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L-Theanine Reduces Epileptiform Activity in Brain Slices

L-Theanin Beyin Kesitlerinde Epileptiform Aktiviteyi Azaltır

ABSTRACT

Objective:

L-theanine is one of the main amino acids of tea plant which can cross the blood brain barrier. In the central nervous system, L-theanine has certain effects on a number of neurotransmitter systems. In this study we tested whether acute application of L-theanine could induce any seizure like or inhibitory activity in the brain slice.

Materials and Methods:

Effects of L-theanine on epileptiform activity were investigated in two different brain regions. Horizontal hippocampal-entorhinal cortex slices were obtained from 30-35 days old C57BL/6 mice. Extracellular field potentials were recorded from medial entorhinal cortex (EC) and CA3 region of hippocampus. Epileptiform activity was induced by application of 4 Aminopyridine (4AP, 100 µM) in the brain slices and 50 µM L-theanine was applied. From the recordings, the duration and frequencies of ictal like events as well as frequency and amplitude of inter-ictal like activities were calculated.

Results:

L-theanine alone did not initiate any synchronized activity. Although, bath application of L-theanine after 4AP-induced epileptiform acitivities, did not significantly alter the duration and frequency of ictal discharges, L-theanine attenuated the interictal discharges. In EC the frequencies of interictal discharges were significantly decreased. In CA3, the amplitudes of interictal activities were significantly reduced.

Conclusion:

The present findings suggest that L-theanine do not induce any seizure like event but rather had a suppressive effect on ongoing epileptiform activity.

Key Words:

Theanine, 4 Aminopyridine, Brain Slice, Epileptiform activity, CA3, Entorhinal cortex

ÖZ

Amaç:

L-theanine, kan beyin bariyerini geçebilen çay bitkisinin ana amino asitlerinden biridir. Merkezi sinir sisteminde, L-theanine'in bir dizi nörotransmiter sistemi üzerinde belirli etkileri vardır. Bu çalışmada, L-theanine'nin akut uygulamasının beyin kesitlerinde herhangi bir nöbet benzeri veya inhibe edici aktiviteye yol açıp açmayacağını test ettik.

Gereç ve Yöntemler:
L-theanine'in epileptiform aktivite üzerindeki etkileri iki farklı beyin bölgesinde araştırıldı. Yatay hipokampal-entorinal korteks dilimleri, 32-36 günlük C57BL/6 farelerinden elde edildi. Hücre dışı alan potansiyelleri medial entorinal korteks (EC) ve hipokampusun CA3 bölgesinden kaydedildi. Epileptiform aktivite, beyin dilimlerine 4 Aminopiridin (4AP, 100 µM) uygulanarak indüklendi ve 50 µM L-theanine uygulandı. Kayıtlardan iktal benzeri olayların süresi ve sıklığı ile interiktal benzeri aktivitelerin frekansı ve genliği hesaplandı.

Bulgular:
L-theanine tek başına herhangi bir senkronize aktivite başlatmadı. 4AP ile indüklenen epileptiform aktivite sonrası L-theanine'in banyo uygulaması iktal deşarjların süresini ve sıklığını önemli ölçüde değiştirmese de, L-theanine interiktal deşarjları azalttı. EC'de interiktal deşarjların sıklıkları anlamlı ölçüde azaldı. CA3'te, interiktal aktivitelerin amplitüdleri anlamlı ölçüde azaldı.

Sonuç:
Bulgularımız, L-theanine'in herhangi bir nöbet benzeri olay uyarmadığını, bunun yerine devam eden epileptiform aktivite üzerinde baskılayıcı bir etkiye sahip olduğunu göstermektedir.

Anahtar Sözcükler:
Theanine, 4 Aminopiridin, Beyin Kesiti, Epileptiform aktivite, CA3, Entorinal Korteks

INTRODUCTION
L-theanine, a nonprotein derivative amino acid, is abundantly found in tea leaves and also named as γ-glutamilethylamid, 5-N-ethylglutamin, γ-glutamic-L-ethylamide, γ-ethylamino-L-glutamic acid, and γ-L-glu-ethylamide (1). Tea has been consumed for over thousands of years and spread to all around the world. It has been shown that L-theanine can cross the blood brain barrier in rats (2). L-theanine has both psychological and physiological effects such as reduction in the heart rate and salivary immunoglobulin-A which most likely depends on the effects on sympathetic nervous system (3). L-theanine showed neuroprotective effects against dopamine induced neurotoxicity via raising glutathione supply in astrocytes (4). L-theanine also reduces Amyloid Beta (Aβ) (1-42) levels which resulted in Aβ (1-42) induced neuronal cell death in the cortex and hippocampus (5).

Glutamine participates in a variety of metabolic pathways which is synthesized from glutamate and ammonia in astrocytes, so it participates the amino acid needs in the synaptic neurotransmitter pools (6). It is thought that L-theanine might be able to take a part like glutamine in the brain because of the structural similarities (7). Accumulation of theanine in the brain is selectively inhibited by glutamine, thus theanine could suppress spontaneous and/or exocytotic release of glutamine (8).

In the rat brain ischemia-reperfusion model, L-theanine has been shown to reduce the damage in the hippocampus, and it has been reported that this is achieved by inhibiting heme oxygenase-1 (HO-1) expression and activating the extracellular signal-regulated kinase 1/2 (ERK1 / 2) pathway (9). In addition, L-theanine has been reported to have a neuroprotective effect by modulating GABAergic transmission (through the Gamma-Aminobutyric Acid-A (GABA-A) receptor) in the mouse ischemia model (10). L-theanine has been reported to increase the exocytosis of various neurotransmitters such as GABA, glycine and dopamine in the rat striatal neurons (11).

Epilepsy is a neurological disorder, characterized by recurrent spontaneous, unpredictable, synchronous neuronal discharges (12). In neuronal circuitry, the imbalance between synaptic inputs of excitation/inhibition and neuronal excitability have been suggested as two major neuronal mechanisms leading to synchronized discharges (13). In spite of its neuroprotective effects and being involved in various neurotransmitter systems, the effects of L-theanine on seizure susceptibility has been reported in only few studies. It has been shown that acute and chronic administration of tea extracts, but not pure L-theanine, exerted proconvulsive effects (14). In a more recent study, the effects of 2 weeks application of L-theanine before the experiments were investigated. The results showed that L-theanine changed the convulsion thresholds of two seizure models. While increasing the threshold for limbic seizures, L-theanine partially protected against generalized seizures (15). In the present study, for the first time, we described the acute effects of L-theanine on epileptiform activity in ex-vivo brain slices. We investigated whether (I) the acute application of L-theanine could induce any seizure like activity and (II) L-theanine has the potential to manipulate ongoing seizure like activity in two different brain regions.

MATERIALS and METHODS
The study was approved by Karadeniz Technical University, Animal Care and Ethics Committee (Approval number: 53488718-108 and date: 08.01.2021) and complies with the Guide for the Care and Use of Laboratory Animals principles and tenets of the Helsinki Declaration. All the chemicals were purchased Sigma-Aldrich unless specified.

Slice Preparation
Acute brain slices were obtained from 30-35 days-old 14 C57BL/6 female mice as described elsewhere (16, 17). Briefly, following decapitation, brain was rapidly transferred into chilled (1.5–2 °C) artificial cerebrospinal fluid (ACSF) containing; 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2-PO4, 25 mM NaHCO3, 25 mM d-glucose, 2 mM CaCl2 and 1.5 mM MgCl2. After the whole brain is immersed in cold ACSF solution for 3-4 minutes, 370 µm thick horizontal hippocampal-EC slices were obtained by cutting the whole brain at the microtome (Leica VT100S, Germany) in ice-cold ACSF. Brain slices were transferred into the 30 ± 1 °C ACSF containing chamber which was oxygenated with 95% O2 and 5% CO2 at pH 7.4, for recovery at least 1h. Brain slices were kept at the same ACSF conditions during the experiment.

Electrophysiological Recordings

The submerged recording chamber was used to record electrophysiological signals. The chamber was continuously perfused with oxygen saturated ACSF and the temperature of the chamber was maintained at 30 ± 1 °C. For the recordings, glass micropipette electrodes were used. The pipettes were positioned to two recording sites on the same slice (EC and hippocampus CA3 regions) (Fig. 1 A). Following the transfer, the slice was allowed to accommodate for 10-15 minutes and then 10 min of recording was monitored. During this period none of the studied slices showed abnormal activity or discharges. After that 100 µM 4-Aminopyridine (4AP) were applied to induce epileptiform activities and the epileptiform activities were observed within 20 min.

To evaluate the effects of L-theanine on epileptiform activity, 30 min of 4AP induced activities were recorded. Then the bath solution was replaced by ACSF containing 100 µM 4AP + 50µM L-theanine. After 20 min incubation, the activity was recorded for another 30 min. 50µM L-theanine concentration indicated significant effect on rats brain slices against Alzheimer diseases (18). Therefore same concentration was applied as a single dose for this experiment.

Borosilicate glass pipettes were prepared for recordings. Pipettes were pulled by a puller (Sutter P-1000, Japan) from borosilicate glass capillaries (GB 150F-8P, Sutter, Japan). The pipettes were filled with ACSF and the resistances were around 1 MΩ. Extracellular field potentials were recorded simultaneously from two channels both from the upper layers of medial EC and CA3 region of hippocampus (Figure 1). The analog biological signals were fed to a differential amplifier (A-M systems Model 1700). The records were obtained under AC mode and filtered between 0.1 Hz – 5 kHz and amplified at x1000. Data were digitized at 5 kHz using a Digidata-1400 acquisition system. pCLAMP10 software (Molecular Devices, Sunnyvale, CA, USA) was used for acquisition and off-line analysis.

The recordings were analyzed off-line. Similar to previous studies, 4AP induced epileptiform activities classified as ictal and interictal like events (17, 19). The sharp rise deflections with an amplitude at least 4 times higher than baseline amplitude were accepted as epileptiform activity (Fig. 1 B). Those activities which were lasted less than 4 s was named as "inter-ictal" (Fig. 1 D). However the duration of events which were longer than 4s were accepted as "ictal" (Fig. 1 C). The time points between initial deflection and return to baseline potentials were defined as the duration of synchronous events.

Statistical Analysis

Paired t-tests were performed for statistical analysis by using GraphPad Prism software. Data were presented as mean ± SEM and $p < 0.05$ was accepted as significant.

RESULTS

Field potentials of epileptiform activities were obtained from both EC and CA3 of the hippocampus (Fig. 1 A). 4AP induced either only interictal like activities (Fig. 2 B and C) or both interictal and ictal like activities (Fig 1. B). At the outset, we tested whether L-theanine triggers any spikes or synchronous discharges that could be interpreted as an epileptiform activity. In six slices, bath application of 50 µM L-theanine did not induce any such activities (Fig-2A). Then the effects of acute application of L-theanine on ongoing epileptiform activity were investigated.

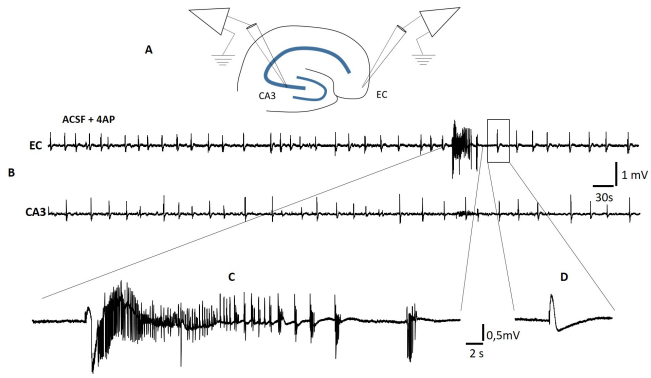

Figure 1. Location of electrodes and field potential recordings samples from EC and CA3 of hippocampus. A. Position of recording electrodes. B. The sample records of interictal and ictal like activities after 100 µM 4AP induced epileptiform discharges. (C) Ictal like activities. (D) Interictal-like activity.

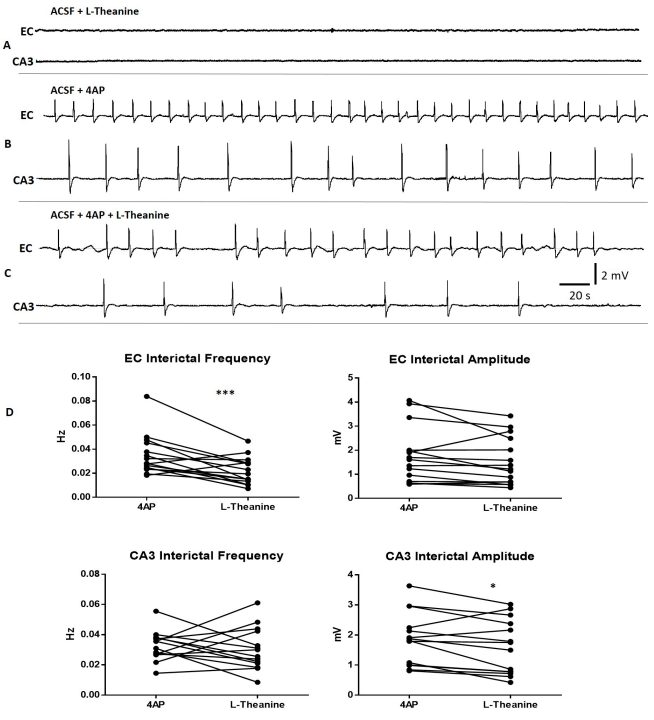

Figure 2. Sample traces of basal and only interictal like events. A. Effect of L-theanine (50µM) alone without discharges which were similar to the basal activities in normal ACSF. Only interictal like events records before (B) and after the application of L-theanine (C). D. Amplitudes and interictal frequencies at the EC and CA3 before and after L-theanine application (n=14), (***: p<0,005; *: p<0,05).

in EC interictal event frequency significantly reduced by L-theanine application (Figure 2.D). The average frequency was 0,0349 ± 0,004 Hz before and reduced to 0,0218 ± 0,003 Hz (p= 0,0024; n=14) after L-theanine application. The amplitudes of interictal events were not affected (1,78 ± 0,30 mV before and 1,52 ± 0,25 mV after). In CA3, L-theanine had no effect on frequency of interictal discharges; before 0,0336 ± 0,003 Hz and after 0,031 ± 0,004 Hz., yet, diminished the amplitudes of interictal event from 1,86 ± 0,24 to 1,59 ± 0,24 mV significantly (p=0,025 and n=14) (Figure 2D).

Ictal events were observed only in 6 slices in EC and 5 in CA3. The durations and frequencies of ictal events were analyzed. L-theanine did not change the ictal parameters. In EC, the durations of ictal events were 25,30 ± 5,7 s and 33,78 ± 11,62 s after L-theanine. The frequencies were 0,0023 ± 0,001 Hz and 0,0032 ± 0,001 Hz after L-theanine application (n=6). Similarly, in CA3, the durations (19,58 ± 5,5 s before and 15,98 ± 7,1 s after) and frequencies (0,0025 ± 0,0013 Hz before and 0,0019 ± 0,0009 after) was not changed by L-theanine (n= 5).

DISCUSSION

In this study, we report for the first time the acute effects of L-theanine on brain slices by employing extracellular field potential records. Our results demonstrated two main findings. First, L-theanine application alone did not initiate any synchronized discharges. Second, when applied following induction to epileptiform activity, L-theanine attenuated selectively the interictal like activities in EC and hippocampus.

Brain slice models constitute ideal preparations to study the functions of neuronal circuitry as the cellular structures, synapses and projections are protected. 4AP model of brain slice seizure model has been commonly used as in vitro model of epilepsy. 4AP is a Potassium channel blocker and induces mainly two types of synchronous discharges (20). Ictal like events are observed in EC and spread to hippocampus. The ictal like events generally last longer (>4s). In the present study we show that L-theanine did not alter the ictal duration and frequency. Previous studies suggested that fast ionotropic glutamatergic transmission and GABAA receptor mediated signaling is required for interictal discharges (21). Interictal-like events corralate with paroxysmal depolarizing shift which is subnormal fluctuation of membrane potential and requires activation of glutamate receptors (22). In this study, L-theanine could have interacted with glutamatergic transmission at a mild to moderate level. A previous study has shown that Theanine mildly inhibits the binding of glutamate to AMPA, Kainate and other Glutamate receptors (23).

In the literature there are few studies that investigated the link between L-theanine and seizures. Tea consumption has been suggested to potentiate Pentilentetrazole induced convulsions (14). Schalier et al. investigated the effects of 14 days of L-theanine administration, not tea extract, on seizure susceptibility. The doses of convulsant chemicals need to induce seizure are altered by L-theanine (15). The application period and the experimental models of the mentioned studies make it difficult to compare with our present findings. However, researchers showed that, L-theanine application decreased the susceptibility for limbic seizures (15). This particular finding supports our results as the slice model we have used is ideally mimics the limbic seizures (24). Another study that evaluated the acute effect of L-theanine is in line with our findings. Yu X. et al. showed that, L-theanine application enhanced the anticonvulsion capacity of pentobarbital sodium in a strychnine model of seizure (25). An earlier study also showed that, intraperitoneal injection of Theanine inhibited the convulsive action of caffeine (26). Our findings showed suppressed interictal activity which is parallel to the reports where the acute effects of L-theanine were evaluated. In the present study we have investigated the effects on of L-theanine 50 µM. Repeating experiments with different doses would certainly give a better idea about the L-theanine on epileptiform activity. However in the literature, it is shown that L-theanine is an active molecule in the concentration range (1-100 µM) on cultured hippocampal neurons and induces an influx of Ca2+ depending on the NMDA receptors on mature hippocampal neurons (27). It is also stated that 50 µM L-theanine in brain slices contributes to NMDA receptor mediated synaptic transmission and modulates LTP (18). As can be seen from these reports, and also from our present findings, 50 µM L-theanine is sufficient to modulate synaptic and neuronal circuitry function.

CONCLUSION

In this study L-theanine indicated mild to moderate effects in the 4AP induced epileptiform activity. Reduction in the interictal frequency at EC can be explained as L-theanine might increase extracellular GABA which resulted in Cl influx and inhibitions in the cells. Similarly in the rat brain, L-theanine modulates neurotransmitter concentrations and can cause increased exocytosis of GABA. However L-theanine has no effect on neuronal network function under normal physiological conditions which suggests that the effect of theanine is somehow eliminated in healthy neurons. Amplitude reduction at the hippocampus possibly related with the decrease in the number of stimulated neurons due to the activation of inhibitory neurons. In this case either inhomogeneity of inhibitory neurons might cause this result or L-theanine might affect GABA, Parvalbumin or type 3 serotonin receptor. Nevertheless extensive future studies are required to identify which inhibitory neurons are activated or which ionic pathway causes these results.

L-theanine has an effect on neuronal cells that tend to be stimulated, therefore L-theanine might be considered as a supplementary molecule to control seizures.

Ethics Committee Approval:
The study was approved by Karadeniz Technical University, Animal Care and Ethics Committee (Approval number: 53488718-108 and date: 08.01.2021) and complies with the Guide for the Care and Use of Laboratory Animals principles and tenets of the Helsinki Declaration. All the chemicals purchased from Sigma-Aldrich unless specified.

Author contribution statement
H.B, S.A.A. and I.A.: Conceptualization
H.B., H.O., H.K. and I.A.: Investigation
H.B. and S.A.A.: Formal Analysis and visualization
H.B. : Writing- Original draft preparation
S.A.A. and I.A.: Writing- Reviewing and Editing
I.A.: Supervision

Informed Consent:
All the participants' rights were protected and written informed consents were obtained before the procedures according to the Helsinki Declaration.

Conflict of Interest:
The authors have no conflict of interest to declare.

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