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Multifunctional Liposomes modulate Purinergic Receptor-induced Calcium Wave in Cerebral Microvascular Endothelial Cells and Astrocytes

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ABSTRACT

Our previous results show that multifunctional liposomes (mApoE-PA-LIP) reduce brain Aβ burden and ameliorate memory impairment in Alzheimer's disease (AD) mouse models (Balducci et al., 2014). In light of these results, we assessed liposomes functionalized with ApoE-derived peptide (mApoE) and phosphatidic acid (PA) at neurovascular unit. In particular, we evaluated their activities on cultured human cerebral microvascular cells (hCMEC/D3), as an in vitro human blood brain barrier model, and on cultured astrocytes (iAstro-WT).

By means of calcium imaging measurements, we aimed to study the intracellular calcium dynamics triggered by purinergic receptors activation. Our result show that the interaction of mApoE-PA-LIP with the hCMEC/D3 and astrocytes actively induced a modulation in the calcium waves duration of ATP evoked response. In particular, we find an increase of the duration of the ATP evoked calcium waves in presence of mApoE-PA-LIP in comparison to untreated cells. After the mApoE-PA-LIP pre-treatment also the area under the curve (AUC) is increased in comparison to controls both in hCMEC and iAstro-WT.

Furthermore, we found that the pre-treatment with mApoE-PA-LIP in absence of extracellular calcium significantly increased ATP evoked calcium waves in comparison to controls. Also under this condition, the AUC increased in comparison to control. We also found that when the Sarco-Endoplasmic Reticulum Calcium ATPase (SERCA) was inactive, due to its specific blockage with cyclopiazonic acid, both in presence or in absence of extracellular calcium, ATP failed to activate calcium wave also after a pre-treatment with mApoE-PA-LIP both in hCMEC and iAstro-WT.

In conclusion, mApoE-PA-LIP modulate calcium dynamics evoked by ATP when SERCA is active. In light of the protective role of the purinergic receptor activation (Weisman et al., 2012), our obtained results would provide an additional support to promote mApoE-PA-LIP as putative therapeutic tool for AD treatment.

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1. INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder, characterized by alterations in memory formation and storage (Popugaeva et al. 2017). It is a progressive and incurable neurodegenerative disease with not fully understood aetiology. AD is the most frequent type of dementia in humans older than 65 years of age. As a matter of fact, age is the greatest risk factor, even if a combination of genetic, lifestyle and environmental factors also contribute to disease development. AD is associated with neurovascular dysfunction (Sagare et al., 2012), cognitive decline (Cummings 2004), and a characteristic pathomorphological picture. The leading candidate for the trigger of Alzheimer's disease is the accumulation of amyloid b peptide (Aβ) in the brain (that contains 39 to 42 amino acids) (Querfurth et al., 2010), tau-related lesions in neurons termed neurofibrillary tangles (Ballatore et al., 2007; Ittner et al., 2011), microgliosis, neurite dystrophy, loss of neurons and synapses, reactive processes including activation of astrocytes and microglia, oxidative stress, metal ion dysregulation, and chronic neuroinflammation (Cieślak et al., 2018).

Aβ peptide is produced by the proteolytic processing of the amyloid precursor protein. Its primacy has been manifested in the 'amyloid-cascade hypothesis', which posits that the accumulation of Aβ (resulting from overproduction, altered processing or a failure of clearance mechanisms) is the initiating molecular event that triggers neurodegeneration in sporadic and familial AD. A parallel concept that has been developed in the last decades (Shi et al. 2000; Iadecola 2004; Zlokovic 2011) is that the reduction of cerebral blood flow in patients with AD may thus cause ischaemic damage to neurons, suggesting that neurovascular dysfunction may lead to AD initiation and progression (Iadecola, 2004; Takano et al. 2007; Zlokovic, 2011).

NEUROVASCULAR UNIT

Brain disorders such as AD may have a vascular origin according to Zlokovic and colleagues which provided evidences that the aged brain develops a functional uncoupling at the neurovascular unit.

The neurovascular unit (NVU) (Figure 1) was firstly described by Harder (Harder et al., 2002) as a functional unit constituted by neurons, interneurones and astrocytes. The close proximity of different non-neuronal cell types with each other and with neurons, allows paracrine regulations that are critical for normal central nervous system (CNS) functioning and disease processes (Zlokovic 2008). These include regulation of hemodynamic neurovascular coupling, microvascular permeability, matrix

interactions, neurotransmitter inactivation, neurotrophic coupling. Angiogenic and neurogenic coupling are in close proximity and are functionally coupled to smooth muscle cells, pericytes, endothelial cells and extracellular matrix. Each component is intimately and reciprocally linked to each other, establishing an anatomical and functional whole, which results in a highly efficient system of cerebral blood flow (CBF) regulation (Armstead et al, 2011, Abbott et al., 2012). Such system is also involved in the transport of oxygen and metabolites into the brain, blood brain barrier (BBB) permeability and regulation of the inflammatory response.

NVU controls BBB permeability and CBF, maintaining constant neuronal environment ('milieu') and chemical composition which is essential for the function of neuronal circuits. CBF is regulated by local neuronal activity and metabolism, known as neurovascular coupling (Iadecola et al., 2004); progressive CBF reductions have increasingly serious consequences for neurons. Moderate to severe CBF reductions and hypoxia affect ATP synthesis, acting on Na^+/K^+ ATPase activity, with neurons gradually failing to generate action potentials (Kalaria, 2010). In addition, these reductions can modify pH with alterations in electrolytes content and water gradient, facilitating the development of oedema and white matter lesions, glutamate and toxic proteins accumulation (for example, amyloid-β and hyperphopshorylated tau) in specific brain regions. A reduction of greater than 80% in CBF results in neuronal death (Zlokovic et al., 2011).

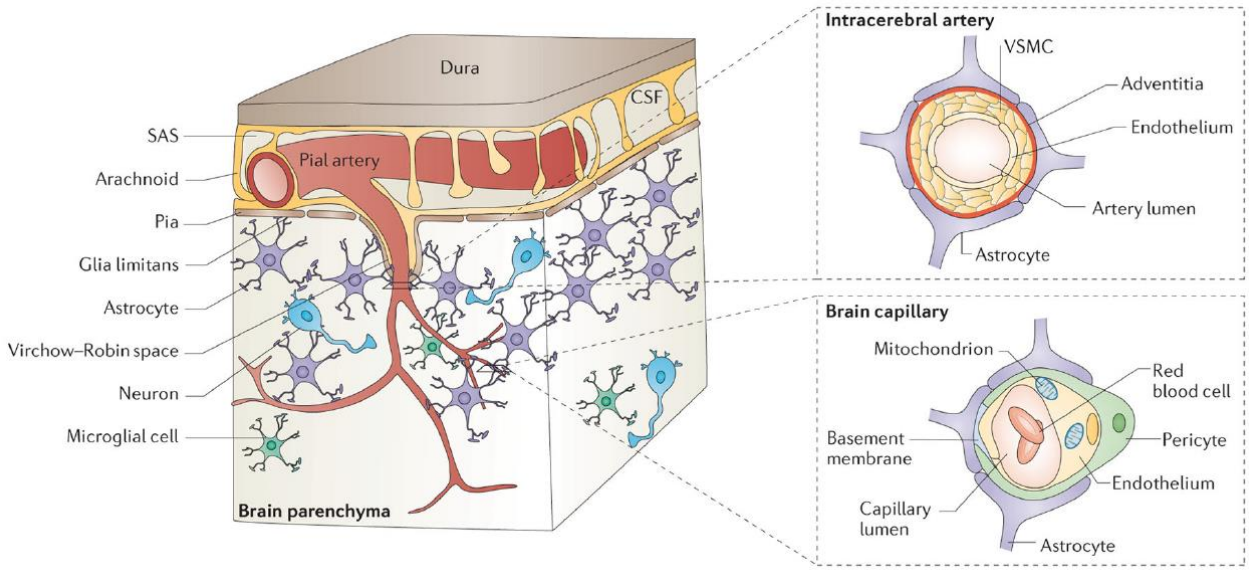


Figure 1. Cerebral microcirculation and neurovascular unit

NEUROVASCULAR UNIT DYSFUNCTION IN AD

Recent studies have elucidated specific cellular and molecular mechanisms within the NVU and at the blood-brain barrier (BBB) mediating neurovascular defects in AD. In particular, NVU dysfunction could be an early event in AD and could provide a potential link between this disorder and cerebral ischemia (Benarroch 2007). AD is associated with profound changes in cerebrovascular structure and function, and functional MRI studies suggest that alterations in CBF regulation in response to cognitive tasks may be a predictor of risk for developing AD (Lee et al. 2003).

AD patients or other dementia diseases frequently show focal changes in brain microcirculation. These changes include the appearance of string vessels, a reduction in capillary density, a rise in endothelial pinocytosis, a decrease in mitochondrial content, loss of tight junctions and/or adherens junctions (Zlokovic 2005), and BBB breakdown with leakage of blood-borne molecules (Brown et al., 2011). It remains ununderstood the time course of these vascular alterations and how they relate to AD pathology.

ASTROCYTES IN AD

Different studies demonstrate that patients with AD show hypertrophic reactive astrocytes. (Medeiros et al., 2013). Numerous gliotransmitters released from astrocytes control synaptic plasticity in different brain structures (Yang et al. 2003; Pascual 2005; Panatier et al. 2006) such as cortex (Ding et al. 2007) and hippocampus (Araque et al. 1998; Jourdain et al. 2007), and are involved in the modulation of memory and learning processes. In AD astrocytes there is a major expression of glial fibrillary acidic protein (GFAP) and protein fibrillary and functional impairment (Olabarria et al. 2011); however, they maintain their morphology without any scarring formation.

Astrocytes due to their specific transporters on processes are able to take up efficiently neurotransmitters, particularly glutamate released by pre-synaptic elements (Petit et al., 2016). One of the main functions of astrocytes is to decrease rapidly glutamate concentration in the synaptic cleft to prevent its excitotoxicity through activation of N-methyl-d-aspartate (NMDA) receptors and to reduce its “spill-over”. Two subtypes of glutamate transporters, namely the glutamate–aspartate transporter (GLAST or EAAT1) and the glutamate transporter type 1 (GLT1 or EAAT2), are expressed by astrocytes where they are responsible for glutamate uptake (Danbolt 2001, Zhou et al., 2013). In astrocytes, glutamate uptake activates the Na^+, K^+-ATPase to maintain the Na^+ homeostasis (Pellerin et al., 2002). Moreover, the glutamate taken up by astrocytes is converted to glutamine (Gln) through the astrocytic enzyme glutamine synthase (GS) and transferred back to the neurons to be

converted to glutamate. This last mechanism known as the “glutamate–glutamine cycle” (Glu–Gln cycle) is fundamental to maintain an efficiently synaptic activity. In AD animal models, early response is marked with astroglial atrophy that could have different effects on synaptic connections due to their role in maintaining synaptic transmission, hence they could be linked with cognitive impairment. Together with endothelial cells and reactive microglia, there is astrocytes accumulation around senile plaques, but their activation could be a first step before Aβ accumulation in insoluble aggregates. Astrocytes also participate in the degradation and removal of Aβ as they express different types of proteases involved in its enzymatic cleaving.

As in the case of microglia, also astrocytes, after Aβ exposition, start releasing cytokines, interleukins and cytotoxic molecules, exacerbating the neuroinflammatory response.

THE BLOOD BRAIN BARRIER

Astrocytes contribute to generate and maintain the blood–brain barrier (BBB) through interactions with the pericytes and endothelial cells.

The BBB is a highly selective lipophilic barrier that separates the systemic blood circulation from the CNS (Figure 2). The ECs which line the microvasculature of the CNS form a dynamic interface between the blood tissue and the brain parenchyma. ECs are responsible for the maintenance of ionic and metabolic homeostasis in the brain (Drewes, 2001). They differ fundamentally from other endothelia by the presence of tight junctions which allow them to constitute the BBB. To supply the brain with nutrients while maintaining the selective barrier function, the ECs express active transport systems for ions and nutrients, such as glucose and amino acids, and transporters which are responsible for the removal of metabolic waste from the brain parenchyma into the blood circulation. The transport across the BBB ECs is controlled by inputs from the nervous tissue as well as the blood (Iadecola et al.,2007).

Many diseases, such as AD, and acute conditions, such as ischemic stroke and hypertension, are able to modify the BBB permeability and dysregulate the proteins contents of the tight junctions (Girouard et al., 2006, Leffler et al.,1989, del Zoppo et al.,2003). Non-neuronal cells and neurons together regulate BBB permeability and CBF. CSF homeostasis and its chemical composition is guaranteed by vascular cells and glia, while the BBB and the blood-spinal cord barrier (BSCB) work together with pericytes to prevent the entry in the CNS of various potentially neurotoxic and vasculotoxic macromolecules, and to promote clearance of these substances from the CNS (Zlokovic et al., 2008). The low paracellular permeability of the BBB is conferred by the presence of tight junctions and adherens junctions that together permit ECs to form the BBB. Normal brain endothelium lacks

fenestrae and has limited vesicular transport; only oxygen, carbon dioxide and small lipophilic molecules are able to diffuse freely across the ECs.

A high energy demand is required to activate ATP-dependent transport, such as the sodium pump (Na^+/K^+ ATPase) and the ATP-binding cassette (ABC) efflux transporters; for this reason, ECs are enriched with a high number of mitochondria. Na^+/K^+ ATPase pumps control the sodium influx and potassium efflux across the abluminal side of the BBB. Changes in sodium and potassium levels in the CSF influence the generation of action potentials in neurons and thus directly affect neuronal and synaptic functions.

Disruption of tight and adherens junctions, an increase in paracellular fluid permeability, and/or enzymatic degradation of the capillary basement membrane cause physical breakdown of the BBB.

Another component of the BBB is represented by Apolipoprotein E (ApoE) receptors, which also have fundamental functions and play a role in the pathophysiology of AD.

Multiple studies have linked the ApoE genotype with BBB function, with E4 in particular leading to higher BBB permeability, decreased cerebral vascularization, and thinner vessel walls and reduced CBF (Bell et al. 2012; Alata et al. 2015). Importantly, these E4-associated vascular defects were observed as early as 2 weeks of age in the mouse model (Bell et al., 2012) largely preceding the neuronal and synaptic dysfunction that is observed in these mice in late age. Apolipoprotein E (ApoE) plays a critical role in the metabolism of lipoproteins and redistribution of cholesterol and has long been studied in relation to atherosclerosis and cardiovascular disease (Mahley et al., 2000; Eichner et al. 2002; Pendse et al. 2009). In the periphery, ApoE is primarily produced by the liver, but it is also expressed by a number of other tissues (Driscoll and Getz 1984; Zechner et al. 1991). In the CNS, apoE is primarily produced by astrocytes and it plays a critical role in neuronal maintenance and repair (Xu et al. 2006; Xu et al. 1996; Mahley et al., 2000). In humans, there are three major isoforms of apoE: E2, E3 and E4 (Mahley et al., 2000). E3 is the major isoform expressed in humans, and the effects of E2 and E4 are typically compared to those of E3 to determine relative risk (Phillips 2014). Importantly, ApoE is the strongest genetic risk factor for late AD onset, with E4 conferring between a 3- (heterozygous) to 15-fold (homozygous) increase in risk of AD (Farrer et al. 1997; (Raber et al., 2004). Conversely, E2 is associated with increased longevity and a decreased risk of AD (Farrer et al., 1997; (Garatachea et al. 2014).

Several clinical trials demonstrate that ApoE could be an interesting therapeutic target. For example, recent trials revealed that the beneficial effects of intranasal insulin treatment on cognition are modulated by ApoE genotype status in AD patients (Zhao et al. 2017). Short-acting intranasal insulin acute treatment leads to cognitive improvement in AD patients who are APOE4 non-carriers, but not in APOE4 carriers (Reger et al. 2006). Long-acting intranasal insulin improves cognition in APOE4

AD patients but worsens the outcome for APOE4 non-carriers (Claxton et al. 2015). In addition, a Phase 3 trial using passive immunization with a humanized anti-Aβ antibody, bapineuzumab, prevents the increase of Aβ deposition and reduces CSF phosphorylated tau in AD patients who are APOE4 carriers, but not in APOE4 non-carriers, although this antibody did not benefit the clinical outcomes (Salloway et al. 2014).

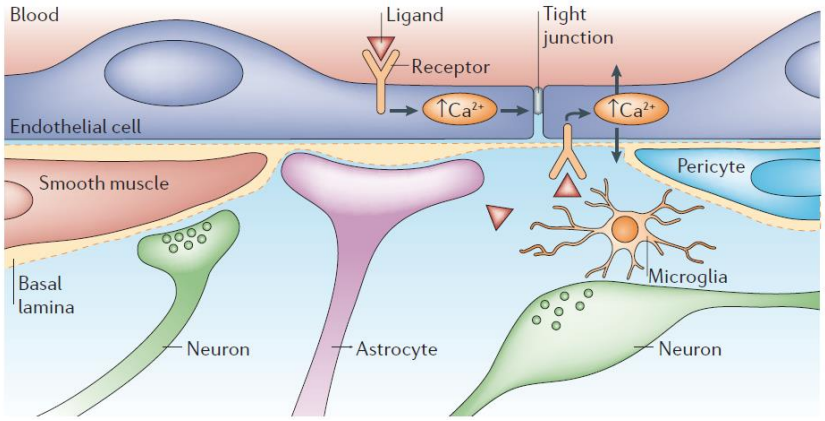


Figure 2. Blood Brain Barrier

BBB DYSFUNCTION IN AD

Changes in the expression of several BBB transporters mediating nutrient transport, ion pumps, ABC transporters, and/or receptors mediating transport of peptides and proteins, including blood-to-brain and brain-to-blood exchanges of Aβ, have been described in AD and AD models (Sagare et al., 2012). In particular, impairment in glucose transporter 1 (GLUT1), in the receptor for advanced glycation products (RAGE) and lipoprotein receptor-related protein 1 (LRP) may cause functional dysfunction linked with AD.

- **GLUT1**

GLUT1 is a BBB specific required glucose transporter with a diminished expression in AD individuals (Mooradian et al., 1997), suggesting deprivation in glucose supply to the brain. Positron emission tomography (PET) studies with 18F-2-fluoro-2-deoxy-D-glucose (FDG), have demonstrated diminished glucose uptake by the brain in individuals at increased risk for dementia (Hunt et al. 2007; Herholz 2010). Several studies have suggested that reduced glucose uptake across

the BBB as seen by FDG-PET can precede brain atrophy (Mosconi et al. 2006; Hunt et al. 2007; (Samuraki et al. 2007; (Mosconi et al. 2008; Herholz 2010) and may be used as a potential biomarker for AD (Miller 2009; Perrin et al.,2009).

- **RAGE**

RAGE is a multiligand receptor of the immunoglobulin superfamily (Neeper et al. 1992). It mediates Aβ re-entry into the brain from circulation and the neurovascular inflammatory response.

RAGE binds distinct classes of ligands including AGE proteins, S100/calgranulins, Aβ, amphoterin, and the family of crossed b-sheet macromolecules (Yan et al., 2010). RAGE interaction with ligands activates signal transduction pathways, leading to sustained cellular stress as shown in chronic diseases such as diabetes, inflammation, and AD (Bucciarelli et al. 2002; Bierhaus et al. 2005; Schmidt et al. 2009). The extracellular domain of RAGE contains one V-type and two C-type immunoglobulin domains (Yan et al. 2010). Most ligands bind to RAGE's V-domain. A single, transmembrane spanning domain is followed by a short, charged cytoplasmic domain-mediating signal transduction after ligand binding to RAGE (Yan et al. 2010).

As a cell surface receptor for Aβ (Yan et al. 1996), RAGE binds monomeric and oligomeric Aβ via its V-domain and aggregated Aβ via its C1 domain (Sturchler et al. 2008; Yan et al. 2010). RAGE directly mediates Aβ-induced neurotoxicity by causing oxidant stress and indirectly by activating microglia (Yan et al. 1996). In addition, intraneuronal Aβ transport via RAGE leads to mitochondrial dysfunction (Takuma et al. 2009). RAGE over-expression is found in cerebrovascular ECs under pathological conditions, including those seen in AD models and AD (Yan et al. 1995, 1996; Deane et al. 2003). RAGE, at the BBB, mediates transport of circulating Aβ into the brain (Mackic et al. 1998; Deane et al. 2003), EC activation resulting in neuroinflammatory response and generation of endothelin-1 suppressing the CBF (Deane et al. 2003). In addition, expression of RAGE in brain endothelium initiates cellular signalling, leading to monocyte trafficking across the BBB (Giri et al. 2000). It is of note that RAGE expression is increased in both neurons and endothelium in an Aβ-rich or AGE-rich environment as in AD (Yan et al. 1995), which amplifies Aβ-mediated pathogenic responses. The cellular events triggered by RAGE at the BBB, neurons and microglia may be implicated in the onset and progression of AD. In the human hippocampus, an increased RAGE expression in brain endothelium of the BBB has been shown in advanced AD compared with early stage AD and/or individuals with mild cognitive impairment (MCI; Miller et al. 2008).

Therefore, RAGE is a potential therapeutic target in AD and blocking RAGE might contribute to the control of Aβ-mediated brain disorder.

- **LRP**

LRP is a major Aβ clearance receptor at the BBB mediating Aβ efflux from the brain and its systemic clearance. It is a LDL receptor with a dual role as a rapid cargo endocytotic cellular transporter and a transmembrane cell signalling receptor (Zlokovic 2010). LRP regulates transport and metabolism of apoE-associated cholesterol (Herz 2001; Herz et al., 2001; Herz et al. 2009).

At the NVU side, LRP is expressed in brain endothelium, pericytes, astrocytes, and neurons (Herz et al., 2002; Polavarapu et al. 2007). LRP internalizes its ligands and directs them to lysosomes for proteolytic degradation. Recent studies have demonstrated that LRP also transports its ligands transcellularly across the BBB, including Aβ (Shibata et al. 2000; Deane et al. 2004).

LRP and many of its ligands are deposited in senile plaques (Rebeck et al. 1995; Arélin et al. 2002)); besides, it interacts with APP, which influences Aβ formation (Pietrzik 2004; Waldron et al. 2008). LRP interacts with g-secretase, an APP processing enzyme, which results in inhibition of the inflammatory response, suggesting a potential for modulating inflammation (Zurhove et al. 2008).

Different studies have demonstrated that LRP has a key role in a clearance mechanism mediating Aβ elimination from brain and peripheral side (Zlokovic et al. 2010). Studies using in vitro BBB models (Nazer et al., 2008; Yamada et al. 2008)have confirmed the role of LRP in Aβ endothelial cellular uptake and endocytosis, respectively, resulting in clearance of Aβ.

Reduced LRP levels in brain microvessels correlate with endogenous Aβ deposition in a chronic hydrocephalus model in rats (Klinge et al. 2006) and Aβ cerebrovascular and brain accumulation in AD patients (Shibata et al. 2000; (Donahue et al. 2006). Several studies have indicated that LRP expression in brain endothelium decreases with normal aging in rodents, non-human primates and humans, as well as in AD models and AD patients (Kang et al. 2000; Shibata et al. 2000; Bading et al. 2002; Deane et al. 2004; Donahue et al. 2006; Bell et al. 2009). LRP reductions have been reported in cerebral vascular cells associated with Aβ accumulation in the wall of small pial and intracerebral arteries (Bell et al. 2009). Therefore, LRP down-regulation at the BBB and in vascular cells may contribute to cerebrovascular and focal parenchymal Aβ accumulations.

NEUROVASCULAR SYSTEM

The brain receives up to 20% of cardiac output in humans and its functions stop in seconds when it is not perfused by CBF, with neurons damage that may occur in minutes (Girouard et al., 2006).

The normal neuronal-vascular relationship is critical for appropriate brain functioning. It has been estimated that nearly every neuron in the human brain has its own capillary (Zlokovic, 2005). The

total length of capillaries in the human brain is about 400 miles and the capillary surface area available for molecular transport is about 20 m^2 (Begley et al., 2003). The thickness of the cerebral endothelial membrane is 0.2 to 0.3 mm. On the other hand, it has been estimated that the length of brain capillaries is reduced in neurodegenerative disorders, as for example in AD (Bailey et al. 2004; Wu et al. 2005). Due to this neurodegeneration there is a decrease in energy substrates and nutrients across the BBB. The former "capillary recruitment" hypothesis, proposing opening of new capillaries from an increase in the CBF and closing of brain capillaries from a decrease in the CBF (Weiss 1988), has thus been modified to a "functional recruitment" hypothesis: brain capillaries are perfused all the time, but they transition from low to high blood flow with an increase in the CBF, or from high to low blood flow with a decrease in the CBF (Kuschinsky et al.,1992).

TWO-HIT VASCULAR HYPHOTHESIS

There are different risk factors that correlate with cerebrovascular disorders and AD (de la Torre 2010). Midlife diabetes (Luchsinger et al. 2007), hypertension and obesity (Whitmer et al. 2008) are vascular risk factors that predispose individuals to AD and vascular dementia. It is now widely recognized that most cases of this neurodegeneration have mixed vascular pathology and small-vessel disease (Marchesi 2011). In addition, brain hypoperfusion–hypoxia (Ruitenberg et al. 2005), silent infarcts (Vermeer et al., 2003), the presence of one or more infarctions, stroke episodes and transient ischaemic or hypoxic attacks all increase the risk of AD (Zlokovic, 2011). In this disorder, although the molecular and cellular events for each step in the disease process and for each risk factor are not clear, there is a common final disease pathway that involves brain microvascular dysfunction and/or degeneration, as well as Aβ and tau accumulation.

According to the two-hit vascular hypothesis of AD, vascular risk factors (hit one) lead to blood–brain barrier (BBB) dysfunction and to a reduction in cerebral blood flow (oligaemia), initiating a cascade of events that precedes dementia. In the non-Aβ pathway (Figure 3), toxic accumulation and capillary hypoperfusion induce early neuronal dysfunction. Vascular injury also reduces amyloid-β clearance at the BBB and increases production of this peptide from the amyloid-β precursor protein (APP), leading to Aβ accumulation (the amyloid-β pathway; see the figure, shown in red boxes) (Zlokovic, 2011). The increase in amyloid-β (hit two) amplifies neuronal dysfunction, accelerates neurodegeneration and dementia. Aβ and/or hypoperfusion can induce hyperphosphorylation of tau (p-tau), leading to neurofibrillary tangle formation.

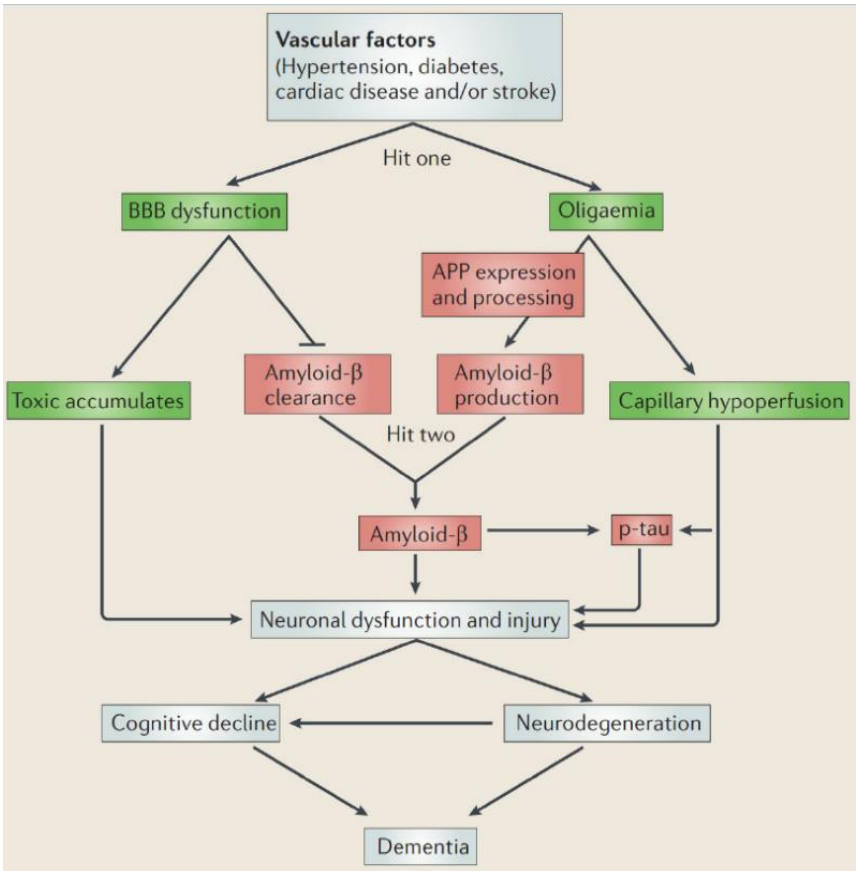


Figure 3. Two-hit vascular hypothesis for Alzheimer's Disease

PHOSPHATIDIC ACID AND CALCIUM: vesicular trafficking and signal transduction

Phosphatidic acid (PA) is a minor component of biological membranes (about 1% of phospholipids) (Buckland et al., 2000). Nevertheless, its presence is critical due to the multiple roles played by this lipid within the living cell. For example, PA is a central element in the synthesis and turnover of glycerophospholipids and it is essential in numerous cellular functions, such as vesicular trafficking, signal transduction, cytoskeletal organization and cell proliferation (Yang et al., 2012; Carman et al., 2007; Pleskot et al. 2013). PA derived from the phospholipase D (PLD) pathway has second messenger functions: for instance, it is a potent activator of cardiac inositol phosphate production. It is evident that it is an important regulator in potentiating the phospholipase C-mediated generation of intracellular second messengers involved in the regulation of cardiac cells (Kurz et al.,1993).

PLD, a phospholipid phosphohydrolase, catalyses the hydrolysis of phosphatidylcholine and other membrane phospholipids to PA and choline. PLD, ubiquitous in mammals, is a critical enzyme in intracellular signal transduction. PA locally changes membrane topology and may thus be a key

player in membrane trafficking events, especially in membrane fusion and fission steps, where lipid remodelling is believed to be crucial. (Tanguy et al. 2019).

PA critically regulates vesicle budding from the Golgi (Yang et al. 2008), autophagy (Holland et al. 2016), and exosome release (Ghossoub et al. 2014). Mechanisms by which PA promotes membrane rearrangements remain unknown. PA could induce membrane curvature and promote fusion, but it be has also able to regulate the activity of different proteins involved in the vesicle docking and/or recruit crucial fusion proteins (Tanguy et al. 2016, 2018).

In addition, PA could accumulate and form microdomains highly negatively charged, which potentially serve as membrane retention sites for several proteins key for exocytosis, such as the SNARE protein syntaxin-1 (Lam et al. 2008), or other membrane remodelling processes (Jenkins et al.,2005). Finally, as a precursor for DAG and PI(4,5)P2, both known to contribute to numerous membrane remodeling events, PA could also have indirect effects. PA contribute in membrane fusion (Chasserot-Golaz et al. 2010; Ammar et al. 2013, 2014; Tanguy et al., 2016), but the heterogeneity of PA pathways lead to further investigate its activity to better understand its pleiotropic action in different physiological processes.

PA generated by agonist- or reactive oxygen species (ROS)-mediated activation of the PLD1 and PLD2 isoforms can be subsequently converted to lysoPA (LPA) or DAG by phospholipase A1/A2 or lipid phosphate phosphatases. In vascular endothelial cells, a wide variety of agonists stimulate PLD and involve Src kinases, p-38 mitogen activated protein kinase, calcium and small G proteins.

In endothelial cells, PA regulates NAD[P]H oxidase activity and barrier function. In airway epithelial cells, sphingosine-1-phosphate and PA-induced IL-8 secretion and ERK1/2 phosphorylation is regulated by PA. PA can be metabolized to LPA and DAG, which function as first- and second-messengers, respectively. PA and its metabolic products play a central role in modulating EC barrier functions. The signalling pathways downstream of PLD leading to EC permeability changes have not been clearly defined. However, PA can directly activate PKC ζ, alter the actin cytoskeleton and modify the actomyosin contractile apparatus (Garcia et al., 1995, Cross et al. 1996). PIP2 and phosphatidylinositol-3,4,5-trisphosphate are other key regulators of the actin cytoskeleton. Subsequently, the PA-mediated activation of kinases can alter intracellular levels of PIP2, in turn modulating interactions between actin and actin binding proteins such as vinculin and filamin (Wakelam et al. 1997). PIP2 can also stimulate PLD activity (Hammond et al. 1997), so amplifying PA production and PA-dependent kinases.

The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C is an important pathway in hormone-regulated signal transduction in a variety of tissues (Rana et al., 1990). The initial products of phospholipase C-mediated hydrolysis of PIP2, inositol 1,4,5-trisphosphate and

DAG, mediate pivotal roles as intracellular second messengers through the mobilization of calcium from intracellular stores and activation of protein kinase C (M J Berridge 1987). Although the hydrolysis of PIP2 by phospholipase C and the intracellular effects of the second messengers have been studied extensively, little is known regarding the functional role of PA.

Indeed an important role of PA in cell signaling is the increase of intracellular Ca^{2+} ($[Ca^{2+}]i$) (Moolenaar et al. 1986). It is known that $[Ca^{2+}]i$ is regulated by the release of Ca^{2+} from intracellular stores and influx from extracellular sources (Berridge et al., 1989). PA has been known to increase $[Ca^{2+}]i$ by the activation of Ca^{2+} efflux from internal stores (Sakano et al. 1996), even though there have been reports suggesting the activation of Ca^{2+} influx by PA. However, there have been contradictory reports on the mechanisms by which PA triggers Ca^{2+} release from internal stores (Sakano et al.,1996). In A431 cells, PA was shown to elevate $[Ca^{2+}]i$ by stimulating the hydrolysis of phosphoinositides ($[Ca^{2+}]i$) (Moolenaar et al., 1986). Contrarily to this report, PA could increase $[Ca^{2+}]i$ in the presence of heparin in Jurkat cells, suggesting that PA stimulated Ca^{2+} efflux from inositol 1,4,5-trisphosphate (IP3)-insensitive stores. However, the detailed mechanisms by which PA increases $[Ca^{2+}]i$ are not still known (Lee et al. 1998)

Phospholipid synthesis, homeostasis and signalling activity are cellular functions completed by PA, perturbations in its metabolism could lead to neurological disorders such as AD.

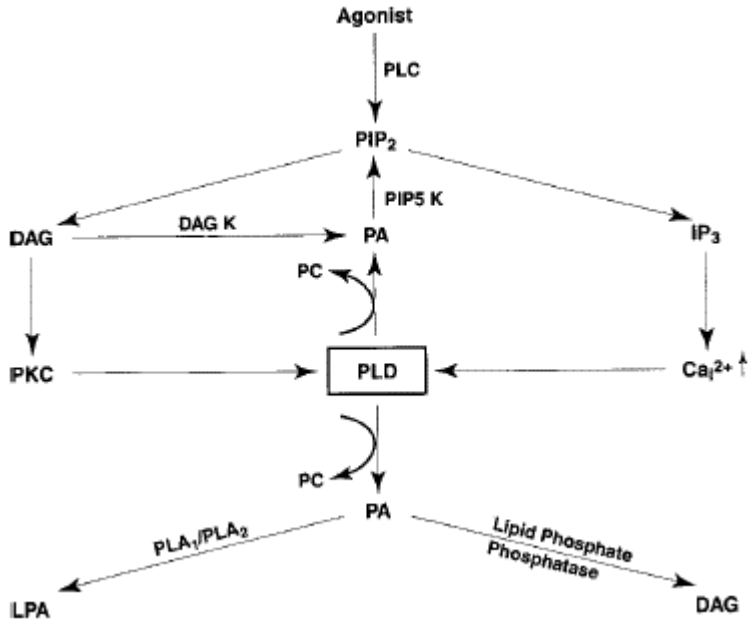


Figure 5. Phospholipase D/phosphatidic acid signal transduction

PHOSPHATIDIC ACID AND ALZHEIMER'S DISEASE

The PLD pathway has also been shown to play a role in amyloidogenesis (Oliveira et al. 2010). Most studies on the link between PLD and amyloidogenesis have so far focused on PLD1 which, like the related family member PLD2, hydrolyses phosphatidylcholine to generate PA and free choline (Oliveira et al., 2010, Jenkins et al., 2005).

Lipid-mediated signalling regulates a lot of physiological processes, including multiple aspects of brain function. Dysregulation of lipid pathways has been involved in different neurodegenerative disorders (Landman et al. 2006, Sanchez-Mejia et al. 2008). Lipid analysis of post-mortem brain tissue in AD-affected patients revealed that the anterior temporal cortex has significantly lower levels of phosphatidylinositol (PI) and trends for lower levels of PI phosphate (PIP) and PI-4,5-bisphosphate [PI(4,5)P2] (Stokes et al., 1987) Nitsch et al., observed that brains of AD patients had decreased levels of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and PA (Pettegrew et al. 2001).

In summary, studies on phospholipids, such as phosphoinositides and PA, show that these lipids are involved in several processes controlling Aβ generation (Di Paolo et al., 2011). This feature has been first pointed out by the susceptibility of the ApoE4 allele to AD, but more recently PLDs have also been proposed to contribute to the development of the pathology (Tanguy et al., 2019). PLD1 is involved in the vesicular trafficking of βAPP (Cai et al. 2006) and later that increased expression of APP promoted PLD activity in human astroglioma cells (Jin et al. 2007). Data show that defects in PA production by PLDs are involved in AD. In this study, the authors found that PLD2 knockout fully rescued AD-related synaptic dysfunction and cognitive deficits in a model of AD (Oliveira et al., 2010). It is necessary to further investigate how to modulate PA imbalance that can interfere with AD condition, this could provide new ideas for the treatment of neurological disorders linked to PA metabolism.

BRAIN TARGETED NANOCARRIERS

Nanotechnological devices, nanoparticles in particular, have been suggested as potential tools for the therapy of CNS diseases. In the last few decades, many drugs have been functionalized with pharmaceutical carriers to enhance the *in vivo* efficiency (Torchilin 2012). Surface modification of pharmaceutical nanocarriers, such as liposomes, micelles, nanocapsules, polymeric nanoparticles and others (Rolland et al. 1993, Alonso 2004)is normally used to control their biological properties and perform various therapeutically or diagnostically important functions. One of the most difficult goals is to reach and overcome the BBB. Nowadays, this goal has been achieved using multi-functionalized

nanoparticles (NPs) that easily reach BBB receptors and, interacting with them, are able to cross it and reach the targeted region. As in the case of other neurological disorders, the treatment of AD is now promising by using NPs that are able to enhance the penetration of therapeutics from the blood into the brain because of their high chemical and biological stability, ability to incorporate different types of molecules and the possibility to be administered by virtually all routes (Sancini et al. 2016). Liposomes have been used as transport systems for a long time. Typically, phospholipids that form mono- and multi-lamellar structures under various technological conditions are used for liposome development (Alyautdin et al. 2014). They are simple to prepare, with a high bioavailability, low toxicity and relatively low cost. Due to their characteristics, liposomes are attractive transport systems, especially for delivering drugs to the brain. However, liposomes are captured relatively quickly by the reticuloendothelial system (RES) cells, restricting their usage as a transport system. Liposomes are able to interact with different proteins and peptides, for example also with Aβ, but they need a specific functionalization on their surface to guarantee high affinity with the target and a complete BBB over-crossing. Targeting of cerebral Aβ1e42 in all its aggregation forms has been suggested for therapeutic and/or diagnostic purposes of the disease (Gobbi et al. 2010). Balducci and colleagues (Balducci et al. 2014) conducted an *in vivo* study to look at the ability of multifunctional liposomes to target Aβ and interacting with aggregates promoting peptide removal across the BBB, facilitating its peripheral clearance. These liposomes were bi-functionalized with mApoE (to enhance crossing of the BBB) and with phosphatidic acid (PA), which is a high affinity ligand for Aβ (Ross et al. 2018). These bifunctional liposomes (mApoE-PA-LIP) were able to disaggregate Aβ fibrils in vitro, a property that was not exhibited by liposomes mono-functionalized with either mApoE or PA alone. This synergistic effect could be due to simultaneous interaction of the negatively charged PA phosphate group with positively charged amino acid residues on Aβ and of positively charged amino acids on mApoE with negatively charged regions of Aβ (Bana et al. 2014). Results from *in vivo* studies, showing a reduction in amyloid plaque load only with mApoE-PA-LIP, are supportive of this idea. However, the uptake of mApoE-PA-LIP in the brain was very low, despite of the mApoE modification. Mancini et al. conducted a follow-up investigation on the mechanism behind this therapy and a strategy known as the "sink effect" was proposed, according to which there is a reduction of Aβ burden by peripheral administration of a binding agent that draws excess Aβ out of the brain (Matsuoka et al. 2003). The study showed that peripheral administration of mApoE-PA-LIP increased the level of plasma Aβ without significant amounts of mApoE-PA-LIP entering the brain. Aβ oligomers were found to be transported out of the brain, across the BBB, with mApoE-PA-LIP acting in the periphery to mediate an increase in this efflux (Mancini et al. 2016). In addition, data in literature that confirm the use of NPs properly functionalized could be useful in the Aβ clearance

pathway. In fact, data suggest that nanoliposomes containing phosphatidylcholine, cholesterol and PA (NLPA) prevent Aβ-42 fibrillation and Aβ-42-induced human arteriole endothelial dysfunction (Truran et al. 2016).

Use of functionalized liposomes as a therapy system provides a valuable insight into future research studies that may effectively prevent amyloid plaque formation and restore vascular impairment that could occur with AD.

hCMEC/D3: **AN *IN VITRO* BBB MODEL**

To evaluate NPs interaction and their putative therapeutic effect it is necessary to model the BBB *in vitro* so that it is able to mimic the *in vivo* phenotype. Couraud and colleagues (Weksler, Romero, and Couraud 2013)developed an innovative immortalized cell line of brain microvascular ECs (hCMEC/D3) that is useful to overcome the availability of fresh human cerebral tissue necessary to isolate cerebral microvessel ECs.

The hCMEC/D3 cell line derived from human temporal lobe microvessels isolated from tissue excised during surgery for control of epilepsy (Weksler et al., 2013). These cells express efflux and trans-cellular transport systems so that they represent really useful tools to better study drugs interaction at the BBB. They contain ATP-binding cassettes for active transport, previously observed in isolated human brain microvessels. The hCMEC/D3 model is useful for NPs transport studies due to its structure and literature results confirm their efficacy (Pinzón-Daza et al. 2012).

Following the vascular dysfunction hypothesis of AD pathogenesis, hCMEC/D3 cell line is widely used to study the toxic effects of Aβ peptides on brain microvasculature.

Aβ peptides have been shown to decrease the activity of efflux transporters in hCMEC/D3 cells (Kania et al. 2011). Besides, these cells have also been used to investigate Aβ clearance mechanisms from the CNS to prevent both neuro- and vasculo-toxic effects.

In summary, results from literature indicate that hCMEC/D3 cells, which have been well characterized in recent years, retain the expression of most transporters and receptors expressed *in vivo* at the human BBB. Due to these advantages, hCMEC/D3 represent a distinctive model for investigating the biology of human brain endothelium and may, in the future, constitute an interesting tool to investigate BBB permeability to different drugs for the pharmaceutical industry.

CALCIUM CONTENT IN ENDOTHELIAL CELLS

Increasing calcium content [Ca^{2+}]i plays an important role in signal transduction pathways in which vascular ECs are involved (Adams et al. 1989, Himmel et al., 1993).

The resting [Ca^{2+}]i, in absence of a trigger stimulus, in vascular ECs is set at around 100–200 nM by the concerted interaction of three Ca^{2+}-transporting systems, which extrude Ca^{2+}across the plasma membrane (PM). They are the Plasma-Membrane Ca^{2+}-ATPase and the Na+/ Ca^{2+}exchanger (NCX), and the SarcoEndoplasmic Reticulum Ca^{2+}-ATPase (SERCA) dealing with cytosolic Ca^{2+} sequestered into the endoplasmic reticulum (ER), the largest intracellular Ca^{2+}reservoir (Moccia 2018) (Figure 4). The endothelium lies at the interface between vascular sidewall and circulatory system. Due to this, it is exposed to numerous transmitters, soluble factors, circulating hormones, cytokines, growth factors and drugs, as well as to mechanical stimuli (Félétou et al., 2006, Nilius et al., 2001).

The opening of Ca^{2+}-permeable channels located in the ER causes an increasing in [Ca^{2+}]i. The channels are activated by extracellular chemical and physical stimuli and allow the passive passage of the ions. In extracellular medium and in the ER lumen Ca^{2+}concentration is very high (in the range of mM and μM, respectively), in comparison to cytoplasm concentration. Calcium entry from outside and its release from ER lumen represent the main ways to increase intracellular calcium content. The most common Ca^{2+} way of entry in endothelial cells from the extracellular space is called store-operated calcium entry (SOCE), which is activated following depletion of the ER Ca^{2+}pool (Zuccolo et al. 2016, Abdullaev et al. 2008, Parekh 2010, Smyth et al. 2010). Up to 1 μM of increase in [Ca^{2+}]i represents a trigger signal that activates vascular ECs, following recruitment of either tyrosine-kinase linked receptors (TRKs) or G-protein coupled receptors (GPCRs) by growth factors and vasoactive agents (Moccia et al. 2010, (Tiruppathi et al. 2006, Munaron 2006, Tran et al., 2006, Nilius et al., 2001). In particular, the β isoform of PLC (PLCβ) is engaged by GPCRs, while TKRs recruit PLC-γ (PLCγ) (Berridge et al., 2003, Moccia 2012). PLCβ and PLCγ are part of a greater family of PLC isoforms that includes PLCδ. It is activated by [Ca^{2+}]i in the range of 0.1–10 mM (Rebecchi et al. 2000; Vines 2012). PLC, in turn, cleaves phosphatidylinositol-4,5-bisphosphate (PIP2) into InsP3 and DAG.

The InsP3Rs represent the most important channels for the release of Ca^{2+} from the ER: at least three isoforms of InsP3Rs exist both in animal and human cells (Yamada et al. 1994). ECs, in particular, express the 1 and 3 subtypes (InsP3R1 and InsP3R3). The InsP3-mediated Ca^{2+}release is a cooperative process, since it requires the intervention of all subunits for the channel to open (Meyer

et al., 1988). Ca^{2+}dependence of InsP3Rs is biphasic and it is regulated by InsP3, $Ca^{2+}$and ATP. Ca^{2+} (at nanomolar concentrations) activates the channel, while greater increases inhibit InsP3R activity. Endothelial InsP3Rs are mainly distributed in the perinuclear region: the recruitment of a InsP3Rs cluster generates a localized Ca^{2+}signal which is then transformed in a global Ca^{2+} wave by the opening of the adjacent receptors following diffusion of InsP3 and Ca^{2+} itself. This mechanism has been given the name of Ca^{2+}-induced Ca^{2+}release (CICR) (Foskett et al., 2007).

Indeed, in resting conditions, there is a constant leakage of Ca^{2+} from ER. This leakage is generally sequestered back into ER lumen by SERCA pumps, whose activity could be blocked by specific inhibitors such as thapsigargin and cyclopiazonic acid (CPA), resulting in the slow accumulation of Ca^{2+} in the cytosol.

CICR processes empty the ER Ca^{2+}stores leading to the opening of the so-called "store-operated" channels (SOCs), which are located on the PM and mediate Ca^{2+}responsible for store refilling via SERCA (Clapham 2007). The best characterized SOC is the Ca^{2+}release-activated Ca^{2+}(CRAC) channel, which was first identified in lymphocytes, mast cells and other immune cells (Feske 2009), then in many other cells, including ECs (Lodola et al. 2012, Sánchez-Hernández et al. 2010) and mouse neuronal progenitor cells (Somasundaram et al. 2014).

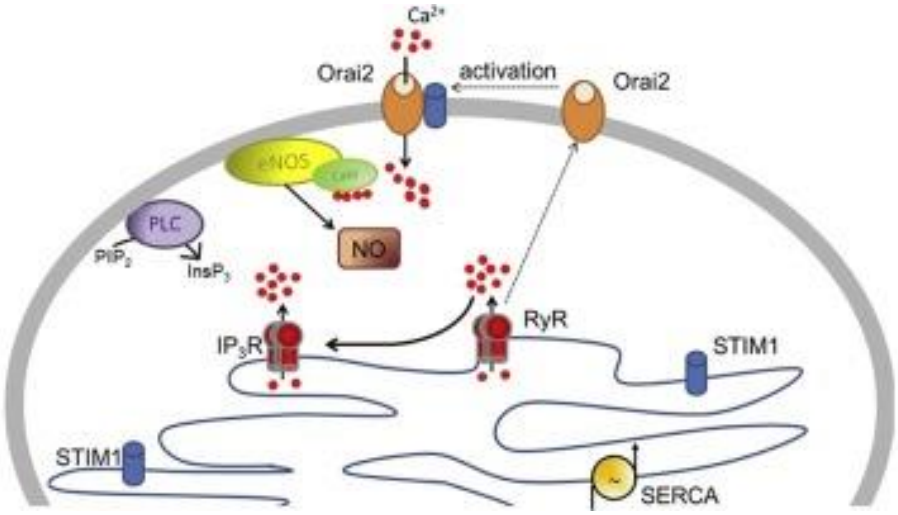


Figure 4. Calcium toolkit in endothelial cells. Figure from Zuccolo et al., 2017

BBB physiological function is modified whenever extracellular Ca^{2+} concentration decreases under the steady condition: around 1.5 mM at the blood side and 1 mM at the brain interstitial side (Redzic 2011) and/or when the intracellular free Ca^{2+}concentration rises up to 50–100 nM (Hess et al., 1989, Koenig et al. 1989).

There are few data in literature about the vasoactive outcomes of endothelial Ca^{2+} signals in CBF. Intracellular calcium modification in brain endothelial cells can involve different pathways. The increase of $[Ca^{2+}]i$ could be localized (the so called 'calcium puffs'): they result from the activation of single or clustered InsP3Rs and they could generate an intracellular Ca^{2+} wave. Calcium waves are not restricted to one cell but may pass through neighbour cells and spread as an intercellular Ca^{2+}wave (Leybaert et al., 2012). In brain endothelial cells there are high frequency Ca^{2+} oscillations generated by chemical stimulation. Ca^{2+} mobilization from the intracellular store and Ca^{2+} entry from the extracellular space support them. Variations in Ca^{2+}spike duration, frequency and amplitude, induce the activation of different Ca^{2+}-dependent pathways. In addition, a continued oscillation pattern is important to avoid the possible toxic effect of a prolonged increase of the $[Ca^{2+}]i$ and in the meantime may exert temporal control over cellular functions (Dupont et al., 2007).

Ca^{2+} signals in endothelial cells spread and ultimately lead to vasodilation of arterioles (Socha et al., 2012, Tallini et al. 2007). Ca^{2+} oscillations in BBB endothelial cells occur in response to bradykinin, ATP and histamine (Revest et al.,1991) and are inhibited by hypoxia (Kimura et al., 2000), which reduces both ER Ca^{2+}levels and SOCE.

Although the observation of Ca^{2+}oscillations and intercellular Ca^{2+} wave propagation in brain capillary endothelial cells indicate that all the essential elements of the Ca^{2+}signaling toolkit are available, the molecular architecture of the Ca^{2+} toolkit in brain microvascular endothelial cells are yet to be fully elucidated (De Bock et al. 2014).

CALCIUM HYPHOTHESIS OF ALZHEIMER'S DISEASE

The accumulation of toxic amyloid-beta (Aβ) peptide aggregates in AD brain are thought to trigger the extensive synaptic loss and neurodegeneration linked to cognitive decline, an idea that underlies the 'amyloid hypothesis' of AD aetiology in both the familiar (FAD) and sporadic forms of the disease. Mutations causing FAD also result in the dysregulation of neuronal calcium (Ca^{2+}) handling and this may contribute to AD pathogenesis, an idea termed the 'calcium hypothesis' of AD. The hypothesis that Ca^{2+}homeostasis perturbation could play a pivotal role in cascading events leading to AD was introduced more than 20 years ago (Khachaturian.1987). Following this hypothesis, the involvement of calcium in AD was further confirmed with the recognition that clinical mutations in

the presenilin (PS1/2) genes associated with FAD, profoundly affect calcium release from the endoplasmic reticulum (ER) stores as well as calcium entry through store operated channels (LaFerla 2002). Rare mutations in the APP and PS1/PS2 genes cause FAD by affecting the accumulation of the Aβ peptide. AD cases (89%) are sporadic (SAD) and advanced age is a prerequisite to develop the disease. Like FAD, SAD is thought to be caused by the accumulation of the Aβ peptide that occurs because of unknown complex genomic, proteomic, lipidomic and environmental interactions. For many years, it has been known that changes in calcium levels and dynamics alter the metabolism and production of Aβ (Green et al., 2008). Such disruption of calcium homeostasis may represent one of the possible causative factors of SAD. Different evidences support early pre-symptomatic roles for dysregulated cellular Ca^{2+}homeostasis in promoting amyloidogenesis, cytoskeletal pathologies, mitochondrial dysfunction, synaptic transmission and plasticity dysfunction and oxidative stress. In fact, Aβ aggregates lead to the formation of reactive oxygen species that can induce membrane-lipid peroxidation (Hensley et al. 1994). This process can adversely affect the function of membrane ion-motive ATPases and other transporters, leading to an elevation of basal intracellular calcium levels (Mark et al. 1995). Inhibition of lipid peroxidation by antioxidant treatments stabilizes calcium homeostasis and prevents the death of neurons exposed to Aβ (Goodman et al., 1994, Keller et al. 1997). These studies indicate that calcium dysregulation reveals as an early molecular defect in AD pathogenesis.

PURINERGIC RECEPTORS AT NEUROVASCULAR UNIT

Purinergic signalling, meaning nucleotides as extracellular signalling molecules, was proposed in 1972 (Burnstock 1972). However, this concept was not well accepted until the 1990's when receptor subtypes for purines and pyrimidines were cloned and characterised. The result was a classification including four subtypes of the P1 (adenosine) receptor, seven subtypes of P2X ion channel receptors and eight subtypes of the P2Y G protein-coupled receptor (Ralevic et al. 1997). Early studies were largely concerned with the physiology, pharmacology and biochemistry of purinergic signalling (Burnstock 2008). Adenosine triphosphate (ATP) is a co-transmitter with classical transmitters in both the peripheral and central nervous systems. In addition, purines are powerful extracellular messengers to non-neuronal cells, including secretory, exocrine and endocrine, endothelial, immune, musculo-skeletal and inflammatory cells (Burnstock et al., 2004).

Purinergic signalling can act rapidly in neurotransmission, neuromodulation and in secretion, but can also act in the long-term as in the case of proliferation, differentiation, migration, development and

regeneration (Burnstock 2016). Purinergic signalling was identified as a very important signalling system for the normal function of the neurovascular unit (Iadecola et al., 2007), where purine receptors participate in the regulation of vasodilatation and are involved in inflammatory reactions (Peterson et al. 2010). On abluminal side, ECs purine receptors could be stimulated by a release of purines or pyrimidines like adenosine triphosphate (ATP) or uridine triphosphate (UTP) from astrocytes in response to stimulation by neurons (Lazarowski et al., 1999). On the luminal side, agonists released from blood cells could stimulate purine receptors. Moreover, under pathophysiological conditions, such as the inflammatory state, the ATP release could be affected. receptors are membrane-bound receptors for extracellular nucleosides (P1-receptors) or nucleotides such as ATP or UTP (P2-receptors). The P2- receptors are subdivided into P2X receptors which are ligand gated ion channels and G-protein-coupled P2Y receptors with seven transmembrane regions (Abbracchio et al., 2006). The binding of ATP to the ionotropic P2X receptors allows the flux of cations (mainly Na+ and Ca^{2+}) across the membrane. The different P2Y receptors are activated by di- or triphosphates of the nucleosides adenosine and uridine; the P2Y1, 11, 12 and 13 receptors respond mainly to adenine nucleotides, the P2Y2 and P2Y4 receptors respond equally to adenine and uracil nucleotides, the P2Y6 receptor subtype is predominantly sensitive to uracil nucleotides, while P2Y14 is stimulated mainly by uridine diphosphate (UDP)-glucose (Fischer et al., 2007). The P2Y1, 2, 4, 6 and 11 receptor subtypes are coupled to PLC via Gq/11 proteins located at the intracellular side of the membrane, resulting in an IP3- mediated Ca^{2+}release from internal stores. The P2Y11 also mediates stimulation of adenylyl cyclase (AC) via Gs protein. The P2Y12, 13 and 14 subtypes are linked to AC inhibition via Gi protein (Erb et al. 2006). In particular, in human microvascular ECs (hCMEC/D3) receptors expression regarding subtypes P2Y2, P2Y6 and P2Y11 as well as P2X4, P2X5 and P2X7 are expressed at the mRNA level (Bintig et al. 2012). At a functional level, purinergic stimulation of the hCMEC/D3 cells induces Ca^{2+}signalling only by the P2Y2 receptor, which activates Ca^{2+}release from intracellular stores, even if they express also ionotropic receptors (Bintig et al., 2012).

At the NVU also astrocytes express purinergic receptors. ATP released from astrocytes activates P2 receptors on astrocytes and other brain cells, allowing a form of homotypic and heterotypic signalling, which also involves microglia, neurons and oligodendrocytes. Multiple P2X and P2Y receptors are expressed by both astrocytes and microglia (Abbracchio et al., 2006). Astrocytic P2Y1 and P2Y2,4 are primarily involved in short-term calcium-dependent signalling, while multiple P2 receptor subtypes seem to cooperate to astrocytic long-term changes. Activation of astrocytes and microglia may originally start as a defence mechanism to protect neurons from cytotoxic and ischaemic insults; dysregulation of this process in chronic inflammatory diseases eventually results in neuronal cell

damage and loss. P2Y receptors are coupled to several different transduction pathways, including activation of phospholipase C (PLC), inositol-phosphate formation and release of calcium from intracellular stores in the case of P2Y1,2,4,6,11 receptors (Abbracchio et al. 2003) and inhibition of cAMP formation in the case of P2Y12,13,14. P2X and P2Y receptors are both expressed on all types of brain cells, including neurons, astrocytes, microglial cells and oligodendroglia. P2X receptors mediate fast synaptic responses, while P2Y receptors have been proposed to mediate slow changes of neuronal membrane potential in response to non–synaptically released ATP. Localisation of these receptors may be at the axon terminals (pre-synaptic) or at the somato–dendritic region (post-synaptic). In the last few years there is an increase in studying purinergic implication in neurological disorders such as Parkinson's Disease, epilepsy and AD, such as a new tool in develop therapeutic strategies.

PURINERGIC RECEPTORS AND ALZHEIMER'S DISEASE

The potential of purinergic drugs for the treatment of AD has attracted a lot of interest in recent years. All subtypes of P2Y receptor present in the CNS (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11–14) are activated by ATP, UTP, adenosine 5′-diphosphate (ADP), uridine 5′-diphosphate (UDP), and UDP-glucose (Burnstock et al., 2004). P2Y receptors are expressed ubiquitously at the NVU side. In the brain, P2Y receptors affect neuronal activity and function of the neurovascular system, but also participate in the neuroinflammatory processes. Their important beneficial effect is induction of the neuro- protective processes, especially during neuroinflammation (Cieślak et al., 2018).

Besides, purinergic receptors (in particular the P2Y subtype) affect the conversion of non-amyloidogenic APP, release of cytokines and chemokines, microglia migration, endocytosis of cells by microglia, degradation of neurotoxic Aβ. P2Y receptors also participate in the cellular immune responses to oxidative stress, axonal outgrowth and neurite extension in neurons, regulation of neurotransmission and vasodilatation dependent on endothelium (Peterson et al., 2010).

Activation of P2Y1,2,4,6 receptors present on ECs results in vasodilation via the release of nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostaglandins (Burnstock et al., 2014). Therefore, stimulation of these receptors might prevent the vasoconstrictive action of Aβ. Activation of P2Y2 on ECs causes binding of monocytes to the endothelial wall and their diapedesis, and that process enhances the neuroprotective action of the microglia cells (Weisman et al. 2012). In AD, the reactive astrocytes are frequently found near plaques, and astrogliosis intensifies with the progression of pathological changes. Aβ plays an important role in their activation. Currently,

research on AD is focused on the role of reactive astrocytes in the degradation of extracellular Aβ deposits and in immunological processes (Boison et al., 2015). One of the roles of astrocytes is preventing the penetration of the brain/blood barrier by noxiousness substances that might participate in neurodegeneration (Sugama et al. 2009). Astrocytes release the neurotrophic factors involved in neuronal survival (Giaume et al. 2010); during neuroinflammation, astrocytes undergo morphological and functional transformation, known as reactive gliosis. The oligomeric β-amyloid peptide Aβ1–42 causes the increased release of nucleotides from astrocytes, that results in the stimulation of the neuronal P2Y2 receptors. In the brain of AD patients, the reactive astrogliosis is not only accompanied by activation, migration and proliferation of glia cells, but also activation of phagocytosis of cells damaged during apoptosis. By activation of Gq-dependent P2Y2 receptor on glia cells, ATP and UDP affect different metabolic processes, but also cell migration and proliferation, which are associated with the reactive astrogliosis.

Previous and recent studies have led to the proposal that both P2X7R and P2Y4R antagonists are potential therapeutic targets for the treatment of AD (Erb et al. 2015; Miras-Portugal et al. 2015; Woods et al. 2016). Stimulation of P2Y2 would therefore be the efficient way of the α-secretase activation, in particular activate P2Y2 receptors present in neurons, glia cells, and endothelial cells may have a positive neuroprotective effect in AD (Cieślak et al., 2018). It has been suggested that the blockade of P2Y1R may have therapeutic potential against cognitive disturbances in AD (Guzman et al., 2016); the glycosylphosphatidylinositol- anchored prion protein, which may be involved in AD, binds to and modulates the expression of P2X4R (Carneiro et al. 2016). These different targets and their implication in AD could be useful to further investigate different processes implicated in AD pathogenesis.

AIM OF THE STUDY

Neurodegenerative brain disorders may have a vascular origin, which provided evidences that the aged brain develops a functional uncoupling at the neurovascular unit (Zlokovic et al., 2017, 2011, 2008). In Alzheimer's disease (AD), we know that Aβ formation and its subsequent accumulation lead to neuronal injury and loss associated with cognitive decline, thus supporting the so call "amyloid hypothesis". According to the Zlokovic's "two hit vascular hypothesis of AD pathogenesis", Aβ accumulation in the brain is a second insult (hit 2) that is indeed initiated by vascular functional impairment and damage (hit 1).

Our previous results show that multifunctional liposomes reduce brain Aβ burden and ameliorate memory impairment in AD mouse models (Balducci et al., 2014); in light of these results, we assessed liposomes (mApoE-PA-LIP) functionalized with ApoE-derived peptide (mApoE) and phosphatidic acid (PA) at neurovascular unit.

We aimed to study the intracellular calcium dynamics triggered by purinergic receptors activation in both brain microvascular endothelial cells and astrocytes when treated with mApoE-PA-LIP by means of calcium imaging techniques.

In particular, the specific aims of the study are:

i) To evaluate mApoE-PA-LIP activities on intracellular calcium dynamics in cultured human cerebral microvascular cells (hCMEC/D3).

ii) To evaluate mApoE-PA-LIP activities on calcium intracellular dynamics in cultured astrocytes (iAstro-WT).

iii) To confirm mApoE-PA-LIP activity on intracellular calcium dynamics in absence of extracellular calcium both in endothelial cells and astrocytes.

iv) To evaluate mApoE-PA-LIP activity on intracellular calcium dynamics when SERCA activity is blocked.

2. MATERIALS AND METHODS

2.1 Cell culture

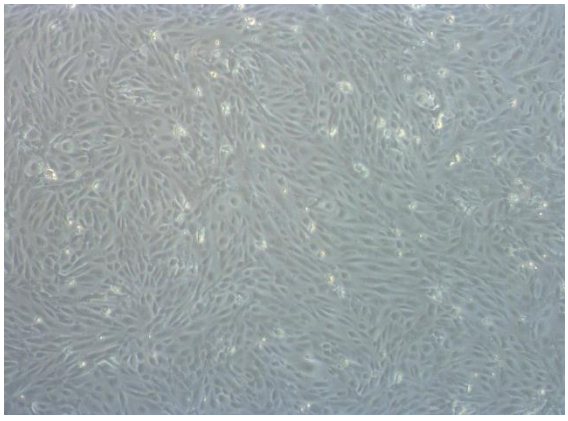
Endothelial cells

Human cerebral microvascular endothelial cells (hCMEC/D3) (Figure 5-A) were obtained from the Institute Cochin (INSERM, Paris, France). Cells at passages between 27th and 33rd were grown on tissue culture flasks, covered with 0.1 mg/ml rat tail collagen type 1, in EndoGRO-MV complete medium (Merck Millipore) supplemented with 1 ng/ml basic FGF (bFGF) and 1% Penicillin–Streptomycin (Life Technologies). Cells were seeded at a density of 24,000–33,000 cells/cm^2 in T75 flasks and cultured at 37 °C, 5% CO_2. For calcium imaging experiments, cells were cultured on type 1 collagen-coated coverslips in Petri dishes (p35) at a density of 150,000-200,000 for each Petri containing three coverslips; confluent hCMEC/D3 monolayers were obtained typically by days 3/4.

Astrocytes

Immortalized hippocampal astrocytes (iAstro-WT) (Figure 5-B) were gently provided by Dmitry Lim (Department of Pharmaceutical Sciences, University of Piemonte Orientale, Novara, Italy). Cells at passages between 16th and 22nd were grown on tissue culture flasks in DMEM complete medium (Euroclone) supplemented with 1% Penicillin–Streptomycin (Life Technologies), 10 % fetal bovine serum (FBS – Gibco) and 2mM glutamine (Euroclone) (Rocchio et al., 2019). Cells were seeded at a density of 6000–7000 cells/cm^2 in T75 flasks and cultured at 37 °C, 5% CO_2. For calcium imaging experiments, cells were cultured in Petri dishes (p35) at a density of 15,000-20,000. Confluent WT-iAstro monolayers were obtained typically after 2 days of seeding.

A



B

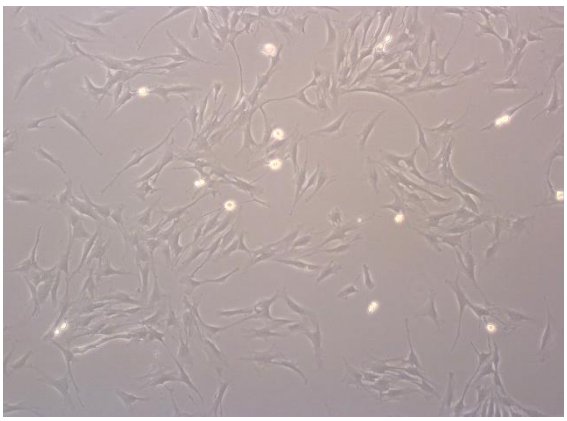


Figure 5. (A) hCMEC/D3 (B) WT iAstro

2.2 Preparation and characterization of mApoE-PA-LIP

mApoE-PA-LIP are composed of sphingomyelin from bovine brain (Sm) and cholesterol Sm/Chol (chol) (1:1 molar ratio) mixed with 2.5 molar% of mal-PEG-PE (Re et al. 2011), and containing either 5 molar% of PA.

Briefly, lipids were mixed in chloroform/methanol (2:1, v/v) and dried under a gentle stream of nitrogen followed by a vacuum pump for 3 hours to remove traces of organic solvent. The resulting lipid film was rehydrated in PSS, vortexed and then extruded 10 times through a polycarbonate filter (100-nm pore size diameter) under 20 bar nitrogen pressure, with an extruder.

mApoE peptide was added to NL to give a final peptide: mal-PEG-PE molar ratio of 1.2:1, and incubated overnight at room temperature to form a thioether bond with mal-PEG-PE. mApoE peptide (CWGLRKLRKRLLR, MW 1698.18 g/mol) was synthetized by Karebay Biochem (Monmouth Junction, NJ, USA).

NL and ApoE-NL size and polydispersity index were obtained using a ZetaPlus particle sizer (Brookhaven Instruments Corporation, Holtsville, NY, U.S.A.) at 25 °C in H2O by DLS with a 652 nm laser beam. For these experiments we use mApoE-PA-LIP (diameter = 122,7± 4,85 nm; PDI= 0,1 ± 0,02).

2.3 Solutions

hCMEC/D3

hCMEC/D3 were cultured on coverslips, maintained in a low-profile chamber with physiological salt solution (PSS) (NaCl 150 mM; KCl 6 mM; MgCl2 1mM; $CaCl_2$ 1.5mM; HEPES 10mM; Glucose 10mM). Ca^{2+}-free solution ($0Ca^{2+}$) was obtained by substituting Ca^{2+} with 2 mM NaCl and by adding 0.5 mM EGTA.

ATP (50µM) was added to the PSS and $0Ca^{2+}$ solutions. Cyclopiazonic acid (10µM) was added to the PSS and $0Ca^{2+}$ solutions. Then, mApoE-PA-LIP were dissolved at a final concentration of 0.01mg/ml in PSS and $0Ca^{2+}$ solutions.

iAstro-WT

iAstro Krebs Ringer Buffer (KRB) solution (125mM NaCl, 5mM KCl, 1mM NaH_2PO_4, 1mM MgSO4, 5.5mM glucose, 20mM HEPES, pH 7.4) was supplemented with 2mM $CaCl_2$. ATP (100µM) was added to the solution (both KRB and $0Ca^{2+}$ KRB). Cyclopiazonic acid (10µM) was added to the

PSS solution (both KRB and $0Ca^{2+}$ KRB). mApoE-PA-LIP were dissolved at a final concentration of 0.01mg/ml in PSS (both KRB and $0Ca^{2+}$ KRB).
All solutions were titrated to pH 7.4 with NaOH.

2.4 $[Ca^{2+}]_i$ measurements

Our experimental set-up (Figure 7) is equipped with wide field fluorescence time lapse microscopy to perform calcium imaging measurements.
hCMEC/D3 and iAstro-WT (Figure 6) were loaded with Fura-2AM (4 µM) in PSS for 30 minutes at 37°C away from light. The coverslip, after being washed in PSS was disposed in a low-profile chamber and maintained in physiological solution at 37°C for the entire duration of the experiments. Fluorescence ratios (excitation at 340 and 380 nm; emission at 510 nm) were observed by wide field fluorescence time lapse Nikon Eclipse FN1 upright microscope (Nikon Corp., Tokyo, Japan) equipped with a 60X Nikon objective (water-immersion, 2.0 mm working distance, 1 numerical aperture). For experiments with astrocytes we used 40X Nikon objective (water-immersion, 3.5 mm working distance, 0.80 numerical aperture). The excitation filters were mounted on a filter wheel (Lambda 10-2, Sutter Instrument, Novato, CA, USA). The fluorescent signal was collected by means of a Coolsmap Photometrics CCD camera through a bandpass 510nm filter.
By using MetaFluor (Molecular Devices, Sunnyvale, CA, USA) software, it is possible to measure and plot online, every 1200 seconds, the fluorescence from 8-12 regions of interest (ROI) inside each loaded cell; each ROI was identified by a number. Intracellular calcium concentration was monitored by measuring, for each ROI, the ratio (340/380nm) of the mean fluorescence. For the entire duration of the experiment, ratio measurements were performed and plotted online every 1200s with 800ms exposure time. Duration and area values were measured using Origin tools per each response in different conditions.

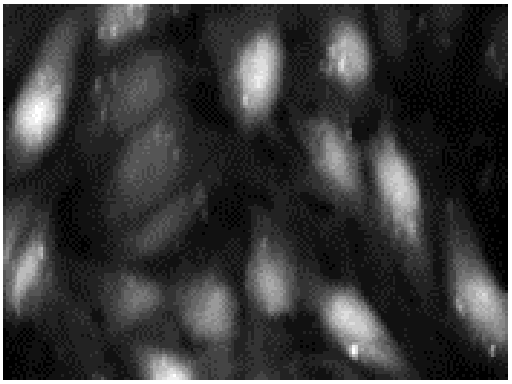
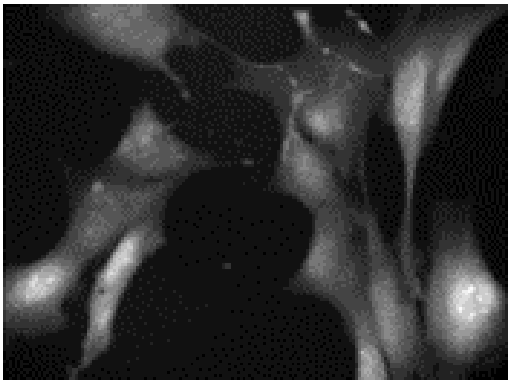
A  B 

Figure 6. (A) hCMEC/D3 (B) WT iAstro loaded with Fura-2AM

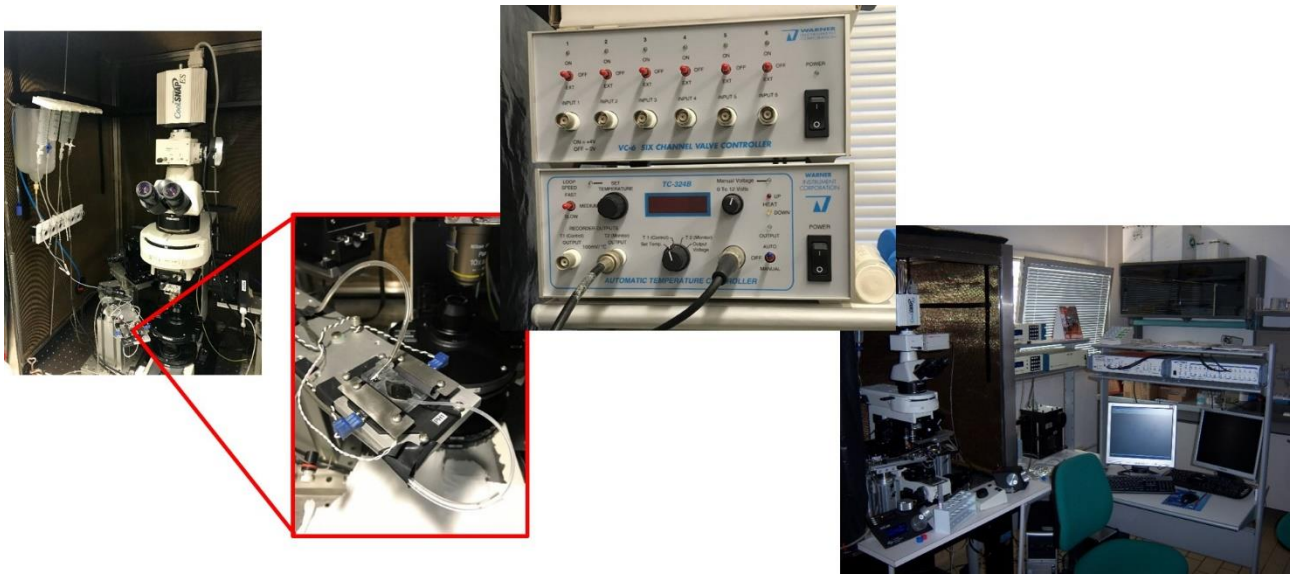


Figure 7. Experimental set-up

2.5 Chemicals

Cholesterol (chol), phosphatidic acid (PA) and adenosine 5′-triphosphate disodium salt hydrate (ATP) were obtained from Sigma–Aldrich.

1,2-Distearoyl-sn-glycero-3-phospho-ethanolamine-N [maleimide(polyethyleneglycol)-2000] (mal-PEG-PE) and sphingomyelin from bovine brain (Sm) were purchased from Avanti Polar Lipids, Inc (Alabaster, AL, USA).

mApoE peptide (CWGLRKLRKRLLR, MW 1698.18 g/mol) was synthetized by Karebay Biochem (Monmouth Junction, NJ, USA).

Fura-2 acetoxymethyl ester (Fura2/AM - 1 mM stock in dimethyl sulfoxide - DMSO) was obtained from Thermofisher. This indicator has an emission peak at 505 nm and changes its excitation peak from 340 nm to 380 nm in response to calcium binding.

Cyclopiazonic acid (CPA - 1 mM stock in DMSO) was obtained from Sigma Aldrich (C1530 – 5MG).

2.6 Statistics

All the data have been collected from hCMEC/D3 and iAstro-WT. The mean response values to ATP was measured considering the value from the baseline before and after the stimulus trigger. A.U.C was obtained using Origin Integration function. Statistical analysis was performed using Microsoft Office Excel.

Pooled data were given as mean ± SE and statistical significance (P-value <0.001) was evaluated by the Student's T-*test* for unpaired observations.

3. RESULTS

3.1 SOCE IS ACTIVE AND CONTROL ENDOPLASMIC RETICULUM CALCIUM CONTENT IN hCMEC/D3 CELLS

In order to study the calcium dynamics in hCMEC/D3 and starting from the evidence that in bEND5 cells SOCE regulate Ca^{2+} entry pathway (Zuccolo et al., 2017), we applied Ca^{2+} "add-back" protocol (Figure 8) to confirm if in hCMEC/D3 is expressed a functional SOCE. This protocol consists in incubating the cells with cyclopiazonic acid (CPA), a selective inhibitor of the SERCA under 0 calcium condition.

As the figure 8 shows there is a first transient increase in $[Ca^{2+}]i$ which is followed by the depletion of the ER Ca^{2+} pool and by the activation of the store-operated calcium channels, as indicated by the second larger increase of intracellular Ca^{2+} concentration arising after extracellular Ca^{2+} restitution. In conclusion, hCMEC/D3 cells express an operated calcium entry from the intracellular stores.

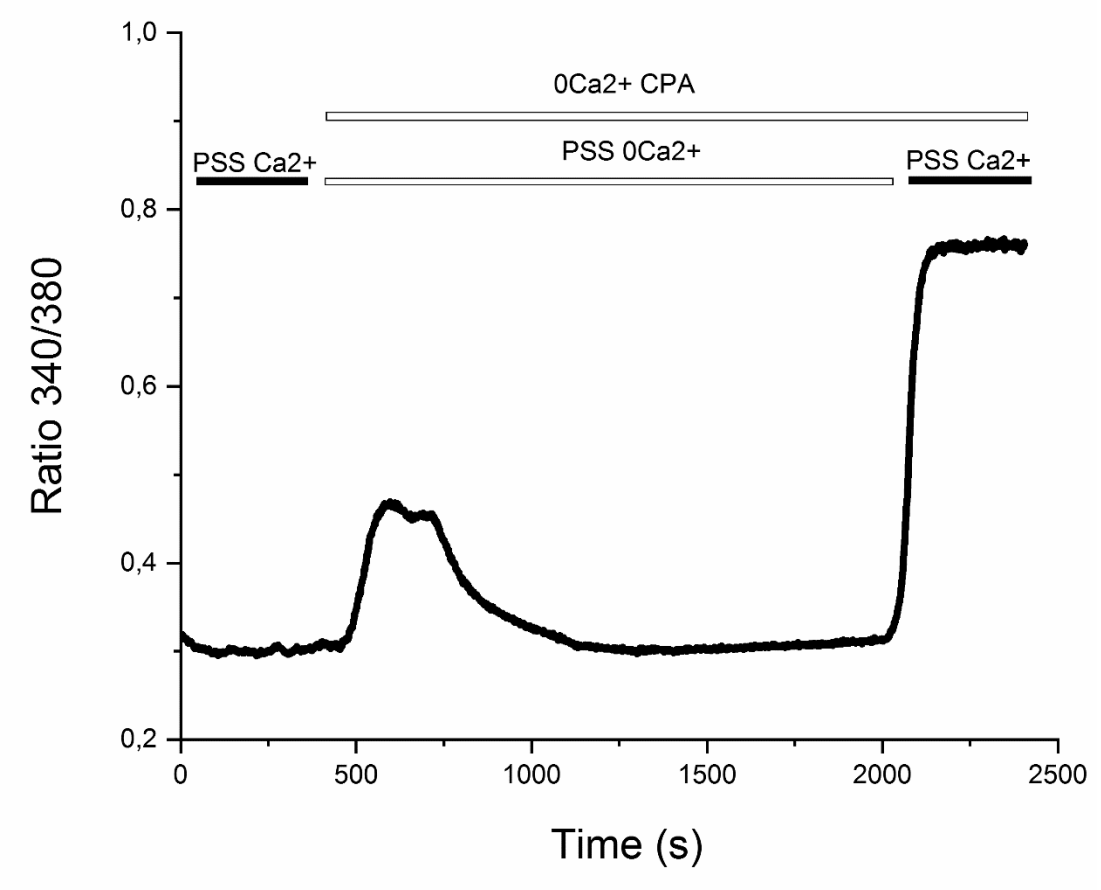


Figure 8. ''add-back'' protocol. In the Ca^{2+} ''add-back'' protocol, CPA (10μM) was administered under $0Ca^{2+}$ conditions to deplete the ER Ca^{2+} pool and activate store-operated calcium channels, as indicated by the second increase in $[Ca^{2+}]i$ arising after restitution of after restitution of external Ca^{2+}.

3.2 mApoE-PA-LIP PRE-TREATMENT INCREASES hCMEC/D3 cells RESPONSE TO ATP

In standard PSS buffer a pre-treatment with 0.01mg/ml mApoE-PA-LIP of the duration of 5 minutes increased the calcium dynamics evoked by ATP stimulus in comparison to control conditions (Figure 9-Aa), in particular, we found an increase of 12.5% (Figure 9-Ab).

A 5' minutes pre-treatment with 0.01mg/ml mApoE-PA-LIP in PSS with [0Ca^{2+}] increased the calcium dynamics evoked by ATP in comparison to control (Figure 9-Ba), in particular, we found an increase of 36% (Figure 9-Bb).

A

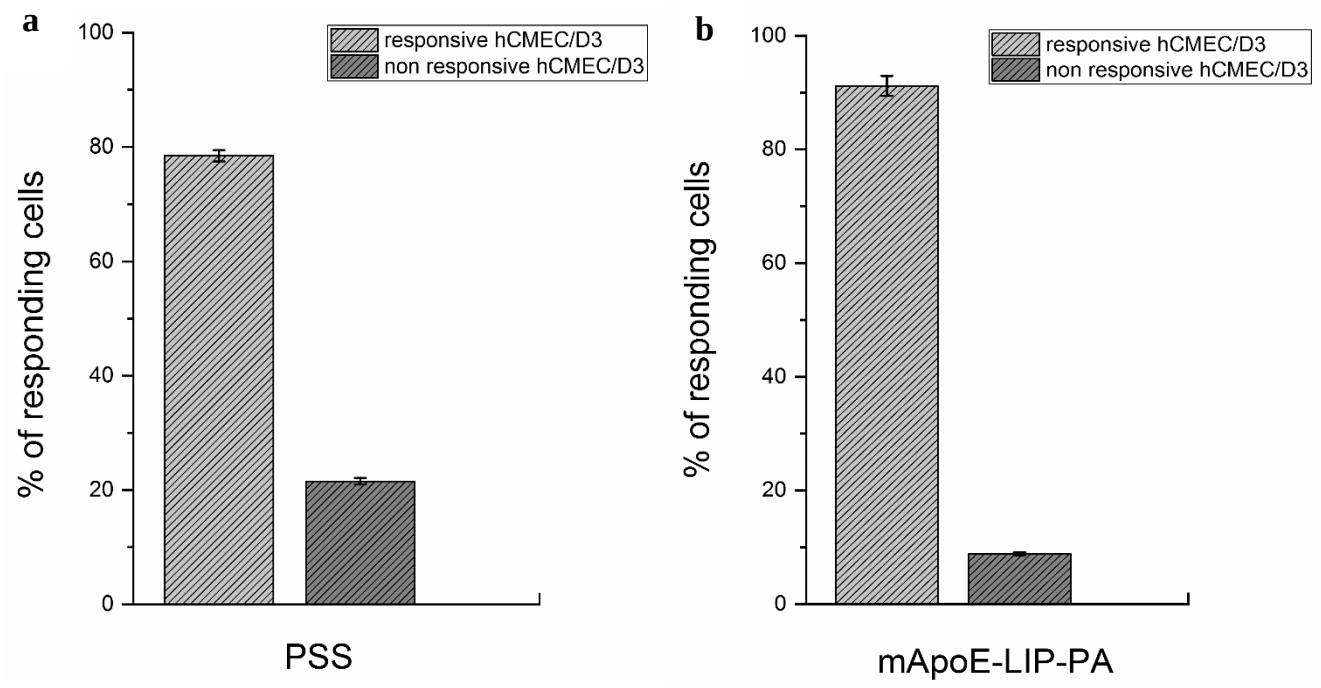


Figure 9. Percentage of responsive hCMEC/D3 cells to 30" ATP (50µM) as a trigger stimulus, in PSS maintaining solution in the presence of calcium is 78,5%. (A-a) Bar histogram shows the percentage ± SE of ATP (50µM) responding cells in PSS, (B-b) bar histogram shows the percentage ± SE of ATP (50µM) responding cells after a pre-treatment with 0,01mg mApoE-PA-LIP, the increase is 12.5%

B

a **b**

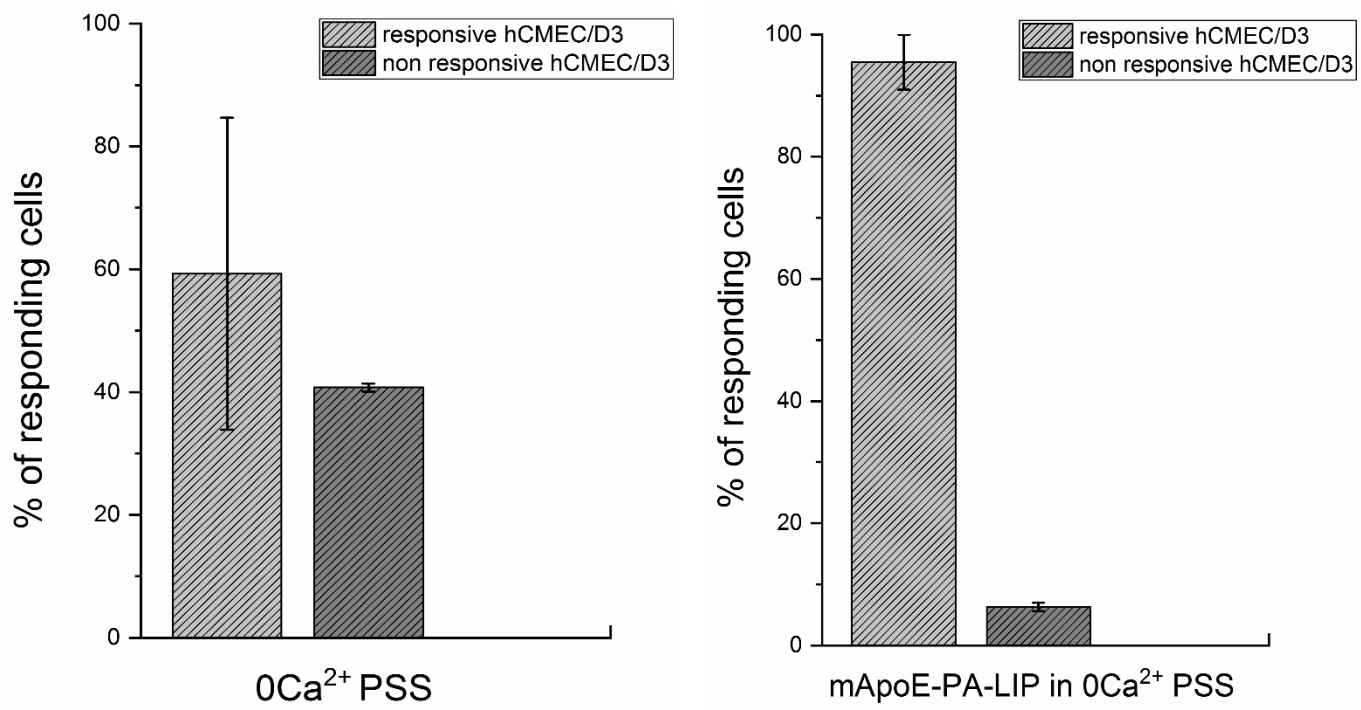


Figure 9. Percentage of responsive hCMEC/D3. (B-a) Bar histogram shows the percentage of ATP (50µM) responding cells in 0Ca²⁺ PSS is 59%, (B-b) bar histogram shows the percentage ± SE of ATP (50µM) responding cells after a pretreatment with 0,01mg/ml mApoE-LIP-PA 0Ca²⁺ PSS, which induced 36% increase.

3.3 PRE-TREATMENT WITH mApoE-PA-LIP INCREASE ATP RESPONSE AND AMPLITUDE IN hCMEC/D3 CELLS.

We after analysed 30" ATP response mean values under control condition and after a 5' pre-treatment with 0.01mg/ml mApoE-PA-LIP (Figure 10Aa-b). Bar histogram of the ATP response mean values ± SE show that there was a significant increase (mean ± se, 144 ± 3.03 sec, n=87, p-value <0.001) of the duration of the ATP evoked calcium waves in presence of mApoE-PA-LIP in comparison to cells without no treatment (mean ± se, 130 ± 2.19 sec, n=139) (Figure 10B-a).

The pre-treatment with mApoE-LIP without PA functionalization did not increase the mean value duration of the ATP induced response in hCMEC/D3 cells, (mean ± se, 125 ± 1.95 sec, n=52) (Figure 10B-b). We analysed also A.U.C values, and as indicated in Fig. 10B there is a significant increase of the response duration after pre-treatment with mApoE-PA-LIP in PSS with calcium. After the pre-

treatment also the area under the curve (A.U.C) is increased (A.U.C ± se 38.26 ± 5.06) in comparison to control (A.U.C ± se 25.44 ± 2.82) (Figure 10B-c).

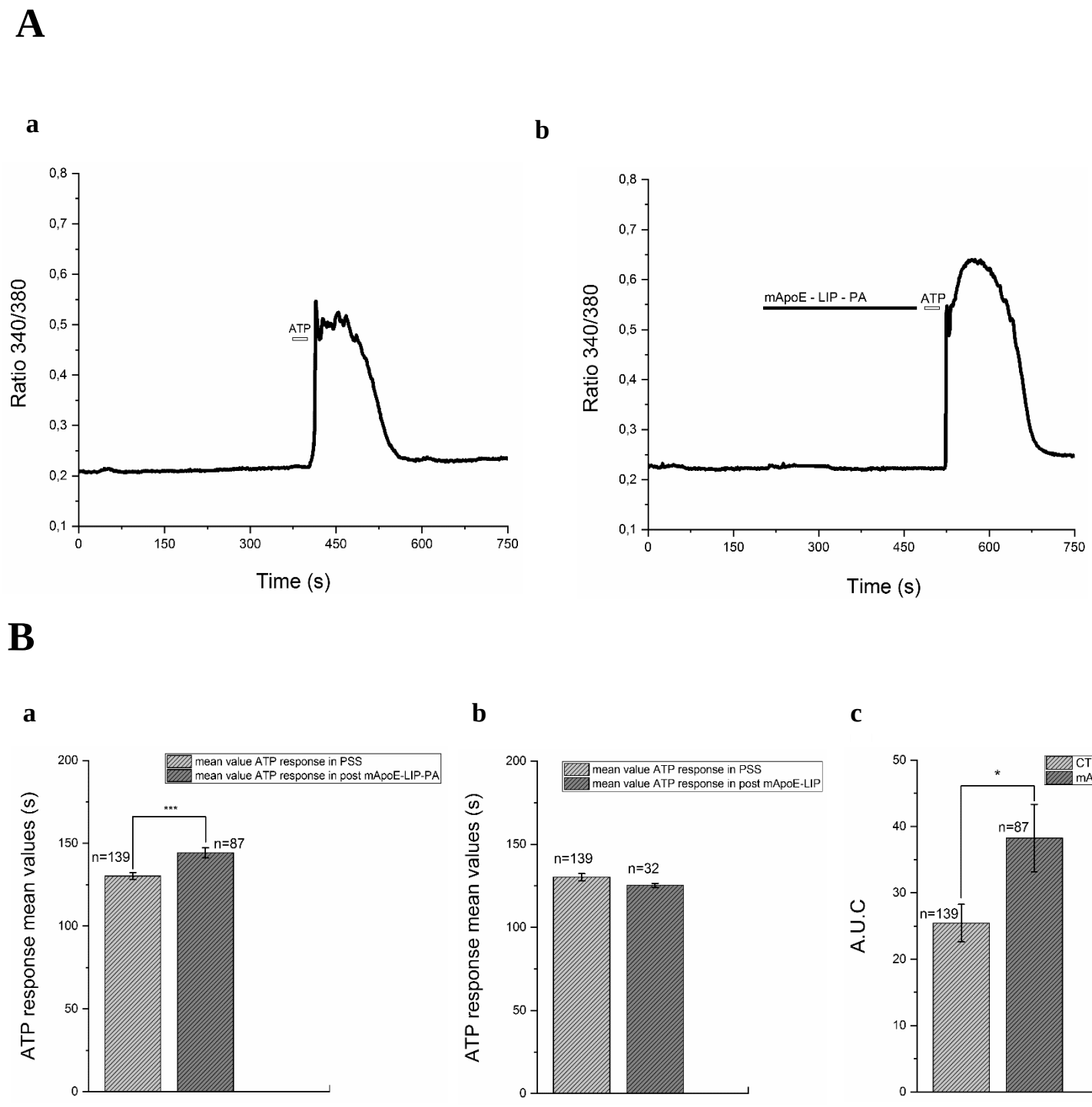


Figure 10. (A-a) hCMEC/D3 ATP (50µM) response. (A-b) hCMEC/D3 ATP (50µM) response after pretreatment with 0,01 mg/ml mApoE-PA-LIP in Ca^{2+} PSS; (B-a) bar histogram of the ATP response mean values ± SE in PSS and after a mApoE-PA-LIP pretreatment. (B-b) bar histogram of the ATP response mean values ± SE in PSS and after a mApoE-LIP pretreatment. (B-c) bar histogram of the A.U.C mean values ± SE in PSS and after a mApoE-PA-LIP pretreatment. The asterisk indicates p-value < 0.05.

Furthermore, we found that the pre-treatment with mApoE-PA-LIP in absence of extracellular calcium increased ATP evoked calcium waves in comparison to control (Figure 11Aa-b) As the histogram shows, the ATP response duration is significantly increased (mean ± se, 192.7 ± 6.38 sec, n=21) in comparison to control (mean ± se, 101.5 ± 9.2 sec, n=16) (Figure 11B-a). Under this condition also the A.U.C is increased (A.U.C ± se 26.97 ± 5.88) in comparison to control (A.U.C ± se 13.29 ± 0.33, p-value < 0.05) (Figure 11B-b).

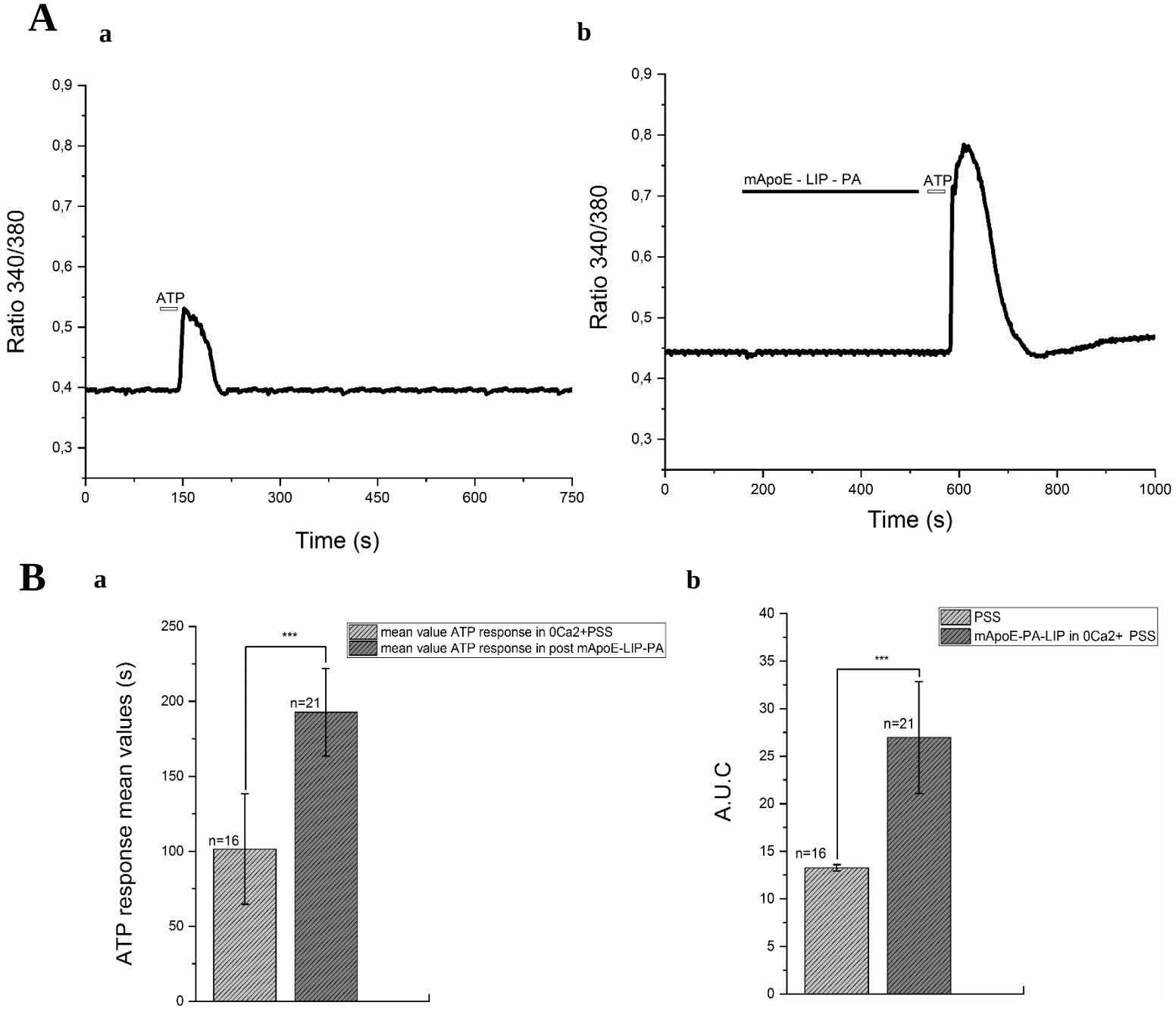


Figure 11. (A-a) hCMEC/D3 ATP (50µM) response in 0Ca²⁺ PSS. (A-b) hCMEC/D3 ATP (50µM) response after pretreatment with 0,01 mg/ml mApoE-LIP-PA in 0Ca²⁺ PSS; (B-a) bar histogram of the ATP response mean values ± SE in PSS and after a mApoE-LIP-PA pretreatment 0Ca2 PSS. The asterisk indicates p-value < 0.05. (B-b) bar histogram of the A.U.C mean values ± SE in PSS and after a mApoE-LIP-PA pretreatment. The asterisk indicates p-value < 0.05. Also, in 0Ca²⁺, the pre-treatment with mApoE-PA-LIP increased the calcium dynamics evoked by ATP stimulus in comparison to control.

3.4 ASTROCYTES PRE-TREATMENT WITH mApoE-PA-LIP INCREASE ATP RESPONSE AND AMPLITUDE

We stimulate iAstro-WT with ATP in control condition and after a 10' pre-incubation with 0.01mg/ml mApoE-PA-LIP (Figure 12Aa-b). Bar histogram of the ATP response (mean values ± SE) shows that there was a significant increase (mean ± se, 277 ± 26.63 sec, n=34) of the duration of the ATP evoked calcium waves in presence of mApoE-PA-LIP in comparison to controls (mean ± se, 137 ± 4.65 sec, n=56, p-value <0.001) (Figure 12B-a).

We then confirmed that also the A.U.C, (Figure 12B-b) of the ATP evoked response increased after pre-treatment with mApoE-PA-LIP in PSS with calcium. After the pre-treatment A.U.C value is increased (A.U.C ± se 4.35 ± 0.41) in comparison to control (A.U.C ± se 2 ± 0.09).

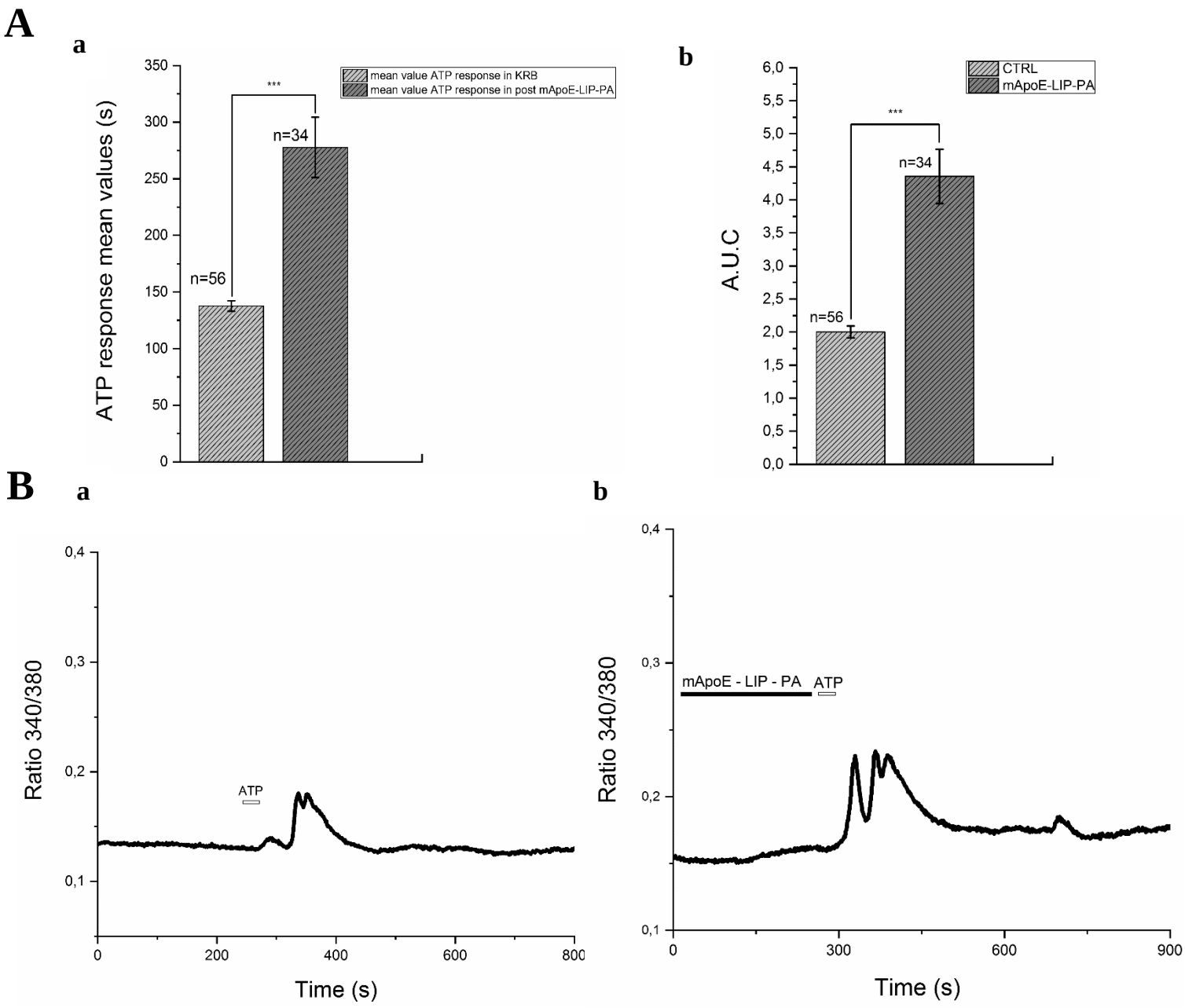


Figure 12. (A-a) iAstro-WT ATP (100µM) response. (A-b) hCMEC/D3 ATP (50µM) response after pretreatment with 0,01 mg/ml mApoE-LIP-PA in Ca^{2+} KRB; (B-a) bar histogram of the ATP response mean values ± SE in KRB and after a mApoE-LIP-PA pre-treatment. (B-b) bar histogram of the A.U.C mean values ± SE in PSS and after a mApoE-LIP-PA pretreatment. The asterisk indicates p-value < 0.05.

We confirmed that the pre-treatment with mApoE-PA-LIP in absence of extracellular calcium increased ATP evoked calcium waves in comparison to control (Figure 13Aa-b). The ATP response duration (Figure 13B-a), is significantly increased (mean ± se, 130.68 ± 3.25 sec, n=21) in comparison to control (mean ± se, 102.47 ± 5.98 sec, n=38). Under this condition also the A.U.C value is increased (A.U.C ± se 1 ± 0.07) in comparison to control (A.U.C ± se 1.71 ± 0.08, p-value < 0.001) (Figure 13B-b).

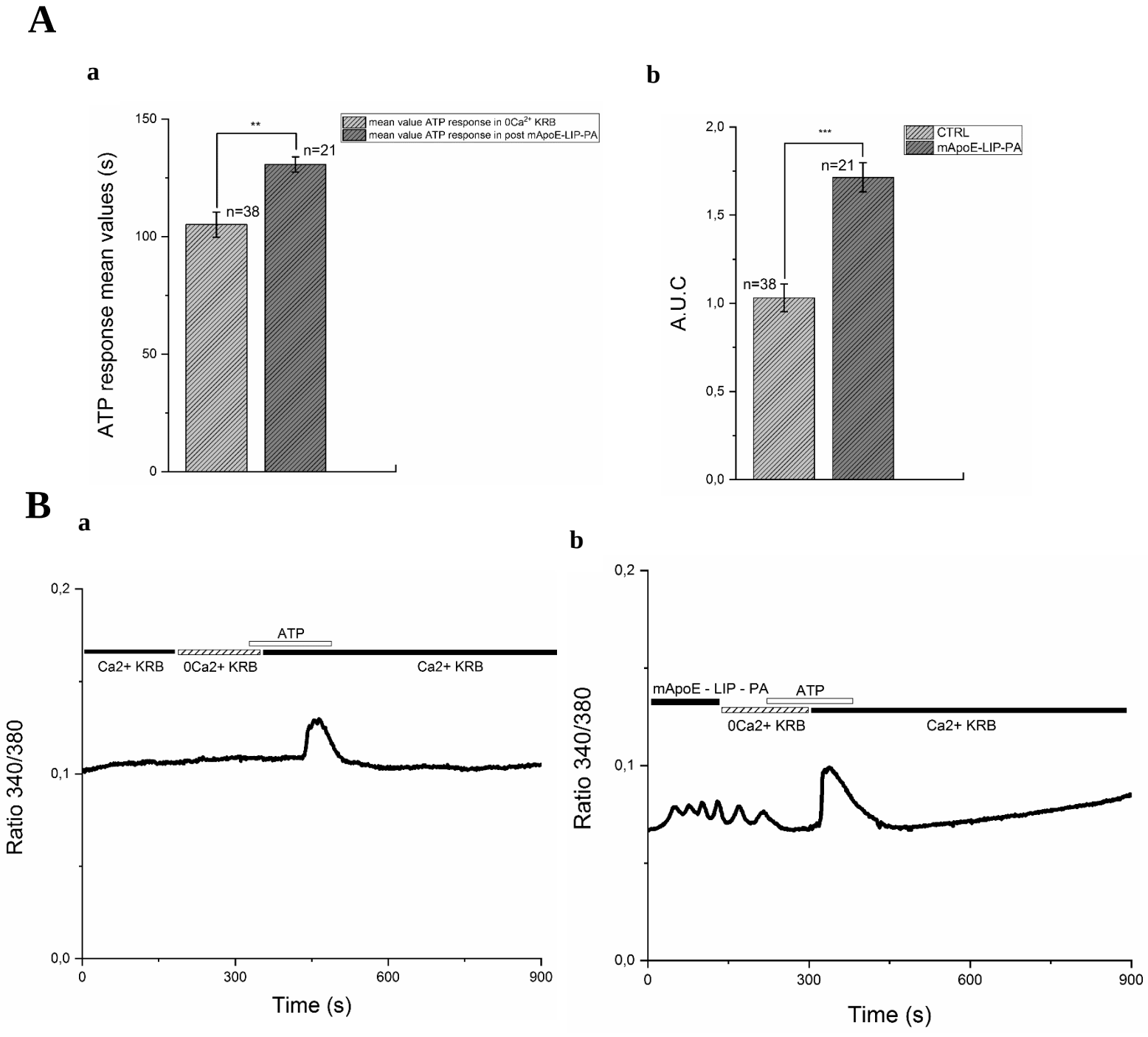


Figure 13. (A-a) iAstro-WT ATP (100µM) response in 0Ca²⁺ KRB. (A-b) iAstro-WT ATP (100µM) response after pretreatment with 0,01 mg/ml mApoE-PA-LIP in 0Ca²⁺ KRB; (B-a) bar histogram of the ATP response mean values ± SE and after a mApoE-PA-LIP pretreatment in 0Ca²⁺ KRB. The asterisk indicates p-value < 0.05. (B-b) bar histogram of the A.U.C mean values ± SE and after a mApoE-PA-LIP pretreatment 0Ca²⁺ KRB. The asterisk indicates p-value < 0.05. Also, in 0Ca²⁺, the pre-treatment with mApoE-PA-LIP increased the calcium dynamics evoked by ATP stimulus in comparison to control.

3.5 mApoE-PA-LIP PRE-TREATMENT MODULATE CALCIUM DYNAMICS WHEN SERCA IS ACTIVE BOTH IN hCMEC/D3 CELLS AND iASTRO-WT

We then confirmed the non-responsiveness of ATP in presence of SERCA blockage induced by CPA both in PSS/KRB and in 0 calcium PSS/KRB both in hCMEC/D3 cells (Figure 14) and in iAstro-WT (Figure 15). We pre-treated cells with mApoE-PA-LIP in presence of CPA in 0 calcium and in this condition ATP failed to activate the calcium wave (Fig. 14B-b; Fig. 15B-b).

hCMEC/D3

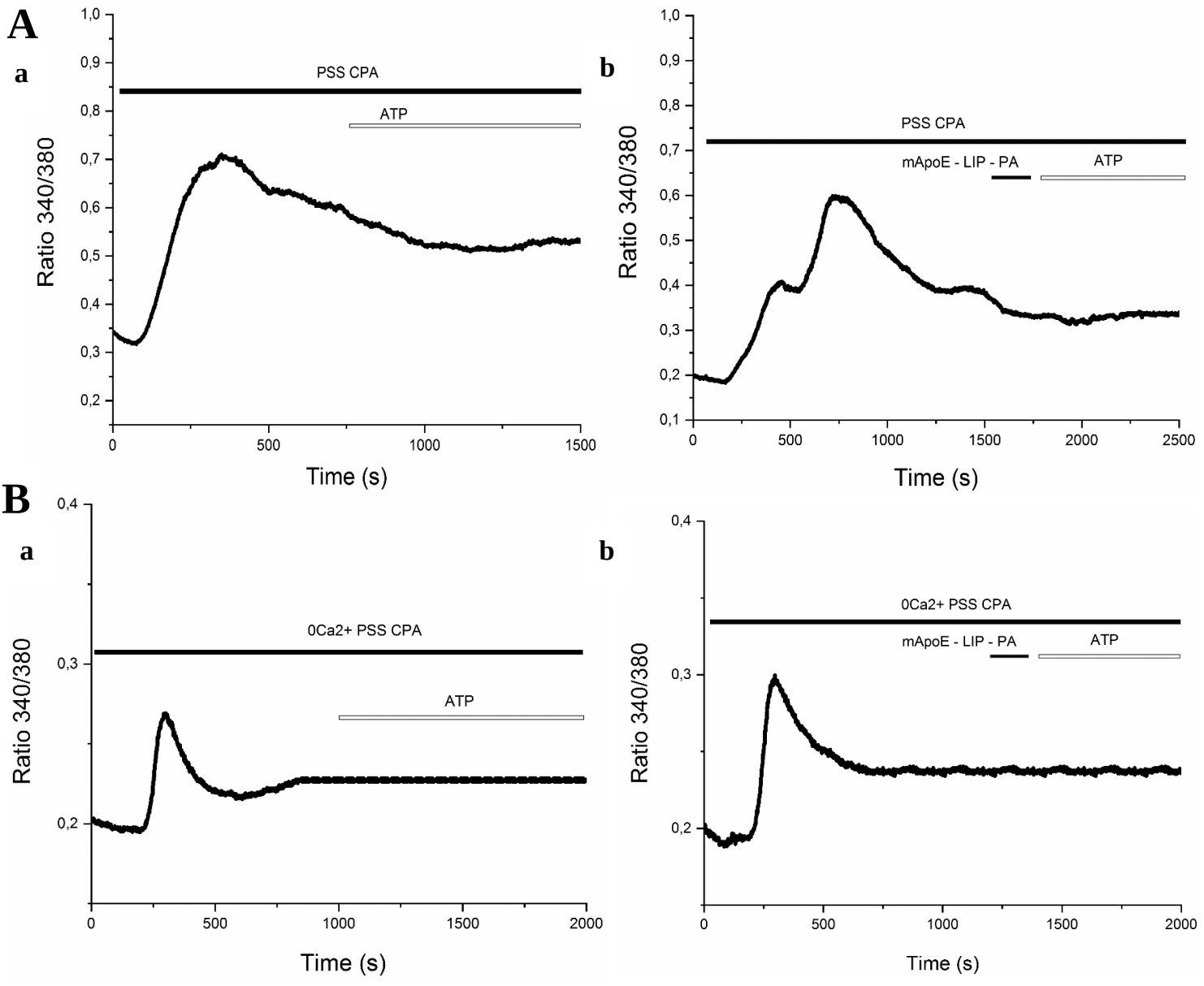


Figure 14. (A-a) CPA (10μM) response under extracelllar Ca^{2+} conditions. ATP evoked response is blocked by CPA perfusion. (A-b) CPA (10μM) response after pretreatment with 0,01 mg/ml mApoE-PA-LIP, also in these conditions there is no ATP response. (B-a) CPA (10μM) response under extracelllar $0Ca^{2+}$ conditions. (B-b) CPA (10μM) response after pretreatment with 0,01 mg/ml mApoE-PA-LIP in $0Ca^{2+}$ PSS. In presence of CPA both under calcium and 0 calcium ATP failed to activate calcium wave.

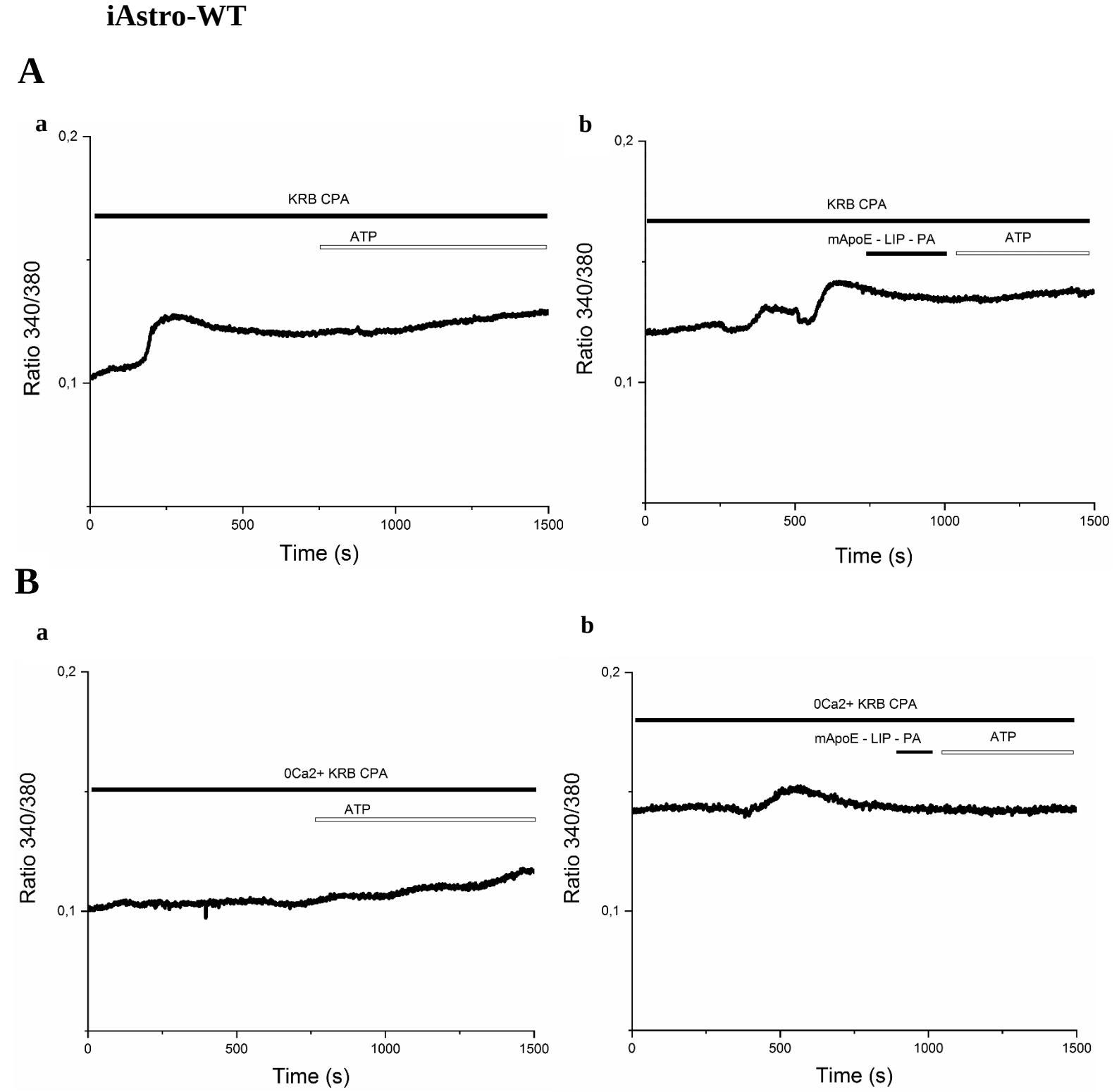


Figure 15. (A-a) CPA (10μM) response under extracelllar Ca^{2+} conditions. ATP evoked response is blocked by CPA perfusion. (A-b) CPA (10μM) response after pretreatment with 0,01 mg/ml mApoE-LIP-PA, also in these conditions there is no ATP response. (B-a) CPA (10μM) administration under extracelllar $0Ca^{2+}$ conditions. (B-b) CPA (10μM) response after pretreatment with 0,01 mg/ml mApoE-LIP-PA in $0Ca^{2+}$ KRB. In presence of CPA both under calcium and 0 calcium ATP failed to activate calcium wave.

4. DISCUSSION

Previous findings promoted mApoE–PA–LIP, bifunctionalized liposomes composed of sphingomyelin (Sm) and cholesterol (Chol) with phosphatidic acid (PA) with the task of binding Aβ (Gobbi et al., 2010) and with a peptide (mApoE), derived from the receptor-binding domain of apolipoprotein E, with the task of targeting and crossing the BBB (Re et al., 2011; Bana et al., 2014), as a well-tolerated valuable new nanotechnological means for AD therapy (Balducci et al., 2014). It has been reported the therapeutic effectiveness of mApoE–PA–LIP in transgenic (Tg) AD mouse models, demonstrating their effects on both the reduction of amyloid burden and memory improvement (Balducci et al., 2014). In addition, our previous results also show that pulmonary administration of functionalized nanoparticles significantly reduces Aβ in the brain of an AD murine model (Sancini et al., 2016).

Starting from these evidences we assessed mApoE-PA-LIP activities on human cerebral microvascular cells (hCMEC/D3) as an *in vitro* human blood brain barrier model and on cultured astrocytes in order to evaluate mApoE-PA-LIP ability of modulating the intracellular calcium dynamics.

The results here outlined proved that mApoE-PA-LIP actively modulate the intracellular calcium waves triggered by extracellular ATP in cultured hCMEC/D3 and astrocytes. Indeed, a trigger stimulus of 50 and 100 µM of ATP increased the duration and the A.U.C of the intracellular calcium waves when both hCMEC/D3 and astrocytes were pre-treated with mApoE-PA-LIP at the final concentration of 0.01 mg/ml for 5 min. It is worth to note that the pre-treatment with mApoE-LIP without PA functionalization failed to increase both the duration and the A.U.C of the calcium wave triggered by ATP.

Indeed, PA is a potent activator of inositol phosphate production and an important role of PA in cell signaling is the increase of intracellular Ca^{2+} ($[Ca^{2+}]i$) (Moolenaar et al., 1986). It is evident that it may serve a key regulatory role in potentiating the phospholipase C-mediated generation of intracellular second messengers (Kurz et al., 1993).

The hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase C is an important pathway in hormone-regulated signal transduction in a variety of tissues (Rana et al., 1990). The initial products of phospholipase C-mediated hydrolysis of PIP2, inositol 1,4,5-trisphosphate and diacylglycerol, mediate pivotal roles as intracellular second messengers through the mobilization of calcium from intracellular stores and activation of protein kinase C (Berridge et al., 1987).

It is known that [Ca^{2+}]i is regulated by the release of Ca^{2+} from intracellular stores and influx from extracellular sources (Berridge et al., 1989). However, there have been contradictory reports on the mechanisms by which PA triggers Ca^{2+} release from internal stores (Sakano et al.,1996). In A431 cells, PA was shown to elevate [Ca^{2+}]i by stimulating the hydrolysis of phosphoinositides ([Ca^{2+}]i) (Moolenaar et al.,1986). Contrary to this report, PA could increase [Ca^{2+}]i in the presence of heparin in Jurkat cells, suggesting that PA stimulated Ca^{2+} efflux from inositol 1,4,5-trisphosphate (IP3)-insensitive stores. However, the detailed mechanisms by which PA increases [Ca^{2+}]i are not still known (Lee et al, 1998).

In our hands mApoE-PA-LIP increased the ATP evoked intracellular calcium waves in cultured hCMEC/D3 and astrocytes even in 0 mM of extracellular calcium, thus indicating that the increased intracellular calcium concentration triggered by bath application of ATP is basically due to the release of Ca^{2+} from intracellular stores. Indeed, when SERCA activity was blocked by CPA, the extracellular application of ATP failed to trigger any intracellular calcium waves.

In our previous studies we found that in hCMEC/D3 histamine-induced intracellular Ca^{2+} oscillations were initiated by endogenous Ca^{2+} mobilization through inositol-1,4,5-trisphosphate- and nicotinic acid dinucleotide phosphate-sensitive channels and maintained over time by store-operated Ca^{2+} entry. In addition, histamine evoked robust NO release that was prevented by interfering with the accompanying intracellular Ca^{2+} oscillations, thereby confirming that the endothelial NO synthase is recruited by Ca^{2+} spikes also in hCMEC/D3 cells (Berra-Romani et al. 2019). These data provide evidence that histamine evokes NO production from human cerebrovascular endothelial cells through intracellular Ca^{2+} oscillations, thereby shedding novel light on the mechanisms by which this neuromodulator controls cerebral blood flow.

Balducci and colleagues (Balducci et al. 2014) conducted an *in vivo* study to look at the ability of mApoE-PA-LIP to target Aβ and interacting with aggregates promoting peptide removal across the BBB, facilitating its peripheral clearance. These liposomes were able to disaggregate Aβ fibrils in vitro, a property that was not exhibited by liposomes mono-functionalized with either mApoE or PA alone. This synergistic effect could be due to simultaneous interaction of the negatively charged PA phosphate group with positively charged amino acid residues on Aβ and of positively charged amino acids on mApoE with negatively charged regions of Aβ (Bana et al. 2014). Results from *in vivo* studies, showing a reduction in amyloid plaque load only with mApoE-PA-LIP are supportive of this idea. Though phosphatidic acid (PA) is a minor component of biological membranes (about 1% of phosphoLIPids) (Buckland et al., 2000), nevertheless, its presence is critical due to the multiple roles played by this lipid within the living cell. For example, PA is a central element in synthesis and turnover of glycerophospholipids and is essential in numerous cellular functions, such as vesicular

trafficking, signal transduction, cytoskeletal organization and cell proliferation (Yang et al., 2012; Carman et al., 2013; Pleskot et al., 2013; Tanguy et al., 2019).

Different studies confirm that PA could act as a positive modulator in different physiological mechanisms; it locally changes membrane topology and may thus be a key player in membrane trafficking events, especially in membrane fusion and fission steps, where lipid remodeling is believed to be crucial (Tanguy et al. 2019). PA could induce membrane curvature and promote fusion, but it also regulates the activity of different proteins involved in the vesicle docking and/or recruit crucial fusion proteins (Tanguy et al. 2016, 2018).

In addition, PA could accumulate and form microdomains highly negatively charged, which potentially serve as membrane retention sites for several proteins key for exocytosis, such as the SNARE protein syntaxin-1 (Lam et al. 2008), or other membrane remodeling processes (Jenkins et al.,2005). Finally, as a precursor for DAG and PI(4,5)P2, both known to contribute to numerous membrane remodeling events, PA could also have indirect effects. PA contribute in membrane fusion (Chasserot-Golaz et al. 2010; Ammar et al. 2013, 2014; Tanguy et al., 2016), but the heterogeneity of PA pathways lead to further investigate its activity to better understand its pleiotropic action in different physiological processes.

Our mApoE-PA-LIP could at the end act as PA confined to biological membranes thus promoting the transcellular trafficking of Aβ and at the Aβ clearance. This evidence could indeed provide new insight to explain the “sink effect” in charge to mApoE-PA-LIP as well established by in vitro and in vivo study in mice models of AD (Balducci et al, 2014; Bana et al. 2014).

Despite the substantial evidence indicating early vascular contributions to AD pathophysiology and dementia, vascular disease very commonly accompanies AD and may also be in the causal pathway (Sweeney et al., 2019). Neuropathological studies have shown that cerebrovascular pathology is a major risk factor for clinically diagnosed AD-type dementia (Arvanitakis et al., 2016). Structural arterial changes leading to functional changes in cerebral blood flow (CBF) are associated with the rate of accumulation of cerebral Aβ over time and the overlap of cerebrovascular and cerebral Aβ pathologies in older adults (Hughes et al., 2018). Indeed Vascular dysfunction appears early in AD, as shown using different imaging biomarkers of BBB integrity, brain microbleeds, cerebrovascular reactivity, resting cerebral blood flow, and increased cerebrovascular resistance (Sweeney et al. 2019).

The presence of β amyloid is detected not only in the brain, but also in other organs and is referred as misfolding diseases. Along this view the essence of AD is not the formation and aggregation of Aβ, which is resistant to the enzymatic proteolysis, but rather disorders in processes of its elimination, changing the equilibrium between the β-amyloid formation and elimination. The PA related to

mApoE-PA-LIP could modulate the membrane curvature and promote membranes fusion, thus regulating the activity of different proteins involved in the vesicle docking and this would again indeed improve the Aβ clearance and elimination as evidenced in previous studies (Balducci et al., 2014).

There are changes to different components of Ca^{2+} handling with aging and such alterations lead to the augmented susceptibility to the induction of long-term depression (LTD) and to an increase in the threshold frequency for induction of long-term potentiation (LTP) in aging neurons. LTD and LTP refer to activity-dependent changes to synaptic strength and remodeling and are proposed to be the basis for memory formation and storage. Recent evidence has revealed new insight into potential importance of enhanced Ca^{2+} release from neuronal ER in the context of AD (Foster, 2007).

Some forms of dysregulated ER Ca^{2+} signaling are a response to the adverse conditions of AD, such as for example the accumulation of Aβ peptides or increased neuronal excitability and are an effort to maintain intracellular Ca^{2+} and cellular homeostasis. If such compensatory mechanisms exist, they may slow down the progression of disease (Foster, 2007).

Our results show that the pre-treatment with mApoE-PA-LIP both in presence and in absence of extracellular calcium, modulates calcium dynamic evoked by ATP when SERCA is active.

In agreement with our findings related to mApoE-PA-LIP activities on intracellular calcium waves a recent paper by Krajnak et al.,2018 provides evidence that agents which actively modulate SERCA repairing calcium unbalance, could exert neuroprotective effects and improves memory and cognition in AD model mice. Ca^{2+} dysregulation by the endoplasmic reticulum (ER) in AD mouse models results in augmented cytosolic Ca^{2+} levels, which can trigger signaling cascades that are detrimental to neuronal function and health. However, there is growing evidence to suggest that not all forms of Ca^{2+} dysregulation in AD neurons are harmful and some of them instead may be compensatory. These changes may help modulate neuronal excitability and slow AD pathology, especially in the early stages of the disease. Clearly, a better understanding of how dysregulation of neuronal Ca^{2+} handling contributes to neurodegeneration and neuroprotection in AD is needed as Ca^{2+} signaling modulators are targets of great interest as potential AD therapeutics (Supnet et al., 2010). Indeed also neurons expressing purinergic receptors could be considered as natural targets of the positive modulation of the calcium waves induced by mApoE-PA-LIP pretreatment thus restoring the dysregulation of the intracellular calcium handling and contributing to the improved cognitive performance evidenced in in vivo model of AD (Balducci et al. 2014).

Occurrence of AD symptoms is sometimes preceded by pathological changes in the brain vascular system, including accumulation of Aβ in the walls of blood vessels (cerebral amyloid angiopathy) and lowering of cerebral blood flow (CBF) (Sagare et al., 2013). Research on humans suggests that

Aβ causes vasoconstriction of brain vessels triggered by free radical formation. These disorders lead to the brain hypoxia and the damage of the blood-brain barrier. Purinergic signaling participates in both vasoconstriction (vasospasm) and vasodilatation. ATP released from endothelial cells and blood platelets participates in the microcirculation in the brain by activation of P2X and P2Y receptors and the release of the endothelium-derived relaxing factor (EDRF) into the blood. (Cieślak et al., 2018). Activation of P2Y2 on the brain core neurons causes the increased degradation of APP assisted by α-secretase, what results in a formation of the soluble sAPPα protein rather than the neurotoxic Aβ1–42 peptide (Erb et al., 2015). Increased activity of the neuron P2Y2 caused by proinflammatory cytokines assists the neuroprotective responses, including activation of the non-amyloidogenic APP processing. (Kong et al., 2009). Research with the animal model of AD (TgCRND8 mice) showed the increased expression of P2Y2 in microglia during initial 10 weeks of life, which diminished after 25 weeks. In the brains of TgCRND8 mice, the P2Y2 receptor is important for activation of microglia cells and might affect the neuroprotective mechanisms via clearance of fibrillar Aβ1–42 (Kim et al., 2012). Activation of P2Y2 on endothelial cells causes binding of monocytes to the endothelial wall and their diapedesis, and that process enhances the neuroprotective action of the microglia cells (Weisman et al., 2012). Thus a positive modulation of the activities of purinergic receptors may have a positive neuroprotective effect in AD. Indeed, we can thus speculate that the increased of the duration and A.U.C of the calcium wave triggered by ATP when both hCMEC/D3 and iAstro-WT were pre-treated with mApoE-PA-LIP would at the end increase these neuroprotective actions.

The oxidative stress may also be counteracted via the purinergic signalling. Indeed, ADP and its non-hydrolysable analogues activate P2Y13 receptors, leading to the increased activity of heme oxygenase, which has a cytoprotective activity. Adenosine, via A1 and A2A receptors, affects the dopaminergic and glutaminergic signalling, the brain-derived neurotrophic factor (BNDF), and also changes the synaptic plasticity (e.g., causing a prolonged excitation or inhibition) in brain regions responsible for learning and memory. Such modulating activity may be advantageous in the Alzheimer's disease (Cieślak et al., 2018) and mApoE-PA-LIP could indeed at the end amplify it. Purine and pyrimidine receptors are of particular interest in relation to the function of the blood-brain barrier, having been shown not only to regulate the release of prostacyclin (PGI2) and nitric oxide from the brain endothelium (Boarder et al., 1998), but also to control blood-brain barrier permeability (Olesen, 1989). In previous studies, it has been evidenced that mApoE-PA-LIP increased the NO synthesis and release from cultured endothelial cells (Orlando et al., 2013). We have recently demonstrated that in hCMEC/D3 histamine evoked robust NO release that was prevented by interfering with the accompanying intracellular Ca^{2+} oscillations, thereby confirming that the endothelial NO synthase is recruited by Ca^{2+} spikes also in hCMEC/D3 cells. These data provided

the evidence that histamine evokes NO production from human cerebrovascular endothelial cells through intracellular Ca^{2+} oscillations (Berra-Romani et al.2019). Taking into account that mApoE-PA-LIP increased the duration and the A.U.C of the calcium wave induced by ATP activation of the purinergic receptor we could indeed speculate that this positive modulation will end to increase NO production thus shedding novel light on the mechanisms by which mApoE-PA-LIP may interfere with the cerebral blood flow.

Natural ligands for nucleotide receptors, including ATP, ADP and UTP, can be released from a number of cell types in the region of the vessel wall, such as platelets on the blood side, and smooth muscle cells and neurons on the brain side. Moreover, the endothelium itself can release ATP (Gordon, 1986) able to act on nucleotide receptors on astrocytes and neurons (reviewed in Nobles et al., 1995), so the endothelium is able to act as both target and source of nucleotide signals (Sipos et al., 2000). It could be of great impact the fact that our mApoE-PA-LIP induced a positive modulation of the ATP triggered calcium waves both in cerebral microvascular endothelial cells and astrocytes.

Indeed the term tripartite synapse describes that, besides the presynaptic nerve terminal and the postsynaptic part of the neuron, processes of astrocytes also participate in the synaptic signaling by bidirectional regulation of neuronal communication (Araque, et al., 1999; Araque, et al., 2014; Gundersen, et al., 2015). Astrocytes detect synaptic activity via ionotropic or metabotropic neurotransmitter receptors (Porter et al., 1996) which cause changes of astrocytic intracellular Ca^{2+} inducing the release of various signalling molecules, such as glutamate, ATP, and D-serine (Guzman et al., 2016).

Gliotransmitters have been shown to act on neurons in a timescale of seconds to minutes to regulate synaptic transmission and plasticity. ATP has a twofold role in the bidirectional neuron-glia communication. First, ATP released from neurons upon activity or during pathological conditions stimulates astrocytes by activation of P2Y1 receptors. Second, ATP released from astrocytes can influence the function of neurons via activation of P2X and P2Y neuronal receptors (Pascual et al., 2005). Moreover, P2Y1 receptors on neighboring astrocytes are able to amplify the astrocyte stimulation by mediating the propagation of Ca^{2+} waves within the astrocytic network (Koizumi et al., 2010).

Depending on the subcellular expression, P2Y receptors acting on voltage-gated membrane channels are able to inhibit neurotransmitter release, modulate dendritic integration, facilitate neuronal excitability, or affect other various neuronal functions such as synaptic plasticity or gene expression. (Guzman et al., 2016).

Activation of other P2Y receptors was described to have neuroprotective effects in neuroinflammatory processes such as AD (Woods et al., 2015). While activation of the P2Y2 receptors stimulated neurite outgrow and non-amyloidogenic processing of amyloid precursor protein (Peterson, et al.,2010) as well as uptake of Aβ, knockdown of the receptors was shown to increase AD pathology (Ajit, et al., 2014). Similarly, P2Y4 receptors present on microglia were also found to play a role in the uptake of Aβ (Li, et al., 2013) and P2Y12 receptors were described to stimulate microglial migration towards neuronal damage (Haynes et al., 2006).

Taking in to account that the activation of purinergic receptors may have a such distinctive protective effect and might be beneficial in the treatment of neurodegenerative diseases. The positive modulation of the purinergic response exerted on astrocytes by mApo-PA-LIP could be strictly related to the high efficacy of these bi functionalized liposome in counteracting the progression of the Abeta deposits and the cognitive impairment in AD animal models (Balducci et al 2014).

Indeed P2Y receptors are activated by ATP released from astrocytes and neurons upon increased neuronal activity or under pathophysiological conditions. Purinergic receptors are able to modulate synaptic transmission and plasticity by interactions with voltage-activated calcium and potassium channels, as well as ionotropic receptors. In the hippocampus and the cerebellar cortex, P2Y receptors activate inhibitory GABAergic interneurons playing a key role in timing and organization of principal cell firing. The modulatory effects of P2Y receptors on membrane channels and receptors are sufficient to influence synaptic transmission and plasticity which may sustainably affect the connectivity between different excitatory and inhibitory cell types and thus the network activity in different brain areas. Therefore, P2Y receptors represent important pharmacological targets to treat cognitive dysfunctions and neuropsychiatric diseases, such as Alzheimer's disease and schizophrenia (Guzman et al., 2016). We have so far investigated mApoE-PA-LIP activities in endothelial cells and astrocytes. Positive results in this sense would give additional support to promote mApoE-PA-LIP as putative therapeutic tool for AD treatment.

Targeting the neurovascular unit in AD instead of a classical neuron-centric approach in the development of neuroprotective drugs may result in improved clinical outcomes. Further study are deserved in order to disclose the specificity of mApoE-PA-LIP in modulating neuronal synaptic transmission.

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