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Transcranial low-level laser therapy (810 nm) temporarily inhibits peripheral nociception: photoneuromodulation of glutamate receptors, prostatic acid phophatase, and adenosine triphosphate

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Abstract. Photobiomodulation or low-level light therapy has been shown to attenuate both acute and chronic pain, but the mechanism of action is not well understood. In most cases, the light is applied to the painful area, but in the present study we applied light to the head. We found that transcranial laser therapy (TLT) applied to mouse head with specific parameters (810 nm laser, 300 mW/cm^2, 7.2 or 36 J/cm^2) decreased the reaction to pain in the foot evoked either by pressure (von Frey filaments), cold, or inflammation (formalin injection) or in the tail (evoked by heat). The pain threshold increasing is maximum around 2 h after TLT, remains up to 6 h, and is finished 24 h after TLT. The mechanisms were investigated by quantification of adenosine triphosphate (ATP), immunofluorescence, and hematoxylin and eosin (H&E) staining of brain tissues. TLT increased ATP and prostatic acid phosphatase (an endogenous analgesic) and reduced the amount of glutamate receptor (mediating a neurotransmitter responsible for conducting nociceptive information). There was no change in the concentration of tubulin, a constituent of the cytoskeleton, and the H&E staining revealed no tissue damage. [DOI: 10.1117/1.NPh.3.1.015003]

Keywords: transcranial low-level laser therapy; photobiomodulation; pain threshold; nociception; Von Frey filaments; tail flick test; cold plate test; formalin injection; adenosine triphosphate; prostatic acid phosphatase; metabotropic glutamate receptor.

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1 Introduction

Photobiomodulation (PBM) or low-level laser therapy (LLLT) has been shown to have multiple beneficial therapeutic effects including pain relief. The absorption of red to near-infrared (NIR) photons by mitochondrial cytochrome c oxidase (Cox) leads to an increase in electron transport, and thus an increase adenosine triphosphate (ATP), NO, ROS, and Ca^{2+}, enhancing cellular energy supply and stimulating signal transduction.[1] These biochemical changes lead to macroscopic effects with medical applications such as: promotion of cellular proliferation, wound healing, decrease in inflammation, and pain inhibition.[2]

When the target cells are neurons, the process can be termed photoneuromodulation. In this study, we show that photoneuromodulation of the central nervous system (CNS), alters the release of mediators of nociception resulting in reducing pain.

The efficacy of PBM in clinical relief of pain has been confirmed in several literature reviews and systematic studies: chronic neck pain,[3] tendonitis,[4] chronic injuries to ligaments,[5] lower back pain,[6,7] and myofascial pain.[8] For clinical pain relief, the usual wavelengths are in the red range ($\lambda = 632.8$ and 670 nm) and in the NIR ($\lambda = 780$; 810 to 830; 904 nm).[3]

In principle, PBM can reduce pain by two distinct mechanisms: light can interact directly with neurons[9] and light can have an anti-inflammatory action.[10] Light with appropriate parameters, including wavelength, energy density, intensity, time, and other dose components activates signaling pathways, leading to a cascade of metabolic effects and a reduction in

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inflammatory markers such as prostaglandins and interleukins.[11] These markers of inflammation stimulate C-fibers; therefore, their inhibition leads to pain attenuation.[12,13] Treatment with PBM, using relatively high-energy densities, is often used to elicit an analgesic effect.[14] Light is usually applied to the painful area and is thought to cause temporary inhibition of axonal transport in small diameter nerve fibers (Aδ and C).

Chow et al.[9] proposed that temporary structural changes were generated in neurons that absorbed the photons. Such changes in the architecture of the neurons were called "varicosities," involving clustering of mitochondria and disruption of microtubules, associated with reduced mitochondrial membrane potential and the blockade of axonal transport in small diameter fibers.

The mechanism of pain reduction for PBM in the peripheral nervous system is supported by reports such as: (1) increased activity of acetylcholinesterase in the synapses[15] (acetylcholinesterase degrades the nociceptive neurotransmitter acetylcholine); (2) increase in serotonin synthesis[16] and β-endorphin synthesis;[9] (these are neurotransmitters related to pain relief); (3) temporary suppression of action potentials evoked by bradykinin[17] (bradykinin is a substance present in inflammatory processes); 4) inhibition of Na^+/K^+-ATPase,[15] (Na^+/K^+-ATPase is responsible for maintaining the resting membrane potential of the neuron). The speed of transmission is reduced and the latency period is increased in nerves continuously irradiated by a laser ($\lambda = 830$ nm).[18]

Recently, remarkable results have been found using transcranial laser therapy (TLT) as a noninvasive treatment for brain injuries or diseases. TLT improved motor recovery after stroke in rats[19] and humans;[20] significantly reduced recovery time in traumatic brain injuries,[21] with little evidence of side effects.[22] Encouraging results have been obtained for some degenerative diseases of the CNS such as familial amyotrophic lateral sclerosis,[23] Parkinson's disease,[24] and Alzheimer's disease.[25]

The evidence for pain reduction connected with modulation of neural activity and release of neurotransmitters, in addition to the recent findings of positive outcomes of TLT in many brain diseases led us to hypothesize that TLT could modulate brain activity related to the perception of painful stimuli. In this report, we quantify the pain attenuation and photoneuromodulation of biochemical markers after TLT in several models of pain in mice.

2 Material and Methods

2.1 *Animals*

All animal procedures were approved by the Institutional Animal Care and Use Committees of Massachusetts General Hospital (protocols # 2014N000040 and 2014N000255) and met the guidelines of the National Institutes of Health. Adult male BALB/c mice (weight 20 to 24 g; Charles River Laboratories, Wilmington, Massachusetts) were used in this study. The animals were housed with five mice per cage and were maintained on a 12-h light–12-h dark cycle with access to food and water *ad libitum*.

2.2 *Illumination Parameters*

The laser irradiation was performed using a diode laser at 810 nm (Micro DioDent 810 Hoya ConBio, Fremont, California) equipped with a silica fiber. The mice were placed in a transparent immobilizer (Flat Bottom Rodent Holders, Kent Scientific, Torrington, Connecticut). TLT was performed with the tip of the optical fiber in contact with the animal's skin and positioned perpendicularly to the bregma point. The laser was set to continuous wave with an irradiated area of 1 cm^2 and 300 mW/cm^2 power density (measured with an optical power meter sensor model S145C, ThorLabs); the irradiation time and energy density, varied between animal groups (Table 1). There are no well-established parameters for TLT in mice. Therefore, we chose parameters taking into account the attenuation of light passing through the layers of skin and skull bone as described by Sousa et al.[26]

Table 1 Transcranial laser therapy parameters.

Groups	GC	GL24	GL120
Local		Head, transcranial	
Application type		In contact	
Irradiated area (cm^2)	0	1.0	1.0
Power (mW)	0	300	300
Irradiation time (s)	0	24	120
Energy (J)	0	7.2	36
Energy density (J/cm^2)	0	7.2	36

The experimental groups were classified according to the dose of light that each animal received. Thus, the animals treated with TLT for 24 s (7.2 J/cm^2) are referred to as laser-treated group #1 (GL24); in turn, animals treated with TLT for 120 s (36 J/cm^2) are referred to as laser-treated group #2 (GL120) and those not receiving laser irradiation as the control group (GC). Mice in GC group underwent the immobilization procedure and the laser was positioned in contact with the animal's head for 120 s; however, the laser was not turned on. Each group consisted of 15 animals: five animals were used to quantify the pain threshold [von Frey filaments (vFF)] before TLT and 1, 2, 3, 6, and 24 h after TLT; 5 were used for nociceptive tests (cold, heat, formalin) 2 h after TLT; and 5 were used for the quantification of ATP, immunofluorescence staining and hematoxylin and eosin (H&E). For the biochemical assays, 5 of the 15 animals of each group were randomly chosen for tissue fixing and extraction 2 h after TLT.

2.3 *Pain Threshold Evaluation with von Frey Filaments*

The mice were placed individually in a cage ($10 \times 20 \times 15$ cm) whose floor was constructed with meshed metal wire (squares of 5×5 mm). The stimulus was applied when vFF were pressed perpendicularly upward, through the cage floor, against the plantar hind paw skin and held for approximately 3 s, until it slightly buckled.

In order to assess pain threshold, the animals were placed and stimulated as described above. The mice were successively stimulated starting with the weakest and progressing to the strongest filaments. The sequence of stimuli was stopped when the mouse reacted with immediate movement or licking of the hind paw. The force of the last used filament was considered the pain threshold. The group pain threshold was the average of individual thresholds.

The vFF, used in this study to estimate pain threshold, were manufactured for our research team. While the applied force of a set of commercial filaments ranged from 0.008 to 300 gf (gram-force), the set we developed had 20 tips with forces ranging from 7.6 to 117.3 gf; this made our set of filaments fit better to use on mice; therefore, the pain threshold was more precisely determined. The filaments were made of inexpensive materials available in stores, and information about manufacturing, calibration, and validation of this set of filaments were recently published.[27]

2.4 *Nociceptive Tests: Cold Plate, Tail-Flick Using Radiant Heat, and Formalin Injection*

Two hours after the laser irradiation of the 15 animals (subgroups of five mice from each group) as described previously, the nociceptive tests were conducted. One single test was performed per day, on consecutive days, in the following order: cold plate, radiant heat, and formalin injection.

The cold plate test evokes moderate pain due to low temperature (4°C ± 1°C) and the evaluation of nociception can be indirectly measured by the latency period until the reaction of the animal. The cold plate used in this experiment consisted of a layer of ice covered with a sheet of aluminum foil inside a Styrofoam box. Mice were confined in a region of 10 × 10 × 10 cm inside the box. The temperature of this apparatus was constantly monitored by a thermometer in the aluminum surface in the region where the mice were confined, and the experiments were conducted only when the temperature was between 3°C and 5°C. When the temperature was higher than 5°C, the ice was replaced and another mouse was placed in the box only when the temperature was again between 3°C and 5°C. At the time when the animals were placed in contact with the surface of the cold plate, the timer was triggered. The tests were terminated when the animals reacted by licking the paw or moving the front legs. The time to response of each animal was recorded and used to calculate the mean latency for the group.

The tail flick from a radiant heat source is a pain sensitivity test characterized by elevation of temperature in the midpoint of the tail surface. For this test, we used a high-powered laser (1 W, 1 mm^2, 730 nm) beam pointed at the midpoint of the tail of the mice placed in a transparent immobilizer (Flat Bottom Rodent Holders, Kent Scientific, Torrington, Connecticut) allowing only the motion of the animal's tail. The time until the animal withdrew the tail out of the beam was recorded with a timer, and was used to calculate the average latency time for the group.

In order to evaluate inflammatory nociception, 10 μL of 2% formalin was injected into the right hind paw, causing inflammatory pain in two stages. The first phase is acute pain (0 to 15 min after injection), characterized by a short duration and visible inflammation of the paw. The second phase is tonic pain (15 to 60 min after injection); it lasts longer than the acute pain, however, it cannot be classified as chronic pain since it lasts for only a few tens of minutes. After the tonic phase is finished, there is no further pain and the animal shows a behavior similar to that of an animal in which there was no injection of formalin. The time that the animals remained with right hind paw raised was measured in two intervals of observation: from 0 to 5 min postformalin (for acute pain), and from 15 to 20 min after the injection (for tonic phase). Table 2 summarizes the information on the assessment of pain threshold and nociceptive tests.

Table 2 Description of the experiments for assessment of pain threshold and nociceptive tests.

Experiment	Pain threshold	Nociception tests
Nociceptive stimulus	von Frey filaments	Cold plate, radiant heat and formalin injection
Response to stimuli	Flinch	Time to react
Stimuli times	Before PBM and 1, 2, 3, 6, and 24 h after PBM	2 h after PBM

2.5 *Tissue Fixing and Extraction*

The euthanasia and extraction of tissues was performed 3 h after transcranial laser irradiation in animal subgroups ($n = 5$) which had not participated in any nociceptive stimulation that day. In order to perform the extraction, the animal was anesthetized by intramuscular injection of 30 μL ketamine and xylazine solution (ratio 10:1). The heart of the animal was accessed by surgery to open the chest. The internal tissue fixation was carried out by two transcardiac infusions: the first with 10 mL of phosphate buffered saline (PBS) and the second with 10 mL of 4% paraformaldehyde. The heart pumps PBS and paraformaldehyde around the circulation, promoting whole body fixation and leading to the death of the animal. After euthanasia of the animal, the whole mouse brain was extracted and incubated for 48 h in 4% paraformaldehyde.

2.6 *Tissue Staining and Image Acquisition*

After fixation, the brains were grossly cut in two along the medial fissure, and the right hemispheres were embedded in paraffin and sectioned sagittally in the medial region, to produce eight samples with 5-μm thickness from each brain. Thus, the brain of each animal generated four pairs of samples, and each pair of samples was assigned to one of three antigens or H&E (in duplicate). The brain slices stained with H&E were used to verify the possible presence of lesions or inflammation resulting from transcranial laser irradiation. The slices were adhered to transparent slides and received the standard protocol for fluorescence immunohistochemistry or staining with H&E practiced in the Photopathology Laboratory, Wellman Center for Photomedicine, Massachusetts General Hospital. The samples stained with H&E were digitally imaged with a NanoZoomer (Hamamatsu, Japan) to form digital images with 40-fold magnification in each dimension, that is, 1600 times magnification.

2.7 *Immunofluorescence and Confocal Microscopy*

Immunofluorescence staining was indirect. The mouse tissue antigens chosen were: tubulin, to look for changes in the cytoskeleton, as described by Chow et al.;[9] metabotropic glutamate receptor type 1 (Glut1 antibody), because this mediates a neurotransmitter involved in the conduction of nociceptive information; and prostatic acid phosphatase (PAP) as an endogenous analgesic. The primary antibodies used were from chicken and specifically bound to each of the antigens. The secondary antibodies were from goat antichicken fluorescein labeled (excitation at 494 nm and emission at 521 nm).

Samples labeled tubulin, GLUT1 and PAP were digitally imaged with a confocal microscope (Olympus FV1000, multiphoton confocal microscope Olympus Corporation of the Americas). The resolution of these images was enough to observe changes in the amounts or distribution of GLUT1, PAP, and tubulin.

The immunofluorescence images captured by confocal microscopy presented in this paper were taken from the somatosensorial regions of the cortex. The images had the fluorescence digitally colored and the colors were arbitrarily chosen such as red, yellow, green, and blue represented, respectively, GL1, PAP, tubulin and the dye DAPI, a marker of cell nuclei used to facilitate and quantifying cell numbers (Fig. 1).

The quantification of the antigens was analyzed by fluorescence intensity in the images. All the images were the same size (1×1 mm) and same resolution (pixel size). As these images are from the tissue surface, there is a particular area of the image without tissue and, therefore, without fluorescence. To enable the comparison of several images, regardless of the amount of tissue present, the quantification of fluorescence was performed in a square region (0.2×0.2 mm) in the center of the images, which always presented biological tissue.

2.8 *Adenosine Triphosphate Quantification*

The ATP present in the brain of mice was quantified with a luminescence assay from the reaction of luciferin and luciferase (Cell Titer Glo assay, Promega). The left hemisphere of the brain (opposite to the hemisphere that was used for tissue imaging) was macerated and centrifuged (4000 rpm, 15 min) immediately after the extraction. After centrifugation, the sample was divided into two parts: the liquid supernatant and turbid pellet. The supernatant was frozen in liquid nitrogen to preserve the biochemical characteristics and used for ATP quantification.

2.9 *Statistical Analyses*

Initially, all data was tested with the Shapiro–Wilk and Levene's tests, that proved the hypothesis of normal distribution and homoscedasticity (homogeneity of variance), respectively. Comparisons between groups (GC versus GL24; GC versus GL120, GL120 versus GL24) at each evaluation time were performed by analysis of variance using one-way ANOVA. Significant differences were determined by the posthoc Tukey test honestly significant difference. For all tests was set a value of 5% for significance, i.e., a 95% confidence that the difference was real and not by chance.

3 Results

3.1 *Temporary Increase in Pain Threshold*

The pain threshold was evaluated immediately before (0 h) and after (1, 2, 3, 6, 24 h) laser irradiation. We found that the two groups receiving TLT showed an increase in pain threshold; however, the pain threshold of the control group remained unchanged (Fig. 2). It can be seen in this graph that the effect

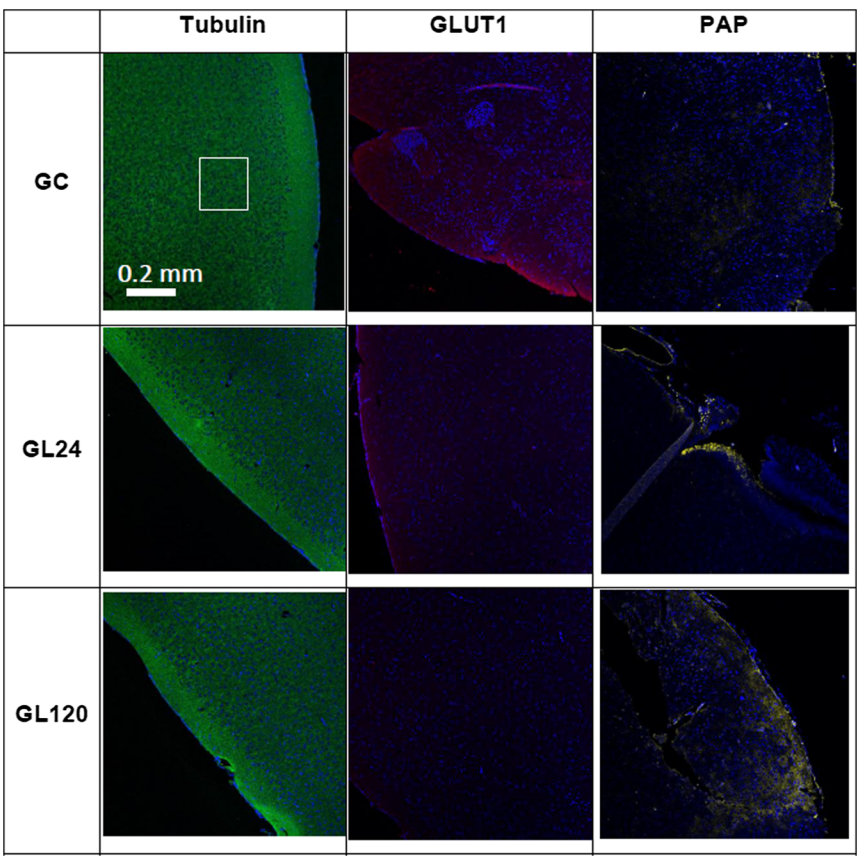

Fig. 1 Examples of images from tissues of each animal group with each one of the marked antigens: tubulin (green), PAP (yellow) GLUT1 (red) and cell nuclei (blue, marked in all images). The GC/tubulin image shows the square (200×200 μm) where the fluorescence quantification was performed.

of laser irradiation was not immediate, since there was a continuous increase in the pain threshold with time till a peak effect at 2 or 3 h post-PBM. The mean pain threshold GC, GL24, and GL120 immediately before irradiation with laser (808 nm) was respectively 21.5, 17.8, and 24.4 gf; this difference was not statistically significant. The second measurement was performed 1 h after transcranial irradiation, and the two treated groups had small increases compared with pre-irradiation values when compared with the control group. The highest value of the pain threshold for GL24 was 38.3 gf, 3 h after irradiation; and for GL120 was 65.2 gf, 2 h after irradiation. The values at 6 h postirradiation showed that GL24 presented a similar response to mechanical stimuli to the control group; nevertheless, GL120 continued to show an increased pain threshold. The final measurement of the series, at 24 h after irradiation, indicated that all groups returned to the baseline pain threshold (0 h), showing that the effect was temporary in nature.

There were significant differences ($p < 0.05$) in pain threshold between the GL120 and GC at measurements performed 1, 2, 3, and 6 h after transcranial irradiation; comparing GL120 and GL24 there was significant differences 1, 2, 3, and 6 h after transcranial irradiation; comparing GL24 and GC there were no significant differences at any time point.

3.2 *Cold Plate Test*

The latency period until the animal's reaction in response to low-temperature thermal stimulation was evaluated 2 h after the TLT; and the averages and SD of the groups ($n = 5$) are shown in Fig. 3. Animals in the GC took 30 s in average to respond to nociception evoked by low temperature by licking forepaws. In GL24 group, the animals took longer to react to nociception (72 s); however, this difference was not statistically significant when compared with GC. The GL120 group showed a mean latency of 161 s, which was significantly higher than both the other groups.

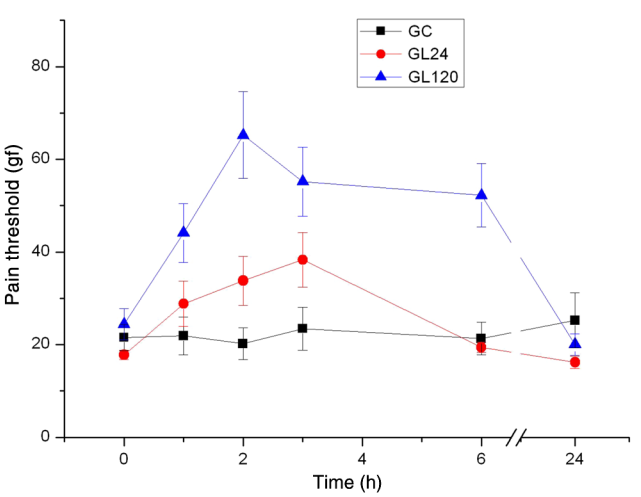

Fig. 2 Average pain threshold and standard error of the mean (SEM) before irradiation and 1, 2, 3, 6, 24 h after the irradiation for the control group (sham irradiation) and those receiving transcranial irradiation of 7.2 or 36 J. Maximum average threshold occurred between 2 and 3 h after irradiation. After 24 h the pain threshold returned to baseline in all groups.

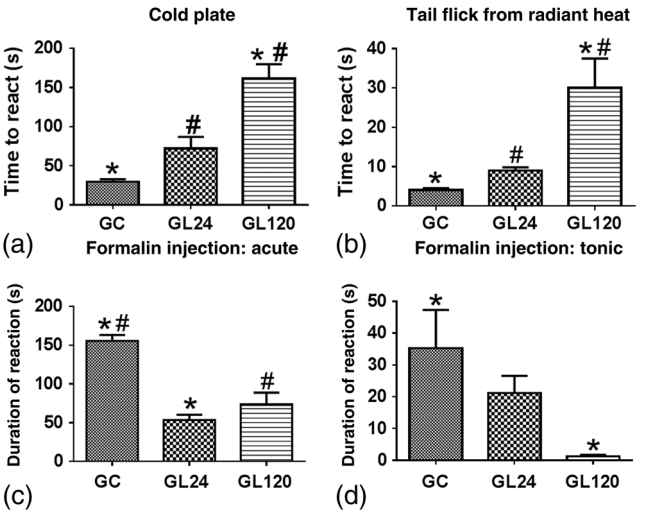

Fig. 3 Mean and standard deviation (SD) of the groups ($n = 5$) for nociception tests: (a) time to reaction of the mice in response to low temperature stimulus to the foot; (b) time to reaction of the mice in response to high temperature stimulus to the tail; (c) time which the mice remain with the inflamed paw raised during the acute phase after formalin injection; (d) time which the mice remain with the inflamed paw raised during the tonic phase after formalin injection. The symbols * and # indicate pairs of statistically different groups, $p < 0.05$.

3.3 *Tail-Flick Using Radiant Heat*

The animals from the control group moved the tail away from the radiant heat spot more rapidly than the groups which received TLT. This shows that TLT could increase the tolerance for pain evoked by high-temperature thermal stimulus. The control group took, on average, 4.1 s to remove the tail while GL24 and GL120 took 9.0 and 30.1 s, respectively, to remove the tail. The GL120 group had a significantly higher latency than other two groups (Fig. 3).

3.4 *Formalin Injection in Hind Paw*

During the acute phase of the inflammatory pain induced by formalin injection in the right hind paw, the time that the animal remained with the inflamed paw raised, thus avoiding contact thereof with the surface, is indicative of the hyperalgesia experienced by the animal. In the first 5 min (300 s), just after formalin injection, the control group had an average time of 155.4 s with the swollen paw raised. The duration of the foot-raising response in the groups that received TLT, were 53.4 s for GL24 and 73.6 s for GL120 (Fig. 3) and was significantly shorter than the GC group.

During the tonic phase, the GL120 group hardly manifested any behavior that indicated pain. The average time that this group remained with the paw raised was only 1.2 s, a result compatible with the absence of pain. The GC and the GL24 groups had times of 35.3 and 21.2 s with the paw raised and this difference was significant only between GC and GL120 (Fig. 3).

3.5 *Effects on Pain Neuromarkers and Adenosine Triphosphate*

3.5.1 *Glutamate*

Glutamate is an excitatory neurotransmitter involved in nociceptive signaling. The decrease in metabotropic glutamate receptors

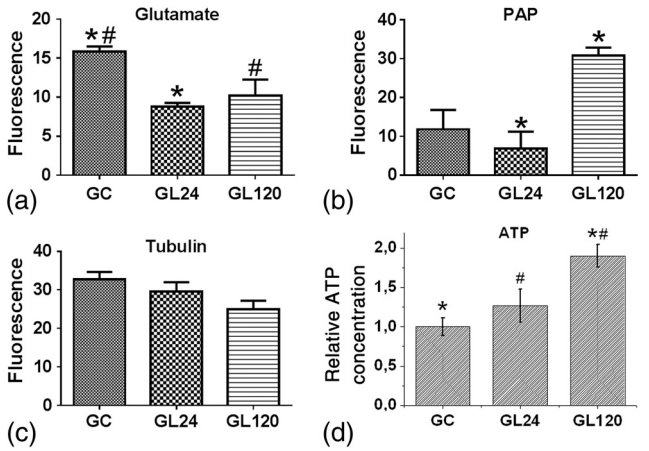

Fig. 4 Mean and SD of the groups ($n = 5$) for neuromarkers fluorescence and relative ATP concentration: (a) glutamate, (b) PAP, (c) Tubulin, (d) ATP. The symbols * and # indicate pairs of statistically different groups, $p < 0.05$.

in the tissue, quantified with marked GLUT1 in immunofluorescence images, leads to decreased conduction of nociceptive stimuli due to the reduced frequency of action potentials.

The amount of GLUT1 was significantly higher in the control group than in the groups receiving TLT (Fig. 4). This effect is consistent with the increase in pain threshold observed in GL24 and GL120 groups when compared with the GC.

3.5.2 *Prostatic acid phosphatase*

PAP is an enzyme responsible for hydrolyzing AMP (adenosine monophosphate) to extracellular adenosine. The amount of PAP in the cerebral cortex of the GL120 mice was significantly higher than in GL24 and GC animals (Fig. 4). The highest amount of the endogenous analgesic PAP occurred in the GL120 group. This result is also consistent with the decrease in nociception in this group as demonstrated in the behavioral tests.

3.5.3 *Tubulin cytoskeleton*

No significant variations in the amount of tubulin were found in the animal groups of this study. Also, we did not observe formation of varicosities (tubulin clusters) in the brain samples of the animals submitted to transcranial laser irradiation, as the ones observed by Chow et al.,[9] with studies *in vitro* in ganglion neurons. For the detection of varicosities, we used the same criterion as Chow et al.[9]: tubulin clusters were defined as fluorescence intensities higher than 20 and sizes larger than 150 μm^2, and we developed a process in ImageJ software[28] in order to analyze the images.

The failure to find varicosity formation can be interpreted as further evidence that analgesia by light therapy may have more than one mechanism of action depending on the cells and tissues irradiated. Another explanation for not finding varicosity formation is that the fluence which effectively reached the cortical neurons in transcranial irradiation was much lower than the fluence used in the *in vitro* work of Chow et al.[9]

3.5.4 *Adenosine triphosphate*

The ATP concentration in the cerebral hemispheres was quantified and normalized to the concentration in the control group. The ATP concentration of the GL24 group was 27% higher than the concentration in the control group; however, this difference was not statistically significant. The GL120 showed a concentration of ATP 90% higher than the control group and 56% higher than GL24, and both differences are statistically significant (Fig. 4).

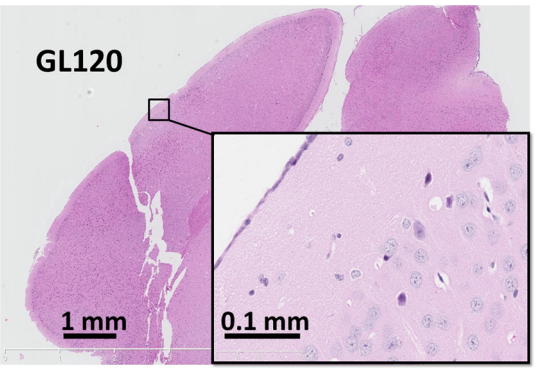

Fig. 5 Sagittal section of the right hemisphere of the mouse brain and detail of the sample with zoom 40×. We found no morphological changes caused by transcranial irradiation even in the point of the highest intensity in animals of GL120 (group with the highest dose).

3.5.5 *Hematoxylin and eosin staining*

The digital images obtained from H&E stained tissue samples could show in the evaluation of the morphology, any changes in tissue such as thermal damage, inflammation and disruption that could be caused by TLT. However, as can be seen in the images in Fig. 5, we found no morphological changes caused by transcranial irradiation. These images show that the doses used in these experiments were sufficient to produce photoneuromodulation without any damage to the brain.

4 Discussion

The experiments described in this article show the effectiveness of photoneuromodulation using transcranial NIR ($\lambda = 808$ nm) to suppress nociception in mice. The assessment of pain threshold in mice demonstrated that photoneuroinhibition of pain was a temporary and reversible process, with a peak between 2 and 3 h and finished by 24 h after transcranial laser irradiation. Other nociception tests showed that the attenuation of pain due to photoneuromodulation in CNS occurred in various parts of the animal body (front paws, hind paws, and tail) and for different kinds of stimuli such as mechanical stress, cold, heat and inflammation.

Various markers have been used in the CNS to monitor analgesic effects. Glutamate is an excitatory neurotransmitter present in the CNS and is correlated with synaptic plasticity and cell death and chronic pain.[29] Glutamate binds to the ionotropic glutamate receptors (NMDA, AMPA), and also to mGluR (G protein-coupled)[30] lowering the excitation threshold of synaptic transmission. The concentration of free glutamate in the extracellular environment and the number of mGluR on the synapses determines the degree of excitatory stimulus.[31] Glutamate may become toxic if the concentration rises excessively high. When the concentration of glutamate increases in the spinal cord dorsal horn, AMPA receptors are activated and rapidly depolarize the membranes of neurons in this region. Therefore, there is a decrease in the threshold to trigger the transmission of pain signal to higher-order neurons.[32] When

NMDA receptors are activated by glutamate the intensity of the stimuli response is potentiated and long-term potentiation is elicited, leaving the organism in a hyperalgesic state.[33] The clearance of glutamate from the synaptic region is extremely important to maintain the capacity of neurons to respond to different stimuli.

PAP is an enzyme whose transmembrane isoform is a marker of nociceptive stimuli.[34] PAP is present mainly in the nonpeptide afferent fibers of type C nociceptors and can dephosphorylate extracellular AMP producing adenosine and downregulate painful sensations. The analgesic effect of PAP only occurs in the presence of adenosine receptor type A1. Type A1 receptors are highly expressed in nociceptive fibers of PNS and in the CNS, promoting inhibition of calcium cellular intake and reducing glutamate release.[35] Consequently, PAP modulates nociceptive responses related to thermal and mechanical stimuli. Moreover, Sowa et al.[36] showed that PAP gene knockout mice showed a thermal and mechanical hyperalgesia when compared to the control group of wild-type mice. When PAP is found in higher concentrations, it indicates that there was a decrease in nociceptive signaling.[34] Thus, PAP is an important marker for analgesia in studies with assessment of nociception and chronic pain models.

ATP is a very important molecule since it is the direct energy source in cells; it is synthesized by the mitochondria during the process of oxidative phosphorylation. Several studies on cultured cells and in isolated mitochondria demonstrated the influence of red and NIR low-level light in mitochondrial physiology and ATP synthesis. The PBM process starts when Cox, a molecule associated with energy production, absorbs photons in the red-NIR region of spectrum.[37] Increasing the amount of ATP could be indirectly responsible for mediating analgesic process since its leads to production and release of endogenous analgesics like PAP. Our results corroborate these previous findings.

Nociception tests in animals are very important to evaluate new drugs, therapies or procedures. One of the difficulties in assessing pain is that it cannot be quantified directly—can only be estimated by analyzing the response to a specific stimulus. There are various models designed to measure nociceptive response to various stimuli or to monitor potential of nociceptive signaling pathways.[38] One of the simplest models for assessment of pain is mechanical stimulation with vFF.[39]

The cold plate test is intended to measure the sensitivity of experimental animals to physical nociceptive stimuli, caused by low temperatures. On the other hand, the radiant heat test evaluates nociception related to different receptors that are stimulated by high temperature. Another stimulus that causes pain is contact with irritant agents (substances that initiate the inflammatory process). Formalin injection is one of the most traditional models to evaluate the inflammatory pain and to test procedures to reduce its effects.

In this study we present evidence of the efficacy of TLT to reduce pain sensation in the whole body evoked by various types of stimuli. The laser irradiation to the brain cortex, specifically in the somatosensory cortex, was supported by evidence of photoneuromodulation of neuromakers related to nociception.

The mechanisms for the suppression of nociception can be explained by biochemical changes initiated by the absorption of photons. The release of the endogenous analgesic PAP is upregulated by ATP since its synthesis requires sufficient energy to be available. An increased amount of endogenous analgesics PAP could reduce the pain signaling; moreover, the decrease in the number of pain receptors (mGluR) reinforces this antinociception route.

The effectiveness of photoneuromodulation depends on the dose used as evidenced in our experiments, where GL24 shows intermediate results between GC and GL120. The effects of transcranial irradiation take some time to occur and there is a possibility that the timing of the tissue extraction was not the ideal time to observe the optimum manifestation of the biochemical changes.

Another mechanism that has been evaluated for suppression of nociception is the formation of varicosities of tubulin. In our studies, we found no significant change in concentrations of tubulin and or the formation of agglomerates. This finding supports the view that pain reduction by photoneuromodulation may occur by more than one mechanism and that there may be fundamental differences between photoneuromodulation of the CNS and the PNS. Moreover, the amount of light energy required to cause photoneuromodulation *in vivo* and *in vitro* is likely to be different.

Supported by our previous work on TLT in rodents[26] we decided to use a power density of 300 mW/cm^2, which is relatively high when compared with the power density used in other studies. The power density used in this work still did not increase the brain temperature enough to cause any thermal damage or local inflammatory process.

A major difficulty faced by transcranial phototherapy (or transcranial photodiagnosis) is the impossibility of delivering light to deep regions of the CNS while maintaining a low intensity at the cortical surface. Nevertheless, we believe that transcranial photoneuromodulation could be further studied and developed as a complementary modality for treating various types of pain in humans and animals.

A common concern about applications TLT in humans is that the light intensity needed to elicit photoneuromodulation can be harmful. Light intensity must be enough to go through the skull but should not increase brain temperature. TLT has been applied in humans for stroke,[40] traumatic brain injury,[41] neurodegenerative diseases,[42] depression, and even to augmentation of cognitive brain functions.[43–44] It has been observed that low power at the brain surface is enough to obtain interesting results without brain warming or damaging. We believe that this research could serve as a theoretical and experimental basis for many scientific, translational and clinical studies about transcranial photoneuromodulation to treat pain.

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