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Postembedding immunocytochemical GABA labeling in rat neocortex cultures: applicability in quantitative studies

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The value of γ -aminobutyric acid (GABA) immunogold labeling in the quantification of nerve endings was studied in dissociated rat neocortex cultures. Adjacent sections were processed in 3 series according to the same staining protocol. Of all nerve ending profiles examined, 20% appeared to be GABA-positive, while 7% could not be classified. In cultures pretreated with gabaculine, GABA-positive endings were labeled more heavily. Cell bodies could always be discriminated as GABA-positive or GABA-negative. Of all neuronal cell profiles, 14% appeared to be GABA-positive. These neurons could also be identified by light microscopy using the peroxidase–anti-peroxidase method.

Recently, an antibody against γ -aminobutyric acid (GABA) [11], used in a postembedding immunogold staining method, enabled the electron microscopic demonstration of this neurotransmitter with optimal preservation of ultrastructural detail [3, 12, 14]. Such a method would be useful in research in which the quantification of GABA-positive structures is needed as a parameter. Such studies demand, however, reliable discrimination of labeled and unlabeled structures. The main purpose of this study, therefore, was to investigate whether cell bodies and nerve endings in rat neocortex cultures could be accurately designated as either GABA-positive or GABA-negative.

GABA was visualized in ultrathin sections by means of a postembedding immunogold method, and in semithin sections by postembedding staining, using the peroxidase–anti-peroxidase method. Cultures pretreated with the GABA-transaminase inhibitor gabaculine were compared with controls in order to see if the expected GABA increase [1, 7] would result in a heavier and, therefore, more discriminative labeling of GABA-positive nerve endings. Pilot experiments in which fixation, rinsing and etching times were systematically varied, led to the following protocol.

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Occipital cortex tissue obtained from 19-day-old fetal rats was mechanically dissociated, and the resulting cell suspension inoculated onto the center of poly-D-lysine-coated plastic Petri dishes. Reaggregates were grown in a serum-free, chemically defined medium for 16 days [10, 15]. Two hours prior to fixation, growth medium of two cultures was replaced by medium containing 1.8 mg/ml gabaculine [9], whereas two control cultures were refreshed with drug-free medium. The cultures were fixed in 5% glutaraldehyde in sodium cacodylate buffer (300 mOsm, pH 7.4) for 2 h at 4°C, rinsed 3 times in 0.05 M Tris-0.9% NaCl and stored overnight in this buffer. Postfixation was done in a mixture containing 1% OsO₄ and 1.5% K₃Fe(CN)₆ in sodium cacodylate buffer (300 mOsm, pH 7.4) for 1 h at 4°C. Cultures were dehydrated in a graded alcohol series, embedded in Epon and polymerized at 45 and 60°C, divided over 2 days.

Both semithin (ca. 1 µm) and ultrathin (ca. 70 nm) sections were cut. Semithin sections were mounted on chrome-alum-gelatine-coated slides and alternately stained with Toluidine blue or processed for GABA staining (Table I). Ultrathin sections

TABLE I

POSTEMBEDDING GABA STAINING ON SEMITHIN (1 µm) SECTIONS

Removal of Epon [4, 6]	
Put slide with sections into Na-ethanolate	1 h
Dab with filtering paper	
100% ethanol (1)	5 min
Dab	
100% ethanol (2)	1 min
Dab	
100% ethanol (3)	1 min
Dab	
100% ethanol (4)	1 min
Phosphate-buffered saline (PBS; pH 7)	5 min
Rinse in aquadest (3 ×)	
PBS (pH 4)	5 min
Rinse in tap water	5 min
Removal of osmium	
Etch in 8% H ₂ O ₂	12 min
Rinse in aquadest (3 ×) and in 0.05 M Tris-0.9% NaCl (pH 7.6; once)	
Immunocytochemical staining	
Incubate in normal, goat serum diluted 1:5 in Tris-NaCl	30 min
Rinse in Tris-NaCl (once)	
Incubate in rabbit anti-GABA diluted in Tris-NaCl-0.5% Triton 1:1000	overnight
Rinse in Tris-NaCl (3 ×)	
Goat anti-rabbit antibody 1:100 in Tris-Triton	1 h
Rinse in Tris-NaCl (3 ×)	
PAP complex diluted 1:600 in Tris-Triton	1 h
Rinse in Tris-NaCl (3 ×)	
7.5 mg Diaminobenzidine and 5 µl H ₂ O ₂ (30%) in 15 ml Tris-NaCl	12 min
Rinse in Tris-NaCl (once) and tapwater	

Note: the whole procedure was done at room temperature.

were collected on 300 mesh nickel grids and processed according to the immunogold method as described in Table II. This method was carried out 3 times on different grids with sections obtained from the same cultures. It should be noted that quantitative statements about cell bodies and nerve endings, made in this communication, refer to the number of profiles of structures examined. Unfolding procedures were beyond the scope of this study. The expression 'profile' will not be used further, so as to maintain a uniform terminology.

At both the light and electron microscopic level, a population of heavily labeled cell bodies could be unambiguously discriminated from lightly labeled or unlabeled cell bodies (Figs. 1–3). The heavily labeled cell bodies belonged to neurons only, not to glial cells, and were defined as GABA-positive. Light microscopic screening of several sections revealed an uneven distribution of GABA-positive neurons without any tendency towards a preferential localization within the cultures. The average size of GABA-positive and GABA-negative neuronal cell bodies did not differ. Only cell bodies that showed a nucleus in sections stained for GABA and in adjacent Toluidine blue-stained sections were measured. GABA-positive neurons constituted 14% of 840 neurons that were screened in 4 cultures. White et al. [16] observed 30–50% putative GABA-positive neurons in comparable cultures. The neuronal population in mouse neocortex cultures has been reported to contain only 10% GABA-positive neurons [8].

Labeling was seen to be distributed more or less evenly across the entire cell body, including the nucleus, using both light microscopy and electron microscopy. Nuclear

TABLE II

POSTEMBEDDING GABA IMMUNOGOLD LABELING ON ULTRATHIN SECTIONS

Removal of osmium	
Etch grids in 30 μ l 8% H ₂ O ₂ droplets	12 min
Rinse in aquadest (3 $\times$) 0.05 M Tris–0.9% NaCl (pH 7.6; once)	
Store grids in droplets Tris–NaCl	
Immunolabeling	
Rabbit anti-GABA diluted 1:1000 in Tris–NaCl–0.5% Triton	20 min
Keep grids in 15 μ l droplets	
Rinse in Tris–NaCl (3 $\times$)	
Store grids in Tris–NaCl droplets	20 min
Goat anti-rabbit IgG labeled with gold particles (diameter 15–20 nm; Janssen Pharmaceuticals)	
diluted 1:40 in Tris–Triton	
Keep grids in 15 μ l droplets	
Rinse in Tris–NaCl (twice) and aquadest (twice)	
Grids can be dried	
Contrast with uranyl acetate and lead citrate	

Note: incubation and storage droplets were kept on parafilm in Petri dishes. Rinsing media were stirred in beakers, in which a grid was held by a forceps. The whole procedure was carried out at room temperature.

labeling is a common artefact, probably induced by the glutaraldehyde fixation, which opens the nuclear membranes and allows not yet fixed molecules to diffuse into the nucleus. Intrinsic GABA as well as [^3H]GABA has been localized in the nucleus in other studies too [5, 13].

At the electron microscopic level, both axons and dendrites could be found to be heavily labeled with gold particles (Fig. 4). Since cortex cultures have large intercellular spaces, the presence of background contamination could easily be determined on the basis of gold particles scattered across these spaces (Fig. 7). In cases where the intercellular space was devoid of contamination, slight labeling on some cell bodies and fibers could still be found.

The occurrence of nerve endings, observed as compartments with at least 3 clear vesicles, was screened in 6 mesh holes of each of 14 grids. In sections where the intercellular space showed no gold particles, nerve endings were defined as being indeterminate if a single particle in small endings, or 2–3 in larger ones were seen. In cases where the intercellular space showed signs of contamination, classification standards were adapted: from the number of gold particles over a distinct nerve ending, the number of gold particles over a similar area in surrounding intercellular space was subtracted. Nerve endings classified as GABA-positive formed synapses on GABA-positive as well as on GABA-negative structures. These synapses were always symmetrical. GABA-negative endings, on the other hand, formed symmetrical as well as asymmetrical synaptic junctions. Gold particles in nerve endings were found to be evenly distributed, i.e. they did not appear to be clustered across either vesicles or mitochondria (Figs. 5 and 6).

The results of the classification of nerve endings are presented in Table III. Based on all sections examined ($n=14$), endings were classified as $20 \pm 2.7\%$ GABA-positive (mean $\pm$ S.D.), $73 \pm 1.7\%$ GABA-negative and $7 \pm 1.7\%$ indeterminate. Pretreatment with gabaculine resulted in an increased labeling of the GABA-positive endings. This finding was supported by the increased number of gold particles over a set of randomly chosen GABA-positive endings in gabaculine-treated cultures compared with those in control cultures (Table III). Qualitative observations indicated that the average size of these endings in both pretreated and control cultures was similar. The percentage of GABA-positive endings in gabaculine-treated cultures was slightly increased, whereas the percentage of indeterminate endings tended to decrease.

Thus, pretreatment with gabaculine slightly improved the possibility of discriminating between GABA-positive and GABA-negative nerve endings. Although accurate identification of GABA-positive nerve endings varied between the 3 immunogold staining series that were carried out, this postembedding labeling method appears to be useful in quantifying GABA-positive neurons and nerve endings. Appropriate quantitative methods were recently described by Braendgaard and Gundersen [2]. Postembedding staining also opens the possibility of separately treating adjacent sections, which offers the opportunity of studying the same tissue sample in different ways, thus providing a broad spectrum of information.

TABLE III
CLASSIFICATION OF NERVE ENDING PROFILES

Immunogold labeling was carried out 3 times on different grids covered with tissue sections obtained from 4 cultures. Nerve endings were examined in 14 sections, processed according to Table II. These sections were derived from 2 drug-treated (A, B) and 2 control (C, D) cultures and placed on different grids. In 2 sections (*) endings were examined in 3 mesh holes instead of 6, because these sections appeared to be cut at the edge of the culture, with only 3 tissue-covered mesh holes present.

Staining series	1				2				3					
Cultures from which sections were obtained														
Pretreatment														
Mean number of gold particles in 20 random GABA-positive endings	A	B	C	D	A	B	C	D	A	B	C	D		
	gabaculine				gabaculine				control					
Presence of gold particles in intercellular space														
% GABA-positive	9.6	9.1	6.6	7.1	7.3	7.7	9.5	5.4	5.6	5.9	12.3	14.0	9.2	8.7
% GABA-negative	—	—	—	—	—	—	+	—	—	—	+	+	+	+
% Indeterminable	24.2	24.4	16.8	16.0	21.1	20.0	19.4	21.8	22.0	16.1	21.2	21.9	19.1	19.4
Number of endings examined	72.7	72.3	74.1	76.0	72.6	73.0	73.4	71.3	72.0	76.8	71.6	70.8	72.6	72.5
	3.1	3.2	9.1	8.2	6.3	7.1	7.1	6.9	6.1	7.1	7.2	7.3	8.3	8.1
	256	311	286	208	285	185	255	188	82*	56*	278	219	288	258

Figs. 1 and 2. Cell bodies in two adjacent semithin sections, showing the same area, stained (1) for GABA according to Table I and (2) by Toluidine blue. Bar = 50 μ m.

Fig. 3. Two neighboring cell bodies in an ultrathin section, processed by the immunogold method described in Table II. The GABA-positive neuronal cell body on the left side is characterized by heavy labeling of gold particles. Bar = 500 nm.

Fig. 4. A GABA-positive axon with its terminal (control culture). Bar = 500 nm.

Fig. 5. GABA-positive and GABA-negative nerve endings synapsing on a GABA-negative dendrite in a gabaculine-pretreated culture. Bar = 300 nm.

Fig. 6. A GABA-positive heavily labeled and a GABA-negative synapse in a control culture. Bar = 300 nm.

Fig. 7. Overall picture of an area in which a GABA-negative (arrow) and a GABA-positive ending (arrow-head) form synapses on the same dendrite in a control culture. The absence of gold particles over the remaining fibers and endings reflects the selectivity of this staining procedure. Bar = 400 nm.

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