Retinoic Acid Effect on Cerebral Vasculature

(Versão Final após defesa)

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Dissertação para obtenção do Grau de Mestre em
Bioquímica
(2º ciclo de estudos)

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agosto de 2021

“Our virtues and our failures are inseparable, like force and matter. Whentheyseparate, manis no more.”

Nikola Tesla

Dedication

Quero agradecer aos meus pais e irmão que me apoiaram e me incentivaram em todos os sentidos durante toda esta jornada.

Acknowledgments

Queria agradecer em primeiro lugar à Professora Elisa Cairrão pela confiança que depositou em mim ao aceitar-me no seu grupo de investigação, pela ajuda, pelo apoio e orientação prestada durante a minha dissertação. Um obrigado por todo o conhecimento que me transmitiu e pela paciência que teve comigo.
Agradeço à professora Graça Baltazar pela confiança que teve em mim e pelo apoio e disponibilidade que deu na parte prática da dissertação.
Um grande obrigado aos membros do meu grupo por toda ajuda que me deram, em específico à Melissa Mariano, por toda a ajuda que me deu e pela paciência que teve comigo, à Joana Feiteiro por toda a ajuda e disponibilidade que ofereceu para me ajudar nesta dissertação, nomeadamente na parte prática e por fim à Margarida Lorigo um grande obrigado por todo o apoio, por fazer-me nunca desistir e pela paciência e compreensão que teve comigo.
Agradeço a todos os meus amigos da Covilhã, pela ajuda, pela paciência que tiveram comigo. Em específico queria agradecer ao Nata, ao Francês e ao Júnior Gava por todo apoio que me deram, ajuda e por me terem aturado. Também queria agradecer à Ana Maia e à Ana Fernandes por toda a preocupação, conselhos e ajuda que me deram ao longo desta jornada.
Agradeço a todos os meus amigos da Sertã, em específico ao Vasco, Pedro, Renato pelo apoio que me deram, pelos conselhos e por toda a preocupação que tiveram comigo.
Agradeço ao meu irmão pela força, incentivo e ajuda que proporcionou que eu alcança-se esta etapa tão importante na minha vida.
Por fim agradeço aos pilares, neste caso os meus pais que sempre me apoiaram, que me deram toda a força, que sempre me perceberam e compreenderam e que fizeram com que isto fosse possível.

Resumo

O cérebro representa um dos órgãos que consome mais oxigénio, tendo por sua vez uma função insubstituível no organismo. Assim para a desempenhar corretamente, o cérebroprecisa de estar em homeostase, sendo assim, contém uma rede que é responsável por esta homeostase, a que se dá o nome de unidade neurovascular. Esta é composta por neurónios, astrócitos, células endoteliais e células murais, como os pericitios e as células do músculo liso, que desempenham diferentes funções fulcrais dentro do cérebro.

Este trabalho apresenta dois objetivos principais, o estabelecimento de um protocolo para uma cultura primária de células endoteliais puras de artérias cerebrais. Assim, foi testado o efeito de um antibiótico que induz morte celular nas células do musculo liso, a puramicina tendo sido estudado este efeito em ensaios de imunocitoquímica. Outro dos objetivos foi o estudo do efeito do ácido retinóico nos mecanismos de contração e dilatação presentes nas células do músculo liso utilizando a técnica *Planar Cell Surface Area*.

No primeiro estudo, a cultura de células endoteliais de rato foi obtida a partir da extração das artérias cerebrais médias e da artéria basilar. Já no segundo estudo, a cultura de células musculares lisas de rato foi obtida apenas pela extração das artérias cerebrais médias.

Posto isto, no primeiro estudo, as culturas de células endoteliais incubadas com uma concentração de 2 μg/mL de puramicina durante 24 h, apresentaram uma pureza de 90% e as de 4 μg/mL aproximadamente 20%. Por sua vez, no ensaio de *Planar Cell Surface Area*nos resultados obtidos nos testes genómicos, só uma das concentrações incubadas de ácido retinóico apresenta vasorelaxamento, a de 0.5μmol/L, tendo uma percentagem de 5% de relaxamento. Já nos testes não genómicos, isto é o efeito rápido, à concentração de 10μg/mL o ácido retinóico apresenta uma percentagem de vasorelaxamento na ordem dos 10%.

Assim sendo, no primeiro estudo os resultados obtidos sugerem que a puramicina pode ser utilizada como antibiótico na obtenção de culturas puras de células endoteliais numa concentração específica, neste caso 2 μg/mL. Já os resultados obtidos no estudo do efeito do ácido retinóico nas células do músculo liso, sugerem que numa incubação de 24 h o ácido retinóico a 0.5 μg/mL traz efeitos vasorelaxantes nas células musculares

lisas e que sem incubação, a uma concentração de 10 μg/mL também apresenta efeitos relaxantes nas células musculares lisas.

Palavras-chave

Ácido retinóico; células do musculo liso;células endoteliais;puramicina;unidadeneurovascular

Resumo alargado

O cérebro é um dos órgãos mais importantes do nosso corpo, daí a grande quantidade de energia que é consumida. Apesar deste não a conseguir armazenar, ele é composto por uma estrutura, a que se dá o nome de sistema cerebrovascular, em que esta estrutura contém uma rede de artérias responsáveis pela entrega de oxigénio e glucose essências para uma normal função do sistema cerebrovascular. Esta normalidade, é chamada de homeostasee é mantida por interações entre uma vasta rede de artérias, a que se dá o nome de árvore vascular, e a unidade neurovascular. Esta unidade por sua vez é formada por diversos componentes, sendo eles os neurónios, astrócitos, pericitos, células endoteliais e células do musculo liso. Todos estes componentes desempenham funções fundamentais para esta homeostase, como por exemplo os neurónios e os astrócitos entre si são responsáveis pelo aumento do fluxo sanguíneo cerebral, aumentando assim a entrega de oxigénio e glucose em áreas específicas onde está a haver grande consumo destes. Por outro lado, as células endoteliais fazem parte da composição da barreira hematoencefálica que é responsável pela restrição da entrada de certos componentes para o cérebro, que possam causar alterações na homeostase do cérebro, como por exemplo componentes citotóxicos. Já os pericitos desempenham funções ao nível da manutenção na estrutura vascular dos capilares e da barreira hematoencefálica. Em relação às células do musculo lise, estas estão presentes na parede de vários órgãos como os vasos sanguíneos, estômago, intestino. Além disso por meio dos seus mecanismos de contração e relaxamento que lhe são restritos estas são responsáveis pela regulação do tónus vascular, pressão sanguínea e distribuição do fluxo sanguíneo.

O plano de estudo realizado nesta dissertação teve como foco central a análise do efeito do ácido retínico na unidade neurovascular, em específico nas células endoteliais e nas células musculares lisas. O primeiro passo deste trabalho, foi a obtenção de culturas primárias de células endoteliais provenientes das artérias cerebrais medias e da artéria basilar. Assim sendo, para aumentar a pureza desta cultura foi utilizado um antibiótico, a puramicina. Já no caso das células musculares lisas, o objetivo foi o estudo do efeito de um retinoide, neste caso o ácido retinóico, de modo a ver o impacto que este pode ter nos mecanismos de vasoconstrição e de vasodilatação.

Posto isto, a adição de puramicina às culturas de células endoteliais foi analisada, usando um ensaio de imunocitoquímica, em que foram testados dois anticorpos, neste caso o fator de Von-Willebrand que é específico das células endoteliais e a α-actina que

é especifica na marcação de filamentos de α-actina e miosina expressos em células musculares. Além disso, nesta experiência foram usadas duas concentrações diferentes de puramicina por um período de incubação de 24h. Em relação aos resultados obtidos, a puramicina a uma concentração de 2 μg/mL obteve-se uma percentagem de pureza a rondar os 90%, sendo que na concentração de 4 μg/mL a pureza foi de 20%.

Em relação ao estudo do efeito do ácido retinóico nas células musculares lisas, primeiramente as culturas utilizadas de musculares lisasforam obtidas a partir da extração das artérias cerebrais médias e neste estudo foram feitos tanto estudos genómicos, como não genómicos. Em relação aos estudos genómicos o período de incubação foi de 24h, sendo utilizadas 5 concentrações de ácido retinóico diferentes sendo que nos não genómicos apenas foi testada uma concentração.

A técnica utilizada foi a *Planar Cell Surface Area* em que nesta foi usado a noradrenalina como agente vasoconstritor e o Nitroprussiato de Sódio como agente vasodilatador.

Em relação aos resultados obtidos, nos testes genómicos, das 5 concentrações testadas, o ácido retinóico só apresenta dilatação numa concentração, neste caso a 0.5 μmol/L. Já nas restantes que são 0.1; 1; 5 e 10 μmol/L apenas existe contração. Em relação ao teste não genómico, à concentração de 10 μmol/L o ácido retinóico apresenta dilatação.

Em suma, com a realização deste trabalho conseguimos obter culturas primárias de células endoteliais puras após a adição de puramicina em que os resultados obtidos sugerem que esta a uma concentração de 2 μg/mL pode ser utilizada como forma de purificar culturas de células endoteliais. Já em relação ao ácido retinóico, os resultados indicam que este retinoide pode ter tanto no imediato como incubado efeitos vasodilatadores nas células musculares lisas.

Abstract

The brain represents one of the organs that consumes the most oxygen, having an irreplaceable function in the body. In turn, to perform it correctly needs to be in homeostasis, so it contains a network that is responsible for this homeostasis, which is called the neurovascular unit. This is composedofneurons, astrocytes, endothelial cells, and mural cells, as pericytes and smooth muscle cells that perform different functions within the brain that are essential for good performance on this.

This work presents two aims: establishment of a protocol to a pure culture of endothelial cells from cerebral arteries, in whichthe effect of puromycin, an antibiotic that induce cellular death on smooth muscle cells was analysedthrough immunocytochemical assays. The other was the study of the effect of retinoic acid on the contraction and relaxation mechanisms present on the smooth muscle cells, using the Planar Cell Surface Area technique.

In the first study, endothelial cell cultures of rat were obtained by extracting the middle cerebral arteries and the basilar artery. In the second study, the culture of smooth muscle cells of rat was obtained onlyextracting the middle cerebral arteries.

Thus, in the first study endothelial cells cultures incubated with a concentration of 2µg/ml of puromycin for 24 h, showed a purity of 90% and those of 4 µg/ml approximately 20%. In turn, in the PCSA assay, on the results obtained ingenomic tests, only one of the incubated concentrations of Retinoic Acid presents vasorelaxation, that of 0.5 µmol/L, with a percentage of 5% relaxation. In non-genomic tests, the RA rapid effect, at a concentration of 10 µg/ml, shows a percentage of vasorelaxation in order of 10%.

Therefore, in the first study, the results obtained suggest that puromycin can be used as an antibiotic to obtain pure cultures of endothelial cells at a specific concentration, in this case 2 µg/ml. The results obtained on the effect of retinoic acid on smooth muscle cells suggest that, in a 24-h incubation period, retinoic acid at 0.5µg/mL has vasorelaxant effects on smooth muscle cells. Moreover, without incubation witha concentration of 10µg/ml, the results demonstratea relaxant effect.

Keywords

Endothelial cells;neurovascular unit; puromycin; retinoic acid; smooth muscle cells

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List of Abbreviations

5-HT	Serotonin
AA	Arachidonic acid
AQP4	Aquaporin-4
ASS	Antibiotic-antimycotic solution
ATP	Adenosine triphosphate
atRA	All-trans-retinoic acid
BBB	Blood-brain barrier
BK_{ca}	Big conductance Ca^{2+}activated K^+
BSA	Bovine serum Albumin
cAMP	Cyclic adenosine monophosphate
Cav1.2	L-type voltage-dependent calcium channels
CBF	Cerebral blood flow
CCM	Complete culture medium
cGMP	Cyclic guanosine monophosphate
CNS	Central Nervous System
CSF	Cerebrospinal fluid
DAG	Diacylglycerol
ECs	Endothelial Cells
EDTA	Ethylenediaminetetraacetic acid
EET	Epoxyeicosatrienoic Acid
EGF	Epidermal grow factor
ERK 1/2	Extracellular signal-regulated kinases
FBS	Fetal Bovine Serum
GPCR	G Protein-coupled receptor
ICH	Intracerebral hemorrhage
IGF-1	Insulin-like growth factor-1
IK_{Ca}	Intermediate-conductance K^+ channels
IL	Interleukin
IP3	Inositol triphosphate
ISF	Interstitial fluid
K2p	Two-pore domain K^+ channels
K_{ATP}	ATP-sensitive channels
K_{ca}	Calcium-activated K^+ channels
K_{ir}	Inward rectifier K^+ channels

K_v	Voltage-dependent K^+ channels
MAPK	Mitogen-activated protein kinase
MCA	Middle cerebral artery
MMPs	Matrix metalloproteinases
MT	Mechanical thrombectomy
NA	Noradrenalin
NO	Nitric Oxide
NVU	Neurovascular unit
PBS	Phosphate-buffered saline
PCSA	Planar Cell Surface Area
PFA	Paraformaldehyde fixation
PIP2	Phosphatidylinositol Biphosphate
PKC	Protein Kinase C
PLC	Phospholipase C
RA	Retinoic Acid
RAR	Retinoic acid receptor
ROS	Reactive oxygen species
RXR	Retinoic X receptor
SK_{ca}	Small-conductance K^+ channels
SMCs	Smooth muscle cells
SNP	Sodium nitroprusside
SR	Sarcoplasmic reticulum
TGF-β	Transforming growth factor beta
TJ	Tight Junctions
TNF-α	Tumour Necrosis Factor alpha
tPA	Tissue plasminogen activator
TRP	Transient receptor potential channels
VDCCs	Voltage-dependent channels
VGCCs	Voltage-gated calcium channels
Vm	Membrane potential
VSMCs	Vascular smooth muscle cells
VWF	Von Willebrand factor

Chapter 1

Introduction

1. Introduction

1.1. Cerebrovascular System

The cerebrovascular system is one of the most important structures responsible for the constant supply of oxygen and substrates in the brain [1]. This consumes approximately 15% of total cardiac output, being the blood transported by the trunk, through four vessels, the left and right internal carotid arteries, and the left and right vertebral arteries, that connected intracranially forming the basilar artery [2].In the brain, these arteries form a network, called the circle of Willis, and play a key role in maintaining the brain function. This network is responsible for the regulation of the cerebral blood flow (CBF), also allow collateral flow on hemispheres, and on the situation where there is a flow interruption, provide a compensatory collateral flow mechanism [3].Moreover, in this structure, one of the arteries more important is the middle cerebral artery (MCA), because most ischemic events occur in this area [4].This artery is the largest branch of the internal carotid artery and go in direction of the insular cortex that was in the Sylvian fissure. Moreover, this artery also irrigates subcortical structures, such as, the basal ganglia and the internal capsule with ramifications that occurs along the path. When this artery passes the Sylvian fissure, the branches expand to supply the lateral zones, such as, the frontal lobe, the parietal lobe and the temporal lobe [5].In addition, an ischemic event on this artery, derived from the areas that she supplies, can bring symptoms, like hemiparesis, deviated gaze, and some problems, on language and vision [6].

1.1.1.Vascular tree and structure of the blood vessels

This effective delivery of oxygen and nutrients is derived by the relationship between the vast network of arteries on the brain and the neurovascular unit (NVU). This vast network is called the vascular tree and starts on the pial arteries that extend through the brain, going into the parenchyma and branching into arterioles and capillaries irrigating several zones of the brain [7]; [8]. These walls contain three different layers: the tunica intima, which is the most intern layer composed by endothelial cells (EC), the tunica media composed by smooth muscle cells, and the tunica adventitia composed by connective tissue and giving the circular shape of the vessels [9]; [7]. Moreover, the arteries between the tunica media and intima present a layer of elastic tissue, are responsible for the vasoconstriction and vasodilation events on vessels. Also, the capillaries only present a single layer of cells allowing the exchange of molecules and fluids between the tissues and the blood [9].

1.1.2. Blood-brain barrier (BBB)

Central nervous system (CNS) has 3 types of barriers, that are responsible for control the molecular exchange between the neural tissue, the fluid spaces, and the blood. These barriers are the choroid plexus that regulates the changes between the ventricular cerebrospinal fluid (CSF) and the blood, the arachnoid epithelium, among the subarachnoid CSF and the blood, lastly the BBB constituted by a monolayer of cerebrovascular EC, that regulate the brain interstitial fluid (ISF) exchanges with the blood [10]. Moreover, these BBB also includes tight junctions (TJ), that limits the entry of large macromolecules, cells and pathogens agents on brain,enzymes, transporters, astrocytes, pericytes and extracellular matrix elements responsible for maintaining the CNS homeostasis (Figure 1) [7]; [11].This homeostasis is maintained with several mechanisms, such as, rapid diffusion of O_2 and CO_2 between the blood and the brain, regulation of the transport of energy metabolites and nutrients into the brain and clearance of metabolic end products from brain to venous circulation [7]. Also, this rapid diffusion of O_2 and CO_2 that is crucial for the brain metabolism and pH regulation on brain ISF can open entry for small lipophilic compounds, including drugs, like barbiturates and ethanol [12]. Furthermore, the blood vessels that include the BBB are mainly formed by two cell types: ECs that form the walls and mural cells located on the abluminal surface of the EC layers. BBB properties are intrinsic to the ECs, in which, are regulated and maintained by interactions between mural cells, immune cells and neural cells on the onset of CNS angiogenesis and in adulthood [13].

Moreover, due to a combination of intracellular and extracellular enzymes, there is a formation of a metabolic barrier, in which extracellular enzymes are responsible for peptide and adenosine triphosphate (ATP) metabolism, while intracellular enzymes deactivate neuroactive and toxic compounds. Also, this barrier plays key roles for CNS homeostasis, such as supplying the brain with vital nutrients and controlling waste efflux by regulating the exchange of ions between the blood and the brain with some specific ion carriers and channels, resulting in a brain ISF that allows the correct neuronal functions[10]. In addition, during some neurological diseases, such as stroke, the loss of some BBB properties can cause an ion dysregulation, altering CNS homeostasis, and an influx of immune cells leading to neuroinflammation[13].

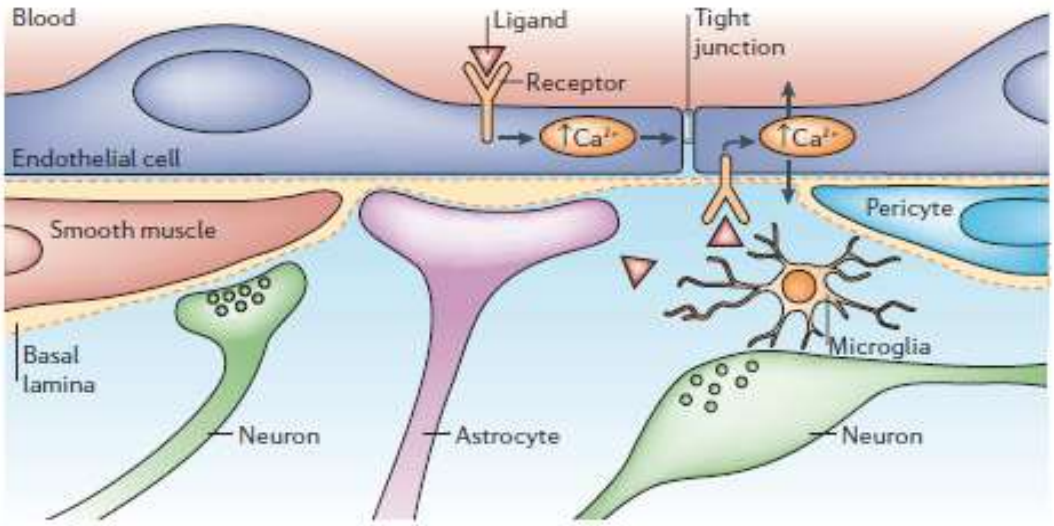

Figure 1: Model of the interactions presented in the Blood-Brain Barrier (BBB). A portion of the BBB, presenting the main cells and the signalling between them. Neurons are interacting with the Smooth muscle Cells (SMCs), contrarily of the astrocyte endfoot that interact directly with the Endothelial Cells (ECs) present in the layer. The microglia are present in the perivascular space, the pericyte and SMCs are presented in the basal lamina, both with closely interactions with the ECs. The ECs are interconnected by tight junction and expresses receptors on their surface, in what, some ligands are responsible for activating pathways which lead to the release of some substances to the blood, such as, Ca^{2+} indicated by the arrows. Adapted from [14].

1.1.3. Neuroinflammation

On healthy brain, peripheral circulation of cellular and molecular components is regulated by the BBB. Nevertheless, a brain damage, will cause the permeability of the TJ between ECs of the BBB causing an influx of peripheral immune cells and infiltration on brain parenchyma [15]. This infiltration will provide an inflammatory response after the brain damage with the migration of numerous inflammatory cells, as well as neutrophils, macrophages, and T cells [16]. Therefore, in the local of injury, will be a rapid inflammatory state, that is provoked by the adhesion and migration of circulating leukocytes. Further, these leukocytes release inflammatory cytokines that are responsible for the tissue damage [17]. In neuroinflammation, activated microglia plays a crucial role and have like the macrophages,: M1 and M2 phenotypes. On ischemic damage, the phenotype expressed is the M1, which is responsible for the release of various proinflammatory cytokines like the Interleukin (IL)-1β, Tumour Necrosis Factor alpha (TNF-α) and Reactive oxygen species (ROS) [18]. On the other hand, M2 phenotype secrete anti-inflammatory molecules like IL-10. Transforming growth factor beta (TGF-β) and growth factors like Insulin-like growth factor-1 (IGF-1) that are responsible for brain repair and control of post-ischemic inflammation [19].On ischemia-reperfusion injury, the larger damage comes from an inflammatory cascade that result an increase in toxic inflammatory mediators on reperfusion. An increase on

these mediators, like IL-1β, TNF-α and nitric oxide (NO) by the increase of ROS induce a neuronal death and irreversible brain damage [15].

1.2. Neurovascular Unit

NVU is a concept of stroke pathophysiology [20], that includes neurons, astrocytes, ECs, SMCs and pericytes (Figure 2). This term is used to correlate the intimate functional relationship between these cells and their reaction to injury[8]. Moreover, this relationship is responsible for maintain CBF under physiological and pathological conditions[20]. These relations are made by gap junctions between vascular and neural components and adhesion molecules, such as, cadherins and integrins that are responsible for the influx and efflux of ions such as Ca^{2+} and K^{+}[21]. In addition, the pathophysiology of the NVU is associated with neurological diseases, and include some features, such as, tissue hypoxia, inflammatory activation and the increase of the blood brain-brain barrier permeability resulting from the start of interactions between cellular (brain ECs, astrocytes, pericytes, inflammatory cells, and neurons) and acellular (basal lamina) parts of the NVU. Furthermore, the communication into the NVU is called neurovascular coupling or functional hyperaemia, and this is characterized by a complex mechanism between neurons, astrocytes and cerebral vessels. These mechanisms, like hyperaemia and autoregulation are responsible for adjusts in the blood supply according to the demands of energy and oxygen of activated neurons [22].

Hyperaemia is the mechanism responsible for regulating the microscopic blood flow in a local level and lead to delivery oxygen and nutrients on specific brain sectors. Also, this phenomenon is activated in physiological situations, such as, reading. Autoregulation is a mechanism behind the macroscopic vascular tone and is responsible for the constant regulation of the brain blood flow in physiological situations, like exercise and in pathological situations, as cardiogenic shock [21]. There are several vasoactive factors underlying these mechanisms, such as, ions, vasoactive neurotransmitters and vasoactive factors released in response to neurotransmitters. An example are the vasoactive ions, like the K^{+} and H^{+} that are released by action potentials and synaptic transmission, causing an increase of K^{+} extracellular, leading a hyperpolarization and a consequent relaxation of SMCs [8].

1.2.1. Neurons

Neurons are particularly sensitive cells, in what, some alterations in the environment surround them, can lead an alteration in their normal function. These, use pumps maintain the internal and external electric gradient, disabling the toxicity of calcium ions outside the cell and allowing the transmission of signals electrically and the communication between them [23]. When neurons detect some changes in the supply of nutrients and oxygen, these convert these signals in electrical and chemical messages to adjacent cells, like interneurons and astrocytes. After these messages, some mechanisms to restore the homeostasis will be activated. Also, the blood supply and vascular tone can suffer alterations, when the neurons communicate with the vessels through the astrocyte, directly or via interneuron [21]. This communication between the blood supply and the neurons are called neurovascular coupling or functional hyperaemia. This mechanisms under normal conditions are responsible for the increase of CBF and O_2 delivery to activate brain structures and to give support in region on the brain where the demand of O_2 is necessary [7]. Moreover, the neuronal response on arterioles is responsible to activate neuronal N-methyl-D-aspartate (NMDA) receptors, synthesis of nitric oxide (NO), resulting in a dilation [24]. In addition, some damage in the neurons membranes or pumps fail, can lead to neuron dead, due to calcium intake and consequently increase of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Moreover, oxygen deprivation events, such as ischemic stroke, can affect the neuron energy requirements and compromise the viability of neurons functions [23].

Some studies prove that in Subventricular zone (SVZ), neural stem cells can stimulate the formation of striatal neurons, replacing some of the neurons that die after stroke. However, some researchers demonstrate in adults' rats, that striatal neuroblasts are produced and continues the production 4 months after stroke. These neuroblasts formed after stroke, will differentiate in mature neurons, or die by apoptosis. Those that survive will migrate for the injury zone proving that after stroke, endogenous neural stem cells continue to supply the brain with new neurons, reducing maybe the damaged caused by an ischemic stroke event [25].

1.2.2. Astrocytes

The astrocyte is a glial cell with peripheral extremities found near the synaptic clefts of neurons and other astrocytes, with endfeet situated in the fashion layer around the vascular smooth muscle cells (VSMCs), forming a space between astrocytic endfeet and VSMCs called perivascular space. These cells have the capacity to communicate simultaneously with neurons, blood vessels and pericytes. Structurally, astrocytes and neurons have a similar organization, with a syncytial structure of 100 units, connect by gap junctions that work through calcium waves, allowing the propagation of electrical messages in large distances and the conduction to the SMCs of the vessels and pericytes bringing alteration in vessel tone [21]. The mainly functions of astrocytes that maintain the neuronal activity are the glutamate uptake, glutamine release, K^+ and H^+ buffering, water transport, and metabolic and trophic support. Moreover, these functions can promote neuronal survival throughout the ischemia and promote neurite growth and regeneration in the post-injury period [26]. Furthermore, this K^+ buffering will be transported to astrocyte end-foot and subsequently, released in perivascular space being uptake by the SMCs of the perfusing arteriole [27]. The glutamate will be converted to glutamine by the glutamine synthetase on astrocytes and will uptake by neurons, being the main precursor for neurotransmitter glutamate synthesis, where will be metabolized to glutamate and stored in synaptic vesicles, reducing the cytotoxic effect of glutamate[26]. In addition, astrocytes can express GABA transports, which is inhibitory neurotransmitter that reduce the effects of glutamate receptor stimulation. Some substances, release by astrocytes, like glycine and D-serine will influence the glutamate receptors having a role on the glutamate excitotoxity in ischemia events. On the other hand, some studies prove that the astrocytes have an important role in neurodegenerative diseases, like, contributing on the neural circuit formation, synapse formation, neuronal Ca^{2+} oscillation, plasticity and memory [26].

1.2.3. Endothelial cells

ECs are mesodermal squamous epithelial cells forming the walls of blood vessels (Figure2)[13]. These cells express multiple functions, and morphological characteristic, organ-to-organ, originating on brain the BBB, being responsible for the maintenance of brain homeostasis (Figure 1) [28]. On BBB, these cells have 4 main functions that are crucial for the maintain of BBB integrity. One of them is the TJ that are among them, responsible to avoid the passage of ions and nutrients into the paracellular space. Second, the CNS-ECs present a low rate of vesicular trafficking between the luminal

and abluminal membranes, comparing with the peripheral endothelium. Third, the regulation of the nutrient's entry, such as, glucose, amino acids and the remotion of neurotoxic compounds from the brain are made by transports instead vesicle trafficking [29]. On CNS-ECs these transports are highly expressed compared with other ECs, derived from the high mitochondria responsible to produce ATP, which is crucial for them functions [13]. Moreover, the transport proteins are located on the luminal and abluminal membranes and are the glucose transporter 1, monocarboxylate transporter 1 and 2, L-system neutral amino acid transporter 1 and the ATP binding protein that transport lipid-soluble complexes out of the brain endothelium [30]. In addition, CNS-ECs express a low rate of immune cells, for example, leukocytes, giving a safeguard space for the immune privileged zones on brain [29].

Junctional complexes formed among the ECs are constituted by two different types: adherents' junctions, like platelet-ECs adhesion, vascular endothelial cadherin and TJ proteins, like claudins, occludins, and zonula occludens (ZO). These cells also maintain the homeostasis by some signals or factors release by astrocytes and other cells of CNS (Figure 1) [30].Moreover, ECs produce some trophic and vasoactive factors, responsible for controlling the vascular tone, but also some dilatory compounds, like NO and vasoconstrictors, like endothelin and thromboxane.Lastly, on the same organ, like brain, these cells, depending on type of vessels can express different characteristics. For example, on arterioles, capillaries and venules, these cells present differences on transporters and levels of transcytosis and enzymes, as Na^+/K^+ ATPase [24].

1.2.4. Pericytes

Pericytes were initially characterized as spatially isolated cells, presented outside the capillaries and in their branches (Figure 2). These cells are also identified by the expression of some components, such as the Platelet-derived growth factor receptor β and the Neural/glial antigen 2 proteoglycan [31]. Moreover, pericytes are cells in close contact with ECs and astrocytes, replacing the SMCs on capillaries [24]. This contact gives an important support to ECs, like, helping in the ECs development, maturation and having part in the metabolic development of myocytes [21]. Furthermore, these cells, do not only act around the capillaries, but also, they can expand their processes for pre-capillary arterioles, post-capillary venules, middle of the capillary bed (Capillary network) and in the end of the arteriole [7]. Also, these cells have an important role in maintaining the vascular structure, BBB, and in flow regulation (Figure 1)[32]. In some studies, released by Kawamura and his collaborators, these cells contracts when the ATP increases and produces some growth and adherence factors. Another study by

Peppiatt, proves that these cells also have functions of controlling capillary diameter and modulating blood flow. In addition, pericytes have some importance, helping with phagocytosis and angiogenesis [21]. In other studies, these cells are responsive to arachidonic acid (AA) metabolites, like the Prostaglandin E2 and 20-Hydroxyeicosatetraenoic (20-HETE) acid causing relaxation or contraction, but, different of VSMCs, these cells do not respond to epoxyeicosatetraenoic acids. Also, these cells can express purinergic receptors (P2X87 and P2Y88), which are activated by ATP, causing an increase of Ca^{2+}, resulting on the pericyte depolarization and contraction. In addition, the activation of big-conductance (BK_{ca}) and small-conductance (SK_{ca})Ca^{2+} activated K^+ channels or ATP-sensitive (K_{ATP}) channels make a reduction of Ca^{2+} entry in voltage-gated calcium channels (VGCCs) resulted by an increase of K^+ efflux and a hyperpolarization of pericytes. Furthermore, compounds released by neurons, like adenosine, can cause pericytes hyperpolarization and relaxation, binding to α1 adrenergic and α2 adrenergic receptors, activating the K_{ATP} channels, and resulting in a release of K^+ from the cells. More studies, suggest that the blocking of NO synthase with Ng-nitro-l-arginine in brain slices, reduce the glutamate-evoked capillary dilation, suggesting NO have a role in pericyte relaxation and capillary dilation [7].

1.2.5. Smooth Muscle cells

SMCs are the main component of the wall structure on diverse organs, such as, blood vessels, stomach, intestines, bladder, airways and uterus[33]. On vessels, these cells present on structure, a cytoskeletal skeleton, composed by structural and contractile proteins. The connection between these cells and neighbour cells is through gap junctions, like connexins, responsible for the control of the ion concentration [34]. Also, on vessels they present two different phenotypes, a quiescent, where they are differentiated and a proliferating, where they are dedifferentiated [35]. Differentiated stage these cells, are specialized on contraction, being responsible for the regulation of vascular tone, blood pressure and blood flow distribution (Figure 2)[36]. Moreover, on this stage, these cells present a low rate of proliferation on blood vessels, expressing a high level of contractile proteins, like α-actin, myosin, calponin, caldesmon and sm22-α, being the α-actin expressed exclusively on these cells and on pericytes[24]; [34]. Also, other components like ions channels and signalling molecules are expressed in these cells, playing a mainly role on contraction mechanisms. On dedifferentiated stage, these cells present a low rate of contractile signs, and a high rate of molecules presenting on cell growth, migration, fibrosis and inflammation, for example, cyclins,

that is a cycle regulator, mitogen-activated protein kinases (MAPK), pro-inflammatory transcriptions elements and matrix metalloproteinases (MMPs) [34]. On this stage, these cells play an important role in the morphogenesis of the blood vessels, with a high productions of extracellular matrix compounds, like collagen, elastin and proteoglycans, giving them the contractile capacity [36]. On the other hand, when these cells are doing is main function, that is contract, they present an extended and fusiform shape, forming a contractile apparatus through filaments, being formed by contractile proteins[24].

The main trigger responsible for the initiation of these mechanisms of contractions is the Ca^{2+}. These mechanisms are initiated with a Ca^{2+}-calmodulin association forming a calcium-calmodulin complex. Subsequently, this complex will activate the myosin light chain kinase, which with the consumption of ATP will phosphorylate the myosin light chain. From this phosphorylation will result a cross-bridge with myosin heads and actin filaments, triggering muscle contraction. Also, the coupled between the actin filaments and cell membrane, by a viscoelastic system, can lead to some variations on the cell length [37].Some studies show that these cells, have the capacity of reverse some issues, like vascular damage, increasing the cells proliferation and the synthetic capacity [36]. In some pathological situations, SMCs differentiated re-enter in the cycle and become dedifferentiated cells with proliferative and migrative properties [34]. In addition, derived from their role on the regulation of the vascular tone, these cells may represent a good target for improving some treatments on diseases caused by chronic elevation of blood pressure, such as stroke[24].

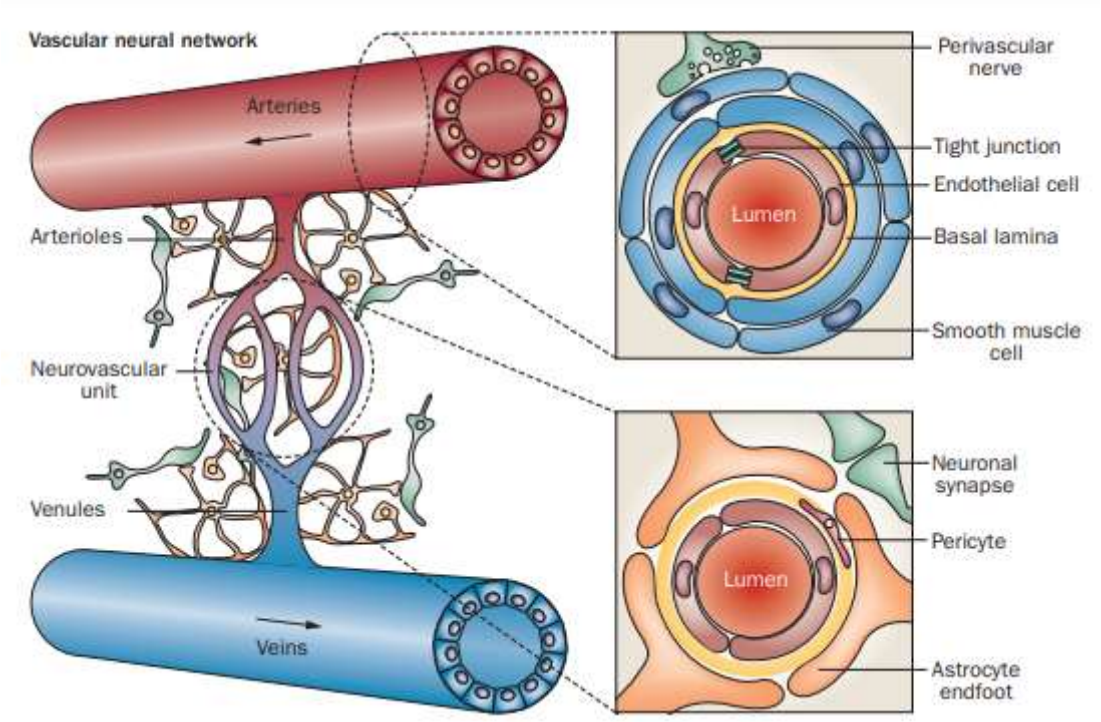

Figure 2: Mainly components of the vascular neural network.The vascular neural network englobes the Neurovascular Unit (NVU) and non-capillary ECs, perivascular nerves, basal lamina. NVU is composed by pericytes, astrocytes endfeet, capillary ECs, SMCs and basal lamina. Therefore, the vascular neural network is composed by the main elements responsible for the maintain of the CBF. Adapted from [20].

1.2.5.1. Contraction mechanisms

On brain, the blood flow is maintained between 60 and 150 mmHg due myogenic response of the arteries. This response is activated by several conditions, such as, alterations on the blood pressure, oxygen pressure, extracellular pH or metabolic activity[24]. Moreover, the SMCs contractions that are present on this response can be started by mechanical, electrical or chemical stimulus resulting in an increase of intracellular Ca^{2+} concentration [38]. This increase can be provoked by two events: the release of Ca^{2+} from inside of intracellular pools or from the opening of voltage-dependent channels (VDCC) located on the plasmatic membrane [39]. VDCC are heteromeric complexes composed mainly by one α1-subunit that form the pore and auxiliary subunits[40].This type of channel appears be expressed on several type of cells, such as, on neurons, cardiac myocytes and SMCs [41].

In SMCs, the L-type voltage-dependent calcium channels (Cav1.2) represent the major calcium entry pathway, being responsible for the regulation of the contractility mechanisms (Figure 3)[42]. Moreover, when activated, the Cav1.2 is responsible to allow the influx of Ca^{2+} that will bind on the calmodulin, resulting in the calcium-calmodulin complex (Figure 3). This complex will be responsible for the myosin light chain phosphorylation, leading on the cellular contraction.Furthermore, in myogenic response, this vasoconstriction represents an important role maintaining constant the

cerebral flow[43].Also, there is other type of channels that have an important role on the vasocontractions that is the transient receptor potential channels (TRP). These channels are non-voltage dependent cationic channels, with a high calcium permeability [44]. Also, are divided into 3 families: TRPV (vanilloid), TRPC (canonical) and the TRPM (melastatin). [45]. This last family, the TRPM, is the most focused, due a subtype, the TRPM4 that appears to have an important role on the SMCs in cerebral arteries, at the level of the membrane potential (Vm) and cellular contraction [46]. Moreover, contrary to VDCC the TRPM4 present specific properties, such as, having a low Ca^{2+}permeability and a high permeability to Na^{+}, promoting the membrane depolarization[24].

TRPM4 is activated due an elevation of the intracellular calcium concentration, resulted from a release in the sarcoplasmic reticulum (SR). This release is started when the diacylglycerol (DAG) and the inositol triphosphate (IP3) are originated from the phospholipase C (PLC) activity [47]. Then the IP3 will be responsible for the activation of the receptors on the SR, resulting in a release of the Ca^{2+} to the intracellular space and the DAG will stimulate the phospholipase C (PKC) activity [48]; [46]. These two paths will originate a translocation of the TRPM4 to the plasma membrane and subsequent activation (Figure 3)[46]. This activation will lead a sodium influx causing a membrane depolarization, activating the VDCC and causing vasoconstriction [45].

G protein-coupled receptors (GPCR) also have an important role on the regulation of the vascular tone. These receptors are activated by some chemical messengers, such as, catecholamines, serotonin (5-HT) and others [49].

On catecholamines, this are release by sympathetic nerve fibres, activating the adrenergic receptors [50]. These receptors belong to the GPCR and are divided in 3 types, being the most present in the brain, the α-1A. [50]; [51]. On 5-HT, the most abundant in brain is the 2A receptor, a receptor coupled on the Gq protein [52]. In both receptors the pathways that allow the contraction are, similarly, this is, after the activation of the receptors, the Gq protein will stimulate the PLC activity, promoting the hydrolysis of the Phosphatidylinositol biphosphate (PIP_2) in IP_3 and DAG. Then, the IP_3 will release the calcium inside the intracellular reservoirs and the DAG will activate the PKC that will originate a cascade of signalling events for the vasoconstriction (Figure 3)[24]. In addition, some searchers prove that MAPK pathways also have importance in the regulation of the vascular tone. For example, in cerebral arteries, one of these pathways is the extracellular signal-regulated kinases

(ERK 1/2) that when activated from the phosphorylation of tyrosine and threonine residues play an important role regulating the tone of this arteries [49].

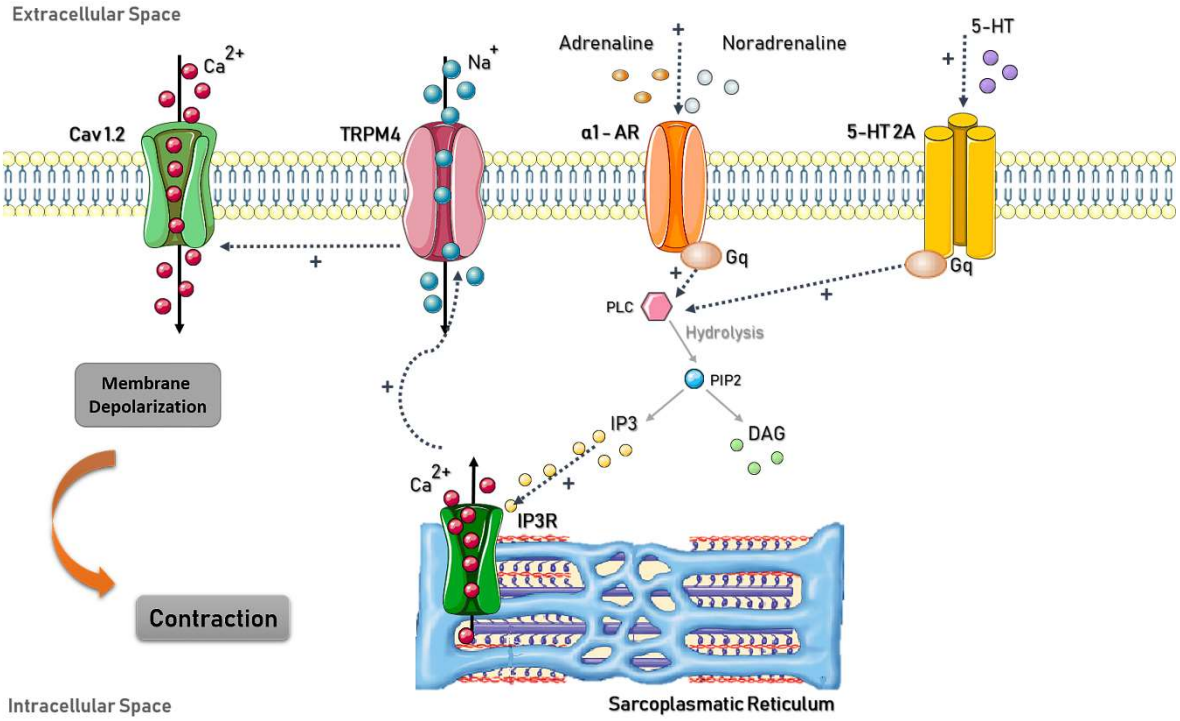

Figure 3: Contractile process on SMCs. G-protein coupled receptors, such as, the adrenergic and 5-HT receptors will activate the Phospholipase C (PLC), causing the hydrolysis of the PIP2 in IP3 and DAG. IP3 molecules will activate the SR receptors causing the release of the SR Ca^{2+}. This release of Ca^{2+} will activate the calcium channels, leading to vasoconstriction. Moreover, this release of Ca^{2+} also will activate the TRPM4 channels promoting the membrane depolarization and activating the VDCC (Cav1.2). The VDCC activation will promote vasoconstriction. Adapted from [24].

1.2.5.2. Relaxation mechanisms

Stroke, congestive heart failure and renal disease are associated with chronic hypertension. This occurs when the regulation of the vascular tone fails, due a malfunction of the vessel relaxation. On SMCs the regulation of the vascular tone and cellular depolarization or hyperpolarization are the ion channels, in this case the K^+ channels responsible for the relaxation mechanisms [53].

1.2.5.2.1. Potassium channels

Potassium channels on SMCs are responsible for the maintenance of the potential membrane and regulation of the vascular tone. When these channels open, occurs an efflux of K^+, resulting in membrane hyperpolarization, leading to the closure of the

voltage dependent Ca^{2+} channels (VDCCs), resulting in vasodilation of arteries [54]. Moreover, the closure of these channels can lead to vasoconstriction due to membrane depolarization. [55].Also, these channels constitute a family of membrane proteins that form aqueous pores in the cell membranes responsible for the K^+ flow. There are five different types of K^+ channels involved in the relaxation process on the SMCs: the voltage-dependent K^+ (K_v) channels; big conductance Ca^{2+}-activated K^+ (BK_{ca}) channels; inward rectifier K^+ (K_{ir}) channels; K_{ATP} channels and two-pore domain (K_{2p}) K^+ channels [56].

- **Voltage-dependent potassium (K_v) channels**

$K_V$channels are a type of channels highly expressed on the SMCs. These channels are activated to contradict a depolarization of the potential membrane, through the activation of the K^+ efflux, resulting in a repolarization of the Vm[56].

Also, depolarization on SMCs can lead an influx of Ca^{2+} into the cells through the Cav1.2, activating contractile mechanisms. This proves, that the activation of the K_V channels is important to regulate the cell excitability and maintain the basal tone.Moreover, the K_V channels is a heteromultimeric protein, composed by four α-subunits in each have six transmembrane domains and cytosolic accessory β-subunits [57].On pial arteries, this channels also present an important role, on determining the level of the myogenic tone in parenchymal arteries.Some studies demonstrate, that K_v channels can be inhibited by the activation of the PKC, derived from a small increase glucose. Another study also proves, that the 4-amino-pyridine also have an inhibited effect on these channels. Furthermore, some researchers demonstrate that these channels have response to vasodilators, as adenosine and β-adrenergic agonists. These vasodilators initiate a Cyclic adenosine monophosphate (cAMP) production, activating the protein kinase A that phosphorylate the $K_v$channels, modulating the $K_v$channels to vasodilatation [58].Lastly, these channels are potent suppressors of neuronal excitability, representing a good target on therapeutic modulation of the neuronal hyperexcitability that occurs on ischemic patients [59].

1.2.5.2.2. Inward rectifier K^+ (K_{ir}) and ATP-sensitive (K_{ATP}) channels

K_{ir} channels are expressed on some SMCs and in excitable and non-excitable cells [56]. Also, this type of channels is known as transmembrane proteins responsible for the regulation of Vm and [K^+] siphoning in glial cells. Moreover, on CNS this type of channels is responsible to control the cell differentiation, the hormone secretion, modulate neurotransmitters on the nigrostriatal system and regulating the artery

dilatation [60]. These channels are composed by seven subfamilies, wherein, their activity is regulated by some intracellular modulators and second messengers. These modulators are the PKC, G-proteins, intracellular Mg^{2+}, PIP_2 and pH. Some of the most important actions of this subfamilies are: $K_{ir}6.X$ controlling the insulin secretion and the myocardial resistance to hypoxia; regulation of the $K_{ir}3.X$ channels by the G-protein responsible for controlling the heartbeat and $K_{ir}1.X$ channels regulated by $[K^+]$ and pH, that control the K^+ secretion in the kidney [61]. Furthermore, some studies also prove that these channels, are inhibited in the presence of Ba^{2+} and Cs^+[54].

K_{ATP} are octamers composed by 4 sulfonylurea (SUR2B) and 4 inward rectifier ($K_{ir}6.1$) subunits [54]. These channels, present a main function on the protection of cardiac cells on ischemic injuries and regulation of the vascular tone. Moreover, K_{ATP} when suffer metabolic changes or exposition on pharmacological vasodilators can affect the vascular tone and blood flow. Also, these channels can be activated by vasodilators, that activate cAMP-dependent protein kinase and inhibited by vasoconstrictors, that activate the PKC. Some studies show that this channels on SMCs do not respond to ATP, being activated by nucleoside diphosphates and inhibited by glibenclamide. Lastly, the opening of these channels with vasodilators, prove to have some therapeutic effects in diseases, such as myocardial ischemia, glaucoma and bronchial asthma [56].

1.2.5.2.3. Calcium-activated K^+ (K_{ca}) channels

Calcium-activated K^+ channels are presented in all tissues and divided in 3 subfamilies: BK_{Ca}, intermediate-conductance K^+ channels (IK_{Ca}) and (SK_{Ca}). Commonly, on VSMCs are more expressed the BK_{Ca} channels being the $IK_{Ca}$and the SK_{Ca}more expressed on the ECs. On VSMCs, BK_{Ca}channels have an important role controlling the vascular tone (Figure 4). Also, these channels, such as K_v are activated by the membrane depolarization and the increase of the $[Ca^{2+}]$ on the VSMCs, due the release from the SR. This activation leads a K^+ efflux, which result in a hyperpolarization in the VSMCs and L-type voltage-gated calcium channels closure causing vasodilation (Figure 4)[56]. Structurally, BK_{Ca} channels present a four α pore-forming subunits and 4 β subunit[55]. The most important is the β-1 subunit responsible to increase the voltage and Ca^{2+}-sensitivity on the channel. A disruption on the β-subunit can lead a hypertension and ventricular hypertrophy [54]. Some studies show that these channels, can be activated by vasodilators, like testosterone and taurine [56]. Other study also prove that these channels can be inhibited by 20-HETE through the PKC activation. Lastly, BK_{Ca} channels represent a good target to treat cardiovascular diseases, due their role, on the

regulation of the vascular tone [55]. Moreover, alteration on their activity, can aggravate pathophysiological states, like vasospasm and ischemia [56].

1.2.5.2.4. Two pore-domain K^+ (K2p) channels

K_{2p} channels are presented in several types of cells, having primordial functions on neuronal and non-neuronal tissues, such as, mechanosensitive, neuroprotector, regulation of resting the Vm and detecting oxygen, pH and changes on the K^+ concentration [62]; [63]. Structurally, these channels have four transmembrane domains and two pore domains [63]. Moreover, these channels are divided in six subfamilies based on their function and structure: TASK; TREK; TWIK; THIK; TRESK and the TALK. For example, the TASK-1, TASK-3 and the TREK-2 have an important role on cerebellar functions, such as, on accuracy, regulation of the muscle tone and acquiring of motor skills. Moreover, the TASK-1 and TASk-3 are associate on some behaviours, like sleep and insomnia. Furthermore, these subfamilies can be inhibited and activated by several compounds, such as TREK subfamily activated by AA, unsaturated fatty acids and mechanical stretch. The TASK-1 and TASK-3 inhibited by extracellular acid pH, being the TASK-2 activated by alkaline pH and the THIK subfamily inhibited by volatile anaesthetics, in which, these anaesthetics activate the TREK-1, TREK-2, TASK-2, TALK-1 and TRESK[62]. Some studies also prove that K_{2p}channels are regulated by pharmacological agent and modulated by some GPCR [64]. Lastly, researchers hypothesise that these channels can represent a good target for the treatment of some brain diseases, like ischemia or other, such as, hypertension and diabetes. [63].

1.2.5.2.5. Transient receptor potential (TRP) channels

TRP channels have an important role on SMCs contraction but also on relaxation mechanism. Specific there is a subfamily, the TRPV4 that is associated SMCs relaxation[24]. Structurally, this subfamily is composed with six transmembrane segments with a pore region, being usually express on tissues, such as skin, brain, kidney and heart [65]. Moreover, Ca^{2+} influx on these channels is called TRPV4 sparklets, that are responsible to create subcellular microdomains with a highly Ca^{2+} level leading to activation of some signalling cascades, endothelium vasodilation and

negative feedback of vasoconstrictor stimuli (Figure 4)[66]. TRPV4 channels also present interactions with other channels, such as, the TRPA-1, $K_{ca}2.3$ and the K_{ca} 1.1. Furthermore, these channels are activated by some factors, such as cell swelling, heat or chemicals, like endocannabinoids, Epoxyeicosatrienoic Acid (EET), AA or 4-α-phorbol esters. In the case of the EET, this cause a Ca^{2+} influx on the cell, stimulating the Ca^{2+} release on ryanodine receptors resulting on an increase of Ca^{2+} sparklets. Due activation of the K_{ca} 1.1, by the Ca^{2+} sparks, will occur a hyperpolarization of the SMCs, resulting in a vascular relaxation (Figure 4). In addition, some studies prove that the activation of these channels are associated with some diseases, such as hypertension, bone disorders, muscle dysplasia and hyponatremia. Other study also considers that TRPV 4 channels can act as an osmolarity sensor in the airways, due to their expression on human airway SMCs [65].

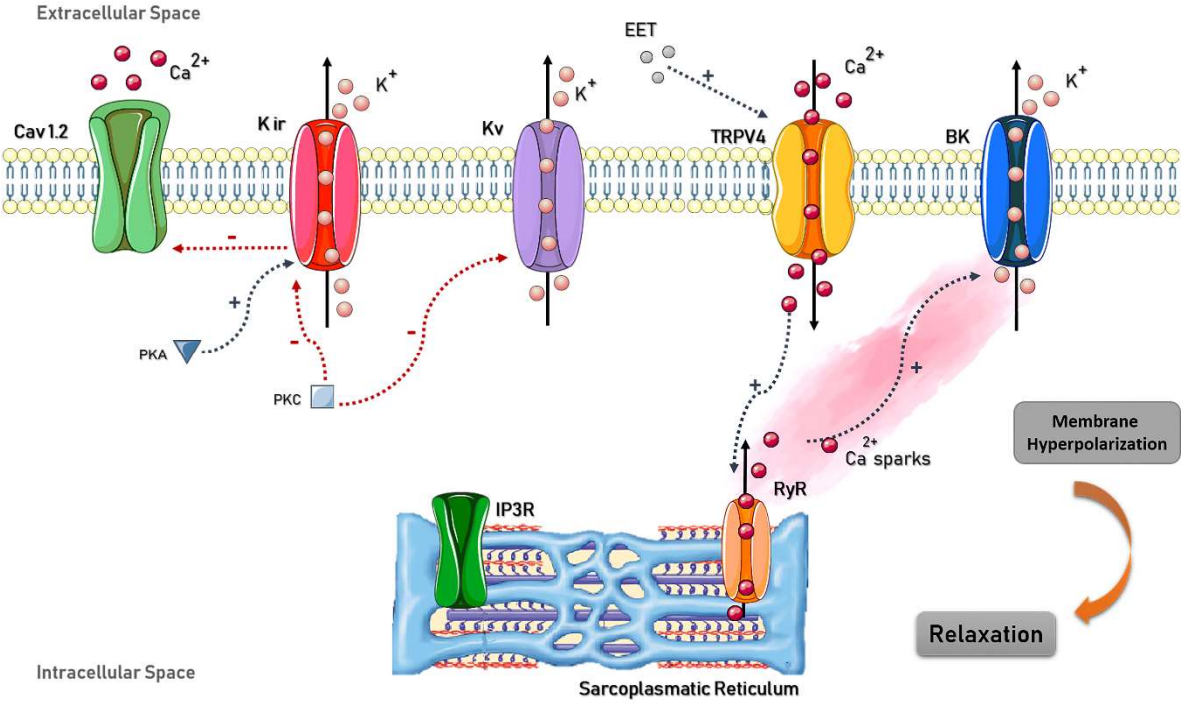

Figure 4: Relaxation process on Smooth Muscle Cells (SMCs). Potassium channels are responsible to promote the output of potassium from the SR to the Intracellular space, inhibiting the VDCC (Cav 1.2) and leading to membrane hyperpolarization. Moreover, TRPV4 channels are activated by the epoxyeicosatrienoic acid (EET) resulting in an influx of Ca^{2+} that will activate the ryanodine receptors (RYR). This activation will generate Ca^{2+}sparks, in which, these will activate the BK Channels, originating relaxation, derived from the membrane hyperpolarization. Adapted from [24].

1.3. Retinoic Acid

Vitamin A is a natural retinol present in some aliments, like fish-liver oils, eggs, milk, butter, plants [67] and in the human body, responsible for regulating the reproduction, immunity, vision, proper function of the lungs, and neuronal system.

This compound is so important for our body because a deficit of Vit A can cause blindness, infections problems, and an iron deficit, lead to anemia. Also, a exacerbate consumption, bring toxicity for the liver, CNS, muscular- skeletal system, internal organs and skin. Retinoids in most of animals are derived from the cleavage of carotenoids in plants and in some animal tissues.A product originated from the metabolism of the Vitamin A (retinol) is the retinoic acid (RA) an active retinoid, which have an important role at the level of the neuronal system, and in inducing pleiotropic effects in the cell growth, differentiation and death. Furthermore, in your organism, to regulate the levels of RA in cells and tissues, there is a balance between synthesis and catabolism [68]. The synthesis of RA starts with the transformation of retinol (Vit A) derived from the dietary, on retinal by a dehydrogenation reaction, and the enzymes involved are the alcohol dehydrogenases, the short chain dehydrogenases (SDR) and the cytochrome P450s. Then the retinal is converted in RA through a non-reversible reaction by the retinal dehydrogenase [67]. On catabolism the enzymes responsible are the CYP26 family that oxidize the RA on 4-hydroxy RA and 4-oxo RA [68].Also, RA can exist in stereo-isomeric forms, such as, all-trans-retinoic acid (atRA), 13-cis RA and 9-cis RA [69].Moreover, RA is a compound that binds to RA receptor (RAR) which forms a heterodimeric complex with the retinoic X receptor (RXR), in the cell nuclei. This complex will modulate the transcription of RA targeting genes by binding to DNA on retinoic acid response element [70]. These receptors present three subtypes: RAR have the RARα, β and γ and the RXR have α, β, γ [71]. The majority of RA targeting genes are involve in cell differentiation, antiproliferation, pro-apoptotic and antioxidant, thus is used as a chemotherapeutic agent and for the treatment of skin diseases [72]. Studies, prove that RA can stimuli the differentiation of progenitor cells on neuronal cells, showing that can help on postinjury neurogenesis [73].

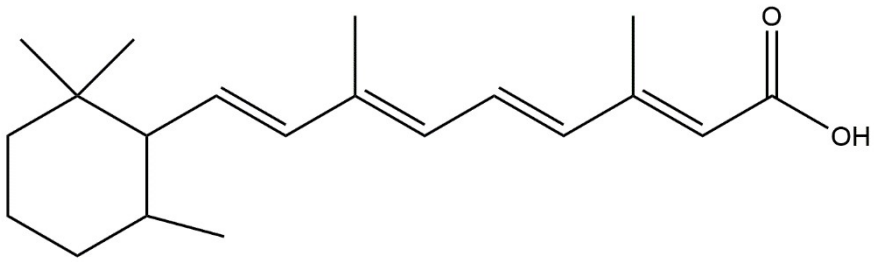

Figure 5:RA chemical structure.

1.3.1. Effects on neurovascular unit

Other studies say that RA also can stimuli the neurite outgrowth and neuronal differentiation on several cells [74]. Moreover, some studies prove that the atRA activating the RAR are responsible for the PI3K and MAPK pathways activation and

stimulation the actin remodelling [75]. Other study also prove that this isomer promotes the Krüppel-like factor 4 acetylation and phosphorylation that will transactivate the SM22α and SM α-actin promoting the SMCs differentiation [76]. Other study also prove that RA appears have another's functions on vasculature, such as, regulating the WNT signalling and controlling the pericyte numbers in the develop of the brain vasculature. This control of the WNT signalling result in an over-recruitment of pericytes being crucial for the vascular stability [77].

On ECs, some studies also prove that the RA have a role modulating the angiogenesis and the vascular endothelial growth factor (VEGF) gene expression [78]. Moreover, other studies also prove, that RA have an important role on proteins expression, such as, the VE-cadherin in BBB, TJ and adherent junctions proteins in both human brain ECs [79], [80]. Furthermore, a study show that RA can have a role on the oxidative stress in inflamed ECs, activating the antioxidant transcription factor nuclear E2 [81].

RA also have an important on the immune system, this is, modulate the T-cell selection, B-cell migration, neutrophil and astrocyte differentiation. Also is responsible to attenuate the neuroinflammation, by the inhibition of the JAK/STAT pathway[82];[83]. Furthermore, RA can modulate the Ca^{2+} levels during homeostatic synaptic plasticity in the hippocampus having an important role on the control of synaptic plasticity and neuronal firing [84]; [85].Additionally, a study proves that one isomer of the RA, the 9 cis RA present neural reparative properties in ischemic events, suggesting that can be a good target on the post stroke therapy [86].

1.4. Stroke

Stroke is one of the major causes of death around the world and disability in adults. Moreover, based on the feature's events, the stroke can be classified in two types: hemorrhagic stroke and ischemic stroke [87]. Most of the time, Hemorrhagic stroke occurs due an intracerebral hemorrhage (ICH), this are a rupture of cerebral vessels, derived by an excessive pressure of blood on arterial walls, previous damaged by atherosclerosis, aneurysm or arteriovenous malformation [88]. ICH can be divided in two different classes, primary and secondary. Primary ICH, that is 88% of all hemorrhages causes, is originated from a spontaneous rupture of small vessels damaged by chronic hypertension or amyloid angiopathy. Secondary ICH, that is the remaining percentual are derived from the bleeding caused by other diseases, like vascular abnormalities, tumours or impaired coagulation [89]. In most of the times, ICH result from rupture of specific arteries, such as, middle or basilar arteries or anterior and posterior cerebral arteries. ICH also triggers some inflammatory

responses, doing changes on vascular level surrounding the local of injury or perihematoma zone, like vasodilation, increasing the permeability of vessels, contraction of ECs, release of numerous substances, like histamine, VEGF, bradykinin, NO. Also, the release of substances, like superoxide, lactoferrin, histamine, IL-1, hydrogen peroxide on the perihematoma zone, tell us that there is a bound between the leukocytes and ECs. In addition, after ICH there is a formation of an oedema, that in some studies on animals have the peak in day 3 or 4, having the decrease on day 10 or 20 [90].

Ischemic stroke or cerebral infarcts have two stages, ischemia and reperfusion [91]. Ischemia is caused by the development of a thrombus and/or an embolism provoking a blockage, leading a deficiency of oxygen on the tissue [89]. This blockage can lead to an irreversible damage on brain that is result from the deprivation of oxygen and essential nutrient that are crucial for the homeostasis of brain (Figure 6). Therefore, if this homeostasis is affected, neurons and another's cells die, and this death will conduct a rapid influx of immune cells, ROS and inflammatory cytokines that will increase the brain damageand lead a brain infarction[15]. This final damage on brain is dependent of many factors, such as, the existence of collateral systems, the location of the infarct and some person features like age, sex, genetic background [92].

Reperfusion is a mechanism, that after a restriction of the blood supply, this is followed by a reoxygenation of downstream tissue and vascular restoration on stroke. But this mechanism also can bring tissue injury, following the reoxygenation of tissues, there is a formation of ROS, calcium overload, mitochondrial permeability transition pore, endothelial dysfunction and inflammatory responses (Figure 6)[93]. Also, there are some issues associated with the incidence of ischemic stroke, like the increasing of the blood pressure, cholesterol, carotid stenosis, atrial fibrillation, diabetes mellitus and some behaviours like smoking [94]. Moreover, in stroke, BBB function is affected and the relationship between astrocytes and ECs is anomalous. Astrocytes begin to secrete TGF-β, which decreases capillary endothelial expression of Tissue plasminogen activator, mechanical thrombectomy, vascular basal matrix proteolysis followed by induction of aquaporin 4 mRNA and protein at the rupture of the BBB [10]. A study shows that the BBB disruption on ischemia, permits that the microvessels let pass some vascular inflammatory cells and proteins that are toxic to neurons, causing is dead [23]. Other studies, also says who suffer ischemic strokes have more chance to survive, that who have hemorrhagic strokes, due the fact, that hemorrhagic strokes not only make a damage in brain, but also increase the pressure on brain or spasms in blood vessels [89]. In addition, there are a zone, called ischemic penumbra, that become the focus of

many researchers for the treatment on stroke. This zone was first defined by Artrup as a region on brain tissue, around an ischemic tissue, where the blood flow is reduced, causing hypoxia, but not severe to causing an irreversible failure of energy metabolism, and dead of the cells, being possible the recovery of this cells, if the reperfusion was good [95]. To finalize, some researchers prove that stroke raise the number of immature neurons in the SVZ, and stimuli the proliferation of endogenous cells in this zone. Moreover, in post-stroke neurogenesis, with reactive astrocytes and blood vessels occur a migration of neuroblasts into damage zones of striatum that border the SVZ [96].

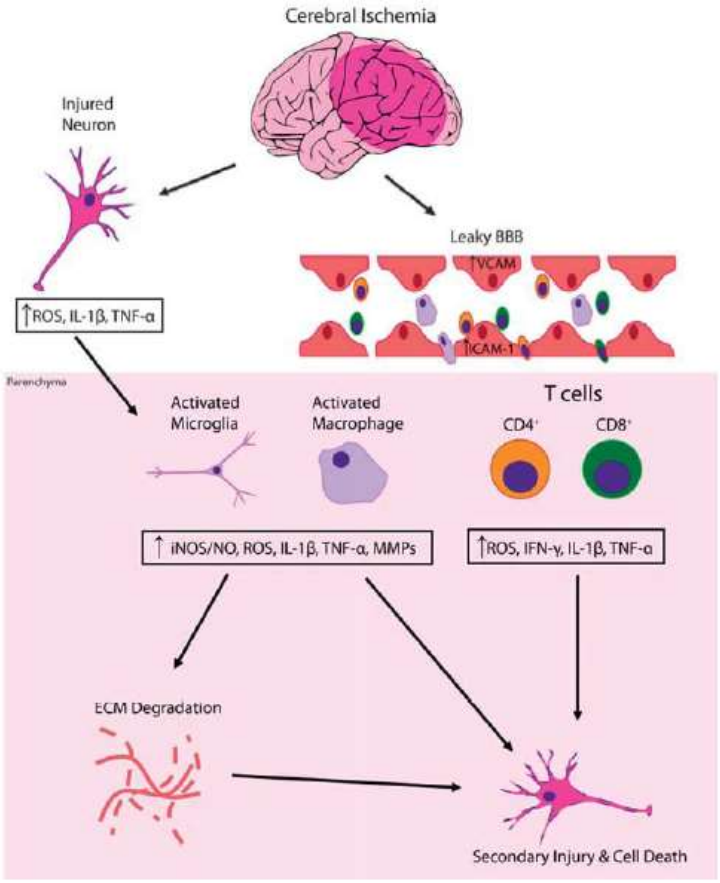

Figure 6: Cascade events after an ischemic injury. Cerebral Ischemia lead two different injuries, one is on injured neurons, generating Reactive Species of Oxygen (ROS) and proinflammatory molecules, such as, IL-1β and TNF-α. Other injury is the BBB disfunction, in which, cell-to-cell Tight Junction (TJ) between the ECs is lost. These will lead to the migration and extravasation of leukocytes into the brain parenchyma originating a variety of proinflammatory molecules, like inducible nitric oxide synthase, MMPs, TNF-α and ROS. These molecules will lead to the extracellular matrix degradation and cell death. Adapted from [15].

Chapter 2

Aims

2.Aims

In the brain, one of the most important things for maintaining his normal function is homeostasis. This is maintained from the interaction of several components that form a network called neurovascular unit. So, this work has two main goals, protocol establishmentfor a primary culture of endothelial cells from cerebral arteries and the retinoic acid effect study in smooth muscle cells mechanisms.

In order to achieve these objectives, several aims were established:

- Evaluation of the puromycin effect to obtain a pure ECs culture from MCA and basilar artery;
- Evaluation of RA effect on SMCs incubated for 24h, long term effects;
- Evaluation of the rapid RA effect on SMCs, non-genomic effects.

Chapter 3

Experimental Section

3. Experimental Section

3.1. Materials and Methods

3.1.1. SMC culture

The Wistar rat females used in the SMCs culture follow the rules related to the protection of laboratory animal for scientific research (Directive 2010/63/EU). The protocol was adapted from a previous method established by Cairrao group [97], [98].For the culture, first, the females were anesthetized with ketamine (87.5 mg/kg) and xylazine (12 mg/ kg) and subsequently sacrificed by cervical dislocation.Then, with surgical material, the middle cerebral arteries from the circle of Willis are extracted and placed on Petri dishes containing PBS and antibiotics. Posteriorly, the arteries are plated on MW coated with collagen (5 uL/Well) and put on the incubator at 37°C and 5% of CO_2 for 5 min. Pass this 5 min, is added 1 mL of complete culture medium containing DMEN F12, Fetal Bovine Serum (FBS 5%), bovine serum albumin (BSA 0.5%), antibiotic (Ab 1%), and GSP ((composed by fibroblast grow factor (FGF 0.5 ng/mL); epidermal grow factor (EGF 5µg/mL); heparin (2µg/mL)) and insulin (5µg/mL), with pH=7.4 (Table 2). After 24h, is added one more milliliter of culture medium. The medium of culture was changed 2 on 2 days, for 1 month being the cells used on the assays on the fourth passage (P4).

3.1.4. PCSA (Planar Cell Surface Area)

On PCSA the protocol used was adapted from Mariana *et al.* [101].This technique allows the study of cell contractility or relaxation through the analysis of the increase or the decrease in the cell area, evidenced by images obtained on different periods.

To initiate this technique, first, the cells used on P4 must be in confluence and then are incubated with a serum-free medium for 48h. After 24h of that 48 h, the cells are incubated with Retinoic acid in different concentrations diluted in a serum-free medium.

After incubation time, the cells are trypsinized and transferred (500 µL) to Petri dishes coated with 5 µL of collagen. Then the Petri dishes with the culture are left for 4h on the incubator at 37 ºC. Once past the 4h, the cells were washed 4 times with Krebs medium, leaving the last wash.

Posteriorly, the cells were observed on an inverted fluorescence microscope (Zeiss Axioobserver Z1). This microscope possesses a hight speed monochrome digital camera (Axio Cam Hsm) and a temperature control system, responsible for maintaining the viability cellular during the assay.

On the microscope, after the first observation, is chosen a good plan of cells and taken the first photo representing a control. Posteriorly, during 20 min, without adding contractile or relaxing agents on the cells, is taken the first video and the second photo, representing the basal contraction. After this second photo, the contractile agent is added for 20 min, in this case, the Noradrenalin (NA) (1 μM) being taken the second video and the third photo. Lastly, is added the relaxing agent, sodium nitroprusside (SNP) (1 μM) for 20 min and at the end of this 20 min, the fourth photo and the third video are taken.

The objective of this technique in this work was to study the retinoic acid effect on cell contractility and relaxation, after the addition of NA and SNP.

Also, the images and the videos were analysed on a supplement tool of the Axion vision 4.8 software, called the "Automatic measurement program" with the study of the differences in the cell area after adding contractile and relaxing agents.

Lastly, contractile and relaxing agents used in this technique were diluted on distilled water and the Retinoic acid in Ethanol 99,8%. All the drugs used in this technique were acquired on the sigma Aldrich.

3.1.2. ECs culture

The Wistar rat males used in the SMCs culture follow the rules related to the protection of laboratory animal for scientific research (Directive 2010/63/EU)For this culture, first, the males were anesthetized with ketamine (87.5 mg/kg) and xylazine (12 mg/ kg) and subsequently sacrificed by cervical dislocation.The procedure used in this culture also was adapted from a previous protocol established by Martin and his associates [99].With surgical material was proceeded the extraction of the middle cerebral and basal arteries from the circle of Willis. Then the arteries were placed on Petri dishes containing lock solution and antibiotics. After this, the arteries suffer mechanical and enzymatic digestion at 36 ℃ for 5 min. Pass this 5 min, the dissociation reaction is stopped with 1 mL of EC medium and the slices of arteries were centrifuged (150 g) and transferred with 2 mL of EC medium to a well without any coating. This medium contains DMEN F12; FBS (20%); Ab (1%) and pH=7.1 (Table 2). After 4h, move the medium for another well and on the previous well, place 2 mL of EC medium.

3.1.3. Immunocytochemistry Assay

On this Assay, the protocol was adapted from Mendes and is Co-workers. [100].This technique was used to differentiate the cell types present on the culture and to study the puromycin effect in these cell types.On this assay, when the cells o P1 cells obtained confluence on the coverslips, the wells were incubated with serum-free medium containing puromycin (2µg/mL and 4µg/mL) for 24h. After this incubation, the first step is the fixation of the cells. This method of fixation consists, after removing the culture medium, wash the cells with PBS and add on the wells a solution containing ethanol/acetone (50/50) for 30 min. After this, to blockage, the nonspecific bindings were added PBS-T (PBS + Tween 0.1% and 20% FBS) during 1h on room temperature. After the blockage, the cells were washed with PBS-T and incubated overnight at 4ºC, with the primary antibodies, Von Willebrand factor (VWF) and α-actin diluted in a solution containing PBS-T + 1% of FBS (Table 1). Pass 24h, washed 3 times the coverslips with PBS-T and incubated with the secondary antibody diluted in the same solution of the primary antibodies for 1h in the dark on room temperature. After this, the coverslips were washed more 3 times and incubated with Hoescht diluted on the same solution of the antibodies, marking the cells cores.Posteriorly, the cells were washed once more time and fixed on the lamina with 5uL of DAKO and sealed with a glaze. On the microscope (Axiobserver Z1, Zeiss) the images were acquired on the objective 40x and each condition had 3 laminas. To analyse the images was used ImageJ.

Table 1: Primary and Secondary Antibodies used in the Immunocytochemistry assay.

Protein	Primary antibody	Dilution	Company	Secondary antibody	Dilution	Company
α-actin	Mouse Monoclonal Anti-α-actin	0.5:300	Sigma Aldrich	Alexa Fluor 488 AffiniPure Goat Anti-Mouse	1:1000	Invitrogen, Molecular Probes
VWF	Mouse Monoclonal Anti-VWF	1:750	Santa Cruz Biotechnology	Alexa Fluor 488 AffiniPure Goat Anti-Mouse	1:1000	Invitrogen, Molecular Probes

Table 2: Solutions used in PCSA and on the preparation of SMC/Endothelial culture

Solutions	Composition
Dulbecco's Modified Eagle Medium F-12 (DMEM-F12)	Lyophilized DMEM (Sigma); $NaCO_3$(1.2g/L) and L-ascorbic acid (20mg/L), pH= 7,4
Antibiotic-antimycotic solution (AAS)	Penicillin Mix (1000 U), Amphotericin (25 mg) and streptomycin (10mg)
Phosphate-buffered saline (PBS)	1.37 M NaCl; 27 mM Na_2HPO4 and 20 mM KH_2PO_4, pH= 7.4
Complete culture medium (CCM)	DMEN-F12 supplemented with bovine fetal serum (FBS-5%); Bovine serum albumin (BSA-0.5%); Epidermic growth factor (EGF-5µg/mL); Fibroblast growth factor (FGF-0.5 ng/mL); heparin (2µg/mL), insulin (5 ug/mL) and AAS (1%), pH 7,4
EC culture medium	DMEM-F12 supplemented with bovine fetal serum (FBS-20%); ASS (1%), pH 7,1
Culture medium with 10% FBS	DMEN-F12 supplemented with bovine fetal serum (FBS-10%); Bovine serum albumin (BSA-0.5%); AAS (1%), pH 7.4
Serum-free medium	DMEM-F12; bovine serum albumin (BSA-0.5%) and AAS (1%), pH7.4
Krebs	NaCl 119 mM; KCl 5mM; $CaCl_2{\cdot}2H_2O$ 1.5 mM; $MgSO_4{\cdot}7H_2O$ 1.2 mM; KH_2PO_4 1.2 mM; $NaHCO_3$ 25 mM; EDTA-$Na_2$0.03 mM; l-ascorbicacid0.6 mMand glucose 11 mM, pH 7.4

3.2. Statistical analysis

The obtained results are expressed in percentage of control, percentage of total cells or percentage of contraction or relaxation induced by NA and SNP, that represent the mean± SEM of n independent experiences indicated on the graphics subtitles, that was realized on triplicates. The statistic analyse was made on the statistic program SigmaStat Statistical Analysis System, version 3.5 (2006) and the graphic design was made on the Software Origin 8.5.1. The statistical significance between the two groups was analysed using the T-Student and to compare more than two groups was used a One-Way ANOVA with the Dunnett test. Furthermore, was considered statistical significance when the probability was inferior to 5% ($P<0.05$).

Chapter 4

Results

4. Results

4.1. Characterization of Primary Endothelial cells culture

Primary Endothelial cells culture was obtained from the explant of the MCA and basal arteryusing a protocol established by Martin and Co-Workers [99]. The culture obtained the confluence between 25/30 days, being used the cells on P1.

4.1.1. Evaluation of puromycin effect on the purity ECs culture

To evaluate the puromycin effect on the culture purity was used an immunocytochemical assay. In this assay, to mark the cells present in the culture, was used antibodies, such as, anti α- actin, which is, a marker of the fibres presented on the SMCs and the VWF, that is specific for a protein present on the ECs membrane. Moreover, due the fact the anti α-actin antibody only marks on the SMCs contractile stage, the cells were incubated 24h with serum-free medium containing puromycin on 2 μg/mL and 4 μg/mL. In the figures 7A, 7B and 7C the green colour is the VWF marking on the ECs and on blue the nuclei of the cells, marked with Hoescht.When incubated with 2 μg/mL and 4μg/mL of puromycin during 24h, the cultures present significant differences between the two concentrations having the culture incubated with 2 μg/mL approximately 90% of cells expressing VWF and 20% the culture incubated with 4 μg/mL (Figure 7D).

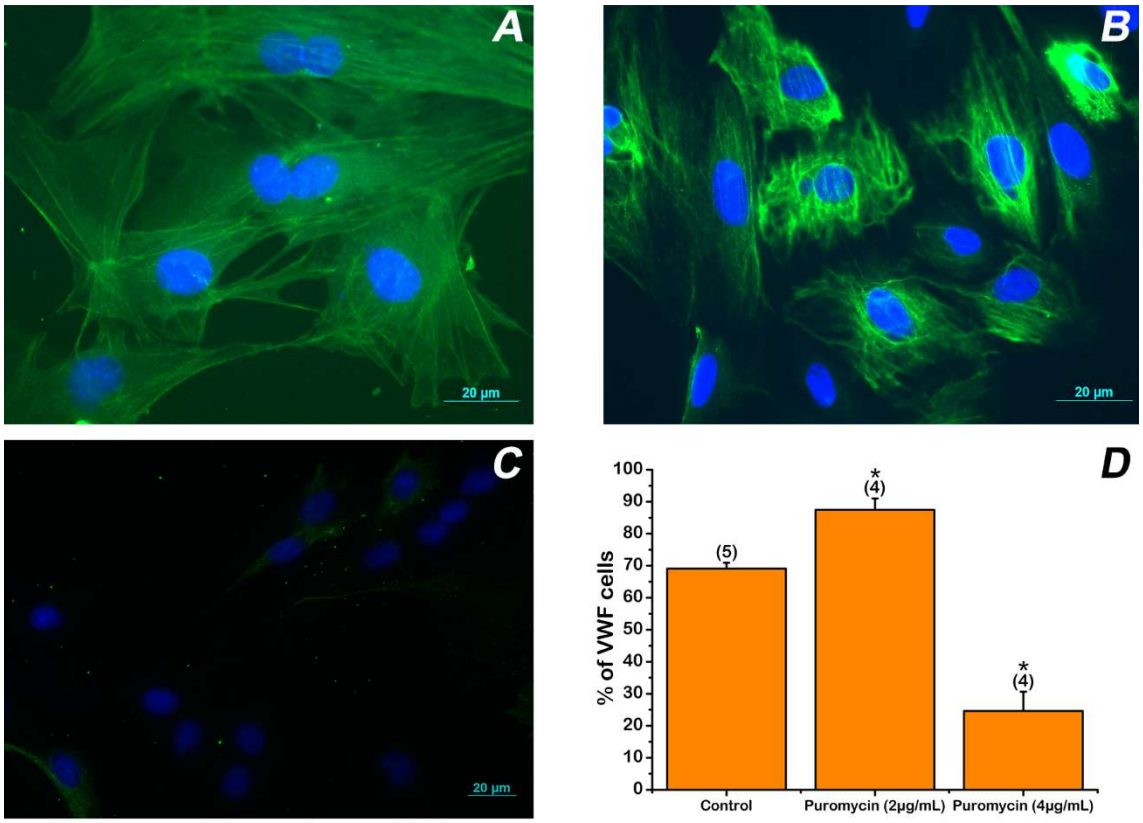

Figure 7: Characterization of the puromycin effect on cultures of Endothelial Cells marked with VWF. A- Representative image of VWF control. B- Representative image of the incubation with 2 µg/mL of puromycin during 24h. C- Representative image of the incubation with 4 µg/mL of puromycin during 24h. All the images were acquired from a fluorescence microscope (Axiobserver Z1, Zeiss) with an objective 40x. D- Quantification of the cell's percentage marked with VWF. Each bar in the graphic represents the media ± SEM of three different experiences, realized in triplicate. * $P<0.05$ versus control and the statistic method used was One-Way ANOVA.

Moreover,was tested the effect of the puromycin in the SMCs, and in this immunocytochemical assay was used the antibody anti α-actin, a marker of the SMCs. On the figure 8A, 8B and 8C we have marked on green, α-actin and on blue the nuclei of the cells. Moreover, on the SMCs also there is a significant difference between the concentrations of puromycin tested, having the culture incubated with 2 µg/mL of puromycin approximately 15% of SMCs and the incubated with 4 µg/mL have about 65% during 24h (Figure 8D).

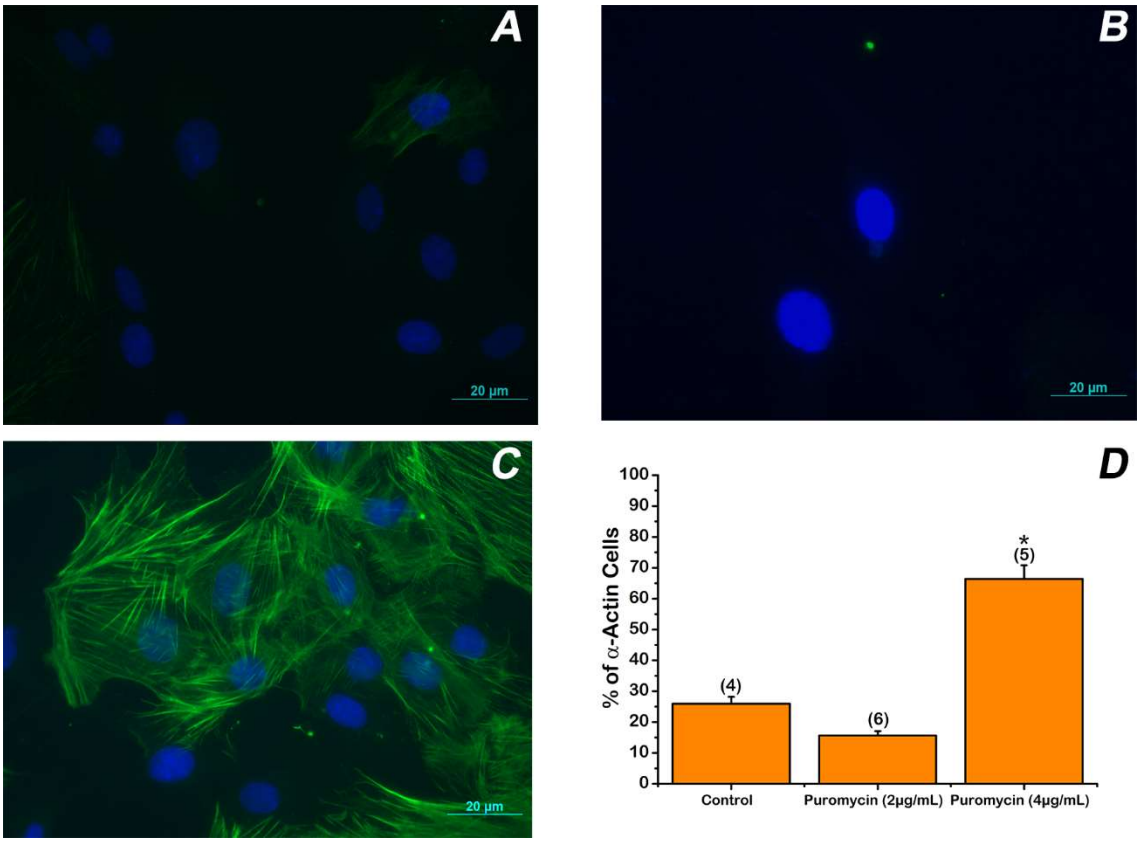

Figure 8: Characterization of the puromycin effect on cultures of Smooth Muscle Cells marked with α-actin. All the images were acquired from a fluorescence microscope (Axiobserver Z1, Zeiss) with an objective 40x. A- Representative image of α-actin control. B- Representative image of the incubation with 2 µg/mL of puromycin during 24h. C- Representative image of the incubation with 4 µg/mL of puromycin during 24h. D- Quantification of SMCs presented in the culture. Each bar in the graphic represents the media ± SEM of three different experiences, realized in triplicate. * $P<0.05$ versus control and the statistic method used was One-Way ANOVA with Dunnett test.

4.2.Characterization of primary Smooth Muscle cells culture

SMCs culture was obtained from the extraction of the MCA from the Circle of Willis, in which, was used a protocol established by Quelhas and his associates [98]. The culture acquired the confluence in 20 days and the cells used was in P4.

4.2.1. Temporal profile of the RA effect on cellular contraction and relaxation

To analyse the temporal profile of the RA effect on the cellular area, was used the PCSA technique. On this technique, the protocol used was pre-established by Mariana and Co-workers [101]. The cells were incubated 48h with serum-free medium to express the contractile phenotype and in this 48h, 24h were incubated with RA in different concentrations. Moreover, was used the NA as contractile agent and the SNP as relaxation agent. The figure 9 shows the temporal profile of the SMCs in different concentrations of RA, and his effect when are added the contractile and relaxation agents. First, on the figure 9A, we have the normal stage of the cells, that is when there is an addition of the NA, on the 5 min pass that addiction, the cell contract, being that contraction more expressive pass 16 min after de addiction. Moreover, after 20 min SNP was add and the cells dilatate.

When added the NA and the SNP, on concentrations of 0.1 μmol/L and 1 μmol/L, 5 μmol/L and 10 μmol/L of RA, in the most of the time the cells only present contraction as shown on the figure 9B, 9D, 9E and 9F. Different is the effect when the cells are incubated with 0.5 μmol/L of RA, and in this concentration, the cells when is added the NA have a slight contraction of 8% and when the SNP is added, pass 18 min, them present a relaxation of approximately 5% (Figure 9C).

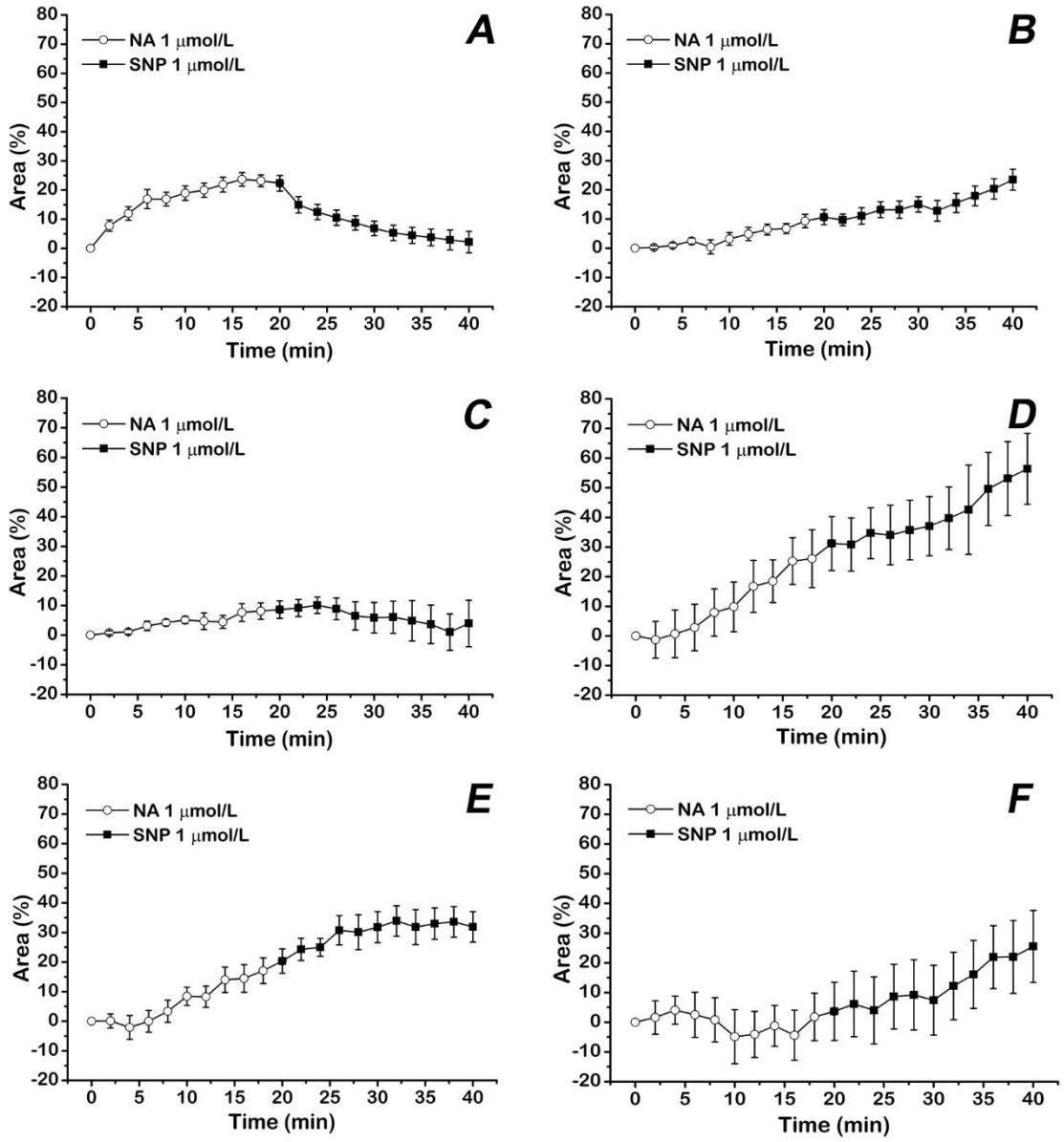

Figure 9: Characterization of the RA effects on SMCs behaviour. A- Representation of the normal SMCs without incubation (Control); B- SMCs incubated with 0.1 μmol/L of RA C- SMCs incubate with 0.5 μmol/L; D- SMCs incubate with 1 μmol/L of RA; E- SMCs incubate with 5 μmol/L of RA; F- SMCs incubate with 10μmol/L of RA.

4.2.2. SNP effect on SMCs with different concentrations of RA

The SNP effect was analysed to see if the RA maintain the SMCs properties of relaxation, after the addiction of the contractile agent. In this case, the figure 10 shows that the RA on concentration of 0.1 µmol/L, 1 µmol/L, 5 µmol/L, 10 µmol/L induce the contraction of the SMCs when is added SNP. Only in one of the concentrations tested, there is relaxation, the 0.5 µmol/L, having approximately 5% of relaxation. (Figure 10) Furthermore, concentrations of 1 µmol/L and 10 µmol/L of RA, have the highest concentration with contraction of 20% and 25%(Figure 10).

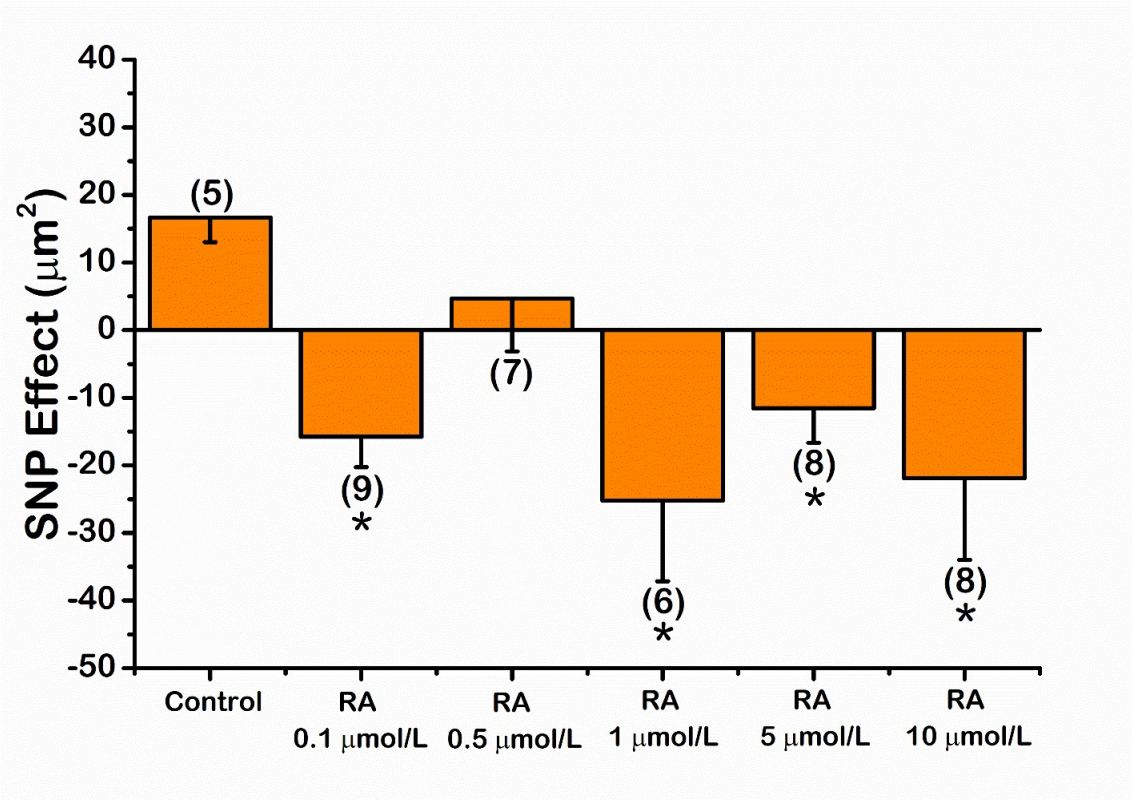

Figure 10: SNP effect at different concentration of RA in SMCs. This figure shows that only one of the concentrations tested, the 0.5 µmol/L maintain the relaxation effects when the addiction of SNP. Moreover, between the other concentrations tested, the 1 µmol/L and 10 µmol/L are the concentrations that have the higher contraction when the addiction of SNP. Each bar represents the media ± SEM of three different experiences, make in triplicate. * $P<0.05$ vs relaxation after a pre-contraction with NA. The SNP effect is expressed from the difference between the areas (Δ Area). The annalistic method was the One-way Nova.

4.2.3. Evaluation of rapid effect of the RA on SMCs

In order to evaluate the rapid effect of the RA on SMCs, the cells were incubated primary with NA. After contracted with NA during 20 min, were administrated 10 μmol/L of RA during 20 min, presenting approximately 10% of relaxation. Moreover, in the same figure is represented the % of relaxation in the SNP which is about 17%, that in comparison with the RA is slightly higher (Figure 11).

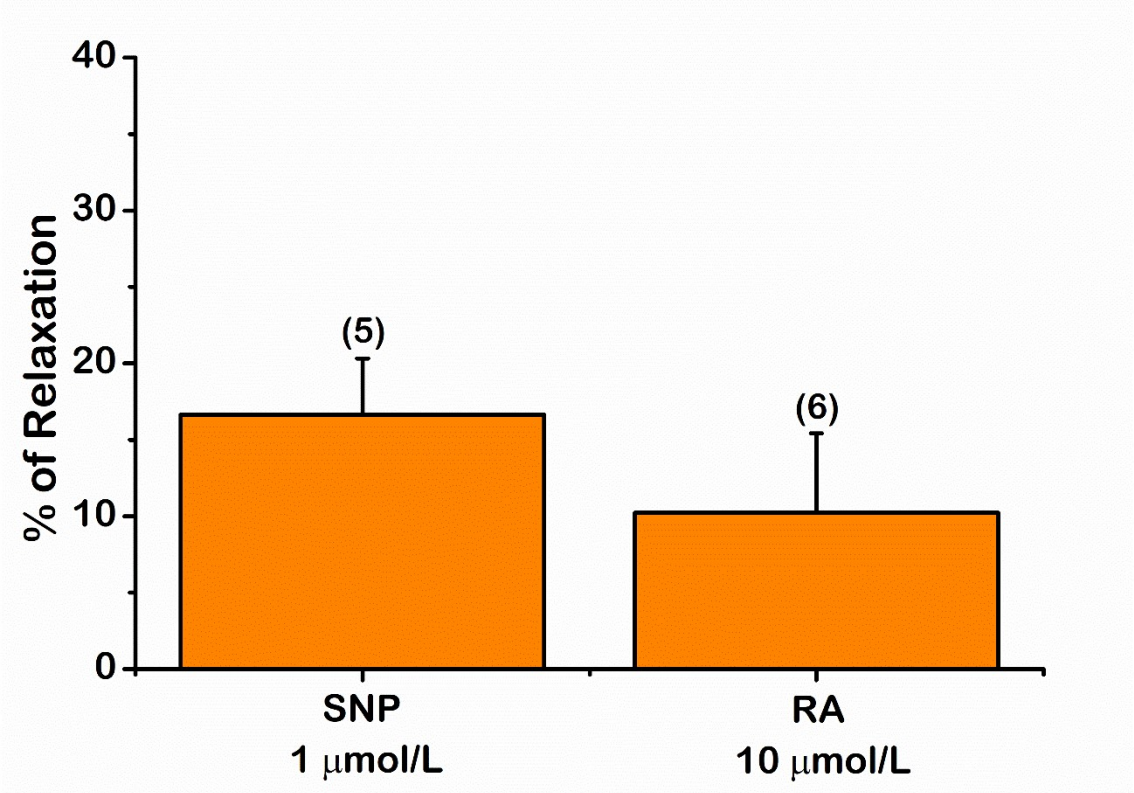

Figure 11: % of Relaxation after a pre-incubation of NAin SMCs. This figure compares the % of relaxation between the SNP and rapid effect of RA, after a pre-incubation of NA during 20 min. Each bar represents the media ± SEM of three different experiences, make in triplicate. The annalistic test used was the T-Student.

Chapter 5

Discussion

5. Discussion

In this work, SMCs culture were obtained from the extraction of the MCA of the circle Willis. The incubation with RA 0.5 μmol/L during 24 h induces a relaxation, having on the other concentrations tested of RA the reverse effect, a contraction when tested with SNP during 20 min. On the other hand, the direct effect of RA with a concentration of 10μmol/L induces a slight relaxation of approximately 10%, but this relaxation is lower than that observed with SNP. Furthermore, a protocol to obtain ECs culture from the basal artery and MCA has been established. The results obtained from the immunocytochemistry assay when incubated with puromycin at 2 μg/mL for 24 h, demonstrated an elevated percentage of cell staining with VWF, this is ECs. On the other hand, when these cells era incubated with a higher concentration of puromycin, such as 4 μg/mL the ECs present in the culture decay drastically comparing with the VWF control.

5.1.Characterization of the purity ECs culture

On Neurovascular Unit, ECs represents a crucial role forming the BBB and maintainthe homeostasis of the brain, with interactions with the other components that form the neurovascular unit, in this case SMCs, astrocytes and neurons [10]. Moreover, these cells present a large glycoprotein, called VWF responsible for mediating the platelet adhesion and being the major responsible for their role in the maintenance of the homeostasis on the brain [102].

On this work, was evaluated the purity of the ECs culture through the characterization of the ECs by immunocytochemistry.First was made a control for the VWF and α-actin, being the VWF described in the literature by Cairrao et.al, as a specific antibody for the ECs and the α-actin antibody for the SMCs [97]; [98] Second, was made an incubation with two different concentrations of puromycin, and the results obtained with the culture incubated during 24 h with 2 μg/mL of puromycin was a purity of 90% and with 4 μg/mL, the purity decay to 20% (Figure 7). Moreover, puromycin, a protein synthesis inhibitor was used due to the fact that induces SMCs apoptosis with the cleavage of the pro-caspase-3 and internucleosomal DNA fragmentation [103]. Comparing the results obtained with the literature, some researchers, such as Bernard-Patrzynski and his co-workers obtain a better purity on the culture incubated with 4 μg/mL, approximately 90%, despite the components used on his study be similarly, with exception of the digestion solution, that was used only collagenase and DNase, and on the fixation was used ethanol on 70% with ethylenediaminetetraacetic acid (EDTA). Also, in this study, the incubation with puromycin on the first day was 10 μg/mL and pass two days, was

switched for 4 μg/mL being maybe one of the reasons, why they obtain a good culture purity [104]. Other study, Zhu Xiao Xiao*et al.* with ECs of the rat aortic obtain without collagenase and puromycin and using the paraformaldehyde (PFA) for fixation has a purity of 94,65% [105]. Furthermore, an assay did by Welser-Alves and his associates, was able to obtain a 99% of purity on the ECs culture, using the papain dissociation method and on the fixation the Acetone/methanol method [106].

Moreover, a study of N.Perrière and is co-workers on the culture purity of rat brain capillary ECs, demonstrate that a culture incubated with a concentration of 3 μg/mL for 3 days and incubated with 4 μg/mL for 2 days, present a similar purity, that is 99%. Also, the compounds use on the isolation of these cells, was the same of used on this work. Furthermore, N.Perrière also test another p-glycoprotein substrates in the same concentration that test the puromycin, such as, the colchicine, resulting in a purity of 70.8% and the doxorubicin presenting purity of 93.1% [107].

According to our results, the cells incubated with 2 μg/mL present high levels of purity compared with the control of VWF and are proximal of the results obtain on other types of ECs for the authors described above and with other concentrations of puromycin (Figure 7). The cultures incubated with 4 μg/mL present results of purity below the VWF control and with the results above mentioned, suggest that perhaps the culture was contaminated with SMCs, due the fact on the cultures with this concentration there is a high percentage of cells mark with α-actin or the puromycin in this concentration during 24h are cytotoxic on ECs from MCA.

5.2. Characterization of SMCs isolated from the MCA of rat brain.

SMCs are present in the structure of arteries and blood vessels, being responsible for the regulation of the flow in several parts such as the brain. On the brain, these cells make part of the neurovascular unit responsible for the homeostasis of the brain [24]. The homeostasis maintenance is given for some unique characteristics adjacent to this cell, such as the mechanisms of contraction and relaxation responsive to some physiological and chemical events present in the brain. This type of mechanism is given by some genes express on these cells, such as α-actin, SM22α, and α-tropomyosin, giving this ability to contrate or dilate [108].

Furthermore, the contraction in most of the times is mediated by the levels of Ca^{2+}, that on the cytosol binds with the calmodulin and this complex activates the myosin light chain kinase with the presence of ATP, phosphorylating, forming bridges between the myosin and the actin filaments, resulting on cell contraction. On the other hand,

relaxation happens when the actin filaments coupled with the cell membrane through a viscoelastic system, in which variations on these filaments result in a relaxation of the vessels [24].

On this study in specifically was evaluate the response of the SMCs culture, previously characterized on the literature by Quelhas et.al to a retinol derivative originate, in this case, the RA [98]. On the brain, according to the literature, this retinol is described as a regulator of cell proliferation, differentiation, and apoptosis [109]. Moreover, was described by Yusheng Wand et.al that the RA present vasorelaxation effects on arteries. This effect is originated from the increase of the production of NO by the endothelium, in which, this increase will active the guanylyl cyclase that will increase the concentrations of cyclic guanosine monophosphate (cGMP) activating the cGMP-dependent protein kinases and provoking the opening of the BK_{ca}, resulting on a vasorelaxation [110].

Through the PCSA technique, the contraction and relaxation of the cells were analysed, with this technique allowing the evaluation of the changes on the cell surface area [101]. Also, in this technique, the cells previously are placed on FBS-free medium for 24h with the objective to change the phenotype from synthetic to contractile. Moreover, the cells as is described in the literature, the cells were incubated with a contractile agent, in this case, the noradrenalin and with a vasodilator agent, the SNP to ensure the relaxation of the cells. How is described, the noradrenaline will act on the adrenergic receptors of the SMCs, such as, the α-1A that is described as the most abundant in the brain. The contraction induced by NA is due the activation of α-1A, this leads to an increase of the PLC activity, that will cause the production of DAG and IP3, increasing the $[Ca^{2+}]$ from the intracellular reservoirs, resulting in contraction [98], [111]. On the other hand, the SNP is responsible for the release of NO, this will activate the cGC, increasing the cGMP and provoking the relaxation of the cells [112].

The RA concentrations used were in concordance with the concentrations described by Tiago Santo et. al on the literature. Moreover, on this work, was amplified the spectrum of RA concentrations and the maximum concentration used was 10 μg/mL since in the article above mentioned, the RA distribution was made with nanoparticles, so the distribution was more local, being 10 μg/mL the limit concentration without cytotoxic effects for the cells [74].

Moreover, some studies, such as the described by Yusheng Wang on the incubation of the mesenteric artery with RA, were obtained with a concentration of 10^{-8} M a percentage of relaxation in order of the 65,5%. Also, in this study was tested the RA

with a concentration of 10^{-8} M with a concentration of 10^{-4} M of L-NG-monomethyl arginine acetate, an inhibitor of the NO synthase, provoking a decay to 20% of relaxation [110].

Comparing the result above mentioned with the obtained in this study, the only concentration that has some similarities with the control, this means 0.5 µmol/L where the RA induces relaxation, the percentage is about 5% (Figure 10). On the other hand, the RA tested on non-genomic comparing with the genomic test the percentage of relaxation is 10% (Figure 11). These results suggest that perhaps the RA has two effects, the rapid effect that is non-genomic and the long-term effect, which is genomic. To our knowledge, this effect non-genomic effect has never been demonstrated.

Additionally, these results show that due the RA effects, such as the increase of the NO release by the endothelium causing SMCs relaxation, on diseases such as the ischemia, can bring value on the treatment or on the prevention. In ischemia, where exist the block of the vessels, interrupting the blood flow and causing cell death, the RA with the capacity to increase the vasorelaxation, can minimize the damage caused by the flow interruption or prevent this interruption.

Chapter 6

Conclusion and Future Perspectives

6. Conclusion and Future Perspectives

Brain is a complex organ, and in this organ, there is a network responsible for maintaining the homeostasis of this, called the neurovascular unit. Among the years, the cells present on this network, have despite the interests of several researchers for this area. On this work, there is a study of two types of cells, that are present in this network, which is the ECs and SMCs. A pure ECs culture have been established. In order to remove the SMCs from the culture 2 μg/mL of puromycin have been add to the culture, and after the incubation with puromycin the ECs culture do not present hight contaminations with SMCs. On the other hand, the ECs cultures incubated with 4μg/mL present a high number of SMCs and a low number of ECs, showing that perhaps the puromycin on this concentration present cytotoxic effect to cells isolated from the MCA.

The results about the effect of the RA on the SMCs, shows the incubation with RA in a certain concentration during 24h have induce a relaxation effects on SMCs. On the other hand, seem that this compound also presents a rapid effect. Moreover, comparing the results of the genomic effect with the non-genomic effect, the RA in the maximum concentration, have contrarily effect, inducing in the first contraction and in the last relaxation.

On the future, to complete this work, these concentrations of RA can be tested on SMCs with Oxygen-Glucose deprivation with the finality to see if the RA can bring some benefit effects from some diseases, like the stroke. For further studies, RA can be tested in pure ECs cultures, using genetic analysis and analysing the effect of secretome on SMCs. The next step is toanalyse this effect in pre and post stroke*in vitro* and posteriorly *in vivo*.

Chapter 7

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7. References

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