THE UNIVERSITY OF SYDNEY

THE EFFECT OF PHOTOBIOMODULATION ON ACTIVITY OF THE HUMAN BRAIN

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Thesis submitted in fulfilment of the requirements for the degree
Doctor of Philosophy (Medicine)

August 2023

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Discipline of Anatomy and Histology
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TABLE OF CONTENTS

STATEMENT OF ORIGINALITY

I certify that this thesis does not contain, without appropriate acknowledgement, any material submitted previously for a degree or diploma in any university. I also certify that this thesis does not contain, to the best of my knowledge, any material previously published or written by another person.

I certify that the intellectual content in this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

I also certify my understanding that if my candidature is successful, my thesis will be lodged with the University Librarian and made available for immediate use.

Hala El Khoury

21 August 2023

LIST OF PUBLICATIONS

All of the four experimental chapters in this thesis have been published in peer reviewed international journals.

CHAPTER 2

El Khoury, Hala & Mitrofanis, John & Henderson, Luke. (2021). Does photobiomodulation influence the resting-state brain networks in young human subjects? Experimental Brain Research. 239. 1-15. 10.1007/s00221-020-05981-x.

CHAPTER 3

El Khoury, Hala & Mitrofanis, John & Henderson, Luke. (2019). Exploring the Effects of Near Infrared Light on Resting and Evoked Brain Activity in Humans Using Magnetic Resonance Imaging. Neuroscience. 422. 10.1016/j.neuroscience.2019.10.037.

CHAPTER 4

Hamilton, Catherine & El Khoury, Hala & Hamilton, David & Nicklason, Frank & Mitrofanis, John. (2019). The “Buckets”: Early Observations on the Use of Red and Infrared Light Helmets in Parkinson's Disease Patients. Photobiomodulation, Photomedicine, and Laser Surgery. 37. 10.1089/photob.2019.4663.

CHAPTER 5

Xie, Kangzhe & El Khoury, Hala & Mitrofanis, John & Austin, Paul. (2022). A systematic review of the effect of photobiomodulation on the neuroinflammatory response in animal models of neurodegenerative diseases. Reviews in the Neurosciences. 10.1515/revneuro-2022-0109.

AUTHORSHIP ATTRIBUTION STATEMENTS

Chapter 2 of the thesis is published as El Khoury et. al., (2019) in the journal *neuroscience.* I was the main drive in the data collection process which included recruiting participants, collecting MRIs, instructing participants on passive resting tasks. I also processed most of the MRI images for early statistical analysis to interpret the effect of PBM on the brain, and for more advanced analysis, I received instruction on what to do. Other authors provided guidance and assisted with interpretation (Mitrofanis, Henderson) and advanced-level statistical analysis (Henderson).

Chapter 3 of the thesis is published as El Khoury et. al., (2021) in the journal *Experimental Brain Research.* I was the main drive in the data collection process which included recruiting participants, collecting MRIs, instructing participants on motor tasks, and providing timed sensory stimulus. I also processed most of the MRI images for early statistical analysis to interpret the effect of PBM on the brain, and for more advanced analysis, I received instruction on what to do. Other authors provided guidance and assisted with interpretation (Mitrofanis, Henderson) and advanced-level statistical analysis (Henderson).

Chapter 4 is published as Hamilton et. al., (2019) in the journal *Photobiomodulation, Photomedicine and Laser Surgery* where I was second author. I contributed to the graphology portion of this study, by analysing the handwriting of the Parkinson's patients and determining the statistical difference in the shape and size of the handwriting. We longitudinally followed each patient's handwriting progress, before, during and after PBM treatment. Other authors collected other signs and symptoms of the patients before and after they administered the photobiomodulation treatments and interpreted other results to assess the effect of PBM in the longitudinal study.

Chapter 5 is published as Xie et. al., (2022) in the journal *Reviews in Neurosciences.* I was joint first author on this publication. My colleague (Xie) and I were equal contributors to the systematic review searches, article selections, data extractions, planning, and interpretation of the results, and writing the manuscript. Our other co-authors (Austin, Mitrofanis) conceived and designed the study design, confirmed article selection criteria, and edited a draft of the manuscript.

In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

Hala El Khoury

21 August 2023

As supervisor for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Paul J. Austin

21 August 2023

ACKNOWELDGEMENTS

Prof. John Mitrofanis, my inspiration, and my rock right from the beginning. I started this journey with you, and you stayed with me through thick and thin, when we were oceans apart and through all sorts of challenging obstacles, you've always been my constant form of support and guidance.

Dr. Paul Austin, for taking a chance on me when I needed to pick up where I left off after 2 long rounds of maternity leave, for helping me with my final stretch to the finish line, allowing me the space to own my accountability & always offering me unique and logical ways of looking at things.

For Kangzhe Xie (AKA Steven), for joining me on the systematic review, long days and long nights pouring over this work together and working as a powerful team. Without our amazing collaboration on the final manuscript, I wouldn't have finished this thesis. Thank you.

A Prof. Luke Henderson, for taking me under your wing in the world of MRI studies, allowing me to join your lab, conduct practical research and collect data, and for your immense patience when teaching me the ropes in the world of MRI image analysis and MATLAB.

Dr. Nabil El-Massri, for setting an example of what is possible as a fellow parent with children, you paved the way and have always been like a mentor to me on the neuroscience PhD journey. You gave me encouragement, answered my questions, and came from a powerful place of experience.

My best friend, Mimosa Wilson, for proofreading the thesis, encouraging me to keep going, cheering me on with an unwavering level of support, and of course, as an intellectual, always asking the most amazing clarifying questions that gave me a run for my money.

My beautiful parents, Ghada & Pierre, for always wanting the best for me & encouraging me to keep going. Whenever I faced intense challenges and my belief in myself wavered a bit, that's when you believed in me even more.

My incredible husband Justin, for constantly cheering me on, being excited for me, and knowing my thesis inside and out – throughout the whole thing I felt like I had a PR representative advocating for my type of work everywhere we went. You've always made me feel how proud you are of me!

My children Alma, Ezra, our third little one on the way, and of course Prue, our little angel with wings. From the moment I conceived all of you, I stopped listening to the voices that told me I can't do or complete things because I was having children. Instead, I spoke to each of you in my womb and told you that I would collaborate with you all, that I will always give my best, and that we'd be a team in making the world a better place.

ABREVIATIONS

PBM – photobiomodulation

DMN – default mode network

EEG – electroencephalogram

PET – positron emission tomography

MRI – magnetic resonance imaging

fMRI – functional magnetic resonance imaging

BOLD – blood oxygen level dependent

ASL – arterial spin labelling

pCASL – pseudo-continuous arterial spin labelling

LED – light emitting diode

BDNF – brain derived neurotrophic factor

GDNF – glial cell derived neurotrophic factor

CcO – cytochrome C oxidase

TRP – transient receptor potential

SNc – substantia nigra compacta

CPu – caudate putamen

GPi – globus pallidus interna

GPe – globus pallidus externa

STN – subthalamic nucleus

ZI – zona incerta

BBB – blood brain barrier

APP/PS1 – amyloid precursor protein/presenilin 1

LPS – lipopolysaccharide

ADAS-cog – Alzheimer's Disease Assessment Scale -cognitive subscale

HAM-D – Hamilton Scale of Depression

TUG – timed up and go test

MCID – minimal clinically important difference

iNOS – inducible nitric oxide synthase

ROS – reactive oxygen species

TNF-α – tumour necrosis factor-α

IL-1β – interleukin 1 beta

IL-6 – interleukin 6

NFkB – nuclear factor kappa B

Bcl-2 – B-cell lymphoma

TGF – transforming growth factor

GFAP – glial fibrillary acidic protein

IBA1 – ionised calcium binding adaptor molecule 1

COX-2 - cyclo-oxygenase enzyme 2

EAE – experimental autoimmune encephalomyelitis

AD – Alzheimer's disease

PD – Parkinson's disease

ABSTRACT

The global aim of this thesis was to explore the effect of photobiomodulation (PBM) on the human brain, with the findings leading hopefully to further establish this treatment as a viable therapeutic option for patients, particularly those with neurodegenerative disease. The results are presented as peer reviewed publications, each with their own chapters (2-5). Chapter 1 contains the literature review, while chapter 6 contains a general discussion.

This thesis included functional magnetic resonance imaging (fMRI) research on the effect of PBM on healthy young brains (chapter 2 and 3), along with clinical case studies of patients with neurodegenerative disease (chapter 4) and a thorough systematic review of the effect of PBM on neurodegeneration-induced neuroinflammation (chapter 5). Together, all these components of the thesis offer a robust and diverse exploration of the effect of PBM on healthy and diseased brains.

In chapter 2, with the use of fMRI, the effect of PBM on human brain activity in young healthy individuals indicated a reduction in default mode network connectivity during the execution of a task (ie, finger tapping). It was suggested that PBM helped focus attention on the sensorimotor task being undertaken by the individuals. In chapter 3, again with the use of fMRI, the effect of PBM indicated no change in resting state brain connectivity in the brains of healthy individuals. From the results in chapter 2 and 3, it was concluded that in a healthy brain state, PBM had a measurable effect such as altering brain connectivity when the brain is in an evoked task state, for example when undergoing a sensorimotor task, but not during passive rest. In Chapter 4, the effect of PBM on the clinical motor signs and non-motor symptoms of elderly patients with neurodegenerative disease (ie Parkinson's disease) was observed and recorded. Almost all the signs and symptoms of the patients showed improvement and none got worse. For example, improvement in motor signs included reduced tremors, improvement in gait and handwriting, whilst non-motor symptoms included improvement in sleep, self-esteem, and reduction of depression. In many of the cases, the improvements were long-term, with some extending over 24 months. It was suggested that these results indicated a slowing down of the disease process (ie neuroprotection), but a proper, large scale, placebo controlled clinical trial is needed for validation. In chapter 5, the effect of PBM on animal models of Parkinson's and Alzheimer's disease was shown to result in significant improvement in behavioural measures of neurodegeneration accompanied with significant decreases in neuroinflammatory markers, including glial markers such as glial fibrillary acidic protein (GFAP), inflammatory cytokines such as interleukin 6 (IL-6) and tumour necrosis factor-α (TNF-α), and oxidative stress such as reactive oxygen species (ROS). This systematic review demonstrated a beneficial relationship between neurodegeneration,

neuroinflammation, oxidative stress and PBM. These beneficial effects were accomplished with a large variation in PBM treatment protocols and dosimetry.

Taking the thesis as whole, there were three key findings that tied the diverse chapters together. First, PBM as a treatment method could be viewed to have preventative qualities (chapter 2, 3, 4 and 5). For example, PBM improved the function of healthy brains and a healthier brain is less likely to suffer dysfunction and disease later in life (chapter 2, 3); with long-term use, many signs and symptoms of patients did not worsen with the use of PBM (chapter 4) and finally, many animal studies on neurodegenerative disease have shown that with pre-treatment, PBM can offer neuroprotection against the toxic insult and/or genetic mutation (chapter 5). Second, PBM seemed to have more of an effect on older diseased brains, such as neurodegenerative disease, compared to younger brains because there are more cellular and molecular targets for PBM to act upon (chapter 2, 3, 4, 5). From the findings in chapters 2 and 3, it appears that PBM also does work on healthy brains, but an effect is measurable when the brain is taken out of its resting baseline and into an evoked state, such as the fingertapping task in chapter 2. Third, PBM can offer long term benefits for neurodegenerative disease with many different dosage variations (chapter 4 and 5).

The results of the thesis have provided a foundation for the concept that PBM can be viewed as an effective therapeutic and preventative treatment for neurodegenerative disease. Further, the results indicated that PBM has the capacity to offer long term benefits for neurodegenerative diseases. This thesis offers a framework for future studies to further support the key findings of PBM as a viable therapeutic treatment, particularly for neurogenerative disease in the clinical setting.

CHAPTER 1: LITERATURE REVIEW

The main aim of this thesis was to investigate the effect of photobiomodulation on the brain, in physiological and pathological conditions. This work was intended to lay down further the foundation for photobiomodulation as a valid non-invasive treatment approach to neurodegenerative diseases in the clinical setting. The major themes explored in the literature review that follows include:

(1) Photobiomodulation: what it is, the mechanisms behind it, and its effect in foundational animal models.

(2) How photobiomodulation affects the human brain: what is currently known about the impact of photobiomodulation on the human brain through imaging and other existing methods .

(3) Photobiomodulation and Parkinson's disease: what is currently known about Parkinson's disease as seen in neuroimaging, such as magnetic resonance imaging (MRI) and electroencephalogram (EEG) techniques, and the effect of photobiomodulation on the brains of Parkinson's-disease patients and in animal models.

(4) The effect of photobiomodulation on neuroinflammation: our current understanding on the relationship between neuroinflammation, neurodegeneration and oxidation; the effect of photobiomodulation on neuroinflammation among different cell types of the brain; and the effect of photobiomodulation on animal models of neuroinflammation.

(5) Aims, rationale and outline of thesis: this section details the aims, rationale and outline of the experimental chapters that includes the publications of the thesis

It should be noted that this literature review chapter does not include the publications that have arisen from the experimental work associated with this thesis. These publications, that populate the main body of chapters of this thesis (chapters 2-5), cover the impact of photobiomodulation on healthy brains, on the clinical signs and symptoms of Parkinson's disease patients, together with a systematic review investigating the effect of photobiomodulation on neurodegeneration-induced neuroinflammation.

1.1 Photobiomodulation: what is it?

Photobiomodulation (PBM), also known as low level light therapy, is the use of light shone through low energy lasers or light-emitting diode (LED) arrays, within the spectrum of approximately 600-1300 nanometres (nm) as a form of therapy (Eells et. al., 2004, Gonzalez-Lima and Barret, 2014; Hamblin, 2016). This range can be split into a portion of visible red light, 600-700nm, as well as a portion of near invisible, near infrared light, 700-1300nm. Additionally, some green and blue wavelengths, between 450nm-570nm, have also been used to modulate different physiological functions, however, won't be discussed in depth, as they are not classified as PBM in the context of this thesis.

The benefits of PBM, were first discovered serendipitously in 1967 by Endre Mester at the Semmelweis Medical University of Hungary. Mester was attempting to repeat an experiment conducted by McGuff in Boston, USA, that utilised ruby lasers to cure malignant tumours (McGuff et. al., 1965). However, he later discovered that the laser built for him had a much lower power density, only a small fraction of the ruby laser. Hence, he did not treat the tumours with this laser. What he did observe was faster wound healing, repair and hair growth in the rats at the wound sites where he surgically implanted the tumours (Mester et. al., 1968; Hamblin, 2016).

Since Mester's time, our knowledge of this method has developed considerably. There is an optimal window of wavelengths, between (but not limited to) 600nm to 1300nm where effective tissue penetration is at its highest (Huang et. al., 2011; Hamblin, 2016). Some wavelength ranges seem to have greater effects than others, for example, light wavelengths between 700-770nm tend to have little beneficial effects, due to minimal biochemical activity, and hence are not used often (Chung et. al., 2012; Hamblin, 2016; Hamblin and Hennessy, 2017). Other wavelengths, for example 600-690nm and 760-950nm have a much greater beneficial impact (Hamblin, 2016; Hamblin and Hennessy, 2017). Wavelengths between 600-690nm are generally used to treat more superficial body tissues, while wavelengths between 760-950nm are used to treat deeper body tissues, due to their ability to penetrate deeper into the tissue (Chung et. al., 2012; Hamblin, 2016), some examples seen in figure 1.1.1a. It is important to note that infrared light can be split into near, middle and far ranges with wavelengths of 700-1400nm, 1400-3000nm and 3000-100000nm, respectively (Vatansever and Hamblin, 2012). Throughout the literature, wavelengths beyond the light range of 600-1300nm have been found to have effects as well as, longer wavelength near infrared (Hoffman et. al., 2007), mid infrared (Zand et. al., 2009), far infrared (Vatansever and Hamblin, 2012; Huang et. al., 2009).

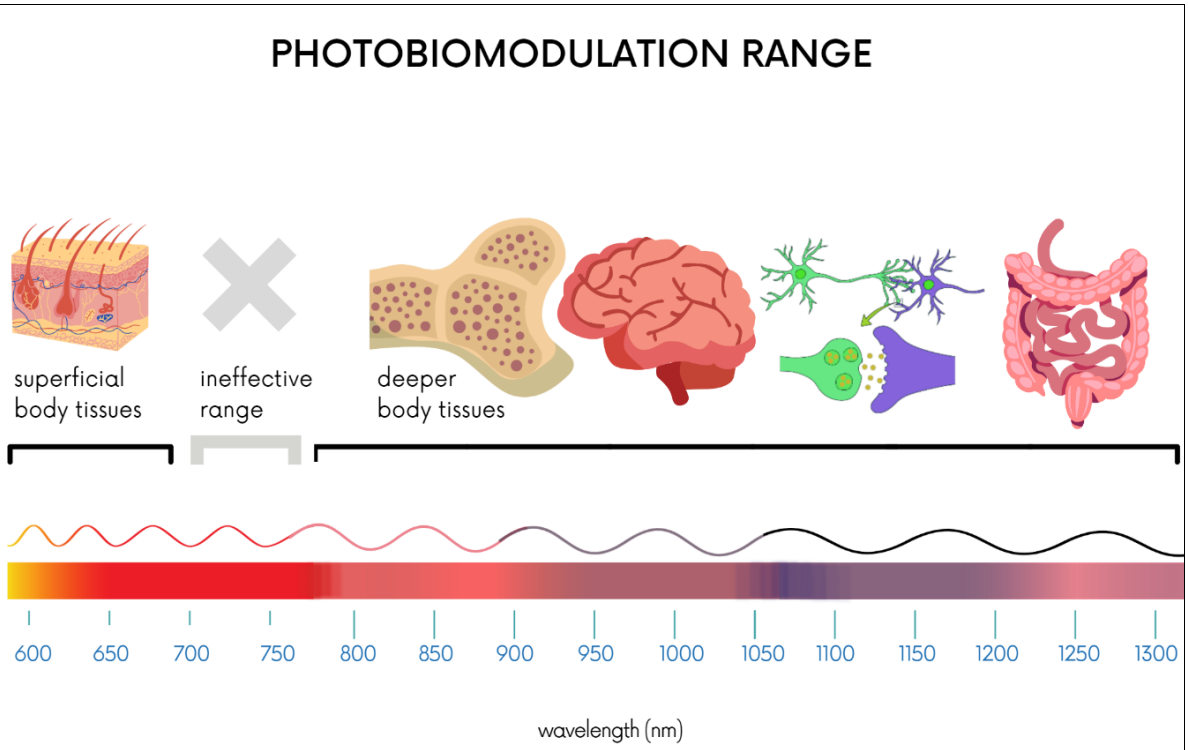

Fig. 1.1.1a. A diagram of the PBM range between 600-1300nm. The general use of the wavelengths for superficial body tissues is mostly utilised in the shorter wavelength ranges (600-690nm) in humans, while the wider wavelengths (780nm-1300nm) are used for deeper body tissues. Wavelength 700 to 770nm seem to have little effect. *Note the organs in the diagram act as examples for tissue that can be penetrated within the 780nm-1300nm range but aren't to scale or directly correspond to an individual wavelength.

Many authors have reported that the beneficial effect of light can also depend on the dose (power density, energy density), the mode of operation (pulsed waves or continuous waves) and treatment duration (Hamblin, 2016; Hamblin and Hennessy, 2017). Power density, also known as irradiance, depends on the wattage of the actual light source and beam size. Irradiance values generally ranging from 5 to 50mW/cm^2 are often used for healing and stimulation, whilst higher values, up to W/cm^2 (1000mW/cm^2 and greater), have been used for nerve inhibition and pain relief (Huang et. al., 2011). In the first instance, the depth of penetration depends on wavelength, as discussed in the previous section. In addition, the amount of power also has an effect on the depth of tissue penetrated by the light. For example, it has been found that at 10-15W, 0.45%-2.9% of 810nm light can penetrate 30mm of tissue, and at 980nm, 1.22% of the light penetrated the 30mm of tissue (Henderson and Morries, 2015). Energy density, also known as fluence, generally is effective between 1 to 20 J/cm^2 (Hamblin, 2016; Hamblin and Hennessy, 2017). Treatment duration (Hamblin, 2016; Hamblin and Hennessy, 2017; Zein et. al., 2018) also has an important impact, for example in a study by Blivet and colleagues (2018), part of their investigation used PBM length of exposure as a dependent variable and demonstrated non-significant effects of PBM in the shorter treatment periods as compared to significant effects of PBM in the longer

treatment periods. In terms of the mode of operation, there have been several studies that showed benefits of PBM from pulsed waves of different frequencies (10Hz and 40Hz) (Blivet et. al., 2018; Zhou et. al., 2021; Hipskind et. al., 2019; Salehpour et. al., 2016; Shan et. al., 2021) as well as continuous waves (Ando et. al., 2011, Johnstone et. al., 2014; Lu et. al., 2017, Yang et. al., 2021,2022). The data is divided, some studies indicate that pulsed waves are more effective / faster-acting compared to continuous waves (Keshri et. al., 2016) whilst other studies suggest that continuous waves are more effective (Khalaj et. al., 2023).

Hence, the parameters of irradiance, fluence, mode of operation and duration have been deemed important to monitor because PBM can have a so-called “biphasic dose response”. A simplified representation of the three parameters: irradiance, fluence and duration can be seen in Fig 1.1.1b whereby the parameters interact three-dimensionally on an xyz plane where their different values contribute to a biphasic dose response. This response has been observed in biological systems treated with any physical or chemical substance. That is, doses that are too low have a negligible effect, optimal levels present a beneficial effect and higher doses have little, sometimes detrimental effects. This pattern of response follows the Arndt-Schulz curve, a standard model used to explain the dose dependent effect of pharmaceuticals (Huang et. al., 2009, 2011; Hamblin, 2016). In the field of PBM, it has generally been found that the negligible, together with the rare detrimental effects, are a result of either too little or excessive power density, energy density or duration of exposure (Huang et. al., 2009, 2011; Hamblin 2016; Hamblin and Hennessy, 2017). Further, for the somewhat detrimental effects, the high laser doses/intensities correlate with a rise in surface temperature ($\geq$45°C) in mice, which is not beneficial to tissue, however this is the greatest extent of the adverse effects (Khan et. al., 2015).

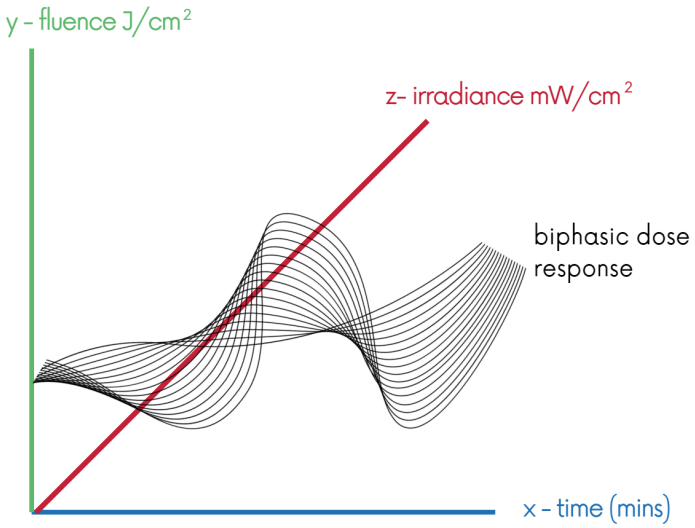

Fig. 1.1.1b. A schematic diagram of the multidimensional interaction between fluence, irradiance and time on the effect of PBM dosage and the subsequent dose response. This diagram shows a three-dimensional rendition the biphasic dose response, similar to the Arndt-Schulz curve.

1.2 Mechanisms of photobiomodulation

Due to the ability of light to penetrate 20-30mm of living tissue at a range of wavelengths within the visible and infrared light spectrum, it indicates that the photons can reach the internal structures of cells, at least within superficial regions of the body. Generally speaking, infrared light penetrates through tissue at a deeper level compared to visible red light (Henderson and Morries, 2015; Hamblin, 2016; Hennessy and Hamblin, 2017). The putative neuroprotective mechanism of light is divisible into two main modes: direct and indirect stimulation (Johnstone et. al., 2016; Mitrofanis, 2017).

1.2.1 Direct effects

The effects of direct stimulation occur from the light hitting the cells "flush". This direct stimulation has been suggested to lead to so-called primary and secondary effects. Primary effects are the effects occurring during light exposure as the photons make direct contact with light receptive cells, whilst secondary effects are the effects that are triggered by cellular signalling pathways after the initial light exposure (Karu, 2010; Rojas and Gonzalez-Lima, 2011; Gonzalez-Lima et. al., 2014; Mitrofanis, 2017), as seen in Fig 1.1.2a.

The primary effects are rather short-term and rely on light being absorbed by a chromophore within a photoacceptor in the mitochondria. One of the most common photoacceptors within these cells is

cytochrome c oxidase (Eells et. al., 2004; Hamblin, 2016), the fourth unit of the mitochondrial respiratory chain located on the membrane of the mitochondria. Cytochrome c oxidase is responsible for the last reduction of oxygen to water using the electrons generated by the process of glucose metabolism (Hamblin, 2016; De Freitas and Hamblin, 2016). Photons cause a dissociation of nitric oxide and this, combined with a photoabsorbance by cytochrome c oxidase results in an increase in mitochondrial membrane potential, greater oxygen consumption, glucose utility and greater production of ATP by the mitochondria (Lane, 2006; Karu, 1999, 2010; Hamblin, 2016). Cytochrome C oxidase primarily has two absorption peaks for photons wavelengths 600-700nm and 760-940nm (Karu, 1999, 2010; Hamblin, 2016). The stimulation of this protein results in an increase of the movement of electrons across the transport chain as well as an increased proton gradient, which ultimately results in the production of ATP. This process results also in a brief increase in production of reactive oxygen species (ROS), as a by-product, which can behave as a signalling molecule that then triggers other signalling pathways, such as the activation of NFkB, a transcription factor (De Freitas et. al., 2016) that regulates a number of gene expressions responsible for cytoprotective, antioxidant and anti-apoptotic effects in the cells (Hamblin, 2016; Waypa et. al., 2016). Nitric oxide, a signalling molecule that triggers a number of cellular pathways, is also released by this process and is thought to be responsible for increasing local blood and lymphatic flow by dilating blood and lymph vessels (Morries et. al., 2015; Cassano et. al., 2016; Hamblin, 2016; Tian et. al., 2016).

The secondary effects follow the primary effects and are more long term. The stimulation of cytochrome c oxidase results in a greater expression of transcription factors for neuroprotective genes. This ultimately results in cellular proliferation, collagen synthesis and the release of growth factors, such as transforming growth factor, platelet-derived growth factor, brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) from cells (Sommer et. al., 2001; Eells et. al., 2004; Hamblin, 2016). These changes result in longer lasting beneficial effects, presumably underpinning findings that a brief exposure to light in animal models of traumatic brain injury or Parkinson's disease results in beneficial effects lasting for days, weeks, even reaching up to months (Ando et. al., 2011; Hamblin, 2016; Darlot et. al., 2016). Hence this long-lasting effect from brief exposure can be explained by the secondary effect involving activation of signalling pathways and transcription factors, causing a lasting change in protein expression (Hamblin, 2016).

A feature worthy of further comment is the previously mentioned phenomenon that cytochrome c oxidase has two absorption peaks for photons, one from 600-700nm and the other from 760-940nm. Many previous studies have reported a beneficial effect of wavelengths outside of these two absorption

peaks, for example 980nm (Skopin et. al., 2009; Ferrante et. al., 2013; Wang et. al., 2016), 1064nm (Barret and Gonzalez-Lima, 2013), 1072nm (Dougal and Lee, 2013), and 3-12µm within the far infrared light band (Vatansever and Hamblin, 2012). These findings suggest that there may be other chromophores, besides cytochrome c oxidase, that exist to account for this beneficial effect of light, such as light gated ion channels and opsins, biological chromophores known as cryptochromes that rely on flavins, water, and heat-gated ion channels (Hamblin, 2017; Hamblin and Liebert, 2022). In cell cultures of adipose tissue, there are clear increases in intracellular calcium levels accompanied by a decrease in mitochondrial calcium levels that accompany the increase in mitochondrial activity and ATP after light application at 980nm (Wang et. al., 2016). These initial benefits can be explained by the above theory of light-mediated calcium channels opening, such as members of the transient receptor potential (TRP) super-family of ion channels found on most animal cell plasma membranes (Wang et. al., 2016). The super-family of the TRP channels were originally discovered to also be light gated in studies that used Drosophila mutants that were defective in visual transduction. Of these "other" chromophores, water has been suggested to be a pivotal photoacceptor. For water to be able to act as a photoacceptor, absorption needs to occur (Santana-Blank et. al., 2016). Water as a photoacceptor is considered to be nanostructured and located in heat or light sensitive ion channels, also on cell plasma membranes (Wang et. al., 2016. The absorption coefficient of water has its first peak at 980nm and keeps increasing until 3100nm (Santana-Blank et. al., 2016), suggesting that water has a large chromophore role. The effect of the above-mentioned light channels opening at 980nm or with the use of calcium blockers was abolished when water structure was disrupted either with cold medium (4°C) or incubation in heat (42°C). Calcium channel blockers, cold medium and incubation did not disrupt the beneficial effect, increase in mitochondrial activity and ATP levels when 810nm light was used, which is a wavelength within an absorption peak of cytochrome c oxidase. Therefore, it appears that the effect of 980nm light is dependent on water structures, unlike 810nm, hence the reason that the beneficial effects are lost with water disruption. There may well be many other, as yet undiscovered, chromophores that are stimulated at the other wavelengths in the infrared range (Wang et. al., 2016).

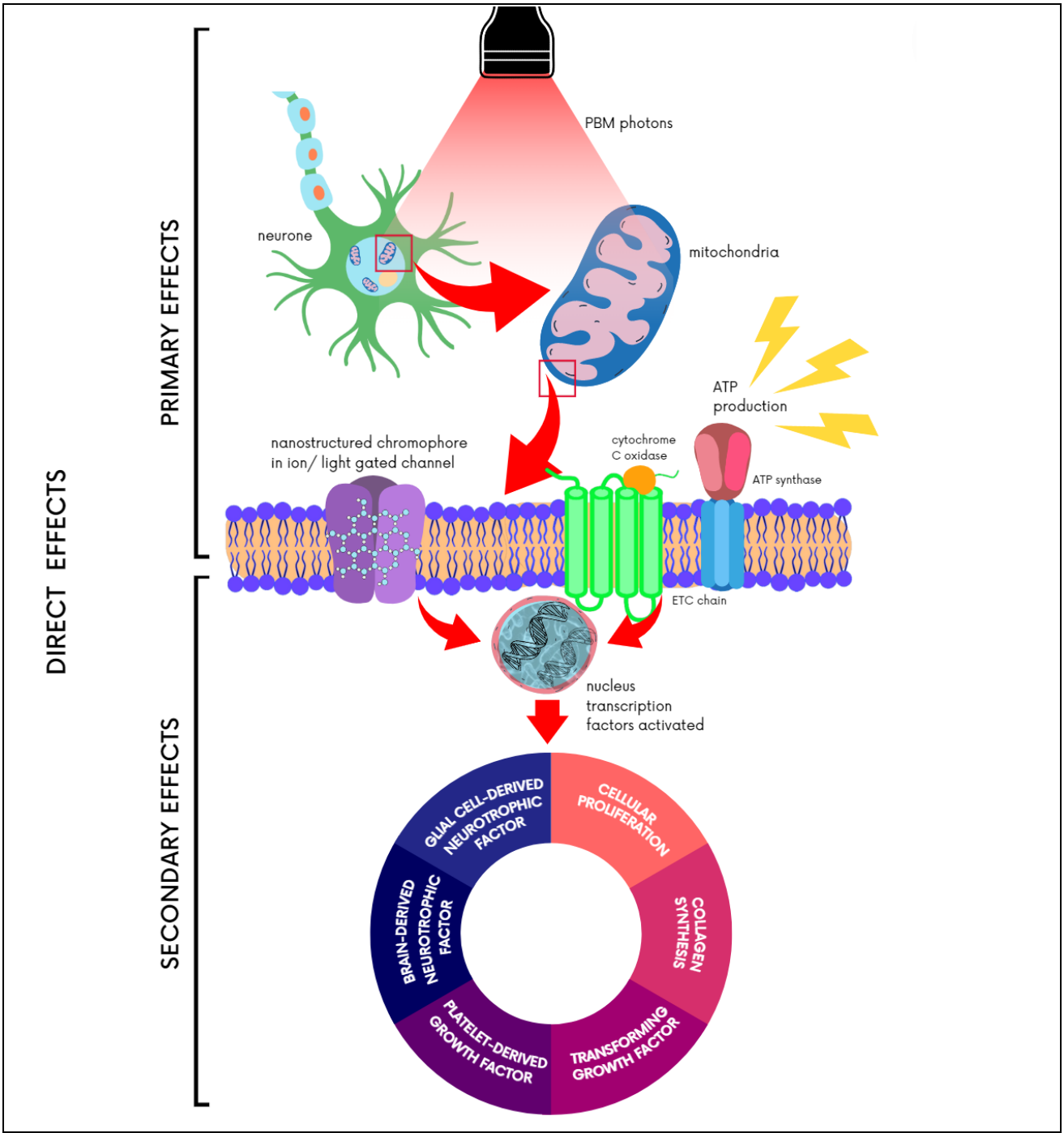

Fig 1.1.2a. A schematic diagram of the primary and secondary effects of PBM when it is working directly on target cells. The primary effects occur while the PBM is being administered and include immediate responses, such as ATP generation in the electron transport chain in the membrane of the mitochondria. The secondary effects do not necessarily happen during exposure, though extend to cellular signalling pathways and transcription factors for the generation of growth factors, cellular repair, and proliferation.

1.2.2 Indirect effects

There is evidence that all the benefits, in particular the neuroprotective ones, of light exposure do not entirely come from direct stimulation. There may be another mechanism at play as well. Several studies have reported that the application of PBM to one area of the body, such as the leg (Fig 1.1.2b.) or dorsum, can result in beneficial effects in another part of the body, such as the brain suggesting that there is an “abscopal” effect (Tuby et. al., 2011; Stone et. al., 2013; Johnstone et. al., 2014; Liebert et. al., 2014; Oron and Oron, 2016; Mitrofanis, 2017, 2019; Kim et. al., 2018). This phenomenon is considered to be the so-called indirect stimulation. It is suggested that this effect relies on a conduit, or "middle-man", such as the immune system, stem cells, or other circulating factors, including hormones or metabolites, which are activated by the light and have the capacity to cross the blood-brain barrier, especially as PBM has been observed to increase BBB permeability (Zhou et. al., 2021). For example, macrophages and stem cells can be stimulated and ultimately act on the distressed region to produce the beneficial effect (Tuby et. al., 2011; Stone et. al., 2013: Liebert et. al., 2014; Hamblin, 2016; Johnstone et. al., 2016; Oron and Oron, 2016; Hennessy and Hamblin, 2017; Mitrofanis, 2017). Pro-inflammatory cytokines such as interferon-ϒ and tumour necrosis factor-α are downregulated whilst anti-inflammatory cytokines, such as interleukin (IL)-4, IL-10 are upregulated by PBM (Muili et. al., 2012; Hennessy and Hamblin, 2017). Additionally, distressed mitochondria have the ability to produce an extracellular signalling molecule, mitokine, which travels globally throughout the body to trigger a systemic mitochondrial stress response (Durieux et. al., 2011; Taylor et. al., 2014; Lee, 2015). Further, PBM may influence the recently discovered free-floating mitochondria in the circulation (Al Amir Dache et. al., 2020) and this influence may help the survival of cells in distress in remote parts of the body (Mitrofanis, 2019).

It appears that both the direct and indirect effects can work independently, that one does not need the other. The direct effect has been shown to be neuroprotective in cell culture, a setting where the indirect effect is not functional (Ling et. al., 2008; Ying et. al., 2008; Trimmer et. al., 2009). The indirect effect has been shown to be beneficial in animal models, in that light is neuroprotective even if applied well away from the area of damage or distress, on another part of the body (see above; Stone et. al., 2013; Johnstone et. al., 2014; Farfara et. al., 2015; Saliba et. al., 2015). When comparing the two effects, the direct effect has been shown to offer more neuroprotection than the indirect; in an animal model of Parkinson's disease, light applied to the head results in more surviving dopaminergic neurones in the substantia nigra compacta (SNc) than after light application only to the dorsum of the mice (Johnstone et. al., 2014). It is more than likely that when in an experimental or clinical setting that the two effects,

both direct and indirect, can operate by working together synergistically to provide the most effective and long-lasting beneficial outcome (Johnstone et. al., 2016; Mitrofanis, 2017, 2019).

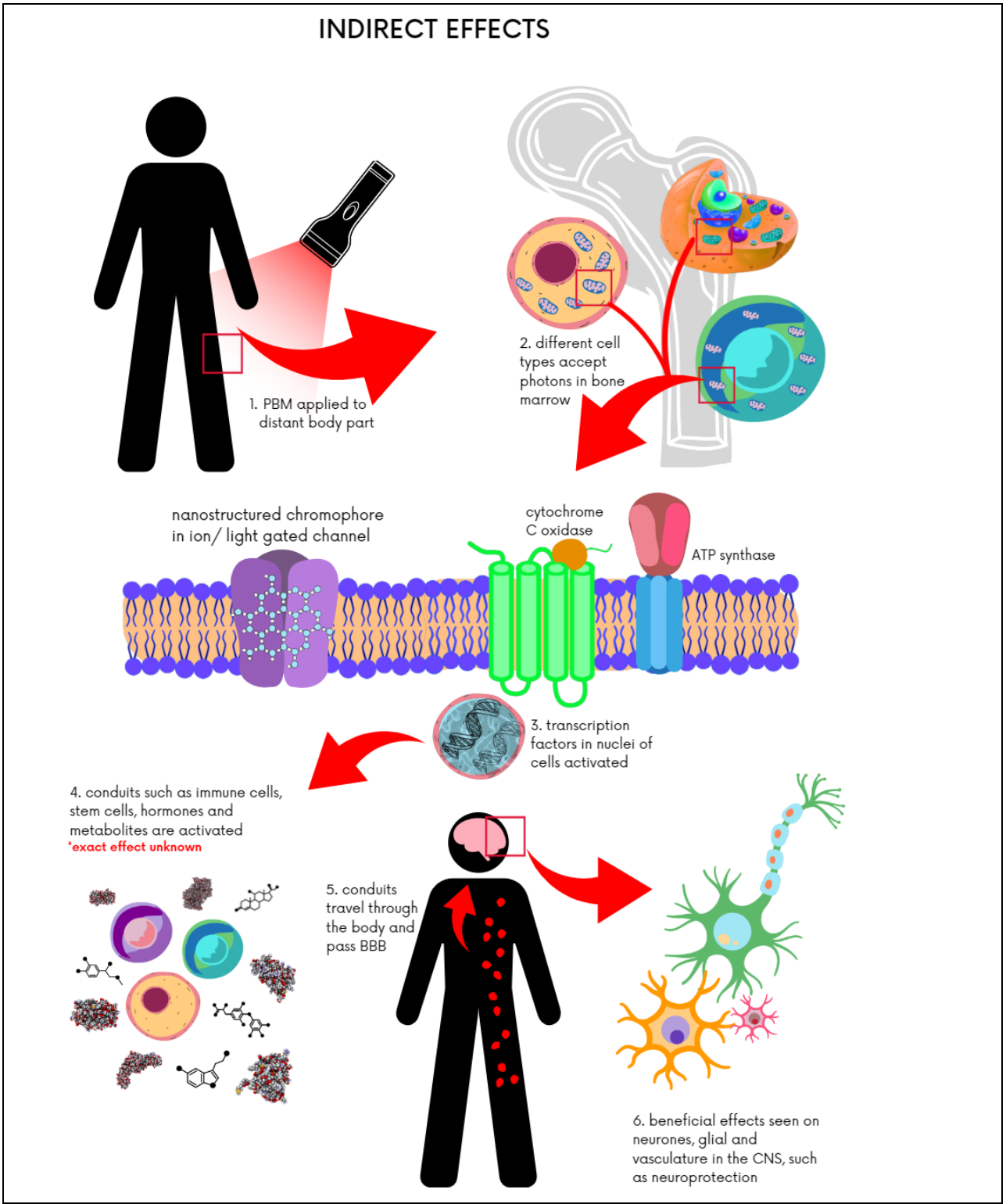

Fig. 1.1.2b. Schematic diagram of the indirect or "abscopal" effect PBM has. For example, the irradiation of the thigh with PBM can result in the activation of conduit factors, such as immune cells, stem cells and metabolites which can migrate to other areas of the body and have a beneficial effect in unrelated areas, such as the brain.

1.2.3 Effect of photobiomodulation on healthy cells

In addition to the direct and indirect effects of light being neuroprotective, that is improving the function and survival of distressed or damaged cells, light has also been reported to influence “normal” or “healthy” cells. Such influences may not necessarily be “neuroprotective” but rather, “modulatory” activating neural circuits and altering brain function.

These effects occur when PBM reaches and stimulates particular regions of the brain that it is being applied to. For example, it has been reported that PBM applied across the central regions of the skull reduces the firing of neurones in the motor cortex of normal healthy humans (Konstantinovic et. al., 2013). This effect may lead to changes in the motor neural circuitry through the basal ganglia and to the spinal cord and could lead to improvements in movement via direct and indirect motor pathways, which can be demonstrated in more skilled movement and improved stamina. This feature may generate, at least in part, some of the improvements in motor function seen in experimental animals (Reinhart et. al., 2016) and human patients (Hamilton et. al., 2018; Liebert et. al., 2021). Similarly, PBM applied in the frontal skull region stimulates the prefrontal cortex, which may trigger improvements in social interaction, mood and depression (Schiffer et. al., 2009; Cassano et. al., 2016, 2018, 2022) together with cognitive and emotional functions (Barret and Gonzalez-Lima, 2013; Gonzalez-Lima and Barret, 2014; Blanco et. al., 2015; Vargas et. al., 2017). Further, light has also been shown to substantially alter the activity of dopaminergic neurones in the SNc of normal mice (Romeo et. al., 2017). Many of the details of these findings will be considered further in a subsequent section dealing with PBM in human subjects.

In summary, the PBM-induced neuroprotective mechanisms on damaged or distressed neurones have been suggested to be either direct or indirect. Direct effects occur as a result of light hitting the target neurone “flush” and producing an immediate primary effect and a longer-term secondary effect after light exposure. Indirect effects occur when light does not make contact with the neurone in question, however the benefits reach the target cell through an intermediate system, for example the circulation, either with the immune, stem or mitochondrial signalling cell systems. In addition to these neuroprotective effects, light impacts on the activity of normal, healthy neurones: for example, stimulating motor, cognitive and emotional functions using both experimental animals and human subjects. The effect of light on healthy cells is of particular interest in this thesis, because many of the human subjects used were considered normal, healthy individuals.

1.3 Photobiomodulation in animal models

There have been a number of studies that have reported neuroprotective and behavioural improvements after PBM treatment on animal models of strokes (Zhang et. al., 2000; Leung et. al., 2002; Streeter et. al., 2004; Lapchak et. al., 2004, 2007; Oron et. al., 2006; Peplow, 2015; Meyer et. al., 2016), wound healing particularly in mouse and rat models of diabetes (Byrnes et. al., 2004; Maiya et. al., 2005, 2009; Carvolho et. al., 2006, 2010; Whelan et. al., 2003; Moradi et. al., 2020; Oyebode et. al., 2021), of oxidative stress in hepatocytes of diabetic mice (Lim et. al., 2009), diabetes-induced retinopathy (Tang et. al., 2013; Saliba et. al., 2015), leakage of retinal capillaries in diabetic models (Cheng et. al., 2018), traumatic brain injury (Oron et. al., 2007; Ando et. al., 2011; Wu et. al., 2012), pain pathways (Chow and Armatti 2016), depression (Wu et. al., 2012; Salehpour et. al., 2016), retinal disease (Eells et. al., 2004; Rutar et. al., 2012; Begum et. al., 2013), Alzheimer's disease (Michalikova et. al., 2008; De Taboada et. al., 2011; Grillo et. al., 2013; Purushothuman et. al., 2014, 2015; Saltmarche et. al., 2017), Parkinson's disease (Shaw et. al., 2010; Peoples et. al., 2012; Moro et. al., 2013, 2014; 2016; Reinhart et. al., 2014, 2015, 2016, 2017; El Massri et. al., 2016a, 2016b, 2017, 2018; Darlot et. al., 2016; San Miguel et. al., 2019), amyotrophic lateral sclerosis (ALS) (Moges et. al., 2009), multiple sclerosis (Muili et. al., 2012), spinal injury (Paula et. al., 2014; Song et. al., 2017; Wang et. al., 2021b, Ma et. al., 2022), even the improvement of microbiome health and diversity in mice models (Bicknell et. al., 2018). Moreover, PBM was observed to stimulate neurogenesis in studies using Alzheimer's animal models (Wu et. al., 2021).

This wide range of effect indicates that PBM holds a healing capacity for many types of pathologies, not restricted to a particular type of cell, but rather, the multi-cellular environments located in multiple physiological systems. However, the current thesis focuses mostly on neurological systems, both healthy and neurodegenerative. Therefore, the effect of PBM on particular animal models will be explored in more detail, including Alzheimer's disease, Parkinson's disease, and some healthy aged animal models, testing cognitive function.

1.3.1 Alzheimer's disease models

Most studies have used transgenic animal models of Alzheimer's to demonstrate the beneficial impact of light therapy in both short-term (weeks) and long-term (months) studies (Johnstone et. al., 2016), in particular with the amyloid precursor protein/presenilin 1 (APP/PS1) transgenic model (De Taboada et. al., 2011; Purushothuman et. al., 2014, 2015), the double transgenic TASTPM model (Grillo et. al., 2013), the CD1 model (Michalikova et. al., 2008), and the tau K369I transgenic model (Purushothuman et. al.,

2014, 2015). In addition, a recent study has measured the effect of PBM which also integrated magnetic emissions (named RGn500) on mice injected with oligomeric amyloid β peptide 25–35 (Blivet et. al., 2018). Using these animal models, a PBM-induced reduction of β-amyloid plaques and hyperphosphorylated tau as a main measure of the improvement within Alzheimer's disease was reported. Other indicators of improvement were noted also, for example reductions in inflammation and oxidative stress, and an increase in ATP and overall improvement in mitochondrial function with both short-term (weeks; Michalikova et. al., 2008; Blivet et. al., 2018), and long-term (months; De Taboada et. al., 2011; Grillo et. al., 2013; Purushothuman et. al., 2014, 2015) light treatment. Some of these studies also measured improvements in behavioural performance after PBM treatment, reporting a reduction in cognitive deficits associated in the CD1 transgenic mouse model (Michalikova et. al., 2008), the APP/PS1 transgenic mouse model (De Taboada et. al., 2011), and the amyloid β peptide 25–35-injected mice (Blivet et. al., 2018). An additional APP/PS1 mouse study demonstrated that PBM was able to reverse Aβ-obstructed interstitial fluid alongside improvement in memory task performance (Yue et. al., 2019).

1.3.2 Parkinson's disease models

A number of animal models of Parkinson's disease have been used to demonstrate the neuroprotective effect of PBM therapy (Hamblin, 2016; Johnstone et. al., 2016; Mitrofanis, 2017). In MPTP-treated mice (Shaw et. al., 2010; Peoples et. al., 2012; Moro et. al., 2013; Johnstone et. al., 2014; Reinhart et. al., 2014, 2015; El Massri et. al., 2016a, 2016b, 2017, 2018; Ganeshan et. al., 2019) and monkeys (Darlot et. al., 2016; El Massri et. al., 2016a, 2016b), 6-OHDA-lesioned rats (Reinhart et. al., 2016), α-synuclein AAV virus-treated rats (Ouselati et. al., 2015), and the transgenic mouse model of K369I (Purushothuman et. al., 2013), PBM treatment resulted in more surviving dopaminergic neurones in the SNc than in the animals that were not treated. Further, in the MPTP-treated mice (El Massri et. al., 2016b, 2017) and monkeys (El Massri et. al., 2016a, 2018) there are reports of a reduction in gliosis, and in MPTP-treated monkeys, an increase in growth factor expression (GDNF) and dopaminergic terminations in the striatum (El Massri et. al., 2017). The greater number of surviving dopaminergic neurones was evident regardless of whether the PBM was applied before, at the same time, or after the MPTP treatment, showing neuroprotective effects of light (Peoples et. al., 2012; Reinhart et. al., 2015). There were further investigations demonstrating that PBM has the capacity to pre-condition the brain by activating neuroprotective transcriptomes that mitigate MPTP insult (Ganeshan et. al., 2019). In transgenic-disease models, far infrared light exposed mice showed significantly increased levels of nerve growth factor and brain derived neurotrophic factor protein in the cerebrum, cerebellum and hippocampus

(Fukui et. al., 2021). Furthermore, an investigation by San Miguel and colleagues (2019) used MPTP injections on mice to demonstrate cerebrovascular compromise that was significantly mitigated by PBM treatment. They used fluorescent-labelled albumin to track the level of vascular leakage in the SNc and the caudate-putamen complex (CPu). Firstly, this investigation was the first of its kind to confirm that MPTP induces vascular leakage, and secondly it demonstrated the capacity of PBM in reducing the leakage, which was evident with significantly lower levels of fluorescent-labelled albumin within the SNc and CPu (San Miguel et. al., 2019).

In addition to above mentioned anatomical features of neuroprotection, Shaw and colleagues (2012) have reported that PBM attenuated the abnormal parkinsonian neural activity in some basal ganglia nuclei such as the SNc and subthalamic nucleus (STN), and a structure associated with the basal ganglia, the zona incerta (ZI) (Lanciego et. al., 2012). This measure was obtained by discovering a lowered Fos expressions in neurones located in the above-mentioned regions. This improvement did not quite reach control levels, indicating partial restoration, and has been suggested to be due to the neuroprotection of the dopaminergic neurones in the SNc (Shaw et. al., 2012).

There have also been studies that have reported clear PBM-induced improvements in locomotive behaviour in MPTP-treated mice (Whelan et. al., 2008; Moro et. al., 2013; Reinhart et. al., 2015), and monkeys (Darlot et. al., 2016), and a reduction in clinical signs in MPTP-treated monkeys (Darlot et. al., 2016). In the MPTP-treated monkeys, such improvements remained up to three weeks post PBM therapy which suggests that the therapeutic effects of PBM therapy are long lasting, beyond the primary effects observed while the light is being applied (Darlot et. al., 2016; Moro et. al., 2016). Finally, in 6-OHDA lesioned rats, there is evidence for a reduction in apomorphine-induced rotational behaviour after PBM treatment (Reinhart et. al., 2016).

Transcranial applications of PBM are thought to be sufficient for reaching the striatum and the SNc in the midbrain which is near enough the cranial surface in rodents (mouse 2-3mm, rats 4-5mm), as PBM has the ability to penetrate 20-30mm of tissue; in monkeys it can reach the striatum (15-20mm) but not the SNc (40-50mm). Similarly, in the human brain the striatum is 50-60mm away from the cranial surface, and the midbrain containing the dopaminergic cells of the SNc 80-100mm below the cranium indicating that transcranial PBM will probably not reach either structure directly in humans. For this reason, an intracranial application of PBM has been developed, with the use of an optical fibre device connected to a laser (Darlot et. al., 2016; El Massri et. al., 2017). Of the already mentioned studies,

several utilised an intracranial method of application on animal models of Parkinson's disease; these studies have reported improvement in dopaminergic cell survival (Moro et. al., 2014; Darlot et. al., 2016), improved behaviour (Reinhart et. al., 2015) and a reduction in clinical signs (Darlot et. al., 2016), in both rodents and monkeys. These studies generally found that the intracranial device was stable in freely moving animals, without toxic side effects, showing promising progress for possible use in humans (Moro et. al., 2014; Darlot et. al., 2016).

1.3.4 Cognitive effects on healthy animals

Photobiomodulation is known to produce effects on healthy aged animals, and some studies have demonstrated that effect in cognitive performance (Gonzalez-Lima and Barret, 2014; Hamblin, 2016). Middle-aged (12 months) and otherwise healthy CD1 female mice were treated with 1072nm light and consequently they showed improved performance in a 3D maze in comparison to sham-treated age-matched controls (Michalikova et. al., 2008). In another study on rats using PBM therapy, measured an increase in oxygen consumption within the prefrontal cortex, accompanied by enhanced extinction memory and prevention of re-emergence of extinguished conditioned fear responses when compared to controls (Rojas et. al., 2012). Young and aged rats were exposed to near infrared light for 58 consecutive days, and results showed that metabolic pathways for neurotransmission were significantly enhanced, while in aged rats, altered metabolic pathways were restored and closer to resembling those of the young rats (dos Santos Cardoso et. al., 2021). These results are indicative of the potential of PBM to have a clear beneficial effect on natural ageing. These results, albeit on animal models, are most encouraging for future use on humans. A caveat to acknowledge is that the ageing brain is a "grey area" when considering healthy vs. diseased categories. Although an ageing brain is "normal" it is still a deviation from "healthy" circuitry, however it is not necessarily "diseased" either.

2. How does PBM affect the healthy human brain?

2.1 fMRI and EEG: what do they tell us about the human brain

Over time, brain imaging has evolved to allow researchers to understand more about the brain's activities, with devices such as magnetic resonance imaging (MRI) machines, and electroencephalogram (EEG) devices. Among the MRI approaches, functional magnetic resonance

(fMRI) offers excellent three-dimensional spatial resolution to visually represent the brain's neuroanatomy, as well as localised brain activity through analysis of blood oxygen level dependent (BOLD) contrast (Voos et. al., 2013). BOLD relies on the concept that neurones do not contain their own reserves of glucose and oxygen, therefore, active neurones demand a delivery of these resources via the bloodstream. This results in a measurable ratio change of oxyhaemoglobin and deoxyhaemoglobin, creating a localised representation of neuronal activity, with a 2-3mm voxel using 3T MRI (Voos et. al., 2013; Zaca et. al., 2014). Arterial spin labelling (ASL) is another MRI approach which labels the protons within arterial water in the brain and tracks their spin. This process provides an absolute measurement of cerebral blood flow and change in its flow, with a high temporal and spatial resolution. A particularly useful derivative of ASL is pseudo-continuous arterial spin labelling (pCASL) which maximises the efficiency and combability of ASL (Borogovac and Asllani, 2011). However, it is important to recognise that blood oxygenation and flow, measured in fMRI and pCASL respectively, does not provide a direct measure of brain activity (Siexas and Lima, 2011).

EEG isanother form of brain imaging whereby electrodes are placed on different surfaces on the scalp to represent different lobes and lobe sections of the brain. The electrical potential is measured by these electrodes in response to naturally generated electrical brain signals. The frequencies that are processed are visually represented in waveforms, and particular ranges of frequency are associated with different types of activity. EEG is considered to have excellent temporal resolution as it almost instantaneously picks up brain wave activity, however, the spatial resolution is limited (Burle et. al., 2015). Collecting EEG readings of the brain offers information on the neural oscillations during different types of tasks and in different types of resting states, which will be explained below. EEG is a widely used method of brain imaging because of its financial accessibility and its universal applicability for measuring brain activity. Generally speaking, fMRI has excellent spatial resolution with limited temporal resolution, whilst EEG has opposite resolution, providing excellent temporal resolution but limited spatial resolution. Therefore, it is understandable that researchers have examined EEG and fMRI integration. This type of integration gives more spatial and temporal resolution as well as a more comprehensive measurement of brain activity, in both healthy and pathological brain environments (for example, epilepsy, and neurodegenerative diseases), during resting state and active states. However, as it currently stands, not all MRI functions are compatible with EEG functions and not all EEG functions are useable within an MRI machine (Laufs, 2012). Before we analyse fMRI scans and EEGs of the brain in response to PBM, we need to understand the six main large-scale networks of the brain, which are collections of brain regions that have functional connectivity, in other words presenting high levels of interaction based on fMRI BOLD signals (Bellec et. al., 2006). These large-scale networks seem to exist

universally amongst humans. There's the "salience network" which presents as connectivity between the midcingulo-insular regions, the "attention network" with dorsal frontoparietal connectivity, the "control network" showing connectivity between the lateral frontoparietal regions, the "sensorimotor/somatomotor network" presenting as connectivity amongst the pericentral region, and the "visual network" with connectivity between the occipital lobe regions. Finally, there's the Default Mode Network (DMN), which was first discovered during fMRI scans of the brain during resting state (Raichle, 2015). The DMN is essentially a map of the brain in which activity is high at certain locations of the brain, representative of functions that never stop, they are either enhanced or attenuated in response to a shift in task. There are three main sections of the DMN, the ventral medial prefrontal cortex, considered to be responsible for emotional processing; the dorsal medial prefrontal cortex, often associated with self-referential mental activity; and the posterior cingulate cortex, known to drive the recollection of previous experiences.

For EEG in particular, it's important to understand the baseline of brain waves and the context in which they are typically found. For example, delta waves (0.1-4 HZ) are associated with the grey matter activity of the brain and often recorded during sleep stages 3 and 4. Theta waves (4-8 Hz) are related to subconscious activity and meditation, not often measured in the typical adult brain. Alpha waves (8-13 Hz) often represent white matter brain activity and are usually recorded while subjects are awake and relaxed with closed eyes. Beta waves (12-30 Hz) are usually seen while individuals are in conscious states, partaking in active behaviour, whilst gamma waves (30-100 Hz) are associated with hyper alertness and while sensory inputs are being deeply integrated (Kumar and Bhuvaneswari., 2012).

Therefore, the DMN for the human brain in its resting state, and the brain waveforms according to associated activities are one way to assess the effect of the healthy human brain in response to PBM, using the neuroimaging tools fMRI and EEG.

2.2 Current data in the literature – do we know anything about the effect of PBM on healthy human brains?

Notwithstanding investigations of the effect of PBM on pathological systems, whether it be animal models or patients with neurodegenerative disorders, the explorations of the effect of PBM on healthy humans also has its merit. Having a better understanding for how PBM affects the healthy brain will allow us to make more accurate comparisons to the effect on diseased brains (Moro et. al., 2022). This in turn lays a foundation for future clinical trials for PBM as a treatment approach for example, in

neurodegenerative disease. In essence, studying the effect of PBM on healthy human brains with neuroimaging sets a template for normal function, that later studies on disease can build on; further, if PBM improves the function of the brain than it could be viewed as a preventative treatment, because many previous studies have shown that an increase in cognitive brain function during middle-age can reduce the likelihood of developing neurodegenerative disease later in life (Gonzalez-Lima and Barret, 2014; Hamblin, 2016; Michalikova et. al., 2008; Rojas et. al., 2012). Many studies have reported that PBM can affect the human brain in both young and older individuals, and beneficial effects on high level cognitive functions have been reported by many research teams (Barrett and Gonzalez-Lima, 2013; Gonzalez-Lima and Barrett, 2014; Blanco et. al., 2017a,b; Grover et. al., 2017; Jahan et. al., 2019). Studies have also shown that PBM is likely to stimulate neuroplasticity in a similar manner to non-invasive brain stimulation such as repetitive transcranial magnetic stimulation (rTMS), and transcranial direct current stimulation (tDCS). This benefit has shown promise for neurological conditions like drug addiction (Mahoney et. al., 2021), and post-traumatic stress disorder (PTSD) (Florian et. al., 2023). Furthermore, a recent study has reported that functional near infrared spectroscopy (fNIRS) devices often used for non-invasive optical imaging were noted to induce cognitive enhancement effects (Waight et. al., 2023). Therefore, contrary to previous assertions, fNRIS devices may in fact change brain activity and hence interfere with the effect of PBM since they behave as PBM devices by emitting near infrared light.

Several studies have revealed emotional and cognitive improvement from PBM treatment including memory retrieval tasks and more positive emotional states in PBM-treated individuals compared to the placebo control group (Barret and Gonzalez-Lima, 2013; Gonzalez-Lima and Barret, 2014). The DMN was also shown to be affected by PBM treatment, accompanied with an improvement in cognitive tasks in two retired football players, albeit they technically suffered from chronic traumatic encephalopathy due to the high contact sport (Naeser et. al., 2019). Executive functions tested via the Wisconsin card sorting task (Blanco et. al., 2017a) and rule-based category learning (Blanco et. al., 2017b) presented fewer errors in the PBM-treated groups. Further, fMRI studies combined with cellular analyses demonstrated increases in cytochrome oxidase C oxidation with elevated haemoglobin oxygenation in response to PBM treatment (Wang et. al., 2017; Saucedo et. al., 2021). Vargas and colleagues (2017) recruited older adults for PBM treatment and conducted both EEG and fMRI analysis while participants engaged in psychomotor vigilance tasks and verbal memory tasks; they found PBM-induced improvements in reaction times and correct responses were statistically significant in comparison to placebo control groups. Together with the improved task performances in these cases, was a reduction in BOLD-fMRI activity in areas of the DMN, such as the posterior cingulate cortex, medial prefrontal cortex, and the inferior parietal lobe (Fig 1.2.2). BOLD fMRI receives its signal based on increased

cerebral blood flow, the reduction of oxygen consumption and cerebral blood volume. Since PBM increases oxygen consumption and haemoglobin oxygenation (see above), a reduced BOLD fMRI signal (Rojas et. al., 2012; Tian et. al., 2016, Wang et. al., 2017) is not so surprising. Contrastingly, there were also instances where PBM was observed to increase connectivity between the DMN structures such as the posterior cingulate cortex and lateral parietal nodes in resting Alzheimer's patients, accompanied with improved performance in the Alzheimer's Disease Assessment Scale-cognitive subscale (ADAS-cog). These results indicate an effect on the DMN in response to PBM, but more thorough exploration on the effect of PBM for neurodegenerative diseases such as Alzheimer's will be addressed in the next section. The EEG results also indicated an increase in alpha, beta and delta amplitudes in response to PBM (Fig 1.2.2.), that were present during the reaction time tasks and even 10 minutes after the tasks. There are a number of other investigations that show similar EEG results, whereby PBM increased alpha, beta and delta waveform amplitudes in accompaniment with improved reaction times and attention performance (Grover et. al., 2017; Jahan et. al., 2019; Wang et. al., 2019, 2021; Zommordi et. al., 2019; Shan et. al., 2021). At the time of candidature commencement (2017) there were few fMRI studies investigating the effect of PBM on resting and motor-tasking brains in healthy individuals. The aim of chapters two and three is to address in fMRI studies of the effect of PBM on resting and motor-active healthy brains.

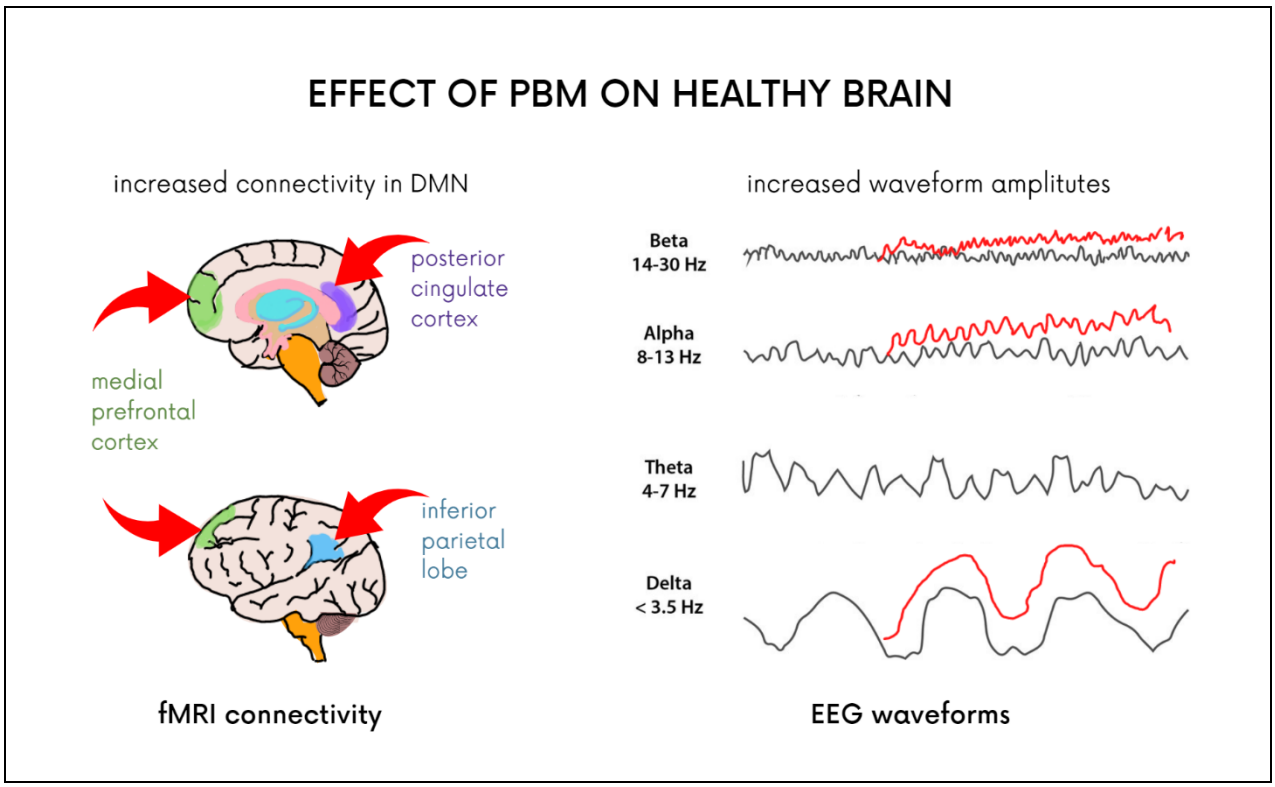

Fig. 1.2.2. Diagram offers a visual representation of the effect of PBM on healthy human brains, according to fMRI, PBM has shown an increase in connectivity between structures of the DMN, such as the posterior cingulate cortex, the medial prefrontal cortex and the inferior parietal lobe (Vargas et. al., 2017). According to EEG readings, PBM resulted in an increase in amplitudes for alpha (8-13Hz), beta (14-30Hz) and delta (<3.5Hz) waveforms, depicted with red lines (Grover et. al., 2017; Jahan et. al., 2019; Wang et. al., 2019, 2021; Zommordi et. al., 2019; Shan et. al., 2021).

3. Photobiomodulation and Parkinson's disease

3.1 Parkinson's disease as we know it

Parkinson's disease is the second most common neurodegenerative diseases, with Alzheimer's disease being the first, and its incidence is steadily growing with the ageing population (Canonico et. al., 2022). Together with the development of distinct motor signs - for example, resting tremor bradykinesia and lead-pipe rigidity - associated with primarily with the degeneration of midbrain dopaminergic cells, many patients develop dementia and loss of cognitive function as the disease progresses. Cross sectional studies showed that dementia occurs in 40% of Parkinson's disease cases, however, longitudinal studies estimate that dementia is present in 75 – 90% of patients (Kehagia et. al., 2010). Within the clinical setting, Parkinson's disease can be assessed with different types of tests, including but not limited to observing a patient's gait, using the timed up and go (TUG) test which is a test based on simple motor tasks, and graphology. The scores on these tests help patients and medical professionals assess the severity of the condition, as well as any potential improvements seen in response to treatment.

Traditional treatment for Parkinson's disease has revolved around managing the motor signs, and sometimes the behavioural components (Antonini and Cilia, 2009). This includes dopaminergic drug therapy, deep brain stimulation, some antidepressants, mood stabilisers and atypical antipsychotics. However, the problem with the pharmacological treatments is that they often have adverse side effects, such as nausea, headache, somnolence, dizziness, agitation, impulsive behaviour and confusion (Engmann, 2011; Ramic et. al., 2020; Gandhi and Saadabadi, 2021). Over time, patients treated with dopaminergic drug therapy eventually develop motor complications, including involuntary movements (i.e., dyskinesias) and hallucinations (Watts et. al., 2004). The dopamine overdose hypothesis has also been introduced (Kish et.al., 1988; Swainson et. al., 2000) whereby a seesaw effect is witnessed in patients on drug therapy (e.g. levodopa) who are reported to have poor probabilistic reversal learning and improvement in their set switching performance. However, when the patients come off of levodopa, there are reports of the opposite effect happening: improved probabilistic reversal learning and worsened set-switching (Vaillancourt et. al., 2013). This trade-off effect indicates that that patients

on levodopa are experiencing challenging short term and long-term side effects, hence the interest in the non-invasive approach of PBM that's known to have very little, if any, side effects.

3.2 Neuroimagining for Parkinson's disease

Parkinson's disease can be visualised and analysed in many different ways with different neuroimaging technologies. Neuroimaging for Parkinson's disease can be approached from an anatomical perspective using structural methods of MRI, such as conventional T1 and T2 weighted imaging, neuromelatonin imaging, voxel-based morphometry, diffusion tensor imaging, magnetic resonance spectroscopy, functional MRI methods, such as BOLD fMRI, pCASL. EEG and positron emission tomography (PET) have also been used to measure baseline activity of Parkinson's disease (Schuff et. al., 2009; Lehericy et. al., 2012; Kahan et. al., 2014). PET imaging in particular is widely used to assess the condition of dopaminergic systems through the use of PET radioligands that target dopamine terminals and dopaminergic receptors.This offers a measurement of both pre- and post- synaptic functions is Parkinson's patients (Gopalakrishnan and Stoessl, 2011; Niccolini et. al., 2014). However, of all these methods, the most appropriate and commonly used have been MRI and EEG methods due to being safer, less invasive and more accessible (Kahan et. al., 2014). Parkinson's disease has been analysed and visualised through the use of MRI with focus on the substantia nigra (Lehericy et. al., 2012), MRI T1 quantitative imaging of the hippocampus (Agosta et. al., 2013), EEG pattern changes in participants with early onset Parkinson's disease (He et. al., 2017) and Parkinson's disease (Geraedts et. al., 2018; Gong et. al., 2021), voxel-based morphometry (Agosta et. al., 2013; Fioravanti et. al., 2015)**.**

In an MRI study that utilised quantitative T1 imaging, a comparison was made on the grey matter tissue of patients with Parkinson's disease and Parkinson's disease with dementia (Haris et. al., 2011). Quantitative T1 imaging (T1p) is a method which measures the amount of time it takes for water molecules to "relax" after magnetisation, the macromolecular composition of the water alters the T1p relaxation time. Macromolecular changes are present in diseased tissue, for example in plaques and tangles (Agosta et. al., 2013). In the study by Haris and colleagues (2011), T1q levels were measured in the hippocampus of Parkinson's patients with normal cognitive function, Parkinson's patients with dementia, and healthy controls. T1p was significantly higher in the Parkinson's patients with dementia compared to healthy controls, whilst the Parkinson's patients without dementia symptoms had significantly lower T1p values compared to healthy controls.

Voxel-based morphometry studies have been used to analyse grey matter atrophy in Parkinson's patients (Beyer et. al., 2007; Lee et. al., 2010; Melzer et. al., 2012; Fioravanti et. al., 2015). Grey matter atrophy is well established in Lewy body dementia (Beyer et. al., 2007; Melzer et. al., 2012). Fioravanti and colleagues (2015) studied grey matter atrophy in Parkinson's disease patients as the disease progressed over 2 years, initially noting reduced grey matter in the right putamen and parietal cortex, and then after 2 years additional atrophy in the putamen and parietal cortex within the same patients, as well as the pedunculopontine nucleus and the SNc. However, Parkinson's disease patients can also have early onset mild cognitive impairment, or late onset mild cognitive impairment. Grey matter atrophy of the orbitofrontal and parietal cortices was significantly more severe in Parkinson's patients with early mild cognitive impairment, compared to Parkinson's with late mild cognitive decline (Lee et. al., 2012). Patients with early cognitive impairment presented with more severe grey matter atrophy in the superior temporal areas, but patients with late cognitive impairment presented more severe grey matter atrophy of the anterior and cingulate cortex (Lee et. al., 2010).

The BOLD fMRI resting state connectivity of unmedicated Parkinson's patients offers useful information for clinical diagnosis and understanding brain disorders (Kahan et. al., 2014), and together with the resting state connectivity of the healthy human brain offer a powerful set of foundations to compare the effect of PBM on the brain and its restorative impact on resting state connectivity. Baseline BOLD fMRI studies for treatment naïve Parkinson's patients showed a reduced connectivity at the premotor cortex and putamen (Wu et. al., 2009; Esposito et. al., 2013), reduced striato-thalamic connectivity (Hacker et. al., 2012), increased connectivity between the motor cortex and cerebellum (Wu et. al., 2009) as well as increase in the motor cortex and subthalamic nucleus (Baudrexel et. al., 2011). The reduced connectivity between the premotor cortex and putamen, along with the connectivity between striatum and thalamus are an indicator of the nigrostriatal dopaminergic connections that are being compromised in PD. Meanwhile, the increased connectivity directly from the motor cortex to the cerebellum and the subthalamic nucleus are an indication of an established compensation for the reduced connectivities (Fig 1.3.2.). For a long time, the putamen (which makes up the striatum along with the caudate nucleus) has been known to work with these other basal ganglia structures to influence a variety of motor behaviours, such as planning, learning and execution (Delong et. al., 1984a; Alexander and Crutcher, 1990) and movement sequences (Marchand et. al., 2007). All these processes are affected in Parkinson's disease as the putamen's innervation to the globus pallidus interna (GPi) in the direct motor pathway pathologically reduces due to lack of dopamine, while its innervation to the globus pallidus externa (GPe) in the indirect pathway increases. through disinhibition. In physiologically normal

conditions the effects are opposite due to sufficient dopamine levels balancing the direct and indirect pathways (Delong et. al., 2007).

EEG patterns are also affected in patients with Parkinson's disease with a general consensus for slower waves in comparison to healthy participants (He et. al., 2017; Geraedts et. al., 2018; Gong et. al. 2021). Furthermore, since patients with Parkinson's disease can have either normal cognitive function, or cognitive impairment with dementia, EEG patterns show that EEG brain wave patterns are slowed down in Parkinson's disease patients with normal cognitive function, however brain waveforms are even slower in Parkinson's disease patients with dementia (Geraedts et. al., 2018; Gong et. al., 2021). A study by He and colleagues (2017) investigated the waveform patterns of the brain of patients with early onset Parkinson's disease, without dementia, and found more "diffuse slow waves" in 44% in comparison to the healthy brain control group. Additionally, almost 29% of these patients showed a lower frequency of beta background waves in the bilateral occipital, bilateral posterior temporal, bilateral parietal and left central regions, down to 7 - 9 Hz, while normal resting beta brain waves in healthy brains are 12 – 30 Hz (Kumar and Bhuvaneswari, 2012).

3.3 Neuroprotective / neuro-regenerative effects of PBM on Parkinson's disease

Numerous studies on animal models of Parkinson's disease have shown time and time again the beneficial effect of PBM, with protection to the dopaminergic cells and restoration of motor and cognitive behaviour (see section 1 Parkinson's disease animal models). Although these animal models are helpful, they do not replicate the human condition in terms of exact pathologies and timescale (Torres et. al., 2017; Hamilton et. al., 2018a). Nevertheless, many different types of animal models have been used - for example, toxin-induced and transgenic in a range of different experimental animals, from fly to monkeys - and each of these contribute to a better understanding of the idiopathic forms of the disease in humans (e.g. Mitrofanis, 2019).

In vitro studies on human neuroblastoma cells that overexpressed α-synuclein and exposed to MPP+, demonstrate PBM treatment increases mitochondrial function and reduced oxidative stress (Trimmer

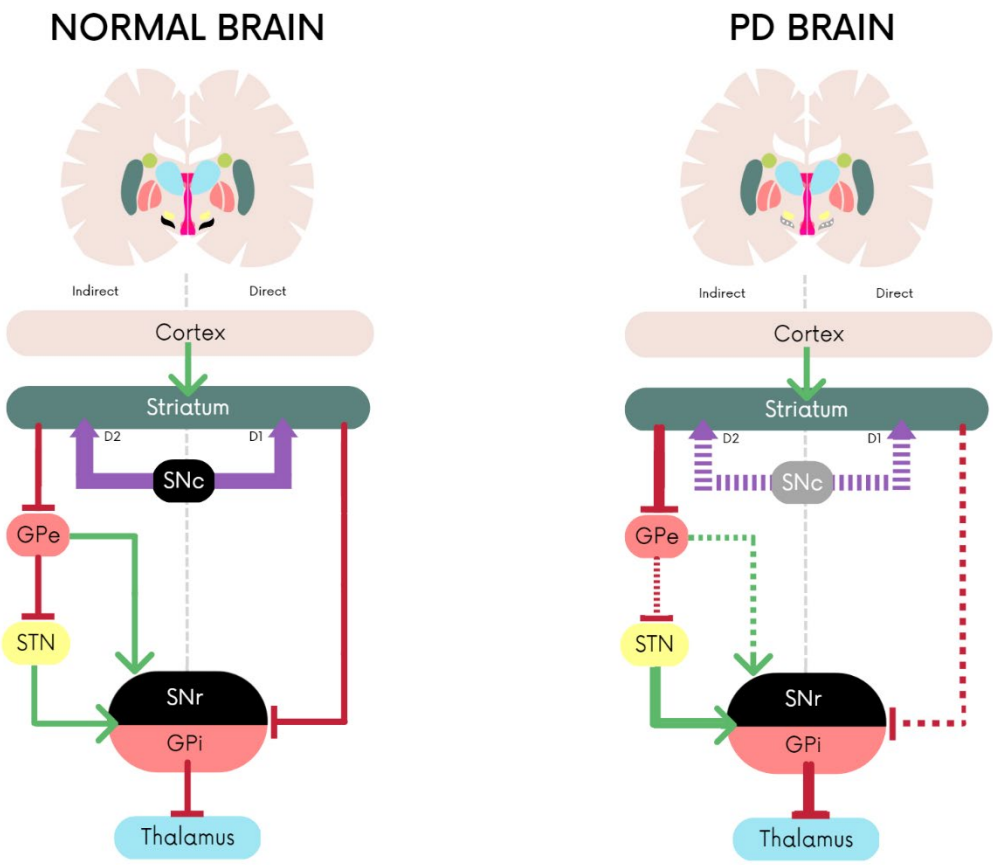

Fig 1.3.2. A schematic diagram to compare the direct and indirect motor pathways in a normal brain versus a brain with Parkinson's disease (PD). The diagram shows the effect that dopaminergic cell death has on the innervation pathways of the direct and indirect motor pathways, hence explaining the unrefined and disrupted motor behaviour. (Adapted from the diagram in Pretegiani and Optican, 2017).

et. al., 2009; Quirk et. al., 2012). In a study by Trimmer and colleagues (2009), PBM treatments were also conducted on hybrid cells that contained mitochondrial DNA from Parkinson's patients. In response

to PBM the pattern of mitochondrial movement along axons was almost restored to healthy control levels. These results suggest that PBM can be effective on human cells considering the above-mentioned results came from studies that used human neuroblastomas and hybrid cells with DNA from Parkinsonian patients.

A number of clinical case reports and one small scale placebo-controlled trial have tested the effects of transcranial PBM, applied with handheld lasers, light emitting devices or helmets lined with LED strips for head coverage, in Parkinson's disease patients. These reports have documented changes in the signs and symptoms of the disease after and during PBM treatment on a number of patients (Hamilton et. al., 2018a; Santos et. al., 2019; Liebert et. al., 2021). A 9-week treatment protocol showed statistically significant improvement in the gait of PBM treated Parkinson's disease patients in comparison to those treated with placebo sham, moderate improvements in the TUG test were non-significant, albeit marginally ($p = 0.094$) (Santos et. al., 2019). In another study, four patients were observed individually, three of which had Parkinson's disease and one progressive supranuclear palsy (PSP), using a helmet lined with LED strips that shone red to near infrared light (670nm, 820nm, 850nm, 940nm). These patients reported improvements in their day to day lives. This measure was based on tremor, gait, difficulty in swallowing and speech, akinesia, difficulty with facial animation, reduced motor skills, diminished sense of smell, and lower social confidence. Signs and symptoms were often reported by carers and medical practitioners. Of these signs and symptoms, approximately 75% showed improvement, 25% remained the same, and none worsened. These improvements were slow in onset and lasted for the entire duration of the longitudinal study which was between 12 and 14 months long for two of the participants. This indicates that there was not a placebo phenomenon, furthermore, none of the participants developed adverse reactions, even though treatment was long term. All of this information suggests that the benefits of PBM are applicable to clinical settings (Hamilton et.al. 2018b).

Other studies have also shown an improvement in parkinsonian signs in response to PBM. A study with thirty-six Parkinson's disease patients involved a PBM treatment protocol of 30 minutes for ten days with an intranasal device and found improvement in approximately 90% of Parkinsonian signs (Zhao et. al., 2003). Speech, cognition, gait and freezing episodes in a two-week study with eight Parkinson's disease patients with transcranial PBM delivery were also improved (Maloney et. al., 2010**).** A promising clinical trial by Liebert and colleagues (2021) recruited 12 participants with Parkinson's Disease. Half the group immediately started their 12-week PBM treatment protocol, while the remaining half waited for 14 weeks before commencing the same treatment protocol. The treatment protocol in question included PBM administered transcranially, intranasally, and over the neck and abdominal regions. Measurements of the effect of PBM on their cognition, mobility, balance and fine motor skills were taken before PBM treatment commenced, 4 weeks after PBM commencement and at the end of the

12-week treatment protocol. Results averaged across all participants showed a significant improvement in all tested categories, such as mobility tests, dynamic balance tests, cognition tests, fine motor tests, and static balance tests. In fact, most results achieved the Minimal Clinically Important Difference (MCID), revealing effectiveness to PBM as a treatment for Parkinson's disease. Liebert and colleagues conducted another series of studies the following year (2022) to elaborate on the concept of PBM as a promising treatment for PD, the main difference in this study being the remote nature of the treatment and the ability to self-administer PBM during the COVID-19 lockdown seasons. Once again, results similarly indicated significant improvement in various PD clinical signs, such as sense of smell, cognition, mobility, dynamic balance and the spiral test. The 2022 study adds an additional layer to the benefits of PBM, such as accessibility to the treatment type away from the clinic, as well as versatility in the treatment to withstand challenging medical pandemics.

Therefore, based on these case studies,incidental findings and reports, there is an early, but promising indicator that PBM has a beneficial and neuroprotective effects on Parkinson's disease patients, at the time of writing this thesis. As it currently stands, there are few trials with large patient numbers with Parkinson's disease and other neurodegenerative conditions. The trajectory of the research seems to be leading in t direction.However for PBM to become a more established treatment for Parkinson's disease, many more trials are needed over varying lengths of time with varied PBM treatment protocols, and the measurement of clinical signs and symptoms of Parkinson's disease. This will allow PBM to progress as a treatment, and for more clarity on the most effective parameters of the PBM treatment protocol.

4. Photobiomodulation, neuroinflammation and oxidative stress

4.1 Drawing the connection between neurodegeneration, neuroinflammation and oxidative stress.

There is a long-standing literature in the neurodegenerative field that has established a clear relationship between neuroinflammation and neurodegeneration. In fact, a strong connection between the two pathologies has been established in many experimental studies and reviews (Rogers et. al., 1988; Dickson et. al., 1993; McGreer et. al., 1993; McGreer and McGreer, 1998; Chen et. al., 2016; Kempuraj et. al., 2016; Guzman-Martinez et. al., 2019; Kwon et. al., 2020; Muzio et. al., 2021; Hanna et. al., 2022). Initially, neuroinflammatory responses are a natural and helpful response to stressors, such as the introduction of pathogens, or help repair damaged tissue from neurodegeneration. However, when neuroinflammatory responses are unregulated, excessive, or occurring for prolonged periods of time, they create a toxic environment that contributes to further damage and exacerbation of

neurodegeneration (Chen et. al., 2016). Even in the normal ageing brain, neuroinflammation is expected and typical to an extent (Moore et. al., 2010; Hanna et. al., 2022).

Innate and adaptive immune-mediated neuroinflammatory responses are observed in Parkinsonian and Alzheimer's patients. Innate immune cells such as microglia, dendritic cells, or macrophages recognise pathogens, fragments of damaged cells, or cellular stress and present these to the adaptive immune system. A study on a stroke model demonstrated infiltration of these cells through the BBB into the brain (Schulze et. al., 2021). This has also been suggested in other neurodegenerative diseases such as Alzheimer's and Parkinson's due to increased BBB permeability (Desai et. al., 2007; Rezai-Zadeh et. al., 2009; Rosenberg et. al., 2012), resulting in infiltrating T lymphocytes and B lymphocytes which would target tau, amyloid beta and a-synuclein surfaces and molecules. There are mentions of Aβ -specific T lymphocytes which have a high affinity for surface molecules of amyloid beta, which are acquired through engagement of the adaptive immune system (Yang et. al,. 2013). Over time, as the adaptive immune system is engaged whilst the neuroinflammatory response is out of control, more cells are likely to be targeted and killed (Mietelska-Porowska & Wojda, 2017). For this reason, there has been a growing interest in targeting the immune system and inflammatory response to attenuate effects of neurodegeneration. It has been proposed that non-steroidal anti-inflammatory drugs (NSAIDs) could be used to reduce neuroinflammation and therefore lower the severity of neurodegenerative diseases, such as Alzheimer's, Parkinson's, and amyotrophic lateral sclerosis (Moore et. al., 2010; Hanna et. al., 2022). It is also important to note that the substantia nigra has higher concentrations of microglia, making the area quite susceptible to neurone destruction via microglial activation (Wilshusen and Mosley, 2014).

In a neurodegeneration-induced neuroinflammation environment, oxidative stress can play a role in the perpetual nature of excessive neuroinflammation. In such conditions, the production of arachidonic acid perpetuates both neuroinflammation as well oxidative stress. Neuroinflammatory responses (Fig 1.4.1.) occur due to the production of eicosanoids such as thromboxanes, prostaglandins and leukotrines in response to arachidonic acid (Farooqi, 2014). These signalling molecules stimulate the innate immune system. On the other side, oxidative stress increases as a result of arachidonic acid causing cellular reactive oxygen species (ROS) levels to increase (Fig 1.4.1.), which disrupts the normal redox signalling pathway and causes molecular damage (Farooqi, 2014; Zuo et. al., 2019). As a result, the presence of elevated ROS levels creates a vicious cycle, which contributes to neurodegeneration and additional neurodegeneration-induced neuroinflammation (Cheng et. al., 2021). When this dynamic is targeted in an animal model of epilepsy by neutralising ROS with antioxidants, the neuroinflammatory response appears to be attenuated, thus breaking the cycle(McElroy et. al., 2017).

To add context to the relationship between oxidative stress and Parkinson's disease, it is believed that the oxidative synthesis and metabolism of dopamine (Riederer and Wuketich, 1976; Greenamyre and Hastings, 2004) along with low glutathione levels in the SN (Banerjee et. al., 2008) creates an environment with higher levels of oxidative stress, contributing to neuronal death of dopaminergic neurons (Wilshusen and Mosley, 2014).

Based on the cyclical connections between neurodegenerative diseases, neuroinflammatory responses and oxidative stress, it is reasonable to seek different forms of treatments that can break these cycles. PBM is one such treatment that has been proposed to attenuate neuroinflammation and oxidative stress (Hamblin, 2016; Kumar Rajendran et. al., 2019). Although PBM absorption of photons by CcO and other molecules results in greater production of ATP and brief outburst of ROS (Hamblin, 2017; Wong-Riley et. al., 2005), PBM, in the longer term, reduces the levels of reactive oxygen species. Alongside this process, a number of transcription factors within cells are activated, along with anti-inflammatory cellular signalling pathways (see above).

There is also a "knock on effect" observed whereby the effect of PBM on one type of cell can lead to benefits in other cell types. For example, there are several reported instances whereby PBM had direct beneficial impact on neurones, and this resulted in reduction of glial activity and inflammation (El-Massri et. al., 2016; Johnstone et. al., 2014). Further, studies have found a beneficial effect of PBM on the microbiome of Parkinson's Patients, since gut inflammation has been linked to a-synuclein misfoldings in the enteric nervous system (Bicknell et. al., 2022). There are also direct effects of PBM on different types of cells, such as astrocytes, microglia, oligodendrocytes, and other infiltrating immune cells (Yang et. al., 2021; Yang et. al., 2022). The effect of PBM on different cell types will be explored more deeply in the following section. These effects, direct and indirect, all interact in a synergistic way to amplify the positive effect of PBM on the brain environment, such as clearing waste products, encouraging beneficial immune responses, to promote neuroprotection (Hamblin, 2016; Johnstone et. al., 2016; Mitrofanis, 2019; Moro et. al., 2022).

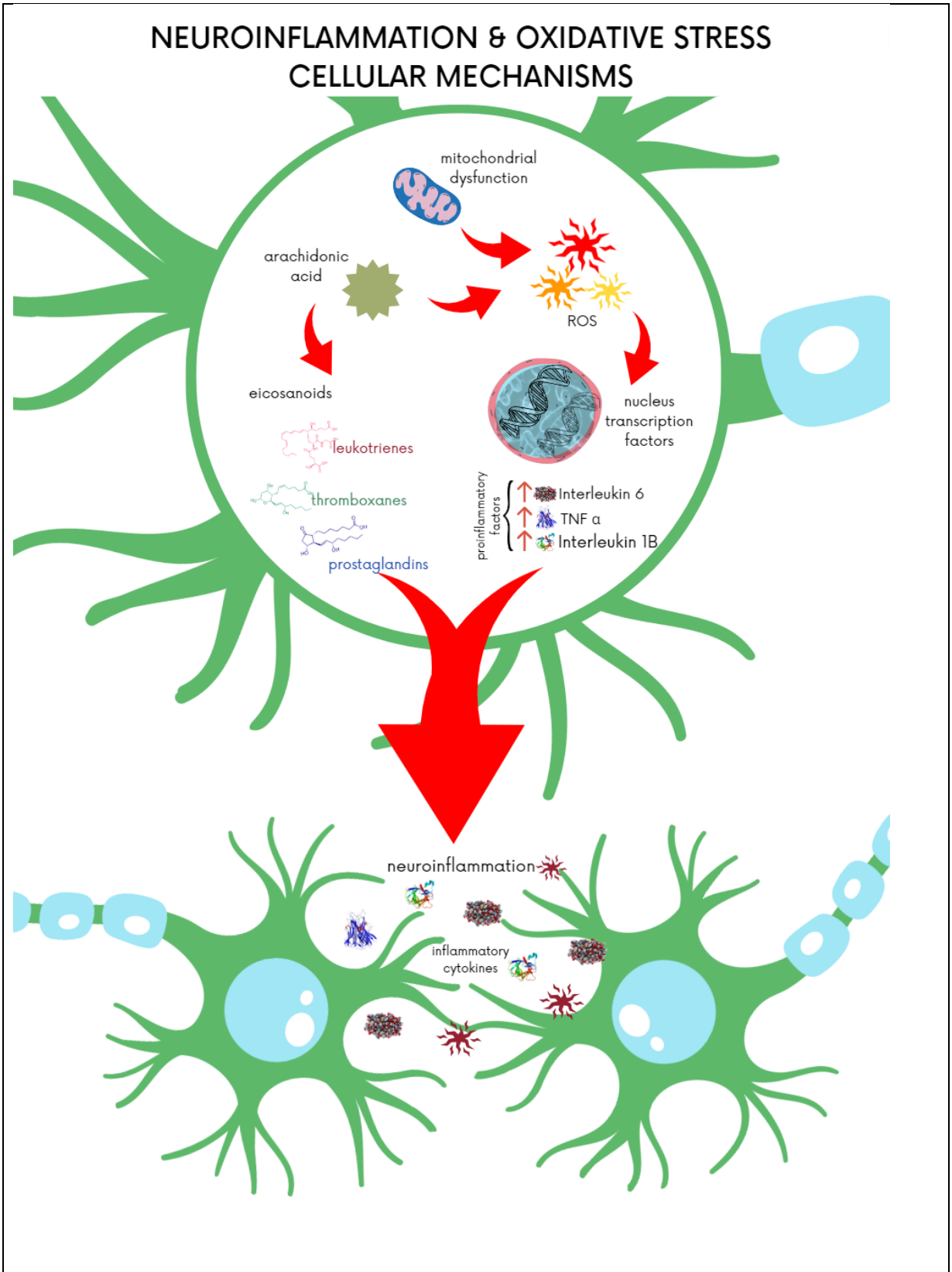

Fig 1.4.1. a simplified schematic diagram of the cellular mechanisms that contribute to both oxidative stress and neuroinflammation. Arachidonic acid contributes to neuroinflammation directly through the production of eicosanoids, and indirectly, through the production of reactive oxygen species (ROS) which stimulates transcription factors for proinflammatory cytokines such as IL-1β, TNF-α, IL-6 (Cheng et. al., 2016). (Diagram adapted from Farooq, 2014)

4.2 Effect of PBM on different cell types within the brain:

Microglia

The microglia would be an ideal target for PBM, considering the potential impact these cells have on creating neurotoxic environments in the brain in neurodegenerative and other disease states (Wang et. al, 2021). Their cellular morphology has an extensive number of protrusions, allowing them to directly contact and influence the function of other cells, such as neurones and other types of glia (Wishusen et. al., 2014). Microglia are derived from myeloid-progenitors and share similar genetic transcriptomes with blood borne monocytes (Ling & Wong, 1993). There are different types of microglia, commonly classified with dichotomous models, such as M1-shifted (inflammatory) and M2-shifted (anti-inflammatory) states, as amoeboid microglia, which are proliferative, migratory, and phagocytic, as opposed to ramified microglia, which are less active (Parakalan et. al., 2012). For example, amoeboid microglia contain monocytic genes Cxcr4, Csk, and Rac1 which have been associated with Parkinson's and Huntington's disease and HIV disease pathways, along with phagocytosis and chemokine signalling pathways (Parakalan et. al., 2012). In contrast, ramified microglia express different monocytic genes, such as Hla-c, Cd74, Cd302, LSp1, and RUnx3, which are responsible for lysosome related functions and antigen presentation (Parakalan et. al., 2012). Amoeboid, neurotoxic microglia express inflammatory markers such as inducible nitric oxide synthase (iNOS), tumour necrosis factor-α (TNF-α), interleukins IL-1β and IL-6 (Pisanu et. al., 2014) which have a destructive effect on the neuronal and glial tissue in the brain. For example, in a Parkinson's disease rodent model, the number of ameboid microglia within the SNc have a high correlation with the degree of cell loss (O'Brien and Austin, 2019). However, this dichotomous way of looking at the states of microglia has now been superseded by classification depending on the area of the brain in which they reside and some other variables (Bachiller et. al., 2018). Microglia are gaining recognition for their multidimensional nature as there are multiple factors that influence their states and types of activities, such as epigenetic, morphologic, proteomic, transcriptomic, and metabolic factors (Paolicelli et. al., 2022). Furthermore, these multiple states contribute to the growing list of microglial functions. There are constructive functions that microglia contribute towards such as myelination, synapse remodelling, tissue repair, vasculogenesis. There are also inflammation-related functions microglia contribute to such as inflammation, BBB permeability, phagocytosis, and surveillance (Paolicelli et. al., 2022). All of these functions are present in healthy brain environments, however become unbalanced in pathological states such as neurodegeneration.

PBM has been shown to have beneficial effects on microglia. An in vitro study by Song and colleagues (2019) has reported a muting effect on the activation of neurotoxic (amoeboid) microglia in a lipopolysaccharide (LPS) cell culture. Where previously the LPS would have initiated strong immune

responses by activating microglia via toll-like receptor activation, PBM prevented thisat a direct cellular level. In the study by O'Brien and Austin (2019), LPS injection in a Parkinson's disease model caused a significant increase in the amoeboid microglial cells, however not in the PBM treated group, indicating an anti-inflammatory effect. In two separate traumatic brain injury models, PBM was shown to also inhibit microglial activation along with improvement in cognitive deficits (Khuman et. al., 2012), and even a reduction in the number of caspase 3- expressing cortical neurons (Esenaliev et. al., 2018). Furthermore, PBM modified the expression of enzymes involved in amyloid peptide production and degradation pathways (Song et. al., 2012; Zhang et. al., 2020; Bathini et. al., 2022). However, microglial activity was not completely inhibited, with phagocytosis of the protein aggregates being significantly increased in PBM treatment groups. The animal studies by Yang and colleagues (2018, 2021, 2022) suggest an explanation as to why microglial activation was not completely inhibited, with results indicating that PBM changed the phenotype of the microglia towards constructive functions and results varied depending on different microglial markers measured.

Astrocytes

Astrocytes have a close relationship with microglia and are activated into neurotoxic states in response to inflammatory cytokines, such as IL-1α, component 1q (C1q) and TNF-α (Fig 1.4.2.) released by the microglia (Liddelow et. al., 2017; Wang et. al., 2021). For this reason, several studies have categorised astrocytes and microglia together to measure neuroinflammatory response. These studies in particular used anti-inflammatory treatments different to PBM, such as bexarotene (He et. al., 2018), Ceria nanoparticles (Zheng et. al., 2021), and microRNAs from neurone-derived exosomes (Jiang et. al., 2020). In a functional context, neurotoxic astrocytes have lost most of their normal supportive functions. In a balanced system, they help to clear toxic agents from the CNS. However, in a context such as neurodegenerative disease or other chronic pathology, where astrocytes are activated continually, this results ultimately in disrupted synaptic function, abnormal synthesis and release of neurotransmitters, and premature death of neurones and oligodendrocytes (Liddelow et. al., 2017; McGeer and McGeer 1998).

PBM has been shown to limit the activation of astrocytes directly and indirectly. PBM directly prevents activation of astrocytes by inhibiting the lcn2 and JAK2/JAK3 cellular signalling pathways (Yoon et. al., 2021), thus preventing the production of transcription factors that directly activate astrocytes. PBM also indirectly prevents activation of astrocytes by inhibiting the very same lcn2 and JAK2/JAK3 cellular signalling pathways, therefore preventing the activation of microglia (Yoon et. al., 2021), preventing microglial secretion of inflammatory cytokines which would have activated the astrocytes.

Furthermore, 660nm PBM was found to promote astrocyte proliferation in a cell culture study (Yoon et. al., 2021).

There are also several studies that did not directly measure the effect of PBM on neuroinflammatory response, however they coincidentally recorded indirect beneficial effects of PBM on astrocytes – such as reduction in astrogliosis (El-Massri et. al., 2016; Johnstone et. al., 2014; Yang et. al., 2021; Yang et. al., 2022).

Oligodendrocytes

For a long time, oligodendrocytes were thought to primarily only play a role in myelination to promote saltatory conduction in a neurone's impulse. However, over the last 30 years or so - oligodendrocytes have gained recognition for their high level of involvement in immunological responses, especially in myelin-degenerative diseases such as multiple sclerosis and experimental autoimmune encephalomyelitis (EAE) (Madsen et. al., 2020; Madeira et. al., 2022).

Due to the connected relationship between astrocytes and oligodendrocytes (Carmen et. al., 2007; Nutma et. al., 2020; Guo et. al., 2021), it is likely that PBM has at the very least an indirect effect on oligodendrocytes by having an effect on astrocytes.

Infiltrating lymphocytes

In a healthy, physiological state, lymphocytes are unable to cross the BBB to infiltrate the nervous system. However, in cases of neurodegeneration or damage such as stroke-induced ischemia, lymphocytes have been observed to infiltrate the central nervous system (Schulze et. al., 2021). For example, T cell lymphocytes can be activated via their T cell receptors to relocate for infiltration. A study using an EAE model by Furlan and colleagues (2001) modelled the infiltration of T lymphocytes into the nervous system, and by exposing their models to excessive amounts of the cytokine, interferon Y, the intruding cytotoxic cells underwent apoptosis. To take this effect further, it was demonstrated that PBM upregulates anti-inflammatory markers, such as IL-10 (Fig 1.4.2.) in neurodegenerative animal models, such as the Alzheimer's APP/PS1 and 3xTgAD mice models (Wu et. al., 2022). It is also important to remember that PBM treatment to other sites of the body can stimulate overall improvements including immune system and cognitive performance. For example, PBM treatment targeting the bone marrow of 5XFAD mice was shown to activate different cell types, such as mesenchymal stem cells from the bone marrow to infiltrate the brain and perform phagocytosing behaviours against amyloid beta

particles (Oron and Oron, 2016). These results were accompanied with cognitive improvements, such as longer freezing time in the mice, suggest indirect PBM effects (as mentioned in section 1.2), possibly in combination with direct effects of PBM on infiltrating lymphocytes.

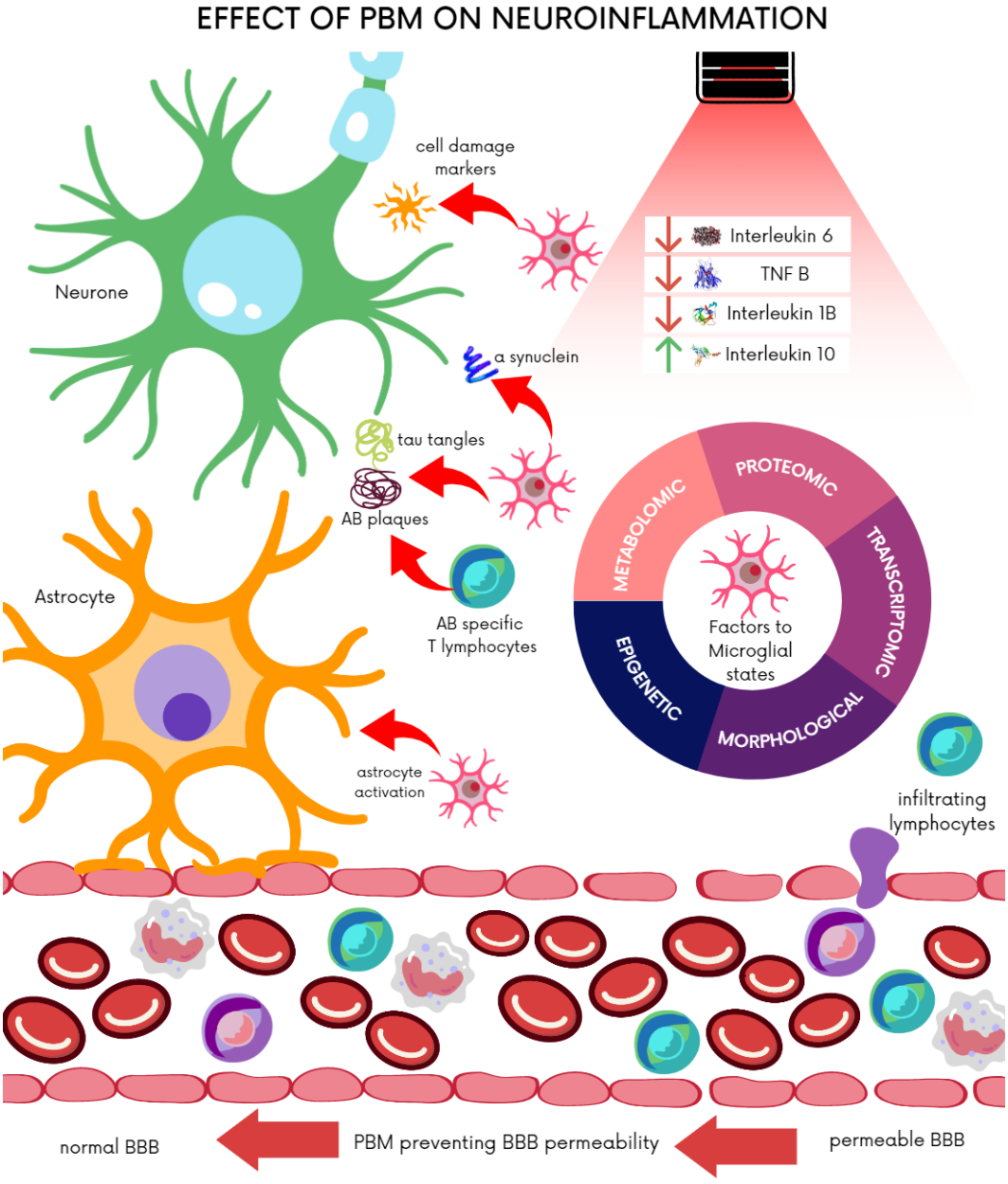

Fig. 1.4.2. A schematic diagram of a neuro-inflamed, neurodegenerative (Alzheimer's disease) brain environment with permeable BBB and the effect PBM has on microglial activity and other infiltrating immune cells. The reduction of destructive inflammatory behaviours and the increase of anti-inflammatory behaviours.

4.3 The relationship between PBM and neuroinflammation.

Recently there has been a consistently growing contribution of studies that investigate the effect of PBM on neurodegeneration that also happen to measure neuroinflammatory and/or oxidative stress markers. However, at the time of writing this thesis, there is a void in the literature that specifically addresses the topic of PBM's effect on neurodegeneration-induced inflammation, therefore a systematic review and summary of the literature will offer insight. There have been several accounts and studies that have demonstrated a beneficial effect of PBM towards neuroinflammatory responses and oxidative stress. Several in vivo animal models and in vitro cellular models have been utilised in studies that measured the effect of PBM on neurodegeneration, neuroinflammation and oxidative stress (Cardoso et. al, 2020; Salehpour et. al., 2021) which will be discussed in greater detail below.

In Vitro Studies

PBM was shown to suppress inflammatory responses, as well as oxidative stress induced by amyloid beta particles in rat astrocytes (Bungart and Lee, 2012). Oxygen and glucose deprivation neurotoxicity was attenuated including neuroinflammatory signals such as iNOS, cyclo-oxygenase enzyme 2 (COX-2), nuclear factor kappa B (NFkB) subunit p65, and B-cell lymphoma 2 (Bcl-2) protein (Gerace et. al., 2021). An in *vitro* model of mesenchymal stem cells treated with PBM demonstrated a heightened capacity to mature into monocytes and phagocytose soluble Aβ particles (Oron and Oron, 2016). PBM reduced apoptosis in PC12 cells with AB25-35 induced neurotoxicity (Duan et. al., 2003; Zhang et. al., 2008; 2012). PBM reduced inflammatory markers IL-1B & iNOS in rat cortical astrocyte cultures that received a toxic dose of amyloid beta particles (Bungart et. al., 2014).

The use of PBM significantly reduced apoptosis in 3 types of cell cultures, Sh-SY5Y, PC12, and HEK293T, that had AB-induced neurotoxicity (Liang et. al., 2012). A similar study by Duggett and Chazot (2014) that used Cath.-a-differentiated (CAD) cells, which are a variant of a CNS catecholaminergic cell line established from a brain tumour in a transgenic mouse (Qi et. al., 1997), and the results demonstrated PBM also significantly decreased apoptosis, even in cells that received a high AB toxic dose of 3.5-25 micromoles (uM). In a study that used primary astrocytes with a toxic dose of amyloid beta particles, PBM also suppressed markers of oxidative stress such as NADPH oxidase & phosphorylation of cPLA, whilst also inhibiting proinflammatory markers IL-1B & iNOS (Yang et. al., 2010). Therefore, through a variety of different cell cultures, and mostly AD in vitro models induced with AB particles, there's a common indication of PBM suppressing inflammatory response and oxidative stress markers.

Animal Models In Vivo:

Several studies have used a variety of Alzheimer's Disease (AD) models to show a relationship between PBM, inflammatory and/or oxidative stress markers. For example, an APP transgenic mouse AD model demonstrated a decrease in brain inflammatory markers such as IL-1, TNF and transforming growth factor (TGF) in response to PBM treatment (De Taboada, 2011). A series of investigations of Purushothuman and colleagues (2014; 2015) used K3 mice to simulate AD and demonstrated PBM causing a significant reduction in oxidative stress markers, such as 8-OHDG and 4-HNE in the neocortex. Another AD model study by Lu and colleagues (2017) with amyloid beta-injected rats demonstrated several significant results on oxidative stress and inflammatory marker. There was an inhibition of oxidative markers such as G6PDH and NADPH oxidase, an enhanced antioxidant capacity, an inhibition of glial activation and a reduction of inflammatory cytokines in response to PBM treatment (Lu et. al., 2017). The investigation by Blivet and colleagues (2018) measured decreases in oxidative stress, shown as a reduction in lipid peroxidation levels; a reduction in inflammatory markers such as IL-1, TNF and IL-6. A decrease in microglial activation was measured in response to PBM treatment of 5XFAD transgenic mice (Cho et. al., 2020), whereas the study by Blivet and colleagues measured astrocyte and microglial activities in a different way, and rather than reporting an increase or decrease in astrocyte and microglial activity, they reported a "modification" of their activities. Studies have also included animal models of Parkinson's disease, such as that of O'Brien and Austin (2019) which demonstrated PBM gave complete neuroprotection for rats treated with a 10ug dose of LPS, accompanied with motor behaviour improvement. Another study that treated monkeys with MPTP demonstrated that PBM effectively reduced MPTP-induced astrogliosis by about 75% as assessed by the measurement of active IBA1 positive astrocytes (El Massri et. al., 2016a). Another animal model of Parkinson's disease demonstrated a prevention of dopaminergic cell death, and although this abscopal effect wasn't completely defined, Johnstone and colleagues (2014) hypothesised that this effect was associated with the potential upregulation of anti-inflammatory cytokines such as IL-4 and IL-10. The idea to conceive is that that these modifications aren't as simple as PBM causing reductions and increases in the immune response. Rather, they are more complex interactions and combinations that assist the immune system in clearing toxins with increases in pro-inflammatory activity, complemented with the upreguation of anti-inflammatory activity to prevent immune response overload. It's also important to note that these papers offer a window into the relationship between PBM and neurodegeneration-induced inflammation, however a thorough systematic review is needed.

Considering that neuroinflammation seems to be reduced in response to PBM, the next question we can ask is whether this change within neurodegenerative diseases can be quantified, and whether the

neurodegenerative conditions can be improved, as assessed by symptom reduction and with imaging. The literature is lacking thorough systematic reviews that directly address the relationship between PBM and the effect it has on neurodegeneration-induced neuroinflammation. Also missing from the literature is the mechanism of neuroinflammation modulation by PBM and specifically how these changes in activities shift the immune responses from inflammatory to anti-inflammatory. This is addressed in the fifth chapter in the form of a systematic review conducted by myself and my colleagues.

5 Aims, rationale and outline of thesis

The main aims of this thesis are three-fold:

a. Does PBM have an effect on healthy human brain function? (Chapters 2 and 3). Using a transcranial approach, the aim was to examine if and how PBM influences the activity of the brain using fMRI. The key issue of the differences in effect in task-positive and task negative situations was explored.

b. Does PBM have a beneficial effect on patients with Parkinson's disease? (chapter 4) Using a transcranial approach again, the impact of PBM was explored on the major signs and symptoms on each patient. Observations were noted and recorded over long-term periods (months to years) on their motor signs and non-symptoms.

c. Does PBM have an effect on neurodegeneration induced neuroinflammation and can this be a viable approach in the future for clinical treatments of a wide range of neurodegenerative diseases (chapter 5)? A systematic review was conducted to examine this key issue.

Chapter 2

Exploring the effects of near infrared light on resting and evoked brain activity in humans using magnetic resonance imaging

NEUROSCIENCE
RESEARCH ARTICLE

H. El Khoury et al. / Neuroscience 422 (2019) 161–171

Exploring the Effects of Near Infrared Light on Resting and Evoked Brain Activity in Humans Using Magnetic Resonance Imaging

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Abstract—We explore whether near infrared light can change patterns of resting (task-negative) and/or evoked (task-positive; eg finger-tapping) brain activity in normal, young human subjects using fMRI (functional magnetic resonance imaging). To this end, we used a vielight transcranial device (810 nm) and compared the scans in subjects after active- and sham-light sessions. Our fMRI results showed that, while light had no effect on cerebral blood flow and global resting state brain activity (task-negative), there were clear differences between the active- and sham-light sessions in the patterns of evoked brain activity after finger-tapping (task-positive). The evoked brain regions included the putamen, primary somatosensory and parietal association cortex, and the overall effect of the light was to suppress or reduce their activity. We also found that while light had no effect on the resting functional connectivity of the putamen and primary somatosensory cortex and the rest of the brain, it did have an effect on the functional connectivity of parietal association cortex. In summary, our fMRI findings indicated that transcranially applied light did have a major impact on brain activity in normal subjects, but only when the brain region was itself functionally active, when undertaking a particular task. We suggest that these light-induced changes, particularly those in parietal association cortex, were associated with attention and novelty, and served to deactivate the so-called default mode network. Our results lay the template for our planned fMRI explorations into the effects of light in both Alzheimer's and Parkinson's disease patients.

Key words: photobiomodulation, 810 nm, transcranial, finger-tapping, parietal association cortex.

INTRODUCTION

Many recent studies have provided compelling evidence that red to infrared light (λ = 600–1070 nm) has a major influence on the structure and function of the nervous system. In particular, light across these wavelengths has been reported to influence both the survival of neurones, helping them to self-protect and -repair against trauma or disease and to maintain their ongoing survival (ie neuroprotection), as well as the function of neurones, being able to change their overall patterns of activity. The application of this type of light on the nervous system, as indeed on all body tissues, is referred to as photobiomodulation (referred to forthwith as "light"). Although the precise mechanisms are not clear, there is a general agreement among authors that the beneficial effects of light are cellular, and not thermal, and that the central, target intracellular organelle is the mitochondria. Light, after being absorbed a photoacceptor within the mitochondria, such as cytochrome oxidase and/or interfacial water, triggers an increase in electron transfer in the respiratory chain and in the mitochondrial membrane potential, resulting in a surge of ATP (adenosine triphosphate) energy. The increase in mitochondrial activity and ATP energy leads to changes in the activity of ion membrane pumps, together with an activation of transcription factors in the nucleus and the expression of genes associated with neuronal survival and function (Eells et al., 2004; Karu, 2010; Rojas and Gonzalez-Lima, 2011; Khan and Arany, 2015; Sommer et al., 2015; Hamblin, 2016; Hennessy and Hamblin, 2017).

In animal models of disease, from traumatic brain injury (Ando et al., 2011; Xuan et al., 2013) to multiple sclerosis (Muili et al., 2012; 2013) and from Alzheimer's (Michalikova et al., 2008; Purushothuman et al., 2014) to Parkinson's (Shaw et al., 2010; Darlot et al., 2016) disease, light has been reported to be neuroprotective of distressed neurones, together with improving the behavioural patterns of the animals, from locomotion to cognition. There have also been some clinical reports in patients indicating that transcranial application of light

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Abbreviations: ASL, arterial spin labelling; ATP, adenosine triphosphate; BOLD, blood-oxygen-level-dependent; CBF, cerebral blood flow; EEG, electroencephalography; fMRI, functional magnetic resonance imaging; LED, light emitting diode; pCASL, pseudocontinuous arterial spin labelling; M1, primary motor cortex; S1, primary somatosensory cortex.

https://doi.org/10.1016/j.neuroscience.2019.10.037
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improves cognition in Alzheimer's disease (Saltmarche et al., 2017), mood in depression (Schiffer et al., 2009; Cassano et al., 2015; Salehpour and Rasta, 2017) and motor signs and non-motor symptoms in Parkinson's disease (Hamilton et al., 2018a,b). In Alzheimer's disease patients (Chao, 2019) and in middle-aged and older human subjects suffering mild memory loss (Vargas et al., 2017), light has been shown to change electroencephalography (EEG) patterns and cerebral blood flow (CBF)/blood-oxygen-level-dependent (BOLD) signal under fMRI (functional magnetic resonance imaging).

Together with influencing the survival and function of distressed neurones in disease, there are also findings indicating that light can effect the function of neurones that are otherwise normal and "healthy". Light has been shown to change the firing rates of neurones in the substantia nigra pars compacta of normal mice (Romeo et al., 2017) and in the motor cortex of normal human subjects (Konstantinovic et al., 2013). Further, in normal human subjects, application of transcranial light improves the executive, cognitive and emotional functions (Barrett and Gonzalez-Lima, 2013; Gonzalez-Lima and Barrett, 2014; Blanco et al., 2017), together with changing EEG patterns (Wang et al 2017; Zomorrodi et al 2019).

In this study, we explored whether light can change patterns of resting state, task-negative, brain activity in normal, young human subjects using fMRI. In addition, we examined if light influenced evoked brain activity when the subjects were undertaking a particular functional task, namely, finger-tapping (task-positive). In essence, we wanted to determine which regions of the brain, if any, changed activity after transcranially applied light. To this end, we used the commercially available light device from vielight (vielight.com) and compared the scans in subjects after active- and sham-light sessions. Previous studies have indicated that transcranially applied light can penetrate 20–30 mm through body tissues (Lapchak et al., 2004; Byrnes et al., 2005; Zivin et al., 2009; Haeussinger et al., 2011; Moro et al., 2014; Henderson and Morries, 2015), so light penetration through the cranium to the brain, at least to the superficial regions, is more than feasible (Hamblin, 2016). Our fMRI study here on the effects of transcranially applied light on brain activity in young normal subjects will lay the template for our planned explorations into the effects of light in both Alzheimer's and Parkinson's disease patients.

EXPERIMENTAL PROCEDURES

Subjects

Twenty four normal, healthy, young subjects (12 of each gender; mean age 32 ± 4 years [±SEM]) were recruited for the study from the general population using an advertisement. Exclusion criteria were the current use of brain altering medication or any neurological disorder. Informed written consent was obtained for all procedures according to the Declaration of Helsinki and local Institutional Human Research Ethics Committees approved the study.

MRI acquisition

Subjects were instructed to lay still, to close their eyes and to not fall asleep. Using a 3 Tesla MRI scanner (Philips Achieva, 32-channel SENSE head coil), three scans encompassing the whole brain were collected while the subject was relaxed and at rest:

(1) Anatomical MRI: a high-resolution 3D T1-weighted anatomical image (200 axial slices, echo time [TE] = 2.5 ms, repetition time [TR] = 5600 ms, raw voxel size = 0.87 mm^3)
(2) Resting state fMRI (task-negative); a series of 180 wholebrain resting state gradient echo echo-planar functional images (37 axial slices, [TE] = 30 ms, [TR] = 2000 ms, raw voxel size = $3 \times 3 \times 4$ mm^3) and,
(3) ASL (arterial spin labelling): a series of 108 pseudo-continuous arterial spin labelling (pCASL) images (50 axial slices, 54 label/control image pairs, [TE] = 12.7 ms, [TR] = 5310 ms, raw voxel size = $2.4 \times 2.4 \times 3.0$ mm^3, labelling time = 1650 ms, slice time = 36.6 ms, post label delay time = 1600 ms, background suppression).

Following these three resting scans, another series of 180 wholebrain gradient echo echo-planar functional images (37 axial slices, [TE] = 30 ms, [TR] = 2000 ms, raw voxel size = $3 \times 3 \times 4$ mm^3) was collected (fMRI with finger-tapping; task-positive). During this fourth scan, following a 30 volume baseline (60 s), subjects were asked to move the pad of their right thumb to touch the pad of their right pointer finger at a frequency of approximately 1 Hz for a period of 6 volumes (12 s). This finger-tapping period was then followed by a 6 volume rest period and this procedure was then repeated a further 12 times for a total of 13 finger-tapping and 12 rest periods.

Light delivery: the device

Each subject was removed from the MRI scanner and sat comfortably in a chair in an adjacent room. They were then asked to wear the vielight-α light device (10 Hz pulse rate, up to 100 mW/cm^2 per diode). This device uses 810 nm near infrared light through 5 non-laser light emitting diodes (LEDs). Each LED was positioned over the frontal (midline) and parietal bones, in line with the ventral medial prefrontal, dorsal medial prefrontal, posterior cingulate/precuneus and lateral parietal and entorhinal cortex, all the regions associated with the so-called default mode network (Zomorrodi et al., 2019). For each of our exposures, all the diodes were used and hence potentially influencing all regions of the network, as well as other regions of the brain. We chose this device for use because it offered a consistency of well-tested parameters, for example the pulse rate and power, that were not available at the time of study commencement. Further, this device was similar to ones used by previous studies, and hence offered a point of comparison (eg, Saltmarche et al., 2017; Chao, 2019; Zomorrodi et al., 2019). The 810 nm near infrared wavelength was

chosen by the manufacturer (vielight.com) because of its ability to penetrate a little deeper into body tissues when compared to other wavelengths (Hamblin, 2016) and hence potentially reaching a greater number of brain areas from a transcranial application in humans.

Subjects then received either (i) a 20 minute active-light vielight session during which time the light was delivered ("active") or (ii) a 20 minute sham session where the vielight was in place but not turned on ("sham"). Subjects were assigned randomly into either the active or sham group and did not know which group they were a part of (all on/off lights on the power device were completely covered/shielded from the subject's view; further, the "sham" process was made much easier by the fact that 810 nm light was near, if not totally, invisible). Immediately following this active- or sham-session, subjects were placed back into the MRI scanner and the same three functional scans collected during the first MRI session, namely resting state fMRI, ASL, and fMRI with finger-tapping, were collected again. Note that the vielight device was not imaging-compatible, hence it had to be used outside of the scanner.

Between 4 and 12 weeks following the first MRI session, each subject retuned for a second MRI session. During this second session, an identical series of procedures to that during the first MRI session were performed. However, if the subject was in the active group during the first MRI session, they were placed into the sham group for the second MRI session and vice versa.

fMRI processing and statistical analysis

fMRI image preprocessing. Using SPM12 and Matlab software, for each subject, both the resting state and finger-tapping fMRI image sets were motion corrected, linear detrended to remove global signal drifts and the effects of movement were modelled and removed from the fMRI signal by LMRP detrending (Macey et al., 2004). There was significant movement (greater than 0.5 mm in any direction) in; 2 subjects during one or more of the four finger-tapping scans; in 4 subjects during any of the four resting state fMRI scans and; in 5 subjects during any of the four pCASL scans and their data was removed from the subsequent analysis. This resulted in 22 subjects for the finger-tapping analysis, 20 subjects for the resting state fMRI and 19 subjects remaining for the pCASL analysis. Each subject's fMRI image sets were coregistered to their own T1-weighted anatomical image set. The T1 images were then normalised spatially to the Montreal Neurological Institute template and the parameters applied to the fMRI image sets so that both the T1-weighted and fMRI images were in the same locations in 3-dimensional space. All images were then smoothed using a 6 mm full-width at half-maximum Gaussian filter. Finally, to remove the potential influence of signal intensity changes within the cerebrospinal fluid, cerebrospinal fluid brain maps were created by segmenting the spatially normalised T1 anatomical images. This map was used to mask the fMRI images so that only grey and white matter remained.

Arterial spin labelling image preprocessing. All pCASL image sets were realigned, co-registered to each individual's T1-weighted image set, the label and control images averaged and a mean cerebral blood flow (CBF) image created using the subtraction method using the ASL toolbox (Wang et al., 2008). Each subject's T1-weighted anatomical image was spatially normalised to a template in MNI space and the parameters applied to the CBF maps. The CBF maps were then smoothed using a 6 mm Gaussian filter. A grey matter mask derived from the T1-weighted anatomical image segmentation was used to restrict the wholebrain analysis to grey matter.

Finger-tapping analysis. Brain activation patterns during finger-tapping prior to active-light were assessed in each subject pre and post active-light and pre and post sham-light. Using a repeated box car model with "on" periods as those during finger-tapping, combined with a haemodynamic delay function, significant changes in signal intensity were assessed in each subject for each of the four finger-tapping fMRI scans. Significant differences in brain activation patterns during finger-tapping pre and post active-light and pre and post sham-light were determined using paired second-level random effects analyses ($p < 0.05$, false discovery rate corrected for multiple comparisons, minimum cluster 5 contiguous voxels). To assess the effect of active compared with sham-light on finger-tapping evoked brain activation, brain contrast maps (representing the strength of signal changes during finger-tapping) of finger-tapping pre active-light were subtracted from those maps post active-light. Thus, positive values represent greater finger-tapping activation after active-light than before active-light. The same procedure was also performed for the pre and post sham-light scans. To determine if the differences observed following active-light were significantly different to those following sham-light, we performed an analysis restricted to those brain regions displaying significant differences pre versus post active-light. That is, the two contrast maps: post-pre active-light and post-pre sham-light, for each subject were placed into a paired random effects analysis to determined significant differences in the effects of active and sham-light on finger-tapping evoked brain activation ($p < 0.05$, false discovery rate corrected). Finally, for each significantly different cluster (active versus sham-light), the individual contrast values (post-pre) were plotted for each subject for the active and sham-light scans.

Resting functional connectivity. The effects of light on resting functional connectivity was assessed using the resting state fMRI scans. Three clusters derived from the finger-tapping analysis were used as "seeding" regions. These clusters were the (i) left putamen (ii) right parietal association cortex and (iii) right primary somatosensory cortex. Mean resting signal intensity changes within these seeds were calculated and a

voxel-by-voxel analyses were performed to determine regions that displayed significant signal intensity covariations with each of the three seed signals. The resulting brain maps were then smoothed using a 6 mm Gaussian filter. Since we derived the seeds from the finger-tapping analysis and were interested in determining if connectivity changes were altered within this circuitry, we restricted our analysis to those regions activated by finger-tapping by applying a mask of the initial analysis of finger-tapping activation prior to active-light. To assess the effect of active- compared with sham-light on resting connectivity of each of the three regions, the resting connectivity strength brain maps pre active-light were subtracted from those maps post active-light. Thus, positive values represent greater resting connectivity strength after active-light than before active-light. The same procedure was also performed for the pre and post sham-light scans. To determine if the differences observed following active-light were significantly different to those following sham-light, the two brain connectivity brain maps: post-pre active-light and post-pre sham-light, for each subject were placed into a paired random effects ($p < 0.05$, false discovery rate corrected). Finally, for each significantly different cluster (active versus sham-light), the individual contrast values (post-pre) were plotted for each subject for the active and sham-light scans.

Arterial spin labelling. The effects of light on resting blood flow was assessed using the CBF maps derived from the pCASL scans. To assess the effect of active compared with sham-light on regional blood flow, CBF brain maps before active-light were subtracted from after active-light CBF maps and the same procedure was performed for the pre and post sham-light maps. To determine if the differences observed following active-light were significantly different to those following sham-light, the two CBF brain maps: post-pre active-light and post-pre sham-light, for each subject were placed into a paired random effects ($p < 0.05$, false discovery rate corrected). Finally, to determine if there were any CBF differences in any of the clusters derived from the finger-tapping or resting state connectivity analyses, the CBF values pre and post light and pre and post sham-light were extracted for each significant cluster and plotted. Significant differences in CBF were then determined using paired t-tests ($p < 0.05$).

RESULTS

In the section that follows, the effects of transcranially applied light on the patterns of brain activity after finger-tapping, resting functional connectivity and CBF will be considered separately.

Finger-tapping

Finger-tapping tasks under the fMRI are among the most commonly used methods to study the patterns of evoked brain, task-positive, activity in the sensorimotor system in humans, in both health and disease (Witt et al., 2008; Lv et al., 2013, 2018). In our subjects, finger-tapping of the right hand evoked increases in signal intensity in regions of the left (contralateral) putamen, primary somatosensory (S1) and motor (M1) cortex, parietal association cortex (adjacent to the postcentral gyrus, in region of intraparietal sulcus), thalamus and cerebellum (not shown). In the subjects that received light treatment (active-light), there was a clear effect on the finger-tapping evoked signal intensities across the brain (Fig. 1A, Table 1). Light reduced (as indicated by the cooler colours in Fig. 1A) finger-tapping evoked activation in the left (contralateral) putamen (mean ± SEM contrast value: pre-light: 0.24 ± 0.04; post-light: 0.08 ± 0.03), right primary somatosensory and motor cortex (pre-light: 0.05 ± 0.05; post-light: −0.15 ± 0.04), right somatosensory/parietal association cortex (pre-light: −0.09 ± 0.05; post-light: −0.27 ± 0.04) and left parietal association cortex (pre-light: 0.11 ± 0.05; post-light: −0.05 ± 0.04). In no brain region did light increase finger-tapping evoked activity.

In striking contrast, in the subjects that received no light treatment (sham-light), there was no significant effect on finger-tapping activation in any brain region (Fig. 1B). Further, a direct comparison of the effects of active-light compared with sham-light on finger-tapping evoked activation revealed significant differences in the left putamen (mean ± SEM contrast value post-pre: active-light: −0.13 ± 0.4; sham-light: 0.04 ± 0.06), right primary somatosensory (active-light: −0.10 ± 0.04; sham-light: 0.05 ± 0.04) and right parietal association cortex (active-light: −0.17 ± 0.03; sham-light: −0.01 ± 0.02) (Fig. 1C). That is, in these three brain regions, active-light reduced finger-tapping activation whereas sham-light had no effect.

The plots of changes in the individual subjects revealed that the direction of difference in the putamen, primary somatosensory and parietal association cortex, were remarkably consistent. In the left putamen 19 of the 22 subjects displayed a reduction in activation during active-light than sham-light, 16 subjects displayed a reduction in the right primary somatosensory cortex and 17 subjects in the right parietal association cortex (Fig. 2).

Resting functional connectivity

To determine if the effect of light on evoked brain activation was accompanied by changes in resting functional connectivity, we assessed the effects of active- and sham-light on resting functional connectivity of the left putamen, right primary somatosensory and right parietal association cortex. The analysis of resting functional connectivity is a well-known means for exploring the patterns of connectivity between different regions of the brain. It represents a measure of the degree of synchrony and correlation between different brain regions, either by direct anatomic projection or by and indirect route, through another region of the brain. In other words, the measure of resting functional connectivity provides information on the density of flow of information traffic between select regions of the brain (van den Heuvel and Hulshoff Pol. 2010; Lv et al., 2018).

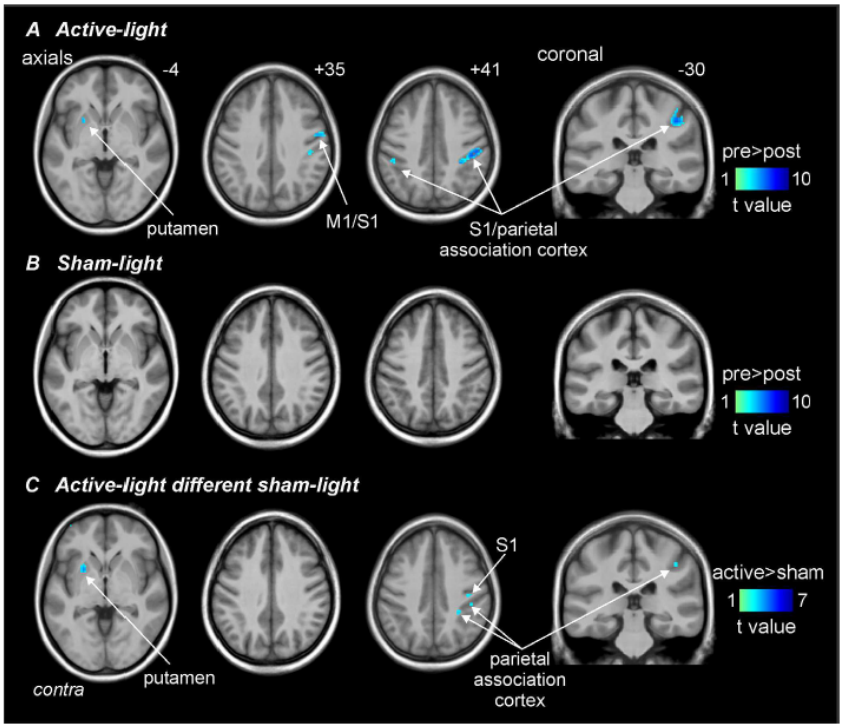

Fig. 1. Effects of active- and sham-light on finger-tapping evoked activity. (A) Significant differences in finger-tapping evoked activation before and after active-light. Regions in which activation was reduced significantly by light are indicated by the cool colour shading and overlaid onto a series of axial and coronal T1-weighted anatomical images. Slice locations in Montreal Neurological institute space are indicated at the top right of each image. Note that active-light significantly reduces activation during finger-tapping in the contralateral (to finger-tapping) putamen, ipsilateral primary motor/somatosensory (M1/S1) cortex and in the bilateral parietal association cortex. (B) Significant effects of sham-light on finger-tapping activation. Note that there is no effect of sham-light on finger-tapping evoked activation. (C) Significant differences in active versus sham-light evoked activation. Note that active-light results in a significantly greater reduction in finger-tapping evoked activations in the contralateral (left) putamen, right primary somatosensory (S1) and right parietal association cortex.

Although active- and sham-light had no significant effect on resting functional connectivity between either the left putamen or right primary somatosensory cortex and the rest of the brain (not shown), it did have a significant effect on the resting functional connectivity of the right parietal association cortex (Fig. 3, Table 1). A direct comparison of the effects of active-light compared with sham-light on resting functional connectivity of parietal association cortex revealed significant differences in the left primary motor cortex (mean ± SEM connectivity strength post-pre: active-light: −0.06 ± 0.2; sham-light: 0.05 ± 0.02), right primary motor cortex (active-light: −0.06 ± 0.2; sham-light: 0.05 ± 0.02), left posterior parietal cortex (active-light: −0.07 ± 0.2; sham-light: 0.04 ± 0.02) and right posterior parietal cortex (active-light: −0.06 ± 0.2; sham-light: 0.05 ± 0.02).

Plots of connectivity strength changes in individual subjects revealed that the direction of difference in these three regions - primary motor, posterior parietal and parietal association cortex - were remarkably consistent. In the left primary motor cortex, 18 of the 20 subjects displayed a greater reduction in connectivity during

Table 1. Location of significant clusters in Montreal Neurological Institute space, cluster sizes and t values for finger-tapping and resting connectivity analyses. The t values relate to the differences seen in each region after finger-tapping (1) pre- compared to post-scans in active-light cases (2) active- compared to sham-light cases and in resting parietal association cortex connectivity in (3) active- compared to sham-light cases.

	MNI co-ordinates			Cluster size	*t* value
	x	y	z		
Finger-tapping					
(1) active-light: pre > post					
left putamen	−24	10	−2	12	5.04
right S1/M1	54	−6	34	37	5.10
left S1/parietal association cortex	−46	−36	40	12	4.77
right S1/parietal association cortex	48	−26	44	198	6.07
(2) active > sham-light					
left putamen	−24	−8	−4	42	4.02
right S1	44	−18	40	3	2.62
right parietal association cortex	44	−30	42	11	2.92
	30	−38	42	3	2.95
resting parietal association cortex connectivity					
(3) active > sham-light					
left M1	−46	−14	34	115	5.68
right M1	46	−10	34	51	5.19
left posterior parietal cortex	−46	−70	8	99	4.88
right posterior parietal cortex	54	−56	14	25	4.48

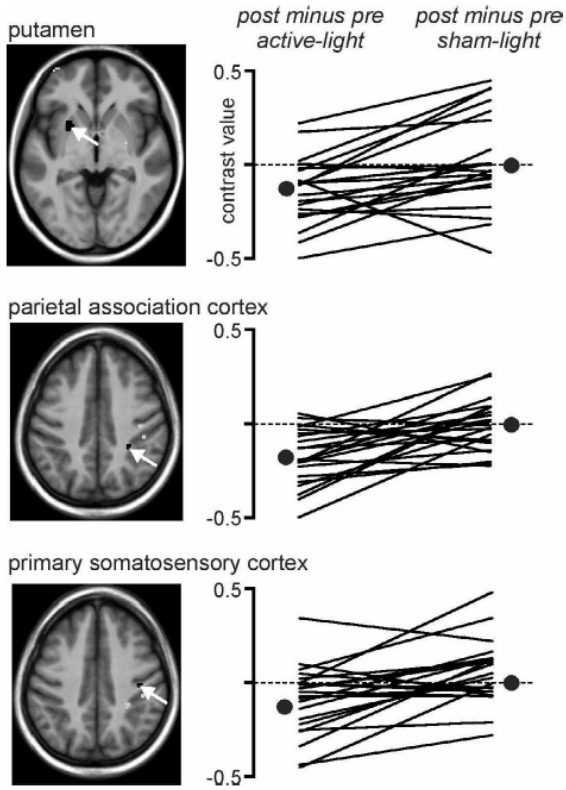

Fig. 2. The effect of active- and sham-light on individual subject brain activation. Plots of individual subject significance measures (contrast values) of finger-tapping activations post active-light minus pre active-light and post sham-light minus pre sham-light for the putamen, parietal association and primary somatosensory cortex. Clusters are shaded black. Negative values indicate that active- or sham-light reduced finger-tapping evoked activations. The large filled black circles indicate means. Note that active-light reduced finger-tapping evoked activations in all three regions whereas sham-light did not.

active-light than sham-light, 17 subjects in the right primary motor cortex, left posterior parietal cortex and right posterior parietal cortex (Fig. 4).

Resting cerebral blood flow

We also explored if light had any effect on resting, task-negative, brain activity levels. To this end, we examined the effects of active- and sham-light on resting regional CBF using ASL, a non-ionising method for measuring tissue perfusion that uses magnetically labeled arterial blood water protons as an endogenous tracer. It is used as a more global measure of brain activity in any given region (Petcharunpaisan et al., 2010). In contrast to finger-tapping evoked brain activity and resting functional connectivity, active- and sham-light had no significant effect on on-going CBF in any region of the brain. Further, direct comparisons of the effects of active- and sham-light on resting CBF in the clusters derived from the finger-tapping scans confirmed no significant CBF change in the left putamen (mean ± SEM cerebral blood flow ml/min/g: active-light pre: 48.2 ± 2.7, post: 51.1 ± 2.4; sham-light pre: 47.8 ± 2.8, post 48.8 ± 2.7), right parietal association cortex (active-light pre: 43.4 ± 2.3, post: 37.3 ± 2.5; sham-light pre: 40.4 ± 3.0, post 40.5 ± 3.4) or right primary somatosensory cortex (active-light pre: 50.7 ± 2.5, post: 47.2 ± 3.5; sham-light pre: 52.1 ± 3.1, post 51.7 ± 3.7; all $p > 0.05$; Fig. 5). Similarly, comparisons of CBF in clusters identified in the parietal association cortex connectivity revealed no significant effects of active or sham-light on CBF in the left primary motor cortex (active-light pre: 54.0 ± 2.5, post: 54.8 ± 2.2; sham-light pre: 52.5 ± 3.0, post 52.1 ± 2.4), right primary motor cortex (active-light pre: 50.8 ± 2.9, post: 50.1 ± 2.6; sham-light pre: 48.9 ± 4.0, post 48.1 ± 3.7), left posterior parietal cortex (in region of angular gyrus) (active-light pre: 75.3 ± 5.7, post: 79.7 ± 4.2; sham-light pre: 78.4 ± 3.8, post 79.4 ± 3.9), or right posterior parietal cortex active-light pre: 80.6 ± 5.2, post: 77.4 ± 4.4; sham-light pre: 73.2 ± 3.8, post 78.1 ± 4.8; all $p > 0.05$)

DISCUSSION

We have three major findings with light use and fMRI. First, that transcranially applied light (810 nm) reduced evoked activation after finger-tapping (task-positive) in

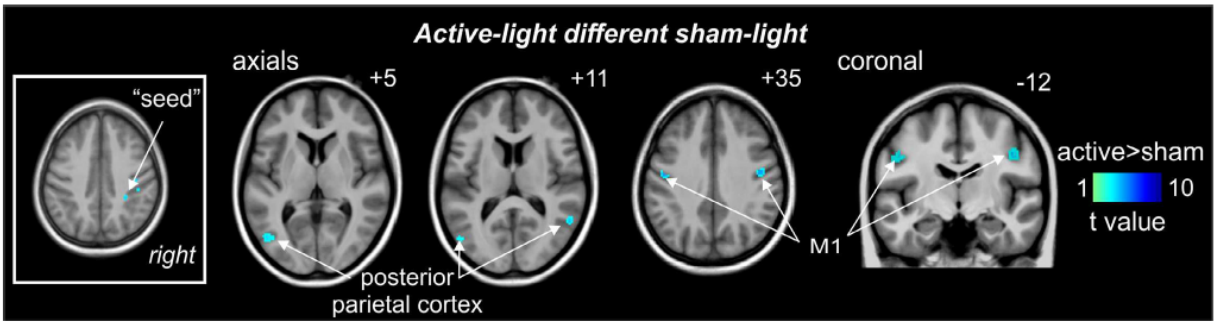

Fig. 3. Effects of active- and sham-light on resting parietal association cortex functional connectivity. Significant differences in right parietal association cortex resting connectivity strengths before and after active compared with sham-light. Region in which resting connectivity was significant reduced by active-light more than sham-light are indicated by the cool colour shading and overlaid onto a series of axial and coronal T1-weighted anatomical images. Slice locations in Montreal Neurological institute space are indicated at the top right of each image. Note that active-light significantly reduces resting connectivity strength between the right parietal association cortex and the left and right posterior parietal cortex as well as the left and right primary motor cortex (M1).

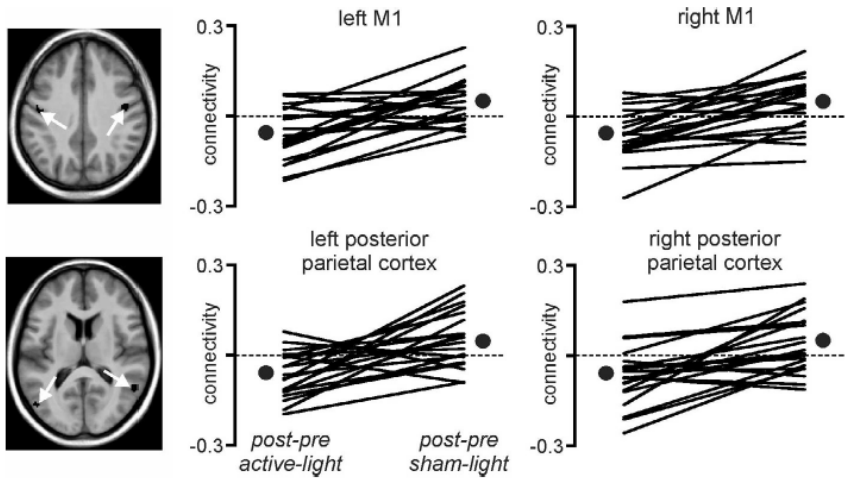

Fig. 4. The effect of active and sham-light on individual subject resting functional connectivity. Plots of individual subject right parietal association cortex resting connectivity strengths post active-light minus pre active-light and post sham-light minus pre sham-light for the left and right primary motor cortex (M1) and the left and right posterior parietal cortex. Clusters are shaded black. Negative values indicate that active or sham-light reduced resting connectivity strengths. The large filled black circles indicate means. Note that active-light reduced resting connectivity strengths in all four regions whereas sham-light did not but instead increased connectivity strengths in all regions.

rather discrete regions of the brain. Second that these light-induced changes in evoked brain activity were accompanied by distinct changes in resting functional connectivity, and third, that the transcranially applied light - in contrast to the finger-tapping evoked activity and resting functional connectivity - had no effect on overall resting state brain activity (task-negative). When taken all together, our fMRI findings indicated that transcranially applied light can have a clear impact on the activity of the brain on normal subjects, but only when a region of the brain was itself functionally active, for example, when undertaking a particular task. These issues will form the focus of the Discussion that follows.

Light impacted on evoked brain activity

One of our major aims was to explore the effect of light on evoked brain activity, namely when subjects were undertaking particular task (task-positive). To this end, we chose the well-known finger-tapping exercise, a test that has been used by many previous fMRI studies examining aspects of the motor system in humans (Witt et al., 2008; Lv et al.,

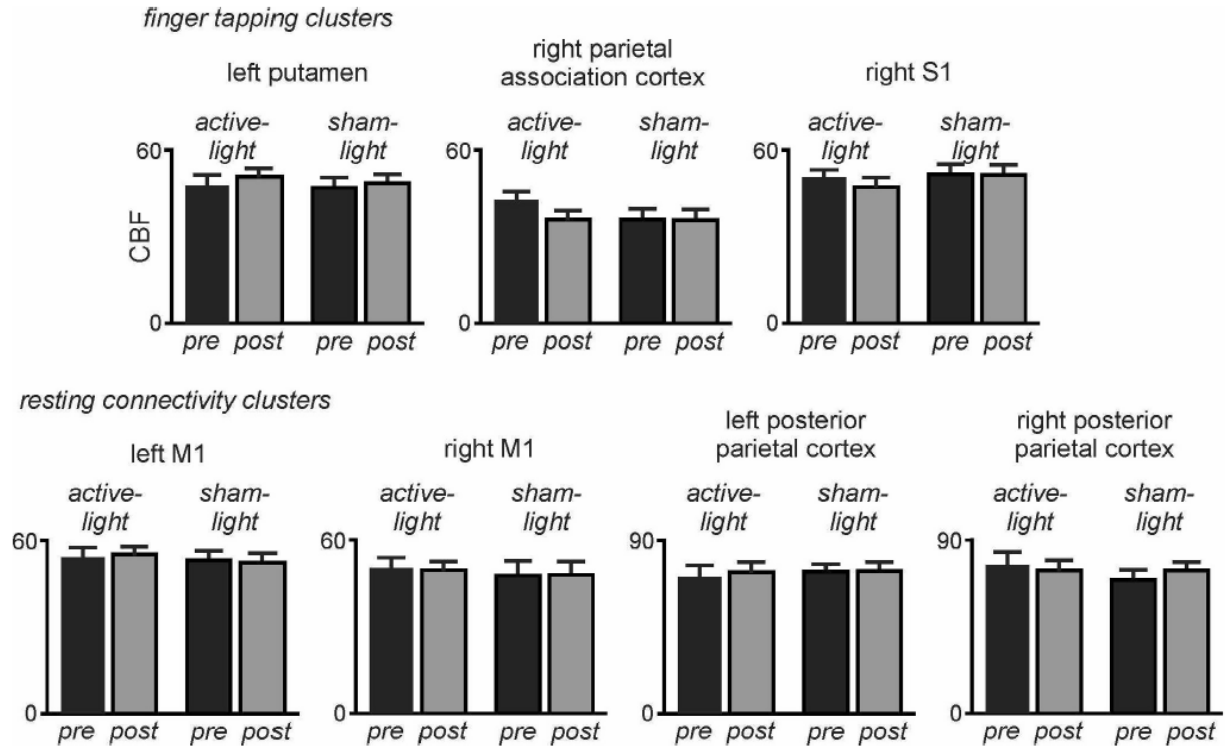

Fig. 5. The effect of active and sham-light on resting cerebral blood flow. Plots of mean (± SEM) cerebral blood flow in 7 clusters, 3 clusters derived from the finger-tapping analysis and 4 clusters derived from the resting connectivity analysis. Note that active and sham-light had no significant effects on resting cerebral blood flow (CBF) in any of the 7 clusters.

2013, 2018). Our finding that finger-tapping evoked activity in primary somatosensory and motor cortex, parietal association cortex, thalamus, putamen and cerebellum was largely consistent with previous ones (Witt et al., 2008). We showed that transcranially applied light - when compared directly to sham-treated - reduced, with remarkable consistency across our 22 subjects, this evoked activation in many of these regions, including the putamen, primary somatosensory and parietal association cortex. By contrast, sham treatments had no effect on finger-tapping activation in any region of the brain. These results here were similar to previous ones that have reported a reduction in BOLD-fMRI signal - a combined measure of increased CBF, cerebral blood volume and reduced oxygen consumption - within regions of prefrontal and parietal association cortex in response to cognitive, working memory tasks after transcranial application of light (λ = 1064 nm) in middle-aged and older subjects (Vargas et al., 2017).

There are several features of our results that are worthy of further comment. First, that light did not have an impact on the activity patterns in all the regions evoked by finger-tapping; there was clear light-induced effect on the putamen, primary somatosensory and motor cortex, and parietal association cortex, but not on the thalamus and cerebellum. This finding may well have reflected on the tissue penetration properties of the transcranially applied light, with the light not being able to reach the more distant and deeper locations of the thalamus and cerebellum, distances over 20–30 mm from cranial surface (see Introduction). Nevertheless, it was striking that light could have such a clear and consistent influence on the specific regions it could reach. Of course, there remains the possibility that at least some of these changes were induced by an indirect route, in that light might have influenced the activity of one region (eg motor cortex), that then lead to a change in activity in another region (eg putamen). Such details remain to be determined. Second, that the overall effect of light was to reduce, rather than increase, the activity of these brain regions. This seemingly counterintuitive finding is not inconsistent with previous reports. For example, transcranially applied light over primary motor cortex, as measured by transcranial magnetic stimulation elicited motor evoked potential amplitudes, has been reported to reduce overall excitability for up to thirty minutes following the application of the light (λ = 905 nm; Konstantinovic et al., 2013). Further, from both in vivo and in vitro studies, light (λ = ~600–1070 nm) can inhibit the activity of many types of peripheral nerves, from radial to saphenous, resulting in a reduced transmission of sensory, in particularly nociceptive, information to the spinal cord and brain (Chow and Armati, 2016).

The precise mechanism of action of the light is not entirely clear, but it appears to involve a disturbance of several ATP dependent ion membrane pumps within the neurones. In particular, light has been reported to stimulate mitochondrial function and ATP levels and the overall activity these pumps, leading ultimately to a greater membrane stability and inhibition of neuronal firing (Konstantinovic et al., 2013; Chow and Armati, 2016). In relation to our study here, the reduction in brain activity could be viewed as a protective measure induced by light (see section below), limiting the overall excitability - and the potential for excitotoxic damage - of the neurones after they have been stimulated, in this case, by finger-tapping. This is particularly poignant for the cortical activations, where most of the neurones are glutamatergic (ie pyramidal neurones). It should be noted that there is one report, on the substantia nigra pars compacta of mice, indicating that light (λ = 710 nm) increases, rather than decreases the activity of neurones (Romeo et al., 2017). The impact of light, from across the broad red to near infrared range, on neuronal activity may therefore be dependent on the type of neurone involved and the transmitter system it may use. In particular, light could have a very different effect on the membrane pumps on the nigral dopaminergic neurones, resulting in excitation, compared to the glutamatergic pyramidal neurones of the cortex, resulting in inhibition (see above; Konstantinovic et al., 2013). Interestingly, as with the inhibition of pyramidal neurones, the excitation of nigral neurones lasts for periods long after the end of light application (Romeo et al., 2017). All in all, the effect of light on neuronal activity appears to thence last well after its application, indicating that light must activate intrinsic cellular pathways, namely transcription factors within the nucleus, that lead to long lasting changes in functional activity and patterns of survival (Hamblin, 2016).

It is also worth nothing at this stage that a limitation of our analysis was that, because the vielight device was not MRI compatible, we could not determine the effect of light on brain activity during the actual light application process. The best we could do was apply the light immediately before the scan, after baseline. Notwithstanding this limitation, a clear light effect was evident from our analysis, indicating that light can alter neuronal activity for a period well after application, in fact, following on from previous studies using either transcranial magnetic stimulation (Konstantinovic et al., 2013) or EEG recordings (Vargas et al., 2017), for at least 10 to 30 minutes thereafter. In addition, a further limitation of our study could include the use of the vielight device itself, that had LEDs placed in particular nodes over frontal (midline) and parietal bones (see Experimental Procedures), possibly biasing the impact of the light to brain regions associated with the default mode network (eg prefrontal, cingulate, precuneus, parietal and entorhinal cortex). This apparent limitation is offset by our findings that the light-induced changes across the brain were very much outside of the location of the LED nodes - for example within the putamen, primary somatosensory and motor cortex - and the reach of the light from the vielight device was not limited to the regions of the default mode network.

Light influenced the resting functional connectivity of parietal association cortex

Our results showed that the light-induced evoked activations across the brain, namely within the putamen, primary somatosensory and parietal association cortex, were not necessarily accompanied by changes in resting functional connectivity in these same regions.

While light had no major effect on the resting functional connectivity between either the putamen or primary somatosensory cortex and the rest of the brain, it did have a considerable effect on the resting connectivity of parietal association cortex. This brain region has been associated with many higher-order circuits such as attention, novelty, sensorimotor integration and visually-guided behaviour (DiMattia and Kesner 1988; Tulving et al., 1996; Whitlock, 2017). It has also been one of the main brain regions associated with the so-called default mode network, a network of highly interconnected regions of the brain that are most active when an individual is awake and at rest (Buckner, 2012; Raichle, 2015; Long et al., 2016). The network is particularly active when an individual has focus on so-called "internal tasks" such as day-dreaming and mind-wandering (Buckner, 2012; Raichle, 2015; Long et al., 2016) and tends to be correlated negatively with neural systems that focus on external visual tasks (Fox et al., 2005) and attention (Broyd et al., 2009), although it has been reported to be active in some goal-oriented functions, such as working memory (Spreng, 2012). With regard to our findings here, it remains to be determined precisely why light was associated with parietal association cortex in particular. As a working hypothesis, we suggest that it was involved in enhancing the "novelty of action and focus of attention" aspect of the finger-tapping task, linking in with the idea that light helps deactivate the default mode network (Naeser et al., 2014; Naeser and Hamblin, 2015; Naeser et al., 2016). In this case, light would deactivate the default mode network so that the novelty and attention circuits of parietal association cortex and other regions of the brain could communicate and function better.

Our findings here on the patterns of resting functional connectivity in normal subjects appear very different to those evident in patients suffering from either dementia, stroke or trauma. In a recent fMRI study in patients with dementia has reported that transcranial light application using the vielight device (λ = 810 nm) increased, after 12 weeks, the resting functional connectivity and cerebral perfusion in the parietal association cortex, as well as posterior cingulate cortex. This increase in functional connectivity was associated with the resulting clinical improvement in cognition in the patients after this long-term time-period (Chao, 2019). Similar findings of increased resting functional connectivity were evident after transcranial light application in stroke patients with chronic aphasia and in retired football players with diffuse brain damage (Marney Naeser, personal communication). It remains to be determined if these differences in normal subjects and patients was due to either the diseased/lesioned state of the brain, or to the different time-periods post light application (short-term vs long-term).

Light had no effect on resting state brain activity using fMRI

In contrast to the major influence of light on the evoked, task-positive, brain activity induced by finger-tapping, we found that light did not alter the global resting state, task-negative, brain activity in normal subjects using fMRI. Further, the effects of light on resting CBF in the clusters derived from the finger-tapping scans also indicated no major change in CBF in the putamen, primary somatosensory or parietal association cortex.

Notwithstanding the limited alteration in global resting state activity evident from our observations of CBF under fMRI, that does not rule out the possibility that neurones may undergo some change in activity after light application. For instance, there are the light-induced changes in resting functional connectivity in parietal association cortex, as noted in the present study (see above) and in patients with dementia (Chao, 2019) and other conditions (see above). Further, transcranial light application with the vielight device (λ = 810 nm) has been shown, using EEG, to influence the connectivity and integration of the brain in normal subjects at rest, together with the resting power spectrum of the different brain waves, with increases evident in the α, β and γ waves, but decreases in the Δ and Θ ones; in addition, consistent with the present findings using fMRI, light application results in an increase in inhibition using EEG (Zomorrodi et al., 2019). Similar patterns of EEG in the resting state have been reported in other studies using normal, healthy subjects (λ = 1064 nm; Wang et al 2017), as well as middle-aged to older ones suffering mild memory loss (λ = 1064 nm; Vargas et al 2017) subjects.

Taken all together, these findings suggest that when in a resting state (task-negative), light has no impact on the CBF and global brain activity, although it can influence the resting functional connectivity of some brain regions (this study; Chao, 2019) and the frequency patterns of brain waves (Zomorrodi et al., 2019). From our results here using fMRI, the impact of light becomes much more evident after a particular brain region becomes functionally active (task-positive). This is in line with the idea that when a neurone undergoes a change in its equilibrium or homeostasis, for example after it has become activated functionally (eg finger-tapping) or it becomes distressed from either disease (eg Parkinson's or Alzheimer's) or damage (eg traumatic brain injury), then an effect of light is clearer (Hamblin, 2016). Light can for example, influence the functional activity of the neurone by suppressing any overactivity (see section above), or it may help distressed neurones survive better against any insult, being neuroprotective (Johnstone et al., 2016; Mitrofanis, 2017). This key property of light, that it can aide neurones to maintain both efficient function and overall survival against insult better, appears to be a feature that may have deep evolutionary links (Mitrofanis, 2017).

Our fMRI results showed that transcranially applied light had a clear influence on brain activity, but only in regions that were functionally active, for example, after undertaking a particular task (task-positive; eg finger-tapping). These regions included the putamen, primary somatosensory and motor cortex and parietal association cortex. Light had no effect on resting state or task-negative brain activity using fMRI, although some light-induced impact on resting activity appears to be evident using other measures, such as EEG. In the evoked brain regions after finger-tapping, the overall effect of light was

to suppress or reduce activity. We suggest that these light-induced changes, particularly those in parietal association cortex, were associated with attention and novelty, and served to deactivate the so-called default mode network (Naeser et al., 2014; Naeser and Hamblin, 2015; Chao 2019; Zomorrodi et al., 2019).

The challenge that remains for future studies would be to explore whether light has similar effects on evoked brain activity when normal subjects are undertaking a series of different functional tasks, such as in memory recall tests (see Vargas et al., 2017) or undergoing somatosensory activation (eg tactile stimulation of a limb). In particular, would there be comparable light-induced changes in brain activity evident in posterior parietal association cortex after such tasks? The potential involvement and significance of light in deactivating the default mode network in these circumstances would also be a most worthwhile consideration for the future. Finally, our results here on normal subjects set a template for our future planned experiments on Alzheimer's and Parkinson's disease patients, and whether similar changes in brain activity are evident after transcranially applied light.

ACKNOWLEDGEMENTS

We thank Marney Naeser and Lew Lim for their many helpful comments on an early version of the manuscript, together with the many volunteers that participated in this study. This research was supported by the NHMRC of Australia and Tenix Corp.

CONFLICT OF INTEREST

The authors declare no competing financial interests. No author was/is associated with vielight, and the device was bought from the company's Australian outlet.

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(Received 28 July 2019, Accepted 21 October 2019)
(Available online 1 November 2019)

Chapter 3

Does photobiomodulation influence the resting state brain networks in humans?

Experimental Brain Research (2021) 239:435–449
https://doi.org/10.1007/s00221-020-05981-x

RESEARCH ARTICLE

Does photobiomodulation influence the resting-state brain networks in young human subjects?

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Received: 10 August 2020 / Accepted: 4 November 2020 / Published online: 19 November 2020

Abstract

Using fMRI (functional magnetic resonance imaging), we explored the effect of transcranial photobiomodulation on four major resting-state brain networks, namely the sensorimotor, salience, default mode and central executive networks, in normal young subjects. We used a vielight transcranial device (810 nm) and compared the scans in 20 subjects (mean age 30.0 ± 2.8 years) after active- and sham-photobiomodulation sessions. Four sets of analysis—independent components, network connectivity, infra-slow oscillatory power and arterial spin labelling—were undertaken. Our results showed that when comparing pre- with post-active and pre- with post-sham photobiomodulation scans, there were no substantial differences in activity across any of the four resting-state networks examined, indicating no clear photobiomodulation effect. When taken together with previous findings, we suggest that the impact of photobiomodulation becomes much clearer only after brain circuitry is altered, for example, after a neurone undergoes some change in its equilibrium or homeostasis, either during pathology or ageing, or during a change in functional activity when individuals are engaged in a specific task (e.g. evoked brain activity).

Keywords 810 nm · Transcranial · Resting-state brain networks · Functional magnetic resonance imaging

Abbreviations

ACC	Anterior cingulate cortex
CBF	Cerebral blood flow
EEG	Electroencephalogram
fMRI	Functional magnetic resonance imaging
ICA	Independent components analysis
MedFCtx	Medial frontal cortex
mPFC	Medial prefrontal cortex
L-PFC	Left prefrontal cortex
L-M1	Left primary motor cortex
L-S2	Left secondary somatosensory cortex
L-MidIns	Left mid-insular cortex
L-OFC	Left orbitofrontal cortex
L-TCtx	Left temporal cortex
L-VentIns	Left ventral insular cortex
PBM	Photobiomodulation
PCC	Posterior cingulate cortex
M1	Primary motor cortex
S1	Primary somatosensory cortex
pCASL	Pseudo-continuous arterial spin labelling
R-CerebCtx	Right cerebellar cortex
R-dlPFC	Right dorsolateral prefrontal cortex
R-MidIns	Right mid-insular cortex
R-PFC	Right prefrontal cortex
SEM	Standard error mean
VentPrec	Ventral precuneus
R-PACtx	Right parietal association cortex
L-PACtx	Left parietal association cortex
L-TCtx	Left temporal cortex
L-MedFCtx	Left medial frontal cortex
R-MedFCtx	Right medial frontal cortex
R-CerebCtx	Right cerebellar cortex
L-PremotorCx	Left premotor cortex
L-vlPFC	Left ventrolateral prefrontal cortex
L-dlPFC	Left dorsolateral prefrontal cortex
R-vlPFC	Right ventrolateral prefrontal cortex
R-PTACtx	Right parieto-temporal association cortex
L-PTACtx	Left parieto-temporal association cortex
SMN	Sensorimotor network
SN	Salience network

Communicated by Melvyn A. Goodale.

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DMN	Default mode network
CEN	Central executive network

Introduction

A number of studies have reported that even when individuals are not involved in a specific task—that is in a task-negative, state of rest—there are numerous on-going functional connectivities occurring between different regions of their brain. Indeed, many authors, principally with the use of functional magnetic resonance imaging (fMRI), have described a range of distinct resting-state neural networks across the brain, including the salience, central executive, sensorimotor and default mode networks. These networks are made up of anatomically separated, but functionally interconnected regions of the brain that show a high level of correlated activity in a state of rest (Biswal et al. 1995; Lee et al. 2012; Moussa et al. 2012; Raichle 2015; Shen 2015). For example, different regions of the default mode network show elevated activity when individuals are not engaged in any specific mental task, they are just "day-dreaming". This elevated activity in the default mode network in the resting-state lowers when individuals become engaged in a particular mental task, such as focusing attention on something in the external (e.g. visual tasks) or internal (e.g. meditation) environments (Raichle 2015).

Photobiomodulation, the use of red to near infrared light ($\lambda = 600–1000$ nm) on body tissues (Hamblin 2016; Mitrofanis 2019), has been shown recently to influence the functional connectivity of these resting-state networks, in particular the default mode network (Naeser and Hamblin 2015; Naeser et al. 2016, 2020; Chao 2019a, b). In young normal subjects, photobiomodulation alters the resting functional connectivity between different cortical regions responding to a simple finger-tapping task exercise (El Khoury et al. 2019). Further, in patients suffering from either traumatic brain injury, chronic stroke or Alzheimer's disease, all of which have abnormally functioning networks, photobiomodulation strengthens functional connectivities within the default mode network itself, together with its connectivity with other networks, for example the salience and central executive ones (Naeser et al. 2011, 2014, 2016, 2020; Naeser and Hamblin 2015; Chao 2019a, b). Although the precise mechanisms are not clear, this process arises after stimulation of mitochondrial activity, that then increases adenosine triphosphate (ATP) levels, leading to the expression of a number of protective and stimulatory genes within the neurones (Hamblin 2016; Johnstone et al. 2016; Mitrofanis 2017, 2019).

In this study, following on from our previous one (El Khoury et al. 2019), we sought to expand and analyse further the effect of photobiomodulation across four of the major resting-state brain networks, namely the sensorimotor, salience, default mode and central executive networks, in normal young subjects. Our results will hopefully provide for a better understanding of how photobiomodulation influences brain activity and in particular, its impact on the resting state of the brain.

Methods

Subjects

Twenty young, healthy subjects (10 of each gender; mean age 30.0 ± 2.8 years [$\pm$SEM]; 10 were Caucasian and 10 were of Asian background) were recruited for the study from the general population using an advertisement. Exclusion criteria were the current use of brain altering medication, any neurological disorder or any recent use of photobiomodulation for any purpose. Informed written consent was obtained for all procedures according to the Declaration of Helsinki and local Institutional Human Research Ethics Committees approved the study. All procedures were approved by the Human Ethics Committee of the University of Sydney.

MRI acquisition

Using a 3 T MRI scanner (Philips Achieva, 32-channel SENSE head coil), a high-resolution 3D T1-weighted anatomical image (200 axial slices, echo time = 2.5 ms, repetition time = 5600 ms, raw voxel size = 0.87mm^3) was collected. Following this, a resting-state fMRI series consisting of 180 whole brain blood oxygen level dependent functional MRI images (37 axial slices, echo time = 30 ms, repetition time = 2000 ms, raw voxel size = 3 × 3x4mm^3) and a resting-state arterial spin labelling series (108 pseudo-continuous arterial spin labelling (pCASL) images; 50 axial slices, 54 label/control image pairs, echo time = 12.7 ms, repetition time = 5310 ms, raw voxel size = 2.4 × 2.4 × 3.0mm^3, labelling time = 1650 ms, slice time = 36.6 ms, post- label delay time = 1600 ms, background suppression) were collected.

Using statistical parametric mapping 12 (Friston et al. 1994) and custom software, the fMRI image sets were slice timing corrected, realigned and movement parameters examined to ensure no subject displayed > 1 mm volume-to-volume movement in the X, Y and Z planes and 0.05 radians in the pitch, roll and yaw directions. Cardiac (frequency band of 60–120 beats per minute + 1 harmonic) and respiratory (frequency band of 8–25 breaths per minute + 1 harmonic) noises were modelled and removed using the Dynamic Retrospective Filtering toolbox (Särkkä et al. 2012). Global signal drifts were then removed using the voxel-level linear model of the

global signal detrending method described by Macey and colleagues (Macey et al. 2004). Any fMRI signal pattern correlated with the movement parameters was removed, using a method similar to the non-linear voxel-level linear model of the global signal detrending method developed by Macey and colleagues (Macey et al. 2004). The fMRI images were then co-registered to each subject's T1-weighted anatomical image, the T1-weighted image spatially normalised to the Montreal Neurological Institute template and the normalisation parameters applied to the fMRI images. This process resulted in the fMRI images being resliced into 2 × 2 × 2mm voxels. The fMRI images were then spatially smoothed using a 6 mm full-width at half maximum Gaussian filter.

All arterial spin labelling image sets were realigned, co-registered to each individual's T1-weighted image set, the label and control images averaged and a mean cerebral blood flow (CBF) image created using the subtraction method using the arterial spin labelling toolbox (Wang et al. 2008). Each subject's T1-weighted anatomical image was spatially normalised to a template in Montreal Neurological Institute space and the parameters applied to the cerebral blood flow maps. The cerebral blood flow maps were then smoothed using a 6 mm full-width at half maximum Gaussian filter.

Photobiomodulation

Each subject was removed from the MRI scanner and asked to wear the vielight-α light device (810 nm; 10 Hz pulse rate, up to 100 mW/cm^2 per diode). For the sake of consistency, all procedures were undertaken in the mornings, the period when photobiomodulation is most effective (Weinrich et al. 2019). Subjects then received either (i) a 20 min active-photobiomodulation session during which time the light was delivered ("active") or (ii) a 20 min sham session where the vielight was in place but not turned on ("sham"). Subjects were assigned randomly into either the active- or sham-group and did not know which group they were a part of (all on/off lights on the power device were completely covered/shielded from the subject's view). Immediately following this active- or sham-session, subjects were placed back into the MRI scanner and the same functional scans collected during the first MRI session.

Between 4 and 12 weeks following the first MRI session, each subject retuned for a second MRI session. During this second session, an identical series of procedures to that during the first MRI session were performed. However, if the subject was in the active-group during the first MRI session, they were placed into the sham-group for the second MRI session and vice versa.

MRI analysis

Four sets of analysis on the effect of photobiomodulation on each of the large scale, resting-state brain networks were undertaken. These analyses were:

Independent components analysis (ICA). To define the four resting-state brain networks of interest, namely the sensorimotor, salience network, default mode and central executive network, we performed an ICA using the Group ICA toolbox (Calhoun et al. 2001). Using Infomax ICA algorithm (Amari et al. 1996), we estimated 30 independent components using all four resting-state fMRI scans from each of the 20 subjects. We selected subsequently the four brain networks of interest by visual inspection. The brain network maps comprise parameter estimate values that essentially quantify the functional connectivity strength between brain regions in each network. The extent of each network was defined using a second level, one-sample random effects analysis ($p < 0.05$, false discovery rate corrected for multiple comparisons, minimum cluster 10 contiguous voxels) using the resting-state fMRI scan collected prior to the sham photobiomodulation light delivery. The resulting network brain maps were used to restrict subsequent analyses of each network. Significant differences in resting-state connectivity strengths between resting fMRI scans prior to compared with post-sham photobiomodulation and also prior to compared with post-active photobiomodulation were then determined for each of the four networks independently using second level, random effects paired groups analyses ($p < 0.05$ false discovery rate corrected for multiple comparisons, minimum cluster 10 contiguous voxels). In addition, parameter estimate values were extracted from each of the clusters in each network for each of the four resting fMRI scans. These values were then plotted and assessed for significant differences between pre- and post-sham photobiomodulation and pre- and post-active photobiomodulation using paired *t*-tests ($p < 0.05$ Bonferroni corrected for multiple comparisons, i.e. $p < 0.002$).

Network connectivity analysis. In addition to assessing differences in connectivity within each brain network using ICA, we also performed a network connectivity analysis to determine connectivity relationships between areas within a single network and between areas in all four brain networks. The mean fMRI time-series signal was extracted from all 28 regions of interest (3 for the sensorimotor, 8 for the salience, 8 for the default mode and 9 for the central executive) and correlation strengths between all possible pairs assessed by means of Pearson's correlation coefficient r-tests. Correlation matrices were obtained for pre-sham photobiomodulation, post-sham photobiomodulation, pre-active photobiomodulation and post-active photobiomodulation resting-state fMRI scans. For between-group analysis—that is, differences between pre- and post-sham photobiomodulation

and between pre- and post-active photobiomodulation—individual correlation scores were converted into z-scores using Fishers r-to-z transformation to improve normality. Parametrical statistical comparisons were then utilised by running a one-way analysis of variance test ($p < 0.05$ false discovery rate corrected).

Infra-slow oscillatory power analysis. Using the resting fMRI scans, we also determined if photobiomodulation altered the on-going pattern of activity in the four brain networks. Using the statistical parametrical mapping data processing assistant for resting-state fMRI toolbox (Chao-Gan and Yu-Feng 2010), we calculated the sum of amplitudes of low-frequency fluctuations between 0.01 and 0.25 Hz for each voxel in the brain for the pre-sham photobiomodulation, post-sham photobiomodulation, pre-active photobiomodulation and post-active photobiomodulation scans. Significant differences between pre- and post-sham photobiomodulation and between pre- and post-active photobiomodulation were determined for each voxel in each of the four brain networks using paired-group random effects procedures ($p < 0.05$ false discovery rate corrected for multiple comparisons, minimum cluster 10 contiguous voxels). In addition, infra-slow oscillatory power values were extracted from each of the clusters in each network for each of the four resting fMRI scans. These values were then plotted and assessed for significant differences between pre- and post-sham photobiomodulation and pre- and post-active photobiomodulation using paired t-tests ($p < 0.05$ Bonferroni corrected for multiple comparisons, i.e., $p < 0.002$).

Arterial spin labelling analysis. The effect of photobiomodulation on resting blood flow was assessed using the CBF maps derived from the pCASL scans. Significant CBF differences between pre- and post-sham photobiomodulation and between pre- and post-active photobiomodulation were determined for each voxel in each of the four brain networks using paired-group random effects procedures ($p < 0.05$ false discovery rate corrected for multiple comparisons, minimum cluster 10 contiguous voxels). In addition, infra-slow oscillatory power values were extracted from each of the clusters in each network for each of the four resting fMRI scans. These values were then plotted and assessed for significant differences between pre- and post-sham photobiomodulation and pre- and post-active photobiomodulation using paired *t*-tests ($p < 0.05$ Bonferroni corrected for multiple comparisons, i.e., $p < 0.002$).

Results

In the section that follows, the four sets of analysis on the effect of photobiomodulation on each of the resting-state brain networks will be presented separately (NB we found no differences in the patterns of activity between genders or ethnicities).

Independent components analysis (ICA). ICA revealed four networks consistent with the sensorimotor, salience, default mode and central executive networks as reviewed by many previous studies (Biswal et al. 1995; Lee et al. 2012; Moussa et al. 2012; Shen 2015; Raichle 2015; Mak et al. 2017). As shown in Fig. 1 and Table 1, the sensorimotor network consisted of clusters located in the left and right primary somatosensory and motor cortices, the left secondary somatosensory cortex and the left primary motor cortex. The salience network consisted of clusters located in the region of anterior cingulate cortex, posterior cingulate cortex, left ventral insula, left mid-insula and right mid-insula. We also noted some clusters within medial frontal cortex, left prefrontal cortex and right prefrontal cortex. The default mode network consisted of clusters located in the medial prefrontal cortex, ventral precuneus, right parietal association cortex, left parietal association cortex and left temporal cortex. There were also clusters evident within the right dorsolateral prefrontal cortex, right cerebellar cortex and left orbitofrontal cortex. The central executive network consisted of clusters located in the left and right medial frontal cortex, left ventrolateral prefrontal cortex, left dorsolateral prefrontal cortex, right ventrolateral prefrontal cortex, right parieto-temporal association cortex and left parieto-temporal association cortex. We also found some clusters within the right cerebellar cortex and left premotor cortex. Direct comparison of ICA maps derived from pre-sham photobiomodulation with those from post-sham photobiomodulation scans revealed no significant differences in any of the four brain networks. Similarly, no significant differences were found when comparing pre- with post-active photobiomodulation scans in any of the four networks. Further, extraction of eigenvariate values from each of the 28 clusters also revealed no significant differences between pre- and post-sham photobiomodulation or between pre- and post-active photobiomodulation scans (Fig. 2; Table 2).

Network connectivity analysis. Figure 3a shows an example correlation matrix in which correlation strengths are colour codes for rho values between each of the 28 clusters. The four black box outlines indicate correlations between areas within the one brain network. Figure 3b shows the correlation matrices for the pre-sham and post-sham photobiomodulation resting-state fMRI scans. Note that almost all areas display some level of positive signal correlations. Direct comparison of all correlations revealed that no significant correlation differences between pre- and post-sham photobiomodulation in any of the between cluster relationships. Similarly, Fig. 3c shows the correlation matrices for the pre-active photobiomodulation and post-active photobiomodulation resting-state fMRI scans. Again almost all relationships between regions are positive and again direct

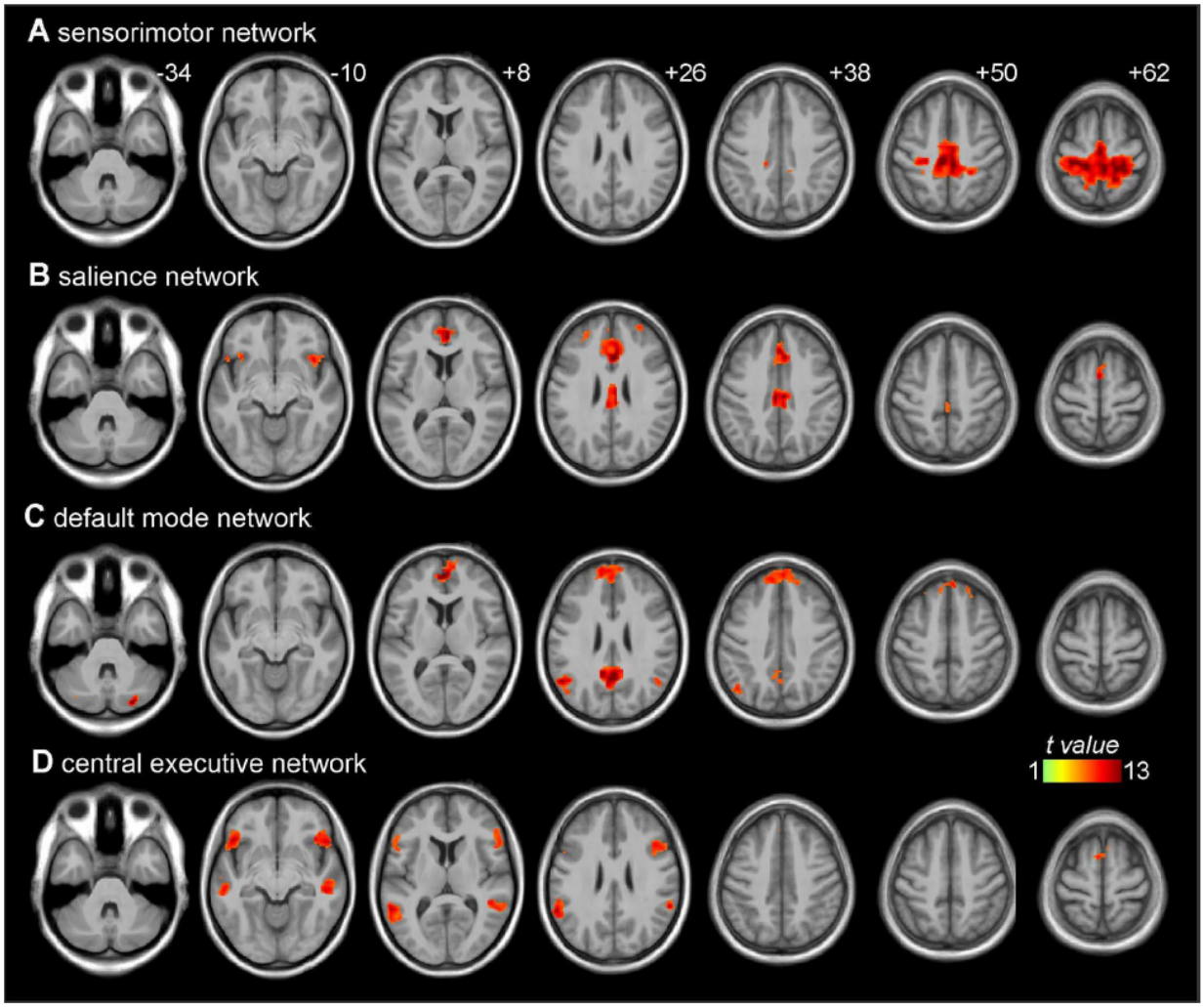

Fig. 1 Overlays of significant clusters derived from an independent components analysis of resting-state functional magnetic resonance imaging scans showing four resting brain networks: **a** sensorimotor; **b** salience; **c** default mode; and **d** central executive networks. Areas are overlaid onto axial slices of a mean T1-weighted anatomical taken from all 20 participants. Location of each axial slices in Montreal Neurological Institute space are indicated at the top right of each slice in **a**

comparison of all correlations revealed that no significant correlation differences between pre- and post-active photobiomodulation in any of the between cluster relationships.

Infra-slow oscillatory power analysis. Assessment of infra-slow oscillation power in which the power at each frequency between 0.01 and 0.025 Hz is calculated and overaged for each voxel can provide a measure of resting activity patterns (Watson 2018). Similar to the voxel-by-voxel ICA analysis, we found no significant differences in infra-slow oscillatory power in any voxel in any of the four brain networks between either pre- and post-sham photobiomodulation or between pre- and post- active photobiomodulation scans. Further, extraction of infra-slow oscillation powers from each cluster in each brain network also revealed no significant differences between pre- and post-sham photobiomodulation or pre- and post-active photobiomodulation in any of the 28 clusters (Fig. 4; Table 3).

Arterial spin labelling analysis. Significant CBF differences between pre- and post-sham photobiomodulation and between pre- and post-active photobiomodulation were determined for each voxel in each of the four brain networks using paired-group random effects procedures ($p < 0.05$, false discovery rate corrected for multiple comparisons, minimum cluster 10 contiguous voxels). Similar to the voxel-by-voxel ICA and infra-slow oscillation analyses, we found no significant differences in CBF values in any voxel in any of the four brain networks between either pre- and post-sham photobiomodulation or between pre- and

Table 1 Locations in Montreal Neurological Institute space and sizes of clusters in the four investigated brain networks: the sensorimotor network (SMN), salience network (SN), default mode network (DMN) and the central executive network (CEN)

	MNI co-ordinate			Cluster size
	X	*Y*	*Z*	
SMN				
M1/S1	6	−14	54	6511
L-S2	−40	−28	18	44
L-M1	−34	−20	44	38
SN				
MedFCtx	0	6	58	170
ACC	−2	22	20	1822
PCC	4	−32	42	932
L-PFC	−24	42	30	109
R-PFC	30	50	24	80
L-VentIns	−34	14	−16	81
L-MidIns	−36	16	2	226
R-MidIns	42	12	−4	322
DMN				
mPFC	2	48	12	1850
VentPrec	−4	−54	18	936
R-dlPFC	20	36	50	106
R-CerebCtx	26	-82	−34	28
L-OFC	−32	20	−24	37
R-PACtx	44	−56	30	104
L-PACtx	−52	−60	28	348
L-TCtx	−64	−18	−14	40
CEN				
L-MedFCtx	−4	6	58	104
R-MedFCtx	8	12	66	20
R-CerebCtx	22	−74	−28	96
L-PremotorCtx	−42	6	40	24
L-vlPFC	−44	24	−10	507
L-dlPFC	−50	16	20	61
R-vlPFC	52	24	2	903
R-PTACtx	52	−30	−8	905
L-PTACtx	−58	−50	28	1045

post-active photobiomodulation scans. Further, extraction of CBF values from each cluster in each brain network also revealed no significant differences between pre- and post-sham photobiomodulation or pre- and post-active photobiomodulation in any of the 28 clusters (Fig. 5; Table 4).

Discussion

Our main finding that photobiomodulation does not influence the activity of the resting-state brain networks, raises several issues worthy of further comment. First, although we did not find any photobiomodulation-induced changes using fMRI, previous studies have, in fact, reported changes in overall resting-state brain activity using electroencephalography (EEG; Vargas et al. 2017, λ = 1064 nm; Wang et al. 2017, λ = 1064 nm; Zomorrodi et al. 2019, λ = 810 nm). In these EEG studies, photobiomodulation was shown to influence the resting power spectrum of the different brain waves, with increases evident in the α, β and γ waves, but decreases in the Δ and Θ ones. It remains to be determined how these photobiomodulation-induced resting-state changes recorded using EEG relate to the limited changes we found in resting-state using fMRI. One possible explanation for these differences could lie in the age of the subjects used; ours were in the 20–30-year-old range while those of the previous EEG studies were much older, mainly in the 60–90-year-old range (Vargas et al. 2017; Zomorrodi et al. 2019), with some even suffering mild memory loss (Vargas et al. 2017). Hence, the ageing brain network could have some impact on how photobiomodulation influences the resting-state activity (see below).

Second, recent fMRI studies on the impact of photobiomodulation on resting-state connectivity in patients with various neurological conditions have reported very different findings to our ones here on normal subjects. In contrast to normal young individuals, patients with either dementia (Chao 2019a, λ = 810 nm), stroke (Naeser et al. 2020, λ = 633 nm, 870 nm) or with diffuse brain damage (retired footballers; Naeser et al. 2019, λ = 633 nm, 810 nm, 870 nm), transcranial photobiomodulation has been shown to increase the resting functional connectivity and cerebral perfusion in some brain regions. It is likely that photobiomodulation may have a more profound impact on the resting-state under neurological conditions, whether neurodegenerative-, vascular- or trauma-related, as well as in normal ageing (see above), compared to the resting-state under normal conditions in young individuals. Photobiomodulation may hence function to normalise the abnormal patterns of functional connectivity evident after pathology and/or ageing (Schiffer et al. 2009; Naeser et al. 2011, 2014, 2016, 2019,2020; Naeser and Hamblin 2015; Saltmarche et al. 2017; Vargas et al. 2017; Chao 2019a, b; Hipskind et al. 2019; Zomorrodi et al. 2019). In this context, a recent study in the retina of normal subjects has shown that, while having a limited effect on younger individuals (< 40 years old), photobiomodulation has a much greater impact on older ones, improving their mitochondrial performance considerably (Shinhmar et al. 2020).

Finally, a limitation of our fMRI study was that, because the photobiomodulation device was not MRI compatible, we could not use it during the scanning process. Hence, we could not measure any "live" changes in resting-state connectivity, frequency or blood flow as the photobiomodulation was being applied. A live measure could have provided a different set of results. Indeed, there is a recent report—using a

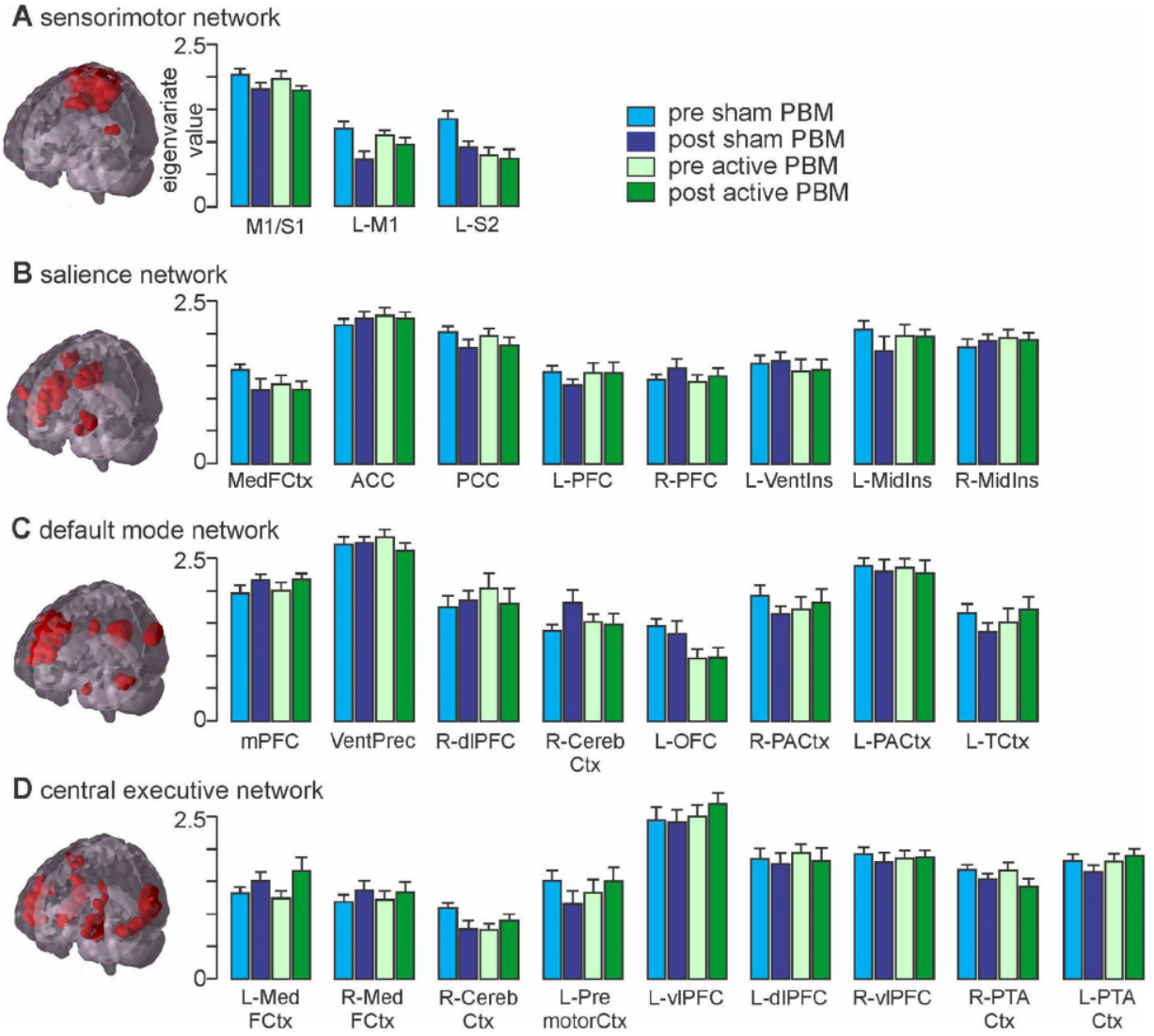

Fig. 2 Plots of mean (±SEM) eigenvariate values representing the strength of resting connectivity for each cluster of each of the four resting brain networks: **a** sensorimotor; **b** salience; **c** default mode; and **d** central executive networks. Values are plotted for the pre-sham photobiomodulation (PBM), post-sham photobiomodulation, pre-active p hotobiomodulation and post-active photobiomodulation resting-state functional magnetic resonance imaging scans. A glass brain view of each brain network is shown to the left. Note that there were no significant differences in connectivity strengths in any region between either pre- and post- sham or pre- and post- active photobiomodulation scans

MRI compatible optical fibre fitted with a ceramic ferrule—that applied photobiomodulation (808 nm) to the forehead of young individuals (mean age = 24.8 ± 4.6 years) and showed an increase in functional connectivity as the light was being applied (Dmochowski et al. 2020). However, unlike in the aged (Vargas et al. 2017; Zomorrodi et al. 2019) or those suffering pathology (Schiffer et al. 2009; Naeser et al. 2011, 2014, 2016,2019, 2020; Naeser and Hamblin 2015; Chao 2019a, b; Hipskind et al. 2019), these live effects fade soon after applications, that is, within 10 min. When taken together with our results here, we suggest that the live effect reflects a "boost" of mitochondrial activity within the neurones while the light is being applied (see below; Hamblin 2016), and can be sustained only if the neurones have a need for it, that is, if they are aged or have some pathology. In the case of young healthy neurones, they are functioning normally and, hence, have no need for a long-lasting increase in mitochondrial activity stimulated by photobiomodulation. It should be noted that a recent study in flies has indicated that effect of photobiomodulation is most profound after three

Table 2 Eigenvariate values for the four investigated brain networks: the sensorimotor network (SMN), salience network (SN), default mode network (DMN) and the central executive network (CEN)

	Sham PBM			Active PBM		
	Pre	Post	Pre vs Post uncorr p value	Pre	Post	Pre vs Post uncorr p value
SMN						
M1/S1	2.04 (0.09)	1.82 (0.10)	0.05	1.99 (0.09)	1.79 (0.07)	0.11
L-S2	1.21 (0.12)	0.72 (0.13)	0.01	1.10 (0.11)	0.96 (0.11)	0.34
L-M1	1.37 (0.12)	0.91 (0.11)	0.01	0.80 (0.14)	0.74 (0.13)	0.69
SN						
MedFCtx	1.46 (0.09)	1.15 (0.16)	0.05	1.25 (0.13)	1.17 (0.13)	0.62
ACC	2.18 (0.10)	2.28 (0.11)	0.51	2.33 (0.12)	2.27 (0.11)	0.59
PCC	2.03 (0.08)	1.79 (0.14)	0.14	1.98 (0.11)	1.82 (0.12)	0.30
L-PFC	1.41 (0.09)	1.21 (0.08)	0.03	1.39 (0.16)	1.40 (0.16)	0.94
R-PFC	1.28 (0.09)	1.45 (0.15)	0.30	1.26 (0.12)	1.33 (0.16)	0.72
L-VentIns	1.55 (0.12)	1.59 (0.13)	0.81	1.43 (0.18)	1.45 (0.16)	0.93
L-MidIns	2.04 (0.14)	1.71 (0.25)	0.21	1.97 (0.16)	1.96 (0.11)	0.94
R-MidIns	1.79 (0.11)	1.87 (0.11)	0.48	1.93 (0.13)	1.89 (0.12)	0.82
DMN						
mPFC	1.96 (0.11)	2.15 (0.08)	0.04	1.99 (0.12)	2.16 (0.09)	0.12
VentPrec	2.74 (0.13)	2.76 (0.12)	0.83	2.86 (0.14)	2.66 (0.12)	0.18
R-dlPFC	1.74 (0.19)	1.84 (0.17)	0.53	2.03 (0.25)	1.80 (0.24)	0.40
R-CerebCtx	1.37 (0.09)	1.82 (0.19)	0.02	1.52 (0.13)	1.48 (0.16)	0.77
L-OFC	1.45 (0.12)	1.34 (0.19)	0.61	1.02 (0.13)	0.97 (0.16)	0.83
R-PACtx	1.93 (0.15)	1.64 (0.12)	0.12	1.79 (0.17)	1.83 (0.20)	0.83
L-PACtx	2.38 (0.14)	2.29 (0.17)	0.55	2.34 (0.14)	2.27 (0.19)	0.61
L-TCtx	1.64 (0.14)	1.36 (0.14)	0.06	1.50 (0.23)	1.69 (0.19)	0.30
CEN						
L-MedFCtx	1.32 (0.10)	1.51 (0.15)	0.21	1.25 (0.11)	1.68 (0.21)	0.06
R-MedFCtx	1.21 (0.10)	1.38 (0.15)	0.39	1.24 (0.13)	1.36 (0.17)	0.39
R-CerebCtx	1.12 (0.07)	0.79 (0.14)	0.06	0.78 (0.10)	0.93 (0.08)	0.13
L-PremotorCtx	1.52 (0.16)	1.16 (0.21)	0.08	1.33 (0.20)	1.51 (0.22)	0.24
L-vlPFC	2.48 (0.20)	2.46 (0.18)	0.93	2.54 (0.19)	2.75 (0.17)	0.17
L-dlPFC	1.89 (0.16)	1.81 (0.16)	0.67	1.98 (0.14)	1.86 (0.21)	0.63
R-vlPFC	1.94 (0.10)	1.83 (0.13)	0.44	1.88 (0.12)	1.90 (0.10)	0.84
R-PTACtx	1.71 (0.07)	1.56 (0.08)	0.14	1.70 (0.12)	1.45 (0.11)	0.07
L-PTACtx	1.84 (0.10)	1.68 (0.09)	0.27	1.83 (0.12)	1.92 (0.11)	0.44

Values are provided for the pre-sham photobiomodulation (PBM), post-sham PBM, pre-active PBM and post-active PBM scans. Cluster location abbreviations are shown in the Results section. Significance values are presented as uncorrected for multiple comparisons. Corrected significant was set at $p < 0.002$

hours, although these were aged flies (Weinrich et al 2018). It remains to be determined if the same effect is evident in younger cases.

Taken all together, these findings indicate that the major benefits of photobiomodulation become evident only after there is some disruption to normal brain circuitry and function, after the individual neurones become aged, distressed, damaged and/or dysfunctional (Hamblin 2016). But are there circumstances, other than those in a pathological or aged neural system, when photobiomodulation has an impact on brain function? Can photobiomodulation influence the neural circuitry of a normal young individual? Recent evidence indicates that it can, in particular, when brain circuits become functionally active during a specific task (task-positive). In our previous study using the finger-tapping exercise in young subjects, we found distinct photobiomodulation-induced changes in functional connectivity between discrete regions of the brain, particularly within the parietal association cortex (El Khoury et al. 2019). We speculated that these changes were associated with attention and novelty, and served to deactivate the default mode network. We have further proposed that such a function has strong evolutionary links, relating to a survival strategy for the organism against any potentially threatening or dangerous

Experimental Brain Research (2021) 239:435–449

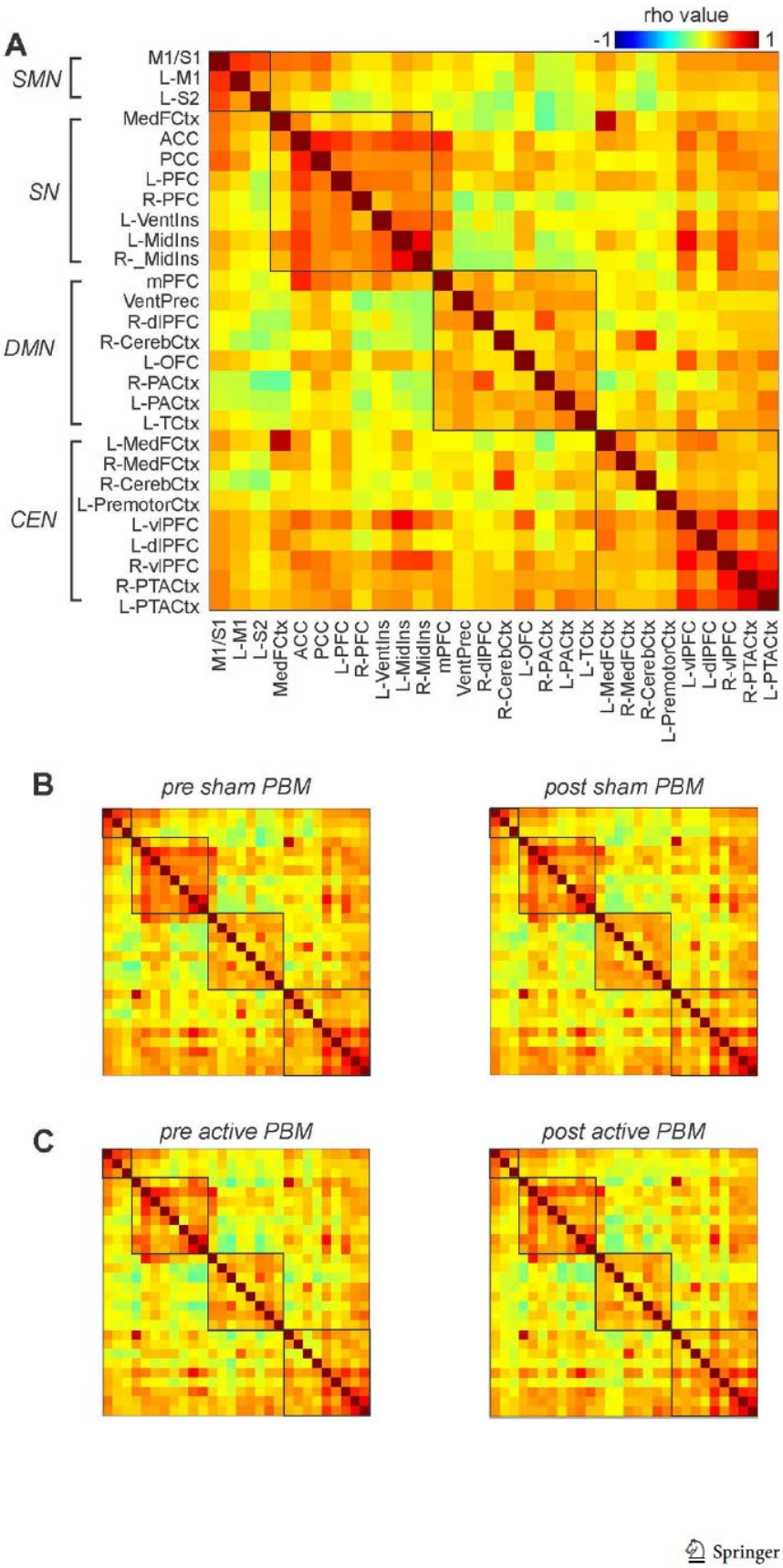

Fig. 3 **a** A correlation matrix representing the strength of resting functional connectivity for defined regions of the sensorimotor network (SMN), salience network (SN), default mode network (DMN) and central executive network (CEN). The correlation matrix is split into 28 × 28 labelled regions of interest that are categorised across each of the four brain networks and colour-coded for rho value. The main diagonal reflects those regions that were self-correlated, resulting in a full correlation of rho = 1. The four black square outlines show correlations between areas of the one brain network. **b** Correlation matrices for the same regions shown in A for the pre- sham photobiomodulation (PBM) and post- sham photobiomodulation resting-state functional magnetic resonance imaging scans. **c** Correlation matrices for the same regions shown in A for the pre- active photobiomodulation and post- sham photobiomodulation resting-state functional magnetic resonance imaging scans

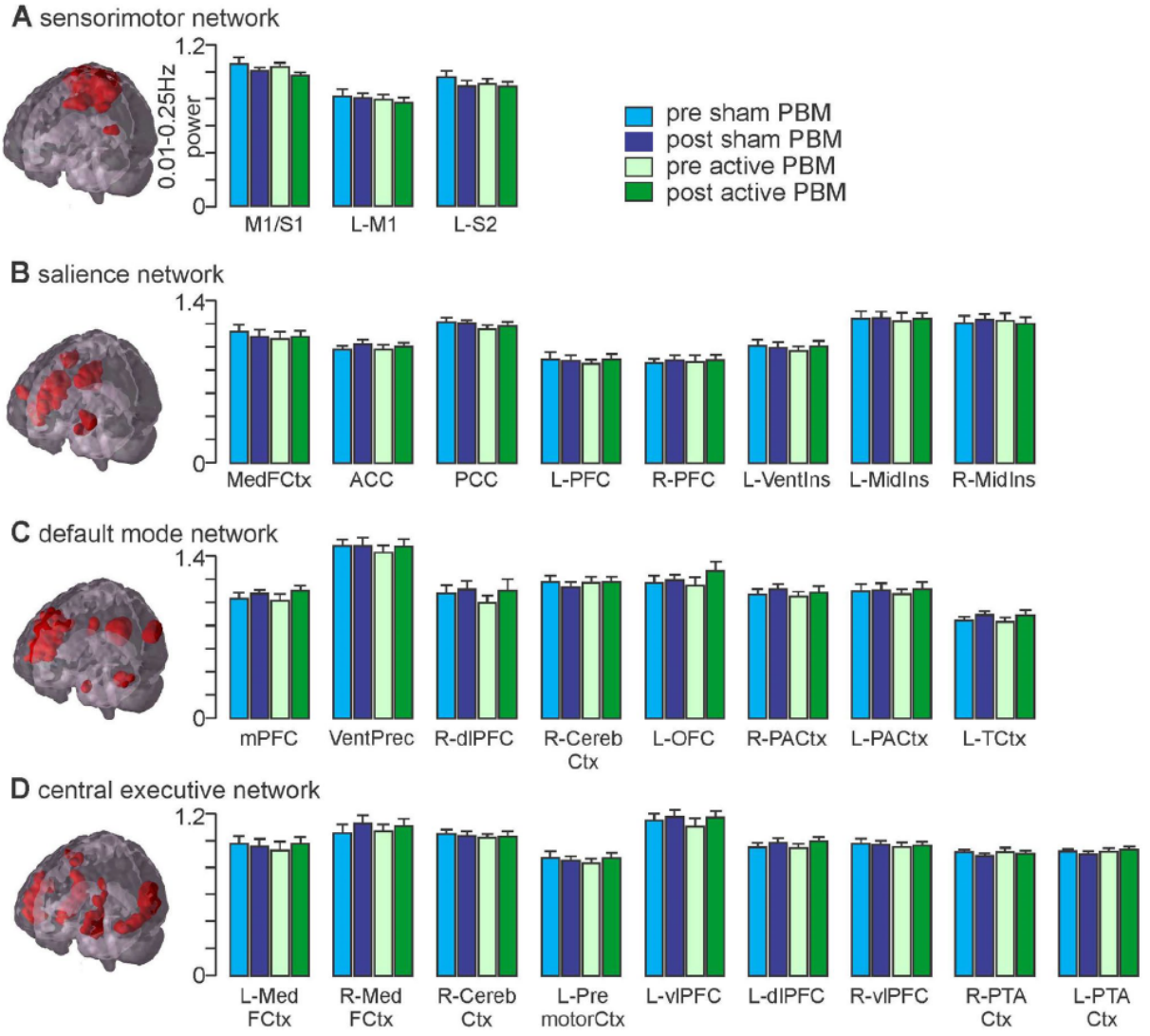

Fig. 4 Plots of mean (±SEM) infra-slow oscillatory power between 0.01–0.25 Hz for each cluster of each of the four resting brain networks: **a** sensorimotor; **b** salience; **c** default mode; and **d** central executive networks. Values are plotted for the pre- sham photobiomodulation (PBM), post-sham photobiomodulation, pre-active photobiomodulation and post-active photobiomodulation resting-state functional magnetic resonance imaging scans. A glass brain view of each brain network is shown to the left. Note that there were no significant differences in infra-slow oscillatory power in any region between either pre- and post- sham or pre- and post-active photobiomodulation scans

situation. That photobiomodulation helps the brain switch from a state of mind-wandering (default mode network) to a state of focused attention (salience and central executive networks) more readily (Mitrofanis and Henderson 2020). Hence, it appears that photobiomodulation can indeed have an influence on normal brain circuitry, in particular, during instances of evoked brain activity, when individuals are undertaking a specific task.

In conclusion, our fMRI results indicated that transcranial photobiomodulation had no effect on the four major resting-state brain networks in young normal individuals. We suggest that the impact of photobiomodulation becomes much clearer only after brain circuitry is altered, for example, after a neurone undergoes some change in its equilibrium or homeostasis, either during pathology (Schiffer et al. 2009; Naeser et al. 2011, 2014, 2016, 2019,

Table 3 Infra-slow oscillation power values for the four investigated brain networks: the sensorimotor network (SMN), salience network (SN), default mode network (DMN) and the central executive network (CEN)

	Sham PBM			Active PBM		
	Pre	Post	Pre vs Post uncorr *p* value	Pre	Post	Pre vs Post uncorr *p* value
SMN						
M1/S1	1.06 (0.04)	1.01 (0.03)	0.22	1.04 (0.03)	0.97 (0.02)	0.08
L-S2	0.82 (0.05)	0.81 (0.03)	0.84	0.80 (0.03)	0.77 (0.03)	0.43
L-M1	0.97 (0.04)	0.90(0.03)	0.18	0.91 (0.04)	0.89 (0.03)	0.47
SN						
MedFCtx	1.14 (0.06)	1.10 (0.06)	0.43	1.08 (0.07)	1.10 (0.05)	0.57
ACC	0.99 (0.04)	1.04 (0.04)	0.17	0.99 (0.04)	1.02 (0.03)	0.31
PCC	1.23 (0.04)	1.21 (0.03)	0.80	1.17 (0.03)	1.20 (0.03)	0.36
L-PFC	0.91 (0.05)	0.89 (0.04)	0.70	0.86 (0.04)	0.91 (0.04)	0.27
R-PFC	0.87 (0.04	0.90 (0.04)	0.45	0.88 (0.06)	0.90 (0.04)	0.74
L-VentIns	1.02 (0.05)	1.01 (0.04)	0.75	0.97 (0.04)	1.02 (0.04)	0.20
L-MidIns	1.26 (0.06)	1.26 (0.06)	0.91	1.24 (0.06)	1.26 (0.05)	0.71
R-MidIns	1.22 (0.05)	1.25 (0.06)	0.42	1.24 (0.06)	1.21 (0.05)	0.54
DMN						
mPFC	1.04 (0.05)	1.08 (0.04)	0.35	1.03 (0.05)	1.11 (0.04)	0.07
VentPrec	1.50 (0.06)	1.51 (0.07)	0.89	1.45 (0.05)	1.51 (0.05)	0.12
R-dlPFC	1.09 (0.07)	1.12 (0.08)	0.59	1.01 (0.06)	1.11 (0.09)	0.08
R-CerebCtx	1.19 (0.05)	1.14 (0.05)	0.42	1.18 (0.06)	1.18 (0.04)	0.91
L-OFC	1.18 (0.06)	1.21 (0.04)	0.69	1.15 (0.07)	1.28 (0.08)	0.02
R-PACtx	1.08 (0.05)	1.12 (0.04)	0.31	1.06 (0.04)	1.10 (0.04)	0.20
L-PACtx	1.10 (0.06)	1.11 (0.06)	0.82	1.07 (0.05)	1.12 (0.06)	0.27
L-TCtx	0.85 (0.04)	0.90 (0.03)	0.02	0.84 (0.04)	0.89 (0.04)	0.12
CEN						
L-MedFCtx	0.99 (0.05)	0.97(0.06)	0.66	0.94(0.06)	0.98(0.05)	0.29
R-MedFCtx	1.07 (0.06)	1.14 (0.06)	0.13	1.08 (0.04)	1.12 (0.06)	0.46
R-CerebCtx	1.06 (0.03)	1.04 (0.03)	0.64	1.03 (0.02)	1.04 (0.04)	0.89
L-PremotorCtx	0.88 (0.04)	0.86 (0.04)	0.45	0.84 (0.04)	0.88 (0.04)	0.14
L-vlPFC	1.16 (0.05)	1.18 (0.05)	0.50	1.11 (0.05)	1.19 (0.04)	0.07
L-dlPFC	0.96 (0.04)	1.00 (0.04)	0.15	0.95 (0.03)	1.00 (0.03)	0.08
R-vlPFC	0.99 (0.03)	0.98 (0.03)	0.62	0.96 (0.04)	0.98 (0.03)	0.61
R-PTACtx	0.92 (0.02)	0.90 (0.01)	0.14	0.93 (0.03)	0.92 (0.02)	0.70
L-PTACtx	0.93 (0.01)	0.91 (0.01)	0.31	0.92 (0.02)	0.94 (0.02)	0.56

Values are provided for the pre-sham photobiomodulation (PBM), post-sham PBM, pre-active PBM and post-active PBM scans. Cluster location abbreviations are shown in the Results section. Significance values are presented as uncorrected for multiple comparisons. Corrected significant was set at $p < 0.002$

2020; Naeser and Hamblin 2015; Chao 2019a, b; Hipskind et al. 2019) or ageing (Vargas et al. 2017; Zomorrodi et al. 2019; Shinhmar et al. 2020), or under normal circumstances during a change in functional activity when individuals are engaged in a specific task (e.g. evoked brain activity; El Khoury et al. 2019).

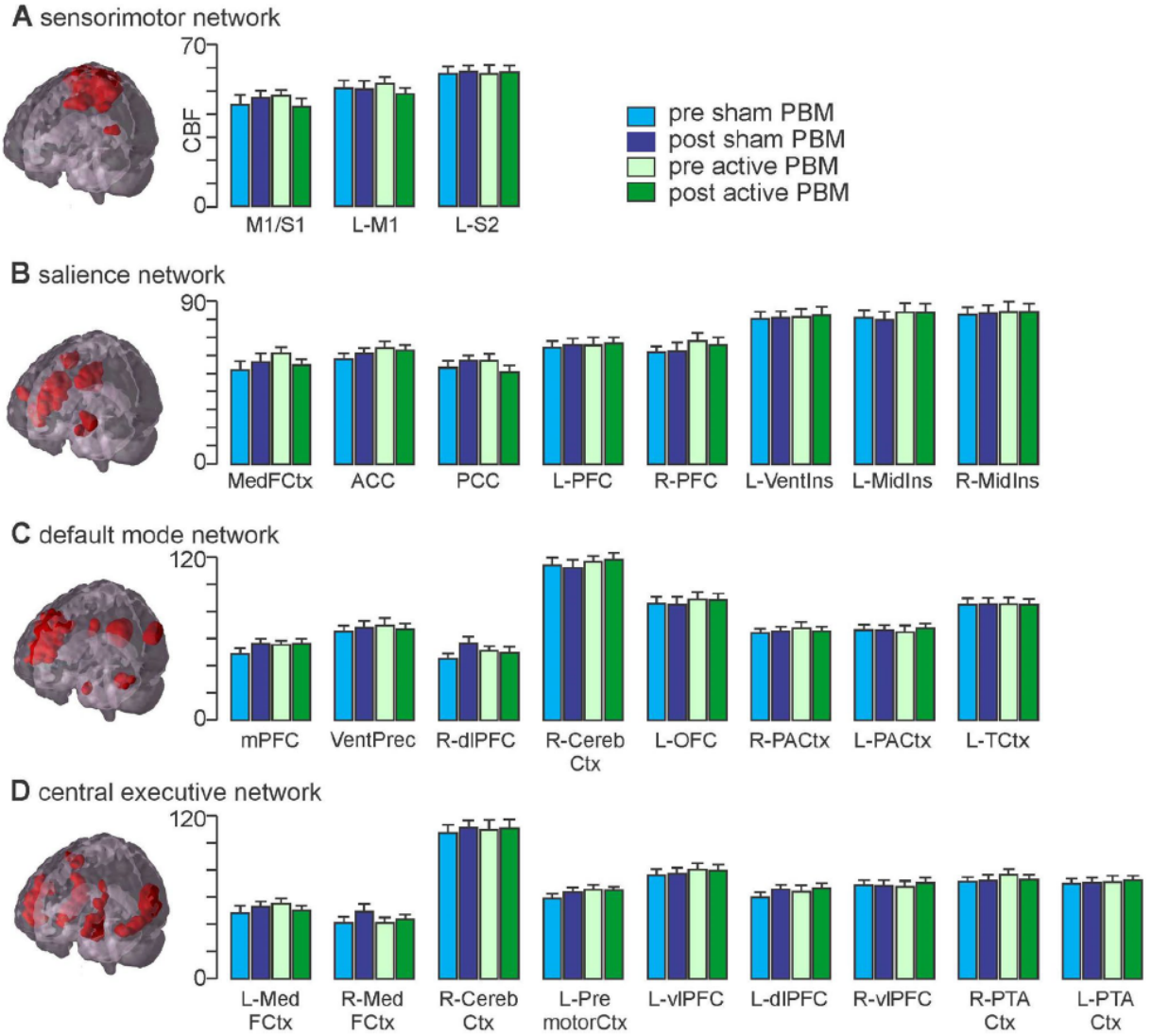

Fig. 5 Plots of mean (±SEM) cerebral blood flow (CBF) in ml/min/100 g for each cluster of each of the four resting brain networks: **a** sensorimotor; **b** salience; **c** default mode; and **d** central executive networks. Values are plotted for the pre-sham photobiomodulation (PBM), post- sham photobiomodulation, pre-active photobiomodulation and post-active photobiomodulation resting-state arterial spin labelling scans. A glass brain view of each brain network is shown to the left. Note that there were no significant differences in connectivity strengths in any region between either pre- and post-sham or pre-and post-active photobiomodulation scans

Table 4 Cerebral blood flow (CBF) values (ml/min/100 g) for the four investigated brain networks: the sensorimotor network (SMN), salience network (SN), default mode network (DMN) and the central executive network (CEN)

	Sham PBM			Active PBM		
	Pre	Post	Pre vs Post uncorr *p* value	Pre	Post	Pre vs Post uncorr *p* value
SMN						
M1/S1	44.4 (4.3)	47.3 (3.2)	0.48	48.1 (2.7)	43.5 (3.7)	0.21
L-S2	51.4 (3.3)	51.0 (3.1)	0.85	53.3 (3.0)	49.1 (2.4)	0.09
L-M1	57.8 (3.1)	58.7 (2.7)	0.67	57.8 (4.0)	58.4 (2.9)	0.84
SN						
MedFCtx	51.9 (5.1)	56.6 (4.5)	0.37	61.4 (3.6)	54.6 (3.5)	0.37
ACC	58.0 (3.3)	61.2 (3.3)	0.17	64.2 (3.7)	62.8 (3.0)	0.67
PCC	53.2 (4.1)	57.1 (2.9)	0.29	57.4 (3.6)	51.0 (3.5)	0.13
L-PFC	64.6 (3.5)	65.9 (3.6)	0.57	66.0 (4.1)	66.8 (3.4)	0.82
R-PFC	61.8 (3.5)	62.3 (5.1)	0.21	68.1 (4.5)	66.0 (4.1)	0.39
L-VentIns	80.2 (4.1)	80.8 (4.2)	0.91	81.5 (4.5)	82.8 (4.1)	0.76
L-MidIns	81.0 (4.1)	80.0 (4.4)	0.68	84.5 (4.5)	84.4 (4.4)	0.98
R-MidIns	82.7 (4.1)	83.3 (4.7)	0.80	84.1 (5.9)	84.3 (4.6)	0.98
DMN						
mPFC	50.0 (3.7)	57.0 (3.7)	0.02	56.3 (2.8)	57.1 (3.7)	0.71
VentPrec	66.0 (4.6)	69.0 (4.8)	0.34	70.5 (6.0)	67.7 (4.2)	0.58
R-dlPFC	45.9 (4.0)	57.3 (4.9)	0.08	52.0 (3.1)	50.4 (4.3)	0.81
R-CerebCtx	115.3 (6.8)	112.9 (7.7)	0.51	117.9 (7.9)	119.5 (6.3)	0.83
L-OFC	86.8 (4.7)	86.5 (5.4)	0.93	89.8 (5.8)	89.1 (5.0)	0.91
R-PACtx	64.7 (3.1)	66.3 (3.2)	0.61	68.7 (4.2)	66.4 (3.5)	0.31
L-PACtx	67.3 (3.8)	67.2 (3.2)	0.96	65.6 (4.7)	68.4 (3.5)	0.46
L-TCtx	86.2 (4.6)	86.3 (4.4)	0.95	86.3 (5.1)	86.1 (3.7)	0.97
CEN						
L-MedFCtx	48.8 (4.4)	53.5 (3.9)	0.34	55.9 (3.3)	50.4 (3.2)	0.15
R-MedFCtx	41.2 (4.4)	49.4 (5.7)	0.19	41.2 (3.8)	43.5 (4.0)	0.63
R-CerebCtx	107 (6.4)	111.7 (5.2)	0.80	109.9 (7.8)	111.7 (6.2)	0.79
L-Premo torCtx	59.5 (3.5)	64.0 (3.7)	0.06	66.0 (3.5)	65.4 (2.8)	0.83
L-vlPFC	76.5 (4.5)	77.4 (4.3)	0.74	80.8 (4.5)	80.0 (4.5)	0.86
L-dlPFC	60.4 (3.5)	66.3 (3.2)	0.02	64.6 (3.8)	67.0 (3.5)	0.41
R-vlPFC	69.1 (3.4)	68.8 (4.2)	0.88	68.0 (4.5)	70.1 (3.9)	0.53
R-PTACtx	71.9 (3.0)	73.0 (3.9)	0.63	76.9 (4.3)	73.3 (3.7)	0.08
L-PTACtx	70.5 (3.3)	71.1 (3.2)	0.74	71.7 (4.6)	73.0 (3.3)	0.69

Values are provided for the pre-sham photobiomodulation (PBM), post-sham PBM, pre-active PBM and post-active PBM scans. Cluster location abbreviations are shown in the Results section. Significance values are presented as uncorrected for multiple comparisons. Corrected significant was set at $p < 0.002$

Acknowledgements We thank Marney Naeser and Paula Martin for their most valuable comments and suggestions on an early version of the manuscript. We also thank the many volunteers in this study.

Funding This research was supported by the NHMRC of Australia and Tenix corp.

Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests. No author was/is associated with vielight, and the device was bought from the company's Australian outlet.

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Chapter 4

The "buckets": the use of red and infrared light helmets in Parkinson's disease patients

Photobiomodulation, Photomedicine, and Laser Surgery
Volume 37, Number 10, 2019

Pp. 615–622
DOI: 10.1089/photob.2019.4663

"Buckets": Early Observations on the Use of Red and Infrared Light Helmets in Parkinson's Disease Patients

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Abstract

Background: Parkinson's disease is a well-known neurological disorder with distinct motor signs and nonmotor symptoms.
Objective: We report on six patients with Parkinson's disease that used in-house built photobiomodulation (PBM) helmets.
Methods: We used "buckets" lined with light-emitting diodes (LEDs) of wavelengths across the red to near-infrared range (i.e., 670, 810, and 850 nm; $n=5$) or an homemade intranasal LED device (660 nm; $n=1$). Progress was assessed by the patients themselves, their spouse, or their attending medical practitioners.
Results: We found that 55% of the initial signs and symptoms of the six patients showed overall improvement, whereas 43% stayed the same and only 2% got worse. We also found that PBM did not target a specific sign or symptom, with both motor and nonmotor ones being affected, depending on the patient.
Conclusions: In summary, our early observations are the first to note the impact of PBM on patients' signs and symptoms over an extended period, up to 24 months, and lays the groundwork for further development to clinical trial.

Keywords: Parkinson's disease, photobiomodulation, LED helmet, 670 nm, 810 nm

Introduction

The application of red to near-infrared light ($\lambda=600$–1070 nm) to body tissues, known also as photobiomodulation (PBM), has been used to treat many neurological conditions in humans, including Parkinson's disease,[1–5] Alzheimer's disease,[6] depression,[7,8] traumatic brain injury,[9,10] age-related macular degeneration,[11] stroke,[12] and lower back pain.[13,14] In each of these explorations, as in all those involving experimental animals,[15] PBM leads to beneficial outcomes, including improved cognition, mood, sight, memory, and movement.

A key feature of PBM, at least from animal models of Parkinson's and Alzheimer's disease, is that it can be neuroprotective, being able to slow the degenerative process.[15–17] This feature makes the treatment most appealing for use in humans, mainly because all of the current treatments for both diseases are symptomatic and not neuroprotective.

In this study, we document six Parkinson's disease patients who used PBM therapy. Five patients used in-house built helmets, buckets lined with light-emitting diode (LED) devices of wavelengths across the red to near-infrared range (i.e., 670, 810, and 850 nm), whereas one patient used an homemade intranasal LED device (660 nm). Two of these patients were reported on previously,[5] but in this study, we provide updates on their progress; the other four were entirely new cases. This report, as with our previous one,[5] was not part of any systematic research study nor randomized clinical trial with placebo controls, it was simply a series of observations made by the patients, carers and, in particular, their attending medical practitioners. In fact, the first bucket helmets were made by one of medical practitioners (C.H.) to potentially help the patients alleviate their signs and symptoms, many of whom, were referred to by a specialist (F.N.). Even though we had a relatively small number of cases ($n=6$), the early findings were most encouraging. Our report serves to alert others with similar conditions of the potential benefits of PBM therapy and forms a template for future clinical trials.[18,19]

Case Descriptions

In the section below, the impact of PBM will be described separately for each patient. The use of PBM was voluntary and progress was assessed by the patients themselves, their carer, and in particular their attending medical practitioners

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(general practitioner and geriatrician). In fact, the medical practitioners (C.H. and F.N.) were in a position to monitor all six patients, hence providing some consistency in the overall assessment. All patients consented for their case to be included in this report.

Tables 1 and 2 show various features of the bucket helmets constructed for each patient, their main signs and symptoms, together with the impact of PBM. As is evident from Table 1, the different patients had a range of different signs and symptoms, reflective of the heterogeneous nature of the disease.[20,21] Further, there were some variations in the construct of the bucket helmets in the different cases, because each was built as each new patient came on board to try them out. Some of the details of power and energy for the bucket helmets were not known, but what we did know is noted in Tables 1 and 2. The one, major factor that the different bucket helmets did have in common, and in fact made them unique, was that they all incorporated two wavelengths, in the red (e.g., 670 nm) and near-infrared (e.g., 810 and 850 nm) ranges. The use of the two wavelengths was because animal experiments have shown that using two wavelengths, sequentially, offers more beneficial outcomes than just the one wavelength alone.[22]

Patient PN

PN, a 63-year-old male, was diagnosed two and a half years previously. His major signs and symptoms included: resting tremor, akinesia, gait change, impaired fine motor skills and facial movement, trouble sleeping and swallowing, persistent cough, fatigue, low self-esteem, and depression (Table 1). His daily medications included levodopa/carbidopa and benztropine.

Nine months after diagnosis, PN started using a PBM helmet, a modified bucket lined internally with strips of 670 and 810 nm LEDs (e.g., Fig. 1A, B; redlightsonthebrain.blog; Table 1). His exposure was 10 min for each wavelength twice daily. Four weeks after first use, there was a noticeable reduction in his tremor. After 8 weeks, he was walking faster, sleeping better, had more facial animation, more energy, coughed less, swallowed more easily, and felt more confident and less depressed. Over the next few months, all of these improvements stabilized, and PN and his family felt that PBM had restored many features of his previous day-to-day activities and self-confidence. His overall fine motor function had improved also and he resumed home renovations.

For a more objective analysis, we measured PN's writing by using ImageJ software. Figure 2A shows a sample of a seven-word sentence that he wrote before PBM therapy began (time point 1) and after 24 months (time point 5). This last time point was a new addition to our analysis; our previous analysis of PN's writing spanned only 10 months.[5] The words were outlined (red lines, Fig. 2A) and the program calculated the area (Fig. 3A) and the perimeter of distance (Fig. 3B) of each word. Our analysis indicated that, although there was a reduction at time point 5, this change did not reach significance for either area (Fig. 3A; ANOVA one-way: $F=0.7$, $p=0.61$) or perimeter of distance (Fig. 3B; ANOVA one-way: $F=0.6$, $p=0.68$) of each word ($n=7$). Hence, over this extensive 2-year period, there was no clear deterioration in his writing, it still being very legible.

In summary, of PN's 12 initial signs and symptoms, including writing, eleven improved (90%) after PBM, while one stayed the same (10%) and none deteriorated (Fig. 4; Table 1). His medication during this period did not change. In recent times, PN has started using a ''Well Red Coronet'' (e.g., Fig. 1C, D), made from thin aluminium sheets lined with red (670 nm) and near-infrared (810 nm) LEDs (www.wellred.com.au).

Patient MH

MH, a 61-year-old male, was diagnosed 6 years previously. His major signs and symptoms included: resting tremor, impaired fine motor skills and facial movement, gait change (reduced stride), fatigue, apathy, difficulty maintaining thoughts, low self-esteem, hesitant speech, and trouble sleeping. His daily medications included levodopa/carbidopa.

Three and half years after diagnosis, MH started using a 670 and 810 nm LED bucket (redlightsonthebrain.blog). His exposure time was 10 min for each wavelength twice daily (Table 1). After a month, MH reported that he had resumed his usual activities, was more confident, socially interactive, and could think more clearly. Over the next few months, improvements in his sleep, speech, and gait became evident, together with his face being more animated. Further, MH reported that he had much more energy and reduced tremor. Over the next 2 years, these improvements have been maintained and he enjoys an active lifestyle. About 12 months previously, he developed dystonia in this right foot, together with a sleep disturbance (dream enactment). These have not however, deteriorated any further over the last 12 months. His fine motor skills have improved also; he recently resumed being able to tie a fly onto a fishing line and he now requires little help doing up his shirt buttons.

Figure 2B shows a sample of a 13-word sentence that MH wrote before PBM therapy began (time point 1) and after 24 months (time point 5). The graphs in Fig. 3 indicate no differences in either area (Fig. 3A; ANOVA one-way: $F=0.4$, $p=0.71$) or perimeter of distance (Fig. 3B; ANOVA one-way: $F=0.3$, $p=0.77$) of each word analyzed ($n=13$). Hence, although no improvements were evident, there was no deterioration over this extensive period. It should be noted that our original analysis of MH's writing—that spanned only 3 months—indicated an improvement in area and perimeter of words.[5] Our current, more extensive analysis of his writing spanning 24 months, showed that his writing stabilized and, most importantly, did not get worse.

In summary, of MH's eleven initial signs and symptoms, including his writing, seven improved ($\sim$90%) after PBM, while one stayed the same ($\sim$10%) and none deteriorated (Fig. 4; Table 1). His medication during this period did not change. MH developed dystonia and dream enactment 12 months ago and they have not deteriorated any further since then. Recently, MH has started using a coronet also (e.g., Fig. 1C and D).

Patient CB

CB, a 64-year-old male, was diagnosed 12 years previously. His daily medications were an apomorphine pump, apomorphine hydrochloride, levodopa, and benserazide. His major signs and symptoms were: slow gait, muscle spasms

Table 1. Table Summarizing the Different Protocols, Bucket Devices, Features of Disease, and Effects of Photobiomodulation on the Six Patients in This Report

Patient	*Disease duration*	*Wavelengths and dose protocol*	*Start of PBM after diagnosis*	*PBM duration*	*Start of beneficial PBM effects*	*PBM impact on motor signs*	*PBM impact on nonmotor symptoms*
PN (63 years old)	2.5 Years	670 + 810 nm 10 min/twice daily	9 Months	24 Months	1 Month	Tremor (↙), akinesia (↙), gait (↙), fine motor skills (↙), facial movement (↙), writing (-)	Sleeping (↙), swallowing (↙), cough (↙), fatigue (↙), self-esteem (↙), depression (↙)
MH (61 years old)	6 Years	670 + 810 nm 10 min/twice daily	3.5 Years	24 Months	1 Month	Tremor (↙), fine motor skills (↙), facial movement (↙), gait (↙), writing (-)	Fatigue (↙), apathy (↙), thinking (↙), self-esteem (↙), speech (↙), sleeping (↙)
CB (64 years old)	12 Years	670 + 850 nm 20 min/daily	11 Years	12 Months	8 Months	Gait (↙), muscle spasms (-), stiffness (-), lockouts (↙), writing (-)	Swallowing (-), speech (↙), bladder urgency (-), itchiness (-), sleeping (-), stress (↙), low self-esteem (↙), social interaction (↙), depression (↙)
SS (64 years old)	4 Years	670 + 810 nm 30 min/daily	2 Years	12 Months	3 Months	Tremor (x), gait (↙), muscle cramps (↙), stiffness (↙), writing (-)	Constipation (-), sweating (↙), swallowing (-)
TU (73 years old)	14 Months	670 + 810 nm 30 min/daily	14 Months	14 Months	14 Months	Tremor (-), writing (-)	Dream enactment (-)
ML (75 years old)	14 Years	660 nm (nasal) 20 min/day	~13 Years	8 Months	8 Months	Tremor (-), cogwheel rigidity (-), facial movements (↙), gait (-), writing (-)	Smell (-), fatigue (↙), anxiety (-), thinking (-), urinary frequency (↙), constipation (↙), memory (-), depression (↙), sleep (↙), restless leg (-), dream enactment (↙)

The symbol (↙) next to a sign or symptom represents an improvement after PBM, while the symbol (-) represents no change and the symbol (x) represents a worsening.
PBM, photobiomodulation.

Table 2. Table to Report Bucket Parameters in Patients

Manufacturer	C & D Hamilton
Model identifier	Eliza
Year produced	2016
No. and type of emitters (laser or LED)	670 nm and 810 ($n=150$); 850 nm ($n=120$) All as LED strips
Wavelength and bandwidth (nm)	670 nm, 810 nm, 850 nm; bandwidth unknown
Pulse mode (CW or Hz, duty cycle)	Continuous wave
Beam spot size at target (cm^2)	N/a
Irradiance at target (mW/cm^2)	N/a
If pulsed peak irradiance (mW/cm^2)	N/a
Exposure duration (sec)	670 nm, 810 nm, 850 nm; 600–900 sec
Radiant exposure (J/cm^2)	670 nm = 6.96 W used, efficiency unknown 810 nm = 26.4 W used, efficiency unknown 850 nm = 6 W used, efficiency unknown
Radiant energy (J)	Variable, range unknown
No. of points irradiated	N/a
Area irradiated (cm^2)	Head
Application technique	Transcranial
No. and frequency of treatment sessions	1–2 Daily
Total radiant energy over entire treatment course (J)	Ongoing

LED, light-emitting diode.

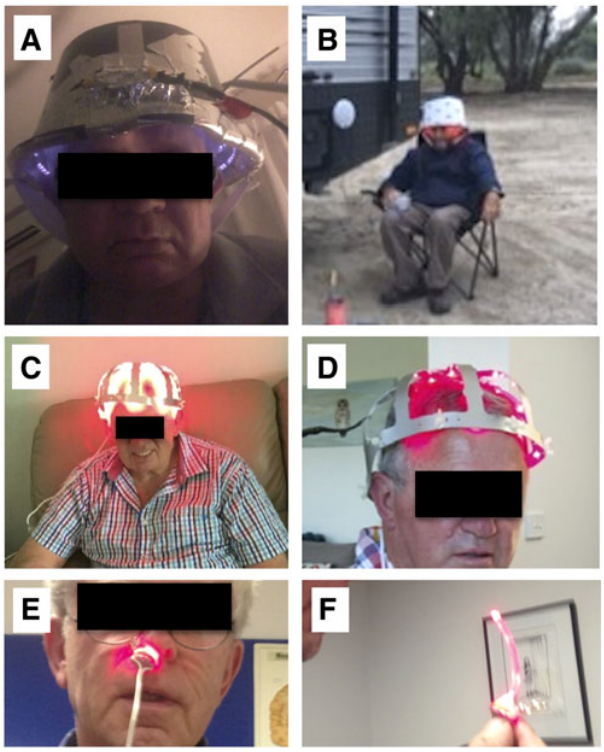

FIG. 1. The light "bucket" helmets, lined with red (670 nm) and near-infrared (810 and 850 nm) LED lights. The original helmets were constructed from either buckets (patient CB) **(A)** or lampshades (patient MH) **(B)**. Some patients—CB **(C)**, PN **(D)**, MH, SS—have recently started using the "coronets," made from thin aluminium sheets lined with red (670 nm) and near-infrared (810 nm) LEDs (www.wellred.com.au). **(E, F)** A homemade intranasal device of 660 nm LED used by one of the patients (ML). LED, light-emitting diode.

and stiffness, trouble swallowing, soft voice, bladder urgency, itchy feet, difficulty sleeping, and stress. He suffered from "lockouts," during which he would feel "frozen with stiffness." These occurred in the periods when the apomorphine pump was switched off, every 2 h. During these lockouts, CB used a rescue dose of apomorphine. His condition had made social interaction very difficult, he had poor tolerance to change in daily routine, his enjoyment of life was limited, and his confidence was low.

Some 11 years after diagnosis, CB started using a 670 nm LED bucket; 4 months later, 850 nm LEDs were added and he used the two wavelengths forthwith (redlightsonthebrain .blog). His exposure time was 20 min/day (Table 1). After 8 months, CB and his wife noted some subtle but distinct changes. They found that his daily number of lockouts had reduced; on most days, where he would suffer three a day, he now suffered none. He still had the occasional lockout however, and on these "bad" days, they tended to be more intense than before. Nevertheless, their frequency was lower. CB also measured the amount of time between his levodopa medication. Before PBM therapy, he would medicate every 75 min, while with PBM, this had increased to 90 min. A small, yet consistent difference. His speech had more volume and was a little quicker than before. His gait improved also, being quicker and with more arm movement. CB's anxiety improved and with it, his ability to tolerate routine changes and social interactions. His wife commented that he was now able to do more and they "have more of a life now."

Figure 3 shows an analysis of CB's writing over a period of 12 months. There was little change in either area (Fig. 3A; ANOVA one-way: $F=0.3$, $p=0.89$) or perimeter of distance (Fig. 3B; ANOVA one-way: $F=1.2$, $p=0.32$) of each word analyzed ($n=11$). Hence, although no improvements were evident, there was no deterioration over this 12-month period.

In summary, of CB's 14 initial signs and symptoms, including his writing, 7 improved (~50%) after PBM, while 7 stayed the same (~50%) and none deteriorated (Fig. 4;

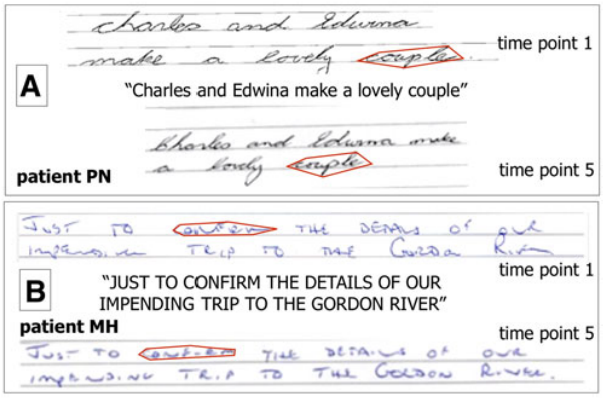

FIG. 2. Analysis of writing from patient PN **(A)** and MH **(B)**: analysis from before light (time point 1) and during its course (time point 5), 24 months after commencement of therapy. Each word of their sample was outlined (red lines) and the program calculated the area and perimeter of distance of the words.

Table 1). His medication during this period did, in fact, reduced slightly. Recently, CB has started using a coronet (e.g., Fig. 1C and D).

Patient SS

SS, a 64-year-old male, was diagnosed 4 years previously. His medications were levodopa and carbidopa twice daily. His major signs and symptoms were: resting tremor, gait change, muscle cramps and stiffness, constipation, profuse sweating on exertion, and difficulty swallowing.

Two years after diagnosis, SS started using a 670 nm LED bucket (redlightsonthebrain.blog) for 30 min daily (Table 1). After 3 months, SS and his wife reported faster times for his daily run. This was followed at 8 months by improvements in his walking, being much quicker and with more arm movement. At about this time, SS added 810 nm LEDs to his bucket, and used the two wavelengths sequentially for 15 min daily. He also noted improvements in sweating, muscle cramps, and stiffness. His tremor however, fluctuated on a day-to-day basis. At 5 months after first use of PBM therapy, his medication was increased from two to three times per day, in response to his tremor fluctuations. The improvements in SS's running, sweating, muscle cramps, and stiffness were evident before this increase occurred.

Figure 3 shows an analysis of SS's writing over a period of 12 months. There was little change in either area (Fig. 3A; ANOVA one-way: $F=0.4$, $p=0.8$) or perimeter of distance (Fig. 3B; ANOVA one-way: $F=0.2$, $p=0.91$) of each word analyzed ($n=12$). Hence, although no improvements were evident, there was no deterioration over this period.

In summary, of SS's eight initial major signs and symptoms, including his writing, four improved (~55%) after PBM, while three stayed the same (~35%) and one deteriorated (10%; Fig. 4; Table 1). Although his medication did increase during this period, many of SS's improvements were evident before this increase occurred. Recently, SS has started using a coronet (e.g., Fig. 1C and D).

Patient TU

TU, a 73-year-old male, was diagnosed over 14 months previously, having developed a resting tremor in his right hand. He also, on occasion, suffered from dream enactment. Otherwise, he was free of the usual Parkinsonian signs and symptoms, for example, akinesia and postural instability. Levodopa was tolerated poorly and discontinued quickly.

Soon after diagnosis, TU started using a 670 and 810 nm LED bucket (redlightsonthebrain.blog), 15 min each wavelength daily (Table 1). After 14 months, TU's tremor was observed by his attending medical practitioners to be no worse than at diagnosis and, on some occasions, not evident at all. The frequency of his dream enactments had not changed also, still being "occasional." Further, even after this extended time period, he had not developed any other Parkinsonian sign or symptom.

Figure 3 shows an analysis of TU's writing over a period of 14 months. There was little change in either area (Fig. 3A; ANOVA one-way: $F=0.3$, $p=0.88$) or perimeter of distance (Fig. 3B; ANOVA one-way: $F=0.2$, $p=0.91$) of each word analyzed ($n=9$). Hence, although no improvements were evident, there was no deterioration over this period.

In summary, although there was no clear improvement in TU's two initial signs and symptoms after PBM, they, including his writing, all stabilized and did not get any worse (Fig. 4; Table 1). He is still not on any medication. Recently, TU has started using a coronet (e.g., Fig. 1C and D).

Patient ML

ML, a 75-year-old male, was diagnosed 14 years previously. His major signs and symptoms were: tremor, cogwheel rigidity, impaired facial movements and gait, diminished sense of smell, fatigue, anxiety, slowed thinking, mild urinary frequency and constipation, memory impairment, depression, troubled sleep, restless leg, and dream enactment. His daily medications included levodopa/carbidopa, rasagiline and pramipexole. He was also using an antidepressant (venlafaxine) at the time and had trialed isradipine (a calcium channel blocker).

ML is a retired general surgeon and an amateur electronics buff. Rather than make a bucket, he developed an intranasal red light (660 nm) device that he could insert a fair distance through the nasal cavity (~7 cm; Fig. 1E, F), placing the PBM source tip close to the bone that covers the brainstem (i.e., sphenoid). His thinking was to get the PBM source as close to the diseased brainstem dopaminergic neurons as possible. With some skepticism, ML commenced treatment with his intranasal device, for 20 min/day, about 8 months ago (Table 1).

Soon thereafter the first use of his device, ML started feeling much better. His wife (who also has a medical degree), and attending medical practitioner, concurred with his much improved status, noting his better mood, improved facial movements, and energy. ML's urinary frequency and constipation resolved, his sleep was much improved, and there was less dream enactment. Over the next few months these improvements continued, so much so that ML felt "on top of the world."

Figure 3 shows an analysis of ML's writing from two sentence samples; one spans a period of about 2 years (ML1), while the other spans a period of 11 years (ML2). The two samples were different sentences, each from time points well before and during PBM therapy. There was little

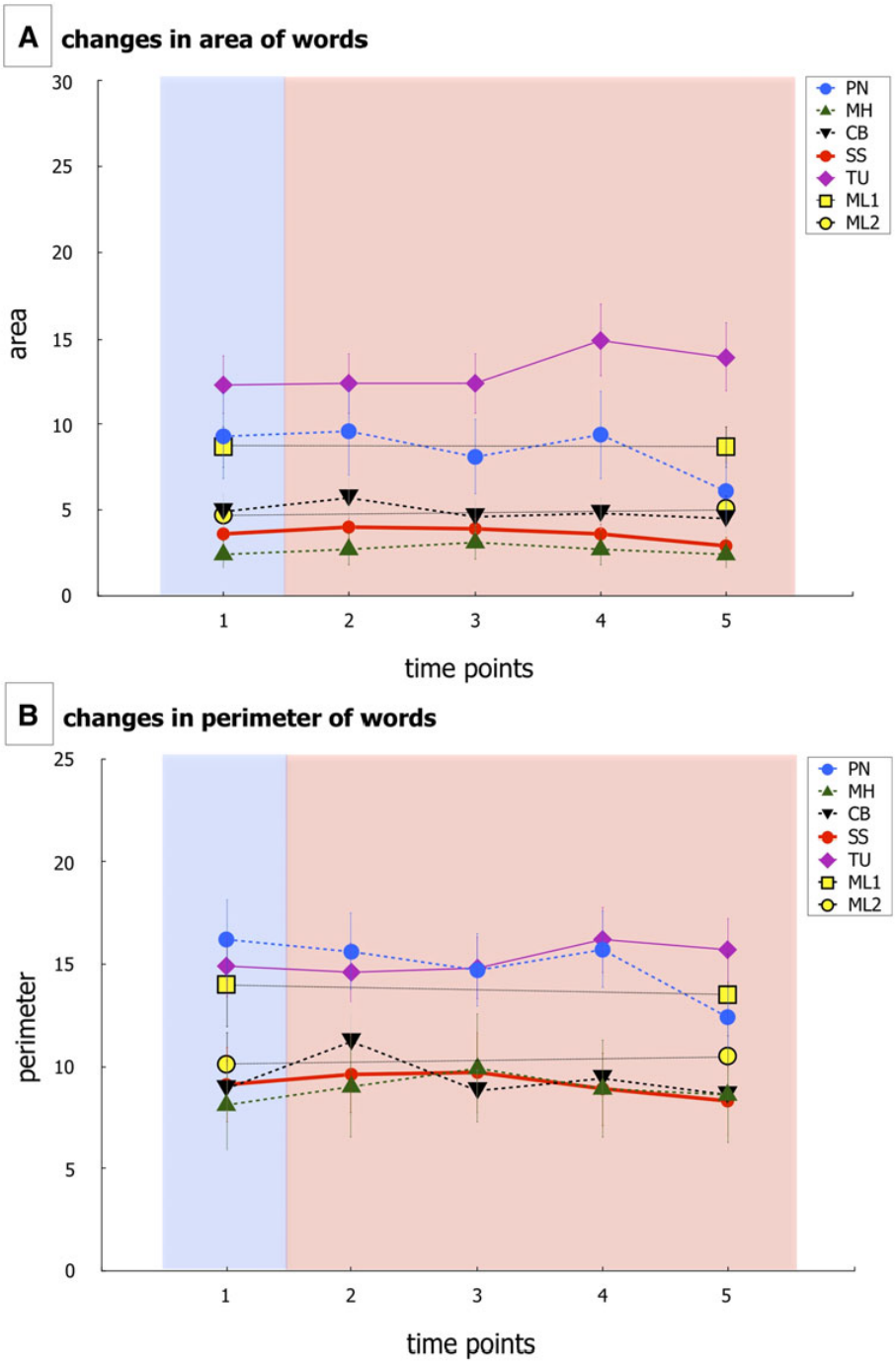

FIG. 3. Graphs show means and SEMs: **(A)** shows changes in area if words while **(B)** shows changes in perimeter of words for each patient. The data for each patient are represented with a different color and/or symbol (see key). The blue shading represents the analysis before onset of light (time point 1), whereas the red shading represents the period during light therapy (time points 2–5). In most cases, the period of light therapy was rather extensive up to 14 months. Note for the patients that we have provided updates for (RP and MH), their data were either reanalyzed (e.g., new words) or data from new samples were added. SEM, standard error of the mean.

change in either area (Fig. 3A; *t*-test, two-tailed, unpaired: ML1 $p=0.98$, ML2 $p=0.65$) or perimeter of distance (Fig. 3B; *t*-test, two tailed, unpaired: ML1 $p=0.70$, ML2 $p=0.77$) of each word analyzed (ML1 $n=28$; ML2 $n=25$). Hence, although no improvements were evident, there was no deterioration over these extended periods.

In summary, of ML's 16 major signs and symptoms, including his writing, 7 improved (~45%) after PBM, while 9 stayed the same (~55%) and none deteriorated (Fig. 4; Table 1). His medication did not increase during this period.

Discussion

This study follows on from our previous exploration into the impact of PBM on four movement disorder patients using PBM buckets.[5] In this study, we describe six cases of Parkinson's disease patients, five using buckets and one, an intranasal device. Two of these patients were cases that we have reported on previously,[5] but we provide further updates on their progress, while the other four were entirely new case reports. Our report is the first to document the impact of

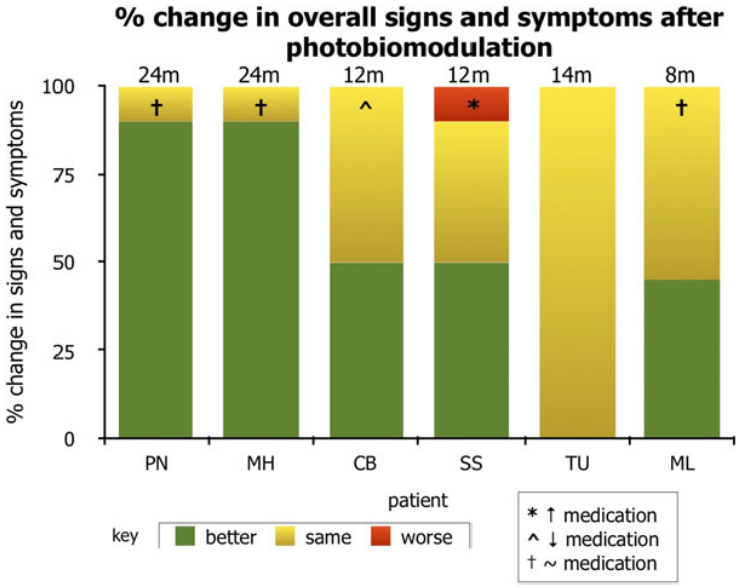

FIG. 4. Graphs showing the % change in overall signs and symptoms of each patient after light therapy. Of the initial signs and symptoms in each patient, the majority showed overall improvement (green regions), a minority stayed the same (yellow), and none got worse (red). Any changes in medication during this time period for each patient is indicated also (see legend). The numbers on top of each column represent the time period (in months) each patient was using during light therapy.

PBM on patients over an extended period, at 8 ($n=1$), 12 ($n=2$), 14 ($n=1$), and 24 ($n=2$) months.

When taken all together, we found that 55% of the initial signs and symptoms of the six patients showed overall improvement, whereas 43% stayed the same and only 2% worsened (Fig. 4). These values assume considerable significance in view of the long-term nature of our analysis and observations, from 8 to 24 months. These findings indicated a stabilization of signs and symptoms that only one sign from all of the six patients (2% of total) worsened during PBM therapy. This feature is highlighted by our objective analysis of their writing, where no patient suffered a major decline in the area and perimeter of words, together with overall writing legibility. Given the progressive nature of the disease,[21,22] this stabilization of writing (and other signs and symptoms) over such a considerable period, was striking. Although one cannot predict the precise time course of the different signs and symptoms of the disease in different individuals, with the disease often not progressing in a straight line,[21,22] the fact that the majority of the signs and symptoms across our six patients did not worsen during PBM was very encouraging. We should note also that many of these PBM-induced changes were not typical of the placebo phenomenon, in that they were slow in onset and sustained. Further, they were often observed by the carer or, in particular, the medical practitioners (general practitioner and geriatrician) rather than the patient themselves.[4]

As with our previous study,[5] we found that PBM did not target a specific sign or symptom, but rather it impacted on both motor signs and nonmotor symptoms, depending on the patient. Indeed, from our experience, Parkinson's disease patients consistently report that among their signs or symptoms, the ones that they most want to improve are the nonmotor symptoms and that these are the ones that very much reduce their quality of life.[21,23] Most patients using PBM experience improvement in nonmotor symptoms, especially mood, anxiety, sleep, confidence, apathy, and fatigue.

We were confident that our findings in the six patients were due to their use of PBM and not to anything else, such as changes in medication. Indeed, during their period of PBM therapy, two patients had no changes in medication (PN, ML), while two had reductions (MH, CB). Patient SS had an increase in medication, but many of his improvements (see Results section), were evident before this increase, suggestive that the improvements were PBM- rather than drug induced.

The factors that generated these stabilizations and improvements are not known, but we suggest the following. For the buckets, the PBM can penetrate to the cerebral cortex and could have influenced the functional activity of its resident neurons directly,[4] by stimulating mitochondrial activity and the expression of various stimulatory genes.[15] Indeed, PBM has been shown to change the activity of seemingly "normal" neurons.[24,25] This mechanism may have underpinned some of the improvements, for example in movement, mood, and confidence, seen in our patients. The buckets covered most of the cranium and hence PBM was in a position to influence a range of functionally distinct cortical areas, from prefrontal to motor.[4] Such a mechanism would however, be more symptomatic, than neuroprotective. The transcranial PBM therapy from the buckets cannot reach the diseased dopaminergic neurons in the brainstem and hence not in a position to influence their survival by direct stimulation.[15–17] PBM from the buckets could, however, have a neuroprotective effect by an indirect stimulation, through the circulation. PBM has been shown to stimulate circulatory cells, for example those of the immune system[26] that may then swarm to the distressed brainstem neurons and helps them survive. We know that in animal models, such a mechanism—the abscopal effect—can be neuroprotective.[15–17,27] It should be noted that for patient ML, who used an intranasal device, PBM would have been much closer to the brainstem than when delivered from the buckets. In his case, PBM may well have penetrated to the brainstem and influenced the diseased neurons directly, stimulating their mitochondria and expression of genes associated with survival,[15] thence being neuroprotective by a direct stimulation.

In conclusion, it is clear that considerably more research, at both basic science and clinical levels, is required to understand better the impact of PBM in Parkinson's disease. For now, our explorations into the PBM-induced effects on

patients were most encouraging (this study[5]) and lay the template for the further development to clinical trial and as a viable therapeutic option.[19,20]

Acknowledgments

The authors thank Light Ahead, Inc. for provision of the PBM devices and Ron Brown for assistance in the development of the PBM devices.

Author Disclosure Statement

C.H. is a cofounder of Well Red Coronet helmets and is the author of redlightsonthebrain.blog. The authors declare no other conflict of interest.

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Received: April 15, 2019
Accepted after revision: June 18, 2019
Published online: September 18, 2019.

Chapter 5

A systematic review of the effect of photobiomodulation on the neuroinflammatory response in animal models of neurodegenerative diseases

DE GRUYTER Rev. Neurosci. 2022; aop

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A systematic review of the effect of photobiomodulation on the neuroinflammatory response in animal models of neurodegenerative diseases

https://doi.org/10.1515/revneuro-2022-0109
Received August 18, 2022; accepted September 18, 2022;
published online October 28, 2022

Abstract: This systematic review examines the effect of photobiomodulation (PBM), the application of red to near infrared light on body tissues, on the neuroinflammatory response and oxidative stress in animal models of neurodegenerative diseases. The research question and search protocol were prospectively registered on the PROSPERO database. Neurodegenerative diseases are becoming ever more prevalent in the ageing populations across the Western world, with no disease-modifying or neuroprotective treatment options being available. Hence there is a real need for the development of effective treatment options for patients. Inflammatory responses and oxidative stress within the central nervous system have a strong correlation with neuronal cell death. PBM is a non-invasive therapeutic option that has shown efficacy and promising effects in animal models of neurodegenerative disease; many studies have reported neuroprotection and improved behavioural outcomes. To the best of our knowledge, there has been no previous study that has reviewed the anti-inflammatory and the antioxidant effect of PBM in the context of neurodegeneration. This review has examined this relationship in animal models of a range of neurodegenerative diseases. We found that PBM can effectively reduce glial activation, pro-inflammatory cytokine expression and oxidative stress, whilst increasing anti-inflammatory glial responses and cytokines, and antioxidant capacity. These positive outcomes accompanied the neuroprotection evident after PBM treatment. Our review provides further indication that PBM can be developed into an effective non-pharmacological intervention for neurodegenerative diseases.

Keywords: cytokine; glia; near infrared light; neurodegeneration; oxidative stress; red light.

Introduction

Neurodegenerative disease is one of the most common chronic neurological disorders in the world. The incidence of neurodegenerative diseases such as Alzheimer's (AD) and Parkinson's disease (PD) is increasing at an alarming rate due to the ageing population of the Western world (National Institute of Environmental Health Sciences 2022). The process of neurodegeneration, characterised by neuronal cell death, is the primary pathological mechanism of neurodegenerative disease. It has been well established that neuroinflammation associates closely with the process of neurodegeneration (Chen et al. 2016). Neuroinflammation is characterised by the activation and proliferation of glial cells as well as the release of inflammatory signalling molecules (Perry and Teeling 2013; Wyss-Coray and Mucke 2002). Presently, therapeutic interventions available for many of the neurodegenerative diseases – in particular AD and PD – are limited, and pharmaceutical treatment options carry significant adverse reactions. Over recent years, many animal models of neurodegenerative disease have been developed, for example, the beta-amyloid (Aβ) peptide injection model for AD, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) injection model for PD, and *Sgsh* transgenic model for Sanfilippo syndrome (Crawley et al. 2006; Facchinetti

Kangzhe Xie and Hala El Khoury contributed equally to this article.

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et al. 2018; Meredith and Rademacher 2011). Thus, research into reducing the degree of neuroinflammation, through the utilisation of these animal models provides a valuable platform to identify and test novel treatments. This, in turn, could reveal new insights for these neurodegenerative disease-targeting therapeutic interventions, with minimal side effects.

Amongst the emerging treatments, photobiomodulation (PBM) has been gaining increasing recognition for its non-invasive therapeutic effect on pathological conditions such as wound healing, cancer and neurodegenerative diseases. It uses low-level light or laser, typically in the visible red or infrared spectrum (600–1200 nm) to produce beneficial physiological changes in the organism (Hamblin 2017). Currently, several chromophores have been put forward to account for the effect of PBM *in vivo*, however, the ways in which they interact with the body remain elusive (Wu et al. 2022). Cytochrome C oxidase, the terminal enzyme (Complex IV) of the mitochondrial electron transport chain, is thought to be one of the main PBM photoreceptors in the body, where absorption of photons by this enzyme results in greater production of adenosine 5′-triphosphate (ATP) and brief outburst of reactive oxygen species (ROS) (Hamblin 2017; Wong-Riley et al. 2005). Collectively, these changes lead to the activation of multiple growth, repairing and anti-inflammatory signalling pathways, which stimulates the migration of stem cells to injured sites, shifts activated macrophages with pro-inflammatory M1 phenotype into the anti-inflammatory M2 phenotype, and promotes neurogenesis in the brain (de Brito Sousa et al. 2020; Feng et al. 2020; Xuan et al. 2015). These beneficial effects are believed to aid the body in repairing itself in response to damage, and thus, suggesting that PBM could dampen the neuroinflammatory responses induced by neuronal cell death, and reduces the disease severity of neurological disorders.

This systematic review aims to investigate the effect of PBM on neuroinflammation in animal models of neurodegenerative disease. The severity of the neuroinflammatory response was determined through the expression levels of astrocyte and microglial markers present at the diseased sites, as well as the amount of inflammatory cytokines released. This review also aims to investigate the effect of PBM on oxidative stress markers, owing to their strong correlation with neuroinflammation. Our findings will establish the link between the effect of PBM in the context of neurodegeneration-induced-neuroinflammation and its clinical applicability as a novel intervention for human neurodegenerative diseases. Such connection will be essential for the further development and progression of PBM as a non-invasive disease-modifying treatment for neurodegenerative disorders in humans. Finally, we will examine the different treatment parameters used in the field, such as wavelength, irradiance, irradiation time and the number of treatment sessions reflective of dosage. We hope to define a "best practice" in relation to reducing inflammation in neurodegenerative disease.

Methods

This systematic review was constructed in accordance with the Preferred Reporting Items for Systematic review and Meta-Analysis Protocols (PRISMA-P) statement (2015) and registered on International prospective register of systematic reviews (PROSPERO) to avoid potential research duplications. All processes of this systematic review (MeSH terms and keywords generation, article collection, study inclusion and exclusion, data extraction and synthesis and quality assessment) were carried out independently by two researchers (KX and HEK). Discrepancy in all of the above processes mentioned were resolved through discussion and consultation with two additional independent researchers (JM and PA).

(Registration is available from https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42022314483).

MeSH terms and keywords generation

The keywords of "photobiomodulation" and "neurodegenerative disease" were entered individually into the advanced search bar on the Ovid SP platform. The "explode" function was used during the keyword search and all related terms generated from this action were considered as "MeSH terms" for the respective keyword. Additionally, MeSH terms of "Alzheimer's disease", "Parkinson's disease", "Ataxia", "Huntington's disease", "Progressive supranuclear palsy", "multiple system atrophy" and "frontotemporal dementia" were also generated to further encapsulate the scope of neurodegenerative diseases (see Supplementary Table S1 for all MeSH terms generated).

Article collection

Published literature that contains the MeSH terms of "photobiomodulation" and "neurodegenerative disease", "Alzheimer's disease", "Parkinson's disease", "ataxia", "Huntington's disease", "progressive supranuclear palsy", "multiple system atrophy" or "frontotemporal dementia" were collected from MEDLINE and EMBASE by using the Ovid SP search platform. In addition, relevant published articles were also obtained by entering the aforementioned search criteria manually in the SCOPUS database (see Supplementary Table S2–S4 for search record). Due to the exporting restriction (maximum 2000 references) in the SCOPUS database, non-primary research articles were filtered (discarded) using the "select article type" tab during the search process. All collected articles were collated into a single library file and stored locally in Endnote X9 throughout the duration of this review.

Study inclusion and exclusion

Using the Endnote X9 software, duplicated studies were identified and discarded by using the "Find Duplicates" function. The title, abstract and full text of the remaining articles was screened against the study inclusion and exclusion criteria in a two-tier process. During the first tier of screening, primary *in vivo* animal research that were published in English language and examined the effect of PBM on neuroinflammatory-related astrocyte or microglia responses in all non-trauma-induced neurodegenerative diseases were included. Manuscripts that did not satisfy the above screening criteria were discarded. The included articles were then screened for the second time in which the full text of the article was reviewed and articles that satisfy the criteria were selected. Articles that did not report relevant glial responses after full text review were also discarded (see Appendix 1 for study selection and exclusion questionnaire).

This systematic review included all PBM treatment with wavelengths ranging between 600 and 1200 nm irrespective of output power, irradiance, irradiation time per session, irradiation schedule, total dosage, irradiation method and site as well as the type of irradiation. Non-primary research articles such as abstracts, literature reviews, editorials, book chapters and conference proceedings were discarded. Additionally, studies that were performed *ex vivo*, *in vitro* or *in silico* were also excluded from the current systematic review.

Data extraction and synthesis

The following information was extracted from each of the selected articles: first author, publication year, animal and species used, gender and age of the animals, neurodegenerative disease modelled, treatment groups and the number of animals in each treatment group.

PBM parameters

This systematic review collected the PBM treatment wavelength (nm), output power (mW) and irradiance (mW/cm^2), irradiation time per session, treatment schedule, total dosage (J/cm^2), irradiation method (transcranial/intracranial/transcutaneous/combined) and site and the type of irradiation (continuous/pulsed) from the selected articles. In addition, output power, irradiance or dosage was calculated using the formula below if it was not reported in the manuscript:

$$\text{Energy (J)} = \text{Output Power (W)} \times \text{Time (s)}$$

$$\text{Irradiance (W/cm}^2\text{)} = \frac{\text{Output Power (W)}}{\text{Area (cm}^2\text{)}}$$

$$\text{Dosage per session (J/cm}^2\text{)} = \text{Irradiance (W/cm}^2\text{)} \times \text{Time (s)}$$

PBM-mediated neuroinflammatory effects

PBM effects on glial cells: The effect of PBM on neuroinflammatory-related astrocyte or microglia responses was the primary outcome of this systematic review. The expression levels of astrocyte and/or microglial markers present at the diseased site(s) were considered as neuroinflammatory-related glial responses. The expression levels could be represented by either the immunoreactivity of glial acidic fibrillary protein (GFAP) or ionised calcium binding adaptor molecule 1 (IBA1) for astrocytes and microglia, respectively. In addition, the number of astrocytes and/or microglia at the diseased site(s) and any other relevant glial specific markers were also extracted. The extracted result was presented as % of change relative to the sham/non-PBM treated disease control animals. The % of change was calculated using the formula below:

If the PBM treated diseased readout is lower than the sham or non-PBM treated diseased readout:

$$\left(1 - \frac{\text{Photobiomodulation Treated Diseased Readout}}{\text{Sham or Non-photobiomodulation Treated Diseased Readout}}\right) \times 100$$

Or

If the PBM treated diseased readout is higher than the sham or non-PBM treated diseased readout:

$$\left(\frac{\text{Photobiomodulation Treated Diseased Readout}}{\text{Sham or Non-photobiomodulation Treated Diseased Readout}} - 1\right) \times 100$$

Furthermore, original representative images were downloaded from the manuscript if no numerical values were reported in the selected study. The number of astrocytes or microglia was obtained from the downloaded images by using the "Analyze Particle" function in ImageJ software. The results were also expressed in % of change relative to the sham/non-PBM treated disease control animals by applying the above formula.

PBM effects on inflammatory cytokine

The protein expression level of inflammatory cytokines was a secondary outcome of this systematic review. The immunoreactivity, brain content or relative protein level of inflammatory signalling proteins measured through immunofluorescence imaging, enzyme-linked immunosorbent assay (ELISA) or Western blot analysis was categorised as the inflammatory cytokine protein level. The extracted data was represented as % of change and calculated using the same formula mentioned in the "PBM effects on glial cells" section.

PBM effects on oxidative stress

This systematic review also included the effect of PBM on oxidative stress and antioxidant markers as a secondary outcome. All relevant changes in oxidative stress and antioxidant markers were extracted and expressed as % of change relative to the sham/non-PBM treated disease control animals using the aforementioned formula.

Risk of bias assessment

The current systematic review employed the Systematic Review Centre for Laboratory animal Experimentation (SYRCLE's) risk of bias tool to examine the quality of the selected articles. The signalling questions and overall risk of bias determination algorithms were derived from Hooijmans et al. (2014), whilst the domains of the signalling questions were simplified to aid reader interpretation (Supplementary material S5). Answers to the signalling questions were also standardised in this review (Supplementary Table S6–S8).

Not reported data and missing information

During the data extraction and synthesis process, the corresponding author(s) of the selected articles were contacted via email to aid data extraction if outcome or risk of bias assessment information was not sufficient in the manuscript (maximum 2 attempts within a 4-week-period). The information was reported as "NR" if no reply was received 4 weeks after the last email.

Results

The database search was performed on the 14th of March 2022 at 21:00 AEDT, and a total of 3076 articles were obtained. Of the 3076 articles, 554 were duplicated and 12 articles met the study inclusion and exclusion criteria (Figure 1). The study characteristics and PBM treatments, PBM-mediated neuroinflammatory effect and PBM-mediated oxidative stress effect are summarised in Tables 1 and 2.

Study characteristics and PBM treatments

Animals and disease modelled

In this review, 9 of the 12 studies used AD animal models, while the remaining 3 were performed on PD animal models. All of the 9 AD animal models used rodents, 6 of which used mice and 3 used rats. Of the 6 AD mouse models, 4 studies used the C57BL/6 strain, 1 study was performed on Swiss mice and 1 used mice with the B6SJLF1.J. background. One selected study used Sprague Dawley rats as their AD model while the remaining 2 AD rat model used Fisher 344 rats. Of the PD animal models, 1 was performed on primate (*Macaca fascicularis*), 1 on BALB/c mice and 1 on Sprague Dawley rats.

To induce neurodegenerative disease-like conditions, 3 of the mouse models and 1 of the rat models induced an AD-like condition by injecting Aβ peptide into the cerebral cortex or hippocampus of the rodents. The remaining 3 mouse models and 2 rat models for AD were transgenic, utilising the mutation of human amyloid beta protein and presenilin 1 gene to mimic AD-like pathologies. With regards to the 3 PD animal models, the primate and mouse studies induced PD-like pathologies by injecting the MPTP compound, while the remaining rat study used supranigral injection of lipopolysaccharide (LPS) to mimic PD-like pathologies.

PBM wavelengths and LED versus laser

The PBM treatment parameters used by the selected studies were examined in an attempt to establish the clinical applicability of PBM as a novel intervention for neurodegenerative diseases. Of the 12 selected articles, 6 studies examined the effect of visible red PBM (625–740 nm) on neuroinflammatory-related glial and cytokine responses and oxidative stress. The study by Cho et al. (2020) used PBM with a wavelength of 610 nm, and O'Brien and Austin (2019) utilised PBM at 675 nm. El Massri et al. (2016) and Johnstone et al. (2014) used 670 nm PBM whereas Shen et al. (2021) and Wu et al. (2021) employed a device that emits 635 nm PBM. Another 4 of the 12 selected studies investigate the effects using PBM in the near-infrared spectra (800–2500 nm). The study by Lu et al. (2017), Yang et al. (2021) and Yang et al. (2022) utilised PBM with a wavelength of 808 nm, whereas 1070 nm PBM was used by Tao et al. (2021). For the remaining 2 studies, Chen et al. (2021) explored the PBM effect of three different wavelengths, in both the visible red and near infrared spectrum (630, 730 and 850 nm) whereas Blivet et al. (2018) employed a PBM device that emits two different wavelengths of light simultaneously (625 and 850 nm).

The emission of PBM was achieved using a light emitting diode (LED) or a laser. Of the 12 selected studies, 5 of them utilised a LED as a source of light emission and 6 studies used a laser emission set up. The study by Blivet et al. (2018) achieved the emission of two different wavelengths of PBM lights through the combination use of LED and laser.

Output power, irradiance and dosage

The output power and irradiance of the PBM treatment were reported in mW and mW/cm^2 in this systematic review. The studies by Blivet et al. (2018), Cho et al. (2020), El Massri et al. (2016), Lu et al. (2017), Shen et al. (2021) and Wu et al. (2021) reported a output power lower than 100 mW (28 , 0.21, 10, 25, 8.75 and 10 mW, respectively). The study by Tao et al. (2021) used a PBM device with an output power of 900 mW and Johnstone et al. (2014) and O'Brien and Austin (2019) reported an output power of 500 mW. The studies by Yang et al. (2021) and (2022) utilised the same output power of 233.33 mW, whilst the output power in the study by Chen et al. (2021) was not reported. With regards to PBM irradiance, all of the 12 selected studies used an irradiance less than 50 mW/cm^2 (Blivet 28 mW/cm^2; Chen 10 mW/cm^2, Cho 1.7 mW/cm^2, Johnstone 50 mW/cm^2, Lu 25 mW/cm^2, O'Brien and Austin 40.84 mW/cm^2, Shen 10 mW/cm^2, Tao 25 mW/cm^2 and Wu 12.74 mW/cm^2) with the exception of the studies by Yang et al. (2021, 2022). They reported PBM irradiance of 350 mW/cm^2 at the scalp of the treated animals. The PBM

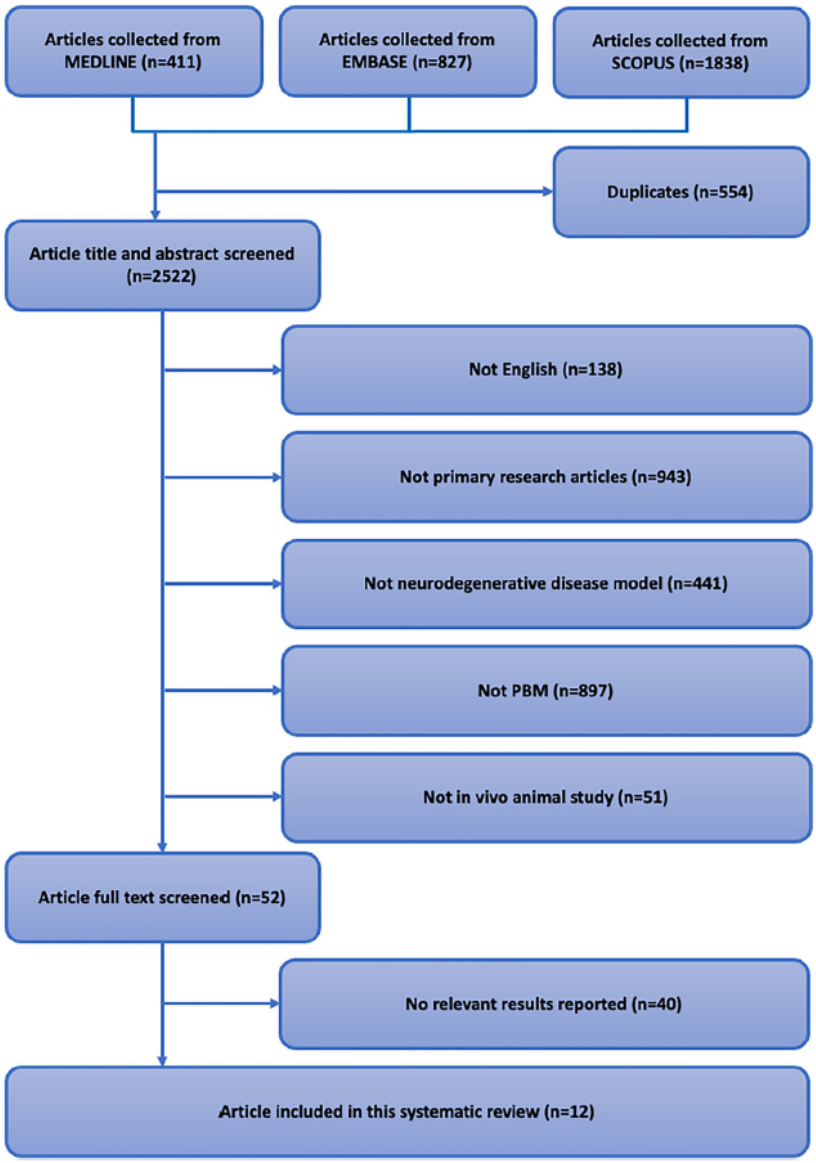

Figure 1: Flow chart of the study inclusion and exclusion screening process. Text in the bubble is indicative of the reason that the article was included or excluded. PBM, photobiomodulation.

irradiance or irradiation area of the PBM device was not reported by El Massri et al. (2016), and as a result, the irradiance parameter could not be extracted in the current review.

The dosage of PBM treatments were represented in J/cm^2. In the current systematic review, 6 of the 12 selected studies reported a dosage less than 100 J/cm^2. The studies by Johnstone et al. (2014); Lu et al. (2017); O'Brien and Austin (2019); Wu et al. (2021); Yang et al. (2021, 2022) delivered a dosage of 24 J/cm^2, 75 J/cm^2, 46.722 J/cm^2, 60 J/cm^2, 42 J/cm^2 and 42 J/cm^2, respectively. The studies that reported a dosage greater than 100 J/cm^2 include: Chen et al. (2021) of 4000 J/cm^2, Cho et al. (2020) of 168 J/cm^2, Shen et al. (2021) of 180 J/cm^2 and Tao et al. (2021) of 270 J/cm^2. The study by Blivet et al. (2018) explored the effect of PBM with different schedules and as a result, reported multiple dosages of PBM given to the treated animals (29.4 J/cm^2, 58.8 J/cm^2 and 117.9 J/cm^2). A similar experimental set up was also observed in the study by El Massri et al. (2016), with a dosage of 25 J/cm^2 and 35 J/cm^2 reported depending on the treatment groups.

Irradiation time and treatment schedules

In the current systematic review, most of the PBM treatments were in the minute range except for the studies by El Massri et al. (2016) and O'Brien and Austin (2019). El Massri

Table 1: Summary table of PBM parameters[a].

Study (Year)	PBM wavelength	Output power and irradiance (measure point)	Irradiation time per session	Treatment schedule (number of treatment sessions)	Total dosage (measure point)	Irradiation method and site	Type of irradiation
Blivet et al. (2018)	850 nm laser combined with 850 and 625 nm LED	28 mW 28 mW/cm^2 (at skin)	2.5min 5min 10min Or 20min	2.5min and 5min: twice daily for 7 days (14) Or 10min and 20min: once daily for 7 days (7)	2.5min: 29.4 J/cm^2 5min: 58.8 J/cm^2 10min: 58.8 J/cm^2 Or 20min: 117.9 J/cm^2 (at skin)	Transcranial: 1 cm above the shaved skin of the head Transcutaneous: centre of the abdomen Or Transcranial and transcutaneous: 1 cm above the shaved skin of the head and centre of the abdomen	Pulsed at 10 Hz frequency when given to head and abdomen or head only Or Pulsed at 1000 Hz frequency when given to abdomen only
Chen et al. (2021)	630 nm LED 730 nm LED Or 850 nm LED	NR for output power 10 mW/cm^2 (NR)	1000 s	Once daily, 5 times a week for 8 weeks (40)	4000 J/cm^2 (NR)	Transcutaneous: on top of the shaved skin of upper abdomen	NR
Cho et al. (2020)	610 nm LED	0.21 mW 1.7 mW/cm^2 (NR)	20 min	3 times a week for 14 weeks (42)	168 J/cm^2 (NR)	Transcranial: on scalp on both sides of the parietal lobe	NR
El Massri et al. (2016)	670 nm laser	10 mW 0.204 mW/cm^2 (at device aperture)	24 h + 5s	1.5 mg/kg: PBM for 5 s before each MPTP injection and PBM for 24 h after each MPTP injection over 5 days (5) 2.1 mg/kg: PBM for 5 s before each MPTP injection and PBM for 24 h after each MPTP injection over 7 days (7)	1.5 mg/kg: 25 J/cm^2 2.1 mg/kg: 35 J/cm^2 (at device aperture)	Intracranial: optical fibres were implanted 1–2 mm to the left of the midline in the midbrain region	Pulsed at 5 s on 5 s off
Johnstone et al. (2014)	670 nm LED	500 mW 50 mW/cm^2 (at device aperture)	90 s	50 mg/kg: Immediately after MPTP injection and 6 h after MPTP injection (4) 75 mg/kg: Immediately after MPTP injection and 6 h after MPTP injection (6) 100 mg/kg: Immediately after MPTP injection and 6 h after MPTP injection (8)	50 mg/kg: 16 J/cm^2 75 mg/kg: 24 J/cm^2 100 mg/kg: 32 J/cm^2 (at device aperture)	Transcranial: directly on the head Or Transcutaneous: directly on the dorsum with infrared-opaque aluminium foil covering the head	Continuous wave
Lu et al. (2017)	808 nm laser	25 mW 25 mW/cm^2 (at target)	2min	Once daily for 5 consecutive days (5)	75 J/cm^2 (at target)	Transcranial: directly on the shaved scalp 3 mm behind the eye and 2 mm in front of the ears	Continuous wave
O'Brien and Austin (2019)	675 nm LED	500 mW 40.84 mW/cm^2 (at target)	88 s[a]	First PBM treatment was 2 h, all subsequent exposures were 88 s performed twice daily for 6 days (13)	46.722 J/cm^2 (at target)	Transcranial: 1 cm above the head	Continuous wave

Table 1: (continued)

Study (Year)	PBM wavelength	Output power and irradiance (measure point)	Irradiation time per session	Treatment schedule (number of treatment sessions)	Total dosage (measure point)	Irradiation method and site	Type of irradiation
Shen et al. (2021)	635 nm laser	8.75 mW 10 mW/cm^2 (at target)	10min	Once daily for 30 days (30)	180 J/cm^2 (at target)	Transcranial: directly on the shaved skin of the head	Continuous wave
Tao et al. (2021)	1070 nm LED	900 mW 25 mW/cm^2 (at device aperture)	6min	Once daily for 60 days (60)	270 J/cm^2 (at device aperture)	Transcranial and transcutaneous: lights were located in the lid of a box, mice inside the box were free to roam during PBM treatments	Pulsed at 10 Hz or 40 Hz frequency
Wu et al. (2021)	635 nm laser	10 mW 12.74 mW/cm^2 (at target)	10min	Once daily for 30 days (30)	60 J/cm^2 (at target)	Transcranial: without skin contact	Continuous wave
Yang et al. (2021)	808 nm laser	NR for output power 25 mW/cm^2 (at target)	2min	3 times a week for 8 months (96)	3 J/cm^2 (at target)	Transcranial: Fibreoptic 35 cm away from shaved scalp to generate a 1.5 cm^2 laser spot size	Continuous wave
Yang et al. (2022)	808 nm laser	NR for output power 25 mW/cm^2 (at target)	2min	3 times a week for 16 months (192)	3 J/cm^2 (at target)	Transcranial: Fibreoptic 35 cm away from shaved scalp to generate a 1.5 cm^2 laser spot size	Continuous wave

LED, light emitting diode; NR, not reported; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PBM, photobiomodulation. [a]Corresponding authors of the manuscript were contacted via email to aid data extraction; parameters were calculated using the formula described in Method section if not provided by the manuscript or the authors.

Table 2: Summary table of PBM treatment outcomes[a].

Study (Year)	Animal and species	Gender and age	Neurodegenerative disease model	Treatment groups (*n*)	Neuroinflammatory Effect		Oxidative stress effect
					Glial effect	Inflammatory cytokine effect	
Blivet et al. (2018)	Mouse Swiss	Male 6 weeks old	AD: intra-cerebroventricular injection of 9 nmol $A\beta_{25\text{-}35}$ peptides	Scramble Aβ ($n = 6$) $A\beta_{25\text{-}35}$ ($n = 6$) $A\beta_{25\text{-}35}$ + 2.5min twice daily PBM to head and abdomen ($n = 6$) $A\beta_{25\text{-}35}$ + 5min twice daily PBM to head and abdomen ($n = 6$) $A\beta_{25\text{-}35}$ + 10min once daily PBM to head and abdomen ($n = 6$) $A\beta_{25\text{-}35}$ + 20min once daily PBM to head and abdomen ($n = 6$) $A\beta_{25\text{-}35}$ + 20min once daily PBM to head only ($n = 6$) Or $A\beta_{25\text{-}35}$ + 20min once daily PBM to abdomen only ($n = 6$)	**2.5min twice daily PBM to head and abdomen:** PBM increased GFAP protein level by 6%, effect not significant **5min twice daily PBM to head and abdomen:** PBM reduced GFAP protein level by 11%, effect not significant **10min once daily PBM to head and abdomen:** PBM significantly reduced GFAP immunoreactivity by 82%; PBM significantly decreased IBA1 immunoreactivity by 51%; PBM significantly lowered GFAP brain content by 37% **20min once daily PBM to head and abdomen:** PBM reduced GFAP protein level by 8%, effect not significant **20min once daily PBM to head only:** PBM increased GFAP protein level by 8%, effect not significant **20min once daily PBM to abdomen only:** PBM increased GFAP protein level by 8%, effect not significant	**2.5min twice daily PBM to head and abdomen:** PBM reduced TNF-α protein level by 22%, effect not significant **5min twice daily PBM to head and abdomen:** PBM significantly reduced TNF-α protein level by 40% **10min once daily PBM to head and abdomen:** PBM significantly reduced TNF-α protein level by 38%; PBM significantly decreased IL-1β protein level by 48%; PBM significantly lowered IL-6 protein level by 52% **20min once daily PBM to head and abdomen:** PBM significantly reduced TNF-α protein level by 31% **20min once daily PBM to head only:** PBM reduced TNF-α protein level by 23%, effect not significant **20min once daily PBM to abdomen only:** PBM reduced TNF-α protein level by 22%, effect not significant	**2.5min twice daily PBM to head and abdomen:** PBM reduced lipid peroxidation by 3%, effect not significant **5min twice daily PBM to head and abdomen:** PBM significantly reduced lipid peroxidation by 28% **10min once daily PBM to head and abdomen:** PBM significantly reduced lipid peroxidation by 34% **20min once daily PBM to head and abdomen:** PBM significantly reduced lipid peroxidation by 25% **20min once daily PBM to head only:** PBM reduced lipid peroxidation by 19%, effect not significant **20min once daily PBM to abdomen only:** PBM reduced lipid peroxidation by 17%, effect not significant
Chen et al. (2021)	Mouse C57BL/6 N	Male NR for age	AD: bilateral injection of 3 nmol $A\beta_{1\text{-}42}$ peptides into the hippocampus	Wildtype ($n = 15$) $A\beta_{1\text{-}42}$ ($n = 15$) $A\beta_{1\text{-}42}$ + 630 nm PBM ($n = 15$) $A\beta_{1\text{-}42}$ + 730 nm PBM ($n = 15$) $A\beta_{1\text{-}42}$ + 850 nm PBM ($n = 15$)	**630 nm:** There is a visible reduction in IBA1 immunoreactivity (71%) **730 nm:** There is a visible reduction in IBA1 immunoreactivity (21%) **850 nm:** There is no visible effect PBM on IBA1 immunoreactivity	NR	NR

Table 2: (continued)

Study (Year)	Animal and species	Gender and age	Neurodegenerative disease model	Treatment groups (*n*)	Neuroinflammatory Effect		Oxidative stress effect
					Glial effect	Inflammatory cytokine effect	
Cho et al. (2020)	Mouse B6SJLF1.J	Male 2 months old and 6 months old	AD: 5XFAD transgenic model with APP_{699} Swedish, London, Florida; and PS1 M146L, L286V mutations	Wildtype + Sham 2 months old 5XFAD + Sham 2 months old 5XFAD + PBM 6 months old 5XFAD + Sham 6 months old 5XFAD + PBM (*n* = 8–12 per group)	**2 months old:** PBM increased GFAP immunoreactivity by 29% in the cortex, effect not significant; PBM produced no change on GFAP immunoreactivity in the hippocampus; PBM significantly reduced IBA1 immunoreactivity by 36% in the cortex; PBM decreased IBA1 immunoreactivity by 47% in the hippocampus, effect not significant **6 months old:** PBM increased GFAP immunoreactivity by 44% in the cortex and 28% in the hippocampus, effects not significant; PBM reduced IBA1 immunoreactivity by 7% in the cortex and 26% in the hippocampus, effects not significant	NR	NR
El Massri et al. (2016)	Monkey *Macaca fascicularis*	Male 4–5 years old	PD: intramuscular injection of 1.5 mg/kg or 2.1 mg kg MPTP over 5 or 7 days period, respectively	Wildtype (*n* = 2) Wildtype + Sham (*n* = 3) MPTP (*n* = 1) MPTP + Sham (*n* = 10) MPTP + PBM (*n* = 6)	PBM significantly reduced GFAP immunoreactivity by 74% in the substantia nigra pars compacta and 58% in the striatum PBM lowered IBA1 immunoreactivity by 1% in the substantia nigra pars compacta, effect not significant; PBM significantly increased IBA1 immunoreactivity by 37% in the striatum	NR	NR
Johnstone et al. (2014)	Mouse BALB/c	Male 8 weeks old	PD: intraperitoneal injection of 50 mg/kg, 75 mg/kg or 100 mg/kg MPTP over 2-, 3- or 4-days period	Saline + shame (*n* = 17) Saline + PBM (*n* = 11) 75 mg/kg MPTP + sham (*n* = 8) 75 mg/kg MPTP + head PBM (*n* = 8) 75 mg/kg MPTP + body PBM (*n* = 8)	**75 mg/kg MPTP + head PBM:** PBM reduced GFAP positive cells by 5%, effect not significant; PBM increased IBA1 positive cells by 5%, effect not significant **75 mg/kg MPTP + body PBM:** PBM reduced GFAP positive cells by 0.5%, effect not significant; PBM decreased IBA1 positive cells by 3%, effect not significant	NR	NR

Table 2: (continued)

Study (Year)	Animal and species	Gender and age	Neurodegenerative disease model	Treatment groups (*n*)	Neuroinflammatory Effect		Oxidative stress effect
					Glial effect	Inflammatory cytokine effect	
Lu et al. (2017)	Rat Sprague Daley	Male 3 months old	AD: bilateral injection of 1 nmol $A\beta_{1\text{-}42}$ into the hippocampus	Vehicle Vehicle + Sham Scramble Aβ $A\beta_{1\text{-}42}$ + Sham $A\beta_{1\text{-}42}$ + PBM (*n* = 4–6 per group)	PBM significantly reduced GFAP immunoreactivity by 70% PBM significantly decreased IBA1 immunoreactivity by 58%	PBM significantly reduced IL-1β level by 50%; PBM significantly decreased IL-6 level by 58%; PBM significantly lowered TNF-α level by 66%	PBM significantly reduced G6PDH enzyme activity by 50%; PBM significantly decreased NADPH level by 23%; PBM significantly lowered NADPH oxidase activity by 54% PBM significantly reduced lipid peroxidation marker 4-HNE immunoreactivity by 60%; PBM significantly decreased peroxynitrite production marker 3-NT by 60%; PBM significantly lowered DNA double-strand breaking marker H2A.X Ser139 by 68%; PBM significantly decreased oxidised DNA damage marker 8-OHdG by 63% PBM significantly reduced superoxide anion by 57% in the mitochondria and 67% in the cytoplasm; PBM significantly increased antioxidant capacity by 100% in the mitochondria and 218% in the cytoplasm
O'Brien and Austin (2019)	Rat Sprague Dawley	Male 6 weeks old	PD: supranigral injection of 10 µg or 20 µg of LPS	10 µg Vehicle + Sham (*n* = 8) 20 µg Vehicle + Sham (*n* = 7) 10 µg LPS + Sham (*n* = 4) 20 µg LPS + Sham (*n* = 9) 10 µg LPS + PBM (*n* = 4) 20 µg LPS + PBM (*n* = 9)	**10 µg LPS:** no difference in IBA1 immunoreactivity. **20 µg LPS:** PBM reduced total IBA1 immunoreactivity by 9%, effect not significant; PBM decreased amoeboid IBA1 immunoreactivity by 18%, effect not significant	NR	NR

Table 2: (continued)

Study (Year)	Animal and species	Gender and age	Neurodegenerative disease model	Treatment groups (*n*)	Neuroinflammatory Effect		Oxidative stress effect
					Glial effect	Inflammatory cytokine effect	
Shen et al. (2021)	Mouse C57BL/6 (B6)	NR for gender 3 months old and 6 months old	AD: APP/PS1 transgenic model with APP_{695} Swedish mutation; and PS1 dE9 mutation	Wildtype + Sham Wildtype + PBM APP/PS1 + Sham APP/PS1 + PBM (*n* = 4–5 per group)	PBM significantly lowered GFAP immunoreactivity by 37% PBM significantly decreased IBA-1 immunoreactivity by 47%	NR	NR
Tao et al. (2021)	Mouse C57BL/6	Female 4 months old and 10 months old	AD: APP/PS1 transgenic model with APP_{695} Swedish mutation; PS1 dE9 mutation	Wildtype APP/PS1 + Sham 4 months old APP/PS1 + 10 Hz PBM 10 months old APP/PS1 + 10 Hz PBM 4 months old APP/PS1 + 40 Hz PBM 10 months old APP/PS1 + 40 Hz PBM (*n* = 5–6 per group)	**4 months old APP/PS1 + 10Hz PBM:** PBM significantly reduced IBA1 immunoreactivity IBA1 by 24%; PBM significantly decreased microglia branch length by 27%; PBM significantly lowered number of microglial branches by 20%; PBM significantly reduced number of microglial terminal processes by 30%; PBM significantly increased microglial volume by 32%; PBM significantly reduced COX2 positive microglia by 16%; PBM reduced COX2 intensity by 3%, effect not significant; PBM reduced GFAP immunoreactivity by 8%, effect not significant; PBM significantly reduced the number of perivascular microglia per vessel by 57%; PBM significantly decreased the % of vessel associated microglia by 11%; PBM significantly decreased the % of vessel associated COX2 positive microglia by 20% **10 months old APP/PS1 + 10Hz PBM:** PBM produced no change on IBA1 immunoreactivity; PBM increased microglial branch length by 2%, effect not significant; PBM increased number of microglial branches by 5%; effect	NR	NR

Table 2: (continued)

Study (Year)	Animal and species	Gender and age	Neurodegenerative disease model	Treatment groups (*n*)	Neuroinflammatory Effect		Oxidative stress effect
					Glial effect	Inflammatory cytokine effect	
					not significant; PBM significantly reduced COX2 intensity by 17%; PBM reduced GFAP immunoreactivity by 2%, effect not significant **4 months old APP/PS1 + 40Hz PBM:** PBM produced no change on IBA immunoreactivity, microglial branch length and microglial volume; PBM reduced number of microglial branches by 11%, effect not significant; PBM decreased number of microglial terminal processes by 14%, effect not significant; PBM reduced COX2 positive microglia by 12%, effect not significant; PBM reduced COX2 intensity by 5%; effect not significant; PBM significantly reduced GFAP immunoreactivity by 24%; PBM significantly reduced the number of perivascular microglia per vessel by 57%; PBM significantly decreased the % of vessel associated microglia by 12%; PBM decreased % of vessel associated COX2 positive microglia by 8%, effect not significant **10 months old APP/PS1 + 40Hz PBM:** PBM produced no change on IBA1 immunoreactivity; PBM increased microglial branch length by 2%, effect not significant; PBM increased number of microglial branches by 5%; PBM significantly increased COX2 intensity by 24%; PBM reduced GFAP immunoreactivity by 2%, effect not significant		

Table 2: (continued)

Study (Year)	Animal and species	Gender and age	Neurodegenerative disease model	Treatment groups (*n*)	Neuroinflammatory Effect		Oxidative stress effect
					Glial effect	Inflammatory cytokine effect	
Wu et al. (2021)	Mouse C67BL/6	Male 6 months old	AD: APP/PS1 transgenic model with APP_{695} Swedish mutation; PS1 dE9 mutation	Wildtype Wildtype + PBM APP/PS1 APP/PS1 + PBM (*n* = 4–6 per group)	PBM significantly reduced GFAP immunoreactivity by 85% PBM significantly reduced GFAP protein level by 28%	NR	NR
Yang et al. (2021)	Rat Fisher 344	Male and female 2 months old	AD: TgF344 transgenic model with APP gene and PS1 gene	wild type (*n* = 8) TgF344 + Sham (*n* = 12) TgF344 + PBM (*n* = 12)	PBM significantly reduced IBA1 immunoreactivity by 66% PBM significantly reduced microglia body diameter by 27%	PBM significantly decreased NFκB levels by 13%; PBM significantly decreased TNF-α levels by 13%; PBM significantly decreased IL-1β by 18%; PBM significantly increased IL-4 by 19%; PBM significantly increased IL-10 by 29%	PBM significantly increased antioxidant capacity by 42%; PBM significantly increased SOD2 intensity by 43%; PBM significantly decreased MDA levels by 32%; PBM significantly decreased 4-HNE by 24%; PBM significantly decreased protein carbonyl levels by 46%; PBM significantly decreased H2AX intensity by 40%
Yang et al. (2022)	Rat Fisher 344	Male 2 months old	AD: TgF344 transgenic model with human amyloid beta (A4) protein (hAPP) gene and human presenilin 1 gene (PS1)	Wildtype + Sham (*n* = 16) Wildtype + PBM (*n* = 16) TgF344 + Sham (*n* = 16) TgF344 + PBM (*n* = 16)	PBM significantly increased microglia count per ROI in the cortex by 60%, 50% and 42% around small, medium and large plaques, respectively PBM significantly increased microglia count per ROI in the hippocampus by 100%, 83% and 36% around small, medium and large plaques, respectively PBM significantly increased microglial/Aβ colocalization per ROI in the cortex by 30%, 30%, and 30% around small, medium and large plaques, respectively PBM significantly increased microglial/Aβ colocalization per ROI in the hippocampus by 40%, 30% and 45% around small, medium and large plaques, respectively	PBM significantly increased IL-3 Intensity in cortex by 125%, 35% and 90% around small, medium and large plaques, respectively PBM significantly increased IL-3 Intensity in hippocampus by 140%, 240% and 283% around small, medium and large plaques, respectively. PBM significantly increased TGF-β in cortex by 150% PBM increased TGF-β in hippocampus by 85%	PBM significantly increased Antioxidant capacity in the cortex by 50% and in the hippocampus by 29% PBM significantly decreased MDA level in the cortex by 30% and the hippocampus by 29% PBM significantly decreased Protein carbonyls levels in the cortex by 23% and in the hippocampus by 29% PBM significantly decreased 8-OHdG levels in the cortex by 25% and in the hippocampus by 28% PBM decreased 4-HNE levels in the cortex by 23%, and in the hippocampus by 36%

Table 2: (continued)

Study (Year)	Animal and species	Gender and age	Neurodegenerative disease model	Treatment groups (*n*)	Neuroinflammatory Effect		Oxidative stress effect
					Glial effect	Inflammatory cytokine effect	
					PBM significantly increased IL-3-Rα/IBA1 positive cell count per ROI in the cortex by 100%, 90% and 100% around small, medium and large plaques, respectively PBM significantly increased IL-3-Rα/IBA1 positive cell count per ROI in the hippocampus by 150%, 160% and 100% around small, medium and large plaques, respectively PBM significantly increased CD206 immunoreactivities in the cortex by 86% and in the hippocampus by 120% PBM significantly increased Arg-1 immunoreactivities in the cortex by 52% and in the hippocampus by 50%		

AD, Alzheimer's disease; Aβ, beta amyloid; PBM, photobiomodulation; GFAP, glial fibrillary acidic protein; IBA1, ionised calcium binding adaptor molecule 1; TNF-α, tumour necrosis factor alpha; NR, not reported; IL, interleukin; PD, Parkinson's disease; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; G6PDH, glucose-6-phosphate dehydrogenase; NADPH, reduced nicotinamide adenine dinucleotide phosphate; 4-HNE, 4-hydoxynonenal; 3-NT, 3-nitrotyrosine; 8-OHdG, 8-hydroxyguanosine; LPS, lipopolysaccharide; APP, amyloid beta protein; PS1, presenilin 1; COX2, cyclooxygenase-2; NFκB, nuclear factor kappa B; SOD2, superoxide dismutase-2; MDA, malondialdehyde; CD206, macrophage mannose receptor cluster of differentiation 206; Arg-1, arginase 1; TGF-β, transforming growth factor beta. [a]Corresponding authors of the manuscript were contacted via email to aid outcome extraction.

and colleagues administered PBM for 24 h consecutively whereas O'Brien and Austin applied PBM 2 h prior disease induction, and then followed by daily treatment of 88 s. With regards to the treatment schedules, both acute (less than or equal to 1 month) and chronic (greater than 1 month) treatment regimens were reported in the selected studies. The studies by Blivet et al. (2018), El Massri et al. (2016), Johnstone et al. (2014), Lu et al. (2017), O'Brien and Austin (2019), Shen et al. (2021) and Wu et al. (2021) used an acute treatment regimen with diseased animals receiving 7, 7, 6, 5, 13, 30 and 30 days of PBM treatments, respectively. Conversely, Chen et al. (2021), Cho et al. (2020), Tao et al. (2021), Yang et al. (2021) and (2022) employed a chronic treatment schedule with diseased animals receiving 40, 42, 60, 96 and 192 days of PBM treatment, respectively.

Irradiation method, site and type

All of the 12 selected studies, with the exception of the studies by Chen et al. (2021) and El Massri et al. (2016), used transcranial PBM. This approach was achieved by placing the PBM device either directly on the scalp of the animals or holding the device above the head at a distance (1–50 cm). The remaining 2 studies by Chen et al. (2021) and El Massri et al. (2016) used transcutaneous and intracranial irradiation methods, respectively. In addition, 3 of the transcranial irradiating studies also examined the effect of transcutaneous PBM administration (Blivet et al. 2018; Johnstone et al. 2014; Tao et al. 2021).

PBM-mediated neuroinflammatory effects

Effects on astrocytes

This review used GFAP immunoreactivity and changes in the numbers of astrocytes as primary indicators of astrocytic neuroinflammatory responses. Eight (8) of the 12 selected studies reported changes on these measures of which, 6 studies explored these changes in AD while the other 2 were in PD. In the models of AD, most studies reported a reduction in GFAP immunoreactivity, except for the study by Cho et al. (2020) which showed an increased GFAP immunoreactivity. However, the change observed by Cho and colleagues did not reach statistically significant level. The studies by Lu et al. (2017), Shen et al. (2021) and Wu et al. (2021) only tested the effect of PBM on astrocyte-related neuroinflammatory response in one condition and reported a statistically significant reduction of GFAP immunoreactivity by 70, 37 and 85%, respectively. The study by Blivet et al. (2018) explored the effect of PBM by varying the irradiation time whereas Tao et al. (2021) examined the effect of different PBM frequencies on different age groups of mice. Under these conditions, only the AD–like mice that received 10 min of daily treatment to the head and abdomen in the study by Blivet et al. (2018) and the 4-month-old AD-like mice that received 40 Hz of PBM in the study by Tao et al. (2021) reported a statistically significant reduction in GFAP immunoreactivity (82 and 24%, respectively). All other treatment conditions from both studies did not report a significant change in GFAP immunoreactivity. In addition, the studies by Blivet et al. (2018) and Wu et al. (2021) also reported a significant reduction of GFAP protein level by 37 and 28%, respectively, after PBM treatment. In the studies using animal models of PD, the PBM-induced reductions in GFAP immunoreactivity or astrocyte number were also observed. In the study by El Massri et al. (2016), GFAP immunoreactivity in the striatum and substantia nigra pars compacta were significantly reduced by 74 and 58%, respectively. Johnstone et al. (2014) reported a trend for a reduction in GFAP immunoreactivity, although this reduction did not reach statistical significance. In summary, these results indicate that PBM can reduce GFAP immunoreactivity and the number of astrocytes near the disease sites in animal models of both AD and PD.

Effects on microglia

In this review, 11 (8 AD studies and 3 PD studies) of the 12 selected studies reported the effect of PBM on IBA1 immunoreactivity or changes to the number of microglia, both markers that represent microglial-related neuroinflammatory responses. Of the 8 AD studies, most observed a reduction in IBA1 immunoreactivity or the microglial number, with the exception of Yang et al. (2022), which showed a significant increase in the number of microglia in both the cortex and hippocampus. In the studies that only examined one treatment condition, IBA1 immunoreactivity was found to be significantly decreased by PBM treatment. Specifically, there was a reduction of 58% by Lu et al. (2017), 47% by Shen et al. (2021), 24% by Tao et al. (2021) and 66% by Yang et al. (2021). In the study by Blivet et al. (2018), that examined different PBM irradiation times, IBA1 immunoreactivity was significantly reduced by 51% in the AD-like animals that received 10 min of treatment to the head and abdomen, while no significant changes were report in other treatment groups. With regard to the PBM-induced microglial responses in mice with different age groups, Cho et al. (2020) showed a significant

reduction of IBA1 immunoreactivity in 2-month-old AD-like mice cortex (36%) while a statistically significant decrease in IBA1 immunoreactivity was reported by Tao and colleagues in 4-month-old AD-like mice (24%). The study by Chen et al. (2021) explored the effect of different PBM wavelengths on the microglial response, in which the representative images showed reductions in IBA1 immunoreactivity in AD-like mice that received 630 and 730 nm of PBM (71 and 21%, respectively). No statistical analysis was performed due to the lack of sample size and PBM effect readouts being obtained from ImageJ (See Method). In addition, the study by Tao et al. (2021) found significant reductions in other microglial-related neuroinflammatory response markers such as microglia branch lengths (27%), number of microglial branches (20%), number of microglial terminal processes (30%) and cyclooxygenase-2 (COX2)-positive microglial population (16%). A significant increase in microglial cell body volume (32%), another marker of microglial activation was also reported by Tao and colleagues. In the animal models of PD, the studies by Johnstone et al. (2014) and O'Brien and Austin (2019) did not observe a significant photobiomodulation-induced alteration to IBA1 immunoreactivity and the microglial population, while El Massri et al. (2016) reported a significant increase of IBA1 immunoreactivity in the striatum (37%). In summary, the result from the selected studies indicates that PBM reduced IBA1 immunoreactivity and microglial population in AD animals, but increased IBA1 immunoreactivity and microglial population in some PD animals.

Inflammatory cytokine effects

Four (4) of the 12 assessed studies reported an effect of PBM on pro-inflammatory cytokines, all of which were investigated in AD-like animals. In the remaining 8 papers, cytokines were not investigated. When considering the results of all four papers, photobiomodulation treatment induced a clear reduction in pro-inflammatory cytokines and downstream signalling molecules, namely IL-1β, IL-6, NFκB and TNF-α. Blivet et al. (2018) and Lu et al. (2017) both measured IL-1B, IL-6 and TNF-α levels and found a significant reduction in these cytokines. Lu et al. (2017) showed a significant reduction in IL-1β by 50%, in IL-6 by 58%, TNF-α by 66%. Blivet et al. (2018) found that 5 min twice daily of PBM applications to head and abdomen significantly reduced TNF-α protein level by 40%. Ten minutes once daily PBM to head and abdomen significantly reduced TNF-α protein level by 38%; IL-1β by 48%; and IL-6 by 52%. Twenty minute once daily PBM significantly reduced TNF-α protein level by 31%. Furthermore, Yang et al. (2021) reported that the pro-inflammatory signalling molecules and cytokines NFκB, TNF-α and IL-1β all had a statistically significant reduction by 13, 13 and 18%, respectively.

Together with, pro-inflammatory cytokines and signalling molecules, anti-inflammatory cytokines have also been investigated. Yang et al. (2021, 2022) observed a photobiomodulation-induced significant increase of anti-inflammatory cytokine levels. In the 2021 study, Yang and colleagues showed a statistically significant increase of the anti-inflammatory cytokines IL-4 and IL-10 by 19 and 29%, respectively. Yang et al. (2022) measured the anti-inflammatory cytokines IL-3 and transforming growth factor (TGF)-β. Significance was also regardless of the size of the plaques the results were measured from. In summary, all their results indicated a statistically significant increase in these anti-inflammatory cytokines, regardless of whether they were measured in the cortex or the hippocampus.

PBM-mediated oxidative stress effect

Oxidative stress effects

Four (4) of the 12 assessed studies examined the effect of PBM on indicators of oxidative stress while the remaining 8 papers did not investigate this toxicity marker. All 4 papers reported a PBM-induced reduction of oxidative markers and an increase of antioxidant capacity.

Blivet et al. (2018) measured lipid peroxidation as an indicator of oxidative stress. Twice daily 5-min head and abdomen applications resulted in significantly reduced lipid peroxidation by 28%; 10 min once daily PBM to head and abdomen resulted in significantly reduced lipid peroxidation by 34%; 20min once daily PBM to head and abdomen resulted in significantly reduced lipid peroxidation by 25%. Lu et al. (2017) found a significant reduction in a range of oxidative stress markers, for example; glucose-6-phosphate dehydrogenase (G6PDH) enzyme activity by 50%, nicotinamide adenine dinucleotide phosphate (NADP) oxidase activity by 54% and its reduced counterpart NADPH level by 23%, 4-hydoxynonenal (4-HNE) immunoreactivity by 60%, peroxynitrite production marker 3-nitrotyrosine (3-NT) by 60%, double-strand breaking marker H2A.XSer139 by 68%, oxidised DNA damage marker 8-OHdG by 63%, superoxide anion by 57% in the mitochondria and 67% in the cytoplasm. Yang et al. (2021) reported that superoxide dismutase (SOD)-2 levels decreased by 43%, malondialdehyde (MDA) levels decreased by 32%, 4-HNE decreased by 24%, protein

carbonyl decreased by 46%, H2AX intensity decreased by 40%. Yang et al. (2022) reported protein carbonyls significantly decreased by 23% in the cortex and 29% in the hippocampus, MDA levels decreased by 30% in the cortex and 29% in the hippocampus, 8-hydroxyguanosine (8-OHdG) levels significantly decreased by 25% in the cortex and 28% in the hippocampus, 4-HNE levels decreased by 23% in the cortex and 36% in the hippocampus.

Three (3) of the four (4) papers also measured antioxidant capacity in response to PBM treatment, all of which showed a significant increase in antioxidant capacity. Lu and colleagues explored the effect of PBM on cellular regions and found a significant increase in antioxidant capacity by 100% in the mitochondria and by 218% in the cytoplasm. Yang et al. (2021) reported a photobiomodulation-induced increase in antioxidant capacity by 42%, while Yang et al. (2022) showed a significant increase of antioxidant capacity by 50% in the cortex and 29% in the hippocampus.

Risk of bias evaluation

The overall quality of the selected studies was assessed using the SYRCLE's risk of bias tool and summarised in Table 3. In this review, 2 studies were determined to carry high risk of bias, 7 studies have an unclear risk of bias and the remaining 3 studies have a low risk of bias. Of the 12 studies, 11 of them attempted randomisation during the process of treatment group allocations. Of the 12 studies, 6 had balanced baseline characteristics; 4 of the studies were unclear as not enough information was provided; and 2 of the studies did not have balanced baseline characteristics because the number of animals in each treatment group was not distributed evenly, and no adjustment was made. Allocation concealment was achieved in 6 of the 12 studies, while the remaining 6 were unclear due to insufficient information. Seven of the 12 studies randomised housing of the animals while 5 of the studies did not provide any

Table 3: Summary table of study risk of bias.

Study (Year)	Random Sequence Generation	Balanced Baseline Characteristics	Allocation Concealment	Random Housing	Blinding During Experiment	Random Outcome Assessment	Blinding During Analysis	No Incomplete Outcome Data	No Selective Outcome Reporting	No Other Sources of Bias	Overall Risk of Bias Comment
Blivet et al. (2018)	✓	✓	✓	✓	✓	✓	✓	?	?	✓	?
Chen et al. (2021)	✓	?	?	?	?	?	?	?	✓	✓	?
Cho et al. (2020)	?	?	?	?	?	?	?	?	✓	✓	?
El Massri et al. (2016)	?	✗	?	?	?	?	?	✗	✓	?	✗
Johnstone et al. (2014)	✓	✗	✓	✓	?	?	✓	?	✓	?	✗
Lu et al. (2017)	✓	✓	✓	✓	✓	✓	✓	?	✓	✓	?
O'Brien and Austin (2019)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Shen et al. (2021)	✓	✓	?	?	?	?	?	✓	✓	✓	?
Tao et al. (2021)	✓	✓	?	?	?	?	?	✓	✓	✓	?
Wu et al. (2021)	✓	✓	?	?	?	?	?	✓	✓	✓	?
Yang et al. (2021)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Yang et al. (2022)	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

✓ = Yes; ? = Unclear; ✗ = No

information on the randomisation of animal housing and were consequently unclear. The ways in which the random sequences were generated was clear in 11 of the 12 studies, and within the remaining 1 study, no information was provided on their attempts at randomising treatment group allocations and housing of the animals. Additionally, 7 of the assessed studies reported blinding during experiment and data analysis while the remaining 5 remained unclear with insufficient information. All 12 of the selected studies avoided selective outcome reporting, in that complete study protocol was available and reported in the manuscripts. Further information with regards to type of bias in each study were summarised in greater detail in Supplementary Table 9.

Discussion

Effect of PBM on glial cells

In this review, we observed a common trend of a general PBM-induced reduction of GFAP expression and astrocyte population in AD- and PD-like animals. These results indicate a potential dampening effect on astroglial activation in the context of neurodegeneration-induced neuroinflammation. However, the involvement of reactive astrocytes in neurodegeneration-induced neuroinflammation is a topic of debate. Further, GFAP expression indirectly represents the magnitude of neuroinflammation; it is worth noting that GFAP is expressed on all astroglial cells, including the ones that are not reactive. To distinguish accurately reactive astrocytes from the resting ones, a more specific marker for reactive astrocytes, such as C3 would provide a more definitive readout of astrocyte behaviour in the context of neurodegeneration-induced neuroinflammation. Therefore, future research investigating the C3 marker will provide a more complete picture on the pathogenesis of neurodegenerative diseases.

Microglia play an integral role in the brain's neuroinflammatory response. The findings of this review demonstrate a general decrease in IBA1 levels after PBM treatment in AD-like animals. However, in the papers that model PD, the effect of PBM on microglia was not as strong. The discrepancies between these results may be attributed to the different activated phenotypes of microglia. Activated microglia have two main phenotypes: the M1 pro-inflammatory phenotype and the opposing M2 anti-inflammatory phenotype. Similar to the duality concept observed in the immune system in general, M1 pro-inflammatory microglia phagocytose and induce cytotoxicity, promoting the clearance of pathogenic substances. Conversely, M2 anti-inflammatory microglia dampen down the pre-existing cytotoxic environment and drive the process of healing and repair through the utilisation of anti-inflammatory cytokines and various trophic factors (Guo et al. 2022). M1 and M2 polarisation is balanced in healthy brains, however, in a neurodegenerative environment there may be an overactive M1 response and underactive M2 response. Although most of the findings presented here are limited by only measuring IBA1, we speculate that PBM is assisting the re-establishment of a balanced M1/M2 neuroinflammatory response. The study by Yang et al. (2022) demonstrated a decrease in M1-like activity as well as an increase in markers that represent M2, such as macrophage mannose receptor cluster of differentiation (CD)-206 and arginase-1. Future studies could investigate the specific markers that represent both M1 and M2 phenotypes such as p38 and IL-1β for M1 and CD206 and arganise-1 for M2 microglia. This will enable us to determine the effect of PBM on polarisation between M1 and M2 microglia from a more multidimensional perspective. In support of the anti-inflammatory effect of PBM, we identified beneficial microglial-vasculature interactions during the data extraction process. Perivascular microglia reside in the brain and are associated with vasculature (Goddery et al. 2021). They are antigen presenting cells that promote cytotoxic T lymphocyte (CD8+) infiltration of the brain, eliminating unhealthy cells from the brain parenchyma. Therefore, in the presence of neuroinflammation an increase in the number of perivascular microglia would be expected. The study by Tao et al. (2021) reported a decrease in vessel associated microglia and M1-like microglia surrounding vessels (presenting with COX2 markers) in response to PBM treatment. Therefore, these findings reinforce the positive PBM effect exerted on microglia in the context of neurodegeneration-induced neuroinflammation.

It was well-established throughout the literature that PBM can have both primary and secondary effects. However, the extent and definition of such primary and secondary effects is somewhat loosely defined. In the context of neurodegeneration-induced neuroinflammation, the primary effect of PBM refers to red and/or infrared light acting directly on astrocytes and microglia by being absorbed by photoreceptors, such as cytochrome C in the glial cells themselves. A convincing piece of evidence was put forward by Yoon et al. (2021), in which an *in vitro* study showed elevated astrocyte proliferation in response to 660 nm PBM treatment. Further asserting the primary effect, *in vitro* studies by Wang et al. (2021) demonstrated

that PBM inhibits the activation of neurotoxic glial cells in astrocytic and microglial cell cultures. On the other hand, the secondary effect of PBM on glial cells is represented by a phenomenon of the photon being accepted by cells other than glia such as neurones, creating an indirect beneficial effect that relieves the nervous system from an inflammatory state and reduces astrocytic and microglial activation (Cardoso et al. 2022; Hamblin 2017; Johnstone et al. 2021). In this review, we are not able to determine whether the positive effects observed are primary or secondary outcomes of PBM treatment. While cell culture studies do not account for the multidimensional closed brain system, *in vitro* co-culture studies of astrocytes and microglia with neurones could answer this question in the future, clarifying the mechanism of action of PBM in the context of neurodegeneration-induced neuroinflammation. This should provide useful information in determining whether glial cells could be a therapeutic target for PBM in neurodegenerative diseases.

Effect of PBM on cytokines

The immune system is balanced between pro-inflammatory and anti-inflammatory immune responses. Such balance is mediated by, but not limited to, secreted signalling peptides called cytokines. In this review, the inflammation-driving cytokines were significantly decreased, while anti-inflammatory cytokine levels were upregulated in response to PBM treatment. These results suggest the potential of a multidimensional effect that PBM exerts on neuroinflammatory responses in neurodegenerative disease models, highlighting the potential of PBM as a disease-modifying intervention to alleviate neurodegeneration-induced neuroinflammation. It is important to note, however, all of the cytokine investigating papers used AD animal models only. Therefore, based on these results, we cannot speculate the effect of PBM on cytokine levels in a broad field of neurodegenerative diseases. While the studies by Moges et al. (2009) and Muili et al. (2012) paved the way for investigating the pathology mitigating effect of PBM in disease models of multiple sclerosis and amyotrophic lateral sclerosis, future studies using different neurodegenerative disease models should examine the effect of PBM on cytokine levels. Additionally, future studies should investigate whether PBM exerts a global effect on the brain or just localising its effect to the inflammatory milieu at disease sites. Only one out of our selected papers (Yang et al. 2022) examined effects on PBM in both hippocampus and the cortex, finding similar anti-inflammatory effects around various sized plaques in both regions. Thus, additional results from future studies are vital in answering this question, which may impact the way we optimise PBM as a clinical treatment for neurodegeneration patients.

Effect of PBM on oxidative stress

ROS such as hydrogen peroxide, superoxide anion radical and hydroxyl radicals are highly reactive molecules generated during the process of aerobic mitochondrial metabolism. These ROS molecules act as secondary signalling messengers and support the cellular adaptations to maintain redox homeostasis (Sies and Jones 2020). Under normal physiological condition, antioxidant enzymes such as peroxiredoxins and glutathione peroxidases restrict the excessive build-up of ROS in the body to maintain physiological balance (Molavian et al. 2015). However, in a neurodegeneration-induced neuroinflammatory environment, cellular ROS levels increase, which disrupts the normal redox signalling pathway and causes molecular damage (Zuo et al. 2019). As a result, the presence of elevated ROS levels creates a vicious cycle, which contributes to neurodegeneration and additional neurodegeneration-induced neuroinflammation (Cheng et al. 2021). The study by McElroy et al. (2017) in a model of epilepsy showed that neutralising ROS with antioxidants attenuates the degree of neuroinflammation, suggesting that this vicious cycle can be disrupted. Multiple reviews propose that PBM attenuates the level of reactive oxygen species (Hamblin 2016; Kumar Rajendran et al. 2019). In this review, we observed a decrease of oxidative stress markers such as lipid peroxidation and an increase of antioxidant capacity markers and SOD-2, an enzyme that actively converts ROS into their non-toxic oxygen counterparts in response to PBM. However, only four out of the 12 selected studies examined the effect of PBM on oxidative stress. Furthermore, out of these four studies, only the two papers by Yang and colleagues (2021 and 2022) had also looked at antioxidant capacity to demonstrate the positive contribution of PBM in restoring the altered redox homeostasis observed in the neurodegenerative disease models. Therefore, we can only tentatively conclude that PBM has a therapeutic capacity to attenuate neurodegeneration-induced inflammation by reducing the degree of oxidative stress in the diseased brain environment.

What is the best set up for the most effective PBM treatment

One of the main issues associated with the therapeutic use of PBM is a lack of consensus on treatment parameters, such as wavelength, irradiance, irradiation time and the number of treatment sessions reflective of dosage (de Freitas and Hamblin 2016). The dose-response curve of PBM is also biphasic. This means that treatment efficacy will reach a maximum value when dosage is increased, while higher dosage beyond the maximum point will diminish the positive effects of PBM or even produce negative outcomes such as excessive heating and fever (Huang et al. 2009; Yue et al. 2019). Whilst this treatment method is not invasive, the heating/thermal effect of PBM should be approached with caution. Thus, modifications to and selection of any of these variables may result in significant changes to the eventual treatment outcomes (Zein et al. 2018). Therefore, it is critical to select optimum PBM parameters in clinical settings to ensure maximum treatment efficacy and minimum side effects.

The wavelength of light is amongst the first parameters to consider when selecting PBM treatment regimen. In a human cadaver model, the studies by Tedford et al. (2015) and Jagdeo et al. (2012) reported that PBM lights in the near-infrared range (808 and 830 nm, respectively) penetrate the skull better than red lights, suggesting that near-infrared lights may be more effective in achieving beneficial physiological effects. In this review, we observed no apparent advantage of either using visible red or near infrared light by the selected papers, with positive PBM outcomes reported across both ends of the spectrum. As a result, we were not able to deduce whether certain wavelengths are superior to others. Secondly, the location of PBM administration is also a major determining factor of PBM effect. The transcranial irradiation approach was the most common method of irradiation in this review. However, this does not confirm that transcranial PBM treatment is the most effective administration route. First put forward by Johnstone et al. (2014), the abscopal neuroprotective effect of PBM refers to the treatment being administered to a secondary location distant to the pathologies, indirectly resulting in the physiological improvement of the conditions at the primary site. Along with Johnstone and colleagues, the studies by Chen et al. (2021) and Tao et al. (2021) further reinforce this concept in this review, highlighting the importance of non-transcranial administered PBM studies in the context of neurodegeneration-induced neuroinflammation. Taken further, Blivet et al. (2018) paved the way in administering PBM in multiple areas of the body. We are excited to see future research exploring the effectiveness of PBM administered to multiple areas of the body. These results could help us to further optimise parameters in future animal studies and make significant contributions in translating this intervention into clinical settings.

Is there more to the story? Continuous versus pulsed

The brain innately has natural rhythms in which the neurones fire in a coordinated fashion at different locations. For example, theta waves are prominent in the hippocampus, firing at 6–10 Hz (Ando et al. 2011; Colgin 2016). Furthermore, the study by Ando and colleagues has shown that PBM pulsed at 10 Hz (theta rhythm) and 40 Hz (γ rhythm) can synchronise with the intrinsic brain rhythm, creating a positive resonance. A beneficial effect from pulsing compared to continuous PBM treatment has been reported in multiple disease models such as traumatic brain injury, somatic pain and acute ischaemic stroke, with various pulsing frequencies (Ando et al. 2011; Lapchak and Araujo 2007; Sushko et al. 2007). In this review, only 2 of the selected studies investigated the effect of PBM using pulsing as a dosage method. With both studies reporting positive effects of 10 Hz PBM, additional research comparing the effect of 10 Hz to other pulsing frequencies with continuously administered PBM will provide greater insight in order to establish more efficacious clinical parameters that can be further optimised for neurodegenerative diseases and neuroinflammation in the future.

Further remarks

This review carries significant impact for therapeutic development to treat neurodegenerative diseases. At the time of writing, to the best of our knowledge, there has been no systematic examination of the effect of PBM on neurodegenerative disease-induced neuroinflammatory and oxidative stress responses. We are also amongst the first teams to establish a foundation for the link between pre-clinical results and clinical applicability of PBM treatment for neurodegenerative conditions.

At this point, we should note several limitations to our review. First, we only selected English primary research articles, while non-English literature was excluded to ensure accurate interpretation of the data and to minimise the risk of translation error. As a result, accurate examination of non-English based literature that satisfy our

selection criteria could expand the strength of our interpretations. Additionally, SRYCLE, the tool used to examine the risk of bias of our selected studies was derived from human clinical trial guidelines. This results in heavy emphasis on the reporting of randomisation and blinding in the author manuscript, which in turn discredits the selected studies that failed to do so. However, the quality of the selected studies could be overshadowed by the word limits and loosely regulated reporting guidelines of many journals. As a result, we believe that stricter standards on methodological write-up should be introduced by the journals, aiding the future reviewing process and maximising study replicability. Furthermore, due to the current animal ethics regulations, the limit placed on the use of animals (especially primates) renders them into the high-risk category for baseline characteristics. However, considering the practical circumstances, we believe that the authors have done everything they can to minimise the presence of bias despite the formal SRYCLE outcomes.

In conclusion, the results from this review indicate that PBM is effective in reducing inflammatory microglial and astrocytic responses, lowering pro-inflammatory cytokines and oxidative stress, whilst promoting anti-inflammatory signalling molecules and increasing antioxidant capacity in AD and PD neurodegenerative animal models. We recommend that future studies measure markers that can quantify the pro-inflammatory and anti-inflammatory polarisation of the immune system. Similarly, we also suggest measuring the oxidative stress-related and antioxidant behaviours to get a better understanding of PBM's effect on homeostatic balance of these two systems in a neurodegenerative environment. Further, additional research could be performed on animal models of other neurodegenerative diseases, such as the childhood dementia, Sanfilippo syndrome. These studies would provide a more robust understanding of the effect of PBM on neuroinflammatory responses, and in particular, further primate studies may provide greater insight into how neuroinflammatory responses translate to clinical outcomes and lay the foundation for the progression of this treatment into human clinical trials.

Acknowledgements: The authors would like to thank the Sanfilippo Children's Foundation, the Cure Sanfilippo Foundation and the Neil and Norma Hill Foundation for funding support for PA and KX.
Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.
Research funding: None declared.
Conflict of interest statement: The authors declare no conflicts of interest regarding this article.

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Supplementary Material: The online version of this article offers supplementary material (https://doi.org/10.1515/revneuro-2022-0109).

Chapter 6: General Discussion

The experimental chapters of this thesis explore four rather diverse aspects of the impact of PBM, from brain activity in young individuals to signs and symptoms in Parkinson's disease, and to neuroinflammation in neurodegenerative disease. There are three key, unifying features of the findings of these chapters (Fig 6.1);

1. That PBM can be viewed as an effective preventative treatment (all chapters)
2. That PBM can have more of an effect on older, diseased brains (e.g. in neurodegenerative disease) than younger healthier ones mainly because there are more cellular and molecular targets for PBM to act upon (chapters 2, 3, 4 and 5)
3. PBM can offer long term benefits for neurodegenerative disease (chapters 4 and 5)

In the section that follows, the results of this thesis and those of others that support each of these key, unifying findings will be discussed in turn. This will be followed by an assessment of the limitations of the four chapters, together with consideration of future endeavours. In general, the findings of this thesis will help propel the field of PBM forward and assist in gaining it recognition as a therapeutic option for the treatment and prevention of neurodegenerative disease.

6.1 PBM can be viewed as an effective preventative treatment

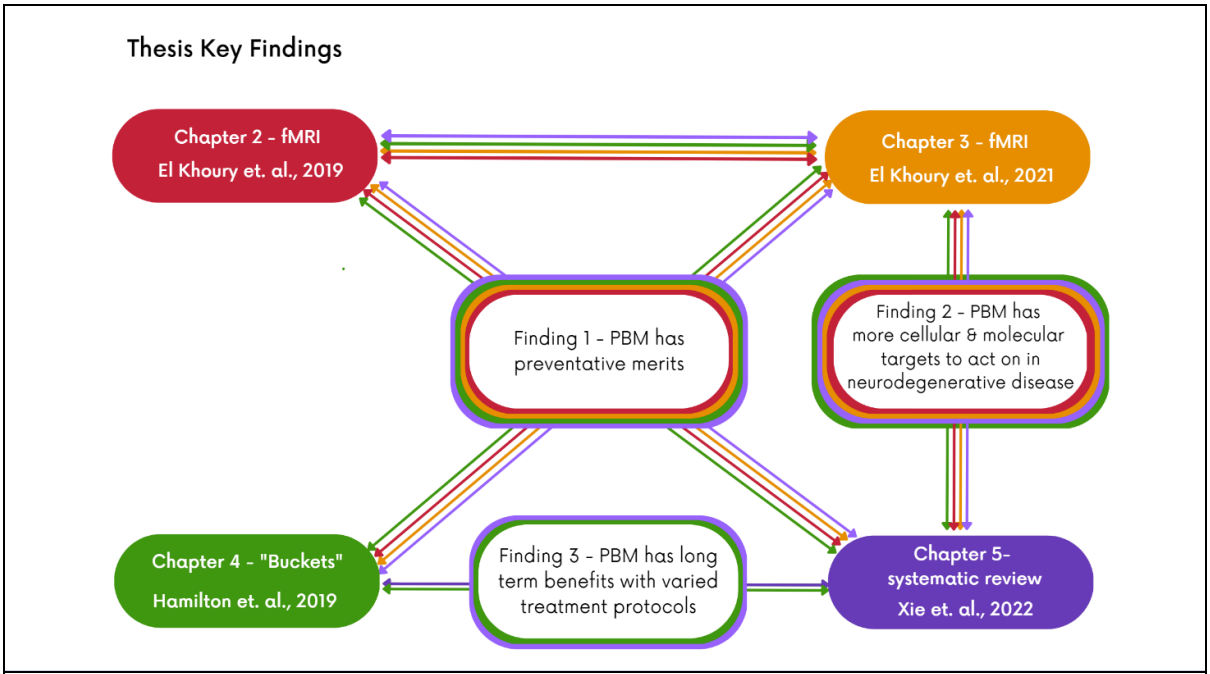

Fig 6.1. A simplified, colour coded diagram of all the interactions between the published thesis chapters and the synthesised key findings of the thesis.

In each of the four experimental chapters of this thesis, there were indications that PBM may be viewed as a preventative treatment (Fig 6.2).

In the fMRI studies (chapters 2 and 3), the results showed that PBM has beneficial effects on the brain circuitry and connectivity of young and healthy brains. In chapter 2, it was demonstrated that brain function was affected and perhaps even improved, as the DMN connectivity was lowered during a task that required a little more focus. This is an improvement because the DMN is responsible for resting brain states, during external goal-oriented tasks, the DMN needs to be switched off in order to improve performance (Fox et. al., 2005). In chapter 3, it was shown that PBM had no impact on resting state activity in brain connectivity signals and cerebral blood flow, suggesting that there needed to be some activity in order for PBM to be effective; one may view this finding as PBM being a treatment for an active brain, that there needs to be active neural circuitry in order for PBM to be effective. These results are supported by other studies that have demonstrated PBM boosting healthy brain functionality in different types of evoked activities, such as cognitive tasks, memory tasks, attention tasks, improved emotional states (Barrett and Gonzalez-Lima, 2013), executive function (Blanco et. al., 2017a), improved prefrontal rule-based learning, and increased learning related cognitive function (Blanco et. al., 2017b), attention and alertness are improved, cognitive function is enhanced, and delta power is decreased simultaneously (Jahan et. al., 2018), frontal brain functions, such as action selection, inhibition ability, and mental flexibility (Chan et. al., 2019). EEG studies have been conducted with relatively healthy ageing brains (Vargas et. al., 2017; Zomorrodi et. al., 2019) that, in contrast to the fMRI findings of chapter 2 and 3, showed a difference in resting states due to PBM, though this could be due to the high temporal resolution available with EEG. There have also been fMRI studies conducted with relatively aged or pathological brains, such as mild cognitive impairment (Vargas et. al., 2017), dementia brains (Chao, 2019), brain damage (Naeser et. al., 2019), stroke (Naeser et. al., 2020) or mild cognitive impairment (Baik et. al., 2021), such studies have demonstrated the PBM helps restore the balance of functional connectivity to “normal” levels, through adjusting ASL perfusion values and some DMN related connectivity, as well as restoration of behaviour measures, such as the ADAS-cog test, to resemble “normal” behavioural scores.

Many previous studies have suggested that a key preventative treatment option for individuals is being cognitively active and using memory skills. In other words, a consistently active brain can be viewed as a "healthy brain" and healthier brains are less likely to suffer disease, later in life, in particular neurodegenerative disease such as Alzheimer's disease. Different types of studies such as epidemiological studies (Ott et. Al., 1999; Wilson et. Al., 2002), randomised clinical trials (Mohs et. al., 1998; Ball et. al., 2002; Valenzuela et. al., 2009), and neurobiological studies using animal models

(Valenzuela et. al., 2003) have indicated that mentally active brains are better protected against neurodegenerative diseases (Gatz, 2005). Other preventative treatment options that have been suggested include controlling vascular risk factors, eating a balanced diet and exercising consistently (Ballard et. al., 2011; Nelson and Tabet 2015; Scheletens et. al., 2016; Crous-Bou et. al., 2017). In essence, if PBM improves brain function then it makes the brain healthier, rendering it less susceptible to dysfunction and disease.

In chapter 4, a range of clinical improvements were observed in many of the patients with Parkinson's disease; in addition, there was no further decline in signs and symptoms evident in the patients. If we consider the writing analysis, which was a test of fine motor skills, each patient showed no deterioration of their writing over extended periods of up to twenty-four months; two of the patients experienced benefits of PBM within the first month of their treatment up to 24 months. This can be viewed as PBM potentially being preventative and slowing or arresting the disease progression. During this time, one would have expected some deterioration of fine motor skills in these patients and in the quality of their writing samples, also referred to as graphology (Fahn et. al., 2004; Burn et. al., 2006; Maetzler et. al., 2009). These findings have been supported by several other clinical case report studies and small-scale clinical trials where many of the patients with Parkinson's disease had little or no worsening of their signs or symptoms during PBM treatment (Maloney et. al., 2010; Hamilton et. al., 2018a; Santos et. al., 2019; Liebert et. al., 2021). The lack of deterioration in the signs and symptoms of the patients (in particular the graphology) suggests that PBM offered protection to the brain cells. If there is protection to the cells, then the cells can be considered to be healthier, and the presence of healthier cells can prevent any further disease, hence by that line of reasoning offering prevention.

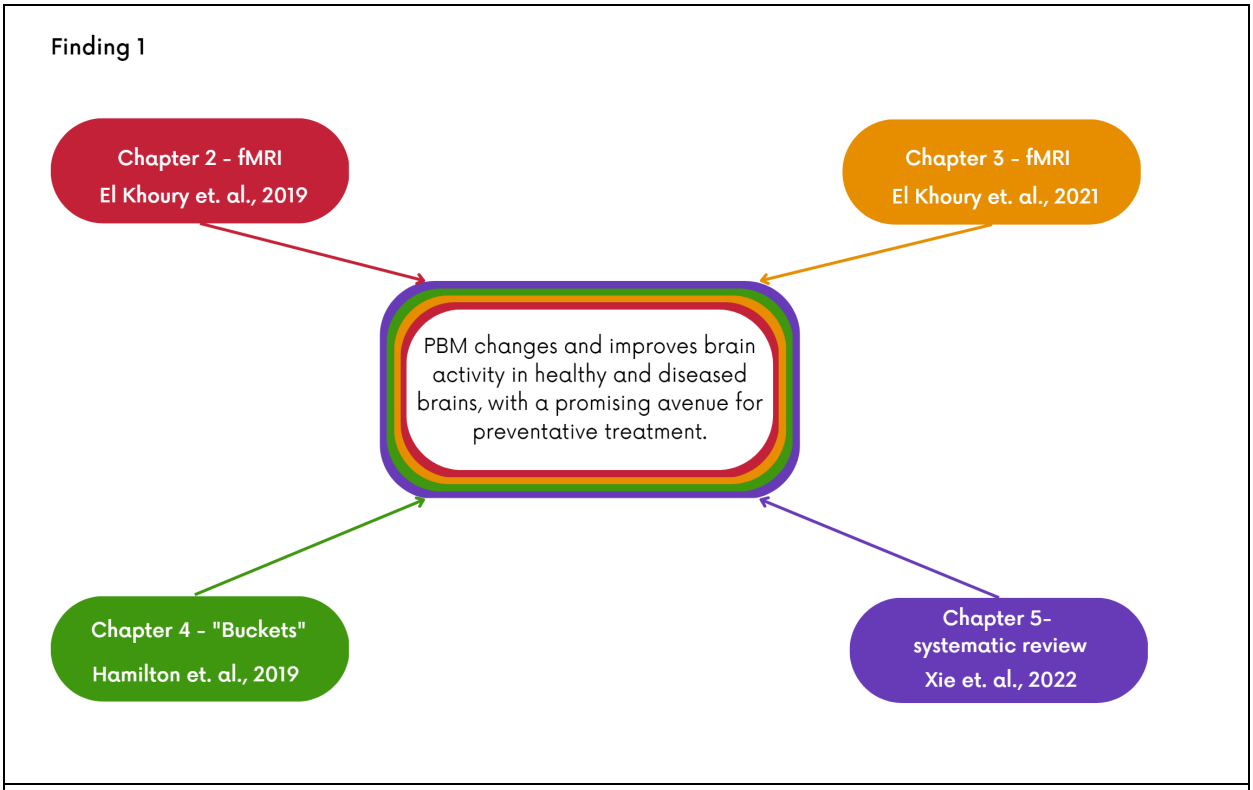

Fig 6.2. A simplified diagram of all the chapters intersecting together to contribute to the main finding. Chapters include fMRI studies on healthy young brains (chapter 2 and 3), case studies with patients that have neurodegenerative disease (chapter 4), and a systematic review of the interaction between PBM (chapter 5), neurodegenerative disease and neuroinflammation. The collection of findings shows a promising avenue for PBM as a preventative treatment.

In chapter 5, the systematic review that explored the effect of PBM on neurodegeneration-induced neuroinflammation, there were many animal studies that have reported that if PBM was applied before the onset, or even during the course of the disease, then less inflammation and neurodegeneration in the brain. For example, studies using MPTP-treated mice and monkeys, 6-OHDA-lesioned rats, α-synuclein AAV virus-treated rats, and the transgenic mouse model of K369I for Parkinson's disease (Shaw et. al., 2010; Peoples et. al., 2012; Moro et. al., 2013, 2014; 2016; Reinhart et. al., 2014, 2015, 2016, 2017; El Massri et. al., 2016a, 2016b, 2017, 2018; Darlot et. al., 2016; San Miguel et. al., 2019), and studies with amyloid precursor protein/presenilin 1 (APP/PS1) transgenic models, double transgenic TASTPM models, CD1 model, and the tau K369I transgenic models of Alzheimer's disease (Michalikova et. al., 2008; De Taboada et. al., 2011; Grillo et. al., 2013; Purushothuman et. al., 2014, 2015; Saltmarche et. al., 2017) have all demonstrated neuroprotection and indicated less inflammation using this pre- or simultaneous treatment approach.

6.2 PBM has more beneficial effects on older brains, particularly those suffering neurodegenerative disease, than in younger brains, potentially as there are more targets.

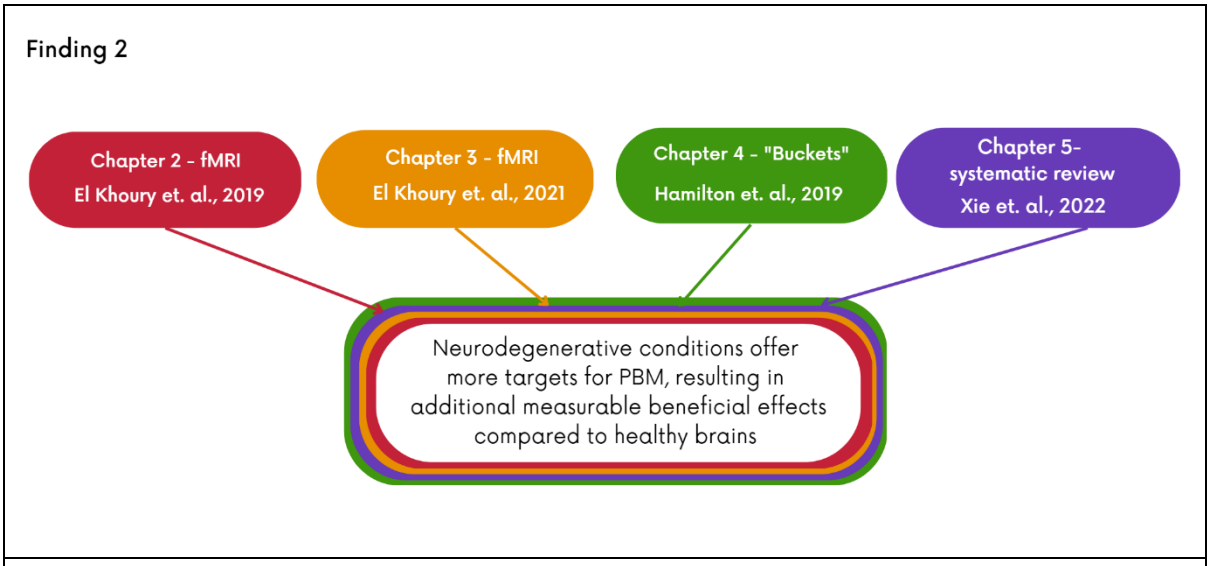

Fig 6.3. A simplified diagram of 4 chapters intersecting together to contribute to the second finding. Chapters include fMRI studies on healthy young brains (chapter 2 and 3), a case study on PD patients (chapter 4) and a systematic review of the interaction between PBM, neurodegenerative disease and neuroinflammation (chapter 5). The collection of findings suggests that PBM has potentially more beneficial effects on the older brains, particularly those suffering neurodegenerative disease, than in younger brains, indicating there may be more targets of PBM in older and diseased brains.

In chapters 2 and 3 of this thesis, it was shown that PBM had an effect on the BOLD connectivities of young healthy brains while they were engaged in a functional activity, namely finger-tapping; no effect was found on the activity of these brains while at rest. In contrast, previous studies - using older individuals or those suffering neurodegenerative disease - have reported that photobiomodulation does have an effect of functional activity while at rest (Vargas et. al., 2017; Chao, 2019; Naeser et. al., 2019, 2020; Baik et. al., 2021). This key difference may be reflective of the fact that older brains, together with those suffering neurodegenerative disease, have considerably more neuroinflammation, oxidative stress and mitochondrial dysfunction, than younger healthier brains. In essence, there appears to be more “targets” for PBM to act upon, resulting in considerably more beneficial outcomes. In this context, the wide range of beneficial outcomes evident in the Parkinson’s disease patients with PBM treatment in the Buckets study (chapter 4) support this hypothesis. One could speculate that if the study was conducted on normal healthy individuals, notable improvements

in issues such as anxiety and fatigue, which are a part of many "normal" people's lives, may not have been as marked.

"Targets" for PBM exist in both healthy and diseased cells, and for the purpose of this thesis, the word "target" can take the form of a range of measurable variables within the brain. It can refer to signs, symptoms and markers of neurodegenerative disease that can be subject to change and hence change the functionality of the brain. In a healthy brain context, targets can represent functional tasks, for example performance in finger-tapping, cognitive memory tasks, and handwriting tasks, as well as circuitry deviations in an ageing but otherwise "healthy" brain. In a healthy brain, we expect these "targets" to show an increase in functional activity influenced by PBM. Whereas in an aged or diseased brain environment "targets" can also include, but are not limited to, markers of neuroinflammation such as inflammatory cytokines, oxidative stress markers (e.g., ROS), mitochondrial dysfunction, neuronal cell death, and signature molecules found in neurodegenerative diseases, such as AB plaques and tau tangles in AD, alpha-synuclein bodies in PD and Lewy body dementia, furthermore, targets can also include clinical signs and symptoms of neurodegenerative diseases measured by cognitive and physical tests, such as ADAS-cog scores, stiffness, number of lockouts, handwriting, and tremors. As a caveat, the ageing brain is not necessarily a so-called "healthy circuitry", as there is often considerable mitochondrial dysfunction, inflammation and loss of grey matter evident, though not as prevalent as brains with neurodegenerative diseases (Sparkman and Johnson, 2008; Hafkemeijer et. al., 2014; Porcher et. at. al., 2021). However, the ageing brain can offer a sub-pathological baseline, as they do house much uncleared waste and inflammation products from the mitochondria, although these dysfunctions have not reached the levels of neurodegenerative disease. Many, if not all of these cellular abnormalities and pathological signatures have been shown to be improved after application of photobiomodulation in a range of animal models of neurodegenerative disease and of ageing (Michalikova et. al., 2008; Shaw et. al., 2010; Gonzalez-Lima and Barret, 2014; Hamblin, 2016; El Massri et. al., 2018; Yang et. al., 2021, 2022; dos Santos Cardoso et. al., 2021). In essence, there needs to be a cellular process to be occurring, whether it be functional activity or dysfunction and pathology, in order for PBM to work. For a healthy brain at rest, there is little functional activity and no dysfunction and pathology and hence PBM is largely ineffective; there is little work to be done. However, while engaged in a task that requires focus and attention, the deactivation of the Default Mode Network (DMN) by PBM indicates an effect that assists the brain even more in focusing on the task at hand (chapters 2 and 3). For a diseased brain that is in the process of enduring neuroinflammation, oxidative stress and/or disease-induced cellular death, PBM may function to correct any dysfunction and pathology by stimulating the cellular processes associated with cell survival; in other words, PBM, tries

to fix the problem, and the measurable clinical benefits aren't restricted to molecular markers, motor or non-motor signs and symptoms, but rather an overall effect (chapter 4).

These findings are mirrored in the animal model studies of the systemic review in chapter 5. Diseased animal brains showed significant benefits of PBM accompanied with measurements of targets returning to baseline. However, in the healthy animal controls that were treated with PBM compared to ones that were not treated with PBM, results showed that there were no significant differences , such as markers of apoptosis, neuroinflammation, and cognitive behavioural tests, as seen in the study by Yang and colleagues (2022). This supports the notion that diseased brains have more targets for PBM to act upon compared to healthy brains.

It is also important to note that neuroinflammation is not necessarily restricted to ageing and neurodegenerative diseases (Moore et. al., 2010; Hanna et. al., 2022). Sometimes in healthy young brains, certain variables may come together temporarily to create an acute inflammation in the brain. For example, in a study of young heathy adult brains, neuroinflammatory markers were discovered during stressful contexts such as the covid-19 pandemic (Brusaferri et. al., 2022) and general social stresses driving higher levels of interleukin 1 (DiSabato et. al., 2020) and microglial activation (Calcia et. al., 2016). In these instances - with an increase in targets, PBM may be effective in younger brains, helping to reduce inflammation and neuronal survival.

For there to be beneficial effects on both healthy and diseased cells, it seems that PBM, via its direct and indirect mechanisms of cells. PBM stimulates mitochondria to produce additional ATP energy to carry out normal functions Additionally, the brief and normal burst of ROS initiated by this process can encourage the cells to release antioxidant enzymes that have a clean-up effect (Kumar et. al., 2019; Fig 6.4), together with the production of many neuroprotective and anti-inflammatory transcription factors and the downregulation of pro-inflammatory transcription factors (Muili et. al., 2012; Hennessy and Hamblin, 2017; da Rocha et. al., 2021). Further, it has been demonstrated that ROS were increased by PBM in healthy cells, while in stressed or diseased cells, the influence of PBM was to reduce ROS levels (Huang et. al., 2013).

This key finding (key finding 2, Fig 6.3), that PBM has more beneficial effects on older brains compared to healthier brains because there are potentially more targets, contributes to the first key finding that supports the prospect of PBM as a preventative treatment as improvement of functionality means improvement of the efficiency of brain processes in the wake of the constant neuronal maintenance and upkeep required to keep the brain healthy and optimal. As an analogy, picture an extremely dirty floor (diseased cells) and a mop (PBM) vs. a moderately clean floor (normal cells with normal amounts of waste) and a mop (PBM). The mop is going to make a much

more visible difference to the dirty floor, but the moderately clean floor can still benefit from the mop (Fig 6.4).

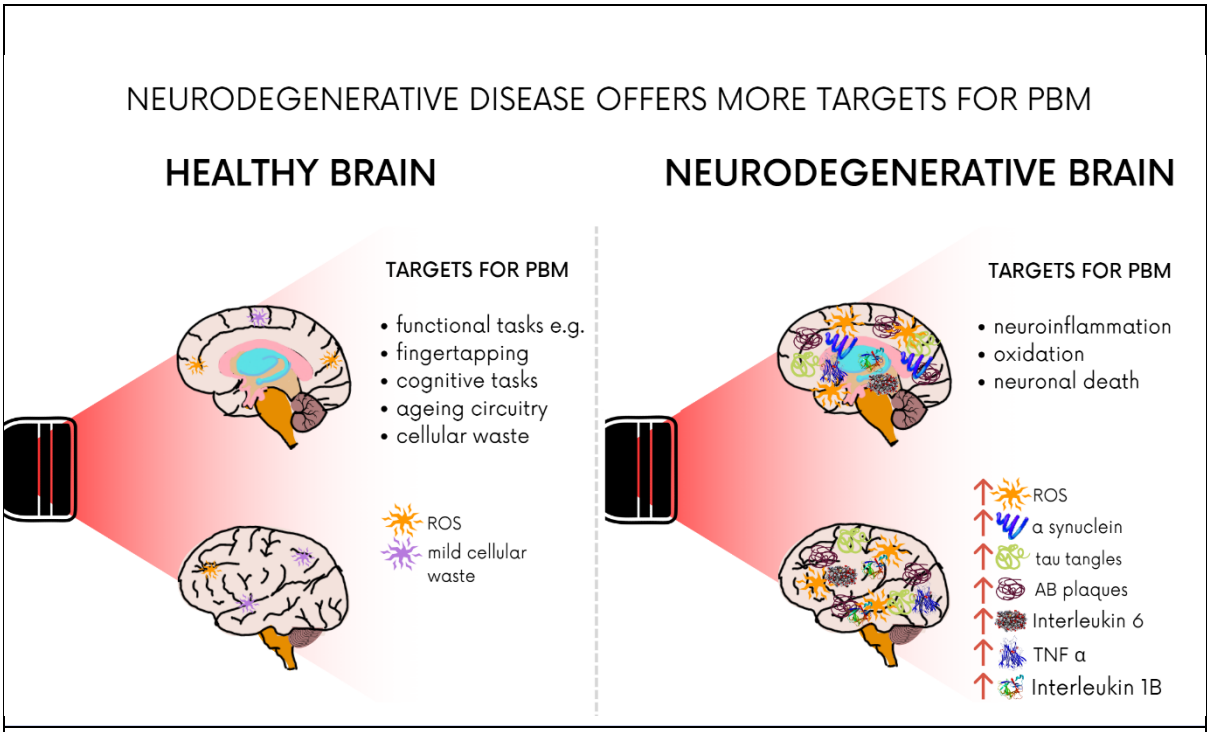

Fig 6.4. A schematic diagram comparing the difference between targets for PBM in healthy brains compared to neurodegenerative brains. "Targets" are variables that can change functionality in the brain – they can be functional tasks, clinical signs and symptoms that are cognitive or motor, as well as markers of pathogens, cellular damage, inflammatory cytokines, or signature proteins specific to neurodegenerative diseases.

6.3 Photobiomodulation offers long-term benefits for neurodegenerative disease

Fig 6.5. A simplified diagram of 2 chapters intersecting together to contribute to the third finding. Chapters include cases studies of patients with neurodegenerative conditions (chapter 4) and a systematic review of the interaction between PBM, neurodegenerative disease and neuroinflammation. The collection of findings suggests that PBM offers long term benefits for neurodegenerative disease.

Regardless of wide range of dosage protocols and wavelengths, the findings in chapter 4 of this thesis showed that PBM can have a long-lasting effect on human participants with neurodegenerative disease (Fig 6.5); whilst chapter 5 showed many previous studies on animal models of AD and PD have shown similar findings of long-term PBM effects. Such long-lasting results have been reported with the use of a range of different wavelengths, irradiance, fluence and length of treatment. In terms of length of treatment, anything starting at 1 month up to 24 months have all showed a retainment of benefits.

From the Buckets study (chapter 4), many of the patients were still reporting improvements in signs and symptoms up to 24 months after starting. An interesting observation is that with the patients aged 63 years and under who started their treatment earlier on in the timeline, within a year of their diagnosis, had a quicker response time to PBM treatment, shown through a reduction of signs and symptoms, within just weeks. By contrast, patients aged 70+ years who had been diagnosed with Parkinson's Disease for more than a decade, only showed improvements in clinical signs and symptoms after 8 months of treatment (Chapter 4). While this is a small study group, it does suggest that an earlier intervention and younger patients results in quicker response to PBM and improvement of symptoms. A further interesting feature of this study in chapter 4, is that even after such a long period of use – that is 24 months in one case – there was little or no decline in impact and no side effects, further highlighting the fact that photobiomodulation treatment is entirely safe to use on human subjects

There are many examples of similar long-lasting effects by photobiomodulation in previous studies using human subjects (Fig 6.6). A study with 8 participants diagnosed with dementia utilised the Vielight Neurogamma device at 810nm for 20 minutes 3 days a week (Chao et. al., 2019). In this 12-week study, benefits of PBM were measured 3 months after baseline indicating that PBM significantly improved Alzheimer's Disease Assessment Scale cognitive (ADAS-cog) and Neuropsychiatric Inventory (NPI) scores, as well as increased cerebral perfusion and increased functional connectivity. Other clinical studies, mainly on Parkinson's disease patients, have been conducted with a remote PBM protocol (i.e., exposing on distant body parts, such as the abdomen and microbiome) and results showed improvements in signs and symptoms lasting up to 45 weeks with continued treatment during a covid lockdown period throughout 2021 (Liebert et. al., 2021; 2022) and an improvement in the profile of the microbiomes themselves (Bicknell et. al., 2022). Further, in a study using patients suffering depression and anxiety, it was shown that a single photobiomodulation treatment to the forehead can result in improvements in depression and anxiety scores and in regional cerebral blood

flow up to 2 weeks post-treatment (Schiffer et. al., 2009). Another study used an 8-week protocol and a total of 16 PBM treatments transcranial to participants who had moderate scores of depression, and their Hamilton Scale of Depression (HAM-D) scores significantly decreased and showed subjective improvements (Cassano et. al., 2022).

As with the human studies, there are many examples of long-lasting effects in animal models. For example, in a non-human primate model of Parkinson's disease, a brief five-day period of photobiomodulation treatment (using an intracranial device) resulted in beneficial outcomes – both behavioural and neuroprotective – up to a month thereafter (Darlot et. al., 2016). Further, there are several studies in animal models of Alzheimer's disease that also demonstrated the long-term benefits of PBM. For example, Cho and colleagues (2018) demonstrated reduced anxiety-like behaviour on the elevated plus maze test, as well as improvements in long term and spatial memory in the Morris Water Maze test sustained over 14 weeks. Cho and colleagues (2018) also noted that these benefits lasted in the mice that started PBM at 2 months of age, compared to those that started treatment at 6 months of age, which supports the idea of earlier PBM intervention having a greater turnaround. Yang and colleagues also demonstrated longer term benefits of PBM, such as 8 months (Yang et. al., 2021) and 16 months (Yang et. al., 2022) in a transgenic rat model of Alzheimer's disease. These lengths of time are particularly noteworthy considering the relatively short lifespan of rodents.

At this point, one should consider the mechanism behind such a long-lasting and consistent phenomenon. As discussed in chapter 1, photobiomodulation has a key influence on mitochondrial function, resulting in the production of more ATP energy to drive cell function. This energy-boost is considered to be short-term however, lasting a matter of minutes (Hamblin 2016; Wang et. A l., 2016). The photobiomodulation impact does however lead to a small increase in reactive oxygen species (ROS) levels, that then regulates the expression of various genes such as NF-kB, NRF2 and PI3K/Akt (Song et. al., 2012; Zhang et. al., 2016), jak/stat signalling pathways (Leyane et. al., 2021), and CXC chemokine receptor 4 genes (Ganeshan et. al., 2019) that are associated with modulation of chronic inflammation. Activation of these pathways can lead to changes in functional activity and neuroprotection for long-lasting periods. Another factor to consider which contributes to the long-lasting effects of PBM is the stimulation of neurogenesis in animal models (Wu et. al., 2021) and in humans (Cassano et. al., 2016).

In summary, this long-lasting feature is exciting because it supports the notion of PBM being an effective and consistent treatment option for neurodegenerative disease. Most PBM treatment protocols within the wavelength ranges of 600 - 680nm and 800 - 1070nm, for dosage protocols between 6 mins to 30 minutes per day can have long lasting effects that can extend up to 24 months.

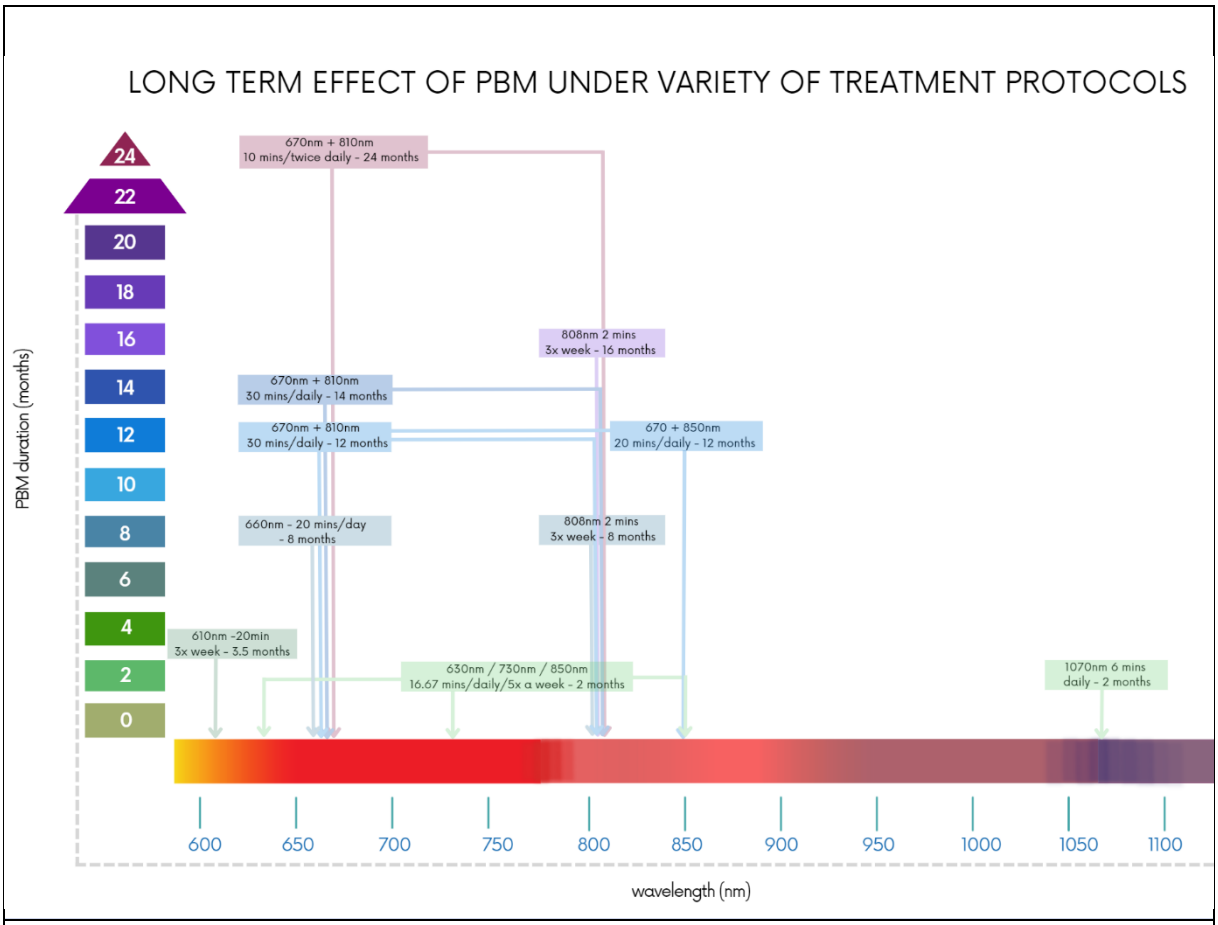

Fig 6.6. A diagram of the varying dosages and treatment protocols for PBM such as wavelength (x axis) and length of time in months that long term effects were measured over (y axis).

6.4 Strengths and limitations of experimental designs

In this thesis the experimental designs of the individual chapters, when taken together, had certain strengths that offered a good foundational basis for further studies and clinical trials on the effect of PBM on the human brain, especially for treatment of the neurodegenerative diseases, such as

Alzheimer's and Parkinson's disease. There were also some limitations that could be improved upon in future studies.

For the fMRI studies on healthy human brains (chapters 2 and 3), there were strengths such as sufficient sample size (20 participants) to measure and statistically analyse a genuine PBM effect on brain function and connectivity. Further, the use of an fMRI machine – which presented a rare and wonderful opportunity - offered accurate spatial resolution to determine exactly which areas of the brain are influenced by PBM.

There were also some limitations to the experimental designs of these chapters. For example, the PBM device was not compatible with the MRI machine. Hence, measurements of resting state connectivity, frequency and cerebral blood flow could not be obtained simultaneously, during the PBM treatment; measurements were possible only before and shortly after the treatment had been applied. A live measure could have provided a different set of results. Another limitation to consider is that the studies were not double blinded when assigning a participant to the treatment or sham group, only participants were blinded but not the administrator of the PBM treatment.

For the Buckets study (chapter 4), the clear strength of the study was that it presented a series of observations – largely objective – on the effect of novel transcranial PBM devices, with wavelengths of 660nm, 670nm, 810, and 850nm on the signs and symptoms of Parkinson's disease patients over long-periods of time, this included graphology measurements. Further, the medical practitioners overseeing the study knew the patients very well and were able to offer rare insights into their progress. Overall, this series of case studies was novel at the time and provided a solid foundation for a large-scale placebo-controlled clinical trial. The clear limitation of this chapter was there was a lack of control group. In addition, the small size of the study, and the diversity of PBM protocols does not provide enough basis for statistical analysis. Future studies with larger group sizes – together with controls using a placebo helmet device - may allow us to identify whether there is a statistically significant correlation between earlier PBM treatment and quicker response.

The systematic review addressing the benefits of PBM on neuroinflammation in neurodegenerative models (chapter 5) had strengths in its design. For example, the search methods were registered in

PROSPERO and the criteria for selection were strict to ensure the most valid studies were included. Every step throughout the data acquisition process was reproduced by two researchers independently. The study provides for a solid reference point for researchers exploring the effect of PBM on neuroinflammation in neurodegenerative disease. However, there were limitations with the systematic review. For example, non-English literature was not included hence some key studies may have been missed out. Further, the strict screening procedure also resulted in many case studies being omitted. Finally, the assessment of the study's overall risk of bias was moderate based on the SYRCLE risk of bias tool derived from human guideline studies.

6.5 Future applications and considerations

When considering future applications to propel the research forward, physical and genetic factors influence effectiveness of treatment and dosimetry. Additionally, improvements of experimental design, including increasing sample sizes and length of studies will expand the research.

In terms of PBM effectiveness, it is important to consider that photoacceptors, like all other cellular molecules and structures are genetically coded. Individuals may have different photoacceptors that have higher affinities for different wavelengths. For example, it has been determined that there are polymorphic variations of cytochrome c oxidase (CcO) that can result in functional variations (Lu et. al., 2010). However, there is very little information about how different genetic variations of CcO and other photoacceptors influence an individual's responsiveness to PBM. Future applications can include studies that sequence for individual participants' photoacceptor types so that PBM treatment protocols can be optimised. For example, future studies that measure the benefits of PBM might include RNA sequencing for the photoacceptors after different treatment protocols. Despite the consistent effect of PBM with varying dosages, some individuals in the case studies have shown quicker and more dramatic effects, while other individuals take a longer time to accumulate enough measurable benefits of PBM (chapter 4). In addition, it would be worthwhile to investigate the difference between the results of ongoing PBM treatments over a continuous period compared to one off treatments, as there has been documentation of long-term effects from ongoing treatments (Liebert et. al., 2021, 2022) as well as one off treatments (Schiffer et. al., 2009).

Physical factors for dosimetry also need to be taken into consideration for future applications. For example, there are several different treatment protocols that have yielded positive responses to PBM. However, there may still be a gold standard to the dosimetry parameters. For example, a study found that the optimal wavelength range of brain penetration was 1000-1100nm (Yaroslavsky et. al., 2002)

while other shorter wavelengths did not necessarily penetrate very deeply into the cranium and brain (Jagdeo et. al., 2012). Penetration is only one variable that contributes to the optimal dosimetry of PBM, especially when considering things like abscopal effects and the minimum power density required for the brain to experience any biological effect (e.g., Hamblin, 2016). Alongside the mentioned parameters, studies that more closely compare the modes of operation of PBM, for example laser vs LED PBM treatment protocols, and a deeper investigation on the difference of outcomes when using continuous vs. pulsed waves of different frequencies (10Hz or 40Hz) need to be explored. There is currently a shortage of studies that accurately compare the different modes of operation, and there is no verdict to whether pulsed or continuous delivery is the most effective. Further advances in dosimetry include the use of the Monte Carlo simulator to determine optimal dose of PBM. The Yaroslavsky study compared the simulator to *in vivo* tests and found different optimal levels depending on the mode of treatment. For example, a different dose needed for intra-oral PBM compared to extra-oral treatment (Yaroslavsky et. al., 2021). This type of research recognises that there may be different gold standards depending on the targets for treatment. There are also varieties in physical application of the PBM, such as helmet devices, some that are specifically manufactured, such as Vielight (www.vielight.com) and the Wellred Coronet (https://wellred.com.au/the-coronet). These helmets have a transcranial delivery, while others have intranasal, and others still have protocols that required multiple body regions to be irradiated (chapters 2, 3, 4). A systematic review by Cassano and colleagues (2022) established that the greater the PBM dose and frequency, the better effects measured on depression and anxiety symptoms without PBM-induced adverse effects. Further, several animal models also included intracranial placement of the PBM devices (chapter 5). A small-scale clinical trial is underway currently testing the efficacy of the intracranial device in human patients (Clinatec, Grenoble). Further investigations can help maximise general PBM dosimetry protocols, however, these investigations may also open up the avenue for customised treatments that are tailored to individuals depending on the protocols they are most responsive to.

Additional improvements in experimental design for future research need to include more longitudinal studies of sufficient length, with both healthy and elderly individuals, including those with mild cognitive decline or suffering neurodegenerative disease. If these studies are designed in a comparable way, then the difference between PBM on healthy versus older and neurodegenerative brains can be more clearly understood and therefore, the preventative potential of PBM can be better defined. Further improvements in experimental design can include larger sample sizes for clinical trials that measure the effect of PBM on clinical signs and symptoms of Alzheimer's and Parkinson's disease

patients. With a larger sample size, there can be a statistical analysis of the relationship between age of patient and early PBM intervention to determine the effectiveness and response time to PBM .

6.6 Conclusions

The results of the thesis have provided a foundation for the concept that PBM can be viewed as an effective therapeutic treatment for neurodegenerative disease. This general concept is supported by key findings that have supported the notion that PBM can be viewed as a preventative treatment and that it has a more beneficial effect when used in older and/or diseased brains compared to younger, healthier ones. This concept (see above) is becoming more established as the sets of results from the thesis chapters have indicated that there are more cellular and molecular targets for PBM to act upon in a diseased state. To further investigate the basis for PBM as a preventative treatment against neurodegeneration, results from this thesis have indicated that PBM has the capacity to also offer long term benefits for neurodegenerative diseases. Whilst the experimental designs in every chapter have limitations, they add to the compilation of knowledge of effects of PBM on the brain and offer a solid template of the concepts. These chapters also provide a framework for improved future studies to further support the key findings of photobiomodulation as a viable therapeutic treatment, particularly for neurogenerative disease.

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