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Original Article

Comparison of influence of lamotrigine and other anticonvulsants on nitric oxide and thiol-disulfide systems in conditions of metrazole kindling

Natalia P. Shastun[1], Valentina I. Opryshko[1], Nina V. Buchtiarova[2], Sergii V. Levich[3]

[1]Department of Pharmacology, Dnepropetrovsk State Medical Academy, Dnepropetrovsk, Ukraine
[2]Department of Pharmacology and Medicinal Preparations, Zaporizhzhya State Medical University, Zaporizhzhya, Ukraine
[3]Department of Biochemistry and Laboratory Diagnostics, Zaporizhzhya State Medical University, Zaporizhzhya, Ukraine

Address for correspondence:
Sergii V. Levich,
Department of Biochemistry and Laboratory Diagnostics, Zaporizhzhya State Medical University, Zaporizhzhya, Ukraine
rshlevas@gmail.com

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ABSTRACT

Objective: Epilepsy and seizure disorders are of the most widespread and serious diseases of the central nervous system (CNS), leading to disability of the patient and to reducing life quality. Seizure disorders are associated with increasing of electrical activity of neurons of the CNS. Heightened electrogenesis of neurons are accompanied with activation of the nitric oxide (NO) system that, by generating of oxidizing agents, leads to damage of the cells and the formation of 'structural footprint' of seizures. This study is focused on the effects of anticonvulsants on the level of NO concentration, NO-synthase (NOS) activity and parameters of the thiol-disulfide system in conditions of equivalents of epilepsy. **Methods:** Research has been carried out on sufficient number of experimental animals. For biochemical and histo-immunochemical study brain tissue of experimental animals was used. We studied: density of inducible (i) and neuronal (n)NOS positive neurons in the sensorimotor cortex layers; content of nitrotyrosine, thiol groups, recovered and oxidized glutathione and activity of glutathione reductase in the brain homogenate. **Results:** The simulation of seizures by metrazole led to increase in the expression of iNOS and nNOS with the background of deficiency thiol recovery. Preventative administration of lamotrigine, carbamazepine, topiramate, sodium valproate or gabapentin normalized the shifts in the NO and thiol systems, but varied from each other. **Conclusions:** Obtained results allowed to suggest that the functional and biochemical system of NO is one of the links of anticonvulsants action. The most pronounced effect that led to decrease in the expression of the two isoforms of NOS was observed with administration of lamotrigine.

KEY WORDS: Anticonvulsants, epilepsy, metrazole kindling, NOS, thiol-synthase

INTRODUCTION

Epilepsy and seizure disorders are among the most widespread and serious diseases of the central nervous system (CNS), leading to disability of the patient and to reducing life quality. Due to this fact, study of the mechanisms and seeking for preventive and therapeutic alternatives is the fundamental of biomedical research in this area.

According to modern concepts, nitric oxide (NO) is a key signaling molecule that regulates the function of the cardiovascular, nervous and immune systems of the body [1]. Seizure disorders are associated with increase of electrical activity of neurons in the central nervous system. Heightened electrogenesis of neurons are accompanied with intensive inflow of calcium ions and the formation of high amplitude of electric waves in glial cooperations [2]. Inflow of calcium ions leads to the activation of NO, which exhibits dual physiological properties: on one hand, NO activates guanylate cyclase and promotes recovery of the concentration gradient of ions; on the other hand, by conjugation with superoxide radical it generates a highly cytotoxic substance, namely peroxynitrite [3]. Peroxynitrite dissociates in acidic medium with formation of the strong oxidant, the hydroxyl radical [4-6]. The formation of relative long-living highly oxidizing agents, which have strong penetrant properties, can lead to damage of the cells and consequently to a 'structural footprint' of seizures. This mechanism largely determines neuron death of the CNS and leads to the development of neuropsychiatric disorders in diseases with epileptic syndrome [7].

Taken together, the potential neuroprotective role of anticonvulsants and their relation with reactive oxygen species (ROS), the NO system and the antioxidant thiol-disulfide system is of great interest. So, studying such activities of anticonvulsants is important in order to develop an effective strategy for the treatment of epilepsy. In the present study, the effects of 5 different anticonvulsants, lamotrigine, carbamazepine, topiramate, sodium valproate and gabapentin, on the level of NO concentration, NOS activity and parameters of the thiol-disulfide system in experimental epilepsy were evaluated.

MATERIALS AND METHODS

Animals

Experiments were carried out on a total of 70 female rats weighing 150-180 g. All animals were housed at standard conditions, with access to food and water *ad libitum* and natural day and night alteration. Rats were obtained from nursery of the Institute of Pharmacology and Toxicology, Academy of

Medical Sciences of Ukraine. All experimental procedures and operative interventions were done in accordance with WMA (World Medical Association) Statement on Animal Use in Biomedical Research.

Experimental procedure

Following drugs were tested: sodium valproate (Depakine 300 mg tablets, Sanofi Winthrop Industria, France) at the dose of 60 mg/kg, carbamazepine (Carbamazepine-Darnitsa 200 mg tablets, Darnitsa, Ukraine) at the dose of 125 mg/kg, topiramate (Topiramax 100 mg tablets, Pharma Start, Ukraine) at the dose of 100 mg/kg, gabapentin (Gabagama 300 mg capsules, Woerwag Pharma, Germany) at the dose of 50 mg/kg, lamotrigin (Lamictal 100 mg tablets, GlaxoSmithKline Pharmaceuticals, Poland) at the dose of 50 mg/kg.

The drugs were given daily for a total of 11 days intragastrically 60 min before daily administration of metrazole (Nizhpharm, Russia) at 40 mg/kg intraperitoneally. Metrazole kindling is one of the most widely used model for epilepsy [8].

Biochemical studies

Brain tissue of experimental animals were taken on the 12th day after beginning of the experiment. The cerebral sensorimotor cortex was used for biochemical investigation. Cerebral tissues were homogenized in ice-cold isotonic salt solution (0.15 M KCl) with the help of glass homogenizer (tissue:salt solution ratio was 1:10). Then, the cytosolic fraction was separated by means of differential centrifugation at 15,000g. Deprivation from proteins was done by 0.6° M $HClO_4$ solution and then the brain homogenates were further neutralized with 5° M solution of potassium carbonate [9].

Nitrotyrosine was determined in the cytosolic fraction of brain homogenate by solid phase immunosorbent method (ELISA, ELISA Kit, Hycult Biotech, The Netherlands) and expressed in nanomoles per gram of tissue [10]. Thiol-disulfide (SH)-groups were defined using the Ellman method modified by Sedlak and Lindsay [11]. Glutathione reductase (GR) activity was estimated by measuring the rate of NADPH oxidation, which was defined spectrophotometrically by decreasing of absorbance at 340 nm; quantity of oxidized and recovered forms of glutathione was assessed fluorometrically [12].

Histoimmunochemical studies

For histological studies the brain parts were fixed in Carnoy's fluid for 24 h and then were filled, following the normal scheme, in blocks by paraplast x100. Then, these blocks were used for preparation of 14° μ histological sections of sensorimotor cortex using Microtome Microm HM 235 (Germany).

For the assessment of inducible- and neuronal-NO-synthase (iNOS and nNOS) expression, histological brain sections were deparaffinized, rehydrated, thrice washed with phosphate buffer (pH 7.4) for 5 min and incubated with 2N hydrochloric acid solution for 30 min at 37°C. Then sections were washed twice with phosphate buffer for 5 min, twice with a borate buffer (pH 8.4) for 5 min, four times again with phosphate buffer (5 min), and incubated with 0.1% trypsin solution in phosphate buffer for 30 min (37°C). After incubation, sections were one more washed four times with phosphate buffer (5 min) and then incubated for 24 h in a humid chamber at 4-6°C with primary rabbit polyclonal antibodies IgC (1:500) for nNOS (Santa Cruz Biotechnology, USA). After incubation, sections were washed four times for 5 min with phosphate buffer and incubated for 1 h (37°C) with secondary goat antibody to fragment of rabbit IgG, conjugated to a fluorescent dye (Sigma-Aldrich). For assessment of iNOS expression, after incubation, sections were washed four times for 5 min with phosphate buffer and then incubated for 24 h in a humid chamber (4-6°C) with primary polyclonal antibodies (Santa Cruz Biotechnology, USA), conjugated to a fluorescent dye (Sigma-Aldrich).

After final washing with phosphate buffer (pH 7.4), sections were embedded in glycerol-phosphate buffer (9:1). Intensity of expression of NOS isoforms was determined by the density of iNOS- and nNOS-positive cells and bcl-2-immunopositive neurons of IV-V layers of the sensorimotor cortex sections using a fluorescent microscope (Axioskop, Zeiss, Germany). Obtained material was processed by a computer image analysis system (VIDAS-386, Kontron Elektronik, Germany).

Statistical analyses

Results of the study were processed using the StatPlus software. The data were represented as mean ± SEM. The control of distribution normality was done in accordance with Shapiro-Wilk criteria. The fidelity of differences between experimental groups was estimated with the help of Whitney-Mann U test. Statistical significance level was set as to be less than 0.05 [13]

RESULTS

Influence of studied drugs on iNOS and nNOS expression and nitrotyrosine content

The simulation of epilepsy via metrazole kindling (MK) led to increased expression of both iNOS and especially nNOS in the sensorimotor cortex of the animals (Table 1). Preventative administration of lamotrigine, larbamazepine, topiramate, sodium valproate or gabapentin mainly reversed these changes but the effects of anticonvulsants were different. Lamotrigine caused the highest decrease rates by 78% for the expression of nNOS (Table 1, Figure 1), and 35% for iNOS (Table 1, Figure 2). In addition, lamotigrine decreased the nitrotyrosine content for 58.9% in comparison to the control group. Other anticonvulsants showed less pronounced effects. Administration of carbamazepine, for example, almost didn't influence the nNOS expression and caused only small decrease for iNOS expression (3.7%) and nitrotyrosine content (5.6%).

Influence of studied drugs on markers of thiol-disulfide system

Metrazole kindling caused a shift of the thiol-disulfide system due to reducing its recovery intermediates, i.e. significant decreasing of cytosolic glutathione level and suppression of glutathione reductase activity (Table 2). Consequently, the oxidized glutathione/recovered glutathione (GSSG/GSH) increased (Figure 3). Administration of anticonvulsants parallel to MK, in most cases, caused normalization of the thiol-disulfide system by increasing the content of recovery thiol intermediates, level of cytosolic glutathione and glutathione reductase activity (Table 2). Again, the most pronounced effect was presented by lamotrigine that increased the content of recovery thiol intermediates and activity of glutathione reductase for 26.5 and 64.6%, respectively (Table 2). This anticonvulsant also normalized the oxidized/recovered glutathione ratio (Figure 3). Other anticonvulsants showed less pronounced effects. Interestingly, carbamazepine and sodium valproate exerted only weak effect on the thiol-disulfide system recovery and even increased the GSSG/GSH ratio (Figure 3).

Table 1. Influence of studied drugs on expression of inducible and neuronal NOS and nitrotyrosine content in the brain of experimental animals with MK-seizures

Groups	Density of nNOS- positive cells (cells/mm^2)	Density of iNOS- positive cells (cells/mm^2)	Nitrotyrosine (nmol/g)
Intact group	338.5 ± 21.6	187.6 ± 19.1	19.2 ± 1.2
Control group	695.5 ± 33.8	312.4 ± 20.5	51.6 ± 3.1
Metrazole + lamotrigin	389.5 ± 22.3*	201.1 ± 18.5*	21.2 ± 1.8*
Metrazole + sodium valproate	451.8 ± 47.2*	230.4 ± 18.1	33.7 ± 2.6*
Metrazole + topiramate	531.3 ± 42.3*	210.2 ± 11.4*	33.3 ± 2.2*
Metrazole + gabapentin	651.3 ± 42.3*	280.2 ± 21.4*	43.3 ± 3.4*
Metrazole + carbamazepine	697.5 ± 57.1*	300.7 ± 28.2	48.7 ± 4.2

*P < 0.05 compared with control group
(n = 10 per each group)

Table 2. Influence of studied drugs on markers of thiol-disulfide system in the brain of experimental animals with MK-seizures

Groups	SH-groups (μmol/g tissue)	GR (μmol/mg protein)	GSH (μmol/g tissue)	GSSG (μmol/g tissue)
Intact group	57.3 ±2.8	14.5 ± 0.82	4.73 ± 0.23	0.033 ± 0.008
Control group	38.4 ± 1.62	8.2 ± 0.64	2.12 ± 0.11	0.056 ± 0.002
Metrazole + lamotrigin	48.6 ± 2.18*	13.5 ± 0.68*	3.14 ± 0.22*	0.035 ± 0.005*
Metrazole + sodium valproate	40.4 ± 1.38	7.4 ± 0.91	1.93± 0.75	0.059 ± 0.003
Metrazole + topiramate	43.2 ± 2.11*	11.4 ± 0.71*	2.88 ± 0.75*	0.043 ± 0.001*
Metrazole + gabapentin	41.2 ± 3.48	8.7 ± 0.77	2.67 ± 0.23	0.052 ± 0.002
Metrazole + carbamazepine	40.7 ± 4.11	7.3 ± 0.64	2 ± 0.33	0.057 ± 0.002

*P < 0.05 compared with control group. **GR,** glutathione reductase; **GSH,** reduced glutathione; **GSSG,** oxidized glutathione.
(n = 10 per each group)

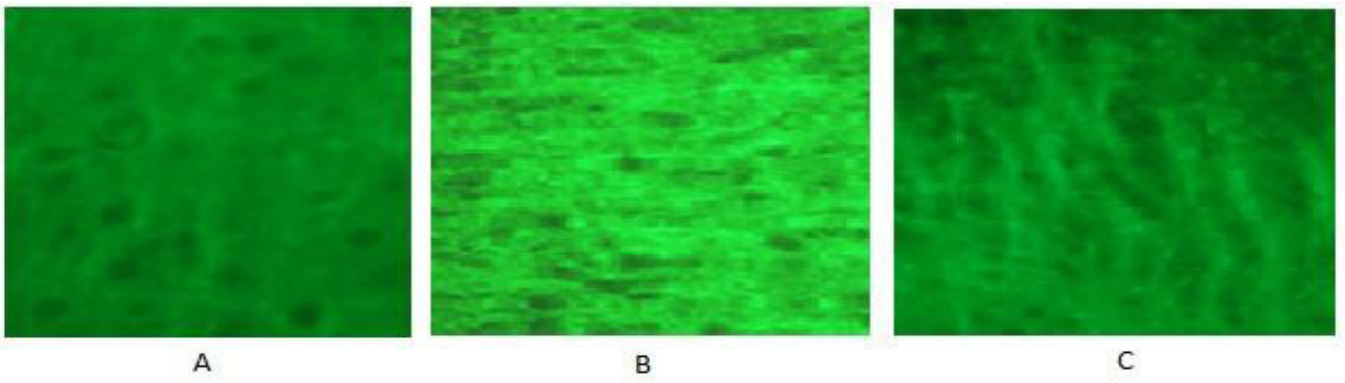

Figure 1. Expression of nNOS in neurons of rats with MK-seizures: **(A)** expression of nNOS in neurons of intact group animals; **(B)** expression of nNOS in neurons of control group animals; **(C)** expression of nNOS in neurons of animals after lamotrigine administration.

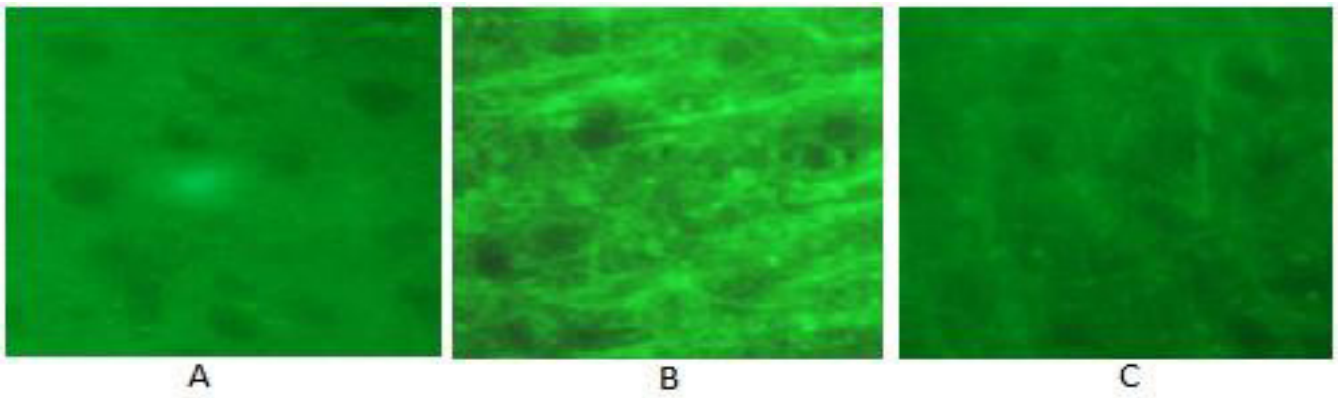

Figure 2. Expression of iNOS in neurons of rats with MK-seizures: **(A)** expression of iNOS in neurons of intact group animals; **(B)** expression of iNOS in neurons of control group animals; **(C)** expression of iNOS in neurons of animals after lamotrigine administration.

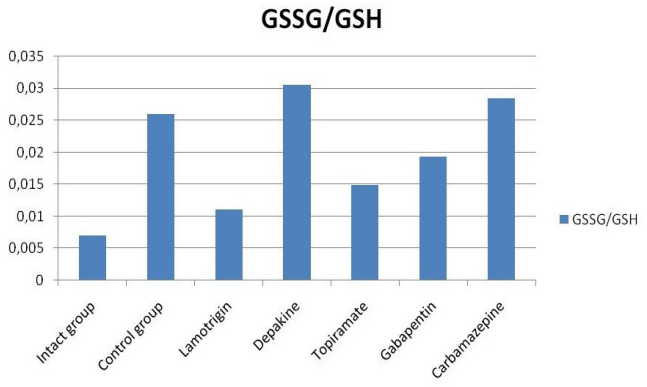

Figure 3. Influence of studied drugs on oxidized glutathione/recovered glutathione ratio.
(n = 10 per each group)

DISCUSSION

Shifts in the system of NO and recovered thiols reduced the bioavailability of NO and stimulated its conversion to peroxynitrite, which is confirmed by the increase of nitrotyrosine in the cytosolic fraction of brain homogenates in the present study. Our data are consistent with the results of other researchers, who had shown hyperproduction of NO and increased activity of NOS exist in rats' brain with MK [14]. Increased activity of NOS accompanied by deficit of L-arginine and deprivation of thiol-disulfide system leads to increase in reactive oxygen species (ROS) synthesis [15].

Increasing of nNOS and iNOS-positive cells density in the sensorimotor cortex of animals with MK confirmed that chronic administration of metrazole (40 mg/kg) increased the expression of nNOS and iNOS, and thus, participating in the nitrosating stress and neuronal damage. Concentration of NO produced by nNOS is not directly a factor which determines neuronal death; it is only sufficient for the activation of phospholipases, intensification of hydroxyl radical forming and modulating activity of N-methyl-D-aspartate (NMDA) receptors [16]. Hyperproduction of NO is caused by the participation of iNOS of glial cells, macrophages and neutrophils. Deferred nature of iNOS expression is associated with a later occurrence of activated astro- and macroglia and inflammatory cells.

No less important consequence of MK-induced seizures is the loss of such NO-mediated effects as suppression of cell proliferation, platelet aggregation and the oppression of the activation of monocytes [17]. The last mechanism is essential for the implementation of immune-inflammatory activation and initiation of NO-dependent mechanisms of apoptosis [18]; changes of expression of the two isoforms of NOS (neuronal and inducible) play important role in the development of the latter. Inducible NOS, unlike nNOS, does not require Ca^{2+} and calmodulin for its synthesis and produce NO in concentrations far exceeding those, which are produced under the influence of nNOS. Furthermore, in contrast to its neuronal and endothelial isoforms, iNOS is expressed only in pathological conditions, in response to activation of pro-inflammatory cytokines (particularly TNF-α). Active synthesis of iNOS is a key factor of hyperproduction of the same TNF-α (on a vicious circle) and other pro-inflammatory cytokines, increased formation of free radicals with subsequent damage and apoptosis of target tissues [19]. Nowadays, taking place an active studying of NO targets and clarification of the question, whether sufficient cytotoxic NO or thereof derivatives are more active. It is known that NO in target cells forms active derivatives, such as nitrosonium (NO^+), nitroxyl (NO^-) and peroxynitrite ($ONOO^-$).

Nitric oxide and its conversion products, such as peroxynitrite, the nitrosonium ion, nitroxyl and diazo trioxide (N_2O_3), are major factors of nitrosative stress, as a result of direct interaction of NO with metals (iron of heme in hemoglobin, myoglobin, iron-containing enzymes, iron-sulfur proteins and DNA, copper and zinc of enzymes active sites) and indirect interaction of nitrosonium with thiol, phenolic, hydroxyl and amino groups (S-, N-, O-nitrosation) of proteins and DNA [20]. Such interaction leads to damaging of receptor, to inhibition activity of mitochondrial enzymes and to the fragmentation of nucleic acids.

Inhibition of GR in conditions of convulsive seizures leads to oxidative modification of low molecular thiols and to the formation of homocysteine, that results in violation of transport NO and generation its cytotoxic derivatives, which further enhances the oxidation of thiols. Deprivation of thiol antioxidant system in neurons is the earliest cause of neurodestructive mechanism in terms of seizures, i.e. incoordination in the NO transport system and reduced resistance of cells to nitrosating stress. The most negative role in neurodestructive action of nitrosative stress plays peroxynitrite leading to nitrosylation of guanine and to DNA strand breaks.

By analyzing the present results of preventative administration of various anticonvulsants, drugs exerted different effects on the different isoforms of NOS. The most pronounced effect, which led to a decrease in the expression of the two isoforms of NOS, was observed in the group which received lamotrigine (Figures 1 and 2, Table 1). Administration of sodium valproate also caused a decrease in the expression of the NOS isoforms, but at less pronounced levels in comparison with lamotrigine. Topiramate caused deprivation of iNOS; gabapentin and, especially, carbamazepine induced moderate effects on the expression of the studied isoforms of NOS. The decreased expression of iNOS and nNOS under the action of anticonvulsants led to inhibition of nitrosative stress reactions, which were manifested as reduction of nitrotyrosine content in the cytosol fraction of brain homogenates of experimental animals one day after the seizures. According to the degree of reduction of the nitrosative stress marker nitrotyrosine, studied anticonvulsants can be arranged in the following order: lamotrigine-sodium valproate-topiramate-gabapentin-carbamazepine.

Decrease of iNOS and nNOS expression under the influence of the gamma-aminobutyric acid (GABA) agonists (sodium valproate and topiramate) might be related to the mechanism of $GABA_b$ receptor activation by anticonvulsants. $GABA_b$ receptor consists of two subunits, R1 and R2, and is able to reduce the expression and activity of nNOS. High activity of topiramate and, especially, lamotrigine by inhibition of nitrosative stress reactions in conditions of modeling seizures are not only associated with the suppression of nNOS and iNOS expression, but also with increase of the thiol-disulfide system activity. Thus, the prophylactic administration of lamotrigine and topiramate ensured the safety of the recovered equivalents of thiol-disulfide system (common thiols, glutathione). Intermediates of the thiol-disulfide system possess transport properties against NO, thereby enhancing its bioavailability. Moreover, glutathione, cysteine, methionine can significantly limit the cytotoxicity of NO and its derivatives, and thus, increase the chance of survival of a neuron at extreme conditions. Sodium valproate, carbamazepine and gabapentin exerted no significant effect on the thiol-disulfide system in rat brain after MK-seizures in the present study. On the contrary, sodium valproate caused activation of gamma-glutamyl transpeptidase, reduced glutathione reductase activity and decreased recovered glutathione level.

We found that the antioxidant effect of topiramate is realized by increased activity of glutathione-dependent antioxidant enzymes and increased levels of recovered thiols. Such effects of topiramate not only limits nitrosative stress damaging action on neurons in a convulsive fit, but also normalizes bioavailability of NO. Other authors also had identified the ROS and NO scavenger properties of topiramate; it was found that topiramate improved the of mitochondrial respiratory chain complex I and cortical neurons in seizures [20]. *In vitro* experiments in isolated rat brain mitochondria reflected activated oxidative phosphorylation and decreased mitochondrial damage with topiramate [19, 20].

In conclusion, the present results allowed to suggest that the NO system is one of the important links of anticonvulsants' action. Metrazole kindling is accompanied with a shift of thiol-disulfide system, due to reducing its recovery intermediates, with significant decline in the level of cytosolic glutathione and with suppression of GR activity. The most pronounced effect, that led to decrease in the expression of the two isoforms of NOS, was observed after lamotrigine administration. Lamotrigine also showed the most pronounced effect on decreasing of nitrosative stress reactions confirmed by reduction of nitrotyrosine content in the brain of experimental animals.

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Conflict of Interest: None declared