Visualization of chandelier cell axons by parvalbumin immunoreactivity in monkey cerebral cortex

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ABSTRACT Antibodies directed against the calcium-binding protein parvalbumin label a subpopulation of γ-aminobutyric acid-releasing neurons in the cerebral cortex that is thought to have particular metabolic and physiological properties. The chandelier cell is a well-characterized morphological type of γ-aminobutyric acid-releasing cortical interneuron, the axon of which possesses very distinctive terminal portions located around the initial axon segments of pyramidal cells. In the pre- and postcentral gyri of the monkey, we found that these distinctive terminal portions of chandelier cell axons were immunocytochemically stained for parvalbumin in a manner that reveals their complete structure. The chandelier cell axons were identified light-microscopically as short, vertically oriented rows of parvalbumin-positive puncta (PV-Rs). The PV-Rs were very numerous in layers II–III, where most pyramidal cells appeared to have a PV-R beneath them. Fewer PV-Rs were found in deeper layers, and in layer VI PV-Rs were rare. With EM all PV-Rs could be seen to form multiple synaptic contacts of the symmetrical type on the initial segments of pyramidal cell axons. Parvalbumin immunoreactivity can therefore be used as a reliable marker for chandelier cell axons.

There is evidence that there is a very large percentage of neocortical interneurons that are likely to use the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (1). In the past, attempts to correlate cell profiles immunoreactive for GABA or its synthesizing enzyme glutamic acid decarboxylase (GAD) with the several morphological types of cortical interneurons have generally relied upon indirect evidence, for the pattern of axonal arborization, which is the most distinctive feature of each type of cortical interneuron, is not revealed fully by immunocytochemistry (2–4). GABA cells can also be subdivided with regard to their content of neuroactive peptides (5–10) and calcium-binding proteins (9), but, again, direct correlation with morphologically defined forms has been difficult.

One of the best-studied GABAergic interneurons in the cerebral cortex is the chandelier cell, which is distinguished by the terminal portions of its axon, which form short vertical rows of terminal boutons resembling candlesticks (11). These terminal portions form multiple synaptic contacts exclusively with the axon initial segments of pyramidal cells and are thought to be a major source of GABAergic synapses on pyramidal cell axons (12–17). Because of this remarkable synaptic specificity and presumably strong inhibitory influence on pyramidal cells, chandelier cells probably play a crucial role in the control of pyramidal cell excitability and, thereby, in the control of cortical afferent activity. Up to the present, all immunocytochemical studies have failed to show, at either light or electron microscopic levels, the presence of peptides or any substance other than GABA or GAD in chandelier cell axons. Hence, chandelier cells appear to constitute a “pure” type of GABA cell.

Recent immunocytochemical studies in the rat and monkey cerebral cortex have shown that the calcium-binding protein parvalbumin (PV) is found almost exclusively in a subpopulation of GABA cells (18, 19), which, at least in the rat, does not contain the peptides cholecystokinin and somatostatin (20). It has also been shown that immunoreactivity for the 28-kDa vitamin D-dependent calcium-binding protein calbindin and PV is present in separate subpopulations of GABA cells in the monkey cortex and that, on the basis of somal size, the large basket cells, another well-characterized type of GABAergic cells, are likely to contain PV (10). Here, we demonstrate that PV can also be used as a reliable marker for chandelier cell axon terminals and, thus, for the chandelier cells themselves.

MATERIALS AND METHODS

This study was made on tissue taken from the cerebral cortex of five Macaca fascicularis brains that have also been used in other studies (19, 21). All animals were normal. Colchicine was not used. Animals were deeply anesthetized with Nembutal and perfused through the heart with 0.1 M phosphate buffer (pH 7.2) followed by a 0.1 M phosphate-buffered mixture of 2–3% paraformaldehyde and 0.1% or 0.2% glutaraldehyde. The most complete immunoreactive staining was obtained when the higher concentrations of paraformaldehyde and glutaraldehyde were used.

The brains were removed and cut into smaller blocks that were postfixed for 2 hr in 2% paraformaldehyde in 0.1 M phosphate buffer. The blocks used in the present study were sagittal slices 3–5 mm thick spanning the pre- and postcentral gyri. After postfixation they were transferred to 7% sucrose in 0.1 M phosphate buffer, in which they remained overnight.

Next day the blocks were sectioned at 20 or 50 μm on a Vibratome and stained immediately by immunocytochemistry. Sections were washed repeatedly in 0.1 M phosphate buffer, preincubated in 3% normal rabbit serum/0.05% Triton X-100, and then transferred to a mixture containing a sheep antiserum made against HPLC-purified PV (P. C. Emson, personal communication). This antiserum was diluted 1:1000 or 1:2000 in 0.1 M phosphate buffer/3% normal rabbit serum/0.05% Triton X-100 for immunocytochemical staining. Sections were incubated for 24 or 48 hr at 4°C. Thereafter, they were washed repeatedly in phosphate buffer and further treated by the ABC-peroxidase (Vectastain) method. The bound immunoglobulin–peroxidase complexes

Abbreviations: PV, calcium-binding protein parvalbumin; PV-Rs, vertical rows of PV-positive puncta; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase.
were visualized by staining in 0.05% diaminobenzidine tetrahydrochloride/0.002% hydrogen peroxide.

Stained sections were washed several times in phosphate buffer and postfixed in 2% osmium tetroxide, dehydrated, and flat-embedded in Spurr’s resin (Polyscience). Serial 2-µm-thick plastic sections were then obtained with a Reichert ultramicrotome, examined with the light microscope, photographed, and then resectioned at 65–70 nm (22). The thin sections were collected on Formvar-coated, single-slot grids, stained with uranyl acetate and lead citrate and examined with a JEOL-100B electron microscope.

RESULTS

Numerous darkly stained immunoreactive cell somata were found in all layers except layer I of all cortical areas examined.

Fig. 1. PV immunostaining through area 4 (A, B, D, E) and area 3b (C). (A) Low-power photomicrograph that includes layer I and the upper part of layer IIIA. No PV-positive neurons are found in layer I. Notice the predominant labeling of PV-Rs (short, vertically oriented rows of puncta) (arrows) in the neuropil of layers II–IIIA. (B) Higher magnification of the PV-R e shown in D, but at a different focal plane, in which the PV-R e is seen surrounding the presumed axon initial segment of an unstained pyramidal cell (p). (C) Three unstained pyramidal cells (p) in layer IIIA that have PV-Rs beneath them with different complexities. (D) Higher magnification of the bracketed area in A. Note the large number of complex PV-Rs. The PV-Rs a–d are also shown in Fig. 2. (E) PV-positive cell (L) in upper part of layer V. Fewer complex PV-Rs are seen (arrows) than in layer II (compare with D). An unstained pyramidal cell covered by puncta probably derived from basket cell axons is also indicated (p). [Bars = 250 µm (A), 10 µm (B), 25 µm (C), and 50 µm (D and E).]
(Fig. 1A). The greatest number of PV-positive neurons were found in layers IIIB and IV. Layers II, IIIA, and V contained a moderate number of PV-positive cells, and there were few PV-positive cells in layer VI. PV-positive neurons showed a variety of somal sizes and shapes (Fig. 1A and E), but none were pyramidal, and where proximal dendrites were stained, the morphology was invariably nonpyramidal. Small- to medium (10–15 μm) PV-positive cells with an ovoid soma seemed to predominate, but from layer IIIB through layer V there was also a considerable number of large (20–30 μm), multipolar, PV-positive cells (Fig. 1E), which closely resemble the large basket cells of the monkey cortex (3, 23).

A large number of immunoreactive processes and punctate elements were found in the neuropil throughout all layers except layer I of both pre- and postcentral gyri. Layer I was characterized by an almost complete lack of stained puncta, and there were only a few beaded processes (Fig. 1A), although these could often be followed to stained parent cells located in layer II. These processes are likely to be dendrites. The density of the plexus of immunoreactive processes and puncta in other layers was related to the number of PV-positive cells found in each layer. Thus, the densest plexus was found in layers IIIB and IV; density was moderate in layers II-IIIA and low in layer VI. Numerous puncta were seen around the unstained somata of pyramidal cells (Fig. 1C and E), especially in layer IIIB and V, where many pyramidal cells were covered by immunoreactive puncta, outlining their somata and proximal dendrites. These puncta have previously been interpreted (1, 23) as the axon terminals of basket cells.

Apart from the above mentioned features of the immunocytochemical staining, the most conspicuous characteristic was the labeling in layers II–VI of short, vertically oriented rows of puncta (Fig. 1), which appeared identical to the terminal portions of the chandelier cell axons identified in Golgi preparations of the monkey sensory–motor cortex (17). As occurs with terminals of Golgi-impregnated chandelier cell axons, the PV-positive vertical rows of puncta (PV-Rs) varied in both their length (from 15 μm to 35 μm) and their complexity: there were PV-Rs made up of only one or two rows of about 10 puncta, whereas others were so complex that they formed tight braid-like structures (Figs. 1C and 2) with large numbers of puncta. Each PV-R was located immediately beneath or at a short distance (no more than 15 μm) below a nonimmunoreactive soma that in many cases could be identified as a pyramidal cell (Figs. 1C and 2). Frequently the PV-Rs outlined shapes that suggested the axon initial segment of the corresponding cell located above them (Fig. 1B).

The complex PV-Rs were not homogeneously distributed throughout all layers; they were very numerous in layers II–III (Fig. 1D). From layer IV to layer VI there was a dramatic decrease in the number of this type of PV-R (Fig. 1E), especially in the deep part of layer VI, where PV-Rs of any kind were only occasionally found. The general low density of the background PV-immunoreactive plexus in layers II–IIIA caused the abundant complex PV-Rs in these layers to be the most overt stained elements of the neuropil (Fig. 1A and D). In any given region of layers II–III, the number of PV-Rs of both single and complex types was so high that virtually every pyramidal cell appeared to have a PV-R beneath it (Fig. 2). Close examination revealed, however, that there were some pyramidal cells that did not have any PV-Rs associated with them.

In previous Golgi-EM studies, the terminal portions of the chandelier cell axons have been found to form symmetrical synaptic contacts with initial segments of pyramidal cell axons (12–17). Therefore, to verify that PV-Rs were indeed the terminal portions of chandelier cell axons, we studied whether the initial segments of pyramidal cell axons were the structures postsynaptic to the PV-Rs. First, all readily identifiable PV-Rs were localized in 2-μm-thick plastic semithin sections (Fig. 3 A and B), and then the same semithin sections were resectioned for EM (Fig. 3C). We found that all PV-Rs studied (n = 32) were made up of round or elongated terminal boutons, many of them joined together by

![Fig. 2.](image-url) (A, B, and C) Photomicrographs at different planes of focus, but at the same magnification, showing four PV-Rs (a–d), also indicated in Fig. 1D. The PV-Rs are located beneath unstained pyramidal cells (p). (Bar = 15 μm.)
cytoplasmic bridges. The boutons contained numerous synapatic vesicles and usually one, two, or more mitochondria, the external surfaces of which were covered with immunocytochemical reaction product (Fig. 3 D and E). All PV-Rs were found surrounding the axon initial segments of pyramidal cells, forming multiple symmetrical synaptic contacts with them (Fig. 3F). Thus, we conclude that chandelier cell axons are indeed the sources of PV-Rs. Tracing the parent axons back to chandelier cell somata is not possible in this type of material, but the selective termination of the PV-Rs on axon initial segments of pyramidal cells confirms their chandelier cell origin.

DISCUSSION
Cortical neurons displaying PV-immunoreactivity have a non-pyramidal morphology with a variety of shapes and sizes and, therefore, constitute a heterogeneous group of interneurons. Colocalization studies have shown that PV is found almost exclusively in a subpopulation of GABA cells (see Introduction). Among the various morphological types of GABA interneurons, it has been recently suggested from correlative evidence that the large basket cells are very likely to be included in the PV-positive group, but others (for example, the double bouquet cells) are more likely to be included in a different subpopulation of GABA cells that are defined by their immunoreactivity for another calcium-binding protein, calbindin (19). In the present study we have demonstrated at both light and electron microscopic levels that the distinctive terminal portions of chandelier cell axons are stained for PV. This staining is as complete as that obtained with Golgi methods, but the staining for PV has the advantage that, instead of labeling only selected terminal ramifications, as does the Golgi stain, PV immunoreactivity is more widespread, homogeneous, and may label virtually all chandelier cell terminations. Apart from the potential physiological significance of the coexistence of PV and GABA in
chandelier cell axons, the importance of the present findings lies in the capacity to quantify these terminations in the normal, experimental, and diseased cortex. It has been suggested, for example, that chandelier cell axons degenerate in cortical epileptic foci (24).

The present results indicated that the number of PV-chandelier cell axons with complex morphology (i.e., those that give rise to a high density of axoaxonic synaptic contacts) are much more numerous in layers II–III than in layers V–VI. This distribution of complex PV-chandelier cell axons according to layers agrees with our previous EM study of chandelier cell axons in monkey sensory–motor cortex (17). We found that initial segments of pyramidal cell axons were, on average, contacted by more synaptic terminals in layers II–III than in layer V. The significance of the variability by layer and/or the variability in number of terminal boutons per cell awaits functional analysis. It is also not yet determined to what extent the number of terminals of a given axon type is fixed for a particular recipient cell type in the cortex or whether variability in number of synapses is reflected in different functional attributes. The present method offers the opportunity of analyzing these issues.

In the present study, we also found that the number of perisomatic PV terminals on pyramidal cell somata was higher in layers IIIb and V than in other layers. This is likely due to the fact that the large basket cells, which are thought to be the major source of pericellular axon terminals on pyramidal cell somata in layers III–V (3, 25), are also PV-positive (19).

It is thought that the GABAergic axoaxonic and axosomatic synapses on pyramidal cells should exert a strong inhibitory effect on pyramidal cell excitability and the generation of axon potentials. PV/GABA cells appear to be electrically and metabolically more active than those GABA cells that do not contain PV (see ref. 18), but the exact function of PV in these two kinds of cortical interneurons remains to be determined.

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