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Research report

# The effect of cycloheximide on the regulation of proenkephalin and prodynorphin gene expressions induced by kainic acid in rat hippocampus

Je-Seong Won, Yung-Hi Kim, Dong-Keun Song, Hong-Won Suh *

*Department of Pharmacology, Institute of Natural Medicine, College of Medicine, Hallym University, 1 Okchun-Dong, Chunchon, Kangwon-Do 200-702, South Korea*

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**Abstract**

The effect of cycloheximide (CHX), a protein synthesis inhibitor, on the regulation of proenkephalin (proENK) and prodynorphin (proDYN) mRNA levels, proto-oncogenes, such as c-*fos*, 35-kDa fra and c-*jun* mRNA, and the levels of their products induced by kainic acid (KA) in rat hippocampus was studied. The proENK and proDYN mRNA levels were markedly increased 4 and 8 h after KA (10 mg/kg i.p.) administration. However, the intracellular proENK protein level was not affected by KA. The elevations of both proENK and proDYN mRNA levels induced by KA were inhibited by pre-administration of CHX (15 mg/kg i.p.). The increases of proENK and proDYN mRNA levels induced by KA were well-correlated with the increases of c-Fos, 35-kDa Fra and c-Jun protein levels. KA administration increased the hippocampal levels of c-Fos, 35-kDa Fra and c-Jun proteins with the time. The increases of c-Fos, 35-kDa Fra and c-Jun protein levels induced by KA administration were also inhibited by CHX pre-administration. KA administration markedly increased both c-*fos* and c-*jun* mRNA levels during 1 and 4 h and the increased levels of these proto-oncogene mRNA were further prolonged by the treatment with CHX. In addition, CHX alone increased both c-*fos* and c-*jun* mRNA levels although the onset times of induction were different. In electrophoretic mobility shift-assay, both AP-1 and ENKCRE-2 DNA-binding activities were increased by KA. KA-induced increases of AP-1 and ENKCRE-2 DNA-binding activities were also attenuated by CHX. In addition, KA-induced AP-1 and ENKCRE-2 DNA-binding activities were diminished by the antibodies against Fos and Jun family proteins. Furthermore, the cross-competition studies revealed that AP-1 proteins actively participated in ENKCRE-2 DNA domain. The results suggest that KA-induced proENK and proDYN mRNA expressions may require on-going synthesis of proteins, such as c-Fos, c-Jun and 35-kDa Fra, which may have a possible role in the up-regulation of proENK and proDYN gene expression through the binding with AP-1 and ENKCRE-2 DNA-binding motifs.

*Keywords:* Kainic acid; Cycloheximide; Proenkephalin; Prodynorphin; Proto-oncogene; Hippocampus

## 1. Introduction

Kainic acid (KA), a unique neuro-excitatory/neuro-toxic substance, produces seizure and neural degeneration in the mammalian CNS and appears to provide a good model for some aspects of human temporal lobe epilepsy [3,43]. KA exerts its action by direct excitatory effects as well as reduction of synaptic inhibition [1,4,22] and by modulating the intrinsic $Ca^{2+}$ and $K^{+}$ conductances [8,25]. In the rat hippocampus, KA receptors are concentrated in the CA3/4 regions, with moderate levels in the other areas [15,29]. It appears that KA has multiple actions, affecting both the properties of individual neurons and synaptic connections. Yet the exact mechanisms by which KA act are not fully understood.

Previously, numerous studies have demonstrated that several proto-oncogene products, such as Fos and Jun family proteins, serve as the third messengers in the regulation of various types of genes [5,12,34,49,61]. These proteins interact with AP-1 domain in the promoter of various types of genes, in turn, modulate the transcriptional activity [41]. Drugs that produce seizure activity induce the expression of AP-1 transcription factors [20,42,50–53,58]. Convulsant doses of KA also induce c-Fos, Fra and Jun proteins in rat hippocampus [32,45,47].

* Corresponding author. Fax: +82 (361) 58-1652; E-mail: hwsuh@sun.hallym.ac.kr


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In addition, the increases of c-Fos, Fra and Jun proteins induced by convulsant dose of KA correlate with AP-1 DNA-binding activity [44,45].

The neurons of the hippocampus express both proENK and proDYN mRNAs and their protein products [24,39]. These proENK and proDYN mRNA levels are also increased by KA [18]. Both proENK and proDYN genes contain an AP-1-like domain, an ENKCRE-2 or DYNCRE-3, in their promoter regions [13,19] which may play a role for the regulation of their gene expression [40,54]. However, it is not yet known whether AP-1 proteins are directly involved in proENK and proDYN mRNA expression and whether these proteins are needed for ENKCRE-2 DNA-binding activities in proENK and proDYN promoters.

In an attempt to examine the involvement of AP-1-binding proteins in the regulation of proENK and proDYN mRNA expression, the effect of cycloheximide (CHX), a protein synthesis inhibitor, on KA-induced proto-oncogene, proENK and proDYN mRNA levels, proto-oncoprotein levels and AP-1 and ENKCRE-2 DNA-binding activities was studied in the present study.

## 2. Materials and methods

### *2.1. Treatment of animals*

Male Sprague–Dawley rats weighing 200–280 g were used. Animals were housed 2/group in a room maintained at $22 \pm 5$°C with an alternating 12-h light–dark cycle. Animals were used only once. CHX was administered i.p. at a dose of 15 mg/kg, 30 min prior to the administration of KA. KA was administered i.p. at a dose of 10 mg/kg. All drugs were dissolved in phosphate-buffered saline [PBS; 10 mM potassium phosphate (pH 7.5), 150 mM NaCl]. Animals were sacrificed 1, 4 and 8 h after KA administration.

### *2.2. Isolation of total RNA and proteins*

Total cellular RNAs and proteins were extracted from pooled rat hippocampi ($n = 3$/group) using a rapid guanidine thiocyanate-water saturated phenol/chloroform extraction and subsequent precipitation with acidic sodium acetate [9]. Total cellular RNAs in the aqueous phase were precipitated with cold isopropyl alcohol. Isolated RNA samples were subjected to spectrophotometric analysis at 260 nm and 280 nm. The separated organic layer was extracted 2× with an equal volume of sterile millipore water and proteins were precipitated by adding 2 volumes of absolute ethanol to the water-extracted organic phase. The protein pellets were washed 2× with cold absolute ethanol and dried. The dried pellets were dissolved in a denaturing buffer [6 M guanidium chloride, 20 mM Tris–HCl (pH 8.0) and 1 mM EDTA]. The protein samples were dialyzed against a renaturing buffer [20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 5 mM $MgCl_2$, 0.4 mM phenylmethylsulfonyl fluoride and 20% glycerol] at 4°C. The concentration of protein was determined with the Coomassie blue protein assay reagent (Pierce Chemical, Rockford, IL) using bovine serum albumin (BSA) as a standard.

### *2.3. Non-isotope northern blot hybridization analysis*

Ten μg of total RNA were denatured and electrophoresed on 1% agarose-formaldehyde gels [31] and transferred to nylon hybond-N hybridization membrane sheets (Amersham, Buckinghamshire, UK). After baking for 1–2 h at 80°C, the membranes were pre-hybridized at 68°C for at least 1 h in pre-hybridization buffer (5 × SSC, 50% formamide, 0.02% SDS, 0.1% sodium *N*-lauroyl sarcosine, 2% blocking reagent). The DIG-labeled proENK, proDYN, c-*fos* and c-*jun* probes were added to pre-hybridization buffer containing 50% formamide. The membranes were incubated overnight at 68°C in a shaking water bath and washed 2× for 10 min/wash in 2× wash solution (2 × SSC, 0.1% SDS) at room temperature. Then, the membranes were washed 2× for 15 min/wash in 0.1× wash solution (0.1 × SSC, 0.1% SDS). After equilibrating in a buffer I [100 mM maleic acid, 150 mM NaCl (pH 7.5)] for 1 min, the membranes were gently agitated in buffer II (1% blocking reagent in buffer I) for 30–60 min. The membranes were hybridized with the diluted anti-DIG-alkaline phosphatase [1 : 10 000 (75 mU/ml)] in a buffer II for 30 min. After washing 2× for 15 min/wash in 0.3% Tween-20 (in buffer I), the membranes were equilibrated in a buffer III [100 mM Tris–HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$] for 2 min. ≈ 0.5 ml/100 $cm^2$ of Lumi-Phos TM 530 was spreaded over the surface of membrane. After incubation at 37°C for 15–20 min, the membranes were exposed to Hyperfilm-MP (Amersham) for detection of the chemi-luminescent signal. For re-hybridization, blots were washed for 20 min at room temperature in sterile millipore water, then further washed overnight at 65°C in 50 mM Tris–HCl (pH 8.0), 50% dimethylformamide and 1% SDS to remove the hybridized probe and re-hybridized to Dig-labeled rat cyclophilin (CPN) cRNA probe, a gene encoding peptidyl-prolyl *cis-trans* isomerase which is constitutively expressed in most mammalian tissues with the exception of skeletal muscle [17,56].

The cRNA probes for proenkephalin [48,60], prodynorphin [10], c-*fos* [16], c-*jun* [5,26] and CPN [17] were synthesized in vitro from linearized expression vector with using DIG-UTP, as suggested by the manufacturer (Boehringer Mannheim, Germany).

### *2.4. Western immuno-blot analysis*

Total cellular proteins (50 μg) were separated by electrophoresis in a 12% polyacrylamide gels as described [33]. A pre-stained rainbow protein mixture (Amersham,

Arlington, USA) was used as a molecular weight standard. Proteins were transferred from acrylamide gel onto nitrocellulose filters according to published procedures [57]. Electro-transferred filters were first incubated in blocking buffer [5% skim milk, 10 mM trizma base (pH 8.0), 150 mM NaCl] and then allowed to interact with antisera against [Met$^5$]enkephalin-Arg$^6$-Phe$^7$ which cross-react with proenkephalin [60] (1:500), c-Fos (1:1000) (Santa Cruz Biotech., CA), 35-kDa Fra (1:1000) (Santa Cruz Biotech.) and c-Jun (1:1000) (Santa Cruz Biotech.), in a blocking buffer for 4 h at room temperature. Filters were then washed 3× with Tris-buffered saline (TBS) containing 0.3% Tween-20 [TBST; 10 mM trizma base (pH 8.0), 150 mM NaCl, 0.3% Tween-20] for 5 min and incubated with 1% normal goat serum in TBS [10 mM trizma base (pH 8.0), 150 mM NaCl] for 20 min. Filters were then reacted with a goat anti-rabbit goat IgG-alkaline phosphatase conjugate at room temperature for 1 h. After washing filters with phosphate buffer solution for 10 min (3×), 5-bromo-4-chloro-3-indolyl phosphate and Nitro blue tetrazolium were added and developed to a desired intensity.

### 2.5. Non-isotope electrophoretic mobility shift-assay

The AP-1 and ENKCRE-2 oligonucleotides used in this study were purchased from Korean Biotech (Dae-Ku, Korea). Annealing was achieved by incubating an equal molar concentration of each single stranded oligo in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA and 200 mM NaCl for 10 min at 95°C for 10 min and then the mixture was allowed to cool to room temperature gradually. The sequence of the oligonucleotide used in the electrophoretic mobility shift-assay are listed below:

AP-1 (TRE) (22-mer) contains the consensus sequence (5′-TGAGTCA-3′).

5′-CTA-GTG-ATG-AGT-CAG-CCG-GAT-C-3′

3′-GAT-CAC-TAC-TCA-GTC-GGC-CTA-G-5′

ENKCRE2 (22-mer) contains the consensus sequence (5′-TGCGTCA-3′).

5′-CTA-GTG-ATG-CGT-CAG-CCG-GAT-C-3′

3′-GAT-CAC-TAC-GCA-GTC-GGC-CTA-G-5′

The DNA-binding assay was done by following the manual provided by DIG-gel shift kit (Boehringer Mannheim). Binding reactions were carried out at room temperature for 15 min and reaction mixtures contained 50 μg of total protein, 20 mM Hepes (pH 7.6), 30 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.2% Tween-20, 50 μg/ml poly(dI-dC) and ≈0.3 pmol of specified probe labeled with DIG-ddUTP by using terminal transferase. Protein-DNA complexes were separated from protein-free DNA by non-denaturing electrophoresis in 5% polyacrylamide (30:1, acrylamide:bisacrylamide) gels. Gels were run at room temperature in 89 mM Tris (pH 8.3), 89 mM boric acid, 2 mM EDTA at a constant voltage (8 V/cm) and electro-blotted onto positive charged nylon membrane. The membranes were baked in 80°C for 15 min, washed with 0.3% Tween-20 in a buffer I and hybridized with the diluted anti-DIG-alkaline phosphatase [1:10 000 (75 mU/ml)] in a buffer II for 30 min. After washing 2× for 15 min with 0.3% Tween-20 (in a buffer I), the membranes were equilibrated in a buffer III [100 mM Tris–HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$] for 2 min. The method of chemi-luminescent detection was identical with the method used for non-isotope Northern hybridization analysis.

## 3. Results

### 3.1. Effects of CHX on KA-induced proENK or proDYN mRNA and proENK protein levels

All data shown in the present study were repeated at least 3× and showed the same tendency of results. The proENK and proDYN mRNA levels and proENK protein levels in rat hippocampus were examined up to 8 h after KA (10 mg/kg) administration. As shown in Fig. 1, the proENK and proDYN mRNA levels began to increase 4 h and were further increased 8 h after KA administration. The proENK mRNA level was elevated ≈12-fold, proDYN mRNA level as much as 4.8-fold 8 h after KA administration (Fig. 1A,B). The elevations of both proENK and proDYN mRNA levels by KA were inhibited by pre-administration of CHX (15 mg/kg). Western immuno-blot assay using antiserum against [Met$^5$]enkephalin-Arg$^6$-Phe$^7$ (MERF) showed that neither the KA

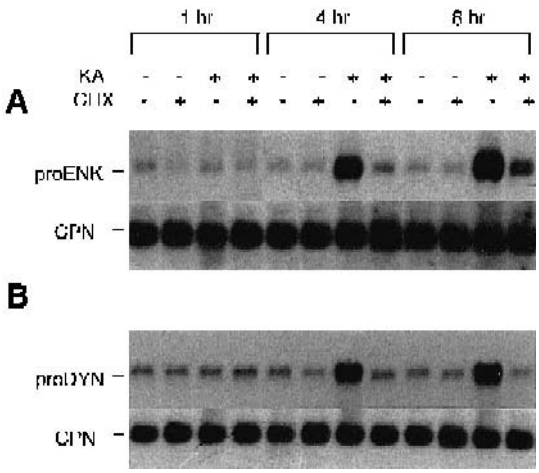


Fig. 1. Effect of CHX on proENK and proDYN mRNA expressions induced by KA in rat hippocampus. After pre-treatment with either PBS or 15 mg/kg of CHX for 30 min, either PBS or 10 mg/kg of KA was administered i.p. The animals were sacrificed at indicated time points (1, 4 and 8 h). 10 μg of total RNA, which were extracted from pooled rat hippocampus ($n$ = 3/group) using a guanidium thiocyanate/phenol/chloroform gradient method, were used for determination of proENK (A) and proDYN (B) mRNA levels. The unregulated mRNA encoding CPN was used as an internal loading control.

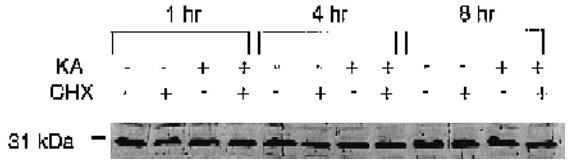


Fig. 2. Effect of CHX and KA on proENK protein level. After pre-treatment with either PBS or 15 mg/kg of CHX for 30 min, either PBS or 10 mg/kg of KA was administered i.p. The animals were sacrificed at indicated time points (1, 4 and 8 h). 50 $\mu$g of total cellular proteins, which were extracted from pooled rat hippocampus ($n = 3$/group), were used for determination of proENK protein level using Western immuno-blot analysis. A polyclonal antiserum against [Met$^5$]enkephalin-Arg$^6$-Phe$^7$ was used at a 1:500 dilution. The 31-kDa band indicates the proENK protein.

administration alone nor pre-treatment with CHX exhibited any significant change in proENK (31 kDa) protein level (Fig. 2).

### *3.2. Effects of CHX on KA-induced c-Fos, 35-kDa Fra and c-Jun protein levels*

Western blot analyses using antibodies against c-Fos, 35-kDa Fra and c-Jun proteins were carried out to examine whether there were correlations with between proDYN and proENK mRNA expressions and proto-oncoprotein levels. The c-Fos, 35-kDa Fra and c-Jun protein levels were increased 1 h and reached a peak 8 h after KA administration. The increases of c-Fos, 35-kDa Fra and c-Jun protein levels were reduced by the pre-administration of CHX at all time points (Fig. 3).

### *3.3. Effects of CHX on KA-induced c-fos or c-jun mRNA levels*

The c-*fos* and c-*jun* mRNA levels were increased 1 and 4 h after KA administration (Fig. 4A,B). Both c-*fos* and

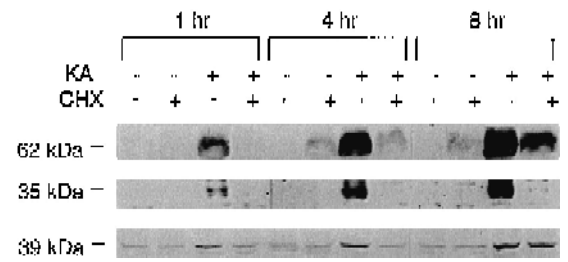


Fig. 3. Effect of CHX on KA-induced proto-oncoproteins expression. After pre-treatment with either PBS or 15 mg/kg of CHX for 30 min, either PBS or 10 mg/kg of KA was administered i.p. The animals were sacrificed at indicated time points (1, 4 and 8 h). 50 $\mu$g of total cellular proteins, which were extracted from pooled rat hippocampus ($n = 3$/group), were used for determination of proto-oncoprotein levels using Western immuno-blot analysis. A polyclonal antibodies against c-Fos, 35-kDa and c-Jun proteins were used at a 1:1000 dilution. The 62-, 35- and 39-kDa bands indicate the c-Fos, 35-kDa Fra and c-Jun proteins, respectively.

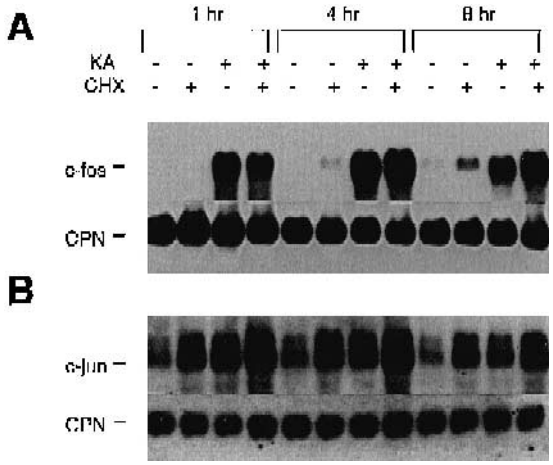


Fig. 4. Effect of CHX on KA-induced c-*fos* and c-*jun* mRNA levels. After pre-treatment with either PBS or 15 mg/kg of CHX for 30 min, either PBS or 10 mg/kg of KA was administered i.p. The animals were sacrificed and hippocampus was dissected at indicated time points (1, 4 and 8 h). 10 $\mu$g of total RNA were used for determination of c-*fos* (A) and c-*jun* (B) mRNA levels. The unregulated mRNA encoding CPN was used as an internal loading control.

c-*jun* mRNA levels were decreased but still remained at higher level than control group at 8 h after KA administration. CHX alone slightly increased c-*fos* mRNA level at 4 and 8 h. However, the c-*jun* mRNA was markedly elevated by CHX alone at all time points (Fig. 4B). The pre-administration of CHX caused a prolongation of c-*fos* and c-*jun* mRNA expression induced by KA administration at all time points (Fig. 4A,B).

### *3.4. Effects of KA and CHX on AP-1 or ENKCRE-2 DNA-binding activity*

As revealed by the electrophoretic mobility shift-assay, both AP-1 and ENKCRE-2 DNA-binding activities began to increase 1 h and were further increased 8 h after the KA administration (Fig. 5A). In addition, the increases of AP-1 and ENKCRE-2 DNA-binding activities induced by KA were inhibited by the pre-administration of CHX at all time points. The diminishment of AP-1 and ENKCRE-2 DNA-binding activities by self-competition with 100 $\times$ excess of unlabeled (cold) probes showed that these binding activities were specific (Fig. 5B). The cross-competitions between AP-1 and ENKCRE-2 showed that the AP-1 or ENKCRE-2 DNA-binding activity was diminished by 100 $\times$ unlabeled cross-competitor. These results imply that the ENKCRE-2 element behaves like AP-1 element.

To determine whether the elevation of AP-1 or ENKCRE-2 DNA-binding activity induced by KA was due to the induction of genuine Fos and Jun family proteins, antibodies that were specific to these proteins were included in the electrophoretic mobility shift-assay. The increases of AP-1 and ENKCRE-2 DNA-binding activities

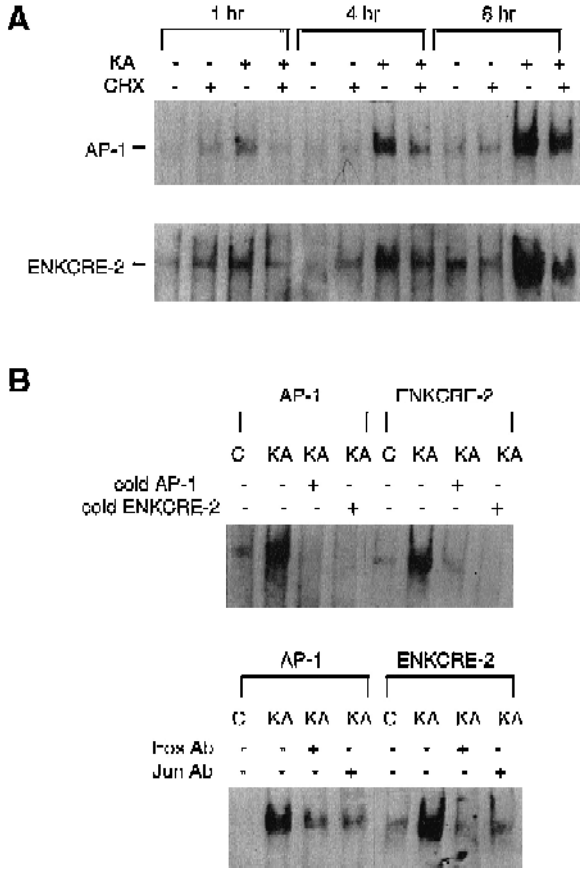


Fig. 5. Effect of CHX on KA-induced AP-1 and ENKCRE-2 DNA-binding activities. (A) After pre-treatment with either PBS or 15 mg/kg of CHX for 30 min, either PBS or 10 mg/kg of KA was administered i.p. The animals were sacrificed at indicated time points (1, 4 and 8 h). 50 $\mu$g of total cellular proteins, which were extracted from pooled rat hippocampus ($n = 3$/group) were used for determination of AP-1 and ENKCRE-2 DNA-binding activities using electrophoretic mobility shift-assay. (B) For self- or cross-competition and antibody interference studies, the electrophoretic mobility shift-assays were performed in the presence or absence of 100-fold molar excess of unlabeled double-stranded oligonucleotide (AP-1 or ENKCRE-2) or 2 $\mu$g of antibodies against Fos or Jun families.

induced by KA were effectively reduced by the antibodies against Fos and Jun family members (Fig. 5B).

## 4. Discussion

The present study demonstrates that KA-induced AP-1 or ENKCRE-2 DNA-binding activity and proENK or proDYN mRNA expression, may require the on-going synthesis of proteins, such as c-Fos, c-Jun and 35-kDa Fra, in rat hippocampus. In Northern blot analyses, proENK and proDYN mRNA expressions markedly began to increase after 4 h and were further increased 8 h after KA administration (Fig. 1A,B). Previous studies have shown that dentate gyrus of hippocampus expressed both proENK and proDYN mRNA and their products [24,39] and both proENK and proDYN mRNA levels were elevated by KA administration. The results of the present study were partially in line with previous studies in that both proENK and proDYN mRNA levels began to increase 3 or 4 h after KA administration [18,45].

The increases of proENK and proDYN mRNA levels induced by KA were inhibited by pre-administration with CHX at all time points (Fig. 1A,B), implying that the increases of proENK and proDYN mRNA levels by KA administration were dependent on de novo synthesis of proteins. This result suggests that pre-existing cellular proteins, such as transcriptional regulatory proteins, appear not to be sufficient for elevations of the proENK and proDYN mRNA levels when KA is administered. This result is in agreement with in vitro experiments which showed that CHX efficiently blocks the increase of proENK mRNA level induced by 12-*O*-tetradecanoylphorbol-13-acetate in cultured bovine adrenal chromaffin cells [37]. However, CHX did not affect the proENK mRNA level induced by norepinephrine plus dexamethasone in C6 rat glioma cells [38]. Therefore, it appears that the blockade of proENK mRNA expressions by CHX may be dependent on types of tissue, cells or drugs tested.

In the results of Western immuno-blot analysis, we did not detect any remarkable change of proENK (31 kDa) protein level (Fig. 2). Although we did not examine the levels of smaller proENK-derived peptides, such as Leu- and Met-enkephalin, it is assumed that the newly synthesized proENK protein may be rapidly processed to smaller enkephalin-containing peptides or released into extracellular space. Our result is similar to in vitro studies of the cultured astrocytes, C6 rat glioma cells and bovine adrenal chromaffin cells [2,37,38].

Previous works have shown that convulsant doses of KA increase immuno-reactivities of Fos and Jun-related proteins in mouse and rat hippocampus [45,52]. In the present study, Western blot analyses showed that the c-Fos, 35-kDa Fra and c-Jun protein levels were induced 1 h and were further increased 8 h after KA administration. In addition, the increases of c-Fos, 35-kDa Fra and c-Jun protein levels were also inhibited by the pre-administration with CHX at all time points (Fig. 3). The kinetics of these proto-oncoprotein expressions were well-correlated with the increases of proENK and proDYN mRNA levels induced by KA. In the study by Pennypacker and co-workers, c-Fos, 35-kDa Fra and c-Jun proteins were detected 1.5 h, intensified 4.5 h and diminished 6 h when 8 mg/kg of KA was administered [45]. Although the reason is not clear, it can be speculated that the differences of kinetics of proto-oncoproteins may due to the different doses of KA used and the diversity of rat species.

In contrast to the effect of CHX on c-Fos and c-Jun protein levels, the KA-induced c-*fos* and c-*jun* mRNA levels were potentiated by pre-administration of CHX (Fig. 3A,B). In addition, CHX alone caused an elevations of c-*jun* and c-*fos* mRNA levels 1 or 4 h, respectively, after KA administration (Fig. 3A,B). Although explanations for

this phenomenon remain unclear, at least four proposed contributory mechanisms were discussed in in vitro experiments. First, the labile proto-oncogene transcripts, such as c-*fos* and c-*jun* mRNAs, may become much more stable in the presence of protein synthesis inhibitor [6,11,23,46,59]. Second, the translational arrest may cause the inability of auto-repression of proto-oncogene transcription by reducing its protein product level [7]. Third, the protein synthesis inhibitor may inhibit the synthesis of labile repressors which may attenuate the proto-oncogene transcription [55]. Finally, some protein synthesis inhibitors, such as anisomycin and CHX, may act as potent intracellular signaling agonists [21,35,36].

The rat proENK and proDYN gene promoters contain an identical AP-1-like element, ENKCRE-2 (also called DYNCRE-3 in proDYN gene) [40], in their 5′-promoter regions [13,19]. In electrophoretic mobility shift-assay, the AP-1 and ENKCRE-2 DNA-binding activities began to elevate 1 h and were further increased 8 h after KA administration (Fig. 5A). These results suggest that KA may increase proENK and proDYN mRNA levels by promoting the interaction of AP-1 and ENKCRE-2 transcriptional factors to their respective binding domains. In addition, the increased AP-1 and ENKCRE-2 DNA-binding activities induced by KA administration were effectively inhibited by CHX, suggesting that the newly synthesized proto-oncoproteins may be responsible for increasing AP-1 and ENKCRE-2 DNA-binding activities.

The time-course study revealed that the increases of AP-1 and ENKCRE-2 DNA-binding activity are further well-correlated with the increases of c-Fos, 35-kDa Fra and c-Jun protein levels. Therefore, the findings from the present study, such as the inhibitions of c-Fos, 35-kDa Fra and c-Jun protein levels, proENK and proDYN mRNA levels, the elevations of AP-1 and ENKCRE-2 DNA-binding activities by CHX strongly suggest that c-Fos, 35-kDa Fra and c-Jun proteins may be actively involved in the regulation of proENK and proDYN mRNA levels during the stimulation by KA by binding to either AP-1 or ENKCRE-2 DNA-binding domains. In support of this hypothesis, we observed that the AP-1 or ENKCRE-2 DNA-binding activities were inhibited by the antibodies against Fos and Jun family proteins 8 h after KA administration (Fig. 5B). In addition, the AP-1 or ENKCRE-2 DNA-binding activities diminished by adding non-labeled ENKCRE-2 or AP-1 oligomer, presuming that the AP-1 proteins may act on the ENKCRE-2 region on the promoters of proENK or proDYN gene in rat hippocampus.

Although AP-1 protein levels and AP-1 or ENKCRE-2 DNA-binding activities are well-correlated with proENK and proDYN mRNA expression, different transcription factors may be involved in the regulation of opioids gene expression. Several lines of evidence have demonstrated that certain factors, such as AP-2, AP-4 and NF-1, are also involved in the regulation of the proENK gene expression [14,27]. In addition, proENK gene promoter has a series of homologous transcriptional control domains, such as ENKCRE-1 and AP-2 in human [13,27] and rat [28,30,48] and proDYN gene has an additional AP-1- or CRE-like sequences, such as DYNCRE-1, DYNCRE-2 and DYNCRE-4, in its promoter region [40]. Although many reports have suggested that ENKCRE-2 (or DYNCRE-3) domain may be a major candidate for the regulation of proENK and proDYN gene expression, the molecular mechanisms involved in proENK and proDYN gene expression may be not as simple as recognition of ENKCRE-2 by AP-1 or CREB proteins. The studies on exact nature of interactions between these transcriptional factors and opioid genes should be further determined in the future study.

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