**Role of single prolonged stress in acquisition of alcohol conditioned place preference
in rats**

Lulu Yu^{1,2}, Lan Wang^{1,2}, Xiaochuan Zhao^{1,2}, Mei Song^{1,2}, Xueyi Wang^{1,2#}

1. Department of Psychiatry, First Hospital of the Hebei Medical University
2. Mental Health Institute of the Hebei Medical University

#Correponding author: Xueyi Wang, Department of Psychiatry, The First Hospital of Hebei Medical University, Mental Health Institute of Hebei Medical University, 89 Donggang Road, Yuhua District, Shijiazhuang 050031, China; Tel: +86031185917113; Fax: +86031185917115; Email: ydyywxy@163.com

Running head: SPS associated with CPP

Abstract

Aims: Previous studies showed that exposure to certain types of stressors enhance the rewarding effects of many drugs of abuse, including alcohol; however, no systematic study has investigated the role of single prolonged stress (SPS) in acquisition of alcohol conditioned place preference (CPP). The purpose of this study was to examine whether SPS would facilitate the acquisition of alcohol CPP in rats.

Main methods: Male Sprague-Dawley rats were randomly assigned to either SPS exposure condition or no exposure condition. Freezing behavior and Elevated plus maze (EPM) were employed to evaluate PTSD-like symptoms induced by SPS. Further, using unbiased procedure, CPP conditioning was conducted with alcohol (2g/kg).

Key findings: SPS significantly enhanced freezing behavior of rats, decreased percentages (%) of both time spent and number of entry into the open arms, and facilitated the acquisition of alcohol CPP without inhibiting rats' activity.

Significance: Our findings suggest that SPS plays an important role in alcohol dependence, and CPP paradigm with SPS may be useful for exploring the rewarding mechanism of alcohol with regard to the interaction between alcohol and post-traumatic stress disorder (PTSD).

Keywords: single prolonged stress, post-traumatic stress disorder, conditioned place preference, alcohol

Introduction

Stress can be a key factor in inducing alcohol dependence. Clinical studies have shown that stressful events increase alcohol intake (Söderpalm and de Wit, 2002) and the risk of relapse to alcohol use by enhancing craving (Overstreet et al. , 2004). Experimentally studies on the relationship between stress and alcohol seeking usually use a variety of types of stress, such as footshock stress, immobilization stress, and isolation stress (Matsuzawa et al. , 2000). However, in experimental animals, studies on the effect of stress on alcohol consumption have produced conflicting results: Some studies have reported that footshock stress increases alcohol intake (van Erp et al. , 2001) whereas other studies have shown that footshock decreases (Funk et al. , 2004) or has no effects on alcohol intake(Boyd et al. , 1989).

Posttraumatic stress disorder (PTSD) is usually resulted from exposure to severe traumatic events, such as war, violent personal assault, or natural disaster. Alcohol dependence is a common comorbidity with profound clinical significance in PTSD. Clinical data reveals that individuals with PTSD are more prone to alcohol use relapse compared to individuals without comorbid PTSD.

Single prolonged stress (SPS) is one of the well-established animal models for study of PTSD (Ding et al. , 2010, Kohda et al. , 2007). Using this model, a range of shared neurological and biochemical mechanisms underlying SPS and PTSD have been identified, including but not limited to, modeified neurocircuitry, altered expression of glucorticoid receptor, glucose-regulated protein 78 (GRP78) and cytochrome oxidase, and abnormally modulated noradrenergic pathway (Kohda et al., 2007, Sabban et al. , 2015, Wan et al. ,

2015, Yu et al. , 2014)

For example, the SPS rats often show enhanced inhibition of hypothalamo-pituitary-adrenal (HPA) axis, which has been frequently demonstrated in patients with PTSD. However, little is known about the effect of single prolonged stress (SPS) on alcohol consumption behaviors in rats.

Conditioned place preference (CPP) paradigm has been widely used to evaluate the rewarding effect of substance abuse of legal and illegal substances including alcohol (Tzschentke, 1998, 2007). In the present study, we used rat alcohol CPP model to investigate the role of SPS in acquisition of alcohol conditioned place preference in rats.

Methods

Animals

Male Sprague Dawley rats, weighing 220g to 240g, were purchased from the Laboratory Animal Center, Hebei Medical University (Shijiazhuang, China). The animals were housed, 5 per cage, with free access to food and water and maintained on a 12-hour light/dark cycle with constant temperature ($23 \pm 2^{\circ}\text{C}$) and humidity ($50 \pm 5\%$). All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Committee for Animal Use and Protection of the institution. Rats with weight of 290 to 310g were used for the following experiments.

Drugs

The 95% alcohol solutions were diluted with saline to 20% to obtain the dose of 2g/kg

before the experiments (Der-Avakian, 2007). All injections were given through the intra-peritoneal (i.p.) route of administration in a volume of 10ml/kg.

Single prolonged stress (SPS)

The SPS model consisted of whole body restraint with an animal holder for 2h followed immediately by 20 min of forced swimming (temperature: 25°C, depth: 40cm). Then the rats were allowed to recuperate for 15 min and subsequently exposed to ether vapor until loss of consciousness. The rats were put into the homemade holder for complete immobilization of the body and left undisturbed for 24 hours until they received the electric foot-shock.

Freezing behavior test

For shock application, the rats were placed into the shock chamber (25 cm long×20 cm wide×20 cm high), and after 3 min, a single 4s foot-shock of 0.8mA current was applied to the feet via the metal grid to induce signs of pain (jumping or vocalizing). The rats remained in the shock chamber for another 60s before being returned to their home cages. For study of conditioned fear, the rats were re-exposed to the shock chamber 24h and 7d later for 3 min without further shock application. Freezing behavior was defined as sustained immobility except for respiratory movement, and was observed by the naked eyes.

Elevated plus maze (EPM)

EPM is based on the conflicting anxiety that the animals explore new environments and fear the hanging open arms. EPM is commonly used to evaluate animal response to external stress stimuli (Der-Avakian et al., 2007). The Plexiglas apparatus consisted of a

plus-shaped platform elevated 60 cm above the floor. Two of the opposing arms (42.5 cm×15 cm) were enclosed by 20 cm high side and end walls (closed arms), whereas the other two arms had no walls (open arms). At the beginning of the test, the rat was placed in the central area (15 cm×15 cm) of the maze facing an enclosed arm. The standardized or baseline behavior was recorded during a 5 min exposure and categorized as percentages (%) of both time spent in and number of entry into the open arms relative to total time spent in all arms and total number of entries into all arms, respectively. The test was carried out 2 hours after freezing behavior test.

Conditioned Place Preference

The apparatus for Conditioned place preference (CPP) training and testing consisted of 5 identical 3-chamber PVC boxes. Two large side chambers (30.5 cm long×25 cm wide×32 cm high) were separated by a smaller one (11 cm long×25 cm wide×32 cm high) with different wall colors (black or white) and floor textures (bar or grid, respectively). Manual guillotine doors separated three distinct chambers. Both large chambers had different visual cue lights on the walls (white chamber with horizontal shape and the black chamber with cross shape).

To determine baseline preference (pre-conditioning test), rats were initially placed in the middle chamber with the doors removed for a period of 15 minutes. Time spent in the previously saline- or alcohol-paired chambers during the 15-minute session was determined using software, which measured time spent in each compartment through interruption of infrared beams by animals. Meanwhile, the animal activity was determined by counting across times (the total number of times rats that crossed 3 chambers).

Conditioning was performed using an unbiased, balanced protocol. The chamber in which alcohol was administered was assigned randomly. During the conditioning period, each rat was trained for 8 consecutive sessions (morning and afternoon for 4 consecutive days) with alternate injections of alcohol (2mg/kg, i.p.) and saline (10ml/kg, i.p.). After each injection, the rats were returned to their home cage for 5 minutes to reduce the initial aversive effects of alcohol. The rats were confined to the conditioning box for 20 minutes. In alternate session, the rats were given saline in the same volume as alcohol and placed into the converse box. The day following the conditioning, the rats were tested for the expression of alcohol (post-conditioning test) CPP under conditions identical to those described in the pre-conditioning test. The rats in the saline group received saline injections throughout the conditioning phase, and the same CPP procedure was measured as described above. The place preference score (CPP score) was defined as the discrepancy between time spent in the alcohol-paired chamber and time spent in the saline-paired chamber (Liebsch et al. , 1998).

PTSD-like symptoms induced by SPS

A total of 16 rats were used in experiment 1 to examine PTSD-like symptoms induced by SPS. Animals were randomly assigned to (1) control group not exposed to SPS (n=8) and (2) SPS group (n=8). Behavior experiments (freezing behavior and EPM test) started at a fixed time during testing days, and animals were always habituated in the testing room for 15 min before behavioral tests. To detect the delayed effect of SPS, we observed the animals at 24h and 7d, respectively. The experimental design for this experiment was illustrated in Fig 1A.

Effect of SPS on acquisition of alcohol CPP

To explore the effect of SPS on the acquisition of alcohol CPP, animals underwent SPS before CPP training with 2g/kg alcohol. A total of 44 rats were randomly assigned to (1) control+saline group not exposed to SPS but trained for CPP with saline; (2) control+alcohol group not exposed to SPS but trained for CPP with alcohol; (3) SPS+saline group exposed to SPS and trained for CPP with saline; and (4) SPS+alcohol group exposed to SPS and trained for CPP with alcohol. The experimental design of this experiment was illustrated in Fig 2A.

Data analysis

Data were expressed as mean $\pm$ SEM. Two-way repeated measure ANOVAs were performed to detect differences in freezing behavior and EPM test with treatment (SPS and control) as a between-subjects factor and test condition as a within-subjects factor. A three-way repeated measures ANOVAs were used to analyze the differences among test conditions in CPP scores with the treatment (SPS and control) and dose (alcohol and saline) as between-subjects factors. All post-hoc comparisons were made using the Turkey test. Difference with $p < 0.05$ was considered statistically significant.

Results

PTSD-like symptoms induced by SPS

A two-way repeated measure ANOVA for freezing time using treatment (SPS and control) as the between-subject factors and test condition (baseline, 24h and 7d) as the within-subjects factor revealed a significant effect of treatment ($F_{(1,47)}=12.426$, $p=0.003$).

Interaction between treatment and test conditions was also significant ($F_{(2,47)}=14.249$, $p<0.001$). Post hoc analysis showed that 24h after the foot-shock, the freezing time increased significantly in both control and SPS groups ($p<0.001$) compared to baseline. However, 7d after the foot-shock, the rats in SPS group ($p<0.001$) but not control group ($p=0.208$) showed a significant increase in the freezing time from baseline. In addition, a significant difference was found between 24h and 7d in freezing time for SPS group ($p=0.007$) (Fig.1B) . The results demonstrated recapulation of features of PTSD by SPS.

EPM

A two-way repeated measure ANOVA for open arm entries (%) using treatment (SPS and control) as the between-subject factors and test condition (baseline, 24h and 7d) as the within-subjects factor revealed a significant effect of treatment ($F_{(1,47)}=5.503$, $p=0.034$). Interaction between treatment and test conditions was also significant ($F_{(2,47)}=3.840$, $p=0.034$). Post hoc analysis showed that compared with baseline, the open arm entries decreased significantly 7d but not 24h after treatment in SPS group ($p=0.002$) (Fig.1C).

Open arm time (%)

A two-way repeated measure ANOVA for open arm time (%) using treatment (SPS and control) as the between-subject factors and test condition (baseline, 24h and 7d) as the within-subjects factor revealed a significant effect of treatment ($F_{(1,47)}=5.718$, $p=0.031$). Interaction between treatment and test conditions was non-significant ($F_{(2,47)}=2.493$, $p=0.101$). Similar to the results of open arm entries, post hoc analysis showed that compared with baseline, the open arm time decreased significantly 7d but not 24h after treatment in SPS group ($p=0.002$) (Fig.1D).

These findings indicate that both conditioned fear and anxiety-like behaviors could be observed after SPS, and they persisted for at least 7-day.

Effect of SPS on acquisition of alcohol CPP

A three-way repeated measure ANOVA for CPP score using treatment (SPS and control) and dose (0 and 2g/kg) as the between-subject factors and test condition (pre-condition and post-condition) as the within-subjects factor showed a significant difference in interaction between dose and test condition ($F_{(1,43)}=4.601$, $p=0.038$). The treatment $\times$ dose $\times$ test interaction ($F_{(1,43)}=0.776$, $p=0.384$) and treatment $\times$ test condition interaction ($F_{(1,43)}=1.176$, $p=0.285$) were non-significant. Post hoc analysis revealed that only rats in SPS + alcohol group showed a significant increase in CPP score from baseline during pre-conditioning test ($p=0.005$). Compared with control + saline group, CPP scores significantly increased in the SPS + alcohol group during the post-conditioning group ($p=0.028$). These results suggest that SPS facilitates the acquisition of alcohol CPP (Fig.2B). In addition, the three-way repeated measure ANOVA indicated no significant main effects or interaction across times for CPP, which demonstrated neither SPS nor alcohol inhibited animal activity (Fig.2C).

Discussion

The rates of PTSD comorbid with alcohol use disorders (AUD) are as high as up to 85% (Baker et al. , 2009, Javidi and Yadollahie, 2012, Kessler et al. , 1995). Recent studies on stress and alcohol CPP have shown that stress can weaken the alcohol CPA in Wistar rats (Funk et al., 2004), and CPP can be induced by conditioned fear stress together with low dose of

alcohol, which may be mediated by the dopamine D1, D2 receptors as well as μ , δ , and κ opioid receptors in the central nervous system (Matsuzawa and Suzuki, 2002, Matsuzawa et al. , 1999a, b). In this study, we used SPS model to study role of PTSD in alcohol CPP.

The SPS animal model is a commonly used animal model for PTSD study (Xiao et al. , 2015). It is most frequently used in the EPM and freezing behavior experiments in order to evaluate the behavior of anxiety and fear memory. In the present study, we found that 24h there was no difference in freezing time, open arm entries (%) and open arm time (%) existing between the SPS & S group and the control group. In contrast, at 7d, the SPS & S group had longer freezing time, significantly reduced open arm entries (%) and open arm time, suggesting significantly delayed fear memory and anxiety in these rats with PTSD, a phenomenon consistent with the characteristic symptoms in patients with PTSD presenting fear memory enhanced flashback and other episodes (Söderpalm and de Wit, 2002). The possible mechanisms underlying the observed delayed fear memory and anxiety may be the enhanced HPA negative feedback occurring on the seventh day (Belda et al. , 2008, Harris et al. , 2013, Overstreet et al., 2004). It is worth mentioning that, EPM is commonly used in anxiety behavior study with a single evaluation, and tolerance can occur easily when making repeated measurements. In order to avoid the bias from repetitive measurements, both the control group and the SPS & S group underwent repeated EPM test for three times.

SD rats are relative insensitive to the rewarding effects of alcohol, and resistant to development of alcohol CPP but prone to produce a dose-dependent conditioned place deviation (van Erp et al., 2001). Our data showed that, the CPP values did not change after training in either the control + alcohol group or the control + saline group, indicating that these SD rats did not

develop alcohol CPP. While the CPP values significantly in the SPS+alcohol group after training, comparing to that of the SPS + saline group, suggesting that the PTSD-like rats developed alcohol CPP. In addition, the CPP values in the SPS + alcohol group were higher than that of the control + saline group, suggestive of facilitated alcohol CPP developed in the PTSD rats. Der-Avakian et al. believe that can-not-escape stress promotes the formation of oxycodone CPP in SD rats, but does not influence of acquisition of alcohol (1g / kg, 2g / kg) CPP (Der-Avakian et al., 2007). This is different from our data, and the difference may be related to different stressors used. In the present study, we used SPS method to establish PTSD, and found that stress induced alcohol CPP. Our results were compatible with the clinical situation in which PTSD is often associated with alcohol dependence (Boyd et al., 1989). In addition, some animal studies on maternal separation showed that this stress could have profound long-term effects on the first alcohol consumption in adult rats (Holter et al. , 2000) or mouse (Cruz et al. , 2008), which is consistent with our findings.

Although we did not investigate the mechanism underlying the comorbidity of PTSD and AUD in the present study, investigators believe that PTSD patients' alcohol expectancies will affect the individuals' drinking behavior (Hruska and Delahanty, 2012, Simpson, 2003). If individuals believe that drinking helps relieve negative emotions (such as anxiety or depression), they will increase alcohol consumption. The mechanisms underlying include changes in expression of neurotransmitters (Pennington et al. , 2014), alterations of gene expression (e.g RXFP3 gene) (Walker et al. , 2015) and synaptic plasticity (Spanagel, 2009) and other factors in nerve system that can modify the function of the neural networking. On the other hand, shared biochemical mechanisms between PTSD/SPS and substance abuse, such as modeified altered expression of

glucocorticoid receptor, glucose-regulated protein 78 (GRP78) and cytochrome oxidase, and abnormally modulated nonadrenergic pathway could also possibly involved in the sensitization of CPP by alcohol. In addition, the endogenous opioid dynorphin and its κ opioid receptors are involved in both the stress response (Land et al. , 2008, McLaughlin et al. , 2003), and participated in the thirst for alcohol as well (Holter et al., 2000, Walker et al. , 2012), which may be one of the factors mediating comorbidity of stress and AUD.

In summary, we found that PTSD promoted alcohol CPP, and underlying biological mechanisms need to be further studied.

Funding

This work was supported by the Natural Science Foundation of Hebei Province (No.H2015206407 and No.H2014206280) and the National Science Foundation of China (No.81271489). The funder had no role in study design, data collection and analysis, decision to publish and preparation of the manuscript.

Acknowledgement

None

Conflict of interest statement

None

Authors' contributions

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Lulu Yu and Xueyi Wang. Acquisition of data: Lan Wang and Xiaochuan Zhao. Analysis and interpretation of data: Lan Wang, Xiaochuan Zhao and Mei Song. Drafting of the manuscript: Lulu Yu. Critical revision of the manuscript for important intellectual content: Xueyi Wang. Obtained funding: Xueyi Wang. Study supervision: Xueyi Wang.

Reference

Baker DG, Heppner P, Afari N, Nunnink S, Kilmer M, Simmons A, et al. Trauma exposure, branch of service, and physical injury in relation to mental health among U.S. veterans returning from Iraq and Afghanistan. *Military medicine*. 2009;174:773-8.

Belda X, Rotllant D, Fuentes S, Delgado R, Nadal R, Armario A. Exposure to severe stressors causes long-lasting dysregulation of resting and stress-induced activation of the hypothalamic-pituitary-adrenal axis. *Annals of the New York Academy of Sciences*. 2008;1148:165-73.

Boyd TL, Callen EJ, House WJ. The effects of post-stress exposure to alcohol upon the development of alcohol consumption in rats. *Behav Res Ther*. 1989;27:35-41.

Cruz FC, Quadros IM, Planeta Cda S, Miczek KA. Maternal separation stress in male mice: long-term increases in alcohol intake. *Psychopharmacology*. 2008;201:459-68.

Der-Avakian A, Bland ST, Rozeske RR, Tamblyn JP, Hutchinson MR, Watkins LR, et al. The effects of a single exposure to uncontrollable stress on the subsequent conditioned place preference responses to oxycodone, cocaine, and ethanol in rats. *Psychopharmacology*. 2007;191:909-17.

Ding J, Han F, Shi Y. Single-prolonged stress induces apoptosis in the amygdala in a rat model of post-traumatic stress disorder. *J Psychiatr Res*. 2010;44:48-55.

Funk D, Vohra S, Lê AD. Influence of stressors on the rewarding effects of alcohol in Wistar rats: studies with alcohol deprivation and place conditioning. *Psychopharmacology*. 2004;176:82-7.

Harris AP, Holmes MC, de Kloet ER, Chapman KE, Seckl JR. Mineralocorticoid and

glucocorticoid receptor balance in control of HPA axis and behaviour. *Psychoneuroendocrinology*. 2013;38:648-58.

Holter SM, Henniger MS, Lipkowski AW, Spanagel R. Kappa-opioid receptors and relapse-like drinking in long-term ethanol-experienced rats. *Psychopharmacology*. 2000;153:93-102.

Hruska B, Delahanty DL. Application of the stressor vulnerability model to understanding posttraumatic stress disorder (PTSD) and alcohol-related problems in an undergraduate population. *Psychology of addictive behaviors : journal of the Society of Psychologists in Addictive Behaviors*. 2012;26:734-46.

Javidi H, Yadollahie M. Post-traumatic Stress Disorder. *The international journal of occupational and environmental medicine*. 2012;3:2-9.

Kessler RC, Sonnega A, Bromet E, Hughes M, Nelson CB. Posttraumatic stress disorder in the National Comorbidity Survey. *Archives of general psychiatry*. 1995;52:1048-60.

Kohda K, Harada K, Kato K, Hoshino A, Motohashi J, Yamaji T, et al. Glucocorticoid receptor activation is involved in producing abnormal phenotypes of single-prolonged stress rats: a putative post-traumatic stress disorder model. *Neuroscience*. 2007;148:22-33.

Land BB, Bruchas MR, Lemos JC, Xu M, Melief EJ, Chavkin C. The dysphoric component of stress is encoded by activation of the dynorphin kappa-opioid system. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2008;28:407-14.

Liebsch G, Linthorst AC, Neumann ID, Reul JM, Holsboer F, Landgraf R. Behavioral, physiological, and neuroendocrine stress responses and differential sensitivity to diazepam in two Wistar rat lines selectively bred for high- and low-anxiety-related behavior. *Neuropsychopharmacology : official publication of the American College of*

Neuropsychopharmacology. 1998;19:381-96.

Matsuzawa S, Suzuki T. [Psychological stress and rewarding effect of alcohol]. *Nihon Arukoru Yakubutsu Igakkai Zasshi*. 2002;37:143-52.

Matsuzawa S, Suzuki T, Misawa M. Ethanol, but not the anxiolytic drugs buspirone and diazepam, produces a conditioned place preference in rats exposed to conditioned fear stress. *Pharmacology, biochemistry, and behavior*. 2000;65:281-8.

Matsuzawa S, Suzuki T, Misawa M, Nagase H. Different roles of mu-, delta- and kappa-opioid receptors in ethanol-associated place preference in rats exposed to conditioned fear stress. *European journal of pharmacology*. 1999a;368:9-16.

Matsuzawa S, Suzuki T, Misawa M, Nagase H. Involvement of dopamine D(1) and D(2) receptors in the ethanol-associated place preference in rats exposed to conditioned fear stress. *Brain Res*. 1999b;835:298-305.

McLaughlin JP, Marton-Popovici M, Chavkin C. Kappa opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2003;23:5674-83.

Overstreet DH, Knapp DJ, Breese GR. Similar anxiety-like responses in male and female rats exposed to repeated withdrawals from ethanol. *Pharmacology, biochemistry, and behavior*. 2004;78:459-64.

Pennington DL, Abe C, Batki SL, Meyerhoff DJ. A preliminary examination of cortical neurotransmitter levels associated with heavy drinking in posttraumatic stress disorder. *Psychiatry Res*. 2014;224:281-7.

Söderpalm AH, de Wit H. Effects of stress and alcohol on subjective state in humans.

Alcoholism, clinical and experimental research. 2002;26:818-26.

Sabban EL, Laukova M, Alaluf LG, Olsson E, Serova LI. Locus coeruleus response to single-prolonged stress and early intervention with intranasal neuropeptide Y. *J Neurochem*. 2015.

Simpson TL. Childhood sexual abuse, PTSD, and the functional roles of alcohol use among women drinkers. *Substance use & misuse*. 2003;38:249-70.

Spanagel R. Alcoholism: a systems approach from molecular physiology to addictive behavior. *Physiol Rev*. 2009;89:649-705.

Tzschentke TM. Measuring reward with the conditioned place preference paradigm: a comprehensive review of drug effects, recent progress and new issues. *Prog Neurobiol*. 1998;56:613-72.

Tzschentke TM. Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. *Addiction biology*. 2007;12:227-462.

van Erp AM, Tachi N, Miczek KA. Short or continuous social stress: suppression of continuously available ethanol intake in subordinate rats. *Behavioural pharmacology*. 2001;12:335-42.

Walker AW, Smith CM, Chua BE, Krstew EV, Zhang C, Gundlach AL, et al. Relaxin-3 receptor (RXFP3) signalling mediates stress-related alcohol preference in mice. *PloS one*. 2015;10:e0122504.

Walker BM, Valdez GR, McLaughlin JP, Bakalkin G. Targeting dynorphin/kappa opioid receptor systems to treat alcohol abuse and dependence. *Alcohol*. 2012;46:359-70.

Wan J, Liu D, Zhang J, Shi Y, Han F. Single-prolonged stress induce different change in the

cell organelle of the hippocampal cells: A study of ultrastructure. *Acta Histochem.* 2015.

Xiao B, Yu B, Liu DJ, Han F, Shi YX. Single prolonged stress induces dysfunction of endoplasmic reticulum in a rat model of post-traumatic stress disorder. *Mol Med Rep.* 2015;12:2015-20.

Yu B, Wen L, Xiao B, Han F, Shi Y. Single Prolonged Stress induces ATF6 alpha-dependent Endoplasmic reticulum stress and the apoptotic process in medial Frontal Cortex neurons. *BMC neuroscience.* 2014;15:115.

Figure 1. PTSD-like symptoms induced by SPS: (A) Diagram outlines behavioral procedures. (B) Freezing time at baseline, 24h, and 7d after stress. A significant difference between SPS group and control group was found in freezing time between SPS and control group (n=10) on day 7. * $P < 0.01$, compared to baseline of the same group. # $P < 0.01$, compared with control group at the same time. (C) Open arm entries (%) at baseline, 24h, and 7d after stress. A significant difference was found on day 7 between SPS group and control group (n=10). * $P < 0.01$, compared with baseline of the same group. # $P < 0.05$, compared with control group at the same time. (D) Open arm times (%) on baseline, 24h and 7d after stress. A significant difference was found on day 7 between SPS group and control group (n=10). * $P < 0.01$, compared with baseline of the same group. # $P < 0.01$, compared with control group at the same time points.

Figure 2. Effect of SPS on acquisition of alcohol CPP: (A) Diagram outlines behavioral procedures. (B) The acquisition of alcohol CPP. Data were depicted as the time spent in the alcohol-paired chamber minus time spent in the saline-paired chamber. A significant difference was found for the post-conditioning CPP scores between SPS + alcohol group (n=12) and control + saline group (n=10). * $P < 0.05$, compared with pre-conditioning of the same group. [#] $P < 0.05$, compared with post-conditioning of control + saline group. (C) The animal activity. Data were depicted as the total number of times rats crossed the 3 chambers. No significant differences were found between any two groups, i.e., Pre-C/pre-conditioning; Post-C/post-conditioning.