

Effect of ketamine on ERK expression in hippocampal neural cell and the ability of learning behavior in minor rats

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Abstract To study the effects of ketamine on ERK expression in hippocampal neural cell and the ability of learning behavior in minor rats. Seventy-two Sprague–Dawley rats of 21 days old were randomly divided into nine groups. The Y-maze was used to test the ability of learning and spatial localization. At the end of training, all rats were killed and the expression levels of ERK1, ERK2 and p-ERK1/2 were tested by immunohistochemistry. The learning times and total reaction time (TRT) of group K2a, K2b, K2c and K3 have significant differences compared with T group ($P < 0.05$). Immunohistochemical staining showed that the level of ERK1, ERK2 and p-ERK1/2 in all rats which received light-electricity integrated training increased remarkably relative to the C group ($P < 0.01$). The expression levels of ERK1, ERK2 and p-ERK1/2 in hippocampal neural cell of group K2a, K2b and K3 significantly decreased when compared with T group ($P < 0.05$). Therefore, the results demonstrate that administration of over-anesthetic ketamine may impair learning ability of 21 days old rats within 24 h. ERK signal transduction pathway may be involved in the ability of learning and spatial localization. The inhibition of ERK signal transduction pathway may be one of the mechanisms of the impairment of learning and memory ability by ketamine.

Keywords Ketamine · Y-maze · Hippocampus · ERK · Rat

Introduction

Signal transduction mechanisms are essential components of the neuronal information processing machinery. Signaling through cellular protein kinase cascades impinges upon targets at the neuronal membrane, in the cytoplasm, and within the nucleus in order to effect changes in synaptic function and connectivity [1]. First and foremost is the extraordinary complexity of biochemical signaling that is involved in triggering long-term potentiation (LTP) and the formation of long-term memories [2]. Substantial evidence has implicated extracellular signal-regulated kinase (ERK) in synaptic plasticity since the initial works of English and Sweatt [3] in LTP and Martin et al. [4] in long-term facilitation in *Aplysia* neurons. Subsequent studies showed the involvement of this kinase in memory processes in animals [5–9].

Ketamine was developed and introduced in 1960s [10]. It could produce a unique anesthetic state of dissociative anesthesia between deep sedation and general anesthesia [11]. The result of ketamine administration is anesthesia, analgesia, suppression of fear and anxiety, and amnesia, which appear to be ideal for the uncooperative child patients [12]. Most of the pharmacologic effects of ketamine appear to be mediated by its interaction with *N*-methyl-D-aspartate (NMDA) receptors [13]. The activation of the NMDAR is essential for long-term potentiation (LTP) at Schaffer collateral-CA1 synapses, and for hippocampal-dependent spatial learning and memory [14, 15]. Blocking the NMDAR in the mouse brain impairs synaptic plasticity and compromises learning and memory [16, 17], whereas conversely, genetic enhancement of NMDAR function results in improved memory [18, 19].

Accordingly, in the present study we attempted to investigate the effects of ketamine on the ability of learning

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and memory in minor rats. Since ERK plays a crucial role in learning and memory [5–9], we also assessed the effects of ketamine on ERK expression in hippocampal neural cell.

Materials and methods

Animals

Seventy-two Sprague–Dawley rats, aged 21 days and weighing 61.0 ± 4.9 gram, were provided by the Laboratory Animal Center of Zhejiang Province. The housing and treatment of the animals were in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee. The mice were randomly divided into nine groups, normal control group (C group), training group (T group), normal saline group (K0 group), 25 mg/kg ketamine group (K1 group), 50 mg/kg ketamine group (K2 group) and 100 mg/kg ketamine group (K3 group), every group has eight rats except K2 group has 32 rats. Group K1, K2 and K3 were separately anesthetized with 25, 50 and 100 mg/kg ketamine i.p., group K0 was received equal volume normal saline, Group K2 was divided into 6, 12, 24 and 72 h subgroups after i.p. (group K2a, K2b, K2c, K2d). The Y-maze was used to test the ability of learning and spatial localization on group K2 at 6, 12, 24 and 72 h. The other groups were tested in 24 h after i.p. Group C adapted in the Y-maze for 5 min, Group T only received training. At the end of training, all rats were killed and the expression of ERK1, ERK2 and p-ERK1/2 were determined by immunohistochemical staining.

The test of learning and memory

The experiment of learning and memory was performed using Y-maze in the quiet, low-light situations in the afternoon. The operation for the Y-maze and the assessment for the behavior of rats were conducted by the fixed personnel, which could exclude the interference with the experimental results by the noise and time factors, and so on. The bottom of Maze is the copper grid interval, the end of each arm have lights, the copper grid in the bottom of Maze with lights bright of one arm has no current, while no light source of two-arms and three-arm were electrified. Adapt the maze for 5 min before the test, the test stimulation voltage was regulated to ensure that rats could run to escape within 10 s. It was considered the correct response that the rats ran directly to the security place after the electric shock, otherwise an error response. When the rats ran to a safe place the light bright was continued to 15 s, then turn off the lights for 45 s, across the next test. The

direction of lighting appeared in accordance with I → II → III → I sequence of transformations, there shall be to reach the learning criteria until 9 times correct response of the 10 consecutive responses. Observed parameters were as follows: (1) measured the learning times when reached to the learning standards; (2) measured the total reaction times (Total Reaction Time, TRT) when reached to the learning standards.

Immunohistochemistry

After anesthetizing with a solution of chloral hydrate (0.4 ml/100 g, i.p.), mice were perfused transcardially with 40 ml of normal saline followed by 30 ml of 4% formaldehyde in PBS (pH 7.4). The brain was fixed in 4% formaldehyde at 4°C for 6 h and kept in a 25% sucrose solution overnight. Coronal sections of 4 μm in thickness were immunochemically stained with anti-ERK1 polyclonal antibody (1:2000; Santa Cruz Biotechnology, CA, USA), anti-ERK2 polyclonal antibody (1:2000; Santa Cruz Biotechnology, CA, USA), and monoclonal antibody p-ERK1/2 (1:2000; Santa Cruz Biotechnology, CA, USA) followed by detection with the EnVision System (Dako, Carpinteria, CA). The sections were washed 3 times in PBS and then developed in 3,3-diaminobenzidine tetrahydrochloride (DAB) solution (0.04% DAB and 0.03% H₂O₂) until appropriate color development. A negative control was included by omitting the primary antibody.

Positive cell counts

To the cytoplasm or the nucleus of cells were stained for yellow or brown as the positive cells, ERK1, ERK2 and p-ERK1/2 immunoreactive cells in the field of hippocampus were counted under the microscope, respectively. Three non-overlapping high power (400 times) vision in each slice of the two similar regions of the hippocampus were selected, and counted, respectively. Then the positive neurons were counted in order to obtain the mean numbers of the positive neurons in the each side of hippocampus, and further obtained the mean number of immunoreactive positive neurons for each rats, and finally calculated and statisted the mean number of immunoreactive neurons for each rat.

Statistical analysis

All statistical analyses were done by the computer program SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SD. LSD t test were used to examine differences among multiple groups. *P* values < 0.05 were considered as statistically significant.

Results

Learning and memory

To evaluate spatial learning and memory in the subjects, a Y-maze test was conducted. In this task, mice had to learn and remember an association between the light and escape. The effect of different doses of ketamine on spatial learning ability of rats showed that the learning times and total reaction time (TRT) of group K2c and K3 have significantly increased when compared with T group and ($P < 0.05$). There was no difference in the learning and memory parameters between the K1 group and the other groups (Table 1; Fig. 1). To further evaluated the effect of 50 mg/kg ketamine at different time points on the spatial learning ability of 21-day-old SD rats, Group K2 was divided into 6, 12, 24 and 72 h subgroups after i.p. (group K2a, K2b, K2c, K2d). The Y-maze was used to test the ability of learning and spatial localization on group K2 at 6, 12, 24 and 72 h. The results showed that the learning times and total reaction time (TRT) of group K2a, K2b and K2c have significantly increased when compared with T group ($P < 0.05$). Unexpectedly, there was no difference in the learning and memory parameters between the K1 group and the T group (Table 2; Fig. 2).

Analysis of ERK protein expression

Next, we determined whether the learning and memory impairment was associated with the ERK expression. ERK expression in the hippocampus neural cell was assessed by immunohistochemical staining analysis. As shown in Fig. 3, the ERK1, ERK2 and p-ERK1/2 expression level in all of the training tests group in rats hippocampus were significantly increased relative to C group ($P < 0.01$), the ERK1, ERK2 and p-ERK1/2 expression level of K3 group were significantly lower than the T group and K0 group ($P < 0.05$). There was no difference in the ERK1, ERK2 and p-ERK1/2 expression level between the K1, K2c group and the T, K0 group (Table 3). Therefore, treatment with sub-anesthetic dose of ketamine yielded lower levels of

Table 1 The learning times and total reaction times (TRT) of each group of rats with different doses of ketamine

Group	Learning Times	TRT (min)
T	20.25 ± 4.89	21.25 ± 5.49
K0	21.00 ± 4.69	22.38 ± 5.32
K1	24.38 ± 5.88	26.38 ± 6.48
K2c	26.63 ± 5.55*	28.75 ± 6.11*
K3	28.13 ± 4.61*	30.63 ± 4.78*

Compared with K0 and T group, respectively; * $P < 0.05$

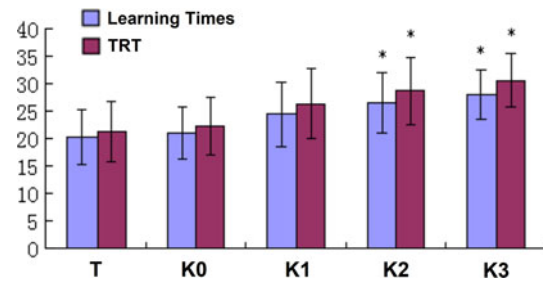


Fig. 1 The effect of different doses of ketamine on spatial learning ability of rats. The learning times and total reaction time (TRT) of group K2c and K3 have significantly increased when compared with T group and ($P < 0.05$). There was no difference in the learning and memory parameters between the K1 group and the other groups

Table 2 The Comparison of learning times and total reaction times (TRT) between the each sub-group of K2 and the T group of rats

Group	Learning Times	TRT (min)
T	20.25 ± 4.89	21.25 ± 5.49
K2a	28.25 ± 4.89*	31.00 ± 5.37*
K2b	27.25 ± 6.08*	28.88 ± 6.81*
K2c	26.63 ± 5.55*	28.75 ± 6.11*
K2d	24.00 ± 7.03	25.75 ± 7.45

Compared with the T group, * $P < 0.05$

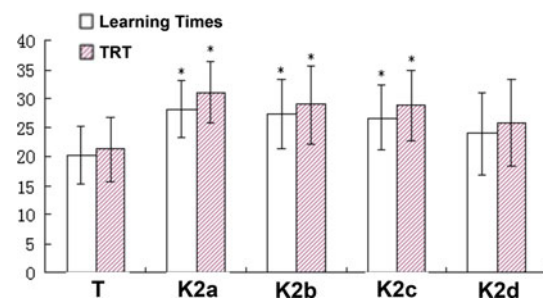


Fig. 2 The effect of 50 mg/kg ketamine at different time points on the spatial learning ability of 21-day-old SD rats. Group K2 was divided into 6, 12, 24 and 72 h subgroups after i.p. (group K2a, K2b, K2c, K2d). The learning times and total reaction time (TRT) of group K2a, K2b and K2c have significantly increased when compared with T group ($P < 0.05$). There was no difference in the learning and memory parameters between the K1 group and the T group

ERK expression in the hippocampus neural cells. In contrast, the influence of anesthetic doses of ketamine on the ERK expression showed that the ERK1, ERK2 and p-ERK1/2 expression level of K2a and K2b group were significantly lower than the T group ($P < 0.05$). There was no difference in the ERK1, ERK2 and p-ERK1/2 expression level between the K2c, K2d group and the T group (Table 4; Fig. 4). The immunohistochemistry of p-ERK1/2 expression of C group and T group were shown in Fig. 5.

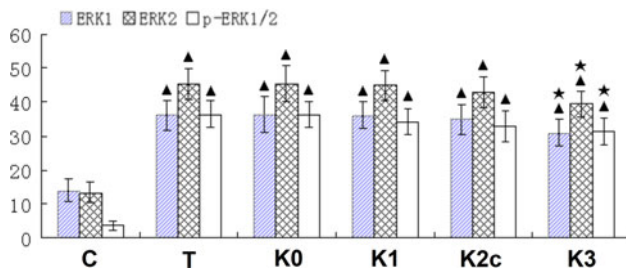


Fig. 3 The effect of different doses of ketamine on the ERK1, ERK2 and p-ERK1/2 expression level of rats. The ERK1, ERK2 and p-ERK1/2 expression level in all of the training tests group in rats hippocampus were significantly increased relative to C group ($P < 0.01$), the ERK1, ERK2 and p-ERK1/2 expression level of K3 group were significantly lower than the T group and K0 group ($P < 0.05$). There was no difference in the ERK1, ERK2 and p-ERK1/2 expression level between the K1, K2c group and the T, K0 group

Table 3 Comparison of the number of positive cells of ERK1, ERK2 and p-ERK1/2 by immunohistochemistry in hippocampus for each group of rats with different doses of ketamine

Group	ERK1	ERK2	p-ERK1/2
C	13.88 ± 3.40	13.25 ± 3.06	3.50 ± 1.41
T	36.13 ± 4.26 [▲]	45.25 ± 4.62 [▲]	36.38 ± 4.03 [▲]
K0	36.38 ± 5.24 [▲]	45.38 ± 5.40 [▲]	36.13 ± 3.87 [▲]
K1	36.00 ± 3.96 [▲]	44.88 ± 4.42 [▲]	34.13 ± 3.72 [▲]
K2c	34.88 ± 4.36 [▲]	42.88 ± 4.55 [▲]	32.88 ± 4.52 [▲]
K3	30.88 ± 4.02 ^{▲*}	39.38 ± 3.81 ^{▲*}	31.38 ± 4.07 ^{▲*}

Compared with the C group, [▲] $P < 0.01$

Compared with the T and K0 group, ^{*} $P < 0.05$

Table 4 Comparison of positive cells of ERK1, ERK2 and pERK1/2 in K2 hippocampus by immunohistochemistry between each subgroup of K2 group and T group, C group

Group	ERK1	ERK2	p-ERK1/2
C	13.88 ± 3.40	13.25 ± 3.06	3.50 ± 1.41
T	36.13 ± 4.26 [▲]	45.25 ± 4.62 [▲]	36.38 ± 4.03 [▲]
K2a	31.25 ± 4.27 ^{▲*}	39.50 ± 3.96 ^{▲*}	31.00 ± 4.00 ^{▲*}
K2b	31.63 ± 4.57 ^{▲*}	40.50 ± 3.66 ^{▲*}	32.13 ± 4.01 ^{▲*}
K2c	34.88 ± 4.36 [▲]	42.88 ± 4.55 [▲]	32.88 ± 4.52 [▲]
K2d	35.75 ± 4.06 [▲]	44.75 ± 4.43 [▲]	33.75 ± 4.09 [▲]

Compared with the T group, ^{*} $P < 0.05$

Compared with the C group, [▲] $P < 0.01$

Discussion

Ketamine, 2-(o-chlorophenyl)-2-(methylamino) cyclohexanone, a phencyclidine (PCP) and cyclohexamine derivative, and a phencyclidine (PCP) and cyclohexamine derivative, was developed and introduced in 1960s [10]. It produces a unique anesthetic state (dissociative anesthesia,

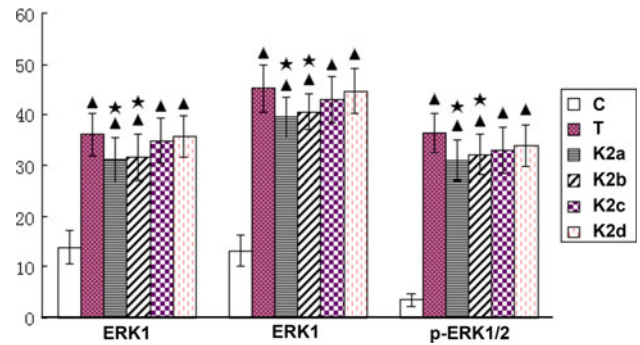
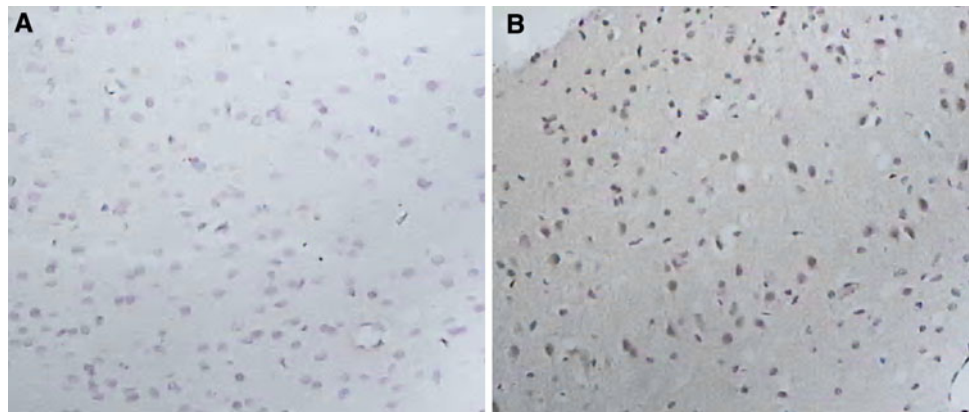


Fig. 4 The effect of 50 mg/kg ketamine at different time points on the ERK1, ERK2 and p-ERK1/2 expression level of 21-day-old SD rats. The ERK1, ERK2 and p-ERK1/2 expression level of K2a and K2b group were significantly lower than the T group ($P < 0.05$). There was no difference in the ERK1, ERK2 and p-ERK1/2 expression level between the K2c, K2d group and the T group

between deep sedation and general anesthesia) characterized by a dissociation between the thalamocortical and limbic systems [11]. Depending on the doses of ketamine, patients are usually unconscious and cataleptic or partially conscious but fail to respond purposefully to physical stimulation. Ketamine has proven effective to achieve truly deep sedation and to relieve physical and psychologic pain in children [20]. However, it can also cause several unwanted effects, especially impairment of memory [21]. In the current study, we aimed to investigate the effects of ketamine on the ability of learning and memory in minor rats, and also assessed the effects of ketamine on ERK expression in hippocampal neural cell.

Y-maze test using light as the conditioned stimulus, while giving the electric shocks to the animal foot in the dark place, resulting in the disgust dark reaction for animals, which could observe the escape reflex ability and spatial ability for the animals. It was a classical method based on the establishment of a conditioned reflex to detect the animals behavior and higher brain function. Using the Y-maze test, we found that ketamine at increasing dose significantly decreased the learning and memory performance of mice relative to the control without treatment. The deleterious effects of ketamine on memory are also demonstrated in several previous studies (10, 24). To further examined the influence of 50 mg/kg ketamine at different time points on the spatial learning ability of 21-day-old SD rats, Group K2 with 50 mg/kg ketamine was divided into 6, 12, 24 and 72 h subgroups after i.p. (group K2a, K2b, K2c, K2d). The results showed that the learning times and total reaction time (TRT) of group K2a, K2b and K2c at 6, 12 and 24 h, respectively, have significantly increased when compared with the training group ($P < 0.05$). The present results have also suggested the timing of such damage, which might be no more than 72 h. Therefore, ketamine have a short-term damage on learning and

Fig. 5 The immunohistochemistry of p-ERK1/2 expression of C group and T group



memory, and its damage decreased over time. The results were in accord with the previous studies [22, 23]. Together the results suggest that anesthetic ketamine may be more suitable for clinical use with regard to the effects on learning and memory with the extension of time.

ERK plays an important role in the process of learning and memory [5–9], which encourages us to determine whether the effects of ketamine on learning and memory associated with the expression levels of ERK. ERK expression in the hippocampus neural cell was assessed by immunohistochemical staining analysis. The results showed that the ERK1, ERK2 and p-ERK1/2 expression level of K3 group with 100 mg/kg ketamine were significantly lower than the training group and K0 group without ketamine ($P < 0.05$). The influence of anesthetic doses of ketamine on the ERK expression showed that the ERK1, ERK2 and p-ERK1/2 expression level of K2a (12 h) and K2b (24 h) group were significantly lower than the training group ($P < 0.05$). As expected, the changes in the ability of learning and memory are nearly in parallel with the variations of the ERK expression; i.e., reduced learning and memory performance upon administration of sub-anesthetic ketamine is accompanied by decreased expression of ERK. This finding suggests that ERK may be involved in the process of learning and memory damage. Previous researches using several behavioral memory paradigms have proved ERK as an important component of the signal transduction mechanisms subserving behavioral memory formation [5–9]. Several studies have proved ERK as a key player in synaptic and neuronal plasticity—a cellular role that is likely to underlie ERK's behavioral role in the animal [24–32]. Molecular studies have indicated the complexities of biochemical regulation of ERK in neurons and have highlighted the variety of likely cellular targets of ERK [1]. However, it is still unknown to what extent ERK participates in this process. Therefore, further research needs to be done to unravel the underlying mechanisms.

Nevertheless, our pilot results demonstrate that administration of over- anesthetic ketamine may impair learning

ability of 21 days old rats within 24 h. ERK signal transduction pathway may be involved in the ability of learning and spatial localization. The inhibition of ERK signal transduction pathway may be one of the mechanisms of the impairment of learning and memory ability by ketamine.

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