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ASSESSMENT OF ACUTE NEUROTOXICITY OF NITROGEN-DOPED MULTILAYER GRAPHENE NANOPARTICLES AND THEIR CAPABILITY TO CHANGE Cd^{2+}/Pb^{2+}/Hg^{2+}-INDUCED INJURY IN BRAIN CORTEX NERVE TERMINALS

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Graphene materials are widely used in different technologies and certainly released into aquatic and air surroundings being environmental pollution components. Nitrogen-doped graphene nanomaterials have great potential for application, in particular, in energy storage, as electrochemical sensors and waste water treatment. The neurotoxic risk of nitrogen-doped multilayer graphene is unknown.

Aim. To evaluate neurotoxic risk of nitrogen-doped multilayer graphene.

Methods. Here, nitrogen-doped multilayer graphene nanoparticles (N-MLG) were synthesized by means of electrochemical exfoliation of high-purity graphite rods in NaN_3-based electrolyte and characterised using TEM, AFM and UV-vis spectroscopy. Neuroactive features of N-MLG were assessed in isolated cortex nerve terminals (synaptosomes) analysing the extracellular level of excitatory neurotransmitter L-[^{14}C] glutamate and inhibitory one [^{3}H]GABA.

Results. It was revealed that N-MLG did not affect the extracellular synaptosomal levels of L-[^{14}C]glutamate and [^{3}H]GABA within the concentration range 0.01–0.5 mg/ml, and an increase in a concentration up to 1 mg/ml caused an insignificant increase (tendency to increase) in these levels for both neurotransmitters. To analyse a capability of interaction with heavy metals in biological system, N-MLG was investigated using model of acute Cd^{2+}/Pb^{2+}/Hg^{2+}-induced neurotoxicity in nerve terminals. It was revealed that Cd^{2+}/Pb^{2+}/Hg^{2+}-induced increase in the extracellular level of L-[^{14}C] glutamate and [^{3}H]GABA was not changed by N-MLG.

Conclusions. N-MLG does not possess neurotoxic signs and is biocompatible within the concentration range 0.01^{-1} mg/ml. In biological system, N-MLG did not mitigate/aggravate Cd^{2+}/Pb^{2+}/Hg^{2+}-induced neurotoxicity in nerve terminals.

Key words: nitrogen-doped multilayer graphene; nanoparticles; heavy metals; neurotoxicity; glutamate; GABA; brain nerve terminals.

Graphene is a carbon 2D crystal consisting of a two dimensional honeycomb-lattice structure made of sp^2-hybridized carbon atoms. Graphene and its derivatives due to their two-dimensional shape and special mechanical, electronic, optical and catalytic properties are widely used in diverse fields, e.g. nanoelectronics, catalysis, and water technologies. Graphene is considered one of the most promising nanomaterials for biomedical applications and nanomedicine [1, 2]. Nitrogen-doped graphene exhibits excellent electrochemical parameters and has great potential for applications in energy storage, as a high-performance catalyst support for fuel cell electrocatalysis and sensor electrochemical applications [2, 3]. Nitrogen-doped graphene nanomaterials are effective for removing toxic species, e.g. organic pollutants, dyes, heavy metal ions from wastewater [4, 5]. In particular, nitrogen-doping enhances the interaction between the active sites and organic or ionic species, thereby improving removal efficiency of these pollutants [2].

Graphene materials during production and application can find ways into the environment in the form of nanoparticles because of its high dispersity in many solvents [6, 7]. In this context, graphene materials can widely pollute water resources and air around the world.

Nowadays, it is clear that carbonaceous airborne particulate matter is the most abandon environmental pollutant that can reach the nervous system of humans and trigger development of neurological and neurodegenerative disorders/diseases [8–10]. Expansion of these disorders/diseases is one of the main reasons of disability and premature death in Europe and linked to air pollution with particulate matter [8, 11–13]. From one hand, particulate matter can reach human organism through lungs and gut to the circulatory system and possibly cross the blood-brain barrier [14–18]. From the other hand, particulate matter can get to the human nervous system along the olfactory axon and overcome the blood-brain barrier [19, 20]. Recently, we have characterised neuroactivity of carbon-based nanoparticles, such as carbon nanodots, nanodiamonds, fullerene C60, carbonaceous smoke particulate matter from combustion of plastics and wood samples. Transport kinetics of key excitatory neurotransmitters, L-[^{14}C] glutamate and inhibitory one [^{3}H] GABA, were changed by above nanoparticles that in turn can provoke presynaptic malfunction and development of neuropathology [21, 22]. Carbonaceous airborne particulate matter possesses adhesive surface and can bind potentially toxic molecules, including heavy metals [23, 24].

The neurotoxic potential of nitrogen-doped multilayer graphene is unknown. In neurobiology study of other graphene derivatives, it was demonstrated that few-layer pristine graphene and monolayer graphene oxide flakes were in contact with the neuronal membrane and free in the cytoplasm, but did not have significant impact on neuron viability. Graphene oxide exposure inhibited excitatory transmission, reduced the number of excitatory synaptic contacts, and concomitantly enhanced the inhibitory activity. This was accompanied by altered Ca^{2+} dynamics in excitatory and inhibitory neurons. So, it was suggested that both graphene preparations affected neuronal transmission [1]. Taking into account abovementioned facts, the aims of the present study were (*) to synthesize nitrogen-doped multilayer graphene nanoparticles (N-MLG) by means of electrochemical exfoliation of high-purity graphite rods; (**) to assess neuromodulatory features of N-MLG in isolated presynaptic rat cortex nerve terminals (synaptosomes) analysing the extracellular level of neurotransmitters L-[^{14}C] glutamate and [^{3}H]GABA; and (***) to examine the effects of N-MLG on the neurotoxic injury caused by heavy metal ions Cd^{2+}, Pb^{2+}, and Hg^{2+} in the nerve terminals.

Materials and Methods

HEPES, EGTA, EDTA, NaN_3, Ficoll 400, High Performance LSC Cocktail salts of the analytical grade were obtained from Sigma, USA; L-[^{14}C] glutamate; [^{3}H] GABA– Perkin Elmer, Waltham, MA, USA. The high-purity graphite rods (99.9995%) were obtained from Alfa Aesar.

Transmission electron microscopy (TEM) images of the synthesized graphene material were recorded using a microscope TEM125K (Selmi) with an accelerating voltage 100 kV. Atomic force microscopy (AFM) of thin film N-MLG samples on the surface of silicon wafers coated with silicon nitride (Agar Scientific) was performed on a Nanoscope IIIa Dimension 3000TM (Digital) instrument. UV-vis-spectra of N-MLG dispersions were registered via UV-visible spectrometer 4802 (Unico).

Synthesis of multilayer graphene doped with nitrogen

Multilayer graphene doped with nitrogen (N-MLG) was synthesized according to previous published procedure by means of electrochemical exfoliation of high-purity graphite rods (Alfa Aesar, 99.9995%) in 1.0 M aqueous solution of NaN_3 using three-electrode undivided cell and potentiostat PI-50-1.1 [25]. Graphite rods were used as working and auxiliary electrodes and Ag/AgCl as reference electrode. Exfoliation procedure was carried out during 20 h via polarization of graphite electrode by +4.0 V (50 s) and 0.0 V (50 s) with multiple changing of polarization potential (pulse mode of electrolysis).

N-MLG particles from the obtained aqueous dispersion were separated on a nylon membrane filter with a pore diameter 0.2 μm (SUPELCO®), rinsed with water, and dried in oven at 60 °C. If necessary, the regeneration of N-MLG dispersion was performed by short-term ultrasound treatment (2 min) of the dried graphene material in appropriate solvent (for example, water) using the ultrasonic washing bath.

Animals and Ethics

Wistar rats (males), 3 months' age, were kept in the vivarium of Palladin Institute of Biochemistry, NAS of Ukraine in a quiet and temperature-controlled room at 22–23 °C. Animals were provided with dry food pellets and water *ad libitum*. All animal-involving procedures were performed in accordance with the guidelines of the European Community (2010/63/EU); "Scientific Requirements and Research Protocols"; "Research Ethics Committees" of Declaration of Helsinki; and "ARRIVE guidelines for reporting experiments involving animal" [26, 27]; and also local Ukrainian laws and policies. The experimental protocols were approved by the Animal Care and Use Committee of Palladin Institute of Biochemistry (Protocol # 1 from 10/01/2023). The total number of animals was 9.

Isolation of the synaptosomes from the rat cortex

The synaptosomes represented ~87% of the particles in electron micrographs of preparations, and did not contain nerve cell bodies and functional glial fragments [28–30]. Nerve terminals were isolated from the cortex regions of rat brain. Isolation procedures were conducted at + 4 °C. The cortex regions were removed and homogenized in the following ice-cold solution: sucrose 0.32 M; HEPES-NaOH 5 mM, pH 7.4; EDTA 0.2 mM. One synaptosome preparation was obtained from one rat. The synaptosomes were isolated according to Cotman method with minor modifications [9, 31–33] by differential/Ficoll-400 density gradient centrifugation. The synaptosomes were fitting for experiments for 2–4 hours after isolation. The standard saline solution contained: NaCl 126 mM; KCl 5 mM; $MgCl_2$ 2.0 mM; NaH_2PO_4 1.0 mM; HEPES 20 mM, pH 7.4; and D-glucose 10 mM. Protein concentrations were monitored according to Larson [34].

The extracellular synaptosomal level of L-[^{14}C] glutamate

The synaptosomes were diluted up to a concentration of 2 mg of protein/ml, and then the synaptosomes were pre-incubated at 37 °C for 10 min, and loaded with L-[^{14}C] glutamate, 1 nmol per mg of protein, 238 mCi/mmol, at 37 °C for 10 min. The synaptosomes after loading were washed with 10 volumes of the ice-cold standard saline solution and centrifuged (10,000×g, 20 s) at +4 °C; the pellets were re-suspended in the standard saline solution up to 1 mg protein/ml. The extracellular L-[^{14}C] glutamate level was assessed in the synaptosome suspensions (125 μl, 0.5 mg of protein/ml). The synaptosome aliquots were preincubated for 8 min to restore the ion gradients, and then Cd^{2+} (1 mM $CdCl_2$), Pb^{2+} (2.5 mM Pb acetatate (PbAc)), and Hg^{2+} (2.5 μM $HgCl_2$) were added to the synaptosomes and they were further incubated at 37 °C during 0 and 6 min; then centrifuged at 10,000 × *g* for 20 s at room temperature. The values of L-[^{14}C] glutamate release were monitored in the supernatant aliquots (100 μl), and the pellets preliminary treated with SDS (100 μl of 10% SDS stock solution) by liquid scintillation counting using the scintillation cocktail ACS (1.5 ml) and liquid scintillation counter Hidex 600SL (Finland) [35]. The experimental data were collected from "*n*" independent experiments carried out in triplicate using different synaptosome preparations.

The extracellular synaptosomal level of [^{3}H] GABA

The synaptosomes were diluted up to 2 mg of protein/ml; after pre-incubation at 37 °C for 10 min, the synaptosomes were preloaded with [^{3}H]GABA (50 nM, 4.7 μCi/ml) in the standard saline solution at 37 °C for 10 min. Aminooxyacetic acid (100 μM) was added

to the incubation medium throughout [^{3}H] GABA experiments. After pre-loading, the suspension was washed with 10 volumes of the ice-cold standard saline solution. The pellets were re-suspended in the standard saline solution up to 1 mg of protein/ml. The synaptosome aliquots were pre-incubated for 8 min to restore the ion gradients, and then Cd^{2+} (1 mM $CdCl_2$), Pb^{2+} (1 mM PbAc) and Hg^{2+} (2.5 μM $HgCl_2$) were added and further incubated at 37 C during 0 and 5 min; then centrifuged at 10,000 × g for 20 s at room temperature [36]. [^{3}H] GABA was measured in the supernatant aliquots (90 μl) by liquid scintillation counting with Sigma-Fluor® High Performance LSC Cocktail (1.5 ml) using liquid scintillation counter Hidex 600SL (Finland), and the extracellular level was expressed as the percentage of total accumulated [^{3}H] GABA [37]. The data were collected from "n" independent experiments performed in triplicate with different synaptosome preparations.

Statistical analysis

The experimental data were expressed as the mean ± S.E.M. of *n* independent experiments. One-way and two-way ANOVA were applied; the accepted significance level was $P < 0.05$. Two-way ANOVA followed by Tukey's post hoc test was applied to assess the interactions between N-MLG and Cd^{2+}, Pb^{2+}, and Hg^{2+} (N-MLG treatment and Cd^{2+}/Pb^{2+}/Hg^{2+} treatment were the independent factors).

Results and Discussion

Characterization of multilayer graphene doped with nitrogen

During pulse mode of electrolysis, multiple (cyclic) azide anions (N_3^-) intercalation/deintercalation into graphite interlayer space occurs, also associated with N_2, NH_3 and O_2 evolution due to partial anodic/cathodic decomposition of N_3^-. This provides exfoliation of the graphite electrode, forming multilayer packages of graphene, doped with nitrogen, and its transition in the electrolyte volume [25].

The obtained graphene material was characterized by several instrumental analysis methods. On the TEM images of N-MLG (Fig. 1, *A*) graphene sheets with lateral size from several hundred nanometers to several microns, forming multiple-layered lamellar structures, can be observed.

According to the data of AFM experiment, shown on the Fig. 1b, the lateral size of N-MLG particles, taken from a dilute dispersion in ethanol, is consistent with the data obtained by TEM. From the cross-sectional profile analysis (Fig. 1, *B*) it can be seen that the thickness of the N-MLG particles present in the AFM image is 4.2 nm. Earlier, on the basis of AFM data, we showed that the thickness of the monolayer in the N-MLG was 0.6 nm [25]. Taking this into account, it can be assumed that the obtained N-MLG particles are multilayered, consisting of the packages of ~7 graphene layers. At the same time, we cannot rule out the presence of a small number of N-MLG particles of both smaller (< 7 graphene layers) and larger (up to 9 graphene layers) thicknesses obtained by the electrochemical approach used herein [25]. Thus, AFM data also confirm the multilayered nature of the obtained graphene material.

UV–vis spectrum of N-MLG dispersions in ethanol is provided on Fig. 2. The presence of a band with an absorption maximum at 267 nm, corresponding to the ππ* transition in the C=C bonds of graphene, indicates in favor of a slight oxidation of the resulting N-MLG.

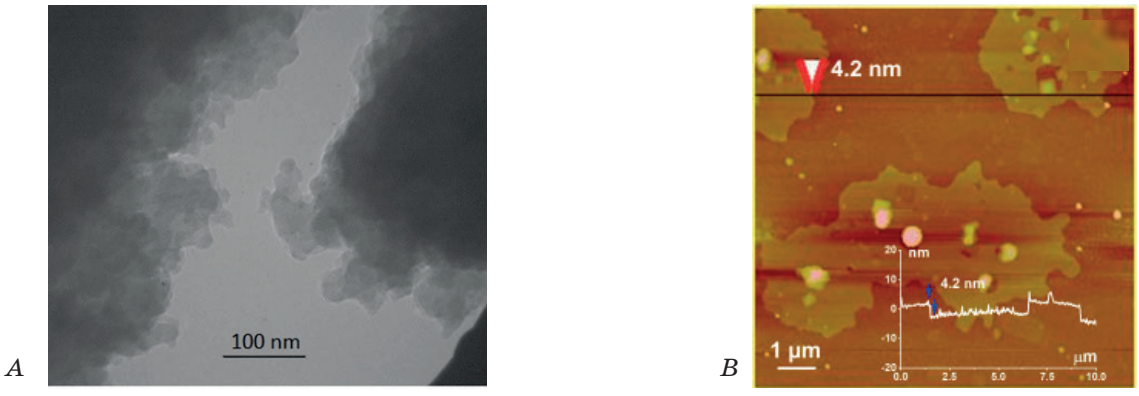

Fig. 1. **TEM image (*A*) and AFM image with corresponding cross-sectional profile (*B*) of N-MLG particles**

Neurotoxicity studies using rat brain nerve terminals

The extracellular levels of neurotransmitters glutamate and GABA are essential synaptic characteristics which show a balance of transporter-mediated uptake and tonic release of neurotransmitters [38, 39].

In the first series of the experiments, the effects of N-MLG on the extracellular levels of excitatory neurotransmitter L-[^{14}C] glutamate and inhibitory one [^{3}H]GABA were assessed in the nerve terminals. It was revealed that N-MLG did not affect the extracellular levels of L-[^{14}C] glutamate and [^{3}H]GABA within the concentration range 0.01–0.5 mg/ml, while an increase in a concentration up to 1 mg/ml caused an insignificant elevation (tendency to increase) of these levels for both neurotransmitters (Fig. 3). Therefore, N-MLG did not possess neurotoxic signs and was biocompatible within the concentration range 0.01^{-1} mg/ml.

In the next series of the experiments, it was investigated whether N-MLG could modulate a Cd^{2+}/Pb^{2+}/Hg^{2+}-induced increase in the extracellular levels of L-[^{14}C] glutamate and [^{3}H]GABA in nerve terminals. Based on above data, a N-MLG concentration of 0.5 mg/ml was chosen for further experiments with heavy metals. It was found that N-MLG (0.5 mg/ml) did not change Cd^{2+}/Pb^{2+}/Hg^{2+}-induced increase in the extracellular levels of L-[^{14}C] glutamate and [^{3}H]GABA in nerve terminals (Fig. 4), and so N-MLG was inert regarding modulation/aggravation of Cd^{2+}/Pb^{2+}/Hg^{2+}-induced neurotoxic effects in nerve terminals.

Two-way ANOVA revealed no interaction between Cd^{2+} and N-MLG [$F_{(1,32)}$ = 0.009; p = 0.92; n = 9], between Pb^{2+} and N-MLG [$F_{(1,32)}$ = 1.55; p = 0.22; n = 9] and between Hg^{2+} and N-MLG [$F_{(1,32)}$ = 1.22; p = 0.27; n = 9] in L-[^{14}C] glutamate experiments.

Two-way ANOVA revealed no interaction between Cd^{2+} and N-MLG [$F_{(1,32)}$ = 0.69; p = 0.41; n = 9], between Pb^{2+} and N-MLG [$F_{(1,32)}$ = 0.22; p = 0.63; n = 9] and between Hg^{2+} and N-MLG [$F_{(1,32)}$ = 0.13; p = 0.72; n = 9] in [^{3}H] GABA experiments.

Graphene and its derivatives have a potential to make a very significant impact on society with applications in the biomedical field. A possibility to engineer graphene-based medical devices at the neuronal interface is of particular interest, making it imperative to determine the biocompatibility of graphene materials with neuronal cells [1]. However, wide production and application of neuroactive graphene and its derivatives can increase

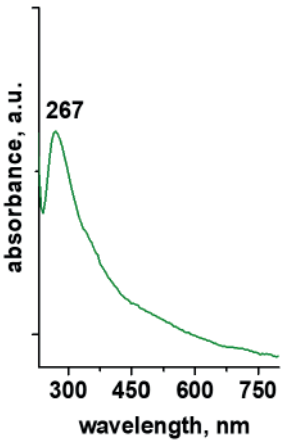

Fig. 2. **UV-vis spectra of N-MLG dispersion in ethanol**

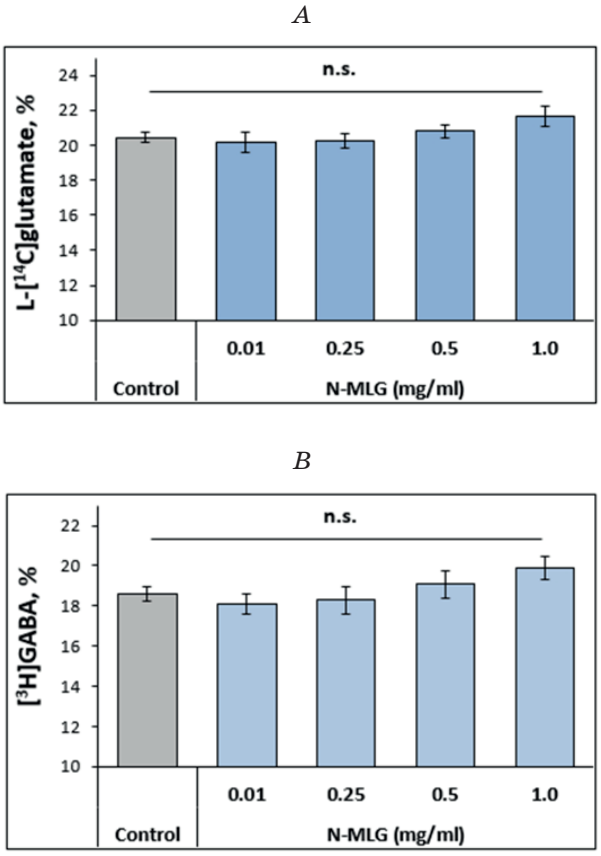

Fig. 3. **The extracellular levels of neurotransmitters L-[^{14}C] glutamate (*A*) and [^{3}H]GABA (*B*) in the nerve terminals in the presence of N-MLG (0.01–1.0 mg/ml)**

Data are the mean ± SEM. n.s., no significant differences as compared to the appropriate control, n = 9

uncontrolled environment pollution with these nanoparticles increasing risks to nervous system. To avoid the problem, technological-oriented graphene and its derivatives should be analysed regarding their neurotoxicity as single pollutants and also in combination with other pollutants, e.g. heavy metals, using multipollutant approach.

Here, we showed that N-MLG did not affect the extracellular synaptosomal levels of L-[^{14}C] glutamate and [^{3}H]GABA within the concentration range 0.01–1 mg/ml. Therefore, N-MLG can be applied in different technologies, including neurotechnologies and waste water treatment, because N-MLG did not possess neurotoxic signs and is biocompatible within this concentration range and it is safe when become environmental pollution component. Among other carbon-based nanoparticles that we have investigated regarding changes in the extracellular levels of L-[^{14}C] glutamate and [^{3}H]GABA in nerve terminals, N-MLG was less neurotoxic as compared to carbon dots [40], nanodiamonds [35], fullerene C_{60} [41], plastic and wood smoke particulate matter [21, 22, 42]. In this contex, N-MLG is more promising for neurotechnologies then other carbon nanoparticles because of absence of neurotoxic signs and its neurocompatibility.

Literature data have demonstrated that due to weak hydrophobic interactions, the negatively charged surfaces of graphene oxide were favorable to interact through electrostatic attractions with organic and inorganic cations [43, 44]. Graphene oxide could remove heavy metal ions as carriers due to its large surface area, pore size and abundant oxygen-containing functional groups [45–47]. In the present study, we revealed that N-MLG did not change Cd^{2+}/Pb^{2+}/Hg^{2+}-induced increase in the extracellular level of L-[^{14}C] glutamate and [^{3}H]GABA and so the nanoparticles did not mitigate/aggravate Cd^{2+}/Pb^{2+}/Hg^{2+}-induced neurotoxicity in nerve terminals. It also means that despite potential application in technologies, in particulate in waste water treatment against heavy metals, N-MLG was

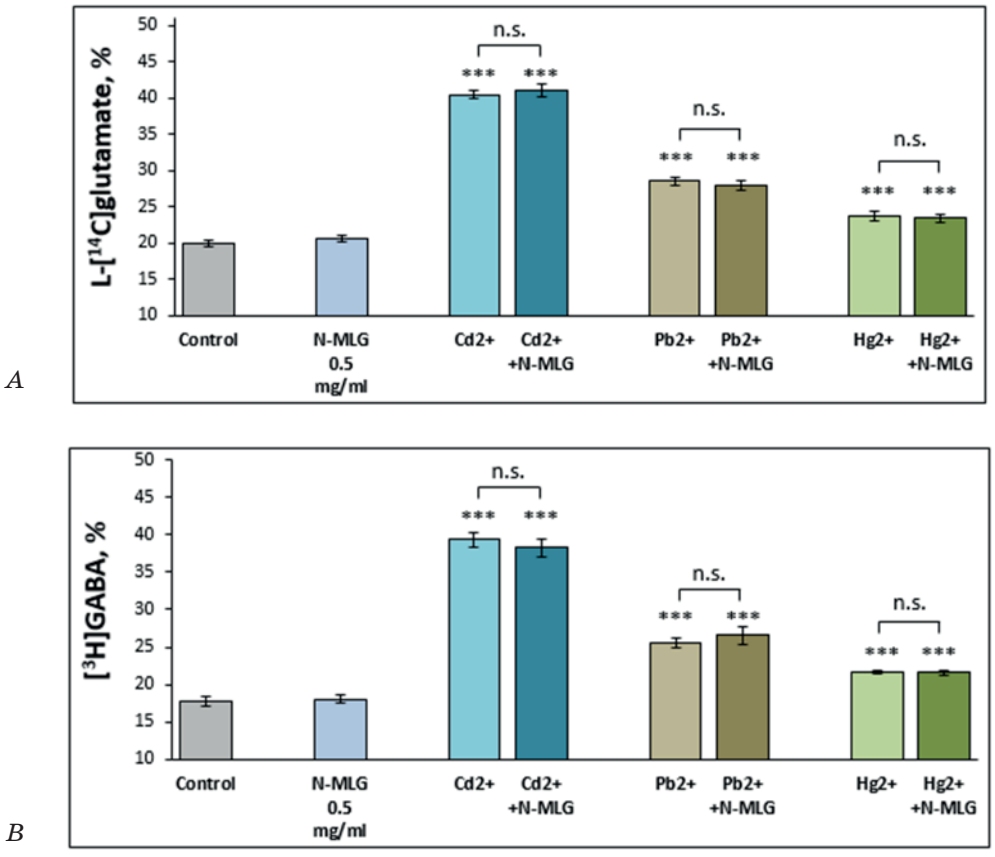

Fig. 4. **Effects of N-MLG (0.5 mg/ml) on Cd^{2+}/Pb^{2+}/Hg^{2+}-induced increase in the extracellular levels of L-[^{14}C] glutamate (*a*) and [^{3}H]GABA (*b*)**

Data are the mean ± SEM. ***, $P < 0.001$; as compared to the control; n.s., no significant differences as compared to Cd^{2+}/Pb^{2+}/Hg^{2+} effects, $n = 9$

not effective against prevention/mitigation of Cd^{2+}/Pb^{2+}/Hg^{2+}-induced neurotoxicity in biological system such as nerve terminals.

Conclusions

N-MLG, prepared by exfoliation of the graphite electrode in azide-containing electrolyte, did not influence the extracellular levels of L-[^{14}C] glutamate and [^{3}H] GABA in the nerve terminals. Cd^{2+}/Pb^{2+}/Hg^{2+}-induced increase in the extracellular levels of L-[^{14}C] glutamate and [^{3}H]GABA was not changed by N-MLG that was shown using model of acute neurotoxicity in nerve terminals. Therefore, N-MLG did not possess neurotoxic signs and is neurocompatible. In biological system, N-MLG did not mitigate or aggravate Cd^{2+}/Pb^{2+}/Hg^{2+}-induced neurotoxicity in nerve terminals.

Competing interests

The authors declare no financial and non-financial competing interests exist.

Authors contributions

N-MLG synthesis and characterisation — OP, YK; synaptosome preparations were obtained by AP & MD, L-[^{14}C] glutamate experiments — AP & KN, [^{3}H] GABA experiments — NP & MD; data analysis and figure preparation — OP, YK, SS, NP, KN, TB, SK; funding acquisitions, project leading, data analysis and paper writing — TB, VK.

Ethical Approval

Animal-involved experiments were performed according to the "Scientific Requirements and Research Protocols" & "Research Ethics Committees" of Declaration of Helsinki; the "ARRIVE guidelines for reporting experiments involving animal" [26, 27], and were preliminary approved by Institutional Animal Care and Use Committee (Protocol # 1 from 10/01/2023).

Consent to Participate

Not applicable.

Consent to Publish

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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ОЦІНЮВАННЯ ГОСТРОЇ НЕЙРОТОКСИЧНОСТІ БАГАТОШАРОВИХ НАНОЧАСТИНОК ГРАФЕНУ, ЛЕГОВАНИХ АЗОТОМ, ТА ЇХНЬОЇ ЗДАТНОСТІ ВПЛИВАТИ НА $Cd^{2+}/Pb^{2+}/Hg^{2+}$-ІНДУКОВАНІ ЗМІНИ ФУНКЦІОНУВАННЯ НЕРВОВИХ ТЕРМІНАЛЕЙ КОРИ ГОЛОВНОГО МОЗКУ

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Графенові матеріали широко використовуються в різних технологіях і, безумовно, потрапляють у водне та повітряне навколишнє середовище та можуть стати його забруднювачами. Графенові наноматеріали, доповані азотом, мають великий потенціал для застосування, зокрема, в накопичувачах енергії, електрохімічних сенсорах і очищенні стічних вод.

Мета. Оцінити нейротоксичний ризик багатошарового графену, допованого азотом.

Методи. У роботі синтезовано наночастинки багатошарового графену, допованого азотом (N-MLG) за допомогою електрохімічної ексфоліації високочистих графітових стрижнів в електроліті на основі NaN_3 та охарактеризовано за допомогою ТЕМ, АСМ і спектроскопії. Нейроактивні властивості N-MLG оцінювали в ізольованих нервових закінченнях кори головного мозку (синаптосомах) шляхом аналізу позаклітинного рівня нейромедіаторів L-[^{14}C] глутамату та [^{3}H]ГАМК.

Результати. Виявлено, що в діапазоні концентрацій 0,5–0,01 мг/мл N-MLG не впливав на позаклітинний синаптосомальний рівень L-[^{14}C] глутамату та [^{3}H]ГАМК, а збільшення концентрації до 1,0 мг/мл викликало незначне підвищення (тенденцію до підвищення) цих рівнів для обох нейромедіаторів. Аналіз здатності N-MLG взаємодіяти з важкими металами в біологічній системі досліджували на моделі гострої $Cd^{2+}/Pb^{2+}/Hg^{2+}$-індукованої нейротоксичності в нервових терміналях. Виявлено, що $Cd^{2+}/Pb^{2+}/Hg^{2+}$-індуковане підвищення позаклітинного рівня L-[$^{2+}$C] глутамату та [$^{2+}$H] ГАМК не змінюється під впливом N-MLG.

Висновки. N-MLG не має нейротоксичних ознак і є біологічно сумісним у діапазоні концентрацій 0,01–1,0 мг/мл. У біологічній системі N-MLG не зменшує/посилює нейротоксичність, спричинену $Cd^{2+}/Pb^{2+}/Hg^{2+}$ у нервових терміналях.

Ключові слова: багатошаровий графен, допований азотом; наночастинки; важкі метали; нейротоксичність; глутамат; ГАМК; нервові терміналі мозку.