Research Paper

Ketamine Promotes Alzheimer's-Like Neurodegeneration by Activating Glycogen Synthase Kinase 3 Beta and Inhibiting Protein Phosphatase 2A

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Fan: Ketamine Promoting Alzheimer-Like Neurodegeneration in Mice

To investigate the mechanism of ketamine promoting Alzheimer's-like neurodegeneration in mice. 24 male 8 w old Kunming mice of specific pathogen-free grade were randomly divided into a blank control group (control group) and a ketamine intervention group (experimental group) according to the random number table, 12 mice in each group. The mice in the experimental group were given ketamine injection at the dose of 30 mg/kg. Mice in the control group were given an equal volume of normal saline injection. The intervention was conducted once a day for consecutive 6 mo. The behavioral changes of the mice in the two groups were compared. Western blot was used to detect the expression levels of tau (phospho Thr231), tau (phospho S396), tau (phospho Ser404), glycogen synthase kinase 3 beta, glycogen synthase kinase 3 beta (phospho ser9) and protein phosphatase 2A proteins in the brain tissue of hippocampus and the ratio of phosphorylated tau protein to tau protein was calculated. The expressions of beta-amyloid protein and tau protein in the hippocampus were observed by immunohistochemistry. The results of the Morris water maze showed that the escape latency of mice in both groups was gradually decreased and the escape latency of mice in the control group was lower than that in the experimental group. Besides, the times of crossing target platform and the proportion of activity time in target quadrant of mice in the experimental group were lower than those in the control group. At the same time, compared with the control group, the expression levels of tau (phospho Thr231) and tau (phospho Ser404) were decreased in the experimental group, and the expressions of tau (phospho S396), glycogen synthase kinase 3 beta, glycogen synthase kinase 3 beta (phospho ser9) and protein phosphatase 2A proteins were increased. Compared with the control group, the expression of beta-amyloid protein in the experimental group was significantly reduced and the opalescence density of beta-amyloid protein was significantly lower than that in the control group ($p<0.05$). Long-term use of ketamine can lead to up-regulated expression of beta-amyloid protein and tau protein in mice hippocampus, which may induce hyperphosphorylation of tau protein at Thr231, S396 and S404 by activating glycogen synthase kinase 3 beta and inhibiting protein phosphatase 2A, causing cognitive function decline in mice.

Key words: Ketamine, cognitive function, tau protein, glycogen synthase kinase 3 beta, beta-amyloid protein, neurodegeneration

Ketamine is a kind of non-phenobarbital intravenous anesthetic. Thanks to the advantages including low respiratory inhibition, short duration, fast absorption and quick anesthesia, it is widely used in clinical practice[1,2]. Due to its mental dependence and hallucinogenic effect, ketamine has become a type of synthetic drug abused in China and has been included in psychotropic drugs. Studies have proven that ketamine can damage the cognitive, executive, visual-spatial ability and other cognitive functions of abusers through N-Methyl-D-Aspartate (NMDA) receptors, monoamine receptors, acetylcholine receptors, voltage-gated calcium channel receptors, opioid receptors and so on[3,4]. Tau protein is a microtubule-associated protein mainly distributed in axons and dendrites of neurons, which has the functions of regulating the growth and development of neurons, maintaining the morphology and stability of microtubules[5]. Beta (β)-amyloid protein is formed by cleavage of β-amyloid precursor protein by secretase, which mainly resides in astrocytes and neurons. It is

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Accepted 10 May 2022
Revised 24 October 2021
Received 25 January 2021
Indian J Pharm Sci 2022;84(3):569-574

neurotoxic and causes cell damage and death by causing hyperphosphorylation and prominent changes of tau protein[6]. Studies have proven that the up-regulation of tau protein and β-amyloid protein expression is related to ketamine-induced cognitive impairment[7]. In this study, a ketamine abuse model was established to explore the mechanism of cognitive impairment caused by abusing ketamine, so as to find a new target for clinical treatment.

MATERIALS AND METHODS

Experimental animals:

Twenty-four 8 w old male Kunming mice of Specific Pathogen-Free (SPF) grade were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd, approval No: SCXK (J) 2016-0011. The mice were fed adaptively in the animal room with a room temperature of 22°±1° and a light/dark cycle of 12 h. They were free to eat or drink.

Main experimental instruments and reagents:

Main experimental instruments: Upright fluorescent microscope (Olympus, Japan), embedding machine (Tianzhirui Medical Technology, China), slicing machine (Leica, Germany), -80° refrigerator (Haier, China), drying oven (Senxin Experimental Instrument Co., Ltd., China), centrifuge (Xiangyi Laboratory Instrument Development Co., Ltd., China).

Main experimental reagents: Preparation of 30 mg/ml ketamine solution by ketamine hydrochloride injection (Fujian Gutian Pharmaceutical Co., Ltd., batch No.: H35020148, specification: 100 mg/2 ml) and normal saline were blended by a ratio of 3:2 and then stored in the refrigerator at 4°. Radioimmunoprecipitation Assay (RIPA) protein lysate (Biyuntian Biotechnology, China), protease inhibitor mixture (Millipore, USA), Glycogen Synthase Kinase 3β (GSK-3β) antibody (Merck, Germany) and Protein Phosphatase 2A (PP2A) antibody (Merck, Germany).

Experimental methods:

Animal grouping and intervention methods: Using the random number table, the mice were randomized into two groups as blank control group (control group) and ketamine intervention group (experimental group), with 12 mice in each, they were intervened following 2 w of adaptive feeding.

The mice were fixed in the position of low tail and high head to migrate their viscera and the needle was inserted at the depth of 5 mm from the white line of their lower abdomen, the syringe was pushed forward subcutaneously 2 cm at an angle of 45°. The feeling of negative pressure attraction of the abdominal cavity without foreign body suction indicates successful insertion and then the injection can be performed. The left and right abdomen was injected alternately. The mice in the experimental group were injected with ketamine at a dose of 30 mg/kg; those in the control group were injected with the same volume of normal saline. Following the injection, the mice in each group were put into a separate cage to observe their skin color to prevent hypoxemia. After the righting reaction was restored in each group, the mice were replaced into the cage. The above-mentioned interventions were performed once a day for consecutive 6 mo.

Morris water maze: After 6 mo of intervention, Morris water maze test was carried out on the mice. A pool with a diameter of 1 m and a depth of 0.4 m was selected and the water temperature was 20°±1°. Note that the experimental environment should be quiet and not affected by external factors. A target platform with a diameter of 5 cm was placed in the target quadrant, about 1 cm below the water surface. 1 d before the formal experiment, each mouse in the two groups was put into the water for swimming for 1 min in order to adapt to the environment. The formal experiment lasted for 6 d, the mice were trained from 1 to 5 d. Facing the pool wall, they were put into in the pool from the entry points in the 1st to 4th quadrants and the time required for the mice to climb the platform in the target quadrant, namely escape latency, was recorded. If the mice did not climb onto the target platform within 60 s, it was recorded as 60 s, and they was artificially guided to the platform and stayed for 10 s. A place navigation test was carried out on the 6th d and the mice were put into water from entry point at the opposite side of the target quadrant, and the escape latency was recorded. Then the platform was removed and the mice were put into water from entry point at the opposite side of the target quadrant. The frequency of crossing the platform within 60 s and the residence time in the target quadrant was recorded.

Brain tissue sampling: The mice were anesthetized by intraperitoneal injection of chloral hydrate at the dose of 300 mg/kg and then their limbs were fixed. The chest was dissected to expose the heart, the needle was inserted at the apex beat of the left ventricle, the right atrial appendage was cut open and normal saline and heparin were injected until the liver turned white.

Finally, they were perfused with 4 % paraformaldehyde solution, the brain was taken out on the ice after the tail and limbs were stiffened and then the head was amputated. With reference to the stereotaxic atlas for mouse brain and the hippocampal tissue was bluntly separated along the coronal plane on the posterior side of the pituitary gland.

If the Western blotting experiment was carried out, the mice would be anesthetized, decapitated and the brain tissue was taken out on the ice to separate the hippocampal tissue. The stripped hippocampal tissue was placed in a cryopreservation tube and frozen at -80° with liquid nitrogen.

Western blotting test: The Phenylmethylsulfonyl Fluoride (PMSF) protease inhibitor of 100 mmol/l concentration was mixed with RIPA protein lysate at the ratio of 1:100 and the hippocampal tissue was weighed. The mixture obtained was added to hippocampal tissue at the ratio of 100 µl lysate per 100 mg tissue and the test tube was placed in ice-water mixture for homogenization. After centrifuging for 15 min at 4° and 12 000 rad/min, the supernatant was collected. The protein concentration of each sample was determined by Bicinchoninic Acid (BCA) assay method and the lysate was used to balance the concentration. The 5x loading buffer was added to the protein sample, at the ratio of 1:4. It was denatured in boiling water at 100° for 10 min, then cooled to room temperature and stored at -20°. After mixing the same amount of protein samples from each group, the sample was injected into the sodium dodecyl sulfate–polyacrylamide gel electrophoresis system with a pipette and treated for 30 min at constant pressure of 85 V, then for 85 min at 115 V and the electrophoresis ended when bromophenol blue ran to 1 cm at the bottom of the glass plate. Then the membrane was transferred and was treated for 35 min with 25 V. Following the transfer, the target protein area was cut in the Polyvinylidene Difluoride (PVDF) membrane, then placed into the confining liquid after washing off the transfer buffer and then oscillated gently on the shaker. It was incubated overnight at 4° after the addition of the primary antibody. After rewarming at 30° at room temperature, the PDVF membrane was rinsed for 3 times, 10 min each and then placed in the secondary antibody and incubated on the shaker for 1 h at room temperature, and then the PDVF membrane was rinse again, 10 min each time, for a total of 3 times. The PDVF membrane was placed in the gel imager; an appropriate amount of chemiluminescence substrate was dripped into on the surface of the membrane and then exposed later. The protein bands were scanned and analyzed by Image J software to calculate the gray level.

Immunohistochemical test: After the separation, the hippocampus was put into 4 % paraformaldehyde and fixed for 48 h at 4°. The tissue was placed in an embedding cassette and washed with running water for 30 min. After gradient dehydration with alcohol, transparent treatment with xylene and paraffin embedding were performed and then it was cooled and solidified for standby. After the tissue was trimmed to expose the observation surface, the slice thickness of the slicer was set to 5 µm and continuous slices were made along the coronal plane. After spreading the slices, they were placed in the thermostat at 65° overnight. The dewaxing and rehydration were performed, then an appropriate amount of sodium citrate repair solution was added to the antigen repair box and then the repair box was heated to above 90° in a pressure cooker. The slices were placed in the box, heated to boiling for 8 min, then cooled at room temperature, rinsed with Phosphate Buffered Saline (PBS) for 5 min each time, a total of 3 times. 3 % hydrogen peroxide was added to the slices, incubated for 10 min at room temperature, then rinsed with PBS for 5 min each time, a total of 3 times. 10 % goat serum was added to the slices and was sealed for 30 min at room temperature. After removing the confining solution, primary antibody was added and sealed at 4° overnight. After rewarming 30 min at room temperature, rinsed with PBS for 3 min each time, a total of 3 times. An appropriate amount of polymer enhancer was added into the slices, incubated at room temperature for 30 min and rinsed for 5 min each time, a total of 3 times. After removing the PBS buffer, an appropriate amount of 3,3′-Diaminobenzidine (DAB) substrate was added to the slices. The staining can be terminated after obvious stain was observed under the microscope. After gradient dehydration with alcohol, the slices were treated transparently with xylene, sealed with neutral sizing agent, then observed and preserved after drying. The optical density of the sample photos was measured semi-quantitatively by Imagine-Pro Plus software.

Statistical method:

Statistical Package for the Social Sciences (SPSS) 20.0 was used for statistical analysis of the data, the counting data was presented by the mean±standard deviation and the t-test was performed. $p<0.05$ means the difference is of statistical significance.

RESULTS AND DISCUSSION

It was shown that the escape latency of the two groups decreased gradually and that of the experimental group was longer than that of the control group, suggesting statistically significant difference ($p<0.05$) as shown in Table 1. The frequency of crossing the target platform and the proportion of activity time in the target quadrant in the experimental group were lower than those in the control group, suggesting statistically significant difference ($p<0.05$) as shown in Table 2.

The findings indicated that compared with the control group, the expression of tau (phospho Thr231) and tau (phospho Ser404) protein decreased, while the expression of tau (phospho S396) protein, GSK-3β protein, GSK-3β (phospho Ser9) protein and PP2A protein increased in the experimental group, and the difference was statistically significant ($p<0.05$) as shown in Table 3.

The results indicated that compared with the control group, tau (phospho Thr231)/tau and tau (phospho Ser404)/tau decreased, while tau (phospho S396)/tau increased in the experimental group as shown in Table 4.

The findings suggested that expression of yellow or brown β-amyloid protein and tau protein were positive in both groups. Compared with the control group, the expression of β-amyloid protein decreased significantly and that of tau protein increased significantly in the experimental group ($p<0.05$). And compared with the control group, the optical density of β-amyloid protein was significantly lower and that of tau protein was significantly higher in the experimental group ($p<0.05$) as shown in Table 5.

Ketamine, a commonly used anesthetic in clinical practice, has become a recreational drug abuse due to its hallucinogenic effect and psychological dependence[8]. Studies have shown that ketamine abuse has obvious toxic side effects on nervous system and mental function, mainly manifested by cognitive impairment, such as memory and cognitive decline, slow response, decreased computing power and other neurodegenerative changes[9].

Cognitive function is one of the most basic and important functions of the nerve center, which is regulated by many brain regions such as cerebral cortex, hippocampus, corpus striatum and so on. When the cognitive function is impaired, the related learning, memory, thinking and other processes are abnormal, such as memory impairment, usually with agnosia, aphasia and other changes[10,11]. The hippocampus is located in the deep layer of the brain tissue and belongs to the limbic system. It is the center of information processing, is closely related to memory, learning and other cognitive functions and focuses on regulating spatial memory and short-term memory[12]. Therefore, the hippocampus was used as the target brain region for this study and the mechanism of Alzheimer's-like neurodegenerative disease in mice was investigated by establishing a ketamine abuse model.

Morris water maze is one of the commonly used behavioral experimental methods, which evaluates the abilities of mice such as learning, spatial positioning and spatial memory by testing the parameters of their movement to the target platform and quadrant[13]. The results showed that compared with the control group, the escape latency of mice was higher and

TABLE 1: COMPARISON OF ESCAPE LATENCY BETWEEN TWO GROUPS OF MICE (x̄±s)

Group	Number of cases	D 1	D 2	D 3	D 4	D 5
Control group	12	41.68±8.63	33.25±7.16	29.25±7.73	25.12±7.06	21.62±6.24
Experimental group	12	49.21±9.08	41.20±7.53	36.72±8.30	32.78±6.81	29.30±6.95
t		2.082	2.65	2.281	2.705	2.880
p		0.049	0.015	0.033	0.013	0.009

TABLE 2: COMPARISON FOR THE FREQUENCY OF CROSSING THE TARGET PLATFORM AND THE PROPORTION OF ACTIVITY TIME IN THE TARGET QUADRANT BETWEEN THE TWO GROUPS (x̄±s)

Group	Number of cases	Frequency of crossing the target platform (times)	Percentage of activity time in the target quadrant (%)
Control group	12	2.75±0.73	25.03±6.83
Experimental group	12	1.58±0.42	18.07±6.18
t		4.812	2.618
p		0	0.016

TABLE 3: RESULTS OF WESTERN BLOTTING TEST IN TWO GROUPS OF MICE (β-actin, x̄±s)

Group	Number of cases	Tau (phospho Thr231)	Tau (phospho S396)	Tau (phospho Ser404)	GSK-3Β	GSK-3Β (phospho Ser9)	PP2A
Control group	12	1.725±0.390	0.513±0.192	1.925±0.365	0.839±0.265	0.473±0.198	0.829±0.131
Experimental group	12	1.389±0.173	1.870±0.375	1.426±0.312	1.152±0.206	1.167±0.251	0.622±0.105
t		2.728	11.158	3.600	3.230	7.520	4.271
p		0.016	0.000	0.002	0.004	0.000	0.000

TABLE 4: MOUSE IN THE TWO GROUPS RESULT RATIO OF TAU (PHOSPHO THR231) PROTEIN, TAU (PHOSPHO S396) PROTEIN, TAU (PHOSPHO SER404) PROTEIN TO TAU PROTEIN (x̄±s)

	Number of cases	Tau (phospho Thr231)/ tau	Tau (phospho S396)/ tau	Tau (phospho Ser404)/ tau
Control group	12	251.905±24.211	186.915±20.614	271.041±.37.038
Experimental group	12	368.242±35.329	391.281±42.630	416.205±59.127
t		9.409	14.951	7.207
p		0.000	0.000	0.000

TABLE 5: COMPARISON OF AVERAGE OPTICAL DENSITY OF HIPPOCAMPAL PROTEIN BETWEEN TWO GROUPS OF MICE (x̄±s)

Group	Number of cases	ß-amyloid protein	Taq protein
Control group	12	0.028±0.008	0.006±0.001
Experimental group	12	0.022±0.003	0.008±0.003
t		2.433	2.191
p		0.029	0.047

the frequency of crossing the target platform and the proportion of activity time in the target quadrant were lower, suggesting that ketamine abuse could damage the cognitive function of mice and reduce their learning ability and spatial ability.

Related studies have shown that ketamine-induced cognitive decline in rats is associated with the increased expression of β-amyloid protein and tau protein and hyperphosphorylation of tau protein in the hippocampus[14]. The injection of β-amyloid protein into the hippocampus can also cause neuronal loss and tissue necrosis at the injection site, accompanied by obvious cognitive impairment. The hyperphosphorylation of tau protein can lead to the decline[15] of cognitive function by destroying microtubule homeostasis and axonal transport function, inducing postsynaptic dysfunction and blocking cell signal transduction. The ratio of phosphorylated tau protein to tau protein can reflect the phosphorylation level of tau protein in brain tissue[16]. Phosphatase and protein kinase are the primary components that regulate the level of tau protein phosphorylation. GSK-3β and Cyclin-Dependent Kinase 5 (CDK5) are the main protein kinases that induce tau protein phosphorylation[17,18]. The former was significantly induced at S396 and S404, while the latter's sites were S202, S235 and S404. The activity of GSK-3β was negatively correlated with the phosphorylation degree of enemy Ser9, which means the higher the dephosphorylation degree of Ser9, the higher the level of GSK-3β and the phosphorylation degree of related tau protein will increase[19]. Tau protein phosphorylation is also regulated by PP2A-based protein phosphatase, which inhibits tau protein phosphorylation more strongly than other protein kinases[20,21]. The results indicated that the phosphorylation degree of tau protein at the sites Thr231, S396 and S404 in the observation group was higher than that in the control group, which may be associated with the increase of GSK-3β protein expression and the decrease of PP2A protein expression[22-24].

In conclusion, long-term use of ketamine can lead to up-regulated expression of β-amyloid protein and tau

protein in the hippocampus of mice, which may induce hyperphosphorylation of tau protein at Thr231, S396 and S404 by activating GSK-3β and inhibiting PP2A, resulting in cognitive impairment in mice.

Conflict of interests:

The authors declared no conflicts of interest.

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