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Adult hippocampal neurogenesis: traumatic brain injury-induced dysregulation and therapeutic potentials of a novel non-invasive nano-pulsed laser treatment

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Adult hippocampal neurogenesis: traumatic brain injury-induced dysregulation and therapeutic potentials of a novel non-invasive nano-pulsed laser treatment.

by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy in Neuroscience

The University of Texas Medical Branch

April, 2019

Dedication

This dissertation is dedicated to my wife Gaia, my parents and my brother for the continuous support even thousand miles away. This dissertation is an important academic achievement and a milestone for my life that I would have never reached without their support for which I cannot thank enough.

Acknowledgements

The first person that I have to thank is my incredible mentor Dr. Maria Adelaide Micci. She has been more than an academic guide for me and is almost impossible to put in words her importance for my academic career and my life. Living overseas thousand miles away from my home country was not easy and Dr. Micci unconditional supported me and pushed me to give my best understanding the difficulties. Her hard work and dedication for education have been source of inspiration and motivation in pursuing my goals. Her curiosity and respect for the science brought the best out of me and helped me to reach a high level of independence. I will never forget "data is data" sentences and the lesson that is behind it every time that I will obtained an unpredicted data (almost 99% of the time). Dr. Micci created a unique alchemy with all the students and the others lab members without which it would have been impossible reach our goals. Therefore, I would thank each lab member of my lab family: Liz, June, Cody, Harris and Debbie. Part of this tremendous achievement is thanks to them. Scientific discussions, coffee time and continuous support have made the work environment a big family that led to important scientific successes for our group.

The development of this promising project on a novel therapy for TBI was not possible without the support of the Laboratory for Optical Sensing and Monitoring and specifically Rinat Esenaliev, Yuriy Petrov, and Irene Petrov that provided the NPLT system used in this research project.

I would also thank each member of my committee for their terrific knowledge and huge help for my project: Drs. Owen Hamill, Rinat Esenaliev, Steven Kerine, Claudia Campanella and Giulio Taglialatela.

I am thankful to the Neuroscience Graduate Program at UTMB in particular to our Program Director Dr. Hamill and the coordinators Aurora Galvan and Debra Moncrief.

A very special thanks goes to my "Italian Family" here in Texas. First of all, Salvo and Filippa with which I started, together with my wife, this incredible (crazy) experience from Palermo. Then my second family here in Galveston: Mauro, Michela and Manfredi. I am very thankful to Mariam and Chiara for their incredible friendship, support and help during hard time.

Another special thank goes to Dr. Claudia Campanella and Dr. Francesco Cappello for giving me this exceptional opportunity and be recruited as part of the combined program.

Finally, I need to thank my wife Gaia for her incredible and unconditioned support during the entire experience and for her "yes" when I asked her to leave our country and start this unique experience.

Adult hippocampal neurogenesis: traumatic brain injury-induced dysregulation and therapeutic potentials of a novel non-invasive nano-pulsed laser treatment.

Publication No._____

Emanuele Mocciaro, MS

The University of Texas Medical Branch, 2019

Supervisor: Maria Adelaide Micci, PhD

Neurogenesis, a physiological process by which new neurons are generated from neural stem cells (NSC), occurs throughout life, subverting the old dogma stating to the inability of the adult brain to replace neurons. In the adult mammalian brain, neurogenesis takes place in the subventricular zone of the lateral ventricle and in the subgranular zone (SGZ) of the hippocampus dentate gyrus. Numerous evidences in the literature demonstrate that neurogenesis decreases during aging and it is impaired in neurodegenerative diseases and after traumatic brain injury (TBI). In this work, I aimed to study TBI-induced dysregulation of neurogenesis and to assess the therapeutic potential of a highly innovative non-invasive device, combining the benefits of both near infrared laser light (808nm) and ultrasound waves, to correct neurogenesis dysfunction in the hippocampus of rats subjected to fluid percussion injury (FPI). I found that a single five minutes transcranial application of NPLT, one hour after FPI, significantly increased neuronal differentiation of DCX^+ neuronal progenitor cells in the SGZ and reduced their aberrant migration into the hippocampus hilus and the dentate gyrus granular layer. Moreover, qRT-PCR analysis of laser capture microdissected (LCM) SGZ and of NSC isolated from the hippocampus of TBI rats showed that NPLT prevented injury-induced upregulation of select miRNA (miR9, miR25, miR29, miR124, miR137) known to regulate migration and differentiation of neuronal progenitor cells. Because NPLT did not prevent TBI-induced activation of microglia (amoeboid-shaped $Iba1^+$ and $CD68^+$) in the hippocampus DG and hilus, our results suggest that NPLT might exert its effect in part by directly modulating NSC and neural progenitor cells in the hippocampus. To further study TBI-induced dysregulation of neurogenesis, I used a rapid stretch injury device to reproduce *in vitro* the effect of mechanical stress, such as the one that occurs during a TBI, on cultured hippocampal NSC. Interestingly, the expression of the same miRNA upregulated by TBI in laser-captured SGZ and TBI-derived hippocampal NSC was significantly increased in NSC subjected to rapid stretch injury. Moreover, rapid stretch injury reduced NSC proliferation and reduced their neuronal and glial differentiation. Inhibition of Piezo1, a mechanoreceptor known to regulate neurogenesis, prevented stretch injury-induced miRNA upregulation, reduced proliferation and increased neuronal differentiation of hippocampal NSC. In conclusion, this work support the use of NPLT for the treatment of TBI-induced dysregulation of hippocampal neurogenesis and suggest that activation of mechanoreceptors is, at least in part, mediating TBI-induced alterations of regulatory miRNA and reduced NSC proliferation and differentiation.

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List of Abbreviations

AD: Alzheimer's disease

ANOVA: Analysis of Variance

BrdU: Bromodeoxyuridine

Ctrl: Control

DCX: Doublecortin

DG: Dentate Gyrus

DMEM: Dulbecco's modified Eagle's medium

EdU: 5-ethynyl-2'-deoxyuridine

FBS: Fetal Bovine Serum

FGF: Fibroblasts Growth Factor

FPI: Fluid Percussion Injury

GABA: γ-aminobutyric acid

GFAP: Glial Fibrillary Acidic Protein

GSBS: Graduate School of Biomedical Science

Hipp: Hippocampus

I.p.: intraperitoneal

IBA1: calcium-binding adaptor molecule 1

IF: Immunofluorescence

LCM: Laser Capture Microdissection

LDH: Lactate dehydrogenase

miRNA: micro RNA

NIL: Near Infrared Light

NPLT: Nano-pulsed laser therapy

NSA: Neuroscience Associates

NSC: Neural Stem Cell

PBS: Phosphate Base Saline

qRT-PCR: Quantitative Real-Time PCR

SGZ: Subgranular Zone

SVZ: Subventricular Zone

TBI: Traumatic Brain Injury

TDC: Thesis and Dissertation Coordinator

US: Ultrasound

UTMB: University of Texas Medical Branch

WB: Western Blotting

CHAPTER 1: BACKGROUND

Adult neurogenesis

Neurogenesis, the physiological process by which new neurons are generated from neural stem cells (NSC), has been demonstrated to occur throughout life in the mammalian brain (Amrein & Lipp, 2009; Kempermann *et al.*, 2015). It peaks during childhood and progressively declines with aging (Galvan & Jin, 2007; Apple *et al.*, 2017). The discovery of neurogenesis in the adult brain has put an end to the central dogma that, until 1990, had dominated the field of neurobiology (Colucci-D'Amato *et al.*, 2006). Particularly, it was believed that, after development, the brain was unable to generate new neurons (Stahnisch & Nitsch, 2002). 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" (Bizzorero, 1893; Ramon y cajal, 1914).

Neurogenesis in the adult rat brain was reported for the first time in the '60 by Joseph Altman thanks to the use of radioactive thymidyne (H^3) (Altman & Das, 1965) (Figure 1). This technique allows to mark only proliferating cells because the radioactive thymidine is incorporated into newly synthesized DNA by cells in the S phase of the cell cycle and detected by autoradiography. 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.

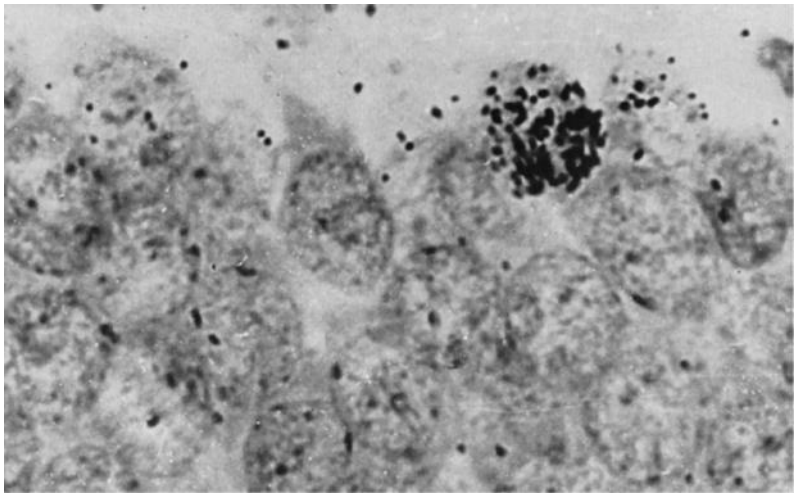

Figure 1.1 Joseph Altman autoradiogram.
Autoradiogram of labeled granule cells of hippocampus dentate gyrus of adult rat two weeks after intraperitoneal injection of H3 thymidine made by Joseph Altman in 1963. (Adapted from Altman et al., 1936).

Michael Kaplan in the ’80 gave a more definite demonstration of the neurogenesis process in mice. Specifically, particularly By using electron microscopy combined with tritiated thymidine (H^3), he showed formation of neurites and synapses of newborn cells with the evidence of neuronal formation in the olfactory bulb of mice, an area in which neurogenesis occurs recurrently (Kaplan *et al.*, 1985). However, even his work was not considered the ultimate evidence of adult neurogenesis for the absence of specific neuronal and glial markers.

Only in the ’90 the persistence of neurogenesis in the adult mammalian brain was accepted by the scientific community thanks to the work carried out by various research

groups in Europe and in the United States that, thanks to the use of specific neuronal markers, unequivocally demonstrated that it was possible to culture NSC isolated from the adult brain and, most importantly, that these NSC could generate functional neurons (Alvarez-Buylla *et al.*, 1990; Goldman *et al.*, 1992). Moreover, in the same decade 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 the dentate gyrus (DG) of the hippocampus (Alvarez-Buylla *et al.*, 1990; Reznikov, 1991). These 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 (Amrein & Lipp, 2009; Van Schepdael *et al.*, 2013).

Adult neurogenesis in the human brain was demonstrated in 1998 by Fred Gage and his collaborators with the bromodeoxyuridine (BrdU) assay (Eriksson *et al.*, 1998). This technique is still currently used to analyze proliferating cells, such as NSC in the hippocampus, because BrdU, a thymidine analogue that is incorporated in the newly synthesized DNA, can be detected using specific antibodies (Kyryachenko *et al.*, 2012) (Figure 1.2)

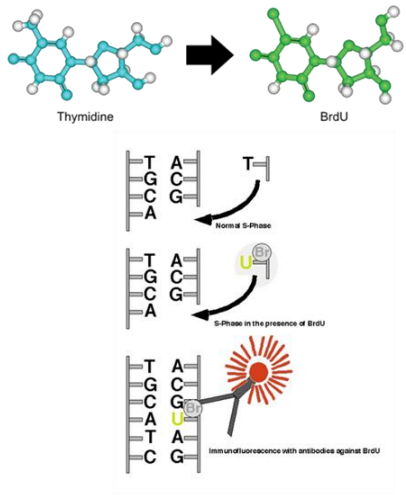

Figure 1.2 The 5-bromo-2'-deoxyuridine (BrdU) cell proliferation assay.
BrdU is a thymidine analogue that is incorporated in the newly synthesized DNA. BrdU can be detected by using specific antibodies anti-BrdU for the detection of the proliferating cells.

Neurogenesis in the adult hippocampus

The hippocampus is a key structure of the brain that plays a pivotal role in memory and cognition. More than 9000 newborn cells are produced every day in rodents and 1.75% is the estimated annual turnover of cells in the human hippocampus, showing that the role of neurogenesis in human hippocampus is still under investigation (Christian *et al.*, 2014).

The hippocampus takes the name from its shape that resembles a seahorse (form the Greek word *hippos* (horse) *kampos* (monster) (Huijgen & Samson, 2015). This structure belongs to the limbic system (from the Latin *limbus*: border, because it is formed by areas and interacting structures of the brain that defines the edge of the cortex), it and is one per hemisphere it plays an important role in memory formation and in the

consolidation of short-term memory into long-term memory (Tatu & Vuillier, 2014). In addition, the hippocampus is the main brain structure responsible for the so called "spatial memory" that is fundamental for orientation (Shrager *et al.*, 2007). These hippocampus-dependent functions are lost after brain damage caused by an external force, such as traumatic brain injury, or by the onset of neurodegenerative diseases, such as Alzheimer's disease, and are extensively studied in various behavioral tests to assess neuronal injury in animal models (Atkins, 2011; Vivar, 2015).

The hippocampus is divided in: **C**ornu **A**mmonis (CA1, CA2, CA3, CA4) and the dentate gyrus (DG) (Piatti *et al.*, 2013) (Figure 1.3). 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. Finally, the DG receive information from layer II of the entorhinal cortex, where episodic memories are formed, thanks to the presence of the granule cells. The DG is composed of three layers: the molecular, the granular and the polymorphic layers. The granule cells forming the granular layer are responsible for the excitatory signals that are further transported by interneurons and pyramidal cells to CA3 (Jonas & Lisman, 2014; Cho *et al.*, 2015; Huijgen & Samson, 2015).

Recently, a different regional function of the hippocampus DG has been shown (Kheirbek & Hen, 2011). Specifically, the ventral DG is responsible for learning and spatial memory while the dorsal region controls stress and emotion and is the area responsible for fear conditioning and is in strict relationship with the amygdala (Fanselow & Dong, 2010).

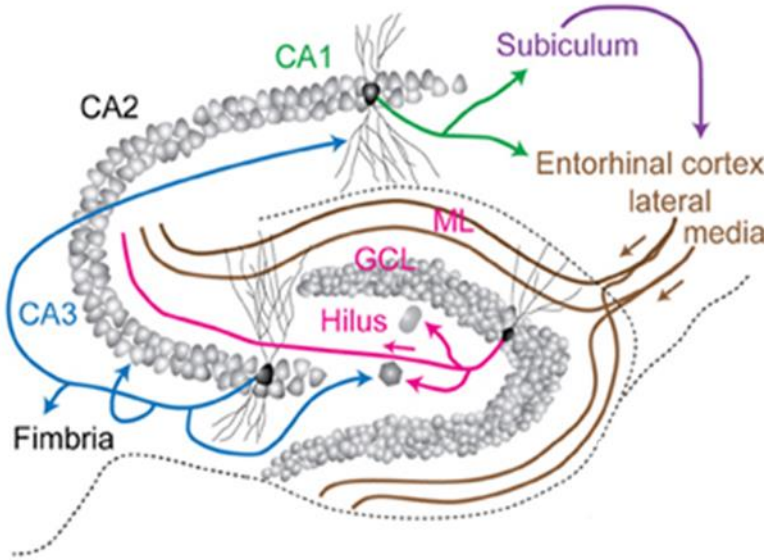

Figure 1.3. Schematic representation of the rat hippocampus.
The excitatory axonal projections are represented with the arrows. These axons carry the signals from different layers to downstream targets. CA: Cornu Ammonis; GCL: Granular cell layer; ML: Molecular layer. (Adapted from Piatti et al., 2013).

Neurogenesis in the hippocampus is a multistep process through which NSC mature into mature cells such as neurons and glia. Each step can be identified by cell morphology and with the expression of specific markers (Figure 1.4) (von Bohlen und Halbach, 2007). Specifically, in the SGZ of the DG there are restricted areas known as "niche" formed by different cell type that create a microenvironment in which NSC cells rarely proliferate. These cells are classified as type I that express nestin, glial fibrillary acidic protein (GFAP) and the transcription factor Sox-2. These cells come from the pool of radial glial like

precursor cells that are actively proliferating during development and that slow down to a quiescent state in the adult brain. Type I cells induced to enter the cell-cycle become type IIa cells (glial precursor cells) that maintain the expression of nestin, GFAP and Sox2. Type IIa starts to differentiate and become dividing neural precursor cells type IIb in which the expression of GFAP is lost and doublecortin (DCX) starts to be expressed in addition to Nestin and Sox2. This critical step is subject to a physiological regulation mechanism through which not all the new born cells proceed to the further differentiation stages but undergoes to programmed cell death (Ryu *et al.*, 2016). The survived Type IIb cells exit the cell cycle and start to migrate into the granular layer becoming type III cells (neuroblast) in which nestin and Sox2 markers are lost and DCX is maintained. Finally, neuroblasts continue to migrate and undergo the final neuronal maturation stage in which the newborn neurons of the DG, called type IV or granular neurons, become fully functional progressively losing the expression of DCX and expressing the nuclear neuronal marker NeuN (Farioli-Vecchioli *et al.*, 2008; Jonas & Lisman, 2014; Kempermann *et al.*, 2015; Koehl, 2015). The entire maturation process differs between species. In rodents takes 4-6 weeks while in primates (macaque) has been shown be much slower (Chapman & Diaz-Arrastia, 2014) (Charvet & Finlay, 2018). Specifically, only after 6 months (28 weeks) new neurons finish the maturation. In human the duration of this maturation process is still debated but it is known that it progressively decreases with aging (Apple *et al.*, 2017; Kempermann *et al.*, 2018). All this complex process is tightly regulated and highly dynamic and can be modified by several factors such as lifestyle, age or pathological conditions (Chastin et al., 2012; Jonas and Lisman, 2014; Yau et al., 2014; Hueston et al., 2017).

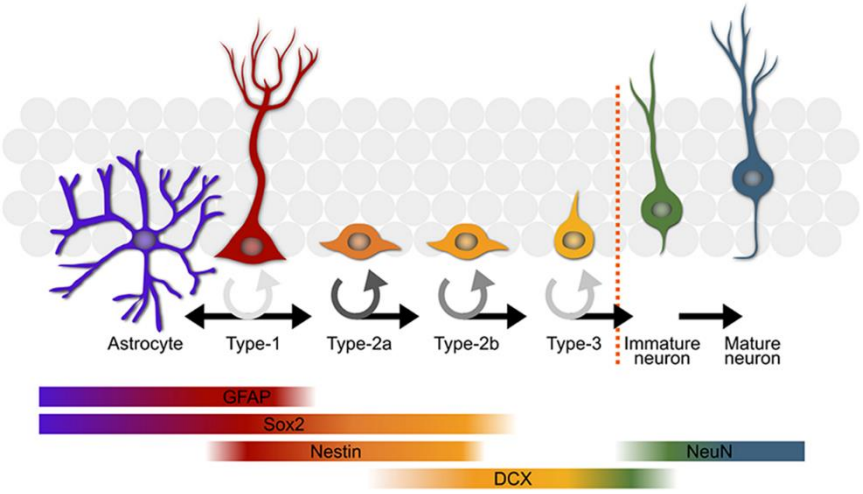

Figure 1.4. Schematic representation of hippocampal neurogenesis in the subgranular zone (SGZ) of the dentate gyrus (DG).
Each step of the differentiation process is identified by transient expression of specific markers.

Regulation of neurogenesis in the hippocampus

In the last 30 years neuroscientists have been studying neurogenesis and in particular they studied the factors that control neurogenesis. The first evidence of neurogenesis as a regulated process was made by Nottebohm in song birds (Nottebohm, 1989). 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 (Alvarez-Buylla *et al.*, 1990). Neurogenesis is influenced by intrinsic and extrinsic factors that determine the fate of the NSC (Aimone *et al.*, 2014). These factors can be modified by lifestyle, age and the presence of pathological conditions (Kempermann, 2015). Several evidence in the literature have shown the positive effects of volunteer physical exercise and the exposure to an enriched environment on

increasing neurogenesis (Kempermann *et al.*, 1997; Fabel *et al.*, 2009). Specifically, increased neurogenesis has been reported in rodents (rats and mice) with an increased social interaction and housed in enriched environments with toys, tunnels and wheels (Monteiro *et al.*, 2014). This increased neurogenesis/survival of neuronal precursor cells correlates with improved cognitive function (Costa *et al.*, 2015). On the other hand, neurogenesis declines with aging and is decreased by the exposure to chronic stress leading to impaired memory and cognitive functions (Figure 1.5) (Zhao *et al.*, 2008; Apple *et al.*, 2017).

Physical Activity			
Voluntary running	Increased proliferation	Improved learning and memory	van Praag et al., 1999
Hindlimb suspension	Decreased proliferation	Not available	Yasuhara et al., 2007
Enriched Environment (EE)			
Exposure to EE	Increased survival	Improved learning and memory	Kempermann and Gage, 1997
presenilin-1$^{-/-}$	Attenuation of the induction of neurogenesis by EE	Reduced memory clearance induced by postlearning EE	Feng et al., 2001
Stress[b]			
Psychosocial stress	Decreased proliferation	Impaired spatial memory	Ohl et al., 1999
Unexpected chronic mild stress	Decreased neurogenesis	No simple correlation	Minuer et al., 2007
Aging			
Aged rats	Decreased proliferation and neurogenesis	Correlated impairment in learning or memory	Drapeau et al., 2003; Driscoll et al., 2006
Aged rats	Decreased proliferation and neurogenesis	Not correlated to cognition	Merrill et al., 2003; Bizon et al., 2004
Aged animals in EE	Increased neurogenesis[c]	Improved learning and memory	Kempermann et al., 1998
Aged animals with running	Increased neurogenesis[c]	Improved learning and memory	van Praag et al., 2005

Figure 1.5. Genetic and environment regulation of adult neurogenesis.
Physical activity and the exposure to an enriched environment has beneficial effect of for neurogenesis and cognition while stress and aging decreases neurogenesis and impairs cognitive function. (Adapted from Zhao et al., 2008).

In particular, excessive stress increases the release of glucocorticoids, important class of steroid hormones that negatively affects neurogenesis (Lucassen *et al.*, 2015). Glucocorticoid receptors are abundant in the hippocampus and are fundamental for maintaining metabolic balance and gluconeogenesis (Kuo *et al.*, 2015). An acute and transitory increase of glucocorticoids, such as after physical activity, has beneficial effect and induce neurogenesis, ameliorating cognitive functions (Chen *et al.*, 2017). On the other hand, chronic increase in glucocorticoids and their receptors (i.e. during aging) reduce hippocampal plasticity in rodents (Landfield *et al.*, 2007; Wosiski-Kuhn *et al.*, 2014).

Recent reports in the literature highlighted the importance of the balance between quiescent and proliferating NSC (Morizur *et al.*, 2018). Indeed, until a few years ago, it was believed that decreased neurogenesis during aging was caused by a depletion of the pool of NSC but recently has been shown that the reduction of neurogenesis is caused by the unbalance toward an excessive quiescent state of the NSC in the aged brain (Song *et al.*, 2012; Ziebell *et al.*, 2018) (Figure 1.6). A prolonged quiescent state leads to fewer differentiating cells resulting in reduced neuronal formation and plasticity (Ninkovic *et al.*, 2018). On the other hand an excessive proliferation leads to depletion of stem cells (Chen *et al.*, 2013). The microenvironment formed by the niche plays a fundamental role in maintaining this equilibrium through the expression of different transcription factors such as TLX, NFIX and CcnD2 (UrbÃ¡n & Guillemot, 2014). Recent studies highlighted the key role of achaete-scute homolog 1 (Ascl1), a transcription factor that controls cell-cycle genes and regulates the proliferation of the NSC in the adult brain (Sueda *et al.*, 2019). Specifically, Ascl1 is mainly expressed in type IIa cells in the hippocampus and although the control mechanism remains to be clarified, Ascl1 is known to be the transcription factor

responsible of the switch between quiescent and proliferating NSC (Kim *et al.*, 2011; UrbÃ¡n & Guillemot, 2014).

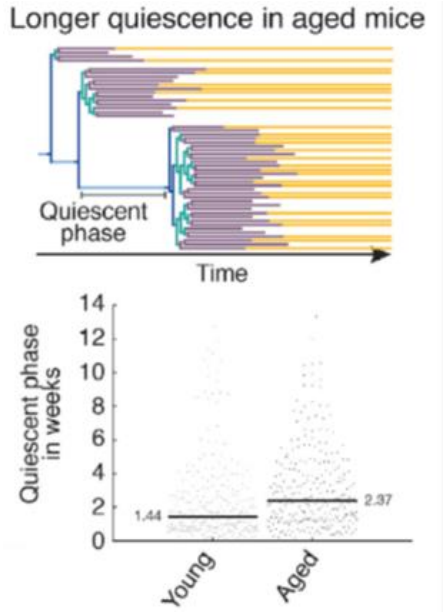

Figure 1.6. Increased neural stem cells quiescence with aging.
Graphical representation of the mathematical model showing the increased quiescent state on the neural stem cells during aging (From Ninkovic et al., 2018)

Microglia and macrophages are among the most active secreting cells and are the main source of cytokines and chemokines present in the hippocampus DG that regulate neurogenesis (Ribeiro Xavier *et al.*, 2015). These primary immune resident cells can be activated by external agents, such as pathogens, or condition of neuronal damage, such as Alzheimer's disease or TBI, in which the damaged cells are cleared by phagocytosis (Loane & Kumar, 2016; Minett *et al.*, 2016). Activated microglia can release both pro and anti-inflammatory factors that regulate neurogenesis (De Lucia *et al.*, 2016). Moreover, the DG is one of the most vascularized brain areas and is subject to different factors released

in the blood stream that can reach the hippocampus and affect neurogenesis (Tatu & Vuillier, 2014).

NSC activity is also regulated by surrounding neurons. GABA (γ-aminobutyric acid) is among the key factors known to regulate hippocampal neurogenesis (Sibbe & Kulik, 2017). Two important aspects are influenced by GABA: NSC maintenance and differentiation of newborn neurons. Specifically, NSC, as immature cells, cannot make synaptic connections with GABAergic interneurons but has been shown that are influenced by a GABAergic tonic activation (Bao *et al.*, 2017). Indeed, GABA depletion in the hippocampus has been shown to enhance the proliferation of the quiescent NSC causing the exhaustion of the NSC pool (Ge *et al.*, 2007; Giachino *et al.*, 2014). On the other hand, during the differentiation, neural progenitor cells (DCX positive) are maintained in a hyperpolarized state until neurons reach the mature stage (NeuN positive) (Faigle & Song, 2013). Several diseases are characterized by the disruption of GABA signals that induces a depolarizing state and hyper-excitability on these neurons with devastating effects (Ting Wong *et al.*, 2003). Epilepsy and TBI are among the most relevant diseases in which there is a dysregulation of GABA signals (Treiman, 2001; Guerriero *et al.*, 2015). Particularly, epilepsy is one of the most common outcomes after brain trauma that seems to be caused by the loss of GABAergic interneurons in the hippocampus hilus (Christensen, 2012).

All these extrinsic factors have an important effect on NSC gene expression. In the last decade several reports have shown a critical role for gene expression in neurogenesis played by micro-RNAs (miRNA) (Wakabayashi *et al.*, 2014). miRNA are small single-stranded non coding RNA (20-22 nucleotides) that are able to bind mRNA in a sequence-specific manner. Pri-miRNA are 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 1.7) (Ha & Kim, 2014). For this reason miRNA constitutes the most important molecules for post-transcriptional regulation in almost all biological processes and are among the most important players in neurogenesis (Stappert *et al.*, 2018). Recently the role of miRNA in regulating NSC differentiation has been studied. In particular, miR9, miR25, miR29, miR124 and miR137 have been shown to be directly involved in the regulation of NSC proliferation and fate specification (Figure 1.8) (Bielefeld *et al.*, 2017).

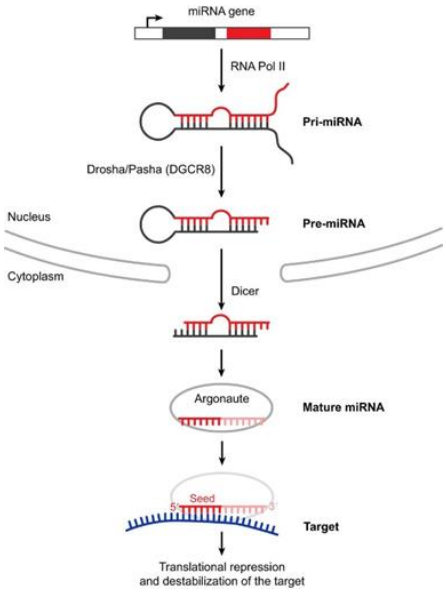

Figure 1.7. miRNA biogenesis and mechanism of targenting of mRNA.
miRNA are non-coding RNA (20-22 nucleotides) that bind mRNA in a sequence-specific manner. Pri-miRNA are synthesized by RNA polymerase II and then processed in pre-miRNA by Pasha/Drosha and further cleaved by DICER in the cytoplasm. The mature miRNA binds to the target mRNA thanks to RNA-induced silencing complex (RISC) silencing gene expression (From Alberti et al., 2017).

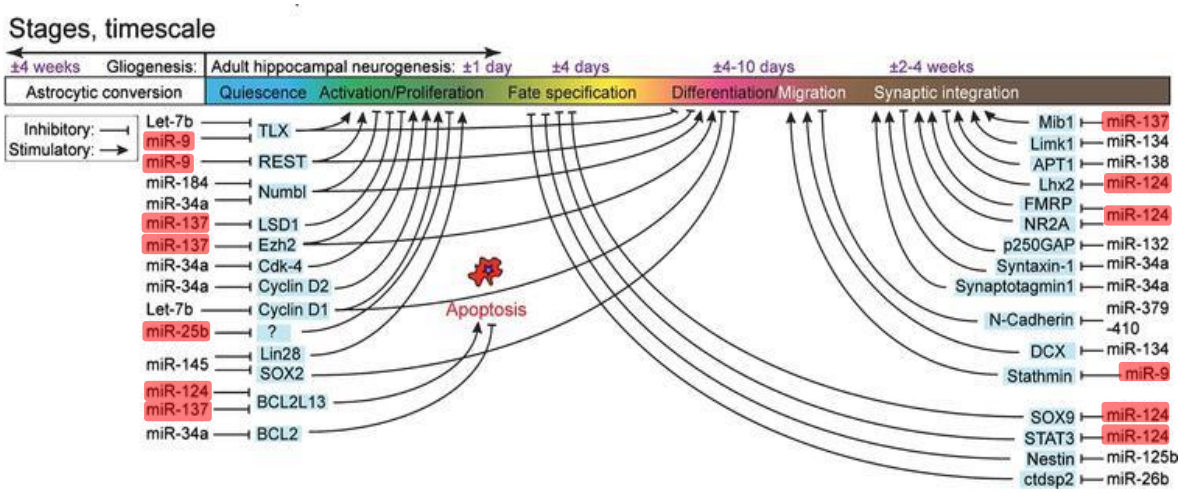

Figure 1.8. miRNA regulation of neurogenesis.
We analyzed the expression of miR9, miR25, miR29, miR124 and miR137 known to regulate both proliferation and differentiation of neural stem cells in the hippocampus. (Adapted from Pascal et al., 2017).

MiR9, is an evolutionary conserved miRNA that has several targets such as the nuclear orphan receptor TLX also known as NR2E1 (Coolen *et al.*, 2012). TLX is one of the most important factors for NSC proliferation and, recently, has been demonstrated to regulate also the differentiation (Anand & Radhakrishnan, 2016). Specifically, TLX plays a pivotal role on the switch from glial to neuronal pathway. Moreover, TLX promotes the terminal differentiation and the migration of new born neurons in the pre-existing circuits of the hippocampus (Anand & Radhakrishnan, 2016). MiR25, is important for maintaining NSC self-renewing property and is involved in insulin/insulin-like growth factor-1 (IGF) signaling (Brett *et al.*, 2011). Interestingly, the regulatory region of miR25 is bound by FoxO3 a member of the FoxO family important for maintaining neural stem cells (Kim *et al.*, 2015).

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) (Shin *et al.*, 2014). miR124, with miR9, is the most abundantly expressed miRNA in the brain. Is one of the most important miRNA for neurogenesis regulation because has hundreds of targets (Cheng *et al.*, 2009). In particular, miR124 is involved in both stemness maintenance and neuronal differentiation (Jiao *et al.*, 2017). The role of this miRNA is still debated because its functions is influenced by the environment and the condition in which is expressed. Indeed, for many years it was believed that the overexpression of miR124 has an anti-inflammatory effect and be beneficial but recently has been shown a pro-inflammatory activity in animal model of epilepsy confirming a context-dependent effect of this miRNA (Gaudet *et al.*, 2018). Not only the environment is critical but even the location plays a key role for the overall effect induced by miR124 (Higuchi *et al.*, 2016). In particular, miR124 is downregulated in the hippocampus after TBI but is significantly unregulated only in the hippocampus DG (Vuokila *et al.*, 2018). Beyond these specific aspects, what is confirmed about miR124 is that it controls proteins involved in chromatin rearrangements, such as DNA methylation, that allows the expression of genes for neuronal differentiation (Neo *et al.*, 2014). miR124 is overexpressed in self-renewing cells and inhibits their differentiation blocking MEK/ERK pathway (Wei *et al.*, 2017). On the other hand miR124 is downregulated in differentiating and migrating NSC (Jiao *et al.*, 2017). Moreover, miR124 is under the control of REST, one of the most important neurodevelopment regulators (Visvanathan *et al.*, 2007). Another critical aspect is the interaction between miR124 and miR137 for the entire neurogenesis process (Santos *et al.*, 2016). miR137 has been

demonstrated to be linked with schizophrenia and is the miRNA that has the largest number common target with miR124 (Curtis & Emmett, 2018). Similar to miR124, miR137 upregulation maintains an undifferentiated state of NSC while is reduced during the differentiation (Mahmoudi & Cairns, 2017).

In addition to miRNA, ion channels, such as mechanoreceptors, have been reported to play a significant role in the control of neurogenesis (Gasparski & Beningo, 2015). 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 in neurogenesis (Figure 1.9) (Ranade *et al.*, 2015). Piezo1 is one of the most studied mechanoreceptors that regulates neurogenesis and act as sensor of stiffness of the surrounding area (Koser *et al.*, 2016). Recent studies have shown the important role played by the physical properties of the niche in cell fate determination (Pathak *et al.*, 2014). Specifically, *in vivo* the increase of brain stiffness, such as during aging, decreases neuronal differentiation (Arani *et al.*, 2015). This reduced differentiation is an additional factor leading to a decreased neurogenesis during aging with a significant impact in memory and cognitive function (Rammensee *et al.*, 2017).

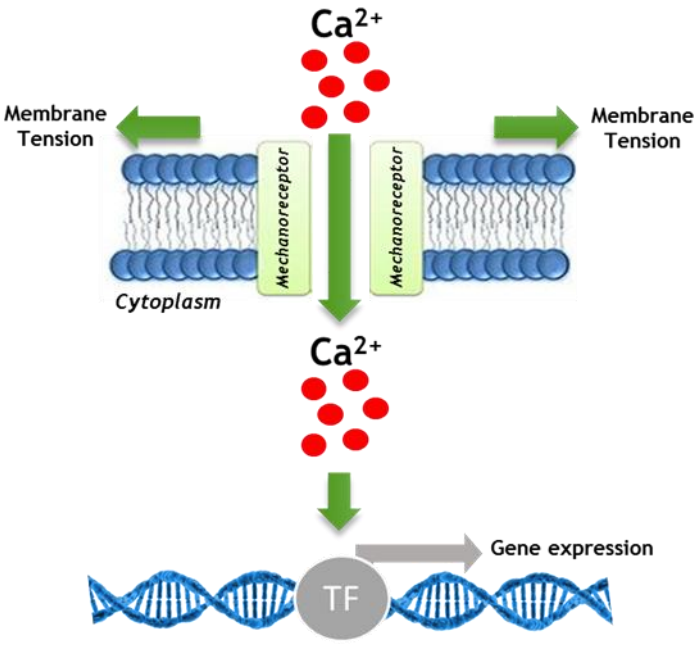

Figure 1.9. Schematic representation of mechanoreceptor regulation mechanism. Mechanoreceptors are mechanosensitive ion channels that are activated by mechanical changes in the environment causing an increase of intracellular Ca2+ affecting the expression of genes involved in neurogenesis. TF: Transcription factor.

TBI and neurogenesis

In addition to normal physiological processes, pathological conditions such as Alzheimer's disease and traumatic brain injury (TBI) are also known to affect neurogenesis (Vivar, 2015; Wang, Gao, *et al.*, 2016). TBI is one of the most important cause of brain dysfunction caused by an external force (Pervez *et al.*, 2018). More than 10 million TBI cases reported each year worldwide with an incredible cost for the health care that is over 50 billion dollars only in the United States (Gardner & Yaffe, 2015). TBI is mostly studied in contact sports players and soldiers that show an incredible increased risk for the development of pathological condition caused by their lifestyle (Zuckerman *et al.*, 2015).

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 (Swanson *et al.*, 2017). This wide range is caused by a difficult diagnosis of TBI especially years after the initial injury. Moreover, some of the symptoms related to TBI disappear with time and for this reason are often underestimated by people who believe they have completely recovered (Carroll *et al.*, 2012). The incidence of this pathology is further increased by accidental falls, car accidents and sport related brain injuries estimated in 1.6-3.8 million per year only in the United States (Zuckerman *et al.*, 2015).

TBI is classified, based on severity, with a score identified by the Glasgow coma scale (Hawryluk & Manley, 2015). This scale goes from 3 to 15 in which the lower is the score the worst is the injury. Three main functions are scored: motor response, verbal response and eye opening. The total score is calculated by adding the single scores of the main functions. A mild-TBI is scored 13-15, a moderate 9-12 and severe 3-8. Despite the presence of this scale, it is not sufficient to characterize TBI due to the complexity of this event for the way in which occurs, the condition and the secondary event that can trigger (Bledsoe *et al.*, 2015). What is known is that TBI is an important risk factor for neurodegenerative disorders such as AD and PD and for epilepsy (Christensen, 2012; DeKosky & Asken, 2017). The relationship between TBI and neurodegenerative disorders is still controversial but data in the literature show an increased phosphorylated Tau, an increased Aβ deposition and increased dementia after TBI thus suggesting that not only TBI increases the probability to develop neurodegenerative disorders but most importantly it seems to anticipate the onset of these pathologies (Cruz-Haces *et al.*, 2017; Ramos-

Cejudo *et al.*, 2018). Taken together, all these factors have contributed to the challenge of finding a cure or a treatment for TBI that, as of today, remains an unmet need.

Memory and cognitive deficits are the most important clinical manifestations among the numerous impairments caused by TBI (Mckee & Daneshvar, 2015). 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. All these studies have shown evidence of neuronal loss in different brain areas such as in the hippocampus DG and an acute increased neurogenesis within days after TBI (Wang, Gao, *et al.*, 2016; Yu *et al.*, 2016). In particular, it has been demonstrated both increased proliferation of NSC and increased presence of neuronal precursor cells, which suggest an attempt of the brain to recover after brain trauma (ROLA *et al.*, 2006). However, the rapid proliferation of NSC induced by the injury can lead to the exhaustion of the pool of stem cells, reducing the ability of the brain to recovery (Ngwenya & Danzer, 2019). This injury-induced increased proliferation is transient and is reduced 1-2 weeks after TBI (Neuberger *et al.*, 2017). Moreover, the differentiation of precursor cells has been shown to occur in a displaced and aberrant way, particularly in the hilar region of the hippocampus and within the granular layer (Ibrahim *et al.*, 2016; Shapiro, 2017). These TBI-induced changes are hallmarks of epileptogenesis (Cho *et al.*, 2015). Indeed, neuronal precursor cells that migrate in a not proper way are among the leading cause of post traumatic epilepsy (PTE), a chronic condition that persists even years from the original trauma (Verellen & Cavazos, 2010). Find a cure for this condition is extremely important because antiepileptic drugs, that are the first treatment to reduce seizures in the acute phase after TBI, are not effective (Zimmermann *et al.*, 2017). This is explained by the nature of seizures, indeed in the acute phase immediately after TBI

there is an injury-induced metabolic dysregulation that causes seizures, while in the chronic phase there is the aberrant migration of neuronal precursor cells that is one of the causes of PTE (Chandel *et al.*, 2015). In addition, after TBI there is loss of interneurons in the hilus of the hippocampus causing a deficiency of GABAergic signal leading to neuronal hyper-excitability (Guerriero *et al.*, 2015; Thodeson *et al.*, 2018) (Figure 1.10).

In conclusion, 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. 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 NSC.

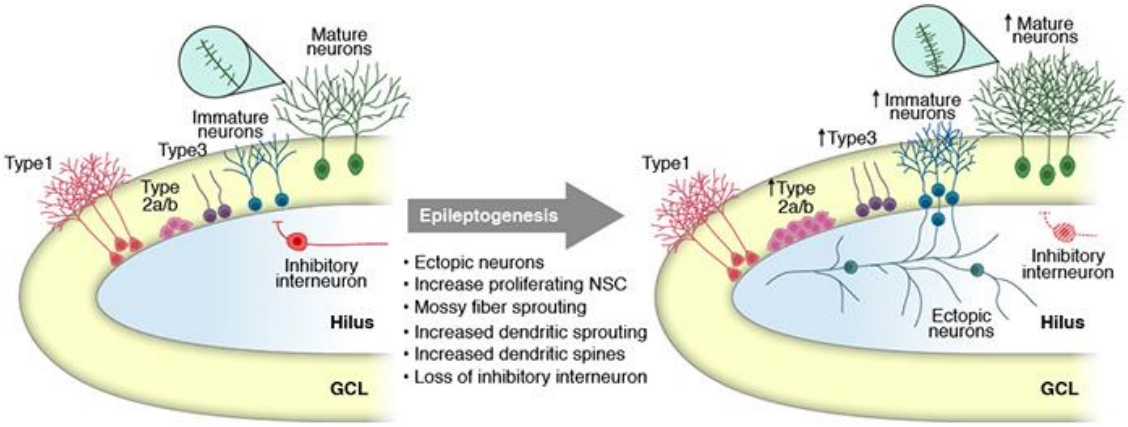

Figure 1.10. Schematic representation of epileptogenesis in the hippocampus dentate gyrus.
Some of the key changes that lead to epilepsy are analogously induced by brain trauma. GCL: Granular cell layer. (From Thodeson et al., 2018).

Non-invasive treatments for TBI

Studying a possible treatment for TBI is challenging due to its nature. TBI has multiple outcomes with a broad variety of damages based on different factors such as sex, age, presence of previous pathology, cause of the injury and intensity (Laskowski *et al.*, 2015). All these factors generate a cascade of secondary events extremely variable that make difficult to find a cure for TBI.

These secondary events lead to 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 (Joseph *et al.*, 2015). Secondary events, in contrast to the primary injury, may be preventable and reversible (Corps *et al.*, 2015). For this reason TBI research is focused on both understanding the molecular events trigged by the primary injury and developing treatments to inhibit or reduce the onset and severity of secondary events.

Recently, non-invasive therapies (delivered transcranially) such as magnetic stimulation, near infrared laser therapy (NIL) (800-1000nm) and ultrasound waves (US) (0.44–0.67 MHz) have received increased interest for their properties: they can spread in the injured area and can be delivered directly to the affected region (Dhaliwal *et al.*, 2015).

Specifically, magnetic stimulation has been demonstrated to be beneficial to the cognitive function due to its ability to induce positive structural changes enhancing neuronal plasticity in the affected cortical areas and deeper areas of the brain such as the hippocampus (Lu *et al.*, 2015).

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 (Naeser *et al.*,

2011). NIL is characterized by well absorption from biological fluids and tissues with an energy that does not cause heating and induce photobiomodulation. It has been reported in the literature that transcranially administration of NIL exerts neuroprotective effects in animal model of TBI, stimulate neurogenesis in mice and produce beneficial cognitive and emotional effects in humans (Oron *et al.*, 2012; Quirk *et al.*, 2012; Henderson & Morries, 2015). 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 (Naeser *et al.*, 2011).

One of the mechanisms by which NIL produces beneficial effects is improving mitochondrial activity (Yu *et al.*, 2015). Mitochondria are ancestral organelles present in the eukaryotic cells that are responsible of ATP production through respiratory chain (Letts & Sazanov, 2017). 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.

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. In addition to this mitochondrial regulating mechanism, NIL can modulate the production of reactive oxygen species (ROS), mitochondrial DNA replication and grow factor expression.

Unfortunately, the big limitation of NIL application is its penetration into the human brain (around 2.5-3 cm) and for this reason is able to stimulate only the superficial cortical layers. Ultrasound waves (US) are commonly used for diagnostic imaging and can be set at different wavelengths. Some of these wavelengths, in the range of low-frequencies (0.44–0.67 MHz), have been shown to stimulate brain circuits, be neuroprotective and reduce edema formation while increasing the permeability of blood vessels (Tufail *et al.*, 2010; Yoon *et al.*, 2012). Moreover, US can induce the production of important neurotrophins such as brain-derived neurotrophic factor (BDNF) (Yang *et al.*, 2015).

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 (Shohayeb *et al.*, 2018). 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 (Wurzelmann *et al.*, 2017).

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 (Yang *et al.*, 2015). 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 (Minnich *et al.*, 2010; Salehi *et al.*, 2017). 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.

Beside the bio-effectiveness the most important advantage of US waves is the 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.

Nano-pulsed laser therapy

Non-invasive therapies seem to be the most promising treatment preventing the secondary brain insults after TBI. Recent evidence in literature showed the beneficial effects in cognitive function and neuroprotection of the different therapies after TBI (Demirtas-Tatlidede *et al.*, 2012; Koski *et al.*, 2015). Here we are testing the effect of a novel approach that combines two different therapies, NIL and US, in a single one. In particular, Dr. Esenaliev and his group in the center for Laboratory for Optical Sensing and Monitoring 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 1.11) (Petrov *et al.*, 2012, 2014).

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. 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 (Esenaliev *et al.*, 1993). The accumulated energy and pressure is released as low intensities ultrasound waves.

Figure 1.11. Nano-pulsed laser therapy device.
Our system consists of an optical parametric oscillator that generates short (10 ns) pulses of near-infrared light (808 nm) at energy of up to 15 mJ and pulse repetition rate of 20 Hz. These pulses generate low-level optoacoustic waves that travel deep into the brain.

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) (Petrov *et al.*, 2005).

AIM OF THE PROJECT

The goals of this work were to study *in vivo* and *in vitro* TBI-induced dysregulation of neurogenesis and to test the therapeutic potential of NPLT to correct it.

I pursued two specific aims

SPECIFIC AIM 1: TO DETERMINE THE EFFECT OF NPLT ON NEUROGENESIS IN A RAT MODEL OF TBI. In this aim adult rats were subjected to fluid percussion injury (a model of TBI) and treated 1 hour post-injury, with NPLT. Four groups of rats were studied: Sham, Sham+NPLT, TBI and TBI+NPLT. In order to observe the proliferation of NSC in the DG of the hippocampus, the rats were treated with bromodeoxyuridine (BrdU), a thymidyne analogue. One day and 2 weeks after injury, the brains were collected, sectioned and stained for BrdU, to assess the number of proliferating cells, doublecortin (DCX) and NeuN (neuronal marker) to evaluate the number of neural progenitor cells and mature neurons. Moreover, total microglia and activated microglia were quantified on the sections stained for calcium-binding adaptor molecule 1 (Iba-1) and Cluster of differentiation 68 (CD68) respectively. Furthermore, DG were isolated using laser capture microdissection and used for RNA extraction in order to evaluate the expression profile of select regulatory microRNAs (miRNA).

In addition I performed *in vitro* studies of proliferation, differentiation and miRNA expression, to determine a possible direct effect of NPLT treatment on NSC isolated from the hippocampus of both naïve and TBI rats.

SPECIFIC AIM 2: TO DETERMINE THE EFFECT OF MECHANICAL STRETCH INJURY ON HIPPOCAMPAL NSC *IN VITRO*. With this aim I specifically assess the effect of mechanical stretch directly on naïve NSC and analyzed possible correlations with TBI. Previous studies have shown the efficacy of the rapid stretch injury device on mature neurons to see the effects of axonal injury, a common situation after TBI that cause neuronal death. I hypothesize that some of the molecular injury-induced changes in NSC that are caused by mechanical stress affect their proliferation and differentiation potential. Naïve NSC were stretch-injured using a single boost of nitrogen at 50 and 30 PSI to test the effect of mechanical stretch directly on NSC and to test whether mechano-induced changes on NSC are replicable *in vitro* with this system. Proliferation (EdU), differentiation (immunofluorescence and western blotting) and miRNA expression (qRT-PCR) were analyzed at various time points after injury. Moreover, in order to test a possible role in mechano-transduction signal played by Piezo1, one of the most studied mechanoreceptors that influence neurogenesis, I used GsMTx4, a peptide isolated from tarantula venom, known to inhibit Piezo1.

CHAPTER 2: THE EFFECT OF NPLT ON NEUROGENESIS IN A RAT MODEL OF TBI

Introduction

Hippocampal neurogenesis is a key event for memory and cognition that is possible thanks to the presence of NSC (Opitz, 2014). The main two characteristics of NSC are their ability to self-renew and, most importantly, their ability to differentiate in multiple brain cell lineage (Kennea & Mehmet, 2002). The latter is a crucial aspect for brain damage recovery following TBI. In fact, although proliferation is fundamental for maintaining the pool of the NSC, differentiation is the process through which the formation of mature cells, such as neurons and glia, occur (Fuchs & Flügge, 2014).

It has been demonstrated that after TBI the repair process takes place and leads to an acute increase of NSC (Yu *et al.*, 2016). However, in order to have a recovery after injury is necessary that quiescent NSC migrate from their niche to the injured area where they develop in mature neurons but this resettlement following TBI is aberrant (Cho *et al.*, 2015; Shapiro, 2017).

Previous data obtained in our laboratory have demonstrated neuroprotective effect on NPLT 10 days after blast injury (Esenaliev *et al.*, 2018). With this aim I first validated the effectiveness of NPLT *in vivo* in a different model of TBI (fluid percussion injury) and analyzed the effect of the treatment on NSC proliferation 24 and two weeks after injury. Moreover, I analyzed the effect of NPLT on the differentiation of NSC into neural progenitor cells and mature neurons. Furthermore, I assessed microglia activation and miRNA expression two weeks after injury and treatment with NPLT.

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.

EXPERIMENTAL DESIGN

The experimental timeline is shown in Figure2.1. Rats were randomized to receive fluid percussion injury (FPI) or sham injury and further randomized to receive NPLT or no treatment one hour later. A total of 18 rats per group were used in the study. Five rats per group were euthanized 24 hours after surgery and the brains processed for immunofluorescence analysis of BrdU incorporation. A second cohort of rats (8 rats/group) were used for immunohistological analysis and five rats/group were used for laser-capture microdissection and qRT-PCR analysis of microRNA expression.

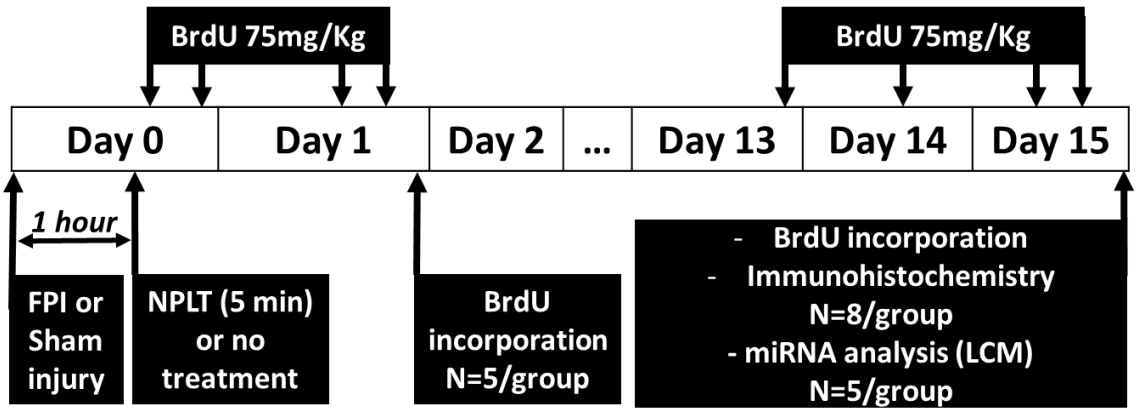

Figure 2.1. *In vivo* experimental design.
Rats were randomized to receive fluid percussion injury (FPI) or sham injury and further randomized to receive nano-pulsed laser therapy (NPLT) or not treatment. One cohort of rats (N=5/group) were treated with bromodeoxyuridine (BrdU) (75mg/Kg i.p.) 2, 4, 20 and 22 hours after surgery and euthanized 24 hours after surgery. A second cohort (8/group) were treated with BrdU (75mg/Kg i.p.) at 48, 24, 4 and 2 hours before euthanasia and the brains processed for BrdU and immunohistochemistry analysis. 5 rats/group were euthanized and the brains immediately dissected out and frozen on dry ice for qRT-PCR analysis of microRNAs expression.

FLUID PERCUSSION 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 for 5 minutes, 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 parasagittal 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. A modified LuerLok syringe hub (Becton-

Dickinson, Franklin Lakes, NJ) was placed on the exposed and intact dura and bonded in place with cyanoacrylic adhesive and covered with dental acrylic. Isoflurane was discontinued. The rats were connected to the trauma device and paw pinch reflexes were tested until a withdrawal response was detected, at which point the pendulum was released and the fluid wave was propagated forward, subjecting the brain to a 2 atm fluid-percussion injury. Sham rats underwent craniotomy and were attached to the FPI device but were not subjected to injury. After fluid percussion or sham injury, rats were disconnected from the device, placed in a supine position and monitored until a righting reflex was observed. Rats were then replaced under isoflurane anesthesia until ready for treatment.

NANO-PULSED LASER THERAPY (NPLT)

Rats were maintained under isoflurane anesthesia for one hour after FPI or Sham procedure and treated for 5 minutes with NPLT. Our system consists of an optical parametric oscillator that generates short (10 ns) pulses of near-infrared light (808 nm) at energy of up to 15 mJ and pulse repetition rate of 20 Hz. These pulses generate low-level optoacoustic waves that travel deep into the brain. Rats were treated 1 h after blast injury for a duration of 5 min to provide a dose of 300 J/cm2. NPLT was delivered through a 3mm diameter optical fiber on the site of the craniotomy (Figure 2.2). For treated animals, the optic fiber was positioned on the craniotomy site for 5 minutes. The animals were allowed to fully recover from the surgery/anesthesia before being returned to their cage with ad libitum food and water.

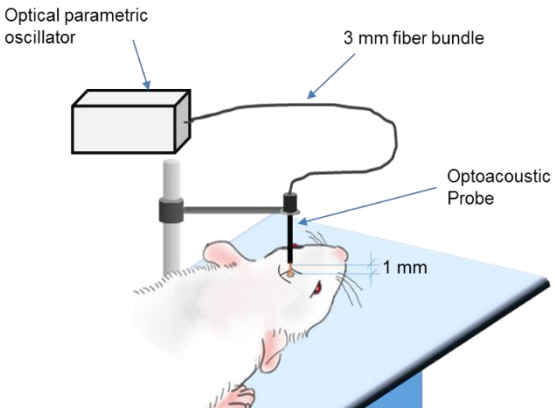

Figure 2.2. Schematic representation on the nano-pulsed laser therapy (NPLT) *in vivo* treatment.
Our optical parametric oscillator generates pulses of near infrared light that is directly delivered though an optoacoustic probe directly on the craniotomy site in both fluid percussion and sham injured rats.

BROMODEOXYURIDINE (BRDU) TREATMENT

Experimental rats received 4 intraperitoneal (i.p.) injections of bromodeoxyuridine (BrdU; Sigma Aldrich; 75mg/Kg). The rats in the 24 hours experimental group were treated on the day of surgery (immediately after surgery and 2 hours later) and the following day at 4 and 2 hours before euthanasia. The rats in the two weeks experimental group were treated with BrdU at 48h, 24h, 4h and 2h before euthanasia.

TISSUE PROCESSING AND IMMUNOSTAINING

One cohort of rats were euthanized 24 hours after surgery using 2-3% isoflurane followed by decapitation. The brains were immediately dissected out, frozen on dry ice and stored at -80 °C until further processing For immunofluorescence analysis of BrdU incorporation, the brains were embedded in OCT and 10µm thick sections were cut on a cryostat and

mounted on superfrost clean slides. Frozen sections were fixed in ice-cold 10% buffered formalin at room temperature for 30 minutes. After two washes in PBS (10 minutes each), sections were incubated with 2N HCl al 37 °C for 30 minutes to denature DNA and unmask the BrdU. The sections were neutralized by washing in 0.1M borate buffer (pH 8.5) 2 times for 5 minutes each and then blocked and permeabilized with a solution of 10% normal goat serum and 0.3% Triton X-100 in PBS for 30 minutes. Sections were incubated with a mouse primary anti-BrdU antibody (1:100 DAKO) and a rabbit anti-NeuN antibody (1:100 Millipore; to label neurons), 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 FluorSave (Millipore) and viewed with a fluorescence microscope BZ-X710 (Keyence) supported by BZ-X analyzer software (Keyence). The slides were stored in the dark at 4 °C.

A second cohort of rats was euthanized two weeks after surgery. For immunohistological and immunofluorescence analysis, rats were anesthetized and cardially perfused with saline followed by freshly prepared phosphate-buffered formaldehyde solution. The brains were dissected and post-fixed in formaldehyde for 12-16 hours at room temperature, transferred to a phosphate buffered solution (PBS) and shipped to NeuroScience Associates (NSA, Knowxville, TN) for tissue processing and immunohistological staining using a patented multibrain® technology. Briefly, 16 rat brains were embedded in one single block (each block containing 4 brains/group for a total of 2 blocks), sectioned on a microtome at 40µm thickness in the coronal plane and collected every 480µm throughout the hippocampus (Figure 2.3). The sections were adhered to glass slides (5x4 cm) and processed for

immunohistochemistry staining of BrdU (to evaluate the number of proliferating cells), doublecortin (DCX; to evaluate the number of neural progenitor cells) and microglia markers (Iba1 and CD68), and for double immunofluorescence staining of DCX and the neuronal marker NeuN (to evaluate the number of neural progenitor cells differentiating into mature neuron). Immunohistochemically stained slides were imaged with a BZ-X710 microscope (Keyence America, Itasca, IL) supported by the BZ-X analyzer software (Keyence America, Itasca, IL). Immunofluorescence stained slides were viewed with an Olympus BX51 fluorescent microscope equipped with a cooled CCD camera. Stereological analyses were performed with the aid of a semi-automated, computer-based microscopy systems (Stereo Investigator, MBF Bioscience). Slides were stored in the dark at 4 °C (immunofluorescence) or at room temperature (immunoperoxidase).

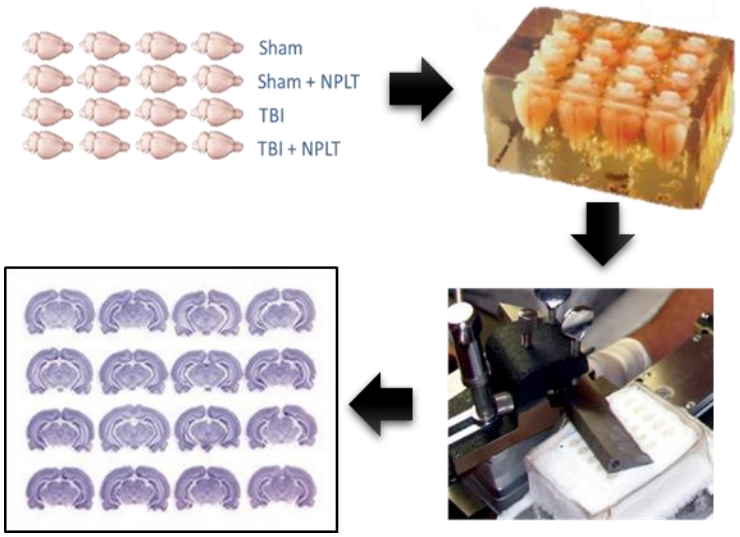

Figure 2.3. MultiBrain® process by NeuroScience Associates (NSA).
16 perfused-fixed rat brains were encased in a gelatin matrix and then sectioned at 40μm thickness. Free-floating immunostaining was performed to allow the antibodies reach the entire thickness of the section. (From www.neuroscienceassociates.com)

CELL COUNTS AND IMMUNOFLUORESCENCE QUANTITATIVE ANALYSES

BrdU incorporation. BrdU+ cells were counted in the subgranular zone (SGZ) of the DG (defined as a two cell-body thickness region below the granular layer) throughout the hippocampus (bregma -2.16mm to -6.12mm; 10 sections per brain) by two independent investigators who were blinded to the experimental groups. The length of the SGZ in each section was measured using ImageJ and used to normalize the mean counts of the two investigators. Total mean count for each brain was calculated by summing the mean of the normalized values of each section.

Aberrant migration of DCX+ cells. DCX-immunoreactivity in the hilus was quantified using Image J and normalized to total hilus area. Specifically, Paxino and Watson's rat brain atlas has been used to determine the boundaries and borders of the components of the dentate gyrus. Then, the area was traced and calculated. DCX+ cells in the granular and sub-granular layers of the DG were counted by two investigators who were blinded to the experimental groups, in coronal sections (bregma -2.40mm to -6.12mm, for a total of 8 sections per brain). The mean of the values obtained by the two investigators for each section was normalized for the area of the granular layer. Total mean count for each brain was calculated by summing the mean of the normalized values of each section.

In order to quantify ectopic migration of the DCX+ cells, the granular layer of the DG was divided in 2 sub-layers (layer I and layer II) arbitrary selected as two cell-body thickness starting from the SGZ. DCX+ cells in each layer were counted by two independent investigators and normalized for the length of the SGZ for each section. The mean of the values of each section were used as total mean count for each brain (8 per experimental group).

CD68+ microglia. CD68+ cells in the DG were counted by two investigators who were blinded to the experimental groups, in coronal sections (bregma -2.40mm to -6.12mm, for a total of 8 sections per brain). The mean of the values obtained by the two investigators for each section was normalized for the area of the hilus and granular layer respectively. Total mean count for each brain was calculated by summing the mean of the normalized values of each section.

Iba1+ microglia. Stereological analysis of Iba1+ microglia in the hilus was performed using StereoInvestigator software (MBF) in coronal sections (bregma -2.40mm to -4.36 mm; total of 4 sections per brain). Specifically, the contour of the hilus and a grid size with 10-12 random squares (containing an average of 5-7 cells) was selected in one coronal section at bregma level -4.16 mm. Mounted section thickness (40µm) and distance from one section to the other (480µm) was insert in the parameters to calculate the total number of cells by the software. Activation of microglia was evaluated at 60X magnification. Amoeboid-shaped microglia was considered activated while ramified not.

Neuronal differentiation of DCX+ progenitor cells. Stereological analysis of DCX+ and DCX+/NeuN+ cells in the DG was performed using StereoInvestigator software (MBF) in coronal sections (bregma -2.40mm to -4.36 mm; total of 4 sections per brain). Specifically, the contour of the SGZ and a grid size with 10-12 random squares (containing an average of 5-7 cells) was selected in one coronal section at bregma level -4.16 mm. Mounted section thickness (40µm) and distance from one section to the other (480µm) was insert in the parameters to calculate the total number of cells by the software.

LASER CAPTURE MICRODISSECTION (LCM) AND TOTAL RNA ISOLATION

Frozen brains were embedded in OCT mounting medium and then sagittal sections (10µm thick) were cut through the hippocampus (bregma -3.16mm to -5.16mm) on a cryostat (Leica Microsystem CM1850) and mounted on superfrost clean glass slides (Superfrost Plus, Thermo Fisher Scientific) The sections were counterstained for 20-30 seconds with 1% cresyl violet and the SGZ of the DG was captured on a thermoplastic film of CapSure Macro LCM caps (1 cap/brain) using a PixCell IIe laser capture microscope with an infrared laser diode. The samples were placed into a 0.5-mL tube containing 100 µL of Lysis buffer, vortexed and stored at -80 °C until isolation of RNA.

Total RNA was isolated from LCM samples using the RNA Aqueous Micro kit (Ambion/Thermo Scientific) according to the manufacturer's protocol before DNase treatment at 37 °C for 20 min to remove any traces of genomic DNA. The concentration and quality of total RNA was assessed using an Agilent Bioanalyzer with the RNA6000 Pico Lab Chip (Agilent Technologies).

MICRORNA RETROTRANSCRIPTION

Ten (10) ng of total RNA were used for the retrotranscription of the pool of miRNA by using TaqMan Advanced miRNA Assay kit (ThermoFisher 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.

QUANTITATIVE REAL-TIME PCR (QRT-PCR)

qRT-PCR was performed on a MX3000P system (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 retrotranscription diluted 1:10, 1µl of predesigned Taqman Advanced miRNA assay primers from Applied Biosystems (Foster City, CA) and 4µl 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).

STATISTICAL ANALYSIS

Data is expressed as mean +/- SEM. Analysis of variance (ANOVA) followed by multiple comparisons post-hoc Tukey's tests were performed using GraphPad 7 Prism software. Differences were considered significant when $p<0.05$.

RESULTS

NPLT DOES NOT INFLUENCE TBI-INDUCED CHANGES IN NEURAL STEM CELL (NSC) PROLIFERATION IN THE HIPPOCAMPUS DENTATE GYRUS (DG).

Rats were subjected to FPI or sham injury and euthanized 24 hours or two weeks post-surgery. In order to evaluate the proliferation rate of hippocampal NSC in the subgranular zone (SGZ) of the DG, the rats were treated with bromodeoxyuridine (BrdU) before sacrifice. We found that, 24 hours after TBI, the number of BrdU+ cells was significantly higher in the SGZ ipsilateral to the injury site (Figure 2.4 A-B). NPLT did not affect the number of BrdU+ cells 24 hours after TBI. No significant differences in the number of BrdU+ cells in the SGZ were observed in the contralateral side 24 hours after FPI or sham injury with or without NPLT (Figure 2.4 C-D).

Contrary to what we observed at 24 hours, BrdU+ cells were significantly reduced in the SGZ two weeks after FPI, in both ipsilateral and contralateral sides of the brain as compared to sham rats (Figure 2.5). NPLT did not prevent the loss of proliferating cells two weeks after TBI (Figure 2.5).

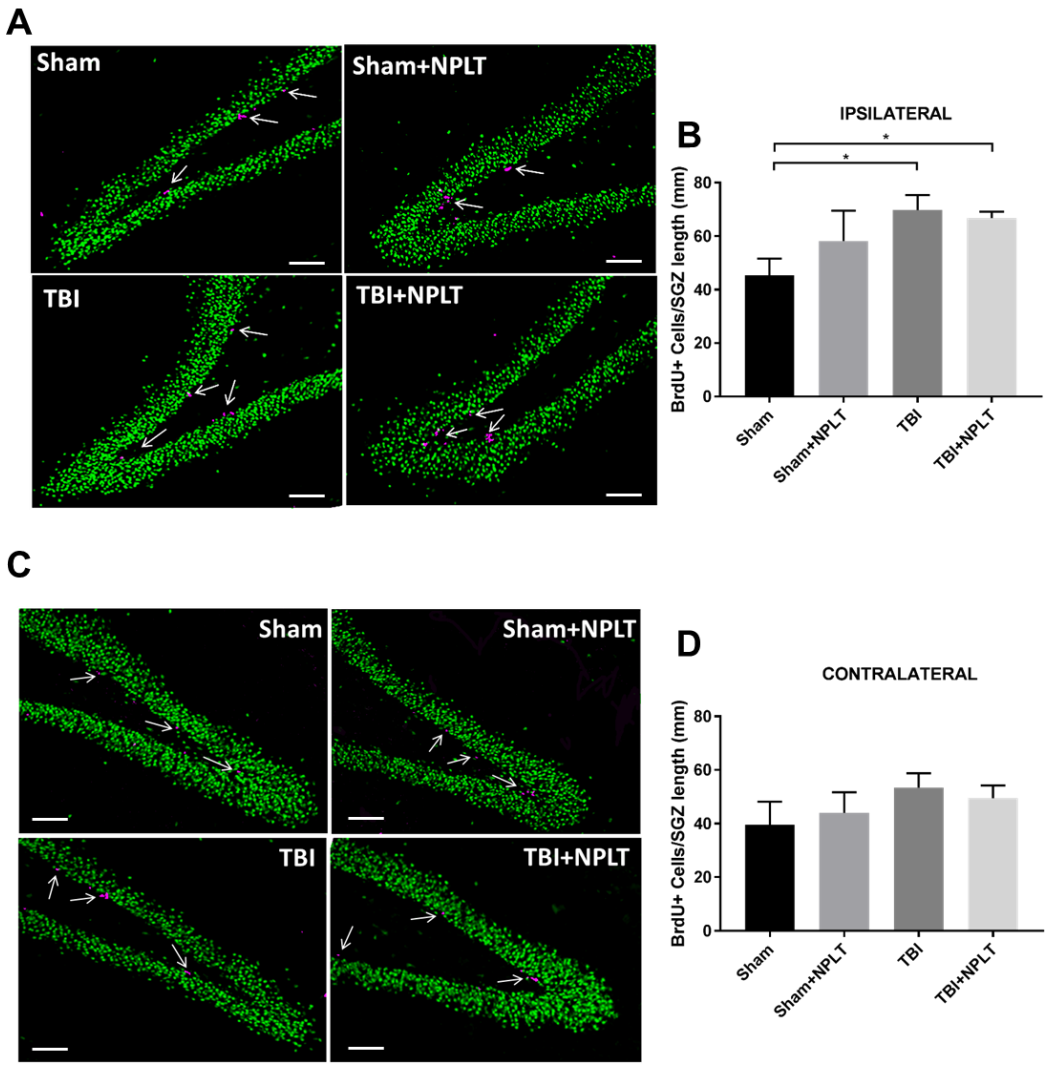

Figure 2.4. Nano-pulsed laser therapy (NPLT) increases cell proliferation in the SGZ of the hippocampus dentate gyrus 24 hours after fluid percussion injury.

(A, C) Representative images of bromodeoxyuridine (BrdU) incorporation in the SGZ of the dentate gyrus of the hippocampus stained with antibodies against BrdU (red) and NeuN (a neuronal marker, green). (B) Quantitative analysis of the number of $BrdU^+$ cells in the ipsilateral SGZ 24 hours post injury. (D) Quantitative analysis of the number of $BrdU^+$ cells in the contralateral SGZ 24 hours post injury. N=5; *$p<0.05$ two-way analysis of variance (ANOVA) with Tukey's post-hoc test; Calibration bar is 100 µm.

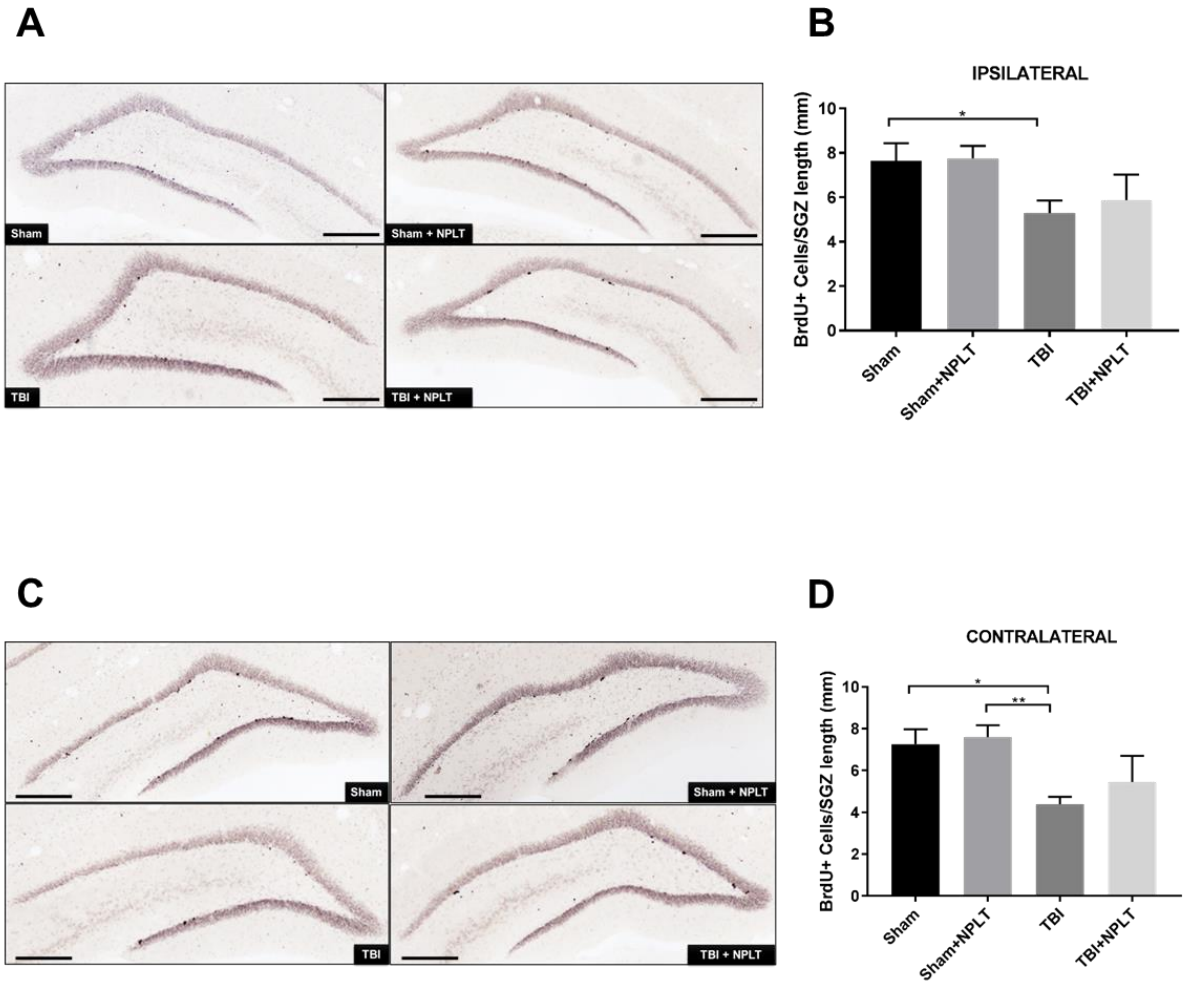

Figure 2.5. NPLT does not prevent decreased cell proliferation of NSC in the SGZ of the hippocampus dentate gyrus two weeks after TBI.

(A, C) Representative images of bromodeoxyuridine (BrdU) incorporation in the SGZ of the dentate gyrus of the hippocampus. BrdU is shown in black. Hematoxylin was used to counterstain nuclei (shown in brown). (B) Quantitative analysis of the number of $BrdU^+$ cells in the ipsilateral SGZ two weeks post injury. (D) Quantitative analysis of the number of $BrdU^+$ cells in the contralateral SGZ two weeks post injury. N=8; *$p<0.05$ two-way analysis of variance (ANOVA) with Tukey's post-hoc test. Calibration bar is 200 µm.

NPLT INCREASES NEURONAL DIFFERENTIATION OF PROGENITOR CELLS IN THE HIPPOCAMPUS DG AFTER TBI.

To evaluate the effect of TBI and NPLT on the differentiation of NSC, we quantified the number of neuronal progenitor cells expressing doublecortin (DCX) and the neuronal marker NeuN in the hippocampus DG two weeks after fluid-percussion or sham injury. Stereological analysis showed that, while the total number of DCX+ cells was significantly increased in the DG of TBI rats ipsilateral to the injury side, as compared to Sham rats (Figure 2.6 B), the number of DCX+/NeuN+ cells was decreased (Figure 2.6 C). At the same time, we found that NPLT treatment prevented TBI-induced increase of total DCX+ cells (Figure 2.6 B) and significantly increased the number of DCX+/NeuN+ cells in the DG of TBI rats as compared to untreated TBI rats and sham rats (Figure 2.6 C). No significant differences were observed in the ipsilateral DG between sham rats and sham rats treated with NPLT (Figure 2.6 B-C). No significant changes in the number of DCX+ cells or DCX+/NeuN+ cells were observed in the contralateral side (Figure 2.6 D-E). These data suggest that, although the number of DCX+ cells is increased after TBI, fewer progenitor cells become neurons, a process that is prevented and reversed by NPLT.

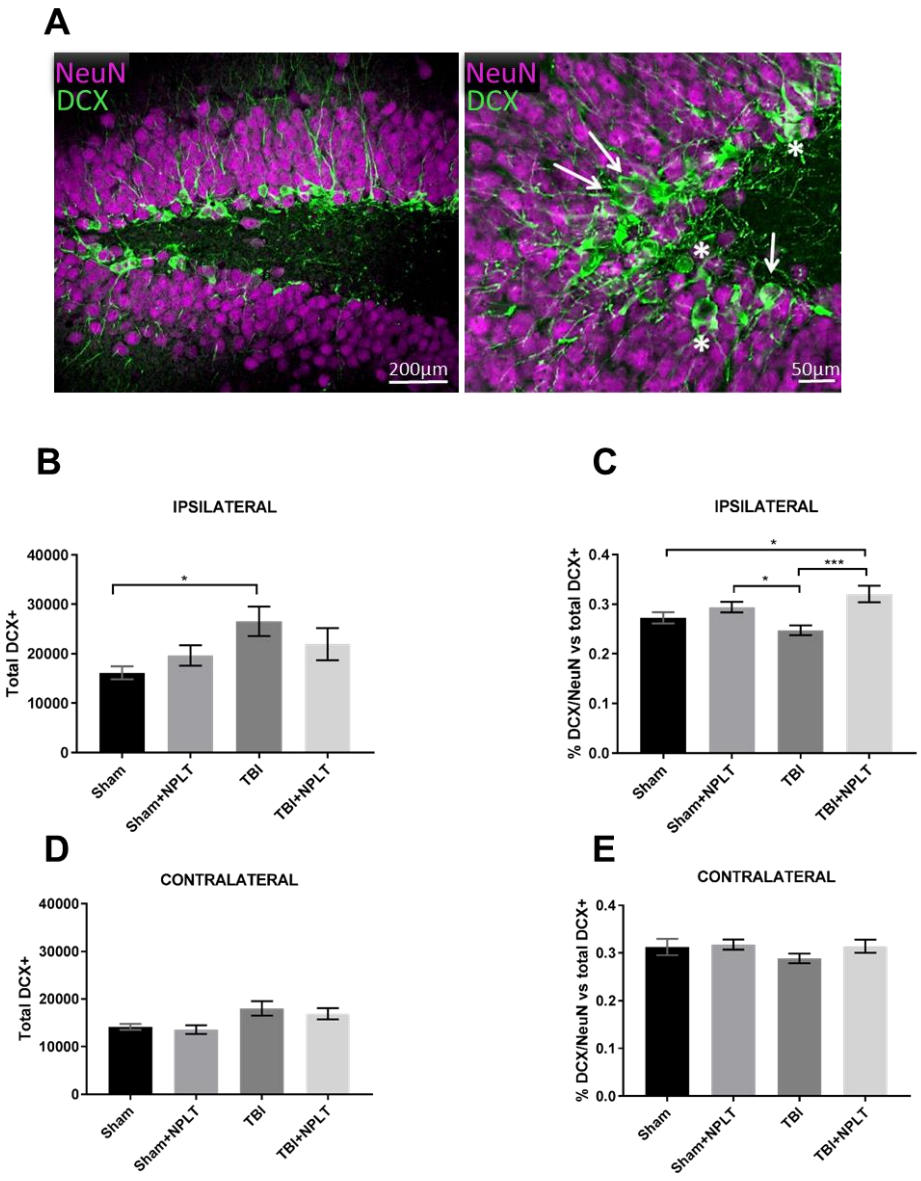

Figure 2.6. NPLT prevents the abnormal increase of neural progenitor cells induced by TBI and potentiates neuronal differentiation in the dentate gyrus of the hippocampus.

(A) Representative images of doublecortin (DCX) and NeuN (a neuronal marker) immunostaining of the hippocampus dentate gyrus. DCX is shown in green. NeuN is shown in magenta. (B, D) Stereological analysis of the number of DCX^+ cells in the ipsilateral (B) and contralateral (D) dentate gyrus two weeks post injury. (C, E) Stereological analysis of the number of $DCX^+/NeuN^+$ cells in the ipsilateral (C) and contralateral (E) dentate gyrus two weeks post injury. N=8; ***$p<0.001$, *$p<0.05$ two-way analysis of variance (ANOVA) with Tukey's post-hoc test.

NPLT PREVENTS ABERRANT MIGRATION OF NEURAL PROGENITOR CELLS IN THE HILUS AND IN THE GRANULAR LAYER OF THE DG AFTER TBI.

Ectopic migration of neural progenitor cells, in particular in the hilar region of the DG, has been previously reported after TBI and is known to be associated with the onset of epileptic seizures (Cho *et al.*, 2015; Shapiro, 2017). To assess the number of neuronal progenitor cells in the hilus, rats subjected to TBI or sham injury and treated or not treated with NPLT, were sacrificed two weeks after surgery and coronal brain sections were immunostained for DCX. Quantification analysis showed a 5-fold increase of DCX+ immunoreactivity in the hilus after TBI that was significantly reduced by NPLT treatment (Figure 2.7 A-B). No significant differences between Sham rats, treated or not treated with NPLT, were observed. The contralateral side showed not significant differences among the groups (Figure 2.7 C-D).

The same sections were used to assess the number of neuronal progenitor cell in the granular layer migrating toward the molecular layer. The granular layer was arbitrarily divided in two sub-layers (namely layer I and II starting from the SGZ) (Figure 2.8 A). Quantification analysis of DCX+ cells in the two layers showed approximately a 5-fold increase of neuronal progenitor cells in layer I and almost 10-fold in the layer II in TBI rats as compared to Sham rats. This increase was significantly reduced by NPLT in both layers. No significant differences between Sham rats, treated or not treated with NPLT, were observed (Figure 2.8 B). The contralateral side showed 2-fold increase after TBI in layer I and a small reduction (not significant) after treatment with NPLT. A similar trend (not significant) was shown in the layer II. Analogously to the ipsilateral side, no significant differences between Sham rats, treated or not treated with NPLT, were observed in the contralateral side of the injury (Figure 2.8 C).

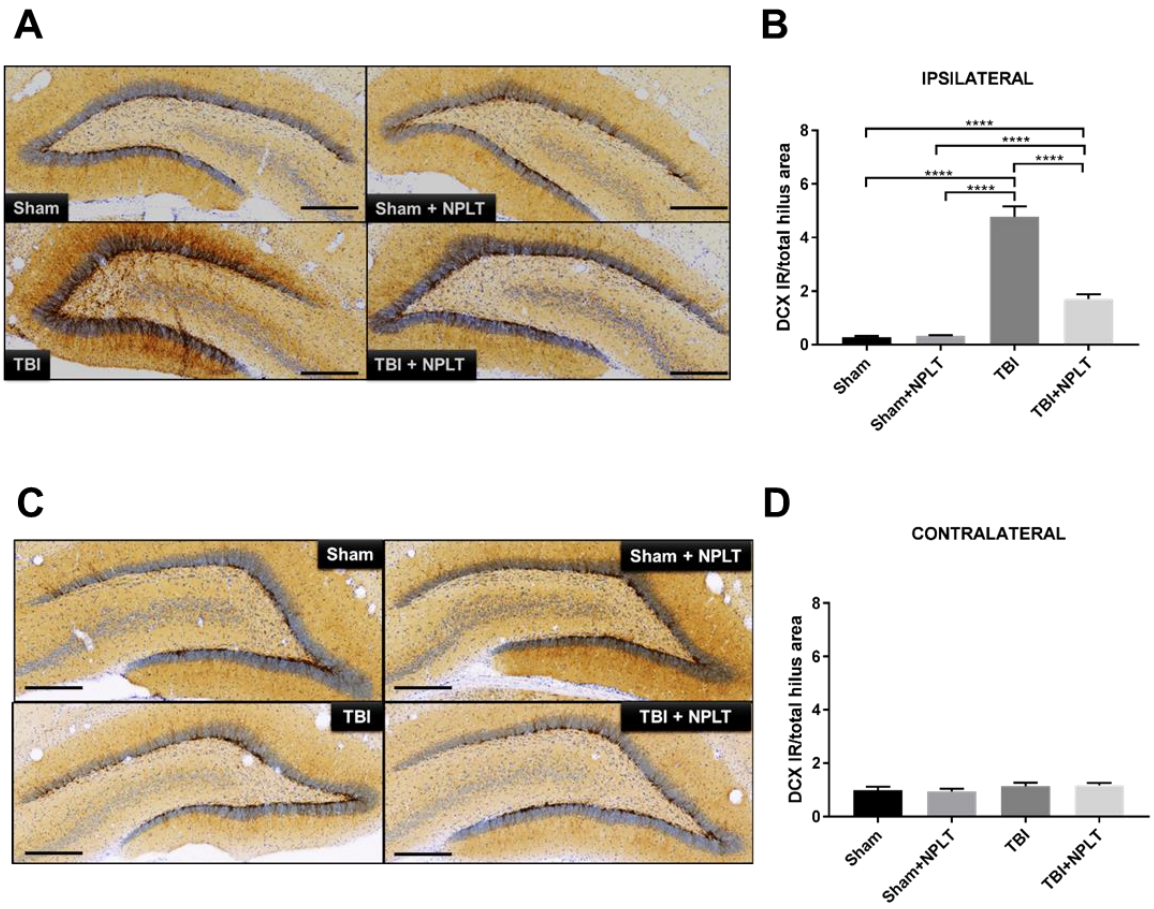

Figure 2.7. NPLT prevents ectopic migration of neural progenitor cells in the hilus of the hippocampus dentate gyrus two weeks after TBI.

(A, C) Representative images of doublecortin (DCX) immunostaining of the dentate gyrus and hilus of the hippocampus. DCX is shown in brown. Thyonine blue was used to counterstain nuclei (shown in blue). (B) Quantitative analysis of the number of DCX^+ cells in the ipsilateral hilus two weeks post injury. (D) Quantitative analysis of the number of DCX^+ cells in the contralateral hilus two weeks post injury. N=8; ****$p<0.0001$ two-way analysis of variance (ANOVA) with Tukey's post-hoc test; Calibration bar is 200 µm.

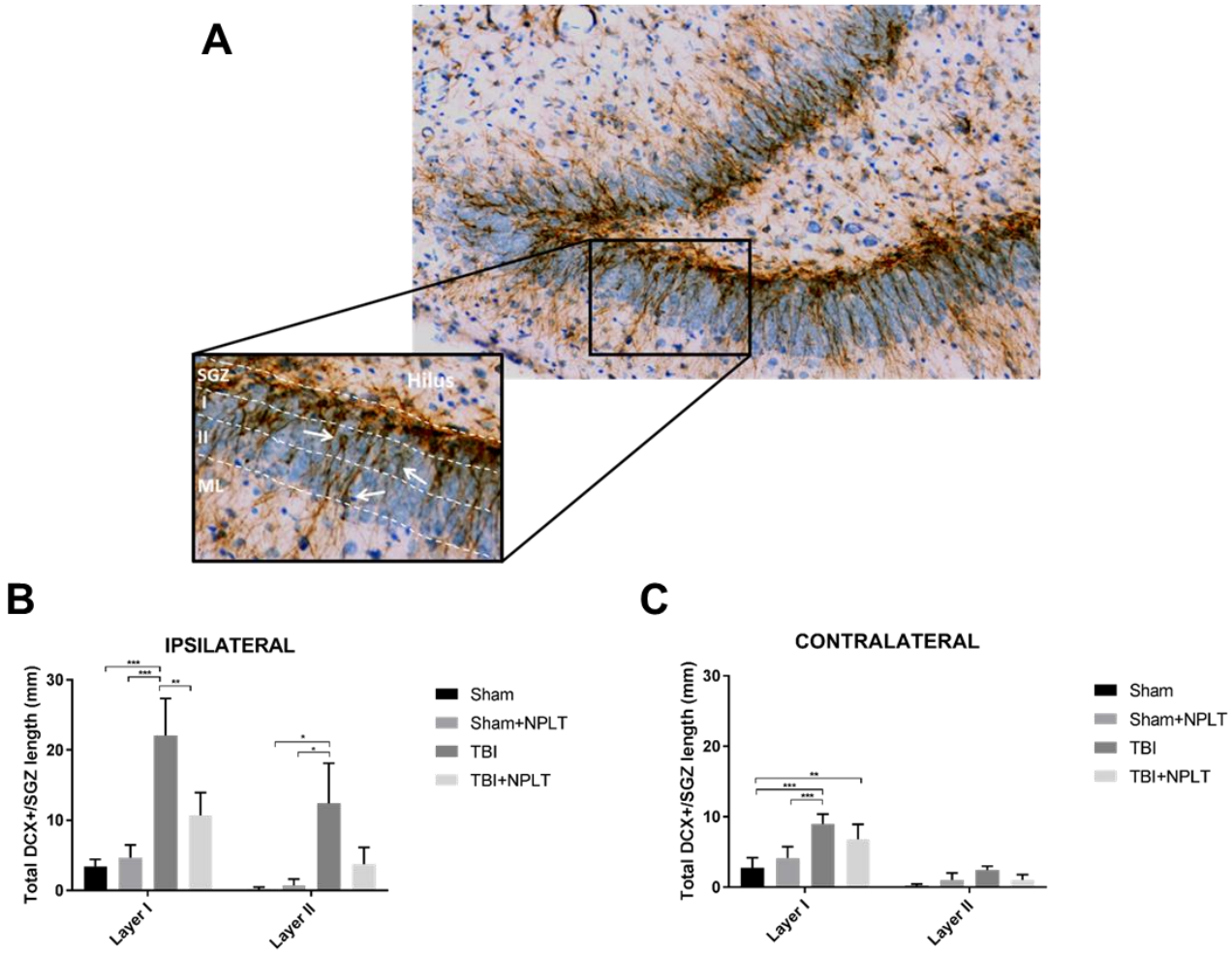

Figure 2.8. NPLT prevents ectopic migration of neural progenitor cells in the granular layer of the hippocampus dentate gyrus two weeks after TBI.

(A) Representative image of doublecortin (DCX) immunostaining of the dentate gyrus and hilus of the hippocampus. DCX is shown in brown. Thyonine blue was used to counterstain nuclei (shown in blue). SGZ: Subgranular zone; I: Layer I; II: Layer II; ML: Molecular layer; (B) Quantitative analysis of the number of DCX^+ cells in the ipsilateral granular layer two weeks post injury. (D) Quantitative analysis of the number of DCX^+ cells in the contralateral granular layer two weeks post injury. N=8; *p<0.05; **p<0.01; ***P<0.001; ****p<0.0001 two-way analysis of variance (ANOVA) with Tukey's post-hoc test; Calibration bar is 200 µm.

NPLT DOES NOT PREVENT MICROGLIA ACTIVATION IN THE HILUS AND GRANULAR LAYER OF THE DG

Microglial activation has been shown to contribute to aberrant migration of neuronal progenitor cells (Loane & Kumar, 2016). Rat brains sections were stained with a specific antibody against Cluster of Differentiation 68 (CD68, a marker for phagocytic microglia) and for calcium-binding adaptor molecule 1 (Iba1) expressed on both resting (ramified, Figure 2.9 A) and activated (amoeboid, Figure 2.9 B) microglia. Stereological quantification showed a significant increase of both resting (ramified) and activated (amoeboid) Iba1+ cells in the hilus of the hippocampus two weeks after TBI, as compared to sham and sham+NPLT rats, that was unchanged in NPLT-treated rats (Figure 2.10 A-C). Moreover, we found that TBI resulted in a significant increase of CD68+ microglia in the hilus and dentate gyrus (Figure 2.11 A). Similarly to what we observed for Iba1+ microglia, NPLT did not reduce the number of activated CD68+ microglia (Figure 2.11 B). Interestingly, NPLT treatment increased the proportion of hypertrophic (active) Iba1+ microglia in the sham group as compared to sham (Figure 2.10 D) but not of CD68+ microglia (that was not detectable in uninjured sham brains) (Figure 2.11 B-D). These results suggest that the observed reduction in the migration of DCX+ progenitor cells in the hilus is not likely to be mediated by NPLT-induced reduction of microglia activation, but rather to a direct effect of NPLT on neuronal progenitor cells.

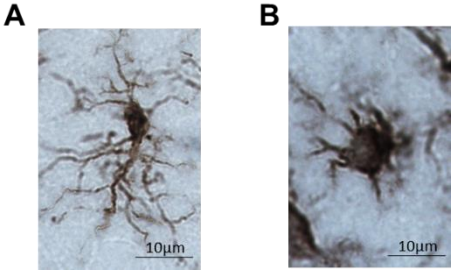

Figure 2.9. Representative images of the microglia morphology stained for Iba1.

(A) Ramified resting microglia. (B) Amoeboid activated microglia.

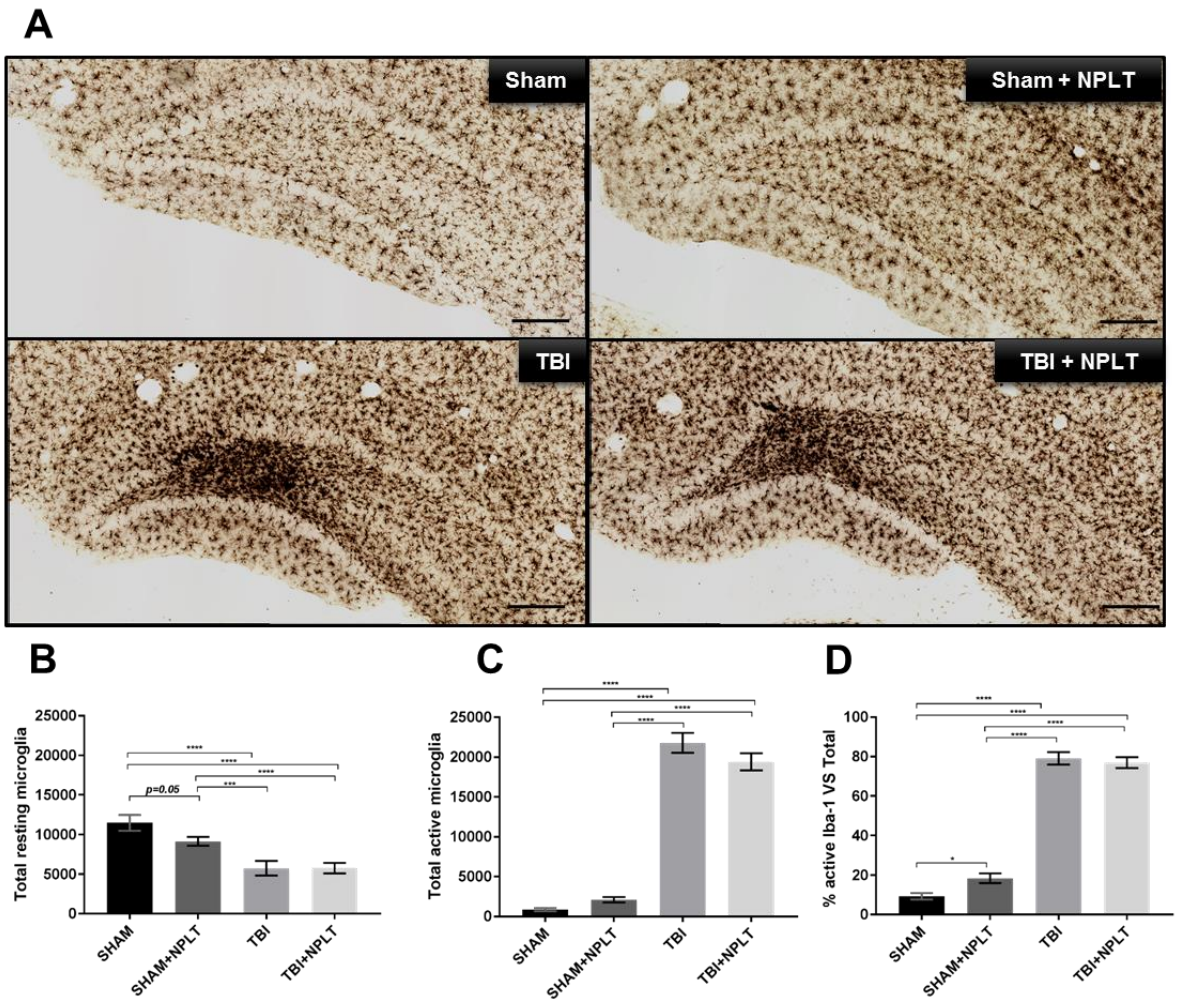

Figure 2.10. NPLT does not prevent TBI-induced Iba1$^+$ microglia activation in the hippocampus hilus.

(A) Representative images of Iba1-positive cells (brown) in the hilus of the hippocampus. (B) Quantification of resting (ramified) Iba1$^+$ cells in the hilus of the hippocampus. (C) Quantification of active (amoeboid) Iba$^+$ cells in the hilus of the hippocampus. (D) Percentage of active Iba1$^+$ microglia versus total (resting+active) Iba1$^+$ microglia. N=8; *p<0.05; ****p<0.0001 two-way analysis of variance (ANOVA) with Tukey's post-hoc test. Calibration bar is 200 µm.

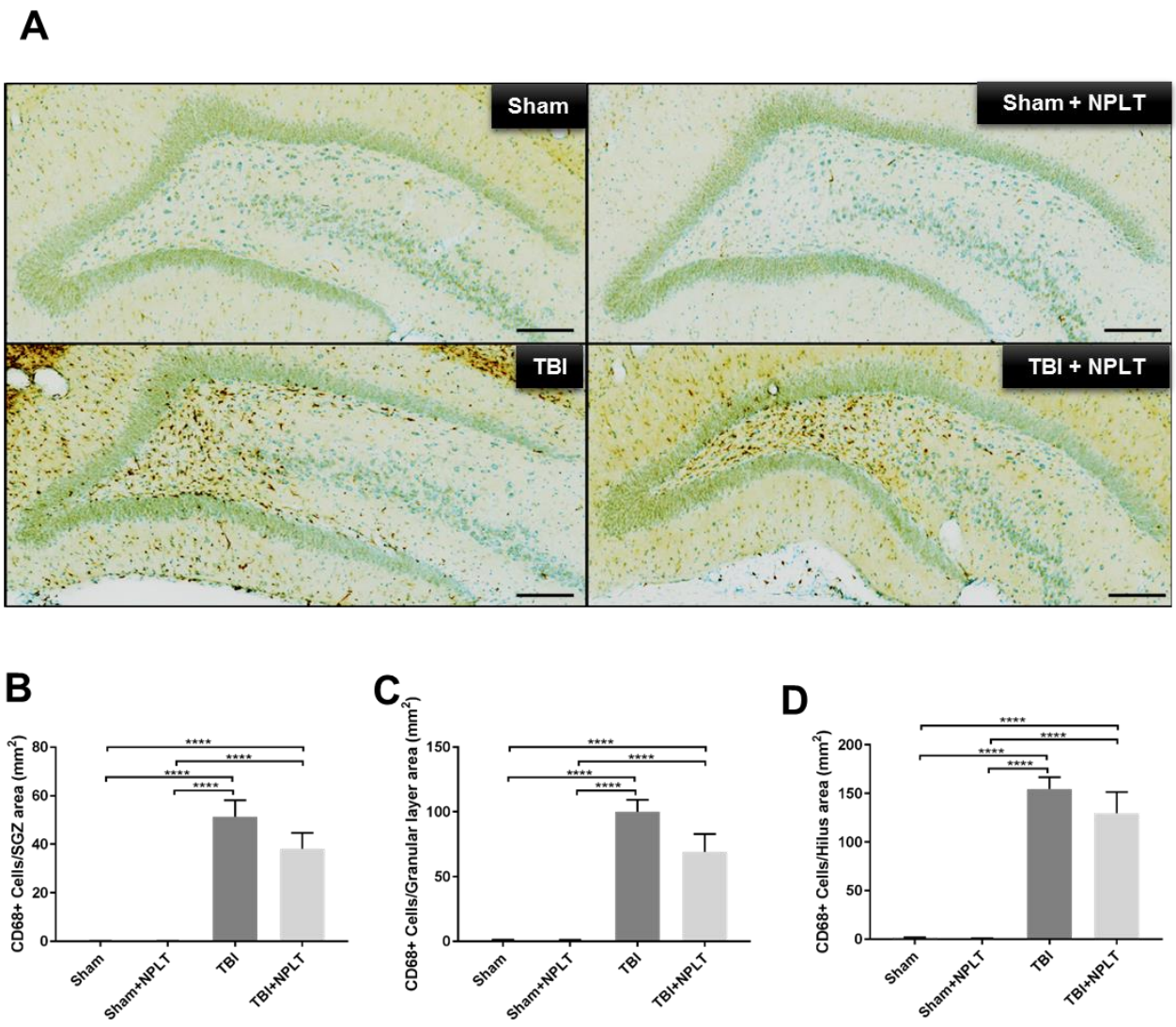

Figure 2.11. NPLT does not prevent TBI-induced upregulation of CD68$^+$ activated microglia in the hippocampus hilus.

(A) Representative images of CD68$^+$ cells (brown) in the DG of the hippocampus. Thyonine blue was used to stain nuclei. (B-D) Quantification of CD68$^+$ cells in the hilus of the hippocampus. N=8; ****p<0.0001 two-way analysis of variance (ANOVA) with Tukey's post-hoc test. Calibration bar is 200 µm.

NPLT MODULATES THE EXPRESSION OF SPECIFIC MIRNA IN HIPPOCAMPAL NEURAL STEM/PROGENITOR CELLS.

Neurogenesis is a process tightly regulated by epigenetic mechanisms (Kawahara *et al.*, 2012). To determine whether NPLT can modulate the expression of specific microRNAs (miRNA), known to regulate proliferation, differentiation and migration of hippocampal neural stem/progenitor cells (Wakabayashi *et al.*, 2014), we used laser capture microdissection (LCM) to selectively isolate the SGZ of the DG of rats two weeks after TBI or sham injury with or without NPLT. We found that the expression of miR9, miR25, miR29, miR124, miR137, assessed by qRT-PCR, was significantly increased in the SGZ of TBI rats as compared to sham rats (Figure 2.12). Interestingly, after TBI, NPLT significantly decreased the expression of the same miRNA (Figure 2.12).

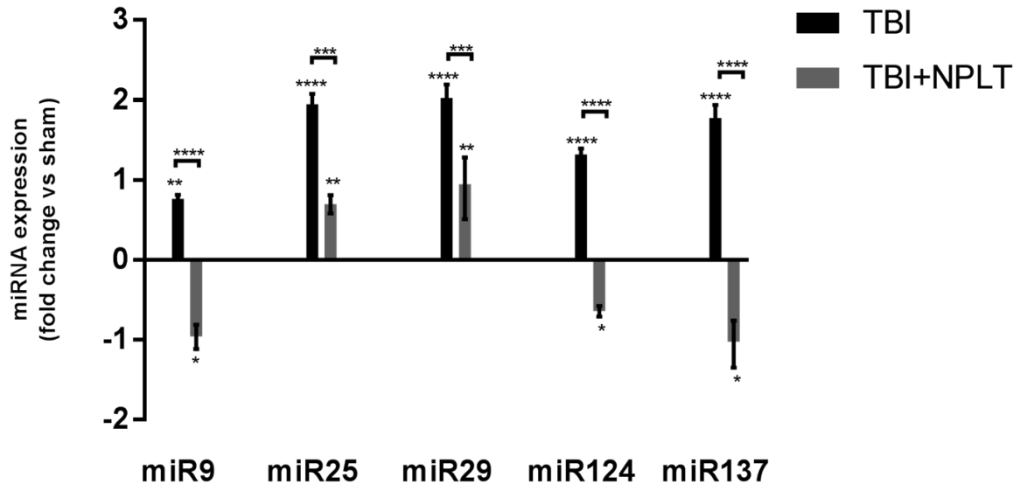

Figure 2.12. NPLT reverts the increased expression of miRNA following TBI in the SGZ of the dentate gyrus of the hippocampus.
qRT-PCR analysis of the expression of miRNA in the SGZ of the dentate gyrus of the hippocampus isolated by laser capture microdissection (LCM). N=8; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 two-way analysis of variance (ANOVA) with Tukey's post-hoc test.

Discussion

Traumatic brain injury (TBI) is characterized by permanent structural and physiological changes that compromise the normal function of the brain (Hemphill *et al.*, 2015). One of the regions most affected by TBI is the hippocampus, located in the temporal lobe and that plays an essential role in learning and memory (Yau & So, 2014). Indeed, memory impairment and disorientation are among the most important symptoms reported by TBI patients (Laskowski *et al.*, 2015). The hippocampus is also a region of the brain in which neurogenesis continues throughout life (Wang, Seekaew, *et al.*, 2016). In this work we report that a highly innovative, non-invasive therapy, that combines near infrared laser light (NIL: 808nm) and ultrasound waves (US), optoacoustically generated with each short (10ns) high-energy (15mJ) laser pulse within the brain, prevents TBI-induced impairments in cognitive function and in neurogenesis in the hippocampus of rats subjected to fluid percussion injury.

We first assessed the proliferation of NSC by measuring the incorporation of BrdU in the SGZ of the DG of the hippocampus. Our data show that, 24 hours after TBI (consistent with published literature (Yu *et al.*, 2016), the proliferation of NSC is significantly increased in the DG ipsilateral to the injury site but not in the contralateral site. Interestingly, two weeks after TBI, the number of proliferating cells in the SGZ was significantly reduced as compared to the sham group, suggesting a depletion of the pool of NSC (that were initially stimulated in response to the injury). These results are consistent with other reports showing that NSC proliferation decreases 1 week and three weeks after (Wang, Gao, *et al.*, 2016). When we looked at the effect of NPLT, we found that, while it did not change the increase in NSC proliferation 24 hours after TBI, it did mitigate the

reduction of NSC proliferation after two weeks. The contralateral side showed a similar trend but without statistical differences, suggesting once again the increased intensity of the changes induced by TBI only in the side of the injury.

Neuronal differentiation is an important functional property of NSC that leads to the generation and integration of newborn neurons into the pre-existing circuit in the hippocampus DG (a critical event for memory formation and consolidation). In order to evaluate how TBI alters this fundamental aspect of neurogenesis, we performed double immunostaining for doublecortin (DCX) and neuronal nuclei (NeuN) and used standard stereological methods to quantify the number of newborn granular neurons in the hippocampus DG. We show that, two weeks after TBI, the number of DCX+ progenitor cells in the TBI group is significantly increased, most likely as result of the increased proliferation of NSC after injury. This abnormal increase is partly prevented by NPLT treatment (Figure 2.6B). Moreover, our findings show that while neural progenitor cells differentiating into mature neurons (DCX+/NeuN+ cells) are reduced in the DG after TBI, they are increased after treatment with NPLT (Figure 2.6C). This effect is only observed in the ipsilateral side thus further confirming a direct effect of injury and NPLT on neural progenitor cells in the DG.

In addition to inducing an abnormal proliferation and differentiation of neuronal progenitor cells, TBI can also influence the migration of NSC. Indeed, the aberrant migration of immature neurons in areas outside the granular layer of the hippocampus DG, such as the hilus, has been previously reported in rodent models of TBI, and has been shown to strongly correlate with the onset of seizures and increased incidence of epilepsy after injury (Cho *et al.*, 2015; Shapiro, 2017). Specifically, seizures have been reported to

occur as early as few weeks following TBI with some manifesting months to years after injury (Uski *et al.*, 2018). Preventing aberrant migration of neuronal progenitors and the presence of displaced neurons in the hilus of the DG is therefore an important therapeutic goal for reducing TBI-induced epilepsy. In the present work we have shown that, in the rat FPI model of TBI, neural progenitor cells (DCX+) aberrantly migrate in the hilus of the hippocampus, where they are found two weeks after injury. This aberrant migration is prevented by NPLT applied one hour after TBI. Moreover, the NPLT treatment corrects abnormal migration of immature neuron in the granular layer of the DG induced by TBI. Taken together, these results strongly support the therapeutic potential of NPTL for preventing the pathological migration of immature neurons, and the associated onset of seizures, following TBI.

In order to assess whether the effects of NPLT on neuronal progenitor cells migration could be, at least partially, due to its anti-inflammatory properties, we performed immunostaining for Iba1 (a marker of microglia) and CD68 (a more specific marker of activated/phagocytic microglia). Interestingly, we found that NPLT did not reduce the numbers of CD68+ microglia (that was significantly increased after TBI as compared to Sham), in the hippocampus SGZ, granular layer and hilus. Thus our data strongly suggest that NPLT prevents aberrant neuronal migration by directly affecting stem cells and neuronal progenitor cells in the hippocampus. Moreover, the quantification of Iba1 (a marker for total microglia) shows, as expected, an increase of both resting and activated (hypertrophic-amoeboid) microglia after injury. Interestingly, the morphological analysis shows an increase of the hypertrophic microglia (activated) in the sham group after treatment with NPLT. This cells Iba1+/CD68- have been previously reported as microglia

immunotype that support neuronal function/growth (Smith *et al.*, 2015). Specifically, an increase in activated Iba1+/CD68- microglia in the hippocampus has been shown to occur after exposure to an enriched environment or after volunteer wheel running (known to support neurogenesis). Our findings show that NPLT-induced increase in active Iba1+/CD68- microglia in the brain, might, at least partially, mediate the neuroprotective effects of NPLT and possibly its ability to reduce aberrant neurogenesis after TBI.

Finally, to further study the changes induced by NPLT on neural progenitor cells, we used LCM to analyze the expression in the SGZ of the DG of specific microRNAs (miRNA) known to be involved in the regulation of neurogenesis (differentiation of stem cells, neurites formation, migration and integration). Our data show that NPLT prevents TBI-induced increase in the expression of regulatory miRNA.

CHAPTER 3: THE EFFECT OF NPLT ON HIPPOCAMPAL NEURAL STEM CELLS

Introduction

Our *in vivo* data previously discussed suggested a possible mechanism of NPLT affecting directly NSC. To confirm this direct effect of NPLT on NSC, I performed *in vitro* analyses on NSC. Specifically, I isolated NSC from the hippocampus of TBI rats and I treated them *in vitro* for 5 minutes with NPLT.

I first analyzed the effect of the treatment on NSC proliferation and miRNA expression 24 hours after treatment. Then, I analyzed the effect of NPLT on the differentiation of NSC. Specifically, I preformed western blotting and immunofluorescence analysis on differentiating NSC 7 days after treatment by using glial (GFAP) and neuronal (βIII-Tubulin) markers.

METHODS

ISOLATION AND *IN VITRO* EXPANSION OF RAT HIPPOCAMPAL STEM CELLS

Reagents for the isolation procedure were obtained from Invitrogen Co. (Carlsbad, CA), except where noted. Adult male Sprague-Dawley rats (200-250g) were subjected to FPI as described above. At one month after injury, the rats were anesthetized with isoflurane (4% by inhalation) and euthanized by decapitation. The brain was rapidly removed, and the hippocampi dissected out. The hippocampi from 4 rats were collected into a 50ml Falcon tube containing sterile DMEM/F12 media with antibiotics (penicillin and streptomycin) and kept on ice. The tissue was minced into small pieces in cold HBSS media containing 1mM EGTA in a sterile petri dish. The tissue pieces were transferred into a sterile 50ml tube containing HBSS (without Ca2+ and Mg2+ with 0.1% collagenase/dispase, 0.01% DNase I, 1 ml for 100 mg of tissue) and incubated for 30 minutes at 37 °C. The tissue was triturated every 10 minutes at 37 °C using a sterile disposable 5 ml pipette. At the end of the incubation, the cell suspension was centrifuged at 200g for 5 minutes at room temperature. The pellet was resuspended in HBSS containing 0.025% trypsin/EDTA and incubated 10 minutes at 37 °C. An equal volume of DMEM/F12, 2mM L-glutamine, 10% FBS and antibiotics was added to stop the cell digestion and the cell suspension was centrifuged at 200g for 5 minutes. The pellet was resuspended in sterile DMEM/F12 with L-glutamine, 10% FBS and antibiotics, triturated using 1ml tip and centrifuged. This step was repeated 2-4 time until single cells suspension was obtained. The cell suspension was filtered through a 70µm Falcon filter, centrifuged at 200 X g for 5 minutes and resuspended in complete growth media: neurobasal A media containing the serum-free supplement B27 (without retinoic acid), 2 mM L-glutamine, 20 ng/ml of epidermal grow factor, 20 ng/ml

of basic fibroblast grow factor 2, penicillin/streptomycin. The cells were plated in an uncoated T25 flask at 1 x 10^6 cells/10ml of complete growth media. The media was changed after 24 hours and every other day after that. Adult naïve rat hippocampal primary NSC (Millipore) were used as control. These cells were plated and cultured as described above.

CHARACTERIZATION OF RAT HIPPOCAMPAL STEM CELLS

To evaluate the stemness of the NSC isolated from TBI rats, we performed Western blotting analysis. For total proteins extraction, NSC isolated from TBI rats (1 month after injury) or control rats growing in suspension, were collected by centrifugation and washed in PBS 1X before being lysed using RIPA lysis buffer (Thermo Fisher) (500µl of buffer per $2x10^6$ cells) containing 1µl of protease inhibitor and 1µl phosphatase inhibitor per 100µl of buffer to prevent degradation and modification of the phosphorylated proteins and incubate for 10 minutes on ice. The samples were pelleted by centrifugation (15 minutes at 14,000 x g) and the supernatant was aliquoted into clean tubes and stored at -20 °C until use. Total protein concentration was determined using the BCA assay kit according the manufacturer's protocol (Thermo Fisher).

Proteins (25µg per sample) were resolved by SDS-PAGE using precasted 4-20% gradient gels (Biorad) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with a 5% non-fat dry milk solution in TBS containing 0.05% Tween-20 (T-TBS) for 1 hour at room temperature and incubated with primary antibodies diluted in 0.5% non-fat dry milk in T-TBS overnight at 4 °C. The following antibodies were used: mouse anti-nestin (1:1000, Millipore), rabbit anti-sox2 (1:1000, CellSignaling)

and mouse anti-βIII−Tubulin (1:1000, Promega). Membranes were washed 3 times for 5 minutes each time with T-TBS and incubated with the corresponding horseradish-peroxidase-conjugated secondary antibody (1:2000, Cell Signaling) diluted in 0.5% non-fat dry milk in T-TBS for 1 hour at room temperature. The signal was detected using BioRad ECL and captured using the ChemiDoc imaging system (Biorad). HRP conjugated Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:3000, Thermo Fisher) was used as loading control.

IN VITRO TREATMENT OF HIPPOCAMPUS NEURAL STEM CELLS WITH NPLT

NPLT was delivered through an optical parametric oscillator probe (3mm diameter) directly to neural stem cells (NSC) plated in a 96-well plate. We used a 96-well plate because the diameter of each well perfectly fit with the optoacoustic probe. Five (5) x 105 of cells in suspension were plated in a single well only for the time of the treatment (5 minutes) and then transferred to a T25 flask at the appropriate density for growth (5 x 105 cells/5 ml) in proliferating media. Because a key feature of NPLT is the production of ultrasound waves (US), generated in the brain by each nano-pulse of near-infrared light (NIL) upon absorption by hemoglobin (ref), we used a black nonporous PVC film attached to the bottom of the plate with an ultrasound gel (Aquasonic 100, Parker Laboratories), to act as chromophore for NIL-stimulated US production. The probe for NIL delivery was positioned at the top of the plate and the US were generated when the laser reached the black nonporous PVC film at the bottom of the plate. The treatment lasted for 5 minutes for a total energy delivery of 4.3 mJ (Figure 3.1).

Figure 3.1. Schematic representation of the nano-pulsed laser therapy (NPLT) *in vitro* treatment of neural stem cells.
The optoacoustic probe for near infrared light (NIL) delivery was positioned at the top of the 96-well plate and the US were generated when the laser reached the black nonporous PVC film at the bottom of the plate. The treatment lasted for 5 minutes for a total energy delivery of 4.3 mJ.

POLY-ORNITHINE-LAMININ COATING

To allow the attachment of the NSC a Poly-Ornithine-Laminin coating was prepared. Poly-Ornithine-Laminin coating (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.

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). EdU was diluted from the stock solution (10mM) in the medium to the final concentration of 10μM. 24 hours after stretch the wells were washed in PBS and fixed with ice-cold 100% methanol on ice for 20 minutes. To remove the fixative, the wells were washed twice with 3% BSA in PBS. The cells were permeabilized with a solution containing 0.5% Triton-X in PBS for 20 minutes and then washed twice with 3% BSA in PBS. The EdU staining was performed by preparing a working solution with different reagents provided by the kit according manufacture's protocol for 30 minutes in the dark. Then the wells were washed once with 3% BSA in PBS and the nuclei stained with Hoechst 33342 (5μg/ml) for 30 minutes. Images were taken with a Confocal microscope (Olympus IX83) supported with FluoView 4.0 software.

IN VITRO DIFFERENTIATION OF RAT HIPPOCAMPAL STEM CELLS

For the differentiation, NSC were plated in Poly-Ornithine-Laminin coated plates at the density of 2 x 10^6/ 10cm or T75 flask. A complete differentiating media, 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 was used. The media was changed every other day for one week.

CELL IMMUNOFLUORESCENCE

Cells were fixed in ice-cold 100% methanol for 20 minutes on ice. Then the wells were washed 3 times with PBS 1X 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. After that the cells were incubated with rabbit anti-GFAP (1:1000, Abcam) and mouse anti-βIII–Tubulin antibody (1:1000, Sigma) overnight at 4°C. The day after the wells were washed 3 times in PBS for 5 minutes each and then incubated 1 hour at room temperature with Alexa-conjugated antibodies diluted in 1.5% normal serum. Then the wells were washed 1 time in PBS and incubated 10 minutes with DAPI (1:1000, Dako) to stain nuclei. Finally, the pictures were taken with fluorescence microscope for the further quantification with ImageJ software. The slides/plates were store in the dark at 4 °C.

CELL COUNT

EdU incorporation. For each group, EdU positive cells were counted from five different field per well and the ratio of the proliferating cells was obtained by dividing the EdU^+ to the total number of cells present in the field (DAPI).

Differentiation. GFAP and βIII–Tubulin positive cells were counted from five different field per well and the ratio of the differentiation was calculated dividing the number of the positive cells to the total number of cell present in the field (DAPI).

All the images were taken with the Olympus BX51 fluorescent microscope equipped with a cooled CCD camera connected to a computer with MBF image analyzer software and quantified with Image J software.

RNA ISOLATION

Cultured NSC were lysed with TRIazol buffer 0.5ml/10^6 cells. The total RNA was isolated using DIRECT-zol miniprep RNA kit (Zymo Research). Specifically, an equal volume of ethanol 100% was added at the TRIazol and mixed by pipetting. The total volume was transferred in a column provided by the kit and centrifuce 30sec at 13,000 x g. The column was washed 2 times with a pre-washed buffer provided by the kit each time followed by a centrifugation at 13,000 x g for 30sec. The pre-washed buffer was removed with the addition of a wash buffer (provided by the kit) and centrifugation 2min at 13,000 x g. The RNA was eluted by adding 50μL drearily on the column and centrifuged 30sec at 13,000 x g. The RNA was quantified with NanoDrop ONE (Thermo) and immediately used or stored at -80 C.

MICRORNA RETROTRANSCRIPTION

Ten (10) ng of total RNA were used for the retrotranscription of the pool of miRNA by using TaqMan Advanced miRNA Assay kit (ThermoFisher Scientific) performed in a thermocycler following the step described above.

QUANTITATIVE REAL-TIME PCR (QRT-PCR)

qRT-PCR was performed on a MX3000P system (Stratagene, Santa Clara, CA) using Taqman reagents from Applied Biosystems (Foster City, CA) as described above.

STATISTICAL ANALYSIS

Data is expressed as mean +/- SEM. Analysis of variance (ANOVA) followed by multiple comparisons post-hoc Tukey's tests were performed using GraphPad 7 Prism software. Differences were considered significant when $p<0.05$.

RESULTS

NPLT DOES NOT CHANGE THE PROLIFERATING RATE OF NEURAL STEM CELLS ISOLATED FROM TBI RATS.

In order to assess whether NPLT can directly affect NSC, we first isolated NSC from the hippocampus of rats 1 month after FPI (TBI-NSC) and assessed their stemness with specific markers (Figure 3.2), then we treated them with NPLT for 5 minutes analogously to the *in vivo* experiment (Figure 3.1). The analysis of the proliferation rate (EdU incorporation) showed not significant differences between NPLT treated and not treated NSC isolated from TBI rats. Moreover, the rate of TBI NSC proliferation is similar to naïve NSC (Figure 3.3). Naïve NSC treated with NPLT shows a significant increase in proliferation (Figure 3.3).

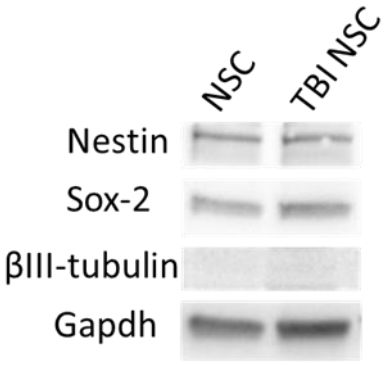

Figure 3.2

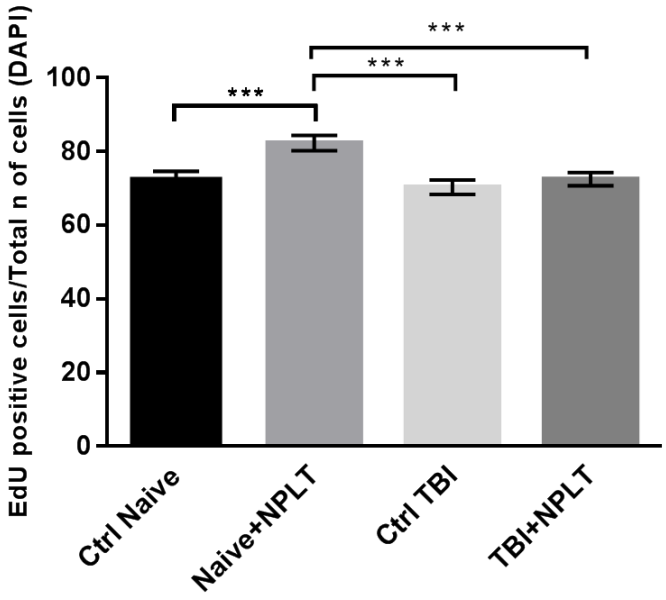

Figure 3.3

Figure 3.2. Western blotting characterization of neural stem cells isolated from TBI and control rats.

Representative blot showing the expression of nestin and Sox-2 (stemness marker), and the absence of the neuronal marker βIII-Tubulin, in NSC isolated from the hippocampus of TBI rats (TBI-NSC) and control uninjured rats (NSC). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is shown as loading control.

Figure 3.3. Analysis of the proliferation rate (EdU incorporation) showed not significant differences between NPLT treated and not treated NSC isolated from TBI rats.

Quantitative analysis of the proliferation (%) of the naïve and TBI NSC -/+ NPLT. N=5; ***$p<0.001$, two-way analysis of variance (ANOVA) with Tukey's post-hoc test.

NPLT IMPROVES THE DIFFERENTIATION OF NEURAL STEM CELLS 7 DAYS AFTER DIFFERENTIATION.

Immediately after treatment with NPLT, NSC were cultured in differentiating media and in coated plate to let the cells attach to the surface. One week after differentiation the expression of GFAP and βIII-Tubulin was assessed by immunohistochemistry to analyze the level of glial and neuronal differentiation respectively. Our analysis shows an overall reduction of the expression of both differentiation markers on TBI NSC as compare to naïve NSC especially for βIII-Tubulin (neuronal marker) (Figure 3.4 A-B). NPLT showed a significant increase of differentiation in both healthy and injured NSC (Figure 3.4 A-B). The expression level of GFAP in TBI-NSC after treatment with NPLT is similar to control naïve. Despite the significant increase of βIII-Tubulin after treatment with NPLT of TBI-NSC, the levels are still dramatically reduced as compare as control naïve (Figure 3.4 B).

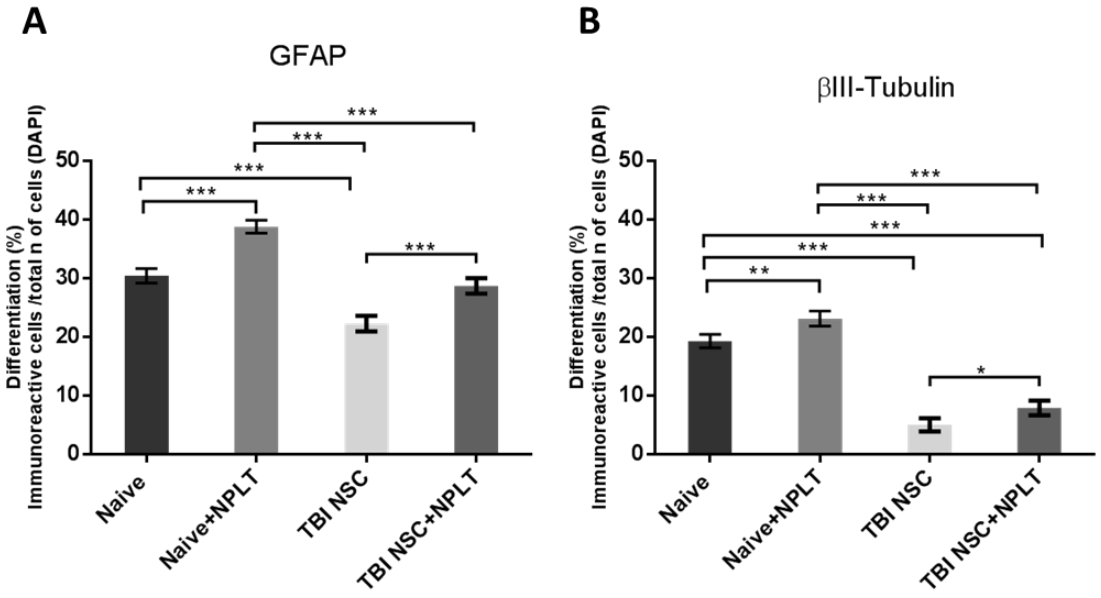

Figure 3.4. NPLT improves the differentiation of neural stem cells 7 days after treatment.

(A-B) Quantitative analysis of the differentiation (%) after 7 days of naïve and TBI NSC -/+ NPLT. (A) GFAP quantitative analysis. (B) βIII-Tubulin quantitative analysis N=5; *p<0.05, **p<0.01, ***p<0.001, two-way analysis of variance (ANOVA) with Tukey's post-hoc test.

NPLT MODULATES THE EXPRESSION OF SPECIFIC MIRNA IN HIPPOCAMPAL NEURAL STEM CELLS ISOLATED FROM TBI RATS.

The expression of miRNA was assessed by qRT-PCR, 24 hours after treatment with NPLT. We found that the expression of miR9, miR25, miR29, miR124, miR137 in TBI-NSC was significantly increased as compared to NSC isolated from naïve rat hippocampus (Ctrl). Moreover, NPLT significantly reduced the expression of all miRNA in TBI-NSC as compared to untreated TBI-NSC (Figure 3.5).

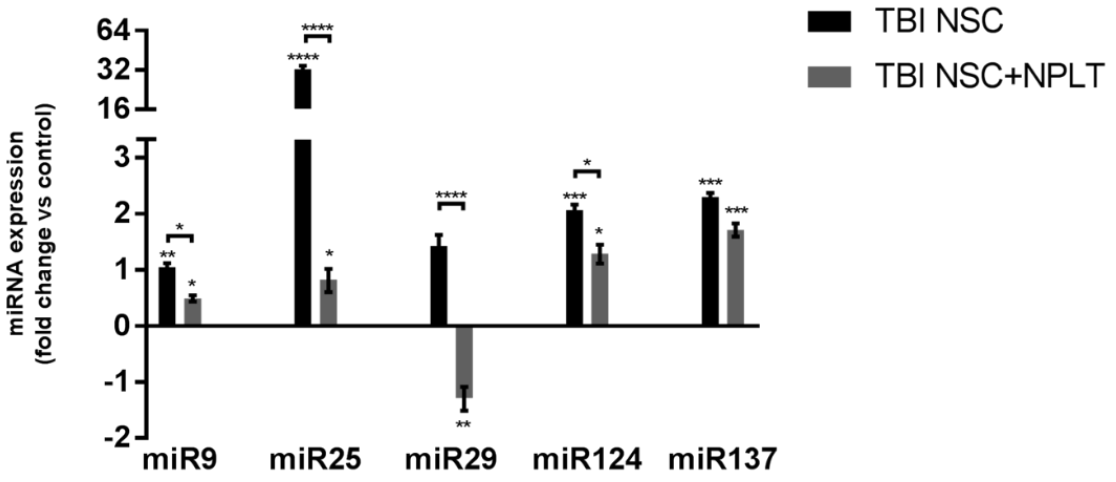

Figure 3.5. NPLT reduces the level of miRNA in hippocampal neural stem cells (NSC) isolated from TBI rats.

qRT-PCR analysis of the expression of miRNA in hippocampal NSC isolated from the dentate gyrus of the hippocampus of TBI rats (TBI-NSC) as compared to TBI-NSC not treated and to NSC from uninjured rats. N=3; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 two-way analysis of variance (ANOVA) with Tukey's post-hoc test.

Discussion

Our *in vivo* data discussed above suggested a possible effect of the NPLT directly on NSC. In order to confirm our hypothesis of a direct effect of NPLT on NSC, we isolated NSC for the hippocampus of TBI rats and we cultured them for a month. We first analyzed the effect on proliferation of NPLT delivered directly on NSC. EdU analysis showed no changes in proliferation of TBI-NSC 24 hours after treatment while is increased in naïve-NSC. These results confirm injury-induced changes that affect directly NSC that are not able to respond to the treatment as the naïve cells probably caused by a metabolic dysfunction. Most of the energy required for neurogenesis are provided by mitochondria (Beckervordersandforth *et al.*, 2017). Mitochondria are the most important organelle for energy production thanks to the cytochrome C, a key enzyme for the electron transport chain, which is stimulated by NPLT because is in the wavelength range of cytochrome C absorption (Yu *et al.*, 2015). NPLT could enhance mitochondrial activity and may increase the rate of proliferation. The absence of the same response in TBI-NSC could be explained by injury-induced changes in NSC that may affect mitochondrial activity. However, further experiments on mitochondrial activity needs to be done to confirm this hypothesis.

The differentiation analysis showed a significant less GFAP and βIII-Tubulin positive cells TBI-NSC as compare to naïve NSC 7 days after differentiation suggesting an impairment/delay in the differentiation after TBI. After treatment with NPLT, the ability to differentiate is improved in naïve NSC and partially recovered in TBI-NSC. However, only glial differentiation of NPLT treated TBI-NSC showed similar level to the naïve cells while neuronal differentiation remains significantly poor.

To further understand the molecular mechanism though which TBI and NPLT directly affect NSC we analyzed miRNA expression. Interestingly, TBI-NSC showed, analogously to LCM samples, a significant increase of the same miRNA (miR9, miR25, miR29, miR124, miR137) indicating a persistence of the modifications trigged by TBI even in daughter cells. This increased expression is reduced after *in vitro* treatment with NPLT, thus further supporting a direct effect on NSC that we discussed above in the *in vivo* experiments. These *in vitro* data further confirmed the therapeutic potential of this novel non-invasive therapy in preventing neurogenesis dysfunction induced by TBI.

CHAPTER 4: THE EFFECT OF MECHANICAL STRETCH INJURY ON HIPPOCAMPAL NSC *IN VITRO*

Introduction

TBI is an episode that trigger many secondary events such as inflammation, increased blood pressure and necrosis. The primary cause of TBI is the mechanical force that lead to brain damage and neuronal loss. The hippocampus is one of the brain region subject to this mechanical stress that affect neurogenesis. Stretch injury model has been extensively used to study *in vitro* the effects of mechanical force on mature neurons but very few information about the effect of mechanical stress on NSC are present in the literature. For this reason, in this aim I tested the effect on proliferation and differentiation of the stretch injury applied directly to the NSC at two different intensities, 30 and 50 PSI with a duration of 50ms to mimic the rapid injury that occurs during a TBI.

Moreover, NSC express several mechano-gated ion channels also known as mechanoreceptors that transduce the mechanical signals from the environment regulating neurogenesis. Piezo1 is one of the most studied mechanoreceptor responsible for mechanical changes detection in the surrounding area and has been demonstrated to regulate neurogenesis. For this reason, I tested the role of Piezo1 on the NSC stretch injury model. Specifically, I used GsMTx4, a peptide isolated from the tarantula venom, to inhibit Piezo1 activity during the stretch injury and analyzed the effect on proliferation and differentiation of stretched NSC.

METHODS

CELL CULTURE

Naïve NSC (Millipore) were cultured in proliferating media (DMEM supplemented with 2% B27 without vitamin A, 20ng/ml FGF, 1% L-Glutamine and 1% antibiotic/antimycotic) and plated onto poly-ornithine and laminin coated plates containing a flexible silicon bottom (BioFlex® Culture Plates, Flex International Corporation). For the differentiation was used a differentiating media (DMEM supplemented with 2% B27 with vitamin A, 1% L-Glutamine, 1% FBS and 1% antibiotic/antimycotic) as described above.

STRETCH INJURY

Twenty-four hours after seeding in proliferating media, Hipp-NSC were stretched using a Cell injury controller II (Earl Ellis laboratory). The device was connected with a nitrogen tank and different parameters were set: duration of the boost 50 ms, high flow output from the nozzle and pressure to 30 or 50 PSI.

To analyze the effect on proliferation immediately before the stretch EdU was added in the media and the cells were fixed 24 hours after stretch. For the miRNA analysis, the cells were lysed 24 hours after stretch with Triazol. For the differentiation, proliferating media was switched to differentiating one immediately before stretch and changed every other day for 1 week.

LACTATE DEHYDROGENASE ASSAY (LDH)

The different pressures will cause different grade of damage in the cells that will be tested by lactate dehydrogenase release. When the integrity of the cell membrane is compromised LDH is released into the media. 50µl of media was mixed with 50µl of tetrazolium salt (INT) according manufacture's protocol (ThermoFisher). INT is converted by LDH into a red formazan product and quantified by measuring the absorbance at 490 nm as measure of damage.

PROLIFERATION

Immediately after stretch the cells were incubated with 5-ethynyl-2′-deoxyuridine (EdU) and 24 hours after injury the cells were fixed with ice-cold 100% methanol on ice. The rate of proliferation of the Hipp-NSC will be assessed through the analysis of EdU incorporation as described above.

CELL TREATMENT WITH PIEZO1 ANTAGONIST

GsMTx4 an antagonist of the mechanoreceptor Piezo1 was dissolved in PBS according manufacture's data sheet (Alomone Labs) to the final concertation of 1mM (Bae *et al.*, 2011). The cells were treated for 30 min before stretch by diluting GsMTx4 in the media to the concentration of 5µM as previously reported (Pathak *et al.*, 2014) and maintained in the media for 24 hours after stretch. 24 hours after stretch the cells were fixed for EdU analysis or lysed with Triazol to collect the total mRNA. For the differentiation, 24 hours after stretch the media with the antagonist was replaced with fresh media without the antagonist.

Moreover, a second set of cells were differentiated after stretch by using standard differentiation media (DMEM supplemented with 2% of B27 with vitamin A, 1% L-Glutamine and 1% antibiotic/antimycotic). To test the level of differentiation will be evaluated the expression of standard marker such as doublecortin (DCX), NeuN, βIII-Tubulin and GFAP through Western Blotting and immunofluorescence after 7 days of differentiation.

PROTEIN ISOLATION AND WESTERN BLOTTING ANALYSIS

Control proliferating NSC (for cell characterization) and NSC one week after injury cultured in differentiating media were lysed with RIPA buffer (with protease inhibitor) and proteins were quantified with BCA kit as described above.

Proteins (25µg per sample) were resolved by SDS-PAGE using precasted 4-20% gradient gels (Biorad) and transferred to polyvinylidene fluoride (PVDF) membranes according the protocol used in the previous experiments in this thesis. The following antibodies were used: rabbit anti-GFAP (1:1000, Abcam); mouse anti-βIII–Tubulin antibody (1:1000, Sigma); mouse anti-Sox2 (1:1000, Cell Signaling); mouse anti-NeuN (1:1000, Millipore); mouse anti-Nestin antibody (1:1000, Millipore) overnight at 4°C. The quantification was performed with Image lab software (BioRad) and HRP conjugated Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:3000, Thermo Fisher) was used as normalizer.

IMMUNOFLUORESCENCE ANALYSIS

To confirm Piezo1 expression in our NSC an immunostaining was performed: attached NSC were fixed in ice-cold 100% methanol on ice and the immunostaining was performed according the protocol described above. The following antibodies were used: rabbit anti-Piezo1 (1:200, AlomoneLab) and mouse anti-Nestin antibody (1:1000, Millipore) overnight at 4°C.

For the differentiation analysis, one week after injury and differentiation in differentiating media, the cells were fixed in ice-cold 100% methanol on ice and the antibody hybridization was performed as described above. The following antibodies were used: rabbit anti-GFAP (1:1000, Abcam) and mouse anti-βIII–Tubulin antibody (1:1000, Sigma) overnight at 4°C.

The images were viewed with a fluorescence microscope BZ-X710 (Keyence) supported by BZ-X analyzer software (Keyence).

CELL COUNT

EdU incorporation. For each group, EdU positive cells were counted from five different field per well and the ratio of the proliferating cells was obtained by dividing the EdU^+ to the total number of cells present in the field (DAPI).

Differentiation. GFAP and βIII–Tubulin positive cells were counted from five different field per well and the ratio of the differentiation was calculated dividing the number of the positive cells to the total number of cells present in the field (DAPI).

Images were taken with a Confocal microscope (Olympus IX83) supported with FluoView 4.0 software and quantified with Image J software.

RNA ISOLATION

Cultured NSC were lysed with Triazol buffer 0.5ml/10^6 cells. The total RNA was isolated using DIRECT-zol miniprep RNA kit following the steps described above.

MICRORNAS RETROTRANSCRIPTION

Ten (10) ng of total RNA were used for the retrotranscription of the pool of miRNA by using TaqMan Advanced miRNA Assay kit (ThermoFisher Scientific) performed in a thermocycler following the steps described above.

QUANTITATIVE REAL-TIME PCR (QRT-PCR)

qRT-PCR was performed on a MX3000P system (Stratagene, Santa Clara, CA) using Taqman reagents from Applied Biosystems (Foster City, CA) as described above.

STATISTICAL ANALYSIS

Data is expressed as mean +/- SEM. Analysis of variance (ANOVA) followed by multiple comparisons post-hoc Tukey's tests were performed using GraphPad 7 Prism software. Differences were considered significant when $p<0.05$.

Results

CELL CHARACTERIZATION

Different substrates have important influences in cell activity and growth (Blaschke *et al.*, 2019). For this reason, we first confirmed that NSC cultured attached on the silicon surface of the BioFlex plates maintain their stem cell phenotype as indicated by the expression of stemness markers (Figure 4.1). Then we confirmed the expression of Piezo1. Specifically, NSC were grown in proliferation media attached to the plate and a qRT-PCR was performed to confirm the presence of Piezo1 mRNA (Figure 4.2 A). Moreover, an immunofluorescence was performed showing the expression of the stemness marker Nestin and the presence of Piezo1 on the cell surface (Figure 4.2 B).

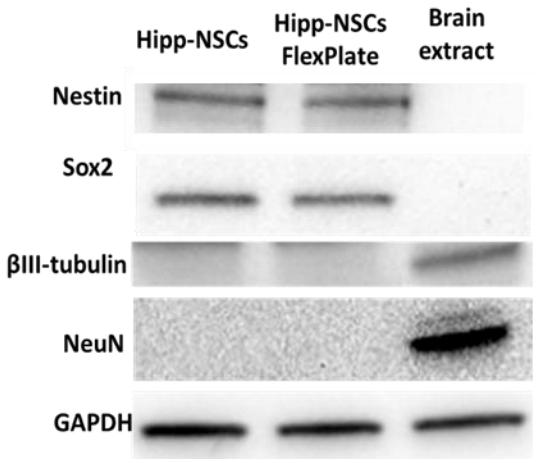

Figure 4.1. Neural stem cells attached to BioFlex plates maintain their stem cell phenotype.

Western blotting showing the expression of stemness markers (Nestin and Sox2) and the absence of differentiating markers (βIII-Tubulin and NeuN) in both NSC in suspension and NSC cultured in BioFlex plate. GAPDH: loading control.

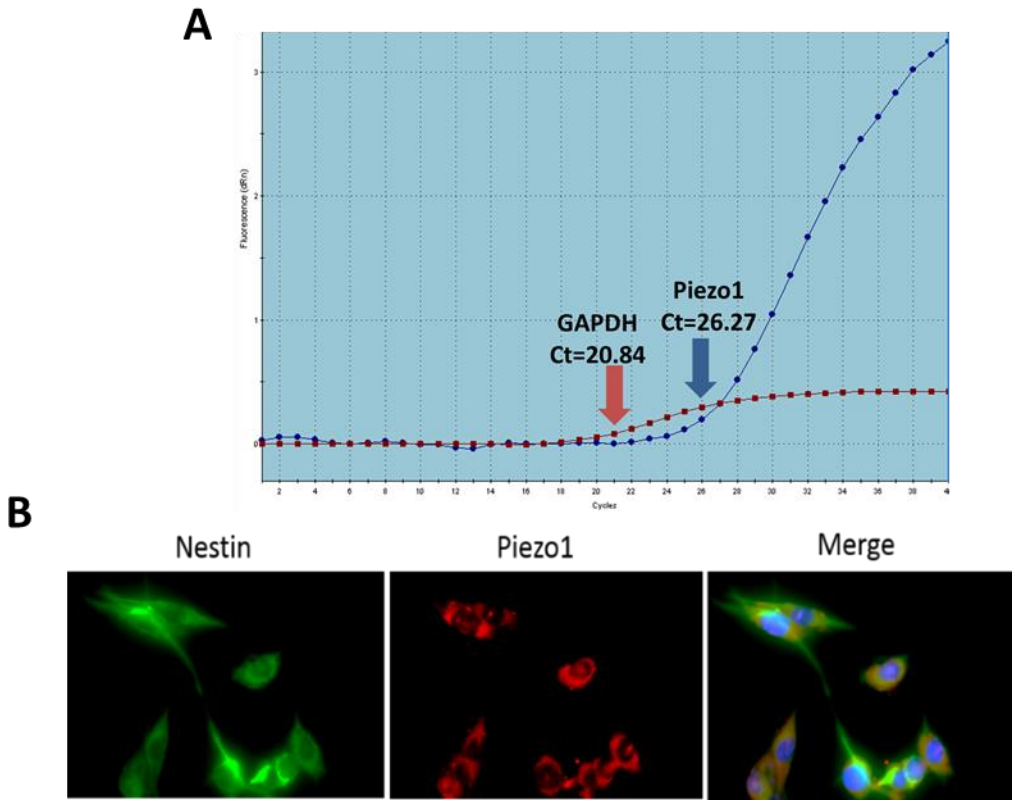

Figure 4.2. Neural stem cells express the mechanoreceptor Piezo1.
(A) qRT-PCR showing the expression of Piezo1 mRNA in NSC (N=8). (B) Immunofluorescence showing the expression of Piezo1 protein on NSC surface. Nestin: marker of stemness. DAPI: nuclei.

Stretch injury induce LDH release of NSC

To validate the effectiveness of our stretch injury model we stretched mature neurons and quantified LDH release to assess the level of injury. Both 30PSI and 50PSI injury levels significantly increased the level of LDH release (Figure 4.3 A). Then we performed the LDH assay in NSC +/- GsMTx4. Our results showed a significant increased level of LDH released from the NSC stretched with 50 PSI but not with 30PSI as compared

to not stretched control (Figure 4.3 B). Stretch injury does not significant increase LDH release on NSC treated with GsMTx4 as compare to not stretched GsMTx4 treated NSC (Figure 4.3 C).

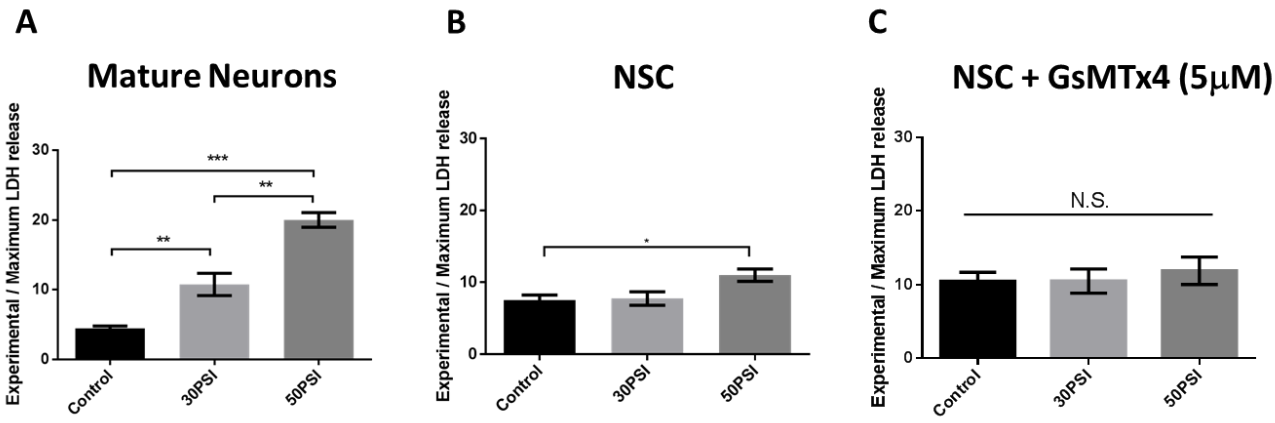

Figure 4.3. Stretch injury induce lactate dehydrogenase (LDH) release of NSC.
(A) LDH release from neurons stretched with 30 and 50 PSI. (B) LDH release from neural stem cells (NSC) stretched with 30 and 50 PSI. (C) LDH release from neural stem cells (NSC) stretched with 30 and 50 PSI + GsMTx4 (5μM). N=3; *$p<0.05$, **$p<0.01$ two-way analysis of variance (ANOVA) with Tukey's post-hoc test.

GsMTx4 prevents stretch injury-reduction of NSC proliferation

To evaluate the effect of stretch injury on NSC proliferation, cells were cultured in proliferating media in presence of EdU for 24 hours after stretch +/- GsMTx4. Immunofluorescence analysis showed a decrease of the proliferating cells in both 30 and 50 PSI in a dose-dependent manner. The injury-induced reduced proliferation is prevented by the treatment with GsMTx4 before stretch (Figure 4.4).

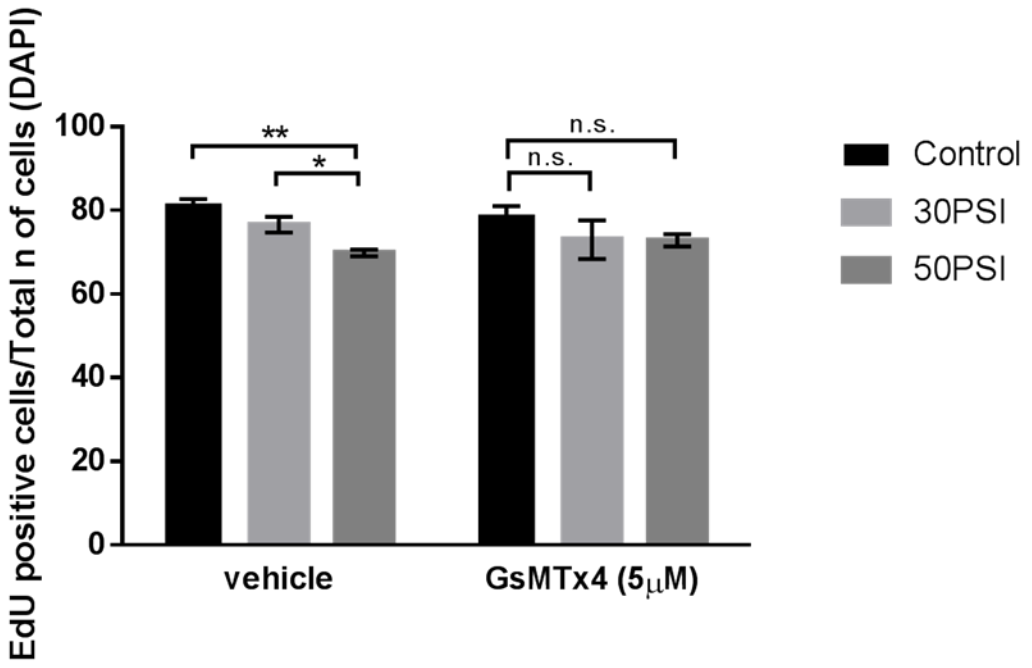

Figure 4.4. GsMTx4 prevents stretch injury-reduction of NSC proliferation (EdU). Quantitative analysis of the proliferation (%) of the neural stem cells (NSC) stretched with 30 and 50 PSI -/+ GsMTx4 (5μM). N=3; *p<0.05, **p<0.01 two-way analysis of variance (ANOVA) with Tukey's post-hoc test.

GsMTx4 prevents stretch injury-reduction of NSC differentiation.

Immediately after stretch, proliferating media was switched to differentiating media to analyze the effect of the stretch injury on NSC differentiation with and without GsMTx4. One week after differentiation cells were lysed and proteins were purified for western blot analysis. Our data shows a reduction of the glial marker GFAP and neuronal marker βIII-Tubulin in a dose dependent manner (Figure 4.5 A and B). This result was further confirmed with immunofluorescence staining. Specifically, one week after differentiation NSC were fixed and immunostained for differentiating markers (GFAP and βIII–Tubulin) (Figure 4.6 A and B).

GsMTx4 treatment prevents this injury-induced reduction of GFAP and βIII–Tubulin markers (Figure 4.7 A and B). Interestingly, immunofluorescence analysis showed that GsMTx4 treatment reverts the reduction of neuronal differentiation and significantly increase it after stretch (Figure 4.8 A and B). The reduction of glial differentiation is not prevented by GsMTx4 treatment. (Figure 4.8 A and B).

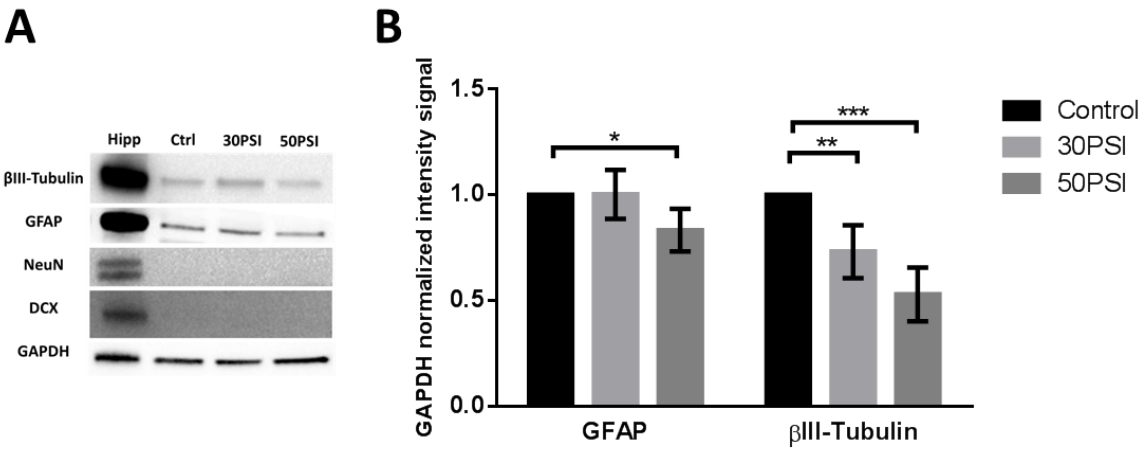

Figure 4.5. Western blotting analysis showed reduced neural stem cells differentiation 7 days after stretch injury.

(A) Western blotting analysis of differentiating markers βIII-Tubulin, GFAP, DCX and NeuN 7 days after stretch of NSC. Hipp; protein extract from rat hippocampus; Ctrl: not stretched neural stem cells; GAPDH: loading control. (B) Quantification analysis of GFAP and βIII-Tubulin normalized by GAPDH intensity signal. N=5; *$p<0.05$, **$p<0.01$. ***$p<0.001$ two-way analysis of variance (ANOVA) with Tukey's post-hoc test.

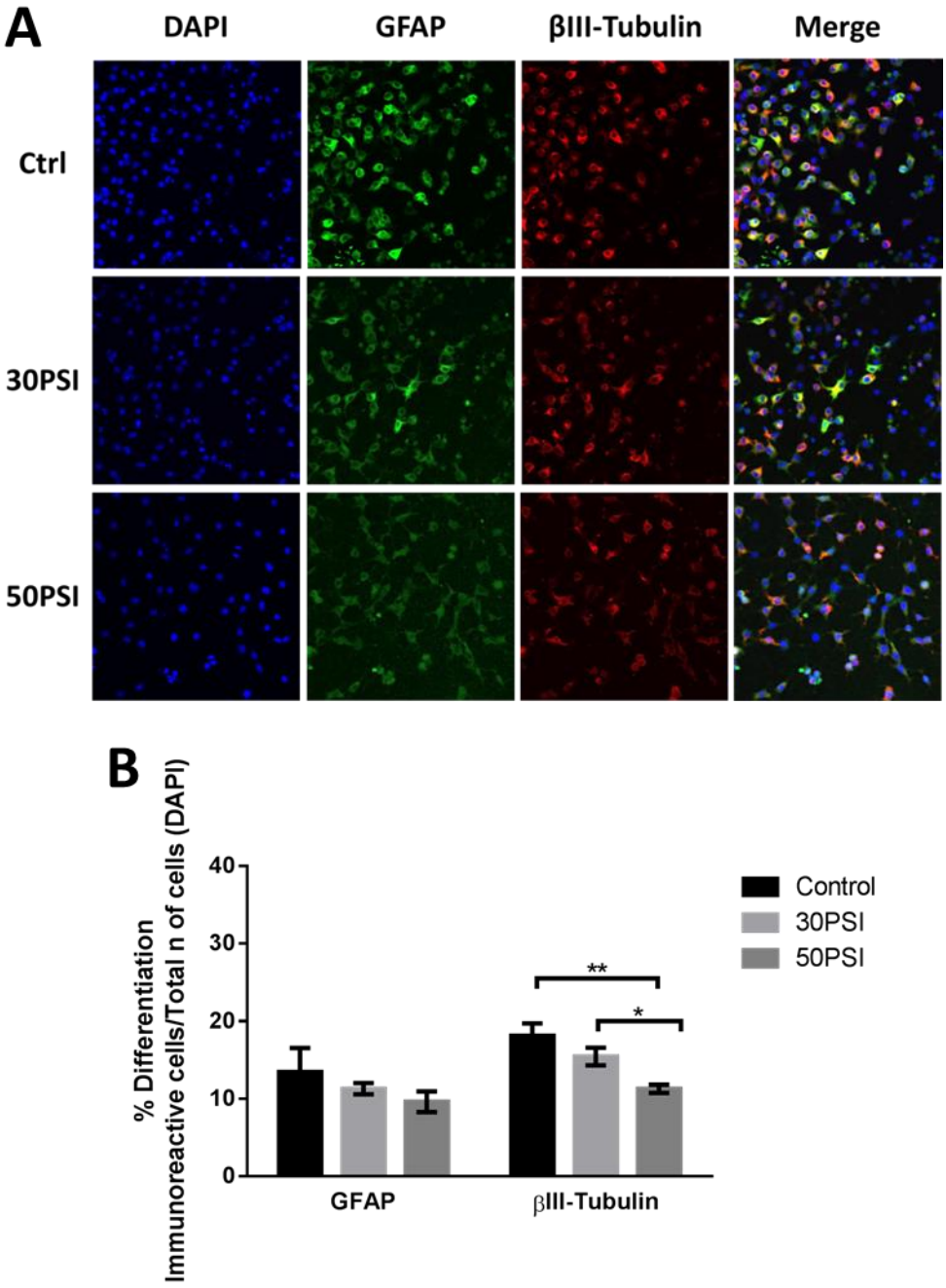

Figure 4.6. Immunofluorescence analysis showed reduced neural stem cells differentiation 7 days after stretch injury.

(A) Representative images of stretched NSC stained for βIII-Tubulin and GFAP 7 days after stretch; Ctrl: not stretched neural stem cells; (B) Quantification analysis of GFAP and βIII-Tubulin normalized by total number of cells (DAPI). N=5; **p<0.01. two-way analysis of variance (ANOVA) with Tukey's post-hoc test.

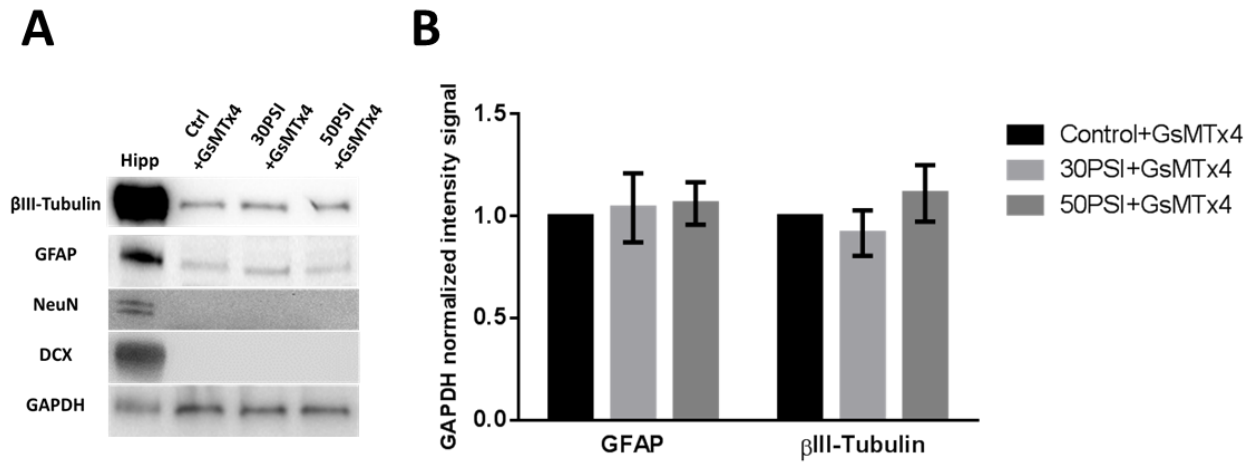

Figure 4.7. GsMTx4 prevents injury-induced reduction of neural stem cells 7 days after stretch injury.

(A) Western blotting analysis of differentiating markers βIII-Tubulin, GFAP, DCX and NeuN 7 days after stretch of NSC treated with GsMTx4 (5μM). Hipp; protein extract from rat hippocampus; Ctrl: not stretched neural stem cells; GAPDH: loading control. (B) Quantification analysis of GFAP and βIII-Tubulin normalized by GAPDH intensity signal. N=3; N.S. two-way analysis of variance (ANOVA) with Tukey's post-hoc test.

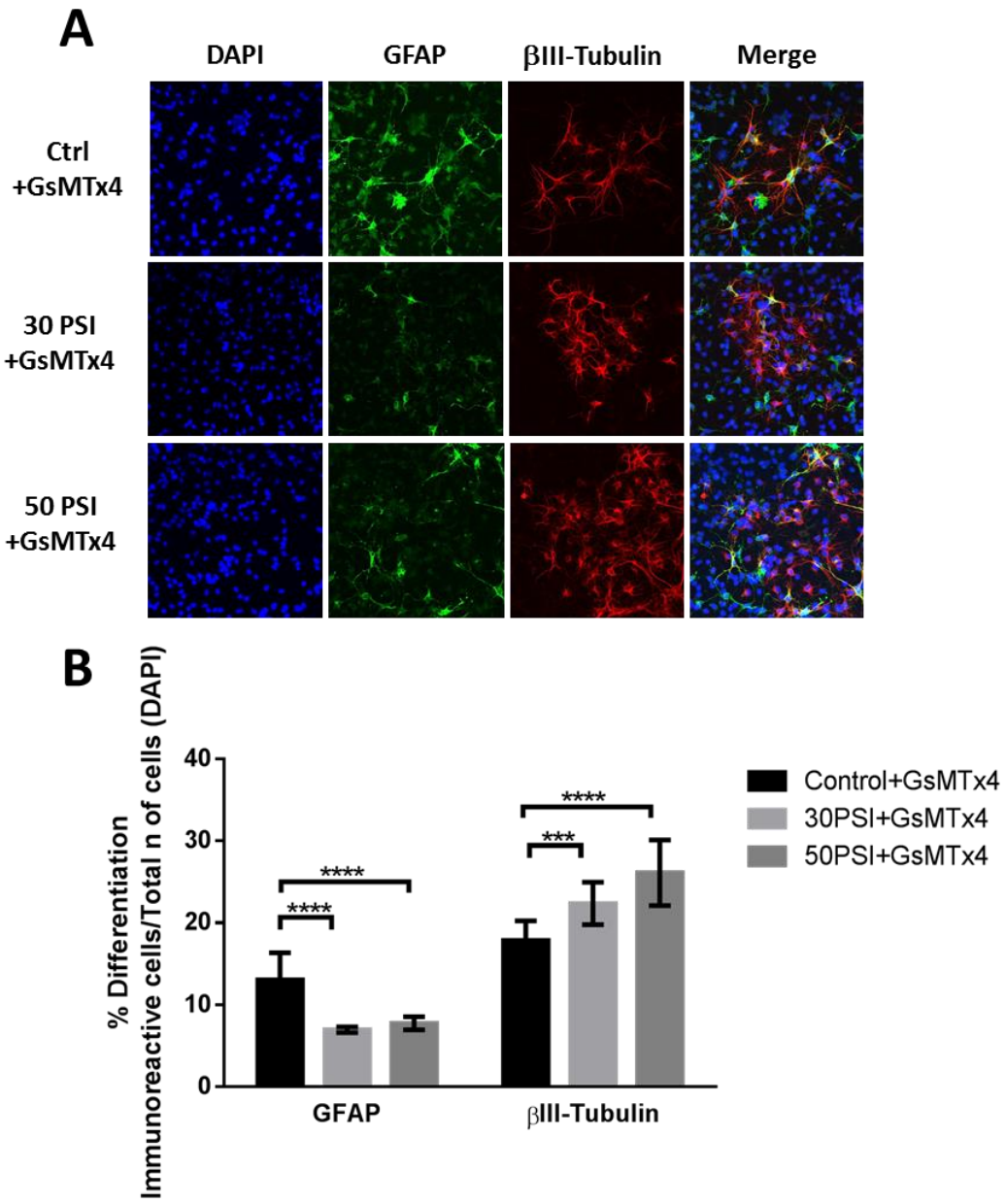

Figure 4.8. GsMTx4 increases neuronal differentiation after stretch and does not prevent the reduction of glial differentiation of neural stem cells 7 days after stretch injury.

(A)Representative images of stretched NSC stained for βIII-Tubulin and GFAP 7 days after stretch of NSC treated with GsMTx4 (5μM); Ctrl: not stretched neural stem cells; (B) Quantification analysis of GFAP and βIII-Tubulin normalized by total number of cells (DAPI). N=3; ***p<0.001, ****p<0.0001 two-way analysis of variance (ANOVA) with Tukey's post-hoc test.

STRETCH INJURY AND GSMTX4 INCREASE THE EXPRESSION OF SPECIFIC MIRNA IN NSC.

Evidences in literature showed the important role of Piezo1 in cell fate determination (Pathak *et al.*, 2014). To determine whether stretch injury can modulate the expression of specific miRNA, known to regulate neurogenesis we isolate RNA from stretched and not stretched NSC +/- GsMTx4. Specifically, 24 hours after stretch injury qRT-PCR was performed to assess miRNA expression. Our data show an increased level of regulatory miRNA induced by the stretch as compare to not stretched controls (Figure 4.9). Interestingly, the presence of GsMTx4 significantly increases miRNA expression without stretch (figure 4.10). Moreover, in the presence of GsMTx4, a 50 PSI stretch injury decreased miRNA expression while a 30 PSI stretch injury did not change their expression as compared to un-injured cells with the inhibitor (Figure 4.10).

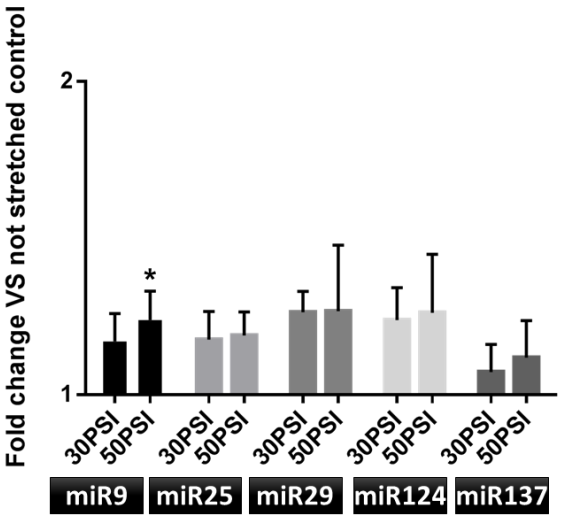

Figure 4.9. Stretch injury increases miRNA expression of neural stem cells.
qRT-PCR analysis of the expression of miRNA of stretched NSC as compared to not stretched NSC. N=8; *p<0.05, two-way analysis of variance (ANOVA) with Tukey's post-hoc test.

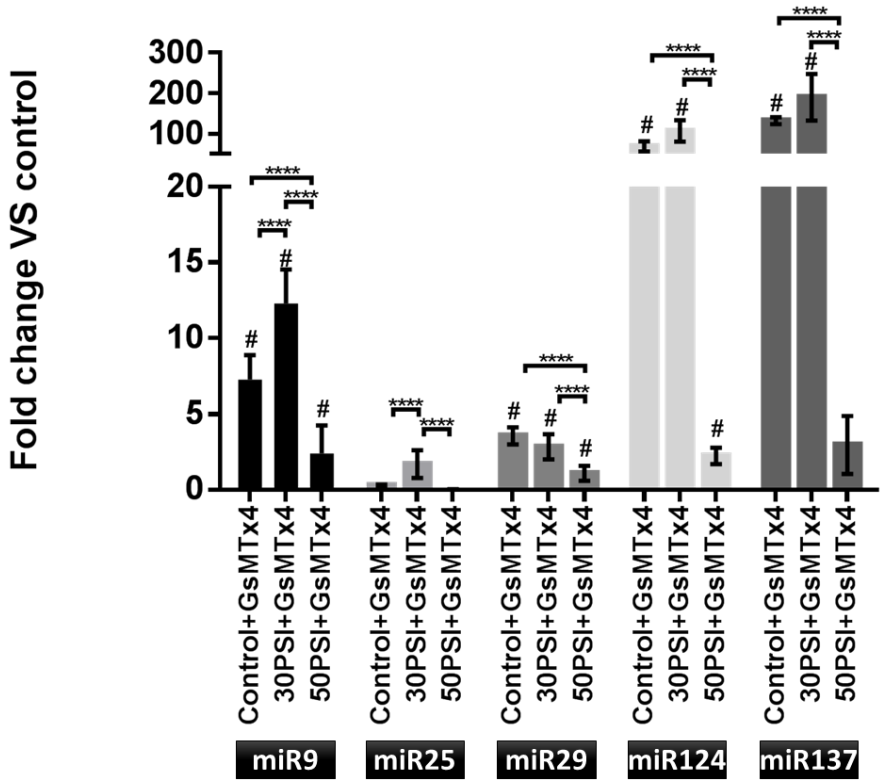

Figure 4.10. GsMTx4 significantly increases miRNA expression of NSC and prevents stretch induced upregulation.

qRT-PCR analysis of the expression of miRNA of stretched NSC as compared to not stretched NSC. N=3; ****p<0.0001, #p<0.0001 as compared to control. Two-way analysis of variance (ANOVA) with Tukey's post-hoc test.

Discussion

TBI is characterized by the disruption of normal brain function caused by an external mechanical force (Hemphill *et al.*, 2015). *In vitro* stretch injury is an establish method to evaluate the effect of the mechanical force applied directly to cells in culture (Tavalin *et al.*, 1995; Geddes *et al.*, 2003). Few reports showed the important role of the activation of Piezo1 on cell fate specification of NSC in neurogenesis (Pathak *et al.*, 2014). Thanks to the stretch injury model we were able to test the effect of mechanical stress directly to hippocampal neural stem cells. Moreover, to test our hypothesis on the importance of mechano-transduction signals in NSC proliferation and differentiation we tested the effect of stretch injury on NSC in the presence of GsMTx4, a peptide isolated from tarantula's venom, known to act as Piezo1 antagonist (Sugimoto *et al.*, 2017).We first confirmed that NSC growth attached to a not hard-plastic supports, such as the stretchable flex bottom plate, maintain their stemness and then we confirmed the expression of Piezo1 on NSC surface.

Two level of injury were tested: 30 and 50PSI. Stretch injured neurons showed a significant increase with both intensities in a dose dependent manner while NSC showed a significant increased level of injury (LDH release) only with the highest intensity (50 PSI). These results confirm the effectiveness of our injury model and suggest a better resistance to the mechanical stress of NSC as compared to mature neurons. The presence of GsMTx4 in the media of cultured NSC significant increases the LDH release highlighting the importance of the basal activity of Piezo1 for the cells. Indeed, a tonic activity of Piezo1, that allows Ca2+ influx important for cell function, has been previously shown in astrocytes and neurons (Maneshi *et al.*, 2015; Toth *et al.*, 2016). The stretch injury also

induces the influx of ions such as Ca2+ that perturbs the ion balance leading to cytotoxicity. Interestingly, the treatment with GsMTx4 does not further increase the level of LDH after stretch confirming an important role of Piezo1 in mechano-transduction and a possible protective effect of the inhibition of Piezo1 during stretch injury. The protective effect of Piezo1 inhibition has been further confirmed with the analysis of cell proliferation through EdU incorporation. Stretched NSC, with 50 PSI but not 30 PSI, showed a decreased level of proliferation that is prevented by treatment with GsMTx4.

In order to investigate a possible effect of the stretch injury on the differentiation, we differentiated stretched NSC for 7 days and then we performed western blotting and immunofluorescence analysis by using specific antibodies against neuronal and glial markers. We found a dose dependent decrease of both neuronal and glial differentiation after stretch injury. Surprisingly, GsMTx4 not only prevented the reduction of neuronal differentiation in stretched NSC but significantly increased differentiation as compared to not-stretched control. Glial differentiation was not prevented by GsMTx4 and is significantly lower with both 30 and 50 PSI stretch. Recent studies showed a "mechanical memory" of cultured stem cells that directs the cell-lineage specification (Yang *et al.*, 2014). Specifically, there are evidences that mechanical changes, induced either by pharmacological inhibition of mechanoreceptors or changes in substrate stiffness, induce neuronal differentiation of NSC. Our findings showed significant reduction of NSC differentiation in response to mechanical stress that is substantially with the inhibition of Piezo1.

As described above, miRNA are known to play a key role in neurogenesis. For this reason we investigated the effect of stretch and Piezo1 inhibition on the expression of

miRNA known to regulate neurogenesis. We demonstrated above that selected regulatory miRNA are up-regulated after TBI in the SGZ of the hippocampus DG. Remarkably, we found the same increased trend of miRNA expression in NSC after stretch. This suggest that the increased miRNA *in vivo* is caused, at least partially, by the mechanical stress to which NSC undergoes during TBI. The presence of GsMTx4 in the media significantly increases miRNA expression confirming the importance of the basal activity of Piezo1 for the cells. Indeed, there are reports showing a tonic activation of this mechanoreceptor that is fundamental for cell activity (Gottlieb & Sachs, 2012). Interestingly, NSC treated with GsMTx4 and subjected to 30 PSI showed the increased expression of miRNA while 50PSI significantly downregulate their expression as compared to the not stretched controls. This finding suggests a different mechanism that is activated in response to the intensity of the stretch that Piezo1 independent that needs further investigation.

All together, these data confirm the key role of Piezo1 in the transduction of the mechanical stress that affect neurogenesis. Some of the injury-induced changes (proliferation and differentiation) are prevented by the inhibition of Piezo1 suggesting a direct role of this mechanoreceptor during stretch. However, other changes are induced by GsMTx4 alone (increase of both LDH and miRNA expression) suggesting the important role of the basal activity of Piezo1 for the cell. For this reason, further experiments are necessary to better understand the molecular mechanism of mechano-transduction signals during stretch injury with different intensities.

CHAPTER 5: GENERAL DISCUSSIONS AND CONCLUSIONS

Neurogenesis is one of the most dynamic process in the adult brain that leads to the formation of new neurons (Fuchs & Flügge, 2014). Neurogenesis occurs throughout life in restricted areas of the mammalian brain such as the hippocampus due to the presence of neural stem cells (Jonas & Lisman, 2014). This tightly regulated event is responsible for hippocampal plasticity and key processes like memory formation and cognitive functions (Costa *et al.*, 2015). Moreover, neurogenesis is a physiological process that is influenced by different factors such as aging, that dramatically reduces it, or physical activity, that stimulates it and leads to beneficial cognitive effects (Kempermann, 2015; Vivar, 2015). Besides physiological condition, neurogenesis is affected by pathological states such as Alzheimer's disease and after traumatic brain injury (TBI) (Yu *et al.*, 2016). TBI is trigged by an external mechanical force that disrupts normal brain function and, in particular, the hippocampus, with dramatic effects for memory and cognition (Mendez *et al.*, 2015). The unpredictability of this event and the secondary effects make difficult the development of an effective cure (DeKosky & Asken, 2017). In the last decade research has been focused on non-invasive therapies that showed to be effective and ameliorate the brain function of TBI patients (Henderson *et al.*, 2015). Among these therapies the most promising are the delivery of near infrared light and ultrasound stimulation. Thanks to our collaborators we developed a novel nano-pulsed laser therapy (NPLT), an innovative device that combines the two therapies through the delivery of short high-energy pulses of near infrared laser that generates low intensity ultrasound waves in the brain.

In this work, we tested the beneficial effect of this novel therapy in a rat model of TBI and analyzed its therapeutic potential.

Our data confirmed the dramatic increase of the inflammatory response after injury and the increase of the ectopic migration of neuronal progenitor cells known as aberrant neurogenesis. The transcranial delivery of NPLT for 5 minutes 1 hour after injury prevents the aberrant migration of neural progenitor cells (DCX) in the hippocampus hilus and in the granular layer. Moreover, my data suggest that these beneficial effects are mediated, at least partially, by the direct effect on NPLT on neural stem cells. Indeed, injury-induced up-regulation of regulatory miRNA are significantly down-regulated after NPLT treatment in both SGZ and NSC isolated from the hippocampus of injured rats.

Furthermore, I demonstrated that some of these changes are induced by the activation of mechanoreceptors in NSC isolated from the hippocampus. Specifically, mechanical stress induces cell toxicity and decreases NSC proliferation. Moreover, rapid stretch injury of NSC reduces/delays cell differentiation. Interestingly, some of these changes are prevented by the blockage of the mechanoreceptor Piezo1 that has been previously shown to play an important role on neurogenesis.

In conclusion, this work demonstrates the beneficial effects of the NPLT device as novel non-invasive treatment for TBI, a condition that is still without a cure. In addition, thanks to our *in vitro* model, it is possible, in future studies, to analyze the effect of TBI on hippocampal neural stem cells leading to neurogenesis dysregulation. Moreover, the treatment of NSC with the Piezo1 inhibitor GsMTx4 prevents some of the stretch-induced changes in NSC and for this reason future studies are necessary to elucidate the effect of Piezo1 blockage preventing secondary effects after brain trauma.

CHAPTER 6: FUTURE DIRECTIONS

This extensive work shows the importance to find a treatment for TBI and how our NPLT device can be a possible candidate to do this. However, there are still uncovered areas that need to be further investigated. First, it will be important to analyze the effect of NPLT on epilepsy. TBI causes the aberrant migration of neural progenitor cells in the hippocampus that can lead to the post traumatic epilepsy (PTE). With this work we show that NPLT prevents aberrant neurogenesis and will be important to test with behavioral studies if NPLT could also reduce or prevent the development of epilepsy after trauma.

My results showed no effect of NPLT on microglia activation, but it is known how microglia plays a critical role on neurogenesis mediated by the release of cytokines and chemokines. Indeed, microglia are a special immune cell type with the ability to switch between pro-inflammatory to anti-inflammatory phenotypes. For this reason, it will be important investigate the cytokines profile after treatment of NPLT to assess a possible switch to cytokines with anti-inflammatory effects.

I showed that NPLT prevents the injury induced upregulation of miRNA involved in neurogenesis regulation. So, will be important to clarify if the beneficial effects of NPLT are mediated by specific miRNA. A possible approach is the inhibition (siRNA) of the expression of specific miRNA and then test if the effect of NPLT is reduced/lost.

The stretch injury model showed important molecular changes in NSC that are induced by mechanical stress. With this model we were able to replicate some molecular changes (miRNA upregulation and decreased differentiation) that we found in TBI rats. For this reason, the stretch injury model will be a powerful tool to further investigate how the mechanical stress affect neurogenesis and NSC. The use of GsMTx4 showed the important role of Piezo1 in the mechano-transduction. It will be interesting to analyze *in vivo* the use of GsMTx4 and determine whether there is an attenuation of the changes trigged by TBI. However, the limitation of the use of GsMTx4 is its specificity because

can block other channels such as TRPV1, TRPV4 and Trek. To confirm the specific role of Piezo1 during stretch injury will be important the use of Piezo1 agonist Yoda1. Yoda1 is a synthetic compound that acts as agonist only for Piezo1. The use of Yoda1 will help to further clarify the role of Piezo1 for the stretch injury.

Another important aspect that needs to be elucidate is the different effect of different stretch injury intensities on miRNA expression. No substantial differences were found in the proliferation and differentiation of NSC stretched with 30 and 50 PSI in presence of GsMTx4. However, miR124 and miR137 are the two most upregulated miRNA with GsMTx4 and the two most affected by the two different stretch intensities. These miRNA are extremely important for the migration of the cells and will be necessary a migration assay to test a possible different migrating potential induced by the two different intensities.

Finally, it will be interesting to test the application of NPLT directly on the model of stretch injury. Our data showed that the mechanical stretch can induce some of the changes present in TBI-NSC such as the increase of miRNA expression. Moreover, NPLT applied to TBI-NSC significantly reduces upregulated miRNA. Therefore, will be important to test whether NPLT reverts stretch-induced miRNA upregulation in NSC.

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Vita

Emanuele Mocciaro was born in Palermo, Italy, on January 10th, 1987. He developed his passion for Biological Sciences since the High School. Emanuele obtained his bachelor's degree in Medical Biotechnology from the University of Palermo in 2011 and then he moved to Bologna where he obtained his master's degree in Pharmaceutical Biotechnology *cum laude* in 2013. Emanuele decide to complete his formation with a doctorate and was selected for a highly competitive combined PhD program establish between the University of Palermo and the University of Texas Medical Branch in Galveston. This program gives the unique opportunity to earn the PhD degree from both universities. Indeed, Emanuele successfully defended his thesis in March 2018 and earned the European PhD from the University of Palermo. Emanuele joined the laboratory in the Anesthesiology Dept. at The University of Texas Medical Branch in September 2015. From the very beginning Emanuele has demonstrated his passion for neuroscience and in particular for his research on traumatic brain injury. Specifically, Emanuele research focuses on a very innovative and highly translational project aimed at studying the therapeutic potentials of transcranial delivery of nano-pulsed near infrared laser light for the treatment of traumatic brain injury. During the three and half years in the Anesthesiology Department, Emanuele presented his remarkable work during national and international meetings. Despite his increasing responsibilities of his graduate training, he was a teacher assistant in the neuroanatomy course for the medical students and has mentored a student in the "Bench mentor program" for which he was awarded "Best mentor of the year". Notwithstanding, he has maintained top grades and passed the written and oral PhD qualifying exams for the admission to candidacy during the Fall semester of 2017. He received the Travel Grant Award from the National Neurotrauma society and scholarships from the UTMB such as the Robert Bennet Scholarship and the Travel Grant Award from the UTMB.

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1. **Emanuele Mocciaro**, Auston Grant, Rinat O. Esenaliev, Irene Y. Petrov, Yuriy Petrov, Jutatip Guptarak, Margaret A. Parsley, Donald S. Prough, Maria-Adelaide Micci. Non-invasive transcranial nano-pulsed laser therapy corrects aberrant hippocampal neurogenesis in a rat model of traumatic brain injury. *In review. Journal of neuroscience*

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Abstracts:

1. **E. Mocciaro**, A. Grant, R. Esenaliev, I. Petrov, Y. Petrov. E. Bishop, K. Johnson, I. Bolding, M.A. Parsely, D.S. Prough, M.A. Micci, Non-invasive transcranial nano-pulsed laser therapy corrects aberrant hippocampal neurogenesis in a rat model of traumatic brain injury, 2nd Neurogenesis conference, Nassau, Bahamas, March 5-8, 2019

2. **E. Mocciaro**, A. Grant, R. Esenaliev, I. Petrov, Y. Petrov. E. Bishop, K. Johnson, I. Bolding, M.A. Parsely, D.S. Prough, M.A. Micci, Non-invasive transcranial nano-pulsed laser therapy corrects aberrant hippocampal neurogenesis in a rat model of traumatic brain injury, *Mission Connect symposium 2018*, Houston, Texas, December 8, 2018

3. **E. Mocciaro**, A. Grant, R. Esenaliev, I. Petrov, Y. Petrov. E. Bishop, K. Johnson, I. Bolding, M.A. Parsely, D.S. Prough, M.A. Micci, Non-invasive transcranial nano-pulsed laser therapy corrects aberrant hippocampal neurogenesis in a rat model of traumatic brain injury, *Society for Neuroscience 2018*, San Diego, California, November 2-7, 2018

4. **E. Mocciaro**, A. Grant, R. Esenaliev, I. Petrov, Y. Petrov. E. Bishop, K. Johnson, I. Bolding, M.A. Parsely, A. Unterainer, C. Szabo, D.S. Prough, M.A. Micci, Modulation of adult neurogenesis by Nano-Pulsed Laser Therapy (NPLT) in a rat model of Traumatic Brain Injury, *International Neurotrauma Symposium 2018*, Toronto, Canada, August 11-16, 2018

5. **E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov. K. Johnson, I. Bolding, M.A. Parsely, A. Unterainer, C. Szabo, D.S. Prough, M.A. Micci, Modulation of adult neurogenesis by Nano-Pulsed Laser Therapy (NPLT) in a rat model of Traumatic Brain Injury, *Mission Connect symposium 2017*, Houston, Texas, December 8, 2017

6. **E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov. K. Johnson, I. Bolding, M.A. Parsely, A. Unterainer, C. Szabo, D.S. Prough, M.A. Micci, Modulation of adult neurogenesis by Nano-Pulsed Laser Therapy (NPLT) in a rat model of Traumatic Brain Injury, *Society for Neuroscience 2017*, Washington DC, November 11-15, 2017

7. **E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov. K. Johnson, I. Bolding, M.A. Parsely, A. Unterainer, C. Szabo, D.S. Prough, M.A. Micci, Modulation of adult neurogenesis by Nano-Pulsed Laser Therapy (NPLT) in a rat model of Traumatic Brain Injury, *Neurotrauma 2017*, Snowbird, Utah, July 9-12, 2017

8. **E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov. M.A. Parsely, D.S. DeWitt, D.S. Prough, M.A. Micci, Modulation of adult neurogenesis by Nano-Pulsed Laser Therapy (NPLT) in a rat model od Traumatic Brain Injury, *National Research Student Forum*, Galveston, Texas

9. **E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov. M.A. Parsely, D.S. DeWitt, D.S. Prough, M.A. Micci, Modulation of hippocampal neurogenesis by Nano-Pulsed Laser Therapy (NPLT), *First Galveston Symposium on Alzheimer's Disease & Related Disorders: Basic, Translational & Clinical Advances*, Galveston, Texas, March 22, 2017

10. **E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov. M.A. Parsely, D.S. DeWitt, D.S. Prough, M.A. Micci, Nano-Pulsed Laser Therapy (NPLT) modulates hippocampal neurogenesis in a rat model of traumatic brain injury, *Mission Connect symposium 2016*, Houston, Texas, December 2, 2016

11. **E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov. M.A. Parsely, D.S. DeWitt, D.S. Prough, M.A. Micci, Nano-Pulsed Laser Therapy (NPLT) modulates hippocampal neurogenesis in a rat model of traumatic brain injury, *Society for Neuroscience 2016*, San Diego, California, November 12-16, 2016

12. **E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov. M.A. Parsely, D.S. DeWitt, D.S. Prough, M.A. Micci, Effect of Nano-Pulsed Laser Therapy (NPLT) on neurogenesis in a rat model of blast-induced neurotrauma, *Texas FreshAir 2016*, Austin, Texas, October 6-7, 2016

13. **E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov. M.A. Parsely, D.S. DeWitt, D.S. Prough, M.A. Micci, Effect of Nano-Pulsed Laser Therapy (NPLT) on neurogenesis in a rat model of blast-induced neurotrauma, *Moody Project TBI symposium*, Galveston, Texas, September 23, 2016

14. **E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov. M.A. Parsely, D.S. DeWitt, D.S. Prough, M.A. Micci, Effect of Nano-Pulsed Laser Therapy (NPLT) on neurogenesis in a rat model of blast-induced neurotrauma, *National Neurotrauma Society*, Lexington, Kentucky, June 26-29, 2016

15. **E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov. M.A. Parsely, D.S. DeWitt, D.S. Prough, M.A. Micci, Short-Pulsed Laser Therapy (SPLT) for Traumatic Brain Injury: Effect on Neurogenesis, *4th Annual Clinical and Translational Forum*, University of Texas Medical Branch, Galveston, Texas, March 21, 2016

16. **E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov. M.A. Parsely, D.S. DeWitt, D.S. Prough, M.A. Micci, Short-Pulsed Laser Therapy (SPLT) for Traumatic Brain Injury: Effect on Neurogenesis, *XI Conference of Italian Researchers in the World*, Houston, Texas, February 26-27, 2016

Oral Presentations:

- Modulation of adult neurogenesis by Nano-Pulsed Laser Therapy (NPLT) in a rat model of Traumatic Brain Injury, **E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov. K. Johnson, I. Bolding, M.A. Parsely, A. Unterainer, C. Szabo, D.S. Prough, M.A. Micci, *Mission Connect symposium 2018*, Houston, Texas, December 8, 2018

- Nano-Pulsed Laser Therapy (NPLT) modulates adult hippocampal neurogenesis, **E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov. D. Prough, M.A. Micci, *NGP Seminar Symposium 2017*, University of Texas Medical Branch, Galveston, Texas, June 28-29, 2017

- Nano-Pulsed Laser Therapy (NPLT) modulates hippocampal neurogenesis in a rat model of traumatic brain injury, **E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov. M.A. Parsely, D.S. DeWitt, D.S. Prough, M.A. Micci, *The Department of Neuroscience and Cell Biology annual retreat 2016*, Galveston, Texas, December 13, 2016

- Nano-Pulsed Laser Therapy (NPLT) modulates hippocampal neurogenesis in a rat model of traumatic brain injury, **E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov.

M.A. Parsely, D.S. DeWitt, D.S. Prough, M.A. Micci, *Mission Connect symposium 2016*, Houston, Texas, December 2, 2016

- Effect of Nano-Pulsed Laser Therapy (NPLT) on neurogenesis in a rat model of blast-induced neurotrauma**, E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov. M.A. Parsely, D.S. DeWitt, D.S. Prough, M.A. Micci, *Moody Project TBI symposium*, Galveston, Texas, September 23, 2016

- Effect of Nano-Pulsed Laser Therapy (NPLT) on neurogenesis in a rat model of blast-induced neurotrauma, **E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov. D. Prough, M.A. Micci, *NGP Seminar Symposium 2016*, University of Texas Medical Branch, Galveston, Texas, June 8-9, 2016

- Short-Pulsed Laser Therapy (SPLT) for Traumatic Brain Injury: Effect on Neurogenesis, **E. Mocciaro**, R. Esenaliev, I. Petrov, Y. Petrov. D. Prough, M.A. Micci, *XI Conference of Italian Researchers in the World,* Houston, Texas, February 26-27, 2016

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