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**ROLE OF ASTROGLIAL GLUTAMATE TRANSPORT ON CEREBRAL
GLUCOSE METABOLISM *IN VIVO*: A PHARMACOLOGICAL CHALLENGE
WITH CLOZAPINE**

*(O papel do transporte glutamatérgico astrocitário no metabolismo de
glicose cerebral in vivo: um desafio farmacológico com clozapina)*

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“The mind is not a vessel to be filled but a fire to be kindled.”

Plutarch

GENERAL PRESENTATION

The following dissertation is organized in conformity with the norms of the Graduate Program in Biological Sciences: Biochemistry of the Universidade Federal do Rio Grande do Sul. The dissertation is divided in three parts:

Part I: comprises the Abstract, *Resumo*, Introduction and Objectives.

Part II: comprises the manuscript in preparation.

Part III: comprises the Discussion, Conclusions and References.

The Introduction presents the objectives and an extended bibliographical review on the relevant themes for the dissertation. The results are presented in Part II, as a manuscript to be submitted in a journal of international circulation, containing an extensive description of the methodology used, as well as a concise introduction, discussion and conclusion. The Part III discusses the themes presented throughout the dissertation and the results described in Part II, with general interpretations and comments. The Bibliographical References at the end of the dissertation represent those used only in Part I and III.

SUMMARY

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PART I

ABREVIATIONS LIST

[¹⁴C]2DG - [¹⁴C]-2-Deoxyglucose

[¹⁸F]FDG - 2-Deoxy-2-[¹⁸F]Fluoroglucose

[³H]2DG - [³C]-2-Deoxyglucose

AMPA - alpha-amino-3-hydroxy-methyl -5-4-isoxazolepropionic acid

ANLS - Astrocyte Neuron Lactate Shuttle

BBB - blood-brain barrier

CNS - central nervous system

dBcAMP - dibutyryl-adenosine 3',5'-cyclic monophosphate

DHK - Dihydrokinic acid

DL-TBOA - dl-threo-β-benzyloxyaspartic acid

EAAC1 - Excitatory amino-acid carrier 1

EAAT4 - Excitatory Amino Acid Transporters 4

EAAT5 - Excitatory Amino Acid Transporters 5

EAATs - Excitatory Amino Acid Transporters

FRET - Forster Resonance Energy Transfer

GABA - Gamma-Aminobutyric Acid

GLAST - Glutamate Aspartate Transporter

GLT-1 - Glutamate Transporter 1

L-β-TBOA - (L-β-benzyl-aspartate),

mTOR - mammalian target of rapamycin

NAD⁺ - nicotinamide adenine dinucleotide

NMDA - N-methyl-D-aspartate

PDC - L-trans-pyrrolidine-2,4-dicarboxylate

PET - Positron emission tomography

vGLUTs - Vesicular Glutamate Transporters

VOIs - volumes of interest

WAY-855 - 3-amino-tricyclo- heptane-1,3-dicarboxylate

β-THA - dl-threo-β-Hydroxyasartic acid

ABSTRACT

After many decades being considered only as neuronal supporters, astrocytes are now fully integrated in the modern neuroscience as very resourceful and valuable brain cells. In this context, the contribution of astrocytes on many brain functions has become an important subject of discussion. In this regard, a topic that still remains under debate is the astrocytic participation on glucose brain metabolism and, consequently, on the uptake of the glucose analogous molecule 2-Deoxy-2- ^{18}F Fluoroglucose (^{18}F FDG), detected by the positron emission tomography (PET).

^{18}F FDG-PET imaging does not offer cellular resolution, although, the resulting image reflects cellular and sub-cellular interactions with the radiopharmaceutical molecule. Until recently, the brain ^{18}F FDG uptake was interpreted mostly as a direct result of neuronal activity, with other cells being only seen as bystanders. However, recent studies have shown important associations between astrocyte function and ^{18}F FDG-PET signal, suggesting a contribution, at least partially, of these cells.

Aiming to evaluate if a decline on an astrocytic function would impact on ^{18}F FDG-PET signal, we performed, in adult rodents, a pharmacological challenge with clozapine. This drug is known to decrease, on astrocytes, glutamate transport and the glutamate transporter 1 (GLT-1) levels. This target was chosen because strong evidence points to the activity of GLT-1 as an important trigger for glucose uptake in astrocytes.

Our work focused on the region where GLT-1 is most abundant: cortex. A six-week treatment with clozapine caused, in adult rats, cortical: ^{18}F FDG hypometabolism, reduced GLT-1 density and expression. Alongside, was found a decline on glutamate transport, indexed by D-aspartate uptake, in cortical slices. A similar outcome was observed in cortical astrocyte primary cultures: a decrease in GLT-1 levels along with reduced D-aspartate and 2-Deoxyglucose uptake.

This work provides evidence that clozapine reduces ^{18}F FDG-microPET signal. Our results indicate that astrocytes are, at least partially, the cells underlying the glucose brain metabolism response to clozapine. Also, it suggests the reduction on GLT-1 levels/glutamate transport in astrocytes as a mechanism by which this response could be generated. These results raise the need for a reevaluation in the way that brain research and clinical ^{18}F FDG-PET data are interpreted, paying particular attention to the potential contribution of cells other than just neurons. Additionally, clozapine is a drug approved for clinical use, therefore, this phenomenon should be carefully investigated in patients under its treatment.

RESUMO

Depois de muitas décadas sendo considerados apenas coadjuvantes dos neurônios, os astrócitos estão agora totalmente integrados na neurociência moderna como células cerebrais muito valiosas. Nesse contexto, a contribuição astrocitária em diversas funções cerebrais se torna um importante ponto de discussão. Acerca disso, um tópico amplamente debatido é a participação dos astrócitos no metabolismo cerebral de glicose e, conseqüentemente, na captação da molécula de [¹⁸F]Fluorodeoxyglucose ([¹⁸F]FDG), detectada pela tomografia por emissão de pósitrons (PET).

O [¹⁸F]FDG-PET não apresenta resolução celular, entretanto, a imagem resultante reflete interações celulares e sub-celulares com o radiofármaco. Até recentemente, a captação de [¹⁸F]FDG no encéfalo vinha sendo interpretada, sobretudo, como um resultado direto da atividade neuronal, com outras células sendo vistas apenas como meras espectadoras. No entanto, estudos recentes demonstraram importantes associações entre a função astrocitária e o sinal do [¹⁸F]FDG-PET, sugerindo uma contribuição, ao menos parcial, dessas células.

Assim, a fim de investigar se o declínio de uma função astrocitária teria impacto no sinal de [¹⁸F]FDG-PET, realizamos, em roedores, um desafio farmacológico com clozapina. Este fármaco já demonstrou reduzir os níveis do transportador de glutamato 1 (GLT-1) e o transporte de glutamato astrocitário. Este alvo foi escolhido pois fortes evidências apontam para a atividade de GLT-1 como um importante gatilho para a captação de glicose em astrócitos.

Nosso trabalho focou na região onde GLT-1 é mais abundante: o córtex. Um tratamento de seis semanas com clozapina causou, em ratos adultos, na região cortical: hipometabolismo de [¹⁸F]FDG, redução na expressão e na densidade de GLT-1. Paralelamente, foi encontrado um declínio no transporte de glutamato, indexado pela captação de D-aspartato, em fatias corticais. Um efeito similar foi observado em culturas corticais primárias de astrócitos: diminuição nos níveis de GLT-1, juntamente com a captação reduzida de D-aspartato e 2-Deoxiglicose.

Este trabalho fornece evidências de que a clozapina reduz o sinal do [¹⁸F]FDG-microPET. Nossos resultados indicam que os astrócitos são, pelo menos parcialmente, as células responsáveis pela resposta do metabolismo cerebral de glicose à clozapina. Além disso, sugerem a redução nos níveis de GLT-1/transporte de glutamato em astrócitos como o mecanismo pelo qual essa resposta possa ter sido gerada. Esses resultados despertam a necessidade de uma reavaliação na forma como os dados do [¹⁸F]FDG-PET são interpretados, salientando a potencial contribuição de outras células além dos neurônios. Além disso, a clozapina é aprovada para uso clínico, portanto, esse fenômeno deve ser cuidadosamente investigado em pacientes sob tratamento com esse fármaco.

1. INTRODUCTION

1.1 Positron Emission Tomography and the use of [¹⁸F]FDG

There are two basic principles that underlie the positron emission tomography (PET) function: the detection/quantification of gamma radiation originated from the annihilation of two beta particles: the positrons (β^+) and electrons (β^-); and the subsequent processing to identify the radiation origin location. Consequently, atoms or molecules emitting β^+ radiation can be measured and have their spatial location determined within structures, such as the human body (Phelps *et al.*, 1975).

In this way, specific molecules radiolabelled with positron emitters - called radiopharmaceuticals - offer the possibility of non-invasive visualization, characterization and quantification of biological structures, molecules and processes occurring at cellular and sub-cellular levels in living biological organisms (Portnow *et al.*, 2013). Therefore, PET can generate a functional image, which in combination with other techniques that generate structural images, such as Computed Tomography or Magnetic Resonance Imaging, allows for the co-localization of biological processes and anatomical structures (Berger, 2003).

Within the positron emitting radiopharmaceuticals developed since the PET conception in 1974 (Phelps *et al.*, 1975), the 2-deoxy-2-[¹⁸F]fluoroglucose ([¹⁸F]FDG) is, by far, the most well known, being even recognized as the "tracer molecule of the century" (Wagner, 2008).

Inspired by the work of Sokoloff and coworkers with [¹⁴C]-2-deoxyglucose ([¹⁴C]2DG) (Kennedy *et al.*, 1975), [¹⁸F]FDG was first synthesized in 1976, emerging with the initial purpose of being used for brain evaluation (Fowler and Ido, 2002) and, later, it was also discovered as an important tool for the identification and management of cancer, as a result of the Warburg effect found in cancer cells (Som *et al.*, 1980; Kelloff *et al.*, 2005). The important contribution of [¹⁸F]FDG on tumoral investigation has stimulated the spread of this molecule and PET itself around the globe. Thus, [¹⁸F]FDG is now the most widely used positron emitting radiopharmaceutical in the world and is found in clinical use in practically all medium and large cities in the developed world. The extensive availability of this

radiopharmaceutical, compared to other radiopharmaceuticals for cerebral evaluation, makes its use in a global scale viable in the neurosciences and establishes great relevance to the correct interpretation of the data generated. In Brazil, for example, [^{18}F]FDG is the only positron emitting radiopharmaceutical approved for clinical diagnosis.

[^{18}F]FDG is a glucose analogue and basically consists of a glucose molecule containing a fluorine-18 radioisotope (half-life: 110 minutes) instead of a hydroxyl group at the C-2 position (**Figure 1a**). Accordingly, when it enters the cells, [^{18}F]FDG behaves exactly like a glucose molecule, presenting equivalent distribution and uptake, and receiving a phosphate by the action of a hexokinase. Phosphorylation of [^{18}F]FDG into [^{18}F]FDG-6-phosphate prevents the release of this molecule from the cell, in the same way as with glucose-6-phosphate (Suolinna *et al.*, 1986). However, unlike glucose, the [^{18}F]FDG molecule cannot follow the metabolic pathways and is effectively retained in the cell in the form of [^{18}F]FDG-6-phosphate, until the decay of the fluorine-18 radioisotope to Oxygen-18, which allows the entry of the molecule into metabolic routes (Reivich *et al.*, 1979) (**Figure 1b-c**).

The radiopharmaceutical [^{18}F]FDG enters cells as if it were a glucose molecule and accumulates; thus, cells using more glucose will have a higher accumulation of [^{18}F]FDG and cells with slower metabolism will accumulate less of it. The intracellular concentration of [^{18}F]FDG is, therefore, representative of the cell glucose uptake rate, and the quantification of [^{18}F]FDG in a tissue over a specific time period, combined with other factors, allows for the calculation of the rate of glucose absorption of the tissue (Wienhard, 2002).

The [^{18}F]FDG value lies on the fact that different cell types take up more glucose than others, creating contrast in the image. Tissues absorb glucose at different rates due their distinct needs. For example, brain tissue is highly active and has glucose as its main source of energy, therefore, requires a high influx of glucose. However, the rate of glucose uptake may change depending on the metabolic needs of the cells in different situations. For instance, skeletal muscle cells elevate their glucose uptake during exercise to sustain increased muscle contractions. These

underlying changes in glucose metabolism influence the intensity of the image. The [^{18}F]FDG-PET is, therefore, sensitive to changes in metabolism and allows its detection with reasonable spatial localization (Phelps *et al.*, 1979).

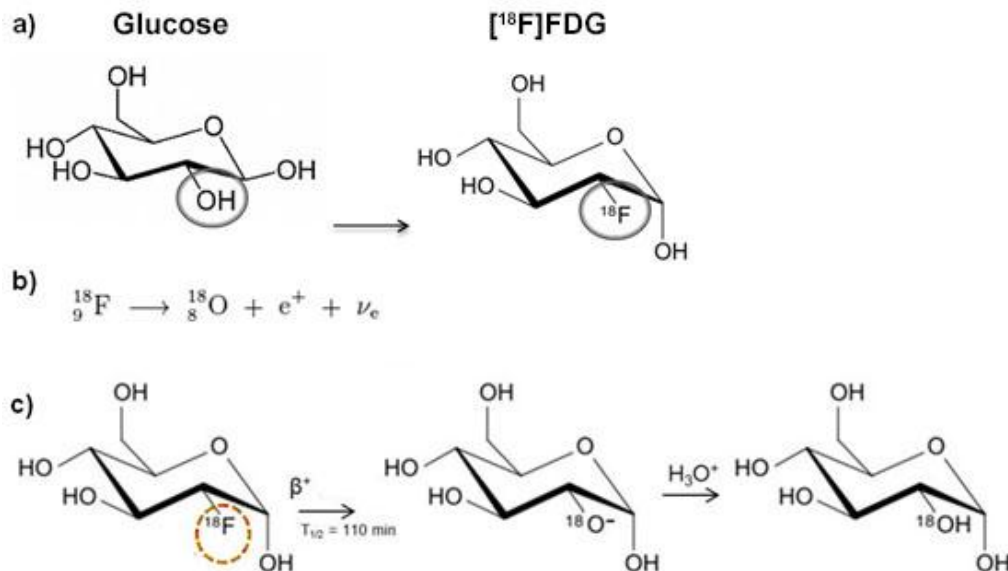


Figure 1. The [^{18}F]FDG molecule. a) [^{18}F]FDG molecule representation in comparison to the glucose molecule. b) Fluor-18 radioisotope β^{+} decay equation. c) [^{18}F]FDG molecule radioisotope β^{+} decay into a glucose molecule (containing an ^{18}O on the C-2 position).

The use of this radiopharmaceutical was validated for brain glucose metabolic rate assessment in 1979 (Phelps *et al.*, 1979) and until today is used to identify brain regions that are more or less active at baseline and in response to different stimuli, changes or damage. [^{18}F]FDG-PET can be applied to perform physiological studies of memory, cognition, among others (Greenberg *et al.*, 1981), as well as, to detect and diagnose various types of tumors and other pathologies of the human brain.

In recent years, the [^{18}F]FDG-PET has become one of the most important tools in the investigation of the brain, with applications in research, diagnosis and monitoring of several cerebral disorders and psychiatric conditions like epilepsy (Sarıkaya, 2015), dementia (Smailagic *et al.*, 2015), Huntington's Disease (Feigin *et al.*, 2001), Parkinson's disease (Poston and Eidelberg, 2010), head trauma (Alavi, 1989), multiple sclerosis (Blinkenberg *et al.*, 1999), ischemic lesions (Heiss *et al.*, 1993), schizophrenia (Seethalakshmi *et al.*, 2006), bipolar disorder (Altamura *et al.*,

2013), depression (Su *et al.*, 2014) and attention-deficit/hyperactivity disorder (Zametkin *et al.*, 1990), among others.

For example, in the case of dementia disorders, [^{18}F]FDG-PET has emerged as a useful tool due its wide applicability and has already been used in clinical practice on a large scale. Studies using [^{18}F]FDG provide not only evidence of the dementia process manifesting in the brain, but also allow the differentiation of dementia disorders types and their staging, since these pathologies have different metabolic patterns (Brown *et al.*, 2014). **Figure 2** presents the different patterns of hypometabolism found in dementia disorders that support the differential diagnoses of these pathologies.

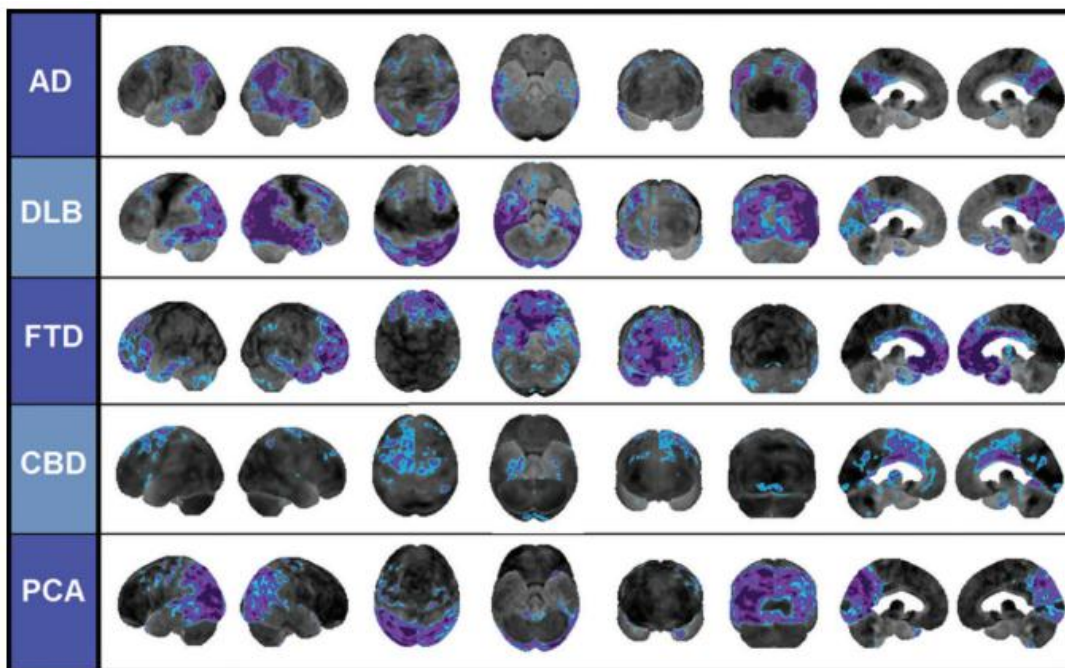


Figure 2. Patterns of hypometabolism in different dementias. Brain areas colored in purple and blue reflect regional patterns of hypometabolism normally found in patients. AD, Alzheimer's disease; DLB, Dementia with Lewis bodies; FTD, Frontotemporal Dementia; CBD, Corticobasal degeneration; PCA, Posterior cortical atrophy. Source: Brown *et al.*, 2014

When it comes to the interpretation of this technique in the brain at a cellular level, neurons are generally considered to have the highest energy consumption during brain activation and, as a consequence, it is assumed that the determination of metabolic rates from the signals of [^{18}F]FDG-PET directly reflects the neuronal use of glucose (Sokoloff *et al.*, 1977). We can again take dementia disorders as an example, on these pathologies the [^{18}F]FDG hypometabolism seen in certain brain

regions is generally interpreted as a result of neuronal dysfunction or death (Morbelli *et al.*, 2015).

The contribution of other brain cells to the [^{18}F]FDG-PET signal is still largely neglected, which becomes a dilemma, since, there are strong evidences pointing to an important contribution of astrocytes in cerebral glucose metabolism (Pellerin and Magistretti, 2012). The possible involvement and implications of astrocytes glucose uptake contribution to the [^{18}F]FDG-PET exam and similar techniques, like 2-deoxy-D-glucose autoradiography, have been denoted since the early 90's (Pellerin and Magistretti, 1994; Magistretti and Pellerin, 1996).

Years passed by and, more than 20 years later, the first *in vivo* evidence of the participation of astrocytes on [^{18}F]FDG-PET has emerged. Zimmer and co-workers (Zimmer *et al.*, 2017) used ceftriaxone to stimulate the glutamate transport on astrocytes, via GLT-1, and analyzed the brain glucose metabolic outcome in rodents by using [^{18}F]FDG-microPET, a PET scanner adapted to examining small animals. The ceftriaxone challenge led to an increase on [^{18}F]FDG signal, mostly in regions where GLT-1 is enriched (**Figure 3**), without changing the cerebral blood flow, suggesting that both responses were uncoupled. Additionally, they performed an *in vitro* approach using adult astrocyte cultures treated with ceftriaxone, observing similar results for [^3H]2DG uptake, alongside to an increase on glutamate transport. Furthermore, a recent study in Alzheimer's Disease patients found an association between astrocytic function, verified by ^{11}C -deuterium-l-deprenyl-PET and glucose metabolism, via [^{18}F]FDG-PET (Carter *et al.*, 2019).

The verdict about the brain glucose metabolism compartmentalization and astrocytes participation on [^{18}F]FDG-PET signal is still open. There are compelling evidences from both sides: a side proposing that astrocytes have an important role on glucose uptake and metabolism (Nehlig and Coles, 2007; Pellerin and Magistretti, 2012; Magistretti and Allaman, 2015); and a side claiming that their contribution is minor (Hertz, 2004; Dienel, 2013; 2017; Tang, 2018; Dienel, 2019).

For the time being, the most accepted interpretation still is that brain glucose metabolism, and, in turn, [^{18}F]FDG-PET signal, are mostly driven by neuronal synaptic activity with minor involvement of glial cells (Mosconi, 2013). At the same

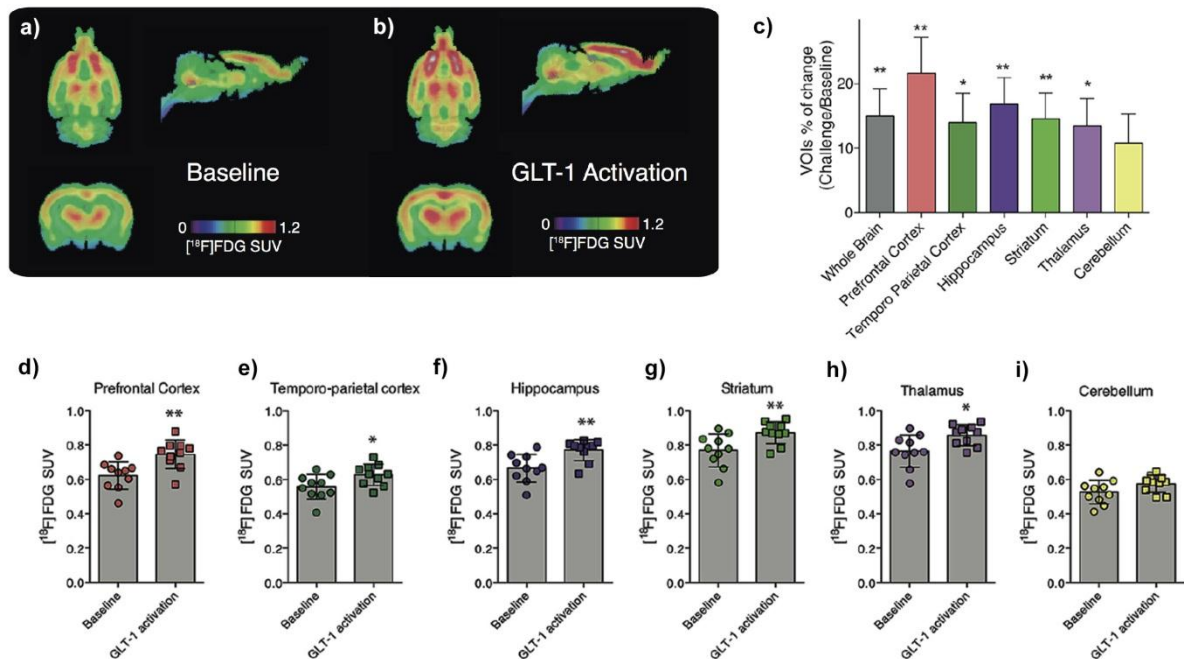


Figure 3. Effects of a ceftriaxone challenge on [^{18}F]FDG-PET results. (a) Averaged [^{18}F]FDG-PET maps of rats before receiving ceftriaxone. (b) Averaged [^{18}F]FDG-PET maps of rats after receiving ceftriaxone. (c) Regional percentage of change between baseline and ceftriaxone challenge on several brain volumes of interest (VOIs). Regional [^{18}F]FDG uptake in the (d) prefrontal (e) and temporo-parietal cortices (f), hippocampus (g), striatum (h), thalamus (i) and cerebellum. Data presented as standardized uptake value (SUV). Source: (Zimmer *et al.*, 2017)

time, astrocytes seem to be, slowly, being inserted into this picture of synaptic activity dictating brain glucose utilization (Stoessel, 2017).

Remains under debate the question whether a dysfunction of astrocytic, or other glial cell, origin could influence this process. The prevailing hypothesis is that the excitatory glutamatergic synapses activity are determinant on [^{18}F]FDG-PET, and therefore, a disturb on glutamatergic astrocytes-neurons coupling could be able to alter the [^{18}F]FDG signal outcomes (Bonvento *et al.*, 2002; Stoessel, 2017).

1.2 Glutamatergic System

The amino acid glutamate, or glutamic acid, is considered the main excitatory neurotransmitter in the central nervous system (CNS) of vertebrates (Orrego and Villanueva, 1993; Platt, 2007). Its importance on brain signaling is so extensive that

often the brain is referred to as a Glutamate/GABA machine (Sanacora *et al.*, 2012), being GABA the main inhibitory neurotransmitter. Glutamate, besides being responsible for much of the rapid excitatory neurotransmission, also seems to be the main mediator of sensory, cognitive, motor and emotional information (Bliss and Collingridge, 1993; Ozawa *et al.*, 1998; Dingledine *et al.*, 1999). It is estimated that about 70-80% of the brain synapses in mammals are glutamatergic (Watkins and Evans, 1981; Fairman and Amara, 1999).

Basically, glutamate is synthesized using, indirectly, the carbon skeleton derived from glucose in conjunction with donors of the amino group, such as other amino acids, ammonia or nucleotides. A major source of glutamate in the brain seems to be glutamine by action of the phosphate-activated glutaminase (Bak *et al.*, 2006). The produced glutamate is then accumulated in synaptic vesicles via vGLUTs (vesicular glutamate transporters) and can then be released at the presynaptic terminals through a calcium dependent mechanism (Birnbaumer *et al.*, 1994; Anderson and Swanson, 2000).

After its release into the synaptic cleft, glutamate is able to bind to pre- and postsynaptic receptors or be uptaken by glial or neuronal glutamate transporters. Glutamic acid exerts its action in basically two classes of receptors: ionotropic (ion channels) and metabotropic (G protein coupled). The class of ionotropic receptors, in turn, is divided into three types: N-methyl-D-aspartate (NMDA) receptors, alpha-amino-3-hydroxy-methyl -5-4-isoxazolepropionic acid (AMPA) and kainate receptors. Along with, the eight identified metabotropic receptors are also subdivided into three types, based on the homology of their sequences, the second messenger and pharmacology (Kew and Kemp, 2005).

Glutamate concentrations on the extracellular space are low and heavily controlled (Danbolt, 2001). Enzymes for degradation of extracellular glutamate have not been identified until the moment, so a phenomenon of extreme importance on the glutamatergic system is the glutamate uptake from the extracellular cleft by astrocytes or neurons. Imbalances on this regulatory system may have deleterious effects inducing hyperexcitability and excitotoxicity in post-synaptic neurons (Choi,

1994; Doble, 1999) or disturbing glutamatergic signaling, by altering the low signal-to-noise ratio required for specific signaling. Another important point in the glutamatergic reuptake are the economy and saving purposes, since the glutamate collected is, for the most part, recycled through the cycle glutamine-glutamate (Danbolt *et al.*, 2016), lessening the need of a new glutamate synthesis.

The reuptake of glutamate is performed by the Excitatory Amino Acid Transporters (EAATs). This process constitutes an important source of energy expenditure as it is coupled to ion transport (H^+ , Na^+ and K^+) and to the maintenance of an electrochemical gradient (Silver and Erecinska, 1997). To date, five distinct types of EAATs have been identified in mammals: GLAST (Glutamate Aspartate Transporter or also known as EAAT1), GLT-1 (glutamate transporter 1, also known

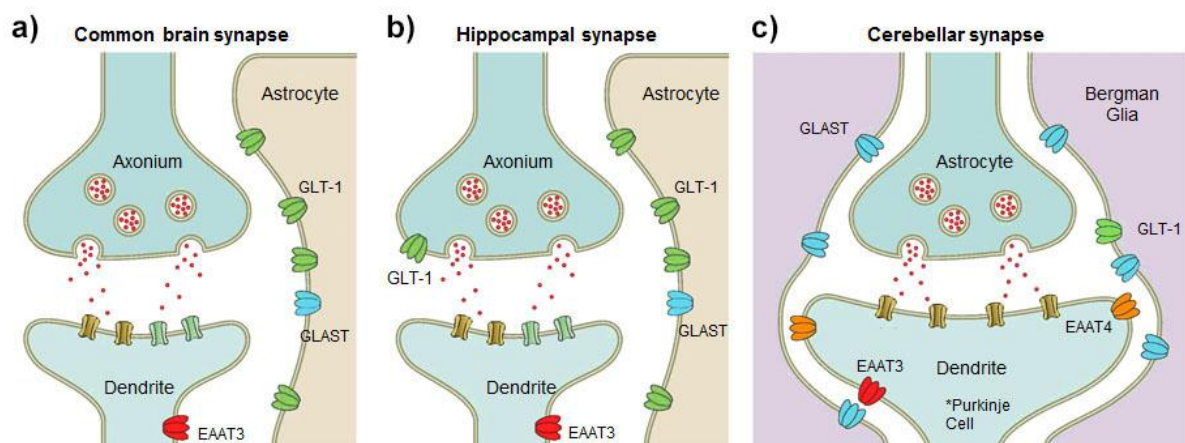


Figure 4. Glutamate transporters cellular distribution. Schematic diagram presenting the different types of EAATs predominant locations in the brain at the cellular level. a) EAATs distribution on most synapses of the brain. b) EAATs distribution on specific hippocampal synapses. c) EAATs distribution on specific cerebellar synapses. Source: modified from Vandenberg e Ryan, 2013.

as EAAT2), EAAC1 (Excitatory amino-acid carrier 1, also known as EAAT3), EAAT4 (Excitatory amino-acid transporter 4) e EAAT5 (Excitatory amino-acid transporter 5) (Tanaka, 2000).

The five types of glutamate transporters are differentially expressed in distinct regions of the brain and also in different cell types (**Figure 4**). Concerning to the transporters cellular expression, GLT-1 is found almost exclusively in astrocytes - with rare neuronal expression in specific regions, as in pyramidal cells of the CA3

region in the hippocampus; GLAST is present only in astrocytes; and EAAC1, EAAT4 and EAAT5 are found only in neuronal cells, with EAAC1 being expressed only in soma and dendrites, but not in axons (Danbolt *et al.*, 2016).

In terms of the CNS regional distribution, the EAAT5 transporter is found exclusively in retinal cells (photoreceptors and bipolar neurons), whereas EAAT4 is predominantly expressed in the cerebellum (in Purkinje cells), although it is also present in some neurons of the forebrain. Differently, EAAT3 is more dispersed, being found in most of the neurons in the CNS (Zhou and Danbolt, 2013). About the astrocytic transporters, GLT-1 is found all throughout the brain, but is significantly more abundant in the cortex, hippocampus and striatum. Meanwhile, GLAST is the main carrier in the cerebellum, inner ear, retina, among other region.

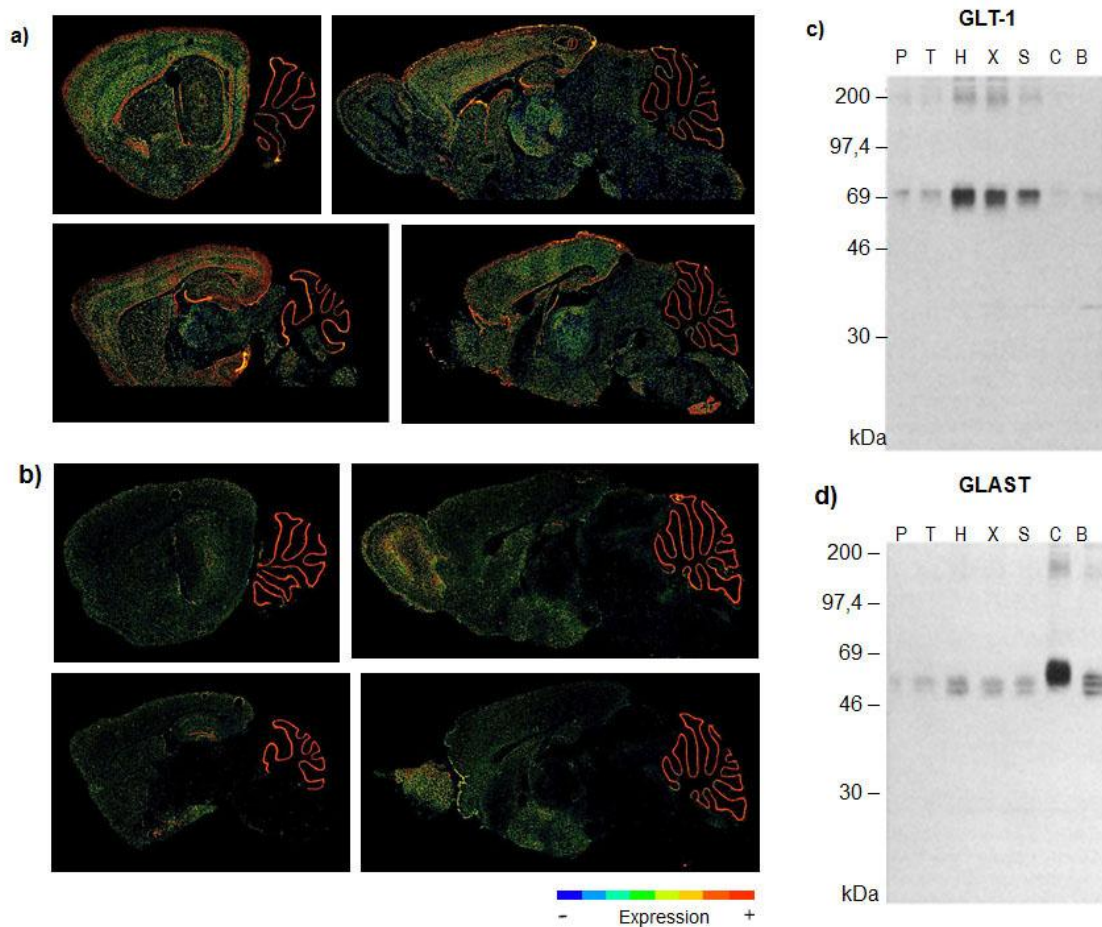


Figure 5. Glutamate transporters GLT-1 and GLAST brain distribution in rodents. Brain representative images of (a) GLT-1 and (b) GLAST mRNA expression in mice identified by in situ hybridization. Western Blot representative images for (c) GLT-1 and (d) GLAST, showing the density of these transporters in different regions on the rat brain. Regions: P, pons; T, thalamus and hypothalamus; H, hippocampus; X, cortex; S, striatum; C, cerebellum; B, olfactory bulb. Source: Allen Mouse Brain Atlas (<http://mouse.brain-map.org>) e Lehre et al., 1995.

In the cerebellum, where it is most concentrated, the expression of GLAST is about six times higher than that of GLT-1. In comparison, at the hippocampus GLT-1 is four times more abundant than GLAST (Lehre *et al.*, 1995; Danbolt, 2001; Vandenberg and Ryan, 2013; Zhou and Danbolt, 2013). A representation of the distribution of these transporters in the rodent brain can be seen on **Figure 5**.

Along with being the most abundant glutamate transporter on the brain - representing 1% of the total brain protein - GLT-1 is, by far, the most important on glutamate reuptake, being responsible for more than 90% of the uptake of this neurotransmitter in the brain (Holmseth *et al.*, 2009).

1.2.1 Astrocytic glutamate transport modulators

As already mentioned, glutamate uptake is a crucial function on brain homeostasis, and the majority of it is carried out by astrocytes, mainly through the transporter GLT-1, but also via GLAST. Over the years, a few molecules were found to be able to alter these transporters expression and function. In terms of stimulators, Andréia Fontana recently unrolled a full review, where she classifies the regulator molecules in three categories: proteins, endogenous molecules, and synthetic molecules (Fontana, 2015).

In the first category we find a group of kinases, the serine–threonine-specific kinases Akt (protein kinase B), mTOR (mammalian target of rapamycin) and GSK3 β (glycogen synthase kinase 3 β), and the tyrosine kinase JAK2 (Janus kinase 2). All showed enhancing effects on the expression of GLT-1.

Among the endogenous molecules we find hormones, second messengers, metabolites and a neurotransmitter: guanosine, epidermal growth factor, dBcAMP (dibutyryl-adenosine 3',5'-cyclic monophosphate), corticosterone, retinol, estradiol and histamine. Dibutyryl-cAMP is a cell-permeable cyclic AMP (cAMP) analog that activates cAMP-dependent protein kinases and is widely used in primary astrocytic cultures derived from newborns rodents, to enhance the expression of GLT-1.

Finally, a few drugs were identified as glutamate transport stimulators: Parawixin1, the corticosteroid Dexamethasone, the anticonvulsant Valproic acid, the

antibiotics Minocycline and Ceftriaxone, the antidepressant Amitriptyline, the β -lactamase inhibitor Sulbactam, Tamoxifen, and Harmine. Within this group, the most studied drug is Ceftriaxone, which the specific effect on GLT-1 was first described in 2005 (Rothstein *et al.*, 2005). Because of its specificity on the brain, strong effect and easy administration, Ceftriaxone was used by Zimmer and co-workers (Zimmer *et al.*, 2017) to test astrocytic glutamate transport effects on [^{18}F]FDG-PET results.

The excitotoxicity caused by excessive glutamate in the extracellular cleft is a common feature in different neurological disorders. Therefore, the above mentioned molecules, and their effects on glutamate transport, are of great relevance, presenting the potential applicability in disease prevention and treatment. Many of them already showed neuroprotective effects on research. The drug Riluzole is the only modulator of glutamate transport already approved for clinical use (Fontana, 2015).

On the other hand, a couple of glutamate transport inhibitors were also described in the literature. No therapeutic applications were found yet, but these molecules are of great interest for experimental approaches to investigate the glutamatergic system influence on many brain functions and disorders.

The list of known glutamate transport negative regulators is short, presenting not even half of members if compared to the positive regulators list. Until now, have been described: Clozapine, Dihydrokinic acid (DHK), DL-TBOA (dl-threo- β -benzyloxyaspartic acid), β -THA (DL-threo- β -Hydroxyaspartic acid), L- β -TBOA (L- β -benzyl-aspartate), PDC (L-trans-pyrrolidine-2,4-dicarboxylate) and WAY-855 (3-amino-tricyclo-heptane-1,3-dicarboxylate) (Soni *et al.*, 2014).

DHK is a GLT-1 specific and competitive inhibitor, being used in research for decades. DHK is the saturated analogue of the naturally occurring and potent neuroexcitatory amino acid kainic acid (Johnston *et al.*, 1979), being so, DHK also presents effects on glutamate ionotropic receptors. It is important to note that DHK permeability to the blood-brain barrier (BBB) is very low, so DHK is capable of crossing the BBB but the concentrations in the brain are low after systemic dosing

(Gynther *et al.*, 2015). For this reason, in many studies using DHK, the intracerebroventricular infusion is chosen as administration route.

β -THA and PDC are broad-spectrum EAATs blockers, with equal affinity for GLT-1, GLAST and EAAT3. L-b-TBOA, however, is EAAT3 selective (Esslinger *et al.*, 2005). DL-TBOA and WAY-855 are also non-substrate broad-spectrum EAATs blockers with effects on GLT-1, GLAST and EAAT3, but presenting a higher affinity for GLT-1 (Shimamoto *et al.*, 1998), (Dunlop *et al.*, 2003). However, WAY-855 is a new molecule and, beyond its characterization, has not yet been used in research. PDC has also presented depressant effects on excitatory synaptic transmission (Maki *et al.*, 1994).

Finally, clozapine is an atypical antipsychotic agent with low affinity for dopamine D1 and D2 receptors, comparatively high affinity for D4 receptors and high affinity for serotonin (5-HT) subtypes 2A, 2C, 6 and 7, histamine H1 receptors and muscarinic acetylcholine receptors (Naheed and Green, 2001). Additionally, clozapine has been shown to reduce up to 60% GLT-1 density and function on the cortex of rats (Melone *et al.*, 2001). Following studies showed that: GLAST and EAAT3 were not affected by clozapine in the rat cerebral cortex (Melone *et al.*, 2003); and that effect of clozapine on GLT-1 density and glutamate uptake is replicated on astrocytic cultures, suggesting that these cells are directly involved in this phenomenon (Vallejo-Illarramendi *et al.*, 2005).

1.3 Astrocytes and glucose metabolism

Astrocytes are abundant glial cells in the brain and participate in a number of important activities to the maintenance of proper brain function. These cells contribute for the BBB and extracellular matrix formation, participate in the maintenance of extracellular ionic and chemical homeostasis, are involved in the response to injury and affect neuronal development, signaling and plasticity (Montgomery, 1994; Markiewicz and Lukomska, 2006). The important roles of astrocytes have been increasingly recognized and new interactions and functions of these cells are identified year after year.

In the mid-1990s, Pellerin and Magistretti were responsible for proposing a new and remarkable astrocytic contribution to brain energetics. They observed in astrocyte primary cultures that the presence of the glutamate neurotransmitter significantly stimulates the uptake of glucose and the release of lactate (Pellerin and Magistretti, 1994).

In this seminal work, they identified that glutamate stimulates aerobic glycolysis by astrocytes and also suggested that the released lactate is sent to neurons, where it will be used by oxidative pathways to generate energy. It is also important to note that the effect of glutamate on astrocytes was not inhibited by glutamate receptors antagonists, but rather by specific inhibitors of glutamate transporters or Na^+, K^+ -ATPase. This findings indicate that the trigger for increased glucose uptake is the glutamate transport and that its effect is Na^+, K^+ -ATPase dependent.

On the following year, the glutamate effect on astrocytic glucose uptake was replicated by other important research group, that also evaluated the influence of electrochemical gradient variants on the process, observing an important role for Na^+, K^+ -ATPase and Na^+ channels (Takahashi *et al.*, 1995).

The theory based initially on these results is called the Astrocyte-Neuron Lactate Shuttle (ANLS) (Pellerin *et al.*, 1998) and in the last 24 years has accumulated a large number of new evidences. **Figure 6** presents a simplified ANLS scheme.

A most recent work, using Forster Resonance Energy Transfer (FRET) glucose sensors, observed a role for potassium as a rapid and transient stimulator for astrocytic glycolysis, while glutamate presents a delayed, but long-lasting, effect (Bittner *et al.*, 2011).

Additionally, the role of glutamate transport as a trigger for glucose uptake in astrocytes has also been observed *in vivo*. For example, studies analyzing the [^{14}C]-2-deoxyglucose ([^{14}C]2DG) metabolism on mice with GLAST downregulation or knockout mice for GLAST and GLT-1, found a decrease on [^{14}C]2DG uptake response to somatosensory activation (Cholet *et al.*, 2001; Voutsinos-Porche *et al.*,

2003). Also, a study using two-photon microscopy and the glucose analog 6-deoxy-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-aminoglucose on rats showed that glucose uptake on astrocytes is increased in response to whiskers stimulation while the neuronal glucose uptake remains unchanged (Chuquet *et al.*, 2010).

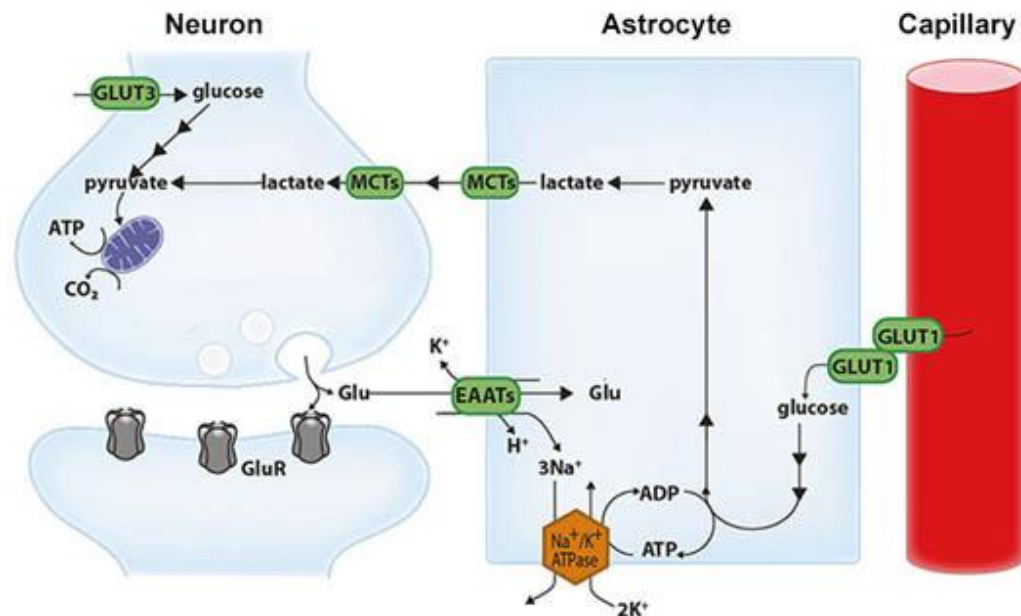


Figure 6. Astrocyte Neuron Lactate Shuttle. ANLS representative scheme demonstrated on a tripartite synapse. Glutamate (Glu) released into the synaptic cleft by the presynaptic neuron can act at the glutamate (GluR) receptors and/or be captured by the astrocyte through the glutamate transporters (EAATs). The transport of glutamate by EAATs is coupled to the transport of Na^+ , H^+ and K^+ ions. The maintenance of these ions gradients is performed by a Na^+/K^+ pump with the ATP expenditure. The astrocyte, located closest to the vessel, captures glucose (GLUT1 transporter) and uses this molecule to generate ATP through a process of aerobic glycolysis, generating lactate. Lactate is released into the extracellular medium by the monocarboxylate transporters (MCTs). Lactate in the extracellular medium can be captured by the neuron also through the MCTs. Once inside the neuron, the lactate can be converted to pyruvate and oxidized to generate energy. The neuron can also uptake glucose (GLUT3 transporter) directly and perform the glycolysis and oxidation of this molecule to generate energy. Source: Modified from (Belanger *et al.*, 2011).

Also, Itoh and co-authors investigated the use of labeled glucose and lactate in cultures of astrocytes and neurons. Neuronal cultures rapidly oxidized both glucose and lactate, while astrocyte cultures oxidized the two substrates moderately and metabolized glucose predominantly to lactate. In addition, a large increase in glucose concentration in the medium inhibited lactate oxidation by astrocytes but not by neurons, indicating a preference for lactate oxidation in neurons (Itoh *et al.*, 2003).

Finally, perhaps the most important study supporting astrocytes participation on glucose uptake was performed by Nehlig and coworkers, using $[^{14}\text{C}]2\text{DG}$ and applying a microautoradiographic imaging procedure, combined with

immunohistochemistry, they observed that brain neurons only take up about half of the [^{14}C]2DG while approximately 53% enters into astrocytes (Nehlig *et al.*, 2004). Ultimately, Barros and co-workers pointed a remarkable and strong interpretation for the ANLS existence assuming an evolutionary perspective: ANLS function would bring the crucial advantage of saving oxygen for neurons (Fernandez-Moncada *et al.*, 2018).

In addition to all functional observations supporting the ANLS, there are also structural evidences. Astrocytes have prolongations enriched in glucose transporters that extend to the blood vessels and embrace them (Leino *et al.*, 1997; Duelli and Kuschinsky, 2001). In this way, glucose uptake appears to be facilitated in these cells, although, glucose can also diffuse easily through the extracellular space. Furthermore, astrocytes appear to have a participation in the regulation of cerebral blood flow (Macvicar and Newman, 2015).

The mentioned studies and several others indicate an important astrocytic role in brain energetic metabolism and corroborate the ANLS theory. On the other hand, there is also a great amount of opposing evidences to the ANSL existence or real importance as an *in vivo* phenomenon, which should be noted. For example, in contrast with the proposed glycolytic metabolism stimulation by glutamate on astrocytes, an early work showed an increase, also Na^+, K^+ -ATPase dependent, on oxidative processes in astrocytes cultures, when exposed to glutamate (Eriksson *et al.*, 1995).

Concerning brain ATP usage, a study, using careful mathematic models, estimates that, in rodents cerebral cortex, the glia is only responsible for 5% of the brain energy consumption in signaling, which is attributed to use on glial resting potential, glutamate uptake and its conversion to glutamine (Attwell and Laughlin, 2001). It is important to note, however, that this value is related to signaling energy and, therefore, not include the estimated 25% of energy expenditure on “housekeeping tasks”. Importantly, they also use an oxidative perspective, neglecting the possibility that some processes may be driven mainly by glycolysis. However, modeling and estimating brain energy usage is very a complex task and there is no consensus in the literature yet, different results were calculated to regions like the

cerebellum (Howarth *et al.*, 2010) and higher values for glia function at the human cortex were also proposed by other studies (Lennie, 2003).

Regarding shuttling of lactate from astrocytes to neurons, a key concept on the ANLS theory, a few studies also proposed preferential glucose consumption in neurons, in detriment of lactate (Patel *et al.*, 2014; Lundgaard *et al.*, 2015). Also, as cleverly recalled by Morelli and Panfoli, the use of large amounts of lactate shuttled to neurons would demand large amounts of the cofactor NAD⁺ (Nicotinamide adenine dinucleotide), what can be, on a biochemical perspective, problematic to rapidly and constantly obtain (Barros and Weber, 2018a).

Moreover, a point always mentioned to refute the ANLS is the existence of evidence showing that glutamate can be oxidized in astrocytes, therefore, providing the energy necessary to its own uptake (Dienel, 2013; Mckenna, 2013; Dienel *et al.*, 2018). As a consequence, it is proposed that an increase on astrocytes glucose uptake as response to glutamate uptake, would not be necessary.

For further considerations about the ANSL subject please see the CrossTalk section recently proposed at The Journal of Physiology, on which an extraordinary debate about this theme was carried out by leading researchers on the field (Bak and Walls, 2018a; b; Barros and Weber, 2018b; a).

2. OBJECTIVE

Investigate the influence of glutamate astrocytic transport on cerebral glucose metabolism and [^{18}F]FDG-PET signal, using a pharmacological challenge with clozapine, in adult rats.

2.1 Specific objectives

- I. Evaluate, via [^{18}F]FDG-microPET, the effects of clozapine on glucose metabolism *in vivo*;
- II. Analyze, *ex vivo*, the impact of clozapine on astrocytic glutamate transporters levels and activity;
- III. Analyze, *in vitro*, the effects of clozapine on glucose uptake, glutamate uptake and glutamate transporters levels on astrocyte primary cultures.

PART II

Manuscript in preparation to be submitted to the Molecular Metabolism Journal as a full-length article.

Note 1: The journal formatting norms can be found in the Attachment A.

Note 2: The journal requires numbered sections. Thus, the numbering of the manuscript does not match the numbering of the dissertation.

PART III

4. Discussion

In the past decades, the importance of glial cells on brain function is being gradually recognized. Astrocytes, for example, are now well established as key players on crucial brain functions such as formation of synapses, ionic and chemical gradients maintenance, signaling regulations, neurotransmitters production, among others (Montgomery, 1994; Araque *et al.*, 1999; Newman, 2003; Markiewicz and Lukomska, 2006). Despite that, their participation on brain energetics seems to be, somehow, a more delicate topic of discussion and astrocytes are still neglected when come to this topic (Bonvento *et al.*, 2005).

However, this debate has implications on the [^{18}F]FDG-PET assessment of brain glucose metabolism. PET, and the use of [^{18}F]FDG as a radiotracer for this technique, were introduced sequentially in 1974 (Phelps *et al.*, 1975) and 1978 (Reivich *et al.*, 1979). From then on, [^{18}F]FDG-PET has become one of the most important tools to brain examination, establishing applicability on several brain disorders study and diagnosis. Through the years, the role of astrocytes on the [^{18}F]FDG-PET signal was questioned a few times (Magistretti and Pellerin, 1999; Magistretti, 2000; Sestini, 2007; Figley and Stroman, 2011), however, at the best of our knowledge, the work of Zimmer *et al.* (Zimmer *et al.*, 2017) and the present study are the first ones to try directly address this question using the technique itself.

Zimmer and coworkers investigation of the astrocytic participation on [^{18}F]FDG-PET was carried out based on the modulation of a signal that acts as trigger to the elevation on glucose uptake by astrocytes (Pellerin and Magistretti, 2012): the glutamate uptake via GLT-1.

GLT-1 is the most abundant glutamate transporter in the brain, being responsible for over 90% of the glutamate uptake in the whole brain (Zhou and Danbolt, 2013). It is found essentially only in astrocytes (see section 1.2 - Part I), therefore, qualifying as proper target for testing the astrocytes contribution on brain energetics. To test their hypothesis, Zimmer used a pharmacological intervention with the drug ceftriaxone, a known stimulator of GLT-1 (see section 1.2.1 - Part I).

Ceftriaxone was able to increase [^{18}F]FDG-PET signal mostly in regions where GLT-1 is enriched. Besides that, until now, the only significant observed effect of ceftriaxone, on the dose and schedule of administration used, is the action on GLT-1. That being sad, the straightforward interpretation is that ceftriaxone caused an increase in the glucose metabolism through its effects on the astrocytic glutamate

transport. Notwithstanding, they also isolated adult astrocytes and tested the effect of ceftriaxone on glucose uptake directly into these cells. The results on the astrocytic culture corroborated the *in vivo* observations. In conclusion, their evidence supports the hypothesis of a significant astrocytic participation on the [¹⁸F]FDG-PET signal.

On the other side, these observations only show one side of the story: astrocytes can increase the [¹⁸F]FDG-PET signal. Then comes the question: could astrocytes decrease the [¹⁸F]FDG-PET signal?

Inspired by the above mentioned work, we searched in the literature a drug that could inhibit or decrease GLT-1. Initially, DHK seemed to be a good choice because it has been investigated for years and is a well-established specific GLT-1 inhibitor. However, DHK needs to be administered directly in the CNS because it has low BBB permeability (Gynther *et al.*, 2015). The need of an intracerebroventricular or intrathecal injection imposes an obstacle to the use of DHK on an imaging approach. The use of an invasive procedure as the intracerebroventricular infusion can cause significant inflammation and can alter the imaging results.

So, as an alternative, the drug clozapine was chosen. Clozapine can be easily administered in the drinking water and provokes an important reduction on glutamate uptake and down-regulation of GLT-1, but not GLAST (Melone *et al.*, 2003), both *in vitro* (Vallejo-Illarramendi *et al.*, 2005) and *in vivo* (Bragina *et al.*, 2006). Meloni and coworkers describe the effect as being stronger in the cortical region but also mention hippocampus, striatum, and thalamus as being slightly affected (Melone *et al.*, 2001).

In our study, clozapine treatment caused an important *in vivo* reduction on [¹⁸F]FDG uptake at the cortex, reflecting a glucose hypometabolism. At the same time, the brain metabolic network was strongly altered, suggesting that the association and organization of glucose metabolism through the brain regions is uncoupled. Being GLT-1 most abundant on the cortical region, the predominant effect on this region corroborates our hypothesis.

The expected clozapine-induced reduction on GLT-1 immunoccontent and expression was confirmed by SDS-PAGE and qRT-PCR. Additionally to the reduction on the transporter levels, the reduction on the glutamate transport function itself was confirmed by the decreased [³H]DAsp uptake on cortical slices.

In parallel, we questioned if the reduced astrocytic glutamatergic transport could impair the glutamate-glutamine cycling and alter the glutamate release on

synaptic terminals. This speculation was ruled out because no alterations on the release of glutamate were verified on synaptosomal preparations. Moreover, the glutamatergic system seems not to be affected in a whole brain scale because changes on LCR glutamate and related aminoacids content were not observed.

We also asked ourselves if the downregulation of the most important glutamate transporter in the brain, as seen on the assessment of the cortex, could have generated a compensatory up-regulation of GLAST, the only other astrocytic glutamate transporter. Even though, being considerable less abundant and significant to glutamate uptake on the analyzed region (Danbolt, 2001), a robust up regulation of GLAST could result in a counterbalance on the astrocytic glucose uptake, confusing our analysis. However, the evaluation of GLAST density and expression showed no differences, which ruled out this compensatory effect.

The *in vitro* evaluation on newborn derived astrocyte cultures treated with clozapine brought us the information that the drug effect on glucose uptake can be observed on astrocytes alone, without the presence of other brain cells. In this way, corroborating our hypothesis by strongly supporting the idea that astrocytes are the cells responsible by the phenomenon seen *in vivo*. The fact that, on cell culture, the reduction on [³H]2DG uptake by clozapine was only observed when in the presence of higher values of glutamate agrees with the ANLS theory. ANLS proposes that astrocytes increase their glucose uptake in situations of neuronal firing. Our observation suggests that clozapine is preventing the triggering effect of glutamate transport on astrocytes on a firing situation but not at rest.

Additionally, following the *in vivo* analysis, we isolated in cultures astrocytes originated from the cortex of the same group of animals receiving clozapine or control solution analyzed previously on [¹⁸F]FDG-PET. It is relevant to raise the fact that clozapine was not added to the culture and that our investigation in adult astrocytes relied on the continuity of clozapine effects through the culture process in the absence of the drug. However, it seems that clozapine effects did not fully persist through the 5 weeks of culture, as no differences were found in any of the carried out analyses.

It is also important to notice the valuable information that the open field test brought us, presenting no differences in the spontaneous activity and implying that no sedative effect (Prut and Belzung, 2003) is associated to our pharmacological manipulation and to the resulting cortical hypometabolism, as could be suggested

(Berti *et al.*, 2014). In addition, it would be expected whole brain hypometabolism if associated to a sedative effect. A more complex behavior was also not altered, as can be seen by the results in the ORT, with no detectable alterations on the memory performance between groups.

Importantly, we are well aware of the effects of clozapine on serotonin, dopamine and histamine receptors (Naheed and Green, 2001). However, because the serotonergic, dopaminergic and histaminergic systems are not recognized as significant contributors to brain energetics, this effect was not judged to be a relevant mechanism explaining our observations. The brain is a big glutamate/GABA machine, with other systems contributing to its function in a much lower extent. There are several studies showing that brain energetic metabolism is mainly driven by glutamatergic and, in a minor degree, gabaergic systems, with the contributions of others neurotransmitter being so small that can be ignored in the overall scenario (Attwell and Laughlin, 2001; Howarth *et al.*, 2010). Regardless the above mentioned information, we cannot completely rule out the possibility that clozapine could be affecting the [¹⁸F]FDG-PET signal through altering these neurotransmitter systems, or via others known or yet unknown effects of the drug, beside the astrocytic mechanism proposed by us. On the other hand, we can argue that [¹⁸F]FDG-PET results being replicated on astrocyte cultures by the [³H]2DG uptake strongly suggests these cells are involved on the process.

We anticipate and encourage our investigation to be a starting point for more sophisticated investigations on the astrocytic contribution to [¹⁸F]FDG-PET and on the possible supporting mechanisms. Other pharmacological challenges with inhibitors of glutamate transport, like Dihydrokinic acid and the new WAY-855 (3-amino-tricyclo- heptane-1,3-dicarboxylate), could be tested to see if they reproduce the same effect of clozapine. Also, to gain more specificity, genic interventions, like the knockdown of GLT-1 and GLAST, would be prominent to further test the cellular origin of [¹⁸F]FDG-PET.

This investigation has direct implication in the clinic, since the [¹⁸F]FDG-PET has been used as a metabolic index of neuronal activity. More specifically, these findings have direct consequences on the interpretation of images with [¹⁸F]FDG-PET in brain diseases. For example, hypometabolic regions captured by [¹⁸F]FDG-PET are features present in several neurodegenerative and neuropsychiatric diseases. Thus, these metabolic signatures may be indicating astrocytic dysfunction and not

necessarily neuronal dysfunction, which potentially challenges the current concept of image interpretation with [^{18}F]FDG-PET.

Furthermore, clozapine is an important antipsychotic drug widely used in patients with schizophrenia who are either intolerant or refractory to classical neuroleptics. Clozapine mechanisms of action are yet not fully understood, therefore, the effect on brain energetics observed in our study can be of importance and should be better studied on this perspective. As matter of fact, it is not the first time that an effect of clozapine on brain [^{18}F]FDG-PET are reported, a hypometabolism was also found in schizophrenic patients under clozapine treatment (Cohen *et al.*, 1997; Molina *et al.*, 2007).

It is relevant to note that we are not proposing that neurons are unimportant on [^{18}F]FDG-PET, or that the synaptic activity, directly or indirectly, is not the main driver of the signal. We are only implying that neurons are not only ones to drive the signal. Based on previous studies and on our observations we suggest that astrocytes can significantly influence the brain metabolism and, as consequence, [^{18}F]FDG-PET. Meaning by that: maybe a dysfunction solely in astrocytes could independently affect the exam results.

Our observations do not question the validity of the [^{18}F]FDG-PET technique and other techniques based on the uptake of glucose analogs in vivo in the brain. Our research, in fact, seeks to clarify the cellular bases for these procedures and to allow a better understanding of the fascinating results that these techniques offer.

5. Conclusion and Perspectives

Finally, as conclusion of our work, we believe that our results strongly suggest that astrocytes can influence [^{18}F]FDG-PET. However, to validate and corroborate our observation in this work, further investigations should be conducted. In this project, we still intend to test clozapine effects on glucose uptake of neuronal cultures. Additionally, experiments using ceftriaxone and clozapine together are being conducted in astrocytes culture to verify if ceftriaxone can counteract the effects of clozapine.

To conclude, we would like to express that we are grateful to the researchers the firstly proposed the ANLS theory, and grateful to all supporters and opposing viewers for the substantially knowledge generated over the years around this discussion. ANLS served as a starting point to a series of great studies that

continues for more than decades. No matter if supporting or refuting the hypothesis, all the studies and discussed ideas, somehow, contributed to a better understanding of the brain, neuronal and astrocytic function. This author hopes that the observations here presented can contribute to this debate.

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ATTACHMENT A – MANUSCRIPT NORMS



Article type

Full-length Article: These present conceptual advances regarding a biological/clinical question of wide interest to the journal's readership. These manuscripts should be around 8500 words for the main text with no more than 8 figures and/or tables. Additional items may be published online as Supplemental Data.

Article structure

Subdivision - numbered sections

Divide your article into clearly defined and numbered sections. Subsections should be numbered 1.1 (then 1.1.1, 1.1.2, ...), 1.2, etc. (the abstract is not included in section numbering). Use this numbering also for internal cross-referencing: do not just refer to 'the text'. Any subsection may be given a brief heading. Each heading should appear on its own separate line.

Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods

Provide sufficient detail to allow the work to be reproduced. Methods already published should be indicated by a reference: only relevant modifications should be described.

Results

Results should be clear and concise.

Discussion

This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions

The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Essential title page information

- Title. Concise and informative. Titles are often used in information-retrieval systems. Avoid abbreviations and formulae where possible.
- Author names and affiliations. Where the family name may be ambiguous (e.g., a double name), please indicate this clearly. Present the authors' affiliation addresses (where the actual work was done) below the names. Indicate all affiliations with a number immediately after the author's name and in front of the appropriate address. Provide the full postal address of each affiliation, including the country name and, if available, the e-mail address of each author.
- Corresponding author. Clearly indicate who will handle correspondence at all stages of refereeing and publication, also post-publication. Ensure that phone numbers (with country and area code) are provided in addition to the e-mail address and the complete postal address. Contact details must be kept up to date by the corresponding author.
- Present/permanent address. If an author has moved since the work described in the article was done, or was visiting at the time, a 'Present address' (or 'Permanent address') may be indicated as a footnote to that author's name. The address at which the author actually did the work must be retained as the main, affiliation address. Superscript Arabic numerals are used for such footnotes.

Structured abstract

A structured abstract, by means of appropriate headings entitled "Objective"; "Methods"; "Results"; "Conclusions", should provide the context or background for the research and should state its purpose, basic procedures or study design (selection of study subjects or laboratory animals, observational and analytical methods), main findings (giving specific effect sizes and their statistical significance, if possible), and principal conclusions. It should emphasize new and important aspects of the study or observations.

Image manipulation

Whilst it is accepted that authors sometimes need to manipulate images for clarity, manipulation for purposes of deception or fraud will be seen as scientific ethical abuse and will be dealt with accordingly. For graphical images, this journal is applying the following policy: no specific feature within an image may be enhanced, obscured, moved, removed, or introduced. Adjustments of brightness, contrast, or color balance are acceptable if and as long as they do not obscure or eliminate any information present in the original. Nonlinear adjustments (e.g. changes to gamma settings) must be disclosed in the figure legend.

General points

- Make sure you use uniform lettering and sizing of your original artwork.
- Embed the used fonts if the application provides that option.
- Aim to use the following fonts in your illustrations: Arial, Courier, Times New Roman, Symbol, or use fonts that look similar.
- Number the illustrations according to their sequence in the text.
- Use a logical naming convention for your artwork files.
- Provide captions to illustrations separately.
- Size the illustrations close to the desired dimensions of the published version.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (not on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and

ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules.

References

Citation in text.

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

Reference style

Text: Indicate references by number(s) in square brackets in line with the text. The actual authors can be referred to, but the reference number(s) must always be given.

List: Number the references (numbers in square brackets) in the list in the order in which they appear in the text.