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Original Article

A comprehensive study on the mechanisms associated with learning and memory impairment induced by etomidate

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Abstract: Objective: To investigate the relationship between learning and memory impairment induced by etomidate, long-term potentiation (LTP) and long-term depression (LTD), and immediate early genes (IEGs). Methods: The effect of etomidate on study and memory of male Wistar rats was investigated through the Morris Water Maze (MWM) test. The effect of etomidate on LTP and LTD in the hippocampal CA1 area was analyzed. The expression levels of IEGs, including *Arc*, *c-fos* and *Egr-1*, were measured through qRT-PCR. Results: Without the condition of affecting athletic ability, etomidate impaired study and memory ability. Etomidate impaired LTP and enhanced LTD in hippocampal CA1 area. LTP in the hippocampal CA1 area increased the expression quantity of *Arc*, *c-fos* and *Egr1*, and LTD in CA1 area inhibited the expression degree of *Arc*, *c-fos* and *Egr1*. The decreased expression degree of *Arc*, *c-fos* and *Egr1* was associated with learning and memory impairment of etomidate. Decreased expression of *Arc*, *c-fos* and *Egr1* was associated with impaired LTP and enhanced LTP. Conclusion: The mechanisms associated with learning and memory impairment induced by etomidate might include two aspects. On the one hand, etomidate could impair learning and memory through directly blocking LTP and enhancing LTD; Etomidate could also inhibit the expression of *Arc*, *c-fos* and *Egr1* through modulating LTP and LTD, which in turn caused blocking of LTP and enhancement of LTD.

Keywords: Etomidate, study and memory, long-term potentiation, long-term depression, immediate early genes

Introduction

Etomidate, also known as (R)-Ethyl 1-(1-phenylethyl)-1H-imidazole-5-carboxylate, is widely applied in sedation and induction of general anesthesia. As the only imidazole among general anesthetics, etomidate has the best beneficial effect after single administration [1]. Similar to plenty of other general anesthesia inducing drugs, etomidate, which may lead to learning and memory impairment at a fraction of concentration, causes other endpoints of anesthesia, including immobility, hypnosis and so on [2-4]. The mechanisms associated with learning and memory impairment induced by etomidate are still not thoroughly elucidated. Studies show that etomidate can block long-term potentiation (LTP) and impair learning and memory through regulating $GABA_A$ receptors ($GABA_ARs$) [4-6]. The formation of study and memory is correlated with the changes of new protein synthesis and gene expression programs, which in turn results in long-lasting status of synapse plasticity, such as long-term depression (LTD) and LTP [7]. Typically, immediate early genes (IEGs) are regarded as the first responders to various cellular activations. Their transcription can be transiently and promptly induced by neural activities [8]. Many reports have demonstrated that the induction of IEGs is essential to the transition from short-term memory to long-term memory [8-10]. In addition, LTP and LTD are associated with the expression changes of IEGs, which contains *Arc*, *c-fos* and *Egr-1* [11, 12]. However, the relationship between learn-

ing and memory impairment induced by etomidate, LTP and LTD, and IEGs has not been systematically investigated.

Materials and methods

Animals, grouping and treatment

There were a total of 40 male Wistar rats that purchased from Experimental Animal Center of Suzhou University. They were 14 days old with an average weight of (48±7) g and maintained under standard laboratory settings. All animal studies were permitted by Animal Experiment Review Committee of Soochow University. Randomize 30 rats to blank group (R), control group (R) and etomidate group (R) with 10 rats respectively in each group. The rats in blank group (R), control group (R) and etomidate group (R) received 2 mL of 0.9% NaCl, 2 mL of fat emulsions and etomidate at a dose of 5 mg/Kg by intraperitoneal injection, respectively. Etomidate group (R) was maintained for 2 h of anesthesia with etomidate. The Morris Water Maze test was performed in the three groups of rats from the 1st day after anesthesia. After the test, brain slices of the hippocampal CA1 area were prepared in all the three groups of rats, and three slices were randomly selected from each rat, and were respectively used for analyzing LTP, analyzing LTD and measuring the expression levels of IEGs in the hippocampal CA1 area. Brain slices of the remaining 10 rats were directly prepared without undergoing the Morris water maze test, and six slices were randomly selected from each rat. Among them, three slices, respectively treated with fat emulsions, etomidate and etomidate + bicuculline, were used for measuring the expression quantity of IEGs after induced by LTP. The remaining three slices, also respectively treated with emulsions, etomidate and etomidate + bicuculline, were used for measuring the expression quantity of IEGs after induced by LTD.

Morris water maze test

The Morris Water Maze, a round pool of 180 cm in diameter and 50 cm in depth, was filled with warm water and had a hidden platform under the water surface in the-third-quadrant. All rats were subjected to the maze for 7 days before the test performed on the 1st day after anesthesia. During each trial, all rats were placed in the fixed position in the pool facing the wall and permitted 1 minute to seek out for the hidden platform. The rats that could not find the platform by 60 s were then guided and permitted to stay for 20 s. The rats involved were tested four times a day. The rats were dried and stayed warm after every trial. Consecutively practiced the rats for 5 days, and removed the platform on 6th day for probe test. The swimming speed, escape latency period, duration, crossing times and total distance traveled were recorded.

Preparation of mouse brain slice

Prepared the mouse brain slices as described above [4]. Briefly, the rats were decapitated quickly, and their brains were removed. Then, the hippocampal hemisphere was removed; cyanoacrylate glue (Quick Adhesive, Westerville, OH) was adopted to stick to a microtome slice tray, and placed the slices into ice-cold cutting artificial cerebrospinal fluid with 95% O_2-5% CO_2. The 400-μm-thickness Brain slices were cut with a vibratome (Leica VT 100S, Leica Microsystems Nussloch GmbH, Nussloch, Germany) and incubated under 37°C for 1 h, and then incubated under 22~25°C for another 1 h.

For brain slices prepared from the 10 rats without undergoing the Morris water maze test, they were randomly allocated to blank group (S), control group (S) and etomidate group (S) with 30 slices in each group. Control group (S), etomidate group (S) and blank group (S) were incubated in 30 μl of artificial cerebrospinal fluid, respectively containing fat emulsions, etomidate (5 μmol/L) and etomidate (5 μmol/L) + bicuculline (10 μmol/L).

LTP and LTD acquisition

Inserted a 16-channel linear array recording electrode (separating recording sites of 50 μm; NeuroNexus Technologies) orthogonally to the hippocampal layers in the middle of the hippocampal CA1 area for performing extracellular recordings. Immersed the brain slices in artificial cerebrospinal fluid at 2.5-3.0 mL/min at the temperature of 30±0.5°C. A tungsten stereotrode stimulated electrode (0.5 M; World Precision Instruments) was employed to evoke field EPSPs (fEPSPs) electrically.

Recordings were conducted for at least 1 h. LTP/LTD was evoked with high frequency stimu-

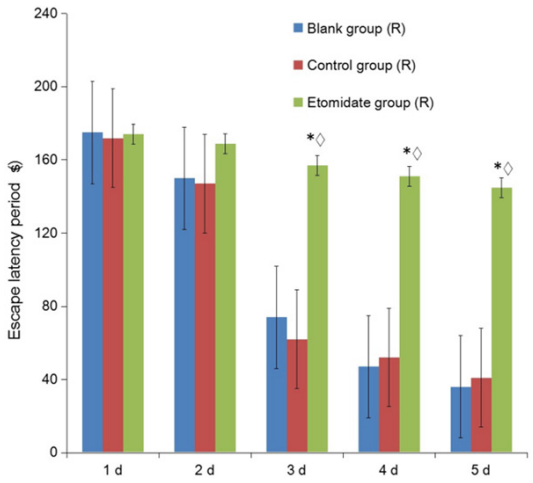

Figure 1. Effect of etomidate on the escape latency period. Note: *P<0.05, vs blank group (R); ◊P<0.05, vs control group (R).

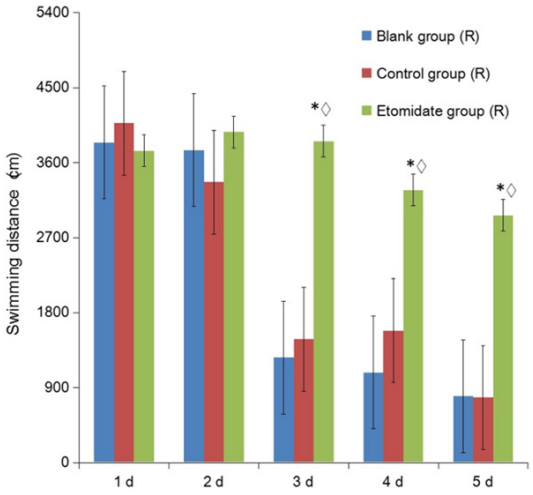

Figure 2. Effect of etomidate on the swimming distance. Note: *P<0.05, vs blank group (R); ◊P<0.05, vs control group (R).

lus/low frequency stimulus after the baseline period stabilized, defined as the slope change fEPSP less than 10% over 30 min. LTP/LTD expressed as a percentage of baseline before tetanus, calculated by the average fEPSP amplitude recorded in the last 10 min divided by the average amplitude of fEPSP detected within 10 min before stimulus.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

The TRIzol® reagent (Ambion, Cat.#: 15596-026, Invitrogen, Carlsbad, USA) for extracting total RNA from tissue samples, and first strand cDNA synthesis kit (Novagen, Cat.#: 69001-3, Merck, New Jersey, USA) was used to synthesize the cDNA. The primer sequences of *Arc*, *c-fos* and *Erg1* were 5'-AGTCTTGGGCAGCATAGCTC-3' (forward) and 5'-GCCGAAGTCTGCTTTTCTTC-3' (reverse), 5'-CAGCCTTTCCTACTACCATTCC-3' (forward) and 5'-ACAGATCTGCGCAAAAGTCC-3' (reverse), and 5'-CAGCGCTTTCAATCCTCAA-3' (forward) and 5'-TGGGATAACTTGTCTCCACCA-3' (reverse), respectively. GADPH was selected as the reference gene (forward primer: 5'-GAAGGGCTCATGACCACAGT-3', and reverse primer: 5'-GGATGCAGGGATGATGTTCT-3'). ABI7500 (Life Technologies, Grand Island, NY, USA) was adopted to perform qRT-PCR, data collection and analysis. The $2^{-\Delta\Delta Ct}$ method was employed for assessing the relative expression quantities of target genes.

Statistical analysis

Conduct statistical analysis by SPSS version 20.0 (SPSS Inc., USA). Kolmogorov-Smirnov test was employed to determine the data distribution. The normally distributed data was expressed as mean ± standard deviation (SD) and compared with Student's *t*-test, and the non-normally data was showed by median (quartile range) and compared with Mann-Whitney U test. Significance setting was P< 0.05.

Results

Etomidate impaired learning and memory

The mouse learning and memory was evaluated by consecutive 5-day training. The escape latency period for the mouse to locate the hidden platform was longer in etomidate group (R) than in blank group (R) and control group (R) on the 3th, 4th and 5th day (**Figure 1**), and the total swimming distance for rats to locate the hidden platform was greater in etomidate group (R) than in blank group (R) and control group (R) on the 3th, 4th and 5th day (**Figure 2**), and the swimming speed was not statistically different between etomidate group (R), blank group (R) and control group (R) (**Figure 3**). After the platform was removed, the times crossing the platform position were fewer in etomidate group (R) than in blank group (R) and control group (R) (2.03±0.78 vs 4.36±

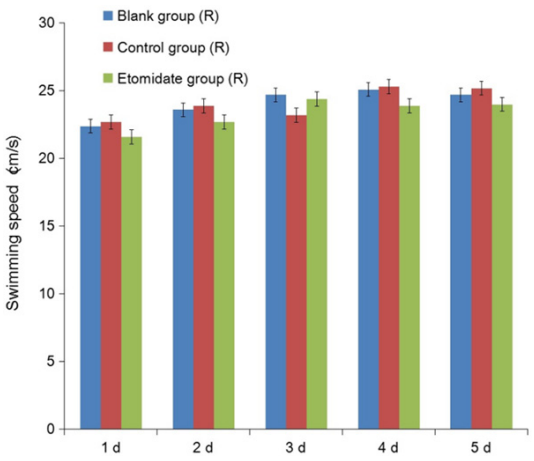

Figure 3. Effect of etomidate on the swimming speed. Note: *P<0.05, vs blank group (R); ◊P<0.05, vs control group (R).

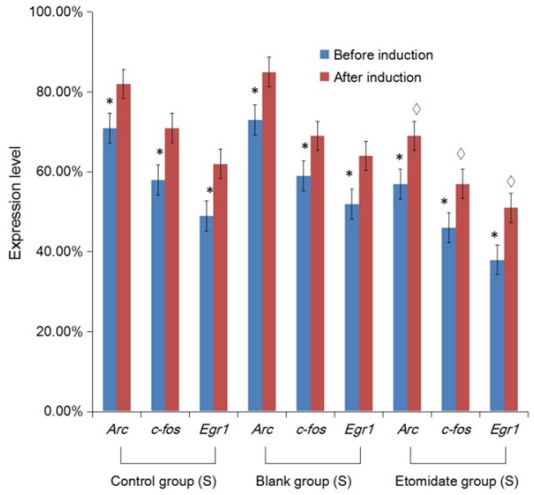

Figure 4. LTP in CA1 area increased the expression of *Arc*, *c-fos* and *Egr1*. Note: *P<0.05, vs after induction; ◊P<0.05, vs control group (S) and blank group (S).

1.12, 4.29±1.17; t=-5.399, -5.082; both P< 0.001), and the duration in the third quadrant was shorter in etomidate group (R) than in blank group (R) and control group (R) (16.94± 2.87 s vs 31.12±5.44 s, 29.85±5.39 s; t= -7.290, -6.686; both P<0.001). All these results suggested that etomidate impaired learning and memory without affecting the motor ability.

Etomidate impaired LTP and enhanced LTD in CA1 area

LTP was induced in control group (S), etomidate group (S) and blank group (S) (154±11%, n=10, one-sample t test, t=4.909, P=0.003; 126± 6%, n=10, one-sample t test, t=4.333, P= 0.009; 151±9%, n=10, one-sample t test, t= 5.667, P=0.000), but etomidate reduced LTP (t=7.066, 7.309; both P<0.001).

LTD was induced in control group (S), etomidate group (S) and blank group (S) (72±9%, n=10, one-sample t test, t=3.114, P=0.021; 49±5%, n=10, one-sample t test, t=10.189, P<0.001; 70±7%, n=10, one-sample t test, t=4.286, P=0.010), but etomidate enhanced LTD (t= 7.064, 7.720; both P<0.001). All these results suggested that etomidate impaired LTP and enhanced LTD in the hippocampal CA1 area.

LTP in CA1 area increased the expression of IEGs

As shown in **Figure 4**, the expression levels of *Arc*, *c-fos* and *Egr1* were higher at 60 min after induction of LTP than before induction in control group (S), blank group (S) and etomidate group (S). Moreover, the expression levels of *Arc*, *c-fos* and *Egr1* were higher in control group (S) and blank group (S) than in etomidate group (S). All these results suggested that LTP in CA1 area increased the expression of *Arc*, *c-fos* and *Egr1*.

LTD in CA1 area inhibited the expression of IEGs

As shown in **Figure 5**, the expression levels of *Arc*, *c-fos* and *Egr1* were lower at 60 min after induction of LTD than before induction in control group (S), blank group (S) and etomidate group (S). Moreover, the expression levels of *Arc*, *c-fos* and *Egr1* were lower in etomidate group (S) than in control group (S) and blank group (S). All these results suggested that LTD in CA1 area inhibited the expression degree of *Arc*, *c-fos* and *Egr1*.

Decreased expression of IEGs was connected with study and memory impairment of etomidate

As the **Figure 6** showed, the expression levels of *Arc*, *c-fos* and *Egr1* were lower in etomidate group (R) than in blank group (R) and control group (R) after the Morris Water Maze test, suggesting decreased expression of *Arc*, *c-fos* and *Egr1* was associated with learning and memory impairment of etomidate.

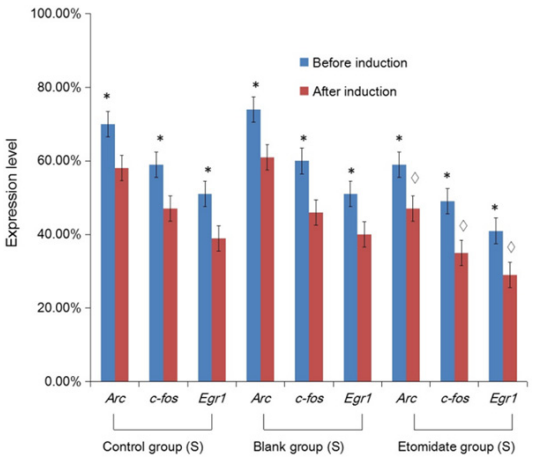

Figure 5. LTD in CA1 area inhibited the expression of *Arc*, *c-fos* and *Egr1*. Note: **P*<0.05, vs after induction; ◊*P*<0.05, vs control group (S) and blank group (S).

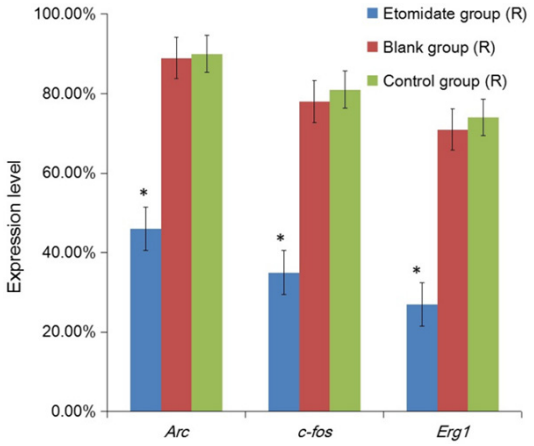

Figure 6. Expression levels of *Arc*, *c-fos* and *Egr1* in etomidate group (R), blank group (R) and control group (R) after the Morris water maze test. Note: **P*<0.05, vs control group (R) and blank group (R).

Decreased expression of IEGs was associated with impairment of LTP and enhancement of LTD

Etomidate group (R) demonstrated impaired LTP compared with blank group (R) and control group (R) (109±5% vs 161±12%, *t*=-12.649, *P*<0.001; 109±5% vs 163±13%, *t*=-12.260, *P*<0.001). In addition, the expression degree of *Arc*, *c-fos* and *Egr1* were lower in etomidate group (R) than in blank group (R) and control group (R). Therefore, decreased expression of IEGs was associated with impaired LTP.

Etomidate group (R) demonstrated enhanced LTD compared with blank group (R) and control group (R) (38±4% vs 81±7%, *t*=-16.866, *P*<0.001; 38±4% vs 83±9%, *t*=-14.449, *P*< 0.001). In addition, the expression degree of *Arc*, *c-fos* and *Egr1* were lower in etomidate group (R) than in blank group (R) and control group (R). Therefore, decreased expression of IEGs was associated with enhanced LTP.

Discussion

Our results demonstrated that etomidate could impair learning and memory of mice, which is consistent with previous studies [2-4]. LTP and LTD, long-term stabilization of synaptic plasticity, are critical for the shape of long-term memory. The underlying mechanisms have been extensively explored. In freely moving animals, the induction of LTP happens concomitantly with learning in the hippocampus and is further discovered to be able to preclude subsequent electrical induction of LTP [13]. A recent report found that the artificial induction of LTD impairs the recall of associative memory in vivo, which can be restored by subsequent induction of LTP [14]. Therefore, LTP and LTD are involved in the processes of learning and memory.

On the one hand, IEGs are associated with the mediation of LTP and LTD [15-17]. Genetic deletion of *Arc*, one of IEGs, is proved to be correlated with impairment of LTD and LTP [18]; and acute knock-down of *Arc* gene has been demonstrated to disrupt LTP in area CA1 [19, 20]. Studies have shown that the expression level of IEGs, including *c-fos*, *Egr1* and *Arc,* are correlated with the processes of learning and memory [3, 8, 21, 22]. Taken together, these results suggest that IEGs may impair learning and memory through regulating LTP and LTD. On the other hand, LTP and LTD can induce the changes in *Arc* gene expression in hippocampal CA1 area in vivo [12]. A rapid and persistent elevation of *Arc* gene expression can be observed following LTP in hippocampal CA1 area in vivo, and a rapid reduction followed by a transient elevation can be observed following LTD. In addition, etomidate can block LTP and impair learning and memory through regulating $GABA_A$Rs [4-6]. Therefore, the mechanisms associated with learning and memory impairment induced by etomidate may be that etomidate inhibits the expression of IEGs through modulating LTP and LTD, which in turn impairs learning and memory through affecting LTP and LTD.

As a single-copy gene, *Arc* gene is predominantly expressed in hippocampal glutamatergic and cortical neurons. Arc is involved in LTP and LTD, and has an important role in consolidation of memory [23]. Arc regulates transcription although it does not encode a typical transcription factor like many IEGs [24]. It can regulate network stability in vivo [25] and is associated with various neuronal signaling pathways [20, 26-28]. At synapses, Arc is associated with regulation of synaptic strength through promoting internalization of AMPA receptor [26].

c-fos gene encodes the Fos protein which dimerizes with transcriptional factors of the Jun family to build up AP-1 [29]. The expression of *c-fos* appears to be activated by Ca^{2+} and cAMP through stimulation of the CREB/CRE complex in neurons. The elevated expression of *c-fos* is usually treated as an indicator of neuronal activation [30]. Studies have shown that the expression of *c-fos* after behavioral training is associated with study and performance [31-34]. The expression changes of c-Fos in different structures can be caused by fear memory paradigms such as an inhibitory avoidance task [35-37]. Moreover, the persistence of fear memory needs expression of c-Fos, and blocking the expression of c-Fos via infusion of *c-fos* antisense oligonucleotide into the dorsal area of CA1 in the retrosplenial cortex or hippocampus leads to deficits in the persistence and consolidation of fear memory [36-38]. In addition, the promoter of *c-fos* has been correlated with optically sensitive proteins to manipulate and mark a specific cell subset associated with contextual learning. Through the above approach, Cowansage et al. demonstrate that c-Fos-expressing neurons in retrosplenial cortex are associated with the acquisition of contextual memories and that behavioral response can be controlled by reactivating this specific set of cells [39].

Egr1 is one of the zinc finger family members of transcription factors. Plenty of signals, such as stress, injury and differentiation factors, can induce the expression of Egr1 [40, 41]. *Egr1* gene modulates the expression of many late-response genes that are associated with varied neuronal processes from plastic changes to growth control [42]. Egr1 is essential to study and memory. Deleting *Egr1* can lead to learning and memory impairment. *Egr1* mutant mice demonstrate intact short-term memory in multiple kinds of behavioral missions, but their long-term memory was dramatically damaged [43-45]. Additionally, studies using antisense oligonucleotides to partially knock down the specific structures of *Egr1* demonstrated that it impairs recognition memory and persistence of inhibitory avoidance memory [46, 47]. At the same time, overexpression of *Egr1* is correlated with the increased resistance of aversive memories to extinction and the improvement of spatial memory [48, 49]. As a high-regulated transcriptional factor, Egr1 has considerable number of target genes [50-52]. These target genes support its role in synaptic plasticity and conduct storage and learning through this mechanism.

In this study, we found that etomidate could block LTP and enhance LTD, down-regulate the expressions of *Arc*, *c-fos* and *Egr1*, and impair learning and memory; and impairment of learning and memory was associated with decreased expression of *Arc*, *c-fos* and *Egr1;* and decreased expression of *Arc*, *c-fos* and *Egr1* could block LTP and enhance LTD. Therefore, the mechanisms associated with learning and memory impairment induced by etomidate might include two aspects. On the one hand, etomidate could impair learning and memory through directly blocking LTP and enhancing LTD; on the other hand, etomidate could inhibit the expressions of *Arc*, *c-fos* and *Egr1* through modulating LTP and LTD, which in turn caused blocking of LTP and enhancement of LTD.

Disclosure of conflict of interest

None.

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