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Selectivity of neuronal [^{3}H]GABA accumulation in the visual cortex as revealed by Golgi staining of the labeled neurons

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[^{3}H]GABA was injected into the visual cortex of rats in vivo. The labeled amino acid was demonstrated by autoradiography using semithin sections of Golgi material. Selective accumulation was seen in the perikarya of Golgi-stained, gold-toned, aspinous stellate neurons. Spine-laden pyramidal-like cells did not show labeling. This method gives direct information about the dendritic arborization of a neuron, and its putative transmitter, and allows the identification of its synaptic connections.

Selective, high affinity uptake of exogenous, radiolabeled putative neurotransmitters has been a valuable tool for the characterization of certain neuron populations (see refs. 5 and 6). In particular, it has been shown that [^{3}H]GABA is selectively accumulated by certain types of neurons in the cerebellum[4,11], olfactory bulb[3] and cerebral cortex[1,4]. Some of the types of neurons accumulating [^{3}H]GABA have been shown in separate immunohistochemical studies to contain the GABA synthetizing enzyme glutamic acid decarboxylase (GAD)[8–10]. This, together with other biochemical evidence[3,6], indicates that the selective uptake of exogenous GABA can be used as evidence of the GABAergic nature of a neuron. However, autoradiographic studies can provide little information about the shape, dendritic and axonal arborization of a single neuron.

Axonal and dendritic arborization are best revealed by single cell staining procedures such as the Golgi method. The Golgi method has gained new impetus with the introduction of new electron microscopic combinations for tracing synaptic connections of identified neurons[2,14,16]. However, Golgi staining gives no information about the transmitter or chemical nature of the stained neuron. Therefore most often indirect correlation is used to evaluate the transmitters of various neuron types.

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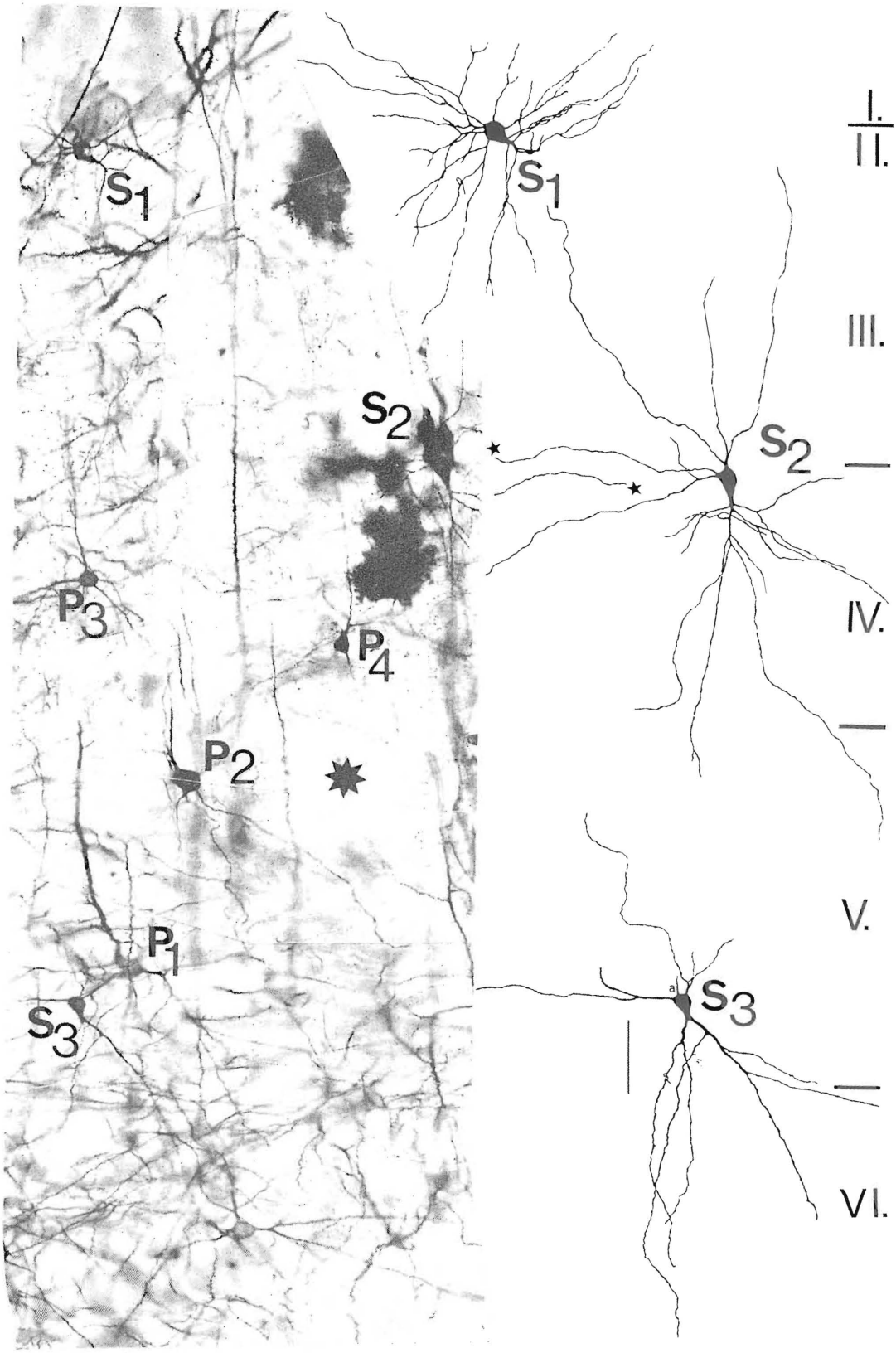
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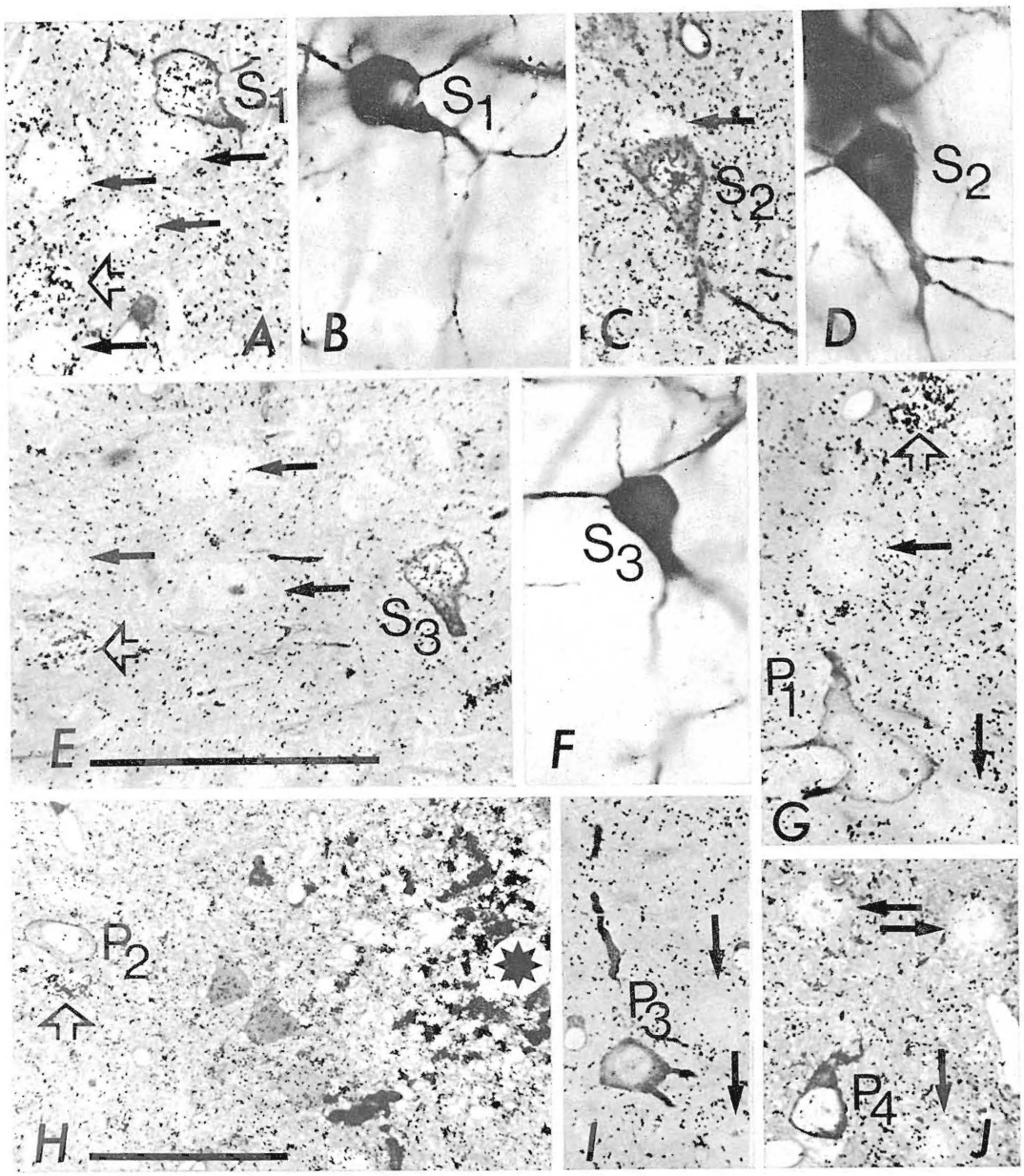

Fig. 2. Semithin sections of the stellate (A, C and E) and pyramidal-like (G–J) neurons, marked in Fig 1, are shown after processing for autoradiography. B, D and F show the gold-toned perikarya of the stellate neurons in the Golgi section. Only the stellate cells have accumulated [^{3}H]GABA preferentially. Other GABA-accumulating but not Golgi-stained neurons (open arrow) and unlabeled neurons (arrow) are also seen. Star marks injection site in H. All figures are the same magnification except H. Scales = 50 μm.

Fig. 1. Golgi-stained, gold-toned sagittal section of the primary visual cortex of the rat is shown on the left. The perikarya of 3 aspinous stellate cells (S_{1-3}) and 4 pyramidal-like spiny cells (P_{1-4}) can be seen. [^{3}H]GABA was injected from a micropipette (track labeled by large star) penetrating nearly perpendicular to the plane of the section. The dendritic arborization of the stellate cells is shown at the same scale in a camera lucida drawing on the right. a, axon initial segment. Scale 50 μm. I–VI correspond to cortical layers.

In order to overcome the limitations of both approaches we have combined the selective labeling of a neuron with exogenously applied putative transmitter and the Golgi staining of the same neuron, revealing its three-dimensional structure.

The visual cortex of male, adult Wistar and CFY strain rats were injected, under chloral hydrate anesthesia (350 mg/kg), with [^{3}H]GABA (Radiochemical Centre Ltd., Amersham, 0.33 mM, 60 Ci/mmol) dissolved in Krebs bicarbonate, containing 134 mM, NaCl; 5 mM KCl; 1.25 mM KH_2PO_4; 2 mM $CaCl_2$; 1 mM $MgSO_4 \cdot 7H_2O$; 22 mM $NaHCO_3$ and 0.18% glucose. The injections were delivered via glass micropipettes (tip diameter 30–50 μm) penetrating 4 mm from the pial surface at an oblique angle of 64° from vertical and perpendicular to the sagittal sinus. The rat (case GA24) illustrated in this paper received two series of injections in the visual cortex, 2 mm apart rostro-caudally. In both sets of injections 0.1 μl (2 μCi) of [^{3}H]GABA was injected at 4 sites; 1 mm apart, over a total time of 5 min. Ten minutes after the last injection the animal was perfused with a fixative containing 3% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Thereafter, sagittal, 1 mm thick slices of the injected cortex were dissected and processed for Golgi staining and gold toning[2], essentially as described previously[14,16]. After silver nitrate treatment, 90 μm thick Golgi sections were placed on slides with a drop of glycerin, illuminated from both sides for 20 min with a Zeiss (Jena) microscope, gold-toned, dehydrated and embedded in DURCUPAN ACM on slides. After drawing and photography, selected cells were re-embedded and 1 μm semithin sections of the perikarya were cut for autoradiography. Slides were covered with Ilford K5 emulsion by the dipping technique and stored for 3–21 days at 4 °C. The autoradiograms were developed with Kodak D19B, fixed, and stained with a mixture of toluidine blue and Azur II.

The Golgi stain obscures all internal detail of a stained perikaryon, usually leaving only the nucleus free of precipitate[14]. After gold toning the precipitate is removed and metallic particles are deposited, mainly along the plasma membrane only[2]. As a result in a semithin section most of the cytoplasm and the nucleus are free of deposit, and a homogenous gray ring marks the boundary of the perikaryon (Fig. 2). Silver grains in the autoradiographic emulsion are easily differentiated from the metallic deposit in the section because of their higher optical density and different focal depth. In addition, the metal particles in the gold-toned neuron appear as a homogenous deposit at light microscopic resolution. Thus silver grains resulting from the decay of ^{3}H-labeled compounds can be recognized over the perikaryon of gold-toned neurons (Fig. 2A, C, E).

Chemical development of the emulsion by the metal deposited in gold-toned neurons or by traces of reagents left in the section can be excluded because certain types of gold-toned neurons never showed accumulation of silver grains over their perikarya (Fig. 2G–J). As shown by electron microscopic studies[3,6,17], silver grains are generally evenly distributed over the nucleus and cytoplasm of [^{3}H]GABA-labeled neurons. In some cases we observed that in labeled, gold-toned neurons, there was a higher grain density over the nucleus than over the cytoplasm, so that in semithin sections not containing the nucleus fewer grains were seen. This suggests that some label might be removed from the cytoplasm during the staining procedure.

Labeling of neuronal perikarya corresponded to that described earlier[1,4]. Labeled perikarya were evaluated relative to other perikarya and not to the neuropil as silver grains over the neuropil represent [^{3}H]GABA accumulated by terminals or present in axons[3,6,17]. The labeled neurons differed greatly in their activity; the most strongly labeled neurons were usually closer to the injection track, but weakly labeled neurons were found there too. As far as they could be identified in semithin sections no typical pyramidal cells were labeled.

Unequivocal evidence for the selectivity of [^{3}H]GABA accumulation comes from the examination of Golgi-stained identified neurons (Figs. 1 and 2). In the illustrated sections all three aspinous stellate cells (S_{1-3}) were labeled but none of the pyramidal-like cells (P_{1-4}) showed activity. The dendrites of P_2 and the axon of P_4 could have been cut by the capillary track, but these neurons did not show [^{3}H]GABA accumulation. This indicates that labeling occurs by an active uptake process rather than by passive diffusion through cut processes.

The value of this approach depends on the acceptance of GABA accumulation as evidence for the GABAergic nature of a neuron. In the cerebral cortex there is complete agreement between results obtained with GAD immmunocytochemistry[8] and [^{3}H]GABA uptake[1,4]. The selective labeling of smooth dendritic stellate cells in the present study also confirms the conclusion obtained with GAD immunocytochemistry: namely, that in the rat visual cortex aspinous stellate cells are GABAergic[8]. However, while immunocytochemistry is the best method for the characterization of the synaptic connections of a chemically defined neuron *population*, it has limitations in revealing the structure and connectivity of *single* neurons because of the difficulty with penetration of the antibodies.

In the visual cortex the functional properties of many neurons depend on the spatial distribution of GABA-mediated inhibition (see, e.g. ref. 12), therefore there is a great need for the three-dimensional reconstruction of *single* inhibitory neurons. The present method is able to reveal these neurons.

It has been suggested that in the visual cortex all aspinous or sparsely spinous stellate cells are inhibitory[7]. This is supported by results that all such cells tested so far establish symmetrical synapses at their terminals[7,13,15]. Some of them, such as the axo-axonic cell[13] or the double bouquet cell[15] are morphologically distinct and they differ in their connections, but the transmitters of these well-defined neurons are not yet known. It will be possible with this method to delineate the three-dimensional structure of individual Golgi-stained neurons, to determine their synaptic afferent and local efferent connections at the electron microscopic level, and also to identify their putative neurotransmitters by autoradiography.

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