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Modulation of hippocampal neurogenesis by Nano-Pulsed Laser Therapy

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"Nulla die sine linea"

Naturalis Historia, Pliny the Elder

ABSTRACT

Neurogenesis is a physiological process through which new neurons are generated and it occurs throughout life, subverting the old dogma stating to the inability of the adult brain to replace neurons. Specifically, neurogenesis take place thanks to the presence of neuronal stem cells (NSCs) located in the subventricular zone of the lateral ventricle and in the subgranular zone of the dentate gyrus of the hippocampus.

Data present in the literature demonstrate that neurogenesis in the hippocampus decreases during aging and it is impaired in neurodegenerative diseases, such as Alzheimer's disease, and after traumatic brain injury (TBI).

Traumatic Brain Injury (TBI) is a chronic disease that occurs after a head trauma and the results of which are permanent. One of the main area affected by TBI is the hippocampus a brain region that plays a pivotal role in learning and memory. For this reason there is an increased interest in TBI research, particularly as it relates to finding a possible cure, which currently does not exist.

The goal of the research I conducted was to stimulate NSCs to proliferate and differentiate so as to restore the neurogenesis process in the brain.

Specifically, I tested the effect of a highly innovative non-invasive device that combines the benefits of both near infrared laser light (808nm) and ultrasound waves, optoacoustically generated with each short (10ns) high-energy (15mJ) laser pulse within the tissue. Dr. Micci's laboratory demonstrated the beneficial effect of NPLT in a rat model of brain trauma. However, the mechanism by which NPLT stimulates NSCs is not well understood.

At the completion of this project I will have gained valuable knowledge of the effects of NPLT on neurogenesis and provided a unique therapeutic strategy based on a self-repair via stimulation of NSCs that are already present in the brain.

RELEVANCE

More than 10 million traumatic brain injury (TBI) cases occur every year worldwide with a significant impact in health care cost. TBI is a chronic disease that occurs after a head trauma with permanent structural and functional damages. So far, no effective therapy for this brain illness is available.

One of the most affected areas in the brain is the hippocampus that is essential for learning and memory processes. Moreover, the hippocampus is one of the two areas in the brain in which neurogenesis occurs. Neurogenesis is the process by which new neurons are generated and occurs throughout life thanks to the presence of neuronal stem cells located in the subventricular zone of the dentate gyrus of the hippocampus. Evidence in literature shows the positive correlation between neurogenesis and improved cognitive function.

My project is highly significant because it has the potential to lead to a novel therapeutic strategy for the treatment of brain trauma and other neurological diseases centered on increasing endogenous neurogenesis.

INTRODUCTION

Neurogenesis

- **History**

Neurogenesis is a physiological process by which new neurons are generated and fully integrated in pre-existing neuronal network in the brain[1]. This complex and tightly regulated event occurs throughout life[2]. The discovery of neurogenesis has put an end to the central dogma that, until 1990, had dominated the field of neurobiology[3]. Particularly, it was believed that, after development, the brain was unable to generate new neurons. This old theory was based on the observation made between the second half of the 19th century and the beginning of the 20th century by the histologist Ramon y Cajal, who stated that "Once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably" and Giulio Bizzozero, mentor of Camillo Golgi, that defined the adult body "labile, stable and perennial" and specifically the central nervous system "composed of life-long lasting cells lacking replicative potentiality"[4].

Some of the observations that supported the old dogma were: the incapacity of patients to recovery after neurological dysfunctions, due to onset of neurodegenerative disorders and lesions, and the ability of the brain to create new stable circuits that can be recalled as memories, in contrast with the thought of the replicative inability of neurons. Now we know that this process is one of the most important examples of neuronal plasticity[5,6].

The proliferation in the adult rat brain was reported for the first time in the '60 by Joseph Altman thanks to the use of the radioactive thymidyne (H^3) (Figure 1)[7].

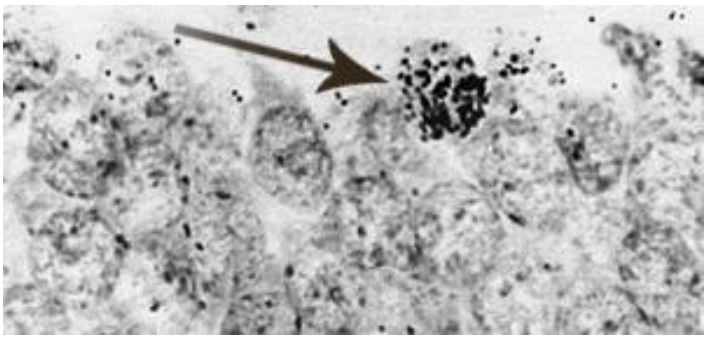

Figure 1. **Autoradiography of adult rat brain sections injected with H^3 made by Joseph Altman in 1965.**

This technique allows to mark only proliferating cells since the radioactive thymidine is incorporated by cells in the S phase of the cell cycle. The newly synthesized DNA is marked and detectable by autoradiography[8]. However this demonstration was considered "heretical" at that time and ostracized particularly for the absence of a specific demonstration that those proliferating cells were neuronal stem cells with the ability to differentiate into mature neurons.

Michael Kaplan in the '80 gave a more definite demonstration of the neurogenesis process with the evidence of neuronal formation, particularly in the olfactory bulb, by using neural markers, however even his theory was rejected[9].

Only in the '90 the theory of neurogenesis was finally accepted thanks to the work carried out by various research groups in Europe and in the United States[10,11]. Specifically, they unequivocally demonstrated that it was possible to culture neural stem cells (NSC) isolated from the adult brain and, most importantly, that NSC could generate functional neurons[12]. Moreover, in the same years, it was demonstrated that neurogenesis occurs *in vivo* in two areas of the adult brain: the subventricular zone (SVZ), a thin layer of the lateral ventricle, and in the subgranular zone (SGZ) of dentate gyrus (DG) of the hippocampus (Figure 2)[13].

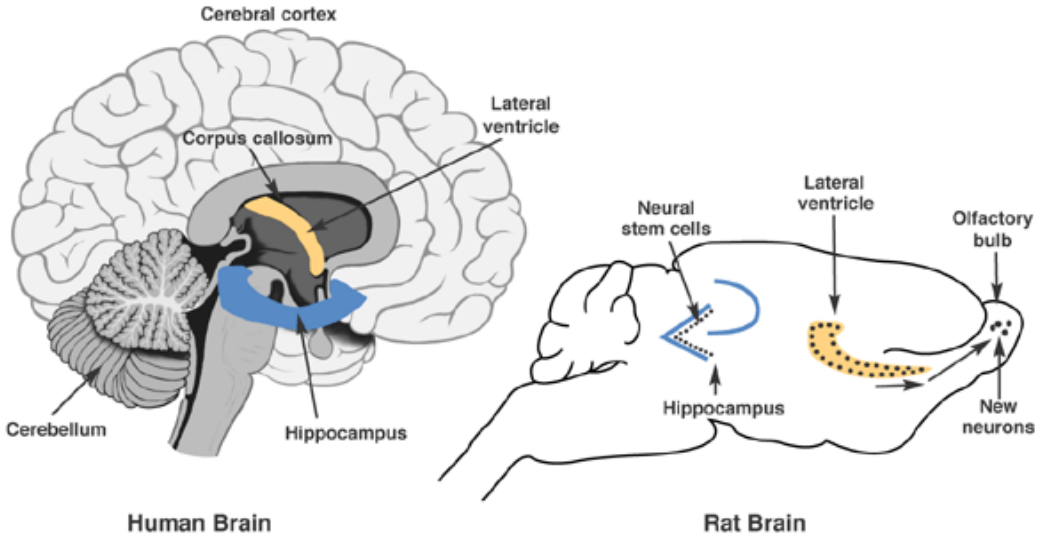

Figure 2. **Neurogenesis process occurs in two areas in the brain: the subventricular zone (SVZ), a thin layer of the lateral ventricle, and in the subgranular zone (SGZ) of dentate gyrus (DG) of the hippocampus.**

The two areas give rise to different specialized neurons: the NSC of the SVZ generate neurons that migrate in the olfactory bulb and the striatum, while NSC of the SGZ integrates in the hippocampal network and thus play a key role in memory, cognitive and emotional processes[14,15].

In 1998 Fred Gage's laboratory has finally demonstrated that neurogenesis occurs in the human hippocampus thanks to the development of the bromodeoxyuridine (BrdU) assay[16]. This technique is still currently used to analyze proliferating cells because BrdU, a thymidine analogue in structure, can be incorporated with the same probability of the thymidine in the newly synthesized DNA since the cell is not able to discriminate those. The advantage of this technique is that BrdU can be detected by using specific antibodies conjugated with enzymes or fluorescent dyes that help the detection of the proliferating cells (Figure 3)[17].

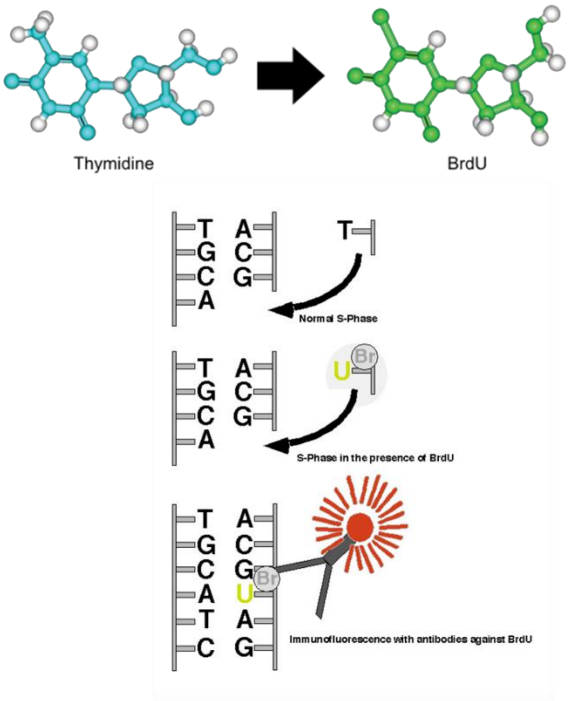

Figure 3. **BrdU is a thymidine analogue that can be incorporated in the newly synthesized DNA of proliferating cells and that can be detected by using specific antibodies conjugated with enzymes or fluorescent dyes for the detection.**

NSC are unique cells with two main characteristics: the ability to self-renew and the ability to differentiate[18]. Self-renewing is the ability to be induced to proliferate and increase the pool of NSC with same properties. Differentiation is the most important property from the functional point of view. Indeed, NSC can be stimulated to differentiate in neuronal and glial progenitor in order to originate the population of neurons, astrocytes and oligodendrocytes present in the adult brain[19].

These properties have been demonstrated by *in vitro* culturing of NSC. Cultured NSC can be induced to proliferate or differentiate by using specific factors that can induce both processes[20]. Some of these processes are well established, however further studies are necessary in order to characterized the regulatory mechanisms of neurogenesis *in vivo*[21].

Understanding the molecular mechanisms by which neurogenesis can be stimulated is important because of its incredible therapeutic potential, particularly for the recovery of brain functions lost during the progress of neurodegenerative disorders or after brain trauma[22].

- **Neurogenesis in the hippocampus**

The hippocampus is a key structure of the brain that plays a pivotal role in memory and cognition[23]. Moreover, is one of the two areas in the brain in which neurogenesis occurs. The hippocampus take the name from its shape of a seahorse (form the Greek word *hippos* (horse) *kampos* (monster)). This structure belong to the limbic system and is divided in: (**C**ornu **A**mmonis, CA) CA1, CA2, CA3, CA4 and the dentate gyrus (DG) (Figure 4)[24].

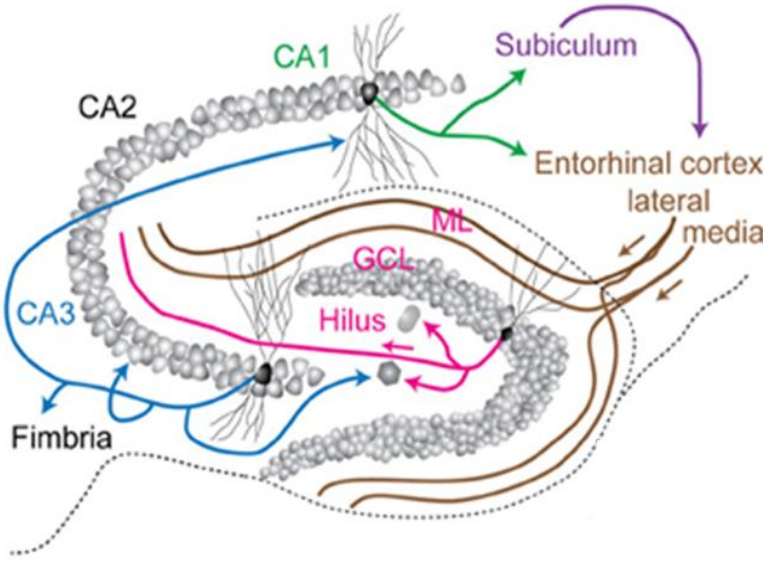

Figure 4. **Structure and network of the hippocampus.**

CA1 is the area of the hippocampus responsible for output signals to the layer V of the entorhinal cortex and the subiculum. CA2 is a very small output area between CA1 and CA3 that often is not included in the hippocampal structures. CA3 is an input structure of the hippocampus that collect the signals from the granule layer of the DG through the mossy fibers. CA3 is the most important relay station of the hippocampus that project some axons back to the DG and mostly to CA1 with Shaffer collateral. CA3 is a very complex structure divided, from the closer to CA1 and the farthest to DG, in CA3a, CA3b and CA3c. CA4 is commonly called hilus and is connected with the granule cells of the DG[25].

Finally, the DG is the most important input structure of the hippocampus that receive information from layer II of the entorhinal cortex and where episodic memories are formed, thanks to the presence of the granule cells. The DG is formed by three layers: the molecular, the granular and the polymorphic layer. The granule cells are present in the granular layer and forms the excitatory signals, transported by interneurons and pyramidal cells, to CA3[26].

The DG is also a fundamental structure of the hippocampus because it is the place in which neurogenesis occurs, in particular the subgranular zone (SGZ)[27].

Neurogenesis is a multistep process characterized by the expression of specific markers. In detail, neurons type I cells, that rarely proliferate and that forms the pool of radial glial like precursor cells, generate type IIa (glial precursor cells) and type IIb (neuronal precursor cells). After the commitments, type III cells (neuroblast) undergoes to the maturation stage in which the newborn neurons become fully functional and make connections with CA3 region (Figure 5)[28].

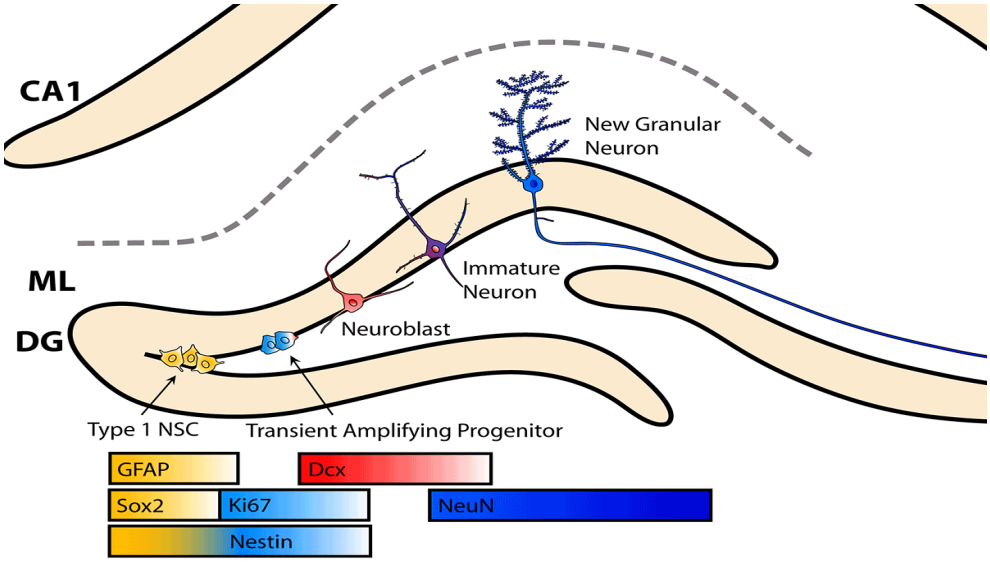

Figure 5. **Neurogenesis in in the SGZ of the dentate gyrus (DG) and the transient expression of specific markers during the differentiation.**

An important recent discovery has highlighted the different roles played by the dorsal hippocampus and the ventral hippocampus. Specifically, there are evidences that the dorsal hippocampus plays an important role in memory formation and retrieval, while the ventral part is critical for emotions[29].

Recently, has been reported a significant role in the control of neurogenesis played by specific ion channels known as mechanoreceptors[30]. Mechanoreceptors are mechanosensitive ion channels that are activated by mechanical changes in the environment causing an increase of intracellular Ca^{2+} affecting the expression of genes involved both in proliferation and differentiation[31,32].

- **Epigenetic regulation of neurogenesis in the hippocampus**

In the last 20 years there has been an increased interest on the epigenetic regulation of neurogenesis mediated by microRNAs (miRNAs)[33]. miRNAs are small 20-22 nucleotides non-coding RNA that have been shown to regulate the expression of more than 60% of genes. Pri-miRNA is synthesized by RNA polymerase II and then processed in pre-miRNA by Pasha/Drosha complex. The pre-miRNA is exported in the cytoplasm in order to be further cleaved by DICER. The mature miRNA binds to the target mRNA thanks to RNA-induced silencing complex (RISC) and the duplex miRNA/mRNA is immediately degraded silencing gene expression (Figure 6)[34].

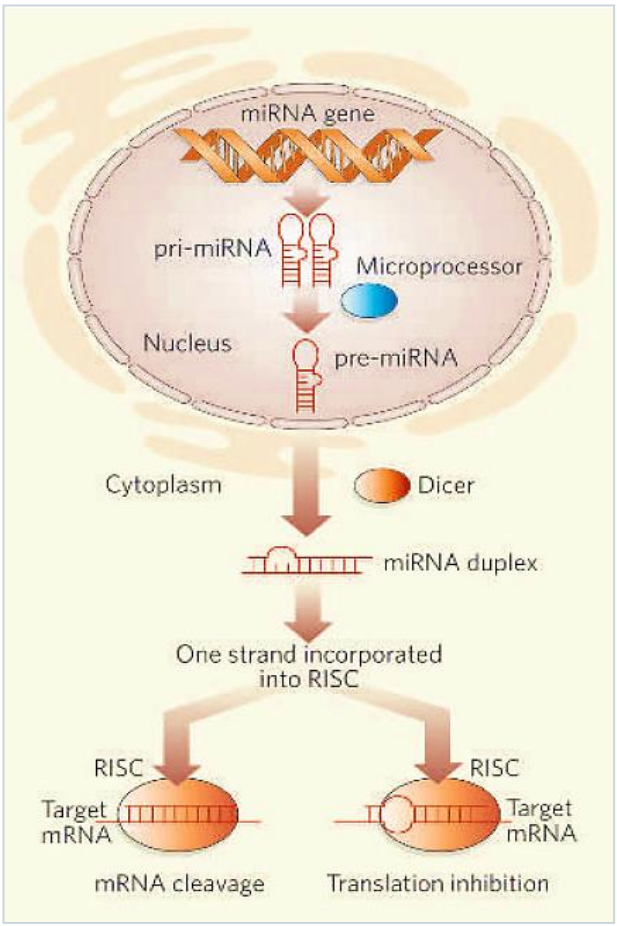

***Figure 6*. miRNA biogenesis/processing and mRNA regulation.**

Recently the role of miRNAs in regulating NSC differentiation has been studies. miR9, miR25 and miR29 have been shown to be directly involved in NSC fate specification[35,36].

miR9, is an evolutionary conserved miRNA that has several targets such as the nuclear orphan receptor TLX also known as NR2E1[37]. miR9 is overexpressed during the late phase of neuronal differentiation suppressing TLX, important for cell proliferation, and promoting the terminal differentiation and the migration of new born neurons in the pre-existing circuits of the hippocampus[38].

miR25, is important for maintaining the self-renewing property and is involved in insulin/insulin-like growth factor-1 (IGF) signaling. Interestingly, the regulatory region of miR25 is bound by FoxO3 a member of the FoxO family important for maintaining neural stem cells[39].

miR29, is upregulated during the differentiation and negatively regulates the proliferation and the self-renewal. In particular miR29 is involved in Wnt/βCatenin signaling by regulating ICAT (inhibitor of β-catenin and TCF-4)[40].

- **Neurogenesis and cognition**

In the last 30 years neuroscientists have been studied neurogenesis and have been demonstrated that this important process is highly regulated and can be stimulated or inhibited by different factors. The first evidence of regulated process was made by Nottebohm in song birds[41]. He observed that seasonal neurogenesis, stimulated by environmental changes, occurs in these birds. Specifically, these birds are able to add newborn neurons in the already formed brain circuit responsible for the control of singing[42].

Most recently, several evidence in the literature have shown the positive effects of physical exercise and the exposure to an enriched environment on increasing neurogenesis[43,44]. This increased neurogenesis/survival of neuronal precursor cells correlates with improved cognitive function. On the other hand, neurogenesis declines with aging and is decreased with the exposure to chronic stress leading to impaired memory and cognitive functions (Figure 7)[45,46].

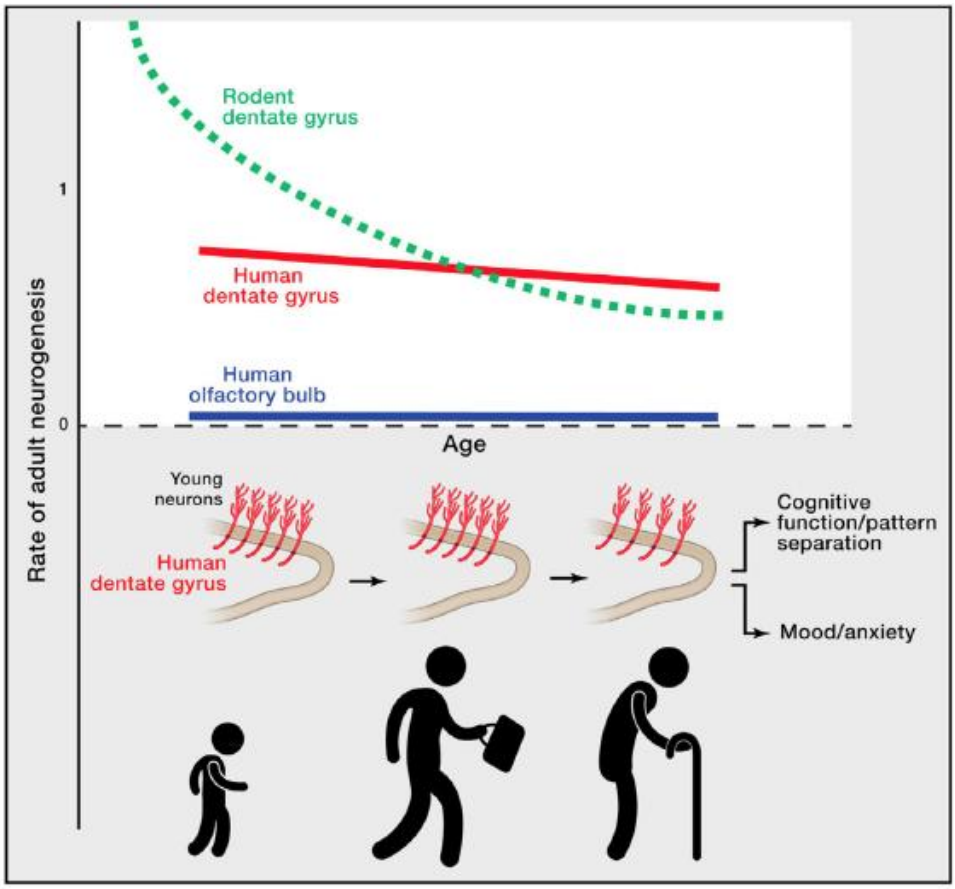

Figure 7. **Comparison between rodent and human neurogenesis. Aging dramatically decreases neurogenesis process in both rodents and human.**

In particular, excessive stress increases the release of glucocorticoids, important class of steroid hormones that negatively affects neurogenesis[47]. Specifically, glucocorticoid receptors

are upregulated in the DG of old mice as compared to younger mice, thus reducing hippocampal plasticity. Moreover, aged brains show increased presence of reactive astrocytes that results in a dramatic reduction of FGF-2, essential for maintaining NSCs[48].

In addition to normal physiological processes, pathological conditions such as Alzheimer's disease and traumatic brain injury (TBI) are also known to affect neurogenesis[49,50].

Alzheimer's disease is a neurodegenerative disorder and the most important cause of dementia worldwide[51]. Alzheimer's disease is a multifactorial brain illness of which the most important risk factor is aging. It is characterized by loss of short-term memory, problems of language and disorientation. This continue decline becomes worst with time and leads to death[52].

TBI is a chronic disease that occurs after brain trauma and often results in permanent disability[53]. TBI is one of the most important brain illness with more than 10 million of cases reported each year worldwide with an incredible cost for the health care that is over 50 billion of dollars only in the United States. TBI is mostly studied in football players and soldiers that show an incredible increased risk for the development of this pathological condition mostly caused by their lifestyle. It is currently estimated that there are more than 300,000 cases of TBI in the United States, of which 2,000 among veterans with a range of 9.6% to 20% of the military personnel. This wide range is caused by a difficult diagnosis of TBI especially years after from the injury[54]. Moreover, some of the symptoms related to TBI disappears with the time and for this reason are often underestimate by people who believe they have completely recovered. The incidence of this pathology is further increased by sport related brain injuries estimated in 1.6-3.8 million per year only in the United States[55].

TBI is an important risk factor for neurodegenerative disorders such as AD and PD[56]. Data in the literature showed that not only TBI increases the probability to develop neurodegenerative disorders but most importantly it seems to anticipate the onset of these pathologies[57].

- **TBI and Neurogenesis**

Memory and cognitive deficits are the most important clinical manifestations among the numerous impairments caused by TBI[58]. In the last 10 years there has been an increased focus on the study of neurogenesis after TBI and its possible role in the recovery process after trauma[59]. All these studies have shown evidence of increased neurogenesis after TBI[60,61]. In particular, it has been demonstrated both increased proliferation of NSC and increased presence of precursor cells, which suggest an attempt of the brain to recover after brain trauma[62]. However, the differentiation of these precursor cells has been shown to occur in a displaced and aberrant way, particularly in the hilar region of the hippocampus[63]. Moreover, TBI causes impaired neurogenesis and gliogenesis pointing to the necessity to better understand the role played by neurogenesis after brain trauma, especially because neurogenesis alone is not able to mitigate the TBI-associated cognitive deficits[64].

Therefore, understanding the molecular mechanism of neurogenesis induced after TBI may provide fundamental information for the development of a potential therapeutic approach for the recovery of neuronal loss after trauma, focused on endogenous NSCs[65].

- **Light and Ultrasound applications in TBI**

Study a possible treatment for TBI is not easy. A pharmacological approach to the management of TBI is very difficult because TBI causes a cascade of secondary events triggered by a primary non-reversible injury[66].

The secondary injury is the cause of several changes in the brain such as, increased inflammation, edema formation, dysfunction at the blood brain barrier, reduction of oxygenation that cause an imbalanced increased angiogenesis, increased necrosis and increased apoptosis[67]. All these events may be preventable and reversible. For this reason TBI research is focused in both understanding the molecular events trigged by the primary injury and the treatment to inhibits or reduce the secondary events[68].

Try to find a single drug that is able to reestablish the normal condition is still considered an infeasible approach and for this reason non-invasive therapies (delivered transcranially) such as near infrared laser therapy (NIL) (800-1000nm) and ultrasound waves (US) (2-20 kHz) have received increased interest for their properties: they can diffuse in the injured area and can be delivered directly in the affected region[69–72].

NIL was for a long time used for pain relief and has recently been applied for TBI treatment showing beneficial effects, in particular in veterans affected by brain injury[73]. It has been reported in the literature that the administration of NIL exerts neuroprotective effects in animal model of TBI, stimulate neurogenesis in mice and produce beneficial cognitive and emotional effects in humans[74]. The application of high-intensity infrared light showed a decrease of headache symptoms, sleep disturbance and irritability in subjects affected by TBI. In particular, in 2011, for the first time it has been reported two cases of chronic TBI treated with light emitting diode (LED) for 6-10 minutes daily for several months showing improvements after 7-9 months[75].

One of the mechanisms by which NIL produces beneficial effects is improving mitochondrial activity[76]. Mitochondria are ancestral organelles present in the eukaryotic cells that are responsible of ATP production through respiratory chain. The respiratory chain involves serial passage of electrons among four protein complexes (Complex I, II, III and IV) leading to

efflux of hydrogen ions from the internal to the external membrane. This proton gradient provides the energy necessary for the ATP-synthase to produce ATP starting from ADP[77].

A key enzyme of these process is the Cytochrome C oxidase that is able to absorb the photons delivered through NIL improving the efficiency of the electron transport that in turn increases the passage of hydrogen ions resulting in a more efficient ATP production[78]. In addition to this mitochondrial regulating mechanism, NIL can modulate the production of reactive oxygen species (ROS), mitochondrial DNA replication and grow factor expression[79].

Unfortunately, the big limitation of NIL application is its penetration into the brain tissue that is limited to around 2.5-3 cm and for this reason is able to diffuse and stimulate only the superficial cortical layers[80].

Ultrasound waves (US) are commonly used for diagnostic imaging and are set at different wavelengths[81]. Some of these wavelengths, in the range of low-frequencies (2 kHz), have been shown to stimulate brain circuits, be neuroprotective and reduce edema formation while increasing the permeability of blood vessels[82]. Moreover, US can induce the production of important neurotrophins such as brain-derived neurotrophic factor (BDNF)[83].

BDNF is an essential neurotrophin involved in neuronal homeostasis by maintaining the survival of existing neurons, inducing the differentiation of new born neurons and maintaining synaptic plasticity[84]. Because of its pleiotropic effects, BDNF was studied for the pharmacological treatment of TBI, however exogenous BDNF administration after TBI did not show protective effects[85].

Glial cell line-derived neurotrophic factor (GDNF) and vascular endothelial growth factor (VEGF) are other factors belonging to the neurotrophins family that can be stimulated by US. In particular, GDNF has neuroprotective effects and reduces the activation of the apoptotic pathway after TBI, while VEGF is the most important angiogenetic factor that improves the

vasculature in damaged brain areas after TBI with extremely positive effects in reducing the swelling of the injured areas[86,87].

The mechanism through which the production/release of these neurotrophic factors is stimulated by US is still under investigation. US produces mechanical force and one of the possible mechanisms involves the transduction of mechanical stimuli into biochemical signals inside the cells. This mechanism can occur in a direct manner through mechanoreceptors or indirect transduction by integrins[88].

Mechanoreceptors are mechano-gated ion channels in a closed conformation that can be opened by mechanical stress[89]. The open state is the direct result of the mechanical force applied: a more intense stimulus results in a more open state of the channels or can results in a longer opening time leading to a greater influx of ion species such as Na^+ and Ca^{2+} [90].

Integrins are proteins linked to the cytoskeleton that allow the cells to be anchored to the external matrix[91]. External mechanical force can be transduced directly to the cytoskeleton and activates downstream proteins[92].

Beside the bio-effectiveness the most important advantage of US waves is their ability to travel through tissues and reach deeper areas in the brain, such as the hippocampus, and for this reason they are candidate for a possible treatment for TBI and other neurodegenerative disorders[93].

- **Nano-pulsed laser therapy (NPLT)**

Dr. Esenaliev and his group in the center for Biomedical engineering at University of Texas Medical Branch have developed an a highly innovative non-invasive device that combines the benefits of both near infrared laser light (808nm) and ultrasound waves, optoacoustically generated with each short (10ns) high-energy (15mJ) laser pulse within the tissue (Figure 8)[94].

Figure 8. **The NPLT device.**

The optoacoustic effect, also known as photoacoustic effects, is a phenomenon by which ultrasound waves are generated following light absorption from a chromophore that undergoes thermo-elastic expansion[95]. In detail, a short pulse (10ns of duration for 20 pulses per second) of near-infrared light is able to induce a very rapid increase in chromophore's pressure because the duration of the pulse is shorter than the time necessary to dissipate the pressure. The accumulated energy and pressure is released as low intensities ultrasound waves[96].

NIL light (808 nm) is produced by an optical parametric oscillator (OPO) (Opolette 532 II, Opotek Inc., Carlsbad, CA) with pulse energy 4 mJ and a repetition rate of 20 pulse per second with a duration of 10ns each. These pulses are delivered through an optoacoustic probe that is composed of a 3mm optical fiber, for the light delivery, and a piezoelectric transducer, for optoacoustic wave detection.

During a 5 minutes treatment (300 seconds 6,000 pulses (20 pulses x 300 seconds) 6,000 pulses are delivered to an area of 0.07cm^2. The energy delivered during a 5 minutes treatment is 24 Joules (4 mJ x 6,000 pulses) with an energy density (J/cm^2) of 343 J/cm^2 (24 J / 0.07 cm^2).

This innovative device not only combines the beneficial effects of each single component, near infrared light and ultrasound (NIL and US waves), but most importantly is able to overcome the limitation of poor light penetration through the skull.

The NPLT device is safe and already tested in a clinical setting to monitor fetal blood oxygenation (this is possible because hemoglobin has two different optoacoustic profile when bound to the oxygen or alone)[97].

AIM OF THE PROJECT

The long-term goal of my research is to stimulate NSCs to proliferate and differentiate so as to repair the brain after TBI.

Previous work in our laboratory has demonstrated that NPLT exerts a significant neuroprotective effect in a rat model of brain trauma and increases the number of proliferating NSC in the hippocampus of blast-injured rats[72]. In this work I tested the effect of a highly innovative, non-invasive device that combines the benefit of both near-infrared laser light (NIL, 808nm) and low-intensity ultrasound waves (US), which are optoacoustically generated with each short (10ns) high-energy (15mJ) laser pulse within the tissue.

I pursued two specific aims:

1. Effect of NPLT *in vivo*: To determine the effect of NPLT on neurogenesis in a rat model of TBI. Stimulating endogenous neurogenesis could represent a therapeutic approach for the treatment of brain trauma and neurodegenerative diseases. For this reason in this aim I tested the effect of NPLT in a rat model of TBI and I analyzed neurogenesis after treatment.

2. Effect of NPLT and its components *in vitro*: To determine the effect of NPLT, and its components, on proliferation and differentiation of hippocampal NSCs *in vitro*. In this aim I analyzed the effect of NPLT on NSC isolated from rat hippocampus in order to assess the effect on proliferation and differentiation after treatment. Moreover, I set up a technique to treat the cells with NIL and US alone in order to test which component is the most effective.

EXPERIMENTAL DESIGN

Effect of NPLT *in vivo*

Four groups of rats were studied in order to cover all the possible combination: Sham, Sham+NPLT, TBI and TBI+NPLT. Anesthetized adult male Sprague-Dawley rats were subjected to Sham or TBI injury in a parasagittal setting, by fluid percussion injury (FPI), and then treated transcranially for 5 minutes, 1 hour post-injury, with NPLT.

These parameters were selected based on previous data present in literature in order induce a biological effect. Particularly, the energy delivered in 5 minutes treatment is consistent with previous work in which was used continuous near-infrared light showing beneficial effect [98]. The treatment applied 1 hour after injury was selected because is a good range of time to prevent the secondary injury. Indeed, it has been demonstrated that the first 24h after injury are critic for the secondary events induced by a non-reversible primary injury. Most of the secondary events are trigged in the first 4 hours.

The rats were euthanized 24 hours and 2 weeks after injury. In order to evaluate the rate of proliferating NSCs, rats were injected with bromodeoxyuridine (BrdU; 75 mg/Kg i.p; SIGMA). BrdU is a thymidine analogue that is incorporated in newly formed DNA and commonly used to mark proliferating cells (Figure 9). The brains were sectioned and immuno-stained for BrdU, to study NSC proliferation and for doublecortin (DCX), to evaluate the presence of neuronal progenitor cells.

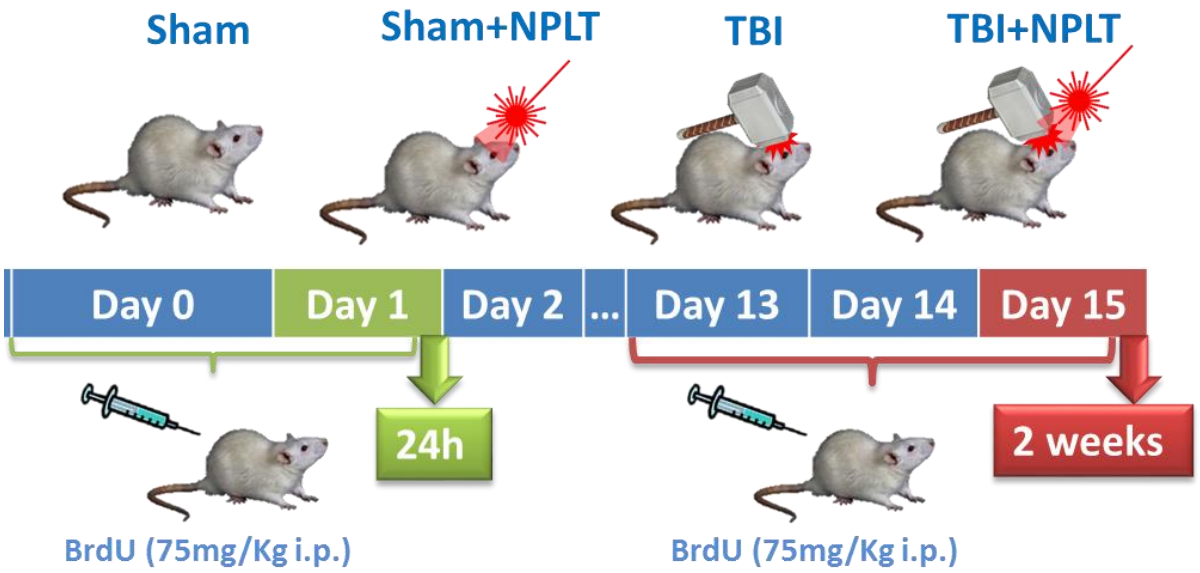

Figure 9. **Experimental design. Four groups of rats were studied: Sham, Sham+NPLT, TBI and TBI+NPLT. Anesthetized adult male Sprague-Dawley rats were subjected to sham or TBI injury, by fluid percussion injury (FPI), and then treated transcranially for 5 minutes, 1 hour post-injury, with NPLT. The rats were euthanized 24 hours and 2 weeks after injury. The rats were injected with bromodeoxyuridine (BrdU) in order to further asses the number of proliferating NSC.**

Effect of NPLT and its components *in vitro*

NSC were isolated from the hippocampus (Hipp-NSCs) of healthy adult rats (Millipore) were cultured in proliferating media or plated onto poly-ornithine-laminin-coated plates and cultured in differentiating media. The cells were characterized by western blotting analysis of stemness and differentiation markers.

In order to evaluate the direct effect of NPLT to NSCs, I treated cultured Hipp-NSCs with NPLT for 5 minutes to maintain the consistency with the *in vivo* experiment. Moreover, Hipp-NSC were treated with near-infrared light (NIL) or optoacoustically generated ultrasound waves (US) in order to study which component of NPLT is the most effective. These two components can be easily separated from NPLT using a special nonporous black plastic film (Figure 10).

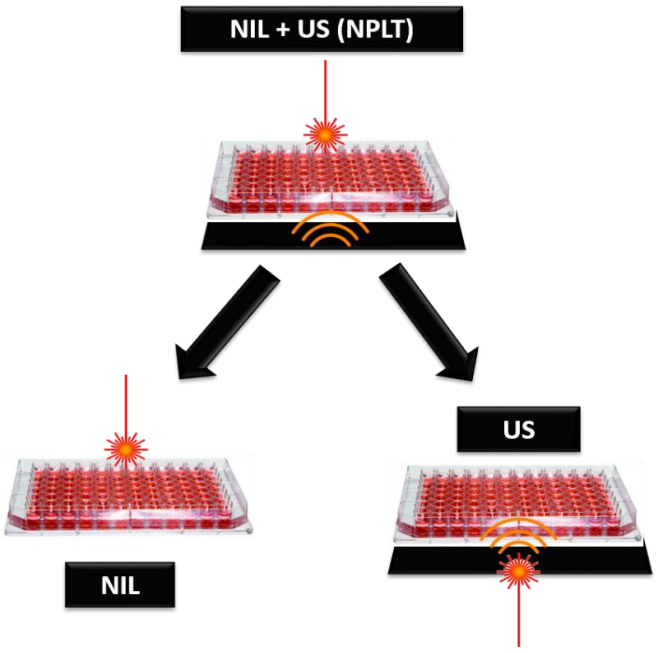

***Figure 10*. NPLT treatment of Hipp-NSCs. NPLT combines the near-infrared light (NIL), delivered with a laser from the top of the well, and ultrasound waves (US), generated when the laser reach the black nonporous plastic filter at the bottom of the plate. NIL is applied without the black nonporous plastic filter at the bottom of the plate. US alone are applied with the beam of the laser set at the bottom black nonporous plastic filter in order to avoid the interference of the NIL.**

To evaluate the effect of NPLT on proliferation, Hipp-NSCs were treated for 5 minutes with NPLT, NIL or US, and cultured in proliferating media. To evaluate the effect of NPLT on differentiation, after each treatment Hipp-NSCs were cultured in differentiating media. After 7 days of differentiation the cells were fixed with 100% ice-cold methanol and processed for immunofluorescence analysis of differentiation markers. A separate set of cells were processed for Western blotting and qRT-PCR analysis.

METHODS

Animals:

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch, Galveston, Texas and conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Fluid percussion model of traumatic brain injury:

Male Sprague-Dawley rats (350 g–400 g) were housed with food and water ad libitum in a vivarium with constant light cycle, temperature and humidity. The rats were anesthetized with isoflurane in an anesthetic chamber, intubated, and mechanically ventilated with 1.5–2.0% isoflurane in O2: room air (70:30) using a volume ventilator (EDCO Scientific, Chapel Hill, NC). Rats were prepared for fluid percussion injury. Specifically, rats were placed in a stereotaxic frame and the scalp was incised along the mid-line. A 4-mm hole was trephined into the skull 2 mm to the right of the mid-sagittal suture, mid-way between lambda and bregma. Sham rats were subject to the surgical procedure without injury.

For the TBI rats, on the exposed dura were placed a modified LuerLok syringe hub and bonded in place with cyanoacrylic adhesive and covered with dental acrylic.

The rats were subjected to moderate (2 Atm) fluid-percussion injury immediately after the return of a withdrawal reflex to paw pinch (spinal reflex that protects from damaging stimuli and that is inhibited by anesthesia). After TBI or sham injury, rats were disconnected from the fluid percussion device and righting reflex was assessed every 60 seconds until a normal righting reflex was observed. This parameter is important in order to measure the degree of injury[99].

NPLT treatment:

The rats were treated for 5 minutes with the NPLT delivered through an optic fiber on the site of the injury. After the animals had fully recovered from the surgery/anesthesia, they were returned to their cage with ad libitum food and water.

Bromodeoxyuridine BrdU treatment:

The 24 hours group of rats were injected i.p. with BrdU (75mg/Kg) immediately after the treatment, 2 hours later, 4 and 2 hours before euthanasia. 24 hours after injury the rats sacrificed. For the 2 weeks group of rats the injections of BrdU were made 72, 48, 24, 4 and 2 hours before euthanasia.

At the appropriate times, the rats were anesthetized with 2-3% of isoflurane and sacrificed by decapitation, brains immediately dissected out, frozen on dry ice and stored at -80 °C until further processing.

Tissue Processing and Immunostaining:

The brains were sectioned on a cryostat and mounted on superfrost clean slides. 10µm thick sections from the 24 hours rat groups were used to perform immunofluorescence staining. The frozen sections were fixed in ice-cold 10% buffered formalin at room temperature for 30 minutes. Then the sections were washed 2 times with PBS 1X for 10 minutes and incubate with 2N HCl al 37 °C for 30 minutes to denaturate the DNA and unmasking the BrdU. The sections were neutralized with 0.1M borate buffer (pH 8.5) 2 times for 5 minutes and then blocked and permeabilized with a solution of 10% normal serum and 0.3% TritonX-100 in

PBS for 30 minutes. The incubation with the anti-BrdU antibody was performed overnight at 4°C. The day after the sections were washed 3 times in PBS for 10 minutes each and then incubated 1 hour at room temperature with Alexa-conjugated antibodies diluted in 1.5% normal serum. Finally, the sections were mounted with fluorosave and the pictures were taken with fluorescence microscope for the further quantification with ImageJ software. The slides were store in the dark at 4 °C.

The brains from the 2 weeks rat groups were shipped to the Neuroscience Associate Company (NeuroScience Associates, Knowxville, TN) that have patented the multibrain® technique. In detail, this particular technique allows to include 16 rat brains in one single block that is sliced on the microtome in sections 40µm thick in the coronal plane and collected every 480µm through the hippocampus. The sections were adhered to glass slides (5x4 cm) ready to be processed for immunohistochemistry staining of BrdU, to evaluate the rate of the proliferating cells, DCX, to evaluate the number of neural progenitor cells, performed by the same company. This technique has several advantages such as the reduced variability of the staining since is made at the same time for the entire slide and the presence of sections at the same bregma level (Figure 11).

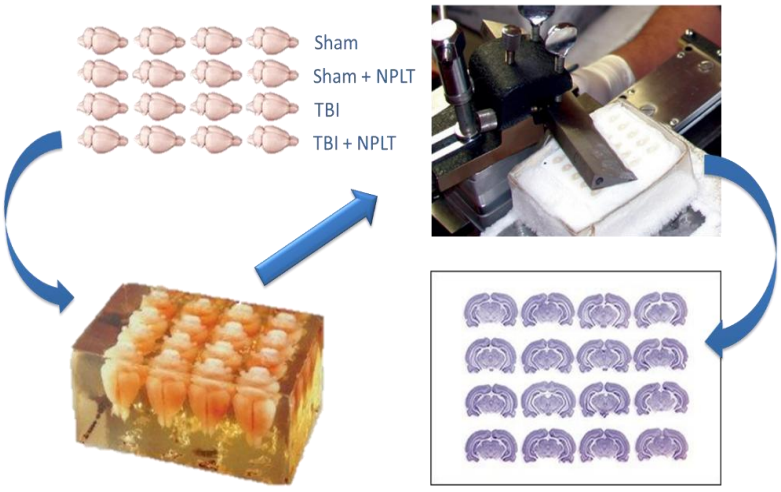

Figure 11. **Neuroscience Associate Multibrain technique.**

Neural Stem cells:

Adult rat hippocampal NSC (Hipp-NSCs, Millipore) were used. These primary NSC were isolated from adult Fisher 344 rats, aged 43-55 days.

Neural Stem cells dissociation:

Hipp-NSC growth is suspension as neurospheres were pelleted 3 minutes at 300g in conical tubes and the supernatant was discarded. Hipp-NSC were then subjected to enzymatic dissociation with 1ml of Accutase solution, pre-wormed at 37°C in the water bath, for 3 minutes at 37°C. The dissociated neurospheres were pelleted and the supernatant discarded. 5ml of fresh medium (DMEM/F12) were added to the tube for the further counts.

Neural Stem cells counts:

In order to plate the correct amount of cells, Hipp-NSC were counted with the hematocytometer and the support of an optic microscope. In details, 150µl of Trypan Blue were added in a tube with 50µl of cells from the 5ml of the cell suspension previously obtained. 10µl of this solution were added to each of the two chambers of the hematocytometer. Only the live cells were counted in order to obtain the correct number of cells of the suspension and plate them at the correct confluency.

Neural Stem cells expansion:

Hipp-NSC were cultured with Dulbecco's modified Eagle's medium/F12 medium (DMEM/F12) containing the serum-free supplement B27 (without retinoic acid), 2 mM L-glutamine, 20 ng/ml of basic fibroblast growth factor-2 (FGF-2) and antibiotics (penicillin,

streptomycin, neomycin). The cells were plated in low-adhesion T75 flasks at $1.7x10^6$ cells per 15ml of media and incubated at 37°C with 5% CO2. Under these conditions Hipp-NSC form neurospheres.

Neural stem cells differentiation:

$1x1.5^5$ cells were plated in poly-ornithine/laminin-coated six-well plates and 10,000 cells/well into chamber slides. To differentiate the cells were cultured in Dulbecco's modified Eagle's medium/F12 (DMEM/F12) medium containing serum-free supplement B27 (with retinoic acid), 2 mM L-glutamine, 1% FBS and antibiotics (penicillin, streptomycin, neomycin) for 7 days.

Neural Stem cells *in vitro* treatment with NPLT:

96-well plates were used for the treatment of Hipp-NSC with NPLT because the diameter of the well perfectly fit with the optoacoustic probe. NPLT combines the near-infrared light (NIL), delivered with a laser from the top of the well, and ultrasound waves (US), generated when the laser reach the black nonporous plastic film at the bottom of the plate that acted as chromophore generating the optoacoustic effect. NIL treatment was made with the laser applied from the top of the well without the black nonporous plastic film at the bottom of the plate. US alone was applied with the beam of the laser set at the bottom of the black nonporous plastic film in order to optoacoustically generate US and avoid the interference of the NIL.

5-ethynyl-2'-deoxyuridine (EdU) assay:

EdU is a thymidine analogue that is used to mark replicated DNA of the proliferating cells. The terminal alkyne group is detected through its cycloaddition “click” reaction catalyzed by copper with fluorescent azides. The reaction was performed following the manufacture’s protocol (Invitrogen).

Polyornithine-Laminin coating:

Poly-L-ornithine (Sigma-Aldrich, catalog number P3655) stock solution was reconstituted with sterile distilled water at a concentration of 10 mg/ml, and aliquots were stored at −20 °C. Laminin (1 mg/ml; Invitrogen) was stored at −20 °C. The poly-L-ornithine stock solution was diluted 1:1000 in sterile distilled water to a concentration of 10 μg/ml. 1.5 ml/well was added in six-well plates, 0.5ml/well was added in the chamber slides. After an overnight incubation at RT, the plates were washed twice with distilled water and incubated with laminin overnight at RT at the concentration 10 μg/ml in Dulbecco’s phosphate-buffered saline (D-PBS) without Ca^{2+} and Mg^{2+}, 1 ml/well was added in six-well plates, 0.4 ml/well was added in chamber slides. After the incubation the plate were used or stored at −20 °C until used.

Total RNA extraction:

TriaZol lysed cells were used for the Total RNA isolation using Direct-zol RNA miniprep (Zymo research, Irvine, CA), following the manufacturer’s protocol. The samples were treated 15 minutes with DNase I in order to remove the genomic DNA and then analyzed with NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer for quantitative and

qualitative analysis. 10ng of total RNA were used for retro transcribe the pool of miRNAs and 300ng of total RNA were used for cDNA synthesis of the mRNA.

miRNA retrotranscription:

10ng of total RNA were used for the retro transcription of the pool of miRNA by using TaqMan Advanced miRNA Assay kit (Thermo Fisher Scientific) performed in a thermocycler following this step: Poly (A) tailing reaction one cycle for 45 min at 37°C, then 10 min at 65°C and hold at 4°C. Ligation reaction one cycle for 60 min and 16°C and hold at 4°C. Reverse transcription reaction one cycle for 15 min at 42°C, then 5 min at 85°C and hold at 4°C. miR-Amp reaction one cycle for 5 min at 95°C and then two-step PCR reaction with 14 cycles each for 3 sec at 95°C and 30 sec at 60°C, then one cycle for 10 min at 99°C and hold at 4°C.

Quantitave Real-Time PCR

qReal-Time PCR was performed on a MX3000P machine (Stratagene, Santa Clara, CA) using Taqman reagents from Applied Biosystems (Foster City, CA). 20µl PCR reaction were made by mixing 10µl of 2X Fast Advanced MasterMix, 5µl of the product of the retrotranscriprtion diluited 1:10, 1µl of Taqman Advanced miRNA assay probes and 4 of nuclease-free water. The thermal profile setup used for the PCR reaction was one cycle 20 sec at 95°C and a two-step PCR with 40 cycles each for 3 sec at 95°C and 30 sec at 60°C. All data from the PCR was collected and analyzed by the MXPro software (Stratagene) and the ΔΔCT fold changes compared to the calibrator (GAPDH).

Total protein extraction:

Total proteins were extracted with RIPA lysis buffer (Thermo Fisher) form whole-cell pellet. After a wash in PBS 1X, 100µl of buffer per $2x10^6$ cells were used to lysed the cells. 1µl of protease inhibitor and 1µl phosphatase inhibitor were added each 100µl of buffer to prevent degradation and modification of the phosphorylated proteins and incubate for 10 minutes on ice. The samples were pelleted by centrifuging 15 minutes at 14,000g and the surnatant transferred in a clean tube for the quantification. The quantification of the samples was performed by BCA assay kit according the manufacturer's protocol (Thermo Fisher).

Western Blot Analysis:

25µg of samples were resolved by SDS-PAGE by using precasted gradients gels 4-20% (Biorad) and then transferred in polyvinylidene fluoride membranes. Monoclonal antibodies Nestin (1:1000) (Millipore), Sox2 (1:1000), bTubulin-III (1:1000) (Promega), NeuN (1:1000) (CellSignaling) were used were used for the hybridization overnight at 4 °C. Membranes were washed wit T-TBS 0.05% and incubated with the corresponding horseradish-peroxidase-conjugated secondary antibody 1 hour at room temperature. The signal was detected using BioRad ECL and captured using a ChemiDoc (Biorad). The protein quantification was normalized to GAPDH (Thermo Fisher).

Statistical Analysis:

Multiple comparisons will be performed among groups of sham/TBI rats treated and not treated with NPLT. GraphPad Prism software was used to perform statistical analysis of variance (ANOVA) among the groups.

RESULTS AND DISCUSSION

Effect of NPLT *in vivo*

The quantification analysis of BrdU incorporation validated the effectiveness of the model by confirming data present in literature of the increased rate of proliferating cells in the DG after TBI in both 24 hours and 2 weeks groups (Figure 12 A-B). Moreover, there is a significant increase in BrdU-positive cells in the subgranular zone of the DG of the hippocampus after treatment with NPLT, particularly 2 weeks after treatment (Figure 12 B).

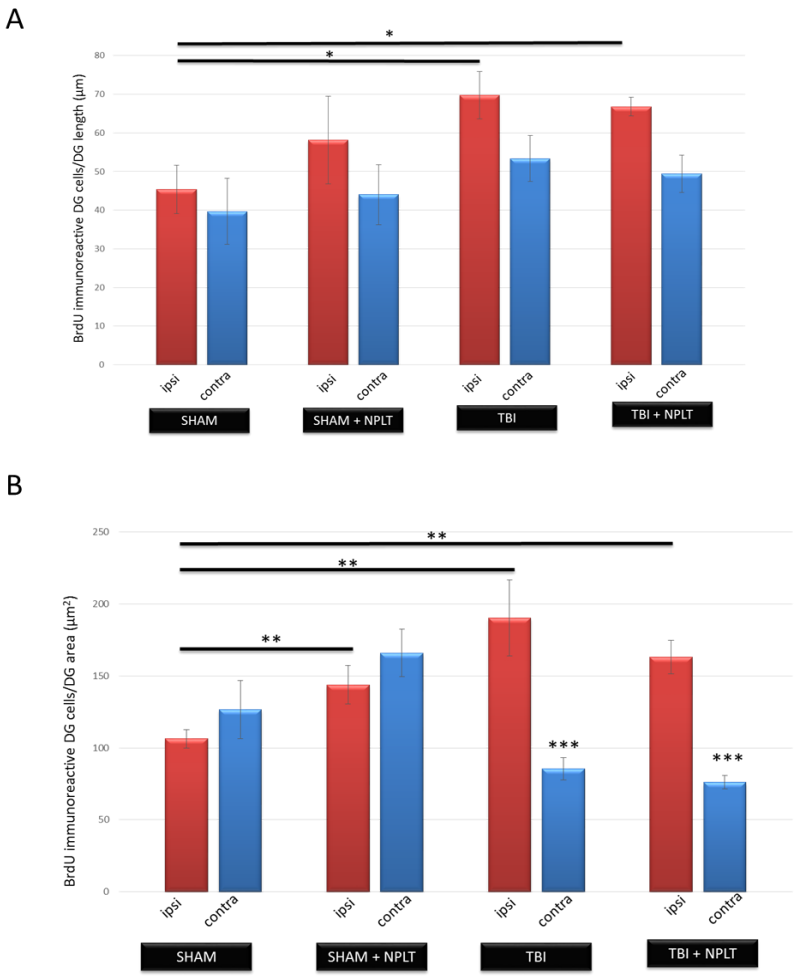

Figure 12. **BrdU quantification. (A-B)There is a significant increase of proliferation after TBI in both groups (24 hours and 2 weeks). (B) There is an increase in BrdU-positive cells in the subgranular zone of the DG of the hippocampus after treatment with NPLT, particularly 2 weeks after treatment. (B) There is a significant reduction of proliferating cells in the contralateral side of the hippocampus of the injured animals after 2 weeks. N=4, *p<0.05, **p<0.01, ***p<0.0001.**

Interestingly, after 2 weeks there is a significant reduction of proliferating cells in the contralateral side of the hippocampus of the injured animals. This result may suggest a compensatory mechanism of the not injured side in favor of the injured side. However, further investigation needs to be performed in order to clarify these results.

Qualitative analysis of the DCX-positive cells (neuronal progenitor) in the ipsilateral side of the hippocampus DG showed a tangible increase after TBI as compared to the sham. Furthermore, ectopic neurogenesis in the hilar region was observed after TBI. Interesting result is the normalization of the DCX-positive cells after treatment with NPLT to a sham levels and most important the disappearance of the positivity in the hilus, suggesting that NPLT treatment dramatically reduced the aberrant neurogenesis (Figure 13 A).

Similarly, the contralateral side showed the same results but less evident, suggesting a response based on different grade of injury and a different regulation in the not-directly injured side of the brain that is mediated mostly by secondary effectors (Figure 13 B).

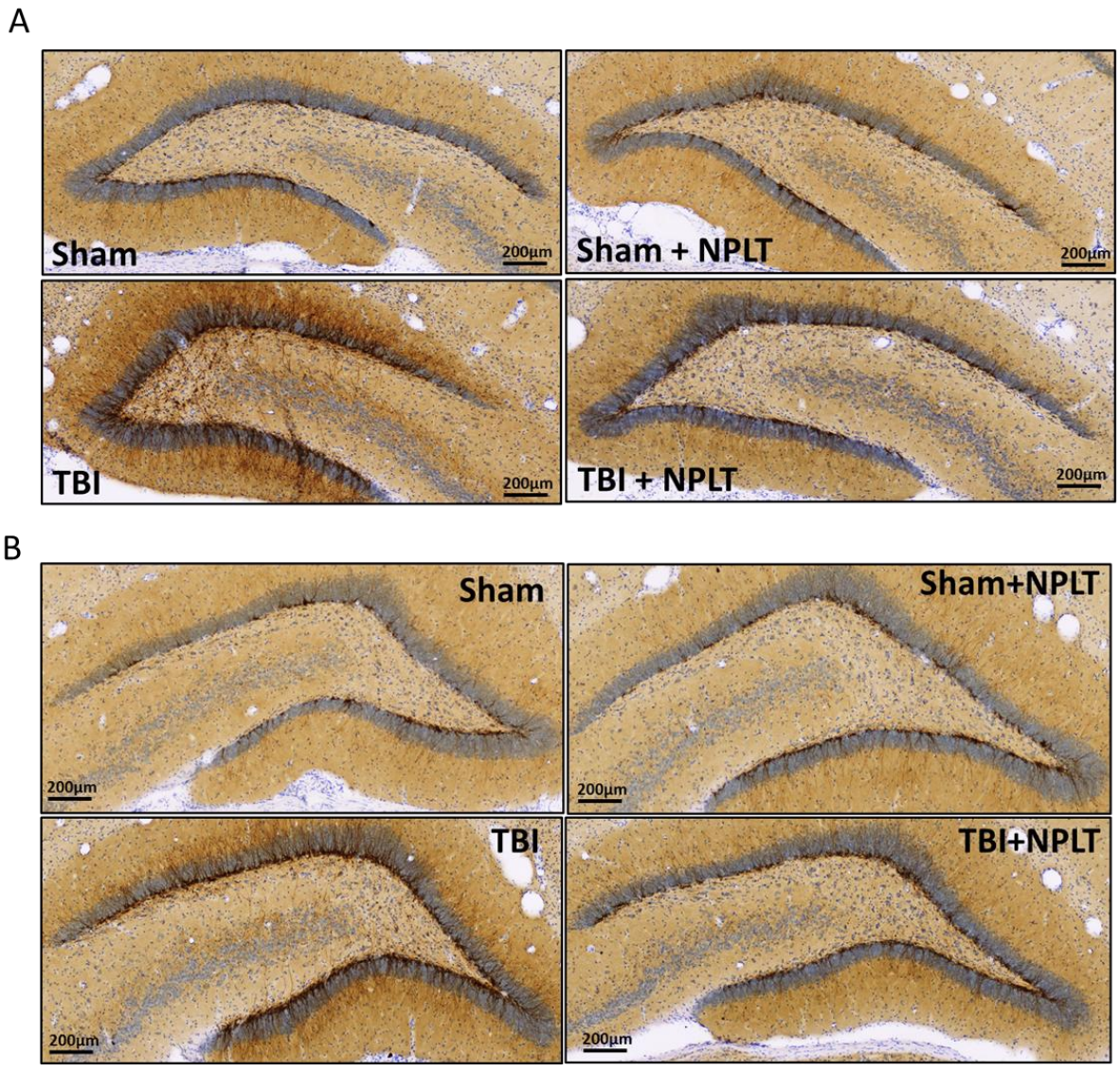

Figure 13. **Doublecortin (DCX) immunostaining. (A)The ipsilateral side showed a tangible increase of DCX after TBI comparing the sham. Furthermore, is evident the ectopic neurogenesis in the hilar region after TBI. DCX-positive cells decreased after treatment with NPLT to a sham level. (B) The contralateral side showed the same results of the ipsilateral. n=4.**

Effect of NPLT and its components *in vitro*

Firstly I characterized the stemness of the Hipp-NSC by western blotting analysis through the expression of stemness and differentiation markers (Figure 14).

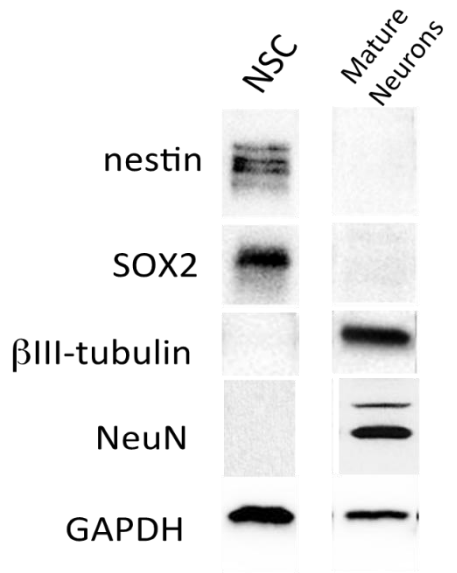

Figure 14. **Wester Blotting characterization of Hipp-NSC in comparison with mature neurons. NSC shows the expression of stemness marker (Nestin and Sox2) but not differentiating markers (βIII-tubulin and NeuN). Vice versa mature neurons shows the expression of differentiating markers but not the one of stemness. GAPDH housekeeping.**

NPLT was directly applied on cultured Hipp-NSC in order to study its direct effect without the mediation of other cell type, such as astrocytes and microglia, and the presence of neurotrophic factors present *in vivo*. Moreover, Hipp-NSC were treated with near-infrared light (NIL) or optoacoustically generated ultrasound waves (US) in order to study which component of NPLT is the most effective. Hipp-NSCs treated with NPLT show an increased number of proliferating cells (EdU-positive) 24 hours and 48 hours after treatment.

Treatment with NIL alone does not affect the proliferation while US increases the number of proliferating cells 48 hours after treatment, suggesting that US is the component of NPLT able to modulates the proliferation (Figure 15).

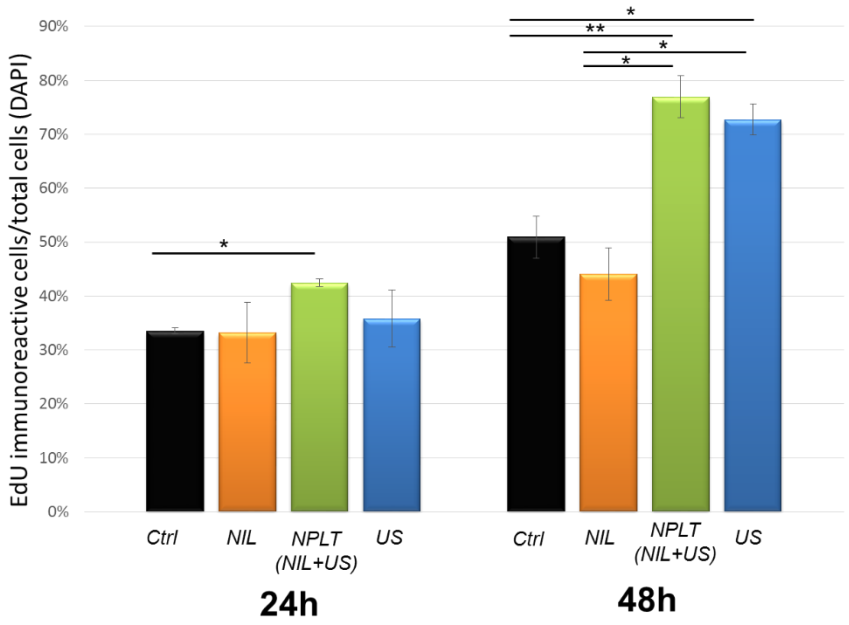

Figure 15. **EdU analysis. Hipp-NSCs treated with NPLT shows an increased number of proliferating cells (EdU-positive) 24 hours and 48 hours after treatment. NIL alone does not affect the proliferation while US increases the number of proliferating cells 48 hours after treatment. n=4, *p<0.01, **p<0.001.**

In order to confirm the modulatory effect of US in Hipp-NSC proliferation, I cultured the US treated cells in differentiating media for 1 week and analyze the degree of differentiation. Immunofluorescence analysis shows that US treatment did not affect the number of GFAP-positive cells but significantly decreased the number of βIII-tubulin-positive cells suggesting a more immature stage of the treated cells in accordance with the proliferation data (Figure 16).

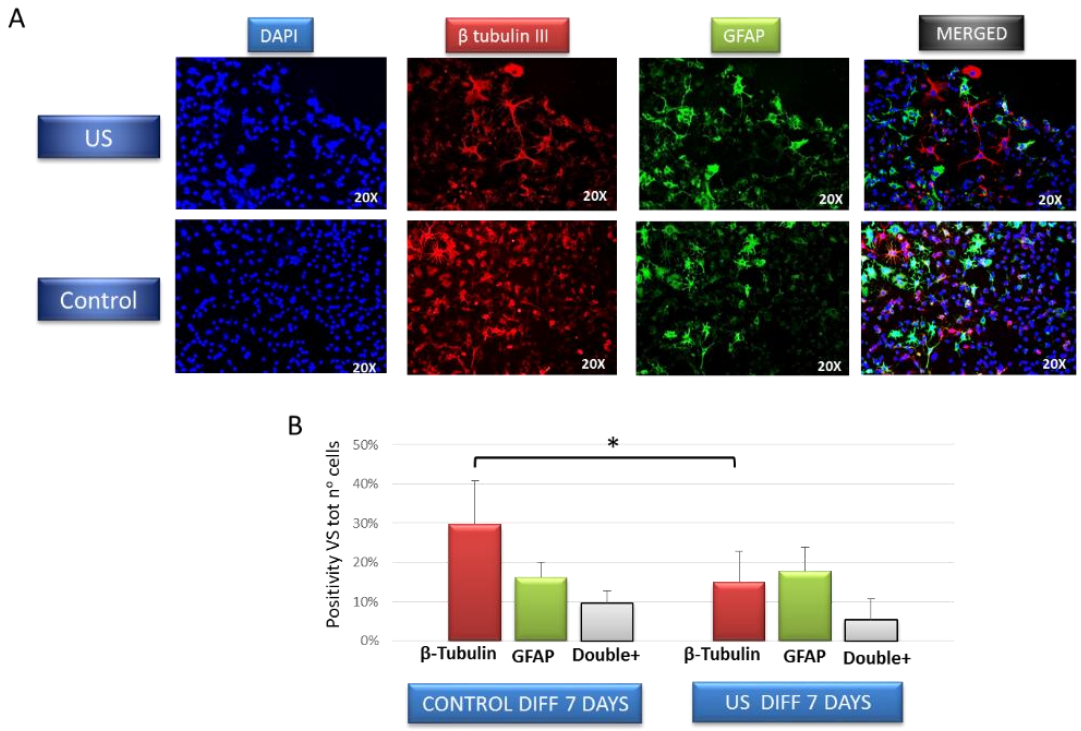

Figure 16. **(A) Representative immunofluorescence of differentiating cells stained with neuronal marker (βIII-tubulin) and glial marker (GFAP) 7 days after treatment with ultrasound VS untreated control (top). Quantification of immunoreactive cells VS total number of cells (bottom). (B) Quantification. US treatment significantly reduced the neuronal differentiation of Hipp-NSCs 6 days after treatment while not affecting glia differentiation. n=3: *p < 0.05.**

Moreover, I analyzed the effect of NPLT on the epigenetic regulation of Hipp-NSCs by measuring the expression of specific miRNAs known to be involved in cell fate specification (miR929, miR25, miR29). NIL does not affect the expression of these miRNA, while US significant decreases their expression. Interestingly, the combination of the two components significant upregulates the expression of these miRNA. This last result further confirm the uniqueness of the NPLT treatment that differentially modulates the epigenetic regulation comparing the treatment by its single components NIL and US (Figure 17).

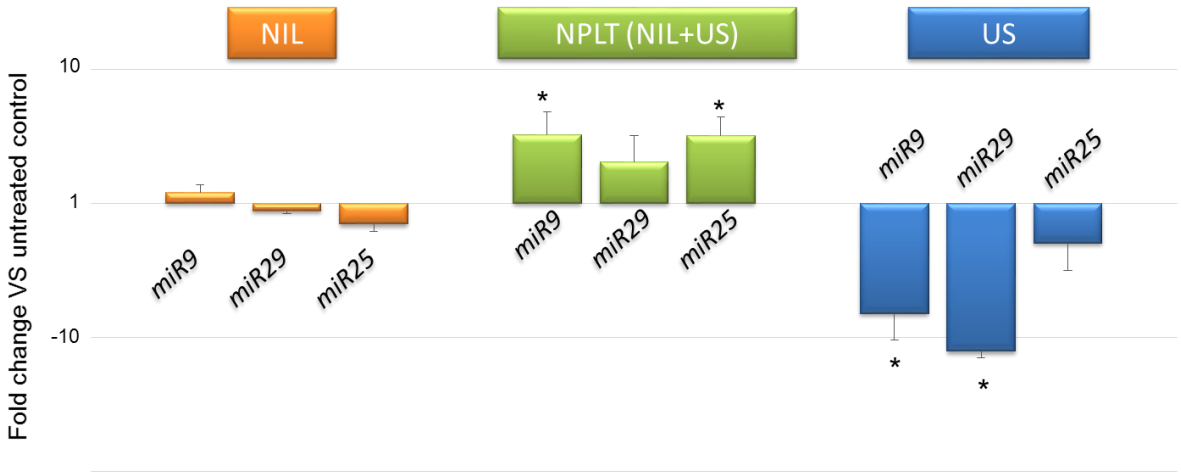

***Figure 17*. miRNA expression analysis. NIL does not affect the expression of these miRNA, while US significant decreases their expression. The combination of the two components significant upregulates the expression of these miRNA. n=3, *p<0.05.**

Taken together the *in vitro* and *in vivo* results demonstrate the beneficial effects of NPLT treatment after brain trauma by increasing the NSCs proliferation, reducing the displaced neurogenesis and by improving the proliferation of Hipp-NSC *in vitro*. If this increased proliferation leads to mature neuron formation needs to be further investigated.

Some of the beneficial effects in the brain are most likely mediated by factors and other cell type such as microglia, however *in vitro* results of this study shows that NPLT may direct stimulates NSCs proliferation.

In literature has been reported the important role of Piezo1, a mechanoreceptor, for NSC lineage specification. I have shown that Piezo1 is expressed on Hipp-NSC and that can be stimulated by US allowing the passage of ions, such as Ca^{2+}, key mediator for the differentiation. Further studies are necessary to demonstrate the role of Piezo1 as critical mediator in NPLT stimulation (Figure 18).

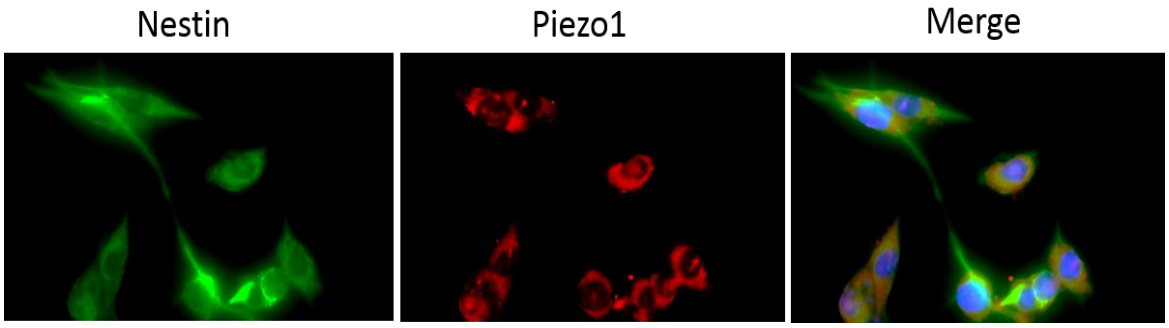

***Figure 18*. Representative immunofluorescence of Hipp-NSCs. Nestin (Green, stemness marker), Piezo1 (Red), Nuclei (Blue). Magnification 40X.**

CONCLUSION

Traumatic Brain Injury (TBI) is characterized by permanent structural and physiological changes that compromise the normal function of the brain[100]. Particularly, the most important affected area is the hippocampus, a region located in the temporal lobe and that belongs to the limbic system[101]. The hippocampus plays an essential role for the process of learning and memory consolidation. Indeed, memory impairment and disorientation are among the most important symptoms shown by TBI subjects[102]. Moreover, in the hippocampus a process takes place called "neurogenesis" through which new neurons are continuously generated, subverting the old dogma that stated the inability of the old brain to replace neurons[103]. Neurogenesis occurs throughout life thanks to the presence of Neural Stem Cells (NSCs) located in only two specific niches in the brain: the subventricular zone of the lateral ventricles and the subgranular zone of the dentate gyrus in the hippocampus[18]. Literature evidences and previous work performed in Dr. Micci's laboratory demonstrate that neuronal differentiation in the hippocampus DG is impaired after TBI[72]. This is associated with the up-regulation of specific miRNAs known to regulate neurogenesis, suggesting that epigenetic changes may be at least in part result in impaired neurogenesis after TBI.

In the last two decades there has been an increased interest in finding an effective treatment for TBI[104]. More specifically, research has focused on non-invasive therapies such as near-infrared light (NIL) therapy and low-intensity ultrasound stimulation (US)[105]. Specifically, NIL has been shown to be neuroprotective, reduce inflammation and improve neurological function in rodent models of TBI, while US has been shown to stimulate neuronal activity in the hippocampus and inhibits edema formation[106,107]. Both treatments have shown encouraging results, however neither have been established as a valid potential neurological

application in part because of the limited ability of NIL to penetrate into the deeper layers of the brain (it has been reported that in the human brain it will only irradiate 3 cm below the skull).

The purpose of the research I conducted is to stimulate neurogenesis so as to repair the brain after TBI.

To pursue this goal, I tested the effect of NPLT, an innovative device that combine the advantages of both near infrared light and low-intensity ultrasound stimulation.

My *in vivo* data on FPI injured rats show increased proliferation of NSC in the DG of the hippocampus after injury and confirmed what was previously shown in Dr. Micci's laboratory in a different model of brain injury. Moreover, this is in accordance with the evidences present in literature of the increased proliferation in the DG most likely as response after brain injury[108]. However, is still debated the role of this increased proliferation since is not able to mitigate the cognitive and memory deficits[64]. For this reason it is essential to follow the differentiation of these proliferative cells in order to assess if they are able to mature in neurons as well as integrate in the pre-existing circuits and re-establish the damaged networks. In this sense, my results of neuronal progenitor doublecortin (DCX) positive cells shown a dramatic increase of the staining in the TBI rat group. However, this increased staining is remarkable in the hilar region confirming the ectopic neurogenesis after TBI previously reported in literature[109].

The innovative part of this project is the use of the NPLT as a novel treatment for TBI. 5 minutes treatment applied 1 hour post-injury show impressive beneficial effects. Specifically, NPLT treated rats show increased proliferation particularly 2 weeks after treatment. Interestingly, at the same time point, TBI rats treated with NPLT show a decrease in proliferation that is similar to the levels found in healthy NPLT treated rats. This result

highlights the positive effects of NPLT that increases the proliferation and, most importantly, moderates the proliferation induced by TBI.

The most important result is the dramatic reduction of the aberrant migration 2 weeks after treatment. Indeed, in the TBI rat group treated with NPLT there is a substantial reduction of the DCX staining in the DG comparing the TBI, even if still increased comparing the Sham. However, the DCX staining in the hilar region of the TBI rats treated with NPLT is almost completely absent as in healthy rats. This result in addition with the previous on the proliferation confirmed the extremely beneficial effect of NPLT that on the one hand improves the proliferation of NSC and on the other hand reduced the pathological migration induced by brain trauma.

Further step for this project was to analyze the effect of NPLT directly on NSC without the mediation of other factors/cell type. With this goal in mind, I cultured hippocampal NSC (Hipp-NSC) and then I treated them with NPLT. The innovation of NPLT is the combination of NIL and US. These two components can be split by using a black nonporous plastic film that I used in order to test which component of NPLT is most effective.

The first analysis that I performed was on the proliferation that I tested through EdU assay. EdU is a thymidine analogue that is incorporated in the newly synthesized DNA of proliferating cells. NPLT treatment increases the proliferation of Hipp-NSCs 24 and 48 hours after treatment. NIL alone does not affect the proliferation while US increases the proliferation 48 hours after treatment at the same level of the NPLT treated cells suggesting an important effect mediated by US. To confirm this evidence I differentiated the US treated Hipp-NSC for 1 week and I analyzed the expression of glial marker (GFAP) and neuronal marker (β Tubulin-III). While GFAP expression is not affected by US treatment, the number of β Tubulin-III stained cells are significant decreased comparing the untreated control. This suggest a

less/delayed differentiation induced by ultrasound, in accordance with the increased proliferation described before. Only this result is not enough to explain the effect on the differentiation of NPLT and for this reason a further step of this project will be analyze the differentiation of NPLT and NIL treated cells.

Epigenetic regulation mediated by miRNA has been shown to be important in NSC differentiation. miRNA are small non-coding RNA that have been shown to regulate the expression of more than 60% of genes. Particularly, miRNA9, miRNA25 and miRNA 29 have been shown to be directly involved in NSC fate specification[110]. I analyzed their expression in the Hipp-NSC treated with NPLT, NIL and US. NIL alone does not affect the expression of this three miRNAs comparing the untreated control while US significant decreases them. Interestingly, the expression of miRNA9, 25 and 29 is increased in the Hipp-NSC treated with NPLT.

This last result is of great importance since is a further confirmation of the uniqueness of the NPLT device. Indeed, NPLT not only is the first device that combines two treatments (NIL and US) that have been shown to be beneficial, but also differentially modulate Hipp-NSC proliferation/differentiation comparing the treatment with its single components.

Based on my data, I hypothesize that NPLT is a valuable approach that can restore neurogenesis in the brain after damage by modulating the proliferation and the differentiation of hippocampal NSC. Moreover, NPLT seems to reduce the misplaced neurogenesis, one of the key factor that, in addition to seizure and epilepsy, leads to the failure of recovery after brain trauma.

Further studies are necessary to better understand the molecular mechanisms and the mediators through which NPLT exerts its effect on NSC. In addition, it is necessary to clarify if NPLT, besides NSC, acts on other cell types *in vivo*, such as microglia, that have been

shown to be important mediators after TBI both as direct effectors or via release of cytokines[111].

In conclusion, NPLT is a unique device that combines the previously known positive effects of both NIL and US in a novel therapeutic strategy based on self-repair by stimulation of the stem cells that are already present in the brain.

FUTURE DIRECTION

This work demonstrated the valuable potential of NPLT for TBI treatment and its possible application for other pathologies, such as Alzheimer's disease, with memory and cognitive impairments in which increasing neurogenesis may be a therapeutic approach.

With this study I gained valuable knowledge on the effects of NPLT on neurogenesis. Particularly, I analyzed the effects of NPLT and its components on Hipp-NSCs.

Dr. Micci's laboratory has demonstrated that neuronal differentiation in the hippocampus DG is impaired after TBI. For this reason, the next step will be to analyze if NPLT treatment increases the number of progenitor cells that differentiate in neurons.

Another important future step will be to analyze the long-term effects of NPLT because one limitation is that the effect of NPLT may disappear with time in proliferating cells. Therefore, in order to clarify the long-term effects of NPLT on NSCs, it will be necessary to use *in vivo* models and analyze the effects after 1, 3, 6 and 12 months.

Finally, it will be important to determine the molecular mechanisms through which NPLT modulates NSC proliferation/differentiation and a possible mediation by mechanoreceptors.

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At the end of a journey so extraordinary, it is difficult and often impossible to thank everyone because everyone has been a part of this path and fundamental in their own way. This thesis represents a milestone in my career, but most importantly for me as a person. Away from home, you can understand the importance of the people around you that became your new family.

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