Universidade Federal do Rio Grande do Sul

Instituto de Ciências Básicas da Saúde

Departamento de Bioquímica

Programa de Pós-Graduação em Ciências Biológicas: Bioquímica

TESE DE DOUTORADO

METABOLISMO ENERGÉTICO HIPOCAMPAL E EFEITOS ELETROFISIOLÓGICOS DA GUANOSINA EM MODELO DE HIPERESTIMULAÇÃO GLUTAMATÉRGICA

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Porto Alegre, RS, Brasil

2014

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, como requisito parcial à obtenção do título de Doutor em Bioquímica.

Porto Alegre, abril de 2014

CIP - Catalogação na Publicação

Vasconcelos Torres, Felipe
Metabolismo energético hipocampal e efeitos eletrofisiológicos da guanosina em modelo de hiperestimulação glutamatérgica / Felipe Vasconcelos Torres. -- 2014.
111 f.

Orientador: Diogo Onofre Gomes de Souza.
Coorientadora: Maria Elisa Calcagnotto.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Porto Alegre, BR-RS, 2014.

1. Epilepsia. 2. Excitotoxicidade. 3. Metabolismo energético. 4. Glutamato. 5. Neuroproteção. I. Gomes de Souza, Diogo Onofre, orient. II. Calcagnotto, Maria Elisa, coorient. III. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

“If our brains were simple enough for us to understand them,

we'd be so simple that we couldn't.”

(Ian Stewart)

“And in the end, it’s not the years in your life that count.

It’s the life in your years.”

(Abraham Lincoln)

"Every dream that you leave behind is a piece of their future that no longer exists.”

(Steve Jobs)

AGRADECIMENTOS

À Fernanda Hansen, pelo amor correspondido, pelo apoio, pela compreensão.

À minha família pelo apoio e por sempre reforçarem a importância da educação e do estudo com dedicação.

Ao Prof. Diogo Souza por ele ser, entre tantos papéis que já exerce na profissão, o meu orientador e apoiador do programa de doutoramento em molde de MD-PhD.

À Profa. Maria Elisa Calcagnotto, coorientadora, amiga, vizinha, e por sempre conseguir me transmitir otimismo e perseverança.

Ao Prof. Adriano Tort, pela ajuda no início da vida científica.

Ao Prof. Manoel Pereira (UFPA) por ter encarado conosco o desafio de iniciar os registros de eletroencefalografia mesmo tendo que cruzar o Brasil.

Ao Lucas Coelho, por ter ajudado (bastante) com os experimentos e pela dedicação.

Ao Prof. Perry e à sua esposa, pelo legado e pelo apoio.

Ao Prof. Renato pelas longas conversas sobre neurociência.

Ao Eduardo Kalinini, à Mayara, ao Ganzella e ao Zimmer pelo apoio.

À Cléia por ter ajudado a mim e a tantos outros pós-graduandos.

Ao Odi, pelas conversas em tantas madrugadas com experimentos.

Ao Alex, da Engenharia Clínica do HCPA pela ajuda nos problemas de interface com os eletrodos.

Ao Prof. Olavo Amaral, pelas festas democráticas na Espel.

Ao CMPA e à FAMED-UFRGS, instituições federais que conseguiram me fornecer uma excelente formação a um custo irrisório, num país onde a competência dos órgãos públicos é sempre posta em xeque.

À Capes e ao CNPq pelo fomento à pesquisa.

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PARTE 1

PREFÁCIO

Dentre o papel do nosso grupo de pesquisa na investigação dos efeitos da guanosina no SNC e frente a distúrbios do sistema glutamatérgico, este trabalho procurou primeiramente preencher uma lacuna existente que seria a de se trabalhar com crises convulsivas sem que houvesse registro eletrofisiológico. Para tanto foi necessário começar do zero, uma vez que foram os primeiros registros de eletroencefalografia realizados no Departamento de Bioquímica da UFRGS, sendo necessária não apenas a padronização dos implantes como a difícil tarefa de conseguir sinais de boa qualidade mesmo em animais que viriam a apresentar uma convulsão tônico-clônica generalizada durante o experimento.

A segunda parte voltou-se à procura de efeitos da guanosina na atividade elétrica cerebral e sua associação com alterações da atividade motora em camundongos, particularmente com a modulação da frequência de pico na banda teta (4-10 Hz).

A terceira parte deste trabalho procurou se voltar ao lado bioquímico da hiperestimulação glutamatérgica, padronizando uma técnica que pudesse mensurar a oxidação de glutamato e glicose com vistas a uma perspectiva de investigação quanto à hipótese de o papel da guanosina no aumento da recaptação do glutamato pelos astrócitos estar correlacionado também com uma alteração do destino do glutamato recaptado. Espero que este sirva de base para próximas investigações e esclarecimentos quanto ao papel da guanosina frente aos distúrbios cerebrais que envolvem hiperestimulação glutamatérgica.

RESUMO

(TORRES FV - EFEITOS ELETROFISIOLÓGICOS DA GUANOSINA CONDIÇÕES NORMAIS E EM MODELO DE HIPERESTIMULAÇÃO GLUTAMATÉRGICA E METABOLISMO ENERGÉTICO HIPOCAMPAL) - O glutamato é fundamental para que ocorram eventos tróficos, desenvolvimentais e de envelhecimento do SNC assim como para que haja cognição e consolidação da memória. O desequilíbrio do sistema glutamatérgico está envolvido na patofisiologia de diversos acometimentos, como epilepsia, mal de Alzheimer, isquemia cerebral e demência microvascular. A guanosina tem demonstrado exercer influência no sistema glutamatérgico quando presente no meio extracelular, sendo liberada principalmente por células gliais após diferentes tipos de estimulação. A administração de guanosina, tanto *in vivo* quanto *in vitro* se mostrou capaz de exercer efeitos neurotróficos, neuromoduladores e neuroprotetores em situações de desequilíbrio do sistema glutamatérgico. No primeiro capítulo desta tese procuramos investigar o efeito da guanosina através da análise do sinal de EEG de ratos submetidos a modelo de convulsão por AQ. Mostramos que a guanosina consegue evitar a convulsão em cerca de metade dos animais e, associado a isto, também foi capaz de minimizar a supressão do ritmo teta ocorrida no período peri-ictal dos animais que convulsionaram. Porém, algum grau de perturbação da sincronia da atividade elétrica inter-hemisférica, bem como algum grau de alteração da onda teta, sugerem que mesmo animais protegidos tiveram alteração de sua fisiologia. No segundo capítulo, investigamos o efeito da guanosina em camundongos enquanto realizavam tarefa de campo aberto. Mostramos que a guanosina é capaz de diminuir a frequência de pico das oscilações teta durante o repouso, mas não durante a atividade locomotora, o que sugere possível modulação de uma via de sinalização intracelular iniciada por um receptor muscarínico M1 e associada à proteína-Gαq. No terceiro capítulo nós investigamos a modulação do metabolismo oxidativo de glicose e de glutamato em hipocampos isolados de camundongos. Mostramos que há um aumento na oxidação de glutamato de mesma ordem que o aumento da concentração extracelular, com uma ampla curva de dose-reposta. Podemos inferir portanto, que as vias responsáveis pela oxidação do glutamato provavelmente permanecem sem se tornar saturadas em condições fisiológicas. Em paralelo, parece ocorrer uma discreta diminuição do uso da glicose quanto maior a concentração extracelular de glutamato, presumidamente pelo uso do glutamato como substrato energético. Pode-se considerar que a oxidação de glutamato talvez tenha um papel de repor a energia celular dispendida num ambiente de excitabilidade celular bem como de poupar a glicose disponível. Com o uso de TFB-TBOA, os astrócitos hipocampais tiveram a recaptação de glutamato impedida, o que impactou significativamente na sua oxidação e evitou que a oxidação de glicose diminuísse. Com estes resultados, ficou mais evidente o papel primordialmente astrocitário na regulação da reserva global de glutamato e na modulação do sistema glutamatérgico. De uma maneira geral, esta tese traz contribuições ao entendimento do papel da guanosina na modulação do sistema glutamatérgico e na atividade elétrica cerebral, além de estabelecer parâmetros de metabolismo oxidativo que poderão ser utilizados para estudo de possíveis efeitos da guanosina e de novas estratégias neuroprotetoras.

ABSTRACT

(TORRES FV - ELECTROPHYSIOLOGICAL EFFECTS OF GUANOSINE UNDER NORMAL CONDITIONS AND IN A MODEL OF GLUTAMATERGIC HYPERSTIMULATION AND HIPPOCAMPAL METABOLISM) - Glutamate plays an essential role for trophic, developmental and aging events of central nervous system, as well as in cognition and memory consolidation. Imbalances of the glutamatergic system are involved in the pathophysiology of diseases, including epilepsy, Alzheimer, brain ischemia and microvascular dementia. Extracellular guanosine seems to modulate the glutamatergic system. It is released from glial cells due to different types of stimulation. Guanosine administration, both *in vivo* and *in vitro*, promotes neurotrophic, neuromodulatory and neuroprotective effects in contexts of glutamatergic system disturbance. In the first chapter of this work we investigate the effect of guanosine using EEG recordings from rats submitted to quinolinic-acid-induced seizures. We show that guanosine prevented seizures in half of the animals, and also partially prevented the EEG theta suppression seen in the peri-ictal period of seizing animals. Nevertheless, some degree of disturbance of the inter-hemispherical synchrony and some degree of theta suppression were still present, even in non-seizing animals that received guanosine, what suggests some alteration of its physiology. In the second chapter, we investigated the effect of guanosine in mice that performed Open Field Test. We show that theta peak frequency slow down when mice are at rest after guanosine administration, but remains unaltered during locomotor activity. This fact may suggest that guanosine acts in a M1-muscarinic receptor or in the associate pathway, an effect mediated by a Gαq-protein. In the third chapter we investigate the modulation of oxidative metabolism of glucose and glutamate in hippocampus isolated from mice. We show there is a proportional increase in the glutamate oxidation according its extracellular concentration, with a large dose-response curve. It suggests that the machinery involved probably does not saturate under physiological conditions. Furthermore, it seems to occur a slight reduction of glucose consumption as the glutamate oxidation rises up, presumably due to the use of glutamate as fuel. Maybe the glutamate oxidation play a role in replenishing energy spent in a excitatory medium and spare the glucose available. When we used TFB-TBOA, we prevent the uptake of glutamate from astrocytes and in consequence the glutamate oxidation in a large amount, and glucose oxidation remains at control level. Together these data reinforce the role of astrocytes in the regulation of the global pool of glutamate and in the modulation of glutamatergic system. Finally, this work makes contributions to the understanding of the role of guanosine over glutamatergic system and brain electrical activity, beyond establishing some parameters of oxidative metabolism able to be used for further experiments looking for mechanisms of action of guanosine and neuroprotective strategies.

LISTA DE ABREVIATURAS

ADP	Adenosina-5’-difosfato
Akt/PKB	Proteína cinase B
AMP	Adenosina-5’-monofosfato
AMPc	Adenosina-3’,5’-monofosfato-cíclico
ApoE	Apolipoproteína E
AQ	Ácido quinolínico
ATP	Adenosina-5’-trifosfato
Bcl-2	(Proteína) célula-B de linfoma 2
bFGF	Fator de crescimento fibroblástico básico
DNA	Ácido desoxiribonucléico
EAAC1	Transportador de aminoácido excitatório
EAAT1-5	Transportador de aminoácido excitatório 1 até 5
EEG	Eletroencefalograma
ERK1-2	MAP quinase 1-2, ou quinase regulada por sinal extracelular 1-2
GDP	Guanosina-5’-difosfato
GLAST	Transportador de glutamato e aspartato
GLT1	Transportador de glutamato 1
GMP	Guanosina-5’-monofosfato
GSK-3β	Glicogênio sintase quinase-3β
GTP	Guanosina-5’-trifosfato
i.c.v.	Intracerebroventricular
Il-1	Interleucina-1
i.p.	Intraperitoneal
LY294002	2-Morpholin-4-yl-8-phenylchromen-4-one
MAPK	Proteína cinase ativada por mitógeno
MK-801	(+)-5-metil-10,11-diidro-5H-dibenzo[a,d]ciclohepteno-5,10-imina
mRNA	Ácido ribonucléico mensageiro

mTLE	Epilepsia do lobo temporal mesial
NGF	Fator de crescimento neuronal
NMDA	N-metil-D-aspartato
PC12	Linhagem de células de feocromocitoma de ratos
p38 MAPK	Proteína cinase p38 ativada por mitógeno
PD98059	2’-amino-3’-metoxiflavona
PI3K	Fosfatidilinositol-3-quinase
PLC-β1	Proteína fosfolipase C-β1
PNP	Purina-nucleosídeo fosforilase
PTX	Toxina pertussis
SH-SY5Y	Linhagem de células de neuroblastoma humano
SNC	Sistema nervoso central
SB202190	4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)-1H-imidazole
SQ22536	9-(Tetrahydro-2-furanyl)-9H-purin-6-amine, 9-THF-Ade
TGFβ1	Fator de crescimento tumoral β1

APRESENTAÇÃO

Os resultados desta tese de doutorado estão apresentados sob a forma de artigos científicos. As seções Materiais e Métodos, Resultados, Discussão e Referências bibliográficas encontram-se nos próprios artigos.

Os itens Introdução, Discussão e Conclusões encontradas nesta tese apresentam interpretações e comentários gerais sobre todos os artigos científicos contidos neste trabalho. As Referências Bibliográficas referem-se somente às citações que aparecem nos itens Introdução e Discussão desta tese.

Detalhes técnicos mais precisos sobre a metodologia empregada em cada trabalho podem ser encontrados nos artigos científicos correspondentes.

1.1 INTRODUÇÃO

1.1.a. SISTEMA GLUTAMATÉRGICO E DISTÚRBIOS DO SNC

O glutamato é um dos aminoácidos não essenciais mais abundantes nos seres vivos e também o neurotransmissor mais abundante no SNC dos mamíferos, exercendo papel fundamental para que ocorram eventos tróficos, desenvolvimentais e de envelhecimento assim como para que haja consolidação da memória e cognição (Van Harreveld and Fifkova 1974, Ng, O'Dowd et al. 1997, Segovia, Porras et al. 2001, Izquierdo, Bevilaqua et al. 2006, Stevens 2008). No entanto, o desequilíbrio do sistema neurotransmissor glutamatérgico pode trazer efeitos danosos ao cérebro (Szatkowski and Attwell 1994, Huang, Sochocka et al. 1997, Allen, Karadottir et al. 2004, Maragakis and Rothstein 2006, Beart and O'Shea 2007, Herman and Jahr 2007, Tilleux and Hermans 2007), e vem sendo alvo de estudos por estar aparentemente envolvido com a patofisiologia de um rol de acometimentos: epilepsia (Meldrum 1994, Bradford 1995, Chapman, Nanan et al. 2000, Meldrum 2000, Moldrich, Chapman et al. 2003, Naylor 2010), isquemia cerebral (Choi and Rothman 1990, Choi 1992, Chao, Fei et al. 2010), traumatismo cranioencefálico (Fei, Zhang et al. 2007), Mal de Alzheimer (Boehm 2013), Doença de Huntington (Sepers and Raymond 2014), demência microvascular (Dirnagl, Iadecola et al. 1999, Segovia, Porras et al. 2001, Dong, Wang et al. 2009, Chao, Fei et al. 2010, Szydlowska and Tymianski 2010) e esclerose múltipla (Stojanovic, Kostic et al. 2014).

O glutamato tem sua ação exercida através de ativação de receptores ionotrópicos e metabotrópicos após ser liberado na fenda sináptica. É sabido que o

glutamato não é inativado enquanto permanece sem ser recaptado, sendo a recaptação realizada basicamente pelas células astrocitárias do SNC. Existem cinco classes de transportadores de glutamato já caracterizados em cérebro de mamíferos (Kanai and Hediger 1992, Pines, Danbolt et al. 1992, Storck, Schulte et al. 1992, Fairman, Vandenberg et al. 1995, Arriza, Eliasof et al. 1997)**.**

- GLAST ou EAAT1: localizado principalmente em astrócitos, é o principal transportador durante o desenvolvimento do SNC e no cerebelo mesmo na forma adulta (Furuta, Rothstein et al. 1997).

- GLT-1 ou EAAT2: localizado principalmente em astrócitos, é responsável por cerca de 90% da atividade de transporte de glutamato no cérebro adulto, principalmente no prosencéfalo (Danbolt 1994, Rothstein 1996, Mallolas, Hurtado et al. 2006, Furness, Dehnes et al. 2008).

- EAAC1 ou EAAT3: localizado principalmente em neurônios hipocampais, cerebelares e dos núcleos da base (Furuta, Martin et al. 1997).
- EAAT4: localizado principalmente em células de Purkinje do cerebelo (Furuta, Martin et al. 1997, Furuta, Rothstein et al. 1997).
- EAAT5: localizado principalmente em fotorreceptores e células bipolares da retina ocular (Pow and Barnett 2000).

A atividade dos transportadores de glutamato parece estar sujeita à regulação e plasticidade (Danbolt 2001, Tzingounis and Wadiche 2007, Eulenburg and Gomeza 2010) e estar modificada em situações de injúria cerebral (Danbolt 1994, Furuta, Rothstein et al. 1997, Tzingounis and Wadiche 2007). Atualmente, diversos estudos já têm considerado que a disfunção de transportadores de glutamato pode ser o evento inicial ou parte de uma cascata implicada na patologia de doenças cerebrais agudas

e crônicas (Maragakis and Rothstein 2004, Robinson 2006, Moussa, Rae et al. 2007, Sheldon and Robinson 2007, Tzingounis and Wadiche 2007, Stevens 2008).

Os transportadores de glutamato parecem ser modulados em níveis da transcrição do DNA, processamento do mRNA, síntese protéica e pós-traducional (Danbolt 2001, Tzingounis and Wadiche 2007, Eulenburg and Gomeza 2010). A modulação pós-traducional pode envolver modulações alostéricas, por exemplo, pelo ácido araquidônico ou pelo Zn+ (Vandenberg, Ju et al. 2004), ou pela translocação dos transportadores entre a membrana plasmática e compartimentos intracelulares o que pode envolver vias de sinalização intracelular, incluindo ativação de proteínas cinases e fosfatases (Davis, Straff et al. 1998, Duan, Anderson et al. 1999, Lortet, Samuel et al. 1999, Gegelashvili, Dehnes et al. 2000, O'Shea 2002, Robinson 2002)

1.1.b. SINAPSE TRIPARTITE E O PAPEL DO ASTRÓCITO

O termo sinapse tripartite se refere ao conceito de que a transmissão de informação dentro do SNC não se dá apenas entre um terminal axonal e outro terminal dendrítico (ou terminais pré e pós sinápticos respectivamente), mas que estes terminais teriam uma triangulação com uma ramificação astrocitária. Esta idéia vem cada vez mais modificando a visão de integração entre neurônios e glia, bem como a de integração da glia com a modulação e participação na transmissão da atividade sináptica. Têm-se cada vez mais atribuído funções aos astrócitos, grupo celular que inicialmente era parte da mera "cola cerebral". Os astrócitos exercem papel fundamental na qualidade da transmissão sináptica uma vez que conseguem manter o nível extracelular de glutamato baixo e otimizar a razão sinal/ruído. Além disso, acumulam funções estruturais (criação da microarquitetura, regulação do volume extracelular, manutenção da homeostase iônica - tamponamento do potássio

extracelular-, regulação do pH extracelular), funções vasculares (formação da barreira hematoencefálica - que evita inclusive a entrada de glutamato presente na corrente sanguínea para o líquor - e regulação da microcirculação/tônus vascular), funções tróficas (suporte metabólico, defesa antioxidante, crescimento axonal, neurogênese), funções moduladoras e plásticas (liberação de neurotransmissores) (Volterra and Meldolesi 2005, Tzingounis and Wadiche 2007, Andersson and Hanse 2010, Stipursky, Romao et al. 2011, Stipursky, Spohr et al. 2012).

Aparentemente, a complexidade da integração astrócito-neuronal acompanhou a evolução das espécies e as necessidades de aprimoramento das funções cognitivas necessárias à sobrevivência. Nas sanguessugas a razão numérica astrócito-neuronal é 1:25, nos roedores 1:3 e nos humanos 3:2; um crescimento exponencial que não parece envolver apenas funções de suporte (Banaclocha 2007, Matyash and Kettenmann 2010). No cérebro humano, os astrócitos parecem conseguir exercer a função de moderador/concentrador (*hub*) local ou central da transmissão de informações e parecem exercer papel na constituição de sensações subjetivas conscientes ao invés da informação explícita que seria atribuída à transmissão neuronal (Oberheim, Wang et al. 2006, Pereira and Furlan 2010). Além disso, a morfologia dos astrócitos parece ter evoluído paralelamente, com os astrócitos presentes em humanos mostrando-se maiores, mais largos e com mais subtipos em relação a roedores e até mesmo outros primatas, o que pode ter sido responsável pelo aumento da capacidade computacional do cérebro humano (Oberheim, Takano et al. 2009). Estima-se que um astrócito protoencefálico humano realize contato com um número de sinapses que pode estar entre 270 000 e 2 000 000. Os fatos aqui levantados já nos fazem pensar que a sinapse tripartite foi apenas o início da compreensão da interação

astrócito-neuronal e do processamento cerebral de informações, uma vez que o termo nos dá inicialmente uma idéia de correlação numérica equivalente entre neurônios pré e pós sinápticos e astrócitos.

Em meio às diversas funções exercidas pelos astrócitos já demonstradas, há um importante papel na integração metabólica cerebral (Sonnewald, Westergaard et al. 1997, Voutsinos-Porche, Bonvento et al. 2003, Hertz, Peng et al. 2007, Belanger, Allaman et al. 2011, Pellerin and Magistretti 2011, Kreft, Bak et al. 2012). Classicamente é postulado que o glutamato é recaptado em sua grande maioria pelos transportadores astrocitários GLT-1 e GLAST, que tem sua presença principalmente nos processos astrocitários perissinápticos (Chaudhry, Lehre et al. 1995, Robinson 1998, Danbolt 2001, Sheldon and Robinson 2007). GLT-1 é o principal transportador no prosencéfalo enquanto GLAST é o principal transportador no cerebelo. Trabalhos utilizando astrócitos, sinaptossomas e fatias de cérebro já analisaram o destino metabólico do glutamato com resultados controversos (Yu, Schousboe et al. 1982, Waniewski and Martin 1986, Farinelli and Nicklas 1992, Rao and Murthy 1993, Yudkoff, Nelson et al. 1994, McKenna, Sonnewald et al. 1996, Sonnewald, Westergaard et al. 1997, Skytt, Madsen et al. 2010, El Hage, Conjard-Duplany et al. 2011), chegando-se à conclusão de que aparentemente as rotas de destino do glutamato são modificadas de acordo com a concentração de glutamato extracelular, com altas concentrações favorecendo o fluxo para o Ciclo de Krebs em razão exponencial com o tempo enquanto o aumento da velocidade de captação parece aumentar linearmente (McKenna, Sonnewald et al. 1996, Sonnewald, Westergaard et al. 1997, McKenna 2007, Genda, Jackson et al. 2011, Bauer, Jackson et al. 2012). Discussão de fatos contraditórios foram elegantemente revisados recentemente por Gerald Dienel (Dienel 2012, Dienel 2013).

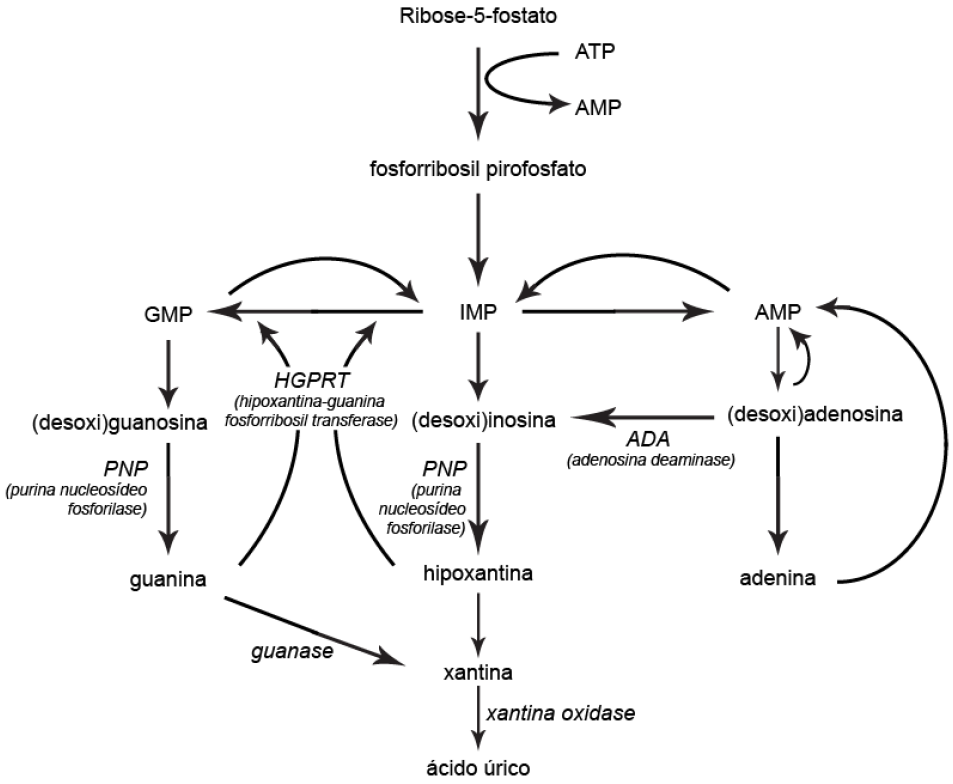
1.1.c. SISTEMA PURINÉRGICO

Neurônios e células gliais liberam nucleotídeos e nucleosídeos derivados das bases púricas adenina e guanina tanto na homeostase quanto em resposta a estímulos. As purinas podem ser classificadas em derivados da adenina (ATP, ADP, AMP, adenosina, adenina) e derivadas da guanina (GTP, GDP, GMP, guanosina e guanina) além dos metabólitos diretos dos derivados da adenina e da guanina: inosina, xantina e hipoxantina. ATP e GTP se co-localizam em vesículas sinápticas de neurônios adrenérgicos, purinérgicos e colinérgicos e são liberados na fenda sináptica após despolarização, por exocitose assim como os neurotransmissores. Nos astrócitos, ATP é liberado provavelmente através de canais de glicoproteína-P. Ambos, neurônios e células gliais possuem sistemas transportadores de nucleosídeos que exercem papel na regulação do nível extracelular de nucleosídeos de purinas. A concentração extracelular de purinas depende de fatores que incluem a quantidade de purinas liberada, o volume extracelular local, mecanismos de recaptação que as reinternalize nas células e da presença de enzimas extracelulares que metabolizam purinas.

Guanosina e guanina são prontamente interconversíveis pela enzima Purina Nucleosídeo Fosforilase (PNP). A Guanina também pode ser transformada irreversivelmente pela guanina deaminase (guanase) originando xantina, que por sua vez pode ser convertida a ácido úrico pela xantina oxidase (Figura 1). A atividade da guanase varia de acordo com a estrutura encefálica envolvida, estando bem presente no hipocampo e no bulbo olfatório, porém em pequena quantidade na ponte, bulbo e cerebelo. Grandes variações na atividade de uma enzima cerebral geralmente sugere que esta está associada com o metabolismo de

neurotransmissores, tanto que no passado se sugeriu que a guanosina fosse um neurotransmissor (McGeer, Singh et al. 1987).

Figura 1. Rotas metabólicas das purinas



As bases púricas derivadas da adenosina já se mostraram como tendo papéis importantes na forma de hormônios, neurotransmissores, fatores tróficos e como moeda energética. No entanto, as bases derivadas da guanina, particularmente a guanosina começaram a ter seus mecanismos de ação e seus diversos papéis explicados há menos tempo, ainda havendo bastantes questões a serem elucidadas.

1.1.d. EFEITOS DA GUANOSINA NO SNC

A guanosina participa como componente estrutural e regulatório nas células, mas também existe no meio extracelular (Ciccarelli, Di Iorio et al. 1999). No cérebro, a origem das bases derivadas de guanina que estão presentes no meio extracelular

é primordialmente glial, provavelmente em grande parte nucleotídeos que são clivados pelas ectonucleotidases até guanosina extracelular (Ciccarelli, Ballerini et al. 2001). A estimulação de culturas de astrócitos por privação de oxigênio e glicose, ou por campos elétricos faz aumentar a concentração extracelular de guanosina (Ciccarelli, Di Iorio et al. 1999). Além disso, dano cerebral focal causa elevação nos níveis extracelulares de guanosina que persistem por até uma semana. A guanosina e os nucleotídeos da guanina exercem papéis não apenas tróficos, mas também neuromoduladores principalmente quando se trata de sistema glutamatérgico, como mostram as tabelas 1 e 2 respectivamente.

Tabela 1. Principais efeitos extracelulares da guanosina

Efeitos Neurotróficos	
Estimula proliferação de astrócitos *in vitro*	(Kim et al. 1991)
Induz a proliferação de células gliais, crescimento axonal e dendrítico em neurônios	(Gysbers and Rathbone 1996)
Induz efeitos tróficos em células neurais (astrócitos)	(Rathbone et al. 1999)
Estimula a síntese e liberação de fatores tróficos do diversos tipos, como o NGF	(Gysbers and Rathbone 1992; Gysbers and Rathbone 1996) (Middlemiss et al. 1995) (Rathbone et al. 1998) (Ciccarelli et al. 2000)
Estimula a síntese de bFGF mRNA em astrócitos	(Gysbers and Rathbone 1992) (Middlemiss et al. 1995) (Ciccarelli et al. 2001)
Estimula a sintese de NGF e TGFβ1 de astrócitos	(Ciccarelli et al. 2001)
Estimula liberaração de Il-1 pela microglia e, consequentemente, a proliferação astrocitária	(Ciccarelli et al. 2000)
Melhora a locomoção e a remielinização em modelo de trauma medular	(Jiang et al. 2003)
Estimula proliferação de células tronco neurais	(Su et al. 2013)
Efeitos Neuromoduladores	
Estimula a liberação de derivados da adenina, incluindo a adenosina	(Ciccarelli et al. 2000)
Aumenta a recaptação de glutamato pelos astrócitos	(Frizzo et al. 2001) (Frizzo et al. 2002)

Previne a diminuição da captação de glutamato induzida pelo AQ	(Vinade et al. 2005)
Aumenta a expressão de ApoE em astrócitos	(Ballerini et al. 2006)
Efeitos Neuroprotetores	
Previne a diminuição da recaptação de glutamato induzida por lesão hipóxico-isquêmica	(Moretto et al. 2005)
Protege fatias cerebrais expostas a hipóxia/hipoglicemia	(Frizzo et al. 2002)
Previne toxicidade induzida por NMDA em neurônios	(Caciagli et al. 2000)
Previne apoptose induzida por β-amilóide	(Pettifer et al. 2004)
Protege contra modelo de Parkinson in vitro	(Giuliani et al. 2012)
Inibe apoptose em astrócitos	(Di Iorio et al. 2002) (Di Iorio et al. 2004)
Inibe vias inflamatórias de serem ativadas por privação de oxigênio e glicose *in vitro*	(Dal-Cim et al. 2013)
Protege contra o estresse oxidativo causado em modelo de isquemia cerebral por termocoagulação	(Hansel et al. 2014)
Efeitos Comportamentais	
Evita convulsões induzidas por diferentes agentes glutamatérgicos	(Lara et al. 2001)
Induz efeito ansiolítico no comportamento de ratos	(Vinade et al. 2003)
Induz amnésia em ratos	(Roesler et al. 2000)
Atenua a hiperlocomoção induzida por MK-801	(Tort et al. 2004)

Tabela 2. Principais efeitos extracelulares dos nucleotídeos da guanina

Efeitos Neurotróficos	
GTP induz a proliferação de astrócitos	(Ciccarelli et al. 2000)
GTP induz síntese e liberação de fatores tróficos	(Middlemiss et al. 1995)
GTP estimula a proliferação de diversos tipos de células	(Rathbone et al. 1992)
Estimulam a proliferação de astrócitos	(Kim et al. 1991)
Efeitos Neuromoduladores	
Inibe a ligação do ácido caínico	(Souza and Ramirez 1991)
Inibe a ligação do glutamato e seus análogos	(Rubin et al. 1996)
Inibe respostas celulares a agentes glutamatérgicos	(Burgos et al. 1998)
Estimula a recaptação de glutamato pelos astrócitos	(Frizzo et al. 2003)

Estimula a recaptação de glutamato por vesículas sinápticas	(Tasca et al. 2004)
GMP evita a liberação de glutamato induzida por AQ em sinaptossomas	(Tavares et al. 2005)
Efeitos Neuroprotetores	
GMP evita a perda de viabilidade celular induzida pelo glutamato	(Molz et al. 2005)
GMP evita a perda de viabilidade celular induzida por hipóxia	(Oliveira et al. 2002)
GMP evita morte neuronal induzida por AQ	(Malcon et al. 1997)
Efeitos Comportamentais	
GMP Evita convulsões induzidas por AQ em roedores	(Schmidt et al. 2005)
GMP induz amnésia em ratos	(Saute et al. 2006)
GMP reverte o efeito facilitador do glutamato na memória em ratos	(Rubin et al. 1996)

Pelos resultados obtidos até o momento é evidente o papel da guanosina e seus nucleotídeos. Porém, ainda há muitas perguntas sobre o mecanismo de ação da guanosina e seus derivados, uma vez que ainda não se sabe através de qual estrutura é desencadeada a ativação das vias de sinalização intracelular já detectadas. O que foi mostrado até o momento é que existem papéis da guanosina e de seus nucleotídeos que são independentes do envolvimento do sistema adenosinérgico e que permanecem acontecendo mesmo com o bloqueio do transporte de purinas para o meio intracelular (Kim, Rathbone et al. 1991, Gysbers and Rathbone 1992, Muller and Scior 1993, Gysbers and Rathbone 1996, Ciccarelli, Di Iorio et al. 2000).

1.1.e. EVIDÊNCIAS ENVOLVENDO MECANISMOS DE AÇÃO DA GUANOSINA

A guanosina parece promover o aumento do AMPc intracelular em astrócitos (Rathbone, Middlemiss et al. 1991) e células PC12 (Gysbers and Rathbone 1996), inibidos parcialmente por SQ22536, um inibidor da adenilato ciclase (Gysbers and Rathbone 1996) , o que sugere que seus efeitos são mediados por mecanismos

dependentes e não-dependentes do AMPc. Os mecanismos ativados pela guanosina que são dependentes de AMPc supostamente são responsáveis por ativar a cascata da via MAPK e talvez outras proteínas cinases (Rathbone, Middlemiss et al. 1991).

A guanosina passou a despertar ainda mais a curiosidade dos grupos que a investigam a partir do momento que foram detectados efeitos biológicos devido à sua presença extracelular, e que não seriam explicados por uma liberação secundária ou em paralelo de bases derivadas da adenina, nem por ativação de purinoceptores da adenina. A procura de sítios distintos de ligação da guanosina à superfície celular então passou a ser foco de pesquisas (Ciccarelli, Ballerini et al. 2001, Traversa, Bombi et al. 2002). Aparentemente os supostos receptores ativados pela guanosina apresentam características de receptores ligados à proteína-G. Dados que sustentam esta hipótese são a inibição do efeito da guanosina na apoptose induzida por β-amilóide em células SH-SY5Y, de neuroblastoma humano quando há pré-tratamento por LY294002, um inibidor da PI3K, e por PD98059, um inibidor da MEK (Dal-Cim, Molz et al. 2012). A guanosina extracelular também promove a fosforilação de Akt/PKB, o que foi evitado também por pré-tratamentos com LY294002 e PD98059 (Pettifer, Kleywegt et al. 2004).

Em astrócitos expostos à staurosporina, o efeito antiapoptótico da guanosina foi antagonizado por pré-tratamento com PTX e também por SB202190, um inibidor da via p38 MAPK. A guanosina também mostrou efeitos sobre a atividade de alvos da via PI3K/Akt/PKB, inibindo os efeitos da enzima pró-apoptótica GSK-3β e promovendo aumento do mRNA e da expressão proteica de Bcl-2, cujo efeito é antiapoptótico (Di Iorio, Ballerini et al. 2004).

Recentemente foi mostrado também que a guanosina aumenta a atividade dos proteassomas em células SH-SY5Y expostas à β-amilóide através da estimulação

das vias de sobrevivência PI3K/Akt/GSK3β e ERK1/2/MAPK (Tarozzi, Merlicco et al. 2010).

Em suma, os efeitos da guanosina são suprimidos por PTX, LY294002 e Wortmannin (inibidores da PI3K) e PD98059 (um inibidor da MEK), bem como por inibidores MAPK, o que sugere que a guanosina age sobre um receptor de superfície celular acoplado a uma proteína-Gi (Gysbers and Rathbone 1992, Rathbone, Middlemiss et al. 1998, Rathbone, Middlemiss et al. 1999, Ciccarelli, Ballerini et al. 2001).

1.1.f. RITMOS DO EEG

As frequências usualmente predominantes no EEG de humanos ou roedores variam de 1 a 30 Hz. Em geral baixas freqüências significam estados menos responsivos e vice-versa. A amplitude das ondas detectadas no EEG indica o quanto de atividade elétrica sincrônica está ocorrendo na área abaixo do eletrodo naquele momento. Por outro lado, se a amplitude for baixa, isto significa que os neurônios estão disparando de forma assíncrona. Em outras palavras, as ondas rítmicas do EEG refletem a tendência de parte do cérebro em gerar oscilações coletivas (Pedley and Traub 2003). Este ritmo pode ser tanto um padrão espontâneo como o ritmo alfa e os fusos de sono, como ondas rápidas induzidas por medicamentos, ou atividade anormal como pontas ictais ou trens de ondas lentas. A forma extrema de sincronicidade da atividade neuronal, que leva a um estado de hiperexcitabilidade, é tratada como crise epiléptica.

Quando utilizamos o EEG de ratos para prospecção de drogas psicoativas, é importante considerarmos que a definição das bandas de frequências do EEG

mostram certa variabilidade de acordo com o autor e entre a literatura de humanos e roedores. A definição clássica de banda teta é entre 4 a 8 Hz (Duzel, Penny et al. 2010, Park, Lee et al. 2014, Vecchio, Miraglia et al. 2014) e alfa entre 8 a 12 Hz. Esta é tipicamente empregada na literatura humana. Na literatura referente a roedores, porém, a banda teta é tipicamente definida por um espectro mais amplo (considerando também frequências um pouco mais rápidas) que em humanos. Diversos artigos consideraram banda teta como oscilações entre 4 a 12 Hz (ou 5 a 12 Hz) em roedores (Fellous and Sejnowski 2000, Whittington and Traub 2003, Dugladze, Vida et al. 2007, Economo, Martinez et al. 2014). Paralelamente, vários outras publicações definiram teta de maneira um pouco diferente, mas ainda mais abrangente que a de humanos, com limite superior de 10 Hz ao invés de 12 Hz (Buzsaki and Draguhn 2004, Arabadzisz, Antal et al. 2005, Jacobs 2014).

1.1.g. EEG E AÇÃO DE DROGAS NO SNC

A análise do EEG pode ser considerada uma ferramenta para estudo de efeitos de drogas psicotrópicas no estado funcional do cérebro (Herrmann and Schaerer 1990). As ondas cerebrais detectadas pelo EEG estão associadas à coordenação de populações neuronais envolvidas no processamento de informações e modulação do comportamento (Engel, Fries et al. 2001, Buzsaki and Draguhn 2004, Sirota, Montgomery et al. 2008).

As alterações de EEG induzidas por fármacos mais comumente são o aumento das oscilações beta e um leve aumento nas oscilações teta - ambas podendo ser provocadas pela administração de benzodiazepínicos em doses

ansiolíticas (Blume 2006). No entanto, doses um pouco maiores de benzodiazepínicos e de antagonistas NMDA como o MK-801 estão associadas a lentificação das ondas teta e sedação e hipnose (Scheffzuk, Kukushka et al. 2013).

Em camundongos, os registros a nível cortical realizados em topografia imediatamente acima do hipocampo são capazes de traduzir de maneira consideravelmente fidedigna o comportamento das oscilações hipocampais, principalmente nas frequências mais proeminentes. Observa-se alto índice de coerência entre sinais colhidos simultaneamente de eletrodos corticais e hipocampais segundo observações nossas (Torres F.V., dados não publicados) e de trabalhos já publicados (Vezzani, Ungerstedt et al. 1985, Brankack, Kukushka et al. 2010). Portanto, o uso de eletrodos epidurais em topografia acima do hipocampo de camundongos é uma ferramenta que pode trazer informações do funcionamento hipocampal de maneira menos invasiva e com metodologia menos complexa do que o uso de eletrodos de profundidade. Essas características fizeram com que considerássemos inicialmente eletrodos epidurais mais adequados do que eletrodos de profundidade para trabalhos de prospecção.

1.1.h. RITMO TETA E NAVEGAÇÃO

A frequência de pico na banda teta parece estar associada à velocidade de locomoção (Slawinska and Kasicki 1998), embora a associação pareça ser fraca e por isso nem sempre detectada (Whishaw and Vanderwolf 1973, Czurko, Hirase et al. 1999). Já foi sugerido que o ritmo teta hipocampal pudesse ser afetado por diferentes classes de drogas ansiolíticas (McNaughton, Kocsis et al. 2007).

As oscilações teta que acompanham a locomoção parecem não ser afetadas pela administração de agonistas muscarínicos, enquanto as oscilações que aparecem durante o repouso (espontâneo ou induzido por drogas) conseguem ser suprimidas por estes (Kramis, Vanderwolf et al. 1975, Leung 1985, Bland 1986, Leung 1998, Shin and Talnov 2001, Buzsaki 2002, Shin 2002, McNaughton, Kocsis et al. 2007).

1.2. OBJETIVO GERAL

Considerando i) a importância dos distúrbios do sistema glutamatérgico nos insultos agudos e crônicos do cérebro; ii) o papel neuroprotetor da guanosina extracelular e sua interferência no sistema glutamatérgico demostrada até o momento, incluindo sua ação anticonvulsivante já descrita comportamentalmente; iii) o grande envolvimento astrocitário na modulação do sistema glutamatérgico e na regulação do meio extracelular e da concentração sináptica do glutamato, o objetivo geral da presente tese de doutorado foi analisar registros de EEG buscando detectar efeitos da guanosina per se e frente à uma situação de hiperexcitabilidade glutamatérgica (crise convulsiva) que pudesse ser evitado pela guanosina, bem como padronizar técnica de mensuração do metabolismo oxidativo de glicose e glutamato em hipocampos de camundongos, com perspectiva de prospecção de um possível efeito da guanosina no metabolismo oxidativo da glicose e do glutamato.

1.3. OBJETIVOS ESPECÍFICOS

- Verificar se o efeito comportamental da guanosina e do MK-801 está associado à diminuição de registros epileptiformes.
- Verificar a influência da guanosina administrada sistemicamente no EEG de camundongos, com análise simultânea da atividade de locomoção.
- Verificar a influência da concentração extracelular de glutamato, na presença ou não do inibidor dos transportadores glutamatégicos astrocitários TFB-TBOA, no metabolismo oxidativo da glicose e do glutamato em hipocampo isolado de camundongos

- Verificar a influência da concentração extracelular de glutamato, na presença ou não do inibidor dos transportadores glutamatégicos astrocitários TFB-TBOA, na viabilidade celular em hipocampo isolado de camundongos

PARTE 2

CAPITULO I

Electrophysiological effects of guanosine and MK-801 in a quinolinic acid-induced
seizure model

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Artigo puglicado no periódico *Experimental Neurology*

Experimental Neurology 221 (2010) 296–306

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Electrophysiological effects of guanosine and MK-801 in a quinolinic acid-induced seizure model

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ARTICLE INFO

Article history:
Received 28 August 2009
Revised 21 October 2009
Accepted 14 November 2009
Available online 4 December 2009

Keywords:
EEG
Seizure
Epilepsy
Theta
Gamma
Neuroprotection
AED

ABSTRACT

Quinolinic acid (QA) is an *N*-methyl-D-aspartate receptor agonist that also promotes glutamate release and inhibits glutamate uptake by astrocytes. QA is used in experimental models of seizures studying the effects of overstimulation of the glutamatergic system. The guanine-based purines (GBPs), including the nucleoside guanosine, have been shown to modulate the glutamatergic system when administered extracellularly. GBPs were shown to inhibit the binding of glutamate and analogs, to be neuroprotective under excitotoxic conditions, as well as anticonvulsant against seizures induced by glutamatergic agents, including QA-induced seizure. In this work, we studied the electrophysiological effects of guanosine against QA-induced epileptiform activity in rats at the macroscopic cortical level, as inferred by electroencephalogram (EEG) signals recorded at the epidural surface. We found that QA disrupts a prominent basal theta (4–10 Hz) activity during peri-ictal periods and also promotes a relative increase in gamma (20–50 Hz) oscillations. Guanosine, when successfully preventing seizures, counteracted both these spectral changes. MK-801, an NMDA-antagonist used as positive control, was also able counteract the decrease in theta power; however, we observed an increase in the power of gamma oscillations in rats concurrently treated with MK-801 and QA. Given the distinct spectral signatures, these results suggest that guanosine and MK-801 prevent QA-induced seizures by different network mechanisms.

Introduction

Quinolinic acid-induced seizures is one of the variety of models in rodents for epilepsy research (Bradford, 1995; Stone, 2001). These models have improved our comprehension about the pathophysiology of epilepsy and anticonvulsant drug effects (Bradford, 1995; Lewis et al., 1997). Quinolinic acid (QA) is a product of tryptophan metabolic route, and acts as an *N*-methyl-D-aspartate (NMDA) receptor agonist, with indirect action as a glutamate releaser and inhibitor of astrocytic glutamate uptake (Connick and Stone, 1988; de Oliveira et al., 2004; Stone, 2001; Tavares et al., 2002, 2005). Given these characteristics, QA is one of the substances used in models studying the effects of overstimulation of the glutamatergic system in the brain. QA has also been suggested to be a neurotoxic endogenous substance involved in the etiology of epilepsy (Nakano et al., 1993), as well as in other diseases like Huntington disease (Ramaswamy et al., 2007) and HIV-associated dementia (Guillemin et al., 2005).

The guanine-based purines (GBPs), namely the nucleotides GTP, GDP and GMP, and the nucleoside guanosine have been shown to modulate the glutamatergic system when administered *in vivo* (Lara et al., 2001; Roesler et al., 2000; Schmidt et al., 2000, 2005, 2007, 2008, 2009; Tort et al., 2004; Vinade et al., 2005). Although the exact mechanisms of action underlying these effects remain unclear, they do not seem to involve a direct modulation of G-proteins (reviewed in Schmidt et al., 2007). GBPs were shown to inhibit the binding of glutamate and analogs (Baron et al., 1989; Paas et al., 1996; Paz et al., 1994), to be neuroprotective under excitotoxic conditions (Frizzo et al., 2002; Malcon et al., 1997; see also Ciccarelli et al., 2001), as well as anticonvulsant against seizures induced by glutamatergic agents, including QA-induced seizures (de Oliveira et al., 2004; Lara et al., 2001; Schmidt et al., 2000, 2005). Of note, the effects of some GBPs have been shown to depend on their conversion to guanosine (Saute et al., 2006; Schmidt et al., 2005; Soares et al., 2004). In line with these anti-glutamatergic effects, it has been shown that guanosine stimulates astrocytic glutamate uptake (Frizzo et al., 2001, 2003; Frizzo et al., 2002), which is the main mechanism of glutamate removal from the synaptic cleft (Anderson and Swanson, 2000; Danbolt, 2001; Beart and O'Shea, 2007).

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0014-4886/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.expneurol.2009.11.013

Although the behavioral effects of guanosine in preventing QA-induced seizures have been well described (de Oliveira et al., 2004; Lara et al., 2001; Schmidt et al., 2000, 2005), little is known about its electrophysiological effects in the brain. In fact, neither the electrophysiological effects of QA nor guanosine when administered alone have been well characterized to date. In this work, we sought to determine the electrophysiological effects of guanosine against QA-induced epileptiform activity at the macroscopic level. We have thus recorded and analyzed epidural electroencephalogram (EEG) recordings of rats under appropriate treatment conditions. We found that QA disrupted a prominent basal theta (4–10 Hz) activity during peri-ictal periods, and that guanosine, when successfully preventing seizures, counteracted this effect. We also observed that MK-801, a known NMDA-antagonist used as positive control, presented different spectral effects than guanosine in rats concurrently treated with QA.

Materials and methods

Animals

Forty male adult Wistar rats weighting 230–280 g were used. Animals were kept in temperature-regulated room (22 ± 1 °C), on a 12 h light/12 h dark cycle (light on at 7:00 am), one per cage, with food and water *ad libitum*. Our institutional protocols for experiments with animals ("Guidelines for Animal Care"), designed to avoid suffering and limit the number of animals sacrificed, were followed throughout.

Chemicals

Guanosine and quinolinic acid were obtained from Sigma Chemicals (St. Louis, MO, USA). 5-methyl-10-11-dihydro-5Hdibenzo [a,b]cyclohepta-5-10-imine maleate (MK-801) was obtained from RBI-Research Biochemicals International (Natick, MA, USA). Guanosine was prepared in NaOH 10 μM (buffered to pH 7.4) and the concentration was limited to 100 mM due to its poor water solubility. The anesthetic ketamine was obtained from Vetbrands (Jacareí, SP, Brazil) and xylazine was obtained from Coopers Brasil Ltda (Cotia, SP, Brazil).

Surgical procedure

Animals were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) i.p. The head of the animal was fixed in a stereotaxic instrument, and the skin covering the skull was cut with a 3-cm-long rostro-caudal incision in the midline. After exposure of the skull bone surface, a 27-gauge 9-mm guide cannula was unilaterally placed at 0.9 mm posterior to bregma, 1.5 mm right from the midline and 1.0 mm above the right lateral brain ventricle. The cannula was implanted 2.6 mm ventral to the superior surface of the skull through a 2-mm hole made in the cranial bone. In addition, five stainless steel screw electrodes (1.0 mm diameter) were placed over the dura mater through holes in the skull bone made with a dental drill. Four electrodes were used as positive electrodes (2.0 mm lateral, right or left, 1.0 mm anterior or 5.0 mm posterior to bregma). The reference (negative) electrode was placed at 4 mm anterior and 2.0 mm right from the midline. The positioning of the cannula and electrodes was fixed with dental acrylic cement, and a screw used for fixation of the dental acrylic helmet to the bone was used as ground.

Experimental design

One week after the surgical procedure, rats were (individually) transferred to an observation cage (Plexiglas chambers). Electrodes were connected to a digital data-acquisition system (Nihon-Kohden, Japan). After an accommodation period of 10 min, a basal EEG activity was recorded for 5 min. After this, rats were pretreated i.p. with one of these: guanosine (10 ml/kg, 0.75 mg/ml), NaCl 0.09% (10 ml/kg), MK-801 (0.5 mg/kg), and had their EEG recorded for 30 min. Next, the animals received an i.c.v. injection of QA (4 μl, 9.2 mM); for this, animals were gently hand-restrained and infusions were made using a 30-gauge injection cannula that was inserted into the implanted guide cannula and connected by a polyethylene tube to a 5-μl Hamilton microsyringe. The animals were then submitted to further EEG monitoring until 10 min after the i.c.v. injection. During this period, rats were observed for the occurrence of wild running, clonic, tonic or tonic–clonic seizures lasting more than 5 s. Animals not displaying seizures during these 10 min were considered "protected". Immediately after this observation, 2% methylene blue was injected i.c.v, followed by anesthesia and sacrifice by decapitation. Brain was sliced to determine the location of the cannula tip, and animals without dye in the lateral brain ventricle were discarded. The epidural EEG recordings were filtered at 0.01–100 Hz and digitally stored at 1 kHz sampling resolution in a computer hard drive for off-line analysis.

Data analysis

All analyses were done using built in and custom written routines in MATLAB (Mathworks, Inc). Both the power and coherence spectra were estimated by means of the Welch periodogram method using a 50% overlapping Hamming window with a length of 1024 points (i.e. 1.024 s), which were obtained using the *pwelch* and *mscohere* functions, respectively, from the Signal Processing Toolbox. These analyses were performed in EEG epochs without motor seizures (i.e., peri-ictally for seizing animals). The time–frequency decompositions used 1024 points sliding windows with 50% overlap, which was obtained using the *spectrogram* function from the Signal Processing Toolbox. The filtering was done by means of a linear finite impulse response (FIR) filter, which was obtained using the *eegfilt* routine from the EEGLAB toolbox (Delorme and Makeig, 2004). The phase time series of a filtered signal was computed from the Hilbert transform, which was obtained using the *hilbert* routine from the Signal Processing Toolbox. The phase difference $\Delta\phi$ between two signals was obtained by using the formula $\Delta\phi = arg(exp(i(\phi_1 - \phi_2)))$, where ϕ_1 and ϕ_2 correspond to the phases of signals 1 and 2, respectively. Signals that were too noisy or that contained too much movement artifact were excluded from the analysis. These were inferred by visual inspection of the traces along with the presence of abnormally high power in lower frequencies (0–3 Hz).

Statistical analysis

Comparisons of coherence and power values were done using a *t*-test or ANOVA followed by Tukey's test, as appropriate. Fisher's exact test was used to compare proportions. A value of $p<0.05$ was considered statistically significant.

Results

Basal epidural EEG traces exhibit prominent theta oscillations

As shown in Fig. 1, we found prominent theta (4–10 Hz) oscillations in the epidural EEG traces of the animals during the basal period, i.e., before the administration of any drug. Such oscillations could be detected by simple visual inspection (Fig. 1A) and were apparently more pronounced in the posterior electrodes (Fig. 1B; but read below). Fig. 1C shows a representative time–frequency decomposition obtained during the basal period, which depicts a clear, relatively steady theta rhythm. Similar to the power spectrum, the coherence spectra between electrodes also exhibited a clear peak at the theta band (Fig. 1D), which was also apparently more pronounced between the posterior electrodes. However, we note that

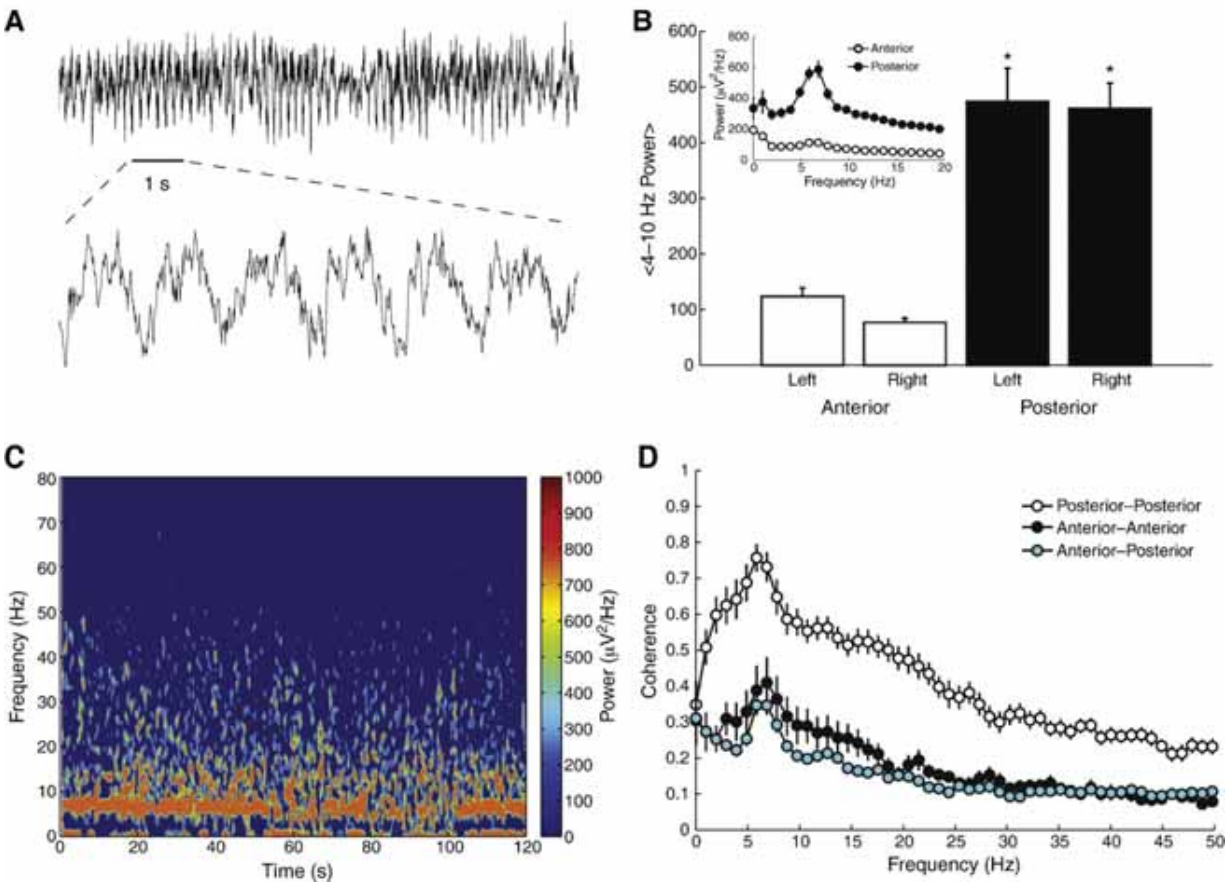


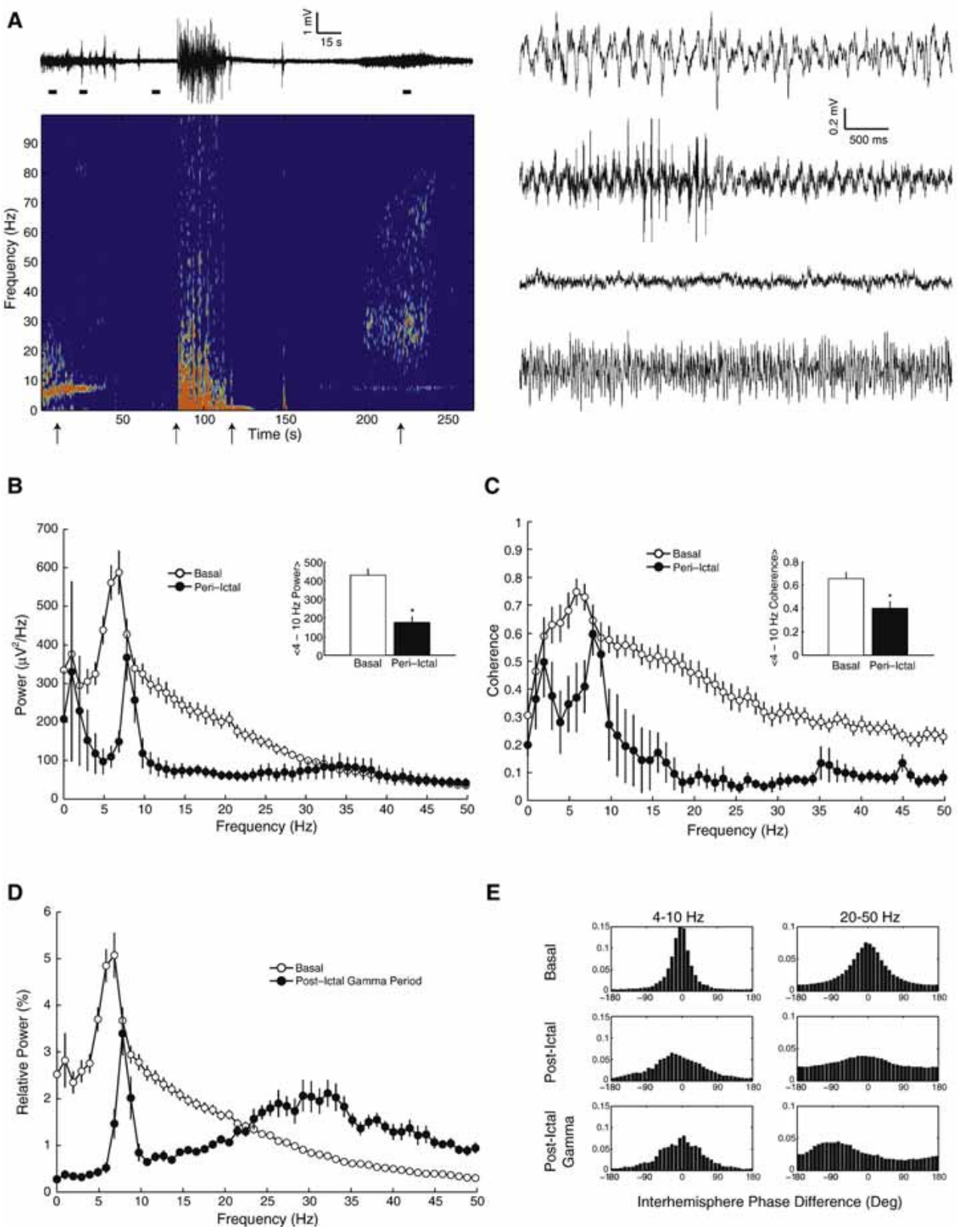
Fig. 1. Epidural EEG traces exhibit prominent theta oscillations. (A) Representative raw signal obtained from a posterior electrode. (Bottom) Zoomed in view (1-s period corresponding to the horizontal black line). (B) Mean theta power levels for each electrode location. Error-bars denote SEM. *$p<0.01$ compared to anterior electrodes (ANOVA followed by Tukey's test; $n=10$–12 per group). Inset shows the mean power spectra over all anterior (white) and posterior (black) electrodes. (C) Time–frequency decomposition of a representative signal. (D) Mean coherence spectra between pair of electrodes. Error-bars denote SEM ($n=13$ pairs per group).

our reference electrode was located anterior to bregma (see Materials and methods), and, by changing the reference electrode to a posterior location, we observed a reversion of these findings; that is, when using a posterior reference, we found that the anterior electrodes present higher theta power and coherence levels than posterior electrodes (not shown). For the rest of this work, we only show results of analyses performed on EEG traces obtained from the posterior electrodes using the anterior reference configuration.

Quinolinic acid disrupts theta and increases gamma oscillations

In Fig. 2A we show a representative EEG trace obtained immediately after QA i.c.v. infusion, along with its time–frequency decomposition. We observed that the basal theta activity becomes extremely reduced both before and after seizure, i.e., during the peri-ictal period (Figs. 2A and B), whereas the seizure event was characterized by a great increase in power of low frequencies, which spilled over higher frequencies (Fig. 2A). The coherence levels between electrodes following QA injection were also dramatically reduced (Fig. 2C). However, although both the overall theta power and coherence levels were reduced, the peak theta frequency exhibited a slight increase following QA infusion (Figs. 2B and C). In addition to these findings, we found that QA treatment induced bursts of gamma (20–50 Hz) activity occurring concurrently with the reduction of theta oscillations (Figs. 2A and D). However, it should be noted that the coherence at the gamma band was actually reduced after QA injection when compared to baseline levels (Fig. 2C). In accordance with these findings, an analysis of phase-differences between bilateral electrodes (i.e., inter-hemispheric) revealed disrupted phase-locking at both theta and gamma bands following QA treatment (Fig. 2E), including when only periods of higher gamma activity were taken into account (Fig. 2E, bottom panel).

Fig. 2. Quinolinic acid (QA) induces seizures and disrupts normal oscillatory activity during the peri-ictal period. (A) (left) Representative EEG trace obtained after QA infusion (top) and its corresponding time–frequency decomposition (bottom; same pseudocolor scale as in Fig. 1C). First arrow points to a theta period still present right after QA infusion (followed by a period of reduced theta power starting at 40 s); second and third arrows indicates the beginning and ending of a seizure event, respectively; fourth arrow points to a high gamma period. (Right) Zoomed-in view of particular epochs corresponding to the horizontal lines under the trace at the top left. From top to bottom, the traces show periods of: normal theta, "disrupted" theta, theta suppression, high gamma. (B) Mean power spectra during the basal and peri-ictal periods after QA infusion. Inset: mean theta power levels. *$p<0.01$ (t-test; basal $n=20$ traces; post-QA $n=10$ traces). Error-bars denote SEM. (C) Mean coherence spectra. Inset: mean theta coherence between bilateral electrodes. *$p<0.01$ (t-test; basal = 13 electrode pairs; post-QA $n=5$ electrode pairs). (D) Percentage power distribution during basal and high gamma periods after QA infusion. (E) Normalized histograms (% of counts) of inter-hemispheric phase difference for theta and gamma oscillations.


A
1 mV
15 s
Frequency (Hz)
Time (s)
0.2 mV
500 ms
B
Basal
Peri-Ictal
Power (μV²/Hz)
Frequency (Hz)
<4 – 10 Hz Power>
C
Coherence
<4 – 10 Hz Coherence>
D
Post-Ictal Gamma Period
Relative Power (%)
E
4-10 Hz
20-50 Hz
Post-Ictal
Post-Ictal Gamma
Interhemisphere Phase Difference (Deg)

Intraperitoneal infusion of guanosine does not alter EEG spectral content

We next studied the effects of i.p. guanosine on the spectral content of epidural EEGs. As shown in Fig. 3A, we found that guanosine did not cause any meaningful spectral alteration in the EEG traces in comparison to baseline findings. The i.p. injection of a saline control also did not cause any alteration in the power spectrum. The coherence spectrum after saline or guanosine administration was also similar to baseline (Fig. 3B), as well as the phase-difference analysis at both theta and gamma bands (Fig. 3C). Of note, we also did not find meaningful alterations in any of these analyses after MK-801 administration (Figs. 3A–C).

Guanosine reduces the incidence of quinolinic acid-induced seizures

In agreement with previous reports (Lara et al., 2001; Schmidt et al., 2000), QA induced seizures in all animals pre-treated with saline ($n=8$ rats; Fig. 4A), whereas seizure episodes were completely prevented by the pre-administration of the NMDA antagonist MK-801 ($n=6$ rats). In addition, we found that guanosine was able to protect seizures induced by QA in around 50% of the cases (protected $n=10$ rats; non-protected $n=9$ rats), which is also consistent with previous findings (Lara et al., 2001; Schmidt et al., 2000, 2005). Animals seizing under guanosine or saline pre-treatment did not differ in the duration of the seizure episode (Fig. 4B).

Animals successfully protected by guanosine present lower reduction in theta power than non-protected animals

In Fig. 5A we show the power spectrum densities of seizing animals that were not protected by guanosine for the three different periods of the experiment (baseline, after guanosine i.p., after QA i.c.v.). As in the case of QA injection alone (see Fig. 2B), we found that seizing animals after the combined treatment with guanosine and QA (the latter administered 30 min after the former) present an overall reduction of the spectral power, and, in particular, at the theta band (Fig. 5A). Interestingly, we found that animals that were successfully protected by guanosine presented a lower decrease in power over multiple frequencies, including the theta band (Fig. 5B). The difference in theta power levels between seizing and non-seizing animals after QA administration was statistically significant ($t(23)=-3.343$, $p=0.0028$). When examining the coherence spectra, we found that both seizing and non-seizing animals presented a reduction of inter-hemispheric coherence over a wide range of frequencies; it should be noted, however, that non-seizing animals exhibited a trend towards higher coherence values at the theta band (Fig. 5C). Similarly, the phase-difference histograms revealed disrupted phase-locking between the right and left hemispheres for both seizing and non-seizing animals (Fig. 5D).

Animals pre-treated with MK-801 present an increase in gamma oscillations after quinolinic acid administration

We have then examined the power spectral densities of animals pre-treated with MK-801 i.p. followed by QA i.c.v. administration 30 min later. As mentioned above, none of these animals presented seizures after QA infusion. We found that the levels of theta power after QA in animals pre-treated with MK-801 did not differ from baseline values, although the peak theta frequency tended to slow down (Fig. 6A). Remarkably, however, we found that the EEG traces of these animals presented a marked increase in gamma oscillations after QA administration (Fig. 6A). The coherence spectrum exhibited a mild decrease of inter-hemispheric theta-coherence (Fig. 6B), which could also be noticed by the phase-difference analysis (Fig. 6C).

The combined pre-treatment with guanosine and MK-801 promotes faster theta oscillations after quinolinic acid administration

Lastly, we examined the power spectral densities of animals pre-treated with both guanosine and MK-801 i.p. followed by QA i.c.v. administration 30 min later. We found that the average theta power level after the combined administration of guanosine and MK-801 was not different from basal levels (not shown), and, as in the case of MK-801 pre-treatment alone, none of these animals ($n=7$) presented motor seizures after QA infusion. Also similarly to the individual pre-treatment with MK-801, we observed a clear increase in gamma oscillations following QA administration (Fig. 7). However, we found that animals pre-treated with both guanosine and MK-801 present a prominent increase in the theta peak frequency following QA infusion (Fig. 7); in fact, as the magnitude of this increase was ~5 Hz, the peak frequency after QA infusion even reached values beyond the definition of theta range (4–10 Hz) employed in this work.

Discussion

We have examined the power spectra of brain electrical activity and signal synchronization (coherence) at baseline as well as in response to a central infusion of QA, which induced acute episodes of motor seizures. In addition to clear EEG changes occurring during the seizure events (Fig. 2A), we found that QA disrupts a prominent basal theta activity during the peri-ictal period; moreover, QA also promoted a relative increase in the power level at the gamma band. These changes in power were accompanied by a reduction of oscillatory coherence between brain hemispheres. Using this same paradigm, we have also examined the electrophysiological effects of a pharmacological intervention that we have previously shown to be neuroprotective against QA-induced seizures, namely the pre-treatment with guanosine. We found that guanosine, when effectively preventing seizures, was able to counteract both the decrease in theta power as well as the appearance of gamma oscillatory activity following QA infusion. Additionally, we also studied the electrophysiological effects of the NMDA antagonist MK-801 in the same seizure model, and we found that whereas MK-801 pre-treatment was able to effectively block the decrease in theta power, this drug was associated with the appearance of large gamma oscillations following QA administration. Finally, we also found that the combined pre-treatment with both guanosine and MK-801 in this model led to qualitatively different results than the observed when each drug was administered alone.

There is a striking resemblance of the spectral changes induced by the QA seizure model we described here to oscillatory alterations found in other experimental models of epilepsy. For instance, recent work studying mice subjected to a mesial temporal lobe epilepsy (mTLE) model secondary to hippocampal kainic acid (KA) injection found an abolishment of the theta rhythm in these chronic, epileptic animals (Arabadzisz et al., 2005; Dugladze et al., 2007). Furthermore, it was also shown that KA-induced epileptic animals exhibit an increase in the power of gamma oscillations (Dugladze et al., 2007; Medvedev et al., 2000). Although the QA model employed here is an acute model of seizure, in contrast to the KA model of mTLE, these similarities suggest that a reduction in theta and an increase in gamma could be a hallmark of an epileptic brain. It should also be noted that these similarities were found at different levels of analysis: while we looked at a more macroscopic electrophysiological scale, Dugladze et al (2007) examined hippocampal local field potentials (LFPs), that is, a more mesoscopic scale (Young and Eggermont, 2009). Moreover, this same work also reported similar spectral disruptions at the *in vitro* cellular level when examining the firing patterns of hippocampal interneurons (Dugladze et al., 2007). Inhibition and GABAergic interneurons were in fact suggested to be important for gamma and theta generation (Gloveli et al., 2005; Tort

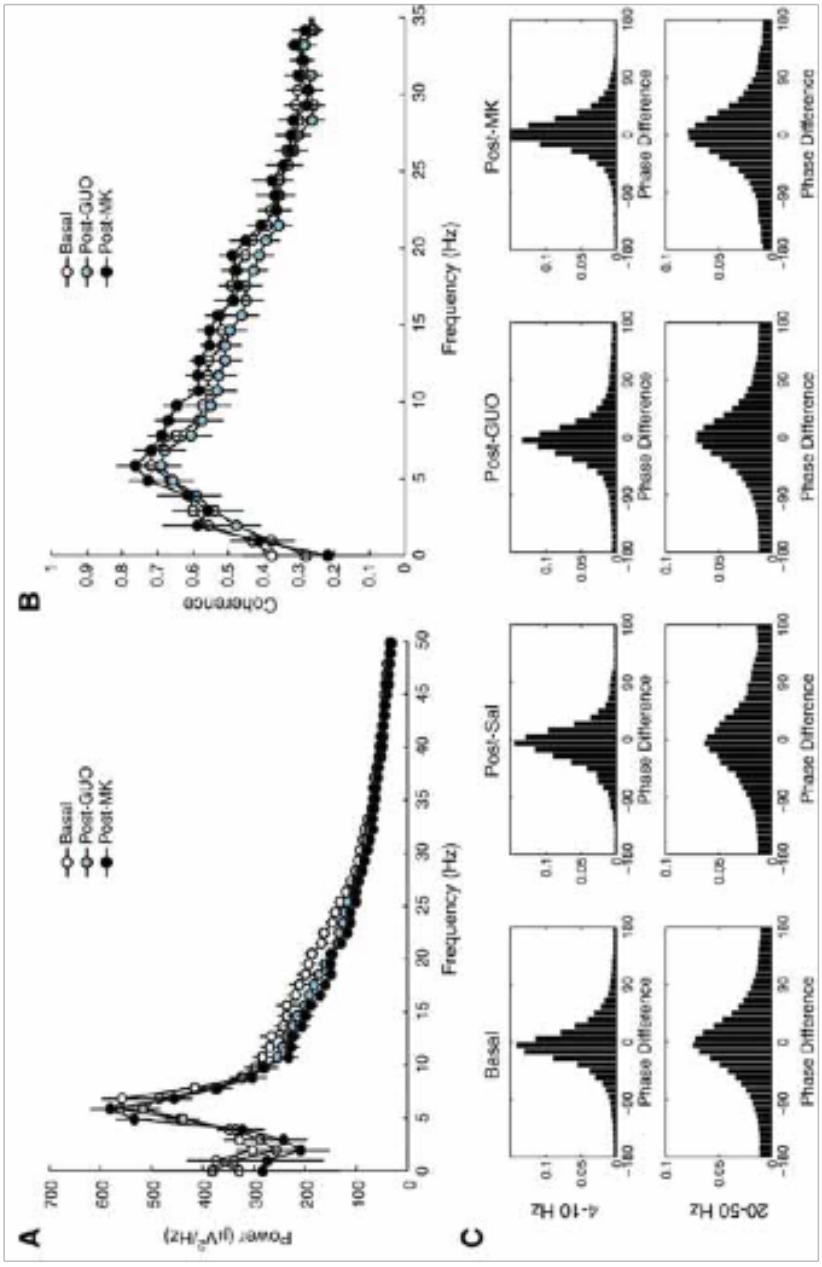


Fig. 3. Neither guanosine nor MK-801 alters EEG spectral content. (A) Mean power spectra. Error bars denote SEM. (B) Mean coherence spectra. Error bars denote SEM. (C) Normalized histograms (% of counts) of inter-hemispheric phase difference for theta and gamma oscillations.

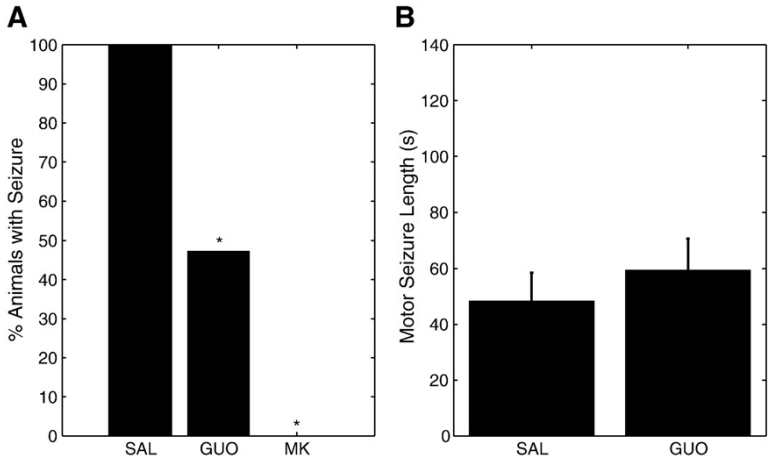


Fig. 4. Guanosine and MK-801 are able to protect against QA-induced seizures. (A) Percentage of seizing animals per pre-treatment group. *$p<0.05$ (Fisher exact test compared to saline group). (B) Mean seizure duration for saline pre-treated rats as well as during unsuccessful guanosine pre-treatment. Error-bars denote SEM.

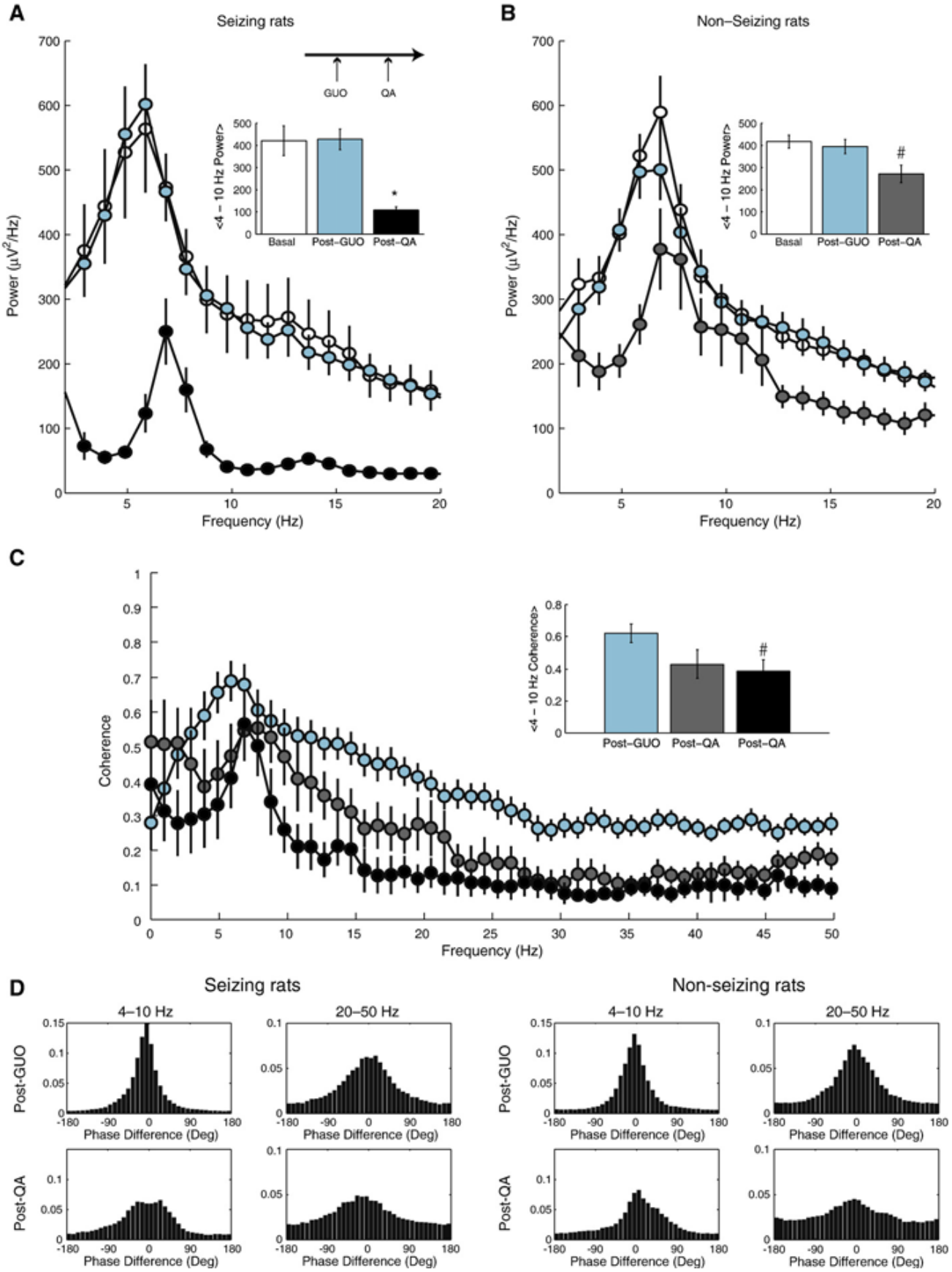
et al., 2007; Wulff et al., 2009), and a disruption of inhibitory activity is associated with epileptiform activity (Cossart et al., 2001; Dinocourt et al., 2003; Kobayashi and Buckmaster, 2003; Morin et al., 1998). However, whether putative alterations of inhibitory networks are playing a role in the QA seizure model remains to be established, but the similarities between the spectral findings suggest that both the KA and QA models may share pathophysiological mechanisms. Of note, these similarities also suggest that the theta oscillations we observed in our epidural recordings were likely volume conducted from the hippocampus.

The work by Arabadzisz et al. (2005) also described an overall reduction in the synchronization of bilateral EEG activity in animals subjected to the KA model of mTLE, similarly to what we found in the present study using the QA model of acute seizures (Fig. 2C). In fact, it should be noted that the inter-hemispheric impairments of synchrony were also observed to some extent even in rats successfully protected against seizures under the pre-treatment of either guanosine (Figs. 5C and D) or MK-801 (Figs. 6B and C). Taken together with the fact that non-seizing animals also presented some degree of alteration in the power of the theta band (Figs. 5B and 6A), our study also shows in particular that protected animals have an altered physiology, which is a fact that could not be hinted only by behavioral studies.

In a recent work, it was shown that the subcutaneous administration of either MK-801 or ketamine induces a marked increase in gamma oscillations in rats (Pinault, 2008), which was discussed in the context of cognitive alterations associated with schizophrenia. Here, we found that the intraperitonial injection of MK-801 (0.5 mg/kg) did not cause alterations in the gamma band; however, the concomitant treatment with QA promoted a marked increase in gamma oscillations (Fig. 6A). Note that this increase was observable in the absolute power levels of these traces (Fig. 6A), and was more prominent than the relative increase of gamma power (assessed by the % of power distribution present in the gamma band; see Fig. 2C) seen after QA administration alone (compare Figs. 6A and 2B). NMDA receptor blockers such as MK-801 were reported to induce paradoxical increases in glutamatergic transmission mediated by non-NMDA receptors activation (Adams and Moghaddam, 2001; Moghaddam et al., 1997; Moghaddam and Adams, 1998), which were postulated to involve disinhibition of glutamatergic principal cells promoted by less activation of GABAergic interneurons(Lopez-Gil et al., 2007; Moghaddam et al., 1997; Schmidt et al., 2009; Tort et al., 2004). It could be therefore that the increase in gamma oscillations induced by MK-801 (Pinault, 2008) is related to non-NMDA receptors activation. In this sense, whereas the MK-801 dose we used (0.5 mg/kg) was unable to promote higher gamma activity, the co-treatment with QA, which can also influence release and uptake of glutamate (de Oliveira et al., 2004; Tavares et al., 2002, 2005), could have synergistically increased the activation of non-NMDA receptors, leading to the observed increase in gamma oscillations. In fact, Pinault (2008) described that the appearance of gamma oscillations with MK-801 treatment was accompanied by ataxic behavior; as we did not observe ataxic behavior under our treatment protocol, this suggests that the effective dose of MK-801 reaching the brain was in fact higher in Pinault (2008) study than in the present work.

Guanosine occurs naturally in the brain and has been reported to present a myriad of biological effects when administered extracellularly, including trophic effects on neural cells (Ciccarelli et al., 2001; Rathbone et al., 1999), stimulation of astrocyte proliferation (Ciccarelli et al., 2000; Kim et al., 1991), and modulation of glutamatergic activity (reviewed in Schmidt et al., 2007). Although these effects might be related to its uptake into the intracellular compartment, a consensus has emerged that some of guanosine actions involve its binding to a specific membrane protein (Traversa et al., 2002, 2003), postulated by some to be a G protein-coupled receptor (Ciccarelli et al., 2001; Traversa et al., 2002, 2003). Indeed, guanosine was reported to present high affinity membrane sites in the brain (Traversa et al., 2002), although the actual existence of its putative specific receptor has yet to be demonstrated. More certain is the fact that guanosine presents clear anti-glutamatergic properties, as demonstrated in several *in vivo* and *in vitro* approaches (reviewed in Schmidt et al., 2007), which places it as a new potential neuroprotective strategy against glutamatergic excitotoxicity. The mechanism of action underlying the modulation of

Fig. 5. Rats successfully protected by guanosine present higher theta power than non-protected rats. (A) Mean power spectra during baseline (white), after guanosine i.p. (cyan), and after QA i.c.v. (black) for non-protected rats are shown. Inset depicts the mean theta power levels. *$p<0.01$ when compared to baseline (ANOVA followed by Tukey's test; $n=10$–14 traces per group). Error-bars denote SEM. Guanosine i.p. pre-treatment was followed by QA i.c.v. 30 min later (arrow scheme). (B) Same as in (A) but for protected rats (QA is shown in dark gray). #$p<0.05$ when compared to baseline (ANOVA followed by Tukey's test; $n=14$–18 traces per group). Error-bars denote SEM. (C) Mean coherence spectra (same color conventions as in A and B). The inset shows the mean theta coherence. #$p<0.05$ when compared to Post-GUO group (ANOVA followed by Tukey's test; $n=9$–14 electrode pairs). Error-bars denote SEM. (D) Normalized histograms (% of counts) of inter-hemispheric phase difference for theta and gamma oscillations.


A
Seizing rats
GUO
QA
Power (μV²/Hz)
Frequency (Hz)
<4 – 10 Hz Power>
Basal
Post–GUO
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B
Non–Seizing rats
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C
Coherence
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Post–GUO
Post–QA
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20–50 Hz
Post-GUO
Post-QA
Phase Difference (Deg)

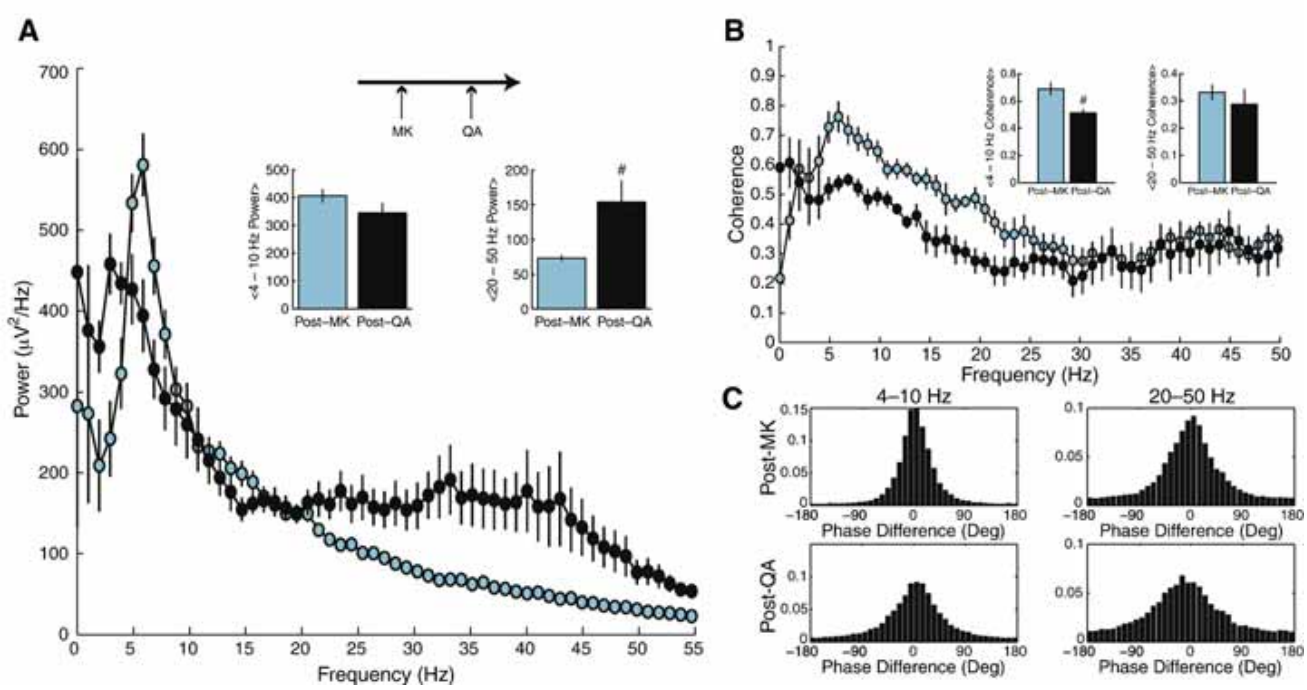

Fig. 6. MK-801 pre-treated rats present normal theta but increased gamma power after QA administration. (A) Mean power spectra after MK-801 i.p. (cyan) and after QA i.c.v (black). Insets depict the mean theta (left) and gamma (right) power levels. #$p<0.05$ (t-test; $n=12$ traces per group). Error-bars denote SEM. MK-801 i.p. pre-treatment was followed by QA i.c.v. 30 min later (arrow scheme). (B) Mean coherence spectra. The insets show the mean theta (left)and gamma (right) coherence. *$p<0.05$ (t-test; $n=6$ electrode pairs per group). Error-bars denote SEM. (C) Normalized histograms (% of counts) of inter-hemispheric phase difference for theta and gamma oscillations.

glutamatergic activity by guanosine is currently under research in our and other laboratories. It has been suggested that astrocytes are crucially involved, since guanosine was shown to stimulate glutamate uptake by astrocytes (Frizzo et al., 2001, 2002, 2003), which is the main mechanism of glutamate removal from the synaptic cleft (Danbolt, 2001; Beart and O'Shea, 2007). In addition, recent works have shown that QA decreases glutamate uptake *in vivo* and that this effect is reverted by guanosine when successfully acting as anticonvulsant (de Oliveira et al., 2004; Tavares et al., 2002, 2005). Taken together, there is strong evidence that the antiglutamatergic action of guanosine occurs in a fundamentally different way than MK-801, which would explain the different electrophysiological results observed here in the network (EEG) level. The finding that both compounds when administered simultaneously promoted qualitatively different effects than when each was tested individually is also consistent with different influences of these drugs in the network level. Noteworthy, since guanosine alone also prevented the increase in gamma oscillations after QA infusion, this result suggests that it might cause less cognitive side effects than MK-801 in the clinical setting.

It is known that guanosine is protective against QA-induced seizures in a dose-dependent manner (Schmidt et al., 2000, 2005). However, it still remains puzzling why guanosine is not effective in preventing QA-induced seizures in 100% of the cases, even in the highest soluble dose possible. In the present study, we found that the pre-treatment with guanosine was an effective anticonvulsant therapy in around 50% of the cases (Fig. 4A), a fraction that was similar to what we found in previous studies (Lara et al., 2001; Schmidt et al., 2000, 2005). Moreover, we observed that this fraction is not related to individual differences among animals: when the same group of animals is subjected twice to guanosine pre-treatment followed by i.c.v. QA infusion (with one week of interval between experiments), we obtained roughly the same fraction of protected animals, and we observed that the seizing/protected rats in the second experiment were not necessarily the same as in the first experiment (Avila TT, Antunes C, Souza DO, unpublished observations). This suggests that dynamic variables within rats are probably determining whether guanosine will successfully prevent seizures or not. It is possible that these variables are related to pharmacokinetic and pharmacodynamic factors (e.g., effective drug distribution), although it could also be related to other influences as the current internal state of the brain. Importantly, here we have demonstrated for the first time that protected and non-protected animals under guanosine pre-treatment can be distinguished electrophysiologically, even before the beginning of the motor seizures. Accordingly, we found that the level of theta power greatly decreased in seizing animals under (unsuccessful) guanosine pre-treatment, both before and after the seizure event (i.e., peri-ictally), similarly to what we observed in saline pre-treated rats. On the other hand, animals successfully protected by guanosine exhibited a statistically higher level of theta power than seizing animals (compare Figs. 5A and B). Although this result adds an extra step towards understanding why guanosine would be anticonvulsive only at times, the factors responsible for these electrophysiological differences are also puzzling at the moment. At any event, as the decrease in theta activity could also be seen previous to seizure onset, the present results suggest that the monitoring of theta activity could be a useful marker of an epileptic brain.

In summary, here we have performed a macroscopic electrophysiological characterization of an acute model of seizure secondary to overstimulation of the glutamatergic system. Using this model, we studied the effect of two neuroprotective pharmacological interventions and we showed that they possess different spectral signatures. While both guanosine and MK-801 are known to exert actions over the glutamatergic system, these results show that the mechanisms of seizure prevention are likely different between these two compounds at the network level. Further electrophysiological studies at the cellular level are warranted and will help in elucidating the precise

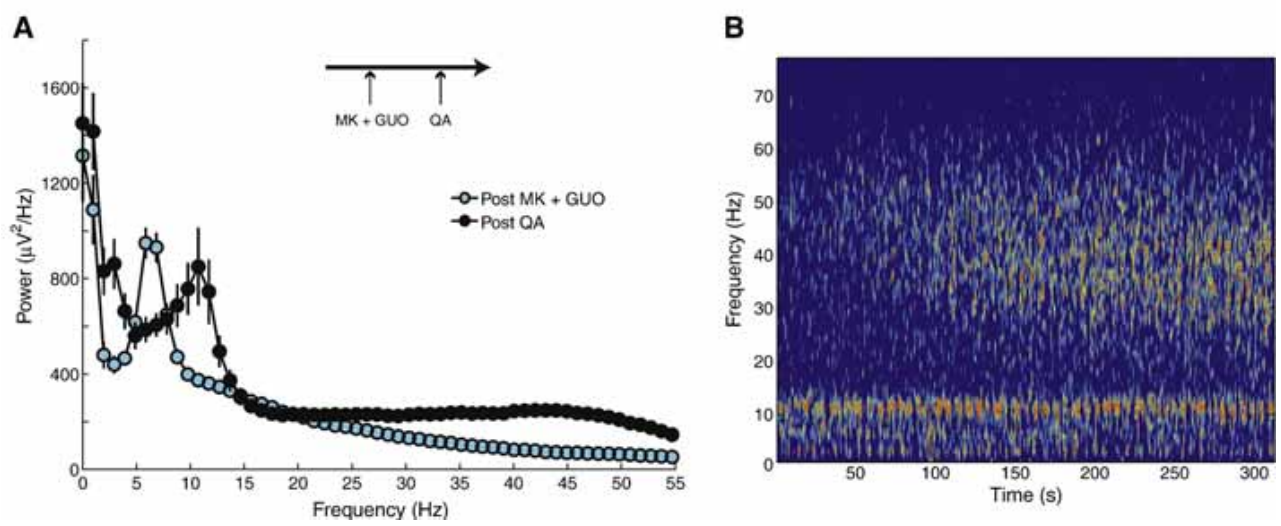

Fig. 7. Rats pre-treated with both guanosine and MK-801 present faster theta oscillations after QA administration. (A) Mean power spectra after guanosine and MK-801 i.p. (cyan) and after QA i.c.v. (black). Error-bars denote SEM ($n = 14$ traces per group). Guanosine and MK-801 i.p. pre-treatment was followed by QA i.c.v. 30 min later (arrow scheme). (B) Time–frequency decomposition of a representative signal after QA infusion (same pseudocolor scale as in Fig. 1C).

mechanism of action of guanosine and how it translates into the present network, macroscopic findings.

Acknowledgments

This work was supported by CNPq-PRONEX/FAPESPA #2268, FINEP research grant "Rede Instituto Brasileiro de Neurociência (IBN-Net)" # 01.06.0842-00, and INCT for Excitotoxicity and Neuroprotection-CNPq. MSF is a CNPq research fellow. A.B.L.T. has received funding from CNPq and CAPES, Brazil. The authors also thank the HCPA Clinical Engineering Section for technical assistance.

References

Adams, B.W., Moghaddam, B., 2001. Effect of clozapine, haloperidol, or M100907 on phencyclidine-activated glutamate efflux in the prefrontal cortex. Biol. Psychiatry 50, 750–757.
Anderson, C.M., Swanson, R.A., 2000. Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia 32, 1–14.
Arabadzisz, D., Antal, K., Parpan, F., Emri, Z., Fritschy, J.M., 2005. Epileptogenesis and chronic seizures in a mouse model of temporal lobe epilepsy are associated with distinct EEG patterns and selective neurochemical alterations in the contralateral hippocampus. Exp. Neurol 194, 76–90.
Baron, B.M., Dudley, M.W., McCarty, D.R., Miller, F.P., Reynolds, I.J., Schmidt, C.J., 1989. Guanine nucleotides are competitive inhibitors of *N*-methyl-D-aspartate at its receptor site both *in vitro* and *in vivo*. J. Pharmacol. Exp. Ther. 250, 162–169.
Beart, P.M., O'Shea, R.D., 2007. Transporters for L-glutamate: an update on their molecular pharmacology and pathological involvement. Br. J. Pharmacol 150 , 5–17.
Bradford, H.F., 1995. Glutamate, GABA and epilepsy. Prog. Neurobiol. 47, 477–511.
Ciccarelli, R., Di Iorio, P., D'Alimonte, I., Giuliani, P., Florio, T., Caciagli, F., Middlemiss, P.J., Rathbone, M.P., 2000. Cultured astrocyte proliferation induced by extracellular guanosine involves endogenous adenosine and is raised by the co-presence of microglia. Glia 29, 202–211.
Ciccarelli, R., Ballerini, P., Sabatino, G., Rathbone, M.P., D'Onofrio, M., Caciagli, F., Di Iorio, P., 2001. Involvement of astrocytes in purine-mediated reparative processes in the brain. Int. J. Dev. Neurosci. 19, 395–414.
Connick, J.H., Stone, T.W., 1988. Quinolinic acid effects on amino acid release from the rat cerebral cortex *in vitro* and *in vivo*. Br. J. Pharmacol. 93, 868–876.
Cossart, R., Dinocourt, C., Hirsch, J.C., Merchan-Perez, A., De Felipe, J., Ben-Ari, Y., Esclapez, M., Bernard, C., 2001. Dendritic but not somatic GABAergic inhibition is decreased in experimental epilepsy. Nat. Neurosci. 4, 52–62.
Danbolt, N.C., 2001. Glutamate uptake. Prog. Neurobiol. 65, 1–105.
Delorme, A., Makeig, S., 2004. EEGLAB: an open source toolbox for analysis of single-trial EEG dynamics including independent component analysis. J. Neurosci. Methods 134, 9–21.
de Oliveira, D.L., Horn, J.F., Rodrigues, J.M., Frizzo, M.E., Moriguchi, E., Souza, D.O., Wofchuk, S., 2004. Quinolinic acid promotes seizures and decreases glutamate uptake in young rats: reversal by orally administered guanosine. Brain Res. 1018, 48–54.
Dinocourt, C., Petanjek, Z., Freund, T.F., Ben-Ari, Y., Esclapez, M., 2003. Loss of interneurons innervating pyramidal cell dendrites and axon initial segments in the CA1 region of the hippocampus following pilocarpine-induced seizures. J. Comp. Neurol. 459, 407–425.
Dugladze, T., Vida, I., Tort, A.B., Gross, A., Otahal, J., Heinemann, U., Kopell, N.J., Gloveli, T., 2007. Impaired hippocampal rhythmogenesis in a mouse model of mesial temporal lobe epilepsy. Proc. Natl. Acad. Sci. U. S. A. 104, 17530–17535.
Frizzo, M.E., Lara, D.R., Dahm, K.C., Prokopiuk, A.S., Swanson, R.A., Souza, D.O., 2001. Activation of glutamate uptake by guanosine in primary astrocyte cultures. Neuroreport 12, 879–881.
Frizzo, M.E., Lara, D.R., Prokopiuk Ade, S., Vargas, C.R., Salbego, C.G., Wajner, M., Souza, D.O., 2002. Guanosine enhances glutamate uptake in brain cortical slices at normal and excitotoxic conditions. Cell Mol. Neurobiol. 22, 353–363.
Frizzo, M.E., Antunes Soares, F.A., Dall'Onder, L.P., Lara, D.R., Swanson, R.A., Souza, D.O., 2003. Extracellular conversion of guanine-based purines to guanosine specifically enhances astrocyte glutamate uptake. Brain Res. 972, 84–89.
Gloveli, T., Dugladze, T., Rotstein, H.G., Traub, R.D., Monyer, H., Heinemann, U., Whittington, M.A., Kopell, N.J., 2005. Orthogonal arrangement of rhythm-generating microcircuits in the hippocampus. Proc. Natl. Acad. Sci. U. S. A. 102, 13295–13300.
Guillemin, G.J., Kerr, S.J., Brew, B.J., 2005. Involvement of quinolinic acid in AIDS dementia complex. Neurotox. Res. 7, 103–123.
Kim, J.K., Rathbone, M.P., Middlemiss, P.J., Hughes, D.W., Smith, R.W., 1991. Purinergic stimulation of astroblast proliferation: guanosine and its nucleotides stimulate cell division in chick astroblasts. J. Neurosci. Res. 28, 442–455.
Kobayashi, M., Buckmaster, P.S., 2003. Reduced inhibition of dentate granule cells in a model of temporal lobe epilepsy. J. Neurosci. 23, 2440–2452.
Lara, D.R., Schmidt, A.P., Frizzo, M.E., Burgos, J.S., Ramirez, G., Souza, D.O., 2001. Effect of orally administered guanosine on seizures and death induced by glutamatergic agents. Brain Res. 912, 176–180.
Lewis, S.M., Lee, F.S., Todorova, M., Seyfried, T.N., Ueda, T., 1997. Synaptic vesicle glutamate uptake in epileptic (EL) mice. Neurochem. Int. 31, 581–585.
Lopez-Gil, X., Babot, Z., Amargos-Bosch, M., Sunol, C., Artigas, F., Adell, A., 2007. Clozapine and haloperidol differently suppress the MK-801-increased glutamatergic and serotonergic transmission in the medial prefrontal cortex of the rat. Neuropsychopharmacology 32, 2087–2097.
Malcon, C., Achaval, M., Komlos, F., Partata, W., Sauressig, M., Ramirez, G., Souza, D.O., 1997. GMP protects against quinolinic acid-induced loss of NADPH-diaphorase-positive cells in the rat striatum. Neurosci. Lett. 225, 145–148.
Medvedev, A., Mackenzie, L., Hiscock, J.J., Willoughby, J.O., 2000. Kainic acid induces distinct types of epileptiform discharge with differential involvement of hippocampus and neocortex. Brain Res. Bull. 52, 89–98.
Moghaddam, B., Adams, B.W., 1998. Reversal of phencyclidine effects by a group II metabotropic glutamate receptor agonist in rats. Science 281, 1349–1352.
Moghaddam, B., Adams, B., Verma, A., Daly, D., 1997. Activation of glutamatergic neurotransmission by ketamine: a novel step in the pathway from NMDA receptor blockade to dopaminergic and cognitive disruptions associated with the prefrontal cortex. J. Neurosci. 17, 2921–2927.
Morin, F., Beaulieu, C., Lacaille, J.C., 1998. Selective loss of GABA neurons in area CA1 of the rat hippocampus after intraventricular kainate. Epilepsy Res. 32, 363–369.
Nakano, K., Takahashi, S., Mizobuchi, M., Kuroda, T., Masuda, K., Kitoh, J., 1993. High levels of quinolinic acid in brain of epilepsy-prone E1 mice. Brain Res. 619, 195–198.

Paas, Y., Devillers-Thiery, A., Changeux, J.P., Medevielle, F., Teichberg, V.I., 1996. Identification of an extracellular motif involved in the binding of guanine nucleotides by a glutamate receptor. EMBO J. 15, 1548–1556.
Paz, M.M., Ramos, M., Ramirez, G., Souza, D., 1994. Differential effects of guanine nucleotides on kainic acid binding and on adenylate cyclase activity in chick optic tectum. FEBS Lett. 355, 205–208.
Pinault, D., 2008. *N*-methyl-D-aspartate receptor antagonists ketamine and MK-801 induce wake-related aberrant gamma oscillations in the rat neocortex. Biol. Psychiatry 63, 730–735.
Ramaswamy, S., McBride, J.L., Kordower, J.H., 2007. Animal models of Huntington's disease. ILAR J. 48, 356–373.
Rathbone, M.P., Middlemiss, P.J., Gysbers, J.W., Andrew, C., Herman, M.A., Reed, J.K., Ciccarelli, R., Di Iorio, P., Caciagli, F., 1999. Trophic effects of purines in neurons and glial cells. Prog. Neurobiol. 59, 663–690.
Roesler, R., Vianna, M.R., Lara, D.R., Izquierdo, I., Schmidt, A.P., Souza, D.O., 2000. Guanosine impairs inhibitory avoidance performance in rats. Neuroreport 11, 2537–2540.
Saute, J.A., da Silveira, L.E., Soares, F.A., Martini, L.H., Souza, D.O., Ganzella, M., 2006. Amnesic effect of GMP depends on its conversion to guanosine. Neurobiol. Learn Mem. 85, 206–212.
Schmidt, A.P., Lara, D.R., Maraschin, J.F., Perla, A.S., Souza, D.O., 2000. Guanosine and GMP prevent seizures induced by quinolinic acid in mice. Brain Res. 864, 40–43.
Schmidt, A.P., Avila, T.T., Souza, D.O., 2005. Intracerebroventricular guanine-based purines protect against seizures induced by quinolinic acid in mice. Neurochem. Res. 30, 69–73.
Schmidt, A.P., Lara, D.R., Souza, D.O., 2007. Proposal of a guanine-based purinergic system in the mammalian central nervous system. Pharmacol. Ther. 116, 401–416.
Schmidt, A.P., Tort, A.B., Souza, D.O., Lara, D.R., 2008. Guanosine and its modulatory effects on the glutamatergic system. Eur. Neuropsychopharmacol. 18, 620–622.
Schmidt, A.P., Tort, A.B., Silveira, P.P., Bohmer, A.E., Hansel, G., Knorr, L., Schallenberger, C., Dalmaz, C., Elisabetsky, E., Crestana, R.H., Lara, D.R., Souza, D.O., 2009. The NMDA antagonist MK-801 induces hyperalgesia and increases CSF excitatory amino acids in rats: reversal by guanosine. Pharmacol. Biochem. Behav. 91, 549–553.
Soares, F.A., Schmidt, A.P., Farina, M., Frizzo, M.E., Tavares, R.G., Portela, L.V., Lara, D.R., Souza, D.O., 2004. Anticonvulsant effect of GMP depends on its conversion to guanosine. Brain Res. 1005, 182–186.
Stone, T.W., 2001. Kynurenic acid antagonists and kynurenine pathway inhibitors. Expert Opin. Investig. Drugs 10, 633–645.
Tavares, R.G., Tasca, C.I., Santos, C.E., Alves, L.B., Porciuncula, L.O., Emanuelli, T., Souza, D. O., 2002. Quinolinic acid stimulates synaptosomal glutamate release and inhibits glutamate uptake into astrocytes. Neurochem. Int. 40, 621–627.
Tavares, R.G., Schmidt, A.P., Abud, J., Tasca, C.I., Souza, D.O., 2005. In vivo quinolinic acid increases synaptosomal glutamate release in rats: reversal by guanosine. Neurochem. Res. 30, 439–444.
Tort, A.B., Mantese, C.E., dos Anjos, G.M., Dietrich, M.O., Dall'Igna, O.P., Souza, D.O., Lara, D.R., 2004. Guanosine selectively inhibits locomotor stimulation induced by the NMDA antagonist dizocilpine. Behav. Brain Res. 154, 417–422.
Tort, A.B., Rotstein, H.G., Dugladze, T., Gloveli, T., Kopell, N.J., 2007. On the formation of gamma-coherent cell assemblies by oriens lacunosum-moleculare interneurons in the hippocampus. Proc. Natl. Acad. Sci. U. S. A. 104, 13490–13495.
Traversa, U., Bombi, G., Di Iorio, P., Ciccarelli, R., Werstiuk, E.S., Rathbone, M.P., 2002. Specific [(3)H]-guanosine binding sites in rat brain membranes. Br. J. Pharmacol. 135, 969–976.
Traversa, U., Bombi, G., Camaioni, E., Macchiarulo, A., Costantino, G., Palmieri, C., Caciagli, F., Pellicciari, R., 2003. Rat brain guanosine binding site. Biological studies and pseudo-receptor construction. Bioorg. Med. Chem. 11, 5417–5425.
Vinade, E.R., Schmidt, A.P., Frizzo, M.E.S., Portela, L.V., Soares, F.A., Schwalm, F.D., Elisabetsky, E., Izquierdo, I., Souza, D.O., 2005. Effects of chronic administered guanosine on behavioral parameters and brain glutamate uptake in rats. J. Neurosci. Res. 79, 248–253.
Wulff, P., Ponomarenko, A.A., Bartos, M., Korotkova, T.M., Fuchs, E.C., Bahner, F., Both, M., Tort, A.B., Kopell, N.J., Wisden, W., Monyer, H., 2009. Hippocampal theta rhythm and its coupling with gamma oscillations require fast inhibition onto parvalbumin-positive interneurons. Proc. Natl. Acad. Sci. U. S. A. 106, 3561–3566.
Young, C.K., Eggermont, J.J., 2009. Coupling of mesoscopic brain oscillations: recent advances in analytical and theoretical perspectives. Prog. Neurobiol. 89, 61–78.

CAPITULO II

Effects of guanosine on EEG theta oscillation and motor activity in mice

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Artigo em preparação.

EFFECTS OF GUANOSINE ON EEG THETA OSCILATION AND MOTOR ACTIVITY IN MICE

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ABSTRACT

Guanosine has several neuroprotective effects in animal models of neurological injuries associated with excitotoxicity. In addition guanosine is amnesic and, like benzodiazepines, presents anxiolytic effects in rodents.

Since several studies have shown changes in EEG profile related to psychotropic drugs such as benzodiazepines its use may be helpful in the prospection of potential drugs with central effect on nervous system. Although guanosine induces neurological behavioral changes, possible concomitant electrophysiological changes in the brain - particularly in theta oscillations that seems to be related with anxiety state - are a field to be explored.

In this work we evaluate the EEG theta oscillation in freely moving mice under guanosine administration during open field test. We found that guanosine reduces the locomotor activity and increase the period at rest in a dose dependent manner and similar to diazepam. Guanosine promoted a decrease in theta peak frequency at 60 mg/kg i.p in periods at rest and diazepam promoted a similar decrease but its effect was independent of the state of activity. Further experiments are needed in order to asses the mechanism of action that underlies the modulation of atropine-sensitive theta rhythms by guanosine.

Keywords: theta, EEG, guanosine, sedative drugs, anxiolytic drugs, motor behavior

ABBREVIATIONS

BDZ	Benzodiazepines
EEG	Electroencephalogram
GABA	Receptor for GABA type A
QA	Quinolinic acid
i.p.	intraperitoneally

INTRODUCTION

Guanosine, a guanine nucleoside, exhibits several neuroprotective effects in animal models of neurological injuries associated with excitotoxicity, such as brain ischemia and seizures (Lara et al. 2001; Oliveira et al. 2002; Schmidt et al. 2005; Schmidt et al. 2000). But beyond the neuroprotective effects, guanosine seems to induce behavioral changes, such as sedation, amnesia and decreased anxiety (Roesler et al. 2000; Tort et al. 2004; Vinade et al. 2003). These effects resemble those usually induced by $GABA_A$ agonists benzodiazepines (BDZ). However, the mechanisms underlying the effects of extracellular guanosine seem to involve modulation of the glutamatergic system without direct effect upon GABAergic system (Lara et al. 2001; Roesler et al. 2000; Schmidt et al. 2005; Schmidt et al. 2000; Schmidt et al. 2008; Tort et al. 2004; Vinade et al. 2005). Some of its neuroprotective and trophic effects seems to occur via surface receptors linked to some Gi-protein (Di Iorio et al. 2004). In the other hand, it has been suggested guanosine could help to prevent injury by high extracellular levels of glutamate enhancing its uptake by astrocytes (Frizzo et al. 2003; Frizzo et al. 2001; Frizzo et al. 2002). Athough these findings give us some clues about the mechanism of action of extracellular guanosine, an specific binding site for extracellular guanosine has not been discovered yet and we have to be judicious in consider the same mechanism of action for different types of effects.

Quantitative electroencephalogram (EEG) analysis is a powerful tool to study the effects of psychotropic drugs on the brain functional state (Herrmann and Schaerer 1990). Brain oscillations are associated with the coordination of distributed neuronal groups believed to underlie behavioral processing (Buzsaki and Draguhn

2004; Engel et al. 2001; Sirota et al. 2008). In mice, the lateral parietal cortex is overlaying the dorsal hippocampus and permits reliable recording of theta hippocampal waves with comparable amplitude and phases between different animals (Brankack et al. 1993).

Several studies have shown EEG changes in acute administration of benzodiazepines (BDZ) in general. Diazepam in particular produces a typical EEG profile at rest and during sustained attention task. This EEG profile is characterized by an increase in beta oscillation (14-30Hz) and decrease in alpha (8-13 Hz) delta (1-4 Hz) and theta (5-10 Hz) oscillation (Ansseau et al. 1984; Bauer et al. 1997; Greenblatt et al. 1989; Lucchesi et al. 2003; Mandena et al. 1992; Munoz-Torres et al. 2011; Romano-Torres et al. 2002; Siok et al. 2012; Urata et al. 1996). Furthermore, diazepam has been consistently reported to decrease theta frequency during active waking and REM sleep (Caudarella et al. 1987; Gottesmann et al. 1998; Monmaur 1981; Siok et al. 2012).

Although guanosine induces neurological behavioral changes, possible concomitant electrophysiological changes in the brain - particularly the changes in theta oscillations that seems to be related with anxiety state - are a field to be explored. In a recent study we described the effect of guanosine 7.5 mg/kg i.p. on the power spectra of brain oscillations and signal synchronization (coherence) under normal conditions (baseline) and during quinolinic acid (QA)-induced seizures in rats (Torres et al. 2009). Guanosine alone prevented the increase in gamma oscillations after QA infusion and decreased seizure activity. However, the effect of progressively higher doses of guanosine on theta oscillations was never explored.

In this work we evaluate the EEG profile and the mean peak frequency of theta in freely moving mice under guanosine administration during motor activity task.

We found that guanosine reduces the locomotor activity and prolongs the period at rest state in a dose-dependent manner and decreases the theta peak frequency at rest 60 mg/kg i.p.. Diazepam 2 mg/kg promoted similar findings, but induced a decrease in theta peak frequency not only at rest but also during motor activity, as expected.

MATERIAL AND METHODS

Animals

Adult (male) Swiss albino mice weighting 35-45 g were used. Animals were kept one per cage, under a 12 h light/12 h dark cycle (light on at 7:00 am), in a heat-regulated room (22 ± 1º C), with relative humidity at 55 ± 5%, and received food and water *ad libitum*. The animals were maintained according to Brazilian Federal Law n.º 11.794/2008 (Arouca Law) and the methodology of this study followed the ethical principles in animal experiments of the Brazilian College of Animal Experimentation. All protocols were approved by the Animal Care and Ethics Committee of the University (approval n.º 5647)**.**

Chemicals

Diazepam was purchased from União Química Nacional S/A (Pouso Alegre, MG, Brazil) and guanosine was obtained from Sigma Chemicals (St. Louis, MO, USA). Guanosine was prepared in NaOH 10 μM (pH 7.4) and all solutions were warmed up to 35˚C previously to administration. The anesthetic ketamine was

obtained from Vetbrands (Jacareí, SP, Brazil) and xylazine from Coopers Brasil Ltda (Cotia, SP, Brazil).

Surgical Procedure

Animals were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) intraperitoneally (i.p.). The head of the animal was fixed in the stereotaxic and the skin covering the skull was cut with a 1.0-cm long rostro-caudal incision in the midline. After exposure of the skull bone surface, three stainless steel electrodes (0.5 mm diameter) were implanted. Two of them were used as positive electrodes (1.2 mm lateral, right or left, 2.0 mm posterior to bregma) and the reference (negative) electrode was placed in the midline of the occipital bone and kept in contact with cerebrospinal fluid. The positioning of the electrodes was fixed with dental acrylic cement, and a screw used for fixation of the dental acrylic helmet to the bone was used as ground.

EEG recording and open field

One week after surgery, mice were (individually) transferred to an observation cage (Plexiglas chambers). Electrodes were connected to a digital data-acquisition system (Nihon-Khoden, Japan). After an accommodation period of ten minutes, a basal EEG activity was registered and observed for 20 minutes in order to ensure that it had good signal to noise ratio and usual characteristics (signal did not suppress, without abnormal electrical discharges). Later, each animals was pretreated (i.p. injection) with one of the following: NaCl 0.09% (2 ml/100 g),

guanosine (15, 30, 60 or 120 ml/kg, diluted to 2 ml/100g), or diazepam (2 mg/kg), and transferred to a square-shaped black arena (50 x 50 x 50 cm) to perform the open field Test with concomitant EEG recording for 90 minutes. During this period, the positions of the animal were acquired with 2 seconds of sampling interval. The epidural EEG traces were filtered at 0.01-100 Hz and digitally recorded to a computer hard drive using 1 kHz sampling resolution for posterior analysis. The distance travelled in the open field and the EEG were recorded for 90 minutes for posterior analysis. We analyzed the total distance travelled to evaluate the motor activity.

Data analysis

The analyses were performed using built in and custom written routines in MATLAB (Mathworks, Inc). The power were estimated by means of the Welch periodogram method using a 50% overlapping Hamming window with a length of 1024 points (i.e. 1.024 s), which were obtained using the *pwelch* and *mscohere* functions, respectively, from the Signal Processing Toolbox. The time-frequency decompositions used 1024 points sliding windows with 50% overlap, which was obtained using the *spectrogram* function from the Signal Processing Toolbox. The filtering was done by means of a linear finite impulse response (FIR) filter, which was obtained using the *eegfilt* routine from the EEGLAB toolbox (Delorme and Makeig 2004).

Statistical analysis

The power values were compared using One-way ANOVA followed by Tukey's Post-Hoc Test, as appropriate. A value of $p<0.05$ was considered statistically significant. All data are presented as mean ± SEM.

RESULTS

Guanosine induces dose-dependent reduction in locomotor activity

In order to evaluate the effect of guanosine in locomotor activity animals were divided in 4 groups based on the dosage of guanosine injected (15, 30, 60 or 120 mg/kg. i.p.). In addition we use as a negative and positive control group animals injected with saline solution and Diazepam (2 mg/kg i.p.), respectively. Each animal was individually placed in the open field apparatus. We observed that the travelled distance after guanosine administration decreases for all groups when compare to controls (Fig. 1A-B, p=0.0003, n=5 per group, One-way ANOVA followed by Tukey's Post-Hoc Test). The decrease in locomotor activity was similar to that induced by DZP (2 mg/kg. i.p.) (Fig. 1B, C).

To evaluate the duration of decrease in locomotor activity we tracked the movement for each animal in the open filed apparatus during 90 minutes. We observed that animals receiving guanosine 30-60 mg/kg i.p., remained at rest for a longer period when compared to control and groups receiving guanosine 15 and 120 mg/kg i.p. (Fig. 2A-B, p=0.0007 n=5 per group, One-way ANOVA followed by Tukey's

Post-Hoc Test). An effect similar to guanosine 30 and 60 mg/kg was observed in animals receiving DZP 2 mg/kg i.p. (Fig. 2A-C).

Guanosine induced decrease in theta peak frequency at rest

To evaluate the changes in brain oscillation during guanosine administration we performed power spectrum analysis (Fig. 3), time-frequency decomposition analysis (Fig. 4A-F top - color scale graphs) and visual inspection (Fig. 4A-F bottom - 5 s sampled signal) of epidural EEG.

During locomotor activity theta oscillation was not affected by guanosine (Fig. 3A), different from Diazepam 2 mg/kg that decrease the theta peak from 7.38±0.06 to 6.25±0.14 ($p<0.0001$, n=5 per group, One-way ANOVA followed by Tukey's Post-Hoc Test). On the other hand, animals receiving guanosine 60 mg/kg and diazepam group exhibited decrease in theta peak at rest from 7.53±0.11 to 5.48±0.46 and 4.68±0.17, respectively (Fig. 3B, $p<0.0001$, n=5 per group, One-way ANOVA followed by Tukey's Post-Hoc Test).

As shown in Fig. 4A, we found prominent theta oscillations (4–10 Hz) in the epidural EEG traces of the control animals during the period normal locomotor activity and at rest. Such oscillations could be detected by simple visual inspection of rough signal (Fig. 4A bottom) and by time-frequency decomposition obtained during this period, which depicts a clear, relatively steady theta rhythm (Fig. 4A color scale graph). Representative time-frequency decomposition graphs and EEG traces of guanosine and Diazepam groups are shown in Fig. 4B-E and 4F, respectively. Note that we can distinguish two different patterns of power distribution in theta

frequencies, one during locomotor activity (shown by black-shadowed graph at the top) and other at rest.

DISCUSSION

In the open field test, we noted an accommodation on locomotor activity observed by the curve of cumulative distance traveled in the open field in the control group over time (dots become closer along time) (Fig 1A). Unfortunately, the number of animals used for this work had not enough power for further analysis of open field measures. Guanosine 60 mg/kg i.p. promoted a decrease in locomotion and period of active movement similar to that induced by diazepam 2 mg/kg. i.p. (Fig. 1). Guanosine promoted a decrease in theta peak frequency at 60 mg/kg i.p in periods at rest and diazepam promoted a similar decrease but its effect was independent of the state of activity. Moreover, neither guanosine or diazepam apparently did have any effect upon oscillatory coherence between brain hemispheres for dosages used in this work (Torres, F.V.; data not shown).

Despite the exuberant effect upon locomotor activity, guanosine affected the EEG theta peak frequency only at the dosage of 60 mg/kg and only at rest, which corresponds to the dosage that showed more prominent effect upon locomotor activity. Interestingly, guanosine at the dosage of 120 mg/kg did not seem to alter the theta peak frequency and showed a trend to have a lower effect upon locomotor activity when compared to guanosine 60 mg/kg. Furthermore, the guanosine effect upon theta peak frequency seems to be restricted to periods at rest. Diazepam showed reductions of the peak frequency independently of the behavioral state, already shown in previous studies (Caudarella et al. 1987; Gottesmann et al. 1998;

Monmaur 1981; Siok et al. 2012). In fact, animal studies have shown that the anxiolytic effects of benzodiazepines are associated only with decreased theta frequency, while anxiogenic drugs caused an increase in frequency of theta activity (McNaughton et al. 2007) and suggest our findings may be in part due to an anxiolytic effect of guanosine already reported (Vinade et al. 2003; Vinade et al. 2005).

Previous works have postulated the existence of two types of theta oscillation: one that could be suppressed and/or slowed independently of the fact to be at rest or not, and another that could de suppressed and/or slowed at rest but return to previous characteristics when animal is navigating, walking or running (Kramis et al. 1975; Shin et al. 2005). Moreover, these two kind of theta rhythms correspond to atropine-sensitive and atropine-resistant theta rhythms. The first can be abolished by administration of muscarinic antagonists (e.g., atropine or scopolamine), but the second is not affected by the same drugs (Bland 1986; Buzsaki 2002; Kramis et al. 1975; Leung 1998; Leung 1985; McNaughton et al. 2007; Shin 2002; Shin and Talnov 2001; Stewart and Fox 1990).

Knock-out experiments showed that atropine-sensitive component of theta seems to be dependent of PLC-β1 (Shin et al. 2005), a second messenger coupled to three muscarinic receptor, namely M1, M3 and M5 via the α subunit of Gq-proteins (being M1 especially predominant in the hippocampus and expressed in the pyramidal cell bodies and apical and basal dendrites of the stratum radiatum and stratum oriens) (Rhee and Bae 1997; Volpicelli and Levey 2004). In contrast, atropine-resistant theta rhythms probably result from the concerted actions of different neurotransmitters on various brain regions, including glutamatergic and GABAergic systems (Shin et al. 2005). Taken together, these facts suggest that

guanosine may play a role modulating the M1-Gαq-PLC-β1-dependent pathway in hippocampal cell bodies and dendrites, despite some of its neuroprotective and trophic effects seem to occur via some Gi-protein (Di Iorio et al. 2004).

The effect of guanosine on theta peak frequency only in resting periods and the fact of two different profiles of power spectrum related to awake behavior show us the important role of EEG analysis to be coupled with animal observation. If not, only a behavioral change could be translated in changes in the mean power and frequencies of theta.

Guanosine apparently shows at least partial loss of effect between 60 and 120 mg/kg (U-shaped dose-effect response). In fact, this effect behavior was already seen in parameters measured in different works and also for guanine nucleotides (Schmidt et al. 2005; Schmidt et al. 2010). Since the CSF levels of guanosine in blood and CSF arise linearly according the dosage used (Schmidt et al. 2010), this could may suggest a loss of specificity or even a context-dependent effect.

In summary, our data suggest that guanosine produces a decrease in peak of theta oscillation depending on the activity state. Guanosine anxiolytic, sedative, anticonvulsant effects are similar to observed for BDZ, however, the mechanisms underlying these various effects are not understood. Still, it remains to be established whether the changes in EEG theta oscillation are related to cognitive and behavioral effects.

Guanosine have been investigated along the last two decades by different groups and seems to have a unquestionable role in the extracellular medium at CNS. Further investigation is required to elucidate its mechanism, always being judicious with the fact that different effects may be occurring bay different pathways and some responses may be context-dependent.

Figure 1.

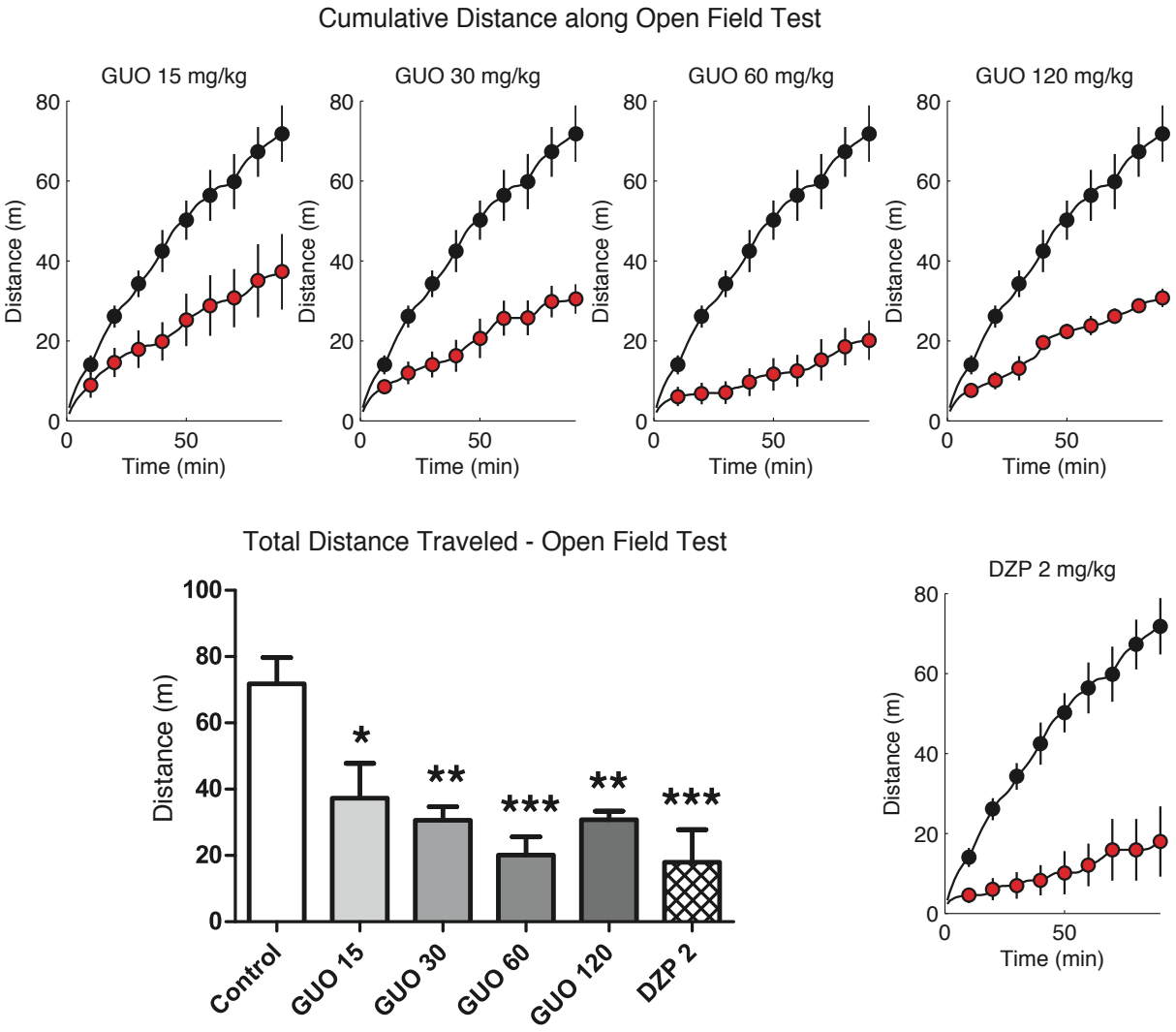

Cumulative Distance along Open Field Test
GUO 15 mg/kg
GUO 30 mg/kg
GUO 60 mg/kg
GUO 120 mg/kg
Distance (m)
Time (min)
0
20
40
60
80
50
Total Distance Traveled - Open Field Test
100
*
**
***
**
***
Control
GUO 15
GUO 30
GUO 60
GUO 120
DZP 2
DZP 2 mg/kg

Figure 2.

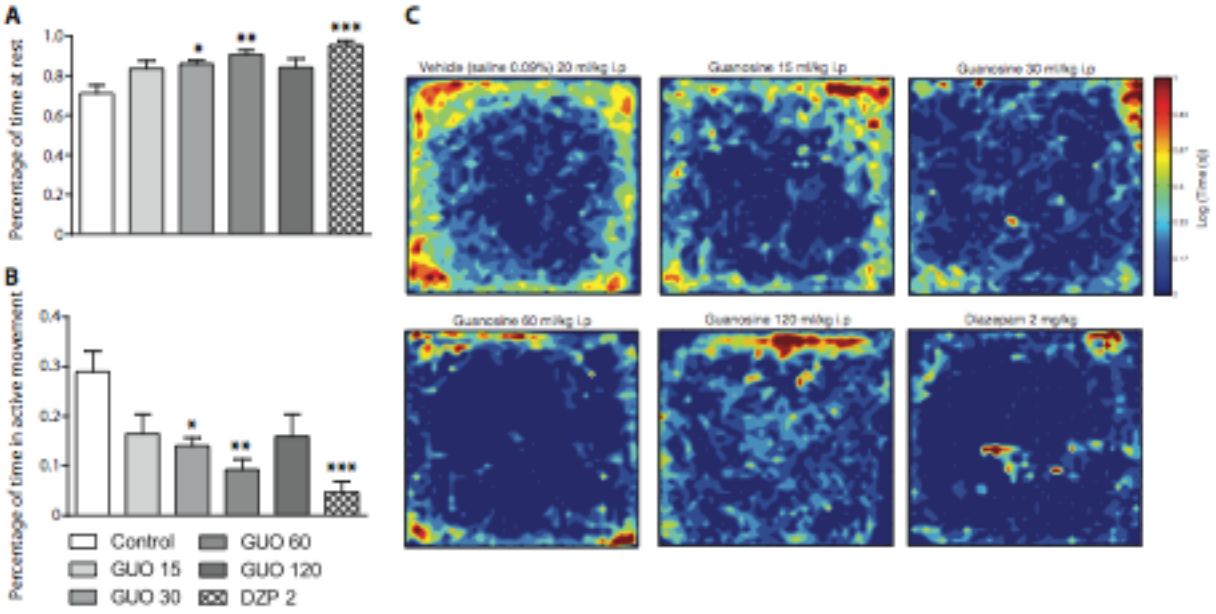

A
Percentage of time at rest
1.0
0.8
0.6
0.4
0.2
0
*
**
***
B
Percentage of time in active movement
0.4
0.3
0.2
0.1
0
*
**
***
Control
GUO 15
GUO 30
GUO 60
GUO 120
DZP 2
C
Vehicle (saline 0.09%) 20 ml/kg i.p
Guanosine 15 ml/kg i.p
Guanosine 30 ml/kg i.p
Guanosine 60 ml/kg i.p
Guanosine 120 ml/kg i.p
Diazepam 2 mg/kg
Log (Time (s))

Figure 3.

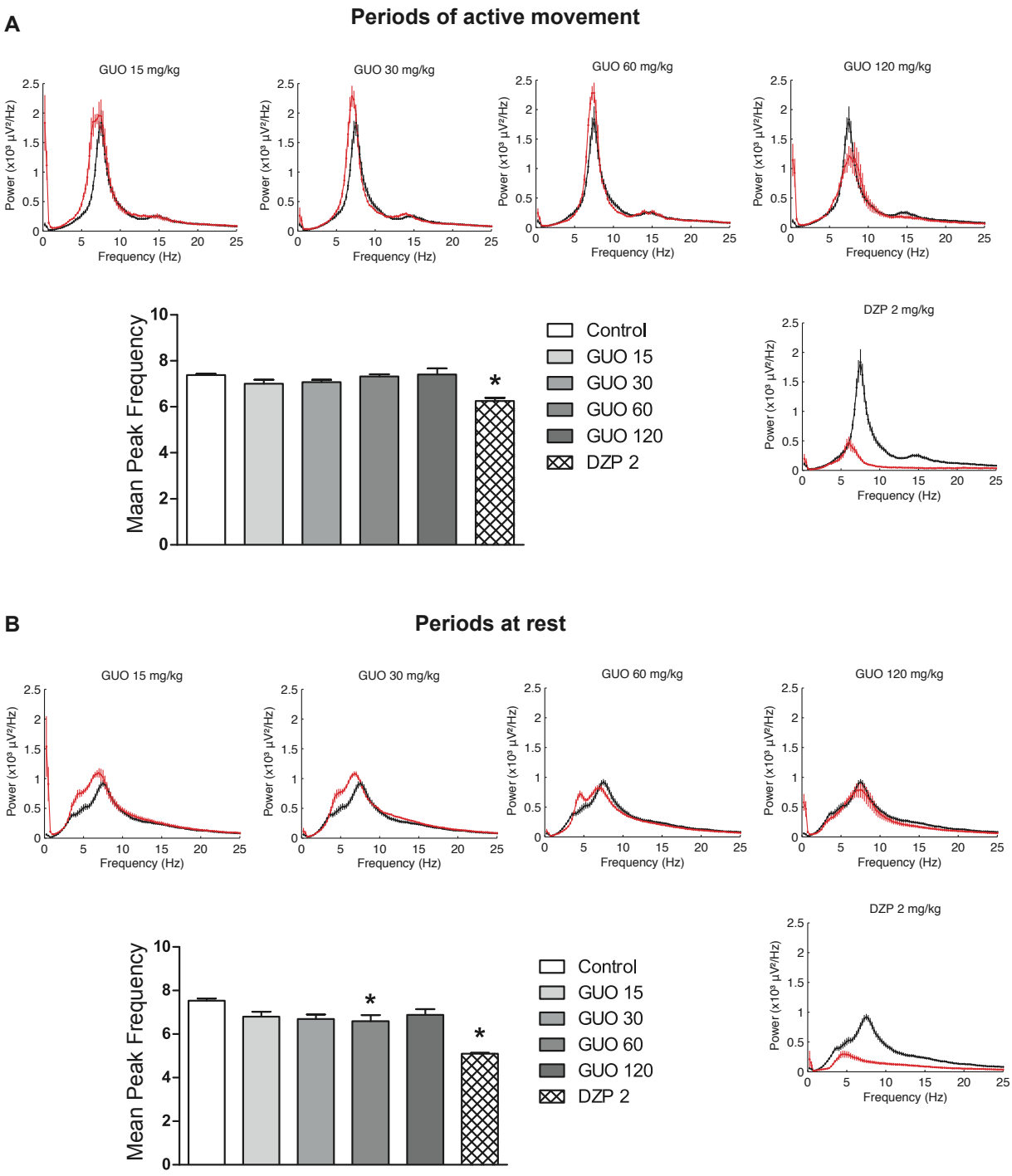
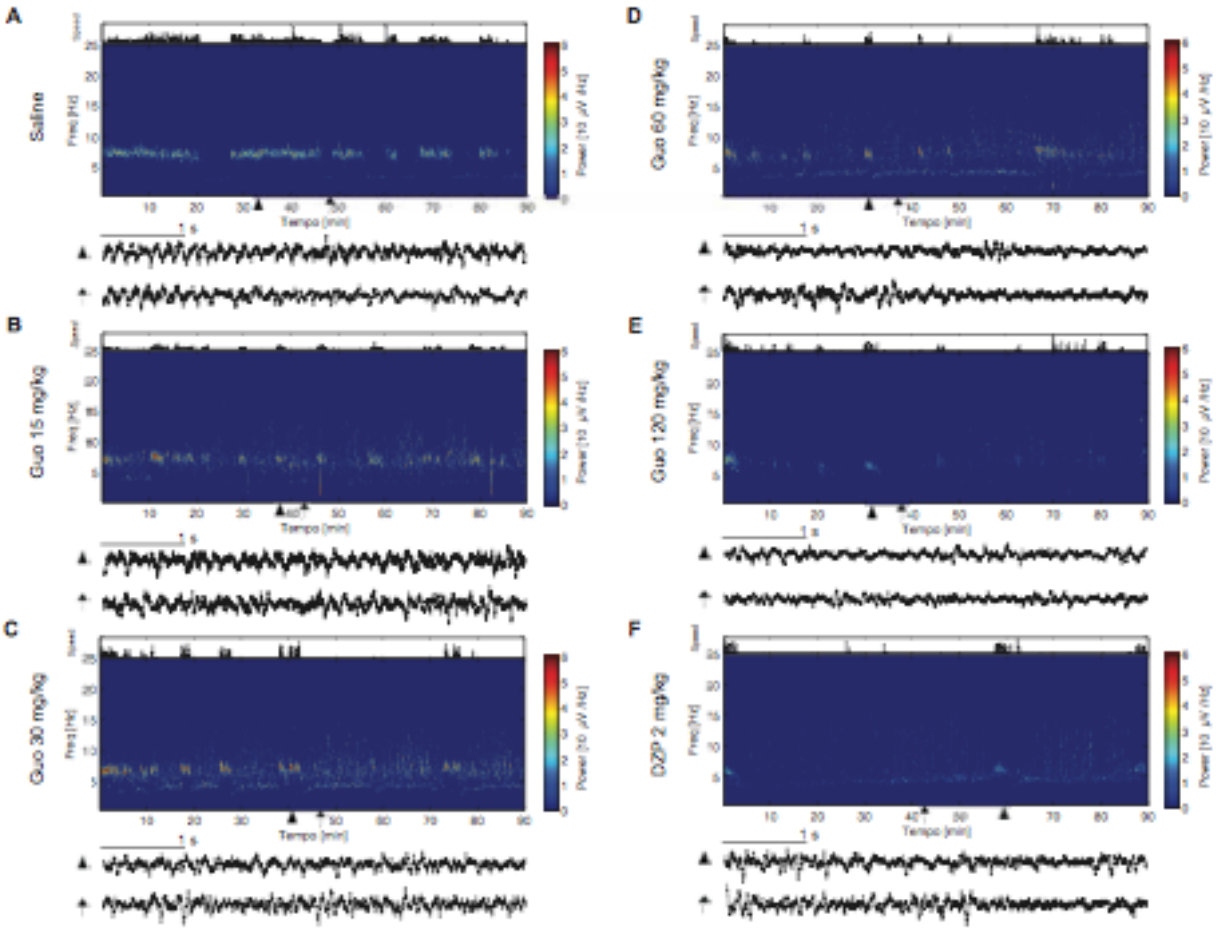

A
Periods of active movement
GUO 15 mg/kg
GUO 30 mg/kg
GUO 60 mg/kg
GUO 120 mg/kg
Power (x10³ µV²/Hz)
Frequency (Hz)
Maan Peak Frequency
*
Control
GUO 15
GUO 30
GUO 60
GUO 120
DZP 2
DZP 2 mg/kg
B
Periods at rest
Mean Peak Frequency

Figure 4.


A
Saline
B
Guo 15 mg/kg
C
Guo 30 mg/kg
D
Guo 60 mg/kg
E
Guo 120 mg/kg
F
DZP 2 mg/kg
Speed
Freq [Hz]
Tempo [min]
Power [10 μV Hz]
1 s

LEGENDS

Figure 1. Decrease in travelled distance induced by guanosine and diazepam. Cumulative distance plots for guanosine and diazepam-treated animals (red-filled dots) compared to control group (black-filled dots) (A). Total distance travelled at 90 min (B). N=5 per group. Error-bars denote SEM. Control differs from all other groups (*p<0.05, **p<0.01, ***p<0.001).

Figure 2. Decrease in time spent in locomotor activity by guanosine and diazepam. Percentage of time at rest during open field test. Period at rest guanosine 30 and 60 mg/kg and DZP 2 mg/kg groups was significantly longer then control group (*p<0.05, **p<0.01, ***p<0.001) (A). Percentage of time in active movement during 90-min of open field test (Mean and SEM). Period of active movement was significantly shorter in rguanosine 30 and 60 mg/kg and DZP 2 mg/kg then control group (*p<0.05, **p<0.01, ***p<0.001) (B). Representative occupancy plots for each group (C). N=5 per group. Error-bars denote SEM.

Figure 3. Decrease in theta peak frequency induced by guanosine and diazepam. Line graphs: Mean Power Spectra for EEG during locomotion (A) and at rest (B) for control (black filled dots) vs guanosine and diazepam-treated animals(red filled dots). Bar graphs: Mean EEG peak frequency during open field test. N=5 per group. N=10 traces per group. Error-bars denote SEM. Theta paek frequency decrease significantly in guanosine 60 mg/kg and DZP 2 mg/kg when compare to control group. DZP 2 mg/kg differs from all groups except guanosine 60 mg/kg(*p<0.05).

Figure 4. Time-frequency decomposition of a representative signal for each group, coupled to respective animal speed (black shadowed graph on the top). Representative traces from EEG at rest (arrow) and during active locomotor activity (triangle) are showed for each group, sampled at the intervals indicated by the same arrow/triangle at the time-frequency graph.

REFERENCES

Ansseau M, Doumont A, Cerfontaine JL, Mantanus H, Rousseau JC, Timsit Berthier M. 1984. Self-reports of anxiety level and EEG changes after a single dose of benzodiazepines. Double-blind comparison of two forms of oxazepam. Neuropsychobiology 12(4):255-259.

Bauer LO, Gross JB, Meyer RE, Greenblatt DJ. 1997. Chronic alcohol abuse and the acute sedative and neurophysiologic effects of midazolam. Psychopharmacology (Berl) 133(3):293-299.

Bland BH. 1986. The physiology and pharmacology of hippocampal formation theta rhythms. Prog Neurobiol 26(1):1-54.

Brankack J, Stewart M, Fox SE. 1993. Current source density analysis of the hippocampal theta rhythm: associated sustained potentials and candidate synaptic generators. Brain Res 615(2):310-327.

Buzsaki G. 2002. Theta oscillations in the hippocampus. Neuron 33(3):325-340.

Buzsaki G, Draguhn A. 2004. Neuronal oscillations in cortical networks. Science 304(5679):1926-1929.

Caudarella M, Durkin T, Galey D, Jeantet Y, Jaffard R. 1987. The effect of diazepam on hippocampal EEG in relation to behavior. Brain Res 435(1-2):202-212.

Delorme A, Makeig S. 2004. EEGLAB: an open source toolbox for analysis of single-trial EEG dynamics including independent component analysis. J Neurosci Methods 134(1):9-21.

Di Iorio P, Ballerini P, Traversa U, Nicoletti F, D'Alimonte I, Kleywegt S, Werstiuk ES, Rathbone MP, Caciagli F, Ciccarelli R. 2004. The antiapoptotic effect of guanosine is mediated by the activation of the PI 3-kinase/AKT/PKB pathway in cultured rat astrocytes. Glia 46(4):356-368.

Engel AK, Fries P, Singer W. 2001. Dynamic predictions: oscillations and synchrony in top-down processing. Nat Rev Neurosci 2(10):704-716.

Frizzo ME, Antunes Soares FA, Dall'Onder LP, Lara DR, Swanson RA, Souza DO. 2003. Extracellular conversion of guanine-based purines to guanosine specifically enhances astrocyte glutamate uptake. Brain Res 972(1-2):84-89.

Frizzo ME, Lara DR, Dahm KC, Prokopiuk AS, Swanson RA, Souza DO. 2001. Activation of glutamate uptake by guanosine in primary astrocyte cultures. Neuroreport 12(4):879-881.

Frizzo ME, Lara DR, Prokopiuk Ade S, Vargas CR, Salbego CG, Wajner M, Souza DO. 2002. Guanosine enhances glutamate uptake in brain cortical slices at normal and excitotoxic conditions. Cell Mol Neurobiol 22(3):353-363.

Gottesmann C, Gandolfo G, Arnaud C, Gauthier P. 1998. The intermediate stage and paradoxical sleep in the rat: influence of three generations of hypnotics. Eur J Neurosci 10(2):409-414.

Greenblatt DJ, Ehrenberg BL, Gunderman J, Locniskar A, Scavone JM, Harmatz JS, Shader RI. 1989. Pharmacokinetic and electroencephalographic study of intravenous diazepam, midazolam, and placebo. Clin Pharmacol Ther 45(4): 356-365.

Herrmann WM, Schaerer E. 1990. Pharmaco-EEG: computer EEG analysis to describe the projection to drug effects on a functional cerebral level in humans. Clinical applications of computer analysis of EEG and other neurophysiological signals. Amsterdam: Elsevier. p 385-445.

Kramis R, Vanderwolf CH, Bland BH. 1975. Two types of hippocampal rhythmical slow activity in both the rabbit and the rat: relations to behavior and effects of

atropine, diethyl ether, urethane, and pentobarbital. Exp Neurol 49(1 Pt 1): 58-85.

Lara DR, Schmidt AP, Frizzo ME, Burgos JS, Ramírez G, Souza DO. 2001. Effect of orally administered guanosine on seizures and death induced by glutamatergic agents. Brain research 912(2):176-180.

Leung LS. 1998. Generation of theta and gamma rhythms in the hippocampus. Neurosci Biobehav Rev 22(2):275-290.

Leung LW. 1985. Spectral analysis of hippocampal EEG in the freely moving rat: effects of centrally active drugs and relations to evoked potentials. Electroencephalogr Clin Neurophysiol 60(1):65-77.

Lucchesi LM, Pompeia S, Manzano GM, Kohn AF, Galduroz JC, Bueno OF, Tufik S. 2003. Flunitrazepam-induced changes in neurophysiological, behavioural, and subjective measures used to assess sedation. Prog Neuropsychopharmacol Biol Psychiatry 27(3):525-533.

Mandena JW, Kuck MT, Danhof M. 1992. Differences in intrinsic efficacy of benzodiazepines are reflected in their concentration-EEG effect relationship. Br J Pharmacol(105):9.

McNaughton N, Kocsis B, Hajos M. 2007. Elicited hippocampal theta rhythm: a screen for anxiolytic and procognitive drugs through changes in hippocampal function? Behav Pharmacol 18(5-6):329-346.

Monmaur P. 1981. Phasic hippocampal activity during paradoxical sleep in the rat: selective suppression after diazepam administration. Experientia 37(3): 261-262.

Munoz-Torres Z, del Rio-Portilla Y, Corsi-Cabrera M. 2011. Diazepam-induced changes in EEG oscillations during performance of a sustained attention task. J Clin Neurophysiol 28(4):394-399.

Oliveira IJ, Molz S, Souza DO, Tasca CI. 2002. Neuroprotective effect of GMP in hippocampal slices submitted to an in vitro model of ischemia. Cell Mol Neurobiol 22(3):335-344.

Rhee SG, Bae YS. 1997. Regulation of phosphoinositide-specific phospholipase C isozymes. J Biol Chem 272(24):15045-15048.

Roesler R, Vianna MR, Lara DR, Izquierdo I, Schmidt AP, Souza DO. 2000. Guanosine impairs inhibitory avoidance performance in rats. Neuroreport 11(11):2537-2540.

Romano-Torres M, Borja-Lascurain E, Chao-Rebolledo C, del-Rio-Portilla Y, Corsi-Cabrera M. 2002. Effect of diazepam on EEG power and coherent activity: sex differences. Psychoneuroendocrinology 27(7):821-833.

Schmidt AP, Avila TT, Souza DO. 2005. Intracerebroventricular guanine-based purines protect against seizures induced by quinolinic acid in mice. Neurochem Res 30(1):69-73.

Schmidt AP, Bohmer AE, Schallenberger C, Antunes C, Tavares RG, Wofchuk ST, Elisabetsky E, Souza DO. 2010. Mechanisms involved in the antinociception induced by systemic administration of guanosine in mice. Br J Pharmacol 159(6):1247-1263.

Schmidt AP, Lara DR, de Faria Maraschin J, da Silveira Perla A, Onofre Souza D. 2000. Guanosine and GMP prevent seizures induced by quinolinic acid in mice. Brain Res 864(1):40-43.

Schmidt AP, Tort AB, Souza DO, Lara DR. 2008. Guanosine and its modulatory effects on the glutamatergic system. Eur Neuropsychopharmacol 18(8): 620-622.

Shin J. 2002. A unifying theory on the relationship between spike trains, EEG, and ERP based on the noise shaping/predictive neural coding hypothesis. Biosystems 67(1-3):245-257.

Shin J, Kim D, Bianchi R, Wong RK, Shin HS. 2005. Genetic dissection of theta rhythm heterogeneity in mice. Proc Natl Acad Sci U S A 102(50):18165-18170.

Shin J, Talnov A. 2001. A single trial analysis of hippocampal theta frequency during nonsteady wheel running in rats. Brain Res 897(1-2):217-221.

Siok CJ, Cogan SM, Shifflett LB, Doran AC, Kocsis B, Hajos M. 2012. Comparative analysis of the neurophysiological profile of group II metabotropic glutamate receptor activators and diazepam: effects on hippocampal and cortical EEG patterns in rats. Neuropharmacology 62(1):226-236.

Sirota A, Montgomery S, Fujisawa S, Isomura Y, Zugaro M, Buzsaki G. 2008. Entrainment of neocortical neurons and gamma oscillations by the hippocampal theta rhythm. Neuron 60(4):683-697.

Stewart M, Fox SE. 1990. Do septal neurons pace the hippocampal theta rhythm? Trends Neurosci 13(5):163-168.

Torres F, da Silva Filho M, Antunes C. 2009. Electrophysiological effects of guanosine and MK-801 in a quinolinic acid-induced …. Exp Neurol 221:296-306.

Tort AB, Mantese CE, dos Anjos GM, Dietrich MO, Dall'Igna OP, Souza DO, Lara DR. 2004. Guanosine selectively inhibits locomotor stimulation induced by the NMDA antagonist dizocilpine. Behav Brain Res 154(2):417-422.

Urata J, Uchiyama M, Iyo M, Enomoto T, Hayakawa T, Tomiyama M, Nakajima T, Sasaki H, Shirakawa S, Wada K, Fukui S, Yamadera H, Okawa M. 1996. Effects of a small dose of triazolam on P300 and resting EEG. Psychopharmacology (Berl) 125(2):179-184.

Vinade ER, Schmidt AP, Frizzo ME, Izquierdo I, Elisabetsky E, Souza DO. 2003. Chronically administered guanosine is anticonvulsant, amnesic and anxiolytic in mice. Brain Res 977(1):97-102.

Vinade ER, Schmidt AP, Frizzo ME, Portela LV, Soares FA, Schwalm FD, Elisabetsky E, Izquierdo I, Souza DO. 2005. Effects of chronic administered guanosine on behavioral parameters and brain glutamate uptake in rats. J Neurosci Res 79(1-2):248-253.

Volpicelli LA, Levey AI. 2004. Muscarinic acetylcholine receptor subtypes in cerebral cortex and hippocampus. Prog Brain Res 145:59-66.

CAPITULO III

Increase of Extracellular Glutamate Concentration Increases Its Oxidation and Diminishes Glucose Oxidation in Isolated Mouse Hippocampus: Reversible by TFB-TBOA

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Artigo publicado no periódico *Journal of Neuroscience Research*

Journal of Neuroscience Research 91:1059–1065 (2013)

Increase of Extracellular Glutamate Concentration Increases Its Oxidation and Diminishes Glucose Oxidation in Isolated Mouse Hippocampus: Reversible by TFB-TBOA

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Glutamate concentration at the synaptic level must be kept low in order to prevent excitotoxicity. Astrocytes play a key role in brain energetics, and also astrocytic glutamate transporters are responsible for the vast majority of glutamate uptake in CNS. Experiments with primary astrocytic cultures suggest that increased influx of glutamate cotransported with sodium at astrocytes favors its flux to the tricarboxylic acid cycle instead of the glutamate–glutamine cycle. Although metabolic coupling can be considered an emergent field of research with important recent discoveries, some basic aspects of glutamate metabolism still have not been characterized in brain tissue. Therefore, the aim of this study was to investigate whether the presence of extracellular glutamate is able to modulate the use of glutamate and glucose as energetic substrates. For this purpose, isolated hippocampi of mice were incubated with radiolabeled substrates, and CO_2 radioactivity and extracellular lactate were measured. Our results point to a diminished oxidation of glucose with increasing extracellular glutamate concentration, glutamate presumably being the fuel, and might suggest that oxidation of glutamate could buffer excitotoxic conditions by high glutamate concentrations. In addition, these findings were reversed when glutamate uptake by astrocytes was impaired by the presence of (3S)-3-[[3-[[4-(trifluoromethyl)benzoyl]amino]phenyl]methoxy]-L-aspartic acid (TFB-TBOA). Taken together, our findings argue against the lactate shuttle theory, because glutamate did not cause any detectable increase in extracellular lactate content (or, presumably, in glycolysis), because the glutamate is being used as fuel instead of going to glutamine and back to neurons.

Key words: brain; oxidation; excitotoxicity; TFB-TBOA; glutamate transporters

Glutamate is the major excitatory neurotransmitter in the central nervous system (CNS), and it has been estimated that glutamate-mediated neurotransmission is responsible for about 80% of the energy expended in the gray matter, with synaptic potentials (more than action potentials) responsible for the high energetic cost in maintenance of excitability (restoration of ion gradients dissipated by signaling; Belanger et al., 2011). Glutamate concentration at the synaptic level must be kept low in order to maintain a high signal-to-noise ratio and to prevent excitotoxicity (Choi, 1992; Szatkowski and Attwell, 1994; Huang et al., 1997; Herman and Jahr, 2007). The concentration of glutamate at the synaptic cleft rises dramatically from low micromolar concentrations up to 1 mM during neurotransmission (Meldrum, 2000), and failure of astrocytes to remove this excess glutamate leads to excitotoxic damage and ultimately neuronal death (Danbolt, 2001; Schousboe and Waagepetersen, 2005; McKenna, 2007). It has been shown that intracerebroventricular administration of (3S)-3-[[3-[[4-(trifluoromethyl)benzoyl]amino]phenyl]methoxy]-L-aspartic acid (TFB-TBOA), a competitive blocker of astrocytic glutamate transporters, namely, GLAST and GLT-1 that did

This article was published online on 29 January 2013. Article revised due to change in authorship. Please see Note Added in Proof for details.

†F.V. Torres and F. Hansen contributed equally to this work.

Contract grant sponsor: Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq); Contract grant sponsor: Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES); Contract grant sponsor: FINEP/Rede IBN; Contract grant number: 01.06.0842-00; Contract grant sponsor: INCT—National Institute of Science and Technology for Excitotoxicity and Neuroprotection.

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Received 11 July 2012; Revised 14 October 2012; Accepted 15 November 2012

Published online 29 January 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jnr.23187

not bind to N-methyl-D-aspartate receptors, induces convulsive behaviors in mice (Shimamoto et al., 2004).

Astrocytes play a key role in brain energetics (Schousboe et al., 1997; Sonnewald et al., 1997; Voutsinos-Porche et al., 2003; Hertz et al., 2007; Belanger et al., 2011; Pellerin and Magistretti, 2011; Kreft et al., 2012), because glutamate is not cleaved extracellularly and the astrocytic glutamate transporters that are enriched in perisynaptic processes are responsible for the vast majority of glutamate uptake. Recently it has been proposed that GLAST and GLT-1 (Genda et al., 2011; Bauer et al., 2012) exist in macromolecular complexes at the level of fine processes of the astrocytes. These macromolecular complexes could be interpreted as local metabolic units to maintain and optimize the electrochemical gradient of sodium necessary for glutamate uptake in a restricted domain. This fact could better explain the efficiency of astrocytes for supporting costly glutamate cotransport (Na^+) in a spatially restricted perisynaptic processes. Despite the classical description of the glutamate–glutamine cycle, evidence from experiments with primary astrocytic cultures suggests alternative metabolic pathways for glutamate (McKenna et al., 1996; Sonnewald et al., 1997; McKenna, 2007). Probably, the metabolic fate of glutamate depends of the active sodium efflux by Na^+/K^+ ATPase (Voutsinos-Porche et al., 2003). It already has been shown that increased influx of glutamate cotransported with sodium at astrocytes favors its flux to the tricarboxylic acid (TCA) cycle instead of the glutamate–glutamine cycle (Dienel and Cruz, 2006; McKenna, 2007; Kreft et al., 2012). In fact, glutamate can be converted to alpha-ketoglutarate and enter the TCA cycle. Thus, the glutamate backbone can be energetically metabolized (producing ATP) to CO_2 and H_2O. Furthermore, experiments with primary astrocytic cultures show that the percentage of glutamate converted to CO_2 nearly doubles from 5 to 15 min, whereas glutamate uptake tends to be linear (Bauer et al., 2012).

Although some data on metabolic coupling support the idea that some portion of the glucose entering the glycolytic pathway in astrocytes is converted to lactate and released in the extracellular space, with the main purpose of being fuel for neurons (Pellerin and Magistretti, 2011), the lactate shuttle remains controversial because of failure in other laboratories to observe this effect and to prove that it takes place in significant quantities (Mangia et al., 2011; Dienel, 2012a,b). In addition, some evidence suggests that glucose oxidation still provides most of the astrocytic energy supply (Hertz et al., 2007; Belanger et al., 2011). Although metabolic coupling can be considered an emergent field of research with important recent discoveries, some basic aspects of glutamate metabolism still have not been characterized for brain tissue, preserving the interconnectivity between astroglia and neurons, and glucose many times is remembered as the main energetic substrate for the brain independent of its state of activity. Therefore, the aim of this study was to investigate whether the presence of extracellular glutamate in different concentrations and TFB-TBOA, a competitive blocker of glial glutamate transporters, is able to modulate the use of glutamate and glucose as energetic substrates. For this purpose, isolated hippocampi of mice were incubated with radiolabeled substrates, and CO_2 radioactivity and extracellular lactate were measured. Cellular integrity and metabolic viability were assessed by extracellular lactater dehydrogenase (LDH) and methylthiazolyldiphenyl-tetrazolium bromide (MTT) assays, respectively.

MATERIALS AND METHODS

Chemicals

L-glutamic acid (monosodium salt) and MTT were purchased from Sigma (St. Louis, MO). TFB-TBOA was purchased from Tocris Bioscience (Bristol, United Kingdom). D-[U-^{14}C]glucose (300 mCi/mmol) and L-[U-^{14}C]glutamic acid (260 mCi/mmol) were obtained from Perkin-Elmer (Boston, MA). All other chemicals were purchased from local commercial suppliers.

Animals

Male Swiss albino mice (60–90 days old, weighing 35–45 g), obtained from our own breeding colony (Federal University of Rio Grande do Sul, Brazil), were used. Animals were kept in a temperature-regulated room (22°C ± 1°C), on a 12-hr light/12-hr dark cycle, five per cage, with food and water ad libitum and air relative humidity was kept at 55% ± 5%.

Tissue Preparation

Mice were killed by decapitation, and the hippocampi were dissected, weighed, and randomly distributed to perform the incubation. Our institutional protocols for experiments with animals (''guidelines for animal care''), designed to avoid suffering and limit the number of animals used, were followed throughout. The methodology of this study was in strict accordance with the National Institutes of Health *Guide for the care and use of laboratory animals*.

Oxidation to CO_2 of Radiolabeled Substrates

For the purposes described above, each intact hippocampus (weighing 12–18 mg) was incubated in 1.0 ml Dulbecco's medium, pH 7.3, 5.0 mM D-glucose plus glutamate and/or TFB-TBOA as adequate. These assays were conducted with radiolabeled substrates, 0.2 μCi/ml D-[U-^{14}C]glucose or 0.2 μCi/ml L-[U-^{14}C]glutamate. Before incubation, the reaction medium was gassed with O_2 for 30 sec. Flasks were sealed with rubber caps and parafilm. Glass center wells containing a folded 60 mm/4 mm piece of Whatman 3 filter paper were hung from the stoppers. Hippocampi were incubated at 37°C in a Dubnoff metabolic shaker (60 cycles/min), according to a previously described method (Schmidt et al., 2010). Incubations were stopped by adding 0.2 ml of 50% w/w trichloroacetic acid solution through the rubber cap. Subsequently, 0.1 ml of 2.0 M sodium hydroxide was injected into the central wells. The flasks were shaken for an additional 30 min at 37°C to trap CO_2 at the central well. Afterward, the CO_2 radioactivity was measured in a liquid scintillation counter.

The calculation of the amount of oxidized glucose was made through the measurement of radioactivity in 20 μl medium (input) in cpm. Once we had the cpm value and with 5

mM the glucose concentration in the medium (100,000 pmol of glucose for each 20 μl), the amount of CO_2 trapped at the central well was calculated as 100,000 × (central well cpm)/(input cpm × weight of tissue). For calculation of the amount of glutamate oxidized to CO_2, the same process was used, with the reservation that the amount of glutamate in each 20 μl of medium was dependent on the group (10, 100, or 1,000 μM glutamate).

Extracellular Lactate Content

To estimate lactate release, hippocampi were incubated under the same conditions of oxidization experiments, and an aliquot of 50 μl of the medium was sampled from each well at 30, 60, and 120 min of incubation. Extracellular lactate content was measured using a lactate assay kit (Katal Biotecnologica), according to the manufacturer's instructions. The calculation of the amount of lactate was made through the calibration factor generated by using a 4.44 μmol/ml lactate standard solution provided by the manufacturer in a proportion of 1:100 (lactate solution:reagent), as

$$\text{Calibration factor} = 4.44 \div (\text{standard solution absorbance})$$

$$\text{Lactate concentration} = (\text{sample absorbance}) \times \text{calibration factor.}$$

Despite the manufacturer's instructions, we also performed a calibration curve (five duplicated points) for each plate used for reading absorbances in order to assess linearity for the range of results found.

Cell Integrity and Metabolic Viability Assays

Cell integrity was assessed by LDH activity in the medium after 120 min of incubation. LDH activity was determined by a colorimetric commercial kit (from Doles), according to the manufacturer's instructions. Viability assay was assessed by the colorimetric MTT method (Hansen et al., 1989). Briefly, at 120 min of incubation, 0.5 mg/mL MTT was added to each well and it kept at a constant temperature of 37°C for 30 min. The formazan product generated during the incubation was solubilized in dimethylsulfoxide and measured at 560 and 630 nm. For these experiments, the data were normalized by the group with glucose and in the absence of glutamate in which the reaction medium was gassed with O_2 for 30 sec before incubation and again at 30, 60 and 90 min to overcome the lack of oxygen supply.

Statistical Analysis

All data were expressed as mean ± SEM, and for all analyses $P < 0.05$ was considered significant.

Oxidation data. The analyses were conducted using one-way or two-way ANOVA as necessary, followed respectively by Tukey's post hoc test or simple main effects test (SMET) if an interaction was present.

Extracellular lactate data. These data were analyzed by repeated-measures ANOVA with between-subjects factors (degrees of freedom were adjusted by Huynh-Feldt epsilon) or one-way ANOVA as necessary.

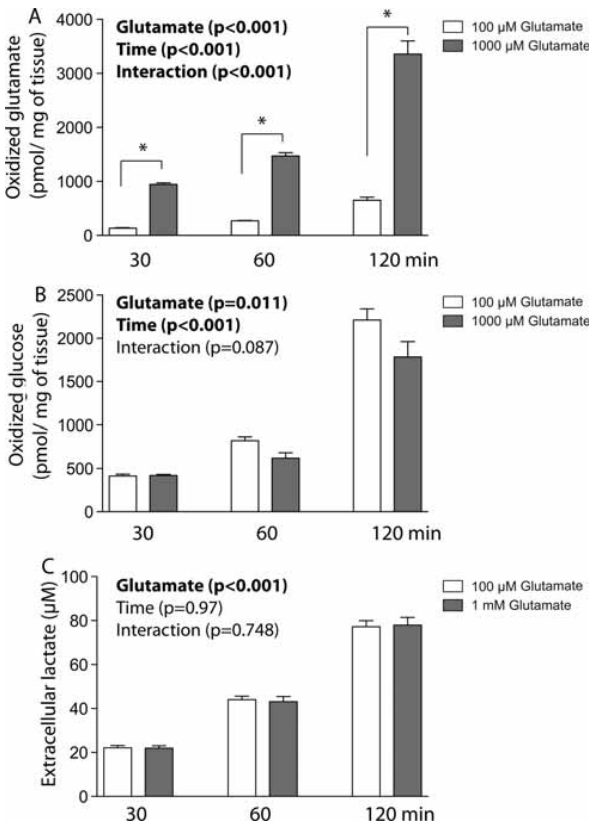

Fig. 1. Glutamate/glucose oxidation to CO_2 and extracellular lactate content at 30, 60, and 120 min of incubation. **A:** Glutamate oxidation. A tenfold increase in extracellular glutamate concentration promotes a similar increase in its oxidation. N = 14 per group in treatments with 100 μM glutamate, and N = 7 per group for treatments with 1 mM glutamate. **B:** Glucose oxidation. A tenfold increase in extracellular glutamate concentration diminishes glucose oxidation, with a trend toward being more prominent after 60 min (interaction time × glutamate, $P = 0.087$). N = 14 per group, except for the group with 1 mM glutamate N = 7. **C:** Extracellular lactate content. Extracellular lactate shows a linear profile and seems not to be altered by an increase in extracellular glutamate. Data are expressed as mean ± SEM and were compared by two-way ANOVA for factors time and glutamate concentration (A,B) and repeated-measures ANOVA (C). None of the measures was normalized by time.

Cell integrity and metabolic viability data. The analyses were conducted by one-way ANOVA, followed by Tukey's post hoc test.

RESULTS

To determine whether extracellular glutamate levels are related to modulation of the oxidative metabolism in mouse hippocampi, we added 100 or 1,000 μM glutamate in Dulbecco's medium and measured CO_2 production from glucose and glutamate at 30, 60, and 120 min of incubation. In response, glutamate oxidation had a linear behavior and was shown to be dependent on time and

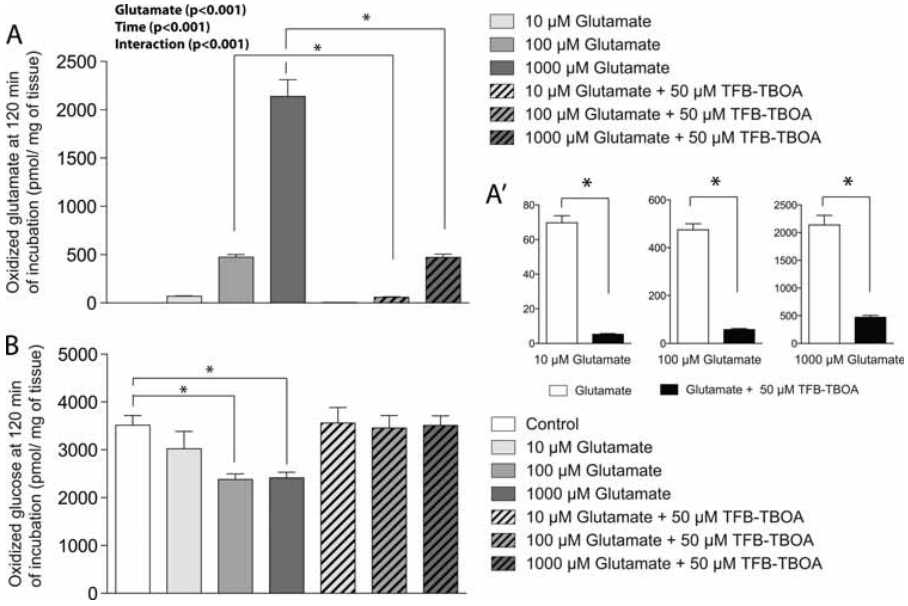

Fig. 2. TFB-TBOA blocks the effect of extracellular glutamate in glucose/glutamate oxidation. **A:** Glutamate oxidation in the presence or absence of TFB-TBOA. The glutamate uptake inhibitor was effective in diminishing glutamate oxidation, being responsible for 92%, 88%, and 78% of inhibition for extracellular concentrations of 10, 100, and 1,000 μM glutamate, respectively. Because of the positive interaction, comparison of data at each level of extracellular glutamate concentration was performed and an effect was found for TFB-TBOA for groups with 100 and 1,000 μM glutamate (*$P < 0.001$) but not for 10 μM glutamate ($P = 0.536$). **A′:** Data from A was split for better visualization of the effect of the competitive inhibitor TFB-TBOA for each glutamate concentration (*$P < 0.001$, independent samples *t*-test). **B:** Glucose oxidation in the presence or absence of TFB-TBOA. The presence of 100 and 1,000 μM glutamate diminished glucose oxidation, which did not occur in the presence of TFB-TBOA (*groups differ from control group by $P < 0.001$). N = 7 per group (for both panels). Data are expressed as mean ± SEM and were compared by two-way ANOVA for factors glutamate concentration and presence or not of TFB-TBOA, followed by SMET (A), independent samples *t*-test (A′), and one-way ANOVA followed by Tukey's post hoc test (B). None of the measures was normalized by time.

extracellular glutamate. The tenfold increase in glutamate concentration caused a proportionate tenfold increase in glutamate oxidation (groups differ by treatment and time in all pairwise comparison, $P < 0.001$, two-way ANOVA followed by SMET; Fig. 1A). Although the glutamate oxidation is increased according to its extracellular levels, our findings point to an inverse effect in the glucose oxidation, with a trend to be more prominent along time (Fig. 1B). At 120 min, the decrease in glucose oxidation was about 20%. Measurement of extracellular lactate levels under the same conditions showed a linear increase along time of incubation, but no effect was associated with the extracellular glutamate concentration (Fig. 1C).

To determine whether this metabolic effect of extracellular glutamate is reversed by TFB-TBOA, we used the incubation period of 120 min, for which the differences in glucose oxidation seemed to be greater than with the briefer periods studied. When the uptake of glutamate by astrocytes was diminished by the presence of 50 μM TFB-TBOA, the glutamate oxidation was strongly suppressed (Fig. 2A,A′) and glucose oxidation to CO_2 returned to levels similar to when there was no additional glutamate in the medium (Fig. 2B).

It is important to note that, in Figure 2A, comparison of data at each level of extracellular glutamate concentration was performed, and an effect for TFB-TBOA was found for groups with 100 and 1,000 μM glutamate ($P < 0.001$), but not for 10 μM glutamate ($P = 0.536$). This can be easily explained because of the huge increase in the absolute difference of means at each level of glutamate concentration (10 μM 65 pmol, 100 μM 420 pmol, and 1,000 μM 1,665 pmol of oxidized glutamate), despite the fact that the more prominent inhibition occurred for the lowest concentration, as seen in Figure 2A′.

Despite the effects of the blockade of glutamate uptake by TFB-TBOA described above, no differences in extracellular lactate content were detected in association with its presence in the media (Fig. 3). Finally, to assess the possibility of cellular damage by the conditions of incubation used in this work, we performed extracellular LDH and MTT assays for 120 min, which did not suggest impairment of cell integrity or metabolic viability of the intact mice hippocampi being caused by glutamate, by TFB-TBOA, or by the hermetic sealing of the wells (Fig. 4).

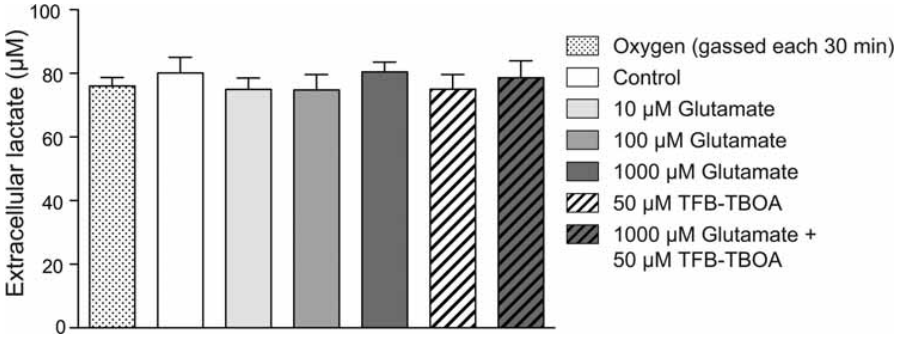

Fig. 3. Extracellular lactate content. None of the treatments promoted a significant change in extracellular lactate concentration at 120 min of incubation. N = 10 per group, except for group with 100 μM glutamate N = 11. Data are expressed as mean ± SEM and were compared by one-way ANOVA.

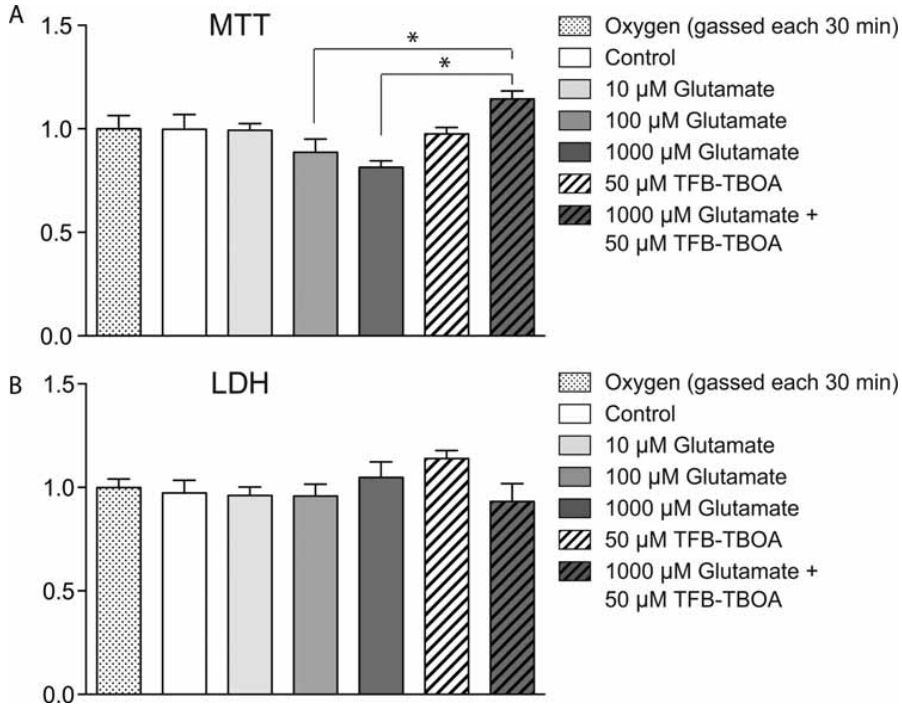

Fig. 4. Cellular integrity and metabolic viability. **A:** MTT assay. Despite 120 min of incubation using hermetic sealing, metabolic viability does not seem to be impaired compared with incubation that receives additional oxygen every 30 min. None of the treatments differs from oxygen or control groups. *Groups differ from 1,000 μM glutamate + 50 μM TFB-TBOA (P = 0.003, one-way ANOVA, followed by Tukey's post hoc test). **B:** Extracellular LDH. Despite 120 min of incubation using hermetic sealing, cell integrity does not seem to be impaired compared with incubation that receives additional oxygen every 30 min. For both assays, all groups did not differ from control groups (with or without additional oxygen).

DISCUSSION

High glutamate concentrations at the synaptic cleft are related to excitotoxic conditions, but elevated glutamate concentrations in the extracellular medium do not necessarily cause alterations from excitotoxicity, because in these cases the glutamate transporters can efficiently perform the clearance, maintaining low glutamate levels in the synaptic cleft (Herman and Jahr, 2007). In addition to the glutamate concentrations in the synaptic cleft during low activity states remaining controversy (if at

μM or nM range), recent studies have demonstrated that the glutamate concentrations in the interstitial fluid inside the tissue (more than 30 μm deep) are very different from those present in the incubation/perfusion medium (Gueler et al., 2007). Our findings using the entire hippocampus isolated from mice and different glutamate concentrations (10 μM to 1 mM) plus the presence or absence of TFB-TBOA demonstrated that the metabolic viability and cellular integrity were not different from control until 120 min (Fig. 4), which was not expected because these are considered potentially excitotoxic conditions.

According to our results, glutamate oxidation increases with time for each glutamate concentration, with time-dependent effects of concentration (progressively from 10 to 1,000 μM; Figs. 1A, 2A). These results suggest that, with increased time and higher amounts of glutamate, glucose oxidation is reduced, presumably because of glutamate being used as fuel (Figs. 1B, 2B). The decrease in glucose oxidation could mean that the flux of glutamate to the TCA cycle may have a role for buffering or for preventing glucose depletion in the brain. Glutamate oxidation could also help to supply the ATP spent for its uptake, i.e., ATP for Na^+ extrusion. Furthermore, TFB-TBOA blocked glutamate oxidation and reversed the glutamate effect on glucose oxidation, suggesting astrocytic participation in this effect. The concentration-dependent increase in glutamate oxidation supports the existence of a very effective glutamate transportation and oxidative system, which seems to have a large response curve, probably not achieving saturation under physiological conditions.

In fact, published data support the idea that even high amounts of extracellular glutamate are efficiently removed by astrocytes, probably to avoid excessive glutamate at the synaptic cleft and prevent excessive neuronal stimulation (Gueler et al., 2007; Herman and Jahr, 2007). Increased glycolysis by astrocytes in the presence of glutamate has been shown using astrocytic culture (Pellerin and Magistretti, 2011), but its relevance remains controversial because of the lack of evidence supporting the idea that the lactate shuttle takes place in significant quantities in the brain (Mangia et al., 2011; Dienel, 2012a,b). Furthermore, the observations regarding astrocytic cultures do not reflect exactly what occurs in the CNS in the presence of neuron–astrocyte integration. Our results point to a diminished oxidation of glucose by increasing the extracellular glutamate concentration (and oxidation). A similar modulation has previously been described, showing that an increase in extracellular glutamate concentration from 0.1 to 0.5 mM in astrocyte cultures stimulates respiration by 50% and inhibits glucose utilization by 20% (Dienel and Cruz, 2006). We could further hypothesize that this is due to recruitment of intra-astrocytic pool of macromolecular complexes to astrocytic processes, regulated by the energy expenditure and/or by the interaction between astrocytes and neurons. This could act as a neuroprotective mechanism to prevent glutamate spillover in the brain; it seems to depend on sustained high extracellular concentrations of glutamate. Moreover, glutamate flux to the TCA cycle is a more energy-efficient mechanism than the glutamate–glutamine cycle.

We found high glycolytic activity in our experiments, but this was not associated with the presence of increased extracellular glutamate. The calculated lactate accumulation rate (analysis of extracellular fluid) for our experiments was about 45 pmol/min/mg; the rate of release of CO_2 coming from glucose was about 23 pmol/min/mg, and for CO_2 coming from glutamate it was 0.5, 4, and 23 pmol/min/mg, for 10, 100, and 1,000 μM of extracellular concentration, respectively. The high glycolytic activity can be explained by post-ischemic tissue being incubated in a large extracellular volume, which contributes to passive transport out of the cell and permanent dilution in the medium. A second candidate explanation is some leakage of lactate to the extracellular space coming from lymphatic vessels that should be draining this lactate out of the brain in vivo, with a purpose other than feeding brain tissue. In fact, there is some evidence that cerebral lymphatic drainage plays a role in the control of oxidative injury (Sun et al., 2011) and that some of the lactate released could travel by perivascular lymphatic vessels (Dienel and Cruz, 2008). However, perhaps the slope of extracellular lactate along time can explain the absence of deterioration of cellular viability for the studied parameters, because some studies have shown a positive effect of high levels of extracellular lactate in brain injury (Levasseur et al., 2006; Alessandri et al., 2012). Taken together, our findings argue against the lactate shuttle theory, because glutamate did not cause any detectable increase in extracellular lactate content, or presumably in glycolysis, and because the glutamate is being used as fuel instead of going to glutamine and back to neurons. This conclusion is also supported by previous work showing that astrocytes were not glycolytic after glutamate addition but were glycolytic if they were exposed to the nonmetabolizable D-aspartate (Peng et al., 2001).

ACKNOWLEDGMENTS

We thank Dr. Marcos Luiz Santos Perry, at UFRGS, for help in the development of the project.

NOTE ADDED IN PROOF

Diogo O Souza has been removed from the list of authors at his request and with the agreement of the authors of this article because he did not make substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data in this study and therefore does not qualify for authorship.

REFERENCES

Alessandri B, Schwandt E, Kamada Y, Nagata M, Heimann A, Kempski O. 2012. The neuroprotective effect of lactate is not due to improved glutamate uptake after controlled cortical impact in rats. J Neurotrauma 29:2181–2191.

Bauer DE, Jackson JG, Genda EN, Montoya MM, Yudkoff M, Robinson MB. 2012. The glutamate transporter, GLAST, participates in a macromolecular complex that supports glutamate metabolism. Neurochem Int (in press).

Belanger M, Allaman I, Magistretti PJ. 2011. Brain energy metabolism: focus on astrocyte–neuron metabolic cooperation. Cell Metab 14:724–738.

Choi DW. 1992. Excitotoxic cell death. J Neurobiol 23:1261–1276.

Danbolt NC. 2001. Glutamate uptake. Prog Neurobiol 65:1–105.

Dienel GA. 2012a. Brain lactate metabolism: the discoveries and the controversies. J Cereb Blood Flow Metab 32:1107–1138.

Dienel GA. 2012b. Fueling and imaging brain activation. ASN Neuro (in press).

Dienel GA, Cruz NF. 2006. Astrocyte activation in working brain: energy supplied by minor substrates. Neurochem Int 48:586–595.

Dienel GA, Cruz NF. 2008. Imaging brain activation: simple pictures of complex biology. Ann N Y Acad Sci 1147:139–170.

Genda EN, Jackson JG, Sheldon AL, Locke SF, Greco TM, O'Donnell JC, Spruce LA, Xiao R, Guo W, Putt M, Seeholzer S, Ischiropoulos H, Robinson MB. 2011. Cocompartmentalization of the astroglial glutamate transporter, GLT-1, with glycolytic enzymes and mitochondria. J Neurosci 31:18275–18288.

Gueler N, Kukley M, Dietrich D. 2007. TBOA-sensitive uptake limits glutamate penetration into brain slices to a few micrometers. Neurosci Lett 419:269–272.

Hansen MB, Nielsen SE, Berg K. 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 119:203–210.

Herman MA, Jahr CE. 2007. Extracellular glutamate concentration in hippocampal slice. J Neurosci 27:9736–9741.

Hertz L, Peng L, Dienel GA. 2007. Energy metabolism in astrocytes: high rate of oxidative metabolism and spatiotemporal dependence on glycolysis/glycogenolysis. J Cereb Blood Flow Metab 27:219–249.

Huang R, Sochocka E, Hertz L. 1997. Cell culture studies of the role of elevated extracellular glutamate and K^+ in neuronal cell death during and after anoxia/ischemia. Neurosci Biobehav Rev 21:129–134.

Kreft M, Bak LK, Waagepetersen HS, Schousboe A. 2012. Aspects of astrocyte energy metabolism, amino acid neurotransmitter homeostasis and metabolic compartmentation. ASN Neuro (in press).

Levasseur JE, Alessandri B, Reinert M, Clausen T, Zhou Z, Altememi N, Bullock MR. 2006. Lactate, not glucose, up-regulates mitochondrial oxygen consumption both in sham and lateral fluid percussed rat brains. Neurosurgery 59:1122–1130; discussion 1130–1121.

Mangia S, DiNuzzo M, Giove F, Carruthers A, Simpson IA, Vannucci SJ. 2011. Response to "Comment on recent modeling studies of astrocyte–neuron metabolic interactions": much ado about nothing. J Cereb Blood Flow Metab 31:1346–1353.

McKenna MC. 2007. The glutamate–glutamine cycle is not stoichiometric: fates of glutamate in brain. J Neurosci Res 85:3347–3358.

McKenna MC, Sonnewald U, Huang X, Stevenson J, Zielke HR. 1996. Exogenous glutamate concentration regulates the metabolic fate of glutamate in astrocytes. J Neurochem 66:386–393.

Meldrum BS. 2000. Glutamate as a neurotransmitter in the brain: review of physiology and pathology. J Nutr 130(Suppl 4S):1007S–1015S.

Pellerin L, Magistretti PJ. 2011. Sweet sixteen for ANLS. J Cereb Blood Flow Metab (in press).

Peng L, Swanson RA, Hertz L. 2001. Effects of L-glutamate, D-aspartate, and monensin on glycolytic and oxidative glucose metabolism in mouse astrocyte cultures: further evidence that glutamate uptake is metabolically driven by oxidative metabolism. Neurochem Int 38:437–443.

Schmidt B, de Assis AM, Battu CE, Rieger DK, Hansen F, Sordi F, Longoni A, Hoefel AL, Farina M, Goncalves CA, Souza DO, Perry ML. 2010. Effects of glyoxal or methylglyoxal on the metabolism of amino acids, lactate, glucose and acetate in the cerebral cortex of young and adult rats. Brain Res 1315:19–24.

Schousboe A, Waagepetersen HS. 2005. Role of astrocytes in glutamate homeostasis: implications for excitotoxicity. Neurotox Res 8:221–225.

Schousboe A, Sonnewald U, Civenni G, Gegelashvili G. 1997. Role of astrocytes in glutamate homeostasis. Implications for excitotoxicity. Adv Exp Med Biol 429:195–206.

Shimamoto K, Sakai R, Takaoka K, Yumoto N, Nakajima T, Amara SG, Shigeri Y. 2004. Characterization of novel L-threo-beta-benzyloxyaspartate derivatives, potent blockers of the glutamate transporters. Mol Pharmacol 65:1008–1015.

Sonnewald U, Westergaard N, Schousboe A. 1997. Glutamate transport and metabolism in astrocytes. Glia 21:56–63.

Sun BL, Xie FM, Yang MF, Cao MZ, Yuan H, Wang HT, Wang JR, Jia L. 2011. Blocking cerebral lymphatic drainage deteriorates cerebral oxidative injury in rats with subarachnoid hemorrhage. Acta Neurochir 110(Suppl):49–53.

Szatkowski M, Attwell D. 1994. Triggering and execution of neuronal death in brain ischaemia: two phases of glutamate release by different mechanisms. Trends Neurosci 17:359–365.

Voutsinos-Porche B, Bonvento G, Tanaka K, Steiner P, Welker E, Chatton JY, Magistretti PJ, Pellerin L. 2003. Glial glutamate transporters mediate a functional metabolic crosstalk between neurons and astrocytes in the mouse developing cortex. Neuron 37:275–286.

PARTE 3

3.1. DISCUSSÃO

Nesta tese, nosso objetivo foi investigar parâmetros eletrofisiológicos e comportamentais em animais saudáveis e em modelo de hiperestimulação glutamatérgica do SNC, possibilitando a avaliação dos efeitos da guanosina em ambas situações. Por último, apresentamos experimentos que investigam o comportamento do metabolismo da glicose e do glutamato no hipocampo, um campo que vem se mostrando promissor para investimento em novas estratégias terapêuticas para as injúrias do SNC e que a guanosina pode estar envolvida uma vez que promove o aumento da recaptação astrocitária do glutamato.

No primeiro capítulo, nosso objetivo foi caracterizar a atividade eletroencefalográfica em animais submetidos a modelo de convulsão por agente glutamatérgico, especificamente o AQ, bem como comprovar a diminuição das crises pela guanosina já descrita comportamentalmente, caracterizando a atividade elétrica cerebral nestes animais.

Ao registrar EEG de ratos com eletrodos epidurais encontramos um ritmo teta proeminente, cuja frequência é equivalente à de origem hipocampal, uma vez que é detectado por condução de volume. O ritmo teta está associado a atividade hipocampal durante a atividade cognitiva e exploratória (navegação) e também surge no sono REM, fase do sono em que há a consolidação da memória.

Ao registrar o EEG de ratos tratados com AQ i.c.v., podemos observar, além do traçado de crise epiléptica, uma supressão da atividade oscilatória das ondas teta e um aumento nas ondas gama. Essas alterações foram evitadas nos animais protegidos pela pré-administração de guanosina, e o aumento na atividade das ondas gama não ocorreu mesmo nos animais que tratados com guanosina

convulsionaram após o AQ. Já os animais tratados com MK-801 apesar de não apresentarem crises nem alterações eletroencefalográficas nos 30 minutos antes de receber AQ, apresentaram aumento da atividade gama após. Interessante notar que em outro estudo, Pinault (Pinault, 2008) detectou aumento da atividade em gama pela administração i.p. de MK-801, com platô após 15 minutos de administração, no entanto, foram utilizados registros de eletrodos em área de córtex frontoparietal de ratos. Esquematicamente, podemos representar os achados na Tabela 3, a seguir:

Tabela 3. Efeitos do pré-tratamento com guanosina e MK-801 nas oscilações teta e gama em modelo de convulsão induzida por AQ em ratos.

	Pré-AQ	Pós-AQ sem crise	Pós-AQ com crise
Salina	--	--	Supressão da atividade em teta, aumento em gama
MK-801	Sem alterações.	Aumento da atividade em gama.	--
Guanosina	Sem alterações.	Diminuição intermediária da atividade em teta	Diminuição da atividade em teta.
MK-801 + Guanosina	Sem alterações.	Aceleração das oscilações teta. Aumento da atividade em gama.	--

As alterações detectadas no período peri-ictal das crises são similares às detectadas em outros modelos de epilepsia em animais e in vitro, como o modelo de mTLE por administração de ácido caínico. Além disso, mesmo nos animais que tiveram sua crise convulsiva evitada pela guanosina ou pelo MK-801 parece haver algum grau de perturbação da sincronia entre a atividade elétrica inter-hemisférica, e

bem como algum grau de alteração da onda teta. Tais observações sugerem que mesmo os animais protegidos têm de certo modo sua fisiologia alterada, o que não poderia ser percebido apenas com experimentos comportamentais.

A atividade gama detectada ao EEG parece vir de interneurônios inibitórios e gabaérgicos hipocampais, e alterações no seu padrão de atividade parecem ser um dos fatores predisponentes à crises epilépticas. Bloqueadores dos receptores NMDA são capazes de induzir um aumento na transmissão glutamatérgica mediada por receptores não-NMDA, o que pode ter sido responsável pelo aumento nas oscilações gama detectadas em animais tratados com MK-801 e AQ. Considerando que a temporalidade da ocorrência das ondas gama parecem estar envolvidas na evocação da memória e no desempenho de tarefas dependentes de contexto (Lisman and Jensen 2013, Koster, Friese et al. 2014), a alteração detectada pode afetar a comunicação coordenada entre diferentes regiões cerebrais, com prejuízos sensoriais e cognitivos.

No segundo capítulo, nosso objetivo foi procurar efeitos da guanosina na atividade elétrica cerebral que pudessem ser indícios da sua ação central como causadora dor efeitos comportamentais já descritos. Para isso realizamos teste de Campo Aberto com camundongos em paralelo com a aquisição de sinais de EEG em curva de dose de guanosina, tendo controle positivo e negativo (salina e diazepam, respectivamente).

Ao analisar o efeito da guanosina na tarefa de Campo Aberto bem como a atividade simultânea do EEG, percebemos uma curva de efeito em U com efeito máximo para guanosina i.p. a 60 mg/kg sobre a distância total percorrida e o tempo em atividade locomotora. Tal comportamento de dose-efeito já havia aparecido em outros trabalhos com guanosina e nucleotídeos da guanina (Schmidt, Avila et al.

2005, Schmidt, Bohmer et al. 2010), porém também já foi mostrado que após uma administração de guanosina i.p. os níveis séricos e no líquor crescem proporcionalmente à dose utilizada (Schmidt, Bohmer et al. 2010). A guanosina se mostrou capaz também de causar lentificação do ritmo teta enquanto os animais estavam em repouso, mas não quando estavam em movimento. Isso sugere que a guanosina pode influenciar a geração do ritmo teta sensível à antagonistas muscarínicos. Uma vez que os principais receptores muscarínicos hipocampais são do tipo M1 (Rhee and Bae 1997, Volpicelli and Levey 2004) e que o antagonista seletivo do receptor M1 pirenzipina é capaz de bloquear as oscilações teta hipocampais no repouso (Williams and Kauer 1997), a guanosina poderia estar modulando a atividade de tal receptor. Também já foi mostrado que a geração de ritmo teta sensível à antagonistas muscarínicos é abolido em camundongos *knockout* para a proteína PLC-β1, um transdutor intracelular do receptor M1 ligado à proteína Gq (Shin, Kim et al. 2005). Portanto é plausível considerar a possibilidade de a guanosina modular a via de sinalização dependente de M1-Gαq-PLC-β1.

No terceiro capítulo nosso objetivo foi investigar quantitativamente o efeito da concentração extracelular de glutamato no destino metabólico da glicose e do glutamato. Para isso incubamos hipocampos isolados de camundongos em banho metabólico com diferentes doses de glutamato no meio e com a presença concomitante ou não de TFB-TBOA, um inibidor seletivo dos transportadores de glutamato astrocitários. Com isso nosso intuito foi além de verificar a modulação do destino metabólico dos substratos, verificar o quanto a oxidação de glutamato e glicose pode ser impactada quando evitamos que os astrócitos sejam capazes de recaptar o glutamato presente no meio extracelular.

Para analisar o metabolismo oxidativo de glicose e glutamato no hipocampo, estrutura cerebral sensível a insultos excitatórios, expusemos a estrutura cerebral a concentrações extracelulares de 10, 100 e 1000 μM. No entanto não foram detectadas alterações em relação ao controle na viabilidade celular nem metabólica dentro do período de 120 minutos de exposição mesmo para as doses mais altas, o que em parte não era esperado. Interessante comentar que a técnica utilizada, com a hipocampo de camundongo, dispensou a utilização de fatias, diminuindo o componente de lesão do tecido por trauma mecânico e debris celulares. A estrutura de hipocampo de camundongo tem em média 15±3 mg, com formato aproximadamente cilíndrico, com dimensão de comprimento superando bastante o diâmetro da secção, o que facilita a perfusão tecidual pelo meio de banho utilizado. A oxigenação do banho com oxigênio a 100% antes do início da incubação é outro fato a ser levado em conta.

Os dados de oxidação nos mostraram que o glutamato parece ser captado e oxidado em proporcionalmente à sua concentração extracelular, tendo um aumento praticamente na mesma ordem de grandeza. Em paralelo, parece ocorrer uma discreta diminuição do uso da glicose, presumidamente pelo uso do glutamato como substrato energético. Pode-se considerar que a oxidação de glutamato talvez tenha um papel de repor a energia celular dispendida num ambiente de excitabilidade celular bem como de poupar a glicose disponível. Com o uso de TFB-TBOA, os astrócitos hipocampais têm a recaptação de glutamato impedida, o que impacta significativamente na sua oxidação e faz com que a oxidação de glicose não diminua. Com estes resultados, fica evidente o papel primordialmente astrocitário de recaptação e oxidação do glutamato hipocampal. Além disso, a curva de dose-resposta vista para na oxidação de glutamato sugere que há ampla reserva no

sistema de recaptação e oxidação astrocitária, não atingindo seu ponto de saturação em condições fisiológicas provavelmente.

Nossos resultados não dão suporte à teoria da lançadeira de lactato, proposta de integração metabólica astrocítico-neuronal através do ciclo glutamato-glutamina (Pellerin and Magistretti 1994), uma vez que em ambiente excitatório não houve aumento da oxidação de glicose, mas sim redução. Apesar de não podermos refutar a teoria da lançadeira de lactato, podemos dizer que ao menos dentro do balanço energético global do hipocampo de camundongos sua contribuição não parece ser de grande impacto. Estudos recentes mostraram que os astrócitos são responsáveis por cerca de 30% do consumo de oxigênio cerebral (Gruetter, Seaquist et al. 2001, Bluml, Moreno-Torres et al. 2002, Lebon, Petersen et al. 2002) e que os astrócitos são responsáveis por no máximo metade da atividade glicolítica cerebral em repouso (Itoh, Abe et al. 2004, Nehlig, Wittendorp-Rechenmann et al. 2004); juntos, estes fatos induzem à conclusão de que caso haja transferência de lactato de astrócitos para neurônios, esta não deve ser quantitativamente relevante. Durante estados de ativação cerebral isto até poderia ser diferente, no entanto nossos resultados apontam no sentido contrário, de que o glutamato sendo progressivamente oxidado seria capaz de diminuir a necessidade de uso de glicose para fins energéticos, mesmo em meio mais excitatório. Além disso, o fato de haver glutamato sendo oxidado mostra que não há como o ciclo glutamato-glutamina ocorrer de maneira estequiométrica. Considerando que o glutamato é recaptado pelo astrócitos em co-transporte com sódio e que a devolução do sódio ao meio extracelular é um processo que espolia ATP, é coerente que ao menos parte do glutamato que seja recaptado destine-se à oxidação. Mais do que isso, o fato de não haver como o cliclo glutamato-glutamina ser estequiométrico nos mostra que há

sempre síntese de glutamato a partir de glicose. De fato, estima-se que 20% da glicose que chega ao cérebro seja utilizada na fabricação de novas moléculas de glutamato, sendo a síntese um processo exclusivamente astrocitário (Hertz and Zielke 2004); no entanto, o fato de boa quantidade de glicose ir a glutamato não significa que há um grande gasto energético para a célula, pois quando o glutamato for oxidado será gerada energia similar à da oxidação da glicose (Hertz 2004).

A oxidação de glutamato aumentada pela adição de glutamato ao meio sugere que esse possa ser um mecanismo de defesa, e vai ao encontro do fato de a quantidade global de glutamato aparentemente ser controlada pelos astrócitos, criando um balanço negativo quando quando há uma tendência a período de menor ativação e um balanço positivo em períodos de maior atividade (Hertz, O'Dowd et al. 2003). Em suma, podemos dizer que quando se trata de cérebro e integração metabólica não podemos falar apenas de glicose nem tampouco apenas de glutamato, e ao mesmo tempo que parece haver uma intercambialidade entre o uso da glicose e do glutamato como substrato energético, parece também haver uma dissociação temporal entre a eliminação e a reposição da quantidade global de glicose/glicogênio e glutamato que pode trazer dificuldades a experimentos quantitativos nesta área.

3.2. CONCLUSÃO

Os resultados obtidos neste trabalho vem a corroborar o papel da guanosina sobre o sistema glutamatérgico e a abrir novas perspectivas para investigação de seus papéis no SNC.

No primeiro capítulo, as diferenças percebidas entre os animais tratados com guanosina e MK-801 sugerem que seus mecanismos de proteção contra a crise induzida por AQ provavelmente se dão por diferente caminhos. E finalmente, podemos assegurar que o efeito de redução pela guanosina das crises tônico-clônicas induzidas por AQ é corroborado pelo registro de EEG. Porém, mesmo animais que foram protegidos das crises pela administração de guanosina ou MK-801 mostraram algum grau de alteração das características basais, não detectadas comportamentalmente nos estudos já realizados.

Estudos farmacodinâmicos e farmacocinéticos também serão necessários para descobrir o motivo de a guanosina não exercer proteção contra crises em todos os animais, mesmo com concentrações crescentes do fármaco, e também para que se possa investigar o fato de nem toda administração ser protetora caso de utilize o mesmo animal novamente após período de eliminação das drogas. Outro fato que permanece instigante é o de a guanosina atingir níveis crescentes no líquor de acordo com a dose administrada mas ter uma curva de dose-resposta em U, encontrada no segundo capítulo desta tese e em outros trabalhos do nosso grupo (Schmidt, Avila et al. 2005, Schmidt, Bohmer et al. 2010).

Os resultados referentes ao segundo capítulo se referem ao achado da diminuição da atividade locomotora durante tarefa de campo aberto com camundongos. Ao analisar o EEG percebemos que a guanosina foi capaz de

produzir lentificação do ritmo teta, o que é compatível com seu efeito ansiolítico que já havia sido descrito. O fato de haver lentificação do ritmo teta apenas em repouso pode sugerir que a guanosina pode pelo menos em parte intervir na via de sinalização M1-Gαq-PLC-β1, que envolve receptor muscarínico associado à proteína-Gq, diferentemente do que já se constatou até o momento para os efeitos neuroprotetores e neurotróficos, cujos mecanismos parecem envolver algum tipo de receptor ou via associada à proteína Gi e/ou o aumento da recaptação de glutamato por astrócitos.

Os achados expostos no terceiro capítulo corroboram a importância da modulação do metabolismo do glutamato quanto maior se torna a concentração extracelular de glutamato, um efeito que deve ser principalmente astrocitário e, apesar disso, parece ser responsável por grande fração do metabolismo oxidativo cerebral.

A guanosina presente no meio extracelular parece modular diversas funções a nível celular e que parecem se exercer por mensageiros secundários ligados à um receptor metabotrópico que ainda não se descobriu, algumas evidências já mostraram efeitos que são compatíveis com modulação de via associada a receptor acoplado à proteína Gi, bem como alguns dos nossos resultados nesta tese apontam para efeitos compatíveis com modulação de receptor (ou via) associado à proteína Gαq. Além disso, postula-se que boa parte do seu efeito neuroprotetor em resposta à a hiperativação e toxicidade glutamatérgica seja astrocitário, por seu efeito na captação de glutamato. Especula-se esteja também na mobilização ou modulação dos transportadores de glutamato astrocitários - GLAST e GLT-1, na superfície celular ou talvez ainda no destino intracelular do glutamato após ser recaptado.

Com os trabalhos ralizados nesta tese, buscamos acrescentar ao grupo de pesquisa o estudo eletrofisiológico do cérebro como aliado à compreensão dos efeitos bioquímicos usualmente trabalhados. György Buzsáki, autor do livro “Rhythms of the Brain” e de longo currículo na eletrofisiologia cerebral, postula que o código neural está embutido nos ritmos elétricos cerebrais; é através de potenciais elétricos que a informação navega dentro do cérebro. No entanto, é através da sinapses químicas que a informação é processada. Nada mais justo que buscar a investigação desses dois mundos em paralelo.

3.3. PERSPECTIVAS

Permanece como perspectiva para próximos trabalhos a investigação dos efeitos da guanosina na atividade elétrica a nível celular, através de implante de eletrodos de profundidade *in vivo* para registro de potenciais de campo locais e de *patch-clamp in vitro*, com possibilidade de administração de fármacos concomitantemente aos registros através de microdiálise ou adição de fármacos ao meio de perfusão. Além disso, há de ser explorado o potencial efeito da guanosina no metabolismo do glutamato (e da glicose, intrinsecamente), dada o grande entrelaçamento de seus efeitos com o sistema glutamatérgico e por promover o aumento da recaptação de glutamato pelos astrócitos.

3.4. REFERÊNCIAS BIBLIOGRÁFICAS

Allen, N. J., R. Karadottir and D. Attwell (2004). "Reversal or reduction of glutamate and GABA transport in CNS pathology and therapy." Pflugers Arch 449(2): 132-142.

Andersson, M. and E. Hanse (2010). "Astrocytes impose postburst depression of release probability at hippocampal glutamate synapses." J Neurosci 30(16): 5776-5780.

Arabadzisz, D., K. Antal, F. Parpan, Z. Emri and J. M. Fritschy (2005). "Epileptogenesis and chronic seizures in a mouse model of temporal lobe epilepsy are associated with distinct EEG patterns and selective neurochemical alterations in the contralateral hippocampus." Exp Neurol 194(1): 76-90.

Arriza, J. L., S. Eliasof, M. P. Kavanaugh and S. G. Amara (1997). "Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance." Proc Natl Acad Sci U S A 94(8): 4155-4160.

Banaclocha, M. A. (2007). "Neuromagnetic dialogue between neuronal minicolumns and astroglial network: a new approach for memory and cerebral computation." Brain Res Bull 73(1-3): 21-27.

Bauer, D. E., J. G. Jackson, E. N. Genda, M. M. Montoya, M. Yudkoff and M. B. Robinson (2012). "The glutamate transporter, GLAST, participates in a macromolecular complex that supports glutamate metabolism." Neurochem Int.

Beart, P. M. and R. D. O'Shea (2007). "Transporters for L-glutamate: an update on their molecular pharmacology and pathological involvement." Br J Pharmacol 150(1): 5-17.

Belanger, M., I. Allaman and P. J. Magistretti (2011). "Brain energy metabolism: focus on astrocyte-neuron metabolic cooperation." Cell Metab 14(6): 724-738.

Bland, B. H. (1986). "The physiology and pharmacology of hippocampal formation theta rhythms." Prog Neurobiol 26(1): 1-54.

Blume, W. T. (2006). "Drug effects on EEG." J Clin Neurophysiol 23(4): 306-311.

Bluml, S., A. Moreno-Torres, F. Shic, C. H. Nguy and B. D. Ross (2002). "Tricarboxylic acid cycle of glia in the in vivo human brain." NMR Biomed 15(1): 1-5.

Boehm, J. (2013). "A 'danse macabre': tau and Fyn in STEP with amyloid beta to facilitate induction of synaptic depression and excitotoxicity." Eur J Neurosci 37(12): 1925-1930.

Bradford, H. F. (1995). "Glutamate, GABA and epilepsy." Prog Neurobiol 47(6): 477-511.

Brankack, J., V. I. Kukushka, A. L. Vyssotski and A. Draguhn (2010). "EEG gamma frequency and sleep-wake scoring in mice: comparing two types of supervised classifiers." Brain Res 1322: 59-71.

Buzsaki, G. (2002). "Theta oscillations in the hippocampus." Neuron 33(3): 325-340.

Buzsaki, G. and A. Draguhn (2004). "Neuronal oscillations in cortical networks." Science 304(5679): 1926-1929.

Chao, X. D., F. Fei and Z. Fei (2010). "The role of excitatory amino acid transporters in cerebral ischemia." Neurochem Res 35(8): 1224-1230.

Chapman, A. G., K. Nanan, M. Williams and B. S. Meldrum (2000). "Anticonvulsant activity of two metabotropic glutamate group I antagonists selective for the mGlu5 receptor: 2-methyl-6-(phenylethynyl)-pyridine (MPEP), and (E)-6-methyl-2-styryl-pyridine (SIB 1893)." Neuropharmacology 39(9): 1567-1574.

Chaudhry, F. A., K. P. Lehre, M. van Lookeren Campagne, O. P. Ottersen, N. C. Danbolt and J. Storm-Mathisen (1995). "Glutamate transporters in glial plasma membranes: highly differentiated localizations revealed by quantitative ultrastructural immunocytochemistry." Neuron 15(3): 711-720.

Choi, D. W. (1992). "Excitotoxic cell death." J Neurobiol 23(9): 1261-1276.

Choi, D. W. and S. M. Rothman (1990). "The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death." Annu Rev Neurosci 13: 171-182.

Ciccarelli, R., P. Ballerini, G. Sabatino, M. P. Rathbone, M. D'Onofrio, F. Caciagli and P. Di Iorio (2001). "Involvement of astrocytes in purine-mediated reparative processes in the brain." Int J Dev Neurosci 19(4): 395-414.

Ciccarelli, R., P. Di Iorio, I. D'Alimonte, P. Giuliani, T. Florio, F. Caciagli, P. J. Middlemiss and M. P. Rathbone (2000). "Cultured astrocyte proliferation induced by extracellular guanosine involves endogenous adenosine and is raised by the co-presence of microglia." Glia 29(3): 202-211.

Ciccarelli, R., P. Di Iorio, P. Giuliani, I. D'Alimonte, P. Ballerini, F. Caciagli and M. P. Rathbone (1999). "Rat cultured astrocytes release guanine-based purines in basal conditions and after hypoxia/hypoglycemia." Glia 25(1): 93-98.

Czurko, A., H. Hirase, J. Csicsvari and G. Buzsaki (1999). "Sustained activation of hippocampal pyramidal cells by 'space clamping' in a running wheel." Eur J Neurosci 11(1): 344-352.

Dal-Cim, T., S. Molz, J. Egea, E. Parada, A. Romero, J. Budni, M. D. Martin de Saavedra, L. del Barrio, C. I. Tasca and M. G. Lopez (2012). "Guanosine protects human neuroblastoma SH-SY5Y cells against mitochondrial oxidative stress by inducing heme oxigenase-1 via PI3K/Akt/GSK-3beta pathway." Neurochem Int 61(3): 397-404.

Danbolt, N. C. (1994). "The high affinity uptake system for excitatory amino acids in the brain." Prog Neurobiol 44(4): 377-396.

Danbolt, N. C. (2001). "Glutamate uptake." Prog Neurobiol 65(1): 1-105.

Davis, K. E., D. J. Straff, E. A. Weinstein, P. G. Bannerman, D. M. Correale, J. D. Rothstein and M. B. Robinson (1998). "Multiple signaling pathways regulate cell surface expression and activity of the excitatory amino acid carrier 1 subtype of Glu transporter in C6 glioma." J Neurosci 18(7): 2475-2485.

Di Iorio, P., P. Ballerini, U. Traversa, F. Nicoletti, I. D'Alimonte, S. Kleywegt, E. S. Werstiuk, M. P. Rathbone, F. Caciagli and R. Ciccarelli (2004). "The antiapoptotic effect of guanosine is mediated by the activation of the PI 3-kinase/AKT/PKB pathway in cultured rat astrocytes." Glia 46(4): 356-368.

Dienel, G. A. (2012). "Brain lactate metabolism: the discoveries and the controversies." J Cereb Blood Flow Metab 32(7): 1107-1138.

Dienel, G. A. (2013). "Astrocytic energetics during excitatory neurotransmission: What are contributions of glutamate oxidation and glycolysis?" Neurochem Int 63(4): 244-258.

Dirnagl, U., C. Iadecola and M. A. Moskowitz (1999). "Pathobiology of ischaemic stroke: an integrated view." Trends Neurosci 22(9): 391-397.

Dong, X. X., Y. Wang and Z. H. Qin (2009). "Molecular mechanisms of excitotoxicity and their relevance to pathogenesis of neurodegenerative diseases." Acta Pharmacol Sin 30(4): 379-387.

Duan, S., C. M. Anderson, B. A. Stein and R. A. Swanson (1999). "Glutamate induces rapid upregulation of astrocyte glutamate transport and cell-surface expression of GLAST." J Neurosci 19(23): 10193-10200.

Dugladze, T., I. Vida, A. B. Tort, A. Gross, J. Otahal, U. Heinemann, N. J. Kopell and T. Gloveli (2007). "Impaired hippocampal rhythmogenesis in a mouse model of mesial temporal lobe epilepsy." Proc Natl Acad Sci U S A 104(44): 17530-17535.

Duzel, E., W. D. Penny and N. Burgess (2010). "Brain oscillations and memory." Curr Opin Neurobiol 20(2): 143-149.

Economo, M. N., J. J. Martinez and J. A. White (2014). "Membrane potential-dependent integration of synaptic inputs in entorhinal stellate neurons." Hippocampus.

El Hage, M., A. Conjard-Duplany, G. Baverel and G. Martin (2011). "Metabolic fate of a high concentration of glutamine and glutamate in rat brain slices: a (1)(3)C NMR study." Neurochem Int 58(8): 896-903.

Engel, A. K., P. Fries and W. Singer (2001). "Dynamic predictions: oscillations and synchrony in top-down processing." Nat Rev Neurosci 2(10): 704-716.

Eulenburg, V. and J. Gomeza (2010). "Neurotransmitter transporters expressed in glial cells as regulators of synapse function." Brain Res Rev 63(1-2): 103-112.

Fairman, W. A., R. J. Vandenberg, J. L. Arriza, M. P. Kavanaugh and S. G. Amara (1995). "An excitatory amino-acid transporter with properties of a ligand-gated chloride channel." Nature 375(6532): 599-603.

Farinelli, S. E. and W. J. Nicklas (1992). "Glutamate metabolism in rat cortical astrocyte cultures." J Neurochem 58(5): 1905-1915.

Fei, Z., X. Zhang, H. M. Bai, X. F. Jiang, X. Li, W. Zhang and W. Hu (2007). "Posttraumatic secondary brain insults exacerbates neuronal injury by altering metabotropic glutamate receptors." BMC Neurosci 8: 96.

Fellous, J. M. and T. J. Sejnowski (2000). "Cholinergic induction of oscillations in the hippocampal slice in the slow (0.5-2 Hz), theta (5-12 Hz), and gamma (35-70 Hz) bands." Hippocampus 10(2): 187-197.

Furness, D. N., Y. Dehnes, A. Q. Akhtar, D. J. Rossi, M. Hamann, N. J. Grutle, V. Gundersen, S. Holmseth, K. P. Lehre, K. Ullensvang, M. Wojewodzic, Y. Zhou, D. Attwell and N. C. Danbolt (2008). "A quantitative assessment of glutamate uptake into hippocampal synaptic terminals and astrocytes: new insights into a

neuronal role for excitatory amino acid transporter 2 (EAAT2)." Neuroscience 157(1): 80-94.

Furuta, A., L. J. Martin, C. L. Lin, M. Dykes-Hoberg and J. D. Rothstein (1997). "Cellular and synaptic localization of the neuronal glutamate transporters excitatory amino acid transporter 3 and 4." Neuroscience 81(4): 1031-1042.

Furuta, A., J. D. Rothstein and L. J. Martin (1997). "Glutamate transporter protein subtypes are expressed differentially during rat CNS development." J Neurosci 17(21): 8363-8375.

Gegelashvili, G., Y. Dehnes, N. C. Danbolt and A. Schousboe (2000). "The high-affinity glutamate transporters GLT1, GLAST, and EAAT4 are regulated via different signalling mechanisms." Neurochem Int 37(2-3): 163-170.

Genda, E. N., J. G. Jackson, A. L. Sheldon, S. F. Locke, T. M. Greco, J. C. O'Donnell, L. A. Spruce, R. Xiao, W. Guo, M. Putt, S. Seeholzer, H. Ischiropoulos and M. B. Robinson (2011). "Co-compartmentalization of the astroglial glutamate transporter, GLT-1, with glycolytic enzymes and mitochondria." J Neurosci 31(50): 18275-18288.

Gruetter, R., E. R. Seaquist and K. Ugurbil (2001). "A mathematical model of compartmentalized neurotransmitter metabolism in the human brain." Am J Physiol Endocrinol Metab 281(1): E100-112.

Gysbers, J. W. and M. P. Rathbone (1992). "Guanosine enhances NGF-stimulated neurite outgrowth in PC12 cells." Neuroreport 3(11): 997-1000.

Gysbers, J. W. and M. P. Rathbone (1996). "Neurite outgrowth in PC12 cells is enhanced by guanosine through both cAMP-dependent and -independent mechanisms." Neurosci Lett 220(3): 175-178.

Herman, M. A. and C. E. Jahr (2007). "Extracellular glutamate concentration in hippocampal slice." J Neurosci 27(36): 9736-9741.

Herrmann, W. M. and E. Schaerer (1990). Pharmaco-EEG: computer EEG analysis to describe the projection to drug effects on a functional cerebral level in humans. Clinical applications of computer analysis of EEG and other neurophysiological signals. Amsterdam, Elsevier: 385-445.

Hertz, L. (2004). "Intercellular metabolic compartmentation in the brain: past, present and future." Neurochem Int 45(2-3): 285-296.

Hertz, L., B. S. O'Dowd, K. T. Ng and M. E. Gibbs (2003). "Reciprocal changes in forebrain contents of glycogen and of glutamate/glutamine during early memory consolidation in the day-old chick." Brain Res 994(2): 226-233.

Hertz, L., L. Peng and G. A. Dienel (2007). "Energy metabolism in astrocytes: high rate of oxidative metabolism and spatiotemporal dependence on glycolysis/ glycogenolysis." J Cereb Blood Flow Metab 27(2): 219-249.

Hertz, L. and H. R. Zielke (2004). "Astrocytic control of glutamatergic activity: astrocytes as stars of the show." Trends Neurosci 27(12): 735-743.

Huang, R., E. Sochocka and L. Hertz (1997). "Cell culture studies of the role of elevated extracellular glutamate and K+ in neuronal cell death during and after anoxia/ischemia." Neurosci Biobehav Rev 21(2): 129-134.

Itoh, Y., T. Abe, R. Takaoka and N. Tanahashi (2004). "Fluorometric determination of glucose utilization in neurons in vitro and in vivo." J Cereb Blood Flow Metab 24(9): 993-1003.

Izquierdo, I., L. R. Bevilaqua, J. I. Rossato, J. S. Bonini, J. H. Medina and M. Cammarota (2006). "Different molecular cascades in different sites of the brain control memory consolidation." Trends Neurosci 29(9): 496-505.

Jacobs, J. (2014). "Hippocampal theta oscillations are slower in humans than in rodents: implications for models of spatial navigation and memory." Philos Trans R Soc Lond B Biol Sci 369(1635): 20130304.

Kanai, Y. and M. A. Hediger (1992). "Primary structure and functional characterization of a high-affinity glutamate transporter." Nature 360(6403): 467-471.

Kim, J. K., M. P. Rathbone, P. J. Middlemiss, D. W. Hughes and R. W. Smith (1991). "Purinergic stimulation of astroblast proliferation: guanosine and its nucleotides stimulate cell division in chick astroblasts." J Neurosci Res 28(3): 442-455.

Koster, M., U. Friese, B. Schone, N. Trujillo-Barreto and T. Gruber (2014). "Theta-gamma coupling during episodic retrieval in the human EEG." Brain Res.

Kramis, R., C. H. Vanderwolf and B. H. Bland (1975). "Two types of hippocampal rhythmical slow activity in both the rabbit and the rat: relations to behavior and effects of atropine, diethyl ether, urethane, and pentobarbital." Exp Neurol 49(1 Pt 1): 58-85.

Kreft, M., L. K. Bak, H. S. Waagepetersen and A. Schousboe (2012). "Aspects of Astrocyte Energy Metabolism, Amino Acid Neurotransmitter Homeostasis and Metabolic Compartmentation." ASN Neuro.

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

Leung, L. S. (1998). "Generation of theta and gamma rhythms in the hippocampus." Neurosci Biobehav Rev 22(2): 275-290.

Leung, L. W. (1985). "Spectral analysis of hippocampal EEG in the freely moving rat: effects of centrally active drugs and relations to evoked potentials." Electroencephalogr Clin Neurophysiol 60(1): 65-77.

Lisman, J. E. and O. Jensen (2013). "The theta-gamma neural code." Neuron 77(6): 1002-1016.

Lortet, S., D. Samuel, L. Had-Aissouni, F. Masmejean, L. Kerkerian-Le Goff and P. Pisano (1999). "Effects of PKA and PKC modulators on high affinity glutamate uptake in primary neuronal cell cultures from rat cerebral cortex." Neuropharmacology 38(3): 395-402.

Mallolas, J., O. Hurtado, M. Castellanos, M. Blanco, T. Sobrino, J. Serena, J. Vivancos, J. Castillo, I. Lizasoain, M. A. Moro and A. Davalos (2006). "A

polymorphism in the EAAT2 promoter is associated with higher glutamate concentrations and higher frequency of progressing stroke." J Exp Med 203(3): 711-717.

Maragakis, N. J. and J. D. Rothstein (2004). "Glutamate transporters: animal models to neurologic disease." Neurobiol Dis 15(3): 461-473.

Maragakis, N. J. and J. D. Rothstein (2006). "Mechanisms of Disease: astrocytes in neurodegenerative disease." Nat Clin Pract Neurol 2(12): 679-689.

Matyash, V. and H. Kettenmann (2010). "Heterogeneity in astrocyte morphology and physiology." Brain Res Rev 63(1-2): 2-10.

McGeer, E. G., E. A. Singh and P. L. McGeer (1987). "Sodium-dependent glutamate binding in senile dementia." Neurobiol Aging 8(3): 219-223.

McKenna, M. C. (2007). "The glutamate-glutamine cycle is not stoichiometric: fates of glutamate in brain." J Neurosci Res 85(15): 3347-3358.

McKenna, M. C., U. Sonnewald, X. Huang, J. Stevenson and H. R. Zielke (1996). "Exogenous glutamate concentration regulates the metabolic fate of glutamate in astrocytes." J Neurochem 66(1): 386-393.

McNaughton, N., B. Kocsis and M. Hajos (2007). "Elicited hippocampal theta rhythm: a screen for anxiolytic and procognitive drugs through changes in hippocampal function?" Behav Pharmacol 18(5-6): 329-346.

Meldrum, B. S. (1994). "The role of glutamate in epilepsy and other CNS disorders." Neurology 44(11 Suppl 8): S14-23.

Meldrum, B. S. (2000). "Glutamate as a neurotransmitter in the brain: review of physiology and pathology." J Nutr 130(4S Suppl): 1007S-1015S.

Moldrich, R., A. Chapman, G. De Sarro and B. Meldrum (2003). "Glutamate metabotropic receptors as targets for drug therapy in epilepsy." European journal of pharmacology 476(1-2): 3-16.

Moussa, C. E., C. Rae, W. A. Bubb, J. L. Griffin, N. A. Deters and V. J. Balcar (2007). "Inhibitors of glutamate transport modulate distinct patterns in brain metabolism." J Neurosci Res 85(2): 342-350.

Muller, C. E. and T. Scior (1993). "Adenosine receptors and their modulators." Pharm Acta Helv 68(2): 77-111.

Naylor, D. E. (2010). "Glutamate and GABA in the balance: convergent pathways sustain seizures during status epilepticus." Epilepsia 51 Suppl 3: 106-109.

Nehlig, A., E. Wittendorp-Rechenmann and C. D. Lam (2004). "Selective uptake of [14C]2-deoxyglucose by neurons and astrocytes: high-resolution microautoradiographic imaging by cellular 14C-trajectography combined with immunohistochemistry." J Cereb Blood Flow Metab 24(9): 1004-1014.

Ng, K. T., B. S. O'Dowd, N. S. Rickard, S. R. Robinson, M. E. Gibbs, C. Rainey, W. Q. Zhao, G. L. Sedman and L. Hertz (1997). "Complex roles of glutamate in the Gibbs-Ng model of one-trial aversive learning in the new-born chick." Neurosci Biobehav Rev 21(1): 45-54.

O'Shea, R. D. (2002). "Roles and regulation of glutamate transporters in the central nervous system." Clin Exp Pharmacol Physiol 29(11): 1018-1023.

Oberheim, N. A., T. Takano, X. Han, W. He, J. H. Lin, F. Wang, Q. Xu, J. D. Wyatt, W. Pilcher, J. G. Ojemann, B. R. Ransom, S. A. Goldman and M. Nedergaard (2009). "Uniquely hominid features of adult human astrocytes." J Neurosci 29(10): 3276-3287.

Oberheim, N. A., X. Wang, S. Goldman and M. Nedergaard (2006). "Astrocytic complexity distinguishes the human brain." Trends Neurosci 29(10): 547-553.

Park, J., H. Lee, T. Kim, G. Y. Park, E. M. Lee, S. Baek, J. Ku, I. Y. Kim, S. I. Kim, D. P. Jang and J. K. Kang (2014). "Role of low- and high-frequency oscillations in the human hippocampus for encoding environmental novelty during a spatial navigation task." Hippocampus.

Pedley, T. A. and R. D. Traub (2003). Physiological Basis of the EEG. Current practice of clinical electroencephalography. J. S. Ebersole and T. A. Pedley. Philadelphia, Lippincott Williams & Wilkins.

Pellerin, L. and P. J. Magistretti (1994). "Glutamate uptake into astrocytes stimulates aerobic glycolysis: a mechanism coupling neuronal activity to glucose utilization." Proc Natl Acad Sci U S A 91(22): 10625-10629.

Pellerin, L. and P. J. Magistretti (2011). "Sweet sixteen for ANLS." J Cereb Blood Flow Metab.

Pereira, A., Jr. and F. A. Furlan (2010). "Astrocytes and human cognition: modeling information integration and modulation of neuronal activity." Prog Neurobiol 92(3): 405-420.

Pettifer, K. M., S. Kleywegt, C. J. Bau, J. D. Ramsbottom, E. Vertes, R. Ciccarelli, F. Caciagli, E. S. Werstiuk and M. P. Rathbone (2004). "Guanosine protects SH-SY5Y cells against beta-amyloid-induced apoptosis." Neuroreport 15(5): 833-836.

Pines, G., N. C. Danbolt, M. Bjoras, Y. Zhang, A. Bendahan, L. Eide, H. Koepsell, J. Storm-Mathisen, E. Seeberg and B. I. Kanner (1992). "Cloning and expression of a rat brain L-glutamate transporter." Nature 360(6403): 464-467.

Pow, D. V. and N. L. Barnett (2000). "Developmental expression of excitatory amino acid transporter 5: a photoreceptor and bipolar cell glutamate transporter in rat retina." Neurosci Lett 280(1): 21-24.

Rao, V. L. and C. R. Murthy (1993). "Uptake and metabolism of glutamate and aspartate by astroglial and neuronal preparations of rat cerebellum." Neurochem Res 18(6): 647-654.

Rathbone, M. P., P. Middlemiss, C. Andrew, F. Caciagli, R. Ciccarelli, P. Di Iorio and R. Huang (1998). "The trophic effects of purines and purinergic signaling in pathologic reactions of astrocytes." Alzheimer Dis Assoc Disord 12 Suppl 2: S36-45.

Rathbone, M. P., P. J. Middlemiss, B. DeLuca and M. Jovetich (1991). "Extracellular guanosine increases astrocyte cAMP: inhibition by adenosine A2 antagonists." Neuroreport 2(11): 661-664.

Rathbone, M. P., P. J. Middlemiss, J. W. Gysbers, C. Andrew, M. A. Herman, J. K. Reed, R. Ciccarelli, P. Di Iorio and F. Caciagli (1999). "Trophic effects of purines in neurons and glial cells." Prog Neurobiol 59(6): 663-690.

Rhee, S. G. and Y. S. Bae (1997). "Regulation of phosphoinositide-specific phospholipase C isozymes." J Biol Chem 272(24): 15045-15048.

Robinson, M. B. (1998). "The family of sodium-dependent glutamate transporters: a focus on the GLT-1/EAAT2 subtype." Neurochem Int 33(6): 479-491.

Robinson, M. B. (2002). "Regulated trafficking of neurotransmitter transporters: common notes but different melodies." J Neurochem 80(1): 1-11.

Robinson, M. B. (2006). "Acute regulation of sodium-dependent glutamate transporters: a focus on constitutive and regulated trafficking." Handb Exp Pharmacol(175): 251-275.

Rothstein, J. D. (1996). "Excitotoxicity hypothesis." Neurology 47(4 Suppl 2): S19-25; discussion S26.

Scheffzuk, C., V. I. Kukushka, A. L. Vyssotski, A. Draguhn, A. B. Tort and J. Brankack (2013). "Global slowing of network oscillations in mouse neocortex by diazepam." Neuropharmacology 65: 123-133.

Schmidt, A. P., T. T. Avila and D. O. Souza (2005). "Intracerebroventricular guanine-based purines protect against seizures induced by quinolinic acid in mice." Neurochem Res 30(1): 69-73.

Schmidt, A. P., A. E. Bohmer, C. Schallenberger, C. Antunes, R. G. Tavares, S. T. Wofchuk, E. Elisabetsky and D. O. Souza (2010). "Mechanisms involved in the

antinociception induced by systemic administration of guanosine in mice." Br J Pharmacol 159(6): 1247-1263.

Segovia, G., A. Porras, A. Del Arco and F. Mora (2001). "Glutamatergic neurotransmission in aging: a critical perspective." Mech Ageing Dev 122(1): 1-29.

Sepers, M. D. and L. A. Raymond (2014). "Mechanisms of synaptic dysfunction and excitotoxicity in Huntington's disease." Drug Discov Today.

Sheldon, A. L. and M. B. Robinson (2007). "The role of glutamate transporters in neurodegenerative diseases and potential opportunities for intervention." Neurochem Int 51(6-7): 333-355.

Shin, J. (2002). "A unifying theory on the relationship between spike trains, EEG, and ERP based on the noise shaping/predictive neural coding hypothesis." Biosystems 67(1-3): 245-257.

Shin, J., D. Kim, R. Bianchi, R. K. Wong and H. S. Shin (2005). "Genetic dissection of theta rhythm heterogeneity in mice." Proc Natl Acad Sci U S A 102(50): 18165-18170.

Shin, J. and A. Talnov (2001). "A single trial analysis of hippocampal theta frequency during nonsteady wheel running in rats." Brain Res 897(1-2): 217-221.

Sirota, A., S. Montgomery, S. Fujisawa, Y. Isomura, M. Zugaro and G. Buzsaki (2008). "Entrainment of neocortical neurons and gamma oscillations by the hippocampal theta rhythm." Neuron 60(4): 683-697.

Skytt, D. M., K. K. Madsen, K. Pajecka, A. Schousboe and H. S. Waagepetersen (2010). "Characterization of primary and secondary cultures of astrocytes prepared from mouse cerebral cortex." Neurochem Res 35(12): 2043-2052.

Slawinska, U. and S. Kasicki (1998). "The frequency of rat's hippocampal theta rhythm is related to the speed of locomotion." Brain Res 796(1-2): 327-331.

Sonnewald, U., N. Westergaard and A. Schousboe (1997). "Glutamate transport and metabolism in astrocytes." Glia 21(1): 56-63.

Stevens, B. (2008). "Neuron-astrocyte signaling in the development and plasticity of neural circuits." Neurosignals 16(4): 278-288.

Stipursky, J., L. Romao, V. Tortelli, V. M. Neto and F. C. Gomes (2011). "Neuron-glia signaling: Implications for astrocyte differentiation and synapse formation." Life Sci 89(15-16): 524-531.

Stipursky, J., T. C. Spohr, V. O. Sousa and F. C. Gomes (2012). "Neuron-astroglial interactions in cell-fate commitment and maturation in the central nervous system." Neurochem Res 37(11): 2402-2418.

Stojanovic, I. R., M. Kostic and S. Ljubisavljevic (2014). "The role of glutamate and its receptors in multiple sclerosis." J Neural Transm.

Storck, T., S. Schulte, K. Hofmann and W. Stoffel (1992). "Structure, expression, and functional analysis of a Na(+)-dependent glutamate/aspartate transporter from rat brain." Proc Natl Acad Sci U S A 89(22): 10955-10959.

Szatkowski, M. and D. Attwell (1994). "Triggering and execution of neuronal death in brain ischaemia: two phases of glutamate release by different mechanisms." Trends Neurosci 17(9): 359-365.

Szydlowska, K. and M. Tymianski (2010). "Calcium, ischemia and excitotoxicity." Cell Calcium 47(2): 122-129.

Tarozzi, A., A. Merlicco, F. Morroni, C. Bolondi, P. Di Iorio, R. Ciccarelli, S. Romano, P. Giuliani and P. Hrelia (2010). "Guanosine protects human neuroblastoma cells from oxidative stress and toxicity induced by Amyloid-beta peptide oligomers." J Biol Regul Homeost Agents 24(3): 297-306.

Tilleux, S. and E. Hermans (2007). "Neuroinflammation and regulation of glial glutamate uptake in neurological disorders." J Neurosci Res 85(10): 2059-2070.

Traversa, U., G. Bombi, P. Di Iorio, R. Ciccarelli, E. S. Werstiuk and M. P. Rathbone (2002). "Specific [(3)H]-guanosine binding sites in rat brain membranes." Br J Pharmacol 135(4): 969-976.

Tzingounis, A. V. and J. I. Wadiche (2007). "Glutamate transporters: confining runaway excitation by shaping synaptic transmission." Nat Rev Neurosci 8(12): 935-947.

Van Harreveld, A. and E. Fifkova (1974). "Involvement of glutamate in memory formation." Brain Res 81(3): 455-467.

Vandenberg, R. J., P. Ju, K. R. Aubrey, R. M. Ryan and A. D. Mitrovic (2004). "Allosteric modulation of neurotransmitter transporters at excitatory synapses." Eur J Pharm Sci 23(1): 1-11.

Vecchio, F., F. Miraglia, P. Bramanti and P. M. Rossini (2014). "Human brain networks in physiological aging: a graph theoretical analysis of cortical connectivity from EEG data." J Alzheimers Dis 41(4): 1239-1249.

Vezzani, A., U. Ungerstedt, E. D. French and R. Schwarcz (1985). "In vivo brain dialysis of amino acids and simultaneous EEG measurements following intrahippocampal quinolinic acid injection: evidence for a dissociation between neurochemical changes and seizures." J Neurochem 45(2): 335-344.

Volpicelli, L. A. and A. I. Levey (2004). "Muscarinic acetylcholine receptor subtypes in cerebral cortex and hippocampus." Prog Brain Res 145: 59-66.

Volterra, A. and J. Meldolesi (2005). "Astrocytes, from brain glue to communication elements: the revolution continues." Nat Rev Neurosci 6(8): 626-640.

Voutsinos-Porche, B., G. Bonvento, K. Tanaka, P. Steiner, E. Welker, J. Y. Chatton, P. J. Magistretti and L. Pellerin (2003). "Glial glutamate transporters mediate a functional metabolic crosstalk between neurons and astrocytes in the mouse developing cortex." Neuron 37(2): 275-286.

Waniewski, R. A. and D. L. Martin (1986). "Exogenous glutamate is metabolized to glutamine and exported by rat primary astrocyte cultures." J Neurochem 47(1): 304-313.

Whishaw, I. Q. and C. H. Vanderwolf (1973). "Hippocampal EEG and behavior: changes in amplitude and frequency of RSA (theta rhythm) associated with spontaneous and learned movement patterns in rats and cats." Behav Biol 8(4): 461-484.

Whittington, M. A. and R. D. Traub (2003). "Interneuron diversity series: inhibitory interneurons and network oscillations in vitro." Trends Neurosci 26(12): 676-682.

Williams, J. H. and J. A. Kauer (1997). "Properties of carbachol-induced oscillatory activity in rat hippocampus." J Neurophysiol 78(5): 2631-2640.

Yu, A. C., A. Schousboe and L. Hertz (1982). "Metabolic fate of 14C-labeled glutamate in astrocytes in primary cultures." J Neurochem 39(4): 954-960.

Yudkoff, M., D. Nelson, Y. Daikhin and M. Erecinska (1994). "Tricarboxylic acid cycle in rat brain synaptosomes. Fluxes and interactions with aspartate aminotransferase and malate/aspartate shuttle." J Biol Chem 269(44): 27414-27420.

A.1. LISTA DE PUBLICAÇÕES DURANTE A TESE

- Paniz LG, Calcagnotto ME, Pandolfo P, Machado DG, Santos GF, Hansel G, Almeida RF, Bruch RS, Brum LM, Torres FV, de Assis AM, Rico EP, Souza DO. Neuroprotective effects of guanosine administration on behavioral, brain activity, neurochemical and redox parameters in a rat model of chronic hepatic encephalopathy. Metab Brain Dis. 2014 May 1. [Epub ahead of print] PubMed PMID: 24788896.

- Torres FV and Hansen F, Locks-Coelho LD. The increase of extracellular glutamate concentration increases its oxidation and diminishes glucose oxidation in isolated mice hippocampus: reversible by TFB-TBOA. Journal of Neuroscience Research. 2013 Aug;91(8):1059-65. doi: 10.1002/jnr.23187. Epub 2013 Jan 29.

• Kolling J, Scherer EB, Siebert C, Hansen F, Torres FV, Scaini G, Ferreira G, de Andrade RB, Gonçalves CA, Streck EL, Wannmacher CM, Wyse AT. Homocysteine induces energy imbalance in rat skeletal muscle: Is creatine a protector? Cell Biochem Funct. 2012 Dec 5. doi: 10.1002/cbf.2938.

• Vettorazzi J, Torres FV, de Ávila TT, Martins-Costa SH, Souza DO, Portela LV, Ramos JG. Serum S100B in pregnancy complicated by preeclampsia: a case-control study. Pregnancy Hypertension: An International Journal of Women's Cardiovascular Health, ISSN 2210-7789, doi: 10.1016/j.preghy.2011.11.004.

• Hansen F, De Oliveira DL, Amaral FUI, Guedes FS, Schneider TJ, Tumelero AC, Schmidt KH, Giacomini ACV, Torres FV. Chronic administration of tryptophan with or

without concomitant fluoxetine did not alter depression-related and anxiety-like behaviors on adult rat. Neuroscience Letters. v. 499, p. 59-63, 2011.

• Torres FV, da Silva Filho M, Antunes C, Kalinine E, Antoniolli E, Souza DO, Tort AB. Electrophysiological e ects of guanosine and MK-801 in a quinolinic acid-induced seizure model. Experimental Neurology. 2010 Feb;221(2):296-306