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ÁCIDO ASCÓRBICO COMO MODULADOR DA AÇÃO NEUROTÓXICA DE DOSE SUB-CONVULSIVANTE DE PILOCARPINA: ANÁLISE ELETROFISIOLÓGICA, COMPORTAMENTAL E BIOQUÍMICA EM RATOS JOVENS EM DISTINTOS ESTADOS NUTRICIONAIS

Recife
2018

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Orientador: Prof. Dr. Rubem Carlos Araújo Guedes

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RESUMO

A pilocarpina, um potente agonista colinérgico muscarínico, pode provocar alterações comportamentais e eletrográficas semelhantes às encontradas na epilepsia do lobo temporal do homem. A vitamina C, ou ácido ascórbico (AA), é um nutriente amplamente conhecido por sua ação antioxidante e neuroprotetora; entretanto, em certas condições, o AA pode facilitar a oxidação e exercer efeitos deletérios sobre o cérebro. Neste trabalho, investigamos a hipótese de que a administração crônica de dose sub-convulsivante de pilocarpina, associada ou não à administração de AA, afeta parâmetros comportamentais, eletrofisiológicos e bioquímicos cerebrais. Ratos nutridos e desnutridos receberam, do 7º ao 28º dia pós-natal, nenhum tratamento, ou solução salina, ou 45 mg/kg/dia pilocarpina e/ou 120 mg/kg/dia de AA, formando, em cada condição nutricional, os cinco seguintes grupos: ingênuo (sem tratamento algum), veículo (salina), pilocarpina (Pilo), ácido ascórbico (AA), e pilocarpina + ácido ascórbico (Pilo+AA). Terminado o tratamento, os animais foram submetidos aos testes de labirinto em cruz elevado (LCE), campo aberto (CA) e reconhecimento de objetos (RO), para avaliar comportamentos de ansiedade e memória. Em seguida, foi registrada a depressão alastrante cortical (DAC) e avaliados os níveis cerebrais de malondialdeído (MDA) induzido por peroxidação lipídica. Em comparação com os grupos controles (ingênuo e veículo), o tratamento com Pilo+AA reduziu o tempo e número de entradas nos braços abertos do LCE e área central do CA. A desnutrição prejudicou o RO nos grupos ingênuo, veículo e Pilo+AA. Nos animais normonutridos, RO foi prejudicado nos grupos AA, Pilocarpina e Pilo+AA. A pilocarpina desacelerou a propagação da DAC; enquanto que o AA acelerou esse fenômeno e aumentou os níveis de MDA no córtex cerebral. A administração de Pilo+AA atenuou o efeito da pilocarpina, porém não reverteu a velocidade da DAC aos valores dos grupos controles. Confirmando achados prévios, a desnutrição aumentou a velocidade de propagação da DAC em todos os grupos estudados. Nossos resultados confirmam a hipótese levantada e auxiliam na compreensão da relação entre efeitos cerebrais do estado nutricional, da pilocarpina e de antioxidantes.

Palavras-chave: Ácido ascórbico. Pilocarpina. Depressão alastrante cortical. Antioxidantes. Desnutrição. Comportamento.

ABSTRACT

Pilocarpine, a potent muscarinic cholinergic agonist, may cause behavioral and electrographic changes similar to those found in human temporal lobe epilepsy. Vitamin C, or ascorbic acid (AA), is a nutrient widely known for its antioxidant and neuroprotective action; however, under certain conditions, AA can facilitate oxidation and exert deleterious effects on the brain. In this work, we tested the hypothesis that chronic administration of a sub-convulsive dose of pilocarpine, combined with, or without AA, affects behavioral, electrophysiological and biochemical parameters in the brain. Well-nourished and malnourished rats received from the 7th to the 28th postnatal day no treatment (naïve group), or 45 mg/kg/day of pilocarpine and/or 120 mg/kg/day of AA, or saline forming, on each nutritional condition, the following five groups: naïve, vehicle (saline), pilocarpine, ascorbic acid (AA), and pilocarpine+ascorbic acid (Pilo+AA). After terminating the treatment, the animals were tested in the elevated plus maze (EPM), open field (OF), and object recognition (OR) paradigm in order to evaluate anxiety-like and memory behavior. This was followed by recording of cortical spreading depression (CSD) and measuring cortical malondialdehyde (MDA) levels induced by lipid peroxidation. Compared with the control groups (naïve and vehicle), the treatment with Pilo+AA reduced the time spent and the entries into the open arms of the EPM and the central area of the OF. Malnutrition impaired OR in the Naïve, Vehicle and Pilo+AA groups. In the well-nourished condition, OR was impaired in the AA, Pilocarpine and Pilo+AA groups. Treatment with pilocarpine decelerated CSD, while AA accelerated CSD propagation and increased the levels of MDA in the cerebral cortex. The administration of Pilo+AA attenuated the effect of pilocarpine but did not reverse the CSD velocity to the values of the control groups. Confirming previous findings, malnutrition increased the CSD velocity in all groups studied. The results confirmed our hypothesis and help in the understanding of the relationship between the brain actions of nutrition, pilocarpine and antioxidants.

Keywords: Ascorbic acid. Pilocarpine. Cortical spreading depression. Antioxidants. Malnutrition. Behavior.

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A pilocarpina, potente agonista colinérgico muscarínico, é utilizada como modelo químico para indução de epilepsia. Injeção intracerebral ou sistêmica deste composto (em doses >300mg/kg) promove episódios comportamentais de natureza epileptogênica, que culminam em um estado de mal epiléptico. A aplicação de pilocarpina pode induzir estresse oxidativo e provocar alterações comportamentais relacionadas à ansiedade e distúrbios de memória, bem como alterações eletrográficas semelhantes às encontradas na epilepsia do lobo temporal do ser humano.

O ácido ascórbico (AA), popularmente conhecido como vitamina C, é um nutriente hidrossolúvel encontrado em altas concentrações nas glândulas adrenais e no cérebro, e amplamente reconhecido por sua ação antioxidante. Apesar da sua atuação neuroprotetora, o ácido ascórbico pode ter ação pró-oxidante sobre o sistema nervoso, o que, em ratos, parece ser dependente da dose utilizada: a aplicação de baixas doses dessa vitamina está relacionada aos efeitos antioxidantes, enquanto que altas doses possuem efeitos pró-oxidantes. Além disso, a administração de AA em doses baixas e altas também é capaz de modular, no rato, a excitabilidade neural de forma bifásica, diminuindo e aumentando, respectivamente, a velocidade de propagação do fenômeno eletrofisiológico denominado depressão alastrante cortical (DAC; MENDES-DA-SILVA et al., 2014).

O organismo precocemente desnutrido apresenta aumento da excitabilidade do córtex cerebral, e possível alteração no equilíbrio redox. A desnutrição precoce pode alterar vários eventos maturacionais neurais, resultando, ao longo da vida, em alterações no funcionamento cognitivo e distúrbios emocionais, de aprendizagem e memória.

Considerando a influência da administração de altas doses de pilocarpina sobre o funcionamento do sistema nervoso (equilíbrio redox do cérebro, alterações comportamentais e eletrofisiológicas), e sabendo que o ácido ascórbico pode ter ações anti e pró-oxidantes sobre o cérebro; considerou-se útil, além de interessante, investigar os efeitos comportamentais, eletrofisiológicos e bioquímicos da associação entre uma dose sub-convulsivante de pilocarpina e ácido ascórbico. Nesse contexto, o presente trabalhou avaliou, em ratos imaturos, as ações da pilocarpina e do ácido ascórbico sobre comportamentos indicativos de ansiedade e memória, bem como sobre a DAC; adicionalmente, tentamos correlacionar alterações desses parâmetros com o nível de peroxidação lipídica no córtex cerebral. Além disso, avaliamos se tais ações foram modificadas pela desnutrição induzida por dieta hipoproteica, durante o período de desenvolvimento cerebral mais intenso.

2 FUNDAMENTAÇÃO TEÓRICA

2.1 EPILEPSIA, SISTEMA GLUTAMATÉRGICO E SISTEMA COLINÉRGICO

A epilepsia é uma das condições neurológicas mais comuns; cerca de 1% da população mundial é portadora de epilepsia, e aproximadamente 30% dos acometidos são resistentes ao tratamento com drogas antiepilépticas (STAFSTROM; CARMANT, 2015). Mais da metade dos casos de epilepsia tem início durante a infância, principalmente antes dos dois anos de idade, pois o cérebro imaturo é mais susceptível à ocorrência de convulsões (RASPALL-CHAURE et al., 2007; SHINNAR et al., 1997).

A *International League Against Epilepsy* (ILAE) definiu a epilepsia como uma desordem cerebral caracterizada por uma predisposição duradoura para gerar convulsões epilépticas, as quais são ocorrências transitórias de sinais e/ou sintomas causados pela atividade excessiva, desordenada e pouco controlada de grupos neuronais no cérebro. Essas convulsões são acompanhadas de consequências neurobiológicas, cognitivas, psicológicas e sociais (FISHER et al., 2005). Em 2014, foi proposto que o diagnóstico de epilepsia deve ser firmado após uma das seguintes condições: 1) ao menos duas convulsões não provocadas (ou reflexas) em menos de 24h; 2) uma convulsão não provocada (ou reflexa) e uma probabilidade de futuras convulsões semelhantes ao risco de recorrência geral após duas convulsões não provocadas, ocorrendo dentro de 10 anos; 3) diagnóstico de uma síndrome epiléptica (FISHER et al., 2014).

As convulsões epilépticas crônicas podem alterar a expressão neuronal e glial de receptores de glutamato e transportadores de sua captação, induzindo elevações no glutamato extracelular, o que contribui para a manutenção e propagação das convulsões (BARKER-HALISKI; WHITE, 2015). Em condições normais, o glutamato age através de três receptores ionotrópicos: receptores de ácido α-amino-3-hidroxi-5-metil-4-isoxazol propiônico (GluA), receptores de N-metil-D-aspartato (GluN) e receptores de cainato (GluK), além dos receptores metabotrópicos acoplados a proteínas G. O glutamato sináptico é recaptado pelos astrócitos via transportadores de glutamato (GLT-1 e GLAST). Na epilepsia, a expressão desses transportadores de glutamato é diminuída, aumentando os níveis sinápticos externos de glutamato, podendo ocasionar danos excitotóxicos (BARKER-HALISKI; WHITE, 2015).

Há uma ligação entre o sistema colinérgico e a epilepsia do lobo temporal. Na epilepsia do lobo temporal, as convulsões são iniciadas no hipocampo ou no córtex entorrinal devido a uma falha no controle colinérgico e/ou GABAérgico (PLATT; RIEDEL, 2011). No sistema colinérgico, a acetilcolina é um importante neurotransmissor excitatório cerebral. A

estimulação colinérgica no cérebro é realizada pela ativação de dois tipos de receptores: nicotínicos e muscarínicos, os quais foram classificados pela capacidade de se ligar à nicotina e muscarina. Os receptores nicotínicos são receptores ionotrópicos (subunidades α e β), enquanto que os receptores muscarínicos são receptores metabotrópicos acoplados à proteína G. Existem cinco subtipos de receptores muscarínicos de acetilcolina (M1 a M5), que estão distribuídos no sistema nervoso central e diferem quanto aos mecanismos de ativação (ABREU-VILLAÇA; FILGUEIRAS; MANHÃES, 2011). Estes subtipos são ainda agrupados com base no seu acoplamento às vias transdutoras de sinais. Quando estimulados pela acetilcolina, os receptores M1, M3 e M5 induzem a liberação de reservas de cálcio intracelular através da ativação da fosfolipase C via Gαq. Enquanto que M2 e M4 se acopla à Gαi/o para regular a adenilil ciclase e canais iônicos (MELANCON et al., 2013). Dentre os cinco receptores muscarínicos colinérgicos, sugere-se que o subtipo M1 seja o principal responsável pelos efeitos sobre processos de aprendizado e memória (BERKELEY et al., 2001; MELANCON et al., 2013). Este parece ser o receptor muscarínico mais abundante no sistema nervoso central, e altamente expresso no córtex, hipocampo, estriado e tálamo (LANGMEAD; WATSON; REAVILL, 2008).

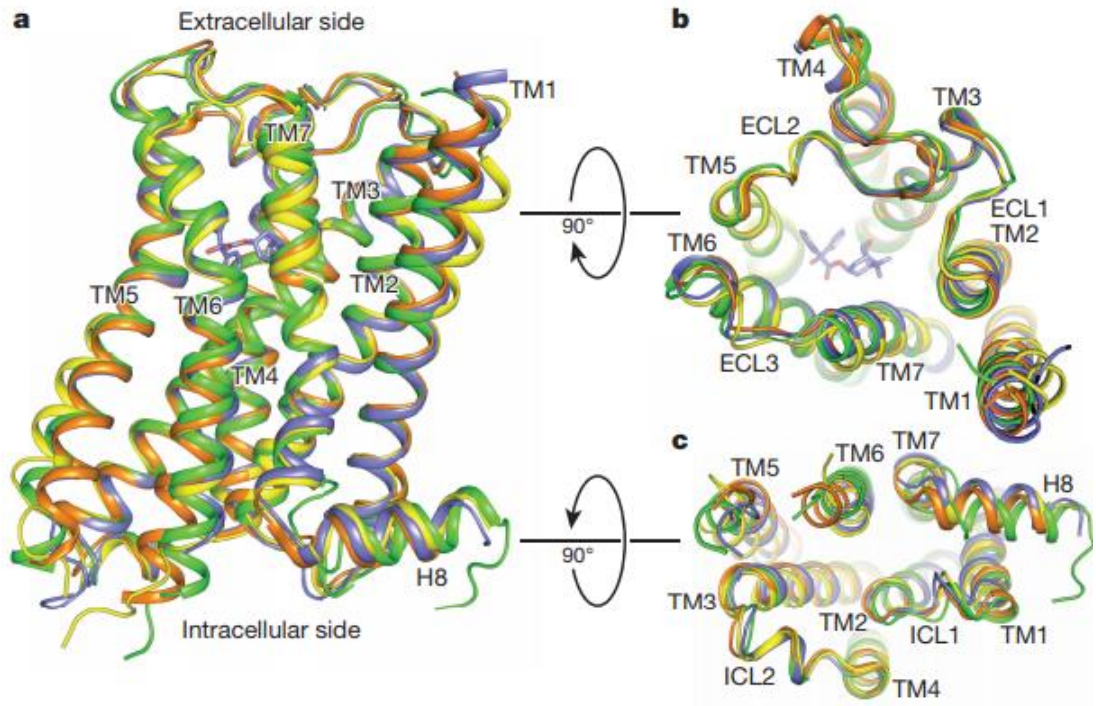


Figura 1 Ilustração esquemática dos receptores colinérgicos muscarínicos M1 – M4. a) Visão geral das estruturas muscarínicas: M1 em verde, M2 em amarelo, M3 em laranja e M4 em azul. b, c: Comparação das vistas do lado extracelular (b) e o lado intracelular (c). (THAL et al., 2016)

2.2 MODELO DE EPILEPSIA INDUZIDO POR PILOCARPINA

A Pilocarpina, um alcalóide derivado do Jaborandi (*Pilocarpus microphyllus*), apresenta ação agonista colinérgica muscarínica. Na clínica, a pilocarpina é indicada como terceira linha de tratamento no controle da pressão intraocular elevada (glaucoma) (LEE; HIGGINBOTHAM, 2005).

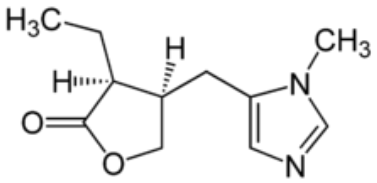
Experimentalmente, a pilocarpina é utilizada como modelo químico para indução de epilepsia em roedores. A injeção intracerebral ou sistêmica deste composto (em doses >300mg/kg) promove episódios comportamentais de natureza epileptogênica, que levam a um estado de mal epiléptico (ou *status epilepticus*), caracterizado por crises encadeadas, que se repetem de forma prolongada (CAVALHEIRO et al., 1991; LEITE et al., 1990). As alterações comportamentais e eletrográficas, que ocorrem no modelo de epilepsia induzido por pilocarpina, são semelhantes às que ocorrem na epilepsia do lobo temporal em humanos (TURSKI et al., 1983a, 1983b). Tais alterações podem ser subdivididas em três períodos distintos: i) período agudo – até 24h; ii) período silencioso (entre 4 a 44 dias) – com temporária normalização eletroencefalográfica e comportamental; iii) período crônico (torna-se permanente durante o resto da vida do animal) – convulsões recorrentes espontâneas (CAVALHEIRO et al., 1991; SCORZA et al., 2009).

A epilepsia do lobo temporal é a forma mais comum de epilepsia parcial complexa. As suas principais características, que podem ser reproduzidas em modelos animais de epilepsia, são: i) localização do foco da convulsão no sistema límbico, particularmente no hipocampo, córtex entorrinal e amígdala; ii) perda de neurônios hipocampais que precede o aparecimento da epilepsia do lobo temporal; iii) intervalo de tempo sem convulsão, conhecido como período latente; iv) presença de esclerose hipocampal levando a reorganização da rede neuronal (CURIA et al., 2008). Os danos hipocampais, assim como déficits cognitivos e de memória que são encontrados nos pacientes de epilepsia do lobo temporal, também estão presentes em ratos submetidos à aplicação de pilocarpina (KANDRATAVICIUS et al., 2014).

Camundongos geneticamente modificados, sem receptor M1, não são capazes de desenvolver convulsões em resposta à aplicação de pilocarpina (HAMILTON et al., 1997). Isto indica que a pilocarpina induz o *status epilepticus* em roedores a partir da ativação do receptor muscarínico tipo M1. O início das convulsões induzidas por pilocarpina pode ser bloqueado pela administração sistêmica de atropina, um antagonista muscarínico (CLIFFORD et al., 1987). Entretanto, após o início das convulsões o uso de drogas antiepilépticas parece ser ineficaz; o que sugere que a manutenção das convulsões está relacionada a outro

mecanismo além do sistema colinérgico, como por exemplo, a ativação do receptor NMDA (quadro 1; NAGAO; ALONSO; AVOLI, 1996). A excitação glutamatérgica aumentada é considerada um dos principais mecanismos celulares para compreender a propagação e manutenção das convulsões epilépticas (MELDRUM, 1994). Clinckers e colaboradores (2005) mostraram que a aplicação de 6,25 µM de glutamato na região CA1-CA3 do hipocampo parece não induzir convulsões; mas quando o glutamato é perfundido antes da aplicação de pilocarpina, as convulsões causadas pela pilocarpina tornam-se mais severas.

Quadro 1. Efeitos desencadeados pela pilocarpina.

<table>
<tr><th colspan="4">Pilocarpina ($C_{11}H_{16}N_2O_2$)</th></tr>
<tr><td>Estrutura</td><td>  </td><td colspan="2">Alvo</td><td>Receptores muscarínicos (agonista)</td></tr>
<tr><td>Mecanismo de geração das crises</td><td>1) Iniciação da crise pela ativação do receptor muscarínico;
2) Manutenção da crise pela modulação de glutamato, GABA ou dopamina;
3) Outras moléculas como receptores de adenosina, citocinas, quimiocinas e canais de cálcio podem também estar envolvidas.</td><td rowspan="2">Patologia</td><td>Axônios e dendritos</td><td>Brotamento axonal e dendritos recentemente brotados, com espinhas dendríticas tipo cone de crescimento, são encontrados no sistema límbico na fase crônica do status epilepticus envolvidos na manutenção do status epilepticus e início das crises recorrentes espontâneas.</td></tr>
<tr><td>Mudanças bioquímicas no cérebro durante e após convulsões</td><td>Aumento de:
Teor de ácidos graxos livres;
Atividade da aspartato aminotransferase, lactato desidrogenase, fosfatase alcalina
Níveis de malondialdeído</td><td>Lesão neuronal</td><td>Perda neuronal, em particular, interneurônios presentes no hipocampo, amígdala, tálamo, subiculum piriforme e córtex entorrinal, neocórtex e substância nigra.</td></tr>
</table>

	Diminuição de: • Atividade da glutationa peroxidase (GPX) • Níveis de glutationa (GSH) • Quantidade absoluta dos principais gangliósidos do cérebro (GM1, GD1a, GD1b e GT1b), esfingomielina, fosfatidilcolina e fosfatidiletanolamina		**Neurogênese**	A neurogênese das células granulares é aumentada na fase inicial (primeiras 3 semanas) após a indução de status epilepticus, mas diminuída logo após.

(Adaptado de TANG; LOKE; LING, 2011 e HOELLER, 2013)

A taxa de mortalidade após a injeção de alta dose de pilocarpina é elevada, atingindo cerca de 40% dos animais. Com o intuito de diminuir essa mortalidade, alguns estudos têm utilizado a combinação de pilocarpina com lítio (MÜLLER et al., 2009) ou aplicações consecutivas de doses subconvulsivantes de pilocarpina, até atingir o status epilepticus (GLIEN et al., 2001). A pilocarpina quando aplicada em baixas doses não induz convulsões, mas é capaz de exercer efeitos nocivos sobre o cérebro. Estudos que retratam os efeitos de doses subconvulsivantes de pilocarpina ainda são escassos (DUARTE et al., 2013; GUEDES; VASCONCELOS, 2008; VASCONCELOS et al., 2004), apesar de serem importantes para verificar a ação deletéria da droga no cérebro antes de ocorrência de convulsões.

As alterações comportamentais e eletrográficas induzidas pela aplicação de pilocarpina parecem ser dependentes da dose utilizada (CURIA et al., 2008). Quando administrada em baixas doses, a pilocarpina não induz convulsões (doses subconvulsivantes); entretanto tais doses ainda são capazes de provocar alterações comportamentais e sobre a excitabilidade neural. Por exemplo, i) uma injeção única de 45, 95 ou 190 mg/kg de pilocarpina foi capaz induzir diminuição na amplitude do eletrocorticograma (GUEDES; VASCONCELOS, 2008; VASCONCELOS et al., 2004); ii) aplicação de 150 mg/kg de pilocarpina promoveu aumento da atividade teta no hipocampo e alteração comportamental, demonstrada por perfil tipo ansiogênico (DUARTE et al., 2010, 2013); iii) única dose de 300mg/kg pilocarpina, em animais que não apresentaram *status epilepticus*, preveniu deficiências de aprendizado relacionadas à idade (DE-MELLO; SOUZA-JUNIOR; CAROBREZ, 2005).

Os mecanismos de ação pelos quais as doses subconvulsivantes de pilocarpina induzem alterações cognitivas e eletrofisiológicas ainda não estão claros. Porém, no modelo de epilepsia induzida por pilocarpina, alguns mecanismos já são conhecidos. Ratos epiléticos, por aplicação de pilocarpina, exibem alto nível de expressão de mRNA para os receptores de neurotrofina p75, e o bloqueio do receptor p75 diminui a frequência e duração das convulsões como também modulam o comportamento relacionado à ansiedade nesses animais (MEHRABI et al., 2017). Anormalidades nos sistemas serotoninérgico e glutamatérgico têm implicações conhecidas sobre a epilepsia do lobo temporal e memória. Durante a epilesia, ocorre um aumento da função do receptor serotoninérgico 5-hidroxitriptamina 2C (5-HT_{2C}), facilitando assim a liberação de IP3. O aumento do receptor 5-HT_{2C} contribui para o aumento da resposta à ansiedade (GIBSON; BARNFIELD; CURZON, 1994; KRISHNAKUMAR et al., 2014). Além disso, foi-se observada uma diminuição na função do receptor NMDA com expressão gênica elevada do receptor metabotrópico de glutamato 5 (mGlu5) e transportador de aspartato/glutamato (GLAST) indicando a possibilidade de excitotoxicidade mediada por glutamato (KRISHNAKUMAR et al., 2014). A classe NMDA, pertencente ao grupo ionotrópico dos receptores de glutamato, exerce papel crítico na indução da potenciação de longo prazo (LTP), uma modificação sináptica que codifica a memória de longo prazo (LISMAN; FELLOUS; WANG, 1998).

2.3 PILOCARPINA E ALTERAÇÕES COMPORTAMENTAIS

Os déficits cognitivos encontrados na epilepsia do lobo temporal em humanos (GUIMÃRAES et al., 2014; PAULI et al., 2006), podem ser observados no modelo de epilepsia induzida por doses elevadas de pilocarpina utilizando testes comportamentais como labirinto em cruz elevado e campo aberto (GRÖTICKE; HOFFMANN; LÖSCHER, 2007).

2.3.1 Labirinto em cruz elevado

O labirinto em cruz elevado (LCE) foi desenvolvido por Handley e Mithani (1984) e validado por Pellow et al. (1985). É considerado o teste “padrão-ouro” na investigação da base psicológica e neuroquímica da ansiedade. O teste do LCE baseia-se no conflito entre a aversão natural dos roedores a espaços abertos e a natureza exploratória/curiosidade do animal.

O equipamento consiste em quatro braços, sendo dois abertos e dois outros envolvidos por paredes laterais. Os braços são dispostos perpendicularmente uns aos outros, formando uma cruz, e são elevados cerca de 50 cm do solo (Figura 2). O arranjo dos braços permite que

os animais percebam simultaneamente dois fatores ansiogênicos: o precipício e o espaço aberto. Nessa situação, os animais demonstram um padrão de comportamento caracterizado por evitar os braços abertos e preferir os braços fechados, nos quais se sentem mais seguros (CRUZ; LANDEIRA-FERNANDEZ, 2012). Esta preferência pelos braços fechados é suprimida por ansiolíticos, e potencializada por agentes ansiogênicos (GRIEBEL; HOLMES, 2013). Normalmente, os parâmetros de ansiedade avaliados são o número de entradas e o tempo gasto nos braços abertos (BOURIN, 2015).

Figura 2. Labirinto em cruz elevado. Aparelho em formato de cruz, 50 cm elevado ao solo. (A) braços abertos; (B) braços fechados.

Estudos demonstram que ratos epilépticos, tratados com 320 e 350 mg/kg de pilocarpina, apresentam perfil ansiolítico nos testes de LCE e campo aberto (CARDOSO et al., 2009; SANTOS et al., 2005), enquanto que ratos não epilépticos, tratados com pilocarpina (75, 150 e 350 mg/kg), parecem apresentar efeito ansiogênico, como observado pelo grupo de Duarte (DUARTE et al., 2010, 2013).

2.3.2 Campo aberto

O campo aberto é um teste experimental bastante utilizado para avaliar respostas vinculadas à ansiedade e a atividade locomotora de roedores. O teste do campo aberto foi primeiramente descrito por HALL (1934), e é realizado em uma arena circular ou quadrada cercada por paredes. O assoalho é subdividido em pequenas áreas, o que permite a quantificação da atividade locomotora do animal contando-se o número de subdivisões que ele percorre (Figura 3-A). A arena é dividida em duas zonas (central e periférica) com mesma área (Figura 3-B).

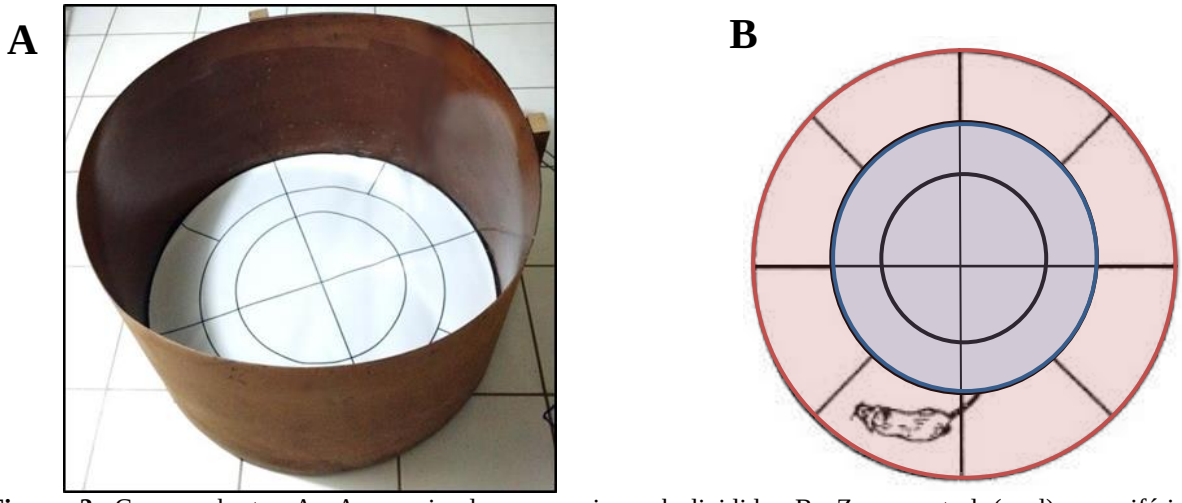


Figura 3. Campo aberto. A- Arena circular, com piso sub-dividido. B- Zona central (azul) e periférica (vermelho) com mesma área.

O animal é posicionado no centro ou próximo às paredes do equipamento e, durante um período de aproximadamente 5 minutos, as atividades comportamentais do animal são monitoradas por uma vídeo-câmera. Os padrões comportamentais que podem ser avaliados no teste de campo aberto incluem:

Subdivisões cruzadas ou distância percorrida – Avaliam a atividade locomotora do animal, e o aumento desses parâmetros indica menor nível de ansiedade;

Entradas no centro – frequência de entradas na zona central. Avaliação do nível de ansiedade: menor número de entradas indica perfil ansiogênico;

Tempo no centro – tempo gasto na zona central. Avaliação do nível de ansiedade: menor tempo no centro indica perfil ansiogênico;

Rearing – comportamento de levantar sobre as patas traseiras. Maior frequência de *rearing* indica aumento do comportamento exploratório e menor nível de ansiedade;

Grooming – limpar-se (com a língua), maior frequência de *grooming* está relacionado ao aumento dos níveis de ansiedade.

Defecação – Número de bolos fecais expelidos durante o teste. Aumento da defecação pode estar relacionado ao aumento dos níveis de ansiedade.

O tempo e número de entradas na área central são os principais parâmetros utilizados para avaliar o nível de ansiedade no teste de campo aberto. A diminuição do número de entradas e tempo na área central indica aumento do nível de ansiedade do animal. Trabalhos relatam que drogas ansiolíticas (que diminuem nível de ansiedade) aumentam o número de entradas e o tempo na área central da arena do campo aberto (CRUZ; LANDEIRA-FERNANDEZ, 2012; PRUT; BELZUNG, 2003).

Além da análise da atividade locomotora e ansiedade, a arena de campo aberto pode ser utilizada para realizar testes de memória baseada no reconhecimento da forma e posição de objetos. KALEMENEV *et al.* (2015) demonstraram que ratos adolescentes, submetidos ao modelo de epilepsia induzida por lítio-pilocarpina, exibiram uma diminuição na atividade exploratória no teste de campo aberto. Além disso, aplicação de >320 mg/kg de pilocarpina provocou déficits na memória de longo prazo no teste de reconhecimento de objetos (PEARSON; SCHULZ; PATEL, 2014; VASCONCELOS DE OLIVEIRA et al., 2015). Entretanto, em animais que não apresentaram *status epilepticus*, dose única de 300mg/kg pilocarpina preveniu deficiências de aprendizado relacionadas à idade (DE-MELLO; SOUZA-JUNIOR; CAROBREZ, 2005).

2.4 PILOCARPINA E ESTRESSE OXIDATIVO

No modelo de epilepsia induzido por pilocarpina, pode-se encontrar diversas alterações cerebrais após as convulsões, tais como: gliose, vacuolização e perda neuronal, em áreas como hipocampo, amígdala e córtex (DONG et al., 2013). Além disso, o excesso de espécies reativas de oxigênio tem sido correlacionado com o desenvolvimento de convulsões induzidas por pilocarpina (FREITAS et al., 2010; SANTOS et al., 2008).

Estresse oxidativo pode ser definido como um distúrbio no equilíbrio entre fatores pró-oxidantes e antioxidantes, com predominância do primeiro (AGUIAR et al., 2012). Sob condições fisiológicas, as vias de defesa antioxidantes se contrapõem à ação de espécies reativas de oxigênio e nitrogênio. Embora originalmente se tenha pensado que as espécies reativas de oxigênio tivessem, no cérebro, um papel exclusivamente nocivo, atualmente se considera que elas desempenham importantes papéis fisiológicos (AGUIAR et al., 2012). . Entretanto, em condições de produção excessiva de radicais livres, ou se as defesas antioxidantes do corpo estão diminuídas, as espécies reativas podem comprometer ácidos graxos, proteínas e DNA. O cérebro é um dos órgãos mais sensíveis a danos oxidativos; dentre os fatores que acarretam esta sensibilidade podemos citar: (1) a presença de aminoácidos excitotóxicos no cérebro (ex.: glutamato); (2) a produção do ânion superóxido (O2-) pela mitocôndria neuronal; (3) diversos neurotransmissores são auto oxidáveis (dopamina, serotonina e noradrenalina podem reagir com O2 e gerar O2-); (4) a presença de ferro em todo o cérebro (ferro e cobre aceleram a peroxidação lipídica); (5) a geração de elevada quantidade de H_2O_2 pelo metabolismo cerebral; (6) defesas antioxidantes relativamente baixas (HALLIWELL, 2006).

O excesso de radicais livres está associado a danos celulares, ao envelhecimento e à patogênese de diversas doenças neurológicas, como Alzheimer, Huntington, Parkinson e epilepsia. Na doença de Alzheimer, o estresse oxidativo contribui para a degeneração do sistema colinérgico do cérebro anterior basal e morte celular em geral (MONTINE et al., 2002; PRATICÒ, 2002), enquanto que na doença de Huntington os radicais livres influenciam na perda de neurônios GABAérgicos no núcleo estriado, ocasionando déficits motores e cognitivos (KUMAR; RATAN, 2016). O estresse oxidativo e a superatividade glutamatérgica são características bioquímicas que acompanham a perda de neurônios dopaminérgicos na substância nigra, o que caracteriza a doença de Parkinson (BALLAZ et al., 2013).

Em relação à epilepsia, as espécies reativas de oxigênio estão envolvidas nos mecanismos de neurodegeneração que ocorrem em modelos experimentais utilizando a pilocarpina (FREITAS, 2009; XUE et al., 2011). Evidências indicam que o dano neuronal no hipocampo é devido a um aumento na peroxidação lipídica e diminuição do teor de GSH que ocorre após o *status epilepticus* (FREITAS et al., 2005). Esses efeitos deletérios causados pelo estresse oxidativo podem ser prevenidos por antioxidantes.

2.5 ÁCIDO ASCÓRBICO

O ácido ascórbico (AA), popularmente conhecido como vitamina C, é um nutriente hidrossolúvel encontrado em altas concentrações nas glândulas adrenais e no cérebro (RICE, 2000), e amplamente reconhecido por sua ação antioxidante. Em sua forma ionizada, o ascorbato, é capaz de doar 2 elétrons (transformando-se em dehidroascorbato, Figura 4) e impedir a oxidação de outros compostos.

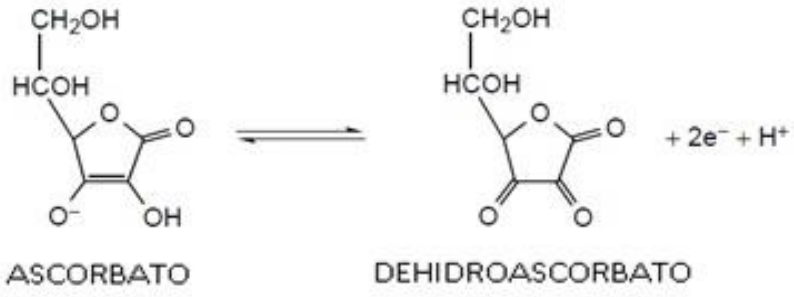


Figura 4 Estrutura molecular do ácido L-ascórbico como ânion monovalente (ascorbato) e o produto de sua oxidação (dehidroascorbato), pela perda de dois elétrons e um próton (modificado de RICE, 2000).

No sistema nervoso, o AA pode exercer também outras funções além da de antioxidante. A neuromodulação é uma das atividades não antioxidantes exercidas pela

vitamina C; a dopamina e o glutamato estão envolvidos na liberação de ascorbato, assim como, em certas regiões do cérebro, o ascorbato pode funcionar como um tipo de molécula de sinalização, regulando a eficácia pós-sináptica desses neurotransmissores (REBEC; PIERCE, 1994). Acredita-se que a vitamina C atue como um neuromodulador que facilita a liberação de neurotransmissores e inibe a ligação do neurotransmissor a receptores, incluindo dopamina, receptores NMDA e canais Cav 3.2 de cálcio do tipo T (KULKARNI; DESHPANDE; DHIR, 2008; SAFFARPOUR; NASIRINEZHAD, 2017).

Os seres humanos e outros primatas são incapazes de sintetizar o AA, pois a perda da enzima gulonolactona oxidase impede a síntese do ácido L-ascórbico a partir da glicose (NAIDU, 2003; ver Figura 5). Sendo assim, a presença de vitamina C na dieta é indispensável para a manutenção do organismo dos seres humanos, e sua carência pode causar a doença escorbuto.

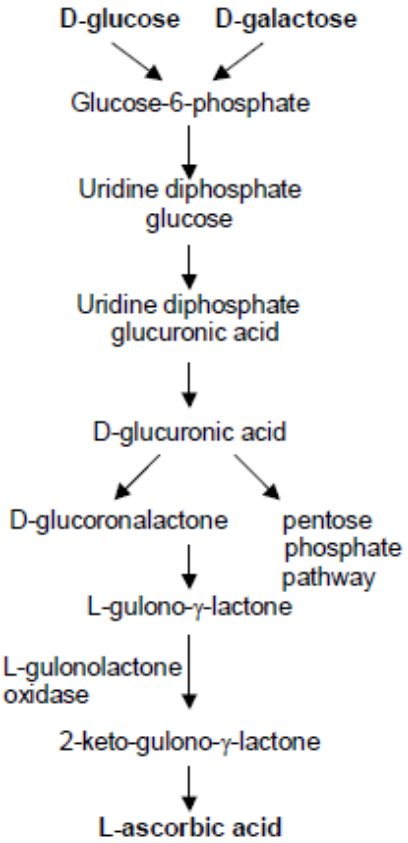


Figura 5 Biossíntese do ácido L-ascórbico em animais (NAIDU, 2003).

A principal via de entrada da vitamina C no sistema nervoso central (SNC) ocorre a partir do "transportador de vitamina C dependente de sódio tipo 2" (SCVT 2). O SCVT 2 é responsável pelo transporte do ascorbato do plasma para o líquido cefalorraquidiano (LCR), através do epitélio do plexo coroide. Além do transporte via SCVT 2, o AA pode entrar

rapidamente no LCR na forma de dehidroascorbato (DHA), a partir do transportador de glicose 1 (GLUT 1), através da barreira hemato-encefálica (HARRISON; MAY, 2009).

A alta concentração intracelular do AA no SNC é mantida pelo transporte via SCVT 2, encontrado apenas em neurônios. Em astrócitos, a entrada de ascorbato é menor, pois ocorre apenas pelo GLUT1 (na forma de DHA), o que pode explicar a diferença de concentração de ascorbato em neurônios e células gliais (respectivamente, 10 mM e 1 mM; ver Figura 6).

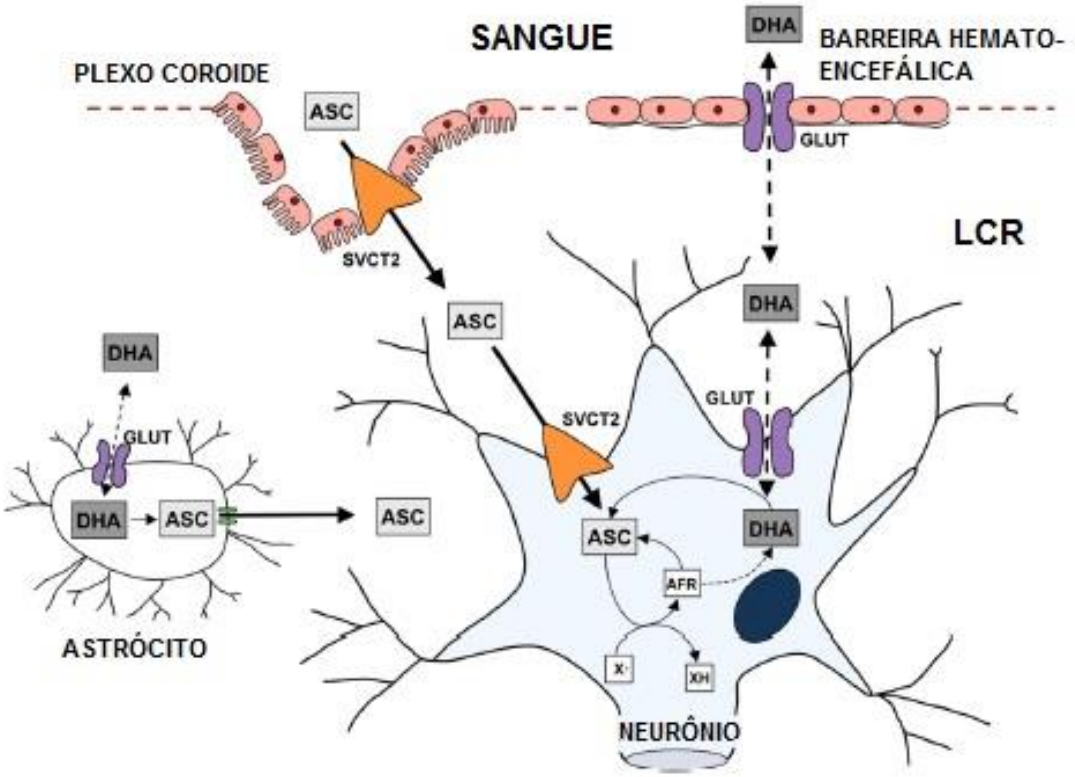


Figura 6 Vias de entrada de ascorbato (ASC) no SNC. ASC: ascorbato; AFR: radical livre ascorbato; DHA: dehidroascorbato; LCR: liquido cefalorraquidiano; X:·espécies de radicais livres oxidantes. O ascorbato entra no LCR via SVCT2 através do plexo coroide, ou como DHA via GLUT através da barreira hemato-encefálica. Similarmente, o ASC entra no neurônio via SCVT2 ou como DHA via GLUT. As células gliais obtêm ascorbato apenas de forma indireta, a partir da entrada de DHA via GLUT, enquanto os neurônios recebem ASC diretamente via SVCT2 (Modificado de HARRISON; MAY, 2009).

As espécies reativas em excesso têm participação direta nos mecanismos que medeiam distúrbios neurológicos, e a administração de ácido ascórbico pode atuar como neuroprotetor. ROSALES-CORRAL et al. (2003) demonstraram que a administração oral de ascorbato protege a área CA1 do hipocampo de ratos contra o estresse oxidativo e liberação de citocinas induzidos por injeção de β-amilóide fibrilar (modelo para doença de Alzheimer). O AA também foi capaz de reduzir os efeitos deletérios da administração de MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; modelo parkinsoniano) em cerca de 20% (WAGNER; CARELLI; JARVIS, 1986); assim como produziu uma melhora nos comportamentos

repetitivos de camundongos geneticamente modificados para apresentarem doença de Huntington (REBEC et al., 2003).

Em ratos epiléticos, a vitamina C inibe a agregação de ácidos graxos no cérebro durante as convulsões, possivelmente pela indução do aumento de enzimas antioxidantes (como a superóxido dismutase e catalase) no hipocampo dos ratos, assim como pela diminuição da peroxidação lipídica (SAWICKA-GLAZER; CZUCZWAR, 2014). Uma administração única de 500 mg/kg de ácido ascórbico em ratos adultos, foi capaz de antagonizar as alterações do equilíbrio redox do cérebro (aumento da produção de radicais livres) e da excitabilidade cerebral (indução de convulsões), produzidas por dose convulsivante de pilocarpina (DONG et al., 2013). Ratos com 10 dias de idade, tratados previamente com 500 mg/kg de ácido ascórbico, por 5 dias, demonstraram retardo, bloqueio ou diminuição das convulsões induzidas por pentilenotetrazol (GONZÁLEZ-RAMÍREZ et al., 2010).

Apesar da atuação neuroprotetora do ácido ascórbico, esta vitamina pode ter ação pró-oxidante sobre o sistema nervoso (AYDOĞAN et al., 2008; SONG; SHIN; ROSS, 2001), que parece ser dependente da dose utilizada e do período de tratamento. Nosso grupo demonstrou que a aplicação crônica de 120 mg/kg/dia de ácido ascórbico aumentou os níveis de malondialdeído no córtex cerebral, enquanto que a dose de 30 mg/kg/dia diminuiu esses valores (MENDES-DA-SILVA et al., 2014). A administração de AA também é capaz de modular a excitabilidade neural de forma bifásica, diminuindo e aumentando a velocidade de propagação da depressão alastrante cortical (ver abaixo a descrição desse fenômeno) de maneira dependente da dose utilizada (MENDES-DA-SILVA et al., 2014; MONTE-GUEDES et al., 2011). A ação bifásica do ácido ascórbico também foi relatada por SCHNEIDER OLIVEIRA et al. (2004), que observou que aplicação única intraperitoneal de 300 mg/kg de ascorbato protege contra convulsões, enquanto que 100 mg/kg prolonga a duração das convulsões e 30 mg/kg não exerce efeito sobre a atividade epiléptica.

Várias drogas antiepilépticas aumentam os níveis extracelulares de dopamina (DA) e/ou serotonina (5-HT) em áreas do cérebro envolvidas na epileptogênese, demonstrando um possível efeito anticonvulsivante dessas substâncias. Assim como o AA, a dopamina e serotonina exercem ações bifásicas sobre a excitabilidade neural dependendo da concentração utilizada. A injeção de 2 nM de DA e 5-HT no hipocampo promove proteção contra as convulsões induzidas pela aplicação de pilocarpina; enquanto que a aplicação de 10 nM de DA e 5-HT tem efeito pró-convulsivante, aumentando os níveis de glutamato extracelular (CLINCKERS et al., 2004).

2.6 DEPRESSÃO ALASTRANTE CORTICAL

A depressão alastrante cortical (DAC) é um fenômeno eletrofisiológico, descrito pela primeira vez pelo neurocientista brasileiro Aristides Azevedo Pacheco Leão. Leão (1944a) se referiu à DAC como uma "onda" reversível e propagável de redução (depressão) da atividade elétrica cerebral, em resposta a estímulos elétricos, químicos ou mecânicos. A DAC é acompanhada do aparecimento de uma "variação lenta de voltagem" (VLV) na região do cérebro invadida pelo fenômeno (LEÃO, 1947).

A propagação da DAC ocorre lentamente de forma concêntrica a partir do ponto estimulado, a uma velocidade de 2 a 5 mm/min. À medida que a resposta ao estímulo atinge regiões corticais mais distantes, a área inicialmente deprimida começa a se recuperar (Figura 7). Ao final de cerca de dez a quinze minutos todo o tecido cortical encontra-se recuperado (GUEDES, 2011). Durante a ocorrência do fenômeno, a variação lenta negativa de voltagem atinge amplitudes entre -5 e -20 mV. Além dessa despolarização, ocorrem outras alterações reversíveis, como a dilatação dos vasos sanguíneos (LEÃO, 1944b), e mudanças na distribuição de íons entre os meios extra e intracelulares (GORJI, 2001).

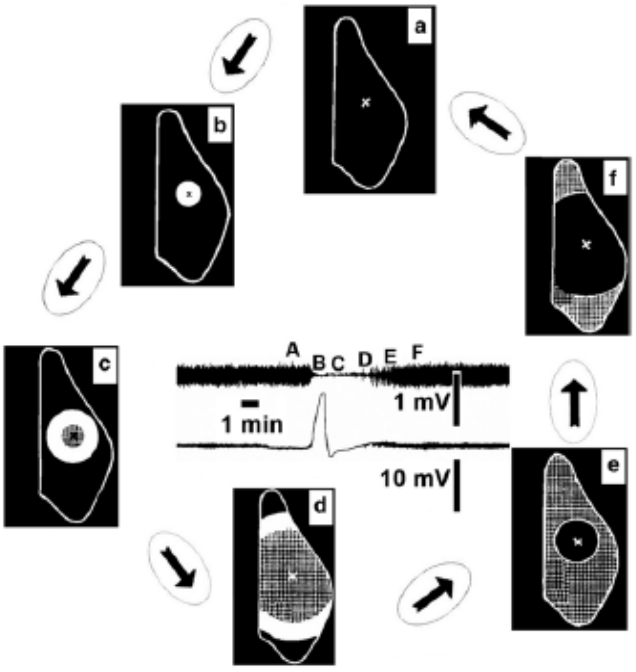


Figura 7 Esquema da sequência temporal cíclica de eventos da depressão alastrante cortical (DAC; adaptado de GUEDES, 2011). Em "a", um córtex normal e nele um ponto (x) é estimulado, iniciando a DAC. Na sequência, indicada pelas setas, "b" a "d", a propagação concêntrica do fenômeno da DAC está ilustrada. As áreas em branco representam porções do tecido cortical invadidas pelo fenômeno em tempos sucessivos. As áreas quadriculadas, "c" a "f" indicam regiões que sofreram a DAC e agora estão se recuperando (áreas refratárias a uma nova estimulação). De "b" a "f", observa-se que propagação (área branca) e recuperação (área escura) dão-se de forma concêntrica, sendo o ponto inicialmente estimulado o primeiro a se recuperar totalmente. Finalmente em "a" todo o tecido se mostra completamente recuperado, retornando à condição inicial. No centro

da figura, um traçado de registro demonstrando o eletrocorticograma (ECoG) e a variação lenta de voltagem (VLV), a qual sempre aparece durante a DAC, quando o ECoG diminui sua amplitude. As letras “A” a “F” correspondem a sequência das etapas representadas nos desenhos externos.

A DAC, por estar relacionada com a excitabilidade neuronal, tem sido usada como modelo experimental para avaliar o funcionamento e desenvolvimento do sistema nervoso central (GUEDES, 2011). Do ponto de vista clínico, características do fenômeno estão relacionadas com importantes doenças neurológicas humanas, como epilepsia, a isquemia cerebral, a enxaqueca com aura, a esclerose múltipla e injúria traumática cerebral. Durante a diminuição da atividade espontânea, “ondas epileptiformes”, semelhantes às encontradas no EEG de pacientes epilépticos, aparecem e se propagam durante a DAC (LEÃO, 1944a).

O tecido nervoso apresenta uma resistência natural à propagação da DAC; entretanto tal resistência pode ser atenuada ou fortalecida em diversas condições clínicas, modificando assim a velocidade de propagação do fenômeno. As tabelas 1 e 2 apresentam diversas condições, estudadas por nosso grupo, que podem dificultar ou facilitar a propagação da DAC.

Tabela 1 Algumas condições que dificultam a propagação da DAC.

Condição experimental	***Autor/Ano***
Tratamento dietético com lítio	(GUEDES et al., 1989)
Hiperglicemia	(XIMENES-DA-SILVA; GUEDES, 1991)
Anestésicos	(GUEDES; BARRETO, 1992)
Hipotireoidismo	(GUEDES; PEREIRA-DA-SILVA, 1993)
Envelhecimento	(GUEDES; AMORIM; TEODÓSIO, 1996)
Epilepsia crônica provocada pela pilocarpina	(COSTA-CRUZ; AMÂNCIO-DOS-SANTOS; GUEDES, 2006; GUEDES; CAVALHEIRO, 1997)
Ativação do sistema serotoninérgico	(AMÂNCIO-DOS-SANTOS et al., 2006; GUEDES et al., 2017)
Condições favoráveis de aleitamento	(ROCHA-DE-MELO et al., 2006)
Tratamento agudo com etanol	(ABADIE-GUEDES; BEZERRA; GUEDES, 2016; ABADIE-GUEDES;
Abolição da função ovariana (castração)	GUEDES; BEZERRA, 2012)

no início da vida	(ACCIOLY et al., 2012)
Dieta hipercalórica	(GERMANO et al., 2013)
Tratamento crônico com 30 mg/kg/dia de ácido ascórbico	(MENDES-DA-SILVA et al., 2014)
Lectina Concanavalina A	(SOARES et al., 2015)
Aminoácidos taurina e alanina	(FRANCISCO; GUEDES, 2015)

Tabela 2 Algumas condições que facilitam a propagação da DAC.

Condição experimental	***Autor/Ano***
Redução do cloreto extracelular	(GUEDES; DO CARMO, 1980)
Privação do sono paradoxal	(VASCONCELOS et al., 2004)
Tratamento crônico com etanol	(ABADIE-GUEDES et al., 2008; ABADIE-GUEDES; BEZERRA; GUEDES, 2016; GUEDES; FRADE, 1993)
Deficiência nutricional pela DBR*****	(ROCHA-DE-MELO; GUEDES, 1997)
Hipoglicemia	(COSTA-CRUZ; GUEDES, 2001)
Privação sensorial periférica	(TENÓRIO et al., 2009)
Arginina durante o desenvolvimento	(MAIA et al., 2017, 2009)
Glutamina durante o desenvolvimento	(LIMA et al., 2009, 2017)
Tratamento crônico com 60 e 120 mg/kg de ácido ascórbico	(MENDES-DA-SILVA et al., 2014; MONTE-GUEDES et al., 2011)
Glutamato monossódico	(LIMA et al., 2013; VITOR-DE-LIMA et al., 2017)
Tratamento com o antagonista serotoninérgico tianeptina	(AMANCIO-DOS-SANTOS et al., 2013; MAIA et al., 2017)
Dexametasona	(LOPES-DE-MORAIS et al., 2014)
Hormônios ovarianos	(ACCIOLY; GUEDES, 2017)

*****DBR= dieta básica regional (Teodósio et al., 1990)

Apesar da relação entre epilepsia e DAC, existem poucos trabalhos nesta área. Primeiramente, em 1997, Guedes e Cavalheiro demonstraram que ratos tornados cronicamente epiléticos pela aplicação de pilocarpina eram muito resistentes à indução e

propagação da DAC, e este efeito podia ser revertido por Diazepam. A amplitude do eletrocorticograma (ECoG) é reduzida após aplicação de pilocarpina (190 mg/kg), em animais nutridos e desnutridos; e a velocidade de propagação da DAC é diminuída apenas nos animais desnutridos. Além disso, verificou-se que a privação de sono REM aumentou o efeito da pilocarpina sobre a DAC (VASCONCELOS et al., 2004). COSTA-CRUZ e colaboradores (2006) demonstraram que 350 mg/kg de pilocarpina diminui a velocidade de propagação da DAC, bloqueando o fenômeno em alguns casos; e o tratamento com pilocarpina não potencializou o efeito da estreptozotocina.

O efeito da pilocarpina sobre a DAC é dependente da dose utilizada, uma vez que doses únicas, intra-peritoneais, de 45, 95 e 190 mg/kg induziram diminuição da amplitude do ECoG, mas apenas os grupos tratados com 95 e 190mg/kg tiveram a velocidade da DAC diminuída (GUEDES; VASCONCELOS, 2008). A resistência à DAC conferida pela pilocarpina também foi observada em fatias de neocórtex de humanos e ratos epilépticos (MASLAROVA et al., 2011). Em ratos geneticamente modificados, submetidos a kindling audiogênico, observou-se efeitos dependentes de gênero; no qual o kindling facilitou a propagação da DAC em animais machos e dificultou a propagação em fêmeas (GUEDES et al., 2009).

2.7 DESNUTRIÇÃO

Os efeitos da desnutrição sob o SNC têm sido extensamente estudados através do fenômeno eletrofisiológico da DAC, demonstrando que esta condição nutricional facilita a propagação do fenômeno, seja a desnutrição induzida por manipulação da dieta (GUEDES; ANDRADE; CABRAL-FILHO, 1987; ROCHA-DE-MELO; GUEDES, 1997) ou por aumento do tamanho da ninhada (BATISTA-DE-OLIVEIRA et al., 2012; FRANCISCO; GUEDES, 2015; LIMA et al., 2009; ROCHA-DE-MELO et al., 2006).

A desnutrição pode ser causada por uma dieta desbalanceada ou deficiente em nutrientes necessários para manutenção do organismo, sendo comum nesta condição clínica a carência de fatores antioxidantes. O estado nutricional do organismo exerce um papel importante durante o crescimento e desenvolvimento do sistema nervoso central, cujas estruturas e funções podem ser alteradas por deficiências nutricionais (MORGANE et al., 1978). Além disso, existem duas hipóteses relacionando a desnutrição e epilepsia: um círculo vicioso em que desnutrição é predisponente a epilepsia ou epilepsia predispondo à desnutrição (CREPIN et al., 2009).

Diversos estudos experimentais demonstram que a desnutrição, durante o período crítico de desenvolvimento, provoca alterações anatômicas e fisiológicas no encéfalo (MORGANE et al., 1993; ROCHA-DE-MELO; GUEDES, 1997), bem como estimula a produção excessiva de radicais livres no tecido cerebral. Tais alterações são refletidas em prejuízos cognitivos e comportamentais, e dependendo da intensidade/tipo de desnutrição causados, os efeitos deletérios podem persistir por longo prazo ou tornarem-se permanentes.

A "Dieta Básica Regional" (DBR) tem sido utilizada como um modelo para provocar desnutrição severa. É caracterizada por níveis reduzidos de minerais, vitaminas e lipídios, e possui uma grande deficiência proteica tanto em quantidade como em qualidade. Foi desenvolvida com base nos alimentos regionais consumidos pela população do nordeste brasileiro na década de 60, época em que a desnutrição, nesta região, acometia boa parte da população de baixa renda (TEODÓSIO et al., 1990). A DBR é composta por feijão, farinha de mandioca, charque e batata doce, apresentando baixos teores de proteína (8%), e níveis elevados de fibras (ver tabela 3).

Tabela 3 Composição percentual da "Dieta Básica Regional" (DBR). Exceto pela última coluna da direita, os números representam g/100g da dieta (adaptado de Teodósio et al., 1990).

INGREDIENTES	**g/100g**	**PROTEÍNA**	**CARBOIDRATO**	**GORDURA**	**FIBRA**	**Kcal % (Kcal em 100g da dieta)**
Feijão mulatinho	18,34	3,99	10,66	0,24	1,09	60,76
Farinha de mandioca	64,81	0,84	48,59	0,12	5,64	198,80
Charque	3,74	2,74	-	0,06	-	11,50
Gordura da charque	0,35	-	-	0,35	-	3,15
Batata doce	12,76	0,30	9,99	0,03	0,48	41,43
	100,00	**7,87**	69,24	0,80	7,21	315,64

Durante o período crítico de desenvolvimento do sistema nervoso a DBR causa o aumento da excitabilidade do córtex cerebral (GUEDES; ANDRADE; CABRAL-FILHO, 1987), promovendo déficit no crescimento e desenvolvimento do animal. O desenvolvimento da prole também é afetado pelo aumento do estresse oxidativo placentário gerado por uma desnutrição materno-fetal induzida pela DBR (VIEIRA-FILHO et al., 2009). Entretanto, foi observado que filhotes amamentados por mães que receberam DBR apenas durante o período

de lactação, não apresentaram elevação nos níveis de malondialdeído no córtex cerebral (MENDES-DA-SILVA et al., 2014).

3 HIPÓTESES

- Baseando-se nos estudos prévios realizados em nosso laboratório, hipotetizamos que o tratamento crônico com dose sub convulsivante de pilocarpina diminui a velocidade de propagação da DAC.

- A dose pró-oxidante de 120 mg/kg/dia de ácido ascórbico, que acelera a velocidade da DAC, intensifica os efeitos prejudiciais da pilocarpina sobre as análises comportamentais e bioquímicas.

- A desnutrição modula negativamente os efeitos da pilocarpina e/ou ácido ascórbico.

4 OBJETIVOS

4.1 GERAL

Avaliar os efeitos da interação entre dose sub-convulsivante de pilocarpina (45 mg/kg/dia) e o ácido ascórbico (120 mg/kg/dia), associados ou não à desnutrição precoce, sobre o sistema nervoso em desenvolvimento, utilizando modelos eletrofisiológicos, comportamentais e avaliação de peroxidação lipídica no córtex cerebral.

4.2 ESPECÍFICOS

- Acompanhar a evolução ponderal ao longo do período de tratamento, como indicador do estado nutricional;
- Avaliar a ansiedade e mobilidade através dos testes do labirinto em cruz elevado e de campo aberto;

- Avaliar memória através do teste de reconhecimento de objetos;
- Investigar as alterações nos parâmetros da depressão alastrante cortical (velocidade de propagação, amplitude e duração) causadas pela administração crônica de pilocarpina e ácido ascórbico;
- Quantificar a amplitude da atividade elétrica cortical antes e após a DAC e compará-las, como forma de demonstrar o efeito de potenciação do eletrocorticograma, que é dependente da DAC;
- Avaliar o peso cerebral, como indicador de efeitos da restrição nutricional sobre o desenvolvimento do cérebro;
- Avaliar o estado oxidativo do córtex cerebral, a partir da análise de peroxidação lipídica;
- Verificar se os efeitos da pilocarpina e do ácido ascórbico são modificados quando o cérebro é precocemente desnutrido;
- Correlacionar os parâmetros da depressão alastrante com os dados obtidos da peroxidação lipídica.

5 RESULTADOS

5.1 THE EFFECT OF PILOCARPINE/ASCORBIC ACID INTERACTION ON BEHAVIORAL IMPAIRMENT IN WELL-NOURISHED AND MALNOURISHED IMMATURE RATS.

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Abbreviations: AA, ascorbic acid; EPM: elevated plus maze; OF: open field; Pilo, pilocarpine.

ABSTRACT

Pilocarpine is a cholinergic agonist that triggers seizures in rodents when applied at a single high dose (300-380 mg/kg i.p.). Ascorbic acid (AA) is an antioxidant that in high doses can facilitate oxidation, which may result in a pro-convulsive role in seizure models. Here, we investigated the memory and anxiety-like effects of chronic treatment with a sub-convulsive dose of pilocarpine in association with or without AA in immature rats. Rats that were born from well-nourished and malnourished dams received from postnatal day 7 to 28 no treatment (Naïve group), a saline solution (intraperitoneal), 45 mg/kg/d of pilocarpine, 120 mg/kg/d of AA (gavage), or 45 mg/kg/d of pilocarpine with 120 mg/kg/d of AA, forming the following five well-nourished and five malnourished groups: Naïve, Vehicle (saline), Pilocarpine, AA, and Pilo+AA. Elevated plus maze (EPM), open field (OF) and object recognition (OR) tests were performed to evaluate anxiety-like and memory behavior. Compared with the control groups, the Pilo+AA groups spent less time and reduced the entries into the open arms of the EPM and the central area of the OF. Malnutrition impaired OR in the Naïve, Vehicle and Pilo+AA groups. In the well-nourished condition, OR was impaired in the AA, Pilocarpine and Pilo+AA groups. In addition to confirming the previously reported effects of malnutrition, our findings showed that pilocarpine interacts with AA, increasing anxiogenic-like behavior and impairing memory in immature rats. We suggest that early and chronic AA administration at high doses facilitates the impairing behavioral action of a sub-convulsive dose of pilocarpine.

Key words: pilocarpine, ascorbic acid, anxiety, memory, malnutrition.

1. INTRODUCTION

Pilocarpine is a muscarinic cholinergic agonist that is widely used to experimentally study the physiopathogenesis of epilepsy. In rodents, a single high dose (>300 mg/kg) of pilocarpine acts on hippocampal cholinergic cells and induces behavioral and electroencephalographic features that are similar to those observed in human temporal lobe epilepsy [1,2].

Anxiety is a common morbidity in children with epilepsy, affecting 15-36% of patients [3]. Epileptic patients are more likely to be anxious than non-epileptic patients; on the other hand, the risk of anxiety is elevated in the years prior to the onset of epilepsy. Furthermore, anxious epileptic patients have cognitive complaints [4].

The immature central nervous system has several neurobiological peculiarities that make it more sensitive for the development of abnormal electrical activity [5,6]. During the period of brain development, processes such as hyperplasia, hypertrophy and myelination occur rapidly [7], and inadequate nutrition can alter the organization of the nervous system both in laboratory animals [8,9] and in humans [10,11].

Ascorbic acid, known also as vitamin C or ascorbate, plays a role in the neuronal differentiation, maturation, myelin formation and modulation of the cholinergic, catecholaminergic, and glutamatergic systems [12]. Despite its known enzymatic roles and antioxidant properties, in some circumstances this vitamin acts as a pro-oxidant in tissues. Ascorbic acid appears to lose its effectiveness as an antioxidant at high concentrations or at partial oxygen pressures [13,14]. Previous reports from our group showed that long-term treatment with a low dose of ascorbic acid acts as an antioxidant, whereas high doses can exert pro-oxidant effect on brain excitability [15] and increase lipid peroxidation [16]; however, little is known about the behavioral alterations associated with chronic treatment with ascorbic acid.

Most studies on the brain effects of pilocarpine have focused only on doses of pilocarpine able to generate *status epilepticus*, which, as a severe form of continuous seizure attacks, is associated with brain damage and significant mortality; however, less attention has been paid to the possible behavioral alterations that occur after treatment with a sub-convulsive dose of pilocarpine. This study aimed to evaluate the effects of long-term administration of a sub-convulsive dose of pilocarpine with and without ascorbic acid on anxiety-like behavior and memory in immature rats under different nutritional conditions.

2. MATERIAL AND METHODS

2.1 Animals

All experimental procedures were previously approved by the Institutional Ethics Committee for Animal Research of our university (approval protocol no. 23076.012755/2014-82), the norms of which comply with those established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Bethesda, MD, USA). Newborn male and female Wistar rats, from litters of 8 to 9 pups, were distributed into two nutritional groups according to the mother's dietary conditions. The well-nourished group was suckled by dams fed a commercial laboratory chow diet (Purina) containing 23% protein, whereas the malnourished group was suckled by dams fed a regional basic diet containing 8% protein, as previously described [17]. After weaning, at post-natal day 21, all of the pups had free access to water and the same commercial lab chow that was offered to well-nourished dams. The weaned pups were housed in polypropylene cages (51 cm × 35.5 cm × 18.5 cm) under controlled temperature at 23 ± 1 °C with a 12-h light:12-h dark cycle (lights on at 6:00 a.m.). In this study, we analyzed data from male pups only: the 67 well-nourished and 65 malnourished rats originated from sixteen and fifteen litters, respectively.

2.2 Pilocarpine and ascorbic acid treatment

From postnatal days 7 to 28, the nutritional groups were subdivided according to the treatments applied: Naïve (no treatment), Vehicle (saline solution), Pilocarpine (Pilo – 45 mg/kg/d, via i.p.), Ascorbic acid (AA – 120 mg/kg/d, via gavage) and Pilocarpine plus ascorbic acid (Pilo+AA). To prevent the peripheral cholinomimetic effects of pilocarpine, scopolamine methyl-nitrate (1 mg/kg/d) was administered 20-30 minutes before pilocarpine injection. L-ascorbic acid, pilocarpine and scopolamine were purchased from Sigma, St. Louis, MO, USA.

2.3 Behavioral tests

2.3.1 Elevated plus maze

The cross-shaped, elevated plus-maze apparatus was raised 55 cm above the floor, and it comprised four arms (two closed arms and two open arms, each measuring 49 cm long × 10 cm wide). A central squared platform (10 × 10 cm wide) connected the open and closed arms. An elevated plus-maze test was performed on the 29th postnatal day. For the 5-min session, the rats were individually placed on the central platform facing an open arm. The following parameters were analyzed: travel distance, number of entries into the open arms and time

spent in the open arms. We considered the animal to have entered one of the arms when the trunk of the animal entered the arm.

2.3.2 Open field

The open field test is based on the natural exploratory instincts of rodents in a novel place, and it has been widely used to monitor locomotion, exploration and anxiety. The test was conducted in a circular arena (90 cm diameter) the floor of which was divided into two equal areas, a peripheral and central region. On the 31st postnatal day, each rat was placed in the center of the field, and its behavior was recorded for 5 min. The variables observed were as follows: distance travelled and time spent in and entries into the central area (away from the walls).

2.3.3 Shape-based object recognition

One day after the open-field test, the animals were subjected to an object recognition test. This comprised two 5-minutes sessions: training (first session) and short-term memory evaluation (second session; 1 hour after training). During the training session, two identical objects were placed in the same circular arena described above (open-field test). For the second session, one of the objects was replaced by another object with a different shape. The parameter analyzed was the time spent by the animal in exploring the familiar versus the novel object.

2.3.4 Spatial placement-based object recognition

The test comprised two 5-min sessions (training and short-term memory evaluation). For the training session, the procedure was the same as described above for the shape-based object recognition test. In the memory evaluation session, one of two identical objects was shifted to a novel location. The test was performed on the 33rd postnatal day, and the variable observed was the time spent by the animal in exploring the object in the novel spatial position.

All behavioral tests were performed under dim light and in a sound-attenuated room. After each session, the corresponding apparatus was cleaned with a 70% ethanol solution. The animal behavioral activity was recorded by a video camera, stored in a computer and subsequently analyzed with the software ANYmaze^{TM} (version 4.99).

2.4. Brain weight

After performing all behavioral tests, the animals were perfused with 0.9% saline solution followed by 4% paraformaldehyde, and their brains were removed and weighed.

2.5 Statistics

The results in all groups are expressed as the means ± standard deviations. All behavioral activities and brain weight were compared between groups using two-way ANOVA. The factors included nutritional condition (well-nourished and malnourished) and treatment (naïve, vehicle, pilocarpine, ascorbic acid and pilocarpine plus ascorbic acid) followed by a post hoc test (Holm–Sidak) when indicated. A p value less than 0.05 was considered significant.

3. RESULTS

3.1 Elevated plus maze

The well-nourished animals that received pilocarpine plus ascorbic acid significantly decreased the time spent in the open arms compared with the control groups (Fig. 1A). The entries in the open arms and distance travelled were not significantly different. In the malnourished condition, the time spent in the open arms (Fig. 1A), entries into the open arms (Fig. 1B) and distance travelled (Fig. 1C) were significantly reduced in the Pilo+AA group compared with the respective control groups.

In the Pilo+AA treatment, a significant difference between well-nourished versus malnourished groups was observed regarding the entries in the open arms and distance travelled (Fig. 1B and 1C). The treatment with only ascorbic acid or pilocarpine did not alter any parameter in the well-nourished or malnourished condition.

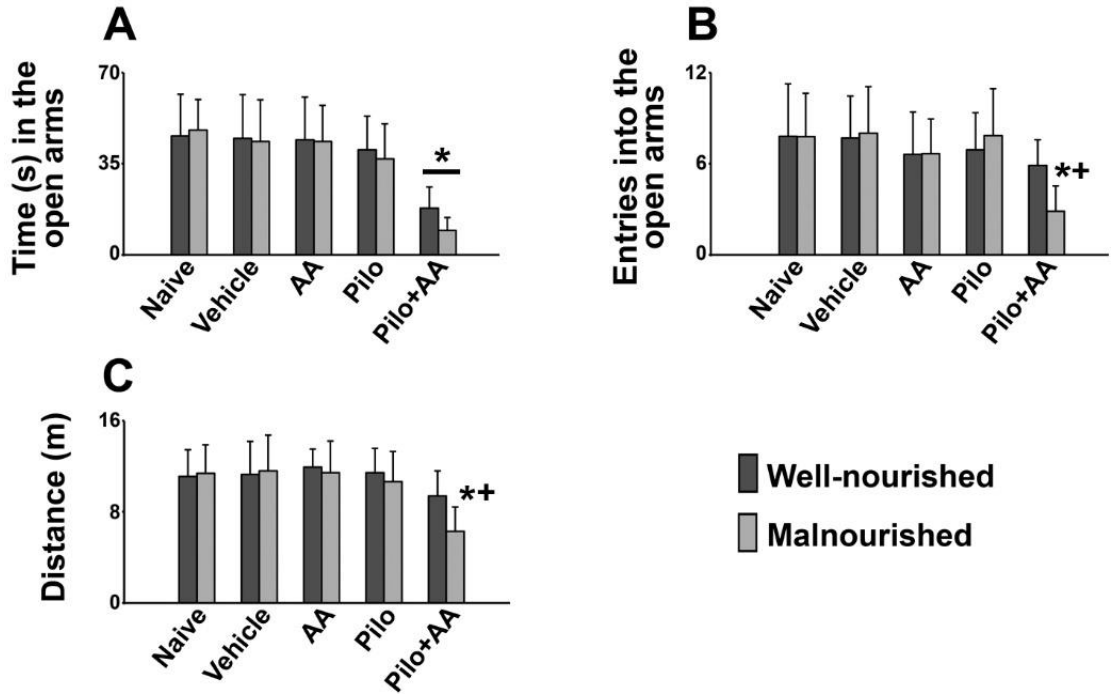

Fig. 1. Behavioral activity of well-nourished and malnourished immature rats in the elevated plus maze test. A, time (in seconds) spent in the open arms. B, number of entries into the open arms. C, distance (in meters) traveled during the 5-min test. Data are presented as the mean ± standard deviation. In each nutritional condition, the animals were subdivided into five groups, as defined in the main text: Naïve, Vehicle, Ascorbic Acid (AA), Pilocarpine (Pilo) and Pilocarpine plus Ascorbic Acid (Pilo+AA). *, different from the corresponding control groups (Naïve and Vehicle; $p < 0.001$). +, different from the respective well-nourished group ($p < 0.05$; two-way ANOVA followed by the Holm–Sidak test).

3.2 Open field

The effect of the administration of pilocarpine and ascorbic acid on the open-field test is shown in Fig. 2. The treatment with Pilo+AA significantly reduced the entries into the central area and distance travelled in both nutritional groups compared with the respective control groups. Only the treatment with pilocarpine did not result in a significant difference compared with the control groups. However, a non-significant trend could be observed (Fig. 2 A, B and C).

Additionally, in the malnourished condition ANOVA revealed significant effects of the Pilo+AA treatment on the time spent in the OF center. There was no statistically significant interaction between the treatment and nutritional condition.

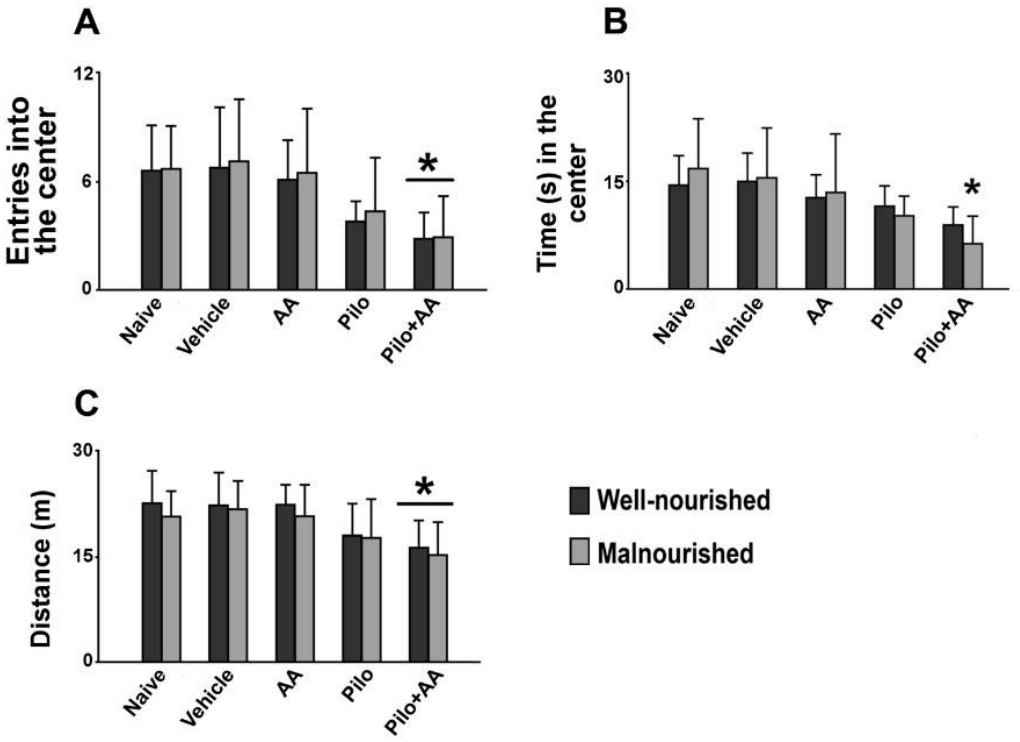

Fig. 2. Behavioral activity of well-nourished and malnourished immature rats in the open field apparatus. A, number of entries into the center of the arena. B, time (in seconds) spent in the center. C, distance (in meters) traveled across the open field. Treatments are as defined in Fig. 1 and the main text. Data are presented as the mean ± standard deviation. * Different from the corresponding control groups ($p < 0.001$; two-way ANOVA followed by the Holm–Sidak test).

3.3 Object recognition memory

In the well-nourished group, the administration of pilocarpine, ascorbic acid and pilocarpine+ascorbic acid reduced the exploration time for an object of novel shape (Fig. 3A). In the test for object placement, a reduced exploration time of an object placed in a novel position was only observed in the Pilo+AA group (Fig. 3B).

In the malnourished condition, only the Pilo+AA treatment reduced the exploration time of an object with a novel shape (Fig. 3A). The comparison between the well-nourished and malnourished condition revealed the impairment caused by malnutrition on the object recognition memory, as evaluated by a shorter exploration time of malnourished controls and the Pilo+AA groups compared with the corresponding well-nourished groups (Fig. 3A).

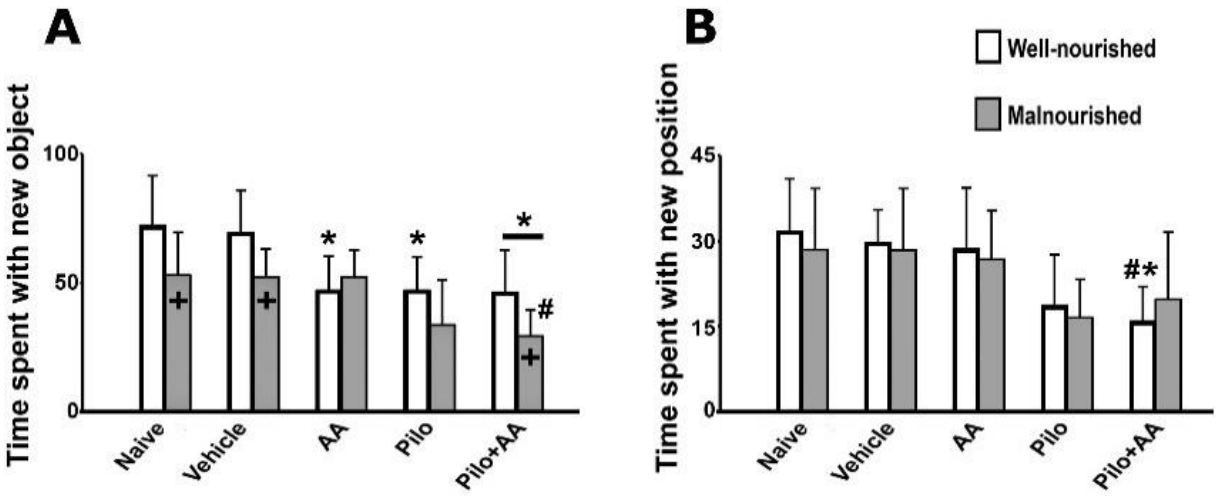

Fig. 3. Effect of treatment with ascorbic acid and/or pilocarpine on well-nourished and malnourished immature rats, in the tests for object recognition memory. A, time spent in the exploration of an object with a novel shape. B, time spent in the exploration of an object with a novel spatial position. Data are the mean ± standard deviation. *, different from the corresponding control groups ($p<0.01$). +, different from the respective well-nourished group ($p<0.05$). #, different from the AA group in the same nutritional condition ($p < 0.01$). All comparisons were made by two-way ANOVA followed by the Holm–Sidak test.

3.4 Brain weight

All malnourished groups displayed a significant reduction in brain weight compared with the corresponding well-nourished group ($p < 0.05$). This weight reduction was independent of the treatment (Fig. 4).

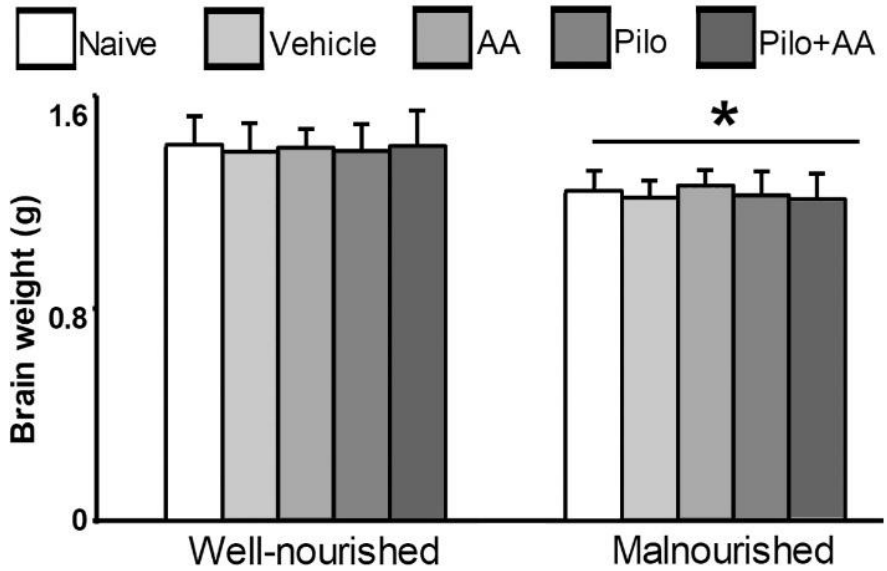

Fig. 4. Brain weight of immature rats suckled by dams fed a 23% protein diet (well-nourished group) or an 8% protein diet (malnourished group). The animals were treated with pilocarpine (Pilo) and/or ascorbic acid (AA) from postnatal days 7 to 28. Data are presented as the mean ± standard deviation. *, different from the corresponding well-nourished group ($p < 0.05$; two-way ANOVA followed by the Holm–Sidak test).

4. DISCUSSION

This study focused on the anxiety-like and object recognition memory effects after chronic treatment with a sub-convulsive dose of pilocarpine and/or ascorbic acid. In addition, we investigated the pilocarpine/ascorbic acid interaction in well-nourished and malnourished immature rats. Our data suggest an interaction between the convulsive drug (pilocarpine) and the redox-active vitamin (ascorbic acid) because the treatment with both compounds did increase the anxiety-like behavior and decreased object recognition memory, whereas the separate administration of ascorbic acid or pilocarpine did not. This last observation is in agreement with that of Duarte and co-workers, who reportedly did not observe alterations in anxiety-like behavior in rats that had been treated with single, systemic low doses of pilocarpine (20, 25 or 50 mg/kg) [18,19].

When applied acutely (single administration), ascorbic acid can counteract cognitive deficits caused by harmful agents, such as monosodium glutamate, lead and methylmercury [20,21,22]. In experimental pilocarpine-induced epilepsy models, the acute administration of ascorbic acid can reverse brain damage, decrease the mortality rate, ameliorate cognitive dysfunction and reduce oxidative stress in the hippocampus after seizures [23-26]. To the best of our knowledge, the present study provides the first pieces of evidence on the effects of chronic treatment (22 day-long) with ascorbic acid in immature, non-seized rats under the chronic action of a sub-convulsive dose of pilocarpine. In contrast with the acute condition, our results suggest that the association between pilocarpine and ascorbic acid may produce anxiogenic-like behavior in the elevated plus maze and open field tests, as evaluated by the distance traveled and time spent in and entries into the open arms of the EPM (Fig. 1) and in the central area of the open field (Fig. 2). Regarding the chronic administration of ascorbic acid, it is interesting to note that under certain conditions it can facilitate oxidative damage [14] and alter brain excitability in an age-dependent manner [27] or dose-dependent manner [28]. Furthermore, in doses comparable with that of the present study, ascorbic acid can facilitate convulsive episodes [29] and excitability-dependent brain phenomena [16]. Of note, intrastriatal injection with ascorbic acid acutely injured the dopaminergic neurons in mice through the generation of oxidative stress, and this toxicity was related to GSH depletion in the nigrostriatal dopaminergic system [30]. *In vivo* intrastriatal ascorbic acid injection may also stimulate hydroxyl radical generation in rats, and iron might be involved in this process [31]. A similar pro-oxidant effect of ascorbic acid has been described in hepatic tissue as well [32]. Therefore, we suggest that the anxiogenic-like effect that was observed in our group

treated with pilocarpine plus ascorbic acid may represent the interaction between the sub-convulsive dose of pilocarpine and ascorbic acid. We postulate that the pilocarpine action is enhanced by the application of the high dose of ascorbic acid (120 mg/kg/d) used in this work. In fact, this dose was previously reported as a pro-oxidant when used chronically in immature rats [16]. Finally, the stress of the treatment procedure, mostly represented by intraperitoneal injection and gavage, cannot be the cause of the behavior alterations because the outcomes of the vehicle groups that received saline by both administration routes (injection and gavage) were similar to the naive groups.

Cognitive impairment, which is commonly observed in the temporal lobe of epilepsy patients [33,34], is frequently reported in the pilocarpine epilepsy model [35-38]. Pilocarpine triggers status epilepticus by the activation of cholinergic neurons [39,40], which plays a crucial role underlying memory and cognitive function. Ascorbic acid is present in large concentrations in the brain areas known to be related to attention, learning and memory, such as the cerebral cortex, hippocampus and amygdala [12,41,42]. Interestingly, pilocarpine treatment may produce an increase in ascorbic acid levels in the cortex and striatum of rats [43,44]. The mechanisms of the action of ascorbic acid on cognition are not yet fully clear; however, it is known that ascorbic acid acts on the modulation of the cholinergic, catecholaminergic, and glutamatergic systems [12], which could be involved in the behavioral effects presently observed.

Nutritional deficiency in early life can disrupt the biochemical and morphological organization of the nervous system [45]. Malnutrition can promote long-lasting epigenetic alterations associated with impairments in attention and cognition [46]. In this study, the brain weights were lower in all malnourished groups compared with the corresponding well-nourished groups, confirming the effectiveness of the low-protein diet in producing malnutrition. Our results suggested that early malnutrition promotes impairment in the object recognition test, corroborating the results of previous experimental [9, 47] and human studies [46, 48]. The object recognition memory outcomes (Fig. 3) allow us to suggest that malnutrition may modulate the memory impairment effects of early and chronic treatment with pilocarpine plus ascorbic acid. Although in this work we did not analyze hippocampal alterations, pilocarpine has been shown to mainly impact the hippocampus, promoting neuronal loss and anatomical changes [49, 50], which imply cognitive deterioration.

In summary, the present study demonstrated that the chronic administration of a sub-convulsive dose of pilocarpine combined with ascorbic acid promotes anxiogenic-like behavior and memory deficit in immature rats. Moreover, the modulation of these effects by

malnutrition is also suggested. Our findings in rats may help to understand the effects of subconvulsant doses of pilocarpine on cognition. We suggest that the chronic administration of a high dose of ascorbic acid may interact with pilocarpine to promote behavioral impairment. However, additional studies are necessary to understand the role of redox imbalance in the development of behavioral alterations associated with anxiety and memory disturbances. As nicely remarked, “the effects of ascorbic acid are complex and may involve other actions unrelated to its antioxidant activity” [29].

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5.2 PILOCARPINE/ASCORBIC ACID INTERACTION IN THE IMMATURE BRAIN: ELECTROPHYSIOLOGICAL AND OXIDATIVE EFFECTS IN WELL-NOURISHED AND MALNOURISHED RATS

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Abbreviations: AA, ascorbic acid; CSD, cortical spreading depression; ECoG, electrocorticogram; MDA, malondialdehyde.

Abstract

Ascorbic acid (AA) administration has been associated with neuroprotection against oxidative stress, although at high doses it can facilitate oxidation and acts like a proconvulsing drug. The pilocarpine-induced epilepsy model has been widely studied. However, less is known about the effects of sub-convulsive doses of pilocarpine on brain activity in immature animals under normal or deficient nutritional conditions. Herein, we investigated the effects of chronic pilocarpine administration in a sub-convulsive dose, with or without AA, on the excitability-related phenomenon denominated as cortical spreading depression (CSD) and levels of lipid peroxidation-induced malondialdehyde in well-nourished and malnourished rats. At postnatal days 7-28, rats received no gavage treatment (naïve group), saline (vehicle group), 45 mg/kg/d of pilocarpine and/or 120 mg/kg/d of AA. CSD propagation and malondialdehyde levels were analyzed at 34-40 days. The pilocarpine group presented with lower CSD velocities, while AA groups exhibited higher CSD velocities and augmented malondialdehyde levels compared with controls. The co-administration of AA partially antagonized the pilocarpine CSD effects, but did not revert it to control levels. Malnutrition increased CSD amplitude and velocity in comparison to the well-nourished condition. The electrocorticogram (ECoG) amplitude increased after CSD (ECoG potentiation) when compared with the baseline amplitude before CSD. However, no intergroup difference was observed in this CSD-related ECoG potentiation. The results support the hypothesis of a pilocarpine/ascorbic acid interaction in the immature rat brain and might help further the understanding of this interaction on neuronal electrical activity and oxidative stress.

Key words: ascorbic acid; pilocarpine; cortical spreading depression; pro-oxidant; malnutrition; brain development.

1. Introduction

Pilocarpine is a cholinergic muscarinic M1 agonist that has been widely used as a model to study the physiopathogenesis of epilepsy (Cavalheiro, 1995; Kandratavicius et al., 2014; Leite et al., 1990). In this model, first described by Turski et al. (1983a,b), pilocarpine is systemically administered at a single high dose or consecutive low doses until the onset of *status epilepticus* (Glien et al., 2001; Gröticke et al., 2007; Schauwecker, 2012) in order to promote neuronal injury, electroencephalographic changes and behavioral features similar to human temporal lobe epilepsy. The alterations induced by pilocarpine are known to be dose-dependent and several studies have compared doses of pilocarpine ranging from 100 to 400 mg/kg (Curia et al., 2008), but not much attention is given to possible effects of lower doses of pilocarpine.

A correlation between pilocarpine-induced seizures and an increase in the production of reactive oxygen species has been shown in several studies (Freitas et al., 2010; Tejada et al., 2007; Xue et al., 2011). Elevated levels of free radicals or a decrease in the activity of antioxidant defense systems can promote the peroxidation of membrane lipids, which may be causally involved in seizure control (Hamed and Abdellah, 2004; Maertens et al., 1995). The relationship between oxidative stress and epilepsy is certainly the basis for the growing use of antioxidants to protect the brain against excitability-induced damage in experimental models (Carmona-Aparicio et al., 2016; McElroy et al., 2017; Tomé et al., 2010; Zhang et al., 2008).

Ascorbic acid (AA) is probably the most known water-soluble antioxidant and is involved in electrophysiological changes, such as modulation of epileptiform activity (Ayyildiz et al., 2007) and long-term potentiation (Vereker et al., 2000). AA administration has been associated with neuroprotection against seizures by retarding the onset of seizures in immature rats subjected to the pentylenetetrazole epilepsy model (González-Ramírez et al., 2010), decreasing the frequency of penicillin-induced epileptiform activity (Tutkun et al., 2015; Yildirim et al., 2010) and increasing the latency to the first seizure induced by pilocarpine (Santos et al., 2008; Xavier et al., 2007). However, evidence indicates that AA may exert a biphasic effect on pentylenetetrazol-induced convulsions (Schneider Oliveira et al., 2004) and can increase the oxidative stress when administered long-term in high doses (Aydoğan et al., 2008; Mendes-Da-Silva et al., 2014).

In early life, nutritional deficiency can induce alterations in brain structure and function in a long-lasting manner, since the immature brain is more vulnerable than the mature adult brain (Guedes, 2011). In humans, a ketogenic diet is used as an anti-seizure treatment for drug-resistant epilepsy in children (Elia et al., 2017). Additionally, there is a

two-way relationship between malnutrition and epilepsy studied in animals and humans (Crepin et al., 2009). On one hand, it is hypothesized that malnutrition influences the susceptibility to seizures and, on the other hand, epilepsy predisposes one to malnutrition. In rats, malnutrition has been shown to facilitate the induction of seizures (Cabral et al., 2011; Stern et al., 1974) and to increase the velocity of the propagation of the electrophysiological phenomenon known as cortical spreading depression (CSD; Batista-de-Oliveira et al., 2012; De Luca et al., 1977; Guedes et al., 1987; Rocha-de-Melo and Guedes, 1997).

CSD is widely used to evaluate brain processes that depend on neural excitability. It was described as a reversible and propagated wave of reduction in the spontaneous electrical activity of the cerebral cortex that occurs in response to electrical, chemical or mechanical stimulation applied to one point of the cortical surface (Leão, 1944). Simultaneously, with the depression of brain activity, there appears a slow direct current (DC) potential change in the tissue (Leão, 1947) and usually electrographic events that resemble tonic-clonic seizure discharges appear (Leão, 1944), suggesting a relationship between CSD and the epileptic phenomenon. Koroleva et al. (1993) demonstrated that epileptic kindling promoted by repeated daily application of 40 mg/kg pentylenetetrazol reduces the incidence of CSD. Additionally, in the pilocarpine epilepsy model, adult rats showed resistance to the elicitation and propagation of CSD after the injection of a single convulsing dose (320 mg/kg; Guedes and Cavalheiro, 1997) or sub-convulsive dose (190 mg/kg; corresponding to 59.4% of the convulsing dose; Vasconcelos et al., 2004).

Considering the relationship between the epilepsy-inducing action of pilocarpine and oxidative stress, as well as the need to better understand the action of nonconvulsing doses of pilocarpine, the current *in vivo* study aimed to investigate the possible action of the long-term administration of a very low-dose of pilocarpine with or without ascorbic acid on the changes in CSD features, electrocorticogram (ECoG)-potentiation and lipid peroxidation analyses. We also evaluated if early malnutrition could modulate the above parameters.

2. Material and methods

2.1. Animals

Wistar rat pups constituted two nutritional groups according to the mother's dietary conditions. The well-nourished group suckled dams fed a commercial laboratory chow diet (Presence, Purina, Ltd.) containing 23% protein. The malnourished group suckled dams fed a regional basic diet containing 8% protein. This diet mimics the diet of low-income human populations of Northeastern Brazil (Teodósio et al., 1990). After weaning, at post-natal day

21, all the pups were fed the control chow diet. In this study, we analyzed data from male pups only: 62 well-nourished and 63 malnourished rats originated from sixteen and fifteen litters, respectively.

Each nutritional group was divided into five subgroups according to the treatment that was applied from 7 to 28 postnatal days: (1) Naïve Control (without any treatment); (2) Vehicle Control (treated with saline solution – via i.p. and gavage); (3) Ascorbic acid (AA – 120 mg/kg/d, via gavage); (4) Pilocarpine (Pilo – 45 mg/kg/d, via i.p.); and (5) Pilocarpine plus ascorbic acid (Pilo+AA – via i.p. and gavage, respectively). Scopolamine (1 mg/kg/d, via i.p.) was administered 20-30 minutes before pilocarpine injection to prevent any peripheral cholinomimetic effect. Ascorbic acid and pilocarpine solutions were prepared daily, shortly before the injections.

The weaned rats were housed in polypropylene cages (51.0 cm×35.5 cm×18.5 cm; 3–4 rats per cage), with food and water ad libitum. The animal room was kept with controlled temperature (23 ± 1° C) and a 12/12-h light/dark cycle (lights on at 06:00 a.m.). Body weight was recorded daily during the treatment and at the CSD recording day.

The experimental protocols were approved by the Ethics Committee for Animal Research of our University (approval protocol no. 23076.012755/2014-82), whose norms comply with the “Principles of Laboratory Animal Care” (NIH; Bethesda, USA).

2.2. Cortical spreading depression recordings

At 34–40 days of age, the animals were subjected to a CSD recording session for 4 hours. Under anesthesia (1 g/kg urethane plus 40 mg/kg chloralose, i.p.), rectal temperature was maintained at 37±1 °C by a heating blanket, and three trephine holes were drilled on the right side of the skull. These holes were aligned in the frontal-to-occipital direction and paralleled to the midline. During the first recording hour, no CSD was elicited (baseline period); during the last 3 h (CSD period), CSD was regularly elicited at 20-min intervals by applying a cotton ball (1–2 mm diameter) soaked with 2% KCl solution (approximately 270 mM) to the anterior hole drilled at the frontal region for 1 min. The other two holes, on the parietal region, served as recording sites. We simultaneously recorded the DC slow potential change typical of CSD and the ECoG using two Ag–AgCl Agar-Ringer electrodes (one in each hole on the parietal region) against a common reference electrode of the same type placed on the nasal bones. The spontaneous cortical electrical activity was amplified and digitized with an MP100 or MP150 system (BIOPAC Systems, Inc, USA), recorded using the acquisition software ACQKnowledge™ and stored in an IBM-compatible computer for

further analysis. The amplitude and duration of the typical CSD-negative DC potential change, as well as the CSD propagation velocity, were calculated.

For each animal, we calculated the mean ECoG amplitude. One 10-min recording sample of the digitized ECoG was selected in the baseline period (first recording hour), and one 10-min sample was selected for each of the three subsequent recording hours (CSD period). Using the ECoG samples, the average ECoG amplitude was calculated with an algorithm implemented with the software MATLAB™ (version R2011b; The MathWorks, Inc.). For each animal, ECoG amplitude data are presented as relative units (values of the normalized amplitudes in relation to the lowest sample value, which was considered equal to 1). The electrode positions and the gains of the amplifiers remained unchanged throughout.

2.3. Lipid peroxidation analysis

After finishing the CSD recording session, the still-anesthetized animals were decapitated; the brains were rapidly removed and frozen. The cortical tissue was homogenized in a cold Tris buffer solution and centrifuged for 10 min at 1000 g at 4° C. Supernatants were used to estimate the lipid peroxidation by measuring malondialdehyde (MDA) levels using a thiobarbituric acid-reactive substances-based method (Ohkawa et al., 1979), which is a parameter to evaluate lipid peroxidation. The reaction was developed by sequential addition of 40 µL of 8.1% sodium dodecyl sulfate, 300 µL of 20% acetic acid (pH 3.5), and 300 µL of 0.8% thiobarbituric acid solutions to the 300 µL homogenate aliquot in a boiling water bath for 50 min. After cooling the tubes with tap water, 300 µL of n-butanol were added to the sample. The tubes were centrifuged at 2500 g for 10 min, and the organic phase was read at 532 nm using a plate reader. Experiments were carried out in triplicate. Total protein concentrations were determined based on a Bradford protein assay; bovine serum albumin was used as a standard. MDA concentrations were determined by using 1,1,3,3-tetraethoxypropane as the standard and expressed as µg/mg protein.

2.4. Drugs

All drugs were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.5. Statistical analysis

Intergroup differences were compared using two-way analysis of variance (ANOVA) including nutritional status (well-nourished and malnourished) and treatment (naïve, vehicle, AA, Pilo and Pilo+AA) as factors. ANOVA was followed by a Holm-Sidak *post hoc* test

when indicated. For intragroup comparisons of the ECoG analysis (baseline versus CSD period in the same animal) the paired t-test was used. Differences were considered statistically significant when $P \leq 0.05$.

3. Results

3.1. Body and cerebral cortex weights

Malnutrition induced by a low protein diet was effective in reducing body and cerebral cortex weight. All five malnourished groups had lower body weights than the corresponding well-nourished groups during the period of pilocarpine and ascorbic acid treatment (figure 1).

The cerebral cortex weight was reduced in four of the malnourished groups (Naïve, Vehicle, Pilo and Pilo+AA) in comparison with the corresponding well-nourished groups, and no difference was observed between AA groups (figure 2).

Within the same nutritional condition, no treatment-associated weight differences were observed.

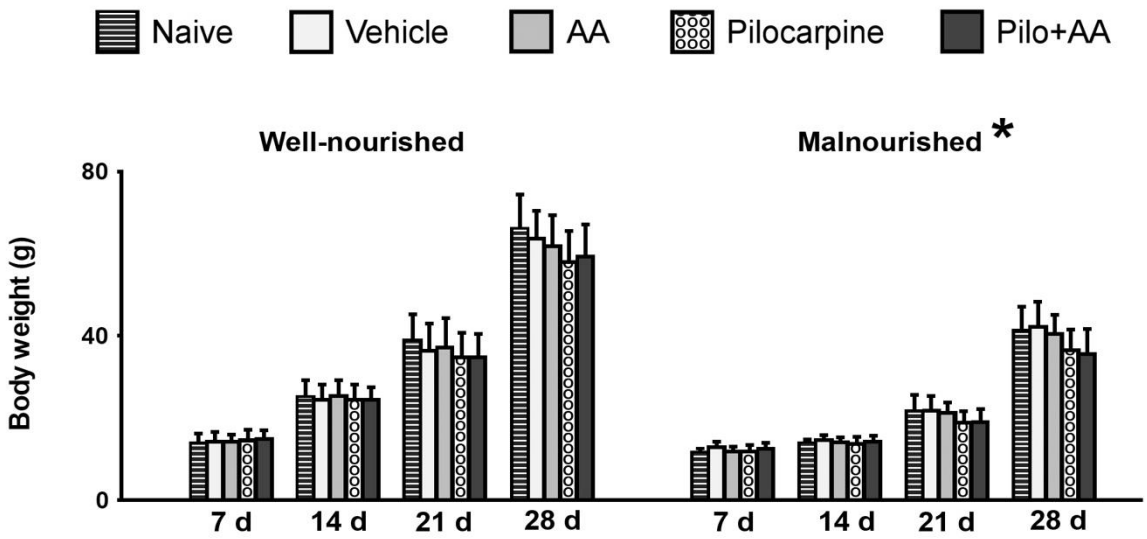

Fig. 1. Body weights of well-nourished and malnourished rats on postnatal days 7, 14, 21, and 28. Mean ± standard deviation is presented. Animals were subdivided into five groups, as defined in the main text: naïve, vehicle, ascorbic acid (AA), pilocarpine (Pilo) and pilocarpine plus ascorbic acid (Pilo+AA). The asterisk indicates that all malnourished groups differed significantly from the corresponding well-nourished groups ($p < 0.05$; ANOVA plus the Holm-Sidak test). Pilo or AA treatment did not influence body weight.

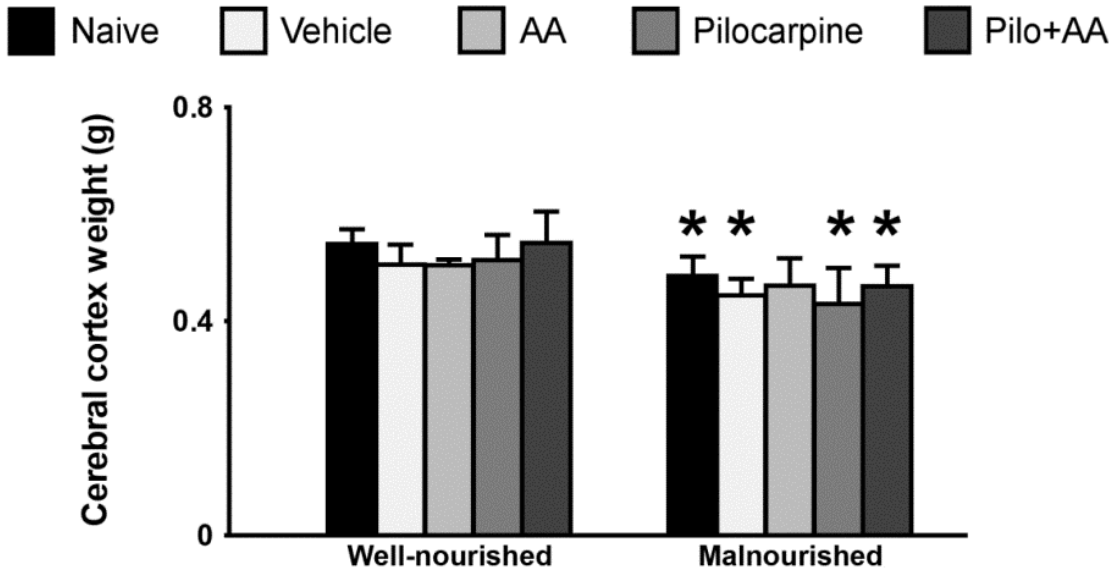

Fig. 2. Weight of the cerebral cortex of well-nourished and malnourished rats after CSD recording. Treatments are as defined in Fig. 1 and the main text. Values are presented as the mean ± standard deviation. *$p < 0.05$ compared with the corresponding well-nourished values (ANOVA plus the Holm-Sidak test).

3.2. CSD parameters

Application of 2% KCl for 1 minute on the cortical point in the frontal region effectively elicited a single CSD episode that propagated without interruption and was sequentially recorded by the 2 recording electrodes on the parietal region of the same hemisphere (Fig. 3).

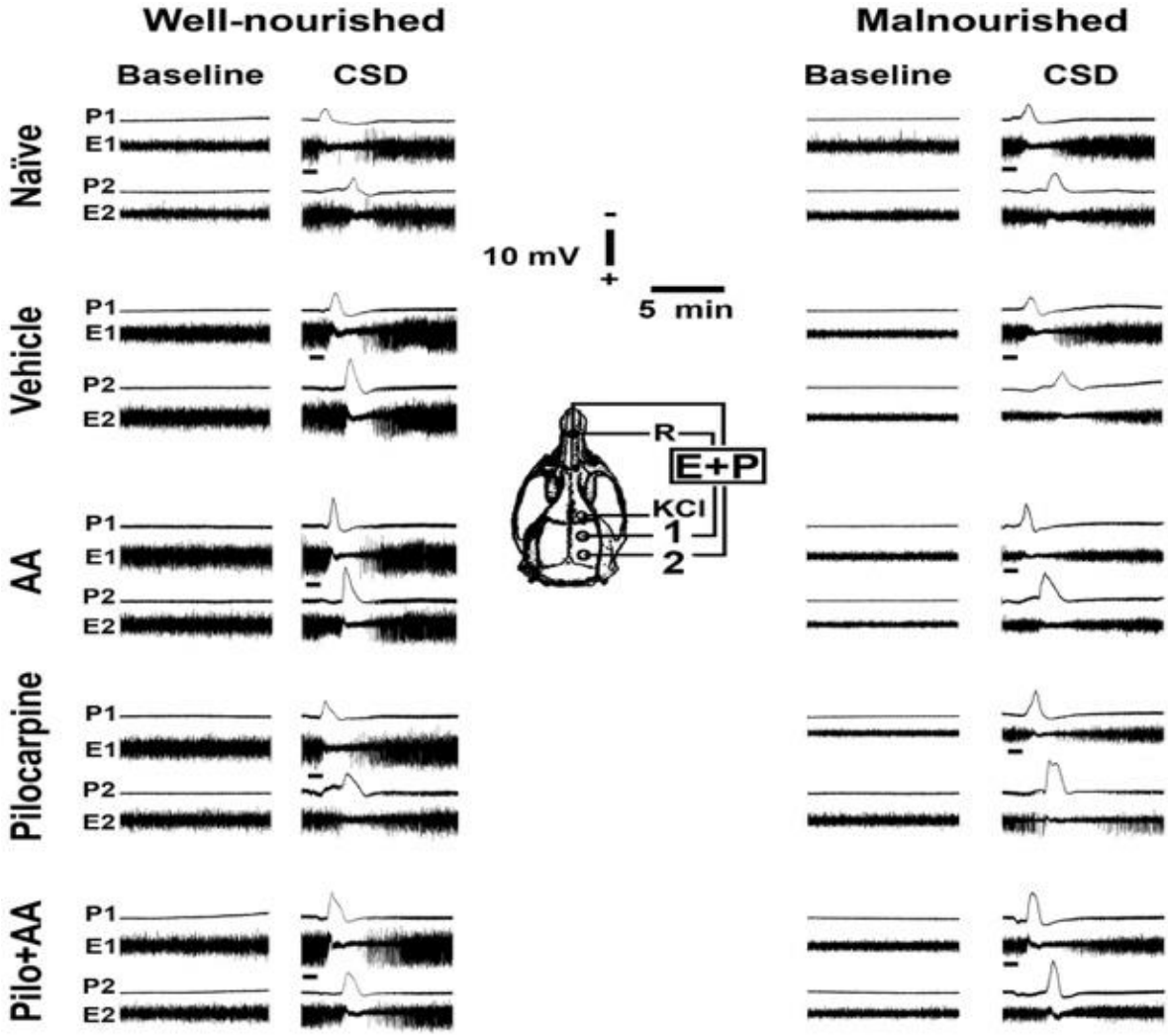

Figure 3. Examples of electrocorticogram (E) and DC-potential (P) recordings in the right hemisphere of five well-nourished and five malnourished 34–40-day-old rats. The diagram of the skull indicates the positions of the common reference electrode (R), the placement of KCl application to elicit CSD, and the 1 and 2 recording locations. Note the increased ECoG amplitude (ECoG potentiation) in the CSD period, compared with the baseline period in the same animal.

Confirming our previous results (Mendes-da-Silva et al., 2014), CSD propagated faster in the cortex of malnourished animals compared with the corresponding well-nourished rats (Fig. 4; $p < 0.001$). As well, rats treated with AA only (120 mg/kg/d) presented increased CSD velocities in well-nourished (4.52 ± 0.19 mm/min) and malnourished (4.65 ± 0.14 mm/min) conditions when compared with the respective control groups (3.78 ± 0.14 mm/min and 3.79 ± 0.08 mm/min for the naïve and vehicle groups, respectively, in the well-nourished condition, and 4.23 ± 0.15 mm/min and 4.26 ± 0.13 mm/min in the malnourished condition).

Pilocarpine administration (groups Pilo and Pilo+AA) was associated with decreased CSD velocity in both nutritional conditions. The CSD velocity in the well-nourished and malnourished Pilo group were 2.98 ± 0.19 and 3.52 ± 0.19 mm/min, respectively, whereas in the Pilo+AA groups, CSD velocity was 3.40 ± 0.18 and 3.73 ± 0.14 mm/min for well-

nourished and malnourished conditions, respectively (Fig. 4). No difference was observed between the two control groups in the same nutritional conditions. For the well-nourished controls, the velocities were 3.78 ± 0.14 mm/min and 3.79 ± 0.08 mm/min for the naïve and vehicle groups, respectively, while for the malnourished naïve and vehicle, the velocities were 4.23 ± 0.15 mm/min and 4.26 ± 0.13 mm/min (Fig. 4), respectively.

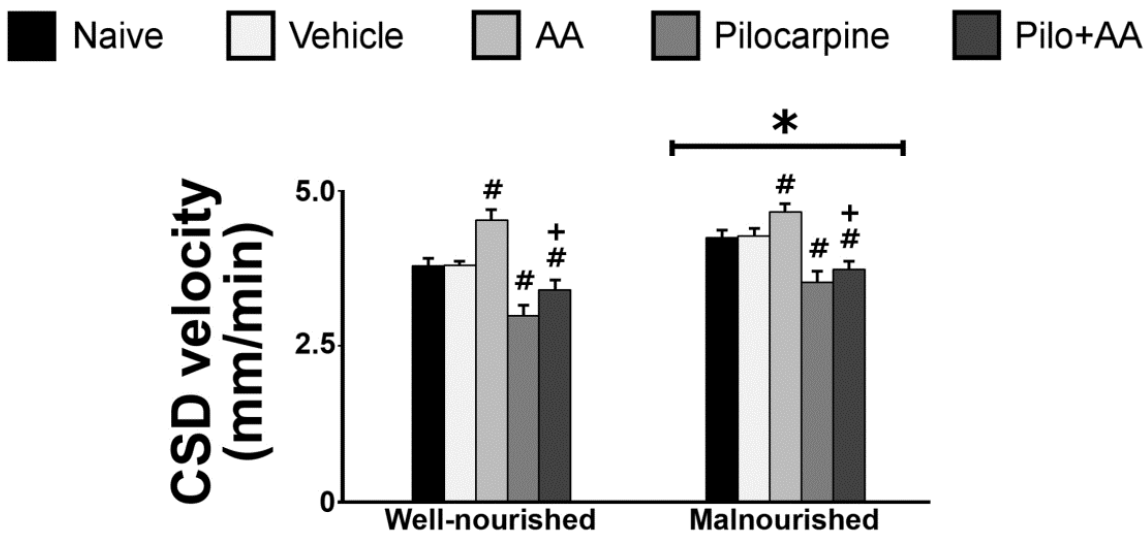

Fig. 4. CSD velocity of propagation in 34 to 40-day-old rats previously suckled by well-nourished or malnourished dams. Treatments are as defined in Fig. 1 and the main text. Values are presented as the mean ± standard deviation. *, $P < 0.05$ compared with the corresponding well-nourished groups. #, $P < 0.05$ compared with the control groups (naïve and vehicle) under the same nutritional conditions. +, $P < 0.05$ compared with the corresponding Pilocarpine group at the same nutritional condition ($p < 0.05$; ANOVA plus the Holm-Sidak test).

The mean amplitude of the CSD negative slow potential shift varied from 6.7 ± 4.5 mV to 7.8 ± 4.8 mV in the well-nourished groups, and from 9.6 ± 4.6 mV to 11.3 ± 4.0 mV in the malnourished groups (Table 1). Analysis byANOVA revealed a significant effect of nutritional status on the amplitude [$F(1,232) = 32.515$; $p < 0.001$]. *Post hoc* comparisons showed that the amplitudes were higher in the malnourished Naïve, Vehicle, Pilo and Pilo+AA animals compared with the corresponding well-nourished rats ($p < 0.05$).

The mean duration ranged from 66.9 ± 13.2 s to 76.3 ± 12.1 s in the well-nourished groups, and from 65.8 ± 12.9 s to 74.2 ± 9.7 s in the malnourished groups. The ANOVA revealed a significant nutrition versus treatment interaction ($F[1, 215] = 3.044$; $p = 0.018$), and *post hoc* comparisons indicated that the duration was shorter in the malnourished AA rats compared with the corresponding well-nourished animals (Table 1).

Table 1. Amplitude and duration of the negative slow potential shifts of CSD in well-nourished and malnourished rats treated with ascorbic acid and/or pilocarpine. Data are expressed as the mean ± standard deviation. The asterisk indicates a significant difference when compared with the corresponding well-nourished values ($p<0.05$).

Treatment groups	CSD Amplitude (mV)		CSD Duration (s)	
	Well-nourished	Malnourished	Well-nourished	Malnourished
Naïve	7.0±4.9	9.7±4.9*****	66.9±13.2	70.1±10.9
Vehicle	7.2±4.3	9.9±4.6*****	69.9±9.9	73.3±12.0
AA	7.8±4.8	10.1±4.6	76.3±12.1	67.6±12.1*****
Pilo	6.7±4.5	9.6±4.6*****	70.7±9.5	65.8±12.9
Pilo+AA	6.7±4.5	11.3±4.0*****	66.9±11.9	74.2±9.7

3.3. ECoG potentiation associated with CSD

In all groups, the amplitudes of the digitized ECoG samples during the baseline period were compared with those of samples from the CSD period. The ECoG amplitude became higher in the CSD period compared with the baseline period. Quantification of ECoG amplitudes revealed a CSD-related increase of approximately 30-70% in the well-nourished groups, and 60–80% in the malnourished condition ($p < 0.05$; paired t-test).

Table 2. Increase of the electrocorticogram (ECoG) amplitude after cortical spreading depression (CSD) in young well-nourished and malnourished rats. ECoG was recorded for 1 h to obtain a baseline value (before regular elicitation of CSD with 2% KCl), followed by 3 h of recording, during which CSD was elicited at 20-min intervals. In each recording hour, one 10-min recording sample was analyzed with an algorithm implemented in the software MATLAB™. Data (mean ± SD) are presented as relative units (values of the normalized amplitudes in relation to the lowest value, which was considered equal to 1). # indicates that the amplitudes after CSD were significantly higher ($p < 0.05$; paired t-test) compared with the baseline values.

Treatment groups	Well-nourished		Malnourished	
	Baseline period	CSD period#	Baseline period	CSD period #
Naïve	1.09 ± 0.05	1.80 ± 0.43	1.09 ± 0.10	1.89 ± 0.51
Vehicle	1.09 ± 0.06	1.74 ± 0.37	1.09 ± 0.06	1.87 ± 0.55
AA	1.12 ± 0.06	1.70 ± 0.31	1.11 ± 0.06	1.82 ± 0.55
Pilo	1.11 ± 0.11	1.39 ± 0.32	1.09± 0.06	1.76 ± 0.48
Pilo+AA	1.10 ± 0.04	1.62 ± 0.63	1.16 ± 0.10	1.77 ± 0.81

3.4. Cortical levels of MDA

The MDA levels in the cerebral cortices were higher in the malnourished AA group in comparison with the corresponding (AA) well-nourished group. Furthermore, in the malnourished condition, the MDA levels of the AA group were higher than the control (naïve and vehicle) and pilo groups (table 3).

Table 3. MDA levels (nmol/mg protein) in the cerebral cortex of 34 to 40-day-old well-nourished and malnourished rats previously treated with ascorbic acid and/or pilocarpine. Data are expressed as the mean ± standard deviation. *$p < 0.05$ compared with the corresponding well-nourished AA group. #$p < 0.05$ compared with malnourished control groups (naïve and vehicle); +$p<0.01$ compared with Pilo group under the same nutritional condition (ANOVA plus the Holm-Sidak test).

Nutritional groups / **Treatment groups**	**Well-nourished**	**Malnourished**
Naïve	2.17±0.29	2.46±0.34
Vehicle	2.15±0.52	2.43±0.51
AA	2.72±0.73	3.45±0.67 ***#+**
Pilo	1.88±0.33	2.17±0.36
Pilo+AA	2.17±0.39	2.69±0.39

4. Discussion

The present study analyzed the electrophysiological and biochemical effects of chronic treatment with a very low dose (45 mg/kg/d) of pilocarpine associated with ascorbic acid. Our data demonstrated that the repeated administration of a sub-convulsive dose of pilocarpine decelerates CSD propagation in immature rats, which is in line with previous studies on pilocarpine administration in a single, higher dose (95 and 190 mg/kg; Guedes and Vasconcelos, 2008; Vasconcelos et al., 2004). The sub-convulsive dose of pilocarpine that was used here represents, on average, approximately 12–15% of the convulsing dose (Guedes and Cavalheiro, 1997). Under such a low dose, neither electrophysiological (epileptic activity) nor behavioral signs of convulsion (motor seizures plus rearing, forelimb clonus, salivation, masticatory movements and loss of erect posture) were detected, confirming the sub-convulsive character of the treatment. Of note, this sub-convulsive pilocarpine treatment was demonstrated to be effective in antagonizing CSD in this study, suggesting a cholinergic modulation on the spontaneous cortical electrical activity involving the activation of muscarinic receptors (Guedes and Vasconcelos, 2008).

Weber et al. (1991) showed that the low free radical scavenging activity of glutathione peroxidase, an antioxidant enzyme, is suggested to be a cause of childhood seizures. Oxidative stress occurs as a consequence of seizures (Sashindranath et al., 2010); and it may increase seizure susceptibility (Liang and Patel, 2004; Patel, 2004). The pilocarpine-induced epilepsy model is associated with elevated MDA levels after *status epilepticus* (Dong et al., 2013; DU et al., 2012; Tsai et al., 2010). Later, after the *status epilepticus*, MDA returns to basal levels (Dal-Pizzol et al., 2000). Tejada et al. (2007, 2006) showed that an intracerebroventricular injection of pilocarpine did not induce oxidative damage while systemic administration of 350 mg/kg increased MDA levels. We suggest that lipid peroxidation is directly associated with the occurrence of the seizures caused by treatment with a high dose of pilocarpine, as chronic treatment with a sub-convulsive dose did not increase MDA levels.

CSD is a helpful electrophysiological index for understanding the influence of the oxidation processes in the brain (Guedes et al., 2012). Evidence suggests that there appears to be a bidirectional relationship between CSD and redox imbalance in the brain: on one hand, CSD induces oxidative stress (Shatillo et al., 2013; Viggiano et al., 2011), and on the other hand the accumulation of reactive oxygen species in the nervous system can trigger CSD (El-Bachá et al., 1998; Malkov et al., 2014; Netto and Martins-Ferreira, 1989). Our group has demonstrated the antagonistic role of several antioxidants on CSD propagation (Abadie-Guedes et al., 2016, 2012; Lopes-De-Morais et al., 2014). In addition to the redox imbalance hypothesis as an explanation of the effects of pilocarpine on CSD, another possibility would be based on an indirect action of pilocarpine on non-cholinergic neurotransmitters (Freitas et al., 2006; 2005), which has to be further explored.

Depending on the dose, administration of AA can exert a neuroprotective effect (González-Ramírez et al., 2010; Tutkun et al., 2015; Yildirim et al., 2010), or might have a proconvulsing action (Schneider Oliveira et al., 2004) on animal models of epilepsy. Our data, resulting from chronic systemic application of AA (120 mg/kg/d), confirmed previous observations that showed an acceleration of CSD propagation and increased MDA levels in developing rats, indicating a pro-oxidant effect of the vitamin (Mendes-da-Silva et al., 2014). Therefore, we suggest that the brain levels of MDA are directly correlated with the CSD velocity of propagation. Compared with the slower CSD velocity of the pilocarpine group, the co-administration of AA plus pilocarpine modified the CSD velocity towards the control levels in both nutritional conditions. However, the CSD velocity of the Pilo+AA groups still

remains slower when compared to the corresponding naïve and vehicle groups, suggesting that the dose of ascorbic acid was not able to completely reverse pilocarpine effects.

The mechanisms underlying interaction between pilocarpine and AA are not completely understood. It is thought that AA modulates brain excitability by alterations on the glutamate system (Kiyatkin and Rebec, 1998), whereas pilocarpine would act via muscarinic receptors. This would promote an imbalance between excitatory and inhibitory transmission, resulting in the generation of *status epilepticus* (Priel and Albuquerque, 2002). It is postulated that, while seizure initiation by pilocarpine is driven by the cholinergic system, the maintenance of the seizures seems be related to a rise in the hippocampus glutamate levels and the activation of NMDA receptors (Curia et al., 2008).

In addition to quantifying the CSD velocity of propagation, we evaluated the ECoG potentiation produced in the cortical tissue by CSD. Our data confirmed the potentiation of the ECoG amplitudes in all groups after the induction of CSD, as previously reported by our group (Lopes-De-Morais et al., 2014; Souza et al., 2015, 2011). No intergroup difference was observed, although the well-nourished pilocarpine group demonstrated a non-significant trend toward a smaller ECoG potentiation in comparison to respective naïve and vehicle groups. This is in line with previous study that demonstrated that 45, 95 and 190 mg/kg pilocarpine acute single injections were followed by reduction of ECoG amplitude (Guedes and Vasconcelos, 2008).

Malnutrition can alter the biochemical and morphological organization of the brain (Morgane et al., 2002, 1993) and promote brain excitability modulation (Guedes, 2011). The body and cerebral cortex weight reductions observed in the malnourished groups of the present study demonstrate that the regional basic diet was effective in inducing malnutrition. Our results confirm that malnutrition facilitates CSD propagation, as previously reported (Francisco and Guedes, 2015; Guedes et al., 1987; Rocha-de-Melo and Guedes, 1997). Additionally, early malnutrition enhanced CSD amplitude, except in the AA group. Although malnutrition influences the susceptibility to seizures, no interaction effect between pilocarpine and malnutrition was observed.

Based on the present data, four main conclusions can be drawn. First, chronic treatment with a sub-convulsive dose of pilocarpine antagonizes CSD propagation but does not change MDA levels in the cerebral cortex. Second, 120 mg/kg/d of AA increases CSD velocity and cortical MDA levels, supporting previous findings (Mendes-da-Silva et al., 2014) and suggesting that the brain levels of MDA are directly correlated with the CSD velocity of propagation. Third, AA co-administration modulates the effect of pilocarpine on CSD

velocity, but does not completely revert it. Fourth, malnutrition accelerates CSD and does not exert an additive effect on pilocarpine. To our knowledge, this is the first study that describes CSD and the lipid peroxidation effects of a sub-convulsive dose of pilocarpine associated with ascorbic acid. The present findings might help further the understanding of the relationship between the pilocarpine epilepsy model, antioxidants, CSD and malnutrition.

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6 CONCLUSÃO

Podemos concluir que:

- A interação da pilocarpina com ácido ascórbico exerceu efeito ansiogênico e prejudicou a memória;
- O tratamento crônico com dose subconvulsivante de pilocarpina inibiu a propagação da DAC, mas não alterou os níveis de MDA;
- O tratamento com 120 mg/kg/dia de AA aumentou a velocidade de propagação da DAC e os níveis de MDA, corroborando estudo anterior;
- A desnutrição aumentou a velocidade de propagação da DAC mas não interagiu com a pilocarpina e ácido ascórbico.
- Nos testes comportamentais, a desnutrição intensificou os efeitos da pilocarpina e ácido ascórbico.

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APÊNDICE A – PROOXIDANT *VERSUS* ANTIOXIDANT BRAIN ACTION OF ASCORBIC ACID IN WELLNOURISHED AND MALNOURISHED RATS AS A FUNCTION OF DOSE: A CORTICAL SPREADING DEPRESSION AND MALONDIALDEHYDE ANALYSIS

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Prooxidant *versus* antioxidant brain action of ascorbic acid in well-nourished and malnourished rats as a function of dose: A cortical spreading depression and malondialdehyde analysis

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ABSTRACT

Although ascorbic acid (AA) is an antioxidant, under certain conditions it can facilitate oxidation, which may underlie the opposite actions of AA on brain excitability in distinct seizure models. Here, we investigated whether chronic AA administration during brain development alters cortical excitability as a function of AA dose, as indexed by cortical spreading depression (CSD) and by the levels of lipid peroxidation-induced malondialdehyde. Well-nourished and early-malnourished rats received per gavage 30, 60, or 120 mg/kg/d of AA, saline, or no gavage treatment (naïve group) at postnatal days 7–28. CSD propagation and malondialdehyde levels were analyzed at 30–40 days. Confirming previous observations, CSD velocities were significantly higher in the early-malnourished groups than in the well-nourished groups. AA dose was important: 30 mg/kg/d AA decelerated CSD and reduced malondialdehyde levels, whereas 60 mg/kg/d and 120 mg/kg/d accelerated CSD and augmented malondialdehyde levels compared with the corresponding saline and naïve groups. Our findings reinforce previous suggestion that AA acts as an antioxidant in the brain when administered at low doses, but as a prooxidant at high doses, as indicated by CSD propagation and malondialdehyde levels.

1. Introduction

The mechanisms of excitability-related brain disturbances include active free radicals of oxygen (Braugher and Hall, 1989; Mori et al., 1990), which is certainly the basis for the growing use of antioxidants to protect the brain against excitability-induced damage (Murashima et al., 1998; Simeone et al., 2014). Ascorbic acid (AA) is one of the most studied antioxidants; it is highly concentrated in the adrenal gland and central nervous system (Miura et al., 2009), with postulated important action during brain development (Zalani et al., 1989). The physiological actions of AA include protection against oxidative stress, with possible protective actions in neurodegenerative diseases (Halliwell, 2006; Padayatty et al., 2003).

Regarding excitability-dependent brain phenomena, experimental evidence indicates that AA can exert biphasic modulating action (Oliveira et al., 2004). For example, the administration of 60 mg/kg/d of AA to rats increased the oxidative stress in the brain induced by bisphenol A, nonylphenol, and octylphenol (Aydogan et al., 2008), suggesting a prooxidant action of AA. Previously, our group demonstrated a similar prooxidant effect in weaned rats treated with 60 mg/kg/d of AA for three weeks (from postnatal days 7–28); compared with saline-treated controls, AA-exposed animals displayed significantly higher-velocity propagation of the brain excitability-related phenomenon known as cortical spreading depression (CSD) (Monte-Guedes et al., 2011).

CSD was first described as a reduction in the spontaneous electrical activity of the cerebral cortex in response to the mechanical, electrical, or chemical stimulation of one point on the cortical surface (Leão, 1944). The phenomenon is characterized by neuronal depolarization (Dreier, 2011), and has been electrophysiologically demonstrated in the brains of many vertebrate species

Abbreviations: AA, ascorbic acid; CSD, cortical spreading depression; sal, saline; DC, direct current; MDA, malondialdehyde; BCA, bicinchoninic acid.

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(Bureš et al., 1974) as well as in the human brain (Fabricius et al., 2008; Gorji and Speckmann, 2004). CSD is modulated by changes in brain excitability (Koroleva and Bures, 1980), and is influenced by the production of reactive oxygen species in brain tissue (El-Bachá et al., 1998). Related to neuronal excitability, CSD has been associated with relevant neurological diseases such as migraine (Bhaskar et al., 2013; Rogawski, 2008), brain ischemia (Busija et al., 2008), and epilepsy (Costa-Cruz et al., 2006; Guedes and Cavalheiro, 1997). Different CSD velocities of propagation along the cortical tissue, which indicate different brain susceptibilities to CSD, are associated with the manipulation of pharmacological, environmental, and nutritional conditions (see Guedes, 2011 for a review).

Malnutrition can disrupt the electrophysiological organization of the nervous system both in laboratory animals (Chen et al., 1997; Morgane et al., 1978) and in humans (Grantham-McGregor, 1995; Levitsky and Strupp, 1995). We previously demonstrated that early malnutrition increases CSD propagation (Rocha-de-Melo et al., 2006), but no information is available regarding the interaction between malnutrition and AA effects on CSD.

In the present work, we addressed two questions via electrophysiological recording of CSD in the brains of weaned young rats subjected to malnutrition during lactation. First, how does the administration of different doses of AA during brain development affect CSD propagation? Second, how is this effect influenced by malnutrition early in life? We demonstrated CSD deceleration and acceleration following the administration of low and high doses of AA, respectively.

2. Material and methods

2.1. Animals

Wistar rat pups ($n = 96$) born from distinct dams were randomly distributed at birth to form litters with eight pups per nurse and assigned to two nutritional groups according to the mother's dietary conditions. The well-nourished group ($n = 53$) was suckled by dams fed a commercial laboratory chow diet (Purina do Brazil LTDA) containing 23% protein. The malnourished group ($n = 43$) was suckled by dams fed a regional basic diet containing 8% protein. This diet mimics the diet of low-income human populations of Northeastern Brazil (Teodósio et al., 1990). After weaning at post-natal day 21, the pups were fed the chow diet.

Both nutritional groups were subdivided into five groups denominated according with the treatment they received, from postnatal days 7–28, per gavage: the saline group (Sal; $n = 9$ Well-nourished rats and 9 Malnourished rats) received saline via gavage. For the AA-treated groups, rats received 30 mg/kg/d (AA-30; $n = 15$ Well-nourished rats and 9 Malnourished rats), 60 mg/kg/d (AA-60; $n = 6$ Well-nourished rats and 6 Malnourished rats), or 120 mg/kg/d (AA-120; $n = 12$ Well-nourished rats and 9 Malnourished rats). AA was purchased from Sigma, St. Louis, MO, USA. A fifth "naïve" group ($n = 11$ Well-nourished rats and 10 Malnourished rats) received no gavage.

Animals were reared 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 AM) with free access to food and water. They were weighed at postnatal days 7, 14, 21, and 28. All experiments were carried out at the Universidade Federal de Pernambuco (Brazil) in accordance with the guidelines of the Institutional Ethics Committee for Animal Research (approval protocol no. 23076.013959/2012-79), which comply with the "Principles of Laboratory Animal Care" from the National Institutes of Health of the United States. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

2.2. Recording of CSD

At 30-40 postnatal days, the animals were subjected to a 4-h CSD recording session, as previously described (Lima et al., 2013). Briefly, under anesthesia (1 g/kg urethane plus 40 mg/kg chloralose, i.p.; both purchased from Sigma, St. Louis, Mo, USA) three trephine holes (2–3 mm in diameter) were made on the right side of the skull, parallel to the midline. The first hole (on the frontal bone) was used to apply the stimulus to elicit CSD. The other two holes (on the parietal bone) were used to record the propagating CSD wave. Rectal temperature was continuously monitored and maintained at 37 ± 1 °C by a heating blanket. During the recording session, CSD was elicited at 20-min intervals by applying, for 1 min, a cotton ball (1–2 mm diameter) soaked in 2% KCl solution (approximately 0.27 M) to the anterior hole drilled at the frontal region. The direct current (DC)-potential change typical of CSD was recorded at the two parietal points on the cortical surface with a pair of Ag–AgCl agar-Ringer electrodes that were connected to a digital data acquisition system (EMG Systems, São Paulo, Brazil). A common reference electrode of the same type was placed on the nasal bones. For all CSD episodes, we calculated the amplitude and duration of the negative slow potential shifts of the CSD waves, as previously reported (Lima et al., 2013). The velocity of CSD propagation was calculated based on the time required for a CSD wave to cross the distance between the two recording electrodes. In the measurement of CSD velocities, the initial point of each DC negative rising phase was used as the reference point.

In each CSD recording session, three or four animals per group were recorded simultaneously. After finishing the recording session, the still-anesthetized animals were decapitated. The brains were removed rapidly and carefully, rinsed in ice-cold saline, and dried on filter paper. The pooled cortical tissue of 3–4 animals was homogenized in a 0.9% (w/v) NaCl solution (1:10) at 4 °C, centrifuged for 10 min at 1000 × g at 4 °C, and the supernatants were stored at −80 °C for the analysis of lipid peroxidation, as recently reported in another publication from our group (Cardoso et al., 2012).

2.3. Analysis of lipid peroxidation

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) levels using a thiobarbituric acid-reactive substances-based method (Ohkawa et al., 1979). The MDA levels were determined in 36 of the well-nourished rats (Sal, $n = 7$; naïve, $n = 6$; AA-30, $n = 10$; AA-60, $n = 5$; AA-120, $n = 8$) and 39 of the malnourished rats (Sal, $n = 8$; naïve, $n = 11$; AA-30, $n = 8$; AA-60, $n = 6$; AA-120, $n = 6$). The reaction was developed by sequential addition of 40 μL of 8.1% sodium dodecyl sulfate, 300 μL of 20% acetic acid (pH 3.5), and 300 μL of 0.8% thiobarbituric acid solutions to the 100-μL homogenate aliquot in a boiling water bath for 30 min. Experiments were carried out in triplicate. After cooling the tubes with tap water, 300 μL of n-butanol were added to the sample. The tubes were centrifuged at 2500 × g for 10 min, and the organic phase was read at 532 nm using a plate reader. Total protein concentrations were determined with the BCA Protein Assay Kit (Pierce, Rockford, IL, USA).

2.4. Statistical analysis

Intergroup differences were compared using two-way analysis of variance (ANOVA) including nutritional status (well-nourished and malnourished) and treatment (saline, naïve, AA-30, AA-60, and AA-120) as factors. ANOVA was followed by a Holm–Sidak post-hoc test when indicated. Differences were considered statistically significant when $p \leq 0.05$.

3. Results

3.1. Body weight

The body weights of the five well-nourished groups and five malnourished groups are presented in Fig. 1. During days 7–28, all malnourished groups had lower body weights than the corresponding well-nourished groups ($p < 0.05$). No weight difference associated with AA treatment was observed.

3.2. CSD recording

Fig. 2 contains typical electrophysiological CSD recordings (slow DC potential change) of five well-nourished and five malnourished rats that were representative of the five treatment groups. Under normal conditions (Sal and naïve groups in Fig. 2), 1-min stimulation with 2% KCl at one point of the right frontal cortex elicited a single CSD wave that propagated without interruption and was recorded by the two electrodes located more posterior on the surface of the parietal cortex (Fig. 2). Recording of the slow potential change confirmed the presence of CSD after KCl application. The electroencephalographic changes caused by CSD always recovered after a few minutes (Fig. 2), and we maintained a 20-min interval between subsequent KCl stimulations. In the majority of AA-120 animals (58.3% and 90% of the well-nourished and malnourished rats, respectively), a single KCl stimulation elicited two CSD episodes (Fig. 2), which may be indicative of increased cortical sensitivity due to the AA-120 treatment.

3.3. CSD parameters

ANOVA showed a significant main effect of nutritional status on the CSD velocity of propagation ($F[1,94] = 89.46$; $p < 0.001$). Post-

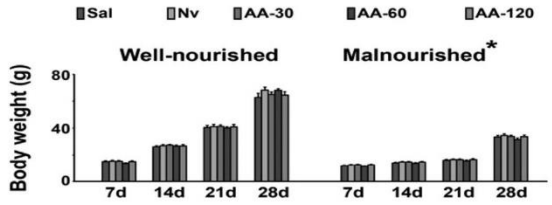

Fig. 1. Body weights of well-nourished and malnourished rats on postnatal days 7, 14, 21, and 28. Mean ± standard error of the mean is presented. Animals were subdivided into five groups, as defined in the main text: naïve, saline (Sal), 30 mg/kg/d AA (AA-30), 60 mg/kg/d AA (AA-60), and 120 mg/kg/d AA (AA-120). The asterisk indicates that all malnourished groups differed significantly from the corresponding well-nourished groups ($p < 0.05$; ANOVA plus the Holm–Sidak test). AA treatment did not influence body weight.

hoc comparisons showed that the velocities were higher in the malnourished naïve, saline, AA-30, and AA-60 animals compared with the corresponding well-nourished animals ($p < 0.001$). Well-nourished and malnourished rats treated with the highest AA dose (120 mg/kg/d) presented with the highest CSD velocities (5.26 ± 0.46 and 5.15 ± 0.33 mm/min, respectively). In both well-nourished and malnourished condition, the lower AA dose (30 mg/kg/d) decelerated CSD compared with the corresponding saline and naïve groups, whereas the higher doses (60 mg/kg/d and 120 mg/kg/d) accelerated CSD (Fig. 3).

The amplitude and duration of the negative slow potential shift, which is the hallmark of CSD, are shown in Table 1. The mean amplitude varied from 9.5 ± 4.6 mV to 12.7 ± 3.4 mV in the well-nourished groups, and from 13.9 ± 2.5 mV to 15.6 ± 4.2 mV in the malnourished groups (Table 1). ANOVA revealed a significant main effect of nutritional status on the amplitude (F [1,95] = 17.79; $p < 0.001$). Post-hoc comparisons showed that the amplitudes were higher in the malnourished naïve and AA-30 animals compared with the corresponding well-nourished rats ($p < 0.001$).

The mean duration of the negative slow potential shift ranged from 81.5 ± 8.0 s to 84.3 ± 8.9 s in the well-nourished groups, and from 73.7 ± 3.7 s to 82.5 ± 7.4 s in the malnourished groups (Table 1). ANOVA revealed a significant main effect of nutritional status on the duration (F [1,95] = 9.83; $p < 0.001$). Post-hoc comparisons showed that the duration was shorter in the malnourished naïve and AA-120 rats compared with the corresponding well-nourished animals ($p < 0.001$).

3.4. Cortical levels of MDA

Table 2 contains measurements of the MDA levels in the cerebral cortices of well-nourished and malnourished animals. ANOVA revealed a significant main effect of treatment on the MDA levels (F [4,85] = 12.53; $p < 0.001$). Post-hoc comparisons showed that in the well-nourished condition the MDA levels were lower in the AA-30 rats compared with the naïve, AA-60 and AA-120 animals ($p < 0.001$). In the malnourished condition, the MDA levels were lower in the AA-30 animals compared with the naïve, saline and AA-60 rats ($p < 0.001$).

4. Discussion

The present results extend our first observations of the CSD-related effects of chronic systemic application of 60 mg/kg/d of AA on the developing rat brain (Monte-Guedes et al., 2011). Previously, we showed that AA accelerated CSD at that dose, which is compatible with a prooxidant effect; other studies also postulated a prooxidant effect at the same AA dose (Aydogan et al., 2008). In the present investigation, we used electrophysiology in rats to identify

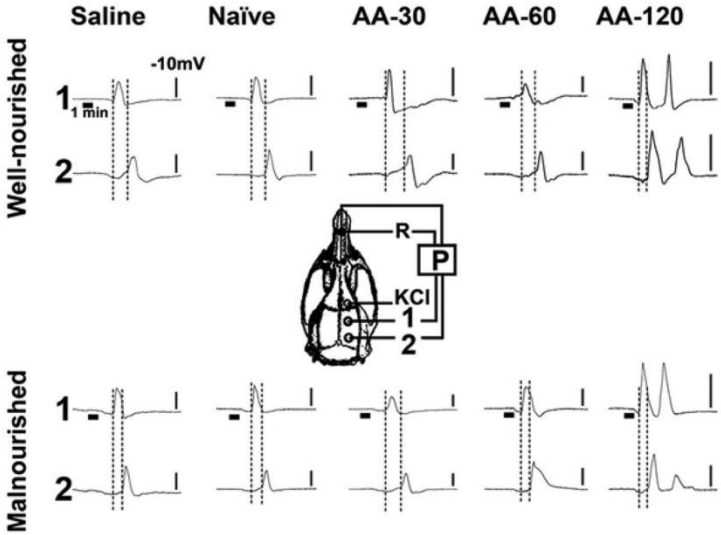

Fig. 2. Electrophysiological recordings (slow potential changes, P) of CSD in five well-nourished and five malnourished rats representative of the five treatment groups defined in the main text and in Fig. 1. The vertical solid bars at the right of the traces indicate 10 mV (negative upwards). The horizontal bars under the traces from the recording point 1 indicate the time (1 min) of stimulation with 2% KCl to elicit CSD. Once elicited in the frontal cortex, CSD was recorded by the two cortical electrodes located at the parietal cortex (central skull diagram, points 1 and 2). A third electrode of the same type was placed on the nasal bones and served as a common reference (R) for the recording electrodes. The vertical dashed lines indicate the latency of a CSD wave crossing the interelectrode distance. This latency was shorter in the malnourished groups than in the well-nourished groups, except for the AA-120 condition. When compared with the control groups in the same nutritional condition, latencies in the AA groups were longer for the lowest AA dose (AA-30) and shorter for the other two AA doses (AA-60>AA-120).

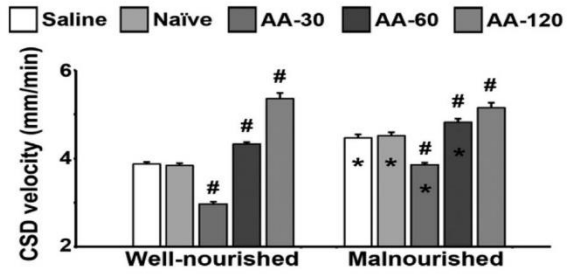

Fig. 3. CSD velocity of propagation in 30–40-day-old rats previously suckled by well-nourished or malnourished dams. Treatments are as defined in Fig. 1 and the main text. Values are presented as mean ± standard error of the mean. *$p < 0.05$ compared with the corresponding well-nourished values. #$p < 0.05$ compared with the control groups (saline and naïve) under the same nutritional conditions (ANOVA plus the Holm–Sidak test).

both antioxidant (CSD deceleration) and prooxidant (CSD acceleration) actions following low (30 mg/kg/d) and high (120 mg/k/d) doses of AA, respectively (Fig. 3). Because we have treated developing animals, we believe that early and chronic AA administration may cause developmental changes in the brain that are associated with the modulation of CSD propagation. Such an effect cannot be attributed to the stress associated with the gavage procedure, because the saline-treated groups were equally submitted to gavage and presented with CSD features similar to those of the naïve controls (Fig. 3).

Although rats can synthesize their own ascorbic acid, behavioral, pharmacological and electrophysiological evidence is available indicating modulation of neuronal function by exogenous AA in rats (see Harrison and May (2009), for a review). In the present study the daily AA treatment was long-lasting (22 days) and AA has been administered during a period of intense neural development. It is tempting to speculate that under such conditions the low and high doses of AA may act respectively as antioxidant and prooxidant in the brain, as suggested by the correspondingly lower and higher CSD velocities of propagation.

Although the mechanisms by which AA modulates CSD propagation are not yet known, we can speculate based on CSD studies of mechanisms that are proposed to be implicated in the neural actions of AA. As a strong water-soluble reducing agent, AA normally acts as an antioxidant (Machlin and Bendich, 1987) that effectively scavenges free radicals, including reactive oxygen species (Retsky et al., 1993), and protects the brain against oxidative stress, particularly in neurodegenerative diseases (Ballaz et al., 2013; Rebec et al., 2003). Increasing the production of reactive oxygen species can result in the elicitation of CSD in vitro (Netto and Martins-Ferreira, 1989) and in vivo (El-Bachá et al., 1998), and antioxidants such as the carotenoid astaxanthin impair the chronic ethanol facilitating effect on CSD propagation that is thought to be due to the formation of reactive oxygen species (Abadie-Guedes et al., 2008). In contrast to this evidence, our previous study revealed a facilitating effect of 60 mg/kg/d AA on CSD propagation, and we postulated that such action would depend on the dose of AA employed. Under certain conditions, AA has been suggested to exert biphasic modulating action on excitability-dependent brain phenomena (Aydogan et al., 2008; Oliveira et al., 2004). The present data support our hypothesis, as a lower dose of AA (30 mg/kg/d) impaired CSD propagation, whereas 120 mg/kg/d, a dose higher than that used in previous studies, accelerated CSD (Fig. 3). Interestingly, there is in vitro (Hisanaga et al., 1992; Song et al., 2001) as well as in vivo (Aydogan et al., 2008) evidence suggesting that AA can exert prooxidant action, leading to neurotoxicity. Furthermore, AA can act as both an anticonvulsant and a proconvulsant in the rat brain (Ayyildiz et al., 2007; Oliveira et al., 2004), and in the presence of transition-metal ions AA can produce reactive radical species in the brain (Halliwell, 1992).

MDA is the final product of lipid peroxidation, generating free radicals and increasing oxidative stress, which can be counteracted by antioxidant molecules (Mehla et al., 2010). In this study, MDA levels were measured via the thiobarbituric acid reactive substances-based method; opposite changes in MDA levels were observed at lower (30 mg/kg/d) and higher (120 mg/kg/d) AA doses compared with the levels in the controls (Table 2). Increased MDA levels in the well-nourished AA-120 group suggest that this dose may have raised the oxidative stress, while the well-nourished AA-30 treatment reduced it, as reflected by decreased MDA levels. These findings are in agreement with the dichotomous CSD results for the AA-30 (CSD deceleration) and AA-120 groups (CSD acceleration), suggesting antioxidant and prooxidant action of AA, respectively. It is important to emphasize that a post-CSD increase in the activity of the enzyme superoxide dismutase, as well as CSD modulation by nitric oxide, has been observed in the rat cortex (Petzold et al., 2008; Viggiano et al., 2011). Collectively, these data support the view of CSD as a valuable, interesting, and very reproducible experimental

Table 2
MDA levels (nmol/mg) in the cerebral cortex of 30–40-day-old well-nourished and malnourished rats previously treated (per gavage) from postnatal day 7–28 with 30 mg/kg/d or 60 mg/kg/d, or 120 mg/kg/d ascorbic acid (AA). Data are expressed as mean ± standard deviation. The groups (left column) are marked with the upper-case letters A to E. In the well-nourished (middle column) and malnourished condition (right column), the values marked with lower case letters differ significantly ($P < 0.05$) from the corresponding values (in the same column) of the groups marked with the same upper case letters in the left column (ANOVA plus Holm–Sidak test). ANOVA indicated that there is not a statistically significant interaction between the nutritional status and the treatments ($F[4,85] = 1.028$; $P = 0.399$).

Treatment groups	Well-nourished	Malnourished
Saline^{A}	2.13 ± 0.30 ($n = 7$)	2.26 ± 0.58 ($n = 8$)
Naïve^{B}	2.36 ± 0.54 ($n = 6$)	2.25 ± 0.38 ($n = 11$)
AA-30^{C}	1.62 ± 0.31^{bde} ($n = 10$)	1.56 ± 0.37 ^{abd} ($n = 8$)
AA-60^{D}	2.86 ± 0.39 ($n = 5$)	2.45 ± 0.69 ($n = 6$)
AA-120^{E}	2.83 ± 0.69^{a} ($n = 8$)	2.32 ± 0.30 ($n = 6$)

Table 1
Amplitude and duration of the negative slow potential shifts of CSD in well-nourished and malnourished rats treated per gavage with three doses of AA. The AA groups were compared to the control groups treated with saline or no treatment (naïve). Data are expressed as mean ± standard deviation.

Treatment groups	CSD amplitude (mV)		CSD duration (s)	
	Well-nourished	Malnourished	Well-nourished	Malnourished
Saline	12.0 ± 4.7 ($n = 9$)	14.6 ± 4.2 ($n = 9$)	84.3 ± 8.9 ($n = 9$)	77.6 ± 5.1 ($n = 9$)
Naïve	10.7 ± 2.6 ($n = 11$)	15.5 ± 2.9^{a} ($n = 10$)	83.7 ± 8.6 ($n = 11$)	75.9 ± 6.2^{a} ($n = 10$)
AA-30	9.5 ± 4.6 ($n = 15$)	15.6 ± 4.2^{a} ($n = 9$)	82.9 ± 8.5 ($n = 15$)	81.6 ± 5.1 ($n = 9$)
AA-60	12.7 ± 3.4 ($n = 6$)	13.9 ± 2.5 ($n = 6$)	83.0 ± 7.8 ($n = 6$)	82.5 ± 7.4 ($n = 6$)
AA-120	11.9 ± 3.7 ($n = 12$)	14.3 ± 3.6 ($n = 9$)	81.5 ± 8.0 ($n = 12$)	73.7 ± 3.7^{a} ($n = 9$)

[a] $p < 0.05$ compared with the corresponding well-nourished group (ANOVA plus the Holm–Sidak test).

phenomenon that can expand our comprehension of the causal relationship between oxidative stress and the generation of some neurological diseases (Guedes et al., 2012).

We have to consider that the brain effects of AA are complex and, as such, it is possible that other mechanisms, not related to its antioxidant ability, may be required to explain its neuroprotective actions. This includes the AA action as neuromodulator acting on the dopaminergic and glutamatergic neurotransmission (Harrison and May, 2009). Perhaps the modulation of CSD propagation involves the action of AA at the glutamate synapse (Smythies, 1999). The balance between AA and glutamate can modulate neuronal vulnerability to degeneration (Ballaz et al., 2013). Glutamatergic stimulation can result in the accumulation of reactive oxygen species and the subsequent release of nitric oxide (Kostandy, 2012). Therefore, nitric oxide excitability-related neuropathological phenomena (Bell et al., 1996; Yildirim et al., 2010) may contribute to the effects of AA on the brain.

All of the above evidence notwithstanding, the role of AA in redox mechanisms at the brain nitrergic and glutamate synapses should be addressed in future investigations. Interestingly, our group has recently demonstrated that administering monosodium glutamate accelerates CSD in a dose-dependent fashion and induces significant microglial reaction in the rat cerebral cortex (Lima et al., 2013).

Early malnutrition impairs brain developmental processes such as synaptogenesis, dendritic development, and myelination (Morgane et al., 1978; Picanço-Diniz et al., 1998). These processes may be involved in facilitating CSD effects, as previously reported in malnourished rats (Rocha-de-Melo and Guedes, 1997) and confirmed in the present investigation. The body-weight reductions observed in the malnourished groups of the present study (Fig. 1) unequivocally demonstrate the effectiveness of the regional basic diet in inducing malnutrition. Deficiencies in the protein content of the maternal diet certainly are crucial in determining the CSD effect in offspring (Guedes, 2011).

The mechanism by which early malnutrition enhances CSD propagation has been the subject of much discussion. Malnutrition reportedly reduces the uptake of extracellular glutamate (Feoli et al., 2006), which may facilitate CSD propagation (Tottene et al., 2009). Malnutrition-induced increases in cell packing density are accompanied by reductions in the volume of the extracellular space, which are thought to increase the CSD propagation rate (Guedes, 2011). Two other conditions that accelerate CSD are present in the early malnourished brain: impairment of glial function (Largo et al., 1997) and hypomyelination (Merkler et al., 2009). Merkler et al. demonstrated an inverse correlation between the degree of cortical myelination and the CSD propagation velocity, using animal models of cuprizone-induced demyelination and genetically modified hypermyelinated animals; dichotomous modulation of CSD propagation based on myelin content was revealed in these studies. In this context, it is interesting that adult rats previously suckled under favorable conditions (reduced litter size, with only three pups) displayed lower CSD propagation velocities than normally suckled controls (Rocha-de-Melo et al., 2006), suggesting links among early nutritional status, myelination, and CSD propagation.

In conclusion, our findings demonstrate that a low dose (30 mg/kg/d) of AA decelerates CSD propagation and decreases MDA levels in the cortex. At higher doses (60 mg/kg/d and 120 mg/kg/d), AA augments CSD velocity and increases cortical MDA levels. These differential, dose-dependent effects are not abolished by malnutrition. We suggest that AA acts as an antioxidant when administered at low doses but as a prooxidant when given at high doses. It is uncertain whether this novel AA effect can be extrapolated from the rat to the human developing brain.

Acknowledgments

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Your co-authored submission

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Para:rosangelafmendes@hotmail.com <rosangelafmendes@hotmail.com>;

Dear Dr. Mendes-da-Silva,

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Journal: Epilepsy & Behavior

Title: The effect of pilocarpine/ascorbic acid interaction on behavioral impairment in well-nourished and malnourished immature rats

Corresponding Author: Rubem Carlos Araújo Guedes

Co-Authors: Rosângela Mendes-da-Silva, Mayara Silva, Glizela Alves

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ANEXO C – PARECER APROBATÓRIO DA COMISSÃO DE ÉTICA

Universidade Federal de Pernambuco
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Recife, 05 de maio de 2014.

Ofício nº 15/14

Da Comissão de Ética no Uso de Animais (CEUA) da UFPE
Para: **Prof. Rubem Carlos Araújo Guedes**
Departamento de Nutrição – CCS
Universidade Federal de Pernambuco
Processo nº 23076.012755/2014-82

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, **"Vitamina C como moduladora da ação neurotóxica da pilocarpina: análise eletrofisiológica e imuno-histoquímica em ratos jovens em distintos estados nutricionais".**

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: Biotério do Departamento de Nutrição da UFPE; Animais: ratos; Linhagem: Wistar; Peso: 2-5 g (nascimento) até 200g (50 dias); Idade: 1-50 dias; Sexo: machos; Número total de animais previsto no protocolo: 80.

Atenciosamente,

Profª Marcia Vasconcelos
Vice-Presidente do CEUA/CCB-UFPE
SIAPE 2199635

CCB: Integrar para desenvolver