European Review for Medical and Pharmacological Sciences 2015; 19: 3865-3872

Comparison of the after discharges induced by electrical stimulation of the prefrontal cortex in urethane- and ketamine-anesthetized mice

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Abstract. – OBJECTIVE: The study aims to compare the effects of urethane and ketamine anesthesia on kindling-induced after discharges (AD).

MATERIALS AND METHODS: We divided forty 4-6 week old female C57BL/6J mice into two groups: one group was anesthetized with urethane (n = 20) and the other with ketamine (n = 20). Kindling was with an electrical stimulation (ES) consisting of a 1-s pulse train of 60 1-ms biphasic pulses at an initial intensity of 700 μA, delivered to the pre-frontal cortex every minute. Ten to fifteen minutes after the first AD was induced, identical electrical stimuli were delivered every 10 minutes for 200 minutes. EEG was continuously recorded from 15 minutes before the first ES until 10 minutes after the last ES. The EEG wave spectrum was analyzed by Fast Fourier Transform and the power spectrum densities (PSDs) of the δ, θ, α, β and γ bands were calculated. EEG wave amplitude was assessed using the root mean square value (RMS).

RESULTS: In the urethane group, an initial AD was induced in 70% of mice following 39 ES. In the ketamine group, an initial AD was induced in 60% of mice following 74 ES. The changes in EEG spectra were similar in both groups. Pre-AD, EEG waves predominantly consisted of δ and θ components. During AD, γ and β components increased significantly ($p < 0.05$). Post-AD, β and γ components decreased and the δ: θ ratio increased to pre-AD levels.

CONCLUSIONS: Our data suggest that AD can be induced by kindling stimulation of the prefrontal cortex in mice under either urethane or ketamine anesthesia.

Key Words:
Urethane, Ketamine, Anesthesia, Electrical stimulation, After discharge.

Introduction

Understanding epileptiform activity is an important challenge for both clinical and scientific researchers. The kindling model is considered to be an ideal animal model of secondary generalized temporal lobe epilepsy as it shares many similarities with human epilepsy[1,2]. During kindling, an electrical stimulus just large enough to trigger a brief after discharge (AD) will generate fully generalized behavioral convulsions. A conscious animal is an ideal subject in which to study AD[3], but in some circumstances, research related to epileptiform AD needs to be performed in an anesthetized animal[4,5]. In such cases, it is important to judge the impact of anesthetics on neuronal measurements made in *in vivo* preparations.

Urethane and ketamine are widely used as anesthetics for *in vivo* electrophysiological animal experiments. Urethane is preferred due to its longer effective duration and its minimal effects on AD generation[4-6]. However, urethane modestly affects multiple neurotransmitter systems (glycine, GABA-A, NMDA, and AMPA receptors) at an anesthetic concentration and has a more obvious influence on normal neural physiological function in some experiments. Huang et al[7] reported stronger inhibition on primary auditory cortex neurons in rats anesthetized with urethane compared to ketamine, as indicated by lower spontaneous firing rates and higher minimal thresholds. Astl et al[8] found urethane may influence the integration of neural outputs on neurons in the inferior colliculus. In addition, urethane's

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demonstrated carcinogenic properties and effects on bone marrow suppression present health risks for both experimental animals and laboratory personnel[9]. Therefore, an alternative anesthetic protocol is required for the study of AD in animal models.

Ketamine has a dual role in both the inhibition and excitation of the central nervous system. Ketamine may inhibit neuronal responses to stimuli by blocking NMDA glutamate receptors[10,11], or it may enhance neuronal excitability through a combination of effects on sigma-type and u-type opioid receptors[12]. Previous studies by Huang et al[13] show that primary auditory cortex neurons keep a relatively good response under ketamine anesthesia, and ketamine has a better analgesic effect compared to urethane due to its inhibition of nitric oxide, which is involved in pain perception[7]. However, ketamine has anti-convulsant effects[14], and is rarely used for anesthesia in animal studies inducing AD.

In this study, we explored the possibility of AD generation under ketamine anesthesia and compared AD electroencephalogram (EEG) patterns and the characteristics of ADs induced in mice under urethane and ketamine anesthesia. We used Fast Fourier Transform (FFT) analysis to assess the energy and frequency distribution of EEGs from urethane- and ketamine-anesthetized mice. The results will direct methodologies used in future neuroscience and electrophysiology research.

Materials and Methods

Forty 4-6 week old female C57BL/6J mice weighing 15-20 g were used in this study. Animal use was in accordance with the Canadian Council on Animal Care, and the protocol was approved by the Animal Care Committee at the University of Calgary.

Animal Preparation

Mice anesthetized with urethane were administered an intraperitoneal injection of urethane (1.2-1.5 g/kg). Additional doses of urethane were given as needed. Mice anesthetized with ketamine were administered an intraperitoneal injection of ketamine and xylazine. The initial doses of ketamine and xylazine were 85 and 15 mg/kg, respectively. Additional doses of ketamine (17 mg/kg, i.p.) and xylazine (3 mg/kg, i.p.) were injected approximately every 40 min to maintain anesthesia, as assessed by the animal's response to tail pinching.

While anesthetized, mice were mounted on a custom-made head holder and their heads were immobilized by clamping the palate and nasal bone. The skull was exposed by making a midline incision and removing connective tissue and muscle. Bregma and lambda points were aligned in the same horizontal plane. For electrode placement, one opening approximately 2 mm in diameter was drilled into the skull to expose the brain surface above the ventral division of the pre-frontal cortex (2.8 mm anterior to bregma, 0.25 mm left of the midline, 1.25-1.5 mm vertically). Two additional openings 1 mm in diameter were made close to bregma and lambda in the right hemisphere for EEG recordings. Body temperature was maintained at 37°C using a feedback-controlled heating pad.

EEG Recording

Two silver wires were placed on the right brain surface close to bregma and lambda for EEG recording. Bioelectrical signals were filtered with a band width of 1-100 Hz and amplified 10,000 times using a conventional biophysical amplifier (Grass-Telefactor, P55 A.C., Astro-Medical, Inc., West Warwick, RI, USA). The output of the Grass amplifier was fed to the DataWave interface (Datawave Sciworks, Datawave Technologies, Longmont, CO, USA), and signals were digitized at a sampling rate of 10 kHz. Online FFT was performed in order to observe variations in the frequency components of the EEG waveform. The original bioelectrical traces and wave frequency distribution were consecutively displayed on a computer screen and oscilloscope throughout the experiment. Data during the period from 15 min before the first electrical stimulus (ES) to 10 min after the last ES were stored to a computer hard-drive for off-line processing.

Induction of After Discharges

A tungsten electrode was placed in the left pre-frontal cortex and an indifference electrode was connected to the scalp and subcutaneous tissue. After 15 min, a stable EEG was observed; this was stored as the baseline EEG. Subsequently, an ES was applied to the pre-frontal cortex via the tungsten electrode. The ES was a 1-s-long train that consisted of 60 1-ms-long biphasic pulses. The electrical current ranged from an initial intensity of 700 μA to a maximum of 900

μA. The electrical train was delivered at a rate of 1 per minute. When the EEG wave showed high-amplitude paroxysmal activity (compared to baseline) of longer than 5 s, AD was considered to be successfully induced. When the initial AD appeared, the mouse was given a 10-15 min break. Following this, an ES at the intensity used to induce the AD was given every 10 min for 200 mins. 10 min after the 20th ES, EEG recording was stopped, and the experiment was terminated.

If at any time an AD was not induced within one hour, the ES was stopped, and the mouse was given a 10-15 min break. The same procedure was applied up to three times with a 100 μA increment of electrical intensity each time. When an AD occurred, the experiment continued as described. If an AD was not induced, the experiment was terminated and data were excluded from the analyses.

Data Processing

EEG data recorded with the silver electrodes from the right hippocampus were analyzed off-line with DataWave software (Datawave Sciworks Version 3 for Windows, DataWave, Inc, Berthoud, CO, USA). Data were digitized at a sampling rate of 10 kHz. Fifteen 5 second long EEG segments recorded before AD, during AD, and after AD, were manually selected to a total of 45 EEG segments selected for each mouse. EEG segments containing breathing and muscle contraction interference were excluded. EEG segments were transformed to frequency functions by FFT and the power spectrum densities (PSDs) of each band (δ [delta, 0.5-3 Hz]; θ [theta, 3.5-7.5 Hz]; α [alpha, 8-12 Hz)]; β [beta, 13-20 Hz]; γ [gamma, 21-50 Hz]) and root mean square value (RMS) levels were calculated with Matlab® software (2008 version, Mathworks, MA, USA).

Statistical Analysis

Values are expressed as the mean ± standard error of mean (SEM). Differences between groups were evaluated using the independent two-tailed *t*-test. Significance was set at $p < 0.05$.

Results

General Properties of the After Discharge in the Urethane and Ketamine Groups

In the urethane and ketamine groups, mean RMS values of baseline EEGs recorded before electrical stimulation were 41.56 ± 1.05 and 31.16 ± 1.02, respectively, which were not significantly different. The predominant oscillation frequencies for both groups were δ and θ, with δ slightly more prominent than θ. FFT analysis showed the mean PSD ratios of the δ / θ / α / β / γ components to total PSD were 33.3%, 20.6%, 11.4%, 13.6%, and 21.1% in the urethane group and 31.5%, 22.5%, 12.7%, 13.2% and 20.1% in the ketamine group, respectively (Figure 1). The differences between the two groups were not significant ($p > 0.05$).

In the urethane group, an AD was induced in 70% (14/20) of mice. In the ketamine group, an AD was induced in 60% (12/20) of mice. Significantly less ES were required to induce an initial AD in mice in the urethane group (38.67 ± 2.88) compared to mice in the ketamine group (74.17 ± 3.39) ($p < 0.05$). Mean duration of the ADs in αsed with increasing numbers of ES. After the 17th ES, mean duration of the ADs was longer in the urethane group than in the ketamine group, but the difference was not significant. There were no significant differences between durations of the ADs after the first ES and the 20th ES in both groups ($p > 0.05$). In the urethane group, the mean number of ADs increased after the 7th, 8th, 9th, 12th and 13th ES, and subsequently decreased. In the ketamine group, the mean number of ADs did not change with increasing numbers of ES. After the 7th ES, the mean number of ADs after each ES was higher in the urethane group than the ketamine group, but the difference was not significant ($p > 0.05$). There were no significant differences between mean numbers of ADs after the first ES and 20th ES in both groups ($p > 0.05$).

FFT Analysis of the ADs in the Urethane and Ketamine Groups

Continuous EEG recordings from the pre-AD period to the AD, followed by the post-ictal period in the urethane and ketamine groups are shown in Figure 2. FFT analysis demonstrated that pre-AD, the dominant frequency was below 10 Hz; during AD, a peak at 10-20 Hz was apparent; post-AD, the dominant frequency returned to below 10 Hz.

In both urethane and ketamine groups, mean RMS values significantly increased during AD, and decreased in the post-AD period; differences in mean RMS values between the urethane and ketamine groups were not significant. In both

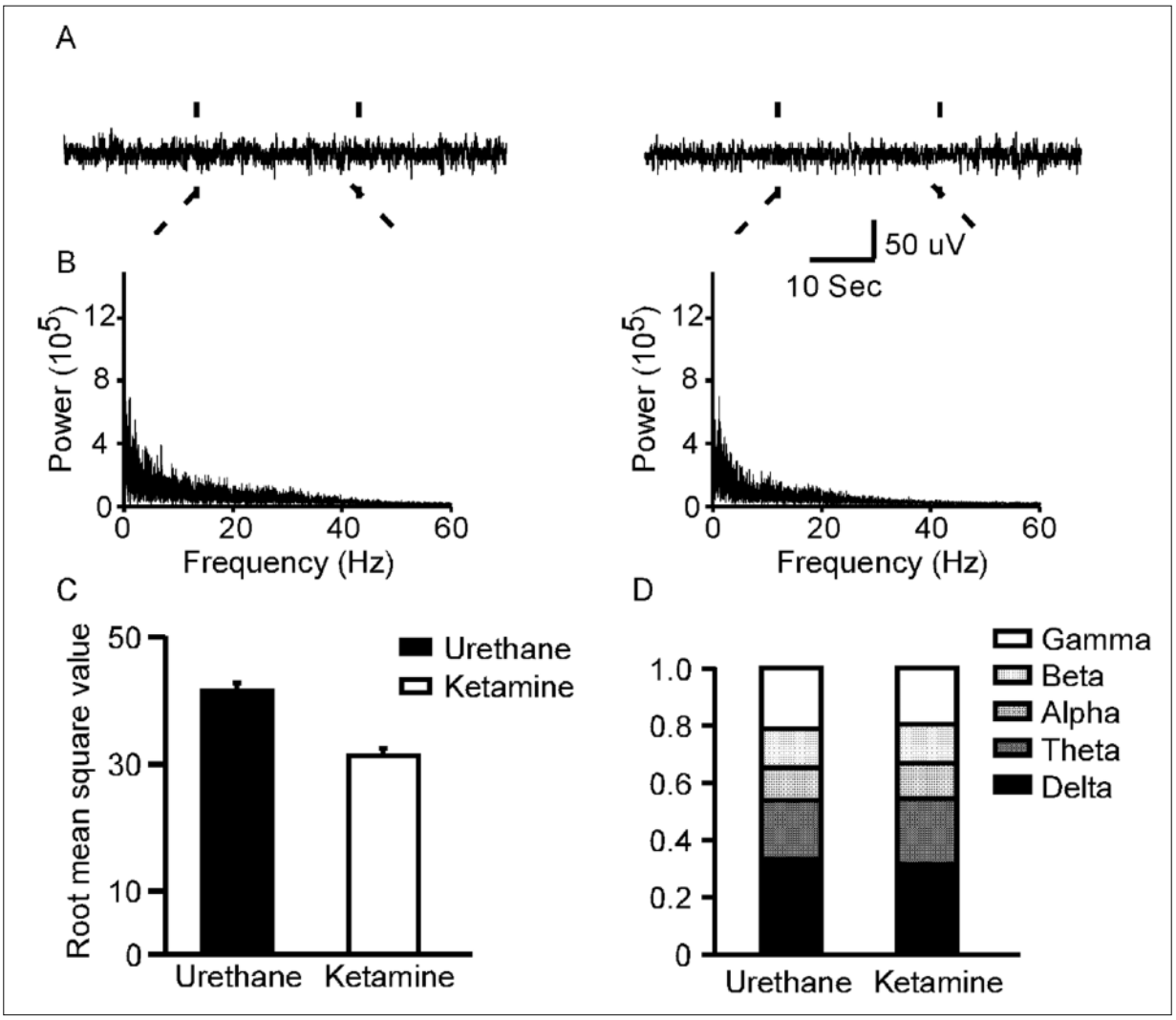

Figure 1. ***A,*** Baseline EEG recordings before electrical stimulation: urethane group, *left panel*; ketamine group *right panel*. ***B,*** FFT analysis of the baseline EEG recording (*between dashlines*) urethane group, *left panel*; ketamine group *right panel*. ***C,*** Mean root mean square values; ***D,*** Power spectrum density (PSD).

urethane and ketamine groups, in the pre-AD period, the EEG had a predominant δ, θ component. During AD, β and γ components increased significantly and δ, θ components decreased; the γ component significantly increased in the urethane group compared to the ketamine group, while the β component significantly increased in the ketamine group compared to the urethane group ($p < 0.05$). In both groups, in the post-AD period, the β and γ components decreased, and the δ, θ ratio increased (Figure 3). There were no significant differences in PSD for each frequency in the pre- and post-AD periods between the urethane and ketamine groups.

Discussion

Kindling is a model of chronic seizures which involves progressive intensification of brain excitability by repeated excitatory stimuli (electrical or chemical) that ultimately induce seizure disorder. If the amplitude of the stimulating signal is sufficiently large, the stimulation elicits a characteristic post-stimulation EEG AD. Racine[15] applied 1 ms biphasic square wave pulses at 60 c/sec with a 1 sec duration of stimulation to the rat posterior hippocampus or amygdala, and successfully induced ADs with a current of 200 μA. Lothman et al[16] applied 1 ms biphasic square-

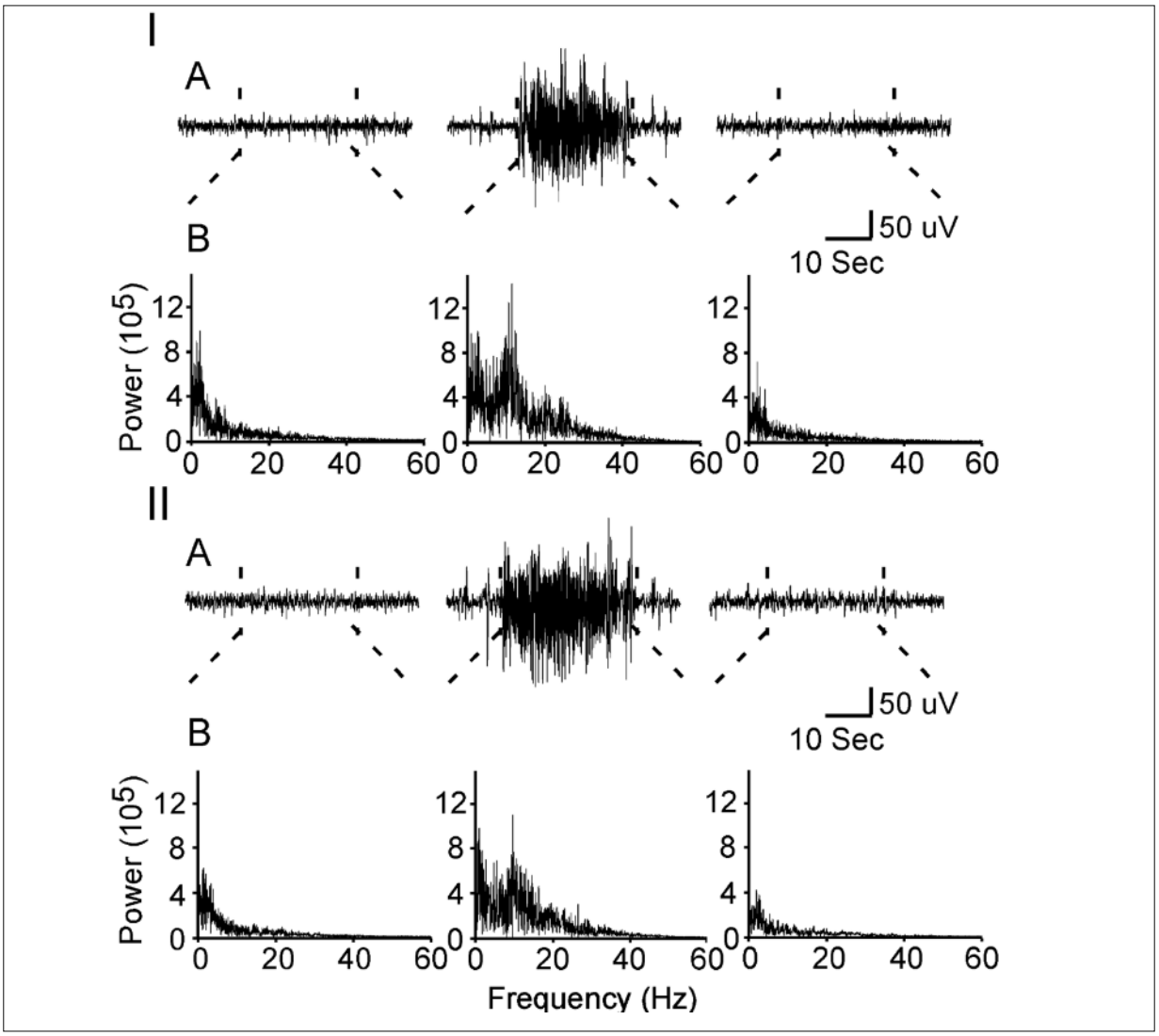

Figure 2. EEG recordings before during and after AD in the urethane group ***(I)*** and ketamine group ***(II)***. ***A,*** Prior to AD *left panel*; during AD *middle panel;* after AD right panel. ***B,*** FFT analysis of the EEG recordings (*between the dashline*).

wave pulses with an intertrain frequency of 10 Hz to the hippocampus of rats, and quickly triggered repetitive ADs with a current of 100 μA; a current of 400-600 μA prevented desensitization that occurred with smaller intensities. In the present study, we used a 1 ms biphasic square wave with an intertrain frequency of 60 Hz, duration of 1 s, and a 1 min interval, and chose current intensities ranging from to 700-900 μA. An initial AD was triggered in 70% of mice in the urethane group and 60% of mice in the ketamine group. Subsequent ES of these mice every 10 min triggered at least one more AD in each. The duration of ADs increased with increasing numbers of ES. These data suggest a stable kindling model was established in urethane- and ketamine-treated rats.

Multiple reports[17] have shown that NMDA receptor inhibitors such as ketamine inhibit the kindling process and delay the appearance and severity of epileptic-like seizures. Our data show that ADs can be triggered in mice under ketamine anesthesia, although ADs were easier to induce in mice under urethane anesthesia. In accordance with these findings, a retrospective study[18,19] of electric shock therapy in psychiatric patients found that ketamine had a less potent anticonvulsant effect and prolonged seizure duration compared to methohexital.

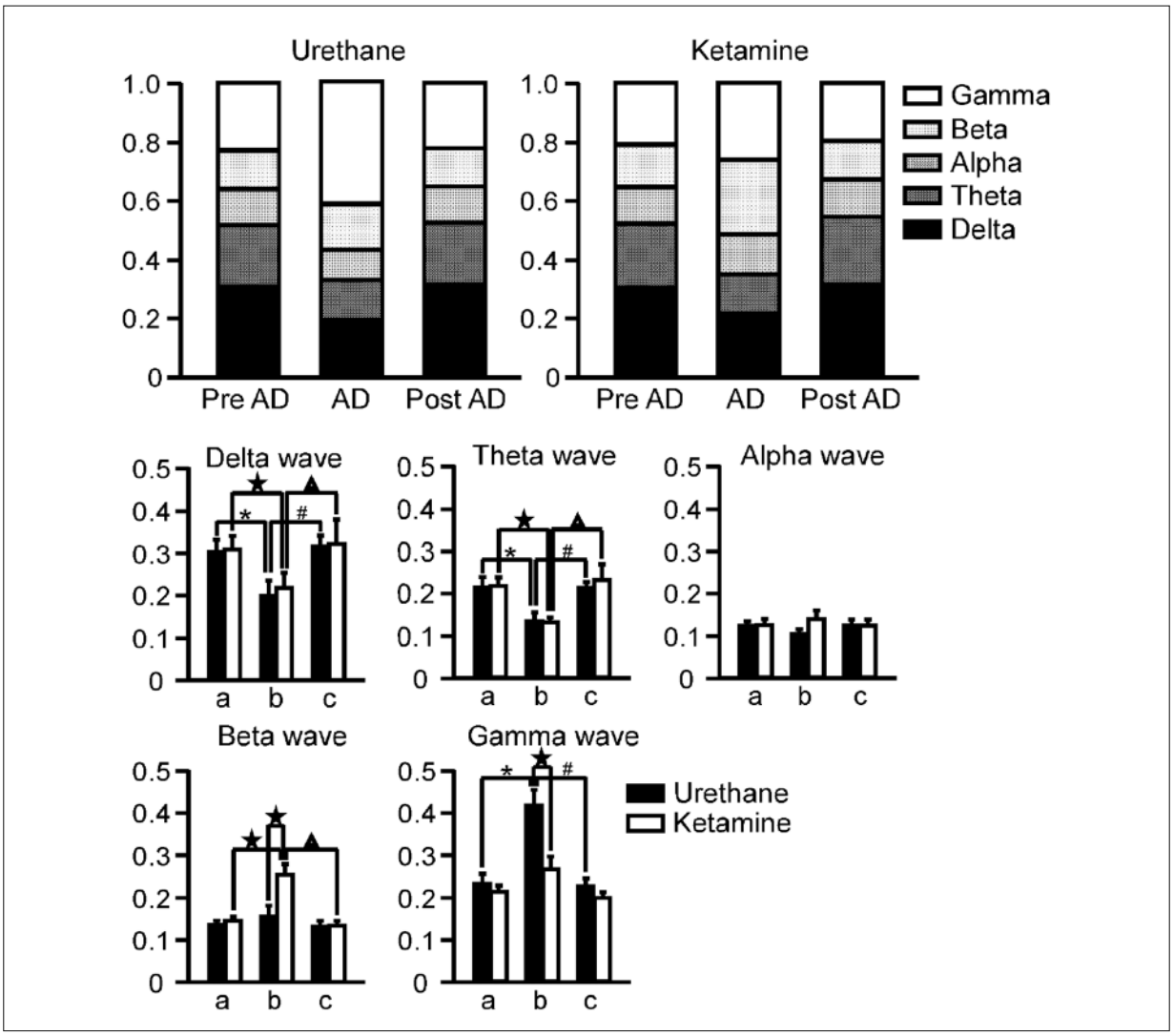

Figure 3. Power spectrum density (PSD) ratios of frequency band distributions pre-AD, during AD, and post-AD in the urethane and ketamine groups. Individual PSD ratios for delta, theta, alpha, beta, and gamma waves for the urethane and ketamine groups. *, ★ vs. pre-AD, $p < 0.05$; #, ▲ vs. post-AD, $p < 0.05$. ★ ketamine vs. urethane group, $p < 0.05$.

We performed FFT to characterize the frequency components of the EEG signals among the urethane and ketamine groups. For both urethane and ketamine groups, baseline EEG recordings predominantly consisted of low frequency δ, θ waves. During AD, the EEG recordings showed an apparent peak at 10-20 Hz band, β and γ components were significantly increased, and δ and θ components were decreased. After AD, the frequency components returned to baseline levels. These results are consistent with previous reports in awake adult rabbits[20].

Extensive activation of the excitatory system in the brain plays an important role in evoking an initial AD. High-frequency ES increases both glutamate and GABA release into the synaptic cleft from pre-synaptic neurons in the limbic system. Glutamate activates post-synaptic AMPA receptors, but the depolarization is immediately reduced by GABA-A receptor-mediated inhibition. If a stimulus is over a certain threshold, GABA-A receptor-mediated inhibition will fail to inhibit AMPA receptor activation and cause a prolonged depolarization. This activates NMDA receptors, facilitates voltage-dependent Ca^{2+} influx, and leads to synchronized bursts and spikes (AD)[21].

Different anesthetic mechanisms may explain the divergent AD threshold and characteristics in mice under urethane and ketamine anesthe-

sia. A previous report showed that 10 mM urethane enhanced the functions of $[alpha]_1[beta]_2[gamma]_{2S}$ GABA-A and $[alpha]_1$ glycine receptors by 23% and 33%, and inhibited the functions of NR1a/NR2A NMDA and GluR1/GluR2 AMPA receptors by 10% and 18%, respectively[9]. Ketamine is a non-competitive inhibitor of the NMDA receptor. Ketamine reduces NMDA receptor function by more than 80% at 10 μM, the anesthetic EC_{50}, but has no effect on GABA-A, glycine, and AMPA receptors[1]. Initial ADs may be easier to induce in mice under urethane, as urethane has a modest effect on both the inhibitory and excitatory systems in the brain, and the magnitude of change is less than that seen with ketamine, which is more selective for one system. With continued stimulation, ADs can be induced under ketamine anesthetic as ketamine has no effect on the GABA-A receptor, yet the failure of GABA-mediated inhibition becomes more obvious in several brain sites, including the hippocampal CA1 region and amygdala.

In our study, repeated ES triggered ADs with increasing durations in mice under urethane and ketamine anesthesia. Previous reports propose that repeated ES can result in a loss of GABA neurons due to structural changes and a decrease in the number of GABA receptors. This may enhance excitatory postsynaptic potentials and prolong the duration of Ads[21]. In the current study, we observed EEG AD patterns of high frequency. The reason for this phenomenon requires further investigation, but it may be explained by findings of Tsuchiya et al[20], who consider oriens-lacunosum molecular neurons and other interneurons have a key role in the formation of the θ rhythm. When ES intensity exceeds a threshold, it may inhibit these interneurons leading to a disinhibition of excitatory neurons. ES may cause an imbalance of inhibitory post-synaptic currents and excitatory post-synaptic currents (EPSCs) towards EPSCs, which could elicit an alteration in frequency distribution[22].

Conclusions

This study showed that repeated ES in mice anesthetized with urethane or ketamine can be used to produce ADs with similar EEG patterns. These results should be considered when selecting methodologies used in future neuroscience and electrophysiology research.

Source of Support

This work was supported by the Campbell McLaurin Chair for Hearing Deficiencies at the University of Calgary.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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