Neuroprotection and Neurocognitive Augmentation by Photobiomodulation

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1 Introduction

This chapter discusses studies with laboratory animals and human volunteers, showing that specific interventions using transcranial light stimulation can cause neuroprotection and neurocognitive augmentation. These neural effects are part of a more general phenomenon known as photobiomodulation, which primarily involves stimulation with red to near-infrared wavelengths of light. Photobiomodulation or low-level light therapy refers to the application of such light to biological systems with the purpose of providing a beneficial effect. The absorption of incident photon energy by tissue chromophores induces electronically excited states that modify their energy state and can promote oxygen consumption and the generation of reactive oxygen species. These two biochemical events have been proposed to activate a variety of intracellular signaling and metabolic pathways that modify cellular functions (Karu 1989, 2000), which in turn modify brain function and behavior because brain cells are critically dependent on oxygen consumption for energy production by mitochondria (Gonzalez-Lima et al. 2014). Photobiomodulation has been found to influence various biological processes in cell cultures, animal models, and clinical conditions (Yu et al. 1994; Eells et al. 2003; Wong-Riley et al. 2005). For example, photobiomodulation is being used clinically in humans to accelerate wound healing (Conlan et al. 1996), to relieve inflammation (Whelan et al. 2002), and to relieve neurogenic pain (Iijima et al. 1991).

Wong-Riley et al. (2001) reported that photobiomodulation using light-emitting diodes (LEDs) was able to reverse the neurotoxic effects of tetrodotoxin on neuronal cultures by a process involving the mitochondrial respiratory enzyme cytochrome

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oxidase (also called cytochrome c oxidase or Complex IV of the Electron Transport Chain). A number of additional in vitro effects of photobiomodulation on nervous tissue have been described. These include, among others, the following: (1) increased expression of the antiapoptotic protein Bcl-2 and reduced expression of the proapoptotic protein Bax (Shefer et al. 2002; Liang et al. 2006); (2) decreased numbers of apoptotic cells after exposure to the amyloid beta protein (Duan et al. 2003); (3) improved function of cortical neurons inactivated by toxins (Wong-Riley et al. 2005; Liang et al. 2006); (4) increased survival and ATP content of striatal neurons after rotenone- and MPP$^+$-induced toxicity and decreased oxidative stress and nitric oxide production (Liang et al. 2008); (5) increased neurite outgrowth (Wollman and Rochkind 1998); (6) regulation of gene encoding for DNA repair proteins, antioxidant enzymes, and molecular chaperones (Eells et al. 2004); (7) increased proliferation of olfactory ensheathing stem cells (Byrnes et al. 2005a), Schwann cells (Van Breugel and Bar 1993), astrocytes, and oligodendrocytes (Rochkind et al. 1990). In vivo, photobiomodulation induces peripheral and central nerve regeneration after trauma (Byrnes et al. 2005b), reduces neuroinflammation (Byrnes et al. 2005b), and prevents methanol-induced photoreceptor degeneration (Eells et al. 2003).

In photobiomodulation, a target tissue or organism is exposed to a low power, high fluency source of directional and monochromatic light, which delivers energy doses that are too low to cause heating, yet high enough to modulate cell functions (Sommer et al. 2001). Photobiomodulation is best done using wavelengths at the end of the visible electromagnetic spectrum (λ = 610–1100 nm). This wavelength range is used because light energy at wavelengths shorter than 600 nm is generally absorbed by the skin pigment melanin, whereas water significantly absorbs energy at wavelengths higher than 1150 nm. This implies the existence of an "optical window" in tissue that covers the red and near-infrared wavelengths, where there is room for a biological effect due to excitation of intracellular photoacceptors (Hamblin and Demidova 2006).

This chapter describes our animal and human research using red to near-infrared lasers and LEDs, a noninvasive and relatively inexpensive intervention, which we are investigating for the augmentation of cognitive brain functions and the treatment of neurodegenerative disorders. Part 1 deals with animal studies on the prevention of neurodegeneration and behavioral deficits by photobiomodulation. Part 1 shows light parameters that prevent optic neuropathy in vivo. These parameters include fractionated exposure to far-red to near-infrared light wavelengths, primarily corresponding to the absorption spectrum of the mitochondrial respiratory enzyme cytochrome oxidase. We also show that such light stimulates cell respiration and upregulates cytochrome oxidase activity and the antioxidant enzyme superoxide dismutase. The translation of this knowledge for human studies in Part 2 shows that this research may have a broad impact, not only relevant for cognitive enhancement in healthy people but also potential for the noninvasive treatment of neurodegenerative and neurocognitive disorders.

2 Part 1: Animal Studies—Prevention of Neurodegeneration and Behavioral Deficits by Photobiomodulation

2.1 Objectives of Animal Studies of Neuroprotection by Photobiomodulation

Targeted stimulation of mitochondrial function with photobiomodulation or low-level light therapy constitutes a sound, yet largely unexplored strategy with great potential for neurocognitive enhancement and neuroprotection from neurodegeneration. Mitochondrial dysfunction, concomitant free radical damage, and decrease in energy production are believed to play a central role in neurodegeneration. Despite the acknowledged central role of mitochondrial dysfunction in the pathophysiology of neurodegeneration, stimulation of mitochondrial function has not been thoroughly considered in the design of neuroprotective interventions (Gonzalez-Lima et al. 2014). Photobiomodulation is a potential noninvasive and safe method to effectively counteract these early events in neurodegeneration induced by mitochondrial dysfunction. Cytochrome oxidase is a potential target for mitochondrial neuroprotective interventions since it is an inducible respiratory enzyme that plays a limiting step role in energy production. Cytochrome oxidase expression is tightly linked to neuronal energetic demands and is mediated through a genome-wide activation of transcription factors with the potential to impact neuronal homeostasis (Gonzalez-Lima and Cada 1998). Being the major photoacceptor in neurons, cytochrome oxidase is an ideal target to promote neuroprotection with photobiomodulation. Although photobiomodulation has been shown to be highly effective at preventing neuronal death in vitro, its in vivo effects and mechanism of action are still poorly characterized (Rojas and Gonzalez-Lima 2011).

Our *long-term research goal* is to develop metabolic interventions that effectively prevent or treat neurodegenerative disorders in humans. The *objective* of Part 1 is to describe beneficial effects of photobiomodulation, which prevent neurodegeneration and behavioral deficits in an in vivo animal model of optic neuropathy induced by mitochondrial dysfunction, and to identify the mechanism of photobiomodulation action. Part 1 is primarily based on our data showing that photobiomodulation reduces retinal damage and associated functional visual deficits induced by mitochondrial dysfunction in vivo (Rojas et al. 2008b). Our *overarching hypothesis* is that photobiomodulation delivered *via* light-emitting diode (LED) arrays will upregulate the mitochondrial respiratory enzyme cytochrome oxidase and the antioxidant enzyme superoxide dismutase, which will be highly effective against mitochondrial optic neuropathy in vivo. Our *rationale* is that determining the parameters to prevent mitochondrial optic neuropathy with photobiomodulation in vivo would establish a strong basis for the design of clinical trials in humans, which we discuss in Part 2 of this chapter. We are especially well prepared to undertake this demonstration because, in addition to our strong data on photobiomodulation, we have developed an efficient in vivo animal model of optic neuropathy induced by mitochondrial dysfunction (Zhang et al. 2002, 2006), and have used it to characterize

neuroprotective effects with the antiexcitotoxic agent memantine (Rojas et al. 2008a) and the metabolic enhancer methylene blue (Rojas et al. 2009). We will explain how using our animal model of optic neuropathy can address the following behavioral, anatomical, and mechanistic specific aims:

Aim 1: Demonstrate an effective photobiomodulation protocol to prevent behavioral deficits induced by mitochondrial dysfunction in vivo. Our hypothesis, based on our in vitro and in vivo data, is that a fractionated photobiomodulation protocol over 6 days with wavelengths aimed at the copper absorption peaks of cytochrome oxidase, will be the most effective in preventing functional visual deficits induced by mitochondrial failure in vivo.

Aim 2: Demonstrate the efficacy of photobiomodulation to prevent neurodegeneration induced by mitochondrial dysfunction in vivo. Our hypothesis, based on our in vitro and in vivo data, is that the most effective photobiomodulation protocol will be able to prevent structural degenerative damage in optic neuropathy induced by mitochondrial failure in vivo.

Aim 3: Demonstrate the mechanism of action of photobiomodulation that prevents neurodegeneration induced by mitochondrial dysfunction. Our hypothesis is that photobiomodulation will exert its neuroprotective effects via upregulation of the mitochondrial respiratory enzyme cytochrome oxidase and the antioxidant enzyme superoxide dismutase.

2.2   Significance of Animal Studies of Neuroprotection by Photobiomodulation

Exposure of neuronal cultures to red or near-infrared light (photobiomodulation) is known to increase survival after neurotoxin exposure in vitro (Wong-Riley et al. 2001). Such intervention might represent a potential preventive strategy to avoid neurodegeneration, an otherwise irreversible process. Despite positive evidence, skepticism about the potential clinical use of photobiomodulation still persists because the scientific rationale for in vivo treatment protocols for particular responses is lacking, and current photobiomodulation use is based on empirical choice of wavelength, total dose, dose fractionation, and other parameters. Nevertheless, the fact remains that photobiomodulation exerts a wide variety of neuronal effects. For example, prophylactic photobiomodulation in vitro has proved to be very effective at protecting neurons from neurodegeneration induced by mitochondrial toxins (Wong-Riley et al. 2001, 2005; Liang et al. 2008). Photobiomodulation has also been successfully employed for nerve repair and reduction of neural injury after stroke in animal models (Lapchak et al. 2004; Byrnes et al. 2005b), and it is clinically used to relieve pain in humans (Iijima et al. 1991). Rojas et al. (2008b) was the first in vivo animal study to use photobiomodulation in the treatment of optic neuropathy. Optic neuropathy is found in conditions with high morbidity for which no effective treatments are available (Abu-Amero

et al. 2006; Iseri et al. 2006; He et al. 2008; Carelli et al. 2009). Furthermore, no previous studies have addressed the effectiveness of photobiomodulation in any model of optic neuropathy and it is also uncertain how energy from photobiomodulation works and what are the optimal light parameters for specific applications in vivo. Our research validated the concept that the mechanism of action of photobiomodulation in vivo involves direct photonic stimulation of the respiratory enzyme cytochrome oxidase. With this animal research, we expect to set the preclinical basis for the design of an effective, noninvasive, inexpensive, and safe intervention against optic neuropathy, which could potentially have a large clinical impact worldwide. We also expect to stimulate research on the applicability of photobiomodulation in other neurodegenerative disorders linked to mitochondrial dysfunction such as Alzheimer's and Parkinson's diseases (Rojas and Gonzalez-Lima 2017).

2.3   Cytochrome Oxidase as a Molecular Target of Photobiomodulation

Virtually, all biological tissues feature photoacceptor molecules adapted to maximize the assimilation of noncoherent electromagnetic radiation from the environment (Alberts 2002). This adaptation reaches its highest degree of efficiency in the thylakoid membrane of chloroplasts in plants, but it is conserved in animal tissue as well. In mammalian tissue, the known photoacceptors are mainly heme-containing metalloproteins. The three most important metalloproteins are hemoglobin, myoglobin, and cytochrome oxidase, but others such as superoxide dismutase, cytochrome *c*, cytochrome *b*, nitric oxide synthase, catalase, guanylate cyclase, flavoproteins, and cryptochromes may also play a role as photoacceptors. Although all these molecules may be involved in the photobiomodulation effects (Wong-Riley et al. 2005), cytochrome oxidase is by far the most abundant photoacceptor in neurons and its absorption peaks have been shown to coincide with the action spectra of photobiomodulation in vitro. For these reasons, *cytochrome oxidase is regarded as the primary photoacceptor of light in the red to near-infrared region of the light spectrum in neural tissue* (Yamanaka et al. 1988; Pastore et al. 2000; Fan et al. 2006).

Cytochrome oxidase is the terminal complex of the mitochondrial electron transport chain and catalyzes the reduction of more than 95% of the oxygen taken up by aerobic organisms. Thus, cytochrome oxidase constitutes an efficient energy-transducing device, acting as a redox-linked proton pump that creates a transmembrane electrochemical gradient and as a rate-limiting step for the synthesis of the energy-storing molecule adenosine triphosphate (ATP) (Hatefi 1985). Cytochrome oxidase is a bigenomically regulated enzyme, whose expression is tightly coupled to neuronal energy demands and glutamatergic activation (Liang et al. 2006; Dhar et al. 2009; Dhar and Wong-Riley 2009). Its activity has been extensively used as a marker of neuronal metabolic activity (Wong-Riley 1989; Sakata et al. 2005).

Cytochrome oxidase is a heme-containing enzyme and the most abundant metallo-protein in neurons. Cytochrome oxidase contains four redox metal centers: Cu_A, Cu_B, Heme a, and Heme a_3. The redox state of the enzyme can vary from fully reduced to fully oxidized, with intermediate states that include oxidation of one, two, or three metal centers. These metal centers determine different light absorption peaks for the enzyme: 613.5–623.5 nm, 667.5–683.7 nm, 750.7–772.3 nm, and 812.5–846 nm, which correspond to Cu_A reduced, Cu_A oxidized, Cu_B reduced, and Cu_B oxidized, respectively (Hamblin and Demidova 2006). These absorption peaks have been shown to coincide with its peaks of catalytic activity and with ATP content (Eells et al. 2004) and, as mentioned above, with the action spectra of photobio-modulation in vitro (Wong-Riley et al. 2005).

2.4   In Vitro Neuroprotective Effects of Cytochrome Oxidase Stimulation by Photobiomodulation

In vitro evidence supports that photobiomodulation directly stimulates the catalytic activity of cytochrome oxidase and further regulates mitochondrial function. For example, photobiomodulation has been shown to increase cytochrome c oxidation in the presence of cytochrome oxidase (Pastore et al. 2000). It also modulates the production of nitric oxide (Liang et al. 2008) and modifies the interactions between cytochrome oxidase and nitric oxide, promoting the generation of free radicals (Karu 1999). Photobiomodulation also increases the rate of oxygen consumption in hepatic mitochondria as well as the mitochondrial phosphate potential, energy charge, catalytic activity of mitochondrial complexes I, II, III, and IV (Yu et al. 1997), and ATP cellular content (Liang et al. 2008; Ying et al. 2008). Further in vitro evidence also supports that besides its immediate effects on neuronal cytochrome oxidase catalytic activity, photobiomodulation induces upregulation of the neuronal cytochrome oxidase pool, implicating an indirect effect of photobiomodulation on gene expression (Wong-Riley and Liang 2017). Rat neuronal cultures exposed to photobiomodulation showed a 12% increase in cytochrome oxidase activity. A similar enhancing effect was also observed when delivered after exposure to tetrodo-toxin, which blocks electrical neural activity and indirectly inhibits cytochrome oxidase activity (Wong-Riley et al. 2001). In addition, photobiomodulation partially restored enzyme activity blocked by potassium cyanide, a cytochrome oxidase inhibitor, and significantly reduced neuronal cell death (Liang et al. 2006). Similar protective effects were observed when striatal and cortical rat neuronal cultures were exposed to rotenone and MPP$^+$, toxins that inhibit mitochondrial complex I. Photobiomodulation significantly increased cellular ATP content, decreased the number of neurons undergoing cell death, and reduced the expressions of reactive oxygen species and reactive nitrogen species in rotenone- or MPP$^+$-exposed neurons as compared to untreated ones (Liang et al. 2008). Furthermore, prophylactic use of photobiomodulation also suppressed rotenone- or MPP$^+$-induced apoptosis in both

striatal and cortical neurons, and pretreatment plus photobiomodulation treatment during neurotoxin exposure was significantly better than photobiomodulation treatment alone during exposure to neurotoxins (Ying et al. 2008). Despite this large body of in vitro compelling evidence supporting a protective role of photobiomodulation against mitochondrial toxins via induction of cytochrome oxidase, there is little evidence regarding its effects against mitochondrial inhibitors in in vivo systems, which prompted the design of our animal studies presented in this chapter.

2.5   *In Vivo Light Delivery and Dosing Considerations*

A number of parameters are known to influence the efficacy, feasibility, and safety of photobiomodulation in vivo (Rojas and Gonzalez-Lima 2011). These should be taken into account since they are expected to vary depending on the specific therapeutic purpose. The main parameters include (1) dosage, (2) wavelength, (3) dosing schedules or fractionation, and (4) source of radiation.

1. *Dose*. There is essentially a very poor characterization of photobiomodulation dosing responses for neuroprotective applications in vivo. Photobiomodulation doses are expressed as radiant energy in Joules per surface area (energy density). Incident energy on a given surface is the product of power density (or light intensity, in watts) and time of exposure (in seconds). Thus, for achieving a desired dose, either power or time of exposure can be varied. Photobiomodulation dosimetry should consider the dose-response phenomenon of *hormesis*. A hormetic dose-response (also known as U-shaped, biphasic, or bell-shaped dose response) involves stimulation of a biological process at a low dose and inhibition of that process at a high dose. Hormetic models are superior to linear threshold models in their capacity to accurately predict responses below a pharmacological threshold (Calabrese and Baldwin 2003; Kaiser 2003). This is important because the stimulatory responses to photobiomodulation are usually modest, being only about 30–60% greater than control values. This contrasts with the several-fold increase in a specific variable expected according to traditional dose-response models (Calabrese 2008). Dose-response hormesis is well documented in photobiomodulation applications since photostimulatory or photoinhibitory effects are obtained with low (0.001–10 J/cm^2) and high (>10 J/cm^2) energy densities, respectively (Brondon et al. 2005). Positive response is expected to vary within this dose range for a particular desired outcome (Hamblin and Demidova 2006). For example, Liang et al. (2008) demonstrated that photobiomodulation daily doses within the stimulatory range (4 and 8 J/cm^2) are more effective than higher doses (12 and 16 J/cm^2) at preventing neurotoxicity induced by mitochondrial complex I inhibitors rotenone and 1-methyl-4-phenylpyridinium in vitro. Therefore, there exist optimal doses of photobiomodulation for neuroprotection in vivo. Doses lower than this optimum value, and more importantly,

doses larger than the optimum value will a have a diminished treatment response. High doses might even produce a negative effect.

2. *Wavelength*. Most photobiomodulation protocols use wavelengths in the range of *620–870 nm* to coincide with maximal absorbance profiles of endogenous chromophores, mainly cytochrome oxidase (Karu 1999). Near-infrared wavelengths offer acceptable penetration into tissues, as they overlap the "optical window". Wavelengths inferior to 600 nm are usually absorbed by abundant chromophores such as melanin and hemoglobin, whereas wavelengths above 1150 nm are usually absorbed by water. This means that the major penetration occurs in a window that includes the red and near-infrared spectrum. Such infrared wavelengths include the absorption peaks of the most important photoacceptor in neurons: the respiratory enzyme cytochrome oxidase. As mentioned before, cytochrome oxidase contains four redox metal centers: Cu_A, Cu_B, Heme *a*, and Heme a_3. The redox state of the enzyme can vary from fully reduced to fully oxidized, with intermediate states that include oxidation of one, two, or three metal centers that have different absorption spectra. The absorption peaks of the two copper centers in cytochrome oxidase (Cu_A and Cu_B) have been shown to coincide with the action spectra of photobiomodulation. In addition, the endogenous antioxidant enzyme superoxide dismutase (SOD) shows absorption peaks at 670–680 nm (Kubota and Yang 1984). SOD scavenges superoxide anions generated during successive reduction of molecular oxygen by single electrons, in electron transport redox reactions. The absorption peaks of SOD fall in the near-infrared window, which makes it an alternate photoacceptor relevant for neuroprotective actions. Hence, near-infrared wavelengths that stimulate cytochrome oxidase might also boost the antioxidant activity of SOD.

3. *Fractionation protocol*. Delivering large total doses of photobiomodulation in a single session produces less favorable outcomes than giving the same doses over several sessions. This dose fractionation has been tested in vitro and in vivo, and it has been shown to be highly effective at preventing neurodegeneration (Liang et al. 2008; Rojas et al. 2008b). Fractionation protocols that deliver the total dose over 6 days or more have demonstrated favorable results, including those that split the daily dose into two sessions (Liang et al. 2006, 2008; Ying et al. 2008). In addition, photobiomodulation fractionation protocols including prophylactic doses given before the neurotoxic lesion are also effective at preventing neurodegeneration (Rojas et al. 2008b; Ying et al. 2008).

4. *Photobiomodulation source*. Light-emitting diode (LED) arrays as well as lasers can produce photobiomodulation. Laser sources allow better penetration than LED sources, and so we prefer lasers for human brain photobiomodulation, as shown in Part 2. However, some lasers have limitations in beam width, wavelength capabilities, and areas of tissues that can be treated. In addition, some lasers are associated with heat production that can induce tissue damage (Eells et al. 2004). In contrast, LED arrays generate negligible amounts of heat reducing the risk of thermal injury. For example, a photobiomodulation source using LEDs with a wavelength of 633 nm, an intensity of less than 10 mW, and divergent beams is considered a safe source because, owing to its divergence, it cannot

damage the eye (Rojas et al. 2008b). In addition, LED arrays are compact and portable and have achieved nonsignificant risk status for human trials by the FDA (Wong-Riley et al. 2005).

In addition to these four variables, the response to photobiomodulation also depends on a number of intrinsic biological properties of the target tissue, including photoacceptor content, cell viability, redox status, susceptibility to induction of genetic expression, metabolic capacity, location in the body with reference to tissues with high absorbances, and conditions of the interstitial milieu. Hence, an optimal neuroprotective response depends on photobiomodulation delivery variables that should be tailored to the properties of the target tissue, and finding an effective protocol requires a careful modification of the relevant parameters.

2.6 Mitochondrial Dysfunction in Neurodegenerative Disorders and Therapeutic Role of Photobiomodulation

Mitochondria play a central role in neuronal physiology. These organelles integrate cell respiration, energy metabolism, and ionic balance into a homeostatic coherent adaptation for energy maintenance and cell survival (Kann and Kovacs 2007). The retina contains neurons with extremely high energy demands that rely mostly on mitochondrial-derived ATP to meet these requirements (Astrup et al. 1981; Ames III et al. 1992; Kann and Kovacs 2007). Thus, similar to other neuronal populations, retinal neurons are very vulnerable to events that lead to oxidative stress and energy depletion, including oxygen or glucose deprivation and dysfunction of the mitochondrial machinery that uses both of them to generate ATP (Beretta et al. 2006; Levin 2007). Evidence accumulated in the last 25 years suggests that mitochondrial dysfunction, induced by both genetic and environmental factors, plays a key role in the pathogenesis of retinal neurodegeneration, the main morphological feature of optic neuropathy.

Common eye disorders, such as glaucoma and age-related macular degeneration, and even neurodegenerative disorders, such as Alzheimer's disease, have been increasingly recognized to feature optic neuropathy induced by mitochondrial dysfunction. Patients with Alzheimer's disease show a reduction in the number of retinal ganglion cells and axons, compared to healthy individuals (Hinton et al. 1986; Valla et al. 2001; Danesh-Meyer et al. 2006; Iseri et al. 2006). In addition, the most common primary mitochondrial disorder, Leber's optic neuropathy, is responsible for approximately 2% of all cases of blindness (Chalmers and Schapira 1999). All these conditions feature retinal ganglion cell degeneration, optic nerve atrophy, and blindness that severely decrease the quality of life of affected individuals and represent a major public health problem. A number of genetic and acquired conditions featuring blindness secondary to degeneration of the retina and optic nerve have been linked to mitochondrial dysfunction. Besides Leber's hereditary optic neuropathy, genetic conditions featuring optic neuropathy and mitochondrial failure

include Leigh syndrome (Borit 1971; DiMauro 1999), Friedreich's ataxia (Carelli et al. 2002), myoclonic epilepsy ragged-red-fibers (MERRF) (Chinnery et al. 1997), mitochondrial encephalomyopathy-lactic acidosis and stroke-like syndrome (MELAS) (Hwang et al. 1997), hereditary spastic paraplegia (Casari et al. 1998), and the deafness-dystonia-optic atrophy syndrome (Tranebjaerg et al. 2000). Similarly, acquired diseases featuring optic neuropathy with an association with mitochondrial dysfunction include the tobacco-alcohol amblyopia and intoxication with chloramphenicol, ethambutol, carbon monoxide, clioquinol, cyanide, hexa-chlorophene, isoniazid, lead, methanol, plasmocid, or triethyltin (Carelli et al. 2002).

We developed and tested a toxicological model of mitochondrial optic neuropathy with a favorable response to methylene blue and photobiomodulation (Rojas and Gonzalez-Lima 2010). Light tissue penetration depends on both the types of target tissues, wavelength and the source of photobiomodulation. Besides being safe, at red to near-infrared wavelengths, light penetration to the eye is maximal, where absorbance by the cornea and lens is negligible (<10%) and high refractive indices favor low light scattering and a high degree of focusing on the retina (Jester et al. 1999). In addition, spectroscopic measures have shown that photons at wavelengths between 630 and 800 nm are able to travel approximately 23 cm even in layers of tissues such as skin, dense connective tissue, muscle, bone, and spinal cord, with about 6% of the total energy density being detectable at the ventral surface of a living rat, when the photobiomodulation source is located on the dorsal surface (Byrnes et al. 2005b; Wong-Riley et al. 2005). Thus, the studies in Part 1 are not only relevant for the treatment of a number of neuro-ophthalmological conditions but also pursue a more general goal of developing therapeutic approaches against neurodegeneration, which focus on counteracting the immediate consequences of mitochondrial failure.

Animal studies relevant for our aims 1, 2, and 3 are briefly presented below. These studies used fixed daily photobiomodulation doses, wavelengths, and power density (3.6 J/cm^2, 633 nm, and 2 mW, respectively) and tested three different photobiomodulation fractionation protocols. The results showed that these variables allow effective neuroprotection in the model of optic neuropathy (Rojas et al. 2008b).

2.7 Photobiomodulation Prevents Impairment of Visual Function in a Rat Model of Optic Neuropathy Induced by Mitochondrial Dysfunction

Rationale. Animals were male Long-Evans rats (40 days-old). Visual function was assessed behaviorally before and after treatment, using a descending method of limits in a 2-choice visual task apparatus designed to determine minute changes in the dark-adapted illuminance sensitivity threshold. This was accomplished to test the in vivo protective effects of photobiomodulation at the functional level.

Model of optic neuropathy. Animals were anesthetized with 1.5% isoflurane and received bilateral intravitreal injections (total volume 3 μL). Subjects were divided into control and experimental groups. In the control group ($n = 7$), the vitreous body in both eyes of each subject was injected with the vehicle dimethyl sulfoxide (DMSO). Subjects in the experimental group ($n = 23$) received bilateral intravitreal injections of 200 μg/kg rotenone in DMSO. Rotenone is a mitochondrial complex I inhibitor shown to induce oxidative stress and energy depletion-mediated neurotoxicity (Sherer et al. 2003; Beretta et al. 2006) and retinal degeneration after a single intravitreal injection (Zhang et al. 2002). Sixteen days after the rotenone injection, the subjects were decapitated, and the eyeballs and brains were rapidly removed and frozen in isopentane (−40 °C).

Photobiomodulation delivery. Photobiomodulation was delivered via two R30-123 narrow-angle light-emitting diode (LED) arrays (radius = 4.4 cm) (LEDtronics, Inc., Torrance, CA) located 3.8 cm above the subjects' head. All photobiomodulation sessions were given at peak $\lambda = 633$ nm, with a power density of 2 mW/cm^2, during 30 min for an energy density dose of 3.6 J/cm^2. This single-session energy density was based on previous studies showing beneficial photobiomodulation effects in in vitro and in vivo models of neurodegeneration induced by mitochondrial dysfunction (Liang et al. 2006). Three different photobiomodulation protocols were assessed in subjects receiving bilateral rotenone intravitreal injections (i.e., the experimental group) for their effectiveness to prevent rotenone-induced retinotoxicity. Protocol photobiomodulation 1 (NILT 1, $n = 7$) consisted of a total dose of 10.8 J/cm^2 fractionated in three sessions of photobiomodulation treatment, each one occurring at 10 min, 24 h, and 48 h after rotenone injections. Protocol photobiomodulation 2 (NILT 2, $n = 5$) consisted of six sessions of photobiomodulation treatment occurring at 10 min, 24 h, 48 h, 72 h, 96 h, and 120 h after rotenone injections, for a total dose of 21.6 J/cm^2. Protocol photobiomodulation 3 (NILT 3, $n = 5$) also consisted of a total dose of 21.6 J/cm^2, similar to protocol photobiomodulation 2, but in this case, treatment sessions occurred at 48 and 24 h before the rotenone injections and continued at 10 min, 24 h, 48 h, and 72 h, after the injections. These dose fractionation schedules were implemented based on studies reporting that multiple photobiomodulation treatment sessions over days are more beneficial than the administration of a single treatment (Brondon et al. 2005; Liang et al. 2008; Ying et al. 2008). Six subjects in the experimental group received bilateral rotenone injections but no photobiomodulation treatment (i.e., rotenone group). Regardless of whether they received rotenone or DMSO injections or whether they were treated with photobiomodulation or not, all subjects were anesthetized with 1.5% isoflurane during similar periods of time at the same intervals.

Results. Our data support that photobiomodulation is able to prevent the visual function deficits induced by mitochondrial dysfunction in vivo. Effective parameters were a daily dose of 3.6 J/cm^2, a wavelength of 633 nm, and a protocol including six sessions given postinjection (protocol NILT 2 in Fig. 1). This protocol prevented the rotenone-induced increase in the illuminance threshold (Fig. 1a), the increase in latency (Fig. 1b), and the decrease in correct choices (Fig. 1c) in the
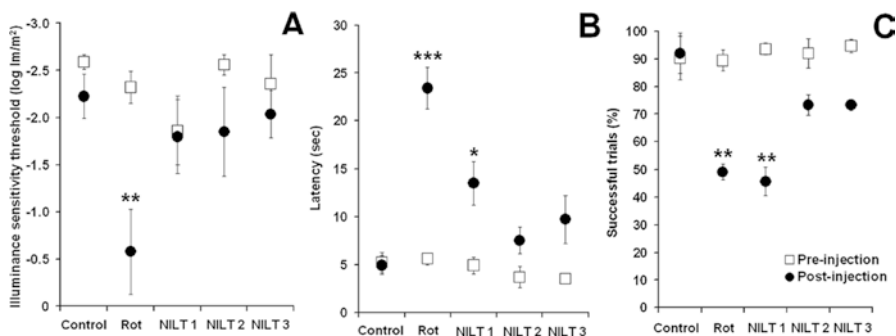
Fig. 1 Effects of photobiomodulation on visually guided behavior. (**a**) Rotenone (Rot) induced a significant decrease in the illuminance sensitivity threshold, but no significant changes postinjection were observed in subjects receiving rotenone plus photobiomodulation. (**b**) Subjects in the Rot group displayed mean postinjection escape latencies across illuminance levels that were significantly higher than control and baseline. A total dose of 21.6 J/cm^2 photobiomodulation at 630 nm given in six daily session postrotenone of 3.6 J/cm^2 (NILT 2) prevented this change, whereas a total dose of 10.8 J/cm^2 photobiomodulation (NILT 1) over three daily sessions of 3.6 J/cm^2 using the same wavelength was not as effective. A third protocol consisted of a total dose of 21.6 J/cm^2 given in six daily doses of 3.6 J/cm^2 at 630 nm, including prophylactic sessions before rotenone injection and four sessions after rotenone injection (NILT 3). This third protocol was also effective at preventing the increase in escape latency. (**c**) Rotenone-treated subjects displayed mean postinjection escape performances across illuminance levels that were not different from chance. This performance was significantly worse than control and baseline. The dose-response effects on performance corresponded to those on escape latency. Asterisks indicate a significant difference compared to control. * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$

visually guided behavioral task. In addition, two prophylactic sessions of 3.6 J/cm^2 photobiomodulation at 633 nm followed by four sessions of postlesion photobiomodulation were also effective at preventing the visual deficits induced by rotenone (NILT 3 in Fig. 1).

2.8 Photobiomodulation Prevents Structural Retinal Damage in the Model of Optic Neuropathy

Rationale. This study tested whether functional effects observed in the behavioral experiment were associated with actual preservation of retinal structure. Measures focus on the retinal nerve fiber layer + ganglion cell layer (RNFL + GCL) because these retinal layers are known to be affected by the intravitreal administration of rotenone and because they constitute the structural basis for the major output pathway from the retina to the brain.

Quantification of retinal structure. Estimates of RNFL + GCL and inner plexiform layer (IPL) thickness were obtained by systematic analyses of NADH dehydrogenase activity-stained retinal sections from animals used in behavioral studies.
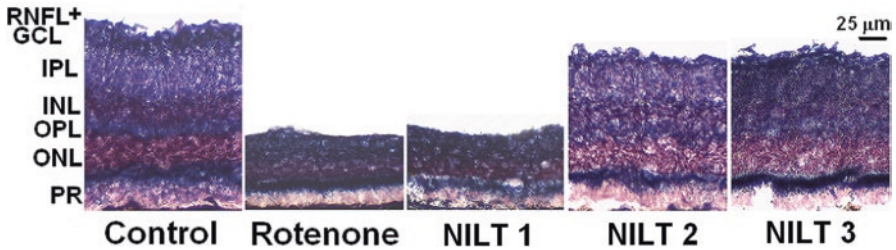
Fig. 2 Neuroprotective effects of photobiomodulation on retinal structure. Layer thinning is a histopathological feature of rotenone-induced neurotoxicity. This is more drastic in the innermost layers including the retinal nerve fiber layer (RNFL), ganglion cell layer (GCL), inner plexiform layer (IPL), and inner nuclear (INL) layer. Whereas structural defects of similar severity were observed in the group treated with the photobiomodulation NILT 1 protocol, photobiomodulation protocols NILT 2 and NILT 3 were effective at preventing the retinotoxic effects of rotenone. NILT 1 = rotenone + 10.8 J/cm^2 photobiomodulation postinjection, NILT 2 = rotenone + 21.6 J/cm^2 postinjection, and NILT 3 = rotenone + 10.8 J/cm^2 preinjection + 10.8 J/cm^2 postinjection. *OPL* outer plexiform layer, *ONL* outer nuclear layer, *PR* photoreceptor layer. Light microscopy, 10×

Results. Photobiomodulation displayed a dose-response effect on the integrity of the retinal layers after rotenone injections (Fig. 2). This is a clear indication that variations in the dose protocol have a large influence on the neuroprotective effects of photobiomodulation, independent of the daily dose and the used wavelength. This supports the need to investigate the protocols that will be most effective at preventing neurodegeneration in different models and disorders.

Mechanistic data relevant for aim 3 are presented below. These studies were aimed at determining the effects of photobiomodulation at effective parameters on cytochrome oxidase activity and oxidative stress. The results support the hypothesis that the mechanism of action of photobiomodulation is mediated through enhancement of the activity of the respiratory enzyme cytochrome oxidase and of the antioxidant enzyme superoxide dismutase.

2.9 Protective Effects of Photobiomodulation Are Not Related to Photodegradation of Rotenone

Rationale. To rule out the possibility that the observed effects of photobiomodulation on visual behavior and retinal structure are due to photodegradation of rotenone by light, we exposed rotenone solutions to 3.6 J/cm^2 633 nm photobiomodulation and characterized the chemical properties of the solutions after the light treatment.

Results. The results showed that rotenone solutions exposed to photobiomodulation do not lose their prooxidant potential and are thus still able to potently induce oxidative stress in brain homogenates, as quantified by generation of lipid peroxides in vitro (Fig. 3).

Fig. 3 Photobiomodulation does not decrease the pro-oxidant potential of rotenone. Rotenone solutions exposed to 3.6 J/cm^2 induce similar levels of lipid peroxides in brain homogenates. This shows that light effects are not mediated through photodegradation of rotenone. * = $p < 0.05$
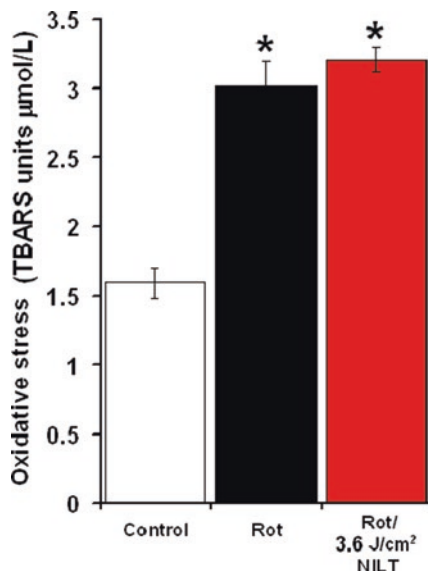
2.10 Preservation of Visual Function and Retinal Structure Are Not Mediated by Isoflurane Exposure

Rationale. We ruled out the possibility that the behavioral and structural effects of photobiomodulation are unrelated to a photobiomodulatory effect and are secondary to a neuroprotective effect of isoflurane instead. Subjects received inhaled anesthesia concomitant to photobiomodulation treatments. This concern was based on suggestion that isoflurane and related anesthetics can prevent neuronal damage secondary to a series of insults. We compared the visually guided behavior (escape latency) of animals intravitreally infused with bilateral rotenone with that of animals given intravitreal rotenone plus 1.5% isoflurane in three 30 min sessions (one session per day). We expected that if isoflurane exposure was responsible for the preservation of visual function after rotenone infusion, isoflurane alone (no photobiomodulation) would prevent the abnormal visually guided behavior induced by rotenone.

Results. Compared to control ($n = 5$) (no rotenone, no isoflurane), rotenone-infused subjects ($n = 5$) showed an overall increase in mean escape latencies across all illuminance levels postlesion (3.2 ± 0.3 s, control vs. 15.6 ± 2 s, rotenone, $p < 0.05$). This effect resulted in a right displacement of the escape latency curve compared to control, which maintained low escape latencies even at low illuminance levels. Rotenone-treated subjects that received isoflurane ($n = 6$) also showed increased escape latencies compared to control across all illuminance levels. In fact, mean escape latencies in the isoflurane group were higher than those of the rotenone-only group (23.4 ± 3, $p < 0.05$) (Fig. 4). These results demonstrate that isoflurane does not prevent the impairments in visually guided
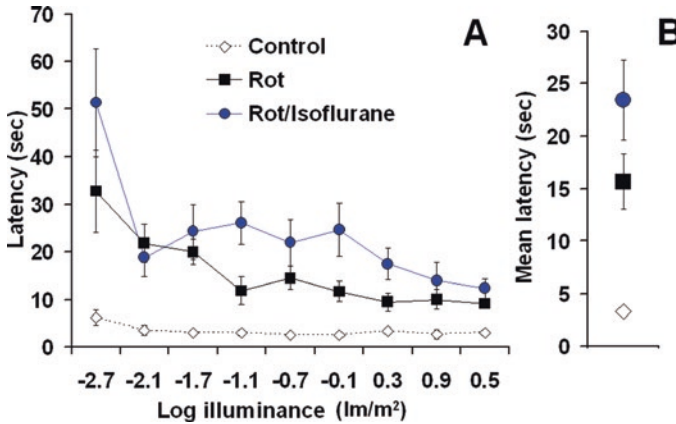
Fig. 4 Isoflurane does not prevent rotenone-induced visual deficits. (**a**) Psychometric curve of escape latencies as a function of platform illuminance. Rotenone increased escape latency compared to control and isoflurane did not prevent this increase. (**b**) Post-lesion mean group latencies pooled across illuminance levels

behavior induced by rotenone and support the fact that the effects of photobio-modulation are due to a photobiomodulatory interaction of light with neural tissue.

2.11   Photobiomodulation Prevents Decreases in Cell Respiration in Brain Homogenates In Vitro

Rationale. We tested the hypothesis that photobiomodulation directly stimulates cell respiration in vitro. Cell respiration is expected to increase if cytochrome oxidase activity is enhanced, since this enzyme is not only the rate limiting step for ATP synthesis but also catalyzes the synthesis of water from molecular oxygen. However, an in vitro system consisting of membrane isolates is uncoupled and oxygen consumption occurs at the maximal possible rate. Inhibition of this system through blockade of the respiratory chain (e.g., addition of the complex I inhibitor rotenone) can provide the conditions to avoid a ceiling effect for oxygen consumption and yet reveal the respiration-enhancing effects of photobiomodulation.

Results. Cell respiration measures in vitro showed that a single session of 0.1 J/cm^2 or 1 J/cm^2 photobiomodulation at 633 nm was able to increase the rate of oxygen consumption in the presence of rotenone (Fig. 5).
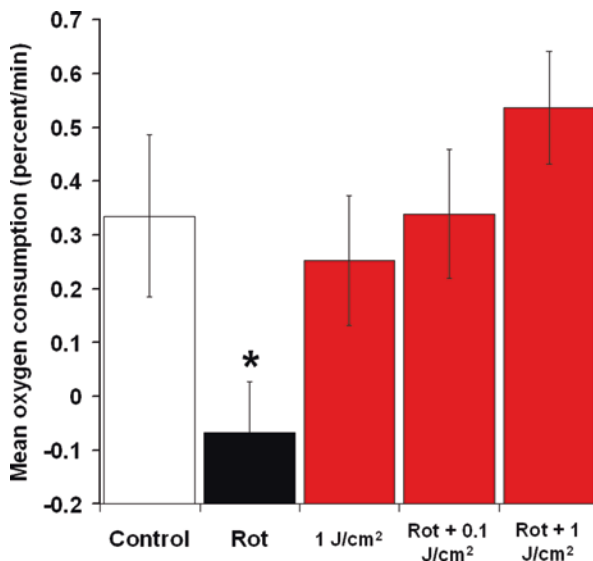
Fig. 5 Photobiomodulation effect on cell respiration in vitro. Rotenone decreased the rate of oxygen consumption. Small (0.1 J/cm^2) and large (1 J/cm^2) light doses reversed the inhibitory effect of rotenone. Rot = 10 μM rotenone, * = significant difference vs. control at $p < 0.05$

2.12 Photobiomodulation Increases Brain Antioxidant Capacity in a Dose-Response Manner In Vivo

Rationale. The effect of photobiomodulation on the antioxidant capacity of neuronal tissue was first shown by Rojas et al. (2008b). Although photobiomodulation is expected to directly stimulate the respiratory enzyme cytochrome oxidase, activation of the latter is expected to allow the expression of enzymatic systems that will coordinate a response for cell survival. Enzymatic systems anticipated to respond to photobiomodulation include inducible endogenous antioxidants such as superoxide dismutase (SOD). In addition, SOD shows absorption peaks at wavelengths similar to cytochrome oxidase. For this reason, SOD expression and activity are anticipated to be affected by photobiomodulation and mediate its neuroprotective effects. In our study, we tested the dose-response effects of 10.8 and 21.6 J/cm^2, 633 nm photobiomodulation on brain SOD activity in vivo. Total doses were delivered in daily sessions of 3.6 J/cm^2 photobiomodulation.

Results. High dose photobiomodulation induced a 50% increase in the whole-brain SOD activity in vivo in a dose-response manner (Fig. 6). The effect reached significance on the cytosolic fraction of the enzyme, but the mitochondrial fraction showed a similar trend. The results support that photobiomodulation increases SOD activity in the brain in vivo and indicate further testing of photobiomodulation to prevent oxidative stress in vivo.
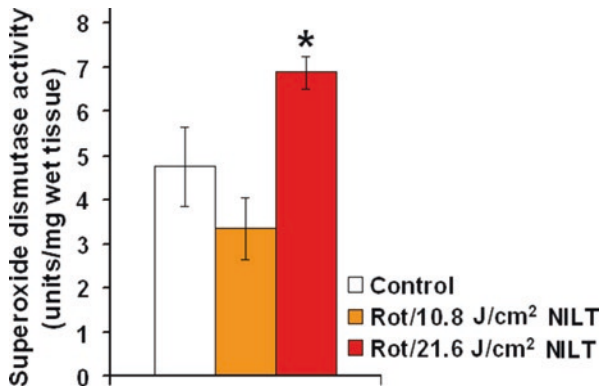
Fig. 6 Photobiomodulation increases brain superoxide dismutase (SOD) activity in vivo. A high dose of 633 nm light given in daily doses of 3.6 J/cm^2 induced a 50% increase in SOD capacity, implicating a transcranial effect. * = $p < 0.05$

2.13 A Single Dose of Photobiomodulation Enhances Brain Cytochrome Oxidase Activity in a Hormetic Fashion In Vivo

Rationale. Previous in vivo experiments tested the effects of a fixed daily dose of photobiomodulation and different fractionation protocols on behavioral and structural variables. This experiment was conducted to test the effects of different daily doses of photobiomodulation delivered in a single session on levels of whole-brain cytochrome oxidase activity in vivo. Unanesthetized animals (male, Sprague-Dawley rats) were exposed to 660 nm light at either 10.9 J/cm^2 ($n = 5$), 21.6 J/cm^2 ($n = 4$), 32.9 J/cm^2 ($n = 4$) or no photobiomodulation (control, $n = 5$) in polycarbonate home cages. Treatments were delivered via four LED arrays with a power density of 9 mW/cm^2 for total treatment times of 20 min, 40 min, and 60 min for each dose, respectively. Twenty-four hours after the single treatment session, animals were decapitated and their brains were extracted, frozen, sectioned, and histochemically stained for quantitative cytochrome oxidase activity (Gonzalez-Lima et al. 1997).

Results. A single session of photobiomodulation at different doses showed enhancement of whole-brain cytochrome oxidase capacity in vivo following a hormetic dose-response pattern. A single dose of 10.9 J/cm^2 photobiomodulation resulted in a 13.6% increase in cytochrome oxidase activity ($p < 0.05$). In turn, a single dose of 21.6 J/cm^2 resulted in an increase of only 10.3%, whereas the highest dose induced no significant change in cytochrome oxidase activity (3%) (Fig. 7). These results suggest that responses of whole-brain cytochrome oxidase levels to photobiomodulation in vivo are characterized by a hormetic pattern, with a low dose given in a single day having a stimulatory effect, while higher doses are less
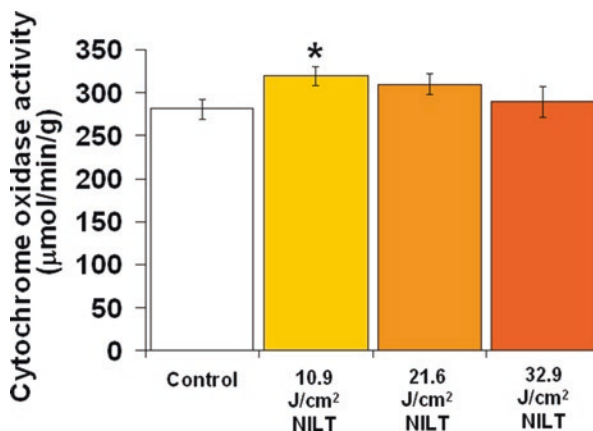
Fig. 7 A single light dose increases whole-brain cytochrome oxidase capacity in unanesthetized rats. Low-dose 660 nm photobiomodulation boosted cytochrome oxidase activity by 13.6%, whereas higher doses were not as effective. This response to a low dose with an absent response to a high dose is typical of hormesis. These data also support a transcranial effect of photobiomodulation. * = $p < 0.05$

effective. Thus, we anticipate that effective photobiomodulation protocols will include daily doses lower than 10.9 J/cm^2.

2.14 Fractionated Photobiomodulation Increases Brain Cytochrome Oxidase Activity in a Dose-Response Manner In Vivo

Rationale. This experiment tested the effects of a fractionated protocol of daily photobiomodulation delivered in vivo to rats on whole-brain cytochrome oxidase activity. Daily doses of 3.6 J/cm^2 633 nm photobiomodulation were given at a power density of 2 mW/cm^2.

Results. With a fractionated protocol, we observed dose-response effects of photobiomodulation on the activity of cytochrome oxidase. While a single-session dose of 21.6 J/cm^2 was ineffective in the previous study, the same cumulative dose of 21.6 J/cm^2 at 633 nm fractionated into six daily sessions of 3.6 J/cm^2 each is sufficient to induce an approximately 20% increase in cytochrome oxidase activity, which is a reflection of increased global energy metabolism (Fig. 8). The results suggest that low daily doses of photobiomodulation provide long-term enhancing effects on whole-brain metabolic capacity. These results further support a transcranial effect of photobiomodulation in living rats. Taken together with the results using 660 and 633 nm photobiomodulation, these data suggest that it is possible that noneffective daily doses trigger a favorable cumulative response in vivo, if wavelengths and power densities are modified.
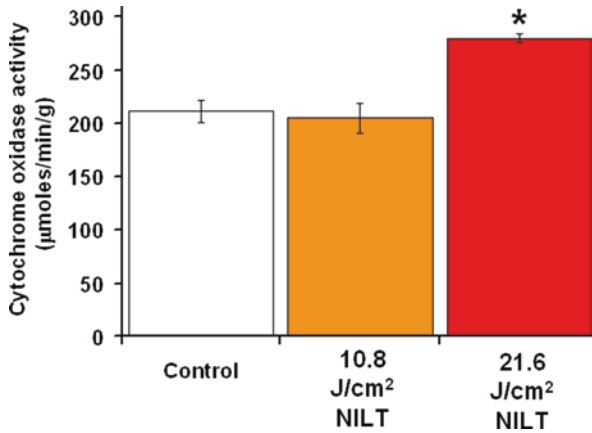
Fig. 8 Fractionated photo biomodulation increased brain cytochrome oxidase in a dose-response fashion in vivo. Rats treated with the low-dose (10.8 J/cm^2) protocol showed no difference to control. Rats treated with the higher-dose protocol (21.6 J/cm^2) showed a 20% increase in cytochrome oxidase activity compared to control. $*$ = $p < 0.01$

In conclusion, the animal experiments reviewed characterized neuroprotective effects of photobiomodulation at three different levels of analysis: *functional, structural, and neurochemical.* The experiments demonstrated optimal photobiomodulation parameters for the treatment of optic neuropathy and its mechanism of action. They served to improve the understanding of the mechanisms mediating the effects of photobiomodulation in neural tissue in vivo. Using in vivo animal models to characterize the effects of photobiomodulation helped us address important questions on how light interacts with neural tissue in a more translational-relevant fashion in the studies in Part 2.

3 Part 2: Human Studies—Augmentation of Neurocognitive Functions by Photobiomodulation

3.1 Introduction and Objectives of the Human Studies

We have conducted randomized controlled trials (RCTs) to test the safety and efficacy of a new noninvasive photobiomodulation intervention, *transcranial infrared laser stimulation (TILS)*, for neurocognitive enhancement in younger and middle-aged adults. Considering that cognition is one of the most important determinants of quality of life and functional ability in middle and older age (Gaugler et al. 2009), it is critical to seek new treatments to prevent or delay cognitive impairment in vulnerable populations. Adults showing early preclinical signs of cognitive decline are prime candidates for interventions intended to enhance cognitive function.

TILS penetrates approximately 40 mm to the brain and improves cognitive functions (Rojas and Gonzalez-Lima 2013; Gonzalez-Lima and Barrett 2014; Tedford et al. 2015). Specifically, TILS of the human prefrontal cortex with a wavelength of 1064 nm and a power density of 0.25 W/cm² upregulates the levels of oxidized *cytochrome oxidase*, the conformation of the enzyme that has the highest oxygen consumption activity, which leads to improved cerebral oxygenation in a nonthermal manner (Tian et al. 2016; Wang et al. 2017). This unique neurophotonics in vivo mechanism is highly relevant for cognitive enhancement because neurons are critically dependent on cytochrome oxidase-mediated oxygen consumption to sustain electrophysiological activity (Ojaimi et al. 1999; Wong-Riley et al. 2005). Since cerebral physiology is critically dependent on oxygen metabolism, the mechanistic action of TILS on cytochrome oxidase has strong potential for cognitive enhancement.

We have shown that TILS is safe and effective for increasing cognitive functions in young adults in five controlled studies using photobiomodulation of the right prefrontal cortex (Barrett and Gonzalez-Lima 2013; Blanco et al. 2015, 2016; Hwang et al. 2016; Disner et al. 2016). Based on our successful TILS studies with young and middle-aged adults, our ongoing project is to determine if TILS may also enhance cognitive performance by improving prefrontal oxygen metabolism in older adults. For TILS to become a mainstream intervention for older adults, it is essential to evaluate quantitatively its cognitive and neurophysiological effects in an older population. However, there are no completed RCTs of TILS in older adults.

The *long-term research goal* of our human studies is to quantify the cognitive and physiological effects of TILS on the human prefrontal cortex using prefrontal-based cognitive tasks and multimodal in vivo evaluation of neurophysiological mechanisms mediating cognitive enhancement, using near infrared spectroscopy (NIRS), electroencephalography (EEG), and functional magnetic resonance imaging (fMRI) techniques.

Aim 1: Demonstrate the neurophysiological effects of transcranial infrared laser stimulation in humans. Our hypothesis, based on our animal studies, is that TILS of the prefrontal cortex will enhance cerebral oxygen metabolism and neurocognitive network function in humans. *Methods*: Using a placebo-controlled design, we were the first to determine the cerebral effects of TILS using noninvasive techniques (NIRS, EEG, and fMRI), including in vivo measures of oxidized cytochrome oxidase, oxygenated hemoglobin (HbO), electrophysiological power spectral density (PSD), cerebral blood flow (CBF), and blood oxygen level-dependent (BOLD) response. We compared cerebral responses with attention/memory tasks, before and after treatment. *Results*: We found that TILS-mediated cytochrome oxidase upregulation promoted better cerebral oxygen metabolism and neurocognitive network function and more efficient prefrontal response posttreatment, relative to participants in the placebo condition.

Aim 2: Demonstrate the cognitive-enhancing effects of transcranial infrared laser therapy in humans. Our hypothesis, based on the animal studies, is that TILS of the prefrontal cortex will enhance cognitive performance in humans. *Methods*:

Using a randomized placebo-controlled design, we are comparing participants' cognitive performance before and after 4–5 week active or placebo treatments and at 1-week and 8-weeks posttreatment. A psychomotor vigilance task for sustained attention and a delayed-match-to-sample working memory task were chosen for weekly evaluation because they engage prefrontal-based attention-memory-executive domains particularly vulnerable to aging, and our data demonstrate that performance in those domains is improved by TILS in younger adults (Barrett and Gonzalez-Lima 2013; Blanco et al. 2015, 2016; Hwang et al. 2016; Disner et al. 2016; Vargas et al. 2017). Weekly simultaneous bbNIRS/EEG and cognitive measures will give us cytochrome oxidase, electrophysiological and behavioral changes to allow us to directly compare how these neural metrics are related to each other and to the cognitive performance. Standard neuropsychological tests will also be administered at baseline and after the treatment. *Results*: We expect that the active treatment group will exhibit better cognitive performance posttreatment, relative to participants in the placebo condition.

This research provided an important translational step between human studies and a large literature utilizing animal models establishing that photobiomodulation improves neuronal oxygenation and upregulates mitochondrial respiration in a way that is safe, noninvasive, and therapeutically beneficial (Anders et al. 2014; Wong-Riley et al. 2005; Rojas and Gonzalez-Lima 2011; de la Torre 2017; Rojas et al. 2008a, b, 2012; Barrett and Gonzalez-Lima 2013; Blanco et al. 2015, 2016; Hwang et al. 2016; Disner et al. 2016; Vargas et al. 2017; Fulop et al. 2010; Lampl et al. 2007; Zivin et al. 2009; Hacke et al. 2014; Wang et al. 2016). In particular, middle-aged and older adults as the study population will provide relevant translational data for early intervention before onset of clinical impairment. The continuous success of this project will also help develop a new way to alleviate or stabilize the cognitive deficits common to aging, Alzheimer's disease, and related dementias.

3.2 Significance of Human Cognitive Enhancement by Photobiomodulation

The potential impact of this research increases as the overall population ages. It is estimated that by 2050, there will be nearly two billion people aged over 60 (He et al. 2016). Along with a rapidly aging population comes the health problems associated with it, including cognitive decline and dementia. While aerobic exercise is effective to support oxygen metabolism, associated with a range of health benefits including cognitive enhancement (Etnier et al. 1997), compliance with exercise is abysmal and only 5% of US adults report the recommended daily physical activity of 30 min (Buford et al. 2013). Aerobic exercise is not a viable option for older patients with severe cardiovascular disease or orthopedic injuries. There is a pressing need for alternatives to aerobic exercise to preserve and enhance cognitive

function for all. Although significance is high for older adults, impairments in atten-tion-memory-executive function are not unique to this population. The success of this project will have far-reaching significance because it will provide relevant data for early intervention before onset of clinical impairment. This may help alleviate or stabilize the cognitive deficits common in aging, Alzheimer's disease, and related dementias. This is also significant to other patients who would benefit from cogni-tive enhancement, e.g., in traumatic brain injury, metabolic, cardiovascular, and many other neurological and mental disorders.

3.3   Cytochrome Oxidase as Molecular Target for Human Cognitive Enhancement

Aging-dependent and neurodegeneration-dependent decline in cytochrome oxidase activity has been well documented, ranging from studies in drosophila (Ren et al. 2010) to the human brain (Ojaimi et al. 1999), and cytochrome oxidase decline is more pronounced in mild cognitive impairment and Alzheimer's disease (Valla et al. 2006) (PubMed lists 1071 reports on 'cytochrome oxidase and aging'). Cytochrome oxidase is the enzyme responsible for utilizing over 95% of the oxygen we breathe for cellular respiration. Nerve cell function is critically dependent on oxygen utilization (i.e., aerobic metabolism). Animal studies from our lab have shown that cytochrome oxidase activity is critical for learning and memory perfor-mance (Gonzalez-Lima et al. 2014). For example, partial inhibition of cytochrome oxidase activity is sufficient to cause cognitive impairment, whereas improvement of cytochrome oxidase activity by metabolic interventions causes cognitive enhance-ment (Callaway et al. 2002, 2004). For the past 20 years, our lab has been searching for a noninvasive, safe, and effective method to upregulate cytochrome oxidase that could be translated to humans for the purpose of cognitive enhancement. We think that photobiomodulation is the answer because it increases cellular oxygen metabo-lism by delivering photons that oxidize cytochrome oxidase, the main intracellular photon acceptor at red-to-near-infrared wavelengths (Anders et al. 2014; Wong-Riley et al. 2005; Rojas and Gonzalez-Lima 2011; de la Torre 2017). High bioavail-ability to brain tissue in vivo is supported by preclinical evidence of increases in brain cytochrome oxidase activity and oxygen consumption that improved behav-ioral outcome in animal models (Rojas et al. 2008a, b, 2012) and by near-infrared light penetration of approximately 40 mm through the human head (Tedford et al. 2015). Recent findings from our team demonstrated that TILS at 1064 nm upregu-lates cytochrome oxidase and produces beneficial effects on human prefrontal cor-tex oxygenation in young adults (Tian et al. 2016; Wang et al. 2017). We found that TILS of the right prefrontal cortex at 1064 nm, 0.25 W/cm² is safe and effective for increasing cognitive functions (sustained attention, working memory, executive skills, and category learning) in young adults in controlled studies (Barrett and Gonzalez-Lima 2013; Blanco et al. 2015, 2016; Hwang et al. 2016; Disner et al. 2016), and we are extending this approach to older adults.

3.4   Need to Investigate How TILS Affects Human Neurocognitive Function

In 2002, the FDA approved low-level light/laser therapy with red-to-near-infrared wavelengths for pain relief in head and neck pain, arthritis, and carpal tunnel syndrome (Fulop et al. 2010). FDA-cleared laser diodes and light-emitting diodes (LEDs) delivering low-power density (low irradiance) but high-energy density (high fluence) light are a highly promising, affordable, and safe alternative for improving human cognitive function (Gonzalez-Lima and Barrett 2014). However, this intervention has not been adopted in spite of safe, portable, effective, and promising outcomes from animal and human studies. One major reason is that the action of TILS in the human brain in vivo has not been quantitatively studied and understood using optical methods to reveal light-tissue interactions. One important example was a large clinical study (*"Neurothera Effectiveness and Safety Trials"; NEST*) sponsored by PhotoThera (Lampl et al. 2007; Zivin et al. 2009; Hacke et al. 2014). NEST had three clinical trials, lasted a total of 7 years, and recruited a total of more than 1000 acute stroke patients. During the studies, an 810-nm laser was applied over the entire surface of the head (20 locations in the 10/20 EEG system) without targeting any particular cortical region regardless of stroke and at a very low laser energy density (1 J/cm^2 over the entire cortical surface) for only 2 min without repeated treatment. While this intervention was found to be safe, without any side effects, and worked well in the first two trials for moderate and moderate-severe acute stroke patients, it did not achieve the expected statistical significance for severe stroke patients. Given our recent results from 1064-nm laser stimulation of the human forearm (Wang et al. 2016) and forehead (Wang et al. 2017) as well as human cognition measures (Barrett and Gonzalez-Lima 2013; Blanco et al. 2015, 2016; Hwang et al. 2016; Disner et al. 2016), it is clear to us that the laser parameters and stimulation setup used in the NEST studies were not chosen effectively, while many other factors could have contributed to the unsuccessful clinical trial. This negative example strongly demonstrates the necessity to investigate how TILS affects neurophysiology both in time and space in our target population, so it can become an effective, noninvasive tool for treating prefrontal-based cognitive decline. We envision a near future with scientifically validated, safe portable devices to conveniently administer TILS in homes, nursing homes, and healthcare facilities.

We propose TILS as an exciting new intervention to enhance neurocognitive function in adults at risk for developing cognitive impairment. Building upon a body of evidence demonstrating increases in brain cytochrome oxidase activity and brain oxygenation, which improve behavioral outcomes and memory in animal models (Rojas et al. 2008a, b; Rojas and Gonzalez-Lima 2017) and healthy humans (Barrett and Gonzalez-Lima 2013; Blanco et al. 2015, 2016; Hwang et al. 2016; Disner et al. 2016; Tian et al. 2016; Wang et al. 2017), we conducted the first randomized clinical trials (RCTs) to test if TILS improves neurocognitive function in humans. We were the first to quantify TILS effects on the human brain in vivo using state-of-the-art neurophysiological techniques (fNIRS, qEEG, and fMRI) and a new

broadband NIRS (bbNIRS) apparatus to quantify cytochrome oxidase in the human brain in vivo. We were enabled in this innovation in the new field of laser-mediated cognitive enhancement by strong interdisciplinary collaborations using all these techniques, including established experts in cerebral metabolism, cognition, and biomedical optics who are coauthors of the studies cited below.

3.5   Overview of Our Approach for Human Cognitive Enhancement by Photobiomodulation

Neurophysiological effects were investigated using a combination of noninvasive NIRS, EEG, and MRI techniques. Cognitive-enhancing effects were investigated in tests of global and prefrontal cortex (PFC)-based cognitive function, with a special emphasis on sustained attention-working memory-executive functions, which show a decline in older adults and patients with chronic neurodegenerative and mental disorders.

Figure 9a shows that the left part of the laser unit has on/off controls and multiple safety interlocks, including key and emergency stop. The center has a screen display and keypad to program output power, number of treatment counts, and exposure time. Output is programmable between 0.1 and 20 W, and optimal parameters for TILS are 3.4–3.9 W and 8 counts of 60-s each (8 min). On screen messages confirm correct handling, calibration, and use of laser. The right side of the laser unit has a calibration port that securely locks the handpiece in place, while the laser is being calibrated before each use. Beam output characteristics are continuously monitored, while laser is active. Since the 1064-nm laser is invisible, a red beam area provides visual confirmation for tissue targeting (Fig. 9b). During operation, the laser is locked into position near the skin and participants are instructed to sit still and keep their eyes closed. Experimenters and participants wear dark safety glasses that block the infrared light from reaching the eyes, as required by the laser manufacturer and the University of Texas Laser Safety Program. Dr. Fenghua Tian and Dr. Hanli Liu are pioneers in the field of optical brain imaging who introduced a realistic brain model and numerical simulation of light propagation through the tissues (Tian and Liu 2014). Based on this model and diffusion theory, Dr. Tian estimated the cortical region of the TILS as shown in Fig. 9c. The model supports that TILS penetrates through the cortical gray matter (Tedford et al. 2015) and effectively targets the dorsolateral PFC, not the ventrolateral or medial PFC regions. We have not had any report of discomfort or adverse events caused by TILS, as low-intensity laser (0.25 W/cm^2) produces negligible heat that most people cannot detect. As shown in Fig. 9d, we have demonstrated that thermal stimulation that matches precisely the heat produced by our laser treatment has no significant effect on cytochrome oxidase in the PFC (Wang et al. 2018). Therefore, TILS-specific molecular action on cytochrome oxidase cannot be explained by thermal effects and cannot be due to a more trivial effect of water heating and increased circulation. In summary, TILS causes 1064-nm photons to oxidize cytochrome oxidase in the dorsolateral PFC and heating would not be a possible explanation for TILS effects (Wang et al. 2018).
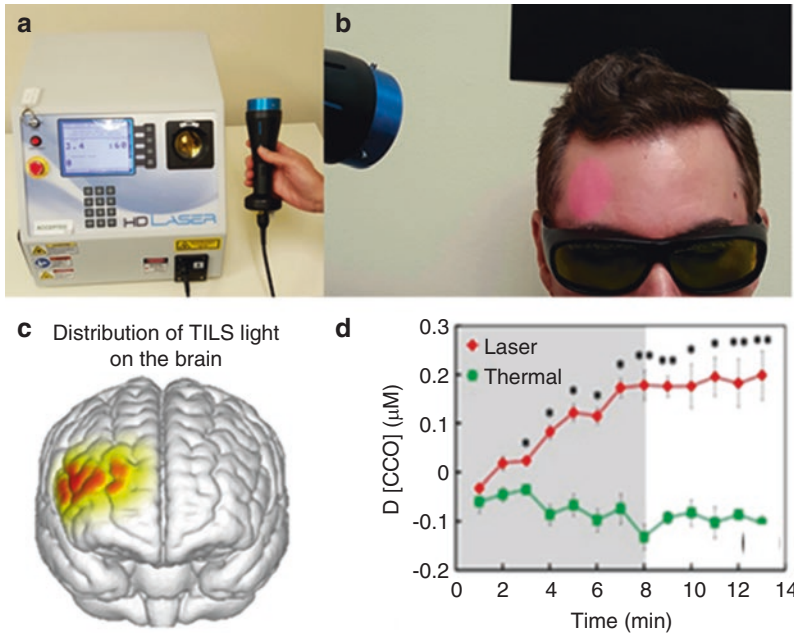
Fig. 9 Transcranial infrared laser stimulation (TILS). (**a**) Laser device for TILS. The FDA-cleared Class IV laser device (HD Laser, Cell Gen Therapeutics, Dallas, Texas) consists of a control unit (16″ × 14″ × 13″) with a fiber optic cable coupled to a handpiece. (**b**). Laser delivery. For illustration purposes, the largest laser aperture aims at the forehead using an internal red diode aiming light. The laser aperture can be adjusted to a desired spot size from 1 to 45 mm diameter. We used a 4-cm diameter laser beam size to match the size of the prefrontal cortex (PFC) area we aim to stimulate. (**c**) Cortical target. Model of intensity distribution of treatment light on the right PFC (orange indicates effective light intensity) made by Dr. Fenghua Tian. (**d**) Molecular target. Oxidized [cytochrome oxidase] increases during laser (red; $n = 11$) but not thermal (green; $n = 11$) stimulation measured in vivo by bbNIRS. "*" $p < 0.05$ and "**" $p < 0.01$ laser vs. thermal stimulation (mean ± SE) (Wang et al. 2018)

Beginning in 2013, we have published twelve studies of TILS neurocognitive effects involving 432 participants, which demonstrated TILS feasibility in humans. First, we briefly review the six studies demonstrating cognitive enhancement and then the six studies demonstrating the neurophysiological effects of TILS.

3.6 Cognitive-Enhancing Effects of TILS of the Human Prefrontal Cortex

We published the first six placebo-controlled studies (333 participants, 177 females, ages 17–40) demonstrating that TILS of the right PFC produces beneficial effects on PFC-modulated attention/memory/executive functions such as sustained attention and working memory, executive skills, attention bias modification, and rule-based category learning (Barrett and Gonzalez-Lima 2013; Blanco et al. 2015, 2016; Hwang et al. 2016; Disner et al. 2016).

1. *Attention and working memory*. In the first study (Barrett and Gonzalez-Lima 2013) (*n* = 40, ages 18–35), a single TILS session improved PFC-based performance (decreased reaction time) in a sustained-attention **psychomotor vigilance task** (PVT) in treated vs. placebo control groups (Fig. 10). Performance (memory retrieval latency and number of correct responses) was also improved in the treated group on a working memory **delayed match-to-sample task** (DMS). In healthy young participants, effect sizes of DMS correct trials were small, about 2–6% due to ceiling effects, but differences in memory retrieval latency were about 20% (Fig. 11). Right PFC was stimulated because PVT and DMS cognitive tasks are predominantly mediated by the right PFC (Barrett and Gonzalez-Lima 2013).

2. *Comparison of right vs. left TILS*. The second study (*n* = 51, ages 18–40) compared TILS effects on **attention bias modification** (ABM) learning. We used this study to directly compare right vs. left PFC stimulation. Participants were randomized to one of the three stimulation conditions: right forehead, left forehead, or sham (Disner et al. 2016). ABM is a cognitive intervention designed to improve depression symptoms by learning to decrease negative attentional bias. The right TILS led to greater symptom improvement among participants whose attention was directed away from negative stimuli. Minimal change was observed in the left and sham TILS, suggesting that the beneficial effects of ABM learning on depression symptoms may be enhanced when paired with right but not left PFC stimulation. This is consistent with the *right PFC being more dominant* in the human brain's attention-memory-executive network, which supports our rationale to continue with stimulation of the right PFC.
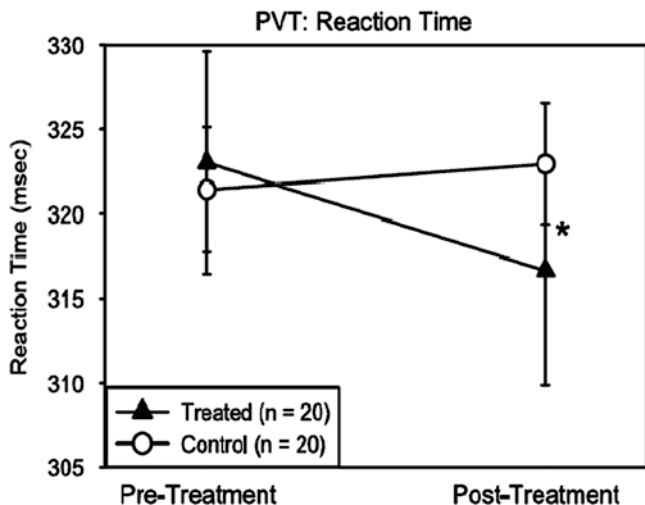

Fig. 10 Improved cognitive performance in the psychomotor vigilance test (PVT) (shorter reaction times) post TILS vs. placebo control group in healthy young adults. (*n* = 40, mean ± SE), *p* < 0.05 (Barrett and Gonzalez-Lima 2013)
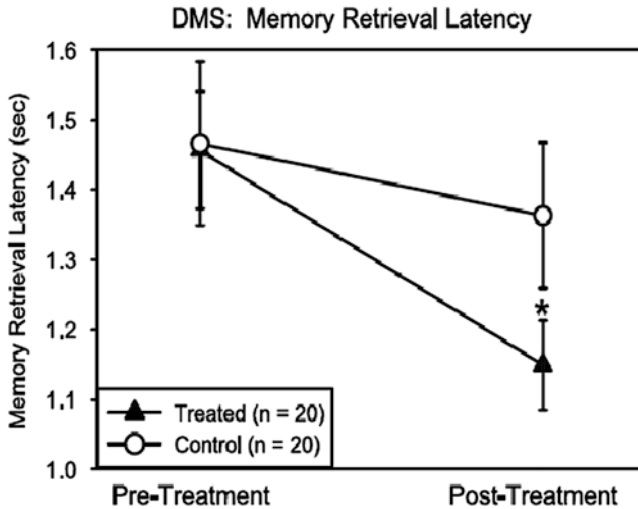
Fig. 11 Improved cognitive performance in the delayed match to sample test (DMS) of working memory (shorter memory retrieval latency) post-TILS vs. placebo control group in the same subjects used for Fig. 10

3. *Executive skills*. In the third study (Blanco et al. 2015), we built on this research by studying the effects of TILS on executive function (*n* = 30, ages 18–40). Executive function is modulated by the PFC and plays a role in several cognitive skills including selective and divided attention, manipulation, task switching, and inhibition of interfering stimuli. Executive dysfunction is characteristic of cognitive aging and mild cognitive impairment (MCI) with deficits worsening as they develop into Alzheimer's disease and related dementias (Kirova et al. 2015). Executive function deficits can be detected by the **Wisconsin Card Sorting Task** (WCST), a gold-standard neuropsychological measure of executive function, and previous studies have shown poor performance in this task by individuals with healthy aging (Salthouse et al. 2003) as well as aging-related diseases such as Parkinson's disease (Monchi et al. 2004) and Alzheimer's disease (Binetti et al. 1996). Participants who received laser treatment aimed at the right PFC made significantly fewer errors and showed improved set-shifting ability relative to placebo controls (Blanco et al. 2015) (Fig. 12). These findings demonstrated the ability of TILS to enhance executive function in healthy people and its potential to improve executive function deficits in elderly and clinical populations. Thus, we will continue to use WCST to evaluate pre- vs. posttreatment effects of TILS on executive skills.

4. *Comparison of TILS with exercise*. The fourth study (*n* = 60, ages 18–30) revealed that compared to placebo, **vigorous aerobic exercise and TILS resulted in the same improvement in cognitive performance in PVT and DMS tasks**. However, the effective vigorous aerobic exercise protocol involved 20 min running in a treadmill at 85–90% maximal oxygen consumption
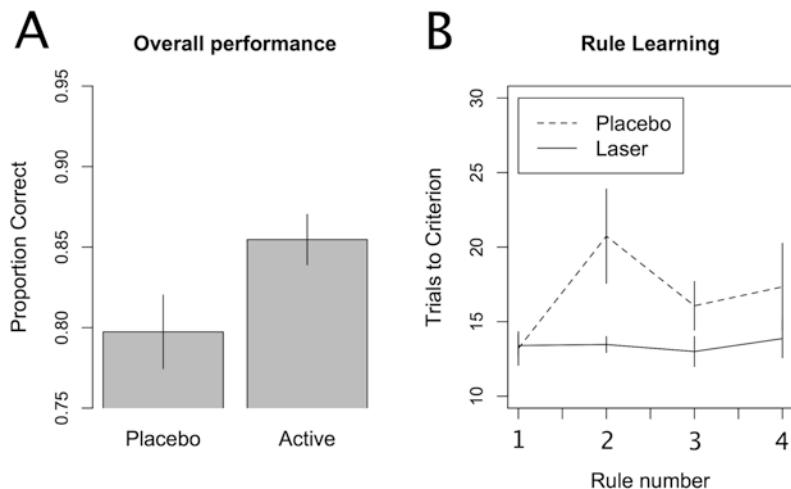
Fig. 12 (**a**) Overall WCST accuracy. The laser group correctly sorted the cards more often than the placebo group. (**b**) Trials to criterion for each of the first four rules learned. The placebo group took significantly longer to reach criterion on the second rule than the laser group, suggesting a benefit in set shifting ability in the laser group. Error bars represent standard errors (Blanco et al. 2015)

(VO$_2$max), while the laser treatment involved 8-min TILS to the right PFC while seating quietly (Hwang et al. 2016). Thus, it is expected that older adults and patients who may be unable to engage in vigorous aerobic exercise to improve cognition could benefit from TILS as an alternative intervention. We intend to answer this question in future studies.

5. *Category learning*. In the fifth study ($n = 118$, ages 17–35) (Blanco et al. 2016), we tested the effects of TILS on category learning, an essential function of everyday life. The study tested two different types of category structures: rule-based and information-integration. **Rule-based category learning** is optimized by a reflective system of learning associated with PFC processing. Information-integration category learning is optimized by a reflexive system of learning associated with processing in the striatum. As hypothesized, participants in the PFC-TILS group had higher learning rates and improved performance in rule-based category learning tasks, but there was no significant effect in information-integration category learning tasks (Fig. 13). These results had two major implications: (1) additional evidence that application of TILS to the right PFC can improve PFC-mediated cognitive functions and (2) different forms of learning can be modulated by TILS without affecting others.

6. *Overall cognitive rate correct score*. In the sixth study, 34 healthy adults (16 males, 18 females; average age: 31, and standard error: 2.5) were recruited (Holmes et al. 2019). The 18 experimental group participants (9 males, 9 females) received full laser stimulation and completed all tasks using a within-subject control design. They performed the PVT and DMS cognitive tasks before and after TILS, with concomitant fNIRS recordings, to reflect the hemodynamic
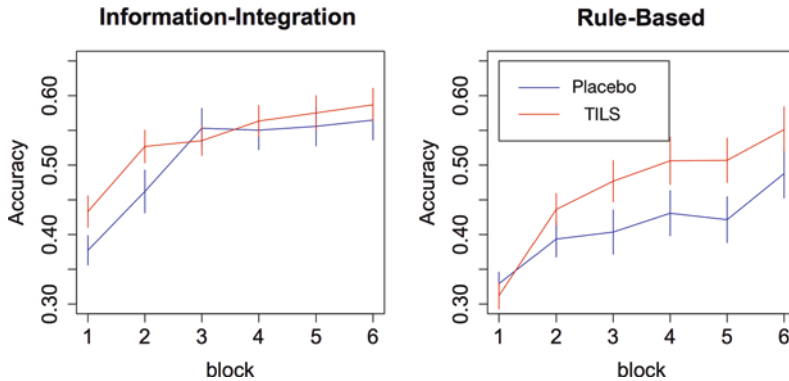
Fig. 13 Accuracy (proportion correct classification). In rule-based learning, the TILS group performed significantly ($p < 0.05$) better than the placebo group in each block after the first block, while information-integration did not show significant group differences ($n = 118$, mean ± SE) (Blanco et al. 2016)

effects of TILS on cognitive performance. Another 16 participants (7 males, 9 females) were matched blind to treatment as sham controls without photobiomodulation (TILS procedure used with light off). Cognitive data were measured in seven of the sham participants (3 males, 4 females) as previously described (Barrett and Gonzalez-Lima 2013). Hemodynamic data were measured in nine of the sham participants (four males, five females) as previously described (Wang et al. 2017). The hemodynamic results are shown below in the discussion of neurophysiological effects. For each subject's cognitive performance in both sessions of the PVT and DMS, an overall cognitive score was calculated, incorporating both speed (reaction time) and accuracy (number of correct responses), using the rate correct score or RCS (Woltz and Was 2006). This overall cognitive score was equal to the number of correct responses divided by the sum of all reaction times. Cognitive results from the PVT and DMS were obtained before and after TILS and sham. As expected, overall cognitive processing improved after TILS, as indicated by the significantly higher rate correct score (Fig. 14a), whereas there were no significant differences after sham (Fig. 14b). This score reflects the speed and accuracy of cognitive processing. The overall rate correct score effect size (Cohen's d) after TILS was $|d| = 0.62$, indicating a medium effect size, given that $|d| < 0.2$ = small effect; $0.2 < |d| < 0.8$ = medium effect; and $|d| > 0.8$ = large effect (Cohen 1988).

The behavioral sham data comparing pre-post tasks without TILS served to confirm the lack of learning/placebo effects for repeated testing of our specific DMS and PVT tasks and replicated the same sham findings from our published studies. The cognitive effects are consistent with our previous two studies in which we compared TILS with sham/placebo groups in the same PVT and DMS tasks (Barrett and Gonzalez-Lima 2013; Hwang et al. 2016). Indeed, pre-post testing of these PVT and DMS tasks in a single session without laser stimulation does not lead to

Fig. 14 Rate correct score
(RCS) for cognitive
performance before and
after TILS (**a**) and Sham
(**b**). Mean ± S.E.,
* = Significant mean
difference between Pre- vs.
Post- TILS scores,
$p \leq 0.01$. * No
randomization occurred for
sham, since we used a
within-subject design in
which the same subjects
are their own control by
directly comparing pre- vs
post- measures statistically.
There were no significant
pre-post differences in the
sham subjects (Holmes
et al. 2019)
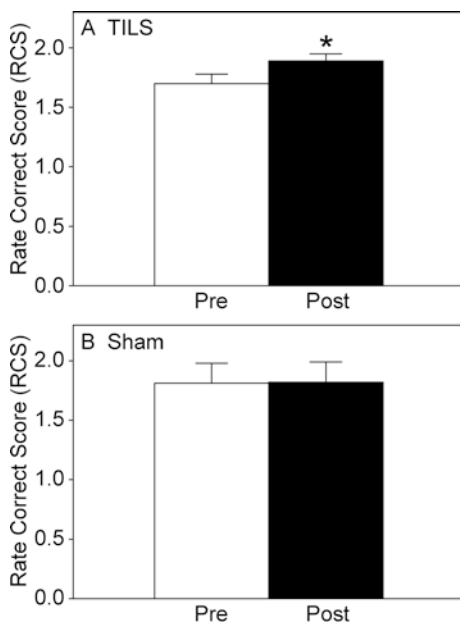
improved cognitive scores (Barrett and Gonzalez-Lima 2013). Therefore, placebo or practice effects cannot explain the cognitive improvement produced by TILS. Overall, the results from these six placebo-controlled trials demonstrate the potential of TILS as a cognitive-enhancing approach.

3.6.1 Cognitive Benefits of TILS in Older Participants

Further, preliminary data with older participants also suggested beneficial neuro-cognitive effects of repeated TILS aimed at the right PFC. In a proof-of-concept open-label pilot study ($n = 21$, 11 females, ages 49–90), we investigated the feasibility of studying older participants using the proposed cognitive protocols ($n = 12$), with qEEG ($n = 6$) and fMRI ($n = 6$) (Vargas et al. 2017). PVT (sustained attention) and DMS (working memory) were conducted for 5 weeks. The first session measured baseline scores, and the second session investigated acute effects of TILS. Chronic effects were investigated after four additional weekly treatments with the proposed TILS. Repeated measure ANOVA using percent change from baseline showed significant ($p < 0.05$) effects: reduced PVT reaction time (Fig. 15a) and failures to respond (Fig. 15b), while increased correct memory retrieval responses in DMS (Fig. 15c). Detailed inspection of the data verified that all older participants improved cognitive performance after single and repeated treatments.

Younger participants had reaction times and correct responses that were consistently better than those of the older cohort, and placebo-treated subjects showed no improvements. However, the relative effect size of TILS in this older group was larger than that in our previous controlled studies with younger participants. Effect sizes (Cohen's *D*) for younger (18–35) vs. older (49–90) were 0.21 vs. 0.65 for PVT
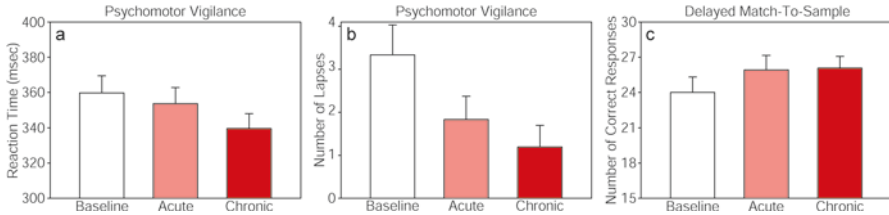
Fig. 15 Cognitive effects of TILS on attention and memory in older adults. (**a**) Reaction time; (**b**) number of lapses (trials in which the subject failed to respond within 500 ms of the stimulus) for the PVT; and (**c**) number of correct responses for the DMS. Bars show group means plus standard errors, *n* = 12 (Vargas et al. 2017)

reaction time, 0.67 vs. 1.05 for PVT lapses, and 0.26 vs. 0.53 for DMS correct responses. For example, the number of trials in which older subjects failed to respond within 500 ms of the stimulus reduced by 55% after a single laser treatment and by 74% after four additional laser treatments. Laser-induced memory improvement was also found in the DMS test. But several participants below 56 (median age of cohort) reached ceiling effects in the correct number of responses after three weeks of treatment. In contrast, the oldest participants (one man and one woman, each 90 years old) continued to improve over weeks, with a mean 15% increase in their number of correct memory retrieval responses as compared to 11% improvement for participants in their 50s. Based on these results, we plan to avoid ceiling effects in future studies by administering two DMS tests, one easier (4-s delay) and one more difficult (8-s delay). The longer the delay before memory retrieval, the more the difficulty involved in this working memory task. In this way, the more difficult task will allow us to evaluate if there are further memory improvements in participants showing ceiling effects in the easier task after repeated laser treatments.

3.7 Neurophysiological Effects of TILS of the Human Prefrontal Cortex

We published the first six placebo-controlled neurophysiological studies (99 participants, 44 females, ages 18–45) demonstrating the effects of TILS on metabolic, hemodynamic, and electrophysiological indices using fNIRS (Tian et al. 2016; Holmes et al. 2019), bbNIRS (Wang et al. 2017), qEEG (Wang et al. 2017a, 2019), and fMRI (Vargas et al. 2017).

1. *Near-infrared spectroscopy (NIRS) studies*. Transcranial infrared laser stimulation of the human prefrontal cortex causes cognitive enhancement. To investigate the hemodynamic effects in prefrontal cortex by which this cognitive enhancement occurs, we used near-infrared spectroscopy (NIRS), which is a safe, noninvasive method of monitoring hemodynamics. We measured concentration changes in oxygenated and deoxygenated hemoglobin, total hemoglobin, and differential effects in healthy adults during resting conditions and during sustained attention and working memory performance, before and after laser of the

right prefrontal cortex. We also measured sham controls without photobiomodulation. NIRS involves optical imaging methods using two-wavelength light (**fNIRS**) or broadband light (**bbNIRS**), which have been developed for mapping human brain functions and functional networks (Hoshi 2007; Ferrari and Quaresima 2012; Boas et al. 2014; Zeff et al. 2007; Eggebrecht et al. 2014; Niu et al. 2012; Niu and He 2014). We conducted two studies using fNIRS and two studies using bbNIRS. The principle of fNIRS is that light absorption of oxy- and deoxy-hemoglobin (HbO and HHb) will vary with cerebral functions, similar to the principle for blood-oxygen-level-dependent (BOLD) signals used in fMRI, but BOLD-fMRI cannot separate HbO and HHb. Utilization of two wavelengths in fNIRS is adequate for quantification of changes (Δ) in concentrations of HbO and HHb (and then $\Delta HbT = \Delta HbO + \Delta HHb$ and $\Delta HbD = \Delta HbO - \Delta HHb$) (Hoshi 2007; Ferrari and Quaresima 2012; Boas et al. 2014; Zeff et al. 2007; Eggebrecht et al. 2014; Niu et al. 2012; Niu and He 2014). TILS is not due to water heating (Wang et al. 2018) or increased circulation (i.e., both HbO and HHb increase). Tian et al. (2016) employed fNIRS and for the first time reported that **TILS increased human cerebral oxygenation (i.e., more HbO but less HHb)** in healthy adults under resting conditions, $n = 18$ (Tian et al. 2016).

In a second fNIRS study, we investigated another 18 participants under cognitive activation. fNIRS revealed large effects on prefrontal oxygenation during cognitive enhancement postlaser and provided the first demonstration that cognitive enhancement by transcranial photobiomodulation is associated with cerebrovascular oxygenation of the prefrontal cortex. Sham control data served to rule out that the laser effects were due to pre-post task repetition or other nonspecific effects. The effects of TILS on the fNIRS signal are illustrated in Figs. 16 and 17, in terms of the temporal and spatial effects of TILS, respectively. The level of oxygenated hemoglobin rose significantly after TILS and maintained a higher
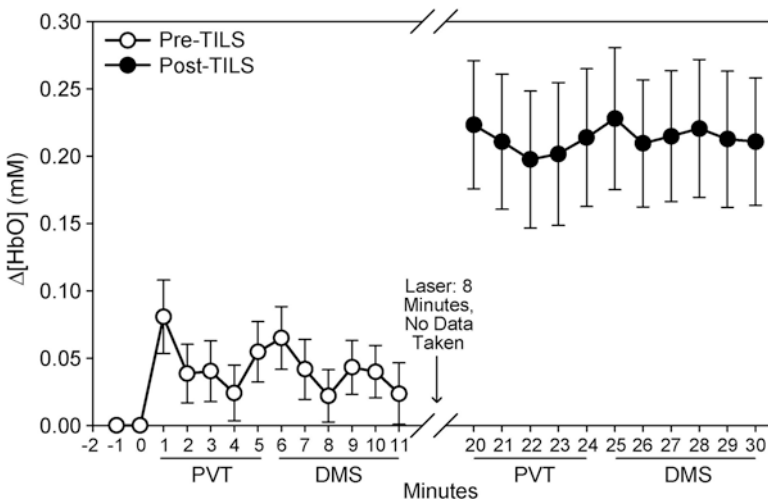

Fig. 16 Temporal effects of TILS on human cerebral oxygenation. Temporal sequence of changes in oxygenated hemoglobin (μM), pre- and postTILS during cognitive processing in the PVT and DMS tasks. Mean ± S.E (Holmes et al. 2019)
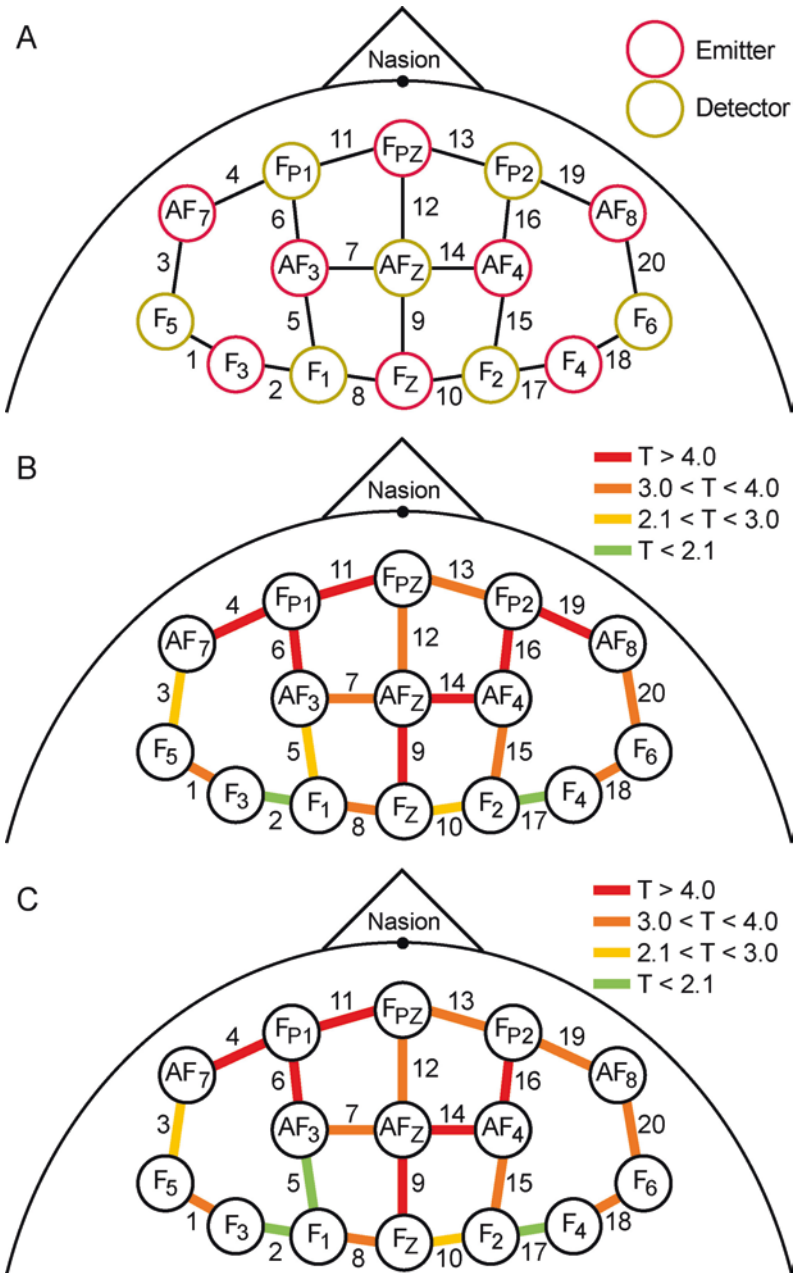
Fig. 17 Spatial effects of TILS on human prefrontal cortex. (**a**) Schematic spatial layout of the frontal montage used for fNIRS recording. Red and yellow circles correspond to emitters and detectors, respectively. The labels inside the circles correspond to the 10–20 EEG system locations used for each emitter and detector. Each adjacent emitter/detector pair formed a channel that gathered fNIRS data (20 labeled lines). (**b**) Colored lines represent the magnitude of *T*-scores from the analysis of mean differences between pre- and postTILS PVT epochs. (**c**) The magnitude of *T*-scores comparing the pre- and postTILS DMS epochs. The affected channels are almost identical, with two exceptions (channels 5 and 19) (Holmes et al. 2019)

level throughout the postTILS period (Fig. 16). The laser treatment resulted in a large increase in oxygenated hemoglobin in the anterior frontal region measured by fNIRS, corresponding to the prefrontal cortex region engaged during the PVT and DMS tasks (Nieder and Miller 2004; Drummond et al. 2005). This large hemodynamic response was about five times greater than that in the preTILS condition, and it was sustained for more than 10 min of cognitive processing after TILS.

Comparing differences between pre- and postTILS in terms of spatial effects, the anterior channels in the fNIRS frontal montage (Fig. 17a) registered higher levels of oxygenated hemoglobin than the posterior channels during cognitive processing of PVT (Fig. 17b) and DMS (Fig. 17c).

It has also become evident that bbNIRS is able to accurately quantify changes in cerebral oxidized cytochrome oxidase (Tachtsidis et al. 2010; Papademetriou et al. 2012; Kolyva et al. 2012, 2014). In a third study, Wang et al. (2017) implemented a bbNIRS system (Fig. 18) and showed in vivo that **cytochrome oxidase increase by 1064-nm laser precedes by minutes the increase in HbO and the decrease in HHb** in forearms ($n = 11$) (Wang et al. 2016) and PFC ($n = 11$) (Wang et al. 2017). In addition, oxidized cytochrome oxidase in PFC is greater than placebo by 2 min after onset of TILS and continues to increase until 8 min of stimulation (Fig. 19) (Wang et al. 2017).

In conclusion, TILS at 1064 nm wavelength enhanced cognitive performance and both fNIRS and bbNIRS responses in human prefrontal cortex. Altogether, the results of the NIRS studies and our previous cognitive studies suggest that TILS is a form of photobiomodulation that can successfully augment cerebrovascular oxygenation and thereby improve human cognitive brain functions.
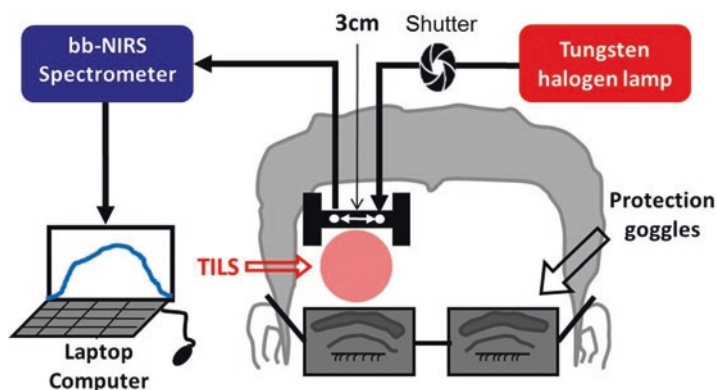

Fig. 18 Schematic of bbNIRS setup. This consisted of a tungsten halogen lamp as the light source and a miniature back-thinned CCD spectrometer as the detector. TILS (red circle) was administered underneath the black probe holder. A laptop computer was used to acquire, display, and save the data from the spectrometer. The shutter controlled the on and off function for the white light from the tungsten-halogen lamp to the subject's forehead. A pair of protection goggles was worn during the whole procedure (Wang et al. 2017)
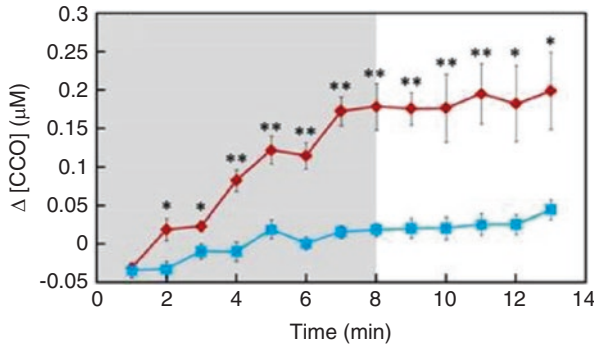
Fig. 19 TILS increases cytochrome oxidase in the human prefrontal cortex. Subject-averaged time courses of TILS (red) vs. placebo (blue) changes (Δ) of oxidized [cytochrome oxidase = CCO] recorded from human foreheads in vivo (mean ± SE, $n = 11$). Time zero ($t = 0$) is the onset of TILS. "*" $0.01 < p < 0.05$ and "**" $p < 0.01$, two sample t-test between TILS and placebo (Wang et al. 2017)

2. *Electroencephalographic (EEG) studies*. We conducted EEG studies using a 64-channel qEEG analysis before, during, and after TILS or placebo, $n = 20$ (Wang et al. 2017a, 2019). A gradual and strong increase of power density in the Alpha band (8–13 Hz) and smaller effects in Beta (13–30 Hz) and Gamma (32+ Hz) bands were observed across time during TILS to right PFC (Fig. 20). Using a novel analysis method and open-source software, eLORETA (Aoki et al. 2015), Dr. Xinlong Wang and Dr. Hanli Liu developed a new spatiotemporal image processing algorithm to estimate TILS-induced EEG responses in time and spectral frequency and focused on specific frequency bands to estimate and reconstruct source localizations induced by TILS (Fig. 21). The results are very promising because they revealed that (a) it takes minutes for the electrophysiological signals to respond (much different from transcranial direct current or magnetic stimulation, tDCS or TMS) and this change manifested after approximately 2 min and peaked by 8 (matching cytochrome oxidase upregulation), (b) the neuromodulation propagated from frontal to posterior cortical regions, (c) TILS modulated ipsilateral activity at the right frontal cortex, while also modulating bilateral activity at the posterior cortex, and (d) TILS modulated an ipsilateral, fronto-parieto-occipital network and a contralateral, parieto-occipital network at the Alpha frequency.

3. *fMRI studies*. BOLD fMRI signal is a combined output of *decreased* oxygen consumption and increased cerebral blood flow (CBF) and blood volume. Since TILS *increases* oxygen consumption in the PFC, as shown in our previous studies (Rojas et al. 2008a, b, 2012; Wang et al. 2017), a reduced BOLD-fMRI signal was expected. BOLD-fMRI responses to a working memory 2-back task were measured in six participants before and after TILS (Vargas et al. 2017). As predicted, TILS-induced cognitive improvement was accompanied by reduced task-evoked BOLD-fMRI signal in right PFC relative to its own baseline, suggesting that TILS-facilitated oxygen consumption promotes more *efficient* PFC function

Fig. 20 TILS increases
EEG power density.
Time-frequency heat plot
of TILS-induced power
density difference with
respect to placebo. Data
were taken from an
electrode near the TILS
site and averaged over 20
subjects. TILS started at
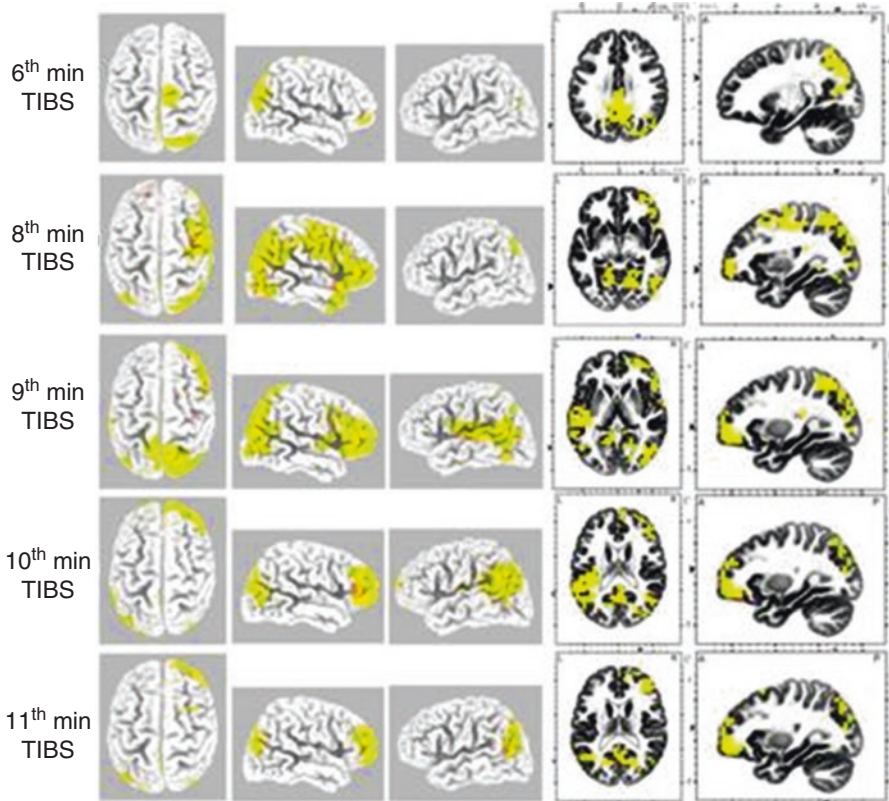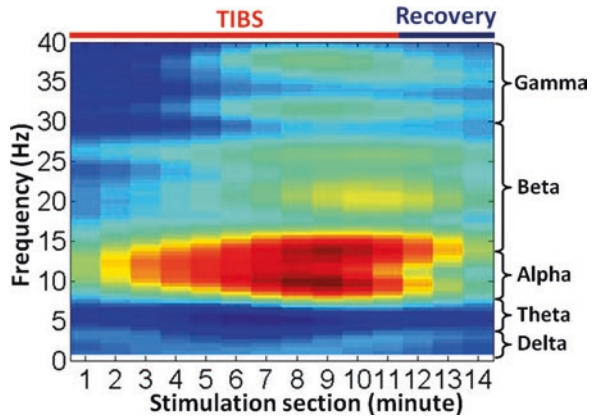time = 0 and ended at 11
(red line on top) (Wang
et al. 2017a)

Fig. 21 TILS modulates a large-scale fronto-parieto-occipital network. 3D views of t-maps (yellow = $p < 0.01$) between TILS and placebo conditions based on Alpha-band (8–13 Hz) power density at 6, 8, 9, 10, and 11 min after right PFC TILS started ($n = 20$) (Wang et al. 2017a)

that enhances cognitive processing. This is consistent with higher cognitive performing older adults showing reduced CBF in right PFC and bilateral neural plasticity (Cabeza et al. 2002). We will conduct more fMRI studies to develop a high-resolution spatial map of the network effects of TILS in the brain. Future fMRI studies of TILS will follow the same PVT and DMS protocols of two recent fMRI studies of methylene blue that we coauthored using BOLD, CBF, and functional connectivity (Rodriguez et al. 2016a, b).

In conclusion, the neurophysiological studies identified a large-scale attention-memory-executive network that corresponds to the network modulated by TILS, including associative areas of frontal, posterior parietal, and occipital cortices, reliably associated with tasks that require high-sustained attention-working memory-executive demands among younger and older adults (Haley et al. 2007a, b, 2008, 2010; Sweet et al. 2008). Our combined cognitive and neurophysiological studies support the hypothesis that TILS of the right PFC modulates a large-scale fronto-parieto-occipital network that promotes cognitive enhancement of attention-memory-executive functions.

4 Conclusions

The studies presented demonstrate that photobiomodulation may be translated from animals to humans to become a noninvasive, safe, nonpharmacological, and cost-effective method for augmentation of brain functions. Noninvasive methods such as fNIRS, bbNIRS, and EEG may be able to monitor hemodynamic and electrophysiological effects on human brains in real time. Combining photobiomodulation with such neurophysiological monitoring allows for calibration of the laser dose for individuals based on their own particular cerebrovascular response, as a new form of "precision medicine". This approach might also permit identifying potential candidates for photobiomodulation before clinical presentation of neurodegenerative effects. This approach is particularly promising for populations with prefrontal hypometabolism, such as in cognitive aging, mild cognitive impairment, Alzheimer's dementia, and many other neurological and psychiatric conditions that lead to neurocognitive decline (Rojas and Gonzalez-Lima 2013).

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