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Immunohistochemical localization of calretinin in the rat lateral geniculate nucleus and its retino-geniculate projection

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In the present study, we examined the distribution of calretinin-immunoreactive neuronal cell bodies and fibers in the lateral geniculate nucleus of the rat. In normal rats, clusters of immunoreactive cell bodies were found in: (i) the rostral portion of the ventral lateral geniculate nucleus pars medialis (VLGM), (ii) the intergeniculate leaflet (IGL), (iii) the intermediate region between the VLGM and the ventral lateral geniculate nucleus pars lateralis (VLGL), (iv) the caudomedial portion of the VLGM, and (v) the caudolateral portion of the VLGM. In the dorsal lateral geniculate nucleus (DLG), immunoreactive cell bodies were rarely observed. After uni- or bilateral eye enucleation, no significant alteration in the morphological features or distribution of immunoreactive cell bodies was detected in the lateral geniculate nucleus. In normal rats, immunoreactive fibers formed dense plexuses in: (i) the DLG, (ii) the external layer of the VLGL, (iii) the internal layer of the VLGL, (iv) the IGL, (v) the caudomedial portion of the VLGM, and (vi) the optic tract. After unilateral eye enucleation, immunoreactive fibers in the external layer of the VLGL and in the optic tract almost totally disappeared on the contralateral side to the lesion. Unilateral eye enucleation caused a significant decrease of immunoreactive fibers in the DLG and in the internal layer of the VLGL, but a substantial number of immunoreactive fibers still remained there. In the IGL and the caudomedial portion of the VLGM, no observable alteration in the distribution of immunoreactive fibers was detected after uni- or bilateral eye enucleation. In conclusion, (i) subpopulations of neurons of the lateral geniculate nucleus are distinguished by the presence of calretinin immunoreactivity, and (ii) calretinin is a chemical component of subpopulations of the contralateral retino-geniculate pathway, which terminate in the DLG and in the external and internal layers of the VLGL.

INTRODUCTION

The lateral geniculate nucleus is one component of the central visual pathways[30]. The antero- and retrograde tracing and degeneration studies have demonstrated that the retinal afferents terminate in the dorsal lateral geniculate nucleus (DLG), ventral lateral geniculate nucleus (VLG) and intergeniculate leaflet (IGL) of the rat (for review see refs. 30 and 37). In the rabbit, a subpopulation of the retino-geniculate projection, which terminates in the superficial portion of the DLG, has been chemically identified by immunohistochemistry of substance P[6]. In the rat, a dipeptide, N-acetylaspartyl glutamate, has been shown as a chemical component of the retinal afferents to the DLG[1]. A retinal afferent to the VLG of the rat, however, has never been characterized by its chemical features.

Distinct populations of neurons and fiber connections in the central nervous system of the rat were distinguished by the location of specific calcium-binding proteins, such as parvalbumin, calbindin-D28K and calretinin[2,8,16,35]. Although the localization of calretinin has never been extensively examined throughout the rat lateral geniculate nucleus, calretinin-immunoreactive cell bodies and fibers in the nucleus have been demonstrated by the studies of brain maps[2,16,35]. Ganglion cells of the rat retina have been shown to contain calretinin immunoreactivity[31,42]. Thus, a possibility can be considered that the retino-geniculate projection system of the rat may contain calretinin.

In the present study, we focused on the lateral geniculate nucleus of the rat and examined thoroughly by use of immunohistochemistry the distribution of calretinin-containing cell bodies and fibers in the nu-

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cleus. In addition, with eye enucleation, we chemically identified subpopulations of the retino-geniculate pathways by calretinin immunoreactivity.

MATERIALS AND METHODS

Animals

Sixteen male Spraque–Dawley rats weighing 140–280 g were used. The animals were divided into two groups: intact rats ($n = 4$) for determining the normal distribution of calretinin-immunoreactive structures in the lateral geniculate nucleus, and unilaterally ($n = 6$) and bilaterally ($n = 6$) eye-enucleated rats for determining the area receiving calretinin-immunoreactive retino-geniculate projections. Eye enucleation was performed under aneasthesia with Chloropent (Fort Dodge Labs.). Eye-enucleated rats were allowed to survive for 7 days.

Tissue preparation

Intact and eye-enucleated rats, under anesthesia with sodium pentobarbital, were perfused transcardially with 100 ml of 0.01 M phosphate-buffered saline (pH 7.4), followed by 200 ml of a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, 4°C). The brains were dissected out and placed in the same fixative for 30 min at 4°C, and then immersed in 0.1 M phosphate buffer containing 20% sucrose for 48 h at 4°C. Serial sections of the lateral geniculate nucleus were cut on a cryostat in a frontal plane at 20 μm and collected in 0.1 M phosphate-buffered saline (PBS). Sections of the lateral geniculate nucleus were divided into four sets, each set consisting of every fourth serial section. Sections of the first set were processed for calretinin immunohistochemistry. Sections of the second set were stained with thionin for demarcation of the anatomical regions. Other sections were used for further observations and for an immunohistochemical control.

Immunohistochemistry

An antiserum was raised in a rabbit against calretinin purified from the guinea pig brain[42]. With the antiserum, calretinin immunoreactivity was detected by use of a streptavidin-peroxidase conjugate[38]. The sections were incubated in the following solutions: (i) 0.3% Triton X-100 in PBS for 1 h at room temperature; (ii) 5% normal goat serum in PBS for 1 h at room temperature; (ii) anti-calretinin antiserum (1: 2,500 in PBS) with 2% normal goat serum for 48 h at 4°C; (iv) biotinylated goat anti-rabbit immunoglobulin G (Vector, 1: 400 in PBS) for 2 h at room temperature; (v) streptavidin-peroxidase (Kirkegaard & Perry Lab., 1:100 in PBS) for 2 h at room temperature. Tissue-bound peroxidase activity was visualized by incubating sections with 0.025% 3,3′-diamino-benzidine (Sigma), 0.6% nickel ammonium sulfate (Fisher) and 0.006% hydrogen peroxide in

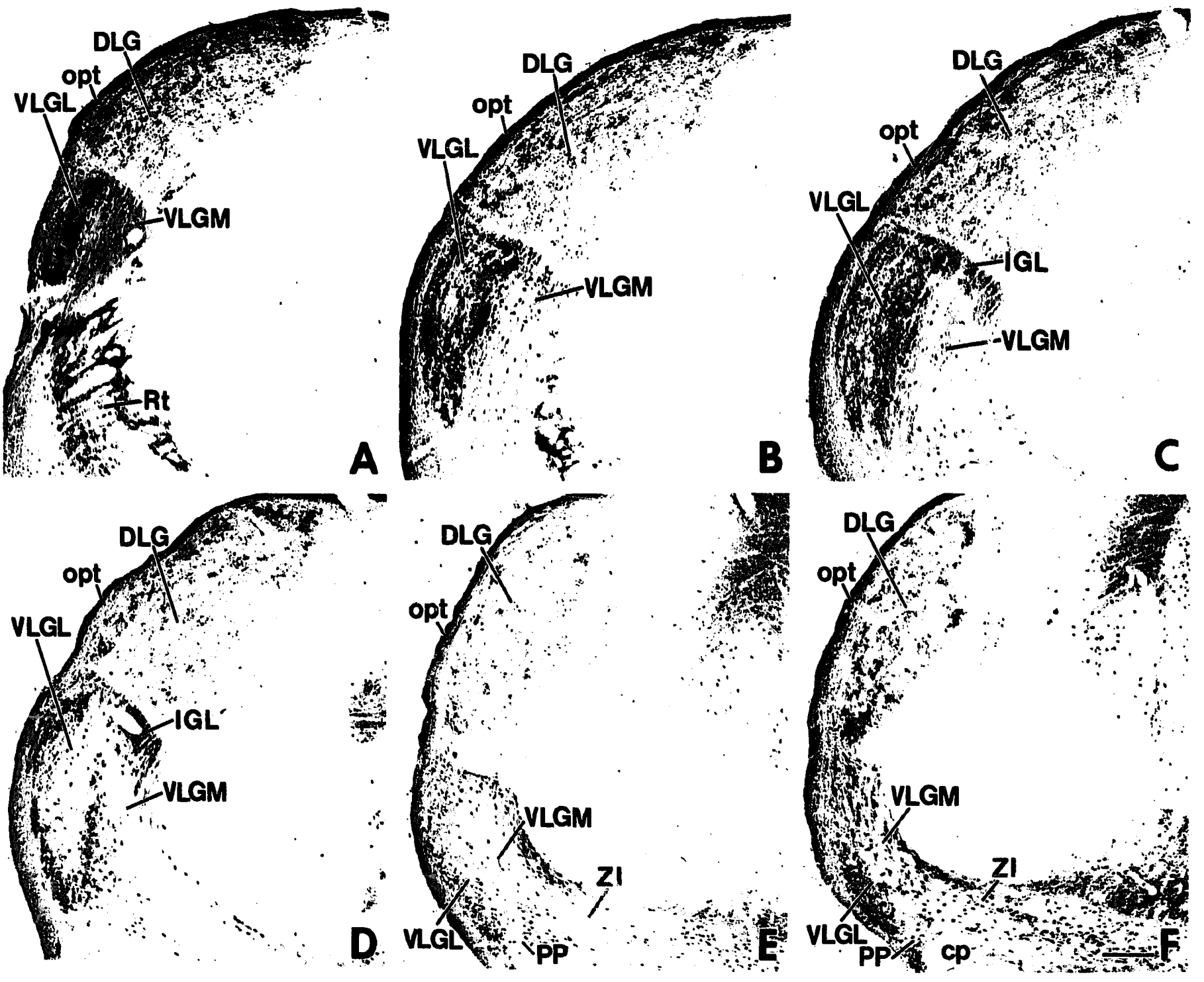

Fig. 1. Calretinin immunoreactivity in frontal sections of the lateral geniculate nucleus of an intact rat. A is rostral; F is caudal. Bar = 200 μm.

0.05 M Tris-HCl buffer (pH 7.6). After each incubation step, the sections were rinsed in PBS for 30 min at room temperature.

In a control for the immunohistochemical staining, we omitted the primary antiserum. No immunolabeling was found under the condition. The possibility has been suggested that anti-calretinin antisera may cross-react with calbindin-D28k because of a high degree of homology between calretinin and calbindin-D28k[34]. In the case of the anti-calretinin antiserum used in the present study, however, this possibility is excluded by the following data: (i) we have previously done the preadsorption control using calretinin purified from the guinea pig brain[16,42] and from the rat brain[2], and no immunohistochemical staining has been found, (ii) the anti-calretinin antiserum has detected only calretinin but not calbindin-D28k on a protein blot of the guinea pig brain sample[42], (iii) the antiserum has not detected cells of the red nucleus, nucleus of the trapezoid body, inferior olive, or Purkinje cells of the cerebellum[2], although these cells were immunostained by anti-calbindin-D28k antisera[4,8,11,17,36]. Furthermore, Purkinje cells of the cerebellum have been also immunostained by anti-parvalbumin antisera[8,36], whereas the cells have not been detected by our anti-calretinin antiserum[2,42].

Data analysis

Localization of calretinin-immunoreactive cell bodies and fibers was determined by examining the immunostained sections and the adjacent thionin-stained sections under a Leitz light microscope. The literature[30,32,40] was consulted to delineate the subdivisions of the lateral geniculate nucleus and the surrounding areas. By comparing the distribution of calretinin-immunoreactive fibers in normal rats and that in eye-enucleated rats, calretinin-containing retino-geniculate pathway terminating fields were examined.

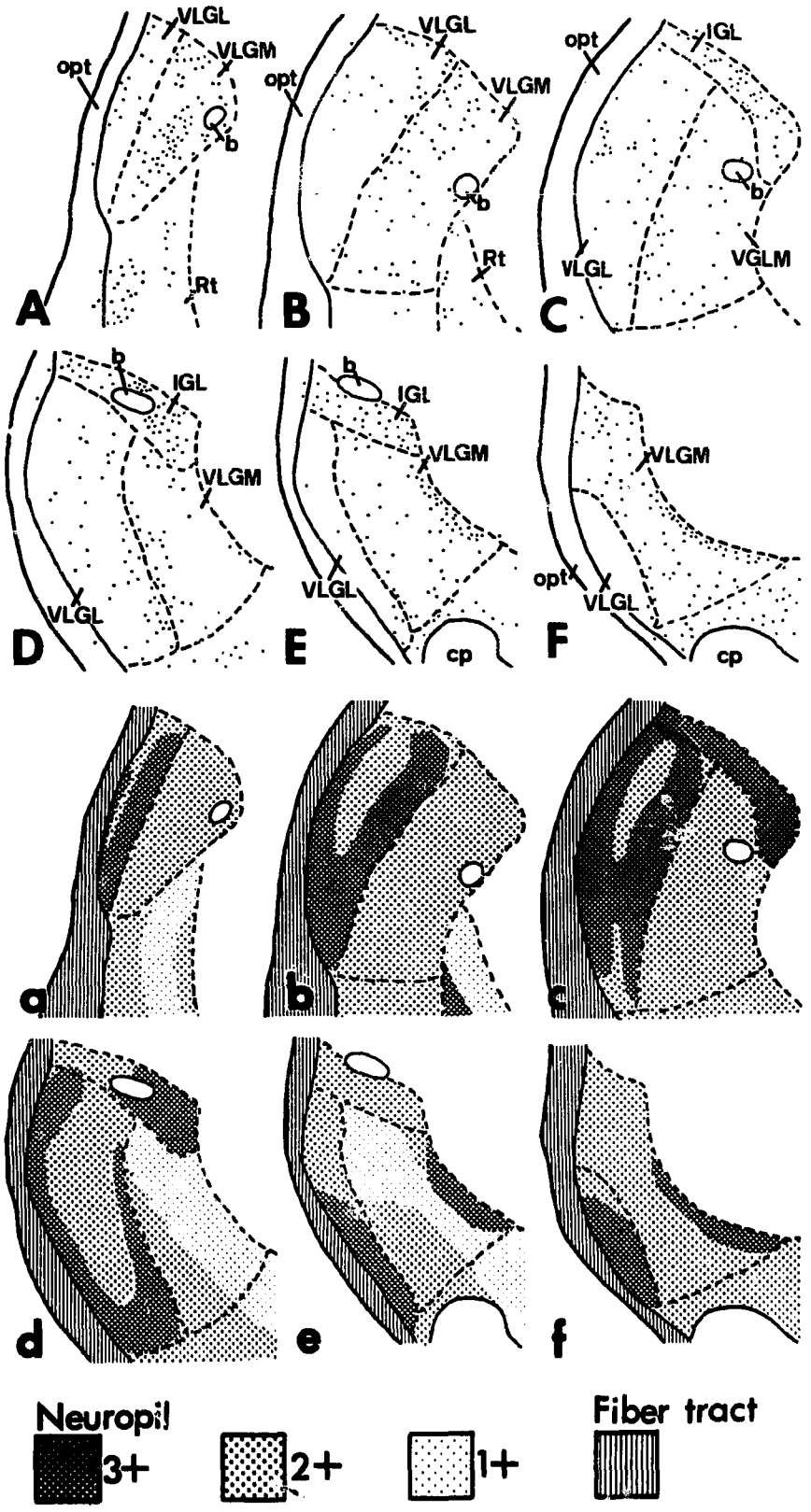

Fig. 2. Top (A–F) shows locations of calretinin-immunoreactive cell bodies in the lateral geniculate nucleus pars ventralis of an intact rat. One symbol indicates one cell. Bottom (a–f) shows calretinin-immunoreactive fibers. Packing density of the fibers is depicted by three variations of compactness of stipple: high-density neuropile (3+), moderate density neuropile (2+), low density neuropile (1+). Fiber tracts are represented by vertical lines. Each drawing corresponds to the sections of Fig. 1: 2A,a – 1A; 2B,b – 1B; 2C,c – 1C; 2D,d – 1D; 2E,e – 1E; 2F,f – 1F.

RESULTS

Localization of calretinin in normal rats

Calretinin immunoreactivity was detected within cell bodies and fibers in the lateral geniculate nucleus of intact rats. The morphological appearance of immunoreactive structures suggests that they are neuronal elements. Fig. 1 shows the perspectives of the lateral geniculate nucleus on immunostained sections of a normal rat at six representative levels. The DLG had a similar distribution pattern of calretinin immunoreactivity over its rostrocaudal extent, whereas the VLG had a great diversity of its localization at the different rostrocaudal levels. Fig. 2 illustrates the distribution of calretinin-immunoreactive cell bodies (Fig. 2A-F) and fibers (Fig. 2a–f) in the VLG and the IGL at the levels corresponding to those of Fig. 1. Fig. 3 shows major calretinin-immunoreactive structures in the lateral geniculate nucleus.

Immunoreactive cell bodies

Clusters of immunoreactive cell bodies were found in: (i) the rostral portion of the VLGM (Figs. 1A and 2A), which was composed of predominantly bipolar cells oriented in a dorsomedial to ventrolateral direction (Fig. 3B), (ii) the IGL (Figs. 1C–E and 2C–E), which was composed of bipolar and multipolar cells (Fig. 3D), (iii) the intermediate region between the VLGL and VLGM (Figs. 1D and 2D), which was composed of bipolar, multipolar and oval cells (Fig. 3C), (iv) the caudomedial portion of the VLGM, which was located in the most medial portion of the VLGM at the caudal level (Figs. 1E,F and 2E,F) and was composed of bipolar cells oriented in a dorsolateral to ventromedial direction (Fig. 3E), and (v) the caudolateral portion of the VLGM, which was located in the lateral aspect of the VLGM at the caudal level (Figs. 1F and 2F) and was composed of predominantly multi-

polar cells (Fig. 3F), and this cluster extended ventromedially and continued to the zona incerta (ZI) (Figs. 1F and 2F). The cluster of the caudomedial VLGM (Figs. 1E,F and 2E,F) had a higher packing density of immunoreactive cells than the cluster of the caudolateral VLGM (Figs. 1F and 2F). A few immunoreactive cell bodies were dispersed in the remaining regions of the VLG (Figs. 1 and 2A–F). In the DLG, immunoreactive cell bodies were rarely observed.

Of all calretinin-immunoreactive cells in the VLG, approximately 20% were found in the IGL, 10% in the caudomedial portion of the VLGM, 9% in the rostral portion of the VLGM, 7% in the intermediate region between the VLGL and VLGM, 4% in the caudolateral portion of the VLGM.

Immunoreactive fibers

Immunoreactive fibers formed dense plexuses in: (i) the DLG (Fig. 1), (ii) the external layer of the VLGL (Figs. 1 and 2a–f), (iii) the internal layer of the VLGL (Figs. 1A–D and 2a–d), (iv) the IGL (Figs. 1C,D and 2c,d), (v) the caudomedial portion of the VLGM (Figs. 1E,F and 2e,f), and (vi) the optic tract (opt) (Figs. 1 and 2a–f). Immunoreactive fibers in the DLG con-

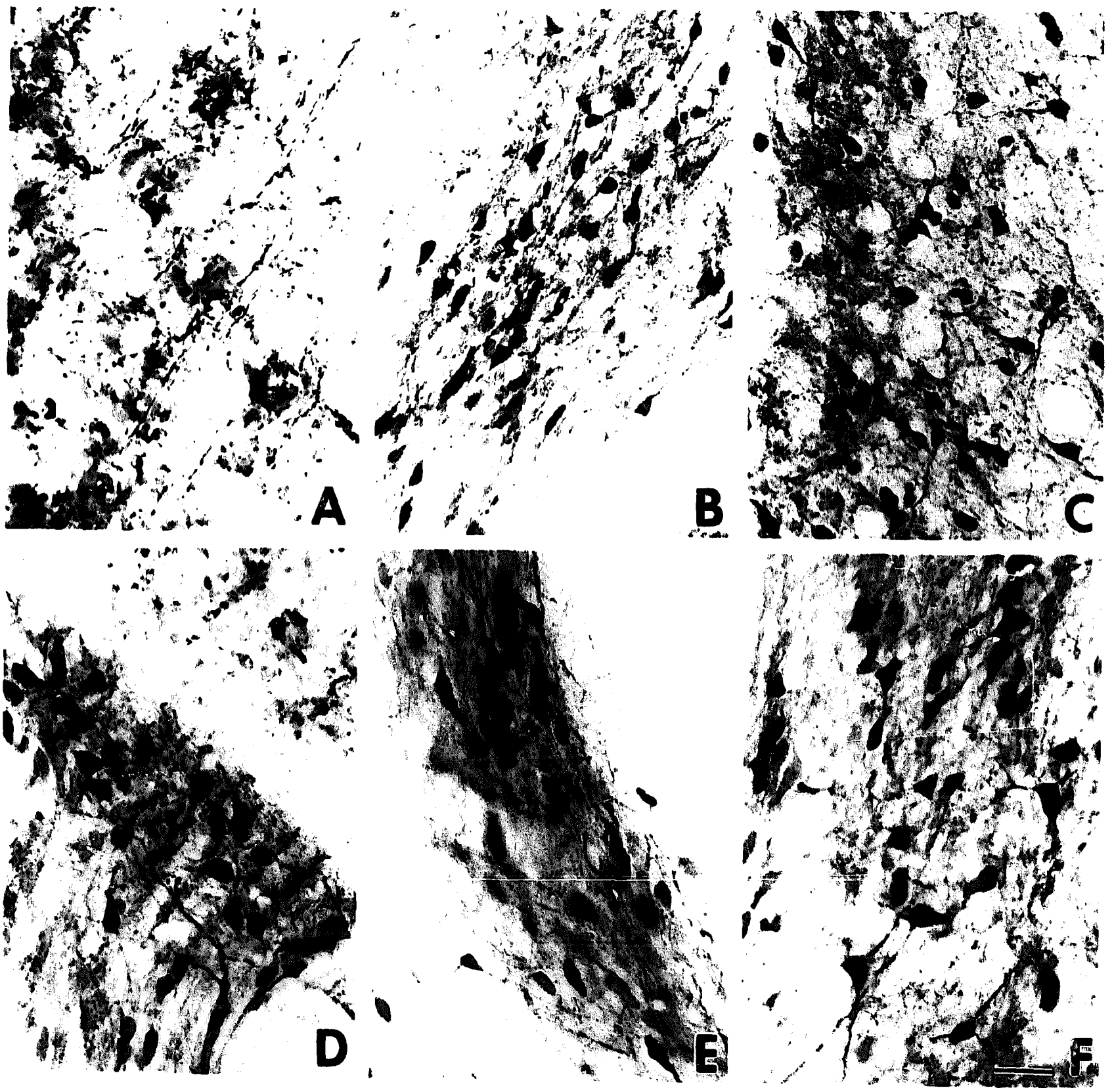

Fig. 3. Calretinin immunoreactivity in frontal sections of the lateral geniculate nucleus of an intact rat. Top of each photograph is dorsal, and right is medial. A: dorsal lateral geniculate nucleus (DLG) at the level of Fig. 1C. B: rostral portion of the ventral lateral geniculate nucleus pars medialis (VLGM) at the level of Fig. 1A. C: intermediate region between the ventral lateral geniculate nucleus pars lateralis (VLGL) and the VLGM at the level of Fig. 1D. D: intergeniculate leaflet (IGL) at the level of Fig. 1C. E: caudomedial portion of the VLGM at the level of Fig. 1E. F: caudolateral portion of the VLGM at the level of Fig. 1F. Bar = 30 μm.

sisted of rod-like varicosities and long fibers extending in a dorsomedial to ventrolateral direction (Fig. 3A), and the density of immunoreactive fibers was higher in the outer portion than in the inner portion (Fig. 1). Immunoreactive fibers in the external and internal layers of the VLGL, in the IGL, and in the caudomedial portion of VLGM were composed of fine dot-like structures. The two layers of the immunoreactive fiber plexuses in the VLGL were prominent with a paucity of immunoreactivity between the two layers at the middle level of the rostrocaudal extent (Figs. 1D and 2d). The two layers were in close proximity at the rostral levels (Figs. 1A–C; 2a–c), and only the external layer remained at the caudal levels (Figs. 1E,F and 2e,f). The optic tract contained a dense bundle of immunoreactive fibers (Fig. 1).

Eye enucleation

Fig. 4 shows the lateral geniculate nucleus of unilaterally (Fig. 4A,B) and bilaterally (Fig. 4C) eye-enucleated rats at the level corresponding to Fig. 1D. Fig. 5 shows the VLG and the IGL of a unilaterally eye-enucleated rat at three different levels. No significant differences in the morphological features or distribution of immunoreactive cell bodies were detected between normal and uni- or bilaterally eye-enucleated rats. The distribution of immunoreactive fibers in the lateral geniculate nucleus was altered by uni- and bilateral eye enucleation as follows:

Unilateral eye enucleation

After unilateral eye enucleation, changes in the distribution of immunoreactive fibers in the lateral geniculate nucleus were obseved only on the contralateral side to the lesion (Figs. 4B and 5B,D,F), but no significant alteration was detected on the ipsilateral side (Figs. 4A and 5A,C,E). Immunoreactive fine dot-like fibers in the external layer of the VLGL and immunoreactive fibers in the optic tract almost totally disappeared on the contralateral side after unilateral eye enucleation (Figs. 4B and 5B,D,F). Unilateral eye enucleation caused a significant decrease of immunoreactive rod-like fibers and long fibers in the DLG (Fig. 4B) and of immunoreactive fine dot-like fibers in the internal layer of the VLGL (Figs. 4B and 5D), but a substantial number of immunoreactive fibers still remained in these regions (Figs. 4B and 5D). In the IGL (Figs. 4B and 5D) and in the caudomedial portion of the VLGM (Fig. 5F), no significant alteration in the distribution of immunoreactive fibers was found after unilateral eye enucleation.

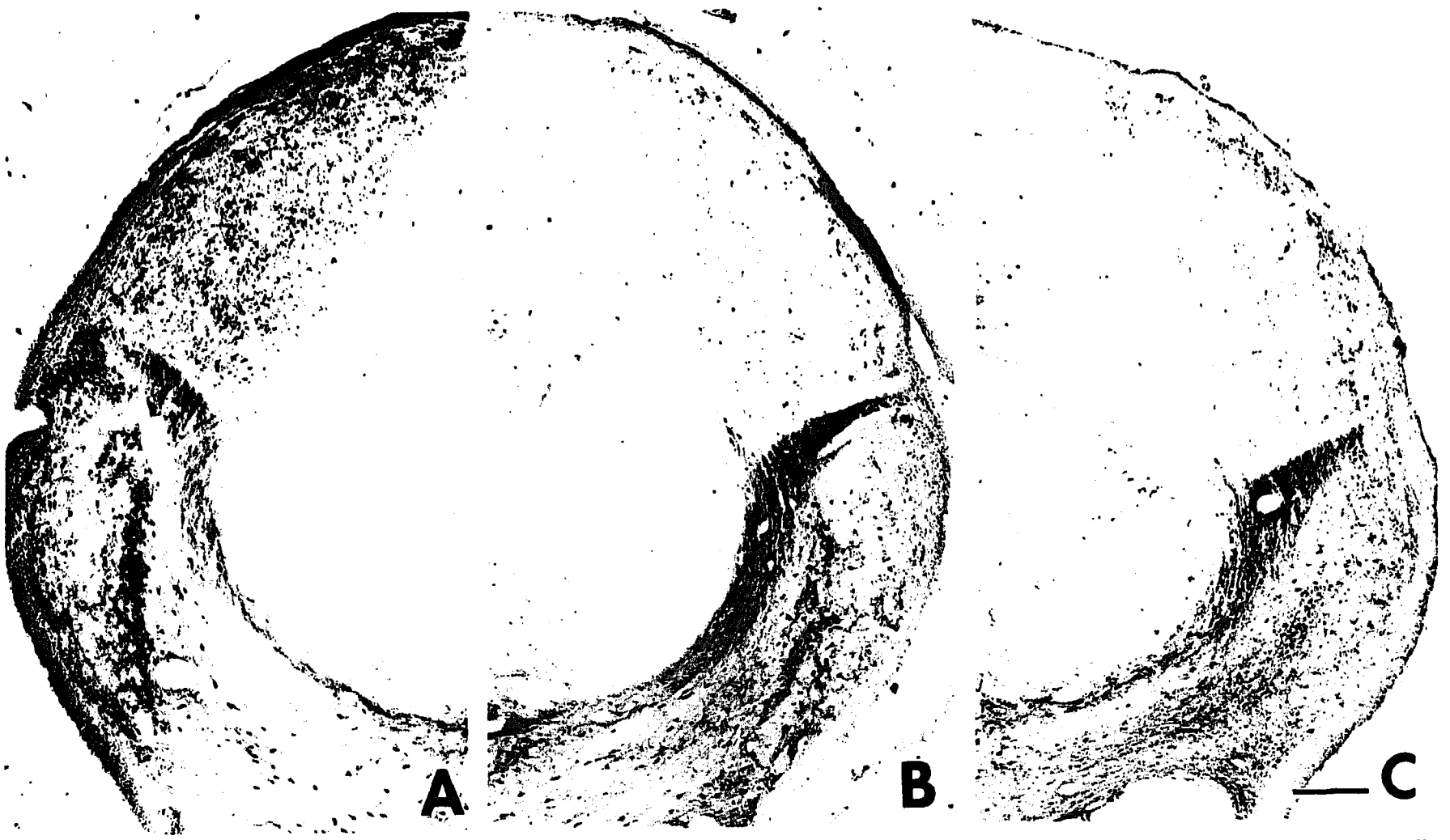

Fig. 4. Eye-enucleated rats. Calretinin immunoreactivity in the lateral geniculate nucleus at the level corresponding to Fig. 1D. A: unilaterally eye-enucleated rat. Ipsilateral to the enucleation side. B: unilaterally eye-enucleated rat. Contralateral to the enucleation side. C: bilaterally eye-enucleated rat. No difference is detected in the distribution of immunoreactive fibers between the left and right lateral geniculate nuclei. Bar = 200 μm.

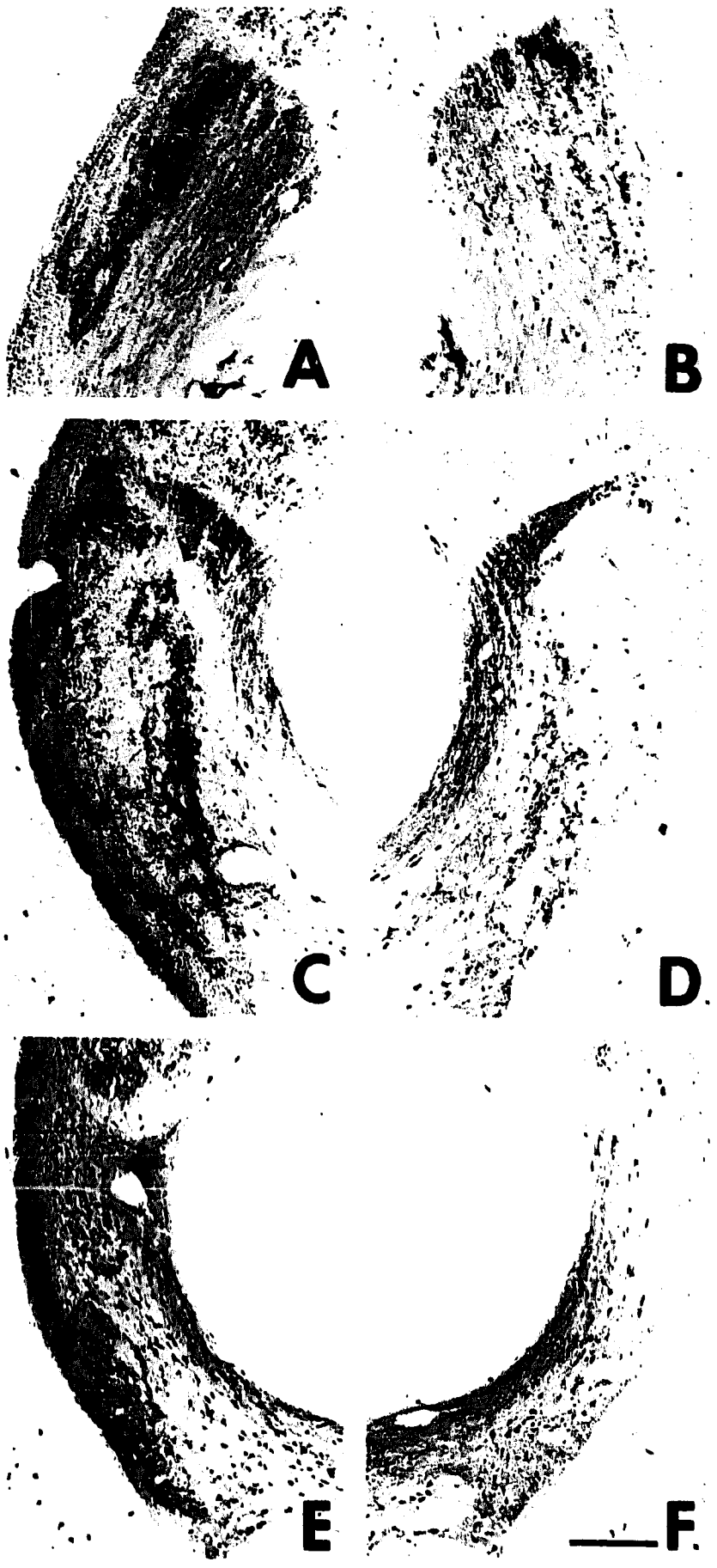

Fig. 5. Unilaterally eye-enucleated rat. Calretinin immunoreactivity in the pars medialis (VLGM) and pars lateralis (VLGL) of the ventral lateral geniculate nucleus and the intergeniculate leaflet (IGL). A and B are on the same section at the level corresponding to Fig. 1A. C and D are on the same section at the level corresponding to Fig. 1D. E and F are on the same section at the level corresponding to Fig. 1F. Left side (A,C,E): ipsilateral to the enucleation side. Right side (B,D,F): contralateral to the enucleation side. Bar = 200 μm.

Bilateral eye enucleation

There was no difference in the distribution of immunoreactive fibers between the left and right lateral geniculate nuclei of bilaterally eye-enucleated rats. In comparison with the lateral geniculate nucleus on the contralateral side of unilaterally eye-enucleated rats, bilateral eye enucleation cause the same alteration in the distribution of immunoreactive fibers in the lateral geniculate nucleus (Fig. 4B,C). As compared with normal rats, the distribution of immunoreactive fibers in the IGL or in the caudomedial portion of the VLGM was not observably changed after bilateral eye enucleation (Fig. 4C). Bilateral eye enucleation caused a significant decrease of immunoreactive fibers in the DLG and in the internal layer of the VLGL, but a substantial number of the fibers still remained there (Fig. 4C). Immunoreactive fine dot-like fibers in the external layer of the VLGL and immunoreactive fibers in the optic tract almost totally disappeared after bilateral eye enucleation (Fig. 4C).

DISCUSSION

We have shown in the present study that: (i) well defined subpopulations of neurons are present within the lateral geniculate nucleus, which exhibit calretinin immunoreactivity, and (ii) calretinin is a chemical component of subpopulations of the contralateral retino-geniculate pathway, which terminate in the DLG and in the external and internal layers of the VLGL.

Comparison of the results with the distribution of other neuroactive substances

A cluster of calretinin-immunoreactive cells were present in the IGL. In the IGL, some neurons having neuropetide Y projected to the suprachiasmatic nucleus, and other cells containing enkephalin sent axons to the contralateral IGL[7]. The morphology of these cells[7] is similar to that of calretinin-containing cells in the IGL. It can be considered that these calretinin neurons may contain neuropeptide Y or enkephalin and may project to the suprachiasmatic nucleus or contralateral IGL. Further studies are necessary to examine this possibility. γ-Aminobutyric acid (GABA)[27,41]- and substance P[41]-containing neurons were also present in the IGL with a distribution that is probably coextensive with calretinin-stained cells.

In addition, the distribution of calretinin-containing cells overlaps that of enkephalin-immunoreactive neurons[24,41] in the intermediate region between the VLGL and the VLGM. The cluster of calretinin-containing cells in the rostral VLGM has never been chemically identified by fluorescence histochemical or immunohistochemical studies for monoamines[9,10,19,22,39], acetylcholine[3,15,25], amino acids[27,28,29] or neuropeptides[20,24,41].

Retino-geniculate pathways

The present results of eye enucleation suggest that subpopulations of the contralateral retino-geniculate

projection contain calretinin, which terminate in the DLG and in the external and internal layers of the VLGL. Thus, calretinin can be used as a marker protein for subpopulations of the retino-geniculate pathway. It would be of interest to identify with calretinin immunoreactivity the retinal afferent terminals in the lateral geniculate nucleus at ultrastructural levels, which so far have been identified by degenerating changes (for example, ref. 21), and new findings may be provided for the synaptic organization of the retinal afferents in the lateral geniculate nucleus. It is worthy of notice, however, that a substantial number of calretinin-containing fibers in the DLG, or in the internal layer of the VLGL, are not of retinal origin.

Anterograde tracing studies have revealed that all of the VLGL is uniformly covered by the contralateral retino-geniculate projection[12,13,33] and that the IGL also receives retinal afferents[13]. In contrast, calretinin-containing retinal afferents terminated only in the two layers of the VLGL with a region of fewer calretinin-immunoreactive fibers between the two layers. In addition, calretinin is not a major component of the retino-IGL pathway, because no change of calretinin-containing fibers in the IGL was observed after eye enucleation. Therefore, the retino-geniculate pathway consists of calretinin- and non-calretinin-containing fibers. It should be noted that, in the rabbit, contralateral retinal afferents terminate in two distinct layers in the alpha sector, which corresponds to the VLGL of the rat, with a small region between the two layers that receives ipsilateral retinal afferents[14].

Retinotopic organization was seen in the VLG[26] as well as in the DLG[23] of the rat. Ganglion cells and their axons in the rat retina were shown to contain calretinin by immunohistochemistry with transverse sections[31,42]. The possibility exists that the calretinin-containing retino-geniculate pathway may originate from restricted ganglion cells that possess calretinin. In order to examine this hypothesis, it is needed to localize calretinin-containing ganglion cells in a whole mount retina.

Our understanding of the possible functions of calretinin is still limited, therefore the physiological role of calretinin in neurons remains speculative. Calretinin has a similar Ca^{2+}-binding site structure (EF-hand) as other calcium-binding proteins including calmodulin, calbindin-D28k and parvalbumin[18]. Among the functions which these calcium-binding proteins are thought to participate in are phosphorylation and Ca^{2+}-buffering (for review, see ref. 5). It seems possible that calretinin is involved in similar functions.

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ABBREVIATIONS

cp	cerebral peduncle
DLG	dorsal lateral geniculate nucleus
IGL	intergeniculate leaflet
opt	optic tract
PP	peripeduncular nucleus
VLG	ventral lateral geniculate nucleus
VLGL	ventral lateral geniculate nucleus, pars lateralis
VLGM	ventral lateral geniculate nucleus, pars medialis
ZI	zona incerta

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