Adaptive regulation of calcium excitability and energy metabolism by CREB-dependent transcription in astrocytes: study of the mechanisms governing astrocyte plasticity

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Memòria de tesi doctoral presentada per Abel Eraso Pichot per optar al grau de doctor en neurociències per l'Institut de Neurociències de la Universitat Autònoma de Barcelona.
Treball realitzat al departament de Bioquímica i Biologia Molecular i a l'Institut de Neurociències de la Universitat Autònoma de Barcelona sota la direcció de la Dra. Roser Masgrau Juanola i la Dra. Elena Galea Rodríguez de Velasco.

Doctorand | Directores de tesi

Abel Eraso Pichot | Dra. Roser Masgrau | Dra. Elena Galea

A la Gabi,
que siga paseando descalza por París

INDEX

I. ABSTRACT

An increasing body of evidence suggests that astrocytes participate in higher-brain functions, controlling from synaptic transmission to global brain waves and learning and memory processes. Different mechanisms have been proposed to mediate these astrocyte-dependent processes, astrocytic lactate release and calcium-dependent gliotransmission being the main known effectors. The existence of control of brain functions by astrocytes suggests that astrocytes may shape brain functions in response to experience as much as neurons, thus constituting the phenomenon of astrocyte plasticity. In neurons, the transcription factor CREB is the best known coordinator of synaptic and intrinsic plasticity. The fact that, in astrocytes, CREB activation is also activity-dependent, positions CREB as an ideal target to promote plasticity-related changes in astrocytes, too. In this thesis, we have analyzed the effect of the activation of CREB-dependent transcription in astrocytes, specifically regarding calcium signals and metabolism. We have demonstrated that activation of CREB-dependent transcription reduces cytosolic calcium events via mitochondria and increases in lactate release, which may have impact on synaptic transmission. An important contribution of the study is the molecular analysis of astrocytic mitochondria, which has revealed that astrocytes may use fuels other than glucose such as fatty acids to meet basic energy metabolic demands. Taken together, our results establish astrocytic CREB as a hub in astrocyte-plasticity and shed light on the interplay between plasticity and energy metabolism in astrocytes; these findings constitute a conceptual and mechanistic advance in the knowledge of astrocytic biology and how these cells may control learning and memory.

II. LIST OF ABBREVIATIONS

AAV	Adenoasociated virus
Ach	Acetylcholine
AD	Alzheimer's disease
Ad5	Adenovirus serotype 5
AG	Aerobic glycolysis
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANLS	Astrocyte neuron lactate shuttle
ANOVA	Analysis of variance
aPKC	Atypical protein kinase C
ATA	System A aminoacid transporter
ATF-1	Activating transcription factor 1
ATP	Adenosine triphosphate
AUC	Area under the curve
BCA	Bicinchoninic acid assay
BDNF	Brain derived neutrotrophic factor
BOLD	Blood-oxygen-level dependent
BSA	Bovine serum albumin
CA	Carbonic anhydrase
CA(1-3)	Cornu ammonis (1-3)
cADPR	Cyclic ADP ribose
CaMK	Ca2+/Calmodulin-dependent kinase
cAMP	Cyclic AMP
CBF	Cerebral blood flow
CBP	CREB-binding protein
CD38	Cluster of differentiation 38
cDNA	Complementary DNA
CEPIA	Calcium-measuring organelle-entrapped protein indicators
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CPT	Carnitine palmitoyl transferase
CRE	cAMP-responsive element
CREB	Cyclic AMP-responsive element-binding protein
CREM	cAMP response element modulator
CRTC	CREB regulated transcription coactivator
DAG	Diacyl glycerol
DMEM	Dulbecco's modified eagle medium
DMN	Default mode network
DNA	Deoxyribonucleic acid
SNARE	Soluble NSF attachment protein receptor
dNTPs	Deoxynucleotide triphosphates
DPL	Day post lesion
DTT	Dithiothreitol
ECAR	Extracellular acidification rate
ECL	Enhanced chemiluminiscence

EDTA	Ethylenediaminetetraacetic acid
EMRE	Essential MCU regulator
ER	Endoplasmic reticulum
ET-1	Endothelin 1
ETC	Electron transport chain
FACS	Fluorescence-activated cell sorting
FAO	Fatty acid oxidation
FCCP	Carbonyl cyanide-*4*-(trifluoromethoxy)phenylhydrazone
FDR	False discovery rate
fMRI	Functional magnetic resonance imaging
FSK	Forskolin
GA	Glutaminase
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GECI	Genetically encoded calcium indicator
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GGBC	Glutamate-Glutamine brain cycle
GLAST	Glutamate aspartate transporter
GLT-1	Glutamate transporter 1
GLUT1	Glucose transporter 1
GO	Gene ontology
GPCR	G-protein coupled receptor
GPN	Glycyl-L-phenylalanine 2-naphthylamide
GRP75	75KDa glucose-regulated protein
GS	Glutamine synthetase
GSEA	Gene set enrichment analysis
GSH	Gluthathione
HAT	Histone acetyltransferases
HBSS	Hank's balanced salt solution
HCAR1	Hydroxycarboxylic acid receptor 1
HIF-1α	Hypoxia inducible factor 1α
HO-1	Heme oxigenase 1
HRE	Hypoxia response element
HRP	Horseradish peroxidase
ICDH	Isocitrate dehydrogenase
IP_3	Inositol 1,4,5 triphosphate
IP_3R_2	Inositol 1,4,5 triphosphate receptor subtype 2
KID	Kinase-inducible domain
Kir	Potassium inward-rectifying channel
KIX	kinase-inducible interacting
KO	Knock-out
KRB	Krebs ringer buffer
LC	Locus coeruleus
LTD	Long-term depression
LTP	Long-term potentiation
MAM	Mitochondrial associated membrane

MCT	Monocarboxylate transporter
MCU	Mitochondrial calcium uniporter
MECP2	Methyl-CpG-binding protein 2
MICU	Mitochondrial calcium uptake protein
MOI	Multiplicity of infection
mPTP	Mitochondrial permeability transition pore
mRNA	Messenger RNA
MSK1	Mitogen- and stress- activated protein kinase
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium bromide
NA	Noradrenaline
NAADP	Nicotinic acid adenine dinucleotide phosphate
NAD	Nicotinamide adenine nucleotide
NADP	Nicotinamide adenine nucleotide phosphate
NALS	Neuronal astrocyte lactate shuttle
NBC	Sodium bicarbonate cotransporter
NCLX	Sodium/calcium/lithium exchanger
NCX	Sodium/calcium exchanger
NEP	Neuroepithelial precursor
NES	Normalized enrichment score
NFκβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Normal goat serum
NKA	Sodium-potassium adenosine triphosphatase
NKCC	Na-K-Cl cotransporter
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
NT-3	Neurotrophin-3
NVU	Neurovascular unit
OCR	Oxygen consumption rate
Orai	Calcium release-activated calcium channel protein
OXPHOS	Oxidative phosphorylation
PAGE	Polyacrilamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCX	Pyruvate carboxylase
PD	Parkinson's disease
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PE	Phenylephrine
PET	Positron emission tomography
Pfkfb3	6-phosphofructo- 2-kinase/fructose-2,6-bisphosphatase 3
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGE2	Prostaglandin E2
PIP2	Phosphatidylinositol 4,5-biphosphate
PKA	Protein kinase A
PLCβ	Phospholipase C beta
PMCA	Plasmalemmal Ca2+ pumps/ATPase

PPP	Pentose phosphate pathway
PVDF	Polyvinyl difluoride
RFP	Red fluorescent protein
RIPA	Radioimmunoprecipitation assay
RMP	Resting membrane potential
RNA	Ribonucleic acis
ROI	Region of interest
ROS	Reactive oxygen species
RPM	Revolutions per minute
RSK2	Ribosomal protein S6 kinase
RT	Room temperature
RyR	Ryanodine receptor
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
SigmaR1	Sigma non-opioid intracellular receptor
siRNA	Small interfering RNA
SOCE	Store-operated calcium entry
STIM	Stromal interaction molecule
TBI	Traumatic brain injury
TBP	TATA box binding protein
TBS	Tris-buffered saline
TCA	Tricarboxilic acid cycle
TE	Tris-EDTA
TGF-β	Transforming growth factor β
TPC	Two-pore channel
TRAP	Translating ribosome affinity purification
TRP	Transient receptor potential channel
TTBS	Tween TBS
tTPA	Tissue plasminogen activator
TTX	Tetrodotoxin
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial growth factor
WT	Wild type
xCT	Cystine/glutamate exchange transporter

INTRODUCTION

III. INTRODUCTION

For many years, the advance in neuroscience has been based on the study of neurons, excitable cells that are considered the main players of brain function. However, advances in neuroscience, although remarkable, are probably lower than expected both in the understanding of higher-brain functions in the normal brain and their dysfunction in neuropathologies.

In our laboratory we firmly believe that this slow rhythm is a consequence of the excessive neurocentrification of the field, which usually forgets other players in brain physiology such as astrocytes. The main goal of my doctoral studies is to understand how astrocytes contribute to higher-brain functions by determining how experience can shape astrocyte functions. To do so, I have studied the role of the transcription factor CREB (cAMP-responsive element-binding protein) in astrocytes, whose activation depends on neurotransmitters released by neurons - that is, CREB activation is experience dependent- (Carriba et al., 2012) and is probably the most studied transcription factor in the brain, with a demonstrated role in memory processes (Benito et al., 2011).

In particular, my investigation is centered in two key aspects of astrocyte physiology: calcium excitability and metabolism, which turned to be more unknown than I previously expected. However, before starting with the results of my investigations, I will introduce astrocytes and how their study and experimental techniques have changed these last years. Then, I will explain the main astrocytic functions, focusing on calcium signaling and energy metabolism. Finally, I will introduce CREB to explain why we think that astrocytic CREB is relevant to astrocytic plasticity.

1. Fundamentals of astrocytes

The study of astrocytes is a thrilling field in neuroscience. Since their first description by Camilo Golgi (Golgi, 1872), and their morphological representation in Cajal's drawings (Ramón y Cajal, 1897), knowledge of their functions, heterogeneity and morphology has changed amazingly thanks to the improvement of experimental techniques.

Astrocytes are one of the most abundant cell types in the so-called glia cells, a concept that may have to be reconsidered for several reasons. The term "glia" comprises many different types of cells from different embryonic origins such as oligodendrocytes, microglia and NG2 cells. Moreover, recent transcriptomic analyses have revealed that each type of glial cell is utterly different from the other cell types and hence it makes no sense to pool them together (Masgrau et al., 2017). Its inclusion as a member of the non-neuronal group has probably slowed down the study of the field; however, research on astrocytic functions has gained much interest in the last years, with a constant increase of the term "Astrocyte" during the last 20 years in published works.

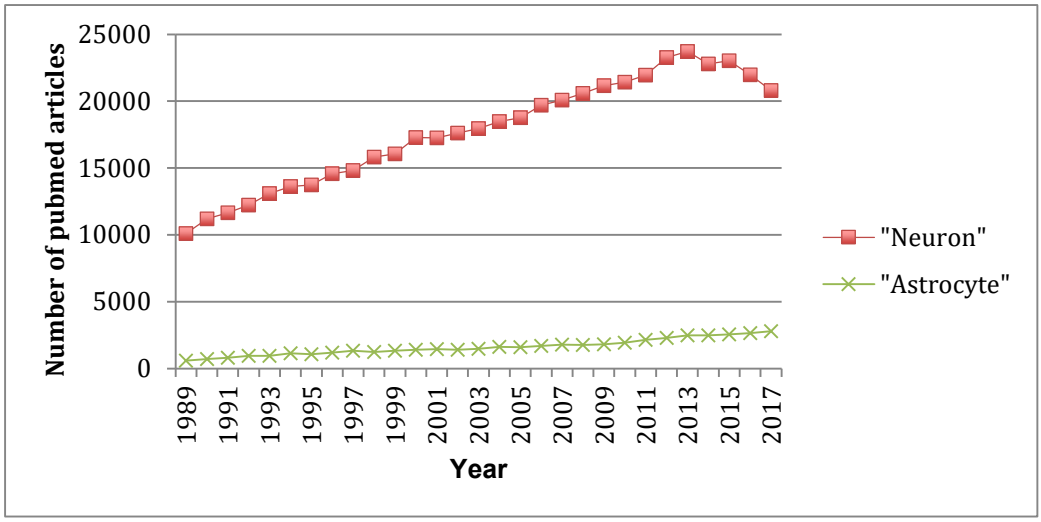


Figure 1. Astrocytes and neurons in the literature. Pubmed articles with the tag "neuron" or "astrocyte" in the last years.

One key unresolved question in the field is the total number of astrocytes in the CNS. Last studies have determined the ratio of neurons:glia cells to be 1:1.4 within the cerebral cortex (Pelvig et al., 2008) and 1:1 in the entire human brain (Azevedo et al., 2009), contrary to what was accepted even in the textbooks, that glial cells represented between 70 – 90% of brain cells (Kandel, 2013) (von Bartheld et al., 2016). Only few studies address the percentage of astrocytes inside the "glial cells", which was found to consist of 20% of the glial component of the total human cortex (Pelvig et al., 2008) or 13-17% of the mature mouse brain except in the cerebellum and olfactory bulb where they represent 3-5% of the cells (Sun et al., 2017).

The fact that the number of astrocytes within the CNS is not as large as expected does not contradict the idea of astrocytes as key elements of the brain. Instead, it opens the possibility that astrocytes are highly specialized cells perfectly integrated in the neural networks. In fact, recent studies have shown that astrocytes increase their complexity upon evolution. Human protoplasmic astrocytes are found to be larger both in diameter (2.6 times higher) and in the number of primary processes (103 more) than rodent astrocytes (Oberheim et al., 2009). This 16.5 fold increase in astrocytic volume allows human astrocytes to contact 10 times more synapses than their mouse counterparts, each astrocyte covering up to 2 million synapses (Oberheim et al., 2009). Moreover, mice transplanted with human astrocytes perform better in LTP and show improved results in behavioral tests, a fact that give us an idea of the importance of these cells in the modulation of higher-brain functions (Han et al., 2013).

In summary, astrocytes do not represent the majority of cells inside the CNS, and their increase in number does not seem to correlate with higher cognitive functions (Herculano-Houzel, 2014), but their complexity and functions are enhanced during evolution, which makes them key players in the homeostatic and higher-functions of the brain.

1.1. Astrocyte definition and classification

The definition of astrocyte is still a matter of debate, and the truth is that the field is changing so fast that there is not a uniform and unequivocal definition of astrocyte (Kettenmann and Verkhratsky, 2013). As an example, not-so-many years ago astrocytes were defined as homogeneous non-excitable brain cells that express glial intermediate filaments (mainly glial fibrillary acid protein –GFAP-) and support neuronal functions (Wang and Bordey, 2008). This definition, however, has completely changed. It is now known that not all the cells that express GFAP are astrocytes, while not all astrocytes express GFAP (Oberheim et al., 2012), that the "non-excitable" definition is not completely correct since their present activity-dependent calcium signals (Khakh and McCarthy, 2015) and that inside the "astrocyte" definition one could see a huge functional and morphological heterogeneity (Zhang and Barres, 2010). Moreover, their attributed functions have changed from being mere neuronal supporters to becoming active participants in brain physiology, as it will be further discussed.

The term "astrocyte" comprises cells with a very heterogeneous morphology. Actually, the first astrocytic classification was done based on morphological features and location. Thus, four different astrocytic types were defined: white matter astrocytes, named fibrous astrocytes, were characterized by unbranched, long and thin processes that envelop nodes of Ranvier, while gray matter astrocytes were named protoplasmic astrocytes and were characterized by many branching processes that occupy a large volume and envelop synapses (Miller and Raff, 1984). Two specialized types of astrocytes in the CNS were added to this classification: Müller cells, which are located in the retina and Bergmann glia, a cerebellum-specific type of astrocyte.

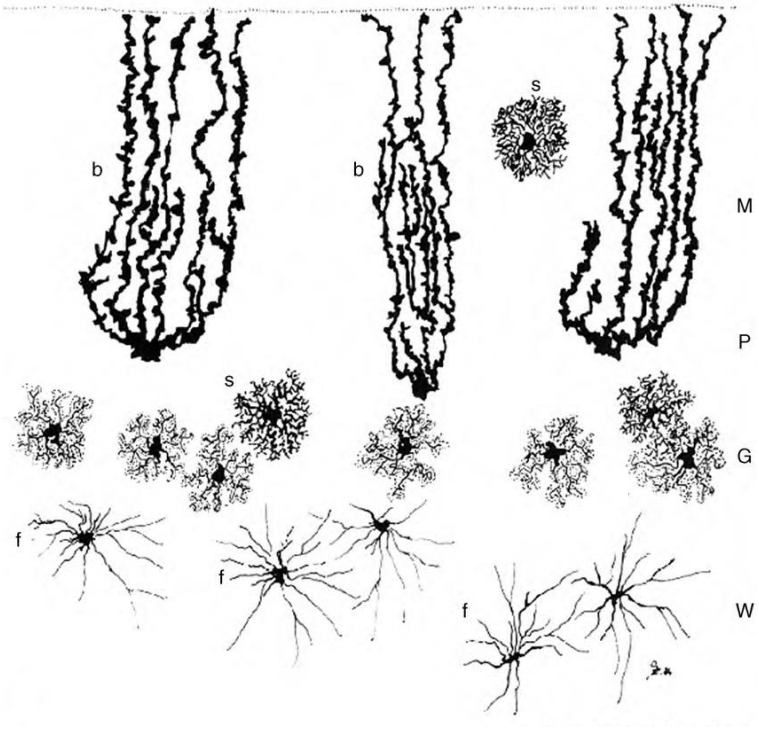


Figure 2. Morphological heterogeneity of astrocytes. Cajal's drawings from Golgi staining of astrocytes in human cerebellum. Lower case letters: b, Bergmann glia. s, Protoplasmic astrocytes. f, Fibrous astrocytes. Right capital letters: M, molecular layer. P, Purkinje cell layer. G, granule cell layer. W, white matter. Adapted from (Zhang and Barres, 2010).

New studies based on the morphology of astrocytes as well as on the expression of astrocyte-enriched proteins, however, clearly outdated the classification of astrocytes in four "types". As an example, Emsley and colleagues described nine putative "astrocyte types" in the murine CNS (Emsley and Macklis, 2006), including the types mentioned above, based on their morphology, astroglial density and proliferation rates. Moreover, two primate-specific astrocytes types were defined: varicose projection astrocytes (Oberheim et al., 2009) and interlaminar astrocytes (Colombo and Reisin, 2004).

Interestingly, recent studies have used transcriptomic analyses to describe different astrocytic populations both in cultured (Yeh et al., 2009) and in *in vivo* astrocytes (Doyle et al., 2008). In these studies, the previous stated morphological differences were associated with changes in gene expression, constituting a novel and interesting field of study trying to answer how many types of astrocytes can we molecularly identify as well as which are the differences and the functions of each astrocyte type.

This new concept of "astrocyte heterogeneity" establishes that each astrocyte population may have different functions depending on their location and type; thus making it difficult to define "astrocyte" and to establish astrocyte-common functions. However, there are some that seem to be consensual within the astrocyte community and could define, *pro tempore,* astrocytic functions.

1.2. Astrocyte functions in CNS development

Astrocytes, neurons and oligodendrocytes share a common neuroepithelial origin and are generated with a specific temporal pattern. Briefly, the neuroepithelial precursor cells (NEPs) give rise to radial glia, which is located in the ventricular zone (VZ). First divisions of radial glia only generate neurons, either neuronal restricted-progenitors or young neurons, but this neurogenesis is followed by gliogenesis. In rodents, around embryonic day 16-18 (E16-18) and until postnatal day 7 (P7), radial glia became gliogenic and starts to give rise to astrocytes, generating both differentiated astrocytes and intermediate cells that can differentiate into astrocytes (Reemst et al., 2016) (Bayraktar et al., 2014).

The study of the differentiation of progenitor cells into astrocytes is complex and incomplete. Difficulties of the study include the lack of astrocyte progenitor-specific markers at every stage, as well as the difficulty to manipulate genes that only affect astrogenesis and not neurogenesis, since all of the discovered ones so far are common to both cell types (Chaboub and Deneen, 2012).

That said, first studies about the development and migration of astrocytes indicate that the initial distribution of astrocytes during development corresponds to their final position in adulthood, suggesting that their location in adulthood is determined during development (Taft et al., 2005).

Moreover, as with neurons, astrogenesis can be found during adulthood in physiological (Zhao et al., 2007) (Rapanelli et al., 2011) and in pathological situations (Benner et al., 2013). Indeed one of the most interesting and trendy topics of the field

nowadays is how to promote this astrogenesis as a target for regeneration-based therapies (reviewed by (Koob, 2017).

Studies have shown three mechanisms by which astrocytes control CNS development. First, astrocytes participate actively in control of synapse formation during development at three stages: promoting synaptogenesis through the release of synaptogenic factors or via direct contact with synapses (reviewed by (Clarke and Barres, 2013), contributing to spine maturation (Chung et al., 2015) and mediating synaptic pruning, which may be accomplished through the release of the transforming growth factor beta (TGF-B) and the induction of expression of the initial protein of the complement cascade C1q in nearby synapses (Stevens et al., 2007) (Bialas and Stevens, 2013). C1q would induce microglial pruning (Schafer et al., 2012) or pruning through intrinsic phagocytic astrocytic mechanisms (Chung et al., 2013).

Second, astrocytes promote angiogenesis through the secretion of angiogenic factors such as vascular endothelial growth factor (VEGF), angiopoietin 1 and 2, endothelin 1 (ET-1) and heme-oxygenase 1 (HO-1) (Reemst et al., 2016). The role of these angiogenic factors in development has been questioned (Weidemann et al., 2010), however, perinatal inhibition of astrogenesis produces a reduction in branching and density of cortical blood vessels (Ma et al., 2012), supporting a role for astrocytes in developmental angiogenesis. Third, astrocytes have also been implicated in axonal guidance and outgrowth, since they secrete axonal guidance molecules and their receptors (Reemst et al., 2016).

The best demonstrations that astrocytes are relevant in development are the known neurodevelopmental disorders such as Rett syndrome. Rett syndrome is caused by the genetic loss of methyl-CpG-binding protein 2 (MECP2), expressed both in astrocytes and neurons. Lioy and colleagues found that expression of MECP2 only in astrocytes in MECP2-deficient mice contributes to partially rescue the dendrite development (Lioy et al., 2011). These findings demonstrate the implication of astrocytic functions during development and how their impaired function could induce pathological situations.

1.3 Astrocyte functions in the adult brain

1.3.1 Neurotransmitter uptake

One of the most critical points in brain homeostasis is the maintenance of low neurotransmitter levels such as glutamate. Glutamate is the main excitatory neurotransmitter in the CNS. The excess of glutamate can lead to excitotoxicity, a well-known component of the pathogenesis of different diseases (Murphy-Royal et al., 2017). Since there is not an extracellular metabolic pathway of glutamate, its degradation is produced in the astrocytic compartment surrounding synapses. To clear the glutamate from extracellular space, astrocytes use two transporters: EAAT1/GLAST (Storck et al., 1992) and EAAT2/GLT-1 (Pines et al., 1992). The genetic ablation of either transporter leads to an increase in glutamate levels, and to either

progressive neurodegeneration and paralysis -GLAST- (Rothstein et al., 1996), or to spontaneous seizures and death -GLT-1- (Tanaka et al., 1997), illustrating the importance of these astrocytic transporters in maintaining glutamate homeostasis and brain functions in general.

Interestingly, the metabolism of glutamate in astrocytes produces glutamine thanks to the astrocytic specific enzyme glutamine synthetase (GS) (Martinez-Hernandez et al., 1977). Glutamine is then transferred to neurons to produce glutamate again, establishing the well-known glutamate-glutamine cycle (Cerdan et al., 1990) (Shen, 2013). In addition, glutamine is used in GABAergic neurons to produce GABA via glutamate decarboxylase; this is known as the glutamine-GABA cycle (Walls et al., 2015).

As a conclusion, astrocytes, on the one hand, maintain brain homeostasis through glutamate uptake creating a proper environment for glutamate signalling and avoiding excitotoxicity and, on the other hand, they are necessary to maintain glutamate and GABA pools in neurons via the glutamate/glutamine/GABA cycles.

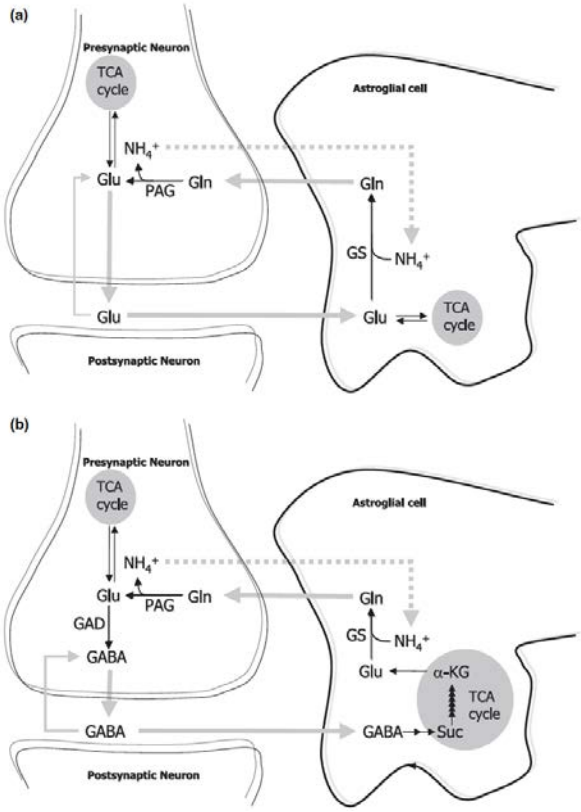


Figure 3. Glutamate/glutamine/GABA cycle. a) In glutamatergic synapses, released glutamate (Glu) is taken up by nearby astrocytes and converted to glutamine (Gln) which is transferred to neurons to produce glutamate again through the phosphate-activated glutaminase (PAG). b) In GABAergic synapses, released GABA is converted to glutamate after entering the tricarboxylic acid (TCA) cycle. Glutamate is converted again to glutamine by glutamine synthetase (GS) and glutamine is transported to nearby GABAergic neurons to synthetize GABA. From (Bak et al., 2006).

Of note is also the role of astrocytes in the uptake of GABA, the main inhibitory neurotransmitter of the CNS. Although it seems clear that neuronal GABA transporter (GABA-1) is the most important transporter to keep GABA levels low (Zhou and Danbolt, 2013), recent evidence suggest that astrocytic GABA transporter (GABA-3) may be implicated in the regulation of tonic GABA current and signalling pathways (Boddum et al., 2016) (Kersante et al., 2013).

1.3.2 Ion homeostasis and pH regulation

Neuronal activity and action potentials lead to the local accumulation of potassium ions (K^+) in the extracellular space, which in turn can alter neuronal membrane potentials and produce hyperexcitability. One important function of astrocytes is the buffering of potassium. Indeed, astrocytes present a strong negative membrane potential and great permeability to potassium due to the expression of a spectrum of K^+ channels (Bellot-Saez et al., 2017). Among these potassium channels, probably the most important one is the NKA pump, which mediates the extrusion of three sodium ions and the entry of two potassium ions against their concentration gradient at the expense of the consumption of one ATP molecule (Schousboe and Hertz, 1971).

In addition to high K^+ permeability, astrocytes are characterized by a strongly negative resting membrane potential (RMP). This allows other "potassium-clearance" mechanisms to import potassium such as the inward-rectifying channels (Kir), whose flow direction depends on the RMP of the cell (Butt and Kalsi, 2006). Moreover, the Na-K-Cl cotransporter (NKCC) has been implicated in the regulation of potassium homeostasis by astrocytes.

Astrocytes are coupled via gap junctions forming a syncytium that allows astrocytes to act cooperatively (Hertz and Chen, 2016). One clear example of this cooperative function is the clearance of potassium. Once inside an astrocyte, the potassium ions are transported through the syncytium and extruded to the extracellular space or the bloodstream in areas where the extracellular potassium concentration is lower. This gap junction-coupled network allows all astrocytes of the syncytium to have "isopotentiality", maintaining the inward driving force of K^+ in areas of high extracellular potassium and increasing its release in distal regions (Ma et al., 2016). One of the most important channels using this conferred-driving force is the Kir4.1, postulated to be the main responsible for the potassium clearance (Larsen and MacAulay, 2014). Interestingly, Kir4.1 expression is reduced in a variety of neurodegenerative diseases such as Huntington disease, Alzheimer's disease, amyotrophic lateral sclerosis, and Alexander disease (Nwaobi et al., 2016).

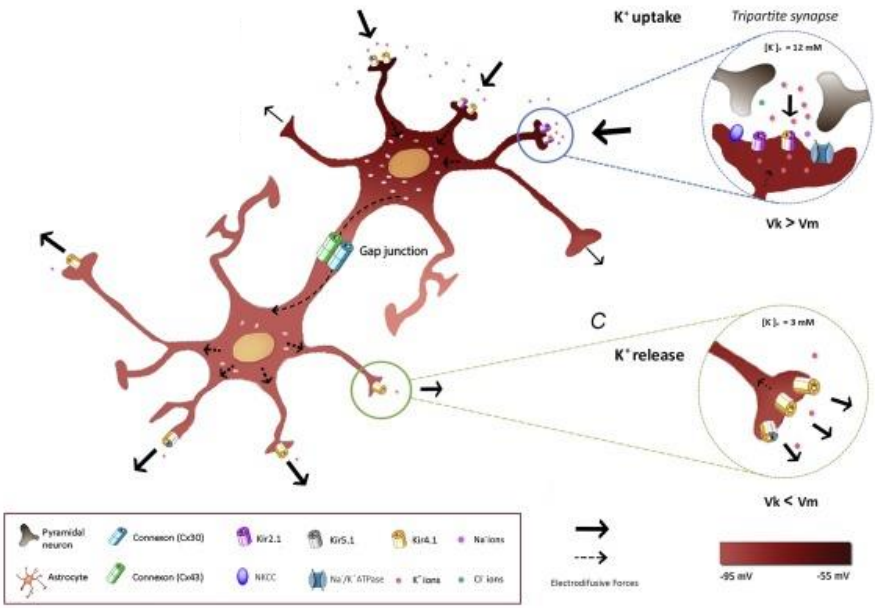


Figure 4. Astrocytic potassium clearance mechanisms. K⁺ uptake form firing neurons flows intracellularly through Gap Junctions of the astrocytic syncytium and is extruded in distal regions with lower extracellular potassium concentrations. Arrows indicate the direction of the K+ driving force. Adapted from (Bellot-Saez et al., 2017).

As with potassium, H^+ buffering and thus pH homeostasis is crucial not only in brain parenchyma but in almost all tissues of living organisms. In the brain, many neuronal phenomena including as metabolism or membrane conductance are affected by small changes in extracellular and intracellular pH (Obara et al., 2008). Astrocytes may play an important role in brain H^+ buffering, since they express a whole range of proteins which can act as pH regulators or cotransport metabolites together with protons such as the carbonic anhydrase (CA), monocarboxylate transporters (MCTs), GLT transporters or the Na^+/HCO exchanger (Liu et al., 2017).

1.3.3 Antioxidant functions

High metabolic activity, high content of unsaturated fatty acids and low antioxidant capacity makes the brain tissue particularly prone to oxidative damage (Belanger et al., 2011). One proof of this statement is that many neuropathological situations show oxidative brain damage: neurodegenerative disorders, stroke and traumatic brain injury (Dringen, 2000).

Curiously, neurons, which are supposed to be the most oxidative cells in the brain and, as a consequence, the ones generating more oxygen radicals, are the ones with less antioxidant capacity. Astrocytes, in turn, present a much more effective defence against reactive oxygen species (ROS) (Wilson, 1997). Thus, in order to have a proper brain function, there is a need to cooperate between these two cell types (Belanger and Magistretti, 2009). Indeed, astrocytes present higher activity of ROS-detoxifying enzymes (including glutathione (GSH) peroxidase, GSH S-transferase, catalase, heme-oxygenase 1 or thioredoxin reductase) and higher levels of antioxidant molecules

such as the same GSH, ascorbic acid or vitamin E (Makar et al., 1994). The high antioxidant capacity of astrocytes is further evidenced by the higher resistance of these cells to oxidative factors in culture and the increased resistance of neurons to these factors when cocultured with astrocytes, suggesting a capacity of astrocytes to protect neurons from oxidative stress (Belanger and Magistretti, 2009).

GSH is the most abundant antioxidant molecule in the brain, and a paradigm of the cooperation between astrocytes and neurons in the antioxidant defence. GSH is a tripeptide composed of glutamate, cysteine and glycine, which is generated in subsequent reactions catalysed by γ-glutamyl cysteine ligase and GSH synthetase. Since the intracellular amount of glutamate and glycine is relatively high, cysteine seems to be the limiting substrate for GSH synthesis (Dringen, 2000). Cysteine is obtained only in astrocytes in its oxidized form (cystine) via the cystine/glutamate exchange transporter (xCT), where it is reduced back to cysteine to produce GSH and release the product in the extracellular space (Seib et al., 2011). Both astrocytes and neurons are able to generate GSH, but since only astrocytes can use cystine, neurons depend on astrocytic GSH for their antioxidant defense. Actually, GSH content in neurons is strongly increased when cocultured with astrocytes (Dringen, 2000), and neuronal resistance to nitric oxide (NO) donors is dramatically reduced in the presence of GSH-depleted astrocytes (Chen et al., 2001).

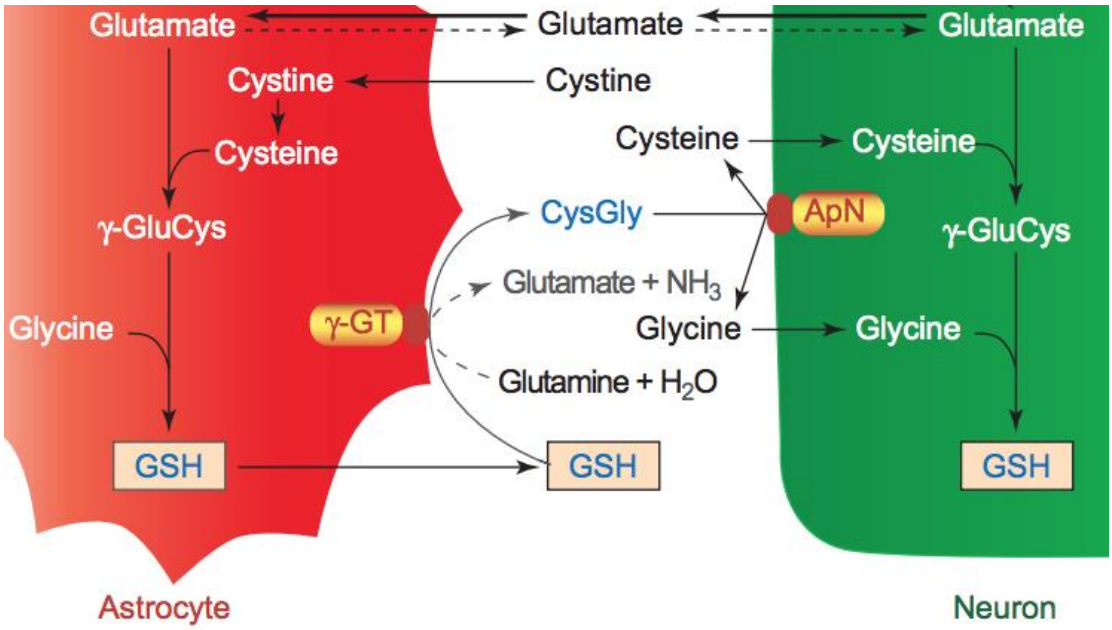


Figure 5. GSH synthesis in astrocytes and neurons. Extracellular cystine is converted to cysteine inside the astrocytic compartment. Together with glycine and glutamate, GSH is formed and released to the extracellular medium, where it is degraded to the three aminoacids again. Neurons take up cysteine and the other two aminoacids to generate GSH themselves. Adapted from (Hertz and Zielke, 2004).

A possible explanation for the differences in antioxidant capacities and responses between astrocytes and neurons is the preferential expression and activation of the Nrf2 factor in astrocytes, a transcription factor that plays a key role in the defence against

oxidative stress. Some studies suggest that Nrf2 is repressed in neurons and activated in neighbouring astrocytes in response to glutamate release (Jimenez-Blasco et al., 2015) (Bolanos, 2016).

Another representative example of astrocyte-neuron cooperation in antioxidant defences is ascorbic acid, another key antioxidant in the brain. Ascorbic acid is used by neurons to scavenge ROS during high activity periods, producing its oxidized form, dehydroascorbic acid. This oxidized form is released by neurons and taken up by astrocytes, where is recycled again to ascorbic acid and used by astrocytes themselves or exported to neurons where it can be used again (Covarrubias-Pinto et al., 2015).

1.3.4 Neurovascular coupling

The neurovascular coupling is defined as the relationship between neuronal activity and subsequent local changes in cerebral blood flow (CBF). This concept is a consequence of many years of study regarding how different stimuli lead to changes in volume, temperature and blood flow in certain cerebral regions (Iadecola, 2017). The neurovascular coupling is the basis of one of the most widely used techniques in neuroscience: functional magnetic resonance imaging (fMRI). In this technique, through the increase of blood flow in specific regions (as well as oxygen consumption in the Blood-Oxygen-Level Dependent –BOLD- studies), one can determine in a non-invasive way the neuronal activity of distinct brain regions (Raichle and Mintun, 2006).

It seems clear that blood flow regulation depends on neuronal activity. The key question here, however, is what is happening at the cellular level: which cells form the neurovascular unit (NVU) that respond to neuronal activities and regulate blood flow and through which signals are these changes produced.

One of the key cellular components of the NVU is the astrocyte. Astrocytes are intimately associated with both synapses and blood vessels, the perfect situation for forming part of this NVU. Moreover, astrocytes have a specialized structure named astrocyte endfeet, which ensheathe blood vessels in the brain, an optimal structure to control cerebral blood flow (Mishra, 2017).

The exact mechanisms by which astrocytes are able to control blood flow are still unknown and are an important field of debate. However, recent studies have shown that capillaries but not arterioles diameter is controlled by the astrocytic release of prostaglandin E_2 (PGE_2) presumably to pericytes, which in turn is produced by neuronal ATP release and calcium rises in astrocytes (Mishra et al., 2016). Changes in blood flow allow active neurons to receive oxygen and metabolites to maintain their activity.

Until here, we have explained some of the most studied astrocyte functions, contributing to the normal homeostasis of the brain. In this PhD, however, we have focused in two astrocyte functions, which, in turn, have been related to "higher"-brain functions in terms of controlling synaptic plasticity, neuromodulation or

synchronization of the entire brain (Masgrau et al., 2017). These functions are metabolism (specially lactate supply to neurons) and astrocyte communication with other cell types through calcium signals and gliotransmitter liberation.

2. Astrocytic metabolism

2.1. Introduction to brain metabolism

Brain energy consumption accounts for at least the 20% of the total body's oxygen consumption, receiving the 15% of total glucose and the 15% of the total cardiac output although this organ represents only the 2-3% of the body weight (Mink et al., 1981) (Magistretti and Allaman, 2015).

It is accepted that most of the ATP consumed in the brain corresponds to the neuronal compartment, basically to maintain neuronal excitability and synaptic processes both presynaptic (vesicle recycling) and postsynaptically (ionic concentration regulation after postsynaptic currents) (Harris et al., 2012) (Rangaraju et al., 2014). However, it is not clear yet which percentage of the total brain ATP is consumed by each cell type (astrocytes, neurons and other glial cells). First modelling studies established that astrocytic cells ATP consumption stands for 5 -15% of energy consumption (Attwell and Laughlin, 2001). Recent studies, however, have demonstrated that some neuronal energy requirement calculations were overestimated (Alle et al., 2009), and that astrocyte energy consumption may be higher than expected.

It has also been observed that increases in brain activity are linked to increases in blood flow and O_2 and metabolite availability. However, the increase in blood flow and glucose consumption associated with brain activation does not correlate with an increase in oxygen consumption (Fox et al., 1988; Lin et al., 2010). This increase in glucose but not in oxygen consumption in an oxygenated environment indicates the presence of aerobic glycolysis (AG), which is the production of lactate in the presence of oxygen. This accounts for 10-12% of glucose consumed in the brain, but is highly variable between regions and development stages (Goyal et al., 2014). Although aerobic glycolysis is also present in the "resting brain", especially in those regions of the default mode network (DMN) (Vaishnavi et al., 2010) (Raichle, 2015), its increase has been principally related to brain activation. Upon regional brain activation (response to imposed tasks), there is a decrease in extracellular glucose and an increase in extracellular lactate (Li and Freeman, 2015). This observation was unexpected, since aerobic glycolysis produces much less energy than the complete oxidation of glucose (2 ATP with AG versus 36 ATP maximum with complete oxidation). However, ATP production from AG is at least two times faster than ATP produced at OXPHOS system (McGilvery and Goldstein, 1983), which may an important characteristic for the fast excitatory synaptic transmissions.

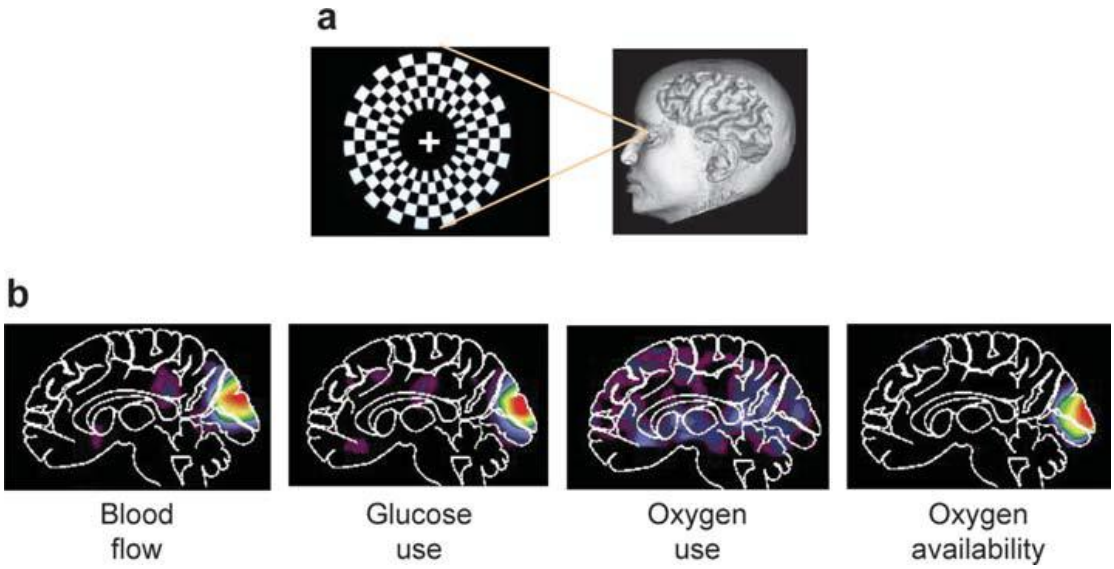


Figure 6. Aerobic glycolysis in brain activation. Stimulation of the human visual cortex produces increases in blood flow and oxygen availability in the area. The increase in blood flow correlates with an increase in glucose use, however, the local demand for oxygen is significantly lower than the availability. From (Belanger et al., 2011)

The existence of AG in the resting brain as well as the activity-induced AG are widely accepted. In fact, the discordance between oxygen consumption and availability is the basis of fMRI-BOLD signals (the ratio of oxygenated/deoxygenated haemoglobin), nowadays probably the most important brain imaging technique (Raichle, 2009). However, no such agreement is found regarding the cellular origin of aerobic glycolysis (Dienel and Cruz, 2016). Which cell type in the brain is consuming glucose and exporting the lactate? How is the glutamate used in neurotransmission recycled? Do astrocytes and neurons use different metabolic substrates? Which cell is consuming oxygen? And how are they integrated in the brain?

2.2 Metabolic compartmentalization between astrocytes and neurons

The first studies that tried to address the metabolic pathways in astrocytes and neurons used cultures of each separated cell type. The general conclusion of these studies is that cultured astrocytes present a highly glycolytic metabolism whereas neurons are mainly oxidative (reviewed in (Belanger et al., 2011), although both cells are capable to fully oxidize glucose and lactate when mixed (reviewed in (Zielke et al., 2009).

Even though some of these studies led to different results, most of them pointed to a "mainly-glycolytic" metabolism in astrocytes and a "mainly-oxidative" neuronal metabolism. These two different and complementary metabolic profiles suggested an important cooperation between both cell types, and established astrocytes as the main glucose consumers in the brain.

Differences in the metabolic preferences between both cell types can be explained by differences in the metabolic pathways. As an example, the entry of pyruvate into

mitochondria is regulated by the pyruvate dehydrogenase enzyme complex (PDH), which converts pyruvate to acetyl-coA. This enzyme has multiple phosphorylation sites that downregulate its activity, controlled by the pyruvate dehydrogenase kinases (PDKs). It has been confirmed that PDH is constitutively phosphorylated in astrocytes (Halim et al., 2010) probably through the pyruvate dehydrogenase kinase 4 (PDK4), which is highly expressed in *in vivo* astrocytes compared to neurons (Zhang et al., 2014).

Another example of differences in metabolism between astrocytes and neurons came from the study of the brain isoform of the enzyme 6-phosphofructo- 2-kinase/fructose-2,6-bisphosphatase 3 (Pfkfb3). This enzyme is responsible of regulating the levels of fructose 2,6-biphospahte, a positive regulator of glycolysis. This brain isoform 3, which has the highest kinase:phosphatase ratio and sustains high glycolytic activity (Shi et al., 2017) is constitutively undergoing proteasomal degradation in neurons, whereas in astrocytes it is fully active. In fact, it has been seen that glucose in neurons enters the pentose phosphate pathway (PPP) instead of glycolysis, a pathway that is crucial to maintain NADPH levels to defeat ROS (Herrero-Mendez et al., 2009).

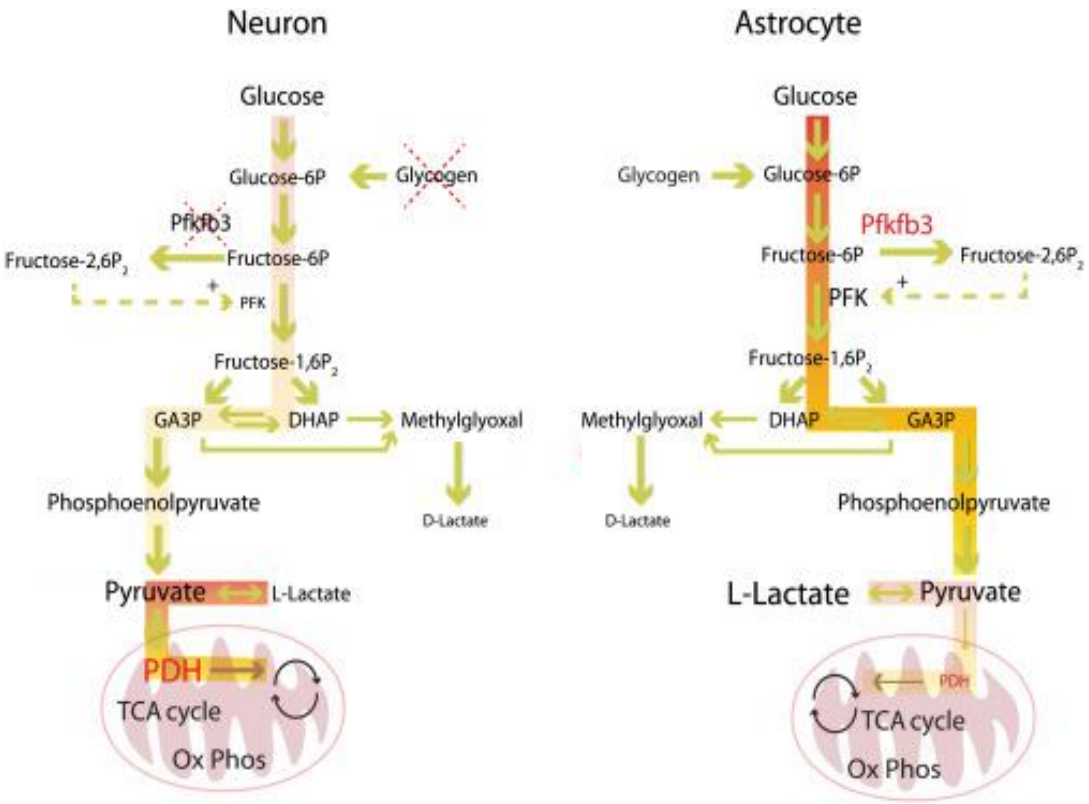


Figure 7. Metabolic differences between astrocytes and neurons. The increased expression and function of the positive regulator of glycolysis Pfkfb3 in astrocytes result in increased glycolytic capacity. Moreover, PDH inhibition by PDK4 in astrocytes reduces pyruvate processing promoting lactate production. Adapted from (Magistretti and Allaman, 2015).

These observations lead to the ANLS hypothesis, where astrocytes take up glucose to produce lactate, which is delivered to neurons for its complete oxidation (Pellerin

and Magistretti, 1994). This is one of the most accepted hypotheses regarding brain metabolic compartmentalization nowadays.

The other accepted brain metabolism hypothesis is the Glutamate/Glutamine cycle, where astrocytes take up neuronal released glutamate and converted it to glutamine. This glutamine, in turn, is used by neurons to produce glutamate again. Again, differences in gene expression can explain the hypothesis. Glutamine synthetase (GS), the enzyme that converts glutamate to glutamine, is found selectively in astrocytes (Norenberg and Martinez-Hernandez, 1979), while Glutaminase (GA), the enzyme that converts glutamine to glutamate is basically expressed in neurons (Akiyama et al., 1990). In this chapter we will review what is known about brain metabolism and which are the unresolved questions.

2.2.1 The astrocyte-neuron lactate shuttle

As stated before, astrocytes present an optimal structure to regulate brain metabolism. Astrocytic endfeet ensheathe blood vessels and are rich in the astrocytic glucose transporter GLUT1, which catalyses the uptake of glucose from the blood borne (Morgello et al., 1995).

Due to glycolytic reactions, glucose is transformed to pyruvate, which can enter the mitochondrial TCA cycle or be converted to lactate in the cytosol in the aerobic glycolysis process.

As explained before, much of this glucose undergoes aerobic glycolysis in astrocytes, although a part can undergo complete oxidation. This produces an accumulation of lactate molecules that need to be released to the extracellular medium. This astrocytic lactate release is mediated by MCT4, a monocarboxylate transporter (Magistretti and Allaman, 2015).

MCT4 (SLC16A3) is a lactate and other monocarboxylates transporter that belongs to the monocarboxylate transporter family (MCTs), a family of 14 transporters encoded by the SLC16A genes. Among them, MCT1-4 are responsible for the transport of different monocarboxylate molecules across the membranes, such as pyruvate, ketone bodies or lactate. This transport is linked to proton transport, so that the extrusion (or intrusion) of one monocarboxylate molecule has to be done together with one proton (Perez-Escuredo et al., 2016).

In the brain, MCT4 is found exclusively in astrocytes (Pellerin et al., 2005), while MCT2 is considered to be the main neuronal lactate transporter (Pierre et al., 2002). CNS expression of MCT1, considered to be almost ubiquitous in the organism, is found in astrocytes, endothelial cells, microglia, oligodendrocytes and some neuronal populations (Perez-Escuredo et al., 2016).

Interestingly, the Km for lactate varies among isoforms. MCT4 has the lowest affinity for lactate ($Km_{lactat\ e}$= 22-28 mM), MCT1 an intermediate ($Km_{lactate}$ = 3.5-10 mM) and MCT2 the highest affinity ($Km_{lactate}$ = 0.5-0.75 mM).

The preference of lactate over other monocarboxylates and the low affinity for pyruvate ($Km_{pyruvate}$ = 150 mM) makes MCT4 the ideal transporter to export lactate. In fact, MCT4 is highly expressed in eminently glycolytic tissues such as tumours, skeletal muscle fibers and white blood cells (Halestrap and Wilson, 2012). Thus, its presence in astrocytes is another proof of the efflux of lactate from astrocytes to neurons.

The mechanism of MCT4 transcription in astrocytes is still unknown. Hypoxia inducible factor 1α (HIF-1α), a transcription factor implicated in hypoxic responses, has been seen to enhance MCT4 transcription in pathological situations such as ischemia (Rosafio and Pellerin, 2014). However, the mechanism by which MCT4 expression is regulated in normoxic conditions is yet to be resolved. Part of my doctoral studies address this key question and found a novel mechanism by which MCT4 is upregulated in response to neurotransmitters, establishing a novel astrocytic pathway that may control neuronal function through metabolic support.

Once extruded from astrocytes, lactate is uptaken by MCT2 in neurons, where it can be enter the TCA cycle and the oxidative phosphorylation system (OXPHOS).

Interestingly, it has been demonstrated that disruption of the ANLS via inhibition of astrocytic export (siMCT4, siMCT1) or neuronal import (siMCT2) produces disturbances in long-term memory (Suzuki et al., 2011b). Moreover, this lactate flux through MCTs is able to maintain excitatory synaptic transmission, even in the presence of enough glucose (Nagase et al., 2014).

ANLS seems to be regulated by glutamate, linking neuronal activity with lactate release. Whenever a glutamate-mediated synaptic activity is produced, part of the neurotransmitter is taken up by the astrocytes, via the glutamate transporters explained before together with sodium ions. The increase in sodium inside the astrocyte activates the Na+/K+ ATPase, consuming ATP to equilibrate the Na^+ concentration inside the cell. This ATP consumption in turn increases glucose uptake and oxidation in astrocytes, producing more lactate that is released to the extracellular space via the monocarboxylate transporters (MCTs) and used by nearby neurons as an energy substrate (Figure). One in vivo proof of this hypothesis is that knocking out glial glutamate transporters (GLAST or GLT-1) reduces glucose utilization in activated areas (Voutsinos-Porche et al., 2003).

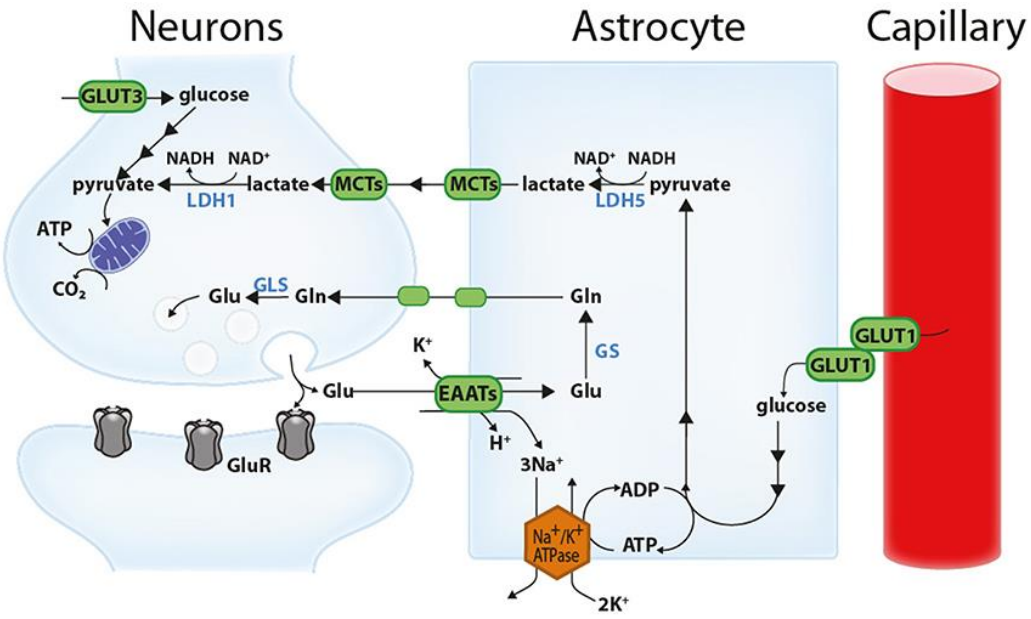


Figure 8. The astrocyte-neuron lactate shuttle. Released glutamate from glutamatergic synapses is taken up by astrocytes in cotransport with sodium. The extrusion of sodium by the Na+/K+ ATPase increases glucose consumption and lactate production in astrocytes, which is shuttled to neurons through monocarboxylate transporters (mainly MCT1 and MCT4 in astrocytes and MCT2 in neurons). This lactate is transformed to pyruvate in neurons and can enter the TCA cycle. From (Belanger et al., 2011).

Other stimuli have been found to increase aerobic glycolysis in astrocytes, such as potassium. As explained before, potassium is released to the extracellular medium after neuronal firing, and astrocytes have a huge permeability for potassium. Recent works suggest that physiological K^+ increases lead to rapid activation of glycolysis in astrocytes, a pathway that precedes glutamate glycolysis activation (Bittner et al., 2011). This effect may be mediated by the electrogenic Na^+/HCO_3^-cotransporter NBCe1 (Ruminot et al., 2011).

All these mentioned data supports the idea of an activity-regulated ANLS, with a net efflux of lactate from astrocytes to neurons. However, as in most science topics, some laboratories refuse the idea of an ANLS. Actually, some works demonstrated exactly the opposite: upon neuronal activation, neurons increase their glycolytic rate and export lactate to the extracellular medium, establishing the Neuronal Astrocyte Lactate Shuttle (NALS). First works were based on modelling studies, taking into account the kinetics of both glucose and monocarboxylate transporters of each cell type (Simpson et al., 2007) (DiNuzzo et al., 2010), but some were confirmed *in vivo* (Mangia, 2009) which generated a huge controversy (Jolivet et al., 2010) (Mangia et al., 2011).

2.2.2 The glutamate-glutamine cycle

As stated before, neuronal-released glutamate is efficiently taken up into astrocytes via glutamate transporters. Once in the astrocyte, this glutamate is transformed to glutamine via the glutamine synthetase enzyme, which is solely expressed in astrocytes, being actually a good marker for *in vivo* astrocytes.

Glutamine is then released to the extracellular medium through the system N aminoacid transporters SN1 and SN2, which are Na^+-dependent transporters expressed in the astrocytic plasma membrane. On the other site, glutamine is taken up by neurons through system A Na^+-dependent aminoacid transporters ATA1 and ATA2 (Rose et al., 2013). Interestingly, approximately 70% of glutamate released by living animals is generated through this cycle (Rothman et al., 2003) and glutamine supply to neurons is necessary to maintain synaptic activity (Tani et al., 2014).

Interestingly, other studies demonstrated that astrocytes also metabolize taken-up glutamate to generate alpha-ketoglutarate and entering TCA to produce other metabolic intermediates and ATP (Sonnewald and Schousboe, 2016). This capacity seems to be regulated by glutamate concentration, with increasing concentrations leading to increased oxidation (McKenna et al., 1996). These oxidative processes are regulated by glutamate transamination and deamination enzymes that are also expressed in astroglial cells. The contribution of this glutamate oxidation in the global astrocytic metabolism, as well as the percentage of oxidation *versus* conversion to glutamine is still unknown and an important matter of debate. In fact, some authors consider that glutamate oxidation is sufficient to maintain astrocytic metabolism during excitatory neurotransmission, instead of the aforementioned increase in glucose consumption (McKenna, 2013).

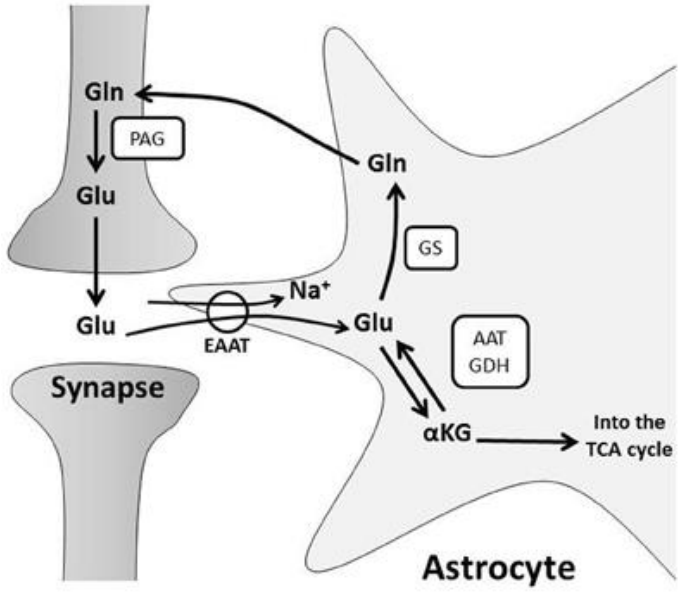


Figure 9. Glutamate/glutamine cycle. Released synaptic glutamate is taken up by astrocytes. This glutamate (Glu) is converted to (a) glutamine by glutamine synthetase (GS) or to (b) alpha-ketoglutarate by glutamate dehydrogenase (GDH) or aspartate aminotransferase (AAT) for subsequent oxidative metabolism in the TCA cycle. From (Stobart and Anderson, 2013).

2.2.3 Glycogen metabolism

Glycogen is a branched polysaccharide of glucose that represents the main mechanism of glucose storage in the body. In humans, glycogen is specially found in the liver and in muscle cells, where it maintains blood glucose levels and meets increased energy demands respectively (Brown and Ransom, 2007).

In the brain, glycogen is almost exclusively stored in astrocytes, establishing these cells as glucose stores for brain metabolism (Cataldo and Broadwell, 1986). Moreover, glycogen degradation is controlled by the enzyme glycogen phosphorylase, which is only expressed in astrocytes. The enzyme catalysing glycogen synthesis, glycogen synthetase, is also found in neurons, where it is supposed to have another function (Pellegri et al., 1996).

Glycogenolysis, the production of glucose from glycogen, has been considered an emergency reaction to provide glucose for the brain during starvation, acting as the liver of the brain. However, the amount of glycogen in the brain is much lower than that of the liver so that, while liver glycogen is able to maintain glucose blood levels for 24h, brain glycogen will be degraded within minutes (Brown and Ransom, 2007).

Taking that into account, recent studies argue in favour of a specific role of glycogen in the brain, especially in the control of learning and memory processes. First observations revealed that glycogen content decreases during early-memory consolidation tests in chicks, together with an increase in glutamate content (Hertz et al., 2003). These observations lead to the study of the inhibition of glycogenolysis during learning processes, where it seems to be crucial for short and long term memory in chicks and rodents (Suzuki et al., 2011b) (Duran et al., 2013) (Newman et al., 2011).

These works establish astrocytic glycogen as an optimal energy buffer, providing neurons with lactate (after its conversion to glucose) to maintain excitatory transmission rapidly.

2.2.4 Mitochondrial metabolism

The ANLS together with the glutamate/glutamine cycle and the glycogen metabolism led to the idea that all astrocytic metabolical functions are cytoplasmic, mitochondrial functions being only marginal. Moreover, some works suggested that *in vitro* astrocytes are able to survive as glycolytic cells without a functional mitochondrial respiration (Almeida et al., 2001). Thus, the general understanding is that metabolic mitochondrial functions in astrocytes may not be as important as in other cell types.

However, it has been seen that astrocytic processes contain many small mitochondria (Derouiche et al., 2015) (Lovatt et al., 2007), and that the expression of mitochondrial genes is comparable between astrocytes and neurons (Lovatt et al., 2007). In addition, it has been seen that astrocytic mitochondria are dynamic organelles that

move to sites of calcium and glutamate uptake, contributing to the ATP production of the cell (reviewed in (Jackson and Robinson, 2017).

Finally, and in addition to glucose oxidative metabolism, some works suggested the use of different substrates in astrocytes as carbon sources for mitochondrial metabolism. As explained before, the most accepted hypothesis is the entry of glutamate to the TCA (Schousboe et al., 2014), however, other carbon sources such as fatty acids (Panov et al., 2014) (Ebert et al., 2003), acetate (Hertz et al., 2007) or lactate (Hertz and Dienel, 2005) have been seen to enter astrocytic TCA.

As a summary, the dogma of brain metabolism that astrocytes are glycolytic cells that transfer lactate to the "oxidative" neurons is now being highly discussed. Different metabolic pathways and possible carbon sources for both astrocytes and neurons have been proposed, emphasizing the complexity of brain metabolism. However, there is a need to understand brain metabolism, not only for the implication that it has in pathological situations, but in normal physiology. Many behavioural experiments are based in the oxygen and glucose consumption of different brain areas, while the truth is that we still do not know which cell is consuming the glucose and/or the oxygen.

Part of my doctoral studies consisted in try to answer these questions regarding brain metabolism. To do so, we generated "mitochondriomes" of astrocytes and neurons using mRNA expression data of nuclear genes encoding for mitochondrial proteins to classify mitochondrial functions in both cells. These studies may shed a light on the metabolic oxidative pathways of astrocytes and neurons and contribute to the knowledge of neurometabolism.

3. Astrocyte calcium signalling

Astrocytes were supposed to be non-excitable cells that serve as the homeostatic regulators of the CNS. However, more than 25 years ago, the discovery that astrocytes were able to respond to neuronal activity via increases in intracellular calcium signals both *in vitro*, *in situ* and *in vivo*, and even produce calcium "waves" among astrocytes, started one of the most controversial topics in neuroscience (Cornell-Bell et al., 1990) (Dani et al., 1992) (Porter and McCarthy, 1996) (Newman and Zahs, 1997) (Wang et al., 2006). This discovery raised the possibility that astrocytic calcium waves could represent a novel signalling pathway in the CNS and many laboratories have focused their studies on the role and the mechanisms of astrocytic calcium signals. Astrocytic calcium is considered today as the basis of astrocyte excitation and hence the communication among astrocytes and with other cells types.

3.1. A brief historical introduction

Calcium is a ubiquitous second messenger in mammalian organisms, controlling the most important activities of all eukaryotic cells (Berridge, 2005) (Clapham, 2007). In the CNS and in neurons in particular, its role is critical as it participates in the spreading of the depolarization signal and the synaptic activity. During the last

phases of neuronal depolarization, cytoplasmic calcium increases induce the liberation of neurotransmitter vesicles, which would act on the postsynaptic neuron. Taking this into account, it is not surprising that the discovery of calcium signals in astrocytes completely changed the perspective of astrocytes as mere homeostasis controllers.

Bazargani and Attwell (Bazargani and Attwell, 2016) described the evolution of the study of astrocyte calcium roles in three waves: the **first wave** includes all the studies arising from the first observations of astrocyte calcium responses to neuronal activity. These observations, however, were linked to the discovery that these increases in intracellular calcium induce calcium rises in neighbouring neurons via the release of neurotransmitters.

These astrocyte-released neurotransmitters were named "gliotransmitters", and their discovery lead to the spreading of the idea of the "tripartite synapse", where not only the two neurons were communicating each other, but the astrocytic processes were also releasing neurotransmitters and modulating the synapses (Araque et al., 1999).

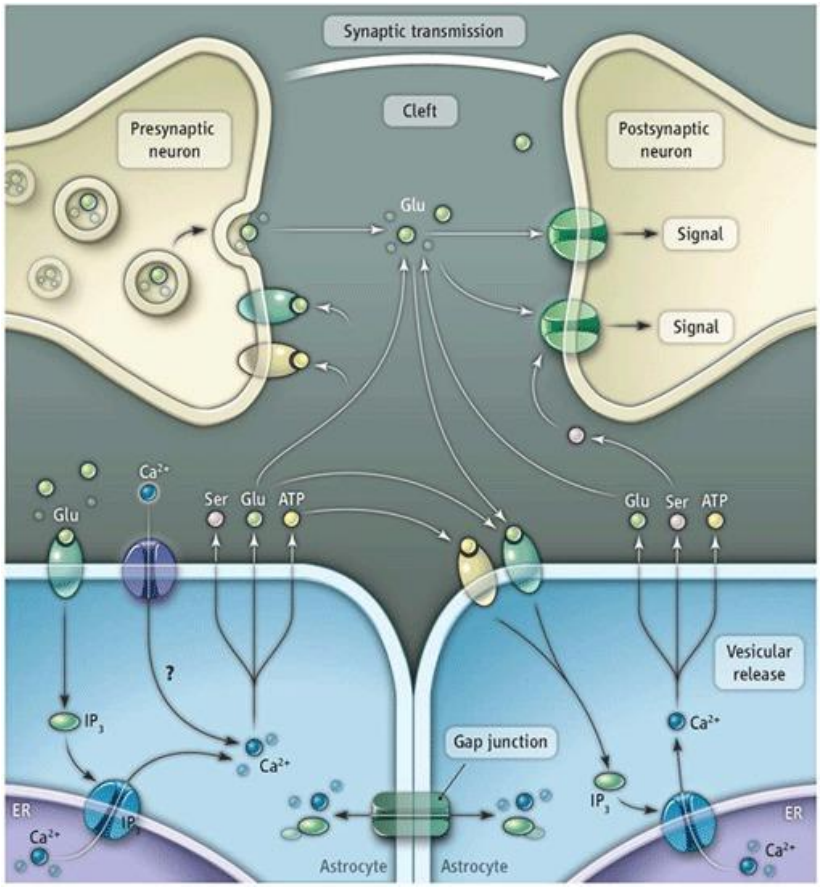


Figure 10. The tripartite synapse. Synaptic glutamate binds to glutamate receptors in the neighbouring astrocytes, generating IP_3, which releases calcium from the endoplasmic reticulum. This calcium, once in the cytosol, induces the liberation of "gliotransmitters" (Glutamate, ATP or D-serine) that in turn modifies the neuronal communication. From (Kirchhoff, 2010).

Different "gliotransmitters" were identified during this first wave: Glutamate (Parpura et al., 1994) (Fellin et al., 2004), ATP (Pryazhnikov and Khiroug, 2008) (Newman, 2001), D-serine (Yang et al., 2003) (Mothet et al., 2005) and GABA (Lee et al., 2010). At the same time the released gliotransmitters produced effects on neighbouring neurons, such as an NMDA-mediated membrane inward current in the case of glutamate (Parri et al., 2001) inducing neuronal synchronization (Fellin et al., 2004), as well as effects on the presynaptic receptors and regulation of neurotransmitters release probability (Jourdain et al., 2007) (Perea and Araque, 2007). Moreover, D-serine release from astrocytes was seen to control NMDA-induced currents (Yang et al., 2003) (Henneberger et al., 2010). Finally, one of the major discoveries during that period was the modulation of sleep homeostasis by accumulation of adenosine, which derives from ATP release during the day (Halassa et al., 2009).

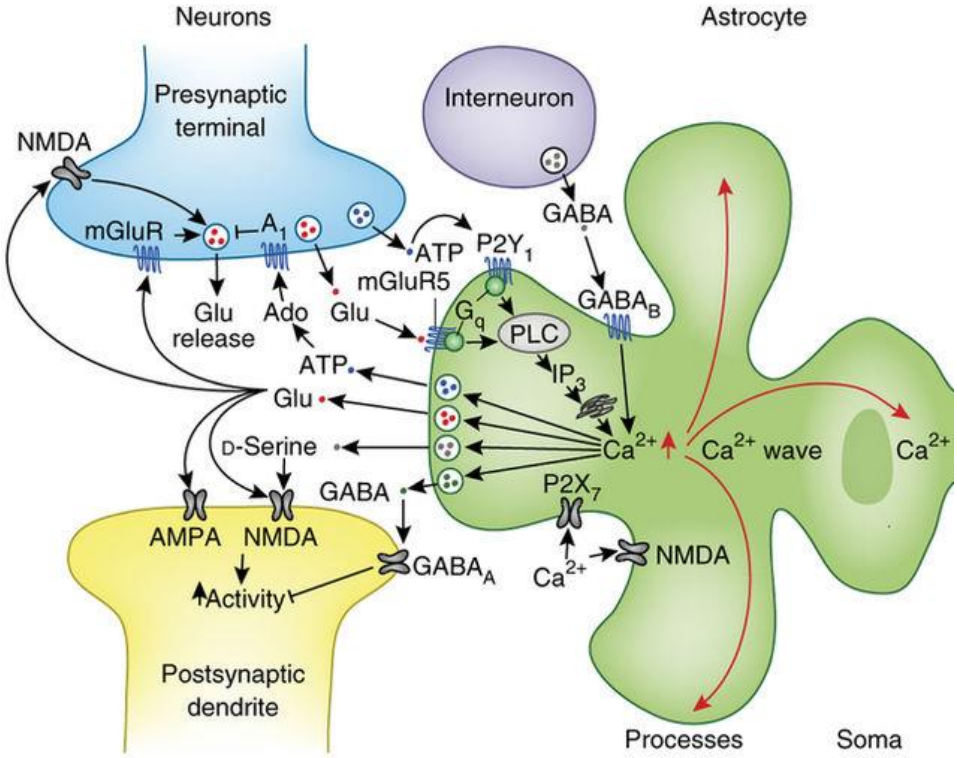


Figure 11. The first wave of astrocytic calcium. Neurotransmitters such as ATP, GABA, and Glutamate were seen to induce calcium responses in astrocytes, leading to a release of gliotransmitters. These gliotransmitters (ATP, Glu, D-Serine, GABA) induced changes in neuronal connectivity. Adapted from (Bazargani and Attwell, 2016).

However, all these exciting discoveries were questioned during the **second wave**. At that time, many laboratories tried to replicate all these results using other techniques with very different results (resumed in Table 1). Moreover, analyses of some widely used models of inhibited gliotransmission, such as the dnSNARE mice demonstrated some neuronal affectation, casting doubts on the effects of gliotransmission seen in these models (Fujita et al., 2014).

Of special importance during this period was the study of IP_3R_2 knockout (KO) mice of McCarthy lab. Although we will discuss in detail calcium signalling pathways in astrocytes in further sections (see mechanisms of astrocyte signals), it is interesting to

introduce the concept to understand the mismatches produced during this wave. One key question regarding calcium signals in astrocytes is which is the main source of calcium rises. In vitro studies demonstrate that calcium signals are generated through activation of phospholipase C beta (PLCβ), which can produce inositol 1,4,5 triphosphate (IP_3). This IP_3, in turn, binds to IP_3 receptors in the ER, promoting a massive calcium release from the store. Among all IP_3 receptors, the subtype 2 seemed to be the one orchestrating the response in astrocytes (Holtzclaw et al., 2002). In fact, IP_3R_2 KO presents a clear reduction of cytosolic calcium rises (Fiacco et al., 2007) (Petravicz et al., 2008).

Topic	First wave discovery	References	Second wave discovery	References
Glutamate stimulates astrocytic calcium	mGluR1 and 5 mediate *in vivo* calcium increases	(Wang et al., 2006) (D'Ascenzo et al., 2007)	mGluR5 is not expressed in adult mice	(Sun et al., 2013)
Astrocytic calcium release from intracellular stores	Calcium events *in situ* depends on ER-release	(Parri et al., 2001)	Astrocyte processes lack intracellular calcium stores	(Patrushev et al., 2013)
Astrocytic calcium modifies synaptic functions	Hippocampal LTP depends on Calcium-dependent astrocytic release of D-serine	(Henneberger et al., 2010)	Mice lacking ER-dependent calcium events showed no effect on neuronal currents, synaptic plasticity or behaviour	(Agulhon et al., 2010) (Petravicz et al., 2014) (Petravicz et al., 2008)
	Astrocytes release glutamate and GABA to modulate neuronal activity in the olfactory bulb	(Kozlov et al., 2006)	Raising astrocytic calcium with a DREADD mice had no effect on neuronal physiology	(Fiacco et al., 2007)
SNARE mediates astrocytic gliotransmission	dnSNARE mice showed altered gliotransmitter release, synaptic transmission and synaptic plasticity	(Pascual et al., 2005)	dnSNARE mice showed expression in neurons	(Fujita et al., 2014)

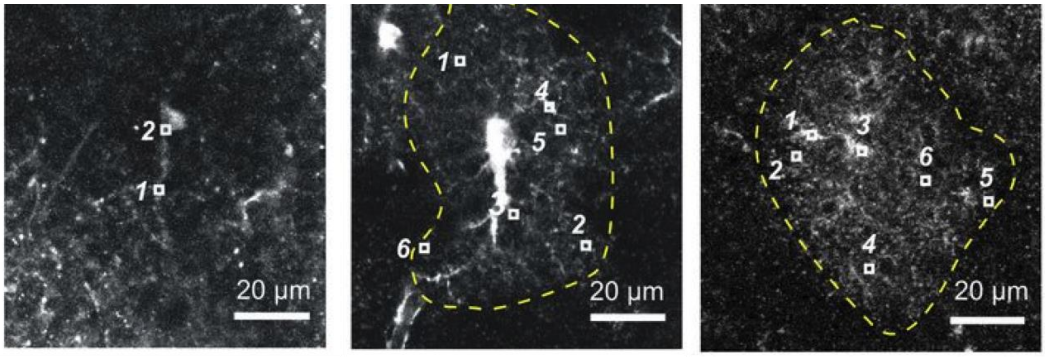
Table 1. Summary of the controversies generated during the second wave of astrocytic calcium.

Unexpectedly, even with a clear reduction on astrocytic cytosolic calcium events, IP_3R_2 KO mice did not present deficits at neuronal excitability (Fiacco et al., 2007), synaptic currents (Petravicz et al., 2008), synaptic plasticity (Agulhon et al., 2010), neurovascular coupling (Bonder and McCarthy, 2014) nor in behavioural responses (Petravicz et al., 2014). The only effect seen in this mouse model was an abolished Acetylcholine (Ach)-evoked Long-term potentiation (LTP) (Takata et al., 2011) (Navarrete et al., 2012). Although one can't discard the hypothesis of a compensatory mechanism on the mouse model, it was clear that most of the astrocytic calcium currents were abolished and no consequences (or low consequences) were found. Moreover, the same group developed

other models to mimic in vivo calcium increases in astrocytes, without a clear effect in neuronal activity nor neurovascular coupling (Fiacco et al., 2007) (Bonder and McCarthy, 2014).

These studies, together with contradictions found in different models (Table 1), lead to the rethinking of the hypothesis of calcium in astrocytes as a mechanism of gliotransmission (Sloan and Barres, 2014).

Finally, some of these controversies were resolved, mainly because of technical advances in the field. Among them, the use of bulk loading membrane-permeable organic calcium dyes (Fura-2, Fluo-4) was substituted by genetically encoded calcium indicators (GECIs), which basically consist in the cellular expression of a calcium fluorescent protein that binds to calcium (Shigetomi et al., 2010). Interestingly, while bulk loading dyes were useful to detect somatic and thick branches events, GECIs expression was able to detect also events from the very thinner branches of the astrocyte. The better signal-to-noise ratio and probably a better diffusion may explain these differences (Tong et al., 2013).



Fluo-4AM **Cyto-GCamp3** **Lck-GCamp3**

Figure 12. Calcium signals with different calcium indicators. (Left) Image of an astrocyte filled with the organic calcium dye fluo-4AM. (Medium) Image of an astrocyte expressing the GECI Cyto-GCamp3. (Right) Image of an astrocyte that expresses the GECI Lck-GCamp3, which is directed to membranes. Adapted from (Shigetomi et al., 2013a).

Thanks to the development of these novel calcium imaging techniques, the field started the **third wave**, in which many of the controversies generated were (partially) resolved. The use of GECIs and other new indicators allowed to distinguish different calcium signals than the ones that were previously described. For the first time, it was seen that inside the complex morphology of an astrocyte there can be different types of calcium signals: somatic events (the ones that were measured before), proximal processes events and distal processes or territories events (Volterra et al., 2014).

The discovery of a spectrum of calcium signals in different compartments of the astrocyte was a real revolution in the field. While it was thought that only neuronal intense firing patterns were able to induce a calcium rise in astrocytes, using these

new techniques small calcium currents in distal processes were seen in response to low levels of synaptic activity (Di Castro et al., 2011) (Panatier et al., 2011).

However, the biggest breakthrough came from a work from Khakh lab. In this work, IP_3R_2 KO mice was analysed using these new GECIs. Interestingly, It was found that only somatic events were disrupted (those that are ER-dependent), but signals in distal processes were not affected, even increased (Srinivasan et al., 2015). This work was a step forward in the field, questioning the findings of McCarthy's lab and at the same time opening a new horizon about the importance of calcium signals in distal processes.

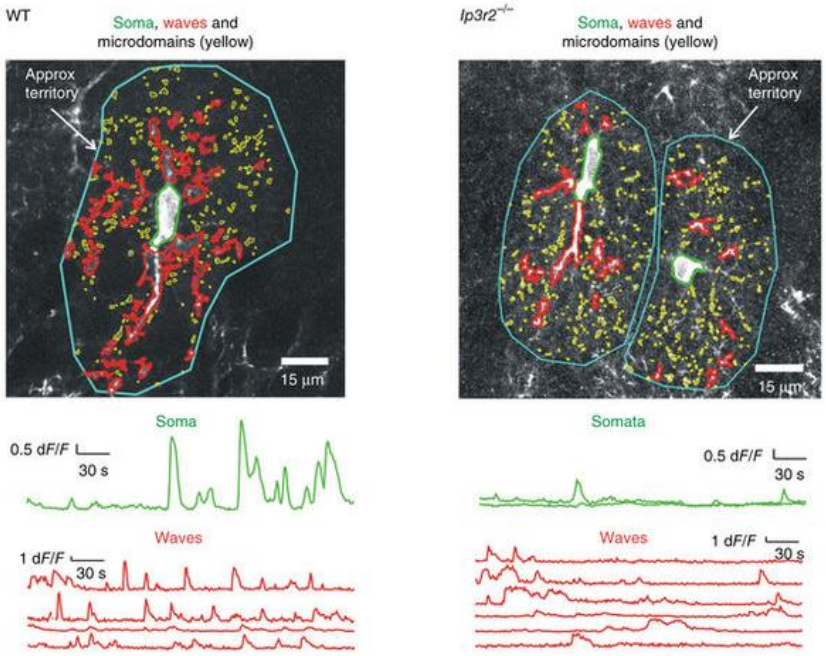


Figure 13. Calcium events in IP_3R_2 KO mouse. Representative images and traces for Ca^{2+} fluctuations of astrocytes from wild type and transgenic mouse expressing the calcium indicator GCamp3. Note that in the IP_3R_2 KO, although somatic calcium events are almost abolished, branches and territories (waves in the image) still present calcium fluctuations. Adapted from (Srinivasan et al., 2015).

3.2. Mechanisms of calcium signals

The main signalling pathway regarding calcium increases is intracellular calcium mobilization through neurotransmitter-induced G protein-coupled receptors (GPCR) activation. These GPCRs activate the Phospholipase C β (PLCβ), which catalyses the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2) into diacyl glycerol (DAG) and inositol 1,4,5 triphosphate (IP_3). This IP_3, in turn, binds to the IP_3 receptors (IP_3R) located in the ER, which, upon activation, releases the calcium content of the ER to the astrocytic cytosol (Cornell-Bell et al., 1990).

Cytosolic calcium increases can induce the liberation of gliotransmitters among other physiological processes (Scemes and Giaume, 2006). Calcium responses are controlled in time, space and magnitude, and differences in any of these characteristics can lead to

different cellular responses. Thus, cytosolic calcium is controlled by many different calcium buffers, channels and pumps that shape the extension and localization of the signal. These are called the "calcium toolkit" of the cell (Berridge, 2005) and can be located in different compartments of the astrocyte.

3.2.1 Endoplasmic reticulum

As stated before, the ER is the main source of calcium rises in cultured astrocytes. This calcium release is mainly through IP_3R, specifically through IP_3R_2, which is predominantly expressed in astrocytes (Sheppard et al., 1997). The other known family of receptors controlling ER calcium release are the ryanodine receptors family (RyR). This family of receptors control calcium release from the ER in response to cyclic ADP ribose (cADPR) or calcium itself (calcium induced calcium release) in many cell types like neurons or muscle cells. In astrocytes, these receptors seem to have a minor role, although their expression at mRNA and protein level is detectable (Matyash et al., 2002).

ER depletion leads to calcium influx to replenish the stores via plasmalemmal opening. This mechanism is known as the store-operated calcium entry (SOCE) or capacitative entrance (Putney, 1986) and is present in the majority of non-excitable cells. At the molecular level, SOCE is mediated by Orai, an ER-adaptor protein, which senses calcium content in the organelle and is in contact with membrane calcium channels. After ER depletion, Orai is able to homodimerize to interact with STIM, a calcium channel in the plasma membrane, which will fulfil the ER with calcium. In astrocytes, the molecular identity of SOCE remains to be identified. Recent works establish Orai1/Orai3 and Stim1 as the molecular players in astrocytic SOCE (Kwon et al., 2017). Previous studies, however, suggested TRPC1 as a key element of SOCE in astrocytes (Malarkey et al., 2008).

The ER also controls cytosolic calcium through the SERCA pump, which buffers calcium into the cytosol in an ATP-dependent manner (Berridge, 2002). Astrocytes seem to express the subtype SERCA2b, the most common subtype in nonexcitable cells (Morita and Kudo, 2010).

3.2.2 Mitochondria

Mitochondria are an important calcium buffer-organelle. Calcium increases in the mitochondrial matrix potentiates mitochondrial metabolism (Griffiths and Rutter, 2009), since they can activate some enzymes from the OXPHOS pathway such as isocitrate dehydrogenase (ICDH) and pyruvate dehydrogenase (PDH). At the same time, however, higher mitochondrial calcium concentrations can lead to apoptosis and cell death (Marchi et al., 2017). Thus, mitochondrial calcium is an important signalling pathway and its regulation is crucial in physiological and pathological processes.

Calcium buffering by mitochondria is mediated by two calcium channels: the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane and the mitochondrial calcium uniporter (MCU) in the inner mitochondrial membrane. The molecular identity of MCU, one of the most controversial topics in the calcium field, has

been recently resolved by two different groups (De Stefani et al., 2011) (Baughman et al., 2011). Thereafter, different regulators of the MCU channel have been found, such as mitochondrial calcium uptake protein (MICU) 1 and 2, MCUb and the essential MCU regulator (EMRE), conforming the mitochondrial calcium uniporter complex (Mammucari et al., 2016).

As with the ER, mitochondria need a way to get rid of calcium, since high and sustained calcium concentrations can lead to cell death. Different mitochondrial calcium efflux pathways have been described in isolated mitochondria, specifically a sodium dependent and a sodium-independent efflux mechanisms (Bernardi, 1999). However, only recently the molecular identity of the mitochondrial sodium/calcium/lithium exchanger NCLX has been found (Palty et al., 2010), whereas the sodium independent mechanism remains elusive. Finally, another mitochondrial calcium release mechanism may be the opening of the mitochondrial permeability transition pore (mPTP). The sustained activation of this mPTP produces swelling, collapse of the mitochondrial membrane potential, ATP depletion and necrotic cell death (Halestrap, 2010), however, transient activation of mPTP may be a signalling pathway implicated in different diseases (Hurst et al., 2017).

In astrocytes, mitochondrial buffering of calcium has been related to changes in cytosolic calcium and in glutamate release as a consequence (Reyes and Parpura, 2008). It has been recently demonstrated that astrocytic mitochondrial dynamics are controlled by calcium in the distal processes, linking these events with mitochondrial metabolism (Jackson and Robinson, 2015) (Jackson et al., 2014).

3.2.3 Mitochondria-associated membranes

An important signalling pathway involving mitochondria and ER has gained interest in the last years; this is the calcium transfer of the mitochondria associated membranes (MAMs). These regions are defined by a close contact between mitochondria and ER (<200nm), and have been implicated in multiple housekeeping functions, such as cholesterol and ceramide synthesis, as well as transport of proteins and ions (Area-Gomez, 2014).

The ER presents IP_3R calcium release channels. At the same time, mitochondria contain calcium buffering mechanisms: VDAC and the MCU complex. Interestingly, once ER calcium is released, cytosolic calcium induces a regulatory feedback to the same calcium releasing channels. This feedback can be inhibitory, activatory or biphasic, depending on the concentration of the liberated calcium (Bezprozvanny et al., 1991). The biophysical characteristics of the ER calcium releasing channels give mitochondrial buffering a key role in the control of their activation. For instance, if calcium from the ER is buffered by nearby mitochondria, IP_3R will not be inhibited and a continuous transfer of calcium through both organelles can be produced. Thus, the proximity of both organelles defined by MAMs is crucial for calcium signalling between both organelles and, as a consequence, for metabolism and cell death. In addition, since this calcium is buffered directly by mitochondria, those functions relying on the cytosolic calcium concentrations can be affected by MAMs.

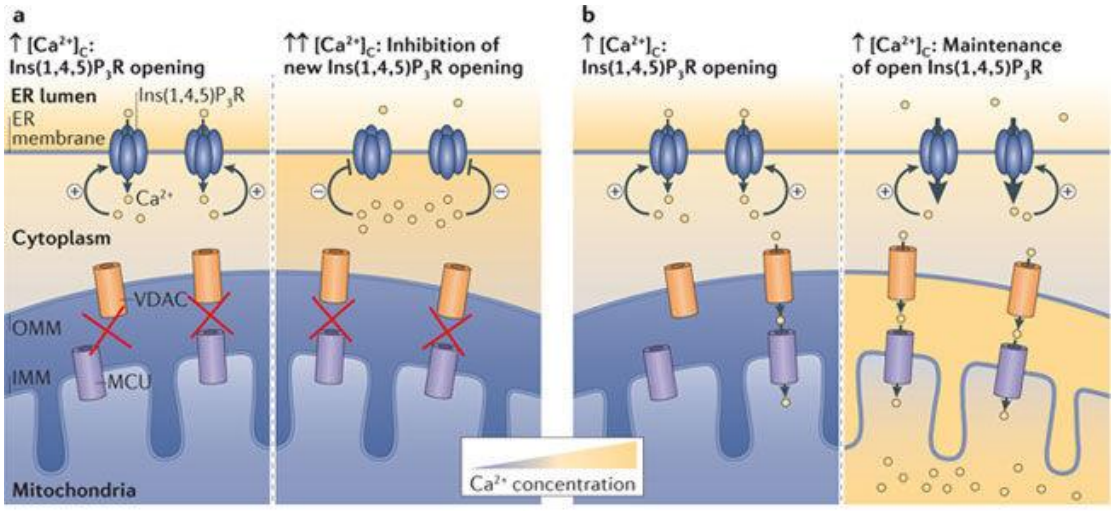


Figure 14. MAMs regulation of calcium fluxes. Calciun release from the IP_3 receptors of the ER could produce an inhibition of the same receptors if mitochondria is not buffering the calcium (a) or produce a potentiation of the release if there is mitochondrial buffering (b). From Rizzuto (Rizzuto et al., 2012)

This pivotal role of MAMs in controlling cellular functions generated special interest in their molecular identity. Biochemical isolation revealed that MAMs are formed by different proteins undergoing distinct functions. On the one hand, MAMs are formed by structural proteins that conform the junction itself such as mitofusin (Mfn) 1 and 2, whose deletion produces an alteration in calcium signals as well as an increase in the ER-mitochondria distance (de Brito and Scorrano, 2008). One the other hand, MAMs contain different proteins involved in calcium transfer, considered the main signalling mechanism between both organelles. Many of these proteins are chaperones that control the stability of calcium channels as well as the junction itself. Among them, Sigma non-opioid intracellular receptor (SigmaR1) is the chaperone that prevents degradation of IP_3R, maintaining mitochondrial calcium buffering (Hayashi and Su, 2007). In fact, SigmaR1 deletion has been related to decreases in mitochondrial calcium entry and impaired responses to stress (Hayashi and Su, 2007).
Other chaperones are enriched in MAMs, such as 75KDa glucose-regulated protein (GRP75), which mediates the direct functional interaction between IP_3R in the ER and VDAC in mitochondria (Szabadkai et al., 2006) or the ER chaperones calnexin and calreticulin, which act as calcium buffers and regulate SERCA activity (Camacho and Lechleiter, 1995) (Roderick et al., 2000).

The role of MAMs in astrocytes has not been investigated in physiological conditions. Only one study addresses the work of MAMs in human astrocytomas, revealing that stem-cell like glioma cells were "MAM-deficient" while well-differentiated glioma cells had a "MAM-rich" phenotype (Arismendi-Morillo et al., 2017). Since MAMs control calcium signalling between the main astrocytic calcium store (the ER) and a crucial organelle for astrocytic metabolism as the mitochondria, regulation of their constituents and physiology can be of special interest to modify and adapt astrocytic functions. Moreover, alterations in astrocytic MAMs can lead to changes in cytosolic calcium, the main communication system of these cells. All in all, the study of these MAMs can be

really important for the understanding of astrocytic physiology and a possible target for pathological processes. Our results revealed a new mechanism by which one character of MAMs (the chaperone SigmaR1) is regulated in astrocytes and which are the consequences of the effect in astrocytic calcium.

3.2.4 Calcium mobilization from acidic stores

One of the last demonstrated signalling pathways involving calcium was the signalling within acidic stores such as lysosomes via nicotinic acid adenine dinucleotide phosphate (NAADP) (Lee, 2005). Although it is not clear which is the pathway that synthetizes intracellular NAADP, different physiological stimuli have been found to increase these second messenger through activation of GPCRs and tyrosine kinase-linked receptors (Galione, 2015).

Once NAADP is generated, probably through CD38 and ADP-ribosyl ciclases, it can bind to and open lysosomal calcium release channels. The molecular identity of these calcium release channels is still under debate, the two-pore channels (TPCs) and the mucolipine family of transient receptor potential channels (TRPML) being the main candidates (Patel et al., 2011).

On the other side, lysosomal calcium entry is mediated by the combined action of the vacuolar (V)-type H^+-ATPase and the Na^+/Ca^{2+} exchanger, the first increasing lysosomal proton content and the former exchanging these protons for calcium ions (Raffaello et al., 2016). (Figure)

In astrocytes, NAADP-dependent calcium signals have been demonstrated in cultures by our research group (Barcelo-Torns et al., 2011). In this work, NAADP is generated in astrocytes in response to ATP, provoking lysosome calcium release.

3.2.5 Extracellular calcium entry

Astrocytes express ionotropic Ca^{2+} channels such as α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) glutamate receptors as well as P2X purinoreceptors (Lalo et al., 2011). The activation of these channels produce calcium influx in astrocytes, which can modify the calcium current. Interestingly, the opening of ionotropic channels produces also extracellular sodium entry, which may also induce calcium increases indirectly via the sodium/calcium exchanger (NCX). In addition, astrocytes express different members of the TRP family channels, and some of them may have a role in extracellular calcium entry. For instance, TRPA1 has been related to the spontaneous local events in astrocytes (Shigetomi et al., 2011), TRPC receptor family has been related to SOCE in astrocytes, and osmotic stimulation of TRPV4 seem to have an important role in ischaemic-induced calcium entry (Butenko et al., 2012) (Verkhratsky et al., 2014).

NCX proteins are expressed in astrocytes, specifically located in perisynaptic processes (Minelli et al., 2007). This exchanger can act both extruding and intruding calcium, although in astrocytes seems to act mainly as a sodium extruder (Lim et al., 2016). Its

action as a calcium entering system has been related to the exocytosis of glutamate from cultured astrocytes (Paluzzi et al., 2007) (Reyes et al., 2012).

Finally, calcium from cytosolic transients can be also extruded to the extracellular space via plasmalemmal Ca^{2+} pumps/ATPases (PMCA), which uses ATP energy (Verkhratsky et al., 2012).

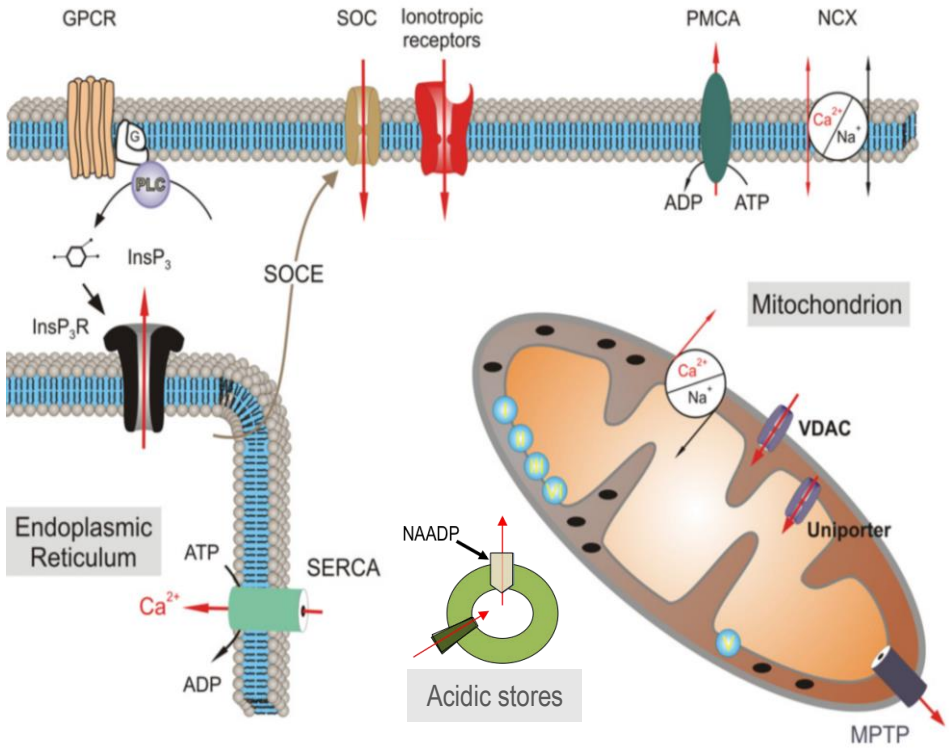


Figure 15. Astrocytic calcium toolkit. Adapted from (Verkhratsky et al., 2012)

3.3. Calcium signals in vivo

Novel molecular tools such as the genetic-encoded calcium indicators have revealed that calcium signals in *in vivo* and *ex vivo* astrocytes are more complex than in cultured astrocytes. Since the *in vivo* studies have just started, it's difficult to classify them correctly, although some reviews have attempted a classification (Volterra et al., 2014). Anyway, in this thesis I will differentiate three types of calcium signals depending on the cell compartment where they take place: focal events in the territories, "extended" events in the processes and somatic events.

Focal events are the most common and the fastest calcium increases in astrocytes, and are produced in the thin processes of astrocytes (Shigetomi et al., 2010). Later studies in *ex vivo* astrocytes demonstrated that some of these "spotted-calcium" signals in the hippocampal CA1 could be explained by extracellular calcium influx through TRPA1 channels (Shigetomi et al., 2011). These signals are linked to the maintenance of basal calcium levels, the constitutive release of D-serine to the extracellular space (Shigetomi et al., 2013b) as well as the expression of GABA transporters in the plasma membrane (Shigetomi et al., 2011).

Similar signals in processes have been discovered in other areas of the brain as well as in *in vivo* experiments. The origin of these focal events, however, seems to be region-dependent, since astrocytes in the CA3 are not affected by a TRPA1 selective antagonist (Haustein et al., 2014) and only partially in the striatum radiatum (Shigetomi et al., 2013b).

The initiating factor of focal events in the processes is still an unresolved question. Some studies reported that calcium signals in processes could be generated either spontaneously (Nett et al., 2002) (Shigetomi et al., 2011) or via the release of different neurotransmitters from neurons such as ATP or glutamate (Di Castro et al., 2011) (Panatier et al., 2011). These differences in the origin of the initiating factor may be explained by different functions in astrocytes: their homeostatic function in the "restless" brain or their functions in response to neural activity (Masgrau et al., 2017). The decoding of different patterns of activity, however, requires much more study to analyse which signals controls which actions.

Extended events in processes are larger than the previous ones, both in time, space and amplitude and are sensible to Tetrodotoxin (TTX), this is, to neuronal firing. These signals are initiated in different regions of the primary and secondary processes and are IP_3-dependent. These signals were found to increase neurotransmitter release probability at local synapses in the hippocampus (Di Castro et al., 2011) (Panatier et al., 2011).

Finally, **somatic** calcium signals are the less common, and are defined by calcium rises in the soma of the astrocyte. They are supposed to happen whenever there is a robust stimulation, such as a global noradrenaline release after locus coeruleus stimulation (Ding et al., 2013)

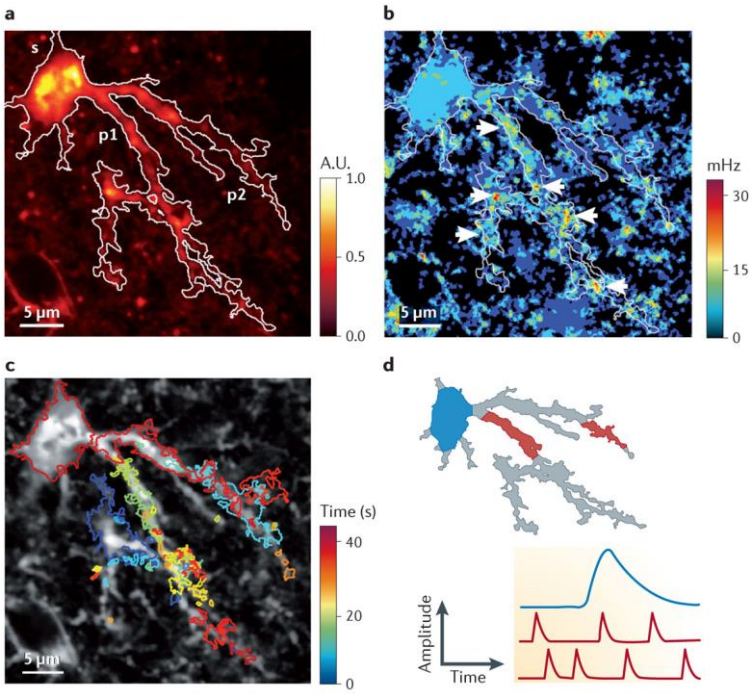


Figure 16. Calcium signals in vivo. Different calcium events in the astrocyte territories in intensity (a), frequency (b) o duration (c). Somatic (blue) and extended (red) signals representations. From (Volterra et al., 2014).

The three calcium signals might represent at the same time a mechanism of integration of different neuronal stimuli. Thus, many focal events in different areas of a process can lead to an extended event. At the same time, different extended events could produce a general somatic calcium event. This could clearly represent a mechanism of integration of the different inputs coming from different synapses at different times in one main "computer".

In summary, the unexpected complexity of astrocyte calcium signals in vivo is probably related to the multiple known (and upcoming) astrocytic functions. From local synapse neurotransmitter liberation to global brain cortical switching and behavioural responses, calcium signals in astrocytes may control more functions than previously expected. Unfortunately, the astrocyte field is one-step back neuronal studies, where calcium signal mechanisms and functions are better understood. My doctoral studies reveal previously unknown mechanisms of astrocyte calcium signalling, and how neurotransmitters and neuromodulators can modulate these pathways. The findings are especially relevant in the field, considering the crucial role of astrocytic calcium in brain physiology.

4. Astrocyte plasticity via CREB

4.1 Astrocyte plasticity

Plasticity (especially neural plasticity, the most studied form of plasticity) is the ability to the cells and networks to modify themselves functionally and structurally, in response to experience (von Bernhardi et al., 2017). The term "plasticity" describing the ability of the brain to modify and adapt itself has been used for more than a century (Berlucchi and Buchtel, 2009); however, the term has been rarely related to astrocytes, with the exception of one review in 2013 (Pirttimaki and Parri, 2013).

In the last years, probably due to the new discovered astrocyte functions, the concept of astrocyte plasticity gained interest. The idea is simple: if the brain is highly adaptable to experience and astrocytes perform key functions in the brain physiology, one could speculate that astrocytes may be able to adapt themselves to experience and modify their functions accordingly.

Since astrocytic functions such as metabolic support (for instance lactate supply) or gliotransmission are able to modify neuronal and brain function, it follows that experience-based modifications of these functions will lead to changes in brain physiology and constitute "astrocyte plasticity".

In neurons, long-term synaptic plasticity and long-term memory requires de novo gene expression and protein synthesis (Kandel, 2001). The mechanism involves the activation of a transcription factor and the consequent increase in expression of target genes. Among other known transcription factors that may develop this function, the best characterized is the cyclic AMP-responsive element-binding protein (CREB)-induced plasticity.

4.2 CREB

CREB is a 43KDa nuclear transcription factor that upregulates the expression of target genes containing cAMP-responsive elements (CRE) sites. It belongs to the basic leucine zipper domain (bZIP) superfamily of transcription factors, comprising the CREB subfamily with their closely related factors activating transcription factor 1 (ATF-1) and cAMP response element modulator (CREM).

All the members of the CREB-family show high similarities in structure, with a basic leucine zipper domain that allows DNA-binding and a dimerization domain, which allows CREB to homo or heterodimerize (Schumacher et al., 2000). In addition, members of the CREB family present two glutamine rich domains (Q1 and Q2) as well as a transactivation kinase-inducible domain (KID) located between them. These domains interact with the components of the basal transcription machinery, as well as with different CREB cofactors (Johannessen et al., 2004).

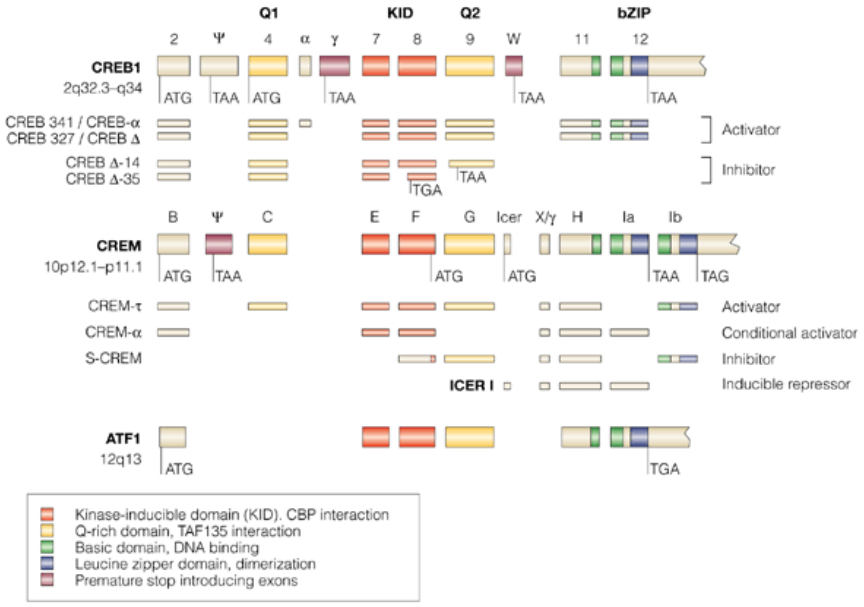


Figure 17. Structure of CREB-family members CREB1, CREM and ATF1. From (Alberini, 2009).

The KID domain is the regulatory region that allows CREB to respond to intracellular signalling pathways. CREB is activated mainly by phosphorylation of serine 133, which can be targeted by different kinases. Once phosphorylated, CREB becomes a target of the kinase-inducible interacting (KIX) domains of transcription co-activators such as CREB-binding protein (CBP) and p300 (Kwok et al., 1994) (Sakamoto et al., 2011).

CBP and p300 are histone acetyltransferases (HAT), thus, their recruitment to the CREB binding site produces a relaxation of the chromatin through acetylation of the histones. In addition, and thanks to their big size, they can act as a link between the transcription factor and the components of the basal transcriptional complex, such as

the RNApol II complex (Valor et al., 2013). Once all the transcriptional machinery is recruited to the promoter, the transcription of the specific gene is produced.

Although serine 133 is the main activator mechanism, other activating or regulatory serines have been found within the KID domain (for review see (Sakamoto et al., 2011). Similarly, other pathways have been found to activate CREB, such as binding of the CRTCs family of coactivators to the bZIP domain, allowing CREB activation independently of their phosphorylation status (Saura and Cardinaux, 2017).

All the CREB family members bind to the same CRE elements, defined by the palindromic consensus sequence (TGACGTCA) or by a half palindromic site (TGACG or CGTCA).

About 750,000 CRE-consensus sequences (both complete and half site) have been found in the human genome, although most of them present a methylation of the cytosine within the site, inhibiting the transcription factor binding (Iguchi-Ariga and Schaffner, 1989). Functional unmethylated CRE-sites are found in the promoter proximal regions of certain genes, within 250 base pairs of the transcription start site. This occurs in about 4,000 genes (Zhang et al., 2005). As it happens with other promoter proximal elements, the presence of a TATA box near the consensus site increases gene transcription, being weaker the further the TATA box from the consensus site (Mayr and Montminy, 2001). However, only one third of the CRE-containing promoters express a TATA box nearby (Zhang et al., 2005).

4.2.1 CREB-activation pathways

CREB activation preferentially relies on Ser 133 phosphorylation. This serine is targeted by multiple kinases that respond to a variety of stimuli, including cAMP and Ca^{2+} increases. As an example, protein kinase A (PKA) mediates Ser 133 phosphorylation upon cAMP increases (Gonzalez and Montminy, 1989), while Ca2+/Calmodulin-dependent kinases II and IV (CaMKII/IV) respond to calcium increases (Sheng et al., 1991). However, other stimuli have been described to activate CREB through other pathways and kinases and this activation pathway seems to change depending on the cell type (Lonze and Ginty, 2002) (Carriba et al., 2012).

CREB activation by cAMP induces binding to the majority of CRE-containing promoters; however, only about 100 genes are upregulated by treatment with cAMP and the signature presents significant differences among cell types (Zhang et al., 2005). The absence of a TATA box in two thirds of the genes that contain CRE sequences, the methylation state of DNA as well as the presence or absence of coactivators may be the explanation of this "low level" and cell-type dependent effect (Altarejos and Montminy, 2011).

In summary, CREB transcriptional activation integrates multiple stimuli and a variety of activation mechanisms that lead to different responses in a cell-type dependent manner, allowing a specific response depending on the activation pathway and on the cellular context.

4.2.2 CREB-dependent functions in the CNS

Probably the best-known CREB function is its role in the CNS, concretely in neurons, where it has been seen to modulate learning and memory. The relationship between CREB and learning-dependent processes was first seen in the mollusc *Aplysia Californica* by Kandel lab, where blocking CREB-dependent transmission blocked long-term facilitation (Dash et al., 1990).

Since then, neuronal CREB has been implicated in a wide range of memory processes in different animal models. In mice, for example, different models addressed the role of CREB through loss or gain-of-function strategies, demonstrating the role of CREB in the CNS. In these models, the impairment or enhancement of long-term memories usually correlates with changes in long-term potentiation processes, where CREB seems to develop its function (Table 2).

Mutant mice	**Genetic system**	**CREB function**	**Physiology**		**Behaviour**			**Reference**
			Basal synaptic transmission	**LTP**	**Memory type**	**Short-term memory**	**Long-term memory**	
CREB α/δ deficient mice	Partial gene deletion	Loss-of-function	Normal	Impaired	Contextual and cued fear memories	Normal (30 min) Impairment (1h)	Impaired	(Bourtchuladze et al., 1994)
					Spatial memory		Impaired	
CREB IR mice	LBD system, forebrain-specific expression	Loss-of-function	ND	ND	Contextual and cued fear memories	Normal (2h)	Impaired	(Kida et al., 2002)
KCREB mice	tTA system, forebrain-specific	Loss-of-function	Normal	Impaired	Object recognition memory	Normal (1h)	Impaired	(Pittenger et al., 2002)
					Spatial memory		Impaired	
VP16-CREB mice	tTA system, forebrain-specific	Gain-of-function	Normal	Enhanced	Spatial memory		Enhanced	(Barco et al., 2002) (Viosca et al., 2009)
Y134F mice	Forebrain-specific expression	Gain-of-function	Normal	Enhanced	Social recognition, contextual fear memories	Normal (5-15 min) Normal (30 min) Enhancement (2h)	Enhanced	(Suzuki et al., 2011a)
					Spatial memory		Enhanced	
CREB DIEDML mice	Forebrain-specific expression	Gain-of-function	Normal	Enhanced	Social recognition, contextual fear memories	Normal (5-15 min) Enhancement (30 min) Enhancement (2h)	Enhanced	(Suzuki et al., 2011a)
					Spatial memory		Enhanced	

ND, not determined

Table 2. Summary of phenotypes of CREB mutant mice. Adapted from (Kida and Serita, 2014)

First studies regarding CREB function discovered its crucial role in **synaptic plasticity**, referred as the ability of synapses to strengthen or weaken in response to changes in their activity (Takeuchi et al., 2014). In this model, CREB is activated in the postsynaptic neuron in response to presynaptic stimuli and induces transcription of genes involved in strengthen of the synapses between both cells.

Probably the most well known gene activated by CREB that contributes to synaptic plasticity is the brain derived neutrotrophic factor (BDNF). CREB activates BDNF transcription in response to neural activity through the CRE element present in its promoter region (Tao et al., 1998).

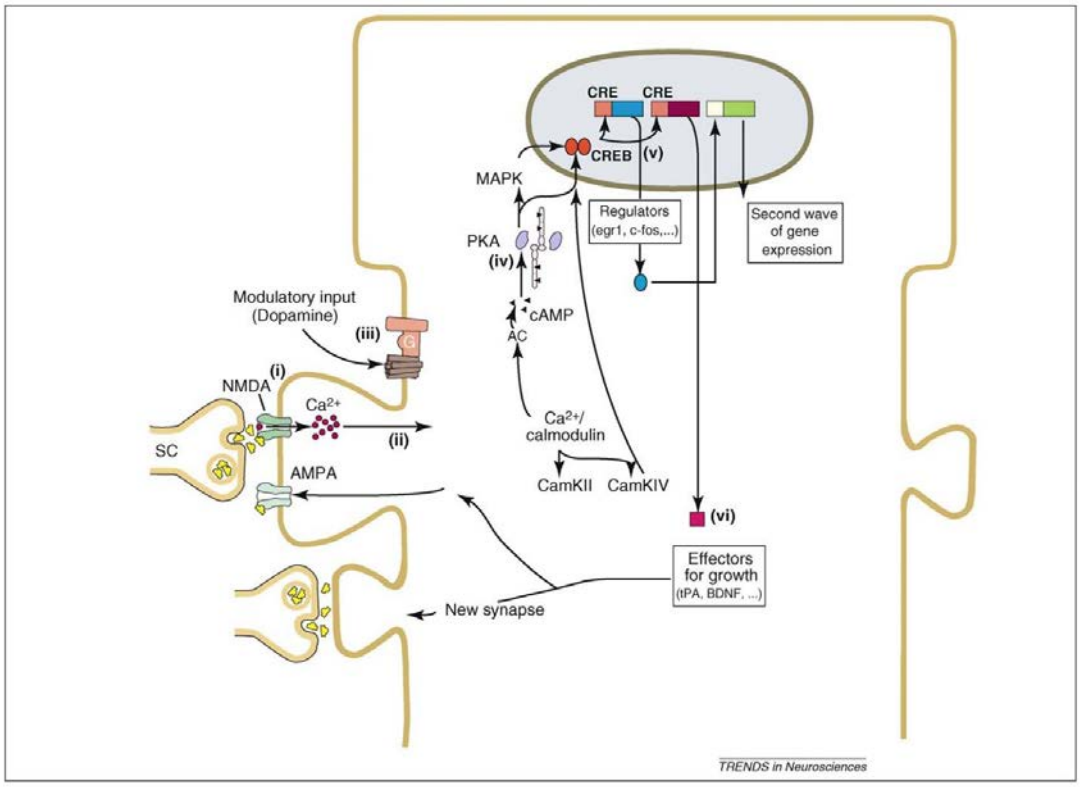


Figure 18. CREB modulation of synaptic plasticity in neurons. Membrane depolarization induces extracellular calcium entry through NMDA receptors. Calcium and cAMP activate CREB kinases, which phosphorylate CREB. Once activated, CREB initiates a transcriptional program which provide the strengthening of synaptic connexions. From Benito (Benito and Barco, 2010).

BDNF, in turn, modulates synaptic plasticity in different neurons and brain regions by functional and structural effects (Lu et al., 2014). In addition to BDNF, many other genes have been involved in CREB-mediated synaptic plasticity such as tissue plasminogen activator (tTPA), synaptotagmin IV or dynorphin, among many others (Benito and Barco, 2010).

More recent studies demonstrated a role of CREB in the regulation of neuronal **intrinsic plasticity**, which are the changes in the neuronal intrinsic excitability. This excitability depends on the electrical properties of the neuron and is defined as the

propensity of the neuron to fire action potentials in response to a specific stimulus (Benito and Barco, 2010).

Different works have detected changes in cellular excitability upon CREB-dependent transcription activation in different neuronal populations: locus coeruleus (Han et al., 2006), nucleus accumbens (Dong et al., 2006), hippocampal CA1 (Lopez de Armentia et al., 2007) (Jancic et al., 2009) and amygdala (Viosca et al., 2009) (Zhou et al., 2009). The mechanisms of CREB-mediated intrinsic plasticity seem to be dependent of the brain region, but all of them imply an increase in the firing rate of neurons (Benito and Barco, 2010).

This gain in intrinsic excitability can be obtained by an increase in sodium-dependent currents, boosting the ability of the neuron to depolarize or a decrease in potassium-dependent currents, diminishing the hyperpolarization produced after a spike. CREB has been proposed orchestrate the transcription of these membrane ion channels and/or modulate their activity through specific kinases and phosphatases that are known to modify the opening and conductance properties of existing channels via posttranslational modifications (Benito and Barco, 2010).

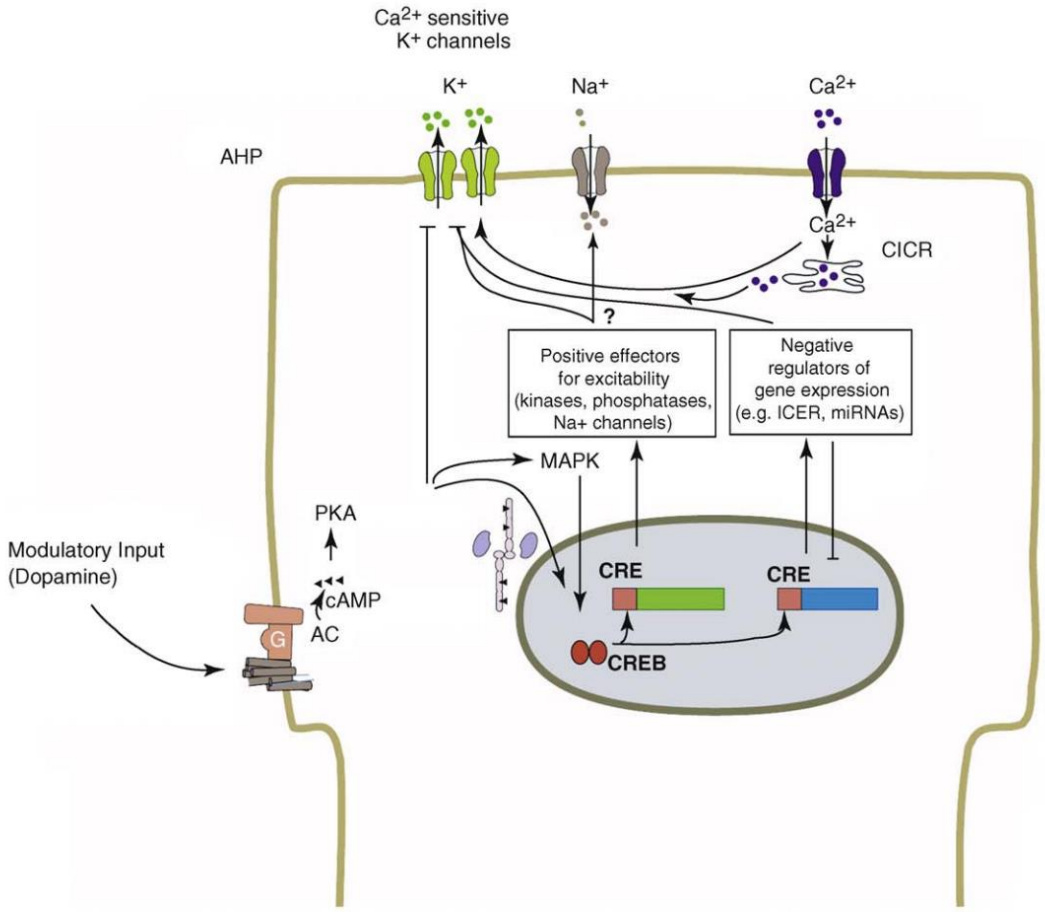


Figure 19. CREB modulation of intrinsic plasticity in neurons. CREB activation in neurons leads to the expression of certain excitability modulators, such as Na^{2+} or K^{+} channels. From (Benito and Barco, 2010).

4.2.3 CREB in astrocytes

Neuronal CREB has gained much interest in neuroscience due to its effects in learning and memory process, being one of the main hubs of activity-driven changes in the brain (Benito et al., 2011). However, CREB functions in astrocytes are just starting to emerge.

The few studies that address the role of CREB in astrocytes have shown that it is activated in response to excitotoxic damage (Ferrer et al., 1996), and in response to neurotransmitters such as ATP (Takasaki et al., 2008) and forskolin, which increases cAMP levels (Murray et al., 2009). Moreover, different pharmacological molecules are able to induce astrocytic CREB activation: aspirin (Modi et al., 2013), sodium benzoate (Jana et al., 2013), sodium phenylbutyrate (Corbett et al., 2013), tamoxifen (Karki et al., 2013) and raloxifen (Karki et al., 2014).

About its functions, most of the studies established CREB as a neurotrophin-inducer, increasing the expression of BDNF (Takasaki et al., 2008) (Corbett et al., 2013), Neurotrophin-3 (NT-3) (Jana et al., 2013) (Corbett et al., 2013) and ciliary neurotrophic factor (CNTF) (Modi et al., 2013). Interestingly, two studies suggest CREB as a regulator of the astrocytic glutamate transporters expression (Karki et al., 2013) (Karki et al., 2014).

Our group has been interested in CREB functions in astrocytes for many years, and have characterized a new pathway of CREB activation (Carriba et al., 2012). In this study, CREB-dependent transcription was activated in primary astrocytes by ATP and Noradrenaline, but not other transmitters, via an atypical protein kinase C (aPKC).

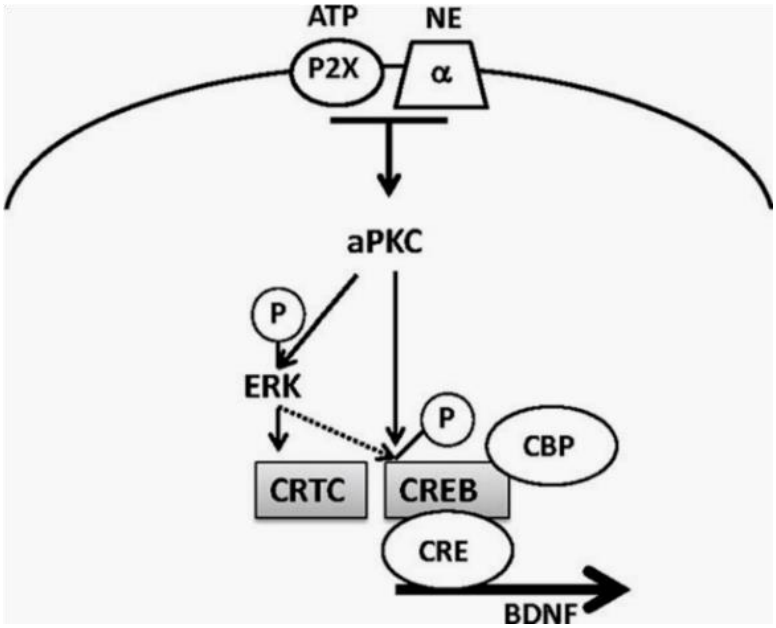


Figure 20. CREB activation in astrocytes. ATP and NE activate an atypical PKC, which in turn phosphorylates CREB in astrocytes. From (Carriba et al., 2012).

This may be probably the best description of the CREB activating pathway in astrocytes, and establishes the possibility of neural-activity dependent changes in the astrocyte transcriptome. Based on these results, one could speculate that neurotransmitters such as ATP or neuromodulators like NA can activate CREB in neighbouring astrocytes, modifying its physiological functions. Thus, it may be possible that CREB activation in astrocytes promotes changes similar to what happens in neurons, facilitating intrinsic astrocytic plasticity or even neural synaptic plasticity.

Moreover, our group has developed the first transgenic mice expressing a constitutively active form of CREB (VP16-CREB) specifically in astrocytes. The model allowed us to discover the potential protective role of astrocytic CREB in traumatic brain injury (TBI), as well as to establish a first transcriptomic analysis of astrocytic CREB-dependent genes (Pardo et al., 2016).

Finally, we have designed an array in cultured astrocytes comparing different pathways to activate CREB (FSK, NA and VP16-CREB) and analysed their own transcriptomic signature, as well as the difference of CREB activation in astrocytes and neurons (Pardo et al., 2017). These transcriptomic arrays are a key element in the development of these doctoral studies, since we can have an idea of which are the CREB-dependent pathways as well as the main characters involved. Interestingly, both transcriptomic analyses pointed to CREB-regulated astrocyte metabolism and lactate release, one of the previously explained functions that regulate brain plasticity (Steinman et al., 2016).

Finally, due to the mentioned importance of calcium signals in astrocytes, one could speculate that changes in calcium excitability in astrocytes may be a target for astrocyte plasticity processes (Pirttimaki and Parri, 2013). Since CREB is able to modify intrinsic and synaptic plasticity of neurons by regulating ionic channels and synapses, we posit that CREB could be an ideal target for astrocytes to adapt to the changing environment via modifications in its "calcium toolkit".

As a conclusion, our hypothesis is that the transcription factor CREB, known to promote plasticity-related changes in neurons, is implicated in astrocyte plasticity in response to neuronal activity.

My doctoral studies aim to explore this hypothesis and discover new CREB-target genes in astrocytes and functional consequences of its action.

References

Agulhon, C., Fiacco, T.A., and McCarthy, K.D. (2010). Hippocampal short- and long-term plasticity are not modulated by astrocyte Ca2+ signaling. Science *327*, 1250-1254.

Akiyama, H., Kaneko, T., Mizuno, N., and McGeer, P.L. (1990). Distribution of phosphate-activated glutaminase in the human cerebral cortex. The Journal of comparative neurology *297*, 239-252.

Alberini, C.M. (2009). Transcription factors in long-term memory and synaptic plasticity. Physiol Rev *89*, 121-145.

Alle, H., Roth, A., and Geiger, J.R. (2009). Energy-efficient action potentials in hippocampal mossy fibers. Science *325*, 1405-1408.

Almeida, A., Almeida, J., Bolanos, J.P., and Moncada, S. (2001). Different responses of astrocytes and neurons to nitric oxide: the role of glycolytically generated ATP in astrocyte protection. Proc Natl Acad Sci U S A *98*, 15294-15299.

Altarejos, J.Y., and Montminy, M. (2011). CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. Nat Rev Mol Cell Biol *12*, 141-151.

Araque, A., Parpura, V., Sanzgiri, R.P., and Haydon, P.G. (1999). Tripartite synapses: glia, the unacknowledged partner. Trends Neurosci *22*, 208-215.

Area-Gomez, E. (2014). Assessing the function of mitochondria-associated ER membranes. Methods Enzymol *547*, 181-197.

Arismendi-Morillo, G., Castellano-Ramirez, A., and Seyfried, T.N. (2017). Ultrastructural characterization of the Mitochondria-associated membranes abnormalities in human astrocytomas: Functional and therapeutics implications. Ultrastruct Pathol *41*, 234-244.

Attwell, D., and Laughlin, S.B. (2001). An energy budget for signaling in the grey matter of the brain. J Cereb Blood Flow Metab *21*, 1133-1145.

Azevedo, F.A., Carvalho, L.R., Grinberg, L.T., Farfel, J.M., Ferretti, R.E., Leite, R.E., Jacob Filho, W., Lent, R., and Herculano-Houzel, S. (2009). Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain. The Journal of comparative neurology *513*, 532-541.

Bak, L.K., Schousboe, A., and Waagepetersen, H.S. (2006). The glutamate/GABA-glutamine cycle: aspects of transport, neurotransmitter homeostasis and ammonia transfer. J Neurochem *98*, 641-653.

Barcelo-Torns, M., Lewis, A.M., Gubern, A., Barneda, D., Bloor-Young, D., Picatoste, F., Churchill, G.C., Claro, E., and Masgrau, R. (2011). NAADP mediates ATP-induced Ca2+ signals in astrocytes. FEBS Lett *585*, 2300-2306.

Barco, A., Alarcon, J.M., and Kandel, E.R. (2002). Expression of constitutively active CREB protein facilitates the late phase of long-term potentiation by enhancing synaptic capture. Cell *108*, 689-703.

Baughman, J.M., Perocchi, F., Girgis, H.S., Plovanich, M., Belcher-Timme, C.A., Sancak, Y., Bao, X.R., Strittmatter, L., Goldberger, O., Bogorad, R.L., *et al.* (2011). Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. Nature *476*, 341-345.

Bayraktar, O.A., Fuentealba, L.C., Alvarez-Buylla, A., and Rowitch, D.H. (2014). Astrocyte development and heterogeneity. Cold Spring Harb Perspect Biol *7*, a020362.

Bazargani, N., and Attwell, D. (2016). Astrocyte calcium signaling: the third wave. Nature neuroscience *19*, 182-189.

Belanger, M., Allaman, I., and Magistretti, P.J. (2011). Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation. Cell Metab *14*, 724-738.
Belanger, M., and Magistretti, P.J. (2009). The role of astroglia in neuroprotection. Dialogues Clin Neurosci *11*, 281-295.
Bellot-Saez, A., Kekesi, O., Morley, J.W., and Buskila, Y. (2017). Astrocytic modulation of neuronal excitability through K+ spatial buffering. Neurosci Biobehav Rev *77*, 87-97.
Benito, E., and Barco, A. (2010). CREB's control of intrinsic and synaptic plasticity: implications for CREB-dependent memory models. Trends Neurosci *33*, 230-240.
Benito, E., Valor, L.M., Jimenez-Minchan, M., Huber, W., and Barco, A. (2011). cAMP response element-binding protein is a primary hub of activity-driven neuronal gene expression. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 18237-18250.
Benner, E.J., Luciano, D., Jo, R., Abdi, K., Paez-Gonzalez, P., Sheng, H., Warner, D.S., Liu, C., Eroglu, C., and Kuo, C.T. (2013). Protective astrogenesis from the SVZ niche after injury is controlled by Notch modulator Thbs4. Nature *497*, 369-373.
Berlucchi, G., and Buchtel, H.A. (2009). Neuronal plasticity: historical roots and evolution of meaning. Exp Brain Res *192*, 307-319.
Bernardi, P. (1999). Mitochondrial transport of cations: channels, exchangers, and permeability transition. Physiol Rev *79*, 1127-1155.
Berridge, M.J. (2002). The endoplasmic reticulum: a multifunctional signaling organelle. Cell Calcium *32*, 235-249.
Berridge, M.J. (2005). Unlocking the secrets of cell signaling. Annual review of physiology *67*, 1-21.
Bezprozvanny, I., Watras, J., and Ehrlich, B.E. (1991). Bell-shaped calcium-response curves of Ins(1,4,5)P3- and calcium-gated channels from endoplasmic reticulum of cerebellum. Nature *351*, 751-754.
Bialas, A.R., and Stevens, B. (2013). TGF-beta signaling regulates neuronal C1q expression and developmental synaptic refinement. Nature neuroscience *16*, 1773-1782.
Bittner, C.X., Valdebenito, R., Ruminot, I., Loaiza, A., Larenas, V., Sotelo-Hitschfeld, T., Moldenhauer, H., San Martin, A., Gutierrez, R., Zambrano, M.*, et al.* (2011). Fast and reversible stimulation of astrocytic glycolysis by K+ and a delayed and persistent effect of glutamate. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 4709-4713.
Boddum, K., Jensen, T.P., Magloire, V., Kristiansen, U., Rusakov, D.A., Pavlov, I., and Walker, M.C. (2016). Astrocytic GABA transporter activity modulates excitatory neurotransmission. Nature communications *7*, 13572.
Bolanos, J.P. (2016). Bioenergetics and redox adaptations of astrocytes to neuronal activity. J Neurochem *139 Suppl 2*, 115-125.
Bonder, D.E., and McCarthy, K.D. (2014). Astrocytic Gq-GPCR-linked IP3R-dependent Ca2+ signaling does not mediate neurovascular coupling in mouse visual cortex in vivo. The Journal of neuroscience : the official journal of the Society for Neuroscience *34*, 13139-13150.
Bourtchuladze, R., Frenguelli, B., Blendy, J., Cioffi, D., Schutz, G., and Silva, A.J. (1994). Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. Cell *79*, 59-68.

Brown, A.M., and Ransom, B.R. (2007). Astrocyte glycogen and brain energy metabolism. Glia *55*, 1263-1271.
Butenko, O., Dzamba, D., Benesova, J., Honsa, P., Benfenati, V., Rusnakova, V., Ferroni, S., and Anderova, M. (2012). The increased activity of TRPV4 channel in the astrocytes of the adult rat hippocampus after cerebral hypoxia/ischemia. PLoS One *7*, e39959.
Butt, A.M., and Kalsi, A. (2006). Inwardly rectifying potassium channels (Kir) in central nervous system glia: a special role for Kir4.1 in glial functions. Journal of cellular and molecular medicine *10*, 33-44.
Camacho, P., and Lechleiter, J.D. (1995). Calreticulin inhibits repetitive intracellular Ca2+ waves. Cell *82*, 765-771.
Carriba, P., Pardo, L., Parra-Damas, A., Lichtenstein, M.P., Saura, C.A., Pujol, A., Masgrau, R., and Galea, E. (2012). ATP and noradrenaline activate CREB in astrocytes via noncanonical Ca(2+) and cyclic AMP independent pathways. Glia *60*, 1330-1344.
Cataldo, A.M., and Broadwell, R.D. (1986). Cytochemical identification of cerebral glycogen and glucose-6-phosphatase activity under normal and experimental conditions. II. Choroid plexus and ependymal epithelia, endothelia and pericytes. J Neurocytol *15*, 511-524.
Cerdan, S., Kunnecke, B., and Seelig, J. (1990). Cerebral metabolism of [1,2-13C2]acetate as detected by in vivo and in vitro 13C NMR. J Biol Chem *265*, 12916-12926.
Chaboub, L.S., and Deneen, B. (2012). Developmental origins of astrocyte heterogeneity: the final frontier of CNS development. Developmental neuroscience *34*, 379-388.
Chen, Y., Vartiainen, N.E., Ying, W., Chan, P.H., Koistinaho, J., and Swanson, R.A. (2001). Astrocytes protect neurons from nitric oxide toxicity by a glutathione-dependent mechanism. J Neurochem *77*, 1601-1610.
Chung, W.S., Allen, N.J., and Eroglu, C. (2015). Astrocytes Control Synapse Formation, Function, and Elimination. Cold Spring Harb Perspect Biol *7*.
Chung, W.S., Clarke, L.E., Wang, G.X., Stafford, B.K., Sher, A., Chakraborty, C., Joung, J., Foo, L.C., Thompson, A., Chen, C., *et al.* (2013). Astrocytes mediate synapse elimination through MEGF10 and MERTK pathways. Nature *504*, 394-400.
Clapham, D.E. (2007). Calcium signaling. Cell *131*, 1047-1058.
Clarke, L.E., and Barres, B.A. (2013). Emerging roles of astrocytes in neural circuit development. Nat Rev Neurosci *14*, 311-321.
Colombo, J.A., and Reisin, H.D. (2004). Interlaminar astroglia of the cerebral cortex: a marker of the primate brain. Brain research *1006*, 126-131.
Corbett, G.T., Roy, A., and Pahan, K. (2013). Sodium phenylbutyrate enhances astrocytic neurotrophin synthesis via protein kinase C (PKC)-mediated activation of cAMP-response element-binding protein (CREB): implications for Alzheimer disease therapy. J Biol Chem *288*, 8299-8312.
Cornell-Bell, A.H., Finkbeiner, S.M., Cooper, M.S., and Smith, S.J. (1990). Glutamate induces calcium waves in cultured astrocytes: long-range glial signaling. Science *247*, 470-473.
Covarrubias-Pinto, A., Acuna, A.I., Beltran, F.A., Torres-Diaz, L., and Castro, M.A. (2015). Old Things New View: Ascorbic Acid Protects the Brain in Neurodegenerative Disorders. Int J Mol Sci *16*, 28194-28217.
D'Ascenzo, M., Fellin, T., Terunuma, M., Revilla-Sanchez, R., Meaney, D.F., Auberson, Y.P., Moss, S.J., and Haydon, P.G. (2007). mGluR5 stimulates gliotransmission in the nucleus accumbens. Proc Natl Acad Sci U S A *104*, 1995-2000.

Dani, J.W., Chernjavsky, A., and Smith, S.J. (1992). Neuronal activity triggers calcium waves in hippocampal astrocyte networks. Neuron *8*, 429-440.
Dash, P.K., Hochner, B., and Kandel, E.R. (1990). Injection of the cAMP-responsive element into the nucleus of Aplysia sensory neurons blocks long-term facilitation. Nature *345*, 718-721.
de Brito, O.M., and Scorrano, L. (2008). Mitofusin 2 tethers endoplasmic reticulum to mitochondria. Nature *456*, 605-610.
De Stefani, D., Raffaello, A., Teardo, E., Szabo, I., and Rizzuto, R. (2011). A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. Nature *476*, 336-340.
Derouiche, A., Haseleu, J., and Korf, H.W. (2015). Fine Astrocyte Processes Contain Very Small Mitochondria: Glial Oxidative Capability May Fuel Transmitter Metabolism. Neurochem Res *40*, 2402-2413.
Di Castro, M.A., Chuquet, J., Liaudet, N., Bhaukaurally, K., Santello, M., Bouvier, D., Tiret, P., and Volterra, A. (2011). Local Ca2+ detection and modulation of synaptic release by astrocytes. Nature neuroscience *14*, 1276-1284.
Dienel, G.A., and Cruz, N.F. (2016). Aerobic glycolysis during brain activation: adrenergic regulation and influence of norepinephrine on astrocytic metabolism. J Neurochem *138*, 14-52.
Ding, F., O'Donnell, J., Thrane, A.S., Zeppenfeld, D., Kang, H., Xie, L., Wang, F., and Nedergaard, M. (2013). alpha1-Adrenergic receptors mediate coordinated Ca2+ signaling of cortical astrocytes in awake, behaving mice. Cell Calcium *54*, 387-394.
DiNuzzo, M., Mangia, S., Maraviglia, B., and Giove, F. (2010). Glycogenolysis in astrocytes supports blood-borne glucose channeling not glycogen-derived lactate shuttling to neurons: evidence from mathematical modeling. J Cereb Blood Flow Metab *30*, 1895-1904.
Dong, Y., Green, T., Saal, D., Marie, H., Neve, R., Nestler, E.J., and Malenka, R.C. (2006). CREB modulates excitability of nucleus accumbens neurons. Nature neuroscience *9*, 475-477.
Doyle, J.P., Dougherty, J.D., Heiman, M., Schmidt, E.F., Stevens, T.R., Ma, G., Bupp, S., Shrestha, P., Shah, R.D., Doughty, M.L.*, et al.* (2008). Application of a translational profiling approach for the comparative analysis of CNS cell types. Cell *135*, 749-762.
Dringen, R. (2000). Metabolism and functions of glutathione in brain. Prog Neurobiol *62*, 649-671.
Duran, J., Saez, I., Gruart, A., Guinovart, J.J., and Delgado-Garcia, J.M. (2013). Impairment in long-term memory formation and learning-dependent synaptic plasticity in mice lacking glycogen synthase in the brain. J Cereb Blood Flow Metab *33*, 550-556.
Ebert, D., Haller, R.G., and Walton, M.E. (2003). Energy contribution of octanoate to intact rat brain metabolism measured by 13C nuclear magnetic resonance spectroscopy. The Journal of neuroscience : the official journal of the Society for Neuroscience *23*, 5928-5935.
Emsley, J.G., and Macklis, J.D. (2006). Astroglial heterogeneity closely reflects the neuronal-defined anatomy of the adult murine CNS. Neuron glia biology *2*, 175-186.
Fellin, T., Pascual, O., Gobbo, S., Pozzan, T., Haydon, P.G., and Carmignoto, G. (2004). Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors. Neuron *43*, 729-743.

Ferrer, I., Blanco, R., Rivera, R., Carmona, M., Ballabriga, J., Olive, M., and Planas, A.M. (1996). CREB-1 and CREB-2 immunoreactivity in the rat brain. Brain research *712*, 159-164.
Fiacco, T.A., Agulhon, C., Taves, S.R., Petravicz, J., Casper, K.B., Dong, X., Chen, J., and McCarthy, K.D. (2007). Selective stimulation of astrocyte calcium in situ does not affect neuronal excitatory synaptic activity. Neuron *54*, 611-626.
Fox, P.T., Raichle, M.E., Mintun, M.A., and Dence, C. (1988). Nonoxidative glucose consumption during focal physiologic neural activity. Science *241*, 462-464.
Fujita, T., Chen, M.J., Li, B., Smith, N.A., Peng, W., Sun, W., Toner, M.J., Kress, B.T., Wang, L., Benraiss, A.*, et al.* (2014). Neuronal transgene expression in dominant-negative SNARE mice. The Journal of neuroscience : the official journal of the Society for Neuroscience *34*, 16594-16604.
Galione, A. (2015). A primer of NAADP-mediated Ca(2+) signalling: From sea urchin eggs to mammalian cells. Cell Calcium *58*, 27-47.
Golgi, C. (1872). Sulla fina anatomia degli organi centrali del sistema nervoso (Firenze: Giunti).
Gonzalez, G.A., and Montminy, M.R. (1989). Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell *59*, 675-680.
Goyal, M.S., Hawrylycz, M., Miller, J.A., Snyder, A.Z., and Raichle, M.E. (2014). Aerobic glycolysis in the human brain is associated with development and neotenous gene expression. Cell Metab *19*, 49-57.
Griffiths, E.J., and Rutter, G.A. (2009). Mitochondrial calcium as a key regulator of mitochondrial ATP production in mammalian cells. Biochimica et biophysica acta *1787*, 1324-1333.
Halassa, M.M., Florian, C., Fellin, T., Munoz, J.R., Lee, S.Y., Abel, T., Haydon, P.G., and Frank, M.G. (2009). Astrocytic modulation of sleep homeostasis and cognitive consequences of sleep loss. Neuron *61*, 213-219.
Halestrap, A.P. (2010). A pore way to die: the role of mitochondria in reperfusion injury and cardioprotection. Biochem Soc Trans *38*, 841-860.
Halestrap, A.P., and Wilson, M.C. (2012). The monocarboxylate transporter family--role and regulation. IUBMB Life *64*, 109-119.
Halim, N.D., McFate, T., Mohyeldin, A., Okagaki, P., Korotchkina, L.G., Patel, M.S., Jeoung, N.H., Harris, R.A., Schell, M.J., and Verma, A. (2010). Phosphorylation status of pyruvate dehydrogenase distinguishes metabolic phenotypes of cultured rat brain astrocytes and neurons. Glia *58*, 1168-1176.
Han, M.H., Bolanos, C.A., Green, T.A., Olson, V.G., Neve, R.L., Liu, R.J., Aghajanian, G.K., and Nestler, E.J. (2006). Role of cAMP response element-binding protein in the rat locus ceruleus: regulation of neuronal activity and opiate withdrawal behaviors. The Journal of neuroscience : the official journal of the Society for Neuroscience *26*, 4624-4629.
Han, X., Chen, M., Wang, F., Windrem, M., Wang, S., Shanz, S., Xu, Q., Oberheim, N.A., Bekar, L., Betstadt, S.*, et al.* (2013). Forebrain engraftment by human glial progenitor cells enhances synaptic plasticity and learning in adult mice. Cell stem cell *12*, 342-353.
Harris, J.J., Jolivet, R., and Attwell, D. (2012). Synaptic energy use and supply. Neuron *75*, 762-777.
Haustein, M.D., Kracun, S., Lu, X.H., Shih, T., Jackson-Weaver, O., Tong, X., Xu, J., Yang, X.W., O'Dell, T.J., Marvin, J.S.*, et al.* (2014). Conditions and constraints for astrocyte calcium signaling in the hippocampal mossy fiber pathway. Neuron *82*, 413-429.

Hayashi, T., and Su, T.P. (2007). Sigma-1 receptor chaperones at the ER-mitochondrion interface regulate Ca(2+) signaling and cell survival. Cell *131*, 596-610.
Henneberger, C., Papouin, T., Oliet, S.H., and Rusakov, D.A. (2010). Long-term potentiation depends on release of D-serine from astrocytes. Nature *463*, 232-236.
Herculano-Houzel, S. (2014). The glia/neuron ratio: how it varies uniformly across brain structures and species and what that means for brain physiology and evolution. Glia *62*, 1377-1391.
Herrero-Mendez, A., Almeida, A., Fernandez, E., Maestre, C., Moncada, S., and Bolanos, J.P. (2009). The bioenergetic and antioxidant status of neurons is controlled by continuous degradation of a key glycolytic enzyme by APC/C-Cdh1. Nat Cell Biol *11*, 747-752.
Hertz, L., and Chen, Y. (2016). Importance of astrocytes for potassium ion (K+) homeostasis in brain and glial effects of K+ and its transporters on learning. Neurosci Biobehav Rev *71*, 484-505.
Hertz, L., and Dienel, G.A. (2005). Lactate transport and transporters: general principles and functional roles in brain cells. J Neurosci Res *79*, 11-18.
Hertz, L., O'Dowd, B.S., Ng, K.T., and Gibbs, M.E. (2003). Reciprocal changes in forebrain contents of glycogen and of glutamate/glutamine during early memory consolidation in the day-old chick. Brain research *994*, 226-233.
Hertz, L., Peng, L., and Dienel, G.A. (2007). Energy metabolism in astrocytes: high rate of oxidative metabolism and spatiotemporal dependence on glycolysis/glycogenolysis. J Cereb Blood Flow Metab *27*, 219-249.
Hertz, L., and Zielke, H.R. (2004). Astrocytic control of glutamatergic activity: astrocytes as stars of the show. Trends Neurosci *27*, 735-743.
Holtzclaw, L.A., Pandhit, S., Bare, D.J., Mignery, G.A., and Russell, J.T. (2002). Astrocytes in adult rat brain express type 2 inositol 1,4,5-trisphosphate receptors. Glia *39*, 69-84.
Hurst, S., Hoek, J., and Sheu, S.S. (2017). Mitochondrial Ca(2+) and regulation of the permeability transition pore. Journal of bioenergetics and biomembranes *49*, 27-47.
Iadecola, C. (2017). The Neurovascular Unit Coming of Age: A Journey through Neurovascular Coupling in Health and Disease. Neuron *96*, 17-42.
Iguchi-Ariga, S.M., and Schaffner, W. (1989). CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation. Genes Dev *3*, 612-619.
Jackson, J.G., O'Donnell, J.C., Takano, H., Coulter, D.A., and Robinson, M.B. (2014). Neuronal activity and glutamate uptake decrease mitochondrial mobility in astrocytes and position mitochondria near glutamate transporters. The Journal of neuroscience : the official journal of the Society for Neuroscience *34*, 1613-1624.
Jackson, J.G., and Robinson, M.B. (2015). Reciprocal Regulation of Mitochondrial Dynamics and Calcium Signaling in Astrocyte Processes. The Journal of neuroscience : the official journal of the Society for Neuroscience *35*, 15199-15213.
Jackson, J.G., and Robinson, M.B. (2017). Regulation of mitochondrial dynamics in astrocytes: Mechanisms, consequences, and unknowns. Glia.
Jana, A., Modi, K.K., Roy, A., Anderson, J.A., van Breemen, R.B., and Pahan, K. (2013). Up-regulation of neurotrophic factors by cinnamon and its metabolite sodium benzoate: therapeutic implications for neurodegenerative disorders. J Neuroimmune Pharmacol *8*, 739-755.

Jancic, D., Lopez de Armentia, M., Valor, L.M., Olivares, R., and Barco, A. (2009). Inhibition of cAMP response element-binding protein reduces neuronal excitability and plasticity, and triggers neurodegeneration. Cerebral cortex (New York, NY : 1991) *19*, 2535-2547.
Jimenez-Blasco, D., Santofimia-Castano, P., Gonzalez, A., Almeida, A., and Bolanos, J.P. (2015). Astrocyte NMDA receptors' activity sustains neuronal survival through a Cdk5-Nrf2 pathway. Cell Death Differ *22*, 1877-1889.
Johannessen, M., Delghandi, M.P., and Moens, U. (2004). What turns CREB on? Cell Signal *16*, 1211-1227.
Jolivet, R., Allaman, I., Pellerin, L., Magistretti, P.J., and Weber, B. (2010). Comment on recent modeling studies of astrocyte-neuron metabolic interactions. J Cereb Blood Flow Metab *30*, 1982-1986.
Jourdain, P., Bergersen, L.H., Bhaukaurally, K., Bezzi, P., Santello, M., Domercq, M., Matute, C., Tonello, F., Gundersen, V., and Volterra, A. (2007). Glutamate exocytosis from astrocytes controls synaptic strength. Nature neuroscience *10*, 331-339.
Kandel, E.R. (2001). The molecular biology of memory storage: a dialogue between genes and synapses. Science *294*, 1030-1038.
Kandel, E.R. (2013). Principles of neural science, 5th edn (New York: McGraw-Hill).
Karki, P., Webb, A., Smith, K., Lee, K., Son, D.S., Aschner, M., and Lee, E. (2013). cAMP response element-binding protein (CREB) and nuclear factor kappaB mediate the tamoxifen-induced up-regulation of glutamate transporter 1 (GLT-1) in rat astrocytes. J Biol Chem *288*, 28975-28986.
Karki, P., Webb, A., Zerguine, A., Choi, J., Son, D.S., and Lee, E. (2014). Mechanism of raloxifene-induced upregulation of glutamate transporters in rat primary astrocytes. Glia *62*, 1270-1283.
Kersante, F., Rowley, S.C., Pavlov, I., Gutierrez-Mecinas, M., Semyanov, A., Reul, J.M., Walker, M.C., and Linthorst, A.C. (2013). A functional role for both -aminobutyric acid (GABA) transporter-1 and GABA transporter-3 in the modulation of extracellular GABA and GABAergic tonic conductances in the rat hippocampus. J Physiol *591*, 2429-2441.
Kettenmann, H., and Verkhratsky, A. (2013). Glial Cells, Vol 36.
Khakh, B.S., and McCarthy, K.D. (2015). Astrocyte calcium signaling: from observations to functions and the challenges therein. Cold Spring Harb Perspect Biol *7*, a020404.
Kida, S., Josselyn, S.A., Pena de Ortiz, S., Kogan, J.H., Chevere, I., Masushige, S., and Silva, A.J. (2002). CREB required for the stability of new and reactivated fear memories. Nature neuroscience *5*, 348-355.
Kida, S., and Serita, T. (2014). Functional roles of CREB as a positive regulator in the formation and enhancement of memory. Brain Res Bull *105*, 17-24.
Kirchhoff, F. (2010). Neuroscience. Questionable calcium. Science *327*, 1212-1213.
Koob, A.O. (2017). Astrogenesis versus astrogliosis. Neural Regeneration Research *12*, 203-204.
Kozlov, A.S., Angulo, M.C., Audinat, E., and Charpak, S. (2006). Target cell-specific modulation of neuronal activity by astrocytes. Proc Natl Acad Sci U S A *103*, 10058-10063.
Kwok, R.P., Lundblad, J.R., Chrivia, J.C., Richards, J.P., Bachinger, H.P., Brennan, R.G., Roberts, S.G., Green, M.R., and Goodman, R.H. (1994). Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature *370*, 223-226.

Kwon, J., An, H., Sa, M., Won, J., Shin, J.I., and Lee, C.J. (2017). Orai1 and Orai3 in Combination with Stim1 Mediate the Majority of Store-operated Calcium Entry in Astrocytes. Exp Neurobiol *26*, 42-54.
Lalo, U., Pankratov, Y., Parpura, V., and Verkhratsky, A. (2011). Ionotropic receptors in neuronal-astroglial signalling: what is the role of "excitable" molecules in non-excitable cells. Biochimica et biophysica acta *1813*, 992-1002.
Larsen, B.R., and MacAulay, N. (2014). Kir4.1-mediated spatial buffering of K(+): experimental challenges in determination of its temporal and quantitative contribution to K(+) clearance in the brain. Channels (Austin) *8*, 544-550.
Lee, H.C. (2005). Nicotinic acid adenine dinucleotide phosphate (NAADP)-mediated calcium signaling. J Biol Chem *280*, 33693-33696.
Lee, S., Yoon, B.E., Berglund, K., Oh, S.J., Park, H., Shin, H.S., Augustine, G.J., and Lee, C.J. (2010). Channel-mediated tonic GABA release from glia. Science *330*, 790-796.
Li, B., and Freeman, R.D. (2015). Neurometabolic coupling between neural activity, glucose, and lactate in activated visual cortex. J Neurochem *135*, 742-754.
Lim, D., Rodriguez-Arellano, J.J., Parpura, V., Zorec, R., Zeidan-Chulia, F., Genazzani, A.A., and Verkhratsky, A. (2016). Calcium signalling toolkits in astrocytes and spatio-temporal progression of Alzheimer's disease. Curr Alzheimer Res *13*, 359-369.
Lin, A.L., Fox, P.T., Hardies, J., Duong, T.Q., and Gao, J.H. (2010). Nonlinear coupling between cerebral blood flow, oxygen consumption, and ATP production in human visual cortex. Proc Natl Acad Sci U S A *107*, 8446-8451.
Lioy, D.T., Garg, S.K., Monaghan, C.E., Raber, J., Foust, K.D., Kaspar, B.K., Hirrlinger, P.G., Kirchhoff, F., Bissonnette, J.M., Ballas, N.*, et al.* (2011). A role for glia in the progression of Rett's syndrome. Nature *475*, 497-500.
Liu, B., Teschemacher, A.G., and Kasparov, S. (2017). Neuroprotective potential of astroglia. J Neurosci Res *95*, 2126-2139.
Lonze, B.E., and Ginty, D.D. (2002). Function and regulation of CREB family transcription factors in the nervous system. Neuron *35*, 605-623.
Lopez de Armentia, M., Jancic, D., Olivares, R., Alarcon, J.M., Kandel, E.R., and Barco, A. (2007). cAMP response element-binding protein-mediated gene expression increases the intrinsic excitability of CA1 pyramidal neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience *27*, 13909-13918.
Lovatt, D., Sonnewald, U., Waagepetersen, H.S., Schousboe, A., He, W., Lin, J.H., Han, X., Takano, T., Wang, S., Sim, F.J.*, et al.* (2007). The transcriptome and metabolic gene signature of protoplasmic astrocytes in the adult murine cortex. The Journal of neuroscience : the official journal of the Society for Neuroscience *27*, 12255-12266.
Lu, B., Nagappan, G., and Lu, Y. (2014). BDNF and synaptic plasticity, cognitive function, and dysfunction. Handb Exp Pharmacol *220*, 223-250.
Ma, B., Buckalew, R., Du, Y., Kiyoshi, C.M., Alford, C.C., Wang, W., McTigue, D.M., Enyeart, J.J., Terman, D., and Zhou, M. (2016). Gap junction coupling confers isopotentiality on astrocyte syncytium. Glia *64*, 214-226.
Ma, S., Kwon, H.J., and Huang, Z. (2012). A functional requirement for astroglia in promoting blood vessel development in the early postnatal brain. PLoS One *7*, e48001.
Magistretti, P.J., and Allaman, I. (2015). A cellular perspective on brain energy metabolism and functional imaging. Neuron *86*, 883-901.
Makar, T.K., Nedergaard, M., Preuss, A., Gelbard, A.S., Perumal, A.S., and Cooper, A.J. (1994). Vitamin E, ascorbate, glutathione, glutathione disulfide, and enzymes of

glutathione metabolism in cultures of chick astrocytes and neurons: evidence that astrocytes play an important role in antioxidative processes in the brain. J Neurochem *62*, 45-53.
Malarkey, E.B., Ni, Y., and Parpura, V. (2008). Ca2+ entry through TRPC1 channels contributes to intracellular Ca2+ dynamics and consequent glutamate release from rat astrocytes. Glia *56*, 821-835.
Mammucari, C., Raffaello, A., Vecellio Reane, D., and Rizzuto, R. (2016). Molecular structure and pathophysiological roles of the Mitochondrial Calcium Uniporter. Biochimica et biophysica acta *1863*, 2457-2464.
Mangia, S. (2009). The in vivo neuron-to-astrocyte lactate shuttle in human brain: evidence from modeling of measured lactate levels during visual stimulation. *109*, 55-62.
Mangia, S., DiNuzzo, M., Giove, F., Carruthers, A., Simpson, I.A., and Vannucci, S.J. (2011). Response to 'comment on recent modeling studies of astrocyte–neuron metabolic interactions': much ado about nothing. J Cereb Blood Flow Metab *31*, 1346-1353.
Marchi, S., Patergnani, S., Missiroli, S., Morciano, G., Rimessi, A., Wieckowski, M.R., Giorgi, C., and Pinton, P. (2017). Mitochondrial and endoplasmic reticulum calcium homeostasis and cell death. Cell Calcium.
Martinez-Hernandez, A., Bell, K.P., and Norenberg, M.D. (1977). Glutamine synthetase: glial localization in brain. Science *195*, 1356-1358.
Masgrau, R., Guaza, C., Ransohoff, R.M., and Galea, E. (2017). Should We Stop Saying 'Glia' and 'Neuroinflammation'? Trends in molecular medicine *23*, 486-500.
Matyash, M., Matyash, V., Nolte, C., Sorrentino, V., and Kettenmann, H. (2002). Requirement of functional ryanodine receptor type 3 for astrocyte migration. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *16*, 84-86.
Mayr, B., and Montminy, M. (2001). Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol *2*, 599-609.
McGilvery, R.W., and Goldstein, G. (1983). Biochemistry, a functional approach, 3rd edn (Philadelphia: Saunders).
McKenna, M.C. (2013). Glutamate pays its own way in astrocytes. Front Endocrinol (Lausanne) *4*, 191.
McKenna, M.C., Sonnewald, U., Huang, X., Stevenson, J., and Zielke, H.R. (1996). Exogenous glutamate concentration regulates the metabolic fate of glutamate in astrocytes. J Neurochem *66*, 386-393.
Miller, R.H., and Raff, M.C. (1984). Fibrous and protoplasmic astrocytes are biochemically and developmentally distinct. The Journal of neuroscience : the official journal of the Society for Neuroscience *4*, 585-592.
Minelli, A., Castaldo, P., Gobbi, P., Salucci, S., Magi, S., and Amoroso, S. (2007). Cellular and subcellular localization of Na+-Ca2+ exchanger protein isoforms, NCX1, NCX2, and NCX3 in cerebral cortex and hippocampus of adult rat. Cell Calcium *41*, 221-234.
Mink, J.W., Blumenschine, R.J., and Adams, D.B. (1981). Ratio of central nervous system to body metabolism in vertebrates: its constancy and functional basis. Am J Physiol *241*, R203-212.
Mishra, A. (2017). Binaural blood flow control by astrocytes: listening to synapses and the vasculature. J Physiol *595*, 1885-1902.

Mishra, A., Reynolds, J.P., Chen, Y., Gourine, A.V., Rusakov, D.A., and Attwell, D. (2016). Astrocytes mediate neurovascular signaling to capillary pericytes but not to arterioles. Nature neuroscience *19*, 1619-1627.
Modi, K.K., Sendtner, M., and Pahan, K. (2013). Up-regulation of ciliary neurotrophic factor in astrocytes by aspirin: implications for remyelination in multiple sclerosis. J Biol Chem *288*, 18533-18545.
Morgello, S., Uson, R.R., Schwartz, E.J., and Haber, R.S. (1995). The human blood-brain barrier glucose transporter (GLUT1) is a glucose transporter of gray matter astrocytes. Glia *14*, 43-54.
Morita, M., and Kudo, Y. (2010). Growth factors upregulate astrocyte [Ca2+]i oscillation by increasing SERCA2b expression. Glia *58*, 1988-1995.
Mothet, J.P., Pollegioni, L., Ouanounou, G., Martineau, M., Fossier, P., and Baux, G. (2005). Glutamate receptor activation triggers a calcium-dependent and SNARE protein-dependent release of the gliotransmitter D-serine. Proc Natl Acad Sci U S A *102*, 5606-5611.
Murphy-Royal, C., Dupuis, J., Groc, L., and Oliet, S.H.R. (2017). Astroglial glutamate transporters in the brain: Regulating neurotransmitter homeostasis and synaptic transmission. J Neurosci Res *95*, 2140-2151.
Murray, P.D., Kingsbury, T.J., and Krueger, B.K. (2009). Failure of Ca2+-activated, CREB-dependent transcription in astrocytes. Glia *57*, 828-834.
Nagase, M., Takahashi, Y., Watabe, A.M., Kubo, Y., and Kato, F. (2014). On-site energy supply at synapses through monocarboxylate transporters maintains excitatory synaptic transmission. The Journal of neuroscience : the official journal of the Society for Neuroscience *34*, 2605-2617.
Navarrete, M., Perea, G., Fernandez de Sevilla, D., Gomez-Gonzalo, M., Nunez, A., Martin, E.D., and Araque, A. (2012). Astrocytes mediate in vivo cholinergic-induced synaptic plasticity. PLoS Biol *10*, e1001259.
Nett, W.J., Oloff, S.H., and McCarthy, K.D. (2002). Hippocampal astrocytes in situ exhibit calcium oscillations that occur independent of neuronal activity. J Neurophysiol *87*, 528-537.
Newman, E.A. (2001). Propagation of intercellular calcium waves in retinal astrocytes and Muller cells. The Journal of neuroscience : the official journal of the Society for Neuroscience *21*, 2215-2223.
Newman, E.A., and Zahs, K.R. (1997). Calcium waves in retinal glial cells. Science *275*, 844-847.
Newman, L.A., Korol, D.L., and Gold, P.E. (2011). Lactate produced by glycogenolysis in astrocytes regulates memory processing. PLoS One *6*, e28427.
Norenberg, M.D., and Martinez-Hernandez, A. (1979). Fine structural localization of glutamine synthetase in astrocytes of rat brain. Brain research *161*, 303-310.
Nwaobi, S.E., Cuddapah, V.A., Patterson, K.C., Randolph, A.C., and Olsen, M.L. (2016). The role of glial-specific Kir4.1 in normal and pathological states of the CNS. Acta Neuropathol *132*, 1-21.
Obara, M., Szeliga, M., and Albrecht, J. (2008). Regulation of pH in the mammalian central nervous system under normal and pathological conditions: facts and hypotheses. Neurochem Int *52*, 905-919.
Oberheim, N.A., Goldman, S.A., and Nedergaard, M. (2012). Heterogeneity of astrocytic form and function. Methods Mol Biol *814*, 23-45.

Oberheim, N.A., Takano, T., Han, X., He, W., Lin, J.H., Wang, F., Xu, Q., Wyatt, J.D., Pilcher, W., Ojemann, J.G., *et al.* (2009). Uniquely hominid features of adult human astrocytes. The Journal of neuroscience : the official journal of the Society for Neuroscience *29*, 3276-3287.
Palty, R., Silverman, W.F., Hershfinkel, M., Caporale, T., Sensi, S.L., Parnis, J., Nolte, C., Fishman, D., Shoshan-Barmatz, V., Herrmann, S., *et al.* (2010). NCLX is an essential component of mitochondrial Na+/Ca2+ exchange. Proc Natl Acad Sci U S A *107*, 436-441.
Paluzzi, S., Alloisio, S., Zappettini, S., Milanese, M., Raiteri, L., Nobile, M., and Bonanno, G. (2007). Adult astroglia is competent for Na+/Ca2+ exchanger-operated exocytotic glutamate release triggered by mild depolarization. J Neurochem *103*, 1196-1207.
Panatier, A., Vallee, J., Haber, M., Murai, K.K., Lacaille, J.C., and Robitaille, R. (2011). Astrocytes are endogenous regulators of basal transmission at central synapses. Cell *146*, 785-798.
Panov, A., Orynbayeva, Z., Vavilin, V., and Lyakhovich, V. (2014). Fatty acids in energy metabolism of the central nervous system. Biomed Res Int *2014*, 472459.
Pardo, L., Schluter, A., Valor, L.M., Barco, A., Giralt, M., Golbano, A., Hidalgo, J., Jia, P., Zhao, Z., Jove, M., *et al.* (2016). Targeted activation of CREB in reactive astrocytes is neuroprotective in focal acute cortical injury. Glia *64*, 853-874.
Pardo, L., Valor, L.M., Eraso-Pichot, A., Barco, A., Golbano, A., Hardingham, G.E., Masgrau, R., and Galea, E. (2017). CREB Regulates Distinct Adaptive Transcriptional Programs in Astrocytes and Neurons. Scientific reports *7*, 6390.
Parpura, V., Basarsky, T.A., Liu, F., Jeftinija, K., Jeftinija, S., and Haydon, P.G. (1994). Glutamate-mediated astrocyte-neuron signalling. Nature *369*, 744-747.
Parri, H.R., Gould, T.M., and Crunelli, V. (2001). Spontaneous astrocytic Ca2+ oscillations in situ drive NMDAR-mediated neuronal excitation. Nature neuroscience *4*, 803-812.
Pascual, O., Casper, K.B., Kubera, C., Zhang, J., Revilla-Sanchez, R., Sul, J.Y., Takano, H., Moss, S.J., McCarthy, K., and Haydon, P.G. (2005). Astrocytic purinergic signaling coordinates synaptic networks. Science *310*, 113-116.
Patel, S., Ramakrishnan, L., Rahman, T., Hamdoun, A., Marchant, J.S., Taylor, C.W., and Brailoiu, E. (2011). The endo-lysosomal system as an NAADP-sensitive acidic Ca(2+) store: role for the two-pore channels. Cell Calcium *50*, 157-167.
Patrushev, I., Gavrilov, N., Turlapov, V., and Semyanov, A. (2013). Subcellular location of astrocytic calcium stores favors extrasynaptic neuron-astrocyte communication. Cell Calcium *54*, 343-349.
Pellegri, G., Rossier, C., Magistretti, P.J., and Martin, J.L. (1996). Cloning, localization and induction of mouse brain glycogen synthase. Brain Res Mol Brain Res *38*, 191-199.
Pellerin, L., Bergersen, L.H., Halestrap, A.P., and Pierre, K. (2005). Cellular and subcellular distribution of monocarboxylate transporters in cultured brain cells and in the adult brain. J Neurosci Res *79*, 55-64.
Pellerin, L., and Magistretti, P.J. (1994). Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. Proc Natl Acad Sci U S A *91*, 10625-10629.
Pelvig, D.P., Pakkenberg, H., Stark, A.K., and Pakkenberg, B. (2008). Neocortical glial cell numbers in human brains. Neurobiology of aging *29*, 1754-1762.
Perea, G., and Araque, A. (2007). Astrocytes potentiate transmitter release at single hippocampal synapses. Science *317*, 1083-1086.

Perez-Escuredo, J., Van Hee, V.F., Sboarina, M., Falces, J., Payen, V.L., Pellerin, L., and Sonveaux, P. (2016). Monocarboxylate transporters in the brain and in cancer. Biochimica et biophysica acta *1863*, 2481-2497.
Petravicz, J., Boyt, K.M., and McCarthy, K.D. (2014). Astrocyte IP3R2-dependent Ca(2+) signaling is not a major modulator of neuronal pathways governing behavior. Front Behav Neurosci *8*, 384.
Petravicz, J., Fiacco, T.A., and McCarthy, K.D. (2008). Loss of IP3 receptor-dependent Ca2+ increases in hippocampal astrocytes does not affect baseline CA1 pyramidal neuron synaptic activity. The Journal of neuroscience : the official journal of the Society for Neuroscience *28*, 4967-4973.
Pierre, K., Magistretti, P.J., and Pellerin, L. (2002). MCT2 is a major neuronal monocarboxylate transporter in the adult mouse brain. J Cereb Blood Flow Metab *22*, 586-595.
Pines, G., Danbolt, N.C., Bjoras, M., Zhang, Y., Bendahan, A., Eide, L., Koepsell, H., Storm-Mathisen, J., Seeberg, E., and Kanner, B.I. (1992). Cloning and expression of a rat brain L-glutamate transporter. Nature *360*, 464-467.
Pirttimaki, T.M., and Parri, H.R. (2013). Astrocyte plasticity: implications for synaptic and neuronal activity. Neuroscientist *19*, 604-615.
Pittenger, C., Huang, Y.Y., Paletzki, R.F., Bourtchouladze, R., Scanlin, H., Vronskaya, S., and Kandel, E.R. (2002). Reversible inhibition of CREB/ATF transcription factors in region CA1 of the dorsal hippocampus disrupts hippocampus-dependent spatial memory. Neuron *34*, 447-462.
Porter, J.T., and McCarthy, K.D. (1996). Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals. The Journal of neuroscience : the official journal of the Society for Neuroscience *16*, 5073-5081.
Pryazhnikov, E., and Khiroug, L. (2008). Sub-micromolar increase in [Ca(2+)](i) triggers delayed exocytosis of ATP in cultured astrocytes. Glia *56*, 38-49.
Putney, J.W., Jr. (1986). A model for receptor-regulated calcium entry. Cell Calcium *7*, 1-12.
Raffaello, A., Mammucari, C., Gherardi, G., and Rizzuto, R. (2016). Calcium at the Center of Cell Signaling: Interplay between Endoplasmic Reticulum, Mitochondria, and Lysosomes. Trends Biochem Sci *41*, 1035-1049.
Raichle, M.E. (2009). A brief history of human brain mapping. Trends Neurosci *32*, 118-126.
Raichle, M.E. (2015). The restless brain: how intrinsic activity organizes brain function. Philos Trans R Soc Lond B Biol Sci *370*.
Raichle, M.E., and Mintun, M.A. (2006). Brain work and brain imaging. Annu Rev Neurosci *29*, 449-476.
Ramón y Cajal, S. (1897). Histology of the nervous system of man and vertebrates (New York: Oxford University Press).
Rangaraju, V., Calloway, N., and Ryan, T.A. (2014). Activity-driven local ATP synthesis is required for synaptic function. Cell *156*, 825-835.
Rapanelli, M., Frick, L.R., and Zanutto, B.S. (2011). Learning an operant conditioning task differentially induces gliogenesis in the medial prefrontal cortex and neurogenesis in the hippocampus. PLoS One *6*, e14713.

Reemst, K., Noctor, S.C., Lucassen, P.J., and Hol, E.M. (2016). The Indispensable Roles of Microglia and Astrocytes during Brain Development. Frontiers in Human Neuroscience *10*.
Reyes, R.C., and Parpura, V. (2008). Mitochondria modulate Ca2+-dependent glutamate release from rat cortical astrocytes. The Journal of neuroscience : the official journal of the Society for Neuroscience *28*, 9682-9691.
Reyes, R.C., Verkhratsky, A., and Parpura, V. (2012). Plasmalemmal Na+/Ca2+ exchanger modulates Ca2+-dependent exocytotic release of glutamate from rat cortical astrocytes. ASN Neuro *4*.
Rizzuto, R., De Stefani, D., Raffaello, A., and Mammucari, C. (2012). Mitochondria as sensors and regulators of calcium signalling. Nat Rev Mol Cell Biol *13*, 566-578.
Roderick, H.L., Lechleiter, J.D., and Camacho, P. (2000). Cytosolic phosphorylation of calnexin controls intracellular Ca(2+) oscillations via an interaction with SERCA2b. J Cell Biol *149*, 1235-1248.
Rosafio, K., and Pellerin, L. (2014). Oxygen tension controls the expression of the monocarboxylate transporter MCT4 in cultured mouse cortical astrocytes via a hypoxia-inducible factor-1alpha-mediated transcriptional regulation. Glia *62*, 477-490.
Rose, C.F., Verkhratsky, A., and Parpura, V. (2013). Astrocyte glutamine synthetase: pivotal in health and disease. Biochem Soc Trans *41*, 1518-1524.
Rothman, D.L., Behar, K.L., Hyder, F., and Shulman, R.G. (2003). In vivo NMR studies of the glutamate neurotransmitter flux and neuroenergetics: implications for brain function. Annual review of physiology *65*, 401-427.
Rothstein, J.D., Dykes-Hoberg, M., Pardo, C.A., Bristol, L.A., Jin, L., Kuncl, R.W., Kanai, Y., Hediger, M.A., Wang, Y., Schielke, J.P.*, et al.* (1996). Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. Neuron *16*, 675-686.
Ruminot, I., Gutierrez, R., Pena-Munzenmayer, G., Anazco, C., Sotelo-Hitschfeld, T., Lerchundi, R., Niemeyer, M.I., Shull, G.E., and Barros, L.F. (2011). NBCe1 mediates the acute stimulation of astrocytic glycolysis by extracellular K+. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 14264-14271.
Sakamoto, K., Karelina, K., and Obrietan, K. (2011). CREB: a multifaceted regulator of neuronal plasticity and protection. J Neurochem *116*, 1-9.
Saura, C.A., and Cardinaux, J.R. (2017). Emerging Roles of CREB-Regulated Transcription Coactivators in Brain Physiology and Pathology. Trends Neurosci *40*, 720-733.
Scemes, E., and Giaume, C. (2006). Astrocyte calcium waves: what they are and what they do. Glia *54*, 716-725.
Schafer, D.P., Lehrman, E.K., Kautzman, A.G., Koyama, R., Mardinly, A.R., Yamasaki, R., Ransohoff, R.M., Greenberg, M.E., Barres, B.A., and Stevens, B. (2012). Microglia sculpt postnatal neural circuits in an activity and complement-dependent manner. Neuron *74*, 691-705.
Schousboe, A., and Hertz, L. (1971). Effects of potassium on concentrations of ions and proteins and on pH in brain-cortex slices from new-born and adult rats. Int J Neurosci *1*, 235-242.
Schousboe, A., Scafidi, S., Bak, L.K., Waagepetersen, H.S., and McKenna, M.C. (2014). Glutamate metabolism in the brain focusing on astrocytes. Adv Neurobiol *11*, 13-30.

Schumacher, M.A., Goodman, R.H., and Brennan, R.G. (2000). The structure of a CREB bZIP.somatostatin CRE complex reveals the basis for selective dimerization and divalent cation-enhanced DNA binding. J Biol Chem *275*, 35242-35247.
Seib, T.M., Patel, S.A., and Bridges, R.J. (2011). Regulation of the system x(C)-cystine/glutamate exchanger by intracellular glutathione levels in rat astrocyte primary cultures. Glia *59*, 1387-1401.
Shen, J. (2013). Modeling the glutamate-glutamine neurotransmitter cycle. Front Neuroenergetics *5*, 1.
Sheng, M., Thompson, M.A., and Greenberg, M.E. (1991). CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases. Science *252*, 1427-1430.
Sheppard, C.A., Simpson, P.B., Sharp, A.H., Nucifora, F.C., Ross, C.A., Lange, G.D., and Russell, J.T. (1997). Comparison of type 2 inositol 1,4,5-trisphosphate receptor distribution and subcellular Ca2+ release sites that support Ca2+ waves in cultured astrocytes. J Neurochem *68*, 2317-2327.
Shi, L., Pan, H., Liu, Z., Xie, J., and Han, W. (2017). Roles of PFKFB3 in cancer. Signal Transduct Target Ther *2*, 17044.
Shigetomi, E., Bushong, E.A., Haustein, M.D., Tong, X., Jackson-Weaver, O., Kracun, S., Xu, J., Sofroniew, M.V., Ellisman, M.H., and Khakh, B.S. (2013a). Imaging calcium microdomains within entire astrocyte territories and endfeet with GCaMPs expressed using adeno-associated viruses. J Gen Physiol *141*, 633-647.
Shigetomi, E., Jackson-Weaver, O., Huckstepp, R.T., O'Dell, T.J., and Khakh, B.S. (2013b). TRPA1 channels are regulators of astrocyte basal calcium levels and long-term potentiation via constitutive D-serine release. The Journal of neuroscience : the official journal of the Society for Neuroscience *33*, 10143-10153.
Shigetomi, E., Kracun, S., Sofroniew, M.V., and Khakh, B.S. (2010). A genetically targeted optical sensor to monitor calcium signals in astrocyte processes. Nature neuroscience *13*, 759-766.
Shigetomi, E., Tong, X., Kwan, K.Y., Corey, D.P., and Khakh, B.S. (2011). TRPA1 channels regulate astrocyte resting calcium and inhibitory synapse efficacy through GAT-3. Nature neuroscience *15*, 70-80.
Simpson, I.A., Carruthers, A., and Vannucci, S.J. (2007). Supply and demand in cerebral energy metabolism: the role of nutrient transporters. J Cereb Blood Flow Metab *27*, 1766-1791.
Sloan, S.A., and Barres, B.A. (2014). Looks can be deceiving: reconsidering the evidence for gliotransmission. Neuron *84*, 1112-1115.
Sonnewald, U., and Schousboe, A. (2016). Introduction to the Glutamate-Glutamine Cycle. Adv Neurobiol *13*, 1-7.
Srinivasan, R., Huang, B.S., Venugopal, S., Johnston, A.D., Chai, H., Zeng, H., Golshani, P., and Khakh, B.S. (2015). Ca(2+) signaling in astrocytes from Ip3r2(-/-) mice in brain slices and during startle responses in vivo. Nature neuroscience *18*, 708-717.
Steinman, M.Q., Gao, V., and Alberini, C.M. (2016). The Role of Lactate-Mediated Metabolic Coupling between Astrocytes and Neurons in Long-Term Memory Formation. Front Integr Neurosci *10*, 10.
Stevens, B., Allen, N.J., Vazquez, L.E., Howell, G.R., Christopherson, K.S., Nouri, N., Micheva, K.D., Mehalow, A.K., Huberman, A.D., Stafford, B., *et al.* (2007). The classical complement cascade mediates CNS synapse elimination. Cell *131*, 1164-1178.

Stobart, J.L., and Anderson, C.M. (2013). Multifunctional role of astrocytes as gatekeepers of neuronal energy supply. Front Cell Neurosci *7*, 38.
Storck, T., Schulte, S., Hofmann, K., and Stoffel, W. (1992). Structure, expression, and functional analysis of a Na(+)-dependent glutamate/aspartate transporter from rat brain. Proc Natl Acad Sci U S A *89*, 10955-10959.
Sun, W., Cornwell, A., Li, J., Peng, S., Osorio, M.J., Aalling, N., Wang, S., Benraiss, A., Lou, N., Goldman, S.A*., et al.* (2017). SOX9 Is an Astrocyte-Specific Nuclear Marker in the Adult Brain Outside the Neurogenic Regions. The Journal of neuroscience : the official journal of the Society for Neuroscience *37*, 4493-4507.
Sun, W., McConnell, E., Pare, J.F., Xu, Q., Chen, M., Peng, W., Lovatt, D., Han, X., Smith, Y., and Nedergaard, M. (2013). Glutamate-dependent neuroglial calcium signaling differs between young and adult brain. Science *339*, 197-200.
Suzuki, A., Fukushima, H., Mukawa, T., Toyoda, H., Wu, L.J., Zhao, M.G., Xu, H., Shang, Y., Endoh, K., Iwamoto, T*., et al.* (2011a). Upregulation of CREB-mediated transcription enhances both short- and long-term memory. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 8786-8802.
Suzuki, A., Stern, S.A., Bozdagi, O., Huntley, G.W., Walker, R.H., Magistretti, P.J., and Alberini, C.M. (2011b). Astrocyte-neuron lactate transport is required for long-term memory formation. Cell *144*, 810-823.
Szabadkai, G., Bianchi, K., Varnai, P., De Stefani, D., Wieckowski, M.R., Cavagna, D., Nagy, A.I., Balla, T., and Rizzuto, R. (2006). Chaperone-mediated coupling of endoplasmic reticulum and mitochondrial Ca2+ channels. J Cell Biol *175*, 901-911.
Taft, J.R., Vertes, R.P., and Perry, G.W. (2005). Distribution of GFAP+ astrocytes in adult and neonatal rat brain. Int J Neurosci *115*, 1333-1343.
Takasaki, I., Takarada, S., Tatsumi, S., Azegami, A., Yasuda, M., Fukuchi, M., Tabuchi, A., Kondo, T., Tabuchi, Y., and Tsuda, M. (2008). Extracellular adenosine 5'-triphosphate elicits the expression of brain-derived neurotrophic factor exon IV mRNA in rat astrocytes. Glia *56*, 1369-1379.
Takata, N., Mishima, T., Hisatsune, C., Nagai, T., Ebisui, E., Mikoshiba, K., and Hirase, H. (2011). Astrocyte calcium signaling transforms cholinergic modulation to cortical plasticity in vivo. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 18155-18165.
Takeuchi, T., Duszkiewicz, A.J., and Morris, R.G. (2014). The synaptic plasticity and memory hypothesis: encoding, storage and persistence. Philos Trans R Soc Lond B Biol Sci *369*, 20130288.
Tanaka, K., Watase, K., Manabe, T., Yamada, K., Watanabe, M., Takahashi, K., Iwama, H., Nishikawa, T., Ichihara, N., Kikuchi, T*., et al.* (1997). Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. Science *276*, 1699-1702.
Tani, H., Dulla, C.G., Farzampour, Z., Taylor-Weiner, A., Huguenard, J.R., and Reimer, R.J. (2014). A local glutamate-glutamine cycle sustains synaptic excitatory transmitter release. Neuron *81*, 888-900.
Tao, X., Finkbeiner, S., Arnold, D.B., Shaywitz, A.J., and Greenberg, M.E. (1998). Ca2+ influx regulates BDNF transcription by a CREB family transcription factor-dependent mechanism. Neuron *20*, 709-726.
Tong, X., Shigetomi, E., Looger, L.L., and Khakh, B.S. (2013). Genetically encoded calcium indicators and astrocyte calcium microdomains. Neuroscientist *19*, 274-291.

Vaishnavi, S.N., Vlassenko, A.G., Rundle, M.M., Snyder, A.Z., Mintun, M.A., and Raichle, M.E. (2010). Regional aerobic glycolysis in the human brain. Proc Natl Acad Sci U S A *107*, 17757-17762.
Valor, L.M., Viosca, J., Lopez-Atalaya, J.P., and Barco, A. (2013). Lysine acetyltransferases CBP and p300 as therapeutic targets in cognitive and neurodegenerative disorders. Curr Pharm Des *19*, 5051-5064.
Verkhratsky, A., Reyes, R.C., and Parpura, V. (2014). TRP channels coordinate ion signalling in astroglia. Rev Physiol Biochem Pharmacol *166*, 1-22.
Verkhratsky, A., Rodriguez, J.J., and Parpura, V. (2012). Calcium signalling in astroglia. Mol Cell Endocrinol *353*, 45-56.
Viosca, J., Lopez de Armentia, M., Jancic, D., and Barco, A. (2009). Enhanced CREB-dependent gene expression increases the excitability of neurons in the basal amygdala and primes the consolidation of contextual and cued fear memory. Learn Mem *16*, 193-197.
Volterra, A., Liaudet, N., and Savtchouk, I. (2014). Astrocyte Ca(2)(+) signalling: an unexpected complexity. Nat Rev Neurosci *15*, 327-335.
von Bartheld, C.S., Bahney, J., and Herculano-Houzel, S. (2016). The search for true numbers of neurons and glial cells in the human brain: A review of 150 years of cell counting. The Journal of comparative neurology *524*, 3865-3895.
von Bernhardi, R., Bernhardi, L.E., and Eugenin, J. (2017). What Is Neural Plasticity? Adv Exp Med Biol *1015*, 1-15.
Voutsinos-Porche, B., Bonvento, G., Tanaka, K., Steiner, P., Welker, E., Chatton, J.Y., Magistretti, P.J., and Pellerin, L. (2003). Glial glutamate transporters mediate a functional metabolic crosstalk between neurons and astrocytes in the mouse developing cortex. Neuron *37*, 275-286.
Walls, A.B., Waagepetersen, H.S., Bak, L.K., Schousboe, A., and Sonnewald, U. (2015). The glutamine-glutamate/GABA cycle: function, regional differences in glutamate and GABA production and effects of interference with GABA metabolism. Neurochem Res *40*, 402-409.
Wang, D.D., and Bordey, A. (2008). The astrocyte odyssey. Prog Neurobiol *86*, 342-367.
Wang, X., Lou, N., Xu, Q., Tian, G.F., Peng, W.G., Han, X., Kang, J., Takano, T., and Nedergaard, M. (2006). Astrocytic Ca2+ signaling evoked by sensory stimulation in vivo. Nature neuroscience *9*, 816-823.
Weidemann, A., Krohne, T.U., Aguilar, E., Kurihara, T., Takeda, N., Dorrell, M.I., Simon, M.C., Haase, V.H., Friedlander, M., and Johnson, R.S. (2010). Astrocyte hypoxic response is essential for pathological but not developmental angiogenesis of the retina. Glia *58*, 1177-1185.
Wilson, J.X. (1997). Antioxidant defense of the brain: a role for astrocytes. Can J Physiol Pharmacol *75*, 1149-1163.
Yang, Y., Ge, W., Chen, Y., Zhang, Z., Shen, W., Wu, C., Poo, M., and Duan, S. (2003). Contribution of astrocytes to hippocampal long-term potentiation through release of D-serine. Proc Natl Acad Sci U S A *100*, 15194-15199.
Yeh, T.H., Lee, D.Y., Gianino, S.M., and Gutmann, D.H. (2009). Microarray analyses reveal regional astrocyte heterogeneity with implications for neurofibromatosis type 1 (NF1)-regulated glial proliferation. Glia *57*, 1239-1249.
Zhang, X., Odom, D.T., Koo, S.H., Conkright, M.D., Canettieri, G., Best, J., Chen, H., Jenner, R., Herbolsheimer, E., Jacobsen, E.*, et al.* (2005). Genome-wide analysis of cAMP-

response element binding protein occupancy, phosphorylation, and target gene activation in human tissues. Proc Natl Acad Sci U S A *102*, 4459-4464.
Zhang, Y., and Barres, B.A. (2010). Astrocyte heterogeneity: an underappreciated topic in neurobiology. Curr Opin Neurobiol *20*, 588-594.
Zhang, Y., Chen, K., Sloan, S.A., Bennett, M.L., Scholze, A.R., O'Keeffe, S., Phatnani, H.P., Guarnieri, P., Caneda, C., Ruderisch, N.*, et al.* (2014). An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. The Journal of neuroscience : the official journal of the Society for Neuroscience *34*, 11929-11947.
Zhao, S., Chai, X., and Frotscher, M. (2007). Balance between neurogenesis and gliogenesis in the adult hippocampus: role for reelin. Developmental neuroscience *29*, 84-90.
Zhou, Y., and Danbolt, N.C. (2013). GABA and Glutamate Transporters in Brain. Front Endocrinol (Lausanne) *4*, 165.
Zhou, Y., Won, J., Karlsson, M.G., Zhou, M., Rogerson, T., Balaji, J., Neve, R., Poirazi, P., and Silva, A.J. (2009). CREB regulates excitability and the allocation of memory to subsets of neurons in the amygdala. Nature neuroscience *12*, 1438-1443.
Zielke, H.R., Zielke, C.L., and Baab, P.J. (2009). Direct measurement of oxidative metabolism in the living brain by microdialysis: a review. J Neurochem *109 Suppl 1*, 24-29.

WORKING HYPOTHESIS AND OBJECTIVES

IV. WORKING HYPOTHESIS AND OBJECTIVES:

Our working hypothesis is that CREB regulates activity-dependent plasticity in astrocytes. Therefore, we study how CREB-dependent transcription modulates the two main mechanisms by which astrocytes contribute to synaptic transmission: calcium signals and lactate release. We will undertake the following objectives:

1. To determine whether CREB regulates calcium excitability in astrocytes by gain and loss-of-function strategies:
    a. Analyze by calcium fluorescence imaging in cultures how CREB modulates calcium signals in astrocytes, for how long and which elements of the calcium toolkit are regulated.
    b. Develop adeno-associated viral vectors to modify CREB function *in vivo.*
    c. Study by confocal calcium imaging *in situ* in hippocampal slices whether modulation of CREB-dependent transcription with the newly generated vectors modifies calcium signals in astrocytes.

2. To determine whether CREB regulates energy metabolism and lactate release in astrocytes by gain and loss-of-function strategies:
    a. Evaluate in cultured astrocytes if the activation of CREB-dependent transcription modifies lactate release and elucidate the mechanisms.
    b. Test in cultured astrocytes if the activation of CREB-dependent transcription enhances glucose oxidative metabolism by analyzing oxygen consumption.
    c. Determine the idiosyncrasies of mitochondrial metabolism in astrocytes by GSEA of mitochondriomes generated from publicly available human and mouse transcriptomic arrays. This analysis will represent the foundations of future studies beyond this thesis on the regulation of mitochondrial functions by CREB.

MATERIALS AND METHODS

V. MATERIALS AND METHODS

1. Models
    1.1 Rat astrocyte cultures
        1.1.1 Neonatal

Astrocyte cultures were obtained from cortices from 1 day-old Sprague Dawley rats. Rats were decapitated and brains were extracted in a laminar flow hood. Under a binocular microscope, meninges were removed and cortices were separated from other brain structures. After isolation, tissue was minced with a scalpel and transferred to a 50 ml tube with Ca^{2+}-free Krebs Ringer Buffer (KRB) containing 120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 14.3 mM glucose, 0.03% Mg_2SO_4 and 0.3% bovine serum albumin (BSA). The tube was centrifuged for 1 minute at 300 g and the pellet was incubated at 37 °C in Ca^{2+}-free KRB with 0.025% of trypsin for 10 minutes, to allow tissue disgregation. Trypsin effect was inhibited by the addition of Ca^{2+}-free KRB containing 0.052% soybean trypsin inhibitor (Gibco), 0.008% DNAse (Sigma) and 0.03% plus of Mg_2SO_4. After stopping the reaction, cells were centrifuged for 1 minute at 300 g. The pellet was then resuspended and cells were mechanically triturated through a glass pipette and filtered through a 40-μm nylon mesh. Filtered cells were centrifugated for 5 minutes at 250 g and, after removal of KRB, resuspended in full culture medium (Dulbecco's Modified Eagle's Medium –DMEM- (Thermo) containing 10% Fetal Bovine Serum (FBS), 20U mL-1 penicillin, and 20 μg mL-1 streptomycin).
Resuspended cells were stained with Trypan Blue and counted in a Neubauer chamber. Cells were plated at different densities (from 200.000 to 500.000 cells/ml) in T150 flasks in full culture medium and maintained in an incubator at 37°C with an humidified atmosphere with 5% CO_2. Medium was changed after two hours to avoid excessive growth of microglia and oligodendrocytes. After this first change, flasks medium was replaced every 7 days.
Once reaching confluence (about ten days), flasks were agitated at 450 rpm in a mechanical shaker, to eliminate the microglia that grew on top of the astrocyte monolayer. Flasks were then washed twice with sterile Phosphate Buffered Saline (PBS) containing 136.87 mM NaCl, 2.5 mM KCl, 0.8 mM NaH_4PO_4 and 1.47 mM K_2HPO_4 at pH 7.4 supplemented with penicillin/streptomycin to remove remaining medium. After washing, 3mL of previously heated Trypsin/EDTA (Sigma) were added to the flask and maintained for 5 minutes at 37 °C in the incubator. Flasks were manually shaken to facilitate cells disattachment, and new full culture medium was added to stop the reaction. Medium containing cells in suspension was centrifuged at 250 g for 5 minutes and the pellet was resuspended and plated at different densities depending on the experiment to be performed (from 80.000 to 120.000 astrocytes/mL). Secondary astrocyte cultures were also maintained in the incubator at 37°C with an humidified atmosphere containing 5% CO_2.

1.1.2 Adult

For those experiments requiring adult cultures, we used 8-week-old Sprague-Dawley rats. Rats were decapitated and their cortices were isolated in a similar way than with neonatal cultures. Cortices were then incubated for 30 minutes at 37 °C in Hank's Balanced Salt Solution (HBSS) containing papain (2U/ml) and DNAse (10U/ml), mixing every 5 minutes. In the middle of the incubation (after 15 minutes), the mixture was mechanically triturated through a Pasteur pipette 5 times and the action was repeated after the whole 30 minutes. Once cells were disgregated, reaction was stopped by adding complete full DMEM medium (same volume as the enzyme mixture) and cells were filtered through a 225 μm nylon mesh. Isolated cells were centrifuged at 250 g for 5 minutes and plated into 24-well plates in DMEM/F12 medium (Thermo) supplemented with 10% FBS, 20U/ml penicillin and 20 μl/ml streptomycin. Plates were incubated at 37°C in 5% CO_2. Full DMEM medium was changed every two days and cultures were used after reaching confluence.

1.2 Mice

All mice used in these experiments were C57/Bl6 (WT and injected animals) or had a C57/Bl6 background (VP16-CREB model). Mouse colonies used in this study were maintained in standard conditions at the Animal Core facility of the Universitat Autònoma de Barcelona, with *ad libitum* food and water, 22+/- 2°C, 50-60% humidity and on a 12 h light/dark cycle. Littermates were housed together with a maximum of five mice per cage, separating males and females after weaning. Experimental procedures were conducted according to the animal experimentation protocols approved and supervised by the Animal Welfare Committee Bioethics Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya (protocol CEEAH 1783, Generalitat de Catalunya 6381).

Regarding *ex vivo* calcium imaging experiments, C57/Bl6 mice were maintained in the animal core facility of the David Geffen School of Medicine of the UCLA. All protocols realized were approved by the UCLA Chancellor's Animal Research Committee (ARC).

1.2.1 VP16-CREB animal model

Part of the *in vitro* CREB effects was confirmed in the VP16-CREB transgenic mice. This mouse model express VP16-CREB in astrocytes (Gfa2-tTa/TetO-VP16-CREB mice). The expression of the transgene is controlled by a TetOff system; however, the expression of the transgene was not inhibited in any of the experiments.

Cryolesions
Since VP16-CREB expression is under the control of the GFAP promoter (Gfa2), basal VP16-CREB expression is really low. In order to increase the transgene expression, GFAP expression was increased by subjecting the animals to cryolesion. For the cryolesion, five-to-seven month-old mice mice were anesthetized with isofluorane and cryolesioned by the application of a dry ice pellet for 30s onto the right frontoparietal

cortex. The protocol is described in (Giralt et al., 2002). Buprenorfin was injected intraperitoneally every 12h during the week after the cryolesion, and animals were sacrificed 1-3 days post-lesion (dpl).

2. Protein analysis

2.1 Protein extraction and quantification

Cultured astrocytes were washed twice on ice with cold PBS and lysed by scrapping in cold RIPA buffer containing 50 mM Tris base at pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% NP40, 0.5% Triton X-100, 0.1% SDS, 1 mM Na_3VO_4, 1 mM PMSF and 50 mM NaF, supplemented with protease (Roche) and phosphatase inhibitors (Sigma). Samples were sonicated for 5 seconds using the sonic dismembrator or for 2 minutes in the ultrasonic bath. After sonication, protein extract was centrifuged for 5 min at 4 °C and supernatant was kept at -20 °C.
Protein extracts were quantified using a BCA reaction kit (Pierce), based on a standard curve made with known albumin concentrations diluted in RIPA buffer.

2.2 Western Blot

Equal amounts of protein extract were diluted in loading buffer containing 62.5 mM Tris HCl pH 6.8, 10% glycerol, 2% SDS, 5% beta-mercaptoethanol and 0.01% bromophenol blue. Samples were then boiled at 95 °C for 5 min and loaded in 8-12% acrylamide polyacrilamyde (PAGE) gels. Electrophoresis was realized at 25 mA per gel in running buffer containing 24 mM TRIZMA base, 190 mM glycine and 10% SDS at pH 8.5.
After electrophoresis of the samples, proteins were transferred to methanol-activated polyvinyl difluoride (PVDF) membranes (Roche) at 100 mV for 90 minutes.
PVDF membranes were then incubated for 1h at room temperature (RT) in blocking buffer containing TBS (20 mM Tris, 137 mM NaCl at pH 7.6) plus 0.1% Tween and 5% skimmed milk or 5% BSA. After blocking, PVDF membranes were washed twice with 0.1% Tween-TBS and incubated overnight at 4 °C with the appropriate primary antibody shown in Table 1, diluted in blocking buffer.

Antibody	Host	Source	Reference	Applications		
				WB	ICC	IHC
Actin	Mouse	Sigma	A5316	x		
GAPDH	Mouse	Life Technologies	AM4300	x		
GFAP	Rabbit	Dako	Z0334		x	
GFAP	Mouse	Sigma	G3893			
GFP	Chicken	Abcam	Ab13970			x
MCT4	Rabbit	Millipore	AB3314P	x		
MCU	Rabbit	Abcam	Ab121499	x		
RFP	Rabbit	Rockland	900-401-379			x
SigmaR1	Rabbit	Invitrogen	42-3300	x		
VP16	Mouse	Santa Cruz	Sc-7545	x	x	

Table 1. Antibodies used in this thesis and their applications

Membranes were then washed three times (10 min/each) in 0.1% Tween-TBS buffer and incubated for 1h at RT with a secondary antibody coupled to the horseradish peroxidase (HRP) enzyme diluted in blocking solution. Finally, membranes were washed three times (10min/wash) in 0.1% Tween-TBS buffer and revealed by chemoluminiscence reaction using ECL substrate, either manufactured (ECL1: 5 mL 1 M Tris-HCL pH 8.5, 250 µL 0.5 M Luminol, 250 µL 79.2 mM p-Coumaric acid in 50 mL H2O. ECL2: 5 mL Tris-HCl pH 8.5, 32 µL 8.8 M Hidrogen Peroxide in 50 mL H2O) or commercial (Promega). Bands were revealed either by autoradiography on X-ray films (Agfa) or using the ChemiDoc imaging system (Bio-rad).

2.3 Immunocytofluorescence

Secondary astrocytes were plated on glass coverslips (13 mm diameter). Medium was removed and cultures were washed twice with PBS. Cells were fixed with 4% paraformaldehyde in PBS for 30 min at RT. Paraformaldehyde was removed and two more PBS washes were done (5 minutes each) before the permeabilization with 0.1% Triton X-100/PBS during 15 minutes. Then, cells were incubated with blocking solution (PBS containing 1% of Normal Goat Serum –NGS-) under agitation for 30 min to prevent non-specific-binding of the antibodies. After blocking, cells were incubated overnight at 4°C with the primary antibody (VP16 and GFAP) in 0.1% NGS/PBS. The following day, primary antibody was removed and astrocytes were washed three times (10 minutes each) with 0.1% NGS/PBS solution at RT. After washing, the cells were incubated for 1 h with the secondary antibodies (1:1000 Mouse and rabbit Alexa-labelled secondary antibodies, Thermo scientific) diluted in 0.1% NGS/PBS. Secondary antibodies were removed and the cells were washed three times in PBS (10 minutes per wash), and incubated for 5 minutes with the nuclear marker DAPI (1:20000) diluted in PBS. DAPI was washed and coverslips containing the cell monolayer were mounted with Fluoromont G (Southern biotech). The staining was visualized using an Eclipse 90i epifluorescence microscope (Nikon).

3. Molecular biology methods

3.1 DNA

3.1.1 Plasmid DNA amplification and purification

Plasmid DNA amplification was performed by mixing 20 µl of DH5α competent bacteria with 1 µg of the DNA plasmid of interest. The incubation was left for 30 min in ice and subjected to a thermal shock (2 minutes at 37 °C) and back to ice for 10 more minutes. After that time, 400 µl of LB medium (25 g LB in 1 l of water at pH 7.4) was added to transformed bacteria and left with stirring at 37 °C for 1 hour. 100 µl of the bacterial suspension were plated in sterile LB + antibiotic plaques (32 g agar in 500 mL LB medium plus antibiotic) and left overnight at 37 °C. The use of the antibiotic depends on the resistance conferred by the plasmid. Once grown, one colony was inoculated in a 15 ml falcon tube containing 3-5 ml of LB + antibiotic and left for 6 hours at 37 °C and under continuous stirring. Bacterial suspension was then added to a sterilized Erlenmeyer containing 250 ml of LB/antibiotic and left overnight in continuous stirring at 37 °C. Once LB media was turbid, bacteria was collected by centrifugation at 4° C and

3200 g for 30 min and the plasmid was purified using the commercial QAFilter Plasmid Maxi Kit (Qiagen). Purified plasmid was diluted in nuclease-free water and the concentration was measured with Qubit fluorometer (Thermo) or Nanodrop 1000 electrophotometer (Thermo).

3.1.2 Genomic DNA extraction and genotyping

To genotype our colony of VP16-CREB transgenic mice, two different mice were considered: the single transgenic mice containing the sequence Gfa2-Tta (considered the wild type mice) and the double transgenic containing both the Gfa2-Tta and the tetO-VP16-CREB trasngenes.

To genotype the colony, a small piece of mouse tail (1-2 mm) was incubated in 500 µl of digestion buffer containing 100 mM Tris HCl at pH 8.5, 200 mM NaCl, 5 mM EDTA, 0.2% SDS and 0.1 mg/ml proteinase K (Roche) and incubated overnight under stirring at 56 °C. The following day, samples were centrifuged at 1000 rpm for 5 minutes and supernatants were mixed with 0.5 ml of isopropanol to precipitate DNA. After a strong centrifugation (12000 rpm for 10 minutes), DNA pellet was washed with 70% ethanol and centrifuged again at same speed. Once precipitated and washed, DNA pellet was resuspended in 50-100 µl of TE buffer () and incubated at 65 °C for 2 hours with stirring.

After DNA extraction, 2 µl of DNA were PCR-amplified for genotyping. DNA was added to a final mixture containing 2.5 µl PCR buffer, 0.5 µl dNTPs, 0.5 µl $MgCl_2$, 0.2 µl of DNA Taq polymerase (5U/ml) (all reagents purchased in Biotools) and 0.5 µM of forward and reverse primers (Table 2). Samples were incubated for 2 minutes at 94 °C followed by 35 cycles of 45 seconds at 94 °C, 25 seconds at 60 °C and 3 minutes at 72 °C. A final step of 15 minutes at 72 °C was also performed.

Name	FWD sequence	RV sequence
Gfa2-Tta	5'-CGG CTG TACG CGG ACC CAC TTT-3'	5'-TCG ACG CCT TAG CCA TTG AGA T-3'
TetO-VP16	5'-AGC TCG TTT AGT GAA CCG TCA GAT-3'	5'-CCT CGC AGA CAG CGA ATT CTA-3'

Table 2. Primers used for the genotyping of the colony

Finally, 15 µl of PCR product was resolved on a 1.2% agarose gel (1.2 g agarose in 100 ml TAE buffer: 40 mM tris-acetate and 1 mM EDTA at pH 8.2) with 1% Sybr Safe (Thermo). DNA electrophoresis was realized at 110V until the dye line was at 75% of the way down the gel. DNA bands were detected in a UV transiluminator (Gene genius, Syngene).

3.2 RNA

3.2.1 RNA extraction and quantification

RNA extraction was made using TRIzol reagent (Thermo). Cells were washed twice with ice-cold PBS before cold TRIzol was added (1 ml for one well of a 6-well plate).pipetting up and down several times to lyse cells. The homogenate was then transferred to a tube and kept at -80 °C.

To purify RNA, TRIzol samples were incubated at RT for 5 minutes and 0.2 ml of chloroform for every 1 mL of TRIzol were added to the tubes. Tubes were agitated manually and centrifuged at 4°C and 12000 g for 15 minutes. After centrifugation, the combination of TRIzol and chloroform separates samples in three layers. The upper layer containing the RNA was collected, mixed with 0.5 mL isopropanol and incubated for 10 minutes on ice. Afterwards, samples were centrifuged for 10 minutes at 12000 g and 4 °C, forming a RNA pellet. The RNA pellet was then washed with 1 ml of ice-cold 75% ethanol and centrifuged again for 5min at 7500 g and 4°C. Once washed, the maximum possible volume of ethanol (without affecting the RNA pellet) was retired and RNA pellet was air dried for about 1 hour. Pellets were resuspended with 30 μl TE at 55 °C for 10 minutes. After extraction, purified RNA was quantified using Nanodrop 1000 or Qubit fluorometer, and kept at -80 °C.

3.2.2 RNA reverse transcription

To reverse transcribe mRNA to cDNA, a reaction mix containing 1 μM Oligo(dT) primers, 1 μM random hexamer primers, 0.5 mM dNTPs, 1x RT buffer, 0.45 mM DTT, 10 U RNAse out and 200 U SuperScript reverse transcriptase (All purchased from Thermo) was added to purified RNA (from 0.5 to 2 μg).

A first mix containing dNTPs, primers and RNA was heated to 65 °C for 10 minutes before adding the rest of the reagents. After this first step, samples were incubated at 25 °C for 10 minutes, 42 °C for an hour and 72°C for 10 minutes to allow reverse transcription in the thermal cycler. Resultant cDNA was kept at -20 °C.

3.2.3 Quantitative PCR

To analyze mRNA expression, reverse transcribed cDNA was diluted (from 1:5 to 1:500) to obtain a detectable range of expression (Ct between 15 and 30). 4 μl of the diluted cDNA were mixed with 1 μl of Taqman primer (Table 3), 5μl of nuclease-free water and 10μl of TaqMan Universal Master Mix (all of them purchased from Thermo). Samples were analyzed in triplicate and were amplified in an 7500 Fast system (Applied biosystems). Results were analyzed with the comparative Cq method (Pfaffl, 2001), using Cq values and PCR efficiencies obtained using LinReg software.

Gene	Model	Reference
18s	Mouse	Mm03928990_g1
Actin	Rat	Rn00667869_m1
GAPDH	Rat	Rn01476455_m1
Hif1a	Rat	Rn01472831_m1
MCU	Rat	Rn01433739_m1
SigmaR1	Rat	Rn00578590_m1
SigmaR1	Mouse	Mm00448086_m1
SLC16A3	Rat	Rn00578115_m1
SLC16A3	Mouse	Mm00446102_m1
Tbp	Mouse	Mm00446973_m1
Tbp	Rat	Rn01455646_m1

Table 3. Taqman primers used for quantitative PCR.

4. Cellular biology methods
   4.1 Transfection
      4.1.1 DNA

Luciferase and renilla plasmids (see below) were transfected using Fugene 9 (Life sciences). In 24-well plates, 1 µl Fugene 9 and 1 or 0.5 µg of the plasmids were incubated in 0% FBS 0% antibiotics DMEM for 3-4 hours. After that time, medium was replaced by full DMEM.
CEPIA plasmids (see below) were transfected using Amaxa nucleofector (Lonza). Primary astrocytes were trypsinized and counted in the Neubauer chamber. 4 millions astrocytes were separated and centrifuged for 4 minutes at 1000 rpm. Astrocyte pellet was resuspended in 100 µl of sterile KRB containing 5 µg of the plasmid of interest. The mix was then transferred to the nucleofection cuvette and submitted to nucleofection (program O-017). Immediately after nucleofection, 900 µl of full medium was added to the cuvette and transfected cells were seeded in new plates.

      4.1.2 siRNA

HIF1α siRNAs were transfected using LipofectamineRNAiMax (Thermo). For 6-well plates, 3 µl/well and 1 µg of the siHIF1α or the siScramble were added to secondary astrocytes in 0% FBS 0% antibodies DMEM and maintained for 3-4 hours. After that time, medium was changed to full DMEM.
HIF1α and siScramble siRNAs were purchased from Thermo.

   4.2 Viral infection

To modify CREB activation, we generated three specific serotype 5 adenoviruses (Table 4). Infections were realized when cells were nearly confluent (70-80%) and in the presence of 1% FBS-DMEM without antibiotics. Different multiplicity of infection (MOI) was used, from 1 to 100. Medium with viral vectors was replaced after 3 h for full DMEM. Experiments were realized after 18 -24 h to allow viral expression.

VP16-CREB	Ad5-VP16CREB	CBATEG-475 own design
NULL	Ad5-Null	CBATEG-638 own design
A-CREB	Ad5-ACREB	CBATEG-471 own design

Table 4. Viral vectors used in cultures.

4.3 Luciferase assays

To detect CREB-dependent transcription, we used the Dual-Luciferase reporter assay (Promega). This assay combines two differnt plasmids in order to assess CREB activation and transfection efficiency. The plasmid pCRE-Luc codifies for the protein luciferin under the control of a promoter containing four CRE sequences. Thus, luciferase expression depends on CREB activation. At the same time, cells were transfected with plasmid pTK-renilla as a control of transfection. Astrocytes were transfected with 1 μg pCRE-Luc and 0.5 μg pTK-Renilla as described above. 48 h after, 25 μl of the cell lysate were mixed with 25 μl of luciferine substrate LAR-II and luminiscensce was measured in a white 96-well plate in a Synergy HT luminometer (Bio-Tek). After the measure, 25 μl of the Stop & Glo reactive, which stops luciferine reaction and contains renilla substrate, were added to detect renilla expression. Results were expressed as the ratio between luciferase and renilla luminescence.

4.4 Intracellular calcium measurements

4.4.1 Cytosolic measurements

For Fluo-4AM measurements, astrocytes were incubated at RT in the dark with KRB containing 10 mM glucose and 4 μM Fluo-4 and 0.02% Pluronic F-127 (both purchased from Thermo). After 1 hour, KRB was replaced for new KRB and responses were measured with a Victor III fluorometer (Perkin Elmer) or a Fluorescence Microscopy System that consist of a fluorescence microscope (Eclipse TE2000-E, Nikon), and a CCD camara (ORCA-ER, Hamamatsu). Emission was established as 520-550nm and excitation at 466-496 nm. Movies were analyzed using Metamorph image processing software (Molecular devices). Background was established with a reference number obtained form empty zones. Each cell was tracked individually and a mean of 15 cells were tracked in each condition.

For ratiometric measurements using Fura-2, cells were incubated with 2 μM of the reagent (Thermo) with BSA in KRB at RT in the dark for 1h. After labeling, media was changed for new KRB and coverslips were mounted in a static imaging chamber. Images were taken by a Calcium Imaging System with an inverted epifluorescence Eclipse TE-2000U microscope (Nikon), a ORCA-ER camera (Hamamatsu), a monochromator (OptoScan, Cairn Research) controlled by the Metafluor Software (Molecular Devices). Cells were excited at 340 nm and 380 nm and emission was detected at 495-520 nm. Same numbers of cells per coverslip were analyzed in each condition.

4.4.2 ER and mitochondrial calcium measurements

ER-calcium release

To analyze ER-calcium release, we used one plasmid encoding for a calcium-measuring organelle-entrapped protein indicator (CEPIA), gift from Masamitsu Lino and available in the Addgene database (pCMV G-CEPIA-1ER, Addgene plasmid # 58215). Two to three days after plasmid nucleofection, astrocytes seeded on glass coverslips were mounted in a static chamber with KRB in the epifluorescence Eclipse TE2000-U microscope (Nikon). The cells were excited at 476 nm and the fluorescence emission was detected at 510-560 nm. Images were analysed using the MetaFluor software (Molecular devices). Between 2 and 6 transfected cells were analysed from each coverslip, with five coverslips per condition in each experiment. F/F_0 was obtained by subtracting fluorescence variations at every time-point after neurotransmitter stimulation.

Mitochondrial calcium entry

For mitochondrial calcium entry measurements, we used two different approaches, using a genetically-encoded CEPIA and an organic calcium dye that gets entrapped into the mitochondria, Rhod-2AM (Thermo).

For CEPIA measurements, we used the plasmid (pCMV CEPIA-3mt, Addgene plasmid # 58219), which detected calcium variations in the desired calcium concentration range. We tried the other two mitochondrial calcium CEPIA indicators (pCMV CEPIA-2mt and pCMV CEPIA-4mt), however, they were outside the range of mitochondrial calcium concentrations in astrocytes. We used the Calcium imaging system described above.
In an alternative set of experiments, astrocytes were loaded with the organic calcium dye rhod-2AM at 10 μM for 5 minutes at RT. After 5 minutes, Rhod-2AM was specifically detecting mitochondrial calcium, since longer times of incubation led to cytosolic contamination of the indicator. Cells were excited at 552 nm and fluorescence emission was recorded at 570-640 nm. Up to 60 single mitochondria were selected for each experiment, determining the mean of calcium increases in each condition.

4.5 Lactate release measurement

To measure extracellular lactate accumulation, we used a commercial kit purchased from Sigma (reference MAK064). The full DMEM medium of secondary astrocytes seeded in 24-well plates was replaced for 200μl of KRB containing 10 mM glucose and cells were kept for 2 h in the incubator. Moreover, oligomycin (1 μg/ml) (Sigma) was added to the KRB in some conditions to increase lactate production. After 2 h, 100 μl of the KRB containing the lactate was collected and stored at -80 °C or processed to lactate detection.
As indicated by manufacturer, dilutions of a lactate standard were prepared in KRB in order to generate a lactate standard curve. 10 μl of the samples (containing the astrocyte-released lactate) were diluted in 40 μl assay buffer and plated into non-treated 96-well plates. A 50 μl mixture of the buffer, the enzyme and the lactate probe

were added to the standard curve and the samples and colorimetric reaction was detected using the Synergy HT plate reader (BioTek).

4.6 Determination of O_2 consumption and medium acidification

To study metabolic activity of the cell, we used the Seahorse XF analyzer (Agilent), which allows the coordinated real-time detection of extracellular medium acidification (-ECAR- which corresponds to the glycolytic activity and lactate release of the cell) and oxygen consumption rate (-OCR- as a measure of the mitochondrial oxidative metabolism).
In our experiment, cortical rat secondary astrocytes were seeded in XF 24-well cell culture microplates (Agilent) and grown until 70-80% confluence. Some wells of the outside of the plate were left empty since previous experience showed too much medium evaporation. After 18-24h of viral infection with VP16-CREB and null Ad5 at MOI25, astrocytes were incubated with XF Assay Medium (Agilent) supplemented with 2 mM L-Glutamine, 5.5 mM glucose and 1 mM sodium pyruvate for 30 min at 37 °C in the absence of CO_2 to reach equilibrium before the first measurement.
Injection ports on the sensor cartridge were loaded with oligomycin (1 μg/ml), FCCP (2 mM) (two ports) and 0.4 mM rotenone plus 1 mM antimycin A, all of them purchased from Sigma. During 3 hours, seahorse XF analyzer made four measures of OCR and ECAR after adding each component. The instrument was calibrated the day before the experiment following manufacturer's instructions.
Means of the four OCR and ECAR values were done to analyze the effect of each component except for the case of FCCP, in which only the four values after the first injection were taken into account. Values were normalized to the protein content of each well detected using BCA.

4.7 Fatty acid oxidation measurement

Beta-oxidation measurements were done using [^{3}H]palmitic acid (Perkin Elmer). The assay consisted in the measurement of extracellular [^{3}H]H_2O, assuming that the mitochondrial oxidation of this fatty acid gives H_2O as an end-product. Astrocytes were labeled overnight with 1 μCi/ml of [^{3}H]palmitic acid, giving a final concentration of 10-30 nM. [^{3}H]Palmitic acid was dissolved in full DMEM culture medium containing 0.5% delipidated BSA (Sigma), to promote the fatty acid dissolution. The day after, labeled astrocytes were washed once with 0.5% delipidated BSA-KRB and twice with KRB in order to wash remaining [^{3}H]palmitic acid of the medium. Astrocytes were then incubated with 0.5 ml of fresh KRB containing different glucose concentrations (Sigma). Each glucose concentration and time-point was done in triplicate. In order to have a control of oxidation, two wells of each condition were treated also with 30 μM etomoxir (Sigma), an inhibitor of the carnitine palmitoyl transferase I and therefore of the beta-oxidation. When appropriate, three hours after the start of the experiment, 100 μM glutamic acid (Sigma) were added.
For each time-point, 400 μl of KRB medium were transferred to tubes containing 1 ml of a 1:2 slurry of Dowey 1 x 2 (chloride form) anionic exchange resin (Sigma) in H_2O. This resin chelated other radioactive metabolic intermediates other than [^{3}H]H_2O. Tubes

with the resin and the KRB medium were vortexed and centrifuged for 2 min at 1,000 rpm. After centrifugation, 100 μl of the supernatant (and containing the resulting $[^3H]H_2O$) were transferred to 6 ml scintillation tubes. 3ml of Emulsifier-Safe scintillation cocktail (Perkin Elmer) were added to the tubes and radioactivity was counted using a Scintillation Counter TRI-CARB 2810TR (Perkin Elmer). Radioactivity of each tube was measured for one minute.

4.8 Induction of hypoxia

Hypoxia conditions were established in a temperature controlled Invivo$_2$ hypoxia workstation (Baker Ruskinn). Workstation was kept at 37°C and with a beaker filled with deionized sterile water inside to maintain humidity.
Secondary astrocytes were seeded in 6-well plates and kept in an oxygenated incubator with full DMEM medium until reaching confluence. The day of the experiment, hypoxic workstation was set up at 0.5% O_2, 5% CO_2 and 94% N_2. Once the workstation reached the gas equilibrium desired, plaques were placed inside. Since after opening of the workstation some gas mixture with outside the chamber is produced, and taking into account that medium deoxygenation requires some time, the experiment started when levels inside the chamber reached the desired values. After hypoxic experiments, plaques were rinsed quickly with ice-cold PBS and processed for RNA extraction.

5. In vivo calcium imaging

5.1 Stereotaxic surgeries

To inject viral vectors in the mouse hippocampus, borosilicate glass pipettes were used. The pipette was beveled at an angle of 40 ° using a pipette grinder. The pipette was autoclaved in a dry bath for 30 s and placed in a glass syringe connected to a liquid pump. All the pump system was filled with mineral oil colored with Sudan red (1 mg/50 ml) using a 1 ml syringe, until the oil filled the entire pipette. This way we removed all the air bubbles of the system.

A piece of clean parafilm containing a 5-10 μl of the viral vector (conserved in small aliquots at -80 °C and stored on ice before the injection) was placed under the glass pipette. The plunger of the syringe was then moved backwards at 0.5 μl/min until the vector filled the syringe. The boundary in the glass pipette between the vector and the oil was marked using a marking pen.

Once the pipette was filled with the vector, 6-week old C57/Bl6 mice were anesthetized in a chamber filled with N_2O and O_2 and 5% isofluorane. After reduction of the respiratory rate, hair of the head was removed using a shaver. One shaved, mice were subjected in the stereotaxic frame, with the head secured by blunt ear bars and the nose placed into a ventilator and anaesthesia system using continuous isofluorane (5% induction, maintenance at 1-2.5%). Artificial tears ointment was applied in both eyes before the surgery.

Before surgery, 0.05 ml Buprenorphin (0.1mg/ml) was injected subcutaneously and the

area of surgery was cleaned three times with 10% povidone iodine and 70% ethanol using cotton swabs. With all surgery utensils previously autoclaved in the dry bath, a skin incision was made between the eyes to have enough space to drill the skull. Periosteum was removed using a cotton swab and the injection site was drilled using a small steel burr powered by a high-speed drill. Once the area of the skull was thin, the bone was removed, leaving the area of the brain to be injected accessible.

Coordinates of the hippocampal viral injections were 2mm posterior to Bregma, 1.5mm lateral to midline and 1.6 from the pial surface, always on the left hemisphere. Once inside the hippocampus, viral vectors (Table 5) were injected at a speed of 0.2 μl/minute, injecting from 1 to 2 μl. After injection, pipette was left for 10 minutes and was retired slowly. Surgical wound was closed with single external 5-0 nylon sutures.

Buprenorphin was administered two times per day for two days after surgery. Mice were feed with antibiotic-containing food (trimethoprim sulfamethoxazole) for 1 week.

Name	**Serotype**	**Source**
gfaABC$_1$D-cyto-GCamp6f	AAV2/5	Baljit Khakh's lab
gfaABC$_1$D-Null	AAV9	Own design
gfaABC$_1$D-VP16-CREB	AAV9	Own design
gfaABC$_1$D-A-CREB	AAV9	Own design

Table 5. Vial vectors used for stereotaxic injections. Own-designed AAV9 expressed the protein TdTomato to identify infected astrocytes.

5.2 Brain dissection

Two to three weeks after injections, mice brains were sliced to do *ex vivo* calcium experiments. Mice were deeply and terminally anesthetized and decapitated. Brain was extracted from the head and the uninjected right hemisphere was removed using a blade. All these processes needed to be fast in order to maintain brain integrity before the experiments. Left hemisphere was then mounted onto the vibratome tray using super glue. The vibratome tray was kept in ice and all the process of slicing was done under ice-cold conditions. Tray with the mounted brain was filled with ice-cold cutting solution containing 194 mM sucrose, 30 mM NaCl, 26 mM NaHCO3, 4.5 mM KCl, 10 mM D-glucose, 1 mM $MgCl_2$, 1.2 mM NaH_2PO_4 and saturated with 95% O_2 and 5% CO_2. New cutting solution was added to maintain O_2 and CO_2 saturation during the cutting process. Brains were sliced coronally at 300 μm thickness and slices were transferred to a slice holding beaker warmed at 34 °C. This beaker contained recording solution: 124 mM NaCl, 26 mM $NaHCO_3$ mM 4.5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 2 mM $CaCl_2$, 1.2 mM NaH_2PO_4 pH 7.3 - 7.4, saturated with 95% O_2 and 5% CO_2. Slices were kept in recording solution at 34 °C under constant gas saturation for 30 minutes, then were maintained for 30 more minutes at RT before starting the experiment.

5.3 Confocal calcium imaging

Between 1 to 5 hours after slicing, a brain slice was placed in the recording chamber

filled with oxygenated recording buffer at RT. The medium was permanently refreshed using a liquid pump at a speed of 1 to 2 ml per minute. It is important to measure the time that liquid lasts to reach the recording chamber in order to know exactly when the different buffers reach the bath. A platinum harp was placed on top of the slice to avoid movement during the experiment.
Different buffers were prepared: Recording buffer containing 0.5 μM Tetrodotoxin – TTX- (Cayman), to measure spontaneous activity and recovery time after ATP and phenylephrine (PE) responses; ATP buffer, consisting of the same recording buffer plus TTX and 300 μM ATP (Tocris) and PE buffer, the same than the previous but containing 10 μM phenylephrine (Tocris) instead of ATP. All buffers have to be permanently saturated with oxygen.
Once the slice was placed, the region of interest was focused using a 40X water immersion objective lens with an aperture of 0.8. An area containing two or more astrocytes, whose GCamp6f fluorescence was not saturated and that expressed TdTomato was chosen for all the experiments. Importantly, this area was about 20 μm below the slice surface to avoid compromised astrocytes.
Once the ideal area was found, digital magnification of 2-3 folds was used and a pilot recording to detect saturation, slice drift or photobleaching was done. If any of the aforementioned problems appeared, argon laser intensity and harp were adapted before starting the experiment.
After this first check, if everything was correct, the imaging experiment was done. Frames were recorded every second for 900s to detect fast responses. During the protocol, liquid pump was filled with the different recording buffers in the indicated times to see the different responses to reagents.

5.4 Image analysis

After the imaging experiment, frames were analyzed using ImageJ software (NIH). Regions of interest (ROIs) containing the entire soma or all the primary branches were detected using a maximal projection of all frames. Somatic fluorescence was extracted of primary branches ROIs in order to analyze them separately. The same ROIs were maintained for the entire time course. Background was extracted using a ROI in a place were no astrocytes were found. If any XY drift was detected during the experiment, images were readjusted using ImageJ program.
Time traces of fluorescence intensity were extracted from ROIs and translated to dF/F. Half width, frequency and AUC were analyzed using Graphpad 6 Prism software after extracting values from ImageJ.

6. Histological methods

6.1 Perfusion, cryogenesis and brain sectioning

To characterize the *in vivo* expression of the newly generated viral vectors, two weeks after AAVs injection mice were euthanized with pentobarbital (i.p.) and the abdominal cavity was opened. 50 U heparin was injected into the heart to avoid blood clotting. Mice were then transcardially perfused with 20 mL PBS followed by 60 mL of PBS containing 10% formalin (Fisher).

Brain was dissected out and kept in 10% formalin PBS overnight at 4 °C. The following day, brain tissue was cryoprotected in 30% sucrose PBS solution for 48 h at 4 °C.
After sucrose, coronal 40 μm brain sections were done using a cryostat microtome (Leica), and resulting slices were processed for immunohistochemistry.

6.2 Immunohistochemistry

Sections were rinsed three times in PBS for 10 min each and incubated in blocking solution containing 10% NGS in 0.5% Triton X-100-PBS for 1h at RT with gentle agitation. After blocking, sections were incubated with primary antibodies chicken anti-GFP (1:1000, Abcam) and Rabbit anti-RFP (1:200, Rockland) diluted in 0.5% Triton X-100-PBS overnight at 4 °C. The following day, sections were washed three times in PBS at 10 min intervals and slices were incubated with secondary antibodies goat anti-chicken Alexa 488 (1:1000, Molecular probes) and goat anti rabbit Alexa 546 (1:1000, Molecular probes) diluted in PBS for 2 hours at RT with gentle agitation. Slices were rinsed 3 times for 10 min each in PBS before being mounted on microscope slides in fluoromont-G.
Images were taken using 20X and 40X oil immersion objective lens on a confocal laser-scanning microscope (Fluoview 1000; Olympus). Sections were excited by 488 nm and 543 nm Argon lasers and the conditions were maintained in all sections. Montages of the whole hippocampus were done by joining different images obtained at 20X using the software of the confocal microscope. Images represent maximum projection intensity of 20 sections with a step size of 1 mm.
After acquisition, images were analyzed using ImageJ software. Colocalization was counted on maximum projection images. 6 different areas of the hippocampus were analyzed to establish colocalization values.

7. Transcriptomic analyses

7.1 Databases

Nuclear DNA-genes codifying for mitochondrial proteins were extracted from the MitoCarta database (Pagliarini et al., 2008). All genes from this database were manually classified into different categories and subcategories, in order to evaluate gene expression of the different mitochondrial functions.

Five different databases from mouse brain transcriptome (Doyle et al., 2008) (Lovatt et al., 2007) (Zhang et al., 2014), one from mouse spinal cord (Anderson et al., 2016) and one from human brain transcriptome (Zhang et al., 2016) were filtered for those genes classified in MitoCarta. The function alias2Symbol of the Bioconductor package for R was used to homogenize names of all different genes between different databases and models. Moreover, gene lists of the different organelles were extracted from the "Cellular Component" ontology on the GO terms using AmiGO 2.0 software.

7.2 Gene Set Enrichment analyses (GSEAs)

To compare the expression of the different mitochondrial functional groups, we used GSEA. GSEA is a computational method that compares two different gene-expression datasets and reveals which genes or groups of genes are enriched in each condition. In our case, the groups of genes were defined by the functional classification of the MitoCarta database, while the different conditions were established in each analysis (e.g. astrocytes vs. neurons or adult vs. fetal astrocytes).
Two different GSEAs were used in order to classify the transcriptomic expression for each database of the genes found in the MitoCarta. Standard GSEA was performed using the software from the Massachusetts Institute of Technology (Subramanian et al., 2005) with R programs when more than two duplicates were found. GSEA analysis gave the enrichment of each group (measured with the Normalized enrichment score –NES-) and the significance of the enrichment (False Discovery Rate –FDR-).
To analyze gene set enrichment when no more than two replicates were published, such as in Lovatt's transcriptomes and Cahoy's culture databases, we designed an in-house GSEA that uses the proportion of genes with a log2FC greater than 2 or lower than -2 in each comparison. This way, we obtained percentages of significantly overexpressed or underexpressed genes in each gene set. Comparison of this percentage with the global proportion of log2FC <2 or >-2 genes in the whole database using an exact binomial test gave us the significance of the differences of each group.

8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software and the statistical functions included in R programming language. In cultures, a minimum of three independent experiments was done for each condition, considering independent experiments astrocytes from a different mouse or pool of mice. In mice, each mouse is considered an independent entity. Number of independent experiments in cultures or the number of mice analysed is indicated in the figure legend of each result. Data are expressed as the mean ± standard error of the mean (SEM). Two datasets were compared using student's *t* test, and multiple groups were compared using analysis of variance (ANOVA). Post hoc test used were Dunnet, Sidack's and Turkey's multiple comparison test. For standard GSEA analysis, significances were obtained from the FDR, whereas in the in-house GSEA, statistical significance was obtained using exact binomial test. Differences with p value < 0.05 were considered significant except for the transcriptome analysis, in which significance was considered for $p < 0.01$.

References

Anderson, M.A., Burda, J.E., Ren, Y., Ao, Y., O'Shea, T.M., Kawaguchi, R., Coppola, G., Khakh, B.S., Deming, T.J., and Sofroniew, M.V. (2016). Astrocyte scar formation aids central nervous system axon regeneration. Nature *532*, 195-200.

Doyle, J.P., Dougherty, J.D., Heiman, M., Schmidt, E.F., Stevens, T.R., Ma, G., Bupp, S., Shrestha, P., Shah, R.D., Doughty, M.L.*, et al.* (2008). Application of a translational profiling approach for the comparative analysis of CNS cell types. Cell *135*, 749-762.

Giralt, M., Penkowa, M., Lago, N., Molinero, A., and Hidalgo, J. (2002). Metallothionein-1+2 protect the CNS after a focal brain injury. Exp Neurol *173*, 114-128.

Lovatt, D., Sonnewald, U., Waagepetersen, H.S., Schousboe, A., He, W., Lin, J.H., Han, X., Takano, T., Wang, S., Sim, F.J.*, et al.* (2007). The transcriptome and metabolic gene signature of protoplasmic astrocytes in the adult murine cortex. The Journal of neuroscience : the official journal of the Society for Neuroscience *27*, 12255-12266.

Pagliarini, D.J., Calvo, S.E., Chang, B., Sheth, S.A., Vafai, S.B., Ong, S.E., Walford, G.A., Sugiana, C., Boneh, A., Chen, W.K.*, et al.* (2008). A mitochondrial protein compendium elucidates complex I disease biology. Cell *134*, 112-123.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res *29*, e45.

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S.*, et al.* (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A *102*, 15545-15550.

Zhang, Y., Chen, K., Sloan, S.A., Bennett, M.L., Scholze, A.R., O'Keeffe, S., Phatnani, H.P., Guarnieri, P., Caneda, C., Ruderisch, N.*, et al.* (2014). An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. The Journal of neuroscience : the official journal of the Society for Neuroscience *34*, 11929-11947.

Zhang, Y., Sloan, S.A., Clarke, L.E., Caneda, C., Plaza, C.A., Blumenthal, P.D., Vogel, H., Steinberg, G.K., Edwards, M.S., Li, G.*, et al.* (2016). Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse. Neuron *89*, 37-53.

RESULTS

Chapter 1:

CREB decreases astrocytic excitability by modifying subcellular calcium fluxes via the sigma-1 receptor

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Cellular and Molecular Life Sciences

ORIGINAL ARTICLE

CREB decreases astrocytic excitability by modifying subcellular calcium fluxes via the sigma-1 receptor

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Abstract Astrocytic excitability relies on cytosolic calcium increases as a key mechanism, whereby astrocytes contribute to synaptic transmission and hence learning and memory. While it is a cornerstone of neurosciences that experiences are remembered, because transmitters activate gene expression in neurons, long-term adaptive astrocyte plasticity has not been described. Here, we investigated whether the transcription factor CREB mediates adaptive plasticity-like phenomena in astrocytes. We found that activation of CREB-dependent transcription reduced the calcium responses induced by ATP, noradrenaline, or endothelin-1. As to the mechanism, expression of VP16-CREB, a constitutively active CREB mutant, had no effect on basal cytosolic calcium levels, extracellular calcium entry, or calcium mobilization from lysosomal-related acidic stores. Rather, VP16-CREB upregulated sigma-1 receptor expression thereby increasing the release of calcium from the endoplasmic reticulum and its uptake by mitochondria. Sigma-1 receptor was also upregulated in vivo upon VP16-CREB expression in astrocytes. We conclude that CREB decreases astrocyte responsiveness by increasing calcium signalling at the endoplasmic reticulum–mitochondria interface, which might be an astrocyte-based form of long-term depression.

Keywords Calcium signalling · Endoplasmic reticulum · Mitochondria · Mitochondria-associated membranes · MCU · VP16-CREB · CEPIA indicators

Abbreviations

ER	Endoplasmic reticulum
IP_3	Inositol 1,4,5-trisphosphate
CRE	cAMP regulatory element
CREB	cAMP regulatory element-binding protein
LTD	Long-term depression
NA	Noradrenaline
PKA	Protein-kinase A
PKC	Protein-kinase C
MSK1	Mitogen- and stress-activated protein kinase
RSK2	Ribosomal protein S6 kinase
FBS	Fetal bovine serum
ET-1	Endothelin-1
Glu	Glutamate
FCCP	Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
GPN	Gly-phe-β-naphthylamide
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
CEPIA	Calcium-measuring organelle-entrapped protein indicators
PBS	Phosphate-buffered saline
NGS	Normal goat serum

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GPCR	Gq protein-coupled receptors
MCU	Mitochondrial calcium uniporter
NAADP	Nicotinic acid adenine dinucleotide phosphate
MAM	Mitochondria-associated ER membranes
WT	Wild-type

Introduction

Calcium signals encode a wide range of physiological responses thanks to a diverse and extensive range of signalling components that can be activated upon receptor stimulation. In astrocytes, cytosolic calcium increases are largely the result of intracellular calcium mobilization due to activation of phospholipase C-β, with the consequent generation of inositol 1,4,5-trisphosphate (IP_3) and the activation of IP_3 receptors in the endoplasmic reticulum (ER). Calcium release from lysosome-related acidic vesicles through the generation of the second messenger nicotinic acid adenine dinucleotide phosphate (NAADP) [1, 2] and extracellular calcium entry also contribute to the final signature of each agonist-induced calcium response in astrocytes [3]. Mitochondrial calcium uptake, which is coupled to IP_3-induced calcium release [4], further shapes cytosolic calcium increases.

The notion that calcium signals are a physiologically relevant sign of responsiveness in astrocytes has been demonstrated both in vitro and in vivo [5], leading to the widely accepted notion that astrocytes display an action-potential independent but calcium-dependent "excitability". This feature allows them to couple neuronal activity to blood flow, maintain the blood–brain barrier, control ion, pH and transmitter homeostasis, provide metabolic support for neurons, and regulate synaptic transmission and memory-related processes [5–9]. It follows that astrocytic calcium signals may be tightly regulated, such that their amplitude may be strengthened or diminished by neuronal or astrocytic activity.

CREB belongs to a large group of transcription factors of the basic leucine zipper domain family and binds to the cAMP regulatory element (CRE) of target genes. In neurons, CREB is a key regulator of synaptic plasticity in various forms, including long-term potentiation (LTP), intrinsic plasticity, and, in some cases, long-term depression (LTD) [10–12], all of which manifest as changes in the threshold, amplitude, or frequency of action potentials, thus changing synaptic strength. CREB is activated by phosphorylation by a variety of kinases, including PKA—the best characterised one—PKC, mitogen- and stress-activated protein kinase (MSK1), and ribosomal protein S6 kinase (RSK2) [13–15]. Moreover, in neurons, CREB can be synergistically activated by increases in intracellular calcium followed by CAMKII activation and by cofactors, such as CTRC [16]. We recently described that CREB is also expressed in astrocytes where it can be activated by neurotransmitters in a calcium-independent mechanism [13].

In this study, we posit that transmitter-dependent long-term changes in cell responsiveness requiring gene expression are not an exclusive property of neurons. To address this hypothesis, we examined whether CREB regulates transmitter-induced calcium responses in astrocytes given the importance of calcium signalling in these cells. CREB-dependent transcription was activated with ATP or noradrenaline (NA) or by expression of VP16-CREB, a constitutive active form of CREB widely used to study CREB-induced LTP and intrinsic plasticity in neurons [17]. Calcium responses were elicited thereafter with different transmitters, including ATP, which has a prominent role in astrocyte physiology. CEPIA calcium dyes targeting subcellular compartments, pharmacological manipulation of signalling pathways, and calcium imaging of single cells were used to dissect out organellar and molecular mechanisms. We found a novel link between CREB and calcium signals, mediated by the sigma-1 receptor at the mitochondria-associated membranes.

Materials and methods

Astrocyte culture

Astrocyte cultures were prepared from 1-day-old Sprague–Dawley rats following a previously described protocol [3]. Cells were plated in T150 flasks in DMEM medium supplemented with 10 % fetal bovine serum (FBS), 20 U/ml penicillin, and 20 µg/ml streptomycin, and incubated at 37 °C in a humidified atmosphere containing 5 % CO_2. After reaching confluence, microglia were removed with mechanical shaking, and pure secondary astrocytes were seeded on new plates in the same medium.

Adult astrocyte cultures

Adult astrocyte cultures were prepared from 8-week-old Sprague–Dawley rats. In brief, rats were sacrified and cerebral cortices isolated. After removing meninges, cortices were incubated with papain (2U/ml) and DNase (10 U/ml) for 15 min at 37 °C. Cell suspension was gentle triturated through a Pasteur pipette and filtered through a 22 µM nylon mesh. Cells were collected by centrifugation at 500 g and plated into 24-well plates in DMEM/F12 medium supplemented with 10 % FBS, 20 U/ml penicillin, and 20 µg/ml streptomycin, and incubated at 37 °C in a humidified atmosphere containing 5 % CO_2. The medium

was replaced every 2 days and the cultures were used at 8–15 days.

Animal model

Transgenic mice with targeted expression of VP16-CREB in astrocytes conditional to gliosis (Gfa2-tTa/TetO-VP16-CREB mice) [18] were used. They were kept under standard laboratory conditions (food and water ad libitum, 22 ± 2 °C, a 12 h dark/light cycle, and 50–60 % humidity and no administration of doxycycline). All experimental procedures were approved by the Animal Welfare Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya, and were in agreement with the European Union Laws for the protection of experimental animals.

Activation of CREB-dependent transcription

Activation in cultures with transmitters

We used ATP (adenosine 5′-triphosphate magnesium salt), L-(-)-norepinephrine bitartrate (NA), endothelin-1 (ET-1), or an L-glutamic acid monosodium salt (Glu), all of them purchased from Sigma Aldrich.

Activation and inhibition in cultures with CREB constructs

We used three adenoviruses of serotype 5 harboring a constitutively active CREB (Ad5-VP16-CREB), a dominant negative CREB (Ad5-A-CREB), or no CREB construct (Ad5-Null). The expression of the transgenes was mediated by the CMV promoter. Infections were carried out in DMEM with 1 % FBS using 1, 5, 30, and 100 multiplicity of infection (MOI). After 3 h, the medium was replaced by DMEM with 10 % of FBS. All experiments were performed within 18–24 h after viral infections. No cellular death was detected up to 24 h after viral infections, as measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction and Hoechst assays (data not shown).

Activation in vivo

To induce expression of VP16-CREB in transgenic mice, we followed a protocol to induce gliosis. Five-seven month-old mice under tribromoethanol anesthesia were cryolesioned with a dry ice pellet following a previously described protocol [18]. Buprenorfin at 0.1 mg/kg was injected i.p. every 12 h until its sacrifice at 1–3 days after the cryolesion. Female and male mice were equally distributed among experimental groups.

Detection of CREB-dependent transcription by luciferase

Astrocytes were transfected with 1 μg of plasmid pCRE-Luc and 0.5 μg of plasmid pTK-Renilla in 1 % FBS-containing DMEM using fugene 9 (Life Sciences). The plasmid pCRE-Luc (Promega) codifies for the luciferase gene under the control of a promoter that contains four CRE-sequences. After 48 h, luciferase luminescence was measured using the dual-luciferase reporter assay system (Promega) following the manufacturer's instructions. Transfection efficiency was normalised by co-transfection with the pTK-Renilla plasmid, which codifies for the Renilla protein under a constitutive promoter.

Stimulation and pharmacological manipulation of calcium signals

To induce calcium responses, we used ATP, NA, or ET-1, all purchased from Sigma Aldrich. To manipulate different signalling pathways, we used carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) and BD-1047 from Tocris, EGTA, and gly-phe-β-naphthylamide (GPN) from Sigma Aldrich. Ned-19 was provided by Dr. Grant Churchill of Oxford University. All these compounds were applied 20 min before calcium imaging.

Intracellular calcium measurements

Cells were loaded with fluo-4AM (Thermo Scientific) at 4 μM and Pluronic F-127 at 0.02 % (Thermo Scientific) in Krebs Buffer with the following composition (in mM): 113 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.2 $MgSO_4$, 10 Hepes, 1.3 $CaCl_2$, and 10 glucose at pH 7.4. After 1 h in the dark at room temperature, the cells were washed with Krebs Buffer, and transmitter-induced calcium responses were measured with a Victor III fluorometer (Perkin Elmer) or an Eclipse TE2000-E fluorescence microscope (Nikon) (excitation at 466–496 nm, emission at 520–550 nm). Microscope images were collected and analysed with the MetaMorph image processing software (Universal Imaging).

When fura-2AM was used, astrocytes were incubated with 2 μM fura-2-AM (Thermo Scientific) and BSA in Krebs Buffer for 1 h in the dark at room temperature. After incubation, the coverslips were mounted in a static imaging chamber of an inverted epifluorescence Eclipse TE-2000U microscope (Nikon). The cells were excited at 340 nm and 380 nm using a monochromator (Cairn Research), and fluorescence emission at 495–520 nm was recorded with an ORCA-ER CCD camera (Hamamatsu). Images were processed and analysed with the MetaFluor image processing software (Universal Imaging). Cell-based calcium

calibration assays were performed to estimate calcium concentrations according to the method of [19].

Imaging of ER and mitochondrial calcium

We used two plasmids encoding for calcium-measuring organelle-entrapped protein indicators (CEPIA), gifts from Masamitsu Lino, one for the ER (pCMV G-CEPIA-1ER, Addgene plasmid # 58215) and the other for the mitochondria (pCMV CEPIA-3mt, Addgene plasmid # 58219). Five micrograms of the CEPIA constructs were transfected using Amaxa nucleofector®. Two or three days after transfection with CEPIA plasmids, cells seeded on coverslips were mounted in a static chamber with Krebs Buffer in the epifluorescence Eclipse TE2000-U microscope stated above. The cells were excited at 476 nm and fluorescence emission was recorded at 510–560 nm. Images were analysed with the MetaFluor software.

Alternatively, in a particular set of experiments, astrocytes were loaded with the mitochondrial calcium dye rhod-2AM (Life technologies) at 10 μM for 5 min at room temperature. The cells were excited at 552 nm and fluorescence emission was recorded at 570–640 nm. For each experiment, up to 60 single mitochondria were selected and mean of calcium increases was determined.

Immunocytochemistry

The cells were fixed with 4 % paraformaldehyde in phosphate buffered saline (PBS) for 30 min at room temperature and permeabilised with PBS/0.1 % Triton X-100 for 15 min. Non-specific-binding blockade was performed with 1 % normal goat serum (NGS, Thermo Scientific) in PBS followed by overnight incubation at 4 °C with anti-GFAP (1/1000 Dako) and anti-VP16 (1/500 Santa Cruz biotechnology) antibodies in 0.1 % NGS-PBS. After washing, the cells were incubated for 1 h with Alexa labelled secondary IgGs (1/1000 in 0.1 % NGS-PBS, Thermo Scientific).

Western blots

The cells were lysed on ice with RIPA solution containing 50 mM Tris–HCl, 150 mM $NaCl_2$, 0.1 % sodium dodecyl sulfate (SDS), 1 % Nonidet P-40, protease inhibitor cocktail (Life Sciences), and phosphatase inhibitor cocktail (Sigma Aldrich). Protein concentrations were determined using Pierce BCA protein assay kit (Thermo Scientific). Equal amounts of protein (15–30 μg) were subjected to SDS 8–15 % polyacrylamide gel electrophoresis and transferred to methanol-activated polyvinylidene fluoride membranes (Life Sciences). The membranes were blocked in 0.1 % Tween 20-Tris buffer solution (TTBS) containing 5 % milk for 1 h and incubated overnight with primary antibodies: 1/1000 sigma-1 receptor (Thermo Scientific), 1/500 VP16 (Santa Cruz Biotechnology), 1/2000 mitochondrial calcium uniporter (MCU, Abcam), 1/50,000 GAPDH (Thermo Scientific), and 1/20,000 β-Actin (Sigma Aldrich), diluted in 1 % BSA-TTBS. Peroxidase-conjugated goat anti-rabbit and anti-mouse (Thermo Scientific) were used as secondary antibodies at 1/10,000 for 1 h at RT. The bands were detected with an enhanced chemiluminescence detection kit (ECL) and developed by autoradiography on X-ray film (AGFA).

Quantitative PCR

Total RNA extraction was done using TRIZOL reagent following manufacturer's instructions. Once purified, the mRNA was reverse-transcribed with RevertAid Reverse Transcriptase. Briefly, a reaction mix containing 1 μg RNA, 2 μM random hexamer primers, 2 μM oligo$(dT)_{18}$ primers, 0.5 mM dNTPs, 0.45 mM 1,4-dithiothreitol, 10 U RNAse-Out, and 200 U RevertAid Reverse Transcriptase was incubated at 25 °C for 10 min, 42 °C for 60 min, and 72 °C for 10 min. All reagents were purchased from Thermo Scientific. After RNA reverse transcription, the resulting samples at 1:25 or 1:100 dilutions were amplified in an Applied Biosystems 7500 Fast system using TaqMan reagents for *SigmaR1*, *Mcu*, *Tbp*, and *Gapdh* genes (Thermo Scientific). We used the LinRegPCR software to obtain average PCR efficiencies for each gene and then compared the Cq values with the comparative Cq method [20]. Gene expression of sigma-1 receptor and *Mcu* in cultures were normalised to *Gapdh* and *Tbp* expression, respectively. In samples from mice, total mRNA extraction was performed after animals were sacrified and the injured hemisphere was excised using a brain matrix as described in [18]; the expression of sigma-1 receptor was normalised to *Tbp* expression.

Statistical analysis

Data are expressed as the mean ± SEM of a minimum of three independent experiments. Two data sets were compared using Student's *t* test, whereas multiple groups were analysed using ANOVA and a Dunnet post-test if all groups were compared with control, or a Turkey post-test when all groups were compared with each other.

Results

Activation of CREB-dependent transcription regulates calcium signals

To determine whether CREB changes astrocyte calcium signalling, we used an experimental paradigm with two

sequential interventions. The first one was applied to induce CREB-dependent transcription and consisted of transmitters (NA, ATP) or virally transduced VP16-CREB, a constitutively active form of CREB previously used in neurons [17] and other cell types [21, 22]. The second intervention was aimed at directly examining excitability. To this end, we administered ATP, NA, or ET-1, three transmitters that are agonists of Gq protein-coupled receptors (GPCR), thereby activating the main intracellular calcium signalling pathways in astrocytes.

We previously showed that long-term incubation with 10 μM NA or 100 μM ATP, but not with 100 μM glutamate, activated astrocytic CREB by an atypical PKC-dependent, calcium-insensitive mechanism [13]. In this study, we resorted to 1-h pulses with the transmitters followed by a 5-h wait before the second stimulus to prevent alterations of calcium responses due to receptor desensitization. Calcium responses were examined with fluorimetry using the calcium dye fluo-4AM (Fig. 1a). Of note, agonist concentrations and duration of stimulation were set to achieve maximal CREB activation in our cultures while mimicking an activity-driven CREB activation in vivo. For example, micromolar concentrations of NA might be achieved under particular physiological and/or pathological conditions in vivo [23, 24]. At these concentrations, NA activates α- and β-adrenergic receptors [23], the latter being linked to gene transcription and plasticity in neurons [25].

Pulses of NA and ATP were able to stimulate CREB as shown with CRE-containing luciferase reporter assays, while glutamate (Glu) and ET-1 had no effect (Fig. 1b). Accordingly, only CREB-activating neurotransmitters induced downregulation of calcium responses (12-39 %), suggesting that CREB mediates the actions of ATP and NA (Fig. 1c–h). Interestingly, the decreased calcium response was observed with all the transmitters tested (NA, ATP, and ET-1) (Fig. 1c–h), suggesting that CREB targets core pathways of calcium signalling in astrocytes. In ensuing experiments, we used 10 μM NA as the first stimulus and 100 μM ATP as the second. We selected the former, because NA is prototypical neurotransmitter acting through volume transmission and modulates the capacity of astrocytes to respond to local neuronal activity [26]. We selected ATP as the second stimulus, because ATP drives intra- and intercellular calcium waves in astrocytes in cultures and in vivo, and is involved in many important physiological events [27–30]. Time-course analysis revealed that the reduction of calcium responses was still present at 12 h but not at 24 h after incubation with NA (Fig. 2a–c), hence it was transient. Finally, we verified that the decreased response was due to CREB activation using virally transduced A-CREB, a well-known dominant negative construct of CREB [31]. Eighteen hours after infection of cells with an empty viral vector (Null), ATP-induced calcium responses were smaller in cells pre-treated with NA; however, no differences were observed between NA and non-treated cells if astrocytes were infected with A-CREB (Fig. 2d–f). In other words, the presence of A-CREB abolished the NA-mediated reduction of ATP-induced calcium responses, confirming that it is a CREB-specific phenomenon.

Before examining whether VP16-CREB reproduced the effect of physiological transmitters on calcium excitability, we confirmed the expression of the transgene with immunocytochemistry and western blot (Fig. 3a, b). The first technique allowed us to calculate that around 85 % of astrocytes were infected by the VP16-CREB viral vector. Next, we examined the effect of VP16-CREB on excitability using different MOIs of the virus. Astrocytes expressing VP16-CREB showed smaller ATP-induced calcium responses (20–30 %) compared to astrocytes infected with the Null viral vector (Fig. 3d, e). The reduction of calcium responses was observed 18–24 h after infection and was independent of the vector concentration, whereas astrocytes infected with the Null vector responded as non-infected astrocytes (data not shown). VP16-CREB also decreased calcium responses to 10 μM NA (Fig. 3f, g). Finally, we explored whether VP16-CREB expression changes basal intracellular calcium levels using the ratiometric calcium indicator fura-2AM. Astrocytes infected with the empty vector had a rest calcium concentration of 117 ± 8 nM, whereas VP16-CREB-expressing cells had 112 ± 5 nM (means of three independent experiments), indicating that CREB did not exert any effect on basal calcium levels.

Overall, this set of experiments demonstrates that CREB activation decreases transmitter-elicited calcium signals in astrocytes, while it does not interfere with basal calcium.

Analysis of calcium fluxes

We next studied the mechanism by which VP16-CREB regulates intracellular calcium responses, analyzing the contribution of calcium mobilization, extracellular calcium entry, and calcium uptake mechanisms, to investigate if there is regulation of a specific signalling pathway or a general alteration of calcium homeostasis. As CREB does not abolish calcium responses but reduces them by 15–30 %, we first analysed extracellular calcium entry and intracellular calcium release from lysosomes, which have an important but not major role in ATP and NA induced calcium responses [3]. We infected astrocytes with VP16-CREB or Null viral vectors at 1–5 MOI and followed calcium responses with real-time fluorescence microscopy after treating astrocytes with 0.5 mM EGTA to chelate extracellular calcium, 50 μM GPN, a lysosomal disruptor,

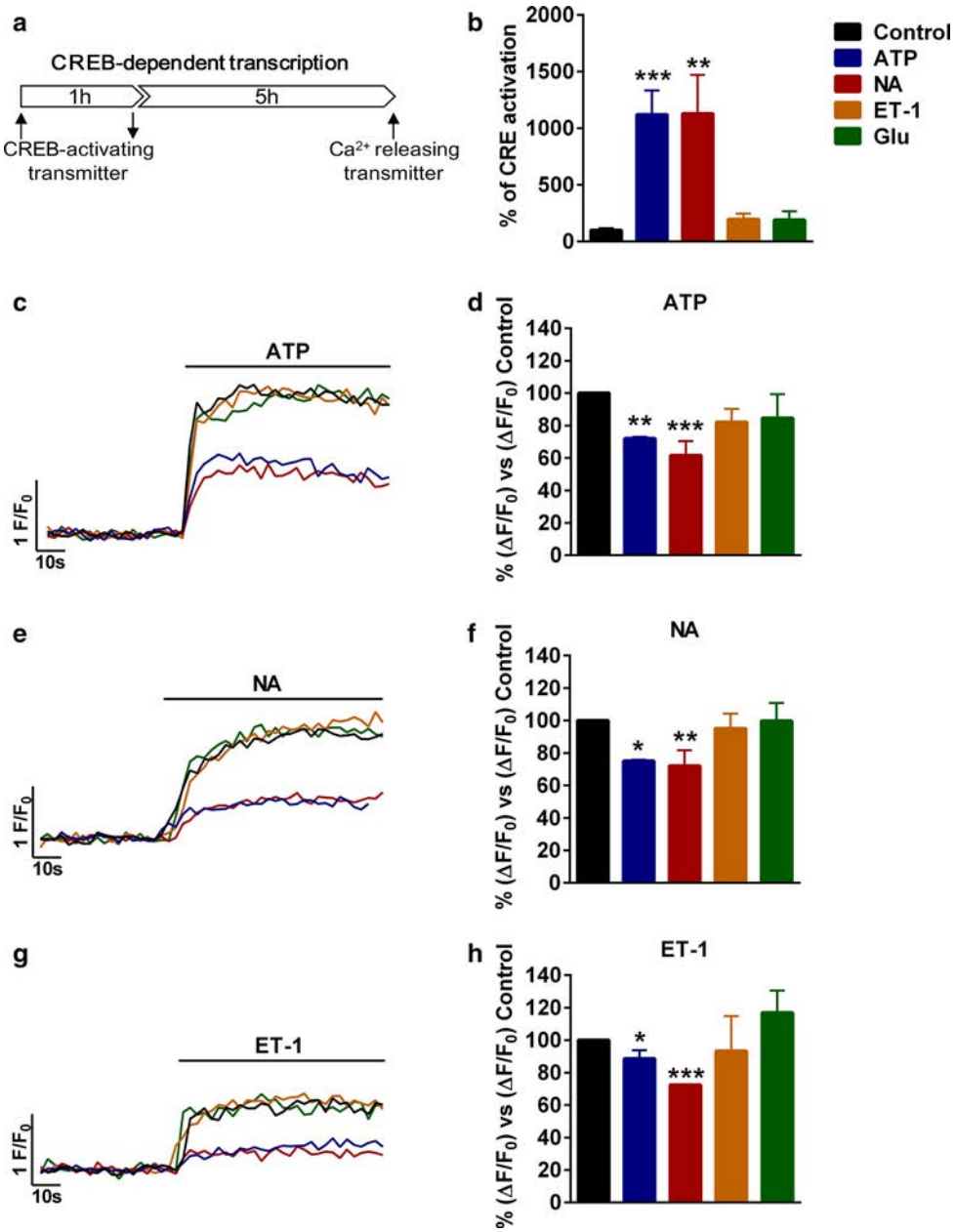

Fig. 1 Pre-stimulation with CREB-activating transmitters reduces transmitter-induced calcium responses. **a** Schematic representation of the protocol used. **b** Activation of CREB-dependent transcription by different transmitters measured with a luciferase gene-reporting assay. Control is cells not treated with any transmitter but with vehicle; otherwise cells were stimulated with 100 μM ATP, 10 μM noradrenaline (NA), 10 nM endothelin-1 (ET-1), or 100 μM glutamate (Glu) and CREB activity measured after 6 h. Representative calcium traces (**c**, **e**, **g**) and quantification (**d**, **f**, **h**) of maximum increases after the addition of 100 μM ATP (**c**, **d**), 10 μM NA (**e**, **f**), or 10 nM ET-1 (**g**, **h**) after CREB activation with ATP, NA, ET-1, or Glu, identified with *color codes*. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

or 100 μM Ned-19, an inhibitor of the NAADP receptor [32]. All these pharmacological treatments diminished intracellular calcium responses in Null-infected astrocytes, as previously described [3], but also in VP16-CREB-expressing astrocytes (Figs. 3d, 4a–c). Therefore, the downregulation of calcium by VP16-CREB compared with Null astrocytes was not altered (Fig. 4a–d). Thus, CREB does not appear to alter extracellular calcium entry or calcium mobilization from acidic vesicles.

We then evaluated calcium release from the ER using G-CEPIA1er, a new calcium protein indicator that specifically localizes in the ER [33]. After co-expression of G-CEPIA1er and VP16-CREB, the cells were treated with 100 μM ATP (Fig. 4e). As shown in Fig. 4f and g, VP16-CREB increased calcium release from the ER. This unexpected result proves that CREB does not reduce purinergic or GPCR expression, nor is there receptor downregulation or desensitization; however, the observation is paradoxical, because greater calcium release from the ER would cause an increase in cytosolic calcium. Since this is not the case, we reasoned that either calcium from the ER is not released to the cytosol, or there is a mechanism of calcium extrusion/uptake potentiated by CREB. We thus focused on analyzing calcium uptake by the mitochondria. First, we recorded the cytosolic calcium increase induced by 100 μM ATP after inhibition of mitochondrial calcium

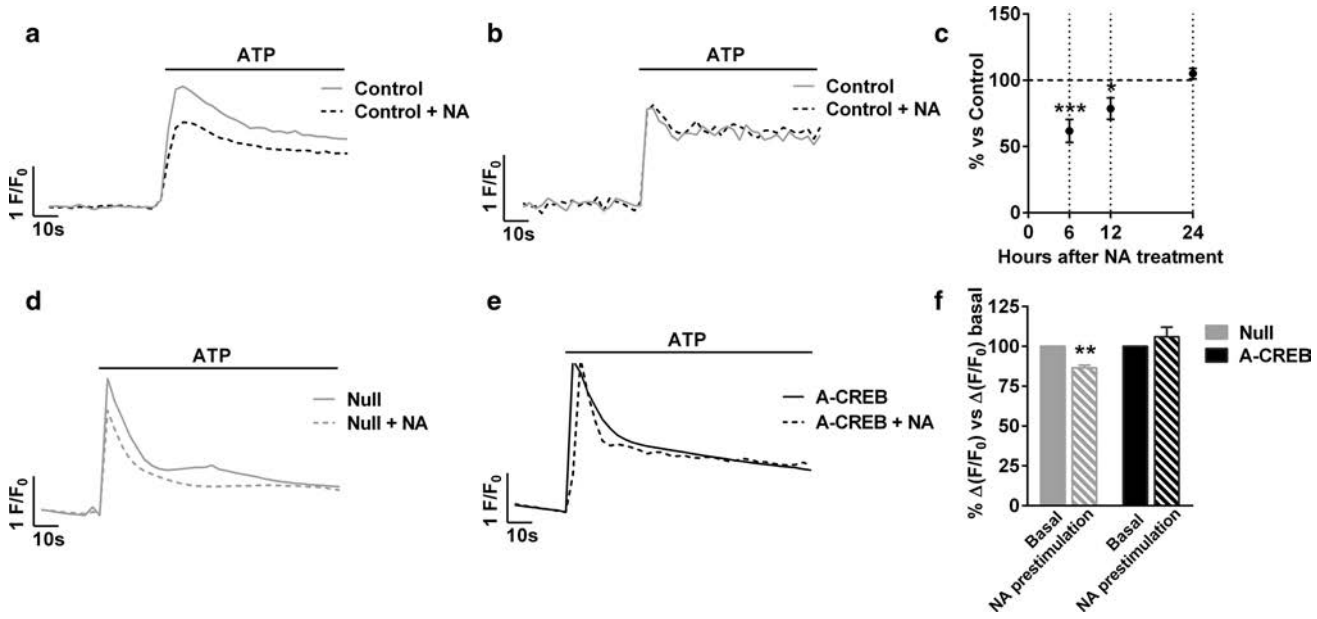

Fig. 2 Transmitter-elicited reduction of cytosolic calcium responses is transient and CREB-dependent. Astrocytes were treated with 10 μM NA to induce CREB activation. Representative traces of calcium responses induced by 100 μM ATP at 12 h (**a**) and 24 h (**b**) after CREB stimulation. **c** Quantification of peak calcium responses induced by ATP after 6, 12, and 24 h after CREB stimulation. The data are the mean ± SEM of 3–4 independent experiments. $*P < 0.05$, $***P < 0.001$. **d**, **e** Representative 100 μM ATP-induced calcium traces in single cells and **f** quantification of calcium responses after 6 h of stimulation of CREB with 10 μM NA in Null and A-CREB-infected astrocytes. The data are the mean ± SEM of 3–4 independent experiments. $**P < 0.01$

uptake with FCCP, an ionophore that transports protons through the inner mitochondrial membrane, and hence disrupts the electrogradient-driven calcium uptake. FCCP increased ATP-induced calcium responses, so that VP16-CREB and Null astrocytes presented calcium responses of equal magnitude (Fig. 5a, b). Second, we directly measured mitochondrial calcium uptake using CEPIA3mt, a new low affinity green fluorescent mitochondrial calcium protein indicator [33] (Fig. 5c). VP16-CREB astrocytes presented a greater mitochondrial calcium increase than Null astrocytes upon stimulation with 100 μM ATP (Fig. 5d, e), in accordance with the FCCP data. Similar results were obtained using rhod-2/AM, a low affinity calcium dye that is preferably entrapped in the mitochondria. Calcium increases in individual mitochondria were 2.89 ± 0.7 times higher in VP16-CREB than in Null-infected cells.

The sigma-1 receptor mediates the effect of VP16-CREB

Taken together, these data indicate that VP16-CREB increases both calcium release from the ER and mitochondrial calcium uptake. These data would be reconciled if CREB activation was potentiating ER-mitochondria interaction, a process that appears to be key for several cellular functions, including calcium signalling [34]. Indeed, calcium transfer from the ER to the mitochondria through mitochondria-associated ER membranes (MAM) is initiated by the release of calcium from the ER following the production of IP_3 [34]. MAMs integrate many different proteins. Among these, sigma-1 receptors have been shown to have a CREB-binding consensus sequence and to interact with the IP_3 receptor [35]. We, therefore, studied whether VP16-CREB expression leads to increased expression of sigma-1 receptor with real-time PCR and western blot. VP16-CREB-expressing astrocytes do have greater sigma-1 receptor mRNA and protein content than Null-infected cells (Fig. 6a, b). We then examined if sigma-1 receptor upregulation contributes to the CREB-mediated effects on calcium fluxes. Pre-treatment with the sigma-1 receptors inhibitor BD1047 at 10 μM diminished the calcium release from the ER (Fig. 6c, d), and reduced mitochondrial calcium increases (Fig. 6e, f) in ATP-stimulated VP16-CREB-expressing astrocytes. All in all, the results provide evidence that the effects of VP16-CREB on calcium signalling can be mediated by the sigma-1 receptor and lend support to the following scenario: VP16-CREB raises the expression of the sigma-1 receptor thus amplifying the functional coupling between ER and mitochondria, increasing mitochondrial calcium uptake and thereby reducing cytosolic calcium.

Sigma-1 receptor in the ER membranes can interact with different proteins of the outer mitochondrial membrane facilitating the flux of calcium, which might then be driven to the mitochondrial matrix through the mitochondrial

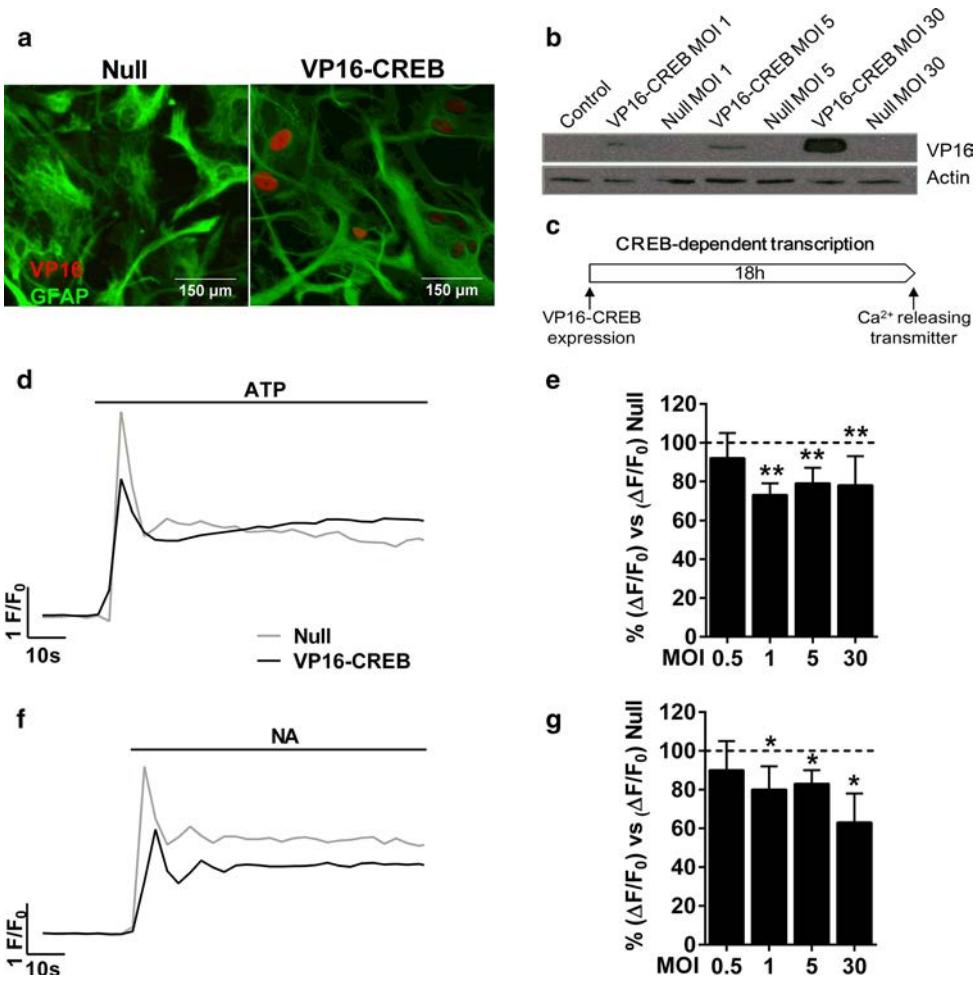

Fig. 3 VP16-CREB decreases calcium responses induced by transmitters. **a** VP16 immunocytochemistry and **b** western blot of VP16 in astrocytes infected with VP16-CREB or Null viral vectors. **c** Schematic representation of the protocol used to measured calcium responses in astrocytes infected with VP16-CREB or Null viral vectors. Representative single cell traces of calcium responses induced by 100 µM ATP (**d**) or 10 µM NA (**f**) in VP16-CREB-infected astrocytes (virus MOI 1). **e**, **g** Quantification of peak calcium responses at different viral vector loads. Data are normalised for the response of Null-infected astrocytes at the same viral vector load. The data are the mean ± SEM of 4–7 (**d**) or 3–4 independent experiments (**f**). $*P < 0.05$, $**P < 0.01$

calcium uniporter (MCU). Very little is known about regulation of MCU at transcription levels [36, 37]. We, therefore, checked if VP16-CREB was also changing MCU expression. As shown in Fig. 7a and b, MCU is expressed in astrocytes but paradoxically, VP16-CREB diminished its expression both at the mRNA and protein level. We consider that this finding is in agreement with our previous observations that, in VP16-CREB-expressing astrocytes, mitochondrial calcium uptake is increased but not to very high levels; therefore, VP16-CREB-induced MCU-down-regulation might be a protective mechanism to avoid mitochondrial calcium overload and hence cell death. Moreover, the changed expression of MCU supports the notion that mitochondrial calcium uptake is exquisitely regulated by transmitter-induced gene expression.

CREB-dependent transcription regulates calcium signals and sigma-1 receptor in adult astrocytes

The first demonstration of the implication of neuronal CREB in synaptic and plasticity was obtained in simple model systems like primary cell cultures from *Aplysia* ganglia [10, 38]. Above, we reported the effect on CREB on astrocyte plasticity using primary cultures from neonatal astrocytes. Next, we sought to confirm key findings in cultured astrocytes from adult rats. Following the protocol stated in Fig. 1a, NA induced CREB activation by 368.7 ± 94.7 % and this resulted in decreased ATP-induced calcium responses, as shown in Fig. 8a and b, much resembling the results in postnatal astrocytes. Reduction of ATP-induced calcium signals to the same extent was also achieved 18–24 h after VP16-CREB infection compared with Null-infected astrocytes (Fig. 8c, d). In this case, the VP16-CREB viral vector infected around 78 % of astrocytes. Finally, sigma-1 receptor was also upregulated upon VP16-CREB overexpression (Fig. 8e). We, therefore, checked if sigma-1 receptor is upregulated by CREB in vivo. To do so, we made use of transgenic mice where VP16-CREB is targeted to astrocytes. In such animals, CREB activation is driven by a GFAP promoter and is not significantly increased in the forebrain in normal conditions (see [18] for a full characterization of the animals and VP16-CREB expression), but it is after a traumatic brain injury

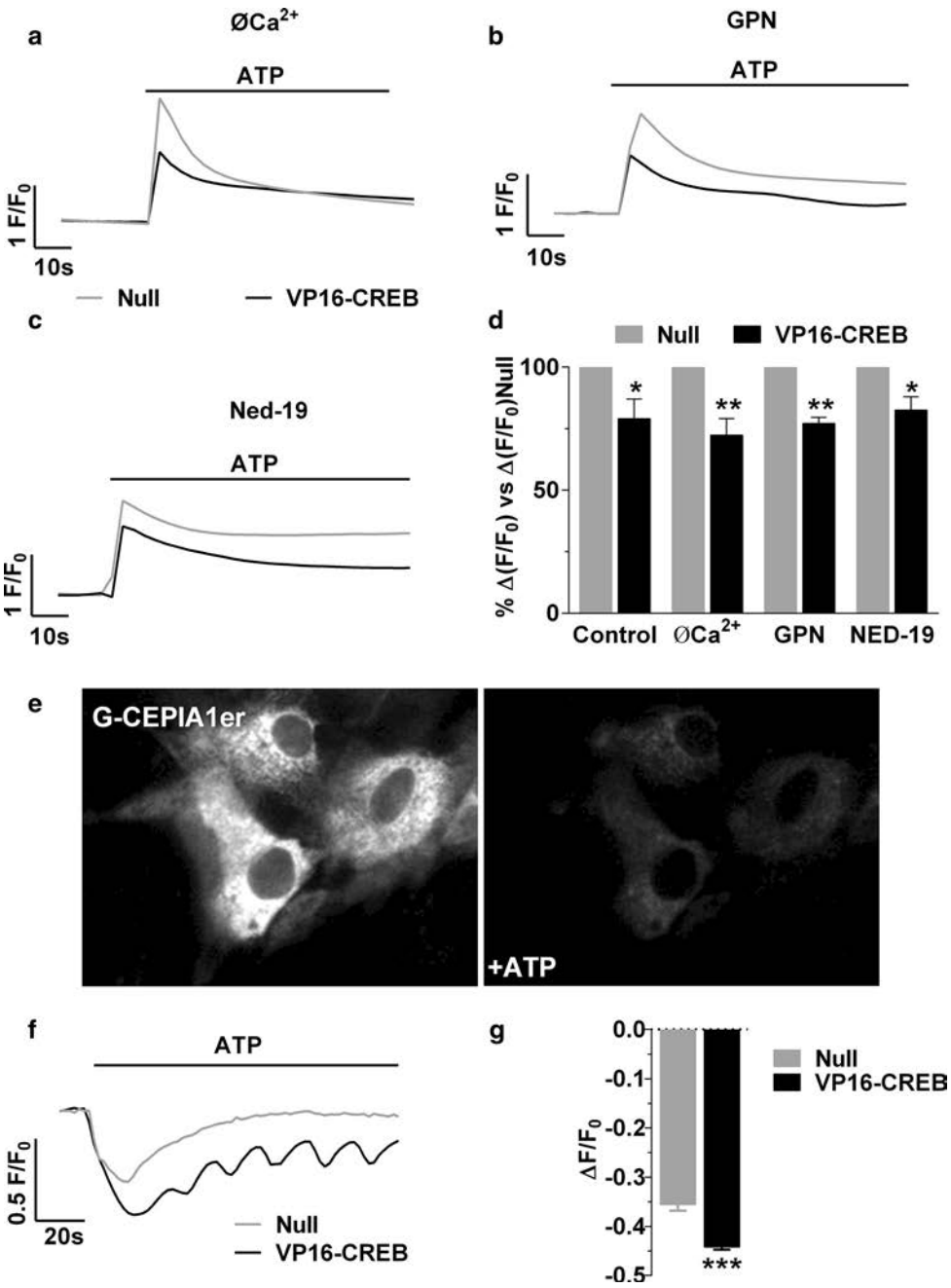

Fig. 4 VP16-CREB action on calcium signalling pathways. ATP-elicited elevation of calcium was tested in astrocytes transducing VP16-CREB with no added calcium and presence of 0.5 mM EGTA ($0Ca^{2+}$), or in the presence of inhibitors of calcium release from acidic lysosomal-related stores: 50 μM GPN or 100 μM Ned-19. Control refers to cells without any treatment other than viral vector infection. Representative traces in single cells (**a**–**c**) and quantification (**d**) of 100 μM ATP-induced calcium responses in Null and VP16-CREB-infected astrocytes. The data are the mean ± SEM of 6–8 independent experiments. $*P < 0.05$, $**P < 0.01$. **e** Representative images of astrocytes transfected with the ER calcium dye G-CEPIA1er before and after 100 μM ATP application. Representative traces (**f**) and quantification (**g**) of the decrease in ER calcium. Data are mean ± SEM of four independent experiments. $***P < 0.001$

(cryolesion), which induces gliosis and hence activates the GFAP promoter. Accordingly, as shown in Fig. 8f, sigma-1 receptor expression is slightly but not significantly different between wild-type (WT) and VP16-CREB mice. Cryolesions upregulate sigma-1 receptor mRNA in both WT and VP16-CREB transgenic mice, and under such paradigm, VP16-CREB mice show a statistically significant higher expression of sigma-1 receptor compared with WT. Overall, these results suggest that VP16-CREB upregulates sigma-1 receptor in astrocytes in vivo.

Discussion

In this study we provide evidence that transmitter-induced intracellular calcium responses, the basis of astrocytic excitability, are regulated by transmitter-triggered gene expression via the transcription factor CREB. Specifically, activation of CREB-dependent transcription in astrocytes by two transmitters, ATP, and NA, or by a constitutively active CREB, reduces the calcium responses triggered by the GPCR agonists ATP, NA, and ET-1. The decrease of calcium responses is transient if the trigger of CREB-

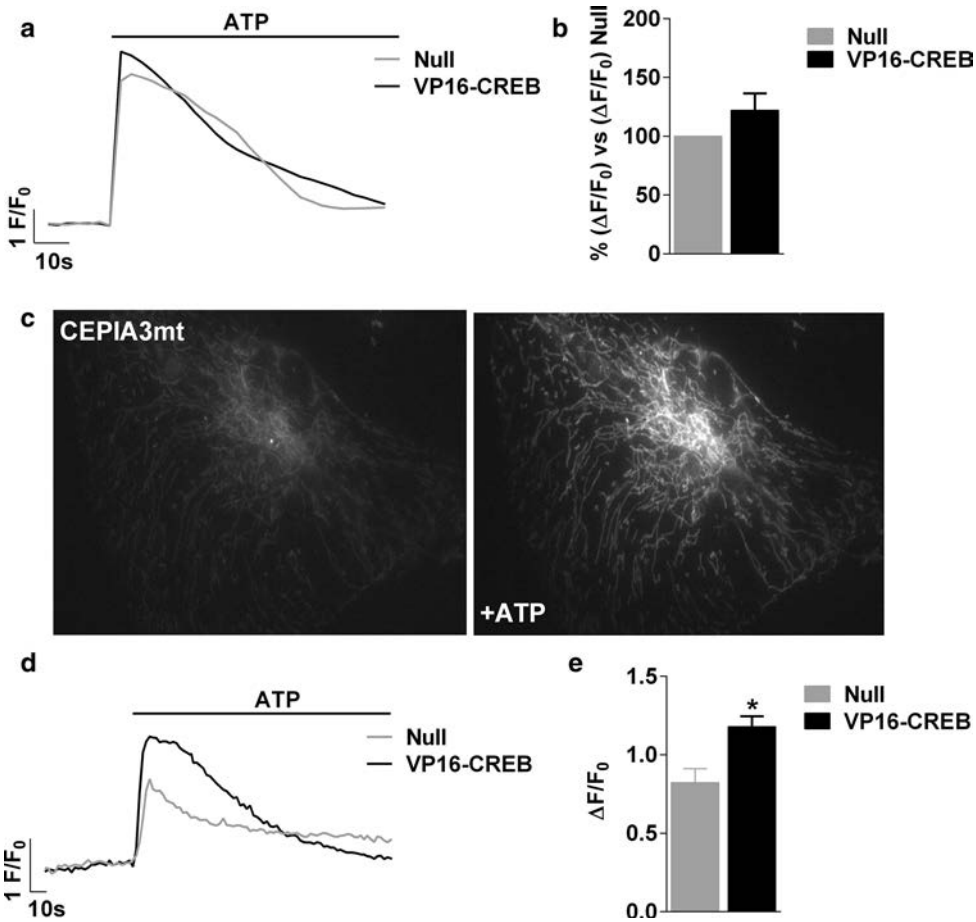

Fig. 5 VP16-CREB increases mitochondrial calcium uptake. **a** Representative traces in single cells and **b** quantification of intracellular calcium responses induced by 100 μM ATP in Null and VP16-CREB-infected astrocytes in astrocytes treated with FCCP to inhibit mitochondrial calcium uptake. The data are the mean ± SEM of four independent experiments. **c** Representative images of the astrocytes transfected with the mitochondrial calcium dye CEPIA3mt before and after 100 μM ATP application. **d** Representative calcium traces and **e** quantification of 100 μM ATP-induced mitochondrial calcium increase in Null and VP16-CREB-infected astrocytes. The data are the mean ± SEM of four independent experiments. $^{*}P < 0.05$

dependent transcription is transmitters given in pulses, consistent with the fact that transmitters produce a transitory activation of signalling pathways leading to CREB activation—namely CREB phosphorylation by kinases—while VP16-CREB bypasses such kinases and constantly activates CREB-dependent transcription. All in all, the data support the novel view that CREB regulates plasticity-like phenomena by targeting calcium signalling. Interestingly, CREB activation is independent of calcium in astrocytes [13], preventing self-depleting feedback negative loops. It will be of value to further extend our results with experimental approaches that contemplate different magnitudes and temporal patterns of CREB activation resembling a range of physiological or pathological conditions.

As to mechanisms, VP16-CREB overexpression does not interfere with basal calcium concentration nor does it exert a wide alteration of GPCR or calcium-channel expression or activation, because there is no change in extracellular calcium entry or NAADP-driven calcium mobilization from acidic stores. Rather, studies with CEPIA-based organellar calcium dyes point to changes in calcium fluxes between ER and mitochondria as a central mechanism. Thus, we found that VP16-CREB enhances purinergic-induced calcium release from the ER, increases mitochondrial calcium uptake, and enhances the expression of sigma-1 receptor, a protein interacting with IP_3-receptors at the MAMs [34]. These observations might not be mere coincidence. First, the inhibition of mitochondrial calcium uptake with FCCP prevents CREB from decreasing ATP-induced calcium responses, suggesting that mitochondria are the main uptake or extrusion loci involved. Second, the inhibition of the sigma-1 receptor abolishes the increased ER calcium release and lowers mitochondrial calcium uptake, demonstrating that sigma-1 receptors—whose gene has a CREB-binding site [35]—mediate the effects of CREB. We also detected upregulation of sigma-1 receptor in cultured astrocytes from adult rats after infection with the VP16-CREB viral vector, and in transgenic mice with targeted activation of CREB in astrocytes. However, we cannot rule out the intervention of other CREB-dependent genes in the modulation of calcium responses in astrocytes.

Importantly, the ATP-mediated mitochondrial calcium increases potentiated by VP16-CREB do not represent mitochondrial calcium overload, because no cell death was detected after VP16-CREB expression, during the

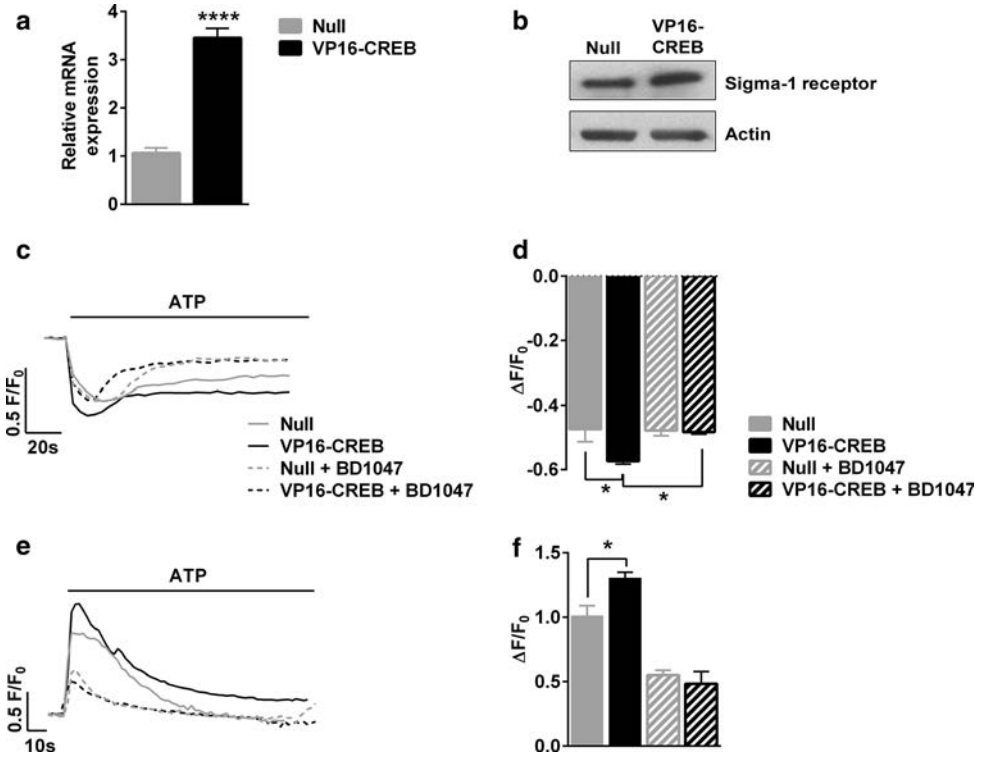

Fig. 6 Sigma-1 receptor mediates the effects of VP16-CREB. **a** Quantification of sigma-1 receptor mRNA expression by quantitative PCR and **b** representative western blot of sigma-1 receptor in astrocytes infected with Null or VP16-CREB. **c** Representative calcium traces in single cells and **d** quantification of 100 μM ATP-induced ER calcium decreases measured using G-CEPIAer in astrocytes infected with null or VP16-CREB viral vectors and in the absence and presence of BD1047, a sigma-1 receptor antagonist. **e** Representative calcium traces in single cells and **f** quantification of 100 μM ATP-induced mitochondrial calcium increases using CEPIA3mt in astrocytes infected with Null or VP16-CREB viral vectors and in the absence and presence of BD1047. The data are the mean ± SEM of 3–4 independent experiments. $*P < 0.05$, $****P < 0.0001$

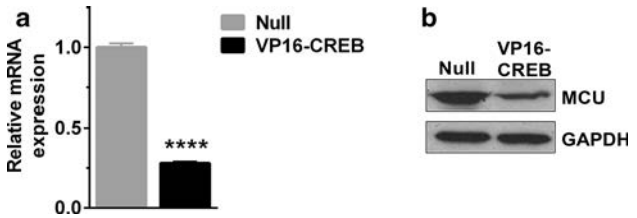

Fig. 7 MCU expression decreases in VP16-CREB-expressing astrocytes. **a** Quantification of MCU mRNA expression in Null and VP16-CREB-infected astrocytes by quantitative PCR (**a**). Data are the mean ± SEM of 3–4 independent experiments. $****P < 0.0001$. **b** Representative western blot of MCU in astrocytes infected by Null and VP16-CREB viral vectors

experimental times used in this study. In this sense, the decrease in the expression of the mitochondrial calcium uptake channel, MCU, induced by CREB may be a protective homeostatic change secondary to a more efficient coupling ER-mitochondria and confirms recently published data showing that CREB binds to the MCU promoter in chicken lymphocytes, although in this case, there is upregulation of MCU [39]. The CREB-dependent mitochondrial calcium rises in astrocytes may potentiate physiological functions, such as oxidative phosphorylation or glutamate recycling and replenishment, since enzymes, such as alpha-ketoglutarate, isocitrate dehydrogenases, pyruvate dehydrogenase phosphatase, and ATP synthase, are activated by mitochondrial calcium [40, 41]. Accordingly, sigma-1 receptor stimulation in cardiomyocytes has been shown to reinforce calcium transport from the sarco/endoplasmic reticulum to the mitochondria and increase ATP production [42]. Although some authors support the idea that astrocytes are exclusively glycolytic, they have functional mitochondria and are capable of oxidizing glucose, fatty acids [43], and glutamate after its uptake from the synaptic cleft [6]. Thus, an adaptive and finely gene-regulated modulation of mitochondrial calcium, and hence of tricarboxylic acid cycle and ATP production, may be more important to astrocyte physiology than previously thought.

It is noteworthy that CREB does not completely abolish intracellular calcium signalling in astrocytes but fine-tunes the response (i.e., decreases are 12–39 %), suggesting that CREB also regulates non-mitochondrial based mechanisms, such as gliotransmission [28]. Thus far, extensive

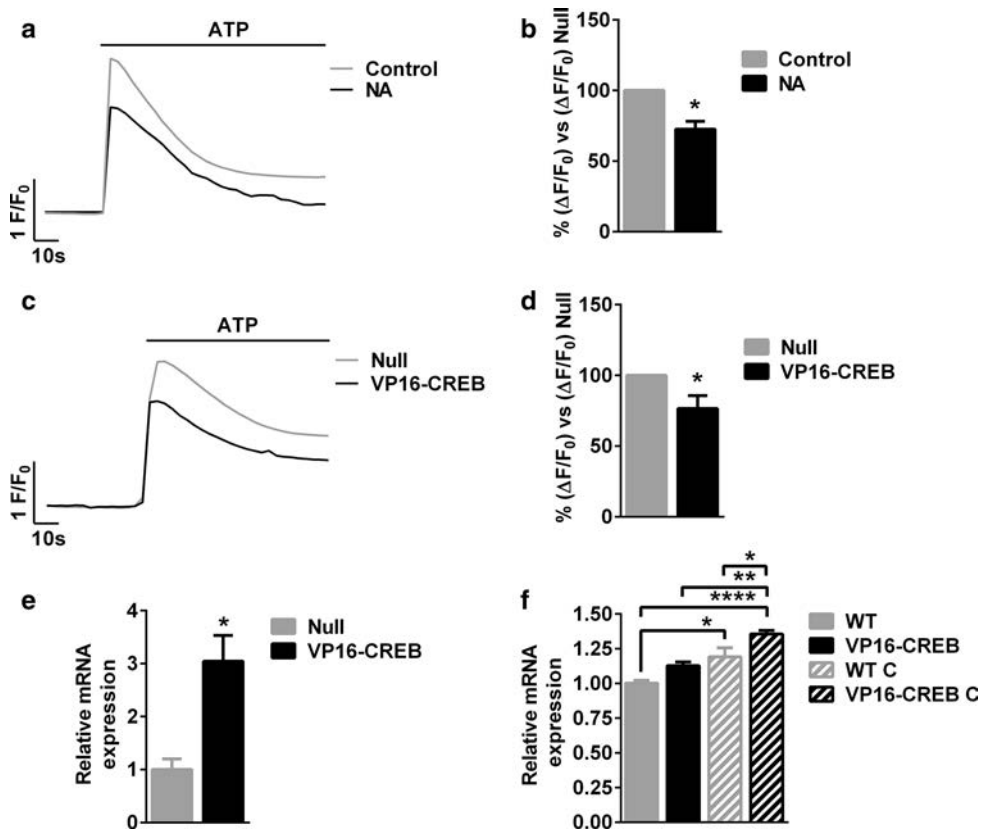

Fig. 8 CREB decreases calcium responses and upregulates sigma-1 receptor in adult astrocytes. **a** Representative traces and **b** quantification of calcium responses induced by 100 µM ATP after 6 h of CREB stimulation (1 h) with 10 µM NA or vehicle (control). **c** Representative traces and **d** quantification calcium responses induced by 100 µM ATP in adult cultured astrocytes infected with Null or VP16-CREB viral vectors. **e** Quantification of sigma-1 receptor mRNA expression by quantitative PCR in Null and VP16-CREB-infected adult cultured astrocytes. In (**b**, **d**, **and e**), data are the mean ± SEM of 4 independent experiments. $*P < 0.05$. **f** Quantification by quantitative PCR of sigma-1 receptor mRNA expression in cortices in WT and VP16-CREB mice before (WT and VP16-CREB) and after cryolesion (WT C and VP16-CREB C). Cryolesions were performed to increase VP16-CREB expression in astrocytes conditional to gliosis. Data are the mean ± SEM of 4–5 animals in each condition. $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$

ex vivo and in vivo evidence implicates calcium-dependent gliotransmission in the regulation of synaptic transmission [44–47]—albeit with some controversy [5], but there was no evidence linking calcium-based astrocyte excitability and long-term plasticity. To our knowledge, this is the first report demonstrating that astrocytes, like neurons, do have long-term and transient intrinsic plasticity-like phenomena or 'cellular' memory in terms of calcium responses. Although the idea of structural and functional plasticity of astrocytes has already been explored in other laboratories [48], the previous reports dealt with short-term changes in gliotransmission (less than 30 min long), and these changes were, moreover, not mediated by a transcription factor and hence gene expression [49, 50]. Because CREB reduces rather than increases astrocyte excitability, it appears that we might have discovered an LTD-like phenomenon that may help memory consolidation by inducing a negative feedback loop that maintains neural networks in stable states or very long transients [51]. As noted in a recent review of 60 years of research in synaptic plasticity, the importance of memory and modulation in behaviour relies on the concept of plasticity per se rather than on the direction of the change in synaptic strength [52]. In any event, our results need further confirmation in vivo.

Finally, it is worth noting the therapeutic interest of the capacity of CREB to modulate calcium signals as these are involved in many and diverse physiological and long-term pathological events, such as cardiac atrophy [53] or neuronal degeneration [54]. In astrocytes, calcium hyperactivity is a hallmark of epilepsy [27] and neurodegenerative diseases [29, 55]. In addition, we have recently reported that the targeted activation of CREB in astrocytes is beneficial in a model of acute brain damage [18], and here, we provide evidence that sigma-1 receptor is upregulated by CREB in such context. Accordingly, disruption of IP_3 receptor-mediated calcium release and activation of sigma-1 receptors are beneficial in animal models of stroke [56, 57]. Therefore, reduction of calcium signalling in

astrocytes may be among the beneficial mechanisms switched on by CREB in several acute and chronic brain pathologies.

In summary, two main conclusions can be drawn from this study: (1) Calcium responses are regulated by transmitter-triggered CREB-dependent transcription, supporting the idea that not only neurons are endowed with plasticity-like phenomena, and (2) CREB-dependent transcription results in increased sigma-1 receptor expression and hence in a more efficient communication between ER and mitochondria. This may potentiate mitochondrial function, thus linking astrocyte energy metabolism and plasticity.

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References

1. Churchill GC, Okada Y, Thomas JM, Genazzani AA, Patel S, Galione A (2002) NAADP mobilizes Ca^{2+} from reserve granules, lysosome-related organelles, in sea urchin eggs. Cell 111:703–708. doi:10.1016/S0092-8674(02)01082-6
2. Yamasaki M, Masgrau R, Morgan AJ, Churchill GC, Patel S, Ashcroft SJH, Galione A (2004) Organelle selection determines agonist-specific Ca^{2+} signals in pancreatic acinar and beta cells. J Biol Chem 279:7234–7240
3. Barceló-Torns M, Lewis AM, Gubern A, Barneda D, Bloor-Young D, Picatoste F, Churchill GC, Claro E, Masgrau R (2011) NAADP mediates ATP-induced Ca2 + signals in astrocytes. FEBS Lett 585:2300–2306. doi:10.1016/j.febslet.2011.05.062
4. Li H, Wang X, Zhang N, Gottipati MK, Parpura V, Ding S (2014) Imaging of mitochondrial Ca^{2+} dynamics in astrocytes using cell-specific mitochondria-targeted GCaMP5G/6s: mitochondrial Ca^{2+} uptake and cytosolic Ca^{2+} availability via the endoplasmic reticulum store. Cell Calcium 56:457–466. doi:10.1016/j.ceca.2014.09.008
5. Bazargani N, Attwell D (2016) Astrocyte calcium signaling: the third wave. Nat Neurosci 19:182–189. doi:10.1038/nn.4201
6. Bélanger M, Magistretti PJ (2009) The role of astroglia in neuroprotection. Dialogues Clin Neurosci 11:281–296. doi:10.1038/nrn1722
7. Verkhratsky A, Nedergaard M, Hertz L (2014) Why are astrocytes important? Neurochem Res 40:389–401. doi:10.1007/s11064-014-1403-2
8. Perea G, Sur M, Araque A (2014) Neuron-glia networks: integral gear of brain function. Front Cell Neurosci 8:378. doi:10.3389/fncel.2014.00378
9. Zorec R, Horvat A, Vardjan N, Verkhratsky A (2015) Memory formation shaped by astroglia. Front Integr Neurosci 9:56. doi:10.3389/fnint.2015.00056
10. Byrne JH, Kandel ER (1996) Presynaptic facilitation revisited: state and time dependence. J Neurosci 16:425–435
11. Deisseroth K, Bito H, Tsien RW (1996) Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity. Neuron 16:89–101. doi:10.1016/S0896-6273(00)80026-4
12. Benito E, Barco A (2010) CREB's control of intrinsic and synaptic plasticity: implications for CREB-dependent memory models. Trends Neurosci 33:230–240. doi:10.1016/j.tins.2010.02.001
13. Carriba P, Pardo L, Parra-Damas A, Lichtenstein MP, Saura CA, Pujol A, Masgrau R, Galea E (2012) ATP and noradrenaline activate CREB in astrocytes via noncanonical Ca^{2+} and cyclic AMP independent pathways. Glia 60:1330–1344
14. Deak M, Clifton AD, Lucocq JM, Alessi DR (1998) Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. EMBO J 17:4426–4441. doi:10.1093/emboj/17.15.4426
15. Xing J, Ginty DD, Greenberg ME (1996) Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase. Science 273:959–963. doi:10.1126/science.273.5277.959
16. Altarejos JY, Montminy M (2011) CREB and the CRTC co-activators: sensors for hormonal and metabolic signals. Nat Rev Mol Cell Biol 12:141–151. doi:10.1038/nrm3072
17. Barco A, Alarcon JM, Kandel ER (2002) Expression of constitutively active CREB protein facilitates the late phase of long-term potentiation by enhancing synaptic capture. Cell 108:689–703. doi:10.1016/S0092-8674(02)00657-8
18. Pardo L, Schlüter A, Valor LM, Barco A, Giralt M, Golbano A, Hidalgo J, Jia P, Zhao Z, Jové M, Portero-Otin M, Ruiz M, Giménez-Llort L, Masgrau R, Pujol A, Galea E (2016) Targeted activation of CREB in reactive astrocytes is neuroprotective in focal acute cortical injury. Glia 64:853–874. doi:10.1002/glia.22969
19. Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260:3440–3450 **(3838314)**
20. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45. doi:10.1093/nar/29.9.e45
21. Reusch JE, Colton LA, Klemm DJ (2000) CREB activation induces adipogenesis in 3T3-L1 cells. Mol Cell Biol 20:1008–1020. doi:10.1128/MCB.20.3.1008-1020.2000
22. Rothem L, Stark M, Assaraf YG (2004) Impaired CREB-1 phosphorylation in antifolate-resistant cell lines with down-regulation of the reduced folate carrier gene. Mol Pharmacol 66:1536–1543. doi:10.1124/mol.104.004135
23. Atzori M, Cuevas-Olguin R, Esquivel-Rendon E, Esquivel-Rendon E, Garcia-Oscos F, Salgado-Delgado RC, Saderi N, Miranda-Morales M, Treviño M, Pineda JC (2016) Locus ceruleus norepinephrine release: a central regulator of CNS spatio-temporal activation? Front Synaptic Neurosci. doi:10.3389/fnsyn.2016.00025
24. Mather S, Sakaki M, Harley CW (2000) Norepinephrine ignites local hot spots of neuronal excitation: how arousal amplifies selectivity in perception and memory. Behav Brain Sci 16:217–265
25. Salgado H, Köhr G, Treviño M (2012) Noradrenergic "tone" determines dichotomous control of cortical spike-timing-dependent plasticity. Sci Rep 2:1–7. doi:10.1038/srep00417
26. Paukert M, Agarwal A, Cha J, Doze VA, Kang JU, Bergles DE (2014) Norepinephrine controls astroglial responsiveness to local circuit activity. Neuron 82:1263–1270. doi:10.1016/j.neuron.2014.04.038
27. Carmignoto G, Haydon PG (2012) Astrocyte calcium signaling and epilepsy. Glia 60:1227–1233. doi:10.1002/glia.22318
28. Zorec R, Araque A, Carmignoto G, Haydon PG, Verkhratsky A, Parpura V (2012) Astroglial excitability and gliotransmission: an

appraisal of Ca^{2+} as a signalling route. ASN Neuro 4:103–119. doi:10.1042/AN20110061
29. Delekate A, Füchtemeier M, Schumacher T, Ulbrich C, Foddis M, Petzold GC (2014) Metabotropic P2Y1 receptor signalling mediates astrocytic hyperactivity in vivo in an Alzheimer's disease mouse model. Nat Commun 5:5422. doi:10.1038/ncomms6422
30. Khakh BS, Sofroniew MV (2015) Diversity of astrocyte functions and phenotypes in neural circuits. Nat Neurosci 18:942–952. doi:10.1038/nn.4043
31. Ahn S, Olive M, Aggarwal S, Krylov D, Ginty DD, Vinson C (1998) A dominant-negative inhibitor of CREB reveals that it is a general mediator of stimulus-dependent transcription of c-fos. Mol Cell Biol 18:967–977
32. Naylor E, Arredouani A, Vasudevan SR et al (2009) Identification of a chemical probe for NAADP by virtual screening. Nat Chem Biol 5:220–226. doi:10.1038/nchembio.150
33. Suzuki J, Kanemaru K, Ishii K, Ohkura M, Okubo Y, Lino M (2014) Imaging intraorganellar Ca^{2+} at subcellular resolution using CEPIA. Nat Commun 5:4153. doi:10.1038/ncomms5153
34. Raturi A, Simmen T (2013) Where the endoplasmic reticulum and the mitochondrion tie the knot: the mitochondria-associated membrane (MAM). Biochim Biophys Acta-Mol Cell Res 1833:213–224. doi:10.1016/j.bbamcr.2012.04.013
35. Zhang Y, Lv X, Bai Y, Zhu X, Wu X, Chao J, Duan M, Buch S, Chen L, Yao H (2015) Involvement of sigma-1 receptor in astrocyte activation induced by methamphetamine via up-regulation of its own expression. J Neuroinflammation 12:29. doi:10.1186/s12974-015-0250-7
36. He J, Shi W, Guo Y, Chai Z (2014) ERp57 modulates mitochondrial calcium uptake through the MCU. FEBS Lett 588:2087–2094. doi:10.1016/j.febslet.2014.04.041
37. Shanmughapriya S, Rajan S, Hoffman NE et al (2015) Ca^{2+} signals regulate mitochondrial metabolism by stimulating CREB-mediated expression of the mitochondrial Ca^{2+} uniporter gene MCU. Sci Signal 8:ra23. doi:10.1126/scisignal.2005673
38. Dash PK, Hochner B, Kandel ER (1990) Injection of the cAMP-responsive element into the nucleus of Aplysia sensory neurons blocks long-term facilitation. Nature 345:718–721. doi:10.1038/345718a0
39. Denton RM, Mccormack JG (1980) On the role of the calcium transport cycle in heart and other mammalian mitochondria. FEBS Lett 119:1–8
40. Jouaville LS, Pinton P, Bastianutto C, Rutter GA, Rizzuto R (1999) Regulation of mitochondrial ATP synthesis by calcium: evidence for a long-term metabolic priming. Proc Natl Acad Sci USA 96:13807–13812. doi:10.1073/pnas.96.24.13807
41. Tarasov AI, Griffiths EJ, Rutter GA (2012) Regulation of ATP production by mitochondrial Ca2+. Cell Calcium 52:28–35. doi:10.1016/j.ceca.2012.03.003
42. Tagashira H, Bhuiyan MS, Shioda N, Fukunaga K (2014) Fluvoxamine rescues mitochondrial Ca^{2+} transport and ATP production through σ1-receptor in hypertrophic cardiomyocytes. Life Sci 95:89–100. doi:10.1016/j.lfs.2013.12.019
43. Panov A, Orynbayeva Z, Vavilin V, Lyakhovich V (2014) Fatty acids in energy metabolism of the central nervous system. Biomed Res Int 2014:472459. doi:10.1155/2014/472459
44. Serrano A, Haddjeri N, Lacaille J et al (2006) GABAergic network activation of glial cells underlies hippocampal heterosynaptic depression. J Neurosci 26:5370–5382. doi:10.1523/JNEUROSCI.5255-05.2006
45. Henneberger C, Papouin T, Oliet SHR, Rusakov DA (2010) Long-term potentiation depends on release of D-serine from astrocytes. Nature 463:232–236. doi:10.1038/nature08673
46. Takata N, Mishima T, Hisatsune C et al (2011) Astrocyte calcium signaling transfmorms cholinergic modulation to cortical plasticity in vivo. J Neurosci 31:18155–18165. doi:10.1523/JNEUROSCI.5289-11.2011
47. Navarrete M, Araque A (2010) Endocannabinoids potentiate synaptic transmission through stimulation of astrocytes. Neuron 68:113–126. doi:10.1016/j.neuron.2010.08.043
48. Pirttimaki TM, Parri HR (2013) Astrocyte plasticity: implications for synaptic and neuronal activity. Neuroscientist 19:604–615. doi:10.1177/1073858413504999
49. Perez-Alvarez A, Navarrete M, Covelo A, Martin ED, Araque A (2014) Structural and functional plasticity of astrocyte processes and dendritic spine interactions. J Neurosci 34:12738–12744. doi:10.1523/JNEUROSCI.2401-14.2014
50. Cheung G, Sibille J, Zapata J, Rouach N (2015) Activity-dependent plasticity of astroglial potassium and glutamate clearance. Neural Plast 2015:109106. doi:10.1155/2015/109106
51. Chaudhuri R, Fiete S (2015) Computational principles of biological memory. Nat Neurosci 19:394–403. doi:10.1038/nn.4237
52. Sweatt JD (2016) Neural plasticity and behavior—sixty years of conceptual advances. J Neurochem. doi:10.1111/jnc.13580
53. Hill JA, Olson EN (2008) Cardiac plasticity. N Engl J Med 358:1370–1380
54. Abeti R, Abramov AY (2015) Mitochondrial Ca^{2+} in neurodegenerative disorders. Pharmacol Res 99:377–381. doi:10.1016/j.phrs.2015.05.007
55. Kuchibhotla KV, Lattarulo CR, Hyman BT, Bacskai BJ (2009) Astrocytes in Alzheimer. Science 323:1211–1215. doi:10.1126/science.1169096
56. Li H, Xie Y, Zhang N, Yu Y, Zhang Q, Ding S (2015) Disruption of IP3R2-mediated Ca^{2+} signaling pathway in astrocytes ameliorates neuronal death and brain damage while reducing behavioral deficits after focal ischemic stroke. Cell Calcium 58:565–576. doi:10.1016/j.ceca.2015.09.004
57. Ruscher K, Wieloch T (2015) The involvement of the sigma-1 receptor in neurodegeneration and neurorestoration. J Pharmacol Sci 127:30–35. doi:10.1016/j.jphs.2014.11.011

Chapter 1. Part2.

CREB inhibition shapes *in situ* norepinephrine-induced calcium responses in astrocytes

Eraso-Pichot A., Srinivasan R., Khakh B.S., Galea E., Masgrau R

Rationale

In the previous, published article we have established a new signaling pathway by which CREB is able to modify astrocytic calcium responses. Specifically, in a culture model of cortical astrocytes, activation of CREB-dependent pathways with the construct VP16-CREB or by neurotransmitters reduces subsequent neurotransmitter-induced cytosolic calcium responses through an increase in mitochondrial calcium entry. The effect is mediated by SigmaR1, a chaperone located in the interface between both organelles whose expression is enhanced by CREB in astrocytes.

Calcium signals differ substantially between cultured and *in situ* or *in vivo* astrocytes. While cultured astrocytes are a valid model to unveil new molecular mechanisms, they only present neurotransmitter-induced somatic events (i.e. IP_3R-dependent events with a global effect), whereas *in vivo* astrocytes present more complex morphology and territorial calcium responses.

The objective of this set of experiments is to analyze the effect of CREB in *in situ* astrocytes using slices of mouse brain.

Results

1. Generation and characterization of serotype 9 adenoviruses expressing VP16-CREB and A-CREB constructs

To analyze *in situ* calcium signals we generated three different serotype 9 adeno-asociated viral vectors (AAV9) containing the constitutively active VP16-CREB, the dominant negative A-CREB, or no construct at all, the so-called control of infection or Null virus.

Gene maps of the two CREB viruses are shown in Fig 1. Transgene expression is under the control of a short variant of the GFAP promoter, ABC1D GFAP, thus, its expression is restricted to astrocytes (Lee et al., 2008). Moreover, the three viruses contain the TdTomato protein to identify the infected astrocytes by microscopy. Importantly, Red TdTomato fluorescence does not interfere with calcium fluorescence indicators. These AAVs contain a 2A sequence, which is a self-cleaving peptide that allows the expression of both transgenes (TdTomato and the CREB-modifier) separately. This 2A technology was recently discovered and it may have important uses in the future due to its high cleavage efficiency (Luke and Ryan, 2018).

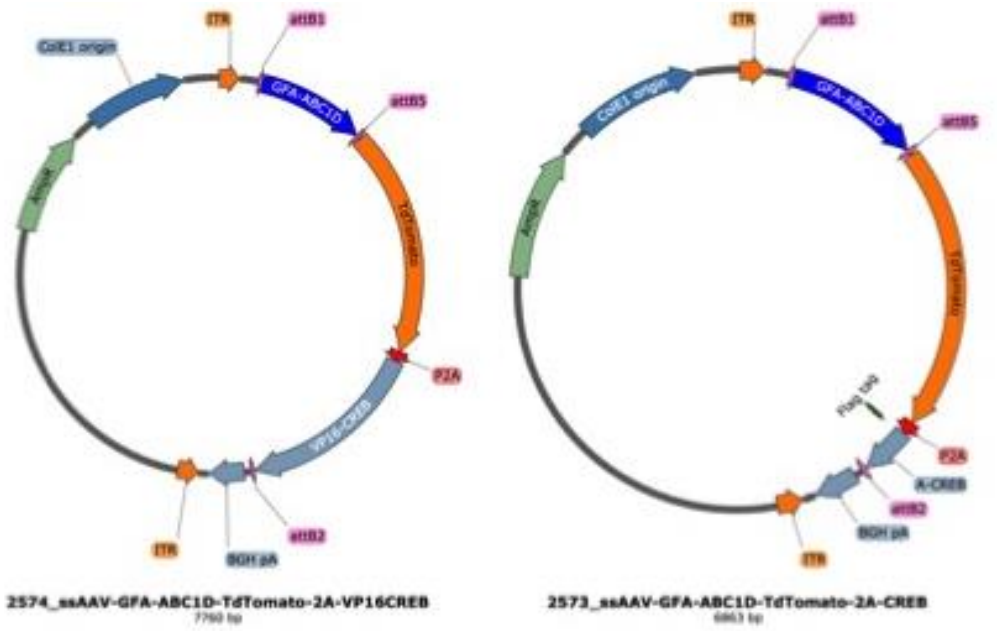

Figure 1: Gene maps of CREB constructs inserted into AAV9 viruses.

In order to characterize the effect of the newly generated viral vectors, 6-week-old C57/Bl6 mice were injected with Null, A-CREB or VP16-CREB AAVs in the hippocampus. Three weeks later, brains were dissected out and viral expression was analyzed (Figure 2). While Null and A-CREB infected astrocytes looked healthy and TdTomato expression was high, VP16-CREB infected astrocytes had very little TdTomato expression and their

morphology resembled those of diseased astrocytes lacking their typical fluffy structure (Figure 2, right panel).

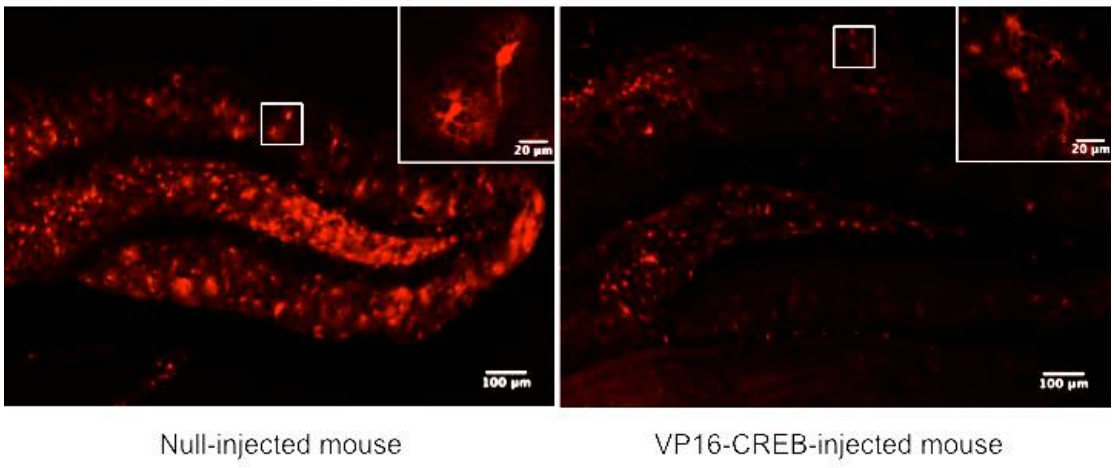

Figure 2: TdTomato expression of Null and VP16-CREB infected hippocampal astrocytes. VP16-CREB astrocytes showed low transgene expression and clear atrophy.

We conclude that virally-transduced CREB overexpression is toxic to astrocytes and probably induce cell death. This is consistent with observations in the bitransgenic VP16-CREB^{high} mice, where high expression of the transgene in neurons causes excitotoxic cell death after three weeks of expression (Valor et al., 2010).

Thus, we decided to analyze only the effects of the inhibition of CREB-dependent transcription in calcium signaling of hippocampal astrocytes. To do so, mice were injected with AAV9-Null and AAV9-A-CREB together with the calcium indicator AAV2/5-gfaABC$_1$D-GCamp6f (Shigetomi et al., 2010).

Since the viruses injected together had different serotypes (AAV9 and AAV2/5), we had to optimize the protocol in order to ensure a good colocalization and that the viral load resulting from infection with two viruses was harmless for astrocytes. We tested viral concentrations ranging from 2xE^{12} to 2xE^{10} vg. In this last concentration (2xE^{10} vg/each virus), after 3 weeks, most of the astrocytes were successfully targeted by both viruses, colocalization being higher than 80% (Figure 3). Moreover, using this viral load, calcium activity showed no differences between Null and GCamp6f-infected astrocytes compared to those astrocytes expressing only GCamp6f, thus allowing us to continue with the calcium experiments. GCamp6f-only controls will be analyzed in parallel with the other viruses in order to detect if our TdTomato viruses affect calcium signals during the protocol.

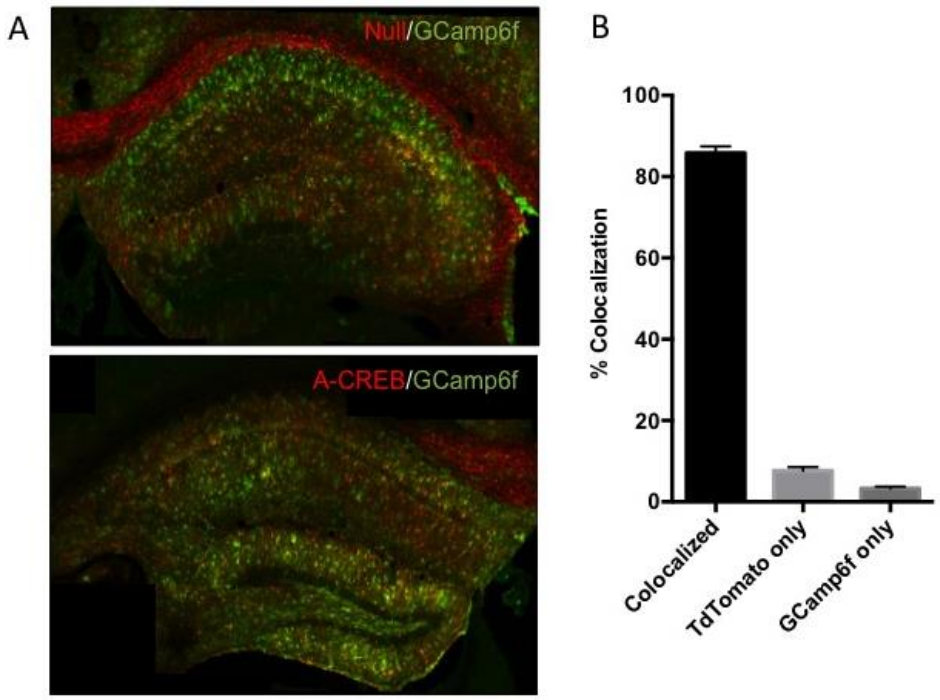

Figure 3: Combined hippocampal transgene and GCamp6f expression in astrocytes. (A) Representative images of Null and A-CREB / GCamp6f injected hippocampus. (B) Percentage of colocalization of both viruses. Data are the mean ± SEM of six different regions of six different animals (three null and three A-CREB).

2. Calcium signals *in situ*

To analyze calcium signals *in situ* we selected those astrocytes expressing both the transgene (TdTomato) and the calcium indicator. In each cell we measured three different calcium signals: spontaneous calcium activity, ATP-induced responses and phenylephrine-induced responses. These three different measurements allow us to detect which type of calcium activity may be affected by CREB inhibition, if any. The exact protocol used is described in Fig 4A. In addition, all the experiments were realized with 0.5 μM tetradotoxin (TTX) in the bath, in order to avoid interferences due to neuronal activity.

Different types of calcium signals have been detected depending on the intracellular location (Volterra et al., 2014). In order to analyze different signals, we measured two different structures of the astrocyte: the soma and the primary branches (Fig 4B). Figure 4C shows representative traces of calcium events in the soma (red) and primary branches (yellow) of the same cell. As it can be seen, although most of the response is similar, some calcium events are really high in the soma and are not translated to the processes (Fig 4C, red arrow) whereas some events are detected only in the branches (Fig 4C, yellow arrow). Analyzing both compartments we will be able to detect subtle differences induced by CREB depending on the area.

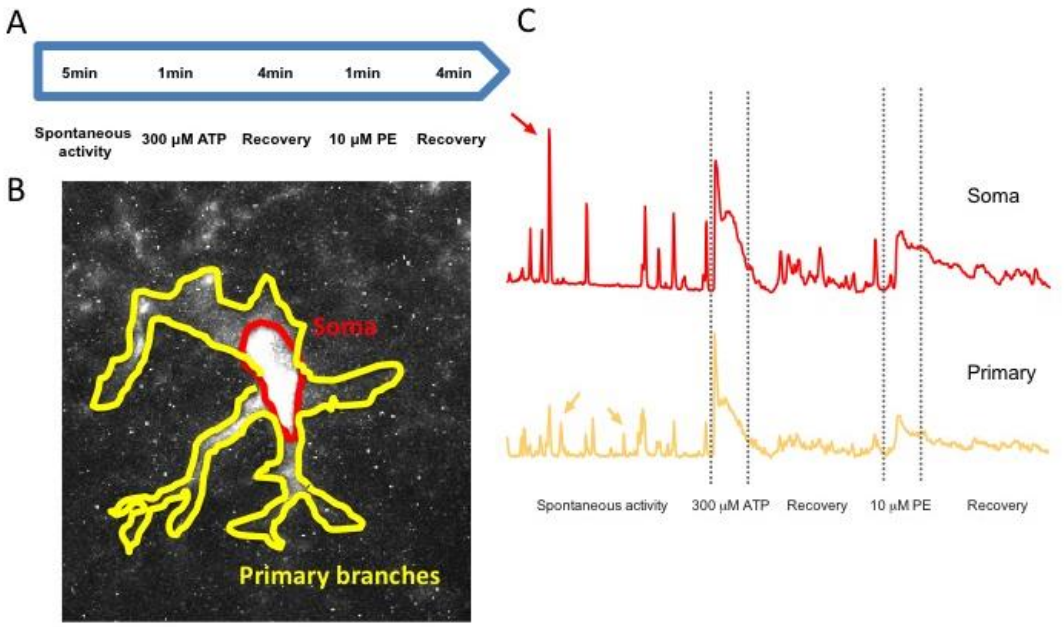

Figure 4: Analysis of astrocytic calcium signals in situ. (A) Protocol used. (B) Analyzed regions of the astrocyte. (C) Representative traces of somatic (red) and primary processes (yellow) events during the protocol in the same null-infected astrocyte.

2.1 Spontaneous activity

Spontaneous activity is defined as the calcium peaks of infected astrocytes that are not induced by an external agonist. To analyze spontaneous activity we measured three parameters: the number of events (sparks) per minute (frequency), the height of the peak (amplitude) and the duration of the event (half-width).

Spontaneous activity was very similar between the three groups of animals. No differences were seen in frequency nor amplitude of calcium responses in the soma (Fig 5A-B) or primary branches (Fig 5D-F). However, differences were seen in the half width parameter of A-CREB peaks compared to controls, with a clear tendency in the somatic events (Fig. 5C) and significantly different in the branches (Fig 5F).

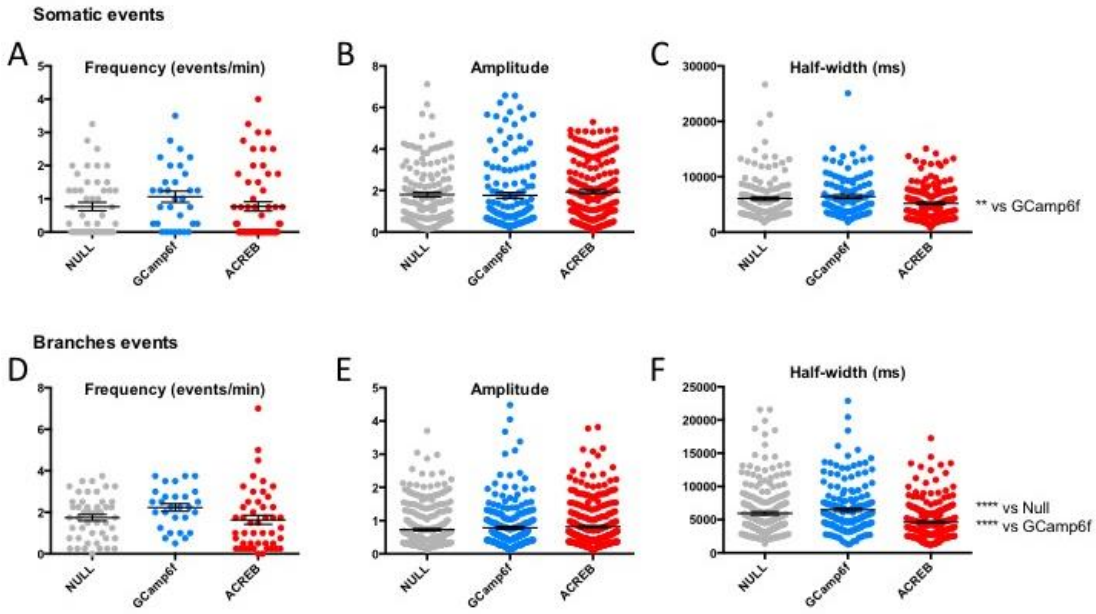

*Figure 5: Analysis of spontaneous activity. (A-C) Somatic spontaneous events characterization. Each point represents a single event. Data are mean ± SEM of 137 events from 37 cells of 4 GCamp6f-injected mice, 147 events of 52 cells of 6 Null-injected animals and 179 events of 63 cells from 6 A-CREB injected mice. **p<0.01 one way ANOVA. (D-F) Spontaneous events characterization in branches. Data are mean ± SEM of 249 events from 37 cells of 4 GCamp6f-injected mice, 327 events of 52 cells of 6 Null-injected animals and 308 events of 63 cells from 6 A-CREB injected mice. ****p<0.0001 one way ANOVA.*

2.2. Analysis of ATP and phenylephrine responses

Our previous *in vitro* results showed differences in agonist-induced calcium events after CREB activation, independently of the agonist used. In these *in situ* experiments, we have used two different agonists targeting different astrocytic receptors and inducing particular calcium responses in time and magnitude. In this way we will be able to analyze if the CREB-induced changes are common for all agonists or agonist-specific. Since we have targeted mainly purinergic and adrenergic receptors in our *in vitro* work, we selected ATP and phenylephrine (α_1-adrenergic agonist) as neurotransmitters.

ATP is a known neurotransmitter in the CNS, although the calcium signals it induces *in situ* are not as strong as the ones elicited by other agonists. Purinergic astrocytic signaling has gained special interest in recent years since it has been related to the abnormal astrocytic calcium signals seen in Alzheimer's disease models (Delekate et al., 2014). In order to analyze the purinergic responses of CREB-inhibited astrocytes, 300 μM ATP was added to the solution after recording spontaneous activity for 300 seconds.

Phenylephrine (PE) is a α_1-adrenergic receptor agonist that mimics the effect of noradrenaline. Adrenergic signaling mediates *in vivo* startle responses and it has been proposed as an important neuromodulator of astrocytic physiology (Ding et al., 2013) (Poskanzer and Yuste, 2016). After a recovery time (240 seconds) for ATP responses, 10 μM PE was added to the bath and responses were analyzed.

Regarding ATP responses, no differences were seen between the three groups with regards to somatic responses, both in amplitude (Fig 6A) or area under the curve (AUC) (FIG 6B). Similarly, no differences were seen in branches responses (Fig 6C-D).

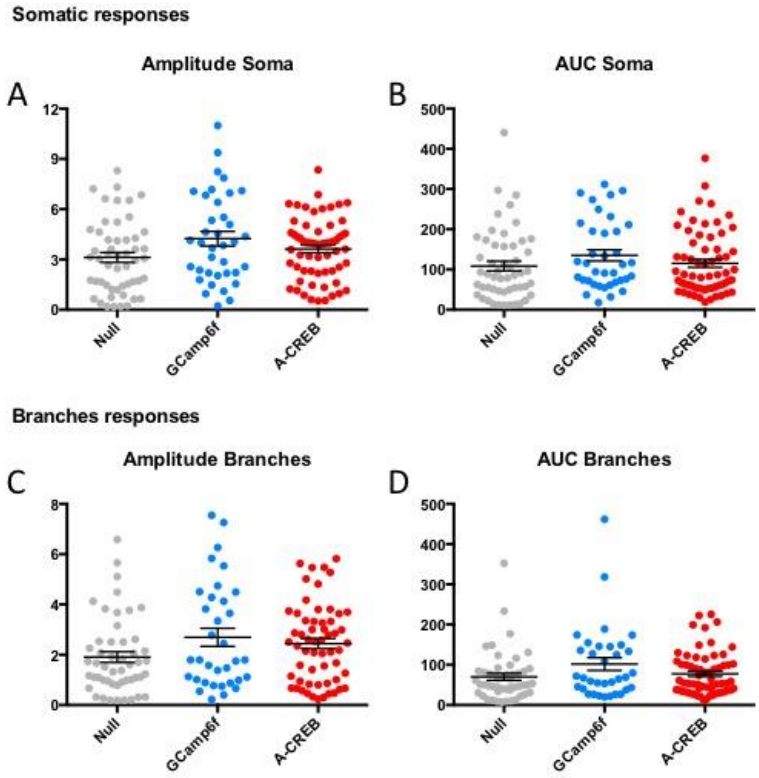

Figure 6: ATP responses of control, Null and A-CREB infected astrocytes. (A-B) Somatic responses to ATP. (C-D) Branches responses to ATP. Each point represents a single response of one cell. Data are mean± SEM of 37 cells of 4 GCamp6f-injected mice, 52 cells of 6 Null-injected animals and 63 cells from 6 A-CREB injected mice.

Likewise, when PE was added, the amplitude of the peak of calcium responses was similar between the three groups both in somatic (Fig 7A) and branches events (Fig 7C). By contrast, significant differences were seen in the AUC of the response, especially in the soma (Fig 7B).

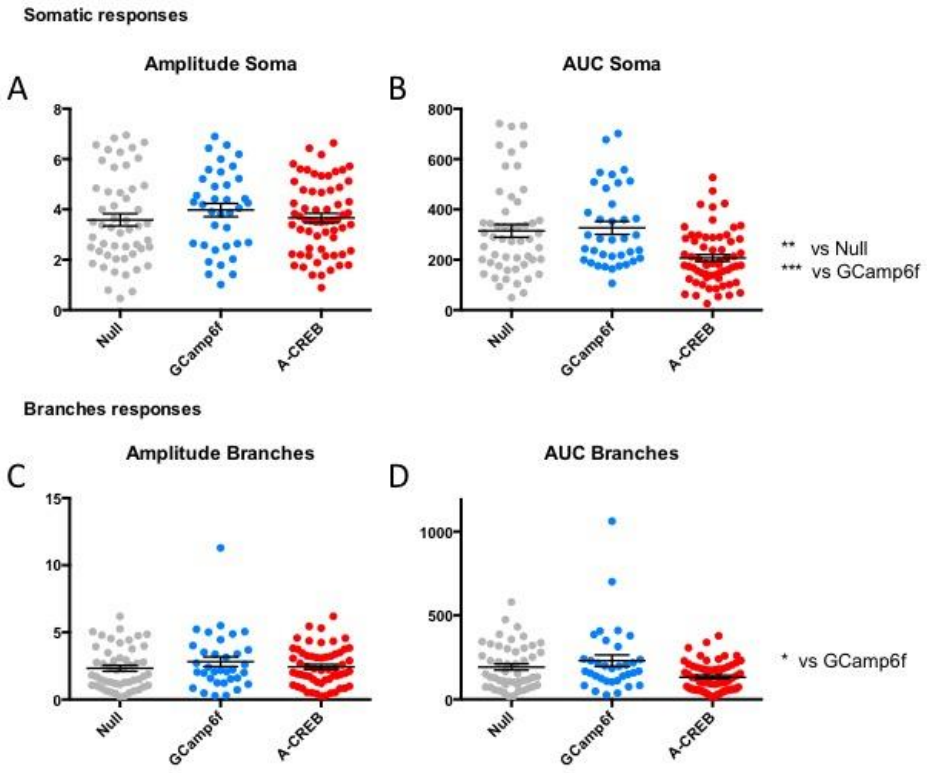

*Figure 7: Phenylephrine responses in infected astrocytes. (A-B) Phenylephrine somatic responses. (C-D) Branches responses to PE. Each point represents a single response of one cell. Data are mean± SEM of 37 cells of 4 GCamp6f-injected mice, 52 cells of 6 Null-injected animals and 63 cells from 6 A-CREB injected mice. *p< 0.05, **p<0.01, *** p<0.001 one way ANOVA.*

Interestingly, reduction in the AUC of the PE responses is produced by a change in the shape of the response. Representative traces exemplify these differences (Fig 8A). First, A-CREB astrocytes present a "peaky" response to PE compared to null astrocytes (smooth arrows), with a similar amplitude peaks but with more oscillated response. Second, A-CREB astrocytes reach basal calcium levels earlier (dotted arrows). The resulting average of all cells shows the decrease in the AUC of A-CREB astrocytes in soma (Fig 8B) and primary branches responses (Fig 8C).

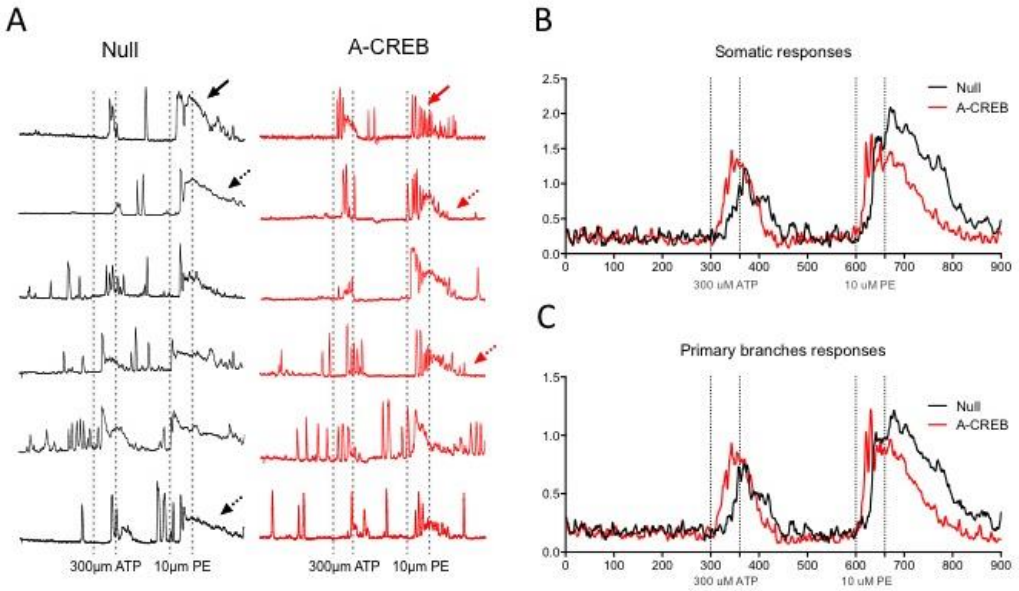

Figure 8: Average and representative traces of the experiment- (A) Representative traces of the entire recordings in Null and A-CREB transfected astrocytes. Each trace is obtained from a different mouse. (B) Average traces of the somatic responses of all cells analyzed. (C) Average traces of the primary branches responses of all cells.

Discussion

Astrocytic calcium is considered the key mechanism of excitability and the way astrocytes communicate with other cell types including neurons. In this regard, *in situ* calcium experiments are necessary to understand how astrocytic calcium is regulated in a more physiological situation. In this preliminary study, we have designed and optimized a protocol to understand how modulation of CREB-dependent transcription may change astrocytic calcium signals. Two different strategies were used to address this question: a gain of function strategy, which consisted in the expression of a constitutively active CREB (VP16-CREB) construct in astrocytes; and a loss of function strategy using expression of the dominant-negative A-CREB. Unfortunately, VP16-CREB expression was harmful to astrocytes and thus our experiments only addressed the effect of the inhibition of CREB-dependent transcription. Importantly, these experiments had an implicit assumption: the existence of a basal CREB-dependent transcription in *in situ* astrocytes.

Taking that into account, our preliminary results showed that A-CREB astrocytes had reductions in the magnitude of PE and spontaneous calcium responses. Interestingly, these reductions in calcium fluxes are related to the duration of the event (half width in spontaneous activity and AUC in PE response) and not its amplitude, which remains unchanged. One first hypothesis to explain these differences in the duration of the response will be that inhibition of CREB-dependent transcription does not change ER-induced release (which may be responsible for the amplitude of the peak), but increases other mechanisms to extrude calcium to intracellular compartments or to the extracellular medium. The differences in shape seen in the PE responses, being more

oscillative and short in A-CREB astrocytes, are in line with this hypothesis of an enhancement of a buffering mechanism that reduces cytosolic calcium peaks faster.

This hypothesis point to differences in the general calcium toolkit of the astrocyte (a buffering mechanism). However, our results showed no differences in the ATP responses, in which calcium signals influx and efflux mechanisms should be similar to those produced during spontaneous and PE responses. Thus, one other possible explanation of our results could be that inhibition of CREB-dependent transcription affects a specific response to the noradrenergic signaling pathway, inhibiting the transcription of the mediators of the response such as the same α_1-adrenergic receptors. If this is the case, adrenergic signaling may be mediating part of the spontaneous responses of astrocytes, since we also see differences in calcium rises without agonist stimulation. This is in line with previous discoveries showing that in awake adult mice, spontaneous events are inhibited by the α_1-adrenergic receptor antagonist prazosin (Ding et al., 2013). Interestingly, noradrenaline release has been seen to be only partially inhibited by TTX (Chiti and Teschemacher, 2007), however, further research is needed to study the integrity and functionality of adrenergic neurons in brain slices.

The data presented here is not sufficient to discern which hypothesis is more plausible. Now that the protocol is optimized, further experiments will be done in order to understand if CREB is affecting a general calcium mechanism (as it does in cultures) or the adrenergic responses specifically.

Moreover, although we have detected a promising phenotype in these preliminary studies on A-CREB astrocytes, our results do not fit the characteristic calcium responses seen in our *in vitro* experiments after CREB activation. In our *in vitro* results, CREB activation by noradrenaline reduced further neurotransmitter calcium responses, a reduction that was abolished by the dominant negative CREB. Although we did not see differences in calcium responses in A-CREB astrocytes in cultures, we expected that CREB might be activated in *in situ* astrocytes. However, the discordance between both results make us think that as in cultures, *in situ* astrocytic CREB may have two different phenotypes: a basal CREB controlling a specific regulon and an activity-dependent CREB, which is the one that we mimicked using noradrenaline or ATP in cultures. Thus, in these *in situ* experiments, we may have studied the role of basal CREB, which is different from the one studied in cultures. Taking that into account, apart from the unraveling of the mechanisms of the inhibition of basal CREB-dependent transcription, further experiments will include a previous CREB activation. As an example, stimulation of the locus coeruleus induces noradrenaline release to different brain areas, including the hippocampus (Hansen, 2017). Thus, it may be interesting to provoke this noradrenaline release from the locus coeruleus before analyzing calcium signals in A-CREB infected astrocytes. Incubation of brain slices with noradrenaline before the calcium experiments may be another possibility to induce astrocytic CREB activation.

Finally, transduction of VP16-CREB in astrocytes using our AAV9 leads to astrocytic atrophy and cell death. Future work should include the design of new strategies to increase CREB signalling in *in vivo* astrocytes to a lower extent, such as astrocytic

overexpression of the wild type transcription factor (Yu et al., 2017) or other CREB active mutants such as DIEDML (Cardinaux et al., 2000) or Y134F (Restivo et al., 2009).

In summary, we have generated two new AAVs to modify CREB *in vivo* and optimized an imaging protocol to study calcium *in situ*. Moreover, we have obtained preliminary results probably regarding basal CREB inhibition in astrocytes, but establishing the first steps to understand which are the situations of CREB activation and how to study them.

References:

Cardinaux, J.R., Notis, J.C., Zhang, Q., Vo, N., Craig, J.C., Fass, D.M., Brennan, R.G., and Goodman, R.H. (2000). Recruitment of CREB binding protein is sufficient for CREB-mediated gene activation. Mol Cell Biol *20*, 1546-1552.

Chiti, Z., and Teschemacher, A.G. (2007). Exocytosis of norepinephrine at axon varicosities and neuronal cell bodies in the rat brain. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *21*, 2540-2550.

Delekate, A., Fuchtemeier, M., Schumacher, T., Ulbrich, C., Foddis, M., and Petzold, G.C. (2014). Metabotropic P2Y1 receptor signalling mediates astrocytic hyperactivity in vivo in an Alzheimer's disease mouse model. Nature communications *5*, 5422.

Ding, F., O'Donnell, J., Thrane, A.S., Zeppenfeld, D., Kang, H., Xie, L., Wang, F., and Nedergaard, M. (2013). alpha1-Adrenergic receptors mediate coordinated Ca2+ signaling of cortical astrocytes in awake, behaving mice. Cell Calcium *54*, 387-394.

Hansen, N. (2017). The Longevity of Hippocampus-Dependent Memory Is Orchestrated by the Locus Coeruleus-Noradrenergic System. Neural Plast *2017*, 2727602.

Lee, Y., Messing, A., Su, M., and Brenner, M. (2008). GFAP promoter elements required for region-specific and astrocyte-specific expression. Glia *56*, 481-493.

Luke, G.A., and Ryan, M.D. (2018). Using the 2A Protein Coexpression System: Multicistronic 2A Vectors Expressing Gene(s) of Interest and Reporter Proteins. Methods Mol Biol *1755*, 31-48.

Poskanzer, K.E., and Yuste, R. (2016). Astrocytes regulate cortical state switching in vivo. Proc Natl Acad Sci U S A *113*, E2675-2684.

Restivo, L., Tafi, E., Ammassari-Teule, M., and Marie, H. (2009). Viral-mediated expression of a constitutively active form of CREB in hippocampal neurons increases memory. Hippocampus *19*, 228-234.

Shigetomi, E., Kracun, S., Sofroniew, M.V., and Khakh, B.S. (2010). A genetically targeted optical sensor to monitor calcium signals in astrocyte processes. Nature neuroscience *13*, 759-766.

Valor, L.M., Jancic, D., Lujan, R., and Barco, A. (2010). Ultrastructural and transcriptional profiling of neuropathological misregulation of CREB function. Cell Death Differ *17*, 1636-1644.

Volterra, A., Liaudet, N., and Savtchouk, I. (2014). Astrocyte Ca(2)(+) signalling: an unexpected complexity. Nat Rev Neurosci *15*, 327-335.

Yu, X.W., Curlik, D.M., Oh, M.M., Yin, J.C., and Disterhoft, J.F. (2017). CREB overexpression in dorsal CA1 ameliorates long-term memory deficits in aged rats. eLife *6*.

Chapter 2:

Astrocytic CREB controls the expression of Monocarboxylate Transporter 4 in normoxia

Article in preparation

Astrocytic CREB controls the expression of Monocarboxylate Transporter 4 in normoxia

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ABSTRACT

Lactate is considered a major communication factor between astrocytes and neurons in the so-called Astrocyte Neuron Lactate Shuttle (ANLS). One of the main elements of lactate export to the extracellular medium is the monocarboxylate transporter 4 (MCT4), which is exclusively expressed in astrocytes. In hypoxic situations, MCT4 expression is upregulated in astrocytes by the hypoxia inducible factor 1 (HIF-1α), giving rise to the idea that MCT4 is only regulated by this transcription factor. However, while HIF-1α is rarely found in normoxia, MCT4 is upregulated in hippocampal training and after β-adrenergic stimulation in normoxic conditions, in correlation with improved performance in learning and memory tests. Our hypothesis is that cAMP Response Element Protein (CREB), which is activated by adrenergic transmitters, may mediate the increase in MCT4 seen in normoxia. In order to test this hypothesis, we evaluated whether CREB-dependent transcription induced by the adrenergic agonist noradrenaline (NA) as well as the constitutively active CREB construct VP16-CREB, increases MCT4 mRNA and protein expression and lactate release. We found that activation of CREB-dependent transcription in astrocytes increases MCT4 expression *in vitro* and *in vivo.* Importantly, MCT4 upregulation is independent from hypoxia and HIF-1α. This represents a novel pathway by which astrocytic CREB mediates the potentiation of adrenergic-induced modulation of learning and memory via the regulation of MCT4 expression.

INTRODUCTION

Astrocytes present a high glycolytic capacity, consuming most of the glucose in the brain and extruding lactate, which is considered the main metabolic substrate for neighbouring neurons (Magistretti and Allaman, 2015). Lactate has been demonstrated to mediate long-term memory formation and decision making in rodents (Suzuki et al., 2011) (Wang et al., 2017), establishing a new pathway by which astrocytes contribute to synaptic plasticity and the formation of new memories. Besides its function as a fuel, lactate may act as signalling molecule by binding to a specific G-protein-coupled lactate receptor in neurons regulating cAMP levels (Morland et al., 2015) (Tang et al., 2014), thus adding another role in lactate-mediated astrocyte-neuron communications. Moreover, extracellular lactate is protective in pathological situations such as traumatic brain injury (Patet et al., 2016), which suggests that the early liberation of lactate from astrocytes may be a promising target in pathological situations.

Lactate release by astrocytes depends on the monocarboxylate transporters 1 and 4 (MCT1 and MCT4). MCT1 is ubiquitously expressed in the brain (Perez-Escuredo et al.,

2016), while MCT4 is exclusively found in astrocytes (Pellerin et al., 2005). Both transporters belong to the family of monocarboxylate transporters, which carry monocarboxylates such as lactate or pyruvate inside and outside the cell. The transport is proton-linked and the members of the family are codified by SLC16A genes (Jones and Morris, 2016). Among all MCTs, MCT4 has the lowest affinity for lactate and pyruvate ($Km_{lactate}$ = 22-28 mM, $Km_{pyruvate}$ = 150mM), MCT4 being the main transporter for the extrusion of lactate. In fact, MCT4 is found in highly glycolytic tissues, such as skeletal muscle fibers, white blood cells and tumours (Halestrap and Wilson, 2012).

MCT4 expression in astrocytes in ischemic situations is controlled by HIF-1α, a transcription factor implicated in protective responses to hypoxia (Rosafio and Pellerin, 2014), in part due to the increase in glycolytic enzymes and lactate release (Sharp et al., 2004) (Li et al., 2017). However, recent evidence suggests a regulation of MCT4 in normoxic conditions. First, MCT4 expression in mouse astrocytes is enhanced after hippocampal training as in inhibitory avoidance (Tadi et al., 2015). Second, administration of the β-adrenergic agonist formoterol in mice increases MCT4 gene expression and improves performance in learning and memory tests (Dong et al., 2017). Since HIF-1α expression is tightly regulated by oxygen contents and is rarely found expressed in normoxic conditions (Suzuki et al., 2017), one could speculate that there is a transcription factor other than HIF-1α governing MCT4 expression in learning-related context.

In this sense, we have demonstrated that the adrenergic transmitter noradrenaline (NA) activates CREB in astrocytes (Carriba et al., 2012), inducing long-lasting dependent changes in calcium signalling (Eraso-Pichot et al., 2017) that might regulate astrocyte-neuron communications. Thus, our hypothesis is that NA-dependent activation of CREB-dependent transcription in astrocytes may be responsible for the changes seen in MCT4 expression under adrenergic treatment or after hippocampal learning.

In this study, we propose a novel mechanism of activity-dependent MCT4 regulation via CREB that is independent from HIF-1α regulation in hypoxia. Activation of CREB-dependent transcription by NA and the constitutively active form of CREB VP16-CREB increases MCT4 expression under normoxic situations. This effect is CREB-dependent and HIF-1α-independent since the dominant-negative form of CREB (A-CREB) abolished MCT4 increases while HIF-1α inhibition does not. Moreover, HIF-1α-dependent increases in MCT4 expression in hypoxia seems to be dependent on CREB. Finally, astrocytic VP16-CREB transgenic mice showed increased expression of MCT4 *in vivo.* As a summary, our results demonstrate that MCT4 is regulated by CREB transcription factor in astrocytes under normoxic conditions, establishing a novel signalling pathway by which astrocytic CREB contributes to astrocyte-neuron communication.

METHODS

Astrocyte cultures

Secondary astrocyte cultures were prepared from one-day-old Sprague Dawley rats. Briefly, brains were dissected out, minced and treated for 10 minutes at 37°C with 0.025% trypsin in Ca2+-free Krebs Ringer Buffer (KRB). After the incubation, the reaction was stopped with soybean trypsin inhibitor and the tissue was mechanically triturated and filtered through a 40-μm nylon mesh. Collected cells were plated in T150 flasks in 10% fetal bovine serum DMEM medium with 50 U/mL penicillin and 50μg/ml streptomycin. Flasks were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The medium was replaced after two hours to prevent other cells from growing. Flasks were plated in 24 and 6-well plates after reaching confluence after a 10-minutes mechanical shaking to eliminate microglial cells.

Mouse model

To analyse MCT4 expression in vivo, we made use of the Gfa2-tTa/TetO-VP16-CREB mice, which expresses the VP16-CREB construct in astrocytes (Pardo et al., 2016). Mice were kept under standard laboratory conditions with ad libitum food and water, 22 ± 2 °C of temperature and 12h light/dark cycle. Doxycycline was not administered to mice to assure continuous expression of the transgene. The Animal Welfare Committee of the Universitat Autònoma de Barcelona and the Generalitat de Catalunya approved all experimental procedures. All the procedures were in agreement with the European Union Laws for the protection of experimental animals.

Cryolesion

To induce robust transgene expression, we performed a cryolesion protocol as previously described (Pardo et al., 2016). Briefly, five-to-seven month-old mice were cryolesioned by the application of a dry-ice pellet directly to the skull under tribromoethanol anesthesia. Buprenorphin (0.1 mg/kg) was injected i.p. every 12h after the lesion and until sacrifice. Female and male mice were equally distributed.

Noradrenaline stimulation

Secondary astrocytic cultures were stimulated with 10 μM NA (Sigma) dissolved in DMEM for the indicated times. NA dilution was freshly prepared every time.

Viral infection

CREB activation/inhibition was done by the viral transduction of CREB constructs. Three serotype 5 adenoviruses have been used: Ad5-VP16-CREB contained a constitutively active CREB, Ad5-A-CREB harboured a dominant-negative form of the transcription factor and Ad5-Null was the control of infection. Infections were carried in a medium with 1% FBS and without antibiotics. Medium was replaced for full DMEM 3h

after. The experiments were conducted at 18-24h after viral infection unless otherwise indicated.

Lactate measurement

Extracellular lactate was detected using a commercial kit (MAK064 from Sigma) following manufacturer instructions. Medium from 24-well plates was replaced by 200µl of Krebs Ringer Buffer containing 10mM glucose. Lactate was measured after two hours of incubation by a colorimetric reaction. Oligomycin (1µg/ml) was added to the KRB when indicated.

Quantitative PCR

Total RNA from cultured cells was extracted using TRIZOL reagent (Thermo) following manufacturer's instructions. After RNA purification, 2 µg were reverse transcribed using RevertAid reverse transcriptase. The mix containing the RNA, 2 µM random hexamer primers, 0.5 mM dNTPs, 2 µM oligo$(dT)_{18}$ primers, 0.45 mM DTT, 10U RNAse-Out and 200U Revertaid reverse transcriptase was incubated at 25°C for 10 min, 42°C for 60 min and 72°C for 10 min. All reagents were purchased from Thermo Scientific. After reverse transcription, cDNA was diluted and amplified in an Applied Biosystems 7500 Fast quantitative PCR system. The primers used were Taqman purchased from Thermo Scientific. After running the qPCR, we obtained efficiencies and Cq values from LinRegPCR software and compared the Cq values using the comparative Cq method. *Actin*, and *18s* were used as reference genes.

For the *in vivo* MCT4 detection, RNA from mice was isolated using RNAeasy spin kit (Capsumlab). Reverse transcription and quantitative PCR was realized in the same conditions that the RNA from cultures. Expression was measured with mouse MCT4 primers and reference genes were *GAPDH* and *TBP*.

Western Blot

Cultured astrocytes were lysed with RIPA solution (150 mM $NaCl_2$, 50 mM Tris-HCl, 0.1 sodium dodecyl sulphate, 1% Nonidet P-40 and phosphatase and protease inhibitor cocktails (Sigma and Enzo respectively). The protein content was determined using Pierce BCA protein assay kit (Thermo). Protein extracts were subjected to SDS-polyacrylamide gel electrophoresis and transferred to methanol-activated polyvinylidene fluoride (PVDF) membranes (Enzo). After transfer, membranes were blocked with 0.1% Tween Tris Buffer Solution (TTBS) with 5% milk for 1 hour. Primary antibodies were incubated O/N at 4°C: 1/500 MCT4 (Santa Cruz Biotechnology) and 1/20000 β-actin (Sigma) diluted in 1% BSA-TTBS. Membranes were incubated with secondary peroxidase-conjugated anti-rabbit and anti-mouse diluted 1/10000 in 1% BSA-TTBS for 1 hour at RT. Finally, bands were detected with an enhanced chemiluminiscence detection kit (ECL) purchased from Thermo and developed by autoradiography on X-ray film (AGFA).

siRNA transfection

Rat HIF-1α siRNAs were purchased from Thermo and were transfected using lipofectamine RNAimax (Thermo) in DMEM medium without FBS and antibiotics. After 4h, medium was replaced for full DMEM.

Hypoxia experiments

Hypoxia experiments were performed in a temperature-controlled Invivo$_2$ hypoxia workstation (Baker Ruskinn). All experiments were realized at 37° C and humidity was maintained by placing a beaker filled with deionized sterile water inside the chamber. Hypoxic conditions were defined as 0.5% O_2, 5% CO_2 and 94% N_2. Cells were kept in full DMEM medium inside the hypoxic chamber for 8h, starting when chamber reached the indicated gas levels.

Statistical analysis

Statistical analysis was perfomed using GraphPad 6 Prism software. Data are expressed as the mean ± SEM of a minimum of three independent experiments or mice. Student's *t* test was used two compare to datasets whereas multiple groups were compared using ANOVA and Turkey's post hoc multiple comparisons test.

RESULTS

CREB upregulates MCT4 expression in astrocytes

To determine whether CREB upregulates MCT4 expression, we made use of two constructs used in previous works intended to modify CREB-dependent transcription (Eraso-Pichot et al., 2017). On the one hand, VP16-CREB is a modified form of CREB that acts as a constitutively active form of the transcription factor (Yan et al., 1994). On the other hand, the construct A-CREB acts as a dominant-negative form by binding to different forms of endogenous CREB thus avoiding their transcriptional activation (Ahn et al., 1998). Both constructs were maintained for 18-24h in order to induce a robust expression of the transgene (Eraso-Pichot et al., 2017).

MCT4 expression was increased upon VP16-CREB infection, both at mRNA (Figure 1A) and protein levels (Figure 1B). Although VP16-CREB increases CREB-dependent expression, the stimulus is not physiological since it can activate a wide range of astrocytic functions that may not be enhanced upon more physiological stimuli (Pardo et al., 2017). To demonstrate that the increase in MCT4 transcription can be produced in a physiological paradigm, cultured astrocytes were stimulated with NA, a known CREB activator (Carriba et al., 2012) whose functions have been determined to be partially orchestrated by astrocytes (Gao et al., 2016). Control (Null) and A-CREB infected cells were treated with 10 μM NA for different times and MCT4 mRNA expression was analysed. NA increased MCT4 expression in a bell shape with its maximum peak at 3h of stimulation, and the effect was totally abolished by A-CREB (Figure 1C), confirming the CREB-dependence of the effect of NA.

Interestingly, A-CREB showed a tendency to decrease basal MCT4 mRNA levels (Figure 1C, under line), suggesting that CREB might be involved also in maintaining basal levels of expression of MCT4. To address this question, and taking into account that the basal turnover rate of the transporter may be slower (Alvarez-Castelao and Schuman, 2015), we decided to maintain A-CREB expression for longer times (30h). After 30h, A-CREB significantly reduced MCT4 mRNA levels by 50% (Figure 1D), pointing to CREB as a maintenance transcription factor for MCT4 expression.

Next, we wanted to determine if the effects of VP16-CREB and A-CREB with regards to MCT4 expression are related to changes in lactate release. VP16-CREB increased lactate release in basal conditions, while A-CREB reduced it (Figure 1E). In order to increase lactate production, we added 1μg/ml of oligomycin, an inhibitor of the ATP-synthetase that increases glycolytic activity of the cell. Under oligomycin treatment, VP16-CREB-expressing astrocytes showed a statistically significant increase in lactate release, while A-CREB showed a statistically non-significant tendency (Figure 1F).

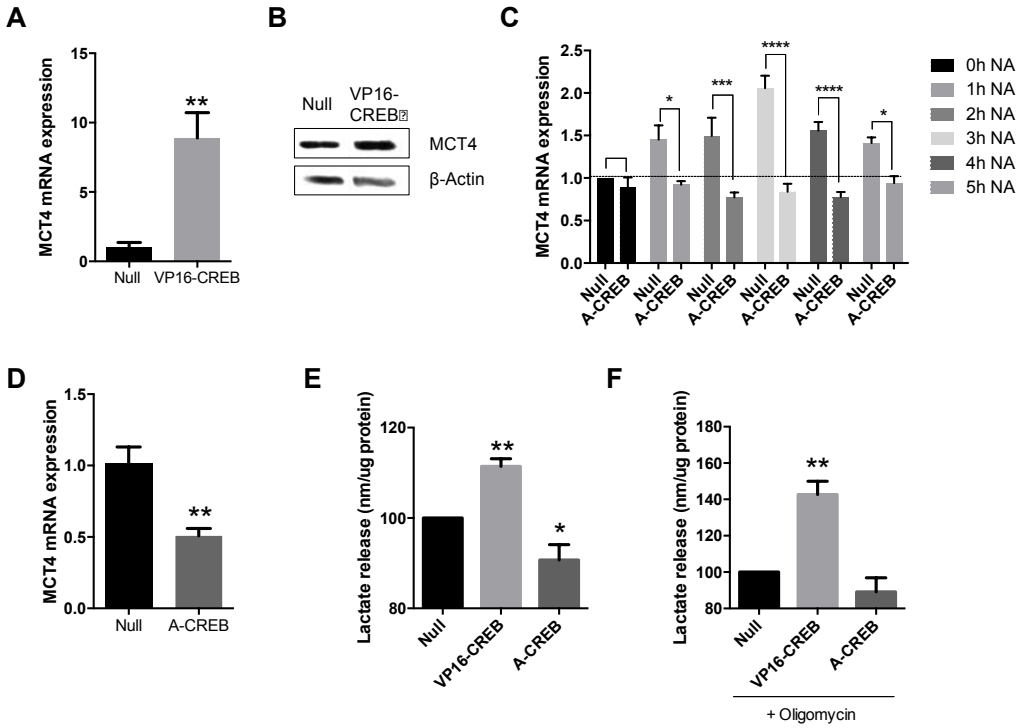

*Figure 1. Regulation of MCT4 expression by CREB. MCT4 mRNA (A) and protein (B) expression after VP16-CREB infection. (C) MCT4 mRNA expression in null and A-CREB infected astrocytes after 10μM NA for the indicated times. (D) MCT4 mRNA expression in null and A-CREB infected astrocytes after 30h of infection. (E) Lactate accumulated in the medium during 2h in Null, A-CREB and VP16-CREB infected astrocytes, in basal conditions and with 1μg/ml oligomycin treatment (F). The data are the mean ± SEM of 5 (A), 4-5 (C), 4 (D) and 3-4 (E-F) independent experiments. * p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.*

MCT4 regulation by CREB is independent of HIF-1α and oxygen tension:

The only known transcription factor regulating MCT4 transcription is HIF-1α (Ullah et al., 2006). In fact, previous studies addressing the mechanism of MCT4 transcription in astrocytes concluded that HIF-1α was activated in astrocytes *in vivo* since brain oxygen tension is lower than expected (Rosafio and Pellerin, 2014), suggesting that astrocytes are slightly hypoxic in normal conditions. However, our results regarding control of MCT4 expression by CREB under normoxic-cultured conditions indicate that MCT4 can be controlled by CREB in conditions in which HIF-1α may not be active. Thus, we analysed if HIF-1α has a role on the regulation of MCT4 by CREB in normoxia.

Importantly, when HIF-1α was inhibited, both NA (Figure 2A) and VP16-CREB (Figure 2B) increased MCT4 mRNA expression to a similar extent. These observations confirm that HIF-1α may not active in our culture conditions and that the regulation of MCT4 by CREB is independent of HIF-1α.

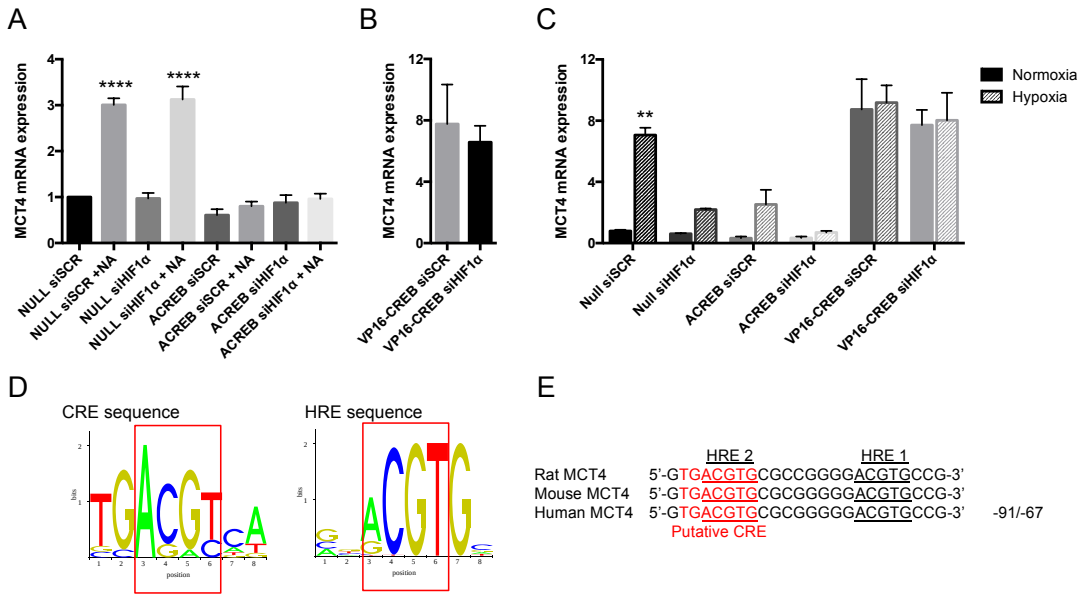

*Figure 2. CREB and HIF-1α interplay in MCT4 regulation in normoxia and hypoxia. (A) MCT4 mRNA expression in null and A-CREB infected astrocytes with HIF-1α or control silencing and with 10μM NA treatment for 3h. (B) MCT4 mRNA expression in VP16-CREB infected astrocytes with or without HIF-1α silencing. (C) MCT4 mRNA expression under hypoxia or normoxia, with or without HIF-1α silencing and in null, A-CREB and VP16-CREB infected astrocytes. The data are the mean ± SEM of 3 independent experiments. **p<0.01 and ****p<0.0001. (D) JASPAR consensus sequences for CREB and HIF-1α transcription factors. (E) Conserved binding HRE/CRE sites in rat, mouse and human DNA.*

Next, we wanted to analyse the opposite way around, the effect of CREB inhibition on the HIF-1α-mediated increase in MCT4 in hypoxia (Figure 2C). First, we observed a significant increase in MCT4 mRNA expression after 8h of hypoxia that is mediated by HIF-1α since it is not produced after the silencing of the transcription factor (Figure 2C, first two paired columns). Second, in the presence of A-CREB, MCT4 upregulation is not produced and MCT4 mRNA expression levels are as low as without HIF-1α (Figure 2C,

third paired columns). Moreover, this inhibition is potentiated when both transcription factors are inhibited (Figure 2C, fourth paired columns). Third, expression of MCT4 under hypoxia can be further increased by VP16-CREB reaching similar levels to the ones observed upon VP16-CREB activation under normoxia (Figure 2C, fifth paired columns). Fourth, HIF-1α inhibition reduces MCT4 expression induced by hypoxia but not by VP16-CREB (either under normoxia or hypoxia), confirming the necessary and sufficient role of CREB in the expression of the transporter.

In summary, our results show that the increase in MCT4 expression in hypoxia and normoxia is regulated by CREB. Unexpectedly, in hypoxic conditions, HIF-1α-induced increase in MCT4 expression is also dependent on CREB, while CREB-induced increase in MCT4 expression is HIF-1α independent.

The MCT4 promoter region presents two hypoxia response elements (HRE) that are crucial for the transcription of the transporter, both in normoxic and hypoxic situations (Ullah et al., 2006). These two HREs are found close to the transcription start site and are conserved between species (Figure 2E). Using JASPAR promoter analysis (Khan et al., 2018), we compared the probabilities of each nucleotide present in HRE sequences and CRE sequences. A consensus sequence is found in the core of both sequences (ACGT) (Figure 2D, red square)(Kvietikova et al., 1995), and most important, a putative CREB/HIF responsive sequence containing those nucleotides that are essential for the binding of the factor. This sequence is –TGACGTG_- and is exactly the sequence found in the second HRE site, known to be the most active (Ullah et al., 2006) (Figure 2E).

CREB-mediated regulation of MCT4 *in vivo*

Regulation of MCT4 by CREB in both hypoxic and normoxic conditions may have important consequences in physiology and pathology. Thus, we wanted to confirm that there is CREB-dependent MCT4 regulation *in vivo*. To do so, we used our VP16-CREB/Gfa2-Tta mouse model, which expresses the VP16-CREB form specifically in astrocytes under the GFAP promoter. This model presents low VP16-CREB expression in basal conditions with clear upregulation of the transgene after a cryolesion, which is a model of traumatic brain injury (Pardo et al., 2016).

To analyse if CREB activation in astrocytes *in vivo* increases MCT4 expression, we examined MCT4 mRNA levels in wild type (WT) and transgenic (Tg) mice in basal conditions and 1 day after cryolesion, when the transgene is highly expressed. While there was no difference in basal MCT4 mRNA levels, once cryolesion was performed, expression in Tg mice was highly increased with respect to all other groups (Figure 3A). These results indicate that MCT4 expression in astrocytes is also regulated by CREB *in vivo*.

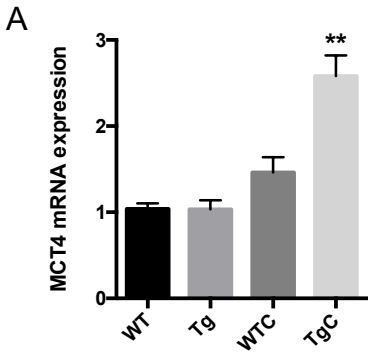

*Figure 3. CREB regulation of MCT4 in vivo. MCT4 mRNA expression of wild-type (WT) or transgenic VP16-CREB/Gfa2-Tta (Tg) mice subjected or not to cryolesion (C). The data are the mean ± SEM of 4-6 mice for each group. **p<0.01.*

DISCUSSION

In the present study, we have demonstrated that under normoxic conditions, the enhancement of CREB-activated transcription by NA and VP16-CREB upregulates MCT4 expression independently of HIF-1α. Moreover, we have seen an interplay between HIF-1α and CREB in hypoxia, which might be carried out through a common HRE/CRE region in the MCT4 promoter. Finally, we have seen increased MCT4 expression *in vivo* after CREB activation in astrocytes. Our results are a step further in the knowledge of the molecular and cellular underpinnings whereby astrocytes may contribute to learning and memory. These results will be discussed in three points.

First, regulation of MCT4 by two transcription factors respectively activated by neuronal activity and by hypoxia opens the possibility of common pathways regulating MCT4 in normal and pathological conditions in astrocytes. Moreover, CREB inhibition in hypoxia led to an impaired hypoxic regulation of MCT4, suggesting a role of the CREB transcription factor also in the regulation of the transporter in hypoxia (HIF-1α-mediated).

Different scenarios could explain the interplay between CREB and HIF-1α regulation of MCT4 expression. First, both transcription factors may bind to the same region in order to transcribe MCT4. CREB can be constitutively bound to the CRE/HRE region found (HRE 2) in the MCT4 promoter even in the absence of stimulation, as it has been seen in neurons (Parra-Damas et al., 2017). This constitutive binding might maintain basal MCT4 transcription, since A-CREB expression reduced the transporter even without CREB stimulation. In addition to basal transcription, neuronal activity leading to noradrenaline release activates CREB in astrocytes, increasing in turn MCT4 expression. In hypoxic situations, where HIF-1α is found active (Semenza, 2007), CREB may be substituted by HIF-1α in the HRE/CRE region, inducing the expression of the transporter. However, when CREB is inhibited (in our case, using the A-CREB construct), the region is not maintained "opened" and HIF-1α is no longer able to increase MCT4 expression producing the impairment seen in the regulation of the transporter in hypoxia. In fact, HIF-1α binding analyses have found that the transcription factor binds

preferentially to HREs found in regions of permissive chromatin and with basal transcriptional activity, determining the cell-specific differences in the response (Xia and Kung, 2009) (Dengler et al., 2014). In a second scenario, the cooperative effect between both transcription factors may be carried out in different binding sites. Here, the presence of CREB in the CRE/HRE responding region is crucial to allow HIF-1α binding to the other HRE sites of the MCT4 promoter. Thus, HIF-1α-mediated regulation of the transporter is done by its binding to the HREs sites 1 and 3 of the promoter, while CREB binding to the site 2 is necessary for the hypoxic increase in MCT4 transcription (Ullah et al., 2006). Anyway, more research needs to be done in order to understand the interplay between these two transcription factors in the MCT4 promoter as well as in other genes that might be controlled by CREB and HIF-1α such as lactate dehydrogenase A (Firth et al., 1995).

Second, the regulation of lactate exporters may be important to understand brain physiology and how astrocytes contribute to synaptic plasticity. Lactate effects on neuronal excitability, plasticity, synapse formation and behaviour and memory coding have been intensely studied and there is an agreement on the role of released lactate as a signaling pathway inducing neuronal plasticity, either as a metabolic substrate or as a signalling transducer (Magistretti and Allaman, 2018) (Bak and Walls, 2018). Our results establish the transcription factor CREB as the major regulator of MCT4 expression in cultured and *in vivo* astrocytes in normoxia. Since neurotransmitters such as ATP and neuromodulators like noradrenaline (Carriba et al., 2012), and more recently dopamine (Koppel et al., 2018), have been seen to activate CREB in astrocytes, one could speculate of an astrocyte plasticity phenomenon, by which potentiation of a specific neural circuit activates CREB in neighboring astrocytes, increasing the expression of the monocarboxylate transporters to increase lactate supply in the synapse. These results are in agreement with previous works, suggesting an increase in the expression of glycolytic genes in astrocytes in response to neuronal activity (Hasel et al., 2017).

Interestingly, astrocytic adrenergic activation leads to an acute enhancement of lactate release, probably by increases in glycogenolysis, lactate being necessary and sufficient to induce long-term memories (Suzuki et al., 2011) (Gao et al., 2016). However, none of these studies addressed if the acute adrenergic effect is maintained for longer times to promote the maintenance of these newly generated memories. We propose a mechanism by which NA, in addition to the acute activation of glycogenolysis and lactate release, primes astrocytes to increase MCT4 expression via CREB and enhance lactate release in ensuing exposures to NA, thus constituting a positive feedback to promote memory consolidation (figure).

Third, the protective effect of CREB activation in astrocytes may be done through an upregulation of MCT4. Our results confirmed the *in vivo* control of MCT4 by CREB using our VP16-CREB/Gfa2-Tta mouse model. This mouse model was found to be more resistant to the damage produced by traumatic brain injury, probably due to a recovery of brain energy metabolism (Pardo et al., 2016). The upregulation of MCT4 transporter may underlie protection (Pardo et al., 2016), since more lactate may be available to neurons, bypassing the known metabolic downregulation produced in traumatic brain

injury processes (Carre et al., 2013). Indeed, exogenous lactate supply has been seen to be protective in TBI (Patet et al., 2016).

Taken together, our results demonstrate CREB dependent regulation of MCT4 transcription, both in normoxic and hypoxic conditions, thus framing astrocytic CREB as a potential key player in synaptic potentiation and a as possible target to promote lactate release in injury.

REFERENCES

Ahn, S., Olive, M., Aggarwal, S., Krylov, D., Ginty, D.D., and Vinson, C. (1998). A dominant-negative inhibitor of CREB reveals that it is a general mediator of stimulus-dependent transcription of c-fos. Mol Cell Biol *18*, 967-977.

Alvarez-Castelao, B., and Schuman, E.M. (2015). The Regulation of Synaptic Protein Turnover. J Biol Chem *290*, 28623-28630.

Bak, L.K., and Walls, A.B. (2018). CrossTalk opposing view: lack of evidence supporting an astrocyte-to-neuron lactate shuttle coupling neuronal activity to glucose utilisation in the brain. J Physiol *596*, 351-353.

Carre, E., Ogier, M., Boret, H., Montcriol, A., Bourdon, L., and Jean-Jacques, R. (2013). Metabolic crisis in severely head-injured patients: is ischemia just the tip of the iceberg? Front Neurol *4*, 146.

Carriba, P., Pardo, L., Parra-Damas, A., Lichtenstein, M.P., Saura, C.A., Pujol, A., Masgrau, R., and Galea, E. (2012). ATP and noradrenaline activate CREB in astrocytes via noncanonical Ca(2+) and cyclic AMP independent pathways. Glia *60*, 1330-1344.

Dengler, V.L., Galbraith, M., and Espinosa, J.M. (2014). Transcriptional regulation by hypoxia inducible factors. Crit Rev Biochem Mol Biol *49*, 1-15.

Dong, J.H., Wang, Y.J., Cui, M., Wang, X.J., Zheng, W.S., Ma, M.L., Yang, F., He, D.F., Hu, Q.X., Zhang, D.L.*, et al.* (2017). Adaptive Activation of a Stress Response Pathway Improves Learning and Memory Through Gs and beta-Arrestin-1-Regulated Lactate Metabolism. Biol Psychiatry *81*, 654-670.

Eraso-Pichot, A., Larramona-Arcas, R., Vicario-Orri, E., Villalonga, R., Pardo, L., Galea, E., and Masgrau, R. (2017). CREB decreases astrocytic excitability by modifying subcellular calcium fluxes via the sigma-1 receptor. Cellular and molecular life sciences : CMLS *74*, 937-950.

Firth, J.D., Ebert, B.L., and Ratcliffe, P.J. (1995). Hypoxic regulation of lactate dehydrogenase A. Interaction between hypoxia-inducible factor 1 and cAMP response elements. J Biol Chem *270*, 21021-21027.

Gao, V., Suzuki, A., Magistretti, P.J., Lengacher, S., Pollonini, G., Steinman, M.Q., and Alberini, C.M. (2016). Astrocytic beta2-adrenergic receptors mediate hippocampal long-term memory consolidation. Proc Natl Acad Sci U S A *113*, 8526-8531.

Halestrap, A.P., and Wilson, M.C. (2012). The monocarboxylate transporter family--role and regulation. IUBMB Life *64*, 109-119.

Hasel, P., Dando, O., Jiwaji, Z., Baxter, P., Todd, A.C., Heron, S., Markus, N.M., McQueen, J., Hampton, D.W., Torvell, M.*, et al.* (2017). Neurons and neuronal activity control gene expression in astrocytes to regulate their development and metabolism. Nature communications *8*, 15132.

Jones, R.S., and Morris, M.E. (2016). Monocarboxylate Transporters: Therapeutic Targets and Prognostic Factors in Disease. Clin Pharmacol Ther *100*, 454-463.

Khan, A., Fornes, O., Stigliani, A., Gheorghe, M., Castro-Mondragon, J.A., van der Lee, R., Bessy, A., Cheneby, J., Kulkarni, S.R., Tan, G.*, et al.* (2018). JASPAR 2018: update of the open-access database of transcription factor binding profiles and its web framework. Nucleic Acids Res *46*, D1284.

Koppel, I., Jaanson, K., Klasche, A., Tuvikene, J., Tiirik, T., Parn, A., and Timmusk, T. (2018). Dopamine cross-reacts with adrenoreceptors in cortical astrocytes to induce BDNF expression, CREB signaling and morphological transformation. Glia *66*, 206-216.

Kvietikova, I., Wenger, R.H., Marti, H.H., and Gassmann, M. (1995). The transcription factors ATF-1 and CREB-1 bind constitutively to the hypoxia-inducible factor-1 (HIF-1) DNA recognition site. Nucleic Acids Res *23*, 4542-4550.

Li, S., Hafeez, A., Noorulla, F., Geng, X., Shao, G., Ren, C., Lu, G., Zhao, H., Ding, Y., and Ji, X. (2017). Preconditioning in neuroprotection: From hypoxia to ischemia. Prog Neurobiol *157*, 79-91.

Magistretti, P.J., and Allaman, I. (2015). A cellular perspective on brain energy metabolism and functional imaging. Neuron *86*, 883-901.

Magistretti, P.J., and Allaman, I. (2018). Lactate in the brain: from metabolic end-product to signalling molecule. Nat Rev Neurosci *19*, 235-249.

Morland, C., Lauritzen, K.H., Puchades, M., Holm-Hansen, S., Andersson, K., Gjedde, A., Attramadal, H., Storm-Mathisen, J., and Bergersen, L.H. (2015). The lactate receptor, G-protein-coupled receptor 81/hydroxycarboxylic acid receptor 1: Expression and action in brain. J Neurosci Res *93*, 1045-1055.

Pardo, L., Schluter, A., Valor, L.M., Barco, A., Giralt, M., Golbano, A., Hidalgo, J., Jia, P., Zhao, Z., Jove, M.*, et al.* (2016). Targeted activation of CREB in reactive astrocytes is neuroprotective in focal acute cortical injury. Glia *64*, 853-874.

Pardo, L., Valor, L.M., Eraso-Pichot, A., Barco, A., Golbano, A., Hardingham, G.E., Masgrau, R., and Galea, E. (2017). CREB Regulates Distinct Adaptive Transcriptional Programs in Astrocytes and Neurons. Scientific reports *7*, 6390.

Parra-Damas, A., Rubio-Ferrarons, L., Shen, J., and Saura, C.A. (2017). CRTC1 mediates preferential transcription at neuronal activity-regulated CRE/TATA promoters. Scientific reports *7*, 18004.

Patet, C., Suys, T., Carteron, L., and Oddo, M. (2016). Cerebral Lactate Metabolism After Traumatic Brain Injury. Curr Neurol Neurosci Rep *16*, 31.

Pellerin, L., Bergersen, L.H., Halestrap, A.P., and Pierre, K. (2005). Cellular and subcellular distribution of monocarboxylate transporters in cultured brain cells and in the adult brain. J Neurosci Res *79*, 55-64.

Perez-Escuredo, J., Van Hee, V.F., Sboarina, M., Falces, J., Payen, V.L., Pellerin, L., and Sonveaux, P. (2016). Monocarboxylate transporters in the brain and in cancer. Biochimica et biophysica acta *1863*, 2481-2497.

Rosafio, K., and Pellerin, L. (2014). Oxygen tension controls the expression of the monocarboxylate transporter MCT4 in cultured mouse cortical astrocytes via a hypoxia-inducible factor-1alpha-mediated transcriptional regulation. Glia *62*, 477-490.

Semenza, G.L. (2007). Hypoxia-inducible factor 1 (HIF-1) pathway. Sci STKE *2007*, cm8.

Sharp, F.R., Ran, R., Lu, A., Tang, Y., Strauss, K.I., Glass, T., Ardizzone, T., and Bernaudin, M. (2004). Hypoxic preconditioning protects against ischemic brain injury. NeuroRx *1*, 26-35.

Suzuki, A., Stern, S.A., Bozdagi, O., Huntley, G.W., Walker, R.H., Magistretti, P.J., and Alberini, C.M. (2011). Astrocyte-neuron lactate transport is required for long-term memory formation. Cell *144*, 810-823.

Suzuki, N., Gradin, K., Poellinger, L., and Yamamoto, M. (2017). Regulation of hypoxia-inducible gene expression after HIF activation. Exp Cell Res *356*, 182-186.

Tadi, M., Allaman, I., Lengacher, S., Grenningloh, G., and Magistretti, P.J. (2015). Learning-Induced Gene Expression in the Hippocampus Reveals a Role of Neuron -Astrocyte Metabolic Coupling in Long Term Memory. PLoS One *10*, e0141568.

Tang, F., Lane, S., Korsak, A., Paton, J.F., Gourine, A.V., Kasparov, S., and Teschemacher, A.G. (2014). Lactate-mediated glia-neuronal signalling in the mammalian brain. Nature communications *5*, 3284.
Ullah, M.S., Davies, A.J., and Halestrap, A.P. (2006). The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. J Biol Chem *281*, 9030-9037.
Wang, J., Tu, J., Cao, B., Mu, L., Yang, X., Cong, M., Ramkrishnan, A.S., Chan, R.H.M., Wang, L., and Li, Y. (2017). Astrocytic l-Lactate Signaling Facilitates Amygdala-Anterior Cingulate Cortex Synchrony and Decision Making in Rats. Cell Rep *21*, 2407-2418.
Xia, X., and Kung, A.L. (2009). Preferential binding of HIF-1 to transcriptionally active loci determines cell-type specific response to hypoxia. Genome Biol *10*, R113.
Yan, G., Chen, X., and Bancroft, C. (1994). A constitutively active form of CREB can activate expression of the rat prolactin promoter in non-pituitary cells. Mol Cell Endocrinol *101*, R25-30.

Addendum to the second chapter:

CREB activation increases lactate release without affecting oxygen consumption

Eraso-Pichot A., Zorzano A., Galea E., Masgrau R

RATIONALE

Results obtained in the previous two chapters seem counterintuitive. In chapter 1 we reported activity dependent increases in mitochondrial calcium in VP16-CREB infected astrocytes, suggesting an increase in mitochondrial metabolism in CREB-activated astrocytes. In chapter 2, VP16-CREB infected astrocytes showed higher lactate release both in basal and under oligomycin treatment, indicating that glucose –considered the main astrocytic fuel- is converted to lactate instead of entering mitochondria as acetyl-coA.

To answer this paradox, we decided to measure lactate release and oxygen consumption at the same time using the Seahorse technology. This method detects medium acidification as a measure of glycolytic activity and lactate release and oxygen consumption rate (OCR) to analyze mitochondrial activity.

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RESULTS

VP16-CREB and Null-infected astrocytes were incubated in *Agilent Seahorse XF* assay medium, containing necessary osmolytes and aminoacids but lacking buffering agents and CO_2 in order to analyze the extracellular acidification. Medium was supplemented with with 2 mM L-glutamine, 5.5 mM glucose and 1 mM sodium pyruvate.

Under these conditions, OCR and proton production rate (equivalent to lactate release) of VP16-CREB and null astrocytes were measured before and after the addition of different inhibitors to study distinct parameters of glycolytic lactate release and mitochondrial respiration. First, before the addition of inhibitors, basal oxygen consumption and proton production rates were measured in null and VP16-CREB astrocytes. Second, 1 μg/ml of oligomycin, a complex V inhibitor were added to the media in order to measure on the one hand, oxygen consumption that is not related to ATP-synthesis, and on the other hand, increases in lactate release after the enhancement of glycolysis. Third, astrocytes were treated with 2mM FCCP, a mitochondrial uncoupler that collapses the inner membrane gradient allowing the electron transport chain to function at its maximal rate. Finally, 0.4mM rotenone and 1mM antimycin A, inhibitors of complexes I and III respectively, were added in order to inhibit mitochondrial metabolism and detect non-mitochondrial oxygen consumption. The proton production rate after the addition of FCCP and the electron transport chain inhibitors was not analyzed since oligomycin represents the maximal glycolytic capacity of the cells.

Regarding lactate release, VP16-CREB showed increased extracellular medium acidification compared to Null-infected astrocytes (Figure 1). The increase in acidification was doubled and the proportion was maintained under oligomycin treatment, confirming the previous observed increase in lactate release.

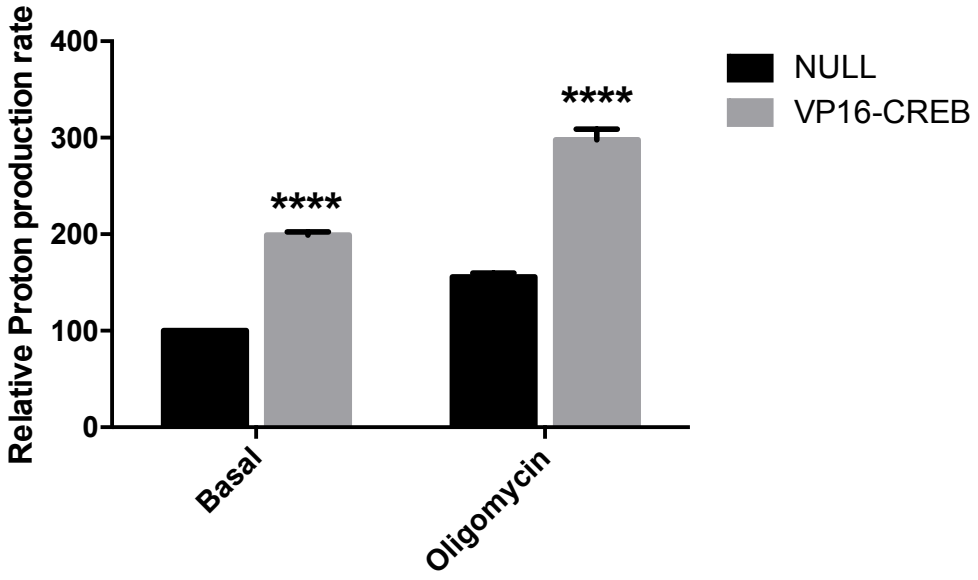

*Figure 1. VP16-CREB over-expressing astrocytes showed increased proton production rate in basal conditions and under oligomycin treatment. Data are mean ± SEM of three independent experiments. ****p<0.001 two-way ANOVA followed by Sidak's post-hoc test.*

Regarding mitochondrial metabolism, VP16-CREB does not change oxygen consumption in the presence of glucose (Figure 2). No differences were seen under basal conditions, following inhibition of ATP production (oligomycin), mitochondrial uncoupling (FCCP) or inhibition of the electron transport chain (antimycin A plus rotenone).

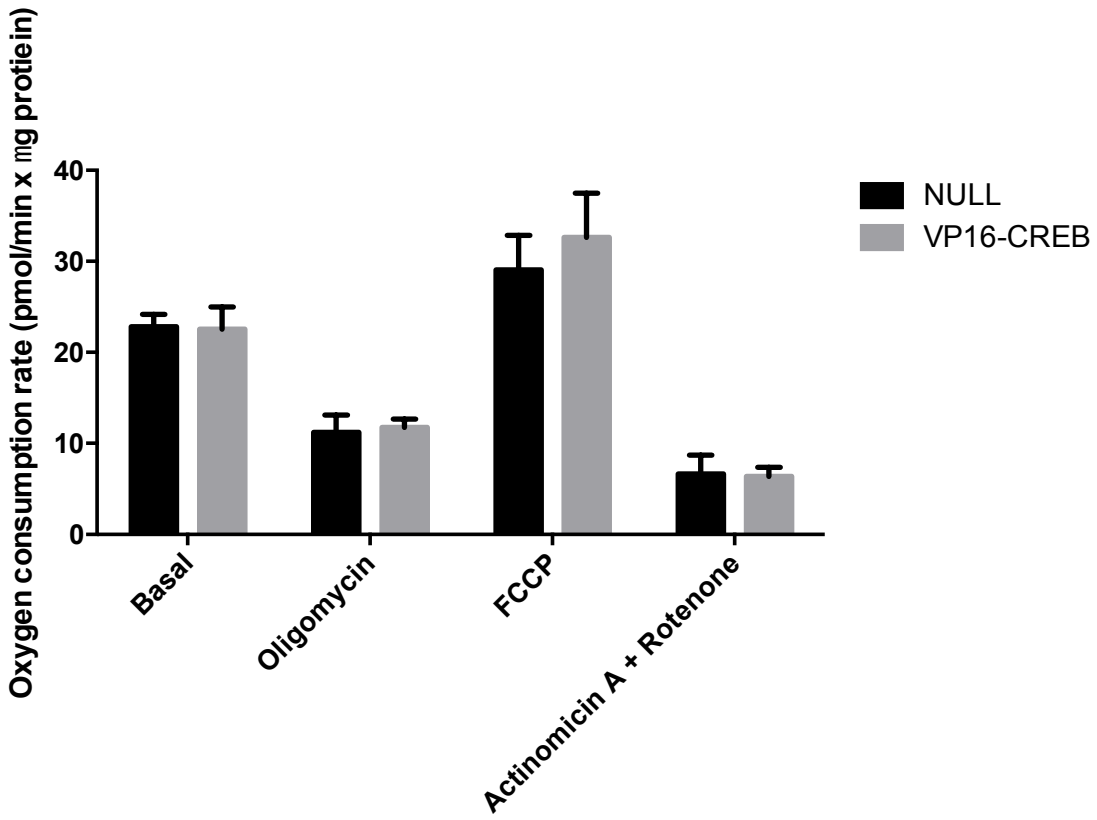

Figure 2. CREB activation in astrocytes does not affect oxygen consumption in the presence of glucose. Oxygen consumption rate of VP16-CREB and Null-infected astrocytes is not significantly different in basal conditions or under oligomycin, FCCP or antimycin A and rotenone treatments. Data are mean ± SEM of three independent experiments.

These results confirm the increase in lactate release seen in chapter 2, since medium acidification is highly increased in VP16-CREB astrocytes. Moreover, we have determined that in basal conditions and in the presence of glucose, oxygen consumption is not changed after activation of CREB-dependent transcription. Further experiments need to address two fundamental questions. First, the results obtained in chapter 1 showed increased mitochondrial calcium entry in VP16-CREB astrocytes. Now that we have determined that basal oxygen consumption is not affected by activation of CREB-dependent transcription, we need to analyze which is the oxygen consumption of these cells after stimulation with a calcium-releasing agent such as ATP. Importantly, OCR depends on mitochondrial calcium entry (Llorente-Folch et al., 2013), thus, one possibility is that mitochondrial metabolism in VP16-CREB astrocytes is increased only after calcium stimulation.

Second, in this experiment, VP16-CREB astrocytes were incubated with glucose, pyruvate and glutamine. Although it is still a matter of debate, some authors suggested that astrocytes might use substrates other than glucose-derived pyruvate for

mitochondrial oxidation in order to maintain ATP production. Aminoacids such as glutamate (Schousboe et al., 2014), the same lactate (Tabernero et al., 1996) or fatty acids (Ebert et al., 2003) have been proposed to fuel astrocytes, however, further research is needed to understand which are the metabolic pathways of astrocytic mitochondria.

REFERENCES

Ebert, D., Haller, R.G., and Walton, M.E. (2003). Energy contribution of octanoate to intact rat brain metabolism measured by 13C nuclear magnetic resonance spectroscopy. The Journal of neuroscience : the official journal of the Society for Neuroscience *23*, 5928-5935.

Llorente-Folch, I., Rueda, C.B., Amigo, I., del Arco, A., Saheki, T., Pardo, B., and Satrustegui, J. (2013). Calcium-regulation of mitochondrial respiration maintains ATP homeostasis and requires ARALAR/AGC1-malate aspartate shuttle in intact cortical neurons. The Journal of neuroscience : the official journal of the Society for Neuroscience *33*, 13957-13971, 13971a.

Schousboe, A., Scafidi, S., Bak, L.K., Waagepetersen, H.S., and McKenna, M.C. (2014). Glutamate metabolism in the brain focusing on astrocytes. Adv Neurobiol *11*, 13-30.

Tabernero, A., Vicario, C., and Medina, J.M. (1996). Lactate spares glucose as a metabolic fuel in neurons and astrocytes from primary culture. Neurosci Res *26*, 369-376.

Chapter 3:

GSEA of mouse and human mitochondriomes reveals fatty acid oxidation in astrocytes

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RESEARCH ARTICLE

GSEA of mouse and human mitochondriomes reveals fatty acid oxidation in astrocytes

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Abstract

The prevalent view in neuroenergetics is that glucose is the main brain fuel, with neurons being mostly oxidative and astrocytes glycolytic. Evidence supporting that astrocyte mitochondria are functional has been overlooked. Here we sought to determine what is unique about astrocyte mitochondria by performing unbiased statistical comparisons of the mitochondriome in astrocytes and neurons. Using MitoCarta, a compendium of mitochondrial proteins, together with transcriptomes of mouse neurons and astrocytes, we generated cell-specific databases of nuclear genes encoding for mitochondrion proteins, ranked according to relative expression. Standard and in-house Gene Set Enrichment Analyses (GSEA) of five mouse transcriptomes revealed that genes encoding for enzymes involved in fatty acid oxidation (FAO) and amino acid catabolism are consistently more expressed in astrocytes than in neurons. FAO and oxidative-metabolism-related genes are also up-regulated in human cortical astrocytes versus the whole cortex, and in adult astrocytes versus fetal astrocytes. We thus present the first evidence of FAO in human astrocytes. Further, as shown *in vitro*, FAO coexists with glycolysis in astrocytes and is inhibited by glutamate. Altogether, these analyses provide arguments against the glucose-centered view of energy metabolism in astrocytes and reveal mitochondria as specialized organelles in these cells.

KEYWORDS
metabolism, mitochondria, neurons, transcriptome

1 | INTRODUCTION

Brain activity requires essential and complex metabolic interactions between neurons and astrocytes. Examples of such crosstalk are the Glutamate–Glutamine Brain Cycle (GGBC) and the Astrocyte-Neuron Lactate Shuttle (ANLS) hypothesis (Belanger, Allaman, & Magistretti, 2011). The GGBC frames astrocytes as key regulators of extracellular glutamate clearance. Glutamate released from synapses is transported into astrocytes and transformed to glutamine by glutamine synthetase. Glutamine is then delivered to neurons and converted again to glutamate by the mitochondrial enzyme glutaminase. The ANLS hypothesis proposes that astrocytes produce lactate from glucose by aerobic glycolysis (in the presence of oxygen). Lactate is then transferred to neurons via monocarboxylate transporters. In neurons, lactate is first converted to pyruvate by lactate dehydrogenase, then to acetyl-CoA by pyruvate dehydrogenase, and further oxidized in the Tricarboxylic Acid Cycle (TCA). The coupling of the TCA to the mitochondrial electron transport chain (ETC) would enable neurons to produce ATP. Interestingly, the conversion of lactate into pyruvate in neurons increases the ratio NADH/NAD^+, which could turn off neuronal glycolysis and facilitate the pentose phosphate pathway for synthesis of glutathione and NADP, without sacrificing neuronal energy requirements

Abel Eraso-Pichot and Marina Brasó-Vives contributed equally to this work.

(Cerdán et al., 2006; Herrero-Mendez et al., 2009). Along these lines, the intrinsic neuronal activity at resting conditions may depend in part on aerobic glycolysis taking place in astrocytes (Raichle, 2015). Lactate availability is necessary to maintain excitatory transmission (Nagase, Takahashi, Watabe, Kubo, & Kato, 2014), and facilitates synapse formation and growth in development (Goyal, Hawrylycz, Miller, Snyder, & Raichle, 2014), albeit the source and levels of lactate are controversial (Dienel, 2017). In fact, the ANLS hypothesis has not been proven in humans. Results in favor of or against the hypothesis have been reported from animal studies *in vivo* (Ashrafi & Ryan, 2017; Diaz-Garcia et al., 2017; Mächler et al., 2016), and illustrate the complexity of brain metabolism.

Whatever the nature and source of the fuels used by neurons may be, the general consensus is that mitochondria are fundamental to neurons, which are oxidative; while astrocytes are glycolytic cells at the service of neuronal energetic needs. This view underestimates the role that mitochondria may play in astrocytes. However, several observations link excitatory neurotransmission with astrocytic anaplerosis and cataplerosis (i.e., reactions that respectively replenish or deplete TCA intermediaries). For example, glutamate taken up by astrocytes is converted in part to alpha-ketoglutarate by oxidative deamination and is incorporated into the TCA cycle (Belanger & Magistretti, 2009; McKenna, 2007; McKenna et al., 2016; Sonnewald, Westergaard, & Schousboe, 1997), to generate pyruvate and lactate by cataplerotic reactions (Schousboe, Scafidi, Bak, Waagepetersen, & McKenna, 2014; Sonnewald, Westergaard, Petersen, Unsgard, & Schousboe, 1993). Also, astrocytes typically express pyruvate carboxylase. This prototypical anaplerotic enzyme produces oxaloacetate, from which glutamate can be generated if there is an operative TCA cycle (Lovatt et al., 2007; Shank, Bennett, Freytag, & Campbell, 1985; Waagepetersen, Qu, Schousboe, & Sonnewald, 2001; Yu, Schousboe, & Hertz, 1982). Moreover, a recent study in isolated mitochondria has revealed comparable oxidative phosphorylation in astrocytes and neurons, despite molecular differences in ETC complexes (Lopez-Fabuel et al., 2016). And, finally, astrocyte mitochondria have an active role in calcium signaling (Agarwal et al., 2017; Eraso-Pichot et al., 2017). This is a key element of astrocyte excitability, mediating astrocyte actions in the so-called cerebrovascular unit that controls the coupling of brain metabolism with blood flow (Belanger et al., 2011).

Overall, the view that astrocytes are only glycolytic is simplistic and underestimates astrocyte metabolic functions. To better understand the complexity of neuroenergetics, we investigated astrocyte mitochondrial functions. On the premise that the functions of mitochondria can be inferred from their molecular makeup, we compared the "mitochondriome" of astrocytes and neurons. The mitochondriome was based on the MitoCarta (Pagliarini et al., 2008) and on transcriptome databases of cells isolated from four sources: normal mouse brains, mouse spinal cords before and after injury, cultured postnatal astrocytes, and fetal and adult human brains. Functional validation was carried out in cultured astrocytes. The findings indicate that the metabolism of astrocytes is versatile and complex and that mitochondrial fatty acid oxidation (FAO) in astrocytes is a key element in brain metabolism. Our results therefore imply that core tenets in neuroenergetics, such as glucose is the main brain fuel, ought to be revised.

2 | MATERIALS AND METHODS

2.1 | Databases and their treatment

We used MitoCarta (Pagliarini et al., 2008), the mouse transcriptome (Anderson et al., 2016; Cahoy et al., 2008; Doyle et al., 2008; Lovatt et al., 2007; Zhang et al., 2014) and the human brain transcriptome databases (Zhang et al., 2016). We applied the alias2Symbol function from Bioconductor organism packages to convert all gene identifiers from all databases to their official gene symbols. We used logarithm base two-fold changes of expression (log2FC) to assess expression differences between conditions. All in-house R scripts are available under request. Organelle gene lists were extracted from the "Cellular Component" ontology of the GO terms using AmiGO 2.0 software. Lists of genes used for each organelle are available in Supporting Information Dataset S5.

2.2 | Standard gene set enrichment analysis (GSEA)

We performed GSEA with the software provided by the Massachusetts Institute of Technology (Subramanian et al., 2005). Normalized enrichment score (NES) and false discovery rate (FDR) were used to quantify enrichment magnitude and statistical significance, respectively.

2.3 | In-house GSEA

We designed an in-house GSEA that uses the proportion of genes with a log2FC greater than 2 (or lower than −2) within a given gene set to determine the magnitude of enrichment under a particular condition or conditions. The statistical significance of the enrichments were assessed post-hoc with an exact binomial test considering the proportion of genes with log2FC greater than 2 (or lower than −2) with respect to the proportion of over-expressed genes of each cell type in the whole database.

2.4 | Tissue culture

Primary cultures of cortical astrocytes were obtained from 1-day-old Sprague-Dawley rats as previously described (Barceló-Torns et al., 2011). In brief, rats were decapitated and the brain was immediately dissected out, minced and incubated for 10 min at 37°C in Ca^{2+}-free Krebs Ringer Buffer (KRB) containing 0.025% trypsin. Tissue was mechanically triturated and cells were filtered through a 40-μm nylon mesh in the presence of soybean trypsin inhibitor and 170 IU/ml DNAse. Collected cells were plated in T150 flasks in DMEM medium supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin and 50 μg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. After reaching confluence, microglia were removed by mechanical shaking and astrocytes were seeded in 24-well plates.

2.5 | β-Oxidation assay

This assay quantifies β-oxidation measuring $[^3H]H_2O$ generated after labeling cells with [3H]palmitic acid, as previously described (Cabodevilla et al., 2013). Rat cortical secondary astrocytes were labeled overnight with 1 μCi/ml [3H]palmitic acid (10–30 nM, final concentration) in culture medium containing 0.5% delipidated bovine serum albumin (BSA). After labeling, cells were washed once with KRB containing BSA and twice with KRB. Cells were then incubated with fresh KRB for the indicated times, at different glucose concentrations. Three hours later, 100 μM glutamate was added, if applicable. Etomoxir (30 μM), an inhibitor of carnitine palmitoyl transferase 1 and therefore of [3H]palmitic acid mitochondrial β-oxidation, was added to establish the extent of $[^3H]H_2O$ production from sources other than mitochondrial β-oxidation. After the incubations, KRB was transferred to tubes containing 1 ml of a 1:2 slurry of Dowex 1 × 2 (chloride form) anionic exchange resin in H_2O. Tubes were vortexed and centrifuged for 2 min at 1,000 rpm. The resin traps radioactive metabolic intermediates other than $[^3H]H_2O$. Finally, 0.1 ml of the supernatants containing $[^3H]H_2O$ were counted for radioactivity. All experiments were done in triplicate and included a blank control where $[^3H]H_2O$ was determined immediately after the addition of KRB buffer without glucose.

2.6 | Reagents

All reagents were from Sigma-Aldrich (St. Louis, MO) except [3H]palmitic acid which was purchased from Perkin Elmer (Waltham, MA).

3 | RESULTS

3.1 | Generation of astrocyte and neuron "mitochondriomes" in healthy adult brains

To identify the functional signature of astrocyte mitochondria with respect to neurons, we used the Mitocarta database—the most comprehensive inventory of the molecular composition of mitochondria, comprising of data obtained from 14 mammalian tissues (Pagliarini et al., 2008). Mitocarta contains 1,098 proteins, which were converted into 919 genes with the Bioconductor mouse package (Supporting Information Dataset S1). We first manually classified the mitochondrial components identified by MitoCarta (Supporting Information Dataset S1) into 12 functional categories (inner circle, Figure 1). Each category was further divided into groups. We thus had 12 categories with two levels of subdivision (Figure 1 and Supporting Information Dataset S1). For example, the functional category Cellular energy homeostasis was divided into ATP-related metabolism, Carbohydrate catabolism, Co-factors metabolism, Ketone body metabolism, Lipid catabolism, Lipid synthesis and Oxidative metabolism. The last group, in turn, was subclassified in Anaplerosis and cataplerosis, Oxidative phosphorylation and TCA. Among all the 919 genes analyzed only 69 fell into more than one functional category. Obviously, categories may contain different numbers of genes. Cellular energy homeostasis is the largest group with 287 genes. This already implies which mitochondrial functions could be more important.

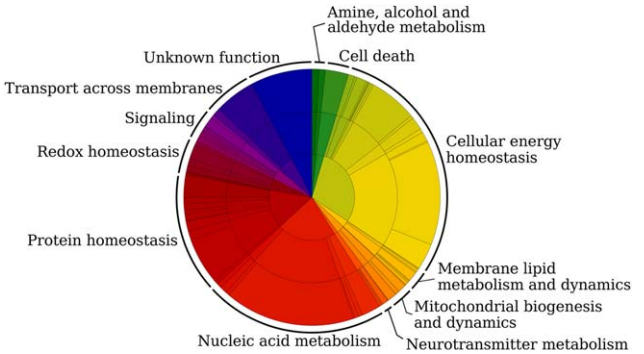

FIGURE 1 Functional classification of mitochondrial molecular components identified by MitoCarta. Genes were classified in 12 functional categories each one represented with a different color in the inner circle and labeled on the outer ring. Each functional group was further divided in subcategories (two outer circles) [Color figure can be viewed at wileyonlinelibrary.com]

Next, mitochondriomes were generated by extracting nucleus-encoded mitochondrial genes and their relative expressions from the transcriptomes of mouse astrocytes and neurons isolated from whole brains or spinal cords. The brain transcriptomes used are available in five repositories (Anderson et al., 2016; Cahoy et al., 2008; Doyle et al., 2008; Lovatt et al., 2007; Zhang et al., 2014), and in the Glia Network website (http://www.networkglia.eu/en). We will henceforth refer to these databases by the name of the first author of the study, viz. Zhang (Zhang et al., 2014), Doyle (Doyle et al., 2008), Cahoy (Cahoy et al., 2008), Lovatt (Lovatt et al., 2007), and Anderson (Anderson et al., 2016). The studies made use of mice at different ages (Zhang, young animals at 7 postnatal days (P7); Doyle, P17 animals; Cahoy, P1–P30 and Lovatt, 10–12-week-old mice). Aldh1/1 BAC (Doyle and Zhang), S100β-EGFP (Cahoy) or GFAP-GFP+/GLT1+ (Lovatt) positive cells were selected as astrocytes, whereas neurons were selected as L1 expressing cells (Zhang), EGFP-negative (Cahoy) and Thy1-YFP-positive (Lovatt). In Doyle, 17 cell types expressing different neuron-specific transgenes under a BAC driven reporter were used. Isolation of cell-specific materials was carried out by a combination of FACS and immunopanning (Zhang and Cahoy), translating ribosome affinity purification (TRAP) (Doyle) and FACS (Lovatt). To determine gene expression, Zhang used RNA-sequencing, whereas the other three studies used the Affymetrix microarray platform. All the mitochondriomes are in Supporting Information Dataset S2. The diversity of cell-selection procedures, isolation protocols and mouse age is a strength of the analysis because it ensures the robustness of the conclusions derived from the comparisons among mitochondriomes.

3.2 | Mouse GSEA

The mitochondriomes of astrocytes and neurons were then compared by standard GSEA (Subramanian et al., 2005), which allows detecting statistically significant expression differences of a given set of genes in different phenotypes or conditions. Because the standard GSEA requires raw expression data, and these are only available in the Cahoy, Doyle and Zhang databases, only these three databases were used at this stage. As in all other analyses in the present manuscript, only

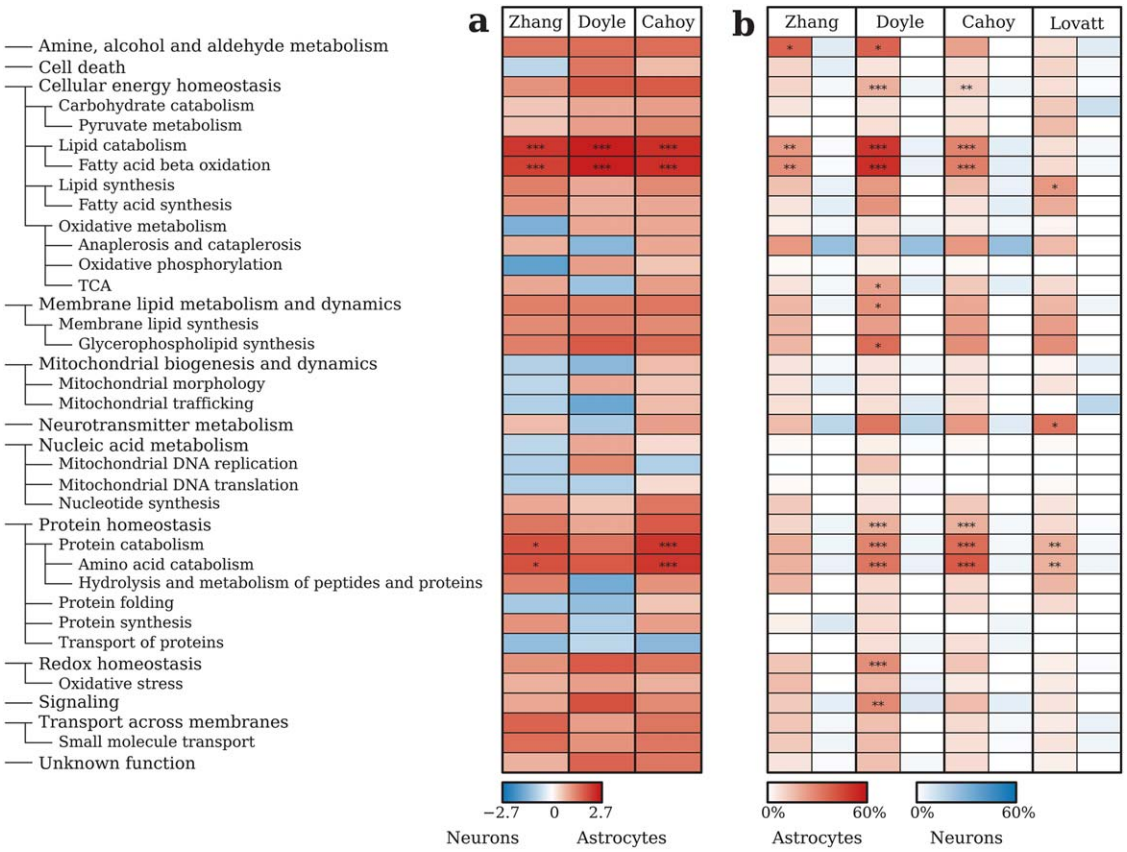

FIGURE 2 Heat maps of mitochondrial functions in mouse brain. (a) Standard GSEA analysis of mitochondrial functions (rows) in astrocytes versus neurons according to Zhang, Doyle, and Cahoy mouse brain transcriptomes (columns). The color scale indicates Normalized Enrichment Score (NES), from dark blue (over-expressed in neurons) to dark red (over-expressed in astrocytes). Statistical significance is given by the False Discovery Rate (FDR). $^{*}p < .01$ and $^{***}p < .0001$. (b) In-house GSEA of astrocyte versus neuronal over-expressed genes in Zhang, Doyle, Cahoy, and Lovatt transcriptomes. The color scale represents the percentage of genes over-expressed (log2FC > 2) in astrocytes (red) or neurons (blue). $^{*}p < .01$, $^{**}p < .001$, and $^{***}p < .0001$, according to the exact binomial test [Color figure can be viewed at wileyonlinelibrary.com]

MitoCarta-based functional groups with 10 or more genes were analyzed. Results clearly show that none of the functional groups are over-expressed in neurons *versus* astrocytes (Figure 2a). By contrast, the two main groups Lipid catabolism/Fatty acid beta-oxidation and Protein catabolism/Amino acid catabolism, are significantly over-expressed in astrocytes versus neurons in all databases except for the second group in the Doyle database. Interestingly, these functional groups belong or are related, in the case of Amino acid catabolism, to the category of Cellular energy homeostasis, suggesting the importance of mitochondrial metabolic functions in astrocytes.

To include Lovatt data in the analyses, we took the log 2 (fold change) of the data (log2FC), and performed an in-house GSEA. In brief, this method gives us the percentage of over-expressed genes (log2FC > 2 or < −2) in each functional group and gene-set percentages in neurons and astrocytes are compared with a binomial analysis. The in-house GSEA showed a high coincidence with the previous standard GSEA. No functional group is over-represented in neurons compared with astrocytes, but three groups are over-represented in astrocytes compared with neurons in at least two transcriptomes: Cellular-energy homeostasis/Lipid catabolism/Fatty acid beta-oxidation, Protein homeostasis/Protein catabolism/Amino acid catabolism, and Amine, alcohol and aldehyde metabolism (Figure 2b). Since the Lovatt database presents slightly different patterns of gene expression, we performed a correlation analysis of gene expression among databases. The Zhang, Doyle and Cahoy databases present high correlation coefficients between each other, and low correlations with Lovatt (Figure 3a). One can speculate that, different subpopulations of astrocytes were selected for the Lovatt study, or that older mice brains perform less oxidative metabolism (discussed below).

Overall, the data indicate over-representation of mitochondrial gene expression in astrocytes versus neurons. Importantly, this over-representation is not due to transcriptome bias, because the distribution of genes according to their relative expression in astrocytes versus neurons is even when all genes are considered. It is shifted to astrocytes in all transcriptomes—except Zhang—when only mitochondrial genes are taken into consideration (Figure 3b). Specifically, 131 genes of mitochondrial proteins are over-expressed (log2FC > 2) in astrocytes compared with neurons versus 24 genes over-expressed in neurons compared with astrocytes (log2FC < −2) in Doyle; 87 versus 20 in Cahoy; 74 versus 25 in Zhang and 50 versus 15 in the Lovatt database. The results contradict the prevailing view that astrocyte mitochondria are of lesser importance than neuronal mitochondria.

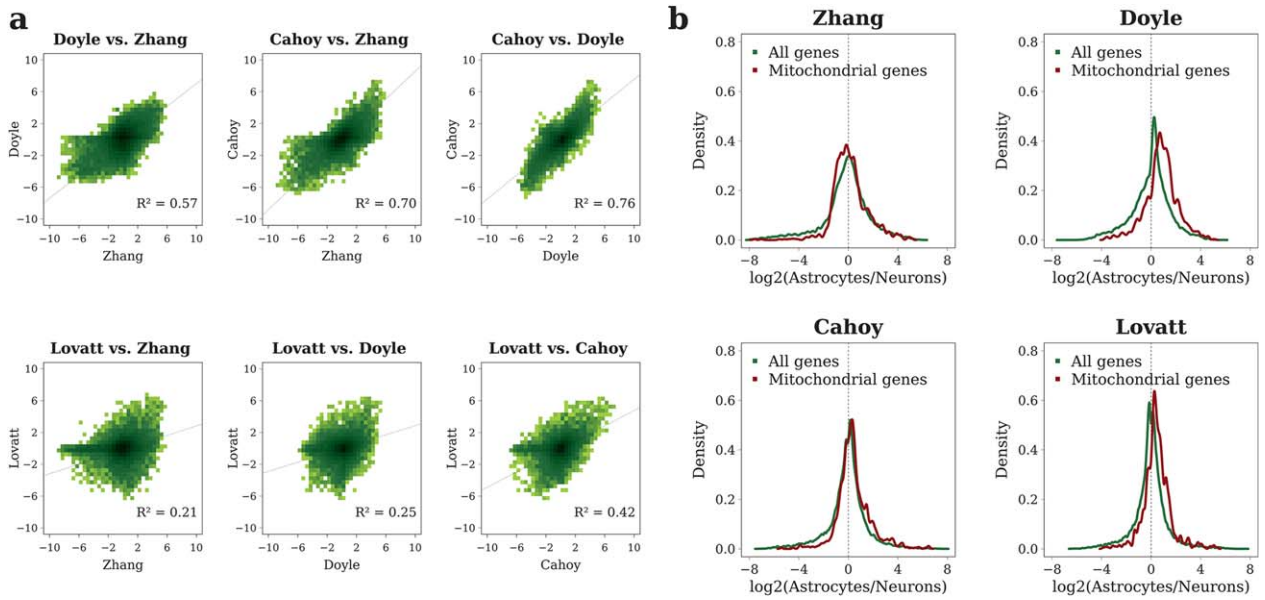

FIGURE 3 (a) Correlation analyses between transcriptomes. We plotted the correlation between the different transcriptomes of mice brain: Lovatt, Zhang, Doyle, and Cahoy. Each comparison is indicated in the title. Correlation coefficient (R^2) is indicated inside the graph and represents all genes present in each database. (b) Distribution of gene expression of each transcriptome between astrocytes and neurons. Representation of the number of total genes (density) versus the log2FC of gene expression (change of expression of each gene in astrocytes versus neurons). Positive values represent genes more expressed in astrocytes and negative values represent genes higher expressed in the neuronal compartment. The green line accounts for all genes whereas the red line represents those genes present in the MitoCarta database [Color figure can be viewed at wileyonlinelibrary.com]

3.3 | Fatty acid oxidation in astrocytes

To gain further insight into the distinct functional signature of astrocyte mitochondria, we examined the individual genes over-expressed in astrocytes in at least two transcriptomes (Table 1). In Cellular energy homeostasis, 19 out of the 36 over-expressed genes are related to canonical FAO. Notably, they encompass all the enzymes associated with this pathway (Figure 4b), including long-chain fatty acid transport to mitochondria by *Ctp1a* and *Ctp2*, in agreement with recent findings

TABLE 1 List of genes over-expressed in astrocytes and neurons in at least two transcriptomes of mice cortex

Main group	Astrocytes	Neurons
Amine, alcohol and aldehyde metabolism	*Aldh7a1*, *Aldh9a1*, *Adhfe1*, *Sardh*	
Cell death	*Aifm3*, *Bcl2*	
Cellular energy homeostasis	*Acaa2*, *Acad11*, *Acadl*, *Acadm*, *Acat3*, *Acot1*, *Acot2*, *Acss1*, *Ak4*, *Car5b*, *Cpt1a*, *Cpt2*, *Crot*, *Decr1*, *Echdc3*, *Eci1*, *Eci2*, *Ehhadh*, *Etfa*, *Gk*, *Glud1*, *Gpam*, *Gpt2*, *Hadh*, *Hadhb*, *Hmgcs2*, *Idh2*, *Pcx*, *Pdk2*, *Pdk4*, *Phyhipl*, *Sfxn5*, *Slc25a18*, *Slc25a20*, *Suclg2*, *Ucp3*	*Acsl1*, *Acsl4*, *Acsl5*, *Atpif1*, *Ckmt1*, *Gls*, *Gls2*, *Got2*, *Me3*, *Pdp1*, *Slc25a22*
Membrane lipid metabolism and dynamics	*Agpat5*, *Gk*, *Gpam*, *Idi1*	*Atp10d*
Mitochondrial biogenesis and dynamics	*Mfn1*	
Neurotransmitter metabolism	*Abat*, *Glud1*, *Maob*	*Gls* ,*Gls2*
Nucleic acid metabolism	*Aldh1l1*, *Fars2*, *Lactb2*, *Mthfd1*	*Nipsnap1*
Protein homeostasis	*Aass*, *Abat*, *Acadl*, *Aldh6a1*, *Aldh7a1*, *Dhtkd1*, *Dnajc19*, *Gcat*, *Gcsh*, *Gldc*, *Glud1*, *Gpt2*, *Hibadh*, *Hibch*, *Ivd*, *Lap3*, *Maob*, *Oat*, *Prodh*, *Prss35*, *Pacrg*, *Sdsl*, *Serhl*, *Suox*	*Alas2*, *Bcat1*, *Gls*, *Gls2*, *Tomm70a*
Redox homeostasis	*As3mt*, *Dhrs4*, *Gstk1*, *Msrb2*, *Prdx6*, *Sqrdl*, *Tst*	
Signaling	*Ddah1*, *Efhd1*, *Gng5*, *Mcur1*, *Ptgr2*	*Dusp26*, *Pak7*
Transport across membranes	*Abcd2*, *Slc25a18*, *Slc25a20*, *Slc25a34*, *Slc25a35*, *Ucp3*	*Slc25a22*
Unknown function	*BC026585*, *Ccdc90b*, *Hsdl2*, *Lyrm5*, *Pnkd*, *Pxmp2*	

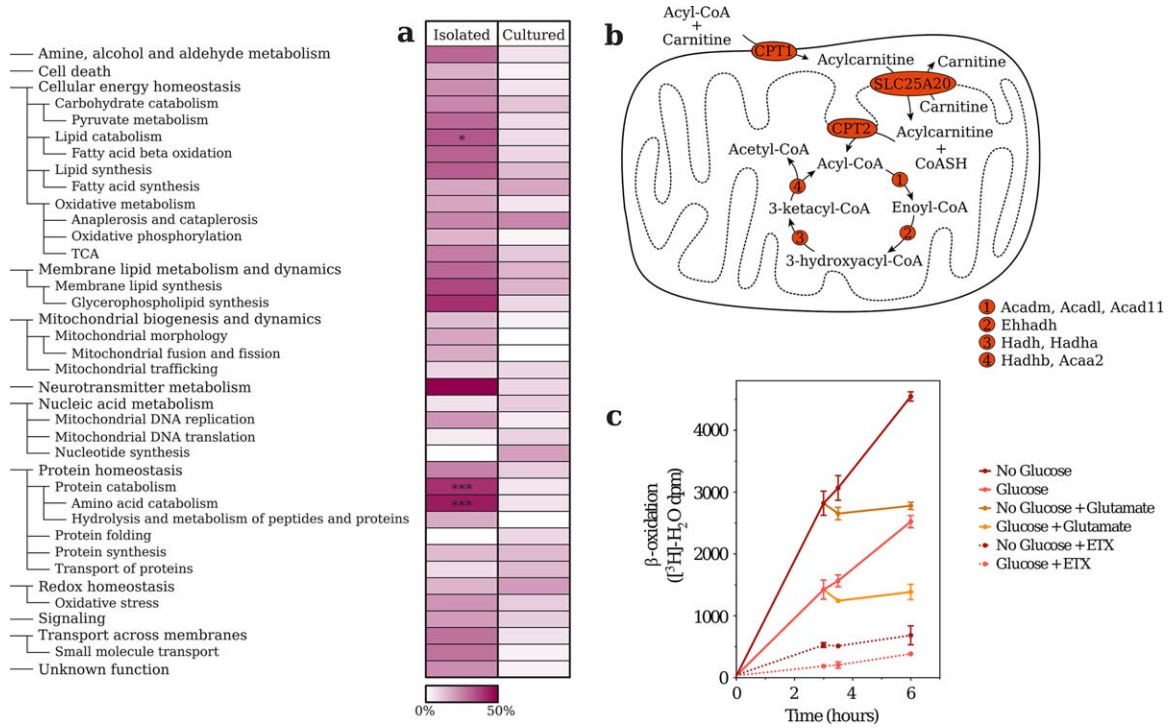

FIGURE 4 Fatty acid oxidation in astrocytes. (a) Heat map of mitochondrial functions in cultured astrocytes. In-house GSEA of over-expressed genes in cultured neonatal cortical and astrocytes acutely isolated from P17 mouse brain. Colors represent percentages of genes over-expressed (log2FC > 2). $^{*}p < .01$ and $^{***}p < .0001$ by exact binomial test. (b) Schematic representation of genes related to FAO over-expressed in astrocytes in at least two mitochondriomes. (c) FAO assay in cultured cortical astrocytes. Cells were incubated with [^{3}H]palmitic acid overnight and [^{3}H]H_2O accumulation, indicative of FAO, was measured incubating the cells with a Krebs-Ringer buffer with 10 mM glucose or without glucose. Etomoxir (ETX) was used to assess [^{3}H]H_2O accumulation from sources other than mitochondrial [^{3}H] palmitic acid oxidation. 100 μM glutamate was added 3 hr after the Krebs-Ringer buffer. Data are mean ± *SD* of one representative experiment of 3 [Color figure can be viewed at wileyonlinelibrary.com]

(Jernberg, Bowman, Wolfgang, & Scafidi, 2017). There is also over-expression of enzymes related to the oxidation of unsaturated fatty acids, namely *Decr1*, *Eci1* and *Eci2* (Table 1). In addition to FAO genes, the over-expression of three groups of genes in astrocytes *versus* neurons supports that FAO does occur in astrocyte mitochondria. First, there are *Pdk4* and *Pdk2*, genes that encode for pyruvate dehydrogenase kinases, which inhibit pyruvate dehydrogenase, preventing pyruvate conversion to acetyl-CoA. As a consequence, malonyl-CoA would not be produced from glucose (Foster, 2012) and would not inhibit the entrance of acyl-CoA (activated fatty acids) into the mitochondrion (Panov, Orynbayeva, Vavilin, & Lyakhovich, 2014). Second, there are genes related to enzymes that, if acetyl-CoA comes from FAO rather than from glucose oxidation, would ensure the replenishment of TCA intermediates through the following reactions: (1) anaplerotic reactions such as the transformation of pyruvate into oxaloacetate by pyruvate carboxylase (*Pcx*); (2) catabolism of glycogenic amino acids such as Glycine (*Gldc*, *Gcsh*), Alanine (*Gpt2*), Valine (*Hibadh*, *Hibch*, *Aldh6a1*), Serine (*Sdsl*, *Serhl*) and Proline (*Prodh*) in astrocytes; and (3) conversion of glutamate to alpha-ketoglutarate, as there is high expression of *Slc25a18* in astrocytes, which transports glutamate to the mitochondria. Also there is high expression of *Glud1*, a glutamate dehydrogenase that catalyzes the oxidative deamination of glutamate to alpha-ketoglutarate (McKenna, 2007). Third, there are genes related to protection against oxidative stress. The concern that FAO may cause excessive oxidative stress (Schonfeld & Reiser, 2017) is unwarranted because there is over-expression of *Prdx6* in astrocytes, an enzyme participating in the reduction of H_2O_2, and *Msrb2*, which provides protection against oxidative stress (Ugarte et al., 2013). This agrees with studies showing that astrocytes have higher levels of a variety of antioxidant molecules (including glutathione, ascorbate, and vitamin E) and display greater activities of cytosolic enzymes that detoxify reactive oxygen species such as glutathione S-transferase, glutathione peroxidase, and catalase (Belanger et al., 2011). Also there are a further five genes related to Redox homeostasis and aldehyde dehydrogenases over-expressed in astrocytes compared with neurons. Moreover, we should highlight that fatty acid availability is not a limiting step for astrocyte FAO as fatty acids are transported into the brain by either rapid passive diffusion and/or facilitated transport across the Brain Blood Barrier (Ebert, Haller, & Walton, 2003; Jernberg et al., 2017; Schonfeld & Reiser, 2013). Further, astrocytes could oxidize fatty acids from intracellular lipid droplets (Cabodevilla et al., 2013; Gubern et al., 2008). All in all, astrocytes appear to be endowed with the necessary molecular tools to carry out mitochondrial FAO.

In this vein, an active TCA cycle fueled by acetyl-CoA from FAO and carbon backbones derived from amino acid catabolism can produce glutamine or pyruvate, from cataplerotic reactions (Lovatt et al., 2007;

McKenna, 2007; Sonnewald et al., 1993). Hence glutamine and/or lactate could be supplied to neurons. Neurons could convert glutamine to glutamate via glutaminases *Gls* and *Gls2*, which have a higher expression in neurons compared with astrocytes (Table 1). Alternatively, acetyl-CoA from FAO could be diverted to ketogenesis, as astrocytes overexpress *Hmgcs2* which encodes HMG-CoA synthase 2, the rate-limiting enzyme of this pathway (Table 1). But some authors claim ketogenesis in the brain of fed animals is minimal (Ebert et al., 2003).

Altogether, the computerized analyses suggest that FAO is a constitutive metabolic pathway in astrocytes, compatible with glycolysis. To validate this prediction experimentally, we measured FAO in astrocytes by quantifying the production of $[^3H]H_2O$ from [^{3}H]palmitic acid. We chose cultured astrocytes to demonstrate unequivocally that astrocytes perform FAO. First, we confirmed that cultured astrocytes were a valid model by comparing the mitochondriomes of cultured astrocytes versus astrocytes isolated from adult mice (Cahoy et al., 2008) with the in-house GSEA (Supporting Information Dataset S3). We observed that there is no significant difference in Fatty acid beta-oxidation between cultured and brain-isolated astrocytes (Figure 4a). Second, we detected FAO in astrocytes. In the presence of 10 mM glucose FAO decreased by ~50%, but it was not abolished (Figure 4c). The data thus demonstrate the coexistence and interplay of FAO and glycolysis. Third, we examined FAO in the context of acutely increased glycolysis, as it occurs *in vivo* during task-elicited excitatory neurotransmission. This entails the release of glutamate that stimulates glucose uptake and glycolysis rate in astrocytes (Pellerin & Magistretti, 1994). To mimic this scenario, 100 μM glutamate was added 3 hr after the initial time of measurement of FAO. We observed that glutamate stops the accumulation of $[^3H]H_2O$, suggesting a complete inhibition of FAO (Figure 4c). The effect is noticeable as early as 30 min after the addition of glutamate, and does not depend on increased glycolysis, because it is observed also in the absence of external glucose (Figure 4c). The data support the capacity of astrocytes to simultaneously use glucose and fatty acids, except under conditions of high excitatory neuronal activity.

3.4 | Astrocyte mitochondria upon injury

The data thus far indicate that FAO is constitutive in normal astrocytes and finely regulated. In order to study what happens in injury, we compared with standard GSEA the mitochondriomes of spinal-cord astrocytes versus non-astrocytic cells 2 weeks after spinal-cord injury caused by severe crush and lateral compression, a model characterized by the appearance of reactive astrocytes (Anderson et al., 2016). Note that under normal conditions, the expression of mitochondrial genes in spinal cord astrocytes compared with other cell types is similar to astrocytes versus neurons in the healthy forebrain (Supporting Information Figure S1 and Dataset S3). Strikingly, the analysis revealed that mitochondrial functions were globally reduced in astrocytes after injury, with a statistically significant decrease in: Cellular energy homeostasis/Pyruvate metabolism, Lipid metabolism/Fatty acid oxidation, Oxidative metabolism/Oxidative phosphorylation and TCA, Mitochondrial DNA translation, Amino acid catabolism, and Small-molecule transport (Figure 5a). Although, we cannot assign these changes of the mitochondriome to a specific astrocyte population among those reported to appear in the injured brain (Wanner et al., 2013), our data support that fatty acid and amino acid based oxidative metabolism is a normal function of mitochondria that is impaired in brain injury.

3.5 | Astrocyte mitochondria in human brains

At present there is no evidence that FAO takes place in the human brain, probably due to the difficulty of measuring such activity in living subjects or in postmortem samples. Therefore, we took advantage of human brain transcriptomes available in Zhang et al., 2016 as a unique resource to elucidate the existence of FAO in human astrocytes. However, we did not perform GSEA-based comparisons between mitochondriomes of human astrocytes and neurons because the neuronal databases are available from only one subject. Instead, we performed standard GSEA comparing astrocyte transcriptomes with transcriptomes from the whole cortex from the same study. The lists of genes used can be found in Supporting Information Dataset S4 and the heat map in Figure 5b. Cellular energy homeostasis/Lipid catabolism/Fatty acid oxidation together with Oxidative metabolism/oxidative phosphorylation and TCA genes, Protein homeostasis and Nucleic acid metabolism/mitochondrial DNA translation are significantly over-expressed in astrocytes compared with whole cortex. The data therefore supports the existence of FAO and oxidative metabolism in human astrocytes.

Mitochondrion activity is associated with cell differentiation (Wanet, Arnould, Najimi, & Renard, 2015). The databases used so far derive from cells isolated from mice and humans at different ages, suggesting that FAO is present in both young and adult brains. But does it increase or decrease with astrocyte maturation? That is, is mitochondrial activity also crucial for astrocyte maturation? As noted, the comparison between cultured postnatal astrocytes and astrocytes isolated from adult brain serves to argue in favor of a potentiation of astrocyte mitochondrial functions with maturation. To further analyze this hypothesis, we compared, with standard GSEA, mitochondriomes from astrocytes isolated from fetal and adult human brains (Zhang et al., 2016). The lists of genes used can be found in Supporting Information Dataset S4. Almost all mitochondrial functions are over-expressed in adult versus fetal human astrocytes (Figure 5c). Specifically, Lipid catabolism/Fatty acid oxidation is, again, overexpressed in a statistically significant manner together with Oxidative metabolism/Oxidative phosphorylation and TCA, Protein catabolism/Amino acid catabolism, and Amine, alcohol and aldehyde metabolism as well as Membrane lipid metabolism and dynamics, Neurotransmitter metabolism and Oxidative stress. All in all, the GSEA suggest that mitochondrial functions are increased in astrocytes during development, and that oxidative metabolism fueled by FAO and amino acid catabolism takes place in adult human astrocytes.

Finally, we wanted to check if the increased expression of genes encoding for mitochondrial proteins upon maturation is exclusive to this organelle or it is a general trait. To this purpose we examined the distribution of genes encoding for components of different organelles with respect to their relative gene expression in adult versus fetal

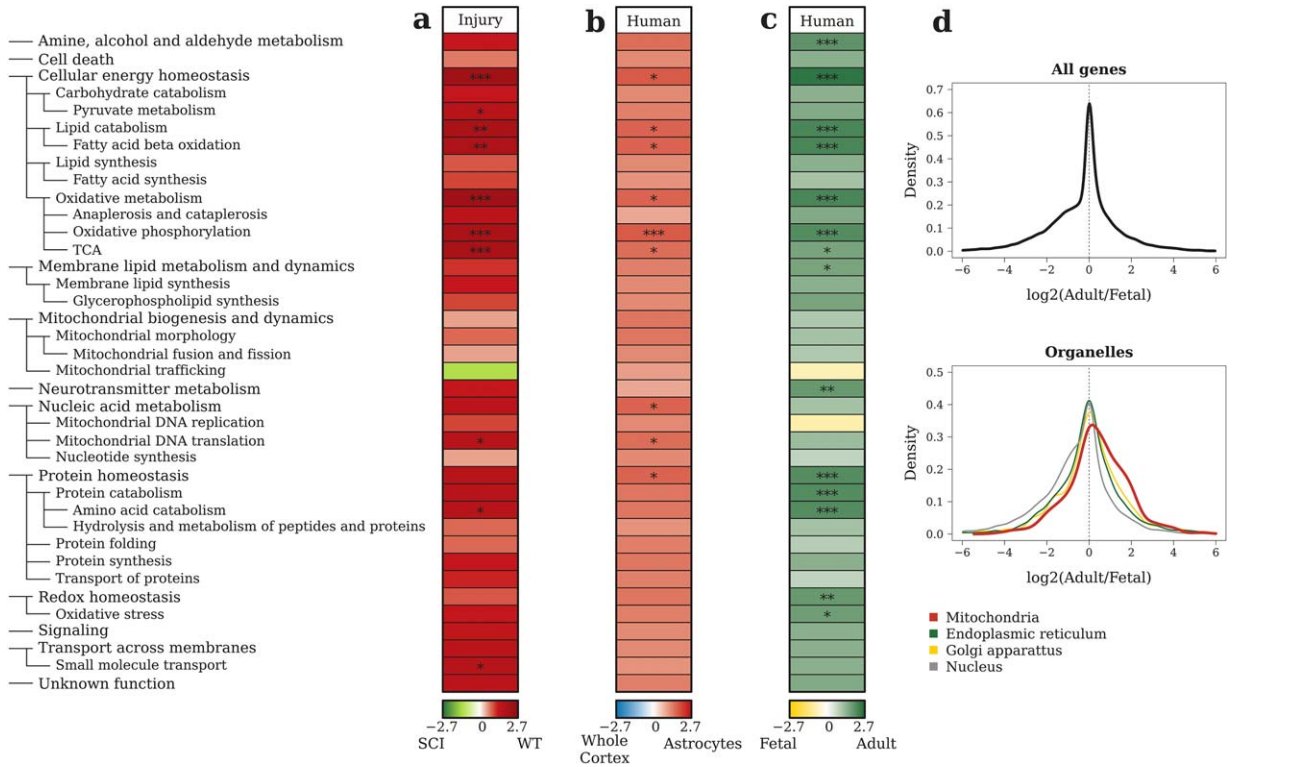

FIGURE 5 (a) Heat map of mitochondrial functions after spinal cord injury. Standard GSEA comparing spinal cord astrocytes before and 2 weeks after injury. Colors indicate Normalized Enrichment Score (NES). Groups enriched in WT astrocytes are represented in red while groups enriched in injured astrocytes are represented in green. $^{*}p < .01$, $^{**}p < .001$, and $^{***}p < .0001$ FDR. (b) Heat map of mitochondrial functions after standard GSEA analysis comparing human astrocytic and whole cortex transcriptomes. The color scale indicates NES, from dark blue (over-expressed in whole cortex) to dark red (over-expressed in astrocytes). $^{*}p < .01$ and $^{***}p < .0001$ FDR (c) Heat map of mitochondrial functions after standard GSEA analysis comparing adult versus fetal human astrocytes. Color indicates NES, green is enrichment in adult astrocytes and yellow in fetal astrocytes. $^{*}p < .01$, $^{**}p < .001$, and $^{***}p < .0001$ FDR. (d) Analysis of log2FC distributions of total gene expression for different organelles in adult versus fetal human astrocytes. Positive numbers represent genes highly expressed in adults, while negative numbers represent genes more expressed in fetal stages [Color figure can be viewed at wileyonlinelibrary.com]

human astrocytes (in log2FC; Supporting Information Dataset S5). The distribution of "total genes," genes related to the "endoplasmic reticulum" and genes related to the "Golgi apparatus" is even between fetal and adult cells. By contrast, genes encoding for 'nuclear proteins' are over-represented in fetal astrocytes, whereas genes encoding for 'mitochondrial proteins' are over-represented in adult astrocytes (Figure 5d). This indicates that the increased production of mitochondrial constituents during development is a unique feature of this organelle, at least in human astrocytes.

4 | DISCUSSION

This study represents the most extensive and integrated analysis of the expression of nuclear-genes encoding for mitochondrial proteins in the nervous system, to date. We have used data from: (1) four databases generated from mouse brains at different ages using different methodologies either to identify and isolate cells or to analyze gene expression; (2) cultured astrocytes; (3) fetal and adult human astrocytes; and (4) spinal cord astrocytes. The results support the relevance of astrocyte mitochondria and define different mitochondrial functional signatures in astrocytes versus neurons. Importantly, we strongly suggest that FAO is active in human astrocytes.

Our analyses show that the expression of TCA and oxidative-phosphorylation-related genes in mice is not different in astrocytes and neurons. Previous studies have reached the same conclusions as our analyses. In fact, Lovatt and colleagues reported that genes related to oxidative metabolism are upregulated in astrocytes versus neurons (Lovatt et al., 2007), but our analyses are more restrictive and do not reach statistical significance. Moreover, activities of complexes I–IV of the mitochondrial ETC have been recently shown to be the same in neurons and astrocytes isolated from mouse brains, despite the fact that complex I is predominantly assembled into super complexes in neurons but not in astrocytes (Lopez-Fabuel et al., 2016). That astrocytes are oxidative in humans is also supported by nuclear magnetic resonance *in vivo* showing mitochondrial glutamate oxidative metabolism after the addition of acetate (Bluml, Moreno-Torres, Shic, Nguy, & Ross, 2002; Lebon et al., 2002). Despite this evidence, the possibility that astrocytes are oxidative has been overlooked. Indeed, most reviews about brain metabolism, even recent ones, consider glucose as the only energy substrate, FAO being ignored (Belanger et al., 2011;

Lopez-Fabuel et al., 2016; Raichle, 2015), or denied (Schonfeld & Reiser, 2013, 2017). However, FAO was calculated to account for 15–20% of total brain oxidative energy production (Ebert et al., 2003; Panov et al., 2014), and direct and indirect evidence argue in its favor (Cabodevilla et al., 2013; Ebert et al., 2003; Edmond, Robbins, Bergstrom, Cole, & de Vellis, 1987; Jernberg et al., 2017; Kawamura, 1988; Panov et al., 2014; Sayre et al., 2017). Nevertheless, which cells perform FAO in whole brains has not been clarified. Our study provides molecular evidence to support that FAO takes place in the CNS, and its compartmentalization is mainly in astrocytes. Unlike previous findings, our conclusions come from an extensive range of transcriptomes of isolated astrocytes, FAO being a significant hit in all the astrocyte mitochondriomes analyzed. Robustness of the conclusions is also supported by the fact that all the FAO enzymes are overexpressed in astrocytes versus neurons at least in two mice transcriptomes, and this coincides with human data. A drawback of our study is that it is mainly based on data from transcriptomic studies, and hence it may not precisely reflect metabolic fluxes (Chubukov et al., 2013). Nevertheless transcriptomic studies have previously led to the identification of metabolic pathways (Corominas et al., 2013; Hu, Gu, Sun, Bai, & Wang, 2016) and they are a sound approach to address brain metabolism in humans (Barros et al., 2017). Moreover, genomic-based models have been extensively used to study metabolic networks (Duarte et al., 2007) and to simulate metabolic-based phenotypes of cancer cells (Gruetter, Seaquist, & Ugurbil, 2001; Lewis & Abdel-Haleem, 2013; Lewis et al., 2010).

Our experiments *in vitro* confirm the co-existence of aerobic glycolysis and FAO in astrocytes, as forecast in a review (Panov et al., 2014). They also agree with recent findings that suggest FAO occurs in the presence of glucose in mouse hippocampus and cortex (Jernberg et al., 2017). We show that FAO in astrocytes is versatile, because it is inhibited by high concentrations of glutamate, resembling states of high excitatory neuronal activity. It is well established that glutamate up-regulates glycolysis in astrocytes (Bittner et al., 2011; Loaiza, Porras, & Barros, 2003; Pellerin & Magistretti, 1994), which may account for [18F]-Fluorodeoxyglucose PET neuroimaging (Zimmer et al., 2017). However, the inhibition of FAO by glutamate does not appear to depend on glycolysis, because it also takes place in the absence of glucose. Without ruling out the possible involvement of glutamatergic receptors, glutamate induced inhibition of FAO might be a result of its uptake leading to: (1) an inhibition of the mitochondrial long-chain fatty acid transporter CPT1, as has been shown in permeabilized hepatocytes (Guzmán, Velasco, Castro, & Zammit, 1994); (2) activation of acetyl-CoA carboxylase, increasing levels of malonyl-CoA, and hence inhibiting CPT1, as shown previously in other tissues (Boone, Chan, Kulpa, & Brownsey, 2000); and/or (3) acidification of mitochondria and reduction of oxygen consumption (Azarias et al., 2011). Whatever the precise mechanism of FAO inhibition might be, it seems paradoxical that glutamate can inhibit FAO while entering the TCA and giving rise to lactate to be delivered to neurons (McKenna, 2007; Sonnewald et al., 1993). However, acetyl-CoA entering the TCA could come from other sources than FAO, namely amino acid catabolism, glucose or glycogen, as previously reported (Dienel & Cruz, 2004; Nissen et al., 2017; Nissen, Pajecka, Stridh, Skytt, & Waagepetersen, 2015; Swanson, 1992).

We suggest that FAO is key for brain metabolism. The fine regulated use of glucose, fatty acids and amino acids by astrocytes provide the brain with high flexibility. FAO also ensures ATP production needed under different physiological or pathological conditions. In this regard, our observations that FAO enzymes are downregulated in spinal cord injury agree with a metabolic depression suggested after acute spinal cord trauma (Levine et al., 2016) and with observations that FAO is protective in stroke (Sayre et al., 2017). Thus we propose a role of FAO in brain disease progression and that its restoration could have a therapeutic benefit. Considering that astrocytes become a heterogeneous population after spinal cord injury including reactive and newly generated astrocytes (Wanner et al., 2013), future studies should clarify the fate of FAO in each astrocyte type at different times after injury, and what happens to astrocyte FAO in neurodegenerative diseases.

A most important implication of the existence of a versatile mitochondrial metabolism in astrocytes of adult human brain, including oxidative metabolism by way of FAO and amino acid catabolism, is the interpretation of imaging techniques that monitor neuronal activity based on oxygen consumption or availability. An example is BOLD-MRI, which is widely used to monitor neuronal activity, and that also depends on blood flow. First, if astrocyte FAO consumes high amounts of O_2 and it is inhibited by glutamate, it follows that the increase in glucose consumption in astrocytes triggered by excitatory neuronal activity would be accompanied by a decrease in O_2 consumption. This might explain one of the big enigmas in neuroenergetics and neuroimaging: why are task-elicited increases in blood flow and glucose consumption only marginally matched by increases in O_2 consumption (Belanger et al., 2011; Fox, Raichle, Mintun, & Dence, 1988)? Second, is astrocyte FAO coupled to blood flow and O_2 availability, as measured by the BOLD signal? Third, beyond task-dependent metabolic requirements—the most studied paradigm in the relationship between brain function and energy metabolism; does astrocyte FAO sustain astrocyte functions during intrinsic activity, the activity of the brain in the absence of external inputs (Raichle, 2015)?

Finally, we demonstrated the over-expression of genes encoding for mitochondrial proteins compared with other organelles in adult human astrocytes including FAO, amino acid catabolism and oxidative metabolism-related genes. Our results are an important addition to findings showing that glycogen metabolism, aerobic glycolysis and lactate release are markers of astrocyte differentiation from neural stem cells (Brunet, Allaman, Magistretti, & Pellerin, 2010; Brunet et al., 2004). Our data confirm that astrocytes also follow the golden rule of increased mitochondrial activity and metabolism during differentiation (Wanet et al., 2015), but reveal cell-specific particularities. For example, when neurogenesis is induced by PGC1alfa-mediated mitochondrial biogenesis, there is no upregulation or downregulation of FAO enzymes (Stoll et al., 2015). However, according to our data, astrocyte maturation implies an increase in FAO and amino acid catabolism, in agreement with the developmental upregulation of carnitine palmitoyl transferases and acyl-CoA dehydrogenases in young mice (Jernberg et al., 2017). We thus argue that the key point in deriving astrocytes

from iPSCs, or neurons from astrocytes, is mitochondrial specialization, and not the switch from a non-oxidative to an oxidative phenotype. Interestingly, a meta-analysis of glucose and oxygen consumption across the human lifespan concluded that anaerobic glycolysis appears to decrease dramatically in the elderly, whereas oxygen consumption is slightly higher (Goyal et al., 2014). This raises the possibility that astrocyte FAO gains importance in aging.

In summary, the study reveals astrocyte mitochondria as key, specialized organelles in the adult brain and broadens the perception that astrocytes are merely glycolytic cells. They are glycolytic and fatty acid and amino acid oxidative cells. This metabolic complexity should be taken into consideration when examining the relationship between brain energy metabolism and function in health and disease, as well as in development.

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REFERENCES

Agarwal, A., Wu, P.-H., Hughes, E. G., Fukaya, M., Tischfield, M. A., Langseth, A. J., … Bergles, D. E. (2017). Transient opening of the mitochondrial permeability transition pore induces microdomain calcium transients in astrocyte processes. *Neuron*, *93*(3), 587. 605 e587. https://doi.org/10.1016/j.neuron.2016.12.034

Anderson, M. A., Burda, J. E., Ren, Y., Ao, Y., O'Shea, T. M., Kawaguchi, R., … Sofroniew, M. V. (2016). Astrocyte scar formation aids central nervous system axon regeneration. *Nature*, *532*(7598), 195–200. https://doi.org/10.1038/nature17623

Ashrafi, G., & Ryan, T. A. (2017). Glucose metabolism in nerve terminals. *Current Opinion in Neurobiology*, *45*, 156–161. https://doi.org/10.1016/j.conb.2017.03.007

Azarias, G., Perreten, H., Lengacher, S., Poburko, D., Demaurex, N., Magistretti, P. J., … Chatton, J. Y. (2011). Glutamate transport decreases mitochondrial pH and modulates oxidative metabolism in astrocytes. *J Neurosci*, *315*(10), 3550–3559. https://doi.org/10.1523/JNEUROSCI.4378-10.2011

Barceló-Torns, M., Lewis, A. M., Gubern, A., Barneda, D., Bloor-Young, D., Picatoste, F., … Masgrau, R. (2011). NAADP mediates ATP-induced Ca2+ signals in astrocytes. *FEBS Letters*, *585*(14), 2300–2306. https://doi.org/10.1016/j.febslet.2011.05.062

Barros, L. F., Bolanos, J. P., Bonvento, G., Bouzier-Sore, A. K., Brown, A., Hirrlinger, J., … Weber, B. (2017). Current technical approaches to brain energy metabolism. *Glia*, https://doi.org/10.1002/glia.23248

Belanger, M., Allaman, I., & Magistretti, P. J. (2011). Brain energy metabolism: Focus on astrocyte-neuron metabolic cooperation. *Cell Metabolism*, *14*(6), 724–738. https://doi.org/10.1016/j.cmet.2011.08.016

Belanger, M., & Magistretti, P. J. (2009). The role of astroglia in neuroprotection. *Dialogues in Clinical Neuroscience*, *11*(3), 281–295.

Bittner, C. X., Valdebenito, R., Ruminot, I., Loaiza, A., Larenas, V., Sotelo-Hitschfeld, T., … Barros, L. F. (2011). Fast and reversible stimulation of astrocytic glycolysis by K+ and a delayed and persistent effect of glutamate. *Journal of Neuroscience*, *31*(12), 4709–4713. https://doi.org/10.1523/JNEUROSCI.5311-10.2011

Bluml, S., Moreno-Torres, A., Shic, F., Nguy, C. H., & Ross, B. D. (2002). Tricarboxylic acid cycle of glia in the in vivo human brain. *NMRi N Biomedicine*, *15*(1), 1–5.

Boone, A. N., Chan, A., Kulpa, J. E., & Brownsey, R. W. (2000). Bimodal activation of acetyl-CoA carboxylase by glutamate. *J Biol Chem*, *275*(15), 10819–10825.

Brunet, J. F., Allaman, I., Magistretti, P. J., & Pellerin, L. (2010). Glycogen metabolism as a marker of astrocyte differentiation. *Journal of Cerebral Blood Flow & Metabolism*, *30*(1), 51–55. https://doi.org/10.1038/jcbfm.2009.207

Brunet, J. F., Grollimund, L., Chatton, J. Y., Lengacher, S., Magistretti, P. J., Villemure, J. G., & Pellerin, L. (2004). Early acquisition of typical metabolic features upon differentiation of mouse neural stem cells into astrocytes. *Glia*, *46*(1), 8–17. https://doi.org/10.1002/glia.10348

Cabodevilla, A. G., Sanchez-Caballero, L., Nintou, E., Boiadjieva, V. G., Picatoste, F., Gubern, A., & Claro, E. (2013). Cell survival during complete nutrient deprivation depends on lipid droplet-fueled beta-oxidation of fatty acids. *Journal of Biological Chemistry*, *288*(39), 27777–27788. https://doi.org/10.1074/jbc.M113.466656

Cahoy, J. D., Emery, B., Kaushal, A., Foo, L. C., Zamanian, J. L., Christopherson, K. S., … Barres, B. A. (2008). A transcriptome database for astrocytes, neurons, and oligodendrocytes: A new resource for understanding brain development and function. *Journal of Neuroscience*, *28*(1), 264–278. https://doi.org/10.1523/JNEUROSCI.4178-07.2008

Cerdán, S., Rodrigues, T. B., Sierra, A., Benito, M., Fonseca, L. L., Fonseca, C. P., & García-Martín, M. L. (2006). The redox switch/redox coupling hypothesis. *Neurochemistry International*, *48*(6–7), 523–530. https://doi.org/10.1016/j.neuint.2005.12.036

Chubukov, V., Uhr, M., Le Chat, L., Kleijn, R. J., Jules, M., Link, H., … Sauer, U. (2013). Transcriptional regulation is insufficient to explain substrate-induced flux changes in Bacillus subtilis. *Molecular Systems Biology*, *9*(1), 709. https://doi.org/10.1038/msb.2013.66

Corominas, J., Ramayo-Caldas, Y., Puig-Oliveras, A., Estellé, J., Castelló, A., Alves, E., … Folch, J. M. (2013). Analysis of porcine adipose tissue transcriptome reveals differences in de novo fatty acid synthesis in pigs with divergent muscle fatty acid composition. *BMC Genomics*, *14*(1), 843. https://doi.org/10.1186/1471-2164-14-843

Diaz-Garcia, C. M., Mongeon, R., Lahmann, C., Koveal, D., Zucker, H., & Yellen, G. (2017). Neuronal stimulation triggers neuronal glycolysis and not lactate uptake. *Cell Metabolism*, *26*(2), 361–374. e364. https://doi.org/10.1016/j.cmet.2017.06.021

Dienel, G. A. (2017). The metabolic trinity, glucose-glycogen-lactate, links astrocytes and neurons in brain energetics, signaling, memory, and gene expression. *Neuroscience Letters*, *637*, 18–25. https://doi.org/10.1016/j.neulet.2015.02.052

Dienel, G. A., & Cruz, N. F. (2004). Nutrition during brain activation: Does cell-to-cell lactate shuttling contribute significantly to sweet and sour food for thought? *Neurochemistry International*, *45*(2–3), 321–351. https://doi.org/10.1016/j.neuint.2003.10.011

Doyle, J. P., Dougherty, J. D., Heiman, M., Schmidt, E. F., Stevens, T. R., Ma, G., … Heintz, N. (2008). Application of a translational profiling

approach for the comparative analysis of CNS cell types. *Cell*, *135*(4), 749–762. https://doi.org/10.1016/j.cell.2008.10.029

Duarte, N. C., Becker, S. A., Jamshidi, N., Thiele, I., Mo, M. L., Vo, T. D., … Palsson, B. O. (2007). Global reconstruction of the human metabolic network based on genomic and bibliomic data. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(6), 1777–1782. https://doi.org/10.1073/pnas.0610772104

Ebert, D., Haller, R. G., & Walton, M. E. (2003). Energy contribution of octanoate to intact rat brain metabolism measured by 13C nuclear magnetic resonance spectroscopy. *Journal of Neuroscience*, *23*(13), 5928–5935.

Edmond, J., Robbins, R. A., Bergstrom, J. D., Cole, R. A., & de Vellis, J. (1987). Capacity for substrate utilization in oxidative metabolism by neurons, astrocytes, and oligodendrocytes from developing brain in primary culture. *Journal of Neuroscience Research*, *18*(4), 551–561. https://doi.org/10.1002/jnr.490180407

Eraso-Pichot, A., Larramona-Arcas, R., Vicario-Orri, E., Villalonga, R., Pardo, L., Galea, E., & Masgrau, R. (2017). CREB decreases astrocytic excitability by modifying subcellular calcium fluxes via the sigma-1 receptor. *Cellular and Molecular Life Sciences*, *74*(5), 937–950. https://doi.org/10.1007/s00018-016-2397-5

Foster, D. W. (2012). Malonyl-CoA: The regulator of fatty acid synthesis and oxidation. *The Journal of Clinical Investigation*, *122*(6), 1958–1959.

Fox, P. T., Raichle, M. E., Mintun, M. A., & Dence, C. (1988). Nonoxidative glucose consumption during focal physiologic neural activity. *Science*, *241*(4864), 462–464.

Goyal, M. S., Hawrylycz, M., Miller, J. A., Snyder, A. Z., & Raichle, M. E. (2014). Aerobic glycolysis in the human brain is associated with development and neotenous gene expression. *Cell Metabolism*, *19*(1), 49–57. https://doi.org/10.1016/j.cmet.2013.11.020

Gruetter, R., Seaquist, E. R., & Ugurbil, K. (2001). A mathematical model of compartmentalized neurotransmitter metabolism in the human brain. *American Journal of Physiology. Endocrinology and Metabolism*, *281*(1), E100–E112.

Gubern, A., Casas, J., Barceló-Torns, M., Barneda, D., de la Rosa, X., Masgrau, R., … Claro, E. (2008). Group IVA phospholipase A2 is necessary for the biogenesis of lipid droplets. *Journal of Biological Chemistry*, *283*(41), 27369–27382. https://doi.org/10.1074/jbc.M800696200

Guzmán, M., Velasco, G., Castro, J., & Zammit, V. A. (1994). Inhibition of carnitine palmitoyltransferase I by hepatocyte swelling. *FEBS Lett*, *344*(2-3), 239–241.

Herrero-Mendez, A., Almeida, A., Fernandez, E., Maestre, C., Moncada, S., & Bolanos, J. P. (2009). The bioenergetic and antioxidant status of neurons is controlled by continuous degradation of a key glycolytic enzyme by APC/C-Cdh1. *Nature Cell Biology*, *11*(6), 747–752. https://doi.org/10.1038/ncb1881

Hu, G., Gu, W., Sun, P., Bai, Q., & Wang, B. (2016). Transcriptome analyses reveal lipid metabolic process in liver related to the difference of carcass fat content in rainbow trout (Oncorhynchus mykiss). *International Journal of Genomics*, *2016*, 1. https://doi.org/10.1155/2016/7281585

Jernberg, J. N., Bowman, C. E., Wolfgang, M. J., & Scafidi, S. (2017). Developmental regulation and localization of carnitine palmitoyltransferases (CPTs) in rat brain. *Journal of Neurochemistry*, *142*(3), 407–419. https://doi.org/10.1111/jnc.14072

Kawamura, N. (1988). Regulation of fatty acid oxidation in rat brain mitochondria: Inhibition of high rates of palmitate oxidation by ADP. *Archives of Biochemistry and Biophysics*, *264*(2), 546–552.

Lebon, V., Petersen, K. F., Cline, G. W., Shen, J., Mason, G. F., Dufour, S., … Rothman, D. L. (2002). Astroglial contribution to brain energy metabolism in humans revealed by 13C nuclear magnetic resonance spectroscopy: Elucidation of the dominant pathway for neurotransmitter glutamate repletion and measurement of astrocytic oxidative metabolism. *Journal of Neuroscience*, *22*(5), 1523–1531.

Levine, J., Kwon, E., Paez, P., Yan, W., Czerwieniec, G., Loo, J. A., … Wanner, I.-B. (2016). Traumatically injured astrocytes release a proteomic signature modulated by STAT3-dependent cell survival. *Glia*, *64*(5), 668–694. https://doi.org/10.1002/glia.22953

Lewis, N. E., & Abdel-Haleem, A. M. (2013). The evolution of genome-scale models of cancer metabolism. *Frontiers in Physiology*, *4*, 237. https://doi.org/10.3389/fphys.2013.00237

Lewis, N. E., Schramm, G., Bordbar, A., Schellenberger, J., Andersen, M. P., Cheng, J. K., … Palsson, B. Ø. (2010). Large-scale in silico modeling of metabolic interactions between cell types in the human brain. *Nature Biotechnology*, *28*(12), 1279–1285. https://doi.org/10.1038/nbt.1711

Loaiza, A., Porras, O. H., & Barros, L. F. (2003). Glutamate triggers rapid glucose transport stimulation in astrocytes as evidenced by real-time confocal microscopy. *Journal of Neuroscience*, *23*(19), 7337–7342.

Lopez-Fabuel, I., Le Douce, J., Logan, A., James, A. M., Bonvento, G., Murphy, M. P., … Bolaños, J. P. (2016). Complex I assembly into supercomplexes determines differential mitochondrial ROS production in neurons and astrocytes. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(46), 13063–13068. https://doi.org/10.1073/pnas.1613701113

Lovatt, D., Sonnewald, U., Waagepetersen, H. S., Schousboe, A., He, W., Lin, J. H.-C., … Nedergaard, M. (2007). The transcriptome and metabolic gene signature of protoplasmic astrocytes in the adult murine cortex. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *27*(45), 12255–12266. https://doi.org/10.1523/JNEUROSCI.3404-07.2007

Mächler, P., Wyss, M. T., Elsayed, M., Stobart, J., Gutierrez, R., von Faber-Castell, A., … Weber, B. (2016). In vivo evidence for a lactate gradient from astrocytes to neurons. *Cell Metabolism*, *23*(1), 94–102. https://doi.org/10.1016/j.cmet.2015.10.010

McKenna, M. C. (2007). The glutamate-glutamine cycle is not stoichiometric: Fates of glutamate in brain. *Journal of Neuroscience Research*, *85*(15), 3347–3358. https://doi.org/10.1002/jnr.21444

McKenna, M. C., Stridh, M. H., McNair, L. F., Sonnewald, U., Waagepetersen, H. S., & Schousboe, A. (2016). Glutamate oxidation in astrocytes: Roles of glutamate dehydrogenase and aminotransferases. *Journal of Neuroscience Research*, *94*(12), 1561–1571. https://doi.org/10.1002/jnr.23908

Nagase, M., Takahashi, Y., Watabe, A. M., Kubo, Y., & Kato, F. (2014). On-site energy supply at synapses through monocarboxylate transporters maintains excitatory synaptic transmission. *Journal of Neuroscience*, *34*(7), 2605–2617. https://doi.org/10.1523/JNEUROSCI.4687-12.2014

Nissen, J. D., Lykke, K., Bryk, J., Stridh, M. H., Zaganas, I., Skytt, D. M., … Waagepetersen, H. S. (2017). Expression of the human isoform of glutamate dehydrogenase, hGDH2, augments TCA cycle capacity and oxidative metabolism of glutamate during glucose deprivation in astrocytes. *Glia*, *65*(3), 474–488. https://doi.org/10.1002/glia.23105

Nissen, J. D., Pajęcka, K., Stridh, M. H., Skytt, D. M., & Waagepetersen, H. S. (2015). Dysfunctional TCA-cycle metabolism in glutamate dehydrogenase deficient astrocytes. *Glia*, *63*(12), 2313–2326. https://doi.org/10.1002/glia.22895

Pagliarini, D. J., Calvo, S. E., Chang, B., Sheth, S. A., Vafai, S. B., Ong, S.-E., ... Mootha, V. K. (2008). A mitochondrial protein compendium elucidates complex I disease biology. *Cell*, *134*(1), 112–123. https://doi.org/10.1016/j.cell.2008.06.016

Panov, A., Orynbayeva, Z., Vavilin, V., & Lyakhovich, V. (2014). Fatty acids in energy metabolism of the central nervous system. *Biomed Research International*, *2014*, 472459. https://doi.org/10.1155/2014/472459

Pellerin, L., & Magistretti, P. J. (1994). Glutamate uptake into astrocytes stimulates aerobic glycolysis: A mechanism coupling neuronal activity to glucose utilization. *Proceedings of the National Academy of Sciences of the United States of America*, *91*(22), 10625–10629.

Raichle, M. E. (2015). The restless brain: How intrinsic activity organizes brain function. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *370*(1668), 20140172. https://doi.org/10.1098/rstb.2014.0172

Sayre, N. L., Sifuentes, M., Holstein, D., Cheng, S. Y., Zhu, X., & Lechleiter, J. D. (2017). Stimulation of astrocyte fatty acid oxidation by thyroid hormone is protective against ischemic stroke-induced damage. *Journal of Cerebral Blood Flow & Metabolism*, *37*(2), 514–527. https://doi.org/10.1177/0271678X16629153

Schonfeld, P., & Reiser, G. (2013). Why does brain metabolism not favor burning of fatty acids to provide energy? Reflections on disadvantages of the use of free fatty acids as fuel for brain. *Journal of Cerebral Blood Flow & Metabolism*, *33*(10), 1493–1499. https://doi.org/10.1038/jcbfm.2013.128

Schonfeld, P., & Reiser, G. (2017). Brain energy metabolism spurns fatty acids as fuel due to their inherent mitotoxicity and potential capacity to unleash neurodegeneration. *Neurochemistry International*, https://doi.org/10.1016/j.neuint.2017.03.018

Schousboe, A., Scafidi, S., Bak, L. K., Waagepetersen, H. S., & McKenna, M. C. (2014). Glutamate metabolism in the brain focusing on astrocytes. *Advances in Neurobiology*, *11*, 13–30. https://doi.org/10.1007/978-3-319-08894-5_2

Shank, R. P., Bennett, G. S., Freytag, S. O., & Campbell, G. L. (1985). Pyruvate carboxylase: An astrocyte-specific enzyme implicated in the replenishment of amino acid neurotransmitter pools. *Brain Research*, *329*(1–2), 364–367.

Sonnewald, U., Westergaard, N., Petersen, S. B., Unsgard, G., & Schousboe, A. (1993). Metabolism of [U-13C]glutamate in astrocytes studied by 13C NMR spectroscopy: Incorporation of more label into lactate than into glutamine demonstrates the importance of the tricarboxylic acid cycle. *Journal of Neurochemistry*, *61*(3), 1179–1182.

Sonnewald, U., Westergaard, N., & Schousboe, A. (1997). Glutamate transport and metabolism in astrocytes. *Glia*, *21*(1), 56–63.

Stoll, E. A., Makin, R., Sweet, I. R., Trevelyan, A. J., Miwa, S., Horner, P. J., & Turnbull, D. M. (2015). Neural stem cells in the adult subventricular zone oxidize fatty acids to produce energy and support neurogenic activity. *Stem Cells*, *33*(7), 2306–2319. https://doi.org/10.1002/stem.2042

Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., ... Mesirov, J. P. (2005). Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(43), 15545–15550. https://doi.org/10.1073/pnas.0506580102

Swanson, R. A. (1992). Physiologic coupling of glial glycogen metabolism to neuronal activity in brain. *Canadian Journal of Physiology and Pharmacology*, *70*(S1), S138–S144.

Ugarte, N., Ladouce, R., Radjei, S., Gareil, M., Friguet, B., & Petropoulos, I. (2013). Proteome alteration in oxidative stress-sensitive methionine sulfoxide reductase-silenced HEK293 cells. *Free Radical Biology & Medicine*, *65*, 1023–1036. https://doi.org/10.1016/j.freeradbiomed.2013.08.008

Waagepetersen, H. S., Qu, H., Schousboe, A., & Sonnewald, U. (2001). Elucidation of the quantitative significance of pyruvate carboxylation in cultured cerebellar neurons and astrocytes. *Journal of Neuroscience Research*, *66*(5), 763–770. https://doi.org/10.1002/jnr.10061

Wanet, A., Arnould, T., Najimi, M., & Renard, P. (2015). Connecting mitochondria, metabolism, and stem cell fate. *Stem Cells and Development*, *24*(17), 1957–1971. https://doi.org/10.1089/scd.2015.0117

Wanner, I. B., Anderson, M. A., Song, B., Levine, J., Fernandez, A., Gray-Thompson, Z., ... Sofroniew, M. V. (2013). Glial scar borders are formed by newly proliferated, elongated astrocytes that interact to corral inflammatory and fibrotic cells via STAT3-dependent mechanisms after spinal cord injury. *Journal of Neuroscience*, *33*(31), 12870–12886. https://doi.org/10.1523/JNEUROSCI.2121-13.2013

Yu, A. C., Schousboe, A., & Hertz, L. (1982). Metabolic fate of 14C-labeled glutamate in astrocytes in primary cultures. *Journal of Neurochemistry*, *39*(4), 954–960.

Zhang, Y., Chen, K., Sloan, S. A., Bennett, M. L., Scholze, A. R., O'Keeffe, S., ... Wu, J. Q. (2014). An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *Journal of Neuroscience*, *34*(36), 11929–11947. https://doi.org/10.1523/JNEUROSCI.1860-14.2014

Zhang, Y., Sloan, S. A., Clarke, L. E., Caneda, C., Plaza, C. A., Blumenthal, P. D., ... Barres, B. A. (2016). Purification and characterization of progenitor and mature human astrocytes reveals transcriptional and functional differences with mouse. *Neuron*, *89*(1), 37–53. https://doi.org/10.1016/j.neuron.2015.11.013

Zimmer, E. R., Parent, M. J., Souza, D. G., Leuzy, A., Lecrux, C., Kim, H. L., ... Rosa-Neto, P. (2017). [18F]FDG PET signal is driven by astroglial glutamate transport. *Nature Neuroscience*, *20*(3), 393–395. https://doi.org/10.1038/nn.4492

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DISCUSSION

VII. GENERAL DISCUSSION

Plasticity is the ability of a specific cell or network to functionally and structurally modify themselves in response to experience (von Bernhardi et al., 2017). In the brain, plasticity is crucial for all brain functions, from learning and memory processes to functional recovery after injury (Pascual-Leone et al., 2005).

In the brain, the most studied form of plasticity is neuronal plasticity, consisting of changes in neuronal functions in response to different stimuli. One of the most well known forms of neuronal plasticity is synaptic plasticity, which in short consists in changes in synaptic strength that are linked with learning and memory (Takeuchi et al., 2014). Interestingly, synaptic plasticity includes different mechanisms performing a broad range of adaptive responses: from short-term plasticity mechanisms lasting milliseconds (Abbott and Regehr, 2004) to long-term plasticity, which includes the typical forms of Hebbian plasticity (LTP and LTD), which last from minutes to months (Abraham, 2003) (Martin et al., 2000).

Our laboratory is interested in how experience changes pivotal functions of astrocytes such as calcium signals or metabolism, in what is called "astrocyte plasticity". These modifications in astrocyte physiology may, in turn, affect neuronal functions, establishing a mechanism of bidirectional communication between both cells. Understanding how astrocyte plasticity is developed -which neuronal signals produce which changes, for how long, and how they affect higher-brain functions- is one of the key questions in the astrocyte field. However, "astrocyte plasticity" is a novel concept rarely used, and it has not been as studied as its importance deserves. Indeed, most of the works addressing astrocytic changes in response to experience (neuronal activation) focused on short-term adaptations, without the need of *de novo* gene expression.

The results presented in this dissertation show a mechanism of long-term "astrocyte plasticity" involving the transcription factor CREB, known to be activated by neuronal activity (Carriba et al., 2012). We have demonstrated that CREB activation in astrocytes in response to neurotransmitters, neuromodulators, or using the viral construct VP16-CREB induces changes in calcium signals and energy metabolism. Concretely, our results point to two specific CREB-dependent targets, the ER chaperone SigmaR1 and the lactate transporter MCT4 as the two proteins that mediate these changes.

In addition to the CREB-mediated response, we have shown that astrocytic metabolism is highly adaptive, with changes in mitochondrial gene expression during development or injury situations and with short-term adaptive responses such as fatty-acid oxidation (FAO) inhibition after glutamate stimulation.

Taken together, our results represent the initial foundation to address "astrocyte plasticity" in increasingly complex paradigms, defining the concept in a wider way and establishing different types of astrocyte adaptive responses.

CREB-induced astrocyte plasticity

Our results show that CREB activation in astrocytes induces a complex and multifunctional response. In the first chapter, we have described that CREB activation in astrocytes reduces astrocytic cytosolic calcium rises, while, at the same time, it increases mitochondrial calcium entry and ER-release through the expression of the MAM protein SigmaR1. In the second chapter, we have discovered that activation of CREB-dependent transcription enhances the expression of the monocarboxylate transporter 4 and increases lactate release. In order to integrate both functions in a physiological environment, we have to understand the state-of-the-art of astrocytic calcium and lactate release, as well as in which situations CREB can be activated:

1. ER-dependent cytosolic calcium rises are implicated in neuromodulator effects

As explained in the introduction, astrocytes release gliotransmitters in response to increases in cytosolic calcium (Araque et al., 2014). Although the exact mechanism is not fully understood (Sahlender et al., 2014), astrocytic calcium effects are no longer questioned, in part thanks to the recent technical advances in the field (Volterra et al., 2014). In fact, astrocytic calcium rises and gliotransmitter release have been implicated in neuronal synaptic plasticity (De Pitta et al., 2016), but also in the neuromodulatory effects of volume transmitters (Bekar et al., 2008) and in the global synchronization of brain electrical activity (Poskanzer and Yuste, 2016).

Interestingly, the recent discovery of different astrocytic signals in different cellular compartments has changed completely our mind about astrocytic calcium functions, since what we previously put together as "calcium rises", now represent different signals and probably different functions (Volterra et al., 2014). In this sense, although it is still being investigated, focal distal events independent from ER-calcium may be related to the control of synaptic activity in close neurons (Stobart et al., 2018), while ER-dependent calcium rises might be implicated in the responsiveness to neuromodulators and in the global control of cortical states.

In our model (cultured astrocytes), calcium signals basically rely on the release from the endoplasmic reticulum. Thus, the reduction in cytosolic calcium rises may represent an adaptive mechanism to functions performed by ER-calcium release inducers, this is, neuromodulators like noradrenaline (NA) or acetylcholine (Ach).

Neuromodulation is the regulation of multiple neurons at the same time via the chemical release of "volume transmitters". These neuromodulators target slow-acting receptors of different neurons thus producing a much global slow-acting response. These pathways include the previous stated NA and Ach, but also serotonin or dopamine, among others. These neuromodulator pathways have been implicated in different brain functions, such as arousal, reward or learning (Lee and Dan, 2012).

Recent studies suggest that part of the effects of these neuromodulators may be performed by astrocytes. The fact that the modulator is released globally, probably targeting surrounding astrocytes and that the time course effect of astrocytic activity

is several orders of magnitude slower than neurons (Vardjan et al., 2016), as it is the effect of the neuromodulation, suggest that astrocytes are the ideal candidates to contribute to the effect.

In fact, both Ach and NA have been implicated in astrocytic calcium responses. In the case of NA, locus coeruleus (LC) stimulation leads to cortical astrocyte activation (calcium transients) in vivo (Bekar et al., 2008). Moreover, physiological stimuli known to activate NA release, such as startle response or sensory stimulation produced a global activation of cortical astrocytes (Ding et al., 2013). Regarding ACh, stimulation of the cholinergic nucleus basalis of Meynert induces global calcium rises in cortical astrocytes. Interestingly these effects are related to cytosolic calcium rises from ER release, since they are not produced in the IP_3R_2-KO (Takata et al., 2011) (Chen et al., 2012).

These ER-dependent calcium signals have been seen to induce changes in Ach-induced synaptic plasticity in the hippocampus (Navarrete et al., 2012), in the visual cortex (Chen et al., 2012) and in the barrel cortex (Takata et al., 2011). In the case of NA effects through astrocytes, its release primes astrocytes to react to neuronal stimuli, probably controlling astrocytic responses according to behavioural states (Paukert et al., 2014). Finally, studies in Drosophila showed that slow-acting modulators (equivalents to NA or Ach in rodents) such as tyramine and octopamine produced increases in astrocytic calcium together with alterations in dopaminergic neuronal firing and behavioural alterations (Ma et al., 2016), confirming the importance of astrocyte calcium in the neuromodulator signalling.

Neuromodulator activity produce ER-dependent calcium rises in astrocytes, which, through release of gliotransmitters, induce changes in synaptic plasticity, neuronal responsiveness and finally behaviour. The reduction in cytosolic calcium seen in our results has to be framed inside this neuromodulator effects.

2. Mitochondrial calcium entry is related to increased metabolism and glutamate oxidation

As stated before, in addition to a decreased cytosolic calcium rises, agonist-induced CREB activation in astrocytes show an increase in mitochondrial calcium entry. Interestingly, mitochondrial calcium entry is related to an increase in mitochondrial metabolism, via activation of multiple mitochondrial enzymes (Griffiths and Rutter, 2009).

In astrocytes, recent reports suggested that increases in mitochondrial calcium entry are related to changes in mitochondrial metabolism (Natarajaseenivasan et al., 2018). At the same time, this mitochondrial calcium entry is related to the oxidation of glutamate, arresting the organelle near sites of glutamate uptake (Jackson et al., 2014) (Jackson and Robinson, 2015) (Stephen et al., 2015). The increase in mitochondrial calcium entry seen in CREB-activated astrocytes, suggest an increase in mitochondrial metabolism that should be integrated in the CREB-mediated response.

3. Lactate release is involved in synaptic plasticity

As explained in the introduction, there are many controversies about the mechanism of lactate-induced effect on synaptic plasticity, although there is consensus regarding the consequences. Recent reports demonstrated that lactate induces plasticity in neighbouring neurons, inducing a plethora of genes related to synaptic plasticity (Yang et al., 2014). In fact, aerobic glycolysis, which is the production of lactate from glucose in the presence of oxygen and seems to be directly related to the astrocytic compartment in the brain (Belanger et al., 2011), is highly increased during early developmental stages in humans, with a peak at 5 years of age, a critical period of brain development (Goyal et al., 2014). Aerobic glycolysis persists in some regions, related to an increase in plasticity related genes in these regions, linking astrocyte lactate release with synaptic remodelling in human adults (Magistretti, 2014).

Regarding the mechanism, lactate may be used as a metabolite, since their uptake impairs long-term memory formation (Suzuki et al., 2011) (Wang et al., 2017) (Steinman et al., 2016), or as a signalling molecule through the newly discovered lactate GPCR receptor: HCAR1 (Bozzo et al., 2013) (Lauritzen et al., 2014). This receptor, previously studied in different tissues, is highly expressed in the CNS (Bergersen and Gjedde, 2012). HCAR1 is coupled to Gi proteins, meaning that, upon activation, cAMP production is inhibited. HCAR1 activation reduces spiking frequency in cultured cortical neurons (Bozzo et al., 2013), and mediates the VEGF production and angiogenesis produced by exercise-derived lactate (Morland et al., 2017).

4. Neuromodulation activates CREB, implications for synaptic homeostasis

Previous studies from our laboratory have demonstrated that NA activates CREB in astrocytes (Carriba et al., 2012), which has been confirmed in our results.

NA release is controlled by the noradrenergic projections from the LC, a small nucleus containing a low number of neurons but with projections to almost all brain areas (Aston-Jones and Waterhouse, 2016). NA release is done through non-junctional varicosities or *en passant* synapses thus allowing broad changes in different cells from the area through volume transmission.

NA functions include maintenance of the arousal and wakefulness state, enhancing memory formation, and mediating the “fight or flight” response. Interestingly, LC firing is related to these different functions. During wake states, LC exerts a tonic firing, with low frequency continuous bursts during the state. However, whenever a relevant stimulus is detected, a phasic high frequency firing from the LC releases NA to different brain areas (Devilbiss and Waterhouse, 2011). This way, LC is able to maintain attentional circuits necessary for working memory and sensory processing, while facilitating shifts in connectivity and coding functions whenever an important stimuli is produced (Sara, 2009) (O'Donnell et al., 2015).

These two firing types may explain two different situations in which astrocytic CREB is activated. On the one hand, high frequency NA release in response to relevant or strong stimuli may lead to a high concentration of the transmitter in a specific brain area, activating CREB transcription in those astrocytes. On the other hand, the tonic NA release may accumulate in the extracellular space activating CREB during the awake state.

Integration of CREB-mediated effects

We can speculate of the biological meaning of CREB activation in astrocytes. First, our results showed a CREB-induced reduction in cytosolic calcium responses. Second, CREB activation in astrocytes leads to an increase in mitochondrial calcium, which has been shown to increase mitochondrial metabolism. Third, CREB activated astrocytes increase the expression of MCT4 and lactate release.

As we have mentioned before, astrocytic CREB may be activated in intense firing situations or due to cumulative NA tonic release. We are going to use the latter as a paradigm of which can be the consequences of CREB-induced changes, but the same hypothesis may be valid for the former in cases of high electrical activity. Tonic NA release during the awake phase may activate CREB in astrocytes. In fact, 20% of genes increased during wakefulness were seen to be under control of LC-released NA and related to an increase in CREB phosphorylation (Cirelli et al., 1996) (Cirelli and Tononi, 2004), without distinguishing between neuronal or astrocytic genes. In fact, transcriptomic analyses of astrocytes from awake compared to sleep mice showed that *Gem*, *Crem*, *Nr4a1* and *Nr4a3* are upregulated during the awake period (Bellesi et al., 2015), all of them targets of CREB activation in astrocytes (Pardo et al., 2017).

According to the synaptic homeostasis hypothesis, during the waking periods there is a continuous evoked activity, which is related to an increased synaptic potentiation throughout the brain. However, during the sleep state, the low brain activity is related to a decrease in global synaptic depression, in order to consolidate critical long-term potentiation (Vyazovskiy et al., 2008) (Tononi and Cirelli, 2014).

As stated before, if astrocytic CREB is activated during the day due to the tonic firing of the LC, the reduction of the calcium raises may happen during the sleep phase, in order to promote memory consolidation. This will fit with the fact that CREB stimulation reduces calcium responses up to 12h, as seen in chapter 1.

Regarding mitochondrial calcium and lactate release potentiation, which seem counterintuitive at first sight, we can speculate of a global metabolic response mediated by astrocytic mitochondria, a highly adaptive organelle as we have seen in the results of the third chapter.

First, neuronal-induced CREB activation in astrocytes produces an increase in glycolytic enzymes (Hasel et al., 2017) in order to increase lactate production and release. Although aerobic glycolysis is energetically inefficient, we have demonstrated that it co-exists with fatty acid and amino acid oxidative metabolism. Therefore, CREB-activated

astrocytes may develop mitochondrial metabolic adaptation to other substrates. In agreement, transcriptomic arrays performed by our group showed mitochondrial functions such as oxidative phosphorylation, TCA cycle or FAO as a clear hit of CREB-activated astrocytes (Pardo et al., 2017). Interestingly, some of the upregulated mitochondrial metabolic functions in astrocytes in response to CREB activation are those that we have described as typically astrocytic in the third chapter of this dissertation. These results are further supported by a recent study suggesting that differentiation of glioblastoma cells into astrocytes is mediated by a metabolic reprogramming towards oxidative metabolism through CREB (Xing et al., 2017).

Thus, CREB activation in astrocytes may lead to potentiation of mitochondrial metabolism, involving the usage of substrates other than glucose while increasing lactate release to promote synaptic plasticity. Whether these phenomena appear at the same time in the same astrocytic processes or it is process-specific needs further study. *In vivo* astrocytes present a very complex morphology, with primary and secondary processes that emerge from the soma and intensely branched ultra-thin prolongations of the cytoplasm that arise from these processes. All these final thin prolongations form the known sponge-like structure. Moreover, these thin processes, which are far beyond diffraction limits, are in close contact with synapses, often enwrapping them (Papouin et al., 2017a). In fact, a single astrocyte can cover up to 100,000 synapses (Oberheim et al., 2012). Thus, it seems plausible that each thin process may have differences in protein expression and, while some of them control lactate release to surrounding synapses, in other processes mitochondrial metabolic oxidation is maintaining the energetic supply of the astrocyte. In fact, a recent study suggests that astrocytic peripheral processes contain the machinery to induce local translation, specifically of mRNAs related to fatty acid and glutamate metabolism (Sakers et al., 2017).

Second, mitochondrial calcium, in addition to be a mechanism of calcium sink and thus reduce cytosolic calcium and glutamate release (Reyes and Parpura, 2008), has been related to glutamate and FAO in astrocytes (Natarajaseenivasan et al., 2018) (Jackson and Robinson, 2015), supporting the idea of this metabolic change in CREB-activated astrocytes. In line with that, astrocytic CREB and the transcription factor NFκβ have been seen also to increase the expression of the glutamate transporter GLT-1, the most important glutamate transporter in astrocytes (Karki et al., 2013). Although we have not studied this pathway, it will perfectly fit in our hypothesis since glutamate uptake can be related to the increase in mitochondrial metabolism while at the same time reduces synaptic glutamate levels to maintain this CREB-induced steady state.

This way, while increasing lactate release and mitochondrial metabolism, CREB downregulates cytosolic calcium and subsequent gliotransmitter release, downscaling the electrical stimulation to neighbouring neurons while potentiating lactate release and maintaining its oxidative metabolism. This way, astrocytic CREB is feeding synapses after their potentiation probably mediating their plasticity. This phenomenon may happen both during high frequency stimulation (acute NA firing or ATP release) or due to the tonic NA release during the awake state.

In line with that, a very recent and surprising report has demonstrated how astrocytes contribute to hippocampal NMDA signalling in response to Ach neuromodulation depending on the time of the day (Papouin et al., 2017b). In this study, hippocampal slices from mouse brains obtained at the end of the active phase had increased D-serine release, which in turn depended on Ach stimulation of the astrocyte. However, during night phase, lower Ach stimulation led to lower D-serine release, contributing to the synaptic downscaling and memory consolidation occurring during sleep. Whether astrocytic CREB reduction in cytosolic calcium is implicated in this night downregulation needs to be addressed.

Astrocytic CREB and neuroprotection

Of special interest is the role that astrocytic CREB may develop in different neuropathologies. Previous studies from our laboratory have demonstrated a therapeutical role of astrocytic CREB in traumatic brain injury, where it maintains mitochondrial stability alleviating energy-metabolism dysfunction (Pardo et al., 2016). However, AD patients show metabolic alterations compared to healthy brains (Thomas et al., 2015), calcium transients in astrocytes are altered in AD models (Kuchibhotla et al., 2009) and noradrenergic dysfunction is one of the main hallmarks of neurodegenerative disorders such as Alzheimer's (AD) and Parkinson's (PD) diseases. In fact, LC pathology often precedes the symptoms of each disorder, probably contributing to their initiation rather than being collateral damage (Weinshenker, 2018).

Thus, the results of my research allow us to frame astrocytic CREB as a target for Alzheimer's disease. In fact, different studies observed downregulated CREB signalling in AD transgenic mouse models (Pugazhenthi et al., 2011) (Qin et al., 2016) and AD patients (Bartolotti et al., 2016), without analysing if there is also CREB downregulation in astrocytes. Taking into account the downregulation of LC-released NA, it seems quite plausible that CREB activation in astrocytes may be impaired as well.

On the one hand, CREB reduces cytosolic calcium rises while increasing mitochondrial calcium entry. The effect is mediated by an increase in SigmaR1 expression, which is reduced in AD patients (Mishina et al., 2008) (Jansen et al., 1993) and its downregulation has been related to AD symptoms (Jin et al., 2015). Thus, the absence of these CREB-induced changes in astrocytic calcium through SigmaR1 may be a cause for the excessive calcium transients in astrocytes of AD models, in addition to previously described mediators (Delekate et al., 2014). On the other hand, metabolic alterations seen in AD patients, such as a reduction in glucose utilization and lactate release (Chen and Zhong, 2013) (Merlini et al., 2011), as well as alteration in FAO (Snowden et al., 2017), may be relieved by astrocytic CREB activation.

It is therefore of capital important to analyse if astrocytic CREB signalling is a neuroprotective agent in neurodegeneration, and treatments addressed to activate CREB in AD have to take into account the astrocytic alterations in AD as well (Teich et al., 2015).

Mitochondrial metabolic plasticity

We have shown that astrocytes have a complex and adaptive mitochondrial metabolism, having roughly the same expression of mRNAs related to TCA cycle or oxidative phosphorylation processes than neurons while containing significantly more transcripts related to other substrates metabolism. Functions such as aminoacid metabolism, FAO or amine, alcohol and aldehyde metabolism are consistently upregulated in astrocytes compared to neurons. Moreover, we have demonstrated that these mitochondrial functions in astrocytes are upregulated during development and inhibited in injury situations. Finally, we have demonstrated that FAO is produced in astrocytes even in the presence of glucose, and that this oxidative metabolism is inhibited in the presence of glutamate.

The results obtained in this study represent a step forward in the field because astrocytic mitochondrial metabolism has been completely ignored in important reviews (Weber and Barros, 2015) (Supplie et al., 2017). Moreover, they establish astrocytic mitochondria as a highly plastic organelle. First, its components are transcriptionally controlled since there is global downregulation of mitochondrial mRNAs in response to injury. The injury-induced downregulation may be transcriptionally directed, in fact, transcription factors such as PGC-1α are able to control mitochondrial biogenesis through target genes (Hock and Kralli, 2009). Interestingly, PGC-1α is a target CREB gene (Herzig et al., 2001). Although the mechanism of downregulation of mitochondrial functions is out of the scope of our interests, it represents another transcriptional adaptive process by which external signalling (in this case from injury processes), modifies astrocytic functions through transcription modelling. Second, neuronal stimulation –glutamate addition- inhibits basal FAO, showing an immediate adaptive response to neural activity. The inhibition of FAO in the presence of glutamate is a paradigm of how astrocytes modify their functions in response to neuronal stimuli. In this context, the adaptive mechanism may not require gene expression, since the effect is seen immediately because $^{3}H_{2}0$ concentration after 30 min is the same than before glutamate stimulation. This means that glutamate stops FAO as soon as it reaches the astrocyte.

This novel metabolic adaptive response is similar to other metabolic astrocytic adaptations to neuronal stimuli, such as the increase in aerobic glycolysis in the presence of glutamate (Pellerin and Magistretti, 1994) or neuronal released potassium (Bittner et al., 2011). Regarding the mechanism to explain glutamate-induced inhibition of FAO, one plausible possibility is that astrocytes present an active mitochondrial metabolism, with acetyl-coA from glucose and FAO and, upon neuronal firing (through released potassium or glutamate), astrocytes change their metabolism towards a glycolytic metabolism to release lactate for neurons. This may explain the subtle increase in oxygen consumption in activated regions compared to the high increases in blood flow and glucose consumption, as discussed in chapter 3.

In summary, results presented in this dissertation show a wide range of astrocyte adaptations to different inputs, from neuromodulation to neurotransmission and

injury processes, and establish astrocytic mitochondrial metabolism as a hub for these plasticity processes.

Future work

Overall, we have made new contributions to understand astrocytic plasticity, breaking dogmas and setting up new concepts. Therefore, our results lead to new important questions and open new fields of research.

Regarding CREB activation, we have shown an astrocyte-based form of long-term depression in which agonist-calcium signaling is reduced. Undoubtedly, it will be interesting to explore if CREB is the only mediator of such phenomena, or it can be activated also by other transcription factors. Of interest, as well, it to know if these mechanisms can be achieved by short and continuous cell-stimulation, if there is a threshold in agonist concentration or if activation of other transcription factors can lead to a similar outcome.

One of the most necessary future directions is to further address effects of CREB activation *in vivo*. Preliminary results of CREB inhibition *in vivo* and analyzing calcium signals in slices point to differences between in vitro and in vivo; three different but equally valid reasons may explain these controversial observations:

First, regional differences. All our in vitro experiments were done in cortical astrocytes; however, the *in vivo* experiments were performed in hippocampal CA1 astrocytes. Many recent transcriptomic and proteomic analyses describe different astrocytic populations, both inter- and intra-regionally. In these studies, morphological differences were associated with changes in gene and protein expression as well as with different properties (John Lin et al., 2017) (Morel et al., 2017). As an example, in a recent work striatal astrocytes were found to be different to hippocampal astrocytes in electrophysiological properties, morphology, synaptic coverage and calcium currents, in parallel with differences in gene and protein expression (Chai et al., 2017). Thus, one possibility is that cortical and hippocampal astrocytes present different protein expression and calcium signals. Finally, age differences could also apply. In our in vitro calcium signalling studies, we used postnatal day 1 astrocytes, whereas adult mouse where used for the ex vivo calcium signalling studies (Cahoy et al., 2008). However, we have seen CREB-dependent SigmaR1 expression in astrocyte adult cultures and in our astrocytic VP16-CREB transgenic mouse model.

Second, it has not been unequivocally demonstrated in which situations CREB is activated in astrocytes *in vivo*. As explained during this discussion, CREB may be activated due to high frequency firing or a constant tonic NA release. However, our first trials were done with the dominant negative A-CREB without a previous CREB stimulation, since the VP16-CREB was affecting *in vivo* astrocyte viability. Thus, one plausible explanation is that we did not analyze the situations in which CREB is found active. Stimulation of the locus coeruleus or incubation of the slices with noradrenaline before the calcium experiment might be a possible mechanism to activate CREB in astrocytes and see the effect of the inhibition in activity-regulated transcription. In line

with that, an interesting and necessary experiment is to analyze *in vivo* situations in which CREB is activated in astrocytes, such as after induction of physiological NA or ATP release or even depending on the active/sleep phase. Interestingly, the recent development of techniques to analyze *in vivo* CREB activation in different tissues may be a highly valuable tool (Akhmedov et al., 2016) (Akhmedov et al., 2017) (Ishimoto et al., 2015).

Third, SigmaR1 is known to have other functions in calcium signals, such as modulation of different membrane calcium channels (Soriani and Rapetti-Mauss, 2017). As explained before, calcium signals differ in vivo and in vitro, being extracellular calcium entry (which is relatively small in cultured astrocytes but really important in in vivo astrocytes) one of the most important differences. Thus, one possibility is that SigmaR1 functions differ between *in vivo* and *in vitro* models.

Regarding CREB-induced astrocytic metabolic changes, again, it will be interesting to understand for how long are they maintained, which other transcription factors might be implicated and how is it produced *in vivo*.

First future steps will need to analyze how in vivo CREB activation in astrocytes may change brain metabolism. My laboratory has done a metabolome analysis of the VP16-CREB mouse model and we have seen interesting differences in metabolites both in basal conditions and under traumatic brain injury processes. *In vitro*, we are interested in analyzing how other metabolic functions can be changed in CREB-activated astrocytes. Oxygen consumption measurements using the *Seahorse* technology experiments showed no differences in mitochondrial metabolism in CREB-activated astrocytes in the presence of glucose (addendum chapter 2). However, results obtained in chapter three suggest that CREB metabolic changes may be related to the usage of other astrocytic specific metabolites, such as aminoacids (like glutamate) or fatty acids, while increasing lactate production from glucose.

Finally, results obtained in the third chapter established FAO as a important metabolic pathway in astrocytes, in turn regulated by neuronal activity. The discovery opens many questions regarding brain metabolism, such as which is the contribution of this FAO in basal and active brain. Does it change after *in vivo* neuronal stimulation? Which would be the consequences of a global inhibition of FAO in all astrocytes? Inhibition of some enzymes from the pathway specifically in astrocytes will be a valuable tool to address this question, as it has been done in *Drosophila* (Schulz et al., 2015).

It is important to mention the value of analyzing big data from other laboratories, which was the strategy followed in the third chapter. Global tendency nowadays in big laboratories is to generate tons of data that will be published in a public repository without being further analyzed. For small laboratories, access to this data allows us to reach important conclusions with no cost from data obtained using last-generation techniques and spending a huge amount of money. Moreover, these data come from different laboratories and different models, enhancing the power of the results.

To finalize, in addition to be a step further in astrocyte knowledge, results presented here involve a wide range of different and original techniques, adding weight to the resulting conclusions.

References

Abbott, L.F., and Regehr, W.G. (2004). Synaptic computation. Nature *431*, 796-803.

Abraham, W.C. (2003). How long will long-term potentiation last? Philos Trans R Soc Lond B Biol Sci *358*, 735-744.

Akhmedov, D., Mendoza-Rodriguez, M.G., Rajendran, K., Rossi, M., Wess, J., and Berdeaux, R. (2017). Gs-DREADD Knock-In Mice for Tissue-Specific, Temporal Stimulation of Cyclic AMP Signaling. Mol Cell Biol *37*.

Akhmedov, D., Rajendran, K., Mendoza-Rodriguez, M.G., and Berdeaux, R. (2016). Knock-in Luciferase Reporter Mice for In Vivo Monitoring of CREB Activity. PLoS One *11*, e0158274.

Araque, A., Carmignoto, G., Haydon, P.G., Oliet, S.H., Robitaille, R., and Volterra, A. (2014). Gliotransmitters travel in time and space. Neuron *81*, 728-739.

Aston-Jones, G., and Waterhouse, B. (2016). Locus coeruleus: From global projection system to adaptive regulation of behavior. Brain research *1645*, 75-78.

Bartolotti, N., Bennett, D.A., and Lazarov, O. (2016). Reduced pCREB in Alzheimer's disease prefrontal cortex is reflected in peripheral blood mononuclear cells. Molecular psychiatry *21*, 1158-1166.

Bekar, L.K., He, W., and Nedergaard, M. (2008). Locus coeruleus alpha-adrenergic-mediated activation of cortical astrocytes in vivo. Cerebral cortex (New York, NY : 1991) *18*, 2789-2795.

Belanger, M., Allaman, I., and Magistretti, P.J. (2011). Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation. Cell Metab *14*, 724-738.

Bellesi, M., de Vivo, L., Tononi, G., and Cirelli, C. (2015). Effects of sleep and wake on astrocytes: clues from molecular and ultrastructural studies. BMC Biol *13*, 66.

Bergersen, L.H., and Gjedde, A. (2012). Is lactate a volume transmitter of metabolic states of the brain? Front Neuroenergetics *4*, 5.

Bittner, C.X., Valdebenito, R., Ruminot, I., Loaiza, A., Larenas, V., Sotelo-Hitschfeld, T., Moldenhauer, H., San Martin, A., Gutierrez, R., Zambrano, M.*, et al.* (2011). Fast and reversible stimulation of astrocytic glycolysis by K+ and a delayed and persistent effect of glutamate. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 4709-4713.

Bozzo, L., Puyal, J., and Chatton, J.Y. (2013). Lactate modulates the activity of primary cortical neurons through a receptor-mediated pathway. PLoS One *8*, e71721.

Cahoy, J.D., Emery, B., Kaushal, A., Foo, L.C., Zamanian, J.L., Christopherson, K.S., Xing, Y., Lubischer, J.L., Krieg, P.A., Krupenko, S.A.*, et al.* (2008). A transcriptome database for astrocytes, neurons, and oligodendrocytes: a new resource for understanding brain development and function. The Journal of neuroscience : the official journal of the Society for Neuroscience *28*, 264-278.

Carriba, P., Pardo, L., Parra-Damas, A., Lichtenstein, M.P., Saura, C.A., Pujol, A., Masgrau, R., and Galea, E. (2012). ATP and noradrenaline activate CREB in astrocytes via noncanonical Ca(2+) and cyclic AMP independent pathways. Glia *60*, 1330-1344.

Chai, H., Diaz-Castro, B., Shigetomi, E., Monte, E., Octeau, J.C., Yu, X., Cohn, W., Rajendran, P.S., Vondriska, T.M., Whitelegge, J.P.*, et al.* (2017). Neural Circuit-Specialized Astrocytes: Transcriptomic, Proteomic, Morphological, and Functional Evidence. Neuron *95*, 531-549.e539.

Chen, N., Sugihara, H., Sharma, J., Perea, G., Petravicz, J., Le, C., and Sur, M. (2012). Nucleus basalis-enabled stimulus-specific plasticity in the visual cortex is mediated by astrocytes. Proc Natl Acad Sci U S A *109*, E2832-2841.
Chen, Z., and Zhong, C. (2013). Decoding Alzheimer's disease from perturbed cerebral glucose metabolism: implications for diagnostic and therapeutic strategies. Prog Neurobiol *108*, 21-43.
Cirelli, C., Pompeiano, M., and Tononi, G. (1996). Neuronal gene expression in the waking state: a role for the locus coeruleus. Science *274*, 1211-1215.
Cirelli, C., and Tononi, G. (2004). Locus ceruleus control of state-dependent gene expression. The Journal of neuroscience : the official journal of the Society for Neuroscience *24*, 5410-5419.
De Pitta, M., Brunel, N., and Volterra, A. (2016). Astrocytes: Orchestrating synaptic plasticity? Neuroscience *323*, 43-61.
Delekate, A., Fuchtemeier, M., Schumacher, T., Ulbrich, C., Foddis, M., and Petzold, G.C. (2014). Metabotropic P2Y1 receptor signalling mediates astrocytic hyperactivity in vivo in an Alzheimer's disease mouse model. Nature communications *5*, 5422.
Devilbiss, D.M., and Waterhouse, B.D. (2011). Phasic and tonic patterns of locus coeruleus output differentially modulate sensory network function in the awake rat. J Neurophysiol *105*, 69-87.
Ding, F., O'Donnell, J., Thrane, A.S., Zeppenfeld, D., Kang, H., Xie, L., Wang, F., and Nedergaard, M. (2013). alpha1-Adrenergic receptors mediate coordinated Ca2+ signaling of cortical astrocytes in awake, behaving mice. Cell Calcium *54*, 387-394.
Goyal, M.S., Hawrylycz, M., Miller, J.A., Snyder, A.Z., and Raichle, M.E. (2014). Aerobic glycolysis in the human brain is associated with development and neotenous gene expression. Cell Metab *19*, 49-57.
Griffiths, E.J., and Rutter, G.A. (2009). Mitochondrial calcium as a key regulator of mitochondrial ATP production in mammalian cells. Biochimica et biophysica acta *1787*, 1324-1333.
Hasel, P., Dando, O., Jiwaji, Z., Baxter, P., Todd, A.C., Heron, S., Markus, N.M., McQueen, J., Hampton, D.W., Torvell, M., *et al.* (2017). Neurons and neuronal activity control gene expression in astrocytes to regulate their development and metabolism. Nature communications *8*, 15132.
Herzig, S., Long, F., Jhala, U.S., Hedrick, S., Quinn, R., Bauer, A., Rudolph, D., Schutz, G., Yoon, C., Puigserver, P., *et al.* (2001). CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. Nature *413*, 179-183.
Hock, M.B., and Kralli, A. (2009). Transcriptional control of mitochondrial biogenesis and function. Annual review of physiology *71*, 177-203.
Ishimoto, T., Mano, H., and Mori, H. (2015). In vivo imaging of CREB phosphorylation in awake-mouse brain. Scientific reports *5*, 9757.
Jackson, J.G., O'Donnell, J.C., Takano, H., Coulter, D.A., and Robinson, M.B. (2014). Neuronal activity and glutamate uptake decrease mitochondrial mobility in astrocytes and position mitochondria near glutamate transporters. The Journal of neuroscience : the official journal of the Society for Neuroscience *34*, 1613-1624.
Jackson, J.G., and Robinson, M.B. (2015). Reciprocal Regulation of Mitochondrial Dynamics and Calcium Signaling in Astrocyte Processes. The Journal of

neuroscience : the official journal of the Society for Neuroscience *35*, 15199-15213.
Jansen, K.L., Faull, R.L., Storey, P., and Leslie, R.A. (1993). Loss of sigma binding sites in the CA1 area of the anterior hippocampus in Alzheimer's disease correlates with CA1 pyramidal cell loss. Brain research *623*, 299-302.
Jin, J.L., Fang, M., Zhao, Y.X., and Liu, X.Y. (2015). Roles of sigma-1 receptors in Alzheimer's disease. Int J Clin Exp Med *8*, 4808-4820.
John Lin, C.C., Yu, K., Hatcher, A., Huang, T.W., Lee, H.K., Carlson, J., Weston, M.C., Chen, F., Zhang, Y., Zhu, W.*, et al.* (2017). Identification of diverse astrocyte populations and their malignant analogs. Nature neuroscience *20*, 396-405.
Karki, P., Webb, A., Smith, K., Lee, K., Son, D.S., Aschner, M., and Lee, E. (2013). cAMP response element-binding protein (CREB) and nuclear factor kappaB mediate the tamoxifen-induced up-regulation of glutamate transporter 1 (GLT-1) in rat astrocytes. J Biol Chem *288*, 28975-28986.
Kuchibhotla, K.V., Lattarulo, C.R., Hyman, B.T., and Bacskai, B.J. (2009). Synchronous hyperactivity and intercellular calcium waves in astrocytes in Alzheimer mice. Science *323*, 1211-1215.
Lauritzen, K.H., Morland, C., Puchades, M., Holm-Hansen, S., Hagelin, E.M., Lauritzen, F., Attramadal, H., Storm-Mathisen, J., Gjedde, A., and Bergersen, L.H. (2014). Lactate receptor sites link neurotransmission, neurovascular coupling, and brain energy metabolism. Cerebral cortex (New York, NY : 1991) *24*, 2784-2795.
Lee, S.H., and Dan, Y. (2012). Neuromodulation of brain states. Neuron *76*, 209-222.
Ma, Z., Stork, T., Bergles, D.E., and Freeman, M.R. (2016). Neuromodulators signal through astrocytes to alter neural circuit activity and behaviour. Nature *539*, 428-432.
Magistretti, P.J. (2014). Synaptic plasticity and the Warburg effect. Cell Metab *19*, 4-5.
Martin, S.J., Grimwood, P.D., and Morris, R.G. (2000). Synaptic plasticity and memory: an evaluation of the hypothesis. Annu Rev Neurosci *23*, 649-711.
Merlini, M., Meyer, E.P., Ulmann-Schuler, A., and Nitsch, R.M. (2011). Vascular beta-amyloid and early astrocyte alterations impair cerebrovascular function and cerebral metabolism in transgenic arcAbeta mice. Acta Neuropathol *122*, 293-311.
Mishina, M., Ohyama, M., Ishii, K., Kitamura, S., Kimura, Y., Oda, K., Kawamura, K., Sasaki, T., Kobayashi, S., Katayama, Y.*, et al.* (2008). Low density of sigma1 receptors in early Alzheimer's disease. Ann Nucl Med *22*, 151-156.
Morel, L., Chiang, M.S.R., Higashimori, H., Shoneye, T., Iyer, L.K., Yelick, J., Tai, A., and Yang, Y. (2017). Molecular and Functional Properties of Regional Astrocytes in the Adult Brain. The Journal of neuroscience : the official journal of the Society for Neuroscience *37*, 8706-8717.
Morland, C., Andersson, K.A., Haugen, O.P., Hadzic, A., Kleppa, L., Gille, A., Rinholm, J.E., Palibrk, V., Diget, E.H., Kennedy, L.H.*, et al.* (2017). Exercise induces cerebral VEGF and angiogenesis via the lactate receptor HCAR1. Nature communications *8*, 15557.
Natarajaseenivasan, K., Cotto, B., Shanmughapriya, S., Lombardi, A.A., Datta, P.K., Madesh, M., Elrod, J.W., Khalili, K., and Langford, D. (2018). Astrocytic metabolic

switch is a novel etiology for Cocaine and HIV-1 Tat-mediated neurotoxicity. Cell Death Dis *9*, 415.
Navarrete, M., Perea, G., Fernandez de Sevilla, D., Gomez-Gonzalo, M., Nunez, A., Martin, E.D., and Araque, A. (2012). Astrocytes mediate in vivo cholinergic-induced synaptic plasticity. PLoS Biol *10*, e1001259.
O'Donnell, J., Ding, F., and Nedergaard, M. (2015). Distinct functional states of astrocytes during sleep and wakefulness: Is norepinephrine the master regulator? Curr Sleep Med Rep *1*, 1-8.
Oberheim, N.A., Goldman, S.A., and Nedergaard, M. (2012). Heterogeneity of astrocytic form and function. Methods Mol Biol *814*, 23-45.
Papouin, T., Dunphy, J., Tolman, M., Foley, J.C., and Haydon, P.G. (2017a). Astrocytic control of synaptic function. Philos Trans R Soc Lond B Biol Sci *372*.
Papouin, T., Dunphy, J.M., Tolman, M., Dineley, K.T., and Haydon, P.G. (2017b). Septal Cholinergic Neuromodulation Tunes the Astrocyte-Dependent Gating of Hippocampal NMDA Receptors to Wakefulness. Neuron *94*, 840-854 e847.
Pardo, L., Schluter, A., Valor, L.M., Barco, A., Giralt, M., Golbano, A., Hidalgo, J., Jia, P., Zhao, Z., Jove, M*., et al.* (2016). Targeted activation of CREB in reactive astrocytes is neuroprotective in focal acute cortical injury. Glia *64*, 853-874.
Pardo, L., Valor, L.M., Eraso-Pichot, A., Barco, A., Golbano, A., Hardingham, G.E., Masgrau, R., and Galea, E. (2017). CREB Regulates Distinct Adaptive Transcriptional Programs in Astrocytes and Neurons. Scientific reports *7*, 6390.
Pascual-Leone, A., Amedi, A., Fregni, F., and Merabet, L.B. (2005). The plastic human brain cortex. Annu Rev Neurosci *28*, 377-401.
Paukert, M., Agarwal, A., Cha, J., Doze, V.A., Kang, J.U., and Bergles, D.E. (2014). Norepinephrine controls astroglial responsiveness to local circuit activity. Neuron *82*, 1263-1270.
Pellerin, L., and Magistretti, P.J. (1994). Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. Proc Natl Acad Sci U S A *91*, 10625-10629.
Poskanzer, K.E., and Yuste, R. (2016). Astrocytes regulate cortical state switching in vivo. Proc Natl Acad Sci U S A *113*, E2675-2684.
Pugazhenthi, S., Wang, M., Pham, S., Sze, C.I., and Eckman, C.B. (2011). Downregulation of CREB expression in Alzheimer's brain and in Abeta-treated rat hippocampal neurons. Mol Neurodegener *6*, 60.
Qin, L., Bouchard, R., and Pugazhenthi, S. (2016). Regulation of cyclic AMP response element-binding protein during neuroglial interactions. J Neurochem *136*, 918-930.
Reyes, R.C., and Parpura, V. (2008). Mitochondria modulate Ca2+-dependent glutamate release from rat cortical astrocytes. The Journal of neuroscience : the official journal of the Society for Neuroscience *28*, 9682-9691.
Sahlender, D.A., Savtchouk, I., and Volterra, A. (2014). What do we know about gliotransmitter release from astrocytes? Philos Trans R Soc Lond B Biol Sci *369*, 20130592.
Sakers, K., Lake, A.M., Khazanchi, R., Ouwenga, R., Vasek, M.J., Dani, A., and Dougherty, J.D. (2017). Astrocytes locally translate transcripts in their peripheral processes. Proc Natl Acad Sci U S A *114*, E3830-E3838.
Sara, S.J. (2009). The locus coeruleus and noradrenergic modulation of cognition. Nat Rev Neurosci *10*, 211-223.

Schulz, J.G., Laranjeira, A., Van Huffel, L., Gartner, A., Vilain, S., Bastianen, J., Van Veldhoven, P.P., and Dotti, C.G. (2015). Glial beta-oxidation regulates Drosophila energy metabolism. Scientific reports *5*, 7805.
Snowden, S.G., Ebshiana, A.A., Hye, A., An, Y., Pletnikova, O., O'Brien, R., Troncoso, J., Legido-Quigley, C., and Thambisetty, M. (2017). Association between fatty acid metabolism in the brain and Alzheimer disease neuropathology and cognitive performance: A nontargeted metabolomic study. PLoS Medicine *14*.
Soriani, O., and Rapetti-Mauss, R. (2017). Sigma 1 Receptor and Ion Channel Dynamics in Cancer. Adv Exp Med Biol *964*, 63-77.
Steinman, M.Q., Gao, V., and Alberini, C.M. (2016). The Role of Lactate-Mediated Metabolic Coupling between Astrocytes and Neurons in Long-Term Memory Formation. Front Integr Neurosci *10*, 10.
Stephen, T.L., Higgs, N.F., Sheehan, D.F., Al Awabdh, S., Lopez-Domenech, G., Arancibia-Carcamo, I.L., and Kittler, J.T. (2015). Miro1 Regulates Activity-Driven Positioning of Mitochondria within Astrocytic Processes Apposed to Synapses to Regulate Intracellular Calcium Signaling. The Journal of neuroscience : the official journal of the Society for Neuroscience *35*, 15996-16011.
Stobart, J.L., Ferrari, K.D., Barrett, M.J.P., Gluck, C., Stobart, M.J., Zuend, M., and Weber, B. (2018). Cortical Circuit Activity Evokes Rapid Astrocyte Calcium Signals on a Similar Timescale to Neurons. Neuron.
Supplie, L.M., Duking, T., Campbell, G., Diaz, F., Moraes, C.T., Gotz, M., Hamprecht, B., Boretius, S., Mahad, D., and Nave, K.A. (2017). Respiration-Deficient Astrocytes Survive As Glycolytic Cells In Vivo. The Journal of neuroscience : the official journal of the Society for Neuroscience *37*, 4231-4242.
Suzuki, A., Stern, S.A., Bozdagi, O., Huntley, G.W., Walker, R.H., Magistretti, P.J., and Alberini, C.M. (2011). Astrocyte-neuron lactate transport is required for long-term memory formation. Cell *144*, 810-823.
Takata, N., Mishima, T., Hisatsune, C., Nagai, T., Ebisui, E., Mikoshiba, K., and Hirase, H. (2011). Astrocyte calcium signaling transforms cholinergic modulation to cortical plasticity in vivo. The Journal of neuroscience : the official journal of the Society for Neuroscience *31*, 18155-18165.
Takeuchi, T., Duszkiewicz, A.J., and Morris, R.G. (2014). The synaptic plasticity and memory hypothesis: encoding, storage and persistence. Philos Trans R Soc Lond B Biol Sci *369*, 20130288.
Teich, A.F., Nicholls, R.E., Puzzo, D., Fiorito, J., Purgatorio, R., Fa, M., and Arancio, O. (2015). Synaptic therapy in Alzheimer's disease: a CREB-centric approach. Neurotherapeutics *12*, 29-41.
Thomas, S.C., Alhasawi, A., Appanna, V.P., Auger, C., and Appanna, V.D. (2015). Brain metabolism and Alzheimer's disease: the prospect of a metabolite-based therapy. The journal of nutrition, health & aging *19*, 58-63.
Tononi, G., and Cirelli, C. (2014). Sleep and the price of plasticity: from synaptic and cellular homeostasis to memory consolidation and integration. Neuron *81*, 12-34.
Vardjan, N., Parpura, V., and Zorec, R. (2016). Loose excitation-secretion coupling in astrocytes. Glia *64*, 655-667.
Volterra, A., Liaudet, N., and Savtchouk, I. (2014). Astrocyte Ca(2)(+) signalling: an unexpected complexity. Nat Rev Neurosci *15*, 327-335.
von Bernhardi, R., Bernhardi, L.E., and Eugenin, J. (2017). What Is Neural Plasticity? Adv Exp Med Biol *1015*, 1-15.

Vyazovskiy, V.V., Cirelli, C., Pfister-Genskow, M., Faraguna, U., and Tononi, G. (2008). Molecular and electrophysiological evidence for net synaptic potentiation in wake and depression in sleep. Nature neuroscience *11*, 200-208.
Wang, J., Tu, J., Cao, B., Mu, L., Yang, X., Cong, M., Ramkrishnan, A.S., Chan, R.H.M., Wang, L., and Li, Y. (2017). Astrocytic l-Lactate Signaling Facilitates Amygdala-Anterior Cingulate Cortex Synchrony and Decision Making in Rats. Cell Rep *21*, 2407-2418.
Weber, B., and Barros, L.F. (2015). The Astrocyte: Powerhouse and Recycling Center. Cold Spring Harb Perspect Biol *7*.
Weinshenker, D. (2018). Long Road to Ruin: Noradrenergic Dysfunction in Neurodegenerative Disease. Trends Neurosci *41*, 211-223.
Xing, F., Luan, Y., Cai, J., Wu, S., Mai, J., Gu, J., Zhang, H., Li, K., Lin, Y., Xiao, X., *et al.* (2017). The Anti-Warburg Effect Elicited by the cAMP-PGC1alpha Pathway Drives Differentiation of Glioblastoma Cells into Astrocytes. Cell Rep *18*, 468-481.
Yang, J., Ruchti, E., Petit, J.M., Jourdain, P., Grenningloh, G., Allaman, I., and Magistretti, P.J. (2014). Lactate promotes plasticity gene expression by potentiating NMDA signaling in neurons. Proc Natl Acad Sci U S A *111*, 12228-12233.

CONCLUSIONS

IIX. CONCLUSIONS

- Astrocytes undergo adaptive functional changes in calcium excitability and lactate release as a consequence of transcriptional programs mediated by CREB. Specifically:

    - Activation of CREB-dependent transcription decreases calcium responses in fetal and adult cultured astrocytes.
    - The reduction in cytosolic calcium events is mediated by the enhanced expression of the chaperon SigmaR1, which increases ER-calcium release and mitochondrial calcium buffering.
    - SigmaR1 transcription is also increased by CREB in adult cultured astrocytes and *in vivo* in a transgenic mouse expressing the active form of CREB, VP16-CREB, in astrocytes.
    - Inhibition of endogenous CREB *ex vivo* in hippocampal astrocytes modulates spontaneous and phenylephrine-induced calcium responses in the soma and primary processes of astrocytes.
    - Activation of CREB-dependent transcription in cultured astrocytes enhances the expression of the lactate transporter MCT4 and hence lactate release.
    - Transcriptional regulation of MCT4 by CREB in normoxia is independent of HIF-1α, whereas MCT4 expression induced by HIF-1α in hypoxia is dependent on CREB.

- We have generated the mitochondriomes of astrocytes and neurons using the relative expression of nuclear genes encoding for mitochondrial constituents available in mouse and human brain-cell transcriptome repositories. GSEA and functional validations have revealed:

    - Greater expression of genes related to oxidative metabolism, namely fatty acid oxidation and aminoacid catabolism, in astrocytes than in neurons.
    - Fatty-acid oxidation coexists with glucose consumption but is inhibited by glutamate in astrocytes.
    - Increased expression of genes related to mitochondrial functions upon astrocyte maturation and downregulation in injury.

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