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Alteration of Apoptotic Protease-Activating Factor–1 (APAF-1)–Dependent Apoptotic Pathway During Development of Rat Brain and Liver

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Brain and liver extracts of rats at different stages after birth were examined for cytochrome *c*/dATP-dependent caspase (DEVDase)-activation (mitochondria pathway) *in vitro*. The caspase-activating activity in the brain extracts rapidly decreased after birth, reaching approximately 50 and 5%, at 1 and 2 weeks, respectively, of that in a 3-days-newborn sample, and essentially no caspase-activation was detected in the adult rat brain extracts. Such a dramatic change was not detected in the liver samples, suggesting that the observed abrogation of the cytochrome *c*–dependent mitochondria pathway after birth is a brain-specific event. In order to determine the factor(s) lacking in adult brain, we separately measured Apaf-1, procaspase 9, and pro-DEVDase activities using a supplementation assay. In adult brain, Apaf-1 activity was scarcely detected, while the tissue retained low but significant amounts of procaspase 9 (16% of that in the fetal tissue) and a pro-DEVDase (3.4%). In contrast, adult liver extracts retained relatively high levels of all of these factors. Immunoblot analyses clearly indicated that the expression of Apaf-1 and procaspase 3 is markedly suppressed within 4 weeks after birth in brain tissue while they are even expressed in adult liver. Considering these results together, we propose that, in the brain, the cytochrome *c*–dependent mitochondria pathway, which is essential for the programmed cell death during normal morphogenesis, is abrogated within 2–4 weeks after birth, whereas the pathway is still active in other adult tissues such as liver.

Key words: Apaf-1, brain, caspase 9, caspase 3, mitochondria pathway.

The cytochrome *c*–dependent mitochondria pathway of apoptosis has been shown to play a major role in the morphogenesis of the normal brain (*1–4*). It is also known that this pathway is one of the two major routes for the caspase activation cascade during the apoptosis of a wide variety of cells induced by various apoptotic stimuli (*5, 6*). An *in vitro* system for activating this pathway has been established by Wang and his group (*7–9*). The formation of an apoptosome composed of Apaf-1 and procaspase 9 in the presence of dATP (or ATP) is triggered by cytochrome *c* released from mitochondria, and then the activated procaspase 9 in the apoptosome processes terminal caspases (*9–13*). Thus, Apaf-1 and procaspase 9 are the essential components of the caspase-activating pathway. In a recent study, we attempted to purify Apaf-1, procaspase 9, and terminal caspases from adult bovine (2 to 3-year-old oxen) tissues including thymus, brain, and liver. We found that the adult bovine brain almost completely lacked Apaf-1 but retained significant amounts of procaspase 9 and procaspase 3 (the details of the study will be presented elsewhere). Since Apaf-1 is known to be highly expressed in the fetal mouse brain and to perform a major role in the programmed cell death during normal morphogenesis (*1, 2*), we examined whether or not the expression of Apaf-1 in the brain varies with the developmental stage. In the present study we found that, in the brain, the expression of Apaf-1, and thus the cytochrome *c*–dependent, mitochondria pathway, is almost completely abrogated within 2 to 4 weeks after birth, whereas the pathway is still active in adult liver tissue. The biological significance of this will be discussed.

MATERIALS AND METHODS

Tissues—Wistar rats were purchased from Japan SLC (Hamamatsu, Shizuoka). Wistar rats pregnant for 20 days (E20) were anesthetized by ether inhalation and the fetuses were removed from the uteri by means of Caesarian section. Brain samples from fetal, 3-days postnatal, 1, 2, 4, and 8-week-old, and adult (14 to 15-week-old) rats were prepared after the cerebellum had been carefully eliminated. Liver samples also were prepared from these animals. These rat tissues were immediately used for the preparation of tissue extracts. The animals were treated according to the guidelines published in the NIH Guide for the Care and Use of Laboratory Animals.

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Abbreviations: Apaf-1, apoptotic protease-activating factor-1; EGTA, ethylene glycol bis(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid.

Preparation of Brain and Liver Extracts—Tissue samples of brain and liver were homogenized with a Teflon-glass homogenizer in 2 volumes of extraction buffer containing 25 mM Tris-HCl buffer, pH 7.4, 0.15 M NaCl, 2 mM EGTA, 5 mM $MgCl_2$, 1 mM DTT (dithiothreitol), and 0.5 mM PMSF (phenylmethylsulfonyl fluoride). Then the homogenates were centrifuged at 13,000 ×*g* for 30 min and the supernatants obtained were used as tissue extracts. The samples could be stored at –85°C without appreciable loss of caspase-activating activity for at least several months.

Preparation of Bovine Thymus Apaf-1, Pro-caspase 9, and Pro-DEVDase—Partially purified Apaf-1, procaspase 9, and pro-DEVDase, without cross contamination, were prepared from bovine thymus as will be described elsewhere.

Caspase Substrate—Synthetic substrate peptides conjugated with 7-amino-4-methyl-coumarin (Ac-DEVD-MCA and other caspase substrates) were purchased from Peptide Institute, Osaka.

Assay for Caspase Activity—The reaction mixture containing 20 μM Ac-DEVD-mca, 25 mM Tris-HCl buffer, pH 7.4, and 10 μl of enzyme sample, in a total volume of 125 μl, was incubated at 30°C for 30 min. After termination of the reaction, the cleavage of the substrate was assayed by measuring fluorescence with a Shimadzu RF 5000 Spectrofluorometer, with excitation at 380 nm and emission at 460 nm. One unit of DEVDase was arbitrarily defined as the amount of enzyme required for the hydrolysis of 14 pmol of the substrate. Assaying of other caspases was performed as above except that Ac-DEVD-MCA was replaced by a synthetic peptide specific to the respective caspase.

Assays for Cytochrome c/dATP-Dependent Caspase-Activating Activity in Tissue Extracts—An appropriate amount of a brain or liver extract (10–150 μg protein) was incubated at 30°C for 30 min in a reaction mixture containing 1 mM dATP, 40 μg/ml cytochrome *c*, 5 mg/ml BSA (bovine serum albumin), and 1 mM DTT, in a final volume of 10 μl. DEVDase activity was measured before and after incubation as described above. One unit of caspase-activating activity was tentatively defined as one unit of DEVDase-activation attained under the conditions used.

Supplementation Assay for Apaf-1, Procaspase 9, and Terminal Pro-DEVDase—Assaying of one of the three factors essential for cytochrome *c*/dATP-dependent caspase-activation was carried out by adding excess amounts (125 units) of the other two factors in place of a tissue extract in the caspase-activating assay as described above. One unit of a factor was tentatively defined as the amount of the factor corresponding to one unit of DEVDase-activation under the conditions used.

Immunoblot Analysis—SDS–polyacrylamide gel electrophoresis (SDS-PAGE: 7.5 or 10% gels) and transblotting onto a PVDF membrane (Pall Biosupport, USA) were carried out as described previously (*14*). The membrane was blocked with 4% skim milk in PBS, and then probed with primary and secondary antibodies successively. The immuno-complex was detected using ECL-plus Western blot detection reagents (Pharmacia-Amersham).

Antibodies—Primary antibodies, rabbit polyclonal anti–human Apaf-1 (Chemicon International, Temecula, CA, USA), rabbit polyclonal anti–human caspase-3 (Pharmingen, San Diego, CA, USA), and mouse monoclonal anti–human caspase 9 (MBL Medical Laboratories, Nagoya) were obtained from commercial sources as indicated in parenthesis. Secondary antibodies, goat anti–rabbit IgG and rabbit anti–mouse IgG conjugated with horseradish peroxidase were obtained from DAKO Japan (Kyoto).

Protein Assay—Protein was assayed using Bio-Rad protein assay kits.

RESULTS

Age-Dependent Change in Cytochrome c- and dATP-Dependent, Caspase-Activating Activity in Rat Brain and Liver Extracts—Brain extracts were prepared from fetal (E20), 3-day newborn, and 1- and 2-week-old, and adult rats. All the extracts showed very low caspase (DEVDase) activity when assayed without activation (data not shown). However, when the fetus (E20) brain extract was incubated with 1 mM dATP and 40 μg/ml cytochrome *c* at 30°C for 30 min in a reaction mixture for caspase-activation, more than 100-fold activation of a terminal caspase (DEVDase) was detected. Both cytochrome *c* and dATP were essential for the activation (data not shown), indicating that the observed activation of the terminal caspase was through the cytochrome *c*-dependent mitochondria pathway (*7–9*). When the change in caspase-activating activity during the development of rats was examined, the activity in brain extracts became maximum at 3 days after birth and then rapidly decreased within 2 weeks (Fig. 1). Surprisingly, essentially no activity was detected in adult rat brain extracts.

Fetal liver extracts showed approximately 12% of the activity in the fetal brain sample, when compared on a protein basis. In contrast to the brain ones, the liver samples showed relatively small variation in the caspase-activating activity throughout life (from E20 to 14 weeks after birth, at least), although a transient decrease was detected at 1 week (Fig. 1). Thus, adult rat liver still retained approximately 70% of the DEVDase-activating activity of the fetal liver. These results suggest that the cytochrome *c*-dependent mitochondria pathway is abrogated at around 2 weeks after birth in rat brain and that this developmental event

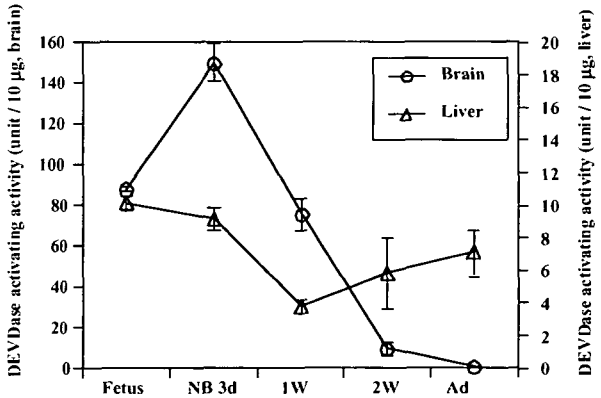

Fig. 1. **Cytochrome *c*/dATP-dependent, DEVDase-activating activity in rat brain and liver extracts.** Brain (40 μg; open circles) and liver (95 μg; open triangles) extracts from fetal, newborn (NB 3d), 1-week-old (1W), 2-week-old (2W), and adult (ad) rats were subjected to the cytochrome *c*/dATP-dependent caspase-activation assay as described under "MATERIALS AND METHODS." The activities are expressed on a protein basis.

is rather specific to the brain tissue.

Adult Rat Brain Extracts Restore the DEVDase-Activating Activity upon Supplementation of Exogenous Apaf-1—In order to identify the caspase-activating factor(s) lacking in the adult rat brain, we supplemented the extract with either partially purified bovine thymus procaspase 9 or Apaf-1, and then the sample was subjected to DEVDase activation. As shown in Table I, the brain extracts of adult rats restored the DEVDase-activating activity after Apaf-1 had been added, while procaspase 9 supplementation was totally ineffective. Similar results were obtained with adult bovine (Table I) and mouse (data not shown) brain extracts. These results indicate that the brain tissues of adult animals commonly lack the cytochrome *c*-dependent mitochondria pathway, mainly due to the lack of Apaf-1 in the tissues.

In contrast to the brain ones, the extracts of adult liver tissues from rats and oxen showed basal DEVDase-activating activity without the supplementation of these factors, although 6 to 7-fold stimulation with supplemented Apaf-1 was observed. This suggests that Apaf-1 is also a major rate-limiting factor of the DEVDase activation pathway in adult liver tissues.

Changes in Apaf-1, Procaspase 9, and Pro-DEVDase Activities during Development of Rat Brain and Liver—In order to separately measure the amounts of the factors involved in the cytochrome *c*-dependent caspase-activating pathway we carried out a supplementation assay, as described under "MATERIALS AND METHODS." As shown in Table II, in the brain, the Apaf-1 activity had decreased to 23% of that in the fetal brain at 2 weeks after birth and had become almost undetectable in the adult rat sample. Although procaspase 9 and pro-DEVDase activities were also markedly decreased in adult rat brain, the tissue still retained 36% of the procaspase 9 activity and 23% of the pro-DEVDase activity when compared to the liver tissue of adult rats on a protein basis (Table II). On the other hand, these three factors did not show such a remarkable change during the development of liver except that the Apaf-1 activity in an adult sample was decreased to 36% that in a fetal sample.

Characterization of the Terminal Caspase Activated in Fetal and Adult Rat Brain Extracts—The terminal procaspases in the fetal and adult rat brains extracts were activated by supplementation of bovine thymus Apaf-1, as described under "MATERIALS AND METHODS." The substrate specificity of the terminal caspase activated under these conditions was examined with the use of various synthetic substrates, which are relatively specific to a caspase class (*15*). As shown in Table III, the caspases in both fetal and adult rat brain extracts showed a quite similar preferences for some of the substrates. The substrate preference was in the order of DEVD>VEID>IETD>others, suggesting that a class of terminal pro-DEVDase, probably caspase 3 judging from the highest affinity for Ac-DEVD-mca, is activated in a cytochrome *c*-dependent manner in both tissues.

Immunoblot Analysis of Apaf-1 and Procaspases of Rat Brain and Liver—Protein expression of Apaf-1 and procaspases in brain and liver extracts of rats at various stages of development was examined by immunoblotting (Fig. 2). All of Apaf-1, procaspase 3, and procaspase 9 in extracts of both brain and liver showed a tendency to decrease gradually with increasing age. However, among them, the changes in the amounts of Apaf-1 and procaspase 3 in brain extracts were most dramatic (Fig. 2, A and B). Both of them disappeared almost completely from the brain extracts after 4 weeks. Note that all of Apaf-1, procaspase 9 and procaspase 3 are detectable in liver extracts of adult rats, while Apaf-1 and procaspase 3 decrease to almost undetectable levels in brain extracts of adult rats.

Characterization of Procaspase 9 of Fetal and Adult Rats—We examined the molecular size and processing of

TABLE I. **Adult rat brain extracts restore the pro-DEVDase activating ability by supplementing Apaf-1.**

Extract		DEVDase activated (units/10 μg)		
		Factor supplemented		
Animal	Tissue	None	Procaspase 9	Apaf-1
Rat	Brain	<1.7	<1.7	24 ± 0.1
	Liver	29 ± 2.8	54 ± 1.8	164 ± 7.7
Bovine	Brain	<1.3	<1.3	62 ± 1.1
	Liver	17 ± 0.9	19 ± 1.1	114 ± 1.4

Appropriate amounts of extracts obtained from the brains and livers of adult rats and oxen were subjected to the DEVDase activation assay without or with supplementation of either purified thymus procaspase 9 (125 units) or Apaf-1 (125 units) as described under "MATERIALS AND METHODS." DEVDase activity attained after activation is shown. The mean of three samples is shown with SEM.

TABLE II. **Apaf-1, procaspase 9, and pro-DEVDase activities in brain and liver extracts of rats of different ages.**

Tissue	Age	Activity (units/10 μg)		
		Apaf-1	Procaspase 9	proDEVDase
Brain	Fetus	145 ± 6.3	40 ± 11	51 ± 12
	2W	33.7 ± 1.6	36	29
	Adult	<0.5	8.4 ± 0.8	3.2 ± 0.1
Liver	Fetus	20 ± 0.1	24.7 ± 2.6	14.3 ± 2.4
	2W	19.7	19.1 ± 1.7	10.2 ± 0.5
	Adult	7.1 ± 0.1	23.1 ± 3.0	14 ± 2.3

Brain and liver extracts were isolated from fetal, 2-week-old (2W), and adult rats. Differential assaying of Apaf-1, procaspase-9, and pro-DEVDase activity was carried out with supplementation of the other 2 factors as described under "MATERIALS AND METHODS." The average of duplicated assays or the mean of three samples with SEM is shown.

TABLE III. **Substrate specificity of a terminal caspase in the extracts of the fetal and adult rat brains.**

Substrate (caspase)	Activity (%)	
	Fetus	Adult
DEVD (caspase 3)	100	100
YVAD (caspase 1)	3	1
VEID (caspase 6)	27	19
IETD (caspase 8)	19	8
WEHD (caspase 1, 4, 5)	7	2
LETD (caspase 9)	6	6

The extracts of fetal rat brain (39 μg) were subjected to activation by incubation in a assay mixture for caspase activation, as described under "MATERIALS AND METHODS." Adult brain extracts (37 μg) were supplemented with 125 units each of purified bovine thymus Apaf-1 and procaspase 9, and then the samples were activated as above. Assaying of a terminal caspase in 2 μg of fetal and 37 μg of adult samples was performed as described under "MATERIALS AND METHODS" except that the substrate (Ac-DEVD-MCA) was replaced by the same concentration of various synthetic substrates, as indicated in the table. Relative activity (%) was calculated from the mean of duplicated assays by setting the activity with Ac-DEVD-MCA as 100%.

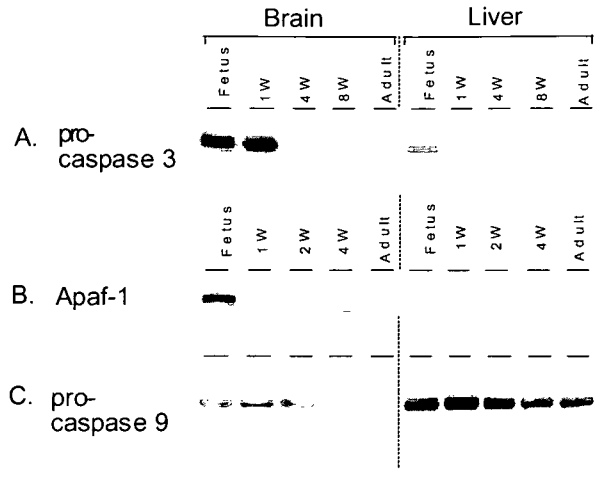

Fig. 2. **Immunoblotting of procaspase 3, Apaf-1, and procaspase 9 in rat brain and liver extracts.** Brain and liver extracts were isolated from fetal, 1-week-old (1W), 2-week-old (2W), 4-week-old (4W), 8-week-old (8W), and adult rats. The samples (30 μg/lane) were analyzed by SDS-PAGE, followed by immunoblotting with anti–caspase 3 (A), anti–apaf-1 (B), and anti–caspase 9 (C) antibodies, as described under "MATERIALS AND METHODS."

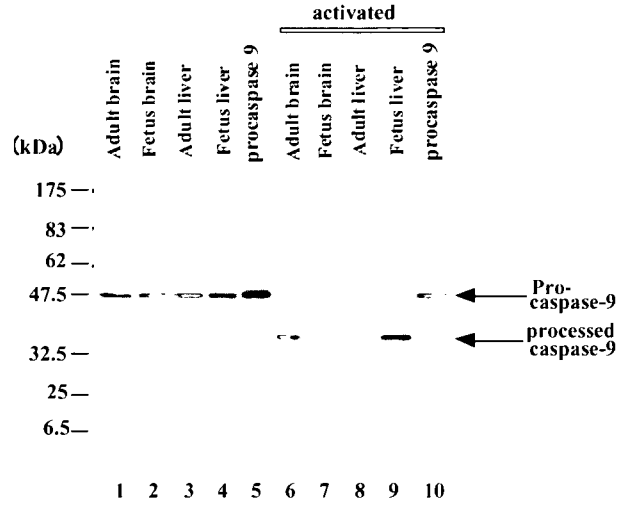

Fig. 3. **Immunoblot analysis of procaspase 9 in brain and liver extracts of adult and fetal rats.** Brain extracts of adult (lane 1, 10 μg) and fetal (lane 2, 5 μg) rats, and liver extracts of adult (lane 3, 10 μg) and fetal (lane 4, 5 μg) rats were analyzed by 10% SDS-PAGE. The processing of procaspase 9 in these samples was carried out as follows. The extracts were supplemented with bovine thymus Apaf-1 (200 units Apaf-1 per 40 μg extract), and then the samples were subjected to activation by incubation in the mixture for cytochrome *c*/dATP-dependent caspase-activation for 15 min as described under "MATERIALS AND METHODS." The processed brain and liver samples were subjected to SDS-PAGE as described above (lanes 6 to 9). Purified bovine thymus procaspase 9 (200 units, lane 5) and a sample activated as above (lane 10) were included as standards.

procaspase 9 in extracts of rat brain and liver by means of immunoblotting. As shown in Fig. 3, immunoblotting of the crude brain and liver extracts of fetal and adult rats with an anti–caspase 9 antibody revealed a single band at the position corresponding to 50 kDa. No cross-reacting band corresponding to a different molecular size was detected, indicating that all of these tissues, including fetal and adult tissues, express a single class of procaspase 9 molecule. When these extracts were subjected to activation by incubation with excessive amounts of purified bovine thymus Apaf-1, cytochrome *c*, and dATP, procaspase 9 was processed into a 36 kDa form in all of the samples examined.

DISCUSSION

The present results clearly demonstrated that the Apaf-1–dependent mitochondria pathway for caspase activation is closed in rat brain within 2 to 4 weeks after birth (Fig. 1). The marked and rapid suppression of the pathway was mainly due to the decrease in the protein expression of Apaf-1 and procaspase 3 in the tissue (Fig. 2). Among them, the down-regulation of Apaf-1 was almost complete (Fig. 2, and Tables I and II), and is considered to be the main reason for the complete suppression of cytochrome *c*/dATP-dependent caspase-activation in the mature rat brain extracts. Similar results were obtained with mature brain samples from mice (data not shown) and oxen (Table I). Although a gradual decrease of Apaf-1 with increasing age was also observed in the liver (Fig. 2 and Table II), the decrease was limited in range and the adult liver still retained 36% of the Apaf-1 activity in the fetal liver (Table II). Thus, abrogation of the cytochrome *c*-dependent mitochondria pathway observed in the normal mature brain seems to be a tissue-specific event. Although the present experiments were carried out with brain samples without the cerebellum, we have also observed that the caspase-activating activity in the cerebellum of adult rats decreased to 1% or less of that in 3-days-newborn (Ota, K., unpublished results). These events may be very important for the mature brain to protect itself from various exogenous apoptotic stimuli considering that regeneration and reconstruction of the central nervous system is restricted in adult animals. Concerning the abrogation of Apaf-1 expression in other tissue, Burgess *et al.* (*16*) have reported that human skeletal muscle cytosol also lacks Apaf-1.

There have been many reports indicating that caspase 3 is activated in the mature brain in response to various kinds of brain damage (*17–19*), or in a human neurodegenerative disease, amyotrophic lateral sclerosis (*20, 21*). However, the involvement of the cytochrome *c*-dependent mitochondria pathway in the caspase activation in the mature brain remains unclear. In this respect, Soengas *et al.* (*22*), and Meinhardt, Roth, and Totok (*23*) recently reported that Apaf-1 could be induced by 5-aza-2′-deoxycitidine and phorbol-12-myristate-13-acetate (PMA) in an Apaf-1 deficient melanoma cell line and U937 cells, respectively. Their findings suggested that, in some tissues, the mitochondria pathway might be regulated through the expression level of Apaf-1, while the caspase-activating factor is constitutively expressed as an inactive form in many other tissues. In fact, we recently found that Apaf-1 mRNA and protein expression were markedly increased after traumatic brain injury in adult rats (*24*).

The mature rat brain contained approximately 20% of the procaspase 9 activity in the fetal tissue (Table II), whereas the tissue contained a negligible amount of Apaf-1 and a very low level of procaspase 3. So far, no detectable difference has been found in the procaspase 9 samples from the fetal and adult rat tissues examined. The procaspase 9

in both samples showed a pro-form of 50 kDa and was processed to a 36-kDa form when activated by supplementation of exogenous Apaf-1 (Fig. 3). Also, no significant difference was found in the DEVDase-activating ability of the fetal and adult brain procaspase 9s (compare the activity and immunoblotting of the fetal and adult brain samples in Table II and Fig. 2C). Although the biological significance of the expression of the procaspase 9 in the normal mature brain remains unclear, the caspase may have some Apaf-1–independent roles as Sperandio, Belle, and Bredesen (*25*) have shown for Apaf-1 null mouse embryonic fibroblasts.

The present finding that the cytochrome *c*–dependent mitochondria pathway for caspase activation is closed in the normal mature brains of three species of animals mainly because of marked suppression of Apaf-1 expression provides useful information for the study of human neurodegenerative diseases. Information concerning Apaf-1 expression in the normal human brain and the change in various human neurodegenerative diseases is quite limited, although Engidawork *et al.* (*26*) recently reported that Apaf-1 detected in postmortem brain samples from patients with Alzheimer disease and controls was not significantly different. Probably, we need more information on the regulation of Apaf-1 expression in the mature brain during the progress of various brain diseases, considering that Apaf-1 expression was markedly enhanced after traumatic brain injury (*24*).

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