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Pain management using photobiomodulation: mechanisms, location, and repeatability quantified by pain threshold and neural biomarkers in mice

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Abstract

Photobiomodulation (PBM) is a simple, efficient and cost-effective treatment for both acute and chronic pain. We previously showed that PBM applied to the mouse head inhibited nociception in the foot. Nevertheless, the optimum parameters, location for irradiation, duration of the effect, and the mechanisms of action remain unclear. In the present study, the pain threshold in the right hind-paw of mice was studied, after PBM (810 nm CW laser, spot size 1 cm^2 or 6 cm^2, 1.2–36 J/cm^2) applied to various anatomical locations. The pain threshold, measured with von Frey filaments, was increased more than 3-fold by PBM to the lower back (dorsal root ganglion, DRG), as well as to other neural structures along the pathway such as the head, neck and ipsilateral (right) paw. On the other hand, application of PBM to the contralateral (left) paw, abdomen and tail had no effect. The optimal effect occurred 2–3 hours post-PBM and disappeared by 24 hours. Seven daily irradiations showed no development of tolerance. Type 1 metabotropic glutamate receptors decreased, and prostatic acid phosphatase and tubulin-positive varicosities were increased as

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shown by immunofluorescence of DRG samples. These findings elucidate the mechanisms of PBM for pain and provide insights for clinical practice.

Keywords

Transcutaneous low level laser (light) therapy; photobiomodulation; photoneuromodulation; nociception; pain threshold; von Frey filaments; dorsal root ganglion; pain biomarkers

1. INTRODUCTION

Throughout the world, injuries, diseases and a variety of chronic or degenerative conditions affect billions of people with acute and/or chronic pain [1]. Better therapies are required for painful conditions such as degenerative orthopedic conditions, neuropathic pain, and chronic central pain. In general, pharmaceutical therapies (painkillers) decrease the intensity and severity of the symptoms but have many undesired side-effects [2]. Moreover, long-term treatment tends to decrease effectiveness as the subject develops drug tolerance [3]. There has recently been an epidemic of opiate overdose-induced deaths, many of which were in individuals originally prescribed opiates for chronic pain [4].

Photobiomodulation therapy (PBMT) also known as low-level laser/light therapy (LLLT), is a rapidly growing approach to stimulate healing [5, 6], increase tissue regeneration [6] and reduce pain [7] and inflammation [8]. PBMT has no known side-effects, and as yet has shown no long-term adaptation to the treatment [9, 10].

Typically, PBMT uses exposure of tissues to low-intensity light (power densities in the region of 1–100 mW/cm^2) for a few minutes [9]. The wavelengths employed are mostly in the red or near-infrared (NIR) spectral regions, and although lasers were originally used exclusively, in recent years light emitting diodes (LED) have become popular [11]. During this exposure, light is absorbed by molecules in the cells called chromophores, such as cytochrome-c-oxidase, located inside mitochondria. Light absorption induces changes in cell signaling pathways and activation of transcription factors. Application of NIR light for pain has clinical advantages such as deeper penetration, which allows noninvasive, transcutaneous delivery [12]. Reports of adverse events in PBMT are extremely rare or non-existent, and this therapy has been approved by FDA (and other national health agencies around the world) for various indications [11].

Over the past decades, much PBM research has focused on demonstrating efficacy in animal models of potentially painful conditions. We previously reported [6] that PBM (810 nm laser, 300 mW/cm^2, 7.2 or 36 J/cm^2) delivered to the head of mice could decrease the reaction to a potentially painful stimulus in the foot, evoked either by light pressure (von Frey filaments), cold plate, or inflammation (formalin injection), or in the tail (evoked by heat). Therefore, the goals of the present study were to establish a reliable treatment regimen (optimum bodily location of irradiation, and the optimum dose or energy density) and to investigate the mechanisms of action underlying the effect of light on pain threshold. The time of onset, duration, and repeatability of the procedure were investigated. This study also

aimed to investigate the effects of PBM on neurological markers (mGluR1 and PAP) involved in pain signaling in the peripheral nervous system (the dorsal root ganglia, DRG).

2. MATERIAL AND METHODS

2.1. Animals

All animal procedures were approved by the IACUC of the Massachusetts General Hospital (protocols # 2010N000202, 2014N000255, 2014N000040) and met the guidelines of the National Institutes of Health. Adult male BALB/c mice (weight 20–24 g; Charles River Laboratories, Wilmington, MA) were used in this study. The animals were housed at five mice per cage and were maintained on a 12-hour light–12-hour dark cycle with access to food and water *ad libitum*.

2.2. Design of the research

We designed a sequence of experiments to find the most suitable anatomical region for applying the light, the best dose (energy density) and exposure time for PBM quantified by pain threshold and variation in expression of neural biomarkers (Figure 1). This was accomplished by four experiments:

Experiment 1: We irradiated each animal in one of 7 different body regions using a $1cm^2$ spot delivering 810 nm CW laser to give 36 J/cm^2 at 300 mW/cm^2 power density and 120 s exposure time. These regions were: head, neck, dorsal root ganglion (DRG), tail, abdomen, ipsilateral right hind-paw, contralateral left hind-paw. The anatomical local of irradiation varied in different animal groups (Table 1). PBM was performed with the tip of the optical fiber positioned perpendicularly to produce a $1cm^2$ spot on the mouse skin. Each group consisted of 5 animals, and we used a set of von Frey filaments to evaluate the pain threshold in the right hind paw. There are no well-established parameters for transcutaneous PBM 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 [12]. A group of mice underwent similar procedures but they did not receive any laser irradiation and they are referred as the Group Laser Sham (GLS).

Experiment 2: We irradiated the DRG by transcutaneous irradiation with a 6 cm^2 spot in order to irradiate a large number of dorsal root ganglions at the same time. Laser irradiation was applied with 4 different energy doses (0, 1.2, 6 and 30 J/cm^2) evaluating the pain threshold at the hind paw at different times 0, 1, 2, 3, 6 and 24 hours post-PBM. Laser treatment was performed using the same diode laser at 810 nm, equipped with quartz-silica fiber and magnifying lens and a collimator which created an irradiation spot with diameter 2.8 cm (area 6 cm^2) onto the lumbar region DRG with a power density of 50 mW/cm^2. Detailed irradiation parameters are given in Table 1.

The experimental groups were classified by duration of irradiation. Thus, the animals treated during 24, 120, 600 s are named, respectively, as Group Laser-treated 24 s (GL24), Group Laser-treated 120 s (GL120), Group Laser-treated 600 s (GL600). Those that received similar procedure but with the laser turned off (sham), receiving 0 s of real laser irradiation

are referred as Group Laser 0 s (GL0). Each group consisted of 5 animals which were tested to quantify the pain threshold by von Frey filament tests at right hind paw.

Experiment 3: Mice received PBM (6 J/cm^2 at 50 mW/cm^2, 6 cm^2 spot) delivered to the DRG and the pain threshold was evaluated (similarly to the other experiments) at 3 hours post-PBM. This procedure was repeated daily for 7 days.

Experiment 4: Mice were sacrificed at three different times (3, 24 hours post single PBM, and 48 hours post 7X daily repeated PBM in Experiment 3). The PBM was delivered to the DRG of mice (spot 6 cm^2, fluence 6 J/cm^2, 50 mW/cm^2). The neuromarkers (tubulin, prostatic acid phosphatase and type 1 metabotropic glutamate receptors) were quantified at the three different times. Sham group had no laser irradiation and tissue extraction occurred 3 h after sham laser irradiation.

2.3. Laser irradiation

The laser irradiation was performed using a diode laser at 808 nm (Micro DioDent 808 Hoya ConBio, Fremont, CA) equipped with a silica fiber. The power densities were measured with an optical power meter (sensor model S145C, ThorLabs - USA). First, all mice were shaved on all the body regions where laser irradiation would be applied. Next, the mice were placed in a transparent immobilizer (Flat Bottom Rodent Holders, Kent Scientific, Torrington - Connecticut - USA). The power densities and irradiation times used in all the experiments did not result in any heating of the skin.

2.4. Pain threshold evaluation with von Frey filaments

The mice were placed individually in a cage (10 × 20 × 15 cm) whose floor was constructed with metal wire mesh (squares of 5 × 5 mm). The stimulus was applied when each von Frey Filament (vFF) was pressed perpendicularly upward, through the cage floor, against the right plantar hind paw skin until the filament slightly buckled and was held in place for approximately 3 s.

Mice were successively stimulated starting with the weakest filament and progressing to the strongest filament. The sequence of applying 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 von Frey filaments, 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 more suitable to use for mice, therefore, the pain threshold was more precise. The filaments were made of inexpensive materials available in stores, and information about manufacturing, calibration and validation of this set of filaments were published in reference [13].

2.5. Tissue extraction

Euthanasia of the mice and extraction of tissues were performed at 3 or 24 hours after a single PBM, or 48 hours after 7 daily repeated laser irradiations (or sham irradiation). To perform extraction, the animal was deeply 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 pumped PBS and paraformaldehyde around the circulation, promoting whole body fixation and leading to the death of the animal. After euthanasia of the animal, the lumbar region of spinal cord and DRG were extracted and incubated for 48 hours in 4% paraformaldehyde.

2.6. Tissue staining and image acquisition

After tissue fixation, the DRG were embedded in paraffin and sectioned in the medial region, to produce eight samples with 5 μm thickness from each DRG. Thus, each DRG generated four pairs of samples, and each pair of samples was assigned to immunofluorescence with antibodies to one of three antigens or H&E (in duplicate). The DRG slices stained with H&E were used to verify the possible presence of lesions or inflammation resulting from laser irradiation. The slices were adhered to transparent slides and were subjected to fluorescence immunohistochemistry or staining with H&E in accordance with protocols of the Photopathology Laboratory, Wellman Center for Photomedicine, Massachusetts General Hospital.

Immunofluorescence staining was indirect. The mouse tissue antigens chosen were: tubulin, to assess changes in the cytoskeleton, as described by Chow et al [14]; metabotropic glutamate receptor type 1 (mGluR1 antibody), because this mediates a neurotransmitter involved in the conduction of nociceptive pain signals; and prostatic acid phosphatase (PAP), an endogenous analgesic. The primary antibodies used were from chicken, with dilution factor of 1:300, and specifically bound to each of the antigens. The secondary antibodies were from goat anti-chicken fluorescein labeled (excitation at 494 nm and emission at 521 nm). Samples labeled tubulin, mGluR1 and PAP were digitally imaged with a confocal microscope (Olympus FV1000 - multi-photon confocal microscope Olympus Corporation of the Americas). The resolution of these images was enough to observe changes in the amounts or distribution of mGluR1, PAP and tubulin using the software ImageJ.

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. Statistical analyses

Initially all data was tested with the Shapiro-Wilk and Levene’s tests, that proved the hypothesis of normal distribution and homocedasticity (homogeneity of variance), respectively. Groups were compared only with the other ones in the same experiment. Comparisons between each pair of group at each evaluation time were performed by analysis of variance using one-way ANOVA. Significant differences were determined by the post-hoc

Tukey test HSD (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 Experiment 1: Increase in pain threshold after PBM to different body regions

The first experiment was designed to determine the best anatomical region of the body to apply the light to affect the pain threshold in the right hind paw and to determine the time course of the analgesic effect. The irradiation parameters were the same in all locations, and the pain thresholds were evaluated before and after the irradiation.

Figure 2 presents data for the anatomical locations where PBM was effective, compared to the sham group. The lower back, where the DRG connected to the right hind paw is located, had a maximal increase in pain threshold 3 hours after irradiation. Even though the increases in threshold found at 1 and 6 hours post-PBM were somewhat lower than those found at 2 and 3 hours post-PBM, they were still statistically different when compared to sham group. Moreover, some other anatomical regions, such as head, neck and ipsilateral right paw, also showed statistically significant increases in pain threshold at 1, 2, 3 and 6 hours. The pain threshold after irradiation at all locations had returned to baseline levels 24 hours post-PBM.

Figure 2 displays the results for tail, abdomen and left paw, which are the locations with no statistically significant increases in pain threshold at any time after irradiation compared to the sham group.

3.2 Experiment 2: Pain threshold increase due to PBM in lumbar region with different energy densities

The fact that the pain threshold accessed at the right hind paw was reversibly increased after PBM to the lower back may be explained by photoneuromodulation at the DRG. Based on the results of Experiment 1, the lumbar region was chosen for varying the energy density for optimal PBM effect. We irradiated the lower back region of the mice with fixed power density laser (810 nm, 50 mW/cm^2) and varied the exposure times. We used a spot with 6 cm^2 area to increase the chances of extracting irradiated DRG from the removed tissue sample. The four groups of mice (exposure times 0, 24, 120, 600 s) were irradiated with total energy densities 0, 1.2, 6.0, and 30.0 J/cm^2, respectively.

As may be seen in Figure 3, the increases in pain threshold were enhanced after higher doses of PBM (6 and 30 J/cm^2) but not for the low dose (1.2 J/cm^2). The total analgesic effect can be defined as the area under the curve of the pain threshold integrated over the entire evaluation time. Both GL120 and GL600 had significant differences in total analgesic effect when compared to GL24 and GL0.

Experiment 3. Daily treatment repetition

To evaluate if any tolerance was developed to the daily repetition of laser irradiation, we carried out PBM once each day for seven consecutive days and measured the increase in pain threshold after 3 hours. The profile of each successive daily increase in pain threshold was remarkably similar to the profile found on the first day. The baselines and peaks

remained statistically the same (Figure 3). The data suggest that there was no adaptation to the analgesic effects evoked by PBM in the DRG.

3.3 Experiment 4: Molecular markers in DRG after PBM

Hematoxylin and eosin staining of DRG showed that there were no gross changes produced in the tissue such as thermal damage, inflammation or disruption of architecture that could be responsible for the neural effects. Therefore, the doses used in these experiments were sufficient to produce PBM effects without causing any DRG damage.

The increase in the pain threshold must be a result of reversible changes at the cytological and tissue levels in the irradiated tissue (DRG). Tubulin is a constituent of the cytoskeleton and is closely connected with axonal transport in neurons.

The mGluR1 is expressed in pre-synaptic and post-synaptic regions of dendrites and since it is a metabotropic glutamate receptor (mGluR), it is related to slow excitatory transmission in the central nervous system and peripheral nervous system, and when activated, amplifies the excitatory response of neurons. The different distribution and expression of mGluR affects the sensation of pain and transmission of pain signaling.

PAP is a nucleotidase that dephosphorylates AMP to form extracellular adenosine, a mechanism that controls the transmission of pain. For this enzyme to exert its full analgesic effect, it requires the presence of adenosine A1 receptors of type (ADORA1). Therefore, it is possible that PAP also has indirect analgesic activity.

Immunofluorescence was used to evaluate the three biomarkers in the extracted tissues, as seen in Figure 5.

3.3.1 Tubulin cytoskeleton—A single transcutaneous laser exposure of the DRG induced reversible β-tubulin aggregation at 3 hours post PBM. The varicosities, β-tubulin aggregations bigger than 150 μm^2, were seen in tissue extracted 3 hours after a single PBM. However, the varicosities were not present 24 hours post-PBM. In addition, DRG tissue irradiated for seven consecutive days and extracted 48 hours after the last irradiation showed the same number of varicosities as sham group (Figure 6).

3.3.2 mGluR1 expression—The expression of mGluR1 decreased 3 hours after laser irradiation and appeared to return to normal levels with only a non-significant decrease at 24 hours. The fluorescence intensity of stained DRG tissue extracted 3 h after single PBM was statistically lower than tissue from the sham irradiation. Seven daily treatments showed an increase in mGluR1 48 h after the last treatment, but it was not statistically different from the sham group (Figure 6).

3.3.3 PAP expression—The concentration of transmembrane PAP showed a large (threefold) increase 3 hours post-irradiation, but at 24 h after PBM the PAP concentration was not statistically different from the sham group. However, 48 h after seven daily treatments the PAP was significantly increased to the same level (threefold) as that found 3 hours-post single PBM (Figure 6).

4 Discussion

We previously showed [7] that applying PBM with an 810 nm laser spot delivered to the head of the mouse, markedly raised the pain threshold in the hind foot with a maximum effect measured 2 hours later. In that study we also showed an analgesic effect for the response to cold, heat and inflammatory stimuli. These remarkable results spurred us on to further investigate what could prove to be a therapeutically useful finding. In the present study we found, when investigating different anatomical sites for the application of light, that an even bigger effect could be achieved when the light was delivered to the lower back as a spot lying over the DRG. The neck, head (as previously shown) and the ipsilateral foot were also all effective anatomical sites for this analgesic effect. Interestingly, anatomical sites that were completely separated (distant) from the nerve conduction pathway, traveling all the way from the foot to the brain, had absolutely no effect (tail, belly and contralateral foot). Another highly relevant finding from the present study was that there was no development of tolerance or adaptation to the analgesic effect of PBM. Many studies have shown that a range of painkillers (especially opioids such as morphine) can rapidly develop tolerance [15] when tested in mouse and rat models. The dose of PBM that produced the best analgesic response was the highest amongst those tested, suggesting that the penetration of the light to the spinal cord and DRG was necessary for the biological effect to occur. Similarly the requirement for some time to elapse (2–3 hours) before the analgesic effect manifested itself, also suggested that gene transcription and changes in protein expression were involved. This was corroborated by the histology findings in the excised DRG tissue that showed upregulation in the expression of PAP, a well-recognized analgesic mediator [17] that operates via NGF-trkA signaling [16]. Moreover changes were also observed in the number of tubulin varicosities and decreases in the expression levels of mGluR1 showing that distinct biochemical changes were occurring in the irradiated tissue.

It is possible to distinguish two broadly different types of pain: a rapid (or acute) painful sensation that occurs during active stimulation of the tissue, and a different painful sensation, which is slower and remains for some considerable time after the stimulation or injury has occurred. Acute pain is caused by stimulation of pain-receptors (nociceptors) connected to Aδ-fibers which have small diameters (2.0 to 5.0 μm), an intermediate conduction velocity (12.0 to 30.0 m/s) and are poorly myelinated. Aδ-fibers form few branches and are usually specific to a stimulus (pressure, temperature, pH, etc.). Aδ-fibers carry acute and localized pain sensations that occur during the noxious stimulus. Often the painful sensation persists even after the harmful stimulus is gone. The continuing painful sensation is transmitted via the peripheral nervous system by C-fibers. These fibers have extremely small diameter (0.3 to 1.5 μm) and are unmyelinated, so the velocity of the action potentials (AP) through these fibers is very slow (0.5 to 2.0 m/s). C-fibers are highly branched, so the sensation of pain is perceived not only in the damaged area, but also in neighboring regions. The nociceptors connected to C-fibers are polymodal i.e. they are sensitive to various types of stimuli. The primary nociceptive neurons are pseudounipolar, with their cell bodies located in the dorsal root ganglia and an axon with two branches, one to the periphery and the other to the posterior horn of the spinal cord. Dorsal root ganglia have a central role in pain perception as signals are conveyed through the spinal cord to the

thalamus where they synapse with neurons projecting to the primary somatosensory cortex (S1) [18–20].

Endogenous analgesics (enkephalins, endorphins and dynorphins) are peptides, which are found in the ascending pain pathway (periaqueductal gray matter, raphe nuclei and the dorsal horn of the spinal cord). These natural opiates block the release of excitatory neurotransmitters by presynaptic terminals, and hyperpolarize the postsynaptic membrane. Therefore, these substances produce a nonspecific inhibition of pain sensations. One of these endogenous analgesics is prostatic acid phosphatase (PAP), an enzyme that can hydrolyze AMP (adenosine monophosphate) to extracellular adenosine. There are reports that PAP, which was originally identified as a secretory protein enriched in the prostate gland and has been used as a marker of diagnosis and therapy of prostatic cancer [21], is an enzyme which is expressed in DRG [22] and whose transmembrane isoform is a marker of nociceptive stimulus [23]. When PAP is found in higher concentrations, it indicates that there is a decrease in AMP which is a sensitizer for nociceptive neurons, and therefore reduces nociceptive signaling producing an indirect analgesic activity [23].

Modulation of the descending pain pathway by endogenous analgesics causes partial and selective inhibition of pain when neurons of the descending pathway synapse with secondary neurons of the ascending pathway. Thus, inhibition of the ascending pain pathway, stimulation of the descending pain pathway and the release of endogenous analgesics may reduce, or even block nociception.

In recent years, there has been a growing number of reports demonstrating promising outcomes of PBM used for pain [24–26]. Mostly NIR wavelengths have been used [27] due to better tissue penetration of the light, and demonstrated clinically relevant improvement [28]. It has been suggested that the mechanism of pain by PBM was by increasing production of serotonin [29], increased synthesis of beta-endorphin [30] and improved synaptic activity of acetylcholine esterase [31]. Moreover, a number of studies have reported that 830 nm continuous wave laser treatments reduced the velocity of AP conduction, increased latencies in the median and sural nerves and produced analgesic effects [31–33]. Tsuchiya et al and Wakabayashi et al reported that 830 nm continuous wave PBM specifically suppressed nerve conduction in myelinated Aδ and unmyelinated C fibers using a rat model [34, 35]. In these studies, the peripheral axons of the DRG neurons in the affected tissue were used as the target for irradiation. The signal was relayed by secretion of a variety of transmitters, including glutamate, substance P and calcitonin-gene related peptide into the synaptic cleft from the DRG neuron to a secondary neuron, which connects to the thalamus through the contralateral ventral horn [18, 36]. Tao et al reported that the glutamate concentration in the synaptic cleft determined the extent of receptor stimulation and excitatory synaptic transmission [37]. This report indicated that controlling glutamate could be the key to reducing painful stimuli.

In 2007, Chow and collaborators [14], found varicosities, described as intensely β-tubulin-positive clusters, indicative of microtubule disruption caused by PBM irradiation to in vitro cultured DRG cells. Although their laser parameters were similar to some of ours (810 nm laser, 300 mW/cm^2, spot size 1.4 cm^2, 1.5 – 30 J/cm^2), the studies differed in one important

aspect. Chow et al found the effects starting to appear 5 minutes after the short irradiation time (few seconds), while our in vivo data suggest that 2–3 hours was optimal. The varicosity formation was proposed to have important functional implications since mitochondria are carried along microtubules to provide ATP for maintenance, generation, and restoration of the axon potential. Microtubule disruption would therefore block ATP supply, which is essential for the delivery of components of the synaptic vesicles, which are required for neurotransmission.

Glutamate is an excitatory neurotransmitter that triggers synaptic transmission, responsible for the transmission of nociceptive stimuli. In the peripheral nervous system, mGluR1 are present in pre- and post-synaptic dendrites of peripheral afferent fibers. This receptor is coupled to a Gq protein, that further sensitizes the neuron to noxious stimuli and continue stimulating the neurons to release more glutamate, then keeping glutamate carriers and AMPA channels active for a longer time. Then, as PBM decreased glutamate channel concentration after 3 hours, it may be suggested that there was a decrease in sensitization of other neurons, or indirectly, a decrease of the opening of the AMDA channels glutamate, which may have contributed to the analgesic effect.

5 Conclusion

NIR (810 nm) laser irradiation to the lower back of mice reversibly increased the pain threshold in the hind paw up to threefold, with a peak at 2–3 hours post-PBM. A dose response was observed, with 6 and 30 J/cm^2 being effective and 1.2 J/cm^2 being ineffective. Irradiation of the head, neck and ipsilateral paw was also effective, but not irradiation of the abdomen, tail or contralateral paw. The treatment was equally effective when repeated daily for one week. mGluR1 was reduced and PAP and tubulin-positive varicosities were increased as shown by immunofluorescence of DRG at 3 hours post-PBM. The data suggest that PBM to the DRG could be tested in human patients with chronic peripheral pain. The wider analgesic applications of PBM should also be further explored. It would be clinically feasible for instance, to deliver PBM to the back or head of patients who are about to undergo a painful procedure (surgery or dentistry for instance).

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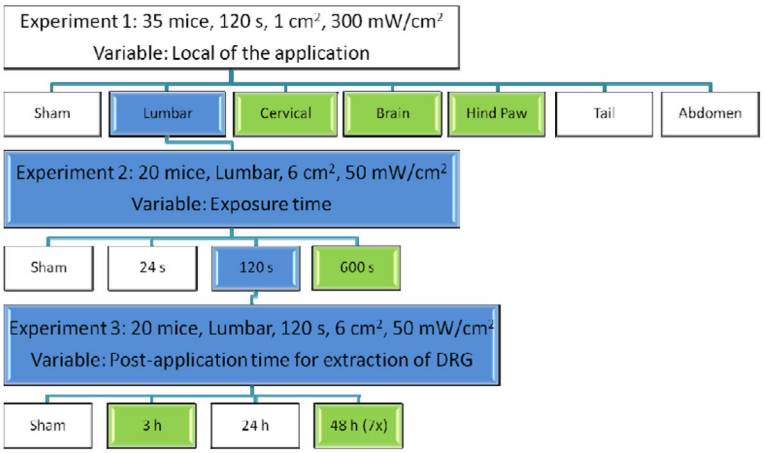

Figure 1. Research design

In the top box, the optical parameters for the first experiment are given, which aimed to find the anatomical site with maximum effectiveness of PBM for increasing pain threshold measured in the right hind paw. The boxes in white depict the situations where there was no effect; the boxes in green are those with observed positive effect. The blue colored boxes show the path that was used to discover the parameters that produced the highest effect.

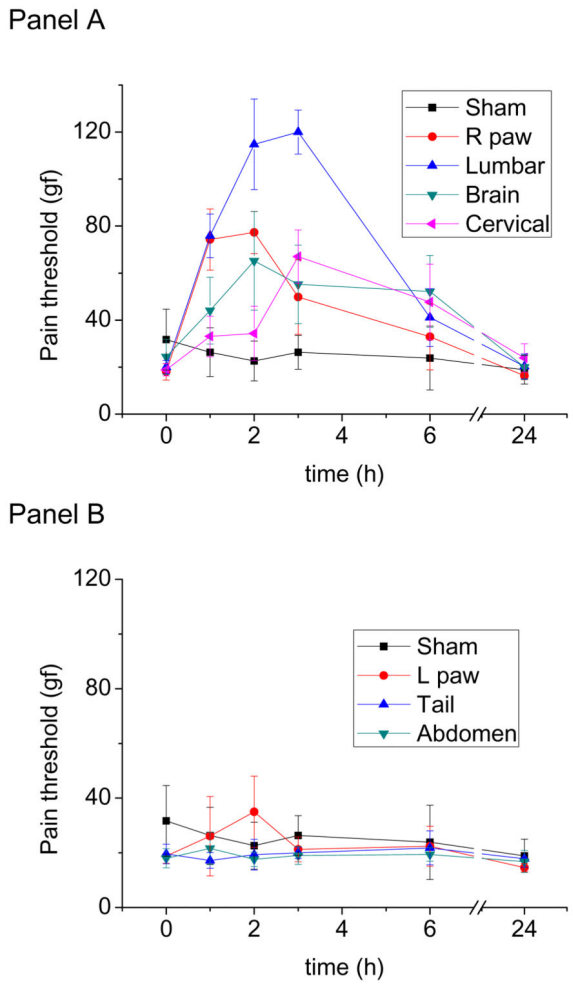

Figure 2. Pain threshold accessed by Von Frey filaments

Time points were before PBM (time 0) and 1, 2, 3, 6, 24 h post-PBM. Panel A presents results for effective sites: lower back, head, neck, ipsilateral right hind paw together with sham group. Panel B presents result for non-effective sites: tail, abdomen, contralateral left hind paw, together with sham group.

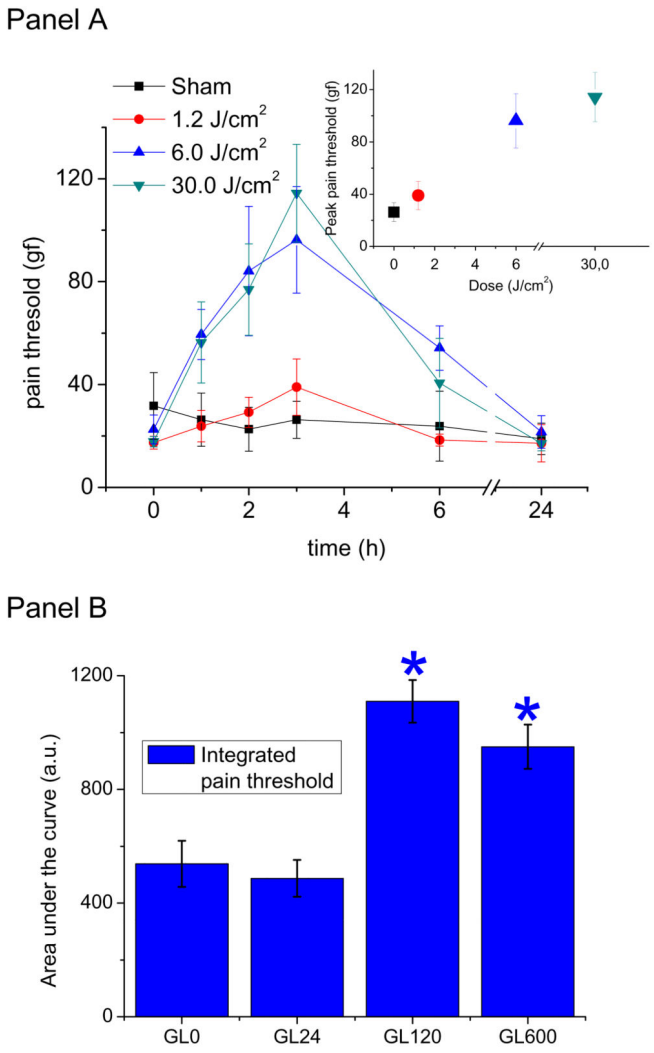

Figure 3.

(A) Pain threshold, means and standard deviations (SD) using Von Frey filaments in groups GL0, GL24, GL120, GL 600 determined before PBM and 1, 2, 3, 6 and 24 hours after exposure to 808 nm laser (total energy densities delivered were 0, 1.2, 6.0, 30.0 J/cm^2) on lower dorsal region and the sham group. B) Integrated pain threshold (Area under the curve of pain threshold, from 0 to 24 h) as an estimation of total pain attenuation effect. The symbols * indicates statistically different from sham group, $p < 0.05$.

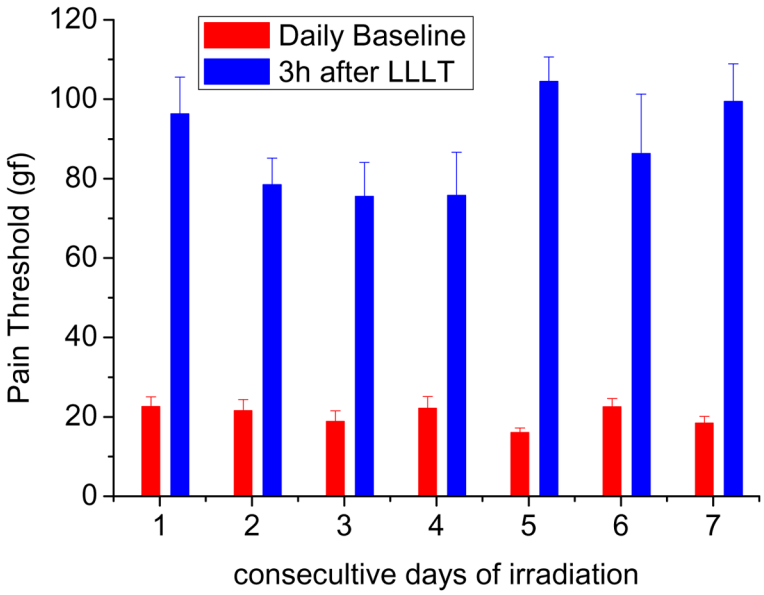

Figure 4.

Increase in pain threshold measured daily, before and 3 hours after PBM, for seven days. The threshold always increased, the baseline average was 20,3 gf and the post-PBM average was 88,0 gf.

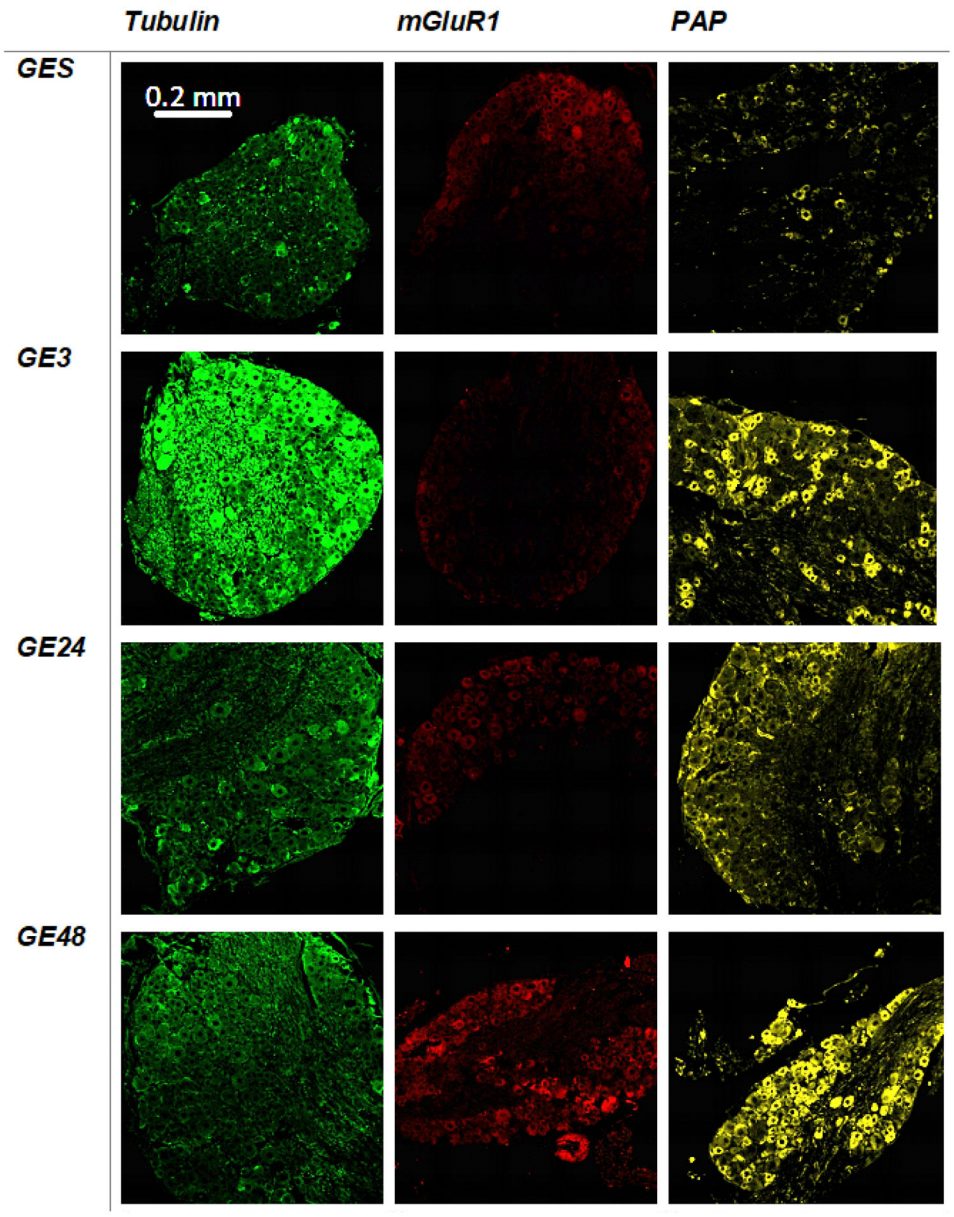

Figure 5.
DRG images with lens 20x and zoom 5X in each group with each of labeled antigens: PAP (yellow), mGluR1 (red), tubulin (green).

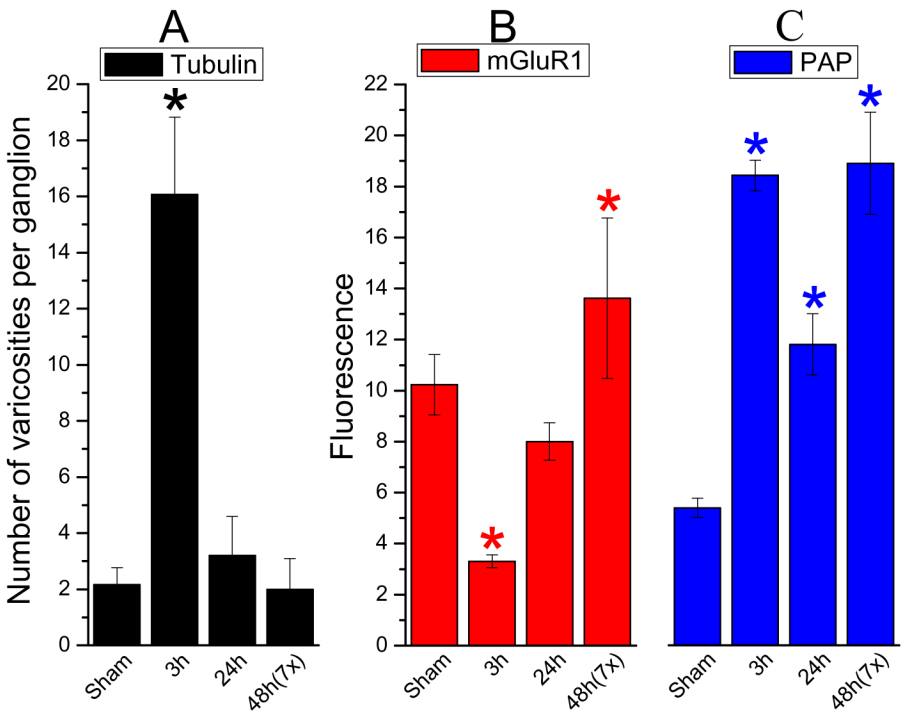

Figure 6.

Mean and SD of the groups (n = 5) for neuromarkers: (a) Tubulin varicosity formation, (b) mGluR1 fluorescence, (c) PAP fluorescence. The symbols * indicate statistically difference to sham group, $p < 0.05$.

Table 1

Parameters of PBM and details about the experiments.

Experiments	**1** **PBM to different regions and time course**	**2** **PBM over lumbar region dose response**	**3** **PBM to lumbar region repeated daily for 7 days**	**4** **PBM to lumbar region repeated once OR daily for 7 days**
Variable parameter	Location of the application	Energy density (exposure time)	Daily repetition	Post-application time for extraction of DRG
Exposure time (s)	120	24; 120; 600		120
Local of the application	Brain, cervical, lumbar, tail, right hind paw, left hind paw, abdomen	Lumbar region	Lumbar region	Lumbar region
Area (cm^2)	1	6	6	6
Type of application	Small spot ($1cm^2$)	Large spot ($6cm^2$)	Large spot ($6cm^2$)	Large spot ($6cm^2$)
Total power (mW)	0; 300	0; 300	0; 300	0; 300
Energy Density (J/cm^2)	0; 36	0; 1.2; 6; 30	6	6
Power density (mW/cm^2)	300	50	50	50
Total energy (J)	**0; 36**	**0; 7.2; 36; 180**	**36**	**36**
Tissue Extracted	None	None	DRG	DRG