

## The Effect of Anti-Fatigue of mGluR4, GABA-AR $\alpha$ 1 and GABA-BR1 on the Substantia Nigra Pars Reticular During Exercise

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### Abstract

*Objective:* The substantia nigra pars reticular (SNR) is the vital nuclei sending out information from basal ganglia and it plays an important part in motor behavior maintenance and motor regulation. It aims to observe the changes of mGluR4, GABA-AR $\alpha$ 1 and GABA-BR1 in substantia nigra pars reticular of rats induced by one single bout of exhaustive exercise, and to discuss the relationship among the expression of mGluR4, GABA-AR $\alpha$ 1, GABA-BR1 and exercise fatigue. *Methods:* Thirty-six male wistar rats were divided into three groups at random including CG, 0EG and 90EG, which having 12 rats. Meanwhile, peripheral blood samples were drawn from the vein of tail at different time points (pre-exercise, exercise for 30 min, exercise for 90 min and exhaustion immediately during exercise, and 30 min and 90 min post exercise), and blood concentrations of lactic acid (LD), blood urea nitrogen (BUN) and activities of creatine kinase (CK) were detected by automatic analyser. Furthermore, the technique of Immunohistochemistry has been used to indicate the protein expression of metabotropic glutamate receptors like mGluR4 and gamma-aminobutyric acid A receptor like GABAAR1, also gamma-aminobutyric acid B receptor like GABAB1. *Results:* The expression level of mGluR4 in SNR in immediately post-exercise group (0EG) and 90 min post-exercise group (90EG) increase significantly than control group (CG), and the expression level of GABA-AR $\alpha$ 1 in SNR at 0EG and 90EG were also significantly higher than control group, and GABA-BR1 in SNR in 0EG and 90EG were both significantly lower than CG. Moreover, it presented significant increases in the levels of LD, BUN and CK in peripheral blood compared with those at rest. *Conclusion:* Exercise fatigue could result in up-regulation of mGluR4 expression, GABA-AR $\alpha$ 1 and down-regulation of GABA-BR1 expression in substantia nigra pars reticular of rats, and it also induce the muscle injury by increase the level of LD, CK and BUN in peripheral blood, which suggested that substantia nigra pars reticular was an important brain region to modulate the motor function, and mGluR4, GABA-AR $\alpha$ 1 and GABA-BR1 were three important receptors related with the muscle injury induced by exercise fatigue. So the mGluR4, GABA-AR $\alpha$ 1 and GABA-BR1 could be the new novel substance produced to prevent from exercise fatigue.

**Keywords:** Exhaustive exercise, anti-fatigue material, substantia nigra pars reticulata, mGluRs, GABA receptors

### I. Introduction

The substantia nigra pars reticular (SNR) plays an essential role of motor circuit in the basal ganglia. The substantia nigra (SN) can be divided into two parts including the substantia nigra pars reticular (SNR) and the substantia nigra pars compacta (SNC), where SNR is a large part of the substantia nigra (SN) and one of the important nuclei sending out information from the basal ganglia (BG). On substantia nigra pars reticular (SNR), GABA ( $\gamma$ -aminobutyric acid) neurons with higher spontaneous firing frequency were mainly distributed, the firing frequency was usually greater than 5 Hz, and the duration of action potential was about 0.6-1.5 ms, in addition, the nigrothalamic GABAergic neurons acted as an important role for the regulating motor activities [1,2]. A variety of animal models of epileptic pain and Parkinson's disease have shown that SNR and its related basal ganglion motor circuits play an important gating role in the onset of epileptic pain and Parkinson's disease, and the excitability of

SNR is inhibited indirectly by directly inhibiting SNR excitability, or by reducing STN's excitability to SNR, through which could both reduce the inhibitory effect of SNR impulse in the anti-epileptic pain brain region, thereby increasing the threshold of epileptic pain seizure [2]. Fatigue is an important reason to reduce the working ability of the body. With the further research on the mechanism of central fatigue, the measures to delay central fatigue have become a hot topic. Glutamate is the most abundant excitatory neurotransmitter and is closely related to central fatigue in central nervous system, currently, metabotropic glutamate receptors such as mGluR5 and mGluR4 may be the novel treatment for Cancer-related Fatigue (CRF) [3, 4]. However, overstimulation of glutamate could result in neuronal cell death, which has been modulated by two major types of glutamate receptors including ionotropic and metabotropic [3]. metabotropic glutamate receptors (mGluRs) are predominantly involved in maintenance of cellular homeostasis of central nervous system and are G-protein-coupled receptors that mediate neuronal excitability and synaptic plasticity in the central nervous system, moreover, they play a 'modulatory' rather than 'mediatory' role in glutamatergic excitatory synaptic transmission, so emerging evidence suggests a role of mGluRs in the biology of cancer, meanwhile mGluR constitute potential therapeutic targets for the development of therapies to treat neurodegenerative diseases [4]. The activity of GABAergic neurons in SNR is mainly controlled by the synaptic inhibition mediated by GABA, and the GABAergic neurotransmission in SNR is mediated by the ionotropic GABAA receptor and the metabotropic GABAB receptor [5].

The question on how exhaustive exercise affects the expression of mGluR4, GABA-AR $\alpha$ 1 and GABA-BR1 in basal ganglia motor circuit especially the substantia nigra pars reticular (SNR) of our central nervous system still remained unclear. Therefore, this study aims to explore the relationship among exercise fatigue, mGluRs and GABA receptors, and the muscle injury indexes such as creatine kinase activity, lactic acid and blood urea nitrogen.

## II. Subjects and methods

### 2.1 Animals and Grouping

In this study, thirty-six clean and healthy male wistar rats aging 8 weeks and weighing  $250 \pm 10$ g, which were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd., animal license number: SCXK (Beijing) 2002-2003. The 36 rats were separated into three groups at random including control group (CG), exhaustive immediately group (OEG), 90 minutes recovery after exercise group (90EG) with 12 rats in each group. The rats were fed normally and separately, and the food and water were supplied with free access. The room for animal was kept constant with conditions at  $(20 \pm 3)$  °C and 40%-60% relative humidity, meanwhile it has a cycle of 12 hr dark and 12 hr light. All the experiments were carried out seriously according to the animal ethics committee of Beijing Normal University.

### 2.2 Exercise-induced Fatigue Model for Rats

During exercise training period, the rats of control group were put on the treadmill without doing exercise. Meanwhile, OEG and 90EG animals had three days of treadmill training for adaptability for 15min every day. Then formal training started from the third day. Exercise training was carried out on treadmills of rats between 9:10 a.m. and 12:10 p.m. And the load programs [6] were as following and it could be seen from the Figure 1: (1) 0 incline of the treadmill; (2) Speeds for the three different levels are 8.2m/min, 15m/min and 20m/min. Time duration for the first two levels is 15 minutes and for the third level until exhaustion. Criteria for exhaustion: animals cannot maintain a predetermined speed and tend to stay for a long time on the fixed tailgate even use of sound, light, electrical stimulation cannot make them move. Meanwhile, there are symptoms of short breath, abdominal lying on the treadmill and head hanging. Meanwhile, athletic ability, weight and activity status of the animals were observed during the model built. The time to exhaustion was recorded and the average exhaustion time was  $155 \pm 14.5$  min

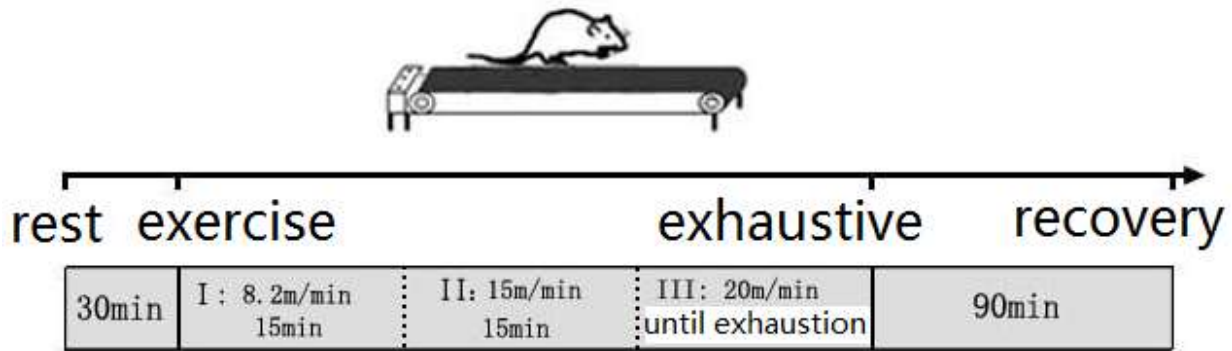


Fig1: Exercise-induced Fatigue Model for Rats

### 2.3 Laboratory instruments and reagents

There are experimental instruments as following: SP-801-type animal treadmill, Olympus microscope camera system, Image-Pro Plus6.0 image analysis system (Canada MediaCybernetics Company). Reagents such as mGluR4, GABA-AR $\alpha$ 1 and GABA-BR1 resistant and anti-kit, and diaminobenzidine (DAB) kit were brought from Beijing Biosynthesis and Bio-Engineering Co., Ltd.; other drugs such as paraformaldehyde, disodium hydrogen phosphate, phosphoric acid, sodium dihydrogen sucrose, sodium chloride, chrome alum, gelatin, methanol, 30% hydrogen peroxide, Triton X-100, ethanol, 95% ethanol and neutral gum, which are all provided by Beijing Biosynthesis Biotechnology Co., LTD.

### 2.4 Perfusion, brain-taking, slicing, staining and filming

After the increasing load treadmill exercise, the control and experimental groups were randomly chosen with 6 rats which were respectively anesthetized with 10% chloral hydrate. The dose is 3.5ml/kg. The anesthetized rats were then fixed supine on the operating table. After carefully cutting their abdominal wall and exposing their diaphragm, then we cut their diaphragm and chest wall and pericardium to make the heart exposed. Puncture needles were used to pierce the left ventricle and the aortic root which was quickly fixed with a hemostat. Then we cut a small hole on the right atrial appendage until blood was seen. After that, approximately 200ml of warm saline was used for perfusion until the liquid flow from the right atrial appendage became clear. And then approximately 200ml and the pre-cooling 4% 4°C paraformaldehyde phosphate buffer solution (0.01M, pH = 7.4) was used for perfusion for no less than 20 minutes until the liver hardened and whitened. Then the brains of rats were taken away from skull, in addition, they were postfixed in the same fixative solution for 24 hours, then embedded in paraffin. The brain tissue containing the SNR was embedded with paraffin and 5  $\mu$ m thickness of brain sections were prepared for immunohistochemistry. Paraffin sections were routinely dewaxed and hydrated, and then microwave-repaired in 0.01 m citrate buffer for 20 min. Each section was incubated at 37 °C for 10 min with 50 l 0.1% Triton solution, PBS was rinsed 3 minutes and 3 times, and animal serum was incubated at 37 °C for 15 min. Then anti-GABA (a) receptor rabbit antibody (1:100) or an anti-GABA (b) receptor rabbit antibody (L: 100) or an anti-mGluR4 rabbit antibody (L: 100) was incubated overnight at 4°C, rinsed at 0.01 mpbs (Ph 7.2) for 5 min, and then a second antibody labeled with a working concentration of biotin was added, goat anti-rabbit IgG labeled with biotin and Rabbit anti-goat IgG labeled with biotin were incubated at room temperature for 20 min, 0.01 mpbs (Ph 7.2) for 3 min, and streptomycin working solution labeled with horseradish enzyme (S-A/HRP) for 20 min at 37°C, then it was rinsed with 0.01 mol/L PBS (Ph 7.2) for 3 min; then it was colored with Diaminobenzidine (Dab) for 10-15 Min; then it was rinsed with 0.01 mol/L pbs (Ph 7.2) for 5 min; then the ethanol with different concentration of 95% I, 95% II, 100% I, 100% II and 100% III to use to be dehydrated for 1 min respectively; then xylene I and Xylene II were used for transparent for 3 min; the neutral resin were used for sealant; the last, the microscopic observation were used for staining results with 40 ~ 400 times and photography [6].

### 2.5 Estimation of Peripheral blood CK, BUN and LD

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During the exhaustion exercise training, twelve rats of the exercise group were randomly chosen for CK, BUN and LD estimation. Peripheral blood samples were taken from the tail vein at different time points (before exercise, 30 min, 90 min and exhaustion immediately during exercise and 30 min and 90 min after exercise), and blood concentrations of lactic acid (LD), blood urea nitrogen(BUN) and activities of creatine kinase (CK) were detected. And creatine kinase (CK) activity, lactic acid (LD) level and blood urea nitrogen (BUN) level were measured for evaluating the cell membrane injury by using automatic analyzer (Hitachi) of the model 7170A with commercial assay reagents (Amresco).

### 2.6 Statistical analysis

Five slices were selected from the same two slices in three experimental groups, and five visual fields (400×) were selected in the substantia nigra pars reticular of each slice according to the stereotactic map of the rat brain, the Positive Cell Count and Integrated optical density (IOD) were analyzed by Image-Pro Plus 6.0 true color pathological image analysis system. The data were expressed as mean ± standard deviation, and the software of SPSS 21.0 was used for statistical analysis. The differences among the groups were tested by One-way Anova. The minimum level of significance was set to be  $P < 0.05$ .

## III. Results

### 3.1 IOD of mGluR4, GABA-AR $\alpha$ 1 and GABA-BR1 post exercise fatigue

The effects of exhaustive exercise on mGluR4, GABA-AR $\alpha$ 1 and GABA-BR1 protein IOD were shown in Figure 2, from which we can see that, The expression level of mGluR4 in SNR at immediately post-exercise group (OEG) and 90 min post-exercise group (90EG) were significantly higher than control group (CG) ( $P < 0.01$  and  $P < 0.001$  respectively), the expression level of GABA-AR $\alpha$ 1 in SNR at OEG and 90EG were also significantly higher than control group (CG) ( $P < 0.001$  and  $P < 0.01$  respectively), the protein expression of GABA-BR1 in SNR at OEG and 90EG were both significantly lower than CG ( $P < 0.05$  and  $P < 0.01$  respectively). In summary, the IOD of mGluR4 and GABA-AR $\alpha$ 1 in SNR increased obviously and it did not return to the rest level of control group. However, the IOD of GABA-BR1 in SNR reduced obviously compared with quiet level after exhaustive exercise.

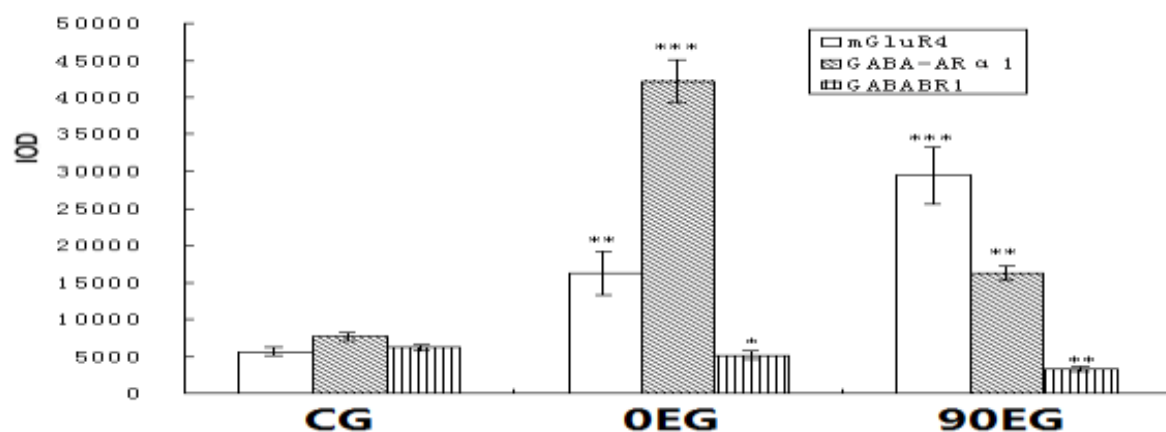
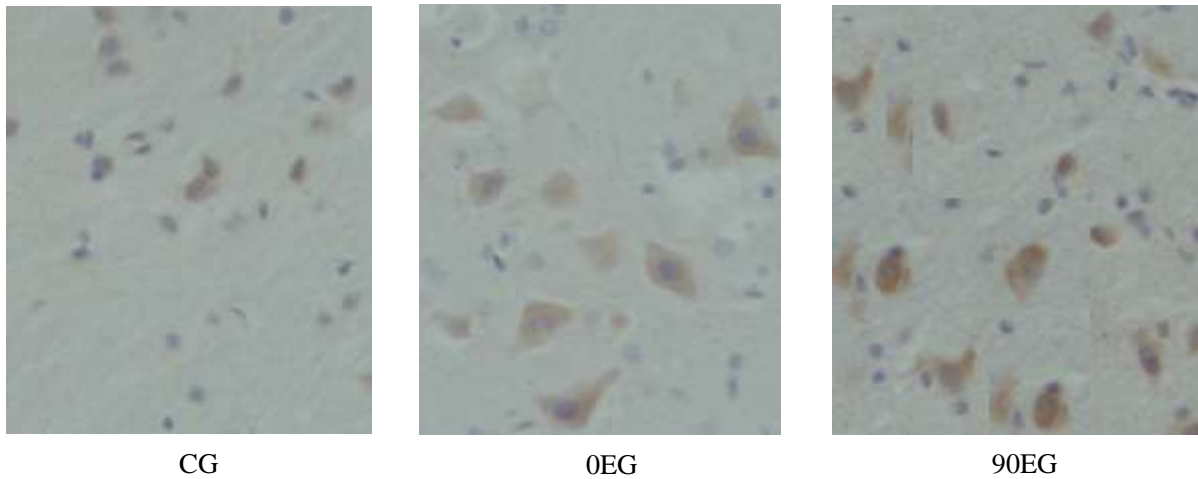
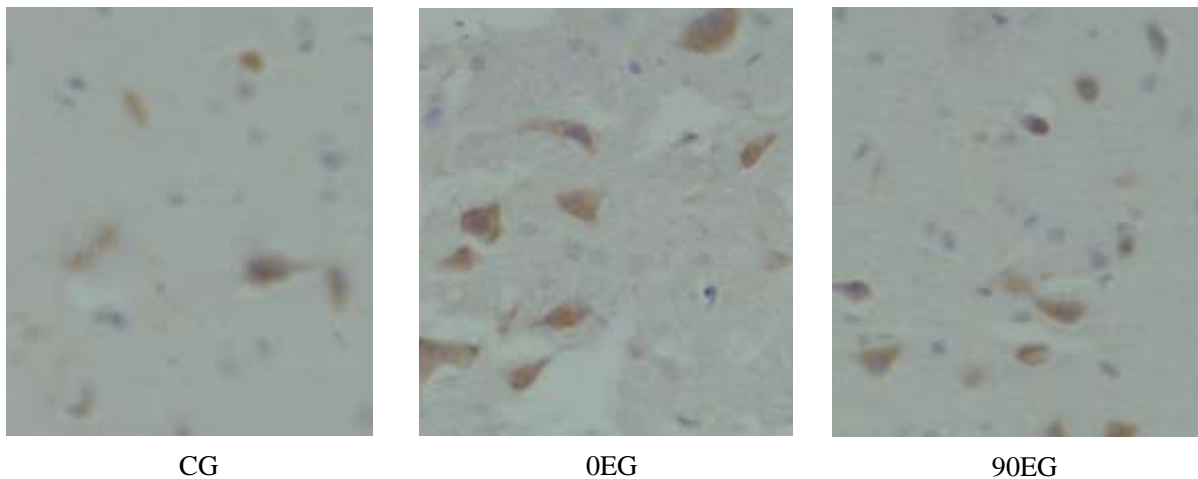


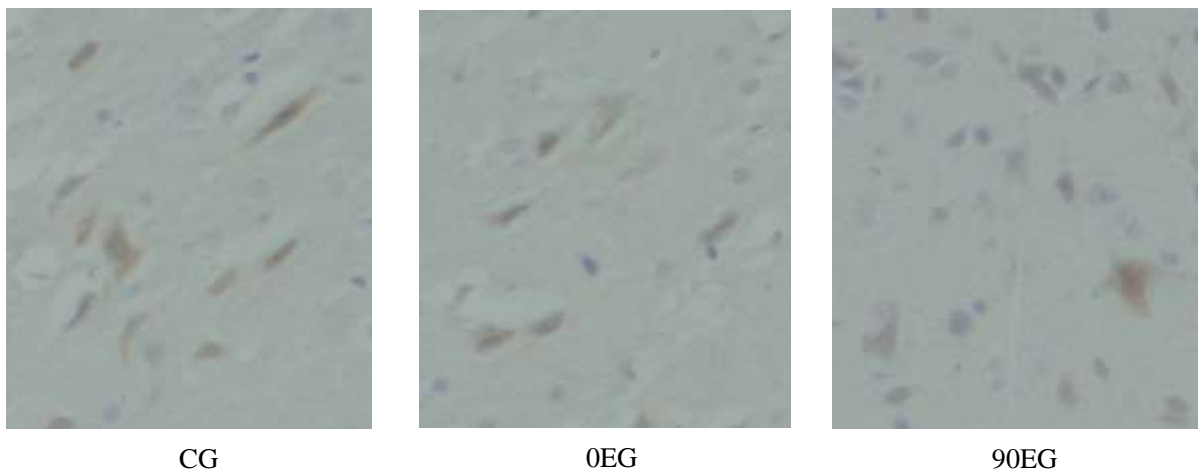
Fig 2: IOD value of mGluR4, GABA-AR $\alpha$ 1 and GABA-BR1 on SNR pre and post exercise ( $\bar{x} \pm s$ ,  $n=6$ )  
All the data was expressed by  $\bar{x} \pm s$ , compared with CG, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .



*Fig 3: Effects of IOD of mGluR4 in SNR after exhaustive exercise on rats (40×10)*



*Fig4: Effects of IOD of GABA-ARα1 in SNR after exhaustive exercise on rats (40×10)*



*Fig 5: Effects of IOD of GABA-BR1 in SNR after exhaustive exercise on rats (40×10)*

From figure 3-5, it showed us that the background of the immunohistochemical sections was light yellow, and the immunopositive cells were brownish yellow in different degrees. From figure 3, the mGluR4 receptors in the

substantia nigra pars reticular were round, oval and polygonal with different size, and there were 2 to 3 cell processes. Moreover, the number of positive cells of mGluR4 in the substantia nigra pars reticular increased significantly immediately post exercise and 90 min post exercise. From figure 4, the GABA<sub>AR</sub>α1 receptors in the substantia nigra pars reticular were round, oval and polygonal with different size, and the contour of the cell body was clearly visible, and the staining intensity was increased at OEG compared with CG. From figure 5, the number of positive cells of GABA-BR1 decreased significantly at OEG and 90EG, and the contour of the cell body was not clear, and the number and staining intensity were decreased compared with CG.

### 3.2 Concentration of LD, CK and BUN in Peripheral blood

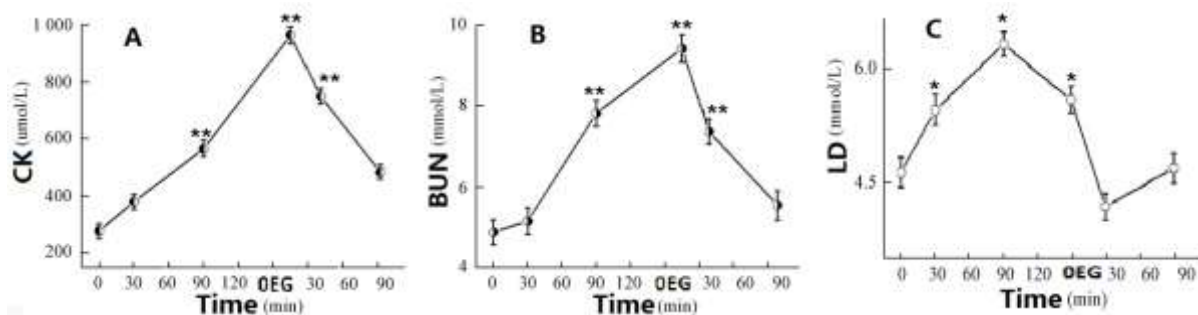


Fig6: Changes of concentrations of CK (A), BUN (B) and LD (C) in peripheral blood of rats during exhaustive exercise and recovery stage

All the data was expressed by  $\bar{x} \pm s$ , compared with CG, \* $P < 0.05$ , \*\* $P < 0.01$ .

From the figure 6-C, it showed that LD concentration was 4.67 mmol/L in peripheral blood of rats in the rest state, which was significantly increased after exercise ( $P < 0.05$ ), and reached the highest value (6.42 mmol/L) after 90 min of exercise, and quickly returned to the quiet level after exercise.

From the figure 6-A, it showed that the average concentration of CK in peripheral blood of rats at rest was 282 U/mL, and the activity of CK increased continuously with the continuous exercise, and reached the peak value of 968.5 U/mL at exhaustion, which was significantly higher than that of rest ( $P < 0.01$ ). After the exhaustive exercise, the activity of CK decreased gradually, but the activity of CK was still significantly higher than the rest level after 30 mins recovery ( $P < 0.01$ ), and the activity of CK decreased to the level of rest after 90 mins during recovery time ( $P < 0.05$ ).

From the figure 6-B, it showed that the variation trend of BUN concentration in peripheral blood of rats during exhaustive exercise was similar to that of CK. The average concentration of BUN at rest was 4.86 mmol/L, and it began to rise after exercise. It was significantly higher than the rest level after exhaustive exercise ( $P < 0.01$ ), and reached the peak value at exhaustion (9.45 mmol/L). After recovery for 30 min, the concentration of BUN was still higher than that of rest ( $P < 0.05$ ). After recovery for 90 min, the concentration of BUN decreased to the pre-exercise level ( $P < 0.05$ ).

## IV. Analysis and discussion

The GABAergic neuron projection of SNR directly regulates the function of thalamus and brain stem motor nuclei, so the change in the activity of GABAergic neurons in SNR will lead to the change of the final outflow information of basal ganglia, leading to the generation of motor dysfunction diseases. Studies have shown that the excessive reduction of wave frequency of substantia nigra pars reticular (SNR) and the occurrence of abnormal waves are closely related to the occurrence of Parkinsonian, which can be used as an indicator for the evaluation of Parkinsonian in rats, moreover, the substantia nigra pars reticular (SNR) regulates posture and motor function, and the increase of SNR electrical activity plays a leading role in autonomous exercise or treadmill exercise, and about 80% of the increased firing of SNR neurons is related to the synchronization of body movement of large muscle

groups [7]. Fatigue is a physiological phenomenon that reduces working efficiency and declines sporting ability, moreover, fatigue may lead to chronic physiological diseases [8]. Lactic acid (LD) has been used as a sensitive index to evaluate the degree of fatigue which is produced under hypoxia in muscles after strenuous exercise, moreover, the increased lactate levels further reduce pH and induce physiological side effects [8]. Urea is a result of metabolism of proteins and causes a significant increase in Peripheral blood nitrogen levels, furthermore it is an important index correlation with protein breakdown, dehydration and stress, which can in turn reduce the endurance and lead to fatigue [8]. Exhaustive exercise may result in muscle cell damage, therefore blood CK activity is often evaluated as a marker for exercise-induced muscle damage, particularly for diagnosis of medical conditions such as myocardial infarction, muscular dystrophy, and cerebral diseases [9]. The rats exercise fatigue model was built up by a single bout exhaustive exercise, and the data in our study presented that the indexes of muscle injury such as CK and BUN all increased significantly post-exercise immediately and post-exercise 30 minutes, and then the level of CK and BUN returned to the rest value post-exercise 90 minutes, however, the activities of LD reached the highest level during exercise at 90 min, and it returned to the rest level at 0EG. From our data, it suggested that the single bout of exhaustive exercise could lead to fatigue and muscle damage.

Glutamate is the largest in number of the excitatory neurotransmitters in the central nervous system. Glutamate excitotoxicity was mediated by its receptors mainly including ionotropic receptors (iGluRs) and metabotropic receptors (mGluRs) [10]. In recent years, glutamate receptor has received considerable attention in the neurological disorder, which is one of the important pathologic markers of central nervous system disease [10], and the up-regulation of mGluR4 expression is closely related to the occurrence and development of depression, which may be one of the causes of depression [10]. Metabotropic glutamate receptor 5 (mGluR5) regulates excitatory glutamate transmitters and postsynaptic signals and is particularly vulnerable to developing chronic inflammation and fatigue after receiving cancer therapy, which has attracted much attention in the field of neurobiology as a target of a variety of neurological disorder drugs [3]. Recent study implicate metabotropic glutamate receptor 4 is highly expressed presynaptically on thalamocortical neuron and glutamate signaling as a promising new molecular target for the treatment of breast cancer [11]. GABA is the mainly inhibitory amino acid neurotransmitter in the central nervous system, which widely exists in the nociceptive pathway. It is combined with GABA receptor and it opens its coupling of chloride ion channels, leading to membrane hyperpolarization by increase of extracellular chloride ion flow and produce inhibitory postsynaptic potential, which results in a variety of causes central inhibition effect including analgesia [11].

Our experimental results showed that the expression of mGluR4 and GABA-AR $\alpha$ 1 protein of substantia nigra pars reticular in 0EG and 90EG both significantly increased, however, the expression GABA-BR1 reduced obviously in 0EG and 90EG, which indicated exhaustive exercise can lead to increasing expression of mGluR4 and GABA-AR $\alpha$ 1. In contrast, it results in decreasing expression of GABA-BR1 in substantia nigra pars reticular. Studies reported [7] that activation of mGluRs receptors could cause neurons excited, which increased neuronal death of glutamate excitotoxicity, and that maybe one of the causes of exercise-induced fatigue. mGluR4, a group III metabotropic glutamate receptor in SNR, is mainly located on various afferent nerve endings. By activating these receptors, it can reduce the release of presynaptic transmitters, thereby inhibiting the excitatory and inhibitory synaptic transmission of GABA neurons in SNR [5].

GABA in SNR plays a regulatory role mainly through ionizing GABAA receptors and metabolizing GABAB receptors, in which rapid postsynaptic inhibition mediated by GABAA receptors plays a dominant role [5]. In conclusion, the GABA-AR $\alpha$ 1 receptor was significantly activated immediately after exhaustion, but it did not return to the level of rest until the recovery time for 90 min. Therefore, the  $\alpha$ 1 subtype of GABAA receptor mediated rapid postsynaptic inhibition to inhibit the excitatory emission of SNR during exhaustion, which was consistent with the role of increased expression of mGluR4 in SNR after exhaustion.

The vital role of GABAB receptor is to control the release of neurotransmitters such as glutamate (Glu) and  $\gamma$ -aminobutyric acid (GABA) from the presynaptic membrane. Enhancing the function of GABAB receptor can inhibit the release of glutamate transmitters and enhance the inhibitory postsynaptic potential (IPSP), thus inhibiting



excitatory transmission [12]. Studies have found that the up-regulation of GABAB1 receptor expression could make presynaptic inhibition of glutamate release, and enhance the GABA inhibitory system, which could prevent the excessive excitement of neurons and has an anti-epileptic effect<sup>[12]</sup>, So it suggested that during the exhaustive exercise the down-regulation of GABAB1 receptor expression results in the occurring of exercise fatigue.

It also has been proved that exhaustive exercise can cause localized ischemia in rat brains and then a series of pathophysiological responses [13]. In our experiment, mGluR4 and GABA-AR $\alpha$ 1 protein content in substantia nigra pars reticular (SNR) of the rat significantly increased in 0EG and 90EG, which may be due to the fact that rats cannot adapt to the stress stimulus at the beginning of acute exercise. And therefore focal ischemia appeared which led to the decline in ATP content in the substantia nigra pars reticular. Simultaneously, large amount of ATP and oxygen was consumed, which caused relative shortage of ATP and oxygen supply in brain. Study found that acute cerebral ischemia could cause metabotropic glutamate receptors mRNA expression increases, suggesting that mGluRs was involved in focal cerebral ischemic injury and probably contributed to the development of ischemic cerebrovascular disease [13]. It had similarities with the results of our study showing out that up-regulation of mGluR4 and GABA-AR $\alpha$ 1, and down-regulation of GABA-BR1 expression was due to exercise-induced ischemia and hypoxia. Therefore, it suggests that the expression mGluR4, GABA-AR $\alpha$ 1 and GABA-BR1 in substantia nigra pars reticular (SNR) may be the other important receptors related with the production of exercise fatigue in the central nervous system.

## V. Conclusion

Exhaustive exercise could lead to exercise fatigue by increasing the level of the indexes of muscle injury such as LD, CK and BUN during exercise, moreover, it results in increasing expression of mGluR4 and GABA-AR $\alpha$ 1 in substantia nigra pars reticular of rats at different time. However, decreasing expression of GABA-BR1 happened in 0EG and 90 EG compared with CG. It suggested that substantia nigra pars reticular is an important brain region to modulate the occurrence of exercise fatigue, which led to the up-regulation of mGluR4 and GABA-AR $\alpha$ 1, and the down-regulation of GABA-BR1 in the central nervous system. The limitation of our study was that we did not have more samples to get the data due to the precious animals. Furthermore, we need to do more experiments to investigate the different time effect until post-exercise 24 hours and 48 hours of these receptors in substantia nigra pars reticular and the other brain regions. Therefore, the mGluR4, GABA-AR $\alpha$ 1 and GABA-BR1 could be the new novel substance produced to prevent from exercise fatigue.

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