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DIFFERENTIAL DISTRIBUTION OF THE mRNA ENCODING TWO ISOFORMS OF THE CATALYTIC SUBUNIT OF CALCINEURIN IN THE RAT BRAIN

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Summary: The distribution of mRNAs encoding two distinct isoforms of the catalytic subunit of calmodulin-dependent protein phosphatase (calcineurin A), designated calcineurin Aα and Aβ, in the rat brain was examined by using Northern blots and *in situ* hybridization histochemistry. The mRNAs for calcineurin Aα and Aβ were both unevenly present in the brain and showed different distribution. The differential distribution between calcineurin Aα and Aβ messages suggests that the individual isoforms are involved in specialized neural functions.

Calcineurin (CaN) is a Ca^{2+}/calmodulin-dependent protein phosphatase and corresponds to protein phosphatase type 2B, one of four principal types of serine/threonine-specific protein phosphatase present in mammalian tissues (1). This enzyme is a heterodimer that consists of catalytic (A) and regulatory (B) subunits and is abundant in the central nervous system. We isolated two complementary DNA (cDNA) clones of CaN A from a rat brain library, and analysis of these cDNA clones has predicted the existence of two distinct isoforms of CaN A, designated CaN Aα and Aβ (2) (3). These two isoforms are thought to be encoded by different genes, whereas recent studies suggest the presence of subspecies of each isoform derived from a single gene by alternative splicing (4) (5). Although the enzymological property of these two isoforms, CaN Aα and Aβ, has not yet been demonstrated, it is possible that each isoform of CaN A has distinct roles in the signal transduction of the neurons.

To investigate the potential significance of two CaN A isoforms, we localized CaN Aα and Aβ transcripts in the rat brain using Northern blots and *in situ* hybridization. We found that the mRNAs encoding two CaN A isoforms are unevenly and differently distributed in the central nervous system.

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The abbreviations used are: CaN, calcineurin; cDNA, complementary DNA; DARPP-32, dopamine and cAMP-regulated phosphoprotein of relative molecular mass 32,000; kb, kilobases.

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MATERIALS AND METHODS

Probes: Four synthetic 45 base oligodeoxynucleotide probes complementary to the two rat CaN A mRNAs (2) (3) were prepared by solid phase synthesis on an Applied biosystems DNA synthesizer and purified with an Oligonucleotide Purification Cartridge (Applied Biosystems). Two probes for CaN Aα mRNA (Aα1 and Aα2) were complementary to bases 1365-1409 and 1710-1754 and probes for CaN Aβ (Aβ1 and Aβ2) to bases 1344-1388 and 1570-1614, respectively. Regions with low homology were selected. The probes used were 5'-labeled using T4 polynucleotide kinase (Takara Biomedicals, Kyoto, Japan) and [γ-^{32}P]ATP (New England Nuclear) for Northern blots and 3'-labeled with terminal deoxynucleotidyl transferase (Takara Biomedicals) and [α-^{35}S]dATP (New England Nuclear) for *in situ* hybridization. Additionally, cDNA probes for CaN Aα and Aβ were labeled using random primer DNA labeling kit (Takara Biomedicals) and [γ-^{32}P]dCTP (New England Nuclear).

RNA extraction and Northern blot analysis: Total RNA was isolated from whole brains and various brain regions of adult male Wistar rats by homogenization in guanidine isothiocyanate buffer (4 M guanidine isothiocyanate, 25 mM sodium acetate, and 0.5% β-mercaptoethanol) and ultracentrifugation through a 5.7 M cesium chloride cushion. RNA content was determined by optical density at a wavelength of 260 nm. Approximately 25μg of denatured total RNA was fractionated on 1.0% agarose gel containing 0.67 M formaldehyde, 0.2 M morpholinopropanesulfonic acid, 0.05 M sodium acetate, and 0.01 M ethylenediamine tetraacetic acid. After electrophoresis, RNA was transferred onto nitrocellulose by capillary action in 10 x SSC [1 x SSC = 0.15 M NaCl, 0.015 M sodium acetate (pH 7.2)]. Baked filters were prehybridized for three hours at 37°C in the hybridization buffer [10mM Tris/HCl (pH 7.4), 40% formamide, 2 x SSC, 1 x Denhardt's solution, 0.02 mg/ml salmon sperm DNA, and 250 μg/ml yeast tRNA]. Hybridization was carried out at 42°C overnight in the same buffer containing 0.5-1 x 10^6 cpm/ml of ^{32}P-labeled probe. Filters were washed twice in 1 x SSC/0.1% SDS at room temperature for 20 min and then three times at 55°C for 20 min. For the cDNA probes, the nitrocellulose filters were further washed in 0.1 x SSC/0.1%SDS at 60°C for 20 min. Autoradiographic localization of bound probes was obtained by exposure of the filters to X-ray film.

***In situ* hybridization:** Anesthetized Wistar rats (4 weeks old) were perfused with ice-cold fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were dissected, immersed in the same fixative for 2-4 hours, and transferred into a 30% sucrose in 0.1 M phosphate buffer. Hybridizations were performed essentially as described by Young et al. (6). Tissues were frozen and sections 20μm in thick were prepared. The sections thaw-mouted on poly-L-lysine-coated slides were rinsed in 0.1 M triethanolamine/0.9% NaCl (pH 8.0) for 10 min at room temperature to reduce the nonspecific binding. These sections were dehydrated through a graded series of ethanol and chloroform, then air-dried. They were then prehybridized at 37°C for 3 hours in 200 μl of the same hybridization buffer as used for Northern blots, with coverslips. Each sections were treated with 10^6 cpm of the ^{35}S-labeled oligonucleotide probes (approximately 0.01 μg) dissolved in 100 μl of the hybridization buffer containing 100 mM dithiothreitol and 14 mM β-mercaptoethanol. For the control study, non-labeled oligonucleotide probes (0.25-0.3 μg) were added to the hybridization buffer. Hybridization was carried out at 49°C overnight in a humid chamber. The coverslips were removed and the sections were rinsed three times in 1 x SSC for 5 min at room temperature, four times in 1 x SSC/20% formamide for 15 min at 51°C and again in 1 x SSC at room temperature for 30 min. After dipping into water and 70% ethanol, these sections were air-dried and autoradiographic localization of the bound probe was performed by exposure to X-ray film for 5-10 days.

RESULTS AND DISCUSSION

Northern Blot Analysis: The oligodeoxynucleotide probes Aα1, Aα2, and cDNA probe for CaN Aα labeled an approximately 4.1kb mRNA, while the three kinds of probe for CaN Aβ labeled an approximately 3.3kb mRNA from the rat brain total RNA (Fig. 1). Co-hybridization with two oligodeoxynucleotide probes for CaN Aα or for CaN Aβ also showed a single band (data not shown).

Fig. 2 shows Northern blot analysis of several brain regions of the rat, using CaN Aα1 (Fig. 2A) and CaN Aβ1 (Fig. 2B) probes. Both CaN Aα and CaN Aβ mRNAs were unevenly distributed through the brain and were most abundant in the hippocampus among the brain

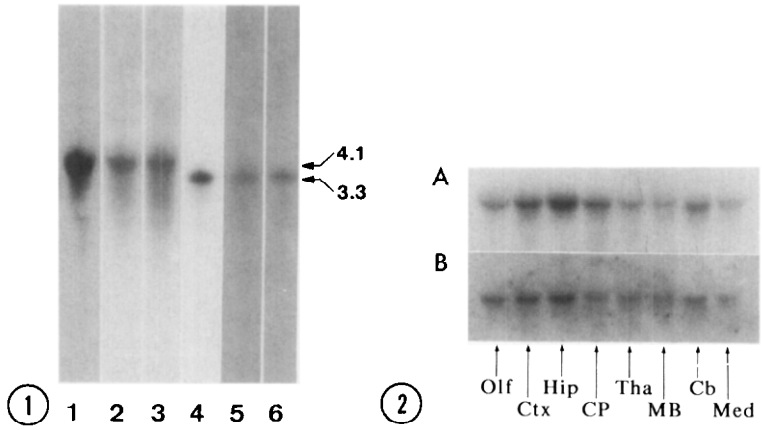

Fig. 1. **Northern blot analysis of rat whole brain using various probes for CaN Aα and CaN Aβ.** Total RNA (25μg) from whole rat brain was electrophoresed and blotted onto a nitrocellulose filter, and the blotted filter was hybridized with the ^{32}P-labeled cDNA probe or oligodeoxynucleotide probe indicated. RNA sizes are given in kilobases. Lane 1, cDNA for CaN Aα; lane 2, Aα1; lane 3, Aα2; lane 4, cDNA for CaN Aβ; lane 5, Aβ1; lane 6, Aβ2.

Fig. 2. **Regional distribution of mRNAs for CaN Aα and CaN Aβ assessed by Northern blot.** Total RNAs (25 μg) from various regions of the rat brain were electrophoresed and transferred to nitrocellulose, and the blotted filters were hybridized with ^{32}P-labeled oligonucleotide Aα1 probe for calcineurin Aα (A) or Aβ1 probe for Aβ (B). Identical RNA preparations were used for each blot. Olf, olfaltory bulb; Ctx, cortex; Hip, hippocampus; CP, caudate-putamen; Tha, thalamus; MB, midbrain; Cb, cerebellum; Med, medulla oblongata.

regions examined. The CaN Aα and CaN Aβ mRNAs were highly expressed in the cerebral cortex and cerebellum. In the midbrain and medulla oblongata, there were low concentrations of mRNAs hybridized with both probes for CaN Aα and Aβ. Different concentrations of CaN Aα and CaN Aβ mRNAs were evident in the caudate-putamen. The concentration of CaN Aα mRNA was higher in the caudate-putamen than in the thalamus or olfactory bulb, while the concentration of CaN Aβ mRNA in caudate-putamen is lower than that in the thalamus or olfactory bulb.

***In situ* hybridization**: Figs. 3 and 4 show regional distributions of the expression of CaN Aα and CaN Aβ mRNAs in the rat brain, determined using *in situ* hybridization histochemistry. Control experiments were performed to examine the specificity of the synthesized probes. Intense signals were seen in the sagittal brain section hybridized with ^{35}S-labeled CaN Aα1 probe (Fig. 3A), while no signal was detected in the section co-hybridized with the ^{35}S-labeled CaN Aα1 probe and an excess amount of non-labeled CaN Aα1 probe (Fig. 3B). However, the intense signals were not altered by adding other non-labeled CaN Aα2, CaN Aβ1 and CaN Aβ2 probes (Fig. 3C). Similar results were obtained in case of CaN Aβ2 (Fig. 3D-F), CaN Aα2 or CaN Aβ1 (data not shown). These findings indicate that the autoradiogram obtained using each probe represents the localization of mRNA for each CaN A isoform but not the non-specific binding to the sections.

The distribution of mRNAs for the CaN Aα and Aβ was examined in various levels of rat brain coronal sections (Fig. 4). The most intense signals of both CaN Aα and CaN Aβ were seen in the pyramidal cell layer of the hippocampus (Fig. 4C). Indusium griseum,

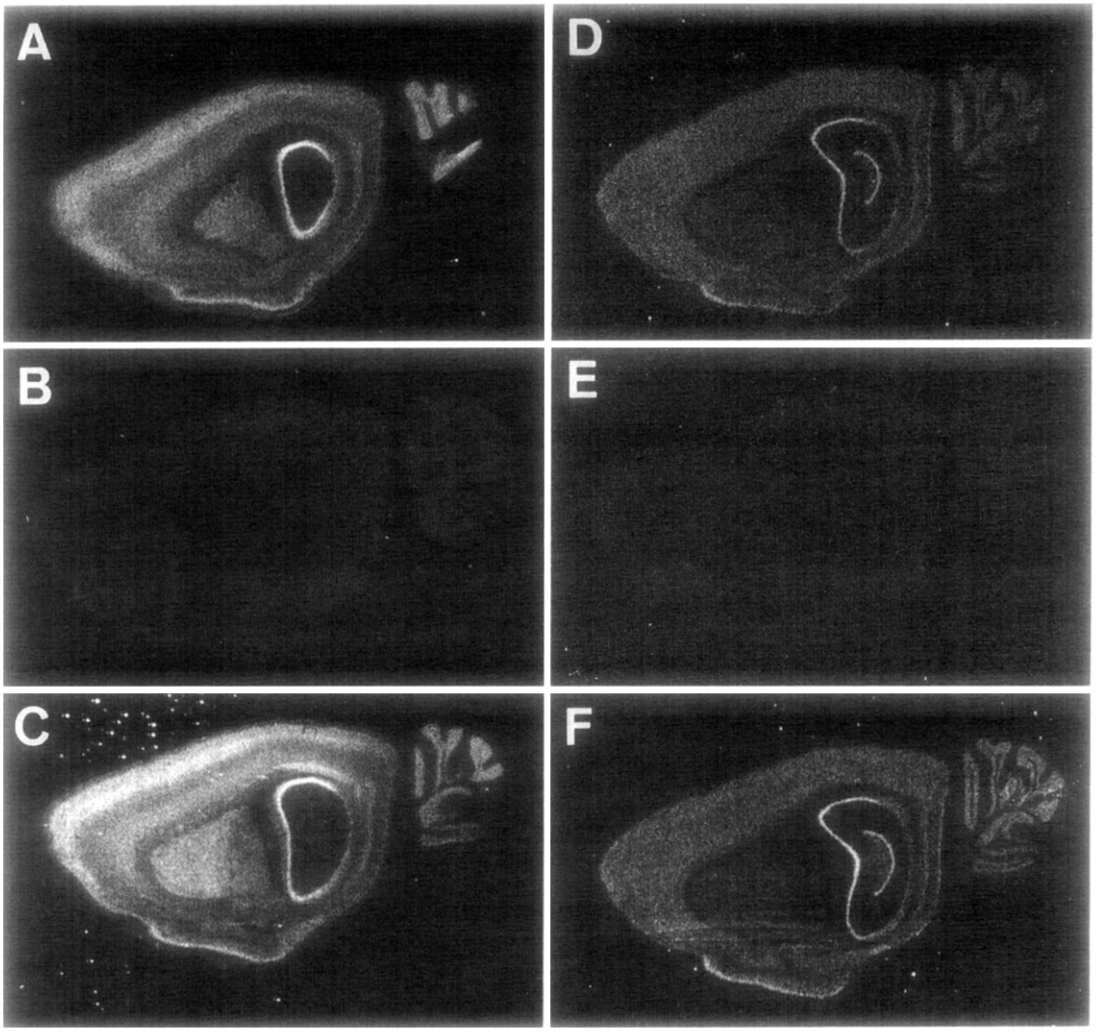

Fig. 3. **Control study for *in situ* hybridization histochemistry.** A, only the ^{35}S-labeled Aα1 probe was applied to a sagittal section; B, the ^{35}S-labeled Aα1 probe and an excess amount of non-labeled Aα1 probe were simultaneously applied to the adjacent sagittal section; C, the ^{35}S-labeled Aα1 probe and an excess amount of non-labeled Aα2, Aβ1, and Aβ2 probe were simultaneously applied to other adjacent sagittal section; D, only the ^{35}S-labeled Aβ2 probe was applied to a sagittal section; E, the ^{35}S-labeled Aβ2 probe and an excess amount of non-labeled Aβ2 probe were simultaneously applied to the adjacent sagittal section; F, the ^{35}S-labeled Aβ2 probe and an excess amount of non-labeled Aα1, Aα2, and Aβ1 probe were simultaneously applied to other adjacent sagittal section.

pyriform cortex, the granular cell layer of the dentate gyrus, and the granular layer of the cerebellum contained moderately high amounts of messages for both CaN A isoforms (Fig. 4C, D, G). The medulla oblongata showed generally lower signals for both CaN A isoforms (Fig. 4G, H). Extremely low levels of the messages for any CaN A were noted in the white matter.

There was a different localization between CaN Aα and Aβ mRNAs in the following areas: (i) in the olfactory bulb, a moderate level of CaN Aα message was detected only in the internal granular layer, while high density of Aβ signals was observed in the mitral cell layer and the internal granular layers (Fig. 4A); (ii) in the anterior olfactory nucleus, intense signals

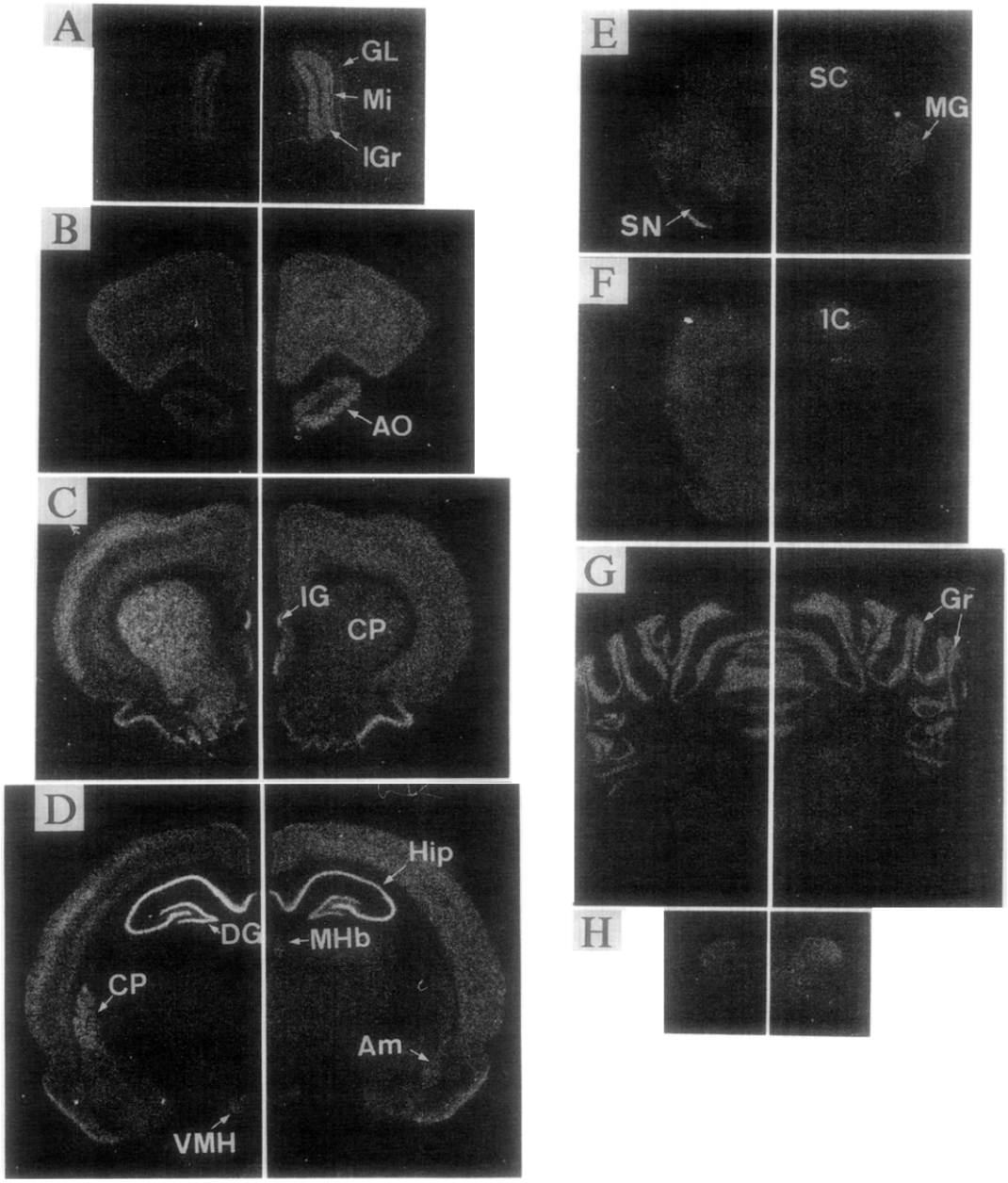

Fig. 4. **Localization of CaN Aα and CaN Aβ in rat brain, by *in situ* hybridization histochemistry.** Sets of coronal sections of the rat brain were hybridized with ^{35}S-labeled oligonucleotide probes directed to mRNAs coding for CaN Aα (right-hand side) and CaN Aβ (left-hand side). GL, glomerular layer; Mi, mitral cell layer; IGr, internal granular layer; AO, anterior olfactory nucleus; IG, indusium griseum; CP, caudate-putamen; DG, dentate gyrus; Hip, hippocampus; MHb, medial habenular nucleus; VMH, ventromedial hypothalamic nucleus; Am, amygdaloid complex; SC, superior colliculus; SN, substantia nigra; MG, medial geniculate nucleus; IC, inferior colliculus; Gr, granular layer of the cerebellum.

of CaN Aβ were observed but the signals of CaN Aα were moderately low (Fig. 4B); (iii) in the cerebral cortex, the CaN Aα message was highly concentrated in the layers II, III, V and VI but not in the layers I and IV, while the laminar pattern of Aβ mRNA was obscure (Fig. 3,

4C,4D); (iv) a high level of the CaN Aα mRNA was present in the caudate-putamen, but the signals of CaN Aβ were moderately low in the caudate-putamen (Fig. 4C).

DARPP-32 (dopamine and cAMP-regulated phosphoprotein, of relative molecular mass 32,000) has been reported as a good substrate for CaN (7). In the caudate-putamen, the D1 receptor and DARPP-32 are present on or in the medium-sized striatal neurons (8) (9). Immunocytochemical studies demonstrated the localization of CaN in medium-sized striatal neurons (10). Although the exact co-localization of these protein in the striatal neurons has not been demonstrated, the differential distribution of the messages described above suggests that CaN Aα is involved in the dephosphorylation of DARPP-32 in these neurons.

The distributions of CaN have been reported using antibodies against purifed CaN from mammalian brain (10) (11). In the preliminary study using specific antibodies for each isoforms, purified CaN from mammalian brain with ordinary method was shown to consists of both CaN Aα and Aβ (data not shown). It is, therefore, suggested that the distribution of CaN in the previous reports represents the sum of the distribution of two or more isoforms, or represents the distribution of the isoform with strong antigenesity. Immunocytochemical localization of CaN in rat brain has been reported by Goto et al. (10) who used antiserum against purified CaN from bovine brain. It seems that the CaN-like immunoreactivity in their study predominantly reflects the localization of the CaN Aα rather than that of CaN Aβ, because the highest immunoreactivity of CaN was seen in the caudate-putamen, layers III, V, and VI of the neocortex and hippocampal pyramidal cells in their reports. Therefore, it is possible that the antiserum raised in their study recognized mainly CaN Aα, or that in the rat brain the amount of CaN Aα mRNA is much higher than that of CaN Aβ.

In conclusion, the differential distribution between calcineurin A isoforms suggests that the individual enzymes are involved in specialized neural functions.

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