# Traumatic stress affects alcohol-drinking behavior through cocaine- and amphetamine-regulated transcript 55-102 in the paraventricular nucleus in rats

JIE LIU, XUE-MING HU, XIAO-JIAN LI and HUI ZHAO

Department of Integrative Medicine and Neurobiology, School of Basic Medical Sciences, Shanghai Medical College, Fudan University, Shanghai 200032, P.R. China

Received February 13, 2017; Accepted August 14, 2017

DOI: 10.3892/mmr.2017.7989

**Abstract.** Cumulative evidence has suggested an association between stress and alcohol self-administration; however, less is known about the role of traumatic stress in alcohol drinking behavior. It has been reported that cocaine- and amphetamine-regulated transcript (CART) 55-102 may be involved in mediating stress responses and regulating reward and reinforcement. The aim of the present study was to evaluate the role of CART 55-102 in alcohol drinking behavior of rats in the presence or absence of traumatic stress. Alcohol drinking behavior was examined using the two-bottle choice drinking paradigm (one bottle contained 10% alcohol and the other contained filtered water), which was initiated 1, 3 and 7 days post-trauma (T1, T3 and T7), for 14 days in rats; the control group was initiated from T0. The results indicated that exposure to trauma significantly increased alcohol consumption and preference, particularly drinking from T3. Immunohistochemistry revealed that the lowest level of CART 55-102 immunoreactivity within the paraventricular nucleus (PVN) was exhibited in the T3 group. Additionally, an intra-PVN injection of CART 55-102 attenuated alcohol-drinking behavior in a dose-dependent manner, in the T3 group. Furthermore, the significant increase in circulating adrenocorticotrophic hormone (ACTH) and corticosterone (CORT) concentrations in the T3 group were inhibited by CART 55-102 administration to the PVN, in particular CORT levels were significantly decreased. Positive correlations between alcohol preference and ACTH and CORT levels were also observed. These results indicated that CART 55-102 in the PVN serves an inhibitory role in traumatic stress-induced alcohol drinking behavior, possibly through disturbing hypothalamus-pituitary-adrenal axis hyperactivity.

*Correspondence to:* Dr Hui Zhao, Department of Integrative Medicine and Neurobiology, School of Basic Medical Sciences, Shanghai Medical College, Fudan University, 138 Yixueyuan Road, Shanghai 200032, P.R. China
E-mail: mayzhao1122@yahoo.com

*Key words:* alcohol drinking behavior, cocaine- and amphetamine-regulated transcript 55-102, traumatic stress, hypothalamus-pituitary-adrenal axis,

## Introduction

Alcohol abuse is characterized by an escalation from low or moderate alcohol consumption to excessive consumption and compulsive alcohol uptake, and is associated with numerous environmental and genetic factors (1,2). Harmful alcohol use is associated with substantial morbidity and mortality, through disrupting the function of the brain, liver, gastrointestinal tract and pancreas (3,4). It has previously been indicated that alcohol consumption is influenced by stressful stimuli that serve as risk factors, including inescapable foot shock, restraint, forced swim and social defeat in animals, and life stressors (such as job loss, divorce, earthquakes or other traumatic events) in humans (5,6). Among these stimuli, trauma is the most common, experienced at least once by 50-70% of people in their lifetimes (7). Trauma not only destroys healthy body tissue, but can also lead to the development of functional disorders within the nerve-endocrine-immune system. Therefore, traumatic stress was used as a stressor in our previous study, which indicated that immunosuppression is accompanied by a decrease in splenocyte proliferation and natural killer cell activity 1-3 days post-trauma; however, complete recovery was observed by day 7 (8). Furthermore, inflammation induced by some factors, such as lipopolysaccharides, has been deemed to result in a persistent increase in alcohol self-administration (9,10). It has thus been speculated that a causal relationship exists between traumatic stress and alcohol consumption; and days 1, 3 and 7 post-trauma appear to be associated with triggering alcohol problems.

Cocaine-and-amphetamine-regulated transcript (CART) was originally described as an mRNA, which is increased in the rat striatum following acute cocaine administration (11). Peptides produced by CART exhibit a high to moderate distribution in the hypothalamus, in particular the paraventricular nucleus (PVN), arcuate and supraoptic nuclei, and in the anterior lobe of the pituitary and adrenal medulla (12). It has been reported that these peptides are involved in the regulation of biological processes, including feeding, stress,

reward, metabolism, anxiety and depression (13,14). Kuhar and Yoho (15) extracted and identified CART 55-102 and CART 62-102 as two major CART fragments in rat tissues, by western blotting. As a bioactive peptide fragment, CART 55-102 has been reported to regulate behavioral and physiological responses to stress (16). CART 55-102 signaling in the PVN may mediate the neuroendocrine response to 2,4,5-trimethyl-3-thiazoline-induced predator stress (17). CART was originally discovered following the acute administration of cocaine and amphetamine into rats (11); however, it is now fully appreciated that CART 55-102 is involved in the actions of other psychostimulants, including cocaine, morphine and alcohol (18-20). As the PVN is an anatomical area that is involved in stress and CART 55-102 signaling, the modulation of CART 55-102 in the PVN in response to traumatic stress and alcohol use is highly plausible.

It is well known that stress responses generally involve the neuroendocrine system, in particular the hypothalamus-pituitary-adrenal (HPA) axis (21,22). These responses are activated by the limbic and ascending brainstem, and the pontine pathways; characterized by a significant increase in the release of neuropeptide and corticotropin releasing factor from the PVN of the hypothalamus, and the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary and glucocorticoids [corticotropin (CORT) in rats, cortisol in humans] from the adrenal glands (21). CART exists abundantly in all three levels of the HPA axis (12), suggesting its role in mediating the stress response. Circulating ACTH and CORT levels can be influenced by intracerebroventricular CART injection in male rats, indicating a marked effect of CART on the regulation of HPA axis-associated activity (23). HPA axis activation has previously been observed to be rapidly evoked by traumatic stress (24); however, it is unknown whether CART 55-102 can regulate the activation induced by trauma.

The present study aimed to determine whether alcohol-drinking behavior may be affected by traumatic stress, to further explore and confirm the role of CART 55-102 within the PVN with the administration of alcohol, and to examine the effects of CART 55-102 within the PVN on the HPA axis during trauma.

## Materials and methods

*Animals*. A total of 120 male Sprague-Dawley rats (weight, ~150 g; age, postnatal day 33; n=6/group) were purchased from the Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). The rats were maintained in a controlled environment under a 12-h light-dark cycle (lights on at 7:00 a.m.), at 23±1˚C and 50% humidity, with *ad libitum* access to food and water. Only male rates were used as female hormones may influence behavioral alterations following surgery. All experimental procedures were approved by the Animal Use and Care Committee of Fudan University (Shanghai, China) and were in accordance with the guidelines of the National Institutes of Health (Institute of Laboratory Animal Resources) on animal care (25).

*Drugs*. Absolute ethanol (concentration ≥99.7% HeYing Chemical Corporation, Shanghai, China) was diluted to 10% (v/v) with filtered water for drinking. For intra-PVN injection, CART 55-102 (3337; Tocris Bioscience, Bristol, UK) was dissolved in normal saline (NS) to produce the following concentrations: 0.025, 0.625 and 1.25 µg/0.5 µl. In the control group, 0.5 µl NS was injected into the PVN.

*Model*. The rats were allowed to acclimate for a minimum of 5 days prior to experimentation. The traumatic stress paradigm was performed as previously described (24,26). In the control group, rats were administered 2% sodium pentobarbital [intraperitoneal injection (i.p.), 62 mg/kg] for anesthesia, but did not undergo trauma. Rats in the traumatic stress group were initially anesthetized in the same manner as the controls, after which a 5 cm incision was made along the abdominal median line. Viscera were exposed for 1 min to verify the absence of tissue damage, and the wounds were subsequently sutured. Similarly, the rats were then incised 6 cm along the dorsal median line and were sutured. Finally, all rats were returned to their cages and kept warm. No post-operative infection occurred.

*Alcohol drinking behavior test*. Rats were provided with 24 h/day access to two-bottle choice drinking (one bottle contained 10% alcohol, the other contained filtered water) for 14 days from 12:00 p.m. on days 1, 3 and 7 post-trauma (T1, T3 and T7, respectively). Non-trauma control rats were provided with two-bottle choice drinking from 12:00 p.m. on T0. The two-bottle choice drinking paradigm was applied, according to the protocol outlined by Beckwith and Czachowski (27). The positions of the alcohol bottle and water bottle were alternated daily to avoid place preference. Spillage and evaporation capacity was measured via a water bottle placed in an empty cage. The volume in the bottle prior to and following drinking activity, and the body weight of the rats were measured daily. These measurements were used to calculate the average alcohol intake over 14 days (g/kg/d). Alcohol preference was calculated by dividing the volume of alcohol consumed by the total volume of alcohol and water consumed. Food was available *ad libitum* during measurements.

*Blood ethanol concentration (BEC) test*. At the end of the 14th day of drinking behavior analysis, rats were anesthetized with sodium pentobarbital (i.p.; 62 mg/kg) then immediately decapitated to collect blood through the carotid artery, which was centrifuged (30 min, 931 x g at 4˚C) to obtain serum. The serum was frozen at -80˚C until use to avoid repeated freeze-thawing. BEC was measured using the EnzyChrom™ Ethanol Assay kit (ECET-100; BioAssay Systems, Hayward, CA, USA) according to the manufacturer's protocol.

*Radioimmunoassay (RIA)*. At 10:00-12:00 a.m. on T0, T1, T3 and T7, rats were first anesthetized with pentobarbital sodium (i.p.; 62 mg/kg), and blood was collected through the carotid artery and then the rats were sacrificed via decapitation. The serum was separated via centrifugation (30 min, 931 x g, 4˚C). Serum ACTH and CORT concentrations were measured with the RIA method (assisted by the Beijing Hua Ying Institute, Beijing, China) to evaluate HPA axis activity. Serum ACTH and CORT levels at 30 and 60 min following intra-PVN injection of saline or 1.25 µg/0.5 µl/side CART 55-102 on T3 were also obtained.

*Immunohistochemistry*. Between 10:00-12:00 a.m. on T0, T1, T3 and T7, rats were fully anaesthetized with pentobarbital sodium (i.p.; 62 mg/kg), and brains were removed following intracardial perfusion with 0.1 M PBS and 4% paraformaldehyde, following with decapitation for euthanasia of the rats, then fixed overnight at 4˚C with 4% paraformaldehyde and dehydrated with sucrose (20-30% gradient sucrose) and embedded with an optimum cutting temperature compound (cat. no. 4583; Sakura Finetek USA, Inc., Torrance, CA, USA) in order to obtain 30-µm sections. Sections were used for immunohistochemical and immunofluorescence staining of CART 55-102. For immunohistochemical staining, sections were washed three times with PBS, antigen retrieval was conducted using buffer P0083 (Beyotime Institute of Biotechnology, Shanghai, China), and the sections were subsequently treated with 3% hydrogen peroxide for 1 h at 37˚C. Following PBS washes and blocking with 5% donkey serum (017-000-121; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) in 0.3% Triton X-100 for 1 h at room temperature, the sections were incubated with a primary rabbit anti-rat antibody against CART 55-102 (1:10,000; cat. no. H-003-62; Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) overnight at 4˚C. The sections were washed three times with PBS and were then incubated with goat anti-rabbit immunoglobulin G antibody (1:150; A0277; Beyotime Institute of Biotechnology) for 1 h at 37˚C, the following day. Sections were washed and incubated with avidin-biotin complex reagent for 1 h at 37˚C (1:100; PK-6100; Vector Laboratories, Inc., Burlingame, CA, USA). Sections were subsequently incubated with DAB (1:100; P0202; Beyotime Institute of Biotechnology). Finally, sections were washed, dehydrated with gradient alcohol, cleared with xylene and mounted with cover glass. Images of the sections were then captured with a light microscope (Zeiss GmbH, Jena, Germany).

*Immunofluorescence*. The sections were washed with PBS and incubated with blocking agent for 1 h, prior to incubation overnight at 4˚C with the following primary antibodies: Rabbit anti-CART 55-102 polyclonal antibody (1:1,000; H-003-62; Phoenix Pharmaceuticals, Inc.), mouse anti-neuronal marker (NeuN) monoclonal antibody (1:1,000; MAB377; EMD Millipore, Billerica, MA, USA), mouse anti-glial fibrillary acidic protein (GFAP) monoclonal antibody (1:400; 3670; Cell Signaling Technology Inc., Danvers, MA, USA), and goat anti-ionized calcium-binding adaptor 1 (IBA1) polyclonal antibody (1:400; ab5076; Abcam, Cambridge, MA, USA). The sections were rinsed three times for 10 min in PBS and incubated with corresponding secondary antibodies (cat. nos. A-11008; conjugated with Alexa Fluor 488 and A-21203; conjugated with Alexa Fluor 594; 1:1000; cat. no. A-11058; conjugated with Alexa Fluor 594; 1:2,000; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 2 h at room temperature. Immunofluorescent images were captured under a confocal scanning laser microscope (FluoView 10-ASW; Olympus Corporation, Tokyo, Japan) following washes of the sections.

*PVN injection of CART 55-102*. When Sprague Dawley rats were ~200 g, they were anesthetized with sodium pentobarbital (i.p.; 62 mg/kg), and maintained on isoflurane then a guide cannula was bilaterally implanted in the PVN of the hypothalamus (anterior/posterior, -1.5 mm; medial/lateral, ±0.4 mm; and dorsal/ventral, -8.0 mm) and cannulas were anchored to the skull with screws and dental cement. For analgesia, fentanyl (10 µg/kg) was intramuscularly injected immediately after the surgery (28). The rats recovered for a period of 8 days; recovery of the circadian rhythm was ensured prior to conducting traumatic stress, performed as described in the *Model* subsection. Between 10:00-12:00 a.m. on T3, rats were injected with 0.5 µl NS or 0.025, 0.625 and 1.25 µg/0.5 µl/side CART 55-102 at a rate of 0.5 µl/min (0.5 µl/side NS was injected into the PVN, and employed as the control group in the experiment). Prior to the 14-day drinking behavior experiment, the injection cannulae were maintained for an additional 1 min. Measurements of alcohol consumption, preference and BEC were conducted as aforementioned.

*Statistical analysis*. Data are presented as the mean ± standard error of the mean of 5 repeated experiments. Data obtained from measuring alcohol drinking behavior, CART 55-102 expression levels, and ACTH and CORT levels post-trauma of the groups were compared by one-way analysis of variance followed by Tukey or Newman-Keuls post hoc tests using Statistical Package for the Social Sciences (SPSS) v19.0 software (IBM Corp., Armonk, NY, USA). To compare levels of ACTH and CORT following CART 55-102 micro-injection, an unpaired Student's t-test was conducted using SPSS. Pearson's Correlation Analysis (2-tailed) between alcohol preference and ACTH and CORT levels was also performed through SPSS. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Traumatic stress enhances subsequent alcohol drinking behavior in rats from T3*. To determine the effects of traumatic stress on subsequent alcohol drinking behavior, rats exposed to trauma were randomly assigned to the T1, T3 and T7 groups, which were given access to 10% alcohol or water on T1, T3 and T7, respectively, for 14 days. As presented in Fig. 1A, alcohol intake in the T1 and T3 groups was significantly increased compared with in the control group ($P=0.016$, $P=0.001$; Fig. 1A); alcohol preference in the T3 group also exhibited a similar increase ($P<0.001$; Fig. 1B). Additionally, the BEC of T3 rats measured near the termination of the alcohol behavior test exhibited a similar trend to intake and preference ($P=0.003$; Fig. 1C). Therefore, initiation of drinking from T3 may increase subsequent alcohol drinking behavior. Conversely, drinking from T7 reversed the rising trend, suggesting that T3 may be a key time point for evoking reinforced alcohol self-administration during stress development.

*CART 55-102 in the PVN exhibits an inhibitory effect on subsequent drinking behavior*. To evaluate the effects of CART 55-102 in the PVN on alcohol drinking behavior, immunohistochemistry and immunofluorescence were performed on samples from T0, T1, T3 and T7. Immunohistochemistry demonstrated that CART 55-102 in the PVN was markedly decreased in the T1 and T3 groups compared with in the

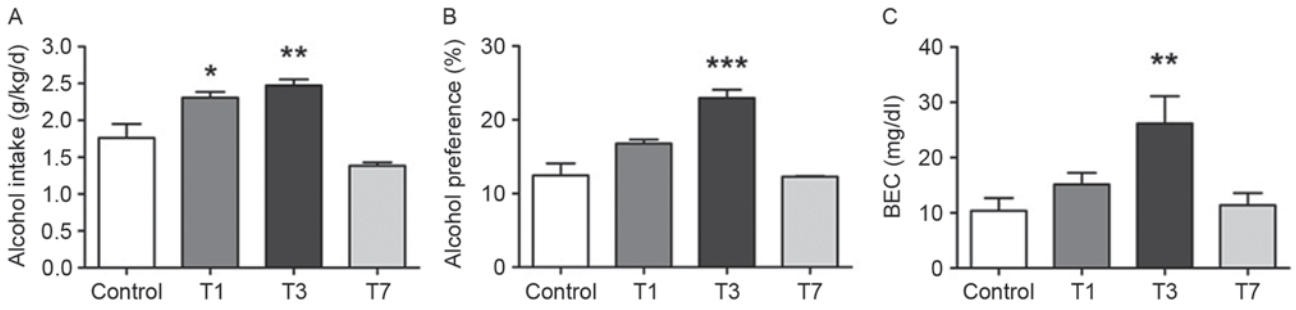


Figure 1. Traumatic stress contributes to alcohol drinking behavior from T3. Rat models of trauma were given access to two-bottle choice drinking on T0, T1, T3 and T7. (A) Average alcohol intake, (B) alcohol preference during 14 days and (C) BEC were significantly increased in the T3 group compared with in the control group (n=6/group). $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001 vs. the control group. BEC, blood ethanol concentration; T, days post-trauma.

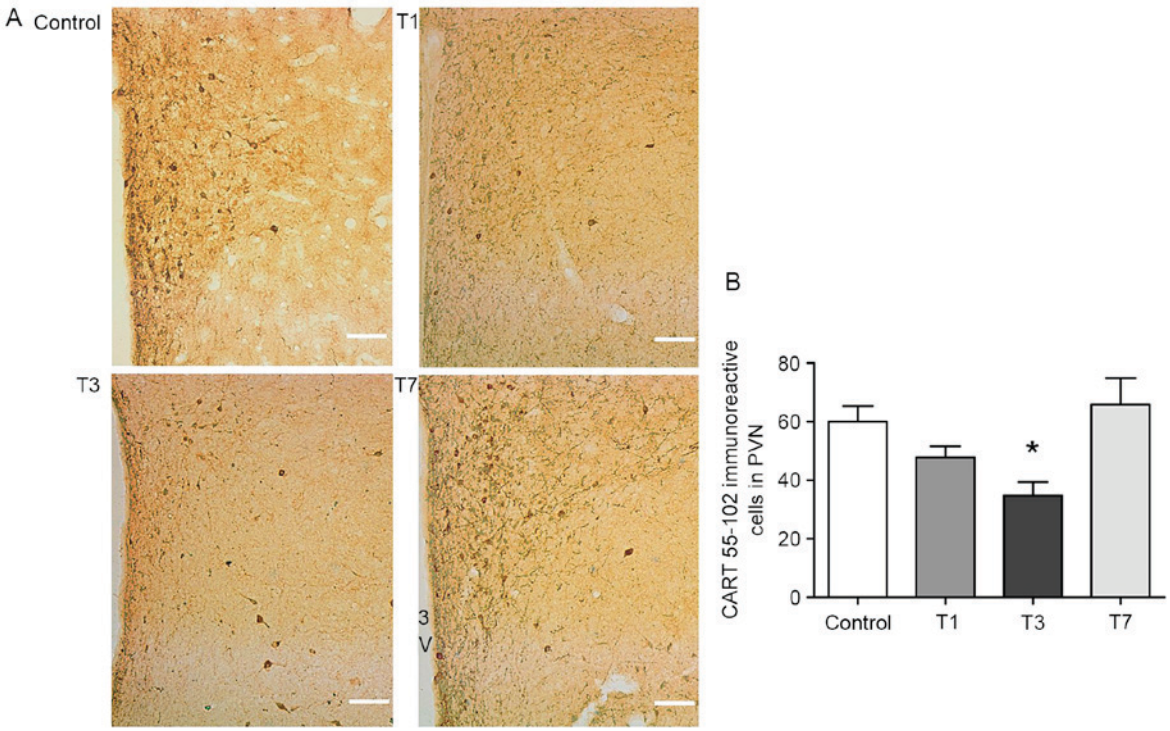


Figure 2. Immunohistochemical staining of CART 55-102 in the paraventricular nucleus reveals that the lowest levels were observed on T3. (A) Immunohistochemical staining of CART 55-102 (n=6/group). (B) Graph showing the results of (A). Scale bar: 100 µm. $^{*}$P<0.05 vs. the control group. 3V, the 3rd ventricle; CART 55-102, cocaine- and amphetamine-regulated transcript 55-102; PVN, paraventricular nucleus; T, days post-trauma.

control group, particularly on T3 (P=0.015; Fig. 2A and B); this appeared to correlate with the concomitant increase in alcohol consumption and preference from T3. The data of the present study suggested that CART 55-102 in the PVN may suppress subsequent and long periods of drinking behavior. Confocal immunofluorescence labeling indicated that CART 55-102 was abundantly expressed in the PVN (Fig. 3A). In the PVN, the majority of CART 55-102 immunoreactive cells were co-localized with NeuN (Fig. 3B), but not with GFAP, an astrocytic marker (Fig. 3C) or IBA1, a microglial marker (Fig. 3D).

Subsequently, recombinant CART 55-102 or saline was bilaterally injected into the PVN on T3, followed by the 14-day drinking behavior test. Repeated analysis of one-way ANOVA indicated that alcohol intake, preference and BEC exhibited a dose-dependent decrease in rats treated with 0.025, 0.625 and 1.25 µg/0.5 µl/side CART 55-102 (Fig. 4). The intake, preference and BEC in the group treated with 1.25 µg/0.5 µl/side CART 55-102 exhibited a marked decrease compared with the saline group (P=0.002, P=0.013, P=0.002; Fig. 4). These findings suggested that CART 55-102 in the PVN may exert a negative role in the regulation of alcohol drinking behavior post-trauma.

*HPA axis hyperactivity may be reversed by CART 55-102 administration into the PVN.* Traumatic stress has been reported to serve a key role in the endocrine system, particularly the HPA axis (29). The results of a RIA indicated that the HPA axis exhibited hyperactivity in the T3 group as measured by serum ACTH and CORT levels (P=0.003, P=0.02; Fig. 5A and B); however, CORT concentration on T1 and T7 also revealed a partial increase. The correlation between CART 55-102 and the HPA axis has been noted previously (23,30). To explore the effects of CART 55-102 administration on HPA axis activity,

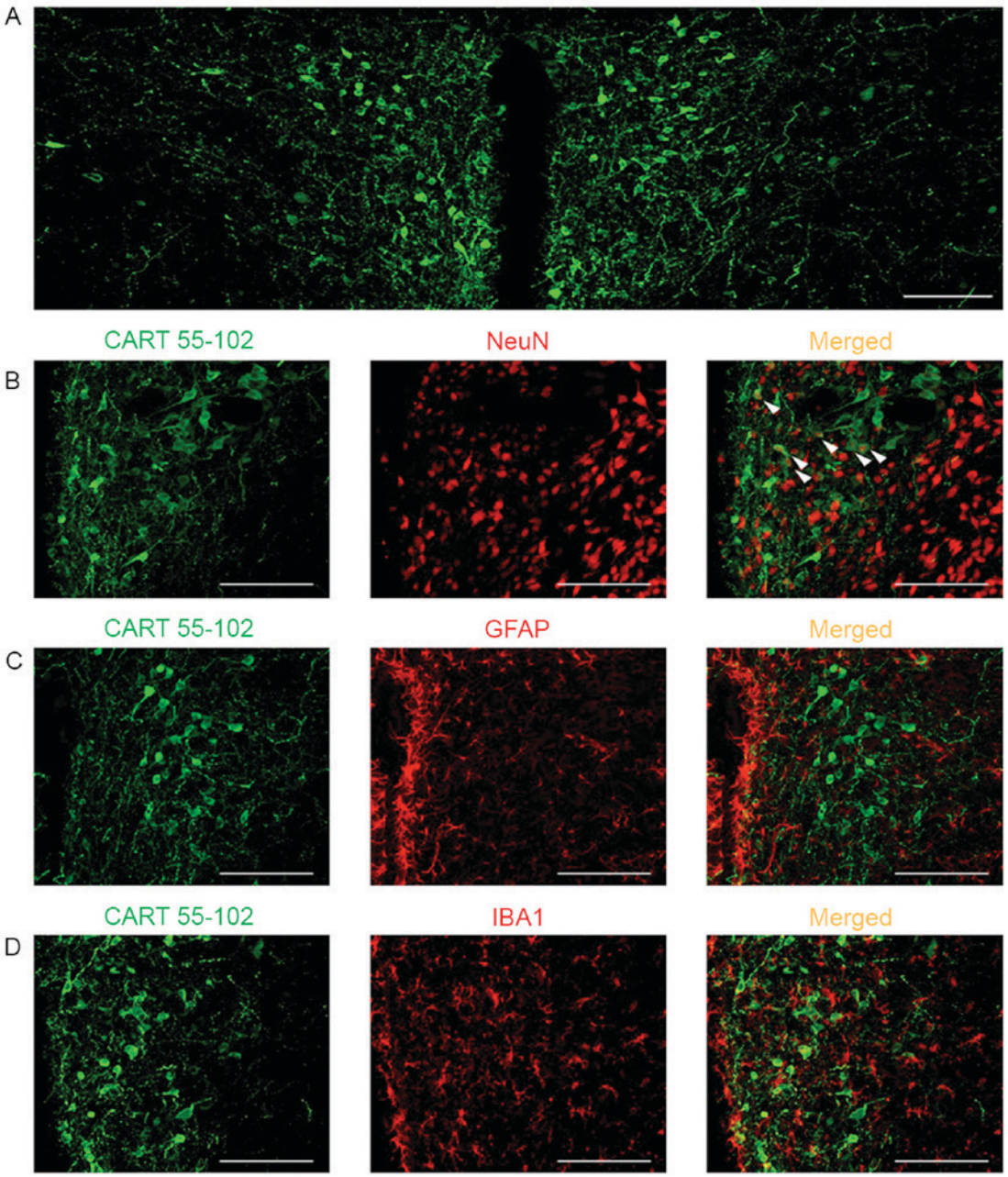


Figure 3. Immunoreactive CART 55-102 cells are mainly localized in PVN neurons. (A) CART 55-102 was abundantly expressed in the PVN. (B) CART 55-102 was mainly co-localized with the neuronal marker NeuN but not with (C) astrocytic marker GFAP or (D) microglial marker IBA1. White arrows indicate double-labeled cells. Scale bar: 100 µm. CART 55-102, cocaine- and amphetamine-regulated transcript 55-102; GFAP, glial fibrillary acidic protein; IBA1, ionized calcium-binding adaptor; NeuN, neuronal marker; PVN, paraventricular nucleus.

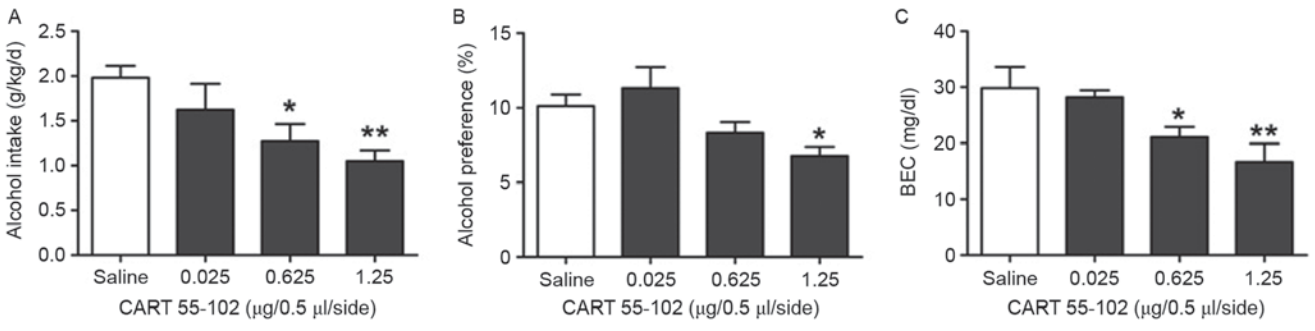


Figure 4. Intra-PVN CART 55-102 injection reduces alcohol drinking behavior in a dose-dependent manner. The rats were injected with saline, 0.025, 0.625 or 1.25 µg/0.5 µl/side CART 55-102 into the PVN on day 3 post-trauma (n=6/group). (A) Alcohol intake, (B) preference, and (C) BEC were significantly decreased in CART 55-102-treated rats compared with saline-treated rats. $^{*}$P<0.05, $^{**}$P<0.01 vs. the saline group. BEC, blood ethanol concentration; CART 55-102, cocaine- and amphetamine-regulated transcript 55-102; PVN, paraventricular nucleus.

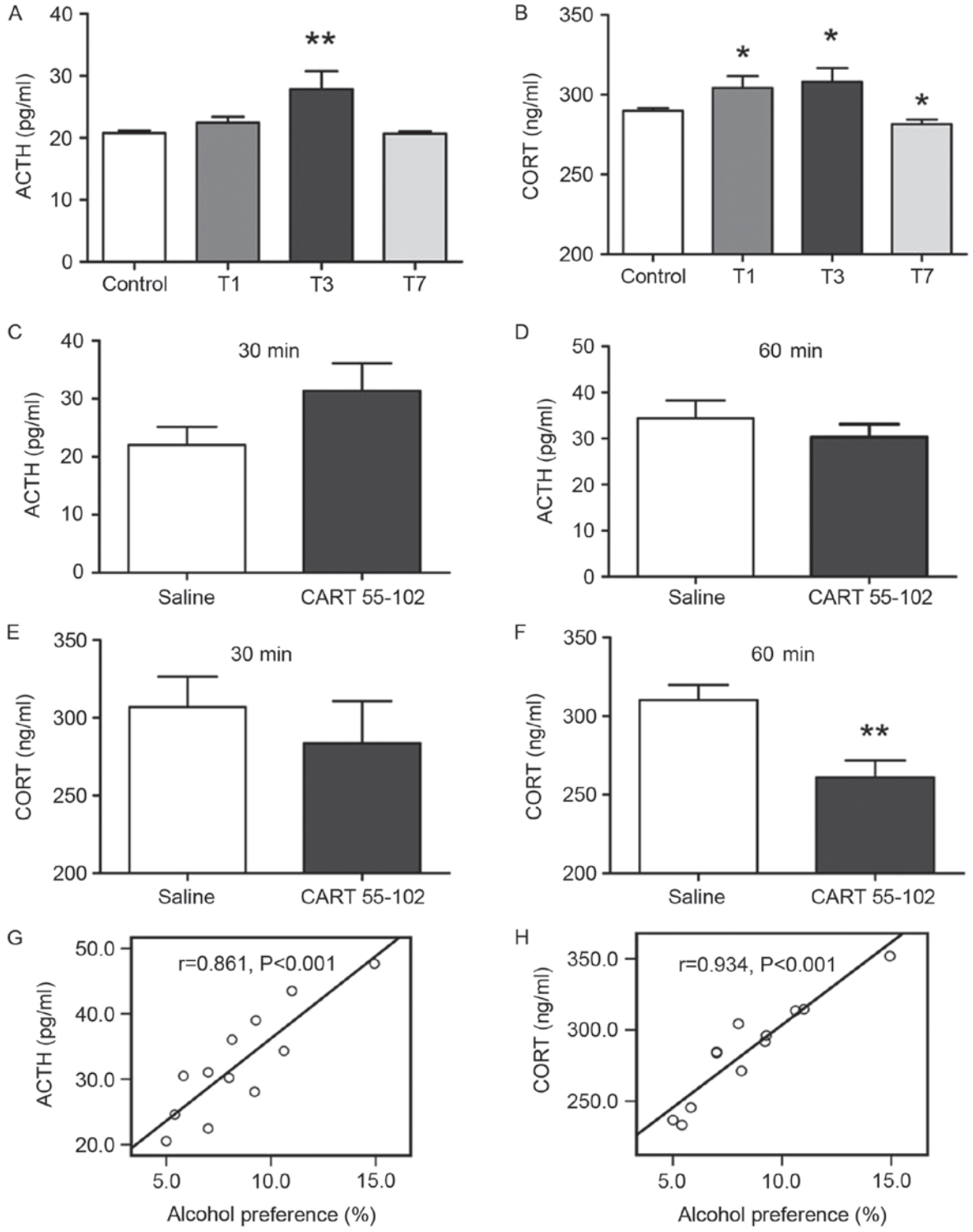


Figure 5. Hyperactivity of the hypothalamus-pituitary-adrenal axis on T3 is reversed by intra-PVN injection of CART 55-102. (A) Serum ACTH and (B) serum CORT concentrations on T0, T1, T3 and T7 were measured by radioimmunoassay; concentration on T3 was significantly increased (n=6/group). CART 55-102 (1.25 µg/0.5 µl/side) was injected into the PVN on T3. (C and D) ACTH and (E and F) CORT levels were evaluated at (C and E) 30 min and (D and F) 60 min post-injection; increased CORT levels on T3 were reversed by CART 55-102 administration at 60 min. The positive correlation between alcohol preference and (G) ACTH and (H) CORT levels at 60 min following CART 55-102 micro-injection was analyzed. $^{*}$P<0.05, $^{**}$P<0.01 vs. the control group. ACTH, adrenocorticotrophic hormone; CART 55-102, cocaine- and amphetamine-regulated transcript 55-102; CORT, corticosterone; PVN, paraventricular nucleus.

the ACTH and CORT concentrations on T3 at 30 and 60 min following intra-PVN CART 55-102 injection (1.25 µg) were examined, since 1.25 µg CART 55-102 had the most significant effect on drinking behavior in rats. A significant alteration in ACTH and CORT levels was not observed at 30 min between the CART 55-102- and saline-treated groups (Fig. 5C and D). However, at 60 min, CORT levels in CART 55-102-treated rats were significantly decreased compared with in the saline group (P=0.0068; Fig. 5F), indicating that CART 55-102 may reverse the excessive CORT levels on T3. In addition, the

correlation analysis between alcohol preference and ACTH and CORT levels at 60 min following CART 55-102 microinjection revealed a positive correlation ($P<0.001$, $P<0.001$; Fig. 5G and 5H). These data suggested that the HPA axis may be involved in the regulation of alcohol drinking behavior.

In summary, administration of CART 55-102 into the PVN may suppress reinforced alcohol drinking behavior induced by traumatic stress, potentially through the disturbance of HPA axis hyperactivity.

## Discussion

The physiological and behavioral response of the body to counteract any factors that perturb and then reinstate homeostasis is known as the stress response. Stress has been reported to have a determinant effect on producing behavioral sequelae (31-33). In humans, traumatic stress is a common stressor, and can include surgical trauma, brain injury and skin damage. The Defense Survey of Health Related Behaviors among Active Duty Military Personnel (HRB Survey) indicated that military trauma can alcohol abuse in soldiers (34). Koob and Le Moal (35) also suggested drug abuse as a pathology induced by direct ties to the stress response via an allostatic mechanism. The present study explored the time-course effect of traumatic stress on subsequent alcohol drinking behavior in Sprague Dawley rats. Notably, traumatic stress markedly increased subsequent alcohol consumption and preference, particularly following drinking from T3. During traumatic stress, the organism undergoes a constantly changing recovery process, from a progressively pathological to a normal state. Wan *et al* (36) reported that hepatolobectomy in rats can trigger astroglial and microglial excessive activation in the brain on day 3, whereas the neuroglia may fully return to a nonreactive state on day 7 post-surgery. Therefore, T3 may be considered a turning point that means turning to the recovery mode. Additionally, other studies support the findings of the present study, since they have demonstrated that stress is an important factor correlated with changes to drinking behavior (32,37). Notably, the present study indicated the important role of traumatic stress in the regulation of voluntary alcohol consumption, and provided a broader line of thought for the treatment of patients that abuse alcohol, although several differences between drinking patterns in humans and research animals exist.

CART is synthesized in neuroendocrine cells of the parvocellular and the magnocellular hypothalamus; CART 55-102 is abundant in the nuclear area of the PVN. The present study suggested that CART 55-102 immunoreactivity within the PVN was prominently reduced on T3, with the majority co-localized within neurons. In addition, the administration of CART 55-102 into the PVN prevented reinforced drinking behavior on T3 in a dose-dependent manner. These results suggested that CART 55-102 within the PVN serves a protective role for the organism by suppressing alcohol consumption. In other studies, it has also been observed that CART 55-102 can negatively modulate drug-seeking behaviors. King *et al* (38) demonstrated the role of CART in mediating the rewarding or motivational properties of ethanol; intracerebroventricular administration of CART 55-102 prevented the context-induced reinstatement of alcohol-seeking behaviors in rats. Intra-accumbens nucleus shell injection of CART 55-102 also attenuated the reinstatement of alcohol-seeking behaviors in a dose-dependent manner; no effect was reported with the CART 1-27 fragment (20). The effects of CART 55-102 on psychostimulants are complex, and brain region- and dose-dependent. Administration of active CART 55-102 into the ventral tegmental area was reported to promote a conditioned place preference, similar to that induced by cocaine or amphetamine (39). In addition, context-induced reinstatement of alcohol-seeking behaviors was reduced with active CART 55-102 administration into the intra-nucleus accumbens (20). The suppressive and reinforcing roles of CART 55-102 in drug abuse are associated with its location and interactions. However, the role of CART 55-102 in supporting and inhibiting the effects of drugs is complex and is not only observed in rodent behavioral studies.

The present study focused on the functional role of the hypothalamic PVN in alcohol consumption. As the primary site of the HPA axis, the PVN harbors the cholinergic basal forebrain neurons that serve a crucial role in regulating the ACTH-CORT system in response to stress (40). Dandekar *et al* (41) revealed a significant increase in CART-immunoreactive cells and fibers following 24 h of alcohol withdrawal and a marked loss of CART-immunoreactivity from cells in the PVN at 48 h. Chen *et al* (42) supported the suggestion that several peptides in the hypothalamic PVN can increase alcohol consumption by promoting distinct aspects of the drinking response. These studies have provided the evidence that suggest the profound role of PVN in alcohol drinking behavior. The results of the present study indicated that CART 55-102 in the PVN may mediate the association between traumatic stress and subsequent alcohol consumption. Therefore, CART 55-102 may be a biological factor in determining vulnerability to drug abuse as well as a potential therapeutic target to post-trauma alcohol abuse.

The modulatory role served by CART 55-102 has been noted to be involved in the stress response (17,43). Expression of CART 55-102 in the PVN was modulated with traumatic stress; the lowest level of expression was reported on T3. HPA axis activity was markedly upregulated on T3, with excessive circulating ACTH and CORT levels. However, the elevated levels of CORT were reversed via CART 55-102 microinjection into the PVN at 60 min; the ACTH levels exhibited an insignificant decrease at 60 min. These data suggested that CART 55-102 may serve a role in the control of adrenal function during trauma. An association between CART 55-102 and the HPA axis has been identified; however, it has also been suggested that CART 55-102 administration into the PVN may stimulate HPA axis activity with an associated increase in plasma ACTH (23,44). This discrepancy may be explained by the use of different animal models. Sprague Dawley rats exposed to trauma were used in the present study compared with healthy Wistar rats used by Stanley *et al* (23). The neurobiological mechanisms differ significantly in different animal models. The HPA axis may be involved in the regulation of alcohol drinking behavior as suggested by the positive correlation between alcohol preference and ACTH and CORT levels; CART 55-102 may serve an inhibitory role in alcohol drinking behavior via the HPA axis in traumatic rats.

In the present study, alcohol intake and preference following treatment with CART 55-102 were measured

over 14 days. The results revealed that a single injection of CART 55-102 on T3 prolonged the 14-day inhibitory effect on drinking behavior. Hyperactivity observed in the HPA axis on T3 was followed by long-term reinforced drinking behavior; however, CART 55-102 reversed the increased serum levels CORT and may regulate long-term alcohol self-administration. The effects of CORT on alcohol consumption were not determined in the present study; however, the results detected a positive correlation between the two factors. Previous studies have revealed that the interaction between CORT and the dopaminergic system in the nucleus accumbens may facilitate the reinforcing effects of alcohol and other drugs (45-47). Therefore, CART 55-102 may serve an inhibitory role in drinking behavior through the disruption of excessive CORT serum levels; however, more evidence is required from future investigations.

In conclusion, the results of the present study demonstrated that traumatic stress significantly increased subsequent alcohol intake and preference from T3. In addition, the majority of CART 55-102 in the PVN was localized to neurons. The results suggested that CART 55-102 may serve an inhibitory effect on alcohol consumption in a dose-dependent manner, which may be due to reduced hyperactivity of the HPA axis. The results suggested that CART 55-102 may negatively modulate the behavioral effect of alcohol abuse and may serve as a therapeutic target to reduce alcohol addiction in the clinical setting.

## Acknowledgements

The present study was supported by grants from the National Natural Science Foundation of China (grant nos. 81471370 and 81573712). The authors would like to thank Dr. Qiu-Qin Han (School of Basic Medical Sciences, Fudan University, Shanghai, China) for her help in revising the manuscript.

## References

1. Koob GF, Ahmed SH, Boutrel B, Chen SA, Kenny PJ, Markou A, O'Dell LE, Parsons LH and Sanna PP: Neurobiological mechanisms in the transition from drug use to drug dependence. Neurosci Biobehav Rev 27: 739-749, 2004.
2. Koob G and Kreek MJ: Stress, dysregulation of drug reward pathways, and the transition to drug dependence. Am J Psychiat 164: 1149-1159, 2007.
3. Federico A, Cotticelli G, Festi D, Schiumerini R, Addolorato G, Ferrulli A, Merli M, Lucidi C, Milani S, Panella C, *et al*: The effects of alcohol on gastrointestinal tract, liver and pancreas: Evidence-based suggestions for clinical management. Eur Rev Med Pharmacol Sci 19: 1922-1940, 2015.
4. Sun C, Shen L, Li X, Liu C and Zhou Y: Risk of pneumonia in central nervous system injury with alcohol intake: A meta-analysis. Int J Clin Exp Med 8: 15738-15744, 2015.
5. Becker HC, Lopez MF and Doremus-Fitzwater TL: Effects of stress on alcohol drinking: A review of animal studies. Psychopharmacology (Berl) 218: 131-156, 2011.
6. Anthenelli R and Grandison L: Effects of stress on alcohol consumption. Alcohol Res Current Rev 34: 381-382, 2012.
7. Ursano RJ, Zhang L, Li H, Johnson L, Carlton J, Fullerton CS and Benedek DM: PTSD and traumatic stress. Brain Res 1293: 2-12, 2009.
8. Xiao S, Wang J, Jiang J, Cao X, Wu G and Zhao H: Characterization of Fyn signaling on the age-dependent immuno-modulation on traumatic rats. Brain Res 1255: 162-169, 2009.
9. Blednov YA, Benavidez JM, Geil C, Perra S, Morikawa H and Harris RA: Activation of inflammatory signaling by lipopolysaccharide produces a prolonged increase of voluntary alcohol intake in mice. Brain Behav Immun 25(Suppl 1): S92-S105, 2011.
10. Blednov YA, Ponomarev I, Geil C, Bergeson S, Koob GF and Harris RA: Neuroimmune regulation of alcohol consumption: Behavioral validation of genes obtained from genomic studies. Addict Biol 17: 108-120, 2012.
11. Douglass J, Mckinzie AA and Couceyro P: PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. J Neurosci 15: 2471-2481, 1995.
12. Koylu EO, Couceyro PR, Lambert PD, Ling NC, DeSouza EB and Kuhar MJ: Immunohistochemical localization of novel CART peptides in rat hypothalamus, pituitary and adrenal gland. J Neuroendocrinol 9: 823-833, 1997.
13. Kristensen P, Judge ME, Thim L, Ribel U, Christjansen KN, Wulff BS, Clausen JT, Jensen PB, Madsen OD, Vrang N, *et al*: Hypothalamic CART is a new anorectic peptide regulated by leptin. Nature 393: 72-76, 1998.
14. Rogge G, Jones D, Hubert GW, Lin Y and Kuhar MJ: CART peptides: Regulators of body weight, reward and other functions. Nat Rev Neurosci 9: 747-758, 2008.
15. Kuhar MJ and Yoho LL: CART peptide analysis by Western blotting. Synapse 33: 163-171, 1999.
16. Koylu EO, Balkan B, Kuhar MJ and Pogun S: Cocaine and amphetamine regulated transcript (CART) and the stress response. Peptides 27: 1956-1969, 2006.
17. Sharma A, Rale A, Utturwar K, Ghose A and Subhedar N: Identification of the CART neuropeptide circuitry processing TMT-induced predator stress. Psychoneuroendocrino 50: 194-208, 2014.
18. Bakhtazad A, Vousooghi N, Garmabi B and Zarrindast MR: Evaluation of CART peptide level in rat plasma and CSF: Possible role as a biomarker in opioid addiction. Peptides 84: 1-6, 2016.
19. Jaworski JN, Vicentic A, Hunter RG, Kimmel HL and Kuhar MJ: CART peptides are modulators of mesolimbic dopamine and psychostimulants. Life Sci 73: 741-747, 2003.
20. Millan EZ and McNally GP: Cocaine- and amphetamine-regulated transcript in the nucleus accumbens shell attenuates context-induced reinstatement of alcohol seeking. Behav Neurosci 126: 690-698, 2012.
21. Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC and Cullinan WE: Central mechanisms of stress integration: Hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. Front Neuroendocrinol 24: 151-180, 2003.
22. Linthorst ACE and Reul JM: Stress and the brain: Solving the puzzle using microdialysis. Pharmacol Biochem Behav 90: 163-173, 2008.
23. Stanley SA, Small CJ, Murphy KG, Rayes E, Abbott CR, Seal LJ, Morgan DG, Sunter D, Dakin CL, Kim MS, *et al*: Actions of cocaine- and amphetamine-regulated transcript (CART) peptide on regulation of appetite and hypothalamo-pituitary axes in vitro and in vivo in male rats. Brain Res 893: 186-194, 2001.
24. Huilin Mou XZXL: NRSF and CCR5 established neuron-glia communication during acute and chronic stresses. Drug Metabol Toxicol 5: 2016.
25. Janet C and Garber RWBJ: Guide for the care and use of lab animals. Washington, DC: National Academy Press, 2010.
26. Zhao H, Huang HW, Wu GC and Cao XD: Effect of orphanin FQ on interleukin-1beta mRNA transcripts in the rat CNS. Neuroscience 114: 1019-1031, 2002.
27. Beckwith SW and Czachowski CL: Alcohol-preferring P rats exhibit elevated motor impulsivity concomitant with operant responding and self-administration of alcohol. Alcohol Clin Exp Res 40: 1100-1110, 2016.
28. Feng XM, Mi WL, Xia F, Mao-Ying QL, Jiang JW, Xiao S, Wang ZF, Wang YQ and Wu GC: Involvement of spinal orexin A in the electroacupuncture analgesia in a rat model of post-laparotomy pain. BMC Complement Altern Med 12: 225, 2012.
29. Dimopoulou I, Tzanela M, Vassiliadi D, Mavrou I, Kopterides P, Orfanos S, Kotanidou A, Kontogiannopoulou S, Vasdekis S, *et al*: Pituitary-adrenal responses following major abdominal surgery. Hormones (Athens) 7: 237-242, 2008.
30. Smith SM, Vaughan JM, Donaldson CJ, Rivier J, Li C, Chen A and Vale WW: Cocaine- and amphetamine-regulated transcript activates the hypothalamic-pituitary-adrenal axis through a corticotropin-releasing factor receptor-dependent mechanism. Endocrinology 145: 5202-5209, 2004.
31. Marco EM, Ballesta JA, Irala C, Hernández MD, Serrano ME, Mela V, López-Gallardo M and Viveros MP: Sex-dependent influence of chronic mild stress (CMS) on voluntary alcohol consumption; study of neurobiological consequences. Pharmacol Biochem Behav 152: 68-80, 2017.

32. Weil ZM, Karelina K, Gaier KR, Corrigan TE and Corrigan JD: Juvenile traumatic brain injury increases alcohol consumption and reward in female mice. J Neurotraum 33: 895-903, 2016.
33. Ostroumov A, Thomas AM, Kimmey BA, Karsch JS, Doyon WM and Dani JA: Stress increases ethanol self-administration via a shift toward excitatory gaba signaling in the ventral tegmental area. Neuron 92: 493-504, 2016.
34. Adams RS, Larson MJ, Corrigan JD, Ritter GA and Williams TV: Traumatic brain injury among U.S. active duty military personnel and negative drinking-related consequences. Subst Use Misuse 48: 821-836, 2013.
35. Koob GF and Le Moal M: Drug addiction, dysregulation of reward, and allostasis. Neuropsychopharmacol 24: 97-129, 2001.
36. Wan Y, Xu J, Meng F, Bao Y, Ge Y, Lobo N, Vizcaychipi MP, Zhang D, Gentleman SM, Maze M and Ma D: Cognitive decline following major surgery is associated with gliosis, β-amyloid accumulation, and τ phosphorylation in old mice. Crit Care Med 38: 2190-2198, 2010.
37. Sperling RE, Gomes SM, Sypek EI, Carey AN and McLaughlin JP: Endogenous kappa-opioid mediation of stress-induced potentiation of ethanol-conditioned place preference and self-administration. Psychopharmacology (Berl) 210: 199-209, 2010.
38. King BJ, Furlong TM and McNally GP: Cocaine and amphetamine related transcript (CART) inhibits context induced reinstatement of reward seeking. Behav Neurosci 124: 423-427, 2010.
39. Kimmel HL, Gong W, Vechia SD, Hunter RG and Kuhar MJ: Intra-ventral tegmental area injection of rat cocaine and amphetamine-regulated transcript peptide 55-102 induces locomotor activity and promotes conditioned place preference. J Pharmacol Exp Ther 294: 784-792, 2000.
40. Cook CJ: Stress induces CRF release in the paraventricular nucleus, and both CRF and GABA release in the amygdala. Physiol Behav 82: 751-762, 2004.
41. Dandekar MP, Singru PS, Kokare DM and Subhedar NK: Transient up-regulation of cocaine- and amphetamine-regulated transcript peptide (CART) immunoreactivity following ethanol withdrawal in rat hypothalamus. Brain Res 1240: 119-131, 2008.
42. Chen YW, Barson JR, Chen A, Hoebel BG and Leibowitz SF: Hypothalamic peptides controlling alcohol intake: Differential effects on microstructure of drinking bouts. Alcohol 48: 657-664, 2014.
43. Balkan B, Gozen O, Yararbas G, Koylu EO, Akinturk S, Kuhar MJ and Pogun S: CART expression in limbic regions of rat brain following forced swim stress: Sex differences. Neuropeptides 40: 185-193, 2006.
44. Stanley SA, Murphy KG, Bewick GA, Kong WM, Opacka-Juffry J, Gardiner JV, Ghatei M, Small CJ and Bloom SR: Regulation of rat pituitary cocaine- and amphetamine-regulated transcript (CART) by CRH and glucocorticoids. Am J Physiol Endocrinol Metab 287: E583-E590, 2004.
45. Galesi FL, Ayanwuyi LO, Mijares MG, Cippitelli A, Cannella N, Ciccocioppo R and Ubaldi M: Role of Hypothalamic-Pituitary-Adrenal axis and corticotropin-releasing factor stress system on cue-induced relapse to alcohol seeking. Eur J Pharmacol 788: 84-89, 2016.
46. Fahlke C, Hård E, Eriksson CJ, Engel JA and Hansen S: Consequence of long-term exposure to corticosterone or dexamethasone on ethanol consumption in the adrenalectomized rat, and the effect of type I and type II corticosteroid receptor antagonists. Psychopharmacology (Berl) 117: 216-224, 1995.
47. Deroche V, Marinelli M, Le Moal M and Piazza PV: Glucocorticoids and behavioral effects of psychostimulants. II: cocaine intravenous self-administration and reinstatement depend on glucocorticoid levels. J Pharmacol Exp Ther 281: 1401-1407, 1997