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Research article

Sub-anesthetic doses of ketamine exert antidepressant-like effects and upregulate the expression of glutamate transporters in the hippocampus of rats

Xianlin Zhu [a], Gang Ye [a], Zaiping Wang [a,*], Jie Luo [b], Xuechao Hao [b]

[a] Department of Anesthesiology, The Central Hospital of Enshi, Autonomous Prefecture, Enshi, Hubei, 445000, China
[b] Department of Anesthesiology, the First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China

HIGHLIGHTS

- Excitatory amino acid transporters (EAATs)-mediated glutamate reuptake dysfunction involved in the pathogenesis of depression.
- Sub-anesthetic doses of ketamine induced antidepressant-like effects in rats.
- Ketamine up-regulated the expression of EAATs and decreased the concentration of extracellular glutamate in the hippocampus of depressive-like rats.

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ABSTRACT

Clinical studies on the role of the glutamatergic system in the pathogenesis of depression found that ketamine induces an antidepressant response, but the molecular mechanisms remain unclear. The present study investigated the effects of sub-anesthetic doses of ketamine on the glutamate reuptake function in the rat hippocampus. Chronic unpredictable mild stress (CUMS) was applied to construct animal models of depression. Sixty adult male Sprague-Dawley rats were randomly assigned to 5 groups and received a different regimen of CUMS and ketamine (10, 25, and 50 mg/kg) treatment. The sucrose preference test and open-field test were used to assess behavioral changes. The expression levels of excitatory amino acid transporters (EAATs) were measured by western blot. Microdialysis and high-performance liquid chromatography (HPLC) were used to detect hippocampal glutamate concentrations. We found that the expression of EAAT2 and EAAT3 were obviously downregulated, and extracellular concentrations of glutamate were significantly increased in the hippocampi of depressive-like rats. Ketamine (10, 25, and 50 mg/kg) upregulated the expression of EAAT2 and EAAT3, decreased the hippocampal concentration of extracellular glutamate, and alleviated the rats' depressive-like behavior. The antidepressant effect of ketamine may be linked to the regulation of EAAT expression and the enhancement of glutamate uptake in the hippocampus of depressive-like rats.

1. Introduction

Depression is a prevalent and disabling psychiatric illness that affects millions of individuals worldwide, resulting in enormous personal suffering and public health costs [1]. Traditional antidepressants such as monoamine oxidase inhibitors (MAOIs) and selective serotonin reuptake inhibitors (SSRIs) usually take weeks to months to produce a therapeutic response, and more than 30% of patients with depression exhibit refractory or intolerant responses to current available antidepressant medications [2]. In contrast, recent clinical studies have demonstrated that the *N*-methyl-D-aspartate (NMDA) antagonist ketamine induces a rapid (within h) antidepressant response [3,4], and is effective in patients with major depressive disorder who are treatment-resistant to traditional antidepressants [5]. However, the molecular mechanisms underlying this process remain unclear.

Multiple lines of evidence have supported a critical role for the glutamatergic system in the pathophysiology of depression, and it is believed to be a key target in mood regulation [6,7]. Glutamate

* Corresponding author.
E-mail addresses: zhuxianlin8@sohu.com (X. Zhu), 343205560@qq.com (Z. Wang).

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is a critical excitatory neurotransmitter in the mammalian brain, and its reuptake is essential for normal synaptic transmission. High levels of extracellular glutamate can mediate excitotoxicity and is implicated in the pathogenesis of many brain diseases [8]. However, the clearance of released glutamate is not assumed by its synaptic degradation. Excitatory amino acid transporters (EAATs), also named glutamate transporters, transport glutamate from the extracellular to the intracellular spaces, thereby efficiently controlling the extracellular concentration of glutamate [9]. Currently, five distinct EAATs (EAAT1-5) that transport glutamate have been cloned. EAAT1 and EAAT2 are predominantly localized on astrocytes and abundant in the hippocampus and cerebral cortex. In contrast, EAAT3 is a neuronal transporter, which is expressed in the pre-and postsynaptic regions of neurons, while EAAT4 and EAAT5 appear mainly restricted to expression on the cerebellum and the retina, respectively [10]. Our previous study found that EAAT2 expression was markedly downregulated in the hippocampus of depressive-like rats [11]. However, whether the antidepressant effect of ketamine is related to regulating glutamate reuptake functions requires further study.

The aim of this study was to investigate the effects of ketamine on depressive behaviors in rats and the potential roles of EAAT-mediated glutamate reuptake function in this process.

2. Materials and methods

2.1. Animals

Healthy adult male Sprague-Dawley rats, weighing 200–250 g, aged 2–3 months, were obtained from the Laboratory Animal Center of Chongqing Medical University. The rats were housed and maintained in standard laboratory conditions (22 ± 2 °C and a 12:12-h light-dark cycle) with free access to feed and water for 1 week before further experiments. All the procedures were approved by the Ethics Committee of Chongqing Medical University and carried out according to the animal care guidelines of the National Institutes of Health. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2.2. Animal models of depressive-like behavior

Chronic unpredictable mild stress (CUMS) was applied to construct animal models of depressive-like behavior as previously described [12]. The rats were housed in individual cages and randomly exposed to one of the following stressors per day for 28 consecutive days: cold water swimming at 4 °C for 5 min; hot stress in an oven at 45 °C for 5 min; pinching the tail for 1 min; food deprivation for 24 h; water deprivation for 24 h; caged in a soiled cage for 24 h; shaking for 20 min (once per second); social crowding (25 rats per cage); cage being tilted to 30° from the horizontal for 24 h; and continuous lighting for 24 h. After the CUMS procedure, 48 rats with depressive-like behavior were obtained.

2.3. Experimental groups and treatments

A group of 12 healthy male rats (with same age and weight) were set as the control group (group C). Forty-eight depressive-like behavior rats were randomly assigned to four groups (n = 12): group D, group DK1, group DK2, and group DK3. Group C did not receive any treatment; rats in group D were treated with normal saline (10 ml/kg, i.p.); rats in group DK1 were treated with i.p. injection of 10 mg/kg ketamine (concentration at 1 mg/ml, No. KH091201, Jiangsu Hengrui Medicine, China); rats in group DK2 were treated with i.p. injection of 25 mg/kg ketamine; rats in group DK3 were treated with i.p. injection of 50 mg/kg ketamine. The aforementioned treatments were given once per day for 5 consecutive days.

2.4. Behavior test

2.4.1. Sucrose preference test

The sucrose preference test was performed as previously described to evaluate the anhedonia in rats (the core symptom of depression) [13]. In the first 24 h, rats were exposed to two bottles of 1% (w/v) sucrose solution to habituate them to consumption of a sucrose solution. In the next 24 h, one bottle of sucrose solution was replaced with a bottle of sterile water. After 23 h of water and food deprivation, each rat was exposed to two identical bottles with one containing 1% sucrose and the other one containing sterile water. All rats were allowed to drink water freely for 1 h. Sucrose preference percentage (SPP) was calculated according to the following formula: SPP (%) = [sucrose solution intake (ml)/(sucrose solution intake (ml)+sterile water intake (ml)] × 100.

2.4.2. Open-field test (OFT)

To evaluate spontaneous locomotor and exploratory activities of rats in a novel environment, the OFT was performed as described previously [14]. The open-field apparatus consisted of a black wooden square arena (100 × 100 × 50 cm) in a quiet room with dim illumination. The floor of the box was marked with a grid dividing it into 25 equal-size squares. Each animal was tested individually and only once in the apparatus. It was placed in the central square and observed for 5 min. Parameters assessed were horizontal ambulation (the number of squares crossed, indicating general locomotors) and the times of rearing (when a rat stood completely erect on its hind legs, indicating exploratory behavior). The OFT was performed and scored by trained and experienced observers who were blind to the diagnoses of the animals. The SPT and OFT was performed twice, once in the 24 h after CUMS treatments was completed, and the other in the 24 h after last time ketamine treatment.

2.5. Microdialysis and tissue preparation

After completion of the behavioral experiments, 6 rats were randomly selected from each group for use in the microdialysis study [15]. Briefly, the rats were anesthetized with 2% pentobarbital sodium (40 mg/kg, i.p.) and then fixed in a stereotaxic frame (Kopf Instruments, California). A microdialysis probe (MAB 4.15.2 Cu, Microbiotech, Sweden) was inserted unilaterally into the dorsal hippocampus. (A/P, −3.6 mm; L, 2.0 mm; D/V, −4.0 mm). The microdialysis pipeline was filled with artificial cerebrospinal fluid (NaCl 147 mmol/L, KCl 2.7 mmol/L, $CaCl_2$ 1.2 mmol/L, $MgCl_2$ 0.85 mmol/L) and continuously perfused at a flow rate of 2.5 μl/min by a microinfusion pump. After allowing the system to equilibrate for 1 h, samples (25 ml) were collected in tubes containing 2 ml of acetic acid and frozen (−20 °C) immediately for further analysis. After the completion of the microdialysis, all rats were killed under anesthesia with 2% pentobarbital sodium (50 mg/kg, i.p.). The bilateral hippocampi were quickly removed and immediately cooled in liquid nitrogen and stored in a refrigerator at −80 °C.

2.6. Western blotting analysis

Frozen hippocampi were weighed and homogenized in protein buffer consisting of 3 ml of radioimmunoprecipitation assay (RIPA) lyses buffer (US Biological, USA) and 30 μl complete cocktail protease inhibitor (Roche Molecular Biochemicals, Germany) per gram of tissue. After centrifugation with 12000 rpm at 4 °C for 10 min, the supernatant was collected and stored at −20 °C until used. Following a bovine serum albumin (BSA) micro assay (Pierce, Rockford, IL) and spectrophotometry to assess protein levels, every

individual supernatant sample containing 50 μg of protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to a polyvinylidene fluoride (PVDF) membrane (Millipore). After blocking with 5% skimmed milk powder in tri-buffered saline with Tween (TBST) solution, the membranes were probed with primary antibodies [GAPDH (1:1000, sc-25778, Santa Cruz Biotechnology), EAAT1 (1:1000, sc-15316, Santa Cruz Biotechnology), EAAT2 (1:1000, sc-15317, Santa Cruz Biotechnology), and EAAT3 (1:1000, sc-25658, Santa Cruz Biotechnology)], and incubated overnight at 4 °C. The transfers were then rinsed with TBST and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies for 1 h at room temperature. The immunoreactive protein was detected with an ECL kit (Santa Cruz).

2.7. High-performance liquid chromatography (HPLC)

The concentration of glutamate was determined with HPLC as previously described [16]. The amino acids were derivatized with mercaptoethanol and o-phthalaldehyde (OPA). The OPA derivatives were then separated on a 5 l m reverse-phase Nucleosil C18 column (250 × 4 mm; Machery-Nagel, Duren, Germany) at 20 °C, using a mobile phase consisting of methanol and potassium acetate (0.1 M, pH adjusted to 5.48 with glacial acetic acid) at a flow rate of 1.0 ml/min in a three linear-step gradient (from 25% to 90% methanol). The analysis was performed with an LC-2010A liquid chromatography system (Shimadzu Seisakusho, Kyoto, Japan). The concentration of glutamate was quantified by comparison with the standard curves for amino acids.

2.8. Statistical analysis

All statistical analysis was performed with SPSS (version 17.0, SPSS Inc., Chicago, Ill). All data are expressed as mean ± standard deviation (SD). Statistical significance was determined with one-way analysis of variance (ANOVA). The Bonferroni correction was used to detect differences between each of the groups. $P < 0.05$ was considered significant.

3. Results

3.1. Sucrose preference test

Before administration of ketamine treatment, there was a significant difference in sucrose preference percentage (SPP) among groups C, D, DK1, DK2, and DK3 ($F = 22.607$, $P < 0.001$), and the SPPs of the four CUMS-treated groups (groups D, DK1, DK2, and DK3) were lower than those of group C ($P < 0.01$, respectively), but no difference was found among groups D, DK1, DK2, and DK3. After ketamine treatment, there was significant difference in SPPs among these five groups ($F = 34.374$, $P < 0.001$), the SPPs of the rats in groups DK1, DK2, and DK3 were significantly higher than those of group D ($P < 0.01$, respectively), but no difference was found among groups DK1, DK2, DK3. Detailed data is shown in Fig. 1(A).

3.2. Open-field test

Before ketamine treatment, there was a significant difference in horizontal ambulation (number of crossed squares) and times of rearing among these five groups ($F = 36.372$, $P < 0.001$ and $F = 42.549$, $P < 0.001$). Both horizontal ambulation and times of rearing in the four CUMS-treated groups (D, DK1, DK2, DK3) exhibited a significant decrease compared to that in group C ($P < 0.001$, respectively), while no difference was observed for horizontal ambulation or times of rearing between the four CUMS-treated groups. After ketamine treatment, there was a significant difference in horizontal ambulation and times of rearing among these five groups ($F = 132.478$, $P < 0.001$ and $F = 113.546$, $P < 0.001$). Compared to group D, groups DK1, DK2, and DK3 exhibited significantly increased horizontal ambulation and times of rearing ($P < 0.05$, respectively). In addition, group DK2 demonstrated higher horizontal ambulation and times of rearing than groups DK1 and DK3. Detailed data are shown in Fig. 1(B, C).

3.3. The expression levels of EAATs in the hippocampus

Western blot analysis was used to analyze and quantify the protein expression of EAATs in the hippocampus. There were significant differences in the expression levels of EAAT1, EAAT2, and EAAT3 among the five groups ($F = 5.829$, $P = 0.036$; $F = 87.593$, $P < 0.001$; $F = 138.354$, $P < 0.001$). Compared to group C, the CUMS-treated group (group D) showed significantly lower levels of expression for both EAAT2 and EAAT3 in the hippocampus ($P < 0.001$, respectively), but no difference was found for EAAT1 ($P = 0.105$). Ketamine can upregulate the expression of EAATs in the hippocampus. Compared to group D, the ketamine-treated groups (groups DK1, DK2, and DK3) exhibited significantly increased levels of expression for both EAAT2 and EAAT3 in the hippocampus ($P < 0.001$, respectively). However, compared to group D, the EAAT1 expression of groups DK1 and DK2 was significantly upregulated ($P < 0.05$, respectively), but no difference was found for group DK3 ($P = 0.187$). In addition, group DK3 demonstrated decreased expression levels of EAAT3 compared with groups DK1 and DK2 ($P < 0.05$, respectively). Detailed data are shown in Fig. 2 (A, B, C).

3.4. Concentration of extracellular glutamate in the hippocampus

There were significant differences in the concentration of extracellular glutamate among these five groups ($F = 153.542$, $P < 0.001$). Compared to group C, the CUMS-treated group (group D) showed significantly higher levels of extracellular glutamate in the hippocampus ($P < 0.001$). Ketamine can effectively reduce extracellular levels of glutamate in the hippocampus of depressive-like rats. Compared to group D, markedly decreased levels of extracellular glutamate were observed for ketamine-treated groups (groups DK1, DK2, DK3) ($P < 0.05$, respectively). However, the levels of hippocampal extracellular glutamate in group DK2 were lower than those of group DK3 ($P < 0.05$). Detailed data are shown in Fig. 2(D).

4. Discussion

The present study demonstrated that glutamate reuptake dysfunction is involved in the pathogenesis of depression. The expression of EAATs, especially EAAT2 and EAAT3, was obviously downregulated, and extracellular glutamate levels were significantly increased in the hippocampi of depressive-like rats. Sub-anesthetic doses of ketamine (10, 25, and 50 mg/kg) upregulated the expression of EAAT2 and EAAT3, decreased the concentration of extracellular glutamate in the hippocampus, and thus effectively alleviated the depressive-like behavior of rats. Additionally, we found that the antidepressant effect of ketamine was associated with its doses. For example, 25 mg/kg of ketamine showed a better antidepressant effect, and the antidepressant effect of ketamine gradually weakened with the increase in doses (50 mg/kg).

CUMS is a common method to establish an animal model of depression [12]. Anhedonia, namely the ability to experience pleasure decline, is a core symptom of depression, which can be evaluated in rodents by sucrose preference percentage (SPP) and exploratory activities [13]. In this study, the SPP and exploratory activities of CUMS-treated rats significantly decreased compared

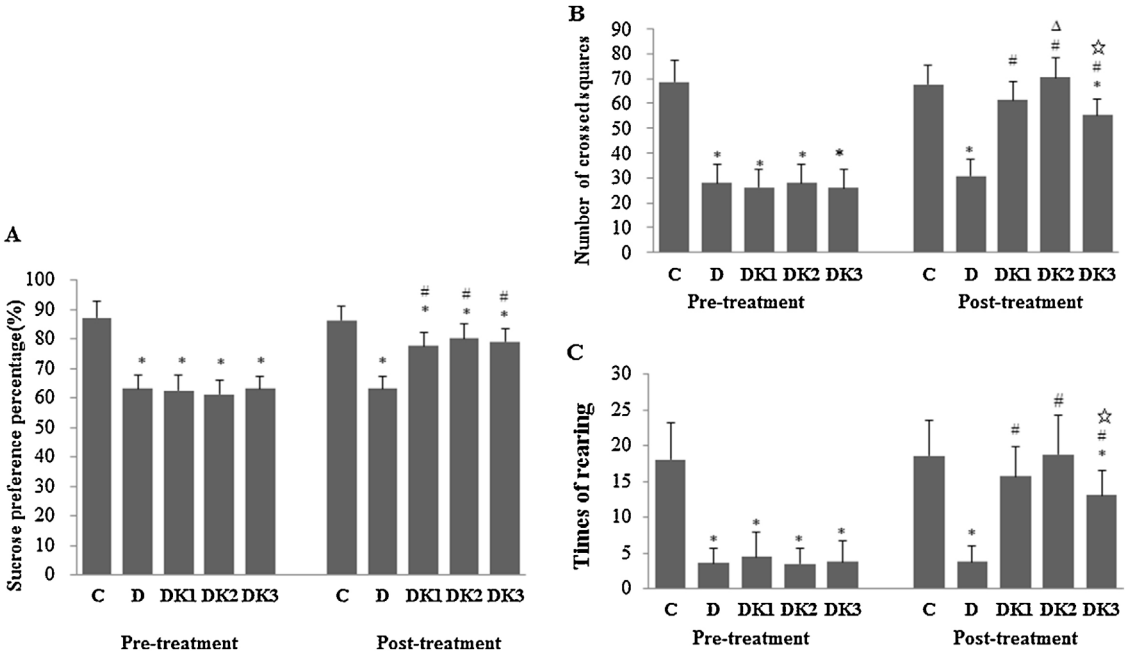

Fig. 1. The effects of ketamine on the behavior test of depressive-like rats. Group C: control group of healthy rats; Group D: CUMS-treated; Group DK1: CUMS + 10 mg/kg ketamine; Group DK2: CUMS + 25 mg/kg ketamine; Group DK3: CUMS + 50 mg/kg ketamine. (A), sucrose preference percentage (%). (B), number of squares crossed (indicating general locomotors). (C), times of rearing (indicating exploratory behavior).
The data for sucrose preference percentage (%) are presented as mean ± SD, n = 12/group. $^{*}P < 0.05$ compared with group C; $^{\#}P < 0.05$ compared with group D. Data are presented as mean ± SD, n = 12/group. $^{*}P < 0.05$ compared with group C; $^{\#}P < 0.05$ compared with group D; $^{\triangle}P < 0.05$ compared with group DK1; $^{\star}P < 0.05$ compared with group DK2.

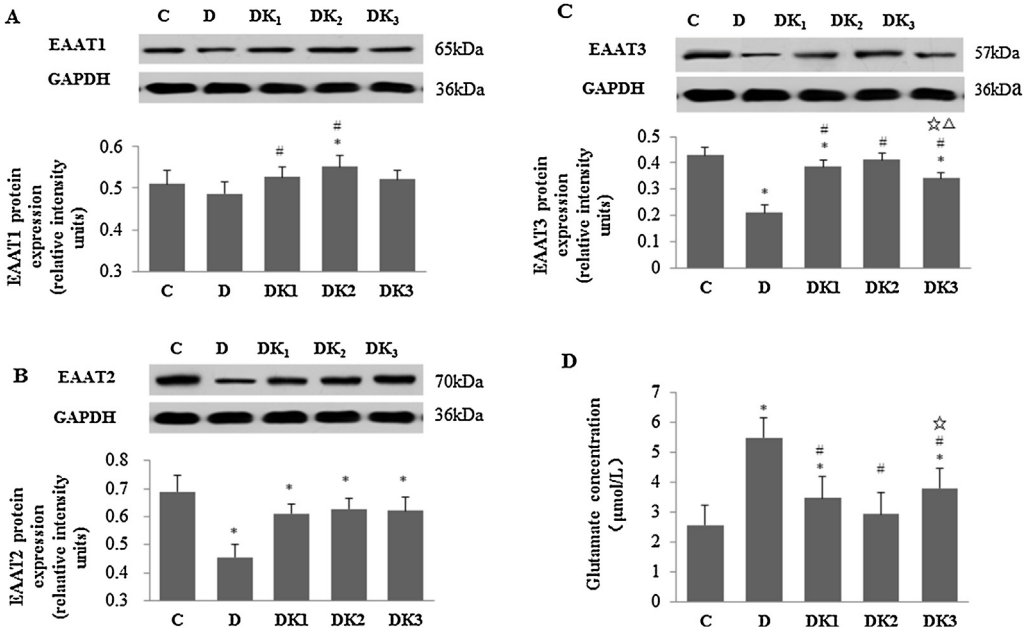

Fig. 2. Effects of ketamine on the protein expression of EAATs and extracellular concentrations of glutamate in the hippocampus of depressive-like rats. Group C: control group of healthy rats; Group D: CUMS-treated; Group DK1: CUMS + 10 mg/kg ketamine; Group DK2: CUMS + 25 mg/kg ketamine; Group DK3: CUMS + 50 mg/kg ketamine. Data are presented as mean ± SD, n = 6/group. GAPDH was used as the internal standard and control for protein loading. (A) The protein expression of EAAT1 in the hippocampus. (B) The protein expression of EAAT2 in the hippocampus. (C) The protein expression of EAAT3 in the hippocampus. (D) The extracellular concentrations of glutamate in the hippocampus. $^{*}P < 0.05$ compared with group C; $^{\#}P < 0.05$ compared with group D; $^{\triangle}P < 0.05$ compared with group DK1; $^{\star}P < 0.05$ compared with group DK2.

to those in the control group, which indicates that the model of depression was successfully established. In addition, our results showed that repeated administration of sub-anesthetic doses of ketamine (10, 25, and 50 mg/kg) significantly increased the SPP and exploratory activities of rats, and improved their depressive-like behavior. Our results are consistent with those of previous studies reporting that a single dose of ketamine possesses rapidly acting antidepressant properties [17].

In vitro studies have shown that some antidepressant drugs bind to NMDA receptors and inhibit the binding of NMDA receptor ligands [18]. Similarly, several research teams have reported that tricyclic antidepressants can modulate the release and/or

uptake of glutamate [19]. In this study, we found that EAAT expression was obviously downregulated, and glutamate uptake function decreased in the hippocampus of depressive-like rats. NMDA receptor antagonist ketamine reversed the depressive disorder-induced downregulation of EAAT expression and effectively alleviated the depressive-like behavior of rats. Our results further strengthened the opinion that glutamatergic system abnormality is an important pathologic basis of depressive disorder. In addition, previous studies have demonstrated that glutamate receptor activity can influence the expression of EAATs, and ionotropic glutamate receptor agonists decreased EAAT1 mRNA and protein levels [20,21]. Therefore, our results cannot rule out the possibility that ketamine's effects on EAAT expression are linked to its antagonistic effect on NMDA receptors. This requires further study.

Concerns about the psychomimetic effects of ketamine have limited its large-scale clinical application, especially for patients with mood disorders. However, preliminary clinical studies have shown the safety of sub-anesthetic doses of ketamine as an antidepressant [17]. Ketamine at 50 mg/kg dose has been considered as a sub-anesthetic or intermediate dose in humans and is most likely at the upper limit of the sub-anesthetic dose in rats[22]. In the present study, 25 mg/kg of ketamine showed a better antidepressant effect. When the dose increased to 50 mg/kg, the antidepressant effect of ketamine weakened. The reasons for this phenomenon may be attributed to the EAAT3 expression changes in the hippocampus. That is, the higher the expression of EAAT3 (25 mg/kg of ketamine), the better is the antidepressant effect.

Although glial transporters (EAAT1 and EAAT2) are believed to be responsible for most glutamate uptake [23], the importance of neuronal transporters (EAAT3) has recently been recognized. In certain brain regions such as the cerebral cortex and hippocampus, a large number of synapses are not surrounded by glial processes, suggesting that neuronal transporters also play an important role in glutamate uptake and synaptic function in these regions [24]. It has been well established that α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) trafficking and redistribution is crucial for synaptic plasticity [25], and the antidepressant effects of ketamine appear to be primarily mediated through regulating AMPA receptors [26]. Recent research has found that the EAAT3 plays a key role in AMPAR distribution and turnover. Inhibition of EAAT3 leads to rapid reduction in synaptic AMPAR accumulation and total receptor amount [21]. Combining this evidence and our results, we believe that neuronal transporters, such as glial transporters, play an important role in the antidepressant effect of ketamine.

Additionally, ketamine 50 mg/kg produced a smaller antidepressant effect than ketamine 10 or 25 mg/kg. A reasonable explanation for this is that higher doses of ketamine may excessively suppress synaptic *N*-methyl-D-aspartate receptor (NMDA) receptor activities, which interfere with normal synaptic transmission. It has been reported that NMDA receptors have dual characteristics: physiological or synaptic NMDA receptor activation induces neuroprotection, while pathological or extrasynaptic NMDA receptor activation mediates neurotoxicity [27]. Therefore, too much NMDA receptor activity is harmful to neurons, but too little is harmful as well. One limitation of this study is that our data cannot effectively reflect the dose-effect relationship of ketamine because the observation was restricted to three dosage groups.

In conclusion, this study revealed that glutamate uptake dysfunction is involved in the pathogenesis of depression, and a sub-anesthetic dose of ketamine has a good antidepressant effect. The antidepressant effect of ketamine may be partially attributed to upregulation of EAAT expression and enhanced glutamate reuptake in the hippocampus of depressive-like rats.

Conflict of interest

None.

Acknowledgements

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