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Inhibition of histone deacetylation by trichostatin A intensifies the transcriptions of neuronal c-*fos* and c-*jun* genes after kainate stimulation

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Abstract

Kainate stimulation induces the expression of immediate early genes, c-*fos* and c-*jun* genes. Trichostatin A (TSA), a potent histone deacetylase (HDAC) enzyme inhibitor was used to test the role of histone hyperacetylation in the transcriptional regulation of c-*fos* and c-*jun* genes in neuronal cells in vivo and in vitro. Intraperitoneal administration of TSA increased histone H4 acetylation in hippocampi. Mice pretreatment with TSA were injected with kainic acid intraperitoneally and sacrificed over a time course of 12 h. Northern blot analysis and in situ hybridization showed that TSA pretreatment caused an increase in pre-existing basal levels of c-*jun* at 0 h and also intensified the maximal expression of both genes especially in the pyramidal layers of the hippocampus, thus demonstrating the inhibition of HDACs subsequently led to histone hyperacetylation to increase these genes expressions. TSA did not prolong the expression of c-*fos* or c-*jun* gene, in contrary to what we expected. Primary hippocampal neuron cell culture also displayed a similar pattern of c-*fos* and c-*jun* mRNA enhancement with trichostatin A pretreatment. This study demonstrated that inhibition of histone deacetylation by TSA in neuronal cells affect the expressions of c-*fos* and c-*jun* genes, suggesting histone acetylation might play a role in the regulation of both genes expressions after kainate stimulation.

Keywords: Kainic acid; Hippocampus; Trichostatin A; Histone acetylation; c-*fos* gene; c-*jun* gene

Histones associate extensively with the DNA in the nuclei of eukaryotic cells to package eukaryotic genomes within the nuclei. An octamer of highly conserved histone proteins wrapped around by DNA forms nucleosomal barriers that prevent transcription [13]. Each core histone is composed of a structured domain with a protruding lysine-rich amino-terminal tail. Histone tails can be covalently modified by acetylation, phosphorylation, methylation, and ubiquitination, resulting in weaken histone:DNA contacts [5]. Among the modification processes, histone acetylation has been linked positively to transcriptional activation. Steady-state levels of histone acetylation is balanced through the interplay between histone acetyltransferases (HATs) and histone deacetylases (HDACs) to either add or remove an acetate group from acetyl-CoA acting on lysine residues of histone tails [5].

Kainate stimulation induces the expression of several immediate early genes (IEGs) including two commonly used markers, c-*fos* and c-*jun* genes in neurons in vivo and in vitro. Crosio et al. reported that kainate stimulation induces histone H3 phosphorylation correlating with c-*fos* upregulation while Huang et al. reported that pilocarpine stimulation alters histone acetylation that downregulated the expression of GluR2 in hippocampal neurons in vivo [4,7]. In this study, we use trichostatin A (TSA), which can inhibit HDAC activity at nanomolar concentration to test the role of histone acetylation in the transcriptional regulation of immediate early genes in neuronal cells.

Materials: Kainic acid (kainate) and trichostatin A were purchased from Wako, Japan (Cat. 114-00611 and 200-11993). Antibodies against c-*fos* (Cat. sc-52) and c-*jun* proteins (Cat. sc-45) were obtained from Santa Cruz

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Biotechnology, Inc (CA, USA) and acetylated histone H4 antibody (Cat. 06-866) was obtained from Upstate Biotechnology (NY, USA).

Animals: Wild-type Std-ddY, 6-week-old adult male mice, were purchased from Sankyo Laboratories, Toyama, Japan. 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.

Cell culture: Embryonic mouse hippocampus was dissected from fetal ICR mice at 18 days of gestation, trypsinized and triturated. Dissociated cell suspensions were plated at 6.67×10^5 cells/cm^2 on plastic tissue culture dishes coated with poly-L-lysine, in defined medium (Neurobasal/B27, Invitrogen) supplemented with 2 mM glutamine, penicillin (25 units/ml) and streptomycin (25 μg/ml). Cultures were maintained in a humidified 5% CO_2 incubator at 37 °C for 9 days before treatment. Under these conditions, cultures contained ∼95% neurons.

Trichostatin A administration: For in vivo administration, trichostatin A was dissolved in dimethyl sulfoxide and animals were injected intraperitoneally (i.p.) with 1 mg/kg of either DMSO or TSA at least 2 h prior to kainate administration as described previously [8]. In order to analyze the accumulation of histone acetylation in hippocampus, Western blot analysis was performed on hippocampi of mice injected i.p. with either TSA or Veh and sacrificed over 8 h. For cell culture, TSA was dissolved in DMSO as 300 μM concentration and cultures were treated with 300 nM of TSA 1 h prior to 100 μM of kainate.

Kainic acid administration: Animals were injected intraperitoneally (i.p.) with 30 mg/kg of kainate in a volume of 0.1 ml/kg. A total of six animals were analyzed from three independent experiments.

Tissue preparation: Mice were fixed by transcardial perfusion of 4% paraformaldehyde (PFA) and cryoprotected in 30% sucrose solution overnight at 4 °C. Ten micrometer- and 40 μm-thick coronal cryosections were cut on a microtome. For northern and Western blots, hippocampal tissue was dissected from mouse brains.

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. All three cDNAs were subcloned into pBlueScript SK vector. Plasmids were linearized and transcriptionally labeled by T3 or T7 RNA polymerases to make the anti-sense digoxigenin (DIG)-labeled riboprobes.

RNA purification and Northern blotting: Total cellular RNA (2.5 μg) was resolved on 1% formaldehyde/agarose gels and transferred onto positively charged nylon transfer membranes (Hybond N+, Amersham Biosciences, UK) and hybridized with the probes of c-*fos*, c-*jun* and GAPDH. The membranes were washed and exposed for 1 min for GAPDH and 5 min for c-*fos* and c-*jun* on X-ray film to standardize exposure time.

In situ hybridization: Cryosections (10 μm) were mounted on silanized slides (DakoCytomation, Japan) in RNase-free conditions. The sections were prehybridized first and followed by layering of 500 ng/ml of DIG-labeled c-*fos* or c-*jun* riboprobe. The slides were treated with RNase A, blocked 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-indolyphosphate/Nitroblue tetrazolium (BCIP/NBT) for 1 h.

Acid extracted histones and Western blotting: Total histones were extracted from hippocampus with H_2SO_4, precipitated with trichloroacetic acid and washed in 0.05 M HCl/acetone and 100% acetone. The protein was loaded and separated by 18% SDS-PAGE followed by electroblotting onto polyvinylidene difluoride membrane (Millipore, MA, USA). Acetylated histone H4 was detected with rabbit polyclonal anti-acetyl H4 antibodies (1:1000) and visualized with enhanced chemiluminescence substrate (Amersham Biosciences, UK).

Immunohistochemistry: Free floating sections of 40 μm thickness were blocked with goat serum and incubated with the primary antibody (1:300), with goat anti-rabbit secondary biotinylated antibody (1:200), followed by avidin–biotin-peroxidase complex solution (ABC solution, Vector Laboratories, Burlingame, CA) and developed with 3–3′ diaminobenzidine DAB (Wako, Japan) and H_2O_2.

Densitometry: Northern blot analysis and immunohistochemistry were quantified with Image J software. For Northern blot, the intensity of each lane was quantified and the data was expressed and normalized as a ratio of mRNA expression for each gene over the GAPDH. For in situ hybridization and immunohistochemistry, the data was represented as a ratio of the measured intensity over the area of the outlined region of hippocampus (average intensity). Significant changes in RNA and protein expressions were determined using the two-tailed Student's *t*-test with unequal variance.

Intraperitoneal administration of TSA in mice increased the level of acetylated histone H4 relative to the total histone H4 in hippocampus by 2-fold (Fig. 1A). Pretreatment with TSA (Fig. 1B top left and right panels and 1C top panel) increased the sensitivity of c-*fos* gene to kainate stimulation. The expression of c-*fos* mRNA was induced in both pretreatments but TSA pretreatment-induced c-*fos* mRNA 1.55- and 1.76-fold more than DMSO pretreatment at 3 h and at 6 h, respectively. c-*fos* mRNA expression was decreased but sustained at 9 h. TSA pretreatment caused a 1.59-fold increase in mRNA expression compared to DMSO pretreatment at 9 h. Pretreatment by TSA induced basal expression of c-*jun* mRNA by 7.29-fold in comparison with DMSO pretreatment (Fig. 1B middle left and right panels and 1C bottom panel). TSA pretreatment induced c-*jun* expression to increase steadily by 1.23-fold over DMSO pretreatment at 6 h, 1.28-fold over DMSO at 9 h after kainate injection. Both genes expressions were decreased back to almost basal levels at 12 h. Both pretreatments are represented graphically as a ratio of mRNA normalized by their respective GAPDH

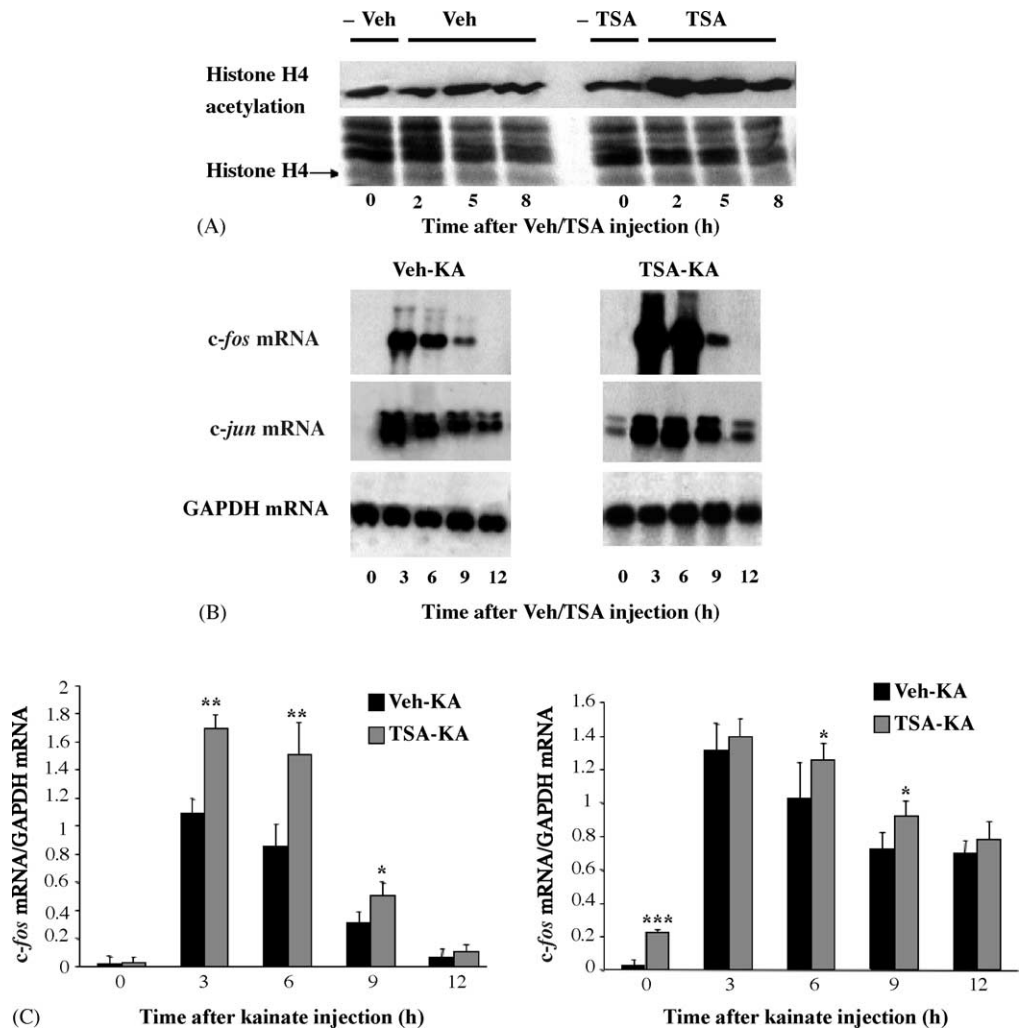

Fig. 1. Increase in expressions of c-*fos* and c-*jun* mRNA after kainate injection with TSA pretreated mice. (A) Western blot analysis on acid-soluble proteins obtained from dissected hippocampi of mice sacrificed 2–8 h after i.p. injection of DMSO (Veh) or TSA. The extracted proteins (20 μg) were separated on 18% SDS/PAGE gels and revealed using acetylated H4 histone antibody (top panel) and the total amount of histones visualized by Coomassie Brilliant blue-staining (lower panel). (B) Total cellular RNA was prepared from hippocampi of mice sacrificed at 0, 3, 6, 9, and 12 h after kainate injection, pretreated 2 h before with DMSO (Veh-KA) or trichostatin A (TSA-KA). Total RNA (2.5 μg) was resolved on formaldehyde/agarose gels and c-*fos*, c-*jun* and GAPDH mRNA were detected by DIG-riboprobes. (C) Quantification of data, normalized to levels of GAPDH, were expressed as mean ± S.E.M. Statistical comparisons were performed with Student's *t*-test between Veh-KA and TSA-KA ($^{*}p < 0.05$ and $^{**}p < 0.01$).

levels. Pretreatment with TSA enhances the mRNA expressions of c-*fos* and c-*jun* genes in hippocampus, in particular, initiation of c-*fos* gene is more sensitive to TSA pretreatment whereas TSA sustained the expression of c-*jun* mRNA (Fig. 1C). We verified this result by TSA-pretreatment of the animals without the injection of kainate over a period of 8 h and found that TSA increased the basal and continual expression of c-*jun* mRNA but only a small increase in c-*fos* mRNA in the absence of kainate stimulation (data not shown).

We also performed in situ hybridization to examine the spatial-temporal expression patterns of c-*fos* and c-*jun* genes mRNA in the hippocampus over a time course of 12 h after kainate treatment (Fig. 2). It was observed that inhibition of histone deacetylation by TSA-pretreatment strongly upregulated c-*fos* mRNA in the dentate gyrus (DG) at 3 h by 1.40-fold over DMSO-KA and continuing to exert its effect at 6 h by 1.75-fold increase over DMSO and sustaining a 3.02-fold increase over DMSO-KA at 9 h. TSA-pretreatment also increased the expression of c-*fos* mRNA by 1.2-fold in cornus ammonis (CA) 1–2 and 3 at 6 h and particularly at 9 h with significant 5.12-fold over DMSO in CA 1–2 and 3.28-fold over DMSO in CA 3 region. However, pretreatment with TSA caused an increase in basal level of c-*jun* mRNA by 2.32-fold over DMSO-KA in the DG and 2.47-fold over DMSO in the CA 3 region. There was only a 1.64-fold increase of c-*jun* mRNA over DMSO in CA 1–2 region at 3 h but no significant increase in DG and CA 3 regions. At 6 h, c-*jun* mRNA expression was significantly increased by 1.13- and 1.20-fold in both DG and CA 1–2 regions, respectively. An apparent significant increase of c-*jun* mRNA was observed in the pyramidal layers of the CA 1–2 and DG regions by 3.40- and 1.50-fold in CA 3 at 9 h. Diffused staining of the

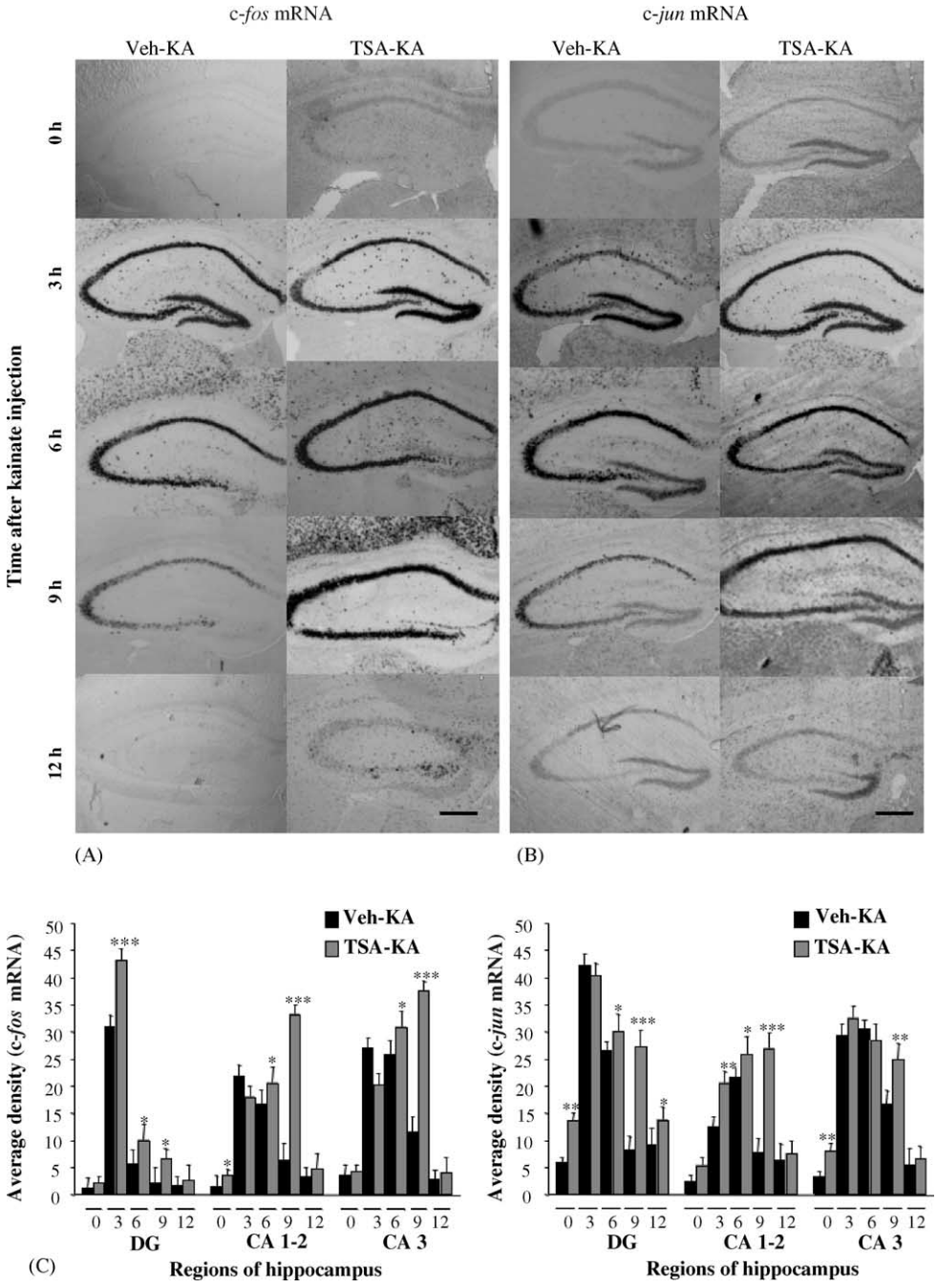

Fig. 2. Spatial and temporal expression pattern of c-*fos* (A) and c-*jun* (B) mRNAs in mice pretreated with DMSO or TSA followed by kainate injection. In situ hybridization using c-*fos* and c-*jun* DIG-riboprobes on hippocampal cryosections obtained from mice sacrificed at 0, 3, 6, 9, and 12 h after kainate injection, pretreated 2 h before with either DMSO (Veh-KA) or TSA (TSA-KA). Scale bars, 500 μm. (C) Quantification of data in (A and B), expressed as a ratio of the intensity over the area of the region (average intensity) ± S.E.M. Regions of hippocampus are abbreviated as dentate gyrus, DG; cornus ammonis, CA; separated as CA 1–2 and CA 3. Statistical comparisons were performed with Student's *t*-test between Veh-KA and TSA-KA ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

cortex of c-*fos* mRNA was observed at 6 h and peaking at 9 h after TSA-KA treatment, in contrast to Veh-KA which cortical expression of c-*fos* mRNA was observed at 6 h. However, it is beyond the scope of this paper to discuss the cortical staining observed.

The enhancement of c-*fos* and c-*jun* mRNA expression resulted in increased intensity of c-*fos* and c-*jun* proteins in immunohistochemistry (Fig. 3A), particularly in the dentate gyrus and the pyramidal layers of the hippocampus. c-*fos* protein increased significantly after TSA-KA treatment by 1.22- and 2.04-fold over DMSO-pretreated KA mice in the DG at 3 and 6 h, respectively and sustaining its expression in the pyramidal layers by 1.90-fold at 3 and 6 h in comparison to vehicle-pretreated mice. Basal levels of c-*jun* protein was increased significantly after TSA-treatment by 1.3–1.4-fold in comparison to vehicle-pretreated mice in all regions of

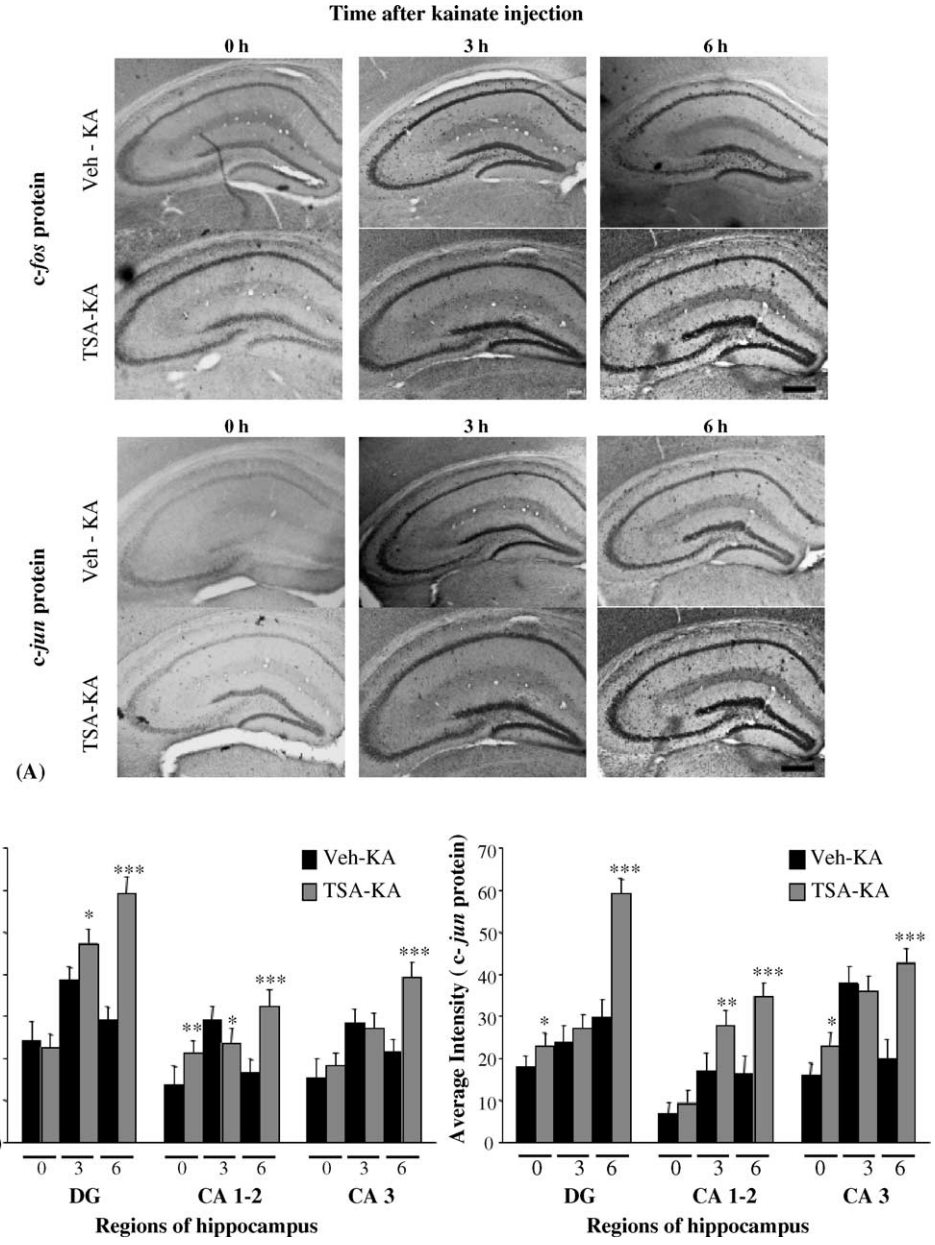

Fig. 3. Increase in c-*fos* and c-*jun* proteins induction after kainate injection with TSA pretreatment. (A) Immunohistochemistry using anti-c-*fos* and c-*jun* proteins antibodies on hippocampal cryosections obtained from mice sacrificed 0, 3, and 6 h after kainate injection, pretreated 2 h before with either DMSO (Veh-KA) or TSA (TSA-KA). Scale bars, 500 μm. (B) Quantification of data in (A), expressed as a ratio of the intensity over the area of the region of hippocampus (average intensity) ± S.E.M. Statistical comparisons were performed with Student's *t*-test between Veh-KA and TSA-KA ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$).

the hippocampus. Significant increase of c-*jun* protein was observed in the pyramidal layers of CA 1–2 by 1.62-fold at 3 h and continued to sustain by 2.0–2.14 fold in all regions of hippocampus by 6 h over vehicle-pretreated mice c-*fos* protein values (Fig. 3B).

Furthermore, we performed the experiment using primary hippocampal neurons to see the effects of histone deacetylation inhibition by TSA. We observed enhancement of c-*fos* and c-*jun* genes expressions with TSA pretreatment after kainate stimulation in hippocampal neurons (Fig. 4). This study demonstrated consistent enhancement of c-*fos* and c-*jun* genes expressions after TSA-kainate treatment in both in vivo and in vitro systems, suggesting a role of histone acetylation in regulating the expression of both genes in neuron cells.

c-*fos* and c-*jun* genes belong to a subgroup of inducible transcription factors, known as immediate early genes (IEGs) [12]. One of the main attributes of this group is the rapid induction and quick cessation of their expression within minutes of cellular stimulation, which could be controlled by pre-existing transcriptional factors [6] and of recent interest, histone modifications at the chromatin level. By maintaining

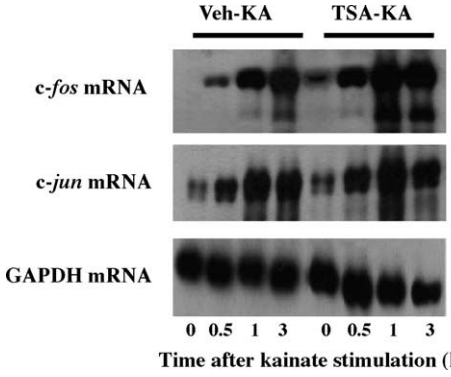

Fig. 4. Intensification in the expressions of c-*fos* and c-*jun* mRNA in primary neuronal culture after kainate stimulation with TSA pretreatment. Total cellular RNA was obtained at 0, 0.5, 1, and 3 h after kainate administration, pretreated 2 h before with either DMSO (Veh-KA) or TSA (TSA-KA). Total RNA was resolved and probed with c-*fos*, c-*jun* and GAPDH DIG-riboprobes.

the dynamic equilibrium of the acetylation status of highly conserved lysine residues on histones, chromatin remodeling and gene expression can be regulated [1]. We used TSA to cause histone hyperacetylation in neuron cells and correlated the findings with the expression of IEGs, c-*fos* and c-*jun* genes, which are common markers upregulated during kainate stimulation.

TSA caused an increase in pre-existing basal levels at 0 h due to greater sensitivity of both genes to kainate stimulation with TSA pretreatment, notably in vitro culture. TSA also intensified the maximal expressions of both genes, demonstrating the inhibition of HDACs subsequently led to histone hyperacetylation to increase and intensify gene expression. However, TSA did not prolong the expression of c-*fos* or c-*jun* gene in contrary to what we had expected, suggesting no involvement of HDACs in cessation of IEGs expression.

We then observed the sequential upregulation of c-*fos* and c-*jun* genes induction after kainate treatment (Fig. 2). This spatial-temporal pattern observed after kainate stimulation is the transmission of neural signals to the hippocampus through an excitatory tri-synaptic chain. The major afferent input to the hippocampus comes from the entorhinal cortex through the perforant path to the dentate gyrus, thus making it the first structure to be affected by kainate. The dentate gyrus connects to the cornus ammonis 3 (CA 3) via mossy fibers and the sequential induction of IEGs is then spread to CA 1 via the Schaffer's collaterals that propagates the onset of seizure to other limbic structures [2,9–11]. We observed the persistent expression of c-*fos* and c-*jun* mRNA at the CA or pyramidal regions at from 6 to 9 h (Figs. 1 and 2) with their effective corresponding protein translation (Fig. 3), as firstly, the CA 3 regions are said to preferentially express high-affinity kainate receptors in the mossy fiber synaptic region [3] and secondly, this result may also suggest the different sensitivity of the pyramidal layer cells compared with dentate gyrus neuronal cells in response to TSA pretreatment.

Expressions of c-*fos* and c-*jun* genes were also enhanced differently. TSA pretreatment enhanced each gene expression in varying degrees in response to kainate stimulation (Figs. 1 and 4). A possible explanation for this could be that histone deacetylation would repress the expression of c-*fos* more than that of c-*jun*. We could further infer from this data that c-*fos* gene could also be more responsive to histone hyperacetylation whereas c-*jun* gene requires more than just HDACs to silence its expression.

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