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Andréia Silva da Rocha

INVESTIGAÇÃO DA INFLUÊNCIA DO TRANSPORTE GLUTAMATÉRGICO ASTROCITÁRIO NO METABOLISMO DE GLICOSE CEREBRAL *IN VIVO*

Porto Alegre
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(Autor Desconhecido)

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1. RESUMO

Nos últimos 20 anos, muito se tem debatido acerca da contribuição das células gliais, especialmente dos astrócitos, ao metabolismo de glicose no encéfalo. Dentre as controvérsias envolvendo o metabolismo cerebral, a influência da glia na captação da [^{18}F]Fluorodeoxiglicose ([^{18}F]FDG), detectada pelo exame de Tomografia por Emissão de Pósitrons (PET), é uma importante questão que permanece não esclarecida. Até o momento, mesmo sem comprovação para tal, esta captação tem sido, majoritariamente, interpretada como apenas resultado de atividade neuronal. Dessa forma, com o intuito de investigar a participação astrocitária no metabolismo cerebral e nos resultados do [^{18}F]FDG-PET, este trabalho utilizou-se do fármaco clozapina para reduzir a densidade do transportador de glutamato GLT-1, encontrado nestas células. A redução na expressão de GLT-1 foi escolhida como estratégia pois fortes evidências apontam a atividade deste transportador como um importante gatilho para captação de glicose nos astrócitos.

Nosso trabalho focou nas duas regiões onde GLT-1 é mais abundante, córtex e hipocampo. Um tratamento de seis semanas com clozapina reduziu significativamente a captação [^{18}F]FDG no córtex de ratos adultos mas não no hipocampo. Concomitantemente, a densidade e expressão de GLT-1 só foi significativamente reduzida no córtex, mas não no hipocampo. Um desfecho semelhante foi observado na cultura primária de astrócitos de ratos adultos. A cultura cortical apresentou uma tendência de redução na densidade de GLT-1, na captação de D-asparto e na captação de 2-Desoxiglucose. A cultura hipocampal não demonstrou alterações aparentes.

Este trabalho fornece a primeira evidência com o uso de [^{18}F]FDG PET sugerindo que a redução na densidade do transportador astrocitário GLT-1 reduz a captação de glicose na região cortical do cérebro de roedores. Estes resultados despertam a necessidade de uma reavaliação na forma como os dados de investigação cerebral com [^{18}F]FDG-PET são interpretados, com especial atenção para a importante contribuição de outras células que não apenas os neurônios.

2. INTRODUÇÃO

2.1 Fluorodeoxiglicose e seu uso na Tomografia por Emissão de Pósitrons

O tomógrafo por emissão de pósitrons (PET) opera com base em dois princípios básicos: a detecção/quantificação da radiação a partir da emissão de radiação γ **(**gama) proveniente da aniquilação de β+ (pósitrons) e elétrons, e o subseqüente processamento para a localização espacial da origem desta radiação. Consequentemente, átomos ou moléculas contendo essa radiação podem ser mensurados e ter sua localização espacial determinada dentro de estruturas, como o corpo humano. Desta forma, utilizando substâncias especificas marcadas com emissores de pósitrons – chamadas radiofármacos – , esta técnica oferece a possibilidade da visualização, caracterização e quantificação de estruturas, moléculas e processos biológicos ocorrendo em níveis celulares e sub-celulares de organismos biológicos vivos, sem a necessidade de processos invasivos (Portnow *et al.*, 2013). Portanto, o PET gera uma imagem funcional, e em combinação com outras técnicas como a Tomografia Computadorizada ou a Ressonância Magnética, permite a co-localização topográfica e funcional (Berger, 2003).

Dentro os radiofármacos emissores de pósitrons desenvolvidos desde a concepção do PET em 1974 (Phelps *et al.*, 1975), o [^{18}F]FDG é, de longe, o mais renomado, sendo, inclusive, reconhecido como “A mólecula do século” (Wagner, 2008). Este radiofármaco surgiu com o propósito inicial de ser utilizado na avaliação cerebral e posteriormente se descobriu também sua importância na identificação e manejo do câncer, graças ao Efeito Warburg encontrado nas células cancerígenas (Kelloff *et al.*, 2005). Esta importante contribuição do [^{18}F]FDG na investigação tumoral estimulou a disseminação desta molécula e do próprio PET ao redor do globo. Dessa forma, o [^{18}F]FDG é hoje o radiofármaco emissor de pósitrons mais utilizado no mundo e é encontrado em uso na clínica em praticamente todas as cidades de médio e grande porte do mundo desenvolvido. A extensa disponibilidade desse radiofármaco, em comparação a outros radiofármacos para avaliação cerebral, torna viável a sua utilização em escala global nas neurociências e

determina grande relevância na correta interpretação dos dados gerados com sua utilização.

O [^{18}F]FDG é um análogo da glicose e consiste, basicamente, em uma molécula de glicose que apresenta um radioisótopo Flúor-18 substituindo um grupo hidroxila na posição C-2 (**Figura 1a**). Sendo assim, em um primeiro momento, o FDG se comporta exatamente como uma molécula de glicose, apresentando distribuição e captação equivalentes, e recebendo, assim como a glicose, um fosfato – pela ação de hexoquinases - ao entrar nas células. A fosforilação do [^{18}F]FDG em [^{18}F]FDG-6-fosfato impede a saída desta molécula da célula, da mesma forma como ocorre com a Glicose-6-fosfato (Suolinna *et al.*, 1986). No entanto, diferentemente da glicose, a molécula de FDG não pode seguir as rotas metabólicas e fica efetivamente retida na célula na forma de FDG-6-fosfato, não podendo sair ou ser metabolizada até que ocorra o decaimento do radioisótopo Flúor-18 para Oxigênio-18, que permite a entrada da molécula em rotas metabólicas (**Figure 1b-c**) (Reivich *et al.*, 1979).

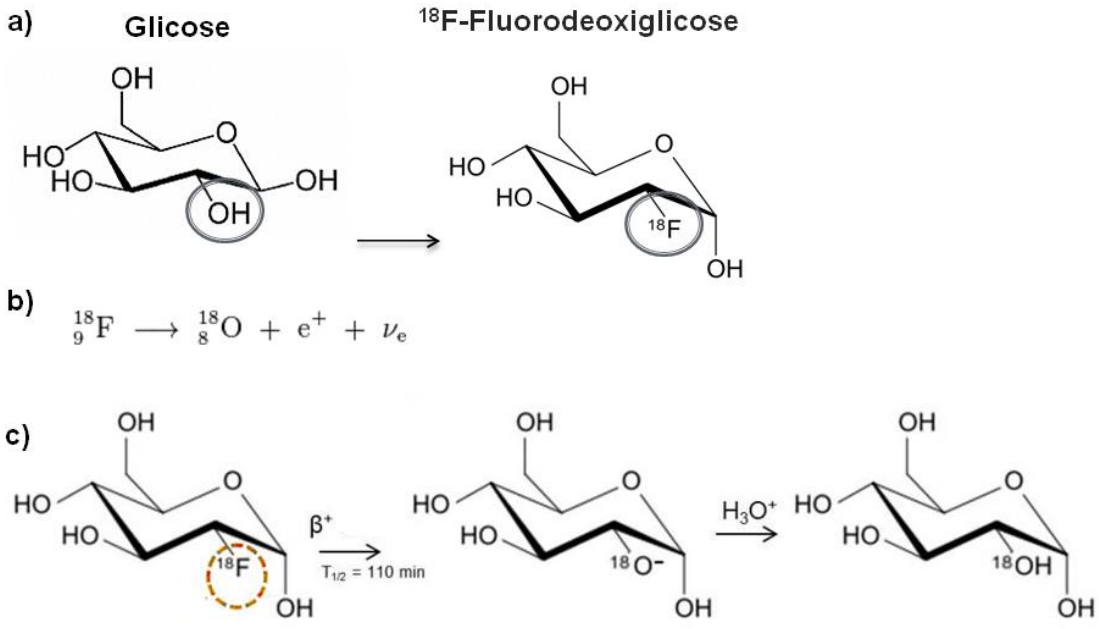

Figura 1. A molécula de [^{18}F]Fluorodeoxiglicose. a) Representação da molécula de [^{18}F]FDG em comparação à molécula de glicose. b) Equação do decaimento β+ do Flúor-18. c) Decaimento β+ do radioisótopo na molécula [^{18}F]FDG e transformação em molécula de glicose (contendo um ^{18}O na posição C-2).

O [^{18}F]FDG entra nas células como se fosse uma molécula de glicose e se acumula, dessa forma, células usando mais glicose apresentarão um maior acúmulo de [^{18}F]FDG e células com metabolismo mais lento acumularão menos FDG. A concentração intracelular de [^{18}F]FDG é, portanto, representativa da taxa de captação de glicose da célula, e portanto, a quantificação de [^{18}F]FDG em um tecido ao longo de um período de tempo específico, em conjunto com alguns outros fatores de correção, permite o cálculo da taxa de absorção de glicose e da taxa metabólica do tecido (Wienhard, 2002).

A utilidade do [^{18}F]FDG encontra-se no fato de que diferentes tipos celulares captam mais glicose do que outros, criando contraste na imagem. Os tecidos absorvem a glicose a taxas diferentes devido às suas diferentes necessidades metabólicas. Por exemplo, o tecido cerebral tem elevada atividade e requer um influxo constante de glicose. No entanto, a taxa de captação de glicose pode mudar dependendo das necessidades metabólicas das células em diferentes situações. Por exemplo, as células musculares aumentam sua absorção de glicose durante o exercício para sustentar o aumento das contrações musculares. Estas alterações subjacentes no metabolismo da glicose aumentam a intensidade da imagem. O [^{18}F]FDG-PET é, portanto, sensível à alterações no metabolismo e permite sua detecção e localização espacial.

No encéfalo, este radiofármaco é utilizado para identificar regiões cerebrais que estão mais ou menos ativas no estado basal e em resposta a diferentes estímulos, alterações ou danos. O [^{18}F]FDG é utilizado para realizar estudos fisiológicos de memória, cognição, entre outros (Greenberg *et al.*, 1981), bem como, para detectar e diagnosticar vários tipos de tumores e outras patologias do cérebro humano.

Nos últimos anos, o [^{18}F]FDG-PET se tornou uma das mais importantes ferramentas na investigação do encéfalo, com aplicação tanto na pesquisa, quanto no diagnóstico e no acompanhamento de diversos distúrbios cerebrais e psiquiátricos como Epilepsia (Sarikaya, 2015), demências (Smailagic *et al.*, 2015), Doença de Huntington (Feigin *et al.*, 2001), Doença de Parkinson (Poston e

Eidelberg, 2010), Trauma (Alavi, 1989), Esclerose Múltipla (Blinkenberg *et al.*, 1999), Lesões isquêmicas (Heiss *et al.*, 1993), Esquizofrenia (Seethalakshmi *et al.*, 2006), Transtorno Bipolar (Altamura *et al.*, 2013), Depressão (Su *et al.*, 2014) e Transtorno do Déficit de Atenção e Hiperatividade (Zametkin *et al.*, 1990) e etc.

Por exemplo, com relação às demências, o [^{18}F]FDG PET tem se destacado pela extensa aplicabilidade, sendo já utilizado na clínica em ampla escala. Os estudos usando o [^{18}F]FDG propiciam não só a constatação do processo de demência se manifestando no encéfalo, mas permitem também a diferenciação dos tipos de demência e seu estadiamento, já que estas patologias apresentam distintos padrões metabólicos (Brown *et al.*, 2014). A imagem abaixo (**Figura 2)** demonstra os diferentes padrões de hipometabolismo encontrados nas demências que, em conjunto com alguns outros fatores, permite identificá-las e diferenciá-las.

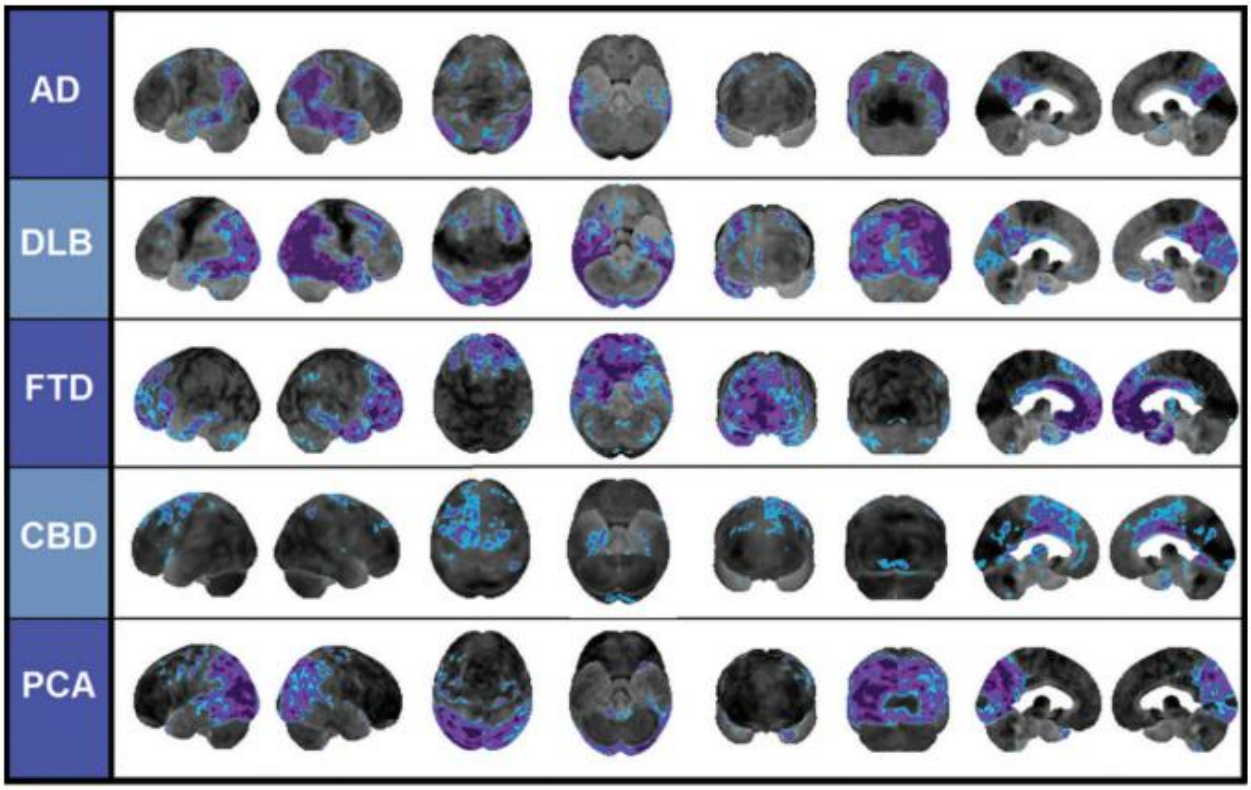

Figura 2. Padrões de hipometabolismo na diferentes demências. AD, Doença de Alzheimer; DLB, Demência com corpos de Lewis; FTD, Demência Frontotemporal; CBD, Degeneração Corticobasal; PCA, Atrofia cortical posterior. Fonte: Brown et al, 2014

Em relação à interpretação a nível celular desta técnica no encéfalo, considera-se geralmente que os neurônios apresentam o maior consumo de energia durante a ativação cerebral e, em consequência, assume-se que a determinação das taxas metabólicas a partir dos sinais do [^{18}F]FDG PET reflete diretamente o uso neuronal de glicose e baseia-se no uso desta molécula sobretudo por processos oxidativos (Sokoloff *et al.*, 1977). Podemos tomar como exemplo novamente as demências, nestas patologias a redução na captação de [^{18}F]FDG vista em certas regiões é geralmente interpretada como disfunção e morte neuronal. A contribuição glial para o sinal do [^{18}F]FDG PET ainda é essencialmente negligenciada, embora fortes evidências apontem uma importante contribuição dos astrócitos no metabolismo de glicose cerebral (Pellerin e Magistretti, 2012).

2.2 Astrócitos e o Metabolismo de Glicose

Os astrócitos são as células gliais mais abundantes do encéfalo e participam de uma série de funções importantíssimas para o bom funcionamento cerebral. Os astrócitos contribuem para a formação da barreira hematoencefálica e da matriz extracelular que rodeia as células cerebrais, participam na manutenção da homeostase química e iônica extracelular, estão envolvidos na resposta à lesão e afetam o desenvolvimento neuronal, a sinalização sináptica e a plasticidade (Montgomery, 1994; Markiewicz e Lukomska, 2006). O importante papel da glia tem sido crescentemente reconhecido e novas interações e funções dessas células são identificadas a cada ano.

Em meados dos anos 90, Pellerin e Magistretti foram responsáveis pela constatação de uma nova e notável contribuição astrocitária em termos energéticos. Estes pesquisadores observaram, em culturas primárias de astrócitos, que a presença do neurotransmissor glutamato estimula a captação de glicose e a liberação de lactato por estas células (Pellerin e Magistretti, 1994). Em outras palavras, estes pesquisadores identificaram que o glutamato estimula a realização de glicólise aeróbica pelos astrócitos e sugeriram, ainda, que o lactato liberado é

encaminhado para os neurônios, onde será utilizado por vias oxidativas para gerar energia. É importante também destacar que o efeito do glutamato na captação e utilização da glicose nos astrócitos não foi inibido por antagonistas dos receptores de glutamato, mas sim por inibidores específicos dos transportadores de glutamato, indicando que o gatilho para o aumento na captação de glicose é o transporte de glutamato.

A teoria baseada inicialmente nestes resultados recebeu o nome de lançadeira de lactato astrócito-neurônio (ANLS, do inglês *Astrocyte Neuron Lactate Shuttle*) (Pellerin *et al.*, 1998) e nos últimos 22 anos acumulou um grande número de novas evidências. A figura abaixo (**Figura 3**) apresenta um esquema simplificado da ANLS.

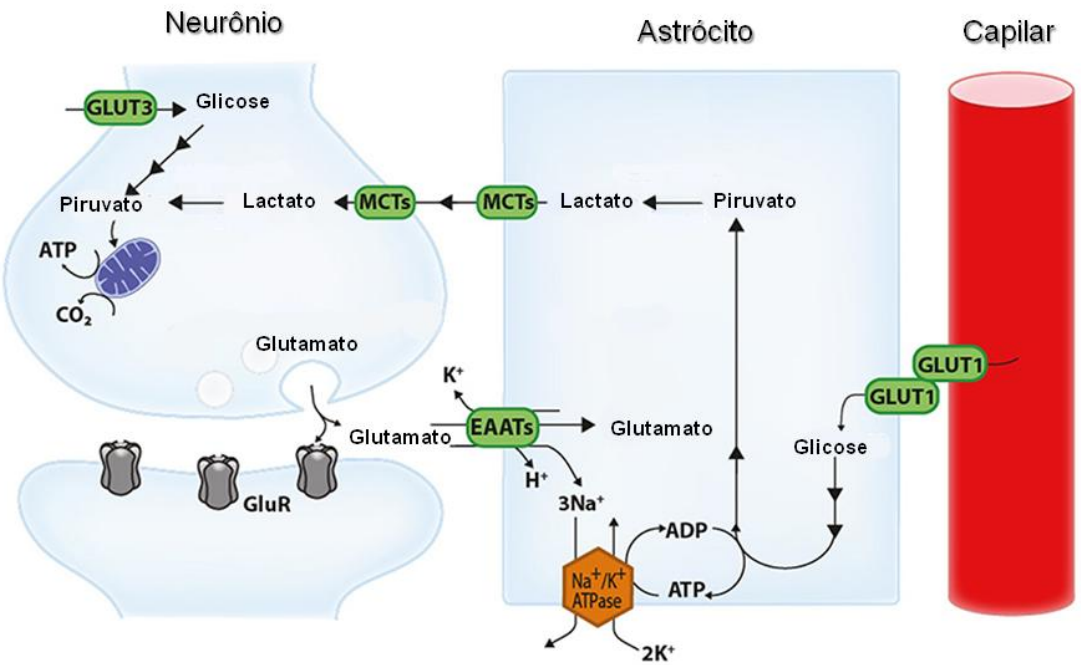

Figura 3. A teoria da lançadeira de lactato astrócito-neurônio. Esquema representativo da ANLS demonstrado em uma sinapse tripartite. O glutamato liberado na fenda pelo neurônio pré-sináptico pode agir nos receptores de glutamato (GluR) e/ou ser captado pelo astrócito através dos transportadores de glutamato (EAATs). O transporte de glutamato pelos EAATs é acoplado ao transporte dos íons Na^+, H^+ e K^+. A manutenção do gradiente destes íons é realizada por uma bomba Na^+/K^+ com o gasto de ATP. O astrócito, localizado mais próximo ao vaso, capta glicose (transportador GLUT1) e utiliza esta molécula para gerar ATP através de um processo de glicólise aeróbica, gerando lactato. O lactato é liberado para o meio extracelular pelos transportadores de monocarboxilato (MCTs). O lactato no meio extracelular pode ser captado pelo neurônio também através dos MCTs. Uma vez dentro do neurônio, o lactato pode ser convertido em piruvato e ser oxidado para gerar energia. O neurônio pode também captar a glicose (transportador GLUT3) diretamente e realizar a glicolise e oxidação desta molécula para gerar energia. Fonte: Modificado de (Belanger *et al.*, 2011)

O papel dos transportadores de glutamato como gatilho para ativar a glicólise aeróbica em astrócitos já foi demonstrado diversas vezes tanto *in vivo*, quanto *in vitro*. Por exemplo, o uso de roedores que não expressam (*knockout)* os transportadores GLAST e GLT-1 evidenciou a necessidade desses receptores para a ativação da resposta glicolítica nos astrócitos (Voutsinos-Porche, Bonvento*, et al.*, 2003). O efeito de alterações no gradiente eletroquímico astrocitário necessário para o funcionamento dos transportadores de glutamato também foi verificado e os resultados foram semelhantes (Takahashi *et al.*, 1995).

Itoh e co-autores investigaram a utilização de glicose e lactato marcados em culturas de astrócitos e neurônios. As culturas neuronais oxidaram prontamente tanto a glicose quanto o lactato, enquanto as culturas astrocitárias oxidaram ambos os substratos com moderação e metabolizaram a glicose predominantemente para lactato. Ademais, um grande aumento na concentração de glicose no meio inibiu a oxidação de lactato pelos astrócitos mas não nos neurônios, indicando uma preferência pela oxidação de lactato nos neurônios (Itoh *et al.*, 2003). Outros trabalhos demonstrando a utilização de lactato pelos neurônios também foram realizados (Bouzier-Sore *et al.*, 2003; Ivanov *et al.*, 2011).

Outra importante evidência, é a de que camundongos *knockout* para GLT-1 e GLAST apresentam captação reduzida de [^{14}C]-2-deoxiglicose no córtex somatossensorial em resposta ao estimulo das vibrissas (Voutsinos-Porche, Bonvento*, et al.*, 2003; Voutsinos-Porche, Knott*, et al.*, 2003). Ainda, outro estudo utilizando estimulo das vibrissas do roedor, verificou que, em resposta a este estimulo, a captação de um análogo de glicose é elevada no astrócitos mas mantém-se no nível basal nos neurônios (Chuquet *et al.*, 2010).

Além de todas as observações funcionais corroborando a ANLS, existem também evidências estruturais. Os astrócitos apresentam prolongamentos que se estendem até os vasos e os envolvem, na extremidade destes prolongamentos (pés vasculares) uma grande concentração de transportadores de glicose é encontrada. Dessa forma, a captação de glicose parece ser favorecida nessas células. Ao mencionar a proximidade e disposição astrocitária ao redor dos vasos, é importante

comentar que os astrócitos parecem tem uma relevante participação na regulação do fluxo sanguíneo cerebral e do acoplamento neurovascular(Macvicar e Newman, 2015).

Essas e muitas outras evidências apontam um importante papel astrocitário no metabolismo energético cerebral e corroboram a teoria da lançadeira de lactato astrócito-neurônio.

2.3 Sistema Glutamatérgico

O aminoácido glutamato, ou ácido glutâmico, é hoje considerado o principal neurotransmissor excitatório no sistema nervoso central dos vertebrados (Orrego e Villanueva, 1993) e a sua importância na sinalização cerebral é tão extensa que muitas vezes o encéfalo é referido como uma máquina Glutamato/GABA (Sanacora *et al.*, 2012), sendo GABA o principal neurotransmissor inibitório. O Glutamato, além de responsável por grande parte da neurotransmissão excitatória rápida, parece também ser o principal mediador de informações sensoriais, cognitivas, motoras e emocionais (Bliss e Collingridge, 1993; Ozawa *et al.*, 1998; Dingledine *et al.*, 1999). Estima-se que cerca de 60-70% das sinapses no encéfalo em mamíferos sejam glutamatérgicas (Watkins e Evans, 1981; Fairman e Amara, 1999).

O glutamato é basicamente sintetizado utilizando indiretamente o esqueleto carbônico derivado da glicose em conjunto com doadores do grupo amino, como outros aminoácidos, amônia ou nucleotídeos. A principal rota de síntese para o glutamato no encéfalo parece ser partindo da glutamina pela ação da glutaminase ativada por fosfato (Torgner e Kvamme, 1990). O glutamato produzido é então acumulado em vesículas sinápticas pelo intermédio de vGLUTs - transportadores vesiculares de glutamato - e pode ser então liberado nos terminais pré-sinápticos através de um mecanismo dependente de íons cálcio (Birnbaumer *et al.*, 1994; Anderson e Swanson, 2000).

Após sua liberação na fenda sináptica, o glutamato pode se ligar a receptores pré e pós-sinápticos ou ser captado por transportadores de glutamato gliais ou neuronais. O acido glutâmico exerce sua ação em basicamente duas classes de

receptores: ionotrópicos, isto é, canais iônicos; e metabotrópicos, isto é, acoplados à proteína G. A classe de receptores ionotrópicos, por sua vez, divide-se em três tipos: receptores N-metil-D-aspartato (NMDA), receptores alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiónico (AMPA) e receptores de cainato. Enquanto isso, os oito receptores metabotrópicos até então identificados também se subdividem em três tipos, com base na homologia de suas sequências, no segundo mensageiro e na farmacologia (Kew e Kemp, 2005).

As concentrações de glutamato no meio extracelular são baixas e fortemente controladas. Enzimas para degradação do glutamato extracelular ainda não foram encontradas, sendo assim, um fenômeno de extrema importância no sistema glutamatérgico é a captação do glutamato no espaço extracelular pela glia ou neurônios. Desequilíbrios deste sistema regulador podem ter efeitos deletérios induzindo hiperexcitabilidade e citoxicidade em neurônios pós-sinápticos (Choi, 1994; Doble, 1999) ou perturbando a sinalização glutamatérgica, por alterar a baixa relação sinal-ruído necessária. Outro ponto importante na recaptação glutamatérgica é a economia, pois o glutamato captado é, em sua grande parte, reciclado através do clico glutamina-glutamato (Danbolt *et al.*, 2016).

A recaptação do glutamato é realizada pelos chamados Transportadores de Aminoácidos Excitatórios (EAATs, do inglês *Excitatory Amino Acid Transporters)* e é acoplada ao transporte de íons (como H^+, Na^+ e K^+) e a manutenção de um gradiente eletroquímico, constituindo, portanto, uma importante fonte de gasto energético (Silver e Erecinska, 1997). Até o momento, cinco tipos distintos de EAATs foram identificados em mamíferos: GLT-1, GLAST, EAAC1, EAAT4 e EAAT5 (Tanaka, 2000).

Os cinco tipos de transportador de glutamato são diferencialmente expressos em regiões distintas do cérebro e também em diferentes tipos celulares (**Figura 4**). Quanto à expressão celular destes transportadores, o GLT-1 é encontrado quase exclusivamente em astrócitos – com rara expressão neuronal em regiões especificas, como nas células piramidais da região CA3 no hipocampo -; o GLAST está presente apenas em astrócitos; e o EAAC1 (EAAT3), o EAAT4 e o EAAT5 são

encontrados apenas em células neuronais, com EAAT3 sendo expresso apenas no soma e dendritos, mas não nos axônios (Danbolt *et al.*, 2016).

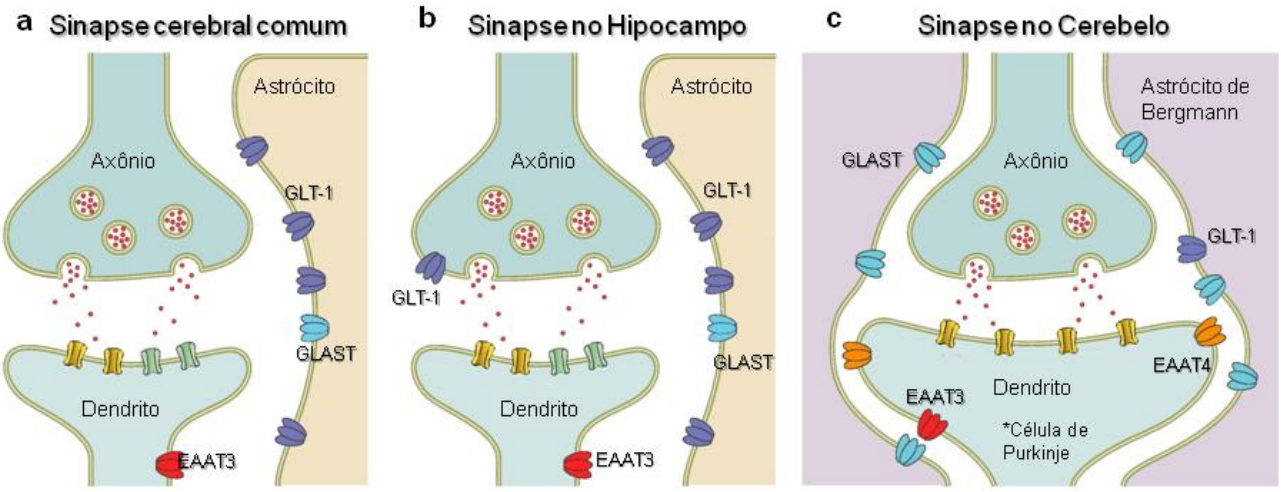

Figura 4. Distribuição celular dos transportadores de glutamato. Diagrama esquemático demonstrando as localizações predominantes dos diferentes tipos de EAATs no encéfalo, à nível celular. a) Distribuição do EAATs na maioria das sinapses do encéfalo. b) Distribuição de EAATs em sinapses especificas do hipocampo. c) Distribuição de EAATs em sinapses especificas do cerebelo. Fonte: Modificado de Vandenberg e Ryan, 2013.

Em relação à distribuição em regiões do sistema nervoso central, o transportador EAAT5 é encontrado exclusivamente em células da retina (fotoreceptores e neurônios bipolares), já EAAT4 é expresso predominantemente no cerebelo (nas células de Purkinje), embora esteja presente em alguns neurônios do prosencéfalo. Diferentemente, EAAT3 é mais disperso, sendo encontrado na maioria dos neurônios no sistema nervoso central (Zhou e Danbolt, 2013).

Acerca dos transportadores astrocitários, GLT-1 é encontrado em todo encéfalo, mas é significativamente mais abundante no córtex e hipocampo, enquanto isso, GLAST é o principal transportador no cerebelo, no ouvido interno, na retina, e algumas outras regiões. No cerebelo, onde é mais concentrado, a expressão de GLAST é cerca de seis vezes maior que a de GLT-1. Em comparação, no hipocampo GLT-1 é quatro vezes mais abundante que o GLAST (Lehre *et al.*, 1995; Danbolt, 2001; Vandenberg e Ryan, 2013; Zhou e Danbolt, 2013). Uma representação da distribuição destes transportadores no encéfalo de roedores pode ser vista abaixo (**Figura 5)**.

Além de ser o mais abundante em praticamente todo cérebro - representa 1% da proteína prosencefálica total -, o Transportador de Glutamato 1 (GLT-1), também chamado EAAT2, é, de longe, o mais importante na recaptação de glutamato, sendo responsável por mais de 90% da captação deste neurotransmissor no encéfalo (Holmseth *et al.*, 2009).

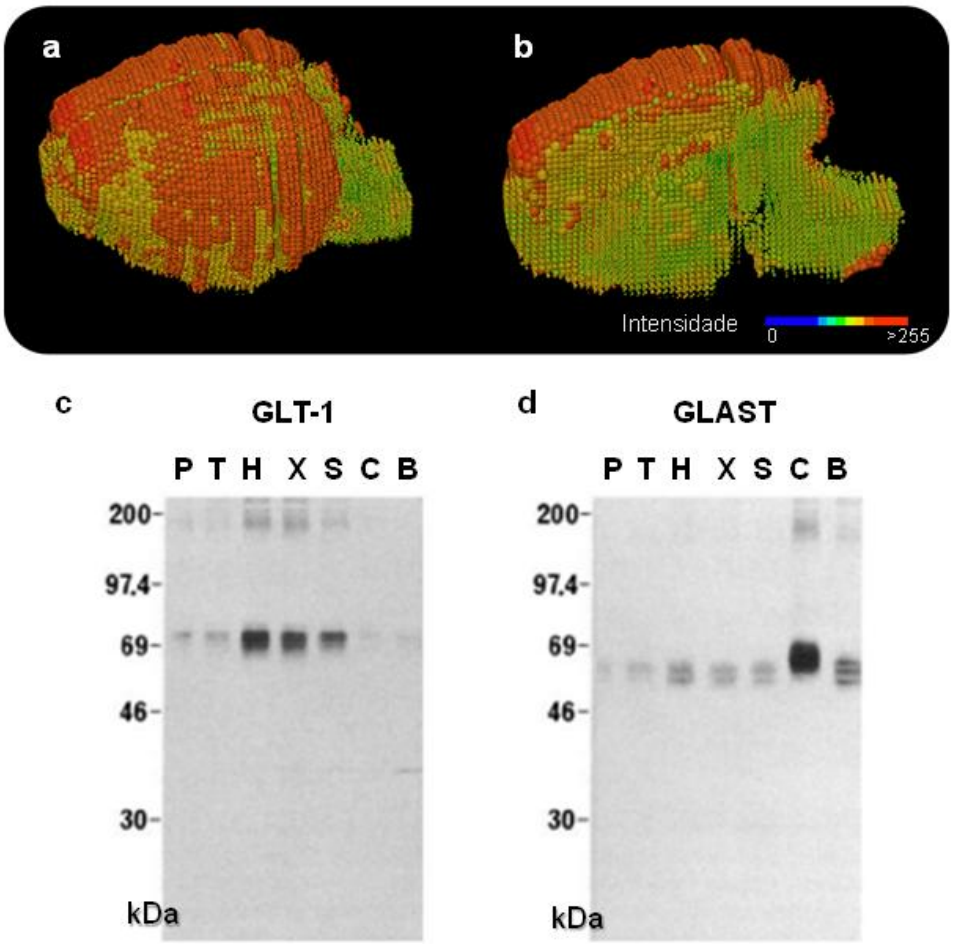

Figura 5. Distribuição encefálica dos transportadores astrocitários de glutamato GLT-1 e GLAST em roedores. Mapa cerebral representativo da expressão do mRNA de GLT-1 em camundongos, a) visão latero-superior do mapa cerebral total e (b) corte sagital na região medial do encéfalo. Imagem representativa do resultado de uma análise por Western Blot para (c) GLT-1 e (d) GLAST mostrando a densidade destes transportadores em diferentes regiões do encéfalo de ratos. Regiões: P, Ponte e Bulbo; T, Tálamos e Hipotálamo; H, Hipoccampo; X, Cortéx; S, Estriado; C, cerebelo; B, Bulbo Olfatório. Fonte: Modificado de *Allen Mouse Brain Connectivity Atlas* (*http://mouse.brain-map.org*) *e* Lehre et al., 1995.

3. ARTIGO CIENTÍFICO

Artigo em preparação com intenções futuras de publicação na revista *Molecular Metabolism* como *full-lenght article*, formatado nos moldes exigidos pela revista (Anexo A).

Nota: A revista exige que as seções do artigo sejam numeradas. Dessa forma, para seguir a formatação da revista, a numeração do artigo não condiz com a numeração do trabalho de conclusão.

Astrocytic GLT-1 determines FDG uptake in cortical layers

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Abstract

Objective

In the past two 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. Interestingly, on *in vivo* methods for analyzing glucose metabolism, such as [^{18}F]FDG positron emission tomography (PET), the participation of these cells is clearly neglected. In this way, we pharmacologically down-regulate GLT-1, the main astrocytic glutamate transporter, to investigate whether these cells impact glucose brain metabolism in vivo indexed by [^{18}F]FDG-PET.

Methods

Adult male Wistar rats received clozapine in the drinking water for six weeks as a strategy for reducing GLT-1. Glucose brain metabolism was longitudinally accessed using [^{18}F]FDG microPET before and after the treatment. Cortical and hippocampal immunocontent and expression of the astrocytic glutamate transporters were also assessed. Moreover, the same regions were used for cultivating adult astrocytes, which were then analyzed for [3H]D-Aspartate uptake and [3H]2-deoxyglucose uptake.

Results

Clozapine treatment reduced [^{18}F]FDG metabolism specifically in the cortex of adult rats. As expected, the same animals presented reduced immunocontent and expression of the glutamate transporter GLT-1 in the cortex. A similar trend was seen in the cortical primary astrocytic culture, with a reduction tendency of GLT-1 density, D-aspartate uptake and 2-Deoxyglucose uptake. On the hippocampus none of the parameters analyzed was significantly changed.

Conclusion

This work provides the first PET evidence that down-regulation of the astrocytic glutamate transporter GLT-1 reduces [^{18}F]FDG signal in cortical layers. These early results suggest that astrocytes need to be integrated in the [^{18}F]FDG data interpretation.

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

1. Introduction

The brain metabolism relies on glucose as its major and primary source of energy for proper functioning, with glucose utilization associated to neuronal activity (Sokoloff, 1993; Gobel *et al.*, 2013; Mergenthaler *et al.*, 2013; Lundgaard *et al.*, 2015). This notion is currently used as a rational for interpreting the accumulation of the radiofluorinated glucose analog 2-deoxy-2-[^{18}F]fluoro-D-glucose ([^{18}F]FDG) (Reivich *et al.*, 1979; Mazzilotta *et al.*, 1981) detected on brain tissue using positron emission tomography (PET) as a consequence of neuronal activity.

The [^{18}F]FDG is the most used radiopharmaceutical on PET imaging (Hofman e Hicks, 2016) and has been widely used to evaluate the brain metabolism in research and clinical settings over the last three decades (Sokoloff, 1981; Alavi *et al.*, 1986; Henry e Votaw, 2004; Mosconi *et al.*, 2008; Berti *et al.*, 2014; Kato *et al.*, 2016). However, the traditionally used perception that the [^{18}F]FDG signal is due to neuronal uptake or directly reflects neuronal activity is currently under debate. In fact, the contribution of other brain cell type(s) to PET [^{18}]FDG signal remains highly controversial (Sestini, 2007; Figley e Stroman, 2011).

In this regard, astrocytes are the most abundant glial cells in the central nervous system and account for approximately half of the mammalian brain cells (Herculano-Houzel, 2014). Analyzing the human brain, glia accounts for nearly 80% of the cortical cells, 60% of gray matter cells, while only 20% of the cerebellar cells (Azevedo *et al.*, 2009). Evidences indicate that these cells participate in several dynamic mechanisms involving synaptic transmission and plasticity, including a critical role in terms of glucose utilization (Volterra e Meldolesi, 2005). As a matter of fact, there are growing evidences suggesting that astrocytes fulfill the energy needs for glutamate recycling by aerobic glycolysis, converting glucose into lactate, which is

then shuttled into neurons for further metabolism (Pellerin e Magistretti, 2012). In particular, the glutamate transport through GLT-1 or GLAST on astrocytes has been shown to act as a trigger, stimulating glucose uptake by these cells (Pellerin e Magistretti, 1994). Another important evidence is that GLT-1 and GLAST knockout mice have reduced [^{14}C]-2-deoxyglucose in the somatosensory cortex after whisker stimulation (Voutsinos-Porche, Bonvento*, et al.*, 2003; Voutsinos-Porche, Knott*, 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). Based on these observations, it seems very likely that [^{18}F]FDG-PET signal may in part reflect glucose consumption in astrocytes (Magistretti e Pellerin, 1996), although no PET evidence exists so far to support this claim. To further test this hypothesis, we conducted a microPET study using [^{18}F]FDG to assess whether clozapine-induced GLT-1 down-regulation (Melone *et al.*, 2001; Melone *et al.*, 2003; Vallejo-Illarramendi *et al.*, 2005; Bragina *et al.*, 2006) was capable of modulating cerebral [^{18}F]FDG consumption in awake adult rats.

2. Methods and Materials

2.1 Animals

Sixteen adult male Wistar rats (200–300g) were maintained two per cage 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. The animals weight and volume of liquid ingestion was recorded once a week and three times a week, respectively. The behavioral tests were performed between 8A.M. and 13P.M. All the experimental

118 procedures were designed to minimize the number of animals used and their
119 suffering, and were approved by the Committee on the Ethics of Animal Experiments
120 of the Federal University of Rio Grande do Sul under the protocol number 30124.
121
122 **2.2 Clozapine treatment**
123 Adult male Wistar rats were randomly divided in two groups, a control group and a
124 experimental (clozapine) group. Experimental rats (n=8) received clozapine in their
125 drinking water (25/35mg $kg^{-1}day^{-1}$) starting at 75 postnatal day (PND75) and
126 continuing for 6 weeks. The clozapine solution was prepared as previously described
127 by Terry *et al.* (Terry *et al.*, 2003), dissolving the drug in a 0.1 N solution of acetic
128 acid and subsequently diluting (1:100) to achieve the final daily dose of drug. Control
129 rats (n=8) received water with the same acetic acid concentration and pH of the drug
130 solution. The solutions were replaced with fresh ones every 2-3 days and protected
131 from light if containing clozapine.
132 Treatment regimen, dose and length of treatment followed Meloni et al (Melone *et al.*,
133 2001), and were based on previous dosing strategies in rats that have been
134 extrapolated from human clinical dosages (See e Ellison, 1990). An oral
135 administration of clozapine was chosen because of its reported rapid elimination and
136 its inability to accumulate in the brain on a chronic intermittent dosing regimen
137 (Baldessarini *et al.*, 1993). Animals were maintained two per cage for monitoring
138 liquid intake.
139
140 **2.3 [^{18}F]FDG microPET scanning**
141 The rats were scanned longitudinally before the treatment (baseline) at PND75, and
142 after 6 weeks of treatment (follow-up), at PND118. The scanning was made between

9:30A.M. - 18:30P.M, in a randomized order. Animals received an intravenous bolus injection (0.2 mL) of [^{18}F]FDG (mean ± s.d.: 1.05 ± 0.072 mCi) into the tail vein. Then, rats were allowed to freely move in their cages (awake) during a 40 minutes of [^{18}F]FDG uptake phase, which was followed by a 10 minutes static acquisition under anesthesia. PET measurements were performed on a *Triumph® II microPET/CT LabPET-4®* scanner (GE Healthcare, Chicago/USA). Rats lay in prone position with the head immobilized by both a body holder and a nose cone of the anesthesia system (2% isoflurane at 0.5 L/min oxygen flow). The brain was positioned in the center of the field of view. The body temperature was maintained at 36.5 ± 1˚C. Images were reconstructed by fully-3D ordered subset expectation maximization (3D-OSEM) algorithm, normalized and corrected for scatter, dead time and decay. Imaging analysis was conducted using PMOD and minc-tools (http://www.bic.mni.mcgill.ca/ServicesSoftware/HomePage). MicroPET images were manually co-registered to a standard rat histological template. Standardized uptake value reference (SUVr) was calculated using pons as the reference region.

2.4 Primary astrocytes culture from adult rats

The adult astrocytes culture was done as described by our group (Souza *et al.*, 2013) with some modifications. Whole cortex and hippocampus were aseptically dissected out from 121-124 days old rats and kept in HBSS (except for some fractions used to Western Blot and PCR) containing 0,05% trypsin (Gibco, Thermo Fischer Scientific, Waltham/USA) and 0,003% DNase (Sigma Aldrich, Saint Louis/USA). Next, this solution was kept at 37°C for 15min. After, tissue was mechanically dissociated with Pasteur Pipette for more 15min, and centrifuged at 1000 RPM for 5 min. The pellet was ressuspended 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 (1000 RPM, 5min), cells were ressuspended in HBSS containing only DNase 0,003% and left for decantation during 40 min. Supernatant was collected and centrifuged for 7 min, 1000 RPM. Cells were ressuspended in DMEM/F12 (Gibco, Thermo Fischer Scientific, Waltham/USA) containing 10% fetal bovine serum (FBS), 15 mM HEPES, 14.3 mM $NaHCO_3$, 1% fungizone and 0,04% gentamicyn, plated in 24-well plates pre-coated with poli-L-lisine and cultured at 37°C in a 95% air/5% CO_2 incubator. 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 experiments.

2.5 2-Deoxy-D-[1,2-3H]glucose ([3H]2DG) uptake

After cells reached confluence, glucose uptake was assessed as previously described (Pellerin e Magistretti, 1994). Briefly, the cells were rinsed once with HBSS and incubated with DMEM/F12 1%FBS containing 1μCi/ml [^{3}H]2DG in the presence of 500μM glutamate for 20min at 37◦C. After incubation, the cells were rinsed with HBSS and lysed overnight with NaOH 0.5M. Incorporated radioactivity was measured in a scintillation counter. Cytochalasin B (10μM) was used as a specific glucose transporter inhibitor. The samples protein content was determined using the bicinchoninic acid assay (Pierce, Sao Paulo/Brazil). Glucose real uptake was determined by subtracting uptake with cytochalasin B from total uptake. The experiments were performed in triplicate.

2.6 D-Aspartate uptake

After the cells reached confluence, the D-aspartate uptake was performed. Briefly, the astrocytes were rinsed once with HBSS and incubated with DMEM/F12 1%FBS containing 0.33mCi/ml [^{3}H]D-Aspartate in the presence of 10μM glutamate for 5min at 37◦C. After incubation, the cells were rinsed with HBSS and lysed overnight in a solution containing 0.5M NaOH. Incorporated radioactivity was measured in a scintillation counter. To determinate the unspecific uptake, the same procedure was repeated with the cells incubated on ice and using all solutions cold. The samples protein content was determined using the bicinchoninic acid assay (Pierce, Sao Paulo/Brazil). The real uptake was then obtained by subtracting the unspecific uptake from the total uptake. The experiments were performed in quadruplicate.

2.7 Western Blot

The Western Blot technique was performed as previously described (Espinosa *et al.*, 2013). This analysis was done both for dissected tissue and for astrocytes culture. In the tissue SDS-PAGE, whole cortex and hippocampus were dissected out from 121-124 days old rats and well distributed representative portions of these regions were immediately homogenized in a lysis buffer (2% SDS, 5mM Tris, 2mM EDTA, pH 7,4) containing a protease inhibitor cocktail (Sigma, Sao Paulo/SP, Brazil) and frozen at −20◦C. In the Western Blot of adult astrocytes culture, a lysis buffer (0,1% SDS, 5mM Tris, 1mM EDTA, pH 7,4) was add to cell cultures of 4th to 5th weeks (after reached confluence) and after 18/20hs on the lysis buffer the samples were frozen at −20◦C. After defrost, the protein content was determined using the bicinchoninic acid assay (Pierce, Sao Paulo/Brazil). Sample extracts were diluted to a final protein concentration of 1 μg/μL or 0,5ug/ul in SDS-PAGE buffer and SDS-PAGE analysis

was carried out with 10µg together with pre-stained molecular weight standards (Bio-Rad, Sao Paulo/Brazil) using a 10% gel with a 4% concentrating gel. After electrotransfer, the membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 and 3% bovine serum albumin for 1h at room temperature (RT) or overnight at 4◦C. The nitrocellulose membranes (Amersham, Sao Paulo/Brazil) were then incubated overnight at 4◦C with the following antibodies: rabbit anti-GLT1 antibody (1:2000; Alpha Diagnostic, Texas/USA) or rabbit anti-GLAST antibody (1:2000; Alpha Diagnostic, Texas/USA). The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1h at room temperature. Finally, using a chemiluminescence ECL kit (Amersham, Sao Paulo/Brazil), the membranes were scanned by an Image Quant system (GE Healthcare, Chicago/USA). Densitometric analyses were performed using NIH ImageJ software. The proteins β-Tubulin or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as a loading control and were quantified using a mouse anti-tubulin antibody (1:10000; from Santa Cruz Biotechnologies, Sao Paulo/Brazil) and a rabbit anti-GAPDH (1: 8000; Alpha Diagnostic, Texas/USA), as described above.

2.8 RNA extraction and quantitative RT-PCR

The whole cortex and hippocampus were dissected out from 121-124 days old rats and well distributed representative portions of these regions were immediately homogenized to 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, CA) 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 real-time RT-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 RT-PCR system. Target mRNA levels were normalized to β-actin levels using the 2-ΔΔCt method (Livak e Schmittgen, 2001). The experiments were performed in triplicates.

2.9 Imunocytochemistry

An imunocytochemistry procedure was performed on the astrocytes cell culture for demonstrative purposes and carried out as previously described by our group (Quincozes-Santos *et al.*, 2009). Briefly, cells cultured were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. After blocking overnight with 4% albumin, cells were incubated overnight with anti-GFAP (1:400) at 4°C, followed by PBS washes and incubation with specific secondary antibody conjugated with Alexa Fluor® 488 for 1 h at room temperature. Nucleus of cells were stained with 0.2 µg/ml of 4’,6’-diamidino-2-phenylindole (DAPI). Cells were viewed with a Nikon inverted microscope and images transferred to a computer with a digital camera (Sound Vision Inc., USA).

2.10 Open Field

At the end of six weeks of treatment, two days before the final imaging procedure (PND116), rats were submitted to an open field (OF) test to evaluate locomotion. The apparatus was made of black-painted Plexiglas measuring 50×50cm 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 measured as the total distance traveled in meters calculated using a computer-operated tracking system (Any-maze, Stoelting, Woods Dale, IL). Procedure done as described previously (Espinosa *et al.*, 2013).

2.11 Novel Object Recognition Task

The object recognition test (ORT) was carried out 24hs after the open field test. 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 5 minutes. 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 2 cm 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. Procedure done as described previously (Espinosa *et al.*, 2013).

2.12 HPLC

Due to the low solubility of clozapine in water, a high performance liquid chromatography procedure was performed to verify its presence in the drinking solution. The chromatographic separation was performed with C18 column (5µm, 250mm × 4.6mm, Sigma Aldrich, Saint Louis/USA). Chromatographic parameters were as follows: mobile phase consisting of methanol and water (800:200); UV detection at 257nm, flow 1 mL per minute; oven temperature 25 °C.

2.13 Statistical Analysis

Data distribution was first tested for normality using Kolmogorov-Smirnov test. A two-tailed unpaired Student's t-test was used for statistical analysis of tissue Western Blot, PCR and open Field data. One-sample t-test (hypothetical value=50%) was used to evaluate the novel object recognition test. Mann–Whitney U test was used analysis of astrocytes culture Western Blot, 2DG uptake and D-aspartate uptake data. 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 hypometabolism in the cortical region of adult rats.

316 To assure that we had an homogeneous group of subjects concerning the brain
317 metabolism, all the rats had its brain glucose uptake assessed using [^{18}F]FDG
318 microPET at the baseline, before starting the clozapine treatment. The results
319 showed no differences in the whole brain SUVr of the animals, representing that both
320 groups of animals had a similar glucose uptake (p= 0.6488; **Figure 1a-b**) at baseline.
321 The [^{18}F]FDG uptake analysis after the clozapine intervention showed a clear
322 reduction in the SUVr in cortical regions in the group clozapine, but not in the group
323 control (**Figures 2a-b**). The group clozapine presented a significant reduction in
324 [^{18}F]FDG uptake widespread in the cortical layers. More specifically, GLT-1
325 donwregulation induced a reduction of more than 20% in some cortical clusters. By
326 contrast, the group control presented only a few small significant clusters when
327 comparing baseline and follow-up scans, with a maximum reduction inferior to 8%
328 (**Figure 2c**). A voxel-wise t-statistical analysis showed a significant glucose
329 hypometabolism almost exclusively in the cortical region of the group clozapine, with
330 a peak effect in the parietal cortex (peak $t_{(7)}$=7.62, p = 0.0001; **Figure 2d, 2f**). On the
331 other side, the t-statistical analysis showed almost no reduction on the group control,
332 presenting just few minimal punctual reductions that could be easily attributed to a
333 limitation on the spatial resolution of the microPET scanner (Moses, 2011), namely
334 artefacts, or due to an age-dependent process. The peak effect on the control group
335 was in the superior colliculus (peak $t_{(5)}$=3.02, p=0.039; **Figure 2d-e**).
336
337 **3.2 Clozapine treatment effects on cortex: tissue and primary astrocytes**
338 **culture**
339 The astrocytic glutamate transporters immunocontent analysis on the cortical tissue
340 revealed a significant reduction for the glutamate transporter GLT-1 (p=0.040; **Figure**

3a) at the clozapine group in comparison to controls, but no differences for GLAST (p=0.920; **Figure 3b**). Consistent results were found on the mRNA expression evaluation, with a reduction on GLT-1 expression (p=0.047; **Figure 3c**) at the clozapine group and no differences on GLAST expression (p=0.184; **Figure 3d**). To assess clozapine sub-chronic treatment effects on astrocytes individually, we used an adult cortical astrocytes primary culture approach (**Figure 4a**). We found no significant differences for GLT-1 (p=0.098; **Figure 4b**) and GLAST (p=0.593; **Figure 4c**) immunocontent on the cultured astrocytes. As well, no statistically significant differences were seen on the astrocytes [^{3}H]D-aspartate (p=0.271; **Figure 4d**) and [^{3}H]2DG uptake (p=0.258; **Figure 4e**). However, a reduction tendency can be noticed for D-aspartate and 2DG uptake, and also GLT-1 density at the clozapine group. Thus, it is important to emphasize that these findings are related to a sample size of 3/4 animals per group, which is substantially low. A Cohen's D analyses demonstrated a large effect size in all three analysis (GLT-1 immunoconten t, Cohen's d=1.549; D-Aspartate uptake, Cohen's d=0.99; 2DG uptake, Cohen's=0.97).

3.3 Clozapine treatment outcome on the hippocampus: tissue and primary astrocytes culture

At the hippocampal tissue no differences were found on the GLT-1 immunocontent (p=0.943; **Figure 5a**) and mRNA expression (p=0.184; **Figure 5b**). As at the cortical region, a hippocampal astrocytes culture (**Figure 6a**) was also performed. The D-aspartate and 2DG uptake evaluation exhibited no differences (p=0.665 and p=0.400, respectively; **Figure 6b-c**). The results using the astrocytic culture here are also preliminary, in consequence of the small sample size (n=3/4 animals per group; D-Aspartate uptake, Cohen's d=-0.88; 2DG uptake, Cohen's d=-0.42).

366

367 **3.4 Behavioral effects and treatment measures**

368 Total distance traveled during the open field test was not significantly different
369 between groups, indicating that the spontaneous locomotor activity of control or
370 clozapine groups was not affected by the pharmacological approach (**Supplemental**
371 **Figure 1a-b**). Analysis of the time spent exploring objects during novel object
372 recognition training revealed that animals on both groups explored equally the two
373 identical objects presented (**Supplemental Figure 1c**). On the other side, during the
374 novel object test session the rats explored significantly more the novel object on
375 groups control ($p<0.0001$) and clozapine ($p=0.0006$), indicating no impairment on the
376 short-term recognition memory (**Supplemental Figure 1d**). Animals had its body
377 weight measured once a week trough the experiment, the results showed only
378 significant effect of time ($p<0.0001$) but not of groups ($p=0.709$). On the other side,
379 the liquid intake measurement demonstrated a lower volume intake on the group
380 clozapine in comparison to controls at second and fourth days of treatment (group
381 effect, $p=0.036$), but normalized from treatment day 9 onwards. Finally, a high
382 performance liquid chromatography confirmed the clozapine presence on the
383 experimental solution (**Supplemental Figure 3**).

384

385 **4. Discussion**

386 The present study shows an important *in vivo* reduction on [^{18}F]FDG uptake,
387 reflecting a glucose hypometabolism, at the cortex when the astrocytic glutamate
388 transporter GLT-1 is pharmacologically down-regulated. This evidence supports the
389 hypothesis of a significant astrocytic participation on the [^{18}F]FDG PET signal.

The Glutamate Transporter 1 (GLT-1), also known as Excitatory Amino-acid Transporter 2 (EAAT2), is responsible for over 90% of glutamate reuptake within the brain (Holmseth *et al.*, 2009) and it is found, almost exclusively in astrocytes (Zhou e Danbolt, 2013). Astrocytic GLT-1 has dominant role on glutamate reuptake and it is considered a signaling trigger for glucose uptake by astrocytes(Pellerin e Magistretti, 2012). Based on aforementioned, GLT-1 seems to be a reliable targeting for testing our hypothesis. Within the goal to manipulate GLT-1, the drug clozapine was chosen because it provokes an important down-regulation of GLT-1 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, caudate-putamen, and thalamus as being slightly affected (Melone *et al.*, 2001). Consequently, our analysis focused on the cortex and hippocampus, since those are the regions where GLT-1 is substantially more abundant, and therefore, are the areas where we would expect its density reduction would have an, bigger and, thus most likely to be observed, effect.

The cortical region results corroborate our hypothesis, exhibiting a significant widespread reduction on FDG signal of about 20%. In agreement, GLT-1 immunocontent and expression reduction confirmed the clozapine's effect previously described by Meloni (Bragina *et al.*, 2006). However, it is important to point out that the degree of GLT-1 density reduction was lower than we expected for a six weeks treatment, since previous works showed a reduction of about 50%-30% on its cortical content in a nine weeks treatment (Melone *et al.*, 2001), in comparison with the approximate 20% we observed.

By contrast, hippocampal data were not correspondent to what we have predicted. The hippocampus showed no apparent difference on the [^{18}F]FDG signal followed by

no differences on GLT-1 density and expression. In fact, the clozapine effect on cortical GLT-1 was replicated several times (Melone *et al.*, 2001; Melone *et al.*, 2003; Bragina *et al.*, 2006), and the resulting effect of clozapine on GLT-1 expression in the hippocampus was not that well established. Additionally, the hippocampus is a highly plastic region (Mcewen, 1999) (Cameron e Gould, 1996) (Mcewen e Alves, 1999), being likely capable to adapt and compensate the effects in a sub-chronic regimen treatment.

We asked ourselves if the down regulation of the most important glutamate transporter in the brain, as seen on the assessment of the cortex, could have generated on this region 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 regions (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.

Following the *in vivo* analysis, we isolated adult astrocytes in cultures. Using these adult cultures of astrocytes, we performed an ex-vivo evaluation of the same brain regions derived from the group of animals receiving clozapine or control solution. It is relevant to raise the fact that clozapine was not added to the culture and that our investigation relies on the continuity of clozapine effects through the culture process, in the absence of the drug. A difference on GLT-1 or GLAST density was not found in the cortical astrocytic culture, as well, no significant differences were found on the D-aspartate uptake - carried out by this glutamate transporters - or in the 2DG uptake, in cultured astrocytes from both cortex and hippocampus. Although, a reduction tendency on GLT-1, 2DG and, especially, D-aspartate can be observed on the

cortical astrocytes. But unfortunately, the sample size on the culture analysis is too small for a proper statistical evaluation, and further studies need to be conducted to delineate a profitable result.

It is also important to notice the valuable information that open field test brought to us, presenting no differences in the spontaneous activity and implying that no sedative effect (Prut e Belzung, 2003) it 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. The results in the ORT also demonstrated no alterations in performance between groups in this hippocampal-dependent task (Cohen e Stackman, 2015).

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). Since then, [^{18}F]FDG PET has became one of the most important tools to brain examination, establishing applicability on several brain disorders as Epilepsy (Sarikaya, 2015), Alzheimer's Disease and other types of dementia (Smailagic *et al.*, 2015), Parkinson's Disease (Poston e Eidelberg, 2010), Schizophrenia (Seethalakshmi *et al.*, 2006), Bipolar Disorder (Altamura *et al.*, 2013), Depression (Su *et al.*, 2014) and Attention Deficit Hyperactivity Disorders (Zametkin *et al.*, 1990). Through the years, the role of astrocytes on the [^{18}F]FDG PET signal was questioned a few times (Magistretti e Pellerin, 1999; Magistretti, 2000; Sestini, 2007; Figley e Stroman, 2011), however, at the best of our knowledge, our group is the first to try directly address this question using the technique itself. As a result of our investigation, we show that the [^{18}F]FDG PET signal being substantially affected by the down-regulation of the main astroglial glutamate transporter.

Then, based on previous works supporting the marked contribution of astrocytes on glucose uptake and on the results found at the present study, it seems that a modified interpretation of [18F]FDG PET data is needed, which requires going beyond the conventional view that these signals are exclusively based on neuronal activity (Welberg, 2009).

Finally, an outstanding information is that a similar outcome - a cortical glucose hypometabolism (Janowsky *et al.*, 1992) – was also found in humans under clozapine treatment. But, it is pertinent to mention that clozapine has other effects and interacts with other molecules and receptors (Lappalainen *et al.*, 1990; Janowsky *et al.*, 1992; Homayoun e Moghaddam, 2007; Lieberman *et al.*, 2008; Kim *et al.*, 2012). Therefore, based only on the results seen in the present study, we cannot rule out the possibility that the reduction on the glucose metabolism seen can result of other pharmacological interactions that not the observed cortical GLT-1 1 downregulation. Further studies need to be conducted to acknowledge this conjecture.

5. Conclusion

Our results suggest that the down-regulation of GLT-1 induces [^{18}F]FDG hypometabolism in the cortical layer. This outcome corroborates the notion that [^{18}F]FDG PET signal reflects not only neuronal activity, but also astrocytic. With this in mind, a reconceptualization in the way we interpret imaging of brain disorders using [^{18}F]FDG PET seems to be imperative. Nevertheless, we recognize that further studies are needed to provide a more reliable conclusion about the role of astrocytes on glucose uptake.

Authors contributions

AR, DGS, AQS, JCC, DS and ERZ participated on the conceptualization, design and interpretation of the experiments. AR performed the treatments, the body weight and liquid intake measurements and analysis, the behavioral procedures and analysis, the imaging analysis, the euthanasia and samples collection, the astrocytes culture preparation and maintenance, the 2DG and D-aspartate uptake procedure and analysis, the Western-Blot procedure and analysis, the PCR analysis and the imunocytochemistry procedure. DGS carried out the astrocytes culture preparation and maintenance, the 2DG and D-aspartate uptake procedure and analysis, and the imunocytochemistry procedure and analysis. BBS performed the PCR procedure and analysis, and the astrocytes culture maintenance. GV and SG performed the imaging procedure. PF performed the HPLC procedure and analysis. IF performed the euthanasia and samples collection. ERZ supervised the whole study and revised the paper.

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References

[1] L. Sokoloff, Sites and mechanisms of function-related changes in energy metabolism in the nervous system, Developmental neuroscience, 15 (1993) 194-206.

[2] B. Gobel, K.M. Oltmanns, M. Chung, Linking neuronal brain activity to the glucose metabolism, Theor Biol Med Model, 10 (2013) 50.

[3] I. Lundgaard, B. Li, L. Xie, H. Kang, S. Sanggaard, J.D. Haswell, W. Sun, S. Goldman, S. Blekot, M. Nielsen, T. Takano, R. Deane, M. Nedergaard, Direct neuronal glucose uptake heralds activity-dependent increases in cerebral metabolism, Nature communications, 6 (2015) 6807.

[4] P. Mergenthaler, U. Lindauer, G.A. Dienel, A. Meisel, Sugar for the brain: the role of glucose in physiological and pathological brain function, Trends in neurosciences, 36 (2013) 587-597.

[5] J.C. Mazzilotta, M.E. Phelps, J. Miller, D.E. Kuhl, Tomographic mapping of human cerebral metabolism: normal unstimulated state, Neurology, 31 (1981) 503-516.

[6] M. Reivich, D. Kuhl, A. Wolf, J. Greenberg, M. Phelps, T. Ido, V. Casella, J. Fowler, E. Hoffman, A. Alavi, P. Som, L. Sokoloff, The [18F]fluorodeoxyglucose method for the measurement of local cerebral glucose utilization in man, Circulation research, 44 (1979) 127-137.

[7] M.S. Hofman, R.J. Hicks, How We Read Oncologic FDG PET/CT, Cancer Imaging, 16 (2016) 35.

[8] V. Berti, L. Mosconi, A. Pupi, Brain: normal variations and benign findings in fluorodeoxyglucose-PET/computed tomography imaging, PET Clin, 9 (2014) 129-140.

[9] L. Mosconi, W.H. Tsui, K. Herholz, A. Pupi, A. Drzezga, G. Lucignani, E.M. Reiman, V. Holthoff, E. Kalbe, S. Sorbi, J. Diehl-Schmid, R. Perneczky, F. Clerici, R. Caselli, B. Beuthien-Baumann, A. Kurz, S. Minoshima, M.J. de Leon, Multicenter standardized 18F-FDG PET diagnosis of mild cognitive impairment, Alzheimer's disease, and other dementias, Journal of nuclear medicine : official publication, Society of Nuclear Medicine, 49 (2008) 390-398.

[10] L. Sokoloff, 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, 1 (1981) 7-36.

[11] T.R. Henry, J.R. Votaw, The role of positron emission tomography with [18F]fluorodeoxyglucose in the evaluation of the epilepsies, Neuroimaging clinics of North America, 14 (2004) 517-535, ix.

[12] T. Kato, Y. Inui, A. Nakamura, K. Ito, Brain fluorodeoxyglucose (FDG) PET in dementia, Ageing Res Rev, 30 (2016) 73-84.

[13] A. Alavi, R. Dann, J. Chawluk, J. Alavi, M. Kushner, M. Reivich, Positron emission tomography imaging of regional cerebral glucose metabolism, Seminars in nuclear medicine, 16 (1986) 2-34.

[14] S. Sestini, The neural basis of functional neuroimaging signal with positron and single-photon emission tomography, Cellular and molecular life sciences : CMLS, 64 (2007) 1778-1784.

[15] C.R. Figley, 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, 33 (2011) 577-588.

[16] S. Herculano-Houzel, The glia/neuron ratio: how it varies uniformly across brain structures and species and what that means for brain physiology and evolution, Glia, 62 (2014) 1377-1391.

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

[18] A. Volterra, J. Meldolesi, Astrocytes, from brain glue to communication elements: the revolution continues, Nat Rev Neurosci, 6 (2005) 626-640.

[19] L. Pellerin, 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, 32 (2012) 1152-1166.

[20] L. Pellerin, 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, 91 (1994) 10625-10629.

[21] B. Voutsinos-Porche, G. Knott, K. Tanaka, C. Quairiaux, E. Welker, G. Bonvento, Glial glutamate transporters and maturation of the mouse somatosensory cortex, Cerebral cortex, 13 (2003) 1110-1121.

[22] B. Voutsinos-Porche, G. Bonvento, K. Tanaka, P. Steiner, E. Welker, J.Y. Chatton, P.J. Magistretti, L. Pellerin, Glial glutamate transporters mediate a functional metabolic crosstalk between neurons and astrocytes in the mouse developing cortex, Neuron, 37 (2003) 275-286.

[23] J. Chuquet, P. Quilichini, E.A. Nimchinsky, G. Buzsaki, 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, 30 (2010) 15298-15303.

[24] P.J. Magistretti, L. Pellerin, The contribution of astrocytes to the 18F-2-deoxyglucose signal in PET activation studies, Molecular psychiatry, 1 (1996) 445-452.

[25] M. Melone, L. Vitellaro-Zuccarello, A. Vallejo-Illarramendi, A. Perez-Samartin, C. Matute, A. Cozzi, D.E. Pellegrini-Giampietro, J.D. Rothstein, F. Conti, The expression of glutamate transporter GLT-1 in the rat cerebral cortex is down-regulated by the antipsychotic drug clozapine, Molecular psychiatry, 6 (2001) 380-386.

[26] M. Melone, L. Bragina, F. Conti, Clozapine-induced reduction of glutamate transport in the frontal cortex is not mediated by GLAST and EAAC1, Molecular psychiatry, 8 (2003) 12-13.

[27] A. Vallejo-Illarramendi, M. Torres-Ramos, M. Melone, F. Conti, C. Matute, Clozapine reduces GLT-1 expression and glutamate uptake in astrocyte cultures, Glia, 50 (2005) 276-279.

[28] L. Bragina, M. Melone, G. Fattorini, M. Torres-Ramos, A. Vallejo-Illarramendi, C. Matute, F. Conti, GLT-1 down-regulation induced by clozapine in rat frontal cortex is associated with synaptophysin up-regulation, Journal of neurochemistry, 99 (2006) 134-141.

[29] A.V. Terry, Jr., W.D. Hill, V. Parikh, J.L. Waller, D.R. Evans, S.P. Mahadik, 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, 28 (2003) 300-309.

[30] R.E. See, 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, 181 (1990) 175-186.

[31] R.J. Baldessarini, F. Centorrino, J.G. Flood, S.A. Volpicelli, D. Huston-Lyons, B.M. Cohen, Tissue concentrations of clozapine and its metabolites in the rat, Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 9 (1993) 117-124.

[32] D.G. Souza, B. Bellaver, D.O. Souza, A. Quincozes-Santos, Characterization of adult rat astrocyte cultures, PloS one, 8 (2013) e60282.

[33] J. Espinosa, A. Rocha, F. Nunes, M.S. Costa, V. Schein, V. Kazlauckas, E. Kalinine, D.O. Souza, R.A. Cunha, L.O. Porciuncula, Caffeine consumption prevents memory impairment, neuronal damage, and adenosine A2A receptors upregulation in the hippocampus of a rat model of sporadic dementia, Journal of Alzheimer's disease : JAD, 34 (2013) 509-518.

[34] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods, 25 (2001) 402-408.

[35] A. Quincozes-Santos, P. Nardin, D.F. de Souza, D.P. Gelain, J.C. Moreira, A. Latini, C.A. Goncalves, C. Gottfried, The janus face of resveratrol in astroglial cells, Neurotoxicity research, 16 (2009) 30-41.

[36] W.W. Moses, Fundamental Limits of Spatial Resolution in PET, Nucl Instrum Methods Phys Res A, 648 Supplement 1 (2011) S236-S240.

[37] S. Holmseth, H.A. Scott, K. Real, K.P. Lehre, T.B. Leergaard, J.G. Bjaalie, N.C. Danbolt, 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, 162 (2009) 1055-1071.

[38] Y. Zhou, N.C. Danbolt, GABA and Glutamate Transporters in Brain, Frontiers in endocrinology, 4 (2013) 165.

[39] B.S. McEwen, Stress and hippocampal plasticity, Annu Rev Neurosci, 22 (1999) 105-122.

[40] H.A. Cameron, E. Gould, Distinct populations of cells in the adult dentate gyrus undergo mitosis or apoptosis in response to adrenalectomy, The Journal of comparative neurology, 369 (1996) 56-63.

[41] B.S. McEwen, S.E. Alves, Estrogen actions in the central nervous system, Endocr Rev, 20 (1999) 279-307.

[42] N.C. Danbolt, Glutamate uptake, Progress in neurobiology, 65 (2001) 1-105.

[43] L. Prut, C. Belzung, The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: a review, European journal of pharmacology, 463 (2003) 3-33.

[44] S.J. Cohen, R.W. Stackman, Jr., Assessing rodent hippocampal involvement in the novel object recognition task. A review, Behavioural brain research, 285 (2015) 105-117.

[45] M.E. Phelps, E.J. Hoffman, N.A. Mullani, M.M. Ter-Pogossian, Application of annihilation coincidence detection to transaxial reconstruction tomography, Journal of nuclear medicine : official publication, Society of Nuclear Medicine, 16 (1975) 210-224.

[46] I. Sarikaya, PET studies in epilepsy, American journal of nuclear medicine and molecular imaging, 5 (2015) 416-430.

[47] N. Smailagic, M. Vacante, C. Hyde, S. Martin, O. Ukoumunne, C. Sachpekidis, (1)(8)F-FDG PET for the early diagnosis of Alzheimer's disease dementia and other dementias in people with mild cognitive impairment (MCI), The Cochrane database of systematic reviews, 1 (2015) CD010632.

[48] K.L. Poston, D. Eidelberg, FDG PET in the Evaluation of Parkinson's Disease, PET Clin, 5 (2010) 55-64.

[49] R. Seethalakshmi, S.R. Parkar, N. Nair, S.A. Adarkar, A.G. Pandit, S.A. Batra, N.S. Baghel, S.H. Moghe, Regional brain metabolism in schizophrenia: An FDG-PET study, Indian journal of psychiatry, 48 (2006) 149-153.

[50] A.C. Altamura, A. Bertoldo, G. Marotta, R.A. Paoli, E. Caletti, F. Dragogna, M. Buoli, V. Baglivo, M.C. Mauri, P. Brambilla, White matter metabolism differentiates schizophrenia and bipolar disorder: a preliminary PET study, Psychiatry research, 214 (2013) 410-414.

[51] L. Su, Y. Cai, Y. Xu, A. Dutt, S. Shi, E. Bramon, Cerebral metabolism in major depressive disorder: a voxel-based meta-analysis of positron emission tomography studies, BMC psychiatry, 14 (2014) 321.

693 [52] A.J. Zametkin, T.E. Nordahl, M. Gross, A.C. King, W.E. Semple, J. Rumsey, S. Hamburger, R.M.
694 Cohen, Cerebral glucose metabolism in adults with hyperactivity of childhood onset, The New England
695 journal of medicine, 323 (1990) 1361-1366.
696
697 [53] P.J. Magistretti, L. Pellerin, Cellular mechanisms of brain energy metabolism and their relevance
698 to functional brain imaging, Philos Trans R Soc Lond B Biol Sci, 354 (1999) 1155-1163.
699
700 [54] P.J. Magistretti, Cellular bases of functional brain imaging: insights from neuron-glia metabolic
701 coupling, Brain research, 886 (2000) 108-112.
702
703 [55] L. Welberg, Neuroimaging: Interpreting the signal, Nat Rev Neurosci, 10 (2009) 166-166.
704
705 [56] A. Janowsky, K.A. Neve, J.M. Kinzie, B. Taylor, T. de Paulis, J.K. Belknap, Extrastriatal dopamine
706 D2 receptors: distribution, pharmacological characterization and region-specific regulation by
707 clozapine, The Journal of pharmacology and experimental therapeutics, 261 (1992) 1282-1290.
708
709 [57] J.A. Lieberman, F.P. Bymaster, H.Y. Meltzer, A.Y. Deutch, G.E. Duncan, C.E. Marx, J.R. Aprille,
710 D.S. Dwyer, X.M. Li, S.P. Mahadik, R.S. Duman, J.H. Porter, J.S. Modica-Napolitano, S.S. Newton,
711 J.G. Csernansky, Antipsychotic drugs: comparison in animal models of efficacy, neurotransmitter
712 regulation, and neuroprotection, Pharmacol Rev, 60 (2008) 358-403.
713
714 [58] J. Lappalainen, J. Hietala, M. Koulu, E. Syvalahti, Neurochemical effects of chronic co-
715 administration of ritanserin and haloperidol: comparison with clozapine effects, European journal of
716 pharmacology, 190 (1990) 403-407.
717
718 [59] H.W. Kim, Y. Cheon, H.R. Modi, S.I. Rapoport, J.S. Rao, Effects of chronic clozapine
719 administration on markers of arachidonic acid cascade and synaptic integrity in rat brain,
720 Psychopharmacology, 222 (2012) 663-674.
721

722 [60] H. Homayoun, B. Moghaddam, Fine-tuning of awake prefrontal cortex neurons by clozapine:
723 comparison with haloperidol and N-desmethylclozapine, Biological psychiatry, 61 (2007) 679-687.

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725

726 ## Figure Legends:

727 **Figure 1: Assessment of brain glucose metabolism by [^{18}F]FDG PET before starting**
728 **the clozapine treatment.** (a) Mean whole brain SUVr of animals assigned to the groups
729 control and clozapine at the baseline. Data represented as groups mean ± S.E.M. (b)
730 Representative images showing the mean SUVr of animals assigned to the groups control
731 and clozapine at the baseline, projected into a standard magnetic resonance imaging (mMRI)
732 image in the axial, sagittal and coronal planes. Standardized uptake value reference (SUVr)
733 was calculated using pons as reference region. (n= 7-8 animals per group).
734
735 **Figure 2: Effects of clozapine treatment on the brain glucose metabolism assessed by**
736 **[^{18}F]FDG** (a) Brain metabolic maps showing the mean SUVr of the control group at the
737 baseline and after the treatment period. (b) Brain metabolic maps showing the mean SUVr of
738 the clozapine group at the baseline and after the clozapine treatment period. (c) Images
739 representing percentage of change between baseline and follow-up at control and clozapine

groups. (d) t-statistical map showing the statistically significant hypometabolism at the groups control and clozapine after the treatment period in relation to the baseline. (e) Representative image of the control group t-statistical in a three dimensional object. (f) Representative image of the clozapine group t-statistical in a three dimensional object. Images are projected into a standard magnetic resonance imaging (mMRI) image in axial, sagittal and coronal planes. (n= 6/8 animals per group)

Figure 3: Density and expression of GLT-1 and GLAST on the cortex following clozapine treatment. Cortical immunocontent of the glutamate transporters (a) GLT-1 (normalized by the β -tubulin immunoreactivity), (b) and GLAST (normalized by the GAPDH immunoreactivity) of the groups control and clozapine after the six weeks treatment period. Cortical mRNA expression of (c) GLT-1 (d) and 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. Data represented as mean ± S.E.M. (n= 7/8 animals per group). *$p<0.05$ (*student's t test*)

Figure 4: Adult cortical astrocytes culture and ex-vivo evaluation of clozapine treatment effects on glutamate and glucose uptake.
(a) Representative confocal images of control and clozapine groups' primary cortical astrocytes culture stained with GFAP (green) and DAPI (blue). Immunocontent of the glutamate transporters (b) GLT-1 (c) and GLAST on the primary cortical astrocytes culture of control and clozapine groups (normalized by GAPDH immunoreactivity). (d) D-aspartate uptake and (e) 2-Deoxy-D-glucose uptake of groups control and clozapine primary cortical astrocytes culture. Uptake values expressed as percentage of control. Data represented as mean ± S.E.M. (n= 3/4 animals per group). 20×magnification and scale bar=50 μm.

Figure 5: GLT-1 Density and expression on the hippocampus following clozapine treatment. Glutamate transporter GLT-1 (a) immunocontent (normalized by the β -tubulin immunoreactivity) and (b) mRNA expression on hippocampus of the groups control and clozapine after the six weeks treatment period. PCR results expressed as fold change in relation to the group control. Data represented as mean ± S.E.M. (n= 7/8 animals per group).

Figure 6: Adult hippocampal astrocytes culture and ex-vivo evaluation of clozapine treatment effects on glutamate and glucose uptake. (a) Representative confocal images of control and clozapine groups' primary hippocampal astrocytes culture stained with GFAP (green) and DAPI (blue). (b) D-aspartate uptake and (c) 2-Deoxy-D-glucose uptake of groups control and clozapine primary hippocampal astrocytes culture. Uptake values expressed as

777 percentage of control. Data represented as mean ± S.E.M. (n= 3 animals per group).
778 20×magnification and scale bar=50 μm.
779
780 **Supplemental Figure 1: Behavioral analysis following clozapine treatment.** (a) Groups
781 control and clozapine representative occupancy plot on the Open Field task after the six
782 weeks treatment period. (b) Total distance traveled in the Open Field task. (c) Objects
783 exploration time percentage on the training session of the Novel Object Recognition Task. (d)
784 Objects exploration time percentage in the test session of the Novel Object Recognition
785 Task. Data displayed as mean ± S.E.M. (n= 8 animals per group) **p<0.01, ***p<0.001,
786
787 **Supplemental Figure 2: Rats body weight and liquid intake across the treatment**
788 **period.** (a) Control and experimental rats body weight across the treatment period. (b)
789 Control and experimental rats liquid intake across the treatment period. Data displayed as
790 mean ± S.E.M (n= 8 animals per group)
791
792 **Supplemental Figure 3: Confirmation of clozapine presence in the experimental**
793 **solution.** (a) Chromatogram (HPLC) showing clozapine presence on the experimental
794 solution and absence in the control solution

Figure 1

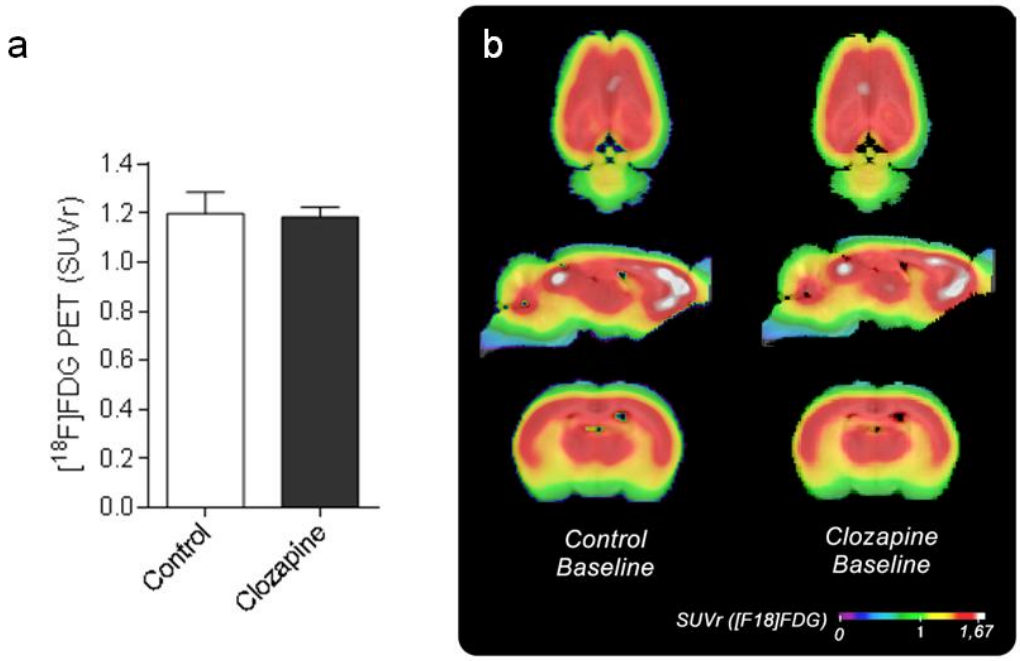

a
[18F]FDG PET (SUVr)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Clozapine
b
Control
Baseline
Clozapine
Baseline
SUVr ([F18]FDG)
0
1
1,67

Figure 2

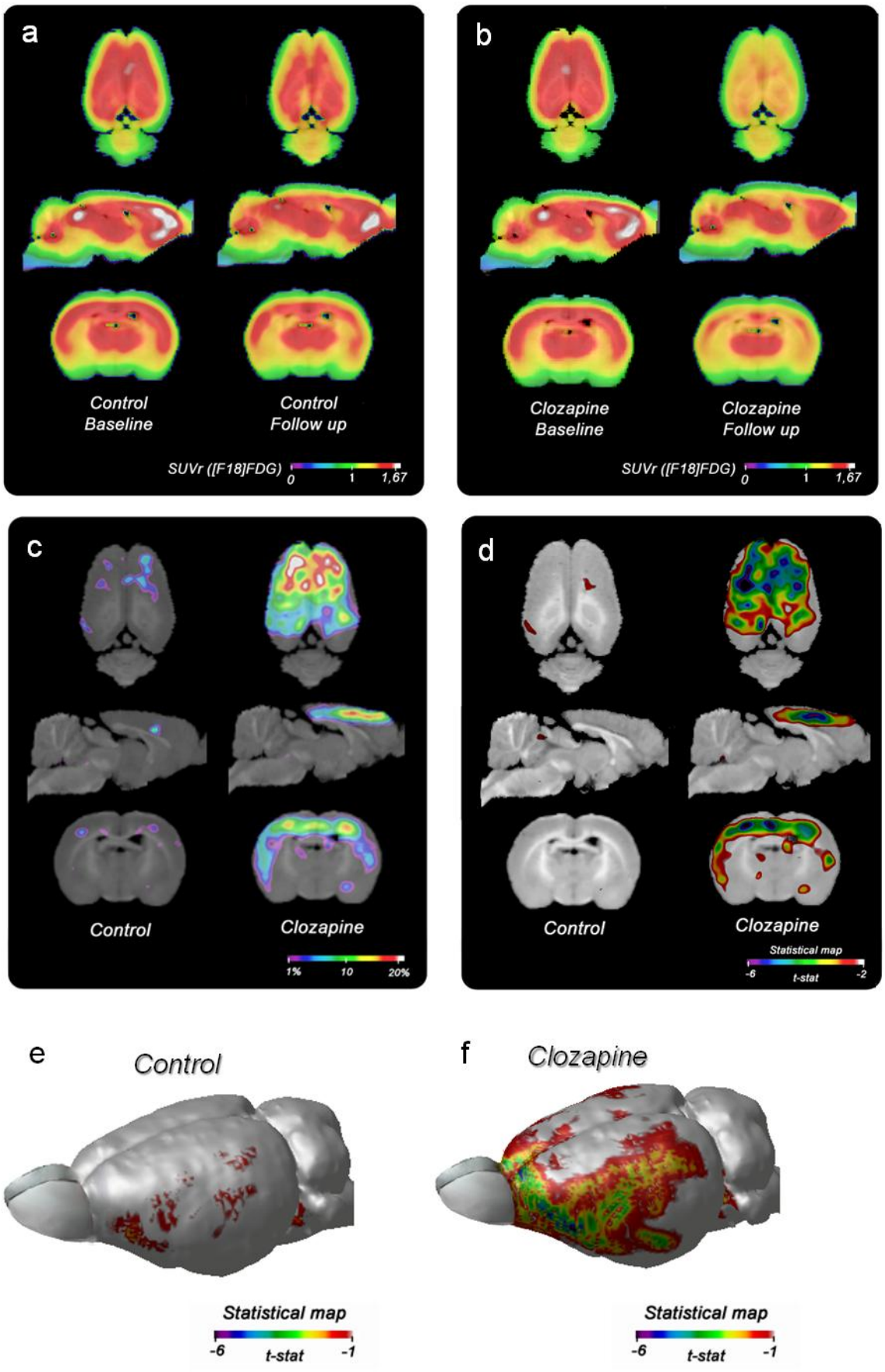

a
Control
Baseline
Control
Follow up
SUVr ([F18]FDG)
0
1
1,67
b
Clozapine
Baseline
Clozapine
Follow up
SUVr ([F18]FDG)
0
1
1,67
c
Control
Clozapine
1%
10
20%
d
Control
Clozapine
Statistical map
-6
t-stat
-2
e
Control
Statistical map
-6
t-stat
-1
f
Clozapine
Statistical map
-6
t-stat
-1

Figure 3

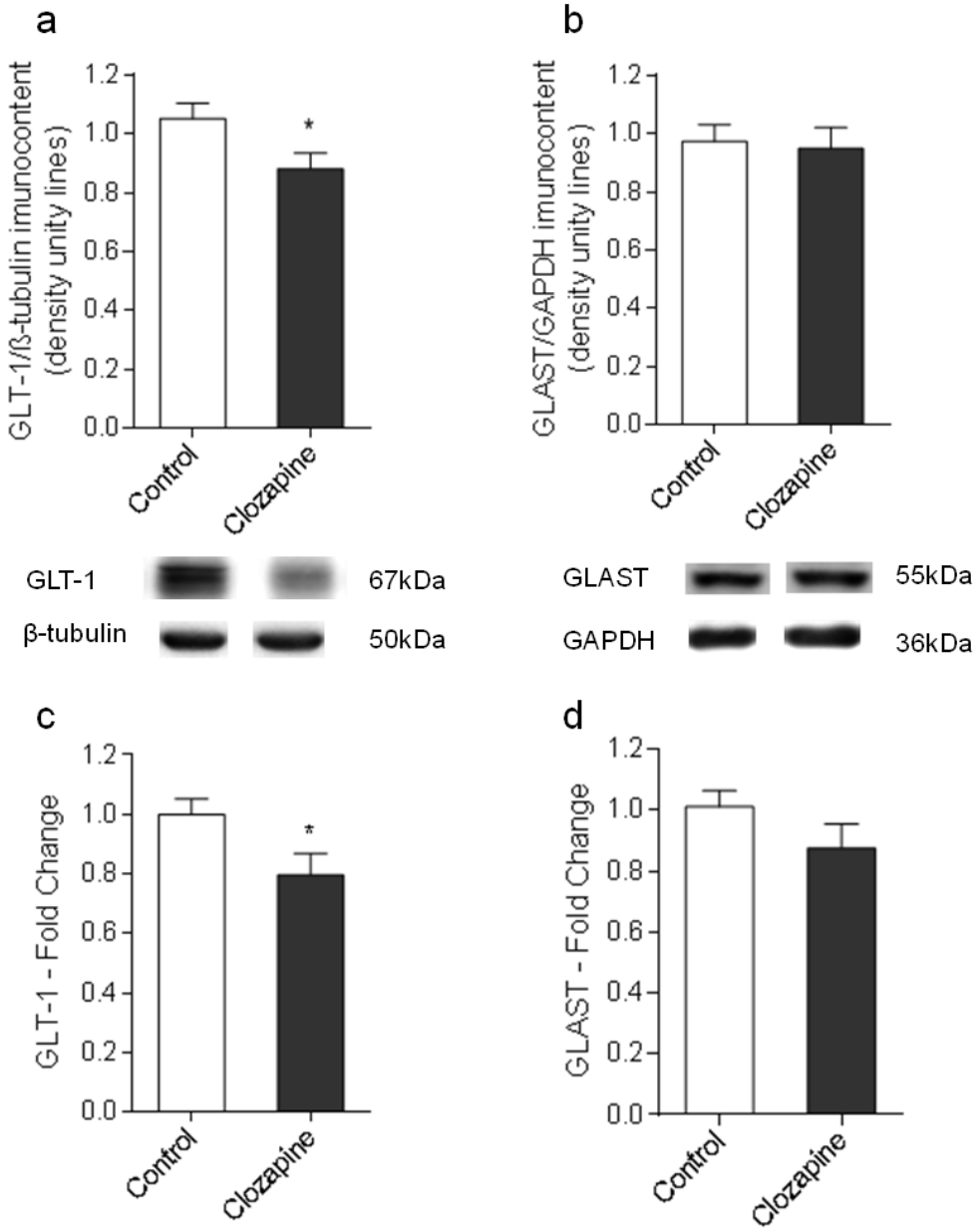

a
GLT-1/ß-tubulin imunocontent
(density unity lines)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Clozapine
GLT-1
67kDa
β-tubulin
50kDa
b
GLAST/GAPDH imunocontent
(density unity lines)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Clozapine
GLAST
55kDa
GAPDH
36kDa
c
GLT-1 - Fold Change
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Control
Clozapine
d
GLAST - Fold Change
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Clozapine

Figure 4

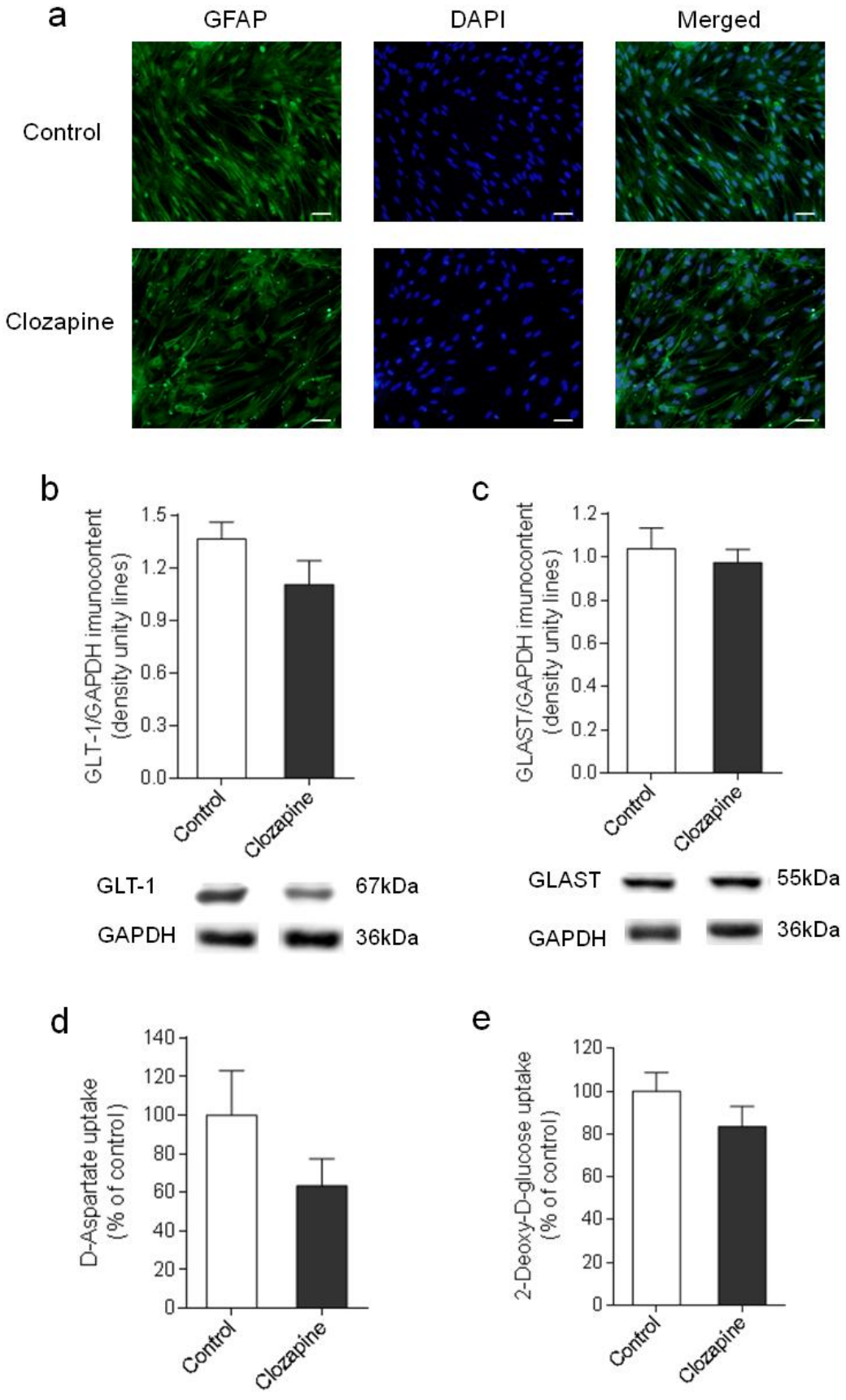

a
GFAP
DAPI
Merged
Control
Clozapine
b
GLT-1/GAPDH imunocontent (density unity lines)
1.5
1.2
0.9
0.6
0.3
0.0
Control
Clozapine
GLT-1
67kDa
GAPDH
36kDa
c
GLAST/GAPDH imunocontent (density unity lines)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Clozapine
GLAST
55kDa
GAPDH
36kDa
d
D-Aspartate uptake (% of control)
140
120
100
80
60
40
20
0
Control
Clozapine
e
2-Deoxy-D-glucose uptake (% of control)
120
100
80
60
40
20
0
Control
Clozapine

Figure 5

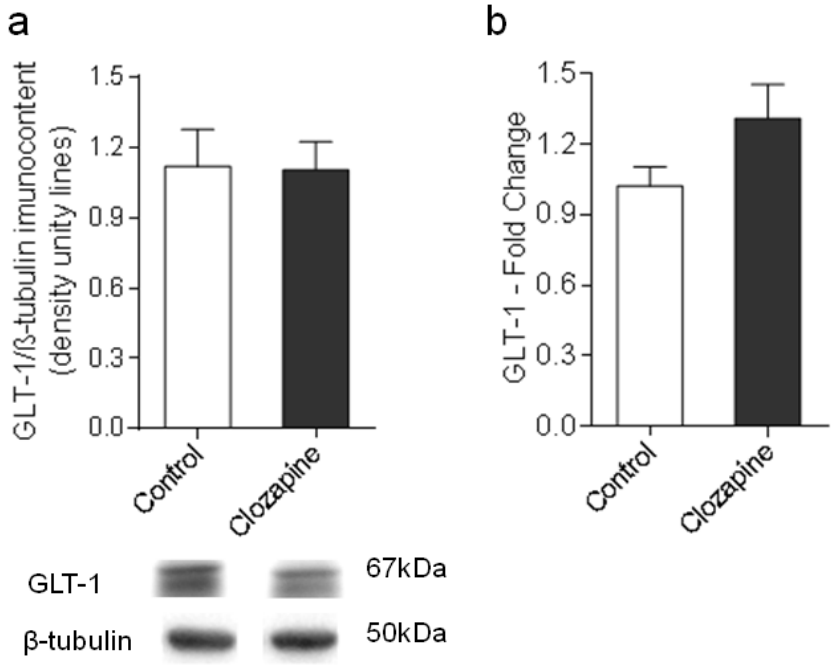
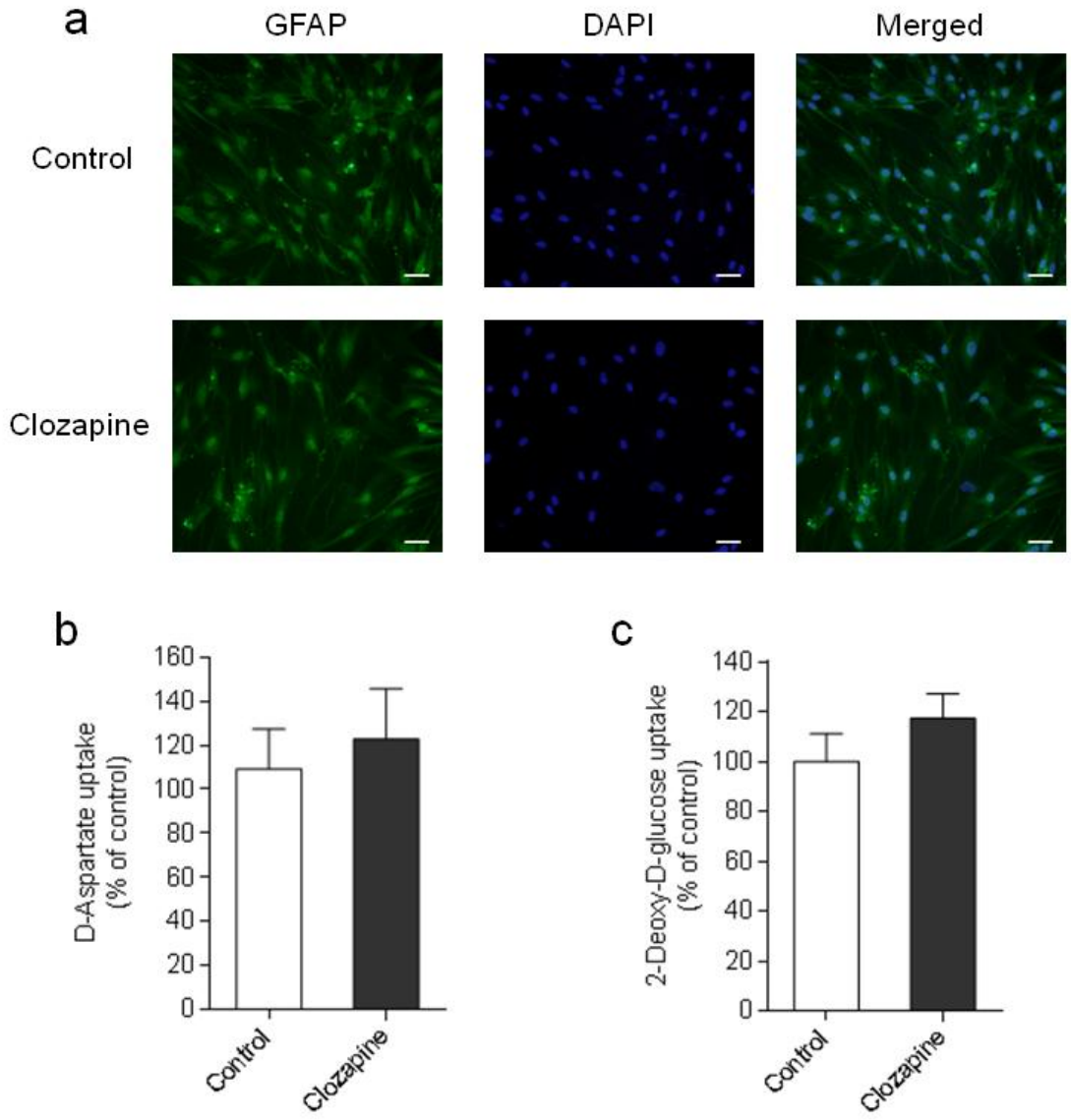
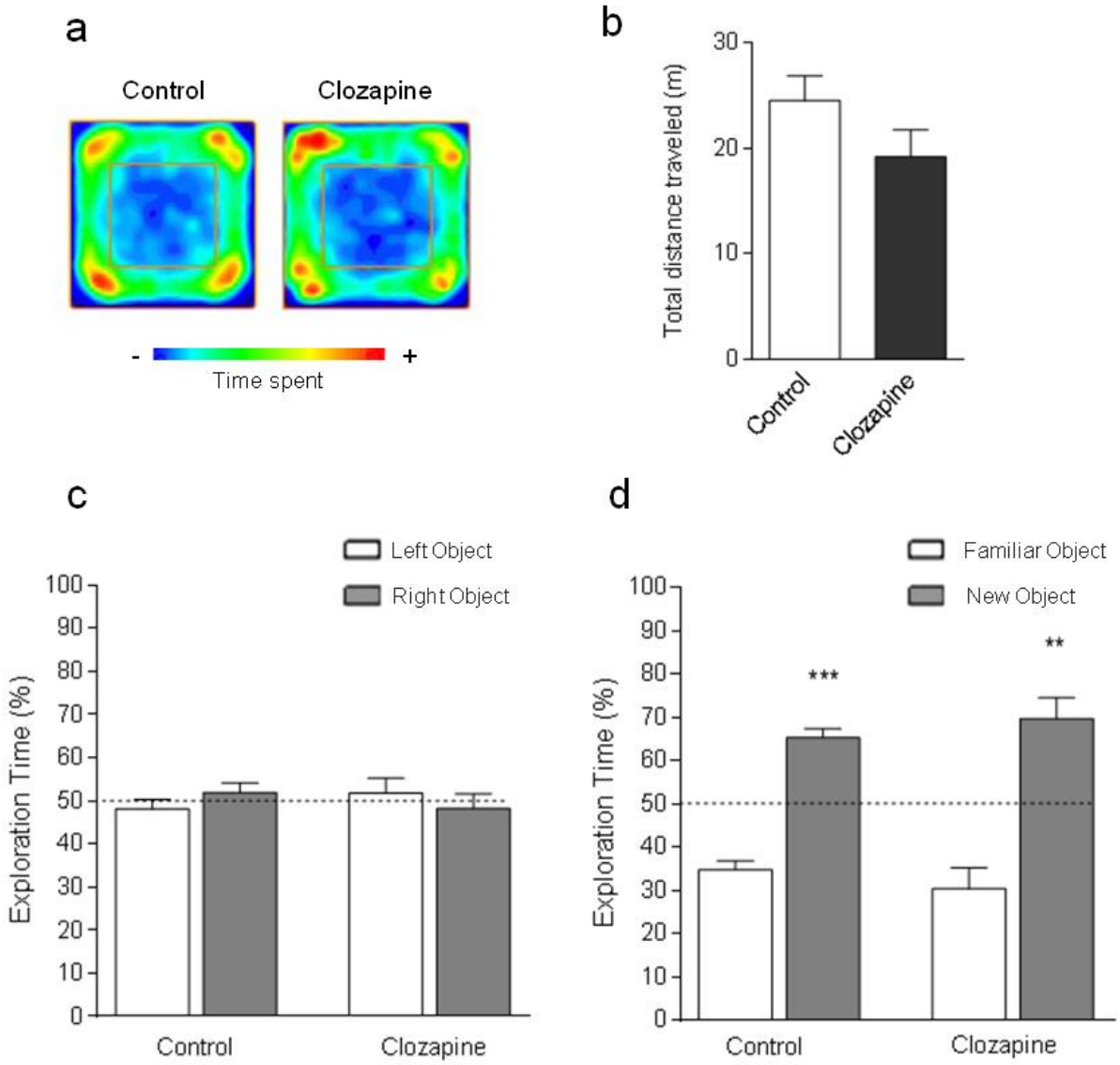

a
GLT-1/β-tubulin imunocontent
(density unity lines)
1.5
1.2
0.9
0.6
0.3
0.0
Control
Clozapine
GLT-1
β-tubulin
67kDa
50kDa
b
GLT-1 - Fold Change
1.5
1.2
0.9
0.6
0.3
0.0
Control
Clozapine

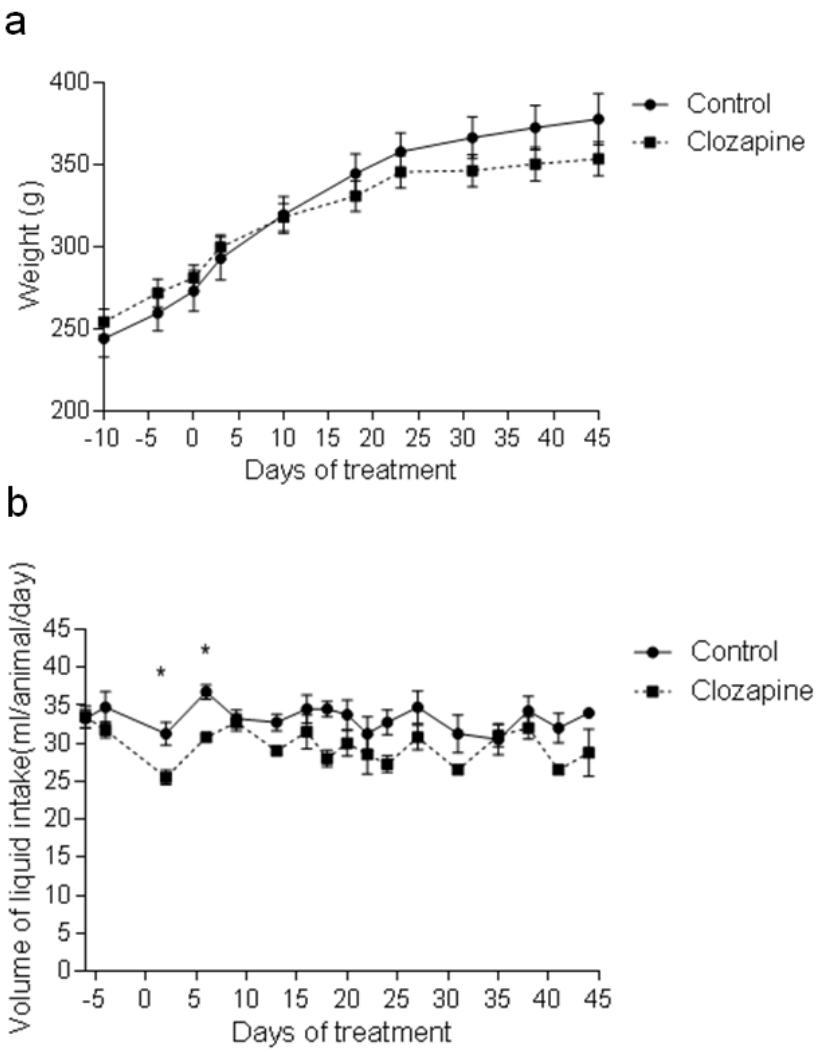
Figure 6


a
GFAP
DAPI
Merged
Control
Clozapine
b
D-Aspartate uptake
(% of control)
160
140
120
100
80
60
40
20
0
Control
Clozapine
c
2-Deoxy-D-glucose uptake
(% of control)
140
120
100
80
60
40
20
0
Control
Clozapine

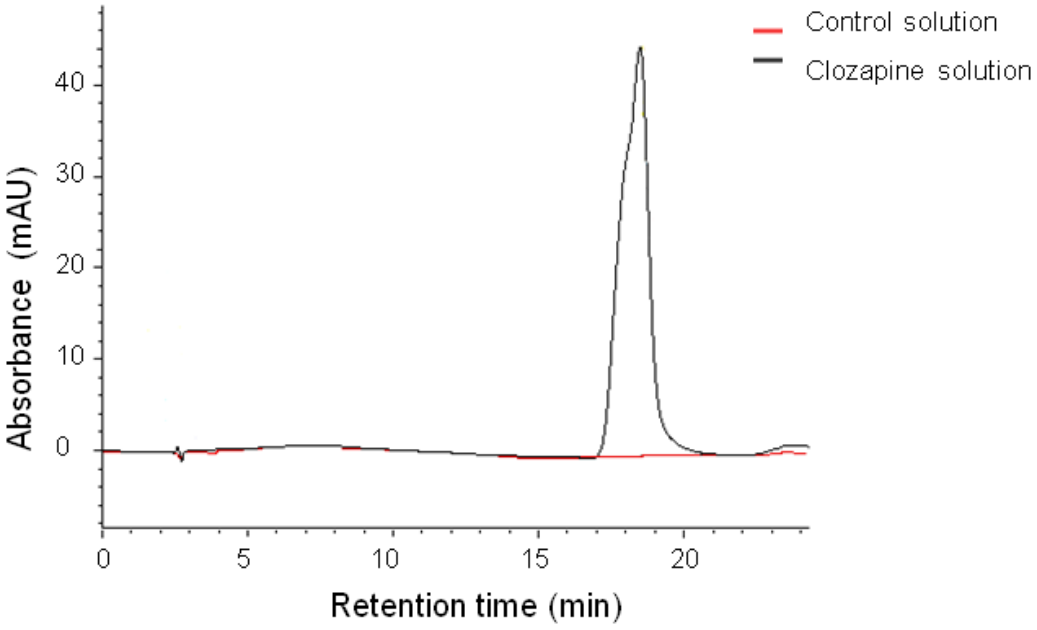
Supplemental Figure 1



Supplemental Figure 2



Supplemental Figure 3



Highlights:

1. Clozapine induced GLT-1 downregulation on cortex reduces [^{18}F]FDG uptake.
2. GLT-1 downregulation might reduce D-Aspartate and 2DG uptake in adult cultured astrocytes.
3. [^{18}F]FDG-PET signal interpretation needs to be reevaluated to include astrocytes contribuition.

4. CONCLUSÕES E PERSPECTIVAS

A técnica de [^{18}F]FDG-PET é utilizada para verificar o metabolismo energético em diferentes regiões do encéfalo. Neste trabalho, nós observamos a redução na captação de [^{18}F]FDG no córtex simultaneamente à redução do transportador astrocitário de glutamato GLT-1 nessa região. A atividade dos transportadores de glutamato astrocitários tem sido reconhecida como um dos gatilhos para captação de glicose por estas células. Portanto, uma redução no sinal de [^{18}F]FDG-PET em resposta à redução nesse transportador sugere que há uma importante contribuição astrocitária nos resultados vistos através dessa ferramenta de imageamento cerebral. Com base em nossas observações e outras evidências prévias da literatura demonstrando a participação astrocitária no metabolismo de glicose cerebral, podemos sugerir que a interpretação tradicional dos sinais da técnica de imagem [^{18}F]FDG-PET como produto direto do funcionamento neuronal deva ser reavaliada.

No entanto, para validar e corroborar nossos resultados neste trabalho, mais investigações devem ser realizadas. Neste projeto, ainda pretendemos repetir os experimentos para verificar a replicabilidade destes em estudos independentes adicionais. Em especial, os nossos estudos com cultura primária de astrócitos devem ser executados novamente, com um maior numero de indivíduos. Análises da captação de glicose e glutamato ainda podem ser realizadas através de outras técnicas, como, por exemplo, captações em fatias de encéfalo, que eliminariam o viés de possíveis efeitos do procedimento de cultura.

Amostras de líquor destes animais foram também colhidas para verificar se existem alterações nas concentrações de glutamato e lactato. Gostaríamos também de investigar o motivo pelo qual a nossa redução na densidade de GLT-1 em resposta ao tratamento farmacológico não foi tão significativa quanta a reportada previamente na literatura. Assim, continuaremos nossas investigações, com o uso do HPLC, na solução de clozapina dada aos animais. No entanto, amostras de soro foram também colhidas dos animais, com o intuito de verificar a concentração sérica do fármaco e se houveram problemas na administração da dose deseja da droga por

via oral. Por fim, eventuais mortes neuronais ou redução na arborização neuronal precisam ser investigadas como possíveis causas na redução do metabolismo de glicose. Com este fim, estudos histológicos serão realizados.

Para corroborar nossa teoria, além da finalização das investigações neste trabalho, nosso grupo está realizando um estudo com ativadores dos transportadores astrocitários de glutamato. Até o momento, os resultados deste estudo demonstraram que o efeito contrário também existe, ou seja, que há aumento na captação de FDG quando há ativação destes transportadores.

A nossa proposta final é de que estudos futuros com o uso de silenciamento genético parcial destes transportadores sejam realizados. Desta forma, eliminando as outras inconvenientes interações que as intervenções farmacológicas exibem.

Por fim, é importante ressaltar que as nossas observações não questionam a validade da técnica de [^{18}F]FDG-PET e outras técnicas baseadas na captação de análogos da glicose *in vivo* no cérebro. A nossa investigação, na verdade, busca esclarecer as bases celulares para estes procedimentos e permitir um melhor entendimento dos fascinantes resultados que estas técnicas oferecem.

REFERÊNCIAS

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)

ALAVI, A. et al. Positron emission tomography imaging of regional cerebral glucose metabolism. **Semin Nucl Med,** v. 16, n. 1, p. 2-34, Jan 1986. ISSN 0001-2998 (Print)

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

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)

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,** v. 513, n. 5, p. 532-41, Apr 10 2009. ISSN 1096-9861 (Electronic)

BALDESSARINI, R. J. et al. Tissue concentrations of clozapine and its metabolites in the rat. **Neuropsychopharmacology,** v. 9, n. 2, p. 117-24, Sep 1993. ISSN 0893-133X (Print)

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 7 2011. ISSN 1932-7420 (Electronic)

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

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)

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)

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)

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)

BOUZIER-SORE, A. K. et al. Lactate is a preferential oxidative energy substrate over glucose for neurons in culture. **J Cereb Blood Flow Metab,** v. 23, n. 11, p. 1298-306, Nov 2003. ISSN 0271-678X

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)

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)

CAMERON, H. A.; GOULD, E. Distinct populations of cells in the adult dentate gyrus undergo mitosis or apoptosis in response to adrenalectomy. **J Comp Neurol,** v. 369, n. 1, p. 56-63, May 20 1996. ISSN 0021-9967 (Print)

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)

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)

COHEN, S. J.; STACKMAN, R. W., JR. Assessing rodent hippocampal involvement in the novel object recognition task. A review. **Behav Brain Res,** v. 285, p. 105-17, May 15 2015. ISSN 1872-7549

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

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)

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

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)

ESPINOSA, J. et al. Caffeine consumption prevents memory impairment, neuronal damage, and adenosine A2A receptors upregulation in the hippocampus of a rat model of sporadic dementia. **J Alzheimers Dis,** v. 34, n. 2, p. 509-18, 2013. ISSN 1875-8908 (Electronic)

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)

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)

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)

GOBEL, B.; OLTMANNS, K. M.; CHUNG, M. Linking neuronal brain activity to the glucose metabolism. **Theor Biol Med Model,** v. 10, p. 50, Aug 29 2013. ISSN 1742-4682 (Electronic)

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 8 1981. ISSN 0036-8075 (Print)

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)

HENRY, T. R.; VOTAW, J. R. The role of positron emission tomography with [18F]fluorodeoxyglucose in the evaluation of the epilepsies. **Neuroimaging Clin N Am,** v. 14, n. 3, p. 517-35, ix, Aug 2004. ISSN 1052-5149 (Print)

HERCULANO-HOUZEL, S. The glia/neuron ratio: how it varies uniformly across brain structures and species and what that means for brain physiology and evolution. **Glia,** v. 62, n. 9, p. 1377-91, Sep 2014. ISSN 1098-1136 (Electronic)

HOFMAN, M. S.; HICKS, R. J. How We Read Oncologic FDG PET/CT. **Cancer Imaging,** v. 16, n. 1, p. 35, Oct 18 2016. ISSN 1470-7330 (Electronic)

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)

HOMAYOUN, H.; MOGHADDAM, B. Fine-tuning of awake prefrontal cortex neurons by clozapine: comparison with haloperidol and N-desmethylclozapine. **Biol Psychiatry,** v. 61, n. 5, p. 679-87, Mar 1 2007. ISSN 0006-3223 (Print)

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)

IVANOV, A. et al. Lactate Effectively Covers Energy Demands during Neuronal Network Activity in Neonatal Hippocampal Slices. **Front Neuroenergetics,** v. 3, p. 2, 2011. ISSN 1662-6427 (Electronic)

JANOWSKY, A. et al. Extrastriatal dopamine D2 receptors: distribution, pharmacological characterization and region-specific regulation by clozapine. **J Pharmacol Exp Ther,** v. 261, n. 3, p. 1282-90, Jun 1992. ISSN 0022-3565 (Print)

KATO, T. et al. Brain fluorodeoxyglucose (FDG) PET in dementia. **Ageing Res Rev,** v. 30, p. 73-84, Sep 2016. ISSN 1872-9649 (Electronic)

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

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)

KIM, H. W. et al. Effects of chronic clozapine administration on markers of arachidonic acid cascade and synaptic integrity in rat brain. **Psychopharmacology (Berl),** v. 222, n. 4, p. 663-74, Aug 2012. ISSN 1432-2072 (Electronic)

LAPPALAINEN, J. et al. Neurochemical effects of chronic co-administration of ritanserin and haloperidol: comparison with clozapine effects. **Eur J Pharmacol,** v. 190, n. 3, p. 403-7, Nov 13 1990. ISSN 0014-2999 (Print)

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)

LIEBERMAN, J. A. et al. Antipsychotic drugs: comparison in animal models of efficacy, neurotransmitter regulation, and neuroprotection. **Pharmacol Rev,** v. 60, n. 3, p. 358-403, Sep 2008. ISSN 1521-0081 (Electronic)

LIVAK, K. J.; SCHMITTGEN, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. **Methods,** v. 25, n. 4, p. 402-8, Dec 2001. ISSN 1046-2023

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)

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)

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)

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)

MAGISTRETTI, P.J.; PELLERIN, L. 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)

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)

MAZZILOTTA, J. C. et al. Tomographic mapping of human cerebral metabolism: normal unstimulated state. **Neurology,** v. 31, n. 5, p. 503-16, May 1981. ISSN 0028-3878 (Print)

MCEWEN, B. S. Stress and hippocampal plasticity. **Annu Rev Neurosci,** v. 22, p. 105-22, 1999. ISSN 0147-006X (Print)

MCEWEN, B. S.; ALVES, S. E. Estrogen actions in the central nervous system. **Endocr Rev,** v. 20, n. 3, p. 279-307, Jun 1999. ISSN 0163-769X (Print)

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)

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)

MERGENTHALER, P. et al. Sugar for the brain: the role of glucose in physiological and pathological brain function. **Trends Neurosci,** v. 36, n. 10, p. 587-97, Oct 2013. ISSN 1878-108X (Electronic)

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)

MOSCONI, L. et al. Multicenter standardized 18F-FDG PET diagnosis of mild cognitive impairment, Alzheimer's disease, and other dementias. **J Nucl Med,** v. 49, n. 3, p. 390-8, Mar 2008. ISSN 0161-5505 (Print)

MOSES, W. W. Fundamental Limits of Spatial Resolution in PET. **Nucl Instrum Methods Phys Res A,** v. 648 Supplement 1, p. S236-S240, Aug 21 2011. ISSN 0168-9002 (Print)

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)

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)

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)

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

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)

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)

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

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

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)

QUINCOZES-SANTOS, A. et al. The janus face of resveratrol in astroglial cells. **Neurotox Res,** v. 16, n. 1, p. 30-41, Jul 2009. ISSN 1476-3524 (Electronic)

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)

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)

SARIKAYA, I. PET studies in epilepsy. **Am J Nucl Med Mol Imaging,** v. 5, n. 5, p. 416-30, 2015.

SEE, R. E.; ELLISON, G. Comparison of chronic administration of haloperidol and the atypical neuroleptics, clozapine and raclopride, in an animal model of tardive dyskinesia. **Eur J Pharmacol,** v. 181, n. 3, p. 175-86, Jun 8 1990. ISSN 0014-2999 (Print)

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)

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)

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)

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)

SOKOLOFF, L. Localization of functional activity in the central nervous system by measurement of glucose utilization with radioactive deoxyglucose. **J Cereb Blood Flow Metab,** v. 1, n. 1, p. 7-36, 1981. ISSN 0271-678X (Print)

SOKOLOFF,L. Sites and mechanisms of function-related changes in energy metabolism in the nervous system. **Dev Neurosci,** v. 15, n. 3-5, p. 194-206, 1993. ISSN 0378-5866 (Print)

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)

SOUZA, D. G. et al. Characterization of adult rat astrocyte cultures. **PLoS One,** v. 8, n. 3, p. e60282, 2013. ISSN 1932-6203 (Electronic)

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)

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)

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)

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

TERRY, A. V., JR. et al. Differential effects of haloperidol, risperidone, and clozapine exposure on cholinergic markers and spatial learning performance in rats. **Neuropsychopharmacology,** v. 28, n. 2, p. 300-9, Feb 2003. ISSN 0893-133X (Print)

TORGNER, I.; KVAMME, E. Synthesis of transmitter glutamate and the glial-neuron interrelationship. **Mol Chem Neuropathol,** v. 12, n. 1, p. 11-7, Jan 1990. ISSN 1044-7393 (Print)

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)

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)

VOLTERRA, A.; MELDOLESI, J. Astrocytes, from brain glue to communication elements: the revolution continues. **Nat Rev Neurosci,** v. 6, n. 8, p. 626-40, Aug 2005. ISSN 1471-003X (Print)

VOUTSINOS-PORCHE, B. et al. Glial glutamate transporters mediate a functional metabolic crosstalk between neurons and astrocytes in the mouse developing cortex. **Neuron,** v. 37, n. 2, p. 275-86, Jan 23 2003. ISSN 0896-6273 (Print)

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)

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)

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

WELBERG, L. Neuroimaging: Interpreting the signal. **Nat Rev Neurosci,** v. 10, n. 3, p. 166-166, 03//print 2009. ISSN 1471-003X..

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

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)

ZHOU, Y.; DANBOLT, N. C. GABA and Glutamate Transporters in Brain. **Front Endocrinol (Lausanne),** v. 4, p. 165, 2013. ISSN 1664-2392 (Linking).

ANEXO A – NORMAS DE PUBLICAÇÃO DA REVISTA

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.

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Journal abbreviations source
Journal names should be abbreviated according to the List of Title Word Abbreviations.

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Supplementary material can support and enhance your scientific research. Supplementary files offer the author additional possibilities to publish supporting applications, high-resolution images, background datasets, sound clips and more. Please note that such items are published online exactly as they are submitted; there is no typesetting involved (supplementary data supplied as an Excel file or as a PowerPoint slide will appear as such online). Please submit the material together with the article and supply a concise and descriptive caption for each file. If you wish to make any changes to supplementary data during any stage of the process, then please make sure to provide an updated file, and do not annotate any corrections on a previous version. Please also make sure to switch off the 'Track Changes' option in any Microsoft Office files as these will appear in the published supplementary file(s).