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EFFECT OF NITRIC OXIDE DONOR SNAP ON GABA RELEASE FROM RAT BRAIN NERVE TERMINALS

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In this work we investigated the effect of nanomolar concentrations of nitric oxide on the release of gamma-aminobutyric acid (GABA) from rat brain nerve terminals using a radioisotope method with [^{3}H]GABA and a spectrofluorimetric method with Ca^{2+}-sensitive probe Fluo-4 AM. It was shown that in the presence of dithiothreitol (DTT), nitric oxide donor SNAP at concentration, in which it produces NO in the nanomolar range, caused Ca^{2+}-independent [^{3}H]GABA release from nerve terminals. The applications of 4-aminopyridine (4-AP) and nipecotic acid (NA), as the inducers of GABA release from vesicular and cytoplasmic pools, showed that the maximum of SNAP/+DTT-induced [^{3}H]GABA release was registered at 10^{th} min of incubation and coincided in time with significant increase (almost double) in NA-induced [^{3}H]GABA release. At this time point, 4-AP-induced release of [^{3}H]GABA was drastically reduced. At the 15^{th} min of incubation of nerve terminals with SNAP/+DTT, the opposite picture was observed: the decrease in NA- and increase in 4-AP-induced [^{3}H]GABA release. Thus, nitric oxide in the form of S-nitrosothiols at nanomolar concentrations causes Ca^{2+}-independent GABA leakage from synaptic vesicles into cytosol with subsequent release from nerve terminals. The reuptake of the neurotransmitter and its re-accumulation in synaptic vesicles occur later.

K e y w o r d s: nitric oxide, GABA release, vesicular and cytoplasmic pool.

Nitric oxide (NO) is a unique signalling molecule that is involved in key body's vital processes. In the central nervous system, nitric oxide modulates the efficiency of synaptic transmission and affects the formation of prolonged connections between neurons that underlies the cognitive brain functions: memory and learning. The physiological function of NO in brain is closely related to its ability to modulate the release of neurotransmitters. Freely diffusing into the presynaptic terminal, nitric oxide affects the release of almost all known classes of neurotransmitters, exhibiting both stimulating [1] and inhibitory [2] effects on neurotransmission. Some authors believe that NO acting on the presynapse exhibits multidirectional effect, namely reducing the stimulated release of neurotransmitters and, at the same time, increasing the spontaneous release of neurotransmitters [3].

GABA is one of the key inhibitory neurotransmitters, which controls neural tissue excitability and affects high integrative brain functions. *In vivo* GABAergic neurotransmission can be modulated by nitric oxide released from NO-containing drugs, glial cells or postsynaptic nerve terminals due to the activation of, for example, postsynaptic NMDA-type glutamate receptors [4]. Concerning the latter, NO acts as a retrograde messenger that diffuses into presynaptic terminals and modulates neurotransmitter release. It was shown that the release of GABA from nerve terminals can occur via the classical pathway, exocytosis [5], as well as via plasma membrane GABA transporter reversal [6]. There is also an opinion that NO, affecting the GABAergic terminals, engages both pathways: inhibiting exocytosis and stimulating the transporter-mediated GABA release [7].

Ambiguity and sometimes contradictory data on the effect of NO on the release of neurotransmitters can be a result from different redox-status of NO molecule ($NO^{\bullet}$, NO^{-} or NO^{+}) of nitric oxide donors generally used in experiments [8]. Therefore, the effects of NO can be mediated by activation of different intracellular signaling pathways, in particular, S-nitrosylation of neuronal protein thiol groups [9] or activation of soluble guanylate cyclase [10]. Concentration of nitric oxide also plays an important role, since at its high concentration mechanisms of oxidative stress, mitochondrial dysfunction and excitotoxicity are activated [11].

Given the aforementioned, the aim of this work was to study the effect of low (nanomolar) concentrations of nitric oxide on GABA release from

isolated rat brain nerve terminals (synaptosomes). As the NO donor, we used SNAP (S-nitroso-N-acetylpenicillamine), which belongs to the class of natural metabolite of nitric oxide. Biological effect of S-nitrosothiols is mediated via direct effect of NO molecules spontaneously released due to homolytic S-N bond cleavage, as well as via a transnitrosation reaction – nitrosonium ion (NO^+) transfer to thiol-containing compounds (peptides, enzymes, etc.). In the range of SNAP concentration from 0.1 to 1 mM, the concentration of free NO is found to be in the nanomolar range from 20 to 100 nM [12].

Materials and Methods

All animal experiments were performed in accordance with the rules and regulations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986). Male white Wistar rats weighing 150-200 g were used in the study. Animals were maintained in standard cages under natural light conditions on standard rodent diet with free access to food and water. In the experimental day, the animals were decapitated under chloroform anesthesia, after that, hippocampus and cerebral cortex were immediately isolated and homogenized in cool isolation medium consisting of: 0.32 M sucrose, 0.2 mM EGTA, 5 mM HEPES, pH 7.4 using glass Poter homogenizer (clearance 0.2 mm). Purified fraction of isolated nerve terminals (synaptosomes) were prepared using Cotman method [13], by suspending precipitate after the last centrifugation in standard cool oxygenated incubation medium composed of (mM): NaCl – 126, KCl – 5, $CaCl_2$ – 1, $MgCl_2$ – 1.4, NaH_2PO_4 – 1, HEPES – 20 (pH 7.4), d-glucose – 10. All the procedures were carried out at 0-4 ºC. Protein concentration was determined by the Lowry method in Larson modifications [14].

GABA release from synaptosomes was determined using radioisotope method with tritiated GABA (5×10^{-7} M). To prevent degradation of neurotransmitter, the experiments were carried out in the presence of aminooxyacetic acid (100 μM), GABA-transaminase inhibitor. The [^{3}H]GABA release from synaptosomes without adding stimulating agents was taken as the basal release. SNAP-induced release was calculated as the difference between the [^{3}H]GABA release in the presence of SNAP and the basal release. GABA release from vesicular pool was induced by addition of 4-aminopyridine (4-AP) and determined as difference between 4-AP-induced [^{3}H]GABA release from control synaptosomes and synaptosomes preincubated with SNAP. GABA release from cytoplasm pool was induced by nipecotic acid (NA). 4-AP/NA was added to synaptosomes at different time points their incubation with SNAP, and after 5 min the aliquots were centrifuged at 12 000 rpm for 15 sec and the supernatants were collected. The content of [^{3}H]GABA in the supernatant was measured using a liquid scintillation counter Tracor Analytic Delta 300 with ACS scintillation liquid, and was expressed as a percentage of total [^{3}H]GABA in synaptosomes. Results are presented as mean ± SEM.

Relative changes of ionized calcium levels within synaptosomes were assessed using fluorescent Ca^{2+}-sensitive probe Fluo-4 AM according to standard procedure. Kinetic measurements were performed using a spectrofluorometer QuantaMaster 40 (PTI, FelixGX-4.1.0) at λ_{ex} = 496 nm and λ_{em} = 518 nm. Criteria for synaptosome functionality were their responses to application of calcium ionophore A 23187 (final concentration 5 μM) and KCl (final concentration 30 mM). Statistical data processing and graphing were performed using Microsoft Origin Pro 9.0 software. Statistical significance was calculated using ANOVA with Bonferroni correction for multiple comparisons. The differences were considered significant at $P < 0.05 / n$ (where n is the number of determined parameters).

The following reagents were used in the experiments: [^{3}H]GABA (94 Ci/mol), ASC scintillation liquid (Amersham, UK), 4-aminopyridine (RBI, USA), SNAP (S-nitroso-N-acetylpenicillamine), dithiothreitol, nipecotic acid, Ficoll-400, aminooxyacetate, d-glucose, HEPES (Sigma, USA), Fluo-4 AM (Invitrogen, USA) and other reagents (Reachim, Ukraine).

Results and Discussion

The first step in our study of the effect of nitric oxide on GABAergic nerve terminals was time measurements of the amount of radiolabeled GABA released from synaptosomes during their incubation with SNAP. As it can be seen (Fig. 1, *A*), 10 min after addition of SNAP (100 μM) to [^{3}H]GABA-loaded synaptosome, the amount of the extrasynaptosomal neurotransmitter (minus basal release) was insignificant (1.1 ± 0.52%). This indicates that SNAP itself, which at this concentration spontaneously releases NO in a concentration of several nM [15], had little effect on the inhibitory neurotransmission. Howe-

ver, the picture drastically changed in the presence of 1 mM DTT in the incubation medium. According to the literature and our data, the biological effect of S-nitrosothiols, including SNAP, is significantly enhanced in the presence of low molecular mass thiols that is associated with the formation of the highly reactive intermediates and additional NO release due to fast SNAP cleavage [15, 16]. As it is seen in Fig. 1, the amount of [^{3}H]GABA released from the synaptosomes at the 5th min of the SNAP action was 5.19 ± 0.52%, and at the 10th min of incubation, this value increased to 8.05 ± 0.24%. However, further elevation in the extrasynaptosomal [^{3}H]GABA level was not observed, and, on the contrary, at the 15th min of incubation the level of [^{3}H]GABA sharply dropped to almost 1.19 ± 0.78% that is likely to reflect the dynamic [^{3}H]GABA uptake into synaptosomes. The assumption about the mechanisms for this SNAP/+DTT effect will be made later. It should be only noted that DTT itself stabilized presynaptic terminals reducing the basal release of radiolabeled neurotransmitter (Fig. 1, the second column).

SNAP/+DTT-induced release of [^{3}H]GABA was found to be insensitive to the absence of calcium ions in the incubation medium (Fig. 1, *B*), indicating that the release does not occur due to classical synchronous exocytosis. Ca^{2+}-independent neurotransmitter release under the action of NO has been described previously [10, 17], and can be a consequence of the involvement of intracellular calcium stores and activation of spontaneous GABA release. The spontaneous process, unlike synchronous, is believed to occur asynchronously and triggered by calcium ions, which are released from intracellular stores into cytosol [18]. Thus, the next step of the study was to clarify the involvement of intracellular calcium stores in SNAP/+DTT-induced [^{3}H]GABA release.

Relative changes in the concentration of ionized calcium inside synaptosomes $[Ca^{2+}]_{in}$ were assessed using a fluorescent probe Fluo-4 AM, which after cleavage by intracellular esterases and binding with calcium ions emits in the green spectral band. As it is shown in Fig. 2, *A*, addition of SNAP (100 μM) to a suspension of synaptosomes preincubated with DTT (1 mM) in Ca^{2+}-free medium (+0.1 mM EGTA) was not accompanied by a change in Fluo-4 fluorescence that indicated a lack of increase in $[Ca^{2+}]_{in}$ upon action of nanomolar concentrations of SNAP. However, the addition to synaptosomes of Ca^{2+}-ionophore A-23187 (5 μM) led to a fluorescence burst due to an increase in the concentration of calcium released from intracellular store – mitochondria and endoplasmic reticulum. Other criteria of synaptosome functionality was the application of KCl (30 mM), which, as expected, did not affect the fluorescence intensity of Fluo-4 in Ca^{2+}-free medium (Fig. 2, *A*), however caused a sharp increase in fluorescence in the presence of 1 mM Ca^{2+} (Fig. 2, *B*). The latter is related to the influx of extrasynaptosomal calcium into synaptosomes through potential-regulated channels due to KCl-induced plasma membrane depolarization. It should be noted that the addition of 100 μM SNAP/+DTT to synaptosomes in the Ca^{2+}-containing medium also did not affect the calcium concentration in synaptosomes (Fig. 2, *B*), and this allows us to hypothesize that SNAP/+DTT-

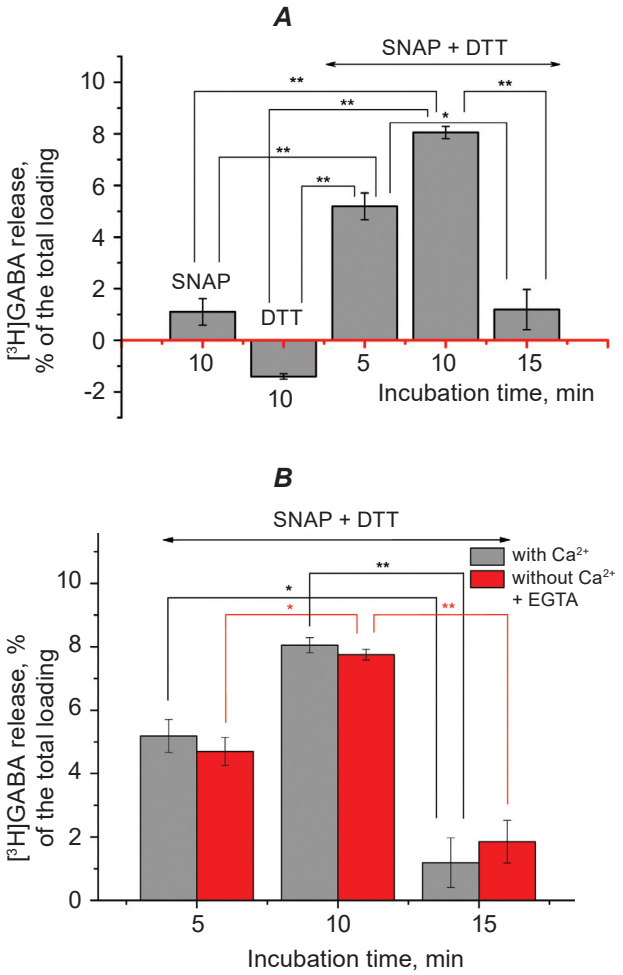

*Fig. 1. [^{3}H]GABA release from synaptosomes under the action of SNAP (100 μM) and/or DTT (1 mM) in the Ca^{2+}-containing (A) and Ca^{2+}-free/+0.1 mM EGTA (B) incubation medium; mean ± SEM, n = 4, * P < 0.05; ** P < 0.01*

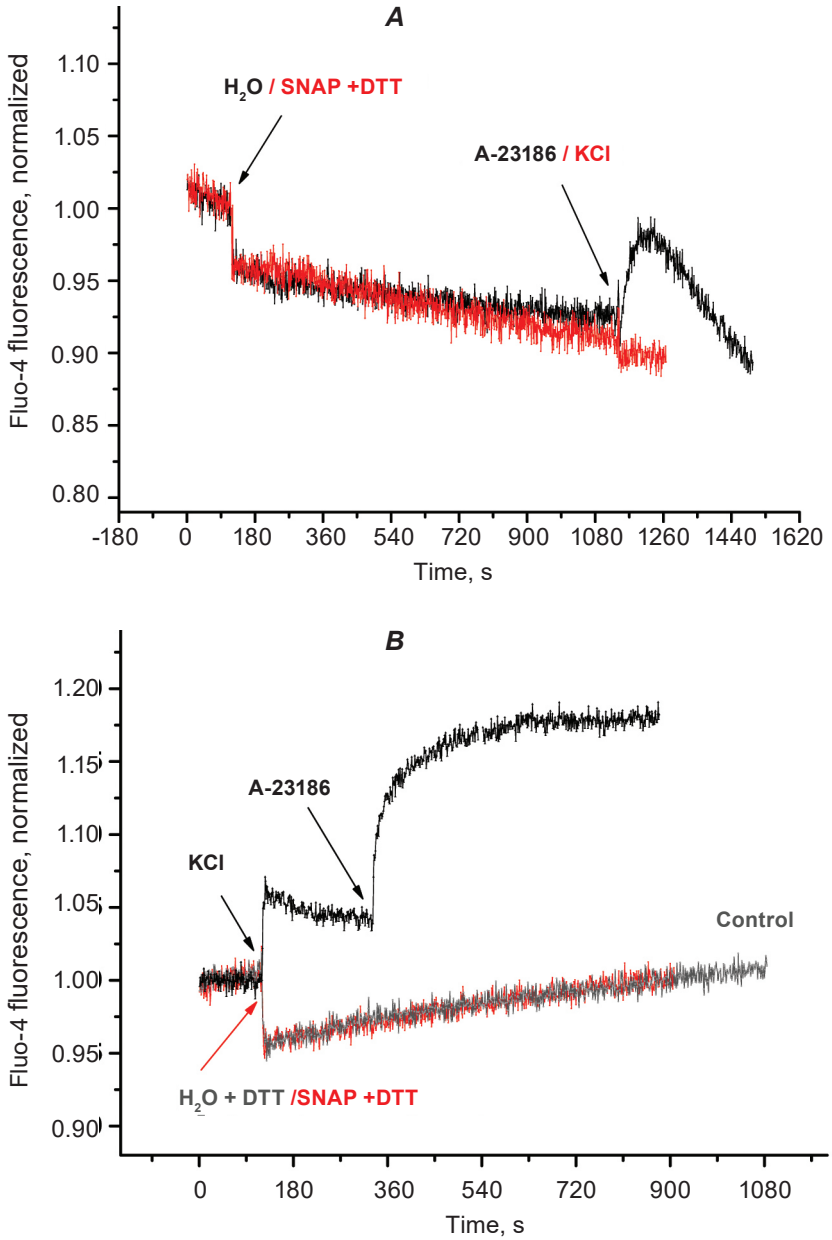

Fig. 2. Relative changes in ionized calcium level inside synaptosomes under the action of SNAP/+DTT (100 μM/1 mM) in Ca^{2+}-free /+0.1 mM EGTA (A) and Ca^{2+}-containing (B) incubation medium. Responses to the addition of KCl (30 mM) and A 23187 (5 μM) served as the criteria of synaptosome functionality. The typical curves are demonstrated

induced [^{3}H]GABA release does not occur by exocytosis (spontaneous and/or synchronous), but rather by engagement of other mechanisms as, fot instance, transporter-mediated.

To check this assumption, we carried out a series of experiments aimed to find out from which pools, vesicular and/or cytoplasmic, GABA is released under the action of SNAP. To this, we used potassium channel blocker 4-AP, which induces Ca^{2+}-dependent GABA release predominantly from vesicular pool [19], and NA, which, having the higher affinity to GABA transporters than GABA, is captured by transporters and transferred into cells in exchange for "cytoplasmic" GABA [20]. As it is seen in Fig. 3 (red columns), the addition of 4-AP (2 mM) to synaptosomes at the 5th and 10th min of their incubation with SNAP/+DTT (100 μM/1 mM) was accompanied by a gradual decrease in the 4-AP-

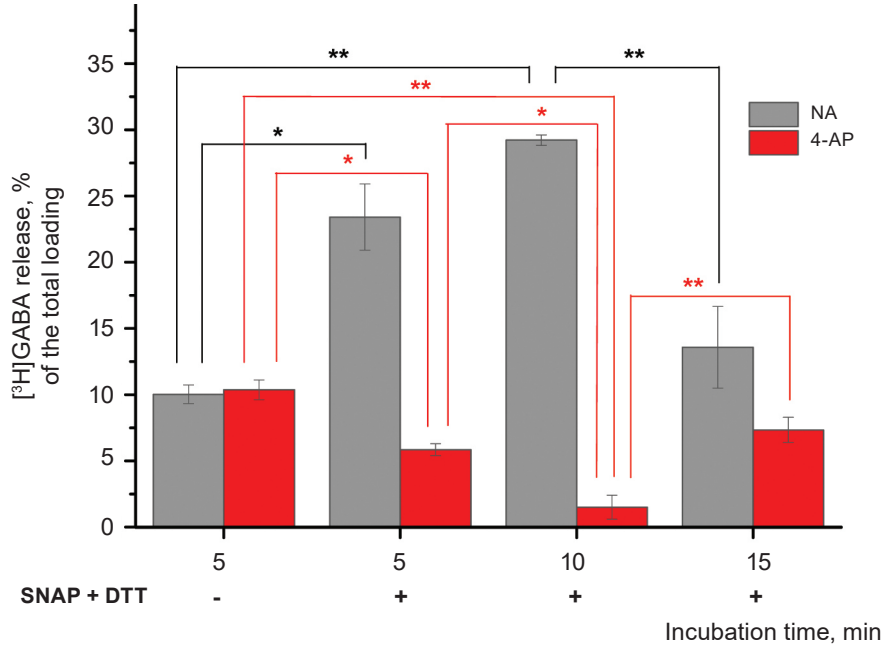

*Fig. 3. NA- and 4-AP-induced [³H]GABA release from synaptosomes preincubated with SNAP/+DTT for various periods of time; mean ± SEM, n = 3, * P < 0.05; ** P < 0.01*

induced [³H]GABA release from vesicular pool, indicating its considerable depletion. However, at the 15th min of incubation, the vesicular GABA pool was significantly restored, as evidenced by further increase in 4-AP-induced [³H]GABA release (compared to the 10th min of incubation).

Thus, the question arises: what happens to vesicular GABA pool at the 5th and 10th min of synaptosome incubation with SNAP/+DTT? We suggest that GABA is likely to leak from vesicles into cytoplasm and then releases out of synaptosomes. This assumption is supported by our data obtained in the experiments with nipecotic acid (100 μM). We observed a gradual increase in NA-induced GABA release from cytoplasmic pool at the 5th and 10th min of SNAP/+DTT action (Fig. 3, grey columns). Analysis of NA- and 4-AP-induced [³H]GABA release from synaptosomes clearly demonstrated that these processes are opposite-directed, that is, a decrease in vesicular GABA content was accompanied by an increase in the [³H]GABA level in cytoplasm. And conversely, a decrease in cytoplasmic [³H]GABA concentration at the 15th min of incubation was correlated with the accumulation of GABA inside synaptic vesicles and, as a consequence, with an increase in the amount of [³H]GABA additionally released from vesicles under the action of 4-AP. Thus, these results allow us to assume that SNAP/+DTT causes GABA release from vesicles into cytosol followed by its release out of synaptosomes, presumably, via reverse operation of GABA transporter.

NO-induced GABA release owing to the reversal of its transporters was previously demonstrated by G. Lonart and K. M. Johnson, who had found that hydroxylamine and S-nitroso-L-cysteine caused the release of dopamine and norepinephrine from striatal and hippocampal slices via pathway that was blocked by the inhibitors of their reverse uptake [21]. It should be noted that if the transporter-mediated neurotransmitter release was previously considered as a pathological process, which occurred, for example, upon ischemic or traumatic brain injury [22], but now there are experimental evidences of the involvement of transporters in the neurotransmitter release under physiological conditions. Thus, Falkenburger et al [23] showed that electrical stimulation of the brain subthalamic nuclei led to the transporter-mediated release of dopamine from dopamineergic dendrites in the substantia nigra, providing communication between neurons. Non-vesicular, transporter-induced GABA release, according to other authors [24], contributes to maintaining of the extracellular GABA concentration at the level sufficient to activate the high affinity GABA receptors and thus, participates in the formation of tonic inhibition.

GABA transporters can operate in the reverse direction due to the disturbance of their dynamic equilibrium. One of the reasons for this is the change in the intracellular neurotransmitter concentration. It was shown that anticonvulsants such as gabapentin and vigabatrin, which irreversibly block GABA transaminase, caused an increase in the cytosolic concentration of GABA and, consequently, its release from the nerve terminals owing to reverse operation of transporters [25]. In our work we also assumed that SNAP/+DTT-induced [^{3}H]GABA release might be transporter-mediated, however an increase in the cytoplasmic concentration of [^{3}H]GABA is probably caused by another reason.

It is known that the accumulation of neurotransmitters in the synaptic vesicles is coupled with proton transfer into the vesicles, so the increase in cytoplasmic GABA concentration might be due to disruption in the function of a vesicular H^+-ATPase, which forms the H^+-gradient. Previously, we have shown that the addition of SNAP/+DTT to synaptosomes caused a dissipation of the synaptic vesicle proton gradient that is most likely the result of cytosolic ATP depletion owing to inhibition of mitochondrial respiration by nitric oxide [15]. The maximum of H^+-gradient dissipation was observed at the 10th min that is fully correlated in time with the maximum of [^{3}H]GABA release from cytosol pool. The vesicular proton gradient was restored at the 15th min of incubation with SNAP+DTT and this process coincided with a decrease in the extrasynaptosomal concentration of [^{3}H]GABA (Fig. 1, *A*) and its re-accumulation inside vesicles (Fig. 3).

Thus, considering the above results, we can conclude that in the nanomolar range of concentrations, nitric oxide in the form of S-nitrosothiols in the presence of SH-reducing compounds may cause Ca^{2+}-independent GABA release from synaptic vesicles into cytosol followed by its release from nerve terminals presumably due to reverse operation of GABA transporters. This process is accompanied by a decrease in the concentration of neurotransmitter within the vesicles and, hence by a decrease in further GABA release by exocytosis. This view on the mechanism of presynaptic action of nitric oxide allows reconciling the contradictory literature data that have shown both stimulating [26] and inhibitory [27] effects of NO on the neurotransmission. A similar opinion about multidirectional action of NO has been previously expressed by other researches [3, 7], in particular, Sequeira et al. believe that NO affecting the GABAergic terminals inhibits the exocytotic and stimulates carrier-mediated GABA release [7]. However, unlike Sequeira, we assume that the decrease in neurotransmitter release by exocytosis occur not so much due to the inhibition of the process itself, but rather to the release of partially depleted vesicles.

In summary, it should be noted that it is quite possible that the effect of nitric oxide on GABAergic neurotransmission may be realized by the involvement of other pathways, in particular in case of higher NO concentrations, since a high oxidative potential of nitric oxide provides this molecule with unique properties.

ВПЛИВ ДОНОРА ОКСИДУ АЗОТУ SNAP НА ВИВІЛЬНЕННЯ ГАМК ІЗ НЕРВОВИХ ЗАКІНЧЕНЬ МОЗКУ ЩУРІВ

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У роботі ми досліджували вплив наномолярних концентрацій оксиду азоту на вивільнення ГАМК (гама-аміномасляної кислоти) з нервових закінчень мозку щурів. Для цього використовували радіоізотопний метод із [^{3}H]ГАМК та спектрофлуориметричний метод із застосуванням Ca^{2+}-чутливого зонда Fluo-4 AM. Показано, що в присутності дитіотреїтолу (ДТТ) донор оксиду азоту SNAP у концентрації, що виділяє NO в наномолярному діапазоні, спричинює Ca^{2+}-незалежне вивільнення [^{3}H]ГАМК із нервових закінчень. Застосування 4-амінопіридину (4-АП) та ніпекотинової кислоти (НК) як індукторів вивільнення ГАМК із везикулярного та цитоплазматичного пулів показало, що максимум SNAP/+ДТТ-індукованого вивільнення [^{3}H]ГАМК припадає на 10-ту хв інкубації і збігається в часі з майже вдвічі збільшеним вивільненням [^{3}H]ГАМК за дії НК. У цей час 4-АП-індуковане вивільнення [^{3}H]ГАМК істотно знижувалося. На 15-й хв інкубації синаптосом із SNAP+ДТТ спостерігалася протилежна картина: зменшення НК- і збільшення 4-АП-індукованого вивільнення [^{3}H]ГАМК. Таким чином, у наномолярному діапазоні концентрацій

оксид азоту у вигляді S-нітрозотіолів зумовлює Ca^{2+}-незалежне витікання ГАМК із синаптичних везикул у цитозоль із наступним його вивільненням із нервових терміналей. У подальшому відбувається зворотне захоплення нейромедіатора і його реакумуляція в синаптичних везикулах.

Ключові слова: оксид азоту, вивільнення ГАМК, везикулярний та цитоплазматичний пул.

ВЛИЯНИЕ ДОНОРА ОКСИДА АЗОТА SNAP НА ВЫСВОБОЖДЕНИЕ ГАМК ИЗ НЕРВНЫХ ОКОНЧАНИЙ МОЗГА КРЫС

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В работе мы исследовали влияние наномолярных концентраций оксида азота на высвобождение ГАМК (гамма-аминомасляной кислоты) из нервных окончаний мозга крыс. Для этого использовали радиоизотопный метод с [^{3}H]ГАМК и спектрофлуориметрический метод с Ca^{2+}-чувствительным зондом Fluo-4 AM. Показано, что в присутствии дитиотреитола (ДТТ) донор оксида азота SNAP в концентрации, выделяющей NO в наномолярном диапазоне, вызывает Ca^{2+}-независимое высвобождение [^{3}H]ГАМК из нервных окончаний. Применение 4-аминопиридина (4-АП) и нипекотиновой кислоты (НК) в качестве индукторов высвобождения ГАМК с везикулярного и цитоплазматического пулов показало, что максимум SNAP+ДТТ-индуцированного высвобождения [^{3}H]ГАМК приходится на 10-ю мин инкубации и совпадает по времени с почти вдвое увеличенным высвобождением [^{3}H]ГАМК под действием НК. В это время 4-АП-индуцированное высвобождение [^{3}H]ГАМК существенно снижалось. На 15-й мин инкубации синаптосом с SNAP+ДТТ наблюдалась противоположная картина: уменьшение НК- и увеличение 4-АП-индуцированного высвобождения [^{3}H]ГАМК. Таким образом, в наномолярном диапазоне концентраций оксид азота в виде S-нитрозотиолов вызывает Ca^{2+}-независимое вытекание ГАМК из синаптических везикул в цитозоль с последующим его высвобождением из нервных терминалей. В дальнейшем происходит обратный захват нейромедиатора и его реаккумуляция в синаптических везикулах.

Ключевые слова: оксид азота, высвобождение ГАМК, везикулярный и цитоплазматический пул.

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