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# Distribution of Calretinin, Calbindin-D28k, and Parvalbumin in the Rat Thalamus

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ARAI, R., D. M. JACOBOWITZ AND S. DEURA. *Distribution of calretinin, calbindin-D28k, and parvalbumin in the rat thalamus.* BRAIN RES BULL 33(5) 595–614, 1994.—The localization of three calcium-binding proteins, calretinin, calbindin-D28k, and parvalbumin, in the rat thalamus was immunohistochemically examined. a) Some thalamic regions revealed cells almost exclusively containing one of the calcium-binding proteins. For example, almost only calretinin-stained cells were found in the central medial and paraventricular nuclei. Calbindin-D28k-stained cells were mostly found in the centrolateral, interanteromedial, anteromedial, and posterior nuclei. Only parvalbumin-positive cells were found in the central part of the reticular nucleus. b) Other regions expressed overlap between the distributions of two cell components composed of different calcium-binding proteins. For example, both calretinin-stained cells and calbindin-D28k-labeled cells were found in the lateroposterior, intermediodorsal, rhomboid, and reunions nuclei. c) Other regions showed no cells stained for any of the calcium-binding proteins. For example, generally no calcium-binding protein was detected in neurons of the anterodorsal, anteroventral, ventrolateral, ventral posterolateral, ventral posteromedial, or gelatinous nuclei, or of the central part of the mediodorsal nucleus. These three proteins serve as useful marker for localizing subpopulations of neurons within the thalamus.

Calcium-binding protein	Calretinin	Calbindin-D28k	Parvalbumin	Thalamus	Rat
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CALRETININ, calbindin-D28k, and parvalbumin are members of the EF-hand family of calcium-binding proteins (37) and have restricted distributions in neurons of the central nervous system of the rat (3,10,23,43). The differential distribution of the two calcium-binding proteins, calbindin-D28k and parvalbumin, has been reported in many regions of the central nervous system of numerous species: of the rat, in the cerebral cortex (51), hippocampus (20,48), thalamus (18), and dorsal horn of the spinal cord (1,55,56); of the cat, in the pulvinar-lateralis posterior complex (5) and superior colliculus (34); of the monkey, in the hippocampal formation (46), basal forebrain and midbrain (14), and thalamus (25,41). In the rat hippocampus, furthermore, other pairs of calcium-binding proteins, calretinin and parvalbumin, and calretinin and calbindin-D28k, have been shown to form largely nonoverlapping cell groups (31). Colocalization of calretinin and calbindin-D28k in the same neurons has been shown in some regions of the rat brain, including the substantia nigra, ventral tegmental area, and triangular septal nucleus (44). In the cat thalamic reticular nucleus, calbindin-D28k-immunoreactive cells are also immunostained for parvalbumin (32).

Although the role of these calcium-binding proteins in neurons is still unknown, their ability to bind  $\text{Ca}^{2+}$  entering the cytosol could buffer intracellular  $\text{Ca}^{2+}$  (8). Côté et al. (14) have revealed that distribution patterns of calbindin-D28k and parvalbumin in the primate basal forebrain and midbrain are complementary and suggested that the two calcium-binding proteins may work synergistically. If the three calcium-binding proteins,

calretinin, calbindin-D28k, and parvalbumin, are differentially distributed in brain regions, it might provide a clue for exploring the possible functions of these proteins.

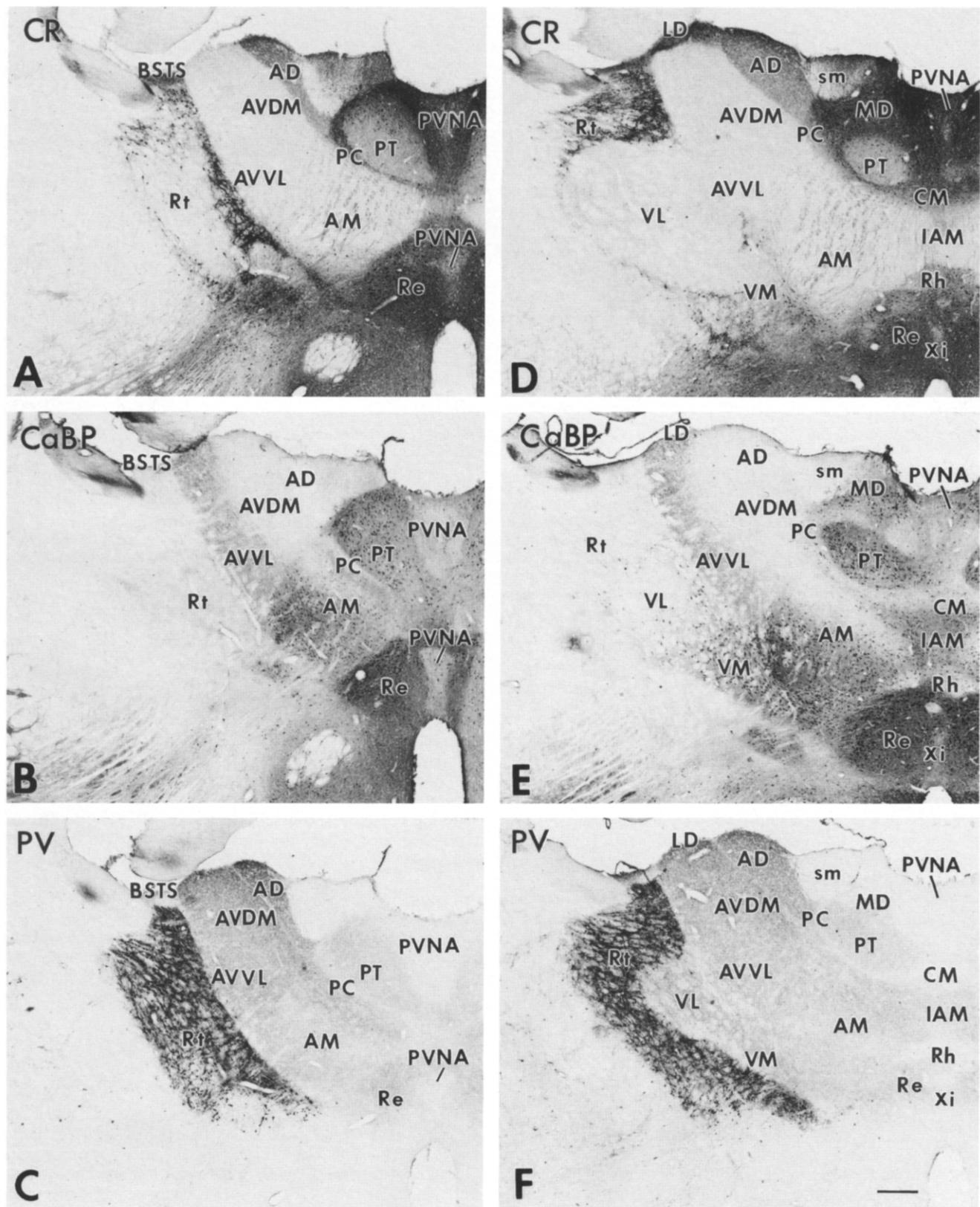
In the thalamus of the rat, previous studies have shown that calretinin (23,43,54), calbindin-D28k (6,10,18) and parvalbumin (10,15,18) are localized in subpopulations of neurons, although their localizations have never been extensively examined. In the present study, we localized immunohistochemically each of the calcium-binding proteins in the rat thalamus using closely matched sections, and compared their distribution patterns to examine whether a complementarity exists. The lateral and medial geniculate nuclei showed rather complicated distribution patterns of the calcium-binding proteins [for example, see (2)]; therefore, a comparison of the distributions in these nuclei will be described elsewhere.

## METHOD

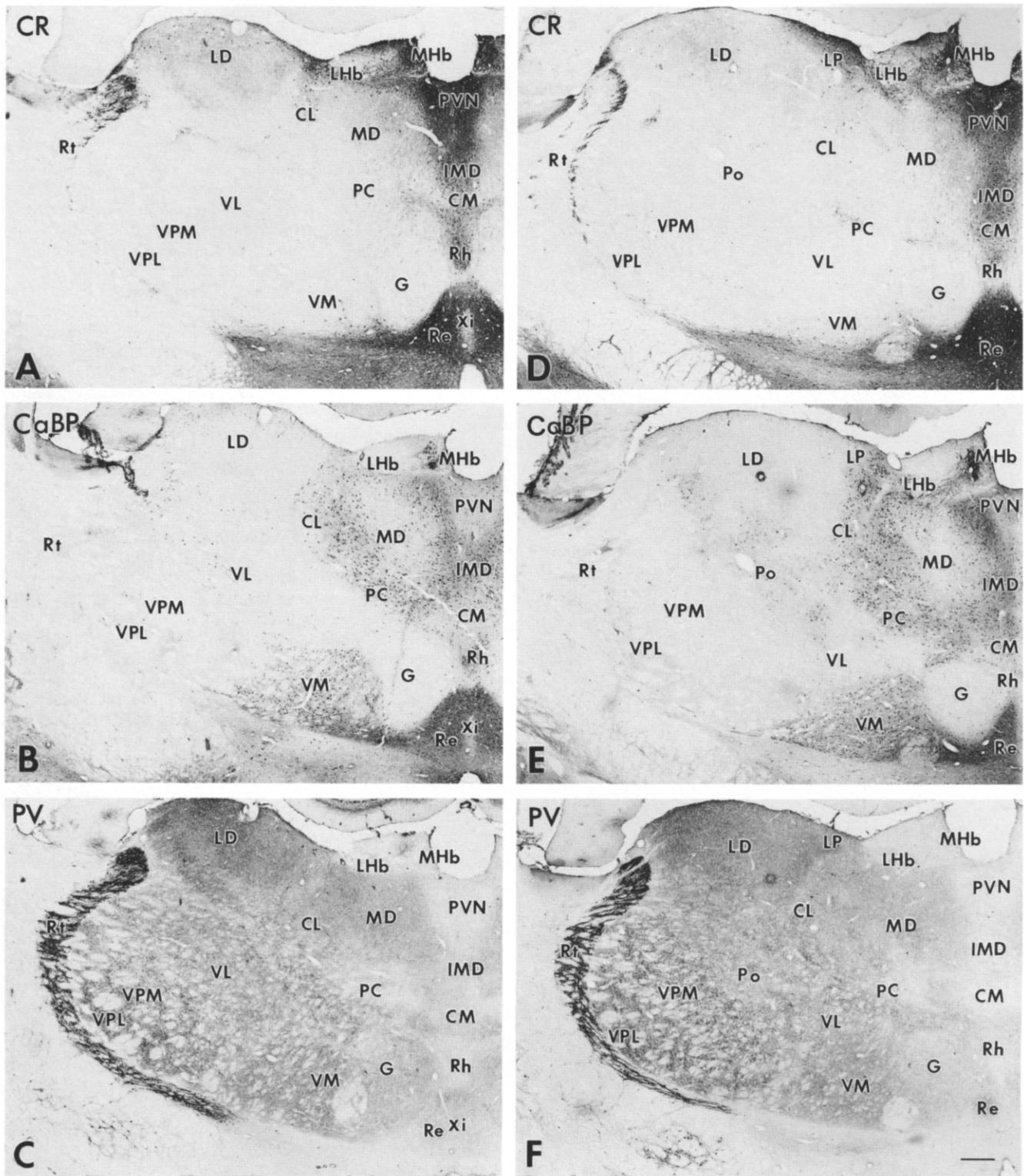
### Tissue Preparation

Male Sprague–Dawley rats (250–350 g,  $n = 5$ ), under anesthesia following an intraperitoneal injection of Somnifer (Richmond Veterinary Supply Co.; 0.2 ml/100 g body weight), were perfused through the ascending aorta with 100 ml of phosphate-buffered saline (PBS, 20 mM, pH 7.4) containing 0.5% (w/v) sodium nitrite, followed by 200–300 ml of formalin (10% v/v in phosphate buffer, pH 7.0, 4°C). The brains were dissected out and placed in the same fixative for 30 min followed by incubation

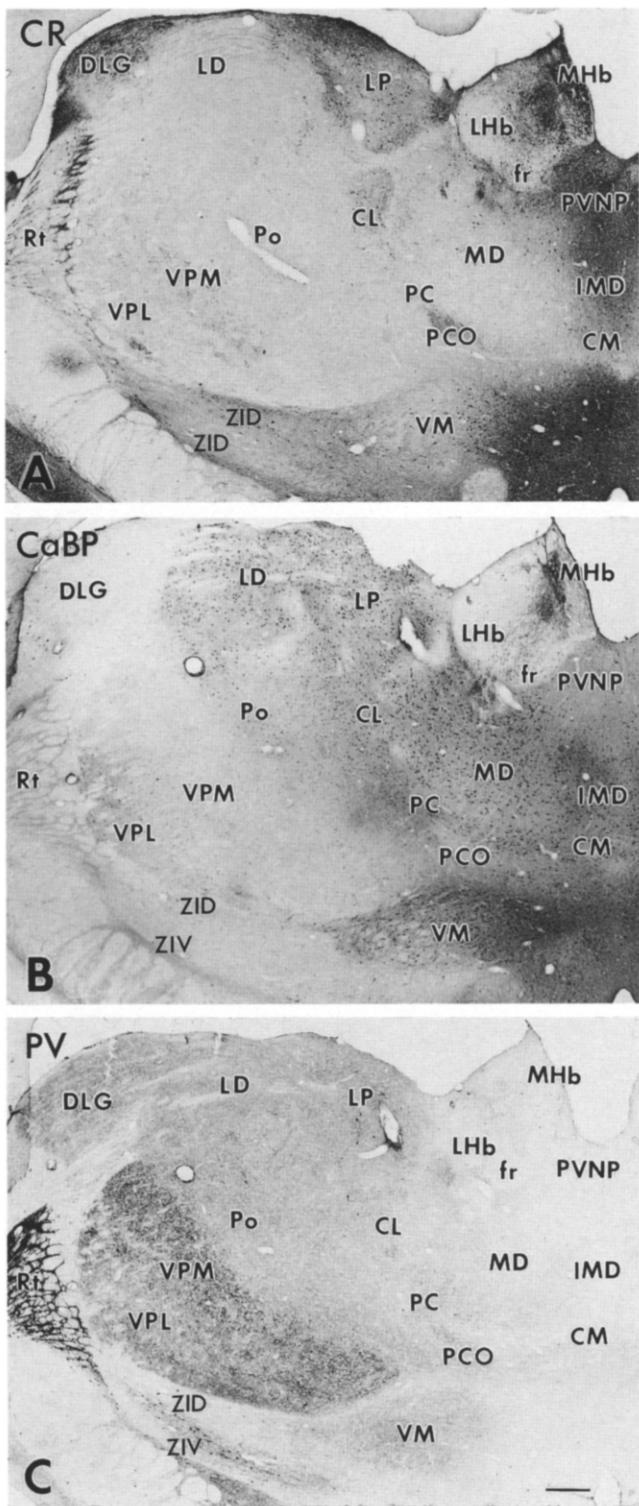
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FIGS. 1–3. Calretinin (CR), calbindin-D28k (CaBP), and parvalbumin (PV) in frontal sections of the thalamus. Top row photographs (Figs. 1A,D; 2A,D; 3A) show calretinin-stained sections. Middle row photographs (Figs. 1B,E; 2B,E; 3B) show calbindin D28k-stained sections. Bottom row photographs (Figs. 1C,F; 2C,F; 3C) show parvalbumin-stained sections. A column of three photographs (Figs. 1A–C, 1D–F, 2A–C, 2D–F, 3A–C) are of closely matched sections. Figure 1A–C is rostral, Fig. 3A–C caudal. The distance from the level of Fig. 1A–C to 1D–F is 300  $\mu$ m, from 1A–



C to 2A–C is 1000  $\mu\text{m}$ , from 1A–C to 2D–F is 1400  $\mu\text{m}$ , and from 1A–C to 3A–C is 2100  $\mu\text{m}$ . Bars = 250  $\mu\text{m}$ . Abbreviations: 3V: third ventricle; AD: anterodorsal nucleus; AM: anteromedial nucleus; AVDM: anteroventral nucleus, dorsomedial part; AVVL: anteroventral nucleus, ventrolateral part; BSTS: bed nucleus of stria terminalis, supracapsular division; CaBP: calbindin-D28K; CL: centrolateral nucleus; CM: central medial nucleus; cp: cerebral peduncle; CR: calretinin; D3V: dorsal third ventricle; DLG: dorsal lateral geniculate nucleus; eml: external medullary lamina; f: fornix; fr: fasciculus retroflexus; G: gelatinosus nucleus; IAM: interanteromedial nucleus; IMD: intermediodorsal nucleus; LD: laterodorsal nucleus; LHB: lateral habenular nucleus; LP: lateroposterior nucleus; MD: mediodorsal nucleus; MHb: medial habenular nucleus; mt: mammillothalamic tract; PC: paracentral nucleus; PCC: paracentral nucleus, ovoid subdivision; PCRDC: paracentral nucleus, rostral dorsal cap; Po: posterior nucleus; PT: paratenial nucleus; PV: parvalbumin; PVNA: paraventricular nucleus, anterior part; PVN: paraventricular nucleus, intermediate part; PVNP: paraventricular nucleus, posterior part; Re: reunions nucleus; Rh: rhomboid nucleus; Rt: reticular nucleus; sm: stria medullaris of thalamus; st: stria terminalis; VL: ventrolateral nucleus; VLG: ventral lateral geniculate nucleus; VM: ventromedial nucleus; VPL: ventral posterolateral nucleus; VPM: ventral postero-medial nucleus; Xi: xiphoid nucleus; Zi: zona incerta; ZID: zona incerta, dorsal part; ZIV: zona incerta, ventral part.



FIGS. 1-3. Continued

for 2 days in PBS containing 20% (w/v) sucrose (4°C). Serial sections of the thalamus were cut on a cryostat in a frontal plane at 25  $\mu\text{m}$  and collected in PBS. Sets of four neighboring sections were made at intervals of 100  $\mu\text{m}$  in a rostrocaudal order, and these sets were used for localization of the three calcium-binding

proteins. Other sections were used for immunohistochemical controls.

#### *Staining Procedure*

In order to localize the immunoreactivity of the calcium-binding proteins, neighboring sections of each set were singly stained for calretinin, calbindin-D28k, parvalbumin, or Nissl substance. The calcium-binding proteins were detected with immunohistochemistry as described below. Nissl substance was stained with thionin to identify the parcellation of the thalamic nuclei.

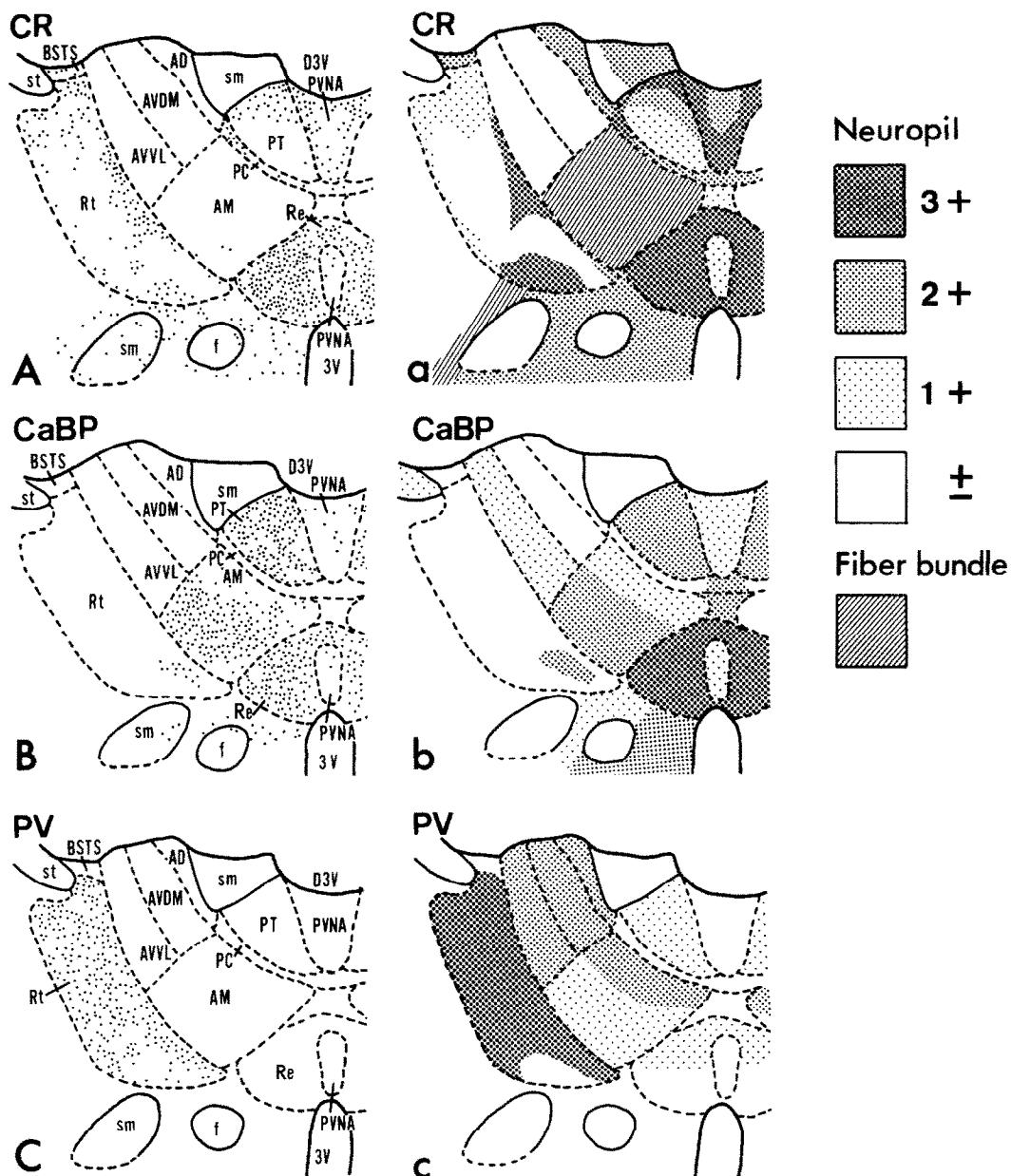
**Immunohistochemistry.** An antiserum was raised in a rabbit against calretinin purified from the guinea pig brain (52). Mouse monoclonal antibodies against calbindin-D28k purified from the chicken gut (clone No. CL-300, lot No. 49F4826) and against parvalbumin purified from the carp muscle (clone No. PA-235, lot No. 49F4824) were obtained commercially (Sigma Chemical Co., St. Louis, MO).

Calcium-binding proteins were detected immunohistochemically with a streptavidin-peroxidase conjugate (47). The sections were incubated as follows: a) 0.3% Triton X-100 in PBS for 1 h at room temperature; b) 5% normal goat serum in PBS for 1 h at room temperature; c) rabbit anticalretinin antiserum (diluted 1:5,000 in PBS), mouse anticalbindin-D28k antibody (1:25,000) or mouse antiparvalbumin antibody (1:10,000), each of which contained 1% normal goat serum, for 48 h at 4°C; d) biotinylated goat antirabbit immunoglobulin G (Vector, Burlingame, CA, 1:400 in PBS) for calretinin, or biotinylated goat antimouse immunoglobulin G (Vector, 1:2,000) for calbindin-D28k and parvalbumin, for 2 h at room temperature; e) streptavidin-peroxidase (Kirkegaard & Perry Lab., Gaithersburg, MD, 1:2,000 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, Pittsburgh, PA) and 0.006% hydrogen peroxide in 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 controls for the immunohistochemical staining, sections were processed for the immunohistochemistry described above with omitting the three primary antibodies. No immunolabeling was found under these conditions with any of the antibodies.

#### *Mapping Procedure*

For the three calcium-binding proteins, schematic drawings of the localization of immunoreactive cell bodies and fibers were made in the following manner. Of twelve sets of closely matched four sections stained for calretinin, calbindin-D28k, parvalbumin, or Nissl substance, photographs were made with an Olympus light microscope at  $\times 60$ . Twelve sets of four montages of the sections were made. Five representative levels were selected from the sets of montages. By use of the montages of the sections stained for calretinin, calbindin-D28k, or parvalbumin, the outline of the sections and immunoreactive cell bodies were first plotted onto translucent tracing papers (series 1, 2 and 3, respectively), and then the outline and immunoreactive fiber-containing regions were plotted onto different translucent tracing papers (series 4, 5, and 6, respectively). By use of the montages of the Nissl-stained sections, the outline of the sections and the borders of brain regions were plotted onto tracing papers (series 7). Six pairs of tracing papers (series 1 and 7, 2 and 7, 3 and 7, 4 and 7, 5 and 7, 6 and 7) were superimposed to make final schematic drawings (Figs. 4-8). The localization and the packing density of both immunoreactive cell bodies and fibers are summarized in Table 1. Packing density was classified by a subjective estimation. To delineate the brain regions, the literature (17,36) was consulted.



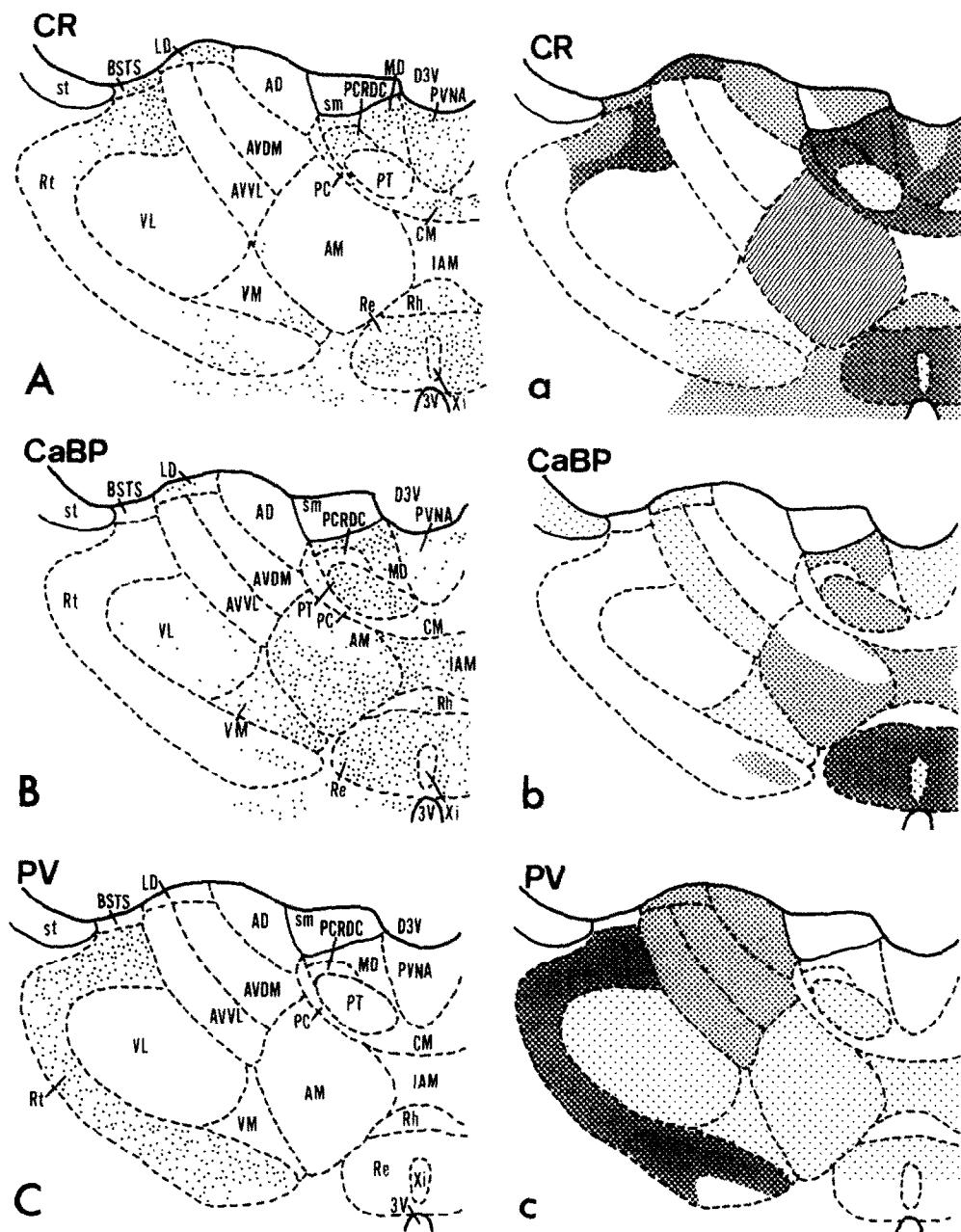
FIGS. 4-8. Calretinin (CR), calbindin-D28k (CaBP), and parvalbumin (PV) in frontal sections of the thalamus. Top row drawings (A,a) are of calretinin-stained sections. Middle row drawings (B,b) are of calbindin D28k-stained sections. Bottom row drawings (C,c) are of parvalbumin-stained sections. Left side drawings (A-C) show locations of immunoreactive cell bodies. One symbol indicates approximately one cell body. Right side drawings (a-c) show immunoreactive fibers. Packing density of neuropil is depicted by four variations of compactness of stipple: high density (3+), moderate density (2+), low density (1+), very low density (±). Fiber bundles are represented by hatching. Left and right drawings of each row are of the same sections. A column of three pairs of drawings (A,a; B,b; C,c) are of closely matched sections. Each drawing represents the section of Figs. 1-3: 4A(a)—1A; 4B(b)—1B; 4C(c)—1C; 5A(a)—1D; 5B(b)—1E; 5C(c)—1F; 6A(a)—2A; 6B(b)—2B; 6C(c)—2C; 7A(a)—2D; 7B(b)—2E; 7C(c)—2F; 8A(a)—3A; 8B(b)—3B; 8C(c)—3C.

#### Specificity of Primary Antibodies

**Anticalretinin antibody.** The possibility has been suggested that anticalretinin antiserum may crossreact with calbindin-D28k because of a high degree of homology between calretinin and calbindin-D28k (42). In the case of the anticalretinin antiserum used in the present study, however, this possibility is excluded by the following data: a) we have previously done the preadsorption control using calretinin purified from the guinea pig brain (23,52) and from the rat brain (3), and no immunohisto-

chemical staining has been found, b) the antiserum has detected only calretinin but not calbindin-D28k on a protein blot of the guinea pig brain sample (52), c) in a study with radioimmunoassay, the anticalretinin antiserum has reacted with calretinin purified from the rat brain, but no significant crossreactivity has been observed between the antiserum and purified rat calbindin-D28k (53). The antiserum has been widely used in immunohistochemical studies of the rat brain (2,3,16,19,23,31,52,54).

**Anticalbindin-D28k antibody.** Immunoblots of the rabbit cerebellum sample, the anticalbindin-D28k antibody used in the



FIGS. 4-8.

present study, has been shown to crossreact with calbindin-D28k, but not to crossreact with other calcium-binding proteins (11). Preadsorption of the antibody with purified chicken intestinal calbindin-D28k has eliminated specific immunostaining of sections of the rat brain (10). The antiserum has been widely used in immunohistochemical studies of the rat brain (9,10,16,18,26,40,45).

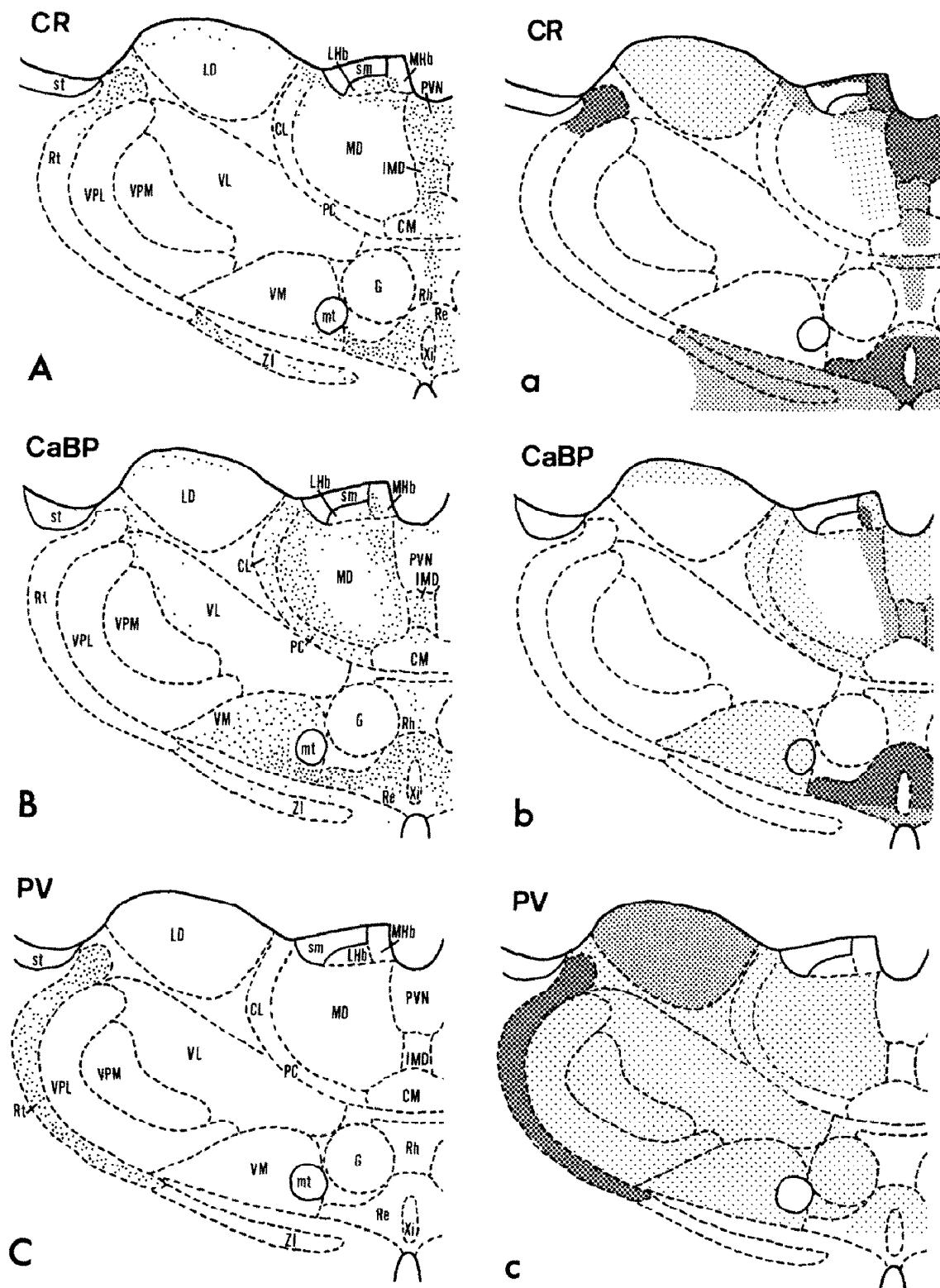
**Antiparvalbumin antibody.** By radioimmunoassay and immunoblots, it has been shown that the antiparvalbumin antibody used in the present study crossreacts with rat parvalbumin, but does not crossreact with calmodulin, calbindin-D28k, or another calcium-binding protein that is considered to correspond to calretinin (12). The antiserum has been widely used in immunohistochemical studies of the rat brain (10,13,15,26,29,39,45).

## RESULTS

In the present study, we localized in detail the three calcium-binding proteins, calretinin, calbindin-D28k, and parvalbumin in the rat thalamus, and compared their distributions. Previous studies have demonstrated immunoreactivity to calretinin (23,43,54), calbindin-D28k (6,10,18), and parvalbumin (10,15,18) in the rat thalamus. The present findings are in general agreement with these reports.

### Localization of Cell Bodies and Fibers Showing Immunoreactivity to Calcium-Binding Proteins

A detailed analysis of the localization of immunoreactive cell bodies and fibers is summarized in Table 1. Figures 4-8 illustrate



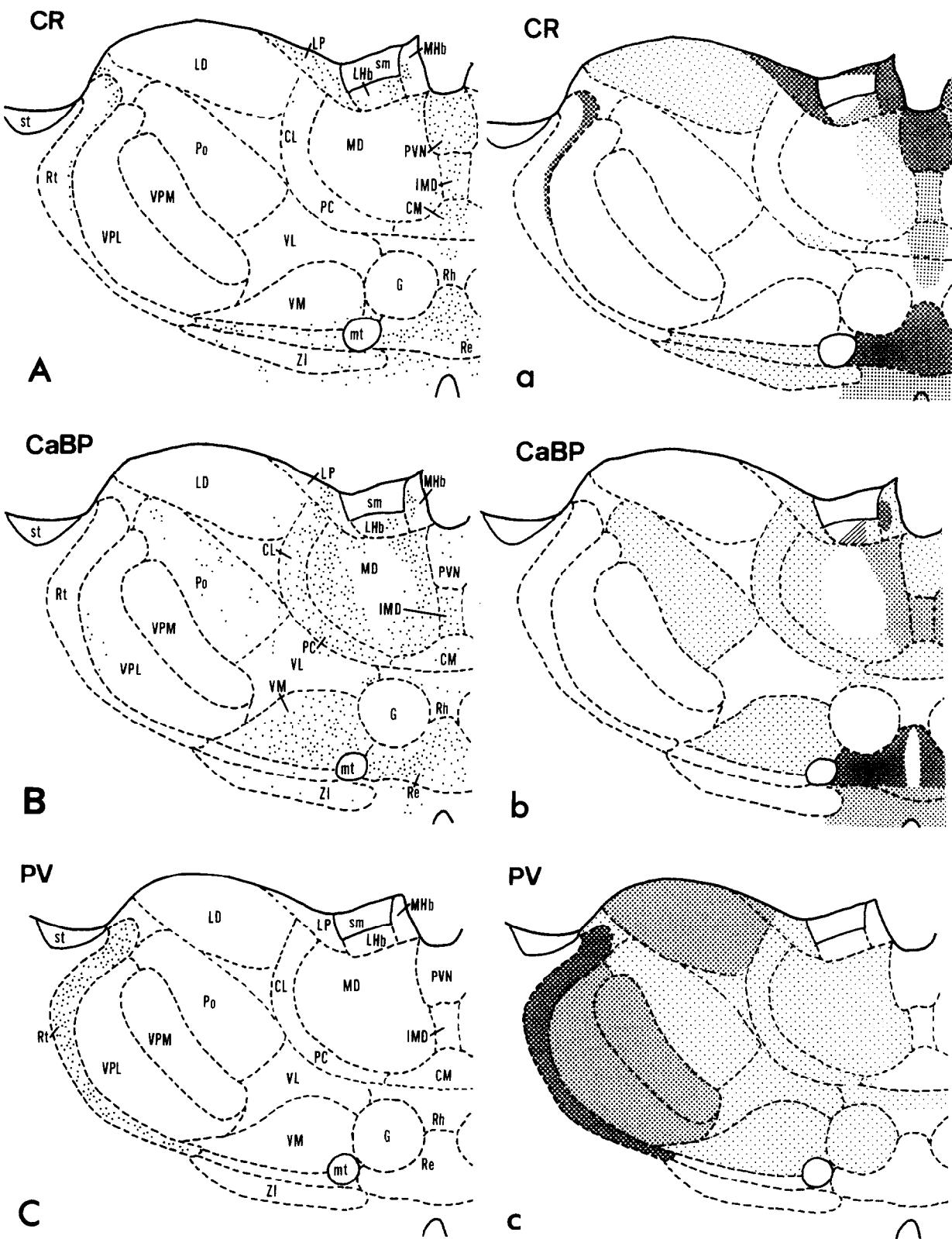
FIGS. 4-8.

the distribution of the immunoreactivity of the three calcium-binding proteins at five representative levels. Figures 1-3 show the perspectives of immunostained sections of the drawings of Figs. 4-8. Figures 9-15 show some regions of interest. In the following text, the major findings of comparison among the localization patterns of the calcium-binding pro-

teins will be described. Other details can be found in Table 1 and Figures 1-16.

#### *Anterior Nuclear Group*

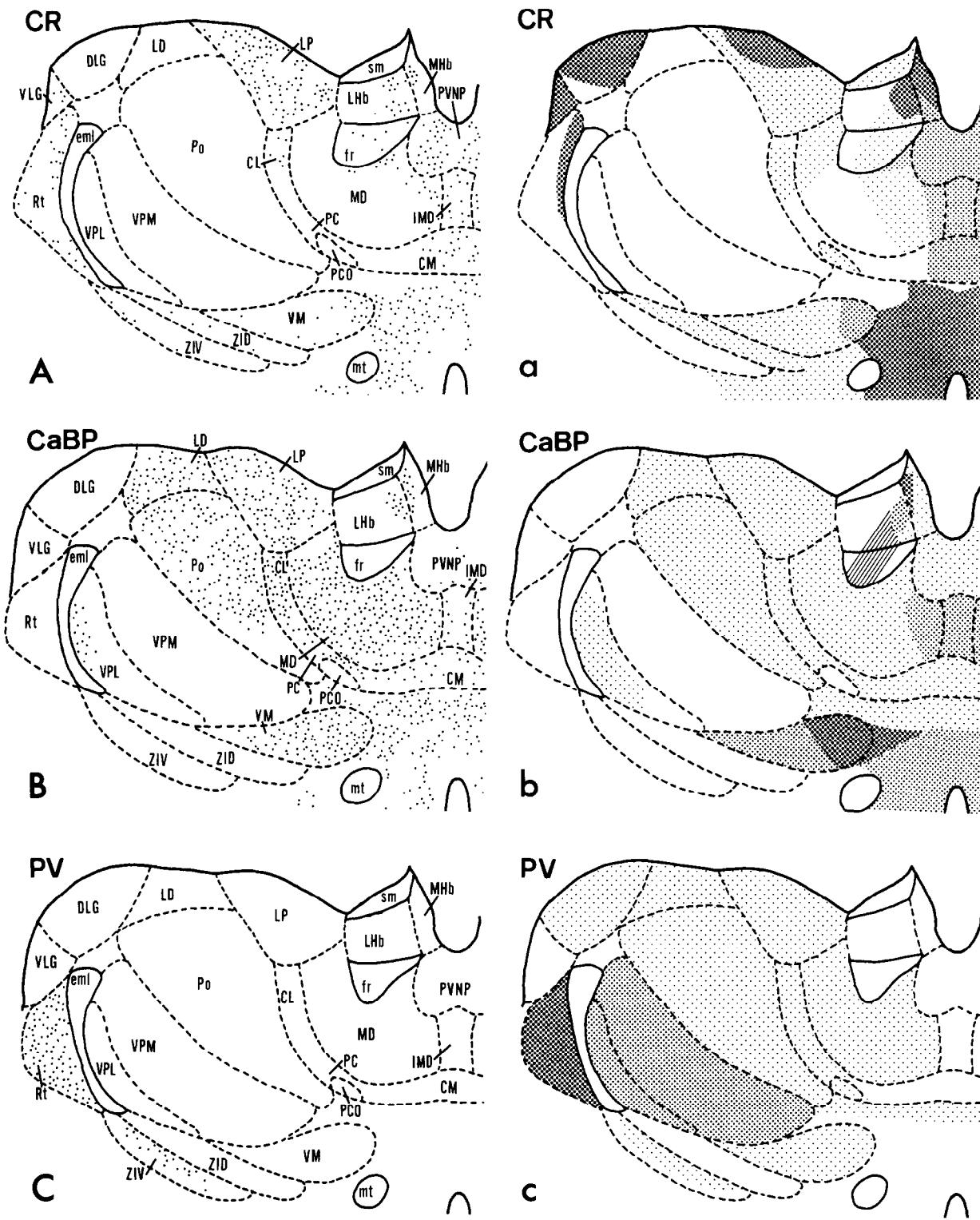
**Cell bodies.** The anteromedial nucleus (AM) showed almost exclusively calbindin-D28k-stained cells in its ventral two-thirds,



FIGS. 4–8.

with an immunoreactive cell poor zone in its dorsal one-third (Figs. 1B,E; 4B; 5B; 12B). In the anterodorsal nucleus (AD), and in the dorsomedial part (AVDM) and ventrolateral part (AVVL)

of the anteroventral nucleus, no neurons revealed immunoreactivity to any of the calcium-binding proteins (Figs. 1; 4A–C; 5A–C; 15D–F).



FIGS. 4-8.

**Fibers.** Neuropil of the AD was stained mainly for both calretinin (Figs. 1A,D; 4a; 5a; 15D) and parvalbumin (Figs. 1C,F; 4c; 5c; 15F). The AVDM showed neuropil more strongly stained for parvalbumin (Figs. 1C,F; 4c; 5c; 15F) than for the other two calcium-binding proteins (Figs. 1A,B,D,E;

4a,b; 5a,b; 15D,E). Neuropil of the AVVL preferentially revealed immunoreactivity to both calbindin-D28k (Figs. 1B,E; 4b; 5b; 15E) and parvalbumin (Figs. 1C,F; 4c; 5c; 15F). Calretinin-stained fiber bundles were found in the AM (Figs. 1A,D; 4a; 5a; 12A).

**TABLE 1**  
**DIFFERENTIAL DISTRIBUTION OF CALRETININ (CR), CALBINDIN D-28k (CB), AND PARVALBUMIN (PV) IN THE THALAMUS OF THE RAT**

Thalamic Regions	Packing Density of Cell Bodies*			Packing Density of Fibers†		
	CR‡	CB§	PV¶	CR#	CB**	PV††
Anterior nuclear group						
Anterodorsal nucleus (AD)	-	-	-	++	±	++
Anteroventral nucleus						
Dorsomedial part (AVDM)	-	-	-	±	±	++
Ventrolateral part (AVVL)	-	-	-	±	+	++
Anteromedial nucleus (AM)						
Dorsal one third	-	±	-	fb	± ~ +	+ ~ ++
Ventral two thirds	±	+++	-	fb	++	+
Mediodorsal nucleus (MD)						
Rostral level	+	+	-	+++	± ~ ++	±
Intermediate level						
Medial part	±	++	-	+ ~ ++	++	+
Central part	-	-	-	±	±	+
Lateral part	-	++	-	±	+	+
Caudal level	±	++	-	± ~ ++	± ~ ++	+
Ventral nuclear complex						
Ventromedial nucleus (VM)						
Lateral part	-	+	-	± ~ +	+	+
Medial part‡‡	+	+ ~ ++	-	± ~ ++	+	+
Ventrolateral nucleus (VL)	-	±	-	±	±	+
Ventral posterolateral nucleus (VPL)						
Rostral part	-	-	-	± ~ +	±	+
Caudolateral part§§	-	+	-	±	+	++
Ventral posteromedial nucleus (VPM)	-	-	-	±	±	+ ~ ++
Gelatinous nucleus (G)	-	-	-	±	±	+
Lateral nuclear group						
Laterodorsal nucleus (LD)						
Rostral part	++	+	-	+ ~ +++	± ~ +	++
Intermediate part	±	±	-	+	±	++
Caudal part	-	++	-	±	+	+
Lateroposterior nucleus (LP)	++	+ ~ ++	-	+ ~ +++	± ~ +	+ ~ ++
Posterior nucleus (Po)	-	+	-	±	+	+
Intralaminar nuclear group						
Central medial nucleus (CM)	+	±	-	+ ~ +++	± ~ +	±
Centrolateral nucleus (CL)	±	+	-	+ ~ +	+	+
Paracentral nucleus (PC)						
Rostrolateral part¶¶	+	±	-	+ ~ +++	±	+
Caudal part	-	+	-	±	+	+
Rostrodorsal cap (PCRDC)##	++	±	-	+++	±	±
Ovoid subdivision (PCO)	-	-	-	+	±	+
Midline nuclear group						
Paraventricular nucleus						
Anterior part (PVNA)						
Dorsally placed PVNA***						
Ventral and lateral regions	++	±	-	+++	+	±
Central region	+	±	-	++	+	+
Ventrally placed PVNA†††	±	±	-	+	+	+
Intermediate part (PVN)	++	±	-	+++	+	+
Posterior part (PVNP)	++	±	-	++	+	+
Paratenial nucleus (PT)						
Rostrodorsal part‡‡	++	++	-	+ ~ +++	++	+
Rostroventral and caudal parts	±	++	-	+	++	+
Interanteromedial nucleus (IAM)	-	++	-	±	++	+
Intermediodorsal nucleus (IMD)	+	+	-	++	++	+
Rhomboid nucleus (Rh)	+	+	-	± ~ ++	± ~ +	+
Reuniens nucleus (Re)	+++	+++	-	+++	+++	+
Xiphoid nucleus (Xi)	±	±	-	+	+	+

TABLE 1  
CONTINUED

Thalamic Regions	Packing Density of Cell Bodies*			Packing Density of Fibers†		
	CR‡	CB§	PV¶	CR#	CB**	PV††
<b>Habenular complex</b>						
Medial habenular nucleus (MHb)						
Medial part	—	—	—	+++	+	±
Lateral part	++	++	—	+++	+++	±
Lateral habenular nucleus (LHb)						
Medial part	++	+	—	+ ~ +++	+ ~ ++	±
Lateral part	±	—	—	±	fb	±
Reticular nucleus (Rt)						
Central part	±	—	+++	±	+	+++
Medial part §§§	++	—	+++	+++	±	+++
Dorsal part ¶¶¶	++	—	+++	+ ~ +++	±	+++
Ventromedial corner###						
Dorsal portion	±	+	++	± ~ +	++	+++
Ventral portion	+	—	±	++ ~ +++	±	±
Surrounding regions						
Zona incerta (ZI)						
Dorsal part (ZID)	±	—	—	+	±	±
Ventral (ZIV)	—	—	+	±	±	+
Bed nucleus of stria terminalis						
Supracapsular division (BSTS)	+	—	—	++	±	±

\* +++, high density; ++, moderate density; +, low density; ±, very low density; —, not found.

† +++, high-density neuropil; ++, moderate-density neuropil; +, low-density neuropil; ±, very low-density neuropil; fb, fiber bundle.

‡ Calretinin-stained cell bodies.

§ Calbindin-D28k-stained cell bodies.

¶ Parvalbumin-stained cell bodies.

# Calretinin-stained fibers.

\*\* Calbindin-D28k-stained fibers.

†† Parvalbumin-stained fibers.

‡‡ Levels of Figs. 5 and 8.

§§ Level of Fig. 8.

¶¶ Level of Fig. 4.

## Level of Fig. 5.

\*\*\* Beneath the dorsal third ventricle (D3V) at the levels of Figs. 4 and 5.

††† Surrounded by the reunions nucleus (Re) at the level of Fig. 4.

‡‡‡ Level of Fig. 4.

§§§ Level of Fig. 4.

¶¶¶ Levels of Figs. 4, 5 and 6.

### Levels of Figs. 4 and 5.

### Mediodorsal Nucleus

**Cell bodies.** The mediodorsal nucleus (MD) contained both calretinin-stained cells (Figs. 1D; 5A; 11A) and calbindin-D28k-stained cells (Figs. 1E; 5B; 11B) at its rostral level. At the intermediate levels, the MD showed predominantly calbindin-D28k-stained cells, which were found in its medial and lateral parts (Figs. 2B,E; 6B; 7B). The central part of the MD revealed no neurons immunostained for any of the calcium-binding proteins (Figs. 2; 6A-C; 7A-C). At the caudal level, the MD contained calbindin-D28k-positive cells throughout the nucleus (Figs. 3B; 8B; 11E) and calretinin-stained cells in its medial part (Figs. 3A; 8A; 11D).

**Fibers.** At the intermediate levels of the MD, the central part of the MD contained neuropil that were preferentially stained for parvalbumin (Figs. 2C,F; 6C; 7C).

### Ventral Nuclear Complex

**Cell bodies.** The ventromedial nucleus (VM) contained calbindin-D28k-positive cells in the whole nucleus (Figs. 1E; 2B,E;

3B; 5B; 6B; 7B; 8B), and the medial part of the VM also included calretinin-stained cells at its rostral and caudal levels (Figs. 1D; 3A; 5A; 8A). The ventral posterolateral nucleus (VPL) had a cluster of faintly stained cells for calbindin-D28k in its lateral part at its caudal levels (Figs. 3B; 8B; 9E). The ventrolateral nucleus (VL), ventral posteromedial nucleus (VPM) and gelatinous nucleus (G) were almost devoid of cells immunostained for any of the calcium-binding proteins (Figs. 1D-F; 2; 3; 5A-C; 6A-C; 7A-C; 8A-C; 12A-C), though dispersed calbindin-D28k-stained cells were found in the VL (Figs. 1E; 2B,E; 5B; 6B; 7B).

**Fibers.** Neuropil of the VL, VPL, VPM, and G showed predominantly parvalbumin immunoreactivity (Figs. 1F; 2C,F; 3C; 5C; 6C; 7C; 8C; 9C,F; 12C). At the level between Figs. 5 and 6, the VPL showed neuropil densely stained for calretinin (Fig. 9A) as well as parvalbumin (Fig. 9C).

### Lateral Nuclear Group

**Cell bodies.** The rostral part of the laterodorsal nucleus (LD) contained both calretinin-immunoreactive cells (Figs. 1D; 2A;

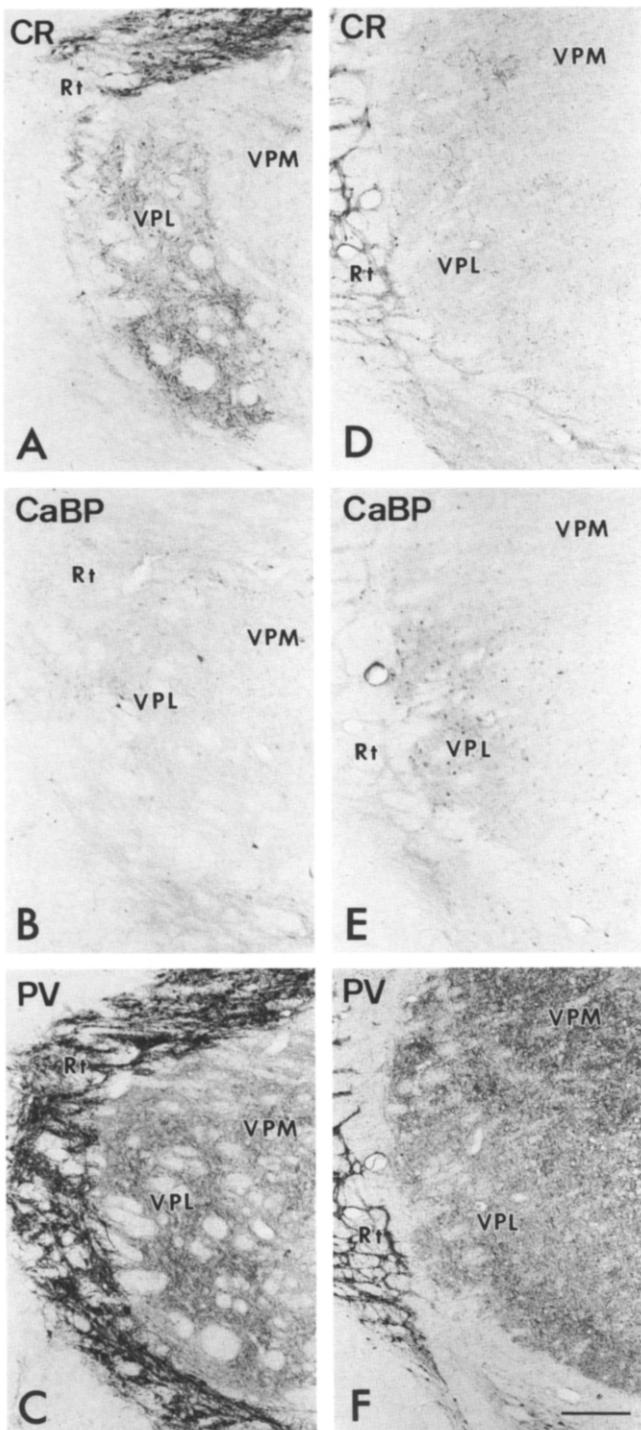


FIG. 9. Calretinin (CR), calbindin-D28k (CaBP), and parvalbumin (PV) in frontal sections through the ventral posterolateral nucleus. (A-C) Level halfway between Figs. 5 and 6. (D-F) Level of Fig. 8. Top row (A,D): calretinin-stained sections. Middle row (B,E): calbindin D28k-stained sections. Bottom row (C,F): parvalbumin-stained sections. A column of three photographs (A-C, D-F) are of closely matched sections. Bar = 200  $\mu$ m (A-F).

5A; 6A; 10A) and calbindin-D28k-stained cells (Figs. 1E; 2B; 5B; 6B; 10B). The most caudal part of the LD contained only calbindin-D28k-stained cells (Figs. 3B; 8B). The lateroposterior

nucleus (LP) at its caudal level showed both calretinin-stained cells (Figs. 3A; 8A) and calbindin-D28k-stained cells (Figs. 3B; 8B). At the rostral level of the LP, calretinin-stained cells were found throughout the nucleus (Figs. 2D; 7A; 10D), and calbindin-D28k-stained cells were observed in its medial part (Figs. 2E; 7B; 10E).

**Fibers.** Neuropil of the LD at its intermediate levels showed preferentially parvalbumin immunoreactivity (Figs. 2C,F; 6c; 7c; 10F).

#### Posterior Nucleus

**Cell bodies.** Only calbindin-D28k-stained cells were found in the posterior nucleus (Po) (Figs. 2E; 3B; 7B; 8B).

**Fibers.** Neuropil of the Po revealed preferentially immunoreactivity of calbindin-D28k (Figs. 2E; 3B; 7b; 8b) and of parvalbumin (Figs. 2F; 3C; 7c; 8c).

#### Intralaminar Nuclear Group

**Cell bodies.** The central medial nucleus (CM) showed predominantly calretinin-stained cells (Figs. 1D; 2A,D; 3A; 5A; 6A; 7A; 8A; 11A; 12A), with a few calbindin-D28k-stained cells included (Figs. 1E; 2B,E; 3B; 5B; 6B; 7B; 8B; 11B; 12B). The centrolateral nucleus (CL) contained almost exclusively calbindin-D28k-stained cells (Figs. 2B,E; 3B; 6B; 7B; 8B; 10E; 11E), though a few calretinin-labeled cells were found in the dorsal part at its caudal level (Figs. 3A; 8A). A cluster of calretinin-stained cells was found in the rostrodorsal part of the CL (Figs. 2A; 6A), and this cluster appeared to be an extension of the calretinin-stained cell group in the LP (Figs. 2D; 3A; 7A; 8A; 10D). The paracentral nucleus (PC) contained calbindin-D28k-stained cells in its caudal part (Figs. 2B,E; 3B; 6B; 7B; 8B). This cell group appeared to continue to the group of calbindin-D28k-stained cells in the CL and in the lateral part of the MD (Figs. 2B,E; 3B; 6B; 7B; 8B). The lateral part of the PC contained predominantly calretinin-stained cells at its rostral level (Figs. 1A; 4A). At the level of Fig. 5, a cluster of calretinin-stained cells (Figs. 1D; 5A; 11A) and only a few calbindin-D28k-stained cells (Figs. 1E; 5B; 11B) were found in a region surrounded by the PC, mediodorsal nucleus (MD), paratenial nucleus (PT) and/or stria medularis of the thalamus (sm). No parvalbumin-stained cells were found in this region (Figs. 1F; 5C; 11C). We named this region the rostrodorsal cap (PCRDC) of the paracentral nucleus. The PCRDC appears to correspond to the region that has been labeled the paracentral nucleus (PC) in Fig. 25 of Paxinos and Watson (36); this term PC has also indicated the region in the internal medullary lamina in the atlas of Paxinos and Watson (36).

**Fibers.** The neuropil of the PCRDC was stained strongly for calretinin (Figs. 1D; 5a; 11A), but weakly for calbindin-D28k (Figs. 1E; 5b; 11B) and parvalbumin (Figs. 1F; 5c; 11C). The ovoid subdivision (PCO) of the paracentral nucleus showed neuropil that contained clusters of both calretinin-stained fibers (Figs. 3A; 8a; 11D) and parvalbumin-stained fibers (Figs. 3C; 8c; 11F) and very sparse calbindin-D28k-stained fibers (Figs. 3B; 8b; 11E). This subdivision was outlined but not labeled in Figure 28 of Paxinos and Watson (36).

#### Midline Nuclear Group

**Cell bodies.** The anterior part (PVNA), intermediate part (PVN) and posterior part (PVNP) of the paraventricular nucleus contained predominantly calretinin-stained cells (Figs. 1A,D; 2A,D; 3A; 4A; 5A; 6A; 7A; 8A). At the level of Fig. 4, the PVNA was split into two portions: the dorsally placed PVNA was found beneath the dorsal third ventricle (D3V), and the ventrally placed

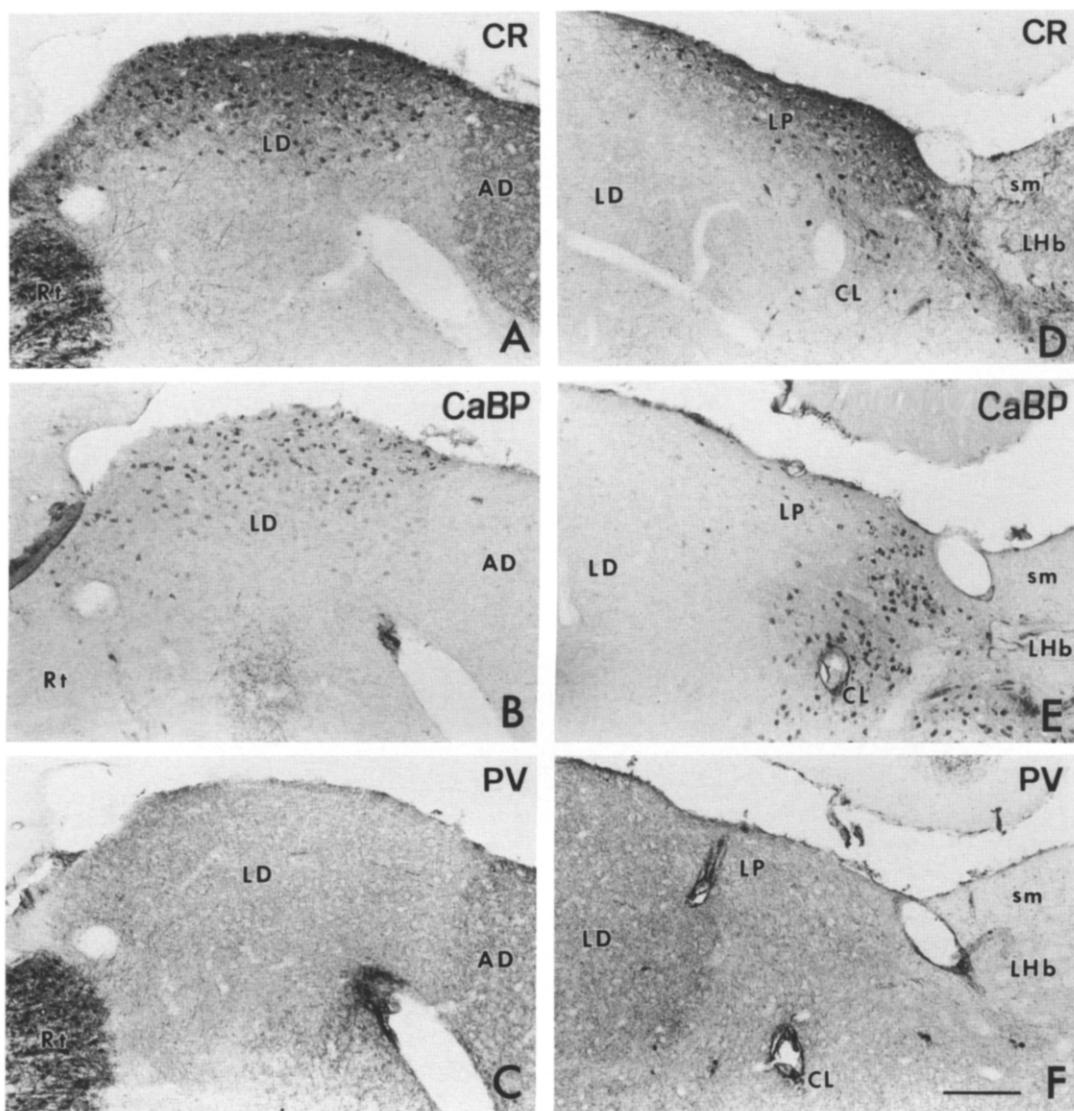


FIG. 10. Calretinin (CR), calbindin-D28k (CaBP), and parvalbumin (PV). (A–C) Frontal sections through the laterodorsal nucleus at the level halfway between Figs. 5 and 6. (D–F) Frontal sections through the lateroposterior nucleus at the level of Fig. 7. Top row (A,D): calretinin-stained sections. Middle row (B,E): calbindin D28k-stained sections. Bottom row (C,F): parvalbumin-stained sections. A column of three photographs (A–C, D–F) are of closely matched sections. Bar = 200  $\mu$ m (A–F).

PVNA was found surrounded by the reunions nucleus (Re). In the dorsally placed PVNA, a larger number of calretinin-stained cells were found in its lateral and ventral regions than in its central region (Figs. 1A,D; 4A; 5A; 11A). The ventrally placed PVNA included a few calretinin-stained cells (Figs. 1A; 4A) and a few calbindin-D28k-stained cells (Figs. 1B; 4B). The paratenial nucleus (PT) contained predominantly calbindin-D28k-stained cells (Figs. 1B,E; 4B; 5B; 11B), and the dorsal part of the PT at its rostral level also revealed calretinin-stained cells (Figs. 1A; 4A). The interanteromedial nucleus (IAM) contained exclusively calbindin-labeled cells (Figs. 1E; 5B; 12B). The intermediodorsal nucleus (IMD) showed both calretinin-stained cells (Figs. 2A,D; 3A; 6A; 7A; 8A) and calbindin-D28k-labeled cells (Figs. 2B,E; 3B; 6B; 7B; 8B). In the reunions nucleus (Re), calretinin-stained cells (Figs. 1A,D; 2A,D; 4A; 5A; 6A; 7A; 12D) and calbindin-D28k-labeled cells (Figs. 1B,E; 2B,E; 4B; 5B; 6B; 7B; 12E) were found with a similar distribution pattern. The rhomboid nucleus

(Rh) contained more calretinin-stained cells (Figs. 1D; 2A,D; 5A; 6A; 7A; 12A) than calbindin-D28k-labeled cells (Figs. 1E; 2B,E; 5B; 6B; 7B; 12B). The xiphoid nucleus (Xi) was almost devoid of cells stained for any of the calcium-binding proteins (Figs. 1D–F; 2A–C; 5A–C; 6A–C; 12D–F).

**Fibers.** The ventrally placed PVNA and the Xi were demarcated by the paucity of immunostained fibers for calretinin (Figs. 1A,D; 2A; 4a; 5a; 6a; 12D) and calbindin-D28k (Figs. 1B,E; 2B; 4b; 5b; 6b; 12E).

#### Habenular Complex

**Cell bodies.** The medial habenular nucleus (MHB) contained both calretinin-labeled cells (Figs. 2A,D; 3A; 6A; 7A; 8A; 14A,D) and calbindin-D28k-stained cells (Figs. 2B,E; 3B; 6B; 7B; 8B; 14B,E) in its lateral part. The lateral habenular nucleus (Lhb) included calretinin-labeled cells (Figs. 2A,D; 3A; 6A; 7A;

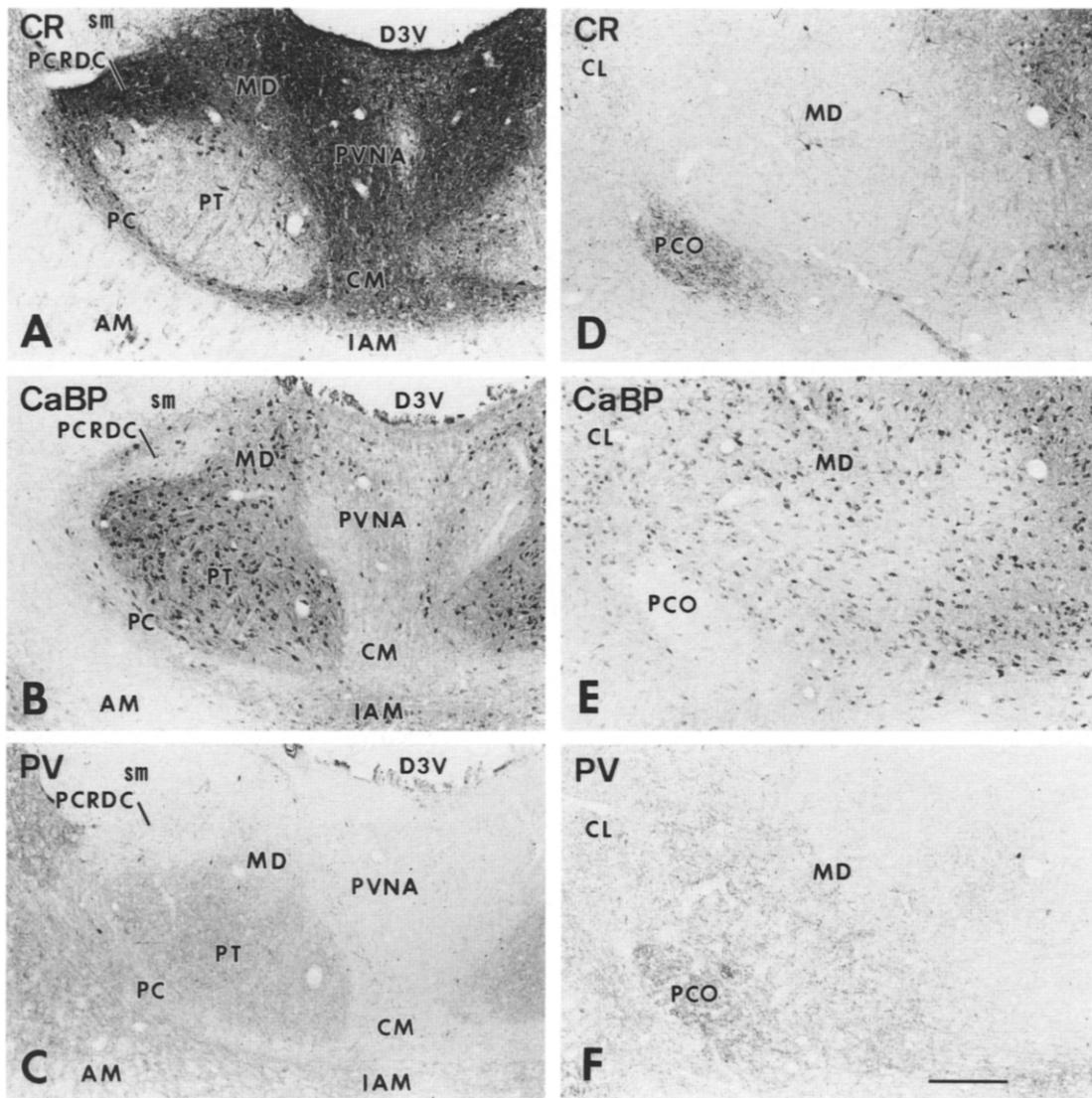


FIG. 11. Calretinin (CR), calbindin-D28k (CaBP), and parvalbumin (PV). (A–C) Frontal sections through the anterior part of the paraventricular nucleus at the caudal one-third level between Figs. 4 and 5. (D–F) Frontal sections through the ovoid subdivision of the paracentral nucleus at the level just rostral to Fig. 8. Top row (A,D): calretinin-stained sections. Middle row (B,E): calbindin D28k-stained sections. Bottom row (C,F): parvalbumin-stained sections. A column of three photographs (A–C, D–F) are of closely matched sections. Bar = 200  $\mu$ m (A–F).

8A; 14A) and calbindin-D28k-stained cells (Figs. 2B,E; 3B; 6B; 7B; 8B; 14B) predominantly in its medial part.

**Fibers.** The medial part of the MHb showed neuropil more intensely stained for calretinin (Figs. 2A,D; 3A; 6a; 7a; 8a; 14A) than for calbindin-D28k (Figs. 2B,E; 3B; 6b; 7b; 8b; 14B) and parvalbumin (Figs. 2C,F; 3C; 6c; 7c; 8c; 14C). In the Lhb, calbindin-D28k-stained fiber bundles extended in a direction from dorsomedial to ventrolateral, and entered the fasciculus retroflexus (fr) (Figs. 2E; 3B; 7b; 8b; 14B).

#### Reticular Nucleus

**Cell bodies.** The reticular nucleus (Rt) was the only region of the thalamus which showed predominantly parvalbumin-stained cells. Parvalbumin-stained cells were found almost entirely in the Rt (Figs. 1C,F; 2C,F; 3C; 4C; 5C; 6C; 7C; 8C). Calretinin-stained cells were found in the medial part of the Rt at its rostral level

(Figs. 1A; 4A) and in the dorsal part at its rostral and intermediate levels (Figs. 1A,D; 2A; 4A; 5A; 6A). A differential distribution pattern was found in the ventromedial corner of the Rt at its rostral levels (Figs. 1; 4; 5). The dorsal portion of the ventromedial corner of the Rt contained both parvalbumin-stained cells (Figs. 1C,F; 4C; 5C; 13F) and calbindin-D28k-labeled cells (Figs. 1B,E; 4B; 5B; 13E), and was devoid of calretinin-stained cells (Figs. 1A,D; 4A; 5A; 13D). In the ventral portion of the ventromedial corner of the Rt, calretinin-labeled cells (Figs. 1C,F; 4C; 5C; 13D) and parvalbumin-stained cells (Figs. 1A,D; 4A; 5A; 13F) were found dispersed. At the level just rostral to Fig. 4, the ventromedial corner of the Rt entirely contained both calretinin-stained cells (Fig. 13A) and parvalbumin-labeled cells (Fig. 13C). Overlap between the distribution of calretinin-stained cells and that of parvalbumin-stained cells was also found in the medial (Figs. 1A,C; 4A, C) and dorsal (Figs. 1A,C,D,F; 2A,C; 4A,C; 5A,C; 6A,C; 9A,C) parts of the Rt. The central part of the

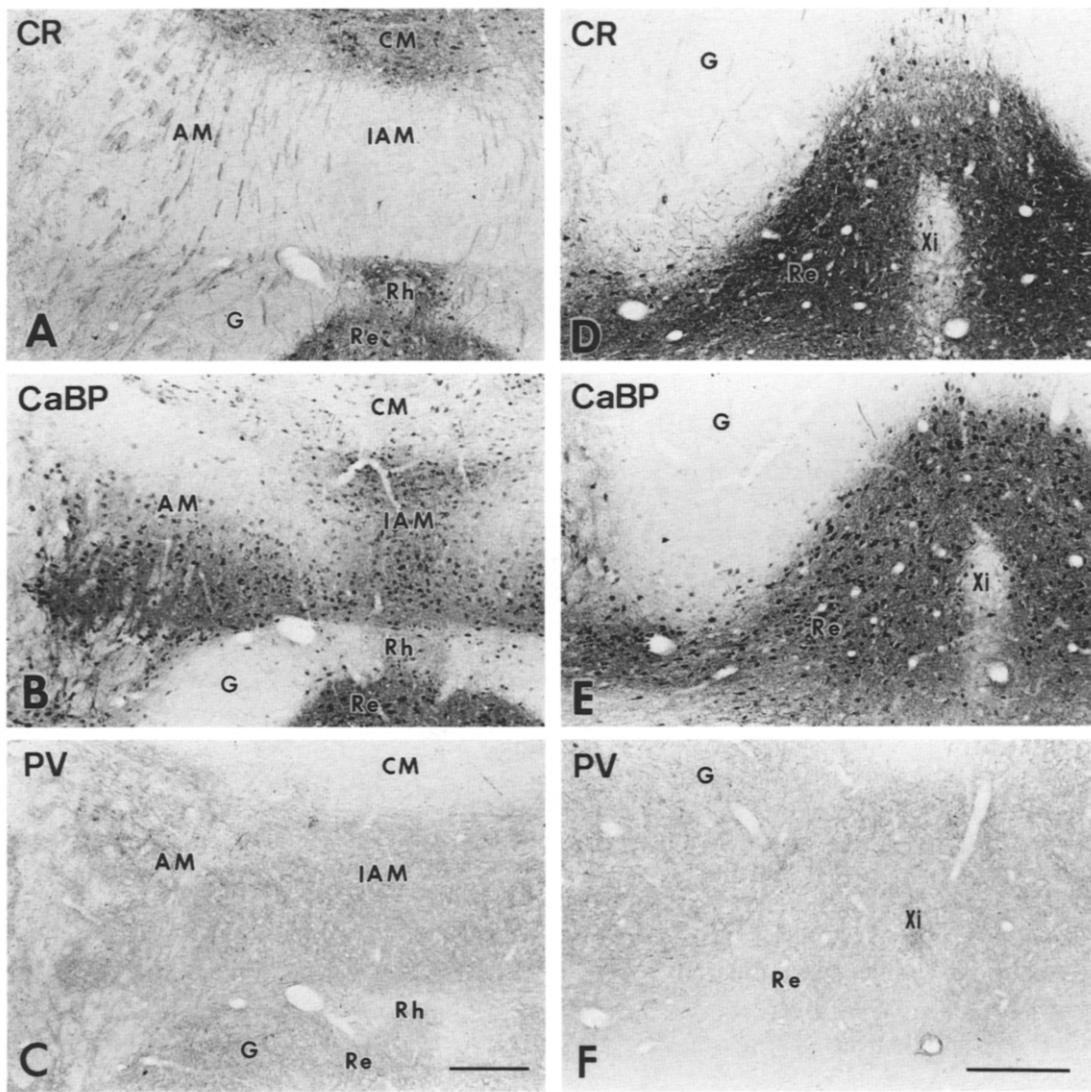


FIG. 12. Calretinin (CR), calbindin-D28k (CaBP), and parvalbumin (PV). (A–C) Frontal sections through the interanteromedial nucleus at the level halfway between Figs. 5 and 6. (D–F) Frontal sections through the reunions nucleus at the level of Fig. 6. Top row (A,D): calretinin-stained sections. Middle row (B,E): calbindin D28k-stained sections. Bottom row (C,F): parvalbumin-stained sections. A column of three photographs (A–C, D–F) are of closely matched sections. Bars = 200  $\mu\text{m}$  (A–C, D–F).

Rt displayed only parvalbumin-stained cells (Figs. 1C,F; 2C,F; 3C; 4C; 5C; 6C; 7C; 8C; 9C).

**Fibers.** Neuropil of the Rt showed preferentially parvalbumin (Figs. 1C,F; 2C,F; 3C; 4c; 5c; 6c; 7c; 8c).

#### Surrounding Regions

**Cell bodies.** The ventral division (ZIV) of the zona incerta expressed only parvalbumin-stained cells in its dorsal portion (Figs. 3C; 8C; 15C). The dorsal division (ZID) of the zona incerta showed dispersed calretinin-stained cells (Figs. 3A; 8A; 15A). The supracapsular division (BSTS) of the bed nucleus of the stria terminalis contained only calretinin-stained cells (Figs. 1A; 4A; 15D).

**Fibers.** Neuropil of the ZIV and of the BSTS preferentially showed immunoreactivity to parvalbumin (Figs. 3C; 8C; 15C) and calretinin (Figs. 1A; 4A; 15A), respectively.

#### DISCUSSION

In this study, we showed that the regions of the rat thalamus were divided into three categories: a) some regions revealed almost exclusively cells containing one of the calcium-binding proteins, b) other regions expressed overlap between the distributions of two cell components composed of different calcium-binding proteins, and c) other regions showed no cells stained for any of the calcium-binding proteins.

#### Methodological Considerations

The specificity of the antibodies against the three calcium-binding proteins has been previously described (see the Method section). Differential distribution patterns of the three calcium-binding proteins demonstrated in the present study, furthermore,

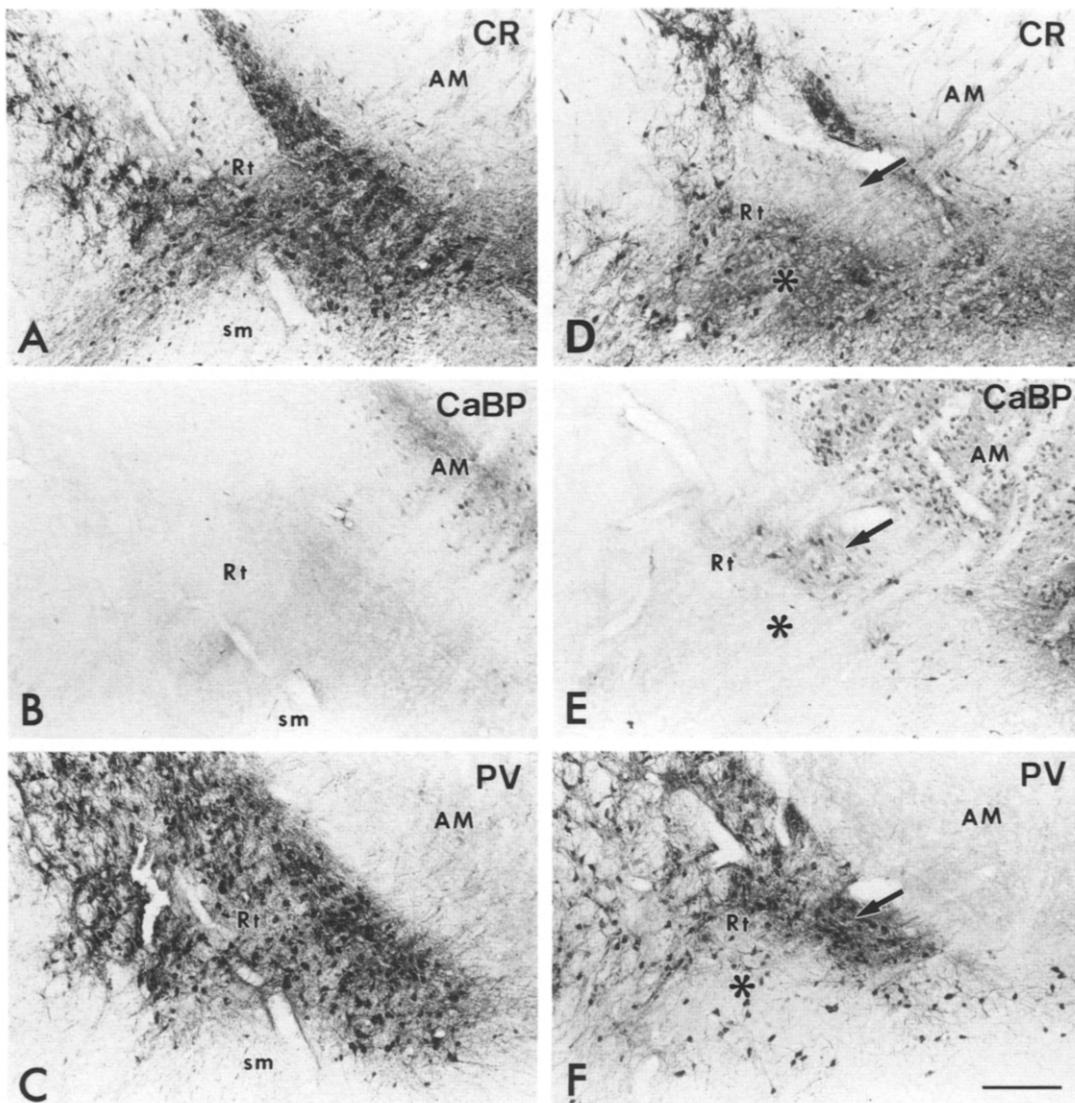


FIG. 13. Calretinin (CR), calbindin-D28k (CaBP), and parvalbumin (PV) in frontal sections through the ventromedial corner of the reticular nucleus. (A-C) Level just rostral to Fig. 4. (D-F) Level just caudal to Fig. 4. Note the dorsal portion (arrows) and ventral portion (asterisks) of the ventromedial corner of the reticular nucleus. Top row (A,D): calretinin-stained sections. Middle row (B,E): calbindin D28k-stained sections. Bottom row (C,F): parvalbumin-stained sections. A column of three photographs (A-C, D-F) are of closely matched sections. Bar = 200  $\mu$ m (A-F).

give evidence that the antibody against any of the proteins does not crossreact with the other proteins: for example, a) the anticalretinin antibody did not crossreact with cells of the dorsal portion of the ventromedial corner of the reticular nucleus, although these cells were immunostained by the anticalbindin-D28k and antiparvalbumin antibodies, b) the anticalbindin-D28k antibody did not show crossreaction with cells of the medial and dorsal parts of the reticular nucleus, whereas these cells were labeled by the anticalretinin and antiparvalbumin antibodies, c) the antiparvalbumin antibody did not immunostain cells of the reunions nucleus, but these cells were revealed by the anticalretinin and anticalbindin-D28k antibodies.

#### *Relationship With Laminar Organization of Thalamocortical Projections*

The thalamic nuclei have been classified into four types according to their major cortical layers of projections (21,22). The

first type nuclei, which include the specific sensory relay nuclei, predominantly project to the middle cortical layers (layers III and IV). The second type nuclei mainly project to the deep cortical layers (layers V and VI). The third type nuclei provide termination confined to a superficial cortical layer (layer I). The fourth type is typified by their terminations both in layer I and in additional (middle and/or deep) layers. The second, third, and fourth types include nonspecific nuclei. There is striking correspondence between this thalamic classification and the distribution pattern of thalamic cells immunoreactive to the calcium-binding proteins, as follows.

The second (central medial, centrolateral, paracentral, and paraventricular nuclei) and third (ventromedial nucleus) type nuclei contained many calretinin- or calbindin-D28k-immunoreactive cells. By contrast, the first type nuclei (ventral posterolateral, ventral posteromedial, mediodorsal, and gelatinosus nuclei) included very few, if any, cells immunostained for any of the cal-

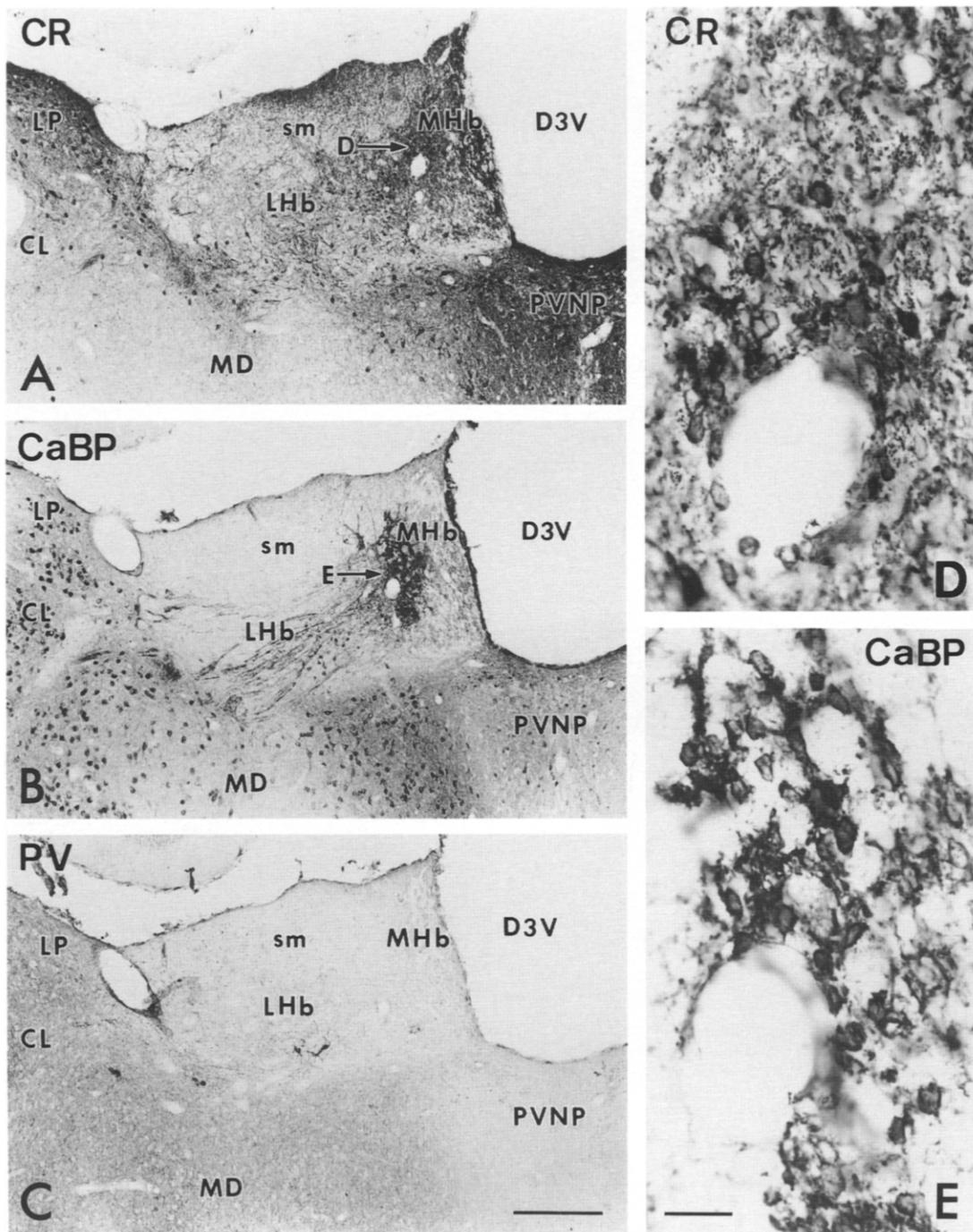


FIG. 14. Calretinin (CR), calbindin-D28k (CaBP), and parvalbumin (PV) in frontal sections through the medial and lateral habenular nuclei at the level of Fig. 7. (A,D) Calretinin-stained sections; (B,E) calbindin D28k-stained sections; (C) parvalbumin-stained sections. (A–C) Closely matched sections; (D) higher power of the lateral part (arrow D in A) of the medial habenular nucleus; (E) higher power of the lateral part (arrow E in B) of the medial habenular nucleus. Bars = 200  $\mu\text{m}$  (A–C), 20  $\mu\text{m}$  (D,E).

cium-binding proteins. Of the fourth type nuclei, some nuclei (lateroposterior, posterior, and reunions nuclei) contained many cells stained for calretinin or calbindin-D28k, and other nuclei (anterodorsal, anteroventral, and ventrolateral nuclei) were almost devoid of the immunostained cells.

Deep layer projections (second type) could profoundly alter excitation of pyramidal neurons, by their terminations on

the proximal dendrites of the pyramidal neurons (22). Superficial layer projections (third type) might affect pyramidal cells in a subtle and universal way, by their contacting the distal ends of apical dendrites of the pyramidal cells (21). On the other hand, middle layer projection (first type) could indirectly lead to thalamic excitation of the pyramidal cells, because their terminals synapse with local-circuit neu-

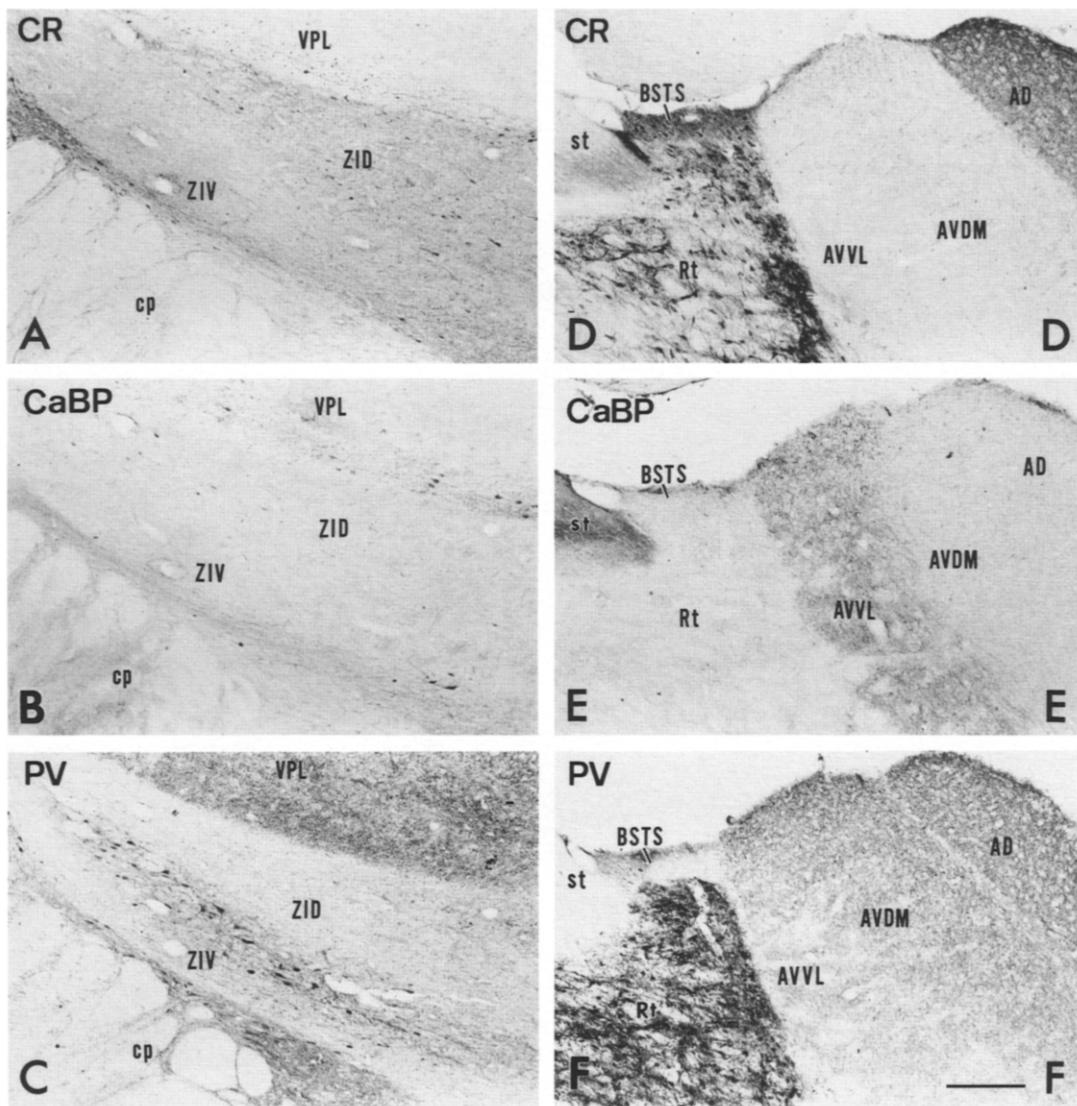


FIG. 15. Calretinin (CR), calbindin-D28k (CaBP), and parvalbumin (PV). (A-C) Frontal sections through the zona incerta at the level of Fig. 8. (D-F) Frontal sections through the supracapsular division of the bed nucleus of the stria terminalis at the level just caudal to Fig. 4. Top row (A,D): calretinin-stained sections. Middle row (B,E): calbindin D28k-stained sections. Bottom row (C,F): parvalbumin-stained sections. A column of three photographs (A-C, D-F) are of closely matched sections. Bar = 200  $\mu$ m (A-F).

rons in the cortical layer IV which in turn synapse with pyramidal cells (38).

It can be assumed, therefore, that calretinin and calbindin-D28k could influence thalamocortical neurons projecting to the deep and superficial cortical layers that could directly modulate pyramidal cell activity. A question of interest is whether calretinin and calbindin-D28k may be contained in the thalamocortical neurons that project to the deep and superficial layers, or in local neurons that contact the thalamocortical neurons.

#### *New Thalamic Regions Revealed by Localization of Calcium-Binding Proteins*

The rostral dorsal cap of the paracentral nucleus (PCRDC) and the ovoid subdivision of the paracentral nucleus (PCO) are outlined in the atlas of Paxinos and Watson (36), but their chemical features have never been described. In the present study, these

regions were clearly demarcated by localization of the calcium-binding proteins. Previous studies have shown that the paracentral nucleus receives afferents from various regions including the prefrontal cortex, cerebellar nuclei, and spinal cord, and mainly projects to the prefrontal cortex and striatum [for references see (7) and (30)]. Anterograde and retrograde tracing will be needed to examine whether the PCRDC and PCO have afferent and efferent connections with more restricted brain regions.

Subdivisions of the reticular nucleus was represented here by the differential distribution of cells immunoreactive to the calcium-binding proteins. It is believed that the reticular nucleus may play a role in gating the relay of information through the thalamus to the cerebral cortex (24). Recent studies have shown that the reticular nucleus is not a uniform structure. For example, in the rat reticular nucleus, several cytoarchitectonic subdivisions are described (49). Developmental expression of parvalbumin and pro- $\alpha$ -thyrotropin-releasing hormone does occur in cells of

different regions of the reticular nucleus at different stages, and these regions appear to correspond to the different sectors of the nucleus which connect with different cortical and thalamic areas (33). It is suggested, therefore, that the reticular nucleus may have more localized and specific functions (32). The present finding further provides evidence for heterogeneity of the reticular nucleus of the rat. It will be necessary to examine fiber connections of the subdivisions of the reticular nucleus revealed by the present study.

#### *Functional Considerations*

Our understanding of the possible functions of the three calcium-binding proteins is still limited; therefore, the physiological role of the proteins in neurons remains speculative. By virtue of their  $\text{Ca}^{2+}$ -buffering capacities, these calcium-binding proteins potentially have a number of different effects on the neurons (4). These effects could include: altering the duration of action potentials; promoting neuronal bursting activity; allowing for a greater contribution of  $\text{Ca}^{2+}$  entry to the overall membrane depolarization; protecting cells against the damaging effects of excessive calcium influx during prolonged periods of high activity (4). A strong correlation has been demonstrated between the occurrence of parvalbumin and the fast firing properties of the hippocampal interneurons (27). Dentate granule cells, depleted of their calbindin-D28k content, had a lower whole-cell  $\text{Ca}^{2+}$  current than cells containing this protein, suggesting that loss of calbindin-D28k from a neuron may reduce  $\text{Ca}^{2+}$  entry and contribute to making the neuron less excitable (28).

Complementarity was found between the distribution of calretinin-containing cells and that of calbindin-D28k-stained cells in both the intralaminar nuclear group and midline nuclear group. Taking into account high homology of amino acid sequence between calretinin and calbindin-D28k (35,50), this complementarity provides a clue for exploring the functions of these proteins in neurons, such as an indication that the two calcium-binding proteins may work synergistically in the regions where the complementarity is found.

Absence of cells stained for any of the three calcium-binding proteins of the EF-hand family was found in some regions of the anterior and ventral nuclear groups and of the mediodorsal nucleus. Most pyramidal cells of the cerebral cortex and hippocampus do not contain calretinin, calbindin-D28k, or parvalbumin (10,19,23,43,48,51), and it is suggested that calmodulin, another member of the EF-hand family of calcium-binding proteins, may be present in these neurons (4). Whether calmodulin is found in cells of the thalamic regions that are free of immunostained cells for calretinin, calbindin-D28k, and parvalbumin, and whether complementarity exists between the distributions of calretinin-, calbindin-D28k-, parvalbumin-, and calmodulin-containing cells in the thalamic regions, will be the objects of future studies.

In conclusion, the present study presents a comparative distribution of the three calcium-binding proteins, calretinin, calbindin-D28k, and parvalbumin in the rat thalamus. These proteins serve as useful neuroanatomical markers that focus on regions that might be useful starting points for further exploration of the calcium-binding proteins.

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