Etomidate Modulates the Tactile Stimulation-Evoked Field Potential Responses in Cerebellar Granule Cell Layer in vivo in Mice

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Keywords
Etomidate · Cerebellar granule cell layer · Cannabinoid 1 receptor · Sensory stimulation · Electrophysiological recording · Protein kinase A

Abstract
Etomidate (ET) produces sedation by binding on the γ-aminobutyric acid type A (GABA_A) receptors. We previously found that ET inhibited cerebellar Purkinje cells activity via both GABA_A and glycine receptors in vivo in mice, suggesting that ET modulated sensory information synaptic transmission in cerebellar cortex. In this study, we investigated the effect of ET on the sensory stimulation-evoked responses in the cerebellar granule layer (GL) in urethane-anesthetized mice, using electrophysiological and pharmacological methods. Our results showed that cerebellar surface perfusion of ET (100 μmol/L) significantly decreased amplitude and area under the curve (AUC) of the sensory stimulation-evoked excitatory component (N1) in the cerebellar GL. Application of GABA_A receptor antagonist, SR95531 (20 μmol/L) significantly attenuated, but not abolished the ET-induced decrease in amplitude and AUC of facial stimulation-evoked responses. However, application of a mixture of SR95531 (20 μmol/L) and cannabinoid 1 receptor (CB1) antagonist, AM-251 (5 μmol/L), completely blocked the ET-induced decrease in amplitude and AUC of facial stimulation-evoked responses. Furthermore, application of the CB1 receptor agonist, WIN55212-2, induced a decrease in amplitude and AUC of N1 in the absence of GABA_A receptors activity, as well occluded the ET-induced depression of N1. Moreover, the ET-induced changes in amplitude and AUC of N1 in absence of GABA_A receptors activity were abolished by a specific protein kinase A (PKA) inhibitor, KT5720. These results indicate that ET facilitates CB1 receptors in the absence of GABA_A receptors activity, resulting in a depression of the sensory stimulation-evoked synaptic transmission via PKA signaling pathway in mouse cerebellar GL.

Introduction
Etomidate (ET) is an intravenous anesthetic that is currently widely used in clinical practice, which is believed to produce sedation by binding on the γ-aminobutyric acid
type A (GABA_A) receptors. Electrophysiological studies have shown that ET either enhanced the amplitude of GABA currents or increased GABAergic inhibitory transmission [1–6]. In cultured hippocampal neurons, ET potentiated GABA currents, resulting in increases in membrane conductance and the open time of single GABA channel [7–8]. In rat spinal dorsal horn neurons, ET at 10 μmol/L potentiated GABA current and slowed activation, desensitization, and deactivation of GABA_A receptors; while ET at 10–1,000 μmol/L directly activated and desensitized GABA_A receptors [1]. In hippocampal pyramidal cells, ET slowed the decay time of miniature inhibitory postsynaptic currents kinetics [9]. The ET inhibited thalamocortical relay neurons activity by potentiation of both synaptic and extrasynaptic GABA_A receptors [10]. In addition, it has been shown that ET specifically modulated beta subunit of GABA_A receptors [5] and exerted its function predominantly through the GABA_A receptor γβ+/α-β and γ+β- subunit interfaces [11]. It has been reported that ET regulated the cannabinoid 1 (CB1) receptor activity in hippocampus and basolateral amygdale [12]. ET-induced sedation was increased and prolonged by activation of the CB1 receptor in vivo in mice [13]. Numerous evidences showed that cerebellar granule cell (GC) expresses abundant CB1 receptors [15–20], suggesting that ET may modulate GC to relay the sensory information via activation of CB1 receptors. Therefore, here we studied the effects of ET on the sensory stimulation-evoked responses in the cerebellar granule layer (GL) in urethane-anesthetized mice, by electrophysiological and pharmacological methods.

Material and Methods

Anesthesia and Surgical Procedures

Experimental procedures were approved by the Animal Care and Use Committee of Yanbian University and were in accordance with the animal welfare guidelines of the U.S. National Institutes of Health. The permit number is SYXX (Ji) 2011–006. Anesthesia and surgical procedures have been described previously [22]. HA/ICR mice were bought from the experiment center of Yanbian University and housed under a 12 h light: 12 h dark cycle with free access to food and water. Either male or female adult (6–8-week-old) mice were anesthetized with urethane (1.3 g/kg body weight i.p.), then tracheotomized to avoid respiratory obstruction. On a custom-made stereotaxic frame soft tissue was retracted to gain access to the dorsal portion of the occipital bone. After a watertight chamber was created, a 1–1.5 mm craniotomy was drilled to expose the cerebellar surface corresponding to Crus II (inferior semilunar lobule). The brain surface was constantly superfused with oxygenated artificial cerebrospinal fluid (ACSF; 125 mmol/L NaCl, 3 mmol/L KCl, 1 mmol/L MgSO_4, 2 mmol/L CaCl_2, 1 mmol/L NaH_2PO_4, 25 mmol/L NaHCO_3, and 10 mmol/L D-glucose) with a peristaltic pump (Gilson Minipulse 3; Villiers, Le Bel, France) at 0.4 mL/min. Rectal temperature was monitored and maintained at 37 ± 0.2 °C using body temperature equipment.

Electrophysiological Recordings, Stimulation, and Drug Application

Local field potential recordings from GC layer were performed with an Axopatch 200B amplifier (Molecular Devices, Foster City, CA, USA). The potentials were acquired through a Digidata 1,440 series analog-to-digital interface on a personal computer using Clampex 10.3 software (Molecular Devices). Patch pipettes were made with a puller (PB-10; Narishige, Tokyo, Japan) from thick-wall borosilicate glass (GD-1.5; Narishige). Recording electrodes were filled with ACSF, with resistances of 3–5 MΩ. Tactile stimulation of the ipsilateral whisker pad was performed using air-puff (10 ms, 50–60 psi) through a 12-gauge stainless steel tube connected to a pressurized injection system (Picospritzer III; Parker Hannfin Co., Pine Brook, Fairfield, NJ, USA). The air-puff stimulations were controlled by a personal computer and were synchronized with the electrophysiological recordings and delivered at 0.05 Hz via a Master 8 controller (A.M.P.I., Jerusalem, Israel) and Clampex10.3 software. ET, 6-imino-3-(4-methoxyphenyl)-1-(6H)-pyridazinobutanoic acid hydrobromide (SR95531), AM-251 and WIN55212-2 were purchased from Sigma-Aldrich (Shanghai, China), while KT5720 were bought from Tocris Cookson (Bristol, UK). All chemicals were dissolved to form solutions and kept frozen as aliquots; they were finally applied to the cerebellar surface at 0.4 mL/min in ACSF. In the experiments involving KT5720, the application of KT5720 was started at least 30 min before recording and continuing throughout the experiments.

Statistical Analysis

Electrophysiological data were analyzed using Clampfit 10.3 software (Molecular Devices, Foster City, CA, USA). All data are expressed as the mean ± SEM. Differences between the mean values recorded under control and test conditions were evaluated with Student paired t test using SPSS software (Chicago, IL, USA). p values below 0.05 were considered to indicate a statistically significant difference between experimental groups.

Results

ET Depressed Facial Stimulation-Evoked Field Potential Responses in Mouse Cerebellar GL

Air-puff stimulation (10 ms, 60psi) of the whisker pad induced a negative component (N1) in the GC layer of the ipsilateral cerebellar cortex folium Crus II (Fig. 1a). Cerebellar surface perfusion of 100 μmol/L ET for 10 min did not significantly change the latency of the response (ET: 13.2 ± 0.26 ms; ACSF: 13.4 ± 0.22 ms; n = 7; p = 0.68). However, application of ET induced a time-dependent decrease in the amplitude and area under the curve (AUC) of N1 (Fig. 1a, c). In the presence of ET, the mean normal-
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The normalized amplitude of N1 was 53.8 ± 6.1% of baseline (ACSF: 100.2 ± 6.8%; n = 7; p < 0.001; Fig. 1d). The mean normalized AUC was 48.9 ± 6.2% of baseline (ACSF: 100.5 ± 8.4%; n = 7; p < 0.001; Fig. 1e). These data indicate that ET time-dependently depressed the sensory stimulation evokes field potential responses in the mouse cerebellar GL.

ET is known to depress neuronal activity through activation of GABA_A receptors or enhancement of GABAergic transmission. Therefore, we employed a specific GABA_A

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**Fig. 1.** ET decreased amplitude and AUC of facial stimulation-evoked field potential responses in mouse cerebellar GL. a Representative traces showing the air-puff stimulation (10 ms, 60psi) evoked field potential responses during treatment with ACSF, 100 μmol/L ET and washout. b Shows the time course of the ET-induced changes in amplitude of N1. c Summary of data (n = 7) showing the time course of ET-induced changes in amplitude of N1. d Individual and mean (± SEM; n = 7) values are showing the amplitude of N1 in each treatment. e Individual and mean (± SEM; n = 7) values showing the AUC of N1 in each treatment. ACSF, artificial cerebrospinal fluid; ET, etomidate; AUC, area under the curve.
receptor blocker, gabazine (SR95531; 20 μmol/L) to examine whether the ET produced depression of sensory stimulation-evoked responses in cerebellar GC layer via activation of GABA_\text{A} receptor. In the presence of gabazine, additional application of ET still depressed the amplitude and AUC of N1 (Fig. 2a, b). The mean normalized amplitude of N1 was 79.22 ± 4.3% of baseline (99.7 ± 4.7%; \( p = 0.02; n = 8 \); Fig. 2c), which was significantly higher than that in the presence of GABA_\text{A} receptor activity (Control: 53.8 ± 6.1% of baseline; \( p = 0.05 \); Fig. 2e). The mean normalized AUC of N1 was 77.3 ± 4.7%, of baseline (99.7 ± 6.7%; \( p = 0.013; n = 8 \); Fig. 2d), which was significant higher than that of N1 was 79.22 ± 4.3% of baseline (99.7 ± 4.7%; \( p = 0.02; n = 8 \); Fig. 2c), which was significantly higher than that in the presence of GABA_\text{A} receptor activity (Control: 53.8 ± 6.1% of baseline; \( p = 0.05 \); Fig. 2e). The mean normalized AUC of N1 was 77.3 ± 4.7%, of baseline (99.7 ± 6.7%; \( p = 0.013; n = 8 \); Fig. 2d), which was significant higher than that
in the presence of GABA_A receptor activity (Control: 48.9 ± 6.2% of baseline; p < 0.05; Fig. 2f). These results indicate that blockade of GABA_A receptors activity significantly attenuated, but not completely prevented the ET-induced decrease in amplitude and AUC of the facial stimulation-evoked field potential responses.

In the Absence of GABA_A Receptors Activity, ET-Induced Depression in Amplitude and AUC of N1 through CB1 Receptor

It has been demonstrated that ET-induced sedation was increased and prolonged by activation of CB1 receptor [13]. Therefore, we used a selective CB1 receptor antagonist, AM-251 (5 μmol/L) to determine whether ET-induced inhibition of N1 occurred via activation of CB1 receptor in the absence of GABA_A receptors activity. In the presence of a mixture of gabazine and AM-251, ET failed to depress the facial stimulation-evoked N1 (Fig. 3a, b). The mean normalized amplitude of N1 was 99.92 ± 6.5%, which was similar to that in control condition (100.23 ± 6.8%; p = 0.75; n = 7; Fig. 3c). The mean normalized AUC of N1 was 100.12 ± 5.9%, which no significant than that in control condition (100.44 ± 5.3%; p = 0.81; n = 7; Fig. 3d). These results indicate that blockade of GABA_A and CB1 receptors completely prevents the ET-induced decrease in amplitude and AUC of facial stimulation-evoked field potential responses in the cerebellar GL, suggesting that the ET-induced depression of N1 through activation of CB1 receptors in the absence of GABA_A receptors activity.

We further examined whether pharmacological activation CB1 receptor induced a depression of N1. Applica-
tion of a specific CB1 receptor agonist, WIN55212-2 (5 μmol/L), induced a time-dependent depression of N1 in the absence of GABA\textsubscript{A} receptors activity (Fig. 4a, b). In the presence of WIN55212-2, the normalized amplitude of N1 was 81.29 ± 4.7% of baseline (100.32 ± 5.1%; \( p = 0.023; n = 8; \) Fig. 4c), and the normalized AUC of N1 was 75.54 ± 5.6% of baseline (100.6 ± 4.9%; \( p = 0.017; n = 8; \) Fig. 4d). In addition, coapplication of WIN55212-2 and ET decreased amplitude of N1 to 80.75 ± 5.8% of baseline (100.74 ± 6.2%; \( p = 0.018; n = 8; \) Fig. 4d). The application of WIN55212-2 and ET also decreased AUC of N1 to 76.26 ± 6.3% of baseline (100.78 ± 7.5%; \( p = 0.013; n = 8; \)), which was similar to application of WIN55212-2 alone (75.54 ± 5.6% of baseline; \( p = 0.76; n = 8; \) Fig. 4d). These results indicate that pharmacological activation of CB1 receptors induces a decrease in amplitude and AUC of N1, as well occludes the ET-induced depression of facial stimulation evoked field potential response in the absence of GABA\textsubscript{A} receptors activity.

**ET-Induced Depression of N1 Was Required Protein Kinase A Signaling Pathway**

We further examined whether protein kinase A (PKA) was necessary for suppression of N1. In the presence of a specific PKA inhibitor, KT5720, there was no significant change in amplitude of N1 following application of ET (Fig. 5a, b). In the presence of KT5720 and ET, the mean normalized amplitude of N1 was 99.89 ± 7.4%, which was...
no significant difference than that in control conditions (100.01 ± 7.2%; p = 0.82; n = 8; Fig. 5c), and the mean normalized AUC of N1 was 99.74 ± 7.3%, which was similar to the control conditions (100.63 ± 7.5%; p = 0.88; n = 8; Fig. 5d). These results indicate that inhibition of PKA abolishes the effects of ET on amplitude and AUC of N1, suggesting that ET-induced depression of N1 through PKA signaling pathway in the absence of GABA<sub>A</sub> receptors activity.

**Discussion**

The main finding of this study is that ET reversely decreased the amplitude and AUC of the facial stimulation-evoked responses in the cerebellar GC layer, in the absence of GABA<sub>A</sub> receptors activity. However, the ET produced depression of facial stimulation-evoked responses was abolished by CB1 receptor antagonist, as well by inhibition of PKA signaling pathway. These results indicate that application of ET facilitates CB1 receptors activity in the absence of GABA<sub>A</sub> receptors activity, resulting in an inhibition of facial stimulation-evoked responses via PKA signaling pathway in mouse cerebellar GL. Our finding suggests that ET acts on CB1 receptors to modulate the sensory information processing in cerebellar GL in vivo in mice.

In the cerebellar cortex, GCs are relay neurons, receiving information from mossy fiber inputs and transferring it to cerebellar Purkinje cells via parallel fibers. Under in vivo conditions, GCs exhibit a low rate of spontaneous spike firing, but they are very sensitive to various sensory
stimulus inputs [23–26], such as passive movement of the forelimbs enough to fire the GCs [27]. Tactile stimulation of the vibrissae evokes high-frequency excitatory responses in GCs, with a maximal instantaneous frequency up to 250 Hz [24]. Furthermore, sensory stimulation can evoke bursts consisting of tens of spikes in GCs at instantaneous frequencies even up to 800 Hz in awake animals [25]. Moreover, cerebellar GCs have been assumed to act as a low-noise sparse coding system, which can reliably relay sensory-evoked signals and filter out the sensory stimulation-unassociated information via mossy fibers [24]. Our present results showed that the cerebellar GCs expressed high-fidelity properties during sensory information transfer, which expressed responses of R_{on} and R_{off} [28].

The general anesthetic action of ET is known to involve the facilitation of GABAergic transmission through presynaptic and postsynaptic pathways. Golgi cells are the inhibitory interneurons of the GL, providing tonic and phasic GABAergic inhibition, and playing an important role in the transfer of sensory information from mossy fibers to GCs [14]. We recently reported that ET inhibited the cerebellar Purkinje cells activity via both GABA_{A} and glycine receptors in vivo in mice [21]. Our present results show that ET inhibited facial stimulation-evoked responses in the cerebellar GL, which was significantly prevented by GABA_{A} receptor antagonist. These results consistent with previous studies [1–6, 21] indicate that ET facilitates GABA_{A} receptor activity during the sensory information transferring in cerebellar GL. However, in the presence of a specific GABA_{A} receptor antagonist, gabazine, ET still induced a depression of the facial stimulation-evoked responses in the cerebellar GL, suggesting that ET-induced depression during the sensory information transfer, which expressed responses of R_{on} and R_{off} [28].

Our results showed that inhibition of PKA abolished the effects of ET on the facial stimulation-evoked field potential responses in cerebellar GL, suggesting that ET-evoked depression of the facial stimulation-evoked field potential responses in the cerebellar GL through PKA signaling pathway. It has been reported that ET reduced glutamate uptake in rat cultured glial cells through PKA signaling pathway [29]. Our results suggest that ET couples to CB1 receptor induces a depression of adenylyl cyclase-cyclic AMP signal-transduction, which leads to downregulation of proteins phosphorylation on synaptic vesicles in presynaptic terminals of MF-GC synapses, resulting in a decrease in glutamate release from mossy fiber onto GCs. Since the cerebellar cortical GL receives transforms and delays input signals coming from many various sources, such as touch, vision, and balance, and that this transformed information then serves as a basis for generating responses that help to control the muscles of the body. Our present results suggest that ET inhibits sensory information processing in GL, resulting in the generation of errors on these muscle control responses during ET administration.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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