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Calcium binding proteins distinguish large and small cells of the ventral posterior and lateral geniculate nuclei of the prosimian galago and the tree shrew (*Tupaia belangeri*)

(thalamus/parallel sensory pathways/fiber size)

I. T. DIAMOND*, D. FITZPATRICK, AND D. SCHMECHEL

Departments of Psychology and Neurobiology, Duke University, Durham, NC 27710

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ABSTRACT **Two different cell types were identified in the thalamus of galago and *Tupaia* by using antibodies to two calcium binding proteins, calbindin and parvalbumin. In each species studied, the lateral geniculate nucleus consists of six layers, two of which have smaller relay cells. Previous studies have shown that the small cell layers receive fibers from the superior colliculus and project to the superficial layers of the striate cortex. These are the only geniculate layers that react to a calbindin antibody but not parvalbumin. The ventral posterior nucleus was included in the study and the results for both nuclei show that calbindin is a marker for thalamic cells that receive small fibers and project to superficial layers of koniocortex.**

The unit of thalamus organization is a nucleus, a group of cells with a certain morphology, limited by medullary laminae and identified by unique connections with cortex and brainstem (1). The relay cells in a nucleus are not, of course, identical, and in some nuclei the difference between projection cell types is striking. For example, in many species, especially the primates, the ventral posterior nucleus (VP) can be divided into clusters of large cells surrounded by a matrix of small cells. In galago, a prosimian primate, the small pale cells in VP constitute two bands, one superior and one inferior, on either side of a central region in which the most conspicuous cells are large and dark (2). In species with a laminated lateral geniculate nucleus (GL), there is usually a correspondence between a layer in a set (i.e., ipsilateral or contralateral set) and the size of the projection neuron. The galago provides a good opportunity to illustrate the separation of cell types according to layer. Layers 1 and 2, which are perfectly matched, are dominated by large cells (mean soma area, 402 μm), whereas layers 4 and 5 (again, one ipsilateral, one contralateral) contain only small pale cells (soma area, 284 μm) (3, 4). Finally, cells in the third pair, layers 3 and 6, are intermediate in size.

One interpretation of cell types within a single thalamic relay nucleus is that they represent several submodalities. In the somatic system, submodalities, notably pain and touch, were identified >60 years ago with pathways of different fiber size; thus, pain is conveyed by a small-fiber path and touch is conveyed by a large-fiber path (5). Though the discoveries of Gasser and Erlanger (5) were a milestone, many years have since been devoted to the complex study of pain, leading to the conclusion that there are several kinds of pain that travel over pathways of different fiber size. Parallel pathways of differing fiber size are also found in the visual system. In galago, large fibers of the optic tract project to GL layers 1 and 2, whereas the smallest optic tract fibers project to layers 4 and 5 (6, 7). However, parallel visual pathways can hardly be called submodalities in the strict sense since, unlike the universal separation of touch and pain, the visual paths do not serve the same functions in different species. In cat, the pathways are named X, Y, and W as a convenient way to signify various complex functions. In the tree shrew, a species we have studied for many years, parallel pathways projecting to layer IV of the striate cortex are distinguished by the difference between ON center and OFF center receptive fields (8). It would be stretching "modality," a term that implies a particular kind of perception, if "ON" and "OFF" were so designated. Only in the monkey do separate visual pathways approximate submodalities—one path seems related to color and a second to motion. But even "color" and "motion" pathways cannot be applied to all primates inasmuch as several species—e.g., owl monkey and galago—are nocturnal. In general, nocturnal mammals such as the domestic cat may have retinal cones but have little or no sensitivity to daylight color.

There must be, or at least we hope to find, some basic principle underlying parallel pathways that is common to all mammals and common to the three major modalities. A step toward such a principle was taken by Bishop in 1959 (9), who argued that in all mammals, the small-fiber pathways are phylogenetically old and terminate in ancient centers of the brainstem before reaching the dorsal thalamus. For example, he presented evidence that these multisynaptic pathways are found in vertebrates long before the evolution of a neocortex. We have tried to contribute to Bishop's principle by showing that small cells in the primary thalamic nuclei relay a small-fiber path to the *superficial* layers of koniocortex. The method was to restrict horseradish peroxidase (HRP) either to the superficial layers (I–III) or to layer IV and then identify cells labeled by retrograde transport of HRP. The results showed that the size of labeled neurons in VP clearly depended on the layer of their cortical target—small cells were identified after injections in cortical layer I, large cells were labeled after injections in cortical layer IV (10). Further, after an injection in the superficial layers of the striate cortex of galago, only small cells were labeled, which is to say GL layers 4 and 5 and small cells between layers (3).

The significance of the "small" cells in VP or GL is that they fit Bishop's idea of a phylogenetically old path, a "non-lemniscal" path. Thus, the small cells in VP are not targets of the lemniscal path (11, 12), and the small cell layers of GL in galago (layers 4 and 5) receive small fibers from the superior colliculus (SC), which, in turn, is the target of the smallest fibers of the optic tract (3, 13). The general principle we seek may simply be that pathways to the *superficial* layers

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Abbreviations: GL, lateral geniculate nucleus; Lat, lateral nuclear group; Po, posterior nuclear group; Pul, pulvinar nucleus; SC, superior colliculus; VP, ventral posterior nucleus; VP_i, inferior division of the VP; VP_s, superior division of the VP.

*To whom reprint requests should be addressed.

of koniocortex have some general function common to the three major modalities—somatic, visual, and auditory.

In the past few years, Jones and his colleagues (11, 12, 14) have shown that large and small cells in the thalamus differ in their staining for calcium binding proteins, calbindin and parvalbumin. The clusters of big cells in VP of the monkey are rich in parvalbumin, whereas the small, pale cells in the matrix are rich in calbindin. In GL all six principal layers show parvalbumin, whereas the small, pale cells in the interlaminar regions contain calbindin. The beauty of this finding is that it shows that different thalamic nuclei can have two kinds of cells, and the *same* two.

In the present paper, large and small cells in GL and VP of the galago will be examined using antibodies to parvalbumin and calbindin. The results will show that the small cells in GL that project to superficial layers of koniocortex are calbindin positive.

Over the years we have compared the tree shrew (*Tupaia glis*) with the primates; the advanced development of GL and layer IV of striate cortex in tree shrew has led some comparative neurologists to consider that arboreal species from southeast Asia are relatives of the primate ancestor. Whether or not this idea is valid, the visual system of the tree shrew has proved to be a fruitful subject (15, 16). It has a conspicuous layer IV in the striate cortex and a six-layered lateral geniculate, two layers of which project above layer IV and receive fibers from the SC (17). The results will show that these two layers are positive for the antibodies to calbindin.

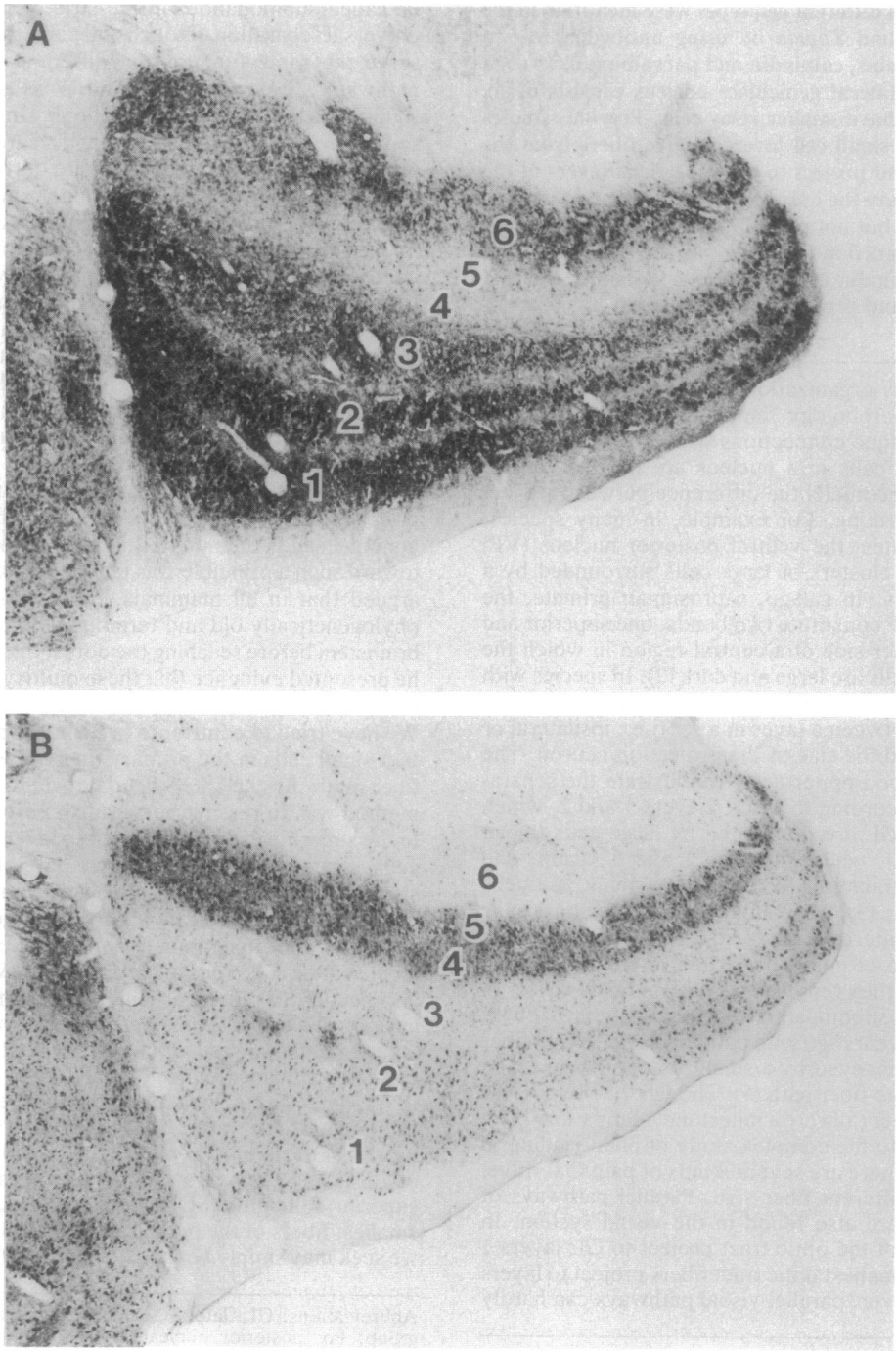

FIG. 1. (*A*) Section through the GL in galago where the stain results from the antibody to the protein parvalbumin. (*B*) Section through the GL in galago where the stain results from the antibody to the protein calbindin. (×30.)

METHODS

Calbindin and parvalbumin immunoreactivity were examined in three tree shrews and two galagos. Animals were given a lethal dose of sodium pentobarbital (70 mg/kg) and perfused through the heart with either 2% paraformaldehyde, 0.15% glutaraldehyde in 0.1 M phosphate buffer, or 4% paraformaldehyde in the same buffer. Thirty minutes after fixation a solution of 10% sucrose in 0.1 M phosphate buffer was flushed through the vasculature. The brain was removed and placed in 20% sucrose/phosphate buffer overnight at 4°C. Sections were cut frozen in the frontal or sagittal planes at 50 μm and collected in 0.01 M phosphate-buffered saline (PBS) for processing. Immunocytochemistry for parvalbumin and calbindin was performed using the standard avidin–biotin staining protocol. Briefly, sections were incubated overnight at room temperature in the primary antibodies. The anti-parvalbumin antibodies were diluted 1:1000, and the anti-calbindin antibodies were diluted 1:500 with 1.0% normal horse serum (NHS) in PBS. After several PBS rinses, the sections were (*i*) incubated in biotinylated anti-mouse IgG diluted 1:50 in PBS containing 1.0% NHS for 30 min, (*ii*) rinsed in PBS, (*iii*) incubated in avidin–biotin complex solution (ABC) (Vector Laboratories) for 45 min, (*iv*) rinsed in PBS, and (*v*) allowed to react in 3,3′-diaminobenzidine hydrochloride plus 0.0001% hydrogen peroxide for 5–10 min. After a final rinse the sections were mounted on gel-coated slides, air dried, and coverslipped after dehydration and clearing. For some sections, normal mouse serum (1:500) was substituted for the primary antibody as a control. Under these conditions, no staining was observed.

RESULTS

The contrast between Fig. 1 *A* and *B* illustrates the main result in the galago. In the parvalbumin section, layers 4 and 5 have no sign of neuropil staining and only a sparse scattering of faint cells—hardly visible in the photograph. In the calbindin section, layers 4 and 5 have the darkest neuropil and a dense population of dark brown cells. It is even possible to distinguish layer 4 from layer 5 since the interlaminar region is largely free of cells. (In the black and white figure it is a gray band.) Layers 3 and 6 also differ in their staining for these two calcium binding proteins. The cells and neuropil in layers 3 and 6 are dark brown in the parvalbumin section, and with antiserum against calbindin, they show the least sign of neuropil staining and the faintest cells.

The contrast between calbindin and parvalbumin is also distinct in layers 1 and 2 but these two layers are different from layers 3 and 6 in showing a substantial population of cells labeled after applying the calbindin antiserum method. At the same time the neuropil in layers 1 and 2 is a very pale tan, not different from that found in layers 3 and 6. The low-power photograph in Fig. 1*B*, however, does not offer a complete picture of the labeled cells in layers 1 and 2. A higher-power photograph (see Fig. 2) shows that the population is not uniform and falls into two sharply distinct classes. The first class consists of small and dark brown cells (see Fig. 2) located close to and above the upper border of layer 2 and below the lower border of layer 1. These cells fall into the class "interlaminar," which implies that they receive fibers from the SC and project above layer IV in the striate area. In both of these respects they are similar to the cells of layers 4 and 5. In the second class the cell bodies are a light tan, intermediate between the very pale cells of layers 3 and 6 (these are too pale to be identified clearly in Fig. 1*B*) and the dark cells in layers 4 and 5.

There are several ways to explain both parvalbumin and calbindin populations in layers 1 and 2. The first possibility is simply that there is colocalization of calbindin and parvalbumin. The second possibility is that these are two distinct populations of projection neurons—one calbindin and one parvalbumin. Using the Golgi method, Conley *et al.* (4) identified a second kind of relay cell but the small number of these does not approximate the large number of intermediate tan cells. A third interpretation is simply that cells can take on a pale stain without calbindin. In support of this is the finding of very faint cells in layers 3 and 6, cells that are unlikely positive for calbindin.

Fig. 3 shows a parvalbumin section through the VP nucleus in galago. It closely resembles the features of VP in the monkey published by Rausell and Jones (11, 12). The "rods"

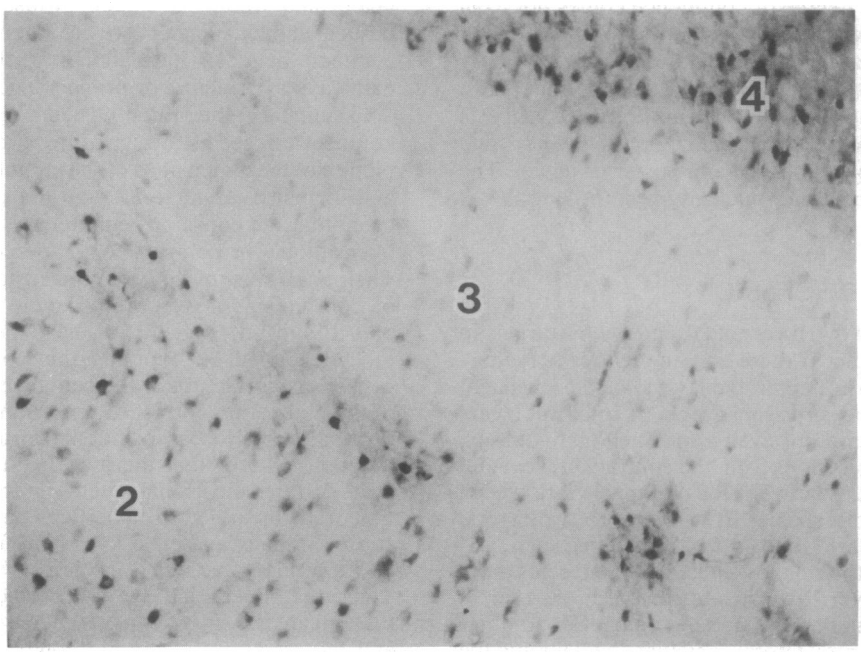

FIG. 2. Layers 2, 3, and 4 after staining for the presence of calbindin. Note that layer 3 is very light and that a small part of layer 4 can be identified because of the dense population of dark cells. The chief point is that below layer 3, there are small dark cells that belong to the intralaminar region or to layer 2 itself. Below the small dark cells are larger light cells that are relay cells to layer IV of striate cortex. (×200.)

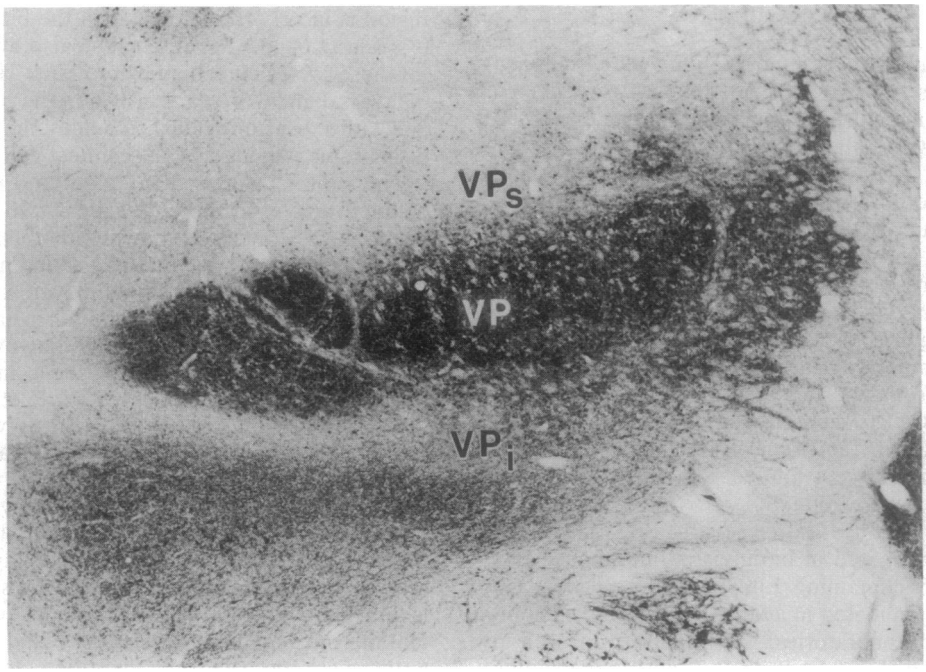

FIG. 3. VP in a parvalbumin section. The dark cells and neuropil are the central and principal part of the nucleus. Above and below are the small cells that project to superficial layers and these show little or no stain for parvalbumin. VP_s, superior division of the VP; VP_i, inferior division of the VP. (×25.)

or subdivisions of VP are dark with cells and neuropil. The regions above and below, VP_s and VP_i, are pale with very faint cells and the absence of neuropil.

Fig. 4 shows parvalbumin and calbindin sections through the GL of the tree shrew. On the right (Fig. 4*B*), calbindin antibody staining is conspicuous in layer 3 and layer 6. Layer 6 is relatively thin but the dark cells are easily seen in the microscope. There are also a very few dark cells in layer 4. The parvalbumin section fails to show sharp borders between layers 2 and 3 and between layers 3 and 4 but these borders are not always sharp in a Nissl stain. There is no question that after staining for parvalbumin, the cells in layers 3 and 6 are slightly paler. But this degree of confidence comes not from the photograph but with the help of the microscope. In any case, cells in layers 3 and 6 in Nissl material appear slightly paler and are smaller than cells in layers 1, 2, 4, and 5 (18). Taking the two calcium binding proteins together suggests that there is a good chance of colocalization in layers 3 and 6. Fig. 4*A* also includes the lateral and posterior nuclei. The contrast between the posterior nuclear group (Po) and VP is obvious.

DISCUSSION

The chief finding is that two layers of GL in *Tupaia* and galago are positive for calbindin. If there was an obvious homology between layers 3 and 6 in Tupaia and layers 4 and 5 in galago, the finding would not be surprising or remarkable. But layers 3 and 6 in tree shrew are not even a matched pair—that is, both layers receive projections from the contralateral eye. Of course certain similarities between the two species are crucial to the argument. In both species SC projects to two layers, layers 3 and 6 in tree shrew and layers 4 and 5 in galago, and these four layers have the smallest cells. In both species these pairs project *above* layer IV in striate cortex. In tree shrew the tectal fibers terminating in GL send collaterals to other centers, notably the ventral lateral geniculate nucleus and the pretectum (19). In both species, the parabigeminal nucleus, which is a target of SC, projects to layers of GL that include 4 and 5 in galago and 3 and 6 in *Tupaia* (20). All of these connections taken together fit Bishop's view of a phylogenetic old path.

The question remains: What is the functional significance of a phylogenetic old path to superficial layers of koniocortex? This is not readily answered and the best we can do is to list a few features of the old pathways and then speculate. (*i*) Small fibers and many synapses in the path to the superficial layers of koniocortex lead to a much slower arrival of impulses. (*ii*) Projections to superficial layers bear some similarity to the thalamic projection to cortex in reptiles, which reaches layer I and terminates diffusely. (*iii*) Cortical projections from the intralaminar nuclei also reach superficial layers and terminals spread diffusely, often over several cortical subdivisions. There are, therefore, some features in common between intralaminar nuclei and the small cell pathways in principal or primary nuclei.

In summary, the primary ("lemniscal") path to layer IV of koniocortex seems designed to produce quickly a clear picture of the dimensions of the stimulus, such as wavelength and intensity. Many years ago the introspectionists would claim that the conscious counterpart of stimulus energy is a "sensation" in contrast to a "perception" of the object. Their point was to show that the basic element of experience or consciousness is entirely devoid of the meaning of the object. Indeed, psychophysical experiments in Tichener's time depended on avoiding the "object-error" (21). The consensus at the turn of the century was that the perception of the object, in contrast to sensation, results from corticortical pathways to "association" cortex (22, 23).

We now know that there are pathways from extrastriate cortex to the superficial layers of striate. Further, a projection from layer IV to superficial layers has been demonstrated. Finally and most important for the present purpose, the secondary pathways project to the superficial layers. Thus, the superficial layers of koniocortex (the striate for certain) are the site of convergence from three major sources and the resulting integration could provide the basis for a judgment of the object. The secondary pathways might then provide answers to the obvious questions: Is the object a basis for a shift in attention; is the object attractive for

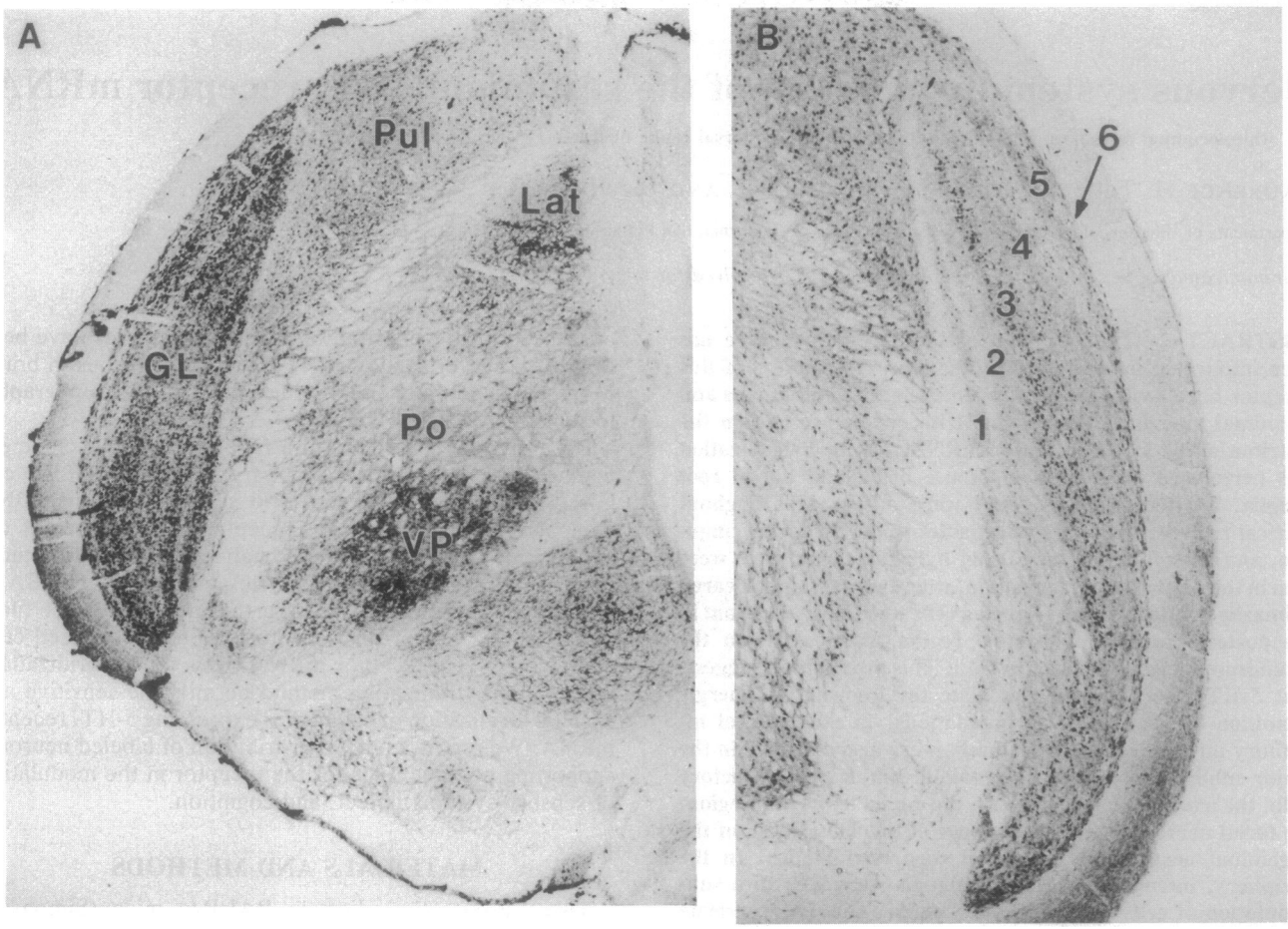

FIG. 4. (*A*) Parvalbumin section of the lateral geniculate in the tree shrew. Also included is a very dark VP. The posterior nucleus dorsal to the VP appears to be without neuropil or cells. Pul, pulvinar nucleus; Po, posterior nuclear group; Lat, lateral nuclear group. (×20.) (*B*) Calbindin section of the lateral geniculate in the nucleus. Layers 3 and 6 stand out by virtue of their dark cells. It is possible that some of the dark cells encroach on layer 4. (×25.)

approach or a danger to avoid? To summarize this speculative argument, the secondary pathways may be taking a role in perceiving the object meaning.

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