PHARMACOLOGY AND TOXICOLOGY

Mechanisms of Action of Ladasten: Activation of Gene Expression for Neurotrophins and Mitogen-Activated Kinases

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We studied the effects of single treatment with ladasten (50 mg/kg) on the content of effector kinases of the mitogen-activated cascade (ERK1/ERK2), pERK1/ERK2 activity (Thr202/Tyr204), and expression of genes for neurotrophic factors BDND and NGF in the striatum, hypothalamus, and hippocampus of rats.

Key Words: *ladasten; NGF; BDNF; gene expression; mitogen-activated kinases ERK1/2*

Ladasten (N-(2-adamantyl)-N-(*p*-bromophenyl)-amine) was synthesized at the V. V. Zakusov Institute of Pharmacology. Experimental and clinical studies showed that this compound has a well-balanced activity (stimulatory and selective anxiolytic effects) [3]. The pharmacological effect of ladasten is manifested in the increase in physical and mental activity, deceleration of fatigue, rapid recovery of activity, and improvement of mnesic performance and learning. Ladasten has a positive effect on cognitive functions (violated in somatogenic asthenia). This drug is effective in the therapy of asthenic depressions [2].

A large body of evidence exists that the effects of various psychopharmacological agents are related to the influence on neurotrophic factors and associated systems of intracellular signaling (*e.g.*, mitogen-activated signal cascades). Antidepressants that belong to a group of selective serotonin reuptake inhibitors (fluoxetine and reboxetine) increase the content of BDNF mRNA, BDNF protein, and TrkB receptor in the hippocampus [14]. Lithium and sodium valproate contribute to neurogenesis in the hippocampus of adult rats, which is associated with their effect on the expression of BDNF mRNA and BDNF protein and further activation of ERK signaling [6]. Moreover, typical (haloperidol) and atypical neuroleptics (clozapine, olanzapine, and risperidon) affect growth factors and their receptors (*e.g.*, modulate the expression of BDNF, neurotrophin-3, TrkB, and TrkC) in brain structures of rats. The neuroprotective effect of long-term treatment with atypical neuroleptics is partially mediated by neurotrophins [10].

Here we studied the effect of ladasten on gene expression for neurotrophins BDNF and NGF in various brain structures. We evaluated whether mitogen-activated protein-kinases (MAP-kinases) are involved in the mechanisms of intracellular signal transduction of ladasten.

MATERIALS AND METHODS

Experiments were performed on male outbred albino rats weighing 200-250 g. The animals were maintained

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in a laboratory vivarium under standard conditions. All manipulations were performed in accordance with the rules on studies with experimental animals. Ladasten was given in a single dose of 50 mg/kg (suspension in Tween 80).

Immunoblotting and hybridization with antibodies (Cell Signaling, Abcam) were conducted according to http://www.cellsignal.com/products/9191.html protocol. Densitometry and quantitative assay were performed by TotalLab. 2.0 software.

The relative content of ERK1/2 protein was standardized by β-tubulin concentration. Total RNA was isolated, cDNA was obtainedm, and real-time quantitative RT-PCR was conducted [1].

Experiments were performed in 3 biological repetitions (2-9 times). The results were analyzed by Student's *t* test. The mean and standard error of the mean were calculated. The differences were significant at $p<0.05$.

RESULTS

ERK1/2 content in the hypothalamus increased 0.5 h after ladasten administration (by 25-40% compared to the control), but returned to the basal level after 1.5 h (Fig. 1, *a*). Other changes were found in the amount of phosphorylated ERK1/2. A 70% increase in the content of phosphorylated ERK1/2 was observed 0.5 h after treatment. The content of pERK2 increased significantly after 1.5 h (by more than 7 times). The content of pERK1 increased less significantly during this period (by 4 times; Fig. 2, *a*). ERK1 content in the striatum increased slightly 0.5 after drug treatment. After 1.5 h the amount of ERK1 and ERK2 did not differ from the control (Fig. 1, *b*). Ladasten had a strong activating effect on pERK2 in the striatum, which was observed 0.5 and 1.5 h after treatment (Fig. 2, *b*). Ladasten had little effect on ERK1/2 content in the hippocampus (0.5-2.5 h; Fig. 1, *c*). Variations in

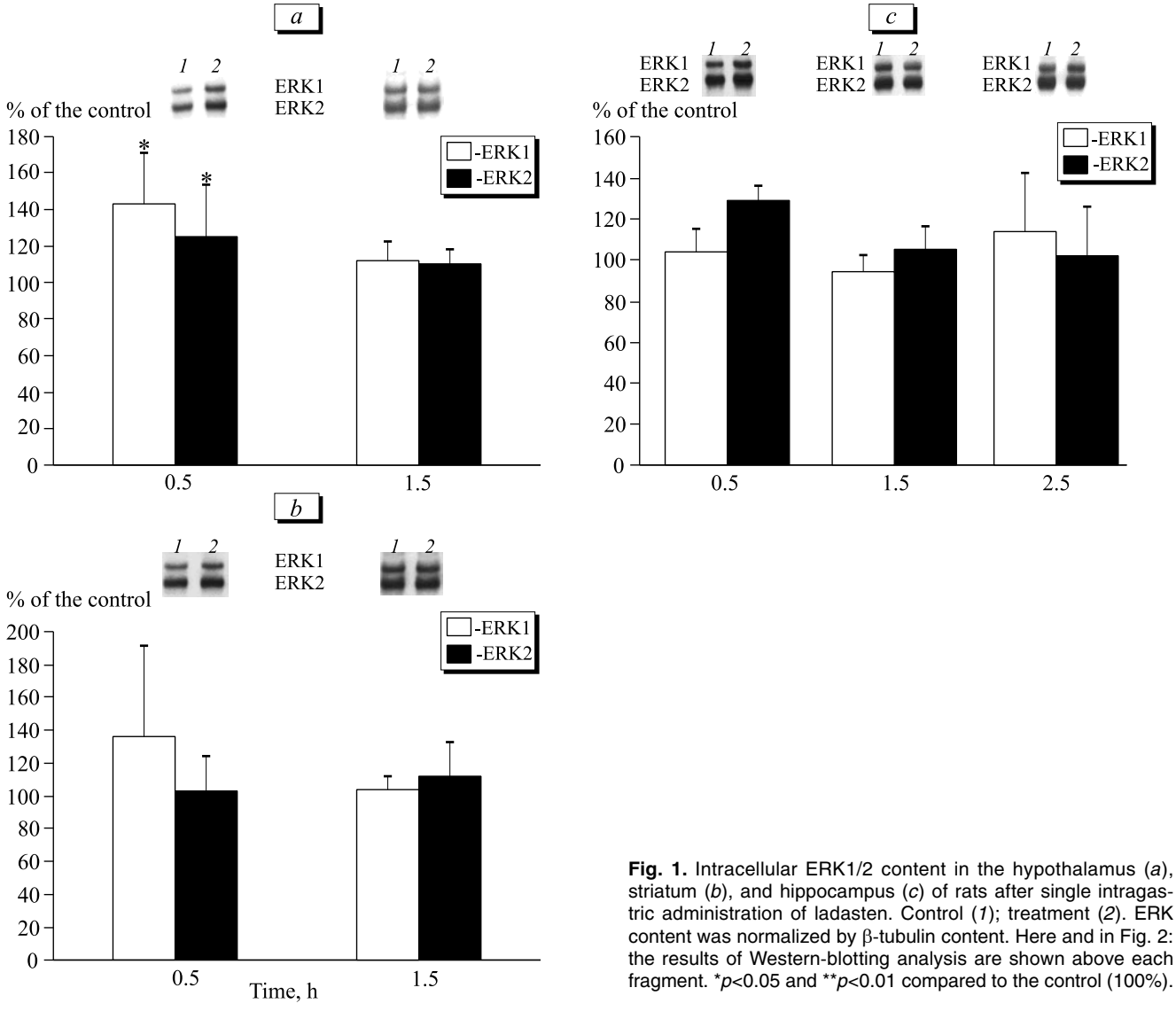

Fig. 1. Intracellular ERK1/2 content in the hypothalamus (*a*), striatum (*b*), and hippocampus (*c*) of rats after single intragastric administration of ladasten. Control (*1*); treatment (*2*). ERK content was normalized by β-tubulin content. Here and in Fig. 2: the results of Western-blotting analysis are shown above each fragment. *$p<0.05$ and **$p<0.01$ compared to the control (100%).

the content of pERK1/2 were most pronounced 0.5 and 1.5 h after ladasten administration (Fig. 2, *c*).

Analysis of the effect of ladasten on the concentration of mRNA for *ngf* and *bdnf* showed that ladasten inhibited transcription of *ngf* and *bdnf* genes in the striatum (0.5 h after treatment). A small, but significant increase in the expression of *ngf* and *bdnf* genes was observed in the hippocampus and hypothalamus, respectively (by 30%; Fig. 3, *a*). The content of *bdnf* mRNA decreased slightly 1.5 h after ladasten administration. The concentration of mRNA for this gene increased slightly in the hippocampus. The content of *bdnf* mRNA in the striatum increased by more than 2 times. However, the content of *ngf* mRNA remained unchanged under these conditions (Fig. 3, *b*).

Our results indicate that ladasten activates the Ras→Raf→MEK1/2→ERK1/2 cascade in brain structures of rats. Ladasten has a strong effect on phosphorylated forms of ERK1/2. It should be emphasized that the content of pERK1/2 in all structures of the brain was highest 1.5 h after ladasten treatment (Fig. 2). Activation of the RAS pathway in neurons is induced by tyrosine kinase receptors for growth factors, G protein-coupled receptors (D_2-D_4 receptors for dopamine), and Ca^{2+} influx through L channels and/or NMDA receptors. Protein kinases of Ca^{2+}- and cAMP-dependent signal cascades are involved in the regulation of ERK1/2 activity [11].

Activation of ERK1/2 and other protein kinases results in phosphorylation of various protein substrates in the cytoplasm and nucleus. They are presented by a variety of transcription factors, including CREB, c-Fos, c-Jun, c-Myc, Elk-1, TAL-1, C/EBP, and ATF-2 [5]. It can be suggested that the increase in pERK1/2 content under the influence of ladasten is associated with activation of protein kinases of cAMP- and Ca^{2+}-dependent signal cascades. MAP kinases mediate the effect of ladasten during the later

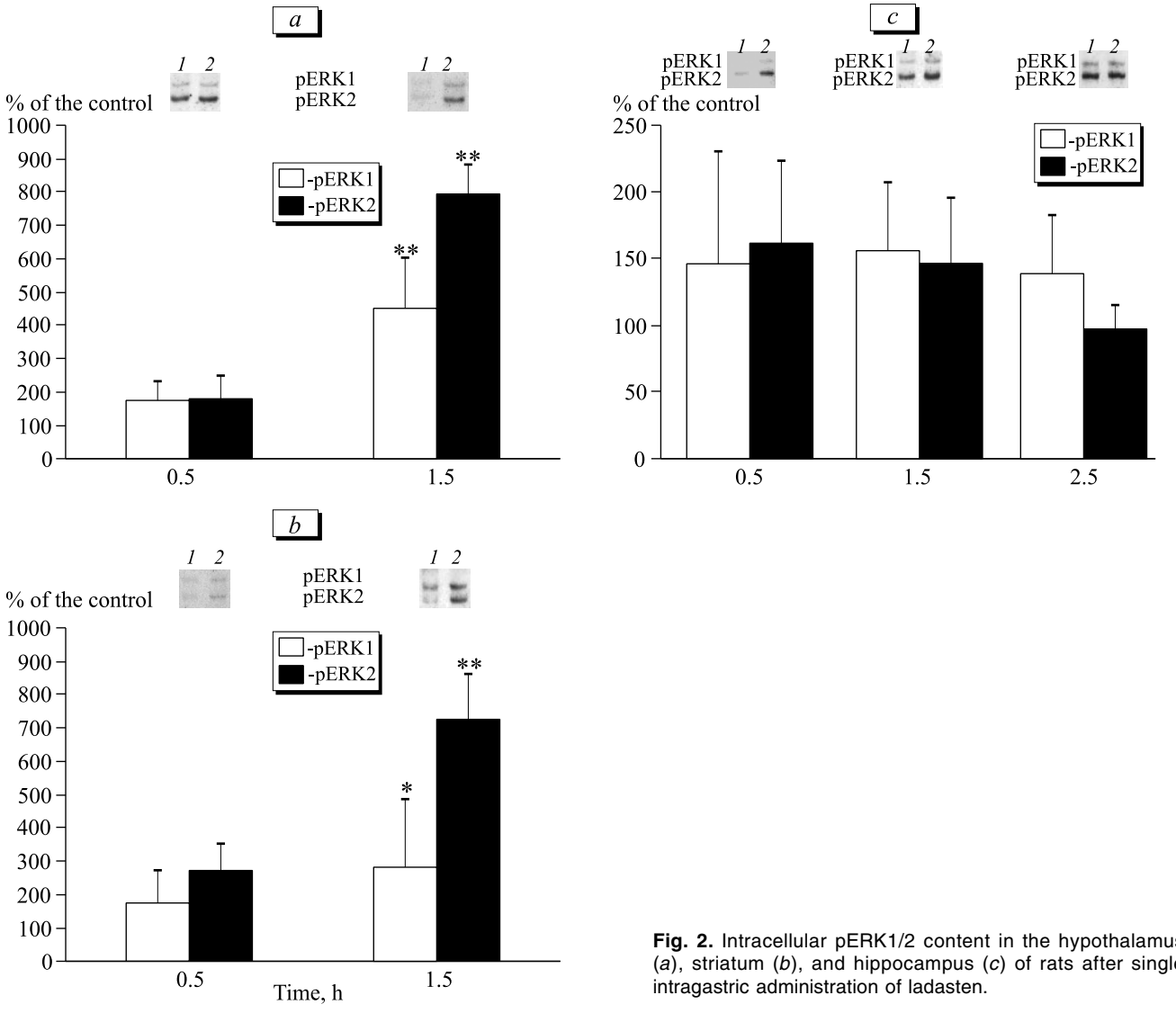

Fig. 2. Intracellular pERK1/2 content in the hypothalamus (*a*), striatum (*b*), and hippocampus (*c*) of rats after single intragastric administration of ladasten.

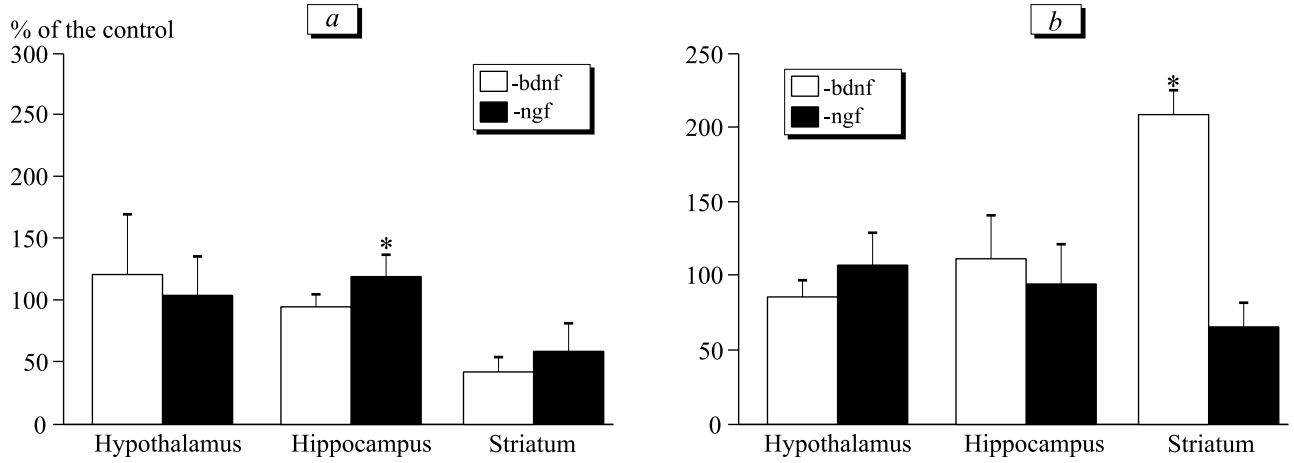

Fig. 3. Expression of mRNA for *bdnf* and *ngf* in the hypothalamus, hippocampus, and striatum of rats 0.5 (*a*) and 1.5 h (*b*) after single treatment with ladasten. *$p<0.05$ compared to the control (100%).

period (1.5 h after drug administration). Our results are consistent with the current concept that MAP-dependent pathways are relatively "slow". Activity of ERK1/2 kinases depends on signal transduction in the Ras→Raf→MEK1/2→ERK1/2 pathway. Each component of this chain is specifically regulated. Moreover, induction of each signal cascade occurs in various periods. These features contribute to differences in the kinetics of activation of transcription factors and, therefore, determine the temporal aspect for regulation of gene expression. For example, CREB phosphorylation by Ser133 is induced by CaMKIV. At the same time, the duration of CREB activation is determined by a further activation of the MAP cascade [13].

It should be emphasized that ERK isoforms in study structures of the brain are characterized by a selective activation. The content of pERK2 in the hypothalamus and striatum is higher than that of pERK1. The cause for differential regulation of ERK isoforms remains unknown. Published data show that stimulation of NMDA receptors and protein kinases A and C is followed by activation of ERK2 (but not of ERK1) in the hippocampus and cortical neurons [4]. It was hypothesized that the tissue-specific mechanisms of regulation are unique for each isoform of ERK.

Some authors believe that the influence of cocaine, morphine, and amphetamine or its derivatives is followed by activation of ERK kinases in mesolimbic structures of the brain (nucleus accumbens, dorsal striatum, amygdala, prefrontal cortex, and ventral tegmental area) [12]. The mechanism of ERK activation in the dorsal striatum under these conditions is associated with stimulation of D_1 receptors and NMDA receptors, protein kinase A-dependent phosphorylation of DARPP-32, and inhibition of protein phosphatase 1. Activation of ERK kinases in the nucleus accumbens after treatment with psychostimulant agents is a key factor for the induction of genes that belong to the families of *fos* (*c-fos*, *fosB*, and *fra2*), *Zif268*, and *Egr3*. These genes encode the corresponding transcription factors. Hence, activation of these genes regulates the expression of other genes that determine the cell response to stimulation and realization of pharmacological effects [8].

A comparative analysis of the results (Figs. 2 and 3) allowed us to reveal a correlation between *bdnf* gene expression and pERK1/2 content in the striatum. These data confirm our suggestion that ladasten treatment is followed by the growth factor-dependent induction of the MAP-dependent signal cascade (at least in this structure of the brain). Activation of the MAP kinase cascade in brain cells can be associated not only with the influence of neurotrophins on gene expression. This effect can be mediated by the ladasten-induced release of neurotransmitters (*e.g.*, dopamine). This assumption is confirmed by published data that the expression of *bdnf* mRNA in cultured astrocytes significantly increases after addition of dopamine at a dose of 150 mM [7]. *In vivo* experiments revealed that acute or chronic exposure to L-DOPA is followed by an increase in the expression of *c-fos* and *bdnf* genes in the subthalamic nuclei and striatum of rat brain [15]. These data illustrate an important role of monoamines in the regulation of *bdnf* expression.

Variations in the content of pERK1/2 kinases reflect "early involvement" and constant level of the MAP kinase-dependent signal cascade in rat brain cells after single treatment with ladasten. Ladasten has a positive effect on the regulation of neurotrophin gene expression. These data elucidate the mechanisms for psychotropic activity of ladasten (*e.g.*, nootropic activity). Recent studies showed that neurotrophins play a role in learning and appearance of memory engrams in the brain of mammals [9].

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