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INTERCELLULAR AND SYSTEM-LEVEL ASPECTS OF THE METABOLIC INTERACTIONS BETWEEN NEURONS AND GLIA

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UNIL | Université de Lausanne
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par

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Résumé grand public

ASPECTS INTERCELLULAIRES ET DE RESEAUX DES INTERACTIONS METABOLIQUES ENTRE NEURONES ET CELLULES GLIALES

Luigi Bozzo

Université de Lausanne, Département des Neurosciences Fondamentales

Quand on parle de l'acide lactique (aussi connu sous le nom de lactate) une des premières choses qui vient à l'esprit, c'est son implication en cas d'intense activité musculaire. Sa production pendant une activité physique prolongée est associée avec la sensation de fatigue. Il n'est donc pas étonnant que cette molécule ait été longtemps considérée comme un résidu du métabolisme, possiblement toxique et donc à éliminer. En fait, il a été découvert que le lactate joue un rôle prépondérant dans le métabolisme grâce à son fort potentiel énergétique. Le cerveau, en particulier les neurones qui le composent, est un organe très gourmand en énergie. Récemment, il a été démontré que les astrocytes, cellules du cerveau faisant partie de la famille des cellules gliales, utilisent le glucose pour produire du lactate comme source d'énergie et le distribue aux neurones de manière adaptée à leur activité. Cette découverte a renouvelé l'intérêt scientifique pour le lactate. Aujourd'hui, plusieurs études ont démontré l'implication du lactate dans d'autres fonctions de la physiologie cérébrale. Dans le cadre de notre étude, nous nous sommes intéressés au rapport entre neurones et astrocytes avec une attention particulière pour le rôle du lactate. Nous avons découvert que le lactate possède la capacité de modifier la communication entre les neurones. Nous avons aussi décrypté le mécanisme grâce auquel le lactate agit, qui est basé sur un récepteur présent à la surface des neurones. Cette étude montre une fonction jusque-là insoupçonnée du lactate qui a un fort impact sur la compréhension de la relation entre neurones et astrocytes.

Abstract

INTERCELLULAR AND SYSTEM-LEVEL ASPECTS OF THE METABOLIC INTERACTION BETWEEN NEURONS AND GLIA

Luigi Bozzo

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Relatively to its volume, the brain uses a large amount of glucose as energy source. Furthermore, a tight link exists between the level of synaptic activity and the consumption of energy equivalents. Astrocytes have been shown to play a central role in the regulation of this so-called neurometabolic coupling. They are thought to deliver the metabolic substrate lactate to neurons in register to glutamatergic activity. The astrocytic uptake of glutamate, released in the synaptic cleft, is the trigger signal that activates an intracellular cascade of events that leads to the production and release of lactate from astrocytes. The main goal of this thesis work was to obtain detailed information on the metabolic and functional interplay between neurons and astrocytes, in particular on the influence of lactate besides its metabolic effects. To gain access to both spatial and temporal aspects of these dynamic interactions, we used optical microscopy associated with specific fluorescent indicators, as well as electrophysiology.

In the first part of this thesis, we show that lactate decreases spontaneous neuronal activity in a concentration-dependent manner and independently of its metabolism. We further identified a receptor-mediated pathway underlying this modulatory action of lactate. This finding constituted a novel mechanism for the modulation of neuronal transmission by lactate.

In the second part, we have undergone a characterization of a new pharmacological tool, a high affinity glutamate transporter inhibitor. The finality of this study was to investigate the detailed pharmacological properties of the compound to optimize its use as a suppressor of glutamate signal from neuron to astrocytes.

In conclusion, both studies have implications not only for the understanding of the metabolic cooperation between neurons and astrocytes, but also in the context of the glial modulation of neuronal activity.

Résumé

ASPECTS INTERCELLULAIRES ET DE RESEAUX DES INTERACTIONS METABOLIQUES ENTRE NEURONES ET CELLULES GLIALES

Luigi Bozzo

Université de Lausanne, Département des Neurosciences Fondamentales

Par rapport à son volume, le cerveau utilise une quantité massive de glucose comme source d'énergie. De plus, la consommation d'équivalents énergétiques est étroitement liée au niveau d'activité synaptique. Il a été montré que dans ce couplage neurométabolique, un rôle central est joué par les astrocytes. Ces cellules fournissent le lactate, un substrat métabolique, aux neurones de manière adaptée à leur activité glutamatergique. Plus précisément, le glutamate libéré dans la fente synaptique par les neurones, est récupéré par les astrocytes et déclenche ainsi une cascade d'événements intracellulaires qui conduit à la production et libération de lactate. Les travaux de cette thèse ont visé à étudier la relation métabolique et fonctionnelle entre neurones et astrocytes, avec une attention particulière pour des rôles que pourrait avoir le lactate au-delà de sa fonction métabolique. Pour étudier les aspects spatio-temporels de ces interactions dynamiques, nous avons utilisé à la fois la microscopie optique associée à des indicateurs fluorescents spécifiques, ainsi que l'électrophysiologie.

Dans la première partie de cette thèse, nous montrons que le lactate diminue l'activité neuronale spontanée de façon concentration-dépendante et indépendamment de son métabolisme. Nous avons identifié l'implication d'un récepteur neuronal au lactate qui sous-tend ce mécanisme de régulation. La découverte de cette signalisation via le lactate constitue un mode d'interaction supplémentaire et nouveau entre neurones et astrocytes.

Dans la deuxième partie, nous avons caractérisé un outil pharmacologique, un inhibiteur des transporteurs du glutamate à haute affinité. Le but de cette étude était d'obtenir un agent pharmacologique capable d'interrompre spécifiquement le signal médié par le glutamate entre neurones et astrocytes pouvant permettre de mieux comprendre leur relation.

En conclusion, ces études ont une implication non seulement pour la compréhension de la coopération entre neurones et astrocytes mais aussi dans le contexte de la modulation de l'activité neuronale par les cellules gliales.

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Abbreviations

3,5-DHBA	3,5-Dihydroxybenzoic Acid
3-HBA	3-Hydroxybenzoic Acid
Acetyl-CoA	Acetyl Coenzyme A
AD	Alzheimer's Disease
ADP	Adenosine Diphosphate
AMPA	2-Amino-3-(3-Hydroxy-5-Methyl-Isoxazol-4-Yl)Propanoic Acid
ANG	Asante Natrium Green
ANLS	Astrocyte-Neuron Lactate Shuttle
ATP	Adenosine-5'-Triphosphate
BBB	Blood-Brain Barrier
Ca^{2+}	Calcium
cAMP	Cyclic Adenosine Monophosphate
CFTR	Cystic Fibrosis Transmembrane Regulator
Cl^-	Chloride
CNQX	6-Cyano-7-Nitroquinoxaline-2,3-Dione
CNS	Central Nervous System
CO_2	Carbon Dioxide
D-AP5	(2R)-Amino-5-Phosphonopentanoic Acid
DHK	Dihydrokainate
EAAT	Excitatory Amino Acid Transporters
$FADH_2$	Reduced Flavin Adenine Dinucleotide
fMRI	Functional Magnetic Resonance Imaging
FRET	Förster Resonance Energy Transfer
GABA	γ-Aminobutyric Acid
GAT	GABA Transporters
GLAST	Glutamate/Aspartate Transporter
GLT-1	Glutamate Transporter-1
GTP	Guanosine-5'-Triphosphate
H^+	Proton
H_2O	Water
HCA	Hydroxycarboxylic Acid
Hz	Hertz
IC_{50}	Half Maximal Inhibitory Concentration
IP_3	Inositol Trisphosphate
K^+	Potassium
K_{ATP}	ATP-Dependent K^+ Conductance
Kir	Inward Rectifier K^+
K_m	Michaelis-Manten Constant
LDH	Lactic Acid Dehydrogenase
MCPG	α-Methyl-4-Carboxyphenylglycine
MCT	Monocarboxylate Transporters
Mg^{2+}	Magnesium
mGluR	Metabotropic Glutamate Receptors
Na^+	Sodium
Na^+/K^+-ATPase	Sodium-Potassium Adenosine Triphosphatase
NAD^+	Oxidized Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
NMDA	N-Methyl-D-Aspartate
O_2	Oxygen
P2X	X-Type Purinergic Receptor
P2Y	Y-Type Purinergic Receptor
PET	Positron Emission Tomography
PKA	Protein Kinase A
pK_a	Proton Dissociation Constant
PTX	Pertussis Toxin
SBFI	Sodium-binding Benzofuran Isophthalate
t-ACPD	trans-1-Aminocyclopentane-1,3-Dicarboxylic Acid
TBOA	DL-Threo-B-Benzyloxyaspartate
TCA	Tri-Carboxylic Acid
TFB-TBOA	(2S,3S)-3-[3-[4-(Trifluoromethyl) Benzoylamino]Benzyloxy]Aspartate
THA	*threo*-β-Hydroxyaspartate
t-PDC	*trans*-Pyrrolidine-2,4-Dicarboxylic Acid

1 Introduction

We have a glutton under our cranium, named brain. The headquarter of our thinking is one of the most energy demanding organs. The human brain, although it constitutes only 2% of body weight, can reach the consumption of 20% of total body glucose, the main energy substrate for the brain (Magistretti, 2006). Large amount of energy is required to maintain neural activity in the central nervous system and prolonged deprivation of glucose leads to irreversible cell damage.

The relationship between neuronal activity and glucose consumption is of prime importance and has been matter of debate within the scientific community. Many clinical and experimental studies have yielded the unquestioned evidence that glucose, in the brain, is fully converted into CO_2 and H_2O in an oxygen-dependent process. However, observations made with functional brain imaging techniques have highlighted some discrepancies between this notion and the apparent way the active brain handles glucose. In active brain regions, the increase in glucose consumption was not mirrored by an enhancement oxygen utilization meaning that glucose was not totally catabolized. In order to explain this particularity from a cellular point of view Luc Pellerin and Pierre Magistretti proposed a model to explain how the brain handles glucose. This hypothesis was called astrocyte-neuron lactate shuttle hypothesis (ANLS). Since its introduction 18 years ago (Pellerin and Magistretti, 1994), the validity of this hypothesis has been debated. Nevertheless, several pieces of experimental evidence that support the lactate shuttle hypothesis have been found *in vivo* and this model remains a valuable paradigm that needs further exploration.

During this thesis work, we focused our attention on the astrocytic output and input signals involved in the ANLS. In particular, we looked more closely at the possibility of a non-conventional role of lactate released by astrocytes and at the mechanisms that trigger the

ANLS. In this introductory part, we provide the basis for a better understanding of the processes involved.

1.1 Cellular and physiological bases of cerebral metabolism

In order to provide energy for its own functioning, the brain receives approximately 10% of glucose and 50% of O_2 from the arterial blood (Zauner and Muizelaar, 1997). Most of the brain's energy consumption goes into sustaining neuronal activity. It was estimated in the grey matter that 75% of the total adenosine-5'-triphosphate (ATP), the major energy currency molecule, available is used for mechanisms that mediate the neuronal signaling (including the maintenance of resting potential) and 25% is consumed for basal activity like organelles transport and turnover of macromolecules (Attwell and Laughlin, 2001).

The purpose of this chapter is to review the main features of brain energy metabolism with a particular focus on the cellular and molecular aspects of it.

1.1.1 Organization of the central nervous system

Neurons are the key elements for the transmission of signals throughout the brain and they represent the principal consumers of energy. With their variety of size and shapes they are the basic elements that constitute the neural tissue, the core element of the central nervous system (CNS). Neurons are not the only cells present in the CNS, they are largely outnumbered by various non-neuronal cellular components. These cells include astrocytes, oligodendrocytes, microglia and endothelial cells. They provide an essential support to neurons in terms of structure, isolation, protection, and many other functions. Among them, astrocytes and blood vessels are essential to provide energy to neurons. The cells that are involved in brain metabolism are described below.

1.1.1.1 Neurons

Neurons are specialized type of cells with peculiar electrochemical properties (**fig.1** representative neurons). They are able to receive, integrate, and transmit neural signals. Two processes mediate signaling between neurons: action potentials that carry electrical signals along the axon, and postsynaptic currents, which are generated by neurotransmitters that are released at the synapses and acts on the target neurons. Both processes require energy for its own functioning.

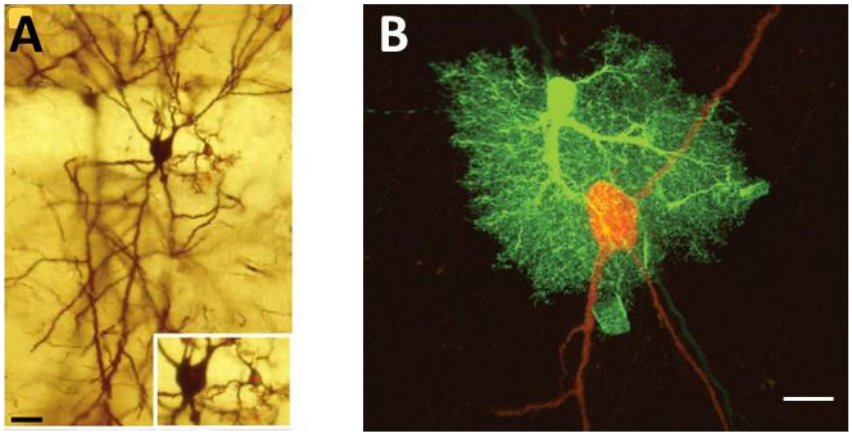

Figure 1: Comparative images of neurons and astrocytes in the brain
A) Neuron and astrocyte stained with the Golgi method. Inset: astrocyte magnification. From (Perea et al., 2009). B) Immunostaining of a neuron (red) and an astrocyte (green). From (Allen and Barres, 2009). Scale bars, 10 μm.

The excitability of neural cells is dependent from the ionic gradient, particularly Na^+ and K^+, across the plasma membrane. The maintenance of the electro-chemical gradients is the main energy-consuming process in neurons. It is achieved by pumping ions against their gradient, via the Na^+/K^+-ATPase, that consumes ATP in the process (Attwell and Laughlin, 2001). During the glutamatergic excitatory signaling in rat cerebral cortex, the pump was estimated to spend 92% of the available ATP excluding the housekeeping functions (Harris et al., 2012). This consumption of energy was predicted to be distributed as follows: 54% for the signaling cascade consecutive to post synaptic effects, 24% for generating action

potential, and 22% for maintaining resting potential (percentages recalculated from Harris et al., 2012, excluding the ATP consumed by glutamate recycle and reversal of presynaptic Ca^{2+} entry).

It is necessary to keep in mind that the values indicated above are only estimates based on a number of assumptions. For instance, they use an average cell size, a fixed firing rate (4Hz), or assume that all neurons are glutamatergic. In the real brain, neurons are a heterogeneous population made of cells with a variety of sizes, number of synapses, rate of activity and kind of neurotransmitters released, thus it is conceivable that the energy consumption is not equivalent for each of them. For example, pyramidal neurons with a regular firing that have a large soma, extended processes and use glutamate as principal neurotransmitter might need a different amount of energy than small local signaling interneurons with a fast firing that use GABA as main neurotransmitter.

Overall, we see that neurons are extremely avid energy consumers; nevertheless, the consuming processes involved are highly optimized to maximize the efficiency with minimum energy cost (Attwell and Laughlin, 2001).

1.1.1.2 Vascular endothelial cells

Vascular endothelial cells, together with connective tissue and smooth muscle, form a network of blood vessels that supply the brain. The cerebral blood flow circulating inside the system delivers oxygen, glucose and other essentials substrates for brain functions. The vascular system is well organized and subdivided in arteries, arterioles, capillaries, venules and veins to distribute and collect the blood through the whole brain (**fig. 2**). The circulating blood is separated from the cerebral extracellular fluid by the blood-brain barrier (BBB). BBB is formed by endothelial cells connected with tight junction, pericytes, basal lamina, and end-feet of astrocytes (**fig. 3**). This particular interface between blood and neurons allows the diffusion of small molecules such as O_2 and CO_2 or the selective transport of metabolic substrates like glucose (Abbott et al., 2006).

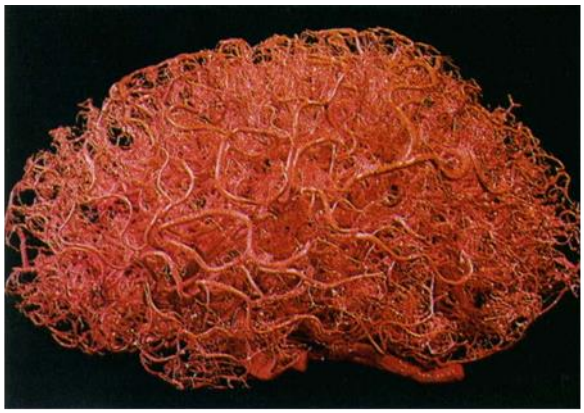

Figure 2: Plastination of the cerebral vascular system. From (Zlokovic and Apuzzo, 1998).

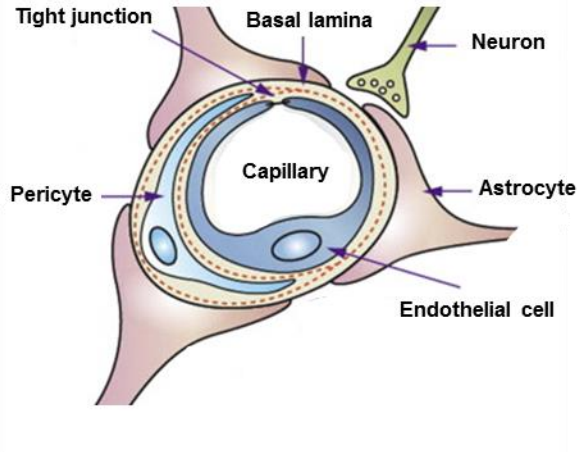

Figure 3: Schematic representation of the blood brain barrier. Adapted from (Chen and Liu, 2012).

The cerebral-blood flow must be maintained in a safeguard range, since the brain is a particularly vulnerable organ. For example, an excess in perfusion rate can increase the blood pressure and damage the neural tissue or an insufficient perfusion rate can lead to neuronal death, due to deprivation of essential elements such as O_2 or glucose.

Beside these pathological conditions, the brain blood flow is carefully regulated in correspondence with the brain activity. It has been demonstrated, using both positron emission tomography (PET) and functional magnetic resonance imaging (fMRI), that in patients involved in a specific task, such as calculation or visual stimulation, the regional blood brain circulation is increased in corresponding activated areas (Raichle and Mintun, 2006).

Intuitively, the reason of this correlation could be that blood-flow changes serve to adjust the glucose-oxygen concentration to match the energy demand. However, this explication can be inexact because it was found that in the human brain monitored with PET, the increase of blood flow induced by the brain activity is accompanied with a high glucose consumption but with a minimal utilization of oxygen (**fig. 4**) (Fox et al., 1988). This uncoupling between glucose and oxygen consumption has particular implications in the relationship between neuronal activity and the metabolism of glucose and is discussed in the next chapter.

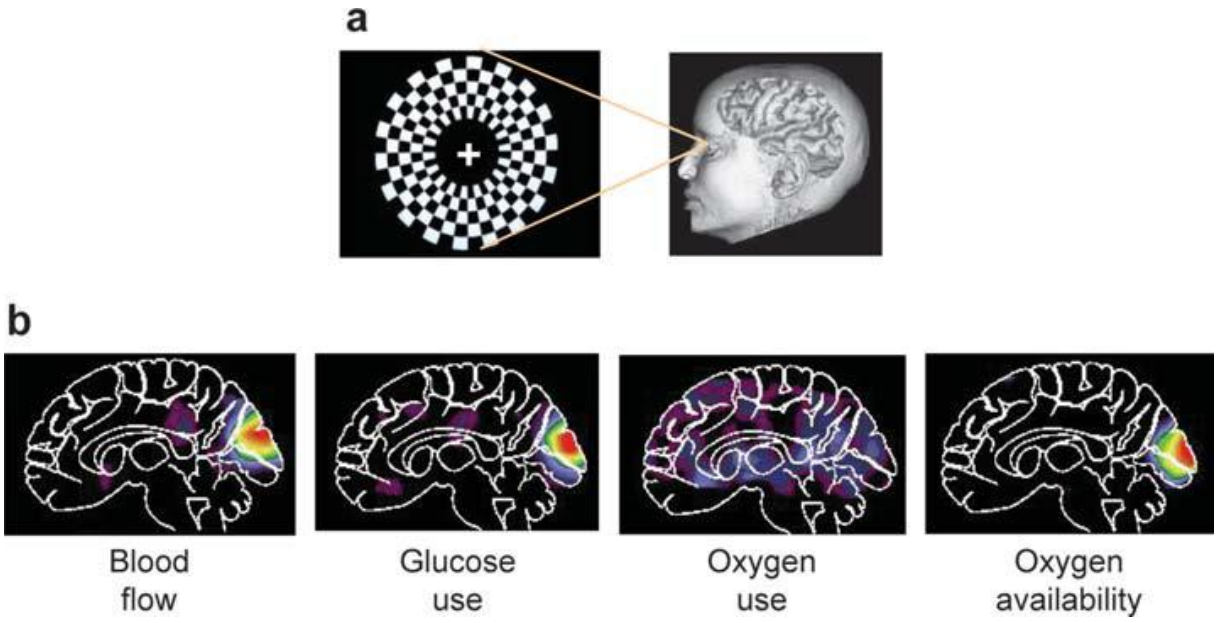

Figure 4: Stimulation of the human visual cortex with a visual stimulus (a) produces an increase in blood flow and glucose utilization but not in oxygen consumption (b). From (Raichle and Mintun, 2006).

1.1.1.3 Astrocytes

As the name suggests, astrocytes (Greek etymology: *astron* = star, *cyte* = cell) in the intact brain has a star morphology (see **fig. 1**). They account for 20 to 50% of the volume of different brain areas (Barres, 2008). Astrocytes are positioned in a way that gives them the potential to interact with neurons, other astrocytes, and/or blood vessels. In addition, with their processes they can enwrap synapses or surround capillaries (**fig. 5**).

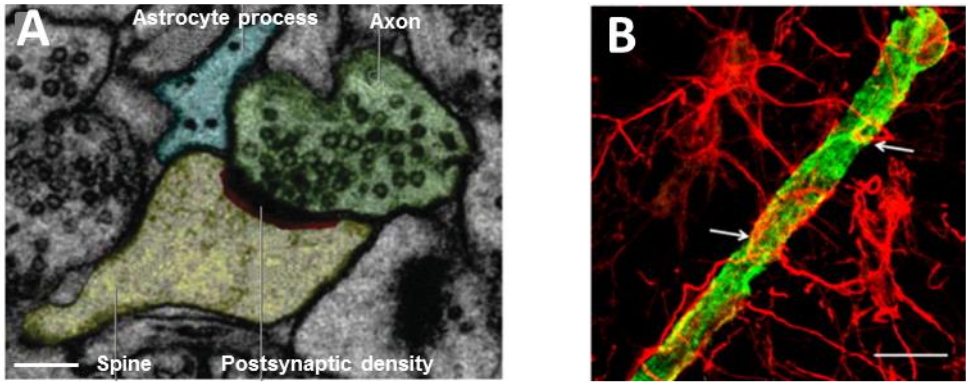

Figure 5: A) Electron microscopy picture showing the process of astrocyte (blue) enwrapping the pre- and post-synapse (green and yellow respectively). Scale bar 200nM. From (Eroglu and Barres, 2010). B) Confocal image of immuno-fluorescent staining of blood vessels (green) and astrocytes (red). White arrows indicate examples of contact between astrocytic processes and vessel. Scale bar 10μM. From (Rajkowska et al., 2013).

This situation gives them the ability to physically form a bridge between neurons and cerebral blood. For a long time, this strategic position between the different components of the brain was interpreted as an isolation and astrocytes were considered as mere brain glue (Volterra and Meldolesi, 2005). With the discovery of the numerous functions of astrocytes, this point of view has now radically changed. Some of these functions are listed below:

- **Blood brain barrier:** As seen in the previous subchapter, the end-feet of astrocyte contribute to form the BBB allowing the metabolism inside the brain to operate differently from the rest of the body.

- **Control of the extracellular fluid:** Astrocytes express a variety of transporters for neurotransmitters and ion channels to regulate the extracellular fluid composition. For example, near the synaptic cleft, they have the capacity to rapidly clear up glutamate and K^+ ensuring the fidelity of the neurotransmission and avoiding excitotoxicity.
- **Energy support to neurons**: They furnish metabolic substrates to neurons such as lactate. Lactate can be derived via glycolysis from glucose or from the glucose store constituted by glycogen.
- **Modulation of synaptic transmission:** Astrocytes release transmitter molecules, named gliotransmitters, able to influence the neural activity such as glutamate, ATP, D-serine (Volterra and Meldolesi, 2005).

Some of these functions will be discussed in more details in following chapters.

1.1.2 Glucose metabolism

The brain typically gets most of its energy, in the form of ATP, from glucose. In normal circumstances glucose is considered as the main energy substrate for the brain and it is substantially converted to CO_2 and H_2O.

The enzymatic reactions for the metabolism of glucose in the brain are the same as in the rest of the body and consist of glycolysis, tri-carboxylic acid (TCA) cycle, and oxidative phosphorylation. These pathways are the principal source of energy, when oxygen is available. With the complete oxidization of glucose, 30-36 molecules of ATP are generated. The pentose phosphate pathway is also involved in the metabolism of glucose. Despite its essential role in the generation of nicotinamide adenine dinucleotide phosphate (NADPH) and pentose, it is not directly producing ATP then it will be not discussed below.

In this chapter, the fundamental bases of the energy production in the nervous tissue, with a particular emphasis on lactate, will be discussed together with the ANLS hypothesis.

1.1.2.1 Glycolysis

In the cytosol, glycolysis is the metabolic pathway that converts glucose in two molecules of pyruvate or lactate. The energy released in this process is used to produce of ATP and NADH. The whole glycolytic process is composed by 10 enzymatic sequences (**fig. 6**). From one molecule of glucose, four molecules of ATP are formed but an initial investment of two ATP is needed to start the process. Therefore, the overall reactions result in the net production of two molecules of ATP.

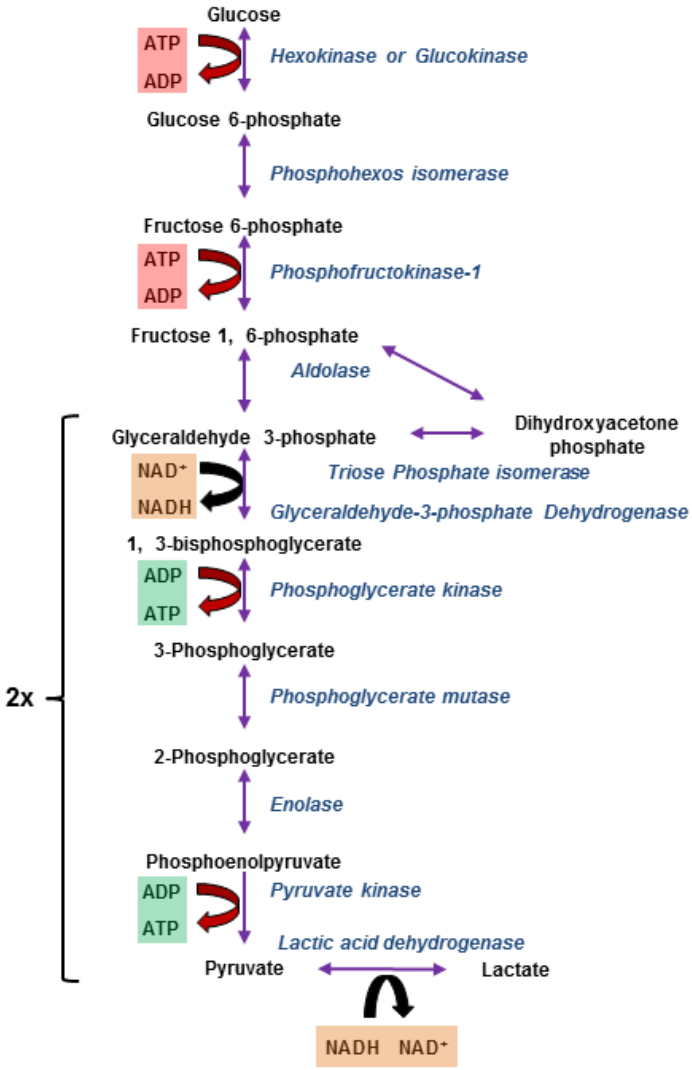

Figure 6: Glycolytic pathway.

To be active, the enzyme glyceraldehyde-3-phosphate dehydrogenase needs NAD^+ as a cofactor for the reaction. This represents a limiting step for the process, if NAD^+ is not available, glycolysis risks to stop at this phase. The regeneration of NAD^+, essential to continue the glycolytic flux, is primarily achieved in the mitochondrion. Under aerobic conditions NADH is oxidized to NAD^+ by electron respiratory chain. Alternatively NADH can be converted in NAD^+ by the ubiquitous lactic acid dehydrogenase (LDH) that catalyzes the conversion of pyruvate in lactate. In this case, the stoichiometry of the glycolysis is:

$$\text{Glucose} + 2\ \text{ADP} + 2\ P_i \rightarrow 2\ \text{lactate} + 2\ \text{ATP} + 2\ H_2O$$

In certain situations, when oxygen availability is absent or in short supply, glucose is not completely oxidized to CO_2 and H_2O. The process stops before entering the TCA cycle and the end product of glycolysis becomes lactate. This is well known during intense muscular activity but a transient mismatch between glucose and oxygen consumption has been also observed during sustained cerebral activity (**fig. 4**) (Raichle and Mintun, 2006). An increase in neuronal activity is followed by an increase in glucose consumption but with a minimal oxygen utilization, meaning that during this phase lactate could be produced in the brain. A temporal increase of extracellular concentration of lactate was indeed seen in different brain regions after stimulation of neuronal activity in rats (Hu and Wilson, 1997).

Glycolysis is the first step for the complete oxidation of glucose. It provides the substrates for the next steps, the TCA cycle and the oxidative phosphorylation.

1.1.2.2 Tricarboxylic acid cycle and oxidative phosphorylation

The TCA cycle and the oxidative phosphorylation are a series of oxygen-dependent reactions that lead to the production of energy. These catabolic pathways represent the last phase for the degradation of carbohydrate, lipids and proteins into CO_2. In addition, the oxidative phosphorylation is responsible for the regeneration of NADH into NAD^+, a cofactor

used in the catalysis of numerous reactions. The whole process occurs in the mitochondrial matrix and is represented in **figure 7**.

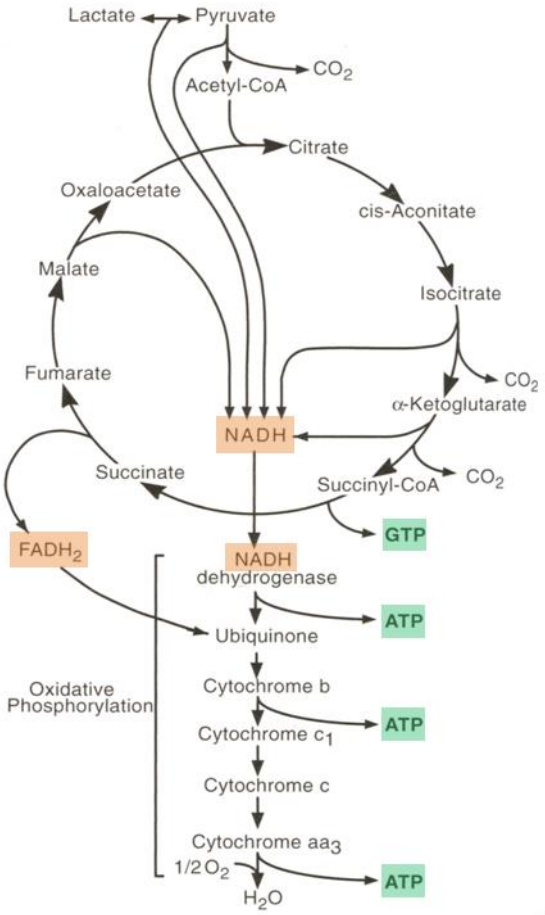

Figure 7: Tricarboxylic acid cycle and the oxidative phosphorylation.
From (Squire et al., 2008).

Pyruvate, produced via glycolysis, enters the mitochondria through the monocarboxylate transporters (MCT) expressed in the inner membrane of mitochondria. Once inside the organelle, it is converted by the enzyme pyruvate dehydrogenase into acetyl coenzyme A (Acetyl-CoA). The acetyl unit enters the TCA cycle and is then completely oxidized into CO_2. The whole process leads to the production of GTP, NADH, $FADH_2$, and coenzyme A with the following stoichiometry:

$$\text{Acetyl-CoA} + 3\ NAD^+ + FAD + GDP + P_i + 2\ H_2O \rightarrow$$

$$2\ CO_2 + 3\ NADH + FADH_2 + GTP + 2\ H^+ + \text{coenzyme A}$$

In this phase, the net production of energy is low, only one GTP per molecule of pyruvate, but the NADH and $FADH_2$ formed have a high energetic potential. In the last process the oxidative phosphorylation, NADH and $FADH_2$ donate their electrons to molecular oxygen through the electron transfer chain. The resulting energy liberated is used to generate ATP. The amount of energy produced in this last step, around 15 ATP, is largely the major source of energy of the metabolism of glucose.

1.1.3 Alternative energy substrates

Glucose is the mandatory energy substrate for the brain. Other substitutes can be used as energy metabolites in absence of glucose. All of these alternative substrates are molecules that can enter the glycolysis and TCA cycle at different steps.

Mannose and fructose can be converted, in one or two enzymatic steps, into fructose 6-phosphate and can be further metabolized by the glycolysis. These sugars are able to maintain the production of neuronal ATP (Yamane et al., 2000). Both molecules can pass the blood brain barrier, mannose via glucose transporters and fructose, most likely, via passive diffusion (Sapolsky, 1986). Once inside the cytoplasm, the enzymatic machinery is responsible to convert them into fructose-6-phosphate, an initial substrate of the glycolysis. However, the normal concentration of both molecules found in the blood is low, meaning that they should be considered as not physiological (Pitkanen and Kanninen, 1994).

Lactate and pyruvate are direct products of glycolysis. These intermediate of glucose metabolism can reach the TCA cycle and maintain the ATP production. It has been found that lactate and pyruvate support the synaptic transmission *in vitro* (Izumi et al., 1997, Rouach et al., 2008). In addition, a growing body of evidence has also confirmed lactate utilization *in vivo* (Gallagher et al., 2009, Boumezbeur et al., 2010, Wyss et al., 2011). Experiments, done with carbon-13 labeled lactate, suggested that blood lactate is able to

reach the neuronal compartment (Hassel and Brathe, 2000). In addition, monocarboxylate transporters were found at the level of the BBB (Pierre and Pellerin, 2005).

This body of information indicates that plasma lactate can be an adequate substitute for glucose. Moreover, if formed inside the brain, lactate and pyruvate can be useful metabolic substrates for neural cells.

The ketone bodies, such as acetoacetate and D-3-hydroxybutyrate, can be processed to Acetyl-CoA, the substrate for TCA cycle. The brain is also able to utilize these alternative molecules. During the suckling period, starvation or diabetes, the blood concentration of ketone bodies is increased (Owen et al 1967). This means that in these conditions, ketone bodies can be used as neuronal metabolic substrates.

1.1.4 The astrocyte-neuron lactate shuttle (ANLS) hypothesis

Experiments performed with PET and fMRI evidenced that an increase of the brain activity is accompanied with a non-oxidative consumption of glucose (see **fig. 4**) (Raichle and Mintun, 2006). This leads to a production of lactate (Bonvento et al., 2005). This brought up the question of why and how lactate is produced in this condition. Around 18 years ago, Pierre Magistretti and Luc Pellerin attempted to answer these questions, from a cellular and mechanistic point of view, proposing a new model for the handling of glucose in the brain. The proposed mechanism, coupling neuronal activity to glucose utilization, is called astrocyte-neuron lactate shuttle hypothesis and introduces astrocytes as pivotal element. In summary, this hypothesis proposes that glutamatergic activity can be sensed by astrocytes. Accordingly, astrocytes are able to increase their glycolysis, leading to the production of lactate. Subsequently, lactate can be delivered by astrocytes to neuron and serve as metabolic substrate. A description of the whole process is reported below and represented in **fig. 8.**

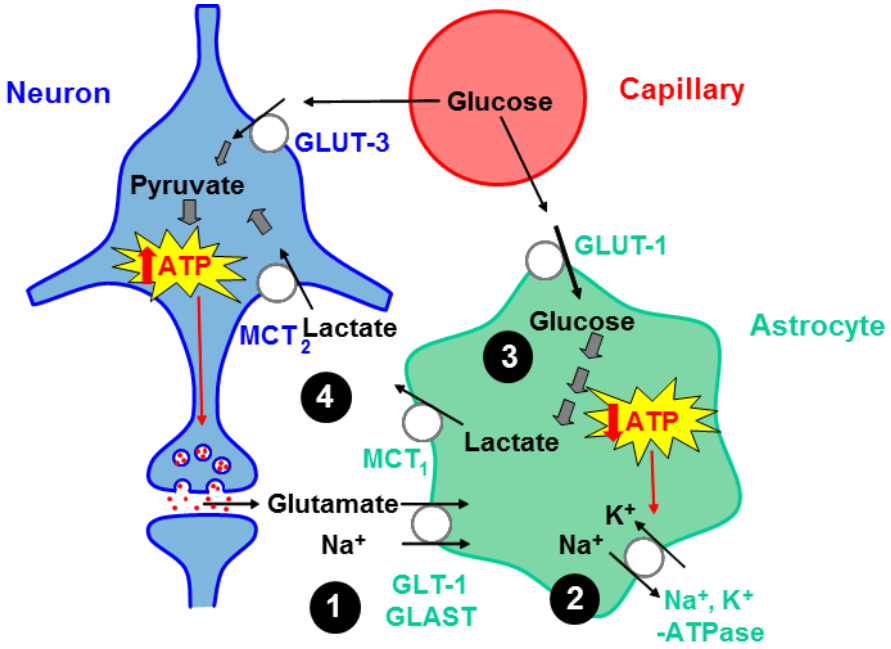

Figure 8: Schematic representation of the astrocyte-neuron lactate shuttle hypothesis. The circled numbers correspond to the key steps of the model explained below.

1. During the glutamatergic activity, glutamate released in the synaptic cleft by neurons is rapidly taken up by astrocytes that express efficient glutamate transporters, named GLAST and GLT-1 (Anderson and Swanson, 2000). The glutamate carriers use the electrochemical gradient of Na^+ as main driving force.
2. The resulting increase of intracellular Na^+ concentration is followed by the activation of the Na^+/K^+-ATPase that recovers the normal Na^+ levels (Chatton et al., 2000). During this phase, the Na^+/K^+-ATPase double or even triple its activity resulting in an augmentation of the metabolic cost.
3. The increased energy demand stimulates glucose utilization. Astrocytes express the glucose transporters GLUT1 (Danbolt, 2001) that facilitate the diffusion of glucose from the capillaries. Glucose in the astrocytic cytosol is converted by glycolysis into lactate (Pellerin and Magistretti, 1994).
4. Once produced in the astrocytes, lactate is delivered to neurons. The transfer is carried out by different isoforms of MCTs: MCT1, expressed by astrocytes are

responsible for the release of lactate in the extracellular fluid and MCT2, primarily expressed by neurons for uptake (Pierre and Pellerin, 2005). Interestingly, in neurons, part of MCT2 are distributed at postsynaptic site where the major amount of energy was estimated to be needed during neuronal activity (Harris et al., 2012). Once inside the neurons, lactate can be used as a metabolic substrate to sustain neuronal activity.

The ANLS hypothesis represents one of the best model of cellular pathway that can explain what is observed in the active brain with PET and fMRI (Bonvento et al., 2005). In this model the brain imaging signal based on glucose utilization could reflect the astrocytic metabolism that is mainly glycolytic. This could explain why we have a glucose consumption with a minimal oxygen utilization. Lactate released by astrocytes is subsequently catabolized by neurons that are mainly oxidative.

1.1.4.1 Controversies on the ANLS hypothesis

Since its introduction, the ANLS model has generated numerous discussions and controversies (Hertz et al., 2007, Dienel, 2012). One of the main points at the center of the debate is the ability of glutamate to induce an increase in astrocyte glycolysis. It was for instance argued that astrocytes have actually high oxidative rates *in vivo* and that glutamate taken up by astrocytes could be metabolized in the Krebs cycle following its degradation into α-ketoglutarate (Yu et al., 1982). The consequence would be to stimulate astrocyte mitochondrial oxidative metabolism instead of the proposed stimulation of glycolysis. It was also argued that lactate could originate from different cell types than astrocytes, including neurons, questioning the existence of a net flux of lactate from astrocytes to neurons (Dienel and Hertz, 2001). In addition, when the model was originally proposed, critiques have been raised because the arguments were largely based on *in vitro* data coming from primary cultures of astrocytes and neurons.

Evidence for stimulation of astrocytic glycolysis after neuronal activity has been brought by studies performed using more complex preparations. In hippocampal slices the neuronal activity was evoked by the stimulation of the Schaffer collaterals, and the NADH autofluorescence was monitored as an indicator of the glycolytic and oxidative metabolism (Kasischke et al., 2004). After the stimulation, a strong increase of NADH fluorescence in astrocytes was observed indicating that an increase of glycolysis occurred. Another *in vivo* demonstration was obtained using knockout mice for the glial glutamate transporters GLT1 or GLAST (Voutsinos-Porche et al., 2003). The authors found that the enhancement of glucose utilization after synaptic activation present in wild type mice was decreased in mutant mice lacking glutamate transporters, suggesting that glutamate uptake is responsible of the increase in astrocytic glycolysis.

It should be finally added that the mechanisms, the applicability, and the regulation of neurometabolic coupling based on the ANSL model are still evolving and being refined. The results presented in the present thesis work are a good example of the evolution of these concepts.

In our laboratory, the metabolic coupling between neuron and astrocytes represents one of the main lines of research. Previous studies performed by my colleagues, contributed to elucidate several aspects of the ANLS hypothesis (Chatton et al., 2003, Morgenthaler et al., 2006, Azarias et al., 2011). For my thesis project, we focused our attention at two different levels of the neurometabolic coupling. In one project, we studied the inhibitors that can block glial glutamate transporters; these pharmacological tools are crucial to refine our understanding of the implication of glutamate transporters in the context of our studies. In the other project that constituted the main work of my thesis, we studied the effects of lactate on neurons beside its role as substrate of the energy metabolism.

Each subject constituted an independent study. The aim of the remaining part of the introduction is to give an overview of the knowledge to better understand the two studies. Chapters 1.2 is for the first project and chapter 1.3 for the second one.

1.2 Focus on the mechanisms that trigger the ANLS

One of the key points of the neurometabolic coupling is the fact that astrocytes are able to sense the level of activity of a majority of neurons as ~85% of neurons are considered to be glutamatergic (Harris et al., 2012). To do so, they need to be in close contact with each other. With their processes astrocytes enwrap the glutamatergic synapses of neurons. The extracellular space that separates the membrane of both cells is very narrow, only few nanometers (see **fig. 5A**). In addition, the surface of astrocytes is equipped with efficient excitatory amino acid transporters that transport glutamate in a Na^+ dependent manner. The glutamate transport and the subsequent intracellular increase of Na^+ in astrocytes are the pivotal elements of the system that trigger the neurometabolic coupling. Beside these carrier proteins, astrocytes express also a variety of other membrane structures such as ion channels, receptors, and transporters that are sensitive to the substances released in the synaptic cleft. With this set of properties, astrocytes are able to control the composition of the intercellular fluid also near the synapses.

Here, we discuss the potential involvement of these structures in enhancing glycolysis. Because the increase in glucose consumption is one of the first steps of the neurometabolic coupling, a special attention is put on those that are related to glutamate or induce an influx of Na^+ in astrocytes. Moreover, for each element, the pharmacological available tools, such as inhibitors or agonists used to demonstrate their involvements, are reported (part of these compounds were used in experiments reported in the results about the TFB-TBOA characterization).

This chapter will be useful to understand parts of my work, as during the thesis we characterized the effects of TFB-TBOA, a novel high affinity glutamate transporter inhibitor. The aim of this study was to characterize an appropriate pharmacological tool that could be

useful to dissect the neuron-astrocyte interactions especially in the context of the neurometabolic coupling.

1.2.1 Excitatory amino acid transporters (EAAT)

EAATs are glutamate transporters that are dependent of the electrochemical gradient of Na^+ to operate the transport. The uptake of glutamate plays a pivotal role for two important functions: first, it clears up the glutamate to avoid excitotoxic effects, and secondly, it triggers the neurometabolic coupling. In humans, the glutamate transporters are termed excitatory amino acid transporters (EAAT) and they are subdivided in five types present in different cells of the CNS. Astrocytes express EAAT1 and EAAT2, for historical reasons their murine homologues are also called GLAST and GLT-1 respectively (Danbolt, 2001). The isoform EAAT3 (also named EAAC in rodent) is present in both the soma and the dendrites of neurons, but the concentration is in a low amount compared to EAAT1 and EAAT2 (Arriza et al., 1994). The other members of the family are the EAAT4 located in the cerebellum in Purkinje neurons and the EAAT5 that appears to be retina specific (Danbolt, 2001).

The majority of the glutamate uptake in the brain is mediated by astrocytic EAAT1 and EAAT2. Both isoforms have a high affinity for glutamate with a half-maximal activity in the range of 20 to 90 µM (Arriza et al., 1994). In the early stage of development, EAAT1 is more strongly expressed than EAAT2. Throughout maturation, this proportion is inverted, the EAAT2 progressively increases and EAAT1 tends to decrease. In adult, EAAT2 is dominant in the CNS and EAAT1 is less concentrated however it is diffusely present in the brain. These different timed expressions of EAATs can explain why *in vitro* only EAAT1 is expressed (Danbolt, 2001), since primary astrocytes cultures are typically prepared from new born animal.

EAATs, use the electrochemical gradient of Na^+ as driving force to transport glutamate. One glutamate is transported with three Na^+ and one H^+ in with a counterpart of one K^+ (Levy

et al., 1998). The subsequent reequilibration of these gradients by the Na^+/K^+-ATPase causes ATP hydrolysis. In addition to glutamate, they can also transport other molecules such as aspartate.

Without the continuous activity of glutamate transporters, neurons risk an increase in excitability and to ultimately encounter a particular type of cellular damage called excitotoxity that can lead to neuronal death. It was demonstrated in rats that the induction of knockout of EAAT1 and EAAT2, using chronic antisense oligonucleotide administration, led to neurodegeneration characteristic of excitotoxicity (Rothstein et al., 1996).

1.2.2 Ion channels

1.2.2.1 K^+ homeostasis

Excitatory synaptic activity releases glutamate in the synaptic cleft and, a few milliseconds later, K^+ is released from the postsynaptic terminal via ionotropic glutamate receptors (Bittner et al., 2011). The resulting excess of interstitial K^+ can interfere with the neuronal activity. Thus, extracellular K^+ composition around synapses have to be maintained at low levels. For this purpose, astrocytes express inward rectifier K^+ (Kir) channels at high density (Butt and Kalsi, 2006). These conductances are essential to ensure the neurotransmission fidelity.

It was suggested that exposure of astrocytes to high K^+ led to an increase in the glycolysis (Brookes and Yarowsky, 1985). Thus, K^+ could be a signal for the neurometabolic coupling. This issue has been investigated by the group of Felipe Barros. Using a glucose sensor based on Förster resonance energy transfer (FRET) to measure the glycolytic rate. They found that K^+ applied extracellularly, was able to increase the glucose consumption likely via the stimulation of Na^+/K^+-ATPase (Bittner et al., 2011). However, a weak point of the study consisted in the fact that they had to use a bulk perfusion of K^+ to induce a metabolic response, not equivalent of local K^+ release that would be mimicking more closely the increase of K^+ in the synaptic cleft. Nevertheless, we can consider that K^+ could have a

concomitant role with glutamate in the coupling between neuronal activity and glucose consumption.

1.2.2.2 Ionotropic glutamate receptors

AMPA, kainate and NMDA receptors are permeable to Na^+ when they open concomitantly to glutamate binding, the resulting sodium influx could induce an increase in the glycolysis. These receptors are generally known for their critical roles in the synaptic transmission, learning and memory when they are expressed by neurons. However, some classes of ionotropic glutamate receptors are also found in astrocytes.

Many groups have demonstrated that astrocytes *in vitro* and *in vivo* express AMPA/kainate receptors (Verkhratsky and Steinhauser, 2000). However, in the continued presence of glutamate the AMPA/kainate current evoked is rapidly inactivated, because of a prominent receptor desensitization process (desensitization time constant 1-10 ms) (Dingledine et al., 1999). This has the consequence that the time window of action is too short to induce a large rise in Na^+ concentration and precludes a significant stimulation of the sodium pump in astrocytes (Chatton et al., 2000). Moreover, the use of the specific AMPA/kainate inhibitor CNQX revealed the minor contribution of AMPA/kainate receptors in the glutamate induced Na^+ rise (Chatton et al., 2000), which is primarily transporter mediated.

NMDA receptors are expressed in astrocytes *in vivo* but this receptor is not expressed on astrocytes in culture. This receptor permits both Na^+ and Ca^{2+} influx and K^+ efflux at opening. In primary astrocytes, the perfusion of NMDA can induce a Ca^{2+} elevation but not Na^+ (Bezzi et al., 1998, Chatton et al., 2000). To dissect the involvement of NMDA receptors in the whole brain, it is possible to use the selective inhibitor D-AP5.

In conclusion, it appears that neither NMDA nor AMPA/kainate receptors are involved in the process of neurometabolic coupling.

1.2.3 Metabotropic glutamate receptors

Metabotropic glutamate receptors (mGluR) are G proteins-coupled receptors that bind the neurotransmitter glutamate. The family of mGluRs includes eight different subtypes, sub-divided into three groups. mGluR5 (group 1) and mGluR3 (group 2) are mainly expressed in astrocytes whereas mGluR1 (Group 1) has been shown to be present in hippocampal astrocytes (Condorelli et al., 1999, Schools and Kimelberg, 1999).

Group 1 mGluRs are coupled to G_q proteins. The binding with glutamate activate a cascade of events that lead to the increase of inositol trisphosphate (IP_3). In turn, IP_3 induce the release of calcium from the endoplasmic reticulum (Petravicz et al., 2008). It has been shown that the stimulation of both mGluRs and AMPA/kainate receptors, is causing an increase in intracellular Ca^{2+} that induced a release of glutamate from astrocytes (Bezzi et al., 1998).

Group 2 mGluRs are associated to G_i protein. This G protein is negatively coupled to adenylate cyclase and lead to a depletion of cyclic adenosine monophosphate (cAMP). One potential effect of such a decrease in cAMP is the reduction of activity of the cAMP-dependent protein kinase A (PKA), an enzyme well known to be associated with exocytosis (Seino and Shibasaki, 2005).

It was argued that the contribution of mGluRs in the neurometabolic coupling is of minor importance because the glutamate transporter substrate D-aspartate, is able to stimulate the metabolic response but does not activate AMPA/kainate receptors of astrocytes (Pellerin and Magistretti, 1994).

In addition, their involvement can be directly investigated with two useful compounds. The trans-1-aminocyclopentane-1,3-dicarboxylic acid (t-ACPD) that stimulates all mGluRs subtypes and the α-methyl-4-carboxyphenylglycine (MCPG) that inhibits all of them.

1.2.4 GABA transporters

γ-Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the CNS. It is released at the level of inhibitory synapses. The clearance of GABA released in the synaptic cleft is achieved by high affinity GABA transporters (GAT). GATs are expressed in GABAergic neurons and surrounding glial cells (Gadea and Lopez-Colome, 2001). GAT1 and GAT3 are exclusively present in the CNS, whereas GAT2 and the low affinity subtype BGT1 are also found in the peripheral tissues. All these transporters are present also in astrocytes. The strategic position of the astrocytic process around the synapses and the presence of GAT suggest that astrocytes can contribute to the cleaning of GABA, helping to terminate the neurotransmission.

GATs belong to the family of Na^+- and Cl^--coupled neurotransmitter transporters. The transport cycle stoichiometry is one GABA, two Na^+, and one Cl^- transported in the same direction. The resulting intracellular influx of Na^+ could, in principle, be followed by an increase in ATP consumption by the Na^+/K^+ -ATPase.

The presence of a GABA uptake system in astrocytes means that the inhibitory neuronal activity could be coupled to glucose utilization as it occurs in the glutamatergic metabolic coupling. This question was tackled in a study performed on primary culture of astrocytes, in which the metabolic cost induced by Na^+ increase, induced by GABA, was compared to that associated with glutamate uptake. The results showed that GABA application at millimolar concentration induced a significant influx of Na^+ however without being able to cause an augmentation of the glycolysis (Chatton et al., 2003). Thus, the inhibitory system is not able to directly increase the consumption of glucose by astrocytes.

1.2.5 Purinergic receptors

ATP can be stored in the synaptic vesicle and released in the synaptic cleft in concomitance with glutamate. Specific receptors for ATP, belonging to the category of purinergic receptors,

exist in the brain. These characteristics lead to consider ATP as a neurotransmitter (Burnstock, 2006).

Purinergic receptors for ATP are divided in ionotropic (P2X) receptors, which are ligand-gated non-selective ion channel and metabotropic (P2Y) receptors. Both categories are expressed in astrocytes (King et al., 1996) and if adequately activated they can lead to an increase of Ca^{2+} with a subsequent release of glutamate (Fellin et al., 2006).

To inhibit their activity it is possible to use suramin, a broad-spectrum antagonist of P2 receptors.

1.2.6 Glutamate transporter inhibitors

The majority of glutamate uptake in the adult brain is principally mediated by EAAT2 (GLT-1 in mouse) and EAAT1 (GLAST in mouse) expressed by astrocytes (Danbolt, 2001). As already discussed at the beginning of this chapter, this function is crucial for the central nervous system.

To elucidate in detail the physiological significances of these transporters, pharmacological tools are needed. For this purpose, inhibitors of Na^+-dependent glutamate transporters have been synthetized. The ideal characteristics for these blockers are that they have to be: selective for the EAATs; potent inhibitor with a high affinity, meaning that they have to work at low concentration; and non-transportable because competitive substrates cause the same flux as glutamate. Several compounds have traditionally been used to inhibit glutamate transporters, such as *threo*-β-hydroxyaspartate (THA) and *trans*-pyrrolidine-2,4-dicarboxylic acid (t-PDC). However, both of them are competitive inhibitors with the side effect to induce a Na^+ increase (Chatton et al., 2001). Dihydrokainate (DHK) is a non-transported inhibitor but has the limitation to selectively acts on EAAT2 only. After a series of possible candidate molecules DL-threo-β-benzyloxyaspartate (TBOA) has been synthetized. TBOA is a non-transported competitive inhibitor of glutamate transporters without EAAT

subtype selectivity (Chatton et al., 2001, Shimamoto, 2008). From then, TBOA has been widely used in numerous studies but the compound suffers of a relative low affinity in the micromolar range (Chatton et al., 2001). More recently, a derivative of TBOA has been produced called (2S,3S)-3-[3-[4-(trifluoromethyl)benzoylamino]benzyloxy]aspartate (TFB-TBOA). It conserves the same positive characteristics of TBOA but in addition it has as a much higher affinity for the transporters (Shimamoto et al., 2004). This would make it an extremely valuable tool to study glutamate transport and neuron-glia interactions. For this reason, one of the projects of my thesis was to characterize the effects of TFB-TBOA on glutamate transport in live astrocytes, as well as its effects on neurons. The results are reported in the chapter 2.3.

Nevertheless, it has to be kept in mind that the inhibition of glutamate transport in the intact tissue causes the accumulation of extracellular glutamate. The enhanced neuronal excitability can lead to epileptic activity or excitotoxicity (Tsukada et al., 2005), making the study of transporters difficult in the intact tissue.

1.3 Lactate beyond its metabolic role

Despite the fact that lactate is an oxidative substrate with a high potential as energy source, it has been considered as a useless and toxic compound that must be eliminated from the brain. Nowadays, evidence indicates that cerebral lactate does play a major role in aerobic energy metabolism. Its utilization, in the human brain and more specifically by neurons, has been confirmed *in vivo* (Boumezbeur et al., 2010). As seen above, lactate is produced by astrocytes and delivered to neurons in response to neuronal activity (Pellerin and Magistretti, 2012). For this reason, the interest in lactate has been renewed.

Beside its function of energy substrate in the neurometabolic coupling, lactate can have other roles. It is implicated in several other physiological situations. Here, we present some of them, in order to have a broader picture of the general effects of lactate.

1.3.1 Biological characteristics of lactate

Lactate (more specifically lactic acid) is a carboxylic acid with the chemical formula $C_3H_6O_3$. It is soluble in H_2O. Its proton dissociation constant (pK_a) is 3.9, meaning that, in H_2O, it can be considered as a weak acid. When lactate is dissolved, the carboxylic group -COOH can lose a proton and produce the lactate ion $CH_3CHOHCOO^-$. In function of the pH of solutions, the proportion of lactate in basic or acid state can vary.

Lactate is commonly known to be responsible for the sour flavour of the old milk that acidifies, leading to the coagulation of the casein, and to be a by-product of the intense and prolonged muscular effort. While the first concept has no impact on the neurophysiological implications (besides the stimulation of the brain's pleasure center, for those who like cheese!) for the second one, a neurological implication cannot be excluded.

During muscular exercise, when glucose consumption is not matched by the oxygen availability, lactate is produced and the excess is released into the blood flow. Following the

blood circulation, it reaches the heart, liver, and other inactive muscles, where it is either metabolized or reconverted into glucose.

The serum concentration at rest is 1-2 mM/L, but during strong muscular activity, such as in competitive sports, it can reach to 30 mM/L (Dalsgaard, 2006).

Lactate transport across the blood–brain barrier is facilitated by the MCT1s that have a K_m of 3.5 mM (Pierre and Pellerin, 2005). Together with this information and the fact that lactate is an energy substrate, lactate might be considered during vigorous activity as an extra energetic stimulus for the brain.

1.3.2 D-lactate

The tridimensional atomic structure of lactate is chiral and has two enantiomers, the L- and D-lactate. In humans, both stereoisomers are produced during normal metabolism with different processes. As we already review, L-lactate is obtained from the conversion of pyruvate, via the enzyme L-lactic acid dehydrogenase whereas D-lactate is produced from methylglyoxal via the glyoxalase pathway.

Small amounts of methylglyoxal are produced from the catabolism of carbohydrates, fats, and proteins. Due to its reactive and toxic nature, it must be eliminated from the body (Kalapos, 1999). The glyoxalase pathway is a biochemical process that catalyses the conversion of methylglyoxal to D-lactate and glutathione in two enzymatic steps, using the glyoxalase I and glyoxalase II (Belanger et al., 2011). This enzymatic pathway, ubiquitously present in the body including the brain, is active in the cell cytosol and in organelles, especially the mitochondria (Thornalley, 1990).

In the normal serum, lactate composition is considered to be entirely L-lactate, with the exception of nanomolar concentration of D-lactate (McLellan et al., 1992). D-lactate is thought to be poorly metabolized by neuron because they lack the appropriate enzymes to convert it into energy (Belanger et al., 2011). As done in several other neural studies, in our

work we have used D-lactate to substitute L-lactate to separate the metabolic implications of lactate.

1.3.3 Lactate and hemodynamic regulation

The neuronal activity is followed by an increase in the cerebral blood flow, which delivers glucose and oxygen to the neural tissue. Although this neurovascular coupling was found more than 100 years ago, the intrinsic mechanisms are not yet definitely established (Attwell and Iadecola, 2002). Different theories were proposed to explain how the vascular supply can be adjusted in correspondence with the neural activity. One proposal is that the regional blood flow is regulated by a mechanism that is directly sensitive to the concentration of the metabolic by-products: CO_2, the final product of the glycolysis; K^+, released during action potentials; nitric oxide and arachidonic acid, both neuronal signaling molecules, are all well-known vasoactive compounds that can be candidate to influence the blood flow. However, this possibility was questioned (Attwell and Iadecola, 2002), based on the fact that the blood flow response after neural activation is typically delayed by 1-2 seconds (Raichle and Mintun, 2006) and that most of the energy consumed for synaptic transmission is used at the postsynaptic level (Attwell and Laughlin, 2001). Thus, the metabolic processes at this level are not fast enough to match the fast response of the neurovascular coupling.

Another theory is that neurons control the blood flow by a dedicated neuronal circuit. In the cerebral cortex, there are neurons that directly innervate the smooth muscle cells around capillaries and induce vasoconstriction (Attwell and Iadecola, 2002).

Finally, the last proposal is that glutamate, released during synaptic transmission, induces an intracellular signaling in another cell that can control the local blood flow (Lauritzen, 2005). Different studies have suggested that astrocytes can be involved in the neurovascular coupling (Raichle and Mintun, 2006). With their strategic position between neurons and capillaries, they can be considered as perfect candidates to carry a signal from neurons to

vasculature. We have seen that in the neurometabolic coupling, astrocytes can respond to glutamatergic activity with a production and release of lactate. In the retina, the delivery of lactate dynamically alters the vascular tone of capillaries (Hein et al., 2006). In addition it has been shown in an experiment, performed in rat brain slices, that the direct stimulation of astrocytic glycolysis induced a contraction of arterioles, via lactate release (Gordon et al., 2008).

The neurovascular coupling is of prime importance for normal brain function. It appears that multiple mechanisms are involved in precisely tuning the local cerebral blood flow.

1.3.4 Lactate involvement in glucose sensing

A few studies have indicated that lactate could modify the neuronal excitability in selected neuron types (Song and Routh, 2005, Shimizu et al., 2007). These findings constituted the starting point of the main study of my thesis about the effects of lactate on neuronal excitability. This chapter presents the most relevant information about the possible mechanisms that could be involved in the neuronal lactate sensitivity.

The availability of energy substrates, in the brain, is of prime importance to ensure the physiological activity and integrity, *i.e.* even a transient deprivation can lead to cerebral damage. Since glucose is the most important source of ATP, the brain is equipped with efficient glucose sensors. These elements are able to monitor and control the glucose concentration to ensure that the brain has an adequate level of energy substrates. Glucose sensors are specialized neurons that can change their firing rate in function of glucose fluctuations (Song et al., 2001). In general, they are located in specific brain regions related to food intake and glucose homeostasis, such as hypothalamus and brainstem (Ainscow et al., 2002, Balfour et al., 2006). Two kinds of these cells exist: the glucose-excited (or glucose-responsive) and glucose-inhibited (or glucose-sensitive) neurons. As their name

suggests, they increase or decrease their firing rate in response of glucose concentration changes.

Because of the importance of lactate as energy source, it is possible that, similar mechanisms of sensing would also be available for it. For this reason, we need to go somewhat deeper in describing intracellular mechanisms that drive the changes in the glucose sensing neurons.

In glucose-excited neurons, the sensing system is reminiscent of the glucose-dependent insulin release of pancreatic *β*-cells. It is essentially based on special K^+ channels sensitive to the ATP/ADP ratio, called K_{ATP} channels. The glucose-excited model is represented in **fig. 9**. Briefly: glucose enters neurons via glucose transporters (GLUT), where it is metabolized and gives a rise in ATP. This increase in the ATP/ADP ratio triggers the closure of the K_{ATP} channels. The subsequent depolarization of the membrane stimulates the electrical activity (Routh, 2002).

The mechanism is less clear in glucose-inhibited neurons. Among several models proposed to explain the glucose inhibition, two are represented in **fig. 9**. In the first one, an increase in glucose leads to an enhancement of ATP production leading to a decrease of the AMP/ATP ratio. This deactivates the AMP-activated protein kinase that normally induces the closure of Cl^- channels, presumably of the cystic fibrosis transmembrane regulator (CFTR) family. This results in an opening of the Cl^- channel inducing a hyperpolarization of the membrane (Murphy et al., 2009) and inhibition of neuronal firing.

In the second one, an ATP independent mechanism has been proposed. Glucose could bind to an unknown receptor. Its activation leads to the opening of a K^+ leak channel that hyperpolarizes the neuron reducing its excitability (Gonzalez et al., 2008).

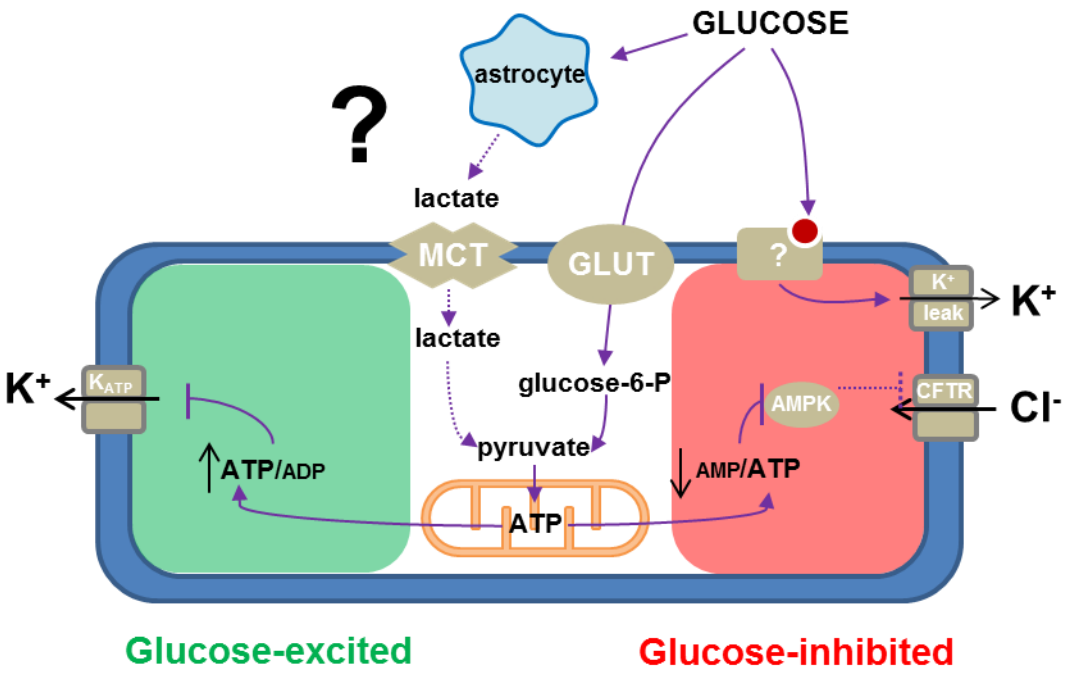

Figure 9: Glucose-sensing mechanisms in neurons. The left part (green) represents mechanisms involved in glucose-excited neurons. The right part (red) depicts some commonly proposed mechanisms for glucose-inhibited neurons. In addition, a possible connection to lactate has been introduced. Lactate, taken up intracellularly, may be transformed into pyruvate, an intermediate step of the mechanisms mediating the glucose sensitivity.

Thus, the ways to alter the neuronal excitability are divided into ATP dependent or independent mechanisms. Lactate, with its demonstrated ability to impact ATP, should be a suitable substrate able to influence the neuronal activity with similar mechanisms. This topic constituted the main project of my thesis and the main questions that we asked were if lactate application in primary cortical neurons could influence their excitability. If it were the case, we need to determine whether it is due to an ATP dependent or independent mechanism. In addition, it was reported that K_{ATP} channel are preferentially expressed by GABAergic interneurons (Zawar et al., 1999). For this reason we also investigated if there were any differences in lactate sensitivity between glutamatergic and GABAergic neurons.

The answers to these questions are discussed in the results section of this manuscript.

2 Results

2.1 Preamble

The main goal of my research was to obtain detailed information on the metabolic and functional interplay between neurons and astrocytes, with a special attention given to the astrocytic input and output elements involved in neurometabolic coupling.

The present result section, that constituted my thesis work, is composed of two parts.

In the first part, we investigated if energy metabolites, in particular lactate, could influence the spontaneous electrical activity of cultured cortical neurons. The study constituted the main subject of my thesis work. This section is composed of results that were recently published (Bozzo L, Puyal J, Chatton JY (2013). Plos One, in press).

In the second part we focused on the characterization of a new pharmacological tool, the high affinity glutamate transporter inhibitor TFB-TBOA that could be useful to study glutamate transport and neuron-glia interactions. This work has been published in Brain Research (Bozzo & Chatton. Brain Res 2010).

The two studies are independent and do not need to be read in particular sequence.

2.2 Lactate modulates the activity of primary cortical neurons through a receptor-mediated pathway

2.2.1 Aim of the study

In the neurometabolic coupling lactate is released form astrocytes in response to glutamatergic activity. The principal function of lactate is to sustain the neuronal metabolism. Whether lactate can have a neuromodulatory function, beside energy substrate, it is not known. The aim of this study was to investigate the influence of lactate on neurotransmission.

2.2.2 Personal contribution

This study is co-authored by Jean-Yves Chatton and Julien Puyal. I performed and analyzed all the experiments in this study except for the Western blot experiment. Moreover, I substantially contributed in the experimental design and wrote the manuscript together with Jean-Yves Chatton.

Lactate modulates the activity of primary cortical neurons through a receptor-mediated pathway

by

Luigi Bozzo[1], Julien Puyal[1], and Jean-Yves Chatton[1,2*]

Abstract

Lactate is increasingly accepted as a major energy substrate of the brain. Beside its metabolic effects, it may have others roles. Here, we demonstrate that lactate can behave like a volume transmitter able to influence the neuronal activity. Neuronal excitability of mouse primary cortical neurons was monitored by calcium imaging. When applied in conjunction with glucose, lactate induced a decrease of the spontaneous calcium spiking frequency of neurons. The effect was reversible and concentration dependent (IC_{50} ~4.2mM). To test whether lactate effects are dependent on metabolism, we applied the closely related substrate pyruvate (5mM) or switched to different glucose concentrations (0.5 or 10mM). None of these conditions reproduced the effect of lactate. In addition, the neuronal activity was also decreased by the stereoisomer D-lactate in the same concentration range as L-lactate (IC_{50} ~4.6mM). We determined that D-lactate, poorly metabolized by neurons, was taken up by neurons, however more than two-fold less efficiently than L-lactate. Recently, a G_i protein-coupled receptor for lactate called HCA1 has been introduced. To test if this receptor is implicated in the lactate sensitivity, we incubated cells with pertussis toxin (PTX) an inhibitor of G_i-protein. PTX prevented the decrease of neuronal activity by L-lactate. Moreover 3,5-dyhydroxybenzoic acid reported to be a specific agonist of the receptor, modulated the neuronal activity in the same manner as lactate. This study indicates that lactate modulates neuronal activity by a receptor-mediated mechanism, independent from its metabolism.

Introduction

The regulation of adequate energy supply is of prime importance for normal brain function. For this reason, the brain is equipped with efficient systems to sense and regulate the concentration of key energy substrates both centrally and peripherally. Lactate is increasingly accepted as a major energy substrate of the brain (Rouach et al., 2008; Wyss et al., 2011). A few studies have documented that lactate can influence the excitability of selected neurons via different metabolic pathways. In glucose-sensing neurons of the ventromedial hypothalamic nucleus (VHN), lactate was found to stimulate the action potential firing frequency (Song and Routh, 2005). In the subfornical organ, center for the control of salt-intake behavior, the firing of GABAergic neurons is regulated by lactate (Shimizu et al., 2007). Lactate and glucose can share common mechanisms for neuronal modulation. Both molecules lead to the production of mitochondrial ATP, which influences selected membrane conductances such as K_{ATP} channels in glucose-excited neurons (Song et al., 2001; Evans et al., 2004), or hyperpolarizing chloride channels in glucose-inhibited neurons (Murphy et al., 2009). Studies have also demonstrated that, in some neurons, lactate and glucose effects are dissociated, such as in VHN glucose-inhibited neurons, where they have opposite effects (Song and Routh, 2005), or in orexin neurons, where only lactate influences the firing frequency (Parsons and Hirasawa, 2010). It is therefore conceivable that energy substrate-sensing systems are able to discriminate between different substrates, or that glucose and lactate do not encompass identical functions.

Recent studies in glucose-excited (Ainscow et al., 2002) and glucose-inhibited neurons (Venner et al., 2011) have found that glucose sensitivity is not always mediated by intracellular variations of ATP. It has even been proposed that a membrane receptor for glucose underlie its effects in glucose-inhibited neurons (Gonzalez et al., 2008).

The composition of extracellular fluid in glucose and lactate differs depending on the brain region. In the hippocampus, lactate concentration was found to be higher than that of glucose and even twice as high in the cortex (Zilberter et al., 2010).

These considerations brought up the question of whether neurons of the cortex can also selectively sense or respond to lactate. This would confer additional roles on lactate such as that of a signaling molecule of metabolic states of the brain, as has been recently proposed (Bergersen and Gjedde, 2012). In support of this hypothesis, a family of G-coupled receptors has been recently identified (Blad et al., 2011) and called hydroxycarboxylic acid receptor (HCA, formerly named GPR81). Among them, HCA1 is considered to be a sensor for lactate in peripheral organs such as the adipose tissue (Cai et al., 2008; Liu et al., 2009). The potential involvement of lactate receptors has to be considered in the lactate sensitivity of neurons in the brain.

To explore these aspects, we investigated the influence of lactate application on the spiking output of mouse primary cortical neurons using rapid calcium imaging. Our results show that lactate can modulate neuronal network activity likely through receptor-mediated mechanisms.

Materials and Methods

Cell culture

All the procedure used to prepare living cells have been approved by the Swiss legislation and follows their guidelines. Mouse cortical neurons in primary cultures were obtained from 17-day-old GAD67 EGFP knock-in C57bl6 or wild type C57bl6 mouse embryos. After removing meninges, entire cortices were first incubated with 180 U/ml trypsin for 20 min at 37°C and then mechanically dissociated in Neurobasal (Invitrogen, Basel, Switzerland) culture medium plus 10% FCS by successive aspiration through sterile glass pipettes. The dissociated cells were filtered using a cell strainer with 40µm nylon mesh and re-suspended in Neurobasal culture medium complemented with 2% B27 and 500 µM glutamax (Invitrogen). Cells were then plated at a density of 20,000 cells per cm^2 on glass coverslips coated with poly-D-lysine and laminin (Invitrogen). Cells were used, for all kind of experiments, at DIV 14-21.

Live microscopy

Experiments were carried out on the stage of an upright epifluorescence microscope (Nikon, Tokyo, Japan) using a 40 × 0.8 N.A. water-immersion objective lens (Nikon). Fluorescence excitation wavelengths were selected using a fast filter wheel (Sutter Instr., Novato, CA) and fluorescence was detected using an Evolve EMCCD camera (Photometrics, Tucson, AZ). Digital image acquisition as well as time series were computer-controlled using the software Metafluor (Universal Imaging, West Chester, PA, USA). Up to 8 individual neurons were simultaneously analyzed in the selected field of view.

pH Measurements

Intracellular pH (pH_i) was measured in single cells on glass coverslips after loading the cells with the pH sensitive fluorescent dye 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF-AM; Teflabs, Austin, TX) as described previously (Chatton et al., 2001). Cell loading was

performed at room temperature for 10min using 1μM BCECF-AM in a HEPES-buffered balanced solution (see composition below). Fluorescence was sequentially excited at 440 and 490nm and detected through a 535nm (35nm bandwidth) emission interference filter. Fluorescence excitation ratios (F490nm/F440nm) were computed for each image pixel and produced ratio images of cells that were proportional with pH_i. In situ calibration was performed after each experiment using a nigericin technique as described before (Chatton et al., 2001).

Calcium Measurements

Intracellular calcium was measured using the indicator Fluo-4 AM 5μM (Teflabs Austin, TX) loaded for 15 min at 37°C. Experiments were performed in CO_2/bicarbonate-buffered solutions (see composition below). Fluorescence was excited at 490nm and detected at >515nm. Acquisition rate of images was varied between 10 and 0.1 Hz to avoid photobleaching. Fluorescence intensity was measured in regions of interest delineating the neuronal soma using Metafluor. Subsequently calcium transient extraction was performed using Minianalysis 6.0.3 (Synaptosoft Inc). The software includes an algorithm for the detection of complex and multiple events giving the possibility to detect overlapping or closely occurring peaks.

In a subset of experiments, to distinguish principal and GABAergic neurons, we used cultures obtained from GAD67 EGFP knock-in mouse. Because green fluorescent protein (GFP) expressed by GABAergic cells and the calcium fluorescent dye Fluo-4 AM have overlapping excitation and emission spectra, we elaborated a strategy to distinguish them. The microscope was equipped with a motorized XY moving stage (Sutter) driven by custom-made software that allowed us to rapidly switch between selected XY positions. Before cells loading, a series of images were recorded in different fields of view in the same culture and their coordinates were stored. Cells were subsequentially loaded with the Fluo-4 AM. By

superimposing images at same XY positions, we were able to distinguish GFP positive and negative cells loaded with Fluo-4.

Electrophysiological recordings

Patch-clamp recordings were made with borosilicate glass pipettes with a resistance of 5.5–8 MΩ. The pipette solution contained (in mM): K-gluconate 130, NaCl 5, Na-phosphocreatine 10, $MgCl_2$ 1, EGTA 0.02, HEPES 10, Mg-ATP 2, and Na_3-GTP 0.5, pH 7.3 (adjusted with KOH).Recordings were made with an Multiclamp 700B amplifier (Molecular Devices). Data were acquired with a Digidata 1440A (Molecular Devices), at 10kHz sampling rate, controlled with Pclamp 10 software and analyzed with Clampfit software (Molecular Devices). A period of 5 min was routinely allowed after establishment of the whole-cell configuration. Experiments were performed using an open perfusion chamber. Control extracellular solutions and solutions containing the tested drugs were gravity fed at 600µl/min and at 35°C on the cultured cells.

Solutions

CO_2/bicarbonate-buffered experimental solutions contained (mM): NaCl 135, KCl 5.4, $NaHCO_3$ 25, $CaCl_2$ 1.3, $MgSO_4$ 0.8, NaH_2PO_4 0.78, glucose 5, bubbled with 5% CO_2/95% air. Glucose 5mM was maintained in all solutions (unless otherwise specified). HEPES-buffered solutions contained (mM): NaCl 160, KCl 5.4, HEPES 20, $CaCl_2$ 1.3, $MgSO_4$ 0.8, NaH_2PO_4 0.78, glucose 5, pH 7,3. For dye loading, this saline solution was supplemented with 0.1% Pluronic F127 (Molecular Probes, Eugene; OR) and glucose was increased to 20mM. pH calibration solutions contained (mM): NaCl 20, KCl 120, HEPES 10, $CaCl_2$ 1.3, $MgSO_4$ 0.8, and NaH_2PO_4 0.78 and were adjusted to their respective pH by addition of NaOH.

Pertussis toxin was from Tocris Bioscience (Zurich). Unless otherwise stated, all other compounds were from Sigma-Aldrich (Buchs, Switzerland).

Immunocytochemistry

Primary mouse cortical cultures grown on coverslips were fixed with 4% paraformaldehyde in phosphate-buffered solution (PBS) for 15 minutes on ice. Cells were pre-incubated in PBS containing 15% serum and 0.05% Triton X-100 and subsequently incubated overnight with the primary mouse anti-NeuN antibody (1:200, Millipore, Temecula, CA, USA) and rabbit anti-Gpr81 (Gpr81 is also known as HCA1) (1:100, GPR81-S-296, Sigma). Cells were washed in PBS and incubated with the appropriate secondary antibodies (Alexa Fluor 488-conjugated donkey anti-mouse IgG and Alexa Fluor 594-conjugated donkey anti-rabbit IgG (Invitrogen). Negative controls were performed in the absence of primary antibodies. Coverslips were mounted in Fluorsave mounting medium (Calbiochem) and analyzed using a Leica SP5 confocal microscope and a 63x PlanApochromat objective lens with fluorescence excitation at 488nm and 543nm.

Western blot

Western blot was performed as described previously (Grishchuk et al., 2011). Briefly, protein samples were harvested in lysis buffer (20 mmol/L HEPES, pH 7.4, 10mM NaCl, 3mM $MgCl_2$, 2.5mM EGTA, 0.1mM dithiothreitol, 50mM NaF, 1mM Na_3VO_4, 1% Triton X-100), and a protease inhibitor cocktail (Roche, 11873580001). Lysates were sonicated and protein concentration was determined using a Bradford assay. Proteins (25µg) were separated by SDS-PAGE on a 12% polyacrylamide gel, incubated with an anti-Grp81-s296 primary antibody (Sigma) and then with a polyclonal goat anti-rabbit IgG conjugated with IRDye 800 (LI-COR, 926-32210). Protein bands were visualized using the Odyssey Infrared Imaging System (LI-COR).

Data analysis

Data are means ± SEM and are represented as percentage of spiking activity or ΔpH change measured during the control condition. Paired Student's t-tests were performed to assess the statistical significance in the same experiments (*P < 0.05). In experiments, were groups were compared non-paired Student's t-test was used. The half-maximum inhibitory concentration (IC_{50}) of L- or D- lactate was determined by non-linear curve fitting using the Levenberg–Marquardt algorithm implemented in the Kaleidagraph software package (Synergy Software, Reading, PA, USA). The concentration-response analysis experiments were fitted using the following equation:

$$R_{obs}=R_{max}[I]/(K+[I])+R_{min} \quad (1)$$

where R_{obs} is the observed response and R_{max}, R_{min} are maximum and minimum parameters of the response. [I] is the concentration of the inhibitor compound and K is the concentration that yields its half-maximum inhibition (*i.e.* IC_{50}).

Results

L-lactate influences the calcium transient frequency in the presence of glucose

The electrical activity of primary cortical neurons was monitored by rapid calcium imaging. We took advantage of the fact that the membrane depolarization that accompanies action potentials leads to an intracellular increase of calcium concentration via the opening of voltage gated calcium channels (Cossart et al., 2005; Sasaki et al., 2008). During the time window of utilization of cells (DIV14-21), spontaneous calcium transients were detected in more than 50% of neurons. To assess to what extent the calcium transients correlate with action potentials in these cells we performed simultaneous recordings in patch clamp and somatic calcium fluorescence. **Fig. 1** shows in parallel example traces of the spiking output recorded in whole-cell current clamp configuration and the corresponding intracellular variation of fluorescence that reflects the calcium variation. Careful visual inspection comparison of electrophysiological and optic recordings indicates an excellent match between both kinds of signals. The main advantage of the calcium imaging method for this study is that it allows monitoring a large number of cells in parallel and avoids altering the cellular solute composition. We therefore used calcium imaging in the following experiments as the main method for monitoring the electrical activity of neuronal population.

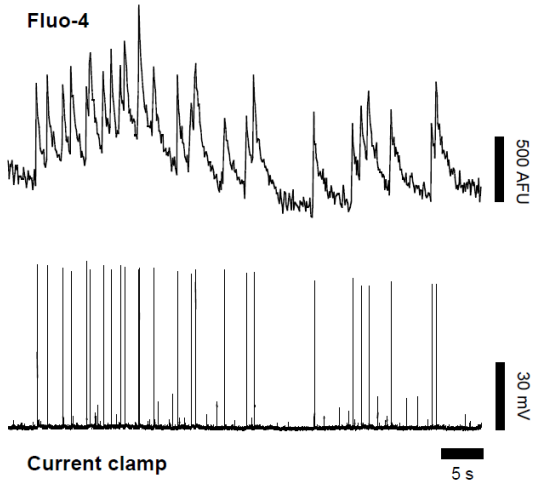

Figure 1. Neuronal activity monitored with calcium imaging
Comparison between simultaneous intracellular calcium imaging sampled at a frame rate of 10Hz and whole-cell patch clamp recordings. A representative experiment out of 15 is shown with the upper trace representing calcium transients (arbitrary fluorescence units, AFU) and lower trace action potentials recorded in current-clamp configuration from the same neuron.

In the context of neurometabolic coupling, lactate is generally considered as an energy source for activated neurons. Recent studies have indicated that in addition lactate could play a role as neuromodulator of certain glutamatergic and GABAergic neurons (Ainscow et al., 2002; Song and Routh, 2005; Shimizu et al., 2007). For this reason, we investigated the effect of L-lactate application on primary cortical neurons obtained from wild-type and GAD67 EGFP knock-in mice, which allowed us to distinguish principal from GABAergic neurons. In order to evidence only a modulatory effect of lactate and not its mere ability to sustain neuronal energy metabolism, experiments were carried out in the presence of 5mM glucose. Recordings were obtained from the same target cells first in control solution, then following 5min of L-lactate or other compounds application and ultimately after 5min washout. **Fig. 2a** shows a typical experimental trace of the calcium transients in control or in the presence of L-lactate in a single cortical principal neuron. Application of L-lactate 5mM reversibly diminished the calcium transient frequency by more than 50% in both principal and GABAergic neurons. **Fig. 2b** summarizes the results obtained in this series of experiments.

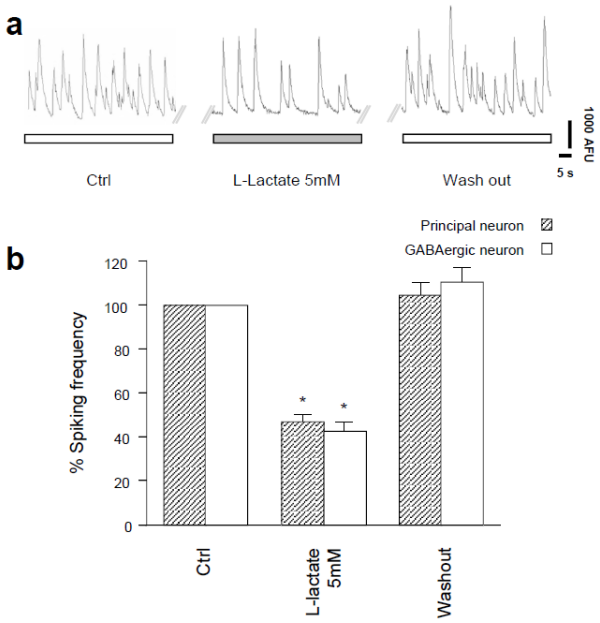

Figure 2. Effects of L-lactate on calcium spiking frequency
(a) Original traces of calcium transients in control or 5mM L-lactate containing solution. (b) Spiking frequency for principal neurons and GABAergic interneurons are shown as percent of activity measured during control solution. Data are obtained from 9 experiments and 35 cells.

In order to understand if the effect was proportional to the concentration, an inhibitory curve of L-lactate was then established **(Fig. 3)**. The graph shows that L-lactate decreased the calcium transient frequency in a concentration dependent manner in both cell types (apparent IC_{50}: principal neurons 4.23±1.9mM; GABAergic neurons 4.18±2.8mM). As the sensitivity to lactate was found identical between principal and GABAergic neurons, cell types were not studied separately in the rest of the study.

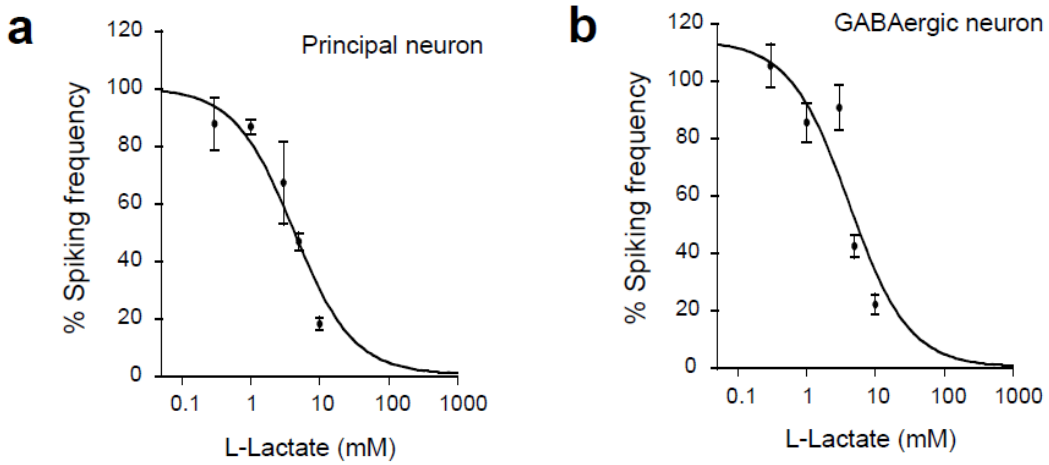

Figure 3. Concentration dependency of L-lactate effects
The decrease in spiking frequency was concentration dependent. Apparent IC_{50} values obtained by nonlinear curve fitting yielded 4.23±1.9mM for principal neurons (n=175 cells, 56 exp) and 4.18±2.8mM for GABAergic neurons (n=83 cells, 35 exp).

Related energy metabolites do not influence neuronal activity

Once inside neurons, L-lactate is converted into pyruvate by lactic dehydrogenase and then can enter the tricarboxylic acid cycle that leads to the production of mitochondrial ATP. The ability of lactate to influence the neuronal activity could arise from the variation of intracellular ATP that influences directly or indirectly membrane conductances such as K_{ATP} or chloride channels We therefore examined whether related energy substrates cause a similar effect as L-lactate. We applied the same experimental protocol using the closely related molecule pyruvate or different concentrations of glucose (0.5 or 10mM). Pyruvate 5mM (in the presence of 5mM glucose) marginally (~7%) influenced the calcium transient frequency **(Fig.**

4a). Glucose at high concentration (10mM) did not replicate the effects of L-lactate, whereas low glucose (0.5mM) tended to somewhat increase the frequency **(Fig. 4b)**. These experiments provided a first indication of the specific nature of lactate effects on neuronal activity.

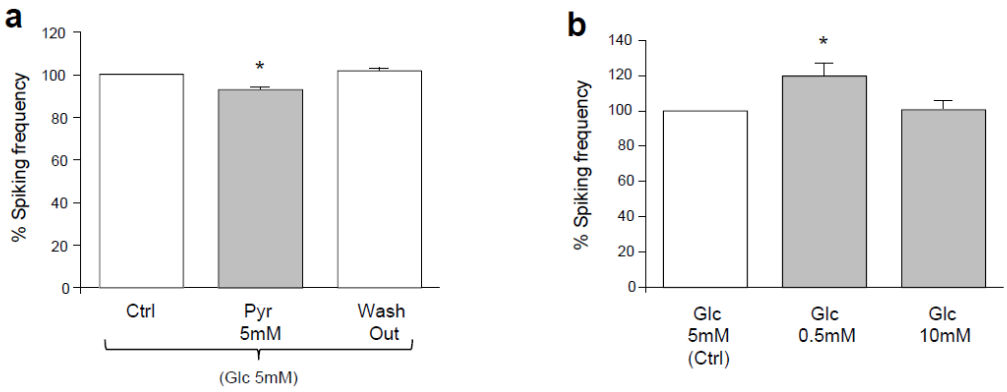

Figure 4. Energy metabolite dependency of calcium spiking frequency
Calcium spikes frequency shown as percent of activity measured during control solution. (a) Effects of pyruvate on calcium spiking frequency (n=188 cells, 24 exp). Glucose (5mM) was present throughout the experiments. (b) Effects of glucose concentration on spiking frequency (n=68 cells, 10 exp).

Effect of the stereoisomer D-lactate

The above results suggest that in our experimental conditions intracellular ATP increase is not involved in the observed modulation of neuronal activity. We further investigated the involvement of metabolism in the lactate effects by applying the stereoisomer D-lactate that is described to be poorly metabolized by neurons (Ewaschuk et al., 2005). **Fig. 5a&b** show that D-lactate application substantially decreased the calcium transient frequency in a reversible manner. This effect was found to be concentration dependent with an IC_{50} of 4.58±1.2mM, *i.e.* approximately the same potency as the L-isomer (see **Fig. 3**).

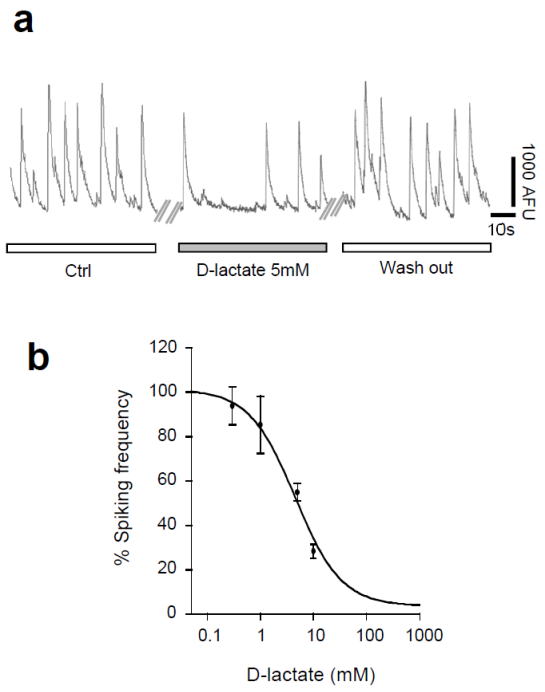

Figure 5. D-lactate effects on neuronal activity
(a) Sample trace of calcium transients in control or 5mM D-lactate containing solution. D-lactate substantially decreased calcium transient frequency. (b) The concentration-response analysis yielded an apparent IC_{50} of 4.58±1.2mM (n=127 cells; 21exp).

Both L-lactate and pyruvate are efficiently transported across the cell membrane of cortical neurons by monocarboxylate transporters (MCTs) (Chatton et al., 2001). It has been reported that neuronal MCTs transport D-lactate less efficiently (Nedergaard and Goldman, 1993). To test to what extent, in our experimental conditions, D-lactate is transported into neurons, we took advantage of the fact that MCTs co-transport lactate with one proton with a stoichiometry of 1:1 (Chatton et al., 2001). The resulting cellular acidification can be used to monitor the transport. The intracellular pH was monitored by loading neurons with the pH sensitive indicator BCECF and we used L-lactate application as control of the transport activity. **Fig. 6** shows that L-lactate application (5mM) resulted in a small and reversible acidification (<-0.1 pH units). The figure shows that the acidification caused by D-lactate was significantly weaker than that caused by L-lactate at the same concentration, indicating that D-lactate is transported less efficiently into neurons than L-lactate as observed before (Nedergaard and Goldman, 1993). These results strengthened the notions that lactate effects on spiking are not solely related to its transport or intracellular metabolism.

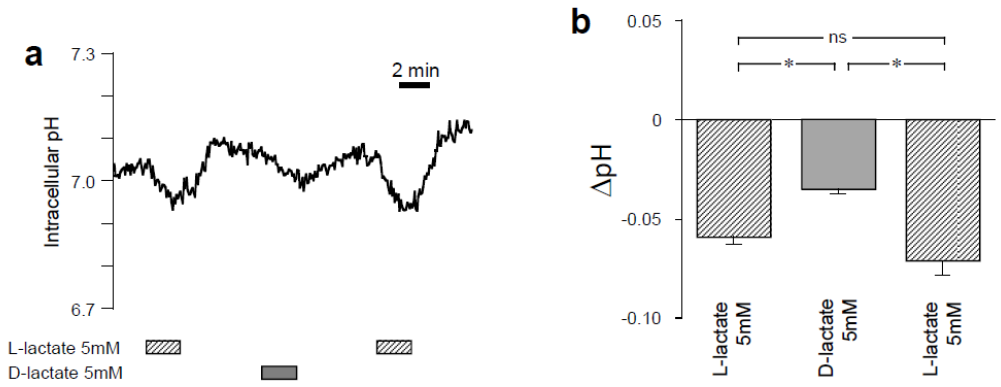

Figure 6. Intracellular pH effects of lactate isomers on cortical neurons
Intracellular pH measured using BCECF and calibrated *in situ* in cortical neurons. (a) Original pH trace during sequences of L- and D-lactate application. (b) Summary of acidification (pH amplitude) measured during L- and D-lactate application. (n=39 cells; 7exp).

Receptor mediated effect of lactate

Recently, a G-protein coupled family of receptors has been identified from a pool of orphan receptors and called hydroxycarboxylic acid receptor (HCA) (Blad et al., 2011). Among them HCA1 (previously known as GPR81) was reported to be activated by lactate in adipocytes (Cai et al., 2008). To determine if this receptor is expressed by mouse primary cortical neurons, we performed an immunohistochemistry analysis using anti-HCA1 antibody. We found that all cells positive for the neuronal marker NeuN show HCA1 immunoreactivity in our primary cortical cultures (**Fig. 7a**). We also verified the antibody specificity and confirm that HCA1 is expressed in mouse cortical neuronal cultures by Western blot (**Fig.7b**).

HCA receptors are reported to be coupled to G_i proteins (Liu et al., 2009). To investigate whether a G_i coupled receptor is implicated in the observed lactate sensitivity we incubated cells with pertussis toxin (PTX), a G_i protein inactivator. Neuronal cultures coming from the same preparation were divided into two equal groups, one used as control and the other incubated with PTX (500ng/ml, 24h). Experiments were performed in parallel on the same day. Importantly, no significant differences in th e spontaneous frequency were found between the two groups (n=123 cells from 16 experiments). In control condition, L-lactate

induced the previously observed reduction of the calcium transient frequency by 46%. However, in the presence of PTX, the effects of lactate were almost abolished (90.8±6% of the initial frequency was maintained, **Fig. 7c**).

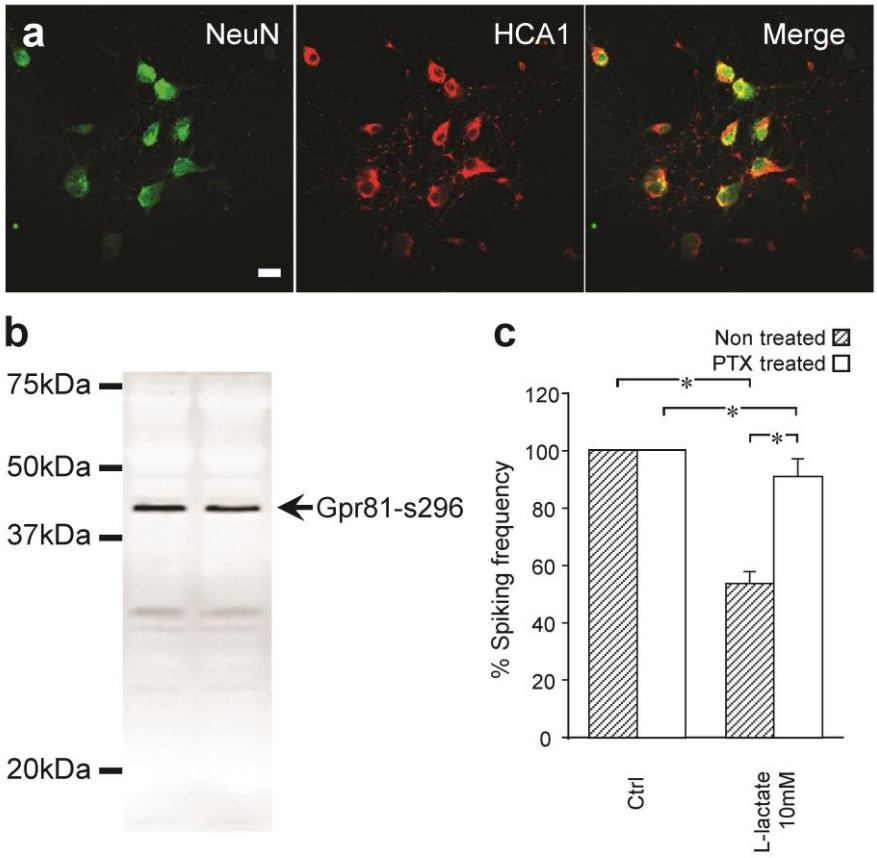

Figure 7. HCA1 receptor involvement in the lactate sensitivity.
(a) Confocal images showing immunostaining for NeuN (green), HCA1 (red) and the merged image in mouse primary cortical neurons. Scale bar, 20µm. (b) Representative Western blot showing that HCA1 is expressed in mouse primary cortical neuronal cultures. (c) Comparison of lactate effect on calcium spiking frequency in cells incubated or not with pertussis toxin (PTX). PTX incubation strongly reduced the effects of lactate on neuronal activity. Data are obtained from 8 experiments and 61 cells for non-treated group and 8 experiments and 62 cells for PTX treated group.

To further investigate the involvement of HCA receptors, we tested the effects of 3,5-dyhydroxybenzoic acid (3,5-DHBA) recently identified as a specific agonist of the lactate receptor HCA1 (Liu et al., 2012) as well as 3-hydroxybenzoic acid (3-HBA) an agonist of HCA1 and HCA2, a receptor highly homologous to HCA1. Both agonists have been reported to have a higher affinity than lactate for these receptors, and were applied at a concentration of 1mM. **Fig. 8** shows that, like L-lactate, both 3,5-DHBA (a) and 3-HBA (b) decreased in a reversible manner the neuronal activity by ~33%.

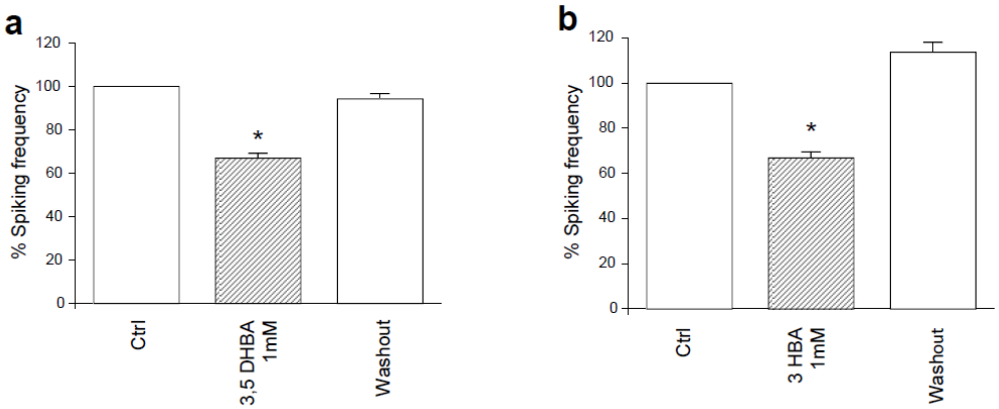

Figure 8. Reduction by 3,5-DHBA and 3-HBA of the calcium transient frequency

Calcium spiking frequency shown as percent of activity measured during control solution. (a) Effects of 3,5-DHBA on calcium spiking frequency (n=155 from 22 experiments). (b) Effects of 3-HBA on spiking frequency (n=10 from 79 experiments).

Discussion

This study shows for the first time that lactate is more than a metabolic substrate for the brain and may be considered as a modulator of cortical neurons. Lactate administration to neurons was found to induce a distinct modulation in a way and magnitude that was not shared by other energy substrates. The observed modulation is driven by a mechanism independent of the production of ATP, likely involving membrane receptors for lactate.

Lactate is produced by aerobic glycolysis in astrocytes and released in register with neuronal activity (Pellerin et al., 2007). It is estimated to be in the low millimolar range in the extracellular fluid (Zilberter et al., 2010). Glucose and lactate are found in different proportions according to brain region, in the hippocampus, lactate concentration was found to be higher than that of glucose and even twice as high in the cortex (Zilberter et al., 2010).

We found that L-lactate application decreased the network activity of neurons in a concentration dependent manner in presence of glucose. Interestingly, modulation of neuronal activity by lactate has been found in GABAergic neurons of the subfornical organ (Shimizu et al., 2007). L-lactate in the concentration range 0-1mM promoted the firing rate whereas in the range 1-10mM the firing rate was progressively suppressed. In that study, the authors highlighted the stimulatory phase that they found to be ATP dependent; however, they did not attempt to explain the suppression of activity by higher lactate concentration, leaving the question open.

Among the different energy substrates used in our study that should bring about a rise in intracellular ATP, L-lactate was the only one able to strongly reduce neuronal firing frequency. High levels of glucose, or pyruvate applied at the same concentration as L-lactate, did not reproduce the effect. It should be noted that pyruvate is not only closely related to lactate but also transported as efficiently in neurons by the same carriers (Chatton et al., 2001). The ability of L-lactate to single-handedly influence neuronal activity was already found in orexin neurons where L-lactate—but not glucose—increased the firing

activity of neurons (Parsons and Hirasawa, 2010). The effects of energy substrates are usually assumed to be mediated by the intracellular variation of ATP. However, the disparity in the effect of these energy substrates led us to consider the possibility that the mechanism involved in the L-lactate sensitivity is not dependent on the levels of ATP produced. A growing body of evidence indicates that mechanisms underlying energy substrate sensitivity are not all dependent on the intracellular ATP concentration changes (Ainscow et al., 2002; Gonzalez et al., 2009). We found that D-lactate, the stereoisomer of lactate that is poorly metabolized by neurons (Flick and Konieczny, 2002), induced the same effect as L-lactate with a very similar IC_{50}. These results suggest that the mechanism of L-lactate sensitivity does not involve cellular energy metabolism.

An interesting parallel can be drawn with a study performed on glucose-inhibited neurons in the lateral hypothalamus (Venner et al., 2011), where only glucose–but not lactate or pyruvate–suppressed the firing activity. Moreover, the non-metabolized glucose analogue 2-deoxyglucose mimicked the effect of glucose, indicating that the glucose-induced hyperpolarization does not require glucose metabolism. In a previous study of the same group on the mechanisms involved in glucose-inhibited neurons (Gonzalez et al., 2008), it was shown that glucose induced a K^+ hyperpolarizing current that was caused only by its extracellular and not intracellular application. To explain this puzzling observation, the authors proposed the involvement of an extracellular glucose receptor.

An alternative mechanism that should be considered for the effects of lactate is intracellular acidification. Lactate is taken up by neuronal MCTs, which co-transport one proton together with lactate, and therefore can bring about cytosolic acidification. In our experiments, the addition of L- or D-lactate (5mM) caused only a minimal acidification of 0.05-0.1 pH units. These pH changes are in agreement with published values of acidification by L-lactate (Chatton et al., 2001) and by D-lactate (Nedergaard and Goldman, 1993) and appear unlikely to affect the spiking activity. The fact that D-lactate decreased neuronal

activity with the same extent and potency than L-lactate but with a two-fold lower acidification indicates that the observed effect is not proportional to the intracellular pH variation. In addition, pyruvate was reported in a previous study on the same cells (Chatton et al., 2001), to induce a larger acidification than L-lactate, whereas on the contrary we found it not to influence neuronal network activity as L-lactate.

Besides being poorly metabolized, D-lactate is less internalized in neurons than L-lactate, which was demonstrated by the lower acidification induced by the monocarboxylate transporter activation. This is consistent with the reported lower affinity of MCTs for D-lactate compared to L-lactate (Nedergaard and Goldman, 1993; Poole and Halestrap, 1993). Inasmuch as both isomers reduced the frequency of spiking to the same extent and with the same potency, it is plausible that L-and D-lactate do not need to enter neurons to induce their effects nor rely on MCT activity, and therefore act as an extracellular ligand.

Recently, a new class of G_i protein-coupled receptors has been identified (Blad et al., 2011) with affinity for several intermediates of energy metabolism. The ligands being all hydroxyl-carboxylic acids (HCA), these receptors have been named HCA receptors. Of particular interest for the present study, the HCA1 isoform (previously known as GPR81) is described as a receptor for lactate with half-maximal affinity of 4.8mM (Liu et al., 2009), very close to our measured IC_{50} value of 4.2mM. The receptor is predominantly expressed in adipose tissue (Liu et al., 2009), but we found that it is also expressed in primary cortical neurons. This finding is in agreement with evidence from *in situ* hybridization that shows HCA1 mRNA expression in neurons in different regions of the brain such as cortex, hippocampus and cerebellum (Bergersen and Gjedde, 2012). To test if such a receptor is implicated in the lactate sensitivity of neurons, we used 3,5-DHBA a specific agonist for the receptor recently discovered (Liu et al., 2012). We found that the application of this agonist decreased the calcium transient frequency by 33% at a concentration of 1mM. In addition, the application of 3-HBA an agonist that has a similar affinity for HCA1 but in addition can

also bind to HCA2 receptor, highly homologous to HCA1, produced the same intensity of effect at equal concentration. These compounds induced a stronger inhibition of activity than lactate at the same concentration, which is consistent with their reported higher affinity for the receptor. Also, these receptors do not bind pyruvate, consistent with our observed lack of effect of pyruvate on spiking activity (Cai et al., 2008; Liu et al., 2009). Another indication of their involvement, in absence of a specific inhibitor currently available, came from PTX experiments. This inhibitor of G_i proteins almost abolished the decrease of neuronal network activity caused by L-lactate without altering the basal rate of spontaneous spiking activity. Taken together, this body of evidence strongly points to the involvement of HCA receptors in the described lactate sensitivity of neurons. Lactate binding to these G_i-coupled receptors reduces the formation of cAMP via inhibition of the adenylate cyclase. A possible downstream effect of decreased cAMP is the reduction of exocytosis via a protein kinase A dependent pathway (Seino and Shibasaki, 2005). Another possible effector for the inhibition is based on the activation of the associated $G_{\beta\alpha}$ subunits that could induce an hyperpolarization by the opening of K^+ conductances or reduce the exocytosis as was reported for the activation of $GABA_B$ receptor, another G_i protein-coupled receptor (Bettler et al., 2004).

The inhibitory activity of lactate could play several roles in the regulation of neuronal activity, and act as a paracrine element that prevents an excess of activity of neurons. In the context of neurometabolic coupling, glutamate released by neurons during activity stimulates the production and the release of lactate in astrocytes (Pellerin et al., 2007). Lactate can be used by neurons to sustain the metabolic activity (Bouzier-Sore et al., 2003). In case of excessive activity, *e.g.* as it occurs during epileptic seizures, the increased levels of lactate may have the beneficial effects of calming down the network. In support of this hypothesis, it has been demonstrated that L-lactate reduced the size of lesion induced by glutamate in rat cortex (Ros et al., 2001). Lactate application has been investigated in several brain

disturbances and induced variable degrees of benefit, *e.g.* in cerebral ischemia (Schurr et al., 2001; Berthet et al., 2009; Berthet et al., 2012), hypoxia (Schurr et al., 1988; Schurr et al., 1997), traumatic brain injury (Alessandri et al., 2012) and hypoglycemia (Maran et al., 1994). It is plausible that the new neuromodulatory role of lactate described in the present study could underlie some of its positive effects.

In conclusion, the results of this study allow us to propose a new role of lactate, besides its role as metabolic substrate, as a cellular signaling element. This signal could act as a metabolic feedback control of neuronal activity.

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2.3 Inhibitory effects of (2S,3S)-3-[3-[4-(trifluoromethyl)benzoylamino]benzyloxy] aspartate (TFB-TBOA) on the astrocytic sodium responses to glutamate

2.3.1 Aim of the study

Removal of neurotransmitters from the extracellular space is crucial for normal functioning of the central nervous system. This task is mainly managed by astrocytes that are equipped with efficient glutamate transporters. In this study the effects of a recently synthetized inhibitor of glutamate transport, the TFB-TBOA, were characterized in astrocytes maintained in culture.

2.3.2 Personal contribution

This study is co-authored by Jean-Yves Chatton. I performed and analyzed all the experiments in this study. Moreover I substantially participated to the experimental design, as well as to the redaction of the manuscript with Jean-Yves Chatton.

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Research Report

Inhibitory effects of (2S, 3S)-3-[3-[4-(trifluoromethyl) benzoylamino]benzyloxy]aspartate (TFB-TBOA) on the astrocytic sodium responses to glutamate

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ABSTRACT

Astrocytes are responsible for the majority of the clearance of extracellular glutamate released during neuronal activity. DL-*threo*-β-benzyloxyaspartate (TBOA) is extensively used as inhibitor of glutamate transport activity, but suffers from relatively low affinity for the transporter. Here, we characterized the effects of (2S, 3S)-3-[3-[4-(trifluoromethyl) benzoylamino]benzyloxy]aspartate (TFB-TBOA), a recently developed inhibitor of the glutamate transporter on mouse cortical astrocytes in primary culture. The glial Na^+-glutamate transport system is very efficient and its activation by glutamate causes rapid intracellular Na^+ concentration (Na^+_i) changes that enable real time monitoring of transporter activity. Na^+_i was monitored by fluorescence microscopy in single astrocytes using the fluorescent Na^+-sensitive probe sodium-binding benzofuran isophtalate. When applied alone, TFB-TBOA, at a concentration of 1 μM, caused small alterations of Na^+_i. TFB-TBOA inhibited the Na^+_i response evoked by 200 μM glutamate in a concentration-dependent manner with IC_{50} value of 43±9 nM, as measured on the amplitude of the Na^+_i response. The maximum inhibition of glutamate-evoked Na^+_i increase by TFB-TBOA was >80%, but was only partly reversible. The residual response persisted in the presence of the AMPA/kainate receptor antagonist CNQX. TFB-TBOA also efficiently inhibited Na^+_i elevations caused by the application of D-aspartate, a transporter substrate that does not activate non-NMDA ionotropic receptors. TFB-TBOA was found not to influence the membrane properties of cultured cortical neurons recorded in whole-cell patch clamp. Thus, TFB-TBOA, with its high potency and its apparent lack of neuronal effects, appears to be one of the most useful pharmacological tools available so far for studying glial glutamate transporters.

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Abbreviations: GLT-1, glutamate transporter 1; GLAST, glutamate-aspartate transporter; TBOA, DL-threo-β-benzyloxyaspartate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; I-V, current–voltage; MCPG, (S)-α-Methyl-4-carboxyphenylglycine; EAAT, excitatory amino acid transporter

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1. Introduction

By rapidly taking up extracellular glutamate, astroglial cells play the critical role of protecting neurons from the excitotoxic buildup of glutamate, thereby ensuring the fidelity of glutamatergic transmission at high frequency (Danbolt, 2001). Astrocytic glutamate uptake also plays an important role in the coupling between synaptic activity and glucose utilization, i.e. neurometabolic coupling (Pellerin et al., 2007). Astrocytes are equipped with efficient glutamate transporters that surround the synaptic cleft and use the electrochemical gradient of Na^+ to take up glutamate against its electrochemical gradient. In these cells, the Na^+-coupled glutamate transport system is so efficient that the astrocytic intracellular Na^+ concentration (Na^+_i) undergoes rapid and large elevations when glutamate is applied extracellularly, even at low micromolar concentration (Chatton et al., 2000). The bulk of glutamate uptake in the adult brain is mediated by astroglia that express mostly the excitatory amino acid transporter (EAAT) isoforms 1 and 2, whereas EAAT3 and EAAT4 are considered to be neuronal transporters, and EAAT5 is retina-specific (Danbolt, 2001).

Inhibitors of Na^+-dependent glutamate transporters are therefore invaluable tools for elucidating the physiological roles of these transporters in detail. Earlier inhibitors of glutamate transporters, such as *threo*-β-hydroxyaspartate (THA) and *trans*-pyrrolidine-2,4-dicarboxylic acid (t-PDC), are transported competitive inhibitors that lead to astrocytic coupled Na^+ influx (Chatton et al., 2001). A more recently synthesized compound, DL-*threo*-β-benzyloxyaspartate (TBOA), has been introduced and since then widely used as a non-transported competitive inhibitor of glutamate transporters (Shimamoto et al., 1998). This compound is a non-selective inhibitor of all EAATs subtypes with activity in the micromolar range. While this compound was a real breakthrough for the study of glutamate transport, its relatively low potency imposes the use of fairly high concentrations, increasing the risk of unwanted effects in particular when used *in situ* (Bernardinelli and Chatton, 2008), or when *in vivo* use is envisaged for instance as a possible treatment for mood disorder pathologies (Lee et al., 2007; Sanacora et al., 2003).

Recently, a series of analogues of TBOA have been reported with both improved potency and selectivity (Shimamoto et al., 2004). Among them, the most promising one appears to be TFB-TBOA with nanomolar affinity for EAATs. In the present study, we characterized the effects of TFB-TBOA on the Na^+_i response to glutamate in primary mouse astrocytes and on the electrical properties of pure cortical neurons in primary culture.

2. Results

Glutamate evokes a robust elevation of Na^+_i in mouse astrocytes in primary culture (Chatton et al., 2000) that is primarily due to Na^+/glutamate cotransport activity. The first set of experiments was aimed at determining whether the new inhibitor of the glutamate transporter TFB-TBOA applied alone influenced baseline Na^+_i levels and at characterizing its inhibitory properties on the Na^+_i responses to glutamate application.

Fig. 1A shows an original experimental trace of the Na^+_i response to 200 μM glutamate superfusion in a single astrocyte. In these experiments, Na^+_i went from a typical resting value of 12 to 33 mM. The baseline Na^+_i as well as the amplitude and kinetics of the response to glutamate in this series of experiments corresponds to what was described in previous studies (Chatton et al., 2000).

TFB-TBOA 1 μM was then applied alone leading to a small change in Na^+_i. When co-applied with glutamate, TFB-TBOA inhibited 83±1% of the glutamate response. After washout of TFB-TBOA, the Na^+_i response to glutamate was restored

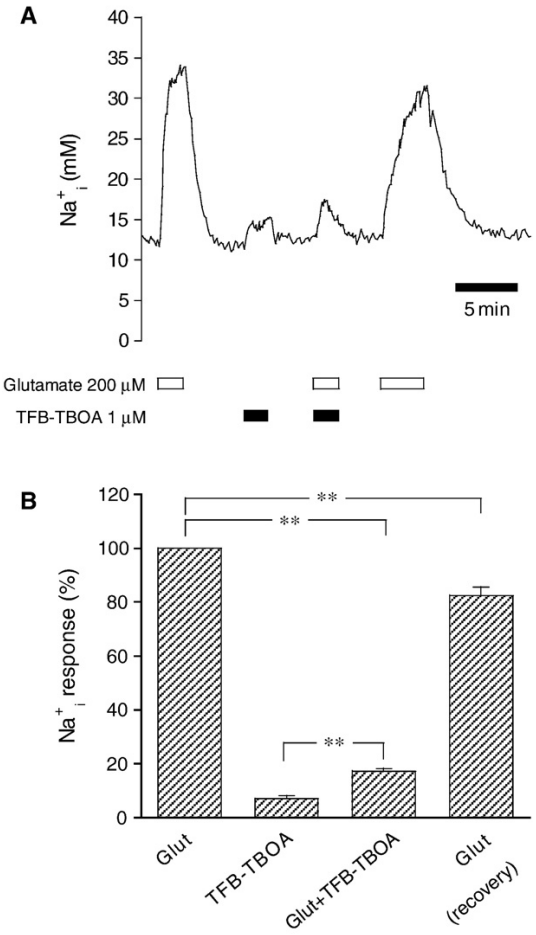

Fig. 1 – Inhibition by TFB-TBOA of the glutamate-evoked Na^+_i response. (A) Representative experimental trace depicting the Na^+_i increase caused by application of 200 μM glutamate followed by the application of TFB-TBOA 1 μM alone and subsequently applied with glutamate. A final application of glutamate shows the reversibility of the response. (B) TFB-TBOA effects on the glutamate-evoked Na^+_i response. Results are expressed as maximum amplitude of Na^+_i and presented as percent of those observed with 200 μM glutamate. Data are means±SEM of 40 cells from 5 independent experiments.

however, with a slower kinetics and lower amplitude. Fig. 1B summarizes the results obtained in this series of experiments.

An inhibition curve of TFB-TBOA on the astrocyte response to 200 μM glutamate was then established (Fig. 2). The graph shows that TFB-TBOA inhibited the Na^+_i response with high affinity, with an apparent IC_{50} of 43±9 nM when measured on the maximal amplitude of the response. In the presence of this maximally effective concentration of TFB-TBOA, about 17% of the Na^+_i response to glutamate subsisted (Figs. 1 and 2). We thus tested whether this residual response was due to AMPA/kainate receptor activation, which also leads to Na^+_i increase in astrocytes (Chatton et al., 2000) rather than to incomplete transporter blockade. Fig. 3 indicates that co-application of TFB-TBOA and CNQX did not further inhibit the Na^+_i response to 200 μM glutamate.

As seen in Fig. 1, TFB-TBOA caused a small Na^+_i response in the absence of glutamate. We thus tested for the potential involvement of three major classes of astrocytic receptors in this response. Fig. 3 shows that addition of the AMPA/kainate receptor antagonist CNQX (50 μM) did not abolish the Na^+_i change caused by 1 μM TFB-TBOA. Application of the metabotropic glutamate receptor antagonist (S)-α-Methyl-4-carboxyphenylglycine (MCPG, 1 mM) or the P2 purinergic receptor antagonist suramin (100 μM) did not influence the response to TFB-TBOA alone, excluding the contribution of these classes of receptors.

In order to have a more direct assessment of the ability of TFB-TBOA to interfere with transport activity, we tested D-aspartate, a substrate of the glutamate transporter that is not metabolized nor activates ionotropic receptors. TFB-TBOA inhibited ~90% of the Na^+_i response to 200 μM D-aspartate in astrocytes (Fig. 4). The reversibility of the inhibition on the response to D-aspartate was of 60±2%, i.e. somewhat lower than was observed with glutamate (see Fig. 1).

We then tested whether the incomplete reversibility of the response to glutamate after TFB-TBOA washout was persistent with time. Fig. 5 indicates that the reversibility improved with time, without however being complete 1 h after washout of the compound. Interestingly, both the amplitude and the initial rate of Na^+_i rise recovered with a similar time course.

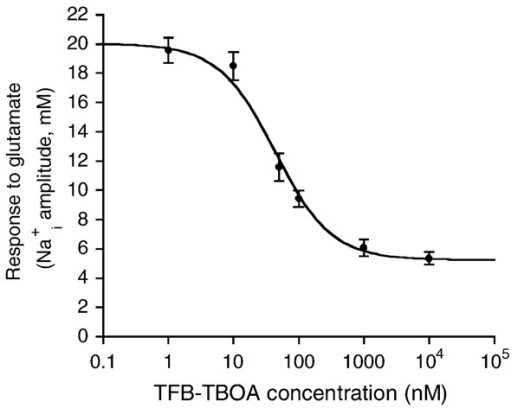

Fig. 2 – Concentration dependency of the inhibitory effects of TFB-TBOA. Inhibitory curve on the maximum amplitude of Na^+_i response to 200 μM glutamate. Non-linear least square fits using 135 cells from 17 experiments yielded IC_{50} values of 43±9 nM. The maximum inhibition was observed at 1 μM TFB-TBOA.

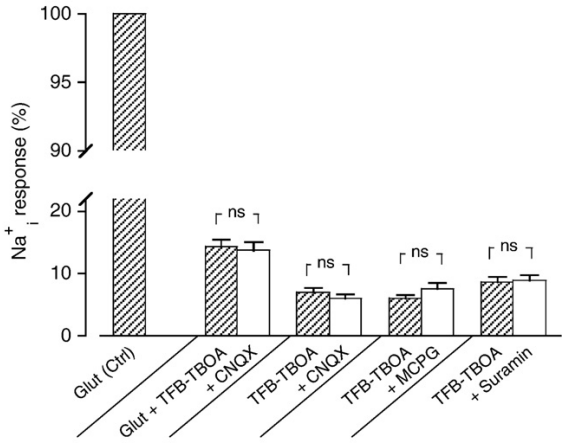

Fig. 3 – Residual Na^+_i increase in the presence of TFB-TBOA. The TFB-TBOA-insensitive residual Na^+_i response to 200 μM glutamate was not altered by the non-NMDA receptor inhibitor CNQX (50 μM, means±SEM of 40 cells from 5 independent experiments). The Na^+_i increases observed upon TFB-TBOA (1 μM) application alone were not abolished by the non-NMDA receptor inhibitor CNQX (50 μM, 52 cells from 7 independent experiments), the metabotropic glutamate receptor antagonist MCPG (1 mM, 40 cells from 5 experiments), or the P2 purinergic receptor antagonist suramin (100 μM, 23 cells from 3 experiments). Results are expressed as maximum amplitude of Na^+_i and presented as percent of those observed with 200 μM glutamate used as a control.

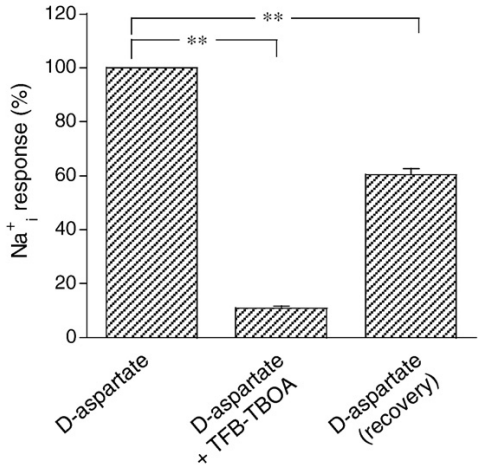

Fig. 4 – Inhibition by TFB-TBOA of the D-aspartate-evoked Na^+_i response. Results are expressed as maximum amplitude of Na^+_i and presented as percent of those observed with 200 μM D-aspartate. Data are means±SEM of 56 cells from 7 independent experiments.

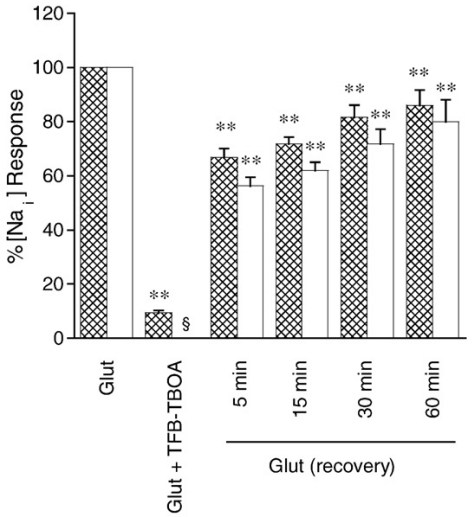

Fig. 5 – Reversibility of TFB-TBOA inhibitory effects. The recovery of the response to 200 μM glutamate was tested consecutively 5, 15, 30, and 60 min after washout of TFB-TBOA (1 μM). Results are expressed as amplitude (hatched bars) or initial rate of rise of Na^+_i responses (open bars) and presented as percent of the response recorded for the first control application of 200 μM glutamate. The significance of recovery was tested against the initial control glutamate application. § not measurable with accuracy. Data are means ± SEM of 46 cells from 6 independent experiments.

In order to investigate the mode of action of TFB-TBOA, a Schild analysis was performed (Fig. 6). The Na^+_i responses to application of glutamate at different concentrations were measured in the absence or in the presence of TFB-TBOA applied at 10, 50 and 100 nM. From a family of three-point dose–response curves, the apparent EC_{50} of glutamate was estimated and used to build the Schild plot (Fig. 6B), which yielded a linear slope of 1.6 (R^2 = 0.96), different from unity and thus not compatible with pure competitive antagonism.

Finally, we investigated the effect of TFB-TBOA on the membrane properties of cortical neurons in pure primary cultures. Neurons were recorded in whole-cell patch clamp in voltage-clamp configuration and the current-to-voltage relationship was analyzed in the presence and in the absence of 1 μM TFB-TBOA. The current–voltage plot shown in Fig. 7 shows that the resting membrane potential was −80 mV regardless of the presence or absence of TFB-TBOA and that the inhibitor did not influence the input resistance. In some recorded neurons, spontaneous or network-induced firing of action potentials was observed and did not appear to be influenced by the presence of TFB-TBOA (*not shown*).

3. Discussion

The aim of this report was to characterize the effects of the new glutamate transporter inhibitor TFB-TBOA on astrocytes in primary culture. As astrocytes are equipped with a high density of Na^+-coupled glutamate transporter, Na^+_i concentration undergoes rapid and robust increases in these cells, which can be measured by microspectrofluorimetric methods (see e.g. Chatton et al., 2000; Rose et al., 1997). This Na^+_i increase is almost entirely attributable to the activity of the Na^+-glutamate cotransporters. Glial glutamate transporters use a complex stoichiometry of 3 Na^+ plus 1 H^+ cotransported (or 1 OH^- exchanged) with 1 glutamate and exchanged against 1 K^+ (Levy et al., 1998). As the transport cycle is electrogenic, glutamate transport leads to a net inward current that can be measured by electrophysiological means. Transport activity

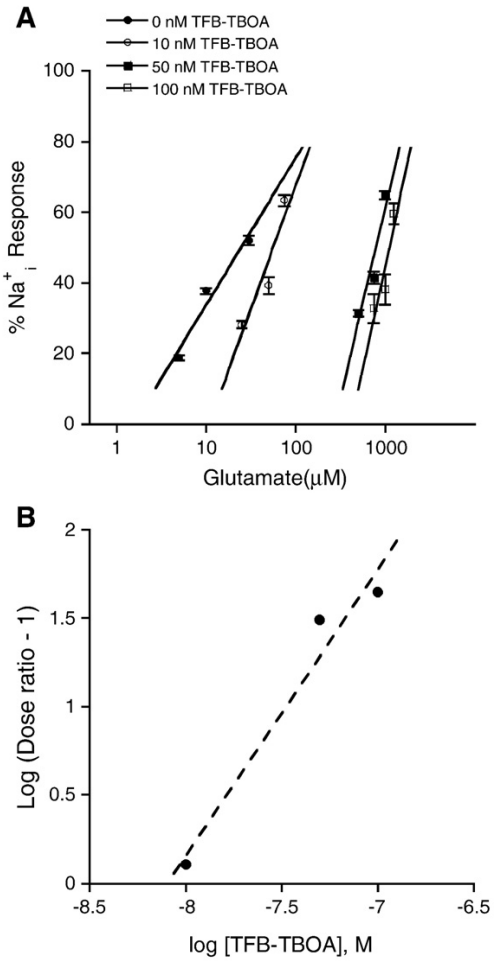

Fig. 6 – Schild analysis of inhibitory action of TFB-TBOA. (A) The Na^+_i responses to applications of glutamate at different concentrations were measured in the absence or in the presence of TFB-TBOA present at three different concentrations indicated in the graph. Data are means ± SEM of 16–24 cells from 13 experiments. (B) Schild plot of the data in panel A. Dose ratio is the ratio of the apparent EC_{50} of glutamate obtained from the family of three-point dose–response curves in panel A in the presence of a given concentration of TFB-TBOA over the EC_{50} of glutamate in the absence of TFB-TBOA. The linear regression yielded a slope of 1.6 (R^2 = 0.96).

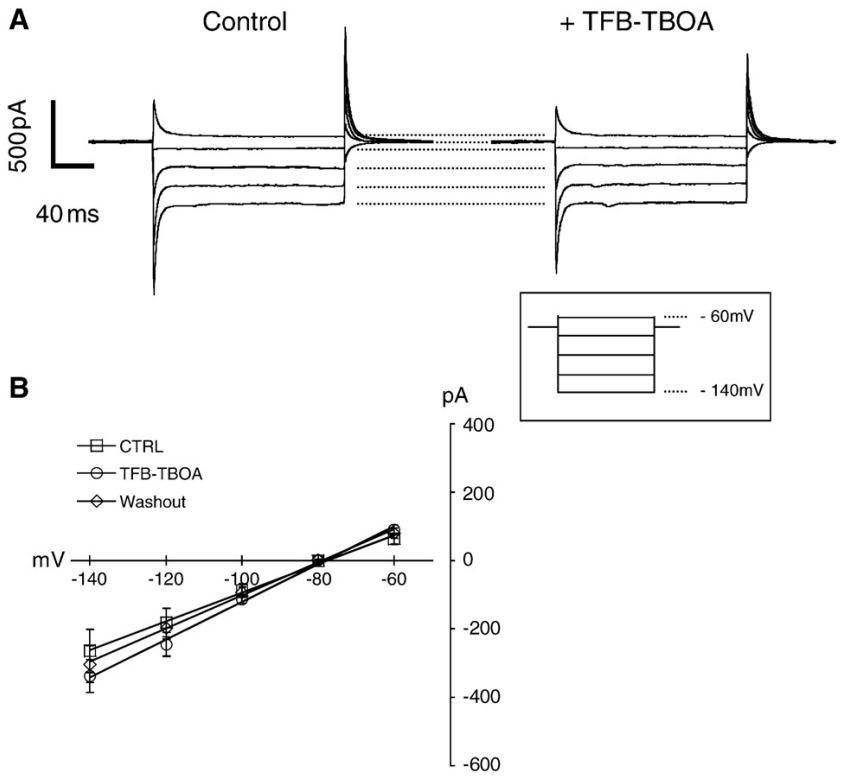

Fig. 7 – Effects of TFB-TBOA on the passive membrane properties of cultured cortical neurons in whole-cell configuration. (A) Representative whole-cell current responses of a neuron to 20 mV voltage steps (−140 to −60 mV, see inset). The cell was clamped at −70 mV and measurements were done with and without 1 μM TFB-TBOA. (B) Current–voltage relationship recorded in the absence and in the presence of TFB-TBOA, as well as after washout of the compound. Input resistances were not significantly different among groups. Data are means ± SEM from nine individually recorded neurons.

can also be measured by radioactive tracers of transporter substrates, *e.g.* glutamate or D-aspartate. Measuring Na^+_i changes, as performed in the present study, offers the advantages of assessing transport activity in real time and at the single cell level.

The first non-transported inhibitors of glutamate transporters were dihydrokainate, with selectivity for EAAT2 (GLT-1), and more recently TBOA, acting on all EAAT subtypes. Both compounds have been widely used but suffer from a relatively low affinity on glutamate transporters and are accompanied with complex unwanted effects (Bernardinelli and Chatton, 2008). The newly released compound TFB-TBOA offers the promise of a high potency of inhibition in the nanomolar range.

The original report characterizing TFB-TBOA (Shimamoto et al., 2004) described the inhibition glutamate transport measured by ^{14}C-glutamate uptake in COS cells expressing EAATs or measured by transporter currents in *Xenopus laevis* oocytes expressing EAATs. The reported IC_{50} vales were 22, 17 and 300 nM for EAAT1, EAAT2, and EAAT3, respectively.

In the present study, TFB-TBOA was tested on mouse astrocytes. The only glutamate transporter functionally expressed in primary mouse astrocytes is the EAAT1 (GLAST) subtype, as the GLT-1 inhibitor dihydrokainate fails to inhibit the Na^+_i response to glutamate (Chatton et al., 2001). We found that TFB-TBOA inhibited Na^+_i responses evoked by 200 μM glutamate in a concentration-dependent manner with an apparent IC_{50} of 43 nM. Previous measurements of inhibition of synaptically activated transporter currents in astrocytes in the hippocampus yielded an IC_{50} value of 13 nM (Tsukada et al., 2005). We found a maximal inhibition of ~80% on the Na^+_i response to glutamate, whereas the inhibition was more pronounced (~90%) when D-aspartate was used as a transport substrate.

TFB-TBOA showed, however, incomplete reversibility of inhibition. Whereas the amplitude of the Na^+_i responses to glutamate measured 5 min after washout of the compound was overall 69% of the original amplitude, the rise-time was significantly slower (~4-fold), possibly because some fraction of the compound remains bound because of its high affinity for the transporter. It is plausible that TFB-TBOA does not only act as a pure competitive inhibitor of transport, but also displays some non-competitive component. Schild analysis of the inhibition of glutamate-mediated Na^+_i responses by TFB-TBOA indeed supported this conclusion. In addition, we observed that reversibility of responses was gradually improving with time after washout of the drug. This result is consistent with observations made on EAAT transporter

currents measured in oocytes (Shimamoto et al., 2004), for which the recovery was incomplete even 1 h after washout of TFB-TBOA. We found that not only did the amplitude gradually recovered, but also the initial rate of Na^+_i rise with a similar time course. Our previous mathematical modeling of the Na^+_i response to glutamate has shown that this parameter best reflects the kinetics properties of the transporter (Chatton et al., 2000). This indicates that TFB-TBOA causes an alteration of transporter function, which persists after washout of the drug. This incomplete reversibility of inhibition is a limitation for the use of TFB-TBOA that has to be taken into account in experimental designs.

Whereas TFB-TBOA applied alone was shown not to elicit detectable currents in oocytes expressing glutamate transporters (Shimamoto et al., 2004), we found, however, that in primary mouse astrocytes, a small but significant Na^+_i increase was evoked by TFB-TBOA, as was found to be the case for TBOA (Chatton et al., 2001). As NMDA receptors are not found in cultured astrocytes (Verkhratsky and Kirchhoff, 2007) and as the AMPA/kainate receptor antagonist CNQX did not prevent this Na^+_i increase, the mechanism of the TFB-TBOA evoked Na^+_i rise does not involve ionotropic glutamate receptors. Metabotropic glutamate and P2 purinergic receptors could also be excluded. Thus, this response to TFB-TBOA could be related to interference with other Na^+ carrier proteins such as the Na,K-ATPase. The observation would also be compatible with TFB-TBOA being a low efficacy transporter substrate, which could elicit a small Na^+_i response.

In previous studies performed under the same experimental conditions, we had found that the maximum inhibition caused by TBOA on the Na^+_i response to 200 μM glutamate was ~70% (Chatton et al., 2001). We show here that the maximal inhibition caused by TFB-TBOA on the response to glutamate was somewhat larger (>80%). As was found for TBOA, the TFB-TBOA-insensitive residual Na^+_i response to glutamate was not mediated by non-NMDA receptors and it is currently unclear what pathways is responsible for this residual signal. By contrast, the EAAT transporter currents measured in oocytes were reported to be fully inhibited by TFB-TBOA (Shimamoto et al., 2004).

It is generally accepted that astroglial cells *in situ* express mostly the EAAT2 (GLT-1) subtype of transporter (Dunlop, 2006), whereas when studied in primary cultures, EAAT1 (GLAST) becomes the almost exclusively functional isoform expressed. As was the case with TBOA, TFB-TBOA has almost identical affinity for both isoforms of the transporter (Shimamoto et al., 2004). However, TFB-TBOA was reported to have an approximately 15-fold lower affinity for EAAT3, a mostly neuronal transporter isoform, which means that this compound could potentially be used to discriminate between the contribution of glial and neuronal glutamate transport in the intact tissue. In addition, we found that TFB-TBOA at its maximally effective concentration did not influence the passive membrane electrical properties of cortical neurons and apparently did not alter their excitability.

Selective inhibition of the EAAT2 (GLT-1) subtype is classically achieved with dihydrokainate, which, however, possesses a fairly low affinity for the transporter (Bridges and Esslinger, 2005) and causes complex effects on astrocytes *in situ* (Bernardinelli and Chatton, 2008). A very recent report described the first selective inhibitor of EAAT1, UCPH-101, with an IC_{50} of ~1 μM and >400-fold selectivity over EAAT2 and EAAT3 (Jensen et al., 2009). With TFB-TBOA acting on both glial isoforms, the latter pharmacological tool could represent an interesting complement for the functional studies of glutamate transport.

Taken together, the present study showed that TFB-TBOA is able to inhibit Na^+-dependent glutamate transport in astrocytes with high potency. Despite a partial reversibility of inhibitory effects that have to be taken into account in experimental designs, TFB-TBOA is to be considered as an extremely valuable tool to study glutamate transport and neuron–glia interactions.

4. Experimental procedures

4.1. *Cell culture and solutions*

Every effort was made to minimize suffering and the number of animals used in all experiments. In addition, all the procedures used to prepare living cells have been approved by the Swiss legislation and follows their guidelines. Primary cultures of mouse astrocytes were prepared as previously described (Chatton et al., 2000). After microdissection of cortices from 1 to 4 day-old C57bl6 mice, tissue was mechanically dissociated by successive aspirations through sterile syringes. The isolated astrocytes were then plated on glass coverslips and cultured in DME medium (D7777, Sigma, Buchs, Switzerland) supplemented with 10% FCS penicillin, streptomycin and amphotericin. Astrocytes were used after 2–3 weeks of culture.

Mouse cortical neurons in primary cultures were obtained from 17-day C57bl6 mouse embryos. After removing meninges, entire cortices were first incubated with 20 U/ml papain for 30 min at 34 °C and then mechanically dissociated in MEM medium plus glucose, glutamine and 10% FCS, by successive aspiration through sterile plastic 2 ml pipettes. The dissociated cells were centrifuged at 1300 rpm for 2 min and then re-suspended at a density of 80–85,0000 cells per cm^2 in Neurobasal (Invitrogen, Basel, Switzerland) culture medium complemented with 2% B27 solution (Invitrogen), 500 μM glutamine according to Brewer et al. (Brewer et al., 1993). Cells were then plated on glass coverslips coated with poly-L-Ornithine (Sigma, Buchs, Switzerland). Cells were used after 7–14 days of culture.

Experimental solutions used during experiments with astrocytes and neurons contained (mM) NaCl 135, KCl 5.4, $NaHCO_3$ 25, $CaCl_2$ 1.3, $MgSO_4$ 0.8, NaH_2PO_4 0.78, glucose 5, bubbled with 5% CO_2/95% air.

TBOA and TFB-TBOA were from Tocris-Anawa Trading (Zürich). Unless otherwise state, all other compounds were from Sigma.

4.2. *Na^+_i fluorescence imaging*

Na^+_i measurements were performed as previously described (Chatton et al., 2000). Briefly, experiments were carried out on the stage of an inverted epifluorescence microscope (Nikon, Tokyo, Japan) and observed through a 40×1.3 N.A. oil-immersion objective lens (Nikon). Fluorescence excitation wavelengths

were selected using fast filter wheel (Sutter Instr., Novato, CA) and fluorescence was detected using a Gen III+ intensified CCD camera (VideoScope Intl., Washington D.C.). Acquisition and digitization of video images, as well as time series were computer-controlled using the software Metafluor (Universal Imaging, West Chester, PA, USA) running on a Pentium computer. Four video frames were averaged at each wavelength and the acquisition rate of ratio images was varied between 0.5 and 0.1 Hz. Up to 10 individual astrocytes were simultaneously analyzed in the selected field of view.

Na^+_i was measured in single cells grown on glass coverslips after loading the cells with the Na^+-sensitive fluorescent dye sodium-binding benzofuran isophthalate (SBFI-AM, Teflabs, Austin, TX). Cell loading was performed at 37 °C using 15 μM SBFI-AM in a HEPES-buffered balanced solution containing (mM) NaCl 160, KCl 5.4, HEPES 20, $CaCl_2$ 1.3, $MgSO_4$ 0.8, NaH_2PO_4 0.78, and glucose 20 and was supplemented with 0.1 % Pluronic F-127 (Molecular Probes, Eugene, OR).

Once loaded with SBFI, cells were placed in a thermostated perfusion chamber designed for rapid exchange of perfusion solutions and superfused at 35 °C. Fluorescence was sequentially excited at 340 and 380 nm and detected at >520 nm. Fluorescence excitation ratios (F_{340nm}/F_{380nm}) were computed for each image pixel and produced ratio images of cells that were proportional with Na^+_i. *In situ* calibration was performed after each experiment by permeabilization of the cell membrane for monovalent cations using 6 μg/ml gramicidin and 10 μM monensin with simultaneous inhibition the Na^+/K^+-ATPase using 1 mM ouabain. Cells were then sequentially perfused with solutions buffered at pH 7.2 with 20 mM HEPES and containing 0, 10, 20 and 50 mM Na^+, respectively, and 30 mM Cl^-, 136 mM gluconate with a constant total concentration of Na^+ and K^+ of 165 mM. A four-point calibration curve was computed for each selected cell in the field of view and used to convert fluorescence ratio values (F_{340nm}/F_{380nm}) into Na^+ concentrations.

4.3. *Whole-cell electrophysiological recordings in neurons*

Whole-cell voltage-clamp recordings were made with borosilicate glass pipettes with a resistance of 5.5–8 MΩ. In voltage-clamp mode, the clamp potential was set at −70 mV. Recordings were made with an Axopatch 200A amplifier (Axon Instruments). Current were filtered at 1 kHz. Data were acquired with a Digidata 1440A (Axon), at 10 kHz sampling rate, controlled with Pclamp 10 software and analyzed with Clampfit software (Axon). A period of 5 min was routinely allowed after establishment of the whole-cell configuration. The patch-clamp intracellular solution contained (in mM) K-gluconate 130, NaCl 5, Na-phosphocreatine 10, $MgCl_2$ 1, EGTA 0.02, HEPES 10, Mg-ATP 2, and Na_3-GTP 0.5, pH 7.3 (adjusted with KOH).

Experiments were performed using an open perfusion chamber. Control extracellular solutions and solutions containing the tested drugs were gravity fed at 600 μl/min and 35 °C on the cultured cells.

4.4. *Data analysis*

Data are means ± SEM and are represented as percentage of the control current or voltage amplitude. Paired Student's *t*-tests or ANOVA tests were performed to assess the statistical significance ($^*P<0.05$; $^{**}P\leq0.01$). The half-maximum inhibitory concentration (IC_{50}) of TFB-TBOA on the response to glutamate was determined by non-linear curve fitting performed using the Levenberg–Marquardt algorithm implemented in the Kaleidagraph software package (Synergy Software, Reading, PA, USA). The dose–response analysis experiments were fitted using the following equation:

$$R_{obs} = R_{max}[I] / (K + [I]) + R_{min} \quad (1)$$

where R_{obs} is the observed response and R_{max}, R_{min} are maximum and minimum parameters of the response. [*I*] is the concentration of the inhibitor compound and *K* is the concentration that yields its half-maximum inhibition (*i.e.* IC_{50}).

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3 Discussion and perspectives

3.1 Lactate modulation of cortical neuron activity

3.1.1 Use of dynamic calcium imaging to monitor neuronal activity

In the first phase of this study, we validated the imaging technique aimed at monitoring the spontaneous neuronal firing activity. Taking advantage of the fact that action potentials induce a strong depolarization, triggering the opening of voltage gated calcium channels, we monitored the intracellular calcium transients as indicators of neuronal activity. Simultaneous measurements of membrane potential recorded in whole-cell patch clamp configuration and somatic calcium fluorescence of the clamped neuron were performed to assess the relationship between the two parameters, similar approaches were presented in several studies (Cossart et al., 2005, Sasaki et al., 2008). We confirmed that action potentials occurred in temporal synchronization with the intracellular calcium transients.

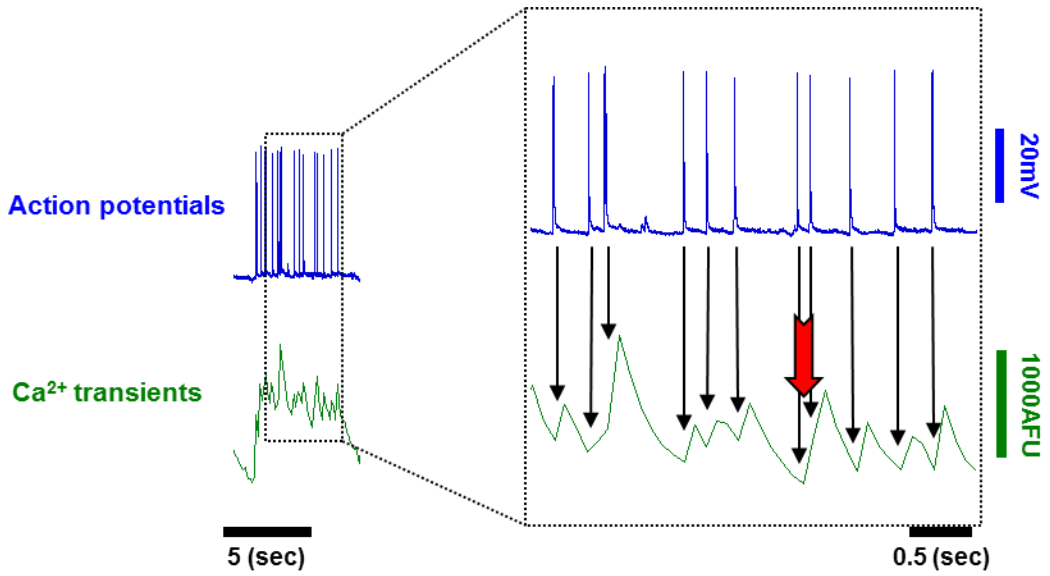

Figure 10: Focus on action potential and calcium transients mechanism. Red arrow indicates an example of two action potentials that are not correctly detected with calcium imaging.

In order to assess the robustness of the technique, we observed calcium transients evoked by relatively regular bursts of action potentials at different frequencies (see **fig. 10**). Single action potentials evoked clearly identifiable calcium transients up to ~5 Hz. Beyond this frequency, the individual calcium spikes were substantially overlapped, which is primarily due to the slow decay of the calcium transients and a narrow interspike interval, rendering the detection of individual spikes unrealizable. Bearing this technical limitation in mind and considering that in our cultures the spontaneous spiking frequency was around 0.1-1 Hz, we considered this calcium imaging method as suitable for our experiments to monitor the spiking activity in the neuronal population.

One of the main advantages obtained from the optical imaging method is to offer the possibility of simultaneous measurements from many neurons. Another benefit brought by this approach is that we can keep the cytosol structure and composition undisturbed, except for the incorporation of the dye. As for other fluorescent ion indicators, Ca^{2+} dye itself can chelate the ions to be monitored. However, if a buffering effect may occur, this will affect the general rate of neuronal activity during all phases of the experiment.

In the context of the energy substrate sensing, the response brought about by the specialized cell such as glucose-excited or glucose-inhibited, involves several intracellular steps. Usually, the electrophysiological recording in perforated patch configuration is a preferred approach rather than conventional whole-cell techniques. Calcium imaging constitutes an additional noninvasive way to carry out this type of investigations, with the benefit of higher throughput.

3.1.2 ATP-independent modulation of the neuronal activity by lactate

In this study, we describe a novel mechanism for the modulation of cortical neuron activity by lactate. Generally, lactate is considered as an important energy substrate of the brain but beside its metabolic function it has an additional modulatory role. We showed that lactate is

able to decrease the spontaneous neuronal activity in a concentration-dependent manner and independently of its metabolism.

The independence of lactate effect from the ATP production, is an important finding of our study, that distinguishes it from lactate effects previously reported in glucose sensing neurons. Here, we comment on selected aspects and results, coming from our experiments, not extensively addressed in the discussion of the manuscript, that indicated the separation between ATP production and neuromodulation by lactate.

- The first set of experiments was carried with a maintained concentration of glucose. The advantage of this condition is that glucose provides neurons with the necessary ATP to sustain their activity, allowing to single-out a neuromodutalory role of lactate.
- The effect of lactate was compared with that of pyruvate that has a quasi-equivalent capacity of ATP production. Once inside the cytosol of neurons, lactate is converted into pyruvate by the LDH with the production of NADH. Pyruvate can enter the TCA cycle and lead to the production of energy. From that point, the formation of ATP is equal for both compounds (lactate and pyruvate) but the NADH produced in the cytosol can be re-oxidized under aerobic conditions by the respiratory chain. NADH is not directly entering mitochondria because the inner membrane is impermeable to it. NADH introduces its electron indirectly to the electron transport chain transferring it to different compounds that are able to shuttle it. Then cytosolic NADH is indirectly oxidized by the respiratory chain and 1.5-2.5 ATP are produced. Considering that, from the degradation of one molecule of pyruvate we obtain ~15 ATP, this means that for one molecule of lactate we can have ~16.5-17.5 ATP, *i.e.* a slightly higher ATP yield.

  In our experiments, we found a profound different intensity in the effect of these compounds. Pyruvate 5mM induced only ~7% of decrease of neuronal activity whereas lactate 5mM more than 50%. Therefore, these effects cannot be explained

based on ATP production, suggesting that the lactate response acts via an ATP-independent pathway.

- Another way that we used to augment the ATP production was to increase the glucose concentration in the medium. Glucose, via glycolysis and oxidative phosphorylation, can supply twice as much energy equivalents per molecule as lactate. However, it is possible that the real rate of catabolism of glucose does not correspond to the theoretical stoichiometry because at some point, the enzymatic machinery could saturate. For example, the hexokinase responsible for the conversion of glucose into glucose-6-P is auto-limited by its product, meaning that the production is regulated by the rate of consumption. Some glucose sensing neurons, as pancreatic β cells, express a special hexokinase, called glucokinase, that is not inhibited by glucose-6-P (Kang et al., 2004). This allows them to produce ATP in register with the extracellular glucose concentration, in a way that is not limited by the end products.

  The expression of glucokinase was not assessed in our neuronal cultures. However, such expression is not expected from cortical neurons but only from specialized neurons of the hypothalamus and brain stem. In our experiments increasing the glucose concentration did not reduce neuronal activity.

- The last way to assess the metabolic independence, was to use a compound similar to lactate but that do not give the same production of ATP. D-lactate is described to be poorly metabolized by neurons because it was reported that mammals lack the D-lactic acid dehydrogenase necessary for its conversion into pyruvate (Ewaschuk et al., 2005). It was nevertheless proposed that the enzyme D-α-hydroxy acid dehydrogenases catalyze at low rate (about fivefold lower than L-isomer) the conversion of D-lactate into pyruvate (Halperin and Kamel, 1996). This means that a small part of D-lactate could possibly lead to some ATP formation but considering

that both isomers induced the same intensity of response, the proportionality of effects does not match.

3.1.3 Receptor-mediated effect

In this study, we identified a receptor-mediated pathway underlying the modulatory action of lactate. Hydroxycarboxylic acid (HCA) receptor-1 is a recently deorphanized G-coupled receptor for which the natural agonist is lactate. This receptor was found to be highly expressed in adipocytes. Compared to the high HCA1 receptor concentration in white and brown fat, the other organs such as liver, muscle and brain showed a limited presence of HCA1 receptors (Liu et al., 2009). However, using anti HCA1 receptor antibody associated with immunostaining and Western blot, we found that this receptor is expressed in primary cortical neurons. In the Western blot experiments, the proportion between the concentration of protein used and the intensity of the signal detected from the sample, indicated that the receptor is not expressed at high levels, confirming the above cited findings (quantification of the expression was not performed). Nevertheless, a high expression of HCA1 is not essential to produce an evident effect, since the signal amplification is one of the main advantages of the metabotropic G protein-coupled receptors.

The functional implication in our cultures of HCA1, a G_i coupled receptor, was confirmed by the action of the agonists 3,5-dihydroxybenzoic acid (3,5-DHBA) and 3-hydroxybenzoic acid (3-HBA). Both compounds have a similar affinity for the receptor. 3,5-DHBA is a specific agonist for only HCA1 whereas 3-HBA is an agonist for HCA1 and HCA2 a receptor belonging to the same family (Liu et al., 2012). The same intensity of effect, deserved with either 3,5-DHBA or 3-HBA, suggested that HCA1 rather than HCA2, is implicated in the lactate associated neuromodulation. These compounds are not substrates of the TCA cycle and cannot produce ATP. They are natural products, present in green tea, grapefruit and

olive oil (Liu et al., 2012). 3,5-DHBA and 3-HBA can represent useful tools to study the HCA1 receptor implications in the intact brain.

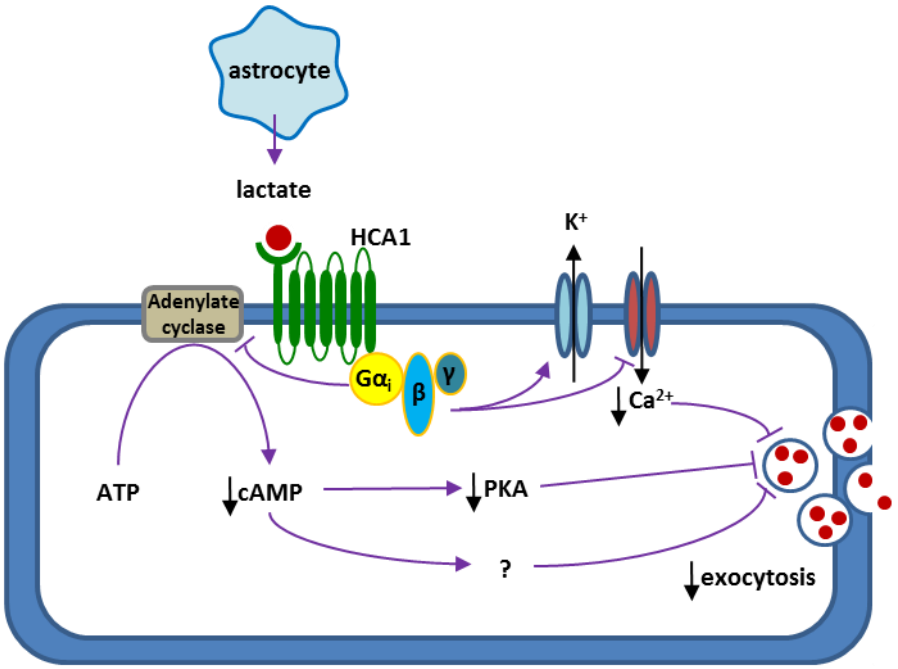

Figure 11: Possible models of the functions of HCA1 receptor in cortical neurons.

Another indication of the functional implication of HCA1 was the reduced effect of lactate on neuronal activity after incubation with pertussis toxin (PTX), an inactivator of G_i protein. This indicated that the lactate neuromodulation works via the activation of the G_i protein. Several mechanisms could explain the reduction of activity. A series of potential pathways and targets are represented in **fig 11**. Lactate binding the HCA1 activates the $G_{\alpha i}$ subunit, this inhibits the adenylate cyclase, resulting in a reduction of cAMP concentration. cAMP is well known to serve as a signal that modulates the neuronal exocytosis by coordinating PKA-dependent and PKA-independent mechanisms (Seino and Shibasaki, 2005). Another possible mechanism involves the $G_{\beta\alpha}$ subunits. This part of the complex triggers the opening of K^+ channels that induce a hyperpolarization of the membrane. This represses the Ca^{2+} influx via the closure of voltage-dependent calcium channels, leading to a decrease of the exocytosis (Bettler et al., 2004).

3.1.4 Pathophysiological implications

In the discussion of the paper, we proposed that the effect of lactate could be implicated in a physiological mechanism that protects neurons against an excess of activity. In the ANLS hypothesis, glutamate liberated during neuronal activity stimulates the production and the release of lactate from astrocytes. During an abnormal neuronal hyperexcitability, the risk of cell damage due to excitotoxicity is increased, *e.g.* in epileptic seizures. In these situations, the concentration of lactate could reach a level that limits network activity and prevent cellular damage. It was recently shown using a mouse model of Alzheimer's disease (AD), that the early AD-related epileptic activity was strongly reduced by the preincubation of brain slices with oxidative energy substrates such as pyruvate or D-3-hydroxybutyrate (Zilberter et al., 2012). In this particular case, the positive effect was obtained just restoring the correct support in energy but with lactate and its inhibitory component we could have an increased efficacy.

3.1.5 D-lactate encephalopathy

We have used D-lactate in the course of our studies as a substitute to L-lactate. Under normal circumstances D-lactate is present only at nanomolar concentration in blood due to the wide distribution of the methylglyoxal pathway. However, D-lactate can have another origin due to the activity of bacteria present in the gastrointestinal tract. Indeed, an abnormal production of D-lactate occurs in patients with short-bowel syndrome or gastric bypass as the bacteria population increases. This increase in D-lactate concentration can cause D-lactate encephalopathy with neurological manifestations such as altered mental status, dysarthria, ataxia, gait disturbance (Uribarri et al., 1998). Until now, the mechanisms of D-lactate encephalopathy are largely unknown. It was proposed that the effects are associated with an interference with the supply of energy metabolites, as D-lactate can compete with L-lactate for the transport into the neurons. Alternatively, D-lactate itself may be neurotoxic. In our

experiments, we found that D-lactate induced a decrease of the spiking frequency with an apparent IC_{50} of ~4.6mM, quite similar to the IC_{50} of L-lactate (~4.2mM). In D-lactate acidosis, serum D-lactate increases to more than 3mM and is capable of crossing the brain blood barrier (Uribarri et al., 1998). Considering that the basal level of L-lactate in the serum is around 1-2mM, it is possible that the resulting sum of L- and D-lactate concentrations could induce a modification of the excitability of neuronal network, thereby leading to the neurological symptoms observed.

3.1.6 Relevance of results

The finding that lactate induces a receptor-mediated neuromodulation confers a new role to this molecule which goes beyond the energy metabolic support. An increase in the neuronal activity is accompanied by an increase in lactate concentration in the extracellular space (Hu and Wilson, 1997). The main cellular source of lactate is thought to be astroglia. Therefore, the results of our study allow us to propose a novel mechanism used by astrocytes to modulate neuronal activity. The notion that astrocytes represent an active partner of synaptic function, is a discussed topic. The existence of a bidirectional communication between neurons and astrocytes is the base of the so-called “tripartite synapse”. This concept includes in addition to the pre- and post- synaptic terminal the surrounding astrocyte processes. We already have seen that there is a tight physical relation between the three elements (**fig. 5a**). Evidence has demonstrated that astrocytes integrate and process synaptic information and are able to release gliotransmitters (neuroactive molecules) such as glutamate, ATP, and D-serine (Volterra and Meldolesi, 2005). The lactate sensitivity that was found in the present study represents a different mechanism than those described in the tripartite synapse field. In general, the gliotransmitter produce an effect at the synaptic level. In our case, lactate could act across wide distances and affect larger volumes of tissue.

However, one has to keep in mind that the cellular source of lactate, its metabolic use, or its role in physiological situations are still debated.

Our study can have also implications in the treatment of the dyslipidemia. We found that the HCA1 receptor mediates the lactate sensitivity of neurons. HCA1, with HCA2 and HCA3, constitutes the family of HCA receptors. They are mainly expressed in adipose tissue and their principal function is to inhibit the lipolysis (Cai et al., 2008). HCA2 is the target for the drug nicotinic acid (also known as niacin), widely used for the treatment of the dyslipidemia. The drug has the positive effects to modify the composition of lipids that results in the reduction of the mortality (Wise et al., 2003). However, this substance induces unwanted side effects, such as cutaneous flushing due to activation of HCA2 receptor in the skin cells (Ahmed, 2011). For this reason HCA1 and HCA3 represent alternative drug targets and several synthetic ligands have been developed. For future research in the field of dyslipidemia, it will be necessary to keep in mind that HCA1 receptor is also expressed in the brain and that candidates could produce unwanted CNS side-effect if they are able to cross the BBB.

3.1.7 Future direction of research

In this study, we found an inhibitory role of lactate mediated by the HCA1 receptor in primary neuronal culture. This finding, as it often happens in science, raised more questions than answers. In this section a series of experiments are proposed in order to drive the future line of research:

- As our entire work was performed using dissociated neuronal culture, the first obvious step is to verify if the lactate sensitivity is also present in the intact brain. Immunostaining, using anti-HCA1 antibody, of the whole brain seems to be an appropriate start. The expression of HCA1 can be verified in different regions and at different stages of maturity. This could help to give an indication of which brain region

and what age to select for functional experiments. In a second phase, in correspondence of the expression of the receptors, the functional implication can be tested on brain slices with either patch clamp techniques or dynamic calcium imaging.

- The intracellular pathway leading to the lactate sensitivity remains to be solved. A series of mechanisms, involving the HCA1 activation, were proposed above. To determine which elements are involved, we can separate the pathways in two main groups, one increasing the cAMP and the other one opening membrane conductances. The cAMP implication can be investigated using an adenylate cyclase inhibitor, such as the SQ-22536, that could mimic the effect of lactate or, in the other way, use forskolin to raise the level of cAMP. In case of the implication a specific conductances, this can be investigated with electrophysiological techniques using the perforated patch configuration to maintain the cytosol intact.
- If the cAMP-PKA signaling is involved in downstream pathways following the activation of HCA1 receptor in neurons, this could have a potential impact on the synaptic plasticity. It has been shown that cAMP-PKA is required for long-term potentiation (LTP) at the hippocampal mossy fibers and for long-term depression at the Schaffer collateral in the CA1 region in the hippocampus (Seino and Shibasaki, 2005). It will be interesting to see if lactate could affect LTP or LTD in those regions. If so, this mechanism could link lactate with learning and memory.
- In the absence of a specific inhibitor for the HCA1, one available way to inhibit its activity was to inactivate the G_i protein incubating cells with PTX. Unfortunately, the effect of PTX is not direct and needs a cascade of intracellular reactions to be effective. The necessity to incubate neurons with PTX for at least 18 hours could constitute a major pitfall for its use in acute brain slices which typically can be kept functional for 6 to 10 hours. This obstacle could be circumvented by using a mouse

model knockout for the HCA1 receptor. This model has been recently produced in another laboratory and will be tested in future studies (Liu et al., 2009).

- Last but not least, it will be interesting to check the effect of lactate in epileptic conditions to see if it is able to decrease the abnormal activity of neurons. Such aim could be achieved by inducing an epileptic condition in primary neuronal culture, simply by incubating them for 1-2h in a Mg^{2+} free solution as described in a previous study (Sombati and Delorenzo, 1995). In this case, to monitor the activity patch clamp technique is recommended, due to high frequency of spiking (not quantifiable with calcium imaging if frequency > 5 Hz). The natural presence of lactate in the brain and even more importantly the development of selective potent agonists could represent an interesting alternative tool for the anti-epileptic treatment, different from currently used therapeutic approaches.

3.2 TFB-TBOA characterization

The presence of glutamate transporters is of primary importance for normal brain function. They actively clear up glutamate from the extracellular space maintaining it at low levels. Removal of such transporters leads to epilepsy, increased glutamate levels and excitotoxic neuronal injury (Tanaka et al., 1997). The majority of the glutamate uptake in the brain is mediated by astrocytic EAAT1 and EAAT2. Glutamate transporters are also involved in the coupling between neurons and astrocytes in the ANLS. The physiological relevance of these carriers highlights the importance to have adequate tools to study their implications. The effort invested in the search for potent and selective inhibitors of glutamate transporter has been substantial over the past years. That research led to the development of TBOA, the most widely used inhibitor. However, this compound suffers from drawbacks such as relatively low affinity increasing the risk to have side-effects. Here we comment the particular characteristics of a new TBOA derivative the TFB-TBOA, highlighted in our study.

The ideal qualities for a useful blocker are: high potency, high affinity, lack of side-effects, and specificity. Different experiments were performed to see if TFB-TBOA fulfills these criteria. We found that TFB-TBOA blocks glutamate-induced changes in intracellular Na^+. This effect was concentration dependent with an IC_{50} ~50nM (TBOA affinity is ~114µM). However, TFB-TBOA did not completely block the change in Na^+ and induced by itself a small Na^+ influx (as also present with TBOA) (Chatton et al., 2001). The source of the residual Na^+ signal remained unclear. The selective action of TFB-TBOA was demonstrated by the block of the D-aspartate-induced increase in intracellular Na^+. We also examined the effect of TFB-TBOA on currents in neurons, and it appeared that TFB-TBOA does not alter neuronal function. This selectivity contrast with other transport inhibitors such as DHK that have electrophysiological effects that are not related to the inhibition of transport (Bernardinelli and Chatton, 2008).

All together this study indicated that TFB-TBOA is a potentially valuable tool to study the implication of glutamate transporters in the relationship between neuron and astrocyte *in vivo*. Experiments performed in our laboratory later on confirmed that TFB-TBOA induced significantly less side-effects in acute slices than TBOA allowing its use in brain slices (Lamy and Chatton, 2011).

It is possible to envisage a use of TFB-TBOA also for our studies about the effects of lactate on neuronal excitability. With the application of the compound one could interrupt the metabolic connection between neurons and astrocytes preventing the release of lactate from astrocytes. However, the appropriate compounds should be added to avoid excitotoxicity caused by the built-up of extracellular glutamate.

3.2.1 Relevance of results

The study indicated that TFB-TBOA has distinct advantages over other inhibitors and is therefore the likely successor of TBOA, extensively used in studies investigating neuron-glia

interactions. Compared to its predecessor, TFB-TBOA has a higher potency and has no obvious side-effect on neurons. These qualities render it a more appropriate compound to use for *in vivo* studies.

At the time of this study, the effects of TFB-TBOA were not tested in intact tissue. This was due to the fact that the most commonly used Na^+ fluorescent dye the sodium-binding benzofuran isophthalate (SBFI) requires UV excitation not ideal for imaging thick living tissues. In addition, the intensity of the signal emitted by SBFI is much weaker compared to some Ca^{2+} indicator *e.g.* Fluo-4. Recently, a new Na^+ dye has been introduced named Asante Natrium Green (ANG) (Lamy and Chatton, 2011).This indicator is excited in the visible spectrum and has a bright fluorescent signal. To push forward the characterization of TFB-TBOA, it could be interesting to test its effect in acute brain slices loaded with ANG.

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