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## **DIFFERENT PATTERNS OF $Ca^{2+}$ SIGNALING DRIVE ACETYLCHOLINE AND GLUTAMATE INDUCED-NO RELEASE IN MOUSE AND HUMAN BRAIN MICROVASCULAR ENDOTHELIAL CELLS**

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## Abstract

Acetylcholine (ACh) and glutamate (Glu) are two of the major excitatory neurotransmitters in the brain which increase cerebral blood flow by releasing nitric oxide (NO) from postsynaptic neurons and astrocytes and causing vasorelaxation in adjacent microvessels. An increase in intracellular  $\text{Ca}^{2+}$  concentration recruits a multitude of endothelial  $\text{Ca}^{2+}$ -dependent pathways, such as  $\text{Ca}^{2+}$ /Calmodulin endothelial NO synthase (eNOS). Surprisingly, the  $\text{Ca}^{2+}$ -dependent mechanisms whereby ACh induces NO synthesis in brain endothelial cells (ECs) is still unclear. On the other hand, Glu stimulates NMDA receptors to activate eNOS, but it is able to cause a metabotropic increase in intracellular  $\text{Ca}^{2+}$  concentration in brain microvascular ECs. The present investigation sought to fill these gaps by analysing murine (bEND5) and human (hCMEC/D3) brain microvascular ECs.

Herein, we first demonstrated that ACh induces NO release by triggering two different modes of  $\text{Ca}^{2+}$  signals in bEND5 and hCMEC/D3 cells. Of note, endoplasmic reticulum  $\text{Ca}^{2+}$  release via inositol-1,4,5-trisphosphate receptors and store-operated  $\text{Ca}^{2+}$  entry shapes the  $\text{Ca}^{2+}$  response to ACh in both cell types but their different  $\text{Ca}^{2+}$  toolkits result in two quite different waveforms, i.e.  $\text{Ca}^{2+}$  oscillations vs. biphasic  $\text{Ca}^{2+}$  elevation. Whatever its waveform, however, ACh-induced intracellular  $\text{Ca}^{2+}$  signals lead to robust NO release in both murine and human brain microvascular ECs. Likewise, we demonstrated for the first time that Glu activated metabotropic intracellular  $\text{Ca}^{2+}$  oscillation in bEND5 cells and a biphasic increase in intracellular  $\text{Ca}^{2+}$  concentration in hCMEC/D3 cells. We further showed that glutamate-dependent  $\text{Ca}^{2+}$  signals drive NO release in both types of cells. This NO signal is delayed as compared to the ACh-induced one and is likely to play a crucial role in the slower vasodilation that often follows brief neuronal activity or that sustains functional hyperemia during persistent synaptic transmission.

This information has a potential clinical relevance as the decrease in neuronal activity-induced cortical CBF is involved in a growing number of neurodegenerative disorders, such as Alzheimer's Disease. Understanding the underlying mechanisms could, therefore, be used in the future as target to rescue local blood perfusion in patients affected by neurodegenerative disorders.

# INTRODUCTION

## Cerebral blood flow regulation: cellular and biochemical pathways

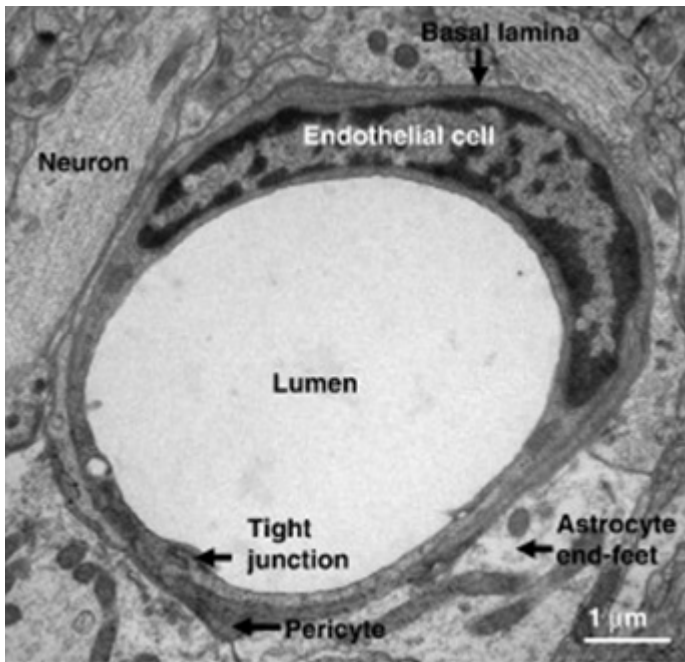
The brain, which constitutes 2% of body mass but consumes 20% of the total energy produced at rest, has a limited capacity to store energy and therefore requires a continuous supply of oxygen and nutrients from the blood stream. Normal brain structure and function integrity thus rely on adequate matching between metabolic needs of neural cells and blood supply ([1], [2], [3], [4]). Normal brain functions depend on the fine regulation of the transport through the blood brain barrier (BBB), the interplay between the different cells that form the functional neurovascular unit (NVU) and the coupling between neurovascular and neurometabolic regulation ([5], [6]). The NVU controls BBB permeability and local cerebral blood flow (CBF), thereby maintaining the chemical composition of the neuronal 'milieu', which is required for proper functioning of neuronal circuits ([7]). Brain functions end within seconds after the interruption of CBF, while irreversible neuronal injury occurs within minutes ([7]). Therefore, local CBF must be very tightly regulated to prevent any unbalance between neuronal activity (NA), oxygen and nutrient delivery, and vessel reactivity. Based on functional and structural observations, it has been proposed that neurons may signal to adjacent blood vessels either directly or through the interposition of glutamate-sensitive glial cells ([8], [9]). This scenario has been further complicated by the recent evidence that CBF may in turn regulate NA, which gives rise to a bidirectional communication between firing neurons and neighbouring vessels ([10], [11]). Moreover, it is now evident that NA does not only control arterial and arteriolar tone, which depends on the contractile status of vascular smooth muscle cells (VSMCs) ([9]). A series of studies have convincingly shown that pericyte-mediated dilation or contraction may alter capillary diameter in response to neuronal activation or silencing ([12], [13], [14]). The control of CBF at capillary level might be indispensable to match the local tissue oxygen supply to local cerebral demand, as their shortest distance ( $\approx 5 \mu\text{m}$ ) from neuronal soma significantly accelerates oxygen and carbon dioxide diffusion as compared to intraparenchymal arterioles ( $\approx 15 \mu\text{m}$ ) ([15]). Surprisingly, it is still unclear whether endothelial signaling actively contributes to NVC ([16], [17], [18]) despite the fact that vascular endothelium has long been known to finely tune blood flow in peripheral [19] and coronary [20] vasculature.

## The neurovascular unit

The concept of neurovascular unit (NVU) was defined by Harder ([21]) as a functional unit where neurons, interneurons and astrocytes 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 CBF regulation ([22], [23]).

In detail, the pial arteries are intracranial vessels on the surface of the brain that give rise to smaller arteries, penetrating arterioles and parenchymal arterioles that go deep into the brain tissues, where they become almost completely surrounded by astrocytic end-feet ([24], [25]). At this level, the vascular basement membrane comes into direct contact with the neurons through the astrocyte-derived glia limitans membrane that forms the outer wall of the perivascular Virchow–Robin space. These arteries branch into smaller arteries and subsequently arterioles, which lose support from the glia limitans and give rise to pre-capillary arterioles and brain capillaries. In an intracerebral artery, the VSMC layer occupies most of the vessel wall. At the brain capillary level, vascular endothelial cells are covered by pericytes, from which they are separated by the basement membrane ([9]). At points of contact, pericytes communicate directly with endothelial cells through the gap junctions and adhesion molecules, such as cadherins and integrins ([26], [27], [28]). These structures not only provide direct contact between these two cell types but also controls vascular tone due to a unique intracellular and extracellular microdomain signaling environment ([12], [13], [14]). Of note, recent evidence has been brought forward to suggest that brain endothelium is a little bit more than an innocent by-stander during NVC ([16], [17], [29], [30]).

Once regarded as an inert barrier between circulation and neuronal tissue, endothelial cells constitute the largest signal transduction platform of the organism ([31]) and, therefore, it is not surprising that they are also essential for the proper functioning of the NVU ([9], [16]). Any alteration in the endothelial capability to detect and decode both physical and humoral stimuli may lead to an astonishing variety of severe diseases ([31]), including Alzheimer's disease ([4], [32]), stroke ([33]), hypertension ([32]) and spinal-cord injury ([34]). In particular, the dynamic interaction between capillary brain endothelial cells and neighbouring cells contributes to their unique characteristics, such as the manifestation of both endothelial and epithelial features ([24], [35], [36], [37]).



**Figure 1.** Electron microscopy (TEM) of rat brain section showing a neurovascular unit. This complex includes microvessel endothelial cells, based on basal lamina, pericytes embedded in basal lamina, astrocytes end-feet and in vicinity some neurons (from Weiss et al.,2008).

## The blood-brain barrier

The blood–brain barrier (BBB) is a highly selective lipophilic barrier that separates the systemic blood circulation from the central nervous system. The BBB maintains the brain homeostasis and provides an optimal chemical environment for cerebral function. These properties are due to the unique characteristic of brain endothelial cells (bECs). Indeed, bECs significantly differ from non-brain ECs by the presence of intracellular tight junctions; a high number of mitochondria, associated with a strong metabolic activity; and the polarized expression of numerous membrane receptors and transporters which are responsible for the active transport of nutrients to the brain or the efflux of potentially toxic compounds from the cerebral to the vascular compartment ([38], [39], [40]). Tight junctions and adherens junctions interconnect bECs and form a highly specialized interendothelial junctional complex that limit paracellular diffusion form blood to brain and vice versa ([24], [41], [42], [43]). Many disease, such as Alzheimer’s disease, 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 ([32], [33], [44]). BBB permeability and CBF are controlled by the concerted action of non-neuronal cells and neurons. The maintenance of the constant chemical composition of the ISF 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 ([45]).

The low paracellular permeability of the BBB is conferred by the presence of tight junctions and adherens junctions that connected the endothelial cells that 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 endothelial cells.

A high energy demand is required for activate ATP-dependent transport, such as the sodium pump ( $\text{Na}^{2+}\text{K}^{+}$  ATPase) and the ATP-binding cassette (ABC) efflux transporters; for these reason endothelial cells are enriched of a high number of mitochondria.  $\text{Na}^{2+}\text{K}^{+}$  ATPase control the sodium influx and potassium efflux across the abluminal side of the BBB. Changes in sodium and potassium levels in the ISF influence the generation of action potentials in neurons and thus directly affect neuronal and synaptic functions.

Disruption to 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. The levels of many tight junction proteins, their adaptor molecules and adherens junction proteins decrease in Alzheimer's disease and other diseases that cause dementia, multiple sclerosis and various animal models of neurological disease. These decreases might be partly explained by the fact that vascular-associated matrix metalloproteinase (MMP) activity rises in many neurodegenerative disorders and after ischaemic CNS injury; tight junction proteins and basement membrane extracellular matrix proteins are substrates for these enzymes. Lowered expression of messenger RNAs that encode several key tight junction proteins, however, has also been reported in some neurodegenerative disorders.



## The neurovascular and neurometabolic coupling

Brain activation is accompanied by a complex sequence of cellular, vascular and metabolic processes that help the brain to maintain an appropriate energy flow to the neural tissue under conditions of increased neuronal activity. Two coupling phenomena can be identified: neurovascular and neurometabolic coupling.

Neurovascular coupling or functional hyperemia refers to the relationship between local NA and changes in vessel diameter and thus in blood flow ([46], [47], [48], [49], [50], [51]). Functional hyperemia is the mechanism by which an increase in NA leads to a local elevation in CBF to adjust oxygen and glucose delivery to the requirements of the activated brain structures.

The mechanism by which an increase in NA activity leads to a corresponding elevation in energy metabolism has been known as neurometabolic coupling ([52], [53], [54], [55]). Glucose is under normal circumstances the main useful energy substrate for the brain energy metabolism ([56]). In the brain, about 90% of glucose is metabolized by oxidative metabolism, which requires oxygen and produces a large amount of ATP, the other 10% is metabolized by glycolysis, which is anaerobic and produces a small amount of ATP. A continuous supply of glucose and oxygen is required and is maintained by changes in CBF in response to variations in neuronal activity ([57], [58], [59], [60]). While it is clear that alterations in NA and metabolism are correlated with changes in CBF, the mechanisms whereby NA control the vascular supply of glucose and oxygen are still a matter of debate.

# The cellular and biochemical pathways of neurovascular coupling

Autoregulation is the mechanism which maintains constant the total blood flow supply to the brain despite for the fluctuations in arterial pressure that occur during normal activities. Autoregulation of cerebral blood flow when pressure fluctuates is most likely due to the myogenic behavior of the cerebral vascular smooth muscle that constrict in response to elevated pressure and *viceversa* ([61], [62]). Locally, blood flow delivery to active brain areas may be finely regulated by neurovascular coupling (NVC) or functional hyperemia. Traditionally, it was thought that local CBF was directly controlled directly by the neuronal demand. In this view, regional blood flow is controlled by feedback mechanisms that are sensitive to variations in the concentrations of metabolic products. This hypothesis suggests that signals related to energy production, such as a rise in carbon dioxide (CO<sub>2</sub>) or adenosine concentration or a drop in glucose or oxygen (O<sub>2</sub>) concentration, may cause an increase in blood flow by triggering vascular smooth muscle cells vasodilation. This model was, however, discarded upon the discovery that regional CBF always overwhelms the local O<sub>2</sub> and glucose requirements; furthermore, the increase in CBF may persist even in the presence of excess O<sub>2</sub> and glucose ([4]). An alternative possibility is that a feedforward mechanism controls the vascular energy supply in response to NA; neurons either signal directly to blood vessels or activate astrocytes to release vasoactive agents onto the vessels. For both of these signaling routes, the coupling mechanisms involve neurotransmitter, particularly glutamate ([63], [64]).

**The arteriolar component** - Synaptic release of glutamate activates neuronal NMDA receptors, thereby resulting in Ca<sup>2+</sup> entry and activation of neuronal nitric oxide synthase (nNOS). The following NO release is able to directly dilate brain arterioles both in brain slices and *in vivo* ([65]). Glial cells are well suited to mediate the vascular effect of glutamate release because of their close contacts with both neurons and blood vessels ([46], [66], [67]). Accordingly, glial cells respond with an increase in intracellular Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>i</sub>) to synaptically released glutamate ([68], [69]), which lead to the Ca<sup>2+</sup>-dependent generation of a number of vasoactive messengers. These include the arachidonic acid metabolites, epoxyeicosatrienoic acids (EETs), which are vasodilating, 20-hydroxyeicosatetraenoic acid (20-HETE), which is vasoconstricting, and prostaglandins, which can be either vasodilating or constricting depending on the target receptor ([70], [71]). In more detail, for instance, glutamate binds to metabotropic glutamate receptor 1

(mGluR1) to stimulate phospholipase C (PLC) and induce an inositol-1,4,5-trisphosphate (InsP<sub>3</sub>)-dependent intracellular Ca<sup>2+</sup> waves which can propagate into the astrocyte endfeet. Glutamate-induced Ca<sup>2+</sup> signals, in turn, recruit the Ca<sup>2+</sup>-dependent effector, phospholipase A2 (PLA2), which releases AA from plasma membrane phospholipids ([8]). Cyclooxygenase (COX) enzymes can then metabolize AA to vasoactive prostaglandins, such as prostaglandin E2 (PGE2), and epoxyeicosatrienoic 21 acids (EETs), which cause vasodilation. Moreover, as shown in rat cerebral arterial microsomes and *ex vivo* brain slices, astrocyte may convert PLA2-derived AA into the vasoconstricting, 20-hydroxyeicosatetraenoic acid (20-HETE), by membrane-bound cytochrome P450 4A, thereby causing local vasoconstriction ([72], [73]).

**The capillary component** - A novel finding in the field has been the discovery that, besides parenchymal arterioles, brain capillaries may also undergo significant changes in their diameter in response to local NA. Accordingly, Attwell's group reported the evidence that pericytes — which are contractile cells present at ~50-µm intervals along capillaries — can markedly alter capillary diameter upon stimulation of glutamatergic synapses. Pericytes express contractile proteins, and their location on capillaries led to the suggestion that they could constrict the microvasculature ([74]). This idea was reinforced with observations that substances that alter arteriole diameter, including arachidonic acid (AA) derivatives and neurotransmitters, can contract and relax pericytes cultured on rubber membranes ([75]). More recently, it has been shown that pericytes constrict in response to noradrenaline and dilate in response to glutamate in brain slices ([76], [13]), thereby demonstrating that endogenous transmitter release can regulate capillary diameter ([76]). Regulation of CBF at the capillary level has yet to be demonstrated *in vivo* in physiological conditions ([74]), although it does occur after ischaemia ([77]). Signals for contraction and perhaps for dilation can propagate from one pericyte to another ([76], [78]). This signal spread may occur through gap junctions between the interdigitating processes of the pericytes themselves, or through gap junctions with ECs. Because active neurons are, on average, closer to pericytes than to arterioles (8–23 µm away versus 70–160 µm), this raises the theoretical possibility that vascular responses to changes in neuronal activity may be initiated by pericytes and then propagated to upstream arterioles ([79]).

**The endothelial enigma** - In addition to these direct and direct interactions between active neurons and brain microvessels, recent studies have discovered that significant vasoactive signals may be directly generated by the endothelium itself, adding a new complex level of interaction in the NVU. ECs release vasodilating (e.g., NO, endothelium-derived hyperpolarizing factor, or EDHF,

prostanoids) and vasoconstricting (e.g., endothelin, TXA<sub>2</sub>, prostaglandin) substances ([18]) in response to either chemical or mechanical stimulation ([31]). It has been shown that Ach stimulates vasodilation in adult mouse middle cerebral arteries by activating Transient Receptor Potential Vanilloid 4 (TRPV4) channels, which leads to Ca<sup>2+</sup> entry and endothelium-dependent EDHF-mediated dilation ([80]), whereas glutamate may stimulate NMDA receptors to locally increase cerebral blood flow in an endothelial and NO-dependent manner ([30]). Moreover, endothelial reactive oxygen species (ROS) act as vasodilators at low concentrations, but at high concentrations they can cause vasoconstriction ([81], [82]). However, the role played by brain microvascular ECs in NVC has been rather neglected ([16]). It is, indeed, still unclear whether and how brain microvascular ECs directly sense neuronal activity by responding to synaptically-released neurotransmitters with the production of vasoactive agents. As mentioned earlier, the best characterized interaction is that described between Ach and brain endothelium. For instance, basal forebrain neurons increase cortical blood flow by releasing Ach, which binds to endothelial muscarinic receptors (mAChRs), thereby leading to NO production and microvessel vasodilation ([83], [84], [85]). In other vascular beds ([86], [87]), Ach stimulates NO production by initiating an oscillatory increase in [Ca<sup>2+</sup>]<sub>i</sub>, which is patterned by the interaction between InsP<sub>3</sub> receptors (InsP<sub>3</sub>Rs) and the so-called store-operated Ca<sup>2+</sup> entry (SOCE). However, the waveform of Ach-induced Ca<sup>2+</sup> signaling in brain microvascular endothelial cells and its relationship with NO release are still unclear. Likewise, recent studies revealed that glutamate may stimulate NMDARs in the presence of the co-agonist d-serine to engage in the eNOS (, [29]), but it remains to be elucidated whether mGluRs are expressed and promote NO release in brain microvascular endothelial cells.

## The neurotransmitter Acetylcholine

Acetylcholine (ACh) is one of the major excitatory neurotransmitters in both the central and peripheral nervous system of CBF in man and in many other species (for a review see [88]). ACh was the first neurotransmitter discovered. The concentrations of ACh in the extracellular fluid range from 0.1 to 6 nM ([89]), while in the brain range from 0.01 to 0.5 nM [90]. ACh is also known to be a powerful dilator of most blood vessels, including cerebral arteries and microvessels ([91], [92]). Stimulation of basal forebrain neurons causes regional increases in CBF which are particularly pronounced in the neocortex and hippocampus ([93], [94]). As anticipated above, basal forebrain cholinergic fibers project to intraparenchymal microvessels, thereby establishing a neurovascular interaction that does not require the interposition of local interneurons and directly controls cortical microcirculation ([93], [94]). Accordingly, ACh released by post-synaptic terminals binds to endothelial muscarinic receptors (mAChRs), thereby leading to NO production and microvessel vasodilation ([83], [94], [85]).

The ACh biosynthesis involves acetyl coenzyme A (Aco-A), choline and ACh biosynthetic enzyme choline acetyltransferase. Primarily, during ACh biosynthesis, choline is taken up from the extracellular space by a high affinity  $\text{Na}^+$ -dependent uptake system present in cholinergic neurons ([95]). Similar to common neurotransmitters, ACh is biosynthesized in the cytosol of nerve terminals and is then stored into synaptic vesicles prior to release by exocytosis ([96]). In response to an action potential, it is released by exocytosis into the synaptic space, from which it diffuses to the postsynaptic site for interaction with appropriate receptors, which results in specific effects according to the receptor type (nicotinic or muscarinic). These receptors are functionally different, the muscarinic type (muscarinic ACh receptor - mAChR) being G-protein coupled receptors (GPCRs) that mediate a slow metabotropic response via second messenger cascades, while the nicotinic type (nicotinic ACh receptor – nAChR) are ligand-gated ion channels that mediate a fast synaptic transmission of the neurotransmitter ([97]).

Muscarinic receptors are involved in a large number of physiological functions, including modulation of heart rate and force, vasorelaxation, and neurotransmitter release. There are five mAChR subtypes which have been distinguished based on pharmacological activity (M1-M5 AChRs) and are all found in the central nervous system (CNS). However, M1-M4 AChRs are also present outside the CNS. M1, M3 and M5 receptors cause the activation of phospholipase C, generating two secondary messengers ( $\text{IP}_3$  and DAG) eventually leading to an increase of intracellular calcium concentration, while M2 and M4 inhibit adenylate cyclase, thereby decreasing the production of

the second messenger cAMP ([97]). M5-AchR is the most abundant endothelial isoform in brain microvessel and mediate Ach-induced vasodilation by activating NO release in a  $\text{Ca}^{2+}$ -dependent manner ([83]). Yet, the signaling mechanisms that couple Ach to the  $\text{Ca}^{2+}$ -dependent activation of the eNOS remain elusive.

## The neurotransmitter Glutamate

The excitatory neurotransmitter glutamate (Glu) is a vasodilator in cerebral circulation *in vivo* ([98], [99], [100], [101], [102], [103]). The glutamatergic synapses *are found throughout the brain and spinal cord in neurons and glia*. Glu increases CBF by activating postsynaptic neurons and pery-synaptic glial cells; as mentioned earlier, Glu can also activate the pericytes that wrap around brain capillary endothelial cells ([13], [14]). Glu concentration in the plasma range between 50-100  $\mu\text{mol/L}$  in human and in other species ([104]), while in the brain it falls to 0.5-2  $\mu\text{mol/L}$  due to BBB activity ([105]). However, during synaptic transmission, Glu concentration within the synaptic cleft may rapidly reach 160-190  $\mu\text{mol/L}$  ([106]), Glu concentration inside the synaptic vesicles being around 60 mM ([107]); Glu can be synthesized by transamination of 2-oxoglutarate, an intermediate of the Krebs cycle ([108]) or by the mitochondrial enzyme glutaminase from the precursor glutamine. Glu act by binding to and activating an array of Glu receptors (GluRs). GluRs in the central nervous system are divided into the ionotropic GluRs, which are ligand-gated ion channel receptors, and metabotropic GluRs, which are associated with G proteins ([109]). Ionotropic GluRs are in turn classified into three groups based on their pharmacology and structural properties. N-methyl-D-aspartate (NMDA)-type receptors are heteromeric complexes comprised of NR1 subunit combined with one or more NR2 subunits ([110]). In the brain, NMDA receptors were detected in cerebral cortex and most other structures. Non-NMDA ionotropic GluRs include AMPA (GluR1–GluR4) and kainate receptors (GluR5–GluR7) that have similar pharmacologic properties. Multiple metabotropic GluRs (mGluR1–mGluR5) that are coupled to diverse second messenger systems, including phosphoinositides, phospholipase D, and cyclic AMP (cAMP), are also widely distributed in the brain ([111]). Glu-mediated signaling leads to an increase in  $[\text{Ca}^{2+}]_i$  in the target cells which is mediated either by the  $\text{Ca}^{2+}$ -permeable NMDA receptors or by the Gq-coupled mGluR1 and mGluR5, which activates the  $\text{Ca}^{2+}$ /Calmodulin-dependent neuronal nitric oxide synthase (NOS) to release NO ([100], [101], [103], [112], [113]). Glu-induced NO release, in turn, causes vasodilation in both cerebellar ([13, 114]) and hippocampal microvasculature ([115]), while it plays a permissive role in AA metabolites-induced

vasodilation in the cortex by suppressing 20-HETE synthesis ([8]). Furthermore, the relative importance of the different Glu-released messengers (i.e. release of NO from neurons and release of arachidonic acid derivatives from astrocytes) varies between brain regions. Actually, it is unclear whether brain endothelial cells also sense Glu through an elevation in  $[Ca^{2+}]_i$  and NO production. As mentioned above, recent publications have showed that NMDA receptor coactivation by glutamate and D-serine increases lumen diameter in pressurized mouse middle cerebral arterioles in an endothelial and eNOS-dependent mechanism ([30]).

## Nitric Oxide

Nitric oxide (NO) is the signaling molecule originally identified as endothelium-derived relaxing factor mediating relaxation of blood vessels ([91]). It is a small, highly diffusible, and reactive molecule with a short lifetime that is generated by NO synthase (NOS) through enzymatic conversion of L-arginine to L-citrulline ([116]). Three NOS genes with distinct tissue localization and properties are known: neuronal (nNOS), inducible (iNOS) and endothelial (eNOS). Activation of eNOS and nNOS is classically  $Ca^{2+}$ /calmodulin dependent, with nNOS being closely coupled to  $Ca^{2+}$ -permeable NMDA receptors at the postsynaptic neurons ([117]), and eNOS tethered to Orai1 in vascular endothelial cells ([118], [119]). NO decreases vascular tone by stimulating cGMP synthesis, thereby leading to the subsequent activation of cGMP-dependent protein kinase (PKG). VSMC contraction is driven by an increase in  $[Ca^{2+}]_i$ , which recruits the  $Ca^{2+}$ /calmodulin-dependent myosin light chain kinase (MLCK). MLCK, in turn, phosphorylates the two light chains of myosin, thus allowing the sliding of these filaments along those of actin and inducing VSMC contraction. The contractile state can also be favored by the action of the RhoA protein, capable of phosphorylate the lateral myosin chain (MLC) and the lateral chain phosphatase of myosin (MLCP) by inactivating it. NO acts by increasing the activity of a protein, termed soluble guanylated cyclase (sGC), which catalyzes the transformation reaction of GTP (Guanosine-5'-triphosphate) into cGMP (guanosine cyclic monophosphate), an intracellular second messenger inside the cells that is capable of activating PKG. PKG phosphorylates its target proteins, thereby inhibiting voltage-gated the  $Ca^{2+}$  entry and activating  $Ca^{2+}$ -dependents  $K^+$  channels, which cooperate to hyperpolarize the plasma membrane and terminate the contraction. Moreover, PKG may also block  $Ca^{2+}$  release from sarcoplasmic reticulum (SR) and the RhoA protein-mediated contraction. In addition to inhibiting VSMC contraction, NO may activate the PGE2 receptor to suppress 20-HETE synthesis and cause arteriolar dilation ([12]). Therefore, if the main cause of the blood flow increase is AA

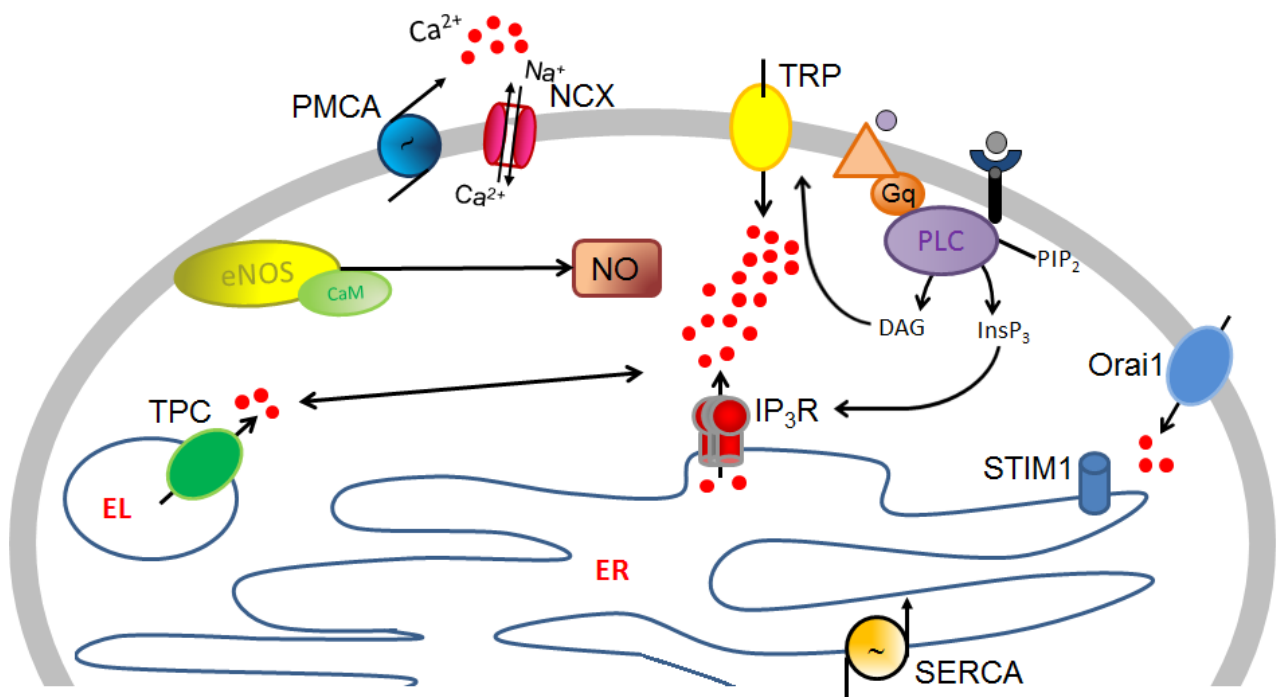
generation by astrocytes, then having NO present to inhibit 20-HETE formation will ensure that only the vasodilatory prostaglandin and EET derivatives of AA will affect arteriole diameter. More in general, it has been proposed that NO induces vasodilation in both cerebellar [13, 114] and hippocampal microvasculature [115], while it plays a permissive role in arachidonic acid (AA) metabolites-induced vasodilation in the cortex by suppressing the synthesis of the vasoconstricting 20-hydroxy-eicosatetraenoic acid (20-HETE) [8]. It should be pointed out that, according to the general agreement, the main NOS isoform responsible for modulating CBF is nNOS. However, as mentioned earlier, eNOS drives arteriolar dilation during cholinergic transmission and has been recently linked also to glutamatergic signalling. In addition, many experiments indicate that eNOS activation can influence synaptic plasticity in the cortex and striatum ([120], [121]). Current literature also strongly supports the major role of eNOS in modulation of synaptic function in the hippocampus which is the first and most severely affected brain region in the pathogenesis of Alzheimer's disease ([122]). Indeed, studies conducted in mice that are doubly mutant in eNOS and nNOS demonstrated that long-term potentiation is dependent on phasic release of NO caused by nNOS activation, as well as tonic release of NO caused by eNOS activity ([123]). This conclusion was confirmed by the results generated by pharmacological and genetic inactivation of eNOS ([124]). Moreover, prior studies indicate that endothelium-derived NO can travel up to 100 mm distance from endothelial cells ([125], [126]). The distance between brain capillaries and neuronal cells is around 40  $\mu\text{m}$  well within the range of NO diffusion. Consistent with these observations, studies in experimental animals (reviewed by [127], [128]) support the concept that intact NO/cGMP signaling is an essential mechanism required for memory formation. Altogether, these results underline a key role of endothelial NO produced by brain endothelial cell in synaptic plasticity and memory formation; therefore, it is becoming apparent that the role of endothelial NO in the control of central nervous system function is very complex.



## The calcium toolkit in vascular endothelial cells

It has long been known that an increase in  $[Ca^{2+}]_i$  plays a key role in the intricate network of signal transduction pathways exploited by vascular ECs to regulate most of the cellular processes ([129], [130]). Due to its strategic location at the interface between vascular wall and blood stream, the endothelium is exposed to a myriad of transmitters (release by automatic and sensory nerves or platelets), circulating hormones, autacoids, cytokines, growth factors and drugs, as well as to mechanical stimuli, such as pulsatile stretch, shear stress and changes in the local osmotic pressure ([131], [132]). An extensive array of proteins, membrane receptors, transporters and ion channels, which are located both on the plasmalemma and within the intracellular  $Ca^{2+}$  reservoirs (namely, endoplasmic reticulum, lysosomes and Golgi), may be utilized by ECs to selectively detect and properly react to the incoming stimulus ([31], [133]). The spatio-temporal profile of the ensuing  $Ca^{2+}$  signal further contributes to enable ECs to select the most suitable response to extracellular inputs, i.e. production of vasoactive mediators (NO, PGI<sub>2</sub>, H<sub>2</sub>S, EDHF and EDCF), biosynthesis of von Willebrand factor and tissue plasminogen activator, control of intercellular permeability, ICAM-1 expression and NF- $\kappa$ B activation, cell proliferation, angiogenesis and wound repair ([129], [134], [135], [136], [137], [138], [139], [140], [141]).

In the absence of extracellular stimulation, the  $[Ca^{2+}]_i$  is very low (100 nM) due to the combined activity of two high affinity/low capacity  $Ca^{2+}$  transporters, that extrude  $Ca^{2+}$  out the cytosol ([142]). More specifically, the plasma membrane  $Ca^{2+}$ -ATPase (PMCA) and the sarco-endoplasmic reticulum  $Ca^{2+}$ -ATPase (SERCA) remove cytosolic  $Ca^{2+}$  by direct ATP hydrolysis, while the Na<sup>+</sup>/  $Ca^{2+}$  exchanger (NCX) clears intracellular  $Ca^{2+}$  by exploiting the Na<sup>+</sup> gradient across PM ([142], [143]). The increase in  $[Ca^{2+}]_i$  is caused by the opening of  $Ca^{2+}$ -permeable channels located both in the PM and in the endoplasmic reticulum (ER), the main intracellular  $Ca^{2+}$  reservoir, the channels are activated by extracellular chemical and physical stimuli and permit the passive passage of the ions that are permeable to. The  $Ca^{2+}$  concentration in the extracellular medium and in the ER lumen is very high (in the range of mM and  $\mu$ M, respectively) when compared to cytoplasm concentration. The entry of  $Ca^{2+}$  from outside and its release from ER lumen represent the main ways to increase  $[Ca^{2+}]_i$  in all cell types. Endothelial cells are considered electrically non-excitable as they do not have voltage-dependent  $Ca^{2+}$  channels which are typical in neurons and muscle, skeleton and cardiac fibres. The most common  $Ca^{2+}$  entry pathway from the extracellular space of those cells is the so-called store-operated calcium entry (SOCE), which is activated following depletion of the ER  $Ca^{2+}$  pool ([144], [145], [146], [128]).



**Figure 2.** Overview of the basic elements of the  $\text{Ca}^{2+}$  signaling toolkit in endothelial cells.

An increase in  $[\text{Ca}^{2+}]_i$  is the physiological response to a variety of extracellular chemical and physical stimuli that bind to their specific receptors. The following activation of PLC cleaves  $\text{PIP}_2$  in  $\text{InsP}_3$  and DAG.  $\text{InsP}_3$ , in turn, releases  $\text{Ca}^{2+}$  from the ER binding  $\text{InsP}_3\text{Rs}$ , while DAG may induce  $\text{Ca}^{2+}$  entry by gating some of TRP channels. The  $\text{InsP}_3$ -dependent  $\text{Ca}^{2+}$  release from ER causes the translocation and clustering of Stim1 with Orai1, an alternative  $\text{Ca}^{2+}$ -permeable route on the PM that obliged to the process of CICR. Another way to increase the  $[\text{Ca}^{2+}]_i$  is due to the depletion of EL reservoir, though the NAADP-dependent opening of TPC.  $\text{Ca}^{2+}$  signals return to pre-stimulated levels through the concerted action of the mitochondrial uniporter, SERCA and PMCA pumps, as well as through NCX. The signalling function of  $\text{Ca}^{2+}$  is carried out by a number of  $\text{Ca}^{2+}$ -sensitive decoders (eNOS, calmodulin, NO) and downstream targets.

ER, endoplasmic reticulum; EL endo-lysosomal system; Gq, Gprotein coupled receptor; PLC, phospholipase C;  $\text{PIP}_2$ , phosphatidylinositol-4,5-bisphosphate;  $\text{InsP}_3$ , inositol-1,4,5-trisphosphate; DAG, diacylglycerol;  $\text{InsP}_3\text{R}$ ,  $\text{InsP}_3$  receptors; TPC, two pore channels, NCX,  $\text{Na}^+-\text{Ca}^{2+}$  exchanger; PMCA, plasma membrane  $\text{Ca}^{2+}$  ATPase; SERCA, Sarco-Endoplasmic Reticulum  $\text{Ca}^{2+}$ -ATPase; NO, nitric oxide; CaM calmodulin; eNOS endothelial NO synthase.

An increase in  $[\text{Ca}^{2+}]_i$  up to  $1 \mu\text{M}$  is the key signal to activate vascular ECs following recruitment of either tyrosine-kinase linked receptors (TRKs) or G-protein coupled receptors (GPCRs) by growth factors and vasoactive agents, respectively ([137], [139], [132], [138], [140]). More specifically, the  $\beta$  isoform of PLC ( $\text{PLC}\beta$ ) is engaged by GPCRs, while TKRs recruit  $\text{PLC}\gamma$  ( $\text{PLC}\gamma$ ) ([142], [31]).  $\text{PLC}\beta$  and  $\text{PLC}\gamma$  are part of a larger family of PLC isoforms that additionally includes  $\text{PLC}\delta$ ,  $\text{PLC}\epsilon$ , and  $\text{PLC}\zeta$ .  $\text{PLC}\epsilon$  is proposed to be activated by small GTPases like Rho and Ras whereas  $\text{PLC}\delta$  is activated by  $[\text{Ca}^{2+}]_i$  in the range of  $0.1\text{--}10 \text{ mM}$  ([147], [148]). PLC, in turn, cleaves phosphatidylinositol-4,5-bisphosphate ( $\text{PIP}_2$ ) into  $\text{InsP}_3$  and diacylglycerol (DAG). DAG remains bound to the PM, where it

recruit both protein kinase C (PKC) and some  $\text{Ca}^{2+}$ -permeable, nonselective cation channels belonging to the canonical transient receptor potential (TRPC) family of ion channels ([142], [149]). The  $\text{InsP}_3\text{Rs}$  represent the most important channels for the release of  $\text{Ca}^{2+}$  from the ER. It is well known that at least three isoforms of  $\text{InsP}_3\text{Rs}$  exist both in animal and human cells ([150]). ECs, in particular, express the subtypes 1 and 3 ( $\text{InsP}_3\text{R1}$  and  $\text{InsP}_3\text{R3}$ ). From the structural point of view,  $\text{InsP}_3\text{Rs}$  are tetramers made up by four subunits, each constituted by approximately 2700 aminoacidic residues and containing a binding site for  $\text{InsP}_3$ . The  $\text{InsP}_3$ -mediated  $\text{Ca}^{2+}$  release is a cooperative process, since it requires the intervention of all subunits for the channel to open ([151]). A characteristic of  $\text{InsP}_3\text{Rs}$  is that they are differentially regulated by  $\text{InsP}_3$ ,  $\text{Ca}^{2+}$  and ATP. The  $\text{Ca}^{2+}$  dependence of  $\text{InsP}_3\text{Rs}$  is biphasic. Nanomolar doses of  $\text{Ca}^{2+}$  activate the channel, while concentrations in the range of hundreds of  $\mu\text{M}$  inhibit  $\text{InsP}_3\text{R}$  activity.  $\text{InsP}_3\text{Rs}$  are associated to various proteins involved in decoding  $\text{Ca}^{2+}$  signals; for instance, calmoduline (CaM) plays an important role in  $\text{InsP}_3$  channels regulation. Similarly, the cytosolic site of the receptor-channels presents consensus sequences for numerous protein kinases, including protein kinase C (PKC), protein kinase A (PKA), and calmodulin-dependent protein kinase II (CaMKII) ([152]). Finally, endothelial  $\text{InsP}_3\text{Rs}$  are mainly distributed in the perinuclear region: the recruitment of a  $\text{InsP}_3\text{Rs}$  cluster generates a localized  $\text{Ca}^{2+}$  signal (termed “ $\text{Ca}^{2+}$  puff”) which is then transformed in a global  $\text{Ca}^{2+}$  wave by the opening of the adjacent receptors following diffusion of  $\text{InsP}_3$  and  $\text{Ca}^{2+}$  itself. This mechanism has been given the name in  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release (CICR).

Intracellular  $\text{Ca}^{2+}$  pools may be also recruited by ryanodine receptors (RyRs), which are stimulated by  $\text{Ca}^{2+}$  itself and modulated by the cyclic ADP-ribose (cADPr). RyRs have been found in different tissues, in particular in striated muscles. Three isoforms have been identified: RyR1, RyR2 and RyR3 which are typical of the skeletal muscles, cardiac tissue and striated muscle respectively. The three isoforms are assembled as homo-tetrameric channels consisting of four 560 kD polypeptides [153]. At the subunit level, each RyR subunit presents with a very large cytosolic  $\text{NH}_2$  domain followed by a central regulatory domain that recognized and binds to several regulatory proteins such as CaM, calstabin, sorcin and presents binding sites for cytosolic ions and modulators, including  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and adenine nucleotides [154]. RyRs may also be modulated PKA- and CaMKII-mediated phosphorylation, may undergo oxidative/nitrosative modifications and undergo pH modulation ([155], [31]).  $\text{Ca}^{2+}$  release from RyRs in ECs, as well as in both excitable and non-excitable cells ([155]), is triggered by CICR ([156]), that occurs 1-10  $\mu\text{M}$   $\text{Ca}^{2+}$  nearby the receptor and is prevented when  $\text{Ca}^{2+}$  is 1-10 mM ([155]). RyRs may be pharmacologically activated by the

methylxanthine derivate, caffeine, which sensitizes the receptor to resting  $\text{Ca}^{2+}$  levels, and by ryanodine, which binds to and locks the receptor in an open state ([157]).

Along with the  $\text{Ca}^{2+}$  release through  $\text{InsP}_3\text{Rs}$  and/or  $\text{RyRs}$ , luminal ER  $\text{Ca}^{2+}$  can be spontaneously released through other mechanisms that have not yet been identified. Indeed, under resting conditions, a continuous leakage of  $\text{Ca}^{2+}$  from RE occurs. This leakage is generally resequenced back into ER lumen by SERCA pumps. As a consequence, the blockade of SERCA activity, which can be accomplished by specific inhibitors such as thapsigargin and cyclopiazonic acid (CPA), results in the slow accumulation of  $\text{Ca}^{2+}$  in the cytosol. The mechanism which is responsible for  $\text{Ca}^{2+}$  leakage from ER is still subject of study, although a number of proteins have been involved, including translocons, pannosins, TRPM8 channels, Orai3 channels,  $\text{InsP}_3\text{Rs}$  and  $\text{RyRs}$ , the latter two channels being constitutively gated by the basal levels of  $\text{InsP}_3$  and  $\text{Ca}^{2+}$ .

The latest addition to the family of intracellular  $\text{Ca}^{2+}$  channels is constituted by two pore channels (TPCs), which sit on the membrane of the endo-lysosomal (EL) system ([158], [159]). TPCs have been regulated by the nicotinic acid adenine dinucleotide phosphate (NAADP) ([160], [161], [162]). NAADP is generated from nicotinamide adenine dinucleotide phosphate (NADP) by CD38 in a base-exchange reaction occurring at acidic pH, targets acidic organelles and mobilizes  $\text{Ca}^{2+}$  from the EL  $\text{Ca}^{2+}$  storage compartment.  $\text{Ca}^{2+}$  is sequestered into EL by a putative  $\text{Ca}^{2+}/\text{H}^+$  exchanger, which is driven by a vacuolar- $\text{H}^+$  ATPase ([158]). TPC1 is expressed on both endosomes and lysosomes, while TPC2 is exclusively present on lysosomes ([163]). Due to the limited amount of  $\text{Ca}^{2+}$  in the endolysosomal compartment, which consists in small vesicles (0.2-1  $\mu\text{m}$  in diameter) trafficking throughout the cytosol, NAADP-induced  $\text{Ca}^{2+}$  signals *per se* appear as scattered, discrete events ([160], [163]). The relatively small quantity of  $\text{Ca}^{2+}$  discharged by EL may, however, be amplified into a global  $\text{Ca}^{2+}$  wave by recruitment of  $\text{InsP}_3\text{Rs}$  and  $\text{RyRs}$  via CICR ([164], [161]).

Emptying of ER  $\text{Ca}^{2+}$  stores through the process of CICR leads to the opening of the so-called "store-operated" channels (SOCs), which are located on the PM and mediate  $\text{Ca}^{2+}$  responsible for store refilling via SERCA ([142], [165]). The best characterized SOC is the  $\text{Ca}^{2+}$  release-activated  $\text{Ca}^{2+}$  (CRAC) channel, which was first identified in lymphocytes, mast cells and other immune cells ([166]), but has since identified in many other cell types, including ECs ([167], [168]) and mouse neuronal progenitor cells ([169]). CRAC channels are activated by  $\text{InsP}_3$ -dependent store depletion due to the physical coupling between the ER  $\text{Ca}^{2+}$ -sensor, Stromal Interaction Molecule-1 (Stim1) and the channel protein, Orai1, on the PM ([170], [171], [145], [146], [172]). Stim1 is a single-pass transmembrane protein endowed with a  $\text{Ca}^{2+}$ -binding EF domain on the amino (N)-terminal ER

luminal portion ([173]). When ER  $\text{Ca}^{2+}$  concentration falls below a threshold level due to  $\text{InsP}_3$ -dependent  $\text{Ca}^{2+}$  release,  $\text{Ca}^{2+}$  dissociates from EF1, Stim1 proteins rapidly redistribute to peripheral ER sites in close proximity to PM, where they multimerized ([173], [174]). Orai1, in turn, is a 33 kDa PM protein with a tetraspanning PM topology and cytosolic  $\text{NH}_2$ - and  $\text{COOH}$ - tails ([173], [174]). Orai1 serves as the pore-forming subunit of CRAC channels: it exists as a dimer in un-stimulated cells, but forms tetramers after the  $\text{InsP}_3$ -dependent store emptying ([173], [174]). Stim1 and Orai1-mediated  $\text{Ca}^{2+}$  entry is gated a current displaying biophysical features similar to those of the  $\text{Ca}^{2+}$  release activated  $\text{Ca}^{2+}$  (CRAC) current recorded in hematopoietic cells, i.e. strong inward rectification, reversal potential ( $E_{\text{rev}}$ )  $>+60$  mV, permeability to  $\text{Ca}^{2+}$ , but not to  $\text{Na}^+$  and  $\text{K}^+$  under physiological conditions, and a single-channel conductance in the order of fS ([144], [175]). This current is characterized by its high selectivity for  $\text{Ca}^{2+}$  ions, an inwardly rectifying current-voltage relationship, and an extremely small single channel conductance in the sub-picoSiemens range ([176], [177]). Other CRAC-related proteins are Stim2, Orai2, and Orai3, which may be involved in store-dependent  $\text{Ca}^{2+}$  influx in heterologous expression systems, although their contribution to SOCs in naïve cells is yet to be fully appreciated. Nevertheless, Orai2 mediates the  $I_{\text{CRAC}}$  in mouse neurons ([178]), whereas Orai3 is one of the main candidates to mediate SOCE in cancer cells ([179]).

An alternative pathway for endothelial SOCE has been reported in a number of vascular beds, as Stim1 may recruit additional  $\text{Ca}^{2+}$ -permeable pathways, which include some members of the Canonical Transient Receptor Potential (TRPC) sub-family of cationic channels, such as TRPC1 and TRPC4 ([180]). The formation of a supermolecular complex involving Stim1, Orai1, TRPC1 and TRPC4 has indeed been reported ([181]). Moreover,  $\text{Ca}^{2+}$  entry may occur through DAG-sensitive channels, such as TRPC3 and TRPC6 ([182], [183]). Of note, DAG itself may be converted by DAG lipase into AA, which may be further metabolized to EETs to activate  $\text{Ca}^{2+}$  influx through TRPV4 channels ([184]). Alternately, extracellular autacoids may recruit phospholipase A2 to cleave off AA from membrane-bound fatty acids ([185]). An additional mode for agonists-induced  $\text{Ca}^{2+}$  entry in vascular ECs is provided by ionotropic receptors, such as nicotinic receptors (nAChRs), ATP-sensitive P2X receptors, and cyclic nucleotide gated channels ([186]). Finally, mechanical stimuli (e.g. pulsatile stretch, laminar shear stress, and changes in the local osmotic pressure) may promote  $\text{Ca}^{2+}$  entry through a number of mechano-sensitive channels, including polycystin TRP 2 (TRPP2) ([187]), TRPV4([184], Piezo1 [188]) and heteromeric TRPC1-TRPP2 ([189]).

## Calcium signals in brain endothelial cells

Calcium ions play an important role in the control of BBB permeability. Every time that the extracellular  $\text{Ca}^{2+}$  concentration is decrease under the normal condition around 1.5 mM at the blood side and 1 mM at the brain interstitial side ([190]) and/or when the intracellular free  $\text{Ca}^{2+}$  concentration rise up the normally 50–100 nM ([191], [192]), normal BBB function may be modified or disturbed. Decreased extracellular  $\text{Ca}^{2+}$  levels result in a disruption of cell–cell and cell–matrix adhesive interactions ([193]), but also give rise to intracellular  $\text{Ca}^{2+}$  signaling pathways ([194]). Less is known about the vasoactive outcome of endothelial  $\text{Ca}^{2+}$  signals inside the cerebral circle. The  $[\text{Ca}^{2+}]_i$  changes in brain endothelial cells can occurs in many different way, that are highly organized in time, localization and duration: usually the increase of  $[\text{Ca}^{2+}]_i$  may appear as a localized elementary blips or puffs that result from the activation of single or clustered  $\text{InsP}_3\text{Rs}$ , that will eventually merge to form a global intracellular  $\text{Ca}^{2+}$  wave. These waves are not restricted to the cytoplasm of one cell but may pass through the cells and spread as an intercellular  $\text{Ca}^{2+}$  wave that is defined as a propagating  $[\text{Ca}^{2+}]_i$  increase that originates in a single cell and sequentially engages neighboring cells ([195]). Often, in brain endothelial cells, chemical stimulation results in a series of high frequency  $\text{Ca}^{2+}$  oscillations, which are supported by  $\text{Ca}^{2+}$  mobilization from the intracellular store and  $\text{Ca}^{2+}$  entry from the extracellular space. The variations in  $\text{Ca}^{2+}$  spike duration, frequency and amplitude, combined with different mechanism that sustain  $\text{Ca}^{2+}$  oscillations, induced the activation of different  $\text{Ca}^{2+}$ -dependent pathways that achieve distinct response. Additionally, a continued oscillation pattern is important to avoid the possible toxic effect of a prolonged increase of the  $[\text{Ca}^{2+}]_i$  and in the meantime may exert temporal control over cellular functions ([196]).

It has long been appreciated that  $\text{Ca}^{2+}$  oscillations result from a coordinated release of intracellular stores and increased  $\text{Ca}^{2+}$  influx across the plasma membrane ([197], [198]). The intracellular release of  $\text{Ca}^{2+}$  most commonly results from  $\text{InsP}_3$ -dependent  $\text{Ca}^{2+}$  release, whereas  $\text{Ca}^{2+}$  entry is mediated by SOCE ([199], [200]). Type 2  $\text{InsP}_3\text{R}$  ( $\text{InsP}_3\text{R}2$ ), which shows the sharpest dependence on cytosolic  $\text{Ca}^{2+}$  and has the highest affinity for  $\text{InsP}_3$ , is the main oscillatory unit as shown in DT40 cells ([201]) and myocytes ([202]). Stimulation of type 1 receptor ( $\text{InsP}_3\text{R}1$ ) can also lead to oscillations, but most often damped rather than sustained ([203], [201]). In contrast, type 3 receptor ( $\text{InsP}_3\text{R}3$ ) tends to suppress oscillations. This surprising result is because  $\text{InsP}_3\text{R}3$ , which is not inhibited by  $\text{Ca}^{2+}$ , provides a constant flux of  $\text{Ca}^{2+}$  without providing the feedback necessary for oscillations to occur ([196]).

With regard to BBB endothelial cells, intercellular  $\text{Ca}^{2+}$  waves have been reported in response to mechanical cell stimulation or photoliberation of caged- $\text{InsP}_3$  in primary rat brain capillary ECs or immortalized cells from the same origin (GP8/3.9 and RBE4) ([204], [205]). In bEnd3 mouse BBB ECs, intercellular  $\text{Ca}^{2+}$  waves were triggered by the production of a localized oxidative insult upon illumination of a photosensitizer probe ([206]). Inter-endothelial  $\text{Ca}^{2+}$  waves propagating in arterioles are believed to strengthen a conducted vasodilation ([207], [208]) while also being involved in hypoxic preconditioning ([209]).  $\text{Ca}^{2+}$  oscillations in BBB endothelial cells occur in response to bradykinin, ATP and histamine ([210]) and were inhibited by hypoxia ([211]), which reduced both ER  $\text{Ca}^{2+}$  levels and SOCE.

Although the observation of  $\text{Ca}^{2+}$  oscillations and intercellular  $\text{Ca}^{2+}$  wave propagation in brain capillary endothelial cells indicate that all essential elements of the  $\text{Ca}^{2+}$  signaling toolkit are available, the molecular architecture of the  $\text{Ca}^{2+}$  toolkit in brain microvascular endothelial cells are yet to be fully elucidated ([212]). For instance, only  $\text{InsP}_3\text{R1}$  has been recently found in human brain ECs ([213]), while there is no report about the other  $\text{InsP}_3\text{R}$  isoforms or RYRs. Moreover, while all PLC subtypes (except  $\text{PLC}\zeta$ ) have been identified in the brain, little is known on their presence and function in brain microvascular ECs ([214]). There is also scarce information as regard to the  $\text{Ca}^{2+}$ -transporting systems. In rat brain capillary ECs, SERCA provides the major pathway responsible for clearing cytosolic  $\text{Ca}^{2+}$ , whereas the contribution of NCX in extruding ER-released  $\text{Ca}^{2+}$  is far less important (around 30%) of the calcium ions mobilized from ER ([215]). Nevertheless, NCX expression *in situ* has been reported in rat brain ECs ([216]).

Surprisingly, while TRP and Orai channels are widely studied in vascular ECs ([31], [217]), little is known about their expression and function in brain ECs. TRPC1 has been found interact with TRPC3 in bovine brain capillary ECs, in which they are responsible for  $\text{Ca}^{2+}$  entry induced by the application of ATP ([218]). Moreover, TRPV1 is expressed in human brain capillary and microvascular eECs and is involved in EDHF-dependent vasodilation induced by AA derivatives ([219]). Interestingly, TRPV4 and TRPV3 are also expressed in ECs from pial arteries and cerebral parenchymal arterioles and they are responsible for  $\text{Ca}^{2+}$  influx in response to EETs and carvacrol, respectively; TRPV4- and TRPV3-mediated  $\text{Ca}^{2+}$  influx can have a robust impact on cerebrovascular tone ([220], [221]). As to SOCE, the genetic down-regulation of Stim1 and Orai1 in a bovine brain endothelial cell line, t-BBEC117, decreases SOCE and reduces cell proliferation. Intriguingly, Orai2 is up-regulated at the G2/M phase of cell cycle and somehow negatively modulates SOCE activity [222]. A subsequent study revealed that hypoxia increases SOCE by inducing the up-regulation of

the Ba<sup>2+</sup>-sensitive inward rectifier K<sup>+</sup> channel, Kir2.1, thereby causing membrane hyperpolarization and boosting cell proliferation ([223]).

Taking together these information, we could give an important input in elucidating the composition and role of the Ca<sup>2+</sup> toolkit of brain ECs. In this work, we first will investigate the Ca<sup>2+</sup> response induced by Ach and Glu in mouse brain endothelial cells (bEND5). Finally, we will compare these results with a human brain cells line (hCMEC/D3).



# MATERIALS AND METHODS

## Cell culture

The bEND5 (American Type Culture Collection, Manassas, VA, USA) is an immortalized mouse cell line derived from mouse brain endothelium of BALB/c mice. Immortalisation has been carried out by infection of primary cells with retrovirus coding for the Polyoma virus middle T-antigen. bEND5 cells are positive for endothelial specific proteins (PECAM-1, Endoglin, MECA-32, Flk-1) tested by FACS. Inflammatory cytokines induce the expression of proteins such as CAM-1, VCAM-1 and E-selectin [224].

The bEND5 cells were used to investigate the  $\text{Ca}^{2+}$  and NO response to Ach in mouse brain microvascular endothelial cells. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 1mmol/L sodium pyruvate, 100 Units/mL penicillin, and 10 ng/mL streptomycin, 1% minimal essential medium nonessential amino acids exactly, as originally described previously [224]. Cells were cultured in a humidified cell culture incubator at 37°C and an atmosphere of 5%  $\text{CO}_2$ /95% air. The bEND5 cells were used for experiments from passages 15 to 25.

Human brain endothelial cells (hCMEC/D3) were obtained from Institut National de la Santé et de la Recherche Médicale (INSERM, Paris, France). hCMEC/D3 cells cultured between passage 25 and 35 were used. The cells were seeded at a concentration of 27,000 cells/cm<sup>2</sup> and grown in tissue culture flasks coated with 0.1 mg/mL rat tail collagen type 1, in the following medium: EBM-2 medium (Lonza, Basel, Switzerland) supplemented with 5% fetal bovine serum (FBS), 1% Penicillin–Streptomycin, 1.4  $\mu\text{M}$  hydrocortisone, 5  $\mu\text{g}/\text{mL}$  ascorbic acid, 1/100 chemically defined lipid concentrate (Invitrogen), 10 mM HEPES and 1 ng/mL basic FGF (bFGF). The cells were cultured at 37 °C, 5%  $\text{CO}_2$  saturated humidity.

## Solutions

Physiological salt solution (PSS) had the following composition (in mM): 150 NaCl, 6 KCl, 1.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 Glucose, 10 Hepes. In Ca<sup>2+</sup>-free solution (0Ca<sup>2+</sup>), Ca<sup>2+</sup> was substituted with 2 mM NaCl, and 0.5 mM EGTA was added. Solutions were titrated to pH 7.4 with NaOH. In Mn<sup>2+</sup>-quenching experiments, 200 μM MnCl<sub>2</sub> was added to the 0Ca<sup>2+</sup> external solution. The osmolality of the extracellular solution, as measured with an osmometer (Wescor 5500, Logan, UT), was 338 mmol/kg.

## Fluorescence microscope

Fluorescence microscopy is probably the most rapidly expanding technique in comparison to the others. The enormous development of fluorescent indicators in the last forty years has contributed to significantly expand the exploration possibilities of this technique. In the biological field its application was initially confined to the localization of structures and/or proteins in cells or tissues and was mainly based on immunofluorescence techniques.

## [Ca<sup>2+</sup>]<sub>i</sub> measurements

bEND5 or hCMEC/D3 cells were loaded with 4 μM fura-2 acetoxymethyl ester (Fura-2/AM; 1 mM stock in dimethyl sulfoxide) in PSS for 20 min at room temperature. After washing in PSS, the coverslip was fixed to the bottom of a Petri dish and the cells observed by an upright epifluorescence Axiolab microscope (Carl Zeiss, Oberkochen, Germany), usually equipped with a Zeiss ×40 Achromplan objective (water-immersion, 2.0 mm working distance, 0.9 numerical aperture). The cells were excited alternately at 340 and 380 nm, and the emitted light was detected at 510 nm. A first neutral density filter (1 or 0.3 optical density) reduced the overall intensity of the excitation light and a second neutral density filter (optical density=0.3) was coupled to the 380 nm filter to approach the intensity of the 340 nm light. A round diaphragm was used to increase the contrast. The excitation filters were mounted on a filter wheel (Lambda 10, Sutter Instrument, Novato, CA, USA). Custom software, working in the LINUX environment, was used to drive the camera (Extended-ISIS Camera, Photonic Science, Millham, UK) and the filter wheel, and to measure and plot on-line the fluorescence from 30-45 rectangular “regions of interest” (ROI) enclosing 30-45 single cells. Each ROI was identified by a number. Adjacent ROIs never superimposed. [Ca<sup>2+</sup>]<sub>i</sub> was monitored by measuring, for each ROI, the ratio of the mean

fluorescence emitted at 510 nm when exciting alternatively at 340 and 380 nm (shortly termed "ratio"). An increase in  $[Ca^{2+}]_i$  causes an increase in the ratio. Ratio measurements were performed and plotted on-line every 3 s. The experiments were performed at room temperature (22°C).

$Mn^{2+}$  has been shown to quench Fura-2 fluorescence. Since  $Mn^{2+}$  and  $Ca^{2+}$  share common entry pathways in the plasmalemma, Fura-2 quenching by  $Mn^{2+}$  is regarded as an index of divalent cation influx ([225]). Experiments were carried out at the 360 nm wavelength, the isosbestic wavelength for Fura-2, and in  $Ca^{2+}$ -free medium supplemented with 0.5 mM EGTA, as previously described ([226], [128]). This avoids  $Ca^{2+}$  competition for  $Mn^{2+}$  entry and therefore enhances  $Mn^{2+}$  quenching.

NO was measured as described in ([119]) and ([227]). Briefly, bEND5 or hCMEC/D3 were loaded with the membrane permeable NO-sensitive dye 4-Amino-5-methylamino-2',7'-difluorofluorescein (DAF-FM) diacetate (10  $\mu$ M) for 60 min at room temperature and washed in PSS for one further hour. DAF-FM fluorescence was measured by using the same equipment described for  $Ca^{2+}$  recordings but with a different filter set, i.e. excitation at 480 nm and emission at 535 nm wavelength (emission intensity was shortly termed "NO<sub>i</sub>"). The changes in DAF-FM fluorescence induced by Ach were recorded and plotted on-line every 3 s. Again, off-line analysis was performed by using custom-made macros developed by Microsoft Office Excel software. The experiments were performed at room temperature.

## **RNA isolation and Real Time RT-PCR (qRT-PCR) of $Ca^{2+}$ -permeable channels**

Total RNA was extracted using the QIAzol Lysis Reagent (QIAGEN, Italy), as recently shown ([228], [229]). The first cDNA copy was synthesized from 1  $\mu$ g total RNA using random hexamers and M-MLV Reverse Transcriptase (Invitrogen S.R.L., Milan, Italy). Real-time PCR was performed using GoTaq qPCR master mix according to manufacturer's instructions (Promega, Milan, Italy) on a SFX96 Real-time system (Biorad, Segrate, Italy). Oligonucleotide primers were obtained from Primer3. Ribosomal S18 subunit was tested as housekeeping gene.

## Immunoblotting

Immunoblotting was performed as previously described [230]. Briefly, the cells were lysed in 100  $\mu$ l RIPA lysis buffer (Santa Cruz Biotechnology, Inc, CA, USA) for 30 min in ice. Lysates were centrifuged at 14,000 g for 10 min at 4°C. Total cell protein extracts were normalized for concentration by the Bradford assay (Bio-Rad Laboratories Segrate Milan Italy). Two different concentrations respectively of 35  $\mu$ g and 20  $\mu$ g of proteins were separated by SDS-PAGE and transferred to polyvinylidenedifluoride membrane (Millipore's Corporate, Billerica, MA, USA). Membranes were incubated with two anti-mouse antibody Stim 1 and Orai 2 according to the manufacturer's instructions (Santa Cruz Biotechnology, Inc, CA, USA). Anti-mouse horseradish peroxidase-conjugated secondary antibody was used 1:500 (Amersham Biosciences, Inc., Piscataway, NJ, USA) and visualized by the ECL detection system (Amersham Biosciences, Inc. Piscataway, NJ, USA) according to the manufacturer's instructions. Each membrane was probed with the monoclonal anti-actin antibody (Fitzgerald Industries International, USA) to estimate equal protein loading.

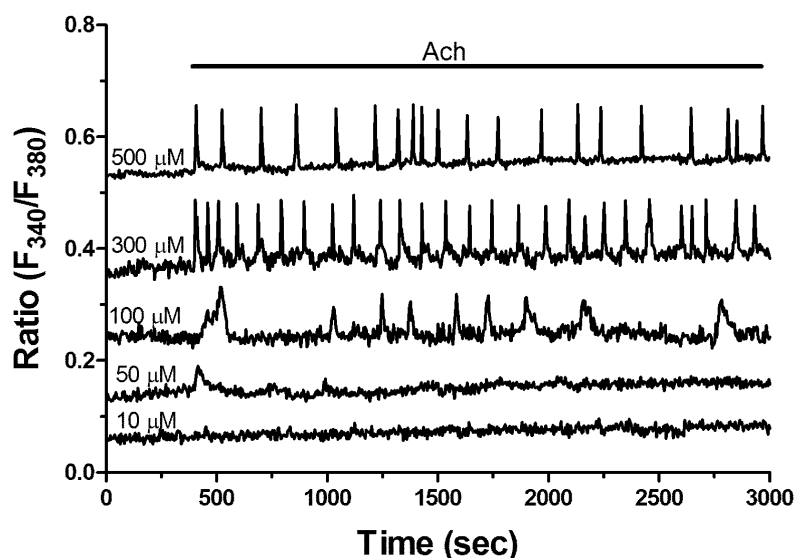
## Statistics

All the data have been collected from bEND5 cells deriving from at least three coverslips from three independent experiments. The amplitude of  $\text{Ca}^{2+}$  release in response to either CPA or Ach (1<sup>st</sup> spike) was measured as the difference between the ratio at the peak of intracellular  $\text{Ca}^{2+}$  mobilization and the mean ratio of 1 min baseline before the peak. The magnitude of CPA-evoked SOCE upon  $\text{Ca}^{2+}$  restoration to the bath was measured as the difference between the ration at the peak of extracellular  $\text{Ca}^{2+}$  entry and the mean ration of 1 in baseline before  $\text{Ca}^{2+}$  readdition. The rate of  $\text{Mn}^{2+}$  influx was evaluated by measuring the slope of the fluorescence intensity curve at 400 sec after  $\text{Mn}^{2+}$  addition. Pooled data are given as mean $\pm$ SE and statistical significance ( $P < 0.05$ ) was evaluated by the Student's t-test for unpaired observations. Data relative to both  $\text{Ca}^{2+}$  and NO signals and to  $\text{Mn}^{2+}$  influx rate are presented as mean $\pm$ SE, while the number of cells analyzed is indicated between parentheses.

## RESULTS

### Acetylcholine induces intracellular Ca<sup>2+</sup> oscillations in bEnd5 cells

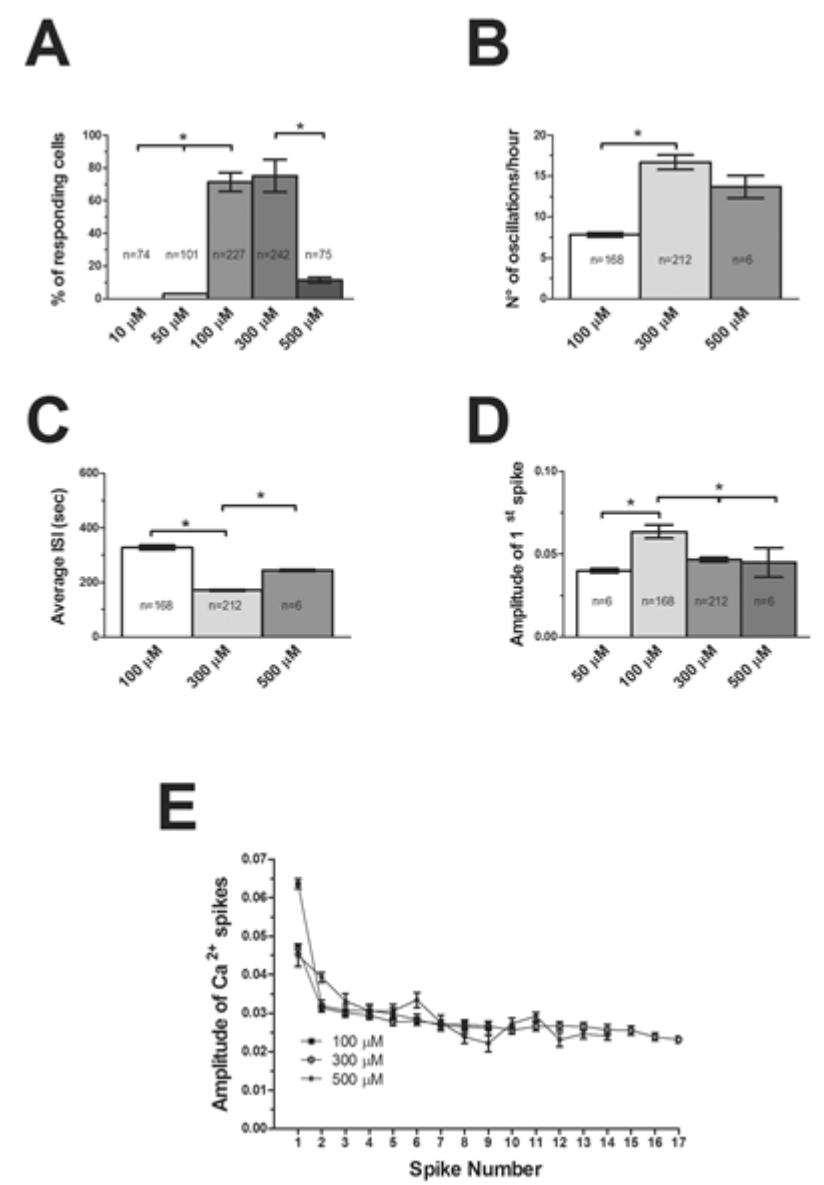
In order to assess whether Ach was able to induce Ca<sup>2+</sup> signals, bEND5 were loaded with the Ca<sup>2+</sup>-sensitive dye Fura-2/AM and were initially imaged with conventional epifluorescence microscopy. A fraction (~45%) of cells showed spontaneous Ca<sup>2+</sup> spikes that arose in the absence of external stimulation. For the following evaluation of Ach-induced Ca<sup>2+</sup> signals, these cells were discarded from the analysis. When Ach was applied to the quiescent cells, we observed a dose-dependent increase in [Ca<sup>2+</sup>]<sub>i</sub> with a different pattern depending on agonist concentration (Fig. 1 and Fig. 2).



**Figure 1.** Acetylcholine evokes repetitive Ca<sup>2+</sup> transients in bEND5 cells. Ach causes the immediate initiation of an oscillatory Ca<sup>2+</sup> response whose duration, amplitude of the 1<sup>st</sup> spike and interspike interval (ISI) was a function of agonist concentration. In this and the following figures, Ach was added at the time indicated by the horizontal bar drawn around the Ca<sup>2+</sup> tracings. The baseline of Ca<sup>2+</sup> tracings has been shifted to avoid their overlapping for representation purposes.

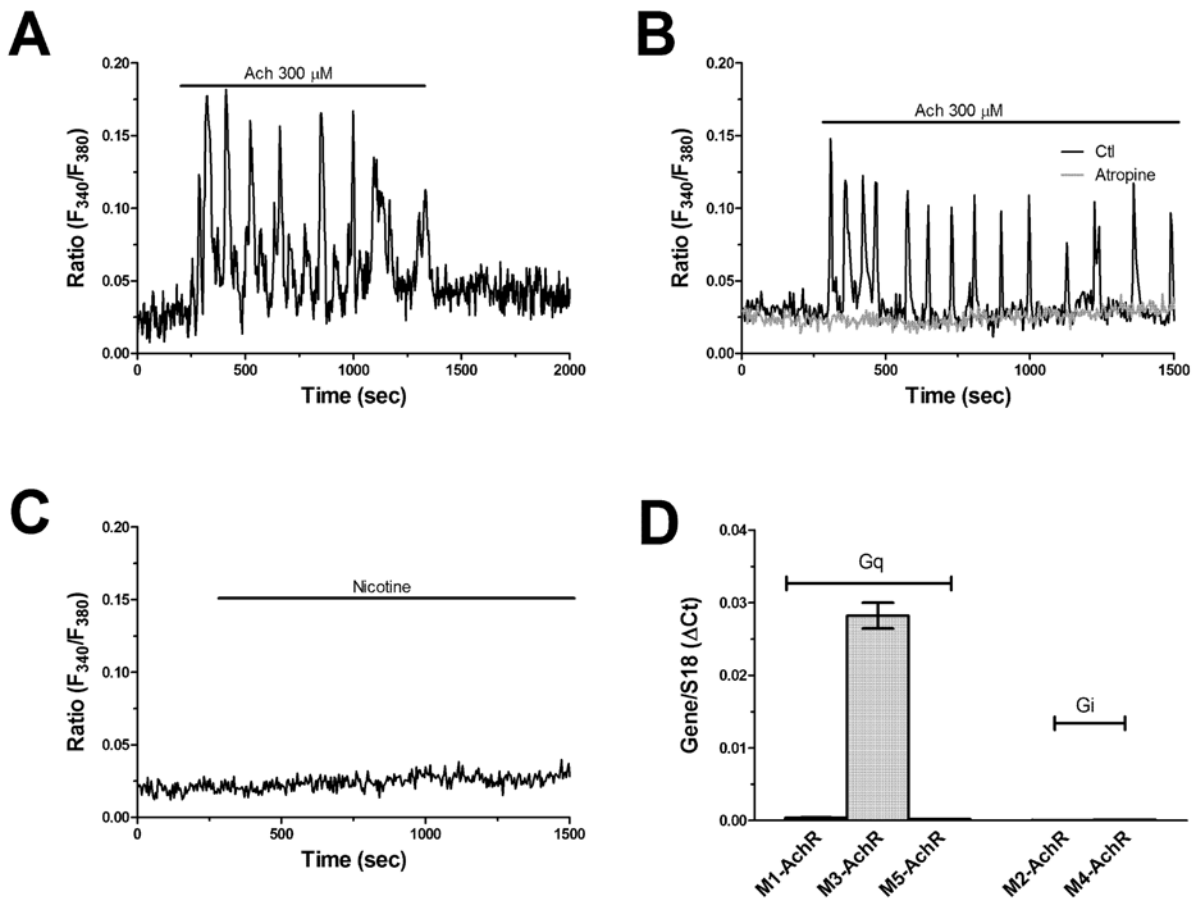
Ach at 100 μM and above, Ach caused repetitive oscillations in [Ca<sup>2+</sup>]<sub>i</sub>, while lower doses of Ach induced only a short Ca<sup>2+</sup> transient (50 μM) or none (10 μM) (Fig. 1 and Fig. 2). Of note, a slow increase in [Ca<sup>2+</sup>]<sub>i</sub> preceded each Ca<sup>2+</sup> oscillation, giving rise to sub-threshold Ca<sup>2+</sup> signal known as pacemaker Ca<sup>2+</sup> ramp ([231]), that led to the regenerative Ca<sup>2+</sup> upstroke and was a hallmark of InsP<sub>3</sub>-driven intracellular Ca<sup>2+</sup> release. According to the statistical analysis, 300 μM was the most

suitable concentration for Ach to generate a prolonged (up to 1 hour)  $\text{Ca}^{2+}$  burst in bEND5 cells. Indeed, at this concentration, the frequency of  $\text{Ca}^{2+}$  transients and the number of oscillations/hour (Fig. 2B) reached their greatest value, while there was no significant ( $p < 0.05$ ) difference in the fraction of responding cells between 100 and 300  $\mu\text{M}$  (Fig. 2A). Notably, while the amplitude of the 1<sup>st</sup>  $\text{Ca}^{2+}$  spike was highest at 100  $\mu\text{M}$  (Fig. 2B), there was no statistically relevant difference in the amplitude of the subsequent  $\text{Ca}^{2+}$  transients at each concentration tested (Fig. 2E). Moreover, the height of the  $\text{Ca}^{2+}$  peaks significantly ( $p < 0.05$ ) declined from the 1<sup>st</sup> to the 2<sup>nd</sup> one, but then it remained constant throughout the  $\text{Ca}^{2+}$  train at both 100 and 300  $\mu\text{M}$  (Fig. 2E). Conversely, the magnitude of the 2<sup>nd</sup>  $\text{Ca}^{2+}$  transient elicited by 500  $\mu\text{M}$  Ach was significantly ( $p < 0.05$ ) lower than the 1<sup>st</sup>, but higher than the following spikes (Fig. 2E).



**Figure 2.** Statistical analysis of Ach-induced intracellular  $\text{Ca}^{2+}$  oscillations in bEND5 cells. Bar histograms show the average  $\pm$  SE of the percentage of responding cells (A), number of oscillations/hour (B), amplitude of the 1<sup>st</sup> spike (C), average ISI (D) and amplitude of the 2-15<sup>th</sup>  $\text{Ca}^{2+}$  spike (E) of Ach-evoked intracellular  $\text{Ca}^{2+}$  oscillations. The asterisk indicates  $p < 0.05$ .

Ach-induced intracellular  $Ca^{2+}$  oscillations required the continuous presence of the agonist as they were rapidly interrupted upon Ach removal from the perfusate (Fig. 3A). Moreover, they were blocked by atropine (100  $\mu$ M, 30 min), a selective mAChR antagonist (Fig. 3B). Conversely, nicotine, a selective agonist of cholinergic nicotinic receptors (nAChRs) ([232]), did not increase  $[Ca^{2+}]_i$  in bEND5 cells (Fig. 3C). Taken together, these results suggest that the most frequent  $Ca^{2+}$  response induced by Ach in bEND5 consists in  $Ca^{2+}$  oscillations that arise upon mAChR activation. Consistently, qRT-PCR revealed that mouse brain microvascular endothelial cells express the transcript encoding for mAChR M3 (M3-mAChR), while those encoding for M1, M2, M4 and M5 are absent (Fig. 3D). The remainder of the experiments was therefore carried out by challenging the cells with 300  $\mu$ M, which is within the same range as that shown to induce vasorelaxation in brain intraparenchymal arterioles ([83], [93]).

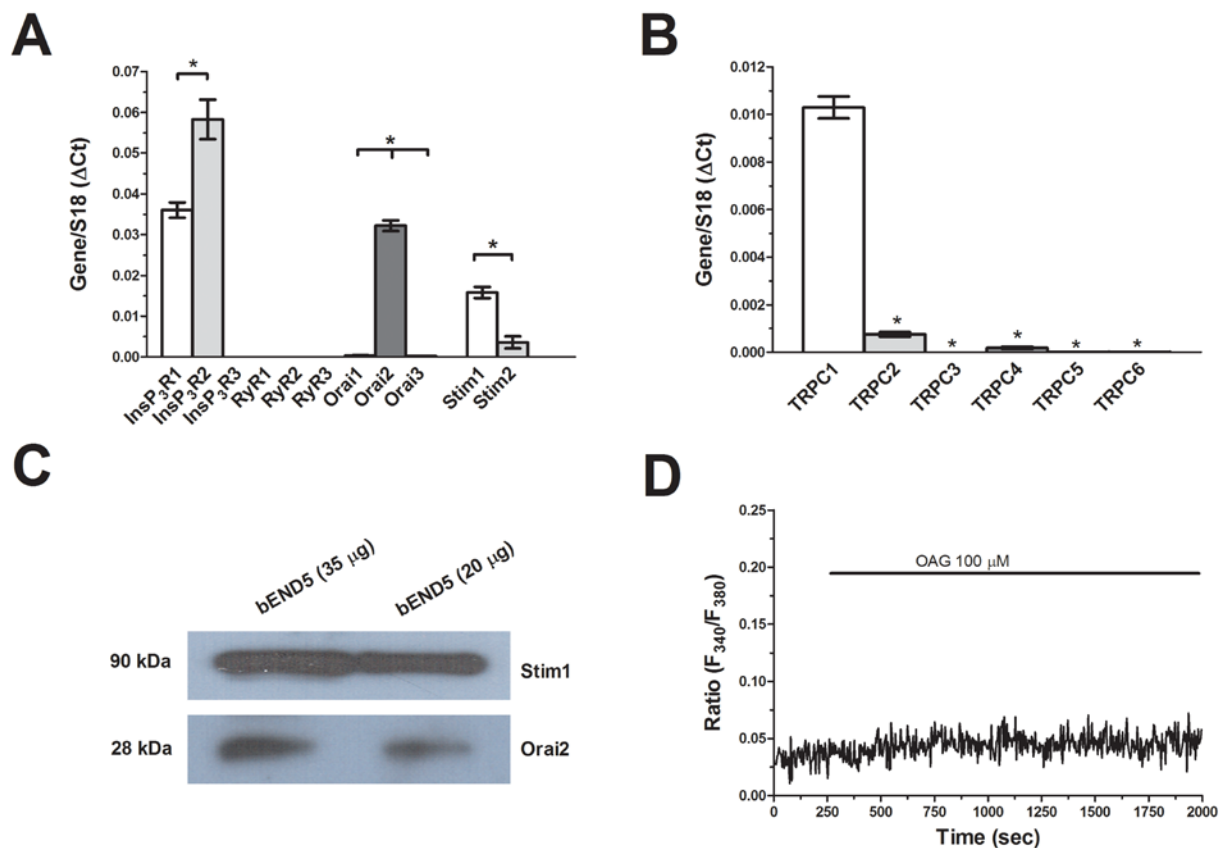


**Figure 3.** Metabotropic muscarinic receptors drive the onset of acetylcholine-induced intracellular  $Ca^{2+}$  oscillations. (A), removal of Ach from the extracellular solution causes the interruption of the ongoing  $Ca^{2+}$  burst. (B), atropine (100  $\mu$ M, 30 min) prevents the initiation of the  $Ca^{2+}$  response to Ach. (C), nicotine (100  $\mu$ M) did not increase  $[Ca^{2+}]_i$  in bEND5 cells, as also observed in other endothelial cell types. (D), qRT-PCR analysis of metabotropic muscarinic receptors (M-AChRs) revealed that only M3-AChR mRNA is expressed in bEND5. Data are expressed as mean  $\pm$  SE of qRT-PCR runs performed in triplicate.

## Expression of the components of the Ca<sup>2+</sup> signaling toolkit in bEND5 cells

As mentioned in the Introduction, the molecular components of the Ca<sup>2+</sup> toolkit in brain microvascular endothelial cells are largely unknown. Therefore, in order to investigate the signalling machinery that shapes Ach-induced intracellular Ca<sup>2+</sup> spikes, we first investigated the expression of the components of the endothelial Ca<sup>2+</sup> toolkit downstream of PLC $\beta$  activation ([128]). We used qRT-PCR by using the specific primers designed with Primer3. The following transcripts were expressed in bEND5 cells: InsP<sub>3</sub>R1 and InsP<sub>3</sub>R2 (Fig. 4A); Stim1 and Stim2 (Fig. 4A); Orai2 (Fig. 4A); TRPC1 (Fig. 4B). As observed in many endothelial cell types (Moccia et al., 2012 WJBC), RyRs were absent (Fig. 4A). TRPC2 levels were significantly low as compared to TRPC1, while TRPC4 was almost undetectable (Fig. 4B). Surprisingly, Orai1 (as well as Orai3) transcripts could not be detected (Fig. 4A), which hints at Orai2 as the pore-forming subunit of store-operated channels in bEND5 cells. As anticipated earlier, Orai2 is the major candidate to mediate both constitutive and agonist-stimulated SOCE also in mouse neurons ([231]). Immunoblotting confirmed that both Stim1 and Orai2 were expressed at protein level in bEND5 cells (Fig. 4C). Moreover, the diacylglycerol (DAG)-gated Ca<sup>2+</sup>-permeable Canonical Transient Receptor Potential 3 and 6 (TRPC3 and TRPC6) were not found in bEND5 cells (Fig. 4B). Consistently, oleoyl-acyl-sn-glycerol (OAG; 100  $\mu$ M), a membrane-permeable DAG analog that mediates Ach-induced Ca<sup>2+</sup> entry through TRPC3 and TRPC6 channels ([233], [234]), failed to evoke any detectable Ca<sup>2+</sup> signal in bEND5 cells (Fig. 4D). Collectively, these results demonstrate that, upon PLC $\beta$  activation, Ach has the potential to induce ER Ca<sup>2+</sup> release only via InsP<sub>3</sub>Rs and to promote extracellular Ca<sup>2+</sup> entry through the store-operated Ca<sup>2+</sup> channel, Orai2.



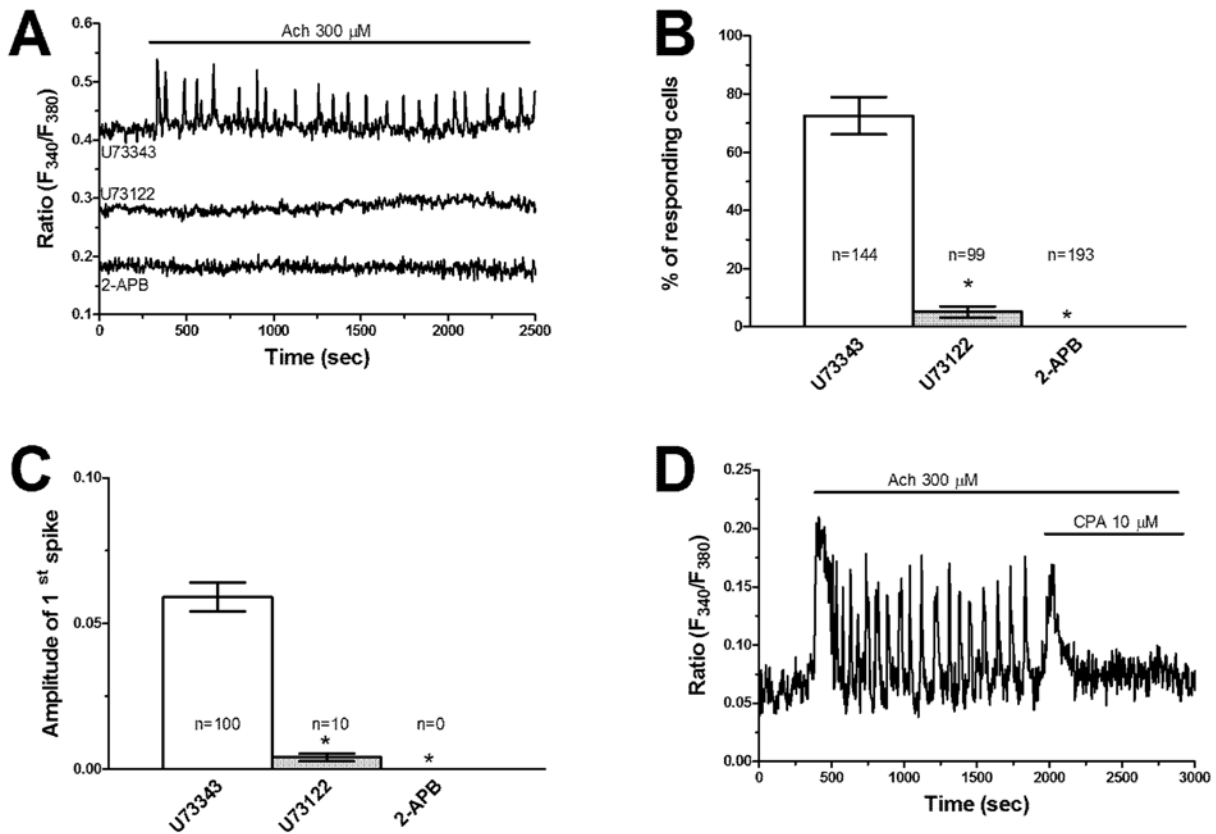


**Figure 4.** The Ca<sup>2+</sup> toolkit of bEND5 cells. (A), mRNA levels of InsP<sub>3</sub>R1-3, RyR1-3, Orai1-3, and Stim1-2 in bEND5 cells. (B), mRNA levels of TRPC1-6 channels in bEND5 cells. In both (A) and (B), data are expressed as mean±SE of qRT-PCR runs performed in triplicate. (C), Stim1 and Orai2 proteins was detected by immunoblotting, as described in Materials and Methods. Lanes were loaded with 35 or 20 μg of proteins and probed with specific rabbit polyclonal antibodies. A band of about 90 kDa was found for Stim1 and of about 28 kDa for Orai2. Blots representative of three were shown. (D), OAG (100 μM) did not increase [Ca<sup>2+</sup>]<sub>i</sub> in bEND5 cells.

## Acetylcholine-induced Ca<sup>2+</sup> oscillations derive from the periodic discharge of intraluminally stored Ca<sup>2+</sup> through InsP<sub>3</sub>Rs

As Ach stimulates Ca<sup>2+</sup> spiking by activating mAChRs in bEND5 cells, we first assessed the signaling pathway whereby PLCβ triggers the Ca<sup>2+</sup> response. U73122 (10 μM, 30 min), a rather selective PLCβ inhibitor ([232]), was able to block Ach-induced intracellular Ca<sup>2+</sup> oscillations, while its inactive structural analog, U73343 (10 μM, 30 min), did not (Fig. 5A-5C). Similarly, the Ca<sup>2+</sup> response to Ach was abolished by 2-aminoethoxydiphenylborate (2-APB) (50 μM, 30 min), a widely employed InsP<sub>3</sub>R inhibitor ([235]), (Fig. 5A-Fig. 5C). Taking together, these results demonstrate that Ach-induced Ca<sup>2+</sup> oscillations are produced by repetitive cycles of ER Ca<sup>2+</sup>

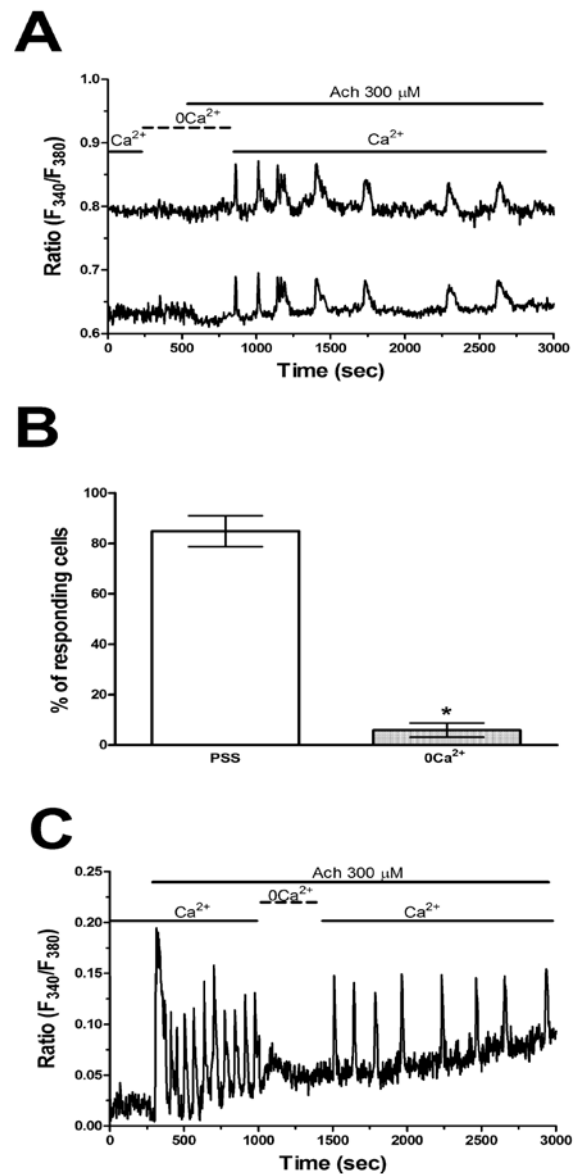
release via  $\text{InsP}_3\text{Rs}$ . In further support of this conclusion, we probed the effect of cyclopiazonic acid (CPA). CPA is a specific SERCA inhibitor, thereby preventing  $\text{Ca}^{2+}$  sequestration into ER lumen. Addition of CPA ( $10\ \mu\text{M}$ ) during ongoing spikes caused an abrupt increase in  $[\text{Ca}^{2+}]_i$ , due to the passive  $\text{Ca}^{2+}$  leak, followed by complete inhibition of the  $\text{Ca}^{2+}$  transients (Fig. 5D). This result confirms that recurrent cycles of ER  $\text{Ca}^{2+}$  release and re-uptake underpin the oscillatory response to Ach.



**Figure 5.** Ach-induced intracellular  $\text{Ca}^{2+}$  oscillations derived from repetitive  $\text{Ca}^{2+}$  release from ER via  $\text{InsP}_3\text{Rs}$ . (A), Ach-induced  $\text{Ca}^{2+}$  oscillations were inhibited by U73122 ( $10\ \mu\text{M}$ , 30 min), an established PLC inhibitor, but not by structurally inactive analog, U73343 ( $10\ \mu\text{M}$ , 30 min), and by 2-APB ( $50\ \mu\text{M}$ , 30 min), which blocks  $\text{InsP}_3\text{Rs}$ . The baseline of  $\text{Ca}^{2+}$  tracings has been shifted to avoid their overlapping for representation purposes. (B), bar histogram shows the average $\pm$ SE of the percentage of responding cells under the designated treatments. The asterisk indicates  $p < 0.05$ . (C), bar histogram shows the average $\pm$ SE of the percentage of the amplitude of the 1<sup>st</sup> spike under the designated treatments. The asterisk indicates  $p < 0.05$ . (D), addition of CPA ( $20\ \mu\text{M}$ ) during Ach-induced  $\text{Ca}^{2+}$  spikes causes an immediate increase in  $[\text{Ca}^{2+}]_i$  due to passive depletion of the ER  $\text{Ca}^{2+}$  pool followed by blockade of ongoing oscillations.

# The acetylcholine-induced intracellular $\text{Ca}^{2+}$ oscillations requires extracellular $\text{Ca}^{2+}$ entry

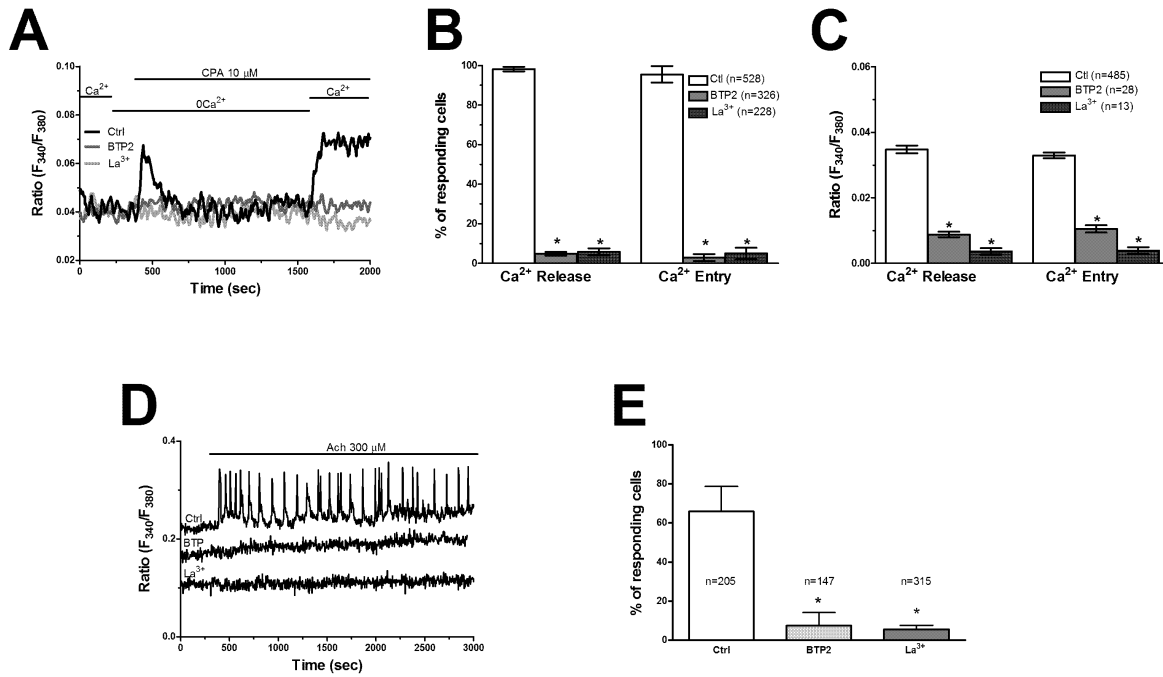
Ach failed to generate any detectable increase in  $[\text{Ca}^{2+}]_i$  in the absence of extracellular  $\text{Ca}^{2+}$  ( $0\text{Ca}^{2+}$ ) in most bEND5 cells (Fig. 6A and Fig. 6B). The oscillatory response to Ach, however, immediately resumed after  $\text{Ca}^{2+}$  addition to the perfusate (Fig. 6A). Similarly, removal of external  $\text{Ca}^{2+}$  during ongoing oscillations caused the stop of the  $\text{Ca}^{2+}$  burst after 1-2 spikes (Fig. 6C). Again, intracellular  $\text{Ca}^{2+}$  oscillations resumed upon  $\text{Ca}^{2+}$  restoration to the bathing solution (Fig. 6C). These findings strongly indicate that the extracellular  $\text{Ca}^{2+}$  entry is required to initiate Ach-induced  $\text{Ca}^{2+}$  oscillations in bEND5 cells. As the  $\text{Ca}^{2+}$  response occurs as a consequence of  $\text{PLC}\beta$  activation, the underlying plasmalemmal channel must be gated by a second messenger generated upon  $\text{PIP}_2$  hydrolysis. Our previous characterization of the  $\text{Ca}^{2+}$  toolkit revealed that DAG-sensitive endothelial  $\text{Ca}^{2+}$  channels (TRPC3 and 6) are absent in bEND5 cells, while the molecular components of SOCE (Stim1, Stim2 and Orai2) are largely expressed. Therefore, SOCE is the major candidate to mediate Ach-induced  $\text{Ca}^{2+}$  entry in bEND5 cells.



**Figure 6.** Extracellular  $\text{Ca}^{2+}$  is necessary for the appearance of Ach-induced intracellular  $\text{Ca}^{2+}$  oscillations. (A), Ach-evoked  $\text{Ca}^{2+}$  oscillations did not arise in the absence of external  $\text{Ca}^{2+}$  ( $0\text{Ca}^{2+}$ ) but rapidly resumed on  $\text{Ca}^{2+}$  restitution to the bath. (B), bar histogram shows the average  $\pm$  SE of the percentage of bEND5 cells displaying an oscillatory response to Ach in the presence and absence of external  $\text{Ca}^{2+}$ . The asterisk indicates  $p < 0.05$ .

## SOCE is the major Ach-activated $\text{Ca}^{2+}$ entry pathway in bEND5 cells

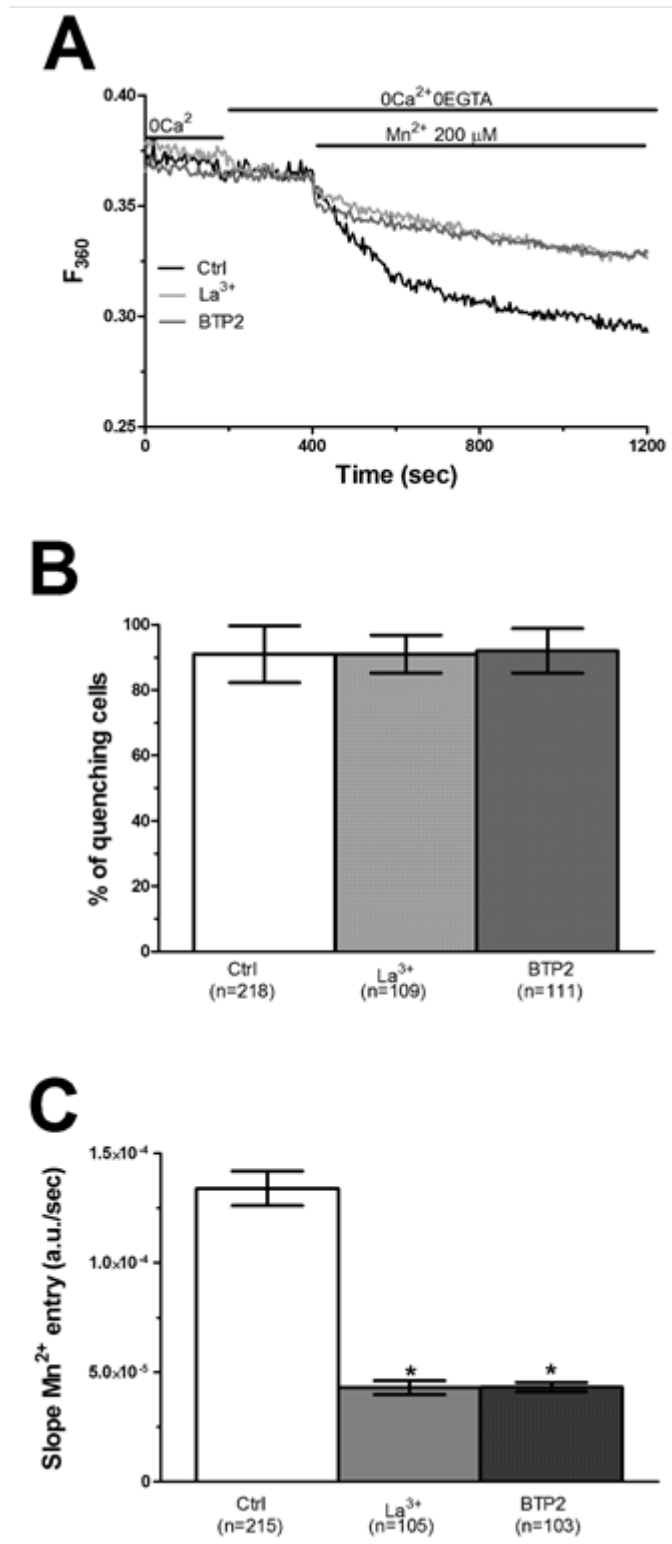
In order to confirm whether a functional SOCE is expressed in bEND5 cells, we exploited the “ $\text{Ca}^{2+}$  add-back” protocol ([167], [236]). This protocol consists in stimulating the cells in  $0\text{Ca}^{2+}$  with a selective SERCA inhibitor, such as CPA or thapsigargin. This treatment depletes the ER  $\text{Ca}^{2+}$  reservoir through yet to be identified  $\text{Ca}^{2+}$  leak pathway and leads to a transient increase in  $[\text{Ca}^{2+}]_i$  due to passive  $\text{Ca}^{2+}$  efflux. Gradually the  $[\text{Ca}^{2+}]_i$  recovers to the baseline due to the concerted action of NCX, PMCA and mitochondria. Once the  $\text{Ca}^{2+}$  reservoir of the ER has been depleted, the ER  $\text{Ca}^{2+}$  sensor Stim1 detects such fall in intraluminal  $\text{Ca}^{2+}$ , oligomerizes and rapidly relocates towards ER-plasma membrane junctions. Herein, Stim1 binds to and gates the  $\text{Ca}^{2+}$ -permeable channel Orai1 to trigger  $\text{Ca}^{2+}$  entry into the cell. Accordingly, subsequent restitution of  $\text{Ca}^{2+}$  to the perfusate causes a second increase in  $[\text{Ca}^{2+}]_i$  driven by  $\text{Ca}^{2+}$  entry through open Orai channels. As shown in Fig. 7A, CPA (10  $\mu\text{M}$ ) caused a robust SOCE in bEND5 cells. Of note, pre-incubating the cells with powerful Orai blockers, such as  $\text{La}^{3+}$  (10  $\mu\text{M}$ ) and BTP2 (10  $\mu\text{M}$ ), prevented both CPA-induced intracellular  $\text{Ca}^{2+}$  mobilization and CPA-induced SOCE. The statistical analysis of these experiments is illustrated in Fig. 7B and in Fig. 7C. This result suggests that SOCE is partially activated under resting conditions and controls ER  $\text{Ca}^{2+}$  refilling in bEND5 cells. Not surprisingly, therefore, both  $\text{La}^{3+}$  (10  $\mu\text{M}$ ) and BTP2 (10  $\mu\text{M}$ ) prevented the onset of Ach-induced  $\text{Ca}^{2+}$  oscillations (Fig. 7D and Fig. 7E).



**Figure 7.** A functional SOCE is present and controls ER Ca<sup>2+</sup> content in bEND5 cells. (A), the Ca<sup>2+</sup> “add-back” protocol was applied to assess whether SOCE is active in bEND5 cells. CPA (10 μM) was administered under 0Ca<sup>2+</sup> conditions to deplete the ER Ca<sup>2+</sup> pool and activate store-operated Ca<sup>2+</sup> channels. The following restitution of Ca<sup>2+</sup> to the external solution caused a second increase in [Ca<sup>2+</sup>]<sub>i</sub> due to SOCE activation. However, pre-incubating the cells with either La<sup>3+</sup> (10 μM, 30 min) or BTP2 (10 μM, 30 min), two rather selective Orai inhibitors, blocked both CPA-induced intracellular Ca<sup>2+</sup> mobilization and SOCE. (B), bar histogram of the average±SE of the percentage of bEND5 cells responding to CPA in the absence and presence of either La<sup>3+</sup> (10 μM, 30 min) or BTP2 (10 μM, 30 min). The asterisk indicates p<0.05. (C), bar histogram of the average±SE of the percentage of the amplitude of CPA-induced Ca<sup>2+</sup> release and SOCE in the absence and presence of either La<sup>3+</sup> (10 μM, 30 min) or BTP2 (10 μM, 30 min). The asterisk indicates p<0.05. (D), Ach-induced intracellular Ca<sup>2+</sup> oscillations were prevented by either La<sup>3+</sup> (10 μM, 30 min) or BTP2 (10 μM, 30 min). (E), bar histogram of the average±SE of the percentage of bEND5 cells displaying a spiking response to Ach in the absence and in the presence of either La<sup>3+</sup> (10 μM, 30 min) or BTP2 (10 μM, 30 min).

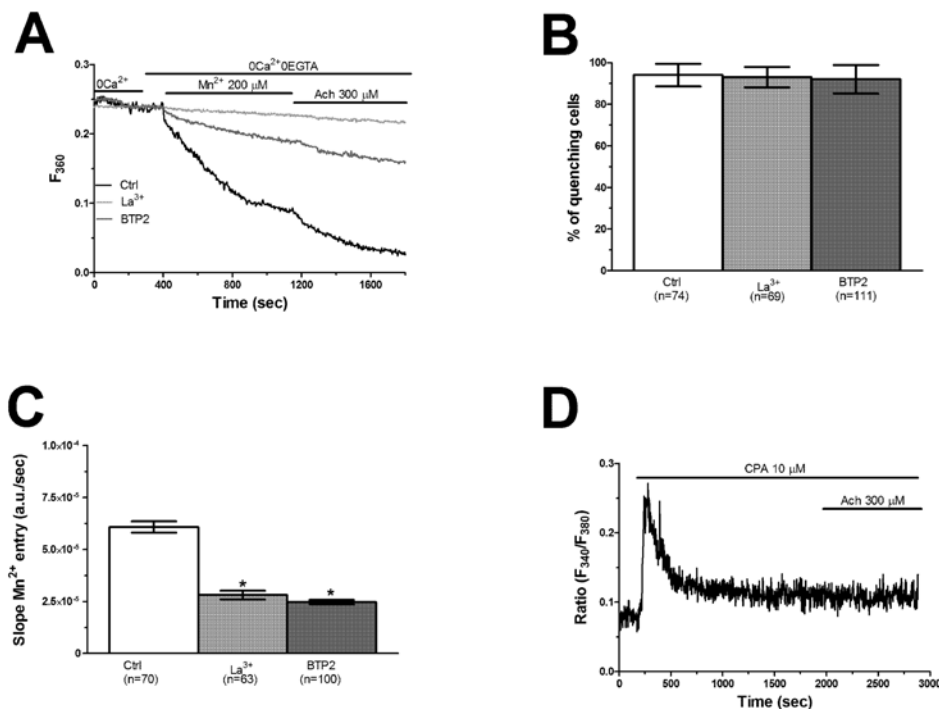
To further corroborate the hypothesis that SOCE is constitutively activated in bEND5 cells, we applied the Mn<sup>2+</sup>-quenching technique. The Mn<sup>2+</sup> quenching technique is an established tool to monitor both constitutive and agonist-induced Ca<sup>2+</sup> entry in vascular endothelial cells and EPCs ([226], [128], [237], [238], [239]). As Mn<sup>2+</sup> and Ca<sup>2+</sup> share common entry pathways in the plasmalemma, Mn<sup>2+</sup> can be used as a reliable surrogate of Ca<sup>2+</sup>. As explained in Materials and Methods, Fura-2 quenching by Mn<sup>2+</sup> is regarded as an index of divalent cation influx. Experiments were carried out at the 360 nm wavelength, the isobestic wavelength for Fura-2, in 0Ca<sup>2+</sup> medium supplemented with 0.5 mM EGTA (0Ca<sup>2+</sup> OEGTA). Figure 8A shows that addition of 200 μM Mn<sup>2+</sup> enhanced the quenching of Fura-2 fluorescence, which is consistent with the expression of a constitutive Ca<sup>2+</sup> entry pathway in bEND5 cells ([226], [128], [240], [241]). Basal Ca<sup>2+</sup> influx was

abolished by pre-treating the cells with either  $\text{La}^{3+}$  (10  $\mu\text{M}$ ) or BTP2 (10  $\mu\text{M}$ ) (Fig. 8A-8C). These data confirm that SOCE is constitutively active and maintains ER  $\text{Ca}^{2+}$  load in brain microvascular endothelial cells ([128]), as also recently demonstrated in mouse brain neurons ([178]).



**Figure 8.** SOCE is constitutively active in bEND5 cells. (A), resting  $\text{Ca}^{2+}$  entry in mouse brain microvascular endothelial cells was evaluated by using the  $\text{Mn}^{2+}$ -quenching technique. 200  $\mu\text{M}$   $\text{Mn}^{2+}$  was added and they caused an immediate decay in Fura-2 fluorescence. This finding indicates that a basal  $\text{Ca}^{2+}$ -permeable pathway is active. The rate of fluorescence decay for each individual tracing was calculated as the slope of a linear regression. Pre-incubating the cells with either  $\text{La}^{3+}$  (10  $\mu\text{M}$ , 30 min) or BTP2 (10  $\mu\text{M}$ , 30 min) caused an evident reduction in rate of  $\text{Mn}^{2+}$  entry. This finding hints at the store-dependent nature of this constitutive  $\text{Ca}^{2+}$  influx pathway. (B), mean  $\pm$  SE of the percentage of bEND5 cells showing constitutive SOCE in the absence or presence of either  $\text{La}^{3+}$  (10  $\mu\text{M}$ , 30 min) or BTP2 (10  $\mu\text{M}$ , 30 min). The asterisk indicates  $p < 0.05$ . (C), mean  $\pm$  SE of the quenching rate of Fura-2 fluorescence induced by  $\text{Mn}^{2+}$  addition in resting bEND5 cells in the absence or presence of either  $\text{La}^{3+}$  (10  $\mu\text{M}$ , 30 min) or BTP2 (10  $\mu\text{M}$ , 30 min). The asterisk indicates  $p < 0.05$ .

Moreover, CPA further increased the rate of Fura-2 quenching induced by  $Mn^{2+}$  in the absence, but not in the presence, of  $La^{3+}$  (10  $\mu M$ ) or BTP2 (10  $\mu M$ ). Therefore, the  $Mn^{2+}$ -quenching technique is also a suitable tool to monitor agonist-induced SOCE. According, in a separate set of experiments, once the rate of basal quenching was established, addition of Ach further increased the slope of the quenching curve (Fig. 9A), which reflects the activation of an Ach-sensitive channel. The pharmacological blockade of SOCE with either  $La^{3+}$  (10  $\mu M$ ) or BTP2 (10  $\mu M$ ) prevented Ach-induced divalent cation entry (Fig. 9A-9C). Taken together, these findings convincingly suggest that Ach stimulates SOCE in bEND5 cells. We finally challenged the cells with CPA (10  $\mu M$ ) in the presence of external  $Ca^{2+}$  (Fig. 9D). The application of CPA induces a rapid elevation in  $[Ca^{2+}]_i$  associated to ER emptying followed by a decay phase to a discernible plateau level, due to SOCE activation ([242]). At 30 min after CPA addition, a time interval sufficient for depleting ER  $Ca^{2+}$  stores and fully activating SOCE ([243], [244]), Ach failed to induce  $Ca^{2+}$  entry (Fig. 9D). This result demonstrates that Ach is unlikely to activate a store-independent channel in brain microvascular endothelial cells ([244], [242]).

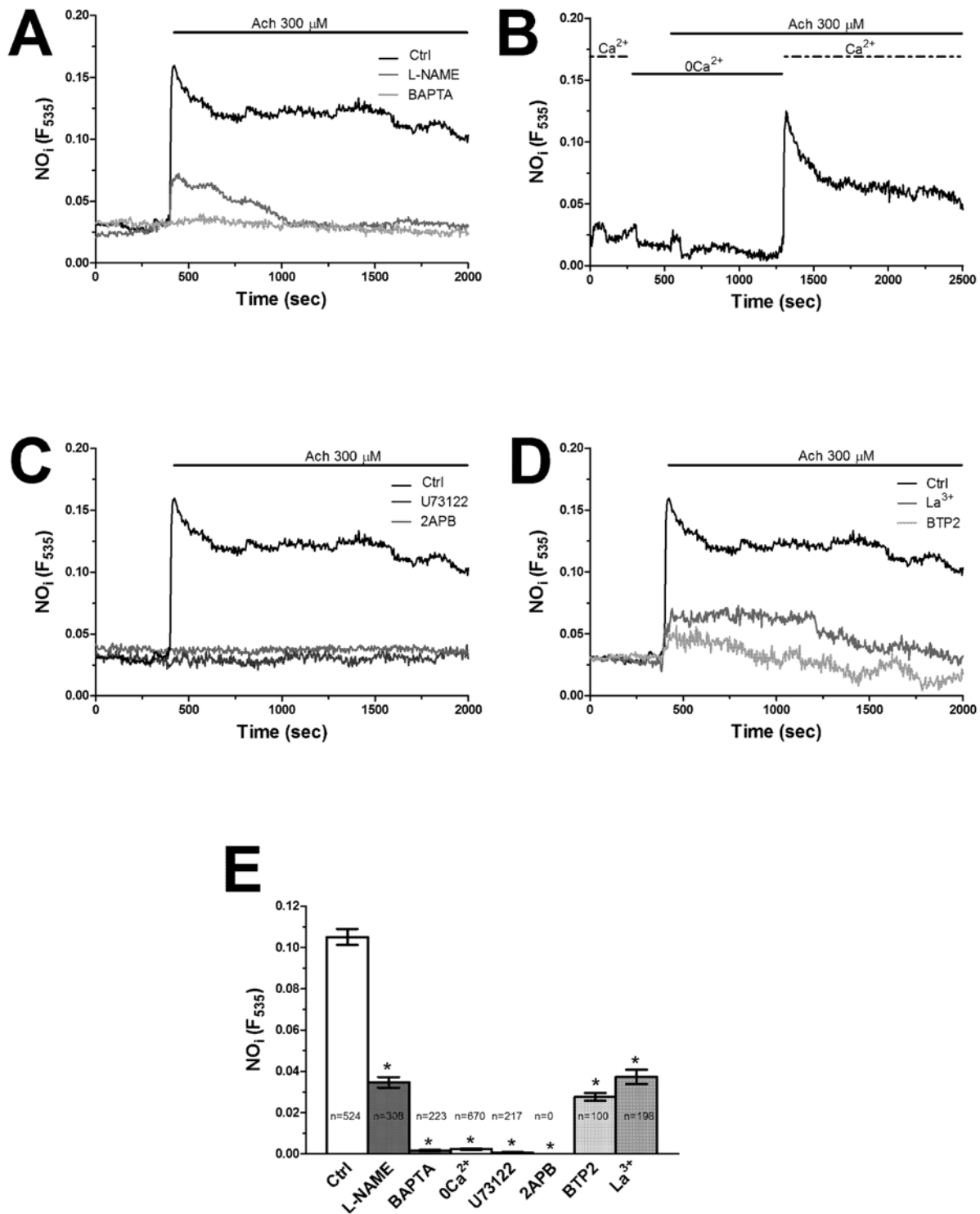


**Figure 9.** Acetylcholine induces SOCE activation in bEND5 cells. (A), Ach (300  $\mu M$ ) caused a clear increase in the rate of  $Mn^{2+}$  entry in bEND5 cells, which was dramatically reduced upon pre-treatment with either  $La^{3+}$  (10  $\mu M$ , 30 min) or BTP2 (10  $\mu M$ , 30 min). (B), mean $\pm$ SE of the percentage of bEND5 cells displaying Ach-induced  $Mn^{2+}$  entry in the absence or presence of either  $La^{3+}$  (10  $\mu M$ , 30 min) or BTP2 (10  $\mu M$ , 30 min). The asterisk indicates  $p < 0.05$ . (C), mean $\pm$ SE of the quenching rate of Fura-2 fluorescence signal measured in bEND5 cells before and after Ach addition. The asterisk indicates  $p < 0.05$ . (D), addition of CPA (10  $\mu M$ ) in the presence of extracellular  $Ca^{2+}$  caused a biphasic increase in  $[Ca^{2+}]_i$  due to passive  $Ca^{2+}$  mobilization followed by SOCE activation. Subsequent administration of Ach at 30 min from CPA application failed to enhance intracellular  $Ca^{2+}$  levels.

## Acetylcholine-induced intracellular $\text{Ca}^{2+}$ oscillations lead to NO synthesis in bEND5 cells

Finally, we assessed whether Ach-induced  $\text{Ca}^{2+}$  oscillations induced NO release by loading the cells with DAF/FM, a NO-sensitive fluorochrome ([128]). Ach caused an immediate elevation in DAF/FM fluorescence, which then decayed to a plateau level (Fig. 10A). Ach-induced NO production was significantly ( $p < 0.05$ ) reduced by pre-incubating the cells with either L-NG-Nitroarginine methyl ester (L-NAME; 100  $\mu\text{M}$ , 1 h), a widely employed eNOS inhibitor, or 1,2-is(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA; 30  $\mu\text{M}$ , 2 h), a membrane-permeable intracellular  $\text{Ca}^{2+}$  buffer (Fig. 10A and Fig. 10D). These findings clearly show that Ach recruits eNOS in a  $\text{Ca}^{2+}$ -dependent manner. Of note, NO release did not occur in the absence of external  $\text{Ca}^{2+}$  ( $0\text{Ca}^{2+}$ ), but rapidly resumed upon restoration of extracellular  $\text{Ca}^{2+}$  concentration (Fig. 10B and Fig. 10D). Moreover, NO production was severely reduced by inhibiting concomitant  $\text{Ca}^{2+}$  oscillations with U73122 (10  $\mu\text{M}$ ), and 2-APB (50  $\mu\text{M}$ , 30 min) (Fig. 10B and Fig. 10D). As expected, Ach-induced NO synthesis was also prevented by  $\text{La}^{3+}$  (10  $\mu\text{M}$ ) and BTP2 (10  $\mu\text{M}$ ) (Fig. 10C and Fig. 10D). These results demonstrate that Ach stimulates NO release from brain microvascular endothelial cells through an oscillatory increase in  $[\text{Ca}^{2+}]_i$ .

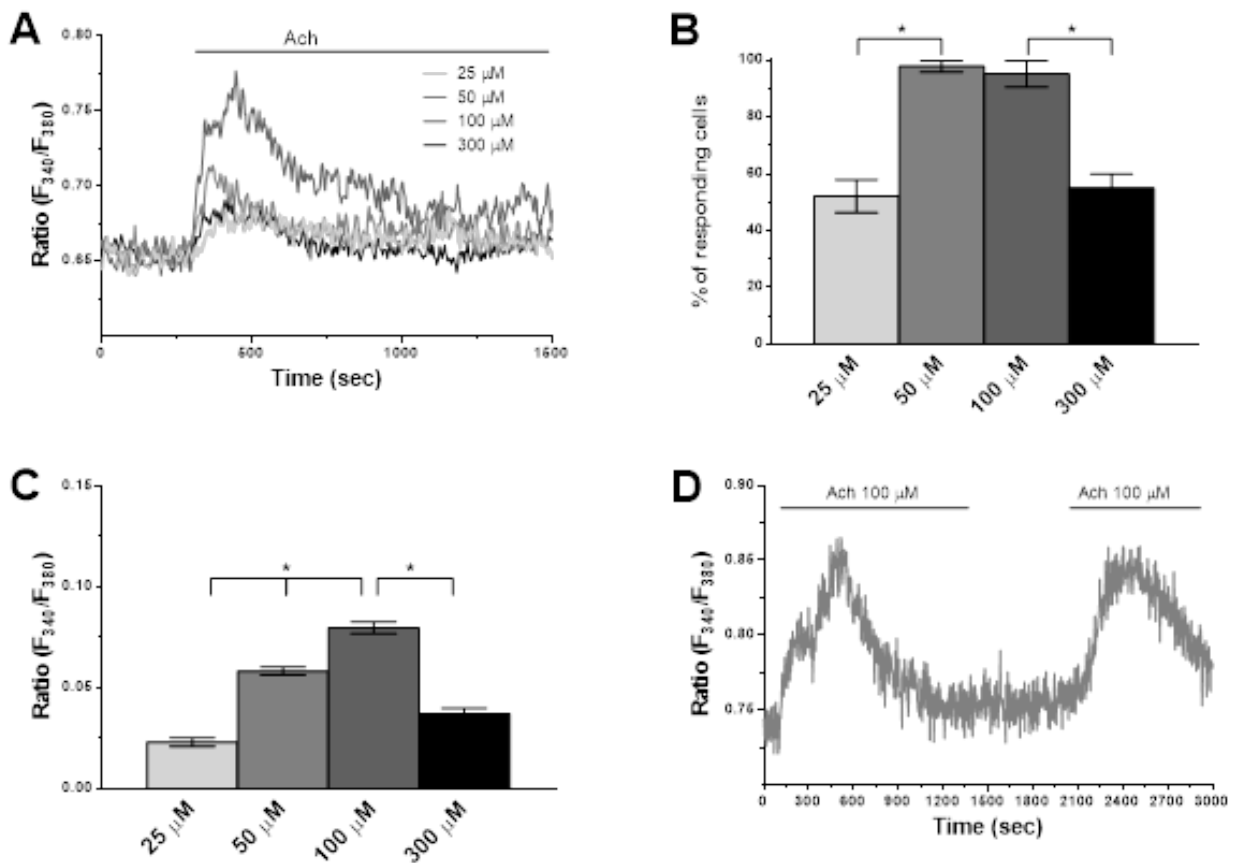




**Figure 10.** Acetylcholine-induced intracellular  $\text{Ca}^{2+}$  oscillations lead to NO release in bEND5 cells. (A), in bEND5 cells loaded with the NO-sensitive fluorophore, DAF/FM, Ach (300  $\mu\text{M}$ ), caused a robust increase NO-dependent signal, which was strongly reduced by either L-NAME (100  $\mu\text{M}$ , 1 h), a selective NOS blocker, or BAPTA (30  $\mu\text{M}$ , 2 h), a membrane-permeable intracellular  $\text{Ca}^{2+}$  chelator. (B), Ach failed to induce NO release under  $0\text{Ca}^{2+}$  conditions, but caused an immediate increase in NO levels upon restoration of the extracellular  $\text{Ca}^{2+}$  concentration. (C), Ach-induced NO release was unaffected by U73343 (10  $\mu\text{M}$ ; 30 min), while it was abolished by U73122 (10  $\mu\text{M}$ , 30 min), and 2-APB (50  $\mu\text{M}$ , 30 min). (D), Ach-induced NO production was inhibited by pre-treating the cells with either  $\text{La}^{3+}$  (10  $\mu\text{M}$ , 30 min) or BTP2 (10  $\mu\text{M}$ , 30 min). (E), mean $\pm$ SE of the magnitude of Ach-induced NO synthesis under the designated treatments. The asterisk indicates  $p < 0.05$ .

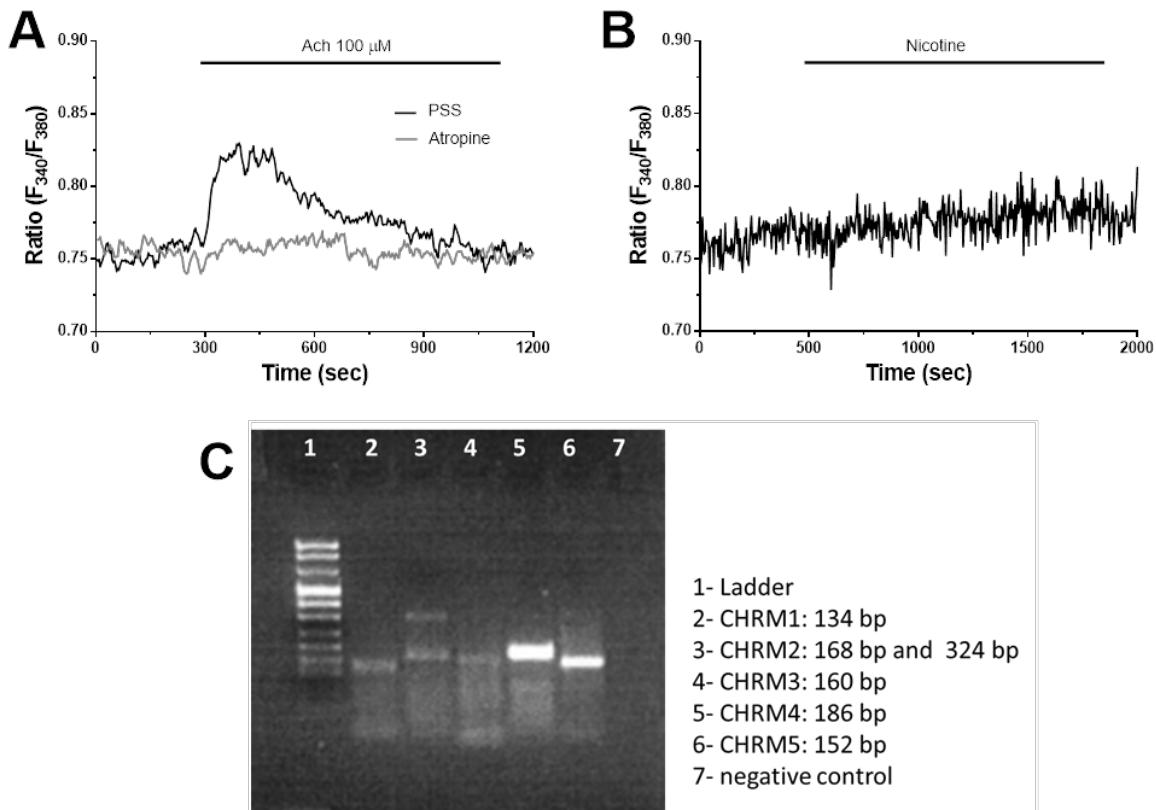
## Acetylcholine induces intracellular Ca<sup>2+</sup> signals in human brain microvascular endothelial cells

In order to assess whether Ach was able to induce Ca<sup>2+</sup> signals also in a human model, we exploited the hCMEC/D3 cell line, a widely established model of human brain microvascular endothelial cells ([245]). hCMEC/D3 were loaded with the Ca<sup>2+</sup>-sensitive dye Fura-2/AM and Ach was applied at different doses (from 25 μM to 300 μM). As shown in Fig. 11A, Ach caused a dose-dependent increase in [Ca<sup>2+</sup>]<sub>i</sub> between 25 μM to 100 μM, while the amplitude of the Ca<sup>2+</sup> response decreased by raising the agonist concentration up to 200 μM (Fig. 11A and 11C). The fraction of responding cells displayed a similar bell-shaped dose-response relationship, as the percentage of Ach-sensitive cells increased from 25 μM to 50-100 μM and further decreased at 200 μM (Fig. 11B and 11C). Conversely, there was no response at Ach concentrations lower than 25 μM (not shown). Of note, the response to 100 μM Ach displayed biphasic kinetics, consisting of an initial Ca<sup>2+</sup> which decayed to a plateau level of intermediate amplitude (Fig. 11A), indicative of SOCE activation ([168]). Conversely, at each of the other doses tested, Ach induced only a transient increase in [Ca<sup>2+</sup>]<sub>i</sub>, which is likely to reflect ER-dependent InsP<sub>3</sub>-mediated intracellular Ca<sup>2+</sup> mobilization ([31]). Finally, the Ca<sup>2+</sup> response to Ach did not desensitize after two 30 min-spaced consecutive applications. Taken together, these data suggest that the 100 μM Ach was the most suitable concentration for Ach to induce a Ca<sup>2+</sup> physiological response in hCMEC/D3 cells.



**Figure 11:** Acetylcholine evokes intracellular Ca<sup>2+</sup> transient increase in hCMEC/D3 cells. (A), Ach causes the immediate rise in [Ca<sup>2+</sup>]<sub>i</sub> followed by a rapid decay in a dose-dependent way. Only 100 μM Ach showed a plateau phase. (B), mean±SE of the percentage of hCMEC/D3 cells displaying Ach-induced Ca<sup>2+</sup> response at different agonist concentrations (from 50 μM to 300 μM). The asterisk indicates p<0.05. (C), mean±SE of the amplitude of Ach-induced Ca<sup>2+</sup> mobilization measured in hCMEC/D3 cell. The asterisk indicates p<0.05. In this and the following figures, Ach was added at the time indicated by the horizontal bar drawn around the Ca<sup>2+</sup> tracings.

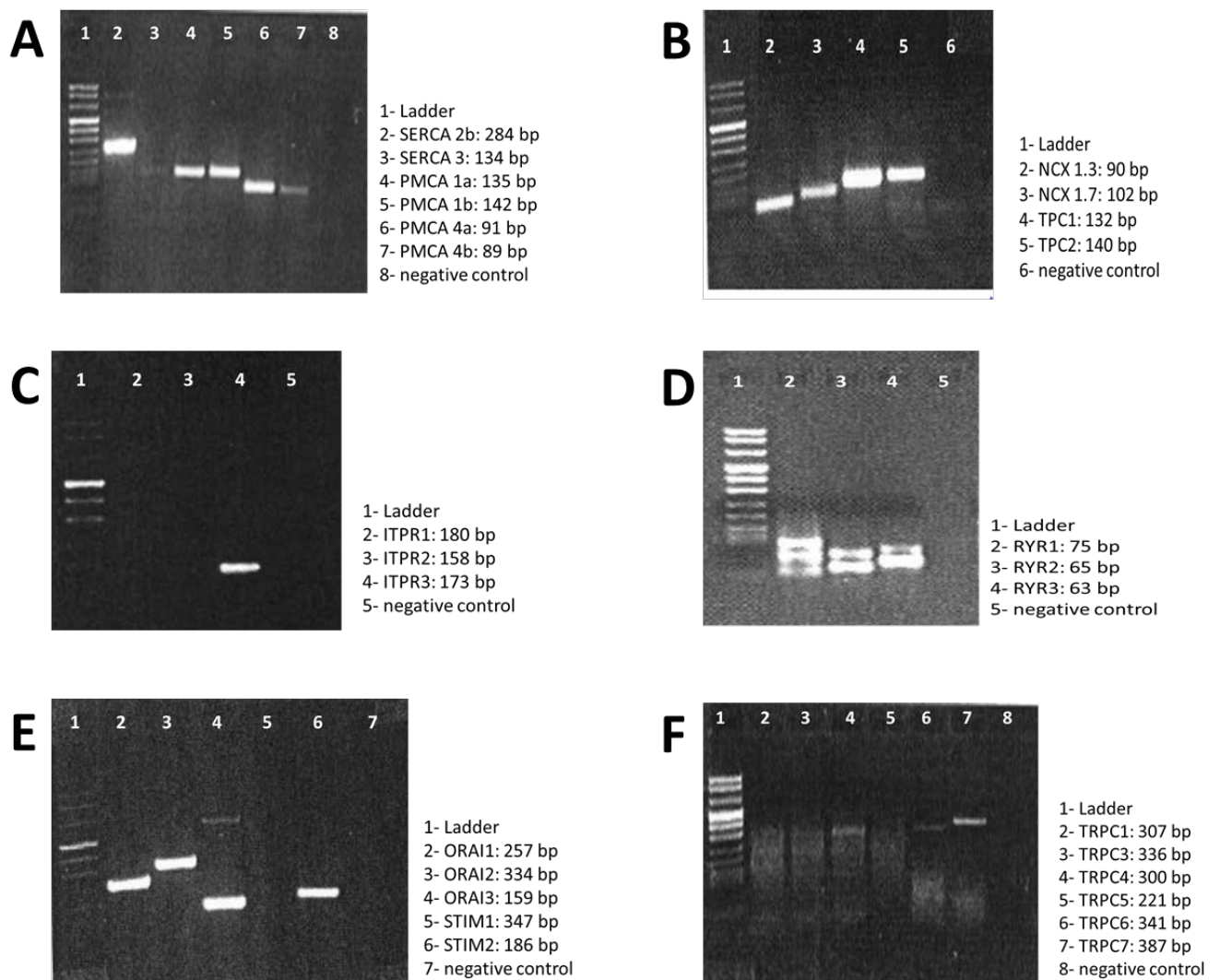
Similar to BEND5 cells, Ach-induced Ca<sup>2+</sup> transient was blocked by atropine (100 μM, 30 min) (Fig. 12A), whereas nicotine did not increase [Ca<sup>2+</sup>]<sub>i</sub> in hCMEC/D3 cells (Fig. 12B). These results suggest that the Ca<sup>2+</sup> response induced by Ach arise upon mAChR activation. Consistently, PCR revealed that human brain microvascular endothelial cells express high levels of M4-mAChR, which is coupled to cAMP production, and M5-mAChR (Fig. 12C), which induces InsP<sub>3</sub> synthesis.



**Figure 12.** Metabotropic muscarinic receptors drive the acetylcholine-induced  $Ca^{2+}$  response. (A), atropine (100  $\mu$ M, 30 min) prevents the initiation of the  $Ca^{2+}$  response to Ach. (B), nicotine (100  $\mu$ M) did not increase  $[Ca^{2+}]_i$  in hCMEC/D3 cells. (c), PCR gel of metabotropic muscarinic receptors (M-AChRs) revealed that, among all the five M-AChR mRNAs expressed in hCMEC/D3, M4 and M5 displayed the highest expression.

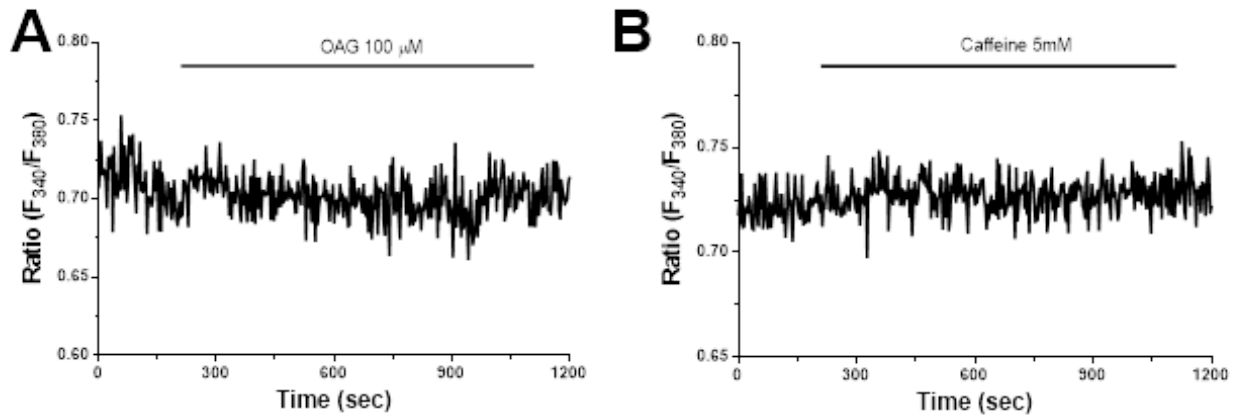
## Expression of the components of the Ca<sup>2+</sup> signaling toolkit in hCMEC/D3 cells

By using a similar approach to that employed for BEND5 cells, we demonstrated that the following transcripts were expressed in hCMEC/D3 cells: SERCA2b and SERCA3 (Fig. 13A); PMCA1a, PMCAb1, PMCA4a and PMCA4b (Fig. 13A); NCX1.3 and NCX1.7 (Fig. 13B); TPC1 and TPC2 (Fig. 13B); InsP<sub>3</sub>R3 (Fig. 13C); Orai 1, 2 and 3 (Fig. 13E); Stim2 (Fig. 13E); and TRPC7 (Fig. 13F).



**Figure 13.** The Ca<sup>2+</sup> toolkit of hCMEC/D3 cells. qRT-PCR gel of (A) SERCA and PMCA, (B) NCX and TPC, (C) InsP<sub>3</sub>R, (D) RyRs, (E) Orai and STIM isoform, (F) TRPC family.

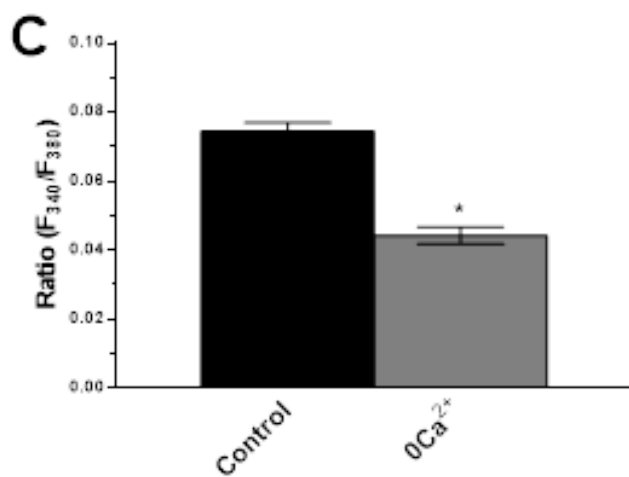
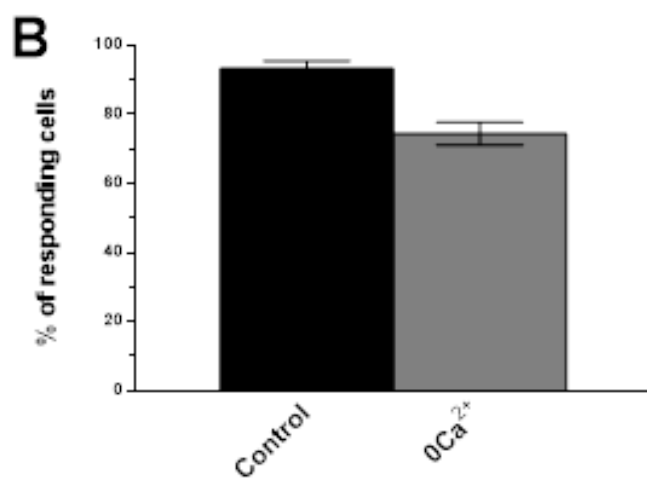
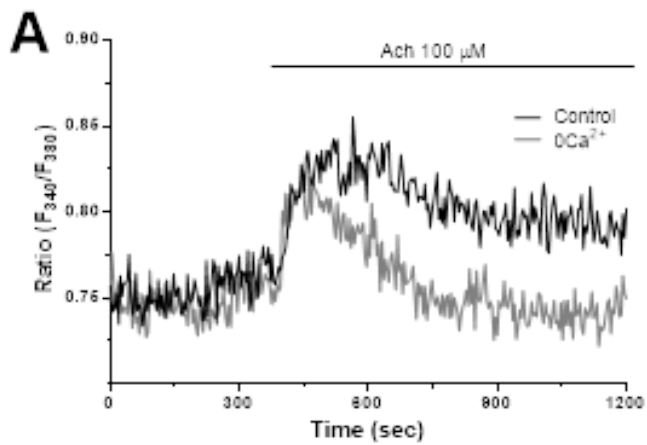
Of note, InsP<sub>3</sub>R1-2, Stim1 and the endothelial DAG-gated TRPC3 and TRPC6 channels were absent in hCMEC/D3 cells. In agreement with this observation, OAG (100 μM) failed to evoke any detectable Ca<sup>2+</sup> signal in these cells (Fig. 14A). Likewise, caffeine (5 mM), a selective RyR agonist, failed to increase [Ca<sup>2+</sup>]<sub>i</sub> in hCMEC/D3 cells (Fig.14B).



**Figure 14.** TRPC3,6-mediated Ca<sup>2+</sup> entry and RyRs-dependent Ca<sup>2+</sup> release are absent in hCMEC/D3 cells. OAG (100 μM) (A) and caffeine (5 mM) did not increase [Ca<sup>2+</sup>]<sub>i</sub> in hCMEC/D3 cells.

## Acetylcholine-induced intracellular Ca<sup>2+</sup> response requires intracellular Ca<sup>2+</sup> release and extracellular Ca<sup>2+</sup> entry

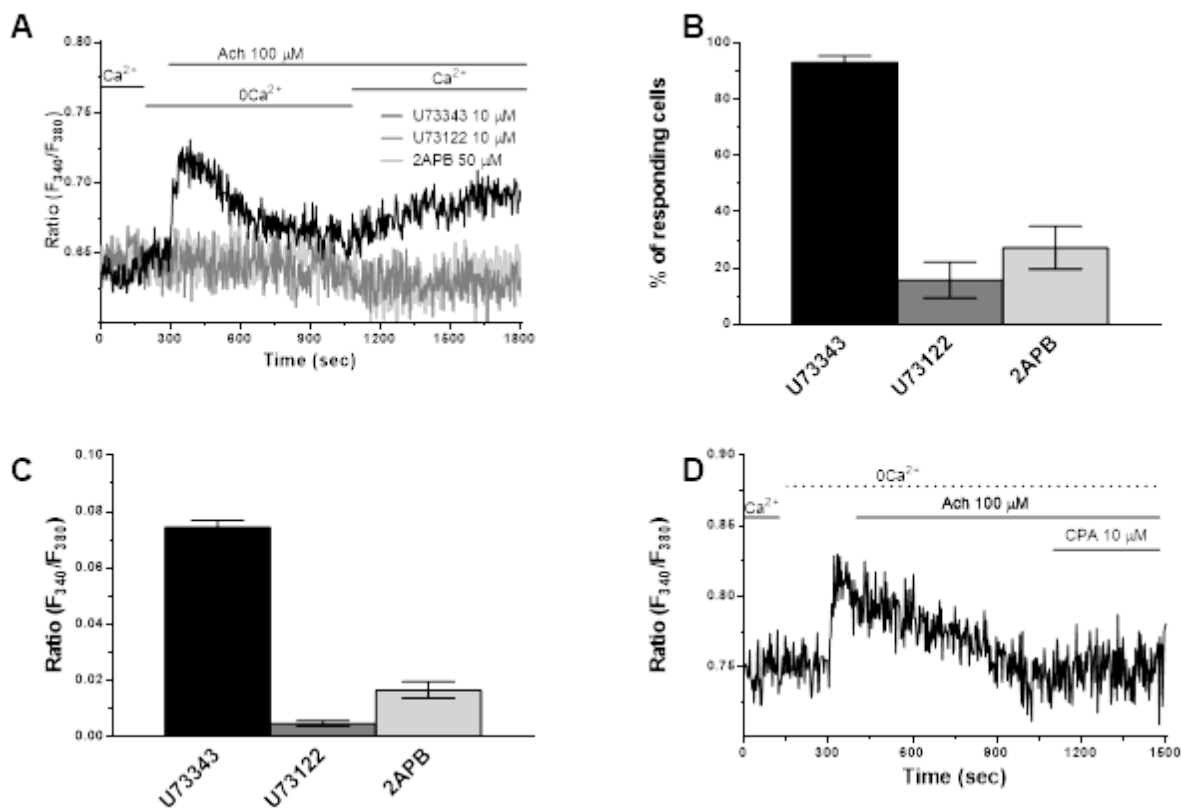
Interestingly, in absent of extracellular Ca<sup>2+</sup> (0Ca<sup>2+</sup>) 100 μM Ach was able to generate a rapid increase in [Ca<sup>2+</sup>]<sub>i</sub> that rapidly declined to the baseline, lacking the plateau phase that characterized the Ach-induced Ca<sup>2+</sup> response in presence of extracellular Ca<sup>2+</sup> (Fig. 15A-C). Taken together these results suggest that Ach-induced intracellular Ca<sup>2+</sup> signalling is triggered by intracellular Ca<sup>2+</sup> release and sustained by extracellular Ca<sup>2+</sup> entry.



**Figure 15.** Intracellular Calcium release and extracellular calcium entry are both involved in the Ach-induced  $Ca^{2+}$  response in hCMEC/D3. (A), Ach-induced increase in  $[Ca^{2+}]_i$  is significantly ( $p < 0.05$ ) lower in  $0Ca^{2+}$  compare with control. (B), bar histogram shows the average  $\pm$  SE of the percentage of responding cells. The asterisk indicates  $p < 0.05$ . (C), bar histogram shows the average  $\pm$  SE of the amplitude of the response. The asterisk indicates  $p < 0.05$ .

## Acetylcholine-induced intracellular $\text{Ca}^{2+}$ release is mediated by the $\text{PLC}\beta/\text{InsP}_3$ signalling pathway

In order to confirm that the  $\text{PLC}\beta/\text{InsP}_3$  signalling pathway drives Ach-induced intracellular  $\text{Ca}^{2+}$  release also in hCMEC/D3 cells, we adopted a similar strategy to that illustrated for BEND5 cells. We found that Ach-induced intracellular  $\text{Ca}^{2+}$  mobilization was abrogated by U73122 (10  $\mu\text{M}$ , 30 min), but not its inactive structural analog, U73343 (10  $\mu\text{M}$ , 30 min), by 2-APB (50  $\mu\text{M}$ , 30 min), and by CPA (Fig. 16A-Fig. 16D). Collectively, these findings demonstrate that M5-mAChRs mediate Ach-induced intracellular  $\text{Ca}^{2+}$  releases by recruiting the  $\text{PLC}\beta/\text{InsP}_3$  signaling pathway.

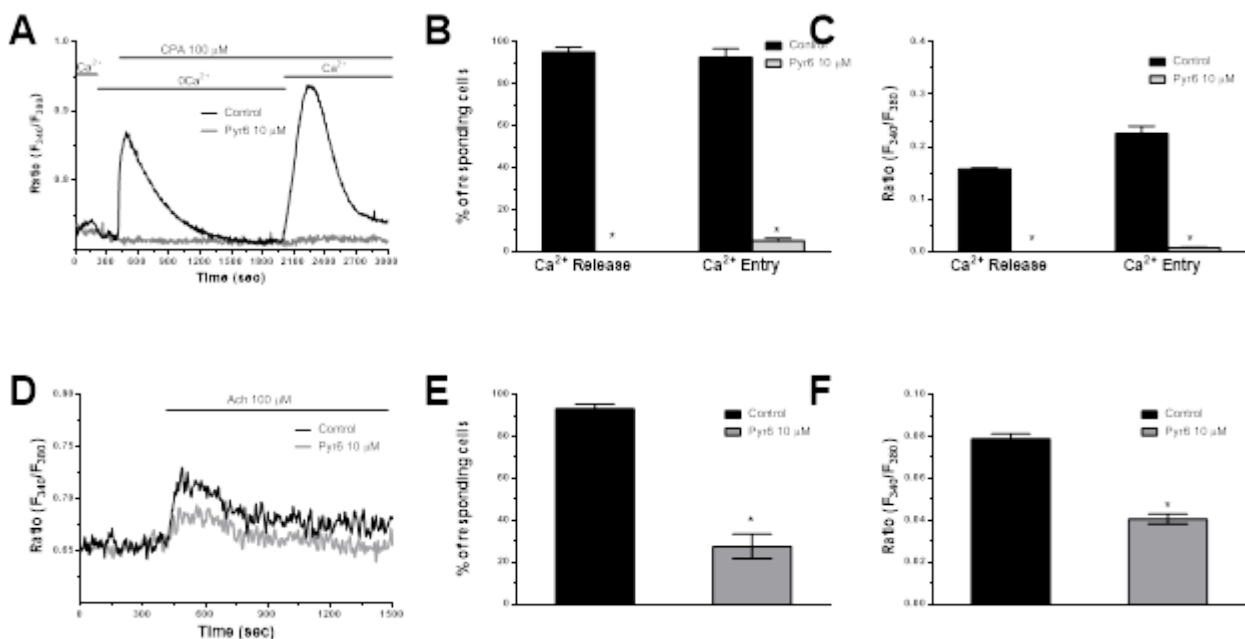


**Figure 16.** Ach-induced intracellular  $\text{Ca}^{2+}$  mobilization requires ER-dependent  $\text{Ca}^{2+}$  release through  $\text{InsP}_3\text{Rs}$ . (A), Ach-induced intracellular  $\text{Ca}^{2+}$  release was inhibited by U73122 (10  $\mu\text{M}$ , 30 min), but not by its structurally inactive analog, U73343 (10  $\mu\text{M}$ ; 30 min), by 2-APB (50  $\mu\text{M}$ , 30 min), and by CPA (to do). (B), bar histogram shows the average $\pm$ SE of the percentage of responding cells under the designated treatments. The asterisk indicates  $p < 0.05$ . (C), bar histogram shows the average $\pm$ SE of the amplitude of the response under the designated treatments. The asterisk indicates  $p < 0.05$ .



## SOCE participates to the $\text{Ca}^{2+}$ response to Ach in hCMEC/D3 cells

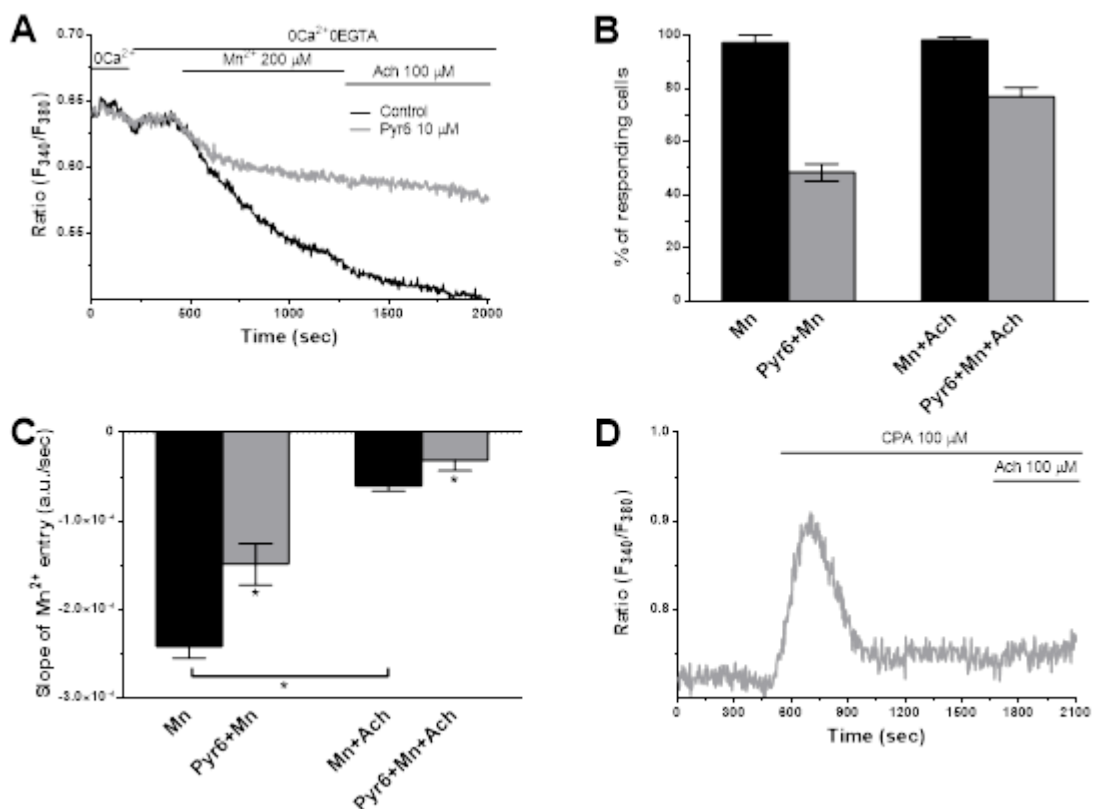
In order to confirm the involvement of a functional SOCE in hCMEC/D3 cells we exploited the “ $\text{Ca}^{2+}$  add-back” protocol ([167], [236]). As shown in Fig. 17A, CPA (10  $\mu\text{M}$ ) caused a robust SOCE in hCMEC/D3 cells. Conversely, pre-incubating the cells with Pyr 6 (10  $\mu\text{M}$ , 30 min), a newly synthesized pyrazole compound that inhibits Orai-mediated  $\text{Ca}^{2+}$  responses, prevented both CPA-induced intracellular  $\text{Ca}^{2+}$  mobilization and CPA-induced SOCE. The statistical analysis of these experiments is illustrated in Fig. 17B and in Fig. 17C. Not surprisingly Pyr6 (10  $\mu\text{M}$ ) also inhibited the  $\text{Ca}^{2+}$  response to Ach in most hCMEC/D3 cells (Fig. 17D-F). Of note, in the responding cells, the  $\text{Ca}^{2+}$  signal recorded in the presence of Pyr6 was similar to that measured in the absence of extracellular  $\text{Ca}^{2+}$ , i.e. it reached lower amplitude as compared to controls and lacked a plateau phase (Fig. 15A). These results suggests that SOCE is activated under resting conditions and controls ER  $\text{Ca}^{2+}$  levels also in hCMEC/D3 cells and that it could be further activated by cholinergic stimulation.



**Figure 17.** A functional SOCE is present and controls ER  $\text{Ca}^{2+}$  content in hCMEC/D3 cells. (A), the  $\text{Ca}^{2+}$  “add-back” protocol was applied to assess whether SOCE was active in these cells. CPA (100  $\mu\text{M}$ ) was administered under  $0\text{Ca}^{2+}$  conditions to deplete the ER  $\text{Ca}^{2+}$  pool and activate SOCs. The following restitution of  $\text{Ca}^{2+}$  to the external solution caused a second increase in  $[\text{Ca}^{2+}]_i$  due to SOCE activation. However, pre-incubating the cells with Pyr6 (10  $\mu\text{M}$ , 30 min), an inhibitor of Orai-mediated  $\text{Ca}^{2+}$  responses, blocked both CPA-induced intracellular  $\text{Ca}^{2+}$  mobilization and SOCE. (B), bar histogram of the average $\pm$ SE of the percentage of hCMEC/D3 cells responding to CPA in the absence

and presence of Pyr6 (10  $\mu$ M, 30 min). The asterisk indicates  $p < 0.05$ . (C), bar histogram of the average  $\pm$  SE of the percentage of the amplitude of CPA-induced  $\text{Ca}^{2+}$  release and SOCE in the absence and presence of Pyr6 (10  $\mu$ M, 30 min). The asterisk indicates  $p < 0.05$ . (D), Ach-induced intracellular  $\text{Ca}^{2+}$  response were inhibited by Pyr6 (10  $\mu$ M, 30 min). (E), bar histogram of the average  $\pm$  SE of the percentage of hCMEC/D3 cells displaying a response to Ach in the absence and in the presence of Pyr6 (10  $\mu$ M, 30 min). (F) bar histogram shows the average  $\pm$  SE of the amplitude of the response. The asterisk indicates  $p < 0.05$ .

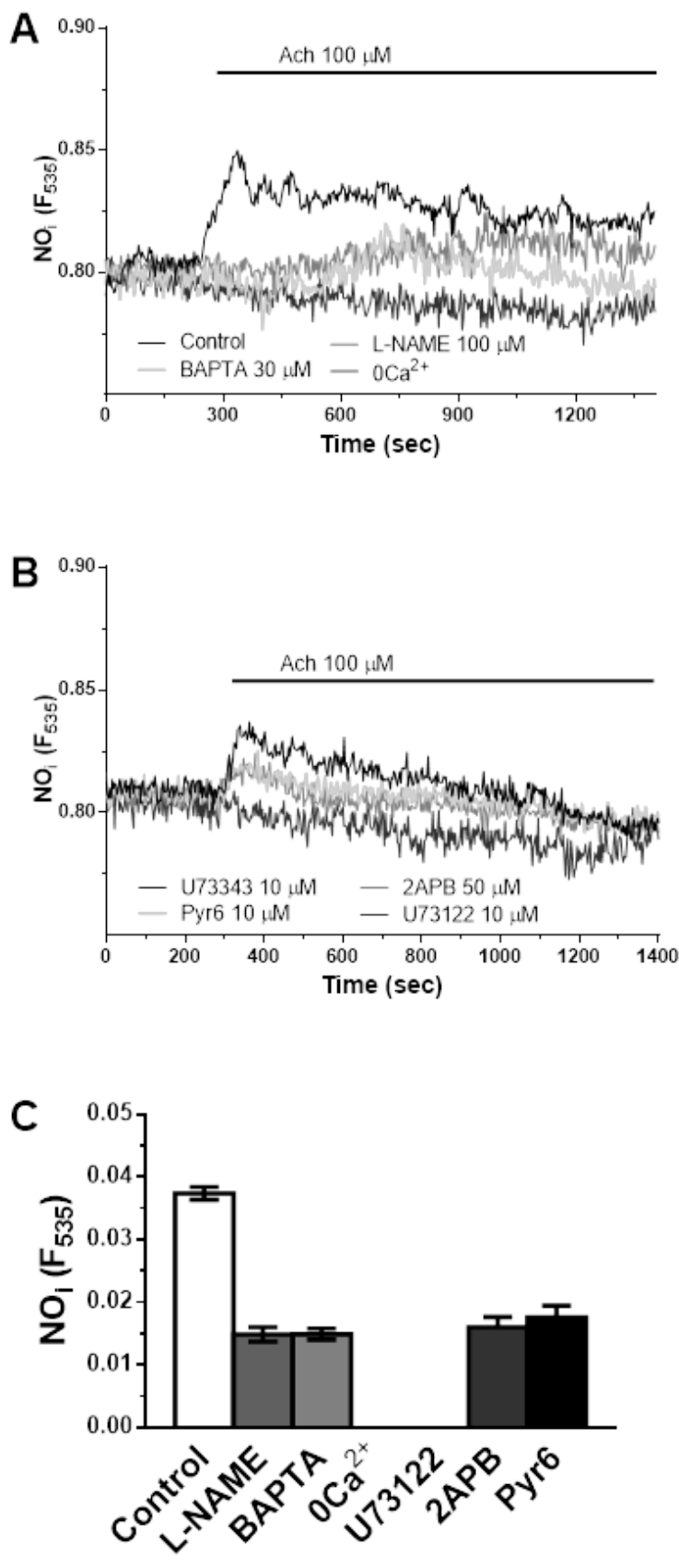
To further corroborate these hypotheses, we exploited again the  $\text{Mn}^{2+}$ -quenching technique (see above), which revealed a Pyr6-sensitive basal  $\text{Ca}^{2+}$  entry also in hCMEC/D3 cells (Fig. 18A-C). Addition of Ach further increased the slope of the quenching curve (Fig. 18A-C), which demonstrated the activation of an Ach-sensitive channel. The pharmacological blockade of SOCE with Pyr6 (10  $\mu$ M) prevented Ach-induced divalent cation entry (Fig. 18A-C). Taken together, these findings convincingly show that SOCE maintains the  $\text{Ca}^{2+}$  response to Ach in hCMEC/D3 cells. We finally challenged the cells with CPA (10  $\mu$ M) in the presence of external  $\text{Ca}^{2+}$  (Fig. 18D). The application of CPA induces a rapid elevation in  $[\text{Ca}^{2+}]_i$  associated to ER emptying followed by a decay phase to a discernible plateau level, due to SOCE activation ([242]). At 30 min after CPA addition, a time interval sufficient for depleting ER  $\text{Ca}^{2+}$  stores and fully activating SOCE ([243], [244]), Ach failed to induce  $\text{Ca}^{2+}$  entry (Fig. 18D). This result clearly demonstrates that Ach is unlikely to activate a store-independent channel in brain microvascular endothelial cells ([244], [242]).



**Figure 18.** Acetylcholine induces SOCE activation in hCMEC/D3 cells. (A), Ach (100  $\mu\text{M}$ ) caused an increase in the rate of  $\text{Mn}^{2+}$  entry in hCMEC/D3 cells, which was dramatically reduced upon pre-treatment with Pyr6 (10  $\mu\text{M}$ , 30 min). (B), mean $\pm$ SE of the percentage of hCMEC/D3 cells displaying  $\text{Mn}^{2+}$  entry and Ach-induced  $\text{Mn}^{2+}$  entry in the absence or presence of Pyr6 (10  $\mu\text{M}$ , 30 min). The asterisk indicates  $p < 0.05$ . (C), mean $\pm$ SE of the quenching rate of Fura-2 fluorescence signal measured in hCMEC/D3 cells before and after  $\text{Mn}^{2+}$  and Ach addition in presence and in absence of Pyr6 (10  $\mu\text{M}$ , 30 min). The asterisk indicates  $p < 0.05$ . (D), addition of CPA (10  $\mu\text{M}$ ) in the presence of extracellular  $\text{Ca}^{2+}$  caused a biphasic increase in  $[\text{Ca}^{2+}]_i$  due to passive  $\text{Ca}^{2+}$  mobilization followed by SOCE activation. Subsequent administration of Ach at 30 min from CPA application failed to enhance intracellular  $\text{Ca}^{2+}$  levels.

## Acetylcholine-induced $\text{Ca}^{2+}$ signalling leads to NO synthesis in hCMEC/D3 cells

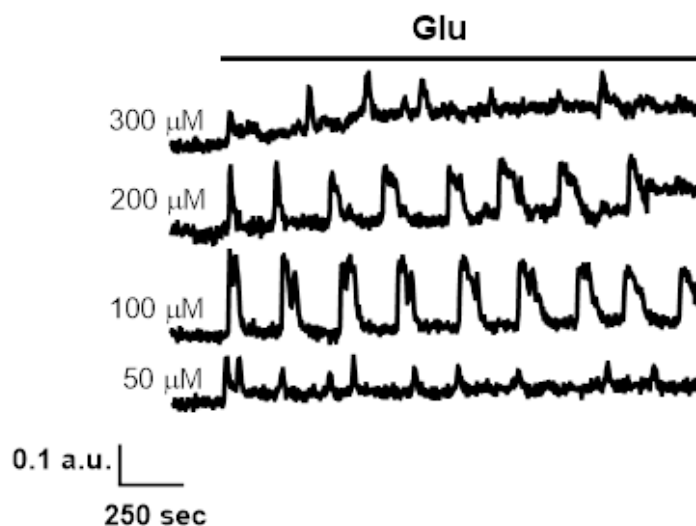
Finally, we assessed whether Ach-induced  $\text{Ca}^{2+}$  increase induced NO release by loading the cells with DAF/FM, the NO-sensitive fluorochrome ([128]). Ach (100  $\mu\text{M}$ ) caused a sustained increase in NO levels. (Fig. 19A). Ach-induced NO production was significantly ( $p < 0.05$ ) reduced by pre-incubating the cells with either L-NAME (100  $\mu\text{M}$ , 1 h) or BAPTA (30  $\mu\text{M}$ , 2 h). These findings clearly show that Ach recruits eNOS in a  $\text{Ca}^{2+}$ -dependent manner. Of note, NO release did not occur in the absence of external  $\text{Ca}^{2+}$  ( $0\text{Ca}^{2+}$ ) (Fig. 19A and Fig. 19C). Moreover, NO production was severely reduced by U73122 (10  $\mu\text{M}$ ), 2-APB (50  $\mu\text{M}$ , 30 min) and Pyr6 (10  $\mu\text{M}$ ) (Fig. 19B and Fig. 19C). These results demonstrate that both  $\text{InsP}_3\text{Rs}$  and SOCE sustain Ach-induced NO release in hCMEC/D3.



**Figure 19.** Acetylcholine-induced increase in  $[Ca^{2+}]_i$  leads to NO release in hCMEC/D3 cells. (A), Ach (100  $\mu$ M) caused a robust increase in DAF/FM fluorescence in hCMEC/D3 cells, that was strongly reduced by either L-NAME (100  $\mu$ M, 1 h) or BAPTA (30  $\mu$ M, 2 h), a membrane-permeable intracellular  $Ca^{2+}$  chelator. Moreover, Ach failed to induce NO release under  $0Ca^{2+}$  conditions. (B), Ach-induced NO release was unaffected by U73343 (10  $\mu$ M; 30 min), while it was abolished by U73122 (10  $\mu$ M, 30 min). 2-APB (50  $\mu$ M, 30 min) and Pyr6 (10  $\mu$ M, 30 min) were also able to inhibit NO release. (C), mean $\pm$ SE of the magnitude of Ach-induced NO synthesis under the designated treatments. The asterisk indicates  $p < 0.05$ .

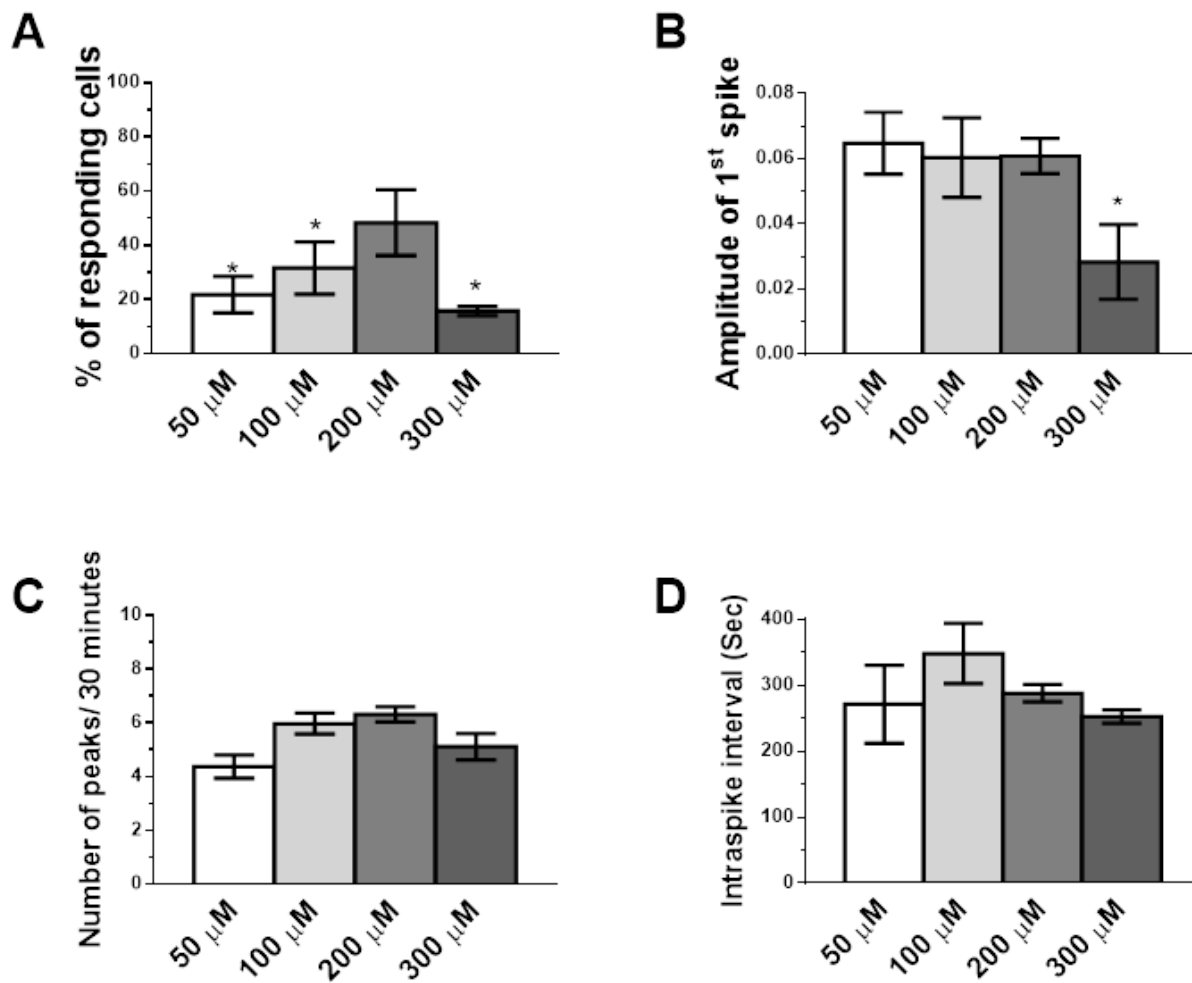
## Glutamate induces intracellular Ca<sup>2+</sup> oscillations in bEnd5 cells

After the characterization of Ach-induced Ca<sup>2+</sup> and NO signals in murine and human brain microvascular endothelial cells, we focused on Glu. Again, bEND5 cells were loaded with with the Ca<sup>2+</sup>-sensitive fluorochrome, Fura-2/AM, in order to assess whether Glu induces intracellular Ca<sup>2+</sup> activity. The addition of glutamate to quiescent cells caused a dose-dependent oscillatory increase in [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 20 and 21). Similar to Ach, each Ca<sup>2+</sup> oscillation was preceded by a pacemaker Ca<sup>2+</sup> elevation, which strongly hints at the involvement of InsP<sub>3</sub>-induced ER Ca<sup>2+</sup> release ([231]).



**Figura 20:** Glutamate evokes repetitive Ca<sup>2+</sup> transients in bEND5 cells. Glutamate causes the immediate initiation of oscillatory Ca<sup>2+</sup> spikes whose duration, amplitude of the 1<sup>st</sup> spike and interspike interval (ISI) was a function of agonist concentration. In this and the following figures, Glut was added at the time indicated by the horizontal bar drawn around the Ca<sup>2+</sup> tracings. The baseline of Ca<sup>2+</sup> tracings has been shifted to avoid their overlapping for representation purposes.

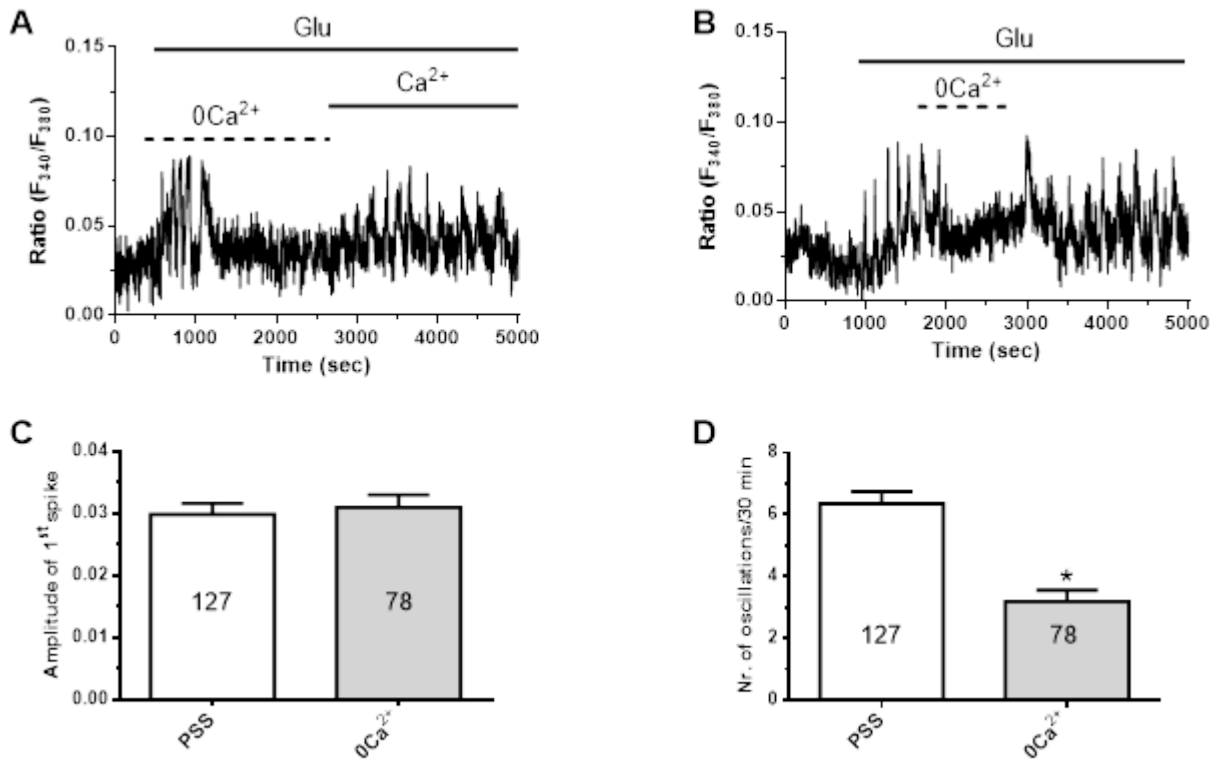
The spiking Ca<sup>2+</sup> response arose at 50 μM and achieved a peak at 200 μM, as the percentage of responding cells (Fig. 21A), the amplitude of the 1<sup>st</sup> Ca<sup>2+</sup> transient (Fig. 21B), the number of Ca<sup>2+</sup> spikes/30 min (Fig. 21C) and the frequency of the Ca<sup>2+</sup> train (Fig. 21D) reached their highest value at this dose, and decreased by further increasing glutamate concentration to 300 μM.



**Figure 21.** Statistical analysis of Glut-induced intracellular  $\text{Ca}^{2+}$  oscillations in bEND5 cells. Bar histograms show the average $\pm$ SE of the percentage of responding cells (A), amplitude of the 1<sup>st</sup> spike, (B), number of oscillations/30 min(D), average ISI. The asterisk indicates  $p < 0.05$ .

## Glutamate-induced intracellular $\text{Ca}^{2+}$ oscillations require both intracellular $\text{Ca}^{2+}$ release and extracellular $\text{Ca}^{2+}$ entry

The analysis of Ach-induced intracellular  $\text{Ca}^{2+}$  oscillations revealed that intracellular  $\text{Ca}^{2+}$  signals in bEnd5 cells may involve ER-dependent  $\text{Ca}^{2+}$  mobilization through  $\text{InsP}_3\text{Rs}$  and SOCE. Therefore, in order to assess the relative contribution of endogenous  $\text{Ca}^{2+}$  release and  $\text{Ca}^{2+}$  entry to the evoked response, we first applied glutamate (200  $\mu\text{M}$ ) in the absence of extracellular  $\text{Ca}^{2+}$  ( $0\text{Ca}^{2+}$ ). Glutamate still induced repetitive  $\text{Ca}^{2+}$  spikes under  $0\text{Ca}^{2+}$  conditions, but these  $\text{Ca}^{2+}$  oscillations rapidly run down (Fig. 22A and Fig. 22C). The subsequent addition of  $\text{Ca}^{2+}$  to perfusate resumed the oscillatory response (Fig. 22A and Fig. 22C). The amplitude of the 1<sup>st</sup>  $\text{Ca}^{2+}$  spike was not different in the presence and absence of external  $\text{Ca}^{2+}$  (Fig. 22B), thus suggesting that  $\text{Ca}^{2+}$  entry is not necessary to initiate the oscillations, but is strictly required to maintain them over time ([246]). Likewise, removal of extracellular  $\text{Ca}^{2+}$  during ongoing oscillations caused the  $\text{Ca}^{2+}$  oscillatory response to persist only for 1-2 spikes before rapidly returned  $[\text{Ca}^{2+}]_i$  to resting levels (Fig. 22D). Again, intracellular  $\text{Ca}^{2+}$  burst quickly recovered when  $\text{Ca}^{2+}$  was restored to the extracellular bathing solution (Fig. 22D). Overall, these findings strongly indicate that glutamate-induced intracellular  $\text{Ca}^{2+}$  oscillations are shaped by the rhythmical mobilization of the intracellular  $\text{Ca}^{2+}$  reservoir and sustained by extracellular  $\text{Ca}^{2+}$  entry.

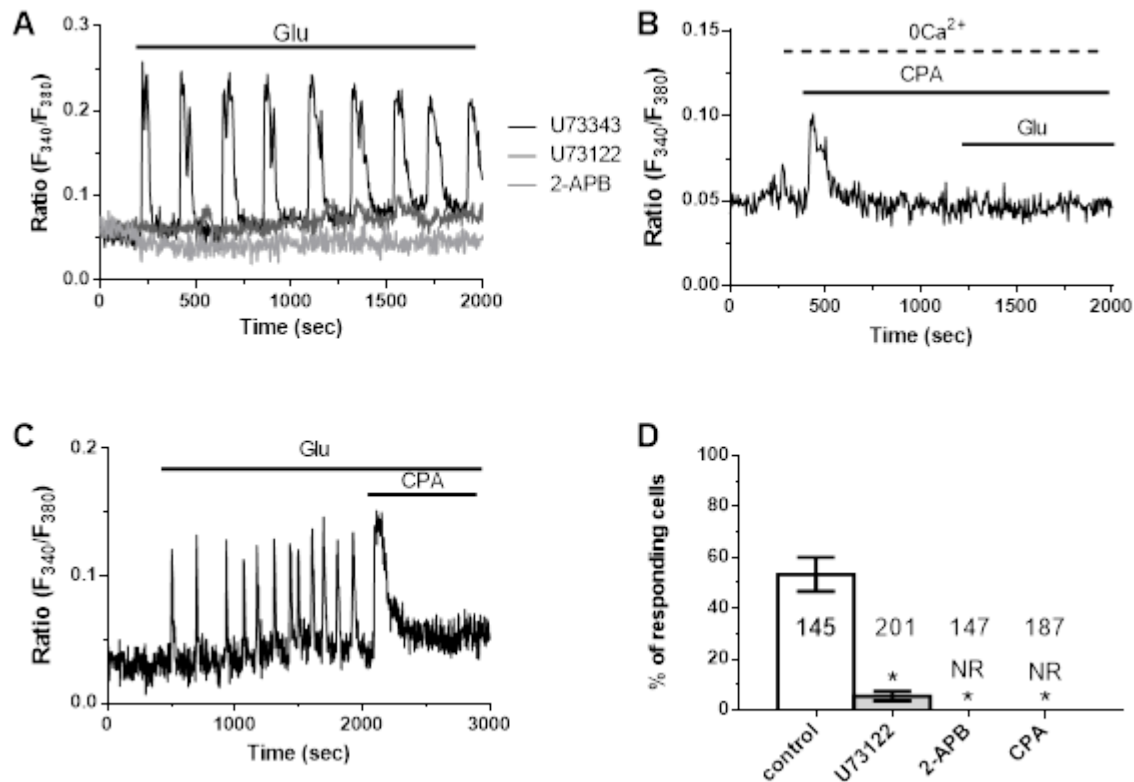


**Figure 22.** Glut-induced intracellular Ca<sup>2+</sup> oscillations require Ca<sup>2+</sup> release and extracellular Ca<sup>2+</sup> entry. (A), Glut induced repetitive Ca<sup>2+</sup> spikes under 0Ca<sup>2+</sup> conditions, but these Ca<sup>2+</sup> oscillations rapidly run down. Glut-induced Ca<sup>2+</sup> oscillations resumed when Ca<sup>2+</sup> was added to perfusate. (B), questa figura dice un'altra cosa, guardala meglio leggendo il testo. (C), bar histogram shows the average±SE of the amplitude of 1<sup>st</sup> spike induced by Glut in the presence and absence of extracellular Ca<sup>2+</sup>. (D), bar histogram shows the number of oscillation/30 min induced by glutamate in bEND5 cells in the presence and absence of external Ca<sup>2+</sup>. The asterisk indicateses p<0.05.



## Glutamate-induced intracellular $\text{Ca}^{2+}$ oscillations are generated by $\text{Ca}^{2+}$ release from the ER

As mentioned earlier, the ER represents the main intracellular  $\text{Ca}^{2+}$  reservoir in bEnd5 cells and delivers periodic  $\text{Ca}^{2+}$  spikes in response to extracellular stimulation through  $\text{InsP}_3\text{Rs}$ . Accordingly, Glut-induced intracellular  $\text{Ca}^{2+}$  oscillations were blocked by U73122 (10  $\mu\text{M}$ , 30 min), while its inactive analogue, U73343 (10  $\mu\text{M}$ , 30 min), had no effect (Fig. 23A and 23D). Likewise, 2-APB (50  $\mu\text{M}$ , 30 min) prevented the initiation of glutamate-induced intracellular  $\text{Ca}^{2+}$  oscillations under  $0\text{Ca}^{2+}$  conditions. Therefore,  $\text{InsP}_3$  plays a key role also in the generation of repetitive  $\text{Ca}^{2+}$  transients induced by Glut in BEND5 cells. To further confirm this hypothesis, we depleted the ER  $\text{Ca}^{2+}$  store with CPA in the absence of extracellular  $\text{Ca}^{2+}$ . As expected, Glut failed to trigger any detectable increase in intracellular  $\text{Ca}^{2+}$  levels under these conditions (Fig. 23B and 23D). According to this model, after each  $\text{Ca}^{2+}$  spike,  $\text{Ca}^{2+}$  must be sequestered back by SERCA to allow the ER to continue releasing  $\text{Ca}^{2+}$  and support the intracellular  $\text{Ca}^{2+}$  waves. If this hypothesis was true, the pharmacological blockade of SERCA should suppress the  $\text{Ca}^{2+}$  spikes as the ER can no longer be replenished. Consistently, addition of CPA during ongoing spikes caused an abrupt increase in  $[\text{Ca}^{2+}]_i$ , due to the passive  $\text{Ca}^{2+}$  leak, followed by complete inhibition of glutamate-induced intracellular  $\text{Ca}^{2+}$  transients (Fig. 23C). The  $[\text{Ca}^{2+}]_i$ , however, decayed to a plateau level which was indicative of SOCE activation. Therefore, these data strongly support the hypothesis that  $\text{InsP}_3\text{Rs}$  mediate the periodic release of ER  $\text{Ca}^{2+}$  in response to glutamate stimulation in bEnd5 cells.

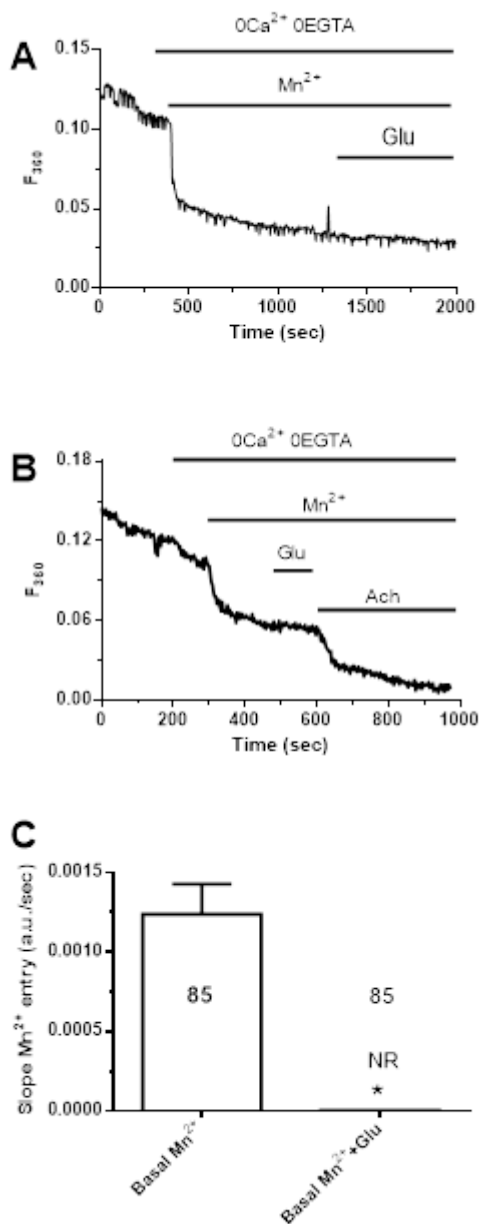


**Figure 23.** Glut-induced intracellular  $Ca^{2+}$  oscillations are mediated by rhythmical ER-dependent  $Ca^{2+}$  release through  $InsP_3Rs$ . (A), Glut-induced  $Ca^{2+}$  oscillations were inhibited by U73122 (10  $\mu M$ , 30 min), but not by structurally inactive analog, U73343 (10  $\mu M$ ; 30 min), and by 2-APB (50  $\mu M$ , 30 min). (B), addition of CPA (10  $\mu M$ ) in absence of extracellular  $Ca^{2+}$  caused an increase in  $[Ca^{2+}]_i$  due to  $Ca^{2+}$  leak from ER. Subsequent administration of glutamate failed to enhance intracellular  $Ca^{2+}$  levels. (C), addition of CPA (100  $\mu M$ ) during ongoing oscillations caused a complete inhibition of Glut-induced  $Ca^{2+}$  transients. (D), bar histogram shows the average  $\pm$  SE of the percentage of responding cells under the designated treatments. The asterisk indicates  $p < 0.05$ .

## Glutamate-induced intracellular Ca<sup>2+</sup> oscillations are maintained by constitutive Ca<sup>2+</sup> entry

The results described in Figure 25A and Figure 25B demonstrate that extracellular Ca<sup>2+</sup> entry is necessary to maintain the oscillatory Ca<sup>2+</sup> response to glutamate over time. As described above, SOCE represents the main pathway for agonist-induced Ca<sup>2+</sup> entry in bEnd5 cells, but is also heavily activated in the absence of extracellular stimulation to replenish the ER Ca<sup>2+</sup> content. Therefore, in order to evaluate whether SOCE is the Ca<sup>2+</sup>-permeable route recruited by glutamate to sustain the intracellular Ca<sup>2+</sup> oscillations, we could not pre-incubate the cells with any SOCE inhibitor.

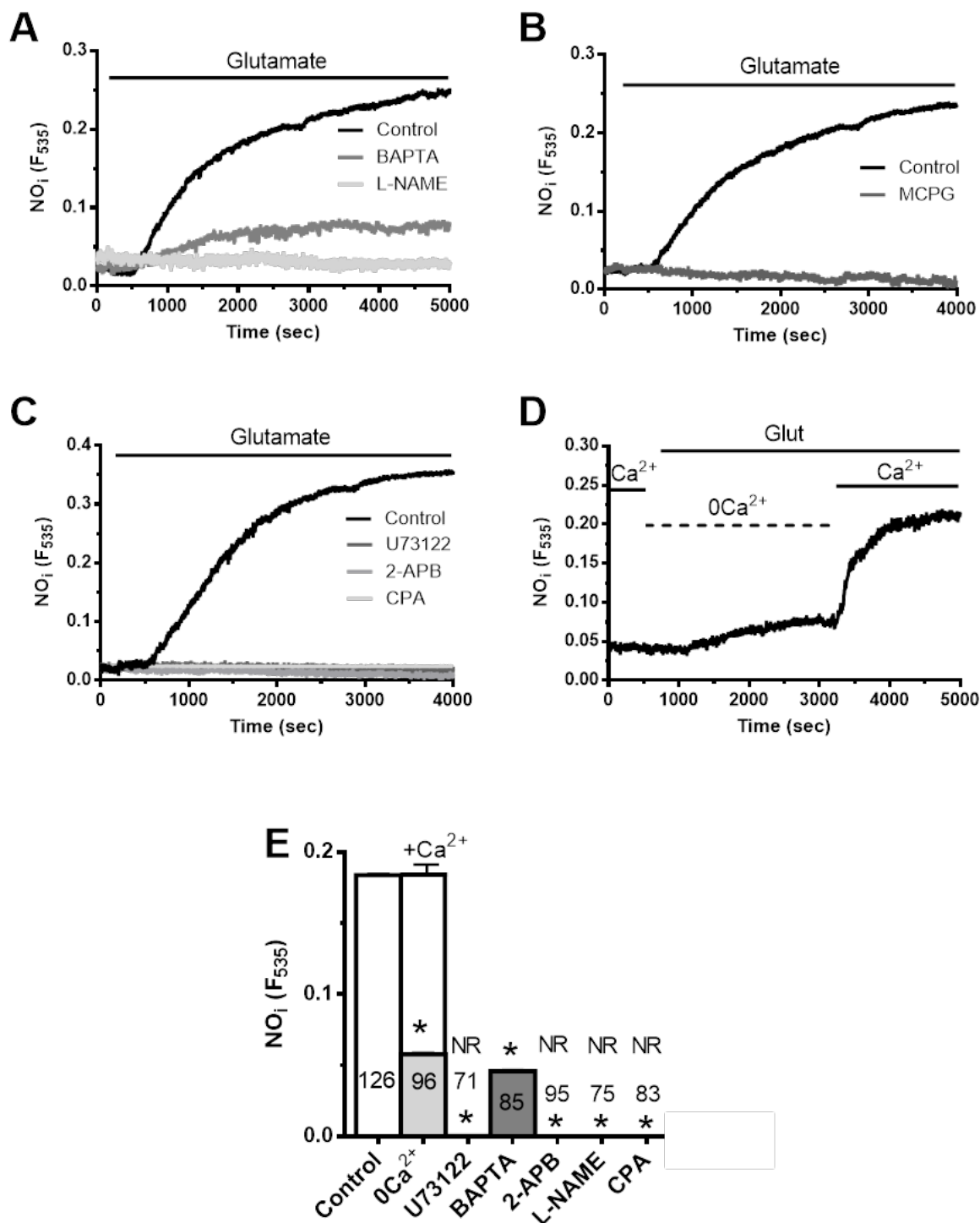
Therefore, we turned to the Mn<sup>2+</sup>-quenching technique, which revealed that Glutamate was not able to enhance the rate of the constitutive Mn<sup>2+</sup> influx in BEND5 cells (Fig. 24A). As expected, in the same batch of cells, acetylcholine (100 μM) increased the rate of Fura-2 fluorescence decline (Fig. 24B). According to the statistical analysis (Fig. 24C), these data provide a strong evidence that glutamate does not activate metabotropic Ca<sup>2+</sup> entry in bEnd5 cells. It turns out that it is the constitutive SOCE to maintain glutamate-induced intracellular Ca<sup>2+</sup> oscillations by providing releasable Ca<sup>2+</sup> to the InsP<sub>3</sub>-sensitive ER stores ([237]).



**Figure 24.** Glutamate induces intracellular calcium entry activation in bEND5 cells. (A), Glut was not able to enhance the rate of constitutive Mn<sup>2+</sup> influx in BEND5 cells. (B), Acetylcholine (100 μM) increased the rate of Mn<sup>2+</sup> entry in bEND5 cells. (C), mean±SE of the slope of basal and Glut-induced in bEND5 cells. The asterisk indicates p<0.05.

## Glutamate-induced intracellular $\text{Ca}^{2+}$ oscillations lead to NO release in bEnd5 cells

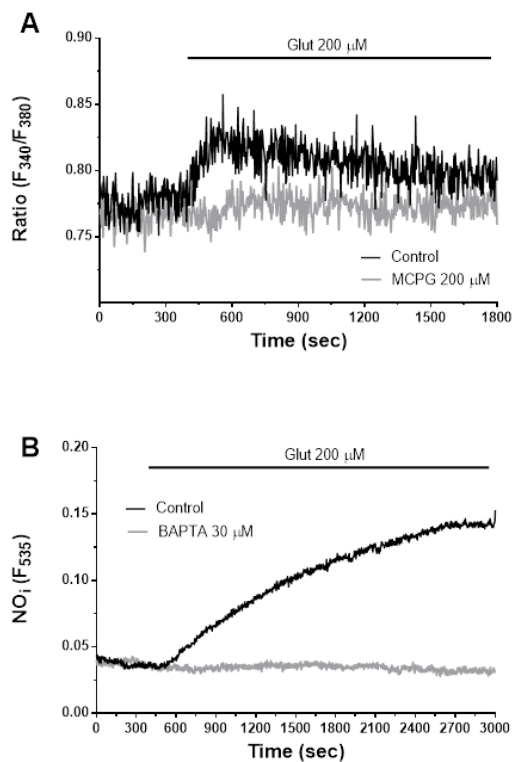
Finally, we assessed whether Glut-induced intracellular  $\text{Ca}^{2+}$  oscillations drive NO release by loading the cells with the NO-sensitive fluorochrome, DAF/FM ([13], [119]). Glut caused a slow, but sustained increase in NO levels, which started with a latency of  $387 \pm 53$  sec ( $n=143$ ) and reached a plateau after about 1000 sec (Fig. 25A). This signal was suppressed by MCPG (200  $\mu\text{M}$ , 10 min) (Fig. 25B and 25E). Moreover, glutamate-induced NO release was abolished by pre-incubating the cells with L-NAME (100  $\mu\text{M}$ , 1 h) or BAPTA (30  $\mu\text{M}$ , 2 h) (Fig. 25A and 25E). These findings demonstrate that glutamate engages eNOS in a  $\text{Ca}^{2+}$ -dependent manner. Also, glutamate failed to elevate NO levels in the presence of either U73122 (10  $\mu\text{M}$ , 30 min), 2-APB (50  $\mu\text{M}$ , 30 min), or CPA (10  $\mu\text{M}$ , 30 min) (Fig. 25C and 25E). Unlike acetylcholine, Glut-induced NO release also occurred in the absence of external  $\text{Ca}^{2+}$  and was further enhanced by the subsequent addition of  $\text{Ca}^{2+}$  to the extracellular solution (Fig. 25D), in agreement with the  $\text{Ca}^{2+}$  imaging data (Fig. 22A). Collectively, these findings clearly show that Glut stimulates NO release by activating intracellular  $\text{Ca}^{2+}$  oscillations in mouse brain endothelial cells.



**Figure 25.** Glut-induced intracellular Ca<sup>2+</sup> oscillations lead to NO release in bEND5 cells. (A), Glut (200 μM), caused an increase NO-dependent signal in bEND5 cells loaded with the NO-sensitive fluorophore, DAF/FM; this signal was strongly reduced by either L-NAME (100 μM, 1 h) or BAPTA (30 μM, 2 h). (B), Glut-induced NO release was abolished by MCPG (200 μM, 10 min). (C) Glut-induced NO release was also abolished by U73122 (10 μM, 30 min), 2-APB (50 μM, 30 min) and CPA (10 μM, 30 min). (D), Glut induced NO production also in absence of extracellular Ca<sup>2+</sup> (0Ca<sup>2+</sup>). (E), mean±SE of the magnitude of Glut-induced NO synthesis under the designated treatments. The asterisk indicates p<0.05.

## Glutamate induces Ca<sup>2+</sup>-dependent NO synthesis in human brain microvascular endothelial cells

As for Ach, we assessed whether glutamate stimulates NO production in a Ca<sup>2+</sup>-dependent manner also in HCMEC/D3 cells. As shown in Figure 27A, Glut (200  $\mu$ M) caused a slow increase in [Ca<sup>2+</sup>]<sub>i</sub>, which then gradually decayed to the baseline. As observed in bEND5 cells, the Ca<sup>2+</sup> response to Glut was abolished by MCPG (200  $\mu$ M, 10 min) (Fig. 26A), which hints at mGluR involvement. Likewise, in hCMEC/D3 cells loaded with DAF/FM, Glut caused a slow and sustained increase in DAF/FM fluorescence that was inhibited by BAPTA (30  $\mu$ M, 2 h) (Fig. 26B). Therefore, Glut is able to induce NO synthesis in a Ca<sup>2+</sup>-dependent manner also in human microvascular endothelial cells, although with a different mode of Ca<sup>2+</sup> signalling.



**Figure 26.** Glutamate causes Ca<sup>2+</sup>-dependent NO release also in human microvascular brain endothelial cells. (A), Glut (200  $\mu$ M) caused an increase in [Ca<sup>2+</sup>]<sub>i</sub> in hCMEC/D3 cells, loaded with Fura-2/AM in the absence, but not in the presence, of MCPG (200 $\mu$ M, 10 min). (B), Glut (200  $\mu$ M) caused an increase in DAF/FM fluorescence in hCMEC/D3 cells in the absence, but not in the presence, of BAPTA (30  $\mu$ M, 2 h).

## DISCUSSION

Acetylcholine and glutamate are two of the most important neurotransmitters of the CNS which regulate CBF by releasing NO and causing vasorelaxation. Ach acts through the stimulation of brain microvascular ECs that engage the activation of the eNOS. eNOS is an archetypal  $\text{Ca}^{2+}$ /Calmodulin-dependent enzyme and the waveform of the  $\text{Ca}^{2+}$  signals that underlie Ach-induced NO release in many vascular bed is well known. Surprisingly, whether and how acetylcholine evokes an increase in intracellular  $\text{Ca}^{2+}$  concentration in brain microvascular cells is still unclear. On the other hand, Glu stimulates NMDARs to activate eNOS, but it remains to be elucidated whether mGluRs are able to promote NO release in brain microvascular ECs. The present investigation sought to fill these gaps by analysing bEND5 and hCMEC/D3 cells, two establish brain endothelial cells lines derived from mouse and human, respectively. This information has a potential clinical relevance as increase in cortical CBF is involved in a growing number of neurodegenerative disorder, such as Alzheimer's Disease. Understand the mechanism and the proteins involved could, therefore, be used in the future as target to rescue local blood perfusion in patients affected by neurodegenerative disorders.

Herein, we first demonstrated that Ach induces NO release by triggering two different modes of  $\text{Ca}^{2+}$  signals in murine (bEND5) and human (hCMEC/D3) brain microvascular endothelial cells. Of note, ER  $\text{Ca}^{2+}$  release via  $\text{InsP}_3\text{Rs}$  and SOCE shapes the  $\text{Ca}^{2+}$  response to Ach in both cell types but their different  $\text{Ca}^{2+}$  toolkits are likely to result in two quite different waveforms, i.e.  $\text{Ca}^{2+}$  oscillations vs. biphasic  $\text{Ca}^{2+}$  elevation. Whatever its waveform however, Ach-induced intracellular  $\text{Ca}^{2+}$  signals lead to robust NO release in both murine and human brain microvascular endothelial cells. Likewise, we demonstrated for the first time that Glut activated mGluRs to trigger intracellular  $\text{Ca}^{2+}$  oscillation in bEND5 and a biphasic increase in  $[\text{Ca}^{2+}]_i$  in hCMEC/D3. We further showed that mGluRs-dependent  $\text{Ca}^{2+}$  signals drive NO release in both bEND5 and hCMEC/D3. This NO signal is delayed compare to the Ach-induced one and is likely to play a crucial role in the slower vasodilation that often follows brief neuronal activity or that sustains functional hyperemia during persistent synaptic transmission ([247], [16]). The glutamate-induced  $\text{Ca}^{2+}$  response is likely to play an important role in the generation of additional endothelial vasorelaxing messengers, such as PGE2 and endothelial-dependent hyperpolarizing factor (EDHF) ([8], [248]), that are also involved in NVC during glutamatergic transmission ([16]).

Future experiments will have to assess: 1) the role of other neurotransmitters, such as GABA and glutamate, or neuromodulators, such as catestatine, in modulating  $\text{Ca}^{2+}$  signaling in bEND5 and hCMEC/D3 cells; and 2) whether Ach- and Glut-induced  $\text{Ca}^{2+}$  and NO signals are deranged in neurodegenerative disorders, such as Alzheimer's Disease (AD). AD indeed is a severe cortical cholinergic deficiency. This disease process is of particular interest, since it seems to be related to distortion in regional brain capillary structure involving endothelial cell shape changes and impairment of NO release which affects signaling between the immune, cardiovascular and nervous systems ([249]).

## **Acetylcholine stimulates intracellular $\text{Ca}^{2+}$ oscillations in bEND5 in a dose-dependent manner**

In bEND5, Ach reliably caused recurrent  $\text{Ca}^{2+}$  oscillations that appeared at 100  $\mu\text{M}$  and were then maintained at 300 and 500  $\mu\text{M}$ . We could not detect any increase in  $[\text{Ca}^{2+}]_i$  at concentrations lower than 50  $\mu\text{M}$ ; however, we cannot rule out the possibility that highly localized sub-cellular  $\text{Ca}^{2+}$  pulses occur at such doses, but are missed by our epifluorescence system. In this view, 0.1  $\mu\text{M}$  carbachol (a non-selective mAChR agonist) elicits spatially restricted  $\text{Ca}^{2+}$  puffs that intermittently arise at no more than two discharging sites located at the ends of rat artery tail endothelial cells in situ ([250]). However, Ach induces vasorelaxation of mouse brain intracortical microvessels in vivo in the mid micromolar range([251]), although in one study it induced vasodilation already at 10  $\mu\text{M}$  ([83]). It has long been known that mAChR expression declines rapidly with successive passages of cultured endothelial cells ([86], [252]), which could account for the reduced sensitivity to Ach observed in our investigation as compared to others ([83]). Unfortunately, the isolation of intact brain microvessels from the mouse is extremely technically challenging and we could not investigate the effect of Ach on  $\text{Ca}^{2+}$  dynamics in brain endothelial cells in situ ([214], [253]). As expected ([254], [248]), Ach-induced  $\text{Ca}^{2+}$  response were indeed prevented by atropine, a wide spectrum inhibitor of mAChRs, in bEnd5 cells. Moreover qRT-PCR analysis revealed that only M3-mAChRs are expressed in bEND5 cells. This finding concurs with the waveform of Ach-induced  $\text{Ca}^{2+}$  signal as M3-mAChRs trigger repetitive  $\text{Ca}^{2+}$  transients in most endothelial preparations studied ([86], [254]). The M3-mAChRs caused ACh-dependent endothelium vasodilation in most vascular preparations studied, although there is evidence implicating a role for M1-mAChRs and M5-mAChRs in some vascular beds ([255]). Accordingly to the statistical analysis, in bEND5 the most suitable concentration for Ach to trigger recurrent  $\text{Ca}^{2+}$  spikes was 300  $\mu\text{M}$ . Accordingly, while the



percentage of responding cells was similar to that measured at 100  $\mu\text{M}$ , the number of oscillations/hour and the average ISI of the  $\text{Ca}^{2+}$  train were significantly higher at this dose. This finding strongly suggests that the biological information encoded within Ach-induced  $\text{Ca}^{2+}$  transients is mainly delivered via frequency rather than amplitude modulation ([256], [257], [258]). Consistent with this hypothesis, while the magnitude of the 1<sup>st</sup>  $\text{Ca}^{2+}$  spike was significantly higher at 100  $\mu\text{M}$ , there was no difference in the amplitude of the subsequent  $\text{Ca}^{2+}$  transients between 100 and 300  $\mu\text{M}$ . A further increase in Ach concentration to 500  $\mu\text{M}$  resulted in the reduction of the percentage of responding cells and in the lengthening of the ISI. This bell-shaped dose-response relationship has also been described for EGF-induced intracellular  $\text{Ca}^{2+}$  oscillations in rat cardiac microvascular endothelial cells ([233]) and hints at partial mAChR desensitization at high Ach concentrations ([259], [260]). For instance, a high dose (1 mM) of carbachol cause rapid desensitization of M3-mAChR-dependent  $\text{InsP}_3$  synthesis and  $\text{InsP}_3$ -dependent  $\text{Ca}^{2+}$  mobilization due to receptor sequestration in chinese hamster ovary (CHO) cells ([259], [261]). Previous studies did not investigate the dose-dependent increase in the endothelial  $\text{Ca}^{2+}$  response to Ach at concentrations higher than 100  $\mu\text{M}$  ([262], [250], [263]). Intriguingly, bEND5 cells displayed a remarkable propensity to generate spontaneous  $\text{Ca}^{2+}$  oscillations, which arose in the absence of any agonist in the bathing solution. Spontaneous intracellular  $\text{Ca}^{2+}$  oscillations are not frequent in vascular endothelial cells, but have also been observed in mouse mesenteric ([264]) and cremasteric arteries ([265]) and in rat lung capillaries ([266]). In most cases, this spontaneous  $\text{Ca}^{2+}$  activity was triggered by  $\text{InsP}_3$ -dependent  $\text{Ca}^{2+}$  release from the ER and sustained by extracellular  $\text{Ca}^{2+}$  entry ([264]).

## The Ca<sup>2+</sup> toolkit of mouse brain endothelial cells

Endothelial cells have two main pathways to generate and regulate intracellular Ca<sup>2+</sup> signals ([87], [31]): Ca<sup>2+</sup> release from endogenous Ca<sup>2+</sup> stores and Ca<sup>2+</sup> entry from the extracellular environment. We performed an extensive qRT-PCR analysis to assess which of the components of the endothelial Ca<sup>2+</sup> toolkit that can be recruited upon PLC $\beta$  activation are present in bEND5 cells. According to our screening, ER stored Ca<sup>2+</sup> could be mobilized via InsP<sub>3</sub>R1 and InsP<sub>3</sub>R2 in bEND5 cells, in which we could not detect any transcript encoding for RyRs. The pattern of InsP<sub>3</sub>R expression in this mouse brain microvascular endothelial cell line supports their propensity to trigger intracellular Ca<sup>2+</sup> oscillations either under resting conditions or in the presence of an extracellular ligand. Indeed, InsP<sub>3</sub>R2, which shows the sharpest dependence on ambient Ca<sup>2+</sup> and is the most sensitive to InsP<sub>3</sub>, has long been regarded as the main oscillatory unit ([201], [267]). As to Ca<sup>2+</sup> entry, we found that bEND5 cells express both Stim isoforms, i.e. Stim1 and Stim2, but only the Orai2 subtype at both mRNA and protein level. Curiously, Orai2 is also the isoform that mediates SOCE in both resting and synaptically-activated mouse neurons ([178]), while Orai1 mediates SOCE in rat brain ([268]). Vascular endothelial cells are extremely sensitive to their surrounding microenvironment ([269]). Their genomic profile may be reprogrammed by signal inputs delivered by the native tissue ([270]). In this perspective, the evidence that Orai2 is the most likely candidate to underlie SOCE in mouse brain microvascular endothelial cells is not surprising. Moreover, Orai2 represents the pore-forming SOC subunit also in a bovine brain capillary endothelial cell line, t-BBEC117 ([222]). The Mn<sup>2+</sup>-quenching technique revealed that a Ca<sup>2+</sup> influx pathway with a pharmacological profile compatible with that of SOCE is activated under resting conditions in bEND5. The slope of this basal Ca<sup>2+</sup> entry was further enhanced by depleting ER Ca<sup>2+</sup> with CPA, thereby suggesting that SOCE could also be recruited by extracellular stimulation. Again, these data are reminiscent of those described in mouse brain neurons, where Orai2 is tonically active due to the partial emptying of the ER Ca<sup>2+</sup> store even in quiescent cells ([178], [271]), but may also contribute to synaptic dependent activity ([272]). It is assumed that Stim2, which displays a lower affinity for ER Ca<sup>2+</sup> levels and may be activated by a modest reduction in intraluminal Ca<sup>2+</sup> concentration, gates Orai2 in unstimulated bEND5 cells ([178], [271]), [273]), while Stim1 is called into action by a massive ER store depletion ([274]). Among TRPC channels, which represent the TRP sub-family preferentially coupled to PLC $\beta$  ([275]), only TRPC1 was expressed in mouse brain microvascular endothelial cells. Interestingly, TRPC1 is a polymodal channel which can mediate SOCE in endothelial cells, but mainly in association with Orai1 ([167], [276]).

## Acetylcholine-induced $\text{Ca}^{2+}$ response is driven by ER $\text{Ca}^{2+}$ release via $\text{InsP}_3\text{Rs}$ and sustained by SOCE in mouse brain endothelial cells

In bEND5, Ach-induced  $\text{Ca}^{2+}$  oscillations were driven by recurrent cycles of ER  $\text{Ca}^{2+}$  release via  $\text{InsP}_3\text{Rs}$  followed by SERCA-mediated sequestration. The first evidence that supports this theory is that inhibiting the synthesis of  $\text{InsP}_3$  with U73122, which selectively blocks  $\text{PLC}\beta$  activity in vascular endothelial cells ([277], [278]), prevents the Ach-induced oscillating  $\text{Ca}^{2+}$  response. This effect was mimicked by 2-APB, which interferes with  $\text{InsP}_3\text{Rs}$ , but it not highly specific as it could also impede  $\text{Ca}^{2+}$  entry ([179]). However, the inactive structural analog of U73122, i.e. U73343, did not exert any significant effect on the  $\text{Ca}^{2+}$  response to Ach. We can therefore conclude that  $\text{PLC}\beta$  triggers the repetitive  $\text{Ca}^{2+}$  oscillations via  $\text{InsP}_3$ -dependent  $\text{Ca}^{2+}$  release. Second, the pharmacological blockade of SERCA activity rapidly interrupted the spiking signal. Indeed, when SERCA activity is blocked,  $\text{Ca}^{2+}$  cannot be sequestered back into ER lumen to refill the store and this prevents the onset of the next  $\text{Ca}^{2+}$  spike. Third, RyRs are absent in bEND5 cells, as reported in endothelial cells from other vascular districts ([31]). However, the presence of extracellular  $\text{Ca}^{2+}$  is a necessary condition to the onset of the regenerative ER  $\text{Ca}^{2+}$  release. Ach-induced  $\text{Ca}^{2+}$  oscillations did not reach the threshold for detection by our epifluorescence system under  $0\text{Ca}^{2+}$  conditions, but suddenly resumed as soon as we restored  $\text{Ca}^{2+}$  to the bathing solution. At the same way, removal of external  $\text{Ca}^{2+}$  led to the rapid decline of the spiking signal, which could not last for longer than 1-2 spikes in the absence of  $\text{Ca}^{2+}$  influx. Likewise, intracellular  $\text{Ca}^{2+}$  oscillations were never observed in the absence of extracellular  $\text{Ca}^{2+}$  in several types of human endothelial colony forming cells ([242], [279]), Ea.hy926 cells ([280]), and bovine atria endothelial cells ([281]).

The  $\text{Mn}^{2+}$  quenching technique revealed that Ach was able to readily activate a  $\text{Ca}^{2+}$  permeable pathway with a pharmacological profile compatible with that of SOCE in bEND5 cells. In the presence of either  $\text{La}^{3+}$  or BTP2, Ach did not cause any significant increase in the slope of the quenching curve, which is a reliable readout of  $\text{Ca}^{2+}$  influx ([237], [239], [241], [282]). Moreover, Ach failed to induce any detectable  $\text{Ca}^{2+}$  response after a prolonged application of CPA, which led to the depletion of the ER  $\text{Ca}^{2+}$  reservoir and to full SOCE activation ([242], [243], [244]). It turns out that a store-independent  $\text{Ca}^{2+}$  channel is not activated by Ach in bEND5 cells. Overall, these data strongly suggest that SOCE is the main  $\text{Ca}^{2+}$  entry route engaged by Ach in bEnd5 cells.

The requirement of SOCE for regenerative  $\text{InsP}_3$ -dependent  $\text{Ca}^{2+}$  release may be interpreted within the framework of the luminal loading mechanism originally proposed by Sir Michael J. Berridge ([283], [284]). According to this model,  $\text{InsP}_3$  causes a highly restricted sub-membranal  $\text{Ca}^{2+}$  release which is not able to result in a regenerative  $\text{Ca}^{2+}$  signal and escapes therefore detection from a conventional epifluorescence system. Nevertheless, local depletion of this peripheral ER sub-compartment is sufficient to promote Stim oligomerization and relocation towards cell periphery, thereby resulting in Orai2 activation. Such a tight coupling between a small superficial compartment of the ER and store-operated Orai channels has been extensively described in many cell types ([274]). Consistently, in bEND5, the ensuing influx of  $\text{Ca}^{2+}$  results in a SERCA-mediated increase in intraluminal  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_{\text{ER}}$ ) that sensitizes  $\text{InsP}_3\text{Rs}$  to ambient  $\text{InsP}_3$  and  $\text{Ca}^{2+}$  and triggers the first regenerative  $\text{Ca}^{2+}$  spike. This coordinated sequence of  $\text{Ca}^{2+}$  fluxes inside the cytosol persists as long as  $\text{Ca}^{2+}$  is present in the extracellular milieu and preserves  $[\text{Ca}^{2+}]_{\text{ER}}$  from depletion. This model is strongly corroborated by the evidence that SOCE maintains ER  $\text{Ca}^{2+}$  levels in bEND5 cells. Accordingly, the pharmacological blockade of constitutive SOCE induces ER  $\text{Ca}^{2+}$  depletion and prevents CPA-dependent  $\text{Ca}^{2+}$  mobilization, as recently shown in EPCs isolated from patients affected by infantile hemangioma ([128]). It is, therefore, conceivable that SOCE is redirected into ER lumen at higher rate following  $\text{PLC}\beta$  activation by Ach in mouse brain microvascular endothelial cells, thereby leading to the onset of the  $\text{Ca}^{2+}$  burst. This model is also supported by a landmark study carried out by Morgan and Jacob twenty years ago, which elegantly demonstrated that agonist-induced SOCE is indispensable for loading up the ER with  $\text{Ca}^{2+}$  and maintaining  $\text{InsP}_3\text{R}$  sensitivity from the luminal side during prolonged  $\text{Ca}^{2+}$  oscillations in human umbilical vein endothelial cells([237]). Once set in motion by Ach administration, the spiking response was not immediately interrupted by extracellular  $\text{Ca}^{2+}$  removal as it persisted for no more than 1 transient. This observation indicated that the  $\text{Ca}^{2+}$  pool available for  $\text{InsP}_3$ -dependent  $\text{Ca}^{2+}$  release rapidly depleted in the absence of  $\text{Ca}^{2+}$  entry and cannot further sustain the  $\text{Ca}^{2+}$  signal.

## Acetylcholine-induced intracellular $\text{Ca}^{2+}$ oscillations drive NO production in bEND5 cells

The functional outcome of Ach-induced  $\text{Ca}^{2+}$  response is the generation of NO in bEND5. The pharmacological inhibition of each signaling pathway involved in the oscillatory signal impaired Ach-induced NO synthesis. This observation suggests that the NO production was driven by  $\text{Ca}^{2+}$  oscillations in bEND5 cells. A frequency modulated system is more suitable to sustain the prolonged activation of a cellular decoder, such as eNOS, that is regulated by  $\text{Ca}^{2+}$  in a highly cooperative manner ([256]). Accordingly, Ach-induced  $\text{Ca}^{2+}$  oscillations have been shown to support NO release in a number of vascular districts ([86]), including rat tail artery ([285]) and mouse aorta ([254]). This information shed further light on the molecular events that take place in the neurovascular unit during neuronal activity ([8]). Moreover, they further highlight the role played by  $\text{Ca}^{2+}$  pathways in brain endothelium ([214]). A periodic increase in  $[\text{Ca}^{2+}]_i$  has long been known to regulate blood-brain barrier permeability([286], [194]), lymphocyte migration across the blood-brain barrier ([287]) and trypanosome infection ([288]). Taken together these data add NO production to the growing list of functions underlain by intracellular  $\text{Ca}^{2+}$  response in brain microvascular endothelium.

## Acetylcholine driven intracellular $\text{Ca}^{2+}$ increase in hCMEC/D3 in a dose-dependent manner

In hCMEC/D3, Ach failed to cause recurrent  $\text{Ca}^{2+}$  spikes, but induced a transient increase in  $[\text{Ca}^{2+}]_i$  with a different pattern depending on agonist concentration. Similar to bEND5 cells, Ach induced bell-shaped dose-response relationship in human brain ECs, as the amplitude of the initial  $\text{Ca}^{2+}$  peak increased when Ach concentration was raised from 25  $\mu\text{M}$  to 100  $\mu\text{M}$  and then decreased upon a further rise to 200  $\mu\text{M}$ . These observations indicate that the mAChRs endowed to hCMEC/D3 cells are more sensitive as compared to those expressed in bEnd5 cells. Accordingly, M5-mAChR was the most abundant isoform detectable in hCMEC/D3 cells, which is consistent with previous findings obtained in cerebral arteries of humans, cattle and mice ([83], [84]). These results are quite surprising, as previous studies in ECs located in other vascular districts expressing both M3- and M5-mAChRs demonstrated that the former is more sensitive to cholinergic stimulation ([255]). We speculate that the signalling coupling between M5-mAChR and PLC is more efficient in bEnd5 cells as compared to that between M3-mAChRs and PLC.

## The Ca<sup>2+</sup> toolkit of human brain endothelial cells

We have screened the components of the Ca<sup>2+</sup> toolkit also in hCMEC/D3 cells. Unlike their murine counterparts, they only expressed InsP<sub>3</sub>R3, which displays the lowest affinity to InsP<sub>3</sub> and Ca<sup>2+</sup> ([267]), tends to curtail the spiking response and therefore functions as an anti Ca<sup>2+</sup> oscillatory unit ([201], [267], [203]). This finding, therefore, could explain why Ach (as well as Glut, as discussed below) does not trigger a spiking Ca<sup>2+</sup> response in hCMEC/D3 cells. Moreover, RYRs are absent, thereby confirming that ER Ca<sup>2+</sup> mobilization is only mediated by InsP<sub>3</sub>R3. As to the Ca<sup>2+</sup> entry pathways, hCMEC/D3 cells only express Stim2, although they are endowed with all the three Orai subtypes. These data suggest that Stim2 is the ER Ca<sup>2+</sup> sensor that drives both constitutive and Ach-induced SOCE, while Orai1 and Orai2 are the most likely candidate to provide the pore-forming subunits of SOCs. Quite surprisingly, hCMEC/D3 cells lack all TRPC channel subtypes with the exception of TRPC7, whose endothelial expression has only barely been reported ([31]). TRPC7 is a DAG-sensitive channel, but OAG failed to cause any evident increase in [Ca<sup>2+</sup>]<sub>i</sub>. Therefore, TRPC7 is unlikely to contribute to Ach-induced Ca<sup>2+</sup> entry in hCMEC/D3 cells.

## Acetylcholine-induced Ca<sup>2+</sup> response are driven by ER Ca<sup>2+</sup> release via InsP<sub>3</sub>Rs and SOCE in human brain endothelial cells

Unlike bEND5, the Ca<sup>2+</sup> response to Ach was detectable also under 0Ca<sup>2+</sup> conditions in hCMEC/D3 cells. Ach was still able to generate a rapid increase in [Ca<sup>2+</sup>]<sub>i</sub> that rapidly went back to the basal line, lacking the SOCE-dependent plateau phase. It is, therefore, possible that hCMEC/D3 possess a higher intraluminal ER Ca<sup>2+</sup> concentration as compared to bEND5 cells, that more InsP<sub>3</sub> is synthesized in response to M5-mAChR activation, or that newly-synthesized InsP<sub>3</sub> is more efficiently transmitted to InsP<sub>3</sub>Rs. These hypotheses are not mutually exclusive among each other. It should, however, be pointed out that the amplitude of CPA-induced ER Ca<sup>2+</sup> leakage was higher in hCMEC/D3 than in bEND5 cells (Fig. 7 and 17). This observation strongly suggests that the Ca<sup>2+</sup> pool available for InsP<sub>3</sub>-dependent Ca<sup>2+</sup> release (see below) is higher in hCMEC/D3 than in bEND5 cells and can give rise to the Ca<sup>2+</sup> signal in absence of extracellular Ca<sup>2+</sup>. Clearly, future experiments with genetic Ca<sup>2+</sup> indicators targeted to ER lumen are necessary to confirm this mechanism. Indeed, we first found that the Ca<sup>2+</sup> response to Ach was inhibited by preventing InsP<sub>3</sub> synthesis with U73122 and by interfering with InsP<sub>3</sub>Rs with 2-APB. Of note, U73343 did not exert

any significant effect on the  $\text{Ca}^{2+}$  response to Ach. Secondly, after the complete emptying of ER  $\text{Ca}^{2+}$  stores obtained after 30 min of treatment with CPA, the  $\text{Ca}^{2+}$  response to Ach was absent. This data further confirms that Ach needs  $\text{Ca}^{2+}$  released from ER to induce the intracellular  $\text{Ca}^{2+}$  response in hCMEC/D3. We can, therefore, conclude that Ach-induced intracellular  $\text{Ca}^{2+}$  release was triggered by  $\text{PLC}\beta$  activation and mediated by  $\text{InsP}_3\text{Rs}$ .

The “ $\text{Ca}^{2+}$  add-back” protocol revealed that Ach was able to activate a SOCE pathway that was completely blocked by Pyr6; similar to bEnd5 cells, Pyr6 was also able to prevent CPA- and Ach-induced intracellular  $\text{Ca}^{2+}$  release. These data indicate that SOCE was also constitutively open in human brain microvascular ECs and could be further recruited upon agonist (i.e. Ach)-induced depletion of the ER  $\text{Ca}^{2+}$  store. Of note, in a minor fraction of hCMEC/D3 cells responding to Ach despite for the presence of Pyr6, the  $\text{Ca}^{2+}$  signal was similar to that measured under  $0\text{Ca}^{2+}$  conditions, i.e. displayed lower amplitude and lacked the plateau phase. This observation corroborates the notion that SOCE sustains Ach-induced  $\text{Ca}^{2+}$  signaling in these cells.

## **Acetylcholine-induced intracellular $\text{Ca}^{2+}$ mobilization drive NO production in hCMEC/D3 cells**

Also in hCMEC/D3 cells, the  $\text{Ca}^{2+}$  response to Ach was able to induce NO synthesis. Accordingly, the pharmacological blockade of  $\text{InsP}_3$ -dependent  $\text{Ca}^{2+}$  release and NO prevented Ach-induced NO release. This effect was mimicked by BAPTA, thereby reinforcing the evidence that cytosolic  $\text{Ca}^{2+}$  is required to recruit the eNOS in Ach-stimulated hCMEC/D3 cells.

## Glutamate stimulates intracellular Ca<sup>2+</sup> oscillations in bEND5 cells

In bEND5, glutamate caused Ca<sup>2+</sup> oscillations that appeared at 50 μM and were then maintained at 100, 200 and 300 μM. We could not detect any increase in [Ca<sup>2+</sup>]<sub>i</sub> at concentrations lower than 50 μM; however, we cannot rule out the possibility that highly localized sub-cellular Ca<sup>2+</sup> pulses occur at such doses, but are missed by our epifluorescence system. The dose-response relationship revealed that 200 μM Glut was the most efficient dose to evoke intracellular Ca<sup>2+</sup> oscillations. This observation gains a strong functional relevance as glutamate concentration rapidly raise well beyond this range in the synaptic cleft in response to high frequency stimulation ([289], [290]). Considering that glutamatergic terminals establish close contacts with parenchymal microvessels ([247], [4]), it is highly conceivable that brain microvascular endothelial cells are exposed to a concentration of glutamate which is able to induce robust Ca<sup>2+</sup> oscillations during neuronal activity.

Glutamate has long been known to induce an NMDARs-mediated increase in [Ca<sup>2+</sup>]<sub>i</sub> in different types of brain microvascular endothelial cells, such as ECV304 ([291]), primary mouse brain endothelial cells ([292]), and human brain endothelial cells ([293]). However, this increase in [Ca<sup>2+</sup>]<sub>i</sub> has always been observed at a rather high glutamate concentration, i.e. 1 mM, as the recording solutions were lacking the glutamate co-agonists, glycine or D-serine, which are strictly required by NMDARs to mediate Ca<sup>2+</sup> entry at physiological glutamate levels also in non-neuronal tissues ([294]). Accordingly, a recent series of studies demonstrated that, in the presence of D-serine, 100 μM was sufficient to cause NMDARs-mediated NO-dependent vasodilation in mouse brain microvessels ([30]). In the present investigation, we did not add either glycine or D-serine to prevent NMDAR activation and rule out any contribution from ionotropic glutamate receptors. We found that glutamate was able to trigger a dose-dependent oscillatory increase in [Ca<sup>2+</sup>]<sub>i</sub> that was, therefore, most likely to due to mGluR activation. The following observations strongly support this conclusion. First, glutamate-induced intracellular Ca<sup>2+</sup> oscillations were inhibited by the broad-spectrum mGluR antagonist, MCPG. Second, the oscillatory response to glutamate arose in the absence of extracellular Ca<sup>2+</sup>, which reflects the engagement of metabotropic receptors ([295]). Third, mGluRs were previously found in brain endothelium ([296], [297], [298]). Unfortunately, qRT-PCR, immunoblotting and immunofluorescence could not confirm mGluR expression in bEnd5 cells. We speculate that, similar to brain neurons, mGluRs could be confined within discrete,



extremely localized, membrane areas, which does not enable their detection with the resolution of our immunofluorescence and immunoblotting assays.

## **Glutamate-induced $\text{Ca}^{2+}$ oscillations are driven by ER $\text{Ca}^{2+}$ release via $\text{InsP}_3\text{Rs}$ and SOCE in bEND5 cells**

In bEND5 cells, Glutamate-induced  $\text{Ca}^{2+}$  oscillations were driven by rhythmical cycles of ER  $\text{Ca}^{2+}$  release via  $\text{InsP}_3\text{Rs}$  followed by SERCA-mediated sequestration and maintained by constitutive SOCE. The first evidence that supports this model is that Glut-induced  $\text{Ca}^{2+}$  spikes were inhibited by preventing  $\text{InsP}_3$  synthesis with U73122 and by interfering with  $\text{InsP}_3\text{Rs}$  with 2-APB ([234]). Again, the inactive structural analog of U73122, i.e. U73343, did not exert any significant effect on Glut-induced  $\text{Ca}^{2+}$  oscillations. We can therefore, conclude, that  $\text{PLC}\beta$  triggers the repetitive  $\text{Ca}^{2+}$  oscillations via  $\text{InsP}_3$ -dependent  $\text{Ca}^{2+}$  release. Second, the pharmacological blockade of SERCA activity prevented the subsequent  $\text{Ca}^{2+}$  response to Glut. Likewise, the application of CPA to ongoing glutamate-induced intracellular  $\text{Ca}^{2+}$  waves resulted in the complete suppression of  $\text{Ca}^{2+}$  activity. Unlike Ach, however, Glut still induced repetitive  $\text{Ca}^{2+}$  spikes under  $0\text{Ca}^{2+}$  conditions, but these  $\text{Ca}^{2+}$  oscillations rapidly run down. The subsequent addition of  $\text{Ca}^{2+}$  to perfusate resumed the oscillatory response. The amplitude of the 1<sup>st</sup>  $\text{Ca}^{2+}$  spike was not different in the presence and absence of external  $\text{Ca}^{2+}$ , thus suggesting that  $\text{Ca}^{2+}$  entry is not necessary to initiate the oscillations, but is strictly required to maintain them over time ([246]). Likewise, removal of extracellular  $\text{Ca}^{2+}$  during ongoing oscillations caused the  $\text{Ca}^{2+}$  oscillatory response to persist only for 1-2 spikes before rapidly returned  $[\text{Ca}^{2+}]_i$  to resting levels. Overall, these findings strongly indicate that glutamate-induced intracellular  $\text{Ca}^{2+}$  oscillations are shaped by the rhythmical mobilization of the intracellular  $\text{Ca}^{2+}$  reservoir and sustained by extracellular  $\text{Ca}^{2+}$  entry. However, the  $\text{Mn}^{2+}$  quenching technique revealed that Glut was not able to activate a  $\text{Ca}^{2+}$  permeable pathway in bEND5 cells. Therefore, we speculate that Glut-induced intracellular  $\text{Ca}^{2+}$  oscillations were maintained over time by the constitutive SOCE. Overall, these findings strongly suggest that the  $\text{InsP}_3$ -dependent ER pools upon which Ach and Glut impinge are quite different. Accordingly, the  $\text{InsP}_3$ -dependent  $\text{Ca}^{2+}$  response to Glut is manifest even in the absence of extracellular  $\text{Ca}^{2+}$  and is maintained by resting  $\text{Ca}^{2+}$  influx, which indicates that the local ER  $\text{Ca}^{2+}$  concentration does not fall below the threshold for Stim activation. Conversely, intraluminal  $\text{Ca}^{2+}$  levels within the ER

sub-region targeted by Ach must be so low that SOCE is required to both trigger and maintain the oscillations over time.

## Glutamate-induced intracellular $\text{Ca}^{2+}$ oscillations drive NO production in bEND5 cells

Similar to Ach, glutamate-induced intracellular  $\text{Ca}^{2+}$  oscillations were able to induce NO synthesis in bEND5 cells. Glut-evoked NO release was abrogated by inhibiting Group I mGluRs with MCPG and by blocking each component of the  $\text{Ca}^{2+}$  toolkit involved in the generation of the  $\text{Ca}^{2+}$  spikes. Unlike Ach (see above), however, the increase in NO levels was detectable at around 300 sec after the onset of glutamate-induced  $\text{Ca}^{2+}$  waves and reached its peak with rather slow kinetics. Therefore, although both agonists induce intracellular  $\text{Ca}^{2+}$  oscillations, eNOS sensitivity to the  $\text{Ca}^{2+}$ -dependent stimulation differs between acetylcholine and glutamate. This difference has already been reported in the endothelial cells *ex vivo* covering pig aortic valves, in which, for a given increase in  $[\text{Ca}^{2+}]_i$ , the extent NO production varied depending on the extracellular agonist (thrombin>ATP>bradykinin>ionomycin) ([299]). The highest frequency of glutamate-induced intracellular  $\text{Ca}^{2+}$  oscillations falls within the same range as that described for acetylcholine ( $\approx 0.0045$  Hz vs.  $\approx 0.0047$  Hz). Therefore, as suggested for endothelial cells *ex vivo* ([299]), we speculate that  $\text{Ca}^{2+}$  transients interact with other signalling pathways to boost eNOS activation in glutamate-stimulated cells ([136]). For instance, Akt/protein kinase B (PKB) promotes NO releases independently on  $\text{Ca}^{2+}$  by phosphorylating eNOS (ref). Interestingly, Group 1 mGluRs may enhance angiogenesis by recruiting the phosphoinositide 3-kinase (PI3K)/PKB pathway ([300]). It should, however, be recalled that DAF/FM is not a true NO detector, but is rather sensitive to several nitrogen derivatives, such as  $\text{N}_2\text{O}_3$ ,  $\text{NO}_2$  or  $\text{ONOO}$  ([301]). More specifically, NO seems to react with the NH radical of the fluorophore which is generated by its non-specific oxidation ([301]). Therefore, NO levels could start to increase before they become detectable through an increase in DAF/FM fluorescence. An additional caveat that should be taken in consideration is that replacing the extracellular perfusate with a glutamate-containing solution does not faithfully mimic the physiological conditions during which glutamate is delivered onto brain microvascular endothelial cells, i.e. synaptic activity. Glutamatergic terminals are in close proximity and functionally coupled to adjoining parenchymal microvessels ([247], [4]). Therefore, brain microvascular endothelial cells are in the most suitable location to directly sense neuronal activity provided that they are

endowed with mGluRs (or NMDARs), as we have shown here. The frequency of the  $\text{Ca}^{2+}$  spikes we observed was even lower of the slow (0.5-1 Hz) brain oscillations that occur during deep sleep ([97]) (but see below). However, we envisage that high frequency (80-100 Hz) synaptic transmission *in vivo* could accelerate the frequency of the endothelial  $\text{Ca}^{2+}$  waves, thereby anticipating the onset of the ensuing NO signal. Nevertheless, glutamate-induced endothelial NO release is unlikely to mediate the fast NO-dependent vasodilation that follows neuronal activity in cerebellum and hippocampus ([247], [13]). Conversely, the delayed endothelial NO signal could underpin the slower component of the vasodilating response to brief neuronal activity in these latter structures ([114], [115], [13]) or play a permissive role in the PGE<sub>2</sub>- or EET-dependent vasodilation that occurs during sustained (up to 1 min) synaptic activation in the cortex ([247]). Future *in vivo* experiments, involving the use of transgenic mice expressing genetic  $\text{Ca}^{2+}$  biosensors (such as GCaMP2) in vascular endothelium ([221]) and of more sensitive tools for NO detection (such as electrochemical probes) ([302]), will be necessary to address the exact role of glutamate-induced eNOS activation in NVC. In must, however, be pointed out that very slow or infra-slow oscillations (ISOs) well below 0.1 Hz (0.005-0.1 Hz) occur in the thalamus, as well as in other cortical structures, and are driven by mGluR activation ([303]). Of note, these ISOs have been associated to the spontaneous <0.1 Hz blood-oxygen-dependent level (BOLD) signals recorded with functional magnetic resonance imaging (fMRI) in resting individuals ([304]). The cellular and biochemical underpinnings of such low frequency BOLD fluctuations, which provide one of the most efficient tools to map brain connectivity, are yet to be elucidated. Intriguingly, the novel observation that low frequency glutamate-induced intracellular  $\text{Ca}^{2+}$  waves in brain microvascular endothelial cells lead to robust NO release could shed new light on the underlying mechanism. Finally, we have to recall that intracellular  $\text{Ca}^{2+}$  drives the synthesis of a plethora of endothelial-derived vasorelaxing messengers, including PGE<sub>2</sub>, EETs, EDHF and hydrogen sulphide ([8], [305], [31]). Therefore, future experiments will be necessary to assess whether the signalling pathway we described in the present investigation contributes to generate PGE<sub>2</sub> and/or EETs in the brain areas, such as the cortex, in which these vasoactive factors trigger the vasodilating response to glutamatergic transmission ([247], [306]).

## Glutamate induces intracellular Ca<sup>2+</sup> signals and NO release in hCMEC/D3 cells

The data obtained from bEND5 cells were partially confirmed in hCMEC/D3 cells. Indeed, Glut was able to trigger an elevation in [Ca<sup>2+</sup>]<sub>i</sub> and NO release also in human brain microvascular ECs. Similar to their murine counterparts, MCPG blocked both signals, thereby hinting at the involvement of mGluRs. As expected from the lack of InsP<sub>3</sub>R1 and InsP<sub>3</sub>R2, the Ca<sup>2+</sup> signal lacked an oscillatory component, but displayed transient kinetics. Our preliminary data, not shown here, suggest that Glut-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> was mainly mediated by InsP<sub>3</sub>R3-mediated Ca<sup>2+</sup> release from the ER with no or only scarce contribution by extracellular Ca<sup>2+</sup> entry.

## CONCLUSIONS

This work showed for the first time the  $\text{Ca}^{2+}$ -dependent mechanisms whereby acetylcholine and glutamate, the two major excitatory neurotransmitters, cause NO release in brain microvascular ECs. This information will shed novel light on the cellular and biochemical pathways that regulate NA-induced CBF.

Ach triggered two different modes of  $\text{Ca}^{2+}$  signals in murine (bEND5) and human (hCMEC/D3) brain microvascular ECs, i.e.  $\text{Ca}^{2+}$  oscillations and biphasic  $\text{Ca}^{2+}$  elevation respectively. The Ach-induced  $\text{Ca}^{2+}$  response involved ER  $\text{Ca}^{2+}$  release via  $\text{InsP}_3$ Rs and SOCE in both cell types. Interestingly, Ach-induced intracellular  $\text{Ca}^{2+}$  signals lead to robust NO release in both murine and human brain microvascular ECs. These results have a remarkable patho-physiological relevance because as NO release drives vasorelaxation upon cholinergic synaptic transmission in the brain. Recent work has shown that the neurovascular coupling between basal forebrain cholinergic nerve fibers and intraparenchymal microvessels declines with aging and in subjects with Alzheimer's type dementia ([94]). Understanding the signaling pathway whereby Ach induces NO synthesis in intracortical arterioles and capillaries could prove essential to design alternative pharmacological approaches to prevent or limit the decline in higher cognitive functions.

Likewise, we demonstrated for the first time that Glut triggered intracellular  $\text{Ca}^{2+}$  oscillations in bEND5 cells and a biphasic increase in  $[\text{Ca}^{2+}]_i$  in hCMEC/D3 cells. We further showed that Glut-dependent  $\text{Ca}^{2+}$  signals drive NO release in both cell types. This NO signal is delayed as compared to the Ach-induced one and is likely to play a crucial role in the slower vasodilation that often follows brief neuronal activity or that sustains functional hyperemia during persistent synaptic transmission ([247], [16]). We also obtained the pharmacological evidence that mGluRs drive the  $\text{Ca}^{2+}$  response to Glut, although future experiment will be necessary to confirm their expression in both types of cells. Nevertheless this data lent further support to the recent observation that glutamate increases CBF by directly activating vascular endothelium, which adds a new layer of complexity to the mechanisms of CBF regulation by NA.

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