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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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Co-advisor *(Coorientador):* **Prof. Dr. Diogo Onofre Gomes de Souza**

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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

PART I

PART I

ABREVIATIONS LIST

[^{14}C]2DG - [^{14}C]-2-Deoxyglucose

[^{18}F]FDG - 2-Deoxy-2-[^{18}F]Fluoroglucose

[^{3}H]2DG - [^{3}C]-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, consequentemente, na captação da molécula de [^{18}F]Fluorodeoxyglucose ([^{18}F]FDG), detectada pela tomografia por emissão de pósitrons (PET).

O [^{18}F]FDG-PET não apresenta resolução celular, entretanto, a imagem resultante reflete interações celulares e sub-celulares com o radiofármaco. Até recentemente, a captação de [^{18}F]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 [^{18}F]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 [^{18}F]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 [^{18}F]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 [^{18}F]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 [^{18}F]FDG-PET são interpretados, salientando a pontencial 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 [^{18}F]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-[^{18}F]fluoroglucose ([^{18}F]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 [^{14}C]-2-deoxyglucose ([^{14}C]2DG) (Kennedy *et al.*, 1975), [^{18}F]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 [^{18}F]FDG on tumoral investigation has stimulated the spread of this molecule and PET itself around the globe. Thus, [^{18}F]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).

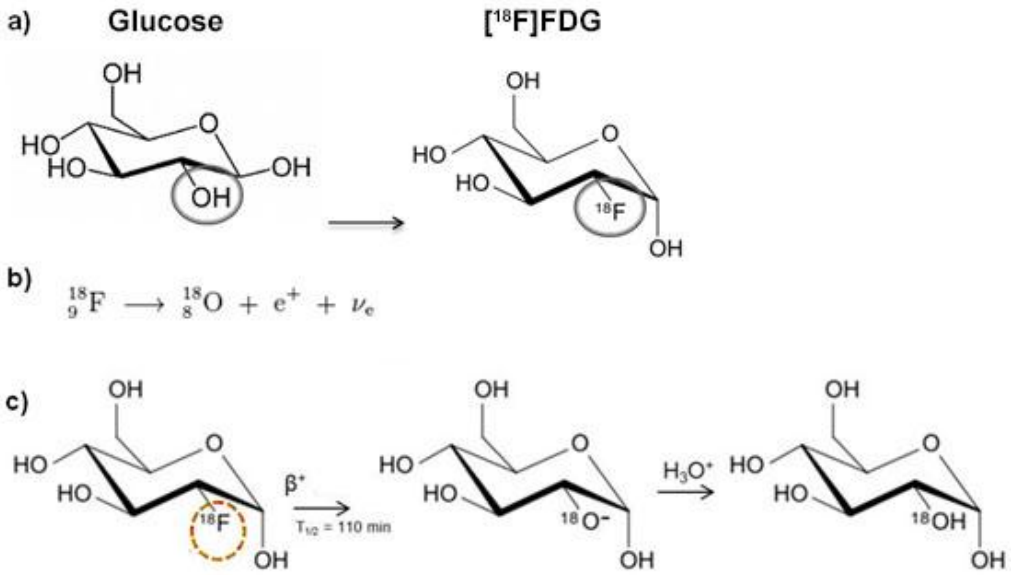

Figure 1. The [^{18}F]FDG molecule. a)[^{18}F]FDG molecule representation in comparison to the glucose molecule. b) Fluor-18 radioisotope β+ decay equation. c) [^{18}F]FDG molecule radioisotope β+ decay into a glucose molecule (containing an ^{18}O on the C-2 position).

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 (Sarikaya, 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.

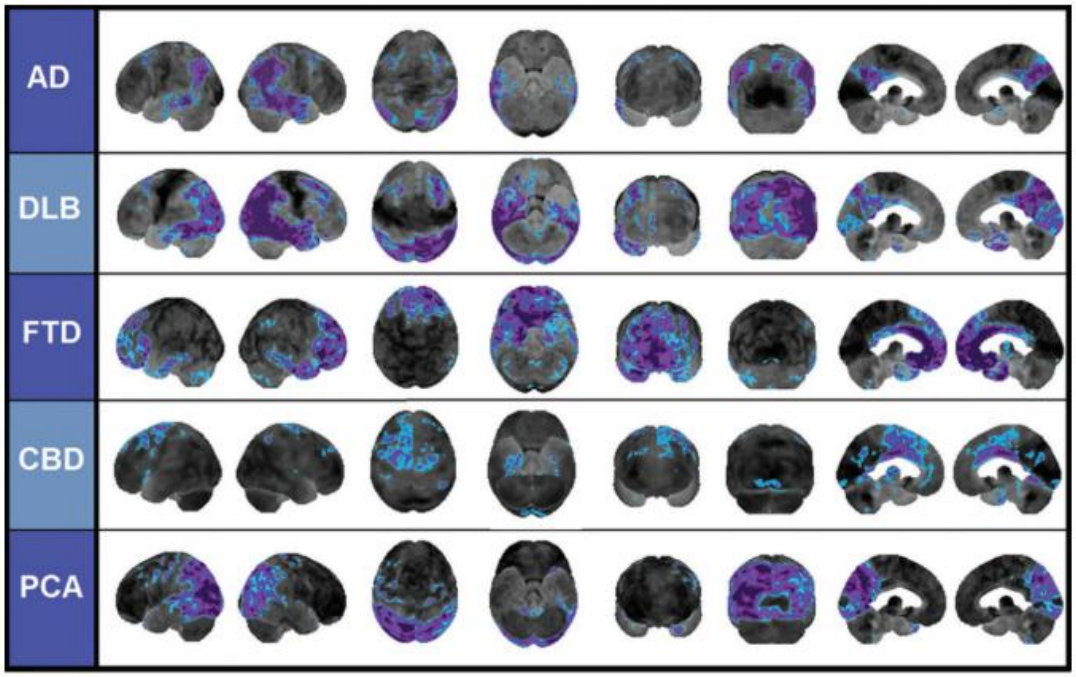

Figure 2. Patterns of hypometabolism in different dementias. Brain areas colored in purple and blue reflect regional patterns of hypometabolism normally found in patients. AD, Alzheimer's disease; DLB, Dementia with Lewis bodies; FTD, Frontotemporal Dementia; CBD, Corticobasal degeneration; PCA, Posterior 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 [^{3}H]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 11C-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

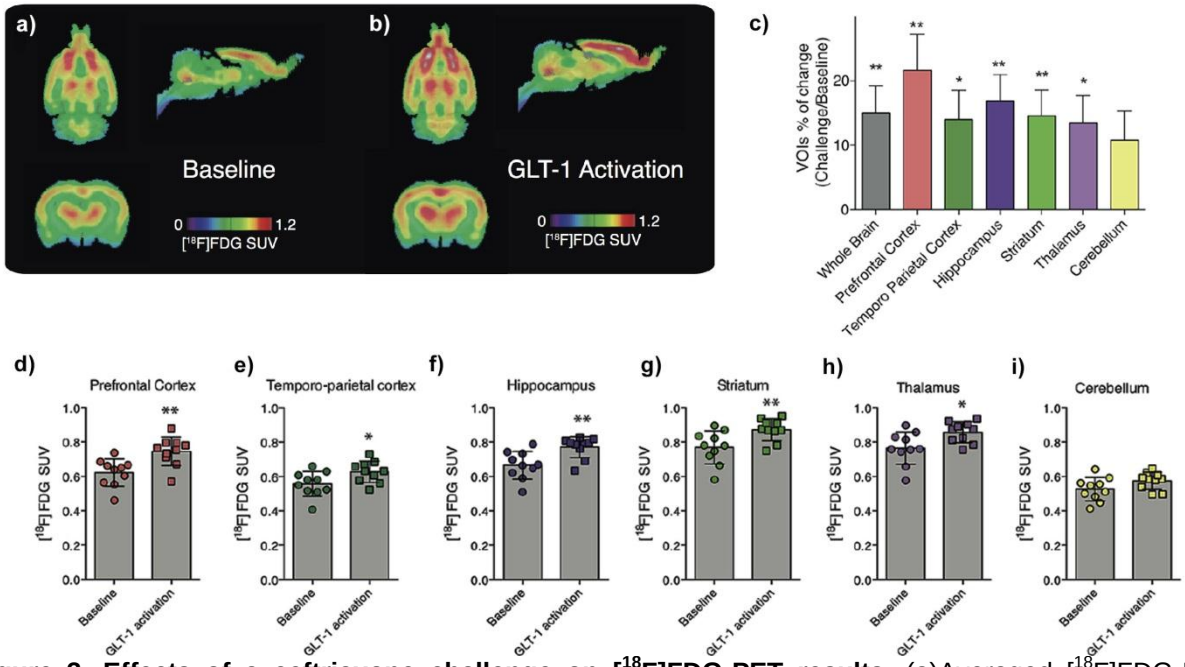

Figure 3. Effects of a ceftriaxone challenge on [^{18}F]FDG-PET results. (a)Averaged [^{18}F]FDG-PET maps of rats before receiving ceftriaxone. (b) Averaged [^{18}F]FDG-PET maps of rats after receiving ceftriaxone. (c) Regional percentage of change between baseline and ceftriaxone challenge on several 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 (Stoessl, 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; Stoessl, 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 excytotoxicity 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

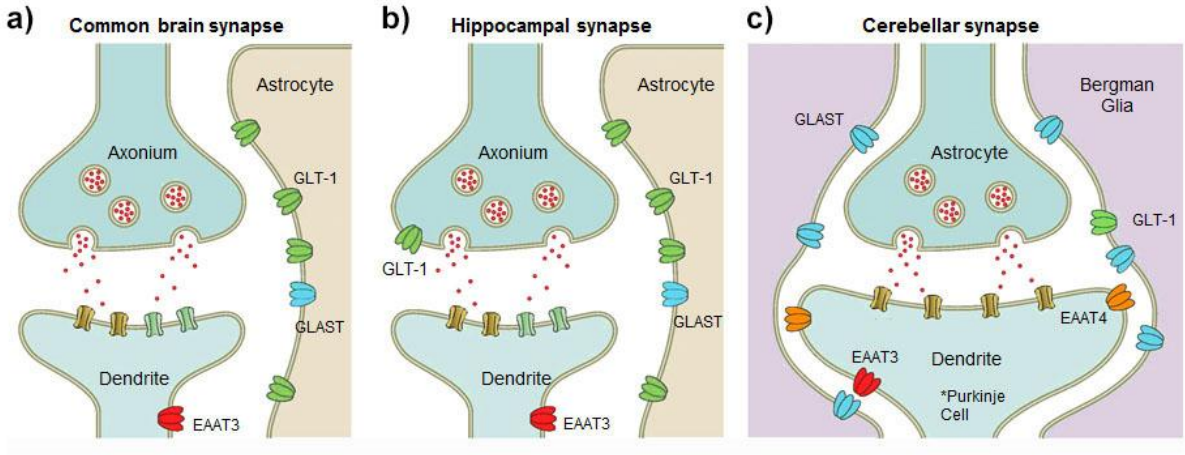

Figure 4. Glutamate transporters cellular distribution. Schematic diagram presenting the different types of EAATs predominant locations in the brain at the cellular level. a) EAATs distribution on most synapses of the brain. b) EAATs distribution on specific hippocampal synapses. c) EAATs distribution 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.

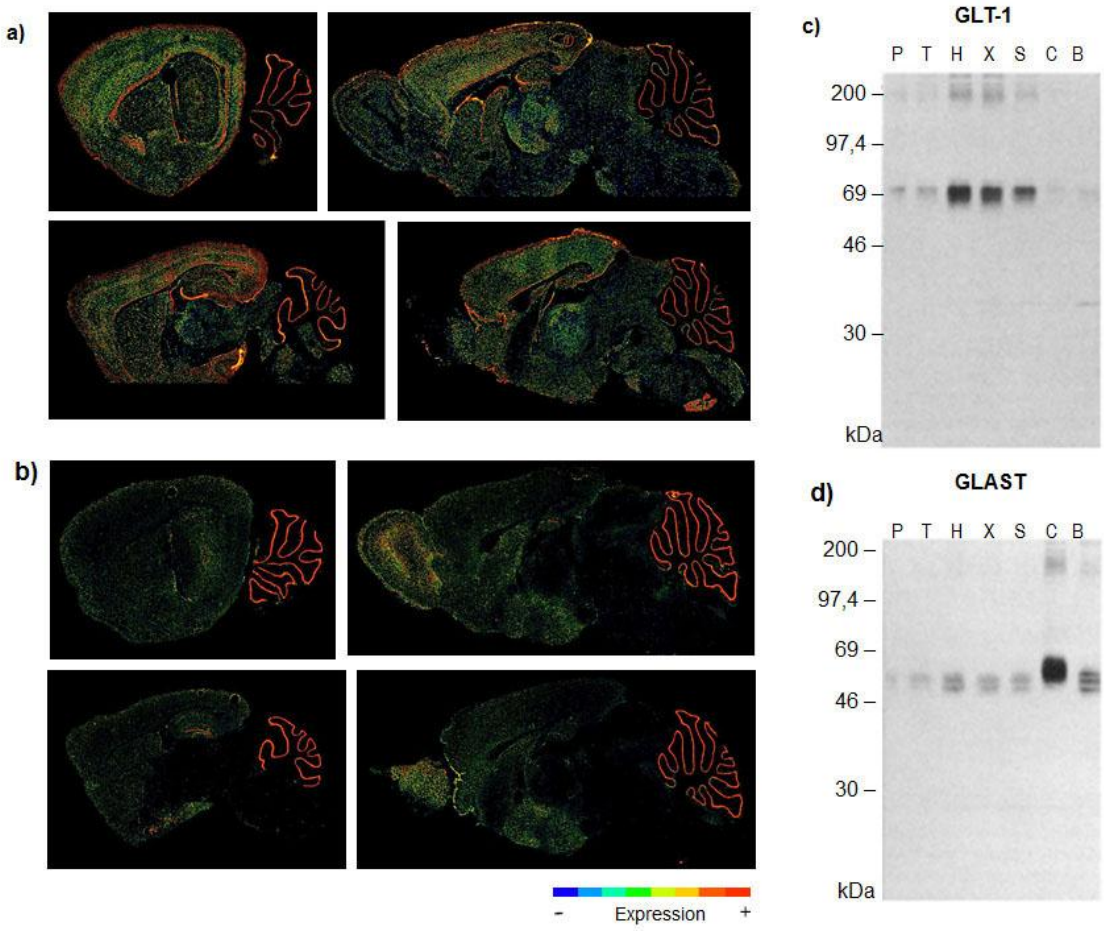

Figure 5. Glutamate transporters GLT-1 and GLAST brain distribution in rodents. Brain representative images of (a) GLT-1 and (b) GLAST mRNA expression in mice identified by in situ hybridization. Western Blot representative images for (c) GLT-1 and (d) GLAST, showing the density of these transporters 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 GSK3β (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 know 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 no affected by clozapine in the rat cerebral cortex (Melone *et al.*, 2003); and that effect of clozapine GLT-1 density and glutamate uptake is replicated on astrocytic cultures, suggesting that these cells are directly involved on 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).

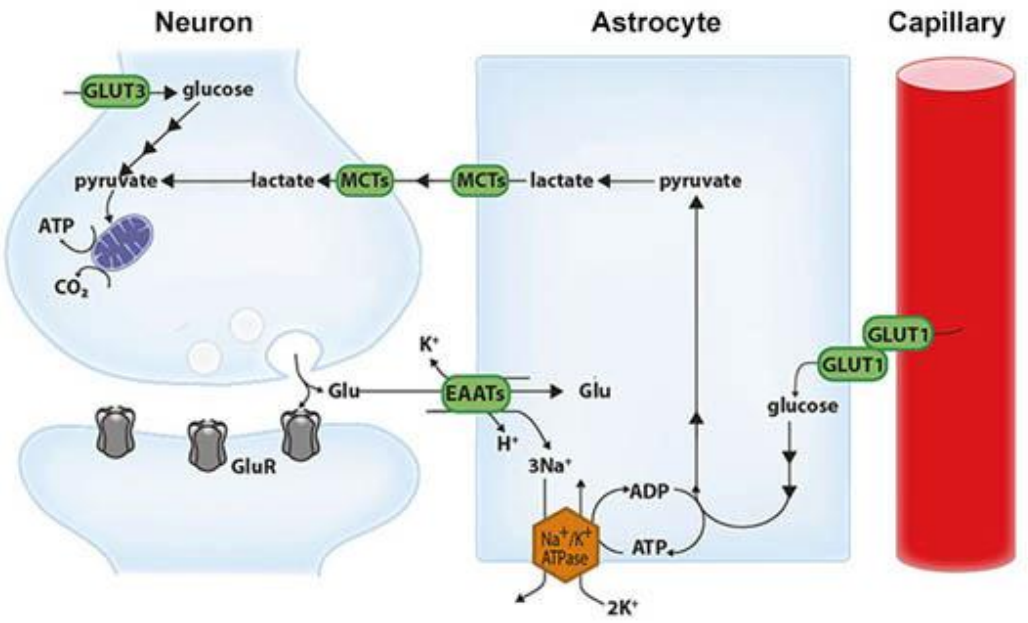

Figure 6. Astrocyte Neuron Lactate Shuttle. ANLS representative scheme demonstrated on a tripartite synapse. Glutamate (Glu) released into the synaptic cleft by the presynaptic neuron can act at the glutamate (GluR) receptors and/or be captured by the astrocyte through the glutamate transporters (EAATs). 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}C]2DG 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 on 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.

Clozapine triggers widespread astroglial glucose hypometabolism in rat cortical layers

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Abstract

Background

In the past decades, the role of glial cells on brain energetics has been an intense focus of discussion. It is hypothesized that glutamate transport acts as a trigger signaling for glucose uptake in astrocytes. However, astrocyte compartment has been neglected in the interpretation of 2-deoxy-2-[^{18}F]Fluoroglucose ([^{18}F]FDG) positron emission tomography ([^{18}F]FDG-PET), an *in vivo* and non-invasive method that allows for estimating brain glucose metabolism. In this way, we performed a pharmacological challenge to investigate whether astrocytes impact on glucose brain metabolism indexed by [^{18}F]FDG-PET. The drug clozapine was used because it has been shown previously to decreases astrocyte glutamate uptake by substantially downregulating its main transporter, GLT-1.

Methods

Adult male Wistar rats received clozapine (25/35mg kg^{-1}) in the drinking water for six weeks. Glucose brain metabolism was longitudinally accessed using [^{18}F]FDG-microPET before and after the treatment. D-aspartate uptake was evaluated in cortical brain slices. Immunocontent and expression of GLT-1 and GLAST were assessed on the cortical tissue and cultures. Primary cortical astrocytic cultures were used for glutamate uptake and deoxyglucose uptake analysis.

Results

Clozapine treatment on adult rats reduced [^{18}F]FDG metabolism mostly in the cortex and disrupted the metabolic network. In addition, clozapine decreased cortical glutamate transport – indexed by D-aspartate uptake – and reduced immunocontent and expression of GLT-1 in the cortex. Astrocytic newborn cultures treated with clozapine presented a decline on GLT-1 density, D-aspartate uptake and deoxyglucose uptake.

Conclusions

This work provides microPET evidence that a treatment with clozapine causes a reduction on [^{18}F]FDG signal mainly on cortical layers and alters brain metabolic network. Our data on the primary cultures indicates that astrocytes might be the cells responsible for this phenomenon. The present results also suggest that astrocytic GLT-1 density and glutamatergic uptake could be part of the mechanism altering astrocytes glucose metabolism. These findings shed light on a potential role played by astrocytes on [^{18}F]FDG-PET signal, which supports further investigations to identify [^{18}F]FDG-PET cellular origin.

Keywords: [^{18}F]FDG, PET, astrocytes, GLT-1, glucose, glutamate, clozapine.

1. Introduction

[^{18}F]FDG is the most used radiopharmaceutical on PET imaging [1] and has been widely applied to evaluate the brain metabolism, both in research and clinics, over the last three decades [2-4]. However, PET does not have cellular resolution and the traditionally used perception that the [^{18}F]FDG-PET signal is due to neuronal uptake or directly reflects neuronal activity is currently under debate [5-8].

As neuroscience advances, astrocytes are being established as key players on crucial brain functions [9]. In this context, growing evidences indicates that these cells play a critical role in terms of glucose utilization. In particular, the glutamate transport through GLT-1 (Glutamate Transporter) or GLAST (Glutamate Aspartate Transporter) and on astrocytes has been shown to act as a trigger, stimulating glucose uptake by these cells [10].

It is hypothesized that astrocytes substantially increase their glucose uptake as a response to glutamate transport, metabolizing the glucose mostly by aerobic glycolysis and then shuttling lactate to neurons to be further oxidized [11]. This theory has receive the name of astrocyte neuron lactate shuttle (ANLS) and has direct implications on the analysis of functional imaging procedures like [^{18}F]FDG-PET [5, 12].

Corroborating the ANSL theory, both *in vitro* and *in vivo* studies have observed an elevation of glucose uptake by astrocytes when in glutamate, and other neuronal signals, presence [13-16]. Also, by using labeled glucose and lactate in cultures of astrocytes and neurons, it was observed a neuronal preference for lactate oxidation and a predominant metabolization of glucose to lactate by astrocytes. Studies *in vivo*, have also observed that reduction/absence of astrocytic glutamate transports reduces glucose uptake [17, 18].

When the brain glucose metabolism and [^{18}F]FDG-PET results are analyzed on the light of the most recent evidences on brain cellular composition, the potential contribution of glial cells seems also suitable. Advanced studies on the brain cellular composition show that glia accounts for nearly 80% of the cortical cells, 60% of gray matter cells, while only 20% of the cerebellar cells [19]. Therefore, regions with a known high glucose consumption and [^{18}F]FDG signal, like the cortex, present a

higher number of glial cells and an inferior number of neurons. Among the glial cells in the neocortex, astrocytes are estimated to account for about 20-40% [20].

Finally, direct [^{18}F]FDG-PET analysis have been used to address if the modulation on the astrocytic functions, like glutamatergic transport, could alter the signal on the exam. Zimmer et al [21] observed an intensification on [^{18}F]FDG signal, mostly in regions where astrocytic transporter GLT-1 is enriched, as effect of a pharmacological challenge with ceftriaxone in rodents. Together, ceftriaxone, which is known for increasing astrocytic glutamate transport via GLT-1, also increased [^{3}H]2DG uptake in adult rat primary astrocyte cultures. In addition, recently, Carter and colleagues showed PET evidences linking astrocytes function and glucose metabolism in Alzheimer's Disease [22].

Based on these observations, it seems very likely that [^{18}F]FDG-PET signal may in part reflect glucose consumption in astrocytes [23]. To further test this hypothesis, we conducted a pharmacological challenge with the drug clozapine, targeting a reduction on astrocytic glutamate uptake, to assess its effects on cerebral [^{18}F]FDG consumption in awake adult rats and [^{3}H]2DG uptake in astrocytic cultures.

2. Methods and Materials

2.1 Animals

Forty-four(44) adult male Wistar rats, 85 days old, were maintained, two per cage (for monitoring liquid intake), under a 12h light-dark cycle (lights on at 7 A.M.), at a constant room temperature (22 ± 1◦C) and with free access to food and water. Animals weight and volume of liquid ingestion were measured. The behavioral tests were performed between 8 A.M. and 13 P.M. All the experimental procedures were approved by the Committee on the Ethics of Animal Experiments of the Federal University of Rio Grande do Sul (approval number 32983).

2.2 Pharmacological treatment

Adult male Wistar rats were randomly divided in two groups, a Control group and a Clozapine group. Rats on the clozapine group received the drug (Cristália, Itapira/Brazil) in their drinking water (25/35mg $kg^{-1}day^{-1}$) for 6 weeks, starting at 85 postnatal day (PND), until euthanasia. The clozapine solution was prepared as previously described by Terry *et al.* [24], dissolving the drug in a 0.1 N solution of

acetic acid and subsequently diluting (1:100) to achieve the final daily dose of drug. Control rats received water with the same acetic acid concentration and pH of the drug solution. The solutions were replaced by fresh ones every 2-3 days and protected from light if containing clozapine. Treatment regimen, dose and length of treatment followed Meloni et al [25], being based on previous dosing strategies in rats that have been extrapolated from human clinical dosages [26]. An oral administration of clozapine was chosen because of its reported rapid elimination [27].

2.3 [^{18}F]FDG microPET scanning

The rats were scanned longitudinally before the treatment (Baseline), at PND 85, and after 6 weeks of treatment (Follow-up), at PND 128. The scanning was made between 9:30A.M. - 18:30P.M, in a randomized order. After overnight fasting, the animals were individually and rapidly anesthetized using a mixture of isoflurane and medical oxygen (3–4% induction dose) and received an intravenous injection (0.4 mL) of [^{18}F]FDG (mean ± s.d.: 1.03 ± 0.07 mCi) into the tail vein. Then, each rat was returned to its home cage for a 40min period of conscious (awake) *in vivo* uptake of [^{18}F]FDG, which was followed by a 10min static acquisition under anesthesia (2% isoflurane at 0.5 L/min oxygen flow). PET measurements were performed on a *Triumph® II microPET/CT LabPET-4®* scanner (GE Healthcare, Chicago/USA). The brain was positioned in the center of the field of view. The body temperature was maintained at 36.5 ± 1˚C. All data was reconstructed using the maximum likelihood estimation method (MLEM-3D) algorithm with 20 iterations. MicroPET images were manually co-registered to a standard rat MRI histological template. Standardized uptake value reference (SUVr) was calculated using pons as the reference region. Mean SUVrs of 14 brain regions and whole brain were extracted using a predefined volume of interest (VOI) template. Imaging analysis was conducted using the minc-tools software (www.bic.mni.mcgill.ca/ServicesSoftware/MINC).

2.4 Metabolic networks

Cross-correlation maps were built using a mask containing 14 VOIs: left (L)-right(R) frontal cortex (FtC), L-R temporo-parietal cortex (TmPtC), L-R thalamus (Th), L-R Hypothalamus (Ht), L-R striatum (St), LR hippocampus (Hip), and L-R cerebellum (Cer). Metabolic brain networks were constructed by computing Pearson correlation

coefficients based on 2000 bootstrap samples and were corrected for multiple comparisons using Bonferroni ($P<0.005$).

2.5 D-[2,3-^{3}H]-Aspartic Acid (D-[^{3}H]Asp) uptake on cortical brain slices

D-[^{3}H]Asp is a metabolically inert substrate for the high-affinity sodium-dependent glutamate transport system, therefore, its uptake was used as a proxy of the system function [28]. Slices (300 µm) of the cerebral cortical region (right hemisphere) were rapidly obtained using a McIlwain Tissue Chopper and immersed in a modified Hanks' balanced salt solution (HBSS; in mM: 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 5.55 glucose, pH 7.2) at 4°C. Cortical slices were preincubated with HBSS at 37°C for 30min, followed by the addition of 0.1µCi/mL of D-[^{3}H]-Asp in the presence of 100µM of L-glutamate. Incubation was stopped after 10 min with three ice-cold washes with HBSS and slices were lysed overnight with NaOH 0.5M. Na^+ independent uptake was measured using the aforementioned protocol with modifications on the temperature (4°C) and composition of the HBSS medium (in mM: 137 N-methyl-D-glucamine-HCl, 0.82 N-methyl-D-glucamine-HEPES, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 5.55 glucose, pH 7.2). Na^+ dependent uptake was calculated as the difference between the total uptake and the Na^+ independent uptake. Incorporated radioactivity was measured using a liquid scintillation counter (Hidex 300 SL, Mikrotek Laborsysteme, Overath, Germany). The experiments were performed in quadruplicate.

2.6 Synaptosomal preparation and L-[^{3}H]glutamic acid (L-[^{3}H]Glut) release

Cortical tissue samples (left hemisphere) were homogenized (manual small capacity Teflon/glass homogenizer in 10× volume/weight) in a buffer sucrose solution (0.32 M sucrose, 10mM HEPES, 1mM EDTA and 1mM DTT; pH = 7.4). The homogenates were centrifuged at 1000g for 10min at 4°C. The resulting supernatant was centrifuged at 11000g for 20 min at 4°C. The synaptosomal-enriched pellet was then washed three times with HBSS-low K^+ (in mM: 133 NaCl, 2.4 KCl, 1.20 KH^2PO^4, 1.0 $CaCl^2$, 1.20 $MgSO^4$, 27.72 HEPES and 12 glucose, pH 7.4) by centrifugation at 14,000g for 10min at 4°C, to remove excess sucrose. The final pellet was resuspended in HBSS-low K^+ containing 0,33µCi/mL L-[^{3}H]Glut and 100µM of L-glutamate, followed by a 15min incubation at 37°C, for uptake. The reaction was

slowed down by putting the tubes on ice and the synaptossomal preparations were rapidly washed three times with ice-cold HBSS-low K^+, by centrifugation at 16,000g for 10min at 4°C, to remove excess L-[^{3}H]Glut. The final pellet was resuspended in HBSS-low K^+ (Uptaken) and partially divided in tubes containing HBSS-low K^+ and tubes containing HBSS-high K^+ (in mM: 95.48 NaCl, 40.4 KCl, 1.20 KH_2PO_4, 1.0 $CaCl_2$, 1.20 $MgSO_4$, 27.72 HEPES and 12 glucose, pH 7.4). The HBSS-low K^+ and HBSS-high K^+ tubes were incubated for 1min at 37°C for release. The release reaction was slowed down by putting the tubes on ice, followed by a centrifugation at 16,000g for 1min at 4°C. The supernatant radioactivity was measured using a liquid scintillation counter. The remaining Uptaken was also measured using a liquid scintillation counter. The samples protein content was determined using the bicinchoninic acid assay (Thermo Fischer Scientific, Waltham/USA). The potassium dependent release of L-[^{3}H]Glut was calculated in relation to the Uptaken radioactivity and subtracting the HBSS-low K^+ release. Experiments were performed in triplicate.

2.7 Primary cortical astrocytes cultures: newborn rats

Newborn (1-2 days old) male Wistar rats cortices were aseptically dissected out. The tissue was enzymatically - with trypsin 0.05% (Gibco, Thermo Fischer Scientific, Waltham/USA) and DNase 0.003% DNase (Sigma Aldrich, Saint Louis/USA) - and mechanically (Pasteur pipet) dissociated. The samples were incubated for 15min at 37°C for enzymatic action, followed by the addition of 10% fetal bovine serum (FBS) and centrifugation at 1000g for 5min. Cells pellet was resuspended in HBSS (Gibco, Thermo Fischer Scientific, Waltham/USA) containing DNase 0.003% and centrifuged for 7min at 1000g. The cells pellet was resuspended in DMEM (Gibco, Thermo Fischer Scientific, Waltham/USA) supplemented with 10% FBS, 15 mM HEPES, 14.3 mM $NaHCO_3$, 1% Fungizone and 0.04% gentamicin, and plated onto 24-well plates at a density of 3–5×105 cells/cm^2. The cells were cultured at 37°C in an atmosphere with 5% CO_2. The first medium exchange occurred 24h after obtaining the culture. The following medium changes occurred once every four days. After cells reach the confluence the protocol followed Vallejo-Illarramendi et al[29]. First, 0.2mM dibutyryl-cAMP (dBcAMP) was added for 6 days to induce GLT-1 expression. Subsequently, dBcAMP was removed and astrocytes were treated with 50mM clozapine (Sigma

Aldrich, Saint Louis/USA), in medium supplemented with 1% FBS, for 48h and used for immunofluorescence, western blot or uptake procedures.

2.8 2-deoxy-D-[^{3}H]glucose ([^{3}H]2DG) uptake in astrocytic cultures

After cells reached confluence, [^{3}H]2DG uptake was assessed as previously described [10] with some modifications. Briefly, the cells were rinsed once with a modified HBSS (in mM: 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 5.55 glucose, pH 7.2) and incubated with DMEM/F12 1%FBS containing 1µCi/mL [^{3}H]2DG in the absence (Basal) or in the presence of 200µM glutamate (Stimulated) for 20min at 37°C. After incubation, the cells were rinsed three times with HBSS and lysed overnight with NaOH 0.5M. Incorporated radioactivity was measured in a scintillation counter. The samples protein content was determined using the bicinchoninic acid assay (Thermo Fischer Scientific, Waltham/USA). Experiments were performed in triplicate.

2.9 D-[2,3-^{3}H]-Aspartic Acid uptake in astrocytic cultures

After cells reached confluence, the astrocytes were rinsed once with a modified HBSS (in mM: 137 NaCl, 0.63 Na_2HPO_4, 4.17 $NaHCO_3$, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 5.55 glucose, pH 7.2) and incubated with HBSS containing 0.33mCi/mL D-[^{3}H]Asp in the presence of 10µM glutamate (Basal) or in the presence of 100µM glutamate (Stimulated) for 7min at 37°C. After incubation, the cells were rinsed three times with HBSS and lysed overnight in a solution containing 0.5M NaOH. Incorporated radioactivity was measured in a scintillation counter. To determinate the Na^+ independent uptake, the same procedure was repeated using the aforementioned protocol with modifications in the temperature (4°C) and composition of the HBSS medium (in mM: 137 N-methyl-D-glucamine-HCl, 0.82 N-methyl-D-glucamine-HEPES, 5.36 KCl, 0.44 KH_2PO_4, 1.26 $CaCl_2$, 0.41 $MgSO_4$, 0.49 $MgCl_2$ and 5.55 glucose, pH 7.2). The samples protein content was determined using the bicinchoninic acid assay (Thermo Fischer Scientific, Waltham/USA). The Na^+ dependent uptake was then obtained by subtracting the Na^+ independent uptake from the total uptake. Experiments were performed in triplicate. As glutamate and D-aspartate are uptaken by the same transporters, the 10 times higher glutamate concentration on Stimulated experiments, versus Basal ones, was accounted in the results measurements.

2.10 SDS-PAGE

For protein immunocontent evaluation the Western Blot technique was performed both for the dissected tissue and for the astrocytes cultures. For the tissue analysis, whole cortex was dissected out from 129-132 days old rats and well distributed representative portions were immediately homogenized in a lysis buffer (2% SDS, 5mM Tris, 2mM EDTA, pH 7,4) containing a protease inhibitor cocktail (Sigma Aldrich, Saint Louis/USA). For the newborn and adult astrocytes cultures, RIPA buffer (Bio-Rad, Hercules/USA) containing a protease inhibitor cocktail (Sigma Aldrich, Saint Louis/USA) was added to cell cultures after reached confluence. The protein content was determined using the bicinchoninic acid assay (Thermo Fischer Scientific, Waltham/USA) and samples were standardized in sample buffer (Bio-Rad, Hercules/USA) with addition of 5% β-mercaptoethanol and boiled at 95°C for 5min. Samples were separated by SDS-PAGE (10 µg protein/well) and transferred to a nitrocellulose membrane (GE Healthcare, Amersham/UK). After electrotransfer, the membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 (TTBS) and 3% bovine serum albumin for 1h at room temperature (RT). Subsequently, membranes were incubated with primary rabbit or mouse antibody overnight at 4°C [GLT-1, 1: 1000 dilution; GLAST, 1: 2000 dilution; β-actin, 1:10000 dilution; and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 1:4000 dilution], washed with TTBS and incubated with horseradish peroxidase conjugated donkey anti-rabbit or anti-mouse IgG (1:5000 dilution, GE Healthcare, Amersham/UK) secondary antibody for 2h at RT. Chemiluminescent bands were detected in the ImageQuant LAS4000 system (GE Healthcare, Amersham/UK) using an Immobilon™ Western chemiluminescence kit (Merck Millipore, Burlington/USA). Densitometric quantification was performed with the ImageJ software (NIH, Bethesda/USA). The results are expressed in percent of control levels after normalization using β-actin or GAPDH as an internal standard.

2.11 RNA extraction and quantitative real time PCR (qRT-PCR)

The whole cortex was dissected out from 129-132 days old rats and well distributed representative portions were immediately homogenized to qRT-PCR. Total RNA was extracted using TRIzol Reagent according to manufacturer's instructions (Invitrogen, Carlsbad/USA). The concentration and purity of the RNA were spectrophotometrically determined at a ratio of 260/280. Subsequently, 1µg of total RNA was reverse

transcribed using the Applied Biosystems™High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City/USA) in a 20 μL reaction, according to the manufacturer's instructions. The mRNAs of glutamate transporter-1 (GLT-1) and glutamate-aspartate transporter (GLAST) were quantified using the TaqMan qRT-PCR system with inventory primers and probes purchased from Applied Biosystems (Foster City/USA). β-actin mRNA levels (primers 5′-CAACGAGCGGTTCCGAT-3′ 5′-GCCACAGGATTCCATACCCA-3′) was also quantified using the TaqMan real-time qRT-PCR system. Target mRNA levels were normalized to β-actin levels using the 2-ΔΔCt method [30]. Experiments were performed in triplicates.

2.12 Immunofluorescence

Immunofluorescence was performed as described previously by our group [31]. Cell cultures were fixed with 4% paraformaldehyde for 20min and permeabilized with 0.1% Triton X-100 in PBS for 5min at RT. After blocking overnight with 4% albumin, the cells were incubated overnight with anti-GFAP (1:400) at 4ºC, followed by PBS washes and incubation with a specific secondary antibody conjugated with Alexa Fluor® 488 or 594 for 1h at RT. For all the immunostaining-negative control reactions, the primary antibody was omitted. No reactivity was observed when the primary antibody was excluded. Cell nuclei were stained with 0.2 mg/ml of 4',6'-diamino-2-phenylindole (DAPI; Merck Millipore, Billerica/USA) for 10min.

2.13 Aminoacids high-performance liquid chromatography

High-performance liquid chromatography (HPLC) procedure was performed as previously described [64] using cerebrospinal fluid (CSF) to quantify three amino acids: glutamate (Glut), aspartate (Asp) and glutamine (Gln). The analysis was performed in a Shimadzu Class-VP chromatography system with fluorescence detector using a Supelco Supelcosil LC-18 column (250 x 4.6mm, 5μm particle size) Chromatographic parameters were set as follows: flow rate 1.4mL/min; temperature 24°C; injection volume 40μL; mobile phase consisting of: buffer A = H4NaO5P pH 5.5 (0.04mol/L):methanol (20:80); buffer B = H4NaO5P pH 5.5 (0.01mol/L):methanol (80:20). The gradient profile was modified according to the content of buffer B in the mobile phase:100% at 0.10min, 90% at 15min, 48% at 10min, 100% at 60min. Detection was performed at 360nm and 455nm, excitation and emission

respectively. For glutamine determination, samples were diluted 10x. The experiments were performed in duplicates.

2.14 Statistical Analysis

A Two-way ANOVA with repeated measures and multiple comparisons analysis was used for whole brain and regional SUVr data. A two-tailed unpaired Student's t-test was used for statistical analysis of the tissue SDS-PAGE, PCR, LCR amino acids, brain slices D-[^{3}H]Asp uptake, L-[^{3}H]Glut release and OF. A two-tailed unpaired Student's t-test was also used for the statistical analysis of the adult astrocytes culture data: SDS-PAGE, Basal and Stimulated D-[^{3}H]Asp uptake, Basal and Stimulated [^{3}H]2DG uptake. A two-tailed paired Student's t-test was used for the newborn astrocytes culture data: SDS-PAGE, Basal and Stimulated D-[^{3}H]Asp uptake, Basal and Stimulated [^{3}H]2DG uptake. Percentage of control data of the D-[^{3}H]Asp and [^{3}H]2DG uptake in cell assays was analyzed by Two-way ANOVA with multiple comparisons. One-sample t-test (hypothetical value=50%) was used to evaluate the novel object recognition test. Two-way ANOVA with repeated measures was used for the weight and liquid intake data analysis. Data were expressed as means ± SEM and differences were considered statistically significant at $p< 0.05$.

3. Results

3.1 Sub-Chronic clozapine treatment causes cortical *in vivo* brain glucose hypometabolism

The [^{18}F]FDG-microPET analysis after the clozapine intervention showed a clear SUVr reduction mainly on cortical brain regions in the clozapine group (**Fig. 1a, 1b**; n=11-13). When evaluating the mean difference between Baseline and Follow-up scans on a percentage of change map, the clozapine group presented a cortical widespread hypometabolism of [^{18}F]FDG, with values between 10-20% (**Fig. 1c**). By contrast, the control group presented only a few small clusters of glucose metabolism reduction that are likely just noise related (**Fig. 1c**). A voxel-wise t-statistical analysis showed a significant glucose hypometabolism mostly in the cortical region of the clozapine group, with a peak effect in the parietal cortex (peak $t_{(23)}=7.62$, $p<0.0001$; **Fig. 1d-e**). On the other side, the t-statistical analysis showed almost no significant differences on the control group, presenting just few minimal punctual reductions that could be easily attributed to a limitation on the spatial resolution of the microPET

scanner [32]. The peak effect on the control group was in the superior colliculus (peak $t_{(23)}$=3.03, p=0.0066; **Fig. 1d-e**. The SUVr analysis of the whole brain showed an effect of time [p=0.0026; F(1, 22)=11.50] and a decline for the clozapine group (p<0.05; **Fig. 1h**) but not for the control group. A brain mask with 14 VOIs, based on the Paxinos and Watson Rat Brain Atlas, was used to obtain mean regional [^{18}F]FDG SUVr values (**Fig. 1f**). A Two-Way ANOVA test with multiple comparisons test identified significant [^{18}F]FDG SUVr decline in the frontal cortex (p<0.0001; **Fig. 1i**), temporo-parietal cortex (p<0.001; **Fig. 1j**) striatum (p<0.05; **Fig. 1k**) and hippocampus (p<0.01; **Fig. 1l**) on the clozapine group follow-up, in comparisons to its baseline. Consistently, those are the main brain regions to present a high content of GLT-1 on rats [33]. The thalamus (**Fig.1m**), hypothalamus (**Fig. 1n**) and cerebellum (**Fig. 1o**) showed no significant differences in both groups. Additionally, a metabolic network analysis across previously delineated VOIs was performed to identify brain reorganization patterns after the clozapine treatment. The clozapine group showed multiple altered connections within the metabolic network, presenting a metabolic a widespread hyposynchronicity among the brain areas (P<0.005, Bonferroni corrected, **Fig. 1g**).

3.2 Cortical GLT-1 and D-[3H]Asp uptake are reduced after clozapine treatment

The astrocytic glutamate transporters immunocontent analysis on the cortical tissue revealed a significant reduction for GLT-1 levels (p=0.0043; $t_{(24)}$=3.16; **Fig. 2a, 2c**) at the clozapine group (n=12) in comparison to controls (n=14), but no differences for GLAST (p=0.635; $t_{(25)}$=0.48; **Fig. 2d, 2f**). The GLT-1 downregulation was of about 18%. Consistent results were found on the mRNA expression evaluation with clozapine reducing GLT-1 expression (p=0.025; $t_{(13)}$=2.52; n=8; **Fig. 2b**) in comparison to controls (n=7) but not altering GLAST expression (p=0.27; $t_{(13)}$=1.14; **Fig. 2e**). Glutamatergic transport was also evaluated by using a D-[^{3}H]Asp uptake assay on fresh cortical brain slices. The clozapine group (n=5) showed a significant (p=0.0489; $t_{(9)}$=2.27; **Fig. 2g**) decline, around 30%, in the brain slices D-[^{3}H]Asp uptake if compared to the control group (n=6). In parallel, the L-[^{3}H]Glut release was assessed in synaptosomal preparations and no differences were observed (p=0.769; $t_{(14)}$=0.23; n=8; **Fig. 2h**). Glutamate(n=9-12) and related aminoacids, aspartate (n=9-12) and glutamine (n=6-7), content were quantified in the CSF. CSF glutamate

(p=0.629; $t_{(19)}$=0.49), aspartate (p=0.486; $t_{(19)}$=0.70) and glutamine (p=0.194; $t_{(11)}$=1.38) content were not altered as well (**Fig. 2i-k**).

3.3 Clozapine treatment effects on newborn primary astrocytic cultures

To assess clozapine effects on cortical astrocytes, we administered the drug on newborn astrocytes primary culture preparations (**Fig. 3a**). We found significant differences for GLT-1 (p= 0.0019; $t_{(7)}$=4.81; **Fig. 3b-c**) and GLAST (p=0.0118; $t_{(7)}$=3.38; **Fig. 3d-e**) immunocontent on the clozapine group (n=8) if compared to the control group (n=8). GLT-1 decline was of about 36% and GLAST 18%. In parallel to the reduction on the glutamatergic transporters content, the clozapine group showed a decline on [^{3}H]D-Asp uptake for, both, the Basal (p=0.0005; $t_{(5)}$=7.97; **Fig. 3f**) and Stimulated assays (p= 0.0357; $t_{(5)}$=2.85; **Fig. 3g**). When comparing Basal and Stimulated [^{3}H]D-Asp uptake from control and clozapine groups (**Fig. 3h**), both a treatment [p=0.0019; F(1, 20)=102.3] and a glutamate concentration [p<0.0001; F(1, 20)=12.76] effects were identified, but no interaction effect [p=0.065; F(1, 20)=3.79]. A multiple comparison analysis showed that Basal to Stimulated differences were significant for both groups (p<0.0001), and that clozapine uptake was reduced if compared to control group only on the stimulated approach (p<0.01). Alongside, the results on the [^{3}H]2DG uptake revealed no differences on the Basal uptake (p=0.60; $t_{(5)}$=2.85; **Fig. 3i**) and a significant decrease on the Stimulated uptake for the clozapine group (p=0.61; $t_{(7)}$=0.54; **Fig. 3j**). An analysis comparing the [^{3}H]2DG uptake across the four test outcomes (**Fig. 3k**) showed effects of treatment [p<0.0001; F(1, 28)=22.54], glutamate concentration [p=0.0061; F(1, 28)=8.79] and interaction [p<0.0001; F(1, 28)=21.34]. A multiple comparison analysis showed that Basal to Stimulated differences were significant only for the control group (p<0.0001), and that clozapine uptake was reduced if compared to control group only on the Stimulated approach (p<0.0001).

3.4 Clozapine treatment effects on adult primary astrocytic cultures

Additionally, we also isolated and cultivated the cortical astrocytes (**Fig. S1a**) of the same animals submitted to the clozapine six weeks treatment and PET imaging. However, the differences identified on the tissue did not consistently persist on the astrocytes culture without the addition of clozapine. We found no significant differences for GLT-1 (p=0.959; $t_{(16)}$=1.77; **Fig. S1b-c**) or GLAST (p=0.141;

$t_{(16)}$=1.552; **Fig. S1d-e**) immunocontent. No statistically significant differences were observed between groups control and clozapine on, both, Basal (p=0.699; $t_{(19)}$=0.39) and Stimulated (p=0.0513; $t_{(19)}$= 2.08) [^{3}H]DAsp uptakes (**Fig. S1f-g**). As well, [^{3}H]2DG uptake showed no differences when comparing control and clozapine groups, either in Basal (p=0.615; $t_{(19)}$= 0.51) or Stimulated (p=0.293; $t_{(19)}$= 1.08) experimental conditions (**Fig. S1i-j**). In a Two-way ANOVA analysis of the four conditions (Control Basal, Control Stimulated, Clozapine Basal and Clozapine Stimulated) for the [^{3}H]DAsp uptake, an effect of the glutamate concentration was identified [p<0.0001; F(1, 38)=51.89], no effects for treatment group [p=0.129; F(1, 38)=2.40] or interaction [p=0.203; F(1, 38)=1.67] (**Fig. S1h**). The same analysis for the [^{3}H]2DG uptake (**Fig. S1k**) showed no effects of treatment group [p=0.586; F(1, 38)=0.30], glutamate concentration [p=0.065; F(1, 38)=3.6] or interaction [p=0.176; F(1, 38)= 1.89].

3.5 Behavioral effects and treatment measures

Total distance traveled (p=0.17; $t_{(26)}$=1.42) and total immobile time (p=0.13; $t_{(26)}$=1.56) during the open field test were not significantly different between groups, indicating that the spontaneous locomotion of control or clozapine groups was not affected by the pharmacological approach (**Fig. S2a-c**) (n=12-14). Analysis of the time spent exploring objects during the NOR (novel object recognition) task revealed that both groups explored equally the two identical objects on the training session and spent more time exploring the new object on the test session (control p=0.0004; $t_{(6)}$=7.22; clozapine p=0.0004; $t_{(4)}$=10.86), indicating no impairment on the short-term recognition memory (**Fig. S2d-f**; n=5-7). Animals body weight was measured once a week through the experiment, the results showed only significant effect of time (p<0.0001; F(7, 196) = 336.6; **Fig. S3a**), as animals gained weight, but not of groups (p=0.35; F(1, 28)=0.89). On the other side, the liquid intake measurement showed not only an increase over time (p<0.0001; F(11, 121)=7.85; **Fig. S3b**), but also a lower volume intake on the clozapine group in comparison to controls (group effect, p=0.036; F(1, 11)=11.73), probably due to differences on the drinking water palatability. In future experiments, the addition of a compound such as sucrose to the drinking water, in order to make the drinking water containing the experimental drug more palatable, should be considered. Finally, a HPLC procedure confirmed that the animals drinking solution preparation was wielding the expected concentration with

high reproducibility, presenting a relative standard deviation (RSD) of 1,86% (**Fig. S3c**). The experimental drinking solution also showed a high stability, with no noticeable modifications on clozapine concentration in a 24 h period at RT (**Fig. S3c**).

4. Discussion

The present study shows an important *in vivo* reduction on [^{18}F]FDG uptake at the cortex, reflecting a glucose hypometabolism, when the astrocytic glutamate transporter GLT-1 is pharmacologically down-regulated by clozapine. Conjointly, the metabolic network was substantially disrupted suggesting that disturbances in astrocytes can alter glucose metabolism coupling among regions.

GLT-1 is the most abundant glutamate transporter in the brain and it is found, almost exclusively, in astrocytes [34]. Astrocytic GLT-1 has a dominant role on glutamate reuptake, over 90% [35], and it is considered a signaling trigger for glucose uptake by astrocytes [11]. Based on the aforementioned points, GLT-1 was a reliable target for testing our hypothesis. Within the goal to manipulate GLT-1, the drug clozapine was chosen. Clozapine reduces glutamate uptake and down-regulates GLT-1 [36], both *in vitro* [29] and *in vivo* [37]. Meloni and coworkers describe the effect as being stronger in the cortical region but also slightly affecting hippocampus, striatum, and thalamus [25]. Consequently, our analysis focused on the cortex, since this is the region with higher GLT-1 density [33].

The cortical region results corroborate our hypothesis, exhibiting a significant widespread reduction, about 20%, on [^{18}F]FDG-PET signal. In agreement, GLT-1 immunocontent and expression decrease confirming the clozapine's effect previously described by Meloni [37]. Additionally to the reduction on the transporter levels, the reduction on the glutamate transport function itself was confirmed by the decreased [^{3}H]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 amino acids 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. Even though, being considerable less abundant and significant to glutamate uptake on the analyzed region [38], a robust up regulation of GLAST could result in a counterbalance on the astrocytic glucose uptake. However, the evaluation of GLAST density and expression showed no differences, which ruled out a potential 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 [^{3}H]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 [^{18}F]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 analysis.

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 [39] is associated to our pharmacological manipulation and to the resulting cortical hypometabolism, as could be suggested [2]. In addition, if the clozapine effect was associated to a sedative impact it would be expected to encounter, as a consequence, a whole brain hypometabolism, not just regionalized as seen. 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 [40]. However, serotoninergic, dopaminergic and histaminergic systems are probable contributing very little to brain energetics. Of course, they can modulate glutamate/GABA system but a direct activation/deactivation of these systems is much more likely to alter glucose metabolism. There are several studies showing that brain energetic metabolism is manly 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 [41, 42].

Regardless the above mentioned information, we cannot completely rule out the possibility that clozapine could be affecting the [^{18}F]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 [^{18}F]FDG-PET results being replicated on astrocyte cultures by the [^{3}H]2DG uptake strongly suggesting these cells involvement in the process.

We anticipate and encourage our investigation to be a starting point for more sophisticated investigations on the astrocytic contribution to [^{18}F]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 [^{18}F]FDG-PET.

Since its inception, in 1978 [44], the role of astrocytes on the [^{18}F]FDG-PET signal was questioned a few times [5-8], however, at the best of our knowledge, the work of Zimmer et al [21] and the present study are the first ones to try directly address this question using the technique itself. Beholding the results of both studies, we suggest that the [^{18}F]FDG-PET signal can be substantially influenced by astrocytes.

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 [45, 46]. Remains unclear if this is an important phenomenon in patients on a clozapine treatment, and more importantly, remains unclear if it could result in a beneficial or prejudicial outcome on a long-term basis.

5. Conclusion

Our results provide microPET evidence that a treatment with clozapine is able to cause a reduction on [^{18}F]FDG-PET signal mainly on cortical layers and to alter the brain metabolic network. The primary cultures data indicates that astrocytes are, at least partially, the cells responsible for this phenomenon. The present results also suggest that astrocytic GLT-1 density and glutamatergic uptake could be part of the mechanism altering astrocytes glucose metabolism. This outcomes corroborate the notion that [^{18}F]FDG-PET signal might reflect not only neuronal activity but also astrocytic metabolism, supporting a reconceptualization in the way we interpret imaging of brain disorders using [^{18}F]FDG-PET.

Authors contributions

AR, DGS, BB, DS and ERZ participated on the conceptualization, design and interpretation of the experiments. DGS and BB carried out the astrocytes cultures preparation and maintenance. BB performed the qRT-PCR procedure. GTV and SG performed the PET imaging procedure. GS performed the metabolic network analysis. FUF and DM performed the HPLC procedures. AR carried out all the remaining experiments with ICF, MLS and LSM assistance. AR supervised the whole study and drafted the paper. ERZ revised the paper. All authors have discussed the results and seen the final version of the paper before submission.

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References

1. Hofman, M.S. and R.J. Hicks, *How We Read Oncologic FDG PET/CT.* Cancer Imaging, 2016. **16**(1): p. 35.
2. Berti, V., L. Mosconi, and A. Pupi, *Brain: normal variations and benign findings in fluorodeoxyglucose-PET/computed tomography imaging.* PET Clin, 2014. **9**(2): p. 129-40.
3. Sokoloff, L., *Localization of functional activity in the central nervous system by measurement of glucose utilization with radioactive deoxyglucose.* Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism, 1981. **1**(1): p. 7-36.
4. Teune, L.K., et al., *Typical cerebral metabolic patterns in neurodegenerative brain diseases.* Movement disorders : official journal of the Movement Disorder Society, 2010. **25**(14): p. 2395-404.
5. Magistretti, P.J. and L. Pellerin, *Cellular mechanisms of brain energy metabolism and their relevance to functional brain imaging.* Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 1999. **354**(1387): p. 1155-63.
6. Magistretti, P.J., *Cellular bases of functional brain imaging: insights from neuron-glia metabolic coupling.* Brain research, 2000. **886**(1-2): p. 108-112.
7. Sestini, S., *The neural basis of functional neuroimaging signal with positron and single-photon emission tomography.* Cellular and molecular life sciences : CMLS, 2007. **64**(14): p. 1778-84.
8. Figley, C.R. and P.W. Stroman, *The role(s) of astrocytes and astrocyte activity in neurometabolism, neurovascular coupling, and the production of functional neuroimaging signals.* The European journal of neuroscience, 2011. **33**(4): p. 577-88.
9. Volterra, A. and J. Meldolesi, *Astrocytes, from brain glue to communication elements: the revolution continues.* Nat Rev Neurosci, 2005. **6**(8): p. 626-40.
10. Pellerin, L. and P.J. Magistretti, *Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization.* Proceedings of the National Academy of Sciences of the United States of America, 1994. **91**(22): p. 10625-9.
11. Pellerin, L. and P.J. Magistretti, *Sweet sixteen for ANLS.* Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism, 2012. **32**(7): p. 1152-66.
12. Bonvento, G., N. Sibson, and L. Pellerin, *Does glutamate image your thoughts?* Trends in neurosciences, 2002. **25**(7): p. 359-64.
13. Bittner, C.X., et al., *Fast and reversible stimulation of astrocytic glycolysis by K+ and a delayed and persistent effect of glutamate.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2011. **31**(12): p. 4709-13.
14. Nehlig, A., E. Wittendorp-Rechenmann, and C.D. Lam, *Selective uptake of [14C]2-deoxyglucose by neurons and astrocytes: high-resolution microautoradiographic imaging by cellular 14C-trajectography combined with immunohistochemistry.* Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism, 2004. **24**(9): p. 1004-14.
15. Chuquet, J., et al., *Predominant enhancement of glucose uptake in astrocytes versus neurons during activation of the somatosensory cortex.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2010. **30**(45): p. 15298-303.
16. Choi, H.B., et al., *Metabolic communication between astrocytes and neurons via bicarbonate-responsive soluble adenylyl cyclase.* Neuron, 2012. **75**(6): p. 1094-104.
17. Voutsinos-Porche, B., et al., *Glial glutamate transporters and maturation of the mouse somatosensory cortex.* Cerebral cortex, 2003. **13**(10): p. 1110-21.
18. Voutsinos-Porche, B., et al., *Glial glutamate transporters mediate a functional metabolic crosstalk between neurons and astrocytes in the mouse developing cortex.* Neuron, 2003. **37**(2): p. 275-86.

19. Azevedo, F.A., et al., *Equal numbers of neuronal and nonneuronal cells make the human brain an isometrically scaled-up primate brain.* J Comp Neurol, 2009. **513**(5): p. 532-41.
20. von Bartheld, C.S., J. Bahney, and S. Herculano-Houzel, *The search for true numbers of neurons and glial cells in the human brain: A review of 150 years of cell counting.* The Journal of comparative neurology, 2016. **524**(18): p. 3865-3895.
21. Zimmer, E.R., et al., *[(18)F]FDG PET signal is driven by astroglial glutamate transport.* Nature neuroscience, 2017. **20**(3): p. 393-395.
22. Carter, S.F., et al., *Longitudinal association between astrocyte function and glucose metabolism in autosomal dominant Alzheimer's disease.* European journal of nuclear medicine and molecular imaging, 2019. **46**(2): p. 348-356.
23. Magistretti, P.J. and L. Pellerin, *The contribution of astrocytes to the 18F-2-deoxyglucose signal in PET activation studies.* Molecular psychiatry, 1996. **1**(6): p. 445-52.
24. Terry, A.V., Jr., et al., *Differential effects of haloperidol, risperidone, and clozapine exposure on cholinergic markers and spatial learning performance in rats.* Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 2003. **28**(2): p. 300-9.
25. Melone, M., et al., *The expression of glutamate transporter GLT-1 in the rat cerebral cortex is down-regulated by the antipsychotic drug clozapine.* Molecular psychiatry, 2001. **6**(4): p. 380-6.
26. See, R.E. and G. Ellison, *Comparison of chronic administration of haloperidol and the atypical neuroleptics, clozapine and raclopride, in an animal model of tardive dyskinesia.* European journal of pharmacology, 1990. **181**(3): p. 175-86.
27. Baldessarini, R.J., et al., *Tissue concentrations of clozapine and its metabolites in the rat.* Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 1993. **9**(2): p. 117-24.
28. Taxt, T. and J. Storm-Mathisen, *Uptake of D-aspartate and L-glutamate in excitatory axon terminals in hippocampus: autoradiographic and biochemical comparison with gamma-aminobutyrate and other amino acids in normal rats and in rats with lesions.* Neuroscience, 1984. **11**(1): p. 79-100.
29. Vallejo-Illarramendi, A., et al., *Clozapine reduces GLT-1 expression and glutamate uptake in astrocyte cultures.* Glia, 2005. **50**(3): p. 276-9.
30. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
31. Souza, D.G., et al., *Anti-aging effects of guanosine in glial cells.* Purinergic Signal, 2016.
32. Moses, W.W., *Fundamental Limits of Spatial Resolution in PET.* Nucl Instrum Methods Phys Res A, 2011. **648 Supplement 1**: p. S236-S240.
33. Lehre, K.P., et al., *Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 1995. **15**(3 Pt 1): p. 1835-53.
34. Zhou, Y. and N.C. Danbolt, *GABA and Glutamate Transporters in Brain.* Frontiers in endocrinology, 2013. **4**: p. 165.
35. Holmseth, S., et al., *The concentrations and distributions of three C-terminal variants of the GLT1 (EAAT2; slc1a2) glutamate transporter protein in rat brain tissue suggest differential regulation.* Neuroscience, 2009. **162**(4): p. 1055-71.
36. Melone, M., L. Bragina, and F. Conti, *Clozapine-induced reduction of glutamate transport in the frontal cortex is not mediated by GLAST and EAAC1.* Molecular psychiatry, 2003. **8**(1): p. 12-3.
37. Bragina, L., et al., *GLT-1 down-regulation induced by clozapine in rat frontal cortex is associated with synaptophysin up-regulation.* Journal of neurochemistry, 2006. **99**(1): p. 134-41.

38. Danbolt, N.C., *Glutamate uptake.* Progress in neurobiology, 2001. **65**(1): p. 1-105.
39. Prut, L. and C. Belzung, *The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review.* European journal of pharmacology, 2003. **463**(1-3): p. 3-33.
40. Naheed, M. and B. Green, *Focus on clozapine.* Current medical research and opinion, 2001. **17**(3): p. 223-9.
41. Attwell, D. and S.B. Laughlin, *An energy budget for signaling in the grey matter of the brain.* Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism, 2001. **21**(10): p. 1133-45.
42. Howarth, C., C.M. Peppiatt-Wildman, and D. Attwell, *The energy use associated with neural computation in the cerebellum.* Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism, 2010. **30**(2): p. 403-14.
43. Phelps, M.E., et al., *Application of annihilation coincidence detection to transaxial reconstruction tomography.* Journal of nuclear medicine : official publication, Society of Nuclear Medicine, 1975. **16**(3): p. 210-24.
44. Reivich, M., et al., *The [18F]fluorodeoxyglucose method for the measurement of local cerebral glucose utilization in man.* Circulation research, 1979. **44**(1): p. 127-37.
45. Cohen, R.M., et al., *The brain metabolic patterns of clozapine- and fluphenazine-treated patients with schizophrenia during a continuous performance task.* Archives of general psychiatry, 1997. **54**(5): p. 481-6.
46. Molina, V., et al., *Marked hypofrontality in clozapine-responsive patients.* Pharmacopsychiatry, 2007. **40**(4): p. 157-62.
47. Souza, D.G., et al., *Characterization of adult rat astrocyte cultures.* PloS one, 2013. **8**(3): p. e60282.

Figure Legends:

Figure 1: Effects of clozapine treatment on the brain glucose metabolism assessed by [^{18}F]FDG. Brain metabolic maps showing the mean SUVr of the groups **(a)**control and **(b)**clozapine at the Baseline and after the treatment period. **(c)** Images representing the percentage of change between Baseline and Follow-up at the groups control and clozapine. **(d)** T-statistical maps showing the statistically significant hypometabolism at the groups control and clozapine after the treatment period in comparison to the Baseline. **(e)** Groups control and clozapine T-statistical maps represented in three dimensional representative brains images. **(f)** Brain mask showing the volumes of interest (VOIs). **(g)** Cross-correlation matrices and maps displaying inter subject cross correlation region-to-region (VOIs) associations in groups control and clozapine at the Follow-up. Data presented as correlation values with False Discovery Rate (FDR) correction at $p<0.05$. Groups control and clozapine mean regional [^{18}F]FDG SUVr values in the whole brain **(h),** prefrontal cortex **(i)**, temporo-parietal cortex **(j)**, striatum **(k)**, hippocampus **(l)**, thalamus **(m)**, hypothalamus **(n)** and cerebellum **(n)** at the Baseline and Follow-up. **(a-d)** Images are projected into a standard magnetic resonance imaging (mMRI) image in axial, sagittal and coronal planes. (n=11-13). Data represented as mean ± S.E.M. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$ (multiple comparisons - Two-Way ANOVA with repeated measures)

Figure 2: Effects of clozapine treatment on the cortical tissue.

Cortical immunocontent (normalized by the β-actin immunocontent) of the glutamate transporters **(a, c)** GLT-1 and **(d, f)** GLAST on the groups control and clozapine, after 4the six weeks treatment period; (n=12-14). Cortical mRNA expression of **(b)** GLT-1 and **(e)** GLAST on the groups control and clozapine after the six weeks treatment period. PCR results are expressed as fold change in relation to the group control; (n=7-8). **(g)** [^{3}H]D-Asp uptake on cortical brain slices at the groups control and clozapine; (n=5-6 animals per group). **(h)** L-[^{3}H]Glut release on cortical synaptosomal preparations at the groups control and clozapine (n=8). **(i)** Glutamate (n=9-12), **(j)** aspartic acid (n=9-12) and **(k)** glutamine (n=6-7) cerebrospinal fluid concentrations at the groups control and clozapine. Data represented as mean ± S.E.M. *$p<0.05$; **$p<0.01$ (unpaired *Student's t test*)

Figure 3: Cortical newborn astrocytes culture and in-vitro evaluation of clozapine treatment effects on [^{3}H]DAsp and [^{3}H]2DG uptake.

(a) Representative images of the newborn primary cortical astrocytes cultures stained with GFAP (red) and DAPI (blue). 20×magnification and scale bar=50 µm. Immunocontent of the glutamate transporters **(b,c)** GLT-1 **(d,e)** and GLAST on the newborn primary cortical astrocytes cultures (normalized by GAPDH immunoreactivity); (n=8). **(f)** Basal (10µM glutamate) and **(g)** Stimulated (100µM glutamate) [^{3}H]D-Asp uptake on newborn astrocytes cultures; (n=6). **(i)** Basal (10µM glutamate) and **(j)** Stimulated (200µM glutamate) [^{3}H]2DG uptake on newborn astrocytes cultures (n=8). Comparisons of **(h)** [^{3}H]D-Asp and **(k)** [^{3}H]2DG uptake results on both conditions (Basal and Stimulated) at groups control and clozapine. Uptake values expressed as percentage of control. Data represented as mean ± S.E.M. *p<0.05; **p<0.01; ***p<0.001 (paired *Student's t test*); ××p<0.01; ××××p<0.0001, differences between groups control e clozapine; ####p<0.0001, differences between Basal and Stimulated (multiple comparisons - Two-Way ANOVA)

Supplemental Figure 1: Cortical adult astrocytes culture and ex-vivo evaluation of clozapine treatment effects on [^{3}H]DAsp and [^{3}H]2DG uptake.

(a) Representative images of the adult primary cortical astrocytes cultures stained with GFAP (red) and DAPI (blue). 20×magnification and scale bar=50 µm. Immunocontent of the glutamate transporters **(b,c)** GLT-1 **(d,e)** and GLAST on the adult primary cortical astrocytes cultures (normalized by GAPDH immunoreactivity); (n=8-10). **(f)** Basal (10µM glutamate) and **(g)** Stimulated (100µM glutamate) [^{3}H]D-Asp uptake on adult astrocytes cultures; (n=10-11). **(i)** Basal (10µM glutamate) and **(j)** Stimulated (200µM glutamate) [^{3}H]2DG uptake on adult astrocytes cultures (n=9-12). Comparisons of **(h)** [^{3}H]D-Asp and **(k)** [^{3}H]2DG uptake results on both conditions (Basal and Stimulated) at groups control and clozapine. Uptake values expressed as percentage of control. Data represented as mean ± S.E.M. *p<0.05; **p<0.01; ***p<0.001 (paired S*tudent's t test*)

Supplemental Figure 2: Behavioral analysis following clozapine treatment.

(a) Representative occupancy plot of groups control and clozapine on the Open Field task after the treatment period. **(b)** Total distance traveled on the Open Field task.

(c) Total time immobile on the Open Field task; (n=12-14). **(d)** Representative occupancy plot on the NOR task of groups control and clozapine on the training and test sessions. **(e)** Percentage of time exploring the objects on the training session at NOR task. **(f)** Percentage of time exploring the objects on the test session at NOR task; (n=5-7). Data displayed as mean ± S.E.M. ***p<0.001 (One-sample t-test - hypothetical value=50%)

Supplemental Figure 3: Body weight and clozapine treatment measures

(a) Control and clozapine rats body weight across the treatment period. **(b)** Control and clozapine rats liquid intake across the treatment period. (n=12-14). Data displayed as mean ± S.D. **(c)** Chromatogram (HPLC) showing reproducibility of clozapine solutions on three different preparations in comparison to a standard sample. **(d)** Chromatogram (HPLC) showing the stability of the clozapine solutions on a 24 h period at RT.

Supplemental Data

Sup1. Methods and Materials

Sup1.1 Primary cortical astrocytes cultures: adult rats

The adult astrocytes culture was done as previously described by our group [47]. Whole cortex was aseptically dissected out from 129-132 days old rats after the pharmacological treatment (groups Control and Clozapine) and kept in HBSS (except for some fractions used to Western Blot and PCR) containing 0,05% trypsin and 0,003% DNase. Next, this solution was kept at 37°C for 15min. After, tissue was mechanically dissociated with a Pasteur Pipette for more 15min, and centrifuged at 1000g for 5 min. The pellet was resuspended in a solution of HBSS containing 40 U papain/mL (Merck Millipore, Billerica/USA), 0,02% cysteine and 0,003% DNase and again mechanically dissociated for 15min, gently, with a Pasteur Pipette. After another centrifugation (1000g, 5min), cells were ressuspended in HBSS containing only DNase 0,003% and left for decantation during 40min. Supernatant was collected and centrifuged for 7 min, 1000g. Cells were ressuspended in DMEM/F12 (Gibco, Thermo Fischer Scientific, Waltham/USA) containing 10% FBS, 15mM HEPES, 14.3mM $NaHCO_3$, 1% fungizone and 0,04% gentamicyn, plated in 24-well plates pre-coated with poli-L-lisine and cultured at 37°C in an atmosphere with 5% CO_2. Were seeded 3 – 5 x 10^5 cells/cm^2. For cells culture maintenance the first medium exchange occurred 24h after obtaining the culture. After that, the medium change occurred once every three days. From the third week on, cells received medium supplemented with 20% FBS. Around 4^{th} to 5^{th} week, cells were used for immunofluorescence, western blot or uptake procedures. No clozapine was added to the adult cultures.

Sup1.2 Open Field

At the end of the six weeks of treatment, two days before the final imaging procedure (PND126), rats were submitted to an open field (OF) test to evaluate locomotion. The apparatus was made of black-painted Plexiglas measuring 50×50 cm and was surrounded by 50cm high walls. Each rat was placed in the center of the arena and allowed to walk and explore it for 5min. The experiments were conducted in a sound-attenuated room under low-intensity light. Rats activity was recorded with a video

camera positioned above the arena. Locomotion was analyzed using a computer-operated tracking system (Any-maze, Stoelting, Woods Dala/USA).

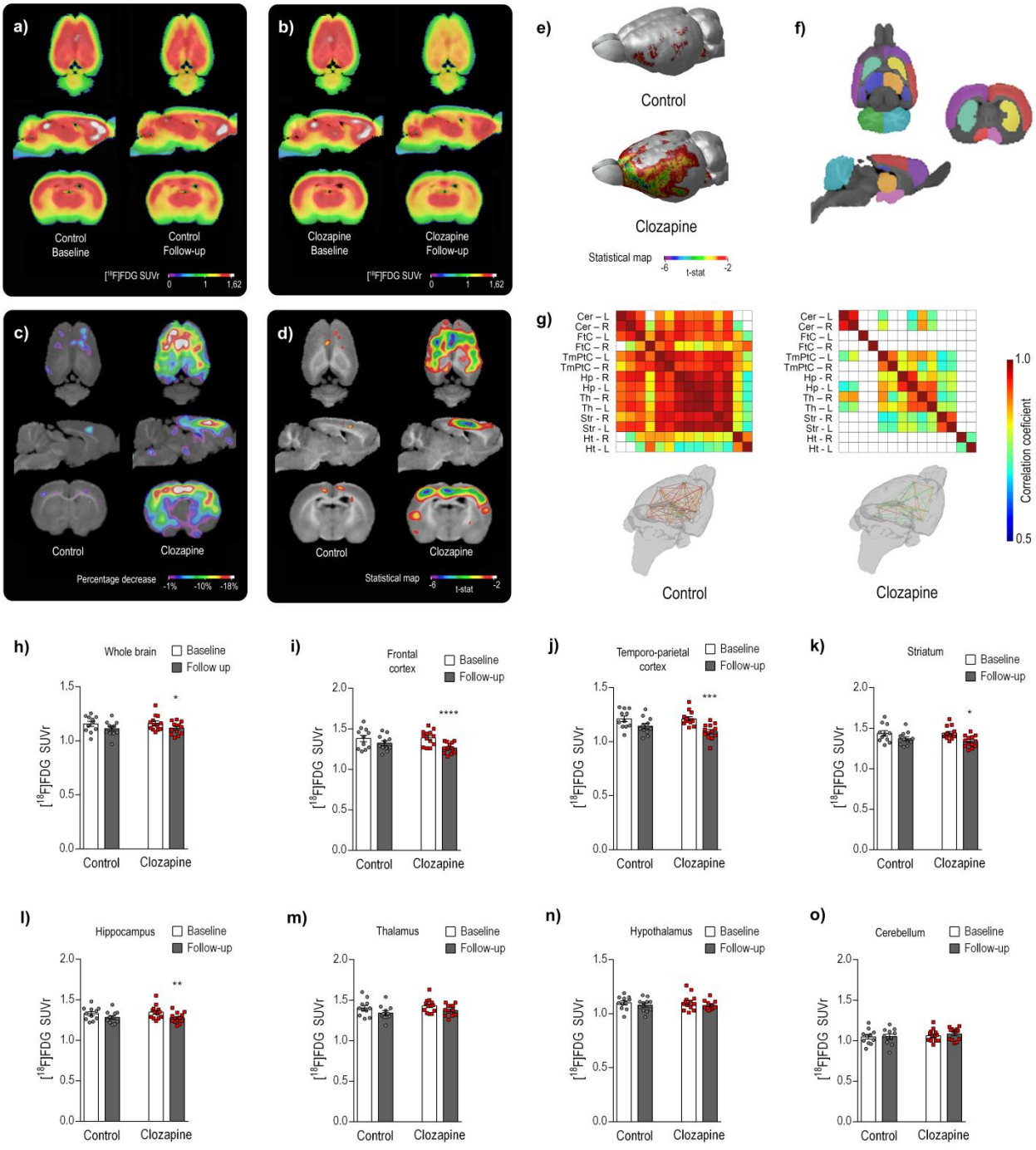
Sup1.3 Novel Object Recognition Task

The novel object recognition (NOR) test was carried out 24hs after the OF. Rats first underwent a training session in which two identical objects were placed in parallel in one side of the arena. Rats were placed individually into the open field facing the center of the opposite wall and allowed to explore the objects for 5min. The test session was performed 90min after training and two dissimilar objects were presented, a familiar one and a novel one. Rats activity was recorded with a video camera positioned above the arena and analyzed by an observer blind to the treatment of the animals. Exploration was defined by directing the nose to the object at a distance of at least 2cm and/or touching the object with the nose or forepaws. Rearing on to object was not considered exploratory behavior. Animals that exhibited a total exploration time of less than 10sec or showed an evident preference for an object in the training session were excluded from the analysis.

Sup1.4 Clozapine high-performance liquid chromatography

A HPLC with ultraviolet detection (UV) method was applied to verify clozapine presence and stability on the animals drinking solution. The chromatographic separation was conducted in a column Agilent zorbax SB-C18 (2.1 x 50mm, 1.8µm, particle size) with a C-18 pre-column. Chromatographic parameters were set as follows: flow rate 0.2mL/min; temperature 25°C; injection volume 2µL; mobile phase consisting of phosphate buffer pH 3.5 (0.5% KH_2PO_4, 5% H_3PO_4):acetonitrile:methanol (63:25:12 v/v); UV detection at 292 nm. Three independent solutions of clozapine were prepared as previously described on section 2.2 and analyzed concerning reproducibility and expected concentration. To assess the stability, the same solutions were verified 24h later, after staying at RT.

Figure 1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Control Baseline
Control Follow-up
[18F]FDG SUVr
0
1
1,62
b)
Clozapine Baseline
Clozapine Follow-up
[18F]FDG SUVr
0
1
1,62
c)
Control
Clozapine
Percentage decrease
-1%
-10%
-18%
d)
Control
Clozapine
Statistical map
-6
t-stat
-2
e)
Control
Clozapine
Statistical map
-6
t-stat
-2
f)
g)
Cer – L
Cer – R
FtC – L
FtC – R
TmPtC – L
TmPtC – R
Hp - R
Hp - L
Th – R
Th – L
Str - R
Str - L
Ht - R
Ht - L
Control
Clozapine
1.0
Correlation coeficient
0.5
h)
Whole brain
Baseline
Follow up
[18F]FDG SUVr
Control
Clozapine
i)
Frontal cortex
Baseline
Follow-up
[18F]FDG SUVr
Control
Clozapine
j)
Temporo-parietal cortex
Baseline
Follow-up
[18F]FDG SUVr
Control
Clozapine
k)
Striatum
Baseline
Follow-up
[18F]FDG SUVr
Control
Clozapine
l)
Hippocampus
Baseline
Follow-up
[18F]FDG SUVr
Control
Clozapine
m)
Thalamus
Baseline
Follow-up
[18F]FDG SUVr
Control
Clozapine
n)
Hypothalamus
Baseline
Follow-up
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Control
Clozapine
o)
Cerebellum
Baseline
Follow-up
[18F]FDG SUVr
Control
Clozapine

Figure 2

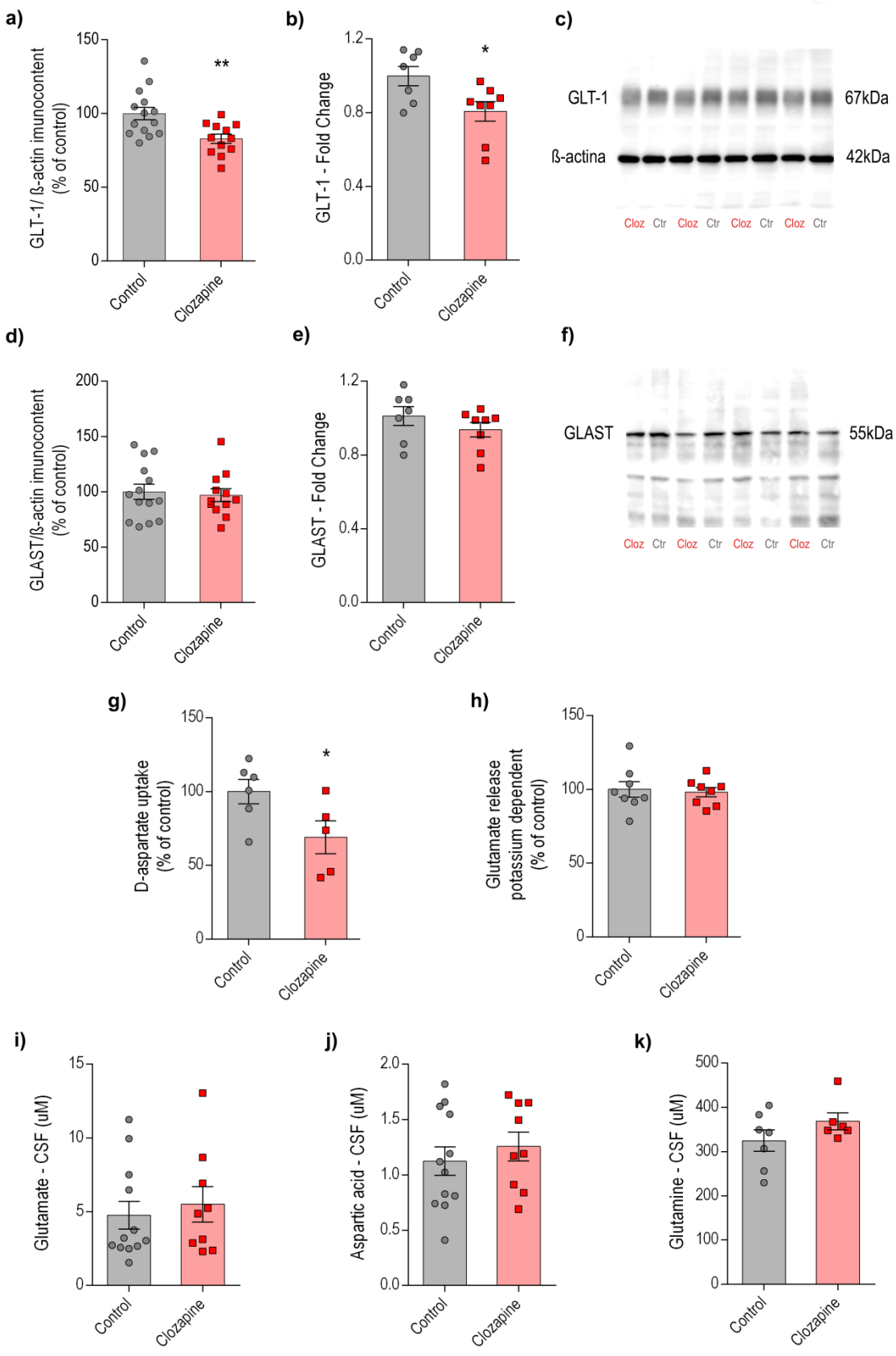
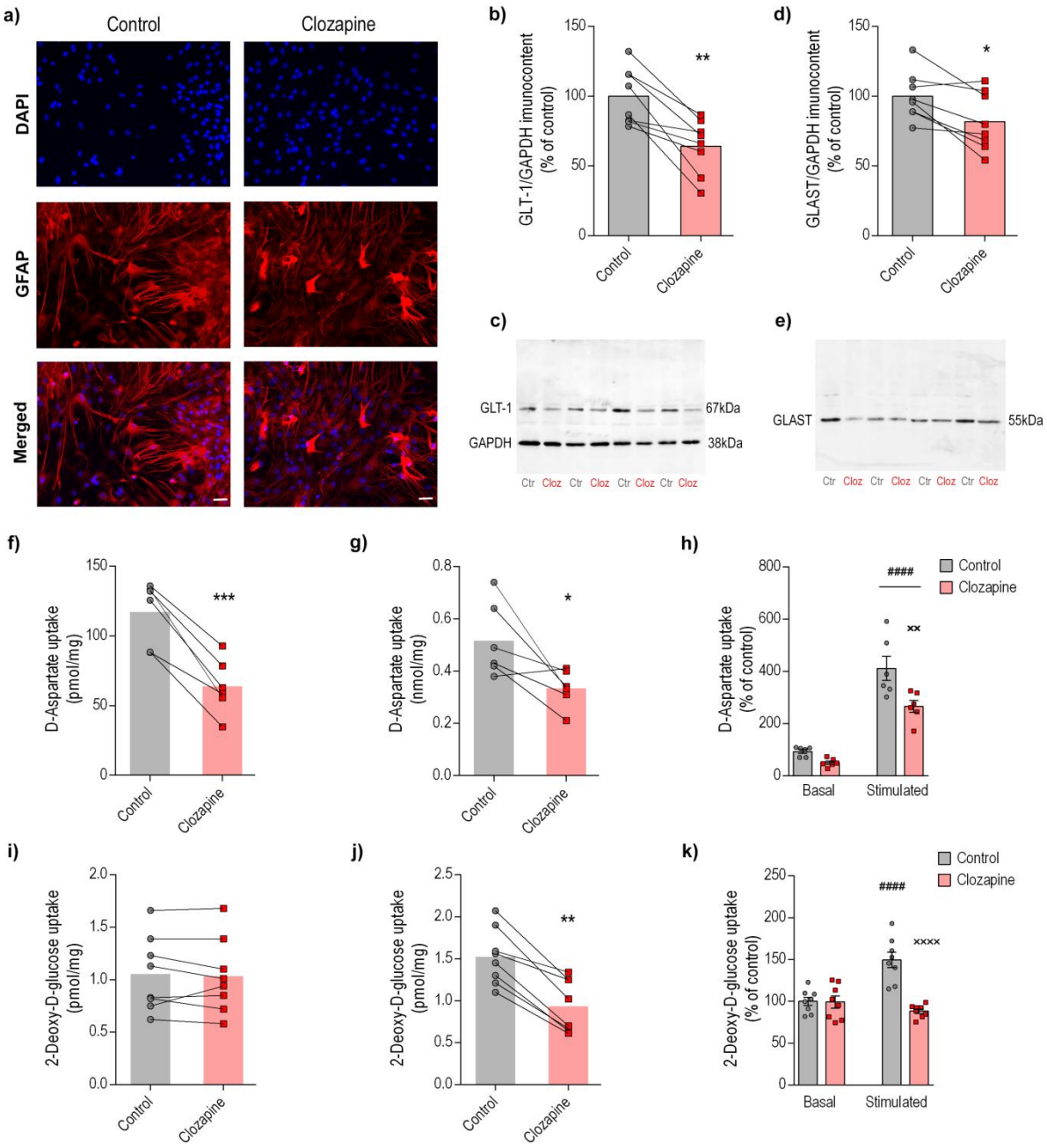

a)
GLT-1/ ß-actin imunocontent (% of control)
150
100
50
0
**
Control
Clozapine
b)
GLT-1 - Fold Change
1.2
0.8
0.4
0.0
*
Control
Clozapine
c)
GLT-1
67kDa
ß-actina
42kDa
Cloz Ctr Cloz Ctr Cloz Ctr Cloz Ctr
d)
GLAST/ß-actin imunocontent (% of control)
200
150
100
50
0
Control
Clozapine
e)
GLAST - Fold Change
1.2
0.8
0.4
0.0
Control
Clozapine
f)
GLAST
55kDa
Cloz Ctr Cloz Ctr Cloz Ctr Cloz Ctr
g)
D-aspartate uptake (% of control)
150
100
50
0
*
Control
Clozapine
h)
Glutamate release potassium dependent (% of control)
150
100
50
0
Control
Clozapine
i)
Glutamate - CSF (uM)
15
10
5
0
Control
Clozapine
j)
Aspartic acid - CSF (uM)
2.0
1.5
1.0
0.5
0.0
Control
Clozapine
k)
Glutamine - CSF (uM)
500
400
300
200
100
0
Control
Clozapine

Figure 3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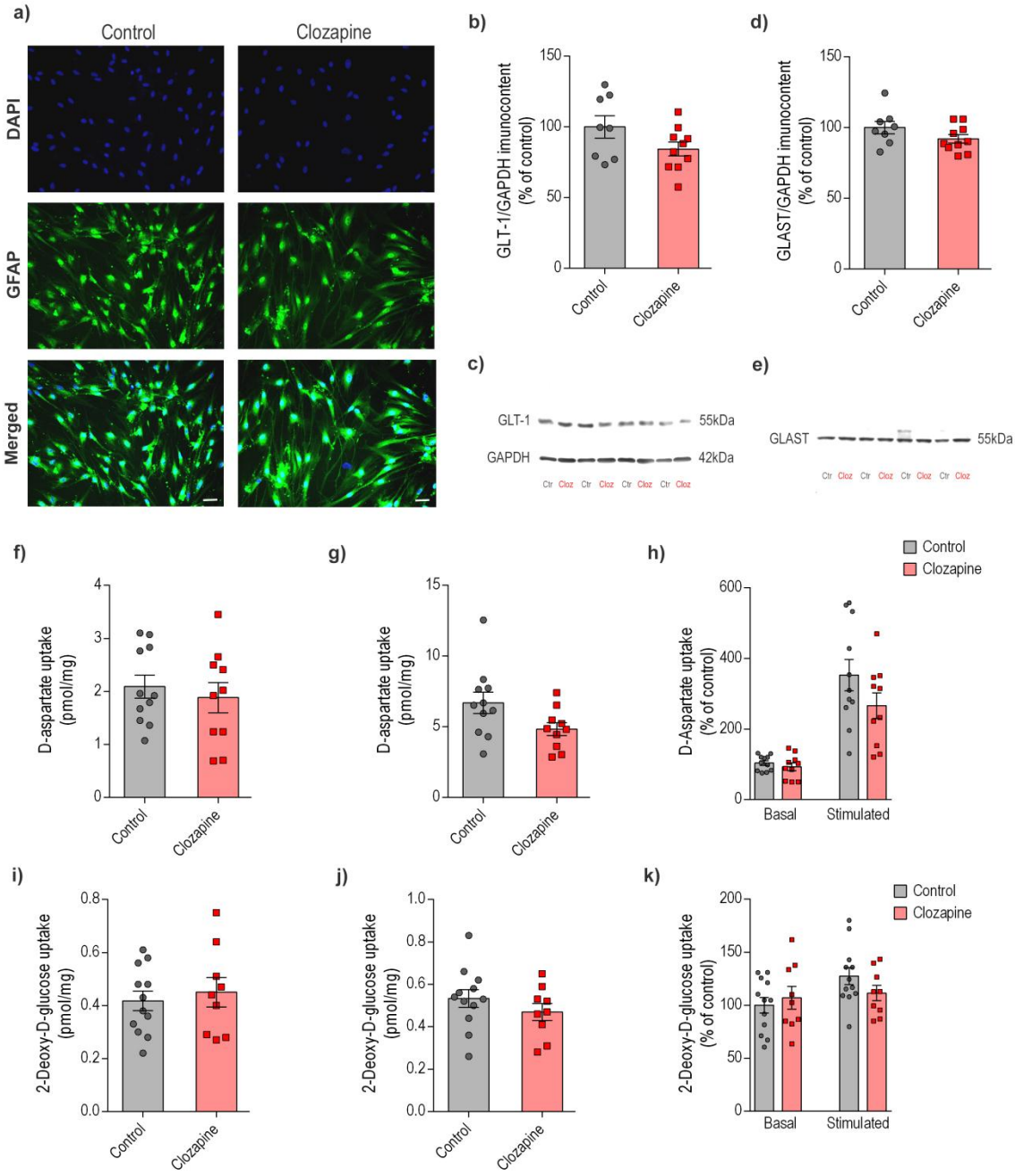
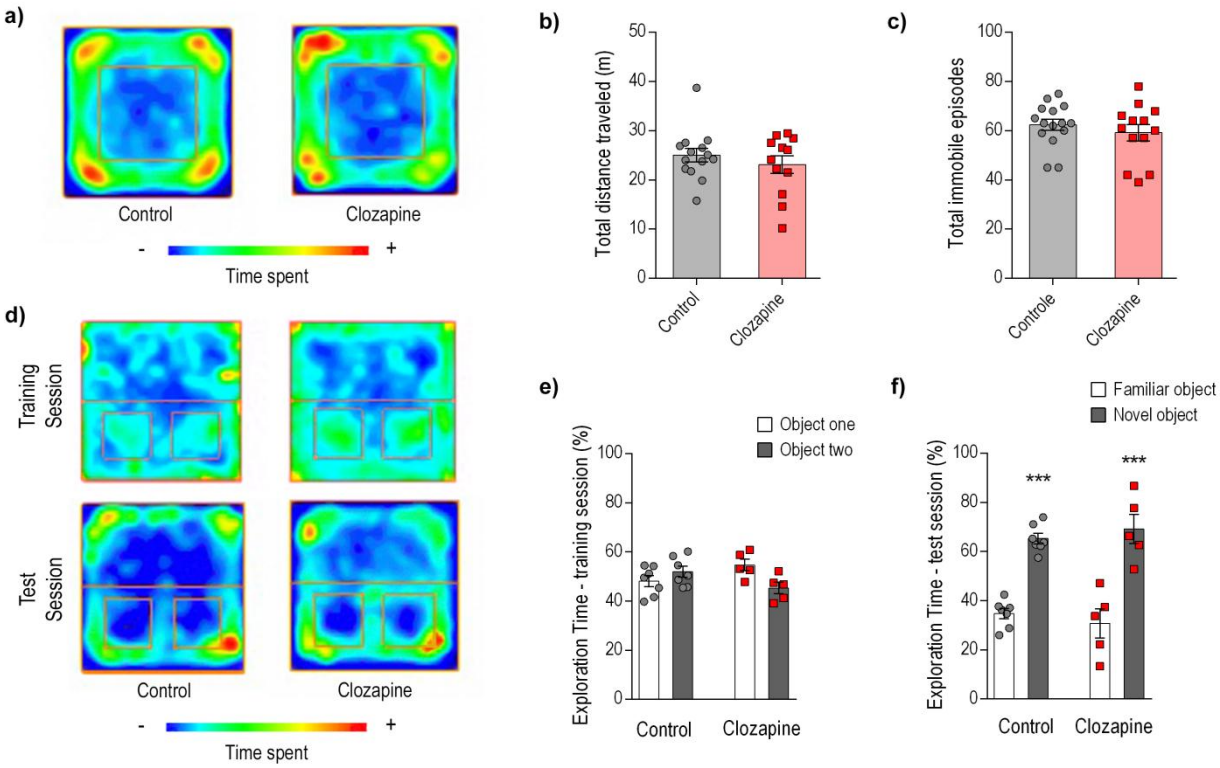
Control
Clozapine
DAPI
GFAP
Merged
b)
GLT-1/GAPDH imunocontent (% of control)
Control
Clozapine
d)
GLAST/GAPDH imunocontent (% of control)
c)
GLT-1
GAPDH
67kDa
38kDa
Ctr Cloz Ctr Cloz Ctr Cloz Ctr Cloz
e)
GLAST
55kDa
f)
D-Aspartate uptake (pmol/mg)
g)
D-Aspartate uptake (nmol/mg)
h)
D-Aspartate uptake (% of control)
Basal
Stimulated
i)
2-Deoxy-D-glucose uptake (pmol/mg)
j)
2-Deoxy-D-glucose uptake (pmol/mg)
k)
2-Deoxy-D-glucose uptake (% of control)

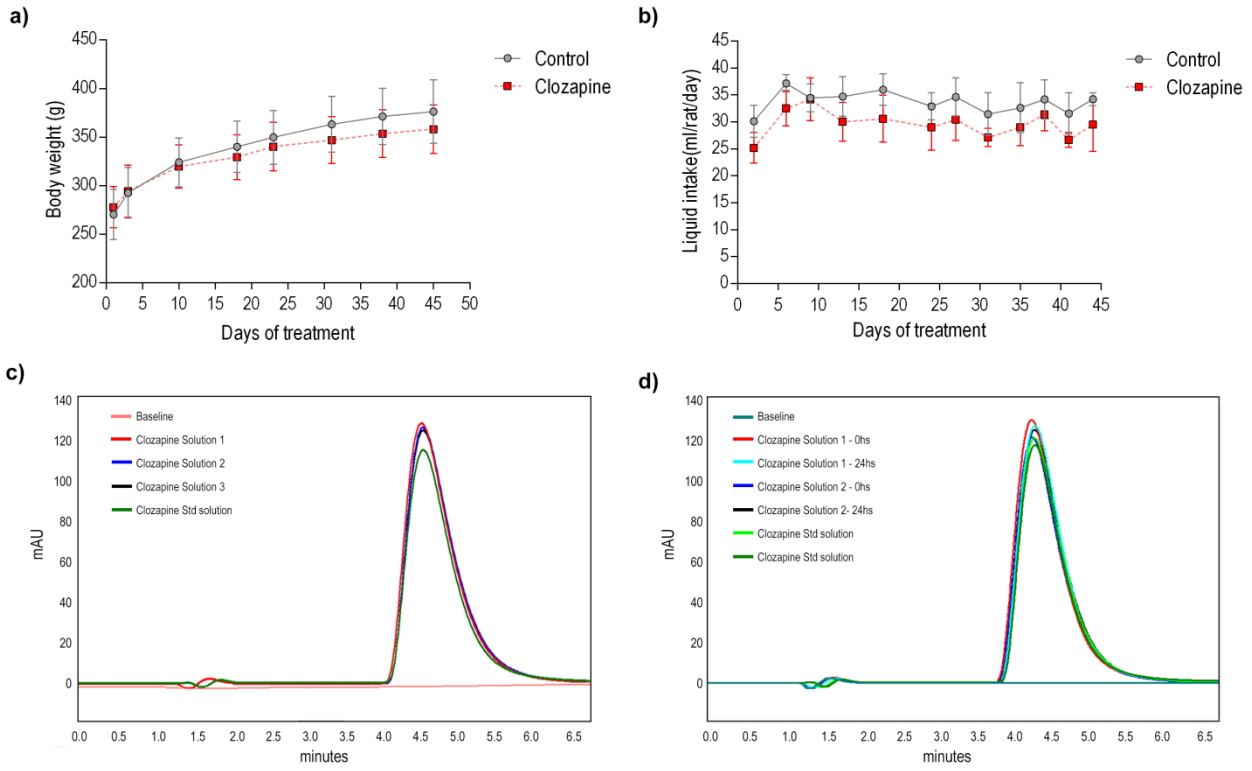
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



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 this cells. The results on the astrocytic culture corroborated the *in vivo* observations. In conclusion, their evidences supports the hypothesis of a significant astrocytic participation on the [^{18}F]FDG-PET signal.

On the other side, this observations only show one side of the story: astrocytes can increase the [^{18}F]FDG-PET signal. Then comes the question: could astrocytes decrease the [^{18}F]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 [^{18}F]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 immunocontent 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 [^{3}H]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 [^{3}H]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 [^{18}F]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 serotoninergic, 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 manly 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 [^{18}F]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 [^{18}F]FDG-PET results being replicated on astrocyte cultures by the [^{3}H]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 [^{18}F]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 [^{18}F]FDG-PET.

This investigation has direct implication in the clinic, since the [^{18}F]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 [^{18}F]FDG-PET in brain diseases. For example, hypometabolic regions captured by [^{18}F]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.

REFERENCES

ALAVI, A. Functional and anatomic studies of head injury. **J Neuropsychiatry Clin Neurosci,** v. 1, n. 1, p. S45-50, Winter 1989. ISSN 0895-0172 (Print)

0895-0172 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/2535420 >.

ALTAMURA, A. C. et al. White matter metabolism differentiates schizophrenia and bipolar disorder: a preliminary PET study. **Psychiatry Res,** v. 214, n. 3, p. 410-4, Dec 30 2013. ISSN 1872-7123 (Electronic)

0165-1781 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24144506 >.

ANDERSON, C. M.; SWANSON, R. A. Astrocyte glutamate transport: review of properties, regulation, and physiological functions. **Glia,** v. 32, n. 1, p. 1-14, Oct 2000. ISSN 0894-1491 (Print)

0894-1491 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/10975906 >.

ARAQUE, A. et al. Tripartite synapses: glia, the unacknowledged partner. **Trends Neurosci,** v. 22, n. 5, p. 208-15, May 1999. ISSN 0166-2236 (Print)

0166-2236 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10322493 >.

ATTWELL, D.; LAUGHLIN, S. B. An energy budget for signaling in the grey matter of the brain. **J Cereb Blood Flow Metab,** v. 21, n. 10, p. 1133-45, Oct 2001. ISSN 0271-678X (Print)

0271-678X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11598490 >.

BAK, L. K.; SCHOUSBOE, A.; WAAGEPETERSEN, H. S. The glutamate/GABA-glutamine cycle: aspects of transport, neurotransmitter homeostasis and ammonia transfer. **J Neurochem,** v. 98, n. 3, p. 641-53, Aug 2006. ISSN 0022-3042 (Print)

0022-3042 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16787421 >.

BAK, L. K.; WALLS, A. B. CrossTalk opposing view: lack of evidence supporting an astrocyte-to-neuron lactate shuttle coupling neuronal activity to glucose utilisation in the brain. **J Physiol,** v. 596, n. 3, p. 351-353, Feb 1 2018a. ISSN 1469-7793 (Electronic)

0022-3751 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/29292507 >.

_____. Rebuttal from Lasse K. Bak and Anne B. Walls. **J Physiol,** v. 596, n. 3, p. 357, Feb 1 2018b. ISSN 1469-7793 (Electronic)

0022-3751 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/29292517 >.

BARROS, L. F.; WEBER, B. CrossTalk proposal: an important astrocyte-to-neuron lactate shuttle couples neuronal activity to glucose utilisation in the brain. **J Physiol,** v. 596, n. 3, p. 347-350, Feb 1 2018a. ISSN 1469-7793 (Electronic)

0022-3751 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/29292516 >.

_____. Rebuttal from L. F. Barros and B. Weber. **J Physiol,** v. 596, n. 3, p. 355-356, Feb 1 2018b. ISSN 1469-7793 (Electronic)

0022-3751 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/29292518 >.

BELANGER, M.; ALLAMAN, I.; MAGISTRETTI, P. J. Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation. **Cell Metab,** v. 14, n. 6, p. 724-38, Dec 07 2011. ISSN 1932-7420 (Electronic)

1550-4131 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/22152301 >.

BERGER, A. How does it work? Positron emission tomography. **BMJ,** v. 326, n. 7404, p. 1449, Jun 28 2003. ISSN 1756-1833 (Electronic)

0959-535X (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/12829560 >.

BERTI, V.; MOSCONI, L.; PUPI, A. Brain: normal variations and benign findings in fluorodeoxyglucose-PET/computed tomography imaging. **PET Clin,** v. 9, n. 2, p. 129-40, Apr 2014. ISSN 1879-9809 (Electronic)

1556-8598 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/24772054 >.

BIRNBAUMER, L. et al. The naming of voltage-gated calcium channels. **Neuron,** v. 13, n. 3, p. 505-6, Sep 1994. ISSN 0896-6273 (Print)

0896-6273 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/7917287 >.

BITTNER, C. X. et al. Fast and reversible stimulation of astrocytic glycolysis by K+ and a delayed and persistent effect of glutamate. **J Neurosci,** v. 31, n. 12, p. 4709-13, Mar 23 2011. ISSN 1529-2401 (Electronic)

0270-6474 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/21430169 >.

BLINKENBERG, M. et al. A longitudinal study of cerebral glucose metabolism, MRI, and disability in patients with MS. **Neurology,** v. 53, n. 1, p. 149-53, Jul 13 1999. ISSN 0028-3878 (Print)

0028-3878 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10408551 >.

BLISS, T. V.; COLLINGRIDGE, G. L. A synaptic model of memory: long-term potentiation in the hippocampus. **Nature,** v. 361, n. 6407, p. 31-9, Jan 7 1993. ISSN 0028-0836 (Print)

0028-0836 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/8421494 >.

BONVENTO, G.; HERARD, A. S.; VOUTSINOS-PORCHE, B. The astrocyte--neuron lactate shuttle: a debated but still valuable hypothesis for brain imaging. **J Cereb Blood Flow Metab,** v. 25, n. 10, p. 1394-9, Oct 2005. ISSN 0271-678X (Print)

0271-678X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15843788 >.

BONVENTO, G.; SIBSON, N.; PELLERIN, L. Does glutamate image your thoughts? **Trends Neurosci,** v. 25, n. 7, p. 359-64, Jul 2002. ISSN 0166-2236 (Print)

0166-2236 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12079764 >.

BRAGINA, L. et al. GLT-1 down-regulation induced by clozapine in rat frontal cortex is associated with synaptophysin up-regulation. **J Neurochem,** v. 99, n. 1, p. 134-41, Oct 2006. ISSN 0022-3042 (Print)

0022-3042 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16987241 >.

BROWN, R. K. et al. Brain PET in suspected dementia: patterns of altered FDG metabolism. **Radiographics,** v. 34, n. 3, p. 684-701, May-Jun 2014. ISSN 1527-1323 (Electronic)

0271-5333 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/24819789 >.

CARTER, S. F. et al. Longitudinal association between astrocyte function and glucose metabolism in autosomal dominant Alzheimer's disease. **Eur J Nucl Med Mol Imaging,** v. 46, n. 2, p. 348-356, Feb 2019. ISSN 1619-7089 (Electronic)

1619-7070 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/30515545 >.

CHOI, D. W. Glutamate receptors and the induction of excitotoxic neuronal death. **Prog Brain Res,** v. 100, p. 47-51, 1994. ISSN 0079-6123 (Print)

0079-6123 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/7938533 >.

CHOLET, N. et al. Local injection of antisense oligonucleotides targeted to the glial glutamate transporter GLAST decreases the metabolic response to somatosensory activation. **J Cereb Blood Flow Metab,** v. 21, n. 4, p. 404-12, Apr 2001. ISSN 0271-678X (Print)

0271-678X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11323526 >.

CHUQUET, J. et al. Predominant enhancement of glucose uptake in astrocytes versus neurons during activation of the somatosensory cortex. **J Neurosci,** v. 30, n. 45, p. 15298-303, Nov 10 2010. ISSN 1529-2401 (Electronic)

0270-6474 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/21068334 >.

COHEN, R. M. et al. The brain metabolic patterns of clozapine- and fluphenazine-treated patients with schizophrenia during a continuous performance task. **Arch Gen Psychiatry,** v. 54, n. 5, p. 481-6, May 1997. ISSN 0003-990X (Print)

0003-990X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9152102 >.

DANBOLT, N. C. Glutamate uptake. **Prog Neurobiol,** v. 65, n. 1, p. 1-105, Sep 2001. ISSN 0301-0082 (Print)

0301-0082 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11369436 >.

DANBOLT, N. C.; FURNESS, D. N.; ZHOU, Y. Neuronal vs glial glutamate uptake: Resolving the conundrum. **Neurochem Int,** v. 98, p. 29-45, Sep 2016. ISSN 1872-9754 (Electronic)

0197-0186 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/27235987 >.

DIENEL, G. A. Astrocytic energetics during excitatory neurotransmission: What are contributions of glutamate oxidation and glycolysis? **Neurochem Int,** v. 63, n. 4, p. 244-58, Oct 2013. ISSN 1872-9754 (Electronic)

0197-0186 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/23838211 >.

_____. Lack of appropriate stoichiometry: Strong evidence against an energetically important astrocyte-neuron lactate shuttle in brain. **J Neurosci Res,** v. 95, n. 11, p. 2103-2125, Nov 2017. ISSN 1097-4547 (Electronic)

0360-4012 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/28151548 >.

_____. Brain Glucose Metabolism: Integration of Energetics with Function. **Physiol Rev,** v. 99, n. 1, p. 949-1045, Jan 1 2019. ISSN 1522-1210 (Electronic)

0031-9333 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/30565508 >.

DIENEL, G. A.; BEHAR, K. L.; ROTHMAN, D. L. Cellular Origin of [(18)F]FDG-PET Imaging Signals During Ceftriaxone-Stimulated Glutamate Uptake: Astrocytes and Neurons. **Neuroscientist,** v. 24, n. 4, p. 316-328, Aug 2018. ISSN 1089-4098 (Electronic)

1073-8584 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/29276856 >.

DINGLEDINE, R. et al. The glutamate receptor ion channels. **Pharmacol Rev,** v. 51, n. 1, p. 7-61, Mar 1999. ISSN 0031-6997 (Print)

0031-6997 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/10049997 >.

DOBLE, A. The role of excitotoxicity in neurodegenerative disease: implications for therapy. **Pharmacol Ther,** v. 81, n. 3, p. 163-221, Mar 1999. ISSN 0163-7258 (Print)

0163-7258 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/10334661 >.

DUELLI, R.; KUSCHINSKY, W. Brain glucose transporters: relationship to local energy demand. **News Physiol Sci,** v. 16, p. 71-6, Apr 2001. ISSN 0886-1714 (Print)

0886-1714 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11390952 >.

DUNLOP, J. et al. WAY-855 (3-amino-tricyclo[2.2.1.02.6]heptane-1,3-dicarboxylic acid): a novel, EAAT2-preferring, nonsubstrate inhibitor of high-affinity glutamate uptake. **Br J Pharmacol,** v. 140, n. 5, p. 839-46, Nov 2003. ISSN 0007-1188 (Print)

0007-1188 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14517179 >.

ERIKSSON, G. et al. Sodium-dependent glutamate uptake as an activator of oxidative metabolism in primary astrocyte cultures from newborn rat. **Glia,** v. 15, n. 2, p. 152-6, Oct 1995. ISSN 0894-1491 (Print)

0894-1491 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/8567066 >.

ESSLINGER, C. S. et al. The substituted aspartate analogue L-beta-threo-benzyl-aspartate preferentially inhibits the neuronal excitatory amino acid transporter EAAT3. **Neuropharmacology,** v. 49, n. 6, p. 850-61, Nov 2005. ISSN 0028-3908 (Print)

0028-3908 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16183084 >.

FAIRMAN, W. A.; AMARA, S. G. Functional diversity of excitatory amino acid transporters: ion channel and transport modes. **Am J Physiol,** v. 277, n. 4 Pt 2, p. F481-6, Oct 1999. ISSN 0002-9513 (Print)

0002-9513 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/10516269 >.

FEIGIN, A. et al. Metabolic network abnormalities in early Huntington's disease: an [(18)F]FDG PET study. **J Nucl Med,** v. 42, n. 11, p. 1591-5, Nov 2001. ISSN 0161-5505 (Print)

0161-5505 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11696626 >.

FERNANDEZ-MONCADA, I. et al. Neuronal control of astrocytic respiration through a variant of the Crabtree effect. **Proc Natl Acad Sci U S A,** v. 115, n. 7, p. 1623-1628, Feb 13 2018. ISSN 1091-6490 (Electronic)

0027-8424 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/29378955 >.

FIGLEY, C. R.; STROMAN, P. W. The role(s) of astrocytes and astrocyte activity in neurometabolism, neurovascular coupling, and the production of functional neuroimaging signals. **Eur J Neurosci,** v. 33, n. 4, p. 577-88, Feb 2011. ISSN 1460-9568 (Electronic)

0953-816X (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/21314846 >.

FONTANA, A. C. Current approaches to enhance glutamate transporter function and expression. **J Neurochem,** v. 134, n. 6, p. 982-1007, Sep 2015. ISSN 1471-4159 (Electronic)

0022-3042 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/26096891 >.

FOWLER, J. S.; IDO, T. Initial and subsequent approach for the synthesis of 18FDG. **Semin Nucl Med,** v. 32, n. 1, p. 6-12, Jan 2002. ISSN 0001-2998 (Print)

0001-2998 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11839070 >.

GREENBERG, J. H. et al. Metabolic mapping of functional activity in human subjects with the [18F]fluorodeoxyglucose technique. **Science,** v. 212, n. 4495, p. 678-80, May 08 1981. ISSN 0036-8075 (Print)

0036-8075 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/6971492 >.

GYNTHER, M. et al. Blood-brain barrier permeability and brain uptake mechanism of kainic acid and dihydrokainic acid. **Neurochem Res,** v. 40, n. 3, p. 542-9, Mar 2015. ISSN 1573-6903 (Electronic)

0364-3190 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25488153 >.

HEISS, W. D. et al. Cerebral glucose metabolism as a predictor of recovery from aphasia in ischemic stroke. **Arch Neurol,** v. 50, n. 9, p. 958-64, Sep 1993. ISSN 0003-9942 (Print)

0003-9942 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/8363450 >.

HERTZ, L. The astrocyte-neuron lactate shuttle: a challenge of a challenge. **J Cereb Blood Flow Metab,** v. 24, n. 11, p. 1241-8, Nov 2004. ISSN 0271-678X (Print)

0271-678X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15545919 >.

HOLMSETH, S. et al. The concentrations and distributions of three C-terminal variants of the GLT1 (EAAT2; slc1a2) glutamate transporter protein in rat brain tissue suggest differential regulation. **Neuroscience,** v. 162, n. 4, p. 1055-71, Sep 15 2009. ISSN 1873-7544 (Electronic)

0306-4522 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19328838 >.

HOWARTH, C.; PEPPIATT-WILDMAN, C. M.; ATTWELL, D. The energy use associated with neural computation in the cerebellum. **J Cereb Blood Flow Metab,** v. 30, n. 2, p. 403-14, Feb 2010. ISSN 1559-7016 (Electronic)

0271-678X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/19888288 >.

ITOH, Y. et al. Dichloroacetate effects on glucose and lactate oxidation by neurons and astroglia in vitro and on glucose utilization by brain in vivo. **Proc Natl Acad Sci U S A,** v. 100, n. 8, p. 4879-84, Apr 15 2003. ISSN 0027-8424 (Print)

0027-8424 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/12668764 >.

JOHNSTON, G. A.; KENNEDY, S. M.; TWITCHIN, B. Action of the neurotoxin kainic acid on high affinity uptake of L-glutamic acid in rat brain slices. **J Neurochem,** v. 32, n. 1, p. 121-7, Jan 1979. ISSN 0022-3042 (Print)

0022-3042 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/759563 >.

KELLOFF, G. J. et al. Progress and promise of FDG-PET imaging for cancer patient management and oncologic drug development. **Clin Cancer Res,** v. 11, n. 8, p. 2785-808, Apr 15 2005. ISSN 1078-0432 (Print)

1078-0432 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/15837727 >.

KENNEDY, C. et al. Mapping of functional neural pathways by autoradiographic survey of local metabolic rate with (14C)deoxyglucose. **Science,** v. 187, n. 4179, p. 850-3, Mar 7 1975. ISSN 0036-8075 (Print)

0036-8075 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/1114332 >.

KEW, J. N.; KEMP, J. A. Ionotropic and metabotropic glutamate receptor structure and pharmacology. **Psychopharmacology (Berl),** v. 179, n. 1, p. 4-29, Apr 2005. ISSN 0033-3158 (Print)

0033-3158 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/15731895 >.

LEHRE, K. P. et al. Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. **J Neurosci,** v. 15, n. 3 Pt 1, p. 1835-53, Mar 1995. ISSN 0270-6474 (Print)

0270-6474 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/7891138 >.

LEINO, R. L. et al. Ultrastructural localization of GLUT 1 and GLUT 3 glucose transporters in rat brain. **J Neurosci Res,** v. 49, n. 5, p. 617-26, Sep 1 1997. ISSN 0360-4012 (Print)

0360-4012 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9302083 >.

LENNIE, P. The cost of cortical computation. **Curr Biol,** v. 13, n. 6, p. 493-7, Mar 18 2003. ISSN 0960-9822 (Print)

0960-9822 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12646132 >.

LUNDGAARD, I. et al. Direct neuronal glucose uptake heralds activity-dependent increases in cerebral metabolism. **Nat Commun,** v. 6, p. 6807, Apr 23 2015. ISSN 2041-1723 (Electronic)

2041-1723 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25904018 >.

MACVICAR, B. A.; NEWMAN, E. A. Astrocyte regulation of blood flow in the brain. **Cold Spring Harb Perspect Biol,** v. 7, n. 5, Mar 27 2015. ISSN 1943-0264 (Electronic)

1943-0264 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/25818565 >.

MAGISTRETTI, P. J. Cellular bases of functional brain imaging: insights from neuron-glia metabolic coupling. **Brain Res,** v. 886, n. 1-2, p. 108-112, Dec 15 2000. ISSN 0006-8993 (Print)

0006-8993 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/11119692 >.

MAGISTRETTI, P. J.; ALLAMAN, I. A cellular perspective on brain energy metabolism and functional imaging. **Neuron,** v. 86, n. 4, p. 883-901, May 20 2015. ISSN 1097-4199 (Electronic)

0896-6273 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25996133 >.

MAGISTRETTI, P. J.; PELLERIN, L. The contribution of astrocytes to the 18F-2-deoxyglucose signal in PET activation studies. **Mol Psychiatry,** v. 1, n. 6, p. 445-52, Dec 1996. ISSN 1359-4184 (Print)

1359-4184 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9154245 >.

_____. Cellular mechanisms of brain energy metabolism and their relevance to functional brain imaging. **Philos Trans R Soc Lond B Biol Sci,** v. 354, n. 1387, p. 1155-63, Jul 29 1999. ISSN 0962-8436 (Print)

0962-8436 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/10466143 >.

MAKI, R.; ROBINSON, M. B.; DICHTER, M. A. The glutamate uptake inhibitor L-trans-pyrrolidine-2,4-dicarboxylate depresses excitatory synaptic transmission via a presynaptic mechanism in cultured hippocampal neurons. **J Neurosci,** v. 14, n. 11 Pt 1, p. 6754-62, Nov 1994. ISSN 0270-6474 (Print)

0270-6474 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/7965076 >.

MARKIEWICZ, I.; LUKOMSKA, B. The role of astrocytes in the physiology and pathology of the central nervous system. **Acta Neurobiol Exp (Wars),** v. 66, n. 4, p. 343-58, 2006. ISSN 0065-1400 (Print)

0065-1400 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/17265695 >.

MCKENNA, M. C. Glutamate pays its own way in astrocytes. **Front Endocrinol (Lausanne),** v. 4, p. 191, Dec 16 2013. ISSN 1664-2392 (Print)

1664-2392 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24379804 >.

MELONE, M.; BRAGINA, L.; CONTI, F. Clozapine-induced reduction of glutamate transport in the frontal cortex is not mediated by GLAST and EAAC1. **Mol Psychiatry,** v. 8, n. 1, p. 12-3, Jan 2003. ISSN 1359-4184 (Print)

1359-4184 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12556903 >.

MELONE, M. et al. The expression of glutamate transporter GLT-1 in the rat cerebral cortex is down-regulated by the antipsychotic drug clozapine. **Mol Psychiatry,** v. 6, n. 4, p. 380-6, Jul 2001. ISSN 1359-4184 (Print)

1359-4184 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11443521 >.

MOLINA, V. et al. Marked hypofrontality in clozapine-responsive patients. **Pharmacopsychiatry,** v. 40, n. 4, p. 157-62, Jul 2007. ISSN 0176-3679 (Print)

0176-3679 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17694479 >.

MONTGOMERY, D. L. Astrocytes: form, functions, and roles in disease. **Vet Pathol,** v. 31, n. 2, p. 145-67, Mar 1994. ISSN 0300-9858 (Print)

0300-9858 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/8203078 >.

MORBELLI, S. et al. A Cochrane review on brain [(1)(8)F]FDG PET in dementia: limitations and future perspectives. **Eur J Nucl Med Mol Imaging,** v. 42, n. 10, p. 1487-91, Sep 2015. ISSN 1619-7089 (Electronic)

1619-7070 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/26067090 >.

MOSCONI, L. Glucose metabolism in normal aging and Alzheimer's disease: Methodological and physiological considerations for PET studies. **Clin Transl Imaging,** v. 1, n. 4, Aug 2013. ISSN 2281-5872 (Print)

2281-5872 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24409422 >.

NAHEED, M.; GREEN, B. Focus on clozapine. **Curr Med Res Opin,** v. 17, n. 3, p. 223-9, 2001. ISSN 0300-7995 (Print)

0300-7995 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/11900316 >.

NEHLIG, A.; COLES, J. A. Cellular pathways of energy metabolism in the brain: is glucose used by neurons or astrocytes? **Glia,** v. 55, n. 12, p. 1238-50, Sep 2007. ISSN 0894-1491 (Print)

0894-1491 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17659529 >.

NEHLIG, A.; WITTENDORP-RECHENMANN, E.; LAM, C. D. Selective uptake of [14C]2-deoxyglucose by neurons and astrocytes: high-resolution microautoradiographic imaging by cellular 14C-trajectography combined with immunohistochemistry. **J Cereb Blood Flow Metab,** v. 24, n. 9, p. 1004-14, Sep 2004. ISSN 0271-678X (Print)

0271-678X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15356421 >.

NEWMAN, E. A. New roles for astrocytes: regulation of synaptic transmission. **Trends Neurosci,** v. 26, n. 10, p. 536-42, Oct 2003. ISSN 0166-2236 (Print)

0166-2236 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/14522146 >.

ORREGO, F.; VILLANUEVA, S. The chemical nature of the main central excitatory transmitter: a critical appraisal based upon release studies and synaptic vesicle localization. **Neuroscience,** v. 56, n. 3, p. 539-55, Oct 1993. ISSN 0306-4522 (Print)

0306-4522 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/7902967 >.

OZAWA, S.; KAMIYA, H.; TSUZUKI, K. Glutamate receptors in the mammalian central nervous system. **Prog Neurobiol,** v. 54, n. 5, p. 581-618, Apr 1998. ISSN 0301-0082 (Print)

0301-0082 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/9550192 >.

PATEL, A. B. et al. Direct evidence for activity-dependent glucose phosphorylation in neurons with implications for the astrocyte-to-neuron lactate shuttle. **Proc Natl Acad Sci U S A,** v. 111, n. 14, p. 5385-90, Apr 8 2014. ISSN 1091-6490 (Electronic)

0027-8424 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/24706914 >.

PELLERIN, L.; MAGISTRETTI, P. J. Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization. **Proc Natl Acad Sci U S A,** v. 91, n. 22, p. 10625-9, Oct 25 1994. ISSN 0027-8424 (Print)

0027-8424 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/7938003 >.

_____. Sweet sixteen for ANLS. **J Cereb Blood Flow Metab,** v. 32, n. 7, p. 1152-66, Jul 2012. ISSN 1559-7016 (Electronic)

0271-678X (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/22027938 >.

PELLERIN, L. et al. Evidence supporting the existence of an activity-dependent astrocyte-neuron lactate shuttle. **Dev Neurosci,** v. 20, n. 4-5, p. 291-9, 1998. ISSN 0378-5866 (Print)

0378-5866 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/9778565 >.

PHELPS, M. E. et al. Application of annihilation coincidence detection to transaxial reconstruction tomography. **J Nucl Med,** v. 16, n. 3, p. 210-24, Mar 1975. ISSN 0161-5505 (Print)

0161-5505 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/1113170 >.

PHELPS, M. E. et al. Tomographic measurement of local cerebral glucose metabolic rate in humans with (F-18)2-fluoro-2-deoxy-D-glucose: validation of method. **Ann Neurol,** v. 6, n. 5, p. 371-88, Nov 1979. ISSN 0364-5134 (Print)

0364-5134 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/117743 >.

PLATT, S. R. The role of glutamate in central nervous system health and disease--a review. **Vet J,** v. 173, n. 2, p. 278-86, Mar 2007. ISSN 1090-0233 (Print)

1090-0233 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/16376594 >.

PORTNOW, L. H.; VAILLANCOURT, D. E.; OKUN, M. S. The history of cerebral PET scanning: from physiology to cutting-edge technology. **Neurology,** v. 80, n. 10, p. 952-6, Mar 5 2013. ISSN 1526-632X (Electronic)

0028-3878 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/23460618 >.

POSTON, K. L.; EIDELBERG, D. FDG PET in the Evaluation of Parkinson's Disease. **PET Clin,** v. 5, n. 1, p. 55-64, Jan 01 2010. ISSN 1879-9809 (Electronic)

1556-8598 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20689674 >.

PRUT, L.; BELZUNG, C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review. **Eur J Pharmacol,** v. 463, n. 1-3, p. 3-33, Feb 28 2003. ISSN 0014-2999 (Print)

0014-2999 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/12600700 >.

REIVICH, M. et al. The [18F]fluorodeoxyglucose method for the measurement of local cerebral glucose utilization in man. **Circ Res,** v. 44, n. 1, p. 127-37, Jan 1979. ISSN 0009-7330 (Print)

0009-7330 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/363301 >.

ROTHSTEIN, J. D. et al. Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. **Nature,** v. 433, n. 7021, p. 73-7, Jan 6 2005. ISSN 1476-4687 (Electronic)

0028-0836 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15635412 >.

SANACORA, G.; TRECCANI, G.; POPOLI, M. Towards a glutamate hypothesis of depression: an emerging frontier of neuropsychopharmacology for mood disorders. **Neuropharmacology,** v. 62, n. 1, p. 63-77, Jan 2012. ISSN 1873-7064 (Electronic)

0028-3908 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/21827775 >.

SARIKAYA, I. PET studies in epilepsy. **Am J Nucl Med Mol Imaging,** v. 5, n. 5, p. 416-30, 2015. ISSN 2160-8407 (Print). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/26550535 >.

SEETHALAKSHMI, R. et al. Regional brain metabolism in schizophrenia: An FDG-PET study. **Indian J Psychiatry,** v. 48, n. 3, p. 149-53, Jul 2006. ISSN 0019-5545 (Print)

0019-5545 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/20844644 >.

SESTINI, S. The neural basis of functional neuroimaging signal with positron and single-photon emission tomography. **Cell Mol Life Sci,** v. 64, n. 14, p. 1778-84, Jul 2007. ISSN 1420-682X (Print)

1420-682X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/17415517 >.

SHIMAMOTO, K. et al. DL-threo-beta-benzyloxyaspartate, a potent blocker of excitatory amino acid transporters. **Mol Pharmacol,** v. 53, n. 2, p. 195-201, Feb 1998. ISSN 0026-895X (Print)

0026-895X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/9463476 >.

SILVER, I. A.; ERECINSKA, M. Energetic demands of the Na+/K+ ATPase in mammalian astrocytes. **Glia,** v. 21, n. 1, p. 35-45, Sep 1997. ISSN 0894-1491 (Print)

0894-1491 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/9298845 >.

SMAILAGIC, N. et al. (1)(8)F-FDG PET for the early diagnosis of Alzheimer's disease dementia and other dementias in people with mild cognitive impairment (MCI). **Cochrane Database Syst Rev,** v. 1, p. CD010632, Jan 28 2015. ISSN 1469-493X (Electronic)

1361-6137 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25629415 >.

SOKOLOFF, L. et al. The [14C]deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetized albino rat. **J Neurochem,** v. 28, n. 5, p. 897-916, May 1977. ISSN 0022-3042 (Print)

0022-3042 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/864466 >.

SOM, P. et al. A fluorinated glucose analog, 2-fluoro-2-deoxy-D-glucose (F-18): nontoxic tracer for rapid tumor detection. **J Nucl Med,** v. 21, n. 7, p. 670-5, Jul 1980. ISSN 0161-5505 (Print)

0161-5505 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/7391842 >.

SONI, N.; REDDY, B. V.; KUMAR, P. GLT-1 transporter: an effective pharmacological target for various neurological disorders. **Pharmacol Biochem Behav,** v. 127, p. 70-81, Dec 2014. ISSN 1873-5177 (Electronic)

0091-3057 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25312503 >.

STOESSL, A. J. Glucose utilization: still in the synapse. **Nat Neurosci,** v. 20, n. 3, p. 382-384, Feb 23 2017. ISSN 1546-1726 (Electronic)

1097-6256 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/28230843 >.

SU, L. et al. Cerebral metabolism in major depressive disorder: a voxel-based meta-analysis of positron emission tomography studies. **BMC Psychiatry,** v. 14, p. 321, Nov 19 2014. ISSN 1471-244X (Electronic)

1471-244X (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/25407081 >.

SUOLINNA, E. M. et al. Metabolism of 2-[18F]fluoro-2-deoxyglucose in tumor-bearing rats: chromatographic and enzymatic studies. **Int J Rad Appl Instrum B,** v. 13, n. 5, p. 577-81, 1986. ISSN 0883-2897 (Print)

0883-2897 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/3818323 >.

TAKAHASHI, S. et al. Role of sodium and potassium ions in regulation of glucose metabolism in cultured astroglia. **Proc Natl Acad Sci U S A,** v. 92, n. 10, p. 4616-20, May 9 1995. ISSN 0027-8424 (Print)

0027-8424 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/7753851 >.

TANAKA, K. Functions of glutamate transporters in the brain. **Neurosci Res,** v. 37, n. 1, p. 15-9, May 2000. ISSN 0168-0102 (Print)

0168-0102 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/10802340 >.

TANG, B. L. Brain activity-induced neuronal glucose uptake/glycolysis: Is the lactate shuttle not required? **Brain Res Bull,** v. 137, p. 225-228, Mar 2018. ISSN 1873-2747 (Electronic)

0361-9230 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/29273209 >.

VALLEJO-ILLARRAMENDI, A. et al. Clozapine reduces GLT-1 expression and glutamate uptake in astrocyte cultures. **Glia,** v. 50, n. 3, p. 276-9, May 2005. ISSN 0894-1491 (Print)

0894-1491 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/15739191 >.

VANDENBERG, R. J.; RYAN, R. M. Mechanisms of glutamate transport. **Physiol Rev,** v. 93, n. 4, p. 1621-57, Oct 2013. ISSN 1522-1210 (Electronic)

0031-9333 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/24137018 >.

VOUTSINOS-PORCHE, B. et al. Glial glutamate transporters and maturation of the mouse somatosensory cortex. **Cereb Cortex,** v. 13, n. 10, p. 1110-21, Oct 2003. ISSN 1047-3211 (Print)

1047-3211 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12967927 >.

WAGNER, H. N., JR. Advancing a molecular theory of disease. **J Nucl Med,** v. 49, n. 8, p. 15N-34N, Aug 2008. ISSN 0161-5505 (Print)

0161-5505 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/18672466 >.

WATKINS, J. C.; EVANS, R. H. Excitatory amino acid transmitters. **Annu Rev Pharmacol Toxicol,** v. 21, p. 165-204, 1981. ISSN 0362-1642 (Print)

0362-1642 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/6112965 >.

WIENHARD, K. Measurement of glucose consumption using [(18)F]fluorodeoxyglucose. **Methods,** v. 27, n. 3, p. 218-25, Jul 2002. ISSN 1046-2023 (Print)

1046-2023 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/12183109 >.

ZAMETKIN, A. J. et al. Cerebral glucose metabolism in adults with hyperactivity of childhood onset. **N Engl J Med,** v. 323, n. 20, p. 1361-6, Nov 15 1990. ISSN 0028-4793 (Print)

0028-4793 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/2233902 >.

ZHOU, Y.; DANBOLT, N. C. GABA and Glutamate Transporters in Brain. **Front Endocrinol (Lausanne),** v. 4, p. 165, 2013. ISSN 1664-2392 (Linking). Disponível em: < https://www.ncbi.nlm.nih.gov/pubmed/24273530 >.

ZIMMER, E. R. et al. [(18)F]FDG PET signal is driven by astroglial glutamate transport. **Nat Neurosci,** v. 20, n. 3, p. 393-395, Mar 2017. ISSN 1546-1726 (Electronic)

1097-6256 (Linking). Disponível em: < http://www.ncbi.nlm.nih.gov/pubmed/28135241 >.

ATTACHMENT A – MANUSCRIPT NORMS

MOLECULAR METABOLISM

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.

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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.

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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

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