Stimulatory and Toxic Effects of Neurotransmitters on the *lux* Operon-Dependent Bioluminescence of *Escherichia coli* K12 TGI

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Abstract: *Background*: The normal functioning of the brain requires *neuromediators,* i.e., substances that transmit messages between nervous cells. Neurochemicals also function as signals that are involved in communication among the microorganisms that inhabit the human organism. While the impact of "classical" neurotransmitters including catecholamines, serotonin, and histamine on microorganisms has been investigated in a number of recent publications, this work provides evidence for the stimulatory and inhibitory (toxic) effects of some other important neurochemicals that have not received sufficient attention in the literature.

Methods: The biosensor was based on a GM *Escherichia coli* K12 strain (TGI) that contained the *lux* operon of the luminescent soil bacterium *Photorhabdus luminescencens* ZMI. The biosensor was exposed to the action of the tested neurotransmitters for 15 mins to 144 hrs. The intensity of bacterial luminescence (counts / second) was monitored in the control and the experimental samples with an 1251 BioOrbit luminometer (Finland).

Results: Neurochemicals such as putrescine, acetylcholine, taurin, and indole were found to stimulate, at low concentrations (0.1-10 μM), the luminescence of the strain *E. coli* K12 TGI containing the *lux* operon from *Photorhabdus luminescencens* ZMI. At higher concentrations, putrescine, taurin, and indole exerted a weak toxic influence, i.e. they marginally attenuated the luminescence of *E. coli* K12 TGI.

Conclusions: Based on the data obtained, a regulatory, presumably receptor-dependent, effect is exerted by the tested neurochemicals on the bacterium *E. coli* K12 TGI, in an analogy to their impact on nervous, immune, and other specialized types of eukaryotic cells. However, high neurochemical concentrations are likely to produce nonspecific effects on the bacterial luciferase system and/or on membrane phosphorylation.

Keywords: Biotesting, bacterial luminescence, toxicity, neurochemicals, biogenic amines, amino acids.

INTRODUCTION

The brain is the central regulator of human neuropsychological processes and social behavior. Its normal functioning requires *neurotransmitters,* i.e., substances that transmit messages between nervous cells. Research on the role of these neuroactive substances in intercellular communication and in the interaction between the microbiota and the host organism forms a part of the field called human microecology. The issue concerning the functional role of amine and amino acid neurotransmitters in microbial systems has recently been addressed in studies conducted by several research teams, including those of Prof. Mark Lyte (Iowa State University, USA), Prof. G. Fraikin and Prof. A. Oleskin (Faculty of Biology, Moscow State University) [1-6]. It was established that biogenic amines, such as catecholamines (norepinephrine, epinephrine, and dopamine) produce a strong stimulatory effect on the growth of a large number of microorganisms [7-10]. Interestingly, norepinephrine, as well as serotonin and histamine, stimulate cell aggregation, the initial stage of biofilm formation, in a non-pathogenic strain of *E. coli* [6]. However, the development of this non-pathogenic strain is more significantly stimulated by serotonin and especially by histamine than by catecholamines [5, 6]. In *Pseudomonas aeruginosa*, serotonin behaves as the pheromone of the QS-system *lasIR*. It enhances virulence and biofilm formation both *in vitro* and *in vivo* [11].

Microbial cells produce their own neurochemicals, which were detected by high-performance liquid chromatography in a variety of pro- and eukaryotic microorganisms. These measurements were originally performed during the late exponential growth phase of microbial cultures [12]. Subsequently, the growth dynamics of neurochemical synthesis and release into the medium was investigated in the bacteria *E. coli* and *Bacillus cereus,* as well as the yeast *Sacchromyces cerevisiae* [13-15]. Recently, similar studies on the intra- and extracellular concentrations of amine neurochemicals have been conducted in a number of lactobacilli and bifidobacteria [16-19].

Despite the data obtained, the role of neurochemicals in microorganisms has not been

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completely understood. Moreover, recent studies are mostly concerned with a limited number of neurochemicals as exemplified by catecholamines, serotonin, and histamine. However, it is imperative that other neuroactive substances, including ptomaines (amino acid decarboxylation products) that are currently considered important regulatory agents, should be assessed with respect to their microbial effects.

The human organism is a complex "superorganism" including numerous eukaryotic, eubacterial and archaean cells, and neurochemicals function as signals that are involved in communication among them. From the perspective of human microbial ecology, estimating the regulatory and toxic effects of neurochemicals on the representatives of the gastro-intestinal tract (GIT) microbiota and their *in vitro* analogs is of paramount importance in neurophysiological and medical terms.

In our previous paper, some of the most important ("major") neurotransmitters of the brain, such as dopamine, norepinephrine, serotonin, and histamine, were revealed to produce a toxic effect on the bioluminescence of the biosensor strain *Escherichia coli* K12 TGI that contained the *lux* operon of *Photorhabdus luminescencens* ZMI. This effect manifested itself at high concentrations of these neurotransmitters; the same neurotransmitters except norepinephrine were found to cause bioluminescence stimulation at low (micromolar) concentrations [20].

The present work addresses the microbial effects of some other ("minor") neurochemicals that have received less attention with regard to their effects on microorganisms.

Of special interest in this context is *acetylcholine (AChl).* This important neurotransmitter is an evolutionarily conserved chemical that fulfills a wide variety of functions in diverse forms of life [21]. In the brain, AChl fulfils functions associated with motivation, locomotive behaviour (particularly at the initial stage of a new movement), the formation of locomotive stereotypes, memorization, learning, plasticity, and the regulation of the wakeful state; this involves the reticular formation of the brainstem and basal ganglia.

Presumably, choline, the direct precursor of acetylcholine, was employed by unicellular organisms over one billion years ago: it was converted by them into membrane phospholipids, including phosphatidylcholine [22]. Subsequently, choline was incorporated in other metabolic pathways, and also became the "raw material" for the synthesis of acetylcholine. The evolutionary prehistory of this signal substance is also highlighted by the presence of acetylcholine receptors in unicellular eukaryotes and by its regulatory effect on the conjugation process in infusorians [23] and on the growth and proliferation of *Acanthamoeba* sp. that possesses a homologue of the neuronal muscarine receptor for AchI and can synthesize its own AChl [21, 24]. As for the role of AChl in prokaryotes, it is only known that AChl is synthesized by various bacteria, including bacilli and lactobacilli [25, 26].

Our work also addresses the effects of *putrescine* that is formed via enzymatic decarboxylation of amino acids, similar to other ptomaines (cadaverine and agmatine). Ptomaines are receiving increasing attention currently because they are considered potential hormones and/or neurochemicals. For instance, agmatine is likely to perform the function of a "non-classical" neurochemical that is synthesized in the brain, released upon membrane depolarization, and inactivated by agmatinase [27].

Some indirect data point to a possible neurochemical role of putrescine, which encourages research on its impact in microbial systems, including the human microbiota and symbiotic *E. coli* strains as its components. Nevertheless, the data on putrescine available in the literature are scanty. Putrescine together with cadaverine was detected in a number of bacterial strains that grow in pheasant meat [28]; putrescine is present in many kinds of wine [29].

The neuroactive amino acid *taurine* is regarded as a neurotransmitter in the literature. It is known to beneficially influence the visual sensory system and to promote athletic training. Taurine is involved in the functioning of insect nervous systems [30]. The data on its microbial effects are limited. The probiotic strain *Lactobacillus casei* K_3III_{24} releases its micromolar concentrations into the medium [17, 18]. The strain *L. brevis* BJ20 that grows on sea weed enriches the medium in taurine, along with other neuractive amino acids, such as glycine and β-alanine [31].

Finally, *indole* is of special interest because this chemical, the bicyclic backbone of the neurotransmitter serotonin, is produced by a large number of bacterial species, including those inhabiting the animal/human gut (*E. coli, Bacteroides ovatis,* and *Clostridium bifermentus*) [32]. In the gut, it is present at high concentrations (up to 0.6 mM) [33]. Indole inhibits biofilm formation in *E. coli* [34, 35] and, in contrast,

stimulates this process in *Pseudomonas aeruginosa* and *P. fluorescens* [36]. Indole accelerates the growth of *Salmonella enterica* var. *enteritidis* [37] and stimulates the formation of antibiotic-tolerant persister cells [38].

In the present work, it was established for the first time that low concentrations of the neurochemicals putrescine, acetylcholine, and taurine, as well as of the serotonin "backbone" indole, stimulate the luminescence of the strain *Escherichia coli* K12 TGI that contains the *lux* operon of *Photorhabdus luminescencens* ZMI. Higher concentrations of these neurochemicals produce a weak toxic effect: they marginally inhibit the luminescence of the tested strain.

MATERIALS AND METHODS

As pointed out in our previous publication [20], a widely used biological test for the primary assessment of the effects of chemicals, their mixtures, and physical factors is based upon bacterial luminescence [39-41]. Intact cells of luminescent bacteria are used in this test, and one of its advantages is that it enables estimating the response to the tested factor from luminescence intensity that is correlated with the bacterial cells' metabolic state. In this work, we used the genetically engineered strain *Escherichia coli* K12 TGI as the biosensor. Its luminescent phenotype was created by cloning the *lux* operon of the luminescent soil bacterium *Photorhabdus luminescencens* ZMI in this *E. coli* strain. The strain was obtained from the Microbiology Department of the Faculty of Biology, Moscow State University. It is known as the biosensor of the *Ecolyum-08* test system [41].

Growing and storing luminescent bacteria, which are generally non-pathogenic, requires conventional microbiological techniques. A convenient, fairly simple technique for the lyophilization of luminous bacteria was developed at the Department of Microbiology, Lomonosov Moscow State University.

Lyophilized bacteria are stored at + 4 °C for a long time (about a year). They represent a standard biosensor preparation, whose luminescence is measured after rehydration with cold (~ 4-12 °C) distilled water. Rehydrated pre-lyophilized biosensor cells are added to the experimental system immediately before the experiment.

The intensity of bacterial luminescence (counts / sec) was concomitantly monitored in the control and the experimental samples with a 1251 BioOrbit (Finland) Luminometer at room temperature (20—22 ^{o}C); the biosensor was exposed to the action of the tested neurotransmitters for a period of 0.5–144 hours.

The toxicity index (T) of the neurotransmitters was determined automatically by the luminometer that was programmed to use the formula:

T = 100 x (Ik - I) / Ik, where Ik and I are the illumination intensity of the control and the experimental samples, respectively. The tested neurotransmitters were classified into three groups according to their toxicity level: T <20, the agent is non-toxic; T >20 < 50, the agent is toxic; T> 50, the agent is very toxic [20]. In some studies, the stimulation of luminescence of the test organism occurred, and the T value was considered to be negative.

In this study, freeze-dried biosensor cells were rehydrated in cold distilled water for 30 minutes. The working suspension contained 2.3 x 10^7 cells/ml. The density of the bacterial suspension was determined nephelometrically (λ = 670 nm) with a KF77 photoelectrocolorimeter, and the number of cells per ml was determined by plotting a calibration curve.

Putrescine, taurine, acetylcholine, and indole (Sigma, USA) were used in this work; their concentrations were calculated based on neurotransmitter content in each of the chemicals.

To determine the bacterial luminescence of the aqueous solutions of the neurotransmitters, 0.1 ml of the bacterial biosensor suspension were applied to 1.5 ml flasks; 0.9 ml of sterile distilled water (pH 7.0) or of the sample solution was added to the control and the experimental flasks, respectively. The number of bacteria was determined with a KF77 photoelectrocolorimeter (Poland) at 590 nm based on a previously plotted standard curve.

The intensity of bacterial luminescence (counts/sec) was concomitantly monitored in the control and the experimental samples. The results of the studies were treated statistically. Three repeats of each experiment were conducted. The statistical validity of the differences between experimental and control values was assessed using the t-Student criterion.

RESULTS AND DISCUSSION

In the present work, the influence of the neurochemicals acetylcholine, putrescine, taurine, and indole on bacterial cell luminescence within the 10^{-8} – 10^{-4}M concentration range was investigated. The

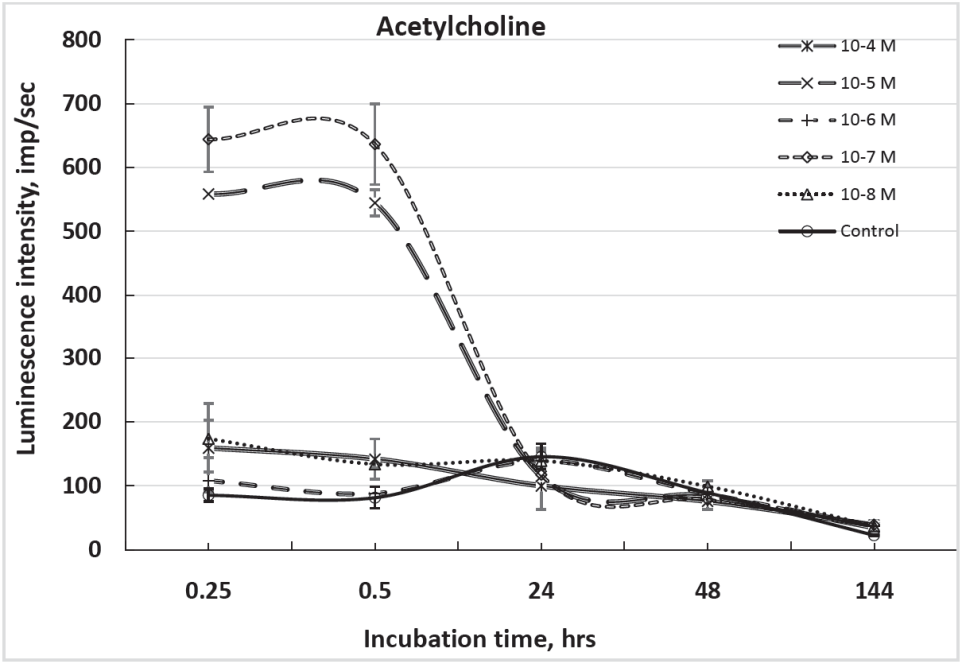

Figure 1: Effects of acetylcholine on the luminescence of the strain *E. coli* K12 TGI.

tested neurochemicals exerted both stimulatory and inhibitory effects on the luminescence of *E. coli* TGI cells.

Acetylcholine (Figure **1**) produced a significant stimulatory effect on bacterial luminescence, an integrated metabolic activity indicator, at low concentrations (10^{-7}-10^{-6} M) and with short incubation times (15-30 min), which is likely to be due to its regulatory, presumably receptor-dependent, influence on cell metabolism. Interestingly, higher AChl concentrations were almost without effect in this system (receptor oversaturation?). However, incubating with AChl for a longer time resulted in slightly inhibiting bacterial luminescence.

A very similar pattern was characteristic of the effect of *taurine* (Figure **2**). The low concentrations that efficiently stimulated luminescence were within the 10^{-8}-10^{-7} M range. After longer incubation times, a marginal inhibitory effect of taurine was observed.

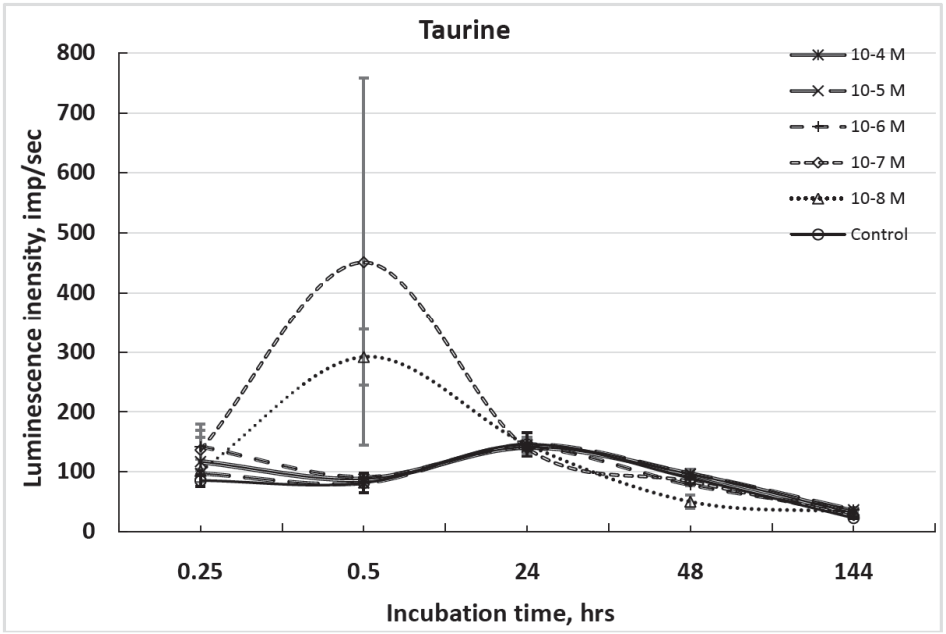

Figure 2: Effects of taurine on the luminescence of the strain E. *coli* K12 TGI.

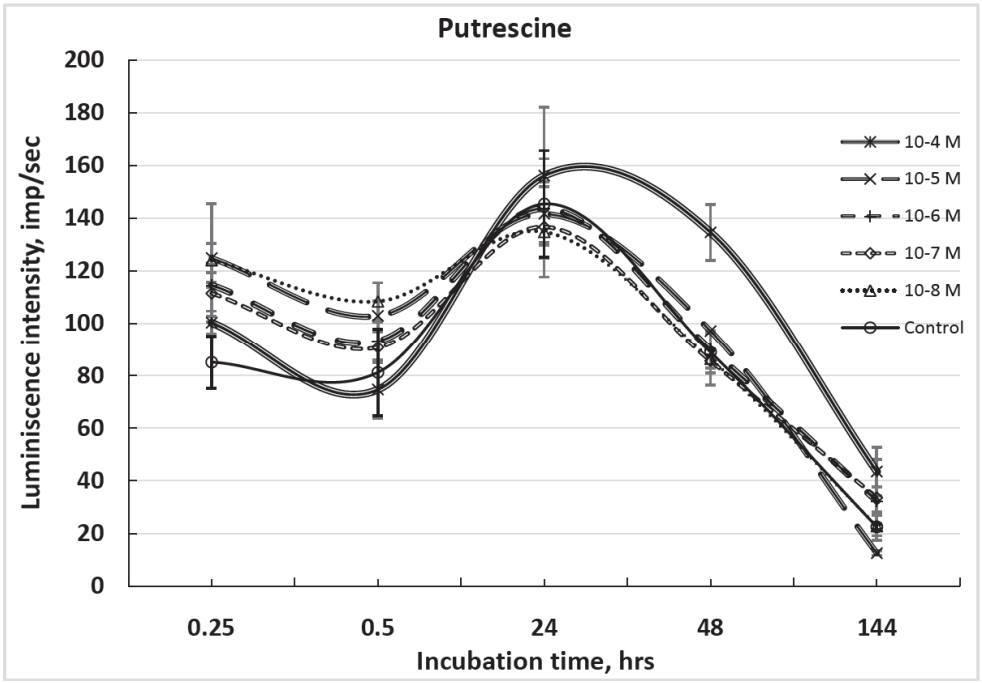

Figure 3: Effects of putrescine on the luminescence of the strain *E. coli* K12 TGI.

Putrescine significantly stimulated bacterial luminescence not only at low (10^{-7}-10^{-6}M) but also at maximally high concentrations (10^{-4}M); in contrast to AChl and taurine, the stimulatory effect increased with an increase in incubation time (Figure **3**).

Indole was an efficient luminescence stimulator after 15-30 min of incubating *E. coli* TGI cells with it. However, a longer period of incubation resulted in luminescence inhibition. Plausibly, such transient luminescence stimulation ("negative toxicity") enables potentially pathogenic *E. coli* strains, e.g., O157:H7, to quit an area with a high indole concentration before the onset of the toxic effect (Figure **4**). This may be the mechanism used by the symbiotic indole-producing microbiota of the gut to oust opportunistic pathogens. It is relevant that the intestinal microbiota normally produces indole concentrations as high as 0.6 mM, which might contribute to its protective action in the gut.

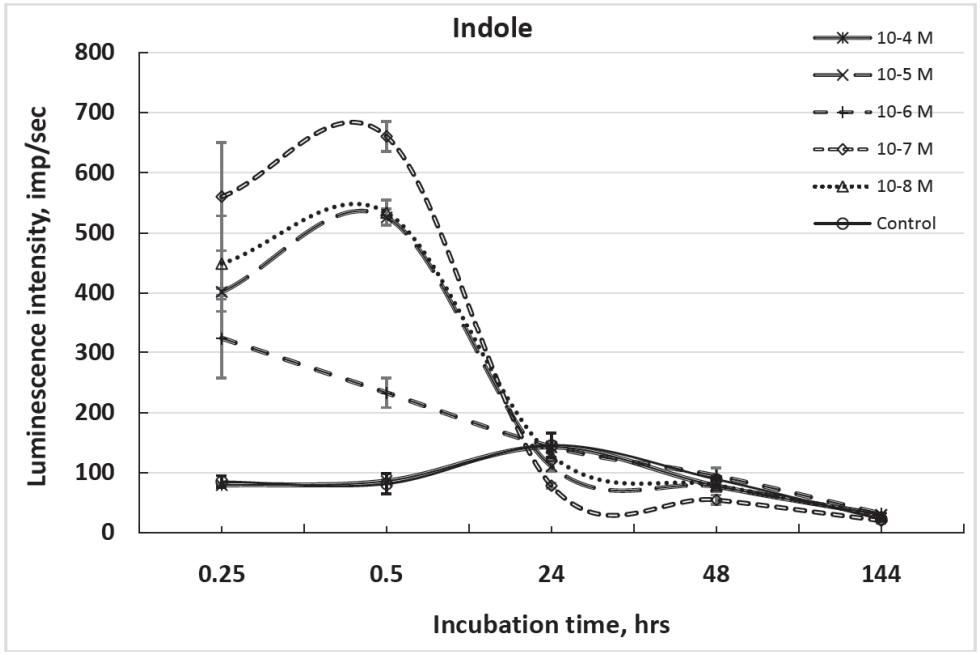

Figure 4: Effects of indole on the luminescence of the strain *E. coli* K12 TGI.

Table 1: Toxicity Index of the Tested Neurotransmitters

Neurotransmitters	Toxicity index (1μM)		Toxicity index (10 μM)		Toxicity index (100 μM)	
	30 min	48 hours	30 min	48 hours	30 min	48 hours
Acetylcholine	-23.8	4.2	-92.6	12.3	-86.9	13.9
Taurine	-60.0	6.1	-66.7	12.0	-37.1	14.6
Putrescine	-31.0	3.8	-34.7	-1.3	-17.9	-50.6
Indole	-279.1	-6.47	-557.8	30.5	6.8	12.4

The above plots demonstrate that all the four tested substances exhibit significant toxicity, i.e., suppress luminescence by over 20%, after a long incubation period. The toxic effect develops in time and fully manifests itself after 48 hours with indole and after 24 with AChl and taurine. Putrescine is significantly toxic only at a concentration of 10^{-5}M and after the maximum incubation time (144 hs). Overall, the stimulatory effect is stronger than the toxic effect. To re-iterate, the stimulatory effect manifests itself at low concentrations, which seems to suggest a regulatory (receptor-dependent) mechanism of action of the tested chemicals (Table **1**).

From the table, it is evident that all the tested neurotransmitters caused the stimulation of bioluminescence after 30 min of incubation, i.e., the toxicity was negative. After 48 hs, all the substances developed slight positive toxicity. The error in all repeats did not exceed 5%. The most pronounced toxic effect was exerted by indole.

It should be emphasized that both the stimulatory and the toxic effect can be conveniently measured with the bioluminescent strain *E. coli* K12 TGI containing the *lux* operon of *Photorhabdus luminescencens* ZMI. The technique of assessing the toxicity of neuroactive compounds with respect to micro-ecosystem components actually forms a part of modern *bio-estimation* technology. In general terms, bio-estimation is aimed at diagnosing the effects of detrimental factors and normalizing the functioning of ecosystems as well as predicting the future dynamics of these effects on the ecosystems under study.

Many neurochemicals stimulate the growth of a wide range of prokaryotic species. These stimulatory effects are based on specific interactions. For instance, catecholamines bind to receptors QseB and QseC in *E. coli* (reviewed, [42]). These functional analogs of eukaryotic adrenoreceptors form a part bacterial quorum sensing systems.

Indole, apart from inhibiting potentially pathogenic microorganisms in the gut, exerts an influence on the entero-endocrine cells (EECs) of the intestinal epithelium. Indole induces EECs to produce the appetite-suppressing glucagon-like peptide (GLP-1). Owing to its similarity both to serotonin and to melatonin, indole produces the soporific effect on humans that is also exerted by high concentrations of these two chemicals [43].

The results of the experiments with the biosensor based on the *lux* operon-containing *E. coli* strain demonstrate that the effects of the neurochemicals can be assessed using standard biological tests. Therefore, it is to be hoped that employing such tests in basic and applied research on the role of neurochemicals in natural and human-made biological systems will help us obtain theoretically and practically (industrially) important data. Since stimulatory effects are predominantly characteristic of relatively low concentrations of the neuroactive substances, we suggest a regulatory (receptor-dependent) mode of action of the tested neurochemicals on *E. coli* K12 TGI, in an analogy to their impact on eukaryotic cells, including specialized nervous and immune cells. Presumably, high neurochemical concentrations exert nonspecific effects that may affect the luciferase enzyme system and/or result in uncoupling membrane phosphorylation. The second option is possible because the molecules of the tested neurochemicals contain ionizable groups in the vicinity of aromatic structures. This facilitates the transfer of electric charges and/or hydrogen ions across energy-transducing membranes and the dissipation of the membrane potential.

Taken together, the results of this work indicate that the bacterial biosensor can be used to estimate the toxicity level (or, alternatively, the stimulatory effect) of the tested neurochemicals. Moreover, the data suggest that these neurotransmitters, at low (physiological) concentrations, function as sufficiently strong

stimulators of the metabolism of the tested prokaryote and, presumably, produce specific, receptor-dependent, effect on its cells. Therefore, they are likely to form a part of the evolutionary conserved chemical "language" invented by living nature over a billion years ago, as the biosphere was exclusively composed of microorganisms.

CONFLICT OF INTEREST DISCLOSURES

The authors do not have any conflicts of interest.

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