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Original Article

Involvement of Bax and caspase-9 in heroin-induced apoptosis in cerebellar granule neurons via C-Jun pathway activation

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Abstract: Background: The cerebellum neuronal apoptosis cell is characteristic lesions of heroin spongiform leukoencephalopathy (HSLE), and the underlying mechanism of cerebellums usceptibility remains to be clarified. C-Jun signal pathway plays a vital role in apoptosis process. C-jun, Bax and Caspase-9, new identified proteins are highly associated with metastasis of many kinds of cell apoptosis, which were never reported in HSLE. Methods: The expression of C-jun, Bax and caspase-9 was detected by Hoechst 33342 staining, flow Cytometry, RT-PCR, Western blotting in normal group and inhibitor groups with Lentiviral, JNK inhibitor SP600125 or CEP1347 to further observe the morphological of cerebellum changes by electron microscopic scanning and studying on the protein expressions of C-jun, Bax and caspase-9 by immunohistochemistry and Western blotting in the rat model of heroin addiction. Results: The study found C-Jun pathway participated in the process of activating neuronal apoptosis by heroin-induced apoptosis. Inhibitions of C-Jun/JNK pathway by Lentiviral, JNK inhibitor SP600125 or CEP1347 upstream blocker could lead to decrease Bax and caspase-9 factors expression. And cerebellum cytoplasm and nucleus appeared obvious morphological changes. C-jun, Bax and caspase-9 factors also showed high expression trend under the effect of heroin in rat. Conclusion: To explore the role of C-Jun pathway in the heroin-induced neuronal apoptosis, C-Jun protein involving in intrinsic apoptotic signaling pathway suggested that C-Jun signal pathway was activated by heroin, Bax and caspase-9 which were candidates target genes of C-Jun signal pathway in the apoptosis process.

Keywords: HSLE, C-jun, Bax, caspase-9, apoptosis

Introduction

Heroin (Also known as diacetylmorphine) large quantities of heroin can cause serious organic disease of the central nervous system-spongiform leukoencephalopathy SLE is more typical [1]. Based on the change of demyelination, HSLE is mainly involved the small white matter, corpus callosum, internal capsule limb, brain stem and cerebral hemispheres after deep white matter. In recent years, with the deep understanding of HSLE and growing drug problem in China, the incidence rate had showed a gradual upward trend. This disease pattern was first reported in 1982 [2]. In the following years, a number of cases were described [3-6]. An increasing number of heroin addicts prefer inhaling the drug to intravenous injection. This trend is especially dominant in first-time users and young addicts [7, 8]. Heroin can rapidly affect the blood-brain barrier and then interfere with the physiological function of the central nervous system. Whether the pathogenesis relates to the central nervous system demyelinating and the exact mechanism is unclear, such as changes in the release of neurotransmitters, nerve transmission depressions in sensory pathways of the spinal cord and brain that

apoptosis signal [9]. Studies have proved that radiation exposure, ischemia and hypoxia can cause the apoptosis of oligodendrocyte cells, resulting in the axon demyelinated and conduction properties loss [10]. Regulation of apoptosis factor c-jun/JNK pathway could lead directly to neuronal apoptosis (bcl-2/bax ratio reduction) [11, 12]. C-jun/JNK signaling pathway is activated by stimulation, and a part of C-jun can enter the nucleus from cytoplasm by phosphorylating and activating the transcription factor AP-1 protein (C-Jun, c-Fos, etc.), ATF-2, so as to regulate expression of apoptotic protein and the related downstream target genes transcribed [13-15]. When the activated JNK enters the nucleus and activates the corresponding transcription factor to induce the expression of related proteins, activated other pro-apoptotic protein Bax prompts Bax from the cytosol into the mitochondria and damaged mitochondrial membrane permeability, so that the cytochrome C (Cytc-c) was released into the cytoplasm and mediated cell apoptosis through the mitochondrial pathway [16]. Then the other part of the JNK can also be left in the cytoplasm after activated, and the activation of Bcl-2 family members (Bim, Bax, Bcl-2, etc.) can be directly regulated by phosphorylation to mediate the cell apoptosis of mitochondrial pathway. Bcl-2 family proteins are mainly three types: pro-apoptotic proteins such as Bak and Bax, anti-apoptotic proteins such as Bcl-2 and Bcl-xl, the BH3-only protein such as Bim and Bid etc. Bax is the main mediator of mitochondrial pathway and also as mitochondrial apoptotic pathway mainly regulator [17, 18]. JNK is equipped with an active role and also phosphorylated Bax factor, activation of Bax was translocated to the mitochondrial outer membrane to increase the mitochondrial membrane permeability, resulting in the release of mitochondrial apoptosis protein such as cytochrome c, Smac/Diablo and apoptosis inducing factor (apoptosis inducing factor, AIF) [19-21]. Caspase, a dependent mitochondrial apoptosis pathway, can be combined with cytochrome C released into cytoplasm, format apoptotic bodies which can block apoptosis by Smac/DIABLO, inhibit the activity of IAPs, and start the apoptosis of caspase dependent mitochondrial apoptosis pathway [21].

Increased C-Jun protein, a C-Jun N-terminal kinase (JNK) signaling pathway component elements, may activate related proteins mediated by JNK pathway to cause extracellular signal-regulated kinase (ERK) and JNK pathway activation when nerve cells injured is ischemia-reperfusion, so the activation of JNK pathway is closely related to the apoptosis. C-Jun factor not only plays a role in promoting neuronal apoptosis in the process of neuron cell apoptosis, but also participates in other biological activities such as cell proliferation, survival, and regeneration of neuron axon. Studies have found that JNK is activated and the process of apoptosis is essential in the 5K treatment CGC, sympathetic neurons withdrawal of NGF treatment, cerebral cortical neurons by amyloid processing, MPP +/MPTP induced substantia nigra neurons apoptosis. But the explanation for this phenomenon is still only in “neuron type level, developing stages or different models”, and the molecular mechanism need continue to be studied. Some researchers have shown that heroin can produce a decrease in the number of splenic leukocytes and increase the apoptotic death of splenic mononuclear cells; heroin can cause a dose-dependent decrease in cell viability in cerebellar granule cells by activation of ATF3 and mitochondrial cytochrome c release [22]. It is also demonstrated that street heroin promoted apoptosis of primary cultured rat cortical neurons characterized by down-regulation of Bcl-2 [23], which suggests the involvement of mitochondrial-dependent apoptosis in the loss of neuronal function induced by heroin. Syndromes of cerebellum impairment and neuronal death, like ataxia, dysmetria and dysarthria, were reported to be typical features of heroin inhalation [24-26].

We previously confirmed that P-C-Jun factor in the role of heroin treatment CGNs in a concentration and time dependent, suggesting that P-C-Jun factors play an important role in the process of heroin induced neuronal apoptosis. However, P-C-Jun transcription, mainly triggering the expression of some target genes to achieve its pro-apoptotic neuronal mechanism of action, is not clear. Relevant studies have shown that using potassium withdraw neurons processed Hrk, Puma, Fasl which are C-Jun pro-apoptotic factor target genes, found Fasl gene mutation can make it inactive in mice in a subsequent study, but all this does not affect neuronal apoptosis [27].

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FasI and C-Jun pro-apoptotic factor target genes, found in a subsequent study in mice FasI gene mutation can make it inactive, but all these do not affect neuronal apoptosis. Bim, the member of the BH3-only adapter protein family (Bcl-2 imcracting mediator of cell death, Bim) is regulated by JNK in the process of withdraw potassium. However Shi et al. laboratory findings increased expression of Bim and C-Jun were irrelevant [28]. Until now, there has been no evidence for a functional relationship between JNK/c-Jun and heroin induced CGNs apoptosis. This study, using immunofluorescence, Hoechst 33258, qRT-PCR, Western blot, Electron microscopy method specificity of C-jun/JNK inhibitor SP600125 group, MLKs inhibitor CEP1347 group and viral transfection group (three interferenced cerebellar granule neurons), detected C-Jun and related pro-apoptotic candidate target genes Bax and caspase-9 protein and mRNA expression levels to explore its role in neuronal apoptosis induced by heroin target point and its significance.

Materials and methods

Materials

Dulbecco's Modified Eagle Media: Nutrient Mixture F-12 (DMEM/F12), Neurobasal™-A medium, Trypsin, penicillin, streptomycin and B27 supplement were supplied by Gibco (Paisley, UK). Cytosine b-D-arabinofuranoside (Ara-c), Dnase-I protease, Heroin, Hoechst 33258 were supplied by Sigma Chemical Co (St. Louis, MO, USA). FITC Annexin V Apoptosis Detection Kit I were supplied by BD Biosciences (USA). SP-600125 and pLenti-14MR0063 (pLenti-DEST) were supplied by life technologies (USA); CEP 1347 was synthesized by Tocris Bioscience (Birmingham, UK). The primers of c-jun, Bax, caspase-9, GAPDH were synthesized by life technologies. The antibodies anti-c-jun, anti-P-c-jun, anti-caspase 9, anti-Bax and anti-β-tubulin were obtained from Abcam company (Cambridge United Kingdom). Durcupan resin was supplied by Fluka company (Fluka, Buchs, Switzerland). Naloxone was purchased in China (Lot number: H20055758; Beijing Sihuan Pharmaceutical Factory).

Cell culture

The cerebellar granule cells (CGNs) were prepared from 7-day-old Sprague Dawley rat pups as previously described [23]. Briefly, neurons were dissociated from freshly dissected cerebella by mechanical disruption in the presence of 0.125% trypsin and DNase-I and then seeded at a density of 1.5~2.0 × 10^6 cells/ml in DMEM/F12 medium containing 10% fetal bovine serum and potassium at concentrations that cause membrane depolarization (25 mM). The basal medium was changed to the NeurobasalTM-A medium with Ara-c (5 μM) after 24 h post plating to prevent the proliferation of nonneuronal cells. The culture medium was changed on 48 h in vitro using NeurobasalTM-A medium supplemented with 2% B-27. Until the seven day, all animals use protocols were approved by the Creighton University Institutional Animal Care and Use Committee (IACUC).

Hoechst 33342 staining

When nerve cells were differentiated by maturation at DIV7, 10 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml and 120 μg/ml dosages of heroin were separately added into the CGNs container for 24 h. Then Phosphate buffer solution washed the different groups, we kept adding 5 μg/mL Hoechst 33258 dye (cell permeable) in buffer and observing the change of nuclear morphological. The neurons were then incubated for 15 min at 37°C in the dark. Images were then taken using fluorescence microscope.

Treatment with c-jun pathway (JNK) inhibitor

For the administration of inhibitors, after 7 days in vitro, CGNs were incubated in the presence of the following reagents: 80 μg/ml heroin group (H group), 10 μmol/l SP600125 + Heroin (H + S group), 8 μmol/L CEP-1347 + Heroin (H + C group), 100 MOI pLenti-14MR0063 + Heroin (H + V group) cells that did not receive drugs received a control vehicle (Control group). To avoid toxicity, the final concentration of DMSO remained < 0.1%.

Annexin V-FITC double staining detect the apoptosis

We detected the rate of apoptosis in different inhibitors groups (C, H, H + S, H + C, H + V) and observed their intervention effects. We washed nerve cells thrice with cold PBS and then resuspended cells in 1× Binding Buffer at a concentration of 1 × 10^6 cells/ml. Transfered 100 μl

of the solution (1 × 10^5 cells) to a 5 ml culture tube; added 5 μl of FITC Annexin V and 5 μl PI. Gently vortex the cells and incubate for 15 min at RT (25°C) in the dark; added 400 μl of 1 × Binding Buffer to each tube; analyze by flow cytometry within 1 hr. These are three independent repeated experiments.

Reverse transcription-polymerase chain reaction (RT-PCR)

Nerve cells in different inhibitors treatment (C, H, H + S, H + C, H + V) were collected. Extracted the total RNA of CGNs, and measured the mRNA expression of c-jun, bax, caspase-9 with RT-PCR. Their primer sequences were designed and synthesized by Invitrogen Co. C-jun primer: upstream: 5'GAGTCTCAGGAGCGGATCAA3', downstream: 5'CTGTTCCCTGAGCATGTTGG 3', amplified fragment length of 161 bp. Bax primer upstream: 5'ACGCATCCACCAAGAAGC3', downstream: 5'CCAGTTGAAGTTGCCGTCT3', amplified fragment length of 164 bp; Caspase-9 primer upstream: 5'GGAAGAGCTGCCAGTTTCTG3', downstream: 5'CTCCCGTGCTTTGCTGAAAT3', amplified fragment length of 101 bp. β-actin: primer upstream: 5'CAACTGGGAGATATGGAGAAG3', downstream: 5'TCTTCCTTCTGATCCTGTCAG3', amplified fragment length of 285 bp, c-jun annealing temperature of 61°C, Bax annealing temperature is 56°C, caspase-8 annealing temperature of 58°C, 35 cycles, through the RT-PCR Kit to detect related RNA. The amounts of c-jun, bax, caspase-9 RNA (mRNA) were analyzed by RT-PCR using the AccessQuick TM RT-PCR System (Promega) according to the manufacturer's instructions.

Western blotting

Nerve cells in different inhibitors treatment and addiction rat cerebellum were collected; the total proteins was extracted by RIPA lysate; Bovine serum albumin (BSA, V fraction) was used as the standard protein for protein quantification in 5% cattle. The 20 μg protein sample was sampled on acrylamide sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), wet transfer method electrically transferred to PVDF membranes, 5% skimmed milk powder closed at room temperature for 2 h. Then the membrane was incubated overnight at 4°C with rabbit polyclonal antibodies against p-c-jun, c-jun, Bax, caspase-9 and β-tubulin diluted in TBST (Dilution: 1:5000, 1:2000, 1:2000, 1:8000) followed by incubating for 1 h at 37°C with secondary antibodies. Membrane was washed three times, chromogenic. The image processing apparatus (Gene Company) were used to analyze the protein expressions of four factors. The optical band densities were quantified by using Gel-Pro Analyzer Image software.

Animal model

40 adult male Sprague-Dawley (SD) rats weighing 200-220 g were purchased from the Experimental Animal Center of the Experiment Animal Department of First Affiliated Hospital of Xinjiang Medical University [SYXK (Xin) 2013, IACUC, accredited number: 20131224002]. In the study, rats were randomly assigned into 5 groups: control group was treated with normal saline; the 10-day heroin-addicted group; the 20-day group heroin-addicted group; the 30-day heroin-addicted group; and the last group, the 40-day heroin-addicted group (n = 12 per group).

Rats in the addiction groups were given subcutaneous injection with heroin twice a day (at the time of 10:00 am and 20:00 pm) with an escalating dose. The regimen of chronic (for instance, 10-day model) escalating dose heroin administration including a dose increase every second day: the first two days dose administered was 7.5 mg/kg/day and was increased to 15 mg/kg/day on the 3rd and 4th day. On day 5 and day 6 the dose was 30 mg/kg/day; and 45 mg/kg/day on the day of 7th and 8th; the dose went up to 60 mg/kg/day on day 9 and 10. This pattern of exposure using heroin has been found to induce physiological dependence in rats. In control group, rats received an equal volume of normal saline. Randomly selected five heroin-addicted rats were then treated with naloxone at 5 mg/kg to induce abstinence for 30 mins. The recognizable abstinence symptoms were observed including standing (1, 1-5 times; 2, 6-10 times; 3, > 11 times), wet-dog shaking, stretching, teeth chatter, jumping, cunnilingus (1, 1-3 times; 2, 4-6 times; 3, > 7 times). The abstinence symptoms were scored. Once the heroin addicted model have built, rats in experimental groups were injected with the heroin (dose = 60 mg/kg/day)

rats for another 10 days, 20 days, 30 days, 40 days, respectively.

Electronic microscope

We get the rat cerebellum from the control group, addiction Group, 40 days addiction Group. The apex of cerebellum material, 1 mm^3 size as electron microscopy, was taken to be saline washed and fixed with 4% glutaraldehyde for 1 week. The samples were dehydrated by the gradient acetone and soaked for 2 h in a mixture of propylene oxide: epoxy resin, and then overnight stored in epoxy resin. Samples flatted on transparent entrapped basket and added propylene oxide: epoxy resin for 48 h at 60°C; removed entrapped baskets and re-embedded the preliminary tissues in propylene oxide: epoxy resin within 60°C, then polymerized for 2 d. Copper mesh salvaged 60-80 cm thick slices, using uranyl acetate and lead citrate dyeing; examined the grids with thin sections in a transmission electron microscope.

Immunohistochemistry for c-jun, p-c-jun, Bax and caspase-9

Immunohistochemistry for c-jun, P-c-jun, cytc and Bax was done with detection kit. Paraffin-embedded sections were deparaffinized and dehydrated. After washing in PBS thrice (3 mins for each), then were treated with 3% H_2O_2 at room temperature for 10 mins to inactivate endogenous peroxidase. After washing in PBS thrice (5 mins for each), antigen retrieval was performed at 98°C (12 mins for each). Sections were kept cool as the room temperature. After washing in PBS (5 mins for each), they were incubated with normal goat serum at room temperature for another 30 mins. Then, these sections were separately treated with primary antibodies (c-jun: 1:50, P-c-jun: 1:50, Bax: 1:200 and caspase-9: 1:100) at 4°C refrigerator overnight. Followed by washing in PBS thrice (5 mins for each), sections were incubated with HRP conjugated streptavidin at 37°C for 40 mins. Followed by washing in PBS thrice (5 mins for each), observation was done with DAB, terminated with water. After washing in water, counter-staining was done with hematoxylin followed by mounting with neutral resin. In blank control, PBS was employed. In alternative control, normal serum was used instead of primary antibody. The known positive control served as a positive control. These two sections were randomly selected from each group, and three fields were randomly selected at a high magnification. The proportion of positive cells was calculated.

Statistical analysis

Data are the mean ± SEM from at least three independent experiments, performed in duplicate or triplicate. Statistical analysis was performed by one-way ANOVA with Bonferroni post hoctest (*$P < 0.05$ or #$P < 0.01$ was considered significant).

Results

Heroin changes nuclear morphology in cerebellar granule neurons

An additional characteristic of apoptotic cell death is the morphological change occurring to nuclear chromatin or fragmentation that manifests as nuclear condensation. Therefore we evaluated the effects of heroin on nuclear morphology in cerebellar granule neurons (CGNs). CGNs displayed significant nuclear morphological changes after 24 h exposure to different dosages of heroin (**Figure 1A-F**). Heroin produced a robust and concentration-dependent stimulation of nuclear condensation. After different concentrations of heroin intervention, CGNs can be caused bright blue of specific nuclear apoptotic bodies at low concentrations of heroin (10 μg/ml), mainly showing condensation nuclei, condensed or fragmentation-like changes (**Figure 1B**); Nuclear condensation were produced many (**Figure 1E**). With heroin concentration gradually increased, the number of neuronal apoptosis were gradually increased (**Figure 1C-F**); with the increase of the number of heroin concentration of neuronal apoptosis was significantly increased ($P < 0.05$) (**Figure 1**, down panel), we confirmed that these cells were undergoing the apoptosis. With the criteria, the results consistently demonstrated that heroin in our model system triggered CGNs apoptosis. Heroin induced CGN apoptosis rates were almost as same as morphological changes which indicated that the apoptosis rates are rapidly increasing when their concentration of heroin are increasing. It shown that apoptosis of nerve cells depend on heroin dose trend.

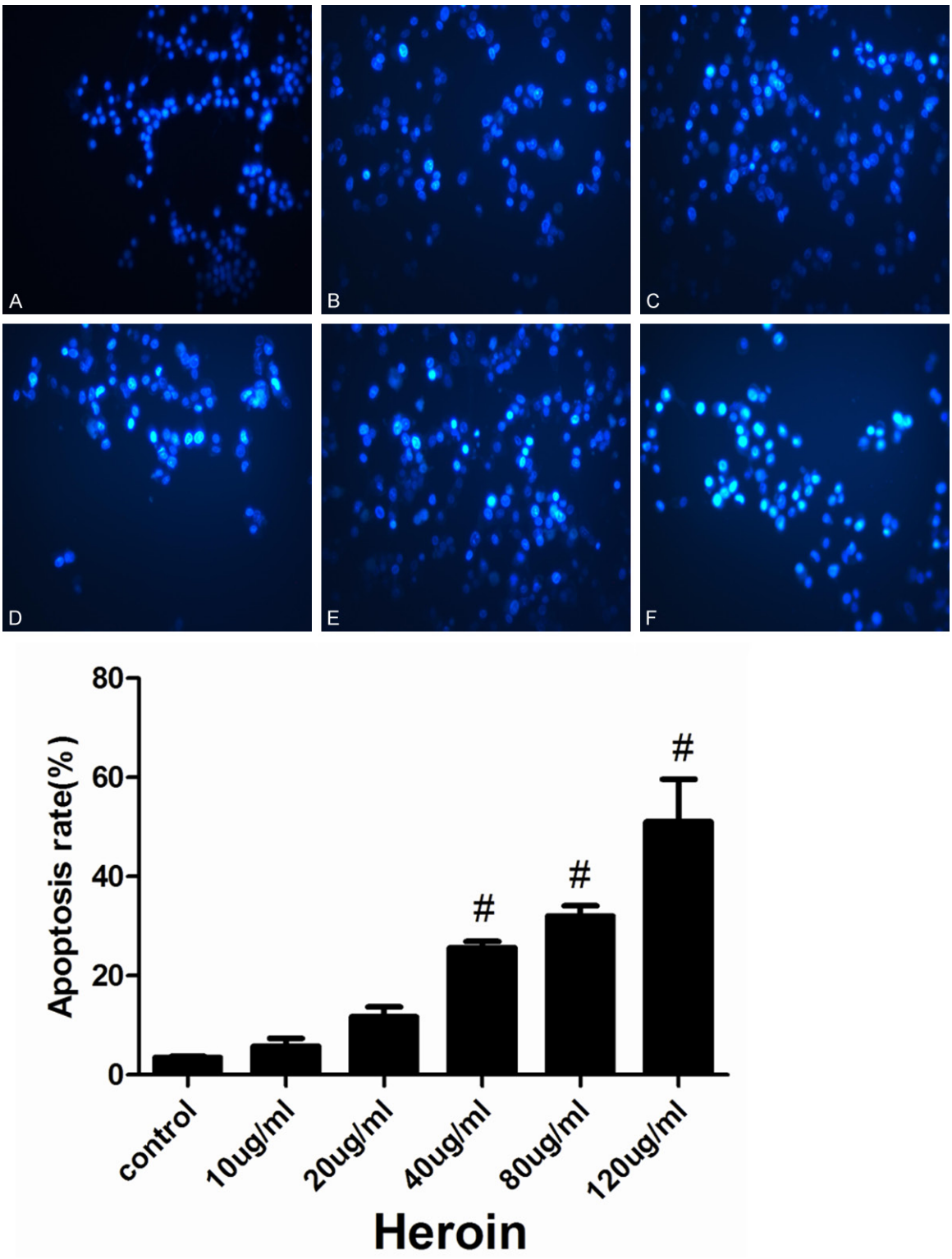

Figure 1. Cerebellar granule cells (CGNs) die when maintained in different dose of heroin (× 200). DIV7 CGNs were then switched to medium containing 10 μg/ml, 20 μg/ml, 40 μg/ml, 80 μg/ml, 120 μg/ml heroin. Phase-contrast micrographs show neurons maintained in 10 μg/ml (B), 20 μg/ml (C), 40 μg/ml (D), 80 μg/ml (E), 120 μg/ml (F) heroin for 24 h. Control cells (A) were maintained for 24 h in heroin-free medium. The percentages of nuclear condensation under the treatments indicated were quantified (down panel). Scale bar = 10 um. Data represent the means ± SEM of three independent experiments. *$P < 0.05$, #$P < 0.01$.

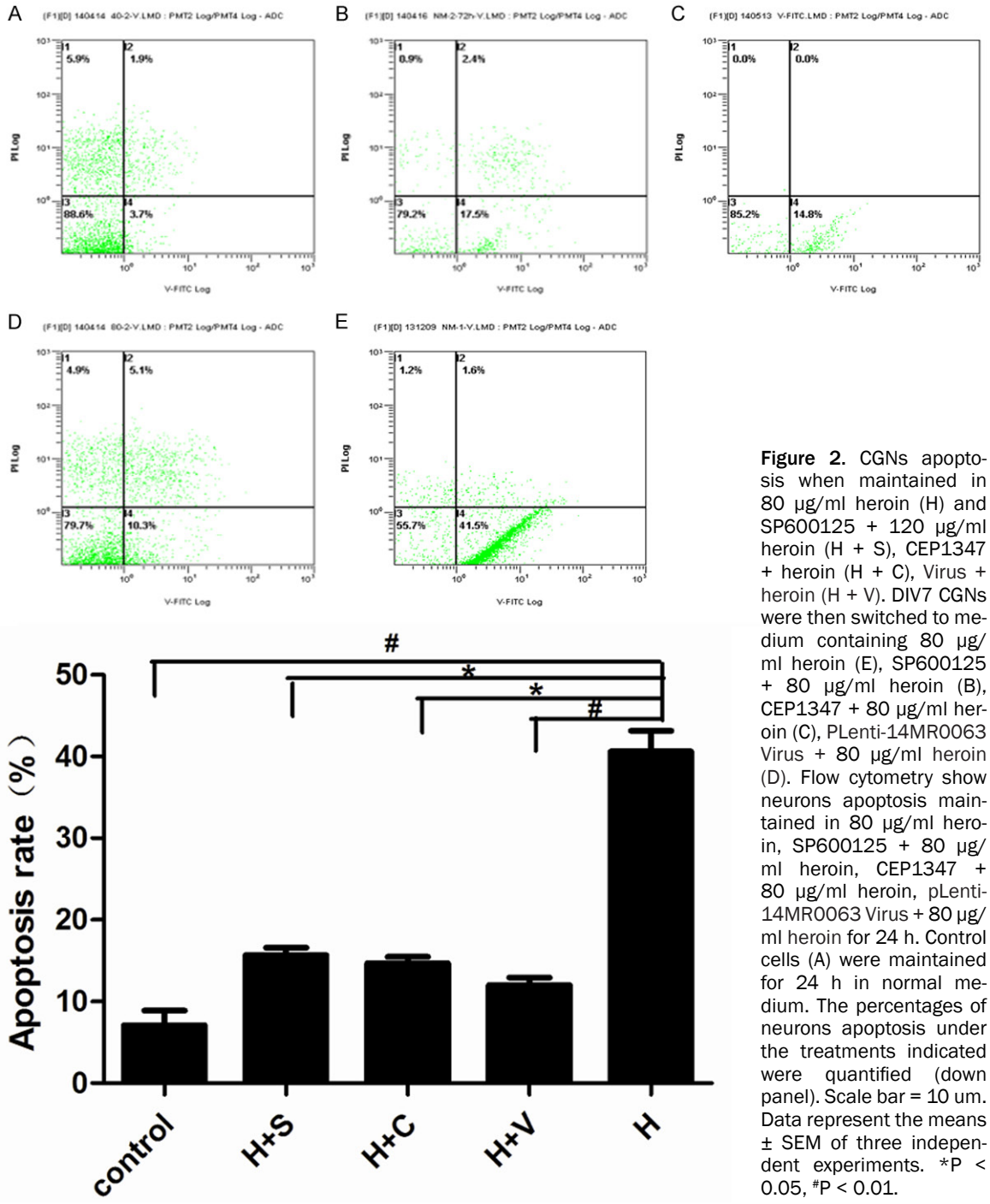

Figure 2. CGNs apoptosis when maintained in 80 μg/ml heroin (H) and SP600125 + 120 μg/ml heroin (H + S), CEP1347 + heroin (H + C), Virus + heroin (H + V). DIV7 CGNs were then switched to medium containing 80 μg/ml heroin (E), SP600125 + 80 μg/ml heroin (B), CEP1347 + 80 μg/ml heroin (C), PLenti-14MR0063 Virus + 80 μg/ml heroin (D). Flow cytometry show neurons apoptosis maintained in 80 μg/ml heroin, SP600125 + 80 μg/ml heroin, CEP1347 + 80 μg/ml heroin, pLenti-14MR0063 Virus + 80 μg/ml heroin for 24 h. Control cells (A) were maintained for 24 h in normal medium. The percentages of neurons apoptosis under the treatments indicated were quantified (down panel). Scale bar = 10 um. Data represent the means ± SEM of three independent experiments. *P < 0.05, #P < 0.01.

C-jun signal pathway is involved in heroin-induced CGN apoptosis

The rate of neuronal cell apoptosis were detected by flow cytometry: This research set up five groups: control group, H + C (80 μg/ml heroin + CEP1347 inhibitor group), H + S (80 μg/ml heroin + SP600125 inhibitor group), H + V (80 μg/ml heroin + pLenti-14MR0063 virus), H (80 μg/ml heroin group), after 24 h in CGN. The results were shown in **Figure 2**. The rate of apoptotic in H group was significantly higher 4 times than other group apoptosis rates. However the groups H + S, H + C, H + V were lower apoptosis rate than H group in which H + V group is relatively lowest apoptosis rate compared with others inhibitor. The pLenti-14MR0063 virus could directly inhibit the expression of c-jun factor as the important apoptosis pathway target. Meanwhile SP600125 and CEP 1347 were JNK

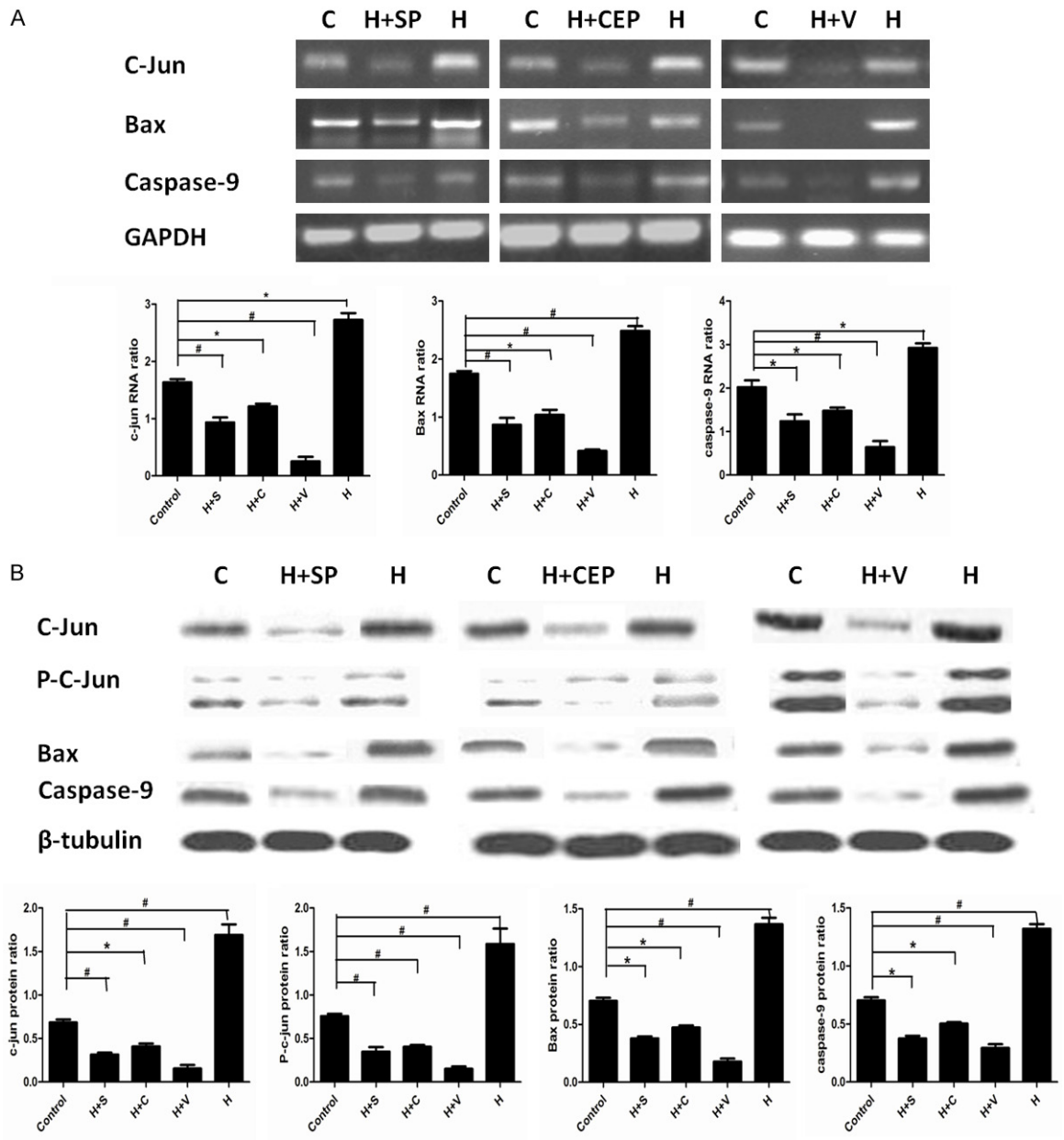

Figure 3. CGNs apoptosis when maintained in 80 μg/ml heroin (H), SP600125 + 80 μg/ml heroin (H + S), CEP1347 + 80 μg/ml heroin (H + C) and pLenti-14MR0063 virus + 80 μg/ml heroin (H + V). A: DIV7 CGNs were transferred to media containing 80 μg/ml heroin in the absence or presence of SP600125, CEP1347 and 100 MOI pLenti-14MR0063 virus. After 24 h, neurons were assessed by PCR using the indicated Semi-quantitative (up panel) and RT-PCR using the c-jun, Bax, caspase-9 and GAPDH were quantified (lower left panel, lower middle panel and lower right panel). B: DIV7 CGNs were transferred to media containing 80 μg/ml heroin in the absence or presence of SP600125, CEP1347 and 100 MOI pLenti-14MR0063 virus. After 24 h, neurons were assessed by Western Blotting using the c-jun, p-c-jun, Bax, caspase-9 and β-tubulin protein were quantified (lower left panel, lower middle panel and lower right panel). *P < 0.05, #P < 0.01.

and MLKs downstream inhibitors (**Figure 2**). Previous studies have shown that they can decrease the cell death. It indicated that C-jun signal pathway have an important effect in Heroin-induced CGNs apoptosis. The apoptosis rate of four groups' difference has statistical signiptosis induced by heroin.

Bax and Caspase-9 mRNA and protein is up-regulated by c-jun signal pathway under heroin treatment in CGN

In previous studies, Bax and Caspase-9 participated in regulator of the apoptosis pathway which is closely related to apoptosis mecha-

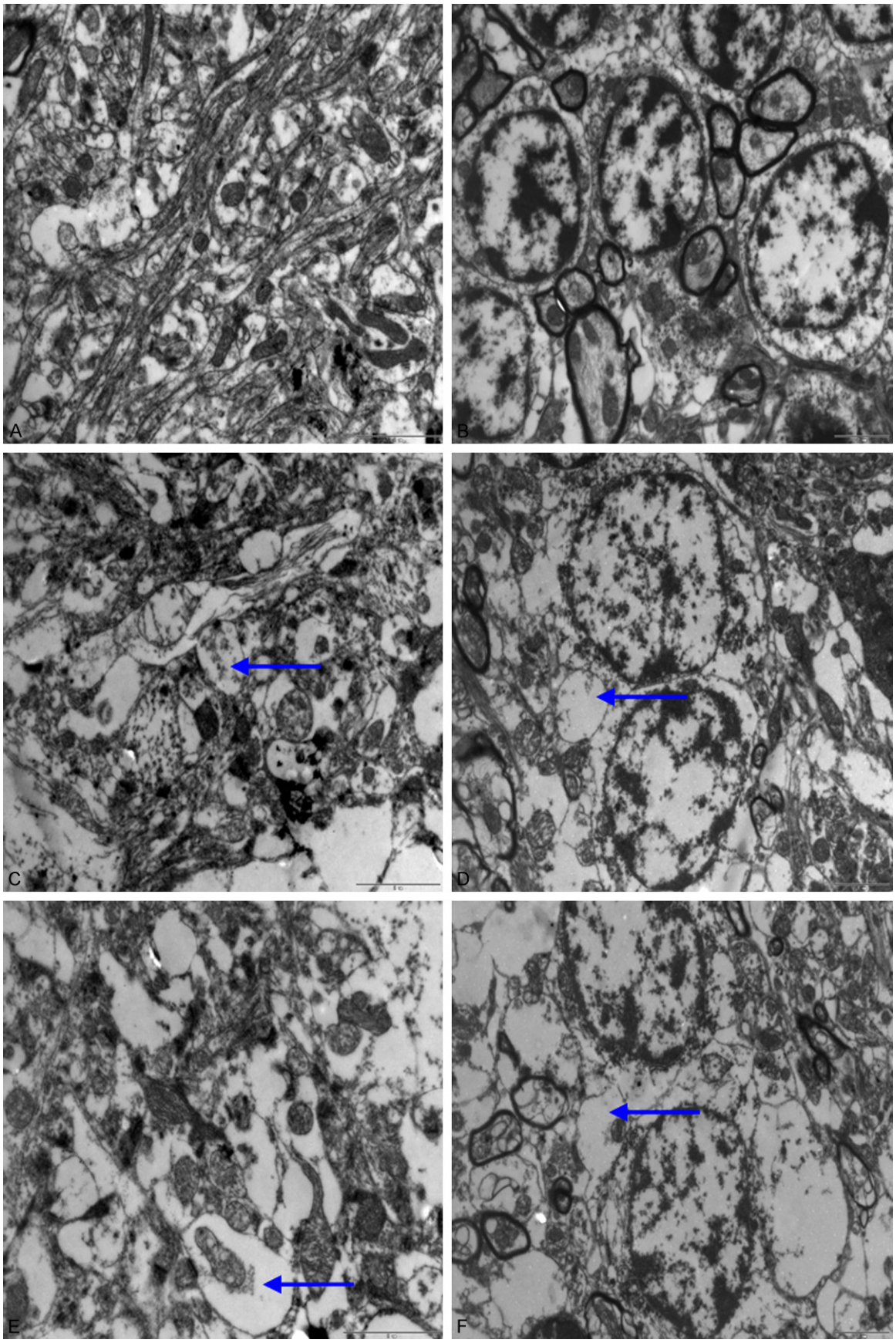
A
B
C
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Figure 4. Neuronal cell alterations in Heroin-affected rat cerebellar tissue (TEM 8.0kvX4000~8000). A, B: The rat's cerebellar granule cells in control group (12 kV~8 Kv); C, D: The rat's cerebellar granule cells in heroin addiction group (12 kV~8 Kv); E, F: The rat's cerebellar granule cells in heroin addiction group of 40 day rat (12 kV~8 Kv).

nism. C-jun acted as an important pro-apoptotic target gene in JNK pathway. Therefore, we studied the mechanism of how Bax and Caspase-9 were regulated under heroin intoxication through JNK pathway: we firstly detected the expression level of c-jun, Bax and Caspase-9 mRNA and protein in different c-jun signal pathway inhibitors effected by 80 μg/ml heroin in CGNs. Semi quantitative PCR had shown (**Figure 3A**, Up panel): It was found that c-jun, Bax and caspase-9 mRNA expression levels began to up-regulate in H group. By reverse transcription polymerase chain reaction (RT-PCR) detected, c-jun mRNA expression level was significantly higher (**Figure 3**) compared with the control group, In H group, Bax mRNA expression levels was far higher than control group (**Figure 3A** middle panel), caspase-9 mRNA expression levels was obviously up-regulated (**Figure 3A**, right panel). These three factors had the lowest mRNA expression in H + V group compared with H + S and H + C groups. The mRNA expression of c-jun, Bax and Caspase-9 in H + C were relatively higher than the H + S group, meanwhile the three inhibitors groups were lower mRNA expression than H group with GAPDH gene as internal reference basis. The same result applies to the effect of the Western blotting. C-jun, p-c-jun, Bax and caspase-9 protein expression levels began to up-regulate in H group (**Figure 3B**). After adding inhibitors of C-jun signal pathway SP600125, CEP1347 and pLenti-14MR0063 virus, C-jun, p-c-jun, Bax and caspase-9 protein expression levels had downward trend compared the single factor of heroin (80 μg/ml) to effect CGNs with β-tubulin gene as internal reference basis (**Figure 3B**). However, these four factors had the lowest mRNA and protein expression in H + V group compared with H + S and H + C groups. It was shown that C-jun, p-c-jun, Bax and caspase-9 factors involved in the process of neuronal apoptosis by heroin when C-jun, p-c-jun, Bax and caspase-9 mRNA and protein level were changed, which also indicated Bax and caspase-9 played a vital role in promoting apoptosis as C-jun candidate genes through c-jun (JNK) signal pathway.

Morphologyical alterations of neuron cells in heroin addicted analyzed

By observing the control group, heroin addicted group, heroin addicted 40 d group cerebellar cell had different morphology in an animal model of heroin addicted, which is helpful to look for the microscopic evidence of heroin spongiform leukoencephalopathy disease. Cerebellum showed ultrastructural features suggesting General swelling of neurons, glia, and neuropil. Cerebellar granule cell morphology control rats, the cells intact, arranged in neat rows, grid cell processes in a cross (**Figure 4A**, **4B**). The heroin-addicted group shown cerebellar granule neuron nuclear morphology: condensation smaller, nuclear chromatin dense, cytoplasmic organelles densely, the mitochondria and rough endoplasmic reticulum slight swelling (**Figure 4C**, **4D**). Heroin-addicted group 40 days shown: nerve cell processes reducing, cell body shrinkage, neuronal cytoplasmic fragmentated, mitochondrial swelling or disintegration, disorder or broken ridge, some vacuolization, the chromatin deepen myelin edema. Cell protrusion height edema, around the capillaries or distributed in the neuropil synaptic edema make neuropil loose mesh shape, spongy appearance (**Figure 4E**, **4F**). These indicated that heroin could induce nerve cells alteration and spongiform leukoencephalopathy disease, which is a typical characteristic lesion in heroin induced apoptosis.

Protein expressions of P-c-jun, c-jun, Bax and Caspase-9 in neurons of cell with heroin addiction

In the immunohistochemistry, cells with yellow-brown granules in the cytoplasm or nucleus were regarded as positive (**Figure 5**). Results showed, comparing with control group, the expressions of P-c-jun, c-jun, Bax and caspase-9 increased dramatically correspond to the increases dose of heroin in heroin addiction groups (10 d, 20 d, 30 d and 40 d) ($P < 0.05$). However, significant differences in protein expressions of P-c-jun, c-jun, Bax and caspase-9 were observed in heroin addiction groups (10 d,

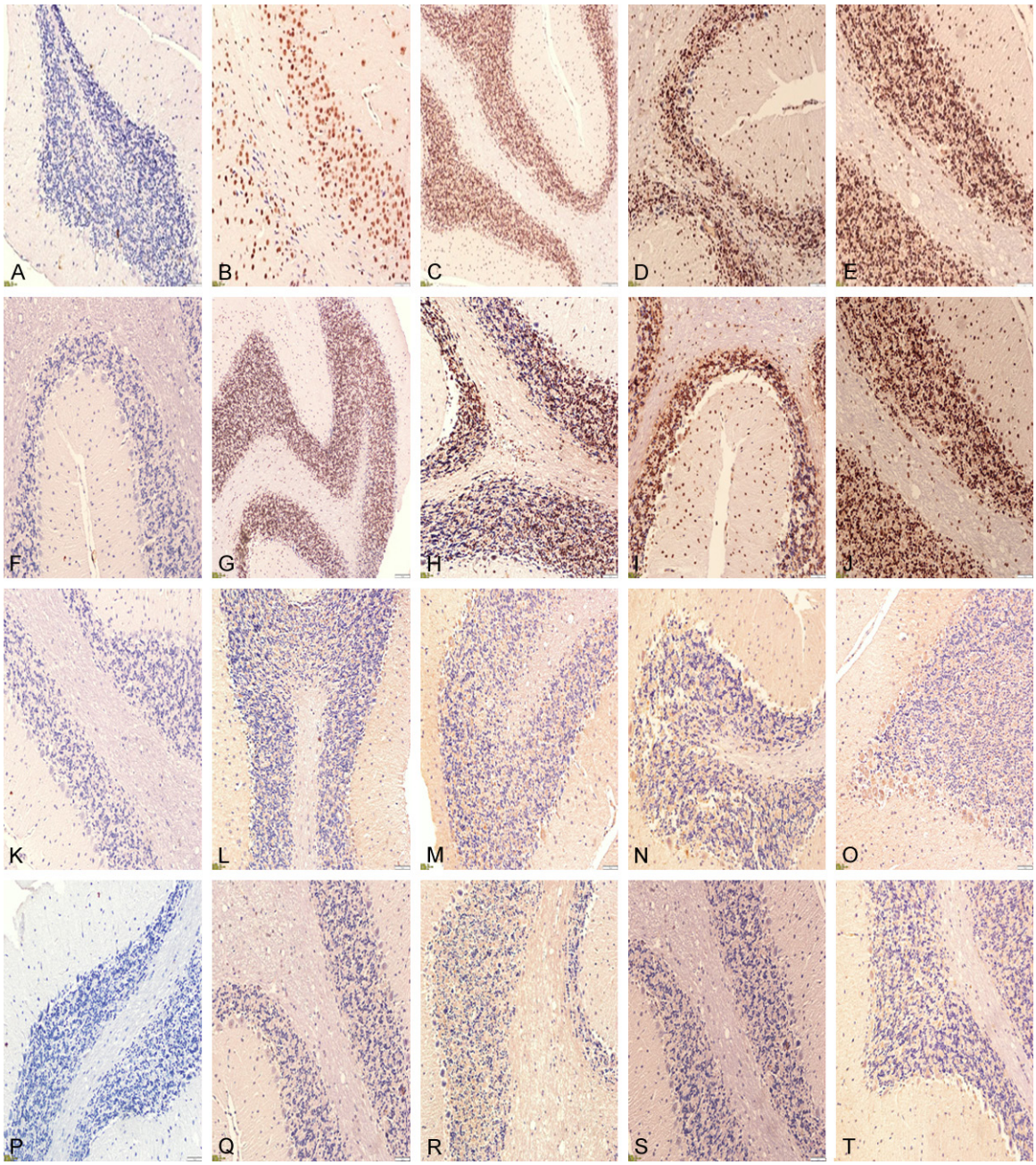

Figure 5. Protein expressions of P-c-jun, c-jun, Bax and caspase-9 in cerebellum neurons of rats with heroin addiction. (Immunohistochemistry, Original magnification: ×200). A-E: P-c-jun protein expression in control group, heroin addiction groups 10 d, 20 d, 30 d and 40 d; F-J: C-jun protein expression in control group, heroin addiction groups 10 d, 20 d, 30 d and 40 d; K-O: Bax protein expression in control group, heroin addiction groups 10 d, 20 d, 30 d and 40 d; P-T: Caspase-9 protein expression in control group, heroin addiction groups 10 d, 20 d, 30 d and 40 d.

20 d, 30 d and 40 d) ($P < 0.05$) continuing to use heroin drugs (**Table 1**).

Discussion

The specific pathogenesis of Heroin spongiform leukoencephalopathy, a serious disease after taking heroin, is still not clear. Presumably heroin containing impurities produced some harmful substances which can damage the nervous system white matter in the heating process and specificity [29]. The pathological features of HSLE is distinctive that the most important feature of site of the lesion is equipped

Table 1. Percentage of cerebellum neurons cell positive of P-c-jun, c-jun, Bax and caspase-9 in rats with heroin addiction (n = 8, $x \pm s$, %)

Group	P-C-Jun	C-Jun	Bax	Caspase-9
NM	6.667±1.528	3.067±1.901	3.3±2.364	4.167±2.754
10 d	14.330±2.080	17.530±6.577*	6.3±0.8544*	5.833±1.255
20 d	23.000±5.290*	25.130±4.801#	17.33±2.082*	13.67±1.317*
30 d	38.330±3.510#	45.330±7.506#	23.33±3.512#	21.67±2.517#
40 d	49.330±4.040#	70±13.230#	42.67±2.32#	37.33±2.07#

Compared with control group, *P < 0.05; #P < 0.01.

with highly selective and imaging examination showed: with progression of the disease, cerebellar hemisphere lesions was appeared firstly in patients, secondly the involvement of the splenium of the corpus callosum, internal capsule limb, brain stem and other parts. It is the important issue of clinical and basic research to find out why HSLE white matter damage has such a highly selectivity, how the extent of the damage in different white matter lesion is, how the nature of the white matter lesions is, how different essence in other common white matter encephalopathy is [2]. It has been reported [2, 3] that heroin can cause neuronal demyelination and ventral tegmental area and nucleus accumbens neurons ultrastructure obviously can occur vacuolar pathology in addicted rats. The neurons cells can occur in a large number of apoptosis under the influence of high dosage of heroin and long time effect [4, 5]. At present, there are few various studies on the mechanism of apoptosis in the mechanism of heroin addiction, the mechanism of brain tissue lesion, the mechanism of treatment. The mechanism of neuron apoptosis induced by heroin for further study can provide a theoretical basis for revealing the mechanism of sudden death and HSLE [24, 30, 31].

Apoptosis process involves a series of complex signaling pathways composed by the extrinsic pathway and the endogenous pathway: JNK/c-jun signaling pathway mainly activating mitochondria is a typical endogenous apoptosis pathway releasing cytochrome, activating caspase-3, and changing the Bax/Bcl-2 ratio in cortical neurons and CGNs. Bcl-2 and Bax are important products of gene regulation in apoptosis process, the Bcl-2 inhibits cell apoptosis, while the Bax is antagonistic role. The ratio of Bcl-2 and Bax was the key factor to determine the cell apoptosis or inhibition. The expression of Bax was correlated with the survival of the cells after cerebral ischemia and reperfusion. The ratio of Bax and Bcl-2 was positively correlated with the apoptosis rate after stimulation [32]. The pro-apoptotic mechanisms of Bax may be active calcium channels involved in mitochondrial permeability transition and process of activating protease in apoptosis, Bax is also possible controlled by releasingcytochromecinthischannelandtriggerapoptosis [33]. Cysteine aspartate (caspases) family has been considered to be the final common pathway of apoptosis, its activation and abnormal expression caused cell apoptosis, so it is also known as the death of protease. Caspase is divided into three major categories which constitute the amplification effect of the cascade: apoptosis initiating factor, apoptosis inducing factor and inflammation mediated factor. Cell apoptosis initiating factor in the upstream region of the cascade reaction including Caspase-8, Caspase-2, Caspase-9, etc. can identify and activate the downstream area of the apoptosis factor Caspase-3, etc to induce reversible apoptosis. However, caspase-9 is starting promoter in process of activating mitochondrial apoptotic pathway. After released from the mitochondria Cytc and apoptotic protease activating factor (Apaf-1) form a polymer compound which can raise the precursor of Caspase-9, then the Caspase-9 was cleavaged and further activated Caspase-7 and Caspase-3 factors resulting in initiating Caspase cascade [34-36]. Neuronal cell necrosis is the earliest and most common pathological changes in the brain tissue damage of HSLE. There have been reported in the literature that the adult rat hippocampal granule cell layer injecting heroin for a long time can cause neurons to generate decline, both function of learning and memory have a direct correlation effects in hippocampus. Electron microscope observed: The oligodendrocytes appeared a lot of vacuolar degeneration, cell edema, height swelling mitochondria, significant expansion of endoplasmic reticulum, appearing vacuoles in the myelin sheath, occurring majority of myelin vacuoles cleavage.

The role of JNK/C-Jun signaling pathway played a very important in the research of all signal apoptotic transduction pathways [37]. Because the mutations of c-jun factor after injecting the specific c-jun antibody or using a transcriptional activation domain reach retained DNA-binding domain of C-Jun dominant negative mutant to block C-Jun transcription factor activity caused neurons obstacles generating stimulus by apoptosis, which could effectively intervene in the occurrence of neuronal apoptosis to protect neurons [38, 39]. Saporito et al. [40] found that JNK/C-Jun signaling pathway at low potassium-induced CGC apoptosis model is early activated, JNK or MLK inhibitors block C-Jun transcriptional activation can effectively protect neurons, indicated JNK/C-Jun signaling pathway in heroin-induced cerebellar granule neurons apoptosis process plays a very key role [41, 42].

We studied the specific JNK inhibitor SP600-125, JNK upstream kinases MLK inhibitors CEP1347 and the lentivirus of silencing C-Jun factor intervened CGNs for 24 h. The effects of SP600125 and CEP1347 can reduce the relative low rate of apoptosis, but lentivirus lead to the lowest rate in CGNs. The three groups had the effect of reducing the apoptosis rate. It indicated that JNK/C-Juns indeed play an important pivotal role in heroin-induced apoptosis of cerebellar granule neuron. At same times, the three groups (the SP600125 group, CEP1347 group and lentivirus group compared with the model of heroin-induced CGNs apoptosis) had the effect of reducing the apoptosis rate and protected nerve cell. The efficiency of inhibiting apoptosis rate by lentivirus was obviously higher than CEP1347 and SP600125 effects. It shown the number of neurons was significantly decreased after lentivirus pLenti6.3/V5-DEST treatment which indicated that the protective effect of the virus on the neuron was more important. Thus further confirmed the transcriptional activity of C-Jun increased neuronal apoptosis is also essential, simultaneously detecting the role of its pro-apoptotic protein in the process of apoptosis. Therefore it is crucial to search the C-Jun apoptotic candidates target genes for revealing the essence of apoptosis and intervening occurrence of apoptosis.

JNK can be phosphorylated and increase the activity of apoptosis protein Bax through damaged the integrity of the mitochondrial membrane the integrity after activated to exert the effect of proapoptotic. Pro-apoptotic protein Bax can gather on the outer membrane of mitochondria and open permeability transition pore [43], releasing the Cytc from the mitochondria into the cytoplasm to activate caspase-9, 3 to promote apoptosis [44, 45]. Therefore, in this study, we will take Bax as a candidate target gene, and the expression of Bax gene was significantly up-regulated in the apoptosis model of heroin in cerebellar granule neurons while the expression level was down regulated after the inhibition effect which was consistent with previous reports. By the same method, we detected the changes of the molecular level of the caspase-9 factor, and the results showed the same variation trend with Bax factor. It was shown Bax and caspase-9 was involved in JNK signaling pathway which plays an important role in the process of neuron apoptosis.

In the animal model of heroin addiction, we found that Cerebellum appeared obvious demyelination or vacuolar degeneration with prolong the time of heroin intervened. This is a characteristic lesion of heroin-induced apoptosis in neurons which is consistent with the results of the VK Khurdayan study [46].

Heroin can induce apoptosis of many kinds of cells, especially neurons. However, no significant work has been done in this filed. Our studies showed that there were a large number of apoptotic cells significantly dependent on dosage heroin and confirmed C-jun signal pathway involved in the process of heroin-induced neurons apoptosis. Bax and caspase-9 factors had an obvious upward trend at high concentration heroin, while both of expression levels can be reduced by JNK/c-jun inhibitor SP60-0125, MLKs inhibitor CEP1347, c-jun silence virus. This research indicated that Bax and caspase-9 are involved in the process of heroin-induced neuronal apoptosis as the candidate target genes of the c-jun signaling pathway. The results provide a platform for further study on the mechanism of heroin induced neuronal apoptosis.

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Disclosure of conflict of interest

None.

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References

[1] Bach AG, Jordan B, Wegener NA, Rusner C, Kornhuber M, Abbas J, Surov A. Heroin spongiform leukoencephalopathy (HSLE). Clin Neuroradiol 2012; 22: 345-349.

[2] Wolters EC, van Wijngaarden GK, Stam FC, Rengelink H, Lousberg RJ, Schipper ME, Verbeeten B. Leucoencephalopathy after inhaling "heroin" pyrolysate. Lancet 1982; 2: 1233-7.

[3] Sempere AP, Posada I, Ramo C, Cabello A. Spongiform leucoencephalopathy after inhaling heroin. Lancet 1991; 338: 320.

[4] Tan TP, Algra PR, Valk J, Wolters EC. Toxic leukoencephalopathy after inhalation of poisoned heroin: MR findings. AJNR Am J Neuroradiol 1994; 15: 175-8.

[5] Weber W, Henkes H, Moller P, Bade K, Kuhne D. Toxic spongiform leucoencephalopathy after inhaling heroin vapour. Eur Radiol 1998; 8: 749-55.

[6] Gupta PK, Krishnan PR, Sudhakar PJ. Hippocampal involvement due to heroin inhalation: "chasing the dragon". Clin Neurol Neurosurg 2009; 111: 278-81.

[7] Keogh CF, Andrews GT, Spacey SD, Forkheim KE, Graeb DA. Neuroimaging features of heroin inhalation toxicity: "chasing the dragon". AJR Am J Roentgenol 2003; 180: 847-50.

[8] Hagel J, Andrews G, Vertinsky T, Heran MK, Keogh C. "Chasing the dragon"--imaging of heroin inhalation leukoencephalopathy. Can Assoc Radiol J 2005; 56: 199-203.

[9] Egan PJ, Becker FW, Schumm F. Spongiform leucoencephalopathy after inhaling illicit heroin and due to carbon monoxideintoxication. Fortschr Neurol Psychiatr 2004; 72: 26-35.

[10] Romero AA, Gross SR, Cheng KY, Goldsmith NK, Geller HM. An age-related increase in resistance to DNA damage-induced apoptotic cell death is associated with development of DNA repair mechanisms. J Neurochem 2003; 84: 1275-87.

[11] Osterhout DJ, Marin-Husstege M, Abano P, Casaccia-Bonnefil P. Molecular mechanisms of enhanced susceptibility to apoptosis in differentiating oligodendrocytes. Neumsci Res 2002; 69: 24-29.

[12] DeLuca GC, Nagy Z, Esiri MM, Davey P. Evidence for a role for apoptosis in central pontine myelinolysis. Acta Neumpathol 2002; 103: 590-598.

[13] Carboni S, Antonsson B, Gaillard P, Gotteland JP, Gillon JY, Vitte PA. Control of death receptor and mitochondrial-dependent apoptosis by C-Jun N-terminal kinase in hippocampal CA1 neurones following global transient ischaemia. J Neurochem 2005; 92: 1054-60.

[14] Pan J, Zhao YX, Wang ZQ, Jin L, Sun ZK, Chen SD. Expression of FasL and its interaction with Fas are mediated by C-Jun N-terminal kinase (JN) pathway in 6OHDA-induced rat model of Parkinson disease. Neurosci Let 2007; 428: 82-7.

[15] Tan BM, Zammit NW, Yam AO, Slattery R, Walters SN, Malle E, Grey ST. Baculoviral inhibitors of apoptosis repeat containing (BIRC) proteins fine-tune TNF-induced nuclear factor κB and C-Jun N-terminal kinase signalling in mouse pancreatic beta cells. Diabetologia 2013; 56: 520-532.

[16] Guan QH, Pei DS, Xu TL, Zhang GY. Brain is ischemia/reperfusion-induced expression of DP5 and its interaction with Bcl-2, thus freeing Bax from Bcl-2/Bax dimmers is mediated by C-Jun N-terminal kinase (JNK) pathway. Neurosci Lett 2006; 393: 226-30.

[17] Perier C, Bové J, Wu DC, Dehay B, Choi DK, Jackson-Lewis V, Rathke-Hartlieb S, Bouillet P, Strasser A, Schulz JB, Przedborski S, Vila M. Two molecular pathways initiate mitochondria-dependent dopaminergic neurodegeneration in experimental Parkinson's disease. Proc Natl Acad Sci U S A 2007; 104: 8161-6.

[18] Kim BJ, Ryu SW, Song BJ. JNK and p38 kinase-mediated phosphorylation of Bax leads to its activation and mitochondrial translation and to apoptosis of human hepatoma HepG2 cells. J Biol Chem 2006; 1: 21256-65.

[19] Giordano G, Klintworth HM, Kavanagh TJ, Costa LG. Apoptosis induced by domoic acid in mouse cerebellar granule neurons involves activation of p38 and JNK MAP kinases. Neurochem Int 2008; 52: 1100-1105.

[20] Tsutsui H, Ide T, Kinugawa S. Mitochondrial oxidative stress, DNA damage, and heart failure. Antioxid Redox Signal 2006; 8: 1737-1744.

[21] Solovyan VT. Characterization of apoptotic pathway associated with caspase-independent excision of DNA loop domains. Exp Cell Res 2007; 313: 1347-60.

[22] Pu H, Wang X, Su L, Ma C, Zhang Y, Zhang L, Chen X, Li X, Wang H, Liu X, Zhang J. Heroin

Activates ATF3 and CytC via c-jun N-Terminal Kinase Pathways to Mediate Neuronal Apoptosis. Med Sci Monit Basic Res 2015; 21: 53-62.
[23] Cunha-Oliveira T, Rego AC, Garrido J, Borges F, Macedo T, Oliveira CR. Street heroin induces mitochondrial dysfunction and apoptosis in rat cortical neurons. J Neurochem 2007; 101: 543-554.
[24] Tan M, Li Z, Ma S, Luo J, Xu S, Lu A, Gan W, Su P, Lin H, Li S, Lai B. Heroin activates bim via C-jun N-terminal kinase/c-jun pathway to mediate neuronal apoptosis. Neuroscience 2013; 233: 1-8.
[25] Cordova JP, Balan S, Romero J, Korniyenko A, Alviar CL, Paniz-Mondolfi A, Jean R. 'Chasing the Dragon': new knowledge for an old practice. Am J Ther 2014; 21: 52-5.
[26] Kass-Hout T, Kass-Hout O, Darkhabani MZ, Mokin M, Mehta B, Radovic V. "Chasing the dragon"--heroin-associated spongiform leukoencephalopathy. J Med Toxicol 2011; 7: 240-242.
[27] Du H, Sun X, Guma M, Luo J, Ouyang H, Zhang X, Zeng J, Quach J, Nguyen DH, Shaw PX, Karin M, Zhang K. JNK inhibition reduces apoptosis and neovascularization in a murine model of age-related macular degeneration. Proc Natl Acad S U S A 2013; 110: 2377-2382.
[28] Song B, Xie B, Wang C, Li M. C-Jun induction is independent of early growth response factor during cerebellar granule neuron apoptosis. Neuroreport 2012; 23: 67-72.
[29] Bach AG, Jordan B, Wegener NA, Rusner C, Kornhuber M, Abbas J, Surov A. Heroin spongiform leukoencephalopathy (HSLE). Clin Neuroradiol 2012; 22: 345-349.
[30] Wei XL, Ye J, Liang Y, et al. Observation of the ultastructural changes of neuronal apoptosis in the brain of heroin-addicted rata. Guangxi Yike Daxue Xuebao 2004; 21: 31-33.
[31] Lai B, Pu H, Cao Q, Jing H, Liu X. Activation of caspase-3 and c-Jun NH2-terminal kinase signaling pathways involving heroin-induced neuronal apoptosis. Neurosci Lett 2011; 502: 209-13.
[32] Guan QH, Pei DS, Xu TL, Zhang GY. Brain ischemia/reperfusion induced Expression of DP5 and its interaction with Bcl-2 thus freeing Bax from Bcl-2/Bax dimmers are mediated by c-Jun-terminal kinase (JNK) pathway. Neurosc Lett 2006; 393: 226-230.
[33] Mertens HJ, Heinerman MJ, Evers JL. The expression of apoptosis related proteins Bcl-2 and Ki67 in endometrium of ovulatory menstrual cycles. Gynecol Obstet Invest 2002; 53: 224-230.
[34] Oshitari T, Yamamoto S, Hata N, Roy S. Mitochondria and caspase-dependent cell death pathway involved in neuronal degeneration in diabetic retinopathy. Br J Ophthalmol 2008; 92: 552-556.
[35] Tang W, Wang W, Zhang Y, et al. Tumour necrosis factor related apoptosis inducing ligand (TRAIL) induced chemokine release in both TRAIL resistant and TRAIL sensitive cells via nuclear factor kappaB. FEBS 2009; 276: 581-593.
[36] Yoshida H, Kong YY, Yoshida R, Elia AJ, Hakem A, Hakem R, Penninger JM, Mak TW. Apaf-1 is required for mitochondrial pathways of apoptosis and brain development. Cell 2002; 94: 739-750.
[37] Ma C, Ying C, Yuan Z, Song B, Li D, Liu Y, Lai B, Li W, Chen R, Ching YP, Li M. dp5/HRK is a C-Jun target gene and required for apoptosis induced by potassium deprivation in cerebellar granule neurons. J Biol Chem 2007; 282: 30901-30909.
[38] Li Y, Wang F, Liu C, Zeng M, Han X, Luo T, Jiang W, Xu J, Wang H. JNK pathway may be involved in isoflurane-induced apoptosis in the hippcampi of neonatal rats. Neurosci Lett 2013; 545: 17-22.
[39] Brahim S, Aroui S, Abid K, Kenani A. Involvement of C-Jun NH2-terminal kinase and apoptosis induced factor in apoptosis induced by deglycosylated bleomycin in laryngeal carcinoma cells. Cell Biol Int 2009; 33: 964-970.
[40] Saporito MS, Brown EM, Miller MS, Carswell S. CEP-1347/KT-7515, an inhibitor of C-Jun N-terminal kinase activation, attenuates the 1-methyl-4-phenyl tetrahydropyridine-mediated loss of nigrostriatal dopaminergic neurons in vivo. J Pharmaeol Exp Ther 1999; 288: 42 1-427.
[41] Valesio EG, Zhang H, Zhang C. Exposure to the JNK inhibitor SP600125 (anthrapyrazolone) during early zebrafish development results in morphological defects. J Appl Toxicol 2013; 33: 32-40.
[42] Jung EJ, Park HC, Chung KH, Kim CW. Proteomic analysis of SP600125-controlled TrkA-dependent targets in SK-N-MC neuroblastoma cells: Inhibition of TrkA activity by SP600125. Proteomics 2014; 14: 202-215.
[43] Yamagata H, Shimizu S, Nishida Y, Watanabe Y, Craigen WJ, Tsujimoto Y. Requirement of voltage-dependent anion channel 2 for pro-apoptotic activity of Bax. Oncogene 2009; 28: 3563-3572.
[44] Jia Y, Zuo D, Li Z, Liu H, Dai Z, Cai J, Pang L, Wu Y. Astragaloside IV inhibits doxorubicin-induced cardiomyocyte apoptosis mediated by mitochondrial apoptotic pathway via activating the PI3K/Akt pathway. Chem Pharm Bull (Tokyo) 2014; 62: 45-53.

[45] Hüttemann M, Pecina P, Rainbolt M, Sanderson TH, Kagan VE, Samavati L, Doan JW, Lee I. The multiple functions of cytochrome c and their regulation in life and death decisions of the mammalian cell: From respiration to apoptosis. Mitochondrion 2011; 11: 369-381.

[46] Khurdayan VK, Buch S, El-Hage N, Lutz SE, Goebel SM, Singh IN, Knapp PE, Turchan-Cholewo J, Nath A, Hauser KF. Preferential vulnerability of astroglia and glial precursors to combined opioid and HIV-1 Tat exposure in vitro. Eur J Neurosci 2004; 19: 3171-82.