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Research Report

# Low-Level Light Therapy Improves Cortical Metabolic Capacity and Memory Retention

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**Abstract**. Cerebral hypometabolism characterizes mild cognitive impairment and Alzheimer's disease. Low-level light therapy (LLLT) enhances the metabolic capacity of neurons in culture through photostimulation of cytochrome oxidase, the mitochondrial enzyme that catalyzes oxygen consumption in cellular respiration. Growing evidence supports that neuronal metabolic enhancement by LLLT positively impacts neuronal function *in vitro* and *in vivo*. Based on its effects on energy metabolism, it is proposed that LLLT will also affect the cerebral cortex *in vivo* and modulate higher-order cognitive functions such as memory. *In vivo* effects of LLLT on brain and behavior are poorly characterized. We tested the hypothesis that *in vivo* LLLT facilitates cortical oxygenation and metabolic energy capacity and thereby improves memory retention. Specifically, we tested this hypothesis in rats using fear extinction memory, a form of memory modulated by prefrontal cortex activation. Effects of LLLT on brain metabolism were determined through measurement of prefrontal cortex oxygen concentration with fluorescent quenching oximetry and by quantitative cytochrome oxidase histochemistry. Experiment 1 verified that LLLT increased the rate of oxygen consumption in the prefrontal cortex *in vivo*. Experiment 2 showed that LLLT-treated rats had an enhanced extinction memory as compared to controls. Experiment 3 showed that LLLT reduced fear renewal and prevented the reemergence of extinguished conditioned fear responses. Experiment 4 showed that LLLT induced hormetic dose-response effects on the metabolic capacity of the prefrontal cortex. These data suggest that LLLT can enhance cortical metabolic capacity and retention of extinction memories, and implicate LLLT as a novel intervention to improve memory.

Keywords: Cytochrome oxidase, fear extinction, memory enhancement, mild cognitive impairment, mitochondrial respiration, neurotherapeutics, photobiomodulation

## INTRODUCTION

Low-level light therapy (LLLT) with red to near-infrared light is a promising and novel neurotherapeutic intervention in animals and humans [1–3]. LLLT *via* light-emitting diodes (LEDs) or lasers uses low-energy irradiation that avoids ablative effects on tissues, yet such energy is high enough to modulate cell functions. LLLT has well-established beneficial effects in nervous tissue *in vitro* and *in vivo*, including enhancement of gene expression [4] and nerve regeneration [5], and protection against traumatic injury [6–8], ischemic damage [9–11], and neurodegeneration induced by mitochondrial dysfunction [12–16]. The mechanism of action of LLLT implicates light absorption by chromophores in the mitochondrial respiratory enzyme cytochrome oxidase (also called cytochrome *c* oxidase or cytochrome *a–a3*), which contains chromophores with high absorbance in the

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λ range 600 to 830 nm [2, 17–20]. LLLT facilitates cell respiration and energy production in metabolically active tissues [14, 21, 22]. Cytochrome oxidase activity is a reliable marker of neuronal metabolism [23] and its expression is enhanced by LLLT in neuronal cultures [13, 24]. Tissues with high oxidative metabolic rates such as brain and muscle show increases in cytochrome oxidase after *in vivo* LLLT [12, 25]. Enhancement of cell respiration and cytochrome oxidase upregulation are believed to underlie a series of secondary effects that promote neuroprotection [1–3].

A largely unexplored area of photobiology comprises the effects of LLLT on higher-order cognitive functions. Recent reports suggest that LLLT can be used non-invasively to exert transcranial brain metabolic effects in rodents and humans [12, 26, 27]. However, LLLT exhibits a hormetic dose-response effect (a quadratic function also called biphasic or U-shaped dose-response) characterized by stimulation of a biological process at a low dose and inhibition of that process at a high dose [1, 3]. Thus LLLT may be anticipated to facilitate memory at low doses but show opposite effects at high doses. We hypothesized that, by enhancing mitochondrial respiration and energy metabolic capacity in specific neural networks, low-dose LLLT may also have a beneficial impact on processes such as learning and memory. For example, rats develop a conditioned fear behavior (freezing) to a neutral tone after pairings of the tone (conditioned stimulus) with a subsequent footshock (unconditioned stimulus). But this conditioned behavior extinguishes after repeated presentations of the conditioned stimulus by itself. Fear extinction is a form of associative learning that is clinically relevant because it constitutes the basis for exposure therapy of phobias and post-traumatic stress disorder [28]. The neurobiology of fear extinction is well-characterized [29], which makes this learning paradigm ideal for the brain-behavior characterization of LLLT. In fact, it has been shown that pharmacological enhancement of extinction memory can be achieved by facilitation of brain energy metabolism during consolidation, a critical period of memory processing that features high energy consumption and protein synthesis [30]. Improvement of extinction memory is mediated by increases in energy metabolism in medial prefrontal cortical regions [31–33]. We thus hypothesized that LLLT aimed at enhancing the metabolic activity of such prefrontal regions during consolidation would modulate extinction memory. This study tested the hormetic dose-response effects of LLLT on extinction memory with two behavioral experiments (extinction and renewal effects) and two neurometabolic experiments (prefrontal oxygen consumption and cytochrome oxidase activity). It was expected that LLLT low-dose effects on the prefrontal cortex would feature *in situ* increases in cell respiration and cytochrome oxidase activity, and that this would be associated with facilitation of extinction memory.

## MATERIALS AND METHODS

Experiments used a total of 62 adult male rats obtained from Harlan (Houston, TX). The number of rats in each group is given for each experiment below. Each rat was handled daily for 3 min for 7 days prior to the start of the behavioral experiments. Rats were given food and water *ad libitum*. They were housed three to four rats per cage and kept on a 12-h light/12-h dark cycle in a facility accredited by the Association for the Assessment of Laboratory Animal Care International. All procedures were done in accordance with NIH guidelines and approved by the Institutional Animal Care and Use Committee of the University of Texas at Austin. Unless otherwise specified, all chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO).

### *Experiment 1. LLLT effects on oxygen consumption in the prefrontal cortex in vivo*

Radiant exposure doses were expressed as energy density (fluence) in J/cm$^2$ given that energy (Joules) = power (Watts) × time (s). LLLT at 660 nm was used because cytochrome oxidase is the main photon acceptor in cells at this wavelength of light [2]. LLLT was delivered *via* narrow-angle GaAlAs light-emitting diodes (LEDs) (peak $\lambda = 660$ nm) from LEDtronics, Inc. (Torrance, CA), 49 of which were assembled in our laboratory on fiberglass circuit boards to build 5.5 × 9.5 cm arrays, as described previously [25]. The light output (irradiance) of the LEDs was measured as power density in mW/cm$^2$ using a Newport (Irvine, CA) model 1916-C power meter attached to a Newport model 918D-SL photodiode detector, prior to the onset of each treatment. The immediate postmortem scalp skin (shaved) plus the dorsal skull of rats ($n = 10$) were used for verification of percent transmittance using this apparatus. This allowed us to establish the light reaching the prefrontal cortical surface. The effect of LLLT on cortical oxygen consumption was assessed *in vivo* by means of fiber optic fluorometric oxygen quenching. For the *in vivo*

measurements, anesthesia was induced in Long Evans rats ($n = 3$) with 5% isoflurane (Baxter, Deerfield, IL) for 3 min and maintained with isoflurane 3% using an E-Z anesthesia vaporizer system (Euthanex, Palmer, PA). Each rat's head was secured in a stereotaxic apparatus, the dorsal surface shaved, and the skull exposed by a mid-sagittal incision. Trephinations were done at stereotaxic coordinates + 3.7 mm A-P and + 2.0 mm M-L, relative to Bregma level [34]. A fiber optic fluorometric oxygen sensor (Ocean Optics, Dunedin, FL) was slowly lowered through the burr holes to a depth of 2 mm ventral to the dura, targeting the dorsomedial prefrontal cortex. The *in situ* oxygen concentration in this region was measured for 1 min immediately after alternating periods of either no treatment in the dark (control, 1 min), LLLT 1 J/cm$^2$ (1 min 51 s) or LLLT 5 J/cm$^2$ (9 min 25 s). At least 3 measurements per condition were obtained and LLLT was off during measurements. LEDs arrays were positioned at 1 cm from the dorsal head surface for irradiation at 9 mW/cm$^2$ in all of the experiments.

*Experiment 2. LLLT effects on extinction memory*

LEDs arrays were mounted on small plexiglass boxes (10 × 14 × 20 cm) to deliver LLLT *via* the non-contact modality above the subjects' heads with an irradiance of 9 mW/cm$^2$. After 7 days of handling, Long Evans rats were habituated for 2 days prior to behavioral training or LLLT delivery. This procedure avoided completely restraining subjects, while still ensuring complete irradiation of the dorsal head surface while each rat rested on the box. We also verified that this light delivery produced negligible amounts of surface heat. LLLT was delivered to one subject at a time 15 min after each extinction session targeting brain tissues during the critical period of memory consolidation. Post-extinction LLLT fractions lasted 10 min, for a radiant exposure of 5.4 J/cm$^2$ (to approximate the most effective treatment used in experiment 1) and were similarly delivered individually to each rat ($n = 11$) in a dark room at $\lambda = 660$ nm and 9 mW/cm$^2$. Subjects treated with LLLT were matched with control subjects ($n = 10$) housed in a similar plexiglass box for 10 min without light delivery.

A behavioral apparatus for extinction training was used as described before [30]. Behavioral training lasted 9 days (Table 1). On days 1 and 2, subjects were exposed to an operant chamber (MED Associates, St. Albans, VT) enclosed in a sound-attenuated box with a soap scent (context A). They were allowed to explore the chamber for 1 h, after which they were placed back in their home cage. On days 3 and 4, subjects underwent acquisition training consisting of four tone (conditioned stimulus)-shock (unconditioned stimulus) presentations. Each tone (15-s, 65 dB, 1- to 2-kHz frequency modulated tones, generated by two Wavetek Sweep/Modulation generators) co-terminated with a 0.5-mA, 0.75-s footshock. Each acquisition session lasted 15 min, with an average pseudorandom intertrial interval of 3 min. Animals were placed back in their home cages after the completion of each session. On days 5 to 8, 30-min extinction sessions of nine 15-s tone presentations at 3 min intervals were conducted in context B. Context B consisted of a clear plastic cage (25.4 × 19 × 15.2 cm) with an iodide scent and a black speaker mounted on top. Freezing behavior was defined as a rat having all four feet on the floor; with shallow, rapid breathing and minimal head and vibrissae movement for 3 s. Freezing for 15 s during tone presentation was the maximal score (100% freezing), whereas zero represented no freezing during tone presentation. Subjects were returned to their home cages following extinction training. After 15 min, subjects were transferred to the LLLT delivery boxes (context C) and received either LLLT treatment or no treatment in the dark. A total of 4 daily extinction sessions were done. Freezing values in the second acquisition session were used as a post-acquisition baseline before extinction.

Table 1
Pavlovian fear extinction training

| Timeline | Behavioral procedure |
|---|---|
| Days 1-2 | *Habituation*, 1 h in conditioning context A |
| Days 3-4 | *Acquisition*, 15 min, 4 paired tone-shock presentations in context A |
| Days 5–8 | *Extinction*, 30 min, 9 tone presentations in context B, LLLT follows after 15 min in context C |
| Day 9 | *Renewal* probe session, 3 tone presentations, context A |

*Experiment 3. LLLT effects on fear renewal after extinction*

Recovery of the acquisition performance demonstrated as high levels of freezing is expected when tone exposure is accomplished not in a neutral context but in the context of original acquisition. This phenomenon is known as renewal. Renewal is clinically relevant for exposure therapy of phobias and other mental disorders, since fears that are extinguished in the context of a therapist's office can easily return

when the subject moves to a different context. Long Evans male rats trained as described in experiment 2 (Table 1) received LLLT delivered as before to one subject at a time 15 min after the first extinction session for a single dose of 5.4 J/cm$^2$ ($n = 5$). On day 9, subjects were exposed to 3 tone presentations in the original acquisition context A and freezing behavior was quantified during tone presentation, which represented a renewal probe. This renewal probe was important to test the effects of an effective LLLT treatment at preventing the return of fear after extinction training, when subjects were reintroduced in the context where the initial tone-footshock association was made.

*Experiment 4. LLLT effects on the metabolic capacity of the prefrontal cortex*

Dose-response hormetic effects of *in vivo* LLLT on oxidative metabolic capacity were evaluated in different naïve male rats by measuring brain cytochrome oxidase activity [35]. LLLT was delivered as before at $\lambda = 660$ nm in different doses (radiant exposure as single fractions of different time lengths) of 10.9 J/cm$^2$ (20 min, $n = 5$), 21.6 J/cm$^2$ (40 min, $n = 4$), and 32.9 J/cm$^2$ (60 min, $n = 4$) administered in a dark room. Rats in the control ($n = 5$) group were housed in the dark for 20 min and received no LLLT. LLLT light intensity (power density) for all the experiments were equivalent in that they implicated delivery of LLLT *via* a non-contact modality above the subjects' heads with an irradiance of 9 mW/cm$^2$, as determined by means of a Newport 1830 C power meter.

At 24 h after LLLT treatment, subjects were decapitated and their brains quickly extracted and frozen in –40°C isopentane. Forty-micrometer-thick brain coronal sections were obtained at –18°C using a CM300 cryostat (Leica, Buffalo Grove, IL) and were mounted on glass slides to create three adjacent series. Sections were stained for quantitative measurement of cytochrome oxidase activity as previously described [36–38]. Frozen sections were submerged in 0.5% glutaraldehyde/10% sucrose in phosphate buffer (PB), pH 7.6, for 5 min at 4°C, followed by three baths of 10% sucrose in PB (5 min each, allowing for gradual increases in temperature). Sections were incubated for 10 min in a solution containing 50 mM Tris, 1.1 mM cobalt chloride, 10% sucrose, and 0.5% dimethyl sulfoxide (DMSO). After a 5 min PB rinse at room temperature, sections were stained for 60 min at 37°C in PB containing 1.3 mM 3,3′-diaminobenzidine tetrahydrochloride, 75 mg/L cytochrome *c*, 20 mg/L catalase, 5% sucrose, and 0.25% DMSO. Sections were fixed in 10% formalin in PB at room temperature for 30 min, dehydrated in series of 30–100% ethanol, cleared in xylene (two times, 5 min each), and coverslipped with Permount®.

Quantitative histochemistry was performed with a combination of image-based densitometry and spectrophotometrically-determined cytochrome oxidase activity, as previously described [36, 38]. This method yielded a linear relationship ($r > 0.95$) between biochemical cytochrome oxidase activity units and tissue optical density. A calibration step tablet (optical density range 0.05–3.05) and cytochrome oxidase activity–stained sections were placed on a DC-powered light box and digitized using a CCD camera and a Targa-M8 digitizer (Javelin Electronics, Torrance, CA). Digitized images were analyzed for optical density using JAVA imaging software (Jandel Scientific, San Rafael, CA). This software allows creating a logarithmic calibration curve of optical density units as a function of pixel (gray level) values in each image. Before analysis, images were corrected for slide and light box artifacts using background subtraction. The median of optical density values were converted to units of cytochrome oxidase activity using calibration curves based on standards of tissue thickness and spectrophotometrically-determined cytochrome oxidase activity. For this purpose, naïve rats ($n = 10$) were used to make brain homogenates that were cryosectioned at different thicknesses (10, 20, 40, 60, and 80 μm) to obtain increasing gradients of cytochrome oxidase reactivity. Group comparisons used cytochrome oxidase activity values expressed as mmol/min/g.

*Statistical analyses*

Analyses were conducted with analysis of variance (ANOVA) using SPSS 11.5 for Windows. Experiment 1 used factorial ANOVA with Dunnett test correction for comparisons of mean percent decreases of *in situ* oxygen concentration. Experiment 2 evaluated behavioral effects (% freezing) with repeated measures ANOVA to determine session × treatment effects and quadratic contrast for the hormetic dose-response effects. This was followed by simple effects tests for pairwise contrasts. Experiment 3 used ANOVA to compare the renewal effects in the control and LLLT group. Experiment 4 compared mean cytochrome oxidase activity values in the control versus the different LLLT groups with Dunnett test-corrected ANOVA. A two-tailed $p$ value $<0.05$ was considered significant.

## RESULTS

### *Experiment 1 showed that LLLT increased prefrontal cortex oxygen consumption in vivo*

Experiment 1 verified that LLLT penetrated through the head and had transcranial metabolic effects on the prefrontal cortex. The light output (irradiance) of the diodes had a power density of 9 mW/cm$^2$ measured using a Newport model 1916-C power meter attached to a Newport model 918D-SL photodiode detector. Verification of percent transmittance using this apparatus allowed us to establish the light level at the cortical surface. Our results established a 5.8% light transmittance through the scalp skin (shaved) plus skull, equivalent to 1.24 optical density and 0.35 absorption coefficient ($\alpha$). In addition, the light transmittance across the entire rat brain (from dorsal to ventral surface) was established at 8.5%, corresponding to 1.07 optical density and 0.20 absorption coefficient ($\alpha$). Enhancement of oxygen consumption by LLLT has been previously demonstrated *in vitro* and has been shown to underlie facilitation of neuronal metabolism and a variety of beneficial biological effects [3, 12]. In experiment 1, *in situ* oxygen concentration in the prefrontal cortex of naïve rats was measured with fluorescent quenching oxymetry immediately following LLLT exposure (Fig. 1). The *in situ* prefrontal cortex oxygen concentration in control conditions (i.e., following no light exposure) decreased only $1 \pm 0.7\%$. In contrast, LLLT induced a dose-dependent decrease in *in situ* oxygen concentration of approximately $5 \pm 1\%$ after LLLT 1 J/cm$^2$ and $15.8 \pm 2\%$ after LLLT 5 J/cm$^2$. These data support a clear physiological effect of LLLT on the rate of cortical oxygen consumption *in vivo*.

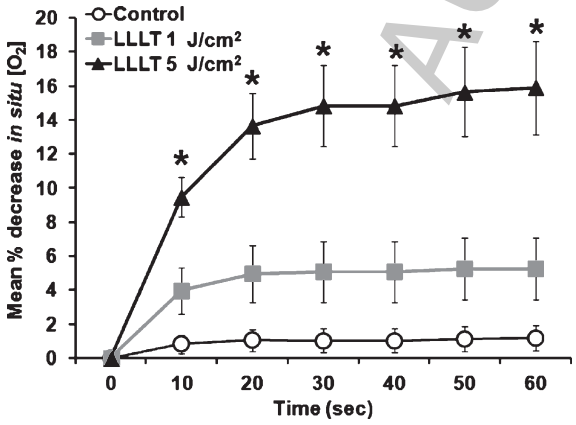


Fig. 1. Enhancement of *in vivo* cortical oxygen consumption by LLLT. *In situ* cortical oxygen concentration decreased in response to LLLT in a dose-response manner, as measured by a fluorescence-quenching oxygen probe placed in the rat prefrontal cortex. Data points are the average of at least 3 measurements. Measurements post-treatment alternated 1 : 1 with measurements after no treatment (control). $*p < 0.001$.

### *Experiment 2 showed that LLLT at low-dose was able to facilitate fear extinction memory*

To test the hypothesis that LLLT facilitates extinction memory in a dose-dependent manner, subjects undergoing extinction training were treated with daily fractions of LLLT delivered 15 min after an extinction session consisting of exposure to the conditioned stimulus (tone) in a neutral context. LLLT fractions were administered after each extinction session with the goal of improving brain oxidative metabolism during the memory consolidation phase of extinction, which is characterized by increased energy expenditure secondary to protein synthesis [39–41] and synaptic strengthening and outgrowth [42, 43]. Table 2 shows that, after acquisition, both control and treatment groups displayed similar high levels of freezing (59–64%). This reflects effective fear conditioning after two sessions of tone-shock pairings. Note also that freezing scores on Extinction 1 are similar in control and LLLT groups (40–43%). This was not unexpected since the first LLLT fraction was delivered 15 min after this first extinction training session. In control subjects, tone exposure during Extinction 1–4 progressively decreased mean freezing scores. This contrasted with the pattern found in LLLT-treated rats, which had an enhanced reduction in freezing scores followed by an increase afterwards. Hence on Extinction 4, LLLT-treated subjects tended to have higher freezing scores than the control group. Repeated measures ANOVA showed a significant interaction of extinction session X treatment ($F(4,76) = 2.5$, $p = 0.047$). The test of within-subject quadratic contrast was also significant ($F(1,19) = 5.3$, $p = 0.032$), as would be expected from a hormetic dose-response effect. On Extinction 2, control subjects showed a mean freezing score of $34.5 \pm 4.6\%$ that contrasted with a mean freezing score of only $18.64 \pm 4\%$ in the LLLT-treated group. This was a significant 46% reduction ($p < 0.05$) in the mean freezing score in the LLLT group compared to control. The statistically significant results support the efficacy of low-dose (but not high-dose) LLLT in the facilitation of extinction in a behaviorally meaningful manner.

Table 2
Behavioral effects of LLLT delivered 15 min after each extinction session

| Training session | Total dose | Control mean (% freezing) | SEM | LLLT mean (% freezing) | SEM | *p* value |
|---|---|---|---|---|---|---|
| Acquisition | | 64.00 | 8.02 | 59.09 | 7.65 | 0.66 |
| Extinction 1 | 5.4 J/cm$^2$ | 40.00 | 7.24 | 42.73 | 6.91 | 0.79 |
| Extinction 2 | 10.8 J/cm$^2$ | 34.50 | 4.60 | 18.64 | 4.39 | 0.02 |
| Extinction 3 | 16.2 J/cm$^2$ | 39.63 | 3.55 | 40.11 | 3.38 | 0.92 |
| Extinction 4 | 21.6 J/cm$^2$ | 12.50 | 6.93 | 30.45 | 6.61 | 0.08 |

*Experiment 3 showed that LLLT low-dose after extinction prevented fear renewal*

This experiment showed the memory enhancing effect of LLLT using the fear renewal test. It was hypothesized that LLLT would not only facilitate extinction but would also make subjects resistant to renewal. To test this hypothesis, subjects that underwent extinction training were exposed to tone presentations alone in the original acquisition context (context A) one day after the last extinction session. In this renewal probe, control subjects showed a mean freezing score of $58 \pm 11\%$, which was similar to acquisition levels of freezing. In contrast, 5.4 J/cm$^2$ LLLT-treated subjects showed a mean freezing score of only $28 \pm 4\%$, which represented a 52% reduction in the mean freezing score ($p < 0.05$) (Fig. 2). Taken together, behavioral experiments 2 and 3 suggested a dose-dependent LLLT improvement of neural processes mediating long-lasting extinction memory.

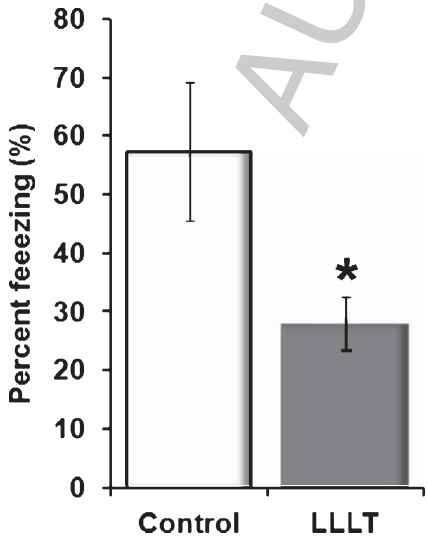


Fig. 2. LLLT after extinction prevented fear renewal. One day after extinction training, subjects underwent tone presentations in the original acquisition context. The memory enhancing effects LLLT were evident as a 50% reduction in freezing behavior compared to control subjects in this fear renewal probe. *$p < 0.05$.

*Experiment 4 showed that LLLT increased the metabolic capacity of the prefrontal cortex*

Secondary effects of LLLT on neural tissue have been postulated to include long-lasting neurochemical changes that persist beyond the period of light exposure and translate into changes of neuronal physiology. These changes include activation of second messenger cascades and gene expression [2]. To test the hypothesis that *in vivo* LLLT induces secondary effects in the brain, mean changes in prefrontal cortex cytochrome oxidase activity were determined in naïve animals 24 h after exposure to LLLT. Cytochrome oxidase is the terminal enzyme in the respiratory chain and its expression is tightly coupled to neuronal energy demands [23, 44]. Cytochrome oxidase activity is a sensitive marker of brain metabolic capacity and histochemical determination of its activity depends on upregulation of the holloenzyme pool [37, 45, 46]. A hormetic dose-response increase in cytochrome oxidase activity was detected at 24 h in the prefrontal cortex of rats exposed to LLLT (Fig. 3). Control subjects showed a mean cytochrome oxidase activity of $281 \pm 12$ mmol/min/g. In contrast, the mean prefrontal cortex cytochrome oxidase activity in subjects treated with LLLT 10.9 J/cm$^2$ was $319 \pm 10$ mmol/min/g. This represented a significant 13.6% increase in metabolic capacity ($p < 0.05$). Higher LLLT doses of 21.6 J/cm$^2$ and 32.9 J/cm$^2$ were less effective at inducing cytochrome oxidase activity, with values of $310 \pm 12$ mmol/min/g and $289 \pm 19$ mmol/min/g, respectively. These values were 10.3% and 3% higher than control, which were not significantly different. These results demonstrate that the lower dose had a significant stimulatory effect on cortical metabolic capacity. The data also show a tendency towards reduced enhancement of metabolic

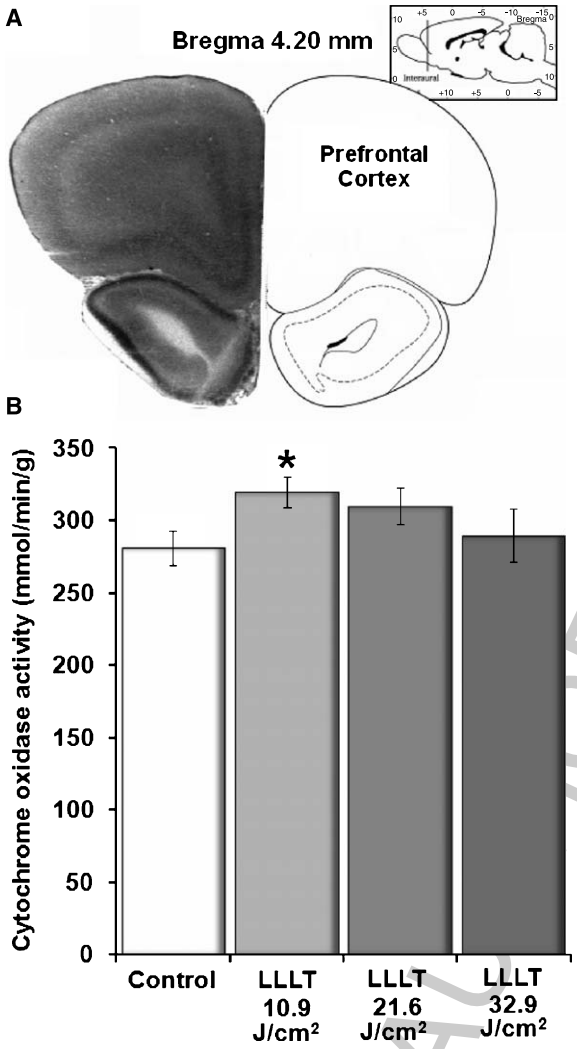


Fig. 3. LLLT induced a hormetic dose-response on cortical metabolic capacity. A) Rat prefrontal cortex stained with cytochrome oxidase histochemistry at the A-P brain level used to measure cytochrome oxidase activity 24 h after LLLT. B) The lower LLLT dose induced a significant 13.6% increase in mean cytochrome oxidase activity, compared to control. Higher LLLT doses resulted in no significant differences compared to control. $*p < 0.05$.

capacity with increasing doses, supporting a hormetic dose-response effect.

## DISCUSSION

This study is the first demonstration that LLLT can improve extinction memory. This study also presents evidence that both primary and secondary LLLT effects occur in the brain *in vivo*. The results add to the growing body of *in vitro* and *in vivo* evidence supporting that LLLT can be used non-invasively to modulate neural function. Red and near-infrared light transmittance has been measured through the mouse skull and its transcranial neuroprotective effects have been demonstrated in the mouse, rat, and rabbit [7, 26, 47–49]. Human experiments have also demonstrated changes in frontal cortex regional cerebral blood flow after exposure to LLLT in the forehead [27]. Several reports have described beneficial effects of LLLT on cognition. Michalikova et al. [50] demonstrated improvement in working memory after LLLT in middle-aged mice tested in an appetitive spatial navigation task. Similarly, Naeser et al. [51] reported improvement of attention, executive function, and memory in two patients with chronic traumatic brain injury with daily use of LLLT to the head. Reports in rats and humans provide further evidence that LLLT modulates mood and decreases depressive symptoms [8, 27]. Taken together, the data supports that LLLT to the head constitutes a promising neurotherapeutic tool to modulate behavior in a non-invasive manner. Future projects should determine the effects of variation in LLLT parameters such as wavelength, radiant exposure, irradiance, and wave type on transcranial applications to affect behavioral and brain metabolic variables.

This study is also the first demonstration that both primary and secondary mechanisms of action of LLLT occur in the brain *in vivo*. The mechanisms of action of LLLT have been previously described in cell cultures. Primary LLLT effects occur during light exposure and refer to the direct photochemical change of the photoacceptor (e.g., cytochrome oxidase) upon excitation by light. The most important primary effect is a redox change of the components of the respiratory chain that affects electron flow. Support that primary effects of LLLT occur in the brain *in vivo* is provided by the dose-response increase in oxygen consumption measured in the frontal cortex of rats exposed to LLLT in this study. Another possible primary effect of LLLT is the generation of reactive oxygen species (ROS), including singlet oxygen via direct photodynamic action and superoxide ion via one electron auto-oxidation [17]. The significance of this effect is that ROS are not only damaging by-products of respiration but they have an important role in cellular signaling. For example, Chen et al. [52] demonstrated that LLLT not only enhances mitochondrial respiration, but also activates the redox-sensitive nuclear factor kappa B signaling via generation of ROS in fibroblast cultures. Another possible secondary effect of LLLT is the generation

of nitric oxide (NO). Cytochrome oxidase has a key role in neuronal activity because it is a rate-limiting enzyme for oxidative energy production in the mitochondrial electron transport [53–55], but cytochrome oxidase can also catalyze the production of NO under hypoxic conditions [56]. LLLT *in vivo* can increase NO and cerebral blood flow in mice with carotid occlusion [57]. Photobiomodulation of cytochrome oxidase with LLLT enhances brain oxygen utilization and NO production, both of which may protect against reduced cerebral vascular perfusion. Photobiomodulation of brain cytochrome oxidase is expected to reverse the pathophysiologic consequences of cerebrovascular hypoperfusion via the vasodilator effect of NO resulting in increased oxygen and nutrient delivery that will increase neuronal respiration. Although this study did not directly test the role of ROS and NO as signaling molecules in the brain *in vivo*, it is possible that ROS and NO also play a role in the memory enhancing and neuroprotective effects of LLLT.

In addition, this study offers evidence of secondary *in vivo* LLLT effects supporting induction of metabolic neuroplasticity of prefrontal brain regions implicated in extinction memory. Secondary LLLT effects implicate the activation of signaling pathways and regulation of gene expression that occur after light exposure. Secondary effects are expected to be pleiotropic and have a significant impact in neuronal function and survival. In this study, secondary LLLT effects are reflected by increases in cytochrome oxidase expression 24 h after LLLT delivery, which represent a non-immediate improvement in neuronal metabolic capacity. Taken together, these results support the hypothesis that LLLT promotes increases in brain energy metabolism that may mediate its memory-enhancing effects.

As mounting evidence indicates an antecedent and possibly pivotal role of mitochondrial bioenergetic deficits and brain hypometabolism in Alzheimer's disease (AD) pathogenesis, the ability of LLLT to increase mitochondrial energy metabolism could be utilized to recover brain processes impacted by regional brain hypometabolism associated with AD. Furthermore, LLLT could be used as a preventive intervention in people who present risk factors for AD, such as those with chronic cerebrovascular hypoperfusion, mild cognitive impairment, or a history of head trauma. In such patients, LLLT could be combined with cognitive intervention approaches, similar to the strategy of applying LLLT during the memory consolidation period described in this study, to attenuate metabolic decline in cortical regions commonly affected by neurodegenerative AD effects. While there is only one clinical study published to date [51] that reports improvement of higher-order cognitive functions in patients with chronic traumatic brain injury after LLLT, and no published studies of LLLT in AD patients to date, other studies aimed at maintaining mitochondrial bioenergetics have shown that a metabolic intervention approach can be useful in postponing and attenuating progression of AD. For example, a meta-analysis examining the effects of acetyl-L-carnitine in mild cognitive impairment and early AD showed beneficial effects on both clinical scales and psychometric tests with improvements after 3 months and even stronger enhancements with longer treatment [58]. Treatment with acetyl-L-carnitine and similar compounds may be augmented by LLLT, which can further increase mitochondrial energy production and cerebral blood flow. LLLT increases cytochrome oxidase and superoxide dismutase activities, without inducing any apparent adverse effects at radiant exposure doses described in this and previous studies [3]. Since memory functions are extremely sensitive to oxidative energy deficits, it is likely that cytochrome oxidase inhibition linked to impairments in cerebral vascular perfusion may underlie memory deficits and eventually contribute to brain cell atrophy and degeneration. Therefore, LLLT should be tested in people with cognitive and memory impairment as a safe and potentially effective alternative or complement to existing pharmacological interventions.

The data presented here support that memory consolidation offers an optimal temporal window to be used as a target to improve brain energy metabolism and modulate memory. Almost thirty five years ago, Martinez et al. [59] demonstrated for the first time that memory improvement can be achieved by methylene blue, a potent mitochondrial metabolic enhancer, during memory consolidation but not before acquisition. More recent experiments demonstrated that extinction memory is effectively improved by brain metabolic enhancement with methylene blue administered 15 min after tone exposure during extinction training [30, 60]. The similarities in the effects of LLLT and methylene blue on extinction become even more remarkable when their common mitochondrial mechanism of action is noted. This common mechanism implicates enhancement of the electron transport chain, an important observation also made by others [61, 62]. Given the memory-improving effects of methylene blue in alternate paradigms such as habituation [63], spatial memory [64–66], object recognition [63], and discrimination learning [67], it is expected

that the beneficial effects of LLLT will have applications in other forms of learning and memory besides extinction.

It is possible that LLLT potentially reaches all brain regions but will selectively enhance those with higher energy demands due to task-dependent activation. This hypothesis is supported by observations that absorption of red and near-infrared light by cytochrome oxidase, and any subsequent photobiological effect, is low in the fully reduced or fully oxidized forms of the enzyme. In contrast, light absorption by cytochrome oxidase has been demonstrated to be maximized in the presence of a mixed valence enzyme state (i.e., partially reduced or oxidized) [18, 68]. The probability of finding a mixed valence enzyme is higher with higher respiratory chain electron flow. In turn, this condition is expected to occur in states of increased energy consumption, such as those of neuronal networks activated during a particular task. In other words, greater photoneurostimulation is expected in highly metabolically active regions such as the prefrontal cortex after extinction training in our study. This potential selectivity represents an important mechanistic insight that deserves validation in future experiments.

To overcome the confounding brain effects intrinsic to the behavioral tasks and the putative neuronal selectivity of LLLT based on energy demand, metabolic effects were determined in brains of naïve rats. This approach allowed the demonstration of a rather interesting hormetic dose-response of LLLT on brain cytochrome oxidase activity. Lower LLLT doses, but not higher doses, induced a significant increase in brain cytochrome oxidase 24 h after treatment. It is believed that far from being a spurious result, this hormetic response on neurometabolic stimulation is representative of the distinctive and well-characterized dose-response effect of LLLT. Hormesis describes a dose-response effect in which there is stimulation of a biological process at a low dose and no effect or even inhibition of that process at a high dose. Hormetic responses are typically illustrated as an inverted U-shaped curve and are useful to describe effects below typical pharmacological threshold, with peak values 30–60% greater than control [69]. Hormetic effects have been regarded at times as negligible, although there is compelling evidence that their presence is biologically meaningful. Hormetic responses have been described for numerous agents including antibiotics [70], antineoplastic drugs [71], antioxidants [72, 73], steroids [74], and radiation [75]. In the case of LLLT, extensive *in vitro* data support photostimulatory effects with lower (0.001–10 J/cm$^2$) doses and inhibition with higher (>10 J/cm$^2$) doses [1, 76, 77]. *In vivo*, hormetic responses of LLLT have been observed in tissues with high oxidative potential such as skeletal muscle [25] and healing connective tissue [78, 79]. Thus, our data can be interpreted as supportive of the current mechanistic paradigm of LLLT that implicates hormetic enhancement of energy metabolism.

In conclusion, this study demonstrated that LED-based LLLT is a non-invasive intervention without significant side effects that can enhance cortical metabolic capacity and facilitate retention of extinction memory. These results implicate LLLT as a potential intervention to improve memory in humans.



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## REFERENCES

[1] Hamblin MR, Demidova TN (2006) Mechanisms of low level light therapy. *Proc SPIE* **6140**, 1-12.

[2] Karu T (1999) Primary and secondary mechanisms of action of visible to near-IR radiation on cells. *J Photochem Photobiol B* **49**, 1-17.

[3] Rojas JC, Gonzalez-Lima F (2011) Low-level light therapy of the eye and brain. *Eye Brain* **3**, 49-67.

[4] Byrnes KR, Wu X, Waynant RW, Ilev IK, Anders JJ (2005) Low power laser irradiation alters gene expression of olfactory ensheathing cells *in vitro*. *Lasers Surg Med* **37**, 161-171.

[5] Anders JJ, Borke RC, Woolery SK, Van de Merwe WP (1993) Low power laser irradiation alters the rate of regeneration of the rat facial nerve. *Lasers Surg Med* **13**, 72-82.

[6] Oron A, Oron U, Streeter J, de Taboada L, Alexandrovich A, Trembovler V, Shohami E (2007) Low-level laser therapy applied transcranially to mice following traumatic brain injury significantly reduces long-term neurological deficits. *J Neurotrauma* **24**, 651-656.

[7] Wu Q, Xuan W, Ando T, Xu T, Huang L, Huang YY, Dai T, Dhital S, Sharma SK, Whalen MJ, Hamblin MR (2011) Low-level laser therapy for closed-head traumatic brain injury in mice: Effect of different wavelengths. *Lasers Surg Med* **6**, e26212.

[8] Wu X, Alberico SL, Moges H, De Taboada L, Tedford CE, Anders JJ (2012) Pulsed light irradiation improves behavioral

outcome in a rat model of chronic mild stress. *Lasers Surg Med* **44**, 227-232.

[9] Lampl Y, Zivin JA, Fisher M, Lew R, Welin L, Dahlof B, Borenstein P, Andersson B, Perez J, Caparo C, Ilic S, Oron U (2007) Infrared laser therapy for ischemic stroke: A new treatment strategy: Results of the NeuroThera Effectiveness and Safety Trial-1 (NEST-1). *Stroke* **38**, 1843-1849.

[10] Lapchak PA, Salgado KF, Chao CH, Zivin JA (2007) Transcranial near-infrared light therapy improves motor function following embolic strokes in rabbits: An extended therapeutic window study using continuous and pulse frequency delivery modes. *Neuroscience* **148**, 907-914.

[11] Oron A, Oron U, Chen J, Eilam A, Zhang C, Sadeh M, Lampl Y, Streeter J, DeTaboada L, Chopp M (2006) Low-level laser therapy applied transcranially to rats after induction of stroke significantly reduces long-term neurological deficits. *Stroke* **37**, 2620-2624.

[12] Rojas JC, Lee J, John JM, Gonzalez-Lima F (2008) Neuroprotective effects of near-infrared light in an *in vivo* model of mitochondrial optic neuropathy. *J Neurosci* **28**, 13511-13521.

[13] Wong-Riley MT, Liang HL, Eells JT, Chance B, Henry MM, Buchmann E, Kane M, Whelan HT (2005) Photobiomodulation directly benefits primary neurons functionally inactivated by toxins: Role of cytochrome c oxidase. *J Biol Chem* **280**, 4761-4771.

[14] Ying R, Liang HL, Whelan HT, Eells JT, Wong-Riley MT (2008) Pretreatment with near-infrared light via light-emitting diode provides added benefit against rotenone- and MPP+-induced neurotoxicity. *Brain Res* **1243**, 167-173.

[15] Liang HL, Whelan HT, Eells JT, Meng H, Buchmann E, Lerch-Gaggl A, Wong-Riley M (2006) Photobiomodulation partially rescues visual cortical neurons from cyanide-induced apoptosis. *Neuroscience* **139**, 639-649.

[16] Liang HL, Whelan HT, Eells JT, Wong-Riley MT (2008) Near-infrared light via light-emitting diode treatment is therapeutic against rotenone- and 1-methyl-4-phenylpyridinium ion-induced neurotoxicity. *Neuroscience* **153**, 963-974.

[17] Karu T (2000) Mechanisms of low-power laser light action on cellular level. *Proc SPIE* **4159**, 1-17.

[18] Karu TI, Pyatibrat LV, Kolyakov SF, Afanasyeva NI (2008) Absorption measurements of cell monolayers relevant to mechanisms of laser phototherapy: Reduction or oxidation of cytochrome c oxidase under laser radiation at 632.8 nm. *Photomed Laser Surg* **26**, 593-599.

[19] Pastore D, Greco M, Passarella S (2000) Specific helium-neon laser sensitivity of the purified cytochrome c oxidase. *Int J Radiat Biol* **76**, 863-870.

[20] Pastore D, Greco M, Petragallo VA, Passarella S (1994) Increase in <–H+/e- ratio of the cytochrome c oxidase reaction in mitochondria irradiated with helium-neon laser. *Biochem Mol Biol Int* **34**, 817-826.

[21] Mochizuki-Oda N, Kataoka Y, Cui Y, Yamada H, Heya M, Awazu K (2002) Effects of near-infra-red laser irradiation on adenosine triphosphate and adenosine diphosphate contents of rat brain tissue. *Neurosci Lett* **323**, 207-210.

[22] Yu W, Naim JO, McGowan M, Ippolito K, Lanzafame RJ (1997) Photomodulation of oxidative metabolism and electron chain enzymes in rat liver mitochondria. *Photochem Photobiol* **66**, 866-871.

[23] Wong-Riley MT (1989) Cytochrome oxidase: An endogenous metabolic marker for neuronal activity. *Trends Neurosci* **12**, 94-101.

[24] Wong-Riley MT, Bai X, Buchmann E, Whelan HT (2001) Light-emitting diode treatment reverses the effect of TTX on cytochrome oxidase in neurons. *Neuroreport* **12**, 3033-3037.

[25] Hayworth CR, Rojas JC, Padilla E, Holmes GM, Sheridan EC, Gonzalez-Lima F (2010) *In vivo* low-level light therapy increases cytochrome oxidase in skeletal muscle. *Photochem Photobiol* **86**, 673-680.

[26] Helm PJ, Ottersen OP, Nase G (2009) Analysis of optical properties of the mouse cranium–implications for *in vivo* multi photon laser scanning microscopy. *J Neurosci Methods* **178**, 316-322.

[27] Schiffer F, Johnston AL, Ravichandran C, Polcari A, Teicher MH, Webb RH, Hamblin MR (2009) Psychological benefits 2 and 4 weeks after a single treatment with near infrared light to the forehead: A pilot study of 10 patients with major depression and anxiety. *Behav Brain Funct* **5**, 46.

[28] McLean CP, Foa EB (2011) Prolonged exposure therapy for post-traumatic stress disorder: A review of evidence and dissemination. *Expert Rev Neurother* **11**, 1151-1163.

[29] Quirk GJ, Pare D, Richardson R, Herry C, Monfils MH, Schiller D, Vicentic A (2010) Erasing fear memories with extinction training. *J Neurosci* **30**, 14993-14997.

[30] Gonzalez-Lima F, Bruchey AK (2004) Extinction memory improvement by the metabolic enhancer methylene blue. *Learn Mem* **11**, 633-640.

[31] Barrett D, Shumake J, Jones D, Gonzalez-Lima F (2003) Metabolic mapping of mouse brain activity after extinction of a conditioned emotional response. *J Neurosci* **23**, 5740-5749.

[32] Bruchey AK, Gonzalez-Lima F (2006) Brain activity associated with fear renewal. *Eur J Neurosci* **24**, 3567-3577.

[33] Bruchey AK, Shumake J, Gonzalez-Lima F (2007) Network model of fear extinction and renewal functional pathways. *Neuroscience* **145**, 423-437.

[34] Paxinos G, Watson C (1997) *The Rat Brain in Stereotaxic Coordinates*, Academic Press, San Diego.

[35] Wong-Riley M (1979) Changes in the visual system of monocularly sutured or enucleated cats demonstrable with cytochrome oxidase histochemistry. *Brain Res* **171**, 11-28.

[36] Gonzalez-Lima F, Cada A (1994) Cytochrome oxidase activity in the auditory system of the mouse: A qualitative and quantitative histochemical study. *Neuroscience* **63**, 559-578.

[37] Gonzalez-Lima F, Cada A (1998) Quantitative histochemistry of cytochrome oxidase activity: Theory, methods, and regional brain vulnerability. In: *Cytochrome oxidase in neuronal metabolism and Alzheimer's disease*, Gonzalez-Lima F, ed. Plenum Press, New York, pp. 55-90.

[38] Gonzalez-Lima F, Jones D (1994) Quantitative mapping of cytochrome oxidase activity in the central auditory system of the gerbil: A study with calibrated activity standards and metal-intensified histochemistry. *Brain Res* **660**, 34-49.

[39] Lin CH, Yeh SH, Lu HY, Gean PW (2003) The similarities and diversities of signal pathways leading to consolidation of conditioning and consolidation of extinction of fear memory. *J Neurosci* **23**, 8310-8317.

[40] Riedel G, Casabona G, Platt B, Macphail EM, Nicoletti F (2000) Fear conditioning-induced time- and subregion-specific increase in expression of mGlu5 receptor protein in rat hippocampus. *Neuropharmacology* **39**, 1943-1951.

[41] Santini E, Ge H, Ren K, Pena de Ortiz S, Quirk GJ (2004) Consolidation of fear extinction requires protein synthesis in the medial prefrontal cortex. *J Neurosci* **24**, 5704-5710.

[42] Herry C, Garcia R (2002) Prefrontal cortex long-term potentiation, but not long-term depression, is associated with the maintenance of extinction of learned fear in mice. *J Neurosci* **22**, 577-583.

[43] Santini E, Muller RU, Quirk GJ (2001) Consolidation of extinction learning involves transfer from NMDA-independent to NMDA-dependent memory. *J Neurosci* **21**, 9009-9017.

[44] Gonzalez-Lima F, Valla J, Cada A (1998) Brain cytochrome oxidase activity and how it relates to the pathophysiology of memory and Alzheimer's disease. In: *Free Radicals, Oxidative Stress and Antioxidants: Pathological and Physiological Significance*, Ozben T, ed. Plenum Press, New York, pp. 205-227.

[45] Liang HL, Ongwijitwat S, Wong-Riley MT (2006) Bigenomic functional regulation of all 13 cytochrome c oxidase subunit transcripts in rat neurons in vitro and in vivo. *Neuroscience* **140**, 177-190.

[46] Yang SJ, Liang HL, Wong-Riley MT (2006) Activity-dependent transcriptional regulation of nuclear respiratory factor-1 in cultured rat visual cortical neurons. *Neuroscience* **141**, 1181-1192.

[47] Detaboada L, Ilic S, Leichliter-Martha S, Oron U, Oron A, Streeter J (2006) Transcranial application of low-energy laser irradiation improves neurological deficits in rats following acute stroke. *Lasers Surg Med* **38**, 70-73.

[48] Lapchak PA, Wei J, Zivin JA (2004) Transcranial infrared laser therapy improves clinical rating scores after embolic strokes in rabbits. *Stroke* **35**, 1985-1988.

[49] Shaw VE, Spana S, Ashkan K, Benabid AL, Stone J, Baker GE, Mitrofanis J (2010) Neuroprotection of midbrain dopaminergic cells in MPTP-treated mice after near-infrared light treatment. *J Comp Neurol* **518**, 25-40.

[50] Michalikova S, Ennaceur A, van Rensburg R, Chazot PL (2008) Emotional responses and memory performance of middle-aged CD1 mice in a 3D maze: Effects of low infrared light. *Neurobiol Learn Mem* **89**, 480-488.

[51] Naeser MA, Saltmarche A, Krengel MH, Hamblin MR, Knight JA (2010) Improved cognitive function after transcranial, light-emitting diode treatments in chronic, traumatic brain injury: Two case reports. *Photomed Laser Surg* **29**, 351-358.

[52] Chen AC, Arany PR, Huang YY, Tomkinson EM, Sharma SK, Kharkwal GB, Saleem T, Mooney D, Yull FE, Blackwell TS, Hamblin MR (2011) Low-level laser therapy activates NF-kB via generation of reactive oxygen species in mouse embryonic fibroblasts. *PLoS One* **6**, e22453.

[53] Wong-Riley M (1989) Cytochrome oxidase: An endogenous metabolic marker for neuronal activity. *Trends Neurosci* **12**, 94-101.

[54] Wong-Riley MT, Nie F, Hevner RF, Liu S (1998) Brain cytochrome oxidase: Functional significance and bigenomic regulation in the CNS. In: *Cytochrome oxidase in neuronal metabolism and Alzheimer's disease*, Gonzalez-Lima F, Ed, Plenum Press, New York, pp. 1-53.

[55] Dhar SS, Wong-Riley MT (2009) Coupling of energy metabolism and synaptic transmission at the transcriptional level: Role of nuclear respiratory factor 1 in regulating both cytochrome c oxidase and NMDA glutamate receptor subunit genes. *J Neurosci* **29**, 483-492.

[56] Poyton RO, Castello PR, Ball KA, Woo DK, Pan N (2009) Mitochondria and hypoxic signaling: A new view. *Ann N Y Acad Sci* **1177**, 48-56.

[57] Uozumi Y, Nawashiro H, Sato S, Kawauchi S, Shima K, Kikuchi M (2010) Targeted increase in cerebral blood flow by transcranial near-infrared laser irradiation. *Lasers Surg Med* **42**, 566-576.

[58] Montgomery SA, Thal LJ, Amrein R (2003) Meta-analysis of double blind randomized controlled clinical trials of acetyl-L-carnitine versus placebo in the treatment of mild cognitive impairment and mild Alzheimer's disease. *Int Clin Psychopharmacol* **18**, 61-71.

[59] Martinez J Jr, Jensen RA, Vasquez B, McGuiness T, McGaugh JL (1978) Methylene blue alters retention of inhibitory avoidance responses. *Physiological Psychology* **6**, 387-390.

[60] Wrubel KM, Barrett D, Shumake J, Johnson SE, Gonzalez-Lima F (2007) Methylene blue facilitates the extinction of fear in an animal model of susceptibility to learned helplessness. *Neurobiol Learn Mem* **87**, 209-217.

[61] Karu TI (2008) Mitochondrial signaling in mammalian cells activated by red and near-IR radiation. *Photochem Photobiol* **84**, 1091-1099.

[62] Karu TI, Pyatibrat LV, Kalendo GS (2001) Cell attachment modulation by radiation from a pulsed light diode (lambda=820nm) and various chemicals. *Lasers Surg Med* **28**, 227-236.

[63] Riha PD, Bruchey AK, Echevarria DJ, Gonzalez-Lima F (2005) Memory facilitation by methylene blue: Dose-dependent effect on behavior and brain oxygen consumption. *Eur J Pharmacol* **511**, 151-158.

[64] Callaway NL, Riha PD, Bruchey AK, Munshi Z, Gonzalez-Lima F (2004) Methylene blue improves brain oxidative metabolism and memory retention in rats. *Pharmacol Biochem Behav* **77**, 175-181.

[65] Callaway NL, Riha PD, Wrubel KM, McCollum D, Gonzalez-Lima F (2002) Methylene blue restores spatial memory retention impaired by an inhibitor of cytochrome oxidase in rats. *Neurosci Lett* **332**, 83-86.

[66] Riha PD, Rojas JC, Gonzalez-Lima F (2011) Beneficial network effects of methylene blue in an amnestic model. *Neuroimage* **54**, 2623-2634.

[67] Wrubel KM, Riha PD, Maldonado MA, McCollum D, Gonzalez-Lima F (2007) The brain metabolic enhancer methylene blue improves discrimination learning in rats. *Pharmacol Biochem Behav* **86**, 712-717.

[68] Karu TI, Pyatibrat LV, Kalendo GS, Esenaliev RO (1996) Effects of monochromatic low-intensity light and laser irradiation on adhesion of HeLa cells *in vitro*. *Lasers Surg Med* **18**, 171-177.

[69] Calabrese EJ, Stanek EJ 3rd, Nascarella MA, Hoffmann GR (2008) Hormesis predicts low-dose responses better than threshold models. *Int J Toxicol* **27**, 369-378.

[70] Calabrese EJ, Hoffmann GR, Stanek EJ, Nascarella MA (2010) Hormesis in high-throughput screening of antibacterial compounds in E coli. *Hum Exp Toxicol* **29**, 667-677.

[71] Nascarella MA, Stanek EJ 3rd, Hoffmann GR, Calabrese EJ (2009) Quantification of hormesis in anticancer-agent dose-responses. *Dose Response* **7**, 160-171.

[72] Bruchey AK, Gonzalez-Lima F (2008) Behavioral, Physiological and Biochemical Hormetic Responses to the Autoxidizable Dye Methylene Blue. *Am J Pharmacol Toxicol* **3**, 72-79.

[73] Calabrese EJ, Mattson MP, Calabrese V (2010) Resveratrol commonly displays hormesis: Occurrence and biomedical significance. *Hum Exp Toxicol* **29**, 980-1015.

[74] Lupien SJ, Buss C, Schramek TE, Maheu F, Pruessner J (2005) Hormetic influence of glucocorticoids on human memory. *Nonlinearity Biol Toxicol Med* **3**, 23-56.

[75] Vaiserman AM (2010) Radiation hormesis: Historical perspective and implications for low-dose cancer risk assessment. *Dose Response* **8**, 172-191.

[76] Chung H, Dai T, Sharma SK, Huang YY, Carroll JD, Hamblin MR (2012) The Nuts and Bolts of Low-level Laser (Light) Therapy. *Ann Biomed Eng* **40**, 516-533.

[77] Huang YY, Chen AC, Carroll JD, Hamblin MR (2009) Biphasic dose response in low level light therapy. *Dose Response* **7**, 358-383.

[78] Demidova-Rice TN, Salomatina EV, Yaroslavsky AN, Herman IM, Hamblin MR (2007) Low-level light stimulates excisional wound healing in mice. *Lasers Surg Med* **39**, 706-715.

[79] Lanzafame RJ, Stadler I, Kurtz AF, Connelly R, Peter TA Sr, Brondon P, Olson D (2007) Reciprocity of exposure time and irradiance on energy density during photoradiation on wound healing in a murine pressure ulcer model. *Lasers Surg Med* **39**, 534-542.