

Activation of γ -aminobutyric Acid (A) Receptor Protects Hippocampus from Intense Exercise-induced Synapses Damage and Apoptosis in Rats

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Abstract

Background: Our previous study has confirmed that one bout of exhaustion (Ex) can cause hippocampus neurocyte damage, excessive apoptosis, and dysfunction. Its initial reason is intracellular calcium overload in hippocampus triggered by N-methyl-D-aspartic acid receptor (NMDAR) over-activation. NMDAR activation can be suppressed by γ -aminobutyric acid (A) receptor (GABA_AR). Whether GABA_AR can prevent intense exercise-induced hippocampus apoptosis, damage, or dysfunction will be studied in this study.

Methods: According to dose test, rats were randomly divided into control (Con), Ex, muscimol (MUS, 0.1 mg/kg) and bicuculline (BIC, 0.5 mg/kg) groups, then all rats underwent once swimming Ex except ones in Con group only underwent training. Intracellular free calcium concentration ([Ca²⁺]_i) was measured by Fura-2-acetoxymethyl ester; glial fibrillary acidic protein (GFAP) and synaptophysin (SYP) immunofluorescence were also performed; apoptosis were displayed by dUTP nick end labeling (TUNEL) stain; endoplasmic reticulum stress-induced apoptosis pathway was detected by Western blotting analysis; Morris water maze was used to detect learning ability and spatial memory.

Results: The appropriate dose was 0.1 mg/kg for MUS and 0.5 mg/kg for BIC. Ex group showed significantly increased [Ca²⁺]_i and astrogliosis; TUNEL positive cells and levels of GFAP, B cell lymphoma-2 (Bcl-2) associated X protein (Bax), caspase-3, caspase-12 cleavage, CCAAT/enhancer binding protein homologous protein (CHOP), and p-Jun amino-terminal kinase (p-JNK) in Ex group also raised significantly compared to Con group, while SYP, synapse plasticity, and Bcl-2 levels in Ex group were significantly lower than those in Con group. These indexes were back to normal in MUS group. BIC group had the highest levels of [Ca²⁺]_i, astrogliosis, TUNEL positive cell, GFAP, Bax, caspase-3, caspase-12 cleavage, CHOP, and p-JNK, it also gained the lowest SYP, synapse plasticity, and Bcl-2 levels among all groups. Water maze test showed that Ex group had longer escape latency (EL) and less quadrant dwell time than Con group; all indexes between MUS and Con groups had no significant differences; BIC had the longest EL and least quadrant dwell time among all groups.

Conclusions: Activation of GABA_AR could prevent intense exercise-induced synapses damage, excessive apoptosis, and dysfunction of hippocampus.

Key words: Endoplasmic Reticulum Stress-induced Apoptosis; γ -aminobutyric Acid (A) Receptor; Hippocampus; Intense Exercise; Synapse Plasticity

INTRODUCTION

Intense exercise can cause excessive apoptosis in a variety of tissues, for instance, renal tubular cell^[1] and lymphocyte.^[2] Recently, exercise-induced central nervous system (CNS) apoptosis and dysfunction had been focused with plenty of interest. The first study regarding exercise-induced neuron apoptosis was reported in 2011, which indicated that exercise can activate apoptosis pathway in hippocampus 24 h after exercise.^[3] A study from Japan also indicated

severe exercise can cause morphological apoptosis in hippocampus, but not in mild exercise.^[4] What's more,

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a cohort research confirmed exercise-induced CNS dysfunction, which declared the explicit memory of marathon runner was impaired (hippocampus dysfunction) immediately on completing this extreme exercise.^[5] Our previous study had proved one bout of swimming exhaustion (Ex) can cause notable apoptosis and synapse damage in rat hippocampus, which may lead to impaired learning and memory ability.^[6]

The initial reason for the intense exercise-induced hippocampus injury and apoptosis is Ca^{2+} overload. Intense exercise causes glutamate (main excitatory transmitter) accumulating in synaptic clefts, and activates postsynaptic ion type glutamate receptor – N-methyl-D-aspartic acid receptor (NMDAR), which has high permeability to Ca^{2+} .^[7] This above pathological process is called excitotoxicity of glutamate, which is considered as the major mechanism for CNS injury and apoptosis.^[8] The earliest apoptotic pathway is endoplasmic reticulum stress (ERS)-induced apoptosis pathway. It is a newly defined pathway happened before the mitochondria apoptosis pathways, and it is distinct from it. A study from Japan found that severe exercise group showed hippocampus apoptosis together with activated ERS pathway.^[4] In our previous study, we also confirmed once Ex can cause hippocampus excessive apoptosis though ERS-induced apoptosis pathway activated by intracellular calcium overload.^[6]

γ -aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the CNS, it can antagonize excess excitement of neurons caused by NMDAR hyperactivation, thus GABA-energetic system plays an important role in CNS homeostasis, this function of GABA is mainly mediated through GABA (A) receptors ($GABA_A R$) – a ligand-gated Cl^- channel. By activation of $GABA_A R$ situated in postsynaptic membranes, the influx of Cl^- flows into cells along electrochemical gradients, which can subsequently induce hyperpolarization and suppress the release of excitatory neurotransmitters. Hence, activation of $GABA_A R$ can reduce calcium overload caused by glutamate-induced excitotoxicity on many CNS pathological process. According to literature, $GABA_A R$ selective agonist muscimol (MUS) can inhibit NMDAR-induced neurotoxicity in primary cell cultures,^[9] it can also reduce cell death in global or focal cerebral ischemia.^[10,11]

Hippocampus belongs to the limbic system that is particularly important for learning, forming new memories, and spatial navigation. Apoptosis is a programmed cell death, which can be partially reversed at early stage of cell death process, but necrosis occurs late and is irreversible. In this study, we studied whether activation of $GABA_A R$ can protect hippocampus from intense exercise-induced damage, excessive apoptosis, and dysfunction through decreasing intracellular free calcium concentration ($[Ca^{2+}]_i$) and inhibiting ERS-induced apoptosis pathway.

Bicuculline (BIC) is a selective antagonist of $GABA_A R$, which can inhibit the influence of chloride ions. We chose MUS as the selective agonist and BIC as the antagonist of $GABA_A R$. Glial fibrillary acidic protein (GFAP) and synaptophysin (SYP) were selected as the marker for estimating hippocampus injury and function. GFAP is a predominantly expressed marker of astrocytes. GFAP expression increases during astrocytes activation, which is an early marker of CNS damages. SYP is a major integral transmembrane protein of synaptic vesicles and is also a marker for showing and quantification of synapses. SYP is related to synaptic plasticity,^[12] so it serves as a functional marker of the brain.^[13] In our previous study, we found that one bout of swimming Ex can lead to astrocytes activation and impaired synaptic plasticity.^[6] So whether activation of $GABA_A R$ can improve astrocytes activation and protect synaptic plasticity was studied in this study. Behavior was tested in Morris water maze (MWM), given the abundant literature on its relationship to spatial learning and hippocampus function.

The number of intensely exercised athletes and exercisers is huge. Overtraining syndrome (OTS) is a universal phenomenon caused by intense exercise, it can occur at least once in an athlete's career. OTS symptoms such as insomnia, amnesia, depression, and bad mood can last several days to weeks. All of the OTS symptoms are about hippocampus dysfunction. Hitherto, a study on exercise-induced CNS apoptosis and dysfunction is very limited, let alone the therapeutic intervention. This study first studied therapeutic intervention for intense exercise-induced hippocampus damage and dysfunction. Thus, this study was very significant to maintaining brain health for athletes and exercising people, and may provide methods and directions for subsequent studies.

METHODS

Animals

One hundred and twenty male Sprague-Dawley rats (8 weeks; 300 ± 20 g) were obtained from Medical Experimental Animal Center of Peking University. The rats were adopted to the laboratory environment ($22 \pm 1^\circ C$, relative humidity $55 \pm 3\%$) and allowed free access to food and water under a 12:12 h light-dark cycle. All experimental procedures were carried out according to the *National Institutes of Health Guide for the Care and Use of Laboratory Animals*. Our study was approved by the Ethics Committee for Laboratory Animal Care of the Health Science Centre of Peking University.

Swimming protocol

All animals were adopted for 3 days, and then subjected to adaptive training consisting of four days (15 min/d with ascending overload weights from 0% to 3% body weight) at the base of tails. Animals swam in transparent Plexiglas Tank (80 cm in tall, 50 cm in diameter, filled with water ($31 \pm 1^\circ C$) to a depth of 60 cm). Each group

was subject to one bout of swimming Ex with 3% body weight. Control (Con) group only underwent training, but not swimming Ex. Ex is determined by continuously three times submerged and beyond 10 s each time, and cannot hang itself by limbs.^[14]

Dose test of drugs

Dose and grouping

The dose of MUS and BIC must not affect animal exercise ability. The doses we referred in this experiment cannot affect physical ability, according to the literature.^[15-17] After swimming adaptation, 80 rats were randomly divided into 8 groups ($n = 10$ in each group): Low dose of MUS group (L_{MUS} , 0.05 mg/kg^[15]), middle dose of MUS group (M_{MUS} , 0.1 mg/kg^[15]), high dose of MUS group (H_{MUS} , 0.2 mg/kg), and low dose of BIC group (L_{BIC} , 0.25 mg/kg^[16,17]), middle dose of BIC group (M_{BIC} , 0.5 mg/kg^[15,16]), and high dose of BIC group (H_{BIC} , 1 mg/kg^[17]) together with Ex and Con groups. MUS and BIC were dissolved in saline. All the drugs were administered intraperitoneal (i.p.) injection in same volume 30 min before swimming Ex. Ex group was given saline i.p. on the same volume. The time from beginning to Ex was recorded.

Assay of plasma and tissue indexes

Rats (8 groups, $n = 10$) were immediately deeply anesthetized with 50 mg/kg pentobarbital sodium i.p. after Ex. Blood was taken from the aorta abdominalis into chilled heparinized (1 mg) tube. The blood samples were centrifuged at 4°C, 1000 × g for 15 min, and plasmas were collected and stored at -80°C. The rectus femoris of the right leg was taken and frozen in liquid nitrogen (LN) quickly, then stored at -80°C. Muscle from each animal was cut into small pieces and diluted 1:10 (w/v) in ice-cold 0.86% saline. The muscles were mechanically homogenized with a polytron homogenizer (ULTRA TURRAX IKA T18 basic, USA) at 6000 r/min for 3 × 30 s bursts, and separated by 2 × 30 s breaks. The homogenates were centrifuged for 15 min at 4°C, 1000 × g , liquid supernatant obtained from each sample was rapidly frozen in LN and stored at -80°C.

Plasma and muscle total-superoxide dismutase (T-SOD), maleic dialdehyde (MDA), plasma lactate, muscle creatine kinase (CK) and glycogen were tested by spectrophotometry. Plasma and muscle T-SOD activity was detected with SOD assay reagent kit (Keygen, KGT00100-1, Nanjing, China) using xanthine oxidase assay method and expressed as U/ml in plasma, while U/mg protein in muscle tissue. Concentration of MDA was determined by MDA assay reagent kit (Keygen, KGT003, Nanjing, China) through thiobarbituric acid method and expressed as nmol/ml in plasma, while nmol/mg protein in muscle. Plasma CK activity was detected using CK assay reagent kit (Keygen, KGT035, Nanjing, China) by enzymology method and expressed as U/ml. Plasma lactate content was measured with lactic acid assay kit (Keygen, KGT023, Nanjing, China) and expressed as mmol/L. Muscle glycogen was determined by glycogen

assay reagent kit (Keygen, KGT568, Nanjing, China) and expressed as mg/g tissue.

Measurement of the intracellular free calcium concentration ($[Ca^{2+}]_i$)

Based on the previous dose test, we had chosen the most appropriate drug dose (did not affect exercise ability). From now on, we randomly divided the other 40 rats into 4 groups: MUS, BIC, Ex, and Con groups ($n = 10$). After Ex, rats were wipe-dried and housed for 24-h then were anesthetized with pentobarbital sodium i.p. Hippocampus was taken and mechanically dissociated passing through nylon mesh (250 μ m, 136 μ m). $[Ca^{2+}]_i$ was determined as described previously.^[18] Cells were collected and incubated with 5 μ mol/L Fura-2-acetoxymethyl ester in complete medium containing at 37°C for 45 min, then washed twice and suspended with cold phosphate buffered saline (PBS) containing 0.2% bovine serum albumin and incubated for another 5 min at 37°C. $[Ca^{2+}]_i$ was determined by excitation wavelengths at 340/380 nm and emission wavelength at 510 nm by fluorescence spectrophotometer (F-4500, Hitachi, Japan).

Immunofluorescence of glial fibrillary acidic protein and synaptophysin

Rats (4 groups, $n = 10$) were sacrificed 24-h after Ex with anesthesia then were perfused transcardially with 2% heparin for 2 min followed by ice-cold 4% paraformaldehyde (PFA) (in PBS, pH 7.2-7.4) for 13 min.^[19] Brain was removed on ice and postfixed in 4% PFA at 4°C overnight; then hippocampus was cryoprotected in 30% sucrose for 48 h. Hippocampus was embedded and frozen in LN calmly. Tissues were sectioned with freezing sliding microtome (Leica CM3050S, Germany) in 20- μ m coronal sections. Serial sections were made.

Sections were incubated with specific GFAP and SYP rabbit polyclonal antibody (1:100, Santa Cruz Biotechnology, sc-6171-R, sc-9116, USA) at 4°C overnight. Donkey anti-rabbit IgG FITC secondary antibody (1:200, Abcam, ab6798, Hong Kong, China) was applied at room temperature (RT) for 60 min. Sections were visualized using epifluorescence microscope (DM300, Leica, Germany).

Terminal dUTP nick end labeling stain

Terminal dUTP nick end labeling (TUNEL) stain was performed with Roche *in situ* death detection kit (Roche, 11684817910, Mannheim, Germany) according to the direction. Diaminobenzidine substrate was used to display the apoptosis cell. TUNEL-positive cells were counted under light microscope (Nikon E600, Japan). Hippocampus has two main parts: Ammon's horn (CA) and the dentate gyrus (DG) regions, CA includes CA1, CA2, and CA3 regions. CA1, CA3, and DG regions are extremely important to hippocampus function. A grid on eyepiece composed of 20 × 20 μ m² was positioned over those hippocampus regions (200-fold), and apoptotic cells were counted in the frames. The number of apoptotic cells in each region

was normalized with the region area according to squares number,^[20] and expressed as number/mm².

Western blotting for endoplasmic reticulum stress-induced apoptosis pathway

Rats (4 groups, $n = 10$) were sacrificed 24-h after Ex. Hippocampus was separated on ice and frozen in LN quickly. Hippocampus was diluted 1:10 (w/v) with ice-cold RIPA Lysis Buffer (50 mmol/L Tris-HCl, pH = 7.4, 150 mmol/L NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS]) with 1% phosphatase inhibitors mixture (Applygen Technologies Inc., P1260, China) and protease inhibitor (Roche, No. 04693159001, Mannheim, Germany). Protein concentration was determined by the BCA method (Pierce BCA Protein Assay Kit, 23227, Thermo, USA). Aliquots containing 30 μ g total protein were loaded and separated by 12% SDS-polyacrylamide gel electrophoresis, then transferred to nitrocellulose membranes. The membranes were blocked and incubated at 4°C overnight with specific primary antibody against caspase-3, B cell lymphoma-2 (Bcl-2) associated X protein (Bax), Bcl-2, CCAAT/enhancer binding protein homologous protein (CHOP), caspase-12, and p-Jun amino-terminal kinase (p-JNK). Goat anti-mouse and anti-rabbit IgG secondary antibodies were used for incubating for 1 h at RT. Blots were visualized and quantified by Odyssey system (LI-COR, USA). All values were normalized to β -actin. GFAP level and SYP level were also measured by Western blotting.

CHOP, p-JNK, and β -actin were from Cell Signaling Technology Company (9665, 2772, 2895, 9255, 3700, 1:1000). Caspase-12 and Bcl-2 were from Santa Cruz Biotechnology Company (sc-5627, sc-7382, 1:200). Goat anti-mouse and anti-rabbit IG secondary antibodies were from LI-COR (IRDye IM, 926-32210, 926-32211, 1:10,000).

Morris water maze test

After Ex, rats (4 groups, $n = 10$) were rested for 5 min then subjected to water maze test. The water maze was consisted of a dark circular pool (diameter 200 cm) filled with

water ($25 \pm 1^\circ\text{C}$) to a depth of 50 cm and divided into four equivalent quadrants, including north-east, north-west (NW), south-east (SE), and south-west. A transparent plexiglas platform (diameter 11 cm) was located 1 cm below the water surface in the center of the designated quadrant (SE). Behavioral testing was performed in MWM.^[21] In each training session of navigation test, the rat was put into water randomly at one of four starting positions. Latency to escape onto the hidden platform was recorded. Each animal underwent 3 continuous trainings every day for 3 days. If the rat was unable to find the platform in 120 s, we placed it on the platform for 30 s and then performed another. Retention testing (probe trial) was performed 24 h after the final training. In probe trial, the hidden platform was removed, and the rat was placed on the NW quadrant and allowed to swim for 60 s. The percentage of time spent in the quadrant of the target platform was calculated.

Statistical analysis

Quantitative data were expressed as mean \pm standard error (SE), with n denoting the rat number of each experiment. Quantitative data were tested by the *post-hoc* least-significant difference or Student-Newman-Keuls test, Tamhane or Dunnett T3 test were practiced when equal variances were not assumed. Differences were considered statistically significant if P value was <0.05 . All statistical tests were performed with SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) for windows.

RESULTS

Physiological and biochemical indexes for assessment of exercise ability

Only the H_{MUS} and H_{BIC} groups had significantly different indexes compared with Ex group: Muscle glycogen elevated in H_{MUS} group (Table 1, $n = 10$ in each group, $P < 0.05$), but lessened in H_{BIC} group (Table 2, $n = 10$ in each group, $P < 0.05$), and plasma T-SOD in H_{BIC} group also lessened (Table 2, $P < 0.05$). So we chose the middle doses 0.1 mg/kg for MUS and 0.5 mg/kg for BIC in the following experiments.

Table 1: Body weight, exercise time, and biochemical indexes in three MUS dose groups, Con, and Ex groups

Items	L_{MUS} group	M_{MUS} group	H_{MUS} group	Con group	Ex group
Body weight (g)	286.80 \pm 17.20	290.00 \pm 16.90	287.50 \pm 17.53	285.10 \pm 16.82	287.13 \pm 17.12
Exercise time (min)	160 \pm 17	156 \pm 7	171 \pm 18	—	165 \pm 16
Plasma T-SOD (U/ml)	0.53 \pm 0.19 [†]	0.51 \pm 0.13 [†]	0.48 \pm 0.11 [†]	0.82 \pm 0.01*	0.50 \pm 0.05 [†]
Plasma MDA (nmol/ml)	9.31 \pm 2.96 [†]	7.66 \pm 2.08 [†]	9.47 \pm 2.57 [†]	2.53 \pm 1.07*	7.53 \pm 2.02 [†]
Plasma CK (U/ml)	1.19 \pm 0.52 [†]	1.48 \pm 0.44 [†]	1.71 \pm 0.57 [†]	0.41 \pm 0.13*	1.39 \pm 0.60 [†]
Plasma lactate (mmol/L)	15.66 \pm 3.05 [†]	15.10 \pm 3.75 [†]	15.09 \pm 4.15 [†]	4.09 \pm 0.87*	15.09 \pm 4.07 [†]
Muscle MDA (nmol/mg)	6.83 \pm 1.65 [†]	6.92 \pm 1.15 [†]	7.43 \pm 2.07 [†]	2.63 \pm 1.02*	7.53 \pm 1.02 [†]
Muscle T-SOD (U/mg)	0.93 \pm 0.11 [†]	0.91 \pm 0.08 [†]	0.98 \pm 0.12 [†]	1.50 \pm 0.26*	0.90 \pm 0.06 [†]
Muscle glycogen (mg/g)	0.76 \pm 0.04 [†]	0.86 \pm 0.07 [†]	1.78 \pm 0.05* [†]	3.04 \pm 0.21*	0.94 \pm 0.13 [†]

* $P < 0.05$, compared with Ex group; [†] $P < 0.05$, compared with Con group. Con group did not undergo exhaustive swimming; $n = 10$ in each group.

MUS: Muscimol; L_{MUS} group: Low dose of MUS group (0.05 mg/kg); M_{MUS} group: Middle dose of MUS group (0.1 mg/kg); H_{MUS} group: High dose of MUS group (0.2 mg/kg); Con group: Control group; Ex group: Exhaustion group; T-SOD: Total-superoxide dismutase; MDA: Maleic dialdehyde; CK: Creatine kinase.

Table 2: Body weight, exercise time, and biochemical indexes in three BIC dose groups, Con, and Ex groups

Items	L _{BIC} group	M _{BIC} group	H _{BIC} group	Con group	Ex group
Body weight (g)	288.75 ± 17.27	286.80 ± 17.10	280 ± 17.10	285.10 ± 16.82	287.13 ± 17.12
Exercise time (min)	155.62 ± 11.36	172.12 ± 27.23	166.75 ± 21.72	–	165.12 ± 16.26
Plasma T-SOD (U/ml)	0.51 ± 0.14 [†]	0.48 ± 0.08 [†]	0.23 ± 0.07 ^{†,*}	0.82 ± 0.01 [*]	0.50 ± 0.05 [†]
Plasma MDA (nmol/ml)	9.46 ± 3.88 [†]	8.17 ± 2.34 [†]	7.61 ± 2.6 [†]	2.53 ± 1.07 [*]	7.53 ± 2.02 [†]
Plasma CK (U/ml)	1.14 ± 0.61 [†]	1.64 ± 0.28 [†]	1.41 ± 0.37 [†]	0.41 ± 0.13 [*]	1.39 ± 0.60 [†]
Plasma lactate (mmol/L)	14.98 ± 3.65 [†]	14.49 ± 3.38 [†]	16.24 ± 2.39 [†]	4.09 ± 0.87 [*]	15.09 ± 4.07 [†]
Muscle MDA (nmol/mg)	6.07 ± 0.69 [†]	7.14 ± 1.75 [†]	6.89 ± 1.82 [†]	2.63 ± 1.02 [*]	7.53 ± 1.02 [†]
Muscle T-SOD (U/mg)	0.91 ± 0.07 [†]	0.88 ± 0.10 [†]	0.93 ± 0.08 [†]	1.50 ± 0.26 [*]	0.90 ± 0.06 [†]
Muscle glycogen (mg/g)	0.84 ± 0.06 [†]	0.95 ± 0.09 [†]	0.51 ± 0.14 ^{*,†}	3.04 ± 0.21 [*]	0.94 ± 0.13 [†]

**P* < 0.05, compared with Ex group; [†]*P* < 0.05, compared with Con group. Con group did not undergo exhaustive swimming; *n* = 10 in each group. BIC: Bicuculline; L_{BIC} group: Low dose of BIC group (0.25 mg/kg); M_{BIC} group: Middle dose of BIC group (0.5 mg/kg); H_{BIC} group: High dose of BIC group (1 mg/kg); Con group: Control group; Ex group: Exhaustion group; T-SOD: Total-superoxide dismutase; MDA: Maleic dialdehyde; CK: Creatine kinase.

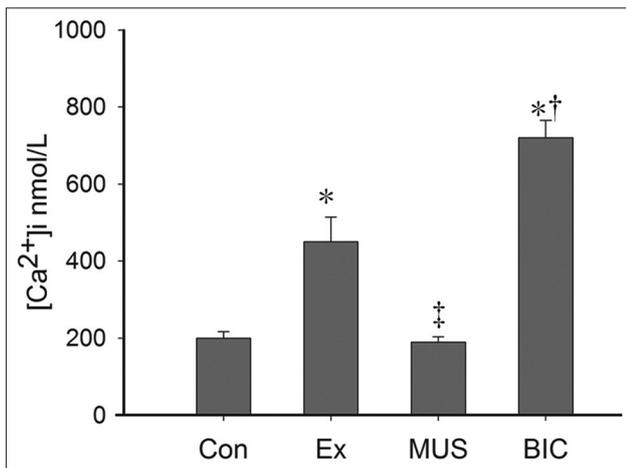


Figure 1: Intracellular free calcium concentration ([Ca²⁺]_i) of each group 24-h after one bout of swimming exhaustion. **P* < 0.01 compared with Con group, [†]*P* < 0.05, [†]*P* < 0.01 compared with Ex group. Con: Control; Ex: Exhaustion; MUS: Muscimol; BIC: Bicuculline.

Intracellular free calcium concentration ([Ca²⁺]_i) in hippocampus

[Ca²⁺]_i in Ex group increased 127% compared with Con group (Figure 1, *n* = 10 in each group, *P* < 0.01). [Ca²⁺]_i in MUS group decreased in contrast with Ex group (Figure 1, *P* < 0.01) but was not significantly different with Con group. BIC group had the highest [Ca²⁺]_i among all groups, which raised 65% compared with Ex group (Figure 1, *P* < 0.05).

Astrocytes activation level in hippocampus

Con group presented very weak GFAP immunofluorescence signal [Figure 2a and 2b], while Ex group showed an increased GFAP immunofluorescence signal and notable astrogliosis (enlarged cell bodies and thick processes) in CA1 and DG regions [Figure 2c and 2d]. GFAP immunofluorescence level seemed to recover to normal state in MUS group [Figure 2e and 2f]. BIC group showed the highest level of astrocytes activation among four groups [Figure 2g and 2h]. Western blotting showed that compared to Con group (3.47 ± 0.59), GFAP protein level elevated 33% in Ex group (4.61 ± 0.8) (Figure 2i, *n* = 10 in

each group, *P* < 0.05), while it down-regulated (3.12 ± 0.4) in MUS group without significant difference. BIC group had the highest GFAP level (6.34 ± 1.02) among four groups, which increased 38% compared with Ex group (*P* < 0.01).

Synaptophysin immunofluorescence and expression level in hippocampus

Con group had a typical SYP immunoreactivity, which was shown as punctate-like or bouton-like distribution pattern with high immunofluorescence signal [Figure 3a and 3b]. Ex group showed weak immunofluorescence signal with less typical distribution pattern in CA3 and DG regions [Figure 3c and 3d]. SYP immunoreactivity in MUS group had recovered and tended to be normal [Figure 3e and 3f]; while in BIC group, it gained the weakest immunofluorescence signal with least typical distribution pattern of SYP among all groups [Figure 3g and 3h]. Western blotting showed that SYP protein level decreased 45% in Ex group (2.05 ± 0.36) compared with Con group (3.74 ± 0.71) (Figure 3i, *n* = 10 in each group, *P* < 0.01), while SYP in MUS group (3.48 ± 0.69) raised without statistical difference between Con group. BIC group gained the lowest SYP level (1.48 ± 0.32) among four groups, which down-regulated 28% (*P* < 0.01) in contrast with Ex group.

Apoptosis cell count in hippocampus

Quantified areas were 16,000 μm² in DG, 12,000 μm² in CA1, and 12,000 μm² in CA3. No obvious apoptosis was observed in Con group [Figure 4a, 4e, and 4i]. Ex group had a notably increased number of TUNEL-positive cells in all regions [Figure 4b, 4f, and 4j]. In MUS group, only physiological apoptosis can be observed [Figure 4c, g, and k]; however, BIC group had the largest amount of TUNEL-positive cells [Figure 4d, 4h, and 4l], especially in DG region. Cell count also indicated that DG region had the most apoptosis in this experiment, TUNEL-positive cells in this region increased 803% in Ex group (235 ± 31/mm²) by comparison with Con group (26 ± 4/mm²) (Figure 4m, *n* = 10 in each group, *P* < 0.001), while it lessened to Con level in MUS group, and significantly raised 58% in BIC group (372 ± 62/mm²) comparing to Ex group (Figure 4m, *P* < 0.05).

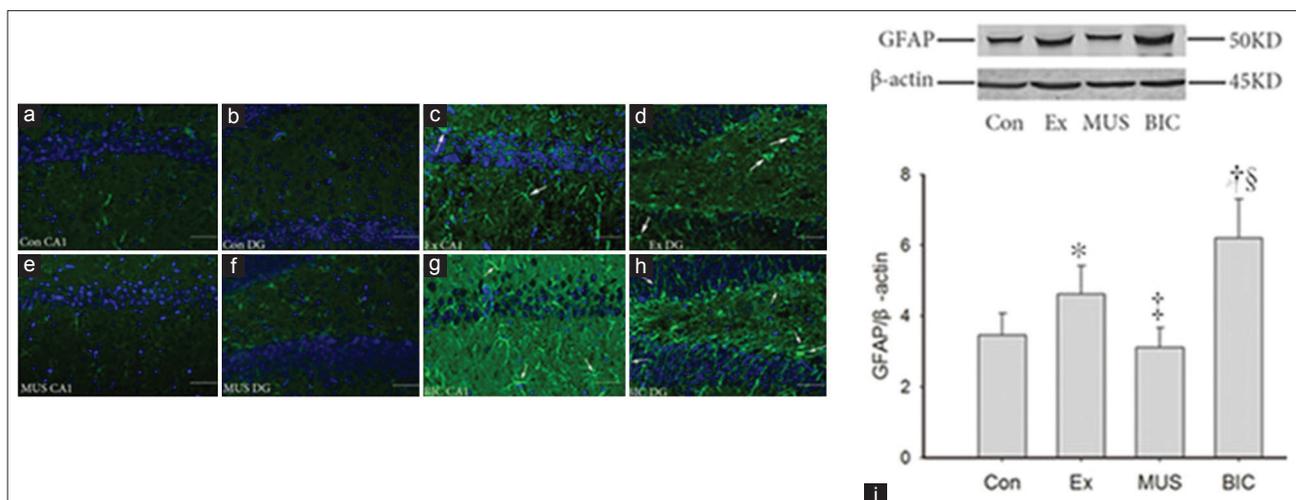


Figure 2: Glial fibrillary acidic protein immunofluorescence in hippocampus of each group 24-h after once exhaustion. Scale bar: 50 μ m. (a and b) Ammon's horn 1 and dentate gyrus regions of control group. (c and d) Ammon's horn 1 and dentate gyrus regions of exhaustion group, the arrows displayed enlarged cell bodies and thick processes of astrogliosis. (e and f) Ammon's horn 1 and dentate gyrus regions of muscimol group. (g and h) Ammon's horn 1 and dentate gyrus regions of bicuculline group, the arrows showed astrogliosis and enlarged cell bodies with thick processes. (i) Western blotting analysis showed glial fibrillary acidic protein levels. * $P < 0.05$, † $P < 0.01$ compared with control group, ‡ $P < 0.05$, § $P < 0.01$ compared with exhaustion group.

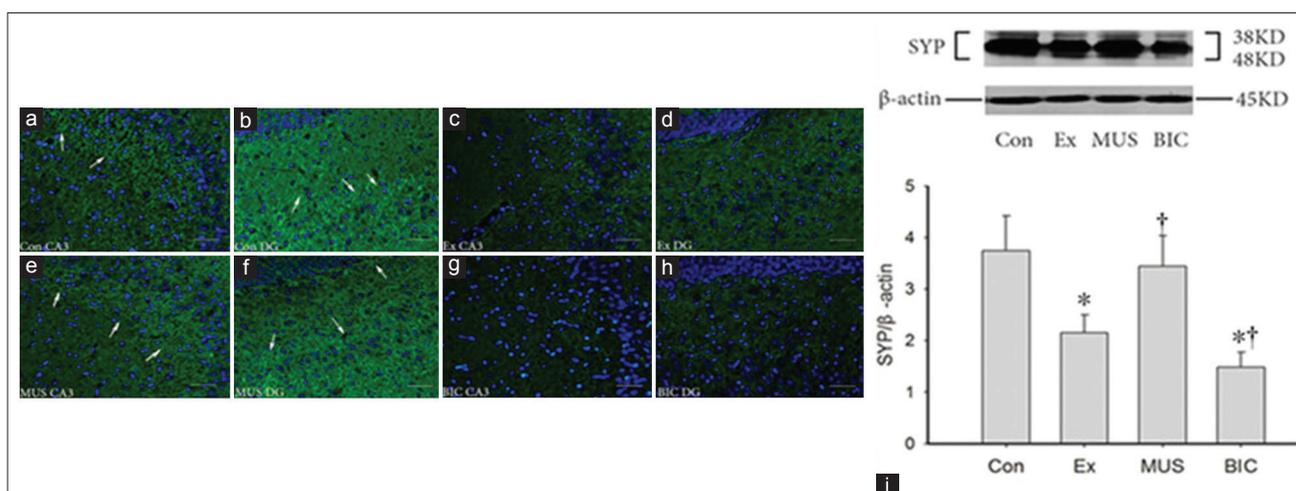


Figure 3: Synaptophysin immunofluorescence 24-h after once exhaustion on hippocampus of each group. Scale bar: 50 μ m. (a and b) synaptophysin in Ammon's horn 3 and dentate gyrus regions of control group, the typical punctate-like or bouton-like distribution pattern was shown by arrows. (c and d) Ammon's horn 3 and dentate gyrus regions of exhaustion group. (e and f) Ammon's horn 3 and dentate gyrus regions of exhaustion group of muscimol group. (g and h) Ammon's horn 3 and dentate gyrus regions of bicuculline group. (i) Western blotting analysis showed synaptophysin protein level in four groups. * $P < 0.01$ compared with control group; † $P < 0.01$ compared with exhaustion group.

Western blotting for endoplasmic reticulum stress-induced apoptosis pathway

In Ex group, the levels of Bax, caspase-3 and caspase-12 cleavage, CHOP, and p-JNK, increased 44% (Figure 5a, $n = 10$ in each group, $P < 0.05$), 84% (Figure 5c, $P < 0.01$), 331% (Figure 5d, $P < 0.001$), 165% (Figure 5e, $P < 0.01$), 82% (phospho-p46, Figure 5f, $P < 0.01$), and 98% (phospho-p54, Figure 5g, $P < 0.001$), respectively compared with Con group. All of the apoptotic proteins significantly down-regulated to Con level in MUS group. All of apoptotic proteins raised higher in BIC group compared to Ex group: 78% on Bax (Figure 5a, $P < 0.01$), 52% on caspase-3 cleavage (Figure 5c, $P < 0.01$), 73% on caspase-12

cleavage (Figure 5d, $P < 0.001$), 49% on CHOP (Figure 5e, $P < 0.01$), 37% on phospho-p46 (Figure 5f, $P < 0.05$), and 30% on phospho-p54 (Figure 5g, $P < 0.05$).

Bcl-2 in Ex group decreased 40% comparing to Con group (Figure 5b, $P < 0.01$), while it raised in MUS group without significant difference between Con group. BIC group had the lowest Bcl-2 level, which lessen 18% in contrast with Ex group ($P < 0.05$).

Behavior in water maze test

Escape latency (EL) from long to short 5 min after Ex (Figure 6a, $n = 10$ in each group) was: BIC group ($P < 0.05$, compared with Ex group; $P < 0.01$, compared with Con

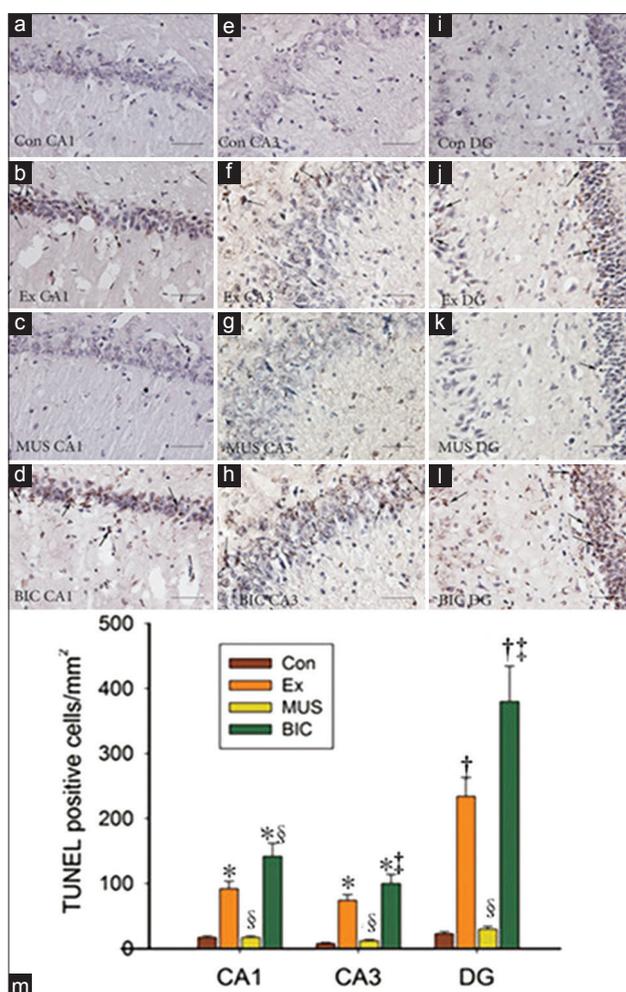


Figure 4: dUTP nick end labeling stain of hippocampus in each group 24-h after once exhaustion. Scale bar: 50 μm . (a, e, i) Ammon's horn 1, Ammon's horn 3, and dentate gyrus regions in hippocampus of control group. (b, f, j) dUTP nick end labeling positive cells in Ammon's horn 1, Ammon's horn 3, dentate gyrus regions as arrows showing in exhaustion group. (c, g, k) Muscimol group only had physiological apoptosis as arrows showing. (d, h, l) Ammon's horn 1, Ammon's horn 3, and dentate gyrus regions in bicuculline group. (m) Numbers of dUTP nick end labeling positive cells in different regions of hippocampus in each group. * $P < 0.01$, † $P < 0.001$ compared with control group; ‡ $P < 0.05$, § $P < 0.01$ compared with exhaustion group.

group), Ex group ($P < 0.01$, compared with Con group), MUS group ($P < 0.05$, compared with Ex group; $P < 0.05$, compared with Con group), Con group. EL tended to shorten in the next two days. From day 1, EL in MUS group tended to lessen and finally had no difference with Con group (Figure 6a, $P > 0.05$). Ex group still gained a longer EL on day 1 in contrast with Con group, however, on day 2, there was no significant difference between Con and Ex groups (Figure 6a; $P < 0.05$ for day 1, $P > 0.05$ for day 2). EL in BIC group was always longer on 3 days. Finally, only EL in BIC group did not reach the Con level on day 2 (Figure 6a; $P < 0.05$, compared with Ex group).

Time spent in the target quadrant decreased 22% (Figure 6b, $n = 10$ in each group, $P < 0.05$) in Ex group by comparison to Con group, while it raised to Con state in MUS group.

BIC group had the lowest percentage of quadrant time, which diminished 31% compared to Ex group (Figure 6b, $P < 0.05$).

DISCUSSION

Although gentle or modest exercise is beneficial to health, intense or prolong exercise is considered to be harmful to body. For example, intense exercise can cause damage and apoptosis in lymphocyte^[2] and renal tubular cell.^[1] CNS is a new-found target of exercise-induced damage and apoptosis. Nowadays, intense exercise-induced CNS damage, apoptosis, and dysfunction have attracted many scientists' interests. Our previous study had proved that once Ex can lead to significant apoptosis and synapse damage in rat hippocampus.^[6]

The initial reason for intense exercise-induced CNS damage and apoptosis is over-activation of NMDAR and Ca^{2+} overload (excitotoxicity of glutamate). NMDAR locates in the most "fast" excitatory synapses in CNS,^[22] it is privileged in regulating postsynaptic Ca^{2+} entry, which can trigger both normal and pathophysiological intracellular signal.^[23] Our results showed that $[\text{Ca}^{2+}]_i$ raised 127% 24-h after once Ex, which corresponded with our previous result. This was because hippocampus is rich in NMDAR, so it is actually very vulnerable to intense exercise-induced excitotoxicity and Ca^{2+} overload. Ca^{2+} overload is particularly neurotoxic, resulting in cell injury and apoptosis.^[24] GABA_A is a ligand-gated Cl^- channel. By activating GABA_A , the influx of Cl^- flows into the cell along their electrochemical gradients, which can subsequently induce hyperpolarization and suppress excitatory neurotransmitter release, hence reduces Ca^{2+} overload during glutamate-induced excitotoxicity. We found using MUS i.p. before Ex can effectively diminish $[\text{Ca}^{2+}]_i$ in hippocampus while using BIC can raise $[\text{Ca}^{2+}]_i$. It revealed that activation of GABA_A can effectively protect hippocampus from intense exercise-induced Ca^{2+} overload.

$[\text{Ca}^{2+}]_i$ overload is also a trigger for astrocytes activation,^[25] which is regarded as an early marker of CNS damage. Astrocytes activation is consisted of GFAP up-regulation and astrogliosis (hypertrophy of astrocyte with enlarged cell bodies and thick processes). Our results showed that astrocytes were activated notably in CA1 and DG regions of hippocampus within 24-h after once Ex, which was corresponding to our previous result. MUS group regained normal state of astrocytes, but using BIC made the level of astrocytes activation much severe than Ex group. Hence, activation of GABA_A can protect hippocampus from astrocytes activation of early stage damage-induced by intense exercise through inhibiting Ca^{2+} overload.

SYP plays an important role in synapses formation and stabilization,^[26] it is considered as a marker of synaptic plasticity^[12] and a functional index of the CNS.^[13] We found once Ex can significantly cause collapsed synapses plasticity in CA3 and DG regions, which was corresponding to our previous study. SYP in MUS group had recovered

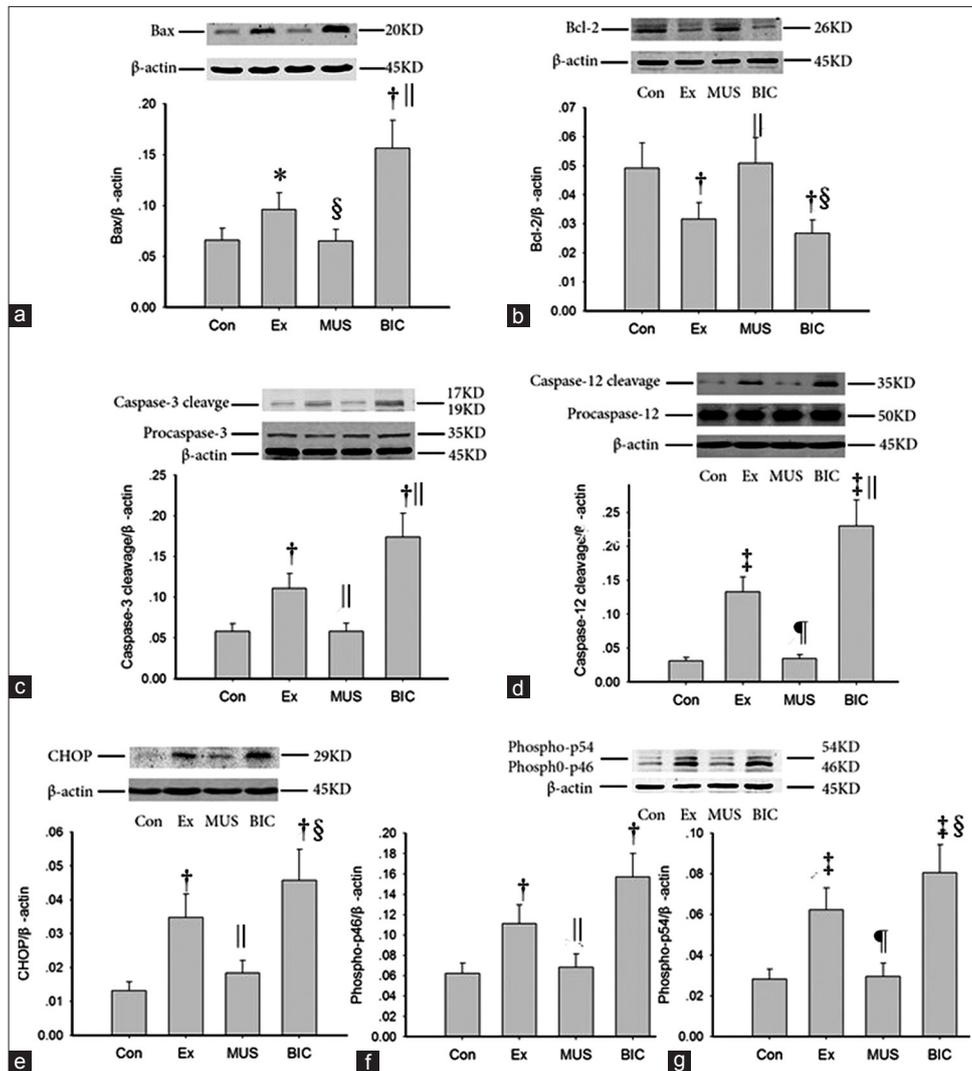


Figure 5: Western blotting of apoptotic pathway in hippocampus 24-h later after one bout of swimming exhaustion in four groups. (a-g) Protein levels of B cell lymphoma-2 associated X protein, B cell lymphoma-2, caspase-3 cleavage, caspase-12 cleavage, CCAAT/enhancer binding protein homologous protein, phospho-p46 and phospho-p54 of Jun amino-terminal kinase in each group were shown correspondingly. * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$ compared with control group; § $P < 0.05$, ¶ $P < 0.01$, § $P < 0.001$ compared with exhaustion group.

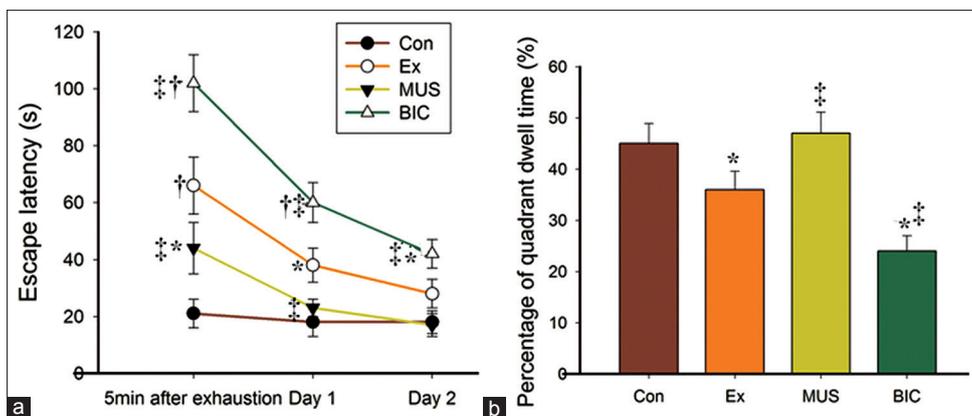


Figure 6: Morris water maze test for each group. (a) Escape latency at three different days. (b) The percentage of time spent in the target quadrant in probe trial. * $P < 0.05$, † $P < 0.01$ compared with control group; § $P < 0.05$ compared with exhaustion group.

to the normal state while in BIC group synaptic plasticity collapsed even worse than Ex group. It revealed activation of

GABA_A could protect synaptic plasticity of hippocampus from intense exercise-induced damage.

Aberrant apoptosis in CNS is related to many diseases, for example, Parkinson disease, and Alzheimer's disease. In this study, once Ex can cause numerous apoptotic cells in CA1, CA3, and DG regions, and activate the ERS-induced apoptosis pathway (CHOP, p-JNK, caspase-12) and the common pathway (Bax and Bcl-2, caspase-3), this result corresponded with our previous study.^[6] These results can also be supported by a Japanese study, which indicated severe exercise can cause morphological apoptosis in hippocampus with activated ER-stress pathway, but not in mild exercise.^[4] In this experiment, the number of apoptotic cells was much higher beyond the physiological level together with activated apoptosis pathway, so we thought this kind of apoptosis-induced by intense exercise was excessive and aberrant, it may relate to some CNS dysfunction and diseases. As apoptosis can be partially reversible at early stage of cell death, and ER stress-induced apoptosis is the earliest activated pathway. We were trying to find a medical intervention that can effectively inhibit ERS-induced apoptosis pathway after intense exercise. In this study, we found using MUS before Ex can effectively inhibit the activation of CHOP, p-JNK, and caspase-12 of ERS-induced apoptosis pathway, together with Bax and caspase-3 in common pathway, besides, Bcl-2 in MUS group had increased to the normal level. Apoptotic cells in all regions in MUS group had diminished and recovered to the normal state. But in BIC group, ERS-induced apoptosis pathway had been activated even more intensely than Ex group, and it gained the most apoptotic cell count in every hippocampus region.

As we know, CHOP, p-JNK, and caspase-12 belong to ERS-induced apoptosis pathway, CHOP, and p-JNK can relay the pro-apoptotic signal to the final execution phase. In the execution phase, all upstream signals can lead to activation of the caspase family, resulting in the programmed cell death.^[27] Caspase is a family of cysteine proteases. It is widely activated in neurons and gliocytes in injured brain^[28] and is always considered to be a reason for inducing apoptosis. Caspase-12 is the characteristic molecule of ER stress-induced apoptosis. Thus, activation of GABA_AR can inhibit ERS-induced apoptosis pathway activated by exhaustive exercise. Another activated caspase was caspase-3. Caspase-3 is the executioner caspase. In the execution phase, all pro-apoptotic signals such as CHOP and p-JNK can cause activation of caspase family, in this process, caspase-3 can be activated by initial caspase and proteolytically cleaved; finally, the activated caspase-3 cleavage degrades structural proteins, signaling molecules, and DNA repair enzymes.^[29] Our results indicated the caspase-3 level raised notably 24 h after Ex, which is similar to Kerr and Swain's study.^[3] It proved that the common pathway of apoptosis had been activated, and apoptotic execution phase had happened. Using MUS can effectively down regulate caspase-3 to normal level. So, activation of GABA_AR may inhibit caspase-3 (the common pathway) through inhibiting ERS-induced apoptosis pathway in intense exercise-induced hippocampus apoptosis.

Both Bax and Bcl-2 are components involved in mitochondrial apoptosis pathway. As we know, ERS-induced apoptosis pathway happens before the two classic apoptosis pathways and interacts with them. So, activation of GABA_AR might inhibit mitochondrial apoptosis pathway by inhibiting ERS-induced apoptosis pathway. To sum up, activation of GABA_AR can inhibit caspase-3 activation (common pathway) through inhibiting the ER stress-induced apoptosis and mitochondrial apoptosis pathway, thus protect hippocampus from intense exercise-induced excessive apoptosis.

CA and DG regions are the structural basis of hippocampus function. Our results indicated that CA1 and DG regions were the sensitive area of astrocytes activation after once Ex, while CA3 and DG regions were the sensitive area for collapsed synapses plasticity, also excessive apoptosis appeared in CA1, CA3, and DG regions after once Ex. These regions are extremely important for hippocampus function: When memory is forming, the information flow is firstly transmitted and propagated to DG, then to the CA3 layer, then to the CA1 layer and finally out of the hippocampus, this is called trisynaptic circuit.^[30] Hence, intense exercise-induced hippocampus damages and apoptosis in those regions might lead to many hippocampus dysfunctions, and GABA_AR activating before Ex may help hippocampus maintain those important dysfunctions. These were confirmed by our MWM consequences.

Our MWM results indicated that once Ex can cause hippocampus dysfunction. EL is an index for estimating hippocampus function of learning and forming new memory. In navigation test, EL of Ex group was beyond the Con level at 5 min or 24 h after once Ex, but it had recovered to normal state at 48-h (day 2). So, once Ex-induced hippocampus dysfunction can appear immediately after exercise and last at least 24 h. This result was supported by a cohort study in 2009, which revealed that marathon runners showed explicit memory impairment immediately upon completing intense exercise, and this kind of impaired explicit memory is similar to amnesia in organic brain damage.^[5] As we know, the explicit memory is the hippocampus function. Nonetheless, using MUS before Ex can significantly shorten EL immediately after exercise and help EL decrease to normal level 24 h later. BIC was the only group that still had raised EL 48-h after once Ex. In the probe trail (Day 3, 72 h later), we found once Ex can damage the memory extraction process of hippocampus, it revealed that intense exercise may have long-term adverse effect on hippocampus function. This result may explain the CNS symptom of the OTS, such as amnesia, depression, bad mood. OTS occurs after intense exercise and can last several days to several weeks. OTS is a universal phenomenon of athletes, which can occur at least once in an athlete's career. However, using MUS before Ex can obviously increase the quadrant dwell time to normal level, which means recovery of memory extraction ability in hippocampus, while using BIC before Ex made the memory ability damaged even worse. Therefore, activation

of GABA_AR can protect hippocampus from learning and memory dysfunction, and even long-term dysfunction caused by intense exercise.

This is the first study for studying therapeutic interventions of intense exercise-caused hippocampus excessive apoptosis and structural and functional damages. We found activation of GABA_AR could effectively protect hippocampus from intense exercise-induced synapses plasticity injury, excessive apoptosis, learning, and memory dysfunction, and each of them can lead to typical symptoms of OTS. OTS is a universal symptom, one epidemiological investigation from China National General Administration of Sport in 2009 revealed that CNS illness had the highest case rate (20.63%) among all nontraumatic diseases. These CNS illness and symptoms were like insomnia, headache, dizziness, and exertional syncope, some of them were related to hippocampus dysfunction. From the possible measures to prevention, this study gives a clue that GABA_AR-targeted drugs or chemicals may be useful, especially for OTS among the intensely exercised athletes and inappropriate exercisers, such as iron-man triathlons athletes and marathon runners. Thus, our study has important significance to maintain brain health for athletes and exercising people.

In conclusion, activation of GABA_AR could protect synapse and astrocytes of hippocampus from intense exercise-induced structural and functional damages through antagonizing intracellular Ca²⁺ overload. Besides, activation of GABA_AR can diminish excessive apoptosis of hippocampus caused by intense exercise through inhibiting the ERS-induced apoptosis, mitochondrial apoptosis, and the common pathways. Moreover, activation of GABA_AR could also protect hippocampus function from intense exercise-caused immediate or long-term impairment of learning and memory ability.

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Conflicts of interest

There are no conflicts of interest.

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