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Original Research Article

Amyrin exerts potent anxiolytic and antidepressant effects via mechanisms involving monoamine oxidase and γ-aminobutyric acid in mouse hippocampus

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Abstract

Purpose: *To investigate the anxiolytic and antidepressant effects of α- and β-amyrins in a mouse model of mild traumatic brain injury (mTBI), and the underlying mechanism(s) of action.*
Methods: *Male Swiss mice (n = 165), weighing 25 - 40 g (mean weight = 32.5 ± 7.5 g), were used in this study. Existing mTBI model was modified and optimized for mild injury to brain capable of producing neurobehavioral changes. Forced swim test (FST) and tail suspension test (TST) were used to measure the antidepressant effects of α- and β-amyrins, while elevated plus maze (EPM) and light-dark model (LDM) were used for anxiety assessment. The probable mechanism of action of amyrin was also investigated through kinetics of serotonin uptake and activities of monoamine oxidases A (MAO A) and B (MAO B) in mouse hippocampus and cortex.*
Results: *Induction of mTBI produced anxiety- and depression-like behaviors in mice. The 5th hitting righting time was 259 ± 25.11 s while duration of apnea was 27.33 ± 3.84 s. Apnea was significantly reduced on 5th and 7th hits, when compared with 4th and 6th hits ($p < 0.05$). The immobility time of mice was significantly reduced in FST and TST. A combination of the two forms of amyrin was more effective in reducing duration of immobility, relative to when each was used alone ($p < 0.05$). Amyrin significantly and dose-dependently increased entries, and time spent in open arms and light zone ($p < 0.05$). Mice in mTBI group exhibited a high degree of hopelessness, when compared with control group ($p < 0.05$). However, amyrin at a dose of 50 mg/kg significantly reduced the degree of hopelessness in the mice ($p < 0.05$). The specific activity of MAO A in hippocampal tissue (265.00 ± 12.07 µmol/min/g protein) was significantly higher than that of cortex (61.85 ± 5.14 µmol/min/g protein). In both tissues, there were no significant differences in the activity of MAO B among the groups ($p > 0.05$). Amyrin significantly reversed the effects of mTBI on the levels of amino acids in mice hippocampus and cortex ($p < 0.05$). The results of synaptosomal uptake of serotonin show that fluoxetin exhibited competitive inhibition of serotonin uptake, while amyrin exhibited mixed type inhibition.*
Conclusion: *The results obtained show that α- and β-amyrins exert potent anxiolytic and antidepressant effects via mechanisms involving MAO and GABA in the hippocampus.*

Keywords: *Anxiety, Depression, Amyrin, Hippocampus, Monoamine oxidase*

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INTRODUCTION

Anxiety is an intense, excessive and persistent worry and fear about everyday situations. Depression is a mental health disorder characterized by persistently depressed mood or loss of interest in activities, causing significant impairment in daily life. According to World Health Organization (WHO), in 2015 alone, the incidence of depression and anxiety disorders increased by 18.4 and 14.9 %, respectively [1]. Even with the emergence of novel antidepressant and anxiolytic drugs, the incidence of depression and anxiety disorders continue to rise [2].

Traumatic brain injury (TBI) whether chronic, mild or severe leads to loss of hippocampal volume [3]. It alters synaptic neurotransmission (neurocholinergic synaptic availability and transmission, and expression of their receptors). Supplementation with trace elements and steroidal hormones constitute common treatment for TBI. The goal of therapy is to promote recovery from injuries, cognition and restoration of normal physiological processes of the brain. The Food and Drugs Administration (FDA) of the United States approved for use, the neuroprotective drug "galantamine". This drug is effective against vascular dementia and Alzheimer's disease (AD). It inhibits acetylcholinesterase and activates nicotinic acetylcholine receptor (nAChR), thereby promoting cognitive function in individuals with TBI [4]. Over the last few decades, drugs of natural origin have shown potential in the treatment of anxiety and depression [5,6]. Alpha- and beta amyrins are triterpenoid positional isomers obtained from a variety of plants usually in the form of resins [7-10]. The aim of the present study was to investigate the anxiolytic and antidepressant effects of α- and β-amyrins in a mouse model of mTBI, and the underlying mechanism(s).

EXPERIMENTAL

Materials

Alpha- and β-amyrins (98.2 and 99.0 % purity, respectively) were obtained from Xian Health Biochem. Technology Co. Ltd. (China). Two-chambered plethysmography was a product of Emka technologies (France). High-performance liquid chromatography machine was purchased from Agilent technologies (USA), while OPA reagent was obtained from Thermo Fischer Scientific Co. Ltd (USA). All the chemicals and solvents used in this study were of analytical grade.

Experimental mice

Male Swiss mice weighing 25 - 40 g (mean weight = 32.5 ± 7.5 g) were used in this study. The mice were housed in plastic cages under controlled conditions at a temperature of 25 ^{o}C, humidity of 55 – 65 %, and 12 h light/12 h dark cycle. They were allowed access to standard rat feed and clean drinking water. Prior to commencement of the study, the mice were acclimatized to the laboratory environment for three months. The mice were handled according to the Guide for Care and Use of Laboratory Animals [11]. The study protocol was approved by the Animal Ethics Committee of Hubei Provincial Hospital of Integrated Chinese and Western Medicine (HHICWM23Q/2018).

Induction of closed head mTBI in mice

The criteria adopted for mTBI were: post-traumatic apnea < 30 sec (synonymous with loss of consciousness in humans) [12]; righting reflex < 6 min; and absence of skull fractures or hemorrhage. Weight drop method was used to induce mTBI , but with slight modifications [13,14]. The system consisted of a metallic pipe (length x inner diameter: 100 cm x 2.9 cm) with small holes at intervals of 2 cm to nullify the effects of back pressure and fracture; cutting disc rubber (thickness x diameter: 0.5 cm x 2.8 cm) and 75 g metallic spherical weight (diameter: 2.8 cm) along with a wooden frame (length x width: 40 cm x 40 cm) with facility to hold the mice (velcro). Each mouse was placed on the wooden frame using velcro under metallic pipe, and anaesthetized with 5 % isoflurane. The cushioning rubber disc was centrally positioned on the bregma of the mouse using dental cement, with the mouse under the metallic pipe. Then, the metallic spherical weight was allowed to fall freely through the metallic pipe from 60 cm height on top of the head of the up to 5 times at intervals of 30 sec. The temperature of the surrounding environment was maintained at 37 ^{o}C using heating pad in order to promote quick and timely recovery of the mouse. After regaining consciousness, the mouse was maintained under standard conditions of care. Mild TBI was not induced in control group mice.

Assessment of righting reflex

The time it took a mouse to return to the upright position after placing it on its back on heating pad was immediately recorded after anesthesia. This was taken as insignificant or recoverable neurological loss or mTBI. Righting reflex < 6 min was used as indicative of mTBI.

Experimental design

A total of 165 mice were used in this study. The optimization study comprised 8 groups of 3 mice each. One of the groups was control, while mice in the other groups received different hits. The EPM consisted of 5 groups of 3 mice each: control group, mTBI group, 5 mg/kg bwt amyrin group, 15 mg/kg bwt amyrin group, and 50 mg/kg bwt amyrin group (25 mg α-amyrin + 25 mg β-amyrin). The LDM (light zone) comprised 5 groups of 3 mice each as described in the EPM. The LDM (despair study) also comprised 5 groups of 3 mice each as described in the EPM. The TST consisted of 5 groups of 6 mice each as described in the EPM. FST consisted of 5 groups of 6 mice each: control group, mTBI group, α-amyrin group, β-amyrin group and (α+ β)-amyrin group. Mice in α- or β-amyrin group received 50 mg/kg bwt α-amyrin or 50 mg/kg bwt β-amyrin, while (α + β)-amyrin group mice received equal doses of α- and β-amyrins (25 mg each). Six groups of mice were each used for determination of activities of MAO A and MAO B (3 groups for cortex and 3 groups for hippocampus): control group, mTBI group and (α + β)-amyrin group. Seven groups of mice were used for estimation of levels of amino acids in hippocampi and cortexes of mice (3 groups per amino acid): control group, mTBI group and (α + β)-amyrin group. Assessment of synaptosomal serotonin uptake made use of three groups of 3 mice each: control group, fluoxetine group and amyrin group. Treatment lasted 14 days in all the tests/models.

Estimation of apnea

Apnea was estimated using respiratory recording method. Whole-body flow-through, two chambered plethysmography fitted with temperature regulator was used in this study. It was calibrated thus: air flow = 180 mL per min; temperature = 30 oC; and air injection = 0.5 mL. Plethysmographic respiratory signals in the form of pressure difference between the two chambers were analyzed using differential pressure transducer. Apnea was defined as cessation of plethysmographic signal for at least twice the mean respiratory cycle duration, and calculated over a 10-sec period of visually identified regular breathing within 20 sec preceding apnea [15].

Anxiety models

Elevated plus maze (EPM)

Elevated plus maze (EPM) is a wooden maze with two covered/closed arms and two open arms alternating along the central platform. These '+' arms and central platform were of dimension 16 cm × 5 cm (open arm), 16 cm × 5 cm × 15 cm (closed arm) and 5 cm × 5 cm, respectively, with 25 cm ground clearance. This model measures the inclination of mice to opt for closed arm, while there is feeling of fear or anxiety for height in the open arm. On the other hand, less anxious mice (mice treated with anxiolytics) will also explore the open arm. An hour after the 14th day treatment with α- and β-amyrin or standard drug, mice were placed individually in the central platform and the number of entries observed and duration of stay in different arms within 5 min were recorded [5].

Light – dark model (LDM)

Another effective model for anxiety is LDM which comprised two opaquely separated compartments of light (40 W light source) and dark (black pasting inside) of same dimensions (5 cm x 25 cm x 25 cm), with a hole (5 cm x 5 cm) in opaque separating wall. Mice were placed individually in light compartment 1 h after the last treatment with α- and β-amyrin, or vehicle. Then, the mice were observed for 10 min for number of entries from light to dark compartment and duration of stay in each compartment. In this model, illumination is used as behavioral feeling of anxiety [16].

Depression models

Forced swim test (FST)

Forced swim test (FST) produces a behavioral situation in mice similar to depression in humans (hopelessness represented by a period of immobility). In FST, an open miniature swimming pool of dimensions 25 cm x 15 cm x 25 cm maintained at a temperature of 25 ± 2 ^{o}C was used. One hour after the 14th day treatment, mice were dropped down in water and their activities were recorded within 4 min after an initial 2 min of vigorous escape attempt [5].

Tail suspension test (TST)

Tail suspension test (TST) is a non-sophisticated and least specific model which uses zero movement or immobility or helplessness of the tail of suspended mice as end-point parameter of depression [17]. In this model, mice were hung through their tails at a height of approximately 70 cm over a shelf, and were observed for activity within 6 min.

Determination of activities of MAO A and MAO b in mice hippocampi and cortexes

At the end of the 14th day of treatment, mice were euthanized, and their brain tissues carefully excised. Brain hippocampus and cortex were isolated and immediately placed on ice. The entire procedure was carried out at low temperature (4 °C) using a stabilizing buffer (20 mM Tris-EDTA buffer, pH 7.4 containing 0.25 M sucrose). Portions of the hippocampus and cortex were then homogenized and centrifuged at 1000 g for 10 min at 4 °C. The resultant supernatant was washed twice with phosphate-buffered saline (PBS). Then, mitochondrial fraction was obtained by subjecting the supernatant to ultracentrifugation at 12,000 g for 20 min at 4 °C. The isolated mitochondrial fractions were used for assessment of activities of MAOs. Protein concentrations in the supernatant and pellets were quantified using Bradford's method [18]. The activities of mitochondrial MAOs were determined using their specific substrates (4 mM serotonin for MAO A, and 100 mM benzylamine for MAO B in 50 mM Tris EDTA buffer, pH 7.4). The reaction lasted 10 min and was terminated with addition of 100 µL 0.5 M HCl. Finally, absorbance of each sample was read at 280 nm (for MAO A) and 250 nm (for MAO B), respectively [19].

Determination of levels of amino acids in specific areas of brain hippocampus and cortex

Estimation of levels of glycine (Gly), glutamate (Glu), aspartate (Asp), taurine (Tau), GABA, serine (Ser), and glutamine (Gln)] was carried out using standard method [19]. Portions of brain hippocampus and cortex (20 mg each) were treated with 100 µL perchloric acid (100 mM) to completely remove free proteins/peptides. Then, the tissues were homogenized with the same volume of perchloric acid, and centrifuged at 17, 000 g for 20 min at 4 ℃. The resultant supernatant was filtered using 0.22 µm Millipore® syringe filter. The acidic supernatant was neutralized with equal volume of 500 mM sodium bicarbonate buffer and then diluted with deionized water. Then, 100 µL of treated sample was derivatized with 50 µL OPA derivatization solution (4 mg/mL OPA, 5 % v/v β thiofluor in 100 mM borate buffer, pH 9.5) by vortex mixing, and an aliquot of the mixture (10 µL) was analyzed using HPLC fitted with FLD detector. Binary pump HPLC system (Agilent 1260) with auto-sampler and a fluorescence detector was used in this study. Reverse phase Ascentis express C-18 column (250 mm × 4.6 mm, 2.7 µm particle size), mobile phase A (0.02 M sodium perchlorate buffer) and mobile phase B (methanol) were used for elution of the amino acids.

Assessment of presynaptic serotonin uptake

Synaptosomal uptake of serotonin (5-hydroxy tryptamine, 5-HT) was assessed based on standard method. Weighed portion of excised brain tissue was homogenized with sucrose solution (0.32 M) and centrifuged at 1000 *g* for 10 min. The resultant supernatant containing synaptosomal membrane was used for assessment of serotonin uptake. The entire procedure was carried out at -4 ^{o}C. The uptake assay solution consisted of 50 µL brain tissue homogenate, 900 µL assay buffer (freshly prepared 10 mM sodium phosphate buffer, pH 7.4, 0.50 mM, $CaCl_2$, 4.0 mM KCl, 0.65 mM $MgSO_4$ and 120 mM NaCl bubbled with mixture of O_2 and CO_2), and 50 µL radioactive 5-HT (ranging from 5 x 10^{-7} to 50 x 10^{-7} M) at 37 ^{o}C. Synaptosomal uptake was stopped after 5 min by rapidly cooling the assay mixture to 4 ^{o}C. The assay mixture was then centrifuged at 5000 g for 20 min and the pellet obtained was washed with assay buffer at 4 ^{o}C. Eluted radioactive material containing buffer was quantitatively monitored for radioactivity using a scintillation counter. Standard inhibitor fluoxetin (1 mg/mL, 50 µL) or test amyrin (10 mg/mL) was used in place of the buffer in order to assess their inhibitory effects. The kinetic parameters (k_m and V_{max}) for receptor-ligand interaction (leading to serotonin uptake) was calculated by plotting using corresponding Lineweaver-Burk plots.

Statistical analysis

Data are expressed as mean ± SEM, and the statistical analysis was performed using GraphPad Prism (7.0). Groups were compared using Tukey-Karmer multiple comparison test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Outcome of mTBI induction

Maximum limit righting time (259) and duration of apnea (27.33) based on the criteria used were gained after the 5th hitting. Apnea was significantly reduced on the 5th and 7th hits, when compared with 4th and 6th hits ($p < 0.05$; **Error! Reference source not found.**).

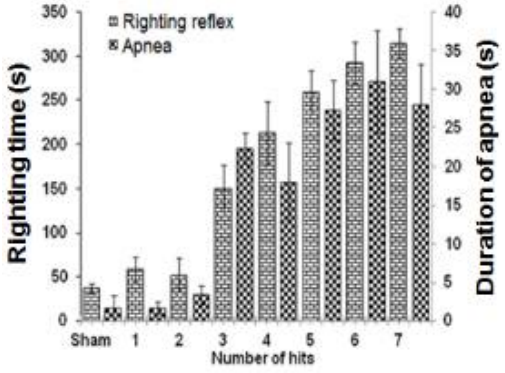

Figure 1: Righting time and duration of apnea as parameters of successfully established mTBI

Effects of α- and β- amyrin on duration of immobility

The duration of immobility was significantly higher in mTBI group than in control group ($p < 0.05$). However, treatment with α- and β- amyrins significantly reduced the duration of immobility ($p < 0.05$). The combination of the two forms of amyrin was more effective in reducing duration of immobility, relative to when each was used alone ($p < 0.05$). These results are shown in Figure 2.

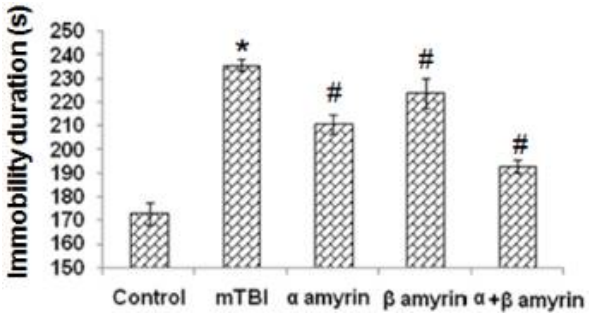

Figure 2: Effects of α- and β-amyrin on duration of immobility; *$p < 0.05$, when compared with control group, #$p < 0.05$, when compared with mTBI group

Anxiolytic effect of amyrin

As shown in Figure 3, entries and time spent in open arms were significantly reduced in mTBI group, when compared with the control group ($p < 0.05$). However, amyrin significantly and dose-dependently increased entries and time spent in open arms ($p < 0.05$).

Effect of amyrin on time spent in light zone

The time spent in light zone was significantly reduced in mTBI group, when compared with the control group ($p < 0.05$). However, amyrin significantly and dose-dependently increased the time spent in light zone ($p < 0.05$; Figure 4).

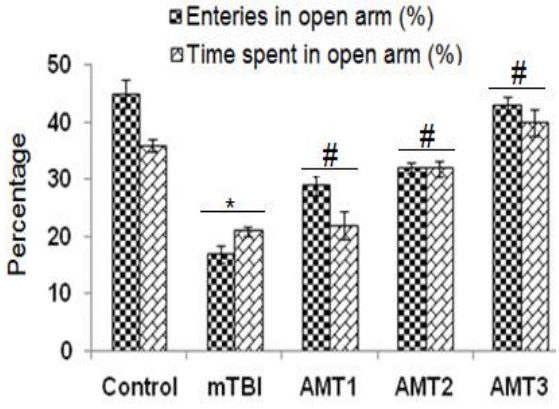

Figure 3: Entries (%) and time spent by mice in open arm; *$p < 0.05$, when compared with control group, #$p < 0.05$, when compared with mTBI group

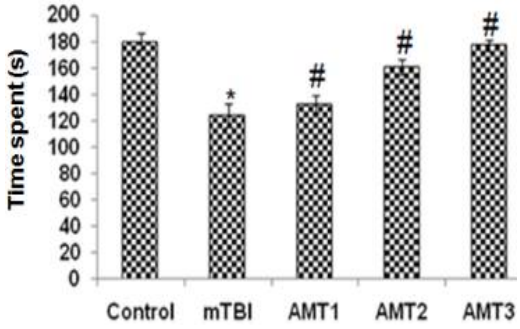

Figure 4: Comparison of time spent by mice in light zone among the different groups; *$p < 0.05$, when compared with control group, #$p < 0.05$, when compared with mTBI group

Effect of amyrin on despair behavior

Mice in mTBI group exhibited high degree of hopelessness, when compared with the control group ($p < 0.05$). However, amyrin at a dose of 50 mg/kg bwt significantly reduced the degree of hopelessness in the mice ($p < 0.05$; Figure 5).

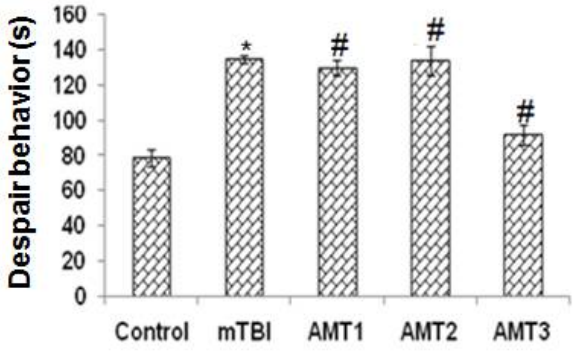

Figure 5: Comparison of despair behavior among the different groups; *$p < 0.05$, when compared with control group, #$p < 0.05$, when compared with mTBI group

TST results

As shown in Figure 6, the duration of immobility was significantly higher in mTBI group than in control group, but was significantly reduced after treatment with varied doses of amyrin ($p < 0.05$).

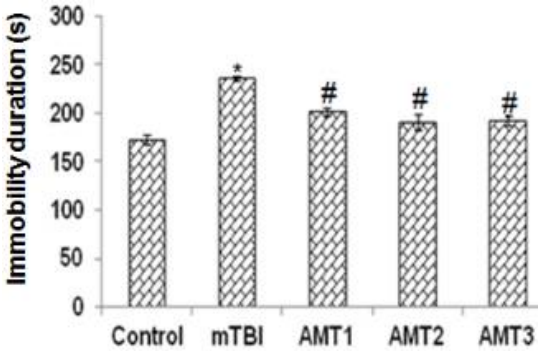

Figure 6: Comparison of duration of immobility among the groups

Effect of amyrin on activities of MAOS A and B in hippocampus and cortex

In hippocampal tissue, the activity of MAO A was significantly lower in mTBI group than in control group, but was significantly increased after treatment with amyrin ($p < 0.05$). However, in the cortex, there were no significant differences in the activity of MAO A among the groups ($p > 0.05$; Figure 7 A). The specific activity of MAO A in hippocampal tissue (265.00 ± 12.07 µmol/min/g protein) was significantly higher than that in the cortex (61.85 ± 5.14 µmol/min/g protein). In both tissues, there were no significant differences in the activity of MAO B among the groups ($p > 0.05$; Figure 7 B).

Effect of amyrin on some amino acid levels in hippocampus and cortex

In the cortex, the levels of GABA, Glu, and Gln were significantly higher in mTBI group than in control group, but were significantly reduced after treatment with amyrin ($p < 0.05$). The corresponding levels of Tau and Asp were significantly lower in mTBI group than in control group, but were significantly increased after treatment with amyrin ($p < 0.05$). However, there were no significant differences in the levels of Gly and Ser among the groups ($p > 0.05$; Figure 8 A).

In the hippocampus, the levels of GABA, Gly, Ser, Tau, and Asp were significantly lower in mTBI group than in control group, but were significantly increased after treatment with amyrin ($p < 0.05$). However, the levels of Glu and Gln were significantly higher in mTBI group than in control group, but were significantly reduced after treatment with amyrin ($p < 0.05$; Figure 8 B).

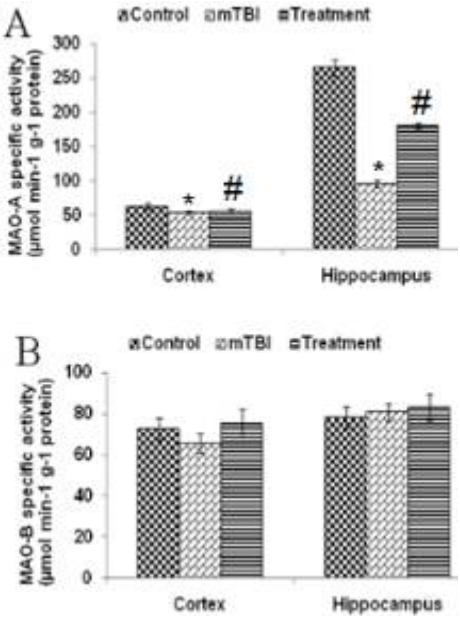

Figure 7: Mitochondrial enzyme activity in hippocampus and cortex. (A): Specific activity of MAO A; and (B): Specific activity of MAO B; $^{*}p < 0.05$, when compared with control group, $^{\#}p < 0.05$, when compared with mTBI group

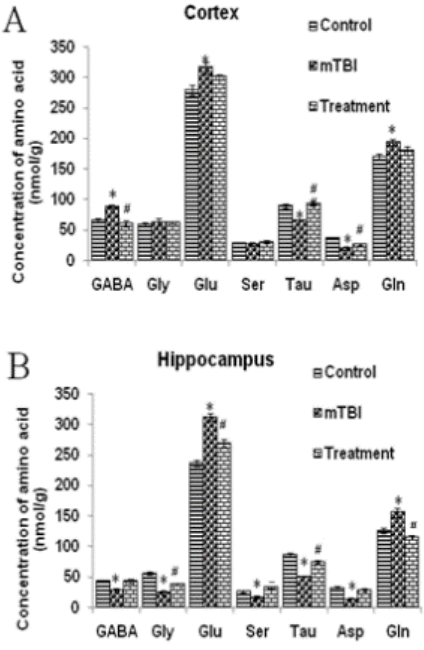

Figure 8: Effect of amyrin on some amino acid levels in hippocampus and cortex. (a) levels of amino acids in the cortex; and (b): levels of amino acids in hippocampal tissue; $^{*}p < 0.05$, when compared with the control group, $^{\#}p < 0.05$, when compared with mTBI group

Synaptosomal uptake of serotonin

The k_m of fluoxetin group was significantly higher than that of control group ($p < 0.05$). However, the v_{max} was not significantly altered ($p > 0.05$). Thus, fluoxetin exhibited competitive inhibition for serotonin. On the other hand, the v_{max} and k_m of amyrin group were significantly increased, when compared with those of control group ($p < 0.05$). Thus, amyrin exhibited mixed type inhibition. These results are shown in Table 1.

Table 1: Kinetic parameters of serotonin uptake obtained from Lineweaver-Burk plot

Group	$1/v_{max}$ (Intercept) x 10^{-4}	v_{max}	k_m/v_{max} (Slope) x 10^{-10}	k_m x 10^{-6}
Control	1.32	7575.76	6.12	4.62
Fluoxetin	1.57	6369.43	20.40	12.70
Amyrin	0.41	24455.86	13.00	31.80

DISCUSSION

Depression and other related mental disorders affect significant percentage of the human population. Globally, the incidence of depression is alarming [1,2]. According to WHO report, in 2015 alone, the incidence of depression and anxiety increased by 18.4 and 14.9 %, respectively [1]. Anxiety and depression affect a large part of the world's population, and there is urgent need for new strategies for their management. Traumatic brain injury (TBI) causes post-recovery anxiety and/or depression in almost all patients.

Non-invasive-closed head mTBI model is suitable for studying real life neurological situations, such as long-lasting learning impairment, loss of memory, anxiety and depression after accidental brain injury [20]. The mTBI model is characterized by post-traumatic apnea < 30 sec (synonymous with loss of consciousness in humans), righting reflex < 6 min, and absence of skull fractures or hemorrhage [14]. In this study, 5 hits were considered sufficient for mTBI induction in the mice.

The pharmacological effectiveness of α- and/or β-amyrin have been reported. In most of the studies, extracts containing both isomers alone or in combination with other compounds were used. The aim of the present study was to investigate the anxiolytic and antidepressant effects of α- and β-amyrins in a mouse model of mTBI, and the underlying mechanism(s).

The results showed that the combination of α- and β-amyrin was more effective in alleviating symptoms of mTBI than when they were used individually. The combination of the two forms of amyrin was more effective in reducing duration of immobility, relative to when each was used alone. Mice in mTBI group exhibited high degree of hopelessness, when compared with the control group. However, amyrin at a dose of 50 mg/kg bwt significantly reduced the degree of hopelessness in the mice.

Studies have shown that the hippocampus is involved in stressful conditions [21]. Structural damage to the hippocampus has been observed in situations of depression, and results in neuronal apoptosis and synaptic dysfunction. Monoamine oxidase (MAO) is an enzyme involved in the metabolism of synaptic neurotransmitters. Reduced expression of this enzyme could be the possible cause of decreased level of neurotransmitters in synapse and slowed neuronal signal transmission. It has been suggested that MAO expression is most likely to be reduced in stressed tissues [21,22].

The results of this study suggest the involvement of MAO-A in mTBI-induced neuronal damage specifically in hippocampal tissue. In the cortex, the levels of GABA, Glu, and Gln were significantly higher in mTBI group than in control group, but were significantly reduced after treatment with amyrin. The corresponding levels of Tau and Asp were significantly lower in mTBI group than in control group, but were significantly increased after treatment with amyrin. However, there were no significant differences in the levels of Gly and Ser among the groups.

In the hippocampus, the levels of GABA, Gly, Ser, Tau, and Asp were significantly lower in mTBI group than in control group, but were significantly increased after treatment with amyrin. However, the levels of Glu and Gln were significantly higher in mTBI group than in control group, but were significantly reduced after treatment with amyrin.

These results are in agreement with those reported previously [23]. Worthy of note is the significant reduction in the level of GABA in hippocampal tissues of mTBI group mice, and a corresponding increase in its level in the cortex. This suggests that mTBI model of anxiety and depression was successfully established, and that amyrin may possess effective anxiolytic and antidepressant effects in mice hippocampi [24]. The results of synaptosomal uptake of serotonin showed that fluoxetin exhibited competitive

inhibition for serotonin, while amyrin exhibited mixed type inhibition.

CONCLUSION

The results obtained in this study show that α- and β-amyrins exert potent anxiolytic and antidepressant effects via mechanisms involving hippocampal MAO and GABA.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them. Xu Kun drafted this manuscript and performed all the experiments. Gong Zuhua assisted in revising this manuscript and gave suggestions in designing the study.

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