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The effects of sub-anesthetic ketamine plus ethanol on behaviors and apoptosis in the prefrontal cortex and hippocampus of adolescent rats

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ABSTRACT

Ketamine has become increasingly popular in adolescent drug abusers worldwide. Meanwhile, alcohol is usually used by ketamine users. However, little work has been conducted to examine the chronic combined effects of ketamine and ethanol on adolescent brain. Here we probed into the effects of chronic administration of ketamine at recreational doses alone or combined with ethanol on behaviors and neuron damage in an adolescent rat model. 28-day old rats were treated with either 20 or 30 mg/kg ketamine plus or not plus 10% ethanol daily for 21 days. Depressive like behaviors, anxiety like behavior and memory impairment were tested using open field test, forced swimming test, elevated plus maze and Morris water maze. Apoptosis in prefrontal cortex (PFC) and hippocampus (HIP) were determined by the TdT-mediated dUTP Nick-End Labeling (TUNEL) and protein and mRNA levels of caspase-3, Bax and Bcl-2. Results show that co-application of ketamine and ethanol significantly increased immobility time in the forced swimming test, up-regulated TUNEL positive cells and both protein and mRNA expressions of caspase-3 and Bax, compared with the control group and ketamine and ethanol use alone groups in the PFC, but not in the HIP. Our study suggests that chronic co-administration of ketamine and ethanol results in depressive-like behavior and the caspase-dependent apoptosis in the PFC of adolescent rats' brains.

1. Introduction

Ketamine, a type of noncompetitive antagonist of ionic glutamate *N*-methyl-D-aspartic acid (NMDA) receptors, is widely used in the pediatric surgery (Dong and Anand, 2013) for induction of anesthesia and analgesia in the past years. Recently, ketamine has acquired drug abuse status due to its psychedelic properties (Corazza et al., 2013). More importantly, the numbers of adolescence abusers are increasing (Li et al., 2011; Morgan et al., 2012). Data reported by US poison centers from 2000 to 2015 indicate that the percentage of male ketamine abusers between the age of 16–25 years was 49% among single agent ketamine exposures (Ni et al., 2018). In addition, alcohol is often co-abused by ketamine users (Dinis-Oliveira et al., 2010; Quek et al., 2013). The simultaneous use of recreational ketamine and ethanol lead to a variety of health problems such as liver fibrosis, deteriorated cardiac functions and kidney dysfunction (Chan et al., 2011; Chan et al., 2012; Wai et al., 2012; Wong et al., 2012). Further, frequent ketamine use at sub-anesthetic doses induces social deficits, memory and executive function impairments, and psychotic-like symptoms (Gama et al., 2012; Morgan et al., 2010) which are associated with compromises in the structural and functional integrity of the prefrontal cortex (PFC) and hippocampus (HIP). Mounting evidence suggested that ketamine increased glutamate release and hyper-metabolism (Holcomb et al., 2005; Olney et al., 2002) and resulted in neuronal cell damage and mental dysfunction. Recent studies further showed that sub-anesthetic doses of ketamine induced apoptosis of neuron in the PFC and HIP of developing brain (Sun et al., 2014; Zuo et al., 2016). More interestingly, pro-apoptotic properties of ketamine are also observed in cultured neurons in vitro (Slikker Jr. et al., 2015), indicating that intrinsic apoptotic degeneration is probably the mechanism responsible for brain injury. With regard to alcohol, chronic alcoholics have significant brain damage (Coutts and Harrison, 2015; de la Monte and Kril, 2014). Acute or chronic heavy drinking leads to neurocognitive deficits (Tiwari and Chopra, 2013) and reduced white matter integrity in adolescents or young adults (Elofson et al., 2013). Exposure to chronic ethanol induces both emotional and memory deficits and the loss of neurons, astrocytes and microglia cells in adolescent rats (Oliveira et al., 2015).

Adolescence is a critical period with higher levels of neuronal plasticity and synaptic remodeling (Crews and Nixon, 2009; Pascual et al., 2009). It is well known that an overproduction of axons and synapses occurs in the PFC and HIP during the pre-pubertal stage

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followed by a rapid pruning stage in late adolescence (Andersen and Teicher, 2004; Andersen et al., 2000). Such dynamic and critical changes in the PFC and HIP may make these regions particularly vulnerable to drugs (Holder and Blaustein, 2014) such as ketamine and ethanol. Despite the potential significance of this issue, little is known about the effects of prolonged ketamine exposure at sub-anesthetic doses alone or combined with ethanol on PFC and HIP in adolescence. Accordingly, the aim of the present study was to test the effects of chronic administrations of ketamine (20 mg/kg or 30 mg/kg) alone, or combined with ethanol (10% ethanol 3.0 g/kg), within an adolescent rat model. Depressive-like behaviors, anxiety-like behaviors and memory were tested using the open field test (OFT), forced swimming test (FST), elevated plus maze (EPM) and Morris water maze (MWM). Meanwhile, apoptotic cells (TUNEL staining) and protein and mRNA expressions of apoptotic proteins including activated caspase-3, Bax and Bcl-2 were examined in the PFC and HIP.

2. Materials and methods

2.1. Animals and grouping

60 male Sprague-Dawley (SD) rats (55–70 g, 21-day old) were obtained from The Animal Experimental Center of Shandong University and allowed to acclimate for one week prior to use in the experiments. Rats were group-housed (4–6 rats/cage) and maintained at a constant temperature (22 ± 1.0 °C) and humidity (55 ± 5%), on an artificial 12 h light/dark cycle with lights on at 06:00 am. Purina Rodent Chow and tap water were available ad libitum and the rats were weighed every 3 days. All procedures were done in accordance with the principles of laboratory animal care of the China Laws for the Protection of Animals and approved by Ethics Committee of Shandong University.

2.2. Drugs and treatments

After a 7-day acclimatization period, the 60 rats were randomly assigned to six groups (10 rats/group): saline control (CTR); low dose ketamine (LK); high dose ketamine (HK); ethanol (E); low dose ketamine plus ethanol (LK + E); high dose ketamine plus ethanol (HK + E).

Control animals received 0.6 ml/kg saline intraperitoneally and tap water intragastricly (in a volume equal to the volume of ethanol administered). Rats in the HK and LK groups were treated intraperitoneally with ketamine (HengRui Medicine Co., Lianyungang, Jiangsu, China) at the recreational dose of 30 mg/kg and relatively lower dose of 20 mg/kg respectively and immediately tap water intragastricly (in a volume equal to the volume of ethanol administered) once a day. E group received 10% *w/v* ethanol solution (Gold Shield Chemicals; Hayward, CA) corresponding to 3.0 g/kg via gavage and 0.6 ml/kg saline intraperitoneally daily. The LK + E groups and HK + E were administrated with ketamine intraperitoneally at the dose of 20 mg/kg (LK + E) and 30 mg/kg (HK + E) respectively, and immediately 10% w/v ethanol at a dose of 3.0 g/kg via gavage.

The high dose of ketamine (30 mg/kg) used in the experiment is considered to be a recreational dose for rodents (Gable, 2004), and low dose of ketamine of 20 mg/kg a sub-recreational dose. The ethanol solution was made by dilution of anhydrous alcohol with tap water. All solutions were freshly prepared prior to use and adjusted every 3 days accordingly to animal's weight changes. All treatments were carried out between 09:00–10:00 am and sustained for consecutively 21 days.

2.3. Behavioral assessments

The behavioral tests were performed 24 h after treatments. The order of behavioral tests in each animal was the same, as follows: (a) open field test; (b) forced swimming test; (c) elevated plus maze test; and (d) Morris water maze test.

2.3.1. Open field test

The open field test (OFT) was performed on a 90 × 90 × 45 cm arena divided into 25 equal squares (18 × 18 cm) (luminance: 150–200 lx). The nine squares comprising the center were defined as the center squares. Each rat was gently placed in the center of the arena and allowed for adaptation for 30 min. After adaptation, activity was recorded for 5 min using a digital camcorder (Panasonic model AF-X8, Secaucus, NJ) located 40 cm above the arena. Five parameters were calculated: (a) latency stage - the cumulative residence time in the central squares; (b) line crossing - the numbers of squares crossed with all four paws; (c) rearing-erect posture with hind-paws on the floor or leaning of their front paws against a wall; (d) grooming- the amount of time grooming, including washing or mouthing of forelimbs, hind-paws, face, body and genitals; and (e) stools- the number of excrement produced. These parameters provide indices of locomotor activity, exploratory and anxiety-like behaviors (Walsh and Cummins, 1976b). All the experiments were conducted in a quiet environment and the arena was wiped with 75% ethanol to avoid olfactory cues before testing another animal.

2.3.2. Forced swimming test

The forced swimming test (FST) was used to evaluate depression-like behaviors (Porsolt et al., 1978). Animals were placed individually in the water tank (22 cm in diameter X 40 cm in height) containing water of 20 cm in depth at 25 °C. These tests were 6 min in duration. Immobility times (in seconds) were recorded using a digital timer. Rats floating motionless without struggling or making only very slight movements were considered to be immobile.

2.3.3. Elevated plus maze

The Elevated plus maze (EPM) test was widely used to assess anxiety-like responses of rodents (Pellow et al., 1985). The apparatus consists of two opposite open arms (luminance: 150–200 lx), two opposite walled arms (luminance: ~50 lx) and a central platform. Each rat was released in the central platform facing an open arm and left to explore the mazes for 5 min. Behaviors of rats in the maze were recorded by a video tracking system interfaced to a compute and the percent time in the open arms was calculated to assess the anxiety level. The apparatus was wiped with 75% ethanol to avoid olfactory cues before testing another animal.

2.3.4. Morris water maze

The Morris water maze (MWM) test is a task that measures spatial learning and memory in an aqueous environment (Morris et al., 1982). The water maze, a cylindrical pool (120 cm in diameter, 50 cm in height), is visually separated into four quadrants and equipped with a platform 2 cm below the waterline in the center of one quadrant (the goal quadrant). During a 5-day training session, each rat was placed at four start location (north, south, east, west) separately facing the wall of the pool and swam for up to 60 s until it found the platform daily. Once the rats located the platform, it remained on it for 20 s. If the rat failed to find the platform within 60 s, it was guided to the platform after 60 s swim period and permitted to stay there for 20 s. During a 2- to 4-min intertrial interval, the animals were kept warm by infrared heating lamps. On the sixth day, the probe test was conducted with the absence of the platform. The rat was placed into the opposite quadrant of the goal quadrant for a free 60-s probe. The percentage of time spent in the goal quadrant was measured by a video tracking system interfaced to a computer. The temperature of the water maze was 20 ± 0.5 °C.

2.4. Immunohistochemistry

2.4.1. Tissue preparation

24 h after the behavioral assessments, 4 rats from each group were sacrificed for immunohistochemical staining. Briefly, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital

(80 mg/kg body weight) and perfused with saline intracardially until the liver turned pale pink, followed by 4% buffered formalin. Then, the brains were removed and fixed in formalin for 24 h. The brain samples were dehydrated and embedded in paraffin wax. Serial sections of the prefrontal cortex and hippocampus were cut at 5 μm intervals.

2.4.2. TUNEL assay

The DNA fragmentation of apoptosis and topographic distribution of apoptotic cells are demonstrated using the TUNEL staining. The TUNEL assays were performed using ApopTag® Peroxidase In Situ Apoptosis Detection Kit (S7100, Millipore Corporation, Billerica, MA, USA) as previously reported (Yeung et al., 2010). According to the manufacturer's instruction, both positive and negative controls were included in the staining process. Briefly, sections were dewaxed in xylene, rehydrated and pre-treated with proteinase K (20 mg/ml) for 15 min at room temperature (RT). After blocking the endogenous peroxidase activity by incubation with 3% hydrogen peroxide in phosphate buffered saline (PBS) buffer for 5 min at RT, the sections were treated with working strength deoxynucleotidyl transferase (TdT) with biotindeoxyuridine triphosphate for 1 h in a humidified chamber at 37 °C. The reaction was stopped with the stop/wash buffer for 10 min. To detect the binding of dioxigenin-11-dUTP, sections were incubated with the anti-digoxigenin conjugate for 30 min at RT in a humidified chamber. Finally, after visualization with DAB kit (3,3′-diaminobenzidine, Invitrogen, Carlsbad, CA, USA) for about 6 min at RT, apoptotic cells exhibited a dark brown nuclear staining under light microscope. In each assay, negative controls were processed similarly but without TdT treatment, while positive controls were performed by pre-treatment of the sections with DNase I (DN 25, 1.0 mg/ml, Sigma, St. Louis, MO, USA) to induce DNA strand breakage before proteinase K treatment. All counting was performed by researchers who were blinded to the groups. Cells in five random fields (400× magnification) across each region of interest prefrontal cortex and hippocampus (CA1, CA2/3, and DG) in three consecutive sections of each rat were quantified. The numbers of cells obtained from the three sections were averaged for each animal, and the results were expressed as the number of cells per mm^2.

2.5. Protein assessments

2.5.1. Tissue preparation

The remaining 6 rats of each group were anesthetized with pentobarbital and euthanized by decapitated, and the brains were rapidly removed from the skulls. The prefrontal cortex and hippocampus were dissected, quickly frozen in liquid nitrogen and stored at −80 °C until use.

2.5.2. Protein extraction

Brain tissue samples were homogenized in lysis buffer (P0013C, Beyotime Institute of Biotechnology, Shanghai, China). The homogenate was centrifuged at 14,000 ×*g* for 30 min at 4 °C. The supernatant was stored at −80 °C until use. Protein concentration was measured using the Protein Quantitative Analysis Kit (k3001-BCA; Shenergy Biocolor, Shanghai, China) from the Bio-Rad DC.

2.5.3. Western blot analysis

The western blot assay was modified as previously reported (Sun et al., 2014). Equal amounts of protein (30 μg) from each sample were boiled in 6 × Laemmli loading buffer for 5 min, run on 10% SDS-polyacrylamide gels and then transferred to 0.2 μm Polyvinylidene-Fluoride (PVDF) membranes (Millipore Corporation). The blots were blocked for 2 h in blocking solution (5%non-fat dry milk, 0.05%Tween-20 in PBS) at 37 °C, then incubated at 4 °C overnight with anti-sera (dilution): β-actin (1:2000) (#4967, Cell Signaling Corp., Beverly, CA, USA), cleaved Caspase-3 (1:1000) (#9661, Cell Signaling Corp.), Bax (1:1000) (#2772, Cell Signaling Corp.), Bcl-2 (1:1000) (#2870S, Cell Signaling Corp.). The blots were then visualized using horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibodies (1:20000) (CW0103, Beyotime Institute of Biotechnology, Shanghai, China) and evaluated using the ECL detection system (Millipore Corporation). The bands corresponding to β-actin, cleaved Caspase-3, Bax, and Bcl-2 were scanned and densitometrically analyzed by image-J software. These quantitative analyses were normalized to β-actin (after stripping).

2.6. Real-time quantitative PCR assay

Total RNA were extracted from homogenized prefrontal cortex and hippocampus using Trizol reagent (Invitrogen, CA, USA). RNA concentration and purity were measured by using the NanoDrop ND-2000 (Nanodrop Technologies, Wilmington, DE, USA). Reverse transcription was performed with corresponding primers according to the manufacturer's instructions (Reverse Transcription System, Promega, Madison, WI, USA), and real-time quantitative PCR was performed using SYBR Green Master Mix Kit (ABI Biosystem, CA) on Real-Time PCR System (Bio-Rad, Hercules, CA, USA). The primer sequences of target mRNA are listed in table below. The RT-qPCR procedure was conducted as follow: cycle 1, 95 °C for 10 min; cycle 2, 40 repeated cycles of 95 °C for 15 s, 60 °C for 45 s. The relative gene expressions of caspase-3, Bax and Bcl-2 were calculated using $2^{-\Delta\Delta CT}$ method with β-actin as an internal reference.

Primers used for RT-qPCT analysis.

Primers	Sequences (5′-3′)
Caspase-3	Forward: AGCTGGACTGCGGTATTGAG
	Reverse: GGGTGCGGTAGAGTAAGCAT
Bax	Forward: TTGCTACAGGGTTTCATCCA
	Reverse: TGTTGTTGTCCAGTTCATCG
Bcl-2	Forward: GAGCGTCAACAGGGAGATGT
	Reverse: CAGCCAGGAGAAATCAAACAG
β-Actin	Forward: CTACAATGAGCTGCGTGTGGC
	Reverse: CAGGTCCAGACGCAGGATGGC

2.7. Statistics

Data were presented as mean ± SEM. One-way ANOVA followed by Tukey's post hoc was used for analyzing individual differences. Two-way ANOVA with ketamine and/or ethanol as independent variables, followed by the Bonferroni post hoc was performed to assess the main effects and the interaction of ketamine and ethanol. All statistical analyses were conducted with SPSS17.0 Software. A $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Co-abuse of ketamine and ethanol effects on depressive-like, anxiety-like behaviors and memory impairment

In order to assess the behavioral change induced by neuronal damage after chronic treatment, not by acute effects of the drugs, the behavioral tests were performed 24 h after treatments.

The OFT can be used to assess locomotor activity, exploratory behavior and anxiety-like behavior of animals in a novel environment (Walsh and Cummins, 1976a). Five parameters were selected: (a) latency stage; (b) line crossing; (c) rearing; (d) grooming and (e) stools. Reduced time spent in the central area (less latency stage), which reflects avoiding exploration of open areas, is indicative of anxiety-like behavior (Lamprea et al., 2008). Line crossings are taken as measures of spontaneous locomotion. Animals with less line crossing indicate having locomotor deficits (Engin et al., 2009). Grooming is an innate self-cleaning behavior of rodent which can be activated by stress state

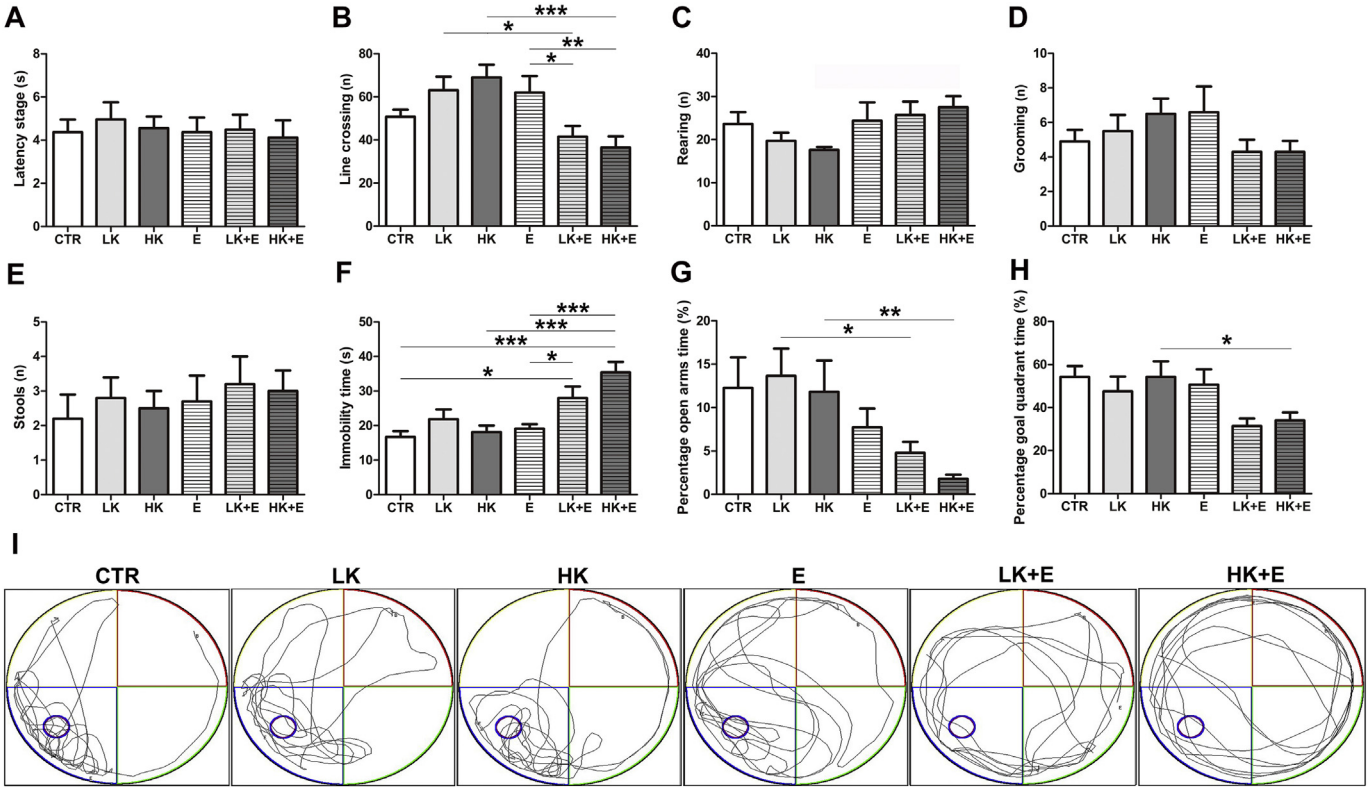

Fig. 1. Behavioral results in the OFT, FST, EPM and MWM.
(A) latency stage in the OFT; (B) line crossing in the OFT; (C) rearing in the OFT; (D) grooming in the OFT; (E) stools in the OFT; (F) the immobility time in the FST; (G) The percentage of open arms time in the EPM test; (H) The percentage of time in the goal quadrant in the MWM test; (I) Tracks of different groups in the MWM probe trial. CTR - control group; LK - low dose ketamine group; HK - high dose ketamine group; E - ethanol group; LK + E - low dose ketamine plus ethanol group; HK + E - high dose ketamine plus ethanol group. Data are presented as the mean ± S.E.M. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

and anxiety-like behaviors, more grooming can be used to indicate anxiety-like behavior (Kalueff et al., 2016). Less rearing and more stools are also usually interpreted as anxiety-like behavior (Lamprea et al., 2008).

The results of OFT are shown in Fig. 1A-E. There was no significant differences on latency stage ($F(5, 54) = 0.211$, $P = 0.956$), rearing ($F(5, 54) = 1.590$, $P = 0.179$), grooming ($F(5, 54) = 1.225$, $P = 0.310$) and stools ($F(5, 54) = 0.257$, $P = 0.934$) among groups. The line crossing in the LK + E and HK + E groups were significantly decreased than that of their ethanol and corresponding LK and HK groups ($F(5, 54) = 5.185$, $P < 0.001$: LK vs LK + E: $P < 0.05$; E vs LK + E: $P < 0.05$; HK vs HK + E: $P < 0.001$; E vs HK + E: $P < 0.01$), but there was no significant difference between any experimental group and the control group (CTR vs LK: $P = 0.649$; CTR vs HK: $P = 0.229$; CTR vs E: $P = 0.733$; CTR vs LK + E: $P = 0.862$; CTR vs HK + E: $P = 0.490$). The two-way ANOVA showed that there was a significant main effect of ethanol ($F(1, 54) = 9.402$, $P < 0.01$) on line crossing while the main effect of ketamine failed to reach significant ($F(2, 54) = 0.307$, $P = 0.737$). However, there was significant interaction between ketamine and ethanol ($F(2, 54) = 7.955$, $P < 0.01$) on line crossing. There was no significant main effect of ketamine and ethanol, as well as interaction effect between ketamine and ethanol on latency stage (ketamine: $F(2, 54) = 0.263$, $P = 0.769$; ethanol: $F(1, 54) = 0.478$, $P = 0.492$; ketamine x ethanol: $F(2, 54) = 0.026$, $P = 0.975$), rearing (ketamine: $F(2, 54) = 0.154$, $P = 0.858$; ethanol: $F(1, 54) = 2.257$, $P = 0.139$; ketamine x ethanol: $F(2, 54) = 1.233$, $P = 0.300$), grooming (ketamine: $F(2, 54) = 0.421$, $P = 0.659$; ethanol: $F(1, 54) = 0.555$, $P = 0.459$; ketamine x ethanol: $F(2, 54) = 2.365$, $P = 0.104$) and stools (ketamine: $F(2, 54) = 0.389$, $P = 0.680$; ethanol: $F(1, 54) = 0.457$, $P = 0.502$; ketamine x ethanol: $F(2, 54) = 0.026$, $P = 0.974$). The results indicated that neither ketamine nor ethanol used alone or combined can induce anxiety-like behaviors or locomotor deficits of rats in OFT.

The forced swimming test (FST) is used to evaluate depression-like behaviors (Porsolt et al., 1978). The longer time of immobility (floating motionless without struggling or making only very slight movements), an indicator of a lower motivation to escape aversive situations, reflects the depressive-like behavior (Aisa et al., 2008). The results of FST are shown in Fig. 1F. The co-abuse of ketamine and ethanol induced significantly longer immobility time compared with the control group ($F(5, 54) = 8.548$, $P < 0.001$: CTR vs LK + E: $P < 0.05$; CTR vs HK + E: $P < 0.001$), either ketamine or ethanol used alone fail to influence the immobility time in the FST (CTR vs LK: $P = 0.687$; CTR vs HK: $P = 0.998$; CTR vs E: $P = 0.983$). Moreover, the HK + E group exhibited a significantly longer immobility time than ketamine or ethanol alone groups (HK vs HK + E: $P < 0.001$; E vs HK + E: $P < 0.001$), and LK + E group had obviously longer immobility time than ethanol alone group (E vs LK + E: $P < 0.05$). The two-way ANOVA showed that there were significant main effects of ketamine ($F(2, 54) = 7.238$, $P < 0.01$) and ethanol ($F(1, 54) = 18.338$, $P < 0.001$), and a significant interaction effect between ketamine and ethanol ($F(2, 54) = 4.972$, $P < 0.05$) on immobility time. The result indicated that co-abuse of ketamine and ethanol induced depressive-like behavior of rats.

EPM is used to assess anxiety-like behaviors of rodents (Pellow et al., 1985). Reduced activity in the open arms (less percentage open arms time) reflects avoiding exploration of open areas, and indicates anxiety-like behavior (Lamprea et al., 2008). The results of EPM are shown in Fig. 1G. Time percentage spent in open arms in LK + E ($P < 0.05$) and HK + E ($P < 0.01$) groups were significantly decreased compared with corresponding ketamine group ($F(5, 54) = 3.200$, $P < 0.01$), no significant difference was observed in the

percentage open arms time between each experimental group and the control group (CTR vs LK: $P = 0.999$; CTR vs HK: $P = 1.000$; CTR vs E: $P = 0.825$; CTR vs LK + E: $P = 0.350$; CTR vs HK + E: $P = 0.071$). The two-way ANOVA showed that there was a significant main effect of ethanol on percentage of open arm ($F(1, 54) = 13.201$, $P < 0.01$). While there was no significant main effect of ketamine ($F(2, 54) = 0.799$, $P = 0.455$) and interaction effect between ketamine and ethanol ($F(2, 54) = 0.601$, $P = 0.552$) on percentage of time in open arm. The results indicated that neither ketamine nor ethanol used alone or combined can induce anxiety-like behavior of rats.

The Morris water maze (MWM) test is used to measure spatial learning and memory (Morris et al., 1982). Reduced time spent in the goal quadrant (less percentage goal quadrant time) indicates spatial memory impairment. The results of MWM test are shown in Fig. 1H and I. The HK + E group had a significantly longer time percentage spent in the goal quadrant than ketamine alone group ($F(5, 54) = 3.041$, $P < 0.05$: HK vs HK + E: $P < 0.05$), there was no significant difference between any experimental group and the control group on percentage goal quadrant time (CTR vs LK: $P = 0.964$; CTR vs HK: $P = 1.000$; CTR vs E: $P = 0.998$; CTR vs LK + E: $P = 0.076$; CTR vs HK + E: $P = 0.156$). The two-way ANOVA showed that there was significant main effect of ethanol on time spent in goal quadrant ($F(1, 54) = 7.898$, $P < 0.01$). However, there was no significant main effect of ketamine ($F(2, 54) = 2.548$, $P = 0.088$) and ketamine x ethanol interaction on time spent in goal quadrant ($F(2, 54) = 1.105$, $P = 0.338$). The result indicated that neither ketamine nor ethanol used alone or combined can influence the spatial learning and memory of rats.

3.2. Co-abuse of ketamine and ethanol effects on apoptosis in the PFC

3.2.1. Co-abuse of ketamine and ethanol effects on TUNEL positive cells in the PFC

TUNEL is widely used to identify apoptotic cells in situ, which relies on the detection of DNA fragments characteristic of apoptotic nuclei (Gavrieli et al., 1992; Rabacchi et al., 1994).

As shown in Fig. 2, compared with control group, the LK + E and HK + E groups had significantly increased numbers of TUNEL positive cells ($F(5, 18) = 245.339$, $P < 0.001$: CTR vs LK + E: $P < 0.001$; CTR vs HK + E: $P < 0.001$), but the number of TUNEL positive cells in LK group, HK group and E group unchanged (CTR vs LK: $P = 0.961$; CTR vs HK: $P = 0.889$; CTR vs E: $P = 0.999$) in the PFC. Moreover, the TUNEL positive cells in the LK + E and HK + E groups were also significantly increased than that of their ethanol and corresponding LK and HK groups (LK vs LK + E: $P < 0.001$; E vs LK + E: $P < 0.001$; HK vs HK + E: $P < 0.001$; E vs HK + E: $P < 0.001$). The two-way ANOVA showed that there were significant main effects of ketamine ($F(2, 18) = 228.936$, $P < 0.001$) and ethanol ($F(1, 18) = 364.265$, $P < 0.001$), and the interactive effect between ketamine and ethanol on TUNEL positive cells in the PFC ($F(2, 54) = 202.280$, $P < 0.001$). The results indicated that co-abuse of ketamine and ethanol induced markedly apoptosis of neurons in the PFC.

3.2.2. Effects of co-abuse of ketamine and ethanol on apoptotic protein and mRNA levels in the PFC

Fig. 3A shows the protein levels of cleaved caspase-3 in the PFC. The LK + E group had higher level of cleaved caspase-3 compared with the control group ($F(5, 30) = 8.097$, $P < 0.001$) ($P < 0.05$) and E group ($P < 0.05$), and the HK + E group had higher level of cleaved caspase-3 compared with the control group ($P < 0.001$), E group ($P < 0.01$) and HK group ($P < 0.01$). There were no significant differences in cleaved caspase-3 protein levels of the LK group, HK group and E group compared with the control group (CTR vs LK: $P = 0.276$; CTR vs HK: $P = 0.191$; CTR vs E: $P = 0.474$) in the PFC. Two-way ANOVA analysis showed that there were major effects of ketamine and ethanol on cleaved caspase-3 expression (ketamine: $F(2, 30) = 11.198$, $P < 0.001$; ethanol: $F(1, 30) = 14.226$, $P < 0.01$), but no significant interaction effect between ketamine and ethanol on cleaved caspase-3 expression ($F(2, 30) = 1.932$, $P = 0.162$).

The mRNA levels of caspase-3 in the PFC are showed in Fig. 3B. In line with the protein expressions of cleaved caspase-3, the HK + E group had higher mRNA level of caspase-3 compared with the control group ($F(5, 30) = 3.458$, $P < 0.05$) ($P < 0.05$), E group ($P < 0.01$), HK group ($P < 0.05$) and LK + E group ($P < 0.05$), there were no significant differences in caspase-3 mRNA levels of LK group, HK group and E group compared with the control group (CTR vs LK: $P = 1.000$; CTR vs HK: $P = 0.951$; CTR vs E: $P = 0.999$) in the PFC. However, inconsistent with protein expression, no significant differences were shown in the mRNA levels of caspase-3 between LK + E group and the control ($P = 0.992$), the corresponding LK ($P = 0.377$) and E groups ($P = 1.000$). Two-way ANOVA analysis showed that there was a major effect of ketamine ($F(2, 30) = 5.568$, $P < 0.01$) on caspase-3 mRNA expression, but no significant main effect of ethanol ($F(1, 30) = 2.564$, $P = 0.120$) and ketamine x ethanol interactions on caspase-3 mRNA expression ($F(2, 30) = 1.620$, $P = 0.215$).

Fig. 3C shows the protein level of Bax in the PFC. Both LK + E and HK + E groups had higher level of Bax compared with the control group ($F(5, 30) = 6.432$, $P < 0.001$: CTR vs LK + E: $P < 0.05$; CTR vs HK + E: $P < 0.01$) and the corresponding ketamine (LK vs LK + E: $P < 0.05$; HK vs HK + E: $P < 0.05$) and E groups (E vs LK + E: $P < 0.05$; E vs HK + E: $P < 0.001$). There were no significant differences in the protein level of Bax in LK group, HK group and E group compared with the control group (CTR vs LK: $P = 0.799$; CTR vs HK: $P = 0.365$; CTR vs E: $P = 1.000$) in the PFC. The major effects of

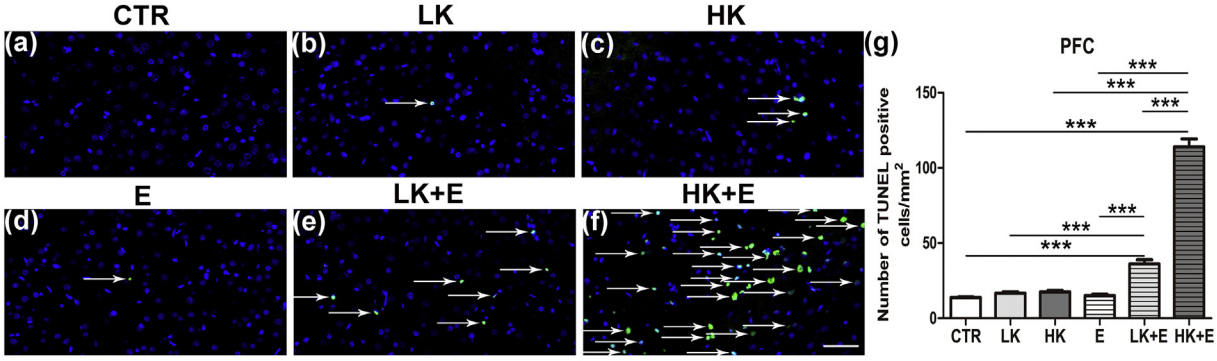

Fig. 2. TUNEL staining in the PFC (×400).
Representative pictures of TUNEL staining (a) – (f) and data analysis of TUNEL positive cells (g) in the PFC. White arrows indicate TUNEL positive cells (olivine) in (a) – (f). CTR - control group; LK - low dose ketamine group; HK - high dose ketamine group; E - ethanol group; LK + E - low dose ketamine plus ethanol group; HK + E - high dose ketamine plus ethanol group. Scale bar = 50 um. Data are presented as the mean ± S.E.M. *** $p < 0.001$.

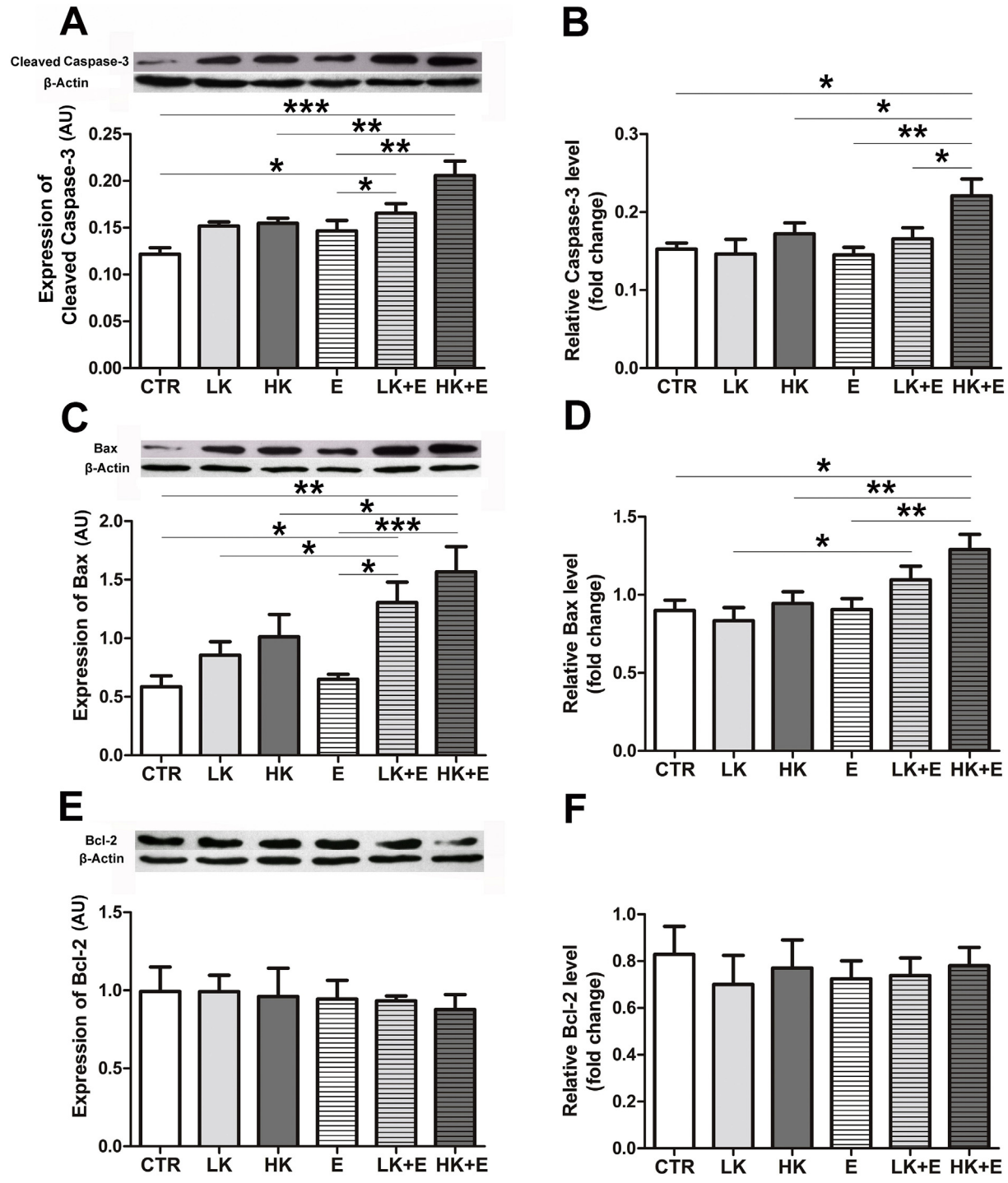

Fig. 3. Protein and mRNA expression of Caspase-3, Bax and Bcl-2 in the PFC.
(A) cleaved Caspase-3 protein expression in the PFC detected by WB; (B) Caspase-3 mRNA expression in the PFC detected by RT-qPCR; (C) Bax protein expression in the PFC detected by WB; (D) Bax mRNA expression in the PFC detected by RT-qPCR; (E) Bcl-2 protein expression in the PFC detected by WB; (F) Bcl-2 mRNA expression in the PFC detected by RT-qPCR. CTR - control group; LK - low dose ketamine group; HK - high dose ketamine group; E - ethanol group; LK + E - low dose ketamine plus ethanol group; HK + E - high dose ketamine plus ethanol group. Data are presented as the mean ± S.E.M. * $p < 0.05$; ** $p < 0.01$. *** $p < 0.001$.

ketamine and ethanol were found (ketamine: $F(2, 30) = 10.433$, $P < 0.001$; ethanol: $F(1, 30) = 8.342$, $P < 0.01$), and the interactive effect between ketamine and ethanol was also significant ($F(2, 30) = 1.475$, $P = 0.245$).

The mRNA levels of Bax in the PFC are showed in Fig. 3D. In accordance with the protein expressions of Bax, the HK + E group showed higher level of Bax mRNA compared with the control, HK and E groups ($F(5, 30) = 4.172$, $P < 0.01$: CTR vs HK + E: $P < 0.05$; HK vs HK + E: $P < 0.01$; E vs HK + E: $P < 0.01$), there were no significant differences in Bax mRNA levels in LK group, HK group and E group compared with control group (CTR vs LK: $P = 0.992$; CTR vs HK: $P = 0.999$; CTR vs E: $P = 1.000$), meanwhile, the LK + E group only exhibited higher Bax mRNA level compared with the LK group ($P < 0.05$). A major effect of ketamine was found in Bax mRNA expression ($F(2, 30) = 3.753$, $P < 0.05$). But, there were no significant main effect of ethanol ($F(1, 30) = 0.469$, $P = 0.630$) and ketamine x

ethanol interaction on Bax mRNA expression (F (2, 30) = 2.392, P = 0.109).

Fig. 3E shows the protein levels of Bcl-2 in the PFC. There were no significant differences in the protein levels of Bcl-2 between groups (F (5, 30) = 0.011, P = 0.986). Moreover, there was no significant main effect of ketamine and ethanol, and interaction effect between ketamine and ethanol on Bcl-2 protein expression (ketamine: F (2, 30) = 0.097, P = 0.908; ethanol: F (1, 30) = 0.401, P = 0.531; ketamine x ethanol: F (2, 30) = 0.011, P = 0.989).

The mRNA levels of Bcl-2 in the PFC are showed in 3F. In accordance with the protein expressions of Bcl-2, no significant differences were observed in the mRNA levels of Bcl-2 between groups (F (5, 30) = 0.201, P = 0.959). Furthermore, there were no significant main effects of ketamine and ethanol and significant ketamine x ethanol interaction on Bcl-2 mRNA expression (ketamine: F (2, 30) = 0.205, P = 0.816; ethanol: F (1, 30) = 0.052, P = 0.822; ketamine x ethanol: F (2, 30) = 0.273, P = 0.763).

All the results indicated that co-abuse of ketamine and ethanol induced apoptosis of neurons in the PFC.

3.3. Effects of ketamine and ethanol on apoptosis in the HIP

3.3.1. Effects of co-abuse of ketamine and ethanol on TUNEL positive cells in the HIP

As shown in Fig. 4, there were no significant differences in the number of TUNEL positive cells among the groups in CA1 (F (5, 18) = 1.688, P = 0.188), CA2/3 (F (5, 18) = 1.523, P = 0.232) and DG (F (5, 18) = 0.830, P = 0.545) regions. Moreover, there were no significant main effects of ketamine and ethanol, and interaction effects between ketamine and ethanol in CA1 (Fig. 4A) (ketamine: F (2, 18) = 1.516, P = 0.246; ethanol: F (1, 18) = 2.379, P = 0.140; ketamine x ethanol: F (2, 18) = 1.516, P = 0.246), CA2/3 (Fig. 4B) (ketamine: F (2, 18) = 2.958, P = 0.077; ethanol: F (1, 18) = 0.669, P = 0.424; ketamine x ethanol: F (2, 18) = 0.515, P = 0.606) and DG (Fig. 4C) (ketamine: F (2, 18) = 1.033, P = 0.376; ethanol: F (1, 18) = 1.866, P = 0.189; ketamine x ethanol: F (2, 18) = 0.108, P = 0.898) subfields of HIP. The results indicated that co-abuse of ketamine and ethanol did not induce apoptosis of neurons in the HIP.

3.3.2. Effects of ketamine and ethanol on apoptotic protein and mRNA levels in the HIP

Fig. 5A shows the protein levels of cleaved caspase-3 in the HIP. There were no significant differences in the protein level of cleaved caspase-3 among the groups (F (5, 30) = 2.148, P = 0.087) in the HIP. Moreover, there were no significant main effects of ketamine and ethanol, as well as ketamine x ethanol interaction on protein expressions of cleaved caspase-3 (ketamine: F (2, 30) = 3.293, P = 0.051; ethanol: F (1, 30) = 4.017, P = 0.054; ketamine x ethanol: F (2, 30) = 0.067, P = 0.935).

The mRNA levels of caspase-3 in the HIP are showed in Fig. 5B. In line with the protein expressions of cleaved caspase-3, no significant differences were observed in the mRNA levels of caspase-3 between any groups (F (5, 30) = 0.771, P = 0.579). Furthermore, there was no significant main effect of ketamine and ethanol and significant ketamine x ethanol interaction on the mRNA levels of caspase-3 (ketamine: F (2, 30) = 1.627, P = 0.214; ethanol: F (1, 30) = 0.293, P = 0.592; ketamine x ethanol: F (2, 30) = 0.265, P = 0.769).

Fig. 5C shows the protein levels of Bax in the HIP. Ketamine or ethanol used alone were capable to induce significant Bax protein expression compared with the control group (F (5, 30) = 9.964, $P < 0.001$: CTR vs LK: $P < 0.01$; CTR vs HK: $P < 0.001$; CTR vs E: $P < 0.05$). Moreover, both LK + E and HK + E groups had higher level of Bax compared with the control group (CTR vs LK + E: $P < 0.001$; CTR vs HK + E: $P < 0.001$) and E groups (E vs LK + E: $P < 0.05$; E vs HK + E: $P < 0.01$). There were significant main effects of both ketamine (F (2, 30) = 19.916, $P < 0.001$) and ethanol (F (1, 30) = 9.556, $P < 0.01$), while no significant ketamine x ethanol interaction (F (2, 30) = 0.216, P = 0.807) on the Bax protein expression.

The mRNA levels of Bax in the HIP are showed in Fig. 5D. Unlike the protein expressions of Bax, no significant differences in the mRNA levels of Bax were observed among the groups (F (5, 30) = 0.440, P = 0.817) in the HIP. Furthermore, there were no significant main effect of ketamine and ethanol and significant ketamine x ethanol interaction on the mRNA levels of Bax (ketamine: F (2, 30) = 0.669, P = 0.505; ethanol: F (1, 30) = 0.559, P = 0.461; ketamine x ethanol: F (2, 30) = 0.123, P = 0.885).

Fig. 5E shows the protein levels of Bcl-2 in the HIP. There were no statistically significant differences in the protein levels of Bcl-2 among the groups (F (5, 30) = 0.049, P = 0.810). Moreover, there was no significant main effect of ketamine and ethanol and a significant ketamine x ethanol interaction on protein expressions of Bcl-2 (ketamine: F (2, 30) = 0.486, P = 0.620; ethanol: F (1, 30) = 1.124, P = 0.297; ketamine x ethanol: F (2, 30) = 0.076, P = 0.927).

The mRNA levels of Bcl-2 in the HIP are showed in Fig. 5F. In accordance with the protein expressions of Bcl-2, no significant differences were observed in the mRNA levels of Bcl-2 among groups (F (5, 30) = 0.876, P = 0.509). Furthermore, there were no significant main effect of ketamine and ethanol and significant ketamine x ethanol interaction on the mRNA levels of Bcl-2 in the HIP (ketamine: F (2, 30) = 0.634, P = 0.538; ethanol: F (1, 30) = 1.517, P = 0.228; ketamine x ethanol: F (2, 30) = 0.740, P = 0.486).

All the results indicated that ketamine and ethanol alone, and co-abuse of ketamine and ethanol failed to induce apoptosis in the HIP.

4. Discussion

OFT, FST, EPM and MWM are typical procedures for assessing spontaneous locomotion, depression-like behaviors, anxiety-like behaviors and spatial learning and memory in rodent (Morris et al., 1982; Pellow et al., 1985; Porsolt et al., 1978; Walsh and Cummins, 1976b). Several studies have reported that ketamine abuse doses produced specific behavioral abnormalities, such as locomotive depression (Becker et al., 2003; Yilmaz et al., 2002) and memory deficits (Gama et al., 2012; Morgan et al., 2010). Other studies have reported that ketamine at sub-anesthetic dose can induce increases in activity (Trujillo et al., 2011) and depression and/or anxiety relieving (Abdallah et al., 2015; Kishimoto et al., 2016). Our study showed that both ketamine and ethanol alone administration failed to produce any abnormal behaviors in OFT, FST, EPM and MWM compared with the control group. However, the immobility times in the FST were significantly longer in the LK + E and HK + E groups compared with the control group. Considering that LK + E group and HK + E group had no change in the spontaneous locomotion compared with the control group, our results demonstrated that co-administration of ketamine plus ethanol induced depressive-like behavior. The results imply that there exists a high risk of depressive disorders in adolescence following co-abuse of ketamine and ethanol (Schobel et al., 2013). Moreover, although there was no significant difference between any experimental group and the control group on percentage goal quadrant time in WMW test, the swimming patterns of CTR, LK, HK and E animals were very different from LK + E and HK + E rats. The search strategy of rats in CTR, LK, HK and E groups was straight-tendency type, while that in LK + E and HK + E groups was edge-random type. This suggested that co-abuse of ketamine and ethanol actually induced memory impairments. Further study should be performed with much longer treatment model or larger dose of ketamine or ethanol in order to examine the effect of LK + E and HK + E on the memory impairments of rats.

TUNEL staining is a reliable method for monitoring apoptosis in laboratory experiments and has been adopted for assessing neurodegenerative effects of drug abuse. Present study showed that neither ketamine nor ethanol were able to independently produce obvious apoptosis in PFC. Moreover, co-administration of ketamine and ethanol

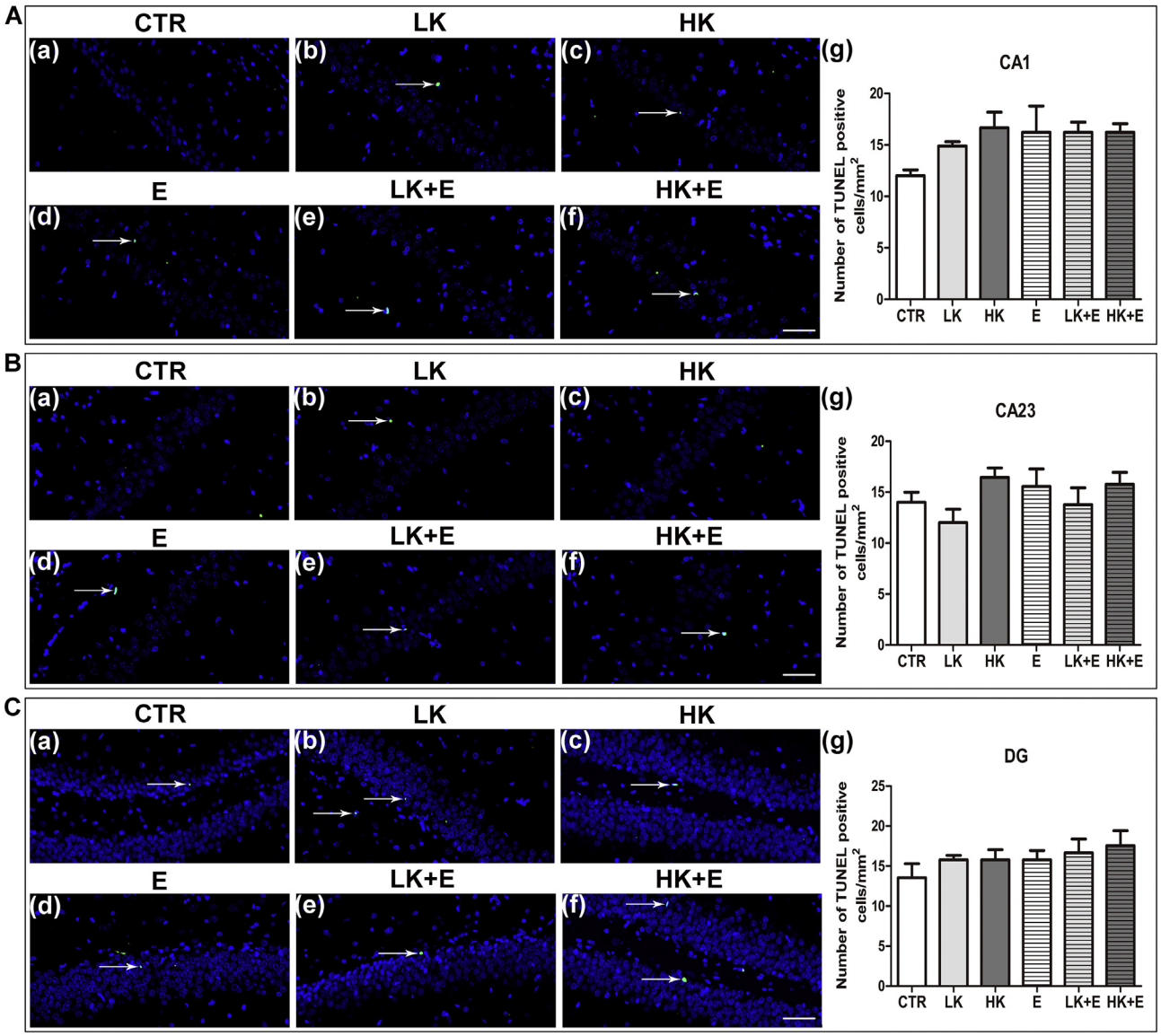

Fig. 4. TUNEL staining in the HIP (×400).
(A) Representative pictures of TUNEL staining (a) – (f) and data analysis of TUNEL positive cells (g) in the CA1 subregion of the HIP; (B) Representative pictures of TUNEL staining (a) – (f) and data analysis of TUNEL positive cells (g) in the CA2/3 subregion of the HIP; (C) Representative pictures of TUNEL staining (a) – (f) and data analysis of TUNEL positive cells (g) in the DG subregion of the HIP. White arrows indicate TUNEL positive cells (olivine) in (a) – (f). CTR - control group; LK - low dose ketamine group; HK - high dose ketamine group; E - ethanol group; LK + E - low dose ketamine plus ethanol group; HK + E - high dose ketamine plus ethanol group. Scale bar = 50 um. Data are presented as the mean ± S.E.M.

markedly aggravated apoptosis as evidenced by greater numbers of TUNEL positive cells in both the low and high dose ketamine plus ethanol groups. This indicates that co-abuse of ketamine and ethanol induced enhanced apoptosis in the PFC.

Caspase-3 is the most important terminal shearing enzyme in promoting apoptosis, which can be cleaved to an active form (cleaved Caspase-3) by either stimulation of tumor necrosis factor (TNF) receptors on the surface of the membrane or the release of cytochrome C from mitochondria. Following activation, the cleaved Caspase-3 plays as an effector protein to degrade downstream targets, and then induce apoptosis (Ananth et al., 2001; McLaughlin, 2004). The pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 are two key members of bcl-2 family, which were demonstrated to correlate with apoptotic cell death. Oligomers of Bax could translocate to the outer mitochondrial membrane, influencing its permeability and triggering cytochrome C release for promoting apoptosis. In contrast, Bcl-2 forms heterodimer with Bax that inhibits the cytochrome C release and thus prevents apoptosis. When apoptosis occurs, the protein levels of cleaved Caspase-3 and Bax are up-regulated, while the protein expression of Bcl-2 is down-regulated. Our findings demonstrated that a co-treatment with ketamine and ethanol significantly increased protein and mRNA expressions of cleaved caspase-3 and Bax in the PFC. Consistent with TUNEL data, it further supported the conclusion that co-abuse of ketamine and ethanol enhanced apoptosis in the PFC.

In the HIP, unlike the results in the PFC, neither the low (20 mg/kg) nor high (30 mg/kg) doses of ketamine use alone or combined with ethanol can significantly increase TUNEL positive cells. Although significantly higher Bax protein expressions were observed in ketamine groups, ethanol group and ketamine plus ethanol groups, however, no significant alterations in protein and mRNA levels of caspase-3 and Bcl-2 were observed in those groups. The results suggest that both ketamine and co-abuse of ketamine and ethanol could not induce apoptosis in the HIP. The current study showed subfield specific response to co-abuse of ketamine and ethanol in adolescent rats.

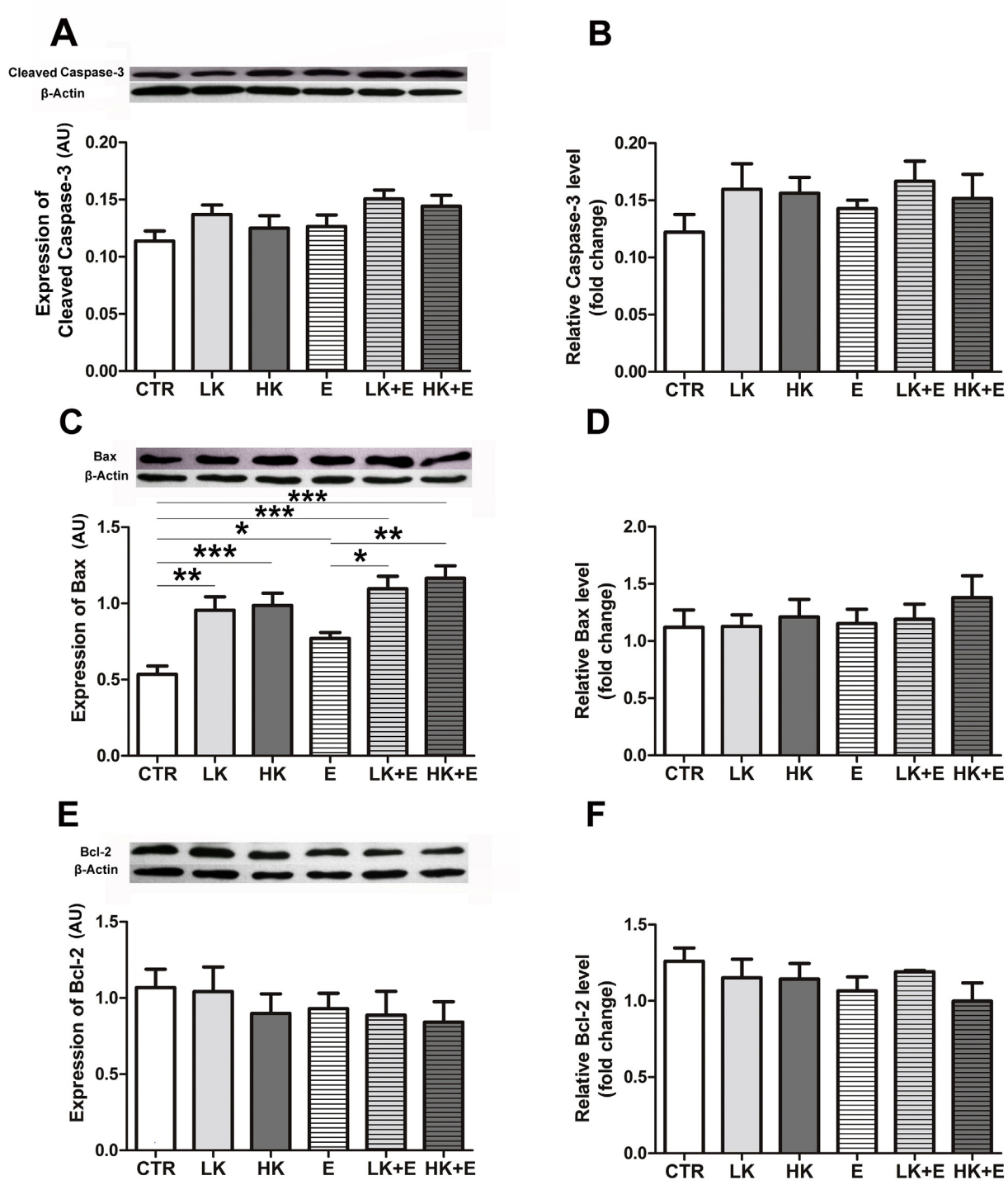

Fig. 5. Protein and mRNA expression of Caspase-3, Bax and Bcl-2 in the HIP.
(A) cleaved Caspase-3 protein expression in the HIP detected by WB; (B) Caspase-3 mRNA expression in the HIP detected by RT-qPCR; (C) Bax protein expression in the HIP detected by WB; (D) Bax mRNA expression in the HIP detected by RT-qPCR; (E) Bcl-2 protein expression in the HIP detected by WB; (F) Bcl-2 mRNA expression in the HIP detected by RT-qPCR. CTR - control group; LK - low dose ketamine group; HK - high dose ketamine group; E - ethanol group; LK + E - low dose ketamine plus ethanol group; HK + E - high dose ketamine plus ethanol group. Data are presented as the mean ± S.E.M. * $p < 0.05$; ** $p < 0.01$. *** $p < 0.001$.

Both ketamine and ethanol increase glutamate release and hyper-metabolism, and result in neuronal damage and mental dysfunction (Holcomb et al., 2005; Hwa et al., 2015; Kim et al., 2011). Ketamine and ethanol block NMDA receptors located on pre-synaptic excitatory amino acid-containing or GABA-containing neurons (Deakin et al., 2008; Homayoun and Moghaddam, 2007). This antagonistic action leads to an increase and excessive amount of excitatory amino acid release within the PFC (Hwa et al., 2015; Kim et al., 2011). In addition, ethanol inhibits the activity of a-ketoglutarate dehydrogenase (aKGDH) in the mitochondria, an enzyme that regulates the glutamate level in the cell, leading to an increase in glutamate and resulting in an adverse effect on cellular integrity (Lin et al., 2014; Vidhya et al., 2013). The excessive release of glutamate can subsequently induce hyper-stimulation of non-NMDA receptors, such as alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA)/kainite (KA) receptors, and result in an increase in intracellular Ca^{2+} levels that triggers a cascade of cellular

response, leading to neuronal cell death (Lan et al., 2014; Zuo et al., 2017). Our findings, demonstrating that a co-treatment with ketamine and ethanol significantly increased TUNEL positive cells, protein and mRNA expressions of caspase-3 and Bax in the PFC, which might in part, results from cumulative increases in extracellular excitatory amino acids. The finding supported the hypothesis that exaggerated excitotoxicity in ketamine and ethanol combined use impaired the PFC via apoptotic pathway. Moreover, co-treatment with ketamine and ethanol did not induce apoptosis in HIP. This may be due to the different concentration of glutamate induced by ketamine and ethanol in the PFC and HIP. Previous studies reported that ketamine induced significantly higher glutamate level in the PFC as compared to the HIP (Lisek et al., 2017; Moghaddam et al., 1997). The level of glutamate in the HIP is too low to promote excitotoxicity and apoptotic pathway. Our study concluded that the PFC is more vulnerable to ketamine and ethanol than the HIP in adolescent period.

It is noticed that ketamine and ethanol use alone or combined significantly up-regulated the Bax protein level, but had no influences on the Bax mRNA level in the HIP. The protein levels are controlled by the expressive level of corresponding mRNA and the mRNA expressions are regulated by transcription process in many forms including DNA methylation, histone modification, chromatin remodeling, transcription factors activation and enhancer activation. In most cases, the proteins and the corresponding mRNAs exhibit the same direction of change. But sometimes only protein expressions changed, while mRNA level unchanged. The main reasons why the protein level of Bax is not consistent with that of mRNA level are explained as follows (Greenbaum et al., 2003): First, there are a number of complex steps involved in turning mRNA into proteins: post-transcriptional processing, degradation of transcription products, translation process, post-translational processing; Second, proteins may differ substantially in their in vivo half-lives; Third, there is a significant amount of error and background noise in both protein and mRNA experiments that limit our ability to get a clear picture; Fourth, the transcription process is negatively regulated by the translation process in eukaryotes.

In conclusion, chronic recreational administration of ketamine (20 mg/kg or 30 mg/kg) combined with ethanol induced obvious depressive-like behavior, TUNEL positive cells and activated caspase-3 and Bax in the PFC, but not in the HIP of adolescent rats which indicate that co-administration of ketamine and ethanol results in behavioral abnormal and the caspase-dependent apoptosis in the PFC of adolescent rats' brains. The conclusion can serve as a warning to the adolescent drug abusers who often co-abuse ketamine with alcohol in the nightclubs.

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Authors' contribution

FP was responsible for the study concept, design and critical review of the whole manuscript. QL assisted with the apoptotic proteins data and drafted the manuscript. HRW, SJF, DXL, HJ and QZ participated in dealing with the animals behavioral tests. All authors critically reviewed content, approved the final manuscript and declared that they have no conflicts of interest.

Declaration of Competing Interest

All authors declared that they have no conflicts of interest.

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