

Ethosuximide Affects Paired-Pulse Facilitation in Somatosensory Cortex of WAG\Rij Rats as a Model of Absence Seizure

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Article info

Article History:

Received: 3 April 2015

Revised: 28 June 2015

Accepted: 27 July 2015

ePublished: 30 November 2015

Keywords:

- Absence seizure
- Paired-pulse stimulation
- WAG\Rij rat
- Somatosensory cortex

Abstract

Purpose: The interaction between somatosensory cortex and thalamus via a thalamocortical loop is a theory behind induction of absence epilepsy. Inside peri-oral somatosensory (S1po) and primary somatosensory forelimb (S1fl) regions, excitatory and inhibitory systems are not balanced and GABAergic inhibitory synapses seem to play a fundamental role in short-term plasticity alterations.

Methods: We investigated the effects of Ethosuximide on presynaptic changes by utilizing paired-pulse stimulation that was recorded from somatosensory cortex in 18 WAG\Rij rats during epileptic activity. A twisted tripolar electrode including two stimulating electrodes and one recording electrode was implanted into the S1po and S1FL according to stereotaxic landmarks. Paired-pulses (200 μ s, 100-1000 μ A, 0.1 Hz) were applied to somatosensory cortex at 50, 100, 400, 500 ms inter-pulse intervals for 50 min period.

Results: The results showed that paired-pulse facilitation was significantly reduced at all intervals in all times, but compared to the control group of epileptic WAG\Rij rats ($p < 0.05$), it was exceptional about the first 10 minutes after the injection. At the intervals of 50 and 100 ms, a remarkable PPD was found in second, third, fourth and fifth 10-min post injection.

Conclusion: These experiments indicate that Ethosuximide has effects on presynaptic facilitation in somatosensory cortex inhibitory loops by alteration in GABA levels that leads to a markedly diminished PPF in paired-pulse stimulation.

Introduction

Non-linear association analysis of spike-wave discharges (SWDs) in line with cortical focus theory supports the idea that epileptic discharges in absence seizure initially begin around the peri-oral region of primary somatosensory cortex during seizures in Wistar Albino Glaxo from Rijswijk (WAG\Rij) rats. They are valid genetic animal models of absence seizure commonly used in the study of the mechanisms involved in epilepsy and the effectiveness of treatment methods.¹ Synchronous and bilateral 7 to 10 Hz SWD² discharges take place automatically in these rats, accompanied by diminished consciousness and immobility, contraction of facial muscles and whiskers during awakening time that lasts from 1 to 30 seconds.³ Functionally, interconnected cortical and thalamic regions of the brain turn out to influence one another, while the direction of this bidirectional coupling can change throughout a single seizure. However, during the first 500 milliseconds, the cortical focus was consistently detected to lead its thalamic counterpart.⁴

Studies on WAG\Rij rats and knockout mice have assumed that imbalance between excitatory and inhibitory

systems results in SWD discharges in absence seizure. As indicated by Polack et al (2009), in Genetic Absence Epilepsy in Rats from Strasbourg (GAERS), cells in layer V and VI of somatosensory cortex increase firing rate before SWDs onset.⁵ These results describe a hot spot region in the peri-oral area where SWDs are started.⁶ The local hyper excitability of somatosensory cortex is in agreement with the cortical focus theory for absence seizures⁷ and further evidence support the fact that it reduces the performance of GABAergic inhibitory system contributing to abnormal function and high excitability of neocortical networks.⁸ Various neurotransmitters such as glutamate and gamma-Amino butyric acid (GABA) which adjust thalamocortical function play major roles in the pathogenesis of absence seizure. GABA_A receptor is inotropic and its endogenous ligand γ -amino butyric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system.⁹ Following an action potential, GABA_A receptor selectively conducts Cl⁻ across its pore, resulting in hyperpolarization of neurons and leading to an inhibitory effect in neurotransmission.¹⁰

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During a seizure activity, as extracellular calcium concentration is reduced,¹¹ the concentration of intracellular calcium increases. It is well known that T-type Ca^{2+} currents are involved in thalamocortical burst firing.¹² Ethosuximide is a T-type Ca^{2+} channel antagonist and first high efficacy option for the treatment of absence epilepsy.¹³ According to theories, this drug affects an interaction between the irritable cortex and a rhythmic operative system which is activated by thalamocortical loop.¹⁴ These bursts are actively conducted via low-threshold T-type calcium current and blocking these channels is the main function of the anti-epileptic effect of Ethosuximide. It has been suggested that SWD begins at pyramidal cells of cortex. The direct infusion of Ethosuximide in cortex leads to a quick suppression in epileptic discharges and it is determined that Ethosuximide reduces the hyper excitability of these neurons.^{12,14} Short-term synaptic plasticity is important for synaptic communication within the brain and is typically assessed with "paired-pulse stimulation" double pulse in close succession. Paired-pulse facilitation (PPF) is generally explained as an enhancement of releasing probability during the second stimulus, arising from prior accumulation of residual Ca^{2+} near active zones. In contrast, paired-pulse depression (PPD) derived from reduced presynaptic release is most often attributed to vesicular depletion.^{15,16}

Considering all of these facts mentioned above, we hypothesized that S1po and S1FL are specific zones with higher excitability and with modifications in GABAergic inhibitory system and synaptic plasticity as compared to other regions of cortex and also probably responsible for generation of SWDs. This study was performed by microinjections of Ethosuximide to reveal the possible effects of T-type calcium channels and subsequent changes in SWDs and short-term plasticity.

Material and Methods

Animal and Drug

In vivo experiments were performed on 18 adult male WAG/Rij rats (250-300 g and 8-10 months old) with 6 rats in each group. Animals were gathered from a maintained colony in Shefa Neuroscience Center, Khatam Hospital, Tehran, Iran and they were kept under standard conditions (light-dark cycle 12-hr: 7:00 am 7:00 pm lights on, at a temperature of 23 ± 1 °C) with food and water available ad libitum. All tests were carried out during the light phase. Experimental procedures were in accordance with the Regional Ethics Committee of Tabriz University of Medical Sciences. Ethosuximide (MW141.2 purchased from Sigma chemicals, USA) was dissolved in 0.9% saline for direct micro-infusion.

Surgical Procedure

Animals were initially anesthetized by an I.P. injection of ketamine (60 mg/kg) and xylazine (12 mg/kg).¹⁷ A twisted tri-polar electrode was prepared from Teflon-coated stainless-steel wire with a diameter of 100 μm as well as two stimulating electrodes and one recording

electrode (stimulating electrodes were 1 mm longer than the recording electrode) which were implanted into the peri-oral somatosensory (S1po) and primary somatosensory forelimb (S1FL) (mm, relative to bregma; AP: -2.1, L: $\pm 0.5.5$, V: 4.0 and AP: -2.1, L: ± 3.0 , V: 2.0 respectively) by a stereotaxic device (Stoelting, USA), while the reference electrode was placed on the occipital cortex. Electrodes were fixed in the sockets by pins. Cannula (23gauge) for drug injection was implanted into the right lateral ventricle (AP: -0.8, L: 1.6 and, V: 3.5 mm below dura) in the case of all of the rats in line with Paxinos atlas.¹⁸ Short pieces of cooper wire were interpolated inside the cannula in order to avoid its shutting. The cap injection needle was selected 1 mm longer than the guide cannula. The cannula and sockets were fixed to the skull by means of dental cement. Before injection, the rats were restrained by the head and the cooper wire was removed and replaced with the injection needle (27gauge) that was connected to a 10 μl Hamilton syringe with small pieces of polyethylene tube. 5 μl Ethosuximide at an effective dose of 900 $\mu\text{g}/\text{kg}$ ¹¹ was injected into the right lateral ventricle within 2 minutes and the needle remained in site for 15 seconds after injection in order to reduce backflow of the solution.

Stimulation and Recording

Experiments were started after a one-week recovery period following surgery. Rats were put into the faraday cage and electrocorticography (ECoG) was recorded after 30 minutes of adaptation to experimental conditions in a freely moving manner. Spike-wave discharges were recorded 60 minutes before and three times with 30-minute-long intervals after drug injections. A mild natural stimulus (moderate sound or soft touch) was carried out to prevent the animal from sleeping. As test pulses, square pulses (200 μs , 100-1000 μA , 0.1 HZ) were applied to somatosensory cortex. Each experiment began by determining stimulus-intensity to elicit threshold and maximum electrical evoked potential. Stimulus intensity was adjusted to be just above threshold to evoke a continuous postsynaptic response. Then, the relationship between stimulus-response was determined and excitation intensity that was raised to 50% maximum electrical evoked potential (EEPs) amplitude was used as the test stimulus during the experiment. Individual EEPs were amplified (100 \times) and filtered (0.1 Hz to 1 kHz band pass) by using a differential amplifier (DAM80, WPI, USA). Signals were passed through an analogue-to-digital interface (National Instruments USB-6221-BNC, USA) to a computer. Data were digitized at a sampling rate of 1 kHz and were analyzed by means of Win LTP software (version 2.01, M-Series, The University of Bristol, UK). The EEP amplitude was measured as the voltage difference between the baseline and EEP wave peak. Later, paired-pulse stimulation was applied to somatosensory cortex at inter-pulse intervals of 50, 100, 400, 500 ms between the conditioning (first) and the test

(second) stimulation. 5 responses were sampled and averaged in each interval. All paired-pulses were delivered in 0.1 Hz frequency.

Statistical Analysis

For statistical analysis of SWDs, SPSS 16 software was used. Variables of SWDs (mean duration, and mean number) were analyzed by one-way ANOVA and repeated measures ANOVA. The Tukey's test was administered for post-hoc analysis. Values were expressed in the form of percentages with respect to baselines; in intergroup comparisons, P values that were smaller than 0.05 were considered to be statistically significant. The baseline was calculated as 100%. In order to compare the effect of variables in paired-pulses (zone-time and interval), mixed models with autoregressive (1) covariance structure and with restricted maximum likelihood (REML) in the form of estimation method were used. The analyses were followed by Sidok post-hoc test where they were needed.

Histology

At the end of electrophysiological experiments, the rats were deeply anesthetized with high doses of ketamine; the brain was removed quickly and fixed with 10% formalin solution for 24 hr at room temperature. Subsequently, coronal slices with 40-micron thickness were cut by vibroslicer (Campden-MA752, UK). The tips of electrode and cannulae location were pointed by cresyl violet staining. Two animals were excluded from the analysis because the electrodes and cannula had not been placed appropriately.

Results

Effects of Ethosuximide on SWDs

To compare the effects of Ethosuximide and saline (control) on SWDs, mean number and mean duration were investigated later within three 30-min-long intervals following drug and saline injection. In the control group, specifically the mean duration and frequency of SWDs within the entire post-injection of saline did not differ significantly from the baseline value ($P > 0.05$). The comparison of control with post injection time revealed significant effects of Ethosuximide on the duration of SWDs ($P < 0.05$). Over the first 30-min after injection of Ethosuximide, the mean duration of SWDs became significantly smaller than the control ($53 \pm 12.0\%$). The difference between the mean duration of SWDs in Ethosuximide and control groups throughout the second and third 30 min was significant ($P < 0.05$). Within the second 30-min phase, the decrease in SWDs duration in the Ethosuximide group was more than the control ($50 \pm 11.6\%$). During the third 30-min interval, there was also a decline in mean duration in comparison to the control group ($45 \pm 7.0\%$). On the other hand, in Ethosuximide-injected animals, a significant reduction in the number of SWDs was seen in comparison to the control ($P < 0.05$) during the first, second and third 30-minute phases. The respective Figures in the first 30

minutes were ($49 \pm 15.1\%$), in the second 30-minute interval they were ($27 \pm 7.9\%$) and in the third period they were ($15 \pm 5.7\%$) (Figure 1-A and B).

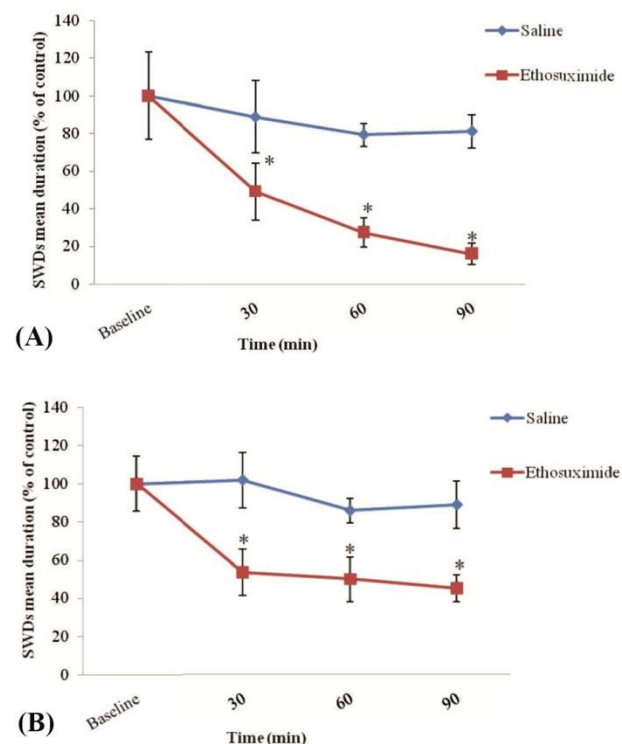


Figure 1. Dynamics of the normalized effects of Ethosuximide (900 µg/kg) (%) on mean Number (A) and (B) Duration of spike-wave discharges within three subsequent 30-min-long post-injection intervals. Baseline indices are taken as 100%. Data were analyzed by One-way ANOVA test, ($*P < 0.05$; $N = 6$ for each group). Asterisks show cases of significant differences from the baseline values.

Effects of Ethosuximide on paired-pulse

EPPs were evoked in somatosensory cortex by paired-pulse stimulation of S1po and S1Fl and paired-pulse ratio which were identified in 50-100-400-500 intervals. When two consecutive stimuli with determined intensity were applied in 50-100-400-500 inter pulse interval (paired-pulse stimulation), a remarkable variability was observed in the EEPs test compared to the baseline. Each EEP was an average of 5 responses at each interval. EEPs which were higher than 1 (PPF: paired pulse facilitation) were more prominent in 50-100-400-500 intervals in the baseline condition. Next, we tested the hypothesis that if in the WAG/Rij rats, the GABAergic inhibitory system efficiency is not enough, changes in GABAergic system by utilizing Ethosuximide will lead to changes in paired-pulse responses that were generated by neocortical neurons. Having said that, evoked responses in the somatosensory cortex following treatment by Ethosuximide ($n=5$) indicated significant differences between intervals and times. The effects of time and interval were significant, so in order to compare these factors, post-hoc analysis Sidak results showed a significant difference for the time variable. Paired-pulse facilitation was reduced significantly at all intervals in all

the times, except for the first 10-minutes after the injection compared to control in epileptic WAG/Rij rats ($p < 0.05$). At the intervals of 50 and 100 ms a remarkable PPD was found at second, third, fourth and fifth 10 min post injection. Also at 400 and 500 ms intervals, a significant reduction was found at all 10-minutes periods after injection in PPI ratio which was remarkable in 20 and 30 minutes post injection. In this study, by mixed model analyses, the zone effect was not significant ($p > 0.05$), therefore the results of the paired-pulse ratio (PPI) in S1fl were similar to those acquired from S1po (Figure 2-A and B).

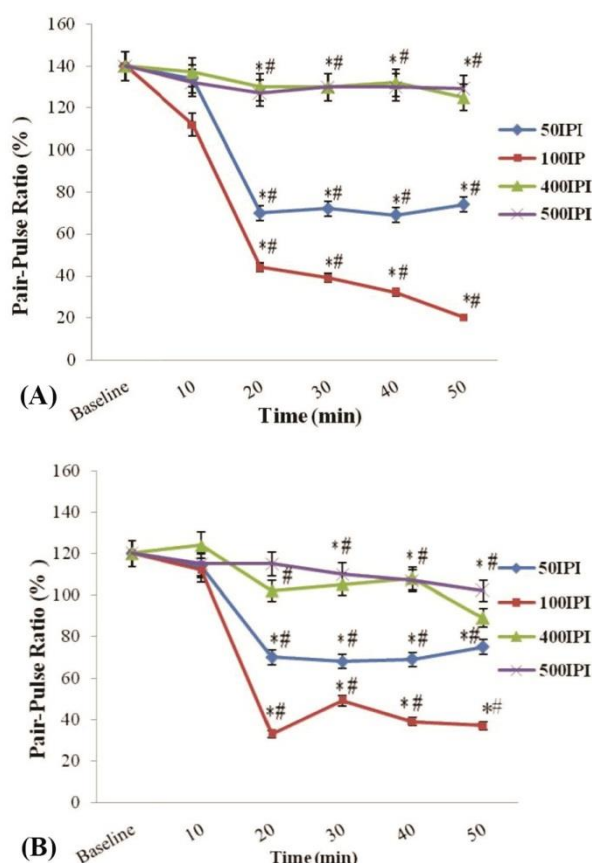


Figure 2. The time course effect of Ethosuximide (900 $\mu\text{g}/\text{kg}$) on PPR in S1po (A) and S1fl (B). Values were analyzed by mixed model test with autoregressive (1) covariance structure in SPSS 16 software. Values are percentage of mean $\text{EEP2}/\text{EEP1} \pm \text{S.E.M.}$ At IPI: 50, 100, 400 and 500 ms within five subsequent 10-min-long post-injection intervals. (* shows significant difference ($P < 0.05$) to compare with control group and # shows significant difference ($P < 0.05$) to compare with first 10-min-long; $N = 6$ for each group).

Discussion

The aim of our study was to discover the role of T-type calcium channels in GABAergic inhibitory systems and alterations in short term synaptic plasticity in somatosensory cortex in WAG/Rij rats. To this end, Ethosuximide, a T-type calcium channel antagonist was used. Our observations after the deep microinjection of the drug revealed that Ethosuximide reduced the mean duration and number of SWDs. Also, after paired-pulse

stimulation in somatosensory cortex, the facilitation decreased remarkably.

Anti-absence drugs such as Ethosuximide probably carry out their function by causing declines in the burst-firing capability of cortical and nRT neurons, and as a result, they create a non-synchronization in thalamocortical loop which inhibits generation of SWDs.¹⁹

The basic mechanism of Ethosuximide performance is still a matter of debate. Huguenardin in 1996 proposed that the low-threshold calcium spike can lead to increase Ca^{2+} -dependent currents through the T-type Ca^{2+} channels to generate consecutive bursts.²⁰ But in WAG/Rij rats, the function of L-type channels were changed, resulting in quick deactivation of these channels and activation of calcium-dependent potassium channels. Potassium channels led to the amplification of the hyperpolarization in neurons and activated T-type calcium channels and in fluxed calcium ions into the cell through these channels leading to burst activity of glutamatergic neurons.¹¹ Sadighi et al (2013) indicated that after PMA injection – an agonist of T-type calcium channels– into the somatosensory cortex, the number and duration of SWDs increased significantly in WAG/Rij rats.²¹ In line with our study, an experiment on thalamus using voltage-clamp techniques implied that Ethosuximide reduces low-threshold calcium currents by cutting down the number of LTCC available channels, or creates a change in transduction across LTCC channels.^{22,23} Another study showed that microinfusion of 20 nmol of ETX into S1po in freely moving manner GAERS resulted in rapid and almost perfect suppression of SWDs in the whole cortex among both hemispheres compared to systemic administration of the drug. As a result, after injection of Ethosuximide, we expected a significant decrease in number and duration of the occurrence of SWDs. In fact, such a finding is also observable in our study.

Paired-pulse stimulation of somatosensory cortex in WAG/Rij rats based on the activity of GABA receptors under control condition revealed both paired-pulse facilitation (PPF) and paired-pulse depression (PPD) at different intervals, although PPF was more prominent.²⁴

In order to investigate the inhibitory circuits, we chose PPF. After micro-infusion of Ethosuximide in the brain, the facilitation was reduced at all the intervals. The maximum depression of the second EEPs occurred around IPI 100 ms and reduction in PPF was observed at intervals up to 500 ms.

Experimental documents from *in vitro* studies on WAG/Rij rats indicated the following: 1: A significant decrease in I_h (a cationic current that modulates interaction between excitatory and inhibitory discharges),⁸ 2: Dysfunction of GABAergic inhibitory system and reduction in the rate of post-synaptic inhibition that was mediated by these receptors,²⁵ 3: Long-time depolarization was mediated by an increase in the number of NMDA receptors and enhancement in the action potential evacuation.²⁶ A study about WAG/Rij rats indicated that the total inhibitory conductance is 50% of

the whole excitatory current. An *in vivo* study on pyramidal neurons in neocortex revealed that GABA receptors play an important role in both excitatory and inhibitory synaptic input. This finding is consistent with other studies that showed decreases in GABA receptor-mediated currents resulting in a decline in PPF at superior colliculus,²⁷ hippocampus²⁸ and neocortex.²⁹ Other experiments showed that PPD converts to PPF when GABA receptors are blocked, suggesting that short-term plasticity is affected by alteration in pre-synaptic receptors. Zilberter et al indicated that when dendritic release of GABA increases, it leads to a reduction in excitatory transmission in layers II/III of pyramidal neurons.³⁰ Some studies that contradict our results indicate that a reduction in the extracellular calcium concentration results in a decrease in neurotransmitter release at presynaptic neurons and diminishes PPF.^{31,32}

When GABA receptors are activated in the neocortex, they perform two prominent functions. First, by using a slow GABA-mediated IPSP, they create flows of post-synaptic potassium currents.³³ The second is that they prevent transmitter release at presynaptic levels.³⁴ Presynaptic GABA receptors were identified in inhibitory and excitatory neurons in somatosensory cortex and their action reduces IPSCs and EPSCs to the normal level. High and low amounts of GABA receptors on excitatory and inhibitory neurons determine the overall irritability of the cells.³⁵

Ethosuximide operates on proprietary neurons of somatosensory cortex and increases the inhibitory postsynaptic conductance. In line with our study, it was stated that Tiagabine— a GABA uptake blocker — diminishes the frequency of IPSCs but enhances the amplitude. In addition, Tiagabine increases the frequency of EPSCs only slightly. As a result, the overall impact was an increase of I:E ratio.¹² Ponnusamy indicated that Ethosuximide can raise the GABA-levels throughout all regions of the brain; specifically in the frontal cortex and thalamus by enhancing GABA neurotransmitter release.³⁶ An *in vivo* study mentioned that chronic application of Ethosuximide has no effect on glutamate release in motor cortex, somatosensory cortex and hippocampus.³⁷ Bailey suggested that an activation of GIRKs (G protein-coupled inwardly-rectifying potassium channel) in the EC could lead to depression of GABA release in inhibitory synapses.³⁸ A former *in vivo* and paradoxical study showed Ethosuximide has no influence on spontaneous secretion of GABA neurotransmitter inside the cortical region of rats and that the administration of this drug does not change extracellular GABA currents in motor cortex.³⁹ Based on other pieces of evidence, Ethosuximide weakly suppresses the somatic T-type conductance.⁴⁰

According to the reports, paired-pulse stimulation on somatosensory cortex suggests that T-type calcium channels do not exist in inhibitory pre-synaptic terminals in EC, layer III and V and somatosensory cortex,^{41,42} and, therefore, the effects of Ethosuximide is probably

controlled by alternative mechanisms in these regions. It is noteworthy that T-type calcium channels (CaV3.2) are present in glutamatergic terminals in excitable synapses of somatosensory cortex and EC, and, under specific conditions, they can increase glutamate release.³⁵ Ethosuximide inhibits persistent Na-channels, Ca-sensitive K-channels and GIRKs.^{43,44} If the activity of GIRKs at inhibitory terminals increases, it would lead to a reduction in GABA release. As the main mechanism of action of Ethosuximide in interneurons, it seems that this drug blocks soma-dendritic K-channels and GIRKs-related interneurons and neutralizes the inhibition of GABA release. It can result in an enhancement in GABA neurotransmitter release and tonic discharges.¹²

Conclusion

The results from our study suggest that Ethosuximide leads to decreases of SWDs by blocking T-type calcium channels. With more follow-up research, such as investigating inhibitory circuits by paired-pulse stimulation, it can be found that the efficacy of Ethosuximide on inhibitory interneurons effects short-term plasticity. Ethosuximide increases GABA levels in inhibitory interneuron terminals and leads to a considerable reduction of PPF in paired-pulse stimulation.

Ethical Issues

Not applicable.

Conflict of Interest

The authors report no conflicts of interest.

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