Denise Sandrelly Cavalcanti de Lima

Ação da Glutamina no Cérebro em Desenvolvimento: Estudo Comportamental, Eletrofisiológico e Imunohistoquímico em Ratos Jovens e Adultos Submetidos a Diferentes Condições de Lactação

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Tese aprovada em 29 de julho de 2016

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Recife
2016

Dedico este trabalho ao meu esposo (George) e aos meus filhos (Heitor, Marina e Lara).

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Paulo Coelho

RESUMO

O aminoácido glutamina (Gln) é precursor dos neurotransmissores cerebrais glutamato e GABA. O aumento de sua disponibilidade pode modular a excitabilidade cerebral. O objetivo deste trabalho foi descrever os efeitos do tratamento com diferentes doses de Gln, durante o desenvolvimento cerebral, sobre o comportamento de ansiedade, a depressão alastrante cortical (DAC) e a ativação da microglia no córtex de ratos recém-desmamados (D) e adultos (A). Os animais foram amamentados em ninhadas com 9 (L_9; lactação normal) e com 15 filhotes (L_{15}; lactação desfavorável). Do 7º ao 27º dia de vida pós-natal (P7-P27), os filhotes machos receberam por gavagem 250, 500 ou 750 mg/kg/dia de Gln (grupos Gln250, Gln500 e Gln750, respectivamente). Os grupos controles foram formados por animais que receberam o veículo (água destilada) no qual a Gln foi dissolvida por gavagem e por animais que não receberam gavagem (grupo ingênuo). Aos P28-P30 (D) e P88-P90 (A), os animais foram submetidos aos testes para comportamentos sugestivos de ansiedade no labirinto em cruz elevado (LCE) e no campo aberto. Dos P30-35 (D) ou P90-120 (A), registrou-se a DAC, obtendo-se dados de sua velocidade de propagação, duração e amplitude. Em seguida, os cérebros de alguns animais foram processados para imunomarcação com anticorpos anti-Iba1, específicos para microglia. No grupo D, os ratos tratados com Gln apresentaram um comportamento menos ansioso, tanto no LCE quanto no campo aberto. Este efeito ansiolítico da Gln foi mais evidente nos animais da condição L_{15}. Na idade adulta (A), os grupos Gln500 e Gln750 da condição L_{15} percorreram uma maior distância e apresentaram menor tempo de imobilidade no LCE. Em relação à DAC, os animais da condição L_{15} apresentaram maior velocidade de propagação do que os correspondentes L_9. Com exceção do grupo Gln250 da condição L_{15} na idade adulta, todos os grupos tratados com Gln apresentaram maior velocidade de propagação quando comparados aos respectivos controles. Além disso, esse efeito acelerador foi dependente da dose, uma vez que os grupos Gln500 e Gln750 apresentaram maiores velocidades de propagação do que os correspondentes Gln250. Quanto à reação da microglia, os animais tratados com Gln apresentaram maior imunorreatividade, tanto no córtex parietal quanto no hipocampo dos grupos D e A. Nos animais A da condição L_9, a imunorreatividade da microglia e o percentual de área marcada foram maiores no grupo Gln500 do que no grupo Gln250. A partir desses resultados, sugere-se que o tratamento com Gln durante o período neonatal module a excitabilidade cerebral, resultando nas alterações eletrofisiológicas, comportamentais e imunohistoquímicas descritas neste estudo. Essas alterações persistem até a idade adulta e são dependentes da dose e da condição nutricional do animal

Palavras-chave: Glutamina. Depressão alastrante cortical. Excitabilidade cerebral. Microglia. Ansiedade.

ABSTRACT

The amino acid glutamine (Gln) is precursor of the brain neurotransmitters glutamate and GABA. Therefore, the increase of its availability can modulate brain excitability. The aim of this study was to describe the effects of treatment with different doses of Gln during brain development on anxiety-like behavior, cortical spreading depression (CSD) and microglial reaction in the cortex of developing (D) and adults (A) rats. Wistar rats were suckled in litters with 9 (L_9; normal condition) or 15 (L_{15}; unfavorable condition) pups. From 7th to 27th postnatal day (P7-P27), male rats received Gln by gavage at the doses of 250 mg/kg/day or 500 mg/kg/day or 750 mg/kg/day (respectively Gln250, Gln500 and Gln750 groups). The control groups were formed by animals that received vehicle which Gln was dissolved (distilled water) and animals that were not submitted to the gavage procedure (naive group). At P28-P30 (D) and P88-P90 (A), animals were tested in elevated plus maze (EPM) and open field. At P30-35 (D) and P90-120 (A), we recorded the CSD, obtaining data from its velocity of propagation, duration and amplitude. The brains of some animals were processed for microglial immunolabeling with anti-Iba-1 antibodies to analyze cortical microglia. In the D group, Gln treated rats showed less anxious behavior, both in EPM and open field. This anxiolytic effect of Gln was more evident in L_{15} condition. In adult rats (A), Gln500 and Gln750 groups of L_{15} condition traveled a greater distance and displayed shorter immobility time in the LCE when compared to controls. Regarding CSD, L_{15} animals presented with higher propagation velocity than the corresponding L_9. Except for the Gln250 group of L_{15} condition in adulthood, all groups treated with Gln showed higher CSD velocity when compared to their respective controls. Moreover, the accelerating effect was dose dependent, since Gln500 and Gln750 groups displayed higher CSD velocity than the corresponding Gln250. Gln treated groups had greater immunoreactivity in both the parietal cortex and hippocampus. In adult rats of L_9 condition, Gln500 group had greater immunoreactivity and higher percentage of labeled area when compared Gln250 group. Our findings suggest that neonatal treatment with Gln modulates brain excitability, resulting in the electrophysiological, behavioral and microglial alterations here described. These alterations persist into adulthood and are modulated by dose and lactation conditions.

Keywords: Glutamine. Cortical spreading depression. Brain excitability. Microglia. Anxiety.

LISTA DE ILUSTRAÇÕES

Figuras do Artigo 1

Figuras do Artigo 2

LISTA DE ABREVIATURAS E SIGLAS

A ..... ratos adultos / adult rats
ANOVA ..... análise de variância
BB ..... tampão de bloqueio / buffer block
CA1 ..... cornus amoni 1
CSD ..... cortical spreading depression
D ..... ratos recém-desmamados / developing rats
DAB ..... 3,3' diaminobenzidina
DAC ..... depressão alastrante cortical
DBR ..... dieta básica regional
EcoG ..... eletrocorticograma
EPM ..... elevated plus maze
GA ..... glutaminase
GABA ..... ácido γ -aminobutírico / γ aminobutyric acid
GAD ..... ácido glutâmico descarboxilase
Glu ..... glutamato
Gln ..... glutamina /glutamine
Gln250 ..... grupo tratado com 250 mg/Kg/dia de L-glutamina
Gln500 ..... grupo tratado com 500 mg/Kg/dia de L-glutamina
Gln750 ..... grupo tratado com 750 mg/Kg/dia de L-glutamina
GS ..... glutamina sintetase
H_2O_2 ..... peróxido de hidrogênio
i.p. ..... intraperitoneal
L_9 ..... lactação normal / normal lactation / normal size litter
L_{15} ..... lactação desfavorável / unfavorable lactation / large size litters
LCE ..... labirinto em cruz elevado
P ..... dia pós-natal / postnatal day
TBS ..... solução salina tamponada com Tris / Tris-buffered saline
UFPE ..... Universidade Federal de Pernambuco
VLV ..... variação lenta de voltagem

SUMÁRIO

1. APRESENTAÇÃO

A glutamina (Gln) é o aminoácido livre mais abundante no plasma e nos fluidos cerebrais de mamíferos (DOLGODILINA et al, 2015), sendo também encontrada em concentrações elevadas em muitos tecidos, onde exerce várias funções biológicas (CURI, 2000). Apesar de ser produzida pelo organismo, é considerada como um aminoácido "condicionalmente essencial", pois em determinadas condições fisiológicas e patológicas, sua demanda pode exceder a capacidade de sua síntese pelo organismo, tornando-se necessária sua suplementação (LACEY e WILMORE, 1990).

Os efeitos benéficos do tratamento com Gln sobre a integridade intestinal, a função imune e o balanço nitrogenado foram extensivamente demonstrados, tanto em animais (BEUTHEU et al, 2014; BEAUFRÈRE et al, 2014; FAN et al, 2015; ZHANG et al, 2016), quanto em humanos (BOLLHALDER et al, 2013; KOKSAL et al, 2014; LORENZ et al, 2015, SAYLES et al, 2016). Praticantes de exercícios prolongados e extenuantes também parecem se beneficiar da suplementação com Gln (SONG et al, 2015). Assim, a suplementação crônica com Gln tem crescido, não apenas entre pacientes hospitalizados e atletas, mas também entre pessoas que acreditam que o seu consumo regular apresente efeitos favoráveis sobre a memória e o sistema imune (HOLECEK, 2013).

Em crianças, os estudos com Gln têm sido direcionados à recém-nascidos pré-termo e/ou com baixo peso em uma ampla variedade de situações clínicas (PAWLIK et al, 2012; WANG et al, 2013; MOE-BYRNE et al, 2016), com destaque para investigação da modulação da resposta inflamatória e estimulação do sistema imune. Apesar do período neonatal ser considerado crítico para o desenvolvimento do sistema nervoso, pouquíssimos trabalhos se propuseram a analisar as repercussões cerebrais da suplementação com Gln no início da vida (KIEVIET et al, 2012a; 2012b; 2014). Além disto, a segurança do uso crônico desse aminoácido tem sido questionada (HOLECEK, 2013).

No cérebro, a Gln participa de inúmeras vias metabólicas, exercendo um papel importante na neurotransmissão, uma vez que é precursora dos aminoácidos excitatórios, glutamato (Glu) e aspartato; e do aminoácido inibitório, ácido γ-aminobutírico (GABA; ALBRECHT et al, 2011). Em roedores, foi demonstrado que o tratamento com Gln, tanto por via oral quanto subcutânea, resulta em aumento nas concentrações de Gln e de GABA no cérebro (WANG et al, 2007; LADD et al, 2010). Além disto, supõe-se que o aumento da disponibilidade de Gln no plasma desequilibre o ciclo glutamina-glutamato e,

consequentemente, eleve as concentrações de Glu e de amônia no cérebro (HOLECEK, 2013). Portanto, pode-se sugerir que o tratamento crônico com Gln promova um desbalanço nos níveis de neurotransmissores no cérebro e, consequentemente, module a sua excitabilidade.

No que concerne à excitabilidade cerebral, o fenômeno da depressão alastrante cortical (DAC) tem sido utilizado em nosso laboratório como modelo para avaliar modificações eletrofisiológicas decorrentes de manipulações ambientais, farmacológicas, clínicas e nutricionais em ratos (GUEDES, 2011). A DAC consiste em uma redução reversível da atividade elétrica (espontânea ou provocada) do córtex cerebral, em resposta à estimulação de um ponto deste tecido (LEÃO, 1944), que se propaga lenta e concentricamente, a partir do ponto estimulado. Considerando que a DAC é modulada por mudanças na excitabilidade cerebral e é influenciada por mecanismos que envolvem o Glu (MARRANES et al, 1988; HOLLAND et al, 2010) e o GABA (GUEDES et al, 1992; HAGHIR et al, 2010), torna-se pertinente a sua utilização neste estudo.

Quanto às repercussões eletrofisiológicas cerebrais do tratamento precoce com Gln, nós demonstramos, em trabalho anterior (LIMA et al, 2009), que o tratamento neonatal crônico com 500 mg/Kg/dia de Gln acelerou a propagação da DAC em ratos recém-desmamados. No presente estudo, ampliamos a investigação acerca das alterações cerebrais resultantes do tratamento precoce com Gln através da análise de parâmetros comportamentais e imunohistoquímicos. Além disto, avaliamos se esses efeitos são dose-dependentes e/ou de longa duração.

Os resultados desta tese estão descritos em dois artigos originais que foram submetidos à publicação (Anexos 1 e 2). O primeiro, intitulado "*Neonatal L-glutamine modulates anxiety-like behavior, cortical spreading depression and microglial immunoreactivity: analysis in developing rats suckled on normal size- and large size litters*", teve como objetivo avaliar os efeitos comportamentais (testes de ansiedade), eletrofisiológicos (DAC) e a reação microglial (imunorreatividade ao anticorpo anti-Iba1) do tratamento neonatal crônico com diferentes doses de Gln em ratos em desenvolvimento, submetidos a condições normais ou desfavoráveis de lactação. O segundo, intitulado "*Long-lasting brain effects of neonatal L-glutamine on the anxiety like behavior, cortical spreading depression, and microglial reaction in adult rats*" propõe-se a avaliar se tais efeitos do tratamento neonatal com Gln permanecem até a idade adulta.

2. REVISÃO DA LITERATURA

2.1 Glutamina

A glutamina (Gln; $C_5H_{10}N_2O_3$) é um L-α-aminoácido, com peso molecular de 146,14 e que pode ser sintetizada por todos os tecidos do organismo. Fazem parte de sua composição química: carbono (41,09%), oxigênio (32,84%), nitrogênio (19,17%) e hidrogênio (6,90%; CURI, 2000). Em humanos, é considerada o aminoácido mais abundante, representando 20% do total de aminoácidos livres no plasma, com uma concentração em torno de 0,8 mM. Sua concentração intracelular é ainda maior, aproximadamente 20 mM, representando 40% do total de aminoácidos nas células musculares (SCALISE et al, 2016).

A existência da Gln como aminoácido foi considerada pela primeira vez por Hlasiwetz e Habermann (1873), quando sugeriram que a amônia encontrada em hidrolisados proteicos era o resultado da sua liberação a partir de Gln e asparagina. Dez anos mais tarde, a Gln foi isolada do suco de beterraba e, em 1932, esse aminoácido foi obtido de um hidrolisado da gliadina (DAMODARAN et al, 1932). Deve-se a Hans Krebs, utilizando rins de cobaias e de ratos, a identificação de enzimas que catalisam a síntese e a hidrólise de Gln (KREBS, 1935). Os conhecimentos dos aspectos relativos às vias de síntese e de degradação da Gln aumentaram significativamente nos anos seguintes. Os trabalhos desenvolvidos por Eagle, na década de 50, evidenciaram a importância da Gln para o crescimento e a manutenção de células em cultura (EAGLE, 1955; EAGLE et al, 1956).

No organismo, a Gln exerce múltiplas funções biológicas. É considerada como um aminoácido fundamental para o crescimento e a diferenciação celular, a transferência de nitrogênio entre os órgãos e o fornecimento de energia para as células de rápida proliferação, como os enterócitos e as células do sistema imune (HALL et al, 1996). Além disso, atua como precursora da gliconeogênese hepática, da amoniogênese renal e de neurotransmissores. Também fornece nitrogênio para a síntese de purinas, de pirimidinas e de nucleotídeos (YOUNG e AJAMI, 2001).

As duas principais enzimas intracelulares que regulam a hidrólise e a síntese da Gln são a glutaminase, que hidrolisa o grupo amina terminal da Gln, produzindo glutamato (Glu) e amônia; e a glutamina sintetase (GS), que catalisa a biossíntese *de novo* da Gln a partir do Glu e amônia. A hidrólise da Gln é o primeiro passo para sua utilização e o Glu gerado por essa

reação pode tomar parte em outras reações, principalmente na via que permite que a Gln seja consumida no ciclo dos ácidos tricarboxílicos. A reação de hidrólise da Gln é crítica para a liberação de amônia para o rim e é uma etapa importante, tanto por contribuir para a homeostase ácido-base, como para a formação de purinas e pirimidinas (SCALISE et al, 2016).

Apesar da grande reserva muscular, os estoques endógenos de Gln podem reduzir-se durante insultos catabólicos, como grandes cirurgias, queimaduras extensas, septicemia e inflamação. Assim, em determinadas condições a demanda metabólica pode exceder a sua capacidade de síntese, sendo necessária a suplementação pela dieta. Devido a esta particularidade, é classificada como um aminoácido condicionalmente essencial (LACEY e WILMORE, 1990).

A suplementação com Gln parece exercer efeitos benéficos em recém-nascidos (VAN DEN BERG et al, 2007; VAN ZWOL et al, 2011; PAWLIK et al, 2012; WANG et al, 2013) e em adultos (KOKSAL et al, 2014; GULL et al, 2016) sob diferentes condições clínico-patológicas. Em uma metanálise relativamente recente, foi visto que a suplementação parenteral de Gln reduziu significativamente o risco de infecções e o tempo de permanência hospitalar de doentes críticos (BOLLHALDER et al, 2013). Lorenz et al (2015) mostraram melhora da resposta imunológica em pacientes que receberam suplementação enteral com Gln após cirurgias de grande porte. Alguns estudos identificaram que a depleção de Gln está correlacionada com pior prognóstico e é considerada um fator de risco independente para mortalidade em pacientes em unidades de cuidados intensivos (OUDEMANS et al, 2001).

No que diz respeito aos estudos em animais, resultados benéficos da suplementação com Gln incluem o melhor desenvolvimento da mucosa intestinal (JIANG et al, 2011; DA ROSA et al, 2015), resposta positiva na função imune (FAN et al, 2015), ganho ponderal em ratos desnutridos em fase de crescimento (TANNURI et al, 2000), efeito protetor sobre os neurônios mioentéricos (ZANONI, 2011) e modulação da resposta inflamatória (TSAI et al, 2012).

Alguns autores mostraram que o consumo de Gln é seguro em animais, sem sinais de toxicidade ou atividade mutagênica (TSUBUKU et al; 2004; WONG et al; 2011). Entretanto, diante do aumento do consumo de Gln, não apenas entre atletas ou em situações clínicas específicas, mas entre indivíduos saudáveis que acreditam que o seu consumo regular não apresenta risco e que pode melhorar a memória e o sistema imune, a segurança do uso crônico de Gln passou a ser questionada (HOLECEK, 2013). Em ratos, Holecek (2011) concluiu que o consumo crônico de uma dieta enriquecida com Gln tem efeito negativo sobre o balanço

proteico no músculo esquelético e altera significativamente as concentrações de aminoácidos no plasma e nos tecidos. Além disto, o uso crônico de Gln alterou a resposta ao jejum, com deficiência de Gln nos fluidos corporais e prejuízo na síntese proteica nos tecidos viscerais. Estudos que avaliem os efeitos da suplementação com Gln sobre o sistema nervoso são escassos (LIMA et al, 2009) e essa questão precisa ser melhor investigada.

2.2 Glutamina e sistema nervoso

A Gln é abundantemente encontrada no sistema nervoso central, onde sua concentração chega a ser superior à de qualquer outro aminoácido nos fluidos intersticial e cérebro-espinhal (ALBRECHT et al, 2007; DOLGODILINA et al, 2015). No cérebro, a Gln participa de inúmeras vias metabólicas, destacando-se a sua função de precursora dos neurotransmissores excitatórios, Glu e aspartato; e do neurotransmissor inibitório, ácido γ-aminobutírico (GABA; ALBRECHT; 2011).

No cérebro, a Gln é produzida a partir do Glu por ação da enzima GS, presente nos astrócitos. A maior parte da Gln astrocitária é captada pelos neurônios, onde é degradada pela enzima glutaminase, originando o Glu. Este é liberado pelos neurônios, recaptado pelos astrócitos e reconvertido a Gln, fechando o "ciclo glutamina-glutamato" (BRÖER e BROOKES, 2001; ALBRECHT et al, 2007). Uma porção da Gln disponível no cérebro serve como metabólito energético e outra segue para o sangue através de carreadores específicos. O Glu, por sua vez, atua como precursor do GABA, através de uma reação catalisada pela enzima neuronal ácido glutâmico descarboxilase – GAD (BAK et al, 2006). Na figura 1 encontra-se uma representação do ciclo glutamina-glutamato/GABA.

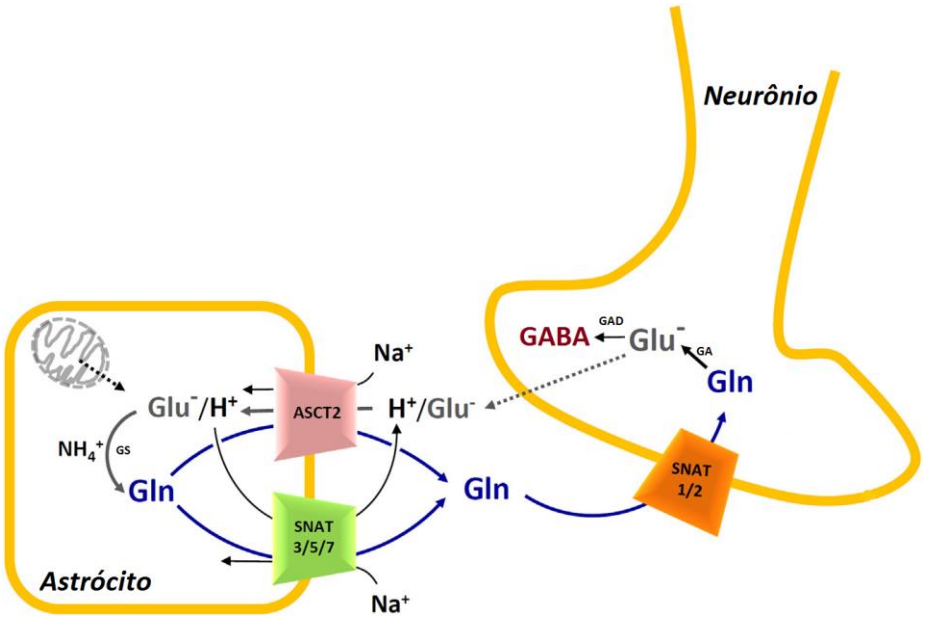

Figura 1. Representação do ciclo glutamina-glutamato/GABA**.** GS = glutamina sintetase, GA = glutaminase, GAD=glutamato descarboxilase. Adaptado de Scalise et al (2016)

O Glu é considerado como o principal neurotransmissor excitatório no cérebro, podendo ser letal para os neurônios e oligodendrócitos em casos de liberação excessiva, o que é conhecido como excitotoxicidade (BAKIRI et al, 2009). Ao contrário do Glu em excesso, a Gln não é tóxica para o cérebro. Entretanto, estudos *in vitro* têm demonstrado ativação da glutaminase em situações específicas, como hipóxia/isquemia, resultando em alterações tóxicas secundárias pelo aumento nas concentrações de Glu (NEWCOMB et al, 1997; 1998)

Segundo alguns autores, o excesso de Gln pode ser, indiretamente, tóxico para o cérebro, o que poderia comprometer a função neural (COOPER et al, 2001; ALBRECHT et al, 2007). Assim, a barreira hematoencefálica está organizada para atenuar a entrada de Gln sanguínea no cérebro e para eliminar do cérebro o excesso de certos compostos ricos em nitrogênio, como a própria Gln. Esse efluxo de Gln do cérebro para o sangue constitui um dos mais importantes mecanismos para a excreção da amônia cerebral (BAK et al, 2006; ADEVA et al, 2012). A toxicidade da Gln sobre os astrócitos e a microglia tem sido sugerida com base na hipótese do “Cavalo de Tróia”. Segundo essa hipótese, a Gln transportada em excesso do citoplasma para a mitocôndria atuaria como um carreador de amônia que, em excesso, ocasiona disfunção mitocondrial, com consequente apoptose dos astrócitos e das células microgliais (ALBRECHT e NORENBERG, 2006; SVOBODA e KERSCHBAUMS, 2009).

A administração oral de Gln pode resultar em um aumento dos níveis plasmáticos (ROGERO et al, 2004) e cerebrais (WANG et al, 2007) deste aminoácido. No cérebro, um estudo realizado em ratos mostrou que a suplementação oral com 500mg/Kg de Gln, resultou em elevação da Gln circulante e no aumento das concentrações de GABA e de Gln no tecido estriatal e no fluido extracelular, sem alterações significativas nos níveis de Glu (WANG et al, 2007).

Considerando que a suplementação com Gln aumenta a sua concentração plasmática (LADD et al, 2010), a liberação de Gln a partir dos astrócitos para a corrente sanguínea poderia ser reduzida, elevando sua concentração no cérebro. Esse aumento da disponibilidade de Gln no cérebro, por sua vez, poderia alterar as interações do ciclo glutamina-glutamato, elevando os níveis de amônia e de Glu. Isso pode ocorrer tanto por ativação da glutaminase nos neurônios, quanto pela supressão da glutamina sintetase nos astrócitos. Além disto, concentrações aumentadas de Gln também podem aumentar sua captação por neurônios GABAérgicos e favorecer a síntese de GABA (HOLECEK, 2011).

Poucos estudos objetivaram demonstrar os efeitos da suplementação com Gln sobre a função cerebral. Um estudo demonstrou que pacientes cirróticos apresentaram comprometimento eletroencefalográfico significativo durante a suplementação oral de Gln (OPPONG et al, 1997). Cocchi et al (1976) avaliaram a função antidepressiva da Gln. Em pacientes com traumatismo craniano, foi demonstrado que a suplementação intravenosa de Gln normalizou a sua concentração plasmática, sem afetar os níveis de cerebrais de Glu (BERG et al, 2006). No entanto, estudos que avaliem efeitos específicos da suplementação com Gln sobre o ciclo glutamina-glutamato ou sobre o equilíbrio entre a transmissão glutamatérgica e GABAérgica não estão disponíveis.

Conforme já foi mencionado no tópico anterior, os efeitos do tratamento precoce com Gln em recém-nascidos pré-termo e/ou de baixo peso ao nascer têm sido amplamente discutidos na literatura (MOE-BYRNE et al, 2016). No entanto, estudos que avaliem as repercussões dessa suplementação sobre o desenvolvimento do cérebro são escassos. Kieviet et al (2012a), em um estudo aleatório e controlado, avaliaram recém-nascidos prematuros ou de peso muito baixo ao nascer que receberam 300mg/Kg/dia de Gln ou um placebo isonitrogenado (alanina) do 3º ao 30º dia pós-natal. Durante o primeiro ano de vida, os recém-nascidos tratados com Gln apresentaram um aumento no crescimento do cérebro (KIEVIET et al, 2014). Aos 8 anos de idade, possuíam um maior volume da substância branca, do hipocampo e do tronco cerebral, quando comparados ao grupo controle (KIEVIET et al, 2012a). Essas diferenças foram fortemente associadas a uma menor incidência de infecções

neonatais sérias no grupo tratado com Gln. Entretanto, no que diz respeito à função cognitiva, motora e comportamental na idade escolar, não houve diferenças entre os grupos suplementados com Gln ou placebo (KIEVIET et al, 2012b).

Em decorrência da escassez de investigações sobre o tema, as repercussões fisiológicas do tratamento com Gln sobre o desenvolvimento neural e a modulação da excitabilidade cerebral não estão bem definidas. Para estudos sobre excitabilidade cerebral, a depressão alastrante cortical (DAC) tem sido utilizada pelo nosso laboratório, como modelo eletrofisiológico (GUEDES, 2011).

2.3. A Depressão Alastrante Cortical

A DAC foi inicialmente descrita por Leão (1944), durante estudos sobre epilepsia experimental, nos quais se registrava a atividade elétrica cortical de coelhos anestesiados. Ela consiste em uma resposta reversível do tecido cortical, provocada por estimulação elétrica, mecânica ou química de um ponto deste tecido. É caracterizada por uma perda da homeostase iônica, com diminuição acentuada da atividade elétrica espontânea ou evocada, a qual se propaga concentricamente a partir do ponto estimulado, numa velocidade de 2 a 5 mm/min. Em seguida, o local inicialmente deprimido começa a se recuperar e em torno de 10 a 15 minutos o tecido encontra-se recuperado (LEÃO, 1972; MARTINS-FERREIRA, 1983). A Figura 1 apresenta a sequência de eventos cíclicos que ocorrem durante a DAC.

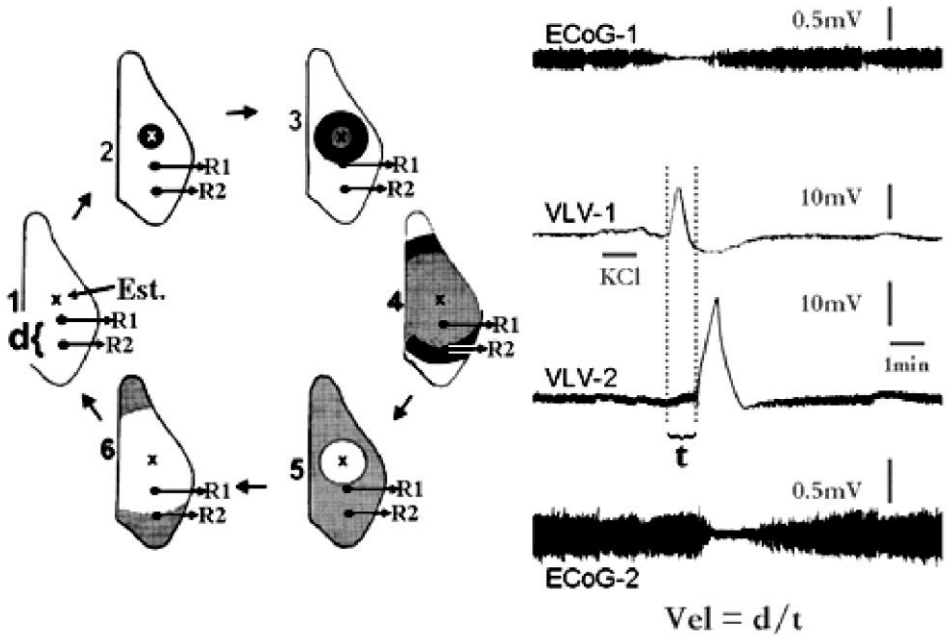

Figura 2. À esquerda, esquema mostrando a sequência temporal cíclica de eventos da depressão alastrante cortical (DAC). Na etapa 1, um ponto cortical (x) foi estimulado (Est.), iniciando a DAC. Dois pontos de registro (R1 e R2) são igualmente mostrados nesta e em todas as outras etapas. A propagação concêntrica da DAC está ilustrada nas etapas 2 a 4, nas quais as áreas escuras representam porções do tecido cortical invadidas pelo fenômeno em tempos sucessivos. As áreas quadriculadas indicam regiões que sofreram a DAC e agora estão se recuperando (áreas refratárias a uma nova estimulação). Nas etapas 5 e 6, observa-se que a recuperação (áreas claras) também se dá de forma concêntrica, sendo o ponto onde a DAC se originou o primeiro a se recuperar totalmente. Finalmente, todo o tecido se recupera, retornando à condição inicial (etapa 1). À direita, mostram-se o eletrocorticograma (ECoG) e a variação lenta de voltagem (VLV), a qual sempre aparece durante a DAC, quando o ECoG diminui sua amplitude. Esses eventos foram registrados simultaneamente nos pontos R1 e R2. Neste exemplo, a depressão do ECoG recupera-se totalmente após cerca de 3 minutos (registros obtidos em nosso laboratório).

Há alguns anos, a DAC vem sendo associada aos mecanismos fisiopatológicos envolvidos na enxaqueca com aura (LAURITZEN, 1994) e na epilepsia (DREIER et al, 2012). Estudos clínicos em humanos, realizados em unidade de terapia intensiva (UTI), têm documentado a presença de DAC após uma variedade de tipos de lesão cerebral aguda, incluindo traumatismo cerebral (HARTINGS et al, 2011), hemorragia subaracnóide (BOSCHE et al, 2010), hemorragia intracerebral e acidente vascular cerebral isquêmico (NAKAMURA et al, 2010; LAURITZEN et al, 2011; DREIER et al, 2011). Mais recentemente, Carlson et al (2016) observaram a presença da DAC em pacientes submetidos a cirurgias neurológicas eletivas. A ocorrência de lesão cerebral induz a DAC e a ocorrência da DAC, particularmente no tecido em risco, resulta em uma maior expansão da lesão, devido as maiores exigências metabólicas que ocorrem no cérebro na tentativa de restaurar os gradientes iônicos (HIZMAN et al, 2015).

Os efeitos negativos da DAC estão relacionados ao aumento da demanda metabólica pelo tecido cerebral. Bioquimicamente, a DAC é caraterizada pelo efluxo celular de potássio, com influxo de cálcio, cloro e sódio; depleção de glicose e aumento do lactato (ENGER et al, 2015; KRAMER et al, 2016). Também está associada ao aumento da liberação de aminoácidos, especialmente o Glu (CARLSON et al, 2012). A restauração dos gradientes iônicos requer a utilização de glicose e ATP, através das bombas de Na/K ATPase. A depressão que ocorre no EcoG é mantida, provavelmente devido a uma incompatibilidade entre o suprimento vascular e a demanda metabólica que ocorre durante a fase de recuperação. A capacidade do tecido para restaurar a sua homeostase metabólica depende de sua integridade e, qualquer condição que prejudique essa capacidade, como hipóxia, hipoglicemia, oligoemia, pode resultar em morte celular e acidente vascular cerebral (LAURITZEN et al, 2011).

Através da implantação de eletrodos subdurais em pacientes que sofreram injúrias cerebrais, a presença de DAC tem sido observada em 70-89% dos casos (DREIER et al, 2006). O aumento do número de DAC está associado a piores resultados neurológicos na hemorragia subaracnoide (DREIER et al, 2006) e no traumatismo cerebral (HARTINGS et al, 2011). Eventos repetidos de DAC também estão associados com a expansão da área de infarto no AVC maligno (NAKAMURA et al, 2010). Esses achados sugerem um papel causal dos eventos repetidos de DAC na hipóxia tecidual, isquemia, comprometimento metabólico e infarto após danos cerebrais (CARLSON et al, 2012).

Estudos em ratos têm demonstrado que o córtex, em algumas situações, pode tornar-se mais vulnerável à DAC, propagando-se com velocidades mais altas. Em outras situações, torna-se mais resistente, com velocidades de propagação mais baixas. Essa susceptibilidade ao fenômeno parece ser influenciada pelas condições em que se encontra o tecido cerebral. As Tabela 1 e 2 apresentam algumas condições e tratamentos farmacológicos/nutricionais que podem influenciar a propagação do fenômeno, resultando em aumento ou redução de sua velocidade.

Os efeitos do tratamento crônico com alguns aminoácidos durante a fase de maior vulnerabilidade de desenvolvimento do SNC sobre a propagação da DAC têm sido avaliados. A L-arginina (FRAZÃO et al, 2008; MAIA et al, 2009) e a Gln (LIMA et al, 2009) aumentaram a velocidade de propagação da DAC, enquanto a taurina e a alanina reduziram (FRANCISCO e GUEDES, 2015). O tratamento agudo de ratos adultos com triptofano, por sua vez, desacelerou a propagação do fenômeno (TRINDADE-FILHO et al 2009).

A desnutrição modifica a ação de certas substâncias sobre a DAC, conforme demonstrado com o diazepam (GUEDES et al, 1992), a glicose (COSTA-CRUZ e GUEDES, 2001), a L-arginina (FRAZÃO et al, 2008; MAIA et al, 2009) e o lítio (DE AGUIAR et al, 2011).

Tabela 1*:* Condições e tratamentos experimentais que facilitam a propagação da DAC em ratos

Condição ou tratamento	**Autores, Ano**
Redução do cloreto extracelular	GUEDES e DO CARMO, 1980
Diazepam (1,5 - 8,0 mg/Kg durante o registro)	GUEDES et al, 1992
Deficiência nutricional pela DBR*****	ROCHA-DE-MELO e GUEDES, 1997
Hipoglicemia	COSTA-CRUZ e GUEDES, 2001
Privação do sono paradoxal	VASCONCELOS et al, 2004
Condição desfavorável de lactação	ROCHA-DE-MELO et al, 2006
Etanol (3g/Kg/18 dias ou 21dias)	ABADIE-GUEDES et al, 2008; ABADIE-GUEDES et al, 2016.
Arginina (300mg/Kg/21 dias)	FRAZÃO et al, 2008
Hipertermia ambiental	FARIAS-SANTOS et al, 2009
Glutamina (500mg/Kg/21 dias)	LIMA et al, 2009
Dipirona (300mg/Kg/7 dias)	AMARAL et al, 2009
Ácido ascórbico (60 ou 120 mg/Kg/22 dias)	MONTE-GUEDES et al, 2011; MENDES-DA-SILVA et al, 2014
Tianeptina (10mg/Kg/23 dias)	AMÂNCIO-DOS-SANTOS et al, 2013
Glutamato monossódico (2 ou 4g/Kg/14 dias)	LIMA et al, 2013; 2014
Dexametasona (doses crescentes 0,5mg/kg, 0,3mg/kg e 0,1mg/kg por 3 dias)	LOPES-DE-MORAIS et al, 2014

*****Dieta Básica Regional

Tabela 2. Condições e tratamentos experimentais que dificultam a propagação da DAC em ratos

Condição ou tratamento	**Autores, Ano**
Lítio (1,5 g/Kg/dia/21 dias)	GUEDES et al, 1989
Hiperglicemia	XIMENES-DA-SILVA e GUEDES, 1991 COSTA-CRUZ et al, 2006
Anestésicos	GUEDES e BARRETO, 1992
Hipotireoidismo	GUEDES e PEREIRA-DA-SILVA, 1993
Envelhecimento	GUEDES et al, 1996
Epilepsia pela pilocarpina (350 mg/kg/10 dias)	COSTA-CRUZ et al, 2006
Estimulação ambiental	SANTOS-MONTEIRO et al, 2000
Fluoxetina (10 ou 20 ou 40 mg/kg/21 dias)	AMÂNCIO-DOS-SANTOS et al, 2006
Condições favoráveis de aleitamento	ROCHA-DE-MELO et al, 2006
Pilocarpina (95 ou 190mg/kg durante o registro)	GUEDES e VASCONCELOS et al, 2008
Triptofano (125 mg/kg durante o registro)	TRINDADE-FILHO et al, 2009
Deficiência, na dieta, de ácidos graxos essenciais	BORBA et al, 2010
Abolição da função ovariana (castração)	ACCIOLY et al, 2012
Dieta hiperlipídica	GERMANO et al, 2013
Naloxona (10 mg/Kg/22 dias)	GUEDES et al, 2013
Ácido ascórbico (30mg/Kg/22 dias)	MENDES-DA-SILVA et al, 2014
Lectina do *Canavalia ensiformis* (1 ou 10 mg/Kg/20 dias)	SOARES et al, 2015
Taurina (300mg/Kg/21 dias)	FRANCISCO e GUEDES, 2015
Alanina (300mg/Kg/21 dias)	FRANCISCO e GUEDES, 2015
Etanol (1g/Kg/1dia)	ABADIE-GUEDES et al, 2016

Poucos estudos foram direcionados a demonstrar a relação entre a Gln e a DAC e seus resultados são controversos. Inicialmente, relatou-se que a aplicação tópica de Gln elicitou a DAC em ratos e em coelhos (VAN HARREVELD, 1959; BURES et al, 1960), mas um outro estudo realizado posteriormente demonstrou que a Gln não possuía tal efeito (DO CARMO e LEÃO, 1972). Subsequentemente, outros autores relataram que na região em que a Gln havia sido aplicada topicamente, a amplitude da variação lenta de voltagem e mudanças na impedância de uma DAC evocada a distância estavam reduzidas (DO CARMO e FERREIRA-FILHO, 1976). Maranhão-Filho e Leão (1991) mostraram que a aplicação tópica de Gln (75 mM) sobre a superfície dorso-lateral do neocórtex de coelhos tornou o tecido reversivelmente refratário a DAC. Tani et al (2007), estudando a atividade elétrica em fatias de córtex de ratos observaram que a adição de concentrações supra fisiológicas (2-5mM) de Gln ao perfusato induziu eventos semelhantes à DAC. Confirmando este achado, An et al (2008) investigaram os efeitos eletrofisiológicos da adição de diferentes concentrações de Gln na composição do fluido cérebro-espinhal artificial (0,5mM e 2-5mM) e demonstrou o surgimento de episódios de DAC quando a concentração de Gln foi maior (2-5mM). Lima et al (2009) demonstraram que o tratamento oral com 500 mg/Kg/dia de Gln, durante o período crítico de desenvolvimento do sistema nervoso, facilitou a propagação da DAC em ratos recém-desmamados.

Conforme foi mencionado, nosso estudo anterior (LIMA et al, 2009) foi pioneiro na investigação de alterações eletrofisiológicas (DAC) resultantes do tratamento neonatal crônico com Gln. Portanto, estudos complementares sobre este tema tornam-se necessários, a fim de que os mecanismos envolvidos com as alterações eletrofisiológicas encontradas sejam elucidados.

2.4 Microglia

A microglia, considerada os macrófagos do sistema nervoso central, está distribuída heterogeneamente por todo o cérebro, representando cerca de 10 a 15% das células cerebrais (YANG et al, 2013). Vários estudos têm demonstrado o papel desempenhado pelas células microgliais tanto em condições fisiológicas, através do controle da homeostase (MARIANI e KIELIAN, 2009; DAVALOS et al, 2005; NIMMERJAHN et al, 2005), quanto em situações patológicas, contribuindo para a manutenção da integridade tecidual após uma grande

variedade de insultos, incluindo lesões, neurotoxicidade e infecções (CHEN e TRAPP, 2016; HUGHES, 2012; GRAEBER, 2010). Nestas circunstâncias, as células microgliais tornam-se ativadas, mostrando padrões de reatividade específicos, os quais são totalmente dependentes da magnitude e da natureza da lesão, bem como do microambiente específico onde este processo de ativação teve lugar.

A ativação microglial envolve mudanças na expressão de genes e do fenótipo das células microgliais, que se manifesta em modificações morfológicas, aumento/diminuição ou na expressão de moléculas de superfície e/ou citoplasmáticas e secreção de uma ampla variedade de substâncias, tais como citocinas e fatores tróficos (CHEN e TRAPP, 2016; RANSOHOFF et al, 2009; DHEEN et al, 2007). Ao produzir essas moléculas, as células microgliais podem modular a progressão da neuroinflamação em curto e em longo prazo (KIGERL et al, 2014; BEYNON et al, 2012). A ativação da microglia está relacionada a algumas doenças neurodegenerativas, como a esclerose lateral amiotrófica, doença de Huntington, esclerose múltipla e doença de Alzheimer (CARTIER et al, 2014).

Na microglia, a Gln pode servir como metabólito energético (KALSBEEK et al, 2016). Embora a Gln seja o produto final do metabolismo do Glu e da amônia, ela também pode ser usada como fonte energética para o cérebro (POCHINI et al, 2014). Quando a Gln é retomada pelos neurônios e/ou astrócitos, ela pode ser convertida em α-cetoglutarato via glutaminase e glutamato desidrogenase. Estas enzimas, bem como os transportadores de Gln, são expressos pela microglia de mamíferos, sugerindo que as células microgliais possuem uma via metabólica ativa da Gln. Vários estudos têm demonstrado neurotoxicidade do Glu produzido pela microglia (JIN et al, 2015; HUANG et al, 2011). Em estudo *in vitro*, Svoboda e Kerschbaum (2009) relataram apoptose da microglia como resultado do aumento da concentração de Gln, sugerindo toxicidade da Gln para o cérebro. Estes autores concluíram que a hidrólise de Gln e, consequentemente, o acúmulo de amônia mitocondrial, induziu a via intrínseca da apoptose. Esse estudo reforça a hipótese do cavalo de Tróia para a toxicidade da Gln, que envolve a astroglia e a microglia (ALBRECHT e NORENBERG, 2006; SVOBODA e KERSCHBAUM, 2009). Mais estudos são necessários a fim de esclarecer a reação microglial resultante do aumento da concentração de Gln no cérebro.

2.5. Nutrição e sistema nervoso

O estado nutricional adequado é condição fundamental para o desenvolvimento e a organização do sistema nervoso, estando diretamente relacionado com a produção de energia e de nutrientes necessários ao desenvolvimento de suas estruturas celulares e aos seus diversos processos metabólicos (MORGANE et al, 1993).

A deficiência de nutrientes no organismo caracteriza a desnutrição. Em humanos, sobretudo na infância, constitui um dos maiores problemas de saúde, enfrentado por países em desenvolvimento. Apresenta etiologia multifatorial, estando normalmente relacionada ao baixo nível social e econômico (OLIVEIRA et al, 2006). A sua prevalência é bem elevada em algumas regiões da Ásia e da África, exercendo grande impacto sobre as despesas públicas com a saúde (NUBÉ e SONNEVELD, 2005). Na América Latina, a desnutrição é manifestada principalmente como nanismo e deficiência de micronutrientes, a despeito da prevalência decrescente da forma aguda severa ou moderada (WEISSTAUB e ARAYA, 2008). No entanto, a desnutrição aguda ainda resulta em uma alta taxa de letalidade (MÜLLER e KRAWINKEL, 2005).

As consequências da desnutrição infantil sobre o sistema nervoso estão bem estabelecidas (ODEBODE e ODEBODE, 2005; BENTON, 2008; MARTINS et al, 2011). Além de afetar a maturação deste sistema, a desnutrição promove alterações bioquímicas, fisiológicas e anatômicas no cérebro, podendo causar prejuízos sobre o desenvolvimento cognitivo e comportamental. Esses efeitos deletérios da desnutrição podem persistir por longo-prazo ou tornarem-se permanentes, dependendo do período, da duração, da intensidade e do tipo de desnutrição (MORGANE et al, 1993; GUEDES et al, 1996).

O período da vida em que a desnutrição ocorre é um fator particularmente importante na determinação do impacto que esta terá sobre o sistema nervoso. Dobbing (1968) relatou que existem períodos especificamente “críticos”, no início da vida, onde o organismo parece estar mais vulnerável aos efeitos da desnutrição. No caso do sistema nervoso, o período crítico ocorre logo no início da vida. Nesse período, o peso do cérebro aumenta de maneira extremamente rápida, como resultado dos processos neuroquímicos e neuroanatômicos próprios do seu desenvolvimento. Segundo Morgane et al (1993), o período crítico está associado ao pico de atividade de eventos específicos da neurogênese, gliogênese, migração e diferenciação celular. Esse período de maior vulnerabilidade do cérebro à desnutrição difere entre as espécies de mamíferos. Em humanos, corresponde ao período que vai do terceiro

trimestre de gestação até o segundo ano de vida (DOBBING, 1968). No rato, ocorre durante as três primeiras semanas após o nascimento (MORGANE et al, 1993), coincidindo com o período de aleitamento. É razoável supor que, durante esses períodos de crescimento rápido, as exigências de nutrientes necessários ao desenvolvimento cerebral são maiores. Portanto, a estrutura e a função cerebral estarão comprometidas se a alimentação for inadequada, ocasionando alterações estruturais e déficits funcionais, com prejuízo para vários processos cerebrais (MORGANE et al, 1993; MORGANE et al, 2002; PENIDO et al, 2012).

Estudos em humanos demonstraram consequências duradouras da desnutrição precoce sobre o desenvolvimento mental e a saúde psiquiátrica (KERAC et al, 2014; ROSEBOOM et al, 2012). A exposição pré-natal à desnutrição tem sido associada a deficiências cognitivas, dificuldades de aprendizagem e esquizofrenia. No entanto, os efeitos biológicos são dependentes do tempo e da duração da desnutrição (ROSEBOOM et al, 2012). Déficit nutricional pós-natal precoce pode impedir o crescimento e a maturação do cérebro.

A natureza do nutriente deficiente determinará o tipo de desnutrição, ou seja, se é uma deficiência de proteínas, de calorias, de vitaminas, de minerais, ou se é uma combinação delas. Os efeitos da desnutrição proteica sobre o sistema nervoso central são os mais ressaltados na literatura (KAR et al, 2008; DURAN et al, 2011; RANADE et al, 2012), com prejuízos morfológicos e neuroquímicos, uma vez que a proteína fornece aminoácidos essenciais para a síntese de enzimas, de neuropeptídeos, de neurotransmissores cerebrais e de proteínas estruturais. Além da proteína, outros nutrientes isolados, tais como os ácidos graxos essenciais (INNIS, 2008; DE VELASCO et al, 2012), o ácido fólico, a vitamina A (JIANG et al; 2012), a vitamina B12 (BLACK, 2008) e o iodo (BERBEL et al, 2008) também foram estudados, no que concerne ao seu papel sobre o desenvolvimento cerebral.

Em modelos animais, a desnutrição durante o período crítico de desenvolvimento do cérebro leva a uma redução nas células cerebrais, na produção de mielina e no número de sinapses, além de alteração nos sistemas de neurotransmissores. O cerebelo e o hipocampo têm demonstrado uma vulnerabilidade particular aos efeitos da desnutrição pós-natal precoce (LEVITSKY e STRUPP, 1995). Embora alguns efeitos da desnutrição sobre a estrutura e o metabolismo cerebral sejam reversíveis pela reabilitação nutricional, outros persistem, com efeitos subsequentes sobre o comportamento e a função cognitiva (LEVITSKY e STRUPP, 1995; PENIDO et al, 2012).

Diversos modelos experimentais vêm sendo utilizados para se provocar desnutrição em animais de laboratório. Dentre estes, encontra-se aquele baseado na manipulação do tamanho das ninhadas, método que será empregado no presente trabalho. De Lucca et al

(1977), Fernandez et al (1993) e Rocha-de-Melo et al (2006) relataram o uso da técnica das grandes ninhadas para impor a subnutrição, baseando-se no aumento do número de filhotes que serão amamentados por uma única mãe. Morgane et al (1978) admitem que neste caso, a qualidade do leite é preservada, reduzindo-se a quantidade recebida por cada filhote, levando-os à deficiência nutricional.

A importância de diversos nutrientes sobre o desenvolvimento do cérebro já está bem documentada na literatura. No entanto, muitos questionamentos ainda precisam ser adequadamente respondidos, especificamente acerca dos efeitos da suplementação com determinados aminoácidos, como a Gln, durante o período de maior vulnerabilidade do desenvolvimento cerebral.

3. HIPÓTESE

- O tratamento crônico com L-Gln durante o período de desenvolvimento do cérebro altera parâmetros comportamentais preditores de ansiedade, facilita a propagação da depressão alastrante cortical e promove ativação microglial. Estes efeitos são dependentes da dose, da condição de lactação e da idade do animal.

4. OBJETIVOS

4.1 Geral

Descrever, em ratos recém-desmamados e adultos, os efeitos comportamentais, eletrofisiológicos e microgliais do tratamento neonatal com diferentes doses de L-Gln, associados a condições normais ou desfavoráveis de lactação.

4.2 Específicos

- Avaliar a evolução ponderal em função do estado nutricional e da suplementação com Gln, como indicador do desenvolvimento corporal sob ação desses tratamentos;
- Avaliar alterações comportamentais relacionadas à ansiedade como efeitos do tratamento com Gln;
- Analisar o efeito da suplementação com diferentes doses de Gln sobre a suscetibilidade cortical à DAC após o desmame e na idade adulta;
- Analisar, através de marcação imunohistoquímica, a reação microglial associada aos respectivos tratamentos;

5. MÉTODOS

5.1 Animais

Foram estudados 224 ratos machos, da linhagem Wistar, oriundos da Colônia do Departamento de Nutrição da Universidade Federal de Pernambuco (UFPE). Estes animais foram mantidos em gaiolas de polipropileno, em ambiente com condições padrão de iluminação (ciclo claro/escuro de 12/12 horas; fase clara com início às 6:00 h) e temperatura em torno de 22±1ºC. As ninhadas eram compostas por machos e fêmeas, mas apenas os machos foram utilizados neste estudo. Esses eram selecionados aleatoriamente para cada tratamento, de modo que em cada ninhada houvesse animais recebendo todos os tipos de tratamento.

Os procedimentos experimentais executados neste estudo receberam aprovação da Comissão de Ética no Uso de Animais da UFPE (Processo n° 23076.010667/2012-84, **Anexo 1**) e encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo *National Institute of Health Guide for Care and Use of Laboratory Animals*.

Após o tratamento com Gln, os ratos foram divididos aleatoriamente em 2 grandes grupos, de acordo com a idade em que seriam submetidos aos testes experimentais, a saber: grupo recém-desmamado (D) e grupo adulto (A).

5.2 Condições de lactação

De acordo com a condição de lactação foram divididos em dois grupos: 1) lactação normal (grupo L_9), ninhadas constituídas por 9 filhotes, e 2) lactação desfavorável (grupo L_{15}; ninhadas com 15 filhotes). Sabe-se que a condição de lactação desfavorável, pelo aumento no tamanho da ninhada, promove competição pelo leite materno, levando a uma deficiência nutricional (FRANCISCO e GUEDES, 2015). O desmame foi realizado, em ambos os grupos, no 25° dia de vida pós-natal (P), considerando o dia do nascimento como dia zero (FRAZÃO et al., 2008). Após o desmame, os filhotes foram alimentados com a dieta-padrão materna (Presence; Purina do Brasil Ltda.), sendo destinados aleatoriamente para compor os grupos D ou A.

5.3 Tratamento com L-glutamina

Do P7 ao P27, os animais foram tratados por gavagem com Gln (Sigma-Aldrich, St Louis, USA) nas doses de 250mg/Kg/dia (grupo **Gln250**), 500mg/Kg/dia (**Gln500**) ou 750mg/Kg/dia (**Gln 750**). A dose de 500 mg/Kg/dia foi definida com base na recomendação de suplementação de Gln já demonstrada em elevar as concentrações plasmáticas e cerebrais em ratos (WANG et al, 2007). Para testar o efeito de outras doses, esta recomendação foi reduzida pela metade (250mg/Kg) ou aumentada 1,5 vez (750 mg/Kg). Dois grupos controles adicionais também foram avaliados, um tratado com água, veículo no qual a Gln foi dissolvida (grupo **Veículo**) e outro que não recebeu gavagem (grupo **Ingênuo**). A representação esquemática dos grupos estudados encontra-se na **Figura 3.**

Para o preparo das soluções, a Gln foi pesada em balança e dissolvida em água destilada imediatamente antes da administração. O procedimento de gavagem foi realizado sempre no mesmo horário do dia, entre 12:00 e 14:00 horas. Durante a primeira semana de

tratamento, a gavagem foi realizada através de uma cânula de polietileno, flexível e de pequeno calibre conectada a uma seringa de vidro de 1,0 ml. A extensão da cânula que deveria ser introduzida pela boca foi marcada para que se tivesse a segurança de que, atingida aquela marca a extremidade da cânula alcançasse a porção distal do estômago, onde a solução seria injetada lentamente. Nas duas semanas seguintes, a cânula de polietileno foi substituída por uma cânula de metal.

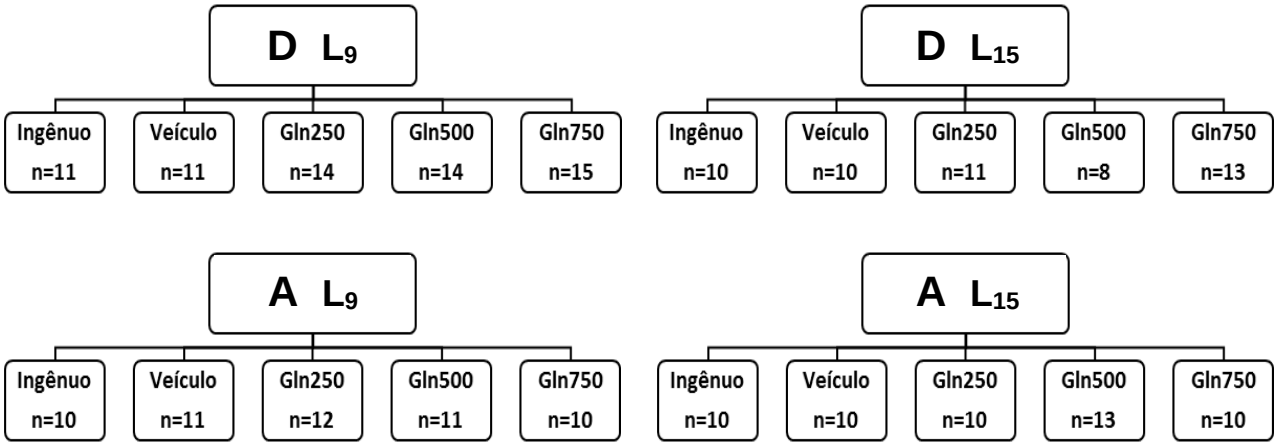

Figura 3. Representação esquemática dos grupos estudados

5.4 Pesos corporais

Os pesos corporais dos animais foram obtidos através de uma balança eletrônica da marca Filizola (capacidade de 3,0 Kg e escala em divisão de 0,5g) aos P7, P14, P21 e P30-35 nos animais do grupo D e aos P7, P14, P21, P60 e P90 no grupo A.

5.5 Testes comportamentais

5.5.1 Labirinto em cruz elevado

Aos P28 (D) e aos P88-P90 (A), os animais foram submetidos ao teste do LCE em uma sala com redução de luminosidade e atenuação de ruídos. O LCE consiste em um aparato de madeira em forma de cruz e com quatro braços (49x10cm cada), elevado a 55 cm do solo. Dois dos braços eram abertos e outros dois braços, dispostos perpendicularmente aos abertos, eram fechados por paredes laterais de 50 cm de altura. Os braços eram unidos por uma plataforma central de 10x10cm. No início do teste, cada animal foi colocado individualmente na área central do labirinto, com a cabeça direcionada para um dos braços abertos, podendo explorar livremente o equipamento durante 5 min. Os testes foram registrados por uma câmera digital localizada verticalmente acima do LCE e conectada a um computador. Entre um animal e outro, o aparato foi limpo com um papel toalha e álcool etílico a 70%. Os testes foram analisados através do *software* AnyMaze (versão 4.99 m). Foram quantificados os seguintes parâmetros: distância percorrida (m), tempo total de imobilidade (s), número de entradas no braço aberto e o tempo de permanência no braço aberto (s).

5.5.2 Campo aberto

Aos P30 (D) e aos P90-P95 (A), os animais foram testados no aparelho de campo aberto. O aparato consiste em uma arena circular com 100 cm de diâmetro e 52 cm de altura, localizado em uma sala com redução de luminosidade e atenuação de ruídos. No início do teste, o animal foi posicionado no centro do aparato, e seu comportamento foi registrado durante 5 min por uma câmera digital localizada verticalmente acima do campo aberto. Após cada teste, o aparelho era limpo com papel toalha e etanol a 70%. A análise dos dados foi realizada pelo *software* ANYmaze (versão 4.99m), que quantificou a distância total percorrida (m), o tempo total de imobilidade (s), o número de entradas na zona central do aparato e o tempo gasto (s) na zona central.

5.6 Registro da depressão alastrante cortical

No dia do registro eletrofisiológico (P30-P35 para o grupo D e P90-P120 para o grupo A), cada animal foi anestesiado com uma mistura de 1000 mg/Kg de uretana + 40 mg/Kg de cloralose (i.p.) e mantido sobre um aquecedor elétrico durante todo o período de registro, para que a temperatura retal se mantivesse em torno de 37 ± 1º C. A cabeça do animal foi fixada à base de um aparelho esterotáxico, a fim de permitir a abertura da pele da cabeça e exposição do crânio. No lado direito do crânio foram trepanados três orifícios alinhados paralelamente à linha média, com 2-3 mm de diâmetro, dois na região parietal e um na região frontal.

O registro eletrofisiológico foi realizado nos dois pontos da região parietal durante 4 horas através de dois eletrodos Ag/AgCl (um em cada orifício) contra um eletrodo comum de referência, localizado no osso nasal. A DAC foi provocada a cada 20 minutos através da estimulação química com cloreto de potássio (KCl) a 2% (aproximadamente 270mM) no orifício frontal durante 1 min. O cálculo da velocidade de propagação da DAC foi realizado com base na distância entre os dois eletrodos registradores e no tempo gasto para a DAC percorrer essa distância. Para cada animal, foram calculadas a velocidade de propagação, a amplitude e a duração de cada DAC. Em seguida, alguns animais, ainda anestesiados, foram submetidos à perfusão transcardíaca para estudo imunohistoquímico, conforme descrito abaixo. Outros animais, enquanto ainda anestesiados, foram submetidos à eutanásia através lesão bulbar provocada pela introdução de uma agulha pela cisterna magna, provocando-se parada cardiorrespiratória, como anteriormente descrito (LIMA et al, 2009).

5.7- Análise imunohistoquímica da microglia

Os encéfalos fixados de 44 animais (22 D e 22 A) foram processados para imunomarcação com anticorpo anti-Iba1. Dos 22 animais de cada grupo, 6 eram controles veículo (3 L_9 e 3 L_{15}), 8 Gln250 (4 L_9 e 4 L_{15}) e 8 Gln500 (4 L_9 e 4 L_{15}).

Os cérebros foram perfundidos com solução salina a NaCl 0,9% seguido por paraformaldeído a 4% diluído em 0,1 M de tampão fosfato (pH = 7,4). Foram conservados em paraformaldeído a 4%, crioprotegidos em solução de sacarose a 30% e armazenados em condições apropriadas (a -80º C) até serem analisados. Para isto, cortes seriados longitudinais

com 40 µm de espessura foram obtidos a -20°C através de um criostato (Leica-1850). Os cortes foram imunomarcados com anticorpo anti-Iba-1 (anti-Iba1, #019-19741; Wako Puré Chemical Industries Ltd., Osaka, Japão), específico para microglia. Os cortes flutuantes livres no meio de incubação foram submetidos ao bloqueio da peroxidase endógena (2% de H_2O_2 em 70% metanol, durante 10 min). Depois, os cortes foram incubados durante 1 h em solução tampão de bloqueio (BB) contendo 0,05 M de solução salina tamponada com Tris (TBS), pH= 7,4, 10% de soro fetal de vitelo, 3% de albumina de soro bovino, e Triton X – 100 a 1%. Em seguida, os cortes foram incubados durante toda a noite a 4°C com anticorpo de coelho anti-Iba1 (1:1.500 diluído em solução BB). Após três lavagens com TBS, mais 1% de Triton, os cortes foram incubados à temperatura ambiente durante 1h com anticorpos secundários anti-coelho biotinilado (1:500). Em seguida, os cortes foram lavados em TBS com Triton 1% e incubados em um conjugado com peroxidase de estreptavidina (1:500). A reação da peroxidase foi visualizada após a incubação dos cortes em tampão Tris, contendo 0,5 mg/ml de 3,3'-diaminobenzidina (DAB) e 0,33 µl/ml de H_2O_2. Por fim, os cortes foram montados em lâminas de vidro, desidratadas em álcoois graduados e xileno, e cobertas com lamínulas e Entellan®.

A análise densitométrica do córtex cerebral e hipocampo (região-cornus Amoni 1-CA1) foi realizada no hemisfério esquerdo para cada animal. Em cada corte, foram analisadas fotomicrografias de quatro campos dentro do córtex parietal e dois campos na região CA1 do hipocampo usando o *software* Image J (National Institutes of Health, EUA; versão 1.46r). Um microscópio Leica-LMG, acoplado a uma câmera de alta resolução Samsung (modelo SHC-410NAD), foi utilizado para obtenção das imagens digitais dos cortes cerebrais. As imagens do córtex e do hipocampo foram obtidas com uma objetiva 20X. Para obtenção das imagens digitais, foram utilizadas as mesmas condições de intensidade de luz, em todos os grupos. Como parâmetros, foram analisadas a percentagem da área ocupada pelas células imunomarcadas, bem como a imunorreatividade total expressa em unidades arbitrárias.

5.8 Análise estatística

Os dados foram expressos como média ± desvio padrão. As comparações intergrupos foram realizadas através da ANOVA de 2 vias, incluindo a condição nutricional (L_9 e L_{15}) e o tratamento (ingênuo, veículo, Gln250, Gln500 e Gln750) como fatores, seguida pelo teste de

Holm-Sidak, quando indicado. Foram aceitas como significantes as diferenças em que $p \leq 0,05$.

6. RESULTADOS

6.1 Artigo 1

Title:

Neonatal L-glutamine modulates anxiety-like behavior, cortical spreading depression and microglial immunoreactivity: analysis in developing rats suckled on normal size- and large size litters

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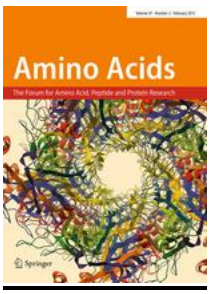

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Abstract

In mammals, L-glutamine (Gln) can alter the glutamate-Gln cycle and consequently brain excitability. Here, we investigated in developing rats the effect of treatment with different doses of Gln on anxiety-like behavior, cortical spreading depression (CSD) and microglial activation expressed as Iba1-immunoreactivity. Wistar rats were suckled in litters with 9 and 15 pups (groups L_9 and L_{15}; respectively normal size- and large size litters). From postnatal days (P) 7 to 27, the animals received Gln per gavage (250 mg/kg/day, 500 mg/kg/day or 750 mg/kg/day), or vehicle (water), or no treatment (naive). At P28 and P30 we tested the animals respectively in the elevated plus maze and open field. At P30-35 we measured CSD parameters (velocity of propagation, amplitude, and duration). Fixative-perfused brains were processed for microglial immunolabeling with anti-IBA-1 antibodies to analyze cortical microglia. Rats treated with Gln presented an anxiolytic behavior and accelerated CSD propagation when compared to the water- and naive control groups. Furthermore, CSD velocity was higher ($p < 0.001$) in the L_{15} compared to the L_9 condition. Gln treatment increased Iba1 immunolabeling both in the parietal cortex and CA1 hippocampus, indicating microglial activation. The Gln effect was dose-dependent for anxiety-like behavior and CSD in both litter sizes, and for microglial activation in the L_{15} groups. Besides confirming previous electrophysiological findings (CSD acceleration after Gln), our data demonstrate for the first time a behavioral and microglial activation that is associated with early Gln treatment in developing animals, and that is possibly operated via changes in brain excitability.

Keywords: L-glutamine, Cortical spreading depression, Brain excitability, Immunoreactivity, Microglia, Anxiety-like behavior.

Introduction

Glutamine (Gln) is found abundantly in the mammalian central nervous system, where it participates in a variety of metabolic pathways. In distinct types of neuronal cells, Gln influences the synthesis of the excitatory amino acids, glutamate (Glu) and aspartate, and the inhibitory amino acid, γ-amino butyric acid (GABA; Albrecht 2011). A neuron-glia metabolic interaction, known as the glutamate-glutamine cycle, controls the extracellular concentration of Glu and enables the detoxification of ammonia that crosses freely from the blood across the blood-brain barrier (Daikhin and Yudkoff. 2000). Enhanced Gln availability could alter the interactions in the glutamate-glutamine cycle and affect ammonia and Glu levels in the brain. Increased availability of Gln may also affect translocation of Gln into GABAergic neurons and GABA is taken up by astroglia (Holecek 2013).

In humans, Gln supplementation may be beneficial in preterm infants, especially in the reduction of infectious complications (Moe-Byrne 2016). However, the potential effects of early Gln supplementation on brain development and subsequent neuron-glia organization and behavioral outcome have not yet been fully elucidated (De Keviet et al. 2012). In addition, few studies have so far evaluated the effect of Gln supplementation on the excitability-related balance between glutamatergic and GABAergic transmission (Lima et al. 2009). These authors reported in developing rats a facilitating effect of Gln (500 mg/kg/day over P7-P27) on the excitability-related phenomenon known as cortical spreading depression (CSD).

CSD is a fully reversible neural response, first described in the rabbit cortex, as a slowly propagating wave of depression of spontaneous neuronal activity produced by electrical, mechanical or chemical stimulation of one point on brain tissue, from which it spreads concentrically to remote cortical regions (Leão 1944). This phenomenon has since been demonstrated in a number of animal studies (Toriumi et al. 2016, Shatillo et al. 2015, Lopes-de-Morais et al. 2014, Guedes et al. 2013). CSD is facilitated under unfavorable lactation

conditions (suckling in large litters; Guedes, 2011), and this condition can also modulate the effect of other treatments (Guedes et al. 2013). Human studies have demonstrated the occurrence of this depolarizing phenomenon in the setting of a variety of pathological states, including migraine (Lauritzen and Strong 2016), cerebrovascular disorders (Nakamura et al. 2010) epilepsy (Dreier et al. 2012), traumatic brain injury (Hartings et al. 2011), and neurosurgical procedures (Carlson et al. 2016).

Measuring CSD velocity of propagation along the cortical tissue is a reasonable and easy way of estimating the brain susceptibility to CSD. This has been experimentally characterized in our laboratory under conditions of environmental, pharmacological, and nutritional manipulations (Abadie-Guedes et al. 2016; Monteiro et al. 2015; Lopes-de-Morais et al. 2014), including treatment with other amino acids (Frazão et al. 2008). Recent studies have also shown possible relationships between alterations of CSD, and behavioral (Francisco and Guedes 2015) and microglial activation (Soares et al. 2015, Lima et al. 2013; 2014). Also, a relationship between litter size and microglia activation and behavior has been reported (Viana et al 2013; Tu et al 2012).

In order to test whether the CSD facilitating effect of early treatment with Gln is dose dependent, we used three different doses of Gln during the brain development period. Furthermore, we investigated the Gln effect on anxiety-like behavior and Iba1-immunoreactivity of microglia. Considering that unfavorable lactation conditions can modulate both CSD and the brain effects of amino acids (Francisco and Guedes 2015), as well as microglia (Tu et al 2012) and behavior (Viana et al. 2013), we analyzed the Gln effects on rats suckled in normal and large litters.

Materials and methods

Animals

The Wistar rat pups of this study (n = 117 males) were handled in accordance with the norms established by the National Institutes of Health Guide for Care and Use of Laboratory Animals (Bethesda, MD, USA). All experimental procedures were previously approved by the Institutional Ethics Committee for Animal Research of our University (Process nº. 23076.010667/2012-84). Animals were housed in polyethylene cages (51 cm X 35.5 cm X 18.5 cm) in a room maintained at 22 ± 1 °C with a 12:12 h light: dark cycle (lights on at 6:00 a.m.). The newborn male and female rats, born from distinct dams, were pooled and assigned to be suckled under favorable or unfavorable conditions, represented respectively by normal size litters, with 9 pups (L_9 groups; n = 65 males), and large size litters with 15 pups (L_{15} groups; n = 52 males). On postnatal day (P) 25, both groups were weaned and switched to the maternal lab chow diet (Purina Ltd.) with 23% protein. Only the male pups (n=117) were used in this study.

L-Glutamine treatment

From P7 to P27, three groups of male pups received per gavage three doses of Gln (Sigma, St. Louis, MO, USA): 250 mg/kg/day (Gln250 group; 14 L_9 and 13 L_{15} pups), or 500 mg/kg/day (Gln500; 14 L_9 and 8 L_{15} pups), or 750 mg/kg/day (Gln750; 15 L_9 and 11 L_{15} pups). Two additional control groups received no gavage (naive group; 11 L_9 and 10 L_{15} pups) or distilled water (vehicle; 11 L_9 and 10 L_{15} pups). The gavage volume of the amino acid solutions or of distilled water ranged from 0.5ml/day (in the second week of life) to 1.0 ml/day (in the third and fourth week of life).

Body Weights

The rats were weighed at P7, P14, P21, and P30–35 (when the electrophysiological recordings were performed).

Elevated plus-maze test

The elevated plus-maze test was conducted on P28. The cross-shaped apparatus consisted of four arms (two closed arms and two open arms, each measuring 49 cm long X 10 cm wide) raised 55 cm above the floor. A central squared platform (10 X 10 cm wide) connected the open and closed arms. For each 5-min session, under dim light and in a sound-attenuated room, the rat was initially placed in the central platform facing an open arm. A video camera recorded the behavioral activity of the animal. The recorded activity was stored in a computer and subsequently analyzed with the software ANYmaze™ (version 4.99 m). After each test, the arms and the central platform were cleaned with a paper cloth soaked with 70% ethanol. The following parameters were analyzed: total distance traveled, number of entries into the open arms, and time spent in the open arms. We considered that the animal entered one open or one closed arm when its four paws entered the arm.

Open field test

On P30, the rats were placed in the center of a circular arena (diameter 100 cm and height 52 cm). The apparatus was placed in a room with a dim light and sound-attenuation. Rats were positioned in the center arena and their movements were captured using a digital camera for 5 min. Between each test the open field was wiped with a paper cloth soaked with 70% ethanol. The recorded activity was stored in a computer and subsequently analyzed with the software ANYmaze™ (version 4.99 m). The following parameters were considered: total distance traveled, the number of entries in the central zone and the time spent in central zone.

CSD recording

On the day of CSD recording (P30–35), the animal was anesthetized with a mixture of 1 g/kg urethane plus 40 mg/kg chloralose injected intraperitoneally. Three trephine holes were drilled on the right side of the skull, aligned in the frontal-to-occipital direction and parallel to the midline. One hole (2mm in diameter) was positioned on the frontal bone and used to apply the stimulus (KCl) to elicit CSD. The other two holes (2 mm in diameter) were positioned on the parietal bone and used to record the propagating CSD wave. CSD was elicited at 20-min intervals by a 1-min application of a cotton ball (1–2 mm in diameter) soaked with 2% KCl solution (approximately 270 mM) to the anterior hole drilled at the frontal region. Rectal temperature was continuously monitored and maintained at 37 ± 1 °C by means of a heating blanket. The DC slow potential change accompanying CSD was recorded for 4 h using two Ag–AgCl agar–Ringer electrodes (one in each hole) against a common reference electrode of the same type, placed on the nasal bones. The CSD velocity of propagation was calculated from the time required for a CSD wave to pass the distance between the two cortical electrodes. In the two cortical recording places, the initial point of each DC-negative rising phase was used as the reference point to calculate the CSD velocities. In addition, amplitude and duration of the CSD waves were calculated, as previously reported (Mendes-da-Silva et al. 2014).

Immunohistochemistry

Rats treated with vehicle (n = 6, of which 3 L_9 and 3 L_{15}), Gln250 (n = 8, of which 4 L_9 and 4 L_{15}), or Gln500 (n = 8; 4 L_9 and 4 L_{15}) were perfused with 0.9% saline solution followed by 4% paraformaldehyde diluted in 0.1 M phosphate-buffered saline (pH 7.4). After being immersed in the fixative for 4h, the brains were transferred to a 30% (w/v) sucrose solution for cryoprotection. Longitudinal serial sections (40-µm thickness) were obtained at −20ºC

using a cryoslicer (Leica 1850). Sections were immunolabeled with a polyclonal antibody against ionized calcium-binding adapter molecule 1 (Iba-1) to detect microglia (1:3000; anti-Iba-1, #019-19741; Wako Pure Chemical Industries Ltd., Osaka, Japan). Free-floating sections were submitted to endogenous peroxidase blocking (2% H_2O_2 in 70% methanol for 10 min) and the sections incubated for 1 h in blocking buffer (BB) solution containing 0.05 M Tris-buffered saline (TBS; pH 7.4), 10% fetal calf serum, 3% bovine serum albumin, and 1% Triton X-100. The sections were then incubated overnight at 4°C with rabbit anti-Iba-1 (1:1500 diluted in BB solution). After three washes with TBS + 1% Triton, sections were incubated at room temperature for 1 h with biotinylated anti-rabbit (1:500) secondary antibodies. Sections were then rinsed in TBS + 1% Triton and incubated with horseradish peroxidase streptavidin (1:500). The peroxidase reaction was visualized by incubating the sections in Tris buffer containing 0.5 mg/ml 3,3'-diaminobenzidine (DAB) and 0.33 µl/ml H_2O_2. Finally, the sections were mounted, dehydrated in graded alcohols, and coverslipped in Entellan® after xylene treatment. Densitometric analysis was performed on four parallel longitudinal sections for each animal. A Leica DMLS microscope coupled to a Samsung high-level color camera (model SHC-410NAD) was used to obtain digital images from brain sections. Images of the parietal cortex and CA1 hippocampus stained for Iba1 were obtained using a 20× microscope objective. In each section, photomicrographs of four fields within the parietal cortex (layer 2) and two fields of CA1 hippocampal region were analyzed, using the Image J software (National Institutes of Health, USA, version 1.46r), which is a worldwide used open tool for the analysis of scientific images (see Schneider et al, 2012 for a historical review). Care was taken to obtain the digital images using the same light intensity. Total immunoreactivity expressed as arbitrary units, as well as the percentage of the area occupied by the Iba1-labeled cells were analyzed.

Statistics

Results in all groups are expressed as means ± standard deviations (SD). Data were compared between groups using ANOVA, including as factors lactation conditions (L_9 and L_{15}) and gavage treatment (naïve, vehicle, Gln250, Gln500 and Gln750) followed by a post hoc test (Holm–Sidak). A *p* value less than 0.05 was considered significant.

Results

Body Weights

As shown in **Fig 1**, L_{15} animals presented with lower ($p<0.05$) body weights, as compared with those of the L_9-groups. In the L_9 condition, the body weights of the control groups ranged from 15.4 ± 1.3 g at P7 to 107.8 ± 6.4 g at P30-35, whereas in the L_{15} condition the weights ranged from 12.5 ± 1.0 g to 74.3 ± 8.4 g. Treatment with Gln did not alter body weights of animals.

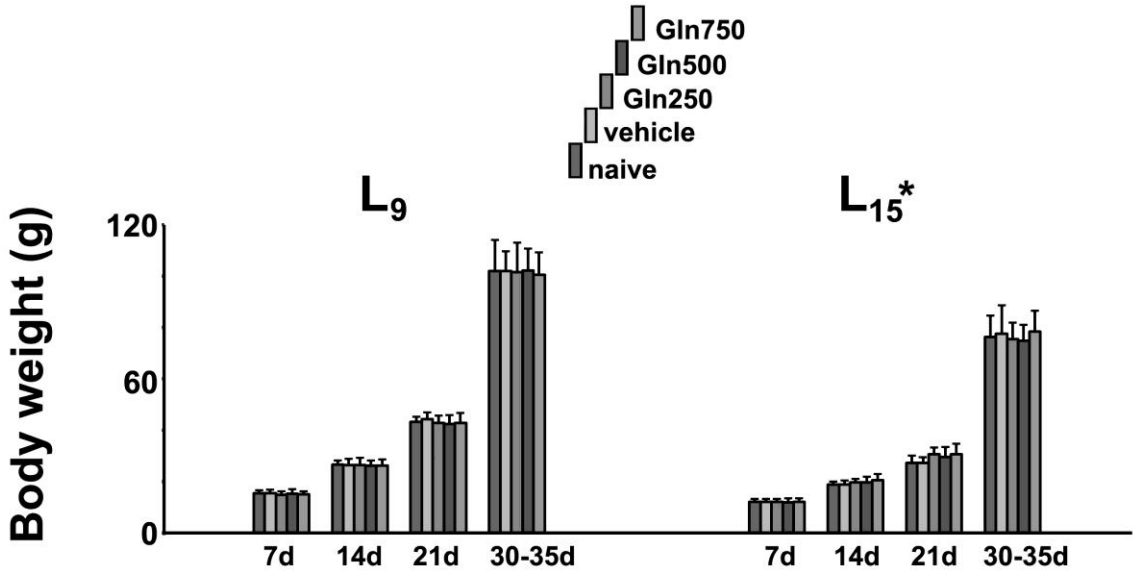

Fig 1. Body weight of male rats (30- to 35-days old) previously suckled in litters with 9 or 15 pups (respectively, L_9 and L_{15} condition). Gln250, Gln500 and Gln750 are rats treated per gavage from postnatal days 7 to 27 with L-glutamine at the doses of 250 mg/Kg/day, 500 mg/Kg/day and 750 mg/Kg/day, respectively. Data are mean ± SD. *Asterisks* indicate significant difference between L_{15} and the corresponding L_9 groups ($p < 0.05$; two-way ANOVA followed by the Holm–Sidak test).

Elevated plus-maze and Open field tests

The effect of Gln treatment on the anxiety-like behavioral activity in the elevated plus-maze, and in the open field tests is shown in **Fig 2**. Regarding the elevated plus maze, in the L_{15} condition, Gln500- and Gln750 groups traveled higher distance than the controls and presented with longer time in the open arms than the L_{15}-Gln250, naive and vehicle groups ($p < 0.05$).

In the open field, the Gln-treated animals of both L_9 and L_{15} condition traveled a higher distance than the corresponding controls. In the L_{15} condition, the Gln-treated animals entered more times in the central zone in comparison with the controls. The Gln500 and Gln750 groups also spent more time in the central zone.

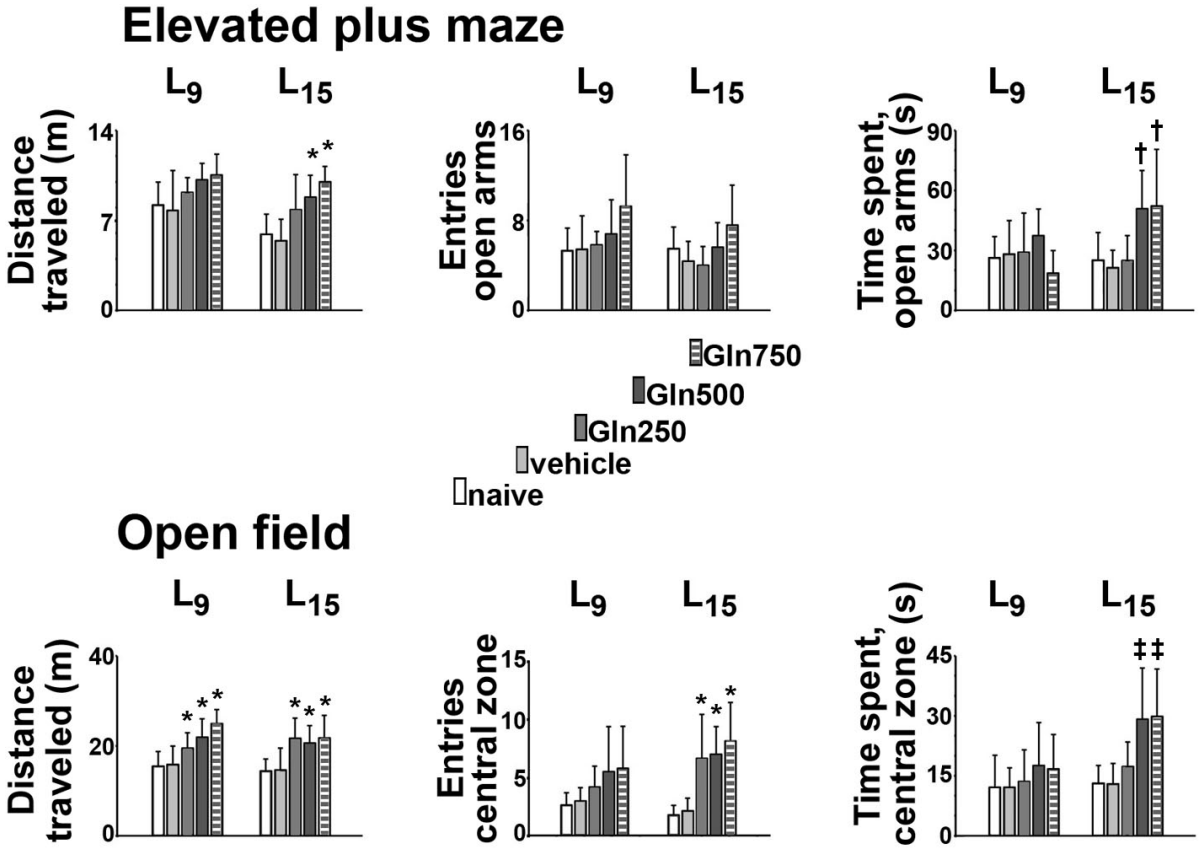

Fig 2. Behavioral activity, in the elevated plus maze and open field apparatus, of young rats that were previously suckled in litters with 9 and 15 pups (respectively L_9 and L_{15} condition). Gln250, Gln500 and Gln750 are rats treated per gavage from postnatal days 7 to 27 with L-glutamine at the doses of 250 mg/Kg/day, 500 mg/Kg/day and 750 mg/Kg/day, respectively. Bars represent mean values ± standard deviation. * indicate values that are significantly different from naive and vehicle-control groups. † significantly different from control groups and from Gln250. ‡ significantly different from the control and from the corresponding L_9 groups ($p < 0.05$; ANOVA followed by Holm-Sidak test).

CSD parameters

In all groups, topical application of 2% KCl for 1 min at the frontal cortex usually elicited a single CSD wave, which was recorded by the two electrodes located more posteriorly in the stimulated hemisphere. **Fig 3** presents electrophysiological recordings on the cortical surface of 5 L_9 and 5 L_{15} animals, illustrating the slow potential change

accompanying CSD. The DC slow potential recordings confirmed the presence of CSD after KCl stimulation.

Regarding CSD velocity of propagation, ANOVA indicated intergroup differences ($F[1,106] = 316.466$; $p < 0.001$), and post hoc (Holm–Sidak) test comparisons showed that the velocities were higher ($p < 0.001$) in the L_{15} groups compared to the L_9 groups. Gln treatment significantly increased the CSD propagation velocities ($F[4,106] = 53.728$; $p < 0.001$) compared with the Naive and Vehicle controls. In both lactation conditions, CSD velocity of the Gln250 animals ($L_9 = 4.12 \pm 0.13$ mm/min; $L_{15} = 4.83 \pm 0.21$ mm/min) was higher than the controls ($L_9 = 3.87 \pm 0.11$; $L_{15} = 4.52 \pm 0.14$ mm/min for the vehicle groups, and $L_9 = 3.85 \pm 0.13$; $L_{15} = 4.61 \pm 0.15$ mm/min for the naive groups), but lower than the Gln500 ($L_9 = 4.51 \pm 0.14$ mm/min; $L_{15} = 5.17 \pm 0.27$ mm/min) and Gln750 rats ($L_9 = 4.52 \pm 0.16$; $L_{15} = 5.26 \pm 0.46$ mm/min). No difference was found between Gln500 and Gln750 groups in both L_9 and L_{15} condition. The CSD velocities for all groups are shown in **Fig 4**.

Data on amplitude and duration of the CSD negative slow potential change are shown in **Table 1**. ANOVA indicated a main effect of the treatment ($F[4, 87] = 15.025$; $p < 0.001$ for amplitude and $F[4, 96] = 14.194$; $p < 0.001$ for duration). The Holm-Sidak test indicated that in L_9 condition all groups treated with Gln presented with larger amplitudes and lower durations when compared to vehicle- and naive controls. In this lactation condition (L_9), the Gln750 group had significantly higher amplitude than the Gln250 group. In L_{15} condition, only the Gln750 group showed significantly higher amplitude and shorter duration when compared to the respective controls. The L_{15} vehicle- and naive groups had a shorter duration when compared to the corresponding L_9.

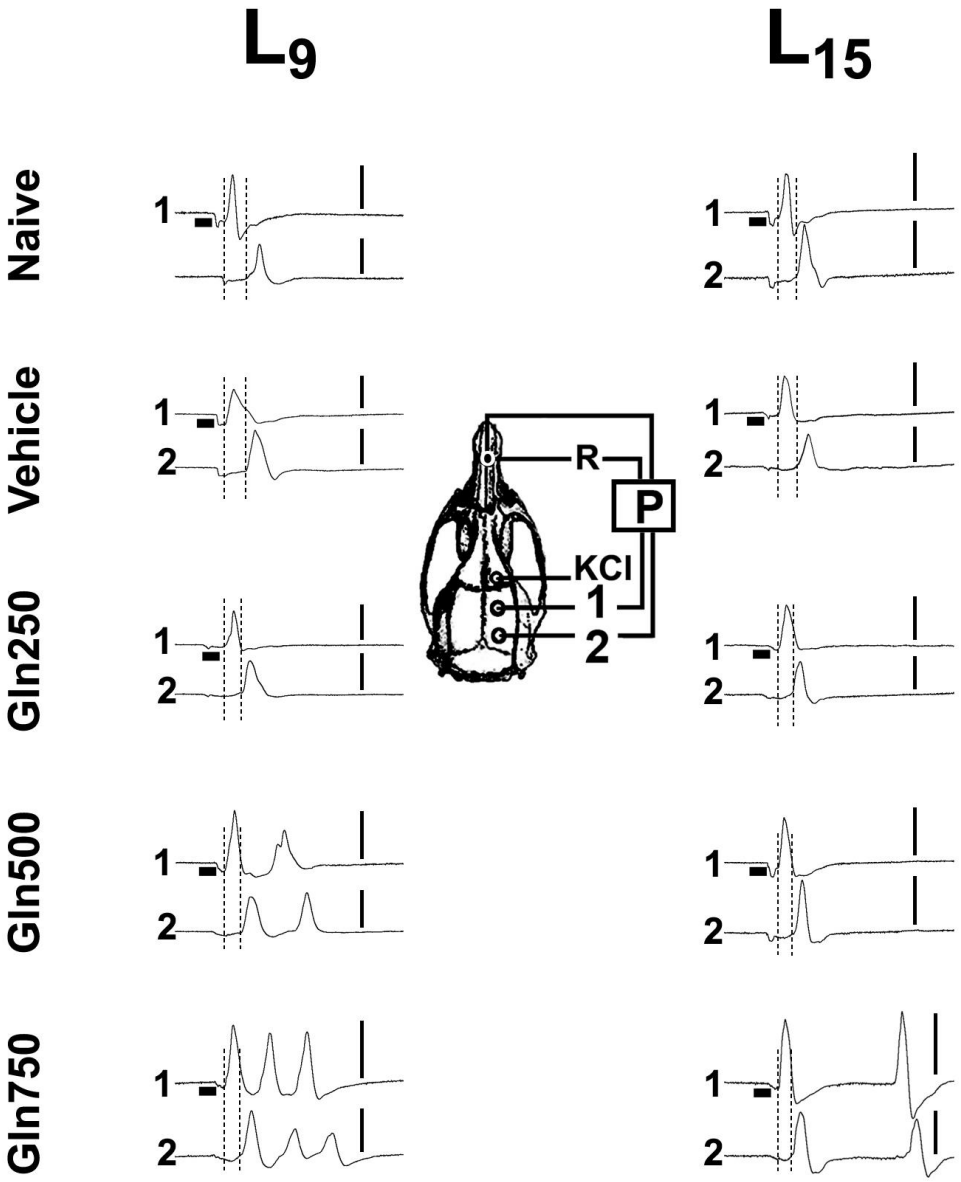

Fig 3. Slow potential changes (P) during cortical spreading depression (CSD), recorded at two cortical points (1 and 2), in 10 developing rats, previously suckled in litters with 9 or 15 pups (respectively L_9 and L_{15} condition). The diagram of the skull shows the recording positions 1 and 2 from which the traces marked at the left with the same numbers were obtained. The position of the common reference electrode (R) on the nasal bones and the application point of the CSD-eliciting stimulus (KCl) are also shown. Gln250, Gln500 e Gln750 are rats treated per gavage with L-glutamine at doses of 250 mg/Kg/d, 500 mg/Kg/d and 750 mg/Kg/d, respectively. The vertical bars indicate 10 mV for P (negative upwards). CSD was elicited in the frontal cortex by chemical stimulation (a 1- to 2-mm diameter cotton ball soaked with 2 % KCl) applied for 1 min on the intact dura mater, as indicated by the horizontal bars. The vertical dashed lines indicate the

latency for a CSD wave to cross the inter-electrode distance. The latencies were shorter in the L_{15} groups compared with the corresponding L_9 groups. In the groups treated with Gln, the latencies decreased when compared with the respective Naive and Vehicle controls. In both L_9 and L_{15} condition, Gln500 and Gln750 groups displayed shorter latencies than the corresponding Gln250 rats.

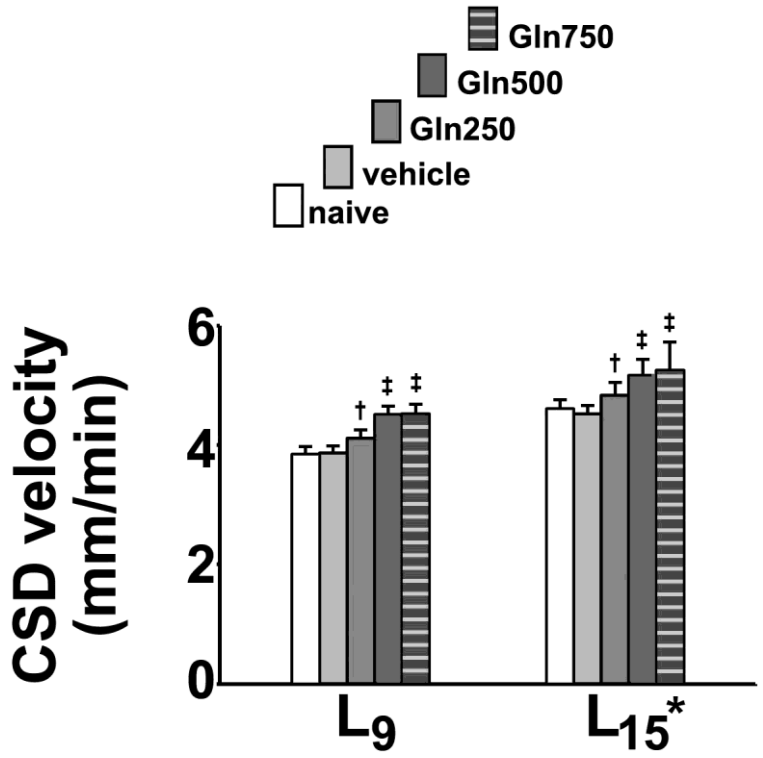

Fig 4. CSD velocity of young rats (30- to 35-days old) that were suckled in litters with 9 and 15 pups, resulting in two distinct lactation conditions (respectively, L_9 and L_{15} groups). Gln250, Gln500 and Gln750 are rats treated per gavage with L-glutamine at the doses of 250 mg/Kg/day, 500 mg/Kg/day and 750 mg/Kg/day, respectively. Data are mean ± SD. ***** significantly different from the corresponding L_9 groups. † significantly different from the naive and vehicle control groups. ‡ significantly different from the Gln250 and control groups ($p < 0.01$; ANOVA followed by the Holm-Sidak test).

Table 1. Amplitude and duration of the negative slow potential shifts of CSD in male developing rats (30- to 35- days old) previously suckled in litters with 9 or 15 pups (respectively L_9 and L_{15} condition). Gln250, Gln500 and Gln750 are rats treated per gavage from postnatal days 7 to 27 with L-glutamine at the doses of 250 mg/Kg/day, 500 mg/Kg/day and 750 mg/Kg/day, respectively.

Group	Amplitude (mV)	Duration (s)
L9		
Naive (n=11)	10.63 ± 2.20	73.01 ± 5.57
Vehicle (n=11)	10.60 ± 2.54	74.17 ± 6.75
Gln250 (n=14)	14.35 ± 2.13*	68.47 ± 5.52*
Gln500 (n=11)	15.92 ± 2.56*	65.43 ± 5.48*
Gln750 (n=14)	18.10 ± 2.80†	64.40 ± 5.81*
L15		
Naive (n=10)	12.51 ± 3.14	68.54 ± 3.29‡
Vehicle (n=10)	12.86 ± 2.73	69.37 ± 1.83‡
Gln250 (n=13)	14.33 ± 2.96	64.73 ± 4.81
Gln500 (n=8)	15.56 ± 3.69	62.90 ± 3.93
Gln750 (n=11)	16.86 ± 3.39*	60.38 ± 2.85*

Data are mean ± SD. *$p < 0.05$ compared with controls groups in the same suckling condition. †$p < 0.05$ compared with Gln250 and controls. ‡ $p < 0.05$ compared with the corresponding L_9 condition (ANOVA plus Holm-Sidak test).

Iba1 immunohistochemistry

Photomicrographs of Iba1- labeled cells in the parietal cortex and CA1 hippocampus are shown in **Fig 5 A and B.** The animals of the L_{15} condition had greater immunoreactivity and higher percentage of marked area when compared to the corresponding L_9 condition **(Fig 5, C and D)**. In both lactation conditions, Gln250 and Gln500 animals showed higher

immunoreactivity compared to vehicle control, both in cortex and hippocampus. Unlike L_9 condition, the L_{15} animals that were treated with Gln500 showed significantly greater immunoreactivity when compared to the Gln250 group. Regarding the percentage of labeled area, in the L_9 condition Gln250 and Gln500 exhibited higher percentage of labeled area when compared to control, while in L_{15} condition only the Gln500 group was different from the control in the parietal cortex. In the hippocampus, only the Gln500 group of the L_9 condition was statistically different from the control group.

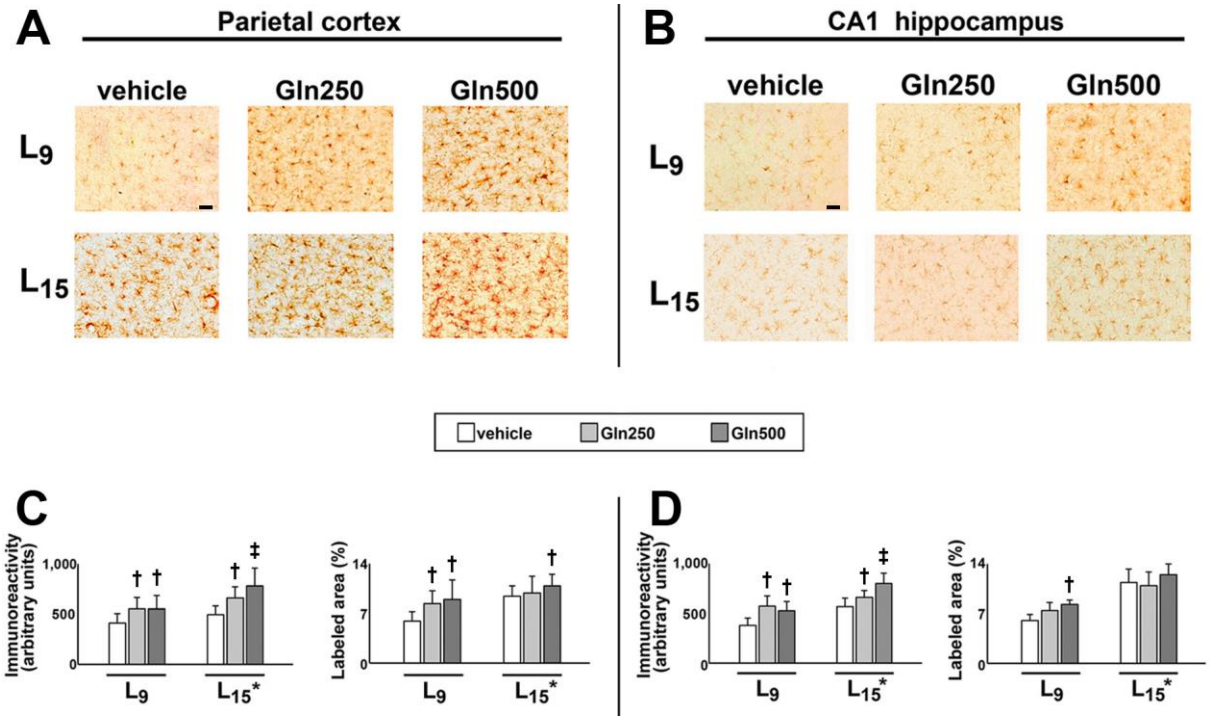

Fig 5. Low magnification photomicrographs of Iba1-labeled microglial cells in longitudinal sections of the parietal cortex (A) and CA1 hippocampus (B) of six young rats (with 30- to 35- days of life) suckled in litters with 9 and 15 pups, resulting in two distinct lactation conditions (respectively, L_9 and L_{15} groups). Gln250 and Gln500 are rats treated per gavage with L-glutamine at the doses of 250 mg/Kg/day and 500 mg/Kg/day, respectively. Scale bars = 20 μm. The bar graphics show immunoreactivity expressed as arbitrary units and the percent area occupied by the Iba1-labeled cells of slices of the parietal cortex (C) and CA1 hippocampus (D) of vehicle control (n=6; $3L_9$ and $3L_{15}$), Gln250 (n=8; $4L_9$ and $4L_{15}$) and Gln500 rats (n=8; $4L_9$ and $4L_{15}$). Data are expressed as mean ± SD. * $p < 0.05$ compared with L_9 condition. † $p < 0.05$ compared with vehicle control. ‡ $p < 0.05$ compared with the other two groups (ANOVA plus Holm–Sidak test).

Discussion

In this study we confirmed our previous results (Lima et al. 2009), which indicated that treatment with Gln during the critical period of the nervous system development accelerates

CSD, regardless of the lactation conditions. Furthermore, we could show, for the first time, changes in anxiety-like behavior and microglial immunoreactivity as a consequence of early treatment with Gln. In some instances, the Gln effects were significantly dose-dependent, and in other instances a non-significant dose-dependent tendency was observed.

Body weights were lower in the L_{15} groups compared with the corresponding L_9 groups, confirming the effectiveness of increasing litter size in producing undernutrition, as previously indicated (Francisco and Guedes 2015; Rocha-de-Melo et al. 2006). In animal models, malnutrition during the critical period of brain development leads to a reduction in the number and/or size of brain cells, in myelin production and in the number of synapses, and changes in neurotransmitter systems (Morgane et al. 2002). Although some effects of malnutrition on the structure and brain metabolism are reversed by nutritional rehabilitation, other effects are not and continue with subsequent electrophysiological, behavioral and cognitive changes (Levitsky and Strupp, 1995; Penido et al, 2012). The treatment with Gln did not alter the animals' body weight **(Fig 1)**. This is in line with previous reports on Gln treatment per gavage (Lima et al. 2009) and via subcutaneous injection (Ladd et al. 2010).

Our results of anxiety tests reveal a less anxious behavior in the L_{15}, but not L_9 groups treated with Gln as indicated by the longer permanence in the open arms in the elevated plus maze, and by a higher number of entries and more time spent in the central zone in the open field apparatus (Fig 2). This lower anxiety-like behavior is in line with previous data on animals that were suckled in large litters (Clarke et al. 2013; Bulfin et al. 2010). In the L9 groups, there was a non-significant tendency for an anxiolytic effect of Gln for all evaluated parameters, except for the time spent in the open arms of the Gln750 group. It is possible that Gln acts at the central nervous system as an anti-anxiety agent by acting as precursor of GABA. Previous studies have already demonstrated an increased GABA level in the brain of rats treated with Gln per gavage (Wang et al. 2007) or subcutaneously (Ladd et al. 2010). This

finding could explain the Gln anxiolytic properties here described. An alternative interpretation of the behavioral data could include Gln-induced enhancement of locomotion, as suggested in humans (McCormack et al, 2015), and also as observed after taurine administration in rats (Francisco and Guedes, 2015).

The higher CSD velocity from the L_{15} animals confirms the facilitating action of early undernutrition, induced by suckling the pups in large litters. Concerning the mechanisms by which malnutrition facilitates CSD propagation, a larger volume of extracellular space in the brain hinders CSD elicitation and propagation (Mazel et al. 2002). Inadequate nutrient intake early in life increases cell-packing density and reduces the extracellular space, leading to facilitation of CSD. Another important factor that modulates CSD propagation is cortical myelination. Previous work has demonstrated an inverse correlation between the degree of cortical myelination and CSD propagation velocity (Merkler et al. 2009). Nutritional deficiency reduces brain myelination and increases CSD propagation velocity (De Luca et al. 1977; Rocha-de-Melo et al. 2006). Furthermore, malnutrition may impair glial function (Morgane et al. 1978) and caloric restriction may suppress induced microglial activation in non-cortical brain areas (Radler et al. 2015; Tu et al. 2012). Furthermore, CSD is facilitated by glial impairment (Largo et al. 1997) and induces potassium currents in microglia (Wendt et al. 2016). In addition, malnourished rats present increased levels of the enzyme glutamic acid decarboxylase (Díaz-Cintra et al. 2007). This condition, in association with reduced brain glutamate uptake (Feoli et al. 2006), enhances extracellular glutamate in the malnourished brain, which might contribute to CSD facilitation (Marrannes et al. 1988).

Our data clearly demonstrate that neonatal treatment with Gln dose-dependently accelerated the propagation velocity of CSD irrespective of the lactation condition (L_9 *versus* L_{15}). These results are coherent with a previous report (Lima et al. 2009), and reinforce the importance of Gln for the proper electrophysiological functioning of the brain, therefore

suggesting that neonatal treatment with Gln can modulate, at least in part, the brain's ability to propagate CSD. The vehicle group, which was treated per gavage with distilled water, presented CSD features similar to the naive control. In view of this observation, we are compelled to conclude that the assumed causal link between Gln treatment and the here described CSD changes is substantial, and cannot be attributed to the gavage stress.

Although in this study amino acid blood levels have not been monitored, it is reasonable to assume that the present long-term (three weeks) neonatal Gln treatment might have caused an amino acid imbalance in plasma and tissues (Holecek 2011) and increased its brain concentration (Szpetnar et al. 2016). Thus, it is tempting to hypothesize that the increase in plasma Gln would impair the output of Gln from the astroglia to the bloodstream. The enhanced Gln availability could alter interactions in the glutamate-Gln cycle and affect ammonia and glutamate levels in the brain. This may occur in neurons by activation of the enzyme glutaminase and in astroglia by suppression of another enzyme, Gln synthetase. Enhanced availability of Gln may also affect translocation of Gln into GABAergic neurons and released GABA into astroglia (Holecek 2013). In fact, glutamate- and GABA mediated mechanisms are important for the phenomenon of CSD (Holland et al. 2010; Haghir et al. 2009; Guedes et al. 1992; Marrannes et al. 1988), and glutamate to GABA imbalance may ultimately lead to a hyperexcitable brain (Becerra et al, 2016), which can help in explaining our findings. Another possibility that deserves future investigation would be the evaluation of the effects of Gln on the activation of astrocytes, which seems to be associated with microglia activation (Guan et al. 2016).

Under CSD accelerating and decelerating conditions, microglial immunolabeling has been found to be respectively enhanced (Lima et al. 2013, 2014) and reduced (Soares et al. 2015). These data suggest a direct relationship between CSD velocity of propagation and microglia activation. Recent evidence from others suggests that microglia is required for

spreading depression (Pusic et al. 2014) and, conversely, CSD activates microglia (Takizawa et al. 2016) and NMDA receptors (Wendt et al. 2016). Our findings are in agreement with those of Lima et al (2013, 2014), and Takizawa et al (2016) as we found CSD acceleration and microglial activation in the Gln-treated animals, which suggests a positive modulating role for Gln in the microglial activation in the brain. All of the evidence notwithstanding, a deeper investigation on the possible directions of this relationship is still required.

Microglial cells are the resident immune cells of the CNS. With their highly dynamic processes they continuously survey the microenvironment, thus being the first cells to be activated in response to injurious tissue demands. These cells are involved in inflammatory responses in the brain, as well as behavioral responses (Viana et al. 2013). It must be remarked that Iba1 immunolabeling is not a specific marker for microglia, as it also labels macrophages, which may infiltrate the CNS and may become involved in inflammatory responses in the brain, as well as behavioral responses (Wohleb et al, 2015).

Microglia help to prevent glutamate-related excitotoxicity by promoting glutamate uptake (Hanisch and Kettenmann 2007). Svoboda and Kerschbaum (2009) reported *in vitro* apoptosis of microglia as a result of the increase in Gln concentration, suggesting Gln toxicity. They concluded that hydrolysis of Gln and, accordingly, accumulation of ammonium in mitochondria induced the intrinsic pathway of apoptosis. In addition, recent evidence suggests that enhancement of Gln levels by glutaminase inhibition blocks glutamate release from stimulated microglia (Thomas et al. 2014). Data collectively support the Trojan horse hypothesis of Gln toxicity, which involves both astroglia and microglia cells (Albrecht and Norenberg 2006; Svoboda and Kerschbaum 2009).

In the Gln-treated groups, microglia activation has been observed in the parietal cortex and, to a lesser extent in the hippocampal CA1 area (Fig 5), which suggests a regional difference in microglia activation. This is interesting, as suckling in large litters may increase

microglia activation in the hippocampus (Viana et al, 2013) and overnutrition (suckling in litters with 4 pups) enhances microglia activation in hypothalamus and cerebellum (Tapia-González et al, 2011).

In conclusion, the results of this study suggest a brain effect of early Gln treatment, expressed as behavioral (anxiety), electrophysiological (CSD) and microglial alterations in developing rats. Regarding on how the Gln-induced anti-anxiety, pro CSD, and pro microglial activation are related to each other, and how unfavorable suckling conditions can modify this relationship, recent evidence from others indicate that microglia is required for CSD (Pusic et al. 2014), and suckling in large litters increases the brain GABA levels after Gln treatment (Ladd et al. 2016), and modulates CSD (Francisco and Guedes 2015), behavior and microglia activation (Viana et a. 2013). Furthermore, the relevance of the CSD phenomenon for important neurological diseases has been well established along the last two decades (Gorji 2001; Dreier 2011; Torrente et al. 2014; Kramer et al. 2016). Most discussed mechanisms for this interplay might include Gln action on the GABA-glutamate balance (Holecek 2013; Wang et al. 2007). Future experiments are needed to further clarify the underlying mechanisms, as well as to explore the possibility of such Gln effects on the adult brain.

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Conflict of interest: The authors declare that they have no conflict of interest.

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6.2 Artigo 2

Title:

Long-lasting brain effects of neonatal L-glutamine on the anxiety like behavior, cortical spreading depression, and microglial reaction in adult rats

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Abstract

Aims: Neonatal L-Glutamine (Gln) can modulate the neurotransmission processes. However, little is known about the long-term effects of neonatal treatment with Gln. Here we have addressed this issue in adult rats by analyzing behavioral (anxiety), electrophysiological (cortical spreading depression; CSD) and microglial (Iba1 immunoreactivity) alterations, associated with the neonatal Gln-treatment.

Main methods: Wistar rats were suckled under favorable and unfavorable lactation conditions (respectively in litters with 9 and 15 pups; groups L_9 and L_{15}). From postnatal days (P) 7 to 27, the animals received per gavage L-glutamine (250 mg/kg/day [Gln250] or 500 mg/kg/day [Gln500] or 750 mg/kg/day [Gln750]). At P88 and P90 we tested the animals in the elevated plus-maze and open field apparatus, respectively. At P90-120 we measured CSD parameters (velocity of propagation, amplitude, and duration). Fixative-perfused brains were processed for microglial immunolabeling with anti-Iba-1 antibodies.

Key findings: CSD velocity was higher ($p < 0.001$) in the L_{15} *versus* L_9 condition. Gln treated groups dose-dependently presented with higher CSD velocity when compared to the corresponding controls, except Gln250 in L_{15} condition. Gln treatment increased Iba1 immunolabeling both in the parietal cortex and CA1 hippocampus. Furthermore, in the elevated plus-maze test the L_{15} Gln500 and Gln750 groups traveled a greater distance and displayed a shorter immobility time, in comparison with the controls.

Significance: The behavioral, electrophysiological and immunohistochemical findings of this study suggest that neonatal treatment with Gln dose-dependently lead to lasting brain alterations, which are modulated by the lactation conditions, and are detectable in adult life.

Keywords: L-glutamine, Cortical spreading depression, Brain excitability, Immunoreativity, Microglia, Anxiety-like behavior.

Introduction

The amino acid L-Glutamine (Gln) is abundant in the mammalian Central Nervous System, where its concentration is higher than any other amino acid in brain fluids [1]. Gln is synthesized exclusively in glial cells from glutamate and ammonia by the enzyme glutamine synthetase. Gln synthesis is considered a brain protecting mechanism against excitotoxicity, as this process reduces the excess of glutamate and ammonia [2]. The synthetized Gln is released back into the extracellular space, shuttled back into neurons and converted to glutamate by another enzyme, glutaminase. The regenerated glutamate may then go on to play a direct role in excitatory neurotransmission, or serve as precursor of GABA [3]. The glutamate/GABA imbalance may lead to a hyperexcitable brain [4]. Disturbances in Gln metabolism are involved in the pathophysiology of some neurological diseases such as epilepsy [5, 6, 7, 8] and migraine [4, 9, 10]. These diseases, in turn, are related to the phenomenon of cortical spreading depression (CSD) [11, 12] that we have analyzed in the present study.

CSD is a fully reversible neural response that consists in a slowly propagating wave of depression of spontaneous neuronal activity. CSD can be produced by electrical, mechanical or chemical stimulation of one point on brain tissue, from which it spreads concentrically to remote cortical regions [13]. This phenomenon has been demonstrated in animal- [14, 15, 16, 17] and human brain [18, 19]. CSD is modulated by changes in brain excitability [20], and is influenced by glutamate- and GABA mediated mechanisms [21, 22, 23, 24]. Our laboratory has used CSD as an experimental model to evaluate the brain excitability associated with pharmacological, environmental, and nutritional manipulations (see [25] for a review). Increased or reduced CSD propagation velocity indicates respectively a greater or lesser CSD susceptibility of the cortical tissue, which are associated with anxiety-like behavior [26] and microglial reaction [27, 28].

Regarding the neuronal excitability modulation by Gln, in a previous study we were able to identify a facilitating effect of oral Gln treatment during the critical period of the nervous system development on CSD propagation in developing rats [29]. In this study, we extended our investigations on the brain repercussions of neonatal Gln treatment in order to answer the following questions: 1) Are the electrophysiological (CSD) effects of Gln long-lasting? 2) Are electrophysiological responses associated with behavioral- (anxiety) and microglial alterations? 3) Are these effects dose-dependent? 4) How are these effects influenced by unfavorable lactation conditions early in life?

Materials and methods

Animals

The Wistar rats used in this study were handled in accordance with the norms established by the National Institutes of Health Guide for Care and Use of Laboratory Animals (Bethesda, MD, USA). The Institutional Ethics Committee for Animal Research, from our University, previously approved all experimental procedures (Process nº.23076.010667/2012-84). Animals were maintained in polypropylene cages (51 cm × 35.5 cm × 18.5 cm) in a room maintained at 22 ± 1 °C with a 12:12 h light: dark cycle (lights on at 6:00 a.m.). The newborn male and female rats, born from distinct dams, were pooled and assigned to be suckled under normal or unfavorable conditions, represented respectively by litters with 9 pups (L_9 groups; n = 54 males) and litters with 15 pups (L_{15} groups; n = 53 males). After weaning at postnatal day (P) 25, both groups were switched to the maternal lab chow diet (Purina Ltd.), with 23% protein until adulthood. Only the male pups (n=107) were used in this study (housed 2–4 rats per polypropylene cage).

L-Glutamine treatment

From P7 to P27, the male pups were treated via gavage with three distinct doses of Gln (purchased from Sigma, St. Louis, MO, USA): 250 mg/Kg/day (Gln250 group; 12 L_9 and 10 L_{15} pups), 500 mg/kg/day (Gln500; 11 L_9 and 13 L_{15} pups) or 750 mg/kg/day (Gln750; 10 L_9 and 10 L_{15} pups). Two additional control groups received no gavage (naive group; 10 L_9 and 10 L_{15} pups) or distilled water (vehicle; 11 L_9 and 10 L_{15} pups). The gavage volume of the amino acid solutions or of distilled water ranged from 0.5ml/day (in the second week of life) to 1.0 ml/day (in the third and fourth week of life). Care was taken to minimize pain and discomfort during the gavage procedure.

Weight Body

The body weights were obtained at postnatal days 7, 14, 21, 60 and 90.

Elevated plus-maze test

We conducted the elevated plus-maze (EPM) test on P88-90 in a room under dim light and sound attenuation. The cross-shaped apparatus consisted of four arms: two closed arms and two perpendicularly-oriented open arms, connected by a central squared platform (10 X 10 cm wide). Each arm measured 49 cm long X 10 cm wide. EPM was raised 55 cm above the floor. For each 5-min session, we initially placed the rat in the central platform facing an open arm. A video camera recorded the behavioral activity of the animal. The recorded activity was stored in a computer and subsequently analyzed with the software ANYmaze (version 4.99 m). Between the tests, we cleaned the EPM with a paper cloth soaked with 70% ethanol. The following parameters were analyzed: total distance traveled, duration of

immobility, number of entries into the open arms, and time spent in the open arms. We considered that the animal entered one open or one closed arm when its four paws entered the arm.

Open field test

On P90-95, the rats were placed in the center of an open field apparatus (a circular arena with 100 cm diameter and 52 cm height), which was located in a room with a dim light and sound-attenuation. After each test, we cleaned the open field with a paper cloth soaked with 70% ethanol. Behavioral reactions were captured for 5 min using a digital camera and the software ANYmaze (version 4.99m) analyzed the following parameters: total distance traveled, total immobility time, number of entries in the central zone of the apparatus and time spent in the central zone.

CSD recording

On the day of CSD recording (P90–P120), the animal was anesthetized with a mixture of 1 g/kg urethane plus 40 mg/kg chloralose injected intraperitoneally. Three trephine holes were drilled on the right side of the skull, aligned in the frontal-to-occipital direction and parallel to the midline. One hole was positioned on the frontal bone (3 mm in diameter) and was used to apply the stimulus (KCl) to elicit CSD. The other two holes were positioned on the parietal bone (3 mm in diameter) and were used to record the propagating CSD wave. CSD was elicited at 20-min intervals by a 1-min application of a cotton ball (2 mm in diameter) soaked with 2% KCl solution (approximately 270 mM) to the anterior hole, drilled at the frontal region. Rectal temperature was continuously monitored and maintained at 37 ± 1

°C by means of a heating blanket. The DC slow potential change accompanying CSD was recorded for 4 h using two Ag–AgCl agar–Ringer electrodes (one in each hole) against a common reference electrode of the same type, which was placed on the nasal bones. We calculated the CSD velocity of propagation from the time required for a CSD wave to pass the distance between the two cortical electrodes. In the two cortical recording traces, we used the initial point of each DC-negative rising phase as the reference point to calculate the CSD velocities. In addition, we calculated amplitude and duration of the CSD waves, as previously reported [30].

Analysis of immunolabeled microglial cells

Twenty-two rats, of which six vehicle (3 L_9 and 3 L_{15}), eight Gln250 (4 L_9 and 4 L_{15}), and eight Gln500 (4 L_9 and 4 L_{15}) were perfused with 0.9% saline solution followed by 4% paraformaldehyde diluted in 0.1 M phosphate-buffered saline (pH 7.4). After being immersed in the fixative for 4 h, the brains were transferred to a 30% (w/v) sucrose solution for cryoprotection. Longitudinal serial sections (40-µm thickness) were obtained at −20ºC using a cryoslicer (Leica 1850). Sections were immunolabeled with a polyclonal antibody against ionized calcium-binding adapter molecule 1 (Iba-1) to detect microglia (1:3000; anti-Iba-1, #019-19741; Wako Pure Chemical Industries Ltd., Osaka, Japan). Free-floating sections were submitted to endogenous peroxidase blocking (2% H_2O_2 in 70% methanol for 10 min) and the sections incubated for 1 h in blocking buffer (BB) solution containing 0.05 M Tris-buffered saline (TBS; pH 7.4), 10% fetal calf serum, 3% bovine serum albumin, and 1% Triton X-100. The sections were then incubated overnight at 4 ◦C with rabbit anti-Iba-1 (1:1500 diluted in BB solution). After three washes with TBS + 1% Triton, sections were incubated at room temperature for 1 h with biotinylated anti-rabbit (1:500) secondary antibodies. Sections were

then rinsed in TBS + 1% Triton and incubated with horseradish peroxidase streptavidin (1:500). The peroxidase reaction was visualized by incubating the sections in Tris buffer containing 0.5 mg/ml 3,3'-diaminobenzidine (DAB) and 0.33 µl/ml H_2O_2. Finally, the sections were mounted, dehydrated in graded alcohols, and coverslipped in Entellan® after xylene treatment. Densitometric analysis was performed on four parallel longitudinal sections for each animal. In each section, we analyzed photomicrographs of four fields within the cortex (layer 2) using the Image J software (National Institutes of Health, USA, version 1.46r). A Leica DMLS microscope coupled to a Samsung high-level color camera (model SHC-410NAD) was used to obtain digital images from brain sections. Images of the parietal cortex- and CA1 hippocampus- stained for Iba1 were obtained using a 20×microscope objective. Care was taken to obtain the digital images using the same light intensity. We analyzed the percentage of the area occupied by the Iba1-labeled cells, as well as their total immunoreactivity expressed as arbitrary units.

Statistics

Data in all groups are expressed as means ± standard deviations (SD). The groups were compared using a two-way ANOVA, including as factors lactation conditions (L_9 and L_{15}) and gavage treatment (naive, vehicle, Gln250, Gln500 and Gln750) followed by a post hoc test (*Holm–Sidak*). A *p* value less than 0.05 was considered significant.

Results

Body Weights

As shown in **Figure 1**, L_{15} animals presented with lower ($p < 0.05$) body weights, as compared with those of the L_9 groups. In the L_9 condition, the body weights of the control groups ranged from 14.1 ± 1.7 g at P7 to 329.9 ± 19.1g at P90, whereas in the L_{15} condition the weights ranged from 12.3 ± 1.7 g to 294.0 ± 18.5 g. Treatment with Gln did not alter body weights of animals.

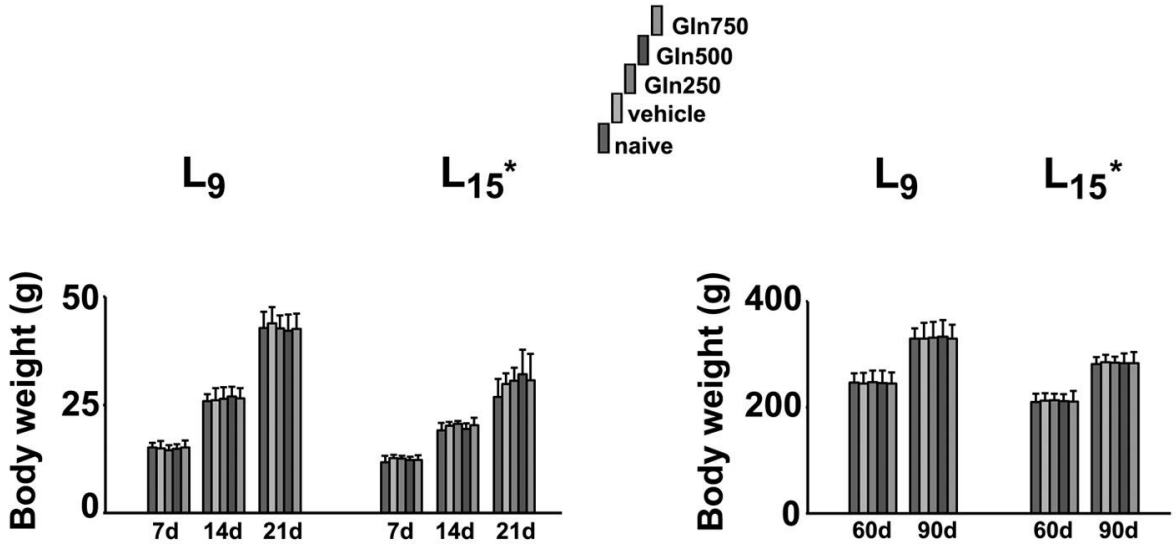

Figure 1. Body weight of male rats (90- to 120-day old) previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} condition). Gln 250, Gln500 and Gln750 are rats treated per gavage from postnatal days 7 to 27 with L-glutamine at the doses of 250 mg/Kg/day, 500 mg/Kg/day and 750 mg/Kg/day, respectively. Data are mean ± SD. *Asterisks* indicate significant differences between L_{15} and the corresponding L_9 groups ($p < 0.05$; two-way ANOVA followed by the Holm-Sidak test).

Anxiety like-behavior (elevated plus-maze and open field tests)

In the EPM test, Gln500 and Gln750 groups of the L_{15} condition traveled greater distances (respectively 12.54 ± 2.15 m and 11.34 ± 2.25 m) when compared to vehicle- (7.61 ± 1.93 m) and naive (7.53 ± 1.92 m) controls ($p < 0.05$). Furthermore, those groups displayed shorter immobility times (Gln500 = 43.38 ± 13.26 s; Gln750 = 40.78 ± 19.70 s) than the controls (vehicle = 86.48 ± 18.07 s; naive = 89.03 ± 34.58 s). No statistically significant difference was observed in the open field test.

CSD parameters

In all groups, topical application of 2% KCl for 1 min at the frontal cortex usually elicited a single CSD wave, which was recorded by the two electrodes located more posteriorly in the stimulated hemisphere. In **Figure 2,** we present electrophysiological recordings taken on the cortical surface of 5 L_9 and 5 L_{15} rats illustrating the slow potential change that accompanies CSD**.** The slow potential recordings confirmed the presence of CSD after KCl stimulation.

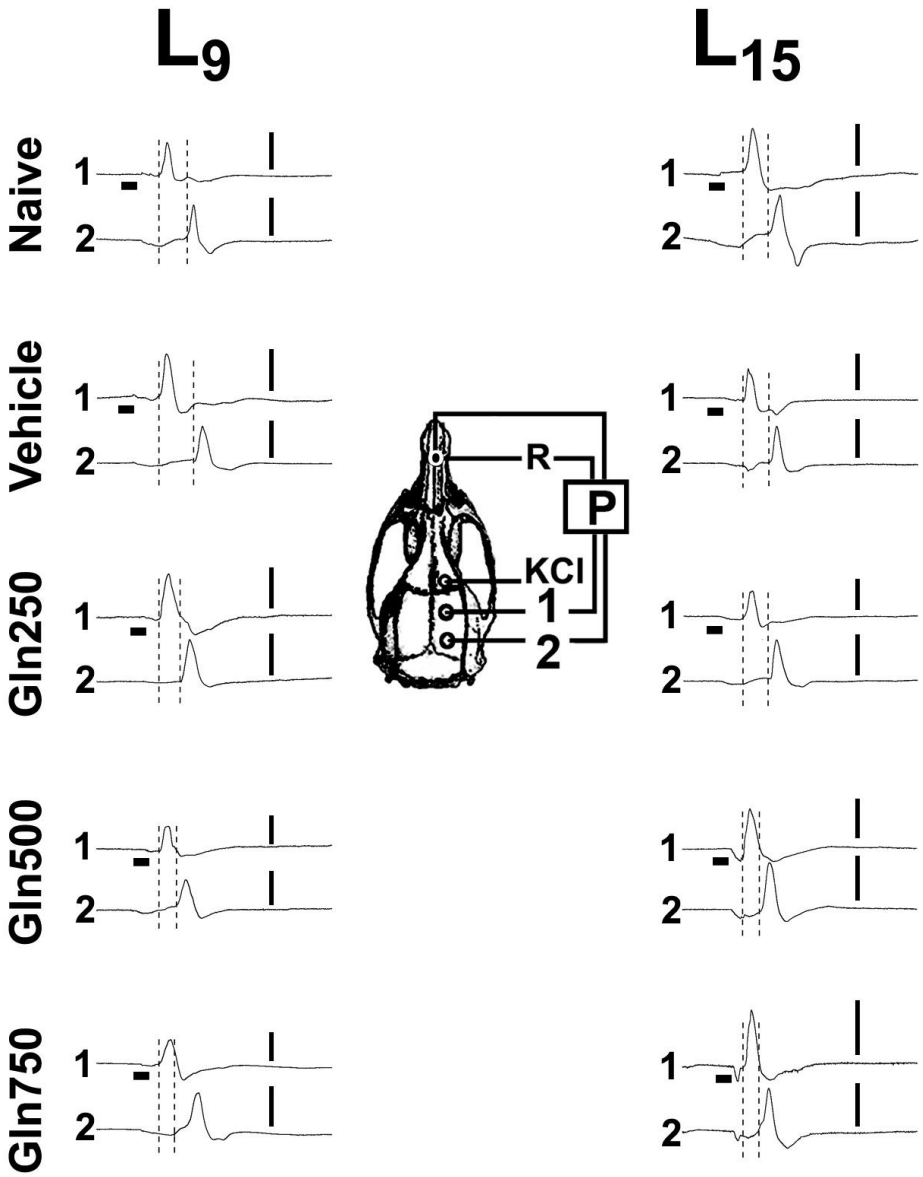

Figure 2. Slow potential changes (P) during cortical spreading depression (CSD), recorded at two cortical points (1 and 2), in 10 adult rats (5 L_9 rats and 5 L_{15} rats). The diagram of the skull shows the recording positions 1 and 2 from which the traces marked at the left with the same numbers were obtained. The position of the common reference electrode (R) on the nasal bones and the application point of the CSD eliciting stimulus (KCl) are also shown. Gln250, Gln500 e Gln750 are rats treated per gavage with L-glutamine at doses of 250 mg/Kg/d, 500 mg/Kg/d and 750 mg/Kg/d, respectively. The vertical bars indicate 10 mV for P (negative upwards). CSD was elicited in the frontal cortex by chemical stimulation (a 1- to 2-mm diameter cotton ball soaked with 2 % KCl) applied for 1 min on the intact dura mater, as indicated by the horizontal bars. The vertical dashed lines indicate the latency for a CSD wave to cross the inter-electrode distance. The latencies were shorter in the L_{15} groups compared with the corresponding L_9 groups. In the groups treated with L-Gln, the latencies decreased when compared with the respective Naive and Vehicle controls, except L_{15} Gln250. In both L_9 and L_{15} condition, Gln500 and Gln750 groups displayed shorter latencies than the corresponding Gln250 rats.

Regarding CSD velocity of propagation, ANOVA indicated intergroup differences ($F[1,104] = 671.054$; $p < 0.001$) and *post hoc* (Holm–Sidak) test comparisons showed that the velocities were higher in the L_{15} groups compared to the L_9 groups. Gln treatment significantly increased the CSD propagation velocities ($F[4,101] = 91.237$; $p < 0.001$) compared with the Naive and Vehicle controls. In L_9 condition, CSD velocity of the Gln250 animals (3.78 ± 0.08 mm/min) was higher than the controls (3.29 ± 0.12 mm/min for the vehicle groups, and 3.32 ± 0.09 mm/min for the naive groups), but was lower than the Gln500 (4.01 ± 0.12 mm/min) and Gln750 rats (4.11 ± 0.18; mm/min). In the L_{15} condition, Gln250 (4.30 ± 0.07 mm/min) was not different from vehicle- (4.20 ± 0.09 mm/min) and naive (4.18 ± 0.09 mm/min) controls, but was lower than Gln500 (4.43 ± 0.18 mm/min) and Gln750 mg (4.47 ± 0.13 mm/min). No difference was found between Gln500 and Gln750 groups in both L_9 and L_{15} condition. The CSD velocities for all groups are shown in **Figure 3**. Data on amplitude and duration of the CSD negative slow potential change are shown in **Table 1**. A significant shortening of the CSD duration was observed in the L_9 Gln500 and Gln750, in comparison with the corresponding naive and vehicle controls. No differences in the amplitude were observed.

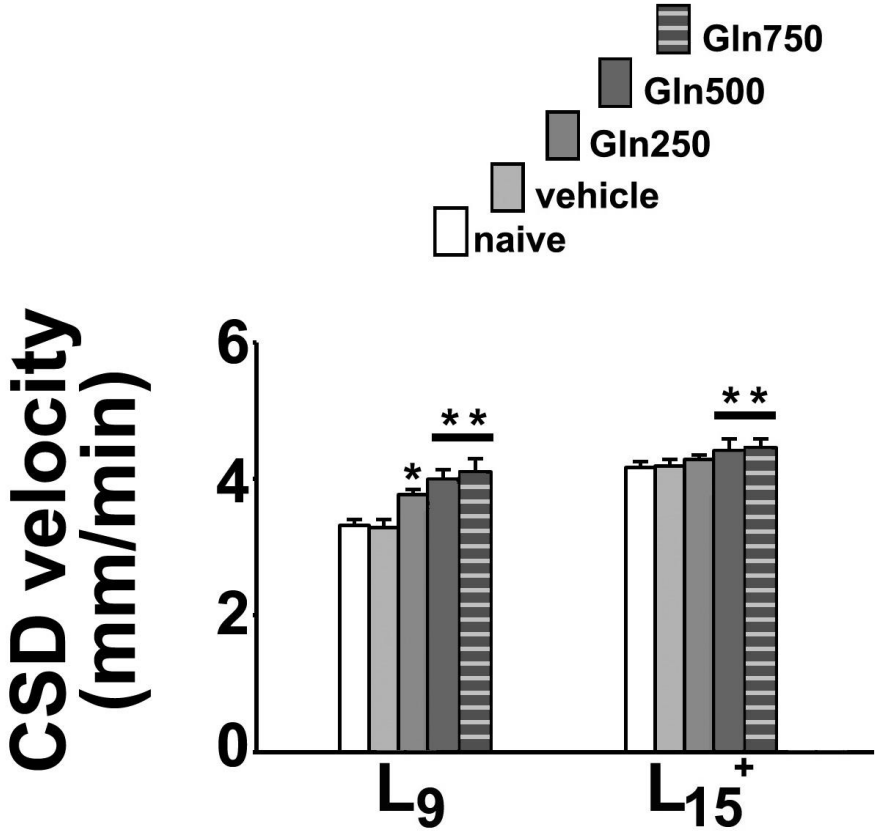

Figure 3. CSD velocity of rats (90- to 120-day old) previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} groups). Gln250, Gln500 and Gln750 are rats treated per gavage with L-glutamine at the doses of 250 mg/Kg/day, 500 mg/Kg/day and 750 mg/Kg/day, respectively. Data are mean ± SD. + indicates all L_{15} groups that were significantly different from the corresponding L_9 groups ($p < 0.001$; ANOVA followed by the Holm-Sidak test). * indicates values that are significantly different from the naive and vehicle control groups ($p < 0.001$). ** indicates values significantly different from the Gln250 group.

Table 1. Amplitudes and durations of the negative slow potential shifts of CSD in male adult rats (90- to 120- day old) previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} condition). Gln250, Gln500 and Gln750 are rats treated per gavage from postnatal days 7 to 27 with L-glutamine at the doses of 250mg/Kg/day, 500mg/Kg/day and 750mg/Kg/day, respectively.

Group	Amplitude (mV)	Duration (s)
L_9		
Naive (n=10)	8.51 ± 2.32	78.12 ± 7.41
Vehicle (n=10)	8.56 ± 1.24	77.11 ± 5.55
Gln250 (n-13)	10.93 ± 1.57	72.46 ± 3.97
Gln500 (n=10)	10.68 ± 2.50	69.48 ± 2.18*
Gln750 (n=10)	11.15 ± 2.72	69.93 ± 5.77*
L_{15}		
Naive (n=11)	10.31 ± 1.96	74.17 ± 5.67
Vehicle (n=8)	10.73 ± 2.51	74.28 ± 7.03
Gln250 (n=9)	10.32 ± 2.23	73.87 ± 5.32
Gln500 (n=12)	11.74 ± 2.97	69.84 ± 2.87
Gln750 (n=10)	12.09 ± 3.01	68.15 ± 3.87

Data are expressed as mean ± SD. *p< 0.05 compared with controls groups (Naive and Vehicle; ANOVA plus Holm-Sidak test).

Iba1 immunohistochemistry

Photomicrographs of Iba1- labeled cells in the parietal cortex and CA1 hippocampus are shown in the upper part of **Figure 4.** Except for the Gln500 treatment in the parietal cortex, L_{15} animals displayed greater Iba1 immunoreactivity and higher percentage of labeled area than the corresponding L_9 groups (**Figure 4**, lower part). In the two lactation conditions

Gln250 and Gln500 animals showed higher immunoreactivity and percentage of labeled area in comparison with the corresponding vehicle group, both in parietal cortex and hippocampus. In contrast to L_{15} condition, the L_9 Gln500 animals showed significantly greater immunoreactivity and higher percentage of labeled area when compared to the Gln250 group.

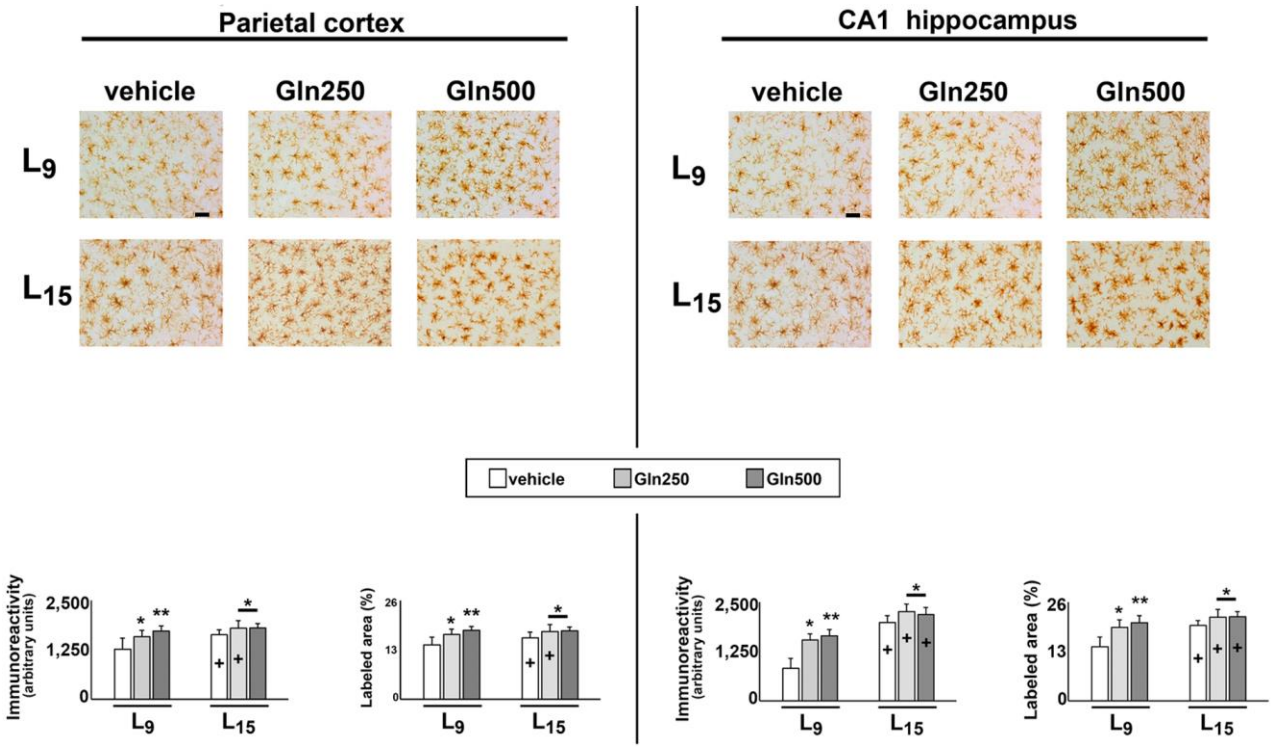

Figure 4. Low magnification photomicrographs of Iba1-labeled microglial cells in longitudinal sections of the parietal cortex (left) and CA1 hippocampus (right) of six adult rats (90- to 120- days of life) suckled in litters with 9 and 15 pups, resulting in two distinct lactation conditions (respectively L_9 and L_{15} groups). Gln250 and Gln500 are rats treated per gavage with L-glutamine at the doses of 250 mg/Kg/day and 500 mg/Kg/day, respectively. Scale bars = 20 μm. The lower bar graphics show immunoreactivity expressed as arbitrary units and the percent area occupied by the Iba1-labeled cells of slices of the parietal cortex (left) and CA1 hippocampus (right) of vehicle control (n = 6; 3 L_9 and 3 L_{15}), Gln250 (n = 8; 4 L_9 and 4 L_{15}) and Gln500 rats (n = 8; 4 L_9 and 4 L_{15}). Data are expressed as mean ± SD. $+p < 0.05$ compared with L_9 condition. $*p < 0.05$ compared with vehicle control. $**p < 0.05$ compared with the other two groups (ANOVA plus Holm–Sidak test).

Discussion

The present data on adult animals reinforce the previous observation on developing rats treated early in life with Gln, regarding the CSD effects [29]. Data also extend our knowledge about the neural actions of Gln by demonstrating behavioral and microglial alterations. As Gln treatment occurred early in life and the anxiolytic effect, CSD acceleration, as well as the increased microglial reaction were observed at adulthood, we suggest that these effects of neonatal Gln are permanent, or at least long-lasting.

The significant reduction in body weights ($L_{15} < L_9$) confirmed the effectiveness of increasing litter size in producing undernutrition, as previously indicated [26, 31]. When occurring during brain development, nutritional deficiency may impair brain growth and maturation [32, 33], as at this period the brain is highly vulnerable to environmental challenges, including unfavorable lactation conditions. Suckling under large litters is a situation that enhances the demand for the dam's milk, therefore resulting in a moderate state of undernutrition [31, 32, 33, 34]. Unfavorable lactation conditions can disrupt the biochemical and morphological organization of the brain [35] and influence CSD propagation [25, 30], behavior [34] and microglia [36]. Regarding CSD, malnutrition can increase the brain cell-packing density and reduce the brain extracellular space and myelination; all these changes accelerate CSD [37]. Regarding behavioral reactions, undernutrition-induced morphological, physiological and biochemical alterations can be also influenced by mother–pup and pup–pup interactions, and this may modulate behavioral development [34, 38].

Although our behavioral findings have indicated only discrete alterations, they point to an anxiolytic role for Gln in the L_{15} condition. It is possible that the behavioral action of neonatal Gln has been attenuated as the animals got old. Further investigation shall address this issue. One interesting possibility to be investigated is based on the action of Gln at the central nervous system as an anti-anxiety agent by acting as precursor of GABA.

Interestingly, increased levels of GABA have been demonstrated in the brain of rodents treated with Gln [39, 33]. Nevertheless, our data are in line with previous evidence that suggests less anxiety in genetically modified mice that present a higher activity for the enzyme glutamine synthetase [40].

In the present study, developing rats received Gln for three weeks (see methods). This long-term treatment is capable of imbalance plasma and brain amino acid levels [41, 42]. Based on this observation, it would be reasonable to postulate that under such condition the Gln availability would be enhanced, modifying the glutamate-Gln cycle and enhancing the brain levels of ammonia and glutamate/GABA [43]. Interestingly, glutamate- and GABA mediated mechanisms are involved in CSD [21, 22, 23, 24], and imbalance between these transmitters may enhance brain excitability [4], which may explain our findings.

Microglial activation (Iba1 immunolabeling) has been directly correlated with CSD propagation [27, 28] and unfavorable lactation [36]. These are two brain energy-demanding conditions in which microglia reportedly can be transformed into reactive glia. The present observations confirm the CSD/microglia correlation as well as the correlation between lactation condition and microglial immunolabeling. Collectively, our data reinforce the suggestion that neonatal Gln long-lastingly modulates both CSD and the Iba1-positive microglia reaction.

Compared with other glial cells, microglia are firstly activated in response to brain tissue demands [44]. Microglia helps preventing glutamate-related excitotoxicity by enhancing glutamate uptake [45]. Svoboda and Kerschbaum [46] reported that increase in Gln concentration in the culture medium can be toxic, and lead to apoptosis of microglia. In this context, the Trojan horse hypothesis of Gln toxicity, which is supported by the present data and involves both astroglial and microglial cells [46, 47] is a very interesting hypothesis to be better investigated in the future.

Conclusion

We conclude that neonatal Gln treatment results in long-lasting changes on the brain excitability (CSD acceleration), behavior like-anxiety and microglial reaction. These effects are dose-dependent and can be modulated by early postnatal undernutrition. Thus, we can advance in the understanding of the cerebral repercussions induced by neonatal Gln. Future investigations should be considered to elucidate the underlying mechanisms involved in the alterations here described, with a particular focus on neurotransmitter pathways.

Conflict of interest

The authors declare that they have no conflict of interest.

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7. CONSIDERAÇÕES FINAIS

Os resultados deste estudo representam um avanço no conhecimento das repercussões cerebrais do tratamento neonatal com Gln. Quanto aos dados eletrofisiológicos, a ação facilitatória da Gln sobre a DAC confirma aquela obtida em trabalho anterior (LIMA et al, 2009). Em relação aos aspectos comportamentais e imunohistoquímicos, os resultados são inéditos, sugerindo propriedades ansiolíticas e de ativação microglial para esse aminoácido. Além disto, foi possível demonstrar que as alterações eletrofisiológicas, comportamentais e imunohistoquímicas aqui descritas, persistem até a idade adulta e são dependentes da dose e da condição nutricional do animal.

Apesar das limitações para a extrapolação dos dados deste estudo para a saúde humana, considera-se o tema relevante, uma vez que a suplementação com Gln em neonatos ainda é observada na prática clínica, a despeito dos resultados controversos referentes aos seus benefícios. Desse modo, sugere-se cautela nas indicações de uso da Gln, bem como se recomenda estudos futuros que considerem os aspectos pertinentes à segurança do tratamento crônico com Gln durante o início da vida.

É importante considerar que a compreensão da relação entre a Gln e seus efeitos sobre o cérebro poderá ser útil na otimização de futuras propostas terapêuticas, visto que a Gln está indiretamente envolvida nos mecanismos fisiopatológicas de algumas neuropatias humanas, como a epilepsia (EID et al, 2012), a enxaqueca (BECERRA et al, 2016), a encefalopatia hepática (BUTTERWORTH, 2015) a doença de Alzheimer (JAHNG et al, 2016) e a esquizofrenia (THAKKAR et al, 2016).

Na tentativa de aprofundar os conhecimentos referentes aos efeitos da Gln sobre o cérebro, sugerem-se as seguintes perspectivas de estudo:

1. Análise dos neurotransmissores e das enzimas relacionadas às vias metabólicas da Gln no cérebro em ratos tratados com Gln;
2. Avaliação dos efeitos cerebrais do tratamento agudo e crônico com Gln, realizado na idade adulta, em distintos pontos temporais, inclusive na senilidade;
3. Análise imunohistoquímica dos astrócitos (anticorpos anti-GFAP);
4. Investigação dos efeitos cerebrais do tratamento com metionina sulfoximina (um inibidor da GS);
5. Análise da modulação dos efeitos cerebrais do tratamento com Gln pela atividade física.

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ANEXO 1

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Recife, 02 de maio de 2012.

Ofício nº 430/12

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof.Rubem Carlos Araújo Guedes**
Departamento de Nutrição
Universidade Federal de Pernambuco
Processo nº 23076.010667/2012-84

Os membros da Comissão de Ética no Uso de Animais do Centro de Ciências Biológicas da Universidade Federal de Pernambuco (CEUA-UFPE) avaliaram seu projeto de pesquisa intitulado, **"EFEITOS ELETROFISIOLÓGICOS DA L-GLUTAMINA NO CÉREBRO EM DESENVOLVIMENTO: ANÁLISE DE UMA CURVA DOSE-RESPOSTA EM RATOS LACTENTES NORMAIS E DESNUTRIDOS".**

Concluímos que os procedimentos descritos para a utilização experimental dos animais encontram-se de acordo com as normas sugeridas pelo Colégio Brasileiro para Experimentação Animal e com as normas internacionais estabelecidas pelo National Institute of Health Guide for Care and Use of Laboratory Animals as quais são adotadas como critérios de avaliação e julgamento pela CEUA-UFPE.

Encontra-se de acordo com as normas vigentes no Brasil, especialmente a Lei 11.794 de 08 de outubro de 2008, que trata da questão do uso de animais para fins científicos e didáticos.

Diante do exposto, emitimos **parecer favorável** aos protocolos experimentais a serem realizados.

Origem dos animais: Departamento de Nutrição-UFPE; Animais: Ratos; Linhagem:Wistar; Sexo: machos; número de animais previsto no protocolo: 160 animais; Peso: 10-350g; Idade:7 a 120 dias.

Atenciosamente,

Profa. Maria Teresa Jansem
Presidente do CEEA
UFPE

CCB: Integrar para desenvolver

ANEXO 2: Comprovante de submissão do Artigo 1

Fwd: AMAC-D-16-00295 - Submission Confirmation (2)

Pessoas
Rubem Guedes <guedes.rca@gmail.com>
Para
Denise Lima
Jul 4 em 8:26 PM

---------- Forwarded message ----------
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Date: 2016-07-04 20:23 GMT-03:00
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To: "Rubem C.A. Guedes" <guedes.rca@gmail.com>

Dear Prof. Guedes,

Thank you for submitting your manuscript, Neonatal L-glutamine modulates anxiety-like behavior, cortical spreading depression and microglial immunoreactivity: analysis in developing rats suckled on normal size- and large size litters, to Amino Acids.

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ANEXO 3: Comprovante de submissão do Artigo 2

Submission Confirmation

Life Sciences <ees.lfs.0.3a7e17.f5a49aab@eesmail.elsevier.com>
Para
denisandrelly@yahoo.com.br
Jul 8 em 11:12 AM
Dear Dr. Lima,

Your submission entitled "Long-lasting brain effects of neonatal L-glutamine on the anxiety like behavior, cortical spreading depression, and microglial reaction in adult rats" has been received by Life Sciences

You will be able to check on the progress of your paper by logging on to Elsevier Editorial Systems as an author. The URL is http://ees.elsevier.com/lfs/.

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