Original Article

Underlying Mechanisms of Memory Deficits Induced by Etomidate Anesthesia in Aged Rat Model: Critical Role of Immediate Early Genes

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Abstract

Background: Etomidate (R-1-[1-ethylphenyl] imidazole-5-ethyl ester) is a widely used anesthetic drug that had been reported to contribute to cognitive deficits after general surgery. However, its underlying mechanisms have not been fully elucidated. In this study, we aimed to explore the neurobiological mechanisms of cognitive impairments that caused by etomidate.

Methods: A total of 30 Sprague-Dawley rats were used and divided into two groups randomly to receive a single injection of etomidate or vehicle. Then, the rats' spatial memory ability and neuronal survival were evaluated using the Morris water maze test and Nissl staining, respectively. Furthermore, we analyzed levels of oxidative stress, as well as cyclic adenosine 3',5'-monophosphate response element-binding (CREB) protein phosphorylation and immediate early gene (IEG, including *Arc*, *c-fos*, and *Egr1*) expression levels using Western blot analysis.

Results: Compared with vehicle-treated rats, the etomidate-treated rats displayed impaired spatial learning (day 4: 27.26 ± 5.33 s vs. 35.52 ± 3.88 s, $t = 2.988$, $P = 0.0068$; day 5: 15.84 ± 4.02 s vs. 30.67 ± 4.23 s, $t = 3.013$, $P = 0.0057$; day 6: 9.47 ± 2.35 s vs. 25.66 ± 4.16 s, $t = 3.567$, $P = 0.0036$) and memory ability (crossing times: 4.40 ± 1.18 vs. 2.06 ± 0.80, $t = 2.896$, $P = 0.0072$; duration: 34.00 ± 4.24 s vs. 18.07 ± 4.79 s, $t = 3.023$, $P = 0.0053$; total swimming distance: 40.73 ± 3.45 cm vs. 27.40 ± 6.56 cm, $t = 2.798$, $P = 0.0086$) but no neuronal death. Furthermore, etomidate did not cause oxidative stress or deficits in CREB phosphorylation. The levels of multiple IEGs (Arc: vehicle treated rats 100%, etomidate treated rats 86%, $t = 2.876$, $P = 0.0086$; c-fos: Vehicle treated rats 100%, etomidate treated rats 72%, $t = 2.996$, $P = 0.0076$; Egr1: Vehicle treated rats 100%, etomidate treated rats 58%, $t = 3.011$, $P = 0.0057$) were significantly reduced in hippocampi of etomidate-treated rats.

Conclusion: Our data suggested that etomidate might induce memory impairment in rats via inhibition of IEG expression.

Key words: Anesthesia; Cyclic Adenosine 3',5'-Monophosphate Response Element-binding Phosphorylation; Etomidate; Immediate Early Genes; Neuronal Death; Oxidative Stress

Introduction

Postoperative cognitive dysfunction (POCD) is a short-term cognitive decline, especially in memory and executive functions, that may last from a few days to a few weeks after surgery.[1] Currently, the underlying mechanisms mediating the development of cognitive impairment after anesthesia and surgery are not yet fully clear. A previous study proposed that general anesthetics play a causal role in POCD because the duration of anesthesia positively correlates with the incidence of POCD in patients.[2] Moreover, a single exposure to an anesthetic can cause retrograde and anterograde memory deficits that last for days to weeks in rodent models.[3,4] For example, halothane and nitrous oxide anesthesia administered during the perinatal period led to learning deficits and delayed behavioral development,[5] as well as N-methyl-d-aspartate

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receptor blockade, which is typical of nitrous oxide and can produce long lasting memory deficits.[6] However, the mechanisms by which anesthetics cause persistent memory deficits in adults are poorly understood.

Etomidate (R-1-[1-ethylphenyl] imidazole-5-ethyl ester), is a unique drug used for the induction of general anesthesia and sedation. Etomidate is the only imidazole among general anesthesia inducing drugs and has the most favorable therapeutic effect following single bolus administration.[7] It is known that the dominant molecular targets that mediate the anesthetic effects of etomidate in the central nervous system are specific γ-aminobutyric acid type A ($GABA_A$) receptor subtypes, which had been strongly implicated in memory processes.[8] Furthermore, etomidate not only causes memory impairment *in vivo* but also abolishes long-term potentiation induced by high-frequency stimulation in the hippocampal slices of wild-type but not $Gabra5^{-/-}$ mice.[9] Thus, etomidate anesthesia impairs synaptic plasticity, which in turn causes memory deficits. However, whether the neuronal death, oxidative stress, loss of cyclic adenosine 3',5'-monophosphate (cAMP) response element-binding (CREB) protein, and immediate early genes (IEGs) are involved in the etomidate-induced memory deficits in the elderly is still unknown.

Here, using behavioral tests and biochemical and immunohistochemical assay, we are trying to understand the underlying mechanisms for cognitive deficits induced by etomidate *in vivo*.

Methods

Animals and treatment

A total of 30 Sprague-Dawley rats, that were 15–18 months old and weighed 350–400 g, were purchased from the Center of Experimental Animal (Zhengzhou University, China) and maintained under standard laboratory conditions (room temperature 22 ± 2°C; relative humidity 60 ± 5%; and 12-h light/dark cycle) with standard rodent food and water *ad libitum*. All the animal experiments were approved by the Review Committee on Animal Experiments of Zhengzhou University, China. The rats were randomly divided into two groups namely the vehicle- (Con) and etomidate-treated (Eto) groups ($n = 15$ in each group). The rats in the Eto and Con groups received a dose of 8 mg/kg etomidate or a dose of the vehicle by intraperitoneal injection, respectively. The body weights were measured before induction of anesthesia and after the Morris water maze test.

Morris water maze test

The maze consisted of a round pool (diameter and depth were 180 cm and 50 cm, respectively) filled with warm water (25 ± 2°C) up to 2 cm above a hidden platform in the third quadrant. The rats were habituated in the test room for 1 week before training, which commenced 1 day after the rats had recovered from the anesthesia. During each trial, the rats were placed in the swimming pool facing the wall in a fixed position and allowed 60 s to find the hidden platform in the third quadrant. Rats that did not locate the platform within this time were guided there and allowed to remain for 20 s. All rats underwent four trials every day in four quadrants. After every trial, the rats were wiped dry and kept warm. The rats were trained for 6 days consecutively, and the hidden platform was removed for the probe test on day 7 when the memory was detected in the rats. A video tracking system was used to record the swimming motions of the rats. The crossing time, duration, and total distance traveled by each rat in the target quadrant were used to evaluate memory retention ability while the swimming speed was used to evaluate motor ability.

Nissl staining

Five rats were randomly selected from each group and euthanized to obtain tissue samples for the Nissl staining using a previously reported method.[10] Briefly, the rats were anesthetized with an overdose of chloral hydrate intraperitoneally and then perfused transcardially with 0.9% sodium chloride at 4°C, followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.40). Then, the whole brains were removed and postfixed in the same fixative at 4°C for another 24 h. The brains were dehydrated in 30% and 40% sucrose until they sank, rapidly frozen in isopentane, and then coronal sections (25-μm thick) were cut on a cryostat (CM1950, Leica, Heidelberger, Germany). All the sections were used Nissl stained with 0.1% cresyl violet (Sigma-Aldrich, St. Louis, MO, USA) to evaluate the hippocampal neuronal damage. The cell counting was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) as previously reported.[11]

Superoxide dismutase and malondialdehyde measurement

The activity levels of superoxide dismutase (SOD) and malondialdehyde (MDA) in brain tissue were assayed using commercial kits (Nanjing Jiancheng Inc., China).[12] The hippocampal SOD activity was expressed as U/mg protein while MDA concentrations were as nmol/mg ($n = 6$ in each group).[13]

Western blot analysis

Protein was extracted from the hippocampi in rats with etomidate treatment or vehicle according to a previously described procedure.[14] Membranes were probed with primary antibodies against CREB, phospho-Ser133-CREB (#9104 and #9191, respectively, Cell Signaling, USA), *Arc*, *c-fos*, and *Egr1* (ab118929, ab53036, and ab55160, respectively, Abcam, USA). Horseradish peroxidase conjugated anti-rabbit or anti-mouse secondary antibodies were used and visualized using the enhanced chemiluminescence kit (Thermo Scientific, USA). The density of each band was quantified using the ImageJ software (National Institutes of Health, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH, ab1603, Abcam) was used as a loading control.

Statistical analysis

The statistical analyses were carried out using SPSS 14.0 (SPSS Inc., Chicago, IL, USA). All the graphic presentations were constructed using the SigmaPlot 10.0

(Systat Software, Inc. San Jose, CA, USA). A paired *t*-test was used to analyze the data, which were shown as a mean ± standard error (SE), and A $P < 0.05$ was considered to be statistically significant.

Results

Etomidate-induced memory deficits

To explore the effects of etomidate on the memory of rats, we first examined the spatial learning and memory ability using the Morris water maze test. The learning ability of the rats was assessed using a consecutive 6 days training with a hidden platform under the water surface. During the learning process, the etomidate-treated rats displayed a longer latency period for locating the platform than the vehicle-treated rats did (day 4: 35.52 ± 3.88 s vs. 27.26 ± 5.33 s, $t = 2.988$, $P = 0.0068$; day 5: 30.67 ± 4.23 s vs. 15.84 ± 4.02 s, $t = 3.013$, $P = 0.0057$; day 6: 25.66 ± 4.16 s vs. 9.47 ± 2.35 s, $t = 3.567$, $P = 0.0036$) [Figure 1a]. Then, the platform was removed for the probe test on day 7, and the etomidate anesthesia led to fewer crossing times (2.06 ± 0.80 vs. 4.40 ± 1.18, $t = 2.896$, $P = 0.0072$) at the platform position, as well as reduced the time spent in the target quadrant from 34% to 11% and the total distance traveled from 41% to 13% (duration: 18.07 ± 4.79 s vs. 34.00 ± 4.24 s, $t = 3.023$, $P = 0.0053$; total swimming distance: 27.40 ± 6.56 cm vs. 40.73 ± 3.45 cm, $t = 2.798$, $P = 0.0086$) [Figure 1b–1e]. No significant difference was found in the swimming speed [Figure 1f] between two groups ($t = 1.430$, $P = 0.1360$). There was no significant difference in the body weight between Con and Eto groups before the injection (372.89 ± 13.97 g vs. 374.07 ± 17.14 g, $t = 0.622$, $P = 0.460$) and after the Morris water maze test (392.35 ± 12.83 g vs. 395.53 ± 17.10 g, $t = 0.593$, $P = 0.650$), suggesting that etomidate anesthesia impaired memory without affecting the motor ability and physiology of the rats.

Etomidate did not induce the neuronal death

To determine the potential mechanisms mediating the memory impairments induced by etomidate anesthesia, we first performed Nissl staining to evaluate the neuronal loss after etomidate anesthesia. However, after carefully counting, we did not detect any significant difference in the hippocampal neuronal numbers between the etomidate- and vehicle-treated rats (CA1: 63.45 ± 6.99 vs. 64.70 ± 4.81, $t = 0.765$, $P = 0.4620$; CA3: 66.18 ± 5.42 vs. 64.60 ± 6.52, $t = 0.833$, $P = 0.4320$; 62.27 ± 5.55 vs. 63.40 ± 5.36, $t = 0.678$, $P = 0.5210$) [Figure 2a and 2b]. Thus, etomidate inducing memory loss may not be due to neuronal loss in the hippocampus.

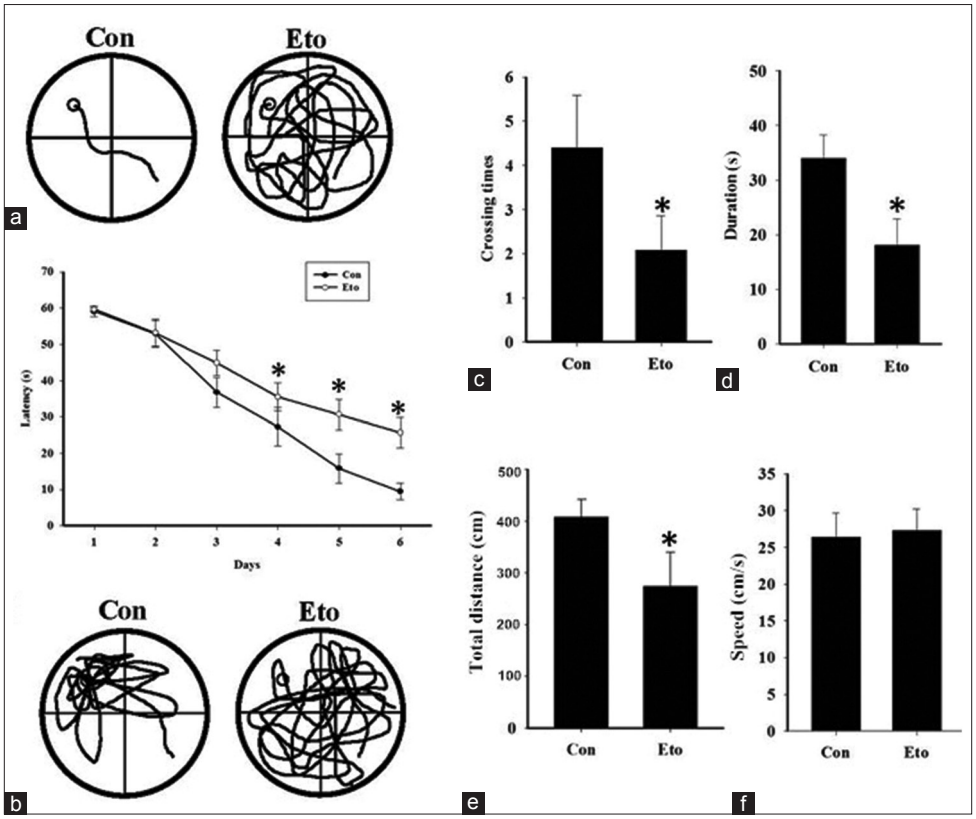

Figure 1: Effect of etomidate on learning and memory of aging rats in Morris water maze test. (a) The representative escape traces (upper) and the escape latencies in the learning days 1–6 (lower; day 4: $t = 2.988$, $P = 0.0068$; day 5: $t = 3.013$, $P = 0.0057$; and day 6: $t = 3.567$, $P = 0.0036$). (b) The swim trace in day 9 for probe test (c) the crossing times at day 9 for probe test ($t = 2.896$, $P = 0.0072$). (d) The duration in the target quadrant ($t = 3.023$, $P = 0.0053$). (e) The total swimming distance in the target quadrant on day 9 ($t = 2.798$, $P = 0.0086$). (f) The swimming speed ($t = 1.430$, $P = 0.1360$). *Compared with vehicle-treated group. Con: The vehicle-treated group; Eto: Etomidate-treated group.

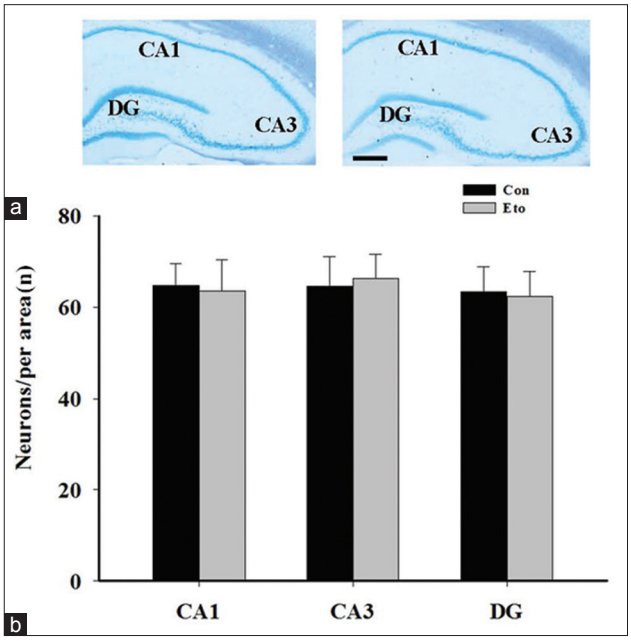

Figure 2: The effect of etomidate on neuronal death by Nissl staining (a) the representative images in the hippocampus; Scale bar = 100 μm. (b) The quantitative analysis between two groups (CA1: $t = 0.765$, $P = 0.4620$; CA3: $t = 0.833$, $P = 0.4320$; DG: $t = 0.678$, $P = 0.5210$). CA1: Region I of hippocampus proper; CA3: Region III of hippocampus proper; DG: Dentategyrus; Con: The vehicle-treated group; Eto: Etomidate-treated group.

Etomidate did not induce oxidative stress

We then asked whether etomidate could induce transient oxidative stress in the hippocampus. We used two commercial kits to determine the expression of SOD and MDA in two groups and found that rats in etomidate-treated group did not show any significant differences in the levels of SOD and MDA compared to the rats in vehicle-treated group (SOD: 105.69 ± 7.40% vs. 100.00 ± 11.59%, $t = 0.975$, $P = 0.3540$; MDA: 96.32 ± 6.78% vs. 100.00 ± 8.29%, $t = 1.112$, $P = 0.2690$), which ruled out the possible role of oxidative stress in etomidate-induced memory deficits [Figure 3].

Cyclic adenosine 3',5'-monophosphate response element-binding phosphorylation was not involved in etomidate-induced memory deficits

Phosphorylation of CREB at the Ser133 site is known to play an important role in regulating the memory process. Therefore, we evaluated the effects of etomidate on CREB phosphorylation using Western blot analysis. However, we did not find significant differences in changes of the protein levels of CREB and phosopho-CREB between etomidate- and vehicle-treated rats [Figure 4a and 4b]. These results excluded the involvement of CREB signaling disruption in the etomidate-induced memory deficits.

Etomidate suppressed the immediate early genes expression

Then, we sought to determine whether the failure of expression of essential IEGs, such as *Arc*, *c-fos*, and *Egr1*, was involved in memory deficits. We found that following treatment with etomidate, the expression of *Arc*, *c-fos*, and *Egr1* was dramatically reduced [Figure 5a], and densitometry analysis revealed the intensities were reduced to 86%, 72%, and 58% of normal levels, respectively [Figure 5b]. Furthermore, by using quantitative real-time polymerase chain reaction we also found that the mRNAs level of *Arc, c-fos,* and *Egr1* was reduced following etomidate treatment [Figure 5c]. These data suggested that etomidate anesthesia suppressed IEGs expression, which may induce the memory deficits.

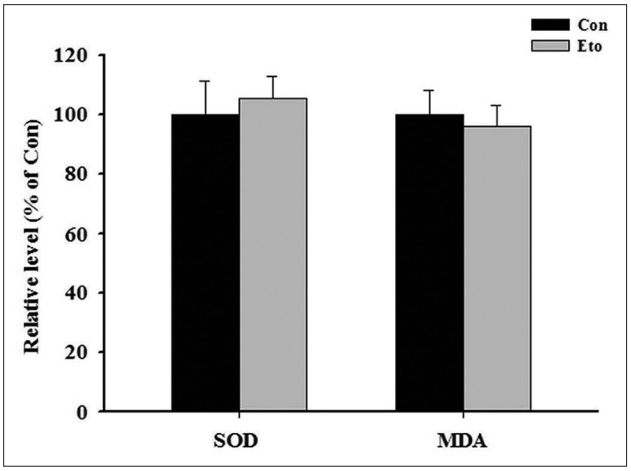

Figure 3: Effect of etomidate on oxidative stress. The SOD and MDA levels were measured by commercial kits, and the raw intensity was finally transferred to the relative levels by setting the control group as 100% (SOD: $t = 0.975$, $P = 0.3540$; MDA: $t = 1.112$, $P = 0.2690$). SOD: Superoxide dismutase; MDA: Malondialdehyde; Con: The vehicle-treated group; Eto: Etomidate-treated group.

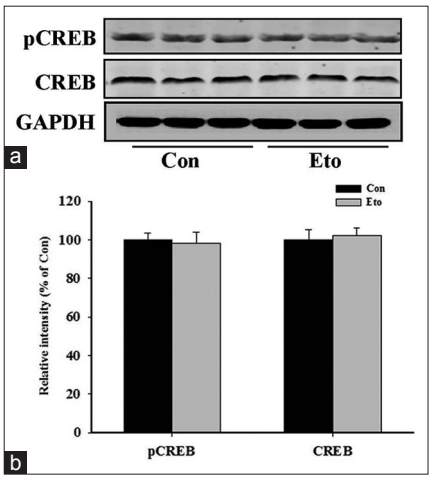

Figure 4: Effect of etomidate on CREB phosphorylation. (a) The representative blots of pCREB at ser133 site, total CREB and the loading control (GAPDH) in the hippocampus. (b) The quantitative analysis between two groups (pCREB: $t = 0.98$, $P = 0.3430$; CREB: $t = 1.021$, $P = 0.2900$). CREB: Cyclic adenosine 3',5'-monophosphate response element-binding; pCREB: Phospho-cyclic adenosine 3',5'-monophosphate response element-binding; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; Con: The vehicle-treated group; Eto: Etomidate-treated group.

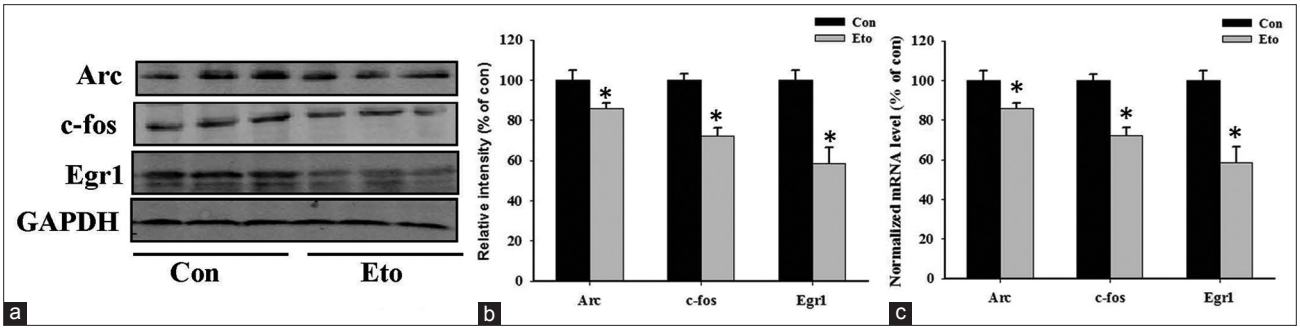

Figure 5: Effect of etomidate on immediate early genes levels. (a) The representative blots of *Arc*, *c-fos*, *Egr1*, and the loading control GAPDH in the hippocampus. (b) The quantitative analysis between two groups (*Arc*: $t = 2.876$, $P = 0.0086$; *c-fos*: $t = 2.996$, $P = 0.0076$; *Egr1*: $t = 3.011$, $P = 0.0057$). (c) The quantitative polymerase chain reaction analysis for the detection of mRNAs of *Arc*, *c-fos*, and *Egr1* (*Arc*: $t = 2.893$, $P = 0.0082$; *c-fos*: $t = 3.213$, $P = 0.0047$; *Egr1*: $t = 3.452$, $P = 0.0034$). *Compared with Con group. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; Con: The vehicle-treated group; Eto: Etomidate-treated group.

Discussion

Previous studies have suggested that anesthesia induces neuronal cell death in the brain of developing rat.[15] Specifically, acute anesthesia exposure downregulated B-cell lymphoma extra-large, upregulated cytochrome C, and activated caspase-9 in 7-day-old rats indicating activation of the intrinsic apoptotic pathway. Furthermore, a parallel upregulation of Fas protein and activation of caspase-8 in 7-day-old rats indicated the activation of the extrinsic apoptotic pathway. Moreover, various studies have reported different results for experiments with etomidate. Acute etomidate treatment reduced the neuronal loss caused by kainic acid[16] but did not alter the hippocampal neuronal loss in rats with traumatic brain injury.[17] In this study, we did not find any significant difference in neuron numbers between etomidate- and vehicle-treated rats, suggesting that etomidate anesthesia did not affect neuronal survival.

Oxidative stress has also been implicated in anesthetic-induced neurotoxicity and memory deficits.[18] The application of oxidative stress blockers including the mitochondrial protector, L-carnitine[19] and melatonin[20] *in vivo*, and specific antioxidants *in vitro*, including the SOD mimetic, M40403, and the nitric oxide synthase inhibitor, 7-nitroindazole, fully, or partially reversed the anesthetic-induced neurotoxicity.[21] Recent gene expression assessments indicated that anesthetic treatment alters genes in the oxidative stress pathway in developing animals.[21] However, in this study, etomidate did not alter the level of MDA and SOD, which are the two principal markers of oxidative stress in the brain, further ruling out the potential role of oxidative stress in the etomidate-induced memory deficits. Previous reports found that etomidate acts on GABAergic neurons to exert its anesthetic activity,[22] and GABAergic neurons are known to be particularly susceptible to oxidative stress.[23] Therefore, the lack of change in the oxidative stress parameters observed here was reasonable and consistent with a previous study.[24]

It is well known that the regulation of gene transcription by cAMP-mediated second messenger pathway plays an important role in learning and memory.[25,26] Both cAMP-dependent protein kinase A (PKA) and CREB are activated in the course of spatial learning. Training of rats using the radial maze resulted in a significant increase in PKA and CREB phosphorylation in the hippocampi during spatial learning, which was followed by spatial memory formation. However, we did not find any increase in phosphorylation of CREB following treatment with etomidate, suggesting that it did not affect the PKA-CREB pathway.

Finally, we discovered that the level of multiple IEGs was decreased following treatment with etomidate. Previous studies have provided direct evidence that IEG expression reflects the integration of information processed by hippocampal neurons. Moreover, IEG expression is not only correlated with neural activity but also plays a critical role in stabilizing recent changes in synaptic efficacy. For example, intrahippocampal administration of antisense IEG oligonucleotides (*Arc* and *c-fos*) or germline disruption of the IEGs *c-fos*, tissue plasminogen activator, or *Egr1* impairs the consolidation of long-term memory formation but does not affect learning or short-term memory.[27,28] Here, etomidate application decreased the level of *Arc*, *c-fos*, and *Egr1* in the hippocampus, which was accompanied with memory deficits and suggested the possible involvement of IEG disruption in etomidate-induced memory decline.

As the disruption of synaptic plasticity, loss of dendritic spines, impairments of membranous receptor trafficking are also important to the memory formation, a further study is necessary to evaluate their roles in etomidate-induced memory deficits.

In summary, we found that etomidate impairs memory by decreasing IEG expression but not by inducing neuronal loss, oxidative stress, or PKA-CREB pathway inhibition.

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Conflicts of interest

There are no conflicts of interest.

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