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Role of astrocytes in an *in vitro* model of ischemic stroke

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Dedicatory

To all those whom might use this knowledge to take a step forward towards something, that really helps people to recover from their illness

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List of Publications:

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Resumo

O Acidente vascular cerebral isquémico representa uma das principais causas de incapacidade em países desenvolvidos, e mesmo após décadas de investigação ainda não existem abordagens terapêuticas eficazes, especialmente nas fases subaguda e crónica da doença. Atualmente, nestes estadios da patologia, não existe uma alternativa que promova a recuperação dos tecidos cerebrais que foram afetados pela isquemia. A maior parte dos tratamentos (fisioterapia, terapia da fala, terapia ocupacional, etc.) são aplicados com o objetivo de reduzir as sequelas ou de controlar os fatores de risco modificáveis (hipertensão, diabetes, coagulopatias, etc.). O que leva a que exista uma necessidade de desenvolver novas abordagens que possibilitem a recuperação desses tecidos, diminuam os défices neuronais e, se possível, promovam a melhoria das funções que são reguladas pelas regiões cerebrais afetadas.

Tendo isto em consideração, este trabalho tem como principal objetivo explorar a ação de duas abordagens distintas na recuperação de lesões isquémicas. A primeira está relacionada com os potentes efeitos fisiológicos do estrogénio no sistema nervoso central e a sua participação em diversos processos como a neurogénese, promoção da expressão de fatores neuroprotetores e ativação de mecanismos antioxidantes, mais precisamente através da avaliação dos potenciais efeitos benéficos induzidos pela ativação seletiva do recetor de estrogénio acoplado à proteína G (GPER). A segunda será através da avaliação dos efeitos induzidos pela estimulação magnética repetitiva de alta frequência (HF-rMS), uma abordagem que já foi descrita como tendo a capacidade de corrigir distúrbios ao nível da neurotransmissão e de melhorar a comunicação neuronal durante o processo de recuperação. Ambas as abordagens já foram descritas como tendo a capacidade de induzir neuroprotecção em patologias neurodegenerativos, como é o caso das doenças de Alzheimer e Parkinson e de perturbações de humor.

De forma a padronizar a lesão isquémica e avaliar os efeitos induzidos por estas duas abordagens, vários modelos *in vitro* foram desenvolvidos e caracterizados. Foram utilizados três tipos de culturas primárias do córtex (cultura de astrócitos, cultura de neurónios e cultura de neurónios e células gliais), as quais foram submetidas à privação de oxigénio e glucose, seguindo-se um período de reperfusão. A avaliação dos efeitos induzidos por estas duas abordagens foi feita através de vários parâmetros relacionados com a sobrevivência e proliferação celular, avaliação do cálcio intracelular, assim como da análise morfométrica das neurites e de modificações sinápticas.

Em relação ao papel do GPER na lesão isquémica, observamos que a privação de oxigénio e glucose não alterou os níveis de expressão deste recetor, nem em neurónios nem em astrócitos. A ativação seletiva do GPER não teve impacto na sobrevivência neuronal mas

promoveu a morte astrocitária através de um mecanismo que envolve a ativação da via da fosfolipase C e o subsequente aumento dos níveis de cálcio intracelular. Estes dados mostram um impacto direto do GPER na viabilidade dos astrócitos e que a ativação do GPER está associada a diferentes vias de sinalização em astrócitos e neurónios.

Os nossos resultados indicam também a HF-rMS reduz alguns dos efeitos negativos desencadeados pela lesão isquémica, tais como a morte neuronal, a degeneração inicial das neurites e a diminuição de marcadores sinápticos. Curiosamente, o efeito protetor da HF-rMS apenas é observável na presença de astrócitos. Estes dados sugerem que a HF-rTMS tem potencial para poder ser utilizada como uma abordagem terapêutica para reduzir a morte neuronal e os danos neuronais, limitando a degeneração das neurites e melhorando a conectividade funcional e a plasticidade sináptica nas áreas afetadas pela isquemia.

Os nossos resultados sugerem também que os astrócitos desempenham um papel crucial na lesão isquémica. Para além de serem mais resistentes a períodos de isquemia do que os neurónios, todos os dados experimentais obtidos mostraram que quando os astrócitos estavam presentes a lesão foi menor, o que indica um papel ativo na proteção neuronal contra a lesão induzida pela isquemia. Tendo em consideração o seu papel preponderante na fisiologia neuronal e o fato de a sua presença ser obrigatória para os efeitos benéficos induzidos pela HF-rMS, parece evidente que os astrócitos podem ter um impacto substancial na proteção e recuperação da lesão induzida por isquemia. Como tal os astrócitos devem ser encarados como potenciais alvos terapêuticos para o tratamento da isquemia cerebral e qualquer metodologia/abordagem que potencialize os seus efeitos protetores pode ser uma abordagem terapêutica bastante promissora.

Palavras-chave:

astrócitos; cálcio intracelular; culturas primárias do córtex; degeneração neurítica; estimulação magnética repetitiva de alta frequência; isquemia; neurónios; plasticidade sináptica; privação de oxigénio e glucose; receptor de estrogénio acoplado à proteína G (GPER ou GPR30);

Abstract

Ischemic stroke (IS) is the leading cause of complex and serious long-term disability in developed countries, and after decades of effort there are no effective clinical treatments for IS, especially in the subacute and chronic phases. Currently, in these stages of the IS there is no alternative to promote the recovery of brain tissues affected by the ischemic injury. Most of the treatments (e.g., physical therapy, speech therapy, occupational therapy) are applied with the aim of reducing the sequelae left, or to controlling modifiable risk factors (e.g., hypertension, diabetes, coagulopathies). This leads to a need to develop new approaches to recover those areas, reduce the neurological deficits and, if possible, enhance the functions regulated by the affected brain regions.

In this context, this work intends to explore two approaches that hypothetically could induce the recovery of the areas affected by ischemia. The first is related to the potent physiological effects of estrogens on central nervous system (CNS) and its participation in several processes such as, neurogenesis, the expression of neuroprotective factors and antioxidant mechanisms, through the evaluation of the potential beneficial effects induced by the selective activation of G protein–coupled estrogen receptor 1 (GPER or GPR30). The second, by evaluating the potential protective effects induced by high frequency repetitive magnetic stimulation (HF-rMS), an approach that has been described as having the ability to correct maladaptive brain plasticity and to enhance neuronal communication during rehabilitation. In both cases the ability to induce neuroprotection in neurodegenerative disorders, such as, Alzheimer´s disease, Parkinson's disease, and mood disorders, was already demonstrated.

To standardize the ischemic damage and evaluate the potential beneficial effects induced by these two approaches several *in vitro* models of ischemia were developed and characterized. Neuron-enriched, neuron-glia, and astrocyte-enriched primary cortical cultures subjected to oxygen and glucose deprivation (OGD) followed by a reperfusion period, were used as models. The evaluation of the effects induced by GPER activation and by HF-rMS was performed through the assessment of several parameters related cell survival and proliferation, GPER expression, calcium imaging, as well as neurite morphometric and synaptic modifications.

Concerning the role of GPER on the ischemic injury, we observe that ischemia did not change the levels of GPER in neurons and astrocytes. Moreover, GPER selective activation had no impact in neuronal survival, whereas it induced the apoptosis of astrocytes, being this effect meditated by the activation of phospholipase C pathway, and the subsequent intracellular calcium rise. These data indicate a direct impact of GPER on the viability of astrocytes, and the coupling of GPER to different signaling pathways in astrocytes and neurons.

Our data also shows that HF-rMS reduces the neuronal loss, the initial neurite degeneration and the loss of synaptic markers triggered by ischemia. Interestingly the

protective effect triggered by HF-rMS required the presence of astrocytes. Taken together the data obtained suggests that HF-rTMS has the potential to be used as a therapeutic approach to reduce neuronal death and neuronal damage, by limiting neurite degeneration and enhance functional connectivity and synaptic plasticity in the areas affected by the ischemia.

Furthermore, our results also suggest that astrocytes play a crucial role on ischemic injury. Astrocytes were more resistant to ischemic periods than neurons in all experiments performed and when they were present the injury was smaller, which indicate an active role in the neuronal protection against ischemia-induced injury. Taking into account their preponderant role in neuronal physiology and the fact that their presence is crucial for the observed beneficial effects induced by HF-rMS it seems evident that astrocytes could have a substantial impact on the protection and recovery of ischemia-induced lesion. Thereby, we hypothesize that astrocytes could be a potential therapeutic target for the treatment of cerebral ischemia and any methodology/approach that potentiate their beneficial effects may be a promising therapeutic approach.

Keywords:

astrocytes; G protein–coupled estrogen receptor 1 (GPER or GPR30); high frequency repetitive magnetic stimulation; intracellular calcium; ischemia; neurite degeneration; neurons; oxygen and glucose deprivation; primary cortical cultures; synaptic plasticity.

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Index of Acronyms:

$[Ca^{2+}]_i$	Intracellular calcium concentration
AD	Alzheimer's disease
ASD	Autism spectrum disorder
ATP	Adenine triphosphate
BBB	Blood-brain barrier;
BDNF	Brain-derived neurotrophic factor
BLA	Basolateral amygdala
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CRE	cAMP response elements
CREB	cAMP response element-binding protein
CT	Computed tomography
DAG	Diacylglycerol
DAT	Dopamine transporter
DGS	Direção-Geral de Saúde
DIV	Day *in vitro*
E2	Estradiol
EAE	Autoimmune encephalomyelitis
ECA	External carotid artery
EGFR	Epidermal growth factor receptor
ER	Estrogen receptors
EREs	Estrogen response elements
ERK1/2	Extracellular signal-regulated kinases 1 and 2
FBS	Fetal bovine serum
GABA	Gamma-aminobutyric Acid
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GPER	G protein–coupled estrogen receptor
HBSS	Hank´s buffered salt solution
HF	High-frequency
HF-rMS	High-frequency repetitive magnetic stimulation
Hz	Hertz
Iba1	Ionized calcium binding adaptor molecule
ICA	Internal carotid artery
ICI-182780	Fulvestrant
IFNγ	Interferon γ
iNOS	Inducible nitric oxide synthase
IP3	Inositol 1,4,5-trisphosphate
IS	Ischemic stroke
JNK	c-Jun N-terminal kinase
LF	Low-frequency
LPS	Lipopolysaccharide
MAP2	Microtubule-associated protein 2
MAPKs	Mitogen-activated protein kinases
MCA	Middle cerebral artery
MCAo	Middle cerebral artery occlusion

MDD	Major depressive disorder
MFI	Mean fluorescence intensity
MMP	Matrix metalloproteinase
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Multiple sclerosis
mTOR	Mammalian target of rapamycin
MTT	3-[4,5-Dimethylthiazol-2-yl] -2,5-diphenyltetrazolium bromide
NBM	Neurobasal medium
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
OCD	Obsessive-compulsive disorder
OGD	Oxygen and glucose Deprivation
OVX	Ovariectomized
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with 0.1% Tween
PD	Parkinson's disease
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PLC	Phospholipase C
PNS	Peripheral nervous system
rMS	repetitive Magnetic Stimulation
ROI	Region of interest
ROS	Reactive oxygen species
rTMS	Repetitive transcranial magnetic stimulation
SCI	Spinal cord injury
SERD	Selective estrogen receptor degrader
SERM	Selective estrogen receptor modulator
SK2	Small conductance calcium-activated potassium channel 2
SSRIs	Selective serotonin reuptake inhibition
TBS	Theta-burst stimulation
tMCAO	Transient middle cerebral artery occlusion
TNFα	Tumor necrosis factor α
tPA	Tissue Plasminogen activator
VEGF-A	Vascular endothelial growth factor A
VMAT	Vesicular monoamine transporter

Chapter I

Introduction

1. - Ischemic stroke

According to the World Health Organization, stroke is a syndrome of rapidly developing clinical signs of focal or global disturbance of cerebral function with vascular origin (1). This disturbance could be caused by ischemic or hemorrhagic imbalance of cerebral blood circulation (2, 3), being ischemic stroke (IS) the most commonly form, with approximately 85% of all stroke cases (2-6).

IS, is a heterogeneous multifactorial neurological disorder characterized by the sudden onset of neurologic signs related directly to the sites of injury in the brain where ischemia occurs (3, 7). It is characterized by the lack of enough blood flow to perfuse the cerebral tissue, leading to irreversible neuronal damage, whose severity is directly proportional to the duration of the ischemic period (4, 8, 9). Even brief ischemic periods can initiate a complex sequence of events that ultimately culminate in cellular death (9). The final infarct size and the neurological outcome depends on multiple factors such as, the duration and severity of ischemia, the existence of collateral systems and an adequate systemic blood pressure (10).

1.1. - Pathophysiology of IS

The decrease or the interruption of the blood supply to brain tissues can have several etiologies, such as thrombosis, embolisms, systemic hypoperfusion and venous thrombosis (3, 11). Each, indicating a different mechanism of blood vessel injury or a reason for decreased blood flow (3).

Cerebral thrombosis refers to the formation of a thrombus inside a cerebral artery, resulting on vascular obstruction (11, 12). Thromboembolic occlusion of major or multiple smaller arteries leads to focal ischemia downstream of blood flow (11, 12). Thrombotic IS occur without warning symptoms in 80% of patients, whereas 20% is heralded by one or more transient Ischemic events (13).

Cerebral embolism refers to a blood clot that is formed at another location in the circulatory system, travels along the vessels and occludes medium sized branching arteries causing ischemia to a localized brain region in the same way that cerebral thrombosis does (3, 11). In fact, the majority of brain embolisms have origin on the heart, aorta, or a proximal artery or vein, for example microemboli can break away from a sclerosed plaque in the carotid artery or from cardiac sources such as atrial fibrillation or a hypokinetic left ventricle and reach the brain causing focal ischemia (3, 11).

Systemic hypoperfusion is characterized by a global decrease in the blood flow to the head (3). Generally, it is the result of complications on the performance of the heart to pump blood adequately to perfuse brain tissues (e.g., myocardial infarction and/or arrhythmia and severe hypotension) or due to inadequate amount of blood and fluid in the vascular compartment of the body (e.g., bleeding, dehydration, and loss of fluid into body tissues) (3). This mechanism induces global ischemia in cerebral tissues and is the worst form of IS (3, 11).

Cerebral venous thrombosis is an uncommon form of IS where a blood clot occludes a dural sinus and/or cerebral veins (14). Usually it affects young individuals and its associated to prior medical conditions (e.g., thrombophilias, inflammatory bowel disease), transient situations (e.g., pregnancy, dehydration, infection), selected medications (e.g., oral contraceptives, substance abuse) and unpredictable events (e.g., head trauma) (14).

Regardless the etiology of the IS on the core of the injury the brain parenchyma undergoes immediate death, while in surrounding areas, the penumbra may only be partially injured with potential to recover (3, 11). The reduction of blood supply associated to low respiratory reserve and complete dependence on aerobic metabolism make brain tissue particularly vulnerable to the effects of ischemia (11). The neurological function is affected by the oxygen and glucose deprivation (OGD) and neuronal injury and cell death begin within 4 minutes of ischemia (3). Then, numerous detrimental effects are triggered, including energy failure, loss of ion homeostasis, acidosis, increased intracellular calcium levels, excitotoxicity, free radical-mediated toxicity, generation of arachidonic acid products, cytokine mediated toxicity, activation of glial cells, disruption of the blood-brain barrier (BBB) and infiltration of leukocytes (3, 9, 15).

1.1.1. - Risk Factors

Several risk factors are associated with an increased risk of IS, and they can be stratified into modifiable and nonmodifiable (12). Modifiable risk factors include those resulting from lifestyle and environment, and can be modified with the help of healthcare professionals, treatment and continuing education, among these the major are: Atherosclerosis, h, blood abnormalities, diabetes mellitus, smoking and obesity (12). Unmodifiable risk factors include factors related to hereditary or natural processes that cannot be modified, such as, age, sex and ethnicity (3, 12).

Atherosclerosis refers to the development of atherosclerotic plaques (atheromas) inside the arteries associated with degeneration of the arterial wall (3). Atheromas develop in the aorta and in the large arteries of the neck and head, and are initiated when high levels of blood lipids (hypercholesterolemia and/or hyperlipidemia) potentiate its accumulation on vascular

smooth muscle cells (3). Lipid, smooth muscle, fibrous tissue, connective tissue, white blood cells, and crystals of cholesterol constitute these plaques. When plaques increase in size they narrow arterial lumen, causing turbulence of flow, and reducing distal brain perfusion (16). The irregular surfaces or cracks on atheromas attract platelets and other blood components that induce the formation of clots (3). Atherosclerotic abnormalities can cause ischemia through three major ways: severe luminal narrowing markedly decreases blood flow; plaques or occlusive thrombus mechanically block branches of the main arteries; propagation and embolization of thrombus cause occlusion of distal branches (16).

Hypertension leads to wear and tear of arteries and accelerates the development of atherosclerotic changes on large arteries. On small arteries of the brain, hypertension leads to thickening of the walls that narrows the lumen of the arteries and can lead to infarcts deep within the brain (3, 12). Longitudinal studies indicate that individuals with high-normal blood pressure (130–139 mm/Hg systolic, 85–89 mm/Hg diastolic, or both) have a twofold increased risk of developing heart disease and IS, than those with normal blood pressure (6). Hypertension can also induce arterial dolichoectasia (dilatative arteriopathy), a condition where the blood vessels become elongated and dilated and follow a tortuous and windy course with frequent loops and curves. These widening and lengthening of arteries can slow blood flow and stimulate blood clotting in the arteries (3).

Several blood components have the purpose of preventing the body from losing blood and a deficiency on these components leads to excessive bleeding, whereas other conditions can lead to excess clotting. Some blood abnormalities that affect the clotting system could be considered risk factors for IS, such as, thrombocytosis (excessive number of platelets), deficiency of some blood proteins (antithrombin III, protein C, and protein S) or excess in clotting proteins (factor VII, VIII, or XII). Medical conditions such as cancer or inflammatory conditions (e.g., inflammatory bowel disease) also increase the tendency of blood to clots (3, 6).

Diabetes mellitus is a well-established independent risk factor for IS. High glucose levels induce pathological changes in blood vessels, which can lead to IS if cerebral vessels are directly affected. These changes include vascular endothelial dysfunction, increased early-age arterial stiffness, systemic inflammation and thickening of the capillary basal membrane (12, 17). It is estimated that nearly 40% of all IS can be attributed to the effects of diabetes either alone or in combination with hypertension (6).

Obesity and abdominal obesity are independent and potent risk factors for IS. This metabolic syndrome correlates with excess body weight and due to its well-known association to other conditions, such as, hypertension, cardiovascular disease, chronic inflammation increases the risk of IS (18, 19).

Several risk factors are associated with lifestyle and daily-life habits, such as smoking, physical inactivity or binge drinking habit. Smoking increases blood levels of carboxyhemoglobin, platelet agregability, and fibrinogen levels and reduces HDL-cholesterol. On the other hand, tobacco compounds induce toxic effects on vessels (12, 20). It is estimated that smoking almost duplicates the relative risk of IS (6). Physical inactivity increase blood pressure, glycaemia levels and body weight, as well as decrease cardiorespiratory capacity and increase the blood pressure at rest (21, 22). The alcohol consumption has been shown to increase blood pressure and heavy or chronic consumption is associated with cardiomyopathy (23).

The genetic predisposition is an unmodifiable risk factor for IS. Over the years several observations linked family clinical history to IS, raising the hypothesis of a genetic basis on IS (24, 25). For example, men whose mother died of IS have a three-fold increased incidence in comparison with men without a maternal history of IS (26). On the other hand, there are also pathologies with genetic predisposition that can lead to IS, as is the case of fibromuscular dysplasia, an uncommon pathological condition that involves the wall of the arteries. In this condition there is an excessive amount of connective tissue and smooth muscle on the wall, this excess narrows and contracts the arterial lumen, which can block blood flow to the brain, causing ischemia (3).

Aging is one of the most significant IS risk factors, with 95% of IS occurring in people with more than 45 years and two-thirds occurring in those over the age of 65 (2, 12). For each consecutive decade after 55 years of age, the risk for stroke approximately duplicates, and the prevalence of IS for individuals older than 80 years of age is approximately 27% (6, 7). Mortality associated with IS also increase with age (2, 12). However, in recent years IS cases are becoming more frequent in younger populations (<45 years) (2, 12).

Sex-specific incidence rates indicate that males until 75 years have a higher risk of IS than females (2, 5), which could be due to a greater prevalence of traditional vascular risk factors (5). However, from this age the risk is similar for both genders (2, 5, 27). Since life expectancy in women is higher, with advancing age the number of woman suffering an IS also increases (2, 5).

The race/ethnicity-specific incidence demonstrates that black individuals have higher risk of suffering an IS than Caucasian, Asian, Hispanic and Indian individuals in any age range or sex (2, 5).

1.1.2. - Prevention

The guidelines for IS prevention are clear and are based on the control and prevention of risk factors that are modifiable (12, 29, 30). For each one of the known modifiable risk factors there are several measures and recommendations to adopt. These guidelines go from simple modifications in diet and lifestyle to pharmacological treatment of pathologies and conditions that are risk factors, such as, hypertension, atrial fibrillation, hyperlipidemia, diabetes mellitus or antiplatelet therapy (29, 30).

1.2. - Signs and symptoms

IS, is a complex neurologic syndrome with sudden onset and with symptoms dependent on the brain region affected by the ischemic injury (3, 7, 31). The most frequent signs and symptoms of patients hospitalized with a confirmed IS diagnosis are paresis (sudden numbness or weakness) on one side of the body. Approximately 87.6% of diagnosed IS subjects presents some kind of paresis, most often of the arms (81.1 %), legs (73.4%) and face (58.7%). Being the location of paresis equally distributed between right and left sides (31).

Sensory deficits are observed on approximately 49% of IS diagnosed patients, most often of the arms (42.3%), legs (37.6%) and face (22.9%). On the face sensory deficits are equal on both sides, whereas on limbs sensory deficits that involve the left side are more frequent (31). Speech deficit (confusion, trouble speaking or difficulty understanding speech) is observed on approximately 26.1% of the patients, severe headache with no known cause on 22.4%, sudden trouble seeing in one or both eyes on 15.4% and gait disturbance on 11.4% of the cases. Convulsions (3.2%) and vertigo (2.5%) are less frequent symptoms (31).

1.3. - Post-Stroke impairments

IS is the leading cause of complex and serious long-term disability in developed countries (2, 32), and data indicate that half of the patients are physically dependent after an IS (32, 33). Functional and clinical problems are persistent with data at five years post-IS demonstrating that approximately two-thirds of patients have some form of neurological impairment and/or disability (2). These impairments can cause significant impact on life quality and restrictions to activities of daily-life, and are closely related to the location, size and severity of the injury and can be classified in physical, cognitive, and emotional.

The physical impairments are associated to motor, visual and somatosensory deficits. Motor impairments affect the balance, coordination and gait, and are the predominant cause of long-term disability (2). Visual impairments can take the form of monocular vision loss, visual field loss on the left or the right side of the midline, or cortical blindness (2, 34). Somatosensory deficits refers to the inability of patients to process and manipulate their environment, and can range in severity from numbness or tingling in one part of the body to complete sensory neglect of a body part or one side of the body (2, 35).

Cognitive impairments can cause several deficits like problems with concentration, attention, memory, orientation, visual spatial perception and apraxia. These type of impairments are linked to long-term mortality and high level of disability (2, 36).

In addition to the effects that are perfectly visible, more subtle symptoms, such as emotional or personality changes may also occur. After an IS patients may experience fear, anxiety, frustration, anger, sadness, and a sense of loss. This may lead to the development of other pathologies such as depression, the most prevalent and the most commonly studied post-stroke mood disorder, with a prevalence of approximately 40% (2, 37, 38). It is also possible that many IS patients develop anxiety. Latest statistics have estimated an occurrence of anxiety of approximately 20-25% (2, 39).

1.4. - Epidemiology

IS is a major global health problem and its significance probably will increase in the future due to ongoing demographic changes, including aged population and health transitions observed in developing countries (5, 32). Until few years ago IS was seen as a pathology of developed countries, because its incidence and prevalence rates were higher than in developing countries, nevertheless is becoming more and more frequent in these countries (5, 32).

The highest prevalence rates are in Eastern Europe, North America, Central Asia, and East Asia (2, 32). Worldwide at every 3 minutes and 45 seconds, someone dies due to an IS (2) being this condition associated to the death of 3.0 million individuals (32). When considered separately from other cardiovascular diseases, IS ranks 5 among all causes of death, behind diseases of the heart, cancer, chronic lower respiratory disease, and unintentional injuries/accidents (32). Due to its well-known unmodifiable risk factors the IS incidence in a defined area is largely influenced by the structure of the population in terms of age, sex and ethnicity distribution (5).

In Europe it is estimated that each year 800.000 people suffer an IS, and the data indicate that the eastern countries have higher rates than southern countries (5). Similarly to

what is observed in the world also in Europe the IS age-standardized incidence is declining, and rates observed in young adults are rising (5). The explanations for these trends have been attributed to the increase of risk factors such as diabetes, hypercholesterolemia, obesity, smoking, alcohol abuse, and the use of illicit drugs in young adults (5).

In Portugal, until 2013 there were relatively few studies on the prevalence and incidence of IS, in fact the few studies that exist were based on stroke in general and do not distinguish between hemorrhagic and ischemic (40). These studies were biased due to the small sample and local or regional nature of the studies (40). For example, the most cited article was carried out in Coimbra in 1992, and reported a prevalence of stroke in males of 10.2% and in females of 6.6% (41). It was also observed that the prevalence increases markedly with age, although always higher in males the difference decreases after the 7th decade of age (41).

In 2007 it was reported a stroke prevalence of 2.1% in primary care patients (42), more recently, in 2013, a cross-sectional study about the prevalence of stroke was developed through a telephone interview having has target the residents in mainland Portugal (40). The results indicate a total prevalence of 1.9%, being this rate higher in males (2.6%) than in females (1.3%) (40). This study also reported several differences in the prevalence at the geographical level, with a higher prevalence in Alentejo (3.6%) and lower prevalence in the North (1.1%) (40). Estimates from 2009 indicate that per hour 6 people suffer a stroke, resulting in 2 deaths, being considered one of the major causes of death in Portugal (43). According to Direção Geral de Saúde (DGS) the standardized mortality rate for stroke decreased between 2007 and 2011 from 79.9 deaths per 100.000 inhabitants to 61.9 (27).

From 2013 these reports from DGS become anual and began to distinguish between hemorrhagic and ischemic. Regarding IS, from 2013 to 2015 the total number of deaths diminished from 6099 per 100.000 inhabitants to 4598, which represent a decrease on age-standardized mortality rate from 61.3 to 46.6 per 100.000 inhabitants(27). This could be explained in part by the increase of aproximately 36.5% of patients subjected to clot-busting drugs (27). It is also observed that individuals over the age of 70 have a higher risk of death due to IS (27). And similar to what is observed in the world until the age of 70 males have higher age-standardized mortality rate than females, from that age forward the rate is similar for both genders (27).

1.5. - Effects of ischemia on brain cells

The human brain is primarily composed by neurons, glial cells, neural stem cells, and blood vessels. Neurons and glial cells are present in similar amounts (44), and establish complex interactions (15, 45). Like any other cell in the organism, to properly perform their functions

brain cells depend on the supply of water, energy, nutrient and oxygen and on the removal of wastes products (e.g., carbon dioxide, nitrogen, phosphates, sulphates), for that, they rely on the bloodstream. If for some reason these exchanges are interrupted, as is the case of ischemia, the cells begin to resent and a cascade of events will begin. Initially a local depletion of oxygen or glucose will occur, causing failure in the production of ATP (11). This will affect energy-dependent processes necessary for cell survival, and sets off a series of interrelated events that may end in cellular injury and death (11).

1.5.1. – Cellular mechanisms triggered by ischemia

During periods of low oxygen or decreased blood flow, the production of ATP by glycolysis and oxidative phosphorylation slows or stops (46-48). Indeed, there are potentially large reserves of alternatives to glucose as substrates for both glycolysis and respiration, such as glycogen, lactate and fatty acids (47, 48). The initial effects induced by ischemia depend on the availability of alternative glycolytic and oxidative substrates and on the rate of ATP consumption (47, 48). In contrast, oxygen is an irreplaceable driver of mitochondrial respiration, the main source of cellular ATP (47, 48). Consequently, lack of oxygen immediately and severely reduces ATP production, which results on a rapid decrease of their levels due to ongoing consumption (47, 48).

The interruption of oxidative phosphorylation triggers ATP synthase to run backward and consume ATP, accelerating the loss of ATP and electron leak and triggering the production of reactive oxygen species (ROS) (47, 48). Finally, when respiration is inhibited but glycolysis persists, protons and lactate generated during glycolysis accumulates, causing rapid intracellular acidification (47, 48). In response to this direct effects of decreased cellular energy and intracellular acidification several detrimental mechanisms are activated, such as the loss of ion pumps function, release of excitatory neurotransmitters, and the production of ROS, all of them promoters of cell death (11, 47).

The loss of ion pumps function is triggered by the decrease of cell membrane potential and leads to the loss of ion gradients. There is an efflux of potassium and influx of sodium, chloride, and calcium ions that is accompanied by the inflow of water, resulting in rapid swelling of cells and the consequent necrosis (11, 47). The necrotic process leads to the loss of membrane integrity resulting in cell lysis and the release of the cellular constituents that in turn promote inflammation in the surrounding tissue (49). The increase in intracellular calcium leads also to the activation of pathways that lead to apoptosis and the release of excitatory neurotransmitters (11).

The release of excitatory neurotransmitters, such as glutamate, accompanied by a dysfunction in the reuptake mechanisms, as a result of ion gradient dissipation, results in glutamate accumulation in the synaptic cleft (11, 47). Excessive glutamate accumulation at the synapse results in the overactivation of glutamate receptors, namely N-methyl-D-aspartate receptors (NMDA-receptors), leading to excitotoxicity (11, 47).

Oxidative stress is another step of the ischemic cascade and is caused by the production of ROS (11, 47). These radical species have the ability to react with and damage almost all cellular and extracellular components, of which vascular endothelium is particularly important (11, 47). ROS-induced modifications lead to cellular impairment through biochemical, functional, and metabolic abnormalities, which ultimately trigger apoptotic mechanisms (11, 47).

In contrast to the ischemic core, where the cells die mostly by necrotic processes, on the penumbra cells die mostly through apoptotic mechanisms (11). The ischemic cascade causes an early response in the expression of genes such as Bax and p53, followed by the release of pro-apoptotic molecules such as cytochrome c and apoptosis-inducing factor from mitochondria (11, 47). This leads to the activation of caspases that potentiate cell death (11, 47). However, in the course of these processes some protective pathways could be activated as a defense against apoptotic and necrotic cell death (e.g., production of Bcl-2, Heat shock protein 70, Neurotrophin-3 or Interleukin-10) (11). The way different cell populations deal with ischemic periods is variable, and depends on its intrinsic characteristics but above all on its metabolic needs (11). Cells that require more energy are more affected by ischemia (11).

1.5.2. - Effects of ischemia on neurons

Neurons, classically considered the most important cells of central nervous system (CNS), play a crucial role on every system of the human body (50). The major function of these cells is to enable the communication within the nervous system, which is done through action potentials and synaptic transmission (49). To receive and send these action potentials it is necessary a shift in the membrane potential caused by the flow of ions through the neuronal membrane (49). In order to maintain the ionic gradients, a constant supply of glucose and oxygen is required, and any imbalance jeopardizes neuronal functions (11). These intrinsic characteristics and the fact that neurons do not have their own energy stores make them extremely sensitive to ischemia (49).

1.5.3. - Effects of ischemia on Glial cells

Glia include different types of cells, such as astrocytes, microglia and oligodendrocytes. Glial cells are seen as the housekeeping cells of CNS and their main function is to support neurons. Their supportive tasks include maintaining homeostasis, providing structural, metabolic, and trophic support to neurons, promote defense against pathogens, regulating inflammatory responses, regulating synaptic transmission, removing metabolites and participating in the formation of the blood-brain-barrier (45, 51-55).

Astrocytes are the most abundant glial cells in the brain, and their characteristics make them less susceptible to ischemic damage than neurons (48). They are able to maintain ATP levels longer than neurons during ischemia, and severe ionic dysregulation proceeds more slowly (48). Firstly because neurons have higher density of ionic channels and a consequent greater energy demand to maintain ionic gradients, and secondly because most of the glycogen stores in the brain is found in astrocytes (48). Additionally, astrocytes express lower levels of ionotropic glutamate receptors than neurons, and have better ionic buffering and antioxidant capacity (48). These attributes presumably underlie the well-known selective loss of neurons over astrocytes (48). However, under these circumstances astrocytes can play two roles, if on one hand these attributes place them in the position of potentially being able to protect neurons, on the other hand they are also stressed by ischemia and may potentiate neuronal death (48).

1.5.4. - Effects of ischemia on brain vasculature

The cerebral blood vessels are endowed with powerful regulatory mechanisms that assure that the brain is perfused according to its needs (56). However, during ischemia these mechanisms become dysfunctional and fail to compensate the reduction in blood flow (56). Ischemia triggers profound modifications in the major mechanisms that control cerebral circulation (endothelial function, autoregulation, vascular reactivity to hypercapnia and neurovascular coupling), these dysregulation undermines the ability of the brain to maintain blood flow and aggravates the intensity of the ischemic insult (56, 57).

The vast majority of these vascular alterations are associated with an increase in ROS levels, which leads to oxidative stress and impairs the function of vascular cells (56, 57). During periods of ischemia there is an impairment of endothelium-dependent regulation of vascular tone and basal receptor-mediated endothelium-dependent vasodilation, being these dysfunction associated to the increase of ROS levels (56, 57).

1.6. - Models for the study of IS

Due to the complexity of IS its study has been made through the combination of several *in vivo* and *in vitro* models. These have been developed, with the aim of identifying the mechanisms that underlie cerebral ischemia and developing new approaches for IS therapy (10, 58, 59). While *in vivo* models enable the study of interactions of all components present in the CNS as a whole, the use of *in vitro* models allows the study of molecular interactions occurring at tissue level (60).

The use of animal models is an indispensable tool for several reasons, in theory experimental models of cerebral ischemia are highly reproducible, well controllable, and standardized, allowing more precise analysis of stroke pathophysiology and drug effects (10, 58, 59). The molecular, genetic, biochemical and physiological studies often require invasive processes to allow direct access to brain tissue, which can be achieved with *in vivo* models (10, 58, 59). It is also possible to evaluate the effects of perfusion and vasculature in the pathophysiology of IS, which cannot be modeled in *in vitro* models (10, 58, 59). However, this may not be enough and frequently the use of cellular models to study specific basic biochemical and molecular mechanisms under conditions of energy deficiency similar to ischemia on specific cell populations is required (10, 61, 62). With the use of *in vitro* models, it is also possible to evaluate the potential therapeutic effect of drugs in specific cell populations (10, 61). Cellular models are easy to use and manipulate allowing a direct control of the environment (10, 62).

The majority of IS experiments, *in vivo* and *in vitro*, are carried out in well-characterized rodent models (10, 58, 59). The lower cost of maintenance, easily monitoring of physiologic parameters, its small brain size, a relative homogeneity within strains, that allow reproducible studies (10, 58, 59), make rodents preferred models in ischemia studies.

1.6.1 - *In vivo* IS models

The purpose of any animal model is to closely mimic the pathophysiologic processes. *In vivo* IS models could be classified taking into consideration the area, global or focal, or the type of occlusion, permanent or transient. On global models, there is a complete interruption of blood flow to the brain and on focal ischemia, the interruption of blood flow affects only a specific part of the brain. In permanent models of ischemia there is a complete interruption of the blood flow, whereas in transient the interruption is temporary and is followed by a reperfusion period. IS is often induced by occlusion of the middle cerebral artery (MCA) or one of its branches (59).

1.6.1.1. - Embolic models

Embolic IS models involves the formation of a thrombus that occludes the vessels, and can be classified into two major categories: microsphere-/macrosphere-induced models and thromboembolic clot models (10, 58). Microsphere-/macrosphere-induced models use a microcatheter to insert the microspheres (20-50 μm) or macrospheres (100-400 μm) into brain arteries. Microspheres are usually inserted via the external carotid artery (ECA) and are flushed passively into the cerebral circulation by the blood flow, inducing multifocal and heterogeneous infarcts (10, 58). The macrospheres are inserted into the internal carotid artery (ICA), and its intra-arterial embolization provides reproducible occlusion of the MCA, resulting in focal ischemic lesions that are comparable to intraluminal suture model (10, 58).

The thromboembolic clot models are based on the application of spontaneously formed clots or thrombin-induced clots from autologous blood or through the injection of thrombin directly into the intracranial segment of the ICA or into the MCA (10, 58). These model closely mimics the mechanism of human IS, and therefore allows the study of thrombolytic agents alone or combined with neuroprotective drugs, as well as thrombolytic processes (10, 58, 59).

1.6.1.2. - Intraluminal suture MCAo model

The MCAo model is the most frequently used experimental model of IS in rodents (10, 58). A monofilament is introduced into the internal carotid artery (ICA) and is positioned at the origin of the MCA inducing its occlusion (10). The model can be permanent or transient. Withdrawal of the filament with subsequent reperfusion allows to develop transient models with variable reperfusion time points (10, 58). The duration of ischemia could range from 60 to 120 min (10, 58).

1.6.1.3. - Craniectomy models

These models are characterized by a direct approach to brain vasculature (10, 58). A craniectomy with incision of the dura mater is made and the MCA is exposed. The focal ischemia could be performed by occlusion of the MCA by electrocoagulation and additional transection, resulting in permanent occlusion, or alternatively a transient occlusion of MCA can be achieved by clamping the artery, followed by subsequent reperfusion (10, 58). It could also involve the occlusion of both common carotid arteries, to reduce the collateral blood flow consolidating the ischemic injury (three-vessel occlusion model) (10, 58).

1.6.1.4. - Photothrombosis models

Photothrombosis, also known as photochemical, are focal models based on intravascular photo-oxidation, which leads to well-defined ischemic injury (10, 58). A photoactive dye (e.g., Rose Bengal, erythrosine B) is injected intraperitoneally (mice) or intravenously (rat) followed by illumination of a specific part of the brain with light of a specific wavelength through the intact skull (10, 58). The activated dye forms singlet oxygen that damages components of endothelial cell membranes, with subsequent platelet aggregation and thrombi formation, leading do the interruption of local blood flow (10, 58).

1.6.1.5. - Endothelin-1 model

Endothelin-1 is a peptide with potent and long-lasting vasoconstrictive properties. It can be applied directly onto an exposed vessel or stereotactically injected into the brain parenchyma leading to vasoconstriction, inducing downstream vessel ischemia (10, 58). The period of ischemia is dose-dependent, and when endothelin-1 effect passes, blood flow is gradually reestablished, thus representing the situation of transient focal ischemia (10, 58).

1.6.1.6. - Global models of ischemia

It is also possible to induce global ischemia on brain tissues. These models are based on the total interruption of blood supply to the brain and usually are used to study the brain damage that occurs in cardio-circulatory resuscitation. Among these the most used are the four vessel occlusion (reversible occlusion of the two common carotid artery (CCA) combined with the permanent interruption of vertebral arteries), the two vessel occlusion (occlusion of the two CCA) and cardiac arrest and resuscitation (63).

1.6.2 - *In vitro* IS models

The application of cellular models provides a simple and highly controlled experimental system that allows detailed high-throughput analyses on how the system, or one particular cell , is affected by ischemia (9, 10). These approaches use primary cultures, cell lines, organotypic cultures and brain slices (9, 10). To induce ischemia in these models OGD or the chemical/enzymatic blockade of the cellular metabolism are the approaches commonly used (10, 61).

1.6.2.1. – Cellular systems

Primary cell cultures are established by the dissociation of brain tissues from embryonic or perinatal rats and mice (9, 60). The initial dissociation can be made from specific anatomical areas of the brain (e.g., cortex, hippocampus, cerebellum), which allow the study of ischemic injury in particular cerebral regions (9). The cultures can be composed of a homogenous population of cells (enriched cultures), or by several populations of cells (mixed-cultures). Mixed-culture systems are set-up with two or more cell types growing with some degree of contact between them and constitute valuable tools to study the interactions between cell populations (64). Whereas enriched cultures, like neuron-enriched cultures, allows assessing how specific cells are affected by an ischemic insult (10, 62).

Immortalized cell lines, such as SH-SY5Y (differentiated into neuronal cells)(65), the human teratoma-derived NT2 (differentiated into neuronal cells, astrocytes, and oligodendrocytes) (66) and the HMO6 (differentiated into microglia) (61, 67), are frequently used on IS studies.

The brain slice method utilizes a thin slice of rodent brain tissue, usually maintained up to 12 hours, and preserving the original architecture of the tissue. This model has been used to evaluate neuronal vulnerabilities under ischemia without cerebrovascular influences (61, 68).

The organotypic brain slice cultures represent a type of cellular system that lies between brain slices and primary cell cultures. They are prepared from different anatomical regions of the brain and allowed to mature *in vitro* (69). With this model the tissue maintains its structural organization (61, 69).

1.6.2.2. – Oxygen and glucose deprivation

Oxygen and glucose deprivation is the most used model of *in vitro* ischemia. It consists on replacing the regular atmosphere (95% air and 5% CO_2) by an anoxic atmosphere (95% N_2 and 5% CO_2), by maintaining cells in a hypoxic chamber. The hypoxic conditions can be associated with the omission of glucose, which is usually referred as *in vitro* ischemia or OGD, which consist on replacing the regular medium for glucose-free buffer (e.g., Hanks balanced salt solution (HBSS) or Locke's solution) (9). The cultures are maintained under these conditions for a previously established period of time and then the reperfusion is made by returning them to the conditions established before the period of OGD (95% air, 5% CO_2 and regular medium) (9). The extent of the ischemic damage will depend on the period of OGD applied, as well as the density of cells present in the culture (9, 70).

1.6.2.3. – Chemical and enzymatic method

It is also possible to induce chemical or enzymatic ischemia on *in vitro* models with the treatment with cyanide (sodium cyanide or potassium cyanide) (61, 71) or cobalt chloride (72, 73). This method consists on the blockade of the cellular metabolism. Cobalt chloride has the ability to induce oxidative damage through the generation of ROS (73) and cyanide is a well-established mitochondrial respiration inhibitor, which leads to the inhibition of oxidative phosphorylation shifting the cellular metabolism from aerobic to anaerobic, both attempt to mimic what happens during an ischemic period (9, 10, 71). The chemical and enzymatic method is considered an alternative approach to study the effects of excitotoxicity on a particular population of cells, like neurons (61). However when compared to the OGD model, the chemical model induces the production of large amounts of free radicals, and has been less used to study cerebral ischemia (9), being more applied on the study of ischemia and reperfusion injury in other parts of the body such as the retina (73), kidney (9, 71) and intestine (72).

2. - G Protein-coupled estrogen receptor (GPER)

Observations demonstrated that during reproductive years females have lower IS incidence than males of the same age. After menopause, and consequent reduction of estrogen levels, the risk of IS in females increase (74-76). These differences were associated to the hypothesis that estrogens could have a neuroprotective role against ischemia, and led to a large focus on the effects induced by estrogens, and particularly estradiol (E2) on the brain (74-76).

E2 is a form of estrogen that regulates multiple functions in human body (50). It controls ovulation and the development of female characteristics, being classically considered a reproductive hormone, due to its well-known role in feedback signaling in the hypothalamic-pituitary-ovarian axis (50, 77, 78). Estrogens refer to any substance, natural or synthetic, that mimics the effects of the natural hormone (79). The three major naturally occurring forms of estrogen are estrone, E2, and estriol, being E2 the most potent and prevalent form, although several metabolites also have estrogenic hormonal activity (79). The actions of estrogens are mediated by estrogen receptors (ER) (80). ERα was first described in the 1960s (81, 82), whereas ERß was described almost 30 years later (83). These homologous receptors, described as ligand-activated nuclear transcription factors (84), are predominately present in nucleus and cytoplasm, with less than 2% on cellular membrane (85, 86). Each ER exhibits differential tissue expression patterns, but both regulate gene transcription through classical genomic pathways (87-89), or by modulating cellular signaling pathways such as the mitogen-activated protein kinases (MAPKs)/extracellular signal-regulated kinases (ERKs) (90), modulation of intracellular calcium (91-93), cyclic adenosine monophosphate (cAMP) production (94, 95), and regulation of phosphatidylinositol 3-kinase (PI3Ks) (91).

In the late 90s, the G protein–coupled estrogen receptor 1 (GPER or GPR30) was identified as a novel estrogen receptor (96). It was described as an orphan receptor belonging to the family of 7-transmembrane spanning G protein-coupled receptors (97, 98). In 2000, Filardo and colleagues demonstrated that E2-mediated activation of ERK1/2 was dependent on the expression of this receptor, and named it GPR30 (96). In 2005, Revankar and colleagues (91) and Thomas and Dong (94) described the binding of E2 to GPR30, suggesting that GPR30 was an E2-binding receptor, which led to its current designation as G protein-coupled estrogen receptor 1 in 2007 (99). Since its identification, GPER has been described in nearly every system of the human body, including reproductive (100, 101), cardiovascular (102, 103), endocrine (104) and nervous system (93, 104).

Estrogens mediate genomic effects through the classical ERs that are characterized by changes in gene transcription and occur in the time frame of hours to days (105). Furthermore, it was also reported that estrogens mediate a variety of “rapid” cellular responses that occur in the time frame of seconds to minutes (105), inconsistent with de novo transcription and

protein synthesis (106). These rapid estrogen-mediated effects have been associated with the activation of membrane-associated ERs, and are referred as "non-genomic" (107, 108). The signaling pathways that trigger these rapid estrogen-mediated effects are diverse and can be induced by ERβ present near or at the plasma membrane (109), by the translocation of ERβ to the plasma membrane after E2 treatment (110), by the interaction of non-membrane ERα and ERβ with integral membrane proteins (111, 112) or through the activation of GPER (96, 113). In addition to this rapid estrogen-mediated effects triggered by GPER it was also described that its selective activation has the ability to modulate gene expression (114). E2, as well as a large number of other compounds that bind to classical ERs bind and activate GPER (88). The discovery of GPER-selective ligands fastered the research into the GPER functions. Bologa and colleagues (2006), using a combination of virtual and biomolecular screening, identified the first selective GPER agonist, a non-steroidal compound named G1. The modulation of GPER was complemented with the identification of two selective antagonists, named G15 (115) and G36 (116). Binding studies about the affinity of these three selective ligands of GPER demonstrated that G1 has a binding affinity of about 11 nM (117) compared to 3-6 nM for estrogen (96). Whereas G15 and G36 presents a similar affinity of approximately 20 nM (115, 116), but with G36 showing a decreased binding and activation of ERa compared to G15 (116). Other compounds were described as having significant affinity to GPER, but in a non-selective manner, include 4-hydroxytamoxifen, the active metabolite of Tamoxifen (118), raloxifene (119), ICI182,780 (91, 95, 96), Genistein (94, 118) and Bisphenol A (94). Since the identification of the GPER-selective ligands, an increasing number of studies addressing the potential cellular and physiological effects of GPER selective activation in numerous systems, including the CNS, were published.

The emerging notion that E2 can act in multiple areas of the brain led to an increased focus on its effects on neuronal physiology and neuroplasticity (120). *In vitro* and *in vivo* studies indicated that E2 is a potent physiological modulator of the CNS and participates in processes such as neurogenesis, regulation of neurotrophic factors expression and regulation of antioxidant mechanisms (76, 121, 122). Estrogens were also associated with the regulation of cognitive processing (123, 124), memory (125-131) and neurological disorders (120, 132).

Selective activation of GPER by G1 enhances cognitive processes, such as learning and memory, in a manner similar to E2 (133). Besides, GPER is highly enriched in the brain and greatly expressed at the synapses, being involved in the rapid regulation of hippocampal dendritic morphology and synaptic plasticity (133). G1 enhances recognition tasks (127-130, 134, 135), learning of specific tasks (136), and social recognition (129, 130). In agreement with this, chronic treatment with the GPER selective antagonist G15 impairs acquisition of a spatial learning task (137).

Over the recent years GPER emerged has a potential therapeutic target to induce neuroprotection. This hypothesis was based on the ability of its selective agents to mimic the

effects of E2 without the feminizing or other adverse effects (99). Activation of GPER may replicate the beneficial effects of E2 in the brain avoiding the side effects associated with estrogen replacement therapies, like increased risk of coronary heart disease, breast cancer and stroke (75, 138).

2.1. - Expression of GPER in the CNS

The expression of GPER is not restricted to traditionally estrogen responsive tissues (89, 99, 139). Indeed, characterization of GPER using immunohistochemistry revealed a ubiquitous expression of this receptor in several tissues (89, 99, 139). High levels of GPER expression are present in numerous organs, including male and female reproductive systems, heart, intestine, ovary, pancreatic islets, adipose tissue and inflammatory cells, and nervous system (89, 99, 139). On nervous system, GPER is similarly expressed throughout the CNS and peripheral nervous system (PNS) of male and female rodents (93, 104, 140-143). GPER immunoreactivity is observed in the forebrain (e.g. cortex, hypothalamus, hippocampus, hypothalamic-pituitary axis and striatum (93, 104, 140, 141, 143-145)), brainstem (e.g. the pontine nuclei locus coeruleus, brainstem autonomic nuclei (93)), cerebellum Purkinje layer (104), spinal cord and autonomic and sensory ganglia (142). In addition, GPER is present in brain vasculature (146, 147). The levels of GPER expression are heterogeneous with GPER presenting high expression in hypothalamic-pituitary axis (93), hippocampus (93, 140, 143), cortex (143) and thalamus (141). The hippocampus and frontal cortex present higher GPER mRNA levels than the septum and striatum (143).

At a cellular level, GPER is expressed by neurons of different regions, such as the pyramidal neurons of the frontal cortex (143), cholinergic neurons in the medial septum, striatum, diagonal band of Broca´s area and nucleus basalis magnocellularis (143), CA1-3 hippocampal neurons (140, 148), GABAergic neurons in the dorsal striatum (149), and dopaminergic neurons from ventral mesencephalon (150). GPER expression was also reported in neurons from paraventricular nucleus (145), luteinizing hormone-releasing neurons (151), neurons of the dorsal and ventral horn of the spinal cord as well as in sensory and autonomic neurons (142, 152). Concerning glial cells, GPER is expressed by cortical and midbrain astrocytes (150), by microglial cells from forebrain (144) and ventral midbrain (153) and by oligodendrocytes of spinal cord, corpus callosum and cortex (154). On brain vasculature, GPER is particularly expressed in the endothelial cell subpopulation of small arterial vessels (146), and in smooth muscle cells (146, 147) and pericytes (146).

At a sub-cellular level, GPER is expressed in the plasma membrane of neurons (96, 143, 148, 155, 156) and glial cells (157). GPER is also present in the cytoplasm, particularly in the

membrane of intracellular compartments such as the endoplasmic reticulum (91, 140, 158) and Golgi apparatus (140).

2.1.1. - Sex differences in GPER expression

The expression pattern of GPER mRNA in human brain tissues (97, 159, 160) is similar to the receptor distribution profile observed in the rat brain, with no differences between sexes (93). In contrast, in the zebra fish brain there is a higher expression in males than in females (161). A clear sexually dimorphic distribution of GPER occurs in some areas of the hamster brain, with higher levels of GPER in the female hypothalamus and amygdala, and moderate and low levels in the male amygdala and hypothalamus, respectively (162).

Interestingly, some pathologies are associated with alterations in the pattern of distribution and expression of the GPER. This is the case of transient focal ischemia, where GPER distribution and expression increases in the brain of male mice, but not of intact or ovariectomized (OVX) females (141).

2.1.2. - Regulation of cell proliferation and differentiation by GPER

It is known that E2 plays an important trophic and protective role in the adult brain, being essential to the maintenance of normal brain functions, and to protect the brain against neural injuries through different mechanisms, including the stimulation of neurogenesis. The first evidence of the modulating effect of estrogens on neurogenesis was achieved when scientists noticed that, in the reproductive cycle of mammals, higher estrogen levels were accompanied by increased cell proliferation in the dentate gyrus of the hippocampus and, contrariwise, a reduction of circulating estrogens resulted in a significant decrease in the proliferation of hippocampal precursors (163).

GPER has also been implicated in the modulation of hippocampal synaptic plasticity (164-166). Although these effects have not yet been demonstrated in pathological models, it was shown that Brain-Derived Neurotrophic Factor (BDNF) expression triggered by GPER selective activation promotes synaptic plasticity (164, 165), being these effect associated to the enhancement of spatial memory (164). GPER activation is also involved in the modulation of neuritogenesis induced by E2 in primary hippocampal neurons (167). To investigate the effect of GPER in modulating neural cell proliferation and differentiation, Okada and colleagues (2010) used E2 conjugated with bovine serum albumin, impeding E2 to permeate the cell membrane. In this way, they showed that GPER is not directly involved in neural cell proliferation induced

by estrogen, but it stimulates oligodendroglial differentiation from neural stem/precursor cells of the telencephalon of 15-day-old rat embryos (168). The same authors reported a couple of years before that administration of E2 or bisphenol A, a xenoestrogen that activates GPER, stimulated the proliferation of neural stem/precursor cells in the absence of mitogens as well as the generation of oligodendrocytes (169).

In intact and OVX adult female rats treatment with E2 or raloxifene, but not with tamoxifen, increased neurogenesis in the ipsilateral subventricular zone following transient middle-cerebral artery occlusion (170). Analysis of the role of GPER in hippocampal cell proliferation in adult female rats showed that treatment with GPER agonist decreased cell proliferation in adult OVX female rats, indicating a GPER-independent role of E2 in hippocampal neurogenesis or, alternatively, an antagonistic effect of intracellular and membrane bound ER activation to maintain the levels of neurogenesis. GPER did not co-localize with progenitor cells in the subgranular zone of the dentate gyrus, indicating that the effects of GPER activation on neurogenesis may be indirect (171). In summary, the scarce information available suggests differential effects of GPER in the two neurogenic niches, with a neurogenesis promoting effect of the receptor restricted to the subventricular area. The existing data also suggest that these effects may be sex-dependent.

2.2. - GPER and aging

Little is known regarding the effect that aging may have on GPER functions. In OVX female rhesus monkeys it was demonstrated that the expression of GPER in gonadotrophin-releasing hormone neurons is not affected by age (172). However, in hypothalamic regions of aged OVX females there were more cells expressing GPER and the expression of the GPER/cell was higher than in young OVX females (172). In contrast, recent findings demonstrated that hippocampal GPER mRNA levels are decreased in aged OVX female mice when compared to young adult (173). Moreover, Wu and colleagues (2018) associated the reduction of GPER expression to the deprivation of E2, since it was demonstrated that low levels of E2 are associated with lower levels of GPER mRNA (173). Extrapolating to what happens in aging these data suggest that as the levels of E2 begin to decrease there is a reduction in the expression of the GPER in females. On males, Xu and colleagues (2018) obtained similar results, since hippocampal GPER expression is decreased in aged male mice compared to young adults (164). In males this reduction does not appear to compromise the effects mediated by GPER, since G1 was capable to enhance memory in aged mice (164). However, in OVX females the results indicate that the beneficial effects induced by GPER selective activation could be related to the critical period hypothesis. G1 exerted a neuroprotective effect after short-term E2 deprivation, whereas after long-term E2 deprivation neuroprotection was not achieved (173). Wu and colleagues (2018) also showed that GPER expression and function can be maintain with

estrogen treatment during aging. Treatment with E2 10 weeks after ovariectomy prevents the reduction of GPER mRNA levels and triggers robust neuroprotective effects on aged females (173). The results also demonstrate that G15 attenuated the neuroprotective effects of E2 within the CA1 region of the hippocampus when administered near the end of E2 treatment (173), indicating that GPER may be an important factor in the loss of the neuroprotection exerted by E2 (173).

The data available indicates that during aging the expression of GPER seems to decrease in both sexes (164, 173) and the expression pattern can be distinctly affected in different brain regions of OVX female (172). Although GPER neuroprotective effects during aging can be maintained in males, on OVX females seems to be more complex. Considering that most of neurological diseases are age-related, it is crucial to develop further research to clarify if aging compromises the protective effects mediated by this receptor.

2.3. - Signaling pathways triggered by GPER activation

The signaling transduction mechanisms triggered by activation of GPER have been studied in various cell types and a large diversity of pathways have been proposed (Figure 1) (174). Besides the mechanisms elicited by the independent activation of GPER, the interactions of GPER with epidermal growth factor receptor (EGFR), and also with the classic ERα and ERβ, have been reported (99, 175). The crosstalk between GPER and ERα/ERβ involves multiple forms of interactions: cooperative, antagonistic and dependent (176). GPER was initially considered to signal via Gαs, leading to activation of adenylyl cyclase and the consequent increase in cAMP levels and PKA activation (95, 113, 156). However, it is known that GPER activation may also lead to inhibition of PKA through Gαi and Gαo (147), and these pathways coexist with other rapid signaling pathways such as the activation of ERK pathway (95), the activation of kinases such as PI3K (91, 119) or PKC (177), intracellular calcium mobilization (91, 113, 178, 179), or activation of ion channels (180). Besides triggering rapid signaling events, GPER activation leads to upregulation of nerve growth factor (NGF) via c-fos expression (181), cyclin D2 and Bcl-2 (114).

Concerning neuronal cells/tissues, activation of the cell survival PI3K/Akt pathway was associated with the protection mediated by GPER activation in models of Alzheimer's disease (AD) (135), Parkinson's disease (PD) (150), spinal cord injury (SCI) (152), and traumatic brain injury (135). Moreover, activation of PI3K signaling by GPER participates in the control of neuritogenesis in developing hippocampal neurons (167) and on the protection of cognitive function (135). Survival promoted by GPER activation was also associated with the regulation of the c-Jun N-terminal kinase (JNK) pathway. In a rat model of global cerebral ischemia G1 exerts significant neuroprotection through the rapid activation of the pro-survival kinases, Akt

and ERK, while decreasing pro-apoptotic effects of JNK activation (182). In addition to regulating cell survival, control of the JNK pathway by GPER also regulates memory since GPER activation in the dorsal hippocampus enhances hippocampal memory in a JNK-dependent manner and independently from ERα and ERß (127), furthermore it was also demonstrated that JNK signaling is triggered via GPER activation during object-in-place learning, and possibly is E2-independent (183).

The phospholipase C (PLC) pathway is also a target of GPER. Our group showed recently that in rat cortical astrocytes, but not in neurons, GPER activation is able to regulate the PLC pathway. Moreover, activation of this pathway promotes the increase in intracellular Ca^{2+} levels and induces the apoptosis of astrocytes (92). In mesencephalic neuron-glia cultures protection induced by G1 against the dopaminergic toxin 1-methyl-4-phenylpyridinium (MPP^{+}) was associated with the involvement of three different pathways: MAPK, PI3K and PLC pathways (150).

Together, the existing data show that GPER has the ability to regulate a wide variety of signaling pathways, which vary between tissues and even between cells of a given tissue.

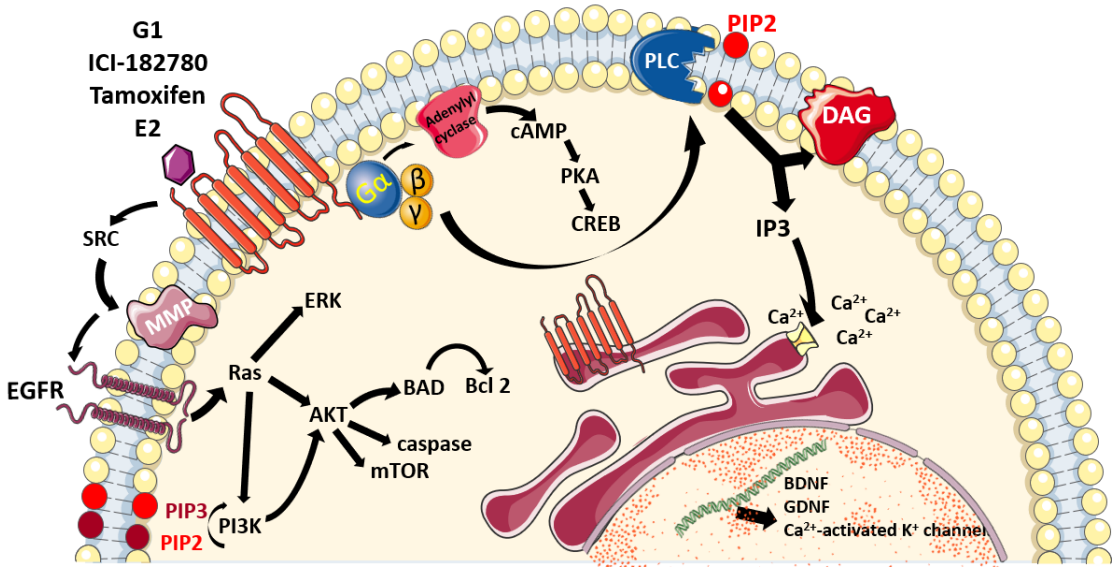

Figure 1: Schematic representation of the diversity of signaling pathways regulated by GPER.

Multiple agonists activate GPER: E2, selective estrogen receptor degraders *(*SERDs*)* such as Fulvestrant (ICI-182780), selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene, and selective agonists such as G1. GPER activation stimulates multiple cellular pathways, part of them mediated by transactivation of EGFR. Abbreviations: brain-derived neurotrophic factor (BDNF); cell-derived neurotrophic factor (GDNF); cyclic adenosine monophosphate (cAMP); diacylglycerol (DAG); epidermal growth factor receptor (EGFR); estradiol (E2); Estrogen receptors (ER); extracellular signal-regulated kinases (ERK); G protein–coupled estrogen receptor 1 (GPER); inositol trisphosphate (IP3); mammalian target of rapamycin (mTOR); matrix metalloproteinase (MMP); phosphatidylinositol 3-kinase (PI3K); phosphatidylinositol 4,5-bisphosphate (PIP2); phosphatidylinositol-3,4,5-triphosphate (PIP3); phospholipase C (PLC); selective estrogen receptor degrader *(*SERD*)*; selective estrogen receptor modulator (SERM);

2.4. - GPER and neurological disorders

Over the past decades, it was demonstrated that E2 has an active role in diseases of the nervous system. Although these effects were initially associated with classical ERs, the identification of GPER and the evidence that GPER mRNA and protein were expressed throughout the CNS and PNS of rodents was accompanied by findings showing that GPER significantly contributes to E2-mediated neurological benefits (Figure 2) (93, 104, 142). The protection mediated by GPER selective activation involves a plethora of mechanisms as diverse as inhibition of pathways mediating apoptosis, stimulation of neurotrophic factors expression, modulation of ion channels, inhibition of neuroinflammatory processes, control of gliosis, and maintenance of BBB and vascular function. These mechanisms are summarized on table I.

Table 1: Protective actions triggered by GPER activation in the brain

Protective actions triggered by GPER (Mechanisms/Pathways)		**References**
Up-regulation of neurotrophic factors	BDNF	Bourque *et al*. 2014
		Bourque *et al*. 2014
	GDNF	Bessa *et al*. 2015
		Cheng *et al*. 2017
Up-regulation of anti-apoptotic proteins	Bcl-2	Bourque *et al*. 2014
		Bourque *et al*. 2015
Up-regulation of pro-survival kinases	Akt and ERK	Bourque *et al*. 2014
		Tang et al. 2014
	PI3K/Akt	Wang et al. 2017
Up-regulation of protective ion channels	SK2	Kosaka *et a*l. 2012
Up-regulation of remyelination	Remyelination	Hirahara *et al*. 2013
Down-regulation of pro-apoptotic kinases	JNK	Tang *et al*. 2014
Modulation of synaptic plasticity	GABAergic and glutamatergic transmission	Tian *et al*. 2013
	BDNF	Briz *et al*. 2015
		Xu et al. 2018
	Neuritogenesis	Ruiz-Palmero *et al*. 2013
		Ruiz-Palmero *et al*. 2011
Modulation of inflammation	IFNγ and IL-17	Blasko *et al*. 2009
	IL-10	Yates *et al*. 2010

Modulation of inflammation	Astrogliosis	Day *et al*. 2013
	IL-1ß and TNFα	Zhao *et al*. 2016
	IL-1ß, TNFα and IL-6	Guan *et al*. 2017
	Phagocytic activity, iNOS expression and NO release	Mendes-Oliveira *et al*. 2017
Restoration of vascular function	Arteriolar dilation	Murata *et al*. 2013
Restoration of the BBB	Regulation of tight junctions and BBB permeability	Lu *et al*. 2016
Increase of cell proliferation	Neural stem/precursor cells Oligodendrocytes	Okada *et al*. 2008
Cell differentiation	oligodendroglial	Okada *et al*. 2010
Increase of neurogenesis		Khan *et al*. 2015

2.4.1 - Cerebral ischemia

The role of GPER in cerebral ischemia has been studied since the identification of GPER selective ligands and the characterization of its expression in the CNS. The potential benefits of GPER modulation was assessed in *in vivo* and *in vitro* studies with conflicting results associated mostly with the amount of circulating estrogens or with the sex (table II).

Initial *in vivo* studies showed that G1 treatment replicates the effects of E2 in promoting neuronal survival following global cerebral ischemia (99, 184). These effects were demonstrated in OVX female rats (182, 184) and mice (185). It was demonstrated that GPER selective activation protected hippocampal CA1 pyramidal neurons exposed to ischemia (182, 184). Lebesgue and colleagues evaluated the effects induced by G1 on young (2 months) and middle aged (9-11 months) Sprague Dawley OVX female rats subjected to transient global cerebral ischemia and Immunohistochemical analysis indicated that G1 prevented hippocampal CA1 pyramidal neuronal loss triggered by ischemia (184). It was also demonstrated that those effects were similar in young and middle aged animals (184). The beneficial effects of GPER selective activation in OXV females were also described in mice. After middle cerebral artery occlusion (MCAo) exposure to G1 reduced the infarct volume (185). Similar effects were reported by Broughton and colleagues (2014), exposure to G1 reduced neurological deficit, apoptosis, and infarct volume in OVX female mice, but had no significant effect in intact females (186). Broughton and colleagues (2014) also argues that in intact females GPER occupancy would presumably be opposed by high levels of circulating and bound estrogen (186).These findings highlight the complex nature of endogenous estrogen signaling and raises the hypothesis that after an IS the effects induced by GPER selective activation could be related to the amount of circulating estrogens.

The work from Broughton and colleagues (2014) also raises the hypothesis that the effects of GPER selective activation after ischemia might be related with the sex (186). Since unexpectedly in young and aged males G1 markedly exacerbates post-stroke neurological deficit and infarct volume, being those effects abrogated by G15 (186). Thus reinforces the body of evidence indicating that effects of estrogen in the female and male brain are not identical (186). Contrary to the data obtained in males, there are evidences that in females exposure to G1 after global cerebral ischemia leads to a reduction of neuronal injury in hippocampal CA1 region and striatum (187). This neuroprotection is similar to the protection induced by E2 treatment (187), which increases the controversy around GPER activation after cerebral ischemia.

The signaling pathways involved in the neuroprotective role of GPER upon an ischemic insult are not completely understood, *in vivo* studies demonstrated that in OVX females neuroprotection is associated to the rapid activation of the pro-survival kinases, Akt and ERK, while decreasing pro-apoptotic JNK activation (182). On males these neuroprotection in hippocampal and striatal neurons is associated to the up-regulation of protective ion channels, such as the small conductance calcium-activated potassium channel 2 (SK2) (187). On the other hand, the detrimental effects induced by G1 in males are associated to the increase in the expression of cleaved caspase-3 in peri-infarct neurons (186).

On *in vitro* studies there are also some controversy, since it was demonstrated that selective activation of GPER with G1 does not induce any protection against an ischemic insult (188). In this study organotypic hippocampal slice cultures were prepared from Sprague Dawley rat pups and exposed to 30 minutes of OGD. After OGD, the cultures were exposed to G1 during a reperfusion period of 24 hours. The results demonstrated that G1 does not protect neurons from ischemic death nor increase the phosphorylation of Akt and/or ERK, unlike E2 (188). Moreover, the beneficial effects induced by E2 after ischemia were maintained after GPER blockade by G15, suggesting that in this case GPER is not involved in E2-induced neuroprotection (188). Interestingly, in primary neuron-glia cortical cultures exposed to 4 hours of OGD, GPER selective activation after ischemia does not induce any effect on neurons, but selectively promotes astrocytes death due to the rise of intracellular calcium levels via PLC (92). These results also show that GPER is coupled to different signaling pathways in neurons and astrocytes (92).

GPER might have an important role in the management of inflammation after an ischemic insult. Using adult female Sprague Dawley rats subjected to a global cerebral ischemia by four vessel occlusion and primary microglial cultures from neonatal rats Zhao and colleagues demonstrated that GPER expressed in microglial cells directly mediates the anti-inflammatory effect of E2 after an IS (144). G1 reduces IL-1β and tumor necrosis factor α (TNFα) levels. Moreover, the specific GPER antagonist G15 was able to abolish the anti-inflammatory effect of E2 (144).

Another interesting effect induced by G1 after hypoxia/reperfusion is the ability to restore the function of arterioles, which points to the protection of the cerebrovasculature against an ischemic insult (189). In this study, rat cerebral penetrating arterioles from both sexes were isolated, cannulated and pressurized. To induce hypoxia, pial sheaths where incubated for 1 hour in the hypoxic bath (PO2<2%), then transferred to the normoxic bath (PO_2=21%) to induce reoxygenation and finally exposed to G1. The results indicate that G1 produces a vasodilatory response, which was partially dependent on endothelium-derived nitric oxide (NO), but not on arachidonic acid cascades and endothelium-derived hyperpolarizing factor. Additionally, G1 treatment after hypoxia/reperfusion injury fully restored endothelium-dependent dilation to ATP (189).

It was also described that GPER activation after stroke can attenuate the BBB disruption and vasogenic edema in early stage of IS in OVX female rats (190). Bilateral intracerebroventricular administration of G1 to female Sprague-Dawley rats subjected to global cerebral ischemia significantly decreased immunoglobulin G extravasation and increased the proteins from the tight junction occludin and claudin-5 in the hippocampal CA1 region. Furthermore, G1 significantly decreased the protein levels of vascular endothelial growth factor A (VEGF-A) in the ischemic hippocampal CA1 region, which suggests that after ischemic injury GPER activation reduces tight junctions disruption via inhibition of VEGF-A expression (190).

Another controversial issue in relation to GPER is its expression pattern after an ischemic insult. In adult female Sprague Dawley rats subjected to global ischemia by four vessel occlusion there was a significant increase of GPER expression in the motor cortex and hippocampal region as demonstrated through immunohistochemical and western blot analysis (144). Using the same techniques, Broughton and colleagues also reported a significant increase in GPER expression after an ischemic insult in hippocampus, somatosensory cortex and hypothalamus of males with no significant changes in intact or OVX females, which suggests a sex-dependent effect of ischemia on GPER expression (141). The same study reported that GPER immunoreactive neurons in the peri-infarct regions appear more intensely labeled (141).

The controversy around GPER expression after an ischemic insult could result, in part, by the use of different stroke models and periods of ischemia. The discrepancies observed between *in vivo* and *in vitro* models may arise from the lack of components in *in vitro* models that can influence GPER expression after stroke, such as the vascular or the immune cells.

Table 2: Effects induced by GPER selective activation in brain ischemia

Major conclusions	Models	Reference
Selective GPER activation increases the number of hippocampal CA1 pyramidal neurons;	Sprague Dawley (OVX females); 4 vessel occlusion (10 min); Exposure to G1;	Lebesgue *et al*. 2010
G1 replacement decreased infarct volume size;	C57Bl/6J mice (OVX females); MCAO (90 min); Exposure to G1;	Zhang *et al*. 2011
Selective GPER activation reduces neuronal injury in the hippocampal CA1 region and striatum following global cerebral ischemia;	C57Bl/6J mice (males); Cardiac arrest and cardiopulmonary resuscitation (8 min); Exposure to G1;	Kosaka *et al*. 2012
Ischemia increases GPER distribution and expression in the peri-infarct brain regions of male mice, but not in intact females or OVX mice;	C57Bl/6J mice (males, intact and OVX females); MCAO (30 min); GPER distribution;	Broughton *et al*. 2013
Selective GPER activation restores vessel function of arterioles after hypoxia/reperfusion;	Male and female rats; Hypoxia (1 hour) and reoxygenation injury; Exposure to G1;	Murata *et al*. 2013
G1 worsened functional outcomes and increased post-stroke infarct volume size in males, effects that were blocked by G15; G15 improved functional outcomes and reduced infarct volume size after stroke in males; G1 reduced neurological deficit, apoptosis, and infarct volume in OVX females, but had no significant effect in intact females;	C57Bl/6J mice (males, intact female and OVX females); MCAO (30, 60 and 90 minutes); Exposure to G1 and G15;	Broughton *et al*. 2014
Selective GPER activation does not induce any protection against an ischemic insult;	Organotypic hippocampal slice cultures prepared from Sprague Dawley rat pups; OGD (30 minutes); Exposure to G1;	Lamprecht and Morrison 2014
G1 exerts significant neuroprotection against ischemia through the rapid enhanced activation of the pro-survival kinases, Akt and ERK, while decreasing pro-apoptotic JNK activation;	Sprague Dawley rats (OVX females); GCI (10 min); Intracerebroventricular administration of G1;	Tang, *et al*. 2014
Ischemia increases GPER expression in the motor cortex and hippocampal region; GPER expressed in microglia mediated the anti-inflammatory effect of E2 after ischemic stroke;	Sprague Dawley rats (intact females); 4 vessel occlusion (15 minutes); Exposure to G1 and G15;	Zhao *et al*. 2016
Selective GPER activation after stroke ameliorates BBB permeability after global cerebral ischemia in OVX rats;	Sprague Dawley rats (OVX females); 4-vessel occlusion (20 min); Intracerebroventricular administration of G1;	Lu *et al*. 2016

2.4.2. - Neurodegenerative disorders

2.4.2.1. - Alzheimer's disease

AD comprises a wide spectrum of alterations, which includes memory loss, functional decline, behavioral disturbances and dementia (191). The hypothesis that GPER modulation could be an effective therapy for reducing cognitive decline associated with aging and AD related dementia emerged from data showing that the GPER has the ability to modulate and enhance cognitive processes such as memory and learning (127, 134, 136, 143, 192), known to be impaired in aging and AD (143).

In the 5XFAD AD mouse model selective activation of GPER with G1 ameliorates memory impairment in the novel object recognition test in female, but not in male mice (128). In females, these effects are similar to the neuroprotection mediated by E2. However, in males, despite the inconsistence in the effects observed, the bulk of evidence demonstrates a beneficial effect of E2 on memory both in intact and gonadectomized male rodents (131).

2.4.2.2. - Parkinson's disease

PD is a neurodegenerative disease characterized by the progressive and selective damage of the dopaminergic neurons from the nigrostriatal pathway. This damage results in a decrease of dopamine in the striatum, which leads to several motor symptoms, such as tremor, slow body movement and postural instability. It has been widely demonstrated that estrogens can exert protective effects on the dopaminergic nigrostriatal neurons against different toxins (193-197). Increasing evidence implicated the activation of the GPER in these protective estrogenic effects. Results from our group demonstrated that selective activation of GPER using G1 protects rat midbrain dopaminergic neurons against MPP^+, a protection similar to that exerted by E2. In addition, we observed that when E2 was used in combination with G15 its protective effect was no longer observed (150). Similar to E2, treatment with G1 increases the concentration of dopamine, its metabolites, and the specific binding to the membrane (DAT) and vesicular (VMAT) dopamine transporters in the striatum of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. These dopaminergic protective effects of E2 and G1 were lost in the presence of G15 (198). Comparable protective effects in the striatum of MPTP-exposed mice mediated by GPER activation were also observed in other studies using treatment with either G1 (199) or raloxifene (200).

The dopaminergic protective actions promoted by GPER activation observed in the above-mentioned studies appear to be related with the ability of G1 to increase the expression of neurotrophic factors. We found that G1 is capable of inducing an increase in glial cell-derived neurotrophic factor (GDNF) protein in midbrain neuron-glia cultures, and that GDNF

neutralization or silencing in these cultures impedes the dopaminergic protective effect of GPER selective activation (150). This was also observed by Cheng and colleagues (2017) on neuroblastoma cell line SH-SY5Y. G1 reduced the MPP^+-induced cell death through the increase of GDNF, effects that were abrogated by G15 (201). In addition, it was observed by others that the protective effects promoted by GPER activation in the striatum of MPTP mice occurred in parallel with an up-regulation of BDNF and GDNF protein levels, increase in the anti-apoptotic Bcl-2 protein and activation of the pro-survival kinases Akt and ERK (199, 200). This suggests that protection mediated by GPER activation involves both inhibition of apoptosis and promotion of dopaminergic survival. Guan and colleagues (2017) showed that protection mediated by G1 in the MPTP mouse model involves also an anti-inflammatory effect. G1 treated mice present a reduction in the number of microglial cells and IL-1ß, TNFα and IL-6 protein and mRNA levels in the midbrain (202). In fact, although PD is essentially an idiopathic disease, it is accepted that inflammation promoted by microglial cells plays a critical role to the progressive dopaminergic neuronal death. GPER selective activation is associated with the modulation of inflammatory responses, with G1 inhibiting Lipopolysaccharide (LPS)-induced IL-6 expression in murine macrophage cells (203). A study from our group, demonstrated that G1 treatment protects dopaminergic neurons in the substantia nigra, an effect accompanied by decreased IL-1ß, CD68 and inducible nitric oxide synthase (iNOS) mRNA levels in this region. Moreover, we also demonstrated that G1 treatment prevents LPS-induced impairment of motor function (153).

The above-mentioned effects were described on males. Data regarding the effects induced by GPER on females are scarce and contradictory. To our knowledge, the few studies that exist in PD models using female models were carried out with the administration of tamoxifen or raloxifene (194, 204), two selective estrogen receptor modulators (SERMs) with antagonistic actions towards ERα and ERß and acting as GPER agonists (96, 205). Dluzen and Mickley (2005) demonstrate a protective role of tamoxifen from dopaminergic toxins, inducing an increase of striatal dopamine and 3,4-dihydroxyphenylacetic acid concentrations on females (204). On the other hand Baraka and colleagues (2011) using a rat model of PD demonstrated that tamoxifen does not induce any protection in OVX females, whereas raloxifene protected striatal dopaminergic neurons against 6-OHDA-induced neurotoxicity (194).

In conclusion, GPER seems to be a promising therapeutic target for the treatment of PD. In males, the activation of GPER promote several protective effects against insults in the dopaminergic nigrostriatal system, namely protecting the dopaminergic neurons in the substantia nigra and the striatal nerve terminals, increasing the concentration of dopamine and its metabolites, as well as DAT and VMAT-2 specific binding. GPER activation can also induce protection of the motor function (table III). These protective actions induced by GPER activation appear to result from an increase in neurotrophic factors, inhibition of apoptosis, promotion of survival and reduction of inflammation. In addition, it would also be important to

evaluate whether its selective activation has the ability to promote recovery or stop the progressive loss of dopaminergic neurons. There is no information on the selective activation of GPER in females, and the scarce information on the modulation of GER refers to the use of non-selective agonists such as raloxifene and tamoxifen. In addition, the existing data is contradictory, making difficult to draw conclusions about the potential effects of GPER in female models of PD.

2.4.2.3. - Multiple sclerosis

Multiple sclerosis (MS) is characterized by multiple focal areas of myelin loss within the CNS called plaques or lesions (206). The hallmarks of MS pathology are axonal or neuronal loss, demyelination, and astrocytic gliosis (206). Among these neuropathological characteristics, axonal loss is particularly relevant because it is the main underlying mechanism of permanent disability (206). This axonal loss was associated with different mechanisms such as the energy deficit linked to mitochondrial dysfunction and the loss of myelin trophic support (206).

Wang and colleagues (2009) reported the ability of GPER activation to promote protection in MS using the rodent experimental autoimmune encephalomyelitis (EAE) model (table III). Selective activation of GPER with G1 reduced clinical and histological EAE signs, whereas E2 mediated protection was significantly impaired in GPER gene-deficient female mice (207). The role of GPER in the EAE model is also supported by the finding that selective activation of GPER with G1 reduced the severity of disease in EAE models of MS and that this effect is concomitant with a G1-mediated decrease in pro-inflammatory cytokines, including Interferon γ (IFNγ) and IL-17 (208). Furthermore, the results also show the ability of G1 to inhibit the production of cytokines such as TNFα and IL-6 in a dose-dependent manner in human primary macrophages and in a murine macrophage cell line (208).

Studies about the influence of ERα and GPER on E2 ability to treat EAE showed that E2 reduced disease severity in wild-type and ERα knockout female mice, but did not alter the disease in the GPER knockout group (209), suggesting that GPER is necessary for the protective effect mediated by E2. Moreover, the effects on disease severity of both receptors were associated with the production of anti-inflammatory IL-10 following E2 treatment (209).

GPER is expressed throughout the oligodendrocyte differentiation and promyelinating stages in primary oligodendrocyte cultures derived from rat spinal cords and brains (154). Additionally, it was also shown that selective activation of GPER with G1 enhanced oligodendrocyte maturation and remyelination after demyelination, suggesting an additional mechanism of protection triggered by GPER selective activation and enhancing the potential of GPER selective agonists as therapies for the treatment of MS (154).

It is also important to note that that results regarding the effects induced by the selective activation of GPER on *in vivo* MS models were carried out only in female models, therefore, it would be important to clarify if these effects also occur in males.

Table 3: Effects induced by GPER selective activation in neurodegenerative disorders

	Major conclusions	Models	Reference
AD	Selective GPER activation ameliorated object recognition memory in female but not male mice;	5XFAD mice (intact female and male); Exposure to G1 and G15;	Kubota *et al*. 2016
PD	Increased concentration of dopamine and its metabolites, and DAT and VMAT2 specific binding in the striatum; Increased DAT specific binding in the substantia nigra;	C57BL/6 mice (male); MPTP model; Subcutaneous injection of G1 twice daily for 10 days - before and after dopaminergic lesion;	Bourque *et al*. 2013
	Increased dopamine and DOPAC concentration and specific binding of DAT and VMAT in the striatum; Increased anti-apoptotic Bcl-2 protein and activation of the pro-survival kinase Akt in the striatum;	C57BL/6 mice (male); MPTP model; Subcutaneous injection of raloxifene twice daily for 10 days - before and after dopaminergic lesion;	Bourque *et al*. 2014
	Increased dopamine concentration and DAT and VMAT-2 specific binding in the striatum; Increased DAT specific binding in the substantia nigra; Increased GDNF, BDNF and Bcl-2 protein levels in the striatum;	C57BL/6 mice (male); MPTP model; Subcutaneous injection of G1 twice daily for 10 days - before and after dopaminergic lesion;	Bourque *et al*. 2015
	Prevention of the dopaminergic neuron loss in a GDNF-dependent process;	Wistar rat midbrain neuron-glia cultures; MPP^+ model; Exposure to G1;	Bessa *et al*. 2015
	Increased dopaminergic fibers density in the striatum; Prevention of the dopaminergic neurons loss in the substantia nigra; Decreased microglial cells number and IL-1β, TNF-α and IL-6 protein and mRNA levels in the midbrain;	C57BL/6 mice (male); MPTP model; Subcutaneous injection of G1 twice daily for 12 days - before and after dopaminergic lesion;	Guan *et al*. 2017
	Prevention of the dopaminergic neuron loss in the substantia nigra; Protection of the motor functions; Decreased IL-1β, CD68 and iNOS mRNA levels in the substantia nigra;	C57BL/6 mice (male); Unilateral injections in the substantia nigra with LPS on 5th day of G1 treatment; Subcutaneous injection of G1 twice daily for 12 days - before and after dopaminergic lesion;	Mendes-Oliveira *et al*. 2017
	G1 reduced the MPP^+-induced cell death through the increase of GDNF, effects that were abrogated by G15;	Neuroblastoma cell line SH-SY5Y; MPP^+ model; Exposure to G1;	Cheng *et al*. 2017

MS	Selective GPER activation mediates protection against MS, which is significantly impaired in GPER gene-deficient mice;	C57Bl/6J mice (female); GPER KO mice; EAE; Exposure to G1;	Wang *et al*. 2009
	Selective GPER activation reduces the severity of MS through the decrease of pro-inflammatory cytokines, including IFNγ and IL-17; G1 inhibits the production of cytokines such as TNFα and IL-6 in a dose-dependent manner;	Primary culture of macrophages, microglia and a murine macrophage cell line (RAW264.7); EAE; Exposure to G1;	Blasko *et al*. 2009
	E_2 reduced disease severity in wild-type and ERα KO mice, but had no impact on GPER KO group; These different effects were associated to the production of anti-inflammatory IL-10; GPER have an important but still undefined role in regulating immune reactivity in MS severity;	C57Bl/6J mice (intact female); Ethinylestradiol treatment; WT, ERαKO and GPERKO mice;	Yates *et al*. 2010
	GPER is expressed throughout oligodendrocyte differentiation and promyelinating stages; Selective GPER activation enhanced oligodendrocyte maturation and remyelination after demyelination;	Primary oligodendrocyte cultures from Wistar rat spinal cord; Demyelination model; Exposure to G1 and G15;	Hirahara *et al*. 2013

2.4.3 - Mood disorders

Mood disorders are common psychiatric illnesses characterized by conspicuous disturbances in emotional disposition, and include diseases such as depression or bipolar disorders (210). In 2009, Xu and colleagues demonstrated that G1 attenuates serotonin receptor signaling in the paraventricular nucleus of the hypothalamus and reduces responses to oxytocin and adrenocorticotropic hormone, rising the hypothesis that GPER could play a role in mood disorders (145). On the other hand, GPER is necessary for E2-induced changes in serotonin 1A receptor signaling (211). Desensitization of serotonin 1A receptor is a key element for selective serotonin reuptake inhibitors (SSRI) efficacy in the treatment of mood disorders, and the expression of GPER shortens the onset of SSRI therapeutic effects in a GPER-dependent manner, thus providing evidences that GPER may accelerate the therapeutic effect of SSRI treatment in mood disorders (Table IV) (211).

In a mouse model of depression, G15 inhibited the anti-depressant effects of G1 (115). Studies on the impact of classical ER and GPER on SSRI treatment of depression in OVX female rats showed that long-term treatment with G1 induced anti-depressant-like effects associated with an increase in the phosphorylation levels of Akt, ERK and TrkB receptor in the hippocampus (Table IV) (212).

Evaluation of serum GPER levels in 38 euthymic bipolar disorder patients showed that both male and female patients had higher GPER levels than the respective control groups, while there were no differences in E2 serum levels, suggesting that GPER may play a role in the pathophysiology of bipolar disorder (213).

Anxiety disorders comprehend a wide range of disturbances that include panic disorders, obsessive-compulsive disorders, post-traumatic stress and generalized anxiety disorders (214). Kastenberger and colleagues (2012) demonstrated that short-term administration of specific agonists of classical ER did not induce any behavioral changes, whereas specific stimulation with G1 in male and OVX female mice induced anxiogenic effects, suggesting that estrogen-induced anxiogenic-like effects were mediated mostly by GPER (Table IV) (215). Studies using wild-type female and male mice and GPER knockout mice demonstrated that alterations in anxiety-like behavior were observed predominantly in male mice (216). In contrast, others reported data supporting anxiolytic effects of GPER in OVX female mice being this associated to the regulation of synaptic transmission in the basolateral amygdala (BLA) (166) and independent of ERK signaling (217). A differential contribution of GPER in the control of anxiety in male and female mice is also supported by data from the elevated plus maze task showing that acute administration of G1 leads to anxiolytic effects in gonadectomized male mice, but not in female mice (Table IV) (218). Somehow, these results establish a parallelism with what has already been described for E2 and ERα/ERβ (218), being the nature of E2 effects on anxiety attributable to the differential effects of specific estrogen receptor subtypes. ERβ activation induces anxiolytic-like effects whereas ERα activation appears to have mainly anxiogenic-like properties (218).

Chronic pain-related anxiety is attenuated by subcutaneous injection or local infusion of G1 in the BLA of OVX female mice, being these effects associated with the prevention of imbalance between excitatory and inhibitory transmissions in the BLA synapses (219).

Increased serum GPER levels might play a role in the etiology of generalized anxiety disorder. In a study involving 40 drug-naïve patients newly diagnosed with anxiety disorder there were significantly higher levels of GPER in the serum of patients with generalized anxiety disorder and a positive correlation between GPER serum levels and anxiety severity (220).

Existing data indicates that for several disorders the effects triggered by GPER selective activation are dependent on the sex of animals, or with the amount of circulating E2 levels. Due to the scarcity of studies regarding the selective activation of GPER in both males and females, it is not possible to establish a clear hypothesis to explain these differences. However, the differential effects can relate with the complex signaling pathways activated by GPER and to the crosstalk between classical ER on males and females (218). Hart and colleagues (2014) showed that G1 increased protein expression of hippocampal phosphorylated ERα in male mice, but not in females (218). These modifications were associated with different anxiolytic effects in males and females (218). Although the differential effects observed upon GPER activation in males and females may involve similar effects the data currently available are insufficient to draw any conclusions.

Table 4: Effects induced by GPER selective activation in mood disorders

	Major conclusions	Models	Reference
Mood disorder	Selective GPER activation attenuates 5-HT1A receptor signaling and accelerates the effects of SSRIs treatment of mood disorders;	Sprague-Dawley rats (intact female); Exposure to G1; GPER distribution:	Xu *et al*. 2009
	GPER is necessary for estradiol-induced changes in the serotonin 1A receptor signaling pathway and desensitization;	Sprague-Dawley rats (intact female): GPR30 siRNAs to decrease GPR30 Expression;	McAllister *et al*. 2012
Depression	Selective GPER activation has antidepressant properties, which were inhibited by G15;	C57Bl6 mice (OVX female); Exposure to G1 and G15;	Dennis *et al*. 2009
	Long-term treatment with G1 induces anti-depressant-like effect;	Sprague Dawley rats (OVX female); Exposure to G1;	Benmansour *et al*. 2016
Bipolar	Serum GPER levels in euthymic bipolar patients are higher than in controls;	38 patients diagnosed with Bipolar disorder (males and females); Quantification of GPER in serum	Orhan *et al*. 2018
Anxiety	Estrogen-induced anxiogenic-like effects are mediated mostly by GPER;	C57Bl6 mice (intact and OVX females); Exposure to G1;	Kastenberger *et al*. 2012
	GPER has a direct involvement in anxiety and stress control, being this impact stronger in male than in female mice;	C57BL/6J mice (male and intact female); GPER KO mice;	Kastenberger *et al*. 2014
	The selective activation of GPER had an anxiolytic effect in the open field test;	C57BL/6J mice (OVX female); Exposure to G1;	Anchan *et al*. 2014
	GPER selective activation has anxiolytic properties in gonadectomised male, but not in female mice;	C57BL/6J mice (gonadectomized males and intact females); Exposure to G1;	Hart *et al*. 2014
	GPER selective activation induced anxiolytic effects in OVX female mice attributed to the maintenance of the balance between excitatory and inhibitory transmissions in the basolateral amygdala;	C57BL/6J mice (OVX female); Exposure to G1 and G15;	Liu *et al*. 2015
	Serum GPER levels were significantly increased in patients diagnosed with generalized anxiety disorder, with a positive correlation between GPER levels and severity of the disease;	40 patients diagnosed with generalized anxiety disorder; Serum GPER quantification;	Findikli *et al*. 2016

2.4.4. - Autism spectrum disorder

To the best of our knowledge, only one study investigated the impact of GPER in Autism spectrum disorder (ASD). Data from the analysis of GPER serum levels in patients diagnosed

with ASD indicate that ASD patients have significantly lower levels of GPER when compared to the control group (221). The results showed also a negative correlation between GPER levels and the Childhood Autism Rating Scale total score rising the hypothesis of a role of GPER in the etiology of ASD (221).

2.4.5 - Spinal cord injury

SCI is a damage to any part of the spinal cord or nerves, causing temporary or permanent changes in strength, sensation and other body functions below the site of the injury (222). GPER selective activation with G1 dose-dependently reduced neuron apoptosis and improved functional recovery following SCI in the weight-drop spinal cord contusion model in male rats, whereas GPER knockdown inhibited the beneficial actions of E2, suggesting that GPER might be the main ER responsible for the neuroprotective effects induced by E2 (223). Similar results were obtained in mice. GPER selective activation with G1 mimicked the effects of E2 treatment and prevented SCI-induced apoptotic cell death and enhanced motor functional recovery after injury, whereas the neuroprotective effects of G1 and E2 were blocked by G15 in adult female C57BL/6J mice (224).

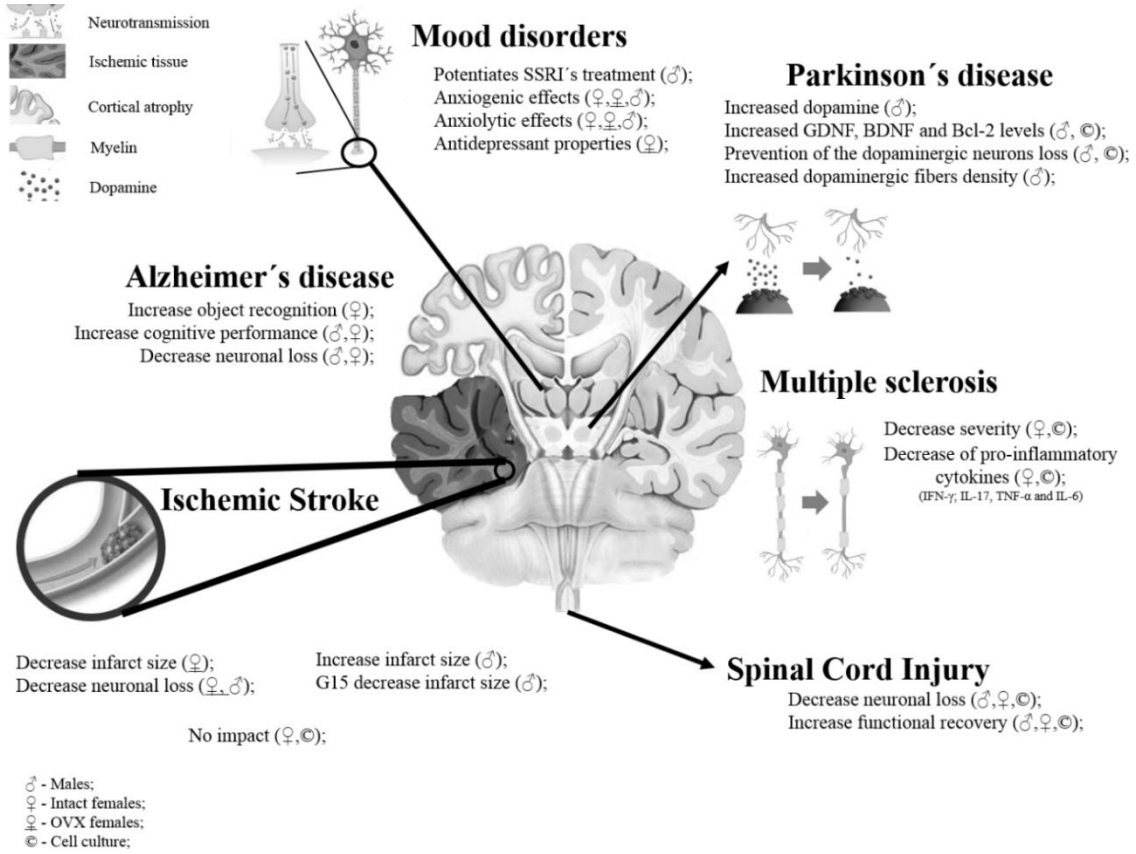

Figure 2: Effects induced by GPER selective activation on brain disorders.

Abbreviations: Brain-derived neurotrophic factor (BDNF); Glial cell-derived neurotrophic factor (GDNF); Selective serotonin reuptake inhibitors (SSRI).

3. - Repetitive transcranial magnetic stimulation (rTMS)

Actually there are relatively few treatment options available to minimize and rehabilitate injured brain tissue following IS, which leads to a urgent need to explore new therapeutic approaches (9, 225). Current therapies are biased, since they are focused on restoring blood flow, neglecting neuronal, glial and vascular repair following ischemia (9, 225). After IS most of the treatments (e.g., Physical therapy, speech therapy, occupational therapy) are applied in the sense of quenching the sequelae that ischemia has left or controlling modifiable risk factors (e.g., hypertension, diabetes, coagulopathies), thus, helping to recover as much as possible and allow the return of patients to independent living (225, 226).

The application of repair-based therapies to post-stroke injured tissues has gained strength, and several approaches are being tested (e.g., growth factors, monoclonal antibodies, cell-based therapies, brain stimulation), some of them already used in human trials (225). The majority of these approaches are regarded as a complement of acute therapies not only in terms of different biological targets but also in terms of treatment time window (days to weeks or larger), and have the potential to help a large number of patients affected by IS (225). A promising therapeutic approach would be to foster neurological recovery by promoting brain remodeling via neurovascular plasticity using the repetitive transcranial magnetic stimulation (rTMS).

Since its development in the early 1980s, the therapeutic potential of rTMS on neurological disorders has been extensively studied (227-230). Moreover, increasing data suggest a therapeutic and neurorestorative role of rTMS in several neurological disorders, such as depression, movement disorders or obsessive–compulsive disorders (227-234). Having effectively received approval for the treatment of depression of national reference entities, such as Health Canada (235), the American Food and Drug Association (236), the American Psychiatric Association, the Canadian Network for Mood and Anxiety Treatments, the World Federation of Societies of Biological Psychiatry, or the The National Health and Care Excellence (United kingdom) (228, 235-237). However, despite the wide application in humans, the cellular and molecular mechanisms underlying rTMS-based therapies are not well characterized (227).

3.1. – Principles of rTMS

rTMS is a noninvasive method to stimulate brain cells and is based on the principles demonstrated by Faraday in 1831, where he reported that an alternating and rapidly changing magnetic field produces electric currents in an adjacent conductor (227, 228, 230, 238), in this case, the adjacent conductor is the brain tissue. In rTMS, an electrical current passes through a wire coil placed over the scalp, the magnetic waves bypass the skull and alters the electrical

activity of brain cells (225, 227-230, 239, 240). The rate at which the circuit is turned on and off determines the frequency of the magnetic field that is produced (241). The magnetic waves bypass the skull without attenuation and interact with the electrical activity of cells in the brain, which then results in the alteration of neuronal excitability (225, 227, 230, 240, 241).

The brain is an electrical organ and expends considerable amounts of energy maintaining a specific cellular resting potential (225). Therefore, electrical and electromagnetic interventions have the potential to induce electric stimulus on brain tissues, which in turn modify brain function and potentially promote neural repair (225). For example, the stimulation of the motor cortex causes contractions in the muscles of the extremities, whereas stimulation of the primary visual cortex induces flashes of light when the eyes are closed (225, 227).

There are two conventional types of rTMS protocols, the low-frequency (LF) and high-frequency (HF) rTMS (227, 229, 238, 240, 241). LF-rTMS is defined by stimulation at frequencies lower than 1 Hertz (Hz), reducing neuronal excitability (227, 229, 238, 240, 241), whereas HF-rTMS stimulation uses frequencies higher than 1 Hz, usually over 5 Hz, and is capable of increasing neuronal excitability (227, 229, 238, 240, 241). These stimulations can be implemented only once or as part of a treatment protocol where they are applied once a day for several days. A modification of rTMS parameters such as stimulus frequency and duration can originate an alternative approach, the theta-burst stimulation (TBS) in which stimuli are applied in short trains (3 pulses) at HF (50hz) repeated at intervals of 200 milliseconds (225, 227-229, 238, 241).

3.2. – Cellular and molecular effects of rTMS on brain cells

Over recent years the neuroprotective effects of rTMS on brain cells has increased. The underlying cellular and molecular effects that support those effects are not well characterized. The most accepted theory indicates that this effect is mediated by the modulation of synaptic plasticity (227, 242). However, the available data indicates that rTMS may also modulate other mechanisms and/or pathways such as neurotransmission, gene expression, neuroprotection, neurogenesis, and inhibition of cell death pathways.

3.2.1. - Synaptic plasticity

Synaptic plasticity is defined has the ability of the brain to reorganize itself, enabling short- and long-term remodeling of neural communication that outlasts an experimental manipulation or period of training (242, 243). This process is complex, but in some way rTMS

has the ability to trigger synaptic modifications, being this changes associated to a shift in ionic balance (242). The most accepted theory indicates that this effect of rTMS is mediated through NMDA-receptors located on the post-synaptic membrane (227, 242). These receptors contain a cationic channel that is blocked by magnesium ions during the resting state, but depolarization of the cell membrane induced by rTMS eliminates this blockade and allows calcium ions to enter to the post-synaptic neuron (227, 242). The calcium entrance in post-synaptic cells activates a calcium-sensitive signaling pathway, which has many downstream targets that induce changes in post-synaptic neurons leading to increased synaptic strength (241). This shift fosters the communication between adjacent neurons (227, 242). There are also data indicating that rTMS increase dendritic spine/synapse numbers, dendritic remodeling, and/or axonal sprouting (244). Other interesting phenomenon that is induced by rTMS is the long-term potentiation, a well-characterized form of synaptic plasticity that fulfils many of the criteria for a neural correlate of memory, which could explain the beneficial effects observed after 6 months of the cessation of treatment (227, 242).

It is important to note that rTMS does not necessarily always produce a beneficial outcome and that these effects largely depend on the stimulation protocol (227). In hippocampal cell cultures, low-intensity stimulation induces dendritic sprouting and growth, and increases the density of synaptic contacts (245). By contrast, excessive high-intensity stimulation induces devastating effects that result in decreased numbers of dendrites and axons, the presence of neuronal lesions, and a diminished number of synapses (245).

3.2.2. - Neurotransmission

The rTMS-induced modifications on synaptic transmission were associated to the increase in the release of several neurotransmitters, such as dopamine (246-250), serotonin (251-254), gamma-aminobutyric Acid (GABA) (250, 255-259), and acetylcholine (260, 261), being these effects associated to the rise in intracellular calcium levels induced by rTMS (249, 262).

Dopamine is a neurotransmitter that participates in several processes such as movement, learning, attention, motivation, and emotional responses, being the changes of its levels associated to illnesses such as Parkinson's disease, schizophrenia, and depression (247, 248, 262, 263). It was demonstrated that rTMS could be a valuable tool to stimulate the production and release of endogenous dopamine (246-250), and to enhance the rehabilitation of dopaminergic system (249, 262).

It was also raised the hypothesis that rTMS could induce changes on neurotransmission pathways such as the serotonergic and glutamatergic/GABAergic systems. Serotonin is an

important neurotransmitter involved in hypothalamic-pituitary-adrenal system regulation (251). Serotonergic system also plays a critical role in depression, being the expression of serotonin A-receptor mRNA decreased in depressive patients (251). It was demonstrated that an increase in serotonergic neurotransmission could underlie the antidepressant effects of rTMS (253). Another interesting study reported that the serotonergic system changes induced by rTMS is correlated positively with serotonin A-receptor binding indices (252). rTMS also has the ability to increase the eficacy of SSRIs, which is useful for SSRI-resistant therapies (254). GABA is the main inhibitory neurotransmitter in the brain, and evidences suggest that a reduction of GABA function contribute to the pathophysiology of depression (255). Research on the effects of rTMS on evoked potentials demonstrates that rTMS is accompanied by changes in local hippocampal inhibitory circuits. On depression the glutamate ratio is decreased and evidences indicate that rTMS increase GABAergic and glutamatergic systems the prefrontal cortex (250, 256-259).

A more recent study demonstrated that rTMS also has the ability to interact with the cholinergic pathway (260). In a rat model of dementia, it was demonstrated that rTMS treatment for 30 days significantly increased acetylcholinesterase and choline acetyltransferase activity, and increased the density of cholinergic neurons, implicating rTMS on the restoration of cholinergic system (260).

3.2.3. - Gene expression

Several data support a role of rTMS in the control of gene expression, such as an increase on the expression of c-Fos (264-267), a well-known transcription factor that regulates several genes and is associated with increased activity, cell proliferation, differentiation, and survival (268). Suggesting that rTMS has the ability to trigger the activation of one or more of these beneficial pathways.

More recently, it was evaluated the impact of different rTMS protocols on gene expression in rat cortices after acute ischemic-reperfusion brain injury (269). The most promising result were obtained with intermittent TBS, on a two week protocol (with a 2-day pause) and single daily stimulation session (269). The results indicate that this stimulation protocol has the ability to upregulate several genes, including those involved in angiogenesis, inflammation, cellular repair, structural remodeling, neuroprotection, neurotransmission, and neuronal plasticity (269). The majority of genes showing increased expression following rTMS are involved in neurotransmission and neuronal plasticity (269). Some of the genes upregulated by rTMS were the Mapk1, BDNF, Fos, Jun, Tubb5, glutamate ionotropic AMPA receptor, NMDA receptor, genes encoding GABA pathway (Gabbr1, Gad1 and Gad2) (269). These results allow hypothesizing that genetic modifications induced by rtMS could underlie the therapeutic effects of this approach.

3.2.4. - Neuroprotection and neurogenesis

BDNF is a well-known neurotrophic factor involved in various functions ranging from enhancement of neuronal survival, neurogenesis, the migration and differentiation of neurons, the growth of dendrites and axons, and synapse formation (270). Data from human serum levels of BDNF after rTMS sessions are contradictory, with results showing an increase (271-273), no changes (274, 275) or a decrease of BDNF levels (276). However, when the effects of rTMS are evaluated on animal models the results indicate that a single HF stimulation increase BDNF signaling (277, 278) whereas LF reduces it (277). On the other hand prolonged exposure to rTMS (5 days) significantly increases BDNF mRNA and protein levels (279). These data indicate that rTMS may induce neuroprotection through the increase of BDNF levels.

Another mechanism by which rTMS may induce its therapeutic effects is neurogenesis, since it was demonstrated that rTMS, both LF and HF, has a positive effect on the differentiation and growth of neural stem cells from the neonatal rat *in vitro* (280-283).

3.2.5. - Prevention of neuronal cell death

rTMS has the ability to prevent neuronal death induced by ischemia, being those effects associated to the increase of ATP content of cells (284) and a higher glucose metabolism (285). In addition to this change in the metabolic pathway, it was also demonstrate that rTMS decreases the number of apoptotic (285, 286) and necrotic cells (286), indicating that rTMS has the ability to prevent cell death. Although a significant amount of studies indicates an impact of rTMS on neurodegenerative disorders, the cellular and molecular mechanisms/pathways by which they are induced remain unclear.

3.2.6. - Glial cells

Research conducted on the cellular and molecular mechanisms induced by rTMS in brain cells have been mostly focused on neurons, forgetting the role that glial cells could have in this process (287). The role of glial cells participate in neurotransmitter uptake, buffering of extracellular potassium, synapse formation, and trophic support (287), in addition to the ability to respond to electrical activity directly or indirectly, makes these cells possible effectors of rTMS (287).

Astrocytes are important regulatory cells within the CNS and are possible mediators of rTMS-induced brain modifications (287). So far, few studies have evaluated the impact of HF-

rTMS in astrocytic cells, being these observations contradictory regarding the effect on the activation status or in the proliferation. On *in vivo* and *in vitro* models, it has been demonstrated that rTMS has either the ability to induce astrocyte activation (288, 289), or does not induce any effect on astrocyte activation (290-294). Evaluation of the proliferative effect of HF-rTMS on astrocytes resulted also in contradictory data with results showing either increase (295) and decrease (292) of astrocyte proliferation. Moreover, several observations raised the hypothesis that astrocytes are only indirect targets of rTMS (287). This theory proposes that the effects observed represent a response of astrocytes to the direct effect of rTMS on neurons, and the consequent release of neurotransmitters and ions (244, 256-259, 287).

Microglial cells are the resident immune cells of the CNS, and they play a multifaceted role in modulating synaptic plasticity (287). The scarce information on the impact of rTMS on these cells indicates that LF rTMS does not affect microglial number in the motor cortex or hippocampus (291). However, application of HF-rTMS following an ischemic injury leads to increased Iba1 expression, suggestive of microglial activation (289, 296). In contrast, HF-rTMS applied to the injured spinal cord reportedly attenuates microglial activation (293).

3.3. – Application of rTMS on neurological disorders

Although there is no molecular basis for explaining all the beneficial effects induced by rTMS, over the past 10–20 years this technique has become widely used in neurology, particularly on depression resistant to pharmacological treatment (227-229). Large, randomized placebo-controlled studies demonstrated the potential effectiveness of rTMS on the treatment of several neurological disorders such as depression, obsessive–compulsive disorders, pain syndromes, and epilepsy (227-229). Interestingly, some of the beneficial effects can persist as long as 6 months after the cessation of treatments (227-229, 242). Additionally, it was demonstrated that rTMS is a good neurorehabilitation method for patients with sequelae of various nervous system disorders such as trauma or stroke (227-229).

3.3.1. – Major depressive disorder

Major depressive disorder (MDD), is a mood disorder that causes a persistent feeling of sadness and loss of interest (297). The pathophysiological mechanisms that induce MDD seems to be related to abnormal synaptic transmission on neuronal circuits implicated in the generation and regulation of emotion, as well as in reward, of the dorsolateral prefrontal cortex (297).

At a therapeutic level, rTMS has been widely used in the treatment of MDD, especially in the acute phase of treatment-resistant depression (228, 229, 232, 234, 236). Moreover, it is accepted as an evidence-based treatment option by the American Psychiatric Association, the Canadian Network for Mood and Anxiety Treatments, and the World Federation of Societies of Biological Psychiatry (228, 235-237). The cellular and molecular mechanisms that are triggered by rTMS are not completely unveiled (227, 228). However, studies indicate that these beneficial effects induced by rTMS on MDD patients are related to improvements of neuronal function comprising the modulation, normalization and optimization of functional connectivity (122, 229, 232, 234, 241, 244, 298), the modulation and improvement of neurotransmission (232, 246-249), modulation of serotonergic system (232, 251-253), modulation of glutamatergic/GABAergic (232, 250, 256-259), and genetic modifications that enhance functional outcomes (232, 264-267, 269).

3.3.2. – Obsessive-compulsive disorders

Obsessive-compulsive disorder (OCD) is an anxiety disorder in which a person has uncontrollable, reoccurring, and unwanted thoughts, ideas or sensations (obsessions) making them feel driven to do something repetitively (compulsions) (299, 300), associated to abnormalities on neuronal circuits that control the behavior (300). Several therapeutic studies have been conducted employing very heterogeneous methodologies, reflecting the various hypotheses on the underlying pathophysiological mechanisms (228, 229). The existing data shows that LF-rTMS of orbitofrontal cortex or supplementary motor area improves this clinical condition (229, 301-305), being these effects associated to inhibitory modulation and normalization of the excitability of motor cortex (229, 301, 304). Similar to the therapeutic approach in patients with MDD, also in cases of OCD the use of this technique for the treatment of patients with pharmacological resistance begins to gain strength (228, 306). Nevertheless, future placebo-controlled rTMS studies in OCD patients should include larger sample sizes and be more homogeneous in terms of demographic and clinical variables, stimulation parameters, and cortical targets (228).

3.3.3. – Pain syndromes

Chronic pain can be neuropathic when originated from a lesion or disease of somatosensory systems, either peripheral or central, non-neuropathic, when caused by excess of nociception secondary to inflammation or tissue lesion, psychogenic, or without proven cause (228, 229). rTMS has been proposed in the treatment of neuropathic pain, where it was demonstrated that rTMS applied contralaterally to the pain side has pain relieving effects (228,

229, 292, 307, 308), being this analgesic effect most robust 2–4 days after an rTMS session (307). The results also indicate that a single session of HF-rTMS of motor cortex induces the reduction of pain scores, whereas LF-rTMS does not have beneficial effects (309, 310). However, the exact mechanism of action of rTMS is still unknown (309). Among the various theories, the modulation of glutamate receptors, due to their role in the pain pathways, is the strongest (309). However, another interesting theory suggest that the effect is mediated by the reduction on the expression of nitric oxide synthase in ipsilateral dorsal root ganglia (292). On non-neuropathic pain, due to small number of studies, no conclusion can be firmly drawn (228, 229).

3.3.4. - Movement disorders

Movement disorders refers to a group of neurological conditions that cause abnormal increased or decreased movements, which may be either voluntary or involuntary (e.g., PD, dystonia, essential tremor, spasticity, ataxia, Huntington's disease, Tourette's syndrome) (311).

PD is the movement disorder more widely studied. It was demonstrated that rTMS of prefrontal cortex improve the motor performance of patients (228, 229, 312, 313) and also cognitive-behavioral functions (314), being these beneficial effects associated to the increased production and release of endogenous dopamine on prefrontal cortex (228, 229, 246, 247).

On dystonia, rTMS has been applied to reduce the excitability of motor cortical regions, thus relieving the symptoms (228, 229, 315, 316). Essential tremors are caused by cerebellar hyperactivity, and it was demonstrated that rTMS on cerebellum has the ability to normalize cerebellar excitability, reducing tremors (316, 317).

Regarding other movement disorders such as spasticity, ataxia, Huntington's disease or Tourette's syndrome rTMS was shown to normalize neuronal activity (228, 229, 318, 319).

3.3.5. – Epilepsy

Epilepsy is a neurological disorder in which brain activity becomes abnormal, causing seizures or periods of unusual behavior, sensations, and sometimes loss of awareness (320). rTMS has antiepileptic effects, decreasing the number of seizures (321) and epileptic discharge frequency (322), being these effects associated to the reduction of interictal electroencephalography abnormalities (321, 322). However, there is still insufficient information to indicate the safety and effectiveness of rTMS on epileptic patients, particularly on the pathophysiological process of seizures (323).

3.3.6. – Spinal cord injury

The application of rTMS to SCI patients is seen as a possible approach to improve motor outcomes by enhancing activity in neuronal pathways involved in the generation of voluntary movements (324). It was demonstrated that this strategy can improve sensory and motor function of the hands (325, 326) and legs (326, 327), being the amplitude of movements higher when higher rTMS frequencies were used (324-327). There are also two other symptoms, frequently associated to SCI, spasticity (60%) and neuropathic pain (80%). The effects of rTMS on these two neurological conditions was discussed above, and the existing data indicates that in SCI rTMS has the ability to reduce both spasticity (327-329) and neuropathic pain (330-332). However, it is fundamental that more studies are developed in order to understand how rTMS modulates and neurorehabilitates motor, sensory and autonomic nervous system functions in SCI patients (324).

3.3.7. – Ischemic Stroke

On the treatment of IS, the application of rTMS is seen as a part of a neurorehabilitation strategy, which should be combined with other approaches such as occupational or speech therapy (228, 333-335). This technique could be used for the correction of maladaptive brain plasticity or enhancing brain plasticity during rehabilitation, which can be translated in improvement of the functions that are regulated by the areas where the IS occurred (228, 335, 336).

In general, after an IS brain compensation mechanisms leads to a reduction of neuronal activity on the affected hemisphere, and to increased activity on the hemisphere non-affected (228, 335, 336). rTMS therapy for IS neurorehabilitation has been designed to normalize this imbalance of cortical excitability induced by IS and enhancing connectivity in neuronal networks (228, 335). The application of this technique on IS patients is being designed to increase the activity of the affected hemisphere with HF-rTMS and to decrease the activity of the non-affected hemisphere with LF-rTMS, thus leading to a normalization on brain activity (228, 335). The effects are reflected mainly in two types of post-IS symptoms, the motor deficits and aphasia.

Motor deficits are characterized by reduced motor-evoked potentials on ipsilesional when compared with contralesional motor area (233, 337). Being that, higher motor-evoked potentials are correlated with better clinical outcome (233, 337). It was demonstrated that HF-rTMS applied to the ipsilesional hemisphere increase the motor-evoked potentials, which correlates with an increase on motor performance (231, 233, 338-342). Whereas on contralesional hemisphere the increase of motor performance, was associated to LF-rTMS,

either through a single session (231, 233, 343-346) or through repeated sessions (231, 233, 347-350). Interestingly, this effect can persist for at least 3 months after the cessation of treatments (231, 342, 350). Nevertheless, the question of whether contralesional LF-rTMS, ipsilesional HF-rTMS, or both should be used still requires further investigation (228, 233).

Aphasia is caused by an ischemic injury on the areas that regulate language, Broca´s and/or Wernicke's areas, or in the connection between these two areas (351). Functional disruption of the language neural system is one possible result of intra- and interhemispheric activity imbalance (351). On IS patients LF-rTMS that targets the triangular part of the right inferior frontal gyrus improves language recovery (333, 334, 351-353), being these effects associated to the inhibition of neuronal excitability (333).

There are also evidences that HF-rTMS on the ipsilesional areas may have other beneficial effects, such as the improvement of learning and memory function (260), enhancement of neurogenesis (278), prevention of neuronal death (289, 354, 355), and priming the brain, enhancing its potential to cope with the injury and to rewire (269). However, more studies are needed.

3.4. - Contraindications

The only absolute contraindication of rTMS is its application on patients who have conductive, ferromagnetic, or other magnetic-sensitive metals implanted in the head or within 30 cm to the discharging coil (e.g., implanted electrodes/stimulators, aneurysm clips or coils, stents, bullet fragments, jewelry, and hair barrettes) (238, 356), due to the risk of inducing malfunctioning of such implanted devices (238). It is also not advisable the application rTMS to patients who have a personal history of syncope and seizure (238, 356). In addition to this, there are other conditions where there is not enough knowledge to be able to say that the application of rTMS is safe, such as pediatric patients and on pregnancy (238, 356). Nevertheless, a conservative view of the use of rTMS in pregnancy might consider to balancing the risk/benefit ratio for each single case (238, 356).

3.5. - Side effects

Although rTMS is considered a safe method for brain stimulation by several medical organizations (228, 235-238), during the application of rTMS some side effects can occur, the most common are transient head or scalp discomfort at or around the location where pulses are applied, acoustic trauma, and seizures (237, 357).

Headache and neck pain are the most commonly reported side effects in an estimated 20–40% of subjects undergoing rTMS (238, 358), particularly in the beginning when there has been no accommodation to the tapping sensation created by the stimulus (237, 357). These side effects are associated to muscle tension, generated by the stimulation itself or by the posture assumed during longer protocols (358). It is also important to note that rTMS does not increase migraine headache risk in healthy participants or those with a history of migraine (237, 357).

During discharge, the rTMS coil produces a deceptively loud clicking noise, which exceeds the recommended safety levels for the auditory system (238, 359). Although, seemingly innocuous the repeated exposure to this intense sound can lead to acoustic trauma (238, 359). In order to prevent these, it is recommended that patients and operators wear earplugs during the full duration of treatment (238, 359).

The induction of seizures is exceedingly rare (237, 238, 356, 360). However, these cases were associated with HF-rTMS and short interval periods between trains of stimulation, particularly on motor cortex or adjacent brain areas with spread of neuronal excitation to motor cortex. It is estimated to occur in 1/30,000 treatments (237, 238, 356, 360), being advised that all programs administering rTMS should have a documented plan for managing seizures (237, 238, 357).

Patients may experience twitching or movements on the extremities coordinated by the areas where stimulation trains are applied, being these associated to the excitation of superficial nerve branches and contraction of superficial muscle groups (237, 357).

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Chapter II

Aims

Aims

The major objective of this work was to explore potential approaches to induce protection and/or the recovery of the cerebral tissues affected by the ischemic injury. To achieve this goal we evaluated the potential beneficial effects induced by GPER selective activation and by HF-rMS, two different approaches that have already been associated to improvements in several ischemic-induced detrimental mechanisms, and on other neurodegenerative disorders such as PD and AD.

To accomplish this major objective several *in vitro* models of ischemic injury were developed and characterized in order to establish controlled and standardized reaction to the ischemic injury (chapter III), with an extent of lesion adequate to study protective and recovery effects. The establishment of these models allowed to analyze the impact that any approach or stimulus has on the ischemic injury or on a particular cell population. The first approach focused on the selective activation of GPER has a potential therapeutic strategy to induce neuroprotection after ischemia, and relies on its ability to mimic the effects of E2 in the brain, without the feminizing effects associated to classical estrogen receptors. GPER was pharmacologically modulated with its agonist G1 and antagonist G15, and several markers related to cell survival and proliferation were assessed. Evaluation of the contribution of two pro-apoptotic pathways triggered by GPER, the PLC and JNK pathways, allowed identifying cell type-specific effect triggered by GPER activation on neurons and astrocytes. These results are present on Chapter IV.

The contribution of HF-rMS to the potential cellular and molecular beneficial effects on the ischemic-induced injury is presented on chapter V. rTMS has been widely used in the treatment of depression and the body of evidences on its neuroprotective effects on other neurological disorders are increasing. The underlying cellular and molecular effects of rTMS are not well characterized, the most accepted theory indicates that this effect is mediated by the modulation of synaptic plasticity, but evidences also point to the modulation of other mechanisms or pathways such as neurotransmission, gene expression, neuroprotection, neurogenesis, and inhibition of cell death. Based on several HF-rTMS protocols that have already been used in human trials an HF-rMS was developed and its effects on ischemia-induced injury were assessed, through the evaluation of several proliferative and survival markers, as well as analysis of neurite morphology and synaptic modifications.

Chapter III

Impact of astrocytes on the injury induced by *in vitro* ischemia

Chapter III

Impact of astrocytes on the injury induced by *in vitro* ischemia

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Abstract:

Cell cultures are characterized by its simplicity, controllability and ability to provide detailed basic information on how a particular cell population responds to specific stimuli or insult. These characteristics led to their extensive application in the study of molecular interactions, and represent a valuable tool in the study of different pathologies. However, due to the lack of interactions between the different components that form an *in vivo* system, the results obtained in pure cell cultures not always translate what occurs in vivo. In this context, the use of mixed-cultures has the advantage of allowing the study of interactions between different types of cells present in a tissue, which in many situations are determinant for the effects obtained.

The present study aimed to characterize cortical neuron-glia and neuron-enriched primary cultures and evaluate their response to an ischemic insult. Cell viability was assessed by the MTT assay and cell number/phenotype was analyzed by immunocytochemistry in control cultures and in cells subjected to 4 hours of OGD. The results obtained demonstrate that astrocytes have a substantial impact on the injury induced by an ischemic insult, thus suggesting that the crosstalk between glia and neurons is crucial to the neuronal protection in conditions of ischemia.

Keywords: astrocytes; ischemia; neuron-glia cultures; neuron-enriched cultures; oxygen and glucose deprivation; microglia.

1. – State of the art

IS is characterized by interruption of the blood supply to a specific part of the brain (1-3), even brief ischemic periods can initiate a complex sequence of events that ultimately culminate in cellular death (2). The pathophysiology of IS is complex and involves several detrimental pathways such as excitotoxicity, oxidative stress, inflammation and endothelial injury, activation of glial cells, disruption of the BBB and infiltration of leukocytes (2, 4). Due to this complexity the study of IS has been made through the combination of several *in vivo* and *in vitro* stroke models. While *in vivo* models enable the study of interactions of all components present in the nervous system as a whole, the use of *in vitro* models allows the study of molecular interactions occurring at tissue level (5). The main advantages of cellular models are the immediate and direct access to the extracellular compartment due to the lack of BBB, direct control of the environment and easiness of using cellular models for quantitative pharmacology, electrophysiology, and imaging studies (6). The application of cellular models provides a simple and highly controlled experimental system that allows detailed basic information on how the system or one particular cell population is affected by a certain stimulus/insult (2). Primary cell cultures are established by the dissociation of original tissues (5), and constitute valuable tools to study the interactions between cell populations (7). The most widely used models to study ischemia-induced injury *in vitro* are organotypic brain slice cultures and primary cultures from cortex, hippocampus and cerebellum of embryonic or perinatal rats and mice (2, 6). In these models the most used approach to induce the ischemic injury is the OGD model (2, 8).

The human cortex is formed by two major cell populations, neurons and glial cells, present in similar amounts (9), and establishing complex interactions (4, 10). Neurons, classically considered the most important cells of CNS, play a crucial role on every system of the human body (11). Glial cells, in turn, provide structural, metabolic and trophic support to neurons (10, 12-14). By maintaining a strait crosstalk with neurons, glial cells control processes such as homeostasis, defense against pathogens and inflammatory responses and synaptic regulation (15, 16). Therefore, neuron-glia cultures represent a valuable tool to explore mechanisms that are regulated or depend on the interaction between neurons and glial cells. Whereas the use of neuron-enriched cultures allows assessing how neurons are affected by an ischemic insult and analyzing the mechanisms involved in their survival/protection.

In the present chapter, primary neuron-glia and neuron-enriched cortical cultures were characterized by immunocytochemistry and the effect of OGD on neuron viability was assessed in both types of cultures. The results obtained demonstrate that astrocytes have a substantial impact in the injury induced by an ischemic insult.

2. – Material and methods

2.1. - Cell Cultures

Primary cortical cultures were prepared from cerebral cortices of 15-day-old Wistar rat embryos. The animals were bred in the animal house of CICS-UBI Health Science Research Center, with free access to water and pellet food, under standard humidity and temperature conditions, at a 12 h light-dark cycle. The colony was raised from Wistar Han IGS animals purchased from Charles River. Females (220-260g) were housed in groups of four, in individually ventilated cages. All procedures were performed 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).

Briefly, pregnant females were anesthetized with ketamine (87.5 mg/Kg, Sigma-Aldrich, catalog number: K-002) and xylazine (12 mg/Kg, Sigma-Aldrich, catalog number: X1126). The abdominal cavity was opened, and the embryos removed. After this procedure, the females were immediately euthanized by exsanguination trough a cut in the aorta. The embryos were placed on a Petri dish with cold phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8.1 mM Na_2HPO_4, pH 7.4). After euthanizing the embryos by decapitation the cerebral, the cortices were isolated and the meninges removed by a method previously described (17). The cortices were chopped into small pieces and pooled together in PBS. Tissue was dissociated mechanically and centrifuged at 400 x g for 3 minutes and the pellet resuspended in Neurobasal medium (NBM, Gibco, catalog number: 21103049) supplemented with 2% B27 (Gibco, catalog number: 17504044), 0.5 mM glutamate (Sigma-Aldrich, catalog number: G8415), 0.5 mM glutamine (Sigma-Aldrich, catalog number: G3126) and 120 µg/ml gentamicin (Sigma-Aldrich, catalog number: G1272).

For neuron-glia cortical cultures, cells were cultured in NBM supplemented with 2% B27, 0.5 mM glutamate, 0.5 mM glutamine, 120 µg/ml gentamicin and 10% heat-inactivated fetal bovine serum (FBS) at a density of 0.14 x 10^6 cells/cm^2 on a 24 well culture plates (Orange) coated with poly-D-lysine (Sigma-Aldrich, catalog number: P6148). For neuron-enriched cortical cultures, cells were cultured in NBM supplemented with 2% B27, 0.5 mM glutamate, 0.5 mM glutamine and 120 µg/ml gentamicin at a density of 0.21 x 10^6 cells/cm^2, also on 24 well culture plates coated with poly-D-lysine. The presence of FBS allows glial cells to proliferate and differentiate. Cell density was defined based on pilot experiments aimed at obtaining similar cell number in neuron-enriched and neuron-glia cultures at DIV 6. The cells were maintained in a 5% humidified CO2 incubator at 37ºC. After five days in culture, the medium was renewed. All experiments started on the 6th day in culture (DIV).

2.2. – OGD and reperfusion

To induce OGD, cells were washed twice in HBSS (1.26 mM $CaCl_2$, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.49 mM $MgCl_2$, 139.9 mM NaCl, 4.17 mM $NaHCO_3$, 3.38 mM Na_2HPO_4, pH 7.4). Cells were then placed on an airtight hypoxia incubation chamber (Stemcell Technologies, catalog number: 27310). The chamber was initially flushed with 20 L/min of a 95% N_2 and 5% CO_2 gas mixture, for 4 minutes, and then sealed and placed in an incubator at 37°C. Cells were maintained under OGD for a previously stablished period of time (4 hours). At the end of the OGD period, the culture plates were removed from the chamber, HBSS medium was replaced by NBM and cells were incubated for further 20 hours under normoxic conditions. For control conditions, cells were washed twice and incubated with HBSS supplemented with 5.56 mM glucose (Sigma-Aldrich, catalog number: G5767) and placed in a 5% humidified CO_2 incubator for the same period as OGD conditions. The HBSS medium was then replaced by NBM and cells were incubated for further 20 hours in a 5% humidified CO_2 incubator.

2.3. – Immunocytochemistry assays

The cells used in the immunocytochemistry assays were cultured on plates containing coverslips previously coated with poly-D-lysine. This was followed by permeabilization with 1% Triton X-100 (Sigma-Aldrich, catalog number: T9284) in PBS for 5 minutes. Non-specific binding was reduced by incubating the cells with 20% FBS in PBS with 0.1% Tween (PBS-T) for 60 minutes at room temperature. To characterize both types of cortical cultures the cells were incubated overnight, at 4ºC, with the antibodies specified in table 5, diluted in PBS-T with 1% FBS.

Table 5: Primary antibodies used on immunocytochemistry assays of impact of astrocytes on the injury induced by in vitro ischemia.

Antibody	Specie	Dilution	Company	Catalog number
Anti-MAP2	Mouse	1:500	Santa Cruz Biotechnology	Sc-74421
Anti-GFAP	Rabbit	1:2000	DAKO	Z0334
Anti-Iba1	Rabbit	1:2000	WAKO	019-19741

The cells were then washed six times with PBS-T and incubated for 1 hour, at room temperature, with the corresponding secondary antibodies, specified in table 6, also diluted in PBS-T with 1% FBS.

Table 6: Secondary antibodies used on Immunocytochemistry assays of impact of astrocytes on the injury induced by in vitro ischemia.

Antibody	Conjugated	Dilution	Company	Catalog number
Anti-rabbit	Alexa Fluor 488	1:1000	Invitrogen	A11034
Anti-mouse	Alexa Fluor 546	1:1000	Invitrogen	A11002

After incubation with secondary antibodies, the cells were washed six times with PBS-T, incubated for 10 min with 2mM Hoechst 33342 (Invitrogen) and washed three times with PBS-T. Finally, coverslips were mounted in fluorescence mounting medium (DAKO). Images were acquired on an epifluorescence microscope (AxioObserver Z1, Zeiss) with a 63x objective for MAP2 and GFAP labelling and with a 40x objective for Iba1 labeling.

2.4. – Cell counting

For quantification of cell number, we performed at least three experiments with different cellular preparations. In each experiment, 3 coverslips/experimental condition and 20 fields/coverslip were analyzed. The cell number/condition was assessed based on the number of nuclei stained with Hoechst 33342. The number of neurons was quantified by assessing the number of cells expressing the neuronal marker MAP2 and the number of astrocytic cells was determined by the number of cells expressing the marker GFAP. Nuclei stained with Hoechst and not labeled for the neuronal marker MAP2 or the astrocytic marker GFAP were assessed separately. The number of microglial cells was quantified by analyzing the cells that express the marker Iba1.

2.5. – Cell viability assessment

Culture viability was assessed through the thiazolyl blue tetrazolium assay (MTT; (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, catalog number: M2128). The culture medium was removed and replaced with MTT solution (0.5 mg/mL), followed by incubation for 1 hour at 37°C. The MTT solution was removed and the formazan crystals were solubilized with acid-isopropanol (0.04 M HCl in isopropanol). Optical density was measured with a spectrophotometer (xMark™ Microplate Spectrophotometer, Bio-Rad, catalog number: 1681150) at 570 nm with background subtraction at 620 nm.

2.6. – Statistical analysis

The results are expressed as the number of cells or as a percentage of values obtained in control conditions and are presented as the mean ± standard error of the mean of at least 3 independent experiments, performed in triplicate. Statistical analysis was performed using the Student's t-test. Values of $P<0.05$ were considered significant. All statistical procedures were performed using GraphPad v.4 (GraphPad Software Inc., San Diego, CA).

3. – Results

3.1. - Characterization of neuron-glia and neuron-enriched cortical cultures

Primary cultures from cortex have been widely used to study mechanisms underlying several brain disorders (2). However, the characterization of these cultures is often absent. Therefore, we first characterized our cell models by immunocytochemistry. In cortical neuron-glia cultures 54 ± 2% of cells expressed the neuronal marker MAP2, 34 ± 3% of cells expressed the astrocytic marker GFAP and only 0.7 ± 0.3% of cells expressed the microglial marker Iba1. Surprisingly, 13 ± 2% of the cells present in the culture were not labeled for either MAP2 or GFAP (Fig. 3A). In neuron-enriched cultures, and as expected, the majority of cells (75 ± 2%) expressed MAP2 and only 3 ± 1% of the cells expressed the astrocytic marker GFAP. Similarly, to the neuron-glia cultures a high percentage of the cells (24 ± 1%) were not positive for either MAP2 or GFAP (Fig. 3A). According to the immunocytochemistry, analysis at the 6th day in culture neuron-glia cultures presented 18 ± 1 cells/field, and neuron-enriched cultures had 17± 1 cells/field. These data indicate that on the day of OGD both types of cultures presented a similar cell number/density (P =0.7598; Student's t-test).

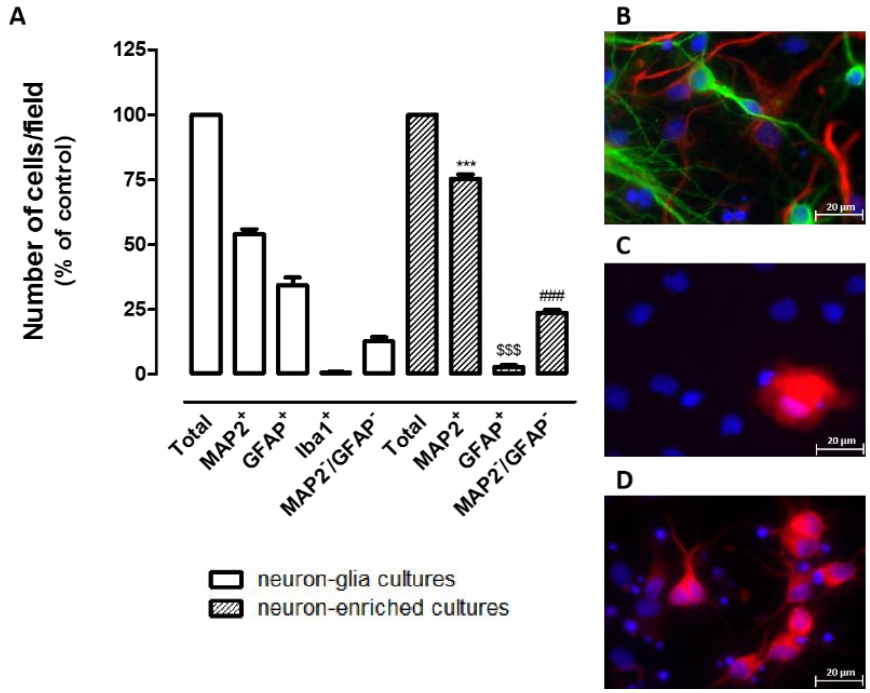

Figure 3: Characterization of neuron-glia cortical cultures and neuron-enriched cortical cultures. (A) The data are presented as percentage of neurons (MAP2$^+$), percentage of glial cells (GFAP$^+$), percentage of microglial cells (Iba1$^+$) and percentage of double negative cells (MAP2$^-$/GFAP$^-$) in culture at the 6th day in culture, and represent the mean ± SEM of 3 independent cell culture preparations performed in triplicate. The total number of cells was assessed by quantifying Hoechst 33342 labeled nuclei. Representative images show the immunostaining for MAP2 (red) and GFAP (green) **(B)**, and for Iba1 (red) **(C)** in neuron-glia cortical cultures. **(D)** Representative image showing the immunostaining for MAP2 (red) in a neuron-enriched cortical culture. Images were obtained with a 63x objective. Statistical analysis was performed using the Student's t-test. ***P<0.001 compared to MAP2$^+$cells on neuron-glia cultures, $^{\$\$\$}$P<0.001 compared to GFAP$^+$cells on neuron-glia cultures; $^{\#\#\#}$P<0.001 compared to MAP2$^-$/GFAP$^-$ cells on neuron-glia cultures.

3.2. - The presence of astrocytes influences the extent of OGD-induced injury in cortical cultures

In order to evaluate the effects induced by ischemia neuron-glia and neuron-enriched cultures were subjected to 4 hours of OGD, and cell viability was assessed by the MTT assay and the number of surviving neurons, astrocytes and microglial cells was assessed by immunocytochemistry. Exposure of neuron-glia cultures to 4 hours of OGD did not have a significant impact on cell survival. OGD led to a decrease in cell number quantified by immunocytochemistry of only 9 ± 4%, and a decrease in MTT reduction of 3.8 ± 1.7%, when compared to control. Astrocytes and neurons were affected by OGD to a similar extent (Fig. 4A). Although microglial cells strongly responded to OGD by increasing its number in the culture to 170 ± 25% of control (Fig. 4B), the number of these cells in control conditions was very low (0.7 ± 0.3%), and even after OGD microglial cells represented only 1.4 ± 0.5% of the cells present in the culture.

In contrast to the reduced impact of OGD in the viability of cells in neuron-glia cultures the results obtained in neuron-enriched cultures indicated that OGD lead to a significant reduction in cell number (30 ± 4% reduction) as assessed by immunocytochemistry (Fig. 4D). This reduction was paralleled by a decrease in MTT reduction (22.6 ± 6.6% decrease when compared to control, Fig. 4E). These results indicate that under the same OGD conditions neuron-glia cultures are more resistant to ischemia than neuron-enriched cultures, thus supporting a protective role of glial cells during ischemia.

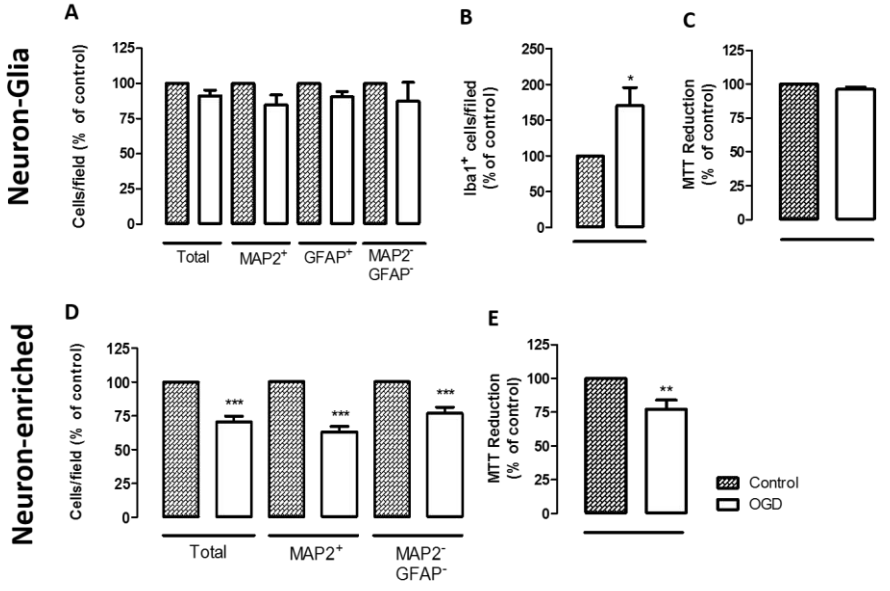

Figure 4: Effect of OGD on neuron-glia and neuron-enriched cortical cultures. Survival of neurons, astrocytes and microglia in neuron-glia (**A and B**) and neuron-enriched cultures (**D**) exposed to 4 hours of OGD. Viability analysis through the MTT assay on neuron-glia (**C**) and neuron-enriched cultures (**E**). The results are expressed as percentage of control and represent the mean ± SEM of 3 independent cell culture preparations performed in triplicate. Statistical analysis was performed using the Student's t-test. *P<0.05 compared to control, **P<0.01 compared to control and ***P<0.001 compared to control.

4. – Discussion

Cell cultures are a valuable tool to study complex cellular mechanisms at tissue level (5). These systems allow manipulations and approaches that are not possible in *in vivo* models. *In vivo* the response to a certain insult is strongly dependent on the interaction between the different components of tissues and organs. When an *in vitro* stroke model is used it is important to take into consideration the contribution of crosstalk between neurons and glial cells. In the present study we characterized the cellular composition of neuron-glia and neuron-enriched cultures prepared from embryonic cortices and analyzed their response to an ischemic insult.

Since animal tissues are formed by different populations of cells, it is necessary to separate the cell population of interest from the others and to determine the composition of the resulting cultures. In the case of neuron-enriched cultures, it is necessary to isolate neurons, as much as possible, from glial cells (18). In this study, we provide a valuable isolation and culture method to obtain neuron-enriched cultures with a contamination of glial cells lower than 4%. The establishment of neuronal cultures with a low percentage of astrocytes is crucial to clearly evaluate the impact of these cells on neuronal survival, and to enable comparison of data in the literature.

In the present study, we observed that in control conditions there was a small percentage of microglial cells. This reduced number can be explained by the age of the embryos used to prepare the cultures (15 days of gestation). According to the literature, microglia migrates and starts to expand and colonize the CNS around embryonic day 14 (19). Although at embryonic day 15 microglial cells are already in the brain, their expansion is still reduced. When we analyzed the effects of an ischemic insult on microglial cells, we found that despite their residual presence in the culture they react to OGD by increasing their number/percentage by approximately twofold, which is in line with the inflammatory response triggered by the neuronal lesion induced by OGD (20).

Both cortical culture types presented a substantial number of cells that did not express either the neuronal marker MAP2 or the astrocytic marker GFAP. MAP2 is a major component of the neuronal cytoskeleton (21, 22). The expression levels of MAP2 normally increase with neuronal maturity (23). The number of MAP2-immunopositive cells increases with time in culture and with the age of the embryos from which the cultures are prepared (21). Published data indicate that at early stages of neural development the expression of MAP2 can be so low that it is not detectable by immunocytochemistry (21). Immunohistochemically, GFAP was found to be associated with reactive astrocytes that respond to CNS injuries in pathological contexts and it became a prototypical marker for immunohistochemical/immunocytochemical identification of glial cells, particularly, astrocytes (24, 25). However, GFAP is not an absolute marker of all non-reactive astrocytes and is often not detectable in astrocytes of healthy CNS

tissue (25). Double staining with multiple glial markers demonstrated that some astrocytes do not express detectable levels of GFAP, and that GFAP expression exhibits both regional and local variability that is dynamically regulated by a large number of inter- and intra-cellular signaling molecules (25, 26). Altogether, these data help to explain the presence of cells in our cultures that do not express either MAP2 or GFAP. Nevertheless, it is noteworthy that, although not labeled for MAP2, these double negative cells behave similarly to neurons when exposed to ischemic injury, thus suggesting that they are in fact neurons.

Here, we provide evidence that with the described culture settings both neuron-enriched and neuron-glia cultures present approximately the same number of cells at the day of OGD exposure. This is especially relevant because *in vitro* stroke models are deeply affected by the total number of cells in culture due to the impact of cell density in the consumption of oxygen and nutrients during ischemia. Thus, this is an essential parameter to consider in order to ensure that the conclusions reached in comparative studies like ours are valid, and might be a limiting factor for direct comparisons between different studies.

Our results indicate that neurons are more susceptible to an ischemic insult than glial cells, which is in accordance to what was previously described in several *in vitro* stroke models (10, 27-30). Possibly related with this, many *in vitro* studies on ischemic injury have a neurocentric approach focusing only on the effects of ischemic insult in neurons and forgetting, in most cases, the role of glial cells (3, 11). Remarkably, the present study proves that, in fact, the presence of glial cells has a strong impact on neuronal viability upon an ischemic injury. In a study using hippocampal cultures Jones and colleagues (2011) also reported that neuron-enriched cultures are more susceptible to the ischemic insult than neuron-glia cultures (30). However, it should be noticed that, in contrast to the 3% reported in the present study, in the work by Jones and colleagues the neuron-enriched cultures exhibited a rather high contamination with astrocytes (24% of astrocytes), which could interfere substantially with the results. On the other hand, knowing the impact that glial cells have on the injury induced by ischemia it is expectable that the ratio glial cells/neurons will be determinant to the response triggered upon ischemia, and any factor that modifies this ratio will influence the final results. Among the main factors that can influence it are: 1) the developmental stage of embryos, 2) the duration of culture, 3) the composition of the culture medium, and 4) medium supplements such as FBS.

The present results led us to hypothesize that glial cells have the ability to secrete molecules that help neurons to survive during ischemia. In fact, our preliminary results indicate that the protection of neurons exerted by astrocytes does not require its physical presence since astrocyte-conditioned media effectively protected neuron-enriched cultures from ischemia-induced lesion. This protection can be mediated by molecules known to be secreted by astrocytes, such as neurotrophic factors and antioxidant molecules (e.g. glutathione), which protective role is well established (31-33).

The ability to limit excitotoxicity by promoting glutamate and K^+ uptake (34-37), to provide energetic support in the form of lactate (38, 39), or to ensure synapse maintenance (40) are some examples of mechanisms pointed out as mediators of the protective effects of astrocytes in cerebral ischemia (10, 41). Although these characteristics make astrocytes an interesting therapeutic target, neuroprotective or neuroreparative strategies that target these cells have been scarcely explored.

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Chapter IV

G protein-coupled estrogen receptor activates cell type specific signaling pathways in cortical cultures: relevance to the selective loss of astrocytes

Chapter IV

G protein-coupled estrogen receptor activates cell type specific signaling pathways in cortical cultures: relevance to the selective loss of astrocytes

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Abstract:

Selective activation of the G protein–coupled estrogen receptor has been proposed to avoid some of the side effects elicited by the activation of classical estrogen receptors α and ß. Although its contribution to neuroprotection triggered by estradiol in brain disorders has been explored, the results regarding ischemic stroke are contradictory, and currently there is no consensus on the role that this receptor may play.

The present study aimed to investigate the role of GPER in the ischemic insult. For that, primary cortical cultures exposed to OGD were used as a model. Our results demonstrate that neuronal survival was strongly affected by the ischemic insult and concurrent GPER activation with G1 had no further impact. In contrast, OGD had a smaller impact on astrocytes survival but G1, alone or combined with OGD, promoted their apoptosis, effect prevented by the GPER antagonist G15. The results also show that ischemia did not change the expression levels of GPER in neurons and astrocytes. In this study, we also demonstrate that selective activation of GPER induced astrocyte apoptosis via the phospholipase C pathway and subsequent intracellular calcium rise, whereas in neurons this effect was not observed. Taken together, this evidence supports a direct impact of GPER activity on the viability of astrocytes, which seems to be associated with the regulation of different signaling pathways in astrocytes and neurons.

Keywords: Ischemia; Astrocytes; GPER; Intracellular calcium; G1.

1. – State of the art

The G protein–coupled estrogen receptor (GPER or GPR30) was first identified in the late 90s (1-3) and was described as an orphan receptor belonging to the family of 7-transmembrane spanning G protein-coupled receptors (4, 5). Filardo and colleagues (2000) demonstrated that E2-mediated activation of ERK1/2 was dependent on the expression of this receptor and named it GPR30 (6). In 2005, Revankar and colleagues (7) and Thomas and colleagues (8) described the binding of E2 to GPR30, confirming that this receptor is an E2-binding receptor, which lead to the designation GPER in 2007 (9).

The expression of this estrogen receptor is not restricted to estrogen responsive tissues (10). In fact, the presence of this receptor was reported in male and female reproductive heart, intestine, ovary, pancreatic islets, adipose tissue and nervous systems (10-15). Indeed, several reports demonstrated that GPER is expressed throughout the CNS and PNS of male and female rodents. These reports described the presence of GPER in almost all anatomical locations of forebrain (11-17), midbrain (14), hindbrain (11, 14), spinal cord and autonomic ganglia and sensory ganglia (18). Particularly, in the cortex, it was demonstrated that GPER is expressed in neurons (13) and astrocytes (19).

In contrast to ERα and ERβ, which mediate genomic effects characterized by changes in gene transcription occurring in the time frame of hours to days (20), GPER mediates rapid non-genomic effects that occur in seconds or minutes (20, 21). These are dependent on ion channels (22, 23) and involve the regulation of kinases such as PI3K (24, 25), PKCε or MAPK (26, 27), cAMP production (21, 28) and intracellular calcium mobilization (7, 24, 28, 29). Additionally, the rapid signaling events initiated by GPER upregulate the expression of genes such as c-fos (30), cyclin D2 and Bcl-2 (31).

The endogenous agonist of GPER is E2, a molecule known to regulate multiple processes in the brain, such as learning, memory, cognition and mood, as well as neurodevelopmental and neuroprotective processes (32-36). The identification of the first GPER-selective agonist G1 (37), and the GPER-selective antagonist G15 (38) lead to a strong increase in the number of studies focusing on the role of this receptor in different physiological systems and pathological conditions. Since then, several reports point to effects induced by the modulation of GPER in brain disorders (9, 39), such as PD (40) or IS(41).

IS is characterized by the interruption of the blood supply to the brain frequently due to the blockade of a blood vessel by a clot. Currently, it is one of the leading causes of death worldwide and the leading cause of adult disability in industrialized countries. The incidence of IS is higher in males than in females (42-44). These sex differences, present in many other brain disorders (32, 34, 43, 45, 46), have been attributed, in part, to the higher serum levels of E2 in women (34, 46). In fact, it was demonstrated that E2 induces neuroprotective effects

through the increase of neurogenesis and reduction of cell apoptosis (47, 48), which, in turn, lead to behavioral recovery (49). The neuroprotective role of E2 is usually ascribed to the activation of the classical ER. However, the identification of GPER raised the hypothesis that some of the effects triggered by E2 could result from GPER activation. In the case of ischemia evidence is not consensual, being GPER activation described either as beneficial (50, 51), detrimental (41), or with different effects depending on the sex (41).

The present chapter aimed to investigate the role of GPER in cultured cortical neurons and glial cells exposed to ischemic conditions. To analyze the role of GPER in the ischemic injury its activity was pharmacologically modulated with its agonist G1 and the antagonist G15. In addition, the pattern of expression and the contribution of GPER activation to cell death induced by ischemic conditions were analyzed in both neurons and glial cells.

2. – Material and methods

2.1. - Cell Cultures

Primary neuron-glia and neuron-enriched cortical cultures were prepared as described on chapter III, section 2.1. For astrocytes-enriched cortical cultures, cells were cultured in supplemented NBM plus 10% FBS at a density of 0.21 x 10^6 cells/cm^2. To remove neurons and obtain an astrocyte-enriched culture, at DIV 4 the dishes were placed on a plate shaker (Grant-bio Orbital Shaking Platform, catalog number: POS-300) in the incubator and shaken at 110 rpm for 6 hours. After shaking, the medium was replaced to remove neurons and cellular debris as previously described (52). Cells were plated on poly-D-lysine (coated 24-well culture plates (Thermo Fischer Scientific, catalog number: 142475), except for calcium imaging (12 mm glass-bottom dishes, Thermo Fischer Scientific, catalog number: 150680). The cells were maintained in a 5% humidified CO_2 incubator at 37°C. After 4 DIV the medium was renewed. All experiments started at DIV 6.

2.2. – Cell culture treatments

Twenty-four hours before exposure to OGD the culture medium was replaced by serum-free NBM and the cultures were incubated with 100 or 200 nM G1 (Tocris Bioscience, catalog number: 3577/10), 100 nM G15 (Calbiochem, catalog number: 271703), 100 nM E2 (Calbiochem, catalog number: 3301) or E2 plus G15 until the end of the reperfusion period. To test the effect of the inhibition of PLC pathway cells were treated with 10 nM U73122(53) (Sigma-Aldrich, catalog number: U6756) and to inhibit the JNK pathway we used 10 μM SP600125(54) (Sigma-Aldrich, catalog number: S5567). Drugs were added 30 minutes prior to the addition of G1 and incubated for further 24 hours.

2.3. – OGD and reperfusion

OGD and reperfusion procedure was realized according to what was described on chapter III, section 2.2.

2.4. – Cell viability assessment

Cell viability assessment was realized according to what was described on chapter III, section 2.5.

2.5. – Immunocytochemistry assays

Immunocytochemistry protocols were realized as described on chapter III, section 2.3, the antibodies specified in tables 7 and 8.

Table 7: Primary antibodies used on Immunocytochemistry assays to evaluate the effects induced by GPER selective activation.

Antibody	Species	Dilution	Company	Catalog number
Anti-GPER	Rabbit	1:200	Santa Cruz Biotechnology	Sc-48525
Anti-MAP2	Mouse	1:500	Santa Cruz Biotechnology	Sc-74421
Anti-GFAP	Rabbit	1:2000	DAKO	Z0334

Table 8: Secondary antibodies used on Immunocytochemistry assays to evaluate the effects induced by GPER selective activation.

Antibody	Conjugated	Dilution	Company	Catalog number
Anti-rabbit	Alexa Fluor 546	1:1000	Invitrogen	A11010
Anti-mouse	Alexa Fluor 488	1:1000	Invitrogen	A11001
Anti-mouse	Alexa Fluor 546	1:1000	Invitrogen	A11002

2.6. – Cell counting

The quantification of cells was performed as described on Chapter III, section 2.5.

2.7. – Quantification of GPER expression

Analyses of fluorescence intensity were performed with the ImageJ software (National Institutes of Health). The cells of interest were delineated and the mean fluorescence intensity (MFI) of the cell was measured. After this, a region without fluorescence was selected and used for background reading. The MFI of each cell was calculated using the formula: MFI = mean density of selected area - mean intensity of background reading.

The intensity of GPER staining was quantified in neurons and astrocytes in control and OGD conditions. For this quantification, experiments were performed in at least 3 distinct cellular preparations. In each experiment, 3 coverslips/condition were prepared and 30 cells of each cell population were randomly selected on each coverslip.

2.8. – Calcium imaging

Neuronal and astrocytic enriched-cultures were incubated with 5 μM Fura-2/AM (Thermo Fischer Scientific, catalog number: F1221) in sodium buffered solution (140 mM NaCl, 5 m MKCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 20 mM HEPES, pH 7.35) for 45 minutes at 37°C. The medium was then replaced by sodium buffered solution followed by further 45 minutes incubation at 37°C to allow complete de-esterification of the probe. After that, the glass-bottom dishes were mounted on the stage of an inverted microscope (Zeiss, AxioObserver Z1) and Fura-2 was alternately excited at 340 and 380 nm and the emitted fluorescence, filtered at 510 nm, and was collected every 10 seconds. This protocol included a 3 minutes period to establish the baseline values, exposure to 100 nM G1 for 10 minutes followed by incubation with 50 m MKCl buffered solution to induce depolarization (145 m MKCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 20 mM HEPES, pH 7.35) for further 10 minutes. To test the effect of blockade of the PLC pathway, U73122 (10 nM) was added to the cultures 30 minutes before incubation with G1.

The collected images for each wavelength were transformed into time-lapse calcium measurements and treated using the ImageJ software. The region of interest (ROI) tool was used to delineate the cells and the 340/380 nm ratio of Fura-2/AM intensity was calculated and representative traces of F340/F380 ratio were drawn with GraphPad Prism Software v.4 (GraphPad Software Inc.; San Diego; CA). To evaluate the intracellular calcium, experiments were performed in 3 distinct cellular preparations. In each experiment, 3 coverslips/condition were prepared and approximately 25 cells/coverslip were analyzed.

2.9. – Statistical analysis

The results are expressed as number of cells, the percentage of values obtained in control conditions, the representative traces of F340/F380 ratio or as the maximal ratio values of F340/F380 in each stimulus (G1 and KCl) and are presented as the mean ± SEM of at least 3 independent cell culture preparations, performed in triplicate. For the identification of outliers was used the Grubb´s method.

Statistical analysis was performed using the Student's t-test and one- or two-way ANOVA as specified in the figure legends, followed by Bonferroni´s post hoc test. Values of $P<0.05$ were considered significant. All statistical analyses were performed using the GraphPad Prism Software. No statistical methods were employed to predetermine sample size of any of the presented experiments and no tests for normal distribution were performed.

3. – Results

3.1. - GPER activation does not protect from an injury induced by OGD

It has been shown that estrogens, particularly E2, can act as neuroprotective agents in ischemic insult (34). In order to determine whether this protection may involve the activation of GPER primary neuron-glia cortical cultures were exposed to G1 (100 and 200 nM) (37) 24 hours prior to the OGD period. Cell viability was assessed by using the MTT assay and the number of cells was analyzed by immunocytochemistry. The exposure of primary neuron-glia cortical cultures to 4 hours of OGD decreased MTT reduction by 36.9 ± 6.8%, when compared to control cultures (Fig. 5A), as well as the total number of cells from 29 ± 2 cells/field to 17 ± 1 cells/field (41.9% decrease, Fig. 5B). On the other hand, the results showed that neurons were more susceptible to OGD (61.2% decrease, Fig. 5C) than astrocytes (36.2% decrease, Fig. 5D). Activation of GPER with G1 did not induce any protection against the ischemic insult, either at 100 nM or 200 nM (Fig. 5A), and thus 100 nM G1 was used in all subsequent experiments. Interestingly, exposure to G1, per se, in the absence of OGD, induced a significant decrease in cell number (31.1% decrease, from 29 ± 2 cells/field to 20 ± 2 cells/field, Fig. 5B). Data from the immunocytochemistry analysis showed that this reduction was due to the loss of astrocytes and did not involve a loss of neurons (Figs. 5C and 5D).

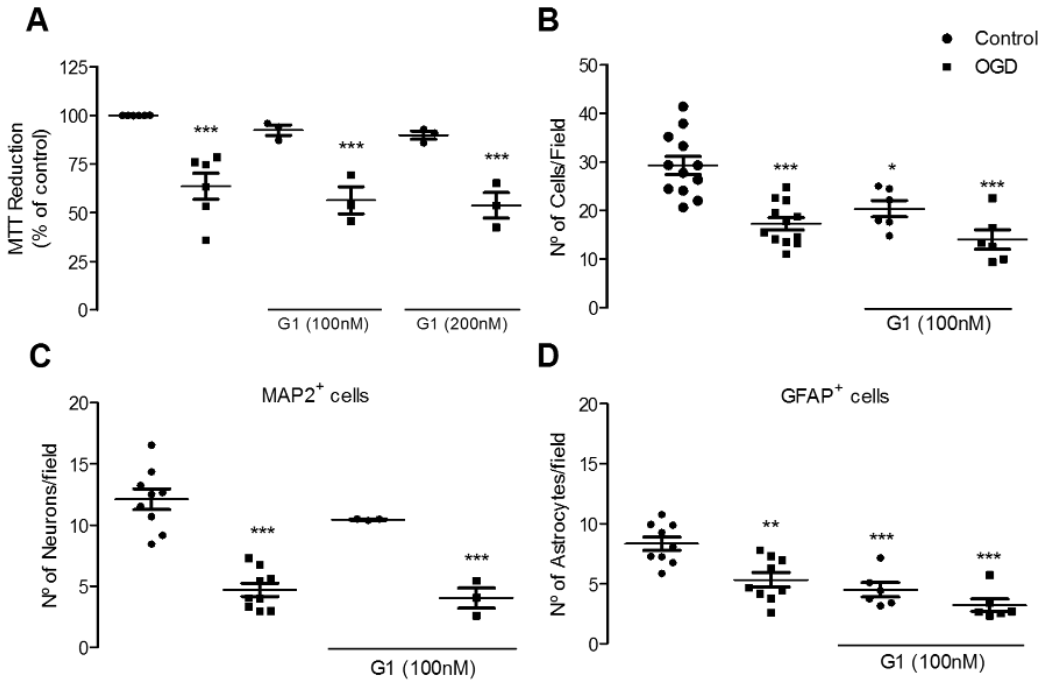

Figure 5: Effect of G1 on rat primary neuron-glia cortical cultures exposed to 4 hours of OGD. A - Evaluation of cell viability through the MTT assay. The results are expressed as percentage of control and represent the mean ± SEM of 3 independent cell culture preparations performed in quadruplicate. **B** - Evaluation of the number of cells through Hoechst 33342 staining. The results are expressed as the number of cells/field and represent the mean ± SEM of 9 (control) or 3 independent cell culture preparations (other experimental conditions) performed in triplicate. **C** - Evaluation of the number of neurons through MAP2 immunocytochemistry. **D** - Evaluation of the number of astrocytes through GFAP immunocytochemistry. The results are expressed as the number of cells/field and represent the mean ± SEM of 3-12 independent cell culture preparations performed in triplicate. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's *post hoc* test. **P*<0.05, ***P*<0.01 and ****P*<0.001 compared to control; *ns*, not significant.

3.2. - GPER blockade protects astrocytes from OGD-induced injury

To further explore the role of GPER under ischemic conditions we analyzed the effect of G15, a GPER selective antagonist (38). Incubation with 100 nM G15 increased MTT reduction in cells exposed to OGD by 14.1 ± 5.6% (Fig. 6A), and increased the cell number by 33.9% (from 17 ± 1 cells/field under OGD conditions to 23 ± 1 cells/field in OGD plus G15, Fig. 6B), suggesting that under ischemic conditions GPER blockade may elicit cell protection.

To assess the cellular targets of the protection afforded by G15 under ischemic conditions we analyzed the impact of G15 treatment on neurons ($MAP2^+$ cells) and astrocytes ($GFAP^+$ cells). Immunocytochemistry analysis showed that GPER blockade selectively protected astrocytes as treatment with G15 completely reduced $GFAP^+$ cell loss to levels similar to control conditions without altering the number of neurons (Figs. 6C and 6D).

Control experiments using 100 nM E2 showed that it improved MTT reduction in OGD-exposed cells (35% increase, Fig. 4A), and promoted a 20.6% increase in cell number (from 17 ± 1 cells/field in OGD to 21 ± 2 cells/field in OGD + E2, Fig. 6C) although, not statistically significant. The protection induced by E2 was not affected by the simultaneous presence of G15 (Fig. 6A), suggesting that E2-mediated protection did not involve GPER. Interestingly, the protective effect of E2 was specific for $GFAP^+$ cells (Fig. 6C and 6D).

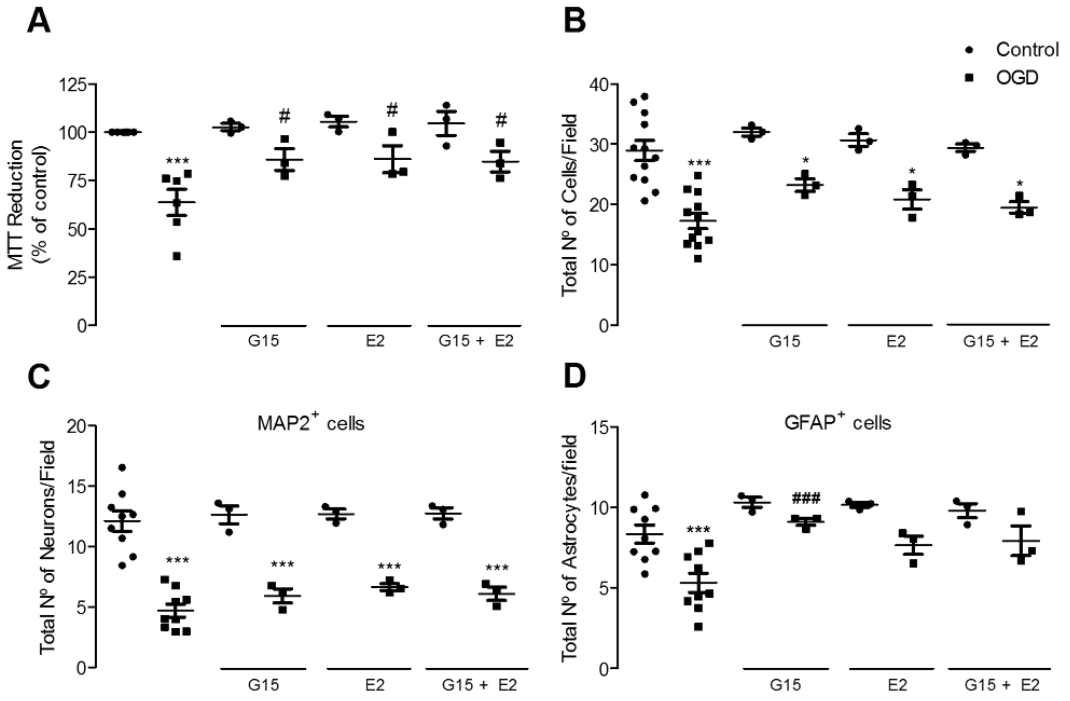

Figure 6: Effect of GPER inhibition on the viability of primary neuron-glia cortical cultures exposed to 4 hours of OGD. A - Evaluation of cell viability through the MTT assay. **B** - Evaluation of the number of cells through Hoechst 33342 staining. **C** - Evaluation of the number of neurons through MAP2 immunocytochemistry. **D** - Evaluation of the number of astrocytes. The results are expressed as the number of cells/field and represent the mean ± SEM of 3-9 independent cell culture preparations performed in triplicate. Statistical analysis was performed using the two-way ANOVA followed by Bonferroni's *post hoc* test. **P*<0.05, ****P*<0.001 compared to control; #*P*<0.05, ### *P*<0.001 compared to OGD; *ns*, not significant.

3.3. - OGD does not induce modifications in GPER expression

To determine if the distinct effects of GPER activation on cell survival in OGD-exposed cultures were related to a differential expression of the receptor in neurons and non-neuronal cells, we analyzed the intensity of GPER staining by immunocytochemistry. The results showed that GPER was expressed by both neurons and non-neuronal cells (Fig. 7A). Furthermore, quantification of fluorescence intensity showed that neurons presented higher levels of GPER staining than non-neuronal cells (Fig. 7B). Additionally, OGD did not alter the pattern of GPER staining, but there was a small, non-statistically significant, decrease in GPER immunoreactivity in non-neuronal cells levels subjected to OGD (20.6 ± 12.2% reduction of MFI, as compared to control). It is also important to note that GPER seems to have a similar expression pattern in both populations, with a more intense labeling in the perinuclear region (Fig. 7A).

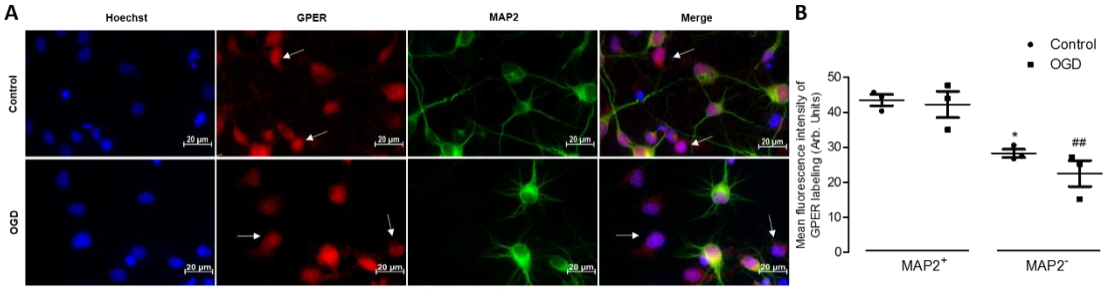

Figure 7: GPER staining in neurons and non-neuronal cells under control and ischemic conditions. A - Representative images of MAP2 (green), GPER (red) and Hoechst 33342 staining (blue) immunocytochemistry, staining magnification 63x. Arrows indicate $MAP2^-$ cells expressing GPER; **B** - Quantification of the MFI of GPER in neurons ($MAP2^+$ cells) and non-neuronal cells ($MAP2^-$ cells). Results represent the mean ±SEM of 3 independent cell culture preparations performed in triplicate. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's *post hoc* test. **P*<0.05 compared to neurons in control conditions; ##*P*<0.01 compared to neurons in OGD condition; *ns*, not significant.

3.4. - GPER activation promotes apoptosis in astrocytes

To get further insight on the effect of G1 on astrocytes and neurons we used different markers of apoptosis. As expected, exposure to OGD lead to a 75.5% increase in the number of non-astrocytic cells with nuclei presenting apoptotic morphology (Figs. 8A and 8C), a 107.6% increase in the number of non-astrocytic cells labeled for annexin V (Figs. 9A and 9C), and a 12.5-fold increase in the number of neurons labeled for activated caspase-3/7. On the contrary, exposure of non-astrocytic cells to G1 did not alter any of the apoptotic markers analyzed (Figs. 8A, 9A and 10A).

OGD promoted a significant increase in the number of astrocytes exhibiting nuclei with apoptotic morphology and a significant increase in annexin V and active caspase-3/7 labeling. In G1-treated cultures we found increased number of astrocytes with nuclei presenting apoptotic morphology (125.9% increase, Fig. 8B and 8D), labeling for annexin V (308.6% increase, Figs. 9B and 9D) and labeling for active caspase-3/7 (282.1% increase, Figs. 10B and 10D). Moreover, G1 aggravated the effect of OGD on astrocytes, with a 126.81% increase in

astrocytes presenting nuclei with apoptotic morphology, a 352.25% increase in annexin V-labeled astrocytes and a 239.7% increase in caspase-3/7-labeled astrocytes, as compared to OGD (Figs. 8B, 9B and 10B).

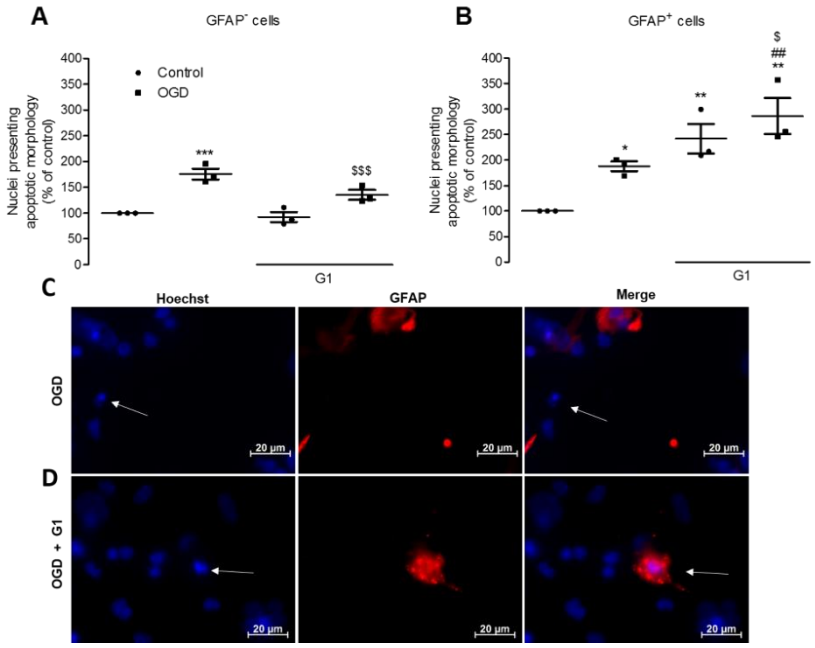

Figure 8: Evaluation of nuclei with apoptotic morphology in primary neuron-glia cortical cultures exposed to OGD. Quantification of non-astrocytic cells (**A**) and astrocytes (**B**) with nuclei presenting apoptotic morphology. **C** - Representative images of immunocytochemistry for GFAP (red) and Hoechst 33342 staining (blue) in cells exposed to OGD in the absence or presence of 100 nM G1. Arrows indicate nuclei with apoptotic morphology. All images were obtained with a 63x objective. The results are expressed as percentage of control and represent the mean ± SEM of 3 independent cell culture preparations performed in triplicate. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's *post hoc* test **P*<0.05, ***P*<0.01 and ****P*<0.001 compared to control; ##*P*<0.01 compared to OGD; $*P*<0.05 and $$$P<0.001compared to control exposed to G1.

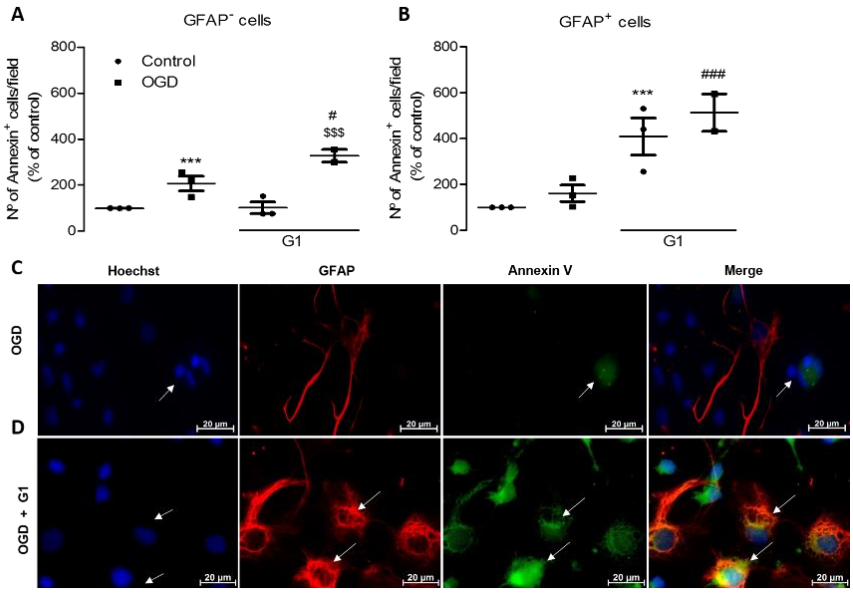

Figure 9: Effect of GPER activation on annexin V labeling in primary neuron-glia cortical cultures exposed to OGD. Quantification of non-astrocytic cells (**A**) and astrocytes (**B**) labeled for annexin V. **C** - Representative images of immunocytochemistry for GFAP (red), annexin V (green) and Hoechst 33342 staining (blue) in cultures exposed to OGD in the absence or in the presence of 100 nM G1. Arrows indicate cells labeled for annexin V. All images were obtained with a 63x objective. Results are expressed as percentage of control and represent the mean ± SEM of 3 independent cell culture preparations performed in triplicate. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's *post hoc* test. ****P*<0.001 compared to control; #*P*<0.05 and ###*P*<0.001 compared to OGD and $$$*P*<0.001 compared to control exposed to G1; *ns*, not significant.

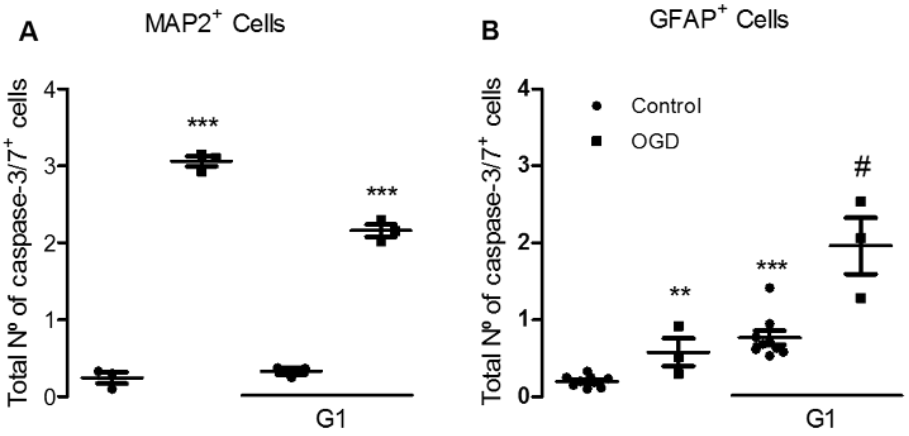

Figure 10A: Effect of GPER stimulation on caspase-3/7 activation in primary neuron-glia cortical cultures exposed to OGD. Evaluation of neuronal (**A**) and astrocytic (**B**) loss through the quantification of $MAP2^+$ and $GFAP^+$ cells labeled with caspase-3/7. Results are expressed as the number of cells/filed and represent the mean ± SEM of 3 independent cell culture preparations performed in triplicate. Statistical analysis was performed using two-way ANOVA test followed by Bonferroni's *post hoc* test. $^{**}P<0.01$ and $^{***}P<0.001$ compared to control and $\#P<0.05$ compared to OGD; ns, not significant.

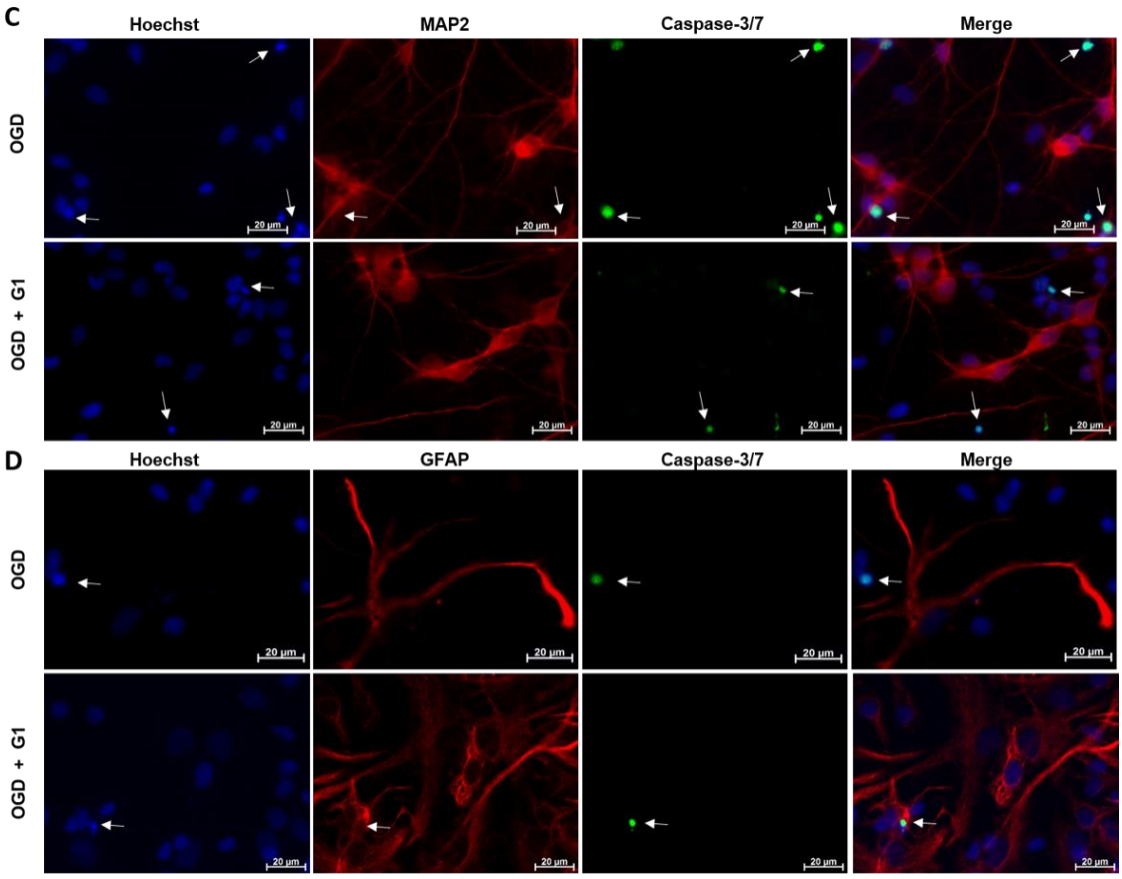

Figure 10B: (**C**) Representative images of immunocytochemistry for MAP2 (red), caspase-3/7 (green) and Hoechst 33342 staining (blue) in cultures exposed to OGD in the absence or in the presence of 100 nM G1. (**D**) Representative images of immunocytochemistry for GFAP (red), caspase-3/7 (green) and Hoechst 33342 staining (blue) in cultures exposed to OGD in the absence or in the presence of 100 nM G1 .Arrows indicate caspase-3/7 labeled cells. All images were acquired with a 63x objective.

3.5. - Blockade of the PLC pathway prevents G1-induced apoptosis in astrocytes

Next, we sought to identify the signaling pathway involved in the harmful effect induced by GPER activation in astrocytes. For that, we have considered two independent pathways activated by stimulation of GPER and reported to have pro-apoptotic effects, the PLC (55, 56) and JNK (57, 58) pathways. The selective blockade of each pathway demonstrated the involvement of PLC since the number of astrocytes labeled for active caspase-3/7 decreased approximately 50% when PLC was inhibited with 10 nM U73122. On the other hand, there was a decrease of 21% in the number of astrocytes labeled for active caspase-3/7 when the JNK pathway was inhibited using 10 μM SP600125, but this beneficial effect did not reach statistical significance. The results also indicate that the activation of GPER seems to activate different intracellular signaling pathways on these two distinct population of cells.

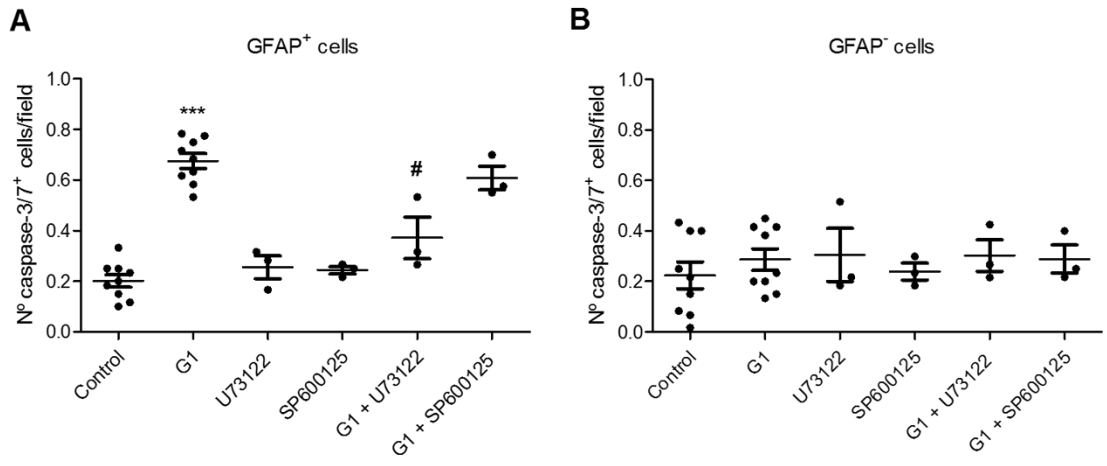

Figure 11: Contribution of PLC and JNK pathways to the deleterious effect of GPER activation in astrocytes. Quantification of GFAP$^+$ cells labeled with caspase-3/7 in cultures incubated with 100 nM G1 in the presence and in the absence of the PLC inhibitor U73122 or the JNK inhibitor SP600125. Results are expressed as the number of cells/filed and represent the mean ± SEM of 3 independent cell culture preparations performed in triplicate. Statistical analysis was performed using the one-way ANOVA test followed by Bonferroni's *post hoc* test. ***P<0.001 compared to control and #P<0.05 compared to G1.

3.6. - Exposure to G1 induces an increase in intracellular calcium levels in astrocytes, but not in neurons

We found that G1 had no effect on neuronal cell viability (Figs. 8A, 9A and 10A), whereas activation of the PLC pathway contributed to G1-induced astrocyte loss (Fig. 11A). Since elevation of cytosolic Ca^{2+} levels plays a role in astrocyte apoptosis (55), we hypothesized that G1 might induce distinct signaling pathways in astrocytes and neuronal cells. To address this possibility, we analyzed changes in $[Ca^{2+}]i$ by Fura-2 imaging in astrocyte and neuronal cell cultures. We observed that exposure to 100 nM G1 promoted a rise in $[Ca^{2+}]i$ in astrocytes (Fig.

12A), but not in neurons, which responded with an increase in $[Ca^{2+}]i$ to a depolarization with K^+ (50 mM) (Fig. 12C). In line with the results concerning the number of active caspase 3/7-positive astrocytes in cultures exposed to G1 (Fig. 11A), the rise of approximately 50% in F340/F380 when compared to baseline values was completely inhibited by the PLC inhibitor U73122 (Fig. 12A), thus indicating that GPER is coupled to the PLC pathway in astrocytes, but not in neurons.

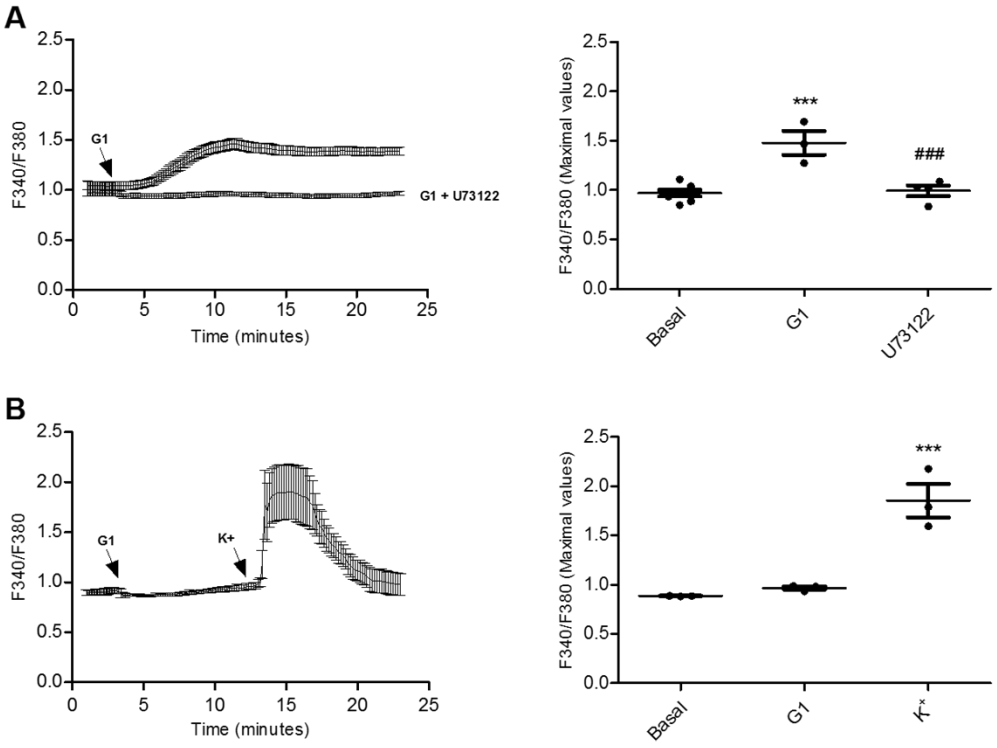

Figure 12: Cell type specific changes in intracellular calcium levels triggered by G1. Representative traces of F340/F380 registered in astrocyte (**A**) and neuronal (**B**) cultures in response to 100 nM G1 and 50 mM K^+. Some astrocyte cultures were previously treated with U73122. The maximal values of F340/F380 obtained in each experimental condition are also shown. Results represent the mean ± SEM of 3 independent cell culture preparations performed in triplicate. Statistical analysis was performed using the using the one-way ANOVA test followed by Bonferroni's *post hoc* test ***P<0.001 compared to basal condition, $^{###}P$<0.001 compared to G1 condition.

4. – Discussion

Neuroprotective effects of estrogens, particularly E2, have been debated for decades and previous reports demonstrate that estrogens can regulate the development, maturation, survival and function of multiple cell populations in different brain regions (32, 33, 59). Several studies were carried out in an attempt to clarify the possible involvement of GPER in the neuroprotective role of E2 in cerebral ischemia, but the results are inconsistent. Selective activation of GPER in vivo has been described as having beneficial (50, 51, 60), detrimental (41) or sex-dependent effects (41).

The results from the present study demonstrate that neuronal survival was strongly affected by an ischemic insult and, in contrast, OGD had a smaller impact on astrocytes survival, which is in accordance with other studies performed in *in vitro* models of ischemia (61, 62). On the other hand, the results indicated that exposure to G1 did not induce any protection against the ischemic insult. These results are similar to what was described by Lamprecht and Morrison (2014) using an organotypic hippocampal slice culture model in which exposure to G1 after OGD did not provide recovery from the ischemic injury (63). In fact, our results demonstrate that exposure to G1 has detrimental effects, similarly to what was described in a tMCAo model subjected to pretreatment with G1 (41). In this model, G1 exacerbated the infarct volume size and worsened functional outcomes after ischemia in male mice. An increase in activated caspase-3 in the peri-infarct area was also reported (41). Our data also indicate that the selective pharmacological blockade of GPER reduces the cell loss induced by the ischemic insult, suggesting that activation of GPER in basal conditions, probably by locally produced E2, contributes to astrocyte loss. In agreement with our findings, Broughton and colleagues (2014) demonstrated that the detrimental effects of G1 were blocked by G15 (41). In fact, G15 improved functional outcomes and reduced infarct volume size after an IS, whether given before or after ischemia (41). Remarkably, our results indicate that GPER blockade had no impact on OGD-induced neuronal loss, but prevented the loss of astrocytes.

Exposure to E2 induced a significant protection against the ischemic insult, as assessed by the MTT assay, but this effect was not altered by the presence of G15, thus indicating that the protection afforded by E2 was not mediated by the GPER pathway. Similar to this, Lamprecht and collaborators showed that GPER activation was not necessary for estrogen-mediated neuroprotection after ischemia (63). However, others demonstrated that G1 exerts significant neuroprotection against ischemia through the rapid activation of the pro-survival kinases, Akt and ERK, while decreasing activation of the pro-apoptotic kinase JNK (64).

Assessment of GPER expression in primary cortical cultures showed that the receptor was expressed by neurons and astrocytes, which is in accordance to what was previously described in rat midbrain neuron-glia cultures (40). Furthermore, neurons presented higher

intensity of labeling for the receptor than glial cells, but the levels of GPER in both cell populations were not affected by OGD. In contrast, a significant increase in the expression of GPER was reported in the hippocampus, somatosensory cortex and hypothalamus of male mice after stroke (15), and in the motor cortex of post-ischemic female rats (17). Such discrepant effects might be related with the stroke model used and its possible limitations on the extrapolation of findings from an *in vitro* model to an *in vivo* situation. On *in vivo* models, cells are exposed to stimuli such as the circulating hormones, such as estrogens, or could be affected by the presence of other cell types, like vascular cells. Although an *in vitro* model provides a more controlled environment of cellular mechanisms it does not contemplate this type of interactions or how they may affect GPER expression.

Exposure of astrocytes to G1 promoted cell death and potentiated the apoptosis triggered by OGD. To the best of our knowledge this is the first time that this detrimental effect is described in astrocytes. However, these pro-apoptotic effects of G1 are in accordance with data from Ding and colleagues (2009) showing that activation of GPER induced apoptosis in rat aortic vascular smooth muscle cells by a process involving activation of ERK and PKA inhibition (65). Contrariwise, there are also studies demonstrating that non-selective activation, with selective estrogen receptor modulators, of GPER in astrocytes induces general beneficial effects through the induction of anti-inflammatory effects (66, 67) and the reduction of extracellular glutamate levels by promoting the expression of the glutamate transporter 1 (19).

The inhibition of the PLC pathway with U73122 reduced G1-induced astrocyte apoptosis by half, suggesting that PLC is highly involved in this detrimental effect induced by GPER activation. On the other hand, analysis of $[Ca^{2+}]i$ levels in neurons and astrocytes indicated that the activation of GPER induced cell type-specific signaling. Astrocytes exhibited a slow rise of $[Ca^{2+}]i$ levels upon GPER selective activation, and these changes were completely prevented by the PLC inhibitor U73122, confirming the involvement of this signaling pathway. These results allow to establish a connection between the stimulation of the GPER, the activation of the PLC pathway and the consequent increase in $[Ca^{2+}]i$ levels as the trigger of this negative effect observed in astrocytes.

PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to the second messenger molecules inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (68, 69), which, in turn, control $[Ca^{2+}]i$ and protein kinase C activity (PKC), respectively(68, 69). The increase in IP3 induces the mobilization of $[Ca^{2+}]i$ by regulating the release of Ca^{2+} from intracellular organelles to the cytosol (68, 69). Several authors have already reported that the increase in $[Ca^{2+}]i$ in astrocytes is associated with the activation of pro-apoptotic pathways (55, 56, 69-71) and cell death (72-74). The mechanisms behind this effects were associated with DNA fragmentation (55), nuclear condensation through the activation of caspase-3 (55, 56, 71), mitochondrial dysfunction(71), reactive oxygen species generation (71) and production of cleaved-poly(ADP-

ribose) polymerase(71). Thus, our results provide further evidence that there is an association between increased $[Ca^{2+}]i$ and the induction of apoptosis in astrocytes.

On the other hand, DAG triggers PKC activation and subsequent activation of the ERK pathway, a known pro-apoptotic signaling mechanism (75). For example, Ding and colleagues (2009) associated the activation of ERK with the pro-apoptotic effects induced by GPER in rat aortic vascular smooth muscle cells (65). We speculate that the same pro-apoptotic cascade may occur in astrocytes since PKC activation is associated with apoptosis of astrocytes through the caspase-9 and caspase-3 cascade, whereas inhibition of PKC pathway decreased cell death (76).

Here we show that stimulation of GPER in glial cells promotes apoptosis and aggravates OGD-induced astrocyte loss. Since we used an *in vitro* model of stroke, where blood circulation, BBB, and other components that interact directly within the CNS are absent, we cannot rule out the possibility that the effect of G1 *in vivo* might be different. Indeed, it was demonstrated that selective activation of GPER induces vasodilatation, an effect which alone may lead to better outcomes after stroke (77). Other studies have shown that G1 administration immediately upon reperfusion decreases BBB breakdown after an ischemic insult (78), and that the GPER agonist has the ability to induce dilation and restore the function of cerebral arterioles after an ischemic injury (79). Other mechanism that might influence the outcome of GPER activation *in vivo* is the inflammation mediated by microglia. Microglial GPER was shown mediate anti-inflammatory effects after IS (17), thus indicating that the protection induced by GPER activation *in vivo* can be partially due to the anti-inflammatory role of GPER.

At a therapeutic standpoint, our results may indicate that an eventual selective activation of GPER may not be an appropriate treatment to protect from an ischemic injury. The GPER selective agonist did not induce neuronal protection after the ischemic insult, contrary to what is observed in other brain pathologies, as it is the case of Parkinson´s disease (40, 80, 81), AD (82) or MS (83, 84). Moreover, we also observe that the selective activation of GPER may induce deregulation of calcium homeostasis in astrocytes, which was associated to its apoptosis, thereby having a detrimental effect. However, more studies will be needed to confirm this theory.

Overall, the results from the present study demonstrated that selective activation of GPER induces different signaling pathways in neurons and astrocytes. GPER activation in neurons has no impact on neuronal viability, either in control or under ischemic conditions. Conversely, in astrocytes, selective GPER activation induces apoptosis and increases the loss of astrocytes triggered by ischemia. Our findings indicate that stimulation of GPER triggers a rise in $[Ca^{2+}]i$ levels and consequent death of astrocytes, whereas the blockade of PLC pathway prevents both processes (Figure 13). To the best of our knowledge, this is the first report

showing that the activation of GPER in astrocytes induces their apoptosis through the PLC pathway.

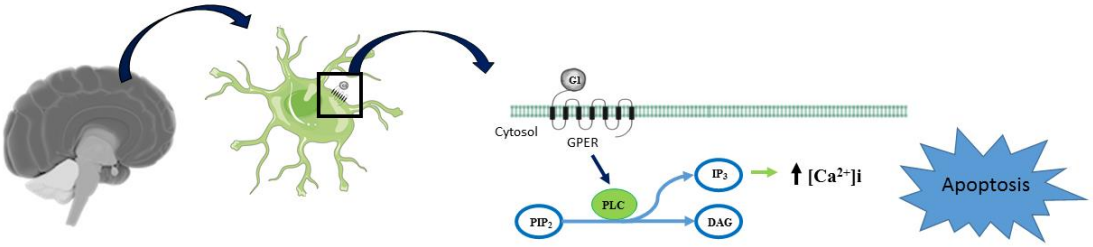

Figure 13: G protein-coupled estrogen receptor activates cell type specific signaling pathways in cortical cultures: relevance to the selective loss of astrocytes. The current data regarding the involvement of G protein-coupled estrogen receptor (GPER) on estradiol-mediated neuroprotection against ischemic stroke is contradictory. Using primary cultures from cerebral cortex subjected to 4 hours oxygen and glucose deprivation (OGD) we found that GPER selective activation with G1 did not promote any protection against OGD. Moreover, G1 induces astrocyte apoptosis via the phospholipase C pathway and subsequent intracellular calcium rise. In neurons, this effect was not observed suggesting that GPER regulates different signaling pathways in astrocytes and neurons.

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Chapter V

High-frequency repetitive magnetic stimulation (HF-rMS) prevents ischemia-induced neurite degeneration

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High-frequency repetitive magnetic stimulation (HF-rMS) prevents ischemia-induced neurite degeneration

Abstract:

IS is the leading cause of complex and serious long-term disability in developed countries, and after decades of effort, there are no effective clinical treatments for IS, especially in the subacute and chronic phases. So, it becomes crucial to develop new approaches to induce the recovery of damaged areas. The application of brain repair-based therapies to post-IS injured tissues has gained strength. In this sense, a promising therapeutic approach would be to foster neurological recovery by promoting brain remodeling using rTMS.

The present study aimed to investigate the effect of HF-rMS on an *in vitro* model of ischemia. For that, primary cortical cultures exposed to 6 hours of OGD were used as a model. Our results demonstrate that HF-rMS reduced the neuronal loss triggered by ischemia, increased the expression of ERK 1/2 and c-Fos, prevented the initial ischemia-induced neurite degeneration and promoted an increase in the number of synaptic puncta. The presence of astrocytes was crucial to the prevention of neuronal death and neurite degeneration.

Taken together, these results suggest that HF-rTMS has the potential to be a valuable therapeutic approach to reduce the neuronal death, as well as to prevent neurite degeneration and enhance functional connectivity and synaptic plasticity in the areas affected by ischemia, thus contributing to the potential neuroprotective effects on motor and cognitive functions.

Keywords: Ischemic Stroke, magnetic stimulation, synaptic plasticity, synaptogenesis, neurons, astrocytes.

1. – State of the art

IS is the leading cause of complex and serious long-term disability in developed countries, being a major global health problem (1-3). Pathophysiologically, IS is characterized by the lack of blood flow in cerebral tissues (4, 5). The decrease or the total interruption of blood supply leads to irreversible neuronal damage, whose severity is proportional to the duration of ischemia (2, 6-10). Current treatments are focused on the acute phase, where its major purpose is to perfuse, as soon as possible, the ischemic cerebral tissues in order to minimize the neuronal loss triggered by ischemia (11). On the subacute and chronic phases, there are no effective clinical treatments for IS.

Application of repair-based therapies to post-IS injured tissues has gained strength, and several approaches are being tested. A promising therapeutic approach would be to induce neurological recovery by promoting brain remodeling via neurovascular plasticity using rTMS. Increasing data suggest a therapeutic and neurorestorative role of rTMS in several neurological disorders, such as depression, movement disorders, and obsessive-compulsive disorders (12-15), being accepted for the treatment of depression by several medical organizations (16-19).

Despite the wide application in humans, cellular and molecular mechanisms underlying rTMS-based therapies are poorly characterized (20). The most accepted theory indicates that the effects of rTMS are mediated by an increase of synaptic plasticity through the modifications on NMDA receptors (20, 21). However, there are also data suggesting that rTMS may also promote neurotransmission (22-25), alter gene expression (26), induce neurogenesis (27-30) and prevents neuronal death (31, 32). However, further studies are necessary to unveil the cellular and molecular mechanisms that underlie the effects of rTMS.

The present chapter aimed to develop an HF-rMS protocol and to evaluate its potential cellular and molecular beneficial effects after ischemia. With this aim, primary cortical cultures subjected to 6 hours of OGD were used as a model and several proliferative and survival markers, as well as the neurite morphometric and synaptic modifications were evaluated.

2. – Material and methods

2.1. - Cell Cultures

Primary neuron-glia and neuron-enriched cortical cultures were prepared as described on chapter III, section 2.1. Cells were plated on poly-D-lysine coated dishes (Thermo Fischer Scientific, catalog number: 130180). The cells were maintained in a 5% humidified CO2 incubator at 37°C. After 4 DIV, the medium was renewed, and all experiments started at DIV 6.

2.2. – OGD and reperfusion

OGD period was realized for 6 hours and was followed by the reperfusion period. All procedures were realized according to what was described on chapter III, section 2.2.

2.3. – High-Frequency repetitive magnetic stimulation (HF-rMS) protocol

Stimulation protocols applied in this study used parameters similar to previously used both in clinical and animal studies (33, 34). HF-rMS was applied with an MCF-B70 figure-8 coil (180x116mm), connected to a MagVentureMagPro G3 X100 5.0.1. This type of coil delivers more focused and defined stimulation, with maximum magnetic field occurring under its center, area where the double-coil intersection is located (34, 35).

Every stimulation session followed the same exact procedure, placing the figure-8 coil directly over each single culture dish, aligning the center of the plate with the center of the coil, hence maximizing magnetic field exposure. The distance between the plates and the coil was approximately 1.5 cm.

Magnetic stimulation was applied using 24 trains of 50 pulses in a biphasic waveform (280μs duration), delivered at 10Hz, with 25-second inter-train interval, totalizing 1200 stimuli. An intensity of 60% of the maximum device output was used and the whole stimulation session lasted approximately 11 and a half minutes for each dish. Stimulation parameters used were within the commonly used practice protocols in stroke and depression treatments (14, 36, 37). Coil temperature was monitored and ranged between 21 and 39°C, thus trying to dismiss any confounding effects of temperature influence.

2.4. – Cell viability assessment

Cell viability assessment was realized according to what was described on chapter III, section 2.5.

2.5. – Immunocytochemistry assays

Immunocytochemistry protocols were realized as described on chapter III, section 2.3, and were used the antibodies described in table 9 and 10.

Table 9: Primary antibodies used on Immunocytochemistry assays to evaluate the effects induced by HF-rMS.

Antibody	Specie	Dilution	Company	Catalog number
Anti-MAP2	Mouse	1:500	Santa Cruz Biotechnology	Sc-74421
Anti-MAP2	Rabbit	1:500	Santa Cruz Biotechnology	Sc-20172
Anti-GFAP	Rabbit	1:2000	DAKO	Z0334
Anti-c-Fos	Mouse	1:200	Santa Cruz Biotechnology	Sc-271243
Anti-ERK 1/2	Mouse	1:250	Santa Cruz Biotechnology	Sc-135900
Anti-Tau	Mouse	1/500	Merck Millipore	MAB3420
Anti-Synapsin	Rabbit	1/4000	Merck Millipore	AB1543P

Table 10: Secondary antibodies used on Immunocytochemistry assays to evaluate the effects induced by HF-rMS.

Antibody	Conjugated	Dilution	Company	Catalog number
Anti-rabbit	Alexa Fluor 546	1:1000	Invitrogen	A11010
Anti-mouse	Alexa Fluor 488	1:1000	Invitrogen	A11001

2.6. – Cell counting

The quantification of cell number was performed as described on Chapter III, section 2.5.

2.7. – Morphometric analysis of neurons

Only neurons whose neurites were clearly distinguishable and not overlapping with other neurons were selected to morphometric analysis. Image analysis was done with FIJI software (Wayne Rasband, NIH). Individual neurons were manually traced and 2-D reconstructions were made using the Simple Neurite Tracer plugin of the FIJI software. The morphometric data of skeletonized neurons was obtained and the length and number of neurites quantified considering the cell body as the origin.

Sholl analysis was realized on each skeletonized neuron considering the soma of the cell as the origin. The Sholl analysis was executed within FIJI using the linear method with a sphere separation of 5 µm, without normalization.

2.8. – Evaluation of synapses

The evaluation of synapses was done on FIJI software (ImageJ), and was based on previously described protocols (38, 39). Images acquired on epifluorescence microscope (Zeiss, AxioObserver Z1) with a 63x objective were imported to FIJI software. The region of interest (ROI) was delineated around each neuron (Tau^+ cells) to calculate the specific area of the cell. Then, using synapsin labeling, the threshold was defined and the ROI was imported to determine, on that specific area, the number of synapsin puncta and their integrated density (area of puncta x intensity) using the "analyze particles" plugin. For this quantification, experiments were performed in at least 3 distinct cellular preparations. In each experiment, 2 coverslips/condition were prepared, and 10 neurons were randomly selected on each coverslip.

2.9. – Statistical analysis

The results are expressed as the number of cells per field, the percentage of values obtained in control conditions, the number of intersections per neuron, the number of neurites per neurons, or as the neurite length per neuron, as mentioned in the legends of figures, and are presented as the mean ± SEM of at least 3 independent cell culture preparations, performed in triplicate. Statistical analysis was performed using one- or two-way ANOVA as specified in the figure legends, followed by Bonferroni´s post hoc test. Values of $P<0.05$ were considered significant. All statistical analyses were performed using the GraphPad Prism Software. No statistical methods were employed to predetermine sample size of any of the presented experiments and no tests for normal distribution were performed.

3. – Results

3.1. - HF-rMS induces beneficial effects on neuron-glia cortical cultures after ischemia

Increasing data suggest a therapeutic and neurorestorative role of rTMS in several neurological disorders (19, 20). To evaluate the possible beneficial effects of HF-rMS on ischemia-induced lesion, primary neuron-glia and neuron-enriched cortical cultures subjected to OGD were exposed to HF-rMS. Cell viability was assessed with the MTT assay and the number of cells was analyzed by immunocytochemistry. The application of HF-rMS to neuron-glia cultures induces a significant reduction, of approximately 23.3%, on cell loss (Fig. 14A), and a decrease by approximately 10% the cell loss induced by ischemia (Fig. 14B). Interestingly, the reduction on the cell loss triggered by HF-rMS was due exclusively to a reduction of neuronal loss (Fig. 14C) with no impact on the astrocytic population (Fig. 14D). Whereas in neuron-glia cultures, HF-rMS did not induce a decrease on the cell loss induced by ischemia (Fig. 15). This suggests that HF-rMS applied after an ischemic insult has the ability to induce beneficial effects on neurons, being those effects dependent on the presence of astrocytes.

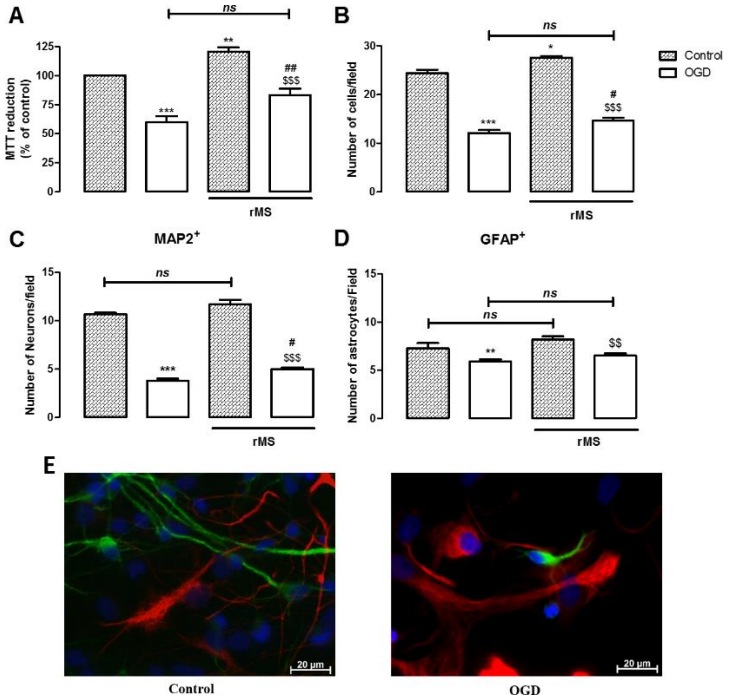

Figure 14: Effect of HF-rMS on rat primary neuron-glia cortical cultures exposed to 6 hours of OGD. A - Evaluation of cell viability through the MTT assay. The results are expressed as percentage of control and represent the mean ± SEM of 3 independent experiments performed in quadruplicate. B - Evaluation of the number of cells through Hoechst 33342 staining. C - Evaluation of the number of neurons through MAP2 immunocytochemistry. D - Evaluation of the number of astrocytes through GFAP immunocytochemistry. E - Representative images of immunocytochemistry for GFAP (red), MAP2 (green) and Hoechst 33342 staining (blue) in control and OGD conditions. The results are expressed as the number of cells/field and represent the mean ± SEM of 3 independent cell culture preparations performed in triplicate. Statistical analysis was performed using Two-way ANOVA followed by Bonferroni's post hoc test. *P<0.05, **P<0.01, and ***P<0.001 compared to control; #P<0.05, and ##P<0.01 compared to OGD;$$ P<0.01, and $$$ P<0.001 compared to control HF-rMS; ns, not significant.

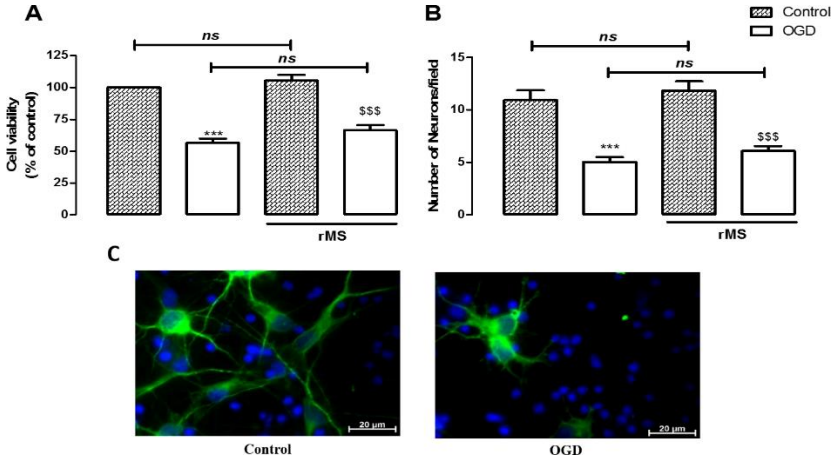

Figure 15: Effect of HF-rMS on rat primary neuron-enriched cortical cultures exposed to 6 hours of OGD. A - Evaluation of cell viability through the MTT assay. The results are expressed as percentage of control and represent the mean ± SEM of 3 independent experiments performed in quadruplicate. B - Evaluation of the number of neurons through immunocytochemistry, with MAP2 and Hoechst 33342 staining. C - Representative images of immunocytochemistry for GFAP (red), MAP2 (green) and Hoechst 33342 staining (blue) in control and OGD conditions. The results are expressed as the number of cells/field and represent the mean ± SEM of 3 independent cell preparations performed in triplicate. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post hoc test. ***P<0.001 compared to control; $$$P<0.001 compared to control HF-rMS; ns, not significant.

3.2. - Glial cells are required for HF-rMS modulation of ERK 1/2 and c-Fos

To get further insight on the cellular and molecular effects triggered by HF-rMS on the ischemic lesion we evaluated different markers associated to cell survival and proliferation. ERK 1/2, regulates cellular responses to a variety of extracellular stimuli, and plays a crucial role on differentiation, plasticity, survival, and neuroprotection (40). c-Fos, is a transcription factor that regulates several genes and is associated with increased metabolic activity, cell proliferation, differentiation, and survival (41), and is upregulated by the activation of ERK 1/2 pathway. In neuron-glia cortical cultures the exposure to OGD lead to an increase on the number of cells expressing ERK 1/2 and c-Fos (Fig. 16A and 17A). The effect of OGD on the expression of these markers was observed in both cell populations, but was more pronounced on non-neuronal cells (Fig. 16B, 16C, 17C and 17D). Moreover, the application of HF-rMS on control conditions induced a significant increase, of approximately 105%, on the number of cells expressing ERK 1/2 (Fig. 16A), and of 29.8% in the number of cells expressing c-Fos (Fig. 16A). On the other hand, when HF-rMS was applied after OGD a significant increase, of approximately 89%, on the number cells expressing ERK 1/2 (Fig. 16A) and of 20% on the number of cells expressing c-Fos was observed (Fig. 17A).

In neuron-enriched cortical cultures, similarly to what was observed on neuron-glia cultures, OGD induced a significant increase in the number of cells expressing ERK 1/2 and c-Fos of approximately 179% and 17%, respectively (Fig. 18A and 19A). Interestingly, the application of HF-rMS induced a small, non-significant, increase on the number of cells expressing ERK 1/2 and c-Fos, either in control or after OGD (Fig. 18A and 19A), once again suggesting that the physical presence of astrocytes is fundamental to the effects induced by HF-rMS.

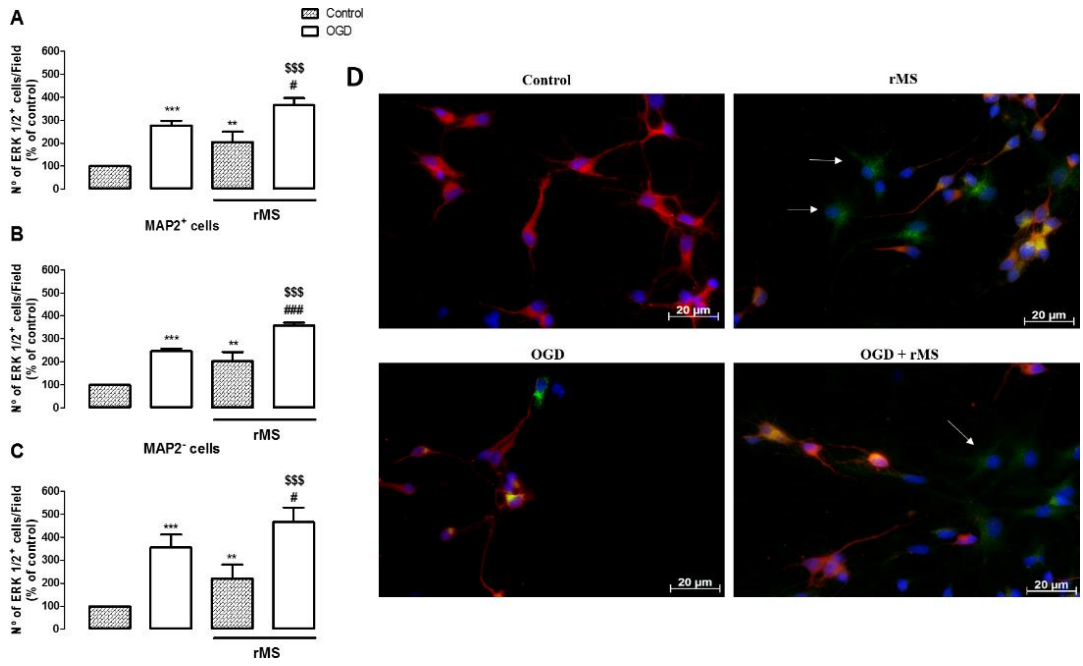

Figure 16: Effect of HF-rMS on the expression of ERK 1/2 in rat primary neuron-glia cortical cultures exposed to 6 hours of OGD. A - Quantification of the percentage of cells expressing ERK 1/2. B - Quantification of the percentage of neuronal cells (MAP2+) expressing ERK 1/2. C - Quantification of the percentage of non-neuronal cells (MAP2-) expressing ERK 1/2. Results are expressed as percentage of control and represent the mean ± SEM of 3 independent cell preparations performed in triplicate. D - Representative images of immunocytochemistry for MAP2 (red), ERK 1/2 (green) and Hoechst 33342 staining (blue) in cultures exposed to OGD with and without the application of HF-rMS. Arrows indicate cells labeled for ERK. All images were obtained with a 63x objective. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post hoc test. **P<0.01, and ***P<0.001 compared to control; #P<0.05, and ###P<0.001 compared to OGD; $$$ P<0.001 compared to control HF-rMS; ns, not significant.

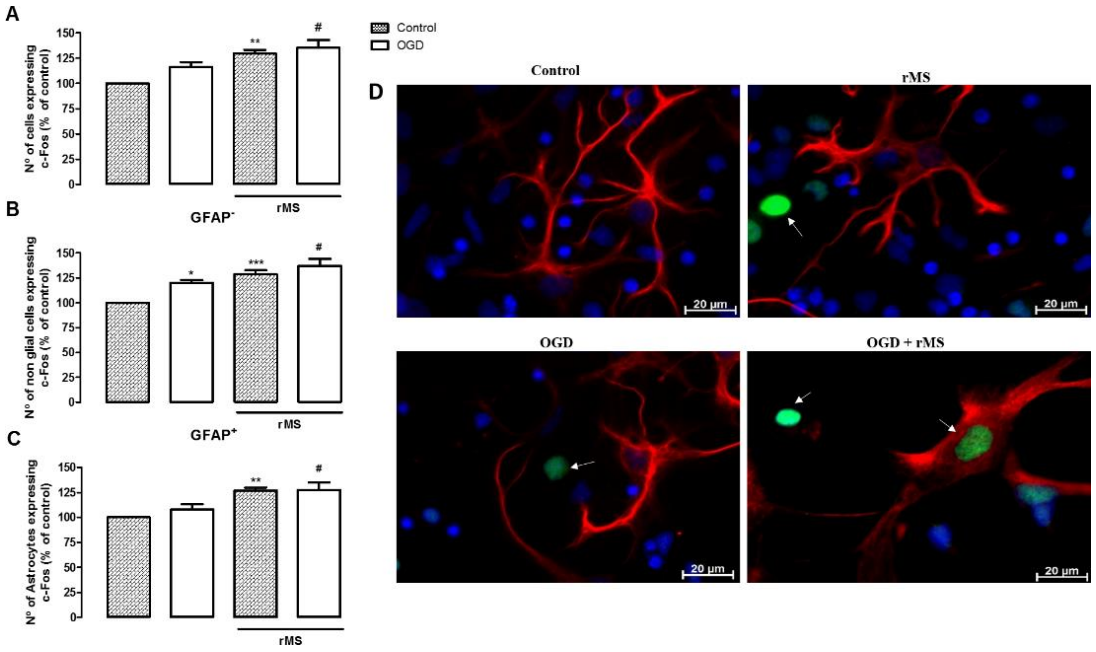

Figure 17: Effect of HF-rMS on the expression of c-Fos in rat primary neuron-glia cortical cultures exposed to 6 hours of OGD. A - Quantification of the percentage of cells expressing c-Fos. B - Quantification of the percentage of non-glial cells (GFAP-) expressing c-Fos. C - Quantification of the percentage of Astrocytes (GFAP+) expressing c-Fos. Results are expressed as percentage of control and represent the mean ± SEM of 4 independent cell preparations performed in triplicate. D - Representative images of immunocytochemistry for GFAP (red), c-Fos (green) and Hoechst 33342 staining (blue) in cultures exposed to OGD with and without the application of HF-rMS. Arrows indicate cells labeled for c-Fos. All images were obtained with a 63x objective. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post hoc test. *P<0.05 and **P<0.01 compared to control; #P<0.05 compared to OGD.

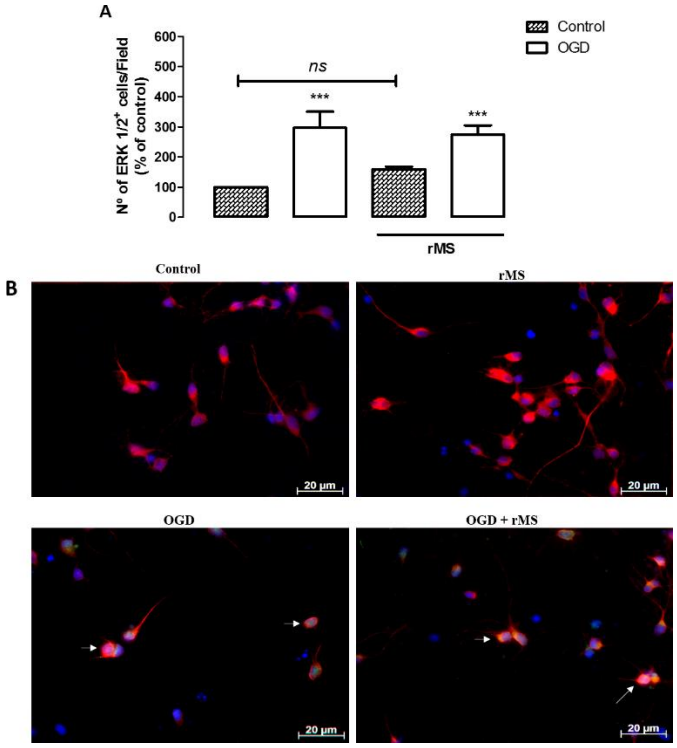

Figure 18: Effect of HF-rMS on the number of cells expressing ERK 1/2 in rat primary neuron-enriched cortical cultures exposed to 6 hours of OGD. A - Quantification of the percentage of cells expressing ERK 1/2. Results are expressed as percentage of control and represent the mean ± SEM of 3 independent cell preparations performed in triplicate. B - Representative images of immunocytochemistry for MAP2 (red), ERK 1/2 (green) and Hoechst 33342 staining (blue) in cultures exposed to OGD with and without the application of HF-rMS. Arrows indicate cells labeled for ERK. All images were obtained with a 63x objective. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's post hoc test. ***P<0.001 compared to control; ns, not significant.

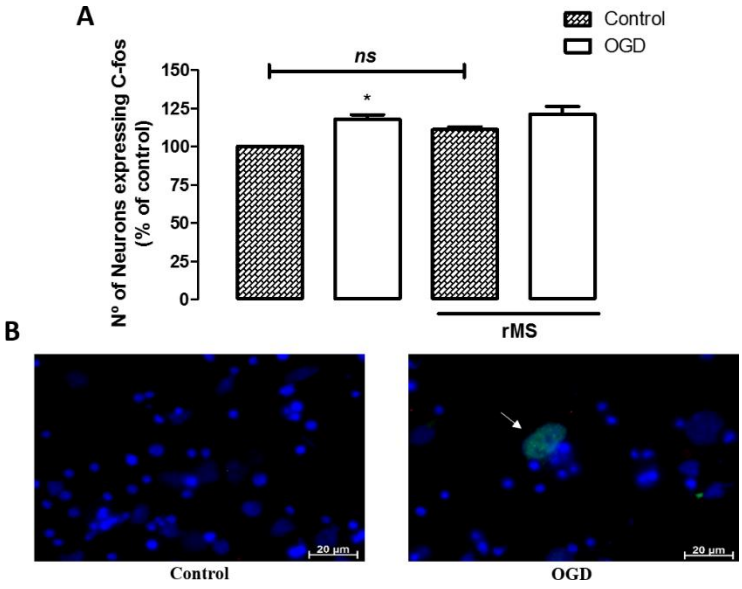

Figure 19: Effect of HF-rMS on the levels of c-Fos-in rat primary neuron-enriched cortical cultures exposed to 6 hours of OGD. **A** - Quantification of the total number of neurons expressing c-Fos. Results are expressed as percentage of control and represent the mean ± SEM of independent cell preparations performed in triplicate. **B** - Representative images of immunocytochemistry for c-Fos (green) and Hoechst 33342 staining (blue) in cultures exposed to OGD with and without the application of HF-rMS. Arrows indicate cells labeled for c-Fos. All images were obtained with a 63x objective. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's *post hoc* test. *P<0.05 compared to control; *ns*, not significant.

3.3. - HF-rMS applied after ischemia prevents neurite degeneration and increases synaptic markers

We evaluated the changes induced by HF-rMS on the number of neurites, its length, and the neuronal arborization and the number of synapsin puncta. In neuron-glia cortical cultures OGD triggered a reduction on the number of neurites (Fig. 20A), the neurite length (Fig. 20B), and global neuronal arborization (Fig. 20C), as well as a decrease on the number of synaptic puncta per μm2 (Fig. 21A) and on synaptic puncta integrated intensity (Fig. 21B). Interestingly, the application of HF-rMS to control conditions does not influence the number of neurites, (Fig. 20A), but induces a significant increase in the neurite length (Fig. 20B) and on neuronal arborization (Fig. 20C), being those effects also observed when HF-rMS was applied after OGD. Application of HF-rMS to cells previously exposed to OGD induce an increase in neurite length from 48.28±2.19 μm to 65.59±2.94 (Fig. 20). This suggests that HF-rMS applied after ischemia did not induce the formation of new neurites, but may prevent the neurite degeneration triggered by ischemia. Moreover, the application of HF-rMS on control conditions induced a significant increase, of approximately 29.6% on the number of synaptic puncta per μm^2 (Fig. 21A), and of 55.0% on their integrated density (Fig. 21 B). On the other hand, when HF-rMS was applied after OGD we observed a significant increase, of approximately 62.9%, on the number of synaptic puncta per μm^2 (Fig. 21A) and of 49.4% on their integrated density of puncta (Fig. 21B). This suggests that HF-rMS has the ability to induce a synaptogenic effect, both in control and when applied after an ischemic injury.

As observed on neuron-glia cortical cultures, on neuron-enriched cultures OGD-lead to a reduction on the number of neurites (Fig. 22A), neurite length (Fig. 22B), neuronal arborization (Fig. 22C), as well as a decrease on the number of synaptic puncta per μm2 (Fig. 23A), and on their integrated intensity (Fig. 23B). Application of HF-rMS to control neuron-enriched cortical cultures does not induce any modification on the number of neurites and on neurite length (Fig. 22A and B) but slightly increase the neuronal arborization (Fig. 22C), being the effect associated to a significant increase on the number of synaptic puncta per μm2 and on their integrated density (Fig. 23A and B). These results suggest that in the absence of glial cells the HF-rMS protocol maintains the synaptogenic effect. However, no significant neuronal modifications on neuronal ramifications were observed, suggesting that the physical presence of glial cells is fundamental to those changes. The application of the HF-rMS protocol after OGD, slightly reduced, although non-statistically significant, the negative effects that were induced by OGD on neuronal ramification as well as on synapsin labelling.

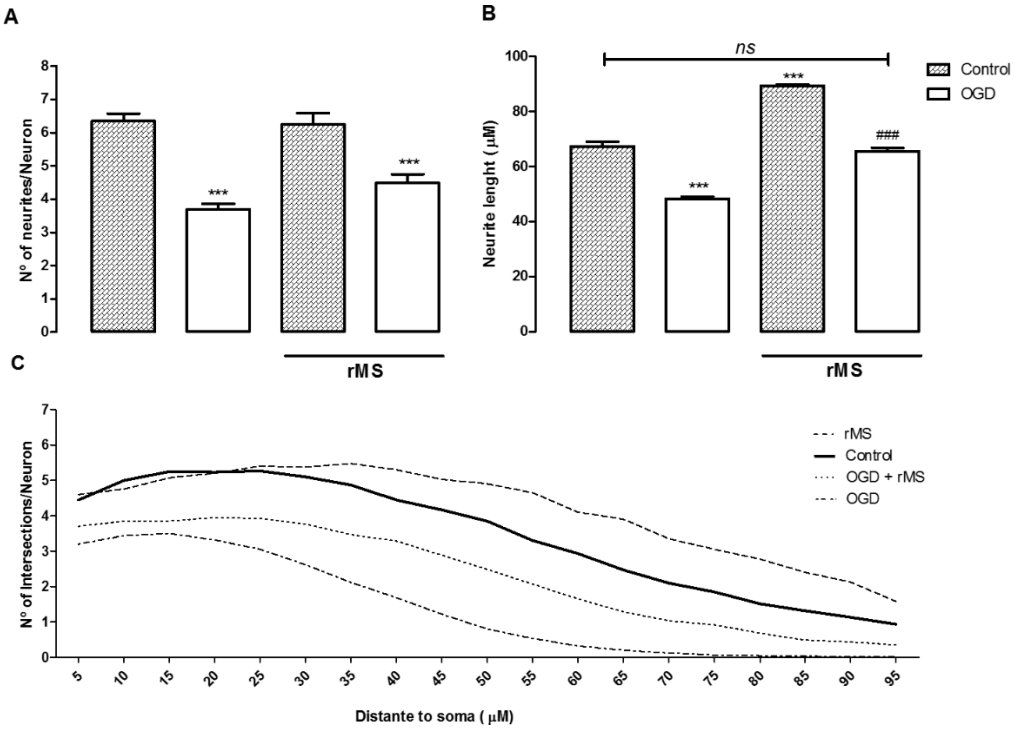

Figure 20: Evaluation of neuronal morphometric changes triggered by HF-rMS in rat primary neuron-glia cortical cultures exposed to 6 hours of OGD. A - Quantification of neurite number. The results are expressed as the number of neurites/neuron. **B** - Quantification of neurite length. The results are expressed as the neurite length/neuron. **C** - Sholl analysis. The results are expressed as the number of intersection/neuron on the distance to soma and represent the mean ± SEM of 3 independent cell preparations performed in triplicate. All images were obtained with a 63x objective. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's *post hoc* test. ***P<0.001 compared to control; ###P<0.001 compared to OGD.

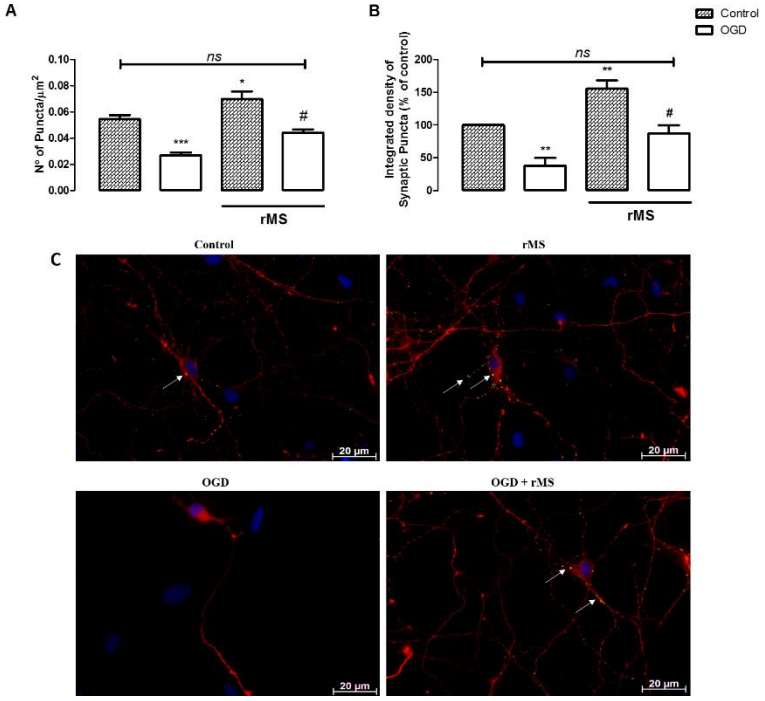

Figure 21: Evaluation of synaptic modifications triggered by HF-rMS in rat primary neuron-glia cortical cultures exposed to 6 hours of OGD. A - Quantification of the number of synaptic puncta per µm^2. **B** - Evaluation of integrated density of synaptic puncta. The results are expressed as the percentage of control and represent the mean ± SEM of 3 independent cell preparations. C - Representative images of immunocytochemistry for Tau (red), Synapsin (green) and Hoechst 33342 staining (blue) in cultures exposed to OGD with and without the application of HF-rMS. Arrows indicate Synaptic puncta. All images were obtained with a 63x objective. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's *post hoc* test. *P<0.05, **P<0.01, and ***P<0.001 compared to control; # <0.05 and ###P<0.001 compared to OGD; *ns* - non-significant.

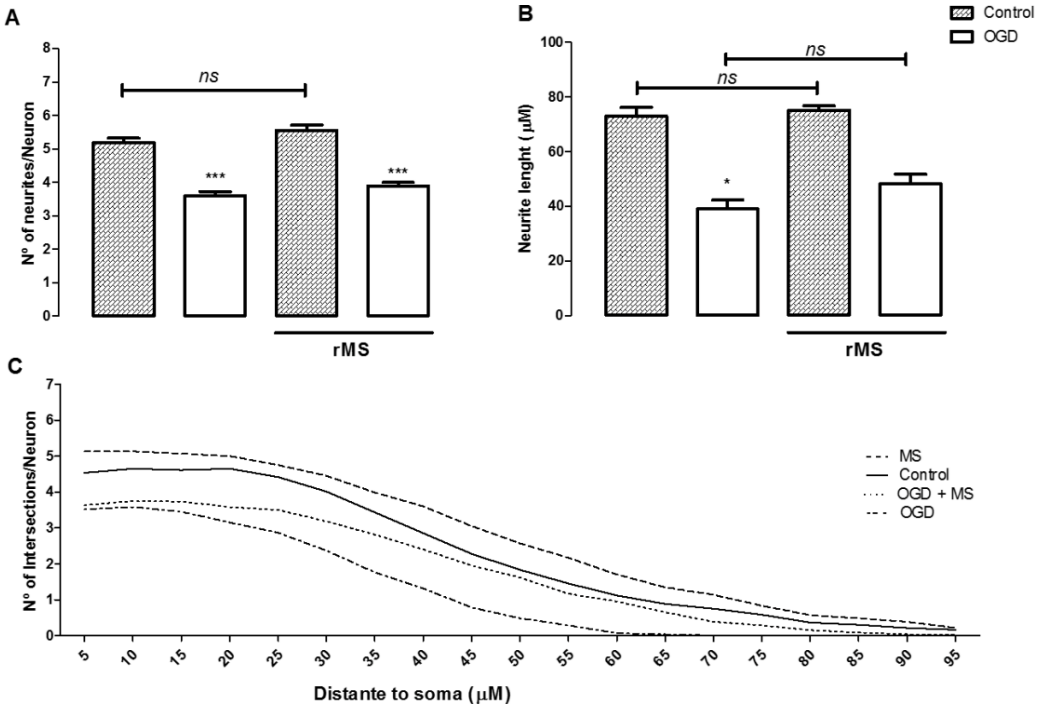

Figure 22: Evaluation of neuronal morphometric changes triggered by HF-rMS in rat primary neuron-enriched cortical cultures exposed to 6 hours of OGD. A - Quantification of neurite number (number of neurites/neuron) **B** - Quantification of neurite length (neurite length/neuron). **C** - Sholl analysis (number of intersection/neuron on the distance to soma). The results represent the mean ± SEM of 3 independent cell preparations with each experimental condition performed in triplicate. All images were obtained with a 63x objective. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's *post hoc* test. **P*<0.05 and ****P*<0.01 compared to control; ###*P*<0.001 compared to OGD.

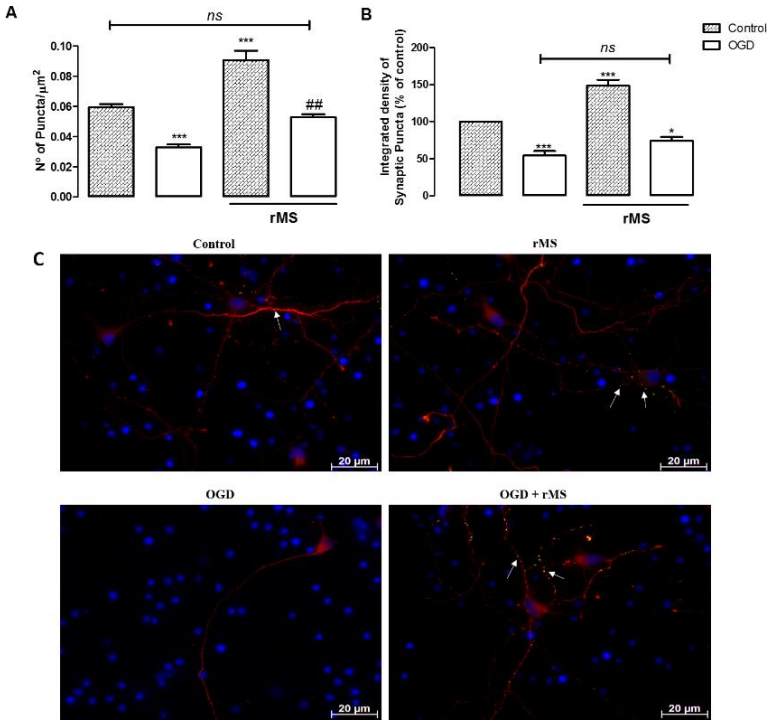

Figure 23: Evaluation of synaptic modifications triggered by HF-rMS on rat primary neuron-enriched cortical cultures exposed to 6 hours of OGD. A - Quantification of the number of synaptic puncta per µm^2. **B** - Evaluation of integrated density of synaptic puncta. The results are expressed as the percentage of control and represent the mean ± SEM of 3 independent cell preparations with each experimental condition performed in triplicate. C - Representative images of immunocytochemistry for Tau (red), Synapsin (green) and Hoechst 33342 staining (blue) in cultures exposed to OGD with and without the application of rMS. Arrows indicate Synaptic puncta. All images were obtained with a 63x objective. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's *post hoc* test. *P<0.05, and ****P*<0.001 compared to control; ## <0.01 compared to OGD; *ns* - non-significant.

4. – Discussion

Increasing data suggest a therapeutic and neurorestorative role of rTMS in several neurological disorders, such as depression, movement disorders or obsessive-compulsive disorders (19, 20, 42, 43). The potential therapeutic application of rTMS on other types of neurodegenerative disorders, such as IS, has been the focus of several studies. However, these studies are associated with a high heterogeneity in the protocols applied and focus essentially on the control of IS-induced symptoms (19, 20). The application of HF-rTMS on IS is seen as a therapeutic approach to correct maladaptive brain plasticity or to enhance brain plasticity during rehabilitation, which can lead to the improvement in functions controlled by the areas affected by ischemia (19, 44, 45). Although very promising effects have been observed, the scientific bases that support them have not been completely clarified, especially at the cellular and molecular levels (45).

The pathophysiological process of ischemia is well known, and even brief ischemic periods can initiate a complex sequence of events that ultimately culminate in cellular death (8). Similarly to what have been previously described on other studies performed in in vitro models of ischemia (46-49), our results also show that neuronal survival was strongly affected by the ischemic insult and, in contrast, OGD had a smaller impact on astrocytes survival. As expected, the OGD-induced lesion lead to an increase in the expression of ERK 1/2 and c-Fos, an effect that is associated to the activation of cellular survival mechanisms in response to ischemia (50-52). This effect is more evident at the neuronal level, and can be associated to the high metabolic needs of neurons that lead to greater impact of the lack of oxygen and/or glucose (53). Moreover, OGD-induced lesion lead to a reduction in the number of neurites, the length of neurites, the neuronal arborization, as well as, to a decrease on the synaptic puncta. This is in accordance with several reports showing that after an ischemic insult neurites shrink and their number decreases due to its rupture (54-56). The neurite degeneration and abnormal synaptic activity is a common early response of neurons to ischemia that occurs before cell death mechanisms are triggered (57). In injured neurons, the initial degeneration of neurites may not be lethal, but when maintained, the progressive loss of dendrites and concomitant synaptic input may lead to neuronal death (56, 57). However, these neurite modifications are reversible in the early phase of ischemia (56, 57). After suffering an injury which results in neurite changes, neurons may retain the potential to survive and injury-induced dendritic degeneration can be blocked (57). Therefore, the application of HF-rMS could be a valuable tool to reduce these OGD-induced cell death mechanisms, as well as to normalize synaptic activity.

Interestingly, HF-rMS applied after OGD lead to a reduction in neuronal loss triggered by ischemia, which, is in accordance to previously studies developed on in vivo models of ischemia, showing that application of HF-rTMS after ischemia lead to an attenuation of the

neuronal loss (58, 59), a reduction in the volume of the infarct area (30, 31), and an improvement of neurological functions (30). We also demonstrate that this beneficial effect is associated to the activation of pathways promoting cell survival, such as ERK 1/2 and c-Fos, which are in accordance with the observations of Ljubisavljevic and colleagues (2015). These authors showed that HF-rTMS protocols applied after ischemia significantly increase the expression of genes related to neuroprotection, cellular repair and remodeling, including the immediate early genes Fos, Jun and JunB (26).

Our data showed also that HF-rMS applied after ischemia increased the number of synaptic puncta, as well as their integrated density. Synapsins are neuronal phosphoproteins that modulate neurotransmitter release at the pre-synaptic terminal. They play a fundamental role in the formation, maintenance and rearrangements of synaptic contacts, being their expression pattern correlated with the time course of synaptogenesis and synaptic transmission (60, 61), the co-localization of synaptic terminals with tau^{+} neurites indicates the presence of active synapses (62). Based on these, our results suggest that after ischemia HF-rMS induce a synaptogenic effect that could also trigger an increase in synaptic transmission (60, 61). Baek and colleagues (2018) also described similar effects, applying an HF-rMS protocol after ischemia resulted in an increase on the expression of the synaptic markers synaptophysin and PSD-95 whereas low frequency-rMS induced a reduction of the same markers (63). These effects were mediated by an increased expression of BDNF (63). Although the ability of HF-rTMS to increase neurite length under non-pathological conditions was already demonstrated (64-66), our results show that HF-rMS protects neurites from the ischemia-induced degeneration. To our knowledge, this is the first time that this effect is demonstrated after ischemia. Stopping neurite degeneration is crucial to prevent the ischemia-induced neuronal death, and to reestablish and normalize the communication, not only with other neurons but also with the surrounding environment, thus leading to significant improvement of the area affected by ischemia.

These results are encouraging and indicate that after ischemia HF-rMS has the ability to increase the number of synaptic puncta and to limit the OGD-induced degeneration of neurites, and possibly to enhance synaptic plasticity on ischemic areas. The increase of functional connectivity induced by HF-rMS has been associated with the brain reorganization after ischemia and consequent improvement of motor recovery (67). Nevertheless, there are evidences indicating that the beneficial effects triggered by HF-rMS after ischemia comprise other mechanisms such as the reduction of apoptosis through the up-regulation of anti-apoptotic proteins (31, 63, 68), the increase of cell proliferation (29, 30, 63, 69), the promotion of differentiation (30), and the increase of BDNF expression (30, 63, 70).

Bearing in mind that rTMS research has been mainly focused on a neurocentric approach there is a lack of data relating to other cell populations (71). The lack of information becomes especially relevant due to the importance of astrocytes on neuronal physiology. Astrocytes

provide a link between neurons and the circulatory system, and regulate many other neuronal mechanisms in which rTMS could induce beneficial effects (72-78). Therefore, it is important to clarify the role of astrocytes on the effects triggered by HF-rMS, as well as the direct impact of HF-rMS on these cells. For this, we proceeded to the same type of analysis but on neuron-enriched cultures. Interestingly in all the parameters evaluated, with the exception of synapse markers, we did not observe the beneficial effect induced by HF-rMS discussed above. This suggests that the physical presence of astrocytes is fundamental for the effects induced by HF-rMS. These results are also very interesting and show that although the focus of rTMS is orientated to neuronal cells we will also have to look at the possible effect that this technique has on glial cells, and particularly astrocytes.

Taken together, the results obtained are encouraging and provide new evidences that may help to unravel the beneficial effects induced by HF-rTMS at the cellular and molecular level after an ischemic injury. We demonstrate that HF-rMS could be a valuable therapeutic approach to reduce neuronal loss triggered by ischemia, and to stop and reverse the initial ischemic-induced neurite degeneration, being unquestionably that astrocytes play a key role in the observed effects. The observed beneficial effects triggered by HF-rMS may contribute to the normalization and enhancement of neuronal communication and synaptic plasticity in the areas affected by the ischemia, and support the beneficial effects that have been reported on motor and cognitive functions.

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Chapter VI

General conclusions and Future perspectives

General conclusions and future perspectives

The research regarding the clinical treatment to the recovery or enhancement of brain regions affected by ischemia on the subacute and chronic phases of IS has not brought enough results (1). Several approaches have been tested, such as neurotrophic factors (1, 2), neural stem cells (1, 3, 4), glutamate antagonists (1, 5), or estrogen therapy (6), however without sufficiently sustained results to allow them to emerge as a therapeutic alternatives. All these efforts have a common major objective; to induce the recovery of brain regions affected by ischemia and consequent improvement of functions regulated by those areas (1). In animal models very promising results were observed, however when the same approaches were tested in humans the beneficial effects were not replicated (1, 7-9). The inability to threat properly this pathological condition is certainly associated to its complex nature (10). Although, the pathophysiological mechanisms of IS are well known and the detrimental mechanisms triggered by ischemia well documented (10-12), it was not possible to establish an approach to recover cerebral tissues (1, 8). The major limitation of the approaches tested is related to the fact that they focus essentially on a single mechanism or signaling pathway. When the system is evaluated as a whole, the beneficial effects are not observed (1, 7). Moreover, the majority of the studies have a neurocentric vision, focusing on neuronal recovery and neglecting the role that other CNS components may play, such as glial cells or the vascular system (1, 13, 14). This led several authors to support that the ideal strategy for IS treatment should be an approach that simultaneously reduces excitotoxicity, oxidative stress, inflammation, endothelial injury and at the same time improves and reinforces communication in injured tissues (1, 15). In this context, we decided to evaluate the potential beneficial effects induced by GPER selective activation and by HF-rTMS, two approaches that have already been associated to improvements in several detrimental ischemic-induced signaling mechanisms.

In neurons, it was reported that GPER activation promotes the activity of pro-survival kinases such as PI3K/Akt (16, 17) and MAPK/ERK (16), and attenuates the pro-apoptotic pathway JNK (16, 17). Besides that, G1 also induces the activation of adenylyl cyclase and the consequent rise in cAMP levels in a dose-dependent manner (18). Hypothetically, through this modulation, it is possible to stop ischemia-induced apoptotic mechanisms and at the same time to induce the recovery of damaged cells making GPER a very interesting target to promote neuroprotection. However, when we explored the mechanisms triggered by GPER activation we observed that after an ischemic injury the selective activation of GPER does not induce neuronal protection, and surprisingly induced the apoptosis of astrocytes, being these effects associated to the activation of different signaling pathways on each type of cells (19). In astrocytes, but not in neurons, G1 activates the PLC pathway which triggers their apoptosis (19).

The notion that ER trigger cell type-specific signaling pathways on neurons and astrocytes is not new and was described in 2006 by Mhyre and Dorsa (24). These authors reported that activation of classical ER on neurons induce the activation of the MAPK and cAMP response element-binding protein (CREB) pathways, whereas on astrocytes does not increase these pathways, but instead activates signaling pathways leading to inhibition of cAMP response elements (CRE) and CRE-mediated transcription (24). In addition, on neurons cAMP activates the MAPK pathway, whereas on astrocytes cAMP has the opposite effect, being this different effect associated to the reduction of cell growth in astrocytes (20, 21). Moreover, in astrocytes cAMP inhibits the PI3K/Akt pathway (22, 23), being this inhibition associated with the presence of several apoptotic markers, such as morphological changes, increase of cleaved caspase-3, condensation and fragmentation of nuclei, and a decrease in the number of cells (23). These data suggests that, in addition to PLC pathway, other factors on neurons and astrocytes influence the cell type-specific signaling activated by GPER (Fig. 24). Considering the crucial role of glial cells in neuronal physiology, it is likely that any condition that interferes with the normal astrocytic and microglial function affects neuronal physiology (14, 24). Since studies on the actions of GPER in glial cells is still very limited, it is of utmost importance to deepen the analysis of the effects triggered by GPER activation on this cell population. Clarification of cell type-specific signaling mechanisms will help to elucidate the potential and possible protective role of GPER activation in brain pathologies and neurodegenerative disorders.

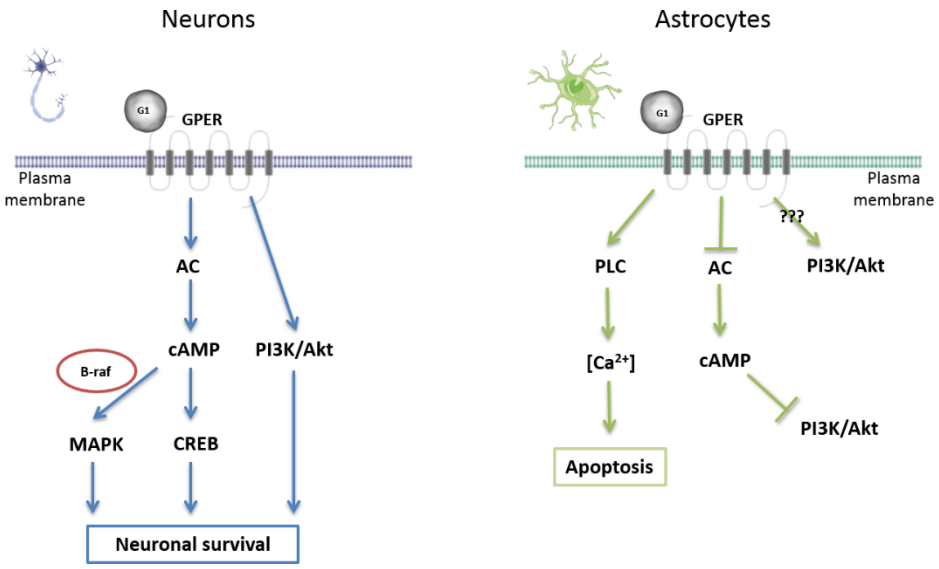

Figure 24: Cell type-specific signaling pathways activated by GPER on neurons and astrocytes. On neuronal cells the activation of GPER is associated to an increase of neuronal survival, via the increase of cAMP levels and the activation of MAPK and CREB pathways, and via the activation of PI3K pathway. On astrocytes, the activation of GPER is associated to a reduction of cAMP levels and to cell death due to the activation of PLC pathway and consequent rise in intracellular calcium levels. Abbreviations: Adenylate cyclase (AC); cAMP response element-binding protein (CREB); cyclic adenosine monophosphate (cAMP); G protein-coupled estrogen receptor 1 (GPER); Intracellular calcium ([Ca^{2+}]); Mitogen-activated protein kinase (MAPK); Phosphoinositide 3-kinase (PI3K); Phospholipase C (PLC).

The importance of glial cells on neurodegenerative disorders is changing the neurocentric approach to these disorders (13, 14). The body of evidences indicates that there is a growing interest in other CNS components to induce neuroprotection after an IS (13, 14). Moreover, there is also a growing interest on the application of repair-based therapies to induce the recovery of injured tissues following an IS, such is the case of rTMS (25). The application of this non-invasive technique is seen as a therapeutic approach to correct maladaptive brain plasticity or to enhance brain communication during rehabilitation, which can lead to the improvement of functions regulated by the areas affected by ischemia (26-29). Interestingly our results demonstrate that HF-rMS reduced the neuronal loss, neurite degeneration and the loss of synaptic markers triggered by ischemia. The stopping and reverse of all these detrimental ischemia-induced mechanisms on neurons is the first step towards the reestablishment and normalization of cellular communication not only between neurons but also with other cells. These promising results suggest that HF-rTMS has the potential to be used as a therapeutic approach to reduce and prevent neuronal damage following an IS. In this sense, it would be interesting to evaluate if these beneficial effects are also observed in an *in vivo* model and if with the application of HF-rTMS on consecutive days these effects can be amplified, thus enhancing the functional outcomes.

Considering the crucial role of astrocytes on the prevention of neuronal death and neurite degeneration induced by HF-rMS, it is important to clarify if those neuroprotective processes are induced directly by HF-rMS on astrocytes, or if they are part of an indirect mechanism triggered by the neuronal reaction to HF-rMS. Furthermore, it is also important to evaluate the astrocyte phenotype present after HF-rMS, as well as to characterize the changes in the secretome, in order to understand how these putative alterations mediate the neuronal protection observed. In addition to these effects of HF-rMS, we showed that the presence of astrocytes leads to a smaller neuronal injury. Our results show that the extent of the injury induced by OGD in neuron-enriched cultures was always higher than the injury induced by OGD on neuron-glia cultures. This suggests an active role of astrocytes in preventing the ischemic-induced injury, therefore astrocytes should be considered as potential therapeutic target in the recovery of tissues affected by ischemia, and any technique or approach that enhances their ability to prevent or induce neuronal repair should be explored.

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Appendix

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ORIGINAL RESEARCH

Impact of Astrocytes on the Injury Induced by In Vitro Ischemia

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Abstract Cell cultures are characterized by their simplicity, controllability, and ability to provide detailed basic information on how a particular cell population responds to specific stimuli or insult. These characteristics led to their extensive application in the study of molecular interactions and represent a valuable tool in the study of different pathologies. However, due to the lack of interactions between the different components that form an in vivo system, the results obtained in pure cell cultures not always translate what occurs in vivo. In this context, the use of co-cultures has the advantage of allowing the study of interactions between different types of cells present in a tissue, which in many situations are determinant for the effects obtained. The present study aimed to characterize cortical neuron–glia and neuron-enriched primary cultures and evaluate their response to an ischemic insult. Cell viability was assessed by the MTT assay and cell number/phenotype was analyzed by immunocytochemistry in control cultures and in cells subjected to 4 h of oxygen and glucose deprivation. The results obtained demonstrate that astrocytes have a substantial impact on the injury induced by an ischemic insult, thus suggesting that the crosstalk between glia and neurons is crucial to the neuronal protection in conditions of ischemia.

Keywords Astrocytes · Ischemia · Neuron–glia cultures · Neuron-enriched cultures · Oxygen and glucose deprivation · Microglia

Abbreviations

BBB	Blood–brain barrier
CNS	Central nervous system
FBS	Fetal bovine serum
GFAP	Glial fibrillary acidic protein
HBSS	Hank's buffered salt solution
Iba1	Ionized calcium-binding adaptor molecule 1
IS	Ischemic stroke
MAP2	Microtubule-associated protein 2
NBM	Neurobasal medium
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
OGD	Oxygen and glucose deprivation
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline with 0.1% Tween

Introduction

Ischemic stroke (IS) is characterized by the interruption of the blood supply to a specific part of the brain (Roger et al. 2011; Woodruff et al. 2011; Scott et al. 2012). The severity of the ischemic event is directly proportional to the duration of blood flow interruption, but even brief ischemic periods can initiate a complex sequence of events that ultimately culminate in cellular death (Woodruff et al. 2011). The pathophysiology of IS is complex and involves numerous pathways, including energy failure, loss of ion

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homeostasis, acidosis, increased intracellular calcium levels, excitotoxicity, free radical-mediated toxicity, generation of arachidonic acid products, cytokine-mediated toxicity, activation of glial cells, disruption of the blood–brain barrier (BBB), and infiltration of leukocytes (Woodruff et al. 2011; Hossmann 1998). Due to this complexity, the study of IS has been made through the combination of several in vivo and in vitro stroke models. While in vivo models enable the study of interactions of all components present in the nervous system as a whole, the use of in vitro models allows the study of molecular interactions occurring at tissue level (Yoshino et al. 2013). The main advantages of cellular models are the immediate and direct access to the extracellular compartment due to the lack of BBB, elimination of contributions from blood components, direct control of the environment, and easiness of using cellular models for quantitative pharmacology, electrophysiology, and imaging studies (Cimarosti and Henley 2008).

The application of cellular models provides a simple and highly controlled experimental system that allows detailed basic information on how the system or one particular cell population is affected by a certain stimulus/insult (Woodruff et al. 2011). Primary cell cultures can be established by the dissociation of original tissues (Yoshino et al. 2013) and can be composed by one homogenous population of cells, or by several populations of cells (co-cultures). Co-culture systems are set-ups in which two or more different cell types grow with some degree of contact between them (Goers et al. 2014) and constitute valuable tools to study the interactions between cell populations.

Ischemia-like damage is usually induced in cellular models by replacing the regular atmosphere (95% air and 5% CO_2) by an anoxic atmosphere (95% N_2 and 5% CO_2). The hypoxic conditions can be associated with the omission of glucose, which is usually referred to as in vitro ischemia or oxygen and glucose deprivation (OGD) (Woodruff et al. 2011). Hypoxia can also be induced chemically, through the treatment with cyanide (NaCN or KCN) (Woodruff et al. 2011) or cobalt chloride (Liu et al. 2012). As chemical hypoxia usually results in higher free radical generation than OGD, it has been less used to induce ischemia in cellular models (Woodruff et al. 2011).

Organotypic brain slice cultures and primary neuronal cultures from cortex, hippocampus, and cerebellum of embryonic or perinatal rats and mice have been widely used to study ischemia-induced damage in vitro (Woodruff et al. 2011; Cimarosti and Henley 2008). The use of neuron-enriched cultures allows assessing how neurons are affected by an ischemic insult and analyzing the mechanisms involved in their survival/protection.

The human brain is formed by two major cell populations, neurons, and glial cells, present in similar amounts (Azevedo et al. 2009), and establishing complex interactions (Hossmann 1998; Barreto et al. 2011). Neurons, classically considered the most important cells of central nervous system (CNS), play a crucial role in every system of the human body (Brann et al. 2007). Glial cells, in turn, provide structural, metabolic, and trophic support to neurons (Ge and Jia 2016; Barreto et al. 2011; Fu et al. 2013; Belanger et al. 2011). By maintaining a strait crosstalk with neurons, glial cells control processes such as homeostasis, defense against pathogens, and inflammatory responses and synaptic regulation (Barreto et al. 2009; Harada et al. 2015). Therefore, neuron–glia cultures represent a valuable tool to explore mechanisms that are regulated or depend on the interaction between neurons and glial cells.

In the present study, primary neuron–glia and neuron-enriched cortical cultures were characterized by immunocytochemistry and the effect of OGD on neuron viability was assessed in both types of cultures. The results obtained demonstrate that astrocytes have a substantial impact on the injury induced by an ischemic insult.

Materials and Methods

Cell Cultures

Primary cortical cultures were prepared from cerebral cortices of 15-day-old Wistar rat embryos. The studies involved the use of 15–20 pregnant Wistar females and were performed 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). Briefly, the pregnant females were anesthetized with ketamine (87.5 mg/kg) and xylazine (12 mg/kg). The abdominal cavity was opened and the embryos were removed. The embryos were placed in a Petri dish with cold phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCL, 1.5 mM KH_2PO_4 and 8.1 mM Na_2HPO_4, pH 7.2). Cerebral cortices were isolated and the meninges were removed as described previously (Zhou and Mei 2013). The cortices were chopped into small pieces and pooled together in PBS. Tissue was dissociated mechanically and centrifuged at $400\times g$ for 3 min, and the pellet was resuspended in Neurobasal Medium (NBM; Gibco) supplemented with 2% B27 (Gibco), 0.5 mM glutamate (Sigma), 0.5 mM glutamine (Sigma), and 120 μg/ml gentamicin (Sigma).

For neuron–glia cortical cultures, cells were cultured in NBM supplemented with 2% B27, 0.5 mM glutamate, 0.5 mM glutamine, 120 μg/ml gentamicin, and 10% heat-inactivated fetal bovine serum (FBS) at a density of 0.14×10^6 cells/cm^2 on 24-well culture plates (Orange) coated

with poly-D-lysine. For neuron cortical cultures, cells were cultured in NBM supplemented with 2% B27, 0.5 mM glutamate, 0.5 mM glutamine, and 120 μg/ml gentamicin at a density of 0.21 × 106 cells/cm^2, also on 24-well culture plates coated with poly-D-lysine. The presence of FBS allows glial cells to proliferate and differentiate. Cell density was defined based on pilot experiments aimed at obtaining similar cell number in neuron-enriched and neuron–glia cultures at the 6th in vitro.

The cells were maintained in a 5% humidified CO_2 incubator at 37 °C. After five days in culture the medium was renewed. All experiments started on the 6th day in culture.

Oxygen and Glucose Deprivation and Reperfusion

To induce OGD, cells were washed twice in Hank's buffered salt solution (HBSS; 1.26 mM $CaCl_2$, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.49 mM $MgCl_2$, 139.9 mM NaCl, 4.17 mM $NaHCO_3$, 3.38 mM Na_2HPO_4, pH 7.4) free of glucose. Then the cells were placed on an airtight hypoxia incubation chamber (Stemcell Technologies). The chamber was flushed with 20 l/min of a 95% N_2 and 5% CO_2 gas mixture, for 4 min, at room temperature. The chamber was then sealed to maintain the gas composition and placed at 37 °C. OGD was carried out for 4 h and afterward the cultures were removed from the chamber, HBSS was replaced by NBM, and the cells were incubated for further 20 h under the conditions previous to OGD. For control conditions, the cells were washed twice in HBSS supplemented with 5.56 mM glucose and placed for 4 h in a 5% humidified CO_2 incubator, at 37 °C. Afterward, the HBSS was replaced by NBM and cultures were incubated for additional 20 h.

Immunocytochemistry Assay

The cells used in the immunocytochemistry assays were cultured in multiwell plates containing coverslips previously coated with poly-D-lysine. After rinsing with PBS, the cells were fixed in 4% paraformaldehyde for 10 min. This was followed by permeabilization of cells with 1% Triton X-100 in PBS for 5 min. To reduce non-specific binding, the cells were incubated with 20% FBS in PBS with 0.1% Tween (PBS-T) for 60 min at room temperature.

To characterize both types of cortical cultures, the cells were incubated overnight, at 4 °C, with anti-glial fibrillary acidic protein (rabbit anti-GFAP; 1:2000; DAKO, Z0334), anti-microtubule-associated protein 2 (mouse anti-MAP2; 1:500; Santa Cruz, Sc-74421), or anti-ionized calcium-binding adaptor molecule 1 (rabbit anti-Iba1; 1:2000; WAKO, 019-19741) antibodies, diluted in PBS-T with 1% FBS. The cells were then washed six times with PBS-T and incubated for 1 h, at room temperature, with the corresponding secondary antibodies: anti-rabbit conjugated to Alexa Fluor 488 (1:1000, Invitrogen, A-11034), anti-mouse conjugated to Alexa Fluor 546 (1:1000, Invitrogen, A-11030), also diluted in PBS-T with 1% FBS. After incubation with secondary antibodies, the cells were washed six times with PBS-T, incubated for 10 min with 2 mM Hoechst 33342 (Invitrogen), and washed three times with PBS-T. Finally, coverslips were mounted in fluorescence mounting medium (DAKO). Images were acquired on an epifluorescence microscope (AxioObserver Z1, Zeiss) with a 63x objective for MAP2 and GFAP labeling and with a 40x objective for Iba1 labeling.

Quantification of Cell Number

For quantification of cell number, we performed at least 3 experiments with different cellular preparations. In each experiment, 3 coverslips/experimental condition and 20 fields/coverslip were analyzed. The cell number/condition was assessed based on the number of nuclei stained with Hoechst 33342. The number of neurons was quantified by assessing the number of cells expressing the neuronal marker MAP2 and the number of astrocytic cells was determined by the number of cells expressing the marker GFAP. Nuclei stained with Hoechst and not labeled for the neuronal marker MAP2 or the astrocytic marker GFAP were assessed separately. The number of microglial cells was quantified by analyzing the cells that express the marker Iba1.

Cell Viability Assessment

Cell viability was assessed through the thiazolyl blue tetrazolium assay (MTT; (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma). The culture medium was removed and replaced with 0.5 mg/ml MTT solution in HBSS, followed by incubation for 1 h at 37 °C. The MTT solution was then carefully removed and the blue formazan dye was solubilized using acid-isopropanol (0.04 M HCl in propan-2-ol). Optical density was measured with a spectrophotometer (xMark™ Microplate Spectrophotometer, Bio-Rad) at 570 nm with background subtraction at 620 nm. Results are expressed as the percentage of control.

Statistical Analysis

The results are expressed as the number of cells or as a percentage of values obtained in control conditions and are presented as the mean ± standard error of the mean (SEM) of at least 3 independent experiments, performed in triplicate. Statistical analysis was performed using the

Student's t test. Values of $P < 0.05$ were considered significant. All statistical procedures were performed using GraphPad v.4 (GraphPad Software Inc., San Diego, CA).

Results

Characterization of Neuron–Glia and Neuron-Enriched Cortical Cultures

Primary cultures from cortex have been widely used to study the mechanisms underlying several brain disorders (Woodruff et al. 2011). However, the characterization of these cultures is often absent. Therefore, we first characterized our cell models by immunocytochemistry.

In cortical neuron–glia cultures, 54 ± 2% of cells expressed the neuronal marker MAP2, 34 ± 3% of cells expressed the astrocytic marker GFAP, and only 0.7 ± 0.3% of the cells expressed the microglial marker Iba1. Surprisingly, 13 ± 2% of the cells present in the culture were not labeled for either MAP2 or GFAP (Fig. 1a). In neuron-enriched cultures, as expected, the majority of cells (75 ± 2%) expressed MAP2 and only 3 ± 1% of the cells expressed the astrocytic marker GFAP. Similarly to the neuron–glia cultures, a high percentage of the cells (24 ± 1%) were not positive for either MAP2 or GFAP (Fig. 1a).

According to the immunocytochemistry analysis at the 6th day in culture, neuron–glia cultures presented 18 ± 1 cells/field, and neuron-enriched cultures had 17 ± 1 cells/field. These data indicate that on the day of OGD both types of cultures presented a similar cell number/density ($P = 0.7598$; Student's t test).

The Presence of Astrocytes Influences the Extent of OGD-Induced Injury in Cortical Cultures

In order to evaluate the effects induced by ischemia, neuron–glia and neuron-enriched cultures were subjected to 4 h of OGD, and cell viability was assessed by the MTT assay and the number of surviving neurons, astrocytes, and microglial cells was assessed by immunocytochemistry.

Exposure of neuron–glia cultures to 4 h of OGD did not have a significant impact on cell survival. OGD led to a decrease in cell number quantified by immunocytochemistry of only 9 ± 4%, and a decrease in MTT reduction of 3.8 ± 1.7%, when compared to control. Astrocytes and neurons were affected by OGD to a similar extent (Fig. 2a). Although microglial cells strongly responded to OGD by increasing its number in the culture to 170 ± 25% of control (Fig. 2b), the number of these cells in control conditions was very low (0.7 ± 0.3%), and even after OGD microglial cells represented only 1.4 ± 0.5% of the cells present in the culture.

In contrast to the reduced impact of OGD on the viability of cells in neuron–glia cultures, the results obtained in neuron-enriched cultures indicated that OGD leads to a significant reduction in cell number (30 ± 4% reduction) as assessed by immunocytochemistry (Fig. 2d). This reduction was paralleled by a decrease in MTT reduction (22.6 ± 6.6% decrease when compared to control, Fig. 2e).

These results indicate that under the same OGD conditions neuron–glia cultures are more resistant to ischemia than neuron-enriched cultures, thus supporting a protective role of glial cells during ischemia.

Discussion

Cell cultures are a valuable tool to study complex cellular mechanisms at tissue level (Yoshino et al. 2013). These systems allow manipulations and approaches that are not possible in in vivo models. In vivo, the response to a certain insult is strongly dependent on the interaction between the different components of tissues and organs. When an in vitro stroke model is used, it is important to take into consideration the contribution of crosstalk between neurons and glial cells. In the present study, we characterized the cellular composition of neuron–glia and neuron-enriched cultures prepared from embryonic cortices and analyzed their response to an ischemic insult.

Since animal tissues are formed by different populations of cells, it is necessary to separate the cell population of interest from the others and to determine the composition of the resulting cultures. In the case of neuron-enriched cultures, it is necessary to isolate neurons, as much as possible, from glial cells (Gordon et al. 2013). In this study, we provide a valuable isolation and culture method to obtain neuron-enriched cultures with a contamination of glial cells lower than 4%. The establishment of neuronal cultures with a low percentage of astrocytes is crucial to clearly evaluate the impact of these cells on neuronal survival and to enable comparison of data in the literature.

In the present study, we observed that in control conditions there was a small percentage of microglial cells. This reduced number can be explained by the age of the embryos used to prepare the cultures (15 days of gestation). According to the literature, microglia migrates and starts to expand and colonize the CNS around embryonic day 14 (Ginhoux et al. 2013). Although at embryonic day 15 microglial cells are already in the brain, their expansion is still reduced. When we analyzed the effects of an ischemic insult on microglial cells, we found that despite their residual presence in the culture they react to OGD by

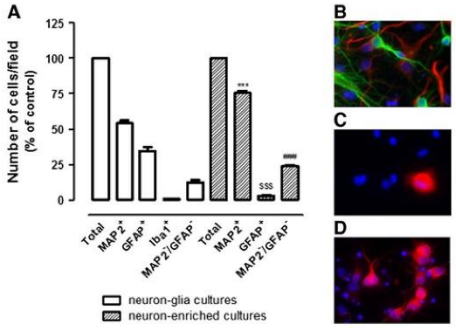

Fig. 1 Characterization of neuron–glia cortical cultures and neuron-enriched cortical cultures. **a** The data are presented as the percentage of neurons ($MAP2^+$), percentage of glial cells ($GFAP^+$), percentage of microglial cells ($Iba1^+$), and percentage of double-negative cells ($MAP2^-/GFAP^-$) at the 6th day in culture, and represent the mean ± SEM of 3 independent experiments performed in triplicate. The total number of cells was assessed by quantifying Hoechst 33342-labeled nuclei. Representative images show the immunostaining for MAP2 (*red*) and GFAP (*green*) (**b**), and for Iba1 (*red*) (**c**) in neuron–glia cortical cultures. **d** Representative image showing the immunostaining for MAP2 (*red*) in a neuron-enriched cortical culture. Images were obtained with a 63x objective. Statistical analysis was performed using the Student's *t* test. $^{***}P < 0.001$ compared to $MAP2^+$ cells on neuron–glia cultures; $^{\$\$\$}P < 0.001$ compared to $GFAP^+$ cells on neuron–glia cultures; $^{\#\#\#}P < 0.001$ compared to $MAP2^-/GFAP^-$ cells on neuron–glia cultures (Color figure online)

increasing their number/percentage approximately twofold, which is in line with the inflammatory response triggered by the neuronal lesion induced by OGD (Iadecola and Anrather 2011).

Both cortical culture types presented a substantial number of cells that did not express either the neuronal marker MAP2 or the astrocytic marker GFAP. MAP2 is a major component of the neuronal cytoskeleton (Chamak et al. 1987; Feldmann et al. 2014). The expression levels of MAP2 normally increase with neuronal maturity (Crandall et al. 1986). The number of MAP2-immunopositive cells increases with time in culture and with the age of the embryos from which the cultures are prepared (Chamak et al. 1987). Published data indicate that at early stages of neural development the expression of MAP2 can be so low that it is not detectable by immunocytochemistry (Chamak et al. 1987). Immunohistochemically, GFAP was found to be associated with reactive astrocytes that respond to CNS injuries in pathological contexts and it became a prototypical marker for immunohistochemical/immunocytochemical identification of glial cells, particularly astrocytes (Eng et al. 2000; Sofroniew and Vinters 2010). However, GFAP is not an absolute marker of all non-reactive astrocytes and is often not detectable in astrocytes of healthy CNS tissue (Sofroniew and Vinters 2010). Double staining with multiple glial markers demonstrated that some astrocytes do not express detectable levels of GFAP and that GFAP expression exhibits both regional and local variability that is dynamically regulated by a large number of inter- and intracellular signaling molecules (Sofroniew and Vinters 2010; Sofroniew 2009). Altogether, these data help explain the presence of cells in our cultures that do not express either MAP2 or GFAP. Nevertheless, it is noteworthy that, although not labeled for MAP2, these double-negative cells behave similarly to neurons when exposed to ischemic injury, thus suggesting that they are in fact neurons.

Here, we provide evidence that with the described culture settings both neuron-enriched and neuron–glia cultures present approximately the same number of cells at the day of OGD exposure. This is especially relevant because in vitro stroke models are deeply affected by the total number of cells in culture due to the impact of cell density in the consumption of oxygen and nutrients during ischemia. Thus, this is an essential parameter to consider in order to ensure that the conclusions reached in comparative studies like ours are valid, and might be a limiting factor for direct comparisons between different studies.

Our results indicate that neurons are more susceptible to an ischemic insult than glial cells, which is in accordance to what was previously described in several in vitro stroke models (Barreto et al. 2011; Swanson et al. 2004; Goldberg and Choi 1993; Giffard and Swanson 2005; Jones et al. 2011). Possibly related with this, many in vitro studies on ischemic injury have a neurocentric approach focusing only on the effects of ischemic insult in neurons and forgetting, in most cases, the role of glial cells (Scott et al. 2012; Brann et al. 2007). Remarkably, the present study proves that, in fact, the presence of glial cells has a strong impact on neuronal viability upon an ischemic injury. In a study using hippocampal cultures, Jones et al. (2011) also reported that neuron-enriched cultures are more susceptible to the ischemic insult than neuron–glia cultures (Jones et al. 2011). However, it should be noticed that, in contrast to the 3% reported in the present study, in the work by Jones et al. the neuron-enriched cultures exhibited a rather high contamination with astrocytes (24% of astrocytes), which could interfere substantially with the results. On the other hand, knowing the impact that glial cells have on the injury induced by ischemia, it is expected that the ratio of glial cells/neurons will be determinant to the response triggered upon ischemia, and any factor that modifies this ratio will influence the final results. Among the main factors that can influence it are as follows: (1) the developmental stage of embryos, (2) the duration of culture, (3) the composition of the culture medium, and (4) the medium supplements such as FBS.

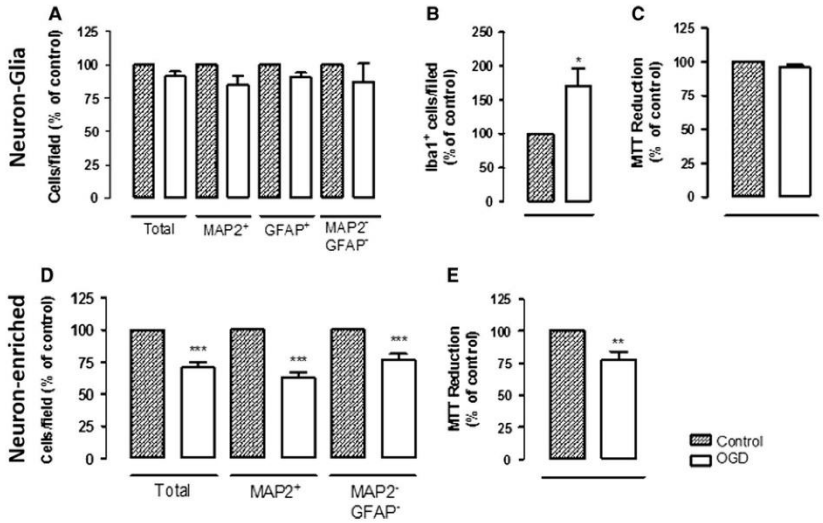

Fig. 2 Effect of OGD on neuron–glia and neuron-enriched cortical cultures. Survival of neurons, astrocytes, and microglia in neuron–glia (**a**, **b**) and neuron-enriched cultures (**d**) exposed to 4 h of OGD. Viability analysis through the MTT assay on neuron–glia (**c**) and neuron-enriched cultures (**e**). The results are expressed as the percentage of control and represent the mean ± SEM of 3 independent experiments performed in triplicate. Statistical analysis was performed using the Student's *t* test. $*P < 0.05$ compared to control, $**P < 0.01$ compared to control, and $***P < 0.001$ compared to control

The present results led us to hypothesize that glial cells have the ability to secrete molecules that help neurons to survive during ischemia. In fact, our preliminary results indicate that the protection of neurons exerted by astrocytes does not require its physical presence since astrocyte-conditioned media effectively protected neuron-enriched cultures from ischemia-induced lesion. This protection can be mediated by molecules known to be secreted by astrocytes, such as neurotrophic factors and antioxidant molecules (e.g., glutathione), which protective role is well established (Giordano et al. 2009; Dringen 2000; Dringen et al. 2000; Rossi 2015).

The ability to limit excitotoxicity by promoting glutamate and K^+ uptake (Leis et al. 2005; Stanimirovic et al. 1997; Mattson and Rychlik 1990; Mattson et al. 1991), to provide energetic support in the form of lactate (Magistretti 2006; Chih et al. 2001), or the ability to ensure synapse maintenance (Ullian et al. 2001) are some examples of mechanisms pointed out as mediators of the protective effects of astrocytes in cerebral ischemia (Barreto et al. 2011; Ricci et al. 2009). Although these characteristics make astrocytes an interesting therapeutic target, neuroprotective or neuroreparative strategies that target these cells have been scarcely explored.

This study demonstrates that an accurate characterization of in vitro stroke models, including cell type composition, is crucial to the correct interpretation of the results obtained. Moreover, we show that the presence of glial cells and the crosstalk between glial cells and neurons are critical to neuroprotection against the injury induced by ischemic conditions.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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ORIGINAL ARTICLE

G protein-coupled estrogen receptor activates cell type-specific signaling pathways in cortical cultures: relevance to the selective loss of astrocytes

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Abstract

Selective activation of the G protein-coupled estrogen receptor has been proposed to avoid some of the side effects elicited by the activation of classical estrogen receptors α and β. Although its contribution to neuroprotection triggered by estradiol in brain disorders has been explored, the results regarding ischemic stroke are contradictory, and currently, there is no consensus on the role that this receptor may play. The present study aimed to investigate the role of GPER in the ischemic insult. For that, primary cortical cultures exposed to oxygen and glucose deprivation (OGD) were used as a model. Our results demonstrate that neuronal survival was strongly affected by the ischemic insult and concurrent GPER activation with G1 had no further impact. In contrast, OGD had a smaller impact on astrocytes survival but G1, alone or combined with OGD, promoted their apoptosis. This effect was prevented by the GPER antagonist G15. The results also show that ischemia did not change the expression levels of GPER in neurons and astrocytes. In this study, we also demonstrate that selective activation of GPER induced astrocyte apoptosis via the phospholipase C pathway and subsequent intracellular calcium rise, whereas in neurons, this effect was not observed. Taken together, this evidence supports a direct impact of GPER activity on the viability of astrocytes, which seems to be associated with the regulation of different signaling pathways in astrocytes and neurons.

Keywords: Astrocytes, G1, GPER, intracellular calcium, ischemia.

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The G protein-coupled estrogen receptor (GPER or GPR30) was first identified in the late 90s (Carmeci *et al.* 1997; O'Dowd *et al.* 1998; Takada *et al.* 1997) and was described as an orphan receptor belonging to the family of 7-transmembrane spanning G protein-coupled receptors (Owman *et al.* 1996; Kvingedal and Smeland 1997). Filardo *et al.* (2000) demonstrated that 17β-estradiol (E2)-mediated activation of ERK1/2 was dependent on the expression of this receptor and named it GPR30 (Filardo *et al.* 2000). In 2005, Revankar *et al.* (2005) and Thomas and Dong (2006) described the binding of E2 to GPR30, confirming that this receptor is an E2-binding receptor, which lead to the designation GPER in 2007 (Prossnitz and Barton 2011).

The expression of this estrogen receptor is not restricted to estrogen-responsive tissues (Shi *et al.* 2013). In fact, the presence of this receptor was reported in male and female reproductive systems, heart, intestine, ovary, pancreatic islets, adipose tissue, and nervous system (Shi *et al.* 2013; Brailoiu *et al.* 2007; Matsuda *et al.* 2008; Hammond *et al.* 2011; Hazell *et al.* 2009; Broughton *et al.* 2013). Indeed, several reports demonstrated that GPER is expressed throughout the central (CNS) and peripheral (PNS) nervous system of male and female rodents. These reports described

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Abbreviations used: BBB, blood–brain barrier; CNS, central nervous system; $[Ca^{2+}]_i$, intracellular calcium concentration; DAG, diacylglycerol; DIV, day *in vitro*; E2, 17β-estradiol; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; GPER, G protein-coupled estrogen receptor; HBSS, Hank's balanced salt solution; IP3, inositol 1,4,5-trisphosphate; IS, ischemic stroke; JNK, c-Jun N-terminal kinase; MAP2, microtubule-associated protein 2; MFI, mean fluorescence intensity; NBM, neurobasal medium; MTT, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; OGD, oxygen and glucose deprivation; PBS, phosphate buffered saline; PBS-T, phosphate buffered saline with 0.1% Tween; PLC, phospholipase C; PNS, peripheral nervous system; RRID, research resource identifier; tMCAO, transient middle cerebral artery occlusion.

the presence of GPER in almost all anatomical locations of forebrain (Brailoiu *et al.* 2007; Matsuda *et al.* 2008; Hazell *et al.* 2009; Xu *et al.* 2009; Hammond *et al.* 2011; Broughton *et al.* 2013; Zhao *et al.* 2016), midbrain (Hazell *et al.* 2009), hindbrain (Brailoiu *et al.* 2007; Hazell *et al.* 2009), and spinal cord and autonomic ganglia and sensory ganglia (Dun *et al.* 2009). Particularly, in the cortex, it was demonstrated that GPER is expressed in neurons (Hammond *et al.* 2011) and astrocytes (Lee *et al.* 2012).

In contrast to estrogen receptors α (ERα) and β (ERβ), which mediate genomic effects characterized by changes in gene transcription occurring in the time frame of hours to days (Prossnitz *et al.* 2008), GPER mediates rapid non-genomic effects that occur in seconds or minutes (Prossnitz *et al.* 2008; Filardo *et al.* 2002). These are dependent on ion channels (Fraser *et al.* 2010; Prossnitz and Barton 2009) and involve the regulation of kinases such as PI3K (Revankar *et al.* 2007; Petrie *et al.* 2013), PKCε or MAPK (Goswami *et al.* 2011; Kuhn *et al.* 2008), cAMP production (Filardo *et al.* 2007, 2002), and intracellular calcium mobilization (Filardo *et al.* 2007; Revankar *et al.* 2007; Tica *et al.* 2011; Revankar *et al.* 2005). Additionally, the rapid signaling events initiated by GPER up-regulate the expression of genes such as c-fos (Kanda and Watanabe 2003) and cyclin D2 and Bcl-2 (Kanda and Watanabe 2004).

The endogenous agonist of GPER is E2, a molecule known to regulate multiple processes in the brain, such as learning, memory, cognition, and mood as well as neurodevelopmental and neurodegenerative processes (Scott *et al.* 2012; Gillies and McArthur 2010; Brann *et al.* 2007; Suzuki *et al.* 2007; Liu and Dluzen 2007). The identification of the first GPER-selective agonist G1 (Bologa *et al.* 2006) and the GPER-selective antagonist G15 (Dennis *et al.* 2009) lead to a strong increase in the number of studies focusing on the role of this receptor in different physiological systems and pathological conditions. Since then, several reports point to effects induced by the modulation of GPER in brain disorders (Prossnitz and Barton 2011, 2009), such as Parkinson's disease (Bessa *et al.* 2015) or ischemic stroke (IS) (Broughton *et al.* 2014).

IS is characterized by the interruption of the blood supply to the brain frequently because of the blockade of a blood vessel by a clot. Currently, it is one of the leading causes of death worldwide and the leading cause of adult disability in industrialized countries. The incidence of IS is higher in males than in females (Roger *et al.* 2011; Pabon *et al.* 2014; Heuschmann *et al.* 2009). These sex differences, present in many other brain disorders (Scott *et al.* 2012; Pabon *et al.* 2014; Brann *et al.* 2007; Liu *et al.* 2009; Gibson *et al.* 2006), have been attributed, in part, to the higher serum levels of E2 in women (Brann *et al.* 2007; Gibson *et al.* 2006). In fact, it was demonstrated that E2 induces neuroprotective effects through the increase in neurogenesis and reduction in cell apoptosis (Ma *et al.* 2013; Suzuki *et al.* 2009), which, in turn, lead to behavioral recovery (Li *et al.* 2011). The neuroprotective role of E2 is usually ascribed to the activation of the classical ER. However, the identification of GPER raised the hypothesis that some of the effects triggered by E2 could result from GPER activation. In the case of ischemia, evidence is not consensual. GPER activation has been described either as beneficial (Kosaka *et al.* 2012; Lebesgue *et al.* 2010), detrimental (Broughton *et al.* 2014), or with different effects depending on the sex (Broughton *et al.* 2014).

The present study aimed to investigate the role of GPER in cultured cortical neurons and glial cells exposed to ischemic conditions. To analyze the role of GPER in the ischemic injury, its activity was pharmacologically modulated with its agonist G1 and the antagonist G15. In addition, the pattern of expression and the contribution of GPER activation to cell death induced by ischemic conditions were analyzed in both neurons and glial cells.

Methods

This study was authorized by the Animal-Welfare Body of the Health Research Centre at the University of Beira Interior (CICS-UBI). Licensing by the Portuguese body regulating the use of animals for scientific purposes (Direcção Nacional de Alimentação e Veterinária) was not required. This study was not preregistered and did not involve randomization or blinding.

Cell cultures

Primary cortical cultures were prepared from cerebral cortices of 15-day-old Wistar rat embryos. The animals were bred in the animal house of CICS-UBI Health Science Research Center, with free access to water and pellet food, under standard humidity and temperature conditions, at a 12-h light–dark cycle. The colony was raised from Wistar Han IGS animals purchased from Charles River (RRID: RGD_2308816). Females (220–260 *g*) were housed in groups of 4, in individually ventilated cages. All procedures were performed 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).

Briefly, pregnant females were anesthetized with ketamine (87.5 mg/Kg, Sigma-Aldrich, St. Louis, MI, USA catalog number: K-002) and xylazine (12 mg/Kg, Sigma-Aldrich, catalog number: X1126). The abdominal cavity was opened and the embryos removed. After this procedure, the females were immediately killed by exsanguination through a cut in the aorta. The embryos were placed on a Petri dish with cold phosphate buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8.1 mM Na_2HPO_4, pH 7.4). After killing the embryos by decapitation, the cerebral cortices were isolated and the meninges removed by a method previously described (Zhou and Mei 2013). The cortices were chopped into small pieces and pooled together in PBS. Tissue was dissociated mechanically and centrifuged at 400 *g* for 3 min and the pellet resuspended in Neurobasal medium (NBM, Gibco, Paisley, UK, catalog number: 21103049) supplemented with 2% B27 (Gibco, catalog number: 17504044), 0.5 mM glutamate (Sigma-Aldrich, catalog number: 49621), 0.5 mM glutamine (Sigma-Aldrich, catalog number: 1294808), and 120 μg/mL gentamicin (Sigma-Aldrich, catalog number: G0200000).

For neuron–glia cortical cultures, cells were cultured in NBM supplemented as above plus 10% heat-inactivated fetal bovine serum (FBS, Biochrom AG, catalog number: S0115) at a density of 0.14×10^6 cells/cm^2. For neuronal cortical cultures, cells were cultured in supplemented, at a density of 0.21×10^6 cells/cm^2. In both cases, cell density was defined based on a previous report from our group (Roque and Baltazar 2017). For astrocyte cortical cultures, cells were cultured in supplemented NBM plus 10% FBS at a density of 0.21×10^6 cells/cm^2. To remove neurons and obtain an astrocyte monoculture, at day *in vitro* 4, the dishes were placed on a plate shaker (Grant-bio Orbital Shaking Platform, catalog number: POS-300) in the incubator and shaken at 110 rpm for 6 h. After shaking, the medium was replaced to remove neurons and cellular debris as previously described (Wolfes *et al.* 2017). Cells were plated on poly-D-lysine (Sigma-Aldrich, catalog number: P6407) (coated 24-well culture plates (Thermo Fischer Scientific, catalog number: 142475), except for calcium imaging (12 mm glass bottom dishes, Thermo Fischer Scientific, catalog number: 150680).

The cells were maintained in a 5% humidified CO_2 incubator at 37°C. After 4 days in culture, the medium was renewed. All experiments started at day *in vitro* 6.

Cell culture treatments

Twenty-four hours before exposure to OGD, the culture medium was replaced by serum-free NBM and the cultures were incubated with 100 or 200 nM G1 (Tocris Bioscience, Bristol, UK, catalog number: 3577/10), 100 nM G15 (Calbiochem, Darmstadt, Germany, catalog number: 271703), 100 nM E2 (Calbiochem, catalog number: 3301), or E2 plus G15 until the end of the reperfusion period. To test the effect of the inhibition of the phospholipase C (PLC) pathway, cells were treated with 10 nM U73122 (Konigame *et al.* 2011) (Sigma-Aldrich, catalog number: U6756), and to inhibit the c-Jun N-terminal kinases (JNK) pathway, we used 10 μM SP600125 (Wu *et al.* 2008) (Sigma-Aldrich, catalog number: S5567). Drugs were added 30 min prior to the addition of G1 and incubated for further 24 h.

OGD and reperfusion

To induce OGD, cells were washed twice and incubated with glucose-free Hank's Balanced Salt Solution (HBSS; 1.26 mM $CaCl_2$, 5.36 mM KCl, 0.44 mM KH_2PO_4, 0.49 mM $MgCl_2$, 139.9 mM NaCl, 4.17 mM $NaHCO_3$, and 3.38 mM Na_2HPO_4, pH 7.4). Cells were then placed on an airtight hypoxia incubation chamber (Stemcell Technologies, catalog number: 27310). The chamber was initially flushed with 20 l/min of a 95% N_2 and 5% CO_2 gas mixture, for 4 min, and then sealed and placed in an incubator at 37°C. Cells were maintained under OGD for 4 h. At the end of the OGD period, the culture plates were removed from the chamber, HBSS medium was replaced by NBM, and cells were incubated for further 20 h under normoxic conditions. For control conditions, cells were washed twice and incubated with HBSS supplemented with 5.56 mM glucose (Sigma-Aldrich, catalog number: G5767) and placed in a 5% humidified CO_2 incubator for 4 h. The HBSS medium was then replaced by NBM and cells were incubated for further 20 h in a 5% humidified CO_2 incubator.

Cell viability

Culture viability was assessed through the thiazolyl blue tetrazolium assay MTT; (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, catalog number: M2128). The culture medium was removed and replaced with MTT solution (0.5 mg/mL), followed by incubation for 1 h at 37°C. The MTT solution was removed and the formazan crystals were solubilized with acid-isopropanol (0.04 M HCl in isopropanol). Optical density was measured with a spectrophotometer (xMark™ Microplate Spectrophotometer, Bio-rad, catalog number: 1681150) at 570 nm with background subtraction at 620 nm.

Apoptosis was assessed with the anti-Annexin V probe (FITC Annexin V Apoptosis detection Kit, Biolegend, catalog number: 640914) and with the anti-caspase 3/7 probe (CellEvent caspase 3/7 green ReadyProbes Reagent, Thermo Fisher Scientific, catalog number: C10423). These analyses were followed by immunocytochemistry in order to identify the phenotype of cells exhibiting apoptotic markers.

Immunocytochemistry

After rinsing with PBS, the cells were fixed in 4% paraformaldehyde (Sigma-Aldrich, catalog number: P6148) for 10 min. This was followed by permeabilization with 1% Triton X-100 (Sigma-Aldrich, catalog number: T9284) in PBS for 5 min. Non-specific binding was reduced by incubating the cells with 20% FBS in PBS with 0.1% Tween (PBS-T) for 60 min at 20–25°C. The cells were then incubated with primary antibodies overnight at 4°C: rabbit anti-GPER (1 : 200; Santa Cruz Biotechnology, RRID: AB_2112494), mouse anti-microtubule-associated protein 2 (MAP2; 1 : 500; Santa Cruz Biotechnology, RRID: AB_1126215), or rabbit anti-glial fibrillary acidic protein (GFAP; 1 : 2000; DAKO, RRID: AB_10013382). The specificity of the GPER antibody was analyzed by western blot.

After a washing step with PBS-T to remove unbound antibodies, the cells were incubated for 1 h at 20–25°C with the secondary antibodies [1 : 1000; anti-rabbit conjugated to Alexa Fluor 546 (Invitrogen, RRID: AB_143156), anti-mouse conjugated to Alexa 488 (Invitrogen, RRID: AB_2633275), and anti-mouse conjugated to Alexa 546 (Invitrogen, RRID: AB_141370)]. The coverslips were further washed with PBS-T and then incubated for 10 min with 2 mM Hoechst 33342 (Invitrogen, RRID: AB_2651133). Finally, the cells were washed with PBS-T and the coverslips mounted in mounting medium (DAKO, catalog number: CS703). Images were acquired in an epifluorescence microscope (Zeiss, AxioObserver Z1) with a 63x objective.

Quantification of cell number

For the quantification of the number of cells, at least three experiments were performed with distinct cellular preparations. In each experiment, three coverslips per condition were prepared and 20 fields per coverslip were analyzed. Cell number was assessed by counting the nuclei stained with Hoechst 33342, the number of neurons was assessed by counting the cells expressing the neuronal marker MAP2, and the number of astrocytes was determined by quantifying the cells labeled for the astrocytic marker GFAP.

Quantification of GPER expression

The analyses of fluorescence intensity were performed with the ImageJ software (National Institutes of Health, RRID: SCR_003070). The cells of interest were delineated and the mean

fluorescence intensity (MFI) of the cell was measured. After this, a region without fluorescence was selected and used for background reading. The MFI of each cell was calculated using the formula: MFI = mean density of selected area – mean intensity of background reading.

The intensity of GPER staining was quantified in neurons and astrocytes in control and OGD conditions. For this quantification, experiments were performed in at least three distinct cellular preparations. In each experiment, three coverslips per condition were prepared and 30 cells of each cell population were randomly selected on each coverslip.

Calcium imaging

Neuronal and astrocytic monocultures were incubated with 5 μM Fura-2/AM (Thermo Fischer Scientific, catalog number: F1221) in sodium buffered solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 20 mM HEPES, pH 7.35) for 45 min at 37°C. The medium was then replaced by sodium buffered solution followed by another 45 min incubation at 37°C to allow complete de-esterification of intracellular AM esters. After that, the glass bottom dishes were mounted on the stage of an inverted microscope (Zeiss, AxioObserver Z1) and Fura-2 was alternately excited at 340 and 380 nm and the emitted fluorescence, filtered at 510 nm, was collected every 10 s. This protocol included a 3-min period to establish the baseline values, exposure to 100 nM G1 for 10 min followed by incubation with 50 mM KCl buffered solution to induce depolarization (145 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 20 mM HEPES, pH 7.35) for further 10 min. To test the effect of blockade of the PLC pathway, U73122 (10 nM) was added to the cultures 30 min before incubation with G1.

The collected images for each wavelength were transformed into time-lapse calcium measurements and treated using the ImageJ software. The region of interest tool was used to delineate the cells and the 340/380 nm ratio of Fura-2/AM intensity was calculated and representative traces of F340/F380 ratio were drawn with GraphPad v.4 (GraphPad Software Inc.). To evaluate the intracellular calcium, experiments were performed in three distinct cellular preparations. In each experiment, three coverslips per condition were prepared and approximately 25 cells per coverslip were analyzed.

Statistical analysis

The results are expressed as number of cells, the percentage of values obtained in control conditions, or the maximal ratio values of F340/F380 in each stimulus (G1 and KCl) and are presented as the mean ± SEM of at least three independent cell culture preparations, performed in triplicate. For the identification of outliers, the Grubb's method was used.

Statistical analysis was performed using the Student's *t*-test and the one- or two-way ANOVA as specified in the figure legends, followed by Bonferroni's *post hoc* test. Values of $p < 0.05$ were considered statistically significant. All statistical analyses were performed using the GraphPad Prism Software (RRID: SCR_002798). No statistical methods were employed to predetermine sample size of any of the presented experiments and no tests for normal distribution were performed.

Results

GPER activation does not protect from an injury induced by OGD

It has been shown that estrogens, particularly E2, can act as neuroprotective agents in ischemic insult (Brann *et al.* 2007). In order to determine whether this protection may involve the activation of GPER, primary neuron–glia cortical cultures were exposed to G1 (100 and 200 nM) (Bologa *et al.* 2006) 24 h prior to the OGD period. Cell viability was assessed by using the MTT assay and the number of cells was analyzed by immunocytochemistry. The exposure of primary neuron–glia cortical cultures to 4 h of OGD decreased MTT reduction by 36.9 ± 6.8%, when compared to control cultures (Fig. 1a) as well as the total number of cells from 29 ± 2 cells/field to 17 ± 1 cells/field (41.9% decrease, Fig. 1b). The results showed that neurons were more susceptible to OGD (61.2% decrease, Fig. 1c) than astrocytes (36.2% decrease, Fig. 1d). Activation of GPER with G1 did not induce any protection against the ischemic insult, either at 100 nM or 200 nM (Fig. 1a), and thus, 100 nM G1 was used in all subsequent experiments. Interestingly, exposure to G1, per se, in the absence of OGD induced a significant decrease in cell number (31.1% decrease, from 29 ± 2 cells/field to 20 ± 2 cells/field, Fig. 1b). Data from the immunocytochemistry analysis showed that this reduction was because of the loss of astrocytes and did not involve a loss of neurons (Fig. 1c and d).

GPER blockade protects astrocytes from OGD-induced injury

To further explore the role of GPER under ischemic conditions, we analyzed the effect of G15, a GPER selective antagonist (Dennis *et al.* 2009). Incubation with 100 nM G15 increased MTT reduction in cells exposed to OGD by 14.1 ± 5.6% (Fig. 2a) and increased the cell number by 33.9% (from 17 ± 1 cells/field under OGD conditions to 23 ± 1 cells/field in OGD plus G15, Fig. 2b), suggesting that, under ischemic conditions, GPER blockade may elicit cell protection.

To assess the cellular targets of the protection afforded by G15 under ischemic conditions, we analyzed the impact of G15 treatment on neurons ($MAP2^+$ cells) and astrocytes ($GFAP^+$ cells). Immunocytochemistry analysis showed that GPER blockade selectively protected astrocytes as treatment with G15 completely reduced $GFAP^+$ cell loss to levels similar to control conditions without altering the number of neurons (Fig. 2c and d).

Control experiments using 100 nM E2 showed that it improved MTT reduction in OGD-exposed cells (35% increase, Fig. 2a) and promoted a 20.6% increase in cell number (from 17 ± 1 cells/field in OGD to 21 ± 2 cells/field in OGD + E2, Fig. 2c) although not statistically significant. The protection induced by E2 was not affected by

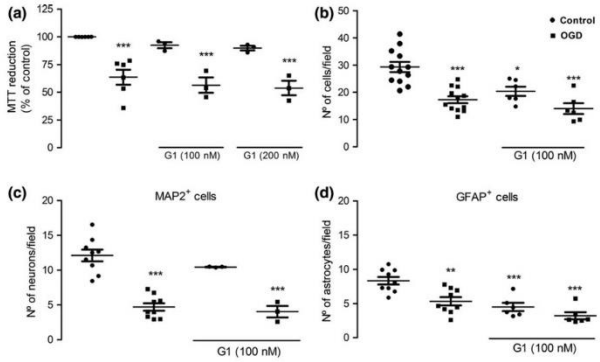

Fig. 1 Effect of G1 on rat primary neuron–glia cortical cultures exposed to 4 h of oxygen and glucose deprivation (OGD). (a) Evaluation of cell viability through the MTT assay. The results are expressed as percentage of control and represent the mean ± SEM of three independent cell culture preparations performed in quadruplicate. (b) Evaluation of the total number of cells through Hoechst 33342 staining. The results are expressed as the number of cells per field and represent the mean ± SEM of nine (control) or three independent cell culture preparations (other experimental conditions) performed in triplicate. Evaluation of the number of neurons (c) and astrocytes (d) through MAP2 and GFAP immunocytochemistry. The results are expressed as the number of cells per field and represent the mean ± SEM of 3–12 independent cell culture preparations performed in triplicate. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's *post hoc* test. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared to control; *ns*, not significant.

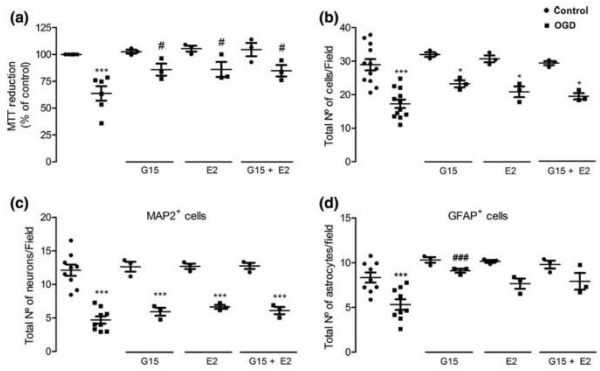

Fig. 2 Effect of G protein-coupled estrogen receptor (GPER) inhibition on the viability of primary neuron–glia cortical cultures exposed to 4 h of oxygen and glucose deprivation (OGD). (a) Evaluation of cell viability through the MTT assay. (b) Evaluation of the total number of cells through Hoechst 33342 staining. Evaluation of the number of neurons (c) and astrocytes (d) through MAP2 and GFAP immunocytochemistry. The results are expressed as the number of cells per field and represent the mean ± SEM of 3–9 independent cell culture preparations performed in triplicate. Statistical analysis was performed using the two-way ANOVA followed by Bonferroni *post hoc* test. $*p < 0.05$, $***p < 0.001$ compared to control; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ compared to OGD; *ns*, not significant.

the simultaneous presence of G15 (Fig. 2a), suggesting that E2-mediated protection did not involve GPER. Interestingly, the protective effect of E2 was specific for GFAP$^+$ cells (Fig. 2c and d).

OGD does not induce modifications in GPER expression

To determine if the distinct effects of GPER activation on cell survival in OGD-exposed cultures were related to a differential expression of the receptor in neurons and

nonneuronal cells, we analyzed the intensity of GPER staining by immunocytochemistry. The results showed that GPER was expressed by both neurons and nonneuronal cells (Fig. 3a). Furthermore, quantification of fluorescence intensity showed that neurons presented higher levels of GPER staining than nonneuronal cells (Fig. 3b). Additionally, OGD did not alter the pattern of GPER staining, but there was a small, nonstatistically significant, decrease in GPER immunoreactivity in nonneuronal cells levels subjected to OGD (20.6 ± 12.2% reduction in MFI, as compared to control). It is also important to note that GPER seems to have a similar expression pattern in both populations, with a more intense labeling in the perinuclear region (Fig. 3a).

GPER activation promotes apoptosis in astrocytes

To get further insight into the effect of G1 on astrocytes and neurons, we used different markers of apoptosis. As expected, exposure to OGD leads to a 75.5% increase in the number of non-astrocytic cells with nuclei presenting apoptotic morphology (Fig. 4a and c), a 107.6% increase in the number of non-astrocytic cells labeled for annexin V (Fig. 5a and c), and a 12.5-fold increase in the number of neurons labeled for activated caspase 3/7. On the contrary, exposure of non-astrocytic cells to G1 did not alter any of the apoptotic markers analyzed (Figs 4a, 5a, and 6a).

OGD promoted a significant increase in the number of astrocytes exhibiting nuclei with apoptotic morphology and a significant increase in annexin V and active caspase 3/7 labeling. In G1-treated cultures, we found increased number of astrocytes with nuclei presenting apoptotic morphology (125.9% increase, Fig. 4b and d), labeling for annexin V (308.6% increase, Fig. 5b and d), and labeling for active caspase 3/7 (282.1% increase, Fig. 6b and d). Moreover, G1 aggravated the effect of OGD on astrocytes, with a 126.81% increase in astrocytes presenting nuclei with apoptotic morphology, a 352.25% increase in annexin V-labeled astrocytes, and a 239.7% increase in caspase 3/7-labeled astrocytes, as compared to OGD (Figs 4b, 5b and 6b).

Blockade of the PLC pathway prevents G1-induced apoptosis in astrocytes

Next, we sought to identify the signaling pathway involved in the harmful effect induced by GPER activation in astrocytes. For that, we have considered two independent pathways activated by stimulation of GPER and reported to have proapoptotic effects, the PLC (Takuma *et al.* 2004, 1999) and JNK (Okamoto *et al.* 2017; Chimento *et al.* 2012) pathways. The selective blockade of each pathway demonstrated the involvement of PLC since the number of astrocytes labeled for active caspase 3/7 decreased approximately 50% when PLC was inhibited with 10 nM U73122. There was a decrease of 21% in the number of astrocytes labeled for active caspase 3/7 when the JNK pathway was inhibited using 10 μM SP600125, but this beneficial effect did not reach statistical significance. The results also indicate that the activation of GPER seems to activate different intracellular signaling pathways on these two distinct populations of cells.

Exposure to G1 induces an increase in intracellular calcium levels in astrocytes, but not in neurons

We found that G1 had no effect on neuronal cell viability (Figs 4a, 5a and 6a), whereas the activation of the PLC pathway contributed to G1-induced astrocyte loss (Fig. 7a). Since elevation of cytosolic Ca^{2+} levels plays a role in astrocyte apoptosis (Takuma *et al.* 2004), we hypothesized that G1 might induce distinct signaling pathways in astrocytes and neuronal cells. To address this possibility, we analyzed changes in $[Ca^{2+}]_i$ by Fura-2 imaging in astrocyte and neuronal cell cultures. We observed that exposure to 100 nM G1 promoted a rise in $[Ca^{2+}]_i$ in astrocytes (Fig. 8a), but not in neurons, which, however, responded with an increase in

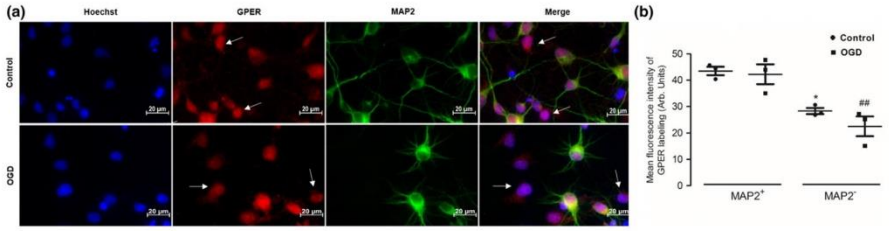

Fig. 3 G protein-coupled estrogen receptor (GPER) staining in neurons and nonneuronal cells under control and ischemic conditions. (a) Representative images of MAP2 (green), GPER (red), and Hoechst 33342 staining (blue), magnification 63x. Arrows indicate $MAP2^-$ cells expressing GPER; (b) Quantification of the mean fluorescence intensity (MFI) of GPER in neurons ($MAP2^+$ cells) and non-neuronal cells ($MAP2^-$ cells). Results represent the mean ± SEM of three independent cell culture preparations performed in triplicate. Statistical analysis was performed using one-way ANOVA followed by Bonferroni *post hoc* test. $^*p < 0.05$ compared to neurons in control conditions; $^{\#\#}p < 0.01$ compared to neurons in oxygen and glucose deprivation (OGD) condition; *ns*, not significant.

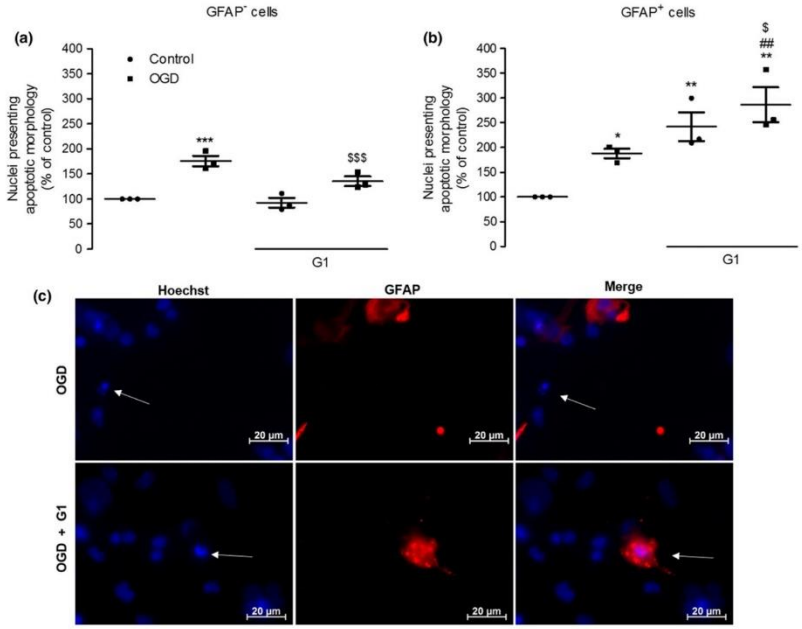

Fig. 4 Evaluation of nuclei with apoptotic morphology in primary neuron–glia cortical cultures exposed to oxygen and glucose deprivation (OGD). Quantification of non-astrocytic cells (a) and astrocytes (b) with nuclei presenting apoptotic morphology. (c) Representative images of immunocytochemistry for GFAP (red) and Hoechst 33342 staining (blue) in cells exposed to OGD in the absence or presence of 100 nM G1. Arrows indicate nuclei with apoptotic morphology. All images were obtained with a 63x objective. The results are expressed as percentage of control and represent the mean ± SEM of three independent cell culture preparations performed in triplicate. Statistical analysis was performed using two-way ANOVA followed by Bonferroni *post hoc* test $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to control; $^{\#\#}p < 0.01$ compared to OGD; $^{\$}p < 0.05$ and $^{\$\$\$}p < 0.001$ compared to control exposed to G1.

$[Ca^{2+}]_i$ to a depolarization with K^+ (50 mM) (Fig. 8c). In line with the results concerning the number of active caspase 3/7-positive astrocytes in cultures exposed to G1 (Fig. 7a), the rise of approximately 50% in F340/F380 when compared to baseline values was completely inhibited by the PLC inhibitor U73122 (Fig. 8a), thus indicating that GPER is coupled to the PLC pathway in astrocytes, but not in neurons.

Discussion

Neuroprotective effects of estrogens, particularly E2, have been debated for decades and previous reports demonstrate that estrogens can regulate the development, maturation, survival, and function of multiple cell populations in different brain regions (Srivastava *et al.* 2013; Scott *et al.* 2012; Gillies and McArthur 2010). Several studies were carried out in an attempt to clarify the possible involvement of GPER in the neuroprotective role of E2 in cerebral ischemia, but the results are inconsistent. Selective activation of GPER *in vivo* has been described as having beneficial (Kosaka *et al.* 2012; Lebesgue *et al.* 2010; Prossnitz and Barton 2014), detrimental (Broughton *et al.* 2014), or sex-dependent effects (Broughton *et al.* 2014).

The results from the present study demonstrate that neuronal survival was strongly affected by an ischemic insult and, in contrast, OGD had a smaller impact on astrocytes survival, which is in accordance with other studies performed in *in vitro* models of ischemia (Choi 1996; Jones *et al.* 2011). The results indicated that exposure to G1 did not induce any protection against the ischemic insult. These results are similar to that described by Lamprecht and Morrison (2014) using an organotypic hippocampal slice culture model in which exposure to G1 after OGD did not provide recovery from the ischemic injury (Lamprecht and

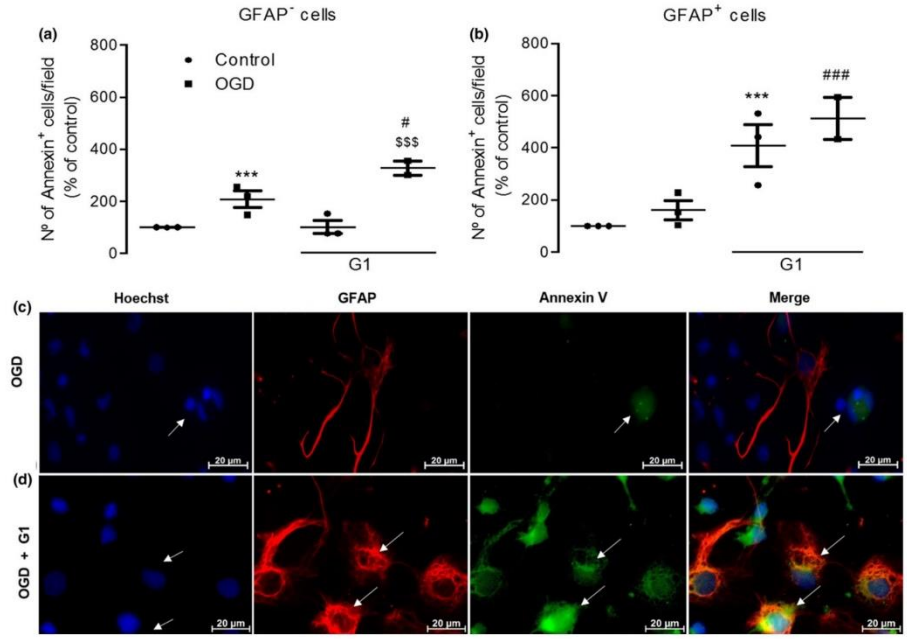

Fig. 5 Effect of G protein-coupled estrogen receptor (GPER) activation on annexin V labeling in primary neuron–glia cortical cultures exposed to oxygen and glucose deprivation (OGD). Quantification of non-astrocytic cells (a) and astrocytes (b) labeled for annexin V. (c) Representative images of immunocytochemistry for GFAP (red), annexin V (green), and Hoechst 33342 staining (blue) in cultures exposed to OGD in the absence or in the presence of 100 nM G1. Arrows indicate cells labeled for annexin V. All images were obtained with a 63x objective. Results are expressed as percentage of control and represent the mean ± SEM of three independent cell culture preparations performed in triplicate. Statistical analysis was performed using two-way ANOVA followed by Bonferroni's *post hoc* test. $^{***}p < 0.001$ compared to control; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ compared to OGD, and $^{\$\$\$}p < 0.001$ compared to control exposed to G1; *ns*, not significant.

Morrison 2014). In fact, our results demonstrate that exposure to G1 has detrimental effects, similar to that described in a transient middle cerebral artery occlusion (tMCAO) model subjected to pretreatment with G1 (Broughton *et al.* 2014). In this model, G1 exacerbated the infarct volume size and worsened functional outcomes after ischemia in male mice. An increase in activated caspase 3 in the peri-infarct area was also reported (Broughton *et al.* 2014). Our data also indicate that the selective pharmacological blockade of GPER reduces the cell loss induced by the ischemic insult, suggesting that activation of GPER in basal conditions, probably by locally produced estradiol, contributes to astrocyte loss. In agreement with our findings, Broughton *et al.* (2014) demonstrated that the detrimental effects of G1 were blocked by G15 (Broughton *et al.* 2014). In fact, G15 improved functional outcomes and reduced infarct volume size after an IS, whether given before or after ischemia (Broughton *et al.* 2014). Remarkably, our results indicate that GPER blockade had no impact on OGD-induced neuronal loss, but prevented the loss of astrocytes.

Exposure to E2 induced a significant protection against the ischemic insult, as assessed by the MTT assay, but this effect was not altered by the presence of G15, thus indicating that the protection afforded by E2 was not mediated by the GPER pathway. Similar to this, Lamprecht and collaborators showed that GPER activation was not necessary for estrogen-mediated neuroprotection after ischemia (Lamprecht and Morrison 2014). However, others demonstrated that G1 exerts significant neuroprotection against ischemia through the rapid activation of the pro-survival kinases, Akt and ERK, while decreasing activation of the proapoptotic kinase JNK (Tang *et al.* 2014).

Assessment of GPER expression in primary cortical cultures showed that the receptor was expressed by neurons

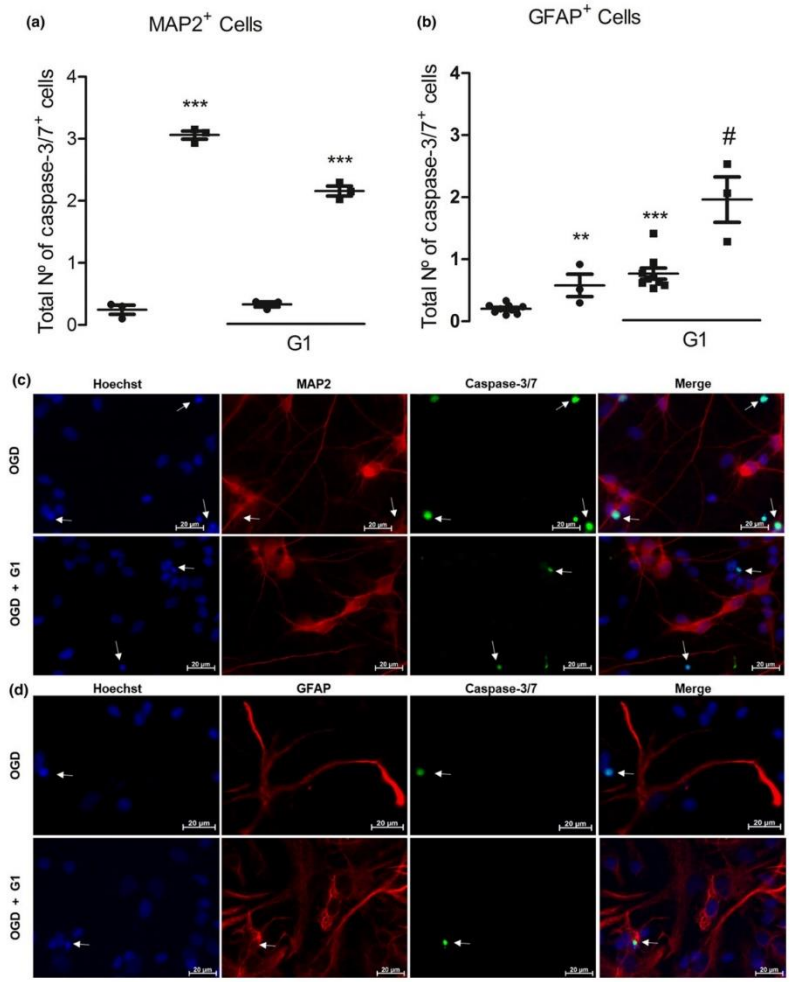

Fig. 6 (a) Effect of G protein-coupled estrogen receptor (GPER) stimulation on caspase 3/7 activation in primary neuron–glia cortical cultures exposed to oxygen and glucose deprivation (OGD). Evaluation of neuronal (a) and astrocytic (b) loss through the quantification of $MAP2^+$ and $GFAP^+$ cells labeled with caspase 3/7. Results are expressed as the number of cells per field and represent the mean ± SEM of three independent cell culture preparations performed in triplicate. Statistical analysis was performed using two-way ANOVA test followed by Bonferroni *post hoc* test. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to control and $^{\#}p < 0.05$ compared to OGD; ns, not significant. (b) Representative images of immunocytochemistry for MAP2 (red), caspase 3/7 (green), and Hoechst 33342 staining (blue) in cultures exposed to OGD in the absence or in the presence of 100 nM G1 (c). Representative images of immunocytochemistry for GFAP (red), caspase 3/7 (green), and Hoechst 33342 staining (blue) in cultures exposed to OGD in the absence or in the presence of 100 nM G1 (d). Arrows indicate caspase 3/7-labeled cells. All images were acquired with a 63x objective.

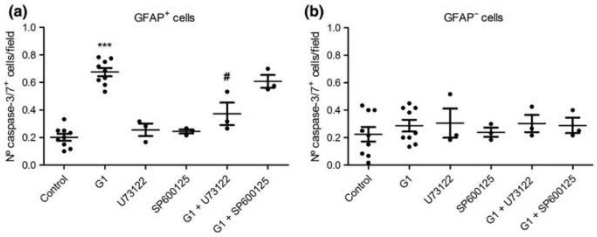

Fig. 7 Contribution of PLC and c-Jun N-terminal kinase (JNK) pathways to the deleterious effect of G protein-coupled estrogen receptor (GPER) activation in astrocytes. Quantification of GFAP$^+$ cells labeled with caspase 3/7 in cultures incubated with 100 nM G1 in the presence and in the absence of the PLC inhibitor U73122 or the JNK inhibitor SP600125. Results are expressed as the number of cells per field and represent the mean ± SEM of three independent cell culture preparations performed in triplicate. Statistical analysis was performed using the one-way ANOVA test followed by Bonferroni's *post hoc* test. $^{***}p < 0.001$ compared to control and $^{\#}p < 0.05$ compared to G1.

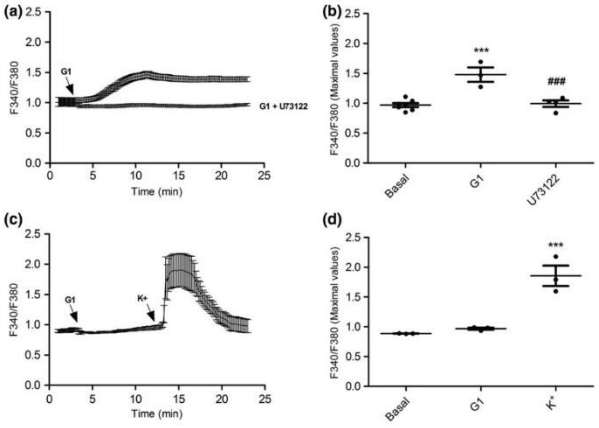

Fig. 8 Cell type-specific changes in intracellular calcium levels triggered by G1. Representative traces of F340/F380 registered in astrocyte (a) and neuronal (b) cultures in response to 100 nM G1 and 50 mM K$^+$. Some astrocyte cultures were previously treated with U73122. The maximal values of F340/F380 obtained in each experimental condition are also shown. Results represent the mean ± SEM of three independent cell culture preparations performed in triplicate. Statistical analysis was performed using the using the one-way ANOVA test followed by Bonferroni's *post hoc* test $^{***}p < 0.001$ compared to basal condition, $^{\#\#\#}p < 0.001$ compared to G1 condition.

and astrocytes, which is in accordance with that previously described in rat midbrain neuron–glia cultures (Bessa *et al.* 2015). Furthermore, neurons presented higher intensity of labeling for the receptor than glial cells, but the levels of GPER in both cell populations were not affected by OGD. In contrast, a significant increase in the expression of GPER was reported in the hippocampus, somatosensory cortex, and hypothalamus of male mice after stroke (Broughton *et al.* 2013) and also in the motor cortex of postischemic female rats (Zhao *et al.* 2016). Such discrepant effects might be related with the stroke model used and its possible limitations on the extrapolation of findings from an *in vitro* model to an *in vivo* situation. On *in vivo* models, cells are exposed to stimuli such as the circulating hormones, like estrogens, or could be affected by the presence of other cell types, like vascular cells. Although an *in vitro* model provides a more controlled environment for the study of cellular mechanisms, it does not contemplate this type of interactions or how they may affect GPER expression.

Exposure of astrocytes to G1 promoted cell death and potentiated the apoptosis triggered by OGD. To the best of our knowledge, this is the first time that this detrimental effect is described in astrocytes. However, these proapoptotic effects of G1 are in accordance with data from Ding *et al.* (2009) showing that activation of GPER induced apoptosis in rat aortic vascular smooth muscle cells by a process involving the activation of ERK and PKA inhibition (Ding *et al.* 2009). Contrariwise, there are also studies demonstrating that nonselective activation (with selective estrogen receptor modulators) of GPER in astrocytes induces general beneficial effects through the induction of anti-inflammatory effects (Suuronen *et al.* 2005; Cerciat *et al.* 2010) and the

reduction in extracellular glutamate levels by promoting the expression of the glutamate transporter 1 (Lee *et al.* 2012).

The inhibition of the PLC pathway with U73122 reduced G1-induced astrocyte apoptosis by half, suggesting that PLC is highly involved in this detrimental effect induced by GPER activation. The analysis of $[Ca^{2+}]_i$ levels in neurons and astrocytes indicated that the activation of GPER induced cell type-specific signaling. Astrocytes exhibited a slow rise of $[Ca^{2+}]_i$ levels upon GPER selective activation, and these changes were completely prevented by the PLC inhibitor U73122, confirming the involvement of this signaling pathway. These results allow to establish a connection between the stimulation of the GPER, the activation of the PLC pathway, and the consequent increase in $[Ca^{2+}]_i$ levels as the trigger of this negative effect observed in astrocytes.

PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to the second messenger molecules inositol 1,4,5-trisphosphate and diacylglycerol (Liao and Carpenter 2009; Agulhon *et al.* 2008), which, in turn, control $[Ca^{2+}]_i$ and protein kinase C activity (PKC), respectively(Liao and Carpenter 2009; Agulhon *et al.* 2008). The increase in inositol 1,4,5-trisphosphate induces the mobilization of $[Ca^{2+}]_i$ by regulating the release of Ca^{2+} from intracellular organelles to the cytosol (Liao and Carpenter 2009; Agulhon *et al.* 2008). Several authors have already reported that the increase in $[Ca^{2+}]_i$ in astrocytes is associated with the activation of proapoptotic pathways (McConkey and Orrenius 1997; Agulhon *et al.* 2008; Takuma *et al.* 1999, 2004; Then *et al.* 2017) and cell death (Matsuda *et al.* 1996; Wang *et al.* 2012; Hirata *et al.* 2006). The mechanisms behind this effects were associated with DNA fragmentation (Takuma *et al.* 2004), nuclear condensation through the activation of caspase 3 (Takuma *et al.* 1999, 2004; Then *et al.* 2017), mitochondrial dysfunction(Then *et al.* 2017), reactive oxygen species generation (Then *et al.* 2017), and production of cleaved-poly(ADP-ribose) polymerase (Then *et al.* 2017). Thus, our results provide further evidence that there is an association between increased $[Ca^{2+}]_i$ and the induction of apoptosis in astrocytes.

Diacylglycerol triggers PKC activation and subsequent activation of the ERK pathway, a known proapoptotic signaling mechanism (Ueda *et al.* 1996). For example, Ding *et al.* (2009) associated the activation of ERK with the proapoptotic effects induced by GPER in rat aortic vascular smooth muscle cells (Ding *et al.* 2009). We speculate that the same proapoptotic cascade may occur in astrocytes since PKC activation is associated with apoptosis of astrocytes through the caspase 9 and caspase 3 cascade, whereas the inhibition of PKC pathway decreased cell death (Kanno and Nishizaki 2011).

Here, we show that the stimulation of GPER in glial cells promotes apoptosis and aggravates OGD-induced astrocyte loss. Since we used an *in vitro* model of stroke, where blood circulation, blood–brain barrier, and other components that interact directly within the CNS are absent, we cannot rule out the possibility that the effect of G1 *in vivo* might be different. Indeed, it was demonstrated that selective activation of GPER induces vasodilatation, an effect which alone may lead to better outcomes after stroke (Lindsey *et al.* 2011). Other studies have shown that G1 administration immediately upon reperfusion decreases blood–brain barrier breakdown after an ischemic insult (Lu *et al.* 2016), and that the GPER agonist has the ability to induce dilation and restore the function of cerebral arterioles after an ischemic injury (Murata *et al.* 2013). Other mechanism that might influence the outcome of GPER activation *in vivo* is the inflammation mediated by microglia. Microglial GPER was shown mediate anti-inflammatory effects after ischemic stroke (Zhao *et al.* 2016), thus indicating that the protection induced by GPER activation *in vivo* can be partially because of the anti-inflammatory role of GPER.

At a therapeutic standpoint, our results may indicate that an eventual selective activation of GPER may not be an appropriate treatment to protect from an ischemic injury. The GPER selective agonist did not induce neuronal protection after the ischemic insult, contrary to what is observed in other brain pathologies, as it is the case of Parkinson′s disease (Bessa *et al.* 2015; Mendes-Oliveira *et al.* 2017; Guan *et al.* 2017), Alzheimer′s disease (Kubota *et al.* 2016), or multiple sclerosis (Wang *et al.* 2009; Blasko *et al.* 2009). Moreover, we also observe that the selective activation of GPER may induce deregulation of calcium homeostasis in astrocytes, which was associated to its apoptosis, thereby having a detrimental effect. However, more studies will be needed to confirm this theory.

Overall, the results from the present study demonstrated that selective activation of GPER induces different signaling pathways in neurons and astrocytes. GPER activation in neurons has no impact on neuronal viability, either in control or under ischemic conditions. Conversely, in astrocytes, selective GPER activation induces apoptosis and increases the loss of astrocytes triggered by ischemia. Our findings indicate that the stimulation of GPER triggers a rise in $[Ca^{2+}]_i$ levels and consequent death of astrocytes, whereas the blockade of PLC pathway prevents both processes. To the best of our knowledge, this is the first report showing that the activation of GPER in astrocytes induces their apoptosis through the PLC pathway.

Acknowledgments and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

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PERSPECTIVE

G protein-coupled estrogen receptor 1 (GPER) activation triggers different signaling pathways on neurons and astrocytes

Estradiol (E2) is the most potent and prevalent form of estrogen, a well-known hormone that regulates multiple tissues and functions in humans. In the brain, E2 regulates processes as diverse as learning, memory, cognition, mood, as well as neurodevelopment and neurodegeneration. The actions of E2 are mediated by classical estrogen receptors (ERs; α and β), and by the G protein-coupled estrogen receptor 1 (GPER or GPR30) (Prossnitz and Arterburn, 2015). Classical ER are predominantly present in the nucleus and cytoplasm, with less than 2% present on the plasma membrane, and mediate genomic cellular effects that occur in the time frame of hours to days (Prossnitz and Arterburn, 2015). GPER is expressed on the plasma membrane, and on intracellular membranes of the endoplasmic reticulum and Golgi apparatus, and mediates rapid estrogen-induced effects that occur in the time frame of seconds to minutes (Prossnitz and Arterburn, 2015).

The cellular and molecular effects triggered by the activation of classical ER on brain cells are well known. However, they cannot explain all the effects induced by E2. It is well established that when E2 binds to classical ER there are modifications in their structure that lead to the formation of homodimers and/or heterodimers (Prossnitz and Arterburn, 2015). These dimers bind to estrogen response elements (EREs) in the DNA and recruit other components of the transcriptional machinery, leading to gene expression (Prossnitz and Arterburn, 2015). Nevertheless, there are also cytoplasmic signaling events or transduction cascades initiated on the plasma membrane, classified as non-genomic, such as, modulation of intracellular calcium, the production of cyclic adenosine monophosphate (cAMP), regulation of phosphoinositide 3-kinase (PI3Ks), and mitogen-activated protein kinases (MAPKs)/extracellular signal-regulated kinases (ERKs), being these non-genomic pathways associated with the activation of GPER (Prossnitz and Arterburn, 2015). Additionally, the rapid signaling events initiated by GPER upregulate the expression of genes such as c-fos, cyclin D2 and Bcl-2 (Prossnitz and Arterburn, 2015).

The signaling mechanisms activated by GPER in neural tissues are not completely understood, and the available data focus mainly on the effects triggered in neurons (Evans et al., 2016). GPER activation in neurons promotes the activity of pro-survival kinases such as PI3K/Akt (Tang et al., 2014; Cheng et al., 2016) and ERK (Tang et al., 2014), and attenuates the pro-apoptotic pathway JNK (Tang et al., 2014; Cheng et al., 2016). Besides that, the GPER agonist G1 also induces the activation of adenylyl cyclase and the consequent rise in cAMP levels in a dose-dependent manner in neurons (Evans et al., 2016). This intracellular messenger has a preponderant role in several signal transduction cascades and thus it is likely that, in addition to the above mentioned signaling pathways, others may also be influenced by the selective activation of GPER.

In contrast, we have previously demonstrated that the selective activation of GPER in astrocytes with G1 induces their apoptosis, while having no impact on neuronal survival (Roque et al., 2018). The differential effects are due to the activation of different signaling pathways on each type of cell (Roque et al., 2018). In astrocytes, but not in neurons, G1 induces the activation of phospholipase C (PLC) and a rise in intracellular calcium levels that triggers astrocytes apoptosis (Roque et al., 2018). These pro-apoptotic effects of G1 were also observed in other cell types, such as the vascular smooth muscle cells (Ding et al., 2009). In this case, the effects were associated with the activation of ERK and inhibition of PKA (Ding et al., 2009). Moreover, GPER could have the ability to control microglial reactivity through the decrease of phagocytic activity, inducible nitric oxide synthase expression and nitric oxide (NO) release (Mendes-Oliveira et al., 2017).

The existence of cell type-specific signaling pathways in response to activation of estrogen receptors was already proposed years ago. Mhyre and Dorsa (2006) reported that the activation of classical ER triggers different rapid signaling pathways in neurons and astrocytes. In neuronal cells the activation of classical ER is coupled to the activation of the MAPK and cAMP response element-binding protein (CREB) pathways (Mhyre and Dorsa, 2006). Conversely, in astrocytes E2 does not increase the phosphorylation of MAPK or CREB pathways, but instead activates signaling pathways leading to inhibition of cAMP response elements (CRE) and CRE-mediated transcription (Mhyre and Dorsa, 2006). Since classical ER and GPER activate common signaling pathways (Prossnitz and Arterburn, 2015), we hypothesize that the same cell type-specific response may be induced by GPER selective activation. Besides the PLC pathway (Roque et al., 2018), other signaling pathways, involving cAMP and/or MAPK, may be involved in these cell type-specific signaling mechanisms.

It is known that MAPK activation provides cell type-specific signals important for cellular differentiation, proliferation, and survival, and that cAMP has divergent effects on MAPK activity (Dugan et al., 1999; Qiu et al., 2000). Activation of the MAPK pathway by cAMP requires the presence of B-raf, and neurons, but not glial cells, express it (Dugan et al., 1999; Qiu et al., 2000). Thus, it is possible that this difference will lead to specific actions of cAMP on the MAPK pathway in these two cell populations. Indeed, this is supported by studies showing that cAMP activates the MAPK pathway in neurons (Dugan et al., 1999; Qiu et al., 2000). In astrocytes, cAMP reduces MAPK activity (Dugan et al., 1999; Qiu et al., 2000), but transfection of B-raf enabled MAPK activation in response to cAMP in these cells (Dugan et al., 1999; Qiu et al., 2000). These effects on MAPK pathway were also associated to the reduction of cell growth (Dugan et al., 1999). Although these observations suggest that neurons and astrocytes respond differently to GPER selective activation due to a differential activation of the MAPK pathway, it is necessary to address directly this hypothesis by evaluating the modulation of MAPK pathway upon selective activation of GPER.

As previously mentioned, the activation of the PI3K/Akt pathway by GPER agonists was consistently associated with protective effects mediated by this receptor (Prossnitz and Arterburn, 2015), with several studies showing that GPER promotes neuronal survival through activation of this pathway (Tang et al., 2014; Cheng et al., 2016). Regarding astrocytes, there is no data about the activation of the PI3K/Akt pathway by GPER. However, it was demonstrated that cAMP inhibits the PI3K/Akt pathway in astrocytes (Wang et al., 2001; Sugimoto et al., 2011), being these effects reversed by the constitutively active form of PI3K (Wang et al., 2001). This inhibition was associated with the presence of several apoptotic markers, such as morphological changes, increase of cleaved caspase-3, condensation and fragmentation of nuclei, and a decrease in the number of cells (Sugimoto et al., 2011). These data suggest a differential effect of cAMP on neurons and astrocytes caused by a different regulation of the PI3K/Akt pathway on these two cell populations. Again, this needs to be evaluated experimentally.

In summary, existing data indicates that selective activation of GPER triggers different signaling pathways in neural cells resulting in cell type-specific responses (**Figure 1**). Activation of GPER in

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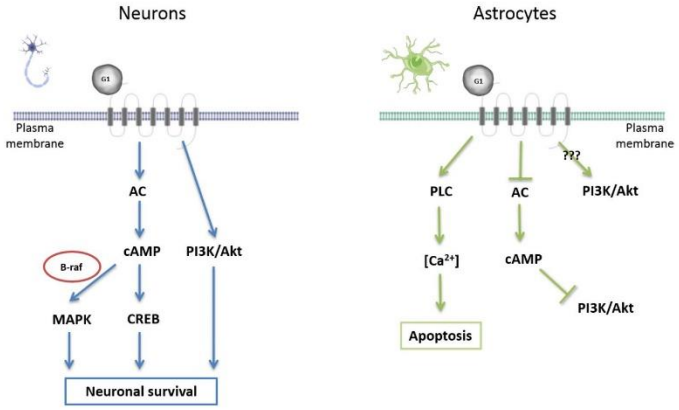

Figure 1 Cell type-specific signaling pathways activated by GPER on neurons and astrocytes.
On neuronal cells the activation of GPER is associated to an increase of neuronal survival, *via* the increase of cAMP levels and the activation of MAPK and CREB pathways, and *via* the activation of PI3K pathway. On astrocytes the activation of GPER is associated to a reduction of cAMP levels and to cell death due to the activation of PLC pathway and consequent rise in intracellular calcium levels. GPER: G protein-coupled estrogen receptor 1; AC: adenylate cyclase; CREB: cAMP response element-binding protein; cAMP: cyclic adenosine monophosphate; GPER: G protein-coupled estrogen receptor 1; $[Ca^{2+}]$: intracellular calcium; MAPK: mitogen-activated protein kinase; PI3K: phosphoinositide 3-kinase; PLC: phospholipase C; Akt: protein kinase B.

neurons is associated with the activation of pathways that lead to the promotion of cell survival. In astrocytes, the signaling pathways activated by which GPER exerts its beneficial role in neurons are either absent, or inhibited. Considering the crucial role of glial cells in neuronal physiology, it is likely that any condition that interferes with the normal astrocytic and microglial function affects neuronal physiology. Since the number of studies on the actions of GPER in glial cells is still very limited, it is of utmost importance to deepen the analysis of the effects triggered by GPER activation on this cell population. Clarification of these cell type-specific signaling mechanisms will help to elucidate the potential, and possibly selective, protective role of GPER activation in brain pathologies and neurodegenerative disorders.

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The role of G protein-coupled estrogen receptor 1 on neurological disorders

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ABSTRACT

G protein-coupled estrogen receptor 1 (GPER) is a membrane-associated estrogen receptor (ER) associated with rapid estrogen-mediated effects. Over recent years GPER emerged has a potential therapeutic target to induce neuroprotection, avoiding the side effects elicited by the activation of classical ERs. The putative neuroprotection triggered by GPER selective activation was demonstrated in mood disorders, Alzheimer's disease or Parkinson's disease of male and female *in vivo* rodent models. In others, like ischemic stroke, the results are contradictory and currently there is no consensus on the role played by this receptor. However, it seems clear that sex is a biological variable that may impact the results. The major objective of this review is to provide an overview about the physiological effects of GPER in the brain and its putative contribution in neurodegenerative disorders, discussing the data about the signaling pathways involved, as well as, the diverse effects observed.

1. Introduction

Estradiol (E_2) is a form of estrogen that regulates multiple functions in human body (Brann et al., 2007). It controls ovulation and the development of female sex characteristics, being classically considered a reproductive hormone, due to its well-known role in feedback signaling in the hypothalamic-pituitary-ovarian axis (Brann et al., 2007; Kelly et al., 2005; Petersen et al., 2003).

Estrogens refer to any substance, natural or synthetic, that mimics the effects of the natural hormone (Liang and Shang, 2013). The three major naturally occurring forms of estrogens are estrone, E_2, and estriol, being E_2 the most potent and prevalent form, although several metabolites also have estrogenic hormonal activity (Liang and Shang, 2013). The actions of estrogens are mediated by estrogen receptors (ER) (Hewitt and Korach, 2003). ERα was first described in the 1960s (Soloff and Szego, 1969; Talwar et al., 1964), whereas ERβ was described almost 30 years later (Kuiper et al., 1996). These homologous receptors, described as ligand-activated nuclear transcription factors (Carroll and Brown, 2006), are predominately present in nucleus and cytoplasm, with less than 2% on cellular membrane (Edwards, 2005; Klinge, 2000). Each ER exhibits differential tissue expression patterns, but both regulate gene transcription through classical genomic pathways (Prossnitz and Barton, 2009, 2014; Schultz-Norton et al., 2011), or by modulating cellular signaling pathways such as the mitogen-activated protein kinases (MAPKs)/extracellular signal-regulated kinases (ERKs) (Wade et al., 2001), modulation of intracellular calcium (Brailoiu et al., 2007; Revankar et al., 2005; Roque et al., 2018), cyclic adenosine monophosphate (cAMP) production (Filardo et al., 2002; Thomas and Dong, 2006), and regulation of phosphatidylinositol 3-kinase (PI3Ks) (Revankar et al., 2005).

In the late 1990s, the G protein–coupled estrogen receptor 1 (GPER or GPR30) was identified as a novel estrogen receptor (Filardo et al.,

Abbreviations: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPP+, 1-methyl-4-phenylpyridinium; AD, Alzheimer's disease; ASD, autism spectrum disorder; EAE, autoimmune encephalomyelitis; BLA, basolateral amygdala; BBB, blood-brain barrier; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; JNK, c-Jun N-terminal kinase; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; DAT, dopamine transporter; EGFR, epidermal growth factor receptor; E_2, estradiol; ER, estrogen receptors; ERK, extracellular signal-regulated kinases; ICI-182780, fulvestrant; GPER, G protein–coupled estrogen receptor 1; GDNF, glial cell-derived neurotrophic factor; iNOS, inducible nitric oxide synthase; IP3, inositol 1,4,5-trisphosphate; IFNγ, interferon γ; IS, ischemic stroke; LPS, lipopolysaccharide; mTOR, mammalian target of rapamycin; MMP, matrix metalloproteinase; MCAO, middle cerebral artery occlusion; MAPKs, mitogen-activated protein kinases; MS, multiple sclerosis; NGF, nerve growth factor; NO, nitric oxide; OVX, ovariectomized; OGD, oxygen and glucose deprivation; PD, Parkinson's disease; PNS, peripheral nervous system; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-triphosphate; PLC, phospholipase C; SERD, selective estrogen receptor degrader; SERM, selective estrogen receptor modulator; SSRI, selective serotonin reuptake inhibitors; SK2, small conductance calcium-activated potassium channel 2; SCI, spinal cord injury; TNFα, tumor necrosis factor α; VEGF-A, vascular endothelial growth factor A; VMAT, vesicular monoamine transporter

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2000). It was described as an orphan receptor belonging to the family of 7-transmembrane spanning G protein-coupled receptors (Kvingedal and Smeland, 1997; Owman et al., 1996). In 2000, Filardo et al. demonstrated that E_2-mediated activation of ERK1/2 was dependent on the expression of this receptor, and named it GPR30 (Filardo et al., 2000). In 2005, Revankar et al. (Revankar et al., 2005) and Thomas and Dong (Thomas and Dong, 2006) described the binding of E_2 to GPR30, suggesting that GPR30 was an E_2-binding receptor, which led to its current designation as G protein-coupled estrogen receptor 1 in 2007 (Prossnitz and Barton, 2012). Since its identification, GPER has been described nearly in every system of the human body, including reproductive (Otto et al., 2009; Wang et al., 2008), cardiovascular (Patel et al., 2010; Recchia et al., 2011), endocrine (Hazell et al., 2009) and nervous system (Brailoiu et al., 2007; Hazell et al., 2009).

Estrogens mediate genomic effects through the classical ERs that are characterized by changes in gene transcription and occur in the time frame of hours to days (Prossnitz et al., 2008). Furthermore, it was also reported that estrogens mediate a variety of "rapid" cellular responses that occur in the time frame of seconds to minutes (Prossnitz et al., 2008), inconsistent with *de novo* transcription and protein synthesis (Falkenstein et al., 2000). These rapid estrogen-mediated effects have been associated with the activation of membrane-associated ERs, and are referred as "non-genomic" (Fu and Simoncini, 2008; Levin, 2009). The signaling pathways that triggers these rapid estrogen-mediated effects are diverse and can be induced by ERβ present near or at the plasma membrane (Mitterling et al., 2010), by the translocation of ERβ to the plasma membrane after E_2 treatment (Sheldahl et al., 2008), by the interaction of non-membrane ERα and ERβ with integral membrane proteins (Boulware et al., 2013; Boulware et al., 2005) or through the activation of GPER (Filardo et al., 2007; Filardo et al., 2000). In addition to the rapid GPER-mediated effects triggered by E_2 it was also described that GPER selective activation has also the ability to alter gene expression (Kanda and Watanabe, 2004). E_2, as well as a large number of other compounds that bind to classical ERs have also been demonstrated to bind to or activate GPER (Prossnitz and Barton, 2009). The discovery of GPER-selective ligands aided the research into the specific activities of GPER. Bologa et al. (2006), using a combination of virtual and biomolecular screening, identified the first selective GPER agonist, a non-steroidal compound named G1. The modulation of GPER was complemented with the identification of two selective antagonists, named G15 (Dennis et al., 2009) and G36 (Dennis et al., 2011). Binding studies about the affinity of these three selective ligands of GPER demonstrated that G1 has a binding affinity of about 11 nM (Bologa et al., 2006) compared to 3–6 nM for estrogen (Filardo et al., 2000). Whereas G15 and G36 presents a similar affinity of approximately 20 nM (Dennis et al., 2009; Dennis et al., 2011), but with G36 showing a decreased binding and activation of ERa compared to G15 (Dennis et al., 2011). Other compounds were described as having significant affinity to GPER, but in a non-selective manner, include 4-hydroxytamoxifen (the active metabolite of Tamoxifen) (Vivacqua et al., 2006), raloxifene (Petrie et al., 2013), ICI182780 (Filardo et al., 2000; Filardo et al., 2002; Revankar et al., 2005), Genistein (Thomas and Dong, 2006; Vivacqua et al., 2006) and Bisphenol A (Thomas and Dong, 2006). Since the identification of the GPER-selective ligands an increasing number of studies addressing the potential cellular and physiological effects of GPER selective activation in numerous systems including the central nervous system (CNS) were published.

The emerging notion that E_2 can act in multiple areas of the brain led to an increased focus on its effects on neuronal physiology and neuroplasticity (Srivastava et al., 2013). *In vitro* and *in vivo* studies indicated that E_2 is a potent physiological modulator of the CNS and participates in processes such as neurogenesis, regulation of neurotrophic factors expression and regulation of antioxidant mechanisms (Li et al., 2011; Ma et al., 2013; Suzuki et al., 2009). Estrogens were also associated with the regulation of cognitive processing (Davis et al., 2005; Hammond et al., 2009), memory (Fortress et al., 2013; Frick et al., 2015; Gabor et al., 2015; Kim et al., 2016; Kubota et al., 2016; Lymer et al., 2017; Zhao et al., 2010) and neurological disorders (Liu et al., 2009; Srivastava et al., 2013).

Selective activation of GPER by its agonist G1 enhances cognitive processes, such as learning and memory, in a manner similar to E_2 (Alexander et al., 2017). Besides, GPER is highly enriched in the brain and greatly expressed at the synapses, being involved in the rapid regulation of hippocampal dendritic morphology and synaptic plasticity (Alexander et al., 2017). G1 enhances recognition memory tasks (Gabor et al., 2015; Hawley et al., 2014; Kim et al., 2016; Kubota et al., 2016; Lymer et al., 2017; Wang et al., 2017), learning of specific tasks (Gibbs et al., 2014), and social recognition (Gabor et al., 2015; Lymer et al., 2017). In agreement with this, chronic treatment with the GPER selective antagonist G15 impairs acquisition of a spatial learning task (Hammond et al., 2012).

Over the recent years this receptor emerged has a potential therapeutic target to induce neuroprotection. This hypothesis was based on the ability of its selective agents to mimic the effects of E_2 without the feminizing or other adverse effects (Prossnitz and Barton, 2012). Activation of GPER may replicate the beneficial effects of E_2 in the brain avoiding the side effects associated with estrogen replacement therapies, like increased risk of coronary heart disease, breast cancer and stroke (Gibson et al., 2006; Pabon et al., 2014). In this review, we explore the expression pattern and signaling pathways of GPER, its role in the CNS, and relevance to neurological disorders.

2. Expression of GPER in the CNS

The expression of GPER is not restricted to traditionally estrogen responsive tissues (Prossnitz and Barton, 2012, 2014; Shi et al., 2013). Indeed, characterization of GPER using immunohistochemistry revealed a ubiquitous expression of this receptor in several tissues (Prossnitz and Barton, 2012, 2014; Shi et al., 2013). High levels of GPER expression are present in numerous organs, including male and female reproductive systems, heart, intestine, ovary, pancreatic islets, adipose tissue and inflammatory cells, and nervous system (Prossnitz and Barton, 2012, 2014; Shi et al., 2013).

On nervous system, GPER is similarly expressed throughout the CNS and peripheral nervous system (PNS) of male and female rodents (Brailoiu et al., 2007; Broughton et al., 2013; Dun et al., 2009; Hammond et al., 2011; Hazell et al., 2009; Matsuda et al., 2008). GPER immunoreactivity is observed in the forebrain (e.g. cortex, hypothalamus, hippocampus, hypothalamic-pituitary axis and striatum (Brailoiu et al., 2007; Broughton et al., 2013; Hammond et al., 2011; Hazell et al., 2009; Matsuda et al., 2008; Xu et al., 2009; Zhao et al., 2016), brainstem (e.g. the pontine nuclei locus coeruleus, brainstem autonomic nuclei (Brailoiu et al., 2007), cerebellum Purkinje layer (Hazell et al., 2009), spinal cord and autonomic and sensory ganglia (Dun et al., 2009). In addition, GPER is present in brain vasculature (Ding et al., 2009; Isensee et al., 2009). The levels of GPER expression are heterogeneous with GPER presenting high expression in hypothalamic-pituitary axis (Brailoiu et al., 2007), hippocampus (Brailoiu et al., 2007; Hammond et al., 2011; Matsuda et al., 2008), cortex (Hammond et al., 2011) and thalamus (Broughton et al., 2013). The hippocampus and frontal cortex present higher GPER mRNA levels than the septum and striatum (Hammond et al., 2011).

At a cellular level, GPER is expressed by neurons of different regions, such as the pyramidal neurons of the frontal cortex (Hammond et al., 2011), cholinergic neurons in the medial septum, striatum, diagonal band of Broca and nucleus basalis magnocellularis (Hammond et al., 2011), CA1-3 hippocampal neurons (Akama et al., 2013; Matsuda et al., 2008), GABAergic neurons in the dorsal striatum (Almey et al., 2016), dopaminergic neurons from ventral mesencephalon (Bessa et al., 2015). GPER expression was also reported in neurons from paraventricular nucleus (Xu et al., 2009), luteinizing hormone-releasing neurons (Noel et al., 2009), neurons of the dorsal and ventral horn of

the spinal cord as well as in sensory and autonomic neurons (Chen et al., 2015; Dun et al., 2009). Concerning glial cells, GPER is expressed by cortical and midbrain astrocytes (Bessa et al., 2015), by microglial cells from forebrain (Zhao et al., 2016) and ventral midbrain (Mendes-Oliveira et al., 2017) and by oligodendrocytes of spinal cord, corpus callosum and cortex (Hirahara et al., 2013). On brain vasculature, GPER is particularly expressed in the endothelial cell subpopulation of small arterial vessels (Isensee et al., 2009), and in smooth muscle cells (Ding et al., 2009; Isensee et al., 2009) and pericytes (Isensee et al., 2009).

At a sub-cellular level, GPER is expressed in the plasma membrane of neurons (Akama et al., 2013; Filardo et al., 2000; Funakoshi et al., 2006; Hammond et al., 2011; Thomas et al., 2005) and glial cells (Almey et al., 2012). GPER is also present in the cytoplasm, particularly in the membrane of intracellular compartments such as the endoplasmic reticulum (Matsuda et al., 2008; Otto et al., 2008; Revankar et al., 2005) and Golgi apparatus (Matsuda et al., 2008).

2.1. Sex differences in GPER expression

The expression pattern of GPER mRNA in human brain tissues (Feng and Gregor, 1997; O'Dowd et al., 1998; Owman et al., 1996) is similar to the receptor distribution profile observed in the rat brain, with no differences between sexes (Brailoiu et al., 2007). In contrast, in the zebra fish brain there is a higher expression in males than in females (Acharya and Veney, 2012). A clear sexually dimorphic distribution of GPER occurs in some areas of the hamster brain, with higher levels of GPER in the female hypothalamus and amygdala, and moderate and low levels in the male amygdala and hypothalamus, respectively (Canonaco et al., 2008).

Interestingly, some pathologies are associated with alterations in the pattern of distribution and expression of the GPER. This is the case of transient focal ischemia, where GPER distribution and expression increases in the brain of male mice, but not of intact or ovariectomized (OVX) females (Broughton et al., 2013).

2.2. Regulation of cell proliferation and differentiation by GPER

GPER have also been implicated in the modulation of hippocampal synaptic plasticity (Briz et al., 2015; Tian et al., 2013; Xu et al., 2018). Although these effects have not yet been demonstrated in pathological models, it was shown that Brain-derived neurotrophic factor (BDNF) expression triggered by GPER selective activation promotes synaptic plasticity (Briz et al., 2015; Xu et al., 2018), being these effect associated to the enhancement of spatial memory (Xu et al., 2018). GPER activation is also involved in the modulation of neuritogenesis induced by E_2 in primary hippocampal neurons (Ruiz-Palmero et al., 2013).

Furthermore, it is known that E_2 plays an important trophic and protective role in the adult brain, being essential to the maintenance of normal brain functions, and to protect the brain against neural injuries through different mechanisms, including the stimulation of neurogenesis. The first evidence of the modulating effect of estrogens on neurogenesis was achieved when scientists noticed that, in the reproductive cycle of mammals, higher estrogen levels were accompanied by increased cell proliferation in the dentate gyrus of the hippocampus and, contrariwise, a reduction of circulating estrogens resulted in a significant decrease in the proliferation of hippocampal precursors (Tanapat et al., 1999).

To investigate the effect of GPER on estrogens action in modulating neural cell proliferation and differentiation, Okada et al. (2010) used E_2 conjugated with bovine serum albumin, impeding E_2 to permeate the cell membrane. In this way, they showed that GPER is not directly involved in neural cell proliferation induced by estrogens, but it stimulates oligodendroglial differentiation from neural stem/precursor cells of the telencephalon of 15-day-old rat embryos (Okada et al., 2010). The same authors reported a couple of years before that administration of E_2 or bisphenol A, a xenoestrogen that activates GPER, stimulated the proliferation of neural stem/precursor cells in the absence of mitogens as well as the generation of oligodendrocytes (Okada et al., 2008).

In intact and OVX adult female rats treatment with E_2 or raloxifene, but not with tamoxifen, increased neurogenesis in the ipsilateral subventricular zone following transient middle-cerebral artery occlusion (Khan et al., 2015). Analysis of the role of GPER in hippocampal cell proliferation in adult female rats showed that treatment with GPER agonist decreased cell proliferation in adult OVX female rats, indicating a GPER-independent role of E_2 in hippocampal neurogenesis or, alternatively, an antagonistic effect of intracellular and membrane bound ER activation to maintain the levels of neurogenesis. GPER did not co-localize with progenitor cells in the subgranular zone of the dentate gyrus, indicating that the effects of GPER activation on neurogenesis may be indirect (Duarte-Guterman et al., 2015). In summary, the scarce information available suggests differential effects of GPER in the two neurogenic niches, with a neurogenesis promoting action of the receptor restricted to the subventricular area. The existing data also suggest that these effects may be sex-dependent.

2.3. GPER and aging

Little is known regarding the effect that aging may have on GPER functions. In OVX female rhesus monkeys it was demonstrated that the expression of GPER in gonadotrophin-releasing hormone neurons is not affected by age (Naugle and Gore, 2014). However, in hypothalamic regions of aged OVX females there were more cells expressing GPER and the expression of the GPER/cell was higher than in young OVX females (Naugle and Gore, 2014). In contrast, recent findings demonstrated that hippocampal GPER mRNA levels are decreased in aged OVX female mice when compared to young adult (Wu et al., 2018). Moreover, Wu et al. (2018) associated the reduction of GPER expression to the deprivation of E_2, since it was demonstrated that low levels of E_2 are associated with lower levels of GPER mRNA (Wu et al., 2018). Extrapolating to what happens in aging these data suggest that as the levels of E_2 begin to decrease there is a reduction in the expression of the GPER in females. On males, Xu et al. (2018) obtained similar results once hippocampal GPER expression is decreased in aged male mice compared to young adults (Xu et al., 2018). In males this reduction does not appear to compromise the effects mediated by GPER, since G1 was capable to enhance memory in aged mice (Xu et al., 2018). However, in OVX females the results indicate that the beneficial effects induced by GPER selective activation could be related to the critical period hypothesis. G1 exerted a neuroprotective effect after short-term E_2 deprivation, whereas after long-term E_2 deprivation neuroprotection was not achieved (Wu et al., 2018). Wu et al. (2018) also shows that GPER expression and function can be maintain with estrogens treatment during aging. E_2 treatment 10 weeks after ovariectomy prevents the reduction of GPER mRNA levels and triggers robust neuroprotective effects on aged females (Wu et al., 2018). The results also demonstrate that G15 attenuated the neuroprotective effects of E_2 within the CA1 region of the hippocampus when administered near the end of E_2 treatment (Wu et al., 2018), indicating that GPER may be an important factor in E_2 neuroprotection loss (Wu et al., 2018).

The data available indicates that during aging the expression of GPER seems to decrease in both sexes (Wu et al., 2018; Xu et al., 2018) and the expression pattern can be distinctly affected in different brain regions of OVX female (Naugle and Gore, 2014). Although GPER neuroprotective effects during aging can be maintained in males, on OVX females seems to be more complex. Considering that most of neurological diseases are age-related, it is crucial to develop further research to clarify if aging compromises the protective effects mediated by this receptor.

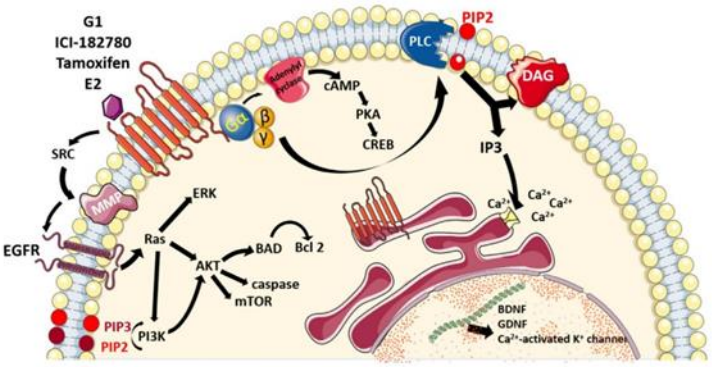

Fig. 1. Schematic representation of the diversity of signaling pathways regulated by GPER. Multiple agonists activate GPER: E2, selective estrogen receptor degraders (SERDs) such as Fulvestrant (ICI-182780), selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene, and selective agonists such as G1. GPER activation stimulates multiple cellular pathways, part of them mediated by transactivation of EGFR. *Abbreviations*: brain-derived neurotrophic factor (BDNF); cell-derived neurotrophic factor (GDNF); cyclic adenosine monophosphate (cAMP); diacylglycerol (DAG); epidermal growth factor receptor (EGFR); estradiol (E2); Estrogen receptors (ER); extracellular signal-regulated kinases (ERK); G protein–coupled estrogen receptor 1 (GPER); inositol 1,4,5-trisphosphate (IP3); mammalian target of rapamycin (mTOR); matrix metalloproteinase (MMP); phosphatidylinositol 3-kinase (PI3K); phosphatidylinositol 4,5-bisphosphate (PIP2); phosphatidylinositol-3,4,5-triphosphate (PIP3); phospholipase C (PLC); selective estrogen receptor degrader (SERD); selective estrogen receptor modulator (SERM);

3. Signaling pathways triggered by GPER activation

The signaling transduction mechanisms triggered by activation of GPER have been studied in various cell types and a large diversity of pathways have been proposed (Fig. 1) (Feldman and Limbird, 2017). Besides the mechanisms elicited by the independent activation of GPER, the interactions of GPER with EGFR (epidermal growth factor receptor), and also with the classic ERα and ERβ, have been reported (Filardo and Thomas, 2012; Prossnitz and Barton, 2012). The crosstalk between GPER and ERα/ERβ involves multiple forms of interactions: cooperative, antagonistic and dependent (Hadjimarkou and Vasudevan, 2018). GPER was initially considered to signal via Gαs, leading to activation of adenylyl cyclase and the consequent increase in cAMP levels and PKA activation (Filardo et al., 2007; Filardo et al., 2002; Thomas et al., 2005). However, it is known that GPER activation may also lead to inhibition of PKA through Gαi and Gαo (Ding et al., 2009), and these pathways coexist with other rapid signaling pathways such as the activation of ERK pathway (Filardo et al., 2002), the activation of kinases such as PI3K (Petrie et al., 2013; Revankar et al., 2005) or PKC (Goswami et al., 2011), intracellular calcium mobilization (Filardo et al., 2007; Revankar et al., 2005; Revankar et al., 2007; Tica et al., 2011), or activation of ion channels (Fraser et al., 2010). Besides triggering rapid signaling events, GPER activation leads to upregulation of nerve growth factor (NGF) via c-fos expression (Kanda and Watanabe, 2003), cyclin D2 and Bcl-2 (Kanda and Watanabe, 2004).

Concerning neuronal cells/tissues, activation of the cell survival PI3K/Akt pathway was associated with the protection mediated by GPER activation in models of Alzheimer's disease (AD) (Wang et al., 2017), Parkinson's disease (PD) (Bessa et al., 2015), spinal cord injury (SCI) (Chen et al., 2015), and traumatic brain injury (Wang et al., 2017). Moreover, activation of PI3K signaling by GPER participates in the control of neuritogenesis in developing hippocampal neurons (Ruiz-Palmero et al., 2013) and on the protection of cognitive function (Wang et al., 2017). Survival promoted by GPER activation was also associated with the regulation of the c-Jun N-terminal kinase (JNK) pathway. In a rat model of global cerebral ischemia G1 exerts significant neuroprotection through the rapid activation of the pro-survival kinases, Akt and ERK, while decreasing pro-apoptotic effects of JNK activation (Tang et al., 2014). In addition to regulating cell survival, control of the JNK pathway by GPER also regulates memory since GPER activation in the dorsal hippocampus enhances hippocampal memory in a JNK-dependent manner and independently from ERα and ERβ (Kim et al., 2016), furthermore it was also demonstrated that JNK signaling is triggered via GPER activation during object-in-place learning, and possibly is E_2-independent (Mitchnick et al., 2019).

The phospholipase C (PLC) pathway is also a target of GPER. Our group showed recently that in rat cortical astrocytes, but not in neurons, GPER activation is able to regulate the PLC pathway. Moreover, activation of this pathway promotes the increase in intracellular Ca^{2+} levels and induces the apoptosis of astrocytes (Roque et al., 2018). In mesencephalic neuron-glia cultures protection induced by G1 against the dopaminergic toxin 1-methyl-4-phenylpyridinium (MPP^+) was associated with the involvement of three different pathways: MAPK, PI3K and PLC pathways (Bessa et al., 2015).

Together, the existing data show that GPER has the ability to regulate a wide variety of signaling pathways, which vary between tissues and even between cells of a given tissue.

4. GPER and neurological disorders

Over the past decades, it was demonstrated that E_2 has an active role in diseases of the nervous system. Although these effects were initially associated with classical ERs, the identification of GPER and the evidence that GPER mRNA and protein were expressed throughout the CNS and PNS of rodents was accompanied by findings showing that GPER significantly contributes to E_2-mediated neurological benefits (Fig. 2) (Brailoiu et al., 2007; Dun et al., 2009; Hazell et al., 2009). The protection mediated by GPER selective activation involves a plethora of mechanisms as diverse as inhibition of pathways mediating apoptosis, stimulation of neurotrophic factors expression, modulation of ion channels, inhibition of neuroinflammatory processes, control of gliosis, maintenance of blood-brain barrier (BBB) and vascular function. These mechanisms are summarized in Table 1.

4.1. Ischemic Stroke (IS)

The role of GPER in cerebral ischemia has been studied since the identification of GPER selective ligands and the characterization of its expression in the CNS. The potential benefits of GPER modulation was assessed in *in vivo* and *in vitro* studies with conflicting results associated mostly with the amount of circulating estrogens or with the sex (Table 2).

Initial *in vivo* studies showed that G1 treatment replicates the effects of E_2 in promoting neuronal survival following global cerebral ischemia (Lebesgue et al., 2010; Prossnitz and Barton, 2012). These effects were demonstrated in OVX female rats (Lebesgue et al., 2010; Tang et al.,

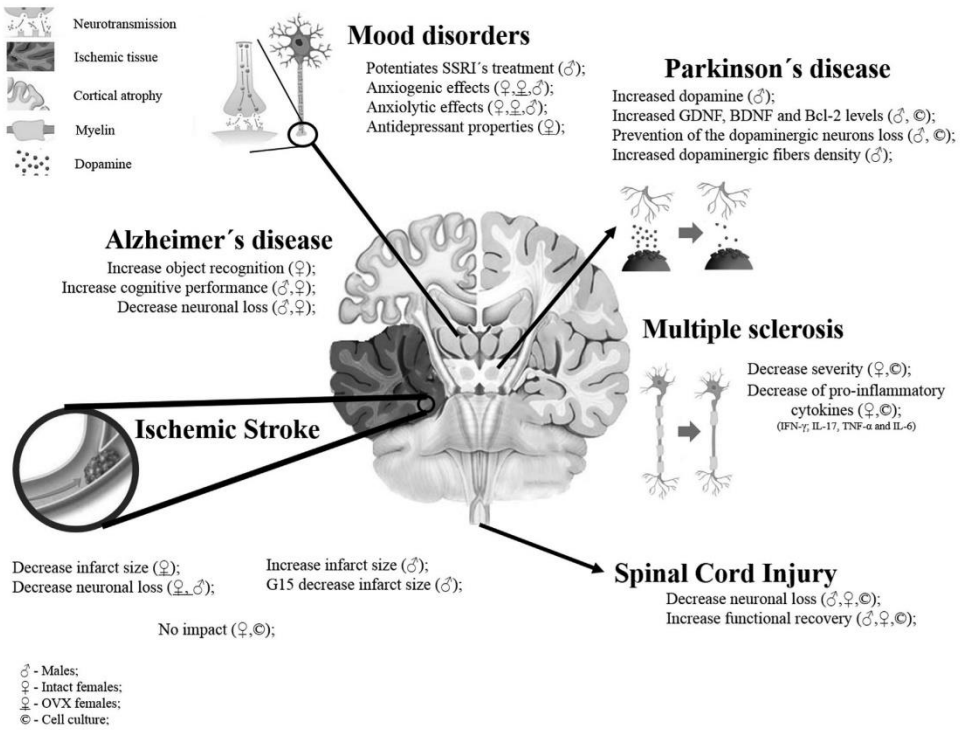

Fig. 2. Effects induced by GPER selective activation on brain disorders. *Abbreviations:* Brain-derived neurotrophic factor (BDNF); Glial cell-derived neurotrophic factor (GDNF); Selective serotonin reuptake inhibitors (SSRI).

2014) and mice (Zhang et al., 2010). It was demonstrated that GPER selective activation protected hippocampal CA1 pyramidal neurons exposed to ischemia (Lebesgue et al., 2010; Tang et al., 2014). Lebesgue et al. (2010) evaluated the effects induced by G1 on young (2 months) and middle aged (9–11 months) Sprague Dawley OVX female rats subjected to transient global cerebral ischemia and (Lebesgue et al., 2010). Immunohistochemical analysis indicated that G1 prevented hippocampal CA1 pyramidal neuronal loss triggered by ischemia (Lebesgue et al., 2010). It was also demonstrated that those effects were similar in young and middle aged animals (Lebesgue et al., 2010). The beneficial effects of GPER selective activation in OXV females were also described in mice. After middle cerebral artery occlusion (MCAO) exposure to G1 reduced the infarct volume (Zhang et al., 2010). Similar effects were reported by Broughton et al. (2014). Exposure to G1 reduced neurological deficit, apoptosis, and infarct volume in OVX female mice, but had no significant effect in intact females (Broughton et al., 2014). Broughton et al. (2014) also hypothesizes that in intact females the effects of GPER selective activation are not observed due to the amount of circulation estrogens (Broughton et al., 2014). These findings highlight the complex nature of endogenous estrogen signaling and raises the hypothesis that after an IS the effects induced by GPER selective activation could be affected by the amount of circulating estrogens.

The work from Broughton et al. (2014) also raises the hypothesis that the effects of GPER selective activation after ischemia might be related with the sex (Broughton et al., 2014). Since in young and aged males G1 unexpectedly and markedly exacerbates post-stroke neurological deficit and infarct volume, being those effects abrogated by G15 (Broughton et al., 2014). Which reinforce the body of evidence indicating that effects of estrogens in the female and male brain are not identical (Broughton et al., 2014). Contrary to the data obtained in males, there are evidences that after global cerebral ischemia exposure to G1 leads to a reduction of neuronal injury in hippocampal CA1 region and striatum (Kosaka et al., 2012). This neuroprotection is similar to the protection induced by E_2 treatment (Kosaka et al., 2012), which increases the controversy around GPER activation after cerebral ischemia.

The signaling pathways involved in the neuroprotective role of GPER upon an ischemic insult are not completely understood, *in vivo* studies demonstrated that in OVX females neuroprotection is associated to the rapid activation of the pro-survival kinases, Akt and ERK, while decreasing pro-apototic JNK activation (Tang et al., 2014). On males these neuroprotection in hippocampal and striatal neurons is associated to the up-regulation of protective ion channels, such as the small conductance calcium-activated potassium channel 2 (SK2) (Kosaka et al., 2012). On the other hand, the detrimental effects induced by G1 in males are associated to the increase in the expression of cleaved caspase-3 in peri-infarct neurons (Broughton et al., 2014).

On *in vitro* studies there are also some controversy, since it was demonstrated that selective activation of GPER with G1 does not induce any protection against an ischemic insult (Lamprecht and Morrison, 2014). In this study organotypic hippocampal slice cultures were

Table 1
Protective actions triggered by GPER activation in the brain.

Protective actions triggered by GPER (Mechanisms/Pathways)		References
Up-regulation of neurotrophic factors	BDNF	Bourque et al. (2014)
		Bourque et al. (2014)
	GDNF	Bessa et al. (2015)
		Cheng et al. (2017)
Up-regulation of anti-apoptotic proteins	Bcl-2	Bourque et al. (2014)
		Bourque et al. (2015)
Up-regulation of pro-survival kinases	Akt and ERK	Bourque et al. (2014)
		Tang et al. (2014)
	PI3K/Akt	Wang et al. (2017)
Up-regulation of protective ion channels	SK2	Kosaka et al. (2012)
Up-regulation of remyelination	Remyelination	Hirahara et al. (2013)
Down-regulation of pro-apoptotic kinases	JNK	Tang et al. (2014)
Modulation of synaptic plasticity	GABAergic and glutamatergic transmission	Tian et al. (2013)
	BDNF	Briz et al. (2015)
		Xu et al. (2018)
	Neuritogenesis	Ruiz-Palmero et al. (2013)
		Ruiz-Palmero et al. (2011)
Modulation of inflammation	IFNγ and IL-17	Blasko et al. (2009)
	IL-10	Yates et al. (2010)
	Astrogliosis	Day et al. (2013)
	IL-1β and TNFα	Zhao et al. 2016
	IL-1β, TNFα and IL-6	Guan et al. (2017)
	Phagocytic activity, iNOS expression and NO release	Mendes-Oliveira et al. (2017)
Restoration of vascular function	Arteriolar dilation	Murata et al. (2013)
Restoration of the BBB	Regulation of tight junctions and BBB permeability	Lu et al. (2016)
Increase of cell proliferation	Neural stem/precursor cells oligodendrocytes	Okada et al. (2008)
Cell differentiation	oligodendroglial	Okada et al. (2010)
Increase of neurogenesis		Khan et al. (2015)

Table 2
Effects induced by GPER selective activation in brain ischemia.

Major conclusions	Models	Reference
Selective GPER activation increases the number of hippocampal CA1 pyramidal neurons;	Sprague Dawley (OVX females); 4 vessel occlusion (10 min); Exposure to G1;	Lebesgue et al. (2010)
G1 replacement decreased infarct volume size;	C57Bl/6J mice (OVX females); MCAO (90 min); Exposure to G1;	Zhang et al. (2010)
Selective GPER activation reduces neuronal injury in the hippocampal CA1 region and striatum following global cerebral ischemia;	C57Bl/6J mice (males); Cardiac arrest and cardiopulmonary resuscitation (8 min); Exposure to G1;	Kosaka et al. (2012)
Ischemia increases GPER distribution and expression in the peri-infarct brain regions of male mice, but not in intact females or OVX mice;	C57Bl/6J mice (males, intact and OVX females); MCAO (30 min); GPER distribution;	Broughton et al. (2013)
Selective GPER activation restores vessel function of arterioles after hypoxia/reperfusion;	Male and female rats; Hypoxia (1 h) and reoxygenation injury; Exposure to G1;	Murata et al. (2013)
G1 worsened functional outcomes and increased post-stroke infarct volume size in males, effects that were blocked by G15; G15 improved functional outcomes and reduced infarct volume size after stroke in males; G1 reduced neurological deficit, apoptosis, and infarct volume in OVX females, but had no significant effect in intact females;	C57Bl/6J mice (males, intact female and OVX females); MCAO (30, 60 and 90 min); Exposure to G1 and G15;	Broughton et al. (2014)
Selective GPER activation does not induce any protection against an ischemic insult;	Organotypic hippocampal slice cultures prepared from Sprague Dawley rat pups; OGD (30 min); Exposure to G1;	Lamprecht and Morrison (2014)
G1 exerts significant neuroprotection against ischemia through the rapid enhanced activation of the pro-survival kinases, Akt and ERK, while decreasing pro-apoptotic JNK activation;	Sprague Dawley rats (OVX females); GCI (10 min); Intracerebroventricular administration of G1;	Tang, et al. (2014)
Ischemia increases GPER expression in the motor cortex and hippocampal region; GPER expressed in microglia mediated the anti-inflammatory effect of estradiol after ischemic stroke;	Sprague Dawley rats (intact females); 4 vessel occlusion (15 min); Exposure to G1 and G15;	Zhao et al. (2016)
Selective GPER activation after stroke ameliorates BBB permeability after global cerebral ischemia in OVX rats;	Sprague Dawley rats (OVX females); 4-vessel occlusion (20 min); Intracerebroventricular administration of G1;	Lu et al. (2016)

prepared from Sprague Dawley rat pups and exposed to 30 min of oxygen and glucose deprivation (OGD). After OGD the cultures were exposed to G1 during a reperfusion period of 24 h. The results demonstrated that G1 does not protect neurons from ischemic death nor increase the phosphorylation of Akt and/or ERK, unlike E_2 (Lamprecht and Morrison, 2014). More, the beneficial effects induced by E_2 after ischemia were maintained after GPER blockade by G15, thus suggesting that, in this case GPER is not involved in E_2-induced neuroprotection (Lamprecht and Morrison, 2014). Interestingly, in primary neuron-glia cortical cultures exposed to 4 h of OGD, GPER selective activation after ischemia does not induce any effect on neurons, but selectively promotes astrocytes death due to the rise of intracellular calcium levels via PLC (Roque et al., 2018). These results also show that GPER is coupled to different signaling pathways in neurons and astrocytes (Roque et al., 2018).

GPER might have an important role in the management of inflammation after an ischemic insult. Using adult female Sprague Dawley rats subjected to a global cerebral ischemia by four vessel occlusion and primary microglial cultures from neonatal rats Zhao et al. demonstrated that the GPER expressed in microglial cells directly mediates the anti-inflammatory effect of E_2 after an ischemic stroke (Zhao et al., 2016). G1 reduces IL-1β and Tumor necrosis factor α (TNFα) levels. Moreover, the specific GPER antagonist G15 was able to abolish the anti-inflammatory effect of E_2 (Zhao et al., 2016).

Another interesting effect induced by G1 after hypoxia/reperfusion is the ability to restore vessel function of arterioles, which points to the protection of the cerebrovasculature against an ischemic insult (Murata et al., 2013). In this study, rat cerebral penetrating arterioles from both sexes were isolated, cannulated and pressurized. To induce hypoxia, pial sheaths where incubated for 1 h in the hypoxic bath ($PO_2 < 2\%$), then transferred to the normoxic bath ($PO_2 = 21\%$) to induce reoxygenation and finally exposed to G1. The results indicate that G1 produces a vasodilatory response, which was partially dependent on endothelium derived Nitric oxide (NO), but not on arachidonic acid cascades and endothelial hyperpolarization factor. Additionally, G1 treatment after hypoxia/reperfusion injury fully restored endothelium-dependent dilation to ATP (Murata et al., 2013).

It was also described that GPER activation after stroke can attenuate the BBB disruption and vasogenic edema in early stage of ischemic stroke in OVX female rats (Lu et al., 2016). Bilateral intracerebroventricular administration of G1 to female Sprague-Dawley rats subjected to global cerebral ischemia significantly decreased immunoglobulin G extravasation and increased the tight junctions occludin and claudin-5 in the hippocampal CA1 region. Furthermore, G1 significantly decreased the protein levels of vascular endothelial growth factor A (VEGF-A) in the ischemic hippocampal CA1 region, which suggests that after ischemic injury GPER activation reduces tight junctions disruption via inhibition of VEGF-A expression (Lu et al., 2016).

Another controversial issue in relation to GPER is its expression pattern after an ischemic insult. In adult female Sprague Dawley rats subjected to global ischemia by four vessel occlusion there was a significant increase of GPER expression in the motor cortex and hippocampal region as demonstrated through immunohistochemical and western blot analysis (Zhao et al., 2016). Using the same techniques, Broughton et al. also reported a significant increase in GPER expression after an ischemic insult in hippocampus, somatosensory cortex and hypothalamus of males with no significant changes in intact or OVX females, which suggests a sex-dependent effect of ischemia on GPER expression (Broughton et al., 2013). The same study reported that GPER immunoreactive neurons in the peri-infarct regions appear more intensely labeled (Broughton et al., 2013).

The controversy around GPER expression after an ischemic insult could result, in part, by the use of different stroke models and periods of ischemia. The later directly influence the extension of the lesion and, consequently, the results. The discrepancies observed between *in vivo* and *in vitro* models may arise from the lack of some components in *in vitro* models that can influence GPER expression after stroke, such as the vascular or the immune cells.

4.2. GPER in neurodegenerative disorders

4.2.1. Alzheimer's disease

AD comprises a wide spectrum of alterations, which includes memory loss, functional decline, behavioral disturbances and dementia (Neugroschl and Wang, 2011). The hypothesis that GPER could be an effective therapy for reducing cognitive decline associated with aging and AD related dementia emerged from data showing that the GPER has the ability to modulate and enhance cognitive processes such as memory and learning (Gibbs et al., 2014; Hammond and Gibbs, 2011; Hammond et al., 2011; Hawley et al., 2014; Kim et al., 2016), known to be impaired in aging and AD (Hammond et al., 2011).

In the 5XFAD AD mouse model selective activation of GPER with G1 ameliorates memory impairment in the novel object recognition test in female, but not in male mice (Kubota et al., 2016). In females these effects are similar to the neuroprotection mediated by E_2. However, in males, despite the inconsistence in the effects observed, the bulk of evidence demonstrates a beneficial effect of E_2 on memory both in intact and gonadectomized male rodents (Frick et al., 2015).

4.2.2. Parkinson's disease

PD is a neurodegenerative disease characterized by the progressive and selective damage of the dopaminergic neurons from the nigrostriatal pathway. This damage results in a decrease of dopamine in the striatum, which leads to several motor symptoms, such as tremor, slow body movement and postural instability. It has been widely demonstrated that estrogens can exert protective effects on the dopaminergic nigrostriatal neurons against different toxins (Baraka et al., 2011; Campos et al., 2012; D'Astous et al., 2003; Jourdain et al., 2005; Sawada et al., 2002). Increasing evidence implicated the activation of the GPER in these protective estrogenic effects. Results from our group demonstrated that selective activation of the GPER using G1 protects rat midbrain dopaminergic neurons against MPP^+, a protection similar to that exerted by E_2. In addition, we observed that when E_2 was used in combination with G15 its protective effect was no longer observed (Bessa et al., 2015). Similar to E_2, treatment with G1 increases the concentration of dopamine, its metabolites, and the specific binding to the membrane (DAT) and vesicular (VMAT) dopamine transporters in the striatum of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice. These dopaminergic protective effects of E_2 and G1 were lost in the presence of G15 (Bourque et al., 2013). Comparable protective effects in the striatum of MPTP-exposed mice mediated by GPER activation were also observed in other studies using treatment with either G1 (Bourque et al., 2015) or raloxifene (Bourque et al., 2014).

The dopaminergic protective actions promoted by GPER activation observed in the above-mentioned studies appear related to the ability of G1 to increase the expression of neurotrophic factors. We found that G1 is capable of inducing an increase in GDNF protein in midbrain neuron-glia cultures, and that GDNF neutralization or silencing in these cultures impedes the dopaminergic protective effect of GPER selective activation (Bessa et al., 2015). This was also observed by Cheng et al. (2017) on neuroblastoma cell line SH-SY5Y. G1 reduced the MPP^+-induced cell death through the increase of GDNF, effects that were abrogated by G15 (Cheng et al., 2017). In addition, it was observed by others that the protective effects promoted by GPER activation in the striatum of MPTP mice occurred in parallel with an up-regulation of BDNF and GDNF protein levels, increase in the anti-apoptotic Bcl-2 protein and activation of the pro-survival kinases Akt and ERK (Bourque et al., 2014, 2015). This suggests that protection mediated by GPER activation involves both inhibition of apoptosis and promotion of dopaminergic survival. Guan et al. (2017) showed that protection mediated by G1 in the MPTP mouse model involves also an anti-

inflammatory effect. G1 treated mice present a reduction in the number of microglial cells and IL-1β, TNFα and IL-6 protein and mRNA levels in the midbrain (Guan et al., 2017). In fact, although PD is essentially an idiopathic disease, it is accepted that inflammation promoted by microglial cells plays a critical role to the progressive dopaminergic neuronal death. GPER selective activation is associated with the modulation of inflammatory responses, with G1 inhibiting Lipopolysaccharide (LPS)-induced IL-6 expression in murine macrophage cells (Okamoto et al., 2017). A study from our group, demonstrated that G1 treatment protects dopaminergic neurons in the *substantia nigra*, an effect accompanied by decreased IL-1β, CD68 and Inducible nitric oxide synthase (iNOS) mRNA levels in this region. Moreover, we also demonstrated that G1 treatment prevents LPS-induced impairment of motor function (Mendes-Oliveira et al., 2017).

The above-mentioned effects were described on males. Data regarding the effects induced by GPER on females are scarce and contradictory. To our knowledge, the few studies that exist in PD models using female models were carried out with the administration of tamoxifen or raloxifene (Baraka et al., 2011; Dluzen and Mickley, 2005), two selective estrogen receptor modulators (SERMs) with antagonistic actions towards ERα and ERβ and acting as GPER agonists (Filardo et al., 2000; Meyer et al., 2011). Dluzen and Mickley (2005) demonstrate a protective role of tamoxifen from dopaminergic toxins, inducing an increase of striatal dopamine and 3,4-dihydroxyphenylacetic acid concentrations on females (Dluzen and Mickley, 2005). On the other hand Baraka et al. (2011) using a rat model of PD demonstrated that tamoxifen does not induce any protection in OVX females, whereas raloxifene protected striatal dopaminergic neurons against 6-OHDA-induced neurotoxicity (Baraka et al., 2011).

In conclusion, GPER is a promising therapeutic target for the treatment of PD. In males, the activation of GPER protects the dopaminergic neurons in the substantia nigra and the striatal nerve terminals, increases the concentration of dopamine and its metabolites, as well as DAT and VMAT-2 specific binding. GPER activation is also able to protect motor functions. (Table 3). These protective actions induced by the GPER activation involve an increase in the production of neurotrophic factors, the inhibition of apoptosis, promotion of survival, and reduction in inflammation. Besides the protective effects, it would be important to evaluate whether GPER activation has the ability to promote recovery or stop the progressive loss of dopaminergic neurons. There is no information on the selective activation of GPER in females, and the scarce information on the modulation of GPER refers to the use of non-selective agonists such as raloxifene and tamoxifen. In addition, the existing data is contradictory, making difficult to draw conclusions about the potential effects of GPER in female models of PD.

4.2.3. Multiple sclerosis

Multiple sclerosis (MS) is characterized by multiple focal areas of myelin loss within the CNS called plaques or lesions (Thompson et al., 2018). The hallmarks of MS pathology are axonal or neuronal loss, demyelination, and astrocytic gliosis (Thompson et al., 2018). Among these neuropathological characteristics, axonal loss is particularly relevant because it is the main underlying mechanism of permanent disability (Thompson et al., 2018). This axonal loss was associated with different mechanisms such as the energy deficit linked to mitochondrial dysfunction and the loss of trophic support (Thompson et al., 2018).

Wang et al. (2009) reported the ability of GPER activation to promote protection in MS using the rodent experimental autoimmune encephalomyelitis (EAE) model (Table 3). Selective activation of GPER with G1 reduced clinical and histological EAE signs, whereas E_2 mediated protection was significantly impaired in GPER gene-deficient female mice (Wang et al., 2009). The role of GPER in the EAE model is also supported by the finding that selective activation of GPER with G1 reduced the severity of disease in EAE models of MS and that this effect is concomitant with a G1-mediated decrease in pro-inflammatory cytokines, including Interferon γ (IFNγ) and IL-17 (Blasko et al., 2009). Furthermore, the results also showed the ability of G1 to inhibit the production of cytokines such as TNFα and IL-6 in a dose-dependent manner in human primary macrophages and in a murine macrophage cell line (Blasko et al., 2009).

Studies about the influence of ERα and GPER on E_2 ability to treat EAE showed that E_2 reduced disease severity in wild-type and ERα knockout female mice, but did not alter the disease in the GPER knockout group (Yates et al., 2010), suggesting that GPER is necessary for the protective effect mediated by E_2. Moreover, the effects on disease severity of both receptors were associated with the production of anti-inflammatory IL-10 following E_2 treatment (Yates et al., 2010).

GPER is expressed throughout the oligodendrocyte differentiation and promyelinating stages in primary oligodendrocyte cultures derived from rat spinal cords and brains (Hirahara et al., 2013). Additionally, it was also shown that selective activation of GPER with G1 enhanced oligodendrocyte maturation and remyelination after demyelination, suggesting an additional mechanism of protection triggered by GPER selective activation and enhancing the potential of GPER selective agonists as therapies for the treatment of MS (Hirahara et al., 2013).

It is also important to note that that results regarding the effects induced by the selective activation of GPER on *in vivo* MS models were carried out only in female models, therefore, it would be important to clarify if these effects also occur in males.

4.3. GPER in mood disorders

Mood disorders are common psychiatric illnesses characterized by conspicuous disturbances in emotional disposition, and include diseases such as depression or bipolar disorders (Marvel and Paradiso, 2004). In 2009, Xu et al. demonstrated that G1 attenuates serotonin receptor signaling in the paraventricular nucleus of the hypothalamus and reduces responses to oxytocin and adrenocorticotropic hormone, rising the hypothesis that GPER could play a role in mood disorders (Xu et al., 2009). On the other hand, GPER is necessary for E_2-induced changes in serotonin 1A receptor signaling (McAllister et al., 2012). Desensitization of serotonin 1A receptor is a key element for selective serotonin reuptake inhibitors (SSRI) efficacy in the treatment of mood disorders, and the expression of GPER shortens the onset of SSRI therapeutic effects in a GPER-dependent manner, thus providing evidences that GPER may accelerate the therapeutic effect of SSRI treatment in mood disorders (Table 4) (McAllister et al., 2012).

In a mouse model of depression, G15 inhibited the anti-depressant effects of G1 (Dennis et al., 2009). Studies on the impact of classical ER and GPER on SSRI treatment of depression in OVX female rats showed that long-term treatment with G1 induced anti-depressant-like effects associated with an increase in the phosphorylation levels of Akt, ERK and TrkB receptor in the hippocampus (Table 4) (Benmansour et al., 2016).

Evaluation of serum GPER levels in 38 euthymic bipolar disorder patients showed that both male and female patients had higher GPER levels than the respective control groups, while there were no differences in serum E_2 levels, suggesting that GPER may play a role in the pathophysiology of bipolar disorder (Orhan et al., 2018).

Anxiety disorders comprehend a wide range of disturbances that include panic disorders, obsessive-compulsive disorders, post-traumatic stress and generalized anxiety disorders (Somers et al., 2006). Kastenberger et al. (2012) demonstrated that short-term application of specific agonists of classical ER did not induce any behavioral changes, whereas specific stimulation with G1 in male and OVX female mice induced anxiogenic effects, suggesting that estrogen-induced anxiogenic-like effects were mediated mostly by GPER (Table 4) (Kastenberger et al., 2012). Studies using wild-type female and male mice and GPER knockout mice demonstrated that alterations in anxiety-like behavior were observed predominantly in male mice (Kastenberger and Schwarzer, 2014). In contrast, others reported data supporting

Table 3
Effects induced by GPER selective activation in neurodegenerative disorders.

	Major conclusions	Models	Reference
AD	Selective GPER activation ameliorated object recognition memory in female but not male mice;	5XFAD mice (intact female and male); Exposure to G1 and G15;	Kubota et al. (2016)
PD	Increased concentration of dopamine and its metabolites, and DAT and VMAT2 specific binding in the striatum; Increased DAT specific binding in the substantia nigra;	C57BL/6 mice (male); MPTP model; Subcutaneous injection of G1 twice daily for 10 days - before and after dopaminergic lesion;	Bourque et al. (2013)
	Increased dopamine and DOPAC concentration and specific binding of DAT and VMAT in the striatum; Increased anti-apoptotic Bcl-2 protein and activation of the pro-survival kinase Akt in the striatum;	C57BL/6 mice (male); MPTP model; Subcutaneous injection of raloxifene twice daily for 10 days - before and after dopaminergic lesion;	Bourque et al. (2014)
	Increased dopamine concentration and DAT and VMAT-2 specific binding in the striatum; Increased DAT specific binding in the substantia nigra; Increased GDNF, BDNF and Bcl-2 protein levels in the striatum;	C57BL/6 mice (male); MPTP model; Subcutaneous injection of G1 twice daily for 10 days - before and after dopaminergic lesion;	Bourque et al. (2015)
	Prevention of the dopaminergic neuron loss in a GDNF-dependent process;	Wistar rat midbrain neuron-glia cultures; MPP^+ model; Exposure to G1;	Bessa et al. (2015)
	Increased dopaminergic fibers density in the striatum; Prevention of the dopaminergic neurons loss in the substantia nigra; Decreased microglial cells number and IL-1β, TNF-α and IL-6 protein and mRNA levels in the midbrain;	C57BL/6 mice (male); MPTP model; Subcutaneous injection of G1 twice daily for 12 days - before and after dopaminergic lesion;	Guan et al. (2017)
	Prevention of the dopaminergic neurons loss in the substantia nigra; Protection of the motor functions; Decreased IL-1β, CD68 and iNOS mRNA levels in the substantia nigra;	C57BL/6 mice (male); Unilateral injections in the substantia nigra with LPS on 5th day of G1 treatment; Subcutaneous injection of G1 twice daily for 12 days - before and after dopaminergic lesion;	Mendes-Oliveira et al. (2017)
	G1 reduced the MPP^+-induced cell death through the increase of GDNF, effects that were abrogated by G15;	Neuroblastoma cell line SH-SY5Y; MPP^+ model; Exposure to G1;	Cheng et al. (2017)
MS	Selective GPER activation mediates protection against MS, which is significantly impaired in GPER gene-deficient mice;	C57Bl/6J mice (female); GPER KO mice; EAE; Exposure to G1;	Wang et al. (2009)
	Selective GPER activation reduces the severity of MS through the decrease of pro-inflammatory cytokines, including IFNγ and IL-17; G1 inhibits the production of cytokines such as TNFα and IL-6 in a dose-dependent manner;	Primary culture of macrophages, microglia and a murine macrophage cell line (RAW264.7); EAE; Exposure to G1;	Blasko et al. (2009)
	E_2 reduced disease severity in wild-type and ERα KO mice, but had no impact on GPER KO group; These different effects were associated to the production of anti-inflammatory IL-10; GPER have an important but still undefined role in regulating immune reactivity in MS severity;	C57Bl/6J mice (intact female); Ethinylestradiol treatment; WT, ERαKO and GPERKO mice;	Yates et al. (2010)
	GPER is expressed throughout oligodendrocyte differentiation and promyelinating stages; Selective GPER activation enhanced oligodendrocyte maturation and remyelination after demyelination;	Primary oligodendrocyte cultures from Wistar rat spinal cord; Demyelination model; Exposure to G1 and G15;	Hirahara et al. (2013)

anxiolytic effects of GPER in OVX female mice being this associated to the regulation of synaptic transmission in the basolateral amygdala (BLA) (Tian et al., 2013) and independent of ERK signaling (Anchan et al., 2014). A differential contribution of GPER in the control of anxiety in male and female mice is also supported by data from the elevated plus maze task showing that acute administration of G1 leads to anxiolytic effects in gonadectomized male mice, but not in female mice (Table 4) (Hart et al., 2014). Somehow, these results establish a parallelism with what has already been described for E_2 and ERα/ERβ (Hart et al., 2014). Being the nature of E_2 effects on anxiety attributable to the differential effects of specific estrogen receptor subtypes. ERβ activation induces anxiolytic-like effects whereas ERα activation appears to have mainly anxiogenic-like properties (Hart et al., 2014).

Chronic pain-related anxiety is attenuated by subcutaneous injection or local infusion of G1 in the basolateral amygdala in OVX female mice, being these effects associated with the prevention of imbalance between excitatory and inhibitory transmissions in the basolateral amygdala synapses (Liu et al., 2015).

Increased serum GPER levels might play a role in the etiology of generalized anxiety disorder. In a study involving 40 drug-naïve patients newly diagnosed with anxiety disorder there were significantly higher levels of GPER in the serum of patients with generalized anxiety disorder and a positive correlation between GPER serum levels and severity (Findikli et al., 2016).

The existing data indicates that for several disorders the effects triggered by GPER selective activation are dependent on the sex of animals, or with the amount of circulating E_2 levels. Due to the scarcity of studies regarding the selective activation of GPER in both males and females, it is not possible to establish a clear hypothesis to explain these differences. However, the differential effects can relate with the complex signaling pathways activated by GPER and to the crosstalk between estrogen receptors on males and females (Hart et al., 2014). Hart et al. (2014) showed that G1 increased protein expression of hippocampal phosphorylated ERα in male mice, but not in females (Hart et al., 2014). These modifications were associated with different anxiolytic effects in males and females (Hart et al., 2014). Although the differential effects observed upon GPER activation in males and females may involve similar effects the data currently available are insufficient to draw any conclusions.

Table 4
Effects induced by GPER selective activation in mood disorders.

	Major conclusions	Models	Reference
Mood disorder	Selective GPER activation attenuates 5-HT1A receptor signaling and accelerates the effects of SSRIs treatment of mood disorders;	Sprague-Dawley rats (intact female); Exposure to G1; GPER distribution:	Xu et al. (2009)
	GPER is necessary for estradiol-induced changes in the serotonin 1A receptor signaling pathway and desensitization;	Sprague-Dawley rats (intact female): GPR30 siRNAs to decrease GPR30 Expression;	McAllister et al. (2012)
Depression	Selective GPER activation has antidepressant properties, which were inhibited by G15;	C57Bl6 mice (OVX female); Exposure to G1 and G15;	Dennis et al. (2009)
	Long-term treatment with G1 induces anti-depressant-like effect;	Sprague Dawley rats (OVX female); Exposure to G1;	Benmansour et al. (2016)
Bipolar	Serum GPER levels in euthymic bipolar patients are higher than in controls;	38 patients diagnosed with Bipolar disorder (males and females); Quantification of GPER in serum	Orhan et al. (2018)
Anxiety	Estrogen-induced anxiogenic-like effects are mediated mostly by GPER;	C57Bl6 mice (intact and OVX females); Exposure to G1;	Kastenberger et al. (2012)
	GPER has a direct involvement in anxiety and stress control, being this impact stronger in male than in female mice;	C57BL/6J mice (male and intact female); GPER KO mice;	Kastenberger and Schwarzer (2014)
	The selective activation of GPER had an anxiolytic effect in the open field test;	C57BL/6J mice (OVX female); Exposure to G1;	Anchan et al. (2014)
	GPER selective activation has anxiolytic properties in gonadectomised male, but not in female mice;	C57BL/6J mice (gonadectomized males and intact females); Exposure to G1;	Hart et al. (2014)
	GPER selective activation induced anxiolytic effects in OVX female mice attributed to the maintenance of the balance between excitatory and inhibitory transmissions in the basolateral amygdala;	C57BL/6J mice (OVX female); Exposure to G1 and G15;	Liu et al. (2015)
	Serum GPER levels were significantly increased in patients diagnosed with generalized anxiety disorder, with a positive correlation between GPER levels and severity of the disease;	40 patients diagnosed with generalized anxiety disorder; Serum GPER quantification;	Findikli et al. (2016)

4.4. Autism spectrum disorder (ASD)

To the best of our knowledge, only one study investigated the impact of GPER in ASD. Data from the analysis of GPER serum levels in patients diagnosed with ASD indicate that ASD patients have significantly lower levels of GPER when compared to the control group (Altun et al., 2017). The results showed also a negative correlation between GPER levels and the Childhood Autism Rating Scale total score rising the hypothesis of a role of GPER in the etiology of ASD (Altun et al., 2017).

4.5. Spinal cord injury

SCI may result in severe dysfunction of motor neurons and consequently the protection and improvement of spinal motor neurons following SCI represents a priority (Thuret et al., 2006). GPER selective activation with G1 dose-dependently reduced neuron apoptosis and improved functional recovery following SCI in the weight-drop spinal cord contusion model in male rats, whereas GPER knockdown inhibited the beneficial actions of E_2, suggesting that GPER might be the main ER responsible for the neuroprotective effects induced by E_2 (Hu et al., 2012). Similar results were obtained in mice. GPER selective activation with G1 mimicked the effects of E_2 treatment and prevented SCI-induced apoptotic cell death and enhanced motor functional recovery after injury, whereas the neuroprotective effects of G1 and E_2 were blocked by G15 in adult female C57BL/6J mice (Cheng et al., 2016).

5. Conclusions

Here we reviewed the body of work that has been conducted over the latest years in an attempt to elucidate the role of GPER selective activation in brain physiology and physiopathology, particularly in neurological disorders. The data demonstrates that for several pathologies, like mood disorders, AD or PD, GPER selective activation could be an interesting therapeutic target to induce neuroprotection. Nevertheless, for others, as is the case of IS, the existing information is not consensual. Despite the promising beneficial effects observed in neurodegenerative disorders, the information that exists is still not enough. It is essential to further explore, clarify and characterize the molecular mechanisms that underlie those effects. In addition, it is crucial to highlight the effect of selective GPER not only on neurons, but also on other cellular populations that are present in the brain like glial and vascular cells.

Further studies are also necessary to elucidate the effects induced by GPER at the tissue level to clarify the impact that its activation has on different types of tissues, also exploring possible cell-type specific interactions within each tissue. On the other hand, it is also evident the need of studies regarding the role of GPER in the human CNS, as well as its modulation with selective or non-selective ligands. It is also important to understand the effects of GPER activation on other organs and to develop studies focusing on the pharmacokinetics and pharmacodynamics of GPER agonists and antagonists.

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