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Effects of High Frequency repetitive Magnetic Stimulation on astrocytes in an ischemic cell model.

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Dissertação para obtenção do Grau de Mestre em
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“Talent is cheaper than table salt. What separates the talented individual from the successful one is a lot of hard work”.

- Stephen King

Dedicatória

Dedico este trabalho a minha mãe Maria Salete Gava, meu pai Genilso Gava, irmãos Raul Marcos, Fabricio e meu afilhado Bryan. O apoio de todos vocês foi fundamental para o desenvolvimento e conclusão deste trabalho.

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Resumo

A estimulação magnética transcraniana repetitiva é uma técnica não invasiva e indolor de neuromodulação, atualmente utilizada como ferramenta auxiliar no tratamento de diversas desordens neurológicas como o AVC isquémico. O AVC isquémico é uma lesão cerebral causada pela interrupção parcial ou total do fluxo sanguíneo, é uma patologia de alta prevalência no mundo e com grande indice de morbilidade e mortalidade. A HF-rTMS vem demonstrando ser capaz de promover recuperação motora, melhoria da hemodinâmica cerebral ipsilateral à lesão, e de promover a diminuição global dos danos causados pelo AVC. Esses efeitos resultam de alterações desencadeadas pela rTMS em mecanismos celulares e moleculares como a plasticidade sináptica, a expressão génica, a neurogénese ou mecanismos de neuroproteção. Porém os mecanismos celulares e moleculares ainda não estão bem descritos.

Nesta dissertação, utilizámos uma cultura celular cortical embrionária submetida a privação de oxigénio e glucose, para avaliar os efeitos desencadeados pela HF-rMS nos astrócitos sujeitos a um insulto isquémico. Os resultados revelam que a lesão isquémica induz aumento de 38% no número de neurónios que expressam ERK1/2, porém houve uma potenciação desses efeitos, com aumento de 75%, quando estes neurónios foram expostos a meio condicionado de astrócitos e meio condicionado por uma cultura neuroglial submetida a HF-rMS (10Hz). Quanto à prevenção da degeneração das neurites, os resultados revelaram que houve uma diminuição da degeneração e manutenção da arborização neuronal, em neurónio sujeitos a ambos os meios condicionados. A HF-rMS também foi capaz de aumentar em aproximadamente 60% a expressão de BDNF pelos astrócitos após lesão isquémica, porém não foi capaz de alterar o fenótipo astrocitário.

Em suma, os resultados obtidos nesta dissertação demonstram que, a HF-rMS pode ser uma ferramenta promissora para o tratamento do AVC isquémico. A HF-rMS atua diretamente sobre os astrócitos promovendo aumento da expressão de BDNF, libertação de fatores que previnem a degeneração das neurites, manutenção da arborização neuronal e aumento do número de neurónios ERK1/2^{+}.

Palavras-chave

AVC isquémico;astrócitos; estimulação magnética repetitiva; neuroproteção

Resumo alargado

A estimulação magnética transcraniana repetitiva é uma técnica não invasiva e indolor de neuro modulação baseada nos princípios demonstrados por Michael Faraday em 1831. Os efeitos desencadeados pela estimulação magnética estão diretamente relacionados com o local da aplicação e à frequência utilizada. Protocolos que utilizam alta frequência (>5Hz) têm demonstrado a capacidade de gerar estímulos excitatórios, ao contrário dos efeitos gerados pela baixa frequência (≤1Hz) que geram respostas inibitórias. Esses protocolos vêm sendo utilizados como ferramenta auxiliar no tratamento de diversas desordens neurológicas como o AVC isquémico.

O AVC isquémico é uma lesão cerebral causada pela interrupção parcial ou total do fluxo sanguíneo, causando a morte neuronal, aumentando a excitotoxicidade, desequilíbrio iónico e stress oxidativo. É uma patologia de alta prevalência no mundo, aproximadamente 5.5 milhões de pessoas morrem todos os anos de AVC, tendo ainda elevado impacto social pelos efeitos incapacitantes. Diversos estudos revelam que a rTMS aplicada a pacientes pós AVC isquémico na fase aguda ou crónica promove efeitos positivos, com melhoria da função motora, da hemodinâmica cerebral ipsilateral à lesão e diminuição global dos danos causados pelo AVC. Os efeitos clínicos observados resultam de alteração desencadeadas pela rTMS sobre mecanismos como a plasticidade sináptica, a regulação da expressão génica, neurogénese e neuroproteção. A ação da rTMS em células gliais como os astrócitos, que desempenham papel chave para conter e reparar o tecido neuronal após o AVC isquémico, têm sido pouco explorada.

Nesta dissertação, utilizando uma cultura celular cortical embrionária submetida a privação de oxigénio e glícose, fomos estudar os efeitos desencadeados pela HF-rMS nos astrócitos sujeitos a isquémia e de que maneira esses efeitos podem contribuir para promover neuroproteção. Os resultados revelam que a lesão isquémica induz aumento de 38% no número de neurónios que expressam ERK1/2, porém houve uma potenciação desses efeitos quando estes neurónios foram expostos a meio condicionado por astrócitos ou meio condicionado por culturas neurogliais estimuladas com HF-rMS (10Hz). Avaliamos também a arborização neuronal e de que modo a HF-rMS age sobre os astrócitos para prevenir a degeneração das neurites causada pela isquímia. Os resultados revelaram uma diminuição da degeneração das neurites e manutenção da arborização neuronal, em neurónios expostos a ambos os meios condicionados. A HF-rMS também foi capaz de induzir aumento de aproximadamente 60% na expressão de BDNF nos astrócitos

após lesão isquémica. Quanto ao fenótipo astrocitário, a lesão isquémica induziu aumento de astrócitos $C3^{+}$, porém a HF-rMS não foi capaz de reverter essa condição.

Em suma, os resultados obtidos demonstram que HF-rMS atua diretamente sobre os astrócitos promovendo aumento da expressão de BDNF, e a libertação de fatores que previnem a degeneração das neurites, a manutenção da arborização neuronal e o aumento do número de neurónios $ERK1/2^{+}$, o que indica que a HF-rMS é uma ferramenta promissora para o tratamento de diversas patologias neurológicas.

Abstract

Repetitive transcranial magnetic stimulation is a non-invasive and painless therapeutic technique, currently used as an auxiliary tool in the treatment of several neuronal disorders such as ischemic stroke. Ischemic stroke is a brain injury caused by partial or total interruption of blood flow to perfuse the cerebral tissue, with a high prevalence in the world and associated with high morbidity and mortality. HF-rTMS is able to promote motor recovery, improve ipsilesional cerebral hemodynamics and decrease global damage caused by stroke. These benefits result from changes triggered by rTMS in mechanisms such as synaptic plasticity, regulation of gene expression, neurogenesis and neuroprotection. Surprisingly, the effect of rTMS in glial cells has been underexplore.

Using rat embryonic cortical cultures submitted to oxygen and glucose deprivation model, we evaluated the effects triggered by HF-rMS on astrocytes subjected to an ischemic insult and how these effects contribute to the promotion of neuroprotection. The results reveal that ischemic injury induces a 38% increase in the number of neurons expressing ERK1/2, but when neurons were exposed to medium conditioned by astrocytes or neuron-glial cultures stimulated with HF-rMS (10Hz) there was a 75% increase in these effects. Regarding the prevention of neurites degeneration, the results demonstrated that there was a decrease in the degeneration and maintenance of neuronal arborization, in neurons subjected to both conditioned media. HF-rMS applied after the ischemic injury was also able to induce an increase of approximately 60% in the expression of BDNF but was not able to alter astrocytic phenotype.

The results obtained show that HF-rMS promotes the increase of BDNF expression by astrocytes, the release of factors that prevent the degeneration of neurites, the maintenance of neuronal arborization and the increase of ERK1/2^{+} neurons. Taken together these results show that HF-rMS is a promising tool for the treatment of neurological disorders.

Keywords

Astrocytes, ischemic stroke, neuroprotection, repetitive magnetic stimulation.

List of Publications

Articles published in international Scientific video journal. Peer reviewed. Multi-disciplinary:

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List of Abbreviations

AD	Alzheimer's Disease
AHRS	Auditory Hallucinations Rating Scale
ANOVA	Analysis of Variance
BBB	Brain Blood Barrier
BDNF	Brain-Derived Neurotrophic Factor
cDNA	Complementary Deoxyribonucleic Acid
CiA	Cyclophilin A
CT	Control
cTBS	Continuous Theta-Burst Stimulation
C3	Complementary Component 3
DEPC	Diethylpyrocarbon
DLPFC	Dorsolateral Prefrontal Cortex
dNTPS	Deoxynucleotidostriphosphate
FAS	Functional Ambulation Scale
FBS	Fetal Bovine Serum
FIM	Functional Independence Measurement
FMA	Fugl-Meyer Assessment
GABA	γ-aminobutyric Acid
GFAP	Glial Fibrillary Acidic Protein
HBSS	Hank's Balanced Salt Solution
HDRS-17	Hamilton Rating Scale for Depression
HF	High-Frequency
HF-rMS	High-Frequency Repetitive Magnetic Stimulation
IS	Ischemic Stroke
iTBS	Intermittent Theta-Burst Stimulation
LF	Low-Frequency
LF-rMS	Low-Frequency Repetitive Magnetic Stimulation
LTD	Long-Term Depression
LTP	Long-Term Potentiation
M1	Primary Motor Cortex

MAO-A	Monoamine oxidase A
MAPK	Mitogen Activated Protein Kinase
MAP2	Microtubule Associated Protein 2
MDD	Major Depressive Disorder
MEM	Minimum Essential Medium Eagle
MMEPs	Magnetic Motor Evoked Potentials
MS	Magnetic Stimulation
NPCs	Neural Progenitor Cells
NSC	Neural Stem Cells
OGD	Oxygen and Glucose Deprivation
OGD+MS	Oxygen and Glucose Deprivation+Magnetic Stimulation
PANSS	Positive and Negative Syndrome Scale
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline + Tween-20
PCR	Polymerase Chain Reaction
PCs	Monoamine Oxidase A
PFA	Paraformaldehyde
PRS	Pain Relief Score
QIDS-SR	Quick Inventory of Depressive Symptomatology
RT- PCR	Reverse Transcription Polymerase Chain Reaction
rTMS	Repetitive Transcranial Magnetic Stimulation
SIAS	Stroke Impairment Assessment Set
SYP	Synaptophysin
TBS	Theta-Burst Stimulation
TMS	Transcranial Magnetic Stimulation
TRD	Treatment-Resistant Depression
Trk-B	Tropomyosin receptor kinase B
VAS	Visual Analogue Scale

Chapter 1

Introduction

1. Introduction

1.1. Principles of repetitive transcranial magnetic stimulation

Repetitive Transcranial Magnetic Stimulation (rTMS) is a painless, non-invasive neuromodulation technique that uses coils placed on specific areas of the brain, altering excitability by means of a magnetic field applied locally. rTMS is currently used as a technique for the treatment of several pathologies[2,3]. The technique is based on the principles demonstrated by Michael Faraday in 1831, where a magnetic field can be converted into electrical energy and vice versa, therefore the coil produces an alternating magnetic field that generates an indirect electrical current. This magnetic field has the ability to promote depolarization of the cellular membrane generating an electrical current that is propagated to adjacent cells[4,5].

Over time, several rTMS approaches have been studied. Since the magnetic field does not propagate from the center of the coil (Figure 1), the formats were modified, seeking to promote a stimulation at a focal point. The most commonly used coils are coils with a 8 shape, where two circulating coils are arranged in parallel, single circular coils and H-shaped coils[1]. In addition to the shape of the coils, other parameters such as stimulation repetition rate, stimulation time, number of pulses and interval between them, stimulus frequency have a substantial impact on the effects obtained[6].

From the protocols used three types stand out: high-frequency (HF), that uses stimuli above 5Hz, low-frequency (LF) that uses stimuli up to 1Hz, and theta-burst stimulation (TBS) that delivers large high-frequency (50Hz) packets in a short period of time[7-9]. A concept has emerged that HF generates an excitatory stimulus and LF an inhibitory stimulus. With respect to theta-burst when continuous it produces an inhibitory effect (cTBS) and when intermittent it generates an excitatory effect (iTBS)[7,10,11]. Therefore, different protocols can be combined in order to recover the excitatory balance of neuronal cells, applying a low-frequency stimulus in the contralateral hemisphere to the lesion, decreasing the compensatory excitability[12].

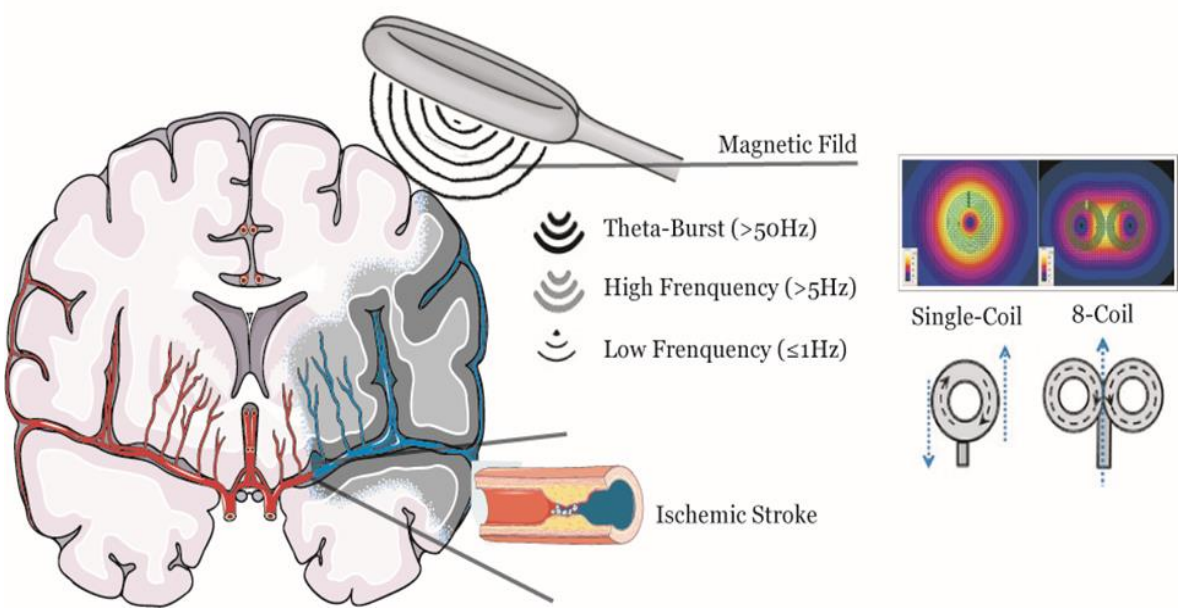

Figure 1: Representative image of the most common transcranial magnetic stimulation protocols applied to ischemic stroke, and coil formats most used with the characteristic magnetic field, adapted from[1].

1.1.1. rTMS applications in the treatment of neurological disorders.

rTMS has been applied in diverse neurologic and neuropsychiatric disorders. The use of rTMS to treat major depressive disorder (MDD) has been widely explored, especially in treatment-resistant depression (TRD) that can reach up to 50% of patients using antidepressant medication[13]. In a randomized controlled trial, twenty-four patients diagnosed with major depression were submitted to HF-rTMS protocols on the left dorsolateral prefrontal cortex (DLPFC) for two weeks, showed significant improvement in the clinical condition, with a decrease in the severe depression score [14]. Similar results were reported in other studies[15-17]. The exact mechanisms responsible for the relief of symptom remain unclear[18]. The combination of HF-rTMS applied to the left DLPFC and LF-rTMS on the right DLPFC to increase and decrease excitability, respectively, has been proposed. Several studies suggest that this combination promotes higher effects than HF-rTMS alone[19]. In protocols using iTBS there were also improvement of symptoms[20], assessed based on several scales Quick Inventory of Depressive Symptomatology (QIDS-SR) and Hamilton Rating Scale for Depression (HDRS-17).

rTMS was also used as an alternative treatment for patients with schizophrenia, with contradictory results. In some studies there was an improvement in the negative effects associated with audiovisual hallucinations[21], in another study rTMS did result in better results than the sham-rTMS group [22]. The effects were evaluated with the score Positive

and Negative Syndrome Scale (PANSS), however the effectiveness of this tool has been questioned[23], therefore the effect of rTMS on schizophrenia is not clear and more robust results are needed.

When used to treat neuropathic pain HF-rTMS is applied in the primary motor cortex (M1) contralateral to the side of pain origin. The data show that it induces a significant decrease in central neuropathic pain, with effects persisting within 12 months. This demonstrates the permanence of the effects in the long term, moreover, no adverse effects of rTMS have been described in patients with schizophrenia, even after 6 years of treatment and with more than 1000 sessions[24,25].

In neurodegenerative diseases such as Alzheimer's disease (AD), rTMS has been applied and studied not only with the aim of improving cognitive ability, but also as a method of predicting the progression of cognitive decline from the beginning of the disease[26-28]. Recent studies aim to understand the cellular and molecular mechanisms that are modulated by rTMS and responsible for the positive effects observed in patients (Table 1). In a study with C57BL/6 mice, it was demonstrated that both HF-rTMS and LF-rTMS were able to decrease the number of apoptotic cells, increase BDNF levels and regulate the levels of β-catenin that is suppressed in Alzheimer's disease[29]. Positive effects on synaptic plasticity were also observed, such as increases in synaptophysin (SYP) and brain-derived neurotrophic factor (BDNF) levels in parallel with cognitive improvement and increased spatial learning[30].

rTMS has been widely studied and applied to several neurological pathologies, one in particular generated a great deal of interest, the application of rTMS in IS. Ischemic stroke is a disease with a high death rate around the world, and when not lethal it frequently results in serious sequelae[31]. Several studies have been conducted with the aim of advancing scientific knowledge on the impact of rTMS and on the cellular and molecular effects promoted by rTMS.

Table 1: Application of the rTMS in different neuronal disorders.

Neurological disorders	**Stimulation frequency and location**	**Major conclusions**	**Article**
major depressive disorders	iTBS (50Hz)	Full remission was achieved, (QIDS-SR) score.	Plewnia, *et al* (2014)
	HF-rTMS (10hz) left DLPFC	Significant decrease in HDRS-17 scores in HF-rTMS vs. sham. significant improvement in the clinical condition.	Kang, *et al* (2016).
	HF-rTMS (10hz) left DLPFC	Significant decrease in HDRS-17 scores in HF-rTMS vs. sham.	Li, *et al* (2016).
	HF-rTMS (10hz) left DLPFC	rTMS group showed significant clinical improvement in HDRS-17 scores compared to the sham group, positive correlation between residual depressive symptoms and connectivity strength after 2-week rTMS was found.	Theleritis, *et al* (2016)
schizophrenia	HF-rTMS (20hz) left DLPFC	There was no significant difference between rTMS and sham group, based on the Average scores of Auditory Hallucinations Rating Scale (AHRS).	Kimura, *et al* (2016)
	HF-rTMS (10hz) left DLPFC	Decrease of PANSS score when compared to sham.	Wagner, *et al* (2019)
neuropathic pain	HF-rTMS (10hz) M1	Decrease in Atypical facial pain, Central post-stroke pain, Neuropathic lower	McLean, *et al* (2018)

		limb pain, Phantom pain of the upper or lower. Based on the visual analogue scale (VAS)	
chronic central pain	HF-rTMS (20hz) M1	Pain relief from the first sessions, results were maintained for up to 12 months, based on the pain relief score (PRS).	Quesada, et al (2018)
Alzheimer's disease	HF-rTMS (20hz)	improvement of cognitive function, memory and language.	Zhao, *et al* (2017)
	HF-rTMS (20hz) Precuneus (PC)	Increased PC cortical activity, clinical improvement, and selective improvement in episodic memory.	Koch, *et al* (2018)
	HF-rTMS (10hz) LF-rTMS (1hz)	LF-rTMS and HF-rTMS reduced cell apoptosis. Effect of rTMS on β-catenin pathways after the treatments with rTMS of both frequencies.	Chen, *et al* (2019)
	HF-rTMS (5hz) HF-rTMS (25hz)	increased expression of BDNF, synaptophysin, phosphorylated CREB (pCREB), improved spatial learning and cognitive function	Ma, et al (2019)

1.2. Ischemic Stroke

IS is a brain injury caused by partial or total disruption of blood supply. This disruption can be caused by a thrombus, that is associated with a dysfunction of the blood vessel, often due to chronic inflammatory disease, atherosclerotic disease, or can be caused by an embolic event, where displacement of some artifact occurs causing the partial or total blockage of the vessel lumen causing sudden loss of function [32,33]. The functional loss is directly related to the site and extent of the injured area. In the core of the lesion the death of the cells occurs by tissue necrosis due to the up-regulation of excitotoxicity pathways, ionic imbalance, oxidative stress, among others. In the area adjacent to the core the cells remain metabolically less active[34].

The social impact of ischemic stroke is enormous. Approximately 5.5 million people die each year from stroke, with up to half of the surviving patients having disabling sequelae[35]. Recent data revealed that almost 800.000 people are victims of stroke each year. Of these approximately 87% are caused by ischemic stroke. Projections suggest an increase in the number of cases due to demographic changes, such as an increase of the elderly population[36]. In Europe, at the beginning of the 21st century approximately 1.1 million people suffered stroke per year, with the 55-60 years age group being the most affected. Due to aging of the European population projections for the future point to an increase of approximately 36% in the number of cases until 2025[37].

The standard approach to the treatment of acute ischemic stroke, involves techniques that aim to reestablish blood flow. The use of fibrinolytic drugs is recommended for patients with known onset of symptoms up to 3 hours. Mechanical techniques such as catheterization are also employed. After the acute event, physiotherapy combined with healthy nutrition and physical activity are frequently recommended, in addition to continuous use of vessel-regulating medication[38,39]. New techniques such as TMS have been studied as an adjunct therapeutic approach with the aim of reducing functional loss and incapacity, since most patients survive the initial ischemic stroke event[40].

1.2.1. Clinical application of rTMS in stroke

Since its presentation in the 80's by Anthony Barker[41], rTMS has been studied and applied as an adjunct tool in the treatment of patients with IS both in acute and chronic phases of the disease. A recent study analyzed the impact of rTMS on patients with acute ischemic stroke by applying HF-rTMS (10Hz) to the ipsilateral motor cortex (M1) and LF-rTMS (1Hz) on contralesionally M1[42]. Results revealed that both HF-rTMS and LF-rTMS significantly increased motor function based on the Fugl-Meyer Assessment (FMA) score,

as well as increased neuronal activity measured by magnetic resonance imaging on ipsilateral M1. Moreover, HF-rTMS was associated with better prognosis and long-term recovery. Chronic and subacute ischemic stroke patients presented similar results[43-45].

Low frequency rTMS applied on M1 contralateral to injury, together with physiotherapy, has the capacity to enhance the recovery of mobility, function and cognitive status results based on the Functional Ambulation Scale (FAS), and Functional Independence Measurement (FIM), showed a significant improvement in all mobility and cognitive function parameters, with the group receiving LF-rTMS presenting the best results[46]. These data indicate the viability of using this technique as an adjunct tool in treatment of stroke.

The differential protocol of intermittent stimulation iTBS, was also effective in the recovery of motor functions in chronic stroke patients[47]. There are few studies in acute patients. A study applied iTBS in in the ipsilateral primary M1 motor zone[48] and LF-rTMS (1Hz) in the contralateral M1 area. The Stroke Impairment Assessment Set (SIAS) score revealed that iTBS has the ability to improve motor function and also showed the safety of the technique since no adverse effects have been observed.

Several other studies demonstrate the ability of rTMS to promote the recovery of motor function and decrease disabling effects after ischemic stroke[49-53] and improving the cerebral hemodynamics ipsilateral of the lesion[54]. rTMS has proven to be a useful tool in the treatment of stroke damage, in addition, TMS can be used as a tool to evaluate the prognosis of post stroke patients, as demonstrated by Dolhaberriague, and collaborators[55]. The magnetic motor evoked potentials (MMEPs) can be obtained using TMS, the time and amplitude of MEP triggered by TMS is a quantitative tool sensitive to the motor function of patients with motor disfunction both in the acute phase and at the beginning of the rehabilitation phase of stroke[56].

1.2.2. Neuronal cellular and molecular mechanisms triggered by rTMS

The clinical benefits of rTMS are widely reported in the literature. New protocols are being tested and studies with higher number of participants are being developed to optimize the gain and evaluate the safety of the technique. However, the cellular mechanisms behind the clinical effects have been insufficiently addressed.

1.2.2.1. Synaptic plasticity

The effects of rTMS on synaptic plasticity are dependent on the frequency and intensity used[57]. Stimulation changes the neuronal excitability that leads to a change in ionic balance. It is believed that changes in ion balance may promote long-term potentiation (LTP) or long-term depression (LTD). Low frequency stimulation promotes LTD whereas high frequency triggers LTP[48,49]. Both LTP and LTD involve the modulation of NMDA receptors in post-synaptic neurons[2,58]. Depolarization promotes the unblocking of the NMDA receptor allowing the influx of Ca^{2+} in the post-synaptic neuron[59]. However, depolarization induced by LF-rTMS is slow and durable whereas HF-rTMS promotes a fast depolarization[57].

Another mechanism recently reported to be promoted by rTMS is the activation of the BDNF/CREB pathway. HF-rTMS applied to the hippocampus of old mice activated the BDNF/CREB pathway and promoted the recovery of the memory deficit caused by age[30]. It is also suggested that rTMS has the ability to increase the expression of the SYP glycoprotein present in synaptic vesicle and also the NR2B functional subunit of the NMDA receptor, which plays an important role in the formation of LTP. The increase of both proteins has a direct relationship with increased expression of brain-derived neurotrophic factor (BDNF) and the recovery of synaptic communication[60].

The effects triggered by rTMS can be observed several months after the last stimulation[2]. A recent study showed that due to the propagation of the synaptic activity triggered by rTMS, the effect of stimulation may be not only focal, and may activate signaling pathways that regulate neuronal plasticity in areas distant from the focal point[61]. Another study with rats submitted to single prolonged stress, showed that the dendritic arborization in anterior cingulate cortex was impaired and that rTMS was able to reverse this effect, specifically the decrease of dendritic length and density[62].

1.2.2.2. Gene expression

Over time, many studies have shown that rTMS has the ability to modulate gene expression[7,29,63]. HF-rTMS regulates the expression of several genes involved in the regulation of cell survival, decreasing the expression of BAX protein, a pro-apoptotic protein X associated with Bcl-2, and increases the expression of anti-apoptotic proteins such as Bcl-2. HF-rTMS was also associated with increased expression of miR106b, a microRNA described as a potential promoter of proliferation of neural progenitor cells (NPCs)[64], suggesting that rTMS may also induce neurogenesis.

rTMS also increases the expression of the HSPA5 gene, that codifies a member of the heat-shock protein-70, GRP78/Bip, a protein that is both a target of the ER stress response, and an essential regulator of the UPR pathway[65].; In addition, HF-rTMS has the ability to increase Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) kinase which contributes to the phosphorylation of the transcription factor CREB that results in the transcription of BDNF[66,67]. Moreover, rTMS induces p-ERK and p-AKT activation, which plays a key role in neurotrophic activity, growth and neuronal survival[66]

Therefore, based on the current literature, rTMS is able to regulate the expression of multiple genes that are relevant to survival pathways, neuroprotection, neurogenesis and neuronal plasticity.

1.2.2.3. Neurotransmission

Several psychiatric and neurologic disorders such as schizophrenia, MDD, Parkinson's disease and chronic neuropathic pain, have in common dysfunctions in the glutamate and monoamine system[65]. rTMS applied to the motor cortex induces changes in γ-aminobutyric acid (GABA)-mediated cortical inhibition in patients with chronic pain[68]. Data from patients with depression revealed that HF-rTMS applied over the left DLPFC area promote an increase in both GABA and glutamate levels. Also in patients with MDD resistant to pharmacological treatment, the application of HF-rTMS improved symptoms and increased glutamate levels[69-71].

Dopamine is a neurotransmitter crucial to the control of motor functions, learning and emotional behavior[72]. Neurological pathologies, such as Schizophrenia and Parkinson's disease, are associated with dysfunctions of the dopaminergic systems[73,74]. Data from the MDD model mice showed that rTMS applied to the region of the nucleus accumbens and to the caudal cortex has the ability to increase the extracellular levels of dopamine[75].

1.2.2.4. Neuroprotection and neurogenesis

As mentioned in the previous sections, both HF and LF rTMS have the ability to modulate neuronal pathways and responses associated with neurogenesis, synaptic plasticity, differentiation and neuronal migration, among other fundamental functions for neuronal homeostasis[64,66,75-77], and all these may contribute to the neuroprotective effects. A recent study demonstrated that rTMS was able to promote neuroprotection in AD mice models by reducing the expression of ApoE, considered one of the main genetic risk factors for AD, and PP2A, and increasing the levels of lc3ii/lc3i autophagy markers[78].

Recent studies explored the role of rTMS in neurogenesis. HF-rTMS applied to mice submitted to stroke, increased NSC proliferation and neuronal differentiation[79], and regulated the neuronal microenvironment by maintaining mitochondrial integrity and inhibiting caspase-9 and caspase-3 apoptotic pathway after stroke[80]. The chronic use of high frequency rTMS increase neurogenesis in the hippocampus. A study with rodents suggest that this process may be linked to the anti-depressant effects of rTMS[81]. Additionally, the BDNF/TrkB pathway plays a key role in neurogenesis and several studies show the potential of rTMS to modulate this pathway, which can be beneficial for the treatment of several pathologies[66,67,82,83]. Another study analyzed the effects of rTMS HF (30Hz)/LF (1Hz) on the differentiation and proliferation of neural stem and progenitor cells (NS/PCs) of rats and the results revealed that both HF and LF rTMS were able to induce increased neuronal differentiation and proliferation[84].

1.2.3. Cellular and molecular mechanisms triggered by rTMS in non-neuronal cells

Inflammation is a process associated with the pathophysiology of ischemic stroke. Microglia plays a key role in the inflammatory response of the brain, secreting several pro-inflammatory cytokines, growth factors and other mediators[85]. Interestingly, HF-rTMS significantly reduced microglial activation in rats submitted to spinal cord injury[86]. A recent study with a mice model of ischemic stroke showed that cTBS also significantly decreases microglial density and reactivity in the periphery of the lesion core. There was also a change in the microglial phenotype, with a decrease of $CD16/32^{+}$ microglia, with M1 phenotype, and an increase of $CD206^{+}$ microglia, M2 phenotype[87]. Together these results suggest that MS may promote neuroprotection by reducing the inflammatory processes. In addition, blood perfusion in the periphery of the ischemic lesion was improved by HF-rTMS and the infarct volume was reduced[88], suggesting that the vascular component may also be a target of this technique.

Presently, efforts to understand the mechanisms underlying the positive effects of rTMS are increasing, but the current literature has a neurocentric character, focusing their efforts on understanding the effects at the neuronal level, with few studies evaluating the effects triggered by rTMS on other cell types such as microglia, astrocytes and oligodendrocytes[89]. In the following topic we will focus on the available data on the role played by astrocytes in ischemic stroke and how rTMS can affect these cells.

1.3. Astrocyte

Astrocytes are specialized glial cells, performing a multiplicity of functions such as release of neurotransmitters, homeostasis of the neuronal environment, control of ion balance, modulation of synapses, neurovascular coupling, structural support, regulation of neuronal communication and energy support[90]. Glial cells are distributed throughout the central nervous system, with almost half of them being astrocytes[91]. Astrocytes are classified into protoplasmatic, present in the grey matter of the brain, and fibrous, found in the white matter[92].

Currently, studies are being developed in order to understand the heterogenicity of astrocytes, and trying to classify them not only by morphology, but also by their profile of gene expression, function or specific location. A study proposed the existence of 5 different astrocyte subtypes, classified by their gene expression profiles, presenting the subtypes AST1-3 small differences in gene expression, whereas subtypes AST4 and AST5 standing out with larger differences, thus forming 3 distinct groups[93]. The authors suggest that AST4 is a subtype with a neurogenic profile, showing increased gene expression related to mitosis and cell cycle control, neurogenesis and neuronal differentiation, located predominantly in the granular zone of the hippocampus, while AST2 is located predominantly in cortical layers and almost absent in the hippocampus[93]. The AST1-3 subtypes present a genetic expression profile characteristic of mature astrocytes. The glial fibrillar acid protein (GFAP) expression in the different subtypes proposed differs greatly, being highly expressed in AST1 and sub-expressed in AST3[93], the GFAP is widely used as an astrocytic marker, but many astrocytes do not express this protein at levels detectable by common techniques such as immunocytochemistry[90].

An important aspect of astrocyte activity is the ability to react to an insult such as ischemic stroke or other neuronal dysfunctions, depending on the site and type of injury, reactive astrocytes may act in different ways[94]. The concept of astrocytic phenotype was introduced recently, with phenotype A1 presenting a neurotoxic profile, with drastically decreased phagocyte capacity no ability to induce synapse formation (and those that are formed are weaker synapses and comparison to non-reactive astrocytes), and also increased expression of complement protein C3, that can be considered a specific marker since it is expressed only by this phenotype[95]. Reactive astrocyte A2 has a protective profile, with increased expression of neurotrophic and neuroprotective factors, maintaining phagocytic capacity and playing a repair function in ischemic injury[95].

1.3.1. Role of astrocytes in ischemic stroke

After a brain injury such as ischemic stroke, several neurodegenerative processes begin to occur, neuronal death, ionic imbalance, increased neurotoxicity, among others[34]. To limit the injury and reverse the effects generated, various cells are recruited such as microglia and other cells from the immune system, and astrocytes. Astrocytes play a key role in the containment and repair of neuronal tissue induced by ischemia. The neuroprotective phenotype A2 acts by re-establishing ionic homeostasis and energy support, decreasing neurotoxicity, re-stabilizing the brain blood barrier (BBB) and secreting molecules that activate neurogenesis and survival pathways[95-97].

After the acute stage of the stroke, reactive astrocytes can trigger the formation of the glial scar, a process that isolates the ischemic area, preventing neurotoxic substances released in the core of the lesion to reach healthy cells in the periphery[98]. The astrocytic response can be very heterogeneous, depending on the site of the injury[93].

1.3.2. Molecular and cellular mechanisms triggered by rTMS in astrocytes

Data from healthy rats showed that HF-rTMS improves neuronal activity and does not induce astrocyte reactivity or induces inflammatory response[99]. Concerning ischemia, a recent study showed that HF-rTMS applied after an ischemic insult has the ability to alter astrocyte phenotype[100]. Using primary cultures from rat embryonic cortex subjected to 6h of oxygen and glucose deprivation (OGD) it was shown that OGD increased the expression of the specific marker of A1 astrocytes C3[95]. However, HF-rTMS (10Hz) drastically decreased the expression of C3 induced by OGD, and increased the expression of the A2 marker, S100A10[95]. In primary cultures of rat cortical astrocytes, rTMS decreased the expression of Sirt1 and monoamine oxidase A (MAO-A), a regulation that may be linked to positive responses obtained in patients with MDD, since Sirt1 is linked to mechanisms that regulate the levels of serotonin in the brain[101].

Although the current data on the effects of rTMS on astrocytes is extremely scarce, it suggests that rTMS can be used to tune astrocytes to a neuroprotective phenotype, making astrocytes a promising target of rTMS in several neurological disorders.

Chapter 2

Objectives

2. Objectives

A previous study of our group that applied HF-rMS in neuron-glia cultures and neuron-enriched cultures, showed that exposure to HF-rMS after OGD has an important role in synaptic plasticity, preventing the degeneration of neurites and the decrease of ramifications, thus maintaining neuronal arborization results that were only observed in neuron-glia cultures and not in neuron-enriched cultures[102]. These data raised questions on the effects of HF-rMS on astrocytes. Taking this into consideration the present work aims to answer the following points:

1. Does HF-rMS induce the secretion of factors by astrocytes that promote increased neuronal survival and plasticity pathways, or is the physical presence of astrocytes essential to the protective effects?

2. Is HF-rMS able to increase BDNF expression in neuron-glia cultures and astrocyte-enriched cultures?

3. Is HF-rMS capable to change the phenotype of astrocytes after an ischemic insult?

Chapter 3

Materials and Methods

3. Materials and Methods

3.1. Animals

To obtain the embryonic cortical primary cultures, Wistar rat embryos at the 15th-16th day of development were used. Animals were bred at the CICS-UBI Health Science Research Center animal facility and were kept in controlled conditions of light and temperature and with free access to food and water. The female was anesthetized with ketamine (87.5 mg/kg) and xylazine (12 mg/kg) and after the removal of the embryos the female was euthanized by cervical dislocation. The embryos were taken to the cell culture room in a falcon tube containing cold phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCL, 1.5 mM KH2PO4 and 8.1 mM Na2HPO4, pH 7.2). All procedures were performed after approval by the CICS-UBI Animal Welfare and Ethics Body, and in accordance with the national ethical requirements for animal research and with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Directive 2010/63/EU).

3.2. Cell culture

The primary cortical cultures were adapted from the previous study[103]. Briefly, the embryos are removed from the yolk sac and kept in cold PBS. The removal was done carefully to avoid damaging the animal. All procedures were performed in sterile conditions, using sterile material inside a laminar flow hood. The cortex was dissected through the incisions shown in (Figure 2). Cells were obtained by mechanical digestion, with the use of tips with progressively smaller diameters (0.8 mm, 0.6 mm and finally 0.5 mm); and. The mechanical digestion was followed by centrifugation at 400 g for 3 minutes. The supernatant containing cell debris was discarded and the pellet was resuspended in culture medium. The number of cells was determined using a Neubauer chamber, trypan blue was used to help identify non-viable cells.

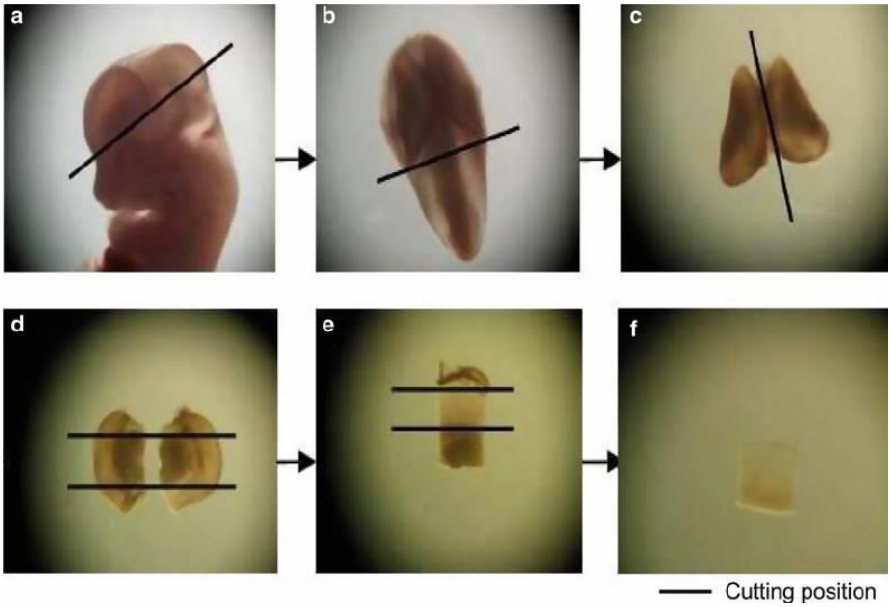

Figure 2: Rat embryonic cortex dissection (from Kim et al 2013[104]).

The cells were kept in culture for seven days at 37°C and with 5%CO_2. To ensure that the cell density was the same for all cultures, the initial density of neuro-glial cultures and neuron-enriched culture of neurons, was established according to a previous study [103]. For the astrocyte-enriched culture, prospective tests were made to establish the initial density and the most appropriate culture medium for the assay, for this, we characterized the astrocytes-enriched culture (Figure 5). The culture medium and the initial cell density is present in Table 02.

Table 2: Conditions used for embryonic cortical cell culture.

Culture	Initial cell density	Culture medium
Neuron-enriched culture	$0.21x10^6$ cell/cm².	Neurobasal medium supplemented with 2% B27 (Gibco, catalog number: 17504044), 0.5mM glutamate (Sigma-Aldrich, catalog number: 1294808), and 120µg/mL gentamicin (Sigma-Aldrich, catalog number: G0200000).
Neuron-glial culture	$0.14x10^6$ cells/cm².	Neurobasal medium supplemented with 2% B27 (Gibco, catalog number: 17504044), 0.5mM glutamate (Sigma-Aldrich, catalog number: 1294808), and 120µg/mL gentamicin (Sigma-Aldrich, catalog number: G0200000) supplemented with 10% heat-inactivated fetal bovine serum.
Astrocyte-enriched culture	$0.28x10^6$ cells/cm².	Minimum Essential Medium Eagle (MEM) (Sigma-Aldrich, M0268) supplemented with insulin from bovine pancreas 5mg/L (Sigma-Aldrich, I5500) 45% anhydrous D-glucose 3.375g/L (Fisher Scientific, G/0450/60) penicillin/streptomycin 12U/ml of both (Biochrom, A2213) 10% heat-not inactivated fetal bovine serum.

3.3. Oxygen and Glucose Deprivation (OGD)

In order to simulate the cellular events that occur during an ischemic period we adapted a protocol previously established by Roque and collaborators[103]. At the 7th day in culture, a brief wash with Hank's Balanced Salt Solution (HBSS, 1.26mM $CaCl_2$, 5.36mM KCl, 0.44 mM KH_2PO_4, 0.49 mM $MgCl_2$, 139.9mM NaCl, 4.17 mM $NaHCO_3$, 3.38mM Na_2HPO_4, pH 7.2) was made, followed by incubation in the same solution. The cells subjected to OGD were placed inside a hermetically sealed hypoxia chamber. The oxygen present in the chamber was previously replaced by a gas mixture containing 95% N_2 and 5% CO_2 for 4 minutes with 20 L/min flow to remove the oxygen present inside the chamber. The flow

was interrupted, and the hypoxia chamber was placed in an incubator at 37 °C for 6 hours (Figure 3 A-B).

3.4. High Frequency repetitive Magnetic Stimulation (HF-rMS)

Cell cultures were subjected once to the High frequency repetitive magnetic stimulation (HF-rMS) protocol in the 7th day in culture immediately after OGD. The MS protocol used throughout the study was based on previous studies of our group[102]. The Petri dishes containing the cell culture were accommodated on a flat surface, a figure-8 MCF-B70 stimulation coil (180x116mm) was placed on top of the petri dish at a distance of approximately 1.5cm with the center of the coil lined with the center of the dish (Figure 1).

The coil was connected to a MagVentureMagPro G3 X100 5.0.1 magnetic field generator. Cell cultures submitted to OGD and MS, were subjected to HF-rMS immediately after the ischemia period, while control cultures were subjected to the same procedure but without MS (Figure 3 A-B).

The parameters defined for MS correspond to 24 trains of 50 pulses in biphasic waveform (280μs duration), delivered at 10Hz, with 25 seconds interval between trains, totaling 1200 stimuli, with maximum duration of 11 minutes and 60% of the total power of the magnetic field generator. The coil temperature was checked during the whole procedure and was kept within 20 and 37°C so as not to interfere in the results.

3.5. Preparation of the Conditioned Medium

To obtain the conditioned medium, two different primary cortical cultures were established, astrocyte-enriched culture and neuron-glia culture, to obtain astrocyte conditioned medium (astrocyte-CM) and neuron glial conditioned medium (neuron glial-CM), respectively. The cells were seeded on 35mm Petri dishes (Orange scientific catalogue: 4450100N) previously coated with poly-D-Lysine and kept in culture medium at 37°C under a 5%CO_2 atmosphere for 7 days.

The experiment included four conditions: control (CT), repetitive magnetic stimulation (rMS), oxygen and glucose deprivation (OGD) and oxygen and glucose deprivation followed by repetitive magnetic stimulation (OGD+rMS) (Figure 3 A). Each experimental condition was always performed in triplicate and each experiment was repeated at least 3 times, with different cell cultures. For the CT and rMS conditions the culture medium was

removed and replaced during the OGD step by HBSS supplemented with 5.56mM of glucose and maintained at 37°C and under an atmosphere with 5%CO_2. After OGD and rMS process the HBSS medium in all experimental conditions was replaced by neurobasal medium. The medium was collected 24 hours after the end of the OGD period and stored at -80°C.

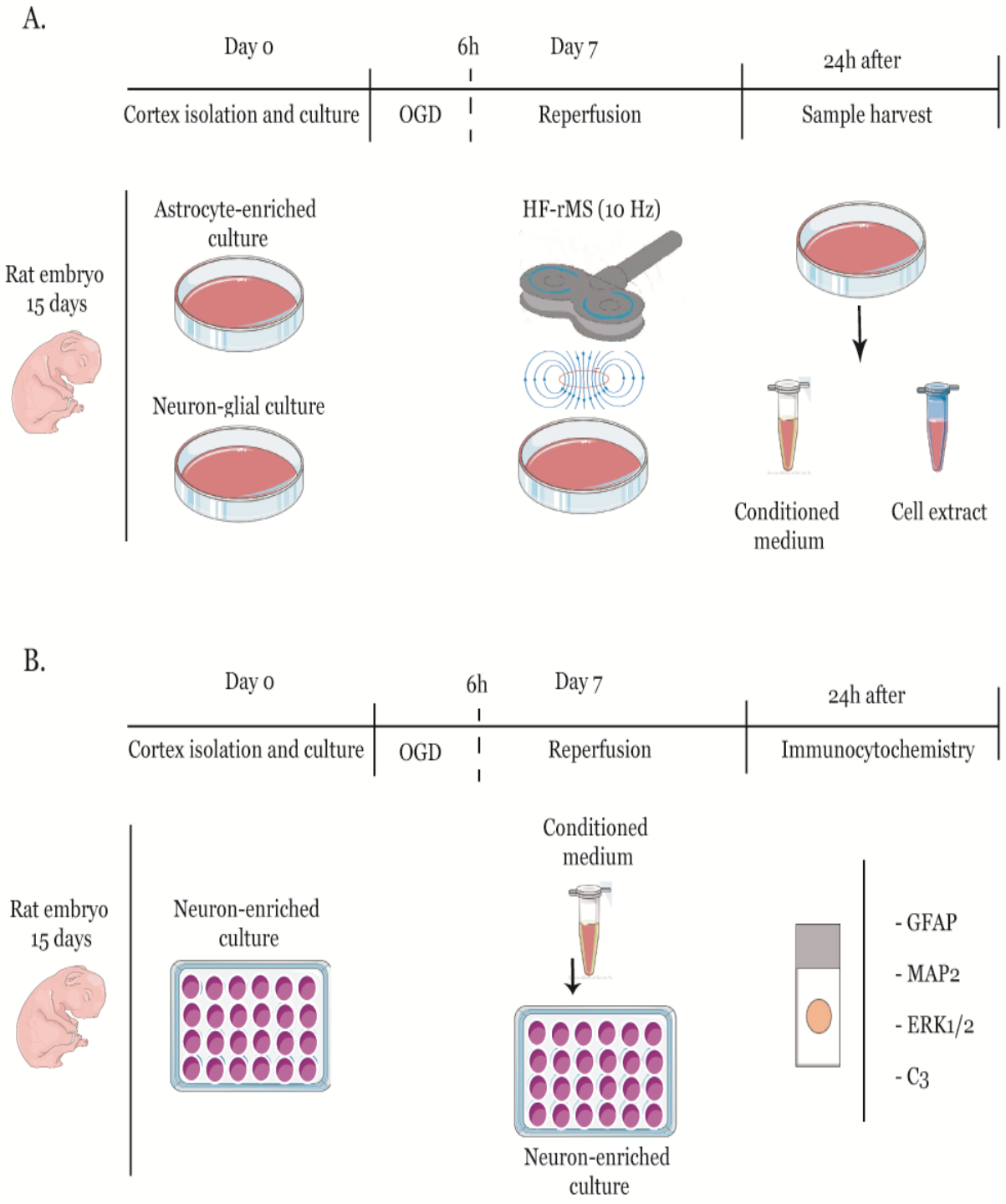

Figure 3: Representative scheme of the experimental paradigm, **A.** Characterization of cell cultures, preparation of astrocyte-CM and neuron glia-CM, cell extract. **B.** Preparation of neuron culture, use of conditioned media and immunocytochemistry.

3.6. Immunocytochemistry

To perform the immunocytochemistry, the cells were seeded in multiwell plates containing 15 mm coverslips coated with poly-D-Lysine. At the end of the culture period, the cells were fixed with paraformaldehyde (PFA) for 10 minutes. After fixation, the cells were permeabilized with PBS supplemented with Triton 1% for 5 minutes. This was followed by the blocking step with a solution containing PBS with 0.1%Tween (PBS-T) and 20% of fetal bovine serum (FBS), for 1 hour at room temperature. Then the cells were incubated with antibodies according to the conditions described in Table. 2. After washing with PBS-T the coverslips were incubated for 10 minutes with Hoechst 33342 (Invitrogen H3569). Finally, the cells were washed with PBS-T and the coverslips were mounted on a microscope slide with DAKO (CS703) mounting medium. The images were acquired using an epifluorescence microscope (Axiobserver Z1, Zeiss) with a 63X objective.

Table 3: Conditions and antibodies used in the immunocytochemical assays.

Antibody	**Biological source**	**Dilution**	**Incubation conditions**	**Reference**
Anti-GFAP	rabbit	1/2000	20h, 4°C	DAKO-Z0334
Anti-MAP2	mouse	1/500	20h, 4°C	SC74421
Anti-MAP2	rabbit	1/500	20h, 4°C	SC-20172
Anti-ERK1/2	mouse	1/250	20h, 4°C	SC135900
Anti-C3	mouse	1/500	20h, 4°C	SC28294
Anti-rabbit conjugated to Alexa 546	goat	1/1000	1hour, room temperature	Invitrogen – A11010
Anti-mouse conjugated to Alexa488	goat	1/1000	1hour, room temperature	Invitrogen – A11001

3.7. Extraction and analysis of the mRNA expression

To lyse the cells and maintain the integrity of the RNA 700 µl of Trizol (Isol-RNA Lysis 2302700 5Prime) was added to a 35mm petri dish. The cell extract with the Trizol was kept frozen at -80°C until the RNA was extracted. For this, the cell extract was unfrozen at room temperature and 130 µL chloroform were added. After being vigorously homogenized by inversion, the samples were incubated for 10 minutes at room temperature, and then the samples were centrifuged at 12.000 g for 15 minutes, at 4°C (Mikro 200R, HettichZentrifugen). After this we obtained a separation of the different

phases, with the total RNA in the aqueous phase, at the top, the DNA in the intermediate phase, and the organic phase at the bottom with the protein remains. The aqueous phase containing the RNA was transferred to an eppendorf and incubated for 10 minutes at room temperature with 350µL isopropanol to precipitate the RNA. After incubation, the sample was centrifuged (12,000 g for 10 minutes, at 4ºC), the supernatant was discarded and 300µL 75% ethanol at -20°C was added to the precipitated RNA. To remove any impurities the sample was centrifuged at 7.500 g for 5 minutes, at 4°C. The supernatant was discarded, and the ethanol was removed. The RNA present in the sediment was resuspended in 10µL of water containing 0.01% diethylpyrocarbon (DEPC). The samples were heated for 5 minutes at 55°C to ensure the solubility of total RNA.

The total RNA concentration was determined using the UV/Vis spectrophotometer NanoPhotometer™ (Implen). When necessary the samples were diluted in DEPC water. The absorbances were read at 260nm and 280nm. To evaluate the integrity of RNA the samples were subjected to a 1% agarose gel with 0.05% GreenSafe. For this 2µL of the sample was mixed with 8 µL of sterile water and 1µL of 10x loading buffer. The electrophoresis was done at 100V for 40 minutes. RNA integrity was evaluated by the presence of two distinct bands of RNA, 18S and 28S.

3.8. Synthesis of cDNA

To obtain the complementary deoxyribonucleic acid (cDNA), 1µg RNA was used. The reaction was prepared with a volume of reagent enough for n+1. Each reaction mix was prepared with 1µL Random Primers 50µg/µL (Random hexamer mix MB12901 - NZYtech), the volume corresponding to 1µg of total RNA, 1µL deoxynucleotidostriphosphate-dNTPs (10mM, #R0141, #R0151, #R0161, #R0171 – Thermo Scientific) and sterile water was added to a final volume of 17µL. For initial denaturation, the mix was incubated at 65°C for 5 minutes in a thermal cycler (T100-Thermo cycle Bio-Rad – 621BR18717). After this incubation 2µL of 10x Buffer and 1µL of reverse transcriptase (NZTtech M-MuLV 20000U) were added to make a total volume of 20µl. For the cDNA synthesis the samples were incubated at 37°C for 50 minutes. Finally, the reverse transcriptase was inactivated with an incubation at 70°C for 15 minutes. All cDNA samples were stored at -20°C to prevent degradation.

3.9. Conventional PCR

We use conventional polymerase chain reaction (PCR) to optimize the protocol used with the primers and sample. The following primers were designed with the PrimerBLAST-NCBI-NIH program and purchased from STAB VIDA: BDNF FW 5'-CTTCTTTGCTGCAGAACAGG-3' RV 5'-CTTCTCACCTGGTGGAACTT-3' fragment size 130pb, Cyclophilin A CyPA (CiA) FW5'- CAAGACTGAGTGGCTGGATGG-3' RV 5'-GCCCGCAAGTCAAAGAAATTAGAG-3' fragment size 163pb.

Each reaction a Mix was prepared with 2.5µl of 10x Buffer (which includes 20 mM MgCl2 and 0.625µl Taq DNAPolymerase, EP0702, Nzytech), 0.5µl dNTPS (10mM, #R0141, #R0151, #R0161, #R0171 Thermo Scientific), 0.75µl Forward primer (10mM) and 0.75 µL Reverse primer (10mM). The reaction mix was completed with sterile water to a volume of 24µl. Two volumes of cDNA (1µl and 2 µl) as well as two annealing temperatures were tested (58 and 60°C). The MultiGeneTM OptiMax Thermal Cycler (Labnet International 1308028) was used for the reaction with the following protocol: 95°C for 3 minutes, followed by 40 cycles of 95°C for 30 seconds, then 45 seconds at the optimal annealing temperature (58 and 60°C), 1 minute at 72°C and finally 5 minutes at 72°C. To ensure specific amplification, the PCR product was subjected to a 2% agarose gel with a 0.05% GreenSafe (100v for 40 minutes). The bands were visualized in a UVITEC transilluminator (UVitec Cambridge, United Kingdom) (Figure. 5A).

3.10. Real Time RT-PCR

To evaluate the expression of the BDNF gene in the samples, we used the real time RT-PCR. To evaluate the efficiency of the primers a dilution in sterile water of the samples (1: 1: 5, 1: 25 and 1: 125) was made. For each reaction we added 1µL of cDNA (with exception of the negative control), 10µL of Sybr Green (Luminaris HiGreen Fluorescein qPCR Master Mix K0983 – Thermofisher), 0.6µL 10mM FW, 0.6µL 10mM RV (BDNF) and 10.2µL 10mM FW, 10.2µL 10mM RV (Cyclophilin A CyPA) and sterile water to reach a final volume of 20µl. In a CFX ConnectTM (Bio-Rad) thermal cycler, the following protocol was used: initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 10 seconds, annealing at 58°C for 45 seconds, extension at 72°C for 10 seconds (40 cycles). The mRNA expression was determined using the cycle time (CT) values and were normalized to the housekeeping gene cyclophilin A. The results are expressed as $2^{-\Delta\Delta CT}$ for the control.

3.11. Morphometric analysis of neurons

The analysis of neuronal arborization provides information about dendritic ramifications and Sholl analysis is a tool commonly used to analyze changes in these ramifications, providing information such as number of neurites, length of neurites and number of intersections at specific distances from the soma. For this analysis we used a neuron-enriched culture. In these cellular preparations only individualized neurons, in which the neurite end did not touch another neuron, or leave the limit of the image, were used for quantification. Image analysis was done with FIJI software (Wayne Rasband, NIH) with a standard distance of 5 µm between each circle (Figure. 4).

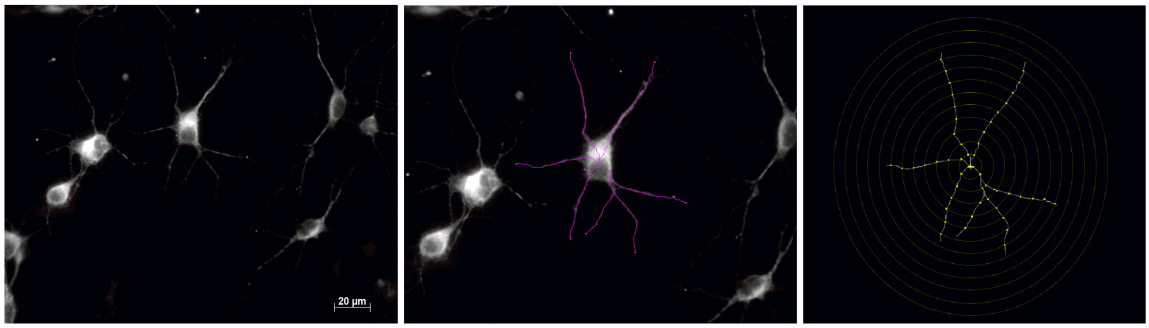

Figure 4: Representative image of the Sholl analysis performed with the FIJI software. The individualized neuron was subjected to 2-D reconstructions using the plugin Simple Neurite Tracer, and Sholl analysis. The intersections were defined at 5µm from the soma.

3.12. Statistical analysis

The results are expressed in number of cells per field, percentage of the control, number of neurites, length of neurites or number of intersections, as specified in the text and in the figure legends. All data represent the mean ± S.E.M. of 3 experiments, performed with different cellular preparations. Each experimental condition was always performed in triplicate and at least in 3 different cellular preparations. Comparisons between three or more groups with only one variable were made with one-way ANOVA followed by Bonferroni's multiple comparison test, while comparisons with more than one variable, when using conditioned medium, were made with two-way ANOVA followed by Bonferroni's multiple comparison test (as indicated in the figure legends). Values of $P<0.05$ were considered significant. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Sotware Inc., San Diego, CA).

Chapter 4

Results

4. Results

In this work we used as experimental model primary cultures obtained from rat embryonic cortex. Both neuron-glia cultures, astrocytes-enriched cultures and neuron-enriched cultures were used. The neuron-glia cultures and neuron-glia cultures were previously characterized by our research group[102,105], whereas the astrocyte enriched culture was characterized during the current project.

4.1. Characterization of the astrocyte-enriched culture

Enriched primary culture of astrocytes were obtained from rat cortex at embryonic day 15-16. Immunocytochemistry against GFAP, an intermediate filament type III protein expressed by astrocytes, and Microtubule Associated Proteins 2 (MAP2), the primary component of the cytoskeletal network, was performed to evaluate the purity of the culture. The total number of cells was assessed by staining the nuclei of all cells with Hoechst 33342 (Figure 5 A). About 97% of the cells present in the culture expressed GFAP and almost 2% expressed MAP2, less than 1% are double-negative (Figure 5 B).

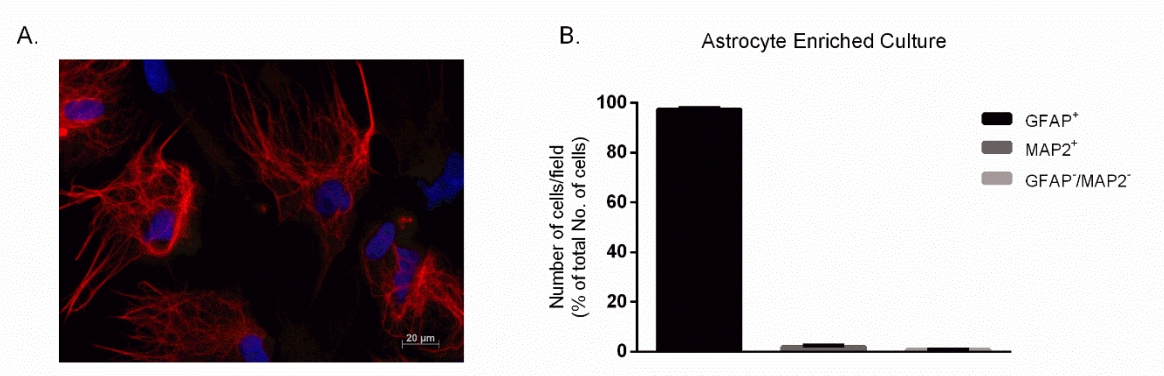

Figure 5: Characterization of astrocyte-enriched cultures from the rat embryonic cortex. **A.** Representative image from the immunocytochemistry performed against GFAP (red) and MAP2 (green) with nuclei stained with Hoechst 33342 (blue). The image was obtained with a 63x objective. **B.** Quantification of the percentage of cells labeled for GFAP and MAP2. Due to the low number of cells in the culture expressing MAP2, the representative image does not show any $MAP2^+$ cell. The graph bars represent the mean ± SEM of 3 independent experiments performed in triplicate. The total number of cells was assessed by quantifying Hoechst 33342-labelled nuclei.

4.2. Effect of medium conditioned by cells subject to HF-rMS and OGD on ERK 1/2 expression

To simulate an episode of ischemia neuron-enriched cultures were subjected to OGD for 6h. To evaluate the impact of HF-rMS in conditions of ischemia, and the role of mediators released by astrocytes, subsequently, during the reperfusion period, the cultures were exposed to media previously conditioned by astrocytes or by neuron-glia cultures in 4 experimental conditions: control, OGD, rMS or OGD + rMS.

Neuron-enriched cultures submitted to OGD were placed in contact with astrocytes conditioned medium (astrocyte-CM) or neuron-glia conditioned medium (neuron glia-CM) for 24h, during the reperfusion period. After this period, the expression of ERK 1/2, a protein with functions in neuronal plasticity, neuroprotection, and survival was determined immunocytochemistry (Figure 6A). Neuronal cultures subjected to OGD presented an increase of 38% in the number of cells that expressed ERK 1/2 and the exposure to either Astrocytes-CM or neuron glial-CM did not alter the number of cells expressing ERK 1/2 (Figure 6 B-C). However, Astrocyte-CM and neuron glial-CM from cells subjected to HF-rMS increased the number of ERK $1/2^{+}$ cells in culture by 74% and 65%, respectively (Figure 6 B-C). When comparing the effect of medium conditioned by cells subject only to OGD with the effect of medium conditioned by cells in which HF-rMS is applied after OGD, there was an increase in the expression of ERK 1/2 of 42% for both astrocyte-CM and neuro glial-CM (Figure 6 B-C).

The expression of ERK $1/2^{+}$ cells in control neuronal cultures was not altered by the exposure to CM, with the exception of the cultures exposed astrocyte-CM+rMS 16% and neuro glial-CM+rMS 53%, astrocyte-CM+OGD+rMS 27% and neuro glial-CM+OGD+rMS 36% (Figure 6 B-C).

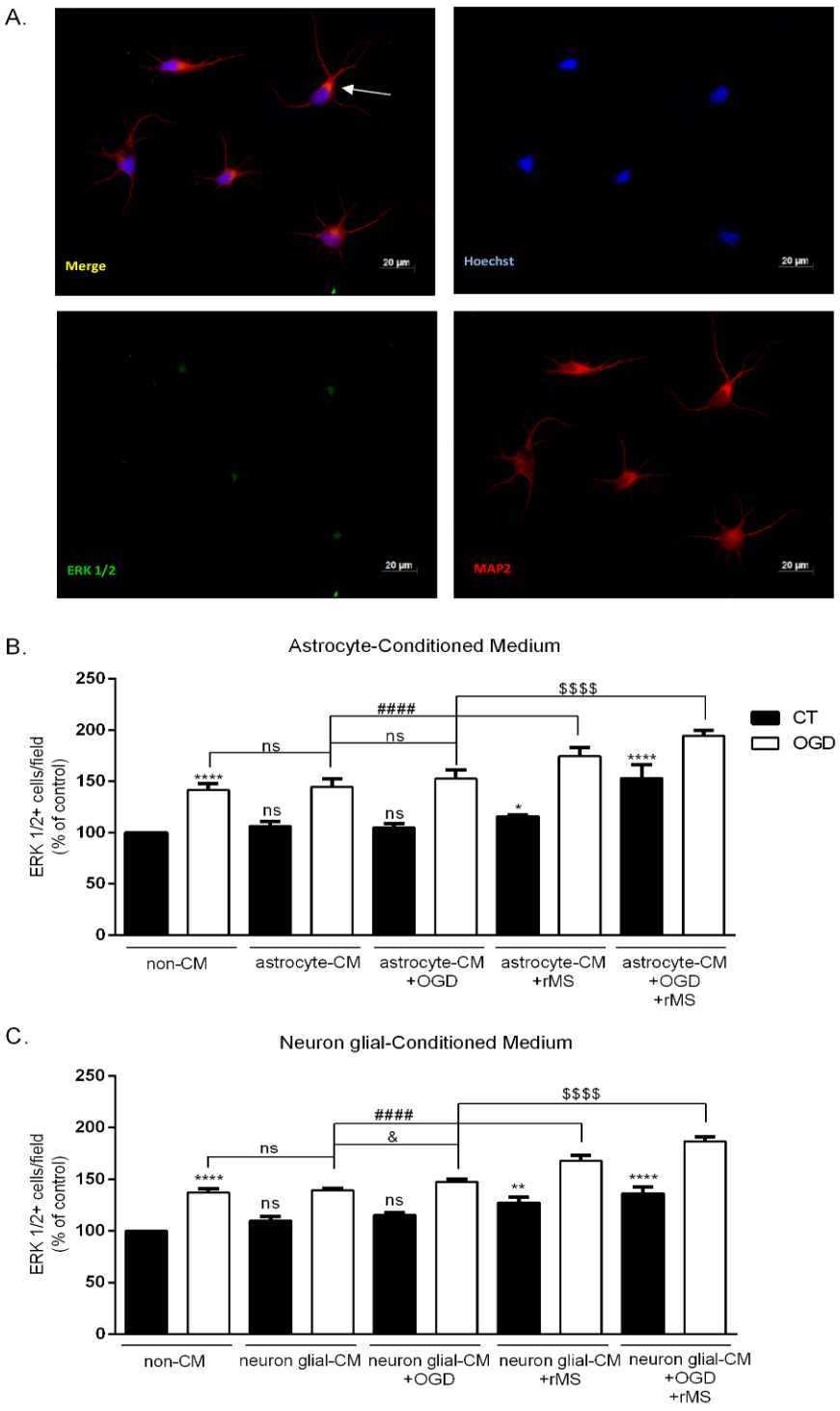

Figure 6: Effect of the medium conditioned by astrocyte or neuron-glial cultures exposed to OGD and HF-rMS, on the number of neurons expressing ERK 1/2. **A.** Representative images of immunocytochemistry performed against MAP2 (red) ERK 1/2 (green) and Hoechst 33342 staining (blue), the image was obtained with a 63x objective. **B.** Quantification of the percentage of cells MAP2$^+$ expressing ERK 1/2 in a neuron enriched culture control and exposed to 6 hours of OGD, which were exposed to Astrocyte CM for 24 hours. Results are expressed as percentage of control and represent the mean ± SEM of 3 independent experiments performed in triplicate. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparison test. ****P<0.0001 and *P<0.05 compared to control non-CM; ####P<0.0001 compared to astrocyte-CM; $$$$ P<0.0001 compared to astrocyte-CM+OGD; ns, not significant. **C.** Quantification of the percentage of MAP2$^+$ cells expressing ERK 1/2 in neuron-enriched culture either in control or exposed to 6 hours of OGD, which were subsequently incubated with non-CM or Neuron-glia Conditioned Medium for 24 hours. Results are expressed as percentage of control and represent the mean ± SEM of 3 independent experiments performed in triplicate. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparisons. ****P<0.0001 and **P<0.01 compared to control non-CM; &P<0.05 compared to neuron glial-CM; ####P<0.0001 compared to neuron glial-CM; $$$$ P<0.0001 compared to neuron glial-CM+OGD; ns, not significant.

4.3. Effects of the neuron glia-conditioned medium on neurite degeneration

To evaluate whether the effect triggered by HF-rMS is due to modulation of pathways that result in the release of neuroprotective factors to the medium, we used neuron glial conditioned medium (neuron glial-CM) of cells subject to 6h OGD and subsequently stimulated with HF-rMS. We evaluated changes in the number of neurites, length and neuronal arborization in neuron-enriched cultures in control conditions and in cultures subjected to 6h of OGD. The ischemic insult lead to a decrease in the number of neurites from 7.27±0.23 to 4.57±0.11 and in the length, from 43.49±0.75 μM to 26.94±0.69 μM. the neuron glia-CM was not able to prevent the neurodegenerative effects triggered by OGD (Figure 7 A-B-C). On the other hand, the neuron glial-CM from cells stimulated with HF-rMS prevented the degeneration of neurites induced by OGD. When comparing the effects of neuron glial-CM and neuron glial-CM+rMS, we observed an increase from 4.66±0.07 to 6.64±0.08 in the number of neurites, and from 28.31±0.78 to 40.80±0.78 in the length of neurites (Figure 7 A-B-C). Similar results were observed when comparing the effects of astrocyte-CM+OGD with the astrocyte-CM+OGD+rMS. Medium from neuron-glia cultures subjected to HF-rMS after an ischemic insult prevented the degeneration of neurites (from 4.57±0.11 to 6.28±0.21) and the length of the neurites (from 28.21±0.41 to 38.30±0.98) as well as of neuronal arborization.

Interestingly, in control neuronal cultures exposure to neuron glial-CM+OGD decreased the number of neurites from 7.27±0.23 to 6.40±0.10, but not their length. On the other hand, neuron-glial media conditioned by cells subject to HF-rMS after OGD increased the length of the neurons from 43.49±0.75 to 52.72±1.30 (Figure 7 A-B-C).

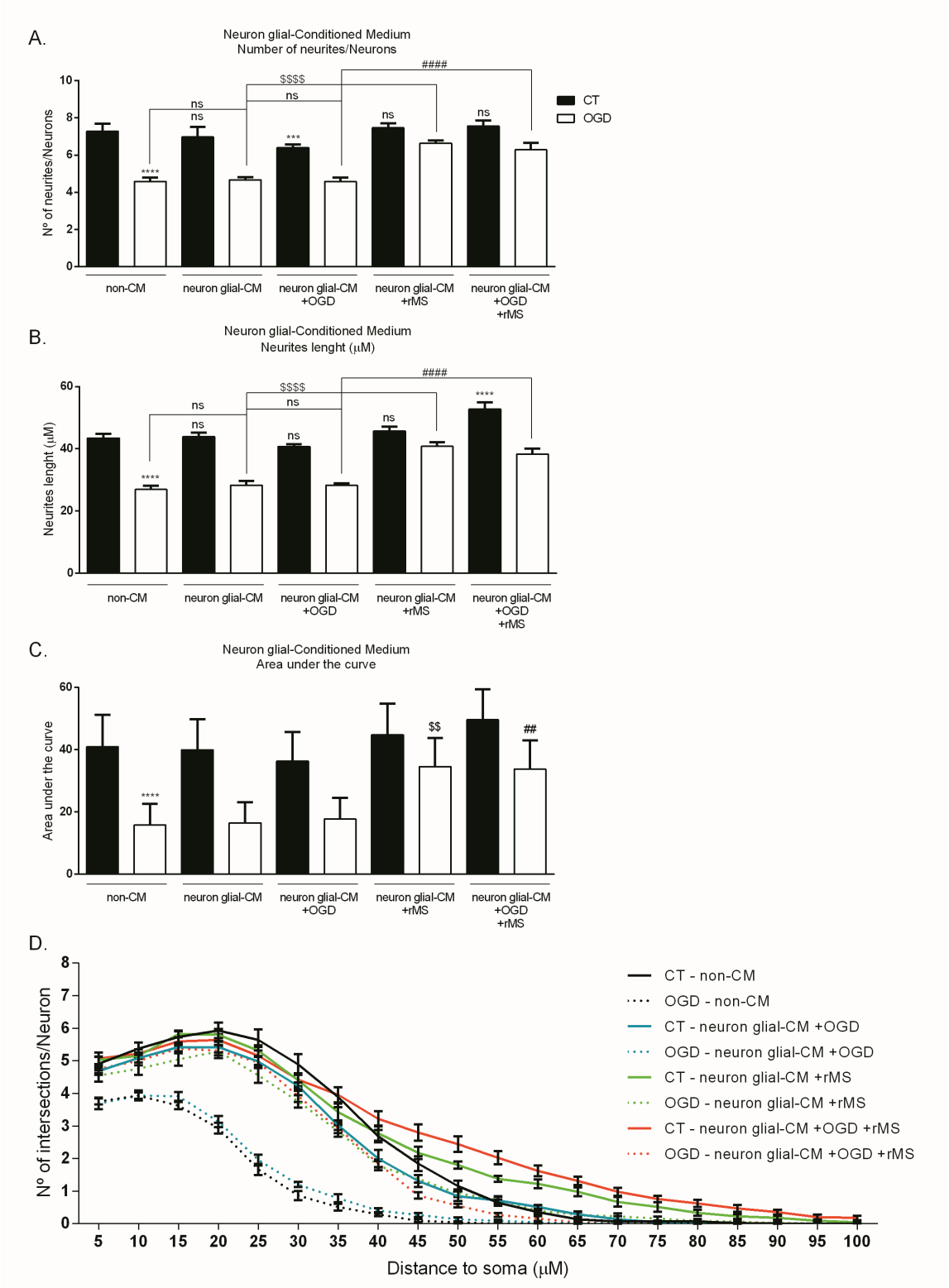

Figure 7: Evaluation of the effects of media conditioned by neuron glial-cultures subject to OGD and HF-rMS on neuronal morphometric changes triggered by 6h of OGD. **A.** Quantification of neurite number (number of neurites/neuron). ****P<0.0001 compared to control non-CM; ####P<0.0001 compared to neuron glial-CM+OGD; $$$$ P<0.0001 compared to neuron glial-CM; ns, not significant. **B.** Quantification of neurite length (neurite length/neuron). ****P<0.0001 to control non-CM; ####P<0.0001 compared to neuron glial-CM+OGD; $$$$ P<0.0001 compared to neuron glial-CM; ns, not significant. **C.** Analysis of the area under the curve. ****P<0.0001 compared to control non-CM; ##P<0.01 compared to neuron glial-CM+OGD; $$ P<0.01 compared to neuron glial-CM; ns, not significant. **D.** Sholl analysis (number of intersections/distance to soma). All results represent the mean ± SEM of 3 independent cell preparations with each experimental condition performed in triplicate. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparisons. All images were obtained with a 63x objective.

4.4. Effect of astrocyte-conditioned medium on neurite degeneration

The results obtained show that HF-rMS may trigger pathways that induce the release of neuroprotective factors. However, concerning the morphometric changes it was unclear whether HF-rMS acted directly on astrocytes, if it was exerted on neurons, or if it requires a crosstalk between the two cell types. To clarify this point, we analyzed the changes induced by the exposure, during the 24h reperfusion period, to astrocyte-CM from cells subject OGD and HF-rMS in the number of neurites, length of neurites and neuronal arborization.

The results obtained were very similar to the results obtained with the neuron-glia CM. The ischemic insult significantly decreased the number of neurites from 7.08±0.25 to 4.75±0.15, the length of the neurites from 41.77±1.10 to 28.81±1.01 and neuronal arborization. The astrocyte-CM and the astrocyte-CM from cells subject to OGD did not prevent the loss of neurites or had the ability to maintain the neuronal arborization in neurons subjected to the ischemic insult (Figure 8 A-B-C). In neurons subjected to OGD that were reperfused with CM from astrocytes stimulated with HF-rMS there was a significant decrease in the loss of neurites (from 4.75±0.15 to 6.15±0.05) and in the decrease of neurites length (from 28.81±1.01 μm to 41.50±1.15 μm (Figure 8 A-B-C). Similar results were observed with CM from astrocytes subjected to HF-rMS after OGD. When compared to the effect of CM from astrocytes exposed only to OGD, there was a decrease in neurite loss from 4.84±0.19 to 6.42±0.14, on the neurite length from 32.02±1.10 μm to 40,97±1.02 μm and on neuronal arborization.

Although in neurons exposed to OGD the effects of media conditioned by astrocytes exposed to HF-rMS only or to HF-rMS after OGD were identical, in the case of control neurons changes in morphology were only observed with media conditioned by astrocytes exposed to HF-rMS after OGD, with an increase in the length of neurites from 41.77±1.10 to 50.49±1.24 (Figure 8 A-B-C).

The fact that the same protective effect was obtained with media conditioned by cultures devoid of neurons suggest that the protective factors are indeed released by astrocytes and does not require the involvement of neurons.

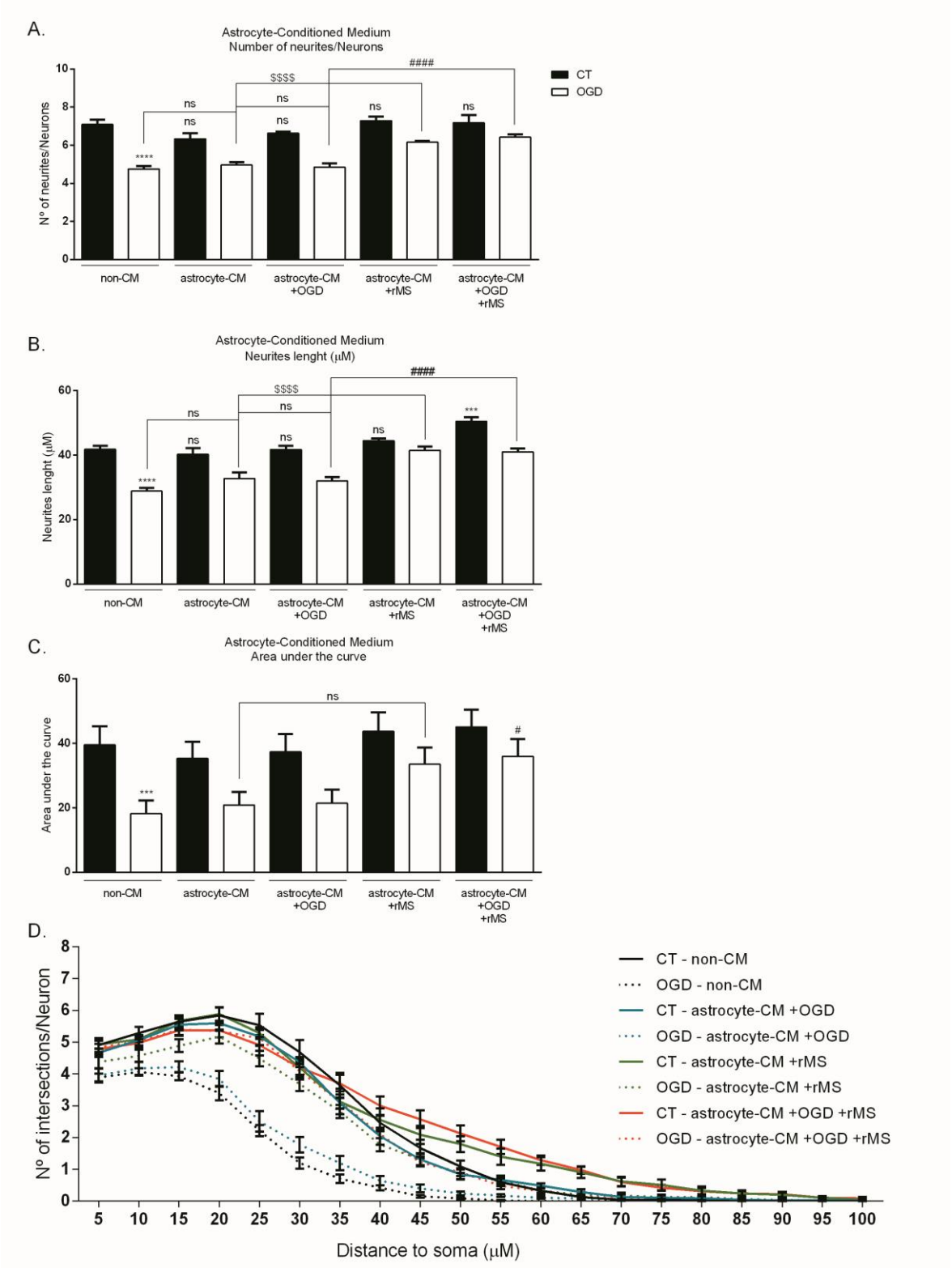

Figure 8: Evaluation of the effects of media conditioned by astrocytes subject to HF-rMS and OGD on neuronal morphometric changes in neuron-enriched cultures exposed to OGD. **A.** Quantification of the number of neurites per neuron. ****$P<0.0001$ compared to control non-CM; ####$P<0.0001$ compared to astrocyte-CM+OGD; $$$$ $P<0.0001$ compared to astrocyte-CM; ns, not significant. **B.** Quantification of neurite length per neuron). ****$P<0.0001$ and ***$P<0.001$ compared to control non-CM; ####$P<0.0001$ compared to astrocyte-CM+OGD; $$$$ $P<0.0001$ compared to astrocyte-CM; ns, not significant. **C.** Analysis of the area under the curve**.** ***$P<0.001$ compared to control non-CM; #$P<0.05$ compared to astrocyte-CM; ns, not significant. **D**. Sholl analysis (number of intersection/neuron over the distance to soma). All results represent the mean ± SEM of 3 independent cell preparations with each experimental condition performed in triplicate. All statistical analysis was performed using two-way ANOVA followed by Bonferroni's multiple comparisons test. All images were obtained with a 63x objective.

4.5. HF-rMS induces BDNF expression in a cellular model of ischemia

The brain-derived neurotrophic factor (BDNF) plays an important role in plasticity, neuronal survival, and neuronal growth[77]. To assess whether the neuroprotective effects induced by HF-rMS are associated to the modulation of BDNF expression, we used cell extracts collected from two distinct cultures, astrocyte-enriched cultures and neuron-glia cultures. Conventional PCR was used for verification of the BDNF gene primers. A PCR product run in 1% agarose gel stained with GreenSafe confirmed that the size of the fragment obtained (130pb) correspond to the BDNF gene (Figure 9 A).

BDNF expression was determined by real time RT-PCR in cultures subjected to HF-rMS, OGD and HF-rMS after OGD. For both cultures, an increase in BDNF expression, of about 60% increase in the neuro-glia culture and of 26% in the astrocyte-enriched culture, was observed when the HF-rMS was applied after the ischemic insult (Figure 9 B-C).

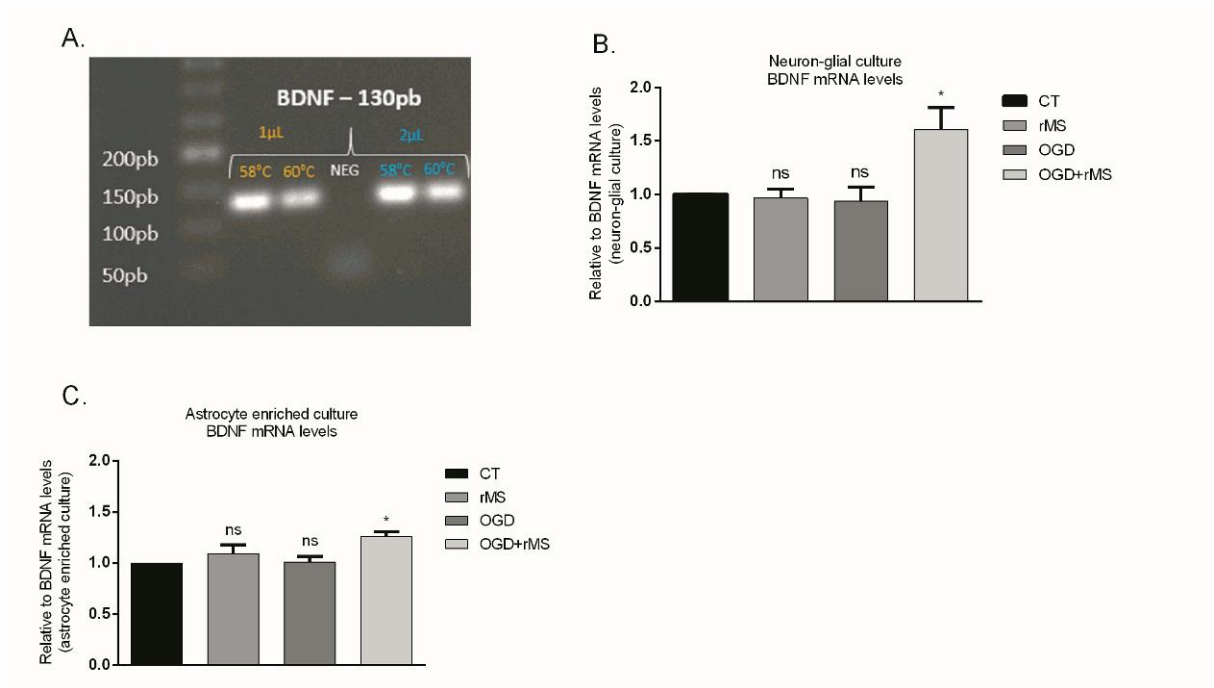

Figure 9: Expression of BDNF in astrocyte-enriched cultures and neuron-glia cultures from embryonic cortex. **A**. Representative image of the product obtained from conventional PCR, with a band at 130pb for BDNF mRNA. From left to right is represented the molecular weight ladder, two temperatures tested with 1µL cDNA, negative control, and a test with 2µL. **B**. Quantification of BDNF expression by real time RT-PCR in neuron-glia culture, **P*<0.05 compared to OGD; ns, not significant **C.** Quantification of the BDNF expression by RT-PCR in astrocyte-enriched cultures, **P*<0.05 compared to OGD; ns, not significant. All results represent the mean ± SEM of 3 independent cell preparations for each experimental condition. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's multiple comparisons test.

4.6. HF-rMS was not able to alter astrocytic phenotype after an ischemic insult

To assess whether HF-rMS has the ability to change the phenotype of astrocytes after an ischemic insult, we use a neuron-glia culture subjected to HF-rMS, OGD or HF-rMS after OGD. In a recent study it was demonstrated that the astrocyte phenotype A1 is present in neuroinflammatory and neurodegenerative diseases, with the complementary component 3 (C3) being highly upregulated specifically in the astrocyte A1 and not the astrocyte phenotype A2. Using immunocytochemistry we quantified the cells co-marked for GFAP and $C3^+$ (Figure 10A). Data showed that the ischemic insult increases the number of astrocytes presenting the A1 phenotype by about 260%, as compared to the control. HF-rMS applied either to control cultures or to cultures injured by OGD did not altered the percentage of $C3^+$-cells (Figure 10 B).

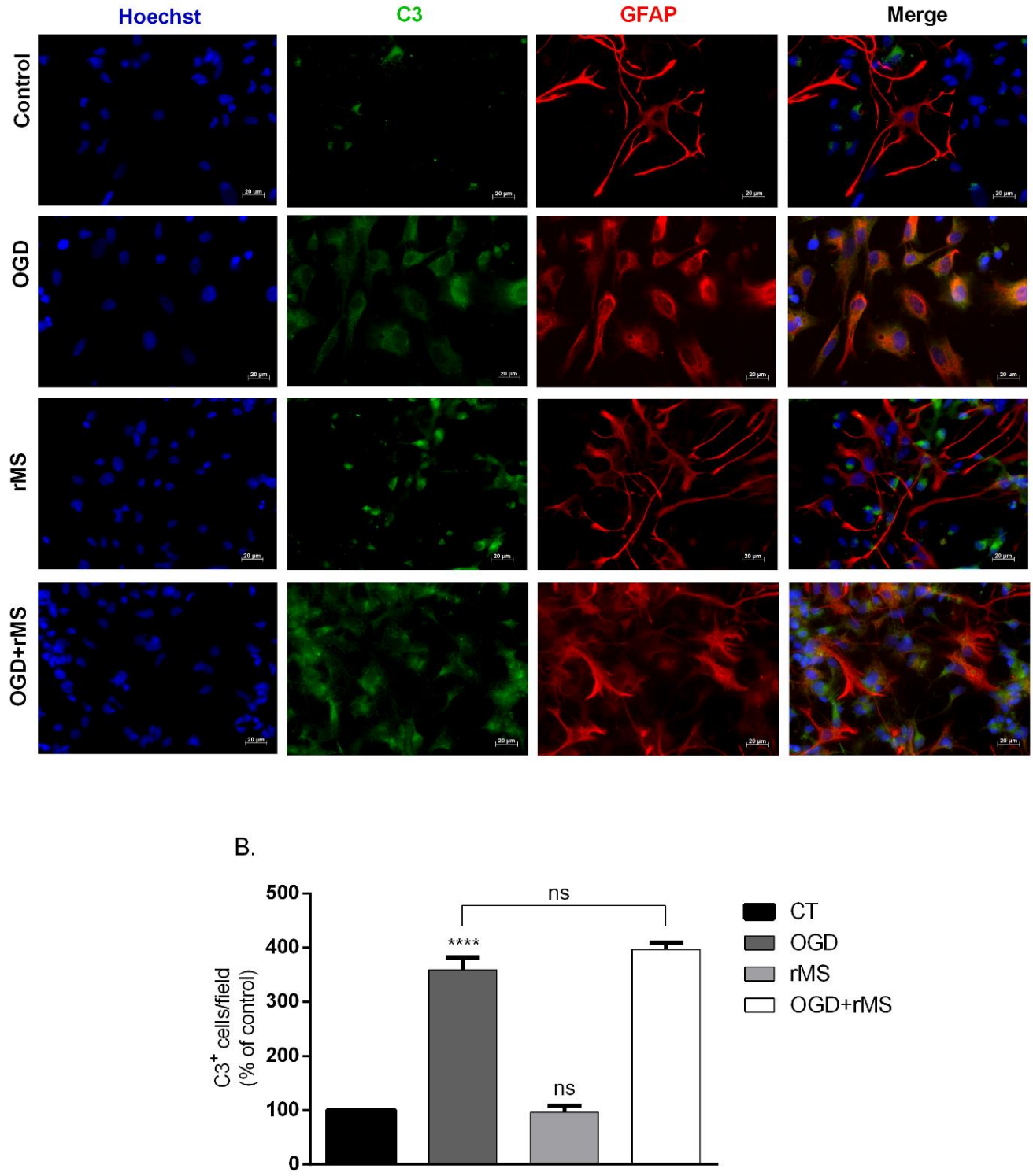

Figure 10: Evaluation of astrocytic phenotype in a neuron-glia culture subjected to rMS, OGD and OGD+rMS. **A.** Representative image shows the immunocytochemistry performed against GFAP (red) C3 (green) with nuclei were stained with Hoechst 33342 (blue), the image was obtained with 63x objective. **B.** Quantification of the percentage of cells labeled for GFAP and C3. Each bar represents the mean ± SEM of 3 independent experiments performed in triplicate. The total number of cells was assessed by quantifying Hoechst 33342-labelled nuclei. Statistical analysis was performed using on-way ANOVA followed by Bonferroni's multiple comparison test. ****P<0.0001 compared to CT, ns, not significant.

Chapter 5

Discussion

5. Discussion

rTMS is accepted as an important modulator of neuronal excitability[107]. Recent data suggest that it affects neuronal plasticity, stimulates survival pathways and decreases the loss of neurons after injury[66,108,109]. Although there are many studies on rTMS, the cellular mechanisms that induce positive effects are not well described[2], most studies evaluate the effects of HF-rTMS on neurons, and there is little data on the effects on astrocytes, currently is seen as an alternative non-invasive method for the treatment of the effects caused by ischemic stroke, recent clinical studies show that the rTMS has positive effects on the control of motor symptoms of acute and subacute stroke patients[42] and also on chronic patients[43,110].

In this work we evaluated the neuroprotective effects triggered by HF-rMS in ischemic injury, and how astrocytes may be involved in these processes. Initially, we observed how the HF-rMS could act on MAPK/ERK1/2 pathway and modulate this survival pathway. The results show that the ischemic injury alone increases the number of neurons that express ERK1/2 and HF-rMS potentiates these effects by inducing an increasing of about 75%, which is in accordance with the current literature[66,111,112]. However, with our approach we did not apply HF-rMS directly to neurons, instead we used media conditioned by astrocyte and neuron-glial cultures and obtained similar results with both. Therefore, our data suggests that HF-rMS triggers pathways in astrocytes that promote the release of neuroprotective factors to the medium, and that physical presence is not essential for the improvement of neuron survival pathways MAPK/ERK1/2 after an ischemic injury, demonstrating that the effects observed are due to changes in the secretome. Another important aspect is that the increase of ERK1/2 pathway triggered by HF-rMS is not dependent on the ischemic insult, since in HF-rMS and OGD conditions alone significant increases on ERK1/2 $^{+}$cells were observed.

To determine whether HF-rMS triggered the expression of BDNF, a key neurotrophic factor for neuronal survival and growth, BDNF mRNA was quantified by RT-PCR. Cells stimulated with HF-rMS after ischemic injury presented an increase of BDNF mRNA by about 60% in the neuron-glia culture and by 26% in the astrocyte-enriched culture. Similar results were obtained in a study with wistar rats[60,76] and in cellular model[111,113,114]. Since no changes of BDNF expression were observed in the rMS and OGD condition alone, this suggest that the increase of BDNF expression triggered by HF-rMS is dependent on the ischemic insult.

The motor recovery observed in studies with animal models, as well as decreased neuronal loss observed in cellular models, may be associated with the modulation of the pathways mentioned above. Importantly, differences in the rMS protocol, such as intensity of magnetic stimulation can influence the amount of BDNF released, as demonstrated by Lin and collaborators[115].

Subsequently, we analyzed whether the decrease in neurite degeneration triggered by HF-rMS observed in a previous study of our group[102] was also mediated by changes in the secretome. Both conditioned media, when stimulated with HF-rMS after an ischemic insult, produced a protective effect, preserving the number of neurites and the length, and providing protection of neuronal arborization. These effects are fundamental to maintain neuronal communication and the homeostasis of the cellular environment that is impaired after an ischemic insult. Other studies show that HF-rMS promotes increased neurites growth[115,116], although the neurons analyzed were stimulated directly. Here we showed that these effects may be linked to factor release by astrocytes, since in neuron-enriched culture stimulated HF-rMS after ischemic insult the neuroprotective effects were maintained[102].

Astrocytes are fundamental cells to maintain the homeostasis of the central nervous system and are very abundant glial cells[117]. A recent study classified reactive astrocytes that lose most of their protective functions as A1. After a CNS lesion these astrocytes become neurotoxic and increase the neurological damage[95]. Our results show that the ischemic lesion significantly increased the number of $C3^+$ astrocytes , a marker of A1 phenotype [95]. However, HF-rMS was not able to alter the number of A1 astrocytes. On the other hand, a recent study has shown that HF-rMS applied after ischemia has the ability to decrease the expression of C3 mRNA in astrocytes[100], these differences may be associated with the time it takes for the mRNA to translate into protein, or to differences in the rMS protocol. The absence of a standard protocol is one of the main difficulties in comparing the effect of rMS between studies.

Chapter 6

Conclusions and Future Perspectives

6. Conclusions and Future Perspectives

Through this work we add some information on the role of astrocytes in ischemia and how HF-rMS can act to reduce the injury. We demonstrate that HF-rMS acts on astrocytes by inducing the release of neuroprotective factors. Our results also showed that BDNF expression is increased when HF-rMS was applied after an ischemic insult. This suggest that the increase in BDNF may contribute to the protective effects observed, both in maintaining the neuronal arborization and increasing the MAPK/ERK1/2 pathway. However, we observed that application of HF-rMS alone, in the absence of OGD, was able to promote protection, but not to induce the expression of BDNF, which suggest that other mechanisms, besides the promotion of BDNF expression and release, are stimulated by HF-rMS.

The HF-rMS protocol used did not have the capacity to decrease the number of A1 astrocytes present in the culture after ischemia. The studies focusing on the action of rMS on astrocytes are still scarce, with many aspects still unclear. Further experiments could be done to advance the understanding of rMS effects and what cellular mechanisms are involved, we can suggest the following approaches:

- To explore whether rMS has the capacity to change the phenotype of astrocytes, using a more extensive panel of markers such as, S100A10, Arg1 and IL-10 as anti-inflammatory markers, and iNOS, TNF-a and IL-12 pro-inflammatory markers.

- To evaluate the impact of rMS on other glial cells such as microglial cells, which have a key role in neuroinflammation, after an ischemic insult and rMS. Markers of microglial reactivity, such as iba1, CD68 and others may be assessed. The impact of the putative modulation of microglia on neuroprotection may also be studied.

- To clarify the contribution of BDNF to the neuroprotective effects of rMS by testing the effect of TRK-B antagonists such as ANA-12, or by silencing BDNF.

Chapter 7

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7. Bibliographic References

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