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Expression of dihydropyridine-sensitive brain calcium channels in the rat central nervous system

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We have localized dihydropyridine (DHP)-sensitive calcium channels in rat brain by in situ hybridization and immunohistochemistry. The mRNA for the dihydropyridine-sensitive calcium channel α1 subunit (DHPR-B) is prominently localized in neuronal cells in the olfactory bulb, dentate gyrus, hippocampus, arcuate nucleus, paraventricular nucleus, ventromedial nucleus, cerebral cortex, superior colliculus and the cerebellar Purkinje cell layer. Strong expression of DHPR-B mRNA was also found in the pituitary and pineal glands. DHP-sensitive calcium channel α1 subunit distribution has also been examined immunohistochemically with polyclonal antibodies raised against synthetic peptides specific for the DHPR-B α1 subunit protein. The results from immunohistochemistry were in good agreement with those from in situ hybridization. Thus, regional distribution and localization of DHPR-B mRNA and α1 subunit protein in rat brain suggest that this type of DHP-sensitive brain calcium channel may play an important role in excitation–secretion coupling functions in the neuroendocrine system.

Ca^{2+}-channel; in situ hybridization; Immunohistochemistry; Brain

1. INTRODUCTION

Voltage-sensitive calcium channels present in vertebrate cells play important roles in excitation–contraction coupling mechanisms and signal transduction. These channels are classified into T, N, L and P-types, based on their electrophysiologic and pharmacologic properties [1,2]. The slowly inactivating, dihydropyridine (DHP)-sensitive, L-type calcium channels from skeletal muscle and heart have been well characterized with respect to their biochemical and molecular characteristics. They are membrane-spanning proteins consisting of several transmembrane subunits (reviewed in [3,4]). The α1 subunit which binds organic Ca^{2+} channel blockers [5–7] can function as a calcium channel, since the α1 subunit cDNA isolated from skeletal and cardiac muscle can direct expression of functional Ca^{2+} channels [8,9]. Recently, several laboratories have isolated cDNA clones corresponding to multiple forms of the α1 subunit of the voltage-sensitive calcium channel from brain [10–12]. Analysis of these brain cDNAs further revealed that the brain α1 subunit isoforms with sequence similarity to the skeletal muscle Ca^{2+} channel, which are produced by a family of related genes [13], encode the DHP-sensitive Ca^{2+} channels [14]. We and others have isolated a novel brain isoform of the DHP-sensitive Ca^{2+} channel α1 subunit cDNA (DHPR-B in [15]; RBα1 in [16]) whose primary structure is significantly different from its skeletal and cardiac muscle counterparts. Here, we describe the distribution in rat brain of the mRNA encoding this form of the DHP-sensitive Ca^{2+} channel α1 subunit by in situ hybridization, and that of the α1 subunit protein by immunohistochemistry.

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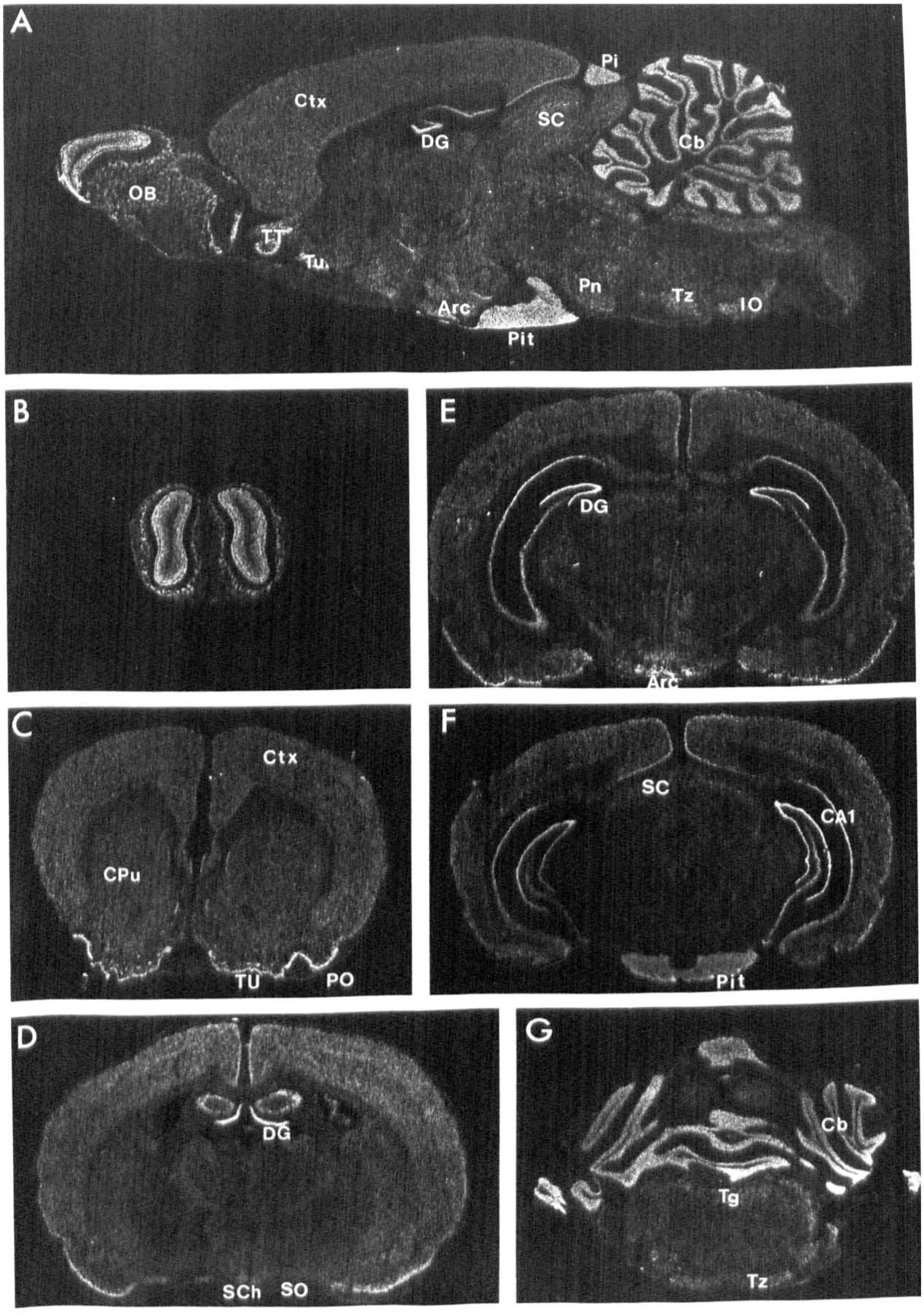
2. MATERIALS AND METHODS

2.1. *cRNA probe preparation and in situ hybridization*

The hybridization probe was prepared from the pGEM plasmid (p60Z) containing a 443 bp insert (residue number 2803–3246 as in [16]) from DHPR-B cDNA, which encodes the cytoplasmic loop between the repeating unit II S6 and III S1. Antisense and sense riboprobes were prepared from the p60Z plasmid, which had been linearized with appropriate restriction enzymes, by transcribing with appropriate RNA polymerases using a Riboprobe System (Promega Corp., Madison, WI) in the presence of α-^{35}S-UTP (1000–1500 Ci/mmol, New England Nuclear).

In situ hybridization was performed essentially as previously described [17]. Briefly, frozen rat brain sections (12 μm thick) were cut, thaw-mounted on gelatin-coated slides, fixed and dehydrated immediately before hybridization. Brain sections were incubated overnight at 54°C in hybridization buffer containing 50% formamide, 10% dextran sulfate, 0.7% Ficoll, 0.7% polyvinyl pyrrolidone, 0.7% BAS, 0.15 mg/ml yeast tRNA, 0.33 mg/ml denatured salmon sperm DNA, and 20 μM dithiothreitol, and the ^{35}S-labeled RNA probe at a density of 1×10^6 cpm per 75 μl of hybridization solution, washed in 2× SSC, treated with RNAse A (20 μg/ml, Boehringer-Mannheim) for 30 min at 25°C, and washed sequentially for 60 min in 2× SSC at 50°C, 60 min in 0.2× SSC at 55°C, and 60 min in 0.2× SSC at 60°C. After drying, the slides were processed for both film (β-max Hyperfilm, Amersham) and emulsion (Kodak NTB2) autoradiography using exposure times of 3 and 8 days, respectively. Following development of emulsion autoradio-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graphy, tissue sections were counterstained with Cresyl violet and coverslipped with Permount.

2.2. *Immunohistochemical staining*

Two oligopeptides from the loop between I S6 and II S1, peptide 197 (DLKGYLDWITQAEDI) and peptide 199 (VKARPRDA-VEVSGAGG), corresponding to residues 366–380 and 413–427 in [15], respectively, were synthesized with a peptide synthesizer (Applied Biosystems Model 430A). The peptides, coupled to keyhole limpet hemocyanin (KLH) [18], were used for immunizing New Zealand white rabbits, and antisera were prepared as described [19].

Immunohistochemical localization of the Ca^{2+} channel α1 subunit in rat brain with HC-2 (immunized with peptide 197-KLH conjugate) and HC-4 antisera (immunized with peptide 199-KLH conjugate) was performed as follows. Under Nembutal (pentobarbital) anesthesia, male Sprague–Dawley rats (200–250 g body weight) were perfused transcardially with 0.9% saline followed by fixative containing 2% paraformaldehyde, 0.075 M lysine, 0.01 M sodium periodate in 0.05 M sodium phosphate buffer (pH 7.4) at room temperature. Following perfusion, the brains were removed and postfixed overnight at 4°C in the same fixative without sodium *m*-periodate. After postfixation, the brains were cut with a vibratome in the frontal plane at 50 μm and washed thoroughly in 0.1 M phosphate buffer. They were then incubated in antisera (1:300 dilution in 0.1 M PBS containing 2% goat serum and 0.5% Triton X-100) at 4°C for 24 h. After washing, they were treated for 2 h with biotin-labeled goat anti-rabbit IgG (BRL, 1:400 dilution in PBS containing 2% goat serum and 0.5% Triton X-100) followed by washing and incubation with streptavidin–horseradish peroxidase (HRP) conjugate (BRL, 1:400 dilution in PBS) for 1 h. After washing, horseradish peroxidase reaction product was developed with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and 0.002% H_2O_2. Sections were then dehydrated, cleared, and mounted.

3. RESULTS

3.1. *Localization of DHPR-B mRNA in rat brain*

The distribution of Ca^{2+} channel α1 subunit mRNA in rat brain was examined by in situ hybridization using radiolabeled riboprobes prepared from DHPR-B cDNA. To establish the specificity of the labeled probe, we conducted the following control experiments. First, there was negligible binding with a labeled sense-strand of p60Z probe. Second, addition of RNAse in the hybridization solution abolished labeling. Third and most important, the distribution pattern of DHPR-B mRNA, identical to that shown in Figs. 1 and 2, was observed with the second probe, p16D-2, which contains the 5′ untranslated sequence of DHPR-B cDNA (data not shown).

The results shown in Fig. 1A with a rat brain sagittal section indicate that the DHPR-B Ca^{2+} channel α1 subunit mRNA was widely expressed throughout the brain. Macroscopic transcript distribution in adult rat brain is shown in Fig. 1 and microscopic distribution is shown in Fig. 2. Specific labeling was detected in neurons but not in glial cells. High levels of DHPR-B mRNA were found in the mitral cell layer and internal granule cell layer of the olfactory bulb (Figs. 1B and 2A), anterior olfactory nucleus, olfactory tubercle (Fig. 1C), and piriform cortex (Fig. 1C,D,E), areas all related to olfactory function. DHPR-B mRNA was also very abundant in the dentate gyrus (Figs. 1D,E and 2B) and moderately abundant in the CA1 region of hippocampus (Fig. 1F). Relatively high levels of expression were observed in the cerebral cortex as well (Figs. 1 and 2C). Low levels of DHPR-B mRNA were found in the caudate and thalamus. Interestingly, the suprachiasmatic nucleus (Fig. 1D) and the pineal gland (Fig. 1A), both of which are important in the control of circadian rhythms [20], contain relatively high levels of DHPR-B transcript. The arcuate nucleus (Figs. 1A,D and 2D), and supraoptic nucleus (Fig. 1D), and the anterior and intermediate lobes of the pituitary gland (Figs. 1A,F and 2E) had high levels of DHPR-B mRNA. Moderate densities were apparent in the intermediate gray layer of the superior colliculus (Fig. 1F). The Purkinje cell layer of the cerebellum (Figs. 1A,G and 2F) also displayed abundant labeling, whereas the granule cell layer was less densely labeled.

Fig. 1. Localization of the DHPR-B mRNA in adult rat brain by in situ hybridization. Negative film image of in situ hybridization of sagittal (A) and coronal sections (B–G). Arc, arcuate nucleus; CA1, CA1 of the hippocampus; Cb, cerebellar cortex; CPu, caudate-putamen; Ctx, cerebral cortex; DG, dentate gyrus; IO, inferior olive; OB, olfactory bulb; Pi, pineal gland; Pit, pituitary gland; Pn, pontine nucleus; PO, piriform cortex; SC, superior colliculus; SCh, suprachiasmatic nucleus; SO, supraoptic nucleus; TT, tinea tecta; Tu, olfactory tubercle; Tz, trapezoid nucleus.

3.2. *Localization Ca^{2+} channel α1 subunit proteins encoded by DHPR-B mRNA in rat brain*

To demonstrate the specificity of immunohistochemical staining, several control experiments were performed in parallel. Either experimental antisera (HC-2 and HC-4) were replaced with the pre-immune sera or anti-KLH antibodies, or were omitted in the staining procedure. In addition, the secondary antibodies were also left out in the immunostaining procedure. Under these conditions, there was only negligible immunoreactivity (data not shown). Staining with HC-2 antisera that had been pre-absorbed with the peptide 197 for 12 h also yielded no specific immunoreactivity as shown in Fig. 3B. A similar result was obtained with HC-4 antiserum pre-incubated with the peptide 199 (not shown).

Strong immunoreactivity was present in the mitral cells of the olfactory bulb (Fig. 4A), olfactory tubercle (Fig. 4B), piriform cortex (Fig. 4B), and pyramidal cells of the cerebral cortex (Fig. 3A). Granule cells of the dentate gyrus and pyramidal cells of the CA3 region of the hippocampus were highly immunoreactive (Fig. 4E). The caudate and thalamus showed relatively low levels of immunoreactivity. The specific areas related to the neuroendocrine system including the suprachiasmatic nucleus (Fig. 4C), supraoptic nucleus (Fig. 4C), arcuate nucleus (Fig. 4D), pituitary and pineal glands, showed relatively high immunoreactivities with HC-2. Cerebellar Purkinje cells (Fig. 4F) were labeled intensely, while the granule cell layer was not. Overall, the results from the immunohistochemical localization of

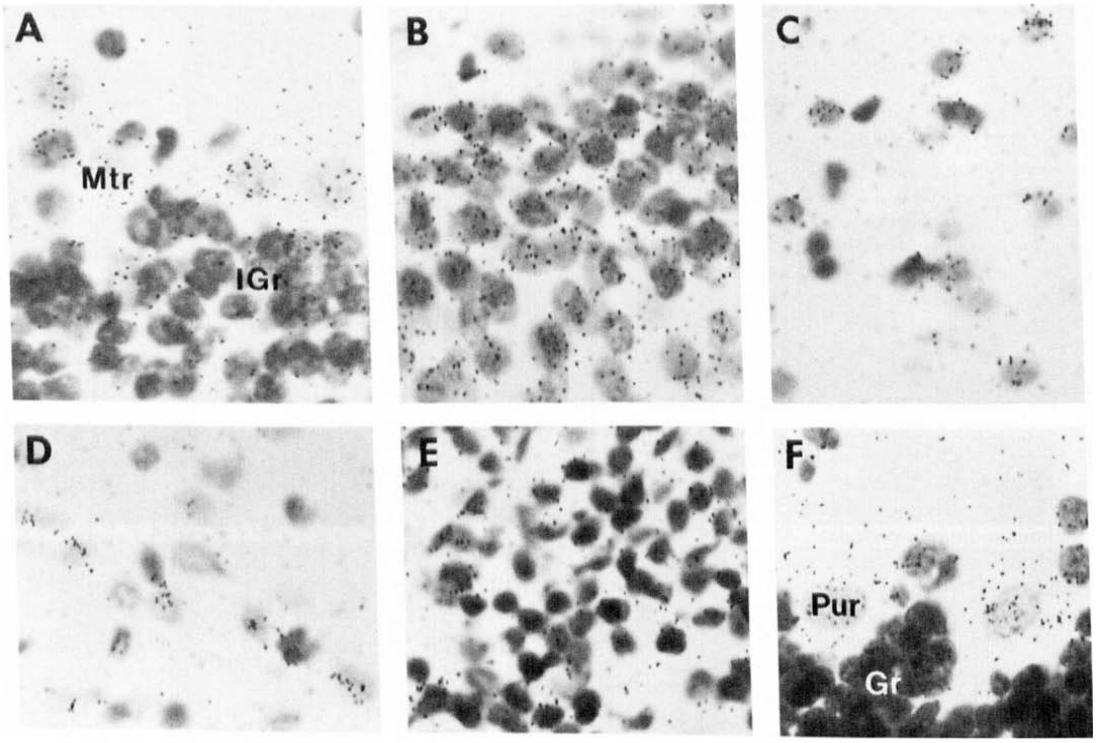

Fig. 2. Localization of the DHPR-B mRNA in rat brain by in situ hybridization. Bright-field photomicrographs of emulsion-dipped sections, showing the olfactory bulb (A), dentate gyrus (B), cerebral cortex (C), arcuate nucleus (D), pituitary gland (E), and cerebellar cortex (F). Mtr, mitral cell; IGr, internal granule cell; Pur, Purkinje cell; Gr, granular cell. Magnification, × 630.

DHPR-B α1 subunit protein were in good agreement with those from the mRNA localization by in situ hybridization.

4. DISCUSSION

Our present findings represent the first study to describe the regional distribution of DHP-sensitive Ca^{2+} channels using specific molecular probes for α1 subunit mRNA and protein in rat brain. The good agreement between our results from in situ hybridization histochemistry and immunohistochemistry, in which rigorous control experiments had been performed in parallel, clearly demonstrate the specificity of the cRNA probes and antibodies used here. The controls included for the in situ hybridization: (i) use of two different probes, (ii) use of labeled sense cRNA probe, and (iii) pretreatment with RNAse; and for the immunohistochemical staining: (i) use of two different antisera obtained from two different peptide antigens, (ii) staining with pre-immune serum, and (iii) blockade of specific immunostaining with the specific peptides.

We have shown that DHP-sensitive Ca^{2+} channels encoded by DHPR-B mRNA are predominantly localized in those brain areas related to neuroendocrine and olfactory functions. Previous investigations on the localization of DHP-sensitive Ca^{2+} channels in brain utilized receptor autoradiography or immunohistochemistry techniques. Visualization of [^{3}H]DHP binding sites [20] and immunostaining with monoclonal antibodies specific for the α2 subunit of DHP-sensitive L-type skeletal muscle Ca^{2+} channel [21] indicate a similar locali-

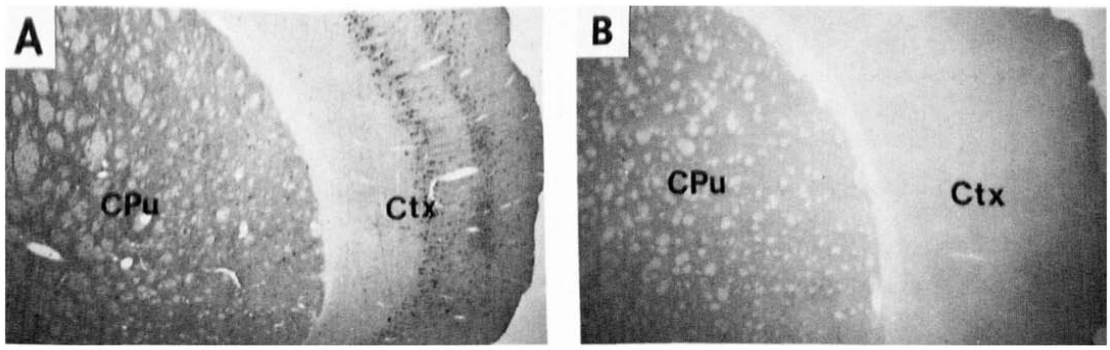

Fig. 3. Photomicrographs of a rat brain section showing dihydropyridine-sensitive Ca^{2+} channel α1 subunit immunoreactivity with HC-2 antiserum (A) and of an adjacent section with HC-2 antiserum pre-absorbed with the peptide 197 (B). Magnification, ×25.

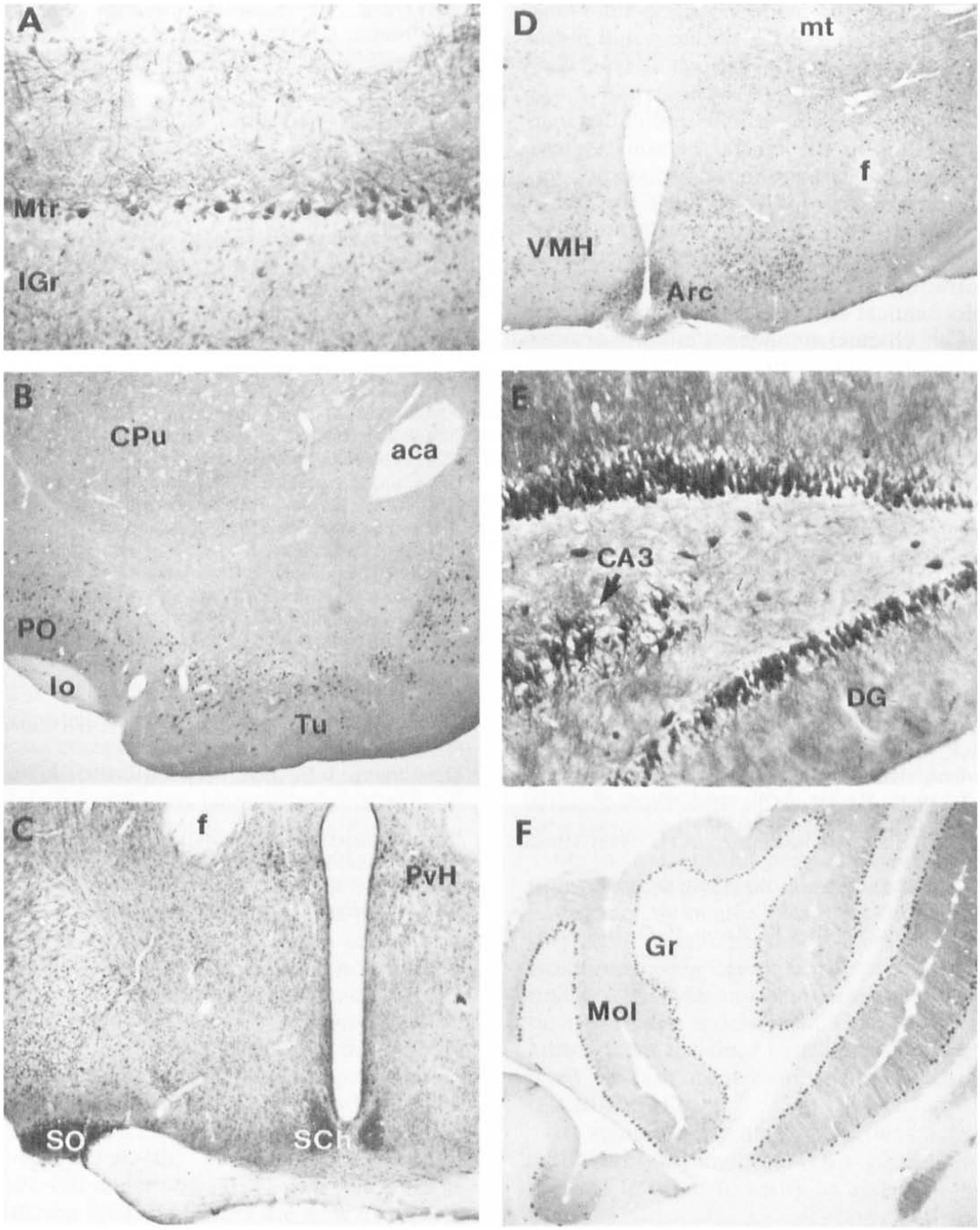

Fig. 4. Localization of the protein encoded by DHPR-B mRNA in the rat brain by immunohistochemical staining. Photomicrographs of rat brain sections showing dihydropyridine-sensitive Ca^{2+} channel $\alpha 1$ subunit immunoreactivity with HC-2 antiserum (A–F). aca, anterior commisure, anterior; Arc, arcuate nucleus; CA3, CA3 of hippocampus; CPu, caudate-putamen; Ctx, cerebral cortex; DG, dentate gyrus; f, fornix; Gr, granule cell layer of cerebellum; IGr, internal granule cell layer; lo, lateral olfactory tract; Mol, molecular layer of cerebellum; mt, mammilothalamic tract; Mtr, mitral cell layer; PO, piriform cortex; PvH, paraventricular nucleus of hypothalamus; SCh, suprachiasmatic nucleus; SO, supraoptic nucleus; Tu, olfactory tubercle; VMH, ventromedial hypothalamic nucleus. Magnification: × 100 (A and F), × 25 (B, C, D, E, G, H).

zation of Ca^{2+} channels in certain brain areas, e.g. hippocampus and cerebellum. However, our present findings indicate that DHP-sensitive Ca^{2+} channels are much more widely distributed in rat brain than previously reported. It is likely that not all Ca^{2+} channels present in brain were disclosed by previously used mapping methods.

The abundance of DHPR-B mRNA and protein in

brain areas responsible for neuroendocrine functions, such as the hypothalamus and the pituitary and pineal glands, suggests that the DHP-sensitive L-type Ca^{2+} channel encoded by DHPR-B mRNA may play a role in excitation–secretion coupling and in modulating neuroendocrine functions in the central nervous system. Generally, N-type Ca^{2+} channels in the presynaptic terminals of neurons are believed to mediate the rise in intracellular calcium that triggers neurotransmitter release, although L-type Ca^{2+} channels can also influence neurotransmitter release [22]. However, evidence from biochemical and electrophysiologic studies indicates that Ca^{2+} channel antagonists inhibit hormone secretion in a variety of endocrine cells, including pancreatic β-cells [23], pituitary cell lines [24] and adrenal cells [25] by blocking DHP-sensitive L-type Ca^{2+} channels. The abundance of DHPR-B mRNA in hypothalamic nuclei, including the suprachiasmatic nucleus and the pineal and pituitary glands, suggests that the L-type Ca^{2+} channel encoded by DHPR-B mRNA may regulate hormone secretion from neurosecretory cells.

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