PHOTOBIOMODULATION OF NEURAL STEM CELL DIFFERENTIATION USING INFRARED (810 NM) LASER STIMULATION

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ABSTRACT

Photobiomodulation of Neural Stem Cell Differentiation Using Infrared (810 nm) Laser Stimulation

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The standardized clinical use of photobiomodulation (PBM) across many applications has been obstructed due to the lack of understanding in how each of the parameters affect the end results. Although the optimal parameters for the therapy have been estimated with a level of uncertainty, the positive effects have been documented on cell differentiation, wound healing, brain modulation, etc. These positive effects observed by PBM have been attributed to the increase in reactive oxygen species (ROS), nitric oxide (NO), and ATP along other molecules generated by the stimulation of Cytochrome-C Oxidase (CCO). Although there is much uncertainty surrounding the coupling mechanisms in the therapy and how they are affected by each parameter, the therapeutic potential has been proven time and time again. Many studies have been published and validated to establish the efficacy of photostimulatory therapeutics.

One of the more recently explored PBM applications has been focused on the brain, including photo-repair of traumatic brain injuries and also enhancing brain functionality. While it is in its early stage of development, translation to clinical settings would require a clear understanding of the PBM parameters. This study is designed to investigate how irradiance affects the outcomes of such potential therapy. We used rat neural stem cells to determine the

extent of photostimulatory neurodifferentiation using an 810 nm laser. This study could lead us to optimization of the PBM parameters to facilitate and enhance neurodifferentiation in the brain.

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I would like to express my gratitude to the following people:

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DEDICATION

I would like to dedicate this work to my family and friends. Their support and understanding have encouraged me to continue and complete this work. They have been a great support system and I could have not done it without them, so for that, I am thankful to each one of them.

TABLE OF CONTENTS

TABLE OF FIGURES

CHAPTER 1:

Introduction

1.1 Photobiomodulation

The use of photobiomodulation (PBM), inclusive of wavelengths between 600 - 1200 nm, has gained popularity for the past 50 years.[1] Photobiomodulation is a form of therapy which uses a visible or near-infrared light source to aid in wound healing, pain and inflammation, stem cell stimulation, nerve repair, trauma injuries, etc.[2] Aside from *in-vitro* studies, there have already been clinical uses of the therapy, some examples in back pain, wound healing, and others; however, there is much debate in the efficacy of the treatment[2]. The standardized use of the therapy has been delayed due to a lack of understanding in the interactions between the light source and tissue/cells.[3] One of the most important factors to consider in photobiomodulation is in the biphasic response observed across many studies, also known as the Arndt-Schulz Law, which states that a low dose will have no positive effect, a higher dose will have a positive effect, an even higher dose will have an even greater positive effect until a plateau is reached, and a dose beyond the threshold will have detrimental effects.[4] The biphasic effect seems to follow the finding from many studies, which all suggest an optimal "range" of stimulus at which the most beneficial effects can be reached. Another important factor to consider is in the first law of photobiology, which states that a photon must be absorbed in order to have any biological effect.[4] Although there is much uncertainty surrounding photobiomodulation, one of the most common mechanism hypotheses is the absorption of photons by Cytochrome-C Oxidase (CCO); As seen in Figure 1-1, CCO is observed to be the primary photoreceptor, through which an increase in ATP, Nitric Oxide (NO), Calcium ions, Reactive Oxygen Species (ROS), and other molecules are observed as a result.[5] Although there has been continuous supporting evidence for the positive effects of photobiomodulation in clinical settings, the inconsistency in results has led

to the effectiveness of the treatment relying in the optimization of parameters, some of which are wavelength, energy density, time, etc.[3]. The successful translation of treatment into the clinical setting will rely on further understanding of the parameters and interaction that result from photobiomodulation; the uncertainty remaining suggests the need for further investigation into the individual components surrounding photobiomodulation.

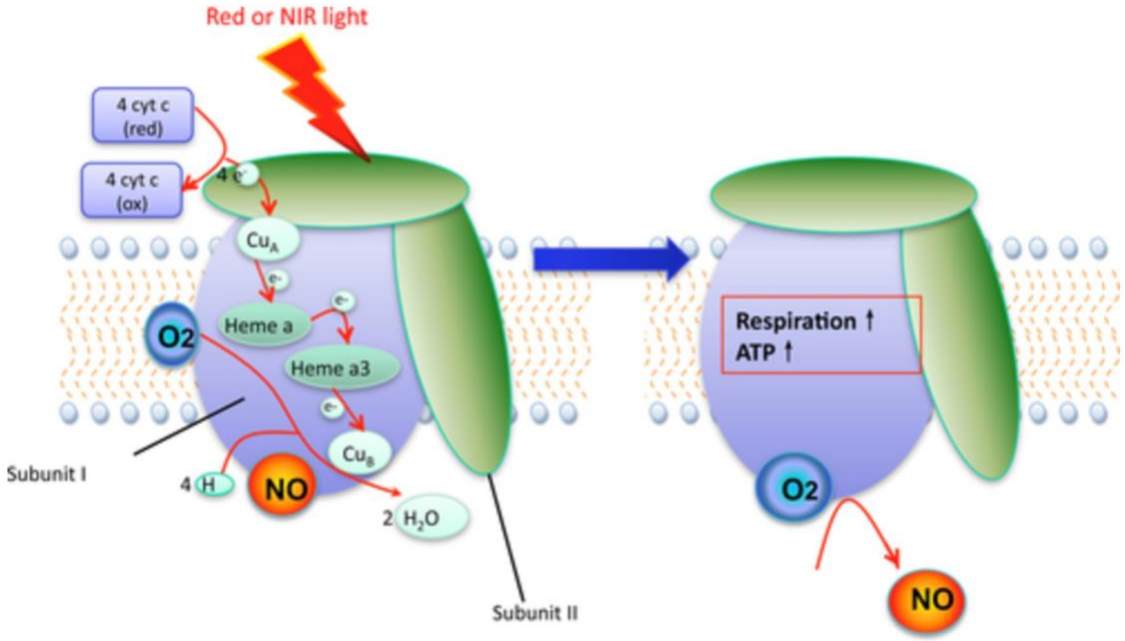

Figure 1-1: Working Coupling Mechanism. Cytochrome C Oxidase (CCO) is presumed to couple to light with appropriate wavelength and increase the electron transfer chain (ETC) activity in the mitochondria. Some consequence of ETC activity should lead to production of ROS and ATP.

[Reference: https://onlinelibrary.wiley.com/doi/full/10.1111/php.12864]

1.2 Photobiomodulation in Stem Cell Differentiation

As discussed previously, some of the already explored applications are in wound healing, pain reduction, and inflammation relief; however, recently, there has been an increase interest in the possible applications for stem cell differentiation, neurological trauma, and cognitive

enhancement.[1] In stem cells, it has been found to promote cell proliferation and differentiation.[6] In a study performed on bone marrow stem cells, an 810 nm laser was found to promote neural differentiation at 3 J/cm^2 and 6 J/cm^2 .[7] A study performed on bovine liver mitochondria (CCO), demonstrated how 810 nm has stimulating effects on CCO; interesting enough, wavelengths 750 and 950 nm had the least effect on CCO.[8] As demonstrated in Figure 1-2, and through previous studies, wavelengths around 810 nm appears to have optimal neuronal differentiation and CCO stimulation in comparison to other NIR wavelengths. Although there is much debate in deciding the optimal parameters for photobiomodulation, Hawkins *et al.* demonstrated how fluences higher than 10 J/cm^2 resulted in decrease cell viability, proliferation, and an increase in DNA damage.[9] The combination of photobiomodulation and differentiation factors has suggested for their use to have a positive, synergistic effect on cell differentiation; extensive research suggests for cell proliferation and differentiation to be competitive processes; however, laser therapy can have potential photothermal effects at high energy densities, which can inhibit neuron differentiation.[7] The combination of already successful components in cell differentiation has been observed to enhance differentiation when compared to the use of the same components individually. To ensure minimal adverse effects and proper use of therapy, an optimal range should be determined to increase success of treatment. Such optimal ranges of phototherapy could depend on the intended differentiation lineage (e.g., neural cells or bone cells) or the source of stem cells (e.g., adult stem cells or embryonic stem cells).

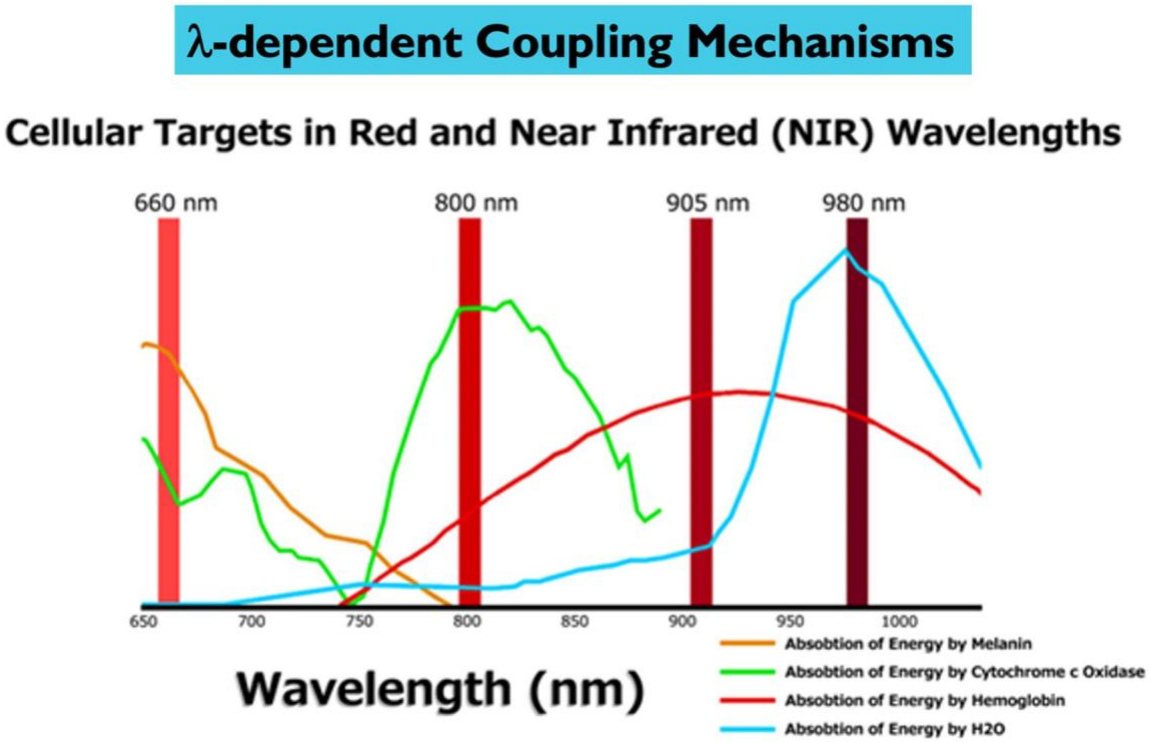

Figure 1-2: Schematic Absorbance Spectra. Light absorptions of several key molecules are depicted in the red and NIR wavelength range. CCO demonstrates a maximum absorbance at 810 nm (green spectra).

[Reference: https://www.foma.org/]

1.3 NeuN and SOX2 as Neural Stem Cell Markers

One of the primary proteins for the development and maintenance of stem cells is SOX2 that belongs to a family of the high-mobility transcription factors in embryonic stem cells. An overexpression of the SOX2 protein has been reported to result in cancer development across different tissue types.[10] A variety of gain-of-function and loss-of-function experiments have demonstrated the role of SOX2 in cell proliferation, survival, renewal, and neurogenesis; the latter being dependent on cellular context and the loss-of-function experiment resulting in increased apoptotic markers and loss of Nestin/GFAP.[11] The expression of SOX2 is maintained until differentiation occurs; forced expression resulting in longer maintenance of stem cell-like

properties.[11] The use of SOX2 as stem cell marker has been widely used; however, it is not only specific to neural stem cells, but to a variety of stem cell types. The NeuN marker is a widely used antigen for the positive identity of postmitotic neurons.[12] The NeuN antibody binds to Fox-3, part of the Fox proteins which bind to RNA and regulate alternative splicing; alternative splicing plays an important role in post-transcriptional regulation of genes.[13] Exclusively to neurons, Fox-3 has been speculated to play a role in the development of the nervous system and be a regulator of differentiation.[14] The expression of NeuN rises during early embryogenesis and is maintained throughout the differentiation process.[15] The higher specificity of NeuN marker is demonstrated in the marker exclusively being present in the nervous system of vertebrates.[16] The localization of NeuN is mainly in the nucleus; however, there have been previous studies which reported cytoplasmic NeuN.[12] The expression levels in nucleic and cytoplasmic NeuN varies within the same neuron type, resulting in slightly unpredictable results.[15] As differentiation takes place, the downregulation of SOX2, followed by the upregulation of NeuN is expected.

1.4 Possible roles of photobiomodulation for cognitive enhancement

The multiple applications of photobiomodulation have been previously discussed. One of the most recently introduced being brain photobiomodulation for neurological regeneration or function modulation.[17] An *in-vivo* study performed on rabbit stroke models, demonstrated the potential neuroprotective and reparative effects of the therapy. The neuroprotective effects of brain photobiomodulation extends to farther than strokes, there has been evidence in many neurological disorders such as Alzheimer's disease, Parkinson's disease, depression, and anxiety.[18] There has been reported increase in cerebral blood flow and NO levels which are deemed to be as a result of Cytochrome-C Oxidase stimulation, the main proposed mechanism of photobiomodulation (as discussed previously).[19] Aside from the neuroprotective effects, there

has been increase interest in the role of photobiomodulation in neuronal function of healthy individuals, not just in damaged and/or injured models. There have been positive cognitive and memory retention effects observed on treatments performed on healthy subjects; the study was a double-blind, placebo study performed on healthy individuals, in which treatment was observed to improve cognition.[20] Brain photobiomodulation is currently in early development and the mechanisms and possible applications are currently being studied.

CHAPTER 2:

Rationale/Overall Hypothesis

The positive effects of photobiomodulation have resulted in consideration of many therapeutics including photostimulation of the brain. Although the brain photobiomodulation is in its early developmental stages, most studies seem to focus on the fluence dependence. As discussed previously, the parameter combinations are too many and can be overwhelming, leading to much uncertainty surrounding the optimal therapy. A further understanding is needed to not only optimize the parameters, but also better elucidate the coupling mechanisms. The focus of this study is to investigate the effects of photon flux on neural stem cell differentiation, and to investigate if the rate at which photons are delivered influences the end result. Previous studies have suggested the positive effects on healthy individuals; however, a clear understanding of the relationship of photon flux must be first understood to establish how photobiomodulation can help cognitive enhancement in healthy individuals. The experimental design of this thesis was developed to investigate the hypothesis that "if the fluence is kept constant, the rate at which the photoenergy is delivered regulates the extent of neural stem cell differentiation."

CHAPTER 3:

Materials and Methods

3.1 Growth and differentiation of neural stem cells

Rat neural stem cell lines (Thermofisher) were used for this study at passage 6. StemPro Accutase cell dissociation reagent (Thermofisher). Culturing media 97% KnockOut DMEM/F12, 2% StemPro Neural Supplement, 2 mM GlutaMAX Supplement, and 20 ng/mL of bFGF and EGF were used in culturing cells. The culturing vessels were coated with CELLstart Substrate, following the protocol given by the supplier. Cells were seeded at 1 x 10^6 cells in a 25 cm^2 flask and allowed to reach confluence. Cells were dissociated and seeded at ~30,000 cells/cm^2 on 35 mm culture dishes and incubated at 37 °C for 2 days. After 2 days, The StemPro NSC complete media was removed, neural and complete media added respectively to each group. Differentiating media, 97% Neurobasal- A Medium (ThermoFisher), 2% B-27 SerumFree Supplement, and 2mM GlutaMAX Supplement. The media was discarded and replenished every 2 days, only half of the differentiating media was removed and replenish to minimize air exposure; complete media was completely discarded and replenished. The cells were differentiated for a total of 15 days. The maintenance and differentiation protocols followed were the ones recommended by the supplier (ThermoFisher).

3.2 Photobiomodulation of stem cells

For the photobiomodulation of rNSCs, a CytonLite laser (Cytonlabs; Austin, TX) was used. The laser's peak wavelength was of 810 nm. The cells were irradiated at 150 mW/cm^2 and 300 mW/cm^2 for 60 seconds and 30 seconds, respectively, for a total of 15 days. As described previously, the upper range of fluence, before any harmful effects, is 10 J/cm^2. To exploit the

upper range, a fluence of 9 J/cm^2 was used. The laser beam was made to fit the entire petri dish. The irradiation set-up can be seen in Figure 3-1. The cells were covered in parafilm to maintain sterility.

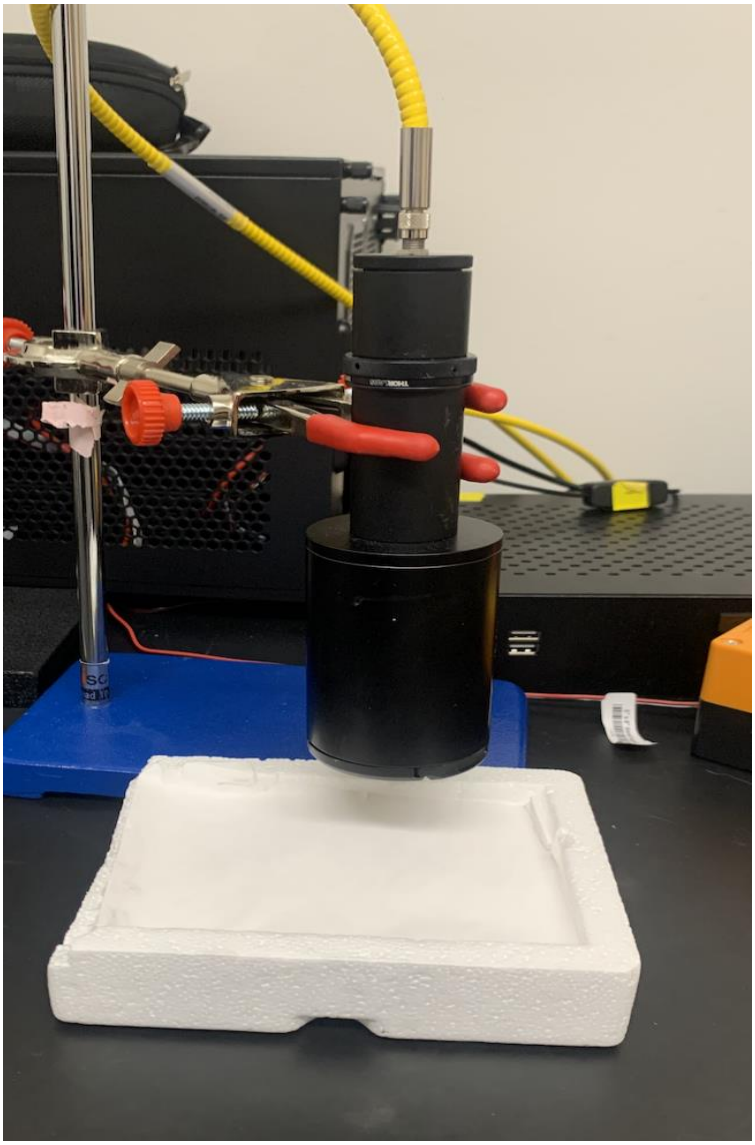

Figure 3-1: Laser irradiation set-up. The laser is seen at the top, being held by a clamp at an optimal distance for beam collimation. Petri dish is typically placed on the top of the white color surface.

3.3 Immunocytochemistry

The cells were stained for SOX2 Monoclonal Antibody (Invitrogen) and NeuN polyclonal antibody (Invitrogen) at 1:1000 dilution. The cells were fixed with 4% Paraformaldehyde and permealized with a 0.25% Triton-X solution for 3 minutes. A 5% BSA solution was used to block cells for an hour at 25 °C. The cells were stained with primary antibodies SOX2 and/or NeuN overnight at 4 °C. The following day, a 5% BSA solution was used to conjugate secondary antibodies as well as stain for NucBlue Live ReadyProbe, Alexa Fluor 488 and Alexa Fluor 555 were conjugated for 1 hour at room temperature. Fluorescence microscopy of cells was performed using Nikon: Ti microscope and NIS Element software.

3.4 Image Analysis

To interpret experiment results, an image processing software was used (ImageJ). A total of 6 images were taken from each sample, for a total of 18 daily images per group. To quantify protein expression, a total of 10 cells were considered from each image, with the background removed. The cumulative pixel intensity was found, and to determine the intensity per cell, the value was divided by 10 (number of cells being analyzed); the value was presented as the mean intensity per cell of each respective day. Each group was analyzed at day 5, 10, and 15. To determine the baseline expression, a control group was analyzed at day 0 of differentiation.

3.5 Temperature rise

Temperature rise is a potential artifact that may obscure the PBM effects. Extensive experiments have been performed in our laboratory to measure PBM-mediate temperature rise. As seen in Figure 3-2, using the maximum laser output, no significant temperature rise was observed at the exposure time of less than 1 minute. Previous *in-vitro* studies have determined

heat stress to occur at around 39-42 °C in cells; A study performed on dental follicle stem cells showed the optimal culturing range to be 39-40 °C.[21,22] No noticeable cell death or adverse effects have been shown in response to a temperate rise of < 1 °C.

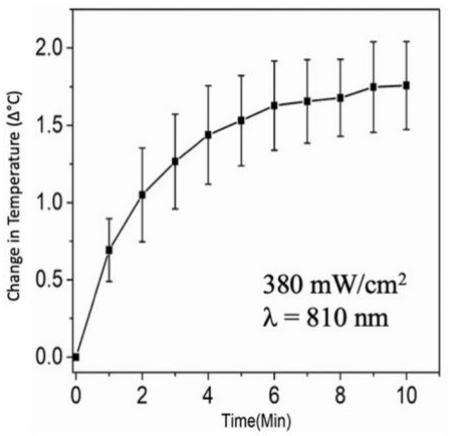

Figure 3-2: Temperature rise continuous monitored over 10 minutes at 810 nm and laser intensity of 380 mW/cm2. [Adopted from the results of Dr. Caleb Liebman].

3.6 Statistical analysis

Unpair t-test was used assuming normal distribution of data. Alternatively, a non-parametric test could be used instead (e.g., Mann-Whitney test)[23], which compares medians of samples instead of means. However, it may lead to differences between groups that are not as recognized.

Analyzed groups had a sample size of n=3 of >180 total cells per group. The standard error of mean (SEM) was calculated for each group, at each day. The values are shown as mean values. Unpaired t-test was used to compare the difference between the groups and a p-value of < 0.05 was interpreted as statistically significant.

CHAPTER 4:

Results

4.1 Neural Differentiation

The experimental design was implemented to test and validate/refute the following hypotheses. First, we hypothesized that photostimulation alone without biologics (e.g., growth factors) is capable of inducing neural stem cell differentiation toward the neuronal lineage. Second, while keeping the total amount of photoenergy constant at 9 J/cm^2, the rates at which the photoenergy is delivered modulate neurodifferentiation. Third, simultaneous stimulation using the combination of growth factors and photostimulation synergistically facilitates the intended neurodifferentiation. These hypotheses will be validated or refuted using two specific markers, including NeuN and SOX2. NeuN has been established as a neuronal marker and SOX2 is thought to be a marker for undifferentiated stem cells which both have been demonstrate by multiple laboratories.[24,25,26,27,28,29]

While neural stem cell differentiation using a cocktail of growth factors has been shown in the past, our laboratory decided to apply the same cocktail (see Methods section) and reproduce the capability to induce neurodifferentiation, Rat NSCs were differentiated for 15 days following standard differentiation procedures using the cocktail growth factors and other molecules. Immunofluorescence was applied to monitor and quantify the NeuN and SOX2 expression. Composite images were created for Days 0 (control), 5, 10, and 15 of neurodifferentiation (Figure 4-1). The diminished number of cells shown in these images are consistent with the notion of stem cells undergoing an initial proliferation, followed by differentiation.

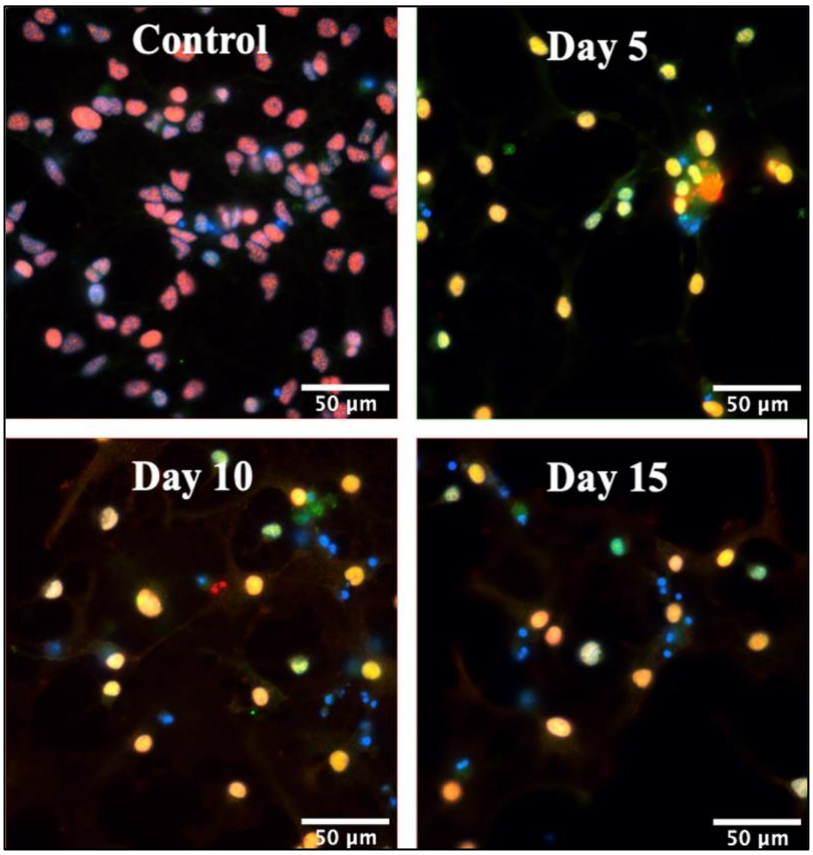

Figure 4-1: Representative images for neural differentiation using the neurogenic biologics over 15 days. A composite image of control cells shown in the top-left panel. Red stained for Sox2, green stained for NeuN, and blue stained nuclei. Purple color would denote co-localization of Sox2, whereas cyan color would indicate co-localization of NeuN.

NeuN immunofluorescence intensity was measured and normalized by the cell number. Quantitative analysis demonstrated significantly elevated protein expressions when compared to control; the highest level was found at Day 5, followed by a decrease at Day 10, with a plateau at Day 15 (Figure 4-2). The NeuN expression of differentiation group among all days was statistically significant. There was no significance difference in the control groups at the same time points.

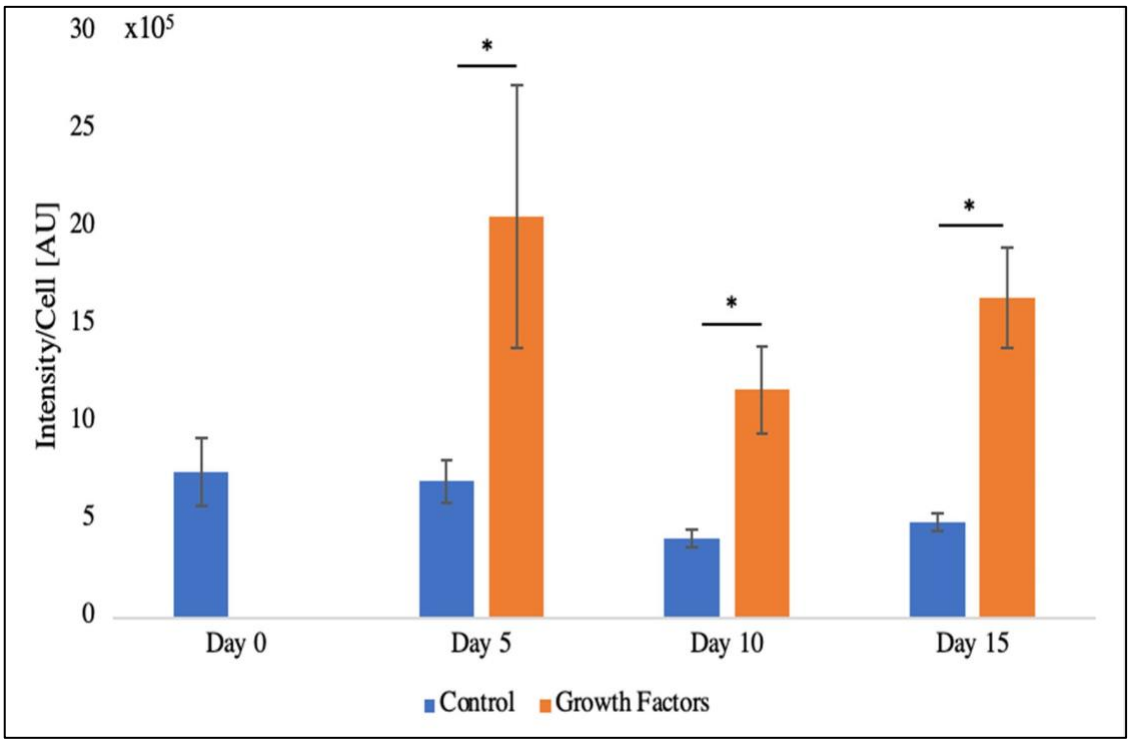

Figure 4-2: Image analysis and quantification of NeuN protein. Total NeuN immunofluorescent intensity was normalized by the number of cells. Data presented as mean ± SEM of n=3. * indicates $p < 0.05$.

While it was anticipated the SOX2 expression should diminish at later stages of differentiation, the SOX2 immunofluorescence quantification demonstrated the protein expression throughout the 15 days of monitoring remained essentially unchanged (Figure 4-3). At a higher level of the expression observed at Day 10, it was not statistically significant in comparison to the control. Therefore, the SOX2 expression was deemed an insufficient marker to determine the extent of undifferentiated neural stem cells.

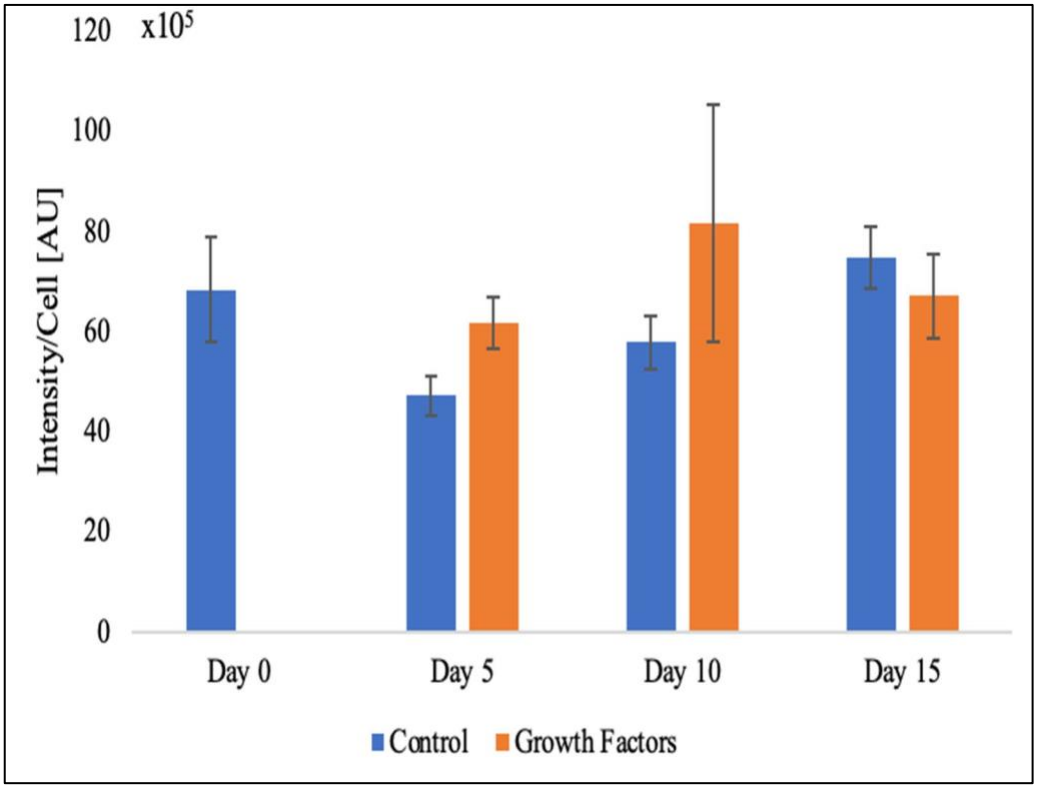

Figure 4-3: Image analysis and quantification of SOX2 protein. Total SOX2 immunofluorescent intensity was normalized by the number of cells. Data presented as mean ± SEM of n=3. No statistical significance was observed.

4.2 Neural Differentiation by Photobiomodulation alone

To determine the differentiation capabilities of PBM alone, we used two sets of the PBM parameters that are designated as PBM 1 (150 mW/cm^2 for 60 sec) or PBM 2 (300 mW/cm^2 for 30 sec). While these two PBM exposure conditions maintained the same fluence (9 J/cm^2), it allowed us to probe whether (1) PBM alone can initiate and induce neurodifferentiation and (2) if the rates at which photoenergy is delivered are involved in the PBM-induced neural differentiation. As shown in Figure 4-4a, composite images of the NeuN expression and nuclei are shown at various days of differentiation using PBM 1 alone. Several interesting observations

are noted here. First, the control cells at Day 0 expressed virtually no NeuN proteins. In contrast, by Day 15, the cells visibly upregulated the NeuN expression using PBM 1. The cell density appears to decrease, which is consistent with the notion of diminished proliferation and increased differentiation.

Similar experiments were performed using PBM 2 (i.e., 300 mW/cm^2 for a shorter exposure). Composite images for using PBM 2 are shown in Figure 4-4b. At Day 5, the NeuN expression appears more pronounced and, interestingly, few morphological features that are unique to neurons were noticeable, including axon-like extensions as well a formation of cell network. At Day 10 and 15, these features were not as visible, and the cell density also appeared to increase. These results are somewhat contradictory in comparison to using PBM 1, raising an intriguing suggestion that the PBM effects are induced and dependent on the parameters.

Quantitative image analyses of the NeuN expressions are shown in Figure 4-5. The use of a higher light intensity (300 mW/cm^2) for a shorter exposure time (30 sec) significantly produced more NeuN proteins at the early stage of neurodifferentiation (Day 5). Sustained increases in the NeuN expression is better achieved using a lower intensity for a longer exposure time (e.g., PBM 1). However, continuous daily treatment of cells with the higher laser intensity suppressed this neuronal marker. An implication may be that an intense light exposure is beneficial but up to a limited time. These results raise some interesting but perhaps contentious debates that, for translational clinical treatment, optimization of the PBM exposure parameters may be one of the important criteria.

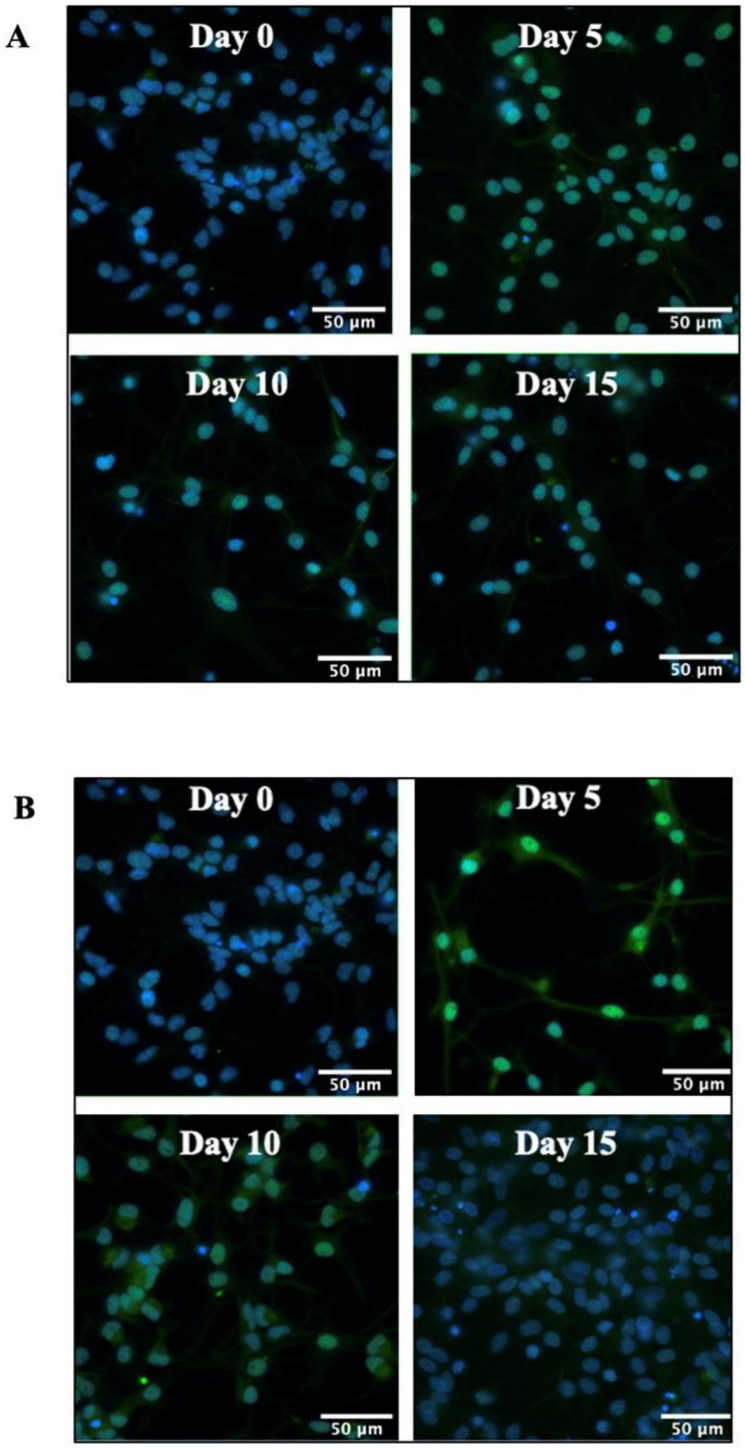

Figure 4-4: Representative images of NeuN expression and distribution over 15 days using either PBM 1 (A) or PBM 2 (B). NeuN is in green and the nuclei are in blue.

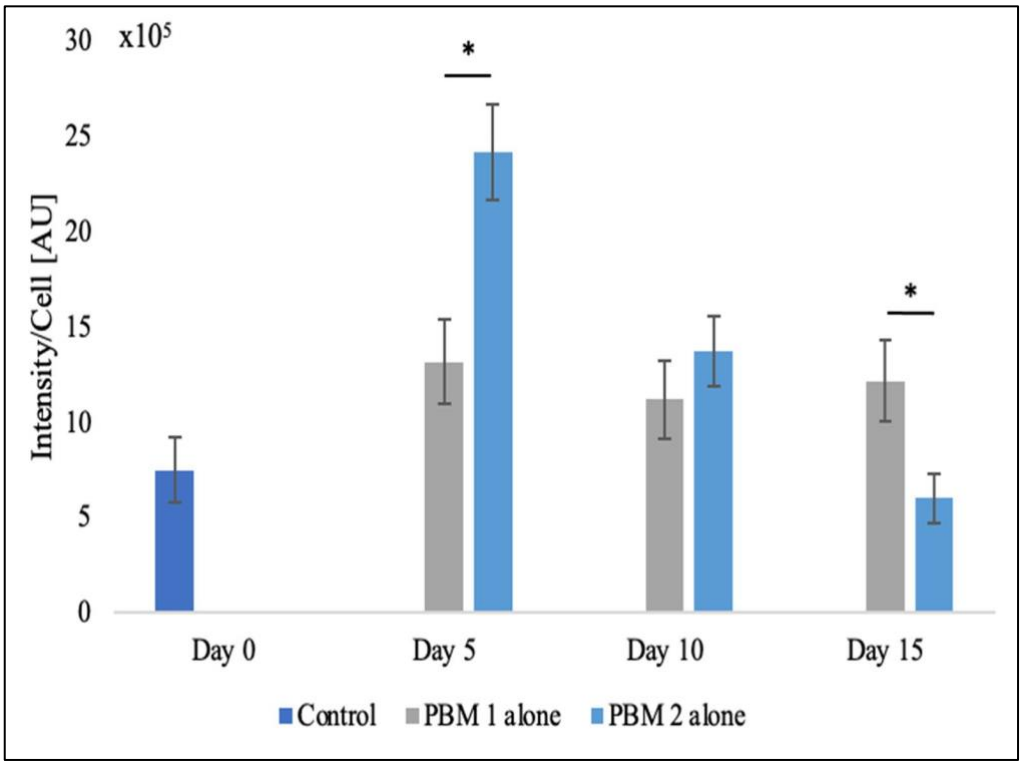

Figure 4-5: Image analysis and quantification of NeuN protein. Two set of experiments using either PBM 1 or PBM 2 are compared and contrasted. Data presented as mean ± SEM of n=3. * indicates $p < 0.05$.

4.3 Potential Synergistic Neurodifferentiation

We next turned to test the hypothesis that a combined use of the growth factors and PBM facilitates neurodifferentiation. As illustrated in Figure 4-5, both PBM 1 and PBM 2 were shown to induce additional NeuN expression. Composite images were again constructed to demonstrate the effects of combined treatment of PBM + growth factors (Figure 4-6). When combined with growth factors, PBM 1 appears to induce a higher level of NeuN expression. In addition, by Day 5, the neuronal morphology was evident (middle column of Figure 4-6), which was not observed when PBM 1 alone was applied. There may be synergistic effects that are induced by the combination stimulations. Similar neuronal morphology is also noticeable when the growth factors are combined with PBM 2 (right column of Figure 4-6).

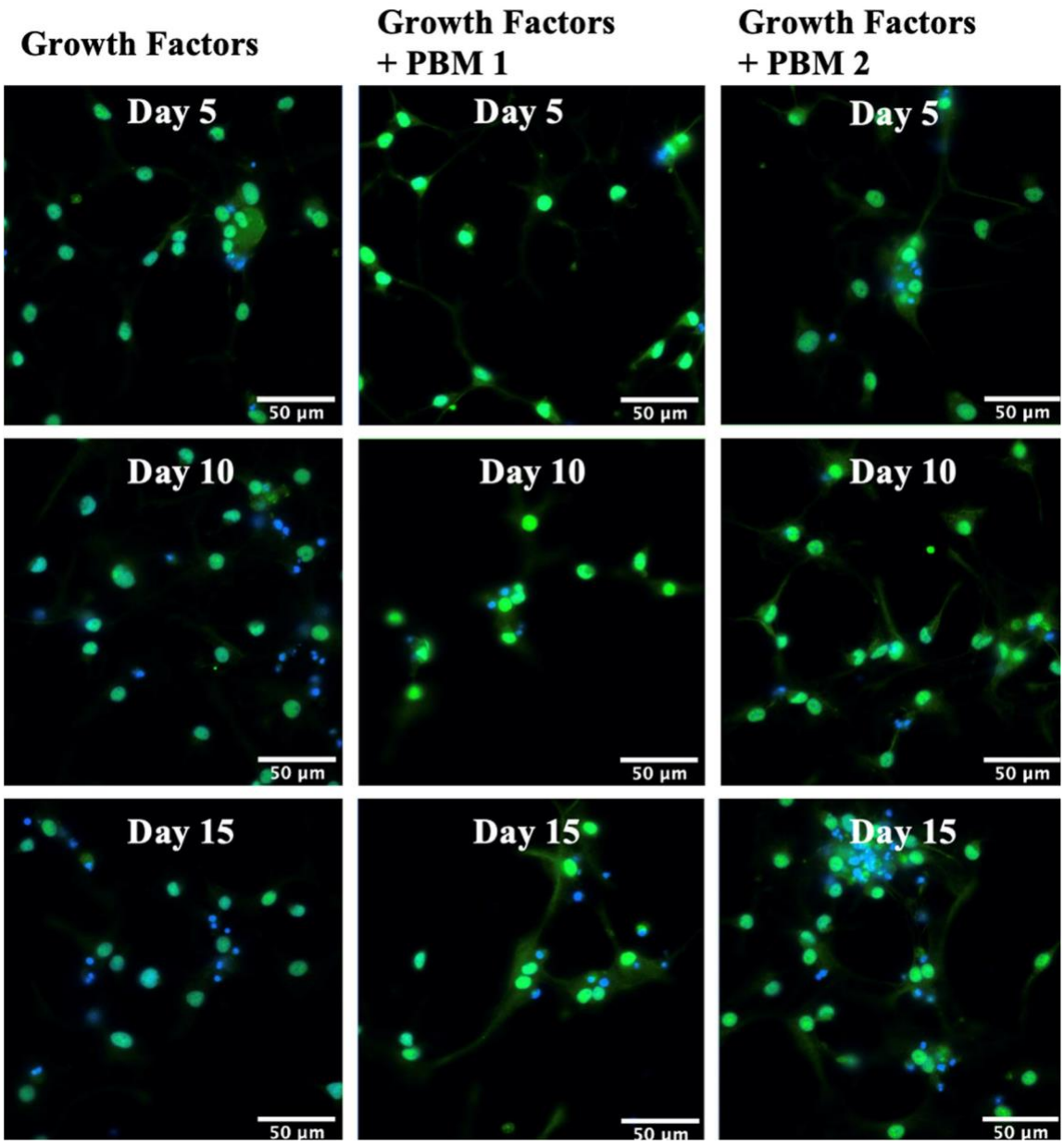

Figure 4-6: Representative images of NeuN expression using the growth factors alone (left column), growth factors + PBM 1 middle column), and growth factors + PBM 2 (right column) over 15 days of neurodifferentiation. NeuN protein is visualized green, and the nuclei are labeled blue.

The NeuN expression was processed and analyzed for the two combinatory sets of experiments. As described in the Methods section, total NeuN fluorescence intensity was normalized by the number of cells. Results are shown in Figure 4-7 (growth factors + PBM 1)

and in Figure 4-8 (growth factors + PBM 2). In the first case, the growth factors + PBM 1 generated more than a 2-fold increase in the NeuN expression by Day 5. Additional PBM 1 treatment for the next following 10 days did not further increase but sustained this marker's level. In the second case of growth factors + PBM 2, the combination was not effective in that the NeuN level induced by PBM 2 alone was comparable at Day 5 (Figure 4-8). Additional PBM 2 treatment alone for the next following 10 days decreased the NeuN expression when PBM 2 alone was applied. However, the decrease in the NeuN expression was reversed and restored when the growth factors were added. These results are consistent that, while neurodifferentiation can enhanced by the combination of growth factors and PBM, the choice of PBM exposure parameters is shown important, and that PBM exposure in the first 5 days appears to promote neurodifferentiation.

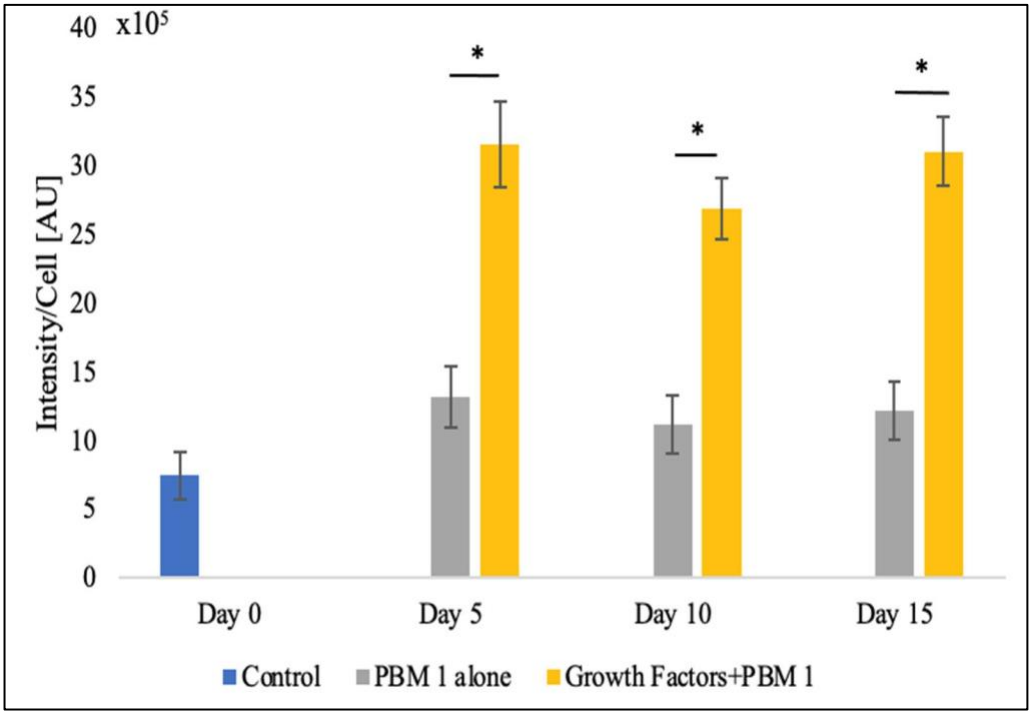

Figure 4-7: Image analysis and quantification of NeuN protein using the combination of growth factors + PBM 1. Total NeuN immunofluorescence intensity was normalized by the number of cells. Data from PBM 1 alone were preproduced from Figure 4-5 for comparison purpose. Data

presented as mean ± SEM of n=3. * indicates $p < 0.05$.

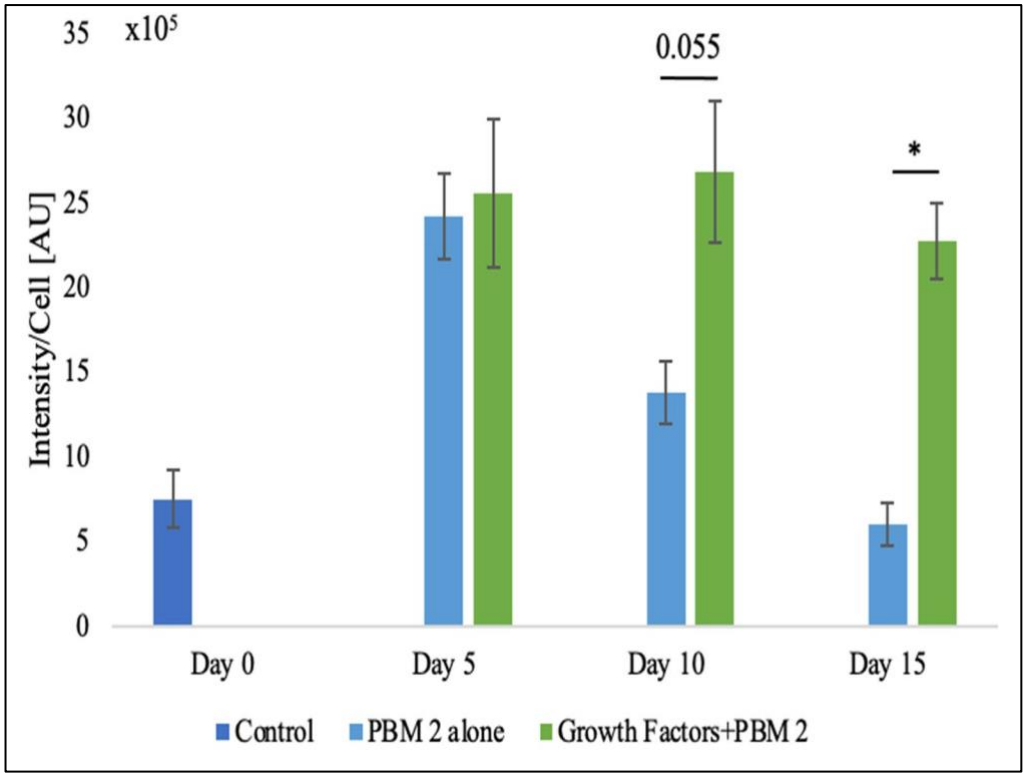

Figure 4-8: Image analysis and quantification of NeuN protein using the combination of growth factors + PBM 2. Total NeuN immunofluorescent intensity was normalized by the number of cells. Data for PBM 2 alone were reproduced from Figure 4-5 for comparison purpose. Data presented as mean ± SEM of n=3. * indicates $p < 0.05$.

CHAPTER 5:

Conclusion

5.1 Aims of Thesis

The aim of the study was to establish whether PBM can induce neurodifferentiation and determine the differences, if at all, in the use of different irradiance parameters [150 mW/cm^2 and 300 mW/cm^2] in the differentiation process. These results may validate/refute if the rate at which photons are delivered will be a determining factor for the intended neurodifferentiation. As discussed previously, the efficacy of photobiomodulation across all application is highly dependent on many variables (i.e., wavelength, fluence, irradiance, etc.).[30] As observed in Figure 4-2, the baseline experiment of differentiation, the significant increase in NeuN of at least 290% in the growth factors group, indicated an ongoing differentiation process. Interestingly, there was an observed expression in NeuN in the undifferentiated control group. As shown by an *in-vitro* study performed on multiple mesenchymal stem cells (MSCs) supporting the expression of the neuronal marker in undifferentiated cells, from which the hypothesis was that due to their predisposition of neuronal differentiation, the cells were NeuN positive.[31,32] Another study performed on rMSCs for 14 days of culture, showed NeuN positive cells which retained their stem cell morphology and no neuronal morphology.[32] It was difficult to find any previously published reports that monitored the NeuN expression in undifferentiated rat NSCs, suggesting our findings may have determined, for the first time, the time-dependent expression of the neuronal marker in rat NSCs. It appears to indicate the predisposition of this type of stem cells to readily undergo differentiation to the neuronal lineage. Although more studies are needed to further characterize the dynamics of NeuN expression in NSCs to accurately represent neurodifferentiation, the NeuN protein along other neuron-specific markers (e.g., β- III

tubulin) can still be utilized to determine the extent of such differentiation. Based on the assessment of NeuN expression to quantify neurodifferentiation, anti-NeuN antibody was used throughout the remaining of the experiment as a suitable early neuronal marker. As presented in Figure 4-3, the expression of SOX2 in both the control and growth factors group was not significant. Although SOX2 expression has been used to signify stem cell characteristic retention, it has been reported in differentiated neurons and glia cells. In thalamic, clock, and hypothalamic neurons, the expression of SOX2 in already differentiated cells did not appear to interfere with the function of neurons.[33,34] Although the presence of the antibody at comparable levels in both the control and differentiation group diminishes the usefulness of SOX2 as a stem cell marker, the presence in differentiating neurons has not been reported to inhibit functionality of developing neurons.

Through the enhancement of differentiation with photobiomodulation, the maximum expression of NeuN in the growth factors + PBM groups appeared to be at Day 5, with PBM 2 parameters showing some competitiveness among growth factors + PBM 2 and PBM 2 alone. The differentiation of both PBM parameters suggest the effectiveness of PBM does not extend past Day 5, after which the growth factors are needed to continue with the intended differentiation process. It appears as though the rate of mitochondrial respiration is influenced by the rate at which the photons are delivered, influencing the results. Further studies are needed to characterize the possible pathways through which photon flux plays a role in differentiation with/without the growth factors.

5.2 Laser Attenuation

For potential clinical translation, the applied laser intensity is likely attenuated by the

skull. As demonstrated by *in-vivo* studies, approximately between 1% to 3.7% of light passes through the skull.[35,36] Although in this study the highest irradiance used was of 300 mW/cm^2, the expected attenuation should deliver ~ 3 mW/cm^2, approximately 1% of the applied irradiation, to the brain. The attenuation of at least 2 orders of magnitude raises concerns that a weak laser intensity may not be able to induce neural stem cell differentiation, although *in-vitro* studies demonstrated efficacy of the treatment. To consider PBM effects *in-vivo*, the studies could be repeated at 1% irradiance which, in turn, would increase the exposure time to close to 2 hours. Alternatively, a more powerful laser could be used for a shorter exposure time. However, we were limited by the maximum output of 810 nm at 380 mW/cm^2. In addition, temperature rise could then become another limiting factor. Nonetheless, *in-vitro* results do suggest that optimized delivery of photoenergy offers an efficacious technique by which neural stem cells can be induced to undergo neurodifferentiation even without the differentiation growth factors and other biologics.

5.3 Postulate

Based on our findings, we formulated several postulates that provide a working model but warrant further validation. A combination of growth factors + PBM could result in a synergistic production of ROS. Because ROS has been associated with intracellular activities, specifically in normal metabolic reactions, survival, and plasticity in neurons, the role of ROS has been elucidated during embryonic development and in adult neurogenic regions.[37] One of the proposed mechanisms of how ROS regulates neuronal development is through calcium channels, which support neurite outgrowth and axon specification.[38] The release of ROS and ATP through mitochondrial respiration (e.g., CCO stimulation by PBM) could enhance neuronal development

by satisfying the high energy demand of developing neurons; the brain consumes ten times more oxygen and glucose than any other tissue.[38,39] The increase in ROS levels as a result of PBM can enhance the differentiation rate and provide neurons with the necessary supplements needed for development at lower irradiance, as observed using 150 mW/cm^2 in our study. However, at a higher irradiance (300 mW/cm^2), the growth factors do not seem to enhance the differentiation process at the early stage. The rate of mitochondrial stimulation between the different PBM parameters yields conflicting results; however, such stimulation appears to depend on the irradiance. The competitiveness in neuronal differentiation by the higher irradiance rate observed at Day 5 may be attributed to the combined ROS production by both PBM and differentiation factors. Through the use of a higher irradiance, mitochondrial ROS production is expected to be higher, as is the expression of NeuN in PBM alone at Day 5. However, after Day 5, the differentiation process is likely dominated by the growth factors, as there is no further NeuN expression increase observed after Day 5 even when the growth factors + PBM combination was applied. These observations seem to suggest the rate at which the photons are delivered to be important and modulate the cellular responses. Through the modulation of ROS levels, the function of neural differentiation can be altered and tuned to yield desired results of potential therapy.

5.4 Future work

The results discussed previously appear to suggest a higher irradiance could overstimulate the mitochondria. Therefore, it does raise the question of how irradiance affects the intended neurodifferentiation. Additional studies are needed to reach more definitive conclusion, as a higher irradiance is expected to produce more ROS, especially during the first 5

days of neurodifferentiation. A possible comparison to clarify the role of ROS in neuronal differentiation is through the use of a 1064 nm laser instead of 810 nm. The use of longer wavelengths in PBM has an increase in the penetration depth; however, studies have demonstrated a decrease absorption of CCO at 1064 nm.[18,40] By the reduced absorption of CCO, a decrease in ROS production is expected. Alternatively, PBM likely affects ATP production, which could be quantified. While the current study focused on one neuronal marker, the intermediate signaling pathways that lead to upregulation of this marker are complex. Likely signaling molecules includes ROS, ATP, calcium dynamics and eventually to the nuclear signaling mechanisms to modulate gene expression.

Through the completion of more functional testing at the subcellular and gene levels, a better understanding is warranted, including additional studies of the rates at which photoenergy is delivered not to overstimulate the intracellular machinery. By acquiring more knowledge into multiple PBM parameters, the efficacious application of light-based therapeutic potential may be standardized. The results from this thesis are expected to contribute toward establishing a framework for PBM to repair traumatic brain injuries and provide a non-invasive and inexpensive technology to enhance the brain function.

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