## Original Article
# Ketamine impairs learning and memory ability in early developing mice via the hippocampal PKA/CREB signalling pathway

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Abstract: Objective: To investigate the effect of ketamine on the PKA/CREB signalling pathway in early developing mice and to explore the molecular mechanism of ketamine on the learning and memory ability impairment. Methods: Sixty Kunming mice weighing 18-25 g were randomly divided into two experimental groups, with 30 mice in each group. These 30 mice were then randomly subdivided into three subgroups (ketamine group, vehicle group and control group). Mice in the ketamine group underwent intraperitoneal injection of ketamine (50 mg/kg), whereas mice in the vehicle group were given an intraperitoneal injection of the same volume of normal saline. And these two groups both received these injections once daily for six consecutive days. However, mice in the control group received no injections. On the seventh day of the experiment, the learning ability of mice was measured via a step-down test and Morris water maze test. Hippocampus proteins were then extracted from the mice, and the activation and expression of proteins involved in the PKA/CREB signalling pathway were detected using Western Blot technology. Results: Inthe ketamine group, the latency period was shortened, error times were increased, the escape latency was prolonged, the expression of PKAcα was decreased and phosphorylated-CREB was reduced, as compared with the corresponding measures in the control group. Conclusion: Ketamine can impair the learning and memory ability in early developing mice, and its mechanism may be related to the inhibition of the hippocampal PKA/CREB signalling pathway.

Keywords: Ketamine, signalling pathway, learning and memory, early development of mice

## Introduction

Through the use of drugs or other methods, anaesthesia can cause a temporary loss of sensation for a patient's whole body or limited regions of the body, thereby making the patient feel no pain, and providing prerequisites for surgical therapies or other medical examinations and treatments [1]. As the derivative of phencyclidine, ketamine is a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist that was developed by Dr. Craig in Wayne State University. Ketamine has been widely used as an intravenous anaesthetic drug in clinical situations for many years, due to its sedative and analgesic properties [2]. Many experimental animal studies, however, have found that repeated application of ketamine can cause short-term and long-term learning and memory dysfunction in early developing mice [3, 4]. Clinical and experimental studies have reported that ketamine can also cause schizophrenia-like symptoms and cognitive impairment in people [5]; however, the molecular biological mechanism of ketamine-induced learning and memory dysfunction remains unclear at present. Some studies have claimed that the learning and memory dysfunction caused by ketamine may be associated with the restraint of hippocampal long-term potentiation (LTP) formation, reduction of the expression of the acetylcholine synthesising choline acetyltransferase enzyme and inhibition of the PKA/CREB signalling pathway [6].

The PKA/CREB signalling pathway has a close relationship with the learning and memory ability. Cyclic adenosine monophosphate (cAMP) can activate protein kinase A (PKA) to phosphorylate the target enzyme, triggering gene

expression. Meanwhile, cAMP response element-binding protein (CREB) is a transcription and transduction factor that is regulated by the cAMP available in cells, and its downstream efficacy includes influencing the survival and growth of neurons, the plasticity of synapses and the formation of long-term memory, which can thereby affect the learning and memory ability [7-10]. In this study, we aimed to investigate the change of PKA/CREB signalling pathway in the mice hippocampi under the repeated ketamine anaesthesia, and its effects on learning and memory ability in early developing mice, so as to explore the hidden connection.

## Materials and methods

### Laboratory animals, main reagents and equipments

Sixty healthy Kunming (KM) mice (weighing 18-25 g, regardless of gender, free diet and water intake) and a ketamine hydrochloride injection (NMPN: H32022820, approval number: KH071004, 2 mL: 0.1 g) were purchased from the experimental animal centre of Southern Medical University. Other laboratory equipments and reagents included a YLS-3T jumping apparatus (Anhui Huaibei Zhenghua Biologic Apparatus Facilities Limited Company), a Morris water maze (MWM; Coulbourn Instruments Inc., USA), rabbit anti-CREB mAb and rabbit anti-p-CREB (pS133) mAb (Abcam, USA, approval number: ab32096), PKAcα antibody (Beijing SinaSun Biological Technology Co., Ltd, approval number: MA119732), mouse anti-βActin mAb (Santa Cruz Biotechnology Inc., approval number: CW0096), peroxidase-conjugated AffiniPuregoat anti-rabbit IgG (approval number: ZB2301) and goat anti-mouse IgG (Millipore Corp., approval number: AP136P), and a BCA Protein Assay Kit (Jiangsu Beyotime Institute of Biotechnology). All antibodies mentioned above were diluted 1:1000.

### Methods

*Animal grouping:* Sixty KM mice were equally divided into two groups at random. The step-down test and MWM test were respectively employed in the two groups. Each of these two groups was then randomly divided into three equally-sized sub-groups (ketamine group, vehicle group and control group), with ten mice

in each small group. Mice in the ketamine group were given an intraperitoneal injection of ketamine (50 mg/kg), whereas mice in the vehicle group underwent an intraperitoneal injection of the same volume of normal saline, and both groups received an injection every morning for six consecutive days. Mice in the control group, however, received no injections.

*Step-down test and MWM test:* In the step-down experiment, we used opaque dark grey plastic to form five chambers, with each chamber being 120 mm by 120 mm by 180 mm, and being equipped with 36 V electric grid voltage and 0.25 mA electric current. On the seventh day after drug application, mice in the step-down group (n=30) were placed in the reaction chamber to adapt for 3 min. The electric grid at the bottom of chamber was then energised. The time that it took for mice to jump to the platform for the first time was within 5 min (latency period of learning) and the frequency of the electrical stimulation caused by the electric grid after jumping down from the platform (error times of learning) were recorded. The next day, these mice were trained for 3 min in line with the above experimental operations after adaptation, and their latency period of learning and error times were also recorded. If the mice did not jump off the platform within 3 min, error times were recorded as 0 and the latency period of learning was recorded as 180 s. The operations mentioned above were repeated at the same time for four consecutive days and the data were recorded accordingly.

Similarly, the MWM experiment was started on the seventh day after drug application to measure the memory consolidation capacity of mice. The water maze was a black circular pool (1 min diameter and 0.6 m deep), filled with water to a depth of 0.3 m, which was divided into four equal quadrants. Moreover, four fixed points set on the inner wall at each compass point (north, south, east and west) were regarded as the starting points for mice. A platform was placed 2 cm below water level in one of the quadrants, and cameras were installed in the maze to record the movement trail of mice synchronously. The water temperature was kept constant at 24±1°C and reference objects remained unchanged. The navigation test was performed four times daily, and every time the mice were required to enter the water from the

**Table 1.** The latency period and error times (mean ± SEM) for learning and memory ability in each of three treatment groups of mice subjected to a step-down test

| Group | Number | Learning | | Memory | |
|---|---|---|---|---|---|
| | | Latency period | Error | Latency period | Error |
| Ketamine group | 10 | 13.42±4.58* | 2.83±1.51** | 55.36±36.67* | 1.60±0.88** |
| Vehicle group | 10 | 19.76±10.16 | 2.03±1.82** | 102.85±59.34 | 1.06±0.96** |
| Control group | 10 | 27.28±19.66 | 0.83±0.92 | 145.16±37.03 | 0.59±0.56 |

Note: Compared with the control group, *P<0.05, **P<0.01.

fixed point and allowed to search for 90 s. If they found the platform successfully, they were required to stay on it for 20 s. But if they failed, they were also guided to the platform and then required to stay on it for 20 s. The average value of time that mice spent reaching the platform in four attempts was taken to be their escape latency for the day. And this test was conducted for four consecutive days.

*WB technology detection:* After finishing the experiments mentioned above, all 60 mice were anaesthetised with chloral hydrate, during which the low concentration oxygen (oxygen fluxes 2 L•min$^{-1}$) was supplied. The skulls of these mice were exposed and brain samples were then taken. The hippocampal tissues of mice were isolated, RIPA lysis buffer and PMSF were added, and then each sample was adequately homogenised on ice using an ultrasonic homogeniser. After being centrifuged at 12,000 rpm at 4°C for 15 min, a supernatant liquid was obtained and stored at -80°C. Protein quantification was then conducted using bicinchoninic acid (BCA). Next, a polyacrylamide gel was prepared, samples were added for electrophoresis, proteins were transferred, and the membranes were washed and blocked. The first antibody was added after rinsing with washing buffer for 3 times*10 min, and the corresponding secondary antibody was added after rewarming for 30 min and rinsing with washing buffer for 3 times*10 min the next day. Samples were then incubated on a slow speed shaker at room temperature for 2 h. Afterwards, they were placed in a dark room to allow ECL exposure after rinsing with washing buffer for 3 min*10 min. The reaction was finally terminated by washing with deionised water after tablet compressing, and then the film was taken out for scanning. The results were analysed using Image ProPlus, the image analysis system. The relative grey of each protein band was analysed, the optical density value of each

group was calculated, and the relative expression level of each target protein was expressed as the ratio of target protein and reference grey. Moreover, the expression levels of PKAcα and P-CREB in the hippocampal tissues of mice in each group were detected.

*Statistical analysis*

SPSS 13.0 software was used to analyze the experimental data, and Graph PAD Prism 5.0 was applied to generate the summary graph. All measurement data were expressed as mean ± standard error (mean ± SEM). Comparison between the two main groups was carried out by using the t test, and the data were tested for homogeneity of variance. Comparison among all groups was performed by using the single factor analysis of variance and comparison of two groups was conducted using the SNK test. Data at different time points in the MWM experiment were compared by using a two-way repeated measure ANOVA design (time was the factor in groups, whereas comparison was the factor between groups). Differences were regarded to be statistically significant when the inspection level was α=0.05 and P<0.05.

**Results**

*Results of step-down experiment*

Based on the average results of learning ability tests during three consecutive days one week after drug application and the memory ability test at the tenth day after drug application, we found that error times were increased in both the vehicle group (P=0.008) and the ketamine group (P=0.0005) in comparison with the control group. In addition, the latency period was shortened in the ketamine group when compared with the control group (P=0.024; **Table 1**).
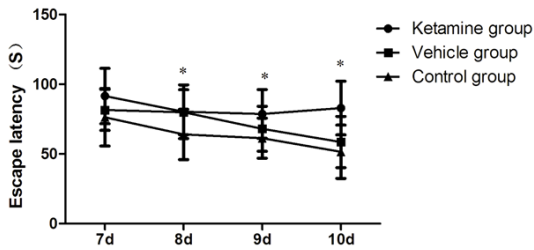
**Figure 1.** A comparison of the escape latency of mice in different treatment groups at different periods of time post injection (7-10 days after), tested using the Morris water maze. *P<0.05, ketamine group compared with control group or with vehicle group.
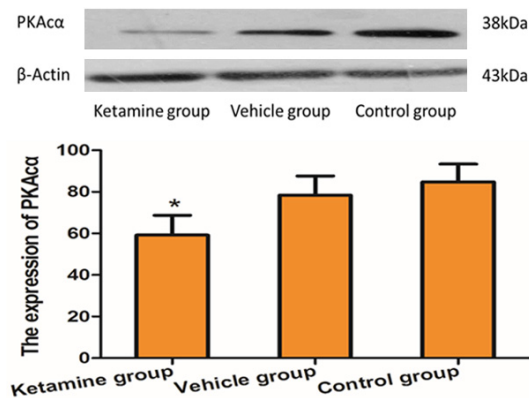


**Figure 2.** The expression of PKAcα (mean ± SEM) in mice in each treatment group (n=10 for each group). *P<0.05, compared with control group.



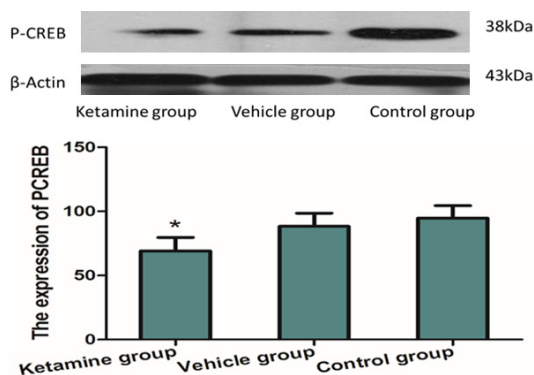**Figure 3.** The expression of P-CREB (mean ± SEM) in mice in each treatment group (n=10 for each group). *P<0.05, compared with control group.

*Results of MWM experiment*

In the water maze experiment that performed a week after drug application, there were no statistically significant differences in the escape latency of mice among groups. From the seventh to tenth day, the indexes were recorded

every day. And it was found that the escape latency of mice in the ketamine group was obviously prolonged as compared with that of those in the control group (P=0.036; **Figure 1**). Additionally, the differences between the ketamine group and the vehicle group were not statistically significant (P=0.803), whereas there was no significant difference between the vehicle group and the control group (P=1.056).

*Results of WB technology detection*

The expressions of both PKAcα proteins and P-CREB proteins in hippocampal tissues of mice were detected by using the WB technology. After ten days of drug application in mice, the expression of PKAcα proteins in hippocampal tissues of mice in the ketamine group was significantly decreased when compared with that of mice in the control group (P<0.05; **Figure 2**). Similarly, compared with the control group, the expression of P-CREB proteins in hippocampal tissues of mice in the ketamine group was significantly decreased (P<0.05; **Figure 3**).

**Discussion**

With the development of anaesthesiology, a variety of narcotic drugs have appeared in the clinical stage. Ketamine in particular has been widely used in various types of anaesthesia for surgical patients, especially in children, since it is a safe drug. Many researches have found, however, that ketamine can impair the learning and memory ability which are advanced functions of the brain. Learning refers to a neural process during which individuals and their movements can depend on experiences to change self-behaviours and adapt to the environment. Memory is a neural process during which individuals store and read out learned information, and it is also a fundamental and significant behavioural function in humans. In recent years, the reasons and mechanisms involved in ketamine-induced learning and memory impairment have therefore been clinical and basic research hotspots.

In this study, we applied the step-down test and MWM experiment to detect the learning and memory ability of mice. When compared with the mice in the control group, mice that underwent repeated intraperitoneal injections of a basic anaesthetic dose of ketamine had the shortened latency period for learning and memory, increased errors times and prolonged

escape latency. These differences were statistically significant, suggesting that ketamine can lead to the obvious learning and memory ability impairment in mice. This result is consistent with the research of Yang et al., who found that after ketamine anaesthesia, the time necessary for learning and memory in mice was longer, and their error times during step-down and MWM experiments increased [11, 12].

Some foreign literature has reported that the process of learning and memory is associated with the connection of a loop between neurons, the transfer of neurotransmitters and the plasticity of synapses. Furthermore, it has been found that LTP can typically result in the change of synapse plasticity, and the PKA/CREB signalling pathway can regulate extensive biological functions, including learning and memory ability [13]. A meta-analysis of related articles conducted by Setlow et al. indicated that cAMP can phosphorylate the target enzyme mainly by activating PKA, and PKA with catalytic activity can play a pivotal role in long-term synaptic plasticity and long-term memory [14]. PKAcα, one of the subunits of PKA, has been found to be abundantly expressed in neurons and essential for synapse plasticity. Activation of CREB phosphorylation is a convergence point for multiple signal transduction pathways, and the phosphorylated CREB (P-CREB) formed after activation can cause various long-term biological effects in cells, including learning and memory ability [15, 16]. The PKA/CREB signalling pathway therefore plays an important role in many aspects, such as neuronal development, synapse plasticity, learning and memory ability, injury and regeneration; in turn, the protein expressed by this signalling pathway can also reflect the conditions necessary for learning and memory ability [17].

In this study, it was found that the expressions of both PKAcα and P-CREB in the ketamine group were significantly decreased when compared with that in the vehicle and control groups. The results suggested that the learning and memory ability impairment of mice in the ketamine group might be related to the ketamine-induced inhibition of the PKA/CREB signalling pathway. Previous studies have indicated that the mechanism may be related to the nature of ketamine, as it is a NMDA receptor antagonist and, as such, can inhibit extracellular calcium ion influx. Additionally, the PKA/

CREB signalling pathway is a calmodulin ($Ca^{2+}$/CaM)-dependent pathway, as $Ca^{2+}$/CaM can inhibit CREB.

The results of our study were in line with those of Axel et al. and Lin et al. [18, 19]. The results of our Western Blot analysis showed that the expressions of P-CREB and PKAcα in the hippocampi of mice in the ketamine group were significantly decreased when compared with those of the vehicle and control groups. The research of Rajagopal et al. also have found that repeated injections of ketamine can likely affect the learning and memory ability in mice, and the reduction of P-CREB expression may be the mediating factor [20]. Furthermore, in our study, we also observed that in the step-down experiment, the error times of mice in the vehicle group were significantly increased. This probably because that the mice had not fully entered the anaesthetic state after acupuncture stimulation and were in a state of stress. Once the mice were released, they would be eager to break free, thereby ignoring the effects of electric shock. The study presented by Arendt et al. found that animals' learning and memory abilities can be affected significantly when they are under strong acute stress or long-term chronic stress [21]; however, the specific mechanism remains unclear at present.

In conclusion, this study found that ketamine may cause the learning and memory ability impairment by inhibiting the expressions of the proteins PKAcα and P-CREB.

**Disclosure of conflict of interest**

None.

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**References**

[1] Liu RJ, Lee FS, Li XY, Bambico F, Duman RS and Aghajanian GK. Brain-derived neurotrophic factor Val66Met allele impairs basal and ketamine-stimulated synaptogenesis in prefrontal cortex. Biol Psychiatry 2012; 71: 996-1005.

[2] Peng S, Zhang Y, Zhang J, Wang H and Ren B. Effect of ketamine on ERK expression in hip-

pocampal neural cell and the ability of learning behavior in minor rats. Mol Biol Rep 2010; 37: 3137-3142.

[3] Zhang LM, Zhou WW, Ji YJ, Li Y, Zhao N, Chen HX, Xue R, Mei XG, Zhang YZ, Wang HL and Li YF. Anxiolytic effects of ketamine in animal models of posttraumatic stress disorder. Psychopharmacology (Berl) 2015; 232: 663-672.

[4] Wang E, Feng Y, Zhang M, Zou L, Li Y, Buys ES, Huang P, Brouckaert P and Chao W. Toll-like receptor 4 signaling confers cardiac protection against ischemic injury via inducible nitric oxide synthase- and soluble guanylate cyclase-dependent mechanisms. Anesthesiology 2011; 114: 603-613.

[5] Wang G, Bochorishvili G, Chen Y, Salvati KA, Zhang P, Dubel SJ, Perez-Reyes E, Snutch TP, Stornetta RL, Deisseroth K, Erisir A, Todorovic SM, Luo JH, Kapur J, Beenhakker MP and Zhu JJ. CaV3.2 calcium channels control NMDA receptor-mediated transmission: a new mechanism for absence epilepsy. Genes Dev 2015; 29: 1535-1551.

[6] Pardue MT, Faulkner AE, Fernandes A, Yin H, Schaeffel F, Williams RW, Pozdeyev N and Iuvone PM. High susceptibility to experimental myopia in a mouse model with a retinal on pathway defect. Invest Ophthalmol Vis Sci 2008; 49: 706-712.

[7] Campos CA, Shiina H, Silvas M, Page S and Ritter RC. Vagal afferent NMDA receptors modulate CCK-induced reduction of food intake through synapsin I phosphorylation in adult male rats. Endocrinology 2013; 154: 2613-2625.

[8] Zhu MJ, Du M, Nijland MJ, Nathanielsz PW, Hess BW, Moss GE and Ford SP. Down-regulation of growth signaling pathways linked to a reduced cotyledonary vascularity in placentomes of over-nourished, obese pregnant ewes. Placenta 2009; 30: 405-410.

[9] Galeotti N, Stefano GB, Guarna M, Bianchi E and Ghelardini C. Signaling pathway of morphine induced acute thermal hyperalgesia in mice. Pain 2006; 123: 294-305.

[10] Peng S, Zhang Y, Wang H, Ren B and Zhang J. Anesthetic ketamine counteracts repetitive mechanical stress-induced learning and memory impairment in developing mice. Mol Biol Rep 2011; 38: 4347-4351.

[11] Yang MY, Ding F, Jiang XG, Gu ZL and Bian SZ. Effects of ketamine on ethanol-induced learning and memory impairment in mice. Journal of Nantong University 2013.

[12] Sharma AC and Kulkarni SK. Effects of MK-801 and ketamine on short-term memory deficits in passive avoidance step-down task paradigm in mice. Methods Find Exp Clin Pharmacol 1991; 13: 155-159.

[13] Song SP, Ma RR, Liu YJ, Jiang YN, Wang HH and Dai TJ. The effect of remifentanil combined with ketamine on the learning and memory function in mice. Acta Academiae Medicinae Xuzhou 2010.

[14] Wang H, Zhang Y, Zhang JN, Ren BX, Liu Y and Zeng YM. Effects on ketamine on learning and memory behavior and BDNF expression in immature mice. Journal of Clinical Anesthesiology 2008.

[15] Yang MY, Ding F, Jiang XG, Wu XX, Gu ZL, Guo CY and Bian SZ. [Effects of ketamine and alcohol on learning and memory impairment in mice]. Fa Yi Xue Za Zhi 2012; 28: 115-119.

[16] Xu XL, Wen J, Dai TJ and Ren XM. Effects of repeated ketamine on weight, autonomic activities, learning and memory in mice. Acta Academiae Medicinae Xuzhou 2008.

[17] Uchihashi Y, Kuribara H, Isa Y, Morita T and Sato T. The disruptive effects of ketamine on passive avoidance learning in mice: involvement of dopaminergic mechanism. Psychopharmacology (Berl) 1994; 116: 40-44.

[18] Guskjolen A, Josselyn SA and Frankland PW. Age-dependent changes in spatial memory retention and flexibility in mice. Neurobiol Learn Mem 2017; 143: 59-66.

[19] Lin L and Huang CP. Research on the ability of learning and memory behavior of mice after using ketamine anesthesia. Journal of Wenzhou Medical College 2006.

[20] Rajagopal L, Burgdorf JS, Moskal JR and Meltzer HY. GLYX-13 (rapastinel) ameliorates subchronic phencyclidine- and ketamine-induced declarative memory deficits in mice. Behav Brain Res 2016; 299: 105-110.

[21] Arendt T. Synaptic plasticity and cell cycle activation in neurons are alternative effector pathways: the Dr. Jekyll and Mr. Hyde concept of Alzheimer's disease or the yin and yang of neuroplasticity. Prog Neurobiol 2003; 71: 83-248.