UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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Padronização da atividade total da Glutamato Descarboxilase em cérebro de ratos

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Monografia elaborada no Laboratório de Sinalização e Comunicação Glutamatérgica, com colaboração do Laboratório de Estudos do Metabolismo Energético do Sistema Nervoso Central e do Metabolismo Intermediário, Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, como pré-requisito para a obtenção do título de Biomédico.

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Biomedicina

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Dedico este triunfo.....

À minha família pelo carinho e compreensão durante este caminho.

À Grande família pelo apoio mesmo nos momentos mais difíceis.

“Poucas vezes chegamos a um lugar... onde se pode parar e simplesmente ser. Ou perguntar-se quem, afinal, é você.”

Ursula K. LeGuin.

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Índice

Abreviaturas

GABA – Ácido γ-Aminobutirico

GABA-T – GABA-oxoglutarato transaminase

GABAa - Receptor GABAérgico ionotrópico

GABAb – Receptor GABAérgico metabotrópico

GABAc – Receptor GABAérgico ionotrópico

GAD - Glutamato Descarboxilase

GAT – Transportador de GABA

GDH – Glutamato Desidrogenase

GS – Glutamina Sintetase

SNC – Sistema Nervoso Central

uCi – micro-curry, unidade de atividade radioativa

VGAT – Transportador de GABA vesicular

p/v – Diluição volume em relação ao peso

Resumo

O ácido γ-aminobutirico (GABA) é o principal neurotransmissor inibitório do sistema nervoso central (SNC) de mamíferos. A sua síntese é mediante a uma reação catalítica envolvendo a descarboxilação do glutamato através da atividade da glutamato descarboxilase (GAD). O Objetivo deste estudo foi padronizar uma técnica capaz de medir a atividade total da GAD, utilizando córtex e hipocampo de ratos. Neste processo, utilizou-se 65 ratos wistar machos (200-300g de peso). Todas as reações foram realizadas sobre as condições de pH 6,4 e temperatura a 37°. O princípio da técnica basea-se na atividade total oxidativa do homogenato, subtraindo-se a oxidação da GAD inibida por penicilinamina. Quatro variáveis foram levadas em consideração, atividade radioativa específica, diluição do tecido, tempo de reação e gradiente de substrato. A menor atividade radioativa específica obtida foi com 0,05 uCi [C-14(U)]Glu, sendo de 0,305 ± 0,022 nmol CO_2/mg/h. A melhor condição de diluição para o hipocampo foi 1:20 p/v e para córtex foi 1:10 p/v. O melhor tempo de reação para ambas as estruturas foi de 1h. Vmax e Km aparente foi de 1,252 nmol CO_2/mg/h e 25,55, e 0,902 nmol CO_2/mg/h e 15,87, para hipocampo e córtex, respectivamente. Nosso estudo demonstrou que é possível obter-se medidas replicáveis e confiáveis da atividade específica da GAD, utilizando-se apenas hipocampo e córtex provenientes de um hemisfério cerebral.

1- Introdução compreensiva

O Ácido γ-Aminobutirico (GABA) é o principal neurotransmissor inibitório do sistema nervoso central (SNC) de mamíferos (SILVILOTTI e NISTRI, 1991). Este aminoácido é sintetizado por aproximadamente 30% de todos os neurônios do SNC, neste caso denominados como neurônios GABAérgicos, ou interneurônios. O GABA apresenta papeis fundamentais para o desempenho de funções cerebrais, como por exemplo, atividade locomotora, aprendizado e ritmo circadiano (VARJU et. al., 2001), podendo ser crítico para o desencadeamento de patologias, como epilepsias (OLSEN et. al., 1999; COULTER, 2001; AVOLI et. al., 2005), convulsões (TREIMAN, 2001), distúrbio de ansiedade (WONG et. al., 2003), entre outras.

Descoberto inicialmente por Eugene Roberts em 1950, o GABA é estocado em vesículas nos neurônios pré-sinápticos. Isto se deve à atividade do transportador vesicular de GABA (VGAT), cuja atividade depende do pH e de gradientes elétricos (McINTIRE et al., 1997; SAGNÉ et. al., 1997). Este transportador esta presente por todas as estruturas do SNC e é encontrado em terminais axonais de interneurônios GABAérgicos, ou Glicininérgicos (CHAUDRY et. al., 1998; DUMOULIN et. al., 1999), estando associados com vesículas sinápticas.

O neurônio GABAérgico, ao ser despolarizado, libera este aminoácido inibitório na fenda sináptica por intermédio de dois processos. O primeiro é relacionado a liberação vesicular, a qual envolve a entrada de cálcio na pré-sinapse e estimulação por altas concentrações de potássio. O segundo é independente do influxo de cálcio e dependente de um posterior influxo de sódio. Neste caso, tendo liberação de GABA

mediante atividade dos transportadores de GABA (GATs), os quais realizam transporte reverso do neurotransmissor (TREIMAN; 2001).

O GABA livre por sua vez, desempenha seu papel por atuar em receptores GABAa, presentes na pré-sinápse e na pós-sinápse, e em receptores GABAb, presentes na pré-sinapse, inibindo o neurônio alvo e modulando a liberação de neurotransmissores, respectivamente.

O receptor GABAa, ionotrópico, é constituído por um pentâmero, cujas subunidade formam um canal de cloreto, tendo como função aumentar o influxo de cargas negativas no neurônio pós-sináptico e hiperpolarizando-o. Até hoje foram isoladas várias subunidades deste pentâmero, as quais são, α1-6, β1-4, γ1-4, δ, π e ρ. O receptor GABAa é mais freqüentemente encontrado sobre a conjunção das subunidades α1, β2 e γ2 (BORNAMANN, 2000; TREINMAN, 2001), contendo duas subunidades α, duas β e uma única subunidade γ (CHANG et. al., 1996; FARRAR et. al., 1999) (Figura 1). Em decorrência da mudança de subunidades, encontramos os vários subtipos de receptores GABAa, os quais apresentam variação em sua expressão por todo o encéfalo (KORPI et. al., 2002), podendo apresentar diferenças não só entre tipos celulares, mas também, no mesmo neurônio (SPERK et. al., 1997).

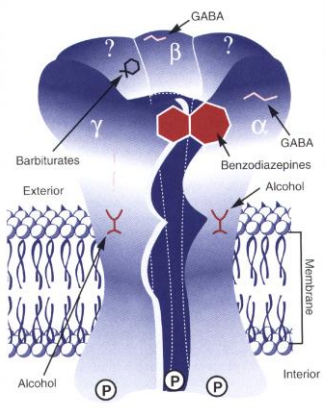

Fig 1. Desenho representativo do receptor ionotrópico GABAérgico (GABAa). É constituído por um pentâmero formado pela junção das subunidades α1-6, β1-4, γ1-4, δ, π e ρ. Ao ser ativado pela ligação de GABA, permite o influxo de cloreto no neurônio pós-sináptico, hiperpolarizando-o.

Um segundo tipo de receptor ionotrópico é o GABAc, o qual foi descrito em retina de vertebrados e é composto de subunidades ρ. Este receptor, ao contrário do GABAa é insensível a bicuculina e baclofen (BORMANN, 2000).

Já no caso dos receptores GABAb, metabotrópicos, a via de sinalização celular desencadeada pela ligação do GABA se dá por intermédio da ativação de proteína G, a qual produz um efeito inibitório lento por diminuir o influxo de cálcio. São encontrados nos terminais axônais de neurônios excitatórios e inibitórios (BORMANN, 1988; BOWERY, 1989). A ativação do mesmo está correlacionada à inibição da liberação de neurotransmissores pelo terminal pré-sináptico (SCHWARTZWELDER et. al., 1986).

Após desempenhar seu papel, o GABA é removido da sinapse por intermédio de transportadores de GABA (GATs) em um processo denominado captação. Ao contrátio do glutamato, o qual é retirado da fenda sináptica por transportadores majoritariamente astrocitários, o GABA é captado pelo terminal pré-sináptico, preferencialmente (SCHOUSBOE, 2003). Decorrente desta característica é possível inferir a importância da reciclagem deste aminoácido no processo de neurotransmissão inibitória (SCHOUSBOE, 2004). Obviamente, temos que levar em consideração o papel da captação astrocitária, o qual é fundamental para a manutenção e disponibilidade do *"pool"* de GABA, tanto em relação à reposição de neurotransmissores, quanto para o papel deste aminoácido no metabolismo (SCHOUSBOE et. al., 2004).

No decorrer da década de 90 foram caracterizados quatro tipos de GATs, os quais são encontrados em membranas plasmáticas celulares e dependentes do gradiente

Na^+/Cl^- (BORDEN et. al., 1992, 1994, 1995; Clark et. al., 1992). Estes quatro transportadores ainda são divididos em dois grupos em relação a sua afinidade ao GABA, o primeiro correspondendo aos GAT1, GAT2 e GAT3 (alta afinidade), e o segundo correspondendo ao BGT-1 (baixa afinidade)(GADEA e LOPEZ-COLOME, 2001) (Figura 2).

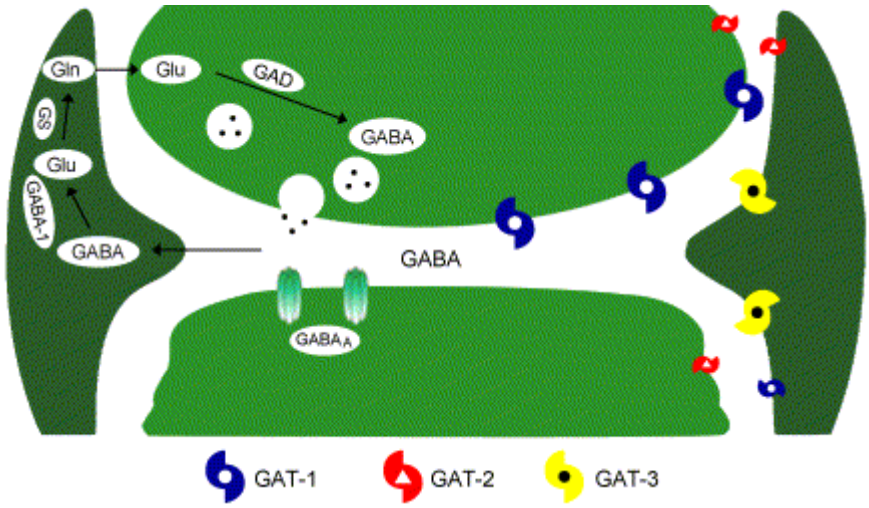

Fig. 2. Figura representativa da distribuição dos GATs, no SNC. GAT-1 representado em azul e majoritariamente expresso em neurônio. GAT-2 em vermelho, baixo grau de expressão pelo SNC. GAT-3 em amarelo, majoritariamente expresso em astrócitos. (CONTI et. al., 2004)

Os principais GATs presentes no SNC são o GAT-1 e o GAT-3 (DURKIN et. al., 1995; YAN e RIBAK, 1998). O GAT-1 é o principal transportador cortical de GABA localizando-se principalmente em neurônios (MINELLI et. al., 1995; LOPEZ-COLUME, 2001), estando co-localizado com proteínas pré-sinápticas, tais como GAD (TAKASHIMA, 2001) e VGAT (MINELLI et. al., 2003). O GAT-3, em contra partida, é tido como o transportador majoritariamente astrocitário (MINELLI et. al., 1996). BGT-1 é distribuído pelo cérebro (BORDEN, 1996), contudo possui uma afinidade

baixa pelo GABA (LIU et. al., 1993), o que leva a pensar em uma atividade não tão efetiva sobre a captação deste (DALBY, 2003). O GAT-2 apesar de ter afinidade pelo GABA semelhante ao GAT-1, apresenta um papel muito menor sobre a captação do mesmo por apresentar uma expressão muito baixa no SNC (EVANS et. al., 1996). O término da sinalização GABAérgica se dá mediante a retirada deste aminoácido da fenda sináptica, resultando no fim da neurotransmissão sináptica (Figura 3).

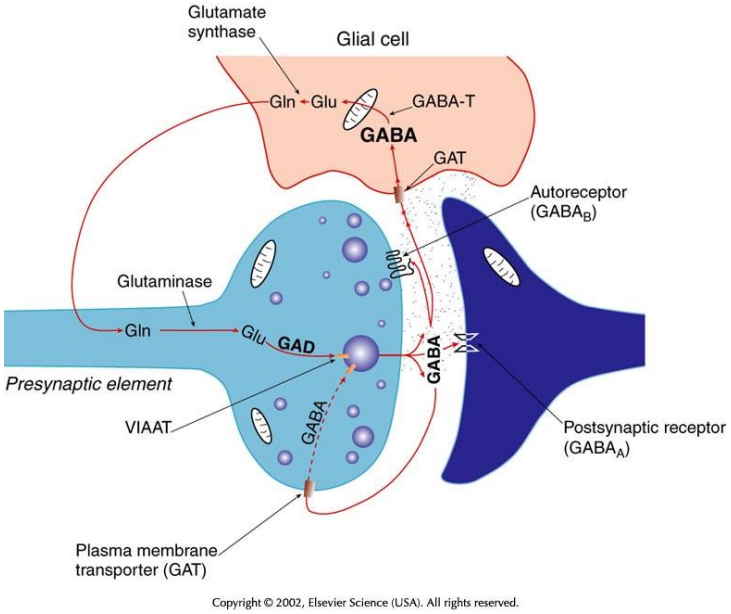

Fig. 3. Imagem representativa da neurotransmissão GABAérgica. O GABA é formado pela atividade da GAD por descarboxilação do glutamato, sendo estocada em vesículas pré-sinápticas pelos VGATs, ou mantendo um *"pool"* de GABA citoplasmático. O neurônio pré-sináptico ao receber um estimulo, libera este neurotransmissor na fenda, o qual se liga tanto a GABAa, quanto a GABAb, desempenhando seu papel. Após realizar sua função, o processo de neurotransmissão é terminado pela atividade de GATs, os quais captam o GABA para o astrócito, onde é convertido a glutamato pela GABA-T, voltando ao neuônio pré-sináptico na forma de glutamina, ou sendo captado diretamente pelo neurônio pré-sináptico, reciclando desta maneira, este aminoácio.

O GABA é um aminoácido formado pelo metabolismo da glicose. No processo oxidativo da glicose ocorre formação de α-cetoglutarato (pelo ciclo de Krebs), o qual pode ser transaminado a glutamato pela atividade da Glutamato Desidrogenase (GDH), o qual é o precursor da reação da GAD(TREIMAN, 2001). Outra via de formação deste neurotransmissor ocorre via GABA "*Shunt"*, processo que ocorre em decorrência da atividade da GABA-oxoglutarato transaminase (GABA-T) (WAAGEPETERSEN et. al., 1999), no qual o α-cetoglutarato recebe um grupamento amino pela transamininação de GABA, originando Glutamato. No primeiro quadro temos a formação de glutamato decorrente do metabolismo neuronal, enquanto no segundo quadro, temos a formação do mesmo decorrente da reciclagem de GABA. Esta reciclagem, por sua vez, pode ter dois objetivos. O primeiro seria reutilizá-lo como fonte energética por intermédio de uma rota anaplerótica em neurônios pré-sinápticos. O segundo seria, disponibilizar no astrócito uma fonte de glutamato para ser convertida a glutamina, pela atividade da glutamina sintetase (GS). Após esta conversão, a glutamina teria livre acesso ao neurônio pré-sináptico, servindo tanto como substrato energético, quanto como substrato para a GAD (BAXTER, 1976) (Figura 4).

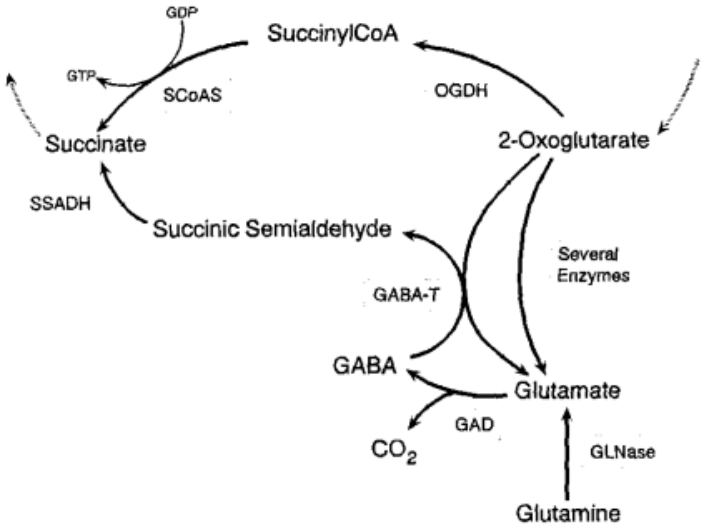

Fig. 4. Quadro esquemático do metabolismo GABAérgico, desde dua formação, até seu consumo como fonte energética. A GAD desempenha papel fundamental para a formação deste aminoácido, decorrente do consumo de glutamato, o qual pode é fruto do processo da oxidação da glicose, ou proveniente da glutamina, a qual é formada nos astrócitos. (MARTIM e RIMVALL, 1993).

O glutamato, principal neurotransmissor excitatório no SNC de vertebrados (DANBOLT, 2001), é então utilizado como substrato para formação de GABA, por intermédio das duas isoformas de Glutamato Descarboxilase, GAD65 e GAD67 (ERLANDER e TOBIN, 1991). GAD65 e GAD67 recebem este nome pelo seu peso molecular aproximado 65.400 Da e 66.600 Da, respectivamente (ERLANDER e TOBIN, 1991), sendo compostos por dois domínios, identificados decorrente de analise molecular. O primeiro domínio está relacionado com a associação à membranas e formação de hetero-dímeros, sendo altamente divergentes em ambas as GADs (apenas 23% de identidade), o segundo está relacionado com a atividade catalítica e é altamente conservado (73% de identidade), respectivamente domínio N-terminal e C-terminal (Figura 5).

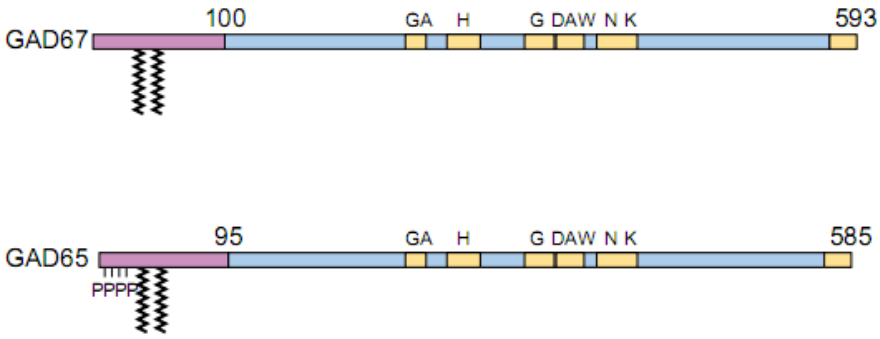

Fig. 5. Comparação entre as proteínas GAD65 e GAD67. Ambas apresentam seis domínios catalítico (amarelo), contudo possuem uma baixa identidade em sua estremidade N-terminal (23%). Existe uma alta identidade na extremidade C-terminal (73%), região caracterizada por apresentar domínio catalítico.

A modulação na síntese de GABA ocorre, a curto prazo, modulando a atividade da GAD ou, a longo prazo, modulando a quantidade de GAD. A atividade da GAD está intimamente relacionada à ligação deste com o seu cofator Piridoxal-P, podendo ter sua atividade rapidamente alterada decorrente da ligação entre ambos (MARTIN et. al., 1993). Em torno de 50% de toda a GAD presente no SNC encontra-se desassociada do seu cofator sendo caracterizada como ApoGAD, e 50% encontra-se associada, sendo caracterizada como HoloGAD, provendo a célula de um *"pool"* de GAD inativa (ITOH e UCHIMURA, 1981). O processo pelo qual ocorre a conversão de um tipo ao outro é altamente controlado, sendo a formação de ApoGAD decorrente de um processo de transaminação, desencadeado pela própria GAD formando ApoGAD e piridoxamina 5'-fosfato (PORTER et. al., 1985). O processo inverso é dependente apenas da ligação do cofator a GAD (PORTER e MARTIN, 1988). Já a forma de controle a longo prazo é mais complexa e muitos estudos demonstram que a expressão gênica das GADs pode ser regulada por mecanismos transcricionais e pós-

transcricionais (YANAGAWA et. al., 1997). Além destes, o próprio GABA pode afetar os níveis de GAD67, por intermédio de um processo pós-transcricionais não muito claro, afetando apenas a quantidade de proteína total mas não a quantidade de mRNA (RIMVALL 1993, 1994).

Apesar de desempenharem a mesma reação, formação de GABA por descarboxilação de glutamato, as reais funções das GAD65 e GAD67 aparentam ter contribuições diferentes para o neurônio GABAérgico. GAD65 é amplamente encontrada em terminais axonais e por estar presente em associação com vesículas sinápticas, propõe-se que sua atividade esteja correlacionada com a formação de GABA para fins de neurotransmissão (SHEIKH e MARTIN 1996). No caso da GAD67, se encontra amplamente distribuída pelo corpo celular, estando correlacionada com a formação de GABA para fins não sinápticos, ou seja, produção deste aminoácido para o *"pool"* metabólico, os quais estão vinculados com o ciclo de Krebs e o *"Shunt"* de GABA (WAAGEPETERSEN et. al., 1999). Estes papéis parecem ser comprovados por experimentos envolvendo camundongos knockout para GAD65 e GA67. No primeiro caso, os camundongos apresentaram susceptibilidade a convulsões (ASADA et. al., 1997) e aumento da ansiedade (STORK et. al., 2000), indicativos de um prejuízo na neurotransmissão GABAérgica. No segundo caso, os animais não sobrevivem (ASADA et. al., 1997), provavelmente devido ao GABA formado pela GAD67 estar vinculado a fontes energéticas ou segundos mensageiras indispensáveis para a sobrevivência celular (JIN et. al., 2003).

Decorrente do papel único que esta enzima apresenta, por converter o principal neurotransmissor excitatório no principal neurotransmissor inibitório presentes

no SNC de vertebrados, l-glutamato e GABA, respectivamente, e por desempenhar um papel fundamental para a sobrevivência celular, o objetivo deste trabalho foi padronizar a atividade total da Glutamato Descarboxilase, e ao contrário de outras metodologias previamente utilizadas na literatura as quais utilizam um homogenizado de encéfalo total, focando a utilização de apenas estrutars provenientes de um hemisfério no processo.

2. TRABALHO EXPERIMENTAL APRESENTADO NA FORMA DE ARTIGO CIENTÍFICO

Trabalho a ser submetido ao periódico Journal of Neuroscience Methods.

Activity pattern of glutamate descarboxylase in hippocampus and córtex of adult rats.

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Summary

γ-Aminobutiric acid is the major inhibitory neurotransmitter in the mammalian CNS. GABA synthesis is meditated by a single enzymatic step, which convert glutamate to GABA by the activity of Glutamate Descarboxylase (GAD). The aim of this study was to standardize a technique that could measure the total activity of GAD in hippocampus and cortex of adult rats. Adult males Wistar rats (270-300 g) were used. All reactions was performed in a homogenized solution (pH 6,4 and temperature 37°C). The GAD Activity was the penicillamine-sensitivite activity from the overall descarboxylase activity. Four variables were taken in account: (i) radioactive specific activity, (ii) dilution of the tissue, (iii) time of reaction and (iv) substrate gradient. The minimum radioactivity specific activity was obtained using 0.05 uCi [C-14(U)Glutamate] 0.305 ± 0.022 nmol CO_2/mg/h. The best dilution condition for hippocampus was 1:20 v/w and for cortex was 1:10 v/w. The better time of reaction for both structures was 60 minutes. Vmax and apparent Km were 1.252 nmol CO_2/mg/h and 25.55 for hippocampus and 0.902 nmol CO_2/mg/h and 15.87 for cortex, respectively. Our study demonstrated a replicable and reliable methodology to measure the total GAD activity using both, or only one hemisphere.

Key words

Glutamate Descarboxylase; activity; hippocampus; cortex.

1. Introduction

Gama-aminobutiric acid (GABA) is the major inhibitory neurotransmitter in the vertebrate central nervous system (CNS) (Sivilotti and Nistri, 1991). GABA modulates every aspect of brain function by activating ionotropic (GABAa) (Bormann, 1986) and metabotropic (GABAb) receptors (Bormann, 2000), which have a ubiquitous expression in the CNS. A disruption of GABAergic transmission is correlated with several neurological (Treiman, 2001; Varju et al., 2001) and psychiatric disorders, including epilepsy (Avoli et al., 2005; Coulter, 2001; Olsen et al., 1999; Schousboe et al., 1983), anxiety disorder (Wong et al., 2003), among others.

Although almost all current studies focuses on its role as a neurotransmitter, its role as a metabolic intermediary is also significant (Sonnewald et al., 2004). In synaptic terminal the mechanism involved must maintain an adequate pool of transmitter GABA, in face of any alteration that could be involved with the change of neuronal activity(Mugnaini, 1985). In none synaptic regions, GABA function is related as a metabolic intermediate, and its mechanism of regulation should be connected with turnover of GABA to succinate mediated by the GABA shunt, in this way correlating GABA with tricarboxylic acid cycle (TCA cycle) (Baxter et al., 1976; Graham et al., 1970; Schousboe et al., 1997). Another process involving GABA is formation of glutamate by GABA transaminase (GABA-T), which in the process of transamination of GABA, confers ammonium to formation of glutamate consumim α-ketoglutarate, being important to inhibitory system, and for the excitatory system in this way, as well as important to production of glutamine in glia, an important precursor to formation of GABA (Schousboe et al., 1997; Waagepetersen et al., 1999a).

In mammalian brain, GABA is synthesized from glutamate in a single step catalyzed by GAD, which uses pyridoxal-P as a cofactor(Martin and Rimvall, 1993; Rimvall and Martin, 1991). GAD is considered unique among other enzymes because both its substrate and product are neurotransmitters that exhibits opposite actions, L-glutamate (excitatory)(Danbolt, 2001), and GABA (inhibitory)(Coulter, 2001). Molecular studies have demonstrated that GAD exists as two major isoforms, called GAD65 and GAD67, which are products of two different genes located on chromosomes 2 and 10(Brilliant et al., 1990; Szabo et al., 1994), respectively.

Observing the role of each function is probably that there are different regulatory mechanisms involving each one, and can help to elucidate the function of each Glutamate Descarboxylase (GAD; EC 4.1.1.15) found in brain (Waagepetersen et al., 1999b). GAD65 is correlated with the activity of GAD to form GABA as a neurotransmitter, and GAD67 is correlated with the activity of GAD to form GABA as an intermediate metabolic(Erlander and Tobin, 1991; Martin and Rimvall, 1993). These affirmations are reasoned in the distribution of each GAD throughout the neuron, GAD65 appears to be exclusively correla ted with terminal nerves(Sheikh and Martin, 1996), although GAD67 is widely distributed among the neuron(Kaufman et al., 1991). Independently of each specific function, both appear to have an important role in the balance of excitatory and inhibitory system.

Considering all this facts, and the crucial role of GAD, in modulating the excitatory and inhibitory system by a process enzymatic of only one step, the aim of this study was to padronizate a technique that could measure the total activity of GAD.

2. Methods

Materials

L-[14C(U)] glutamic acid (specific activity 260mCi/mmol) and liquid schintlizatin Optiphase, were purchased from Perkin Elmer, USA. Pyridoxal 5'-phosphate, L-glutamic acid 2-mercapto etanol and D-Penicillamine were purchased from Sigma Chemical Co., St. Louis, MO, USA. Other chemicals were purchased from Nuclear, Brazil.

Animals

Adult males Wistar rats (270-300 g) were used (n = 65). All animals were maintained under a 12-light/dark cycle with food and water *ad libitum*. The handling and care of the animals were conducted according to the National Institute of Health guide for care and use of laboratory animals (NIH Publications No. 80-23, revised 1978). All procedures in the present study were approved by the Committee of Ethics from Universidade Federal do Rio Grande do Sul.

GAD Activity

The GAD activity standardization was done according to Alberts et. al. (1959), which is determined subtracting the penicillamine-sensitivite activity from the overall descarboxylase activity (in the absence of penicillamine 10 mM). The animals were killed by decapitation and their brains exposed by the removal of the parietal bone. Hippocmapus and cortex were rapidly dissected on an inverted Petri dish placed on ice. Tissues were homogenized in 50mM potassium phosphate, pH 6,4, with a glass homogenizer. Hippocampus and cortex were used to enzymatic assays. To evaluate the best condition of the assays, four variables was taken in account: (i) radioactive specific activity (0.025 uCi, 0.050 uCi, 0.075 uCi, 0.1 uCi), (ii) dilution of tissue (1:10, 1:20,

w/v, for single and couple of structure), (iii) time of reaction (15, 30, 45, 60 and 120 min) and (iv) substrate gradient (1.5mmol to 150mmol).

Incubation were carried out in flaks after contents were gassed with 100% O_2 for 30 seconds and then sealed with rubber caps. The homogenates were incubated at 37° C in a Dubnoff metabolic shaker (60 cycles/min) according to the method of (Dunlop et al., 1975). Incubation was stopped by adding 0.25 ml 50% TCA through the rubber cap. Then 0.1 ml of 1 M sodium hydroxide was injected into the central wells. The flasks were shaken for a 30 min at 37° C to trap CO_2. After, the contents of the flasks were transferred to vials, and assayed for CO_2 radioactivity in a liquid-scintillation counter.

Statistical

Data were expressed as mean±SD and were analyzed by one-way ANOVA followed by Tukey's *post hoc* test for unequal samples. $P<0.05$ was considered significant. All data was measured out by the software GraphPad Prism 5.

3. Results

Radioactivity specific activity

In order to determine the better radioactivity specificity, four concentration of L-[14C(U)]glutamic acid was used: 0.025 uCi, 0.050 uCi, 0.075 uCi, 0.1 uCi. The activities were 0.305 ± 0.022, 0.404 ± 0.040, 0.415 ± 0.057, and 0.427 ± 0.035 nmol CO_2/mg/h, respectively (Fig. 1). There were no differences among the three last concentrations. However, in the lower concentration, there was a decrease at GAD activity. Other conditions were followed as described by Malfatti et al. (2007).

Dilution tissue

In order to establish the conditions of preparation the homogenized tissue (minimum volume of reaction as 400 ul), there were performed three dilutions. For hippocampus, the dilution of 1:40 w/v showed a lower activity when compared with 1:20 w/v, there was no difference between the activities when compared with a dilution of two hippocampus 2:40 w/v, (Fig. 2). For cortex, the dilution of 1:20 w/v showed a lower activity when compared with 1:10 w/v, without demonstration of different activity when was homogenized two cortex for 2:20 w/v, (Fig. 3).

Time of reaction

In order to evaluate the optimal time of reaction criterion time curve was performed (15, 30, 45, 60 and 120 min). The activity obtained to hippocampus was described by linearity with a start point as 0.078 ± 0.016 nmol CO_2/mg/h up to an end point of 0.794 ± 0.121 nmol CO_2/mg/h (Fig. 4). The activity that was found in cortex

was not described by linearity as in hippocampus with a start point as 0.098 ± 0.019 nmol CO_2/mg/h up to an end point of 0.675 ± 0.165 nmol CO_2/mg/h (Fig. 5).

Substrate gradient

To investigate the Vmax (maximum velocity) and K(m) (Michaels constant), there was established a substrate curve composed by the following concentrations of L-glutamic acid: 1.5, 5, 7.5, 10, 15, 20, 30, 50, 75, 100, 120 and 150 mM. The experiment was carried out three times with n=3 for each point. The Vmax as well as the apparent K(m) for hippocampus were 1.252 nmol CO_2/mg/h and 25.55 and demonstrated a Linear-regression of Edie-Hofstee of with Y-intercept = 1.244 ± 0.04655, X-intercept = 0.05030, r^2 = 0.9576 (Fig. 6), and for cortex 0.902 nmol CO_2/mg/h and 15.87 and demonstrated a Linear-regression of Edie-Hofstee with Y-intercept = 0.9280 ± 0.05132, X-intercept = 0.05104, r^2 = 0.8937 (Fig. 7), respectively.

4. Discussion

Gama-Aminobutiric acid is the major inhibitory neurotransmitter in the vertebrate CNS. The GABA synthesis is meditated by a single enzymatic step, which convert glutamate to GABA by the activity of Glutamate Descarboxylase, which requires pyridoxal-P as a cofactor (Martin and Rimvall, 1993). There is well established that the ideal pH of the reaction is 6,4 and the temperature is 37°C, which is the physiologic temperature of the mice (Casu and Gale, 1981; Dunlop et al., 1975; Loscher et al., 1989).

In order to use the minimum radioactivity in the reaction, there was elaborated a comparison between different activity of uCi to perform this protocol. Our results demonstrate, that there would be possible to use a half of the minimum uCi used in another studies without alteration the GAD activity (Fig. 1), demonstrating that in the concentration of 0,05uCi, the reaction reaches saturation point when [C-14(U)]glutamate is used as the radioactivity substrate the reaction, and concentrations lower than this begins to lose sensibility of the role reaction.

In several protocols were observed the necessity to use both hemispheres to perform this technique (Malfatti et al., 2007). However, in order to compare GAD activity and another parameters of CNS, which implicated in using one hemisphere to quantify the activity, and another hemisphere to other analyses, it was necessary to establishe the minimum homogenized volume and the better dilution to quantify gad acitivity. Starting from 200 uL to 500 uL to established the minimum volume necessary to have a replicable result, we choose the volume of 400 uL as the better replicable (data not shown), and taking in account that the principle of the reaction is subtract the penicillamine-sensitivity activity from the overall descarboxylase activity, the minimum

volume necessary was 800 uL per structure. To determine the better dilution for the sample, taking in account the weight of brain structures (50-60 mg for hippocampus and 130-160 mg for cortex) and minimum volume necessary to perform the reaction, the factor of dilution was different for hippocampus (Fig. 2) and for cortex (Fig. 3). However when was duplicate this by duplicating the final volume, the GAD activity was reduced for less than a half, implicating in a possible problem of kinetic reaction , which could be proven when we used both hemispheres for the last factor of dilution. Therefore use one hemispheres leads to obtain only one reaction for each sample.

In order to determine the better time of reaction, there were used five time points. Our results demonstrated that there was a linearity in the activity among times for hippocampus (Fig. 4) and for cortex (Fig. 5). This could be explained by the fact that the Ki of GABA formation by GAD is at a milimolar range, and the activity that was found here was at an nM range. Another fact that contributes to this is the $\Delta G°$ that is extremely negativity (-23Kj/mol) (Martin and Rimvall, 1993). Moreover, glutamate was used in an mM range and the activity was obtained in an nM range, indicating that the precursor is converted and is not a limitation in the reaction. The third point which could elucidate it, is the fact that 50% of GAD is found in brain as HoloGAD (GAD linked with pyridoxal-P), and 50% is found as ApoGAD (GAD inactivite) (Mugnaini, 1985), the former being stabilized by ATP (Meeley and Martin, 1983; Porter and Martin, 1988), which is also as responsible for the inactivation of ApoGAD(Miller et al., 1977). Among time the ATP and creatine-P are degraded letting inorganic phosphurum (Pi) to react with endogenous pyridoxal present in the homogenized solution forming more pyridoxal-P, plus the ApoGAD loosing the stability after a long time of degradation of ATP, reaction with the cofactor in the homogenized solution, adding HoloGAD among

time to the reaction. These facts could explain the absence of a balance in the reaction after a long time of reaction(Miller et al., 1978).

With all this parameters pre-established we could finally determine the GAD apparent Km and Vmax. Our results indicated that the hippocampus had a bigger apparent Km and Vmax (Fig. 6), when compared with cortex (Fig. 7). In the absence of works which demonstrates different population of GAD65 and GAD67 in the two structures, as well as the specific activity of each one, there is not a definitive argument which could explain this difference. We could speculated this as a result of the physiologic activity in each structure, knowing that the hippocampus environment is excitatory, and the cortex environment is inhibitory, is possible that the second had a more refined system for GABA syntheses than the first, however this speculation only will be clarified with further studies to determine the distribution of the two forms of GAD in each structure and their specific activity, which is not possible now a days by the absence of a specific inhibitor for GAD65 or GAD67.

In conclusion, the present study demonstrated the better condition of GAD reaction as 0,05uCi of [C-14(U)-Glutamate], dilution 1:20 to hippocampus and 1:10 to cortex, time of reaction as 60 min, and a linearity of reaction between 1,5 up to 30 mM for hippocampus, and 1,5 up to 20 mM for cortex. The high replicability of the reaction shows that even with only one structure from only one hemisphere, wich implicates in the use of unicate for each sample, the reaction was very reliable.

Acknowledgments

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Figure legends

Fig.1. Radioactivity specific activity. GAD activity showed no variation when were used 0.05, 0.075, and 0.1 uCi, activities 0.305 ± 0.022, 0.404 ± 0.040 and 0.415 ± 0.057 nmol CO_2/mg/h, respectively. Nevertheless there was found a reduction in the same when was used 0.025 uCi, 15.248 ± 1.10 pmol CO_2/mg/h. n=5 for each point, the time used was 60 minutes and the dilution was 1:10 for the hemisphere, the concentration of the substrate was 15mMol. Data represent means ± S.D. *$p<0.05$ (one-way ANOVA followed by Tukey's test).

Fig.2. Comparison of dilution between one hippocampus 1:10 w/v and 1:20 w/v, and two hippocampus 2:20 w/v. As expect the result of the first and the last possibilities showed no difference, 0.443 ± 0.024 and 0.456 ± 0.043 nmol CO_2/mg/h, however the second dilution showed a lesser level in the activity 0.150 ± 0.043 nmol CO_2/mg/h. n=6 for each group, the time of reaction used was 60 minutes, the concentration of the substrate was 15mMol. Data represent means ± S.D.

Fig.3. Comparison of dilution between only one cortex, dilution 1:20 w/v and 1:40 w/v, and two cortex 2:40 w/v. As expect the result of the first and the last possibilities showed no difference, 0.375 ± 0.013 and 0.423 ± 0.031 nmol CO_2/mg/h, however the second dilution showed a lesser level in the activity 0.168 ± 0.027 nmol CO_2/mg/h. n=6 for each group, the time of reaction used was 60 minutes, the concentration of the substrate was 15mMol. Data represent means ± S.D.

Fig.4. Time of reaction. The hippocampus demonstrated linearity in the activity correlated with the time of reaction, 15, 30, 45, 60 and 120 minutes. The corresponded activities for each time were 0.078 ± 0.016, 0.213 ± 0.024, 0.338 ± 0.057, 0.442 ± 0.027 and 0.794 ± 0.121 nmol CO_2/mg/h. n=4 for each point, the concentration of substrate in the experiment was 15 mMol under the condition of 0.050uCi and hippocampus dilution 1:20 w/v. Data represent means ± S.D.

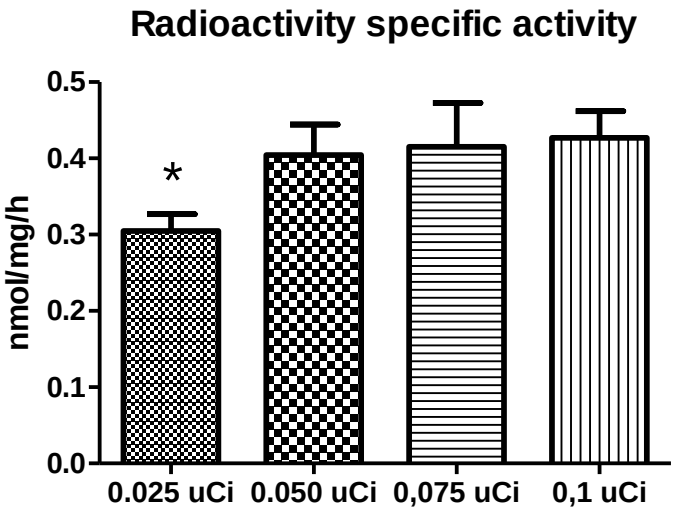
Fig.5. Time of reaction. The cortex demonstrated a linearity in the activity correlated with the time of reaction until the fourth point chosen for test, but there was not the same correlation with the last time, 15, 30, 45, 60 and 120 minutes, respectively. The corresponded activities for each time were 0.098 ± 0.019, 0.240 ± 0.022, 0.364 ± 0.024, 0.461 ± 0.027 and 0.675 ± 0.165 nmol CO_2/mg/h. n= 4 fot each point, the concentration of substrate in the experiment was 15 mMol under the condition of 0.050uCi and cortex dilution 1:10 w/v. Data represent means ± S.D.

Fig.6. Substrate gradient of GAD correlated with twelve points of L-Glutamate concentration, 1.5, 5, 7.5, 10, 15, 20, 30, 50, 75, 100, 120 and 150 mMol. Incert: A linearity profile was identified in hippocampus from 1.5 to 30 mMol, 0.077 ± 0.001 up to 0.739 ± 0.024 nmol CO_2/mg/h, reaching to saturation at 50 mMol and forward, 0.822 ± 0.031 up to 1.057 ± 0.020 nmol CO_2/mg/h. The Vmax as well as the apparent K(m) for hippocampus were 1.252 nmol CO_2/mg/h and 25.55, respectively. Three experiment with n=3 were carried out for each point under the condition of 0.050uCi, hippocampus dilution 1:20 w/v and 60 minutes of reaction. *Insert*: Linear-regression of Edie-Hofstee

of hippocampus; Y-intercept = 1.244 ± 0.04655, X-intercept = 0.05030, r^2 = 0.9576. Data represent means ± S.D.

Fig.7. Substrate gradient of GAD correlated with twelve points of L-Glutamate concentration, 1.5, 5, 7.5, 10, 15, 20, 30, 50, 75, 100, 120 and 150 mMol. A linearity profile was identified in cortex from 1.5 to 20 mMol, 0.069 ± 0.008 up to 0.556 ± 0.012 nmol CO_2/mg/h, reaching to saturation at 30 mMol and forward, 0.633 ± 0.020 up to 0.828 ± 0.023 nmol CO_2/mg/h. The Vmax as well as the apparent K(m) for cortex for 0.902 nmol CO_2/mg/h and 15.87, respectively. Three experiment with n=3 were carried out for each point under the condition of 0.050uCi, cortex dilution 1:10 w/v and 60 minutes of reaction. *Insert*: Linear-regression of Edie-Hofstee of cortex; Y-intercept = 0.9280 ± 0.05132, X-intercept = 0.05104, r^2 = 0.8937. Data represent means ± S.D.

Figure 1


Radioactivity specific activity
nmol/mg/h
0.5
0.4
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0.025 uCi
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0,1 uCi

Figure 2

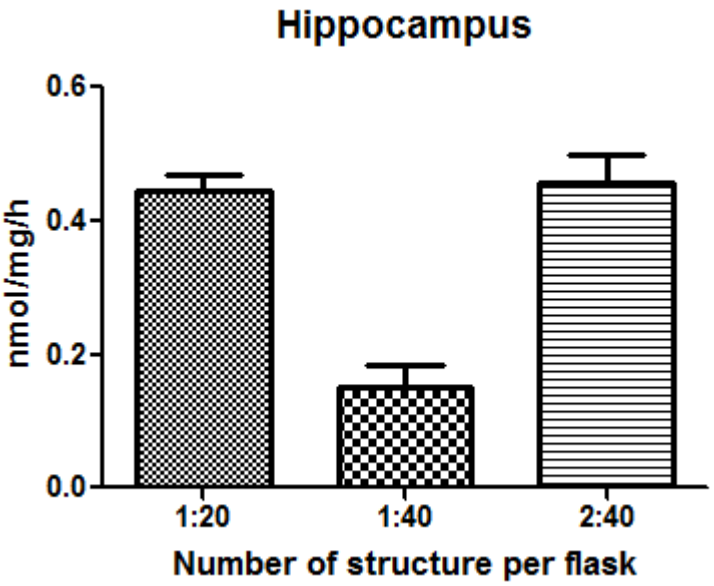


Figure 3

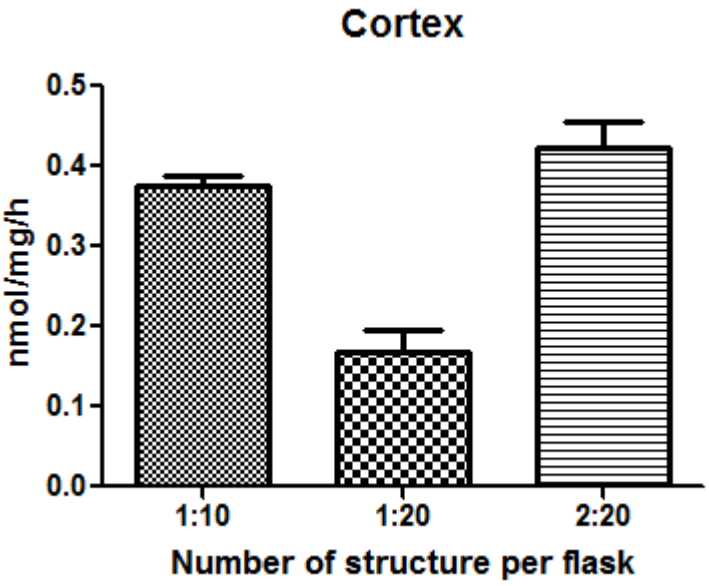


Figure 4

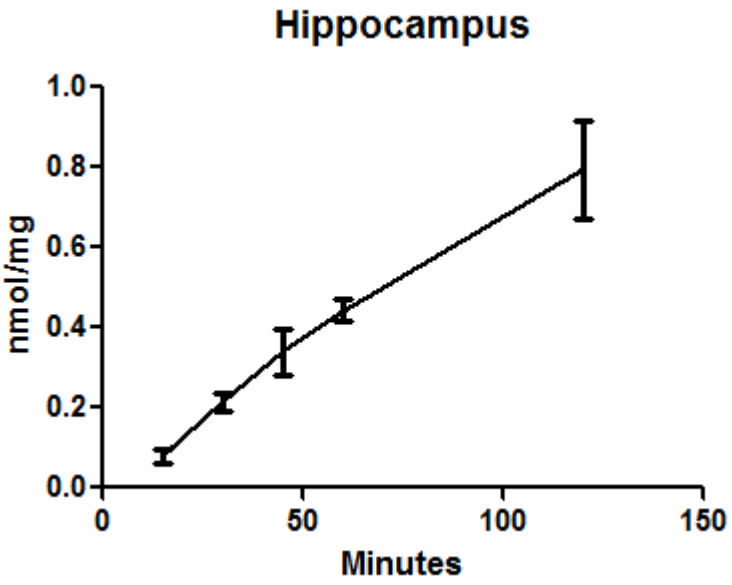


Figure 5

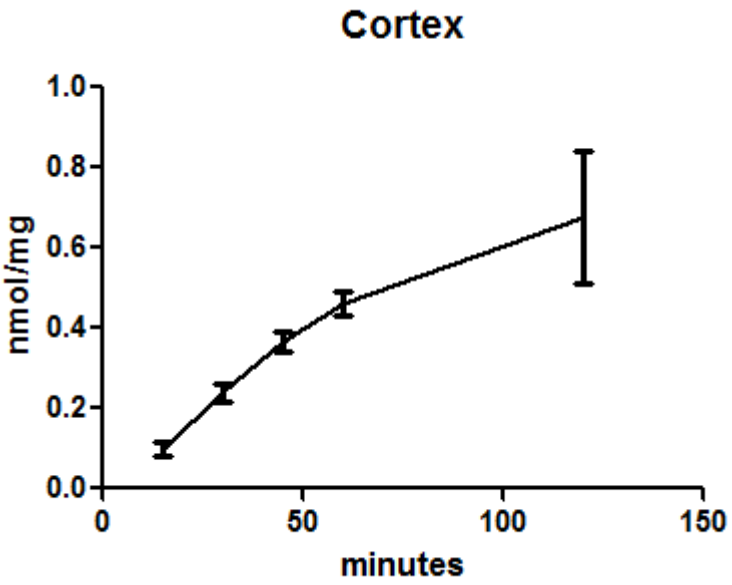


Figure 6

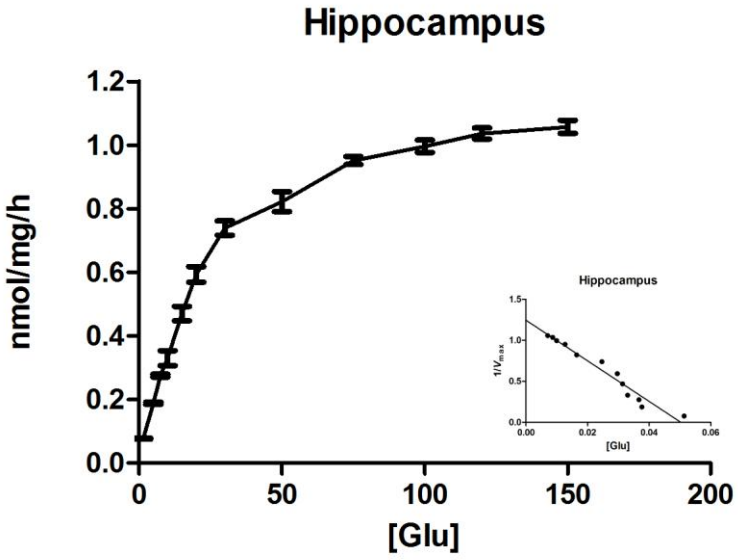
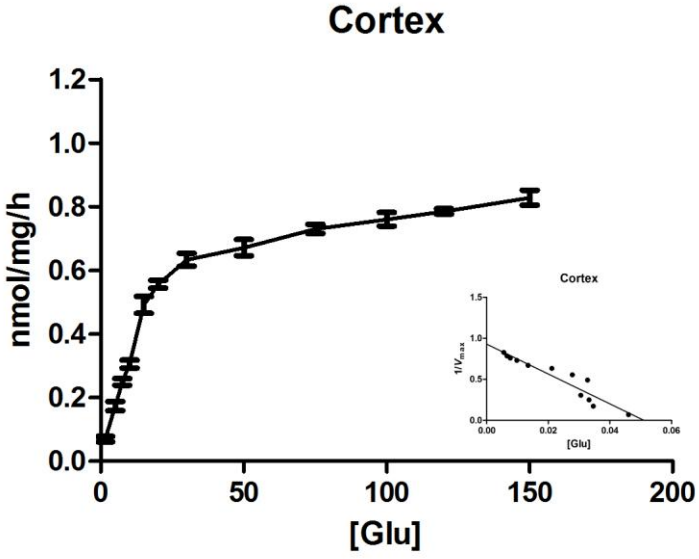


Figure 7



Conclusões e perspectivas

Por intermédio deste estudo, obtivemos uma técnica altamente reproduzível e confiável, e por intermédio da qual, poderemos inferir muitos dados mediante a vinculação desta a estudos envolvendo patologias. Pelo fato da técnica utilizar apenas um hemisfério, existe a possibilidade de desenvolver trabalhos utilizando um hemisfério para medir a atividade da GAD, e outro para medir outros parâmetros, o que não era possível anteriormente, pois as técnicas utilizavam o cérebro total para o mesmo fim. Foi possível atribuir uma diferente atividade global da Glutamato Descarboxilase em córtex e hipocampo de ratos adultos.

Contudo, na tentativa de elucidar esta diferença:

1- Padronizar o wester bloting para GAD total e GAD67, podendo medir o nível proteico em diferentes estruturas;
2- Padronizar a himunohistoquímica tanto da GAD total quanto da GAD67, para estudos envolvendo distribuição celular, atividade e alterações morfológicas;
3- Vincular esta técnica a outros trabalhos que envolvam o sistema GABAérgico;
4- Buscar, por intermédio do somatório deste trabalho e destas perspectivas, compreender o real papel das diferentes formas de GAD e as diferentes atividades encontradas pelo SNC.

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