ORIGINAL RESEARCH

A linear relationship between lamotrigine and GABA in cerebrospinal fluid

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ABSTRACT: The γ-amino butyric acid (GABA)-mediated neurotransmission is useful in treating conditions like anxiety, sleep disturbances, depression and bipolar disorders. The aim of the present study is to supply evidence about neurochemical effects of acute lamotrigine treatment on GABA and L-glutamic acid levels in the cerebrospinal fluid of Wistar Albino rats and the involvement of cholinergic system. The day after the placement of concentric microdialysis probes into the lateral ventricles of rats, microdialysis experiments were performed in conscious rat model. The rats either received intraperitoneal injections of physiological saline or 20 mg/kg lamotrigine. For assessing the cholinergic involvement 0.5 mg/kg physostigmine or 2 mg/kg atropine sulfate pre-treatments were given prior to lamotrigine injection. GABA, L-glutamic acid and lamotrigine concentrations in the dialysates were analyzed using High Performance Liquid Chromatography. Saline produced no change in GABA or L-glutamic acid levels, but lamotrigine treatment significantly elevated GABA concentrations ($p<0.05$). Pre-treatment with physostigmine or atropine sulfate did not affect either GABA or L-glutamic acid levels siginificantly. Physostigmine or atropine sulfate pre-treatments did not affect the lamotrigine-induced GABA levels. The results may imply that lamotrigine-induced GABA plays a role in the pharmacological effects of lamotrigine where a linear relationship exists between lamotrigine and GABA. However, central cholinergic system fails to affect lamotrigine-induced GABA release.

KEY WORDS: Glutamate, cholinergic system, microdialysis, conscious rat model

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INTRODUCTION

Lamotrigine as a comparatively novel antiepileptic agent is now being used widely as a drug of first choice in certain seizure types including Lennaux Gestaut syndrome, grand or petit mal seizures and myoclonus (1-8). Lamotrigine has also been extensively preferred for bipolar disorders (9,10,11). γ-amino butyric acid (GABA) is the major inhibitory neurotransmitter in the brain and low GABAergic activity has been implicated in the pathophysiology of bipolar disorder (12,13). Previous studies indicated that GABAergic potentiation plays role in the effects of lamotrigine in addition to its anti-glutamergic effects (14,15).

Lamotrigine reduces glutamate and aspartate release through inhibiting Na^+ channels and thus causing inhibition of exocytosis of these excitatory amino acids (16). The role for inhibition of Ca^{2+} channels was also demonstrated (17-19).

Prior studies performed in rat entorhinal cortex using whole patch clamped cells demonstrated that lamotrigine produces reductions in glutamate and increases in GABA release without affecting Na^+ or Ca^{2+} channels (20,21). It was also reported that lamotrigine also suppresses $GABA_A$-mediated neurotransmission in rat amygdala cells through affecting presynaptic Ca^{2+} influx (22). Lamotrigine also decreases veratrine- or electrically stimulated release of endogenous glutamate and [^{3}H]-GABA, [^{3}H]-5-hydroxytriptamine and [^{3}H]-dopamine in rat cortical slices (17). However, ex vivo studies documented that acute lamotrigine did not produce an effect on hippocampal tissue content of GABA or taurine but both were increased following chronic treatment with the drug (23).

In central nervous system, the interaction between cholinergic system and GABAergic transmission is long studied. Previous *in vivo* and *in vitro* studies also demonstrated that GABA and its analogues directly inhibit cortical acetylcholine release in the freely moving guinea pig and

in electrically stimulated slices (24,25). Local inhibitory effects of acetylcholine have been ascribed to the excitation of GABAergic inter neurons in the cerebral cortex (26) but some findings suggested that acetylcholine exerts direct inhibitory effects on GABA release at least in other brain areas (27,28) and in the periphery (29).

The aim of this study is to monitor the time-course changes of GABA and L-glutamic acid in rat cerebrospinal fluid produced by lamotrigine treatment by measuring the amino acids dialyzed through microdialysis probes implanted into lateral ventricles of conscious rats and secondly to show the possible modulatory effect of cholinergic system on the lamotrigine-induced amino acid release.

MATERIALS AND METHODS

1. Animals and laboratory

Wistar albino rats weighing 250-275 g of both sexes supplied from Marmara University, Experimental Research and Animal Laboratory were used. An approval of Marmara University Ethical Committee for Experimental Animals was taken before the experiments (16.12.2005 - 63.2005.mar). The animals were kept in a temperature-controlled room with 12-h light and dark cycle and fed with standard animal food and water ad libitum.

2. Drugs used in the study

All drugs were supplied from Sigma Chemical (USA) except lamotrigine (supplied kindly from GlaxoSmithKline, Turkey). Lamotrigine was dissolved physiological saline prior to injection.

3. Stereotaxic surgery and microdialysis

Concentric microdialysis probes were used as as described previously (30). The rats were anesthetized with intraperitoneal ketamine (100 mg/kg) and chlorpromazine (1.0 mg/kg) mixture and placed in a stereotaxic frame (Stoelting, Model 51600, USA). The scalp skin was incised and the periosteum was separated from the cranium. Probe was implanted into the right lateral ventricle (lateral ventricle coordinates; 1.0 mm posterior to bregma, 1.5 mm lateral to midline and 3.8 mm ventral to the skull surface) according to the Paxinos and Watson rat brain atlas (31). Supporting screws were also placed and the microdialysis probe was covered together with the screws with dental acrylic cement. The collection of intracerebral perfusion samples was performed 24 h following surgery.

The day after the placement of microdialysis probes, polyethylene tubings were attached to the inlet of the microdialysis probes to collect the samples in conscious rat model in a plexyglass cage (42X42X20 cm). Artificial cerebrospinal fluid was delivered continuously via 250 µl hamilton syringe which was connected to a microinfusion pump (KD Scientific, USA). The composition of artificial cerebrospinal fluid was 2.5 mM KCl, 125 mM NaCl, 1.26 mM $CaCl_2 2H_2O$, 1.18 mM $MgCl_2 6H_2O$ and 0.2 mM $NaH_2PO_4 2H_2O$ and the pH was set to 7. The artificial cerebrospinal fluid was filtered through 0.4 µm nylon membrane filters.

Two basal samples were collected at 0.5 µl/minute flow rate every 40 min in a 0.25 ml ependorf tubes from Wistar rats after an equilibration period of 1 h. After collection of basal samples, intraperitoneal physiological saline injection was administered and five more consecutive samples were collected. The same protocol was repeated with lamotrigine (20 mg/kg), physostigmine (0.5 mg/kg) or atropine sulfate (2 mg/kg). The dialysates were divided into two equal ependorf tubes for different High Performance Liquid Chromatography analysis methods and kept at -80°C.

Throughout the microdialysis procedure, the rats were observed and atypical behaviors were noted. The rats were anesthetized with ether and methylene blue was injected through the probe and decapitated. The brains were sliced with a blade to observe the dye in the ventricles for verification of probe placement. Only the proper experiments were used in data analysis.

4. Chromatographic system and High Performance Liquid Chromatography analysis of L-glutamic acid and GABA in the cerebrospinal fluid dialysates

Chromatographic system for analysis of L-glutamic acid and GABA (supplied from Sigma, USA) consists of a gradient pump (Agilent 1100, Germany) with four solvent bottles, degasser module, C18 reverse phase nucleosil column (15 cm and 3.9 cm length, 4.6 mm diameter and 5 µm pore size), autosampler unit, fluorescent detector with excitation and emission wave lengths set to 360 nm and 410 nm respectively and a computer. The composition of mobile phase and chromatographic procedures were performed as described previously (32).

5. Lamotrigine and amino acid High Performance Liquid Chromatography analysis

The isocratic High Performance Liquid Chromatography system for lamotrigine analysis consists of a 100 µl loop, rheodyne valve with a pump (Jasco PU 980, Tokyo, Japan), C18 reverse phase colon (15 cm length, 4.6 µm diameter and 5 mm pore size), UV detector (Jasco UV 975, Tokyo, Japan) wave length set to 214 nm and a computer. The chromatographic analysis was carried out with a software (Borwin Chromatograph, version 1.2, France). The mobile phase is a mixture of 0.1 M KH_2PO_4 (pH: 6.7), acetonitrile and methanol (7:2:1, v/v/v). The flow rate of the pump was set to 1.3 ml/min. Manual injections were given within a volume of 10 µl at room temperature. The retention time of lamotrigine was 4.5 min. Total duration of the chromatogram was 15 min.

6. Statistical analysis

All data are expressed as means ± s.e.m. The effect of saline or drug treatment on amino acids were tested using *Kruskal-Wallis* followed by *Dunn's Multiple Comparison Test*. The relationship between the lamotrigine and GABA concentrations in the CSF dialysates was determined by *Pearson's test* (alpha = 0.05). *Two-tailed Student's t-test* for unpaired data was used to determine the differences between the basal percent change of GABA after physostigmine or atropine sulfate injections. Statistical significance was accepted where $p<0.05$.

RESULTS

1. The effect of lamotrigine injection on GABA and L-glutamic acid in cerebrospinal fluid dialysates

The basal levels of L-glutamic acid and GABA in the cerebrospinal fluid dialysates prior to physiological saline injection

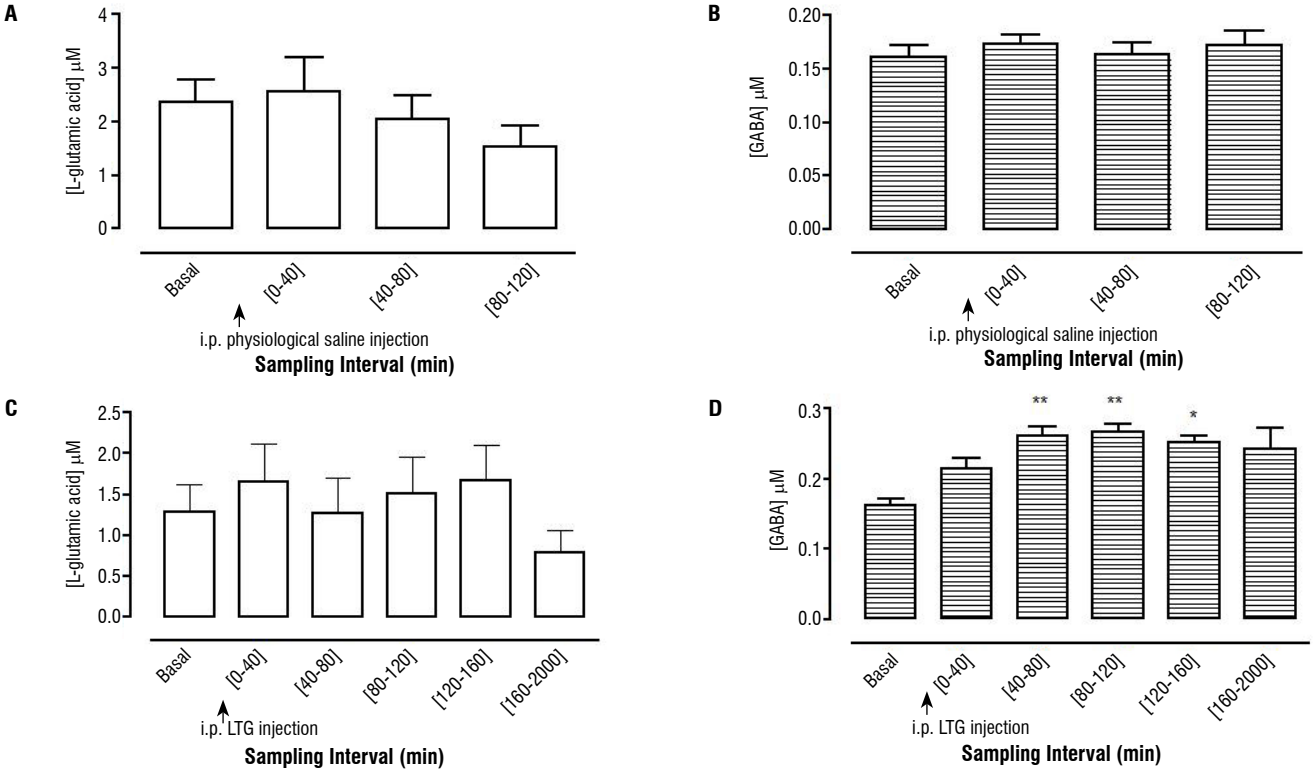

FIGURE 1. The effect of intraperitoneal physiological saline injection on L-glutamic acid (A) and GABA (B) levels in cerebrospinal fluid dialysates collected from the right lateral ventricle of Wistar rats (n=10). Lamotrigine (LTG) was administered at a dose of 20 mg/kg and its effects on L-glutamic acid and GABA are presented in C and D, respectively (n=10). *p<0.01 **p<0.001 (compared to [-40-0]-min (basal) sample)

were found to be 2.35 ± 0.42 mM and 0.16 ± 0.01 mM, respectively. Physiological saline injection produced no significant difference either in L-glutamic acid or GABA levels (Figure 1A and 1B; p=0.565 and p=0.789). Lamotrigine injection did not affect L-glutamic acid levels (Figure 1C; p=0.922) but produced increases in GABA level yielding significant differences in [40-80]-, [80-120] - and [120-160]-min sampling intervals when compared to [-40-0]-min (basal) sample (p<0.001, p<0.001 and p<0.01, respectively; Figure 1D).

2. The relationship between GABA and lamotrigine levels in the cerebrospinal fluid dialysates

Lamotrigine started to appear in [0-40] min samples following intraperitoneal injection, and reached a peak at [80-120]-min period and the levels started to decline in the samples collected afterward (Figure 2). When GABA and lamotrigine concentrations measured within the same sampling periods are plotted and a linear relationship between the drug and GABA was recognized (Figure 3; p=0.0122, alpha=0.05).

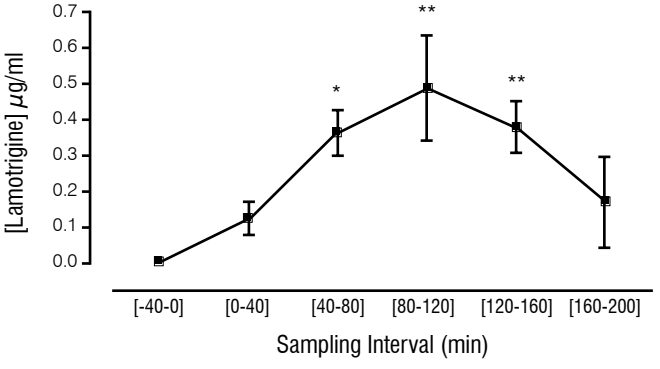

FIGURE 2. The time course change in lamotrigine (LTG) concentrations in cerebrospinal fluid dialysates following intraperitoneal injection. *p<0.01 **p<0.001 (compared to [-40-0] min (basal) sample)

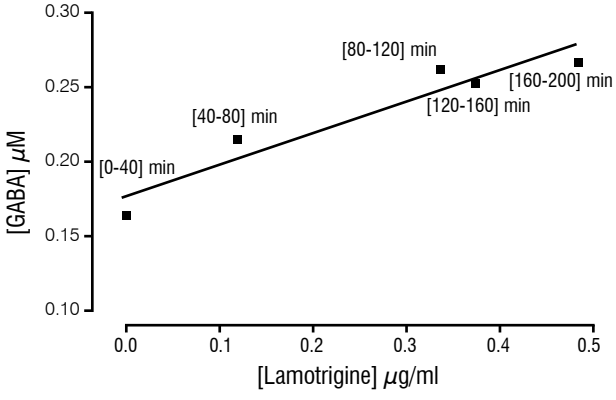

FIGURE 3. The relationship between GABA and lamotrigine (LTG) levels in the cerebrospinal fluid dialysates. Data points represent the mean of concentrations in corresponding sampling intervals (n=10; Pearson test, p=0.0122, alpha=0.05).

3. The effect of physostigmine or atropine on lamotrigine-induced GABA response in cerebrospinal fluid dialysates

When cholinomimetic physostigmine was injected intraperitoneally at a dose of 0.5 mg/kg, a tendency to decrease in the GABA level of [0-40]-min sample was observed, but this did not yield a statistically significant difference ($p = 0.252$). Non-selective muscarinic antagonist atropine (2 mg/kg) also produced non-significant increases in GABA [0-40]-min sample after atropine sulfate injection. Likewise, this increase did not yield a statistically significant difference ($p = 0.1023$).

In order to analyze the involvement of cholinergic system in the lamotrigine induced GABA response, lamotrigine injections were given to physostigmine-pretreated rats (n=6). The percent maximum effects were 78.4 ± 10.2 and 74.7 ± 7.2 in physiological saline- and physostigmine- pretreated groups, respectively. Comparison of these data did not produce a statistical significant difference ($p = 0.8749$).

Another group of rats received atropine sulfate pretreatment before lamotrigine injection (n=6) and the percent maximum GABA response was calculated as 85.5 ± 13.1. No statistical significant difference was found between physiological saline- and atropine sulfate- pretreated groups ($p = 0.6354$).

DISCUSSION

The present study shows that acute lamotrigine treatment elevates GABA levels in cerebrospinal fluid of rats at a dose of 20 mg/kg. Previously, Wheatley et al. reported that this dose was within the anticonvulsive range in the rat (33). In preliminary experiments, we also used the doses of 10 mg/kg, but observed no response in GABA levels (data not shown) and continued the study with the dose as indicated above. Morris et al. also showed that the plasma concentrations of lamotrigine in rats achieved with this dose were also similar to the concentration range proposed for epileptic patients (34). This can account for the discrepancy between human and rat doses.

We also analyzed the lamotrigine concentration in the dialysates collected from lateral ventricles. The increase in GABA concentrations was parallel to the increase in lamotrigine levels in cerebrospinal fluid. This observation is comparable to those previously reported (35,36). A linear relationship appears to exist between the administered doses of lamotrigine and the respective plasma concentrations (35). As with all antiepileptics, lamotrigine needs to cross blood–brain barrier to exert its anticonvulsant effects. Consequently, the interpretation of the lamotrigine plasma levels requires the assessment of the drug concentration at the neuronal sites of action. After intraperitoneal administration, lamotrigine rapidly appears in plasma (peak value at 0.25 h), demonstrating rapid absorption from the peritoneal cavity. In addition, lamotrigine quickly appears in brain (peak value between 0.5 and 2 h), suggesting ready penetration of the blood–brain barrier (35). After peak values, a monoexponential fall is observed in both plasma and brain concentrations. Parallel patterns observed in plasma and brain profiles together with linear relationship established between drug in plasma and drug in brain suggested that the lamotrigine distribution is dependent on blood flow, suggesting that lamotrigine crosses the blood–brain barrier by simple diffusion (37). In our study, there is also a similar pattern for lamotrigine as shown previously.

The results of *in vivo* or *in vitro* studies showed that antiepileptic effect of lamotrigine is due to inhibition of voltage-dependent sodium channels (38-41), which in turn could inhibit release of transmitter glutamate (18,38,42). Interestingly, the therapeutic effect of lamotrigine is different from that of other antiepileptic drugs that act on sodium channels, such as phenytoin and carbamazepine. For instance, lamotrigine is effective in treating childhood absence epilepsy, whereas phenytoin and carbamazepine are not (43). This suggests that lamotrigine can act through multiple mechanisms. Wang et al. reported that lamotrigine produced decreases in glutamate release from rat cortical synaptosomes, but in this current study, we detected no significant change in glutamate levels in response to 20 mg/kg lamotrigine injection (19). Wang et al. suggested the role of inhibition of Ca^{2+} channel activity in the mechanism of action of the drug (19). Cunningham and Jones found an indirect evidence for lamotrigine-mediated amino acid effects by using *in vitro* electrophysiological techniques, where they observed decreased glutamate and enhanced (20) GABA levels. A reciprocal modulation of glutamate and GABA was also demonstrated for phenytoin (21). Braga et al. (22) reported that lamotrigine decreased $GABA_A$-mediated transmission under experimental conditions similar to those reported by Cunningham and Jones (20). With this recent paper, we supply evidence for the linear relationship between lamotrigine and GABA in cerebrospinal fluid. Chronic lamotrigine treatment has been found to elevate hippocampal tissue content of GABA and taurine (23). There are several mechanisms for the elevation of GABA following administration of lamotrigine, one of which is the inhibition of glutamate. One the reasons that we could not show any significant change in L-glutamic acid may be that measured L-glutamic acid does not always reflect the neuronal sources. However, it was also discussed that basal extracellular levels of either transmitters although increased at the synaptic level, such changes may be undetectable by dialysis (44). Another mechanism may be the contribution of cholinergic system. Many studies show that there is a bidirectional GABAergic–cholinergic interaction in the central nervous system. Immunohistochemical findings strongly suggest the co-existence of GABA and acetylcholine in mammalian nerve endings (45). To elucidate the relation between GABAergic and cholinergic systems further, De Boer and Westerink studied the effects of GABAergic drugs on the output of striatal acetylcholine by using *in vivo* brain microdialysis techniques (46). Previously, tonic inhibition of cholinergic system via $GABA_A$ receptors was demonstrated where administration of muscimol either intracerebroventricularly or microinjecting into the dorsomedial hypothalamic nucleus prevented carbachol evoked blood pressure changes in conscious rats (47). The activation of $GABA_A$ receptors by muscimol administration completely prevents the pressor effects of carbachol, possibly by depressing cholinergic activity in the brain (47). In another study, endogenous brain acetylcholine was shown to have a modulator role on $GABA_A$ receptor-mediated blood-pressure control via nicotinic receptors (48). There are also studies performed in local brain regions considering the muscarinic modulation of GABA release. While in some brain regions, like rat globus pallidus (49) and substantia gelatinosa (50), muscarinic receptor activation increases GABA release and in some other regions like rat midbrain dopaminergic neurons, stimulation of muscarinic M_3 receptors cause decreases in GABA-mediated inhibitory effects (51).

Beyin omurilik sıvısında lamotrijin ve GABA arasında lineer ilişki

ÖZET: γ-amino bütirik asit aracılığıyla oluşan sinir iletisi, anksiyete, uyku bozukluğu, depresyon ve bipolar hastalık gibi durumların tedavisinde kullanılmaktadır. Bu çalışmanın amacı Wistar albino sıçanlara akut lamotrijin uygulamasının, beyin omurilik sıvısındaki GABA ve L-glutamik asit düzeylerine nasıl etki ettiğine ve olası etkilerin kolinerjik sistemle ilişkisine dair /nörokimyasal kanıt sağlamaktır. Konsantrik mikrodiyaliz problarının lateral ventriküllere yerleştirilmesinden bir gün sonra uyanık hayvan modelinde mikrodiyaliz deneyleri yapıldı. Sıçanlara fizyolojik tuzlu su veya 20 mg/kg lamotrijin uygulaması yapıldı. Kolinerjik katılımı göstermek için 0.5 mg/kg fizostigmin veya 2 mg/kg atropin sülfat ön uygulamaları lamotrijin enjeksiyonundan önce uygulandı. Toplanan diyalizatlarda GABA, L-glutamik asit ve lamotrijin düzeyleri yüksek performanslı sıvı kromatografisi ile analiz edildi. Fizyolojik tuzlu su, GABA veya L-glutamik asit düzeyinde bir etki oluşturmazken, lamotrijin anlamlı derecede GABA düzeyini artırdı ($p<0,05$). Fizostigmin veya atropin ön uygulamaları lamotrijinle indüklenen GABA atışı üzerine bir etki oluşturmadı. Bu sonuçlar lamotrijinin farmakolojik etkilerinde GABA'nın katılımının olduğunu ve aralarında lineer bir ilişki bulunduğunu göstermektedir. Santral kolinerjik sistem lamotrijin ile indüklenen bu etkiye katılmamaktadır.

ANAHTAR KELİMELER: glutamat, kolinerjik sistem, mikrodiyaliz, uyanık sıçan modeli

In this study, we also aimed to analyze the involvement of cholinergic system in the GABA response of lamotrigine treatment. After pretreating with physostigmine, a cholinesterase inhibitor, lamotrigine injection produced non-significant decreases in cerebrospinal fluid GABA levels. Similarly, a muscarinic antagonist, atropine sulfate produced non-significant increases in GABA.

In conclusion, our results may imply that lamotrigine-induced GABA response participates in the mechanisms of anticonvulsant and mood stabilizing effects of the drug where cholinergic system seems to be not involved at least in healthy rats and the issue remains to be examined further.

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