Picture me high:
Endocannabinoid dynamics on astrocytes

DISSERTATION

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“What if God was wrong?”

Lukas Troberg

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Abstract

The *Cannabis sativa* potential for both medicinal and recreational applications has attracted attention of diverse civilizations throughout the times. With the advent of molecular biology, the study of its components lead to the discovery and characterization of the endocannabinoid system (ECS).

The ECS is a widely distributed, polyfunctional signalling system that is virtually involved in all brain functions. Indeed, the main receptor of the ECS, the cannabinoid receptor type 1 (CB1), is the most abundant G-protein coupled receptor (GPCR) and exhibits remarkable tissue-, cell- and subcellular-specific dynamics. In consequence, stimulation of CB1 receptors may produce extremely different outcomes depending on its particular context. For instance, activation of CB1 receptors present on membranes of astrocytes triggers gliotransmission, an astrocytic version of neurotransmission, which contrasts with the classical role of endocannabinoids (eCBs) as retrograde signals that inhibit the synaptic release of neurotransmitters.

CB1 expression on astrocytes has been initially controversial. However, as a new wave of information expand our perceptions on the astrocytic roles on the complex neuronal networks they integrate, new astroglial eCB mechanisms are also being described. Currently, astrocytes are well recognized as an additional player on the ECS, with functions raging from neuronal metabolism support, to the direct regulation of neuronal spiking and synaptic plasticity. However, given the lack of efficient methods to study the eCB production dynamics on astrocytes, most of our current knowledge on astrocytic ECS mechanisms focus on astroglial CB1.

However, a novel eCB fluorescent sensor (GRABeCB2.0) with the potential to report eCBs at unprecedent spatiotemporal resolutions has been recently developed. In this project, we successfully expressed and validated GRABeCB2.0 on cultured astrocytes to pioneer a detailed study of the eCB dynamics on these cells. Using this method, we found that astrocytes have a constitutively active ECS. Supporting this, our data suggest that astrocytes present a basal eCB tone. Strikingly, we also reported spontaneous eCB events linked with astroglial 2-AG production, that closely remind descriptions of Ca^{2+} oscilations in astrocytes. Indeed, the correlation between Ca^{2+} and eCB dynamics was further verified, which launches a myriad of hypothesis on astroglial eCB physiological roles, given the extensive interest currently being devoted to astrocytic Ca^{2+} oscillations. Furthermore, we successfully developed a eCB SNFR protocol using HEK293 cells, which we used to confirm that astrocytes effectively release eCBs.

Lastly, we briefly share our current achievements on the set up of GRABeCB2.0 on hippocampal acute brain slices. Up to the moment, we were able to report a response to the application of WIN55, a CB1 agonist, which indicates that this sensor is functional on this model. Giving that acute brain slices closely mimic the environment that astrocytes integrate *in vivo*, we expect that this model will bring exciting new insights on the role of astroglial eCBs on physiological functions and, eventually, behaviour.

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Introduction

Cannabis sativa has long been used for medicinal purposes, with early records dating from more than 5000 years ago (Mechoulam, 1986). Early documentation of medicinal Cannabis can be found on ancient Chinese pharmacopoeias (Lin & Li, 1974; Hill *et al.*, 2012), and its applications included the use of fruits, leaves or seeds, sometimes together with other herbs, for rheumatic pains, disorders of the female reproductive tract and malaria (probably for the headache caused by the disease; Mechoulam, 1986). In Western civilizations, Cannabis medicinal properties were not popularly recognized until the 19th century when the Irish physician, William O'Shaughnessy claimed the plant's analgesic, anti-inflammatory, anti-emetic and anti-convulsant properties (O'Shaughnessy, 1843; Hill *et al.*, 2012).

Among its medicinal properties, the potential of Cannabis abuse for recreational purposes was well recognized across times. Cannabis has been deeply involved in several Indian rituals and it is also suggested to play a role in religious practices among some Muslim (the *Hashshashins*) and Christian groups, as well as in several African cultures (Kohek *et al.*, 2011). During colonization, Europeans and enforced African people brought it to the Americas which was, eventually, integrated in the medico-religious practices of some Indigenous groups (Kohek *et al.*, 2011). In consequence, Cannabis is one of the most widespread psychoactive substances in the world.

The psychotropic effects of Cannabis were also well known in ancient China, temporal distortion and hallucinations, with some reporting '*seeing devils*' after taking an excess of hemp fruits (Lin & Li, 1974). In consequence, despite its wide uses, the word "*Ma*" (Cannabis, in Chinese) has a negative connotation in Chinese society. In fact, some authors argue that this plant was scarcely used as a recreational drug in China due to '*a basic incompatibility with Chinese temperament, philosophy of life, and traditions*' (Mechoulam, 1986). It is suggested that Cannabis medicinal use fell out of favour due to those undesirable effects (Lin & Li, 1974).

Regarding the modern world, Cannabis psychotropic effects were also in the origin of the declining of its use (and research) for medicinal purposes. However, this happened in an intricate societal panorama closely related with it recreational consumption. Following the international implementation of drug policies in the early 20th century, consisting of a prohibition and control framework addressing several drugs, the Cannabis consumption was transnationally forbidden (Collins, 2020). Eventually, Cannabis medicinal interest vanished almost completely, and the plant was dropped from the British (1932) and American (1941) pharmacopoeias (Collins, 2020). In the following years researchers complained about restrictive laws and other obstacles that prevented scientific investigation (Mechoulam, 1986; Collins, 2020).

This scenario is in profound contrast with the current panorama. The last decade has seen a dramatic shift in global attitudes toward medicinal Cannabis. Currently, over 50 countries established legal access pathways to Cannabis-derived therapeutical products (United Nations, 2020). However, positioning of Cannabis as a legitimate medical product produces some tensions with other regulatory frameworks (Perkins *et al.*, 2021). Furthermore, the debate in turn of

decriminalization and legalization of recreational cannabis also boomed in recent years, with some countries such as Canada and Uruguay approving its consumption. In consequence, scientific and political debates about Cannabis have sometimes been mis considered by public opinion, causing an hysteria that mirrors the polarized society itself: while some believe it has miraculous properties that will suit every disease, others consider it as a dangerous and harmful drug.

Apart from historical and society perspectives, decades of research have shown the undeniable relevance of the endocannabinoid system (ECS). This domination refers to a widespread molecular signalling system that cannabis hijacks to exert its effects. Importantly, it also plays an important role in the regulation of several biological processes such as development, inflammation and gastrointestinal functions (Fride, 2008; DiPatrizio, 2016; Barrie and Manolios, 2017). The ECS is particularly relevant in the central nervous system (CNS) where it plays a critical role controlling synaptic plasticity, metabolism and neuronal proliferation (Roche, 2010; Laprairie, 2012; Xu, 2015). Indeed, the ECS has a great impact on multiple brain functions such as on memory, appetite, anxiety and rewarding (Zou and Kumar, 2018) and, consequently, its dysregulation is implicated in numerous pathological conditions such neurodegeneration, epilepsy, addiction and schizophrenia (Zhang *et al*., 2004; Dalton *et al*., 2011; Ortega-Alvaro *et al*., 2011; Di Marzo, 2015). Thus, research focusing on understanding ECS mechanisms has a great potential to expand current therapeutical options for a myriad of diseases and other medical conditions. However, as we will further address, the ECS is particularly complex, with subtle nuances that demand a detailed study in order to effectively design strategies to modulate its constituents towards the desired effects.

An introduction to the endocannabinoid system

The ECS has been widely reviewed elsewhere (Castillo *et al*., 2012; Pertwee, Roger G; Zimmer, 2015; Lu and MacKie, 2016; Zou and Kumar, 2018; Cristino *et al*., 2020). Very briefly, this system is constituted by 2 principal G protein-coupled receptors (GPCR), namely type 1 and type 2 cannabinoid receptors (CB1, CB2), their lipidic endogenous ligands, so called endocannabinoids (eCBs), of which anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the best characterized, and the enzymes linked to their synthesis and metabolism: AEA is synthesized from N-acyl-phosphatidylethanolamine (NAPE) by a specific phospholipase D (NAPE-PLD) and then degraded by fatty acid amide hydrolase (FAAH); on the other hand, 2-AG is mainly produced from diacylglycerol (DAG) by DAG lipase α (DAGLα) and further hydrolysed by monoacylglycerol lipase (MAGL).

Contrarily to other neurotransmitters, eCBs are not stored in vesicles prior to release. Instead, all the ECS machinery locates on cellular membranes and eCBs are produced *de novo* on demand, in response to stimuli. More precisely, eCBs are typically produced on the postsynaptic neuron upon its activation and travel backwards, by a mechanism not fully understood, to bind on presynaptic cannabinoid receptors (CBRs). In turn, the activation of CBRs suppresses the calcium (Ca2+) influx either by direct G protein-dependent suppression of voltage-gated Ca2+ channels (VGCCs) or by

inhibition of adenylyl cyclase and downregulation of the cAMP/PKA pathway. As a result, CBRs activation inhibits the fusion of synaptic vesicles to the presynaptic terminal and, consequently, neurotransmitter release. This mechanism, known as retrograde signalling, creates a feedback loop allowing postsynaptic regulation of the presynaptic neuron.

Importantly, the ECS is capable of regulating the plasticity of both excitatory and inhibitory synapses. Short-term forms of eCB-mediated synaptic plasticity includes metabotropic- and depolarization-induced inhibition (MSI/DSI) and excitation (MSE/DSE), corresponding to γ-aminobutyric acid (GABA) and glutamate neurotransmission, respectively (Castillo *et al.*, 2012). It is also known that the ECS has an impact on long-term forms of synaptic plasticity, by mediating long-term depression (LTD) or by repressing long-term potentiation (LTP) (Silva-Cruz *et al.*, 2017). This set of features confers to the ECS very interesting functions in brain circuits. For instance, it has been described that, in some brain regions, CB1 mediate processes of frequency discrimination, acting either as circuit breakers or favouring a selective communication between neurons with a higher firing rates (Busquets-Garcia *et al.*, 2018).

Besides CB1 and CB2 receptors, eCB signalling comprises other targets such as transient receptor potential cation channel subfamily V member 1 (TRPV1), peroxisome proliferator-activated nuclear receptor α and γ (PPARα, PPARγ) and T-type Ca2+ (Cav3.2) channels (Cristino *et al.*, 2020). Less attention has been given to a putative third cannabinoid receptor, the orphan GPCR GPR55, which

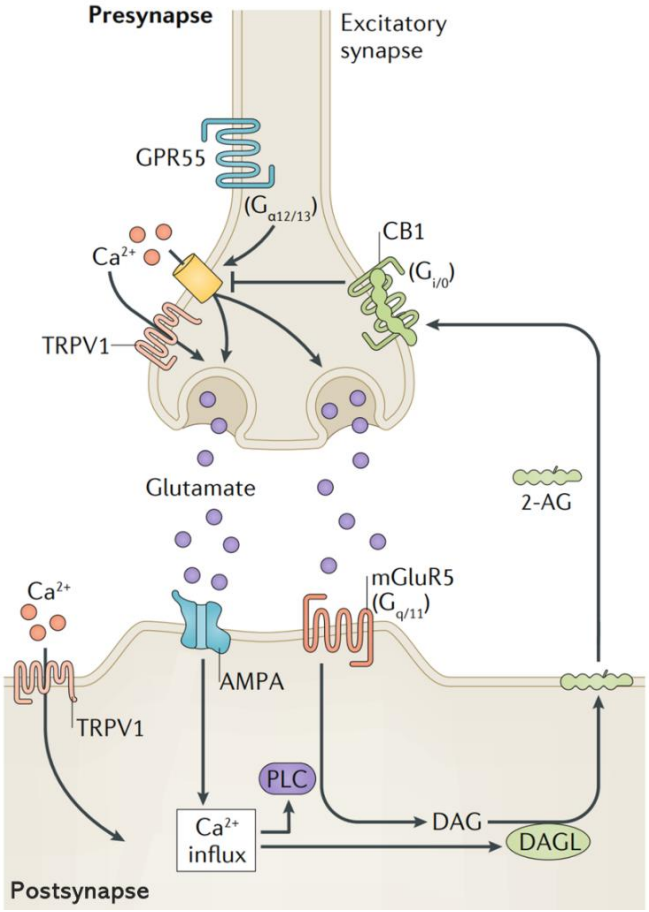

Figure 1 – Endocannabinoid retrograde signalling. The eCB canonical signalling mechanism in the CNS consists of the synthesis of eCBs (2-AG, in the figure) in response to a stimulus that travel backwards, from the postsynaptic neuron, to activate presynaptic CBRs (CB1, in the figure), what ultimately leads to the inhibition of neurotransmitter release. The stimulus triggering eCB production can be either a calcium influx, for instance due to a postsynaptic depolarization, or via $G_{q/11}$ coupled receptors (such as mGluR5, in the figure). Therefore, eCBs are able to mediate both metabotropic- depolarization-induced short forms of synaptic plasticity. Additionally, the ECS is also able to promote long term forms of synaptic plasticity, including LTD and LTP. Besides CB1 and CB2 receptors, other ECS constituents add complexity to this picture such as cation channel (TRPV1) and the putative third cannabinoid receptor GPR55 (in the figure). However, the signalling mechanisms mediated by those additional players are not as well described. Adapted from Cristino *et al.* 2020.

has been shown to be activated by eCBS and to play a role in motor coordination and in neuropathic/inflammatory pain (Wu *et al.*, 2013). Additionally the orphan GPCRs GPR18 and GPR119 might also contribute to eCB signalling, but very little is known about their role in brain physiology (Cristino *et al.*, 2020).

Several other aspects can modulate ECS signalling, allowing a fine tune of its impact on the biological processes it regulates. For instance, eCBs have different basal levels and show contrasting affinity and agonism properties for each CBR (Zou and Kumar, 2018). There are also alternative routes for eCB metabolism depending on the brain region and physiological conditions, what has an impact on their local concentration, and thus, on signalling (Zou and Kumar, 2018). Furthermore, as we will further address, CBRs present several features capable of diversifying their function and impacts on brain functions, such as a dynamic expression in response to specific stimuli (Galán-Ganga *et al.*, 2020), alternative downstream signalling pathways (Nogueras-Ortiz and Yudowski, 2016) and even distinct subcellular localizations (Busquets-Garcia *et al.*, 2015). The functional diversity combined with widespread expression of the ECS constituents results in a potential role of this system in virtually all studied brain functions (Fride, 2005).

The Endocannabinoid system: a glimpse on its complexity

The scientific method is an iterative process which consists of a constant definition, reformulation and substitution of models in an attempt to explain accumulated and new evidences. Paraphrasing Busquets-Garcia and colleagues (2018), while unveiling the mechanisms of such complex and heterogeneous biological system as the brain, it is very tempting to consider its elements as '*static bricks*' with intrinsic properties that combine to form complex networks that underlie all brain functions. In this vision, 'molecular bricks', such as receptors, are thought to exert the same function independently of the cell where they are being expressed. Therefore, if a receptor has a previously described function, it is admitted that it will induce the same effects at the different localizations that it will subsequently be found. However, numerous signalling systems escape to this paradigm and the ECS seems to be particularly defiant to it. In fact, eCB signalling depends on the brain region, the cell type and its state of activation, the receptors subcellular localization and the presence of other interacting proteins (Busquets-Garcia *et al.*, 2018; Jordan and Xi, 2019). Understanding how the ECS properties vary according to its specific spatio-temporal context is a key point for *'extract specificity from ubiquity'*.

CB2 receptor was initially considered to be absent from the brain, with early studies failing to detect its presence in the CNS (Atwood and Mackie, 2010). However, after an 'identity crisis', CB2 receptor was found to be also present and functionally relevant first in glial cells, later in neurons, under both physiological and pathological conditions (Jordan and Xi, 2019). Some authors argue that CB2 may actually be implicated in several behavioural responses. For instance, CB2 knockout in mice induces schizophrenia-related behaviours such as altered locomotor activity, anxiety-like and depressive-like behaviours, and cognitive deficits including impaired sensorimotor gating

(Ortega-Alvaro *et al.*, 2011). However, it is important to be cautious when considering CB2 brain functions, particularly when considering knockout studies since its impact might result either by direct alterations on neuron function or due to developmental impairments, giving the eCB signalling relevance during development (Hashiesh *et al.*, 2021). Furthermore, CB2 levels are incredibly modest when compared to CB1 and it is still considered as the "peripheral" CBR, as it is predominantly expressed in hematopoietic cells with prominent functions on eCB mediated immune modulation (Basu and Dittel, 2011). Therefore, and for the sake of brevity, this introduction will focus on properties of the '*main'* CB1 receptor.

Contrarily to CB2, CB1 is mostly expressed in the brain where it is claimed to be the most abundant GPCR (Cristino *et al.*, 2020). Thus, it is possible to argue that it is the major responsible for eCB mediated signalling in the CNS and most of the attention given to ECS influence in the brain has focused on CB1 receptor. Interestingly, CB1 has very few intrinsic properties and its signalling is pleiotropic, meaning that the receptor has multiple and often opposite or unrelated effects, as we will further address.

CB1 is known to be widespread in different subtypes of cells such as GABAergic, glutamatergic, noradrenergic, serotoninergic and other neurons and even glial cells (Busquets-Garcia *et al.*, 2018). However, the levels of expression of CB1 receptor are astonishingly variable across different brain regions. For instance, CB1 receptor has a pronounced distribution in basal ganglia, hippocampus, cerebellum and cerebral cortex (Ortega-Alvaro *et al.*, 2011), while it is present in relatively low levels in the hypothalamus (Busquets-Garcia *et al.*, 2018). However, early studies reported higher levels of eCB mediated G protein signalling in the hypothalamus than in the hippocampus, for instance, despite the opposite relative abundance of CB1 expression in this regions (Breivogel *et al.*, 1997). Additionally, G protein activation by CB1 receptors in the hippocampus is by far more affected and reduced by a specific CB1 deletion in glutamatergic neurons than in a similar knockout GABAergic neurons, despite CB1 protein levels are more affected in the second case (Steindel *et al.*, 2013). If CB1 signalling was an intrinsic property of the receptor, it would be expected that G protein recruitment and activation was proportional to the levels of expression. This leads us to a first key conclusion on eCB signalling: CBR abundance does not necessarily correspond to functional relevance.

The mechanisms that underlie these cell specific differences remain largely obscure but it is suggested that CBR activity might be modified by several interacting proteins and other molecules. For instance, cannabinoid receptor-interacting protein 1A (CRIP1A) has been proposed to attenuate CB1 signalling in neuronal cells by binding to its distal carboxy terminus (Smith *et al.*, 2015). Similarly to other GPCRs, CB1 activity can also be altered by allosteric modulators, such as the endogenous anti-inflammatory lipid lipoxin A4, the neurosteroid pregnenolone and pepcans, a recently identified family of endogenous peptides. Allosteric modulators act by altering the receptor's affinity to its ligands, by altering the efficacy of the signalling transduction. As an example, the presence of lipoxin A4 is able to increase the affinity of CB1 receptors to AEA, thereby potentiating the signalling and behavioural effects of this eCB (Pamplona *et al.*, 2012).

Adding more complexity to this picture, the classic two components model (one GPCR and one type of associated G protein) might be oversimplistic for a large proportion of GPCR signalling processes (Haack and McCarty, 2011). Increasing evidence has shown that much GPCR signalling involves homo- or heterodimerization which can greatly enrich the diversity of intracellular responses and alter the pharmacology of receptor ligands. It is proposed that a second GPCR might act as a ligand dependent or independent allosteric modulator and, even more interesting, heterodimerization may allow the coupling of different signalling systems by transactivation phenomena (Haack and McCarty, 2011). Strikingly, CB1 has been proposed to form homodimers and also heterodimers with other GPCRS, including CB2, dopamine D_2, μ-opioid, orexin and serotonergic receptors (Hojo *et al.*, 2008; Busquets-Garcia *et al.*, 2018). This could be a possible explanation for the CB1 paradoxical activity in the globus pallidus: in this region, it inhibits neurotransmission through the well-known retrograde signalling mechanism; however, under certain circumstances such as simultaneous dopaminergic activation, cannabinoid induced effects switch to neurotransmission potentiation via other G protein activation, namely G_s, with opposite functions to $G_{i/o}$ (Caballero-Florán *et al.*, 2016): the 'i' and 's' in index refers to the α subunit of the G protein heterotrimeric complex (composed by α, β and γ subunits) and indicates the *inhibitory* or *stimulatory* effects of the activated protein on the cAMP/PKA pathway.

Besides this phenomena, it has been shown that CB1 itself is able to bind G proteins other than $G_{i/o}$, such as G_q and $G_{12/13}$ (Diez-Alarcia *et al.*, 2016). Interestingly, the same authors also reported biased agonism, which is defined as a ligand-dependent mechanism in which ligands selectively confer activity in one signalling pathway over another.

CB1 signalling extends even far beyond G protein signalling: the receptor activation can also result in its phosphorylation mediated by GPCR kinases (GRKs). This modification leads to CB1 desensitization and the recruitment of β-arrestins. These proteins were initially described as key players for GPCR "arrest", acting as a scaffold for endocytic machinery that accomplish receptor removal from the cell surface (Zou and Kumar, 2018). More recently, β-arrestins were found to initiate a second G protein-independent wave of signalling, that can result in the activation of multiple effector proteins. This signalling cascade can be regulated by distinct β-arrestin isoform recruitment, by a biased agonism-compatible mechanism: the 'barcoding hypothesis'. In this model, specific ligands induce differential conformational alterations which are detected by GRKs resulting in a distinct pattern of phosphorylation at the receptor intercellular domains that is subsequently selectively recognized by β-arrestins, leading to specific signalling patterns activation. For instance, regarding CB1 receptor activation, β-arrestin 3 mediates receptor desensitization and internalization whereas β-arrestin 2 is responsible for activation of multiple signalling cascades, including ERK 1/2 pathways, JNK 1/2/3, Src, p38α, and also activation of transcription factors as CREB (Nogueras-Ortiz and Yudowski, 2016). This last example links CB1 activation to gene regulation and protein synthesis, suggesting that long-term effects of eCB signalling may be mediated by β-arrestins.

A progressive perspective on the ECS, beyond retrograde signalling

In accordance with the eCB classical mechanism -retrograde signalling mediated by presynaptic CB1-, it is evident that the receptor must undergo some sort of directional transport towards axon terminals. This idea is supported by the lack of anatomical evidence for CB1 localization on postsynaptic plasma membranes (Busquets-Garcia *et al.*, 2018). The mechanism that regulates this polarity establishment and maintenance remains unclear, although some studies already provided some insights such as the relevance of CB1 C-terminal helix 9 for its transport via the secretory pathway (Fletcher-Jones *et al.*, 2019). In this sense, intracellular CB1 receptors, including receptors localized on somatodendritic compartments, were often interpreted as mere 'trafficking' proteins or by-products of the natural polarization mechanism.

However, relatively recent findings challenged this paradigm by demonstrating that CB1 is present in other subcellular localizations with completely different functional consequences. There is now

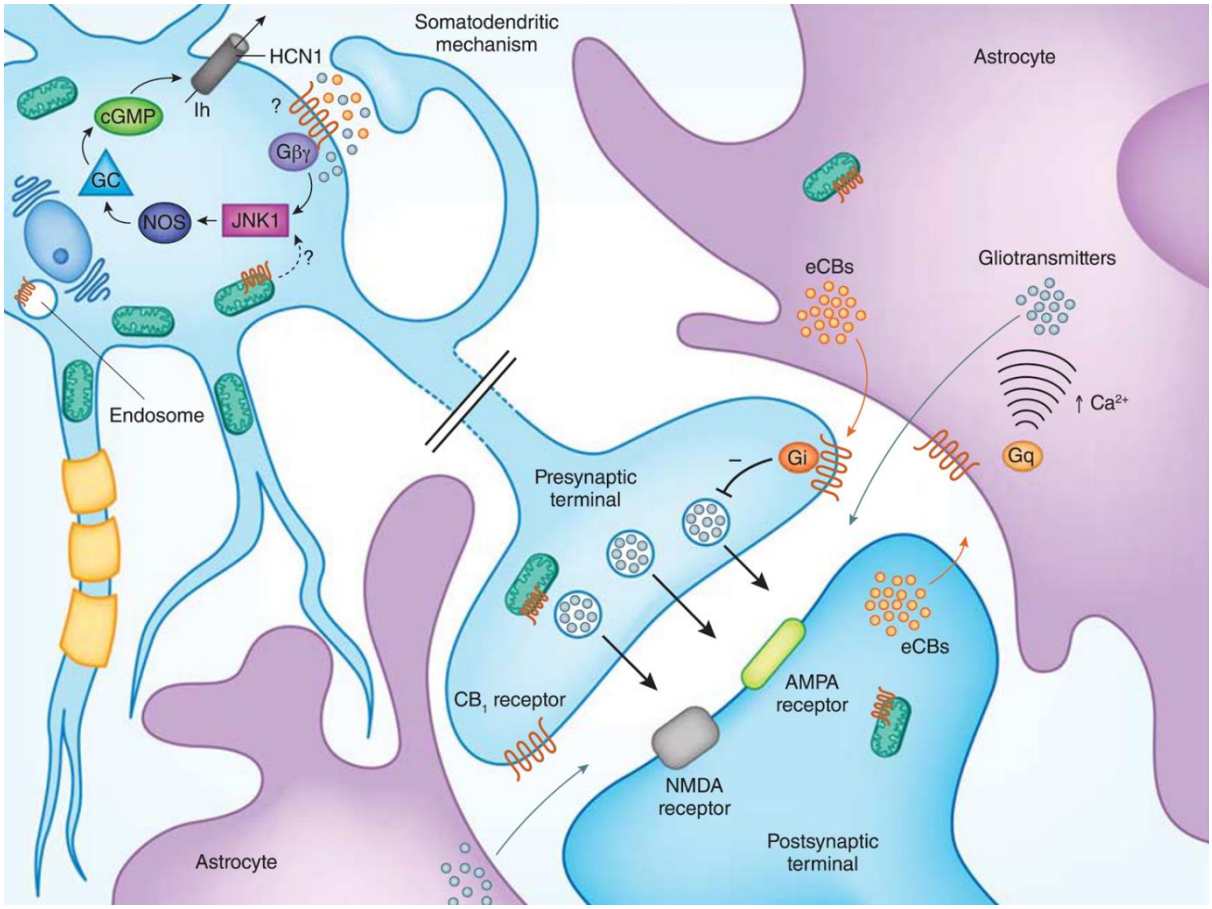

Figure 2 – ECS beyond retrograde signalling. Besides CB1 presence on presynaptic membranes, where it exerts its canonical function, this receptor is present in other subcellular localizations including somatodendritic compartments, with very distinct functions. For instance, CB1 has been shown to be present in endo/lysosomes with functions independent from external ligand binding. Another example is mitochondrial CB1, or mtCB1, which has been shown to decrease mitochondrial respiration following its activation. Astrocytes also present both membrane and mitochondrial CB1, with relevant functions such as CB1 mediated gliotransmission on tripartite synapses (in evidence in the figure). Adapted from Busquets-Garcia *et al.*, 2018.

accumulated evidences that clearly show that CB1 intracellular pools can act independently of extracellular agonist binding (Grimsey *et al.*, 2010; Zou and Kumar, 2018). For instance, activation of endo/lysosomal CB1 receptors by intracellular agonist administration is capable of increasing the release of calcium from the endoplasmic reticulum and lysosomes (Zou and Kumar, 2018). Complementally, it was recently reported that CB1 receptors located on endo/lysosomes in four distinct cell lines do not contribute to the subpopulation expressed at the cell surface, demonstrating that those two groups of receptors can act independently (Grimsey *et al.*, 2010). Although studies using cell-lines must be cautiously considered, especially when extrapolating conclusions for neuronal mechanisms, there is now a strong suggestion that intracellular CB1 receptors must have an active role in brain functions. Supporting this idea, it has been estimated that only ~15% of the Cb1 receptors appear on the cell surface regardless of total receptor number (Grimsey *et al.*, 2010).

Interestingly, intracellular pools of CB1 can add postsynaptic functions to the ECS. For instance, it was demonstrated that CB1 receptors localized on postsynaptic somatodendritic compartments of neocortical interneurons were involved in an autocrine self-inhibition mechanism (Bacci, Huguenard and Prince, 2004). Another very interesting finding was that the activation of a specific pool of postsynaptic CB1 receptors increases a hyperpolarization-activated current (Ih) which is an important modulator of dendritic excitability of a subset of hippocampal pyramidal neurons (Maroso *et al.*, 2016).

In a recent review about CB1 trafficking, Fletcher-Jones and colleagues (2020) suggested that differential internalization is one of the possible mechanisms for the establishment and maintenance of CB1 axonal polarity: CB1 is non-discriminately delivered to both the somatodendritic and axonal membrane (Leterrier *et al.*, 2006) but then it undergoes constitutive internalization by endocytosis at different rates. This proposal is consistent with the idea of a higher endocannabinoid tone on somatodendritic membranes due to DAGLα exclusive presence on dendritic surfaces (Fletcher-Jones *et al.*, 2019). Whether this mechanism contribute for eCB signalling in an autocrine fashion or is just an evolutionary coincidence can be a matter of future studies. Nonetheless, it should not be discarded the hypothesis that low levels of CB1 could be constitutively present on postsynaptic membranes under the level of detection, and that those receptor might actually have relevant functions.

The most fascinating and well-described example of intracellular CB1 is probably mitochondrial CB1 receptors, also referred as mtCB1. It was early reported that cannabinoids affect mitochondrial functions. However, it was thought that those effects resulted from unspecific mitochondrial membrane disruption, due to the lipidic nature of cannabinoids (Busquets-Garcia *et al.*, 2018). It was only recently that a significant proportion of CB1 receptors were found to be present at mitochondrial membranes, including in hippocampal neurons and also in other peripheral tissues (Melser *et al.*, 2017). The behavioural relevance of mtCB1 was further demonstrated by linking its activation to memory impairments (Hebert-Chatelain *et al.*, 2016): both exogenous and endogenous cannabinoids decrease oxygen consumption and mitochondrial mobility through a

mtCB1-dependent mechanism, leading to a reduction in synaptic transmission and memory formation (Harkany and Horvath, 2017). This function introduces a fascinating mechanism of eCB-mediated bioenergetic regulation of cognitive functions with a great potential to impact behaviour. Considering that the brain is the most energy-avid organ of the human organism and that it is the mitochondria what regulate the amount of energy (ATP) available, as well as other functions absolutely necessary for normal synaptic transmission such as the modulation of Ca^{2+} levels and the generation of reactive oxygen species, it is very likely that cannabinoid-mitochondria interactions will be a key topic on the ECS research field during the following years.

Altogether, the role of different intracellular pools of CB1 show how far the ECS extends beyond retrograde signalling. Adding more complexity to this picture, other cell types can also contribute for eCB impacts on synaptic functions, namely astrocytes. In some brain regions, astrocytes are in close anatomical relationship with nerve terminals, which make them perfectly positioned to 'listen' and 'talk' to synapses (Araque *et al.*, 1999; Oliveira da Cruz *et al.*, 2016). One of the well-known mechanisms of neuron-astrocyte interactions in those so-called '*tripartite synapses*' is the CB1 mediated triggering of gliotransmission: activation of astroglial CB1 receptors coupled to $G\alpha_{q/11}$ proteins activate phospholipase C (PLC) and lead to the production of inositol 1,4,5-trisphosphate (IP3) and calcium liberation from the internal stores (Navarrete and Araque, 2008). This ultimately results in the release of neurotransmitters by astrocytes (gliotransmission), such as glutamate (Navarrete and Araque, 2008). Further work on astroglial CB1 implicated this receptor in the release of several types of gliotransmitters that exert a variety of short- and long-term synaptic effects (Covelo *et al.*, 2021) and on behaviour, such as in THC-induced memory impairment (Busquets-Garcia *et al.*, 2015).

Astrocytes: a step back for some perspectives

Astrocytes (or astroglia as they are collectively known) are a type of highly heterogeneous (in form and function) neural cells that sustain homeostasis in the CNS. Astrocytes have a remarkable adaptive plasticity that enables them to be tightly integrated into neural networks and to act according with the context of the neural tissue. In consequence, astrocytes accumulate numerous functions that define aspects of vital importance for CNS operation, not only in the adult brain but also during development and aging (Verkhratsky and Nedergaard, 2018).

Early considerations on astroglia ranged from the perspective of Rudolph Virchow, who first introduced the concept of neuroglia as real connective tissue of the brain, describing these cells as intermediate mass into '*which the nervous system elements are embedded*' (1856-1858), to the ambitious vision of Carl Ludwig Schleich and Ramon y Cajal (1894-1895) who thought that astrocytes might contribute to control information flow in the brain, by swelling and shrinking astroglial fine processes (Navarrete and Araque, 2014; Verkhratsky and Nedergaard, 2018).

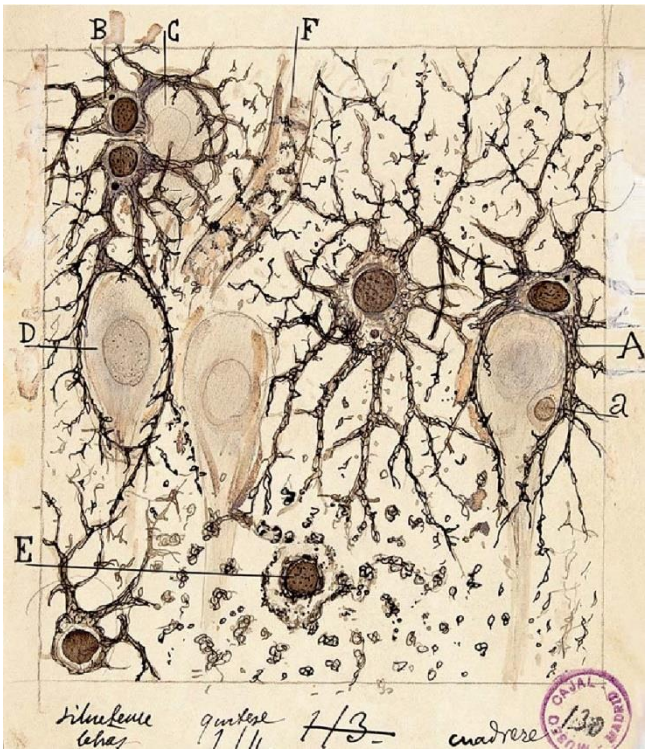

Figure 3 – Cajal's drawing of astrocytes. Astrocytes represent a large proportion of brain cells, with high heterogeneity of function and morphology. Anatomically, astrocytes are found in close relationship with both blood vessels and synapses, which corresponds to an optimal position to receive and integrate signals from different cell-types. In consequence, astrocytes accumulate a myriad of functions, from maintaining general homeostasis in the CNS, to support neurons with metabolic, structural, synaptic and protective features. Original labels, according to Navarrete and Araque, 2014: A, large astrocyte embracing a pyramidal neuron; B, twin astrocytes forming a nest around a cell, C, while one of them sends two branches forming another nest, D; E, cell with signs of "autolysis"; F, capillary vessel. Adapted from Macvicar and Newman, 2015.

Nonetheless, the study of neuroscience evolved in a neuronal-centred perspective: according to the classical view of the nervous system, even though glial cells are present in numeric superiority, they have inferior roles with the simplistic goal of providing an ideal environment for neuronal-cell function (Covelo *et al.*, 2021).

In accordance with this idea, astrocytes orchestrate homeostasis of the brain as an organ, particularly through establishing the glymphatic system, consisting of a waste clearance pathway in the brain (Benveniste *et al.*, 2019). For instance, it is estimated that 65% of β-amyloid, a by-product of neuron activity that is thought to be in the origin of Alzheimer's disease, is cleared by this glymphatic system (Frost and Li, 2017; Verkhratsky and Nedergaard, 2018).

Astrocytes also express numerous ion transporters that control ion homeostasis of the CNS, including potassium (K^+), Chloride (Cl^-), extracellular calcium (Ca^{2+}) and pH (a consequence of proton concentration, H^+) (Verkhratsky and Nedergaard, 2018). Note that ion concentration defines overall neuron excitability and major signalling processes.

Other function of astrocytes is to confer energy support to neurons, which is accomplished by a dynamic neuron-astrocyte interaction. First, astrocytes are able to control microcirculation in response to neuronal stimuli. Some astrocytes present specialized structures called *endfeet*, which are in contact with blood vessels (Gordon, Mulligan and MacVicar, 2007) and regulate blood flow by controlling local blood vessel diameter via several molecular mediators, such as prostaglandins (MacVicar and Newman, 2015). Second, astrocytes '*pre-digest*' glucose to fuel neuron activity. Although brain function lays almost completely on glucose, there is a mismatch between glucose and oxygen consumption during increased brain activity (Fox *et al.*, 1988). Most of the experimental data supports the astrocyte-neuron lactate shuttle hypothesis (ANLSH), in which astrocytes uptake a disproportional amount of glucose and export lactate, which is than used as an alternative energy source by neurons (Barros and Weber, 2018; Verkhratsky and Nedergaard, 2018; Covelo *et al.*, 2021). Lastly, this neuronal energy metabolism results a constant production of reactive oxygen species (ROS) that are further cleansed by astrocytes in order to avoid cellular damage (Verkhratsky and Nedergaard, 2018).

An additional roles of perivascular astrocytes is to be an important constituent of the blood-brain barrier (BBB). The BBB is a semi-permeable diffusion barrier that impedes influx of most compounds from blood into brain parenchyma, except a very limited and tightly regulated molecular exchange (Ballabh *et al.*, 2004; Cabezas *et al.*, 2014). Astrocytes are fundamental for the formation and maintenance of the BBB as they are able to up-regulate various of its features: they contribute to tighter endothelial tight junctions, for the expression and polarized localization of transporters, and with specialized enzyme systems, which are, transport barrier and metabolic barrier, respectively (Liu *et al.*, 2018).

Besides the aforementioned astrocytic roles providing structural and metabolic support for neurons, there is a wave of new evidence suggesting that astrocytes are intimately involved in the active control of synaptic transmission, and thus, contribute for information processing in the CNS.

Indeed, astrocytes have an astonishing role on synaptic connectivity: through a synaptic cradle that embraces the synapse, astrocytes control the emergence, maturation and extinction of synapses (Verkhratsky and Nedergaard, 2018). Supporting this idea: addition of astrocytes to pure neuronal cultures increased the number of synapses sevenfold (Pfrieger and Barres, 1997), by a mechanism that was further clarified and confirmed in vivo (Eroglu and Barres, 2010); astrocytes release important factors for synapse maturation, such as activity-dependent neurotrophic factor (ADNF) and tumour necrosis factor α (TNFα), which regulate the trafficking of glutamate receptors into postsynaptic membranes (Eroglu and Barres, 2010; Verkhratsky and Nedergaard, 2018); astrocytes can label synaptic terminals with complement factor C1q, which is a tag subsequently recognized by microglia to be eliminated by selective phagocytosis (Schafer and Stevens, 2013).

Astrocytes also have an active role on synaptic flow of information. First, they sustain neurotransmission by supplying neurons with neurotransmitter precursors, such as glutamine, precursor of glutamate and GABA, or L-serine, precursor of D-serine (Verkhratsky and Nedergaard,

2018). In addition, astrocytes are able to contribute themselves for neurotransmitter release in the context of tripartite synapses. Interestingly, not only eCBs (as it was previously mentioned) can induce gliotransmission. Other neurotransmitters including glutamate and GABA (Preston, Cervasio and Laughlin, 2019), norepinephrine (Wahis and Holt, 2021) and ATP (Shen *et al.*, 2017) can also induce calcium fluctuations in astrocytes and the consequent release of gliotransmitters. Thus, by releasing gliotransmitters such as glutamate, ATP, D-serine, and GABA (Preston, Cervasio and Laughlin, 2019), astrocytes can directly modulate synaptic transmission and plasticity. Furthermore, due to the extensive cellular branching that allows a single astrocyte to be in contact with thousands of synapses (Covelo and Araque, 2016), gliotransmitters can be released not only in the active synapse but also on neighbouring synapses. This has been proposed to be linked with the phenomena of sustained neuronal synchronization (Pirttimaki, Hall and Parri, 2011), as if astrocytes serve as way of communication between distant non synaptically-connected neurons.

Calcium fluctuations on astrocytes are extremely dynamic and their functional meaning is far from being fully understood. The main signalling pathway concerning Ca^{2+} increases is intracellular Ca^{2+} mobilization following neurotransmitter binding to GPCR: $G_{q/11}$ activation triggers PLC, which in turn catalyses the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP2) into diacyl glycerol (DAG) and IP3. The IP3, sequentially, binds to the IP3 receptors (IP3R) situated in the endoplasmic reticulum (ER), which, after being activated, distributes the calcium of the ER into the astrocytic cytosol (Cornell-Bell *et al.*, 1990). However, cytosolic Ca^{2+} elevation can spread through the activated astrocyte and into nearby astrocytes connected through gap junctions (Preston, Cervasio and Laughlin, 2019). Indeed, the extension of the Ca^{2+} signal depends on the morphological characteristics of astrocytes (Covelo and Araque, 2016), and these calcium responses may take to different cellular responses, depending on their extent in time, space and magnitude (Guerra-Gomes *et al.*, 2018).

Although astrocytes cannot be electrically stimulated like neurons, calcium fluctuations are emerging as an alternative encoding language by which astrocytes could contribute for information processing (Kastanenka *et al.*, 2020). Whether astrocytes could play a central role on cognitive functions and behaviour remains a debatable question. It has been hypothesized that astrocytes represent *'the smallest computing unit of the brain, the microchip, for integration of synaptic input'* (Verkhratsky and Nedergaard, 2018). Interestingly, it is suggested that astrocytic role in neural processing has expanded with evolution: human astrocytes are larger than in rodents and a single astrocytic domain covers many more synapses in the human brain (Han *et al.*, 2013; Verkhratsky and Nedergaard, 2018). In line with this, neonatal immunodeficient mice engrafted with human glial progenitor cells developed astrocytes able to propagate Ca^{2+} signals 3-fold faster, showed a lower threshold for LTP induction, and performed better in learning and memory behavioural tests (Han *et al.*, 2013).

Although it will require further and clearer studies, it is tempting to envision the future of neuroscience transcending the classical neuron-centred model to a new perspective in which astrocytes share or even snatch the limelight from neurons.

Astrocytes: an additional player of the ECS

CB1 presence on astrocytes was initially controversial, due to their relative low expression when compared to neurons (Busquets-Garcia *et al.*, 2018). But although low protein levels are difficult to detect by conventional immunohistochemistry or *in situ* hybridization approaches, RNASeq studies done in the cortex showed that about 20% of total CB1 receptor mRNA is present in astrocytes (Busquets-Garcia *et al.*, 2018). However, as it was mentioned before, CB1 level of expression do not necessarily predict their functional relevance. Indeed, astroglial CB1 was subsequently detected in other regions, including the hippocampus or the caudate putamen, and specific deletion studies using mutant mouse lines demonstrated that CB1 is responsible for some of the cannabinoid induced effects on these cells (Jimenez-Blasco *et al.*, 2020; Covelo *et al.*, 2021).

Interestingly, astrocytes present both membrane and mitochondrial CB1 (Jimenez-Blasco *et al.*, 2020; Covelo *et al.*, 2021). However, while membrane CB1 is thought to be mainly coupled to G_q proteins, mtCB1 activation has been associated with $Gi_{/o}$ proteins and subsequent inhibition of intra-mitochondrial cAMP/PKA pathway and to decreased phosphorylation of the complex I subunit NDUFS4 (Jimenez-Blasco *et al.*, 2020). As a result, mtCB1 activation decreases not only mitochondrial respiration by reducing the stability and activity of complex I (Jimenez-Blasco *et al.*, 2020), but also ROS production. Strikingly, ROS are a critical signal for glycolytic enzymes (Vicente-Gutiérrez *et al.*, 2020). Therefore, mtCB1 activation affected glycolytic production of lactate on astrocytes, linking eCB signalling with astrocytic metabolic support of neurons, with impacts on mice behavioural responses in social interaction assays (Jimenez-Blasco *et al.*, 2020; Covelo *et al.*, 2021).

Furthermore, as previously mentioned, astrocytes are important additional players on eCB effects on neuronal synaptic function. The involvement of astroglial CB1 in the tripartite synapse and gliotransmission was first reported in the CA1 region of the hippocampus (Navarrete and Araque, 2008). However, as it was further clarified, the resultant astrocytic glutamate release was not restricted to the active tripartite synapse and its effects were actually predominant in relative distant synapses, since the effect of astroglial glutamate on the depolarized neuron was overpowered by the direct effects of the presynaptic CB1 activation (Navarrete and Araque, 2010). By this eCB mediated form of *'lateral synaptic regulation'* (Covelo and Araque, 2016), eCBs induce a core of synaptic depression near their source (<60 µm), and a shell of lateral potentiation further away from the source of cannabinoids (>60 µm) (Covelo *et al.*, 2021).

In addition, not only membrane CB1, but also mtCB1 is involved on astrocytic lateral synaptic potentiation (Serrat *et al.*, 2020). It was recently described that mitochondria helps defining calcium dynamics on astrocytes via mitochondria-endoplasmic reticulum contacts (MERCs) (Göbel *et al.*, 2020). In this sense, it was reported that mtCB1 stimulation promotes calcium transfer from the endoplasmic reticulum to mitochondria with further consequences on the duration, spreading and frequency of cytosolic calcium signals (Serrat *et al.*, 2020). The relevance of mtCB1 on astrocyte-dependent synaptic integration was further demonstrated by electrophysiological

recordings in hippocampal slices, since genetic mtCB1 exclusion blocked lateral synaptic potentiation in neighbouring neurons (Serrat *et al.*, 2020).

The given examples on CB1 function in astrocytes exert the wide variety of roles that those receptors assume on these cells, from the impact in metabolism to its contribution on synaptic modulation. Furthermore, the influence of both membrane and mitochondrial CB1 on calcium signalling reinforced the concept of how calcium transients are tightly regulated in astrocytes and might serve as an alternative encoding language for information processing in the brain. However, it seems that this field is only in the beginning, as the impact of astroglial CB1 on behaviour remain largely obscure (Busquets-Garcia *et al.*, 2018). Nonetheless, these recent evidence point to astroglial CB1 studies as a promising topic to unveil and clarify not only how eCBs effects are highly context dependent, but also how astrocytes contribute for higher-brain functions.

eCB production on astrocytes

The vast majority of the studies regarding ECS functions on the CNS have focused on its receptors, and the consequences of the signalling that they mediate. This can be explained in part due to the lack of probes capable of reporting eCBs in real-time, with a satisfactory spatiotemporal resolution (Dong *et al.*, 2020). However, despite how challenging it is to study eCB dynamics, some evidence suggest that astrocytes are also able to produce eCBs, instead of acting only as passive '*receivers*'.

The first suggestion of the astrocytic to produce eCBs came from the early 2000s, as it was demonstrated that cultured astrocytes were able to produce AEA (Walter *et al.*, 2002) and 2-AG (Walter *et al.*, 2004) in response to ATP and endothelin-1 in a process that seems to be calcium dependent (Walter and Stella, 2003; Walter *et al.*, 2004). However, these authors considered eCB production by astrocytes as a mere neuroprotective response to prevent the propagation of harmful neuroinflammation, and these findings remained overlooked regarding their potential to contribute for neuronal activity and behaviour.

Further studies suggested a link between astroglial CB1 activation and the astrocytic production of 2-AG (Belluomo *et al.*, 2015; Hegyi *et al.*, 2018). In cultured spinal cord astrocytes, CB1 seems to be located in close proximity with DAGLα (the main enzyme responsible for 2-AG production) and the stimulation of those receptors was reported to induce a slow intracellular calcium event linked to an increased release of 2-AG (Hegyi *et al.*, 2018). Conversely, analysis of eCB concentration in mouse brains showed that deletion of astroglial CB1 increased 2-AG levels in the frontal cortex, while it had no effect in other regions. Although additional studies are needed to clarify these results, this suggests region specific mechanisms by which CB1 activity might regulate eCB dynamics on astrocytes.

The functional relevance of astroglial eCBs production and release is, however, still a matter of discussion. Transcriptomic analysis of purified *ex vivo* astrocytes detected the mRNA expression of enzymes that drive eCB production and metabolism, in particular enzymes related with 2- AG

pathways, whose levels where higher than in neurons (Zhang *et al.*, 2014). In a recent study, DAGLα expression in mouse brains was detected in astrocytes from cortex, hippocampus, striatum, amygdala and hypothalamic regions, although the levels of the enzyme were lower than in neurons (Schuele *et al.*, 2021). Finally, metabolic labelling experiments in neuron-astrocyte cocultures pointed neurons as the main producers of extracellular 2-AG while astrocytes were responsible to metabolize it and supply neurons with eCB precursors (Viader *et al.*, 2015). Altogether, this set of studies suggest that astrocytes have all the enzymes required to produce and metabolize eCBs, but their role is more implicated in a metabolic astrocyte-neuron crosstalk that contributes to the neuronal eCBs release.

Two recent papers, however, defied this concept of astrocytes as secondary players on eCB production, by relating astroglial 2-AG with astrocytic synaptic modulation and behaviour (Smith, Bekar and Nedergaard, 2020; Schuele *et al.*, 2021). In the first study, pharmacological treatment has shown that following stimulation of astroglial glutamate receptors and the consequent calcium signal, eCBs mediate hippocampal transient heterosynaptic depression (tHSD) (Smith, Bekar and Nedergaard, 2020). tHSD is a recently described mechanism of astrocytic mediated intersynaptic communication by which an active synapse transiently decreases the efficacy of a neighbouring inactive synapse (Andersson, Blomstrand and Hanse, 2007). However, despite this study showed that eCBs mediated this phenomena in hippocampal slices, the hippocampal slice pharmacological treatment with JZL184 (an inhibitor of MAGL and, thus, 2-AG degradation) was not specific for astrocytes (Smith, Bekar and Nedergaard, 2020). Therefore, additional experiments are necessary to elucidate the source of 2-AG, since an alternative astrocyte-neuronal interaction could lead to eCB release (Covelo *et al.*, 2021). In the second study, deletion of DAGLα in a specific subpopulation of adult mouse astrocytes lead to depressive-like behavioural responses and striking effects on maternal behaviour, although alterations on steady-state brain 2-AG levels were not highly pronounced (Schuele *et al.*, 2021). However, due to the prominent roles of astrocytes in neuronal support, those results could be related with general disruption of the neuronal-astrocyte eCB interactions.

Altogether, these studies suggest a great potential for the influence of astroglial eCBs on the control of astrocytic functions, neuronal and synaptic activity and, ultimately, behaviour. However, the lack of suitable techniques capable of detecting real-time eCBs and their origin cause major drawbacks in the conclusions. Thus, the development of new models to study eCB dynamics are imperative to clarify and differentiate the astrocytic role in this type of signalling form neurons.

GRABecb2.0: a new endocannabinoid fluorescent sensor

In the words of the Nobel-winning chemist Roger Y. Tsien, a biosensor *'is a molecular spy […] that will enter a cell or organism and report back to us what the conditions are'*. The basic function of a biosensor is to translate a biological property or state into a measurable data. In the specific case of an encoded fluorescent biosensor, it is designed to be expressed in a determined biological

model and to convert a signalling activity, such as the presence or concentration of a molecule of interest, its localization, configuration or activity into fluorescent signals (Greenwald, Mehta and Zhang, 2018).

In recent years, a series of genetically encoded tools for sensing neurotransmitters and neuromodulators emerged based on GPCR coupling with circular-permutated (cp) fluorescent proteins (Dong *et al.*, 2020). This kind of cp fluorescent proteins allow conformational changes to transduced to robust fluorescent responses in sensors with only one reporting unit (Greenwald, Mehta and Zhang, 2018).

Recently, this highly successful technology was applied to the development of a GPCR activation-based (GRAB) eCB sensor (Dong *et al.*, 2020). The design of this GRABeCB2.0, or simply eCB2.0, consists of the human CB1 receptor coupled with a cp enhanced green fluorescent protein (cpEGFP). The performance of this sensor was validated *in vitro*, using cultured HEK293T and neurons, revealing high sensitivity and specificity for eCBs, and kinetics on the order of seconds (Dong *et al.*, 2020). This sensor exhibits a robust fluorescent response, with moderate bias for 2-AG detection over AEA, as the maximum fluorescent response to bath-applied eCBs was 8 and 5.5 fold increase (comparing to the basal condition), respectively.

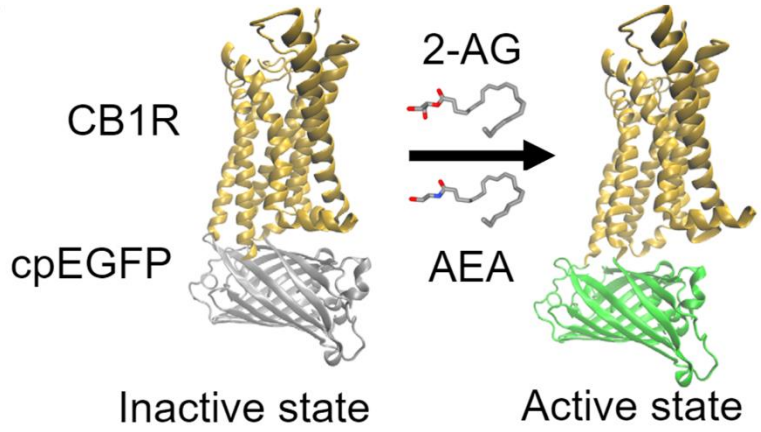

Figure 4 – Structural design of the novel GRABeCB2.0 sensor. This recently developed eCB sensor consists of the human CB1 receptor coupled with a cpEGFP, which allows the sensor to report a fluorescent response following a conformational change. Therefore, following endogenous (2-AG and AEA, in the figure) or exogenous (such as WIN55, a synthetic cannabinoid) cannabinoid binding, the sensor reports a fluorescent signal that can be measured by standard bioimaging techniques.

The potential of eCB2.0 use in preparations with a higher physiological relevance was further demonstrated. First, signals evoked by electrical stimuli were successfully detected in a stimulation frequency-dependent manner, using acute brain slices of mice previously injected in the dorsolateral striatum with a virus encoding the sensor (Dong *et al.*, 2020). Then, *in vivo* protocols were also developed and the authors were able to monitor foot-shock evoked eCB signals in the

basolateral amygdala in freely moving mice and eCB dynamics in the mouse hippocampus during running and seizure activity (Dong *et al*., 2020).

In conclusion, this novel eCB sensor is a promising versatile tool that can be used in multiple *in vitro* and *in vivo* preparations. Due to the widely use of fluorescent sensors in current molecular biology (Greenwald, Mehta and Zhang, 2018), a variety of bioimaging techniques are already well established and can now be used to study eCB dynamics. Lastly, the intrinsic properties of this sensor will overcome previous barriers of ECS research and contribute to unveil its specific dynamics at unprecedented, physiologically-relevant spatial and temporal scales.

Aim of the study

The ECS is a widely distributed signalling system in the CNS that control several molecular functions, including synaptic transmission and metabolism. However, the role of the ECS seems to be extremely context specific, not only at a tissue-level, but also in a cell-specific and even subcellular scale.

In recent years, astrocytes emerged as an important additional player of the ECS. Indeed, astrocytes express both membrane and mitochondrial CB1, accumulating functions that range from its synaptic contribution by mediating gliotransmission, to the control mitochondrial respiration and metabolism. Furthermore, both membrane and mitochondrial CB1 receptors seem to contribute for the tight regulation of calcium in astrocytes, opening the exciting perspective that the ECS might contribute to this possible alternative encoding language for information processing in the brain.

In line with this, most of the studies on the astroglial role on eCB signalling focus on CBRs, the signalling pathways that follow their stimulation and inhibition and their physiological consequences. However, the characterization of those mechanisms seems to be frequently incomplete, as the origin of those eCBs is not clarified. Interestingly, a new wave of information is suggesting that astroglial eCBs may play a role on the control of astrocytic functions, neuronal and synaptic activity and, ultimately, behaviour. However, major drawbacks on the conclusions of these studies are related with the lack of an effective tool to solve the complex eCB dynamics.

Therefore, the aim of this project is to develop a new *in vitro* model to detect eCB on astrocytes with an accurate spatiotemporal resolution, thanks to the intrinsic properties of the novel eCB fluorescent sensor, GRABeCB2.0. Complementary, the development of this *in vitro* model will be accompanied with the establishment of suitable bioimaging protocols to solve and characterize the specific astroglial eCB dynamics.

Once the bioimaging tools are established, we will use this system to determine in a direct way if astrocytes are able to produce and release eCBs and to study which are the mechanisms controlling this eCBs dynamics.

This project will contribute to elucidate specific astroglial eCB dynamics and to generate further hypothesis to be pursued in this regard. I will also contribute for a detailed characterization of the ECS, which will eventually contribute for the design of effective new therapeutical strategies.

Materials and Methods

Animals

CBN WT and DN22 WT (internal breeding) mice were used for both primary cortical brain cell cultures and acute slices preparation.

Animals were housed 2 to 6 per cage under conditions of controlled temperature (22±2˚C) and lighting (12/12 h light/dark cycle, lights on at 07:00 am), with ad libitum access to food and water throughout and a standard environmental enrichment (material for nest and cardboard house). All cultures experiments were run during the light phase (within 07:00 am-07:00 pm).

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council Committee (2011): Guide for the Care and Use of Laboratory Animals, 8th ed. Washington, DC: The National Academic Press.) and the European Communities Council Directive of September 22nd 2010 (2010/63/EU, 74). Experiments were approved by the local ethical committee of the University of Bordeaux (approval number 501350-A) and the French Ministry of Agriculture and Forestry (authorization number 3306369).

Primary cortical brain cell cultures

Wild type one to three day old (P1-P3) pups were used to generate two types of primary brain cell cultures: pure astrocytes and cocultures (astrocytes and neurons).

Concerning primary pure astrocytic cortical brain cell culture, after decapitation, brain was removed and cortex dissected in Hank's medium (dissection medium) at 4 °C in controlled sterile conditions. Hank's medium (dissection medium) contained: 10% of Hanks' Balanced Salt Solution (HBSS) 10X (plus glucose), 1% of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 1X, 1% of amphotericin B, 1% of penicillin-streptomycin (PS), 87% of ultrapure water.

The cortex was enzymatically treated with trypsin/Ethylenediaminetetraacetic acid (EDTA) for 5 min at 37°C and then stopped with complete Dulbecco's Modified Eagle Medium (DMEM) medium at 37°C. Complete DMEM consists of DMEM supplemented with 10% fetal bovine serum (FBS) and 1% of PS. The enzymatically treated tissue was softly dissociated by mechanical force by passing through a P1000 micropipette tip and cells were left in suspension to allow debris to precipitate. Cells were then seeded in T150 cell culture flasks to grow at 37ºC in a humidified atmosphere of 5% CO_2. After approximately 2 hours the media was renewed. This process was repeated twice a week. Two to three weeks later, secondary pure astrocytes were detached using trypsin/EDTA and seeded on 25mm glass coverslips to perform imaging experiments, where they were kept in complete DMEM for less than one week.

Primary cortical brain cell cocultures were generated in a similar way, with only minor changes: after cortex dissection, tissue enzymatic treatment and dissociation, cells were seeded directly on 25-mm glass coverslips treated with poly-l-lysine and left for 2h to allow cell adhesion. After this,

medium was replaced with B-27 supplemented neurobasal medium with 10 mM glucose and 2 mM glutamine at 37 °C in a humidified atmosphere of 5% CO_2. Cells were allowed to grow and develop around two to three weeks before imaging experiments.

Preparation of adenoviruses

Syn-GRABeCB2.0 and Syn-GRABeCBmut (gifts from the Yulong Li lab) were subcloned under the Cytomegalovirus (CMV) promoter and Ad5 packaging plasmids to generate adenoviral particles serotype 5, coding for the original and mutant versions of the eCB-sensitive fluorescent biosensor GRABeCB2.0 (Ad5-CMV-GRABeCB2.0 and Ad5-CMV-GRABeCB2.0mut) by Vector Biolabs (Malvern, PA, USA). Viral vectors were diluted in Serum-free DMEM to reach the concentration of $1x10^9$ plaque forming unit (PFU)/ml.

Adenovirus infection

After 1 to 2 days of coverslip seeding, cells were exposed overnight to $1x10^7$ plaque forming units (PFU) Ad5-CMV-GRABeCB2.0 viral particles. Infection was stopped 16-18 hours later by washing cells with fresh culture media. Experiments were carried out 48-72 hours post-infection.

Transfection (calcium sensor)

Transfection was carried out at 1-2 days after coverslip seeding with 1µg of plasmid CMV-REX-GECO1 using lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Medium was changed to DMEM with no antibiotics nor FBS 1h before the transfection and a mix of the DNA and lipofectamine solutions was added in a dropwise manner. 3h after, medium was changed to full DMEM. Experiments were performed 48h to 72h hours after transfection.

The plasmid CMV-REX-GECO1 was obtained from addgene and codifies the fluorescent calcium sensor REX-GECO1 (hereinafter referred as R-GECO), which emits fluorescence in the red zone of the light spectrum. This enabled a simultaneous detection of eCB (green) and Calcium (red) dynamics.

Drug and pharmacological treatment of the cells

Among the drugs used during imaging experiments WIN55-212-2 (WIN55), DO34 and ATP disodium salt-hydrate (ATP) were purchased from Sigma-Aldrich (St-Louis, USA), JZL195 and AM251 were purchased from Tocris Biosciense (Bristol, UK). Aliquots were prepared by dissolving the drugs in DMSO (WIN55, AM251, JZL195, DO34) or in water (ATP), to be further dissolved in imaging media before drug perfusion.

SNFR cell experiment

HEK293 (Cedarlane, EP-CL-0001) cells were cultured in DMEM supplemented with 10% FBS and 1% PS and maintained at 37 °C in a humidified atmosphere of 5% CO_2. To express the GRABeCB2.0 sensor, HEK293 cells were seeded in a 6-well plate and, 24h later, cells were infected with Ad5-CMV-GRABeCB2.0 viral particles. Infection was stopped 16-18 hours later by washing cells with

fresh culture media. After 24 to 48 hours, cells were detached using trypsin/EDTA and seeded on 25-mm glass coverslips treated with poly-l-lysine (control) or on top of non-infected astrocytes (SNFR) previously seeded on non-treated coverslips of the same kind. Cells were left for 16 hours to allow adhesion, after which experiments were carried out.

Fluorescence Imaging

Endocannabinoid (GRABeCB2.0) and calcium (R-GECO) imaging in cortical astrocytes was recorded using an inverted Leica DMi8 microscope (Leica Microsystems, Wetzlar, Germany) equipped with a flash 4.0 sCMOS camera (Hamamatsu, Hamamatsu, Japan). The illumination system used was a Cool LED PE-4000 (CoolLED, Andover, USA). The objective used was a HC PL APO CS2 63X oil 1.4 NA DIC. The multi-positions were done with a ASI MS-2000-500 motorized stage (Applied Scientific Instrumentation, Eugene, USA). The 37°C atmosphere was created with an incubator box and a gas heating system (Pecon GmbH, Erbach, Germany). This system was controlled by MetaMorph software (Molecular Devices, Sunnyvale, USA). An additional perfusion system was manually coupled to the microscope to allow imaging media renewal and drug perfusion, at a constant rate (2 mL/min).

Four different focal planes were manually selected and recorded every 5 seconds using an auto-focus system to prevent movement artifacts. In the case of simultaneous endocannabinoid and calcium imaging, only two focal planes were selected and the interval between images was also decreased to 2 seconds to avoid disparities between the timepoint in which images were taken in each channel. Imaging medium was perfused constantly with a perfusion pump and the different compounds added to the media when needed. Imaging medium consisted of a buffered solution (pH=7.4, adjusted with NaOH) with the following components (in mM): Hepes 10, KCl 3, $MgCL_2$ 1.25, $CaCL_2$ 1.25, NaCl 136, glucose 10. Zero potassium ($ØK^+$) solution was modified from imaging media by replacing KCl by 3 additional mM of NaCl. WIN55, was perfused as positive control for GRABeCB2.0 expression in the last 5 minutes of every experiment, except in experiments involving AM251, which is an irreversible antagonist for CB1 receptors. Furthermore, every time the experimental designed allowed, ATP was used as a control for cell responsiveness and ability to produce eCBs.

The microscopy was done in the Bordeaux Imaging Center (BIC) a service unit of the CNRS-INSERM and Bordeaux University, member of the national infrastructure France BioImaging supported by the French National Research Agency (ANR-10-INBS-04).

Data analysis

Image processing, analysis and editing were done with ImageJ software (NIH, USA). In all standard experiments, at least 30 cells from more than 3 independent experiments were considered. In the regard of experiments characterizing eCB spontaneous activity, the four areas recorded in each one of the at least 3 independent experiments were considered.

For standard experiments, regions of interest (ROIs) were manually selected around cells expressing the sensors and the average fluorescence was measured using ImageJ multi measure tool. Further data processing and quantifications were performed using Excel (Microsoft®). Briefly, following background subtraction, the variation in fluorescence was normalized with the baseline fluorescence (ΔF/F0) and the values for quantification were selected from the frames of interest, considering the graphic profile and the time interval for each stimuli. When needed, the area under the curve was calculated by a rectangular approximation.

In order to detect and characterize eCB events, a specific custom Image J macro was designed in collaboration with Román Serrat, from our lab, based on his recent unpublished paper (Serrat *et al.*, 2020). Briefly, images were first laterally realigned so as to compensate for slow, micrometric drift of the field of view. Then, calcium events (above mean+5*std) were isolated and selected in 3 dimensions (x, y, t) using 3D object counter and 3D viewer Image J plugins (events bigger than 40 pixels). From each calcium event, the amplitude, frequency, duration and spreading coefficient (max *x* distance * max *y* distance) were calculated. The detected events were further compared with the source images to discriminate between true and false events. Further analysis was performed on Graphpad software (version 5.0 or 6.0).

Statistical analysis

All graphs and statistical analyses were performed using GraphPad software (version 5.0 or 6.0). Results were expressed as means of independent data points ± s.e.m. Parametric test was used for the data and analysed using the appropriate statistical test: in experiments designed to compare sequential stimuli, a paired t-test was used, while for comparisons between independent experiments, a unpaired t-test was used.

eCB detection

Mouse cortical pure astrocytes were seeded in 6-well plates in full DMEM media. The day of the experiment, cells were washed twice and were incubated for 30 min with KRB media plus 10µM glucose containing vehicle or 1µM JZL-195. After 30 min, media was collected to analyse released eCB content and 1 ml of KRB/glucose was added to scratch cells to analyse eCB content inside astrocytes. For eCB detection, samples were homogenized and lipids extracted with chloroform/methanol/Tris-HCl 50mM pH 7.5 (2:1:1,v/v). Samples were then analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the Analytical Chemistry platform of the Neurocentre Magendie.

Preparation of adeno-associated viruses (AAV)

To express the sensor only in astrocytes, GRABeCB2.0 backbone was cloned into a GFAP (0.68) plasmid to generate the GFAP (0.68)-GRABeCB2.0 viral construct. The resulting vector was transfected with PEI into HEK293 cells together with the AAV9-serotypepackaging plasmids, the viruses were then purified by Iodixanol density gradient and titred. Virus titres were between 10^{11} and 10^{12} genomic copies per ml for all batches of virus used in the study.

Stereotaxic surgeries and AVV administration

Mice (8-16 weeks of age) were anaesthetized by isoflurane 5% to induce anesthesia and 2% during the surgery by inhalation and placed into a stereotaxic apparatus (David Kopf Instruments) linked to a digital reader with mouse adaptor and lateral ear bars. Analgesia was achieved by local application of 100 ul of lidocaine (lurocaine, 1%) and subcutaneous (s.c.) injection of buprenorphine (buprécare, 0.05 mg/kg). A heating-pad was positioned underneath the animal to keep the body temperature at around 37 ºC. Eye dehydration was prevented by topical application of ophthalmic gel. The skin above the skull was shaved with a razor and disinfected with modified ethanol 70% and betadine before an incision was made.

For all acute slice preparation and subsequent imaging experiments, viral intrahippocampal AAV delivery were performed. Mice were injected with the astrocytic virus AAV-GFAP-GRABeCB2.0 mixed with the astrocytic virus AAV-GFAP-mCherry as fluorescence control. AAV vectors were injected with the help of a glass pipette attached to a Nanoject III (Drummond, Broomall, USA). Mice were injected 0.4 µl per injection site at a rate of 0.3 µl per min with the following coordinates: anterior hippocampus, anterior-posterior -2.20; medial-lateral -2.00; dorsal-ventral -1.50. The optical fiber (400 µm diameter) was placed 250 µm above the injection site, until dorsal-ventral -1.60, in order to create a site for the virus to express.

Animals were used for acute slice preparation and subsequent imaging experiments 3-4 weeks after surgery and virus injection to allow recovery and protein expression. In addition, immediately after surgery and for two days, mice were systematically intraperitoneally injected with meloxicam (Metacam) 0.1Kg^{-1}, as an anti-inflammatory agent.

Acute Slices preparation and confocal microscopy

Animals were rapidly decapitated and the brain was placed in ice-cold artificial cerebral spinal fluid (ACSF). ACSF contained (in mM): NaCl 124, KCl 2.69, KH_2PO_4 1.25, $MgSO_4$ 2, $NaHCO_3$ 26, $CaCl_2$ 2, and glucose 10, and was oxygenated with 95% O_2 / 5% CO_2 (pH =7.3-7.4). 350mm thick coronal slices containing the hippocampus were made with a vibratome (Leica VT 1200S) and incubated in oxygenated ACSF at room temperature for > 30 min. Slices were placed in an immersion recording chamber and superfused (2 mL/min) with oxygenated ACSF and visualized using Leica DM6000 TCS SP5 multiphoton microscope.

Results

1. GRABeCB2.0 expression in astrocytes and eCB detection

The recently developed fluorescent eCB sensor, GRABeCB2.0, constitutes a new tool of major interest for our lab, in which we approach in a multidisciplinary way the multiple roles of the ECS on brain functions. Given the recent advances on the literature suggesting the potential of astrocytes to contribute for eCB signalling, in particular by producing and releasing eCBs, we considered that the characteristics of this sensor would be particularly useful on the study of eCB dynamics of this specific cell type.

In order to effectively express GRABeCB2.0 on astrocytes, we decided to generate an adenovector containing the sequence of the sensor under the constitutive cytomegalovirus (CMV) promoter (work done by Vector biolabs). Then, we set up an infection protocol on primary cultured pure astrocytes seeded on glass coverslips, suitable for observation under a videomicroscope.

In preliminary experiments, we observed the astrocytes two and three days after infection. In both cases the expression of the sensor was satisfactory (~90% of expression), although at different intensities. Furthermore, the sensor expressed in astrocytes was able to respond to a 5 minutes treatment with 100µM ATP, one of the two stimuli that had been described to increase eCB production in astrocytes (Walter *et al.*, 2004) and 2µM of WIN55, a CB1 cannabinoid agonist that should increase the fluorescence of the sensor, being our positive control . Interestingly, we observed the cells *flashing* in response to ATP treatment, while WIN55 induced an intense and persistent fluorescent response, which is in accordance with eCB production in the first case and CB1 activation by WIN in the second.

In order to effectively measure the signals returned by the sensor, we used ImageJ. ROIs were manually defined around the cells expressing the sensor to determine the average pixel intensity during the experiment. After removing the background and normalizing the fluorescence using the baseline value, we plotted this data in function of time. The resultant graphic showed a sharp

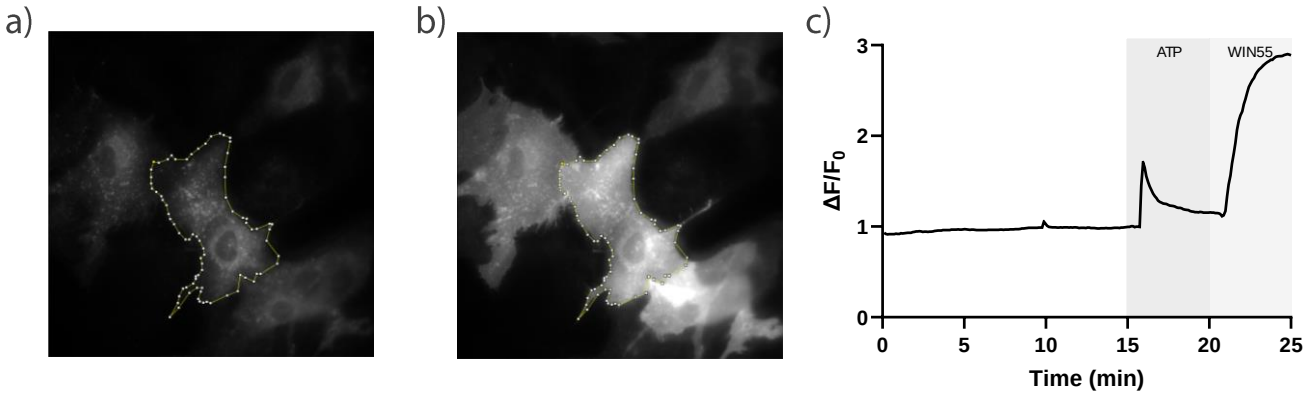

Figure 5 – Methodological strategy. Astrocytes were infected with the GRABeCB2.0 sensor, in inactive (**a**) and active (**b**) state, following WIN55 bath application. Processed data was plotted in function of time (**c**).

peak of fluorescence corresponding to the ATP *flash*, and a sharp curve increasing towards a maximum following WIN55 treatment (figure 5c). This set of preliminary experiments corroborated that our newly generated sensor was able to detect and report both endogenous and exogenous cannabinoids, and it confirmed that physiological concentrations of ATP are able to induce eCBs signals.

2. Validation of eCB detection under different conditions

In order to confirm the results suggested during the preliminary experiments, we decided to replicate the same protocol of together with a negative control in a more representative sampling. To do so, we applied the previously developed protocol to express a mutant version of the GRABeCB2.0, GRABeCBmut (or simply eCBmu), which was developed in parallel to the original sensor. The GRABeCBmut has three missense mutations in the binding pocket of the CB1 domain of the sensor, severely impairing the cannabinoid binding and consequently, the fluorescent response (Dong *et al*., 2020).

The quantification of the fluorescent response to ATP and WIN55 (figure 6b) confirmed that ATP induces eCB production in astrocytes, with an average fluorescent peak of ~1.7 fold-increase in comparison to the basal level of fluorescence. The profile of the WIN55 response among the replicates was also similar to what we previously described, with an average maximum of ~2.5 fold-increase in fluorescence. Noteworthy, during this set of experiments, fluorescence responses were extremely variable following both ATP (~1.1 to ~4.9) and WIN55 (~1.4 to ~4.8), and although there was a general tendency for ATP peaks be less intense than the WIN55 maximum, the proportion between responses was variable (data not shown). Interestingly, during the basal condition we also observed irregular eCB peaks in some cells, variable in terms of frequency and intensity, corresponding to spontaneous *flashes* with a similar profile to ATP responses. Importantly, these responses were almost completely abolished using the mutant sensor, confirming that the observed signals report cannabinoid detection (figure 6a and 6b).

Once we found out that the mutant sensor was not responsive to eCBs-generating stimuli, we next asked whether our model could report differences on the eCB dynamics on astrocytes, as well as to confirm the specificity of the sensor for eCBs. To adress this question, we recorded the fluorescent response of two sequential ATP stimuli, in which the second one was done in the presence of JZL195 (figure 6c), an inhibitor of both 2-AG and AEA degradation (figure 6e). As a result, JZL195 changed the profile of the ATP peak to a relatively long lasting curve with a significant increase of the area under the curve (AUC; figure 6d). Interestingly, in most of the cells, JZL195 treatment produced a broad peak (figure 6c) suggesting that astroglial machinery for eCB production is constitutively active, and thus, regulates a basal tone of eCBs together with the enzymes responsible for their metabolism.

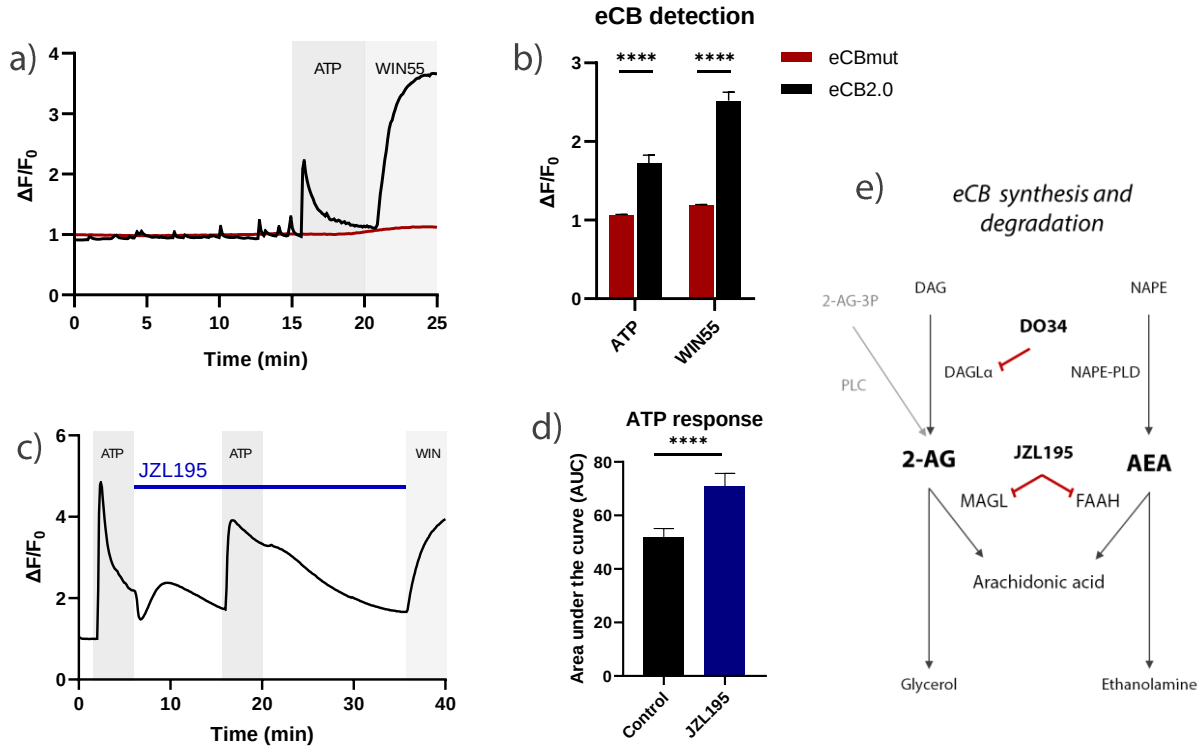

Figure 6 – Validation of eCB detection using GRABeCB2.0. (**a**) Representative trace of the comparison between the response of the GRABeCB2.0 (**black**) and GRABeCBmut (**red**) sensors to ATP and WIN55, and (**b**) respective quantification. Data represented as mean ± SEM of 32-61 cells from 3 independent experiments. **** P<0.001 by unpaired T-test. (**c**) Representative trace of astrogial eCB response to ATP stimuli, with and without JZL195 treatment and (**d**) respective quantification. Data represented as mean ± SEM of 54 cells from 3 independent experiments. **** P<0.0001 by paired T-test (**e**) Schematic representation of the eCB synthesis and metabolic pathways, based on the description of Fezza *et al.* 2014, with emphasis on the effects of JZL195 and DO34.

3. Astrocytes may present a constitutive level of eCBs

Given the hypothesis that astrocytes might have a constitutively active ECS, we asked whether these cells exhibited a basal level of eCBs. To evaluate this possibility, we compared the level of fluorescence under basal conditions and following the treatment with a CB1 irreversible antagonist, AM251, which has been shown to suppress the fluorescent responses of the GRABeCB2.0 (Dong *et al.*, 2020). The rational behind this experiment is that GRABeCB2.0 may be constitutively reporting a basal level of eCBs and that this fluorescence would disappear with AM251, which holds the sensor in a non-fluorescent conformation. In addition, given that AM251 also supresses the WIN55 response (Dong *et al.*, 2020), to maintain a positive control during this experiment, we had to treat cells with WIN55 between the basal condition and AM251 treatment (figure 7a).

The quantification of this experiment showed a barely detectable difference between the two conditions of interest, although it was still statistically significant (figure 7b). Interestingly, once

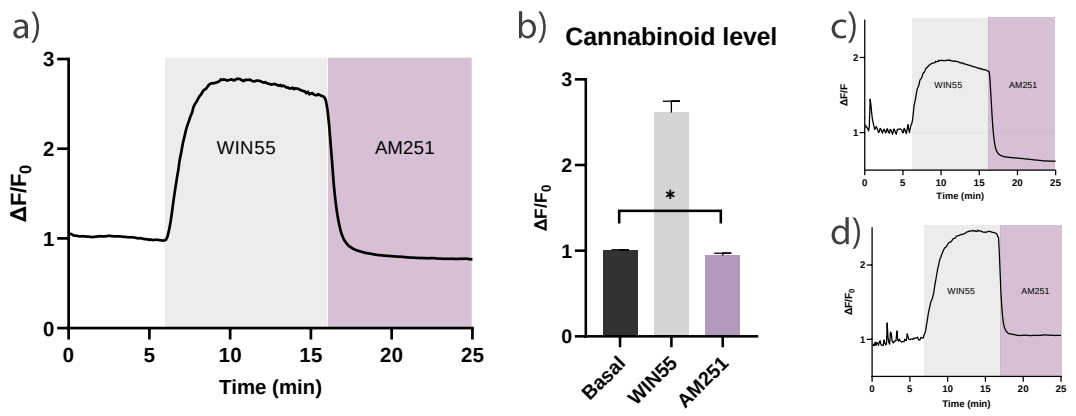

Figure 7 – Astroglial eCB basal level. (**a**) Representative trace of the experiment addressing the basal level of astroglial eCBs and (**b**) respective quantification. Data represented as mean ± SEM of 34 cells from 3 independent experiments. * P<0.05 by paired T-test. Examples of astrocytes exhibiting eCB spontaneous activity with (**c**) and without (**d**) differences on the basal level of eCBs.

again we observed some cells with spontaneous eCB activity during the basal condition (figure 7c and 7d). Furthermore, some cells exhibited a clear eCB level (figure 7c) that cannot be simply explained by the spontaneous activity, since other cells with similar phenomena showed no visible eCB basal level (figure 7d). Taken together, these results were unexpected considering the profile of the experiments. However, although some artifacts can also be in the origin of the reported difference, these results still suggest that astrocytes have a constitutive level of eCBs, in particular the hypothesis that such basal level is presented in a still unidentified subpopulation.

4. Astrocytes exhibit spontaneous eCB events

Next, we focused on the surprising spontaneous eCB activity that we observed in some astrocytes (figure 6a, 7c and 7d). Interestingly, these events had some heterogeneous characteristics. For instance, some of them had a broad spreading throughout the cytosol and even able to travel from one cell to other in some cases, while others were extremely localized, sometimes constricted to tiny astrocytic processes. To properly address these issues, we adapted a custom ImageJ macro that our lab previously developed to automatically detect and characterize calcium events (Serrat *et al.*, 2020).

We tuned the manually defined operating parameters using the data acquired for the validation of the eCB detection, in specific the first 15 minutes under basal conditions. We eventually achieved a satisfactory performance, although imperfect: in order to detect most of the eCB events, several false events were also reported. Therefore, we added a manual filter to the pipeline of this protocol, in which following the automatic detection we discriminate between true and false events by overlapping the original video with the map of events returned by the macro.

Once the protocol was established, we systematically analysed the same data. As a result, we were able to objectively characterize the dynamics of these events, in terms of spreading, duration and intensity (respectively, figure 8a, 8b and 8c).

Half of the events spread between 450 and 2800 (25% and 75% percentile) the average was ~3300 px^2, although the most frequent values were between 350 to 400 px^2, what is consistent with the heterogeneity that we previously described. In terms of duration, the temporal limitation of our method was visible: given that the minimum interval required between frames while recording 4 regions simultaneously was 5s, we were not able to detect events that last less then this interval. In fact, the distribution of the duration of events suggest that we might be losing information with these acquisition parameters. Therefore duration of each event is still to be confirmed, in particular the shorter ones. Taking that into account, the average duration of the events was around 12.15s, with half of the values concentrated between 5s and 15s, and a few extremely long events also reported (>35s). Lastly, the intensity of events presented a distribution similar to the spreading, as the mean, ~0.28, has higher than the 25% and 75% percentiles, ~0.11 and ~0.26 (values of $\Delta F/F_0$). These values are clearly lower than the previously reported responses to ATP and WIN55 suggesting that these spontaneous events may be controlled by relatively low amounts of neurotransmitters.

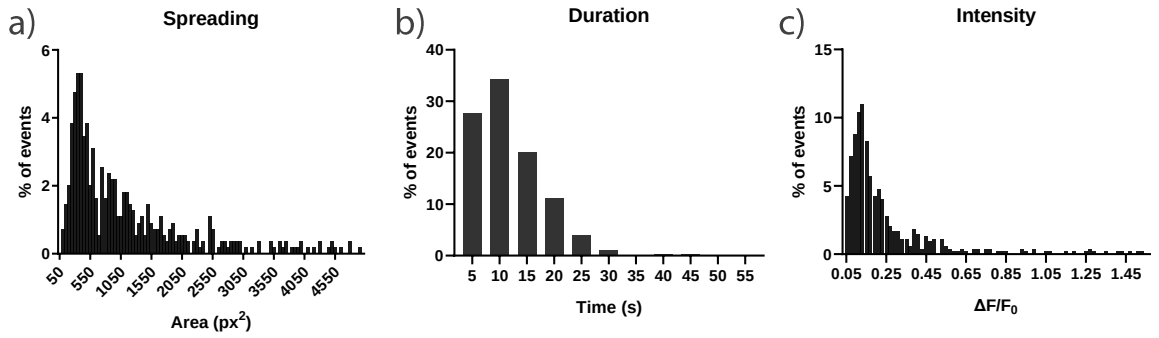

Figure 8 – Characterization of astroglial spontaneous eCB events in terms of spreading (**a**), duration (**b**) and intensity (**c**). Frequency distribution (%) of events acquired from 17 videos from 3 independent experiments. Bin interval was, respectively, 50, 5 and 0.02.

5. Both spontaneous and ATP-induced eCB events are linked to 2-AG production

Once our *in vitro* model was fully established, we proceeded to address some of the questions and hypothesis that our first observations suggested. First, we asked which was the identity of the eCBs being produced by astrocytes. Given our set up and that astrocytes were reported to produce 2-AG, but not AEA, in response to ATP (Walter *et al.*, 2004), we decided to study the eCB dynamics of astrocytes under pharmacological treatment with DO34, which inhibits DAGLα and, consequently, the main 2-AG synthesis pathway (figure 6e).

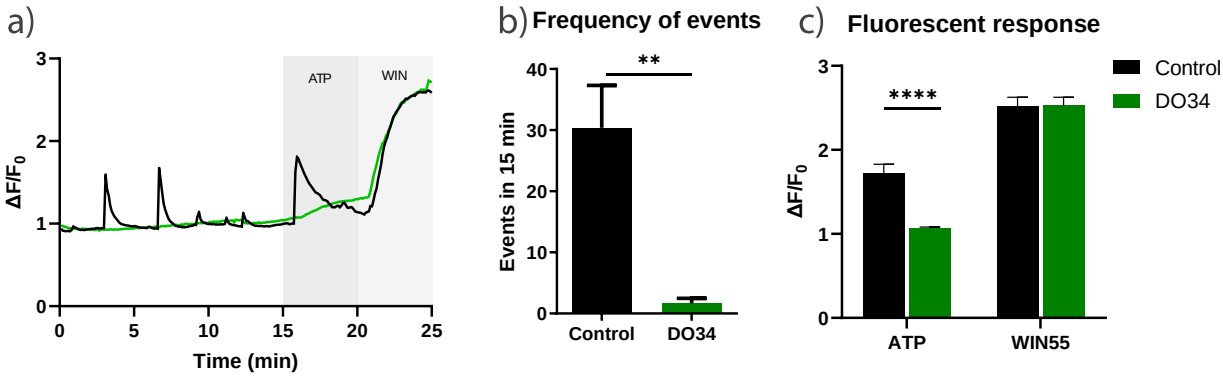

Figure 9 – Effects of astrocytes treatment with DO34 treatment on both spontaneous activity and ATP response. (**a**) Representative trace of the comparison between astroglial eCB responses under control (**black**) conditions and DO34 treatment (**green**). (**b**) Number of events detected in 15 minutes, expressed as an average of the events detected per video. Data represented as mean ± SEM of 12-17 videos from 3 independent experiments. ** P<0.01 by unpaired T-test (**c**) Quantification of the fluorescent responses under control conditions and DO34 treatment. Data represented as mean ± SEM of 51-61 cells from 3 independent experiments. **** P<0.001 by unpaired T-test

To do so, we reproduced the same experimental design of the validation of eCB detection, except this time we perfused DO34 during the first 15 minutes of experiment, and also together with the 5 minute stimulus with ATP (figure 9a). Strikingly, DO34 treatment dramatically decreased the number of events during the first 15 minutes of the experiment (figure 9b) and it also completely abolished ATP responses (figure 9c), while WIN responses remained unchanged. These results provide clear evidence that both eCB spontaneous activity and ATP response in astrocytes are mainly linked to 2-AG production.

6. eCB production and Ca^{2+} transients exhibit coordinated dynamics on astrocytes

We next focused on the involvement of calcium on eCB production in astrocytes, in particular if we could study the interaction between the eCB and Ca^{2+} dynamics using our set-up. It has previously been shown that ATP induction of eCB production in astrocytes is calcium-dependent (Walter and Stella, 2003; Walter *et al.*, 2004), however, we asked if this eCB-calcium association could be broader.

Astrocytes are known to express Kir4.1, a inwardly rectifying K^+ channel that at low concentrations of this ion is capable to mediate Ca^{2+} influx (Härtel *et al.*, 2007). We asked whether this stimulus was able to trigger eCB production in astrocytes. To verify this hypothesis, we performed a preliminary experiment to observe the response of astrocytes expressing GRABeCB2.0 to the absence of K^+, by switching the imaging medium being perfused for an equivalent medium in which K^+ is absent ($ØK^+$). Strikingly, following the switch we assisted a fantastic and uncoordinated *'firework'* of

astrocytes flashing, indicating that the influx of external Ca^{2+} was able to trigger eCB production (figure 10a).

Next, we proceeded to set up a calcium sensor to simultaneously visualize Ca^{2+} and eCB dynamics. In order to do that, we had to set up a sensor that reports Ca^{2+} levels in a different wavelength than GRABeCB2.0 (green), so we could acquire images from different fluorescent channels for each

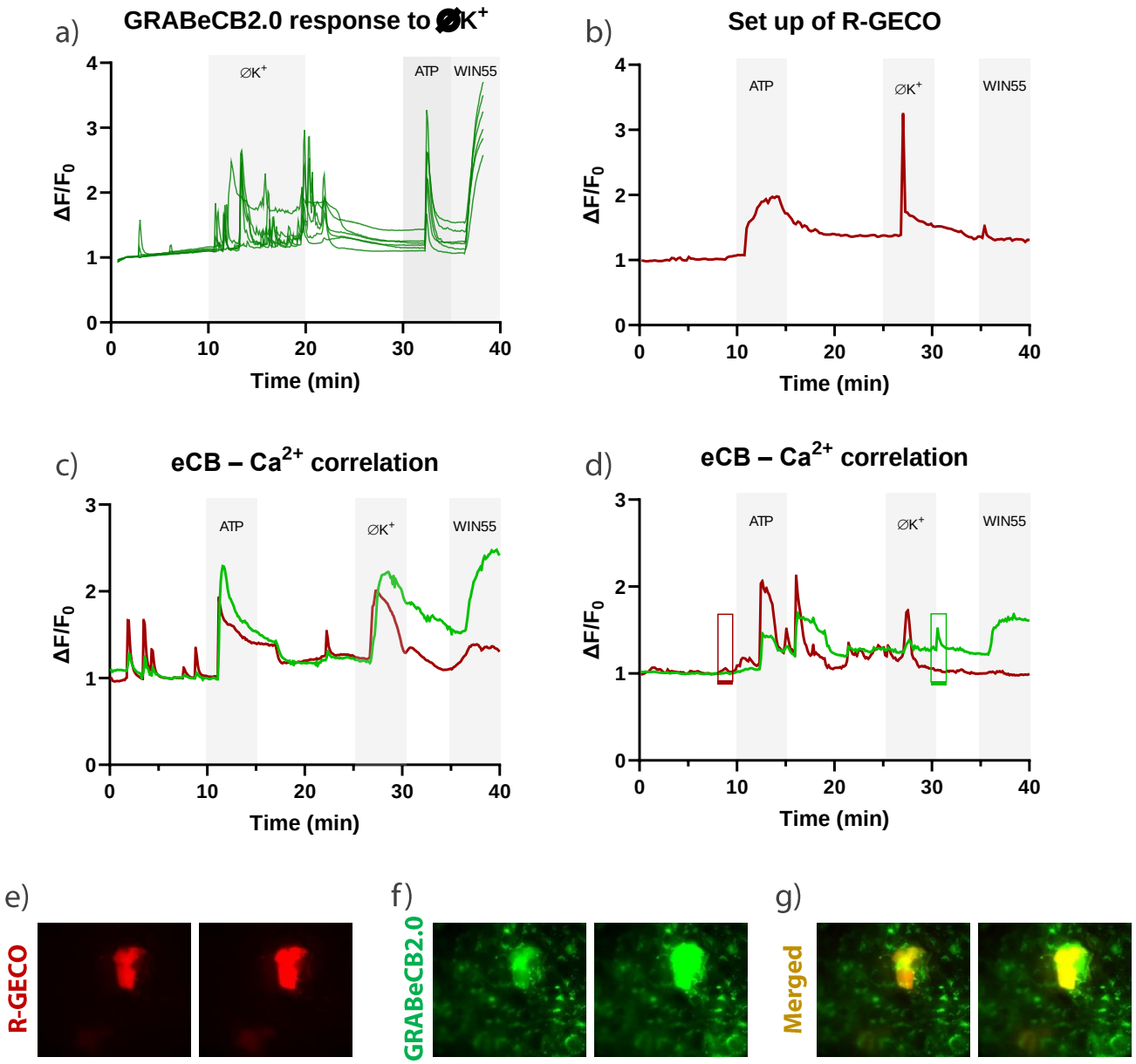

Figure 10 – Relationship between eCB (green) and calcium (red) dynamics on astrocytes. (**a**) Preliminary experiment reporting eCB activity following external calcium influx (several cells). (**b**) Preliminary experiment setting the R-GECO calcium sensor on cultured astrocytes. (**c**-**d**) Examples of single cell analysis of the correlation between Ca^{2+}-eCB dynamics, with emphasis on rare unmatched calcium (red box) and eCB (green box) events (**d**). (**e**-**g**) Example of an astrocyte expressing both R-GECO (**e**) and GRABeCB2.0 (**f**) sensors and correspondent merged images (**g**), before (**left**) and during (**right**) a Ca^{2+}-eCB event.

analyte. Among the options available at our lab, we chose to transfect a plasmid encoding the red fluorescent Ca^{2+} sensor R-GECO (Addgene). Again, in a preliminary experiment, we defined the imaging parameters to observe the fluorescent responses to ATP and $ØK^{+}$ returned by R-GECO. This turn, we switch the order of the stimuli, given that the intense calcium influx in response to $ØK^{+}$ could disrupt the internal calcium dynamics of astrocytes. We successfully observed fluorescent responses to both ATP and $ØK^{+}$ stimuli (figure 10b), which considering our previous observations was a good indicator for the possible eCB-Ca^{2+} interaction.

Once the R-GECO protocol was established, we proceeded to observe astrocytes co-expressing both sensors. Since the method used for delivery of the encoded sensor was different (chemical transfection for R-GECO and viral infection for GRABeCB2.0) the expression efficiency was extremely different. While around 90% of the astrocytes expressed GRABeCB2.0, the expression of R-GECO was rare although it showed a robust intensity. However, this disparity was an advantage from data analysis perspective: we focused on astrocytes expressing R-GECO to define clear since-cell ROIs, to further acquire the measurements of fluorescence.

Although we are still defining an effective strategy to analyse the data we generated, there is a evident visual correlation between calcium and eCB dynamics (figure 10c and 10d). By plotting the fluorescence measurements together, we observed a clear relation between eCB events and Ca^{2+} transients not only in response to both ATP and $ØK^{+}$, but also during spontaneous activity. Interestingly, we also observed a few exceptions in both calcium and eCB dynamics (figure 10d, respectively emphasised with red and green boxes). Furthermore, it seems that Ca^{2+} transients consistently precede eCB production, which is in accordance with the idea that Ca^{2+} triggers the enzymes responsible for eCB production. Lastly, regarding the effect of WIN55, it is not evident whether cannabinoids are able to induce a Ca^{2+} increase on astrocytes, since some cells exhibited some sort of response while others remained silent. As we mentioned before, this can also be a result from a rapid calcium influx induced $ØK^{+}$ by affecting astrocytic internal dynamics or by the fact that not all astrocytes in culture express endogenous CB1(Cahoy *et al.*, 2008). In conclusion, although preliminary, these observations strongly suggest the involvement of Ca^{2+} dynamics on astroglial eCB production. Nonetheless, to our knowledge it is the first time that such a clear evidence of the correlation between calcium transients and eCB production is reported.

7. Astrocytes effectively release eCBs

Although it has been previously shown that astrocytes are able to release eCBs, the contribution of these cells for eCB levels is currently considered secondary, particularly regarding 2-AG (Viader *et al.*, 2015). Therefore, we asked whether the eCB activity we observed so far was mainly regulating internal signalling or if astrocytes could effectively release eCBs to act in a paracrine way on adjacent cells.

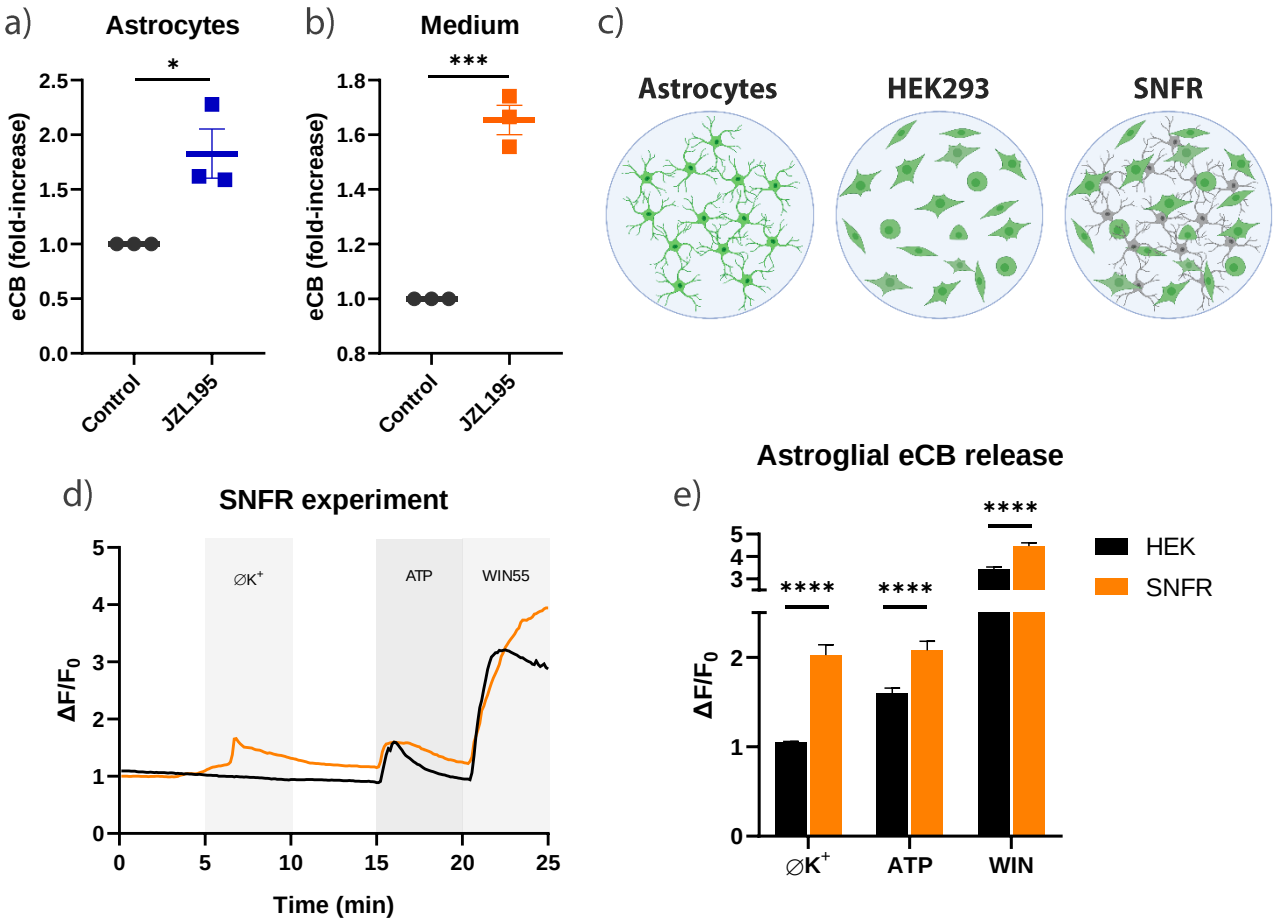

Figure 11 – Releasing of eCBs by astrocytes. (**a**-**b**) eCB quantification by ___ on astrocytes (**a**) and respective medium (**b**). Data represented as mean ± SEM of 3 independent experiments. * P<0.05. *** P<0.001 by unpaired T-test (**c**) schematic representation of the SNFR experiment; cells expressing GRABeCB2.0 represented in green, non-expressing cells represented in grey. (**d**) representative trace of the SNFR experiment (**orange**) and respective control (**black**) and respective quantification (**e**). Data represented as mean ± SEM of 53-79 cells from 3 independent experiments. **** P<0.0001 by unpaired T-test.

First, we verified whether astrocytes were able to release eCBs without any stimuli. To do so, we treated a culture of pure astrocytes with JZL195 for 30 minutes, after which we collected both cells and media, separately for eCB quantification by LC-MS/MS. In result, we saw a constant and statistically significative increase of the eCB levels on both astrocytes and media (1.8 and 1.7 fold-increase, respectively; figure 11a and 11b). This relatively rapid increase on the eCB level in the media suggest that besides having a role on the uptake and clearance of eCBs, astrocytes also contribute for external levels of eCBs without the need of any external stimuli.

Although simple, this method presented the same limitations of previous studies, particularly the lack of insights on the dynamics by which astrocytes release eCBs. Therefore, we decided to explore how could we address these dynamics using the GRABeCB2.0 sensor. We came up with the solution of instead observing directly astrocytes expressing the sensor, infect HEK293 cells and

seed them over-night on top of non-expressing astrocytes to '*sniff*' the eCBs (SNFR) and to compare the fluorescent responses with HEK293 cells alone (figure 11c).

Surprisingly, during preliminary experiments to set up the GRABeCB2.0 sensor on HEK293 cells, we observed that these cells alone produce eCBs in response to ATP, similarly to what happen with astrocytes (data not shown; a similar response can be observed on figure 11d). Indeed, HEK cells are known to express a greater variety of ATP receptors than astrocytes (Fujii, Maekawa and Morita, 2017). To overcome this barrier, we decided to stimulate astrocytes with $ØK^+$, similarly to what we had previously done to observe calcium dynamics. Strikingly, this stimulus was astrocytic specific, as no response was visible nor measurable on HEK293 cells.

Therefore, we proceeded to systematically perform this SNFR experiment with HEK293 cells in the presence and absence of astrocytes (figure 11d). Data analysis and quantification showed that in the presence of astrocytes, HEK293 cells exhibit in average a 2.03 fold-increase in fluorescence while no response (1.05 fold-increase) was reported in HEK293 alone (figure 11e). Surprisingly, both ATP and WIN55 responses were also significantly higher in SNFR cells, what can possibly suggest that there is an astroglial component being added to the cannabinoids being reported. Taken together, these results provide strong evidence that astrocytes effectively release eCBs that reach other cells, contributing for paracrine signalling mechanisms.

8. Ongoing: setting up GRABeCB2.0 on *ex vivo* models more physiologically relevant

Many central astrocytic functions in metabolism, gliotransmission and calcium signalling have been derived from studies conducted with primary cultured astrocytes, that were further confirmed *in vivo* (Lange *et al.*, 2012). However, *in vitro* models do not fully mimic the complex dynamics and interactions that astrocytes present in the brain. *In vivo*, astrocytes are in close contact with blood vessels and synapses, displaying a highly polarized morphology and reclaiming spatially exclusive microdomains (Lange *et al.*, 2012). Such intricate 3-dimensional network is surely not recreated in primary astrocytic cultures, where cells are grown in a contact-inhibited monolayer, even when cocultured with other cell-types (Lange *et al.*, 2012).

To overcome these barriers, we decided to set up the GRABeCB2.0 on more physiologically relevant *ex vivo* models, such as acute brain slices and hippocampal organotypic cultures, both currently being used in the framework of other projects in our lab. Acute brain slices are rapid preparations obtained by serial sectioning of the brain tissue, normally considering the coordinates of a region of interest. This model conserves the original 3-dimensional organization and constitutes a closely anatomically intact and representative are from the brain region they are derived. Indeed, acute brain slices are by far the most common model used on electrophysiological studies. In turn, organotypic cultures are dissected brain regions, typically the hippocampus, cultured on a porous membrane. Organotypic cultures display higher levels of plasticity and resistance to the mechanical trauma caused when neuronal processes are cut.

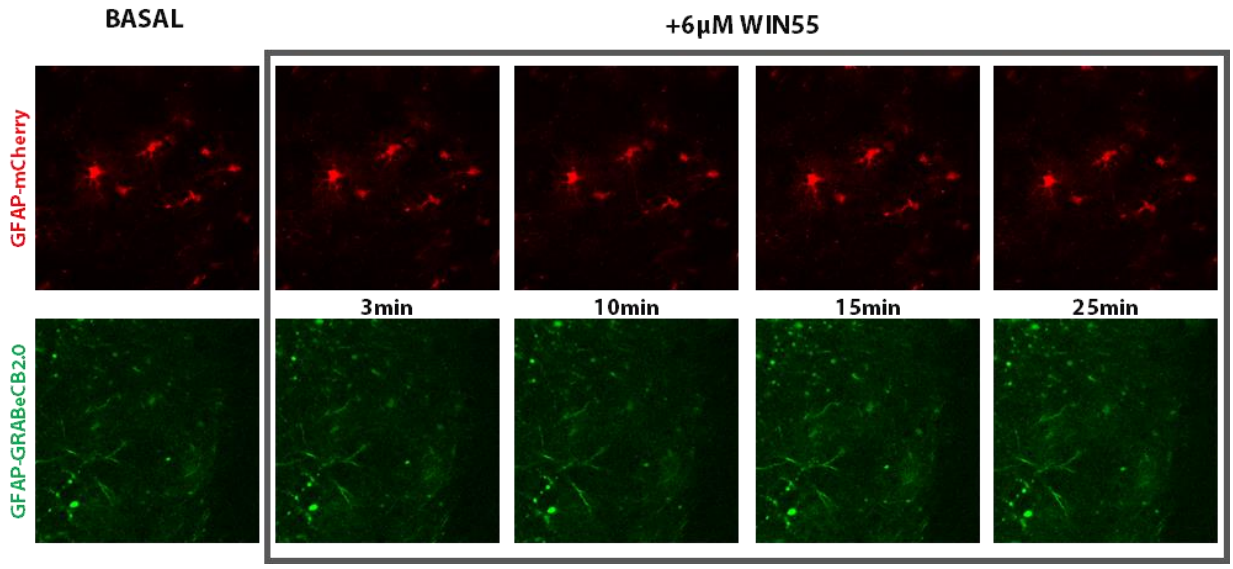

Figure 12 – First preliminary observations with hippocampal acute brain slices expressing mCherry (control) and GRABeCB2.0 under the GFAP promoter. Images acquired before (basal) and following the beginning of perfusion with ACSF containing 6µM of WIN55 (timepoints are specified in the figure). As it can be seen, no alterations following WIN55 application were observed; details in the main text.

Consequently, these cultures can be cultured for extended periods of time compared to acute brain slices (weeks over hours), thus expanding the potential of *ex vivo* models for long-term observations (Lange *et al.*, 2012). Nevertheless, both models display their own advantages and both could be used to answer the main question that has been thrilling us: whether the spontaneous eCB events are physiologically relevant and can be observed in more complex models.

While no relevant results with organotypic cultures were obtained so far, some advances were already registered during the set-up of this eCB sensor on acute brain slices. To express GRABeCB2.0 on hippocampal acute brain slices, we injected the hippocampi of adult mice with a mixture of adeno-associated virus encoding either mCherry (a red fluorescent marker used as a control in the following experiments) or GRABeCB2.0 under the glial fibrillary acidic protein (GFAP) promoter. Although GFAP expression is not accomplished by all astrocytes and it is, in fact, a hallmark of reactive phenotypes, given the absence of a universal astrocytic marker it is still one of the most popular promoters currently available for astrocyte-specific genetic manipulation (Preston, Cervasio and Laughlin, 2019).

Four weeks after injection, we used the mice brains to prepare and observe hippocampal acute brain slices under a confocal microscope (figure 12). We were able to detect a visually satisfactory expression of both mCherry and GRABeCB2.0, although their colocalization was oddly scarce (note that both proteins were expressed under the same promoter). We decided to perform a preliminary experiment to evaluate the responsiveness of the eCB sensor by perfusing ACSF containing WIN55. However, image acquisition was extremely challenging as the focal plane was constantly drifting in the z-axis. To overcome this problem, we had to constantly use mCherry as a

reference to adjust the focal plane before the acquisition of each image, which made impossible to record a video similarly to what we did *in vitro*.

Single image acquisition in multiple timepoints following the beginning of the perfusion with ACSF containing 6µM WIN55 revealed that cells apparently expressing the GRABeCB2.0 did not respond to this stimulus (figure 12). Although WIN55 treatment had previously resulted in rapid fluorescent responses in our *in vitro* experiments, we continued to perfuse WIN55 for up to 25 minutes. Still, no response was reported. However, after 10 minutes perfusing an additional amount of WIN55 (10 µM), we gave a closer look in another region we previously captured containing a group of astrocytes expressing mCherry (but with no apparent GRABeCB2.0). By comparing the images, we saw that this very same group of astrocytes emerged in the green channel. This observation confirmed that it is possible to detect cannabinoids on acute brain slices using the GRABeCB2.0. Furthermore, it suggested an alternative strategy to select regions of interest for future experiments of eCB detection on astrocytes, restabilising the potential of this methodology to validate our previous observations on a more complex and physiologically relevant model.

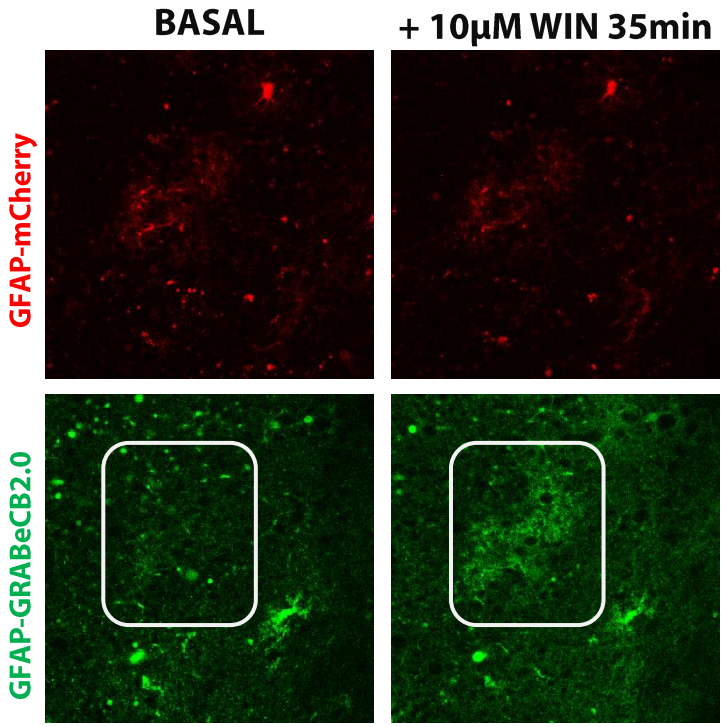

Figure 13 – Additional preliminary observations with hippocampal acute brain slices expressing mCherry (control) and GRABeCB2.0 under the GFAP promoter. Images acquired before (basal) and after 10 additional minutes (total of 35 minutes) perfusion with ACSF containing 10µM of WIN55. Responsive astrocytes pointed with a grey box.

Discussion and future directions

In the present work, we successfully established and validated a new *in vitro* model to study the eCB dynamics on astrocytes, based on standard imaging techniques to detect the activity of a recent eCB fluorescent sensor, GRABeCB2.0. This *in vitro* model proved to be a versatile tool for screening and characterization of astrocytic eCB related mechanisms in normal and altered environments, such as in response to a pharmacological treatment. Due to the intrinsic properties of the sensor, this model overcame previous barriers like spatial and temporal resolution. As an example, during several experiments, we were able to report extremely localized eCB events on tiny astrocytic processes. Not surprisingly, according with the *BioRxiv* metrics, the original GRABeCB2.0 preprint already accounts with 9 citations, even before being officially peer-reviewed. Nonetheless, to our knowledge there is still no work currently being conducted with this sensor on astrocytes. In other words, this project is pioneering the study of eCB dynamics on astrocytes at unprecedent spatiotemporal scales.

During our early experiments, we effectively demonstrated that ATP induces eCB production on astrocytes. Furthermore, we later confirmed that the eCB being produced in those responses was 2-AG, as we suspected. Although ATP-induced 2-AG production in astrocytes has previously been suggested (Walter *et al.*, 2004), most of these observations were done using millimolar concentrations, which can only be found during severe neuroinflammatory responses. In turn, in our experiments we verified that a physiological concentration of ATP (100µM) is enough to drive a rapid production of 2-AG that is quickly released and/or degraded, given the sharp peak reported by the GRABeCB2.0 sensor. Complementary, during preliminary experiments for ongoing work, using our model we observed that even lower concentrations of ATP (10µM) are able to produce the same effect. This suggests that, despite what it has been previously thought, astrocytes can contribute for 2-AG signalling under healthy physiological conditions.

Our work also addressed another well-established eCB dogma: most of the literature produced regarding the ECS refers that eCBs are produced on demand (Lu and MacKie, 2016; Zou and Kumar, 2018). However, most of the work conducted in this field focus on neuronal derived eCB signalling, which is mainly restricted to the synaptic impulse. Conversely to this idea, our results reported a tight, yet statistically significative, difference between the basal level of eCBs and the AM251 condition, which abolishes GRABeCB2.0 eCB detection (Dong *et al.*, 2020). Although, some artifacts could also be in the origin of the reported difference, such as an imperceptible photobleaching or a AM251-induced imperfect inactive conformation of the sensor, our results suggest that cultured astrocytes may present a constitutive level of eCBs, which might be particularly evident in a non-identified subpopulation. We hope that our further characterization of eCB dynamics on astrocytes will shed a light on future hypothesis to clarify the exciting possibility and physiological outcome of an astroglial constitutive basal level of eCBs.

While the idea of basal level of astroglial eCBs is debatable, our results undeniably demonstrated that cultured astrocytes have an active and functional ECS. On top of the observations proving this

perspective, there are the surprising and exciting spontaneous eCB events on astrocytes in the absence of any external stimuli.

Given these unexpected observations, we complemented our model with an additional feature to detect and characterize spontaneous eCB events. Currently, this feature has a satisfactory performance, as we are able to detect spontaneous eCB events in a semi-automatic way. Interestingly, the ratio between true and false events returned by the custom imageJ macro we adapted seems to be consistent among videos from the same experiment while it is extremely variable when comparing independent experiments (note that in our set-up each independent experiment produces 4 videos with similar characteristics, corresponding to the 4 selected regions for image acquisition). This suggests that future optimization of the protocol must focus on variables upstream data analysis. For instance, factors influencing GRABeCB2.0 expression, such as the time following incubation, or the precise circumstances of image acquisition might trigger a *butterfly effect* that manifests itself during data analysis. Therefore, a future assessment of the high performance videos may give some insights on the optimal conditions.

Nonetheless, we were able to characterize these spontaneous eCB events in terms of spreading, duration and intensity. In this regard, the characterization of the duration of events can still be improved given that the interval of image acquisition did not match the temporal scale of these events. Given that we have the right tool, improving the time resolution will be simple: by recording 1 area per coverslip instead of 4, the interval of image acquisition in our set up can be decreased until ~0.5 seconds. However, this means that a trade-off between quantity and quality of information needs to be done, implying time-consuming replicates in order to reach a representative sample.

Despite the possible improvements mentioned above, our current method can already be used to study the intrinsic eCB dynamics of astrocytes, under normal and modulated conditions. For instance, in an ongoing experiment, using primary neuron-astrocytic cocultures we are assessing the influence of the presence of neurons on the characteristics of these spontaneous eCB events. Furthermore, using bicuculine, a proepileptic drug, and tetrodoxine, a blocker of sodium channels and thus neuronal activity, we want to evaluate whether neuronal activity influences eCB dynamics on astrocytes. With these experiments we are hoping that a possible shift on the distribution of the characteristics of these eCB events will provide new insights on the mechanisms triggering and regulating eCB dynamics on astrocytes. Additionally, given that ATP was the only neurotransmitter reported to trigger 2-AG production on astrocytes (Walter *et al.*, 2004) and astrocytes have been described to release ATP among other gliotransmitters, which can further contribute for the propagation of astrocytic calcium waves (Fujii, Maekawa and Morita, 2017), we will assess whether these eCB events are regulated in a ATP cross-talk between astrocytes. To do so, we will treat cells with suramin, an antagonist of ATP receptors, and characterize eCB dynamics on pure astrocytic cultures. Taken together, our method for detection and characterization of spontaneous eCB events in astrocytes is an exciting new tool with the potential to produce major impacts on our perception about astroglial eCB dynamics in the near future.

Although AEA has also been reported to be produced by astrocytes, particularly in response to endothelin-1, a neuropeptide upregulated during brain injury (Walter *et al.*, 2002), our results pointed to 2-AG as the main eCB being produced by astrocytes. Indeed, DO34, a specific inhibitor of DAGLα which is the main enzyme responsible for 2-AG production, dramatically decreased both ATP response and eCB spontaneous activity on astrocytes. However, some cells still exhibited eCB events, although at a lower rate. One interesting hypothesis would be the potential involvement of AEA on these remaining events, although they can also be explained by an incomplete blockage of DAGLα or by 2-AG production by alternative pathways, such as PLC hydrolysis of 2-AG-3P (Fezza *et al.*, 2014). To effectively address whether astrocytes produce AEA, we will use our *in vitro* model to observe fluorescent responses to pharmacological co-treatment with both endothelin-1 and DO34, given that this stimulus may trigger 2-AG production as well (Walter and Stella, 2003).

During this project, we also addressed the previously suggested calcium-mediated mechanism triggering astroglial eCB production. Strikingly, by co-expressing GRABeCB2.0 and R-GECO we saw that eCB dynamics and Ca^{2+} transients are extremely coordinated. To our best knowledge, it is the first time that a Ca^{2+}-eCB interaction is observed at such temporal resolution, particularly in astrocytes.

Intriguingly, spontaneous calcium transients in astrocytes have long been known (Verkhratsky and Nedergaard, 2018). Such events have already been reported in all *in vitro*, *ex vivo* and *in vivo* models, and further studies clarified its involvement on several astrocytic functions, such as the regulation of regulation of neuronal spiking, synaptic plasticity and brain blood flow (Bazargani and Attwell, 2016; Verkhratsky and Nedergaard, 2018; Denizot *et al.*, 2019; Semyanov, Henneberger and Agarwal, 2020). The similarity of the descriptions of these Ca^{2+} transients with the spontaneous eCB events, together with our own observations, particularly the close coordination between Ca^{2+} and eCB dynamics, inspire a great potential for future discoveries on the roles of astroglial eCBs on behaviour. For instance, some studies have now started to suggest the possible involvement of astrocytic calcium transients on memory, depressive-like behaviours and motor coordination (Guerra-Gomes *et al.*, 2018). Strikingly, eCBs have already been reported to participate in all these three behaviour dimensions (Manira and Kyriakatos, 2010; Lutz *et al.*, 2015; Soria-Gomez *et al.*, 2017).

Interestingly, our results showed that the influx of external calcium was also able to trigger astroglial eCB production. Astrocytes are known to propagate calcium waves, resulting in synchronized global Ca^{2+} signals (Verkhratsky and Nedergaard, 2018). While distal propagation of calcium waves seems to be mediated by ATP, it has been described that astrocytes in contact can rapidly propagate calcium increases by direct Ca^{2+} transference via gap junctions (Fujii, Maekawa and Morita, 2017). Other mechanism by which calcium transients occur in astrocytes is the spontaneous opening of transient receptor potential A1 (TRPA1) channels (Bazargani and Attwell, 2016). Curiously, these receptors can be modulated by both endogenous and exogenous cannabinoids (Muller, Morales and Reggio, 2019). Thus, in our future experiments, direct influx of extracellular calcium must be considered as an additional possible mechanism for spontaneous

eCB events. To assess this possibility, further experiments are needed to clarify the calcium mechanisms triggering astroglial eCB productions. For instance, we are planning to discriminate the influence of internal Ca^{2+} dynamics and external Ca^{2+} influxes by observing astrocytes in our *in vitro* model while selectively blocking each Ca^{2+} route, by treating cells with 2-APB, a IP_3 receptor antagonist which blocks Ca^{2+} from the endoplasmic reticulum, or perfusing imaging media with no Ca^{2+} together with EGTA (Ca^{2+} chelator), to prevent the influence of external calcium.

Lastly, our results demonstrated for the first time that astrocytes are capable to effectively release eCBs in the absence of any external stimuli. Additionally, by using HEK293 cells to *sniff* astroglial eCBs, we demonstrated that eCB release by astrocytes can efficiently bind to CB1 receptors in adjacent cells and consequently act in a paracrine signalling mechanism on adjacent cells. Furthermore, besides the astrocyte-specific stimulus ($ØK^+$), ATP and WIN55 treatment also produce a statistically significative increase in the amount of cannabinoids being reported by the GRABeCB2.0 sensor. These observations are in agreement with the aforementioned ATP-induced astroglial 2-AG production and with the well-known Ca^{2+} increase and consequent gliotransmission following astroglial CB1 stimulation. However, during our of calcium imaging experiments, WIN55 frequently failed to produce a fluorescent response. This conflict of observations might be related with the experimental design of the calcium imaging experiments, in which the interval between the $ØK^+$ stimulus and WIN55 treatment is shorter, or by the fact that not all astrocytes in culture express endogenous CB1 (Cahoy *et al*., 2008). Nevertheless, given that our observations demonstrate that HEK293 express a functional ECS and other astrocytic stimuli could be increasing eCB production in these cells, it would be interesting to confirm our results by using HEK293 unable to produce eCBs. Such experiment could be accomplished either by direct genetic manipulation or controlling translation with small interfering RNAs (siRNA).

The characterization we did so far on eCB dynamics on astrocytes inspire great potential on possible physiological roles of astroglial eCBs. However, our current *in vitro* model lacks the intricate 3-dimensional network that astrocytes integrate on the brain, in particular the close anatomical relationship with other cell types. These obstacles pushed us to set-up the GRABeCB2.0 sensor on more complex and physiologically relevant *ex vivo* models. We have been facing similar difficulties in the procedures with both acute brain slices and organotypic slices. Nonetheless, by the time we cease to perform experiments for the scope of this thesis, we already validated that the GRABeCB2.0 sensor is functional on as we were able to report a WIN55-induced fluorescent response. Although this observation was reported only 35 minutes after the beginning of WIN55 application, we believe that quick responses can also be observed if we follow the right imaging strategy.

Given that focally restricted and complex calcium activity patterns have been previously reported in astrocytes using acute brain slices (Semyanov, Henneberger and Agarwal, 2020) and the close coordinated Ca^{2+}-eCB dynamics we observed, we are confident that we will be able to detect eCB spontaneous events using this model. Additionally, local translation in astrocytes has been described to mediate functional compartmentalization of astrocytic perisynaptic and perivascular

processes (Mazaré *et al.*, 2020). Considering that the most important calcium transients occur in fine astrocytic processes (Bazargani and Attwell, 2016), we are excited to see what possible eCB mechanisms will we unveil using acute brain slices.

To conclude, during this project various new methodologies have been developed to study and characterize the eCB dynamics on astrocytes. In consequence, we successfully produced valuable evidences on astroglial eCB dynamics in terms of eCB identity, mechanism and outcome. Furthermore, we are now moving to more complex models that closely mimic the structural and functional organization of the neuronal networks that astrocytes integrate. Given the promising evolution of this project, we are excited to see whether future work will provide new insights on the role of astroglial eCBs on physiological functions and, eventually, behaviour.

Concluding remarks

We successfully produced valuable evidences on astroglial eCB dynamics in terms of eCB identity, mechanism and outcome. In particular:

1. GRABeCB2.0 sensor is efficiently expressed in astrocytes and it successfully detects eCB signals induced by neurotransmitters such as ATP as well as CB1 agonists (WIN).

2. GRABeCB2.0 detects specifically eCB signals since the responses are abolished in the mutated form of the sensor (GRABeCB2.0mut) and increased in conditions with increased eCB concentrations such as in the presence of JZL195.

3. Astrocytes in culture seem to present a basal eCB tone.

4. Cultured astrocytes show spontaneous eCB activity without any external stimuli.

5. Both ATP-mediated and spontaneous eCB signals in astrocytes are mediated by 2-AG.

6. Absence of Potassium induces generation of calcium peaks and eCB signals in astrocytes.

7. Astrocytes show a clear correlation between cytosolic calcium increases and eCBs production, with the second being produced mostly after calcium spikes.

8. Astrocytes release extracellular eCBs and we developed a new tool to assess eCBs release in cultures or slices (HEK SNIFR).

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