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Histone modifications in kainate-induced status epilepticus

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Abstract

To understand the molecular actions of status epilepticus at the chromatin level, we studied the effects of kainate-induced status epilepticus on two different histone modifications at amino terminal tails: histone H3 phosphorylation at serine 10 and histone H4 acetylation. In addition to induction of c-*fos* and c-*jun* immediate early genes (IEGs) expression in mouse hippocampus, we also found the upregulation of acetylation and phosphorylation of histones, coupled with status epilepticus after kainate administration. c-*fos* and c-*jun* mRNA were sequentially induced in response to kainate, in different hippocampal subpopulations, starting from the dentate gyrus (DG) and spreading to the cornus ammonis regions. Immunohistochemical analysis showed that the spatio-temporal distribution of histone H4 hyperacetylation after kainate treatment was well correlated with the expression of c-*fos* and c-*jun* genes. Additionally, there was a transient appearance of phosphorylated histone H3 specifically in the DG region. CREB-binding protein or CBP, a well-known transcriptional co-activator with histone acetyltransferase (HAT) activity, was also induced by kainate and its expression pattern well correlated with histone H4 hyperacetylation in the hippocampus. Chromatin immunoprecipitation analysis showed that both histone modifications were associated with c-*fos* gene promoter after kainate stimulation, but only histone acetylation with c-*jun* gene. Pretreatment with curcumin, which has a HAT inhibitory activity specific for CBP/p300, attenuated histone modifications, IEGs expression and also the severity of status epilepticus after kainate treatment. Our findings suggest the involvement of histone modifications induced by kainate not only in IEGs expression but also in the development of epilepsy.

Introduction

There has been increasing interest that chromatin remodeling might play a critical role in gene regulation, even in non-dividing cells such as neurons (Huang *et al*., 2002; Tsankova *et al*., 2004). One form of chromatin remodeling is histone amino-terminal modification that can generate synergistic or antagonistic affinities for the interactions of transcriptional factors, in turn causing changes in gene activity (Jenuwein & Allis, 2001). Two widely studied histone modification processes are histone acetylation and phosphorylation (Davie & Spencer, 2001; Fischle *et al*., 2003). While histone hyperacetylation indicates an increase in gene activity, its hypoacetylation marks gene repression. Both states are controlled by a dynamic interplay of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Among HATs, CREB-binding protein or CBP is a well-known transcriptional co-activator and also possesses intrinsic HAT activity (Ogryzko *et al*., 1996; Rouaux *et al*., 2003). CBP HAT activity regulates neuronal gene expression and also memory consolidation (Hardingham *et al*., 1999; Korzus *et al*., 2004). On the other hand, histone H3 phosphorylation plays dual roles and has been associated with both interphase chromatin condensation and immediate early genes (IEGs) induction.

Among many IEGS, c-*fos* and c-*jun* genes induction by several different stimulations have been associated with histone modifications *in vitro*. Histone H3 phosphorylation in EGF-treated 10T1/2 cells is associated with c-*fos* induction (Strelkov & Davie, 2002), while both histones H3 phosphorylation and H4 acetylation in anisomycin-treated 10T1/2 cells are associated with both c-*fos* and c-*jun* inductions *in vitro* (Barratt *et al*., 1994; Clayton *et al*., 2000).

Induction of status epilepticus or prolonged seizures either by pilocarpine or kainate can trigger a myriad of gene upregulation that are thought to contribute to the development of epilepsy (Hevroni *et al*., 1998; Ben-Ari & Cossart, 2000; Chiang *et al*., 2001; Elliott *et al*., 2003). Only three papers have described that either drug-induced or electrostimulated seizures increase histone modifications *in vivo*. Tsankova *et al*. (2004) have shown that electroconvulsive seizures increase H4 acetylation at c-*fos* and brain-derived neurotrophic factor (BDNF) gene promoters, but decrease acetylation levels at CREB promoter, correlating with the respective mRNA levels. On the other hand, Huang *et al*. (2002) have described that pilocarpine treatment reduces acetylation of histone H4 in rat hippocampal cornus ammonis (CA)3 neurons at GluR2 promoter, but increases at BDNF promoter as an early event in status epilepticus. Crosio *et al*. (2003) have shown that the induction of histone H3 hyperphosphorylation but not H3 acetylation in the hippocampus was associated with c-*fos* gene expression after kainate administration. However, little is known about the molecular mechanism of the development of epilepsy, and the correlation with histone modifications and gene upregulation.

Here, we examined two histone modifications of H3 phosphorylation and H4 acetylation susceptible to IEGs expression, as reported in kainate-induced status epilepticus *in vivo*. We found that kainate stimulation induces histone H3 phosphorylation and H4 acetylation in

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hippocampus associated with c-*fos* and c-*jun* mRNA expression. Pretreatment of curcumin, a yellow pigment found in curry, which has been reported to have HAT inhibitory activity specific for CBP/p300 (Balasubramanyam *et al*., 2004), suppressed the induction of histone modifications and the expression of IEGs, and furthermore reduced the severity of epilepsy induced by kainate. Our findings provide the possibility of the involvement of histone modifications induced by kainate not only in IEGs expression but also in the development of epilepsy.

Materials and methods

Materials

Kainate was purchased from Wako, Japan (Cat. 114-00611). Curcumin was purchased from Sigma-Aldrich (Cat. C7727). Antibodies against phosphorylated histone H3 at residue serine 10 (P-H3-S10) (Cat. 06-570) and acetylated histone H4 (Ac-H4) (Cat. 06-866) were obtained from Upstate Biotech (Lake Placid, NY, USA). Antibodies against c-*fos* (Cat. sc-52), c-*jun* (Cat. sc-45) and CBP (Cat. sc-369) were obtained from Santa Cruz Biotechnology (CA, USA).

Animals

Wild-type Std-ddY, 6-week-old adult male mice, weighing between 27 and 33 g, were purchased from Sankyo Laboratories, Toyama, Japan. The mice were housed in metallic breeding cages in a room with a light : dark cycle of 12 h and humidity of 55 ± 2% at 25 ± 1 °C, with food and water available *ad libitum* for more than 1 day before use. Animal care was conducted in accordance with the Guidelines of Animal Experimentation of the Japanese Society for Pharmacology, and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University. All efforts were made to minimize the number of animals used.

Kainate administration

Animals were injected intraperitoneally (i.p.) with phosphate-buffered saline (PBS) or 30 mg/kg kainate in a volume of 0.1 mL per kg. Mice were monitored and the extent of seizure activity was scored according previous description (Schauwecker & Steward, 1997; Yang *et al*., 1997). Typically, status epilepticus-associated behaviors were staring, piloerection, rearing and falling, and tail extension, usually accompanied by forelimb clonus.

Curcumin administration

Curcumin was dissolved in dimethyl sulfoxide to make a stock solution of 30 mg per mL. Prior to administration, stock solution was diluted 10-fold in PBS. Animals were injected i.p. with PBS, 3 mg/kg or 30 mg/kg curcumin in a volume of 0.1 mL per kg 18 h prior to kainate administration.

Tissue preparation

Mice were killed with an overdose of sodium pentobarbital and fixed by transcardial perfusion of 4% paraformaldehyde (PFA) in 0.1 M PB, pH 7.5. Whole brains were removed, fixed for 2 h in the same fixative solution and cryoprotected in 30% sucrose solution overnight at 4 °C. The next day, brains were frozen on dry ice, placed in optimal cutting temperature compound and sectioned at 10 μm and 40 μm thickness by a microtome. For Western and Northern blots, and chromatin immunoprecipitation (ChIP) assay, mouse brains were rapidly removed after cervical dislocation and the hippocampal tissue was dissected on an ice-cold plate.

DIG-labeled cRNA probes for Northern blot and in situ hybridization

Mouse c-*fos* (∼ 1.1 kb), c-*jun* (∼ 1.6 kb) and GAPDH (∼ 1.0 kb) cDNAs including their entire coding regions were prepared from mouse brain mRNA by reverse transcription-polymerase chain reaction and confirmed by sequencing to be identical to the reported sequences. All three cDNAs were subcloned into pBlueScript SK vector and transcriptionally labeled by T3 or T7 RNA polymerases to make the antisense digoxigenin (DIG)-labeled riboprobes.

RNA purification and Northern blotting

Total cellular RNA was extracted from mice hippocampus using a rapid/guanidine thiocyanate-water saturated phenol/chloroform extraction and precipitated by isopropyl alcohol. The extracted RNA (2.5 μg) was resolved on 1% formaldehyde/agarose gels and transferred onto positively charged nylon transfer membranes (Hybond N+, Amersham Biosciences, UK). Hybridizations were carried out using DIG-labeled riboprobe of c-*fos*, c-*jun* and GAPDH.

Western blotting

For c-*fos* and c-*jun* protein analysis, the hippocampal tissue was lysed in CSK buffer [(in mM): NaCl, 100; sucrose, 300; PIPES (pH 6.8), 10; $MgCl_2$, 3; EDTA, 1; 95% Triton X-100]. Hippocampal lysates (15 μg per lane) were separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). For histone modification analysis, hippocampus was sonicated in a lysis buffer [(in mM): HEPES (pH 7.9), 10; $MgCl_2$, 1.5; KCl, 10; dithiothreitol, 0.5] with addition of H_2SO_4 (final concentration 0.2 M) to extract basic proteins including histones (Li *et al*., 2004). Acid-soluble proteins were precipitated with trichloroacetic acid (final concentration 33%) and washed in 0.05 M HCl/acetone and 100% acetone. The pellet was resuspended in water and 20 μg of histone proteins was loaded and separated by 18% SDS–PAGE followed by electrophoretic transfer onto polyvinylidene difluoride membrane (Millipore, MA, USA). The membranes were probed with rabbit polyclonal anti-Ac-H4 (1 : 3000), anti-P-H3-S10 (1 : 1000), anti-c-*fos* (1 : 3000), anti-c-*jun* (1 : 3000), anti-CBP (1 : 1000) and anti-tubulin (1 : 1000) antibodies, and visualized with enhanced chemiluminescence substrate (Amersham Biosciences).

In situ hybridization

Cryosections (10 μm) were mounted on silanized slides (DakoCytomation, Japan) in RNase-free conditions. Once dried, mounted sections were fixed with 4% PFA, followed by HCl, proteinase K and acetic anhydride treatment. After prehybridization, the sections were hybridized with 500 ng/mL DIG-labeled c-*fos* or c-*jun* riboprobe at 65 °C for 16 h. The slides were treated with RNase A, blocked with 1.5% blocking buffer and incubated with anti-DIG-AP-Fab fragments (Cat. 1093274, Roche, Germany) at 4 °C overnight. The sections were developed by 5-bromo-4-chloro-3-indolylphosphate/nitro-blue tetrazolium (BCIP/NBT) for 1 h, mounted with 60% glycerol and examined under light microscopy. For double-labeling of

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c-*fos* or c-*jun* and P-H3-S10 or Ac-H4, the sections were not treated with proteinase K and HCl.

Immunohistochemistry

Immunohistochemistry was used to detect P-H3-S10, Ac-H4 and CBP. Briefly, free-floating sections of 40 μm thickness were incubated with each primary antibody (1 : 200) overnight at 4 °C. After washing three times with 0.1 M PB, sections were incubated for 45 min at room temperature with goat anti-rabbit secondary biotinylated antibody (1 : 200). After washing three times with 0.1 M PB, sections were incubated with avidin-biotin-peroxidase complex solution (ABC solution, Vector Laboratories, Burlingame, CA, USA). Sections were developed with 0.1% DAB (Wako) containing 0.02% H_2O_2. The sections were then mounted on gelatin-coated slides, dehydrated through alcohol to xylene and examined under light microscopy.

Chromatin immunoprecipitation assay

Hippocampal tissue was sliced to 300 μm thickness with a vibratome. The slices were incubated in 1% formaldehyde at 37 °C for 15 min to cross-link histones and their associates. The slices were sonicated by a Handy Sonic (Tomy Seiko, Japan) at 60% of maximum power for 10 s on ice, five times in a lysis buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin and 1 μg/mL pepstatin). The sizes of the sonicated genomic DNA were about 300–500 bp. The ChIP assay was performed following the protocol provided by the ChIP assay kit (Cat. 17295, Upstate Biotech), with some modifications. After chromatin immunoprecipitation with anti-Ac-H4 or anti-P-H3-S10 antibody, DNA samples were extracted with phenol/chloroform and precipitated by ethanol. Twenty percent of the precipitated DNA was used for polymerase chain reaction (PCR). The sequences of primers used in this study are: c-*jun* 5′ flanking region (amplified region −732 to −573, upstream 5′-GAGGGCTA CTCTCAAGCCCGC-3′, downstream 5′-GCACGCCCGA-GAAAGGG CTG-3′), as described in Thomson *et al*. (2001); c-*fos* gene 5′ flanking region (amplified region −277 to +10, upstream 5′-CACGGCCGGTCCCTGTTGTTC-3′, downstream 5′-GTCGCGGTT GGAGTAGT AGGCG-3′), as described in Cheung *et al*. (2000). We verified that the PCR was not saturated for amplification using a different amount of genomic DNA. Mock immunoprecipitations were performed in the absence of antibody as a negative control. The PCR for total input DNA was performed using 3% of genomic DNA used for the immunoprecipitation. The PCR products were run on 2% TBE gel.

Quantification and statistics

Images were quantified with Image J software. For Northern blot, the intensity of each lane was quantified and the data were expressed and normalized as a ratio of mRNA expression for each gene over the GAPDH relative to control at 0 h. For Western blot, the intensity of each lane was quantified and the data were expressed and normalized as a ratio of histone modification over its respective histone relative to control at 0 h. For ChIP assay, the intensity of each lane was quantified and the data were expressed and normalized as a ratio of histone modification of each gene over the input. For *in situ* hybridization and immunohistochemistry, the data were represented as a ratio of the measured intensity over the area of the outlined region

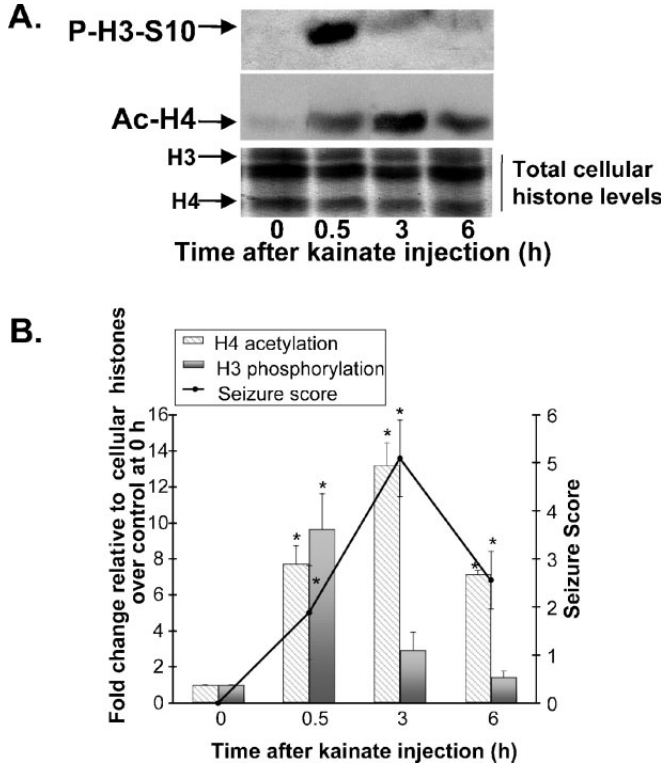

FIG. 1. Induction of histones H4 acetylation and H3 phosphorylation at serine 10 by kainate stimulation. (A) Kainate stimulation increases histone H4 acetylation and transient H3 phosphorylation in the hippocampus. Acid-extracted histones were prepared from hippocampi of mice killed at the time indicated after kainate injection. The levels of histone modifications were assessed by Western blot analysis using anti-phosphorylated H3 at serine 10 (P-H3-S10, top panel) and anti-acetylated forms of H4 (Ac-H4, middle panel) antibodies. Acid-extracted histones were visualized with Coomassie Brilliant Blue (CBB) staining (lower panel). (B) Coupling of status epilepticus and histone modifications induced by kainate. Levels of histone modifications were measured, normalized with the corresponding histone levels visualized with CBB staining, and expressed as fold change over control at 0 h (mean ± SEM, $n = 3$, $^*P < 0.05$ from control at 0 h). Seizure responses of mice were scored as described in Materials and methods, and represented on the same graph by a line (mean scores ± SEM, $n = 10$, $^*P < 0.05$).

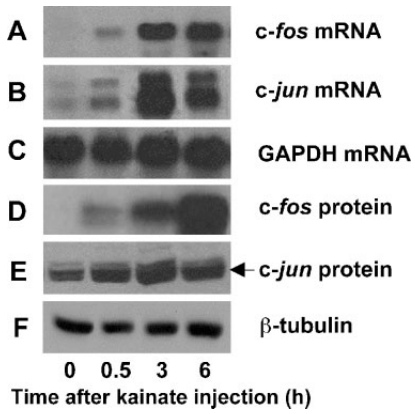

FIG. 2. Kainate stimulation induces the expression of c-*fos* and c-*jun*. Total cellular RNA obtained from hippocampi of mice killed at the indicated time after kainate injection. Total RNA (2.5 μg) was resolved on formaldehyde/agarose gels, and (A) c-*fos*, (B) c-*jun* and (C) GAPDH mRNA levels were detected by DIG-riboprobes. Protein extracts were obtained from hippocampi of mice killed after kainate injection for Western blot analysis. Extracted proteins (15 μg) were separated on 10% SDS-gels and probed with (D) anti-c-*fos* and (E) anti-c-*jun* antibodies. (F) Anti-β-tubulin was used as an internal standard.

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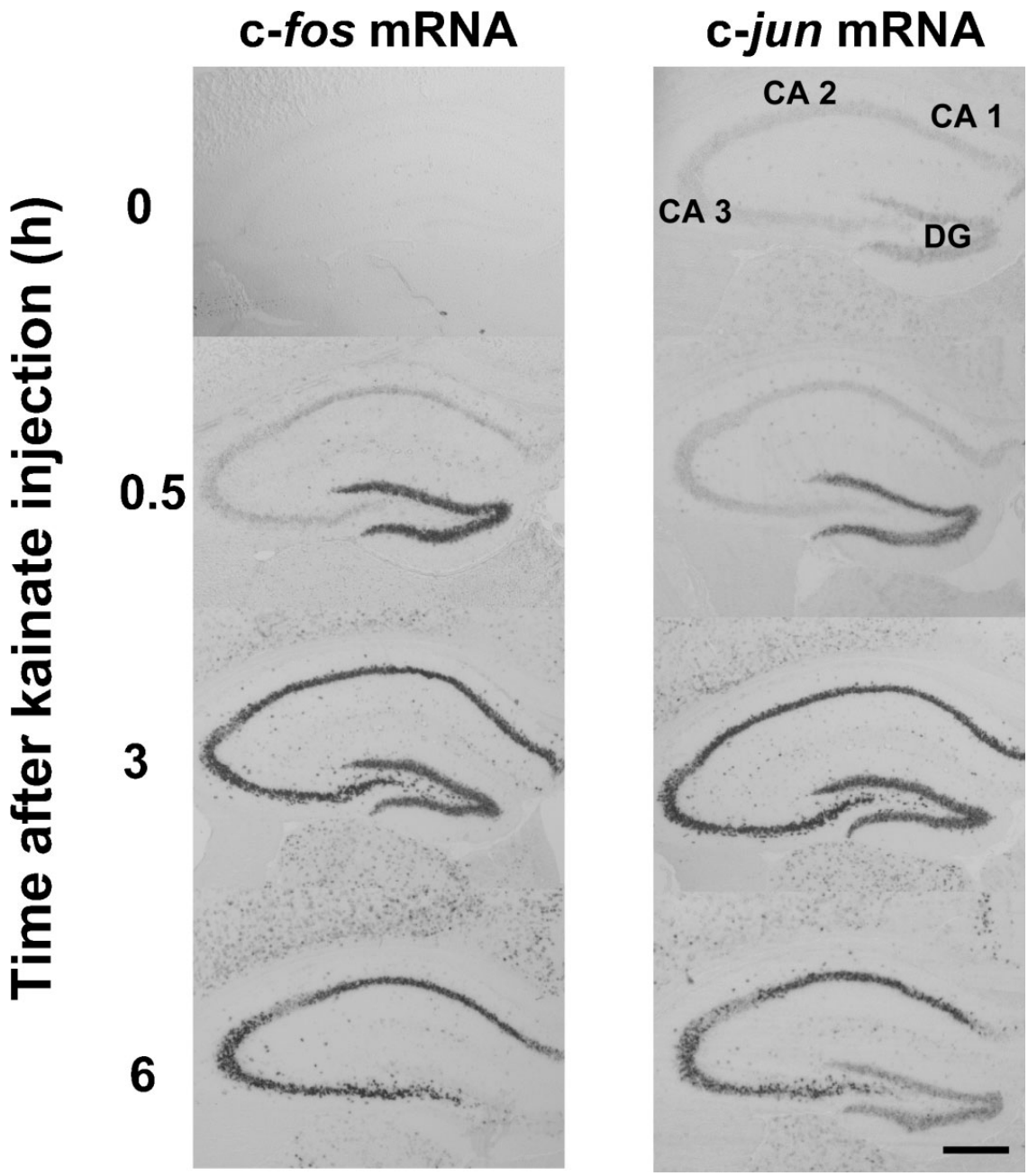

FIG. 3. Spatio-temporal distribution of c-*fos* and c-*jun* mRNA in hippocampus induced by kainate stimulation. *In situ* hybridization using c-*fos* (left column) and c-*jun* (right column) DIG-riboprobes on hippocampal cryosections obtained from mice at the indicated time after kainate injection. CA, cornus ammonis 1–3; DG, dentate gyrus. Scale bar, 500 μm.

of hippocampus (average intensity). Significant changes were determined using the two-tailed Student's *t*-test with unequal variance.

Results

Coupling of status epilepticus with upregulation of acetylation and phosphorylation of histones after kainate stimulation in the hippocampus

Histones associate extensively with the DNA in the nuclei of eukaryotic cells. Each core histone is composed of a structured domain and an unstructured amino-terminal tail of varying lengths from 16 amino acids for H2A, 32 residues for H2B, 44 amino acids for H3, and H4 with 26 amino acids (Davie & Spencer, 2001; Grant, 2001). Histone tails can be covalently modified, such as acetylation, phosphorylation, methylation, ADP-ribosylation and ubiquitination, by upstream mediators. Covalent modifications onto the histone tails regulate cellular processes such as transcription, chromosomal condensation and mitotic condensation (Nowak & Corces, 2000; Strahl & Allis, 2000). To understand the molecular actions of status epilepticus at the chromatin level, we studied the effects of kainate-induced status epilepticus on two different histone amino-terminal tails, histone H3 phosphorylation at serine 10 (P-H3-S10) and histone H4 lysines acetylation (Ac-H4).

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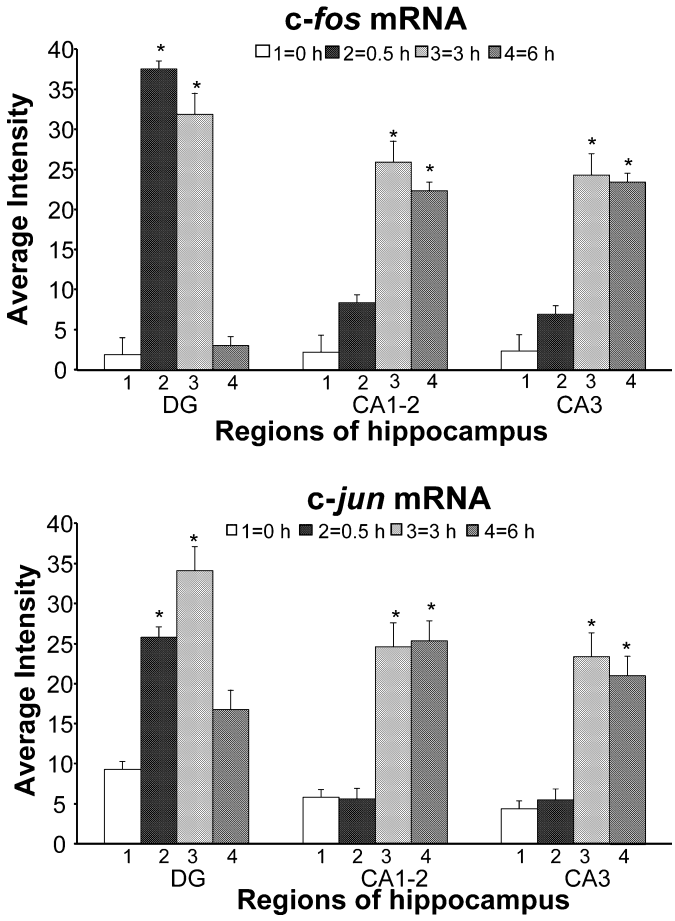

FIG. 4. Spatio-temporal distribution of c-*fos* and c-*jun* mRNA in hippocampus induced by kainate stimulation. Quantification analysis of the data in Fig. 3, represented as average intensity in the different regions of the hippocampus. CA, cornus ammonis 1–3; DG, dentate gyrus. Statistical comparisons were performed with the Student's *t*-test, $*P < 0.05$, $n = 3$ for each time point.

Because chromatin remodeling is a dynamic process, we studied histone modifications at several time points (0.5 h, 3 h and 6 h; $n = 3$ for each time point) after kainate injection and observed the changes in histone acetylation and phosphorylation. Mice were injected i.p. with 30 mg/kg kainate, as this dose produced the highest proportion of mice experiencing status epilepticus but the lowest mortality rate. At the prescribed time points, the hippocampus was dissected and acid-soluble histone proteins were extracted from the hippocampal tissue and the extracted proteins were separated on 18% SDS-gel. After kainate injection, histone H3 phosphorylation at serine 10 was increased significantly by 9.6-fold at 0.5 h, its levels decreased to 2.9-fold at 3 h and returned to control values by 6 h (Fig. 1A, top panel, and B). Histone H4 acetylation was also increased by 7.7-fold at 0.5 h, 13.2-fold at 3 h and sustained by 7.1-fold at 6 h (Fig. 1A, middle panel, and B). The acid-extracted proteins including histones were shown (Fig. 1A, bottom panel).

We then studied the temporal correlation of the changes in histone acetylation and phosphorylation with seizure responses of the mice after kainate injection (Fig. 1B). The seizure parameters were scored as follows: 1, arrest of motion; 2, myoclonic jerks of the head and neck, with brief twitching movements; 3, unilateral clonic activity; 4, bilateral forelimb tonic and clonic activity; 5, generalized tonic–clonic activity with loss of postural tone; 6, death from continuous convulsions (Schauwecker & Steward, 1997; Yang *et al*., 1997). We used a sample size of 60 from all the experiments conducted ($n = 15$, for each time point) for the seizure score analysis. Within 15 min of injection, mice assumed a catatonic posture accompanied by staring behavior proceeding with myoclonic twitching and often frequently accompanied by rearing and falling (score at 0.5 h, 1.88 ± 0.88). After 1 h of kainate administration, mice exhibit continuous tonic–clonic seizures, developing into full seizures in the next 2 h (score at 3 h, 5.10 ± 0.8). Out of 30 mice analysed during 0.5–3 h, six mice died from continuous convulsions. After 4–6 h, the mice were observed in a hunched position and remained immobile (score at 6 h, 2.56 ± 0.6). We observed that the seizure responses concomitantly occurred with histone modifications.

*Induced expressions of c-*fos *and c-*jun *in hippocampal neurons after kainate stimulation*

Kainate injection induced the expression of c-*fos* mRNA from 0.5 h by 1.8-fold and continued to peak at 3 h by 13.1-fold in the hippocampus, measured as a ratio of c-*fos*/GAPDH over control at 0 h (Figs 2A and C, and 9B). Expression kinetics of c-*jun* mRNA was congruent with that of c-*fos*, with its significant increase by 6.0-fold at 3 h in the hippocampus after kainate stimulation (Figs 2B and C, and 9B). Western blot analysis on 10% SDS-gel showed the effective translation of the mRNAs to form elevated levels of c-*fos* and c-*jun* proteins at 3 h, sustaining up to 6 h after kainate treatment (Fig. 2D and E). Both c-*fos* and c-*jun* genes were a direct induction by kainate injection and not caused by stress handling. This was determined from a group of animals injected with PBS instead of kainate, and both the mRNA and protein upregulation were negligible (data not shown).

We performed *in situ* hybridization to examine the spatial and temporal pattern of expression of c-*fos* and c-*jun* genes over a time course of 6 h after kainate injection in the hippocampus (Fig. 3). There was a strong induction of c-*fos* and c-*jun* mRNA in the hippocampus after kainate stimulation, although there was moderate basal expression of c-*jun* without stimulation. We quantified the data in Fig. 3 and expressed them as average intensity in Fig. 4. The induction of c-*fos* mRNA was initiated in the dentate gyrus (DG) at 0.5 h, increasing by 19.2-fold relative to 0 h, sustaining by 16.3-fold at 3 h and back to almost the basal level (1.6-fold) at 6 h. In the CA regions, c-*fos* mRNA was induced significantly at 3 h by 11.6-fold in CA 1–2 and 10.4-fold in CA 3 regions, and continued to maintain its expression by 10-fold in all CA regions up to 6 h. Because the basal level of c-*jun* mRNA expression was detected at 0 h (4.7-fold at 0 h in the DG and 2-fold in the CA regions), the fold increase of c-*jun* was only 2.8-fold (compared with 0 h) at 0.5 h in the DG, increasing by 3.6-fold at 3 h and decreasing its intensity to 1.8-fold by 6 h. The mRNA expression of c-*jun* was increased significantly by 4.4-fold in the CA 1–2 regions and 5.3-fold in the CA 3 region at 3 h. It continued to be maintained by 4.5-fold in the CA 1–2 regions and 4.8-fold in the CA 3 region at 6 h. The sequential inductions of both c-*fos* and c-*jun* genes within the hippocampal subregions were identical: The DG was the first structure to response to kainate followed by the CA regions in the order of CA 3, CA 2 leading to CA 1. This phenomenon can be explained by the neural signals transmission to the hippocampus through perforant pathway, which connects to an excitatory tri-synaptic chain propagating the onset of seizure to other limbic structures (Lothman & Collins, 1981; Le Gal La Salle, 1988; Liu *et al*., 1996). Levels of mRNAs were back to baseline levels within 12 h after kainate injection (Sng *et al*., 2005).

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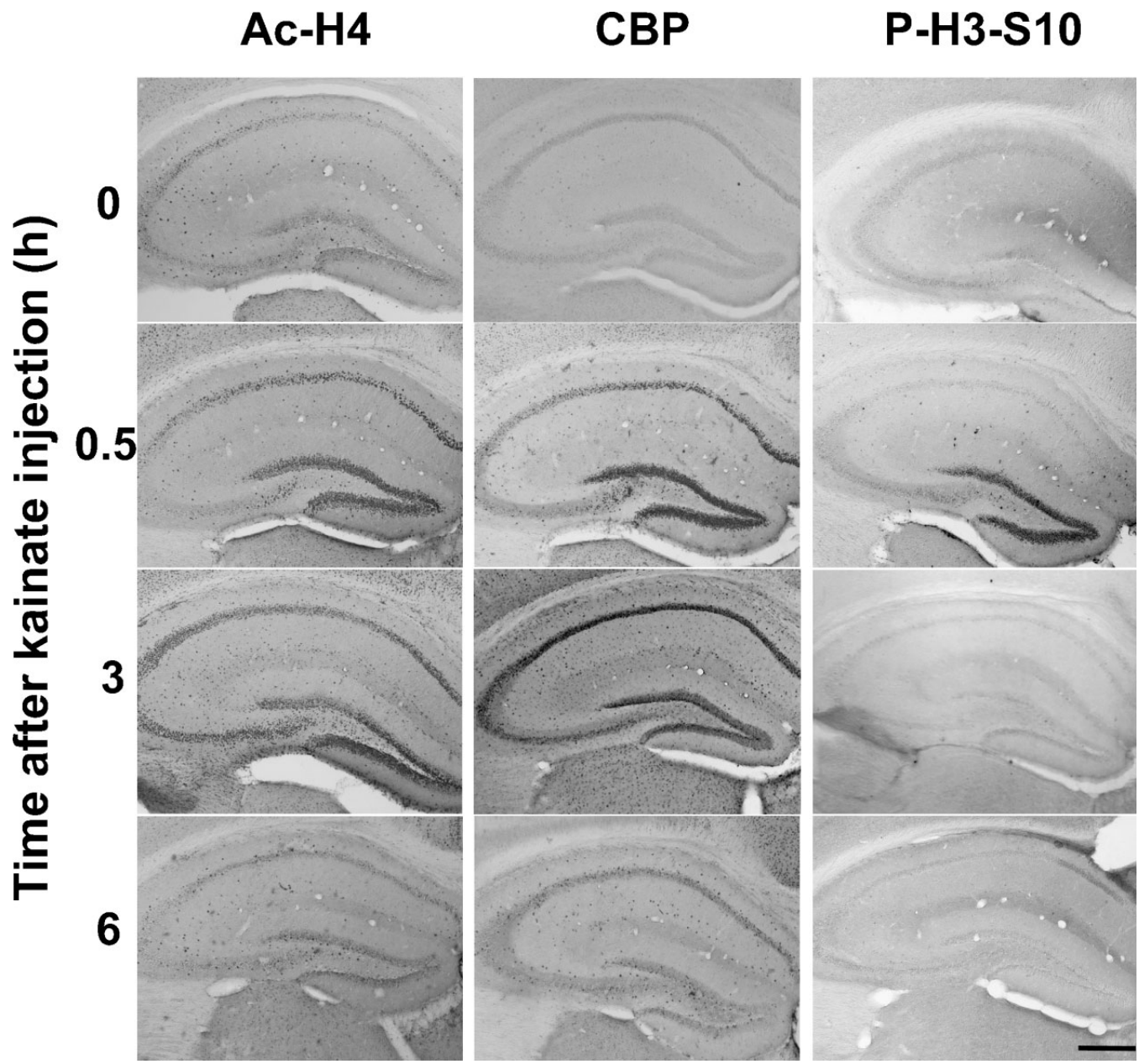

FIG. 5. Spatio-temporal distribution of histone modifications induced by kainate stimulation. Immunohistochemistry using anti-Ac-H4 (left column), anti-CREB-binding protein (CBP) (middle column) and anti-P-H3-S10 (right column) antibodies on mouse hippocampal cryosections obtained from mice killed at the indicated time after kainate injection. Scale bar, 500 μm.

Kainate-induced histone modifications in the hippocampus correlated with IEGs expression

Next, we studied the spatial and temporal upregulation pattern of histone H4 acetylation and H3 phosphorylation after kainate stimulation in the hippocampus by immunohistochemistry. Adjacent sections to those used for *in situ* hybridization were used for immunohistochemical detection of histone modifications. Interestingly, H4 acetylation was upregulated in a similar sequential fashion, as *in situ* hybridization analysis of c-*fos* and c-*jun* expressions. Basal levels of histone H4 acetylation immunostaining were observed at 0 h, followed by a marked 4.6-fold increase in intensity relative to 0 h in the DG and CA regions at 0.5 h that continued to intensify at 3 h (DG, 4.3-fold; CA 1–2, 4.9-fold; CA 3, 3.4-fold) and returned back to almost the basal level by 6 h (Figs 5, left column, and 6, top panel). In contrast, histone H3 phosphorylation at serine 10 transiently appeared at 0.5 h in the DG (3.4-fold, Fig. 5, right column, and 6, bottom panel).

Among HATs, CBP is thought to be a good candidate for the involvement in kainate-induced IEGs expression. CBP is induced by calcium influx via *N*-methyl-D-aspartate (NMDA) receptors or voltage-sensitive calcium channels (Hardingham *et al*., 1999; Impey *et al*., 2002). Transgenic mice expressed mutant CBP, in which the HAT activity was eliminated, and exhibited reduced physiological levels of c-*fos* gene expression *in vivo* (Korzus *et al*., 2004). We examined the spatial and temporal expression of CBP after kainate stimulation in the hippocampus. Induction/accumulation and distribution of CBP immunostaining were well correlated with those of histone acetylation (Fig. 5, middle and left column) after kainate treatment, suggesting a possible role for CBP to have a HAT activity to acetylate histones H4.

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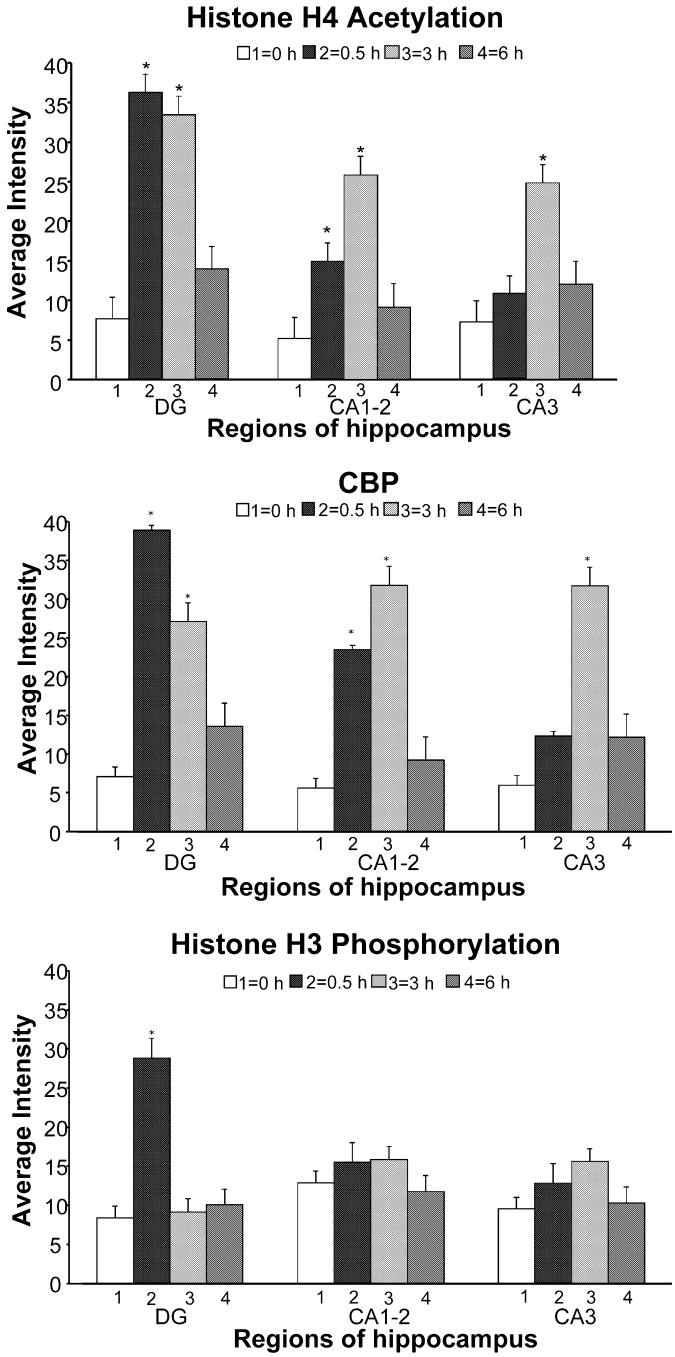

FIG. 6. Spatio-temporal distribution of histone modifications induced by kainate stimulation. Quantification of data in Fig. 5 represented as average intensity in the different regions of the hippocampus. CA, cornus ammonis 1–3; DG, dentate gyrus. Statistical comparisons were performed with the Student's *t*-test, $*P < 0.05$, $n = 3$ for each time point.

*Histone modifications and c-*fos *or c-*jun *mRNA expression occur in the same neurons*

We performed double-labeling on cryosections to determine whether the expression of c-*fos* or c-*jun* mRNA occurs in the same hippocampal neurons in which histone modifications are induced. High-magnification microscopy showed numerous histone H3 phosphorylated-immunopositive dentate granule cells after kainate stimulation. Almost all the DG neurons positive for c-*fos* mRNA expression (NBT/BCIP staining, purple) exhibited histone H3 phosphorylation (DAB staining, brown) concomitantly (94%) at 0.5 h after kainate injection (Figs 7A and 8A, left panel). Only 71% of DG neurons positive for c-*jun* mRNA expression (NBT/BCIP staining, purple) concomitantly exhibited histone H3 phosphorylation (DAB staining, brown) at 0.5 h after kainate injection (Figs 7A and 8A, right panel). The upregulation of histone H4 acetylation and c-*fos* or c-*jun* mRNA were compared in the pyramidal layer cells of the DG and CA regions at 3 h (Figs 7B and 8B). Almost all neuronal cells in the DG showed more than 90% concomitant expression of either c-*fos* or c-*jun* mRNA and histone H4 acetylation. However, in the CA regions, the co-labeling of neuronal cells stained in the nuclei immunopositively for histone H4 acetylation and stained in the cytoplasm with c-*fos* mRNA showed only about 78% (Fig. 8B, top), and with c-*jun* mRNA, 62–68% (Fig. 8B, bottom).

*Histone modifications associate with the c-*fos *and c-*jun *genes activation*

To investigate whether kainate-induced histone modifications are associated with the c-*fos* and c-*jun* genes activation, we performed the ChIP. A simplified diagram of the region of c-*fos* and c-*jun* genes amplified after immunoprecipitation is shown in the top panel of Fig. 9A. The primers chosen covered −277 to +10 of the 5′ flanking region of the c-*fos* gene and the region −732 to −573 of the 5′ flanking region of the c-*jun* gene, as described by Cheung *et al*. (2000) and Thomson *et al*. (2001), respectively. Each PCR for ChIP assay was internally controlled by quantifying the total cross-linked DNA shown in Fig. 9A as input. Mock immunoprecipitations (Fig. 9A as no antibody) were performed in the absence of antibody using cross-linked chromatin to demonstrate the specificity of the ChIP assay.

We found that histone H4 acetylation at the c-*fos* gene in the hippocampus rapidly increased to 5-fold over control values within 0.5 h after kainate treatment. In addition, there was an early transient 6.6-fold increase of histone H3 phosphorylation at 0.5 h. At 3 h, H4 acetylation was 6.6-fold over control values. By 6 h, H4 acetylation levels returned to almost its basal level (1.6-fold over control values, Fig. 9A, left panel and 9B, upper panel). After kainate injection, c-*fos* mRNA expression was induced by 1.6-fold at 0.5 h, continued to increase by 13-fold at 3 h and sustaining 6.0-fold over control up to 6 h. H4 acetylation and H3 phosphorylation levels were significantly changed at 0.5 h, in addition to significant changes of c-*fos* mRNA expression. We concluded that kainate stimulation altered both phosphorylation and acetylation states of histones that associated with the c-*fos* gene activation in hippocampal neurons. In contrast, there was no significant change in histone acetylation (1.2–1.3-fold over control values, Fig. 9A, right panel and 9B, lower panel) at the c-*jun* gene over time course after kainate injection. There was no upregulation of histone phosphorylation detected at the c-*jun* gene. However, the mRNA expression of c-*jun* was elevated significantly by 7.2-fold after 3 h of kainate injection (Figs 2A and C, and 9B, lower panel). We also performed PCR for ChIP assay using different primers covering −296 to −79 of the 5′ flanking region of the c-*jun* gene. Although the primers selected span the major transcription factor binding sites of the c-*jun* gene, we could not detect any PCR products using genomic DNA immunoprecipitated with antibodies either against histone H4 acetylation or H3 phosphorylation (data not shown). We concluded that kainate stimulation did not induce significant changes in preexisting H4 acetylation and H3 phosphorylation at the c-*jun* gene.

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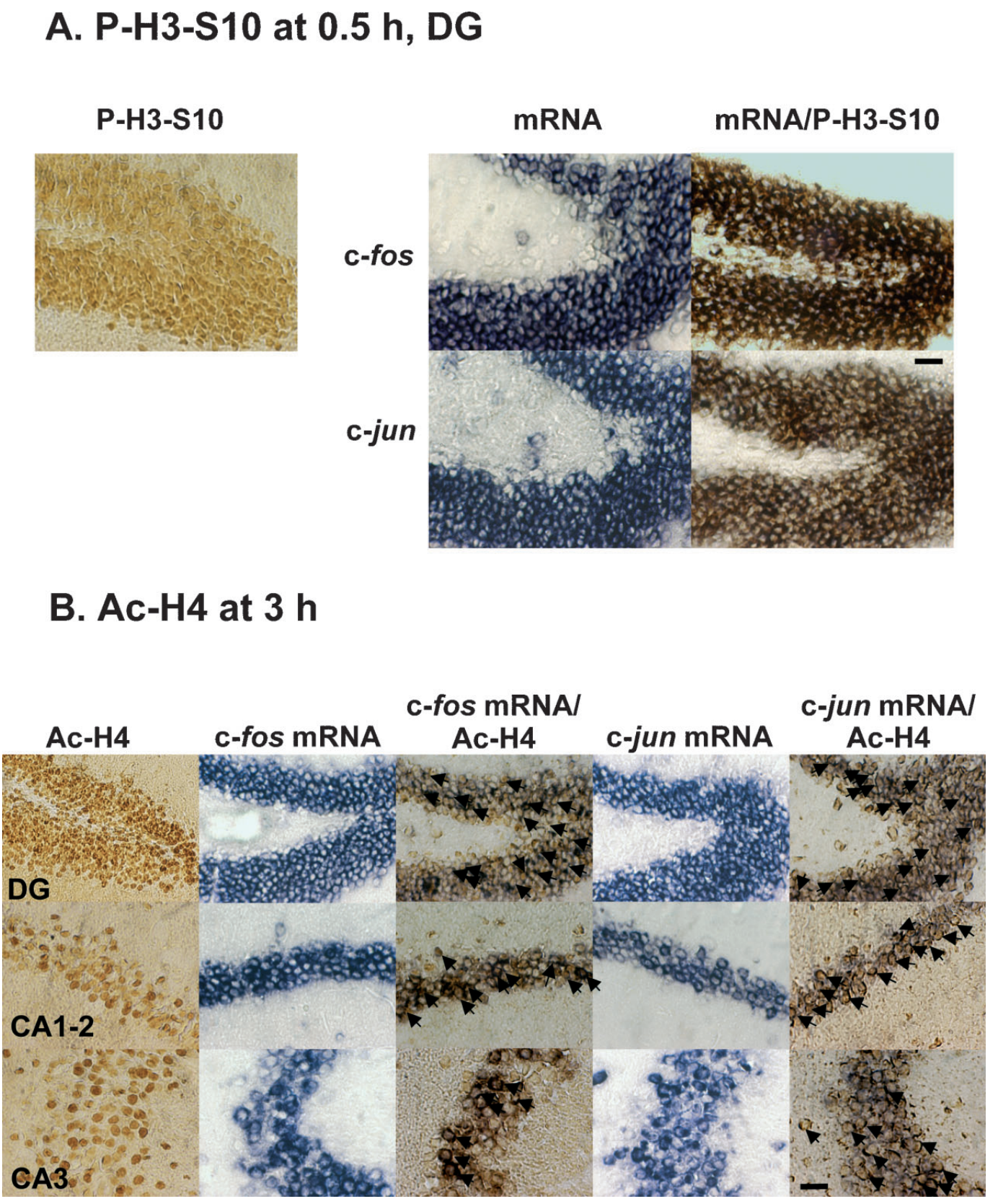

FIG. 7. Histone modifications and expressions of c-*fos* and c-*jun* induced by kainate stimulation in the same hippocampal neurons. (A) High-magnification of dentate gyrus (DG) at 0.5 h after kainate stimulation using a combination of anti-P-H3-S10 antibody (light brown) and c-*fos* or c-*jun* riboprobes (purple). Arrows indicate co-labeling cells. (B) High-magnification of DG and cornus ammonis (CA) regions at 3 h after kainate stimulation using combination of anti-Ac-H4 antibody (light brown) and c-*fos* or c-*jun* riboprobes (purple). Arrows indicate co-labeling cells. Scale bars, 25 μm.

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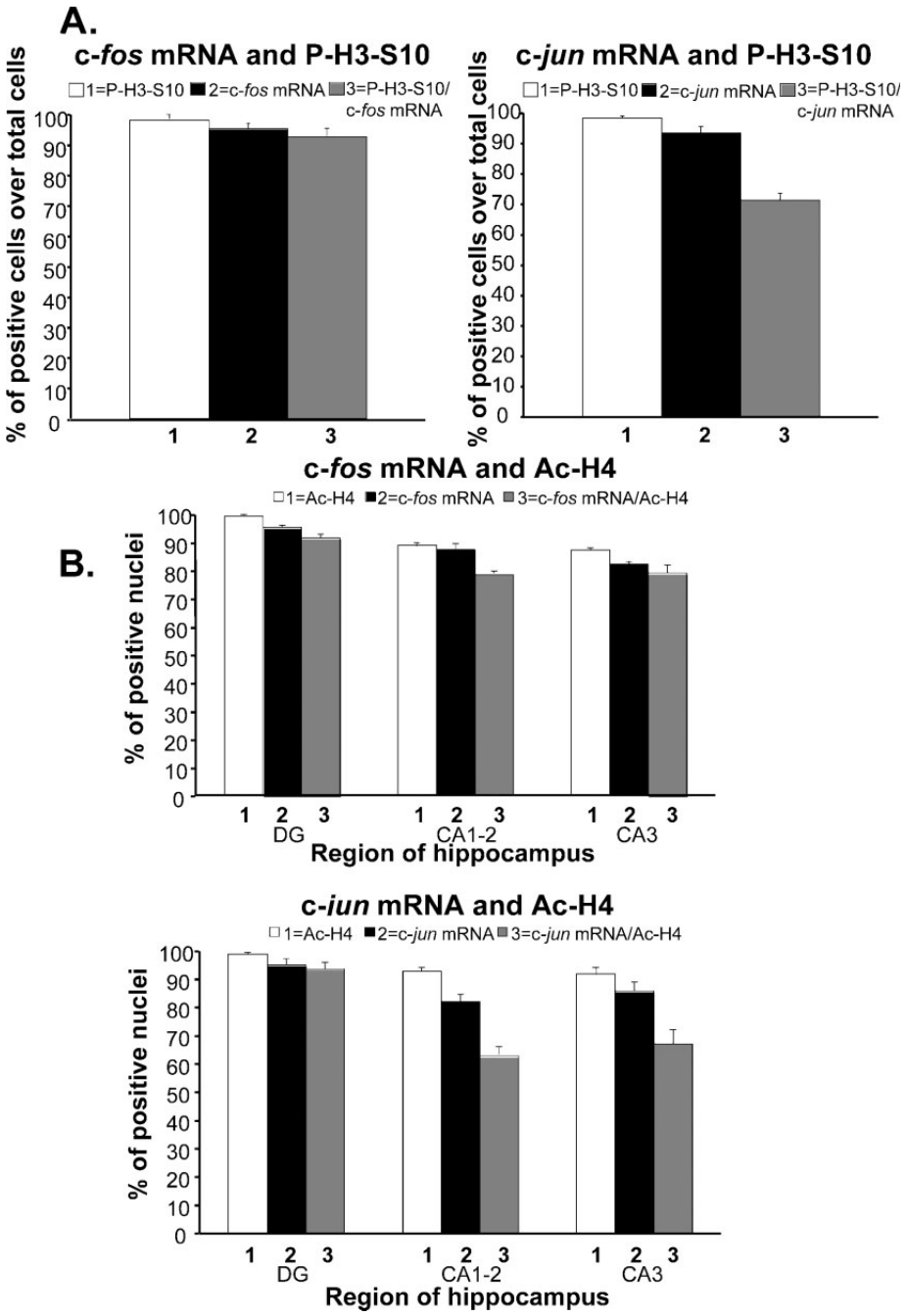

FIG. 8. Histone modifications and expressions of c-*fos* and c-*jun* induced by kainate stimulation in the same hippocampal neurons. (A) Quantification of data in Fig. 7A. (B) Quantification of data in Fig. 7B. The cell count was performed under the microscope.

*Curcumin, a HAT inhibitor specific for CBP, not only prevents the induction of kainate-induced histone modifications and the levels of c-*fos *and c-*jun *mRNA expression but also suppresses the development of epilepsy*

Curcumin is extracted from *Curcuma longa* rhizome, which is used as a yellow pigment in tumeric and curry. Curcumin has been found to inhibit different enzymes including HIV-1 integrase and JNK (Chen & Tan, 1998; Cheng *et al*., 2003). It has also been found to inhibit specifically p300/CBP HAT activity but not other HAT enzymes, and effectively inhibits the acetylation of histones (Balasubramanyam *et al*., 2004). To determine the physiological role of kainate-induced histone acetylation, we examined the effects of curcumin pretreatment on kainate-induced status epilepticus and found: (i) reduction in bulk histone H4 acetylation; (ii) reduction in c-*fos* and c-*jun* mRNA expression; and (iii) finally decreased severity in seizures reflected by seizure scoring.

Curcumin is a lipophilic molecule that distributes to the brain when injected intraperitoneally (Pan *et al*., 1999). Cells pretreated with curcumin for 18–20 h show profound histone acetylation inhibition (Kang *et al*., 2005). To examine the effect of curcumin on histone modifications, mice were pretreated with either dimethylsulfoxide (Veh) or curcumin (Cur, 30 mg/kg) 18 h before kainate injection. Curcumin pretreatment (Cur-KA) inhibited the kainate-induced histone H4 hyperacetylation. Its levels remained between 0.68- and 1.07-fold over Veh-KA control at 0 h and statistically significant from Veh-KA at the corresponding time points (7.6-fold at 0.5 h, 13.7-fold at 3 h and 7.9-fold at 6 h over the control of Veh-KA, Fig. 10A and B).

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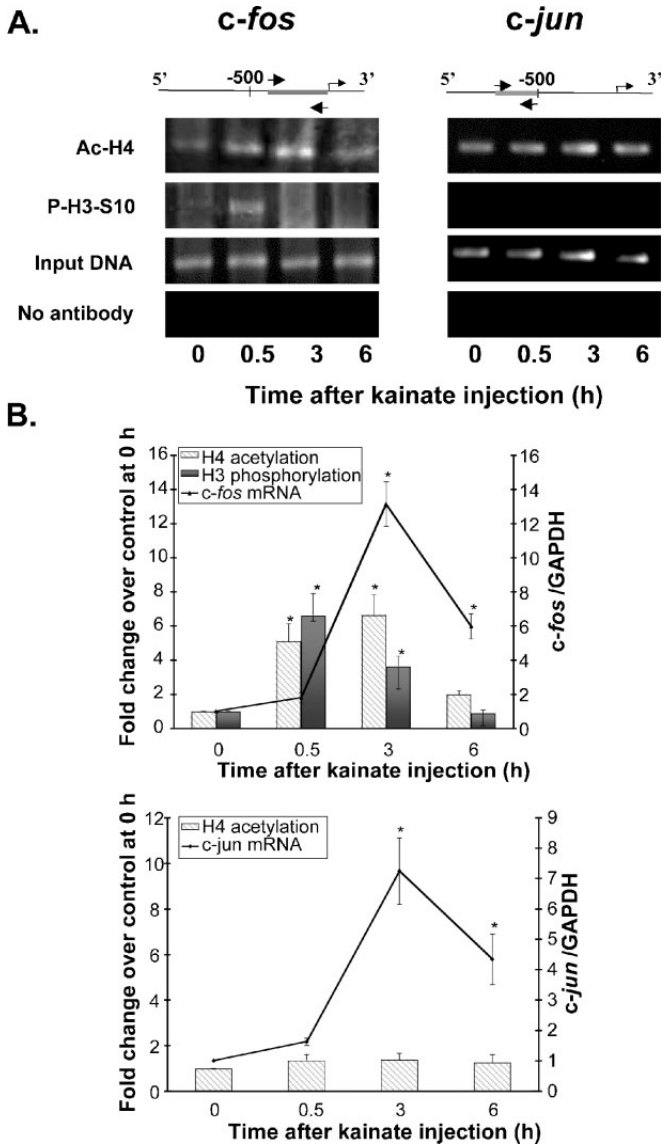

FIG. 9. Histone modifications at the c-*fos* and c-*jun* genes after kainate stimulation. (A) Chromatin immunoprecipitation assay. Genomic DNA was prepared from hippocampi of mice injected with 30 mg/kg kainate at the time as indicated, and immunoprecipitated with anti-Ac-H4 or anti-P-H3-S10 antibodies. The c-*fos* and c-*jun* genes were detected by PCR with primers specific for the region of the indicated genes. Left panel: region of c-*fos* amplified. Right panel: region of c-*jun* amplified. (B) Quantification of data in (A) and in Fig. 2A–C. Relative intensities of amplified c-*fos* and c-*jun* genes products were normalized with those using input DNA and represented as fold change over control at 0 h (mean ± SEM; $n = 4$–5, $^*P < 0.05$ from control). Levels of mRNA expression in Fig. 2A–C were measured, normalized with mRNA levels of GAPDH and represented as fold change over control at 0 h (mean ± SEM; $n = 4$–5, $^*P < 0.05$ from control), a line with closed triangle for c-*fos* mRNA (top panel) or closed diamond for c-*jun* mRNA (bottom panel).

We also investigated the effect of curcumin pretreatment on the levels of histone phosphorylation. Interestingly, curcumin pretreatment also decreased the level of histone H3 phosphorylation at serine 10, retaining between 0.97- and 1.2-fold change over Veh-KA control at 0 h after kainate stimulation (Fig. 10A and B). There was statistical significance between the corresponding time points of Veh-KA and Cur-KA. This suggests that curcumin has either direct or indirect inhibitory effect on histone H3 phosphorylation at serine 10. To examine the effect of curcumin on the induction of CBP, tissue lysates extracted from dissected hippocampus were analysed by Western blot. CBP was induced after kainate injection at 0.5 h by 1.84-fold over Veh-KA at 0 h, increasing by 3.0-fold over control at 3 h, and decreasing at 6 h by 2.6-fold (Fig. 10A, fourth and fifth panel). However, curcumin pretreatment showed no induction of CBP over the time course after kainate stimulation (fold change remaining between 0.62- and 0.81-fold over Veh-KA 0 h), suggesting that curcumin inhibits the induction and/or accumulation of CBP.

Next, to determine whether curcumin pretreatment can prevent kainate-induced upregulation of c-*fos* and c-*jun* mRNA, mice were pretreated with either 30 mg/kg curcumin (Cur-KA) or vehicle (Veh-KA) 18 h before kainate stimulation. While vehicle-pretreated mice showed a higher induction of c-*fos* and c-*jun* mRNA levels compared with no treatment (Figs 2 and 10C), we found that curcumin-pretreated mice showed decreased c-*fos* mRNA expression at 0 h (0.52-fold over Veh-KA at 0 h), 3.37-fold change (in parallel to Veh-KA, 6.2-fold) at 0.5 h, and no or little expression at 6 h (Figs 10C, right top panel, and 11A, top panel). On the other hand, inhibition of histone acetylation seemed to have slight or no effect on the c-*jun* mRNA levels (Figs 10C, right middle panel, and 11A, bottom panel), only a significant decrease of c-*jun* mRNA at 3 h (5.0-fold compared with Veh-KA, 9.1-fold) and 6 h (0.6-fold compared with Veh-KA, 1.3-fold). Our finding suggests that histone modifications are required for upregulation of c-*fos* gene expression, whereas c-*jun* gene expression is less susceptible to histone acetylation than c-*fos* gene.

We scored the seizure severity (Fig. 11B) of kainate-induced status epilepticus on mice pretreated with vehicle (Veh-KA, $n = 15$), 3 mg/kg curcumin (3 mg/kg Cur-KA, $n = 18$) or 30 mg/kg curcumin (30 mg/kg Cur-KA, $n = 15$). We obtained unexpected results on the seizure severity: At 0.5 h, low-dose Cur-KA (1.58 ± 0.53) and high-dose Cur-KA (1.15 ± 0.34) showed decreased seizure scores compared with Veh-KA (2.05 ± 0.37). Curcumin pretreatment also reduced seizure severity at 3 h with low-dose curcumin mice scoring 3.13 ± 0.44 and high-dose curcumin mice scoring 1.04 ± 0.15 as compared with Veh-KA, with an average score of 5.04 ± 1.29. At 6 h, Veh-KA and low-dose curcumin mice displayed almost similar scoring of an average of 3.80 ± 0.63 and 3.67 ± 1.13, respectively, but high-dose curcumin-pretreated mice showed returning back to normal (1.04 ± 0.15). Thus, curcumin pretreatment not only prevents the induction of kainate-induced histone modifications and the levels of c-*fos* and c-*jun* mRNA expression, but also decreases the severity of kainate-induced epilepsy.

Discussion

Transcription of genes is a dynamic process even in non-dividing cells, such as neurons, and is controlled by chromatin remodeling and histone modifications. Changes in the modification status of histone tails are thought to regulate the access by regulatory factors to the underlying DNA. Here, we showed that kainate stimulation induced a rapid but transient phosphorylation of histone H3 in DG neurons, and a more widespread and sustained histone H4 acetylation in whole hippocampal neurons *in vivo* (Figs 5 and 6). Crosio *et al*. (2003) also reported that kainate stimulation induces histone H3 phosphorylation but not H3 acetylation in hippocampal neurons associated with c-*fos* gene expression *in vivo*. In our kainate-induced status epilepticus model mice, the spatio-temporal distribution of histone H3 phosphorylation and histone H4 acetylation in the hippocampus were correlated well with mRNA expressions of c-*fos* and c-*jun* genes (Figs 3–6). Furthermore, histone H3 phosphorylation and histone H4 acetylation at the c-*fos* gene were also well correlated with the c-*fos* mRNA expression profile induced by kainate (Figs 2, and 7–9).

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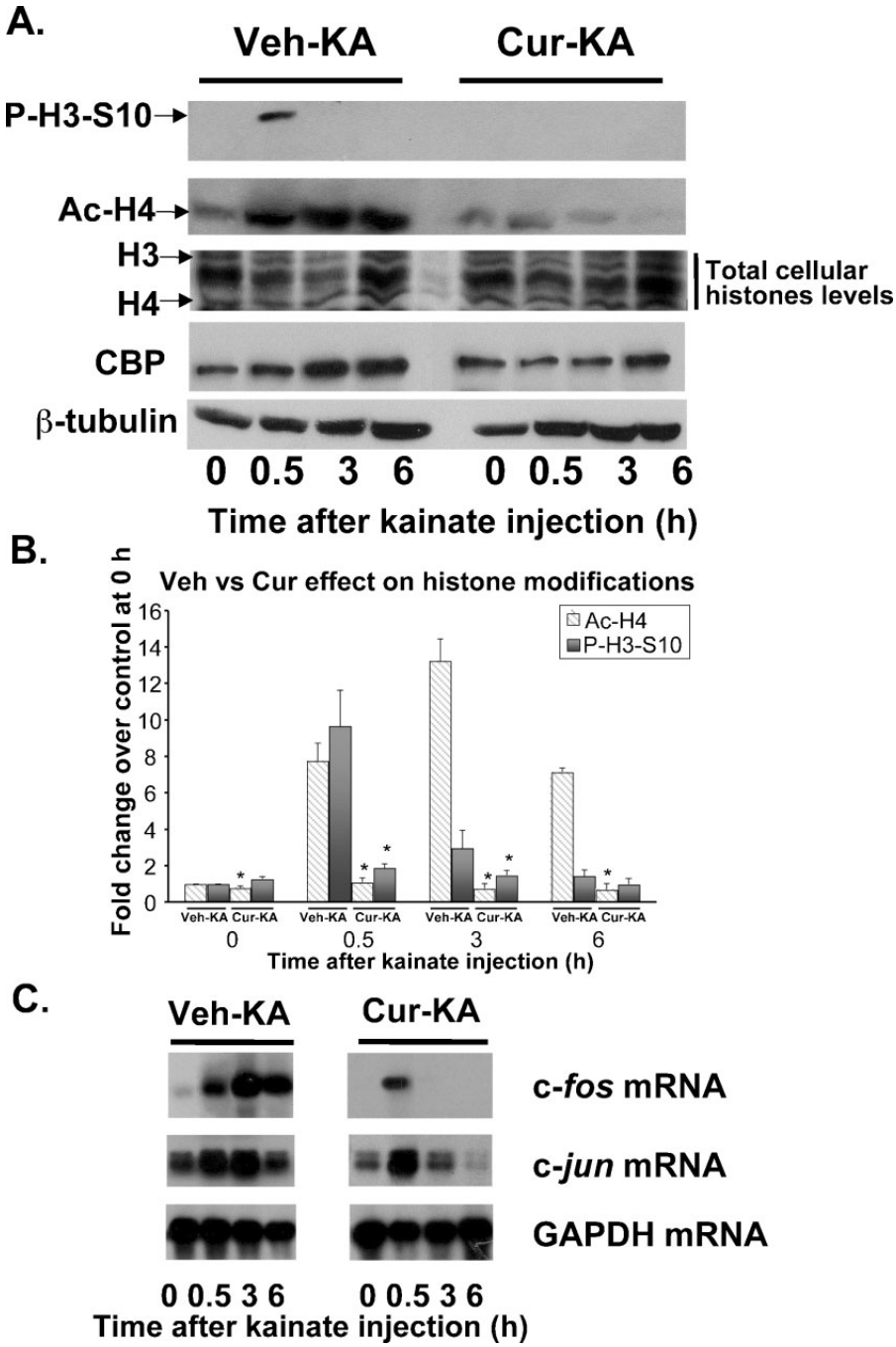

FIG. 10. Curcumin, a HAT inhibitor specific for CREB-binding protein (CBP)/p300 HAT activity, attenuated histone modifications and the expression of IEGs. (A) Curcumin prevents kainate-induced histone modifications. Curcumin (30 mg/kg, Cur-KA) or vehicle (Veh-KA) was injected into mice 18 h before kainate stimulation. The top two panels are Western blot analysis for the histone modifications at the time indicated after kainate stimulation with pretreatment of vehicle (Veh-KA) or curcumin (Cur-KA) using acid-extracted histones detected by anti-Ac-H4 and P-H3-S10 antibodies. The third panel showed acid-extracted histones, visualized with Coomassie Brilliant Blue (CBB) staining. The fourth and fifth panels are Western blot analysis for CBP at the time indicated after kainate stimulation with pretreatment of vehicle (Veh-KA) or curcumin (Cur-KA) using tissue lysates (15 μg protein) detected by anti-CBP and anti-β-tubulin antibodies. (B) Quantitative data analysis in A. Levels of histone modifications were measured, normalized with the corresponding histones levels visualized with CBB staining and represented as fold change over Veh-KA control at 0 h (mean ± SEM, $n = 3$, Student's t-test, $^*P < 0.05$ between the two pretreatment groups). (C) Northern blot analysis of c-*fos,* c-*jun* and GAPDH at time as indicated after kainate stimulation pretreated with either 30 mg/kg curcumin (Cur-KA, right panel) or vehicle (Veh-KA, left panel).

However, we could not detect any significant alterations of these histone modifications at the c-*jun* gene. Histone H4 acetylation seems to be necessary but not sufficient for the activation of c-*jun* transcription in our kainate-induced status epilepticus model because of only minor reduction of c-*jun* mRNA induction by curcumin treatment and a 1.2-fold increase in c-*jun* expression by trichostatin A (TSA) treatment compared with vehicle treatment after kainate administration (Sng *et al*., 2005). Thus, there could be other regulatory factors that may contribute to the c-*jun* induction in kainate-induced status epilepticus.

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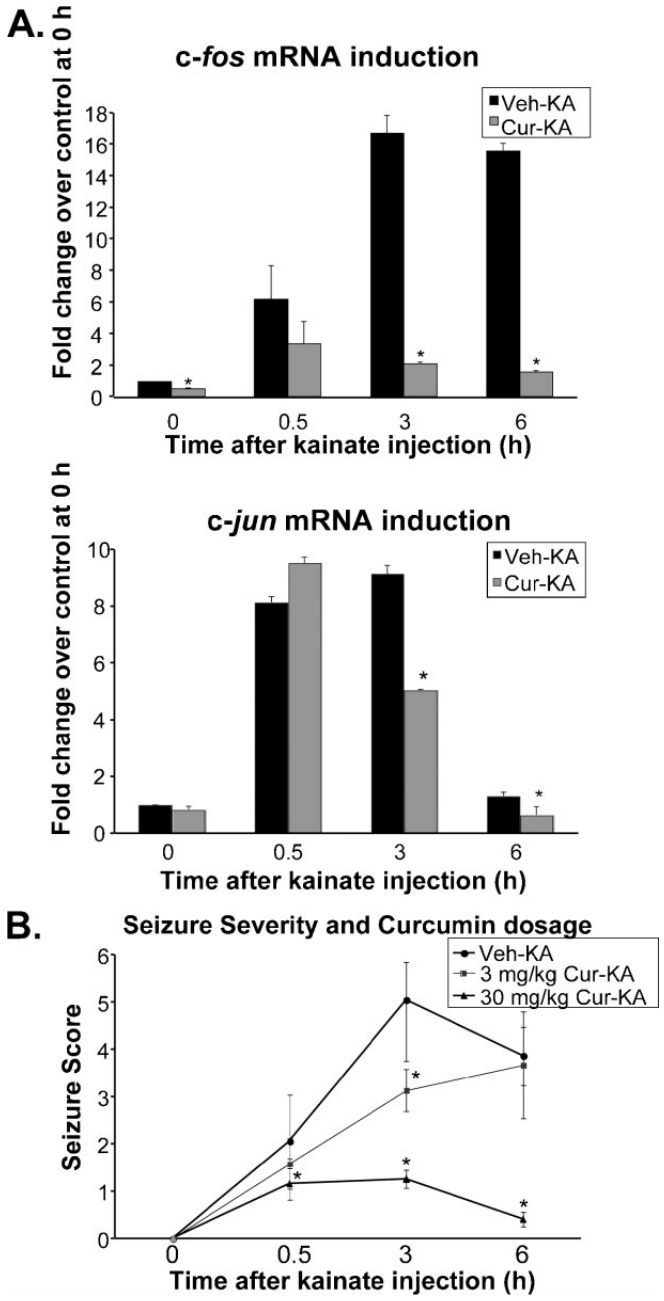

FIG. 11. Curcumin, a HAT inhibitor specific for CREB-binding protein (CBP)/p300 HAT activity, attenuated the expression of IEGs and the severity of status epilepticus induced by kainate. (A) Quantitative data analysis of C. mRNA expression levels of c-*fos* and c-*jun* are measured, normalized with levels of GAPDH and expressed as fold change over Veh-KA control at 0 h (mean ± SEM, $n = 3$, Student's *t*-test, $*P < 0.05$ between the two pretreatment groups). (B) The effect of curcumin on seizure severity. Mice were pretreated with low-dose curcumin (3 mg/kg Cur-KA, black line with closed squares), high-dose curcumin (30 mg/kg Cur-KA, black line with closed triangles) or vehicle (Veh-KA, black line with closed circles), and the seizure severity was scored at the indicated time after kainate stimulation.

As for histone acetylation, different HAT families have been characterized into CBP and p300, the GNAT superfamily, the MYST family, TAF II p250 and TFIIIC family (Sterner & Berger, 2000; Roth *et al*., 2001). Among them, CBP is thought to be a good candidate for the involvement in kainate-induced IEGs expression. CBP is induced by calcium influx via NMDA receptors or voltage-sensitive calcium channels (Hardingham *et al*., 1999; Impey *et al*., 2002). Korzus *et al*. (2004) reported that transgenic mice that expressed mutant CBP, in which the HAT activity was eliminated, exhibit reduced physiological levels of c-*fos* gene expression *in vivo*. We also demonstrated that the kainate-induced expression pattern of CBP in the hippocampus spatio-temporal well correlated with histone H4 hyperacetylation. Recently, curcumin, a major curcumanoid in the spice turmeric, has been found to be a cell-permeable specific inhibitor of the p300/CBP HAT activity among other HAT enzymes *in vitro* (Balasubramanyam *et al*., 2004). Pretreatment of curcumin attenuated histone modifications and IEGs expression by kainate stimulation in the hippocampus, and the severity of kainate-induced status epilepticus (Figs 10 and 11).

Our study provides the first evidence suggesting that histone modifications precede the convulsive behavior after kainate stimulation. We pretreated mice with curcumin for 18 h before kainate stimulation according to the reports. Because curcumin is a lipophilic molecule that distributes to the brain after at least 1 h, we tested different times for curcumin pretreatment (3, 6, 12 and 18 h) and observed similar results of the reduction of seizure scores compared with Veh-KA (data not shown). Curcumin does not cause any sedative effects or loss in locomotion, unlike benzodiazepines, which are commonly used anticonvulsants. Taken together with our data showed that curcumin pretreatment did not decrease the expressions of c-*fos* and c-*jun* significantly at 0.5 h after kainate stimulation (Figs 10C and 11A), this may suggest that curcumin did not prevent the initial excitation of neurons but prevented further propagation and continuation of neuronal excitation correlated with the severity of seizure by alteration of the histone modifications. We have previously reported that TSA, a HDAC inhibitor, pretreatment increases the basal level of histone H4 acetylation by 2-fold, and the expressions of c-*fos* and c-*jun* by 1.8-fold and 1.2-fold, respectively, in the hippocampus after kainate stimulation (Sng *et al*., 2005). We also noted that conversely TSA pretreatment increased seizure score significantly but slightly at 6 h after kainate stimulation compared with Veh-KA (data not shown). Two recent papers clearly reported that seizures induce histone modifications: One demonstrated that electroconvulsive seizures, produced by repetitive electronic stimulation, induce H4 acetylation at c-*fos* and BDNF genes promoters while decreasing at CREB promoters in correlation with mRNA levels of these genes (Tsankova *et al*., 2004). Another showed reduction of histone H4 acetylation at GluR2 promoter but an increase at BDNF promoter after induction of status epilepticus by pilocarpine in rat hippocampal CA3 neurons (Huang *et al*., 2002). Because curcumin has many other cellular effects as reported, we could not rule out that curcumin can inhibit the severity of epilepsy induced by kainate through other pathways resulting in the suppression of stimulation-induced histone modifications. Further investigations are necessary to understand the exact mechanistic action of curcumin when used as a prophylaxis in epilepsy.

Although c-*fos* is a good candidate gene for the involvement in the initiation and development of epilepsy from our results, several papers have reported contrary results. c-*fos*-deficient mice exhibit more severe kainate-induced seizures (Jin *et al*., 2002; Zhang *et al*., 2002), but others show the anticonvulsant properties by antisense c-*fos* oligodeoxynucleotides treatment in kainate-induced seizures (Panegyres & Hughes, 1997). There could also be many other genes, other than c-*fos* or c-*jun*, which are involved in epileptogenesis and could have been induced by kainate stimulation and upregulated by the observed histone modifications.

It is unclear the role of histone H3 phosphorylation in IEGs expression and epilepsy development. There was no correlation between H3 phosphorylation and IEGs expression in CA regions of the hippocampus when IEGs and seizure severity are at their peaks. Pretreatment of mice with curcumin suppressed histone modifications,

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H3 phosphorylation and H4 acetylation during kainate stimulation. Addition of negatively charged phosphate groups may disrupt electrostatic interactions between basic H3 tail and DNA backbone, just like the effects of histone acetylation. Earlier reports have described phosphorylation of histone H3 on c-*fos*- and c-*jun*-associated nucleosomes upon gene activation in both *in vitro* and *in vivo* conditions (Barratt *et al*., 1994; Clayton *et al*., 2000; Strelkov & Davie, 2002; Crosio *et al*., 2003). Our present data also showed well the correlation of H3 phosphorylation and IEGs expression in DG of the hippocampus, and association of H3 phosphorylation on the c-*fos* gene at an early time point after kainate administration, suggesting the role of H3 phosphorylation in c-*fos* expression in DG. Clayton *et al*. (2000) and Cheung *et al*. (2000) demonstrated the synergistic coupling of H3 phosphorylation and H3 acetylation, but Thomson *et al*. (2001) showed independent regulation of histone H3 phosphorylation and H4 acetylation in the activation of c-*fos* and c-*jun* genes. Histone H3 phosphorylation may be necessary for the induction of H4 acetylation, initial induction of IEGs in DG or propagation of H4 acetylation to CA regions of the hippocampus.

We found that inhibition of histone H4 acetylation by curcumin also inhibited the phosphorylation of histone H3 at serine 10, suggesting that either histone H3 phosphorylation is regulated by histone acetylation or curcumin also inhibits directly the histone phosphorylation pathway (Fig. 10A). Curcumin has been reported to inhibit different enzymatic activities, including HIV-1 integrase, JNK activity and p38 MAP kinase (Chen & Tan, 1998; Cheng *et al*., 2003), suggesting that curcumin is not a specific inhibitor for HAT. It has been demonstrated that status epilepticus rapidly induces a transient activation of extracellular regulated kinase (ERK) and p38 MAP kinase signal transduction pathways in the hippocampus (Crosio *et al*., 2003; Jiang *et al*., 2005). Although no report has identified histone H3 kinases in kainate-induced status epilepticus, two H3 kinase candidates have been postulated: MSK1/2, which is phosphorylated and activated by ERK or p38 MAP kinase (Thomson *et al*., 2001). The attenuation of kainate-induced H3 phosphorylation by curcumin may be caused by direct inhibition of p38 MAP kinase. The inhibition of ERK or p38 activation results in a significant reduction of seizure-induced neuronal degeneration. However, these inhibitors did not cause any significant inhibitory effects on acute behavioral seizures induced by either kainate or pilocarpine (Berkeley *et al*., 2002; Kim *et al*., 2004; Jiang *et al*., 2005).

The present finding suggests that histone modifications play a role in kainate-induced epilepsy. There are possibilities that curcumin may find alternative applicability, though we need to understand more of its mechanistic action, and CBP may be a new molecular target to develop the potential therapeutic agents in epilepsy.

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Abbreviations

BCIP, 5-bromo-4-chloro-indolylphosphate; BDNF, brain-derived neurotrophic factor; CBP, CREB-binding protein; ChIP, chromatin immunoprecipitation; DAB, 3,3′-diaminobenzidene; DG, dentate gyrus; DIG, digoxigenin; ERK, extracellular regulated kinase; HAT, histone acetyltransferase; HDAC, histone deacetylase; IEGs, immediate early genes; NBT, nitro-blue tetrazolium; NMDA, *N*-methyl-D-aspartate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PFA, paraformaldehyde; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TSA, trichostatin A.

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