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Etomidate-Induced myoclonus in Sprague–Dawley rats involves the activation of neocortical Calpain-2 and its decrement on KCC2 protein

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Abstract

Background Etomidate-induced myoclonus has become a pressing clinical problem with an incidence of 50–80%. The underlying mechanism involves neocortical glutamate accumulation and N-methyl-d-aspartate (NMDA) receptor activity. However, the therapeutic target remains uncertain.

Methods Adult male Sprague-Dawley (SD) rats were injected with etomidate (1.5 mg/kg), propofol (11.8 mg/kg), and lidocaine (4.0 mg/kg) plus etomidate (1.5 mg/kg), etomidate (3.8 mg/kg), etomidate (6.0 mg/kg) through the tail vein and behavioral scores of the rats were recorded within 5 min after anesthesia to establish the model of etomidate-induced myoclonus and to observe the dose dependence. The in vitro Western blot analysis of NKCC1 and KCC2 proteins and the regulatory effect of N-methyl-d-aspartate (NMDA) receptor were performed to find the potential target of etomidate-induced myoclonus or excitability. Additionally, to verify whether calpain-2 is involved in the process of regulatory effect of NMDAR on the cleavage of KCC2 protein during etomidate-induced myoclonus, muscular tension and KCC2 protein were analyzed in rats microinjected with calpain-2 inhibitor (MDL-28170) or MDL-28170 + NMDA in the neocortical motor cortex during etomidate anesthesia. Finally, MDL-28170 or vitamin E was injected intravenously before etomidate, the muscular tension, KCC2 protein and duration of loss of righting reflex (LORR) of rats were evaluated to verify the neuroprotective effect of vitamin E.

Results Etomidate significantly increased the mean behavioral score at different time points compared with the propofol and lidocaine + etomidate groups within 5 min after anesthesia; the mean behavioral score decreased at different time points with increasing dose of etomidate. 0.5 μM (0.73 ± 0.18 vs. 1.04 ± 0.17, $n = 6$, $p = 0.0096$) and 1 μM (0.73 ± 0.24 vs. 1.03 ± 0.14, $n = 6$, $p = 0.0077$) etomidate induced the decrement of neocortical KCC2 protein compared to the control group. NMDA activated but 2-amino-5-phosphonopentanoic acid (AP5) inhibited 0.5 and 1 μM etomidate-induced decrement of neocortical KCC2 protein. MDL-28170 microinjected into the neocortex during etomidate anesthesia not only inhibited the decrement of KCC2 protein but also blocked the muscular tension induced by etomidate alone or etomidate plus NMDA. Intravenous injection of vitamin E prevented etomidate-induced muscular tension and decrement of the KCC2 protein.

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Conclusion Calpain-2 was involved in the process of etomidate-induced myoclonus and NMDAR activity by promoting the decrement of KCC2 protein and exerting the excitability. Vitamin E, as a natural antioxidant, can effectively prevent etomidate-induced myoclonus and does not affect recovery after etomidate anesthesia in rats.

Keywords Etomidate, Myoclonus, Calpain-2, KCC2, Vitamin E

Introduction

Etomidate, a hypnotic intravenous anesthetic belonging to imidazole derivatives, has been widely used in clinical anesthesia, especially in elderly patients or those with cardiovascular disease due to its rapid induction, recovery, stable respiration and circulation [1]. Nevertheless, etomidate-induced myoclonus with an incidence of 50–80% [2–4] may result in accidental dislocation of the intravenous tube, monitoring devices, reflux aspiration and increased blood potassium levels [5]. Propofol or lidocaine have been reported to prevent etomidate-induced myoclonus [6, 7]. Previous research has demonstrated that etomidate-induced myoclonus in Sprague–Dawley rats involves neocortical glutamate accumulation and N-methyl-d-aspartate (NMDA) receptor activity [8]. To our knowledge, the mechanism of seizures includes impaired glutamatergic signaling, NMDAR activation, calcium overload, and calcium-activated calpain-mediated decrement of the K-Cl cotransporter (KCC2) protein [9–11]. Na-K-2Cl cotransporter isoform 1 (NKCC1) and KCC2 traffic chloride into (NKCC1) or out of (KCC2) the cell to maintain intracellular Cl^- homeostasis and regulate the function of the γ-aminobutyric acid (GABA) receptor and neuronal excitability [12]. However, the effect of etomidate at different concentrations, propofol and lidocaine plus etomidate in vitro on NKCC1 and KCC2 protein remains unknown.

Calpain has been suggested to play a vital role in the development of epilepsy, as this calcium-dependent kinase is activated after seizures, and its activation can lead to various types of neurodegeneration [13]. There are two major isomers of calpain in the brain, calpain 1 and calpain 2, which perform opposite functions in the brain. Micromolar concentrations of calcium activate calpain 1 to play a neuroprotective role, and millimolar calcium concentrations activate calpain 2 to play a neurodegenerative role by regulating the KCC2 decrement [14]. In the previous study, etomidate at concentrations of 0.5 μM and 1 μM activated neocortical intracellular calcium signaling, and NMDA enhanced, whereas AP5 inhibited this effect in vitro; but the intracellular calcium signaling was inhibited when the concentration increased to 5 μM and 10 μM [8]. Calpain-2 may be involved in the process of etomidate-induced myoclonus and modulation of NKCC1 and KCC2 proteins following the activation of NMDA receptors. Vitamin E is a well-known antioxidant that can be safely used in patients [15]. There is some evidence on the association between vitamin E and epilepsy for neuroprotective effects [16]; however, there is no evidence regarding the prevention of etomidate-induced myoclonus in rats and the therapeutic target remains uncertain.

We hypothesized that etomidate-induced myoclonus or excitability would be concentration-dependent and regionally different and the mechanism of etomidate-induced myoclonus would correlate with the activation of neocortical calpain-2 and its decrement of the KCC2 protein. Vitamin E could prevent etomidate-induced myoclonus by reversing the decrement of the KCC2 protein.

Methods

Animals

All animal experiments were approved by the Animal Ethics Committee of West China Hospital (ethical approval number: 20211423 A) and conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health [17]. Adult male (6–8 weeks old) Sprague Dawley (SD) rats weighing 200–340 g and young male rats weighing 78–184 g (3–6 weeks old) were purchased from Dossy Biological Technology Co. Ltd. (Chengdu, China). All rats were housed at 25 ± 1 °C with 60% humidity in the Animal Experimental Center of Sichuan University (Chengdu, China) on a 12-hour light/dark cycle (lights on at 7:00 am) and provided with water and food ad libitum. Rats were randomly assigned to experimental groups using a random number table, and tested in sequential order.

Anesthesia and quantification of behavioral activity in rats

Rats were randomly divided into 5 groups ($n = 10$/group): those that received intravenous doses of etomidate (1.5 mg/kg [2 times 50% effective dose (ED_{50})] [18], equivalent dose of propofol 11.8 mg/kg [19] and, those that received anticonvulsant dose of lidocaine (3 mg/kg) [7] plus etomidate (1.5 mg/kg) through the tail vein, those that received intravenous etomidate at the doses of 3.8 mg/kg [5 times ED_{50}], and 6.0 mg/kg [8 times ED_{50}]) through the tail vein. Rats were placed in individual transparent cages and assessed by a blinded observer at 0, 1, 2, 3, 4, and 5 min after anesthesia for the behavioral changes. The behavioral manifestations were classified according to the Modified Racine Scale [20]: stage 0: no response; stage 1: ear and facial twitching; stage 2: myoclonic jerks without rearing; stage 3: myoclonic jerks and

rearing; stage 4: turning over onto the side and tonic–clonic seizures; and stage 5: turning over onto the back and generalized tonic–clonic seizures. Rats were identified to exhibit myoclonus starting at stage 2.

Reagents and antibodies

All drugs, commercial antibodies and reagents were purchased from the following sources: etomidate (YT200712, Nhwa Pharma. Corporation, Xuzhou, China), propofol (X19055B, AstraZeneca UK, London, UK), lidocaine (2105J05, Zhaohui Pharma. Corporation, Shanghai), isoflurane (C002190101, YIPIN Pharma. Corporation, HuBei), 2-amino-5-phosphonopentanoic acid (AP5) (#GC17206, GlpBio Technology Inc, CA) and NMDA (#R054523, Shanghai Yien Chemical Technology Co., LTD, China). MDL-28170 (calpain-2 inhibitor, MedChemExpress, USA, Cat #:88191-84-8), Vitamin E (Sichuan Jishan Home Pharmaceutical Co. LTD, China), radioimmunoprecipitation assay buffer (#P0013B, Beyotime, China), protease inhibitor cocktail (Roche Diagnostics GmbH, Germany), Multicolor Prestained Protein Ladder (WJ103, Epizyme, China), bicinchoninic acid (BCA) assay (#P0010, Beyotime, China), chemiluminescent ClarityTM Western ECL Substrate (#170–5060, BIO-RAD, China), Omni-ECL™ Femto Light Chemiluminescence Kit (#SQ201, Yamei, China), rabbit anti-KCC2 (Proteintech, China, 1:1000, Cat #:19565-1-AP), rabbit anti-NKCC1 (Proteintech, China, 1:1000, Cat #:13884-1-AP), rabbit anti-calpain 2 (Cell Signaling Technologies, USA, 1: 1000, Cat #: 2539), rabbit anti-α-tubulin (Proteintech, China, 1:4000, Cat #:11224-1-AP).

Sample Preparation for Western blot assay

In vitro, acute brain slices were obtained from young (3–6 weeks) male SD rats. Rats were decapitated after a short period of isoflurane anesthesia (3% for induction, 2% for maintenance), and the brains were quickly removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM):124 NaCl, 4.5 KCl, 1.2 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, 10 glucose, 2 mM sodium pyruvate, and 1 mM sodium ascorbate (adjusted to 300–310 mOsm, pH 7.4, bubbled with O_2 and CO_2 (95% O_2, 5% CO_2). After 30 min of equilibration at room temperature, brain slices were transferred to the carbogenated no-magnesium ACSF containing etomidate (0.5 μM, 1 μM, 5 μM, 10 μM), propofol (30 μM), etomidate (0.5 μM) plus lidocaine (100 μM), etomidate (0.5 μM, 1 μM) plus NMDA (100 μM), etomidate (0.5 μM, 1 μM) plus AP5 (100 μM), respectively for another 2 h of incubation, proteins of KCC2 and NKCC1 were analyzed, samples of tissue from the hemisphere containing the neocortex and hippocampus were dissected. In vivo, adult male SD rats anesthetized intravenously through the tail vein with etomidate (1.5 mg/kg), lidocaine (3 mg/kg) plus etomidate (1.5 mg/kg), and propofol (11.8 mg/kg) were killed at 5 min after anesthesia, and calpain-2 protein was analyzed. Rats that were microinjected with 5 μL (0.09 μg/μL) of MDL-28170, NMDA, MDL-28170 + NMDA and the same volume of DMSO into the bilateral neocortex (from the bregma: 2.0 mm anteroposterior; 2.7 mm mediolateral; 1.8 mm dorsoventral) according to the hindlimb motor cortex map [21] during etomidate administration (1.5 mg/kg induction, 4 $mg{\cdot}kg^{-1}{\cdot}h^{-1}$ maintenance) were killed at 5 min after the microinjection, and the KCC2 protein was analyzed to verify whether calpain–2 was involved in the regulatory effect of NMDAR on the decrement of the KCC2 protein during etomidate-induced myoclonus. In addition, rats that were pretreated intravenously with MDL-28170 (3.0 mg/kg) or vitamin E (20.0 mg/kg) at 10 min prior to etomidate (1.5 mg/kg) were killed at 5 min after anesthesia, and the KCC2 protein was analyzed to verify the anticonvulsant effect of vitamin E. Samples in the neocortex were dissected from hemispheres in vivo.

Samples of the neocortex or hippocampus were subjected to a radioimmunoprecipitation assay with a protease inhibitor cocktail. Tissue was immediately homogenized, and analyzed by the enhanced bicinchoninic acid (BCA) assay by microplate reader (EPOCH2, BioTek Instruments, Inc, USA). Standard protein electrophoresis was performed using Tris-glycine polyacrylamide 8% gels, loading buffer and running a multicolor stained protein ladder. Wet transfer was performed using a Trans-Blot SD device (Bio-Rad). Blots were blocked with 5% nonfat dry milk (Bio-Rad) and then incubated overnight with primary antibodies: rabbit anti-KCC2, rabbit anti-NKCC1, rabbit anti-calpain 2, and rabbit anti-alpha tubulin. Secondary antibody goat anti-rabbit HRP was incubated for 2 h at room temperature. The blot was then cut and processed individually with chemiluminescent ClarityTM Western ECL Substrate or Omni-ECL™ Femto Light Chemiluminescence Kit and immediately imaged on an Amersham Imager 600 (GE, USA). ImageJ and KCC2, NKCC1, and calpain 2 densities were normalized to alpha-tubulin loading controls.

Muscle tension monitoring

A calibration-free biotension sensor (FT-102) was used to monitor muscular tension. The sensor was attached to a holder to ensure that the direction of the force was perpendicular to the spring plate of the force and that the probe was inserted into the left gastrocnemius muscle. The signal was recorded using a BL420N Biological Signal Acquisition and Analysis system (Chengdu Techman Software Co, Ltd, Chengdu, China). The peak muscular tension amplitude and frequency were recorded in adult rats that were anesthetized intravenously with etomidate alone (1.5 mg/kg induction, 4 $mg{\cdot}kg^{-1}{\cdot}h^{-1}$ maintenance),

propofol (11.8 mg/kg induction, 60 mg·kg^{-1}·h^{-1} maintenance) alone, lidocaine 3 mg/kg plus etomidate (1.5 mg/kg induction, 4 mg·kg^{-1}·h^{-1} maintenance), microinjected with 5 μL (0.09 μg/μL) of MDL-28170, NMDA, MDL-28170 + NMDA and the same volume of DMSO in the neocortex during etomidate anesthesia, and pretreated with MDL-28170 (3.0 mg/kg), vitamin E (20.0 mg/kg) and an equal dose of DMSO intravenously through the tail vein at 10 min before etomidate anesthesia.

Loss of righting reflex (LORR) measurement

Rats were pretreated intravenously with MDL-28170 (3.0 mg/kg), vitamin E (20.0 mg/kg) and an equal dose of DMSO at 10 min before etomidate (1.5 mg/kg induction), loss of righting reflex (LORR) was assessed after anesthesia. LORR was considered positive if the mouse remained on its back with at least three paws in the air for 60 s [22]. Recovery of LORR was recorded when the rat was able to roll from lateral to sternal recumbency three times without assistance [23].

Statistical analysis

All data were obtained by researchers blinded to treatment regimens and reported as the means and standard deviations (means ± SD). The mean behavioral scores with different drugs and different doses of etomidate at different time points were compared by two-way analysis of variance (ANOVA) (linear mixed model), with a post-hoc Bonferroni correction test was performed for multiple comparisons. Muscular tension recordings and western blot data were compared using one-way ANOVA, followed by Tukey's test for multiple comparisons between groups or two-sample t test comparisons within groups for normally distributed data and the Kruskal–Wallis test followed by Dunn's multiple comparison between groups or two-tailed Wilcoxon signed rank test comparisons within groups for non-normally distributed data. Sample size was calculated by using G*Power 3 [24] with power (1 – β) set at 0.85, and α = 0.05 (2 side), indicating $n = 10$/group for quantification of etomidate-induced myoclonus, $n = 6$/group for muscular tension recording and Western blot analysis. Statistical analyses were performed using Prism 9.0.0 software (GraphPad Software, San Diego, CA) and SPSS software, version 19.0 (IBM SPSS Statistics). $p < 0.05$ was considered statistically significant.

Results

Etomidate-induced myoclonus was dose-dependent

Behavioral scores of rats in the etomidate, propofol, and lidocaine plus etomidate groups were recorded at different time points. Etomidate increased the mean behavioral score at 1 min (1.80 ± 1.32 vs. 0.00 ± 0.00, $p = 0.0049$; 1.80 ± 1.32 vs. 0.20 ± 0.42, $p = 0.0099$), 2 min (1.70 ± 1.25 vs. 0.10 ± 0.32, $p = 0.0072$; 1.70 ± 1.25 vs. 0.00 ± 0.00, $p = 0.0051$), 3 min (1.70 ± 1.34 vs. 0.00 ± 0.00; 1.70 ± 1.34 vs. 0.00 ± 0.00, both for $p = 0.0076$), 4 min (1.20 ± 1.23 vs. 0.00 ± 0.00; 1.20 ± 1.23 vs. 0.00 ± 0.00, both for $p = 0.0316$), 5 min (1.70 ± 0.95 vs. 0.00 ± 0.00; 1.70 ± 0.95 vs. 0.00 ± 0.00, both for $p = 0.0008$), but did not affect the mean behavioral score at 0 min (0.30 ± 0.48 vs. 0.00 ± 0.00; 0.30 ± 0.48 vs. 0.00 ± 0.00, both for $p = 0.1769$) compared to the propofol and lidocaine plus etomidate groups (Fig. 1A, B). Etomidate at doses of 3.8 mg/kg and 6.0 mg/kg decreased the mean behavioral score at 1 min (0.00 ± 0.00 vs.1.80 ± 1.32; 0.00 ± 0.00 vs. 1.80 ± 1.32, both for $p = 0.0058$), 2 min (0.10 ± 0.32 vs.1.70 ± 1.25, $p = 0.0084$; 0.00 ± 0.00 vs. 1.70 ± 1.25, $p = 0.0060$), 3 min (0.10 ± 0.32 vs.1.70 ± 1.34, $p = 0.0127$; 0.00 ± 0.00 vs. 1.70 ± 1.34, $p = 0.0091$), 5 min (0.20 ± 0.42 vs.1.70 ± 0.95, $p = 0.0018$; 0.00 ± 0.00 vs. 1.70 ± 0.95, $p = 0.0009$), but did not affect the mean behavioral score at 0 min (0.20 ± 0.60 vs. 0.30 ± 0.50; 0.20 ± 0.42 vs. 0.30 ± 0.50, both for $p > 0.9999$) compared to the etomidate at the dose of 1.5 mg/kg. 6.0 mg/kg of etomidate (0.00 ± 0.00 vs. 1.20 ± 1.23, $p = 0.0390$) decreased, 3.8 mg/kg of etomidate did not affect (0.20 ± 0.63 vs. 1.20 ± 1.23, $p = 0.1168$) the mean behavioral score at 4 min compared to the 1.5 mg/kg of etomidate (Fig. 1C, D). This result indicated that etomidate-induced myoclonus was dose dependent.

Effect of NMDAR activity on etomidate-induced changes in neocortical KCC2 expression

Based on the previous research, we found that 0.5 and 1 μM etomidate activated neocortical intracellular calcium signaling [8], which indicated that etomidate induced cell excitability in a concentration-dependent manner. We further conducted in vitro western blot analysis of brain slices at different concentrations of etomidate to verify the effect of etomidate on the NKCC1 and KCC2 proteins. As shown in Fig. 2A-I. In the neocortex, we found that 0.5 μM (0.73 ± 0.18 vs. 1.04 ± 0.17, $n = 6$, $p = 0.0096$) and 1 μM (0.73 ± 0.24 vs. 1.03 ± 0.14, $n = 6$, $p = 0.0077$) etomidate induced, 5 μM etomidate (0.94 ± 0.15 vs. 0.77 ± 0.13, $n = 6$, $p = 0.1627$) did not affect but 10 μM etomidate (1.39 ± 0.17 vs. 0.77 ± 0.13, $n = 6$, $p < 0.0001$) inhibited the decrement of KCC2 protein compared to the control group. Additionally, 0.5 μM etomidate plus NMDA (0.12 ± 0.02 vs. 0.73 ± 0.18, $n = 6$, $p = 0.0006$) and 1 μM etomidate plus NMDA (0.24 ± 0.16 vs. 0.73 ± 0.24, $n = 6$, $p = 0.0182$) activated, 0.5 μM etomidate plus AP5 (1.48 ± 0.33 vs. 0.73 ± 0.18, $n = 6$, $p < 0.0001$) and 1 μM etomidate plus AP5 (1.39 ± 0.36 vs. 0.73 ± 0.24, $n = 6$, $p = 0.0017$) inhibited the decrement of KCC2 protein compared to 0.5 μM etomidate and 1 μM etomidate alone. Both lidocaine plus 0.5 μM etomidate (1.44 ± 0.27 vs. 0.73 ± 0.18, $n = 6$, $p < 0.0001$) and propofol (1.39 ± 0.25 vs. 0.73 ± 0.24, $n = 6$, $p = 0.0018$) inhibited

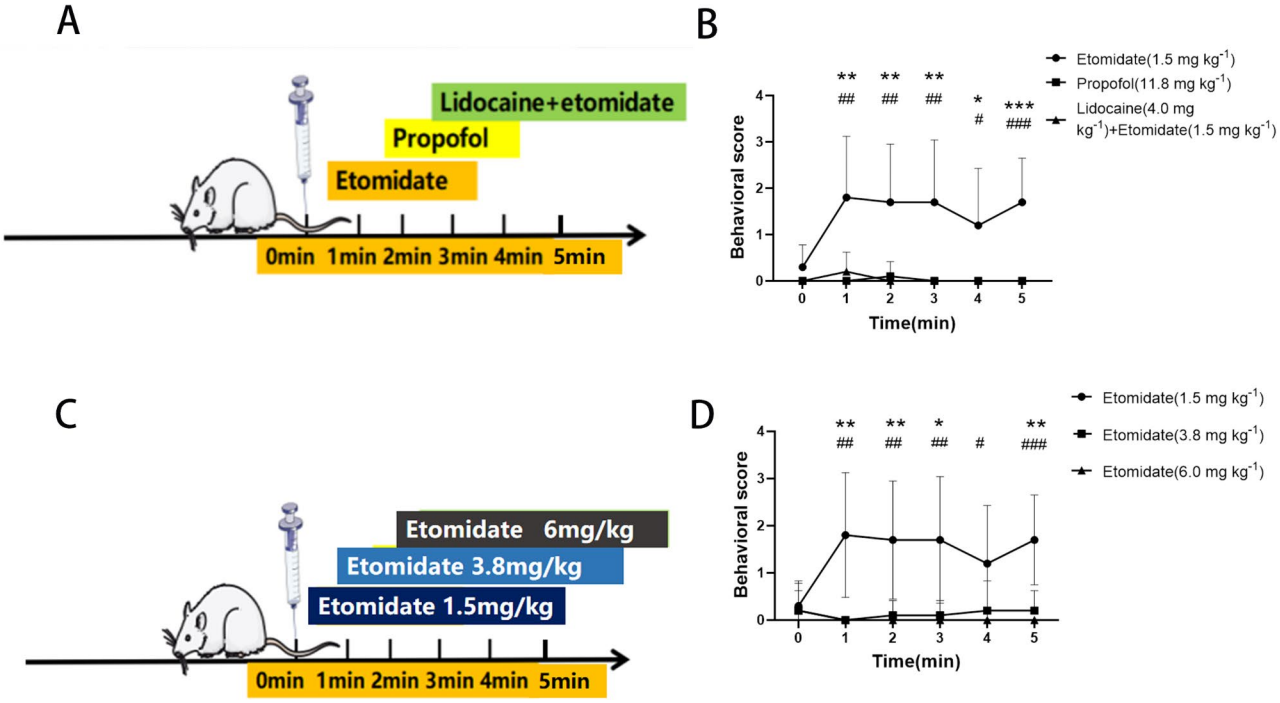

Fig. 1 Quantification of the behavioral activity in rats in vivo. **A** Rats were injected intravenously through the tail vein with etomidate (1.5 mg/kg), propofol (11.8 mg/kg), lidocaine (3 mg/kg) plus etomidate (1.5 mg/kg). **B** The trend of behavioral score at different time points in the etomidate, propofol, lidocaine plus etomidate groups. **(C)** Rats were injected intravenously through the tail vein with etomidate at different dose (1.5 mg/kg, 3.8 mg/kg, 6.0 mg/kg). **(D)** The trend of behavioral score at different time points in the different dose of etomidate groups (1.5 mg/kg, 3.8 mg/kg, 6.0 mg/kg). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ the etomidate group vs. the propofol group; #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$ the etomidate group vs. the lidocaine plus etomidate group by the two way ANOVA (linear mixed-model) analysis and a post-hoc Bonferroni correction test

the decrement of KCC2 protein compared to the 0.5 μM etomidate group and the 1 μM etomidate group, respectively. None of the different concentrations of etomidate (0.5 μM (1.03 ± 0.13 vs. 0.93 ± 0.25, $n = 6$, $p = 0.4331$), 1 μM (0.97 ± 0.15 vs. 0.88 ± 0.07, $n = 6$, $p = 0.1713$), 5 μM (1.04 ± 0.08 vs. 0.98 ± 0.12, $n = 6$, $p = 0.4656$), 10 μM (1.06 ± 0.06 vs. 0.98 ± 0.12, $n = 6$, $p = 0.2665$)) affected the NKCC1 protein compared to the control group. Lidocaine plus etomidate ($p = 0.5170$), propofol ($p = 0.8844$), 0.5 μM etomidate plus NMDA ($p = 0.0595$), 1 μM etomidate plus NMDA ($p = 0.0595$), 0.5 μM etomidate plus AP5 ($p = 0.4941$), and 1 μM etomidate plus AP5 ($p = 0.6661$) did not affect the NKCC1 protein compared to the etomidate alone.

In the hippocampus, etomidate at concentrations of 0.5 μM (1.20 ± 0.14 vs. 0.95 ± 0.08, $n = 6$, $p = 0.0125$), 1 μM (1.21 ± 0.20 vs. 0.71 ± 0.27, $n = 6$, $p = 0.0196$), 5 μM (1.19 ± 0.13 vs. 0.78 ± 0.14, $n = 6$, $p = 0.0002$), and 10 μM (1.01 ± 0.12 vs. 0.78 ± 0.14, $n = 6$, $p = 0.0188$) inhibited the decrement of KCC2 protein compared to the control group. Neither lidocaine plus 0.5 μM etomidate (1.33 ± 0.27 vs. 1.20 ± 0.14, $n = 6$, $p = 0.3085$) nor propofol (1.29 ± 0.32 vs. 1.21 ± 0.20, $n = 6$, $p = 0.3430$) affected the KCC2 protein compared to the 0.5 μM etomidate group and the 1 μM etomidate group, respectively. None of the different concentrations of etomidate (0.5 μM (0.97 ± 0.20 vs. 1.05 ± 0.14, $n = 6$, $p = 0.2490$), 1 μM (0.99 ± 0.29 vs. 1.07 ± 0.20, $n = 6$, $p = 0.6721$), 5 μM (1.03 ± 0.16 vs. 0.93 ± 0.10, $n = 6$, $p = 0.3952$), 10 μM (0.97 ± 0.09 vs. 0.93 ± 0.10, $n = 6$, $p = 0.8599$)) affected the NKCC1 protein compared to the control group. Lidocaine plus etomidate ($p = 0.9871$) and propofol ($p = 0.3726$) also did not affect the NKCC1 protein compared to etomidate alone.

Etomidate increased muscular tension and the level of the calpain-2 protein

We performed muscle tension monitoring combined with Western blot assays to explore the relationship between etomidate-induced myoclonus and the calpain-2 protein. The results showed that etomidate increased the peak muscular tension amplitude (4.87 ± 1.77 vs. 0.80 ± 0.29 g; 4.87 ± 1.77 vs. 0.57 ± 0.34 g, both for $n = 6$, $p < 0.0001$) or frequency (4.61 ± 1.41 vs. 0.46 ± 0.18 Hz; 4.61 ± 1.41 vs. 0.47 ± 0.33 Hz, both for $n = 6$, $p < 0.0001$) compared to the propofol and lidocaine plus etomidate groups. In addition, etomidate increased the level of neocortical calpain-2 protein (1.43 ± 0.16 vs. 0.74 ± 0.23, $n = 6$, $p = 0.0022$; 1.43 ± 0.16 vs. 0.92 ± 0.16, $n = 6$, $p = 0.0148$) but did not affect hippocampal calpain-2 protein (0.86 ± 0.23 vs. 0.74 ± 0.16, $n = 6$, $p = 0.4515$; 0.86 ± 0.23 vs. 1.02 ± 0.18,

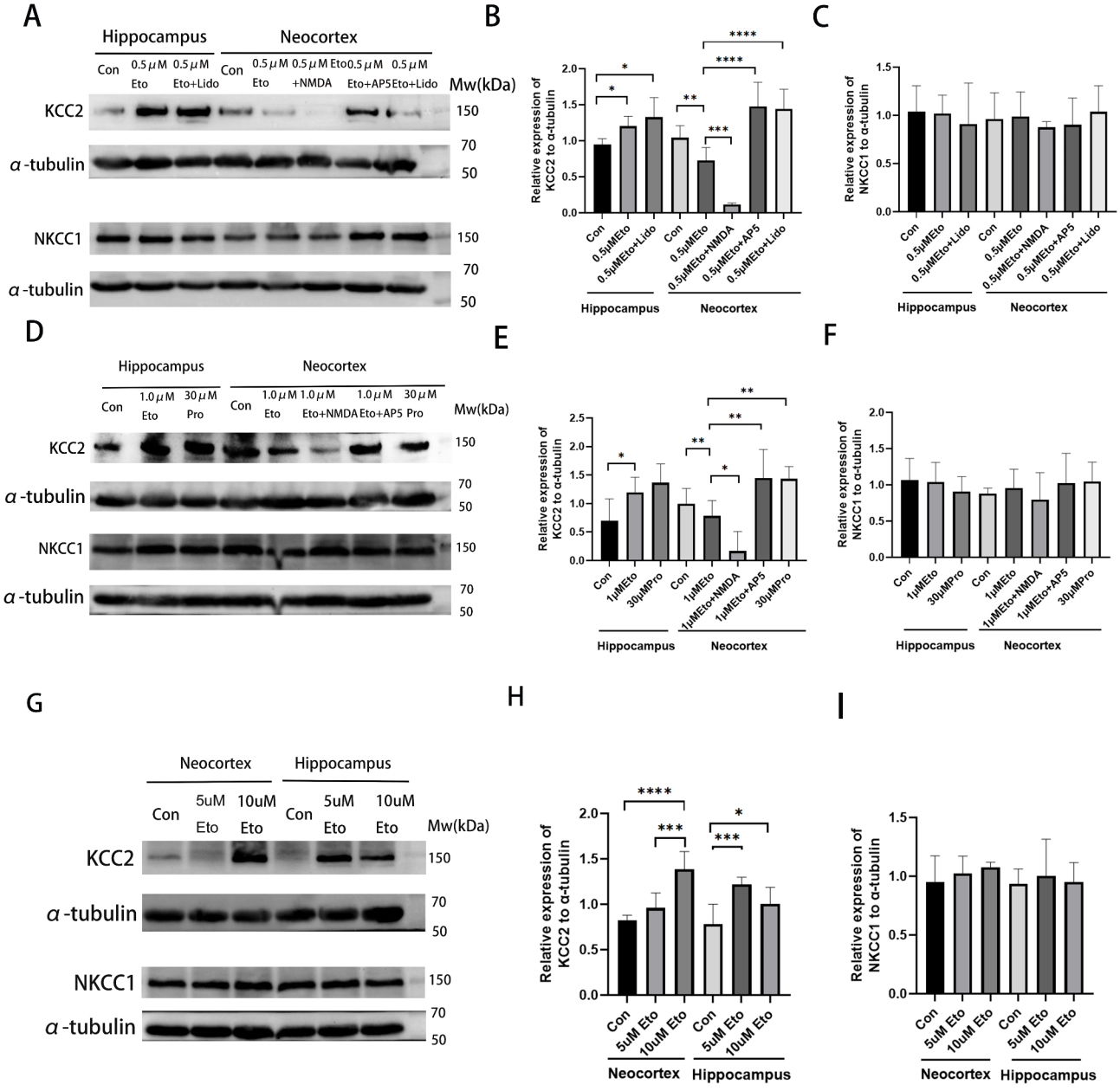

Fig. 2 Western blot analysis for KCC2 and NKCC1 proteins of brain slices in vitro. Western blot analysis for KCC2, NKCC1 at 0.5 μm (**A**), 1μM (**D**), 5μM and 10μM (**G**) of etomidate. Quantification of blot after normalization to α-tubulin of KCC2 (**B**), NKCC1(**C**), in control group, 0.5 μM etomidate group, 0.5 μM etomidate plus 100 μM lidocaine group, 0.5 μM etomidate plus 100 μM NMDA group and 0.5 μM etomidate plus 100 μM AP5 group in neocortex and hippocampus ($n=6$ rats in each group). Quantification of blot after normalization to α-tubulin of KCC2(**E**), NKCC1(**F**) in control group, 1 μM etomidate group, 1 μM etomidate plus 100 μM lidocaine group, 1 μM etomidate plus 100 μM NMDA group and 1 μM etomidate plus 100 μM AP5 group in neocortex and hippocampus ($n=6$ rats in each group). Quantification of blot after normalization to α-tubulin of KCC2 (**H**), NKCC1 (**I**) in control and 5 μM and 10 μM etomidate groups ($n=6$ rats in each group). Con: Control. Eto: Etomidate. Pro: Propofol. Lido: Lidocaine. AP5: DL-2-amino-5-phosphopentanoic acid. NMDA: N-methyl-d-aspartate.*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$ by one-way multiple comparisons ANOVA and two-tailed t-Test

$n = 6$, $p = 0.4201$) (Fig. 3A-C) compared to propofol and lidocaine plus etomidate. Furthermore, there were strong correlations between the peak muscular tension (amplitude or frequency) and the neocortical calpain-2 protein (Spearman's $r = 0.7779$, $p = 0.0001$; Spearman's $r = 0.8039$, $p < 0.0001$, Fig. 3D, E). However, the correlation analysis revealed no significant relationships between the peak muscular tension (amplitude or frequency) and the hippocampal calpain-2 protein (Spearman's $r = 0.1188$, $p = 0.6387$; Spearman's $r = 0.2239$, $p = 0.3717$; Fig. 3F, G.

Microinjection of the calpain-2 inhibitor MDL-28170 in the neocortex alleviated etomidate-induced myoclonus and decrement of the KCC2 protein

Rats were microinjected with 0.5 μg of MDL-28170, NMDA, MDL-28170 + NMDA and DMSO into the neocortex during etomidate anesthesia to explore the role of calpain-2 in the effect of etomidate-induced myoclonus and the modulation of NMDA receptors. Etomidate plus NMDA increased the peak muscular tension amplitude (10.79 ± 3.10 vs. 6.33 ± 2.52 g, $n = 6$, $p = 0.0058$), but did not affect the peak muscular tension frequency (9.75 ± 5.20 vs. 8.18 ± 3.63 Hz, $n = 6$, $p = 0.8456$) compared to the etomidate plus DMSO group. Etomidate plus MDL-28170 significantly decreased the peak muscular tension amplitude (1.74 ± 0.68 vs. 6.33 ± 2.52 g, $n = 6$, $p = 0.0045$) and frequency (2.75 ± 1.71 vs. 8.18 ± 3.63 Hz, $n = 6$, $p = 0.0465$) compared to the etomidate plus DMSO group. Both etomidate plus MDL-28170 and etomidate plus NMDA plus MDL-28170 decreased the peak muscular tension amplitude (1.74 ± 0.68 vs. 10.79 ± 3.10 g, $n = 6$, $p < 0.0001$; 1.36 ± 0.44 vs.10.79 ± 3.10 g, $n = 6$, $p < 0.0001$) and frequency (2.75 ± 1.71 vs. 9.75 ± 5.20 Hz, $n = 6$, $p = 0.0079$; 1.83 ± 0.98 vs. 9.75 ± 5.20 Hz, $n = 6$, $p = 0.0027$) compared to the etomidate plus NMDA group (Fig. 4A). Etomidate plus NMDA induced neocortical decrement of the KCC2 protein (0.71 ± 0.10 vs. 1.04 ± 0.15, $n = 6$, $p = 0.0197$), etomidate plus MDL-28170 suppressed the neocortical decrement of KCC2 protein (1.28 ± 0.22 vs. 1.04 ± 0.15, $n = 6$, $p = 0.0038$) compared to the etomidate plus DMSO group. Both etomidate plus MDL-28170 and etomidate plus NMDA plus MDL-28170 suppressed the neocortical decrement of KCC2 protein (1.28 ± 0.22 vs. 0.71 ± 0.10, $n = 6$, $p = 0.0053$; 1.23 ± 0.18 vs. 0.71 ± 0.10, $n = 6$, $p = 0.0080$) compared to the etomidate plus NMDA group (Fig. 4B, C).

Intravenous injection of vitamin E prevented etomidate-induced myoclonus and alleviated neocortical decrement of the KCC2 protein

The effect of vitamin E on etomidate-induced myoclonus has not been reported. We found that pretreatment

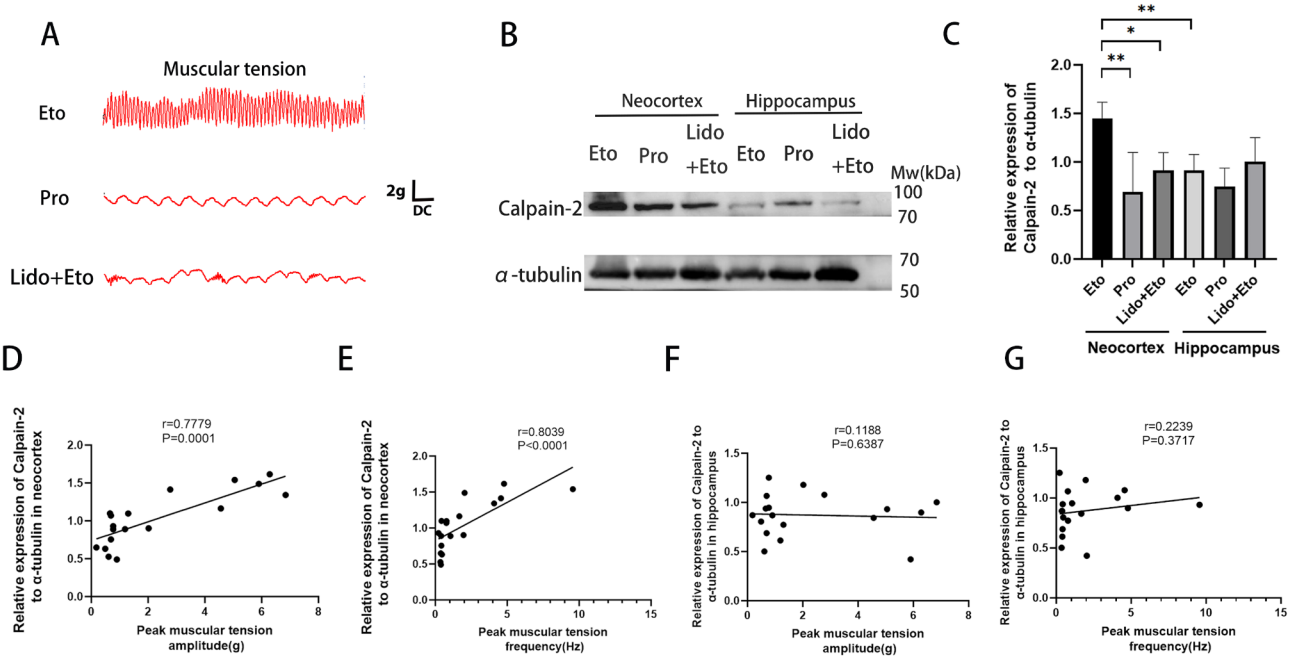

Fig. 3 Etomidate increased the muscular tension and the level of the calpain-2 protein in rats in vivo. **(A)** Muscular tension monitoring in the etomidate, propofol, lidocaine plus etomidate group. **(B)** Western blot analysis for calpain-2 protein of neocortex and hippocampus in the etomidate, propofol, lidocaine plus etomidate group. **(C)** Quantification of blot after normalization to α-tubulin of calpain-2 in the etomidate, propofol, lidocaine plus etomidate group. Spearman correlation analysis of the relationship between the peak muscular tension amplitude and relative expression of calpain-2 to α-tubulin in neocortex ($r = 0.7779$, $p = 0.0001$, **D**) and hippocampus ($r = 0.1188$, $p = 0.6387$, **F**) and the relationship between the peak muscular tension frequency and relative expression of calpain-2 to α-tubulin in neocortex ($r = 0.8039$, $p < 0.0001$, **E**) and hippocampus ($r = 0.2239$, $p = 0.3717$, **G**). *: $p < 0.05$, **: $p < 0.01$ by one-way multiple comparison ANOVA test

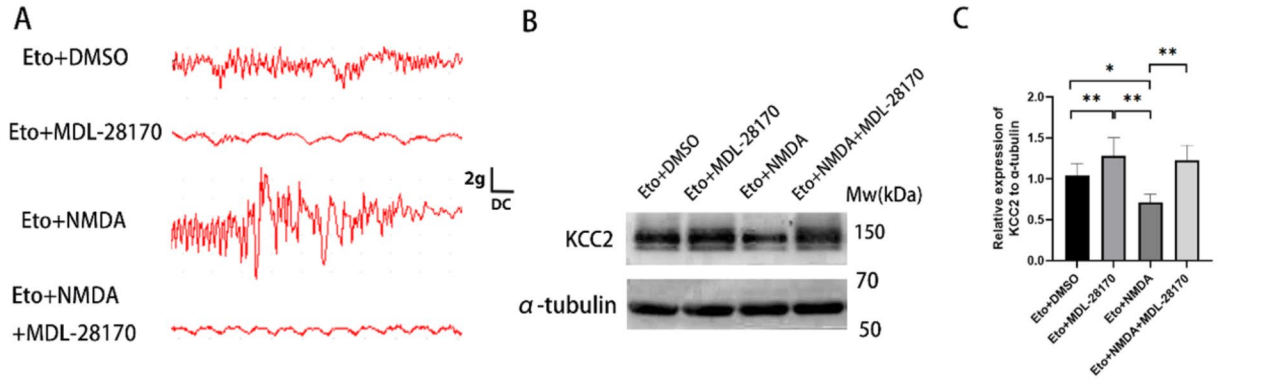

Fig. 4 MDL-28170 microinjected into the neocortex alleviated etomidate-induced myoclonus and the decrement of KCC2 protein in vivo. Muscular tension monitoring (**A**), Western blot analysis for KCC2 protein of neocortex (**B**), and quantification of blot after normalization to α-tubulin of KCC2 (**C**) in the etomidate plus DMSO, etomidate plus MDL-28170, etomidate plus NMDA, etomidate plus NMDA plus MDL-28170 groups. *: $p < 0.05$, **: $p < 0.01$ by one-way multiple comparison ANOVA test

with intravenous injection of MDL-28170 plus etomidate and vitamin E plus etomidate decreased the etomidate-induced peak muscular tension amplitude (1.87 ± 0.75 vs. 6.63 ± 3.00 g; 1.52 ± 0.71 vs. 6.63 ± 3.00 g, both for $n = 10$, $p < 0.0001$) and frequency (2.25 ± 1.07 vs. 7.68 ± 4.13 Hz; 2.00 ± 0.89 vs. 7.68 ± 4.13 Hz, both for $n = 10$, $p < 0.0001$) compared to the DMSO plus etomidate group (Fig. 5A). Moreover, both MDL-28170 plus etomidate and vitamin E plus etomidate deccreased the mean behavioral score at 0 min (0.10 ± 0.32 vs. 0.80 ± 0.63, $n = 10$, $p = 0.0234$; 0.20 ± 0.42 vs. 0.80 ± 0.63, $n = 10$, $p = 0.0128$), 1 min (0.10 ± 0.32 vs. 1.40 ± 0.70, $n = 10$, $p = 0.0005$; 0.00 ± 0.00 vs. 1.40 ± 0.70, $n = 10$, $p = 0.0004$), 2 min (0.30 ± 0.67 vs. 1.50 ± 0.71, $n = 10$, $p < 0.0001$; 0.20 ± 0.63 vs.1.50 ± 0.71, $n = 10$, $p = 0.0094$), 3 min (0.00 ± 0.00 vs. 1.70 ± 1.25, $n = 10$, $p = 0.0051$; 0.10 ± 0.32 vs. 1.70 ± 1.25, $n = 10$, $p = 0.0050$), 4 min (0.00 ± 0.00 vs. 0.80 ± 0.79; 0.00 ± 0.00 vs. 0.80 ± 0.79, both for $n = 10$, $p = 0.0262$), 5 min (0.00 ± 0.00 vs. 1.10 ± 0.88; 0.00 ± 0.00 vs.1.10 ± 0.88, both for $n = 10$, $p = 0.0082$) after anesthesia compared to the DMSO plus etomidate group (Fig. 5B). The neocortical level of KCC2 protein was evaluated among the three groups to verify the calpain-2 inhibitor effect of vitamin E. We found that both MDL–28170 plus etomidate (1.27 ± 0.10 vs. 0.80 ± 0.22, $n = 6$, $p = 0.0011$) and vitamin E plus etomidate (1.09 ± 0.19 vs. 0.80 ± 0.22, $n = 6$, $p = 0.0332$) alleviated etomidate-induced neocortical decrement of KCC2 protein compared to the DMSO plus etomidate group (Fig. 5C, D). For the duration of LORR, we found that neither MDL-28170 (9.59 ± 0.64 vs. 9.47 ± 0.94 min, $n = 10$, $p = 0.9406$) nor vitamin E (10.28 ± 0.65 vs. 9.47 ± 0.94 min, $n = 10$, $p = 0.0621$) affected the duration of LORR after etomidate anesthesia compared to DMSO(Fig. 5E).

Discussion

Etomidate and its analogs, such as ET-26-HCL [18] and ABP-700 [25], can induce myoclonus and have become an urgent clinical problem. In this study, we found that etomidate at concentrations of 0.5 μM and 1 μM induced the decrement of neocortical KCC2 protein, NMDA activated and AP5 inhibited 0.5 μM and 1 μM induced decrement of neocortical KCC2 protein. However, etomidate at concentrations of 5 μM and 10 μM did not affect the decrement of neocortical KCC2 protein, and etomidate at concentrations of 0.5 μM, 1 μM, 5 μM and 10 μM did not affect the decrement of hippocampal KCC2 protein. None of the different concentrations of etomidate (0.5 μM, 1 μM, 5 μM, 10 μM), NMDA and AP5 affected the NKCC1 protein in the neocortex or hippocampus. This indicated that etomidate induced decrement of KCC2 protein in a concentration-dependent and regionally different manner via the modulation of NMDARs. Consistent with a previous study, etomidate at low concentrations (0.1-1 μM) modulated the function of $GABA_A$ receptors, etomidate at moderate concentrations (< 5–10 μM) directly activated $GABA_A$ receptors, and etomidate at high concentrations (> 5–10 μM) suppressed the function of $GABA_A$ receptors [26–29]. Different effects of etomidate on KCC2 protein in the neocortex and hippocampus may be due to the different distribution of GABA receptor subtypes in the neocortex and hippocampus and their different sensitivity to etomidate, resulting in neuronal excitability [30]. Lateral diffusion and aggregation of KCC2 is the key mechanism by which glutamine signaling rapidly and reversibly regulates the expression and function of KCC2 membrane proteins. Calcium-dependent calpain activity causes KCC2 decrement at the C-terminus and has been shown to contribute to the reduction in KCC2 protein activity [31].

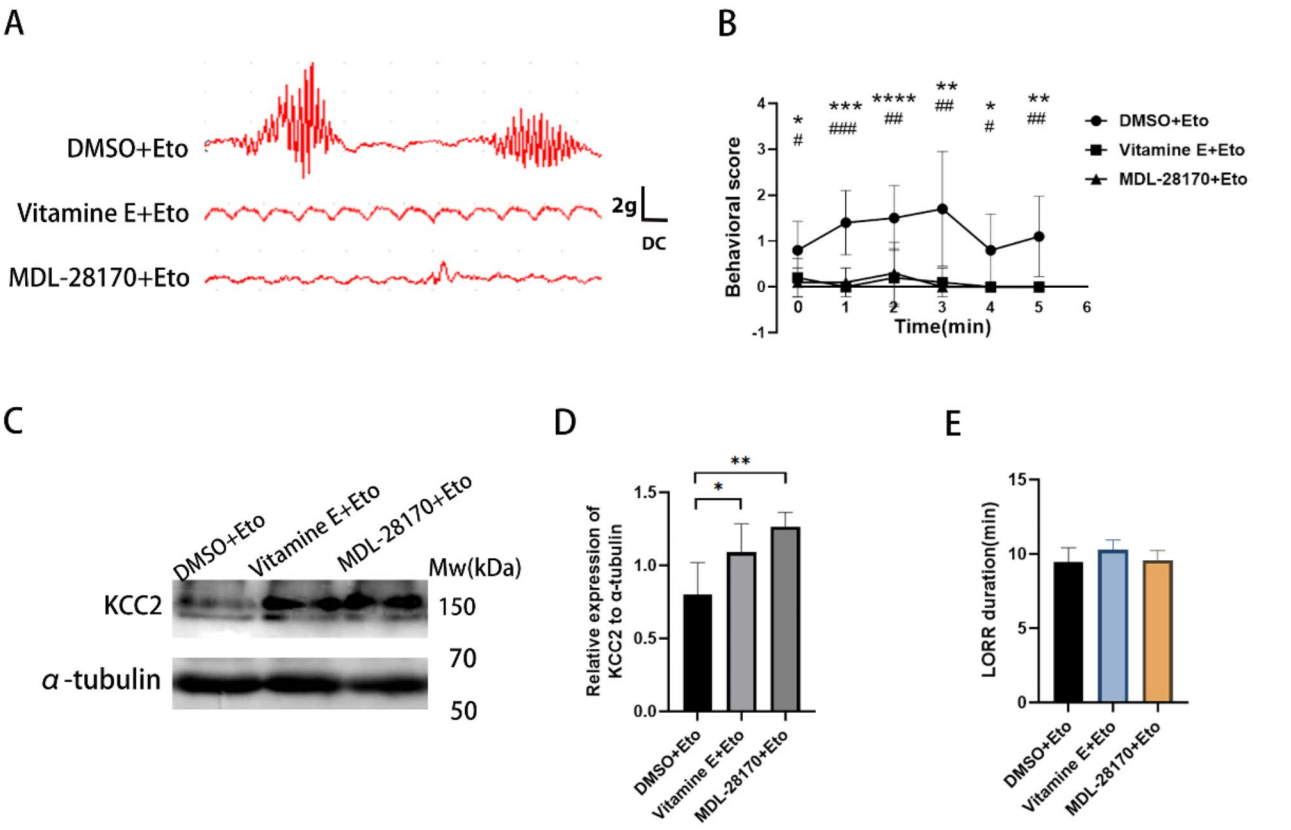

Fig. 5 Vitamin E prevented etomidate-induced myoclonus and alleviated neocortical decrement of KCC2 protein in vivo. **(A)** Muscular tension monitoring in the DMSO plus etomidate, MDL-28170 plus etomidate, vitamin E plus etomidate group. **(B)** The trend of behavioral score at different time points in the DMSO plus etomidate, DMSO plus etomidate, vitamin E plus etomidate groups. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, the DMSO plus etomidate group vs. MDL-28170 plus etomidate group; #: $p < 0.05$, ##: $p < 0.01$, ###: $p < 0.001$, ####: $p < 0.0001$, the DMSO plus etomidate group vs. vitamin E plus etomidate group by two way analysis of variance (ANOVA) (linear mixed-model) analysis and a post-hoc Bonferroni correction test. Western blot analysis for KCC2 protein of neocortex(**C**), quantification of blot after normalization to α-tubulin of KCC2(**D**), and duration of LORR(**E**) in the DMSO plus etomidate, MDL-28170 plus etomidate, vitamin E plus etomidate groups. Eto: etomidate, LORR: loss of righting reflex. *: $p < 0.05$, **: $p < 0.01$ by one-way multiple comparison ANOVA test

Etomidate-induced myoclonus originates in the neocortex, and myoclonus correlates with NMDA receptor-induced downregulation of KCC2 protein expression [8]. Rats were microinjected with the calpain-2 inhibitor MDL-28170 in the neocortex during etomidate anesthesia to verify whether calpain-2 was involved in the regulatory effect of NMDAR on the decrement of KCC2 protein during etomidate-induced myoclonus. We found that MDL-28170 and NMDA + MDL-28170 not only blocked the etomidate and etomidate + NMDA induced muscular myoclonus but also the decrement of KCC2 protein. This indicated that calpain-2 was involved in the process of etomidate-induced myoclonus and NMDAR activity by promoting the decrement of KCC2 protein.

Calpain-2 has long been implicated in neuronal cell damage, such as hyperexcitatory toxicity, and the development of neuropathology associated with epileptic seizures [32]. In our study, intravenous injection of MDL-28170 or vitamin E could prevent etomidate-induced myoclonus, decrease the mean behavioral score at different time points, and reverse the decrement of KCC2 protein, but neither of them affected the duration of LORR after etomidate anesthesia. This result indicated that vitamin E, as a natural antioxidant, can effectively prevent etomidate-induced myoclonus and the mechanism may correlate with the reversion of KCC2 protein decrement. Vitamin E is a natural oxygen free radical scavenger localized in cell membranes that can maintain membrane stability through free radical elimination, inhibition, repair and endogenous defense mechanisms, giving it very rapid and extensive antioxidant capacity [33]. Previous studies have also demonstrated that vitamin E inhibited the expression of calpain-2 or calmodulin-dependent protein kinase II-α in the treatment of epilepsy [34] and Alzheimer's disease [35]. Vitamin E is often administered orally in clinical settings [36]. Etomidate drugs that are compatible with vitamin E or its metabolites for intravenous use may have the potential to be transformed into current

antiepileptic medications [37]. This possibility warrants further investigation.

Our study has several limitations. First, whether vitamin E can prevent etomidate-induced myoclonus in patients remains unknown. The pharmacodynamics and pharmacokinetics of etomidate drugs compatible with vitamin E or its metabolites need to be further investigated. Second, only male rats were included in our study. The effect of etomidate on the function of GABA receptor is sex hormone-dependent [38].

In conclusion, etomidate regulated KCC2 protein and myoclonus by activating NMDA receptors in a concentration-dependent and regionally different manner. Calpain-2 was involved in the process of etomidate-induced myoclonus and NMDAR activity by promoting the decrement of KCC2 protein and exerting the excitability. Vitamin E, as a natural antioxidant, can effectively prevent etomidate-induced myoclonus and does not affect recovery after etomidate anesthesia in rats.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12871-025-03065-3.

Supplementary Material 1

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Not applicable.

Author contributions
Yan Feng: Data curation, Formal analysis, Methodology, Resources, Supervision, Validation, Visualization, Writing-original draft. Yong-xiang Cheng: Writing-review & editing and Preparing the figures. Xing-hao Wang: Data curation, Formal analysis, Methodology, Resources, Supervision, Writing-original draft, Preparing the figures.

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Data availability
The datasets used or analyzed during this study can be made available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Animal experiments were approved by the Animal Ethics Committee of West China Hospital (ethical approval number: 20211423 A) and conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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