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## JNK1 regulates histone acetylation in trigeminal neurons following chemical stimulation

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### Abstract

Trigeminal nerve fibers in nasal and oral cavities are sensitive to various environmental hazardous stimuli, which trigger many neurotoxic problems such as chronic migraine headache and trigeminal irritated disorders. However, the role of JNK kinase cascade and its epigenetic modulation of histone remodeling in trigeminal ganglion (TG) neurons activated by environmental neurotoxins remains unknown. Here we investigated the role of JNK/c-Jun cascade in the regulation of acetylation of H3 histone in TG neurons following *in vitro* stimulation by a neuro-inflammatory agent, mustard oil (MO). We found that MO stimulation elicited JNK/c-Jun pathway significantly by enhancing phospho-JNK1, phospho-c-Jun expression, and c-Jun activity, which were correlated with an elevated acetylated H3 histone in TG neurons. However, increases in phospho-c-Jun and c-Jun activity were significantly blocked by a JNK inhibitor, SP600125. We also found that altered H3 histone remodeling, assessed by H3 acetylation in triggered TG neurons, was reduced by SP600125. The study suggests that the activated JNK signaling in regulation of histone remodeling may contribute to neuro-epigenetic changes in peripheral sensory neurons following environmental neurotoxic exposure.

### Introduction

Trigeminal nerve fibers in nasal and oral cavities are sensitive to various environmental hazardous stimuli originating from chemical irritants and toxins. Oral and nasal trigeminal chemosensory stimuli include volatile chemical toxins present in living and work environments. These neuro-irritating volatile compounds trigger many neurotoxic problems including chronic migraine headache, trigeminal and orofacial pain. It causes patients to seek medical treatment and may causes long-term suffering. Despite various medical advancements, the medical community still faces a significant challenge to successfully relieve chronic pain

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resulted from environmental hazardous exposure. Although multiple cellular pathways have been identified as transmitters of the neuro-stimulant signals [1], the neurochemical pathways relaying this information at the epigenetic level in trigeminal ganglion (TG) neurons in response to environmental neurotoxins remains unclear.

Previous studies have suggested that regulation of histones, and thus remodeling of chromatin structure, plays a critical role in controlling gene transcription and facilitating long-term changes in neuronal plasticity. Different stimuli alter in chromatin structure in the CNS neurons, and chromatin structure is regulated in hippocampal neurons in response to activation of multiple intraneuronal kinase pathways [2,3]. In the present study, an *in vitro* preparation of primary cultured TG neurons was used to evaluate the reaction of c-Jun N-terminal kinase (JNK)—c-Jun cascade and levels in histone acetylation were examined in response to neurotoxic stimulation. Furthermore, whether the JNK cascade has a potential role in association with altered histone modulation was also investigated.

## Materials and methods

### Trigeminal neuron culture

Primary cultures of TG neurons of male adult Sprague-Dawley rats (weighing 260–300 grams, Harlan Sprague-Dawley, TX) were used in this study. The protocol was approved by the Institutional Animal Care Committee and in accordance with the ethical guidelines of NIH. After anesthetized by pentobarbital and decapitated. TG neurons were excised and enzymatically dissociated and plated on poly-L-lysine-coated dishes in F12-medium with 10% fetal calf serum ( $2 \times 10^6$  cells).

The modified concentration of a neurostimulant, mustard oil was 10  $\mu$ M (dissolved in mineral oil, allyl isothiocyanate; Fluka) on a daily basis for a sustained stimulation, since a higher dose demonstrated an acute robust effect [1]. Our preliminary data suggested that current concentration of mustard oil produced a lesser toxicity to TG neurons (data not shown). Mineral oil served as a vehicle control. TG neurons received the DMSO as a control when a JNK inhibitor, SP600125 (1,9-Pyrazoloanthrone, Sigma, MO) was co-administrated at a concentration of 10  $\mu$ M, a concentration significantly to block the phosphorylation of c-Jun without impacting cellular morphology and cell viability [4]

### Extraction of histone protein and Western blot analysis

The pellet was resuspended in TEB (PBS containing 0.5% Triton X 100, 2 mM PMSF, 0.02% NaN<sub>3</sub>). Cells were lysed and centrifuged. The supernatant (cytoplasmic fraction) was removed and nuclear fraction was resuspended. Histone proteins were acid extracted from the nuclear fraction and centrifuged. The concentration of protein was tested by using a BCA kit and was read on a microplate reader [5]. Equal amounts of protein (40  $\mu$ g) were loaded and size fractionated by SDS-PAGE in a 4–20% ready-gel preparation and transferred onto a PVDF membrane. After blocking in buffer, the membranes were incubated with antibodies to phospho-JNK1, phospho-c-Jun, and acetylated H3 (Lys-9) and H3 (Cell Signals, MA) overnight at 4°C. The antibody against H3 histone does not have cross-reactions with acetyl-H3. The blots were rinsed three times with PBS and then incubated with HRP-conjugated anti-rabbit or anti-mouse IgG in 5% non-fat milk. The membranes were then enhanced with a chemiluminescence reagent (Amersham, IL) and exposed. The expression of  $\beta$ -actin served as an internal control. The density of the blotted bands was acquired by using a Doc-It Gel system and analyzed by using an AlphaEase software [6,7]. The densitometric units of bands of detected proteins were expressed relative to the values for  $\beta$ -actin.

## ELISA assessment of c-Jun activity

Biochemical activity of c-Jun in TG neurons was determined using a c-Jun assay kit (Active Motif, CA) according to the instructions. Briefly, 20  $\mu$ L of sample nuclear extract containing 10  $\mu$ g of total protein was loaded into each well of a 96-well plate coated by an oligonucleotide containing c-Jun-responsive element. c-Jun contained in the nuclear extracts binds specifically to this oligonucleotide. After incubations in a primary antibody against c-Jun (1:500) and in a secondary antibody conjugated to horseradish peroxidase, c-Jun-associated reaction was visualized and quantified by a colorimetric reaction and read at 450 nm [8].

## Statistical analysis

The significance of differences between different groups were calculated and compared with the Student's *t*-test. Coefficients of correlation between the expression levels of phospho-c-Jun, c-Jun activity, and acetylated H3 were calculated using the Spearman correlation analysis.  $P < 0.05$  was considered significant. All data were expressed as mean  $\pm$  S.E.M. [7].

## Results

### Mustard oil stimulation enhanced JNK/Jun cascade components in TG neurons

The results demonstrated that the JNK/Jun cascade components such as phospho-JNK1 and phospho-c-Jun were detected in TG neurons. Peripheral TG neurons can be activated through ionic transient receptor potential family by the pungent ingredients in mustard seed [1]. Following a prolonged stimulation by mustard oil (MO), the expression of phospho-JNK1 and phospho-c-Jun showed a significant elevation in TG neurons at Day 1, 3, and 5 post-stimulation (Figure 1) when compared to that of neurons treated with mineral oil (MI), a control agent. The relative density of the phospho-JNK1 significantly was upregulated at Day 1 ( $2.12 \pm 0.34$  vs  $1.20 \pm 0.27$ ;  $p < 0.05$ ), Day 3 ( $2.32 \pm 0.38$  vs  $1.18 \pm 0.21$ ;  $p < 0.05$ ) and Day 5 ( $2.75 \pm 0.41$  vs  $1.23 \pm 0.26$ ;  $p < 0.05$ ) post-treatment of MO (Figure 1, Panels A and B). A downstream target protein of JNK, phospho-c-Jun, was also increased at Day 1 ( $1.74 \pm 0.2$  vs  $0.87 \pm 0.07$ ;  $p < 0.05$ ), Day 3 ( $2.07 \pm 0.24$  vs  $0.86 \pm 0.07$ ;  $p < 0.05$ ) and Day 5 ( $2.05 \pm 0.22$  vs  $0.91 \pm 0.06$ ;  $p < 0.05$ ) when compared to neurons treated with control (Figure 1, Panels A and C). There are no significant changes in expression of total JNK and c-Jun proteins between MO-treated and control treated TG neurons (Data not shown). We also investigated the biochemistry activity of c-Jun in TG neurons after noxious stimulation. c-Jun activity significantly increased at Day 1 ( $153 \pm 17.4$  vs. 100;  $p < 0.05$ ), Day 3 ( $179.3 \pm 19.2$  vs.  $109.7 \pm 10.97$ ;  $p < 0.05$ ), and Day 5 ( $207.6 \pm 19.5$  vs.  $108.4 \pm 16.2$ ;  $p < 0.05$ ) in MO-treated TG neurons when compared to the control treatment (Figure 1, Panel D).

### Mustard oil stimulation increased acetylation of H3 histone

To determine the effect of MO on histone structure re-modeling, acetylated H3 histone (Lys-9) and total H3 histone in TG neurons were assessed. The result showed that levels of acetyl-H3 histone, but not the total H3 histone, was elevated significantly in TG neurons treated with mustard oil at Day 1 ( $2.7 \pm 0.53$  vs.  $1.38 \pm 0.29$ ;  $p < 0.05$ ), Day 3 ( $2.8 \pm 0.52$  vs.  $1.43 \pm 0.28$ ;  $p < 0.05$ ) and Day 5 ( $2.97 \pm 0.39$  vs.  $1.42 \pm 0.34$ ;  $p < 0.05$ ), when compared to TG neurons administered with mineral oil (Figure 2).

### Effects of an inhibitor of JNK, SP600215

To investigate the activity of JNK kinase in regulating its downstream c-Jun expression through its phosphorylation, we assessed the effect of an inhibitor of JNK kinase, SP600125, on the expression phospho-c-Jun, as well as c-Jun activity by ELISA assay. The immunoblot result showed that there was a marked decrease in the phospho-c-Jun expression in mustard oil stimulated-TG neurons treated with SP600125 at Day 1 ( $0.76 \pm 0.06$  vs.  $1.43 \pm 0.14$ ;  $p < 0.05$ ),

Day 3 ( $0.73 \pm 0.06$  vs.  $1.66 \pm 0.12$ ;  $p < 0.05$ ), and Day 5 ( $0.70 \pm 0.05$  vs.  $1.7 \pm 0.16$ ;  $p < 0.05$ ) when compared to DMSO (Figure 3, Panels A and B). Results from ELISA study also support that inhibiting JNK pathway by administrating SP600125 reduced c-Jun activity in mustard-oil-treated TG neurons at Day 1 ( $78 \pm 7.19$  vs.  $148.66 \pm 14.49$ ;  $p < 0.05$ ), Day 3 ( $74.83 \pm 8.97$  vs.  $170.5 \pm 11.71$ ;  $p < 0.05$ ) and Day 5 ( $73.33 \pm 8.47$  vs.  $187 \pm 11.15$ ;  $p < 0.05$ ), when DMSO treatment (Figure 3, Panel C). SP600125 did demonstrated significant effects on phospho-c-Jun expression and c-Jun activity in mineral oil treated neurons (data not shown).

To further confirm that JNK/cascade components are responsible for the increased acetylation of H3 in TG neuron after mustard oil stimulation, we evaluated the effect of SP600125 on the acetyl-H3 histone and total H3 expression. We found that, when compared to DMSO treatment, the mustard oil-induced increase in H3 acetylation was significantly reduced by SP600125 at Day 1 ( $0.45 \pm 0.08$  vs  $0.86 \pm 0.11$ ;  $p < 0.05$ ), Day 3 ( $0.42 \pm 0.11$  vs  $0.81 \pm 0.1$ ;  $p < 0.05$ ), and Day 5 ( $0.42 \pm 0.09$  vs  $0.81 \pm 0.15$ ;  $p < 0.05$ , Figure 3, Panel D). However, the total H3 histone did not show significant changes at Day 1 ( $0.51 \pm 0.08$  vs  $0.5 \pm 0.06$ ;  $p < 0.05$ ), Day 3 ( $0.53 \pm 0.07$  vs  $0.53 \pm 0.09$ ;  $p < 0.05$ ), and Day 5 ( $0.52 \pm 0.08$  vs  $0.51 \pm 0.09$ ;  $p < 0.05$ , Figure 3, Panel E). We did not find remarkable effects of SP600125 on H3 acetylation in TG neurons treated with mineral oil (data not shown). The data suggest that JNK inhibitor block the increased H3 acetylation.

### Relationship of phospho- c-Jun, c-Jun activity, and H3 acetylation

To examine the potential relationship between c-Jun and H3 acetylation, the level of phospho-c-Jun and c-Jun activity were compared to the status in mustard-oil-treated TG neurons. Figure 4 demonstrates that there are significant correlations between phospho-c-Jun as well as c-Jun activity and H3 acetylation level ( $r = 0.806$ ,  $r = 0.906$ , respectively;  $p < 0.05$ , Figure 4, Panels A and B).

### Discussion

The results of this study demonstrate that persistent neurostimulant mustard oil application in TG neurons *in vitro* causes a significant expression of JNK1 kinase as well as subsequent downstream phospho-c-Jun. The application of JNK inhibitor, SP600125, blocked this increase in phospho-c-Jun, as well as c-Jun activity produced by mustard oil stimulation. The data also provide compelling evidence that acetylation of histone H3 is significantly increased in TG neurons after stimulation. Furthermore, the increased H3 acetylation observed with upregulated phospho-c-Jun and its activity were reduced when a JNK inhibitor, SP60012, was administered. Nonetheless, the decreased phospho-c-Jun and c-Jun activity associated with a reduction in H3 acetylation, indicated that JNK signaling pathway regulates histone structure, assessed by histone acetylation in TG neurons following neuro-toxic exposure.

c-Jun is an immediate-early genes and inducible transcription factors that activate the AP-1 DNA-binding complex [9,10]. After binding to the specific site on the DNA close to a promotor or enhancer, these regions are indispensable for initiation of transcription [9]. Phosphorylation of c-Jun is important for its DNA-binding complex formation, translocation and nuclear translocation [11,12]. Its potential to activate target gene transcription strongly depends on phosphorylation of its N-terminal amino acids Serine-63 and Serine-73 by JNKs [13,14]. This phosphorylation represents signal cascades in response to nerve injuries, infection and inflammation in the nervous systems [15,16]. JNK itself needs to be phosphorylated at Thr183 and Tyr185 by the upstream dual specificity kinases for the activation of its kinase domain [17,18,19]. Once activated, active JNK translocates to the nucleus to regulate transcription through phosphorylation of several transcription factors such as c-Jun, ATF-2, and other transcription factors [20,21]. Morphological studies indicate that JNKs are widely and functionally distributed in the nervous system [18,22]. Inhibitors of JNK (SP600125 and

CEP-1347) have been shown to have anti-inflammatory effects in rheumatoid arthritis and other diseases [15]. A few reports have shown its possible neurotransmission following noxious stimulation [22]. Using an *in vitro* setting, we confirmed that mustard oil induced a significant upregulation of phospho-JNK1 in TG neurons (Figure 1). Phospho-c-Jun and its transcription activity were also enhanced following stimulant. Significantly, blocking the JNK activity with administration of a specific JNK inhibitor, SP600125, reduced the phospho-c-Jun protein and its activity induced by mustard oil (Figure 3). These observations suggest a role of the JNK/c-Jun cascade in the signaling transmission of noxious information in TG neurons.

Another important finding of this study is that we revealed a novel neuroepigenetic modulation mechanism of histone protein by JNK kinase in peripheral TG neurons following prolonged environmental stimulation. It is becoming increasingly clear that the cellular mechanisms by which neurons modify gene expression following stressful stimuli depends not only on activation of transcription factors, but also on recruitment of multifunctional coactivators that enhance or repress transcription [10]. The dynamic changes in reorganization of chromatin directing gene expression were critically involved in these coactivators. Histone acetylation is one of mechanisms for the local and global control of chromatin structure.

The regulation of gene expression through posttranslational modifications of histones being present in neurons was reported recently. Histone acetylation, together with DNA methylation, is reported in regulating the effect of postnatal environment on brain response to stressful stimulation [23]. Histone acetylation also controls transcription of genes required for consolidation of long-term memory and LTP [24,25]. Increased acetylation of H3 and H4 histones during the transcriptional activation phase of the circadian rhythm was reported [26]. Histone phosphoacetylation in the striatum was reported to be involved in cocaine-induced neural and behavioral plasticity [27]. In the suprachiasmatic nucleus, it showed that stimuli induced resetting circadian rhythms through phospho-H3 [2,3]. In particular, it has been shown that the histone acetyltransferase activity of c-AMP responsive element binding protein (CREB) binding protein (CBP) is necessary for synaptic plasticity in the hippocampus neurons and activity-dependent gene expression [28,29]. CBP is a transcriptional coactivator important for the action of several transcription factors, including CREB, suggesting that histone acetylation is an important regulatory step of gene transcription mediated by CREB [30]. Furthermore, increased phosphorylation of CREB through the activation of glutamate receptors and the PKA, PKC, CaM kinase, and JNK cascades in peripheral and central sensory neurons was intensively reported in several animal models of painful stimulation [7,31,32]. The results identified that a novel neuroepigenetic mechanism was involved in the peripheral neurons in response to environmental chemical stimulation.

Our study implicates a solid connection between activation of JNK/c-Jun cascade and its regulatory mechanism in histone acetylation since inhibiting JNK activity resulted in reduction in acetylation of H3 histone, paralleled with reduced c-Jun activity (Figure 4). This data support the idea that chromatin acts as a transnuclear platform to integrate JNK cascade signaling in response to extracellular stimuli. The JNK-dependent molecular mechanism paralleled with elevated epigenetic markers in TG neurons in response to peripheral chemical stimuli may be associated with a possible role of epigenetic regulation of long-term gene expression since JNK was reported to mediate neuropeptide NK1 receptor in spinal nociceptive neurons [32]. In future experiments, we will explore the precise epigenetic mechanism through which JNK/c-Jun cascade control chromatin structure contributing to neuronal plasticity in response to environmental chemical stimulation.

In summary, the results of our study are the first to provide strong evidence that epigenetic regulation of chromatin remodeling through JNK signaling cascade play a critical role in neuronal response to environmental chemical stimulation. We envision that future

investigations will decode the epigenetic mechanism underlying neuronal gene regulation and provide insight to develop novel tools based on epigenetic pathways to treat human diseases caused by environmental toxic exposure.

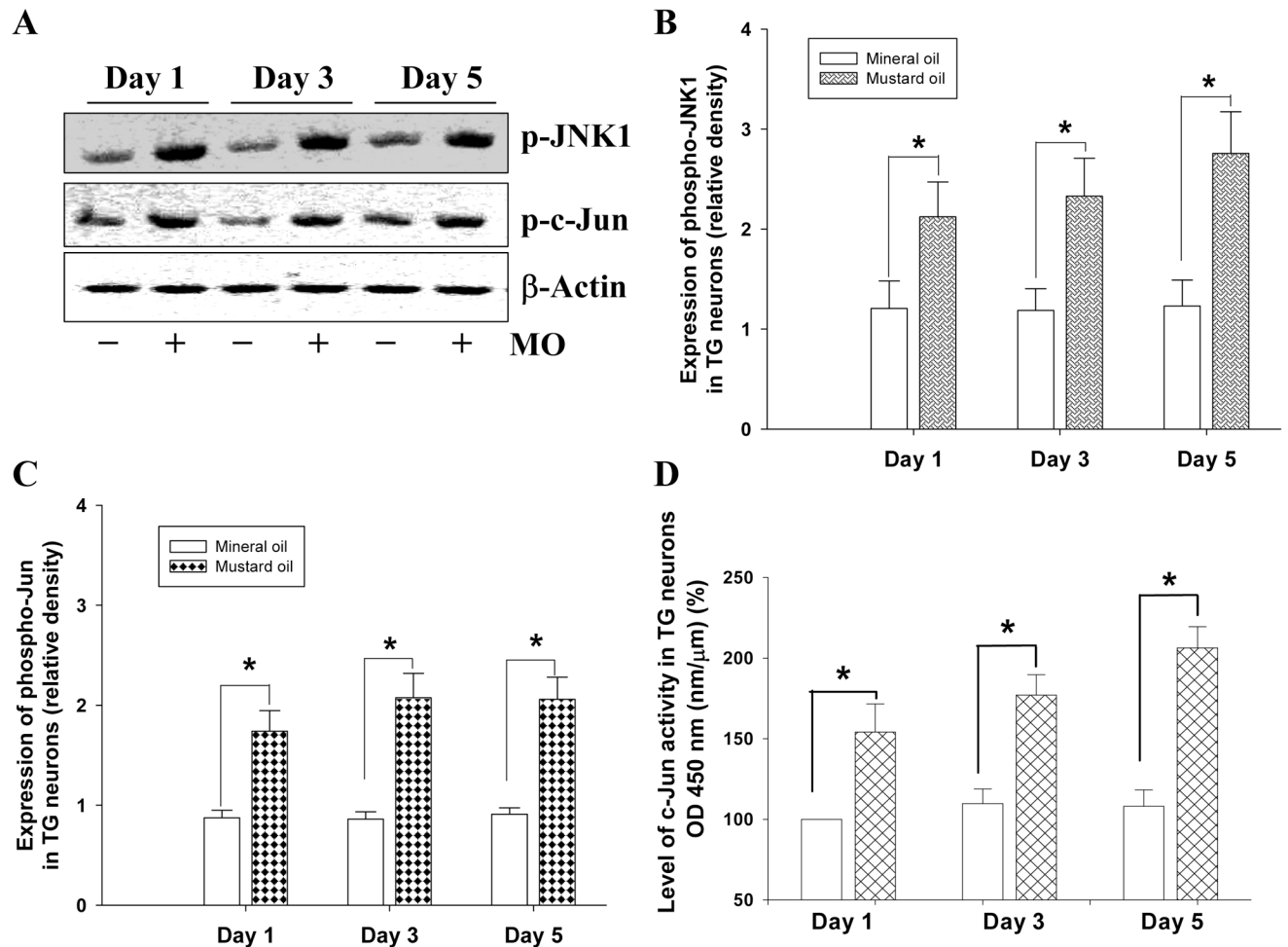
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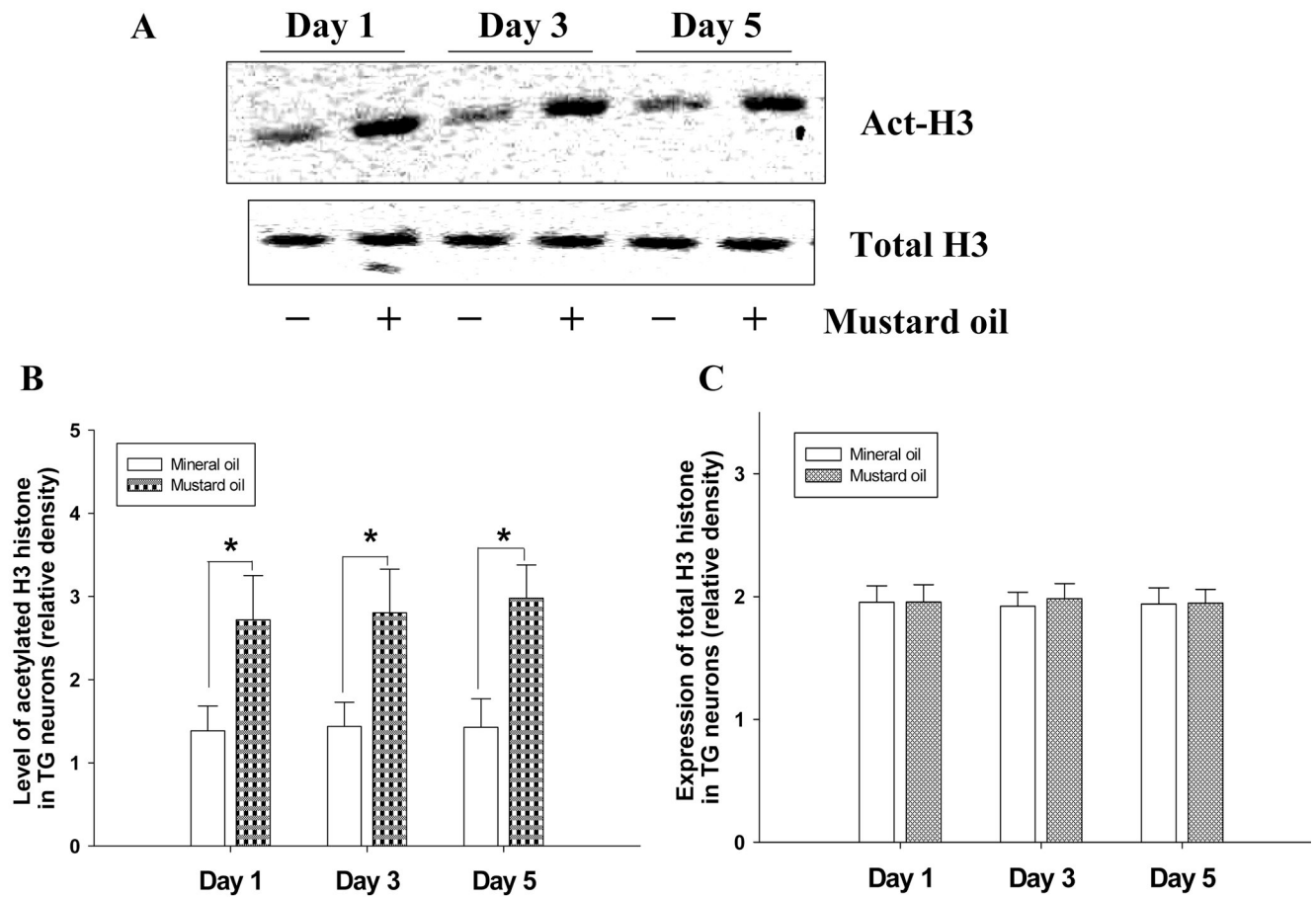
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**Figure 1.**

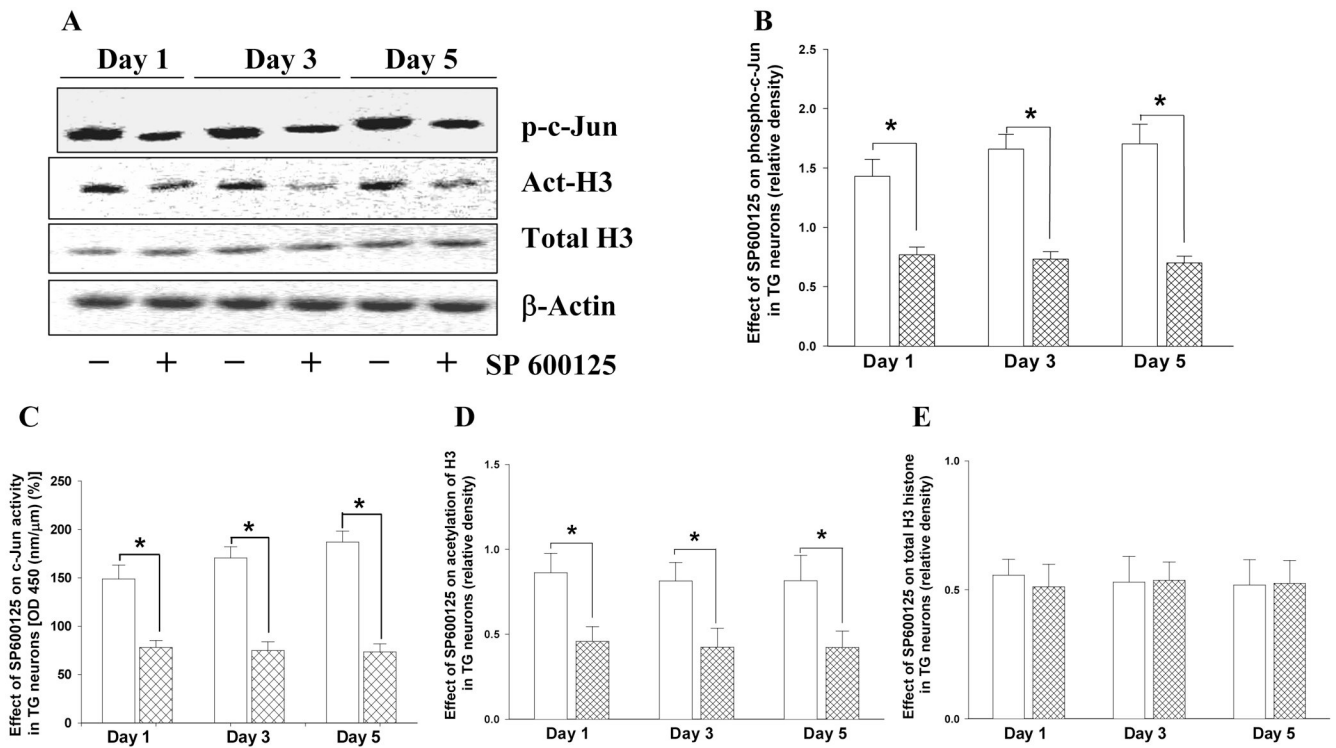
Expression of phospho-JNK1, phospho-c-Jun and c-Jun activity in TG neurons at Day 1, 3, and 5 postadministration of mineral oil and mustard oil (+: present, -: absent). *Panel A*: Representative pictures of phospho-JNK1 (46 KD), phospho-c-Jun (39 KD) and  $\beta$ -actin (40 KD). *Panels B, C, D*: Bar graph summarizing the relative density of phospho-JNK1 (*Panel B*), phospho-c-Jun (*Panel C*) (as the ratio to the value of  $\beta$ -actin) and c-Jun activity (*Panel D*). MO, mustard oil. \*,  $p < 0.05$ ; the value from the group with mustard oil treatment vs. mineral oil treatment ( $n=5$  in each group). Open bar, mineral oil treatment; hatched bar, mustard oil treatment.



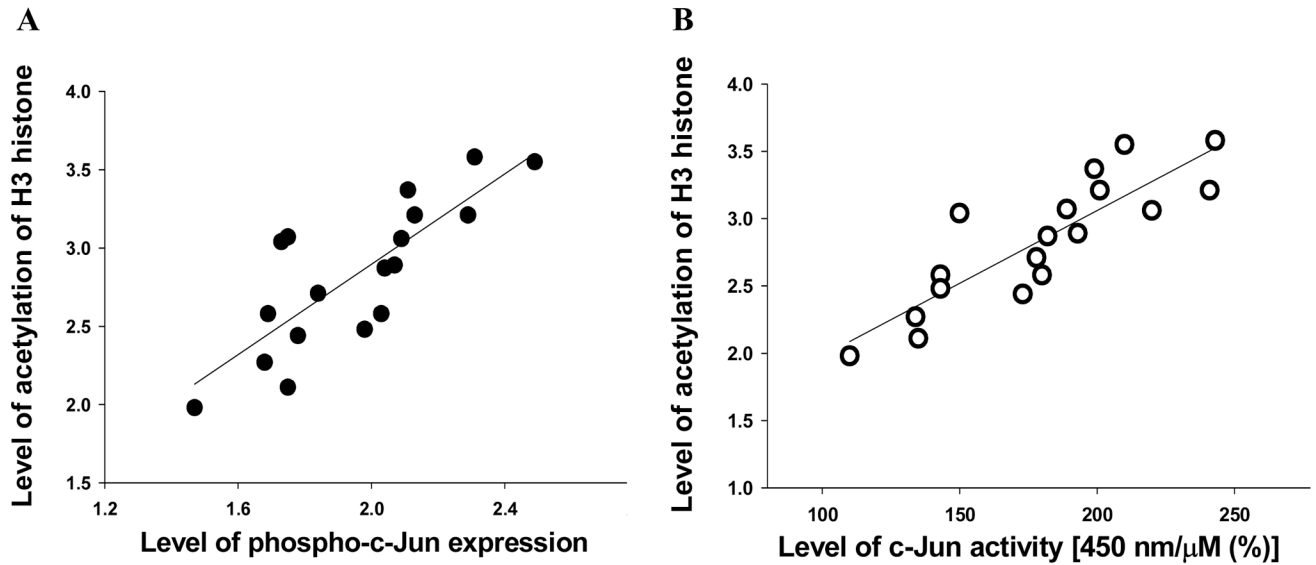


**Figure 2.**

Increased acetylated H3 and total H3 histone expressed in TG neurons at Day 1, 3, and 5). Acetylated H3 histone and total H3 histone could be found on the 17 KD and 15 KD, respectively (*Panel A*). Panels B and C indicate the elevated acetylated H3 histone in TG neurons treated with mustard oil when compared to the control. No difference in total H3 histone expression was detected. (n = 5 in each group). Open bar, mineral oil treatment; hatched bar: mustard oil group.

**Figure 3.**

SP600125 reduces phospho-c-Jun and c-Jun activity, and subsequently the H3 acetylation level in mustard-oil-treated TG neurons. Panel A: Representative immunoblot bands of phospho-c-Jun, acetyl-H3 histone, total H3 histone and  $\beta$ -actin in TG neurons. Panels B–D show the level of phospho-c-Jun (Panels B), acetylated H3 (Panels C) and total H3 histone (Panels D) (ratio to the value of  $\beta$ -actin). No change in total H3 histone affected by SP600125 was detected (Panel D). Increased activity of c-Jun was blocked by SP600125 (Panel E). In mineral-oil-treated TG neurons, no changes in target protein expression impacted by SP600125 were detected (data not shown). (\*,  $p < 0.05$ ; the value from DMSO treatment vs. SP600125 treatment in mustard oil - stimulated TG neurons;  $n=6$  in each group). Open bar, DMSO-treated group; hatched bar, SP600125-treated group.



**Figure 4.**

Levels of phospho-c-Jun expression and c-Jun activity correlated with levels of acetylated H3 histone in mustard-oil-stimulated TG neurons. The levels of phospho-c-Jun (Panel A) and c-Jun activity (Panel B) were significantly correlated with the H3 histone acetylating level in TG neurons (correlation coefficient,  $r = 0.806$ ,  $r = 0.906$ , respectively;  $p < 0.05$ ).