

Photobiomodulation under Electroencephalographic Controls of Sleep for Stimulation of Lymphatic Removal of Toxins from Mouse Brain

Inna Blokina¹, Egor Iluykov², Dmitry Myagkov², Dmitry Tuktarov², Sergey Popov², Timofey Inozemzev², Ivan Fedosov², Alexander Shirokov^{1,3}, Andrey Terskov¹, Alexander Dmitrenko¹, Arina Evsyukova¹, Daria Zlatogorskaya¹, Viktoria Adushkina¹, Matvey Tuzhilkin¹, Maria Manzhayeva¹, Valeria Krupnova¹, Alexander Dubrovsky², Inna Elizarova¹, Maria Tzoy², Oxana Semyachkina-Glushkovskaya¹

¹ Department of Biology, Saratov State University ² Institute of Physics, Department of Optics and Biophotonics, Saratov State University ³ Institute of Biochemistry and Physiology of Plants and Microorganisms, Saratov Scientific Centre of the Russian Academy of Sciences

Corresponding Author

Oxana Semyachkina-Glushkovskaya
glushkovskaya@mail.ru

Citation

Blokina, I., Iluykov, E., Myagkov, D., Tuktarov, D., Popov, S., Inozemzev, T., Fedosov, I., Shirokov, A., Terskov, A., Dmitrenko, A., Evsyukova, A., Zlatogorskaya, D., Adushkina, V., Tuzhilkin, M., Manzhayeva, M., Krupnova, V., Dubrovsky, A., Elizarova, I., Tzoy, M., Semyachkina-Glushkovskaya, O. Photobiomodulation under Electroencephalographic Controls of Sleep for Stimulation of Lymphatic Removal of Toxins from Mouse Brain. *J. Vis. Exp.* (), e67035, doi:10.3791/67035 (2024).

Date Published

June 20, 2024

DOI

10.3791/67035

URL

joVE.com/t/67035

Abstract

The meningeal lymphatic vessels (MLVs) play an important role in the removal of toxins from the brain. The development of innovative technologies for the stimulation of MLV functions is a promising direction in the progress of the treatment of various brain diseases associated with MLV abnormalities, including Alzheimer's and Parkinson's diseases, brain tumors, traumatic brain injuries, and intracranial hemorrhages. Sleep is a natural state when the brain's drainage processes are most active. Therefore, stimulation of the brain's drainage and MLVs during sleep may have the most pronounced therapeutic effects. However, such commercial technologies do not currently exist.

This study presents a new portable technology of transcranial photobiomodulation (tPBM) under electroencephalographic (EEG) control of sleep designed to photostimulate removal of toxins (e.g., soluble amyloid beta (A β)) from the brain of aged BALB/c mice with the ability to compare the therapeutic effectiveness of different optical resources. The technology can be used in the natural condition of a home cage without anesthesia, maintaining the motor activity of mice. These data open up new prospects for developing non-invasive and clinically promising photo-technologies for the correction of age-related changes in the MLV functions and brain's drainage processes and for effectively cleansing brain tissues from metabolites and toxins. This technology is intended both for preclinical studies of the functions of the sleeping brain and for developing clinically relevant treatments for sleep-related brain diseases.

Introduction

Meningeal lymphatic vessels (MLVs) play an important role in the removal of toxins and metabolites from brain tissues^{1,2,3}. Damage of MLVs in various brain diseases, including tumors, traumatic brain injuries, hemorrhages, and neurodegenerative processes, is accompanied by a decrease in the MLV functions leading to the progression of these pathologies^{1,2,3,4,5,6}. Therefore, the development of methods for the stimulation of MLVs opens new horizons in the emergence of effective technologies for the treatment of brain diseases. Recently, non-invasive technology for effective transcranial photobiomodulation (tPBM) has been proposed to stimulate MLVs and remove toxins such as blood and A β from the brain^{5,7,8,9,10,11,12}. It is interesting to note that deep sleep is a natural factor for the activation of lymphatic drainage processes in the brain^{13,14}. Based on this fact, it is logical to assume that the tPBM of MLVs during sleep may have more effective therapeutic effects than during wakefulness^{9,11,12,15}. However, there are currently no commercial technologies for tPBM during sleep¹⁶. In addition, animal experiments to study the therapeutic effects of tPBM are performed under anesthesia, which is required to accurately deliver light to the brain. However, anesthesia significantly affects the brain's drainage, which reduces the quality of research results¹⁷.

A β is a metabolic product of normal neural activity¹⁸. As it was established in cultured rat cortical neurons, A β is released from them at high rates into the extracellular space (2-4 molecules/neuron/s for A β)¹⁹. There is evidence that the dissolved form of A β , located in the extracellular and perivascular spaces, is most toxic to neurons and synapses²⁰. The soluble A β is rapidly cleared from the human brain during 1-2.5 h²¹. MLVs are the tunnels for removal

of the soluble A β from the brain^{1,7} that declines with age, leading to the accumulation of A β in the aged brain^{1,22}. There is evidence that extracellular abnormalities of A β levels in the brain correlate with cognitive performance in aging and are associated with the development of Alzheimer's disease (AD)^{23,24}. Therefore, aged and old rodents are considered alternatives to transgenic models for the study of amyloidosis, including AD^{25,26}.

This study presents an original and portable tPBM technology under electroencephalographic (EEG) control of deep or non-rapid eye movement (NREM) sleep in non-anesthetized male BALB/c mice of different ages to stimulate lymphatic clearance of A β from the brain into the peripheral lymphatic system (the deep cervical lymph nodes, dcLNs).

Protocol

All procedures were performed in accordance with the "Guide for the Care and Use of Laboratory Animals", Directive 2010/63/EU on the Protection of Animals Used for Scientific Purposes, and the guidelines from the Ministry of Science and High Education of the Russian Federation (N° 742 from 13.11.1984), which have been approved by the Bioethics Commission of the Saratov State University (Protocol No. 7, 22.09.2022).

1. Hardware assembly

1. Cut a piece of foil textolite that is 1.5 mm thick to the dimensions of 1.5 mm x 2 mm. This part will be referred to as the light-emitting diode (LED) printed circuit board (PCB) (**Figure 1C**).
2. Solder an LED to a PCB, as shown in **Figure 1**.

3. Solder two stranded copper wires to the pins of an LED and then cover them with a sleeve.
4. Print out the 3D model of the frame (**Figure 1A**); place an LED (**Figure 1B**) and magnets (**Figure 1D**) on the frame; place a washer (**Figure 1E**) on the mouse's head.
5. To assemble the circuit (**Figure 2**), follow these steps.
 1. First, connect the resistor (**Figure 2**; R1) between an LED anode and 5 V port on the Arduino.
 2. Next, connect an LED cathode to the metal-oxide-semiconductor field-effect transistor (MOSFET) (**Figure 2**; Q1) drain. Then, connect the MOSFET source to the ground.
 3. Connect the pull-down resistor (**Figure 2**; R2) between the MOSFET gate and the ground. Finally, connect the MOSFET gate to pin 3 on the Arduino.
6. Connect a liquid crystal display (LCD) keypad shield to the Arduino.
7. Print out the 3D models of the case, the cover plate, and the buttons. Insert the Arduino board with LCD keypad shield, MOSFET, and LED connector into the case, as shown in **Figure 3**.
4. The left-hand column on the screen is the pulse width modulation (PWM) duty cycle selection field. Use the **Up** and **Down** buttons to adjust the duty cycle. Achieve the 10/20/30 Dj/cm² dose of PBM with a corresponding 2%/4%/6% PWM duty cycle in a 17 min session.
5. The right-hand column on the screen is the **RUN/Off** field. To select this column, press the **Right** button on the keypad and then press **Select**. When the process is running, the activity indicator will blink, and the text on the **RUN** button will change to **OFF**.
NOTE. Mice are prepared for the experiments over a period of 10 days, including implantation of EEG electrodes, implantation of a chronic catheter into the right lateral ventricle for injection of fluorescent A β , and placement of a plate for PBM.

3. Implantation of an EEG recording system (**Figure 5**)

1. Weigh the mouse and anesthetize it with a mixture of Zoletil 100 and Xylanite (100 mg/kg; 10 mg/kg, respectively) by intramuscular injection into the thigh.
 2. When the rear foot withdrawal reflexes and tail pinch response cease, place the mouse in a stereotaxic frame over a heating pad (**Figure 5A**).
 3. Apply ophthalmic ointment to the eyelids to prevent drying of the eyeballs during surgery. Repeat this procedure whenever necessary.
 4. Shave the head in the area from the nasal bones to the occipital bones using a shaving machine and sterilize exposed skin first with alcohol.
 5. Using straight dissecting scissors, cut off the scalp, hold it with micro forceps, clean the skull from fascia, and dry
- ## 2. Software guide (**Figure 4**)
1. Download the Arduino sketch (.ino file) and open it via the Arduino integrated development environment (IDE) (**Supplementary Coding File 1**).
 2. Select the correct communication (COM) port and flash the firmware.
 3. The interface includes two columns. Use the buttons **Left** and **Right** to navigate between these columns. The selection indicator is located to the left of the column.

it with cotton swabs. If necessary, use the hemostatic agents.

6. Using a drill with a diameter of 1.3 mm, make 2 holes in the temporal bones on each side along the coordinates: AP = -1 mm for the first pair of screws and AP = -3 mm for the second pair of screws.
7. Place the EEG screws with wire leads in alcohol for 5 min. Afterward, place the EEG screws in the saline solution.
8. Place four silver-plated screws with electrodes into the holes to a depth of 1 mm (**Figure 5B**).
9. Fix the screws on the surface of the skull using dental acrylic so that the electrodes extending from them are located towards the animal's nose (**Figure 5C**). Allow the dental acrylic to harden for 15 min.
10. Attach an EEG recording sensor to the animal's nose using dental acrylic. Allow the dental acrylic to harden for 30 min (**Figure 5D**).
11. Place the EMG electrodes on the back of the orbicularis oculi muscle using curved tweezers and fix them with the dental acrylic. Allow the dental acrylic to harden for 15 min (**Figure 5E**).
12. Connect the EEG electrodes to the silver-plated recess of the sensor and solder them using a soldering station. Afterward, fix the EEG electrodes using the dental acrylic (**Figure 5F**).
13. After surgery, place the mouse on a heating pad to maintain body temperature until the animal fully recovers from anesthesia.
14. Afterward, put the mouse in an individual home cage with free access to food and water with ibuprofen (40 mg/kg in 200 mL of water) for analgesia after surgery for 10 days.

4. Implantation of a plate for PBM

1. Weigh the mouse and anesthetize it with a mixture of Zoletil 100 and Xylanite (100 mg/kg; 10 mg/kg, respectively) by intramuscular injection into the thigh 7 days after implantation of the EEG recording system.
2. When the rear foot withdrawal reflexes and tail pinch response cease, fix the mouse in a stereotactic system.
3. Apply ophthalmic ointment to the eyelids to prevent drying of the eyeballs during surgery. Repeat this procedure whenever necessary.
4. Fix a metal plate with a diameter of 5 mm on the occipital bone of the skull using dental acrylic and Dumont forceps. Allow the dental acrylic to harden for 15 min (**Figure 6**).

5. Preparation of a chronic catheter

1. Mark on the insulin needle a segment 2 cm from the side of the beveled end.
2. Fix the insulin needle in the needle holder from the side of the beveled tip to the marked segment.
3. Place a 2 cm-PE-10 polyethylene catheter over the entire remaining length of the needle.
4. Fill the catheter with a saline solution and cover it with a plastic cap to seal it.

6. Implantation of a chronic catheter into the right lateral ventricle

1. After implantation of the plate for PBM, make a trepanation hole at the coordinates AP = -0.5 mm and ML = 1.2 mm, with a diameter of 1.5 mm, using a drill.

2. Place the PE-10 polyethylene catheter in a stereotactic holder and insert it into the mouse skull (DV = 2 mm). Afterward, fix it using the dental acrylic. Allow the dental acrylic to harden for 15 min (**Figure 7**).
3. After surgery, place the mouse on a heating pad to maintain body temperature until the animal fully recovers from anesthesia.
4. Afterward, put the mouse in an individual home cage with free access to food and water with ibuprofen (40 mg/kg in 200 mL of water) for analgesia after surgery for 10 days.

7. tPBM under EEG control of NREM sleep

1. Connect any commercial EEG recording system to the connector on the mouse's head and set the requirement value of the PWM duty cycle.
2. Monitor the EEG signal and wait for delta rhythm activity. If NREM sleep is seen, initiate the PBM process and stop the process if NREM sleep transitions to rapid eye movement (REM) sleep or wakefulness. The dose increases with each interaction until it reaches the required value.
3. When the required PBM dose is obtained, the session is over.

8. Confocal imaging of lymphatic removal of A β from mouse brain

1. Connect a 10 cm catheter to an insulin needle.
2. Using a Hamilton syringe with a 29 G needle, prepare an infusion of fluorescent beta-amyloid (FA β) in a volume of 5 μ L into the catheter. Let the catheter remain on a Hamilton syringe.
3. Fix the mouse's hand and connect the catheter through a needle to the implanted chronic catheter.

4. Connect the catheter to a microinjector. Afterward, place the mouse in an individual box;
5. Select the injection rate 0.1 μ L/min in the microinjector menu, and press the Start button.
6. Make injection of FA β into the right lateral ventricle.
7. After FA β administration, make PBM using an LED for 61 min following the algorithm: 17 min - light and 5 min - pause over 61 min.
8. After PBM, intravenously inject any tracer for labeling the cerebral vessels via the tail.
9. After injection, euthanize mice using the CO₂ euthanasia chamber.
10. Using sharp, straight scissors, make a small transverse incision in the skin along the trachea, holding the skin with straight non-sharp tweezers.
11. Make a longitudinal incision along the entire length of the neck using straight scissors.
12. Using curved tweezers, take up the salivary glands and carefully separate them from the connective tissue. Place a wound retractor on the open section of the incision and fix it in such a way as to push back the surrounding tissues.
13. Examine the area between the trachea and the cleidomastoid muscle using two curved tweezers from both sides of the neck.
14. When the deep cervical lymph node (dcLN) is detected on each side, take off dcLNs using straight tweezers with blunt ends and cut it from the connective tissue.
15. Place dcLNs in a Petri dish with a saline solution and cover them with horizontally oriented cover glass (25 mm $\times$ 50 mm $\times$ 0.17 mm).

16. Use any commercial confocal microscope to obtain images of whole dcLNs.

9. Analysis of A β in the lysates of brain tissues

1. Prepare the samples for the assay.
 1. Euthanize mice using the CO₂ euthanasia chamber.
 2. Decapitate the mouse, remove the skin from its head, and remove the muscles from the skull.
 3. Make two incisions with sharp, straight scissors from the great occipital foramen to the auditory canal.
 4. Using straight tweezers, separate the ventral part of the skull, the occipital bone, and bones forming the middle ear cavities.
 5. Using tweezers, separate the brain from the parietal and frontal bones.
 6. Using straight scissors, remove the upper jaw and cut off the olfactory bulbs.
 7. Place the brain in the physiological solution.
 8. Rinse the brain in cold phosphate-buffered saline to remove excess blood thoroughly and weigh before homogenization.
 9. Prepare a lysing buffer pH 7.2 containing 1.5 mm KH₂PO₄, 8 mm Na₂HPO₄, 3 mm KCl, 137 mm NaCl and 0.1% Tween20, 10 mM EDTA with a freshly prepared protease inhibitory mixture.
 10. Homogenize the brain in fresh lysis buffer (1 mL of lysis buffer for 200-500 mg tissue sample) with a glass homogenizer on ice.
 11. Sonicate a resulting suspension with an ultrasonic cell disruptor till the solution is clarified.
 12. Centrifuge the homogenates at 10,000 $\times$ g for 5 min.
13. Collect the supernatant using a single channel mechanical pipette (100-1000 μ L) and assay immediately or aliquot and store at $\leq$ -20 $^{\circ}$ C.
2. Prepare the following materials: microplate reader with 450 nm $\pm$ 10 nm filter; microcentrifuge tubes; single or multi-channel pipettes with high precision and disposable tips; absorbent paper for blotting the microplate; container for wash solution; 0.01 mol/L (or 1x) phosphate buffered saline (PBS); and deionized or distilled water.
3. Prepare the reagents.
 1. Bring the components of the kit and samples to room temperature (RT; 18-25 $^{\circ}$ C) before use.
 2. Reconstitute the standard with the 1.0 mL of standard diluent, keep for 10 min at RT, and shake gently (not to foam). The standard stock solution is 300 pg/mL. Prepare 5 tubes containing a volume of 0.6 mL of standard diluent and make a triple dilution series.
 3. Set up 5 points (300 pg/mL, 100 pg/mL, 33.33 pg/mL, 11.11 pg/mL, and 3.70 pg/mL) of the diluted standard, and the last tubes with as blank containing only the standard diluent (0 pg/mL).
 4. Quickly spin down the stock solutions of Detection reagent A and Detection reagent B prior to use. Dilute them 100-fold with Assay Diluent A and B to prepare the working concentration.
 5. Dilute 20 mL of the concentrated wash solution (30x) with 580 mL of deionized or distilled water to make 600 mL of wash solution (1x).

6. Aspirate the needed dosage of the solution with sterilized tips, and do not dump the residual solution into the vial again.
4. Perform the assay.
 1. Determine wells for a diluted standard, blank, and sample.
 2. Prepare 5 wells for standard points and one well for blank.
 1. Add 50 μL each of standard, blank, and sample dilutions into the corresponding wells, respectively. Then, add 50 μL of Detection reagent A to each well immediately.
 2. Shake the plate gently (a microplate shaker is recommended) and cover it with a plate sealer. Incubate the plate at 37 °C for 1 h. Detection reagent A may appear cloudy. Warm the solution to RT and mix gently until it appears uniform.
 3. Aspirate the solution and wash each well with 350 μL of 1x wash solution with the help of a squirt bottle, multi-channel pipette, manifold dispenser, or auto washer. Leave the plate undisturbed for 1-2 min. Snap the plate onto absorbent paper to completely remove the remaining liquid from all wells. Repeat this procedure 3 times.
 4. After the last wash, aspirate or decant any remaining wash buffer. Ensure complete removal of the washing solution by inverting the plate and blotting it against absorbent paper.
 5. Add 100 μL of Detection reagent B working solution to each well and incubate the plate for 30 min at 37 °C after covering it with the plate sealer.
 6. Repeat the aspiration/washing steps for a total of 5 min.
 7. Add 90 μL of substrate solution to each well and cover the plate with a new plate sealer. Incubate for 10-20 min at 37 °C (Do not exceed 30 min) protected from light. After adding the substrate solution, the liquid will turn blue.
 8. Add 50 μL of a stop solution to each well to terminate the reaction. Adding the stop solution will turn the liquid yellow. Tap the plate on its side to mix the liquid. If the color change is inconsistent, gently tap the plate to ensure thorough mixing.
 9. Ensure complete removal of water and fingerprint on the bottom of the plate and no bubble formation on the liquid surface. Then, read the plate in a microplate reader at 450 nm immediately.
5. Calculate the results.
 1. Determine the average of the duplicate readings for each standard, control, and sample. Plot a standard curve with the log of A β 1-42 concentration on the y-axis and absorbance on the x-axis.
 2. Draw a best-fit curve through the points, which can be determined by regression analysis.
 3. If diluted samples were used, multiply the concentration obtained from the standard curve by the dilution factor.
- NOTE:** For ELISA, a kit for determining A β 1-42 was used in this study.

Representative Results

In the first step, the study has focused on establishing the effective light dose (a 1050 nm LED) for stimulation of

lymphatic removal of fluorescent A β from the brain to dcLNs in awake adult (2-3 month old, 26-29 g) male BALB/c mice. The light doses were selected randomly as 10 J/cm², 20 J/cm², and 30 J/cm² based on our previous studies of tPBM effects on the removal of different dyes and the red blood cells from the brain^{7,8,9,10,11,12}. The results of confocal imaging presented in **Figure 8A-E** clearly demonstrate the dose-related effects of tPBM on lymphatic removal of fluorescent A β from the brain to dcLNs. The light dose 30 J/cm² was determined to be the most effective compared to the light doses 10 J/cm² and 20 J/cm² due to a higher intensity of a fluorescent signal from A β in dcLNs.

In the second step, the research was aimed at finding the wavelengths that are most effective for lymphatic elimination of fluorescent A β from the brain of adult (2-3 month old, 26-29 g) awake BALB/c male mice using the portable photo platforms with the different light wavelengths (880 nm, 1050 nm, 1300 nm) and modes (1050 nm in continues and pulse

regimes) with a same dose (30 J/cm²). **Figure 9A-E** shows the confocal imaging results of dcLNs in the tested groups. The findings revealed that the light wavelength 1050 nm in pulse mode vs. other tested wavelengths (880 nm and 1300 nm) and the 1050 nm wavelength in continuous regime caused significantly higher removal of fluorescent A β from the brain to dcLNs.

In the final step, the effects of a 1050 nm LED in pulse mode on the level of soluble A β in the brain of aged mice before and after the 10 day course of tPBM under an EEG control of NREM and wakefulness were studied. The immunoassay analysis revealed that the soluble A β content in the brain was significantly higher in aged male BALB/c mice (16-18 months old, 30-33 g) compared with adult male BALB/c mice (2-3 months old, 26-29 g). The 10 day course of tPBM during deep sleep, but not while awake, effectively reduced the soluble A β level in the brains of aged mice to the level of soluble A β in the brains of adult mice (**Figure 10**).

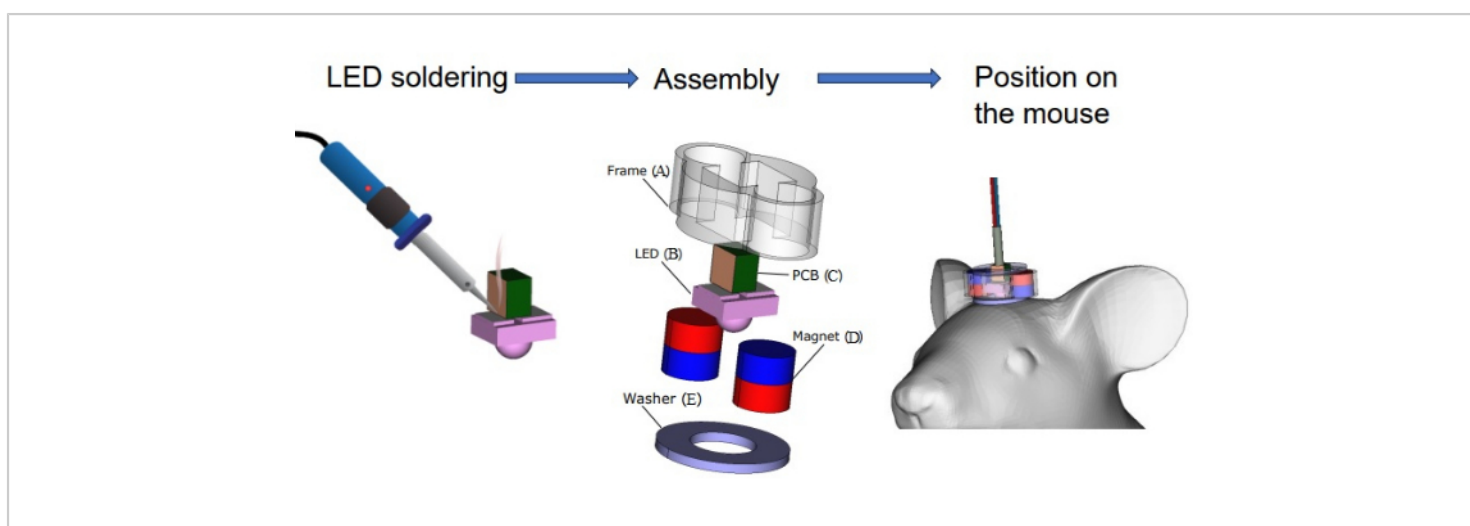


Figure 1: Hardware assembly process. (A) 3D printed frame, (B) LED, (C) LED printed circuit board, (D) magnets, (E) metallic washer. [Please click here to view a larger version of this figure.](#)

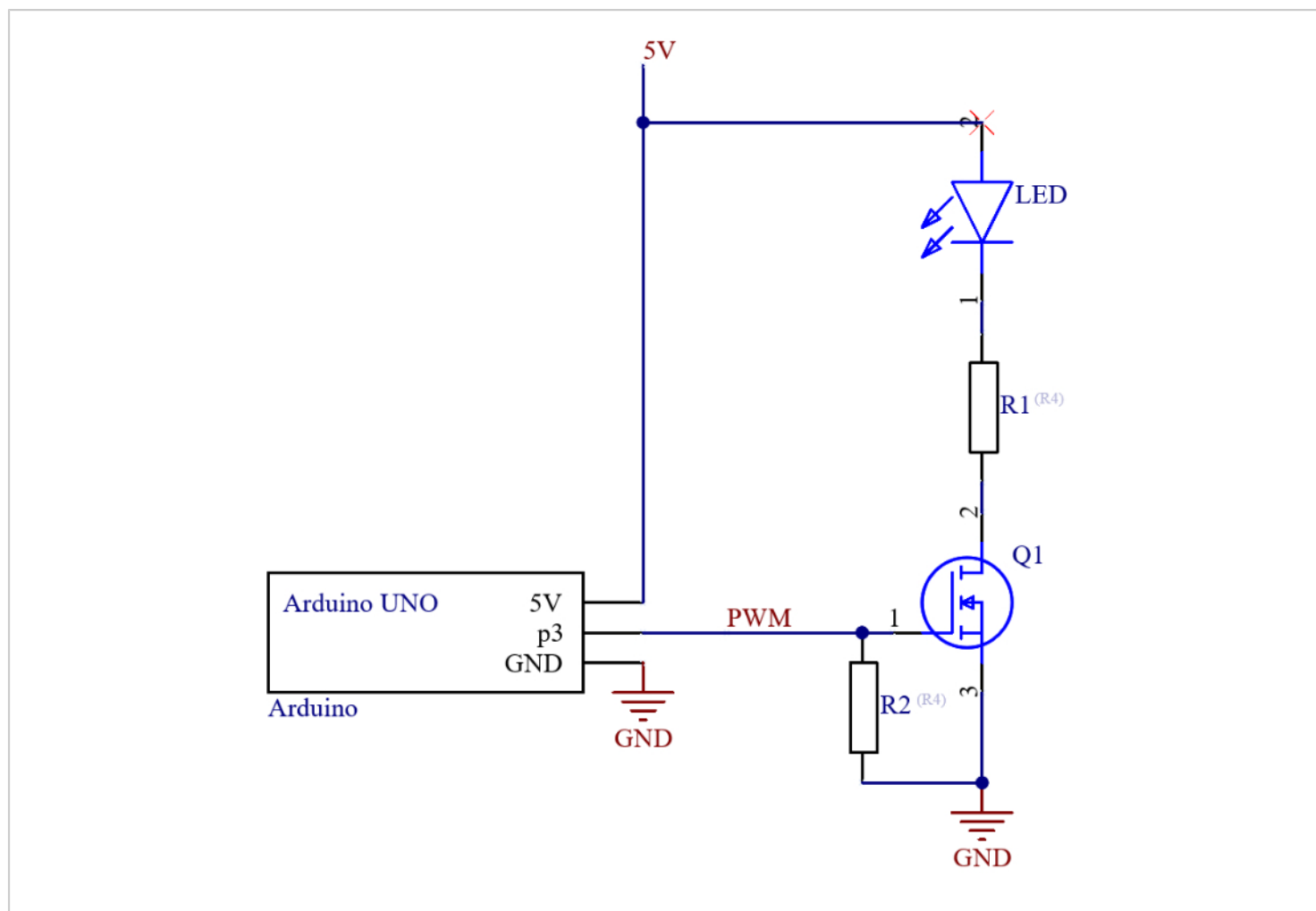


Figure 2: LED control circuit. R1 - current regulation resistor, R2 - pull-down resistor for the MOSFET gate, Q1 - n-channel MOSFET. [Please click here to view a larger version of this figure.](#)

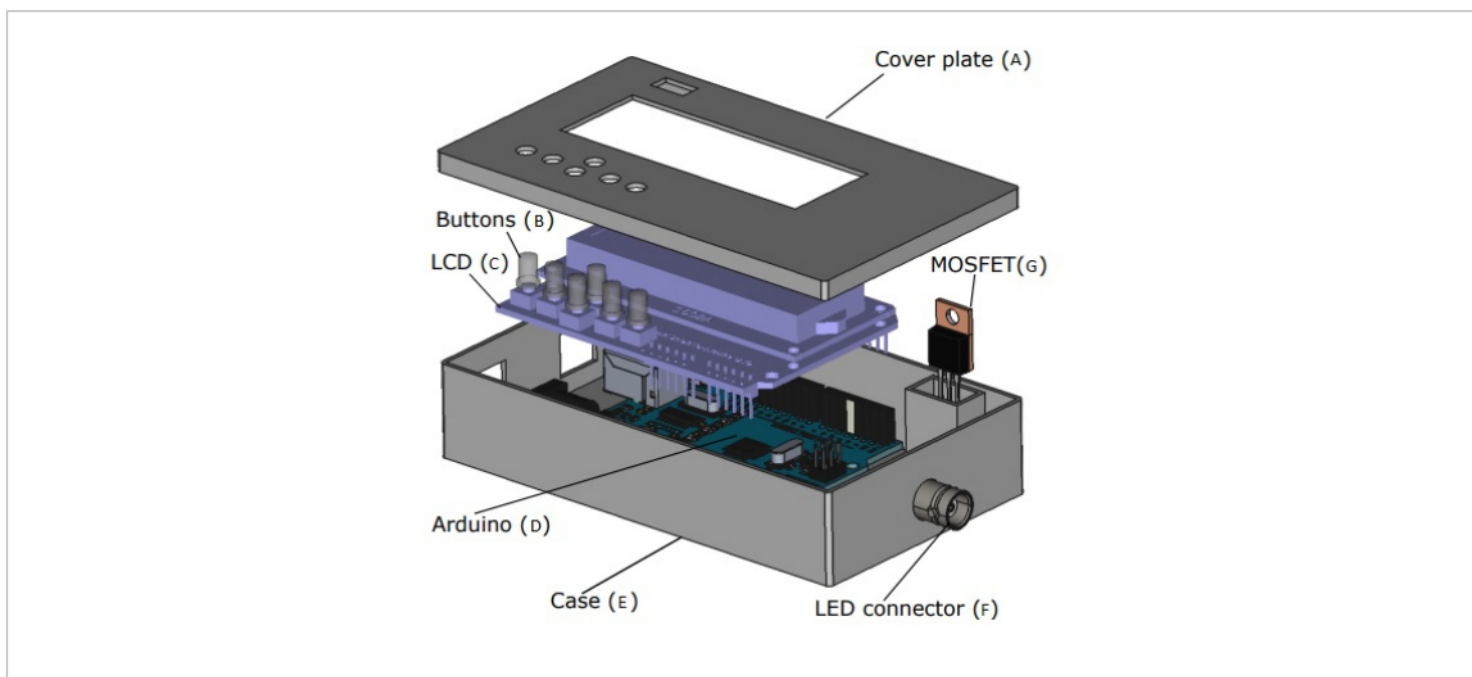


Figure 3: Case 3D view. (A) 3D printed cover plate, (B) 3D printed buttons, (C) LCD keypad shield for Arduino, (D) Arduino, (E) 3D printed case, (F) LED connector, (G) Channel MOSFET. [Please click here to view a larger version of this figure.](#)

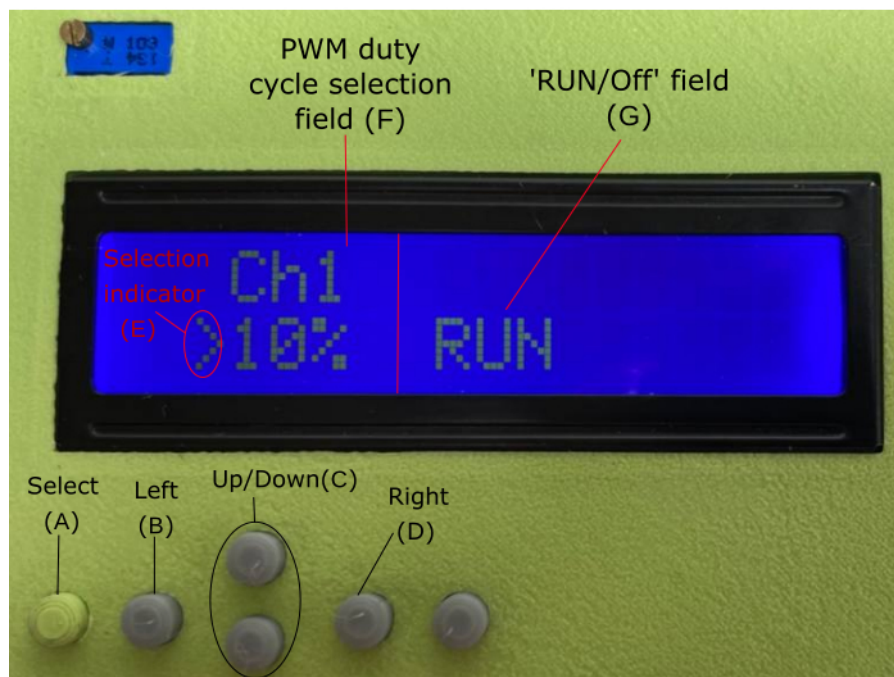


Figure 4: Software. (A) Select button, (B) Left button, (C) Up/Down button, (D) Right button, (E) Selection indicator, (F) PWM duty cycle selection field, (G) RUN/OFF field. [Please click here to view a larger version of this figure.](#)

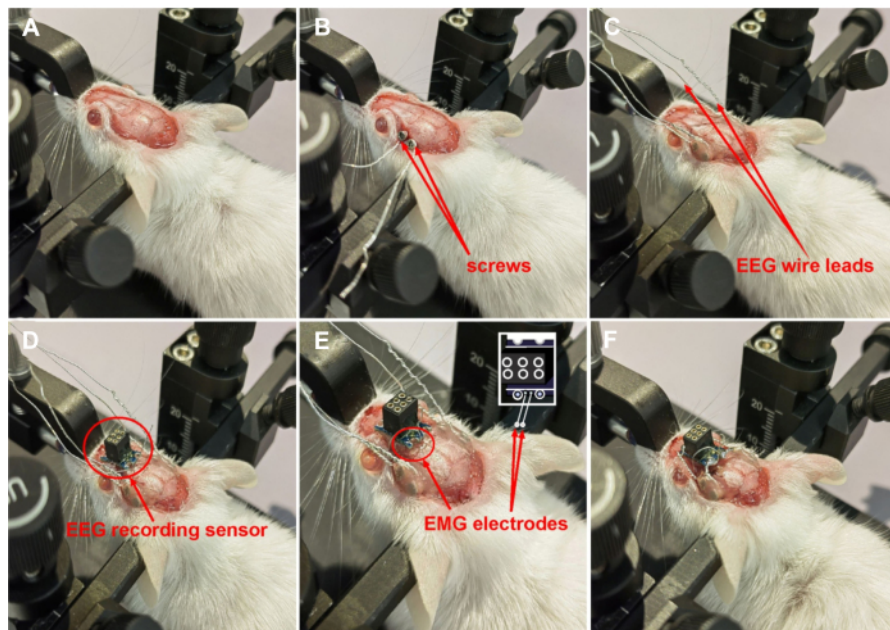


Figure 5: Implantation of an EEG recording system. (A) Skull preparation; (B) Implantation of screws; (C) Fixation of screws with the dental acrylic; (D) Fixation of the EEG recording sensor; (E) Implantation and fixation of the EMG electrodes on the back of the orbicularis oculi muscle; (F) The soldered electrodes to the silver-plated recess of the sensor. [Please click here to view a larger version of this figure.](#)

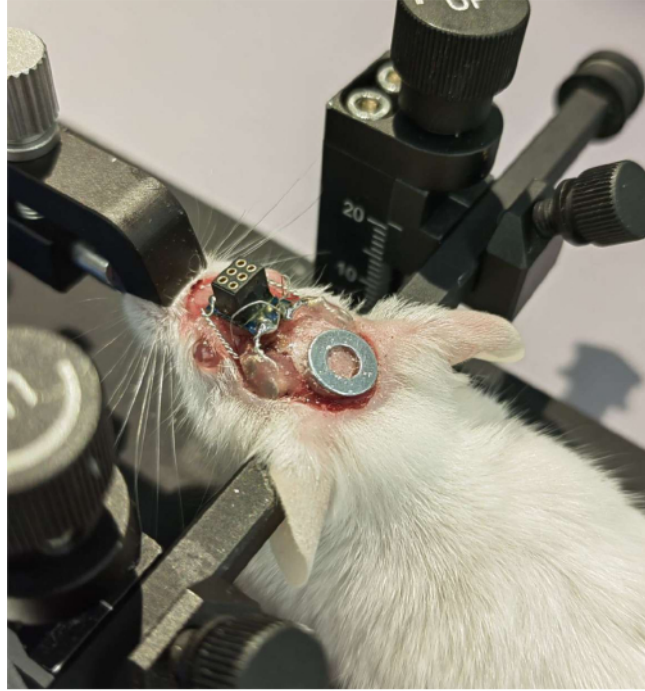


Figure 6: Fixation of a plate for PBM on the occipital bone of the skull. [Please click here to view a larger version of this figure.](#)

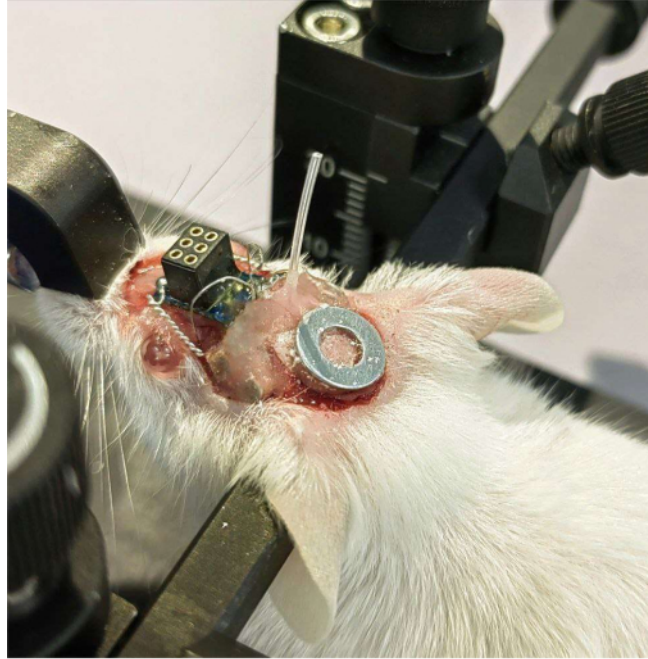


Figure 7: Implantation of a chronic catheter into the right lateral ventricle. [Please click here to view a larger version of this figure.](#)

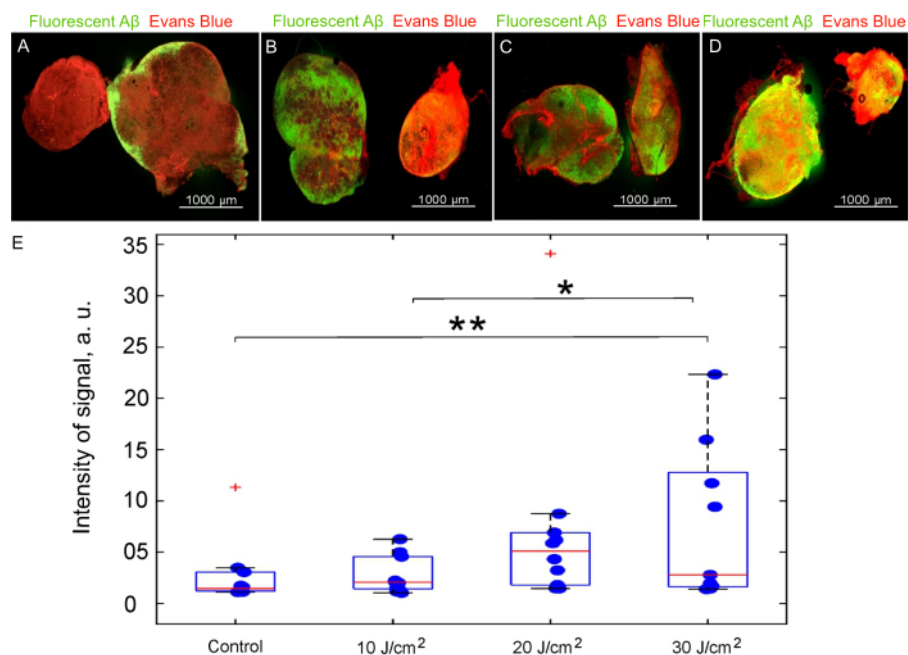


Figure 8: The effects of different LED 1050 nm doses on lymphatic removal of fluorescent A β from the brain to dcLNs. (A-D) Representative images of dcLNs from the (A) control group and the PBM group with (B) 10 J/cm², (C) 20 J/cm², and (D) 30 J/cm²; (E) Quantitative analysis of the accumulation of fluorescent A β in dcLNs from the tested groups (n = 8, one-way ANOVA with Duncan post hoc test, **p < 0.01, *p < 0.05). [Please click here to view a larger version of this figure.](#)

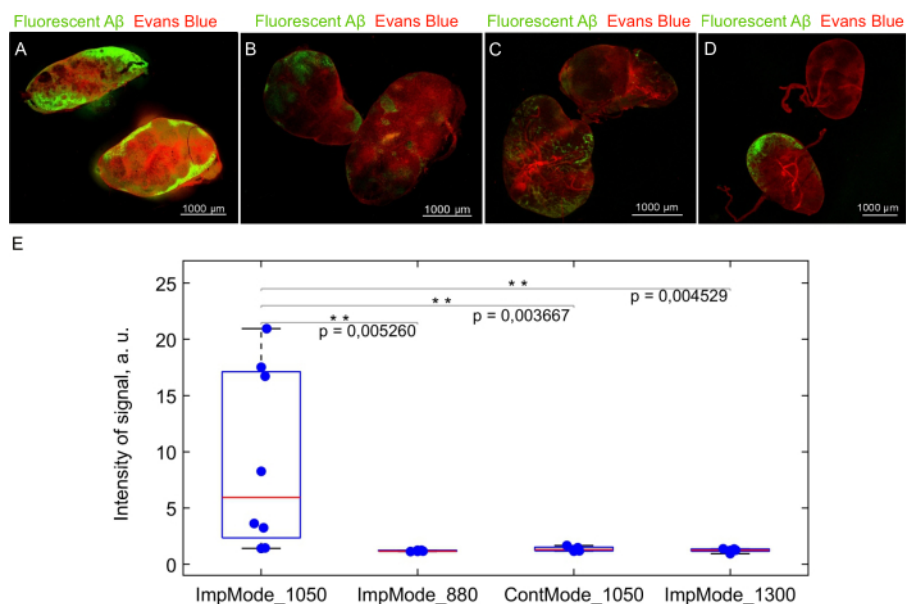


Figure 9: The effects of different LED wavelengths on lymphatic removal of fluorescent Aβ from the brain to dcLNs.

(A-D) Representative images of dcLNs from the PBM group with impulse mode 1050 nm (ImpMode_1050, A), 880 nm (ImpMode_880, B), 1300 nm (ImpMode_1300, C) and continuous mode 1050 nm (ContMode_1050 nm, D); (E) Quantitative analysis of accumulation of fluorescent Aβ in dcLNs from the tested groups (n = 8, one-way ANOVA with Duncan post hoc test, **p < 0.01). [Please click here to view a larger version of this figure.](#)

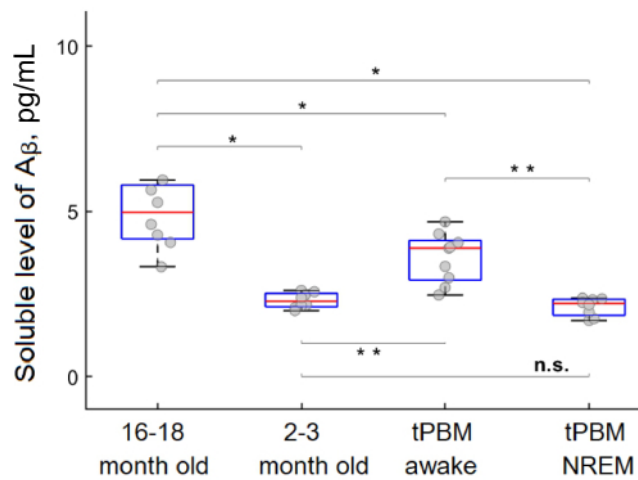


Figure 10: The PBM effects (LED 1050 nm in impulse mode, 30 J/cm²) during NREM and awake state on the level of soluble Aβ (pg/mL) in mouse brains of adult and aged mice. A one-way ANOVA with Duncan post hoc test, **p < 0.01, *p < 0.05. [Please click here to view a larger version of this figure.](#)

Supplementary Coding File 1: Icd1key.ino [Please click here to download this File.](#)

Discussion

MLVs are an important target for the development of innovative technologies for modulation of the brain's drainage and removal of cellular debris and wastes from the brain, especially in aged subjects whose MLV function declines^{1,22}. In a homeostatic state, deep sleep is associated with the natural activation of brain tissue cleansing^{13,14}. Therefore, it is obvious to expect that stimulation of MLVs during deep sleep will be more effective than during wakefulness^{15,16}. The new non-invasive and portable technology of tPBM under EEG control of sleep for stimulation of lymphatic removal of soluble Aβ from the brain of aged male mice is presented here.

The small size of the photo platform (7 mm x 11 mm) and its light weight (1 g) allow it to be firmly and securely mounted

on the heads of mice, maintaining their natural motor activity. This also eliminates the need to use anesthesia during tPBM. The tPBM during deep sleep can be performed using any commercial device for an EEG control of sleep stages. Thus, our technology allows performing the study in a home cage, preserving the most natural conditions for animals.

In the first step, an LED wavelength of 1050 nm was randomly selected for the study of the effective dose of photo-effects on lymphatic removal of fluorescent Aβ from the brain to dCLNs in awake adult (2-3 month old) mice. The choice of this wavelength is related to the previous data on effective stimulation of MLVs with a 1267 nm laser^{8,10,27,28,29,30,31,32,33,34}. This wavelength promotes the direct generation of the singlet oxygen in small quantities in brain tissue and in its meninges, which is one of the mechanisms underlying the PBM of MLVs^{8,28,34}. However, the biological effects of both a 1267 nm laser and an LED 1050 nm are related to the generation of singlet oxygen

excitation³⁵. Indeed, the bands of a 1267 nm $\pm$ 20 nm laser and a 1065 nm $\pm$ 15 nm LED are related to formation of the singlet oxygen from the ground state of an oxygen molecule. A band of a 1267 nm laser pumps an oxygen molecule directly into the first excited singlet state, while a band of 1065 nm LED corresponds to additional vibrational energy, which decays very fast, releasing heat to the environment. Moreover, water absorption in the 1065 nm wavelength is tenfold lesser than in the 1267 nm wavelength, which makes it more favorable for biological research³⁵. Furthermore, a 1050 nm LED is commercially available in the market and much cheaper compared with the rare and expensive 1267 nm laser. Note that the emission band of a used 1050 nm LED in this study was 40 nm wide, and it only partially overlaps with a singlet oxygen band. However, we demonstrated the significant effect of tPBM on the lymphatic removal of fluorescent A β from the brain to dcLNs using a 1050 nm LED (30 J/cm²). The light dose 30 J/cm² was determined to be the most effective compared to the light doses 10 J/cm² and 20 J/cm² based on the higher intensity of a fluorescent signal from A β in dcLNs. Thus, a 1050 nm LED can be very useful for the application of the tPBM technique to humans, which is consistent with the common trend to use LEDs instead of lasers for PBM^{36,37}.

In the second step, the research aimed to answer the question of which light wavelengths are most effective for lymphatic elimination of fluorescent A β from the brain of adult awake mice. This step of study was performed using a portable photo platform with different light wavelengths (880 nm, 1050 nm, 1300 nm) and modes (1050 nm in continuous and pulse regimes) with the same dose (30 J/cm²). These wavelengths were chosen due to the widespread use in clinical practice for tPBM (880 nm) and new expensive LEDs (1300 nm), which could potentially be clinically significant. In addition,

the pulsed and continuous modes only for the 1050 nm LED as a light resource were compared. These data clearly demonstrate that only a 1050 nm LED in pulse mode vs. other wavelengths and the 1050 nm in continuous mode causes a significant lymphatic removal of A β from the brain to dcLNs. These results are consistent with the findings of other researchers, who also indicate the advantage of using a pulsed PBM to effectively achieve biological effects^{38,39,40}.

In the final step, when the effective wavelength, dose and mode were selected, the study of the effects of a 1050 nm LED in pulse mode on the level of soluble A β in the brain of aged mice before and after the 10 day-course of tPBM under an EEG control of NREM and wakefulness was carried out. The immunoassay analysis revealed that the soluble A β content in the brain was significantly higher in aged mice (16-18 months old) compared with adult mice (2-3 months old). It is interesting to note that the 10-day course of tPBM during deep sleep, but not during awake, effectively reduced the soluble A β level in the brains of aged mice to the level of soluble A β in the brains of adult mice. An increase in the A β content in brain tissue of healthy mice and rats with age has also been noted in other studies^{25,26}, which may be associated with an age-related decrease in the MLV functions leading to ineffective cleansing of brain tissue from this toxic protein^{1,22}. The more effective tPBM of removal of soluble A β from brain tissue in sleeping mice compared to awake ones can be explained by the natural activation of brain tissue drainage during sleep. The MLV morphology changes significantly with age, leading to reduced lymphatic removal of metabolites from the aged brain²². However, tPBM during sleep helps restore the lymphatic removal of soluble A β from the brain tissue of aging mice to the level of adult animals.

Considering the scattering of light energy when passing through the skull, tPBM limits its effects only on MLVs without penetrating deep into the brain tissues. However, despite the fact that the lymphatic vessels have not yet been found directly in the brain tissues of humans and animals, growing evidence is emerging indicating the presence of the cerebral lymphatic system^{41,42,43,44}. This explains the results showing the removal of toxins (blood and A β) from the deep structures of the brain (the ventricular system, the hippocampus) to MLVs and further to the periphery (dcLNs)^{5,7}. Over the course of a century, knowledge has accumulated, indicating a close connection between the brain's drainage and the peripheral lymphatic system⁴⁵. Even in the absence of a generally recognized lymphatic network in the central nervous system, there are facts indicating lymphatic removal of macromolecules from the deep parts of the brain to the periphery^{1,2,3,4,5,6,7}. This also explains why tPBM, acting only on MLVs, stimulates the clearance of blood and A β from the brain^{5,7}.

In summary, this study presents a portable technology of tPBM under EEG control of sleep designed to photo-stimulate the removal of toxins using the example of soluble A β from the brain of aged mice with the ability to compare the therapeutic effectiveness of different optical resources. The technology can be used in the natural condition of a home cage without anesthesia, maintaining the natural motor activity of mice. These data open up new prospects for the development of non-invasive and clinically promising photo-technologies for correcting age-related changes in the MLV functions and brain's drainage processes and for the effective cleansing of brain tissues from metabolites and toxins. The proposed technology for photostimulation of the brain's drainage and lymphatic removal of toxins from the brain has limited effects on MLVs due to the scattering of

light energy when passing through the skull. Therefore, the technology can be used to develop new methods for treating brain diseases associated with MLV dysfunction. The most pronounced stimulating photo-effects on MLVs are observed in deep (NREM) sleep, which requires the use of PBM under EEG control. This creates certain difficulties (technical, programming) for simultaneous control of the onset of deep sleep and the supply of light exposure.

Disclosures

The authors have nothing to disclose.

Acknowledgments

This research was supported by a grant from the Russian Science Foundation (No. 23-75-30001).

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