

Developmental Changes in Distribution of Death-Associated Protein Kinase mRNAs

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Death-associated protein kinase (DAP-kinase) is Ca²⁺/calmodulin-dependent serine/threonine kinase that contains ankyrin repeats and the death domain. It has been isolated as a positive mediator of interferon- γ -induced apoptotic cell death of HeLa cells. In order to reveal the physiological role of DAP-kinase, the tissue distribution and developmental changes in mRNA expression of DAP-kinase were investigated by Northern blot and in situ hybridization analyses. DAP-kinase mRNA was predominantly expressed in brain and lung. In brain, DAP-kinase mRNA had already appeared at embryonic day 13 (E13) and was, thereafter, detected throughout the entire embryonic period. High levels of expression were detected in proliferative and postmitotic regions within cerebral cortex, hippocampus, and cerebellar Purkinje cells. These findings suggest that DAP-kinase may play an important role in neurogenesis where a physiological type of cell death takes place. The overall expression of DAP-kinase mRNA in the brain gradually declined at postnatal stages, and the expression became restricted to hippocampus, in which different expression patterns were observed among rostral, central, and caudal coronal sections, suggesting that DAP-kinase may be implicated in some neuronal functions. Furthermore, it was found that the expression of DAP-kinase mRNA was increased prior to a certain cell death induced by transient forebrain ischemia, indicating a possible relationship between DAP-kinase and neuronal cell death. *J. Neurosci. Res.* 58:674–683, 1999. © 1999 Wiley-Liss, Inc.

Key words: DAP-kinase; brain; development; ischemia; neuronal cell death

INTRODUCTION

Death-associated protein kinase (DAP-kinase), which is a novel Ca²⁺/calmodulin-dependent serine/

threonine kinase, was cloned by a functional gene selection approach based on random inactivation of gene expression with antisense cDNA libraries (Deiss et al., 1995; Kimchi, 1998; Kissil and Kimchi, 1998). DAP-kinase protein contains unique domains and motifs including eight ankyrin repeats, two potential ATP/GTP binding sites (P-loops), cytoskeleton binding domain, death domain, and a serine-rich C-terminus tail (Cohen et al., 1997; Feinstein et al., 1995), suggesting that DAP-kinase may play a pivotal role in various biological processes. DAP-kinase has been suggested to act as a positive mediator of apoptosis based on the following lines of evidence: (1) HeLa cells expressing DAP-kinase antisense RNA were rescued from interferon- γ -induced apoptotic cell death (Deiss et al., 1995); (2) overexpression of DAP-kinase killed HeLa cells in the absence of any external stimulus (Cohen et al., 1997); (3) a catalytically inactive mutant which carried a lysine to alanine substitution within the kinase domain, displayed dominant negative features and protected cells from interferon- γ -induced cell death. By itself, the catalytically inactive mutant did not induce cell death, suggesting that the intrinsic kinase activity was critical for the death-promoting properties (Cohen et al., 1997).

DAP-kinase expression was lost in various carcinoma and B-cell lymphoma (Kissil et al., 1997). In lung carcinoma cells assayed in mouse model systems for metastasis, restoration of DAP-kinase suppressed the metastatic activity of the cells by increasing their potential sensitivity to various apoptotic stimuli (Inbal et al., 1997). Therefore, DAP-kinase has been regarded as a tumor suppressor protein (Kissil and Kimchi, 1998).

This study shows that DAP-kinase mRNA is abundantly expressed in brain and lung tissues. However, the

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physiological features of DAP-kinase in the brain, where more than half of the neurons die by apoptotic cell death before reaching maturation (Oppenheim, 1991), were not clear. To understand the involvement of DAP-kinase in neuronal development, we investigated here the distribution of DAP-kinase mRNA in the developing brain by Northern blot and in situ hybridization analyses. Moreover, we demonstrated a possible relationship between DAP-kinase and neuronal cell death caused by transient forebrain ischemia.

MATERIALS AND METHODS

RNA Isolation

Total RNA was extracted from rat brain using RNeasyTM (TEL-TEST Inc., Friendswood, TX), based on the method of Chomczynski and Sacchi (1987). Poly (A) mRNA was isolated using OligotexTM-dT30 (Daiichi Chemicals, Tokyo, Japan).

Digoxigenin (DIG)-Labeled Complementary RNA (cRNA) Probe

The 0.4-kb DAP-kinase cDNA fragment (nucleotide residues 3895–4293), obtained from rat brain by reverse transcriptase-polymerase chain reaction (RT-PCR) using RNA LA PCR kit (Takara Shuzo, Kyoto, Japan) with specific primers 5'-GATATCCATGCGTCAGACCTGA-3' (forward) and 5'-TCACCGGGACACCACTGAGCTAAT-3' (reverse), was amplified by PCR with bipartite primers consisting of sequences of SP6 RNA polymerase promoter (underlined) and DAP-kinase (5'-AGGGGAATTCATTGGATTTAGGTGACACTATA-GAATACGATATCCATGCGTCAGACCTGA-3') and reverse sequences of DAP-kinase (5'-GAAAAAGTCGACTCACCGGGACACCACTGAGCTAATGGAATTGTA-3') or forward sequences of DAP-kinase (5'-GAAAAAGAATTCGATATCCATGCGTCAGACCTG-3') and sequences of T7 RNA polymerase promoter (underlined) and DAP-kinase (5'-AGGGGTCGACAGCTCTAATACGACTCACTATAGGGCGATCACCGGGACACCACTGAGCTA-3'). The resulting cDNA fragments were purified on 1% agarose gel electrophoresis. The cRNA probes were generated from 1 µg of purified cDNA fragment in the presence of DIG-labeled uridine triphosphate with SP6 and T7 RNA polymerase for sense and antisense probes, respectively.

Northern Blots

For tissue distribution analysis, adult rat multiple tissue Northern blots (OriGene Technologies Inc., Rockville, MD) were used. To examine DAP-kinase mRNA expression in the developing rat brain, 2 µg of poly (A)

mRNA were separated on a 1% formaldehyde-agarose gel and transferred onto a positively charged nylon membrane (Nylon Membrane, Boehringer Mannheim, Indianapolis, IN). After incubation at 67°C for 1 hr in hybridization buffer (DIG Easy Hyb, Boehringer Mannheim), hybridization with DAP-kinase-specific DIG-labeled cRNA probe was performed at 67°C for 16 hr in hybridization buffer. Filters were washed twice at 67°C for 15 min in 2 × saline-sodium citrate (SSC), containing 1% sodium dodecyl sulfate (SDS), and were further washed twice at 67°C for 15 min in 0.2 × SSC containing 0.1% SDS. Specific bands were immunologically detected using DIG-luminescent detection kit (Boehringer Mannheim) as indicated in the manufacturer's protocol. Filters were stripped by 50 mM Tris-HCl (pH 8.0) containing 50% formamide and 1% SDS, and reprobed at 42°C with DIG-tailed oligonucleotide of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA corresponding to nucleotides 434–503 as a control for RNA loading and transfer. Signals were quantitated on computer-assisted scanning densitometry (Molecular Analyst, BioRad, Richmond, CA).

In Situ Hybridization

Phosphate-buffered saline (PBS), milliQ water, and 20 × SSC were treated with diethylpyrocarbonate. Anesthetized rats were perfused transcardially with 4% paraformaldehyde in PBS. Brains removed from the skull were immersed at 4°C for 16 hr in PBS containing 4% paraformaldehyde and 30% sucrose, embedded in Tissue-Tek O.C.T. compound (Miles Incon, Elkhart, IN) and frozen quickly in liquid nitrogen. Fresh frozen sections, 10–20 µm in thickness, prepared by cryostat were mounted onto 3-aminopropyltriethoxysilane (APS)-coated glass slides and stored at -80°C until use.

For hybridization, specimens were fixed in 4% paraformaldehyde in PBS. Embryonic and postnatal sections were permeabilized in 0.1% and 0.3% Triton X-100 for 5 min, respectively. Nucleic acids were hydrolyzed partially in 0.2 N HCl for 20 min. Proteins were digested in 1 µg/ml proteinase K at 37°C for 5 min. Following prehybridization at 65°C for 1 hr in 2 × SSC containing 50% formamide, slides were hybridized with DIG-labeled cRNA probe in buffer containing 5 × SSC, 2% blocking reagent, and 50% formamide under parafilm coverage at 65°C for 16 hr. Then, unbound and nonspecific bound probes were removed by rinsing the sections in 2 × SSC containing 50% formamide at 65°C for 1 hr and treating with 20 µg/ml RNaseA at 37°C for 30 min. Sections were washed at 65°C for 20 min in 2 × SSC, and twice in 0.2 × SSC. For immunological detection, blocking was performed at room temperature for 1 hr in solution A composed of 100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2% blocking reagent, and 20% lamb serum.

Sections were incubated at room temperature for 1 hr with alkaline phosphatase-conjugated anti-digoxigenin antibodies in solution A and washed three times in solution A. After rinse in solution B composed of 100 mM Tris-HCl (pH 9.5), 100 mM NaCl and 50 mM MgCl₂, 1:50 diluted nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP) in solution B was overlaid on the specimens. Color development reaction was stopped by placing the specimens in water. Samples were mounted by Crystal / Mount (Biomedica Corp., Foster City, CA).

Induction of Forebrain Ischemia

Male Wistar rats 8–12 weeks old were used for ischemia experiments. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Tokyo University of Pharmacy and Life Science, and were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Forebrain ischemia was induced by four-vessel occlusion (Pulsinelli and Brierley, 1979). For electrocauterization of the bilateral vertebrate arteries, anesthesia was induced with pentobarbital (40 mg/kg, i.p.), and the bilateral vertebrate arteries were electrocauterized using monopolar electrocautery needle, connected to a coagulator (Micro-3E, Mizuho Ika Kogyo, Japan). Then, the neck incision was sutured. After recovery from anesthesia, the rats were housed in cages with free access to tap water. The following day, forebrain ischemia was induced by clamping both common carotid arteries with aneurysm clips for 20 min. Following the ischemic insult, the dissected wound was sutured. The rectal temperature was maintained at 37°C throughout the procedure using a thermal blanket. Then, the rats were housed in cages with free access to pellet food and tap water for indicated time.

Materials

Wistar rats were purchased from Japan SLC (Shizuoka, Japan) with a vaginal plug date considered embryonic day 1 (E1). SP6 RNA polymerase, T7 RNA polymerase, DIG-UTP, blocking reagent, proteinase K, alkaline phosphatase-conjugated anti-digoxigenin antibodies, and NBT/BCIP were from Boehringer Mannheim (Indianapolis, IN). APS-coated glass slide was from Matsunami Glass Ind. (Tokyo, Japan). RNaseA and lamb serum were from Sigma (St. Louis, MO).

RESULTS

DIG-labeled antisense cRNA probe was used to detect DAP-kinase mRNA. The specificity of the signal was confirmed by using DIG-labeled sense cRNA probe. Tissue distribution of DAP-kinase mRNA in adult rat was examined by Northern blot analysis (Fig. 1). A signal 6.3-kb mRNA transcript, similar in its size to human

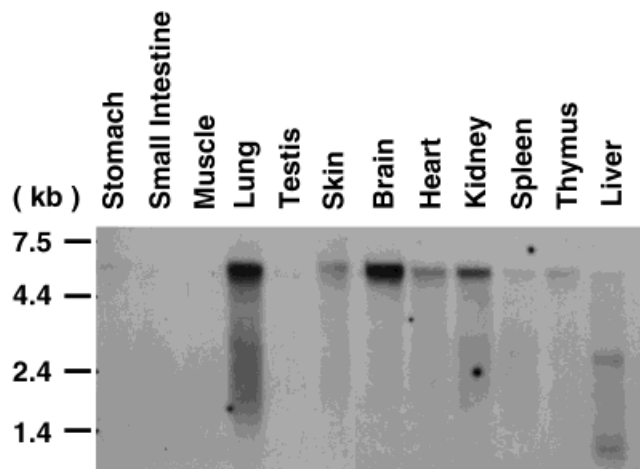


Fig. 1. Northern blots demonstrating tissue distribution of death-associated protein (DAP)-kinase mRNA in adult rat. Multiple tissue Northern blots were hybridized with DAP-kinase-specific digoxigenin (DIG)-labeled cRNA probe. RNA size markers are shown on the left.

DAP-kinase mRNA, was abundantly detected in brain and lung. Low to moderate expression levels were detected in kidney, heart, and skin, and weaker signals were observed in stomach, spleen, and thymus. The signals were hardly detected in small intestine, muscle, and testis. In the liver, 3-kb and 1-kb bands were also observed in addition to a weak 6.3-kb band.

The developmental expression of DAP-kinase mRNA in rat brain was examined by Northern blot analysis (Fig. 2). The mRNAs were extracted from whole embryos at stage E13, from brain at stages E15, E18, E20, E22, and postnatal days (P)4, P7, P14, P21, and adult. The expression of DAP-kinase mRNA was already observed on E13 and was further increased in the brain during the prenatal stages (Fig. 2A). The signal at E20 was approximately sixfold stronger than that at E13 (Fig. 2C). After birth, the expression levels gradually decreased.

The expression pattern of DAP-kinase mRNA in developing rat brain was investigated by *in situ* hybridization analysis. At the embryonic stages, DAP-kinase mRNA was widely distributed in brain. The level of expression was the highest in the cerebral cortex, hippocampus, and cerebellum. The expression of DAP-kinase mRNA was observed in neuronal cells and was hardly detected in glial cells.

In the cerebral cortex, high levels of expression were observed in the cortical plate, subplate, subventricular zone, and ventricular zone at E18–22 (Fig. 3A, B). At the postnatal stage, the expression was detected in the neocortex layers II–VII (Fig. 3C), but the signals decreased gradually as maturation of cerebral cortex proceeded (Fig. 3D). In the adult brain, weak expression was

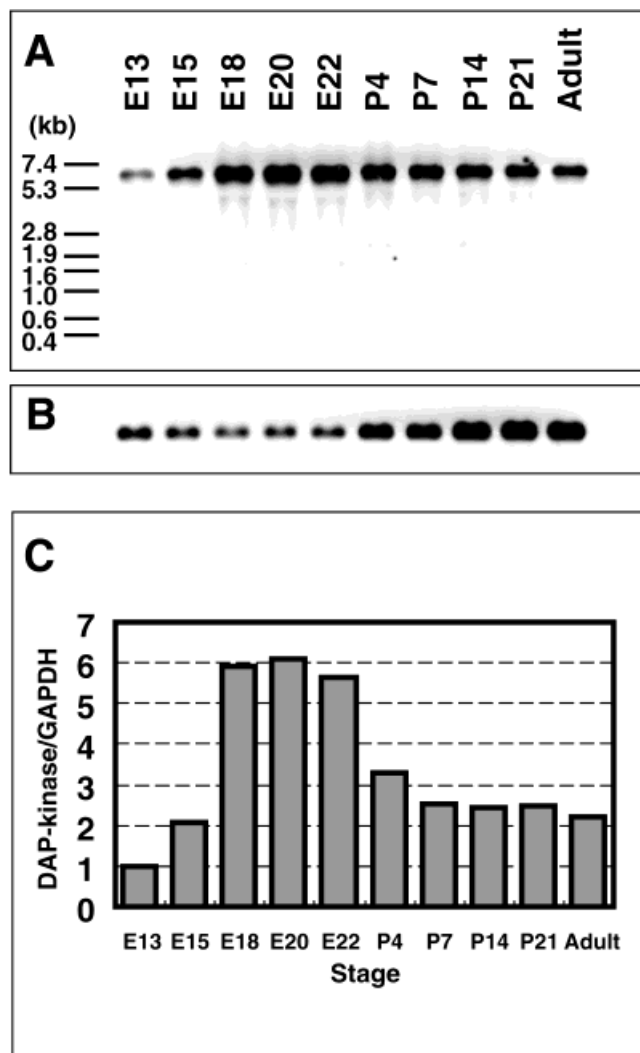


Fig. 2. Northern blot analysis of developmental expression for DAP-kinase mRNA. The mRNAs were extracted from whole embryos at embryonic day 13 (E13), from brain at stages E15, E18, E20, E22, and postnatal days (P)4, P7, P14, P21, and adult. **A:** Poly (A) mRNAs (2 μ g) were subjected to RNA blot analysis using DAP-kinase-specific DIG-labeled cRNA probe. RNA size markers are shown on the left. **B:** The same blot was rehybridized with DIG-tailed oligonucleotide probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as internal control. **C:** The relative expression levels of DAP-kinase mRNA during the development are shown as the ratio of DAP-kinase mRNA/GAPDH mRNA using a densitometer. The ratio of DAP-kinase mRNA / GAPDH mRNA at E13 was defined as 1.

localized in the pyramidal neurons of layers II and III (Fig. 3E). Moderate expression was found in the pyriform cortex throughout the developmental stages (Fig. 3F).

Intense expression was observed in the hippocampal primordia at E18 (Fig. 4A). During development, DAP-kinase mRNA transcripts became localized in CA1–CA4, dentate gyrus and subicular area, and were sus-

tained in the adult brain (Fig. 4B, C). Interestingly, the expression pattern was different between rostral, central, and caudal coronal sections after P21. In the rostral section, remarkable expression was restricted in the medial part of CA1, CA2, and lower lips of dentate gyrus, whereas lower levels of expression were found in CA3 and CA4 (Fig. 4D). In the central section, the angle region of the dentate gyrus showed intensive expression signals in particular. The expression patterns of CA region were the same as those of the rostral section (Fig. 4E). In the caudal section, high levels of expression were detected in the whole CA1, CA2, and dentate gyrus, and moderate expression was shown in CA3 and CA4 (Fig. 4F). These regional expression patterns in hippocampus were maintained into adulthood.

In the cerebellum, DAP-kinase mRNA was expressed in the identifiable Purkinje neuron precursors and precursor cells of cerebellar deep nucleus at E18 (Fig. 5A). Thereafter, the expression became restricted in Purkinje cell layer and deep nucleus by P7 (Fig. 5B–D). Purkinje cells in the rostral part of cerebellum expressed DAP-kinase mRNA higher than those in caudal part at E22 (Fig. 5B). At P3, expression levels in caudal region were as intensive as in the rostral section (Fig. 5C). The expression of DAP-kinase mRNA in Purkinje cells declined after P14 (Fig. 5E, F). In the granular cell layer, low levels of expression were observed at P14 transiently in external granular cell layer, but not in the internal layer (Fig. 5E). No significant expression signals could be detected at other stages. The distribution patterns are summarized in Table I.

In an attempt to examine a possible relationship between DAP-kinase and neuronal cell death, the change of the level of DAP-kinase mRNA after transient forebrain ischemia by four-vessel occlusion was investigated by Northern blot analysis (Fig. 6). The mRNAs were extracted from cerebral cortex and cerebellum at 1 hr, 6 hr, 9 hr, and 24 hr after 20 min of ischemia. The expression of DAP-kinase mRNA in cerebral cortex was apparently observed at sham-operated conditions and was further induced after transient forebrain ischemia (Fig. 6A). The signal at 24 hr after transient forebrain ischemia was approximately three times stronger than that at sham control (Fig. 6E). On the other hand, the expression of DAP-kinase mRNA in cerebellum was faintly detected at sham-operated condition. There was no difference of the level of DAP-kinase mRNA after transient forebrain ischemia in cerebellum (Fig. 6C, E).

DISCUSSION

DAP-kinase has been identified as a novel Ca^{2+} /calmodulin-dependent serine/threonine kinase. Overexpression of intact DAP-kinase, but not of catalytically inactive kinase mutant, induced apoptotic cell death, suggesting that the cell death-inducing activity of DAP-

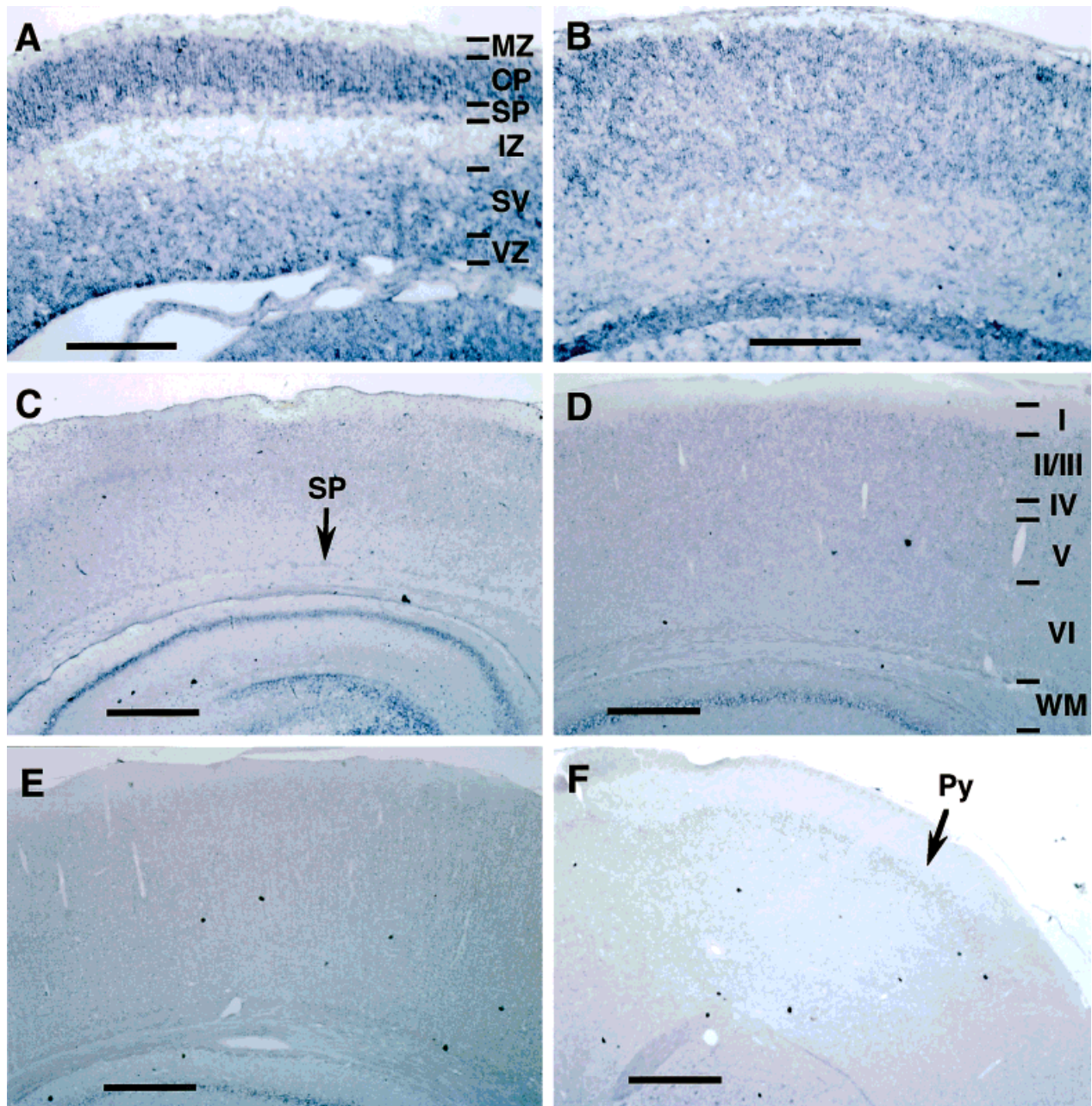


Fig. 3. Developmental change in distribution of DAP-kinase mRNA in the cerebral cortex at E18 (A), E22 (B), P7 (C), P21 (D), adult (E), and adult pyriform cortex (F). Moderate expression of DAP-kinase mRNA was exhibited in cortical subplate (arrow in C) and pyriform cortex (arrow in F). CP,

cortical plate; IZ, intermediate zone; MZ, marginal zone; Py, pyriform cortex; SP, cortical subplate; SV, subventricular zone; VZ, ventricular zone; WM, white matter; I-VI, layer I-VI. Bars = 250 μ m for A, B; 500 μ m for C-F.

kinase depends on its intrinsic kinase activity (Deiss et al., 1995; Cohen et al., 1997; Kimchi, 1998). In this work, we examined the tissue distribution of DAP-kinase mRNA in adult rat. The high levels of expression detected in brain led us to investigate further the expression patterns in the developing brain by in situ hybridization analysis.

DAP-kinase mRNA was widely distributed in developing brain. The intense signals were particularly observed within the proliferative and postmitotic regions of

the cerebral cortex, such as the cortical plate and ventricular zone (Fig. 3; Table I), which show high incidence of apoptotic cell death during neurogenesis (Ferrer et al., 1990a, 1992; Wood et al., 1992; Spreafico et al., 1995; Blaschke et al., 1996, 1998). In these regions, spontaneous fluctuations of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) mediated by Ca^{2+} released from intracellular stores occur during neurogenesis (Owens and Kriegstein, 1998). Moreover, many neurotransmitters have been

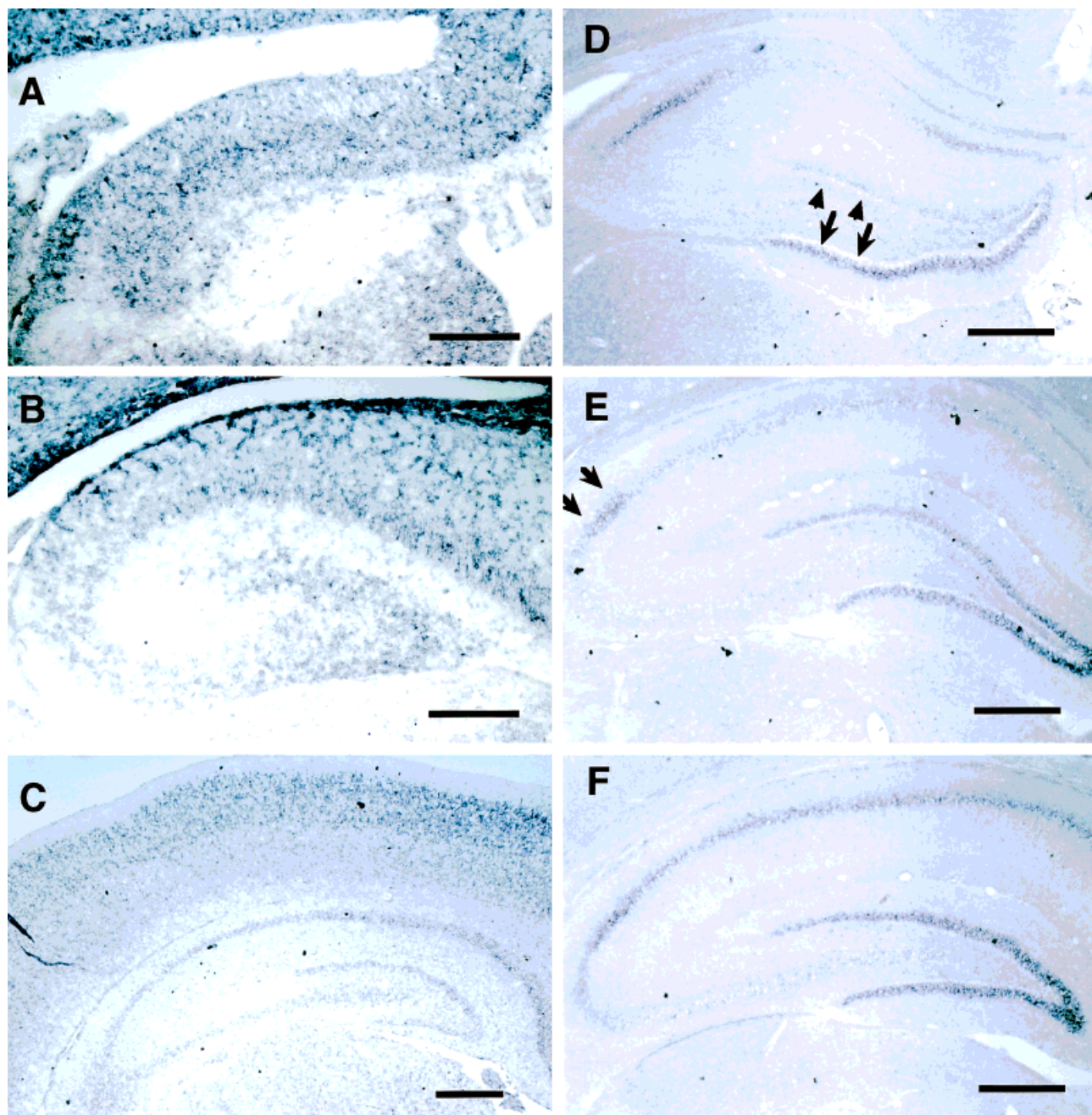


Fig. 4. Developmental change in distribution of DAP-kinase mRNA in the hippocampus at E18 (A), E22 (B), P7 (C), rostral coronal section at P21 (D), central coronal section at P21 (E), and caudal coronal section at P21 (F). In rostral section, signals were restricted in lower lips of dentate gyrus (arrows in D) and

weak expression of DAP-kinase mRNA was observed in upper lips of dentate gyrus (arrowheads in D). In central section, strong expression was shown in CA2 (arrows in E). Bars = 250 μ m for A, B; 500 μ m for C–F.

implicated in causing a rise in the $[Ca^{2+}]_i$ in developing neurons. For instance, glutamate activates the glutamate receptor channels that contribute to fast excitatory synaptic transmission, resulting in an increase in the $[Ca^{2+}]_i$ (LoTurco et al., 1991, 1995). Gamma aminobutyric acid (GABA), the principal inhibitory transmitter in the adult brain, also acts as the excitatory transmitter during neocortical neurogenesis and leads to a rise in the $[Ca^{2+}]_i$ via the

voltage-gated Ca^{2+} channel activation (LoTurco et al., 1995; Owens et al., 1996). Recent study demonstrated that Ca^{2+} /calmodulin-dependent protein kinases II and IV were activated in PC12 cells when depolarized with KCl (Solem et al., 1995). Thus, since DAP-kinase is regulated by Ca^{2+} /calmodulin, it is conceivable that the increase in $[Ca^{2+}]_i$ during neocortical neurogenesis activates DAP-kinase and that the activated DAP-kinase induces apoptotic cell death.

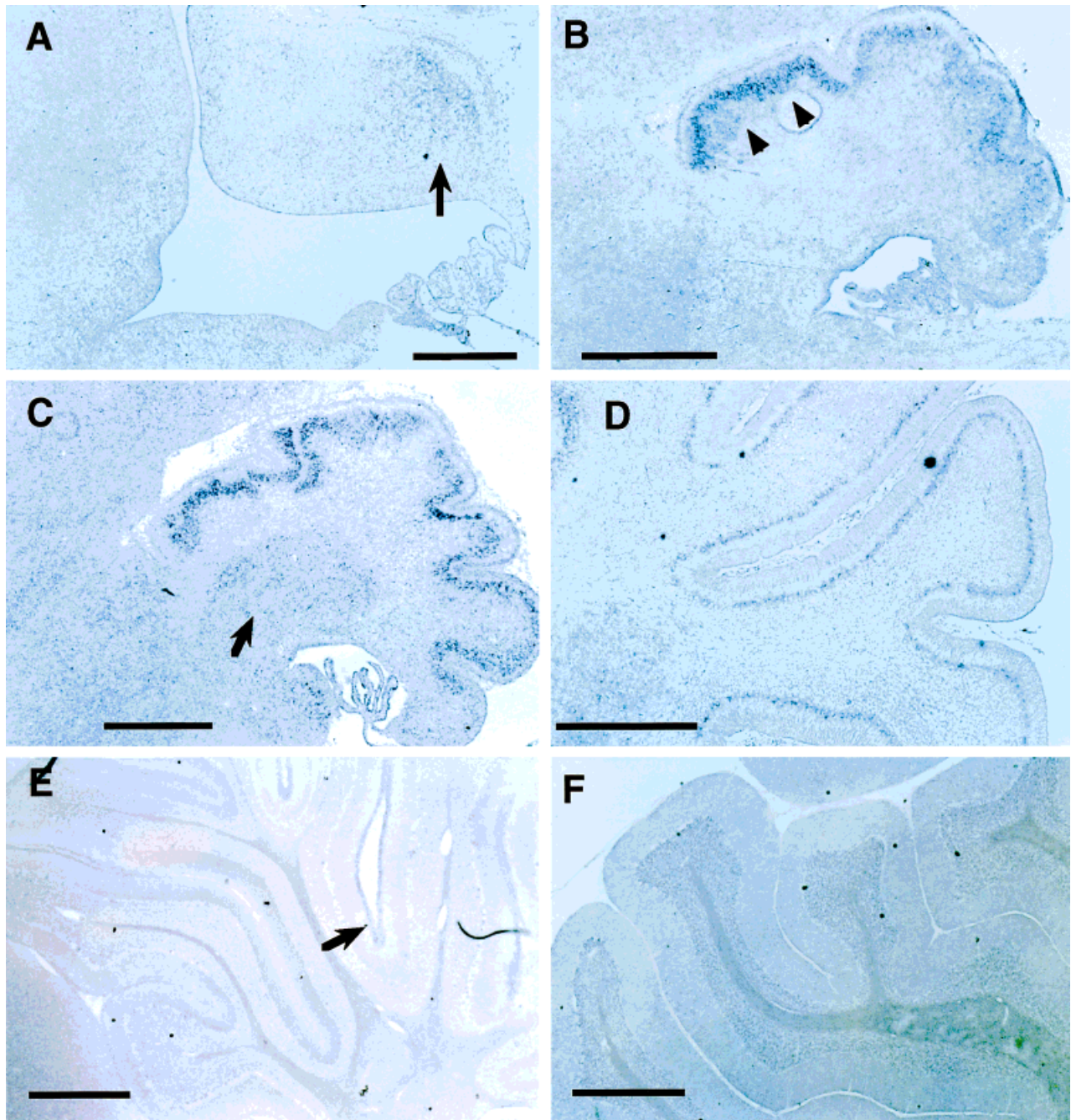


Fig. 5. Developmental change in distribution of DAP-kinase mRNA in the cerebellum at E18 (A), E22 (B), P3 (C), P7 (D), P14 (E), and adult (F). Purkinje neuron precursors and precursor cells of cerebellar deep nucleus expressed DAP-kinase mRNA (arrow in A). Purkinje cells in rostral part of cerebellum

showed intensive signals (arrowheads in B). Deep nucleus expressed DAP-kinase mRNA (arrow in C). Low levels of expression were exhibited in external granular cells (arrow in E). Bars = 500 μ m.

DAP-kinase mRNA was abundantly detected in developing hippocampus (Fig. 4; Table I), where widespread apoptotic cell death occurs during the first postnatal week (Ferrer et al., 1990b; Gould et al., 1991). In hippocampus, activation of GABA_A receptors depolarizes immature neurons, leading Ca²⁺ influx through the

voltage-gated Ca²⁺ channels and *N*-methyl-D-aspartic acid (NMDA) receptor channels (Fiszman et al., 1990; Ben-Ari et al., 1997). Thus, it is possible to speculate that DAP-kinase may be involved in naturally occurring cell death in developing hippocampus. The intense signals of DAP-kinase mRNA expression persist until the adult

TABLE I. Relative Expression Levels of Death-Associated Protein (DAP)-Kinase mRNA in Various Rat Brain Regions by In Situ Hybridization Analysis

Rat brain region	Developmental stage ^d					
	E18	E22	P7	P14	P21	Adult
Cerebral cortex						
Marginal zone						
Neocortex layer 1	+ ^a	+	-	-	-	-
Cortical plate						
Neocortex layer 2	++	+++	+++	++	++	+
Neocortex layer 3	++	+++	+++	++	++	+
Neocortex layer 4	++	+++	+++	+	-	-
Neocortex layer 5	++	+++	++	+	-	-
Neocortex layer 6	++	+++	++	+	-	-
Cortical subplate						
Neocortex layer 7	++	++	++	++	# ^b	#
Intermediate zone						
White matter	-	-	-	-	-	-
Subventricular zone						
White matter	++	++	#	#	#	#
Ventricular zone	+++	+++	#	#	#	#
Pyriform cortex	++	++	++	++	++	++
Hippocampus						
CA1	++	+++	+++	+++	+++	+++
CA2	++	+++	+++	+++	+++	+++
CA3	++	+++	+++	++	+	+
CA4	++	+++	+++	++	+	+
Dentate gyrus	#	+++	+++	+++	+++	+++
Interneuron	#	-	-	-	-	-
Subicular area	++	+++	++	+	+	+
Cerebellum						
Purkinje cell layer	+++	+++	+++	+	-	-
External germinal layer						
Granular cell layer	+++	-	-	+	-	-
Medial deep nucleus	+++	+	+	-	-	-
Fiber tracts	-	-	-	-	-	-
Hypothalamus	++	+	+	-	-	-
Midbrain	++	+	+	-	/	/
Olfactory bulb	++	+	+	+	/	/
Septum	++	++	+	+	/	-
Thalamus	+	++	+	+	+	+
Pons	++	++	++	+	/	+
Medulla oblongata	++	++	++	-	/	/
Spinal Cord	++	++	+	-	-	-
Amygdala, Preoptic area	+++	++	+	+	-	-
Basal ganglia	++	++	+	+	-	-

^aRelative signal intensities were estimated by visual comparison, as follows: +++, high; ++, moderate; +, low; -, background level; /, not determined.

^b#, this region does not exist at the developmental stage.

^c***, expression patterns of DAP-kinase mRNA in hippocampus were different among rostral, central, caudal coronal sections.

^dE, embryonic day; P, postnatal day.

stage. What is the role of DAP-kinase in adult hippocampal neurons? One possibility is that DAP-kinase possesses other functions relating to synaptic transmission, plasticity, and so on. Another possibility is that DAP-kinase provokes the apoptotic signal, but there may be factor(s) which can prevent DAP-kinase-induced cell death. Neurotrophin-3 (NT-3) may be one candidate molecule. NT-3 has important effects in maintenance of function of subpopulations of hippocampal neurons (Col-

lazo et al., 1992; Ip et al., 1993). Furthermore, it is noteworthy that distributions of NT-3 mRNA in hippocampus are strikingly similar to the particular patterns of DAP-kinase mRNA expression (unpublished data).

In the cerebellum, the widespread expression of DAP-kinase mRNA was observed during the neonatal period. After birth, DAP-kinase transcripts were prominent in the Purkinje cells until P7 and declined thereafter to undetectable levels by P21 (Fig. 5; Table I). The period

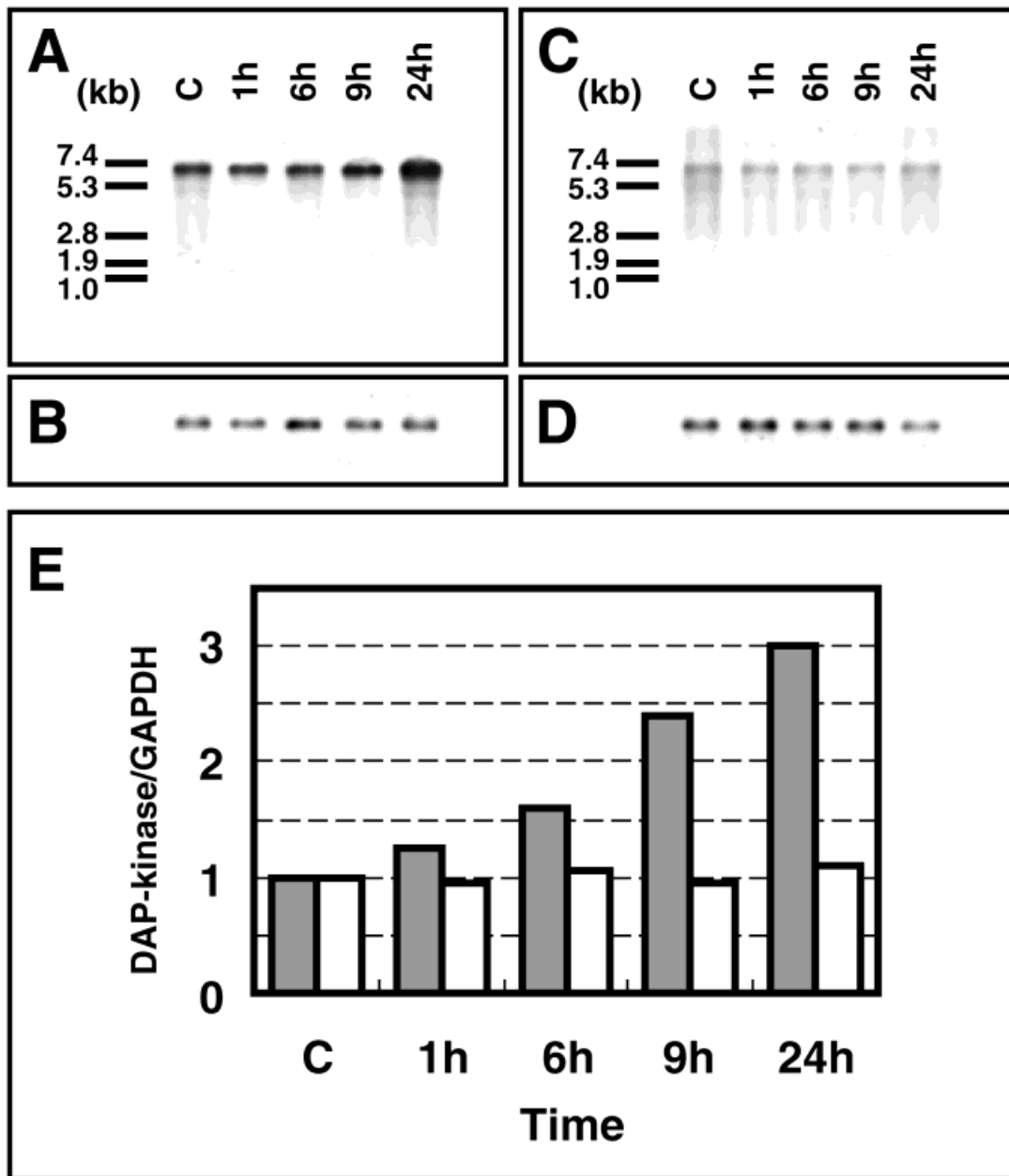


Fig. 6. Northern blot analysis of DAP-kinase mRNA expression after transient forebrain ischemia by four-vessel occlusion. The mRNAs were extracted from cerebral cortex (**A and B**) and cerebellum (**C and D**) at 1 hr, 6 hr, 9 hr, and 24 hr after 20 min of transient forebrain ischemia. A and C: Poly (A) mRNAs (2 µg) were subjected to RNA blot analysis using DAP-kinase-specific DIG-labeled cRNA probe. C represents the sham-operated

control. RNA size markers are shown on the left. B and D: The same blot was rehybridized with DIG-tailed oligonucleotide probe for GAPDH as internal control. E: The relative expression levels of DAP-kinase mRNA in cerebral cortex (filled bars) and in cerebellum (open bars) are shown as the ratio of DAP-kinase mRNA/GAPDH mRNA using a densitometer. The ratio of DAP-kinase mRNA/GAPDH mRNA of sham-operated control was defined as 1.

of DAP-kinase expression corresponds to the time of the maturation of Purkinje cells (Hatten and Heintz, 1995). Early in the postnatal period, migration of granule cells and connections between parallel fibers and Purkinje cells take place. Subsequently, Purkinje cells grow, develop their characteristic dendritic branches, and form synaptic contacts at the dendritic spines. These final steps in Purkinje

cell differentiation occur during the second and third postnatal weeks. It is therefore conceivable that DAP-kinase may be involved in the synaptic maturation in Purkinje cells.

It is important to determine whether or not DAP-kinase is involved in the neuronal cell death. Therefore, the expression of DAP-kinase mRNA was examined prior to a certain cell death by transient forebrain ischemia. The

transient forebrain ischemia by four-vessel occlusion is known to cause the delayed neuronal cell death in cerebral cortex, but not in cerebellum (Pulsinelli et al., 1982). The expression of DAP-kinase mRNA in cerebral cortex gradually increased at 1–24 hr after transient forebrain ischemia, whereas that in cerebellum remained at a low level (Fig. 6). The present findings that the level of DAP-kinase mRNA in cerebral cortex was increased preceding the ischemic cell death surely indicate the possible relationship between DAP-kinase and neuronal cell death.

In conclusion, this study has shown that DAP-kinase mRNA was predominantly detected in proliferative and postmitotic regions in developing brain, and especially expressed in hippocampus until the adult stage. Furthermore, the evidence that the expression of DAP-kinase mRNA was increased prior to a certain cell death by transient forebrain ischemia indicates a possible relationship between DAP-kinase and neuronal cell death. Thus, a better understanding of the role of DAP-kinase could allow new insights for synaptic formation, synaptic plasticity, and neuronal cell death. To reveal the physiological and pathological role of DAP-kinase in detail, further experiments must be undertaken, including work on DAP-kinase-deficient mice.

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