relevant range
(Figure 2b), the particle density is also nearly proportional to the
concentration of fixed glutamate above about 6 mmol/liter, but
falls off curvilinearly below this level. However, even at the lowest
 glutamate concentration tested (0.09 mmol/liter) the net particle
density was significantly higher than that over the "none" conjugate
(8.5 vs 3.5 particles/μm² in the experiment shown in Figure 2b),
indicating a good sensitivity. The non-linearity of the calibration
curve at low concentrations implies that the concentration ratios
between different sites will tend to be underestimated when grain
density is used as a direct measure of glutamate concentration. The
concentration ratios for normal localization and effects of stimula-
tion are therefore likely to be even greater than the particle density
ratios given in the Results.

To be useful for calibration, the model sections must be processed
together with the tissue specimens in the same drops of fluids. Even
so, it should be remembered that the values obtained are only ap-
proximate, since the calibration involves several assumptions and
factors that are difficult to control. The possibility therefore exists
that local variation in the retention of amino acid and in the acces-
sibility of the fixed amino acid by retention of amino acid and in
the accessibility of the fixed amino acid by the antibody could per-
turb the distribution of the immunoreactivity (but see below under
Tissue Localization). The average tissue concentrations estimated
by the method in hippocampus (this work) and cerebellum (17)
agree with those measured biochemically, providing support for the
usefulness of the approach. However, it should be pointed out
that the relationship between average particle density and average
tissue concentration is not a simple one, owing to the local varia-
tions in glutamate concentration and the non-linearity in the lower
concentration range of the calibration curve.

Tissue Localization

The initial pre-embedding light microscopic results on perfusion-
fixed tissue (25,26) were disappointing, insofar as the distribution
of glutamate-like immunoreactivity in the neuropil was rather uni-
form in contrast to that of exogenous radiolabeled glutamate accu-
mulated through high-affinity membrane transport (38). Further-
more, in cortical areas the immunoreactivity in perikarya and
dendrites was generally higher than that in neuropil. This has also
been observed by others (e.g., 8,12). Streit and colleagues (13), using
the post-embedding light microscopic silver-enhanced immunogold
approach, did find staining of nerve terminal-like dots in the hip-
 pocampal region, but the staining and its laminar distribution varied
for reasons that could not be worked out. As reported here, we,
too, found variation in laminar staining pattern and in the ratio
between nerve terminal-like and perikaryal neuron staining in semi-
thin sections, of the same material that electron microscopically
gave consistently high nerve terminal labeling (Figure 4). This sug-
gests that problems related to incomplete and uneven accessibility
of immunoreagents to the antigenic sites remain even when semi-
thin sections are used.

On the other hand, brain slices fixed by immersion in vitro as
in the uptake experiments demonstrated a preferential localization
of glutamate-like immunoreactivity in small dots in the neuropil
(24,33), which showed a laminar pattern similar to that of high-
affinity glutamate uptake sites (36). Electron microscopically, this
proved to be due to a high concentration of glutamate in putative
glutamatergic nerve endings (Figure 3). Moreover, the immunoreac-
tivity in the terminals appeared to increase when tissue was fixed
by immersion rather than by perfusion, as evaluated in experiments
where the two types of specimen were processed parallel in the same
drops of solutions (21). An obvious interpretation of this observa-
tion is that a redistribution occurs into compartments bounded
by membranes endowed with glutamate transporters.

However, the low light microscopic immunoreactivity of nerve
terminals in tissue fixed by perfusion could also be due to masking
of antigenic sites in this type of preparation, which presumably
is more heavily cross-linked than the immersion-fixed slices. This
notion was supported by the finding that treatment of the perfusion-
fixed tissue with trypsin before incubation with antibody (21), a
procedure used to recover antigenicity in specimens fixed by alde-
hydes (5), increased the staining intensity in the neuropil layers,
giving a distribution pattern similar to that in the slices. However,
loss of staining from some structures (perikarya) simultaneously with
the increase of staining in nerve terminals and the difficulty of con-
trolling the process make this approach unsuitable for general use. There thus seems to exist a problem of accessibility of the antigenic epitopes for interaction with the antibodies. The problem appears to be much less in the demonstration of other amino acids, such as GABA, glycine, taurine, or glutamine, than with glutamate. This suggests that it could be related to the high concentration of negative charge in the fixation products.

The electron microscopic post-embedding immunogold approach seems to overcome this type of artifact, since it invariably gives the highest labeling intensities over putative glutamatergic nerve endings and a similar rank order of immunoreactivities of the different structures in perfusion-fixed and immersion-fixed preparations (4, 18, 21, 22, 27, 30). Further support comes from the observation (unpublished) that the nucleolus, a structure of very high mass density, appears much less immunoreactive than the rest of the nucleus in “conventional” light microscopic preparations of perfusion-fixed tissue, but shows about the same particle density as the nucleoplasm in the electron microscopic post-embedding method. In this method there is no penetration of the antibodies into the tissue, the interaction being restricted to the antigenic sites exposed at the cut (actually microfractured) surface. This would eliminate regional differences in penetration and would also abolish steric hindrance of interaction between antibodies and antigen buried inside folds in cross-linked macromolecules.

A further virtue of the electron microscopic post-embedding immunogold method is its ability to produce quantitative results (see above). This is particularly important for the investigation of glutamate, which has roles in general metabolism and protein synthesis in addition to its alleged transmitter role. Our findings confirm the inference based on biochemical results (see ref. 11) of a large neuronal and a small glial pool of glutamate in brain, and a glutamate/glutamine cycle between nerve endings and glia. They further show that the highest concentrations of glutamate-like immunoreactivity occur in nerve terminals that are considered to use glutamate as transmitter on other grounds. The glutamate concentration was estimated to be approximately 15–21 mmol/liter in such terminals in hippocampus and has been estimated at 26 mmol/liter in cerebellum in a previous study (17). Although it remains to be seen whether this is generally valid, our results suggest that a high concentration of glutamate in a nerve terminal is a good indication that the terminal is glutamatergic. On the other hand, the concentration in perikarya and dendrites does not seem useful for distinguishing between glutamatergic and non-glutamatergic neurons. Thus, perikarya of motoneurons and of aminergic neurons in substantia nigra, raphe, and locus coeruleus show glutamate immunoreactivities of the same order as hippocampal and cortical pyramidal cells (25, 26).

Central to the role of glutamate as a transmitter is the question of whether the amino acid is concentrated in synaptic vesicles. The giant reticulo-spinal axons of the lamprey are a particularly favorable preparation for the study of this question. Intracellular recording from the two sides of the synapse have shown that a glutamate-like substance is the transmitter (6). We found here a very clear enrichment of glutamate immunoreactivity over the clusters of synaptic vesicles. The absence of such an enrichment of gold particles after incubation with a glycine antiserum suggests that the finding is not simply due to more favorable conditions for retention of fixed amino acids in the vesicle-rich areas (unpublished). The almost glutamate-free surrounding cytosol and a large distance to mitochondria (where glutamate synthesis is thought to take place) underline the ability of cells to develop large concentration gradients between intracellular compartments, even for small molecules. The vesicular glutamate transporter of mammalian brain has a K_m of about 1 mmol/liter (16). Our results for lamprey have not been calibrated to absolute glutamate concentrations, but the densities obtained suggest that the cytosolic concentrations may be considerably less than 1 mmol/liter. This might indicate that the vesicular transporter of lamprey has a higher affinity than the mammalian one.

Based on our results, likely values for concentrations of glutamate in glutamatergic synaptic vesicles are 30–50 mmol/liter for the excitatory terminals in the hippocampus. (These estimates do not take into account the fact that the available intravesicular water space may be considerably less than the gross vesicular volume.) Cytosolic glutamate is probably less than 10 mmol/liter in the same terminals. The values are in agreement with recent biochemical data on the concentration of glutamate in vesicles isolated under conditions minimizing the loss of glutamate (7), of the vesicular/cytosolic gradient of glutamate (15), and on the concentration of glutamate in cortico-striatal glutamatergic terminals (10).

The demonstration of a high concentration of glutamate in synaptic vesicles of probably glutamatergic terminals is complemented by our observation that such terminals can be depleted of glutamate-like immunoreactivity on prolonged depolarization by K^+. The Ca^{2+} dependence of this effect suggests an exocytotic release mechanism. Previous light microscopic results have shown that similar depletion can be elicited by veratrine, which depolarizes by opening Na^+ channels, and that in either case the depletion can be prevented by supplying the glutamate precursor glutamine (37). The latter observation and the different sensitivities to depletion in the different classes of glutamatergic terminals suggest that intraterminal glutamate levels reflect a balance between release (Ca^{2+}-dependent and -independent) and replenishment [synthesis and (re)uptake], perturbation of which leads to depletion of the transmitter pool of glutamate. The concomitant changes in glial glutamate emphasize the role of glia in removal/recycling of synthetically released glutamate.

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