Long-lasting antinociceptive effects of green light in acute and chronic pain in rats

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Abstract
Treatments for chronic pain are inadequate, and new options are needed. Nonpharmacological approaches are especially attractive with many potential advantages including safety. Light therapy has been suggested to be beneficial in certain medical conditions such as depression, but this approach remains to be explored for modulation of pain. We investigated the effects of light-emitting diodes (LEDs), in the visible spectrum, on acute sensory thresholds in naïve rats as well as in experimental neuropathic pain. Rats receiving green LED light (wavelength 525 nm, 8 h/d) showed significantly increased paw withdrawal latency to a noxious thermal stimulus; this antinociceptive effect persisted for 4 days after termination of last exposure without development of tolerance. No apparent side effects were noted and motor performance was not impaired. Despite LED exposure, opaque contact lenses prevented antinociception. Rats fitted with green contact lenses exposed to room light exhibited antinociception arguing for a role of the visual system. Antinociception was not due to stress/anxiety but likely due to increased enkephalins expression in the spinal cord. Naloxone reversed the antinociception, suggesting involvement of central opioid circuits. Rostral ventromedial medulla inactivation prevented expression of light-induced antinociception suggesting engagement of descending inhibition. Green LED exposure also reversed thermal and mechanical hyperalgesia in rats with spinal nerve ligation. Pharmacological and proteomic profiling of dorsal root ganglion neurons from green LED-exposed rats identified changes in calcium channel activity, including a decrease in the N-type (CaV2.2) channel, a primary analgesic target. Thus, green LED therapy may represent a novel, nonpharmacological approach for managing pain.

Keywords: Green-light phototherapy, Constellation pharmacology, Calcium imaging, Proteomics, Neuropathic pain, Thermal antinociception, Mechanical allodynia

1. Introduction
In the United States, over 100 million patients suffer from chronic pain.\textsuperscript{36} On average, $261$ to $300$ billion dollars are spent annually to manage pain, and $297$ to $336$ billion dollars are lost to work non-productivity.\textsuperscript{36} Additionally, the emotional tax from chronic pain has resulted in life-altering events ranging from shattering family unity\textsuperscript{71} to suicide attempts.\textsuperscript{29,32,59} Options for treatment of acute, postoperative pain, as well as for chronic pain, are limited, and many drugs are marginally effective\textsuperscript{22} and often accompanied by severe side effects.\textsuperscript{19} Nonpharmacological options for the treatment of pain would be highly desirable and advantageous in safety, including lack of tolerance, somnolence, addiction, gastrointestinal disturbances and, perhaps, other factors such as convenience and cost.

Light therapy has been reported to be useful for multiple medical conditions. For example, light therapy, has been used to control depression,\textsuperscript{20,25} to increase daytime circadian stimulation, to improve sleep quality, and mitigate depression in Alzheimer disease.\textsuperscript{21} Additionally, bright light improved the mood of adolescents taking antidepressants compared with those without light therapy.\textsuperscript{55} Exposing patients to bright light of more than $6000$ lux significantly improved seasonal affective disorder, a serious condition with increased risk of suicidality.\textsuperscript{20,25} Pain has sensory, cognitive, and affective dimensions.\textsuperscript{9,59} The emotional state of patients significantly influences pain.\textsuperscript{24} For example, chronic psychological stress and depression increased pain perception in humans when painful and nonpainful stimuli were applied to their forearms.\textsuperscript{76} A similar response is also reported in rodents. In a model of social defeat, rats demonstrated enhanced nociceptive response to subcutaneous formalin administration.\textsuperscript{6} By contrast, elevated emotional status may decrease the perception of pain\textsuperscript{17}, general happiness and laughter therapy significantly decreased chronic pain scores in elderly patients.\textsuperscript{79} Similar findings were observed in primary care patients who were enrolled in positive psychological intervention programs, which decreased their depression and improved overall functionality, as well as decreased pain perception.\textsuperscript{42} It is possible that light therapy may have psychological effects, possibly arising from physiological changes such as increased endogenous opioid release, as shown in this study, which affect pain response or its perception.
Few studies have investigated possible effects of light therapy on pain. A randomized clinical trial investigated the effect of bright light exposure in managing nonspecific back pain where patients received light exposure once a week for 3 weeks reduced pain as assessed by the Brief Pain Inventory suggesting an active role for light in controlling pain. Preclinical studies on the possible modulation of pain by light therapy have not been reported. Here, we investigated the effect of light in modulation of acute nociception as well as in a model of chronic neuropathic pain. We found that green light produced antinociceptive and antihyperalgesic effects that involved descending, opioid-sensitive inhibition.

### 2. Methods

#### 2.1. Animals

Pathogen-free, adult, male and female Sprague Dawley (SD) as well as male Long Evans (LE) rats (weight at testing 250-350 g; Harlan–Sprague–Dawley, Indianapolis, IN) were housed in a climate-controlled room on a 12-hour light/dark cycle and were allowed to have food and water ad libitum. All procedures were approved by the University of Arizona Animal Care and Use Committee and conformed to the guidelines of the National Institutes of Health (publication no. 80–23, 1966) for the use of laboratory animals. All behavioral experiments were conducted by experimenters blinded to the treatment conditions. The experiments were replicated a minimum of 2 times with independent cohorts of animals.

#### 2.2. Chemicals

All experimental compounds, doses, sources, and catalog numbers are described in Table 1.

#### 2.3. Light-emitting diodes

Visible-spectrum light-emitting diode (LED) flex strips were purchased from ledsupply.com (Randolph, VT). The specifications of the LEDs were (1) #LS-AC60-6-BL, 472 nm wavelength (ie, blue), 8 W, 120 V, 120˚ beam angle; (2) #LS-AC60-6-GR, 525 nm wavelength (ie, green), 8 W, 120 V, 120˚ beam angle; and (3) #LS-AC60-66-WH, white, 9.6 W, 120 V, 120˚ beam angle. Light-emitting diode strips were affixed to the outside of clear plastic cages that housed the rats so as to prevent the strips from being chewed. Rats were exposed to the various LEDs in these cages with full access to food and water in a dark room devoid of any other source of light. After behavioral assessment, the rats were returned to their cages for additional LED exposure. At the end of daily testing, the rats were returned to their regular animal room where they were exposed to room light (regular florescent bulbs). A lux meter (Tondaj LX1010B) was used to determine the illuminance and luminous emittance of the LED strips.

Because there are no previously published data reporting the effects of green LEDs on antinociception in rats, as a first step, we performed “pilot” experiments using a fixed amount (330 lux—which represents 1 full “strip”) of green LEDs over time, noting that antinociception developed only after 3 to 5 hours of exposure for at least 3 days. This antinociception continued to gradually increase over the next 2 days of exposure, reaching its zenith between days 4 and 5 (data not shown). The bulk of the studies were thus performed with 8 hours daily exposure for 5 days. Next, we performed a light-intensity vs antinociception analysis, which revealed that lower lux intensity was more effective than higher lux exposure for 8 hours daily for 5 days. These pilot experiments formed the basis of the exposure paradigm used in these studies—lux level, number of hours of exposure per day, and number of days of exposure.

#### 2.4. Thermal sensory thresholds

Paw withdrawal latencies (PWLs) used were determined as described by Hargreaves et al. Rats were acclimated within Plexiglas enclosures on a clear glass plate maintained at 30°C. A radiant heat source (high-intensity projector lamp) was focused onto the plantar surface of the hind paw. When the paw was withdrawn, a motion detector halted the stimulus and a timer. A maximal cut-off of 33.5 seconds was used to prevent tissue damage.

#### 2.5. Tactile thresholds

The assessment of tactile sensory thresholds was determined by measuring the withdrawal response to probing the hind paw with a series of calibrated fine (von Frey) filaments. Each filament was applied perpendicular to the plantar surface of the paw of rats held in suspended wire mesh cages. Withdrawal threshold was determined by sequentially increasing and decreasing the stimulus strength (the “up and down” method), and data were analyzed with the nonparametric method of Dixon, as described by Chaplan et al. and expressed as the mean withdrawal threshold.

#### 2.6. Elevated plus maze

The elevated plus maze (EPM) consists of 4 elevated (50 cm) arms (50 cm long and 10 cm wide) with 2 opposing arms containing 30 cm high opaque walls. Elevated plus maze testing occurred in a quiet testing room with ambient lighting at ~500 lux. On the day of testing, the rats were allowed to acclimate to the testing room for 20 minutes.

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**Table 1:** Experimental compounds used in this study.

<table>
<thead>
<tr>
<th>Experimental compound</th>
<th>Vendor</th>
<th>Catalog number</th>
<th>Target/mechanism of action</th>
<th>Dose</th>
<th>Route</th>
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<tr>
<td>Naloxone</td>
<td>Tocris</td>
<td>0599</td>
<td>Nonselective opioid receptors</td>
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<td>Intraperitoneal (i.p.)</td>
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<td>Phentolamine</td>
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<td>Sigma</td>
<td>P0884</td>
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<td>subcutaneously</td>
</tr>
<tr>
<td>Phenoxybenzamine</td>
<td>Sigma</td>
<td>B019</td>
<td>Alpha 1-adrenergic receptor</td>
<td>1 mg/kg</td>
<td>i.p.</td>
</tr>
</tbody>
</table>
Each rat was placed in a closed arm, facing the entry platform and cage mates started in the same closed arm. Each rat was allowed 5 minutes to explore the EPM and then returned to its home cage. Between animals, the EPM was cleaned thoroughly with Versa-Clean (Fisher Scientific, Waltham, MA). Elevated plus maze performance was recorded using an overhead video camera (MHD Sport 2.0 WiFi Action Camera; Walmart.com) for later quantification. Open- and closed-arm entries were defined as the moment the front paws entered the arm and open-arm time began the moment the front paws entered the open arm and ended on exit. An anxiety index was also calculated; the index combines EPM parameters into one unified ratio with values ranging from 0 to 1, with a higher value indicating increased anxiety. The following equation was used for calculation of the anxiety index:

Anxiety index = \( 1 - (\text{open arm time}/5\text{ min}) + (\text{open arm entry}/\text{total entry}) \).

### 2.7. Implantation of intrathecal catheters

For intrathecal (i.t.) drug administration, rats were implanted with catheters as described by Yaksh and Rudy. Rats were anesthetized with isoflurane and placed in a stereotactic device. The occipital muscles were separated from their occipital insertions and retracted caudally to expose the cisternal membrane at the base of the skull. Polyethylene tubing was passed caudally from the cisterna magna to the level of the lumbar enlargement. Animals were allowed to recover and were examined for evidence of neurologic injury. Animals with evidence of neuromuscular deficits were excluded.

### 2.8. Fabricating rat contact lenses and imaging of eyes postmortem

All plastic materials were purchased from Evergreen Scale Models (Des Plaines, IL). We used the method developed by Levinson et al., with the following modifications. In brief, 0.25 mm sheets were cut into 2 cm² pieces held by forceps over a 6 mm ball bearing, shaped when malleable with a copper pipe of 9 mm internal diameter and then trimmed into a truncated hemisphere with iris scissors and sanded with a fine grit and emery cloth to a depth of 3.5 ± 0.2 mm and a base diameter of 7.0 ± 0.2 mm. We used a Weller 1095-1000 W dual temperature heat gun, instead of the Bunsen burner used by Levinson et al., as a heat source for the fabrication process. The rats where anesthetized using isoflurane just long enough to place the contact lens in their eyes and were allowed to recover from anesthesia. The rats were anesthetized again with isoflurane to remove the contact lens at the end of each 8-hour exposure.

To examine if the contact lens induced any pathologic damage, at the end of the experiment, the corneas were excised with the small rim of the sclera and then fixed for 30 minutes with 4% paraformaldehyde in phosphate-buffered saline as described previously. Next, the corneas were transferred to a 30% sucrose solution until staining with Evans blue solution for 1 minute. The Evans blue dye solution was prepared by mixing 1 mL of a commercially prepared 0.5% Evans blue sterile aqueous solution with 9 mL of normal saline. This yielded a final solution of 0.05% Evans blue with a pH of 6.75. After staining, the excess dye was removed by gently passing the tissue through 2 baths of normal saline solution. Light microscope images of the stained corneas were obtained on an Olympus BX51 microscope with a Hamamatsu C8484 digital camera using a 4X UplanFL N, 0.13 numerical aperture, or a 20X UplanSapo 0.75 numerical aperture objective. The freeware image analysis program ImageJ (http://rsb.info.nih.gov/ij/) was used to generate merged images.

### 2.8.2. Rostral ventromedial medulla cannulation

Rats received a bilateral guide cannula (26GA, #C235-1.2 mm; Plastics One Inc, Roanoke, VA) directed to the rostral ventromedial medulla (RVM). The cannula was placed at: −11.0 from bregma, −7.5 mm from the dura, and 0.6 mm on either side of the midline. Injections were made by expelling 0.5 μL through an injection cannula protruding 1 mm beyond the tip of the guide. Cannula placement was confirmed with 0.5 μL Evans blue injected into both sides of the cannula, and microscopic examination of medullary sections and data from rats with misplaced cannula were eliminated from the experiment. Acute single injections into the RVM were performed by inserting an injector (#C235I-SPC; Plastics One Inc) attached to a 2-μL Hamilton syringe and expelling 0.5 μL at the same coordinates.

### 2.10. Sample preparation for proteomics

The DRG and dorsal horn of the spinal cord from rats exposed to ambient light or GLEDs (4 lux, 5 days, 8 hours/day) were isolated and lysates were prepared by sonication in RIPA buffer (50mM Tris-HCl, pH 7.4, 50mM NaCl, 2mM MgCl₂, 1% [vol/vol] nonidet P40, 0.5% [mass/vol] sodium deoxycholate, and 0.1% [mass/vol] sodium dodecyl sulfate and protease (Cat#B14002; Biotool) and phosphatase inhibitors (Cat#B15002, Biotool), and BitNuclease (Cat#B16002, Biotool). Protein concentrations were determined using the BCA protein assay (Cat#PI23225, Thermo Fisher Scientific, San Jose, CA). Proteins were precipitated using 100% ice-cold acetone and centrifuged at 15,000g at 4°C for 10 minutes. The pellets, containing solubilized proteins, were resuspended in 100 mM Tris pH = 7.4, 8 M urea (to eliminate carrying forward any salt contamination) and their protein content...
analyzed by mass spectrometry at the Arizona Proteomics Consortium after trypsin digestion.

2.10.1. Database searching
Tandem mass spectra were extracted. Charge state deconvolution and deisotoping were not performed. All tandem mass spectrometry (MS/MS) samples were analyzed using Sequest (version 1.3.0.339; Thermo Fisher Scientific, San Jose, CA). Sequest was set up to search RattusNorvegicus_UniprotKB_2016_0406_cont.fasta assuming the digestion enzyme trypsin. Sequest was searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 10.0 PPM. Oxidation of methionine and carbamidomethyl of cysteine were specified in Sequest as variable modifications.

2.10.2 Criteria for protein identification
Scaffold (version 4.5.1; Proteome Software Inc, Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 20.0% probability to achieve a false discovery rate less than 0.1% by the Scaffold False Discovery Rate algorithm. Protein identifications were accepted if they could be established at greater than 100.0% probability and contained at least 5 unique peptides. Protein probabilities were assigned by the Protein Prophet algorithm.9 Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. Proteins were annotated with gene ontology terms from gene_association.goa_uniprot.9

2.11. Semiquantitative real-time polymerase chain reaction
Semiquantitative real-time polymerase chain reaction (RT-PCR) was performed as described previously.5,6,7 After GLED treatment or control, rat spinal cords were dissected and the lumbar region isolated before flash freezing in liquid N2. The cords were kept at −80°C until analysis. RNA was extracted from tissues using Tri reagent (Cat# TR118; MRC, Cincinnati, OH) according to the manufacturer’s protocol. Briefly, Tri reagent was added to the cords before homogenization. The homogenates were centrifuged at 12,000g for 15 minutes at 4°C to eliminate insoluble materials, then chloroform was added to the supernatants at 20% (vol/vol). After centrifugation at 12,000g for 15 minutes at 4°C, the upper, RNA-containing, aqueous fraction was harvested in a new tube and total RNA was subsequently precipitated by adding 1/10 volume of isopropanol and 2.5 volumes of ethanol. The RNA pellets were washed twice with 75% ethanol. Finally, purified RNA was resuspended in ultrapure water and stored at −20°C until analysis. To generate cDNA, 2 µg of purified RNA were retrotranscribed using Maxima Reverse Transcriptase (Cat# EP0743; Thermo Fisher Scientific, San Jose, CA) according to the manufacturer’s instructions. Quantitative RT-PCR analysis was performed using SYBR Green PCR Mastermix (Cat# 4368814; Applied Biosystems, Foster City, CA) on a CFX connect (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Specific primer sequences used are given in Table 2. L27 expression was measured as a housekeeping gene to normalize target mRNA expression between samples. The relative mRNA level for each gene (x) relative to L27 mRNA (internal control) was calculated using the ΔΔCt method.49

2.12. Data analysis
The statistical significance of differences between mean was determined by parametric analysis of variance followed by post hoc comparisons (Student–Newman–Keuls test) using GraphPad Software. Animal numbers required to achieve statistical power for each in vivo experiment were determined by G.Power 3.1, using post hoc variance estimates from pilot experiments. Pharmacological profiling of sensory neuron data was analyzed using SigmaPlot 12.5 and compared by z-test. Differences were considered to be significant if P ≤ 0.05. All data were plotted in GraphPad Prism 6.

3. Results
3.1. Antinociceptive effects of green light-emitting diodes
We first determined if various kinds of light conditions affected responses to a noxious thermal stimulus in naïve rats. Rats were exposed, for 8 hours, to ambient room light that consisted of white fluorescent lights and ambient sunlight through glass windows (600 lux), white LEDs (575 lux), or darkness (0 lux), and PWL was measured immediately after termination of illumination. As shown in Figure 1, the PWLs were unchanged across all conditions. By contrast, naïve rats exposed to GLEDs (525 nm wavelength; 110 lux) but less than in those rats exposed to blue LEDs (472 nm wavelength; 110 lux) but less than in those rats exposed to GLEDs (Fig. 1). Paw withdrawal latencies were also higher in rats exposed to blue LEDs (472 nm wavelength; 110 lux) but less than in those rats exposed to GLEDs (Fig. 1). The increase in PWLs, elicited by GLED exposure (Fig. 2A), plateaued by the second day and was unchanged thereafter until the fifth day (Fig. 2B). The GLED exposure was terminated on day 5 and the PWLs were measured on subsequent days to determine whether the increase in PWLs was transient or long lasting. The increase in PWLs induced by GLED exposure was maintained for 4 days before returning to baseline (Fig. 2B).

The preceding experiments were conducted with 110 lux GLEDs. However, whether this lux level was sufficient to elicit the antinociceptive behavior is not known. Consequently, we exposed

<table>
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the rats to GLEDs spanning several lux intensities. Exposing rats for 8 hours to 4 lux GLED levels was sufficient for increasing PWLs compared with nonexposed rats (Fig. 3). Similar levels of PWLs were observed with rats exposed to 12, 36, or 110 lux GLED levels (Fig. 3). Exposure of rats to GLED 330 lux level significantly increased PWLs compared with baseline ambient light-exposed rats but resulted in only a half-maximal response when compared with the other lux conditions (Fig. 3). As the 4-lux GLED exposure for 8 hours was sufficient for achieving maximal antinociception, we used this lux level for all subsequent experiments.

We also tested if prolonged exposure to GLEDs could elicit tolerance to the antinociceptive effect. Rats exposed to GLEDs for an additional 7 days past the initial 5 days exposure (4 lux, 8 h/day) did not develop tolerance (Fig. 3B). This is in contrast with published data with morphine where analgesic tolerance can be observed within 3 days and causes rats to be hyperalgesic within 5 days.

### 3.2. Characterization of the role of the visual system in the antinociceptive effects of green light-emitting diodes

We determined whether the mechanism of GLED-induced antinociception requires direct activation of the visual pathway by fashioning dark, opaque, plastic contact lenses that permitted no light penetration (confirmed by measuring light intensity). These were fitted onto the rats’ eyes under anesthesia. As a control, transparent clear lenses were also installed onto control rats’ eyes. The application of contact lenses did not alter the baseline PWL. Both groups of rats were then exposed to GLEDs for 8 hours daily for 5 days and their PWLs were monitored. Following this exposure paradigm, rats fitted with the dark, opaque, contact lenses failed to develop antinociception, whereas rats fitted with clear, transparent, contact lenses developed antinociception similar to rats with no contacts (Fig. 4A). Consistent with the importance of the visual system in the development of GLED-induced antinociception, rats fitted for 8 hours with “green” contacts that permit light transmission in the green part of the visual spectrum (Fig. 4B), developed antinociception when exposed to room light (Fig. 4C). Importantly, histological analysis of the eyes of the rats at the end of the experiments revealed no damage caused by either contact lens (data not shown). These results support a role for the visual system in mediating the GLED-mediated antinociception.

To test if pigmented skin is involved in the antinociceptive effects of GLEDs, we selected the pigmented LE rats, which are different from the albino SD rats that lack pigmentation. A similar level of antinociception was observed in SD or LE rats exposed to GLEDs for 8 hours daily for 5 days; the PWLs were significantly higher than the respective strains exposed to ambient light (Fig. 5). The antinociception was not restricted to male rats as female SD rats exposed to the same GLED paradigm also exhibited increased PWLs (Fig. 5). Collectively, these results suggest that pigmentation is not important for developing antinociception.

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### Figure 1

**Effect of light-emitting diode (LED) exposure on thermal analgesia in naive rats.** After measurement of baseline (BL) paw withdrawal latency (in seconds), rats were randomly assigned (n = 6 per group) to exposures of 8 hours daily for 5 days to dark; ambient room light; or white, green (λ = 525 nm), or blue (λ = 472 nm) LEDs. At the end of this exposure paradigm, paw withdrawal latencies were again measured. Blue and green LED exposures resulted in thermal analgesia. *P < 0.05 when comparing with white LEDs (1-way analysis of variance followed by Student–Newman–Keuls test) and #P < 0.05 when comparing between green and blue LED exposures (nonparametric Student's t test). Unless otherwise stated, the data represent mean ± SEM for all figures in this article.

### Figure 2

**Green light-emitting diode (GLED)-induced thermal analgesia—time course and duration of effect.** (A) Schematic representation of the experimental design, LED exposures, and Hargreaves testing. (B) Bar graph showing the paw withdrawal latency (in seconds) of rats (n = 6 per group) treated as shown in the schematic in A. BL indicates the baseline latency before GLED exposure. Green light-emitting diode exposure resulted in thermal analgesia starting at the second day (D2) of phototherapy and lasted 4 days after cessation (D9) of LED exposure. *P < 0.05 when comparing with BL (1-way analysis of variance followed by Student–Newman–Keuls test).

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3.3. Pharmacological characterization of the antinociceptive effects of green light-emitting diodes

The mechanisms that might underlie the antinociceptive effects of GLEDs were investigated by determining possible contributions of endogenous pain transmitters and circuits as well as mediators of stress. Naive rats exposed to GLEDs for 8 hours daily for 5 days received antagonists immediately after light termination, and then PWLs were measured 20 to 30 minutes later. The GLED-induced antinociception was reversed, to baseline levels, after administration of the mu-opioid receptor (MOR) antagonist naloxone (20 mg/kg intraperitoneally [i.p.]). Twenty microgram, i.t., of naloxone also reversed the antinociception (Fig. 6A). These results suggest that GLED exposure likely elicits antinociception through release of endogenous opioids.

Neurons within the RVM are known to project to the spinal or medullary dorsal horns to directly or indirectly enhance or diminish nociceptive traffic. We examined the possible contribution of the RVM in the GLED-induced antinociceptive response by microinjection of 2% lidocaine. Rostral ventromedial medulla lidocaine reversed the development of antinociception when the rats were exposed to GLEDs (Fig. 6B).

Although our data, thus far, suggest a role for the endogenous opioid system, it is possible that the exposure conditions may result in stress-induced analgesia that could involve the sympathetic nervous system.

Figure 3. Green light-emitting diode (GLED)-induced thermal analgesia—effect of level of illuminance—without development of tolerance. (A) Bar graph of paw withdrawal latency (PWL, in seconds) of rats (n = 6 per group) exposed to the indicated illuminance level of GLED for 8 hours daily for 5 days. BL indicates the baseline latency before GLED exposure. Light-emitting diode exposure, GLED exposure as low as 4 lux, resulted in thermal analgesia. (B) Bar graph of PWL (in seconds) of rats (n = 6 per group) exposed to the indicated illuminance level of GLED for 8 hours daily for 5 and 12 days. The PWLs were not different after 5 days and 12 days of consecutive exposure to GLED. *P < 0.05 when comparing with BL (1-way analysis of variance followed by Student–Newman–Keuls test). #P < 0.05 when comparing with other lux values (1-way analysis of variance followed by Student–Newman–Keuls test). n.s., not significant.

Figure 4. Involvement of the visual system in green light-emitting diode (LED)-induced thermal analgesia. (A) Bar graph of paw withdrawal latency (in seconds) of rats (n = 6 per group) before and after treatment with green LEDs as indicated. During the green LED exposure paradigm, the rats were fitted with clear or dark plastic lenses on their eyes. Blocking green LED absorbance to the eyes with dark contacts prevented the development of green LED-induced thermal analgesia. (B) Rats "wearing" green plastic eye contacts and exposed to ambient room light for 8 hours daily developed thermal analgesia on days 3 and 4. *P < 0.05 when comparing with baseline (BL) (1-way analysis of variance followed by Student–Newman–Keuls test). (C) Absorbance spectra, in arbitrary units of clear, dark, or green contacts. Dark contacts absorbed light in all wavelengths while green contacts showed a peak absorbance in the 680 to 700-nm range.
nervous system with engagement of alpha- and beta-adrenergic receptors by norepinephrine or epinephrine. Thus, to elucidate the role of the adrenergic system, several adrenergic receptors antagonists were tested including phenoxybenzamine (1 mg/kg i.p.), a nonselective irreversible alpha-blocker; phentolamine (3 mg/kg i.p.), a nonselective reversible alpha-blocker; and propranolol (10 mg/kg s.c.), a nonselective beta-blocker. All these drugs given immediately after light termination and tested 15 to 20 minutes afterwards failed to prevent or reverse the antinociceptive effect of GLEDs (Fig. 7A). Additionally, rats exposed to GLEDs or ambient light were observed to have normal ambulation and huddling, as well as maintained regular grooming behaviors (data not shown), which is in contrast with diminished grooming observed in stressed rats.38

To address the possibility of the antinociception being the result of anxiety due to overstimulation of the visual system, we performed EPM experiments. A cohort of rats exposed to GLEDs for 8 hours daily for 5 days developed antinociception as before (Fig. 7B), showed no signs of stress/anxiety as there were no differences in closed- or open-arm entries, or in the anxiety index (Fig. 7C, D); the index combines EPM parameters into one unified ratio with values ranging from 0 to 1, with a higher value indicating increased anxiety.34 In fact, rats exposed to GLEDs spent a significantly higher amount of time in the open arms than rats exposed to ambient room light (Fig. 7E), suggesting that GLED exposure was anxiolytic. Collectively, these results show that GLED-induced antinociception is not due to an anxiety-related response in these rats.

3.4. Exposure to green light-emitting diodes does not impair motor performance

If, after exposure to GLEDs, the rats had a reduced motor activity, then this could contribute to the antinociception. To test this possibility, we investigated if the GLED exposure affected motor performance using the rotarod assay. After verification of antinociceptive behaviors induced by exposure to GLEDs for 8 hours daily for 5 days exhibited antinociception, we observed no change in the ability of the rats to stay on a rotating rod (Fig. 8). Thus, repeated GLED exposure does not affect motor performance or sedation.

3.5. Proteomics reveals green light-emitting diode effects in the dorsal root ganglion and spinal dorsal horn

We used an unbiased proteomics approach to identify possible protein alterations, using liquid chromatography–MS/MS, in tissues from ambient vs GLED-exposed rats. Among 559 nonredundant proteins identified in our DRG samples (Lumbar Figure 5. Green light-emitting diode (LED)-induced thermal analgesia does not rely on skin pigmentation and occurs in both genders. Bar graph of paw withdrawal latency (in seconds) of male and female Sprague Dawley (SD) rats (white fur) and male Long Evans (LE) rats (n = 6 per group) before and after treatment with green LEDs as indicated. All rats developed thermal analgesia compared with their own baseline. *P < 0.05 when comparing with baseline (1-way analysis of variance followed by Student–Newman–Keuls test).

Figure 6. Green light-emitting diode (LED)-induced thermal analgesia involves activity of the descending pain pathways through endogenous opioid signaling. (A) Bar graph of paw withdrawal latency (in seconds) of rats (n = 6 per group) before and after treatment with green LEDs as indicated. BL indicates the baseline latency before green LEDs exposure. Inhibiting mu-opioid receptor (MOR) with naloxone (intraperitoneal [i.p.] or intrathecal [i.t.] administration) (Table 1) reversed green LED-induced thermal analgesia. Untreated (No Tx) rats or rats injected i.p. with saline (n = 6 per group) developed green LED-induced thermal analgesia. (B) Inactivation of the descending pathway pain with an injection of a 2% solution of lidocaine into the rostral ventromedial medulla of rats (n = 6 per group) reversed green LED-induced thermal analgesia. *P < 0.05 when comparing with BL (1-way analysis of variance followed by Student–Newman–Keuls test).
levels 4, 5, and 6 were pooled), 65 proteins were detected only in DRG from GLED-treated rats (Fig. 9A). Gene ontology terms corresponding to the biological processes (Fig. 9B), molecular functions (Fig. 9C), and cellular components (Fig. 9D) of these proteins identified in DRG were extracted and compared between ambient and GLED-exposed rats. We noted a higher number of proteins associated with the “response to stimulus” category and a fewer proteins associated with “growth” category in DRG from GLED-exposed rats compared with DRG from ambient room light-exposed rats (Fig. 9B). The molecular functions associated with these proteins suggest a decreased “structural molecule activity” and an increased “antioxidant activity” in DRG from rats with GLED-induced thermal analgesia compared with controls (Fig. 9C). Finally, fewer proteins from GLED DRG appear to be localized in “organelle part” and “membrane” (Fig. 9D).

A total of 430 nonredundant proteins were identified in dorsal horn tissues, of which 43 were found only in the samples from rats with GLED-induced thermal analgesia (Fig. 9A). Gene ontology terms corresponding to the biological processes (Fig. 9B), molecular functions (Fig. 9C), and cellular components (Fig. 9D) of these proteins identified in DRG were extracted and compared between ambient and GLED-exposed rats. We noted a higher number of proteins associated with the “response to stimulus” category and a fewer proteins associated with “growth” category in DRG from GLED-exposed rats compared with DRG from ambient room light-exposed rats (Fig. 9B). The molecular functions associated with these proteins suggest a decreased “structural molecule activity” and an increased “antioxidant activity” in DRG from rats with GLED-induced thermal analgesia compared with controls (Fig. 9C). Finally, fewer proteins from GLED DRG appear to be localized in “organelle part” and “membrane” (Fig. 9D).

Figure 7. Green light-emitting diode (LED)-induced thermal analgesia does not invoke a stress/anxiety response. (A) Bar graph of paw withdrawal latency (PWL) (in seconds) of rats (n = 6 per group) before and after treatment with green LEDs as indicated. BL indicates the baseline latency before green LED exposure. Inhibiting the alpha- (with phenoxbenzamine or phentolamine) or beta- (with propranolol) adrenergic receptors (Table 1) failed to reverse green LED-induced thermal analgesia. (B) After verification that green LED exposure (8 hours X 5D) induces analgesia (B), rats were subjected to the elevated plus maze (EPM) test. (C) Green LED exposure did not change the number of times the rats entered the open or closed arms in the EPM. (D) The anxiety index, an integrated measure of times and entries into the arms of the EPM, was not different between rats at BL and after exposure to the green LED paradigm. (E) The green LED-exposed rats spent significantly more time in the open arms, indicating that green LED exposure was anxiolytic. *P < 0.05 when comparing with BL (1-way analysis of variance followed by Student–Newman–Keuls test). ns, not significant.

Figure 8. Motor function is not affected by green light-emitting diode (LED) treatment. Bar graph of latency of rats (n = 6 per group) to fall off the rotarod at incremental speed. Thermal analgesia induced by green LED exposure did not impair motoric performance as there were no differences between the fall-off latencies between these and rats exposed to ambient room light. P > 0.05 when comparing between the 2 conditions (paired Student t-test).
3.6. Analgesic effects of green light-emitting diode exposure in neuropathic pain

Having determined that GLEDs are antinociceptive in naive animals, we next asked if this nonpharmacological paradigm could be effective in reversing allodynia and hyperalgesia associated with the SNL model of experimental neuropathic pain. Probing the plantar surface of the hind paw ipsilateral to the side of nerve injury in SNL rats, 7 days postinjury, revealed thermal hyperalgesia (Fig. 10A) and tactile allodynia (Fig. 10B). Exposing SNL-injured rats for 8 hours daily to 4 lux GLED levels resulted in complete reversal of thermal hyperalgesia; the latencies were significantly higher than baseline so as to be antinociceptive (Fig. 10A). Paw withdrawal thresholds were also maximally reversed by a 3-day exposure to GLEDs (Fig. 10B). To address the duration of the GLED effect in SNL rats, we terminated GLED exposure and continued to measure antihyperalgesic and antiallodynic behaviors. After termination of GLEDs, it took 10 days for the thermal latencies (Fig. 10C) and 4 days for mechanical thresholds to return to postsurgical values (Fig. 10D).

Using quantitative RT-PCR, we determined expression levels of mRNAs for various endogenous opioids in spinal cords of naive rats as well as rats with SNL or sham in the presence of GLED exposure. Expression of the following genes was measured: (1) prepronociceptin (PNOC) coding for nocistatin, nociceptin, and orphanin; (2) pro-opiomelanocortin (POMC) coding for N-terminal peptide of proopiomelanocortin (NPP), melanotropin gamma, corticotropin, melanotropin alpha, lipotropin beta, lipotropin gamma, melanotropin beta, beta-endorphin, and met-enkephalin; (3) proenkephalin-A (PENK) coding for synenkephalin, met-enkephalin (4 copies), met-enkephalin-arg-gly-leu, met-enkephalin-arg-phe, and leu-enkephalin; (4) proenkephalin-B (PDYN) coding for alpha-neoendorphin, beta-neoendorphin, and met-enkephalin; and (5) mu-type opioid receptor (OPRM1) coding for prepronociceptin and pro-opiomelanocortin. These analyses demonstrate that expression of PENK gene, but not others, is...
significantly increased after GLED exposure (Fig. 10E), suggesting that the increased enkephalin levels in the spinal cord of GLED-exposed rats could be one contributing factor for their analgesia.

4. Discussion

We characterized the antinociceptive and antihyperalgesic effects of GLED phototherapy. In naive rats, the antinociceptive effect of GLEDs involves (1) visual system, (2) mu-opioid receptor pathways and descending pain inhibitory pathways from the RVM, (3) increased spinal cord expression of enkephalins, and (4) alterations in spinal cord and nociceptor proteomes. We were unable to link GLED-mediated antinociception to stress/anxiety. We demonstrate GLED phototherapy’s ability to reverse reduced sensory thresholds in a model of neuropathic pain, supporting its use as a possible novel, nonpharmacological approach in managing chronic pain.

A key issue in interpreting photically induced antinociception is delineating the route of entry of light and its possible relationship with pain modulatory circuits. Here, whole-body illumination with GLEDs for 8 hours resulted in antinociception. By contrast, previous studies reported reversal of pain in mice after 30 to 150 seconds of infrared LED exposure directly touching the skin.15,16 The differences in exposure times (seconds vs hours) and “routes of administration” with either direct skin exposure or whole-body
illumination could result in engagement of different mechanisms. Additionally, lower-intensity GLEDs was more antinociceptive. These results are consistent with a recent study,\(^5\) which reported that low-intensity green light reduced the intensity of migraines by \(~15\)% compared with lights of other colors and higher intensities.

Blocking GLED access to the eyes prevented antinociception, arguing for an important role by the visual system in the development of antinociception. Our data point to a role for the visual system in the antinociceptive and antiallodynic effects of GLEDs. However, exactly how this occurs is unknown. Electrical stimulation of the optic nerves in rats has been reported to increase blood pressure and heart rate, and to decrease baroreceptor-mediated vagal bradycardia. This effect was largely attenuated by inactivation of the periaqueductal grey (PAG), an anatomical region known to modulate pain,\(^6\) suggesting mechanistic neural links between the optic nerve and the PAG.\(^1\)

Light-sensitive melanopsin-containing ganglion cells in the eyes\(^6\) project directly to the circadian rhythm controlling suprachiasmatic nucleus\(^3\)^{31,38} and PAG\(^3\)^{36}; these structures are connected.\(^4\) Importantly, the suprachiasmatic nucleus has been linked with pain as inferred from disruption of the circadian rhythm of thermoregulation in models of chronic inflammatory pain in rats\(^7\) and increased c-fos levels in rats with cystitis pain.\(^8\) Finally, a retrograde labeling of the PAG demonstrated glutamate-like immunoreactivity in several regions of the brain including the occipital cortices,\(^9\) further supporting a link between the visual system and pain response. Martenson et al.\(^1\) have recently identified a possible circuitry for photic stimulation that included pain-modulating “ON-cells” and “OFF-cells” in the RVM which project to the dorsal horn of the spinal cord where they are postulated to modulate somatosensory processing. An imbalance of these may lead to enhanced or diminished pain with ON-cells facilitating nociception and OFF-cells inhibiting nociception. Our studies support a role for the RVM as its chemical inactivation prevented GLED-induced antinociception.

We demonstrated that GLED-induced antinociception involves opioid receptors. However, whether this occurs through a peripheral or central mechanism of action is at present unknown. Suppression of GLED-induced antinociception with the opioid receptor antagonist naloxone, administered systemically, may support a role of peripheral opioid receptors, which is consistent with previous reports demonstrating that administration of naloxone prevents infrared spectrum therapy–induced antinociception in models of postoperative\(^1\)^{16} or inflammatory pain.\(^3\)

However, the ability of naloxone given i.t. to reverse the antinociception effect argues in favor of a central site of action for the GLEDs since this dose and volume would not be expected to have a significant effect if the site of action was in the periphery. It is conceivable that GLEDs may have both central and peripheral effects. Future studies will explore these possibilities.

Although overstimulation of the visual system could cause stress-induced antinociception, our data suggest that GLED-induced antinociception is not linked to stress/anxiety for several reasons. First, pharmacological antagonism of receptors implicated in stress did not affect antinociception. Second, behavioral studies did not link GLED-induced antinociception to anxiety in the EPM experiments. Finally, grooming behaviors of rats with GLED exposures were unaffected, thus implying no link to stress. Future studies will assess neurochemical correlates of stress (e.g., glucocorticoids) in GLED antinociception.

Sensory neuronal adaptations, due to possible changes in activities of voltage-gated calcium and/or sodium channels, may lead to the practical application of GLEDs in the treatment of pain.

### Table 3
Proteins enriched in tissues from rats exposed to green light-emitting diodes.

<table>
<thead>
<tr>
<th>Identified proteins</th>
<th>Gene name</th>
<th>Accession number*</th>
<th>% Sequence coverage</th>
<th>Pain implication</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dorsal root ganglia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxypeptidase</td>
<td>ctsa</td>
<td>QGAYS3_RAT</td>
<td>12</td>
<td>Enkephalin degradation(^{14})</td>
</tr>
<tr>
<td>Alpha-synuclein</td>
<td>snca</td>
<td>SY0A_RAT</td>
<td>46</td>
<td>Expressed in laminae I, II, VII, and X of the dorsal horn(^{81})</td>
</tr>
<tr>
<td>Microtubule-associated protein</td>
<td>mapt</td>
<td>F1LS14_RAT</td>
<td>19</td>
<td>Lost in neuropathic pain(^{39})</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>bnr</td>
<td>THIO_RAT</td>
<td>54</td>
<td>Improves ziconotide-induced analgesia.(^{54}) Expression is changed after surgery(^{20})</td>
</tr>
<tr>
<td>Stathmin</td>
<td>stmn1</td>
<td>STM11_RAT</td>
<td>37</td>
<td>Substrate for Cdk5(^{57})</td>
</tr>
<tr>
<td>Acid ceramidase</td>
<td>asah1</td>
<td>A0A0G2KBT0_RAT</td>
<td>28</td>
<td>Inactivating mutation in Faber disease (patients experience pain)(^{76})</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>hp</td>
<td>A0A0H2UHM3_RAT</td>
<td>18</td>
<td>Serum concentration decreased in abdominal pain in horses(^{47})</td>
</tr>
<tr>
<td>Collillin 2</td>
<td>cti2</td>
<td>MOR065_RAT</td>
<td>46</td>
<td>Phosphorylation is associated with hyperalgesia(^{46,86})</td>
</tr>
<tr>
<td>Caletinrin</td>
<td>calb2</td>
<td>CALB2_RAT</td>
<td>21</td>
<td>Protects against TRPV1-mediated toxicity in pain-sensing neurons(^{56})</td>
</tr>
<tr>
<td><strong>Lactoylglutathione lyase</strong></td>
<td>glo1</td>
<td>LGUL_RAT</td>
<td>45</td>
<td>Expression reduced in diabetic neuropathy(^{57}) Catalyses the detoxification of methylglyoxal, a positive regulator of TRPA1, upregulated during painful neuropathy(^{7})</td>
</tr>
<tr>
<td><strong>Dorsal horn of the spinal cord</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactoylglutathione lyase</td>
<td>glo1</td>
<td>LGUL_RAT</td>
<td>54</td>
<td>Expression reduced in diabetic neuropathy(^{57}) Catalyses the detoxification of methylglyoxal, a positive regulator of TRPA1, upregulated during painful neuropathy(^{7})</td>
</tr>
<tr>
<td>Purine nucleoside phosphorylase</td>
<td>prop</td>
<td>PNH1_RAT</td>
<td>25</td>
<td>Catalyses the breakdown of adenosine into inosine, which induces antinociception in the formalin test(^{55})</td>
</tr>
<tr>
<td>Aspartyl aminopeptidase</td>
<td>dpap</td>
<td>Q4VBH5_RAT</td>
<td>17</td>
<td>Catalyses angiotensin III synthesis which activates inhibitory pain descending pathways from the periaqueductal grey matter(^{56})</td>
</tr>
<tr>
<td><strong>Cathepsin B</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alpha-2 neutral subunit</td>
<td>ctsb</td>
<td>Q6IN22_RAT</td>
<td>22</td>
<td>Expressed in microglia during inflammatory pain(^{77})</td>
</tr>
<tr>
<td>Lactoylglutathione lyase</td>
<td>ganab</td>
<td>D3ZAN3_RAT</td>
<td>14</td>
<td>Deficiency causes Pompe disease with increased mild pain(^{77})</td>
</tr>
</tbody>
</table>

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* Accession number is from Universal Protein Resource (UniProt).
help explain the molecular mechanisms underlying GLED-induced antinociception. N-type voltage-gated calcium channels (CaV2.2) are key for antinociception.73 The overall functional competence of neurons for calcium influx, as assessed by their ability to respond to agonists of various receptors using a high-throughput calcium imaging platform,59 was not different between sensory neurons from rats exposed to ambient light or GLEDs (Supplementary Fig. 1, available online at http://links.lww.com/PAIN/A358). However, we observed a decrease in depolarization-induced Ca2+ influx through CaV2.2 in sensory neurons from GLED-exposed rats (Supplementary Fig. 1E, http://links.lww.com/PAIN/A358). No changes were observed in sodium channels (Supplementary Fig. 2, available online at http://links.lww.com/PAIN/A358) ruling out their involvement in neuronal adaptations and GLED-induced analgesia. It is possible that the prolonged antinociceptive effect of GLEDs may, in part, be attributed to long-term changes in CaV2.2 or other calcium channel subtypes. Further studies will investigate the role of calcium and other channels in mediating the long-lasting effect of GLEDs.

Unbiased proteomics identified a protein signature in the dorsal root ganglia and dorsal horn of GLED-exposed antinociceptive rats. Stratification of proteins based on gene ontology terms corresponding to biological processes, molecular functions, and cellular components revealed an enrichment of proteins related to antioxidant activity in ganglia of GLED-exposed rats, a finding consistent with reported reduction in antioxidant activities (eg, catalase and superoxide dismutase) in rats with inflammatory pain.53 Additionally, low-level light therapy reduces levels of inflammatory mediators (eg, interleukins 6 and 8) associated with intervertebral disc degeneration, a key cause of low back pain.35 We noted a reduction in the number of membrane-localized proteins in the dorsal horn of GLED-exposed rats, which likely correlates with decreased signaling capabilities of neurons. Our findings uncovered proteins enriched in tissues from GLED-exposed rats that are reportedly linked to antinociception (Table 3). For example, purine nucleoside phosphorylase breaks down adenine into inosine, which has antinociceptive properties through actions on the adenosine A1 receptor58 and through pertussis toxin–sensitive G protein–coupled receptors and the subsequent inactivation of calcium channels.50 Aspartyl aminopeptidase, which converts angiotensin II to angiotensin III, is reported to activate inhibitory pain-descending pathways from the PAG.50 Our findings of chemical inactivation of the RVM further support a role of inhibitory pain-descending pathways in GLED-mediated antinociception. Another example is lactoylglutathione lyase (ie, glyoxalase-1), which catalyzes the detoxification of methylglyoxal, a cytotoxic ketoaldehyde, which is directly linked to activating nociceptors.1 Moreover, expression of glyoxalase-1 is reduced in diabetic neuropathy.37 These lines of evidence, along with those listed in Table 3, build a picture consistent with global changes induced by GLEDs to produce antinociception.

We used a model of neuropathic pain to illustrate the possible use of GLEDs as a nonpharmacological treatment. Spinal nerve ligation is a commonly used model of neuropathic pain in rats.60 Common neuropathic pain medications reverse hypersensitivity associated with neuropathic conditions, but do not usually produce antinociception. For example, gabapentin blocks the N-type calcium channel65 to reverse tactile allodynia and thermal hyperalgesia in diabetic neuropathy; however, it failed to produce antinociception.70 Carbamazepine, an antiepileptic medication used for neuropathic pain, blocks sodium channels to reverse thermal hyperalgesia in tail-flick test in a model of cisplatin-induced hyperalgesia, but fails to produce antinociception.72 By contrast, GLEDs reversed tactile and thermal hypersensitivity associated with SNL and produced long-lasting antinociception. We observed that GLEDs decreased calcium influx through the N-type calcium channel (supplementary Fig. 1F, http://links.lww.com/PAIN/A358), which may account for both the antiallodynic and antihyperalgesic effects; however, the exact mechanisms contributing to the long-lasting nature of the effect is presently unknown. We also observed that GLEDs activate opioid receptors, which may account for antiallodynia, antihyperalgesia, and antinociception. The complete reversal of the effects of GLEDs using naloxone also argues for a key role for opioid receptors in producing antiallodynia, antihyperalgesia, and antinociception. It is conceivable that multiple mechanisms of actions may act synergistically to produce the effects seen with GLEDs. In contrast with the side effects observed with opioids, an advantage of the GLED approach is lack of side effects, such as development of tolerance, sedation, respiratory depression, and motor coordination. Equally important was the lack of hyperalgesia after prolonged GLED exposure. The lack of these side effects suggests that using GLEDs may be a safe treatment for neuropathic and other pain states, and may also complement the use of pain medications.

Our findings identify the cellular and molecular basis of GLED-mediated antinociception. From a translational perspective, the discovery that GLED exposure is antinociceptive and antiallodynic, opens up routes for the development of a noninvasive therapeutic approach to pain. Consequently, modulating the duration and intensity of GLEDs may prove useful, clinically, for reducing opioid in pain management. Additionally, the long-lasting and nontolerance-inducing effects of GLEDs may improve patient compliance.

Conflict of interest statement
The authors have no conflicts of interest to declare.

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Appendix A. Supplemental Digital Content
Supplemental Digital Content associated with this article can be found online at http://links.lww.com/PAIN/A358.

Supplemental media
Video content associated with this article can be found online at http://links.lww.com/PAIN/A385

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References


