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Localization of parvalbumin mRNA in rat brain by in situ hybridization histochemistry

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Summary. Parvalbumin mRNA was localized in rat brain by in situ hybridization using a ^{35}S labelled rat parvalbumin cDNA and a synthetic oligodeoxyribonucleotide (corresponding to base sequences 140 to 183 of rat parvalbumin cDNA). Strongest hybridization signals were detected in the Purkinje cells of the cerebellum and in neurones of the reticular nucleus of the thalamus. Signal was also detected in the cerebral cortex, hippocampus, basal ganglia and brain stem in agreement with the distribution of parvalbumin immunoreactivity.

Key words: Ca^{2+}-binding protein – Parvalbumin – Gene expression – In situ hybridization – Histochemistry – mRNA

Introduction

Disturbance of neuronal calcium homeostasis has been implicated in the impairment of brain function with ageing and in the development of the neuropathology of degenerative disease such as Alzheimer's disease (Arai et al. 1987; Crapper McLachlan et al. 1987; Gibson and Peterson 1987; Mayer and Westbrook 1987). The mechanisms underlying these disturbances are essentially unknown because of the complex compartmentation of calcium within the neuron. However, some of the control of calcium-mediated signaling is likely to involve calcium-binding proteins such as calmodulin, calbindin-28K and parvalbumin (Heizmann 1984; Heizmann and Berchtold 1987). The concentration of two of these proteins calmodulin and calbindin have been reported to be reduced in the brains of patients affected with Alzheimer's disease (ATD) (Crapper McLachlan et al. 1987). Further a selective loss of parvalbumin immunoreactive neurons was observed in the frontal and temporal cortices of patients dying with a diagnosis of ATD (Arai et al. 1987). All these various obervations suggest that impairment of neuronal calcium homeostasis may be implicated in the neuropathology of ATD. In this study we used immunohistochemical techniques together with "in situ" hybridization to localize immunoreactive parvalbumin (Heizmann and Celio 1987) and its corresponding parvalbumin mRNA (Berchtold 1987; Berchtold et al. 1988; Berchtold and Means 1988; Epstein et al. 1986) in the normal rat brain. Development of these techniques will enable further studies on parvalbumin gene expression in human neurological illness.

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Methods

In situ hybridization

Male rats (n = 20) 150–300 g in weight were decapitated, the brains rapidly removed and frozen on dry ice. Cryostat sections were thaw mounted onto gelatine/chrome alum coated slides and fixed with 4% buffered paraformaldehyde for 15 min at room temperature. The sections were washed with PBS (0.1 M phosphate buffered saline) for 10 min, dehydrated through 70%, 90%, and absolute ethanol (5 min per change), then air-dried and stored dessicated at –70° C.

For hybridization, these sections were treated sequentially with ethanol, xylene and ethanol again (5 min each) and then rehydrated through a descending alcohol series to sterile PBS. After incubation with prehybridization buffer [50% deionized formamide, 2xSSC (1xSSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.4), 0.2% bovine serum albumin, 0.2% polyvinyl pyrrolidone, 0.2% ficoll-400, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml tRNA (yeast) and 14.3 mM β-mercaptoethanol] for 60 min at 37° C. Sections were then incubated with heat-denatured labelled cDNA probe or labelled oligodeoxyribonucleotides in hybridization buffer. The hybridization buffer was identical to the prehybridization buffer except for the addition of dextran sulphate to a final concentration of 10%. This procedure for in situ hybridization is based on that of Shivers et al. (1987).

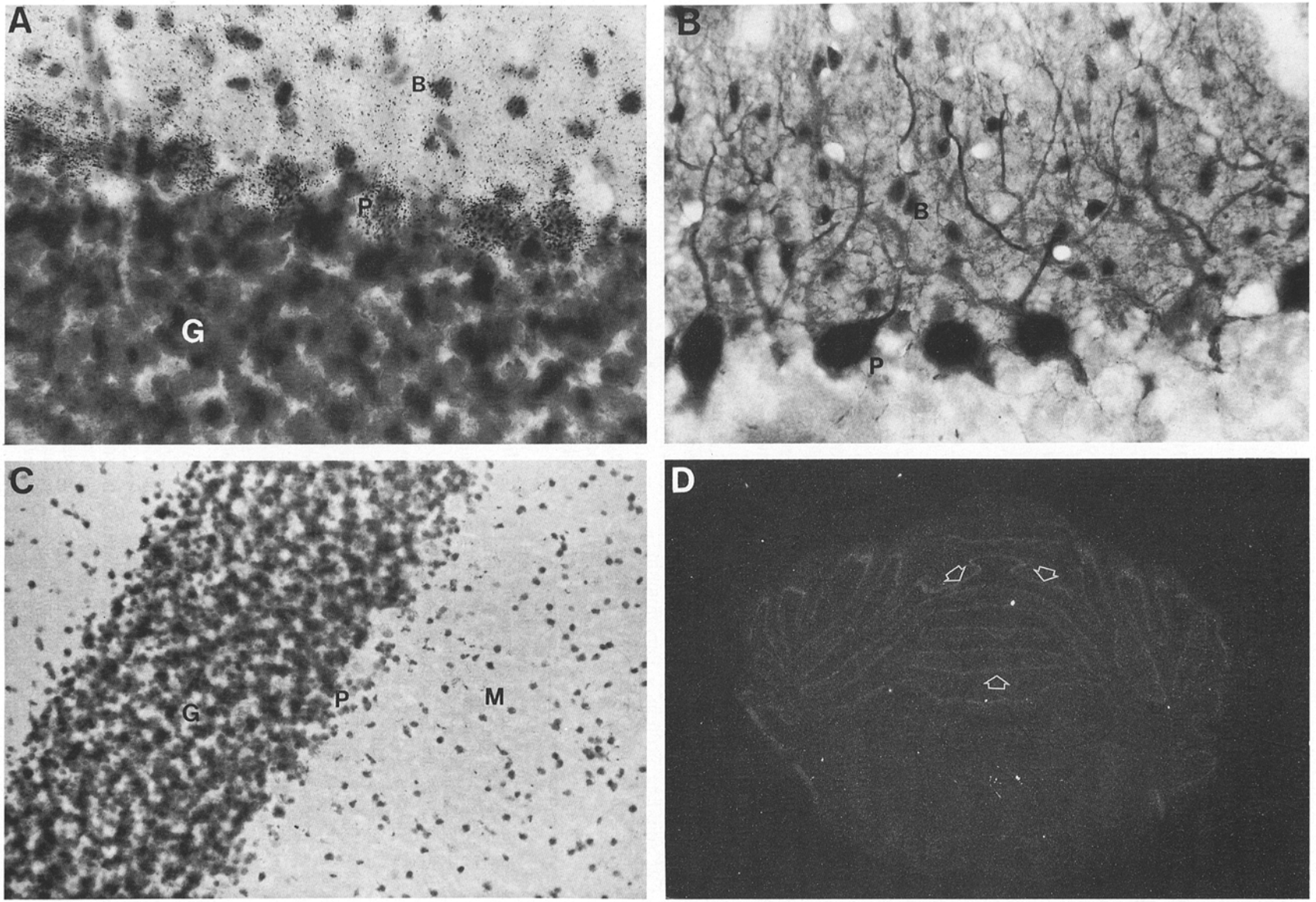

Fig. 1. A High power autoradiograph to illustrate the localisation of specific PV mRNA (×630). **C** Control section pretreated with RNase A to digest mRNA. Note the lack of (silver grains) visualized using the PV cDNA probe over the Purkinje cells (P) and (B) hybridization signal. G granule cell layer. P Purkinje cell layer ×200. **D** Low power dark Basket cells of the molecular layer cerebellum. Note the lack of signal over the granule cell field autoradiography film image showing the localization of ^{35}S-labelled anti sense layer (G). Magnification ×630; **B** localisation of parvalbumin-immunoreactivity in the oligonucleotide probe in the cerebellum. Silver grains are concentrated in the molecular cerebellum. Purkinje cells (P) basket cells and stellate cells are also positively stained layer (arrows) ×25

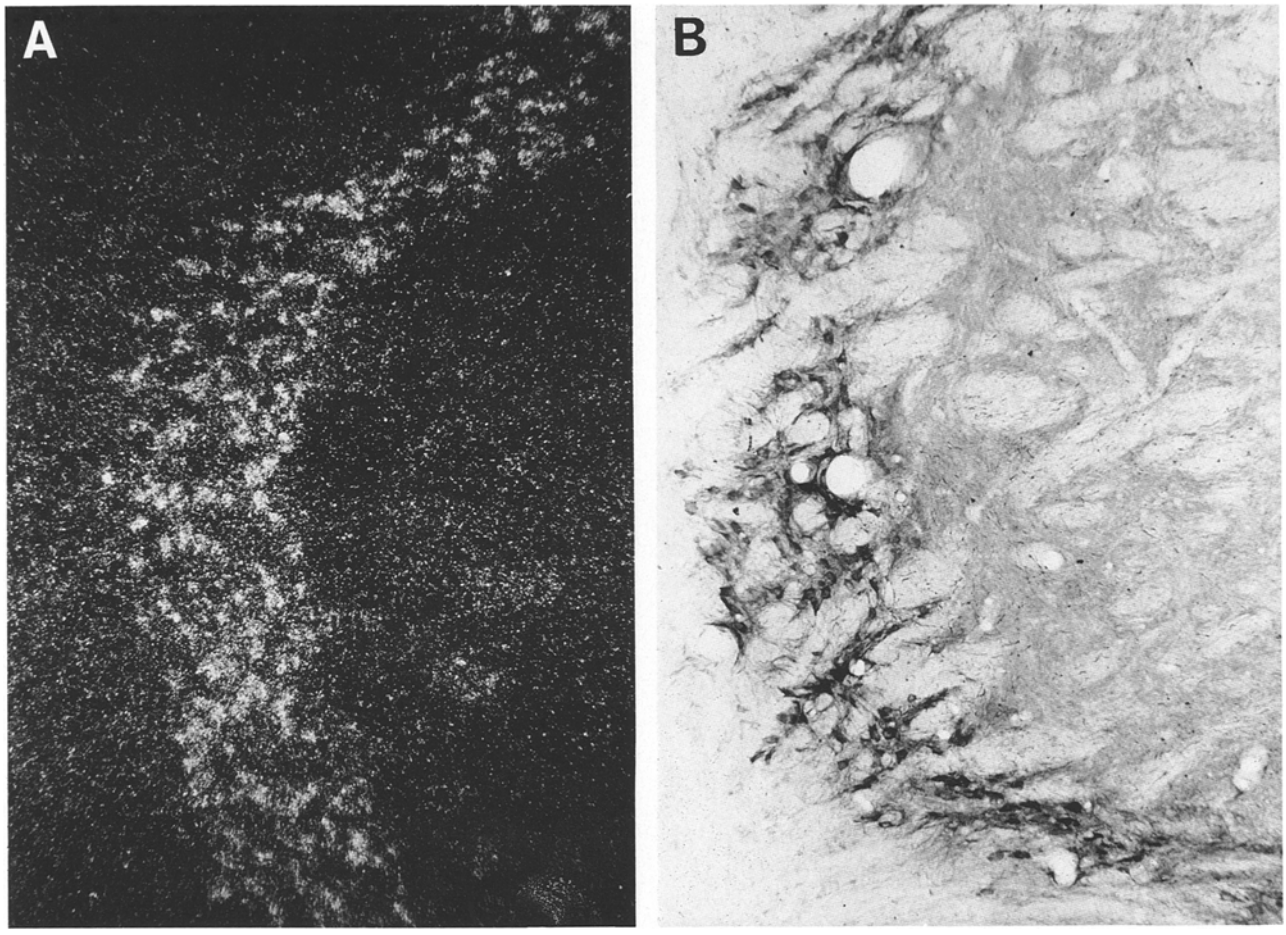

Fig. 2A, B. Co-localisation PV-mRNA and PV immunoreactivity in the reticular thalamic nucleus. **A** PV mRNA signal localized using labelled ^{35}S-cDNA. Dark field photomicrograph (x200). **B** A comparable section processed to visualize PV-immunoreactivity (×200)

The cDNA insert was labelled using the multiprime labelling system (Amersham UK) with PV cDNA and [^{35}S]dCTP (SJ305, 600 Ci/mmol Amersham). Parvalbumin cDNA inserts in a pUC plasmid (Berchtold and Means 1985) were isolated by restrictive digestion with EcoRI and Sal I and purified on low melting point agarose gels. A reaction mixture containing 0.3 pmole of PV cDNA in agarose and 35 pmoles of [^{35}S] dCTP (SJ305, 600 Ci/mmole, Amersham) was incubated overnight at 37° C. The reaction mixture containing labelled cDNA was separated from free [^{35}S] dCTP on a Sephadex G-50 column. The specific activity of the labelled cDNA was $> 5 \times 10^8$ dpm/μg DNA.

Sense and anti-sense oligodeoxyribonucleotides corresponding to the base sequences 140 to 183 of the rat parvalbumin cDNA (Berchtold et al. 1988; Berchtold and Means 1985; Epstein et al. 1986) were synthesized on an Applied Bio-Systems DNA Synthesizer. 10 pmole of each oligodeoxyribonucleotide was 3′ labelled to a specific activity of $> 1 \times 10^8$ dpm/μg using terminal deoxynucleotidyl transferase (Pharmacia) and (^{35}S) dCTP (600 Ci/mmole, SJ305 Amersham). 3′ labelled oligodeoxynucleotides were purified on Sephadex G-50 or NENsorb columns (DuPont).

Labelled cDNA or oligodeoxyribonucleotide probes (5–10,000 cpm/μl) were applied to sections as described above. For control experiments some sections were pretreated with RNase A (20 μg/ml) in 0.1 M Tris-HCl, 5 mM EDTA pH 7.6 before incubation with prehybridization buffer. After hybridization (approx 16 h at 37° C) the sections were rinsed sequentially with 2xSSC, 1xSSC, and 0.5xSSC at 37° C for thirty minutes each. All the wash solutions contained 14.3 mM β-mercaptoethanol. Sections were dehydrated with 70%, 90% and absolute ethanol (5 min each) and air dried. Radioactive signal on the sections was localized by exposure to autoradiography film (Hyperfilm β-max Amersham) and subsequently by dipping sections in Ilford nuclear track emulsion (K2) diluted 1 : 1 with water.

Immunohistochemistry

For histochemistry, rats (n = 10) were perfused via the ascending aorta with 4% paraformaldehyde in 100 mM sodium phosphate buffer pH 7.4, the brains were removed and left overnight in 30% sucrose at 4° C. After sucrose cryoprotection brains were sectioned at 25 μm on a Reichert (Austria) sledge microtome and sections collected into PBS. Sections were incubated overnight in parvalbumin antiserum (raised in rabbits) (Heizmann and Celio 1987; Kagi et al. 1987) diluted 1 : 500 in PBS containing 0.1% Triton X-100 and 1% normal sheep serum (NSS). After overnight incubations, sections were washed (x3) in PBS and then incubated with second antibody sheep-anti rabbit (Miles UK) at 1 : 50 dilution in PBS 0.1% Triton X-100 1% normal sheep serum for 1 h

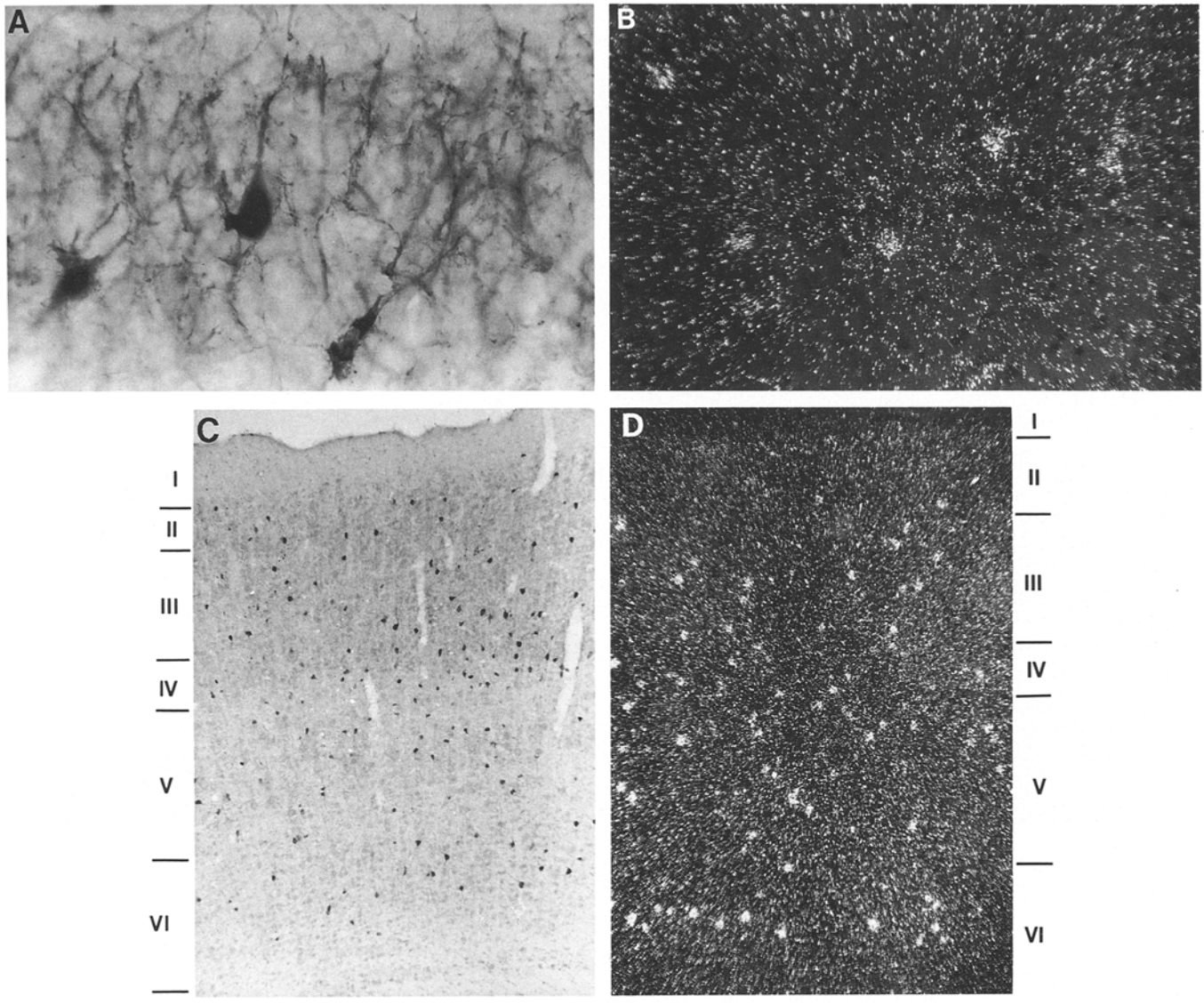

Fig. 3. A Cellular localisation of PV-immunoreactivity in interneurones of CA-1 region of hippocampus (×630). **B** Localisation of PV-mRNA, visualized with ^{35}S-labelled cDNA probe, over cells of the CA-1 region of hippocampus (×400). Dark field photomicrograph. **C** Low power photomicrograph illustrating the distribution of PV-immunoreactive neurones in the rat sensory-motor cortex. The PV-immunoreactive neurones are small multipolar or bipolar cells (×180). **D** Low power dark field photomicrograph illustrating the localisation of PV mRNA (^{35}S cDNA probe) in the rat sensory-motor cortex (×200)

at 37° C. After washing sections they were then incubated in rabbit – PAP (Miles UK) at 1 : 100 in PBS 0.1% Triton X-100 1% normal sheep serum for 1 h at 37° C. After further washes in PBS, sections were added to a solution of 3′3′-diaminobenzidine (1 mg/ml) in PBS containing 0.005% hydrogen peroxide to visualize sites of primary antibody binding. After incubation with the chromogen, stained sections were washed and mounted onto gelatin/chrome alum subbed slides. Specificity of staining was checked by preincubation of the diluted parvalbumin antiserum with parvalbumin protein 10^{-6} M (adsorption control), or omission of the primary antibody from the reaction. Stained sections or developed autoradiographs were photographed using a Leitz Dilaux 22 microscope and a Leitz camera system using Ilford PanF film.

Results

Specific parvalbumin signal (PV mRNA) detected by the ^{35}S multiprime labelled cDNA insert was most concentrated in two brain areas; the Purkinje cells of the cerebellum (Fig. 1) and the reticular thalamic nucleus (Fig. 2). In the cerebellum, in addition to the strong signal over the Purkinje cells, cells in the position expected for basket and stellate cells were also labelled. Granule cells were not labelled. The localisation of PV mRNA signal coincided with those

cerebellar neurons expressing parvalbumin immunoreactivity (Fig. 1B) including basket cells and stellate cells. Pretreatment of sections before hybridization with RNase A to digest cellular mRNA and then hybridization with labelled cDNA eliminated the PV specific signal (Fig. 1C). The ^{35}S-labelled antisense parvalbumin oligodeoxynucleotide also labelled the Purkinje, basket and stellate cells of the cerebellar molecular layer (Fig. 1D). In contrast the complementary sense oligodeoxynucleotide probe did not detect a specific signal in any brain region examined (data not shown).

In the reticular thalamic nucleus the distribution of neurones hybridizing positively with the PV-cDNA probe (Fig. 2A) coincided with the distribution of PV-immunoreactive neurones (Fig. 2B). In the telencephalon the PV-mRNA signal was found over a number of neurones in the hippocampus and dentate gyrus (Fig. 3B) as well as in the cerebral cortex (Fig. 3D). Comparison of these sections with sections processed to visualize PV-immunoreactivity revealed that the localization of neurones labelled by the cDNA probe also matched with the distribution of PV-immunoreactive neurones in these areas (Fig. 3A, C). PV-mRNA containing neurones were found in all layers of the cerebral cortex except layer I. Similarly in the hippocampus and dentate gyrus PV-mRNA signal was localized over neurones in the granule cell layer (dentate gyrus) and in neurones between the pyramidal cells of CA1–CA3 (see Fig. 3B).

A few scattered PV-mRNA containing cells were detected in the caudate-putamen and amygdala. In the brain stem, PV-signal was found in the cells of the mesencephalic trigeminal nucleus and in the superior olive (data not shown).

Discussion

The results presented here demonstrate that sites of PV-gene expression in the rat fore-brain coincide with the known cellular localization of PV-immunoreactivity (Endo et al. 1985; Heizmann 1984; Heizmann and Berchtold 1987; Heizmann and Celio 1987). The intensity of the PV-mRNA signal especially for the Purkinje cells and neurones of the reticular thalamic nucleus seems to reflect the abundance of PV-immunoreactivity in these cells. The specificity of the signal was confirmed by use of RNase A digestion, which eliminated the specific signal and by showing that the antisense oligodeoxynucleotide labelled the same population of cells as the cDNA probe, whereas the complementary sense oligodeoxynucleotide probe did not label any neurones in the rat brain. Further the distribution of PV-mRNA does not coincide with the distribution of other known calcium binding proteins such as calbindin-28K, or calretinin (Feldman and Christakos 1983; Jande et al. 1981; Rogers 1987).

The majority of neurones in the fore-brain containing PV-mRNA are known to be GABA-ergic neurones (Celio 1986; Celio and Heizmann 1981; Endo et al. 1985; Houser et al. 1980; Kosaka et al. 1987; Mugnaini and Oertel 1983; Stichel et al. 1987; Stichel et al. 1988). These include the PV-containing neurones of the cerebral cortex, hippocampus, reticular thalamic nucleus and cerebellum (Celio 1986; Celio and Heizmann 1981; Kosaka et al. 1987; Stichel et al. 1987; Stichel et al. 1988). The role of PV in these neurones has not been established with the exception of a possible role for PV as a calcium buffer in hippocampal interneurones (Kawaguchi et al. 1987). The development of "in situ" hybridization histochemistry for PV offers the possibility of following changes in PV gene expression and such studies may clarify its role in neuronal physiology.

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