Involvement of nuclear factor of activated T-cells (NFATc) in calcineurin-mediated ischemic brain damage *in vivo*

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Abstract: Aim To study the involvements of nuclear factor of activated T-cells (NFATc) and NF-κB in calcineurin-mediated ischemic brain damage *in vivo*. **Methods** The rat transient forebrain ischemia conducted through 15 min ischemia followed by 8, 24, and 72 h reperfusion was induced using the four-vessel method. The rats were divided randomly into five groups; sham control group, ischemia/reperfusion (I/R) group, CsA treated groups (for 8, 24, and 72 h reperfusion). Western blotting was performed to detect changes of FasL, NFATc, I-κB-α, and phospho-I-κB-α protein expression, and gel shift assays for NFAT FasL-DNA binding activities. **Results** Western blotting showed that the expressions of both FasL and NFATc protein were significantly increased in the hippocampus of rat subjected to transient forebrain ischemia in comparison with those of the sham control group, which were markedly reduced by CsA. The I-κB-α protein showed no changes in all groups, and phospho-I-κB-α protein was not observed in this study. Proximal and distal FasL promoter NFAT sites bind NFAT proteins from the hippocampal neurons subjected to transient forebrain ischemia, and DNA-binding activities increased significantly compared with those of the sham control group. CsA markedly inhibited these changes. **Conclusion** NFATc may be involved in calcineurin-mediated ischemic brain damage and transcription factor NF-κB may not be involved.

Key words: calcineurin; nuclear factor of activated T-cells (NFATc); FasL; cyclosporin A; ischemic brain damage

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转录因子 NFATc在钙神经素介导的脑缺血再灌注损伤中的作用

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摘要: 目的 研究转录因子 NFATc及 NF-κB在钙神经素介导的脑缺血再灌注损伤中的作用。方法 Western blotting和 EMSA分子生物学技术。结果 与对照组相比较, CsA明显减低 I/R组 Fas配体和 NFATc的蛋白表达;对照组、I/R组和 CsA处理组 I-κB-α蛋白表达无显著区别;未观察到对照组、I/R组和 CsA处理组有 phospho-I-κB-α蛋白表达;与对照组相比较, CsA明显减低 I/R组 Fas配体启动子远端和 Fas配体启动子近端 NFAT结合位点的 NFAT-DNA结合活性($P < 0.01$)。结论 转录因子 NFATc参与钙神经素介导的脑缺血再灌注损伤,促进 CD95配体分子的转录表达; NF-κB可能未参与钙神经素介导的脑缺血再灌注损伤的作用机制。

关键词: 钙神经素; 转录因子 NFATc; Fas配体; 环孢素 A; 脑缺血再灌注损伤

Brain ischemia can elicit pathogenic cascade that leads to death of susceptible populations of neurons within a few days. However, little is known about the sequence of events that leads to ischemic neuronal

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death. Understanding these events at the molecular level is essential for developing treatments to prevent or reduce ischemic injury to the brain. A considerable amount of evidence suggests that excitotoxic overactivation of glutamate receptors, especially those of the NMDA-type, contributes to ischemia-induced neuronal cell death[1,2]. Since NMDA receptors are highly permeable to Ca^{2+}, influx of extracellular Ca^{2+} is considered to be the primary event responsible for glutamate toxicity. Although the mechanism of this increased Ca^{2+}-induced neurotoxicity is not completely understood, we speculated that calcineurin, a Ca^{2+}-regulated protein, may be associated with the neurotoxicity because the areas of the brain that are most vulnerable to ischemia largely coincide with regions containing a high concentration of calcineurin, such as the cerebral cortex, striatum, substantia nigra, cerebellum, and the hippocampus[3]. Moreover, there is both direct and indirect evidence that calcineurin can mediate apoptosis in certain types of cells, including neurons[4,5].

The known substrates for calcineurin include nitric oxide synthases (NOS), nuclear factor of activated T cells (NFAT)[4,6,7], NF-κB[8,9], the NMDA receptor and others[5]. Among the diverse function of calcineurin, its role in the immune system is relatively well understood. Ca^{2+}/Calmodulin-Calcineurin-NFAT-cytokine expression pathway has been highly focused on in recent years[4,6]. NFAT, as a downstream transcriptional effector of calcineurin, is a family of critical regulators of gene transcription in response to receptor-mediated signals. Four of the five NFAT proteins (NFATc, NFATp, NFAT3, NFAT4) reside in the cytoplasm in unstimulated cells but quickly translocate to the nucleus in response to stimulation that promote Ca^{2+} mobolization[4,6]. The Ca^{2+}/Calmodulin-activated phosphatase calcineurin physically interacts with NFAT members within the cytoplasm, where it directly dephosphorylates multiple serine residues within the N-terminal regulatory domain of NFAT, resulting in the unmasking of two nuclear localization sequences required for nuclear import[10]. Antigen receptor engagement on T lymphocytes activates transcription factors (NFAT) important for stimulating cytokine genes (such as the IL-2, IL-3, TNF-α, FasL *et al*) expression. This is critical for clonal expansion of antigen-specific T cells and propagation of immune response. In addition, the induced expression of FasL initiates subsequently the apoptosis of over-activated T-lymphocytes for its homeostasis[11].

Although a large number of studies have convincingly demonstrated the importance of calcineurin as a mediator of neurons apoptosis *in vivo* and *in vitro*, the importance of the downstream NFAT factors has not been evaluated in ischemic hippocampus *in vivo*. Recent studies found that FasL expressions in protein and mRNA levels increased significantly in ischemic hippocampus[12-14]. Based on above experimental results, we speculated that NFAT may be involved in calcineurin-mediated ischemic brain damage *in vivo* through mediating the FasL expression. Considering NF-κB as substrates for calcineurin, we observed also the expression of NF-κB to identify whether it is involved in calcineurin-mediated ischemic brain damage.

Materials and methods

Animals Experiments were performed on 10 to 12 week-old, 250 to 300 g female Sprague-Dawley rats (Experimental Animal Center of Hebei Medical University, certificate No: 04064).

Drug treatments CsA was from Fujian Kerui Pharmaceuticals, and dissolved in ethanol. CsA was injected intraperitoneally during 2 h after the reperfusion in doses of 10 mg·kg^{-1}. It was administered once each 24 h. Ethanol was injected ip in control group.

Cerebral ischemia model Forebrain ischemia involved occlusion of all four major extracranial arteries ("four-vessel occlusion" model).

The first day, the rats were anesthetized by intraperitoneal injection of 10% chloral hydrate. Body temperature was maintained at 37 ℃. The vertebral arteries were irreversibly occluded by electrocoagulation, after a delay of 1 day for recovery, both common carotid arteries were clamped. Rats lost their righting reflex within 1 min of carotid clamping. After 15 min of ischemia, cerebral circulation was restored by removal of clamp and continued for 8 h, 24 h, and 72 h separately.

Experimental animals grouping Preliminary results showed that Fas ligand protein increased in ischemic hippocampus, with the highest levels of expression at 24 h among three groups. So, the rats were divided into five groups: sham control group, $I_{15\,min}/R_{24\,h}$ group, CsA treatment group ($I_{15\,min}/R_{8\,h}$, $I_{15\,min}/R_{24\,h}$, and $I_{15\,min}/R_{72\,h}$ group separately).

Antibodies and reagents pAb against FasL, I-

κB-α, NFATc (H-110, sc-13033), mAb phospho-I-κB-α (B-9, sc-804), and secondary Ab with horseradish peroxidase conjugated IgG were obtained from Santa Cruz (USA). Gel Shift Assay Core System (E3050) was from Promega, NF-κB consensus oligonucleotide (5′-AGTTG AGGGG ACTTT CCCAG GC-3′) and NFAT consensus oligonucleotide[15] (NFAT distal: - 283 - - 263, 5′-GTGGG CGGAA ACTTC CAGG-3′; NFAT proximal: - 144 - - 126, 5′-TAGCT ATGGA AACTC TATA-3′) were synthesized by Shenggong (Shanghai, China). Following reagents were purchased respectively from: PMSF (Merck), aprotinin (Sigma), leupeptin (Sigma), pepstatinA (Sigma), BSA (Gibco), agarose (Promega), Acr/Bis (Gibco), DTT (Sigma), APS (Sigma).

Preparation of nuclear extracts[15] The whole hippocampus were lysed in 1 500 μL of buffer A (10 mmol· L^{-1} HEPES, pH 7.9, 1.5 mmol· L^{-1} $MgCl_2$, 10 mmol· L^{-1} KCl, 0.5 mmol· L^{-1} DTT, 0.5 mmol· L^{-1} PMSF, 0.5% NP-40) and homogenized immediately, the homogenates were centrifuged for 10 min at 1 850 g. The supernatant was removed and the pellet was lysed in 1 000 μL of buffer B (buffer A without NP-40) and allowed to stand on ice for 10 min, followed by centrifugation for 15 min at 3 300 g. The supernatant was removed and nuclei lysed in both 20 μL of buffer C (20 mmol· L^{-1} HEPES, pH 7.9, 10 mmol· L^{-1} KCl, 1.5 mmol· L^{-1} $MgCl_2$, 0.2 mmol· L^{-1} EDTA, 0.5 mmol· L^{-1} DTT, 0.5 mmol· L^{-1} PMSF, 20% glycerol) and 10 μL of buffer D (20 mmol· L^{-1} HEPES, pH 7.9, 400 mmol· L^{-1} KCl, 1.5 mmol· L^{-1} $MgCl_2$, 0.2 mmol· L^{-1} EDTA, 0.5 mmol· L^{-1} DTT, 0.5 mmol· L^{-1} PMSF, 20% glycerol) by tumbling on ice for 1 h. Lysates were then centrifuged for 30 min at 15 000 × *g*, the supernatants thus obtained were stored at - 80 ℃ until required for use. All procedures were carried out at 4 ℃.

Electrophoretic mobility shift assay EMSA were perfomed with 10 μg of nuclear extract protein in a volume of 9 μL according to Promega kit instruction. The binding reactions were incubated for 30 min at room temperature with double-stranded oligonucleotides end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. A 100-fold excess of unlabeled specific and nonspecific oligonucleotides were used as competitors.

Western blotting analysis Hippocampal tissue was lysed in a pharmalysis buffer (20 mmol· L^{-1} Tris· HCl, 1 mmol· L^{-1} EDTA, 5 mmol· L^{-1} $MgCl_2$, 50 mmol· L^{-1} NaCl, 1 mmol· L^{-1} DTT, 1 mmol· L^{-1} Na_3VO_4, 1 mmol· L^{-1} PMSF, 10 μg· mL^{-1} aprotinin, 2 μg· mL^{-1} leupeptin, 10 μg· mL^{-1} pepstatin A) and homogenized at 4 ℃. After centrifugation at 10 000 × *g* for 20 min at 4 ℃, protein concentrations were determined by the Bio-Rad protein assay. Proteins were boiled for 5 min, and loaded on an SDS-PAGE gel (12%, 50 μg per lane) followed by transference to nitrocellulose (NC) membranes. Membranes were blocked for 2 h at room temperature using 5% nonfat dry milk (for phospho-protein detection, dry milk was substituted with 3% bovine serum albumin) dissolved in Tris-buffered saline (20 mmol· L^{-1} Tris · HCl, pH 7.5, 500 mmol· L^{-1} NaCl). Anti-NFATc1, anti-FasL, anti-I-κB-α pAb was diluted 1∶200, and anti-p-I-κB-α mAb was diluted 1∶100 in blocking buffer. Membranes were incubated overnight at 4 ℃. Secondary Ab were used at a dilution of 1∶2 000 in blocking buffer and incubated for 2 h at room temperature. Signals were detected with DAB staining and scanned by gel analysis imaging system (VILBER LourMAT, France).

Statistical analysis Data are presented as the $\bar{x} \pm s$. Statistical comparisons were made by one-way ANOVA followed by Student′s *t* test (LSD), or Dunnett′s test. $P < 0.05$ was considered statistically significant.

Results

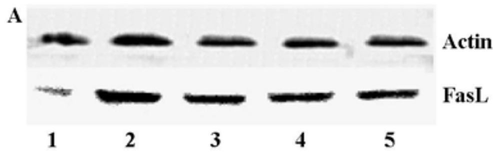
1 Effect of CsA on the expression of FasL, NFATc, I-κB-α, and phospho-I-κB-α proteins

Western blotting showed that the expressions of both FasL and NFATc protein increased significantly in the hippocampus of rat subjected to transient forebrain ischemia in comparison with those of the sham control group, and these increases were markedly reduced by CsA treatment ($P < 0.01$ for FasL, $P < 0.05$ for NFATc, respectively, Figure 1, 3). The I-κB-α protein showed no changes in all groups ($P > 0.05$, Figure 2). The expression of phospho-I-κB-α protein was not observed in this study.

2 Effect of CsA on NFAT FasL-DNA binding activities

We then examined NFAT-dependent regulation of FasL with EMSA method. A ^{32}P-labelled oligo from the FasL NFAT-binding site was used in gel mobility shift assays with nuclear extracts from rat hippocampi (10 μg). FasL-NFAT complex was separated on a 6%

nonreducing polyacrylamide gel, and NFAT in hippocampal nuclear extracts gave rise to specific DNA-binding activity with the FasL NFAT consensus site oligo, and competitor oligo was used at 100-fold excess. Proximal and distal FasL promoter NFAT sites bind NFAT proteins from the hippocampal neurons subjected to transient forebrain ischemia, and DNA-binding activities increased significantly compared with that of the sham control group. CsA treatment ($I_{15\ min}/R_{8\ h}$, $I_{15\ min}/R_{24\ h}$, and $I_{15\ min}/R_{72\ h}$ group) markedly inhibited these changes, respectively ($P < 0.05$ for proximal FasL binding site, $P < 0.01$ for distal FasL binding site, separately, Figure 4, 5).



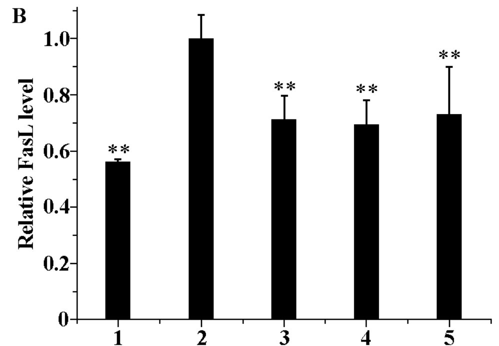
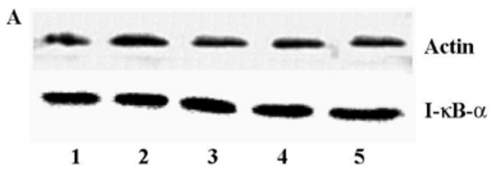


Figure 1　The expression of FasL in the hippocampal neurons subjected to transient forebrain ischemia. A: Whole cell lysates were immunoblotted with anti-FasL pAb (M_W of FasL: 38 kD); 1, 2, 3, 4, and 5 means sham control, $I_{15\ min}/R_{24\ h}$, and CsA treatment group ($I_{15\ min}/R_{8\ h}$, $I_{15\ min}/R_{24\ h}$, and $I_{15\ min}/R_{72\ h}$), respectively; B: Densitometric analysis is shown on the right panel ($n = 4$). ** $P < 0.01$ *vs* I/R group



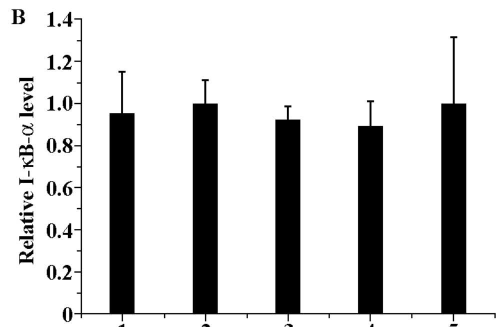
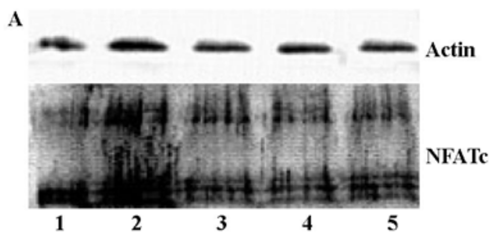


Figure 2　The expression of I-κB-α in the hippocampal neurons. A: Whole cell lysates were immunoblotted with anti-I-κB-α pAb (M_W of I-κB-α: 37 kD); symbols as in Figure 1; B: Densitometric analysis is shown on the right panel ($n = 4$)



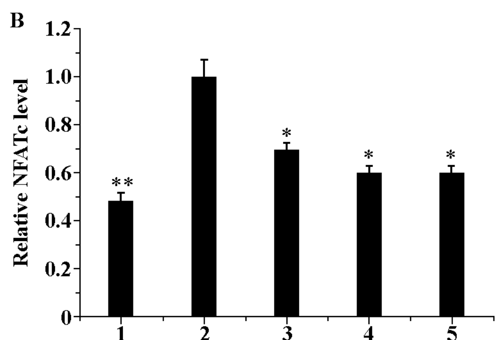


Figure 3　The expression of NFATc in the hippocampal neurons. A: Whole cell lysates were immunoblotted with anti-NFATc pAb (M_W of NFATc: 110 and 86 kD, the latter is predominant form); symbols as in Figure 1; B: Densitometric analysis is shown on the right panel ($n = 4$). * $P < 0.05$, ** $P < 0.01$ *vs* I/R group

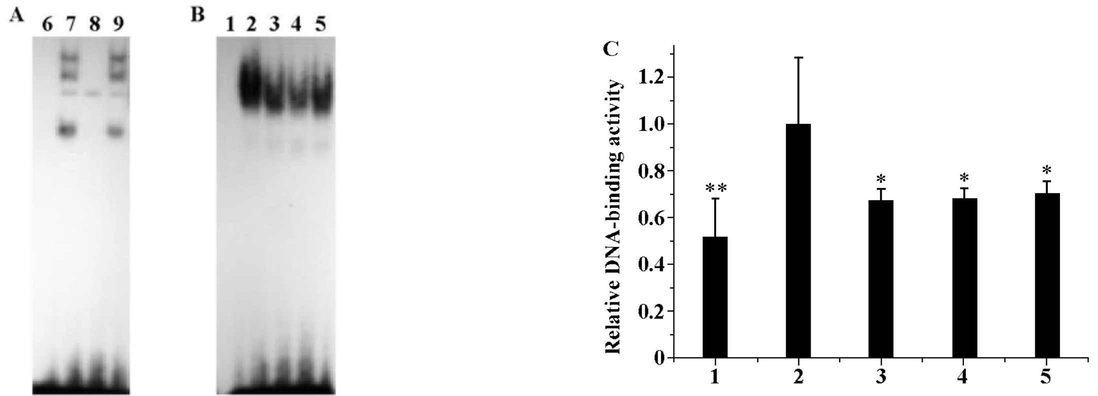


Figure 4 NFAT-dependent regulation of FasL. Proximal FasL promoter NFAT site binds NFAT proteins from rat hippocampi subjected to transient forebrain ischemia. A: Competitor oligo was used at 100-fold excess; Gel shift assays were performed with HeLa nuclear extract using NF-κB consensus oligo; Lane 7, positive control (^{32}P-labelled NF-κB consensus oligo); lane 8, specific competitor (^{32}P-labelled NF-κB oligo plus unlabelled NF-κB oligo); lane 9, nonspecific competitor (^{32}P-labelled NF-κB oligo plus unlabelled AP-1 oligo); lane 6, negative control (without HeLa nuclear extract); B: NFAT in hippocampal nuclear extracts gives rise to specific DNA-binding activity with the FasL NFAT consensus site oligo; symbols as in Figure 1; C: Densitometric analysis is shown on the right panel ($n = 4$). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* I/R group

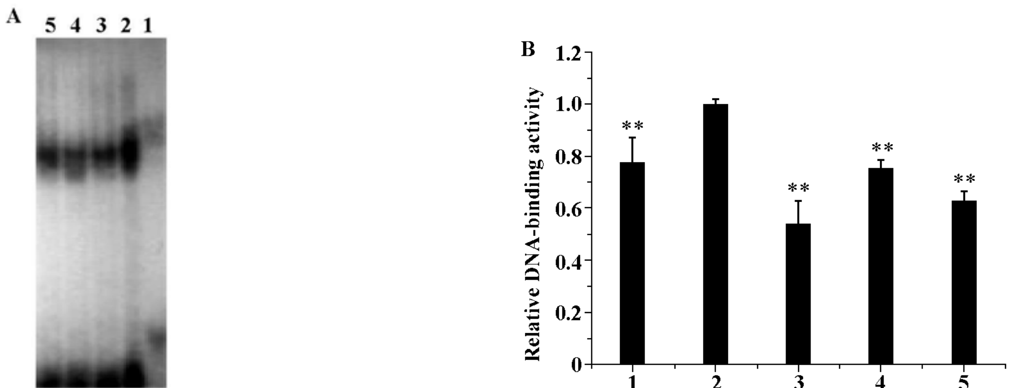


Figure 5 NFAT-dependent regulation of FasL. Distal FasL promoter NFAT site binds NFAT proteins from rat hippocampi subjected to transient forebrain ischemia. A: NFAT in hippocampal nuclear extracts gave rise to specific DNA-binding activity with the FasL NFAT consensus site oligo; symbols as in Figure 1; B: Densitometric analysis is shown on the right panel ($n = 4$). $^{**}P < 0.01$ *vs* I/R group

Discussion

The present study shows that both increments of FasL protein and NFATc protein expression in the whole cell lysates of ischemic hippocampus were inhibited, in part at least, by CsA, which suggests the requirement of calcineurin/NFAT in the expression of FasL. We also show that there is no NFAT FasL DNA-binding activities in sham control group, speculating that a basal level of NFAT activity may not be required for the maintenance of normal cell function. And that forebrain ischemia can cause a substantial up-regulation of both proximal and distal FasL promoter NFAT FasL DNA-binding activities, CsA decreased significantly the tendency of these changes, suggests that calcineurin/NFATc pathway may be involved in the expression of cytokine FasL and induction of apoptosis, and NFATc is required in the calcineurin-mediated ischemic brain damage. The ability of CsA to only partially blocks NFAT FasL DNA-binding activity suggests that calcineurin also may activate NFATc-independent pathways that lead to induction of apoptosis[16].

The NF-κB family of proteins are ubiquitously expressed and are inducible transcription factors that regulate the expression of genes involved in disparate processes such as immunity and inflammation, growth,

development, viral-gene transcription and cell-death regulation[17,18]. In general, activation of NF-κB is the result of I-κB phosphorylation by the multi-subunit I-κB kinase (IKK) complex, which leads to I-κB degradation. This permits NF-κB to translocate to the nucleus and activate transcription of target genes. A number of I-κB proteins have been identified, including I-κB-α, I-κB-β, I-κB-γ and I-κB-ε proteins, of which I-κB-α and I-κB-β are the best studied[17]. The results of our study demonstrate that the expression of I-κB-α protein in the whole cell lysates of ischemic hippocampus showed no significant changes between sham control group and I/R group, and p-I-κB-α protein expression was not observed both in sham control group and I/R group in the same conditions, which suggest that NF-κB transcription factor may not be involved in the ischemic brain damage. This result is not in good agreement with some reports[18,19], which showed that NF-κB activation was associated with neuroprotection against death-inducing stimuli such as oxidative stress and nitric oxide. The reasons for the apparent discrepancy may attribute to technical issues, severity of ischemia, duration of reperfusion currents, or specificity and sensibility of molecular approaches applied. In addition, some other observations[20] found that neurons lack inducible degradation of I-κB-α in response to multiple inducers, and observations that neurons lack significant inducible NF-κB activation may be important in maintaining an immunoprivileged status in neurons[20]. So, further work is needed to determine whether NF-κB is involved in the ischemic brain damage by using advanced, more specific and sensible approaches and controlling more strict experimental conditions.

Studies conducted in neurons have demonstrated either pro- or anti-apoptotic effects of calcineurin activation[5,16,21], depending on the status of p38 MAPK activation[21,22]. Lotem *et al*[21] have shown that calcineurin activation can lead to the activation of p38 MAPK and that p38 MAPK pathway may mediate the induction of apoptosis. CsA can block the activation of JNK and p38 MAPK signaling pathways in addition to mediate the suppression of NFAT pathway[22]. Recent studies[23] indicate that NFATc is a direct target of p38 MAPK. p38 MAPK exerts its stimulatory effect on stages of NFATc activation through activating the promoter and inducing the transcription, increasing the mRNA stability, enhancing the translation, and promoting the interaction with the coactivator CREB-binding protein, respectively. Moreover, inhibition of p38 MAPK leads to selective inactivation of NFATc[23]. We speculate that the cross-talk among these signaling transducing cascades finally converges on the activation of FasL/Fas pathway, which results in the induction of apoptosis in neurons.

It is generally accepted that many kinds of mammalian cells, including neurons, use common apoptotic machinery, such as the FasL/Fas pathway, to execute apoptosis. However, pathways upstream from this common machinery may be quite diverse, depending on cell types and apoptotic stimuli[16]. At the molecular level, calcineurin appears to participate in apoptosis in diverse ways including NFAT, p38 MAPK, even possible NF-κB pathway and cross-talk among these signaling pathways. In considering therapies for central nervous system diseases, it is important to assume that insults that induce neuronal death are multifactorial. However, if a common mechanism underlies the death of neuronal cells from various causes, it might be a good target of treatment. Our results suggest that calcineurin may be a potential target molecule in the treatment of central nervous system diseases.

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