BASES MOLECULARES ENVOLVIDAS NA NEUROPROTEÇÃO CAUSADA PELA EXPOSIÇÃO A UM AMBIENTE ENRIQUECIDO EM MODELO ANIMAL DE ISQUEMIA CEREBRAL

Lara Vezula Gonçalves

Dissertação de Mestrado em Bioquímica e Farmacologia
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Orientador: Prof.ª Dr ª.Cristina Martins e Silva

Co-orientador: Prof.ª Dr ª. Rita Gomes Wanderley Pires

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RESUMO

O acidente vascular encefálico (AVE) é considerado uma das principais causas de morbidade mundial. Essa condição patológica pode resultar em alterações motoras, cognitivas e sensoriais. Atualmente, não há tratamentos eficazes para o comprometimento cognitivo pós-AVE. Dentre os tratamentos disponíveis, o uso de terapias não farmacológicas vem se mostrando eficiente em prevenir e/ou tratar doenças do sistema nervoso central. Uma destas terapias envolve o ambiente enriquecido (AE), um conjunto de estímulos externos que auxilia na recuperação do tecido cerebral após um insulto. Adicionalmente, o AE também pode ser utilizado como um método de pré-condicionamento com o objetivo de induzir tolerância cerebral a um evento isquêmico. Já é sabido que animais pré-condicionados a um AE apresentam tolerância quando submetidos à isquemia com consequente melhora na função motora e em processos mnemômicos, como aprendizagem e memória. Entretanto, os mecanismos moleculares envolvidos nessa tolerância ainda não estão totalmente esclarecidos. De todo exposto, torna-se relevante à identificação dos mecanismos de neuroproteção provocada pelo pré-condicionamento ao AE em modelo murino de AVE. No presente estudo, camundongos C57Bl/6 recém-desmamados foram mantidos por cinco semanas em AE ou ambiente padrão (AP), e posteriormente divididos nos grupos sham ambiente padrão (SS, n=30), sham ambiente enriquecido (ES, n=18), isquemia ambiente padrão (SI, n=39) e iquemia ambiente enriquecido (EI, n=21). Os animais do grupo isquemia foram submetidos à oclusão bilateral das artérias carótidas comum por 30 minutos. Paralelamente, os animais do grupo sham passaram por procedimento cirúrgico semelhante, porém, sem a oclusão das artérias. A área de infarto foi verificada pela coloração ao cloreto de 2,3,5-trifeniltetrazólio. Parâmetros cognitivos foram avaliados por meio dos testes de reconhecimento de objetos e labirinto em Y. A expressão relativa dos genes das subunidades de receptores glutamatérgicos do tipo NMDA (GluN1, GluN2A, GluN2B e GluN2C), dos receptores colinérgicos muscarínico M1 e ionotrópico alfa 7, do marcador de ativação astrocitária GFAP, marcador inflamatório IL-1β e da neurotrofina BDNF foram avaliadas no hipocampo. Adicionalmente, foram analisados os níveis teciduais do neurotransmissor glutamato na região acima mencionada. O paradigma de AE utilizado impediu o déficit de memória de curto

prazo causado pela isquemia, reduzindo significativamente o volume do infarto. Nosso estudo sugere fortemente que o aumento da expressão das subunidades dos receptores glutamatérgicos GluN1, GluN2A e GluN2C e a redução da expressão da citocina inflamatória IL1-β e aumento da expressão de GFAP em animais isquêmicos pode ter contribuído para a melhora cognitiva induzida pelo AE.

Palavras-chave: enriquecimento ambiental, isquemia cerebral, neuroproteção, aprendizagem e memória.

ABSTRACT

Stroke is considered a major cause of global morbidity. This pathological condition can result in motor, cognitive and sensory alterations. Currently, there are no effective treatments for post-stroke cognitive impairment. Among the available treatments, the use of non-pharmacological therapies has shown to be efficient in preventing and/or treating diseases of the central nervous system. One of these therapies involves the enriched environment (EE) which is a set of external stimuli that aid in the recovery of brain tissue after insult. In addition, EE may also be used as a preconditioning method to induce cerebral tolerance in an ischemic event. Animals pre-conditioned to an EE presented tolerance when submitted to ischemia with consequent improvement in motor function and in mnemonic processes, such as learning and memory. However, the molecular mechanisms involved in this tolerance are not yet clear. Therefore, it becomes relevant to the identification of the mechanisms of neuroprotection provoked by the preconditioning to the EE in murine model of AVE. In the present study, freshly weaned C57Bl/6 mice were kept for five weeks in EE or standard environment (SC). After that period they were divided into sham/standard environment (SS, n = 30), sham/enriched environment (ES, n = 18), ischemic/standard environment (SI, n = 39) and ischemic/ enriched environment (EI, n = 21). The animals in the ischemia group underwent bilateral occlusion of the common carotid arteries for 30 minutes. At the same time, the animals of the sham group underwent a similar surgical procedure, however, without the occlusion of the arteries. The infarct area was checked by staining the 2, 3, 5-triphenyltetrazolium chloride. Cognitive parameters were evaluated by means of object recognition (TRO) and Y-maze tasks. The relative expression of the genes of the NMDA glutamatergic receptor subunits (GluN1, GluN2A, GluN2B and GluN2C), the cholinergic receptors muscarinic M1 and ionotropic alpha 7, the astrocytic activation marker GFAP, the inflammatory marker IL-1β and the neurotrophin BDNF were evaluated in the hippocampus. In addition, the tissue levels of the neurotransmitter glutamate in the aforementioned regions were analyzed. The EE paradigm prevented short-term memory deficit caused by ischemia, significantly reducing the infarct volume. Our study strongly suggests that the increased expression of the glutamatergic receptor subunits GluN1, GluN2A and GluN2C and the reduction of the inflammatory cytokine

IL1-β expression and increased expression of GFAP in ischemic animals may be contributed to the cognitive improvement induced by the AE.

Keywords: environmental enrichment, cerebral ischemia, neuroprotection, learning and memory.

LISTA DE ABREVIATURAS

ACh	Acetilcolina
AE	Ambiente enriquecido
AHA	American Heart Association
AMPA	Ácido-amino-3-hidroxi-5-metil-isoxazol-4-propiônico
ANOVA	Análise de variância
AP	Ambiente padrão
AVE	Acidente Vascular Encefálico
BDNF	Fator neurotrófico derivado do cérebro
Ca^{2+}	Íon cálcio
DNA	Ácido desoxirribonucleico
EAAT	Transportador de aminoácido excitatório
GABA	Àcido gama-aminobutírico
GFAP	Proteína glial fibrilar ácida
Glu	Glutamato
iGluRs	Receptores glutamatérgicos ionotrópicos
IL-1β	Interleucina 1 beta
K^{+}	Íon potássio
KA	Cainato
M1	receptores colinérgicos muscarínico do tipo M1
mGluR1- 8	Subtipos 1 ao 8 de receptores glutamatérgicos metabotrópicos
mGluRs	Receptores glutamatérgicos metabotrópicos
NMDA	N-metil-D-aspartato
NMDAR	Receptor N-metil-D-aspartato
SNARE	Soluble N-ethylmaleimide-sensitive fusion protein attachment
SNC	Sistema nervoso central
TRO	Teste de reconhecimento de objetos
tSNARE	SNARE de membrana
vGLUT	Transportador vesicular de Glu

vSNARE	Vesicular soluble N-ethylmaleimide-sensitive fusion protein
WHO	World Health Organization
α7	Receptores colinérgicos nicotínico do tipo alfa 7

SUMÁRIO

1 INTRODUÇÃO

1.1 ACIDENTE VASCULAR ENCEFÁLICO

O acidente vascular encefálico (AVE) é descrito como a segunda maior causa de morte no mundo (WHO, 2016). A ocorrência em pessoas com menos de 40 anos é incomum, sendo causada principalmente por pressão arterial elevada. O AVE também ocorre em cerca de 8% de crianças com doença falciforme (MACKAY; MENSAH, 2004).

Projeções indicam que até 2030, cerca de 3,4 milhões de pessoas com idade ≥18 anos sofrerão um AVE, com um aumento de 20,5% da prevalência a partir de 2012, sendo o maior aumento (29%) previsto para homens latino-americanos (AMERICAN HEART ASSOCIATION - AHA, 2016). O AVE também está descrito entre as maiores causas de morte no Brasil (MINISTÉRIO DA SAÚDE, 2013). Apenas no período de 2007 a 2011, foram registradas cerca de 10.000 internações por AVE/ano no Sistema Único de Saúde na população de 30 a 59 anos (MINISTÉRIO DA SAÚDE, 2012).

1.1.1 Fisiopatologia do AVE

O AVE é causado pela interrupção do fornecimento de sangue para o cérebro, podendo ocasionar prejuízos ao tecido (GHANTOUS et al., 2016). O AVE é classificado em isquêmico, quando ocasionado pelo entupimento dos vasos, ou hemorrágico, quando ocorre rompimento dos mesmos (WHO, 2005). A isquemia cerebral corresponde a aproximadamente 85% dos casos de AVE, enquanto 15% dos eventos são hemorrágicos (SOCIEDADE BRASILEIRA DE DOENÇAS CEREBROVASCULARES, 2002).

Os principais fatores de risco estabelecidos para o AVE são divididos em modificáveis e não modificáveis (SIEGLER et al., 2014). A inatividade física, dislipidemia, dieta desequilibrada, hipertensão, obesidade e má distribuição de gordura corporal, diabetes mellitus, tabagismo e fibrilação atrial são considerados modificáveis, pois são fatores passiveis de intervenções que potencializam a prevenção do AVE (DI LEGGE et al., 2012). O tratamento do diabetes e hipertensão, juntamente com o controle de demais fatores de risco contribuem significativamente para a diminuição da mortalidade por AVE (GO et al., 2014). São considerados fatores de risco importantes para o AVE aspectos não modificáveis, como idade, baixo peso ao nascimento, etnia e fatores genéticos (MESCHIA et al., 2014).

Fisiologicamente, o encéfalo isquêmico pode ser separado em duas regiões: núcleo e penumbra (APPIREDDY et al., 2015). A zona de núcleo é uma área de isquemia grave com perda do fornecimento de oxigênio e glicose, que quando persistente, pode resultar em necrose do tecido cerebral. A região de penumbra é definida como o tecido que envolve o núcleo isquêmico e é onde as intervenções farmacológicas têm maior probabilidade de sucesso (FISHER; BASTAN, 2012). Nessa área, o fluxo sanguíneo é baixo a fim de manter a atividade elétrica, mas suficiente para preservar canais iônicos, permitindo viabilidade celular por um tempo limitado, pois sua irrigação é suprida pelas artérias de regiões adjacentes durante as primeiras horas após a isquemia (CASTILLO et al., 2016).

A lesão tecidual causada pela isquemia gera uma cascata de eventos celulares e moleculares provenientes da falta de suprimento sanguíneo e posterior reperfusão da região isquêmica (LIPTON, 1999). Processos fisiopatológicos como inflamação, estresse oxidativo, excitotoxicidade e apoptose podem levar à necrose dos neurônios (MOSKOWITZ; LO; IADECOLA, 2010).

Considerado uma das principais causas de morbidade mundial (GOTTESMAN; HILLIS, 2010), o AVE isquêmico pode resultar em alterações motoras, cognitivas e sensoriais (WHO, 2005). Segundo Kelly-Hayes e outros (2003), entre pacientes com idade ≥65 anos, 26% tornaram-se dependentes para realizar suas atividades diárias e 46% apresentaram déficits cognitivos seis meses após um evento isquêmico. O

indivíduo afetado pode sofrer acometimentos cognitivos como afasia (comprometimento da linguagem), negligência unilateral (dificuldade em responder a estímulos no lado contralateral ao lesionado), deficiências na memória de trabalho, atenção, aprendizagem, percepção visual ou função executiva, que inclui tomada de decisão, organização e resolução de problemas (GOTTESMAN; HILLIS, 2010). Os déficits cognitivos observados em indivíduos que sofreram um AVE podem estar relacionados a disfunções em vários sistemas neurotransmissores, entre eles, os sistemas glutamatérgico e colinérgico (KATAOKA et al., 1991; ARUNDINE; TYMIANSKI, 2004).

1.1.2 Sistemas neurotransmissores afetados no AVE

1.1.2.1 Neurotransmissão Glutamatérgica

O glutamato (Glu) é o principal neurotransmissor excitatório do sistema nervoso central (SNC) (KRNJEVIC; PHILLIS, 1963) e atua como precursor para a síntese de ácido gama-aminobutírico (GABA) (ZHAO; GAMMIE, 2014). Seus níveis extracelulares são rigorosamente regulados (PITA-ALMENAR, 2012). Funções cognitivas, como memória e aprendizagem, são influenciadas pela via glutamatérgica no hipocampo e neocórtex (TSIEN; HUERTA; TONEGAWA, 1996).

Os receptores glutamatérgicos são classificados em receptores glutamatérgicos metabotrópicos (mGluRs) e receptores glutamatérgicos ionotrópicos (iGluRs). Existem oito subtipos de mGluRs, os quais pertencem a família dos receptores acoplados a proteína G e são classificados em três grupos com base na homologia de sequências e farmacologia: Grupo I (mGlu1 e mGlu5), Grupo II (mGlu2 e mGlu3) e Grupo III (mGlu4, mGlu6, mGlu7 e mGlu8) (NISWENDER; CONN, 2010).

Os iGluRs são classificados em: receptores alfa-amino-3-hidroxi-5-metil-4-isoxazolpropiónico (AMPA), cainato (KA) e *N*-metyl-d-asparate (NMDA) (TSIEN;

HUERTA; TONEGAWA, 1996). Atualmente, sete subunidades de NMDAR (Receptor N-metil-D-aspartato) foram identificadas: GluN1, GluN2A-D e GluN3A-B.

O anabolismo do Glu no citosol do neurônio pré-sináptico se dá a partir da glutamina, quando metabolizada pela enzima mitocondrial glutaminase (THANKI et al., 1983). O Glu é então armazenado em vesículas sinápticas, de modo que seu transporte através da membrana vesicular é realizado por transportadores vesiculares de glutamato (VGLUTs) (MALET; BRUMOVSKY et al., 2015), dos quais foram identificados três tipos: VGLUT 1, 2 e 3 (LIGUZ-LECZNAR; SKANGIEL-KRAMSKA, 2007).

A liberação do Glu para a fenda sináptica ocorre em resposta a um potencial de ação que induz a despolarização da membrana dos neurônios pré-sinápticos, e consequentemente abertura de canais de Ca^{2+} dependentes de voltagem (RAMAKRISHNAN et al., 2012). O processo de exocitose do neurotransmissor depende das proteínas SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptors) (THOMPSON et al., 2010). Proteínas SNARE específicas, localizadas na membrana vesicular (vSNARE), interagem com proteínas alvo presentes na membrana plasmática dos neurônios pré-sinápticos (tSNARE), permitindo a liberação da vesícula (KRISHNAKUMAR et al., 2011).

A regulação dos níveis de Glu extracelular é feita por transportadores de aminoácidos excitatórios (EAATs), que recaptam o Glu da fenda sináptica e impedem a ocorrência de danos neuronais por ativação excessiva dos receptores glutamatérgicos no SNC (WADICHE; AMARA; KAVANAUGH, 1995). São conhecidos cinco tipos de EAATs (EAAT 1-5), de modo que, expressão de EAAT 1 é restrita aos astrócitos, onde também predominam os transportadores do tipo EAAT2, enquanto EAAT3 é especificamente neuronal. Os transportadores EAAT4 e EAAT5 foram identificados nos dendritos dos neurônios cerebelares de Purkinje e na retina, respectivamente (DANBOLT, 2001). No citosol dos astrócitos, o Glu é convertido em glutamina, por ação da enzima glutamina sintetase (MARTINEZ-HERNANDEZ; BELL; NORENBERG,1977). Os astrócitos e os neurônios possuem transportadores

de glutamina, o que permite o tráfego da glutamina para o neurônio pré-sináptico, onde pode ser convertida novamente em Glu (ERECIŃSKA; SILVER, 1990).

A isquemia provoca o aumento da liberação de Glu para o meio extracelular e a falhas na recaptação, levando a excitotoxicidade (WAHLESTEDT et al., 1993). A excitotoxicidade é um processo mediado pelos NMDAR presentes nos neurônios pós-sinápticos (ARUNDINE; TYMIANSKI, 2004), que, quando estimulados excessivamente, geram o influxo descontrolado de cálcio capaz de induzir apoptose (LAI; ZHANG; WANG, 2014).

Os NMDAR são compostos obrigatoriamente por duas subunidades de GluN1 e duas subunidades GluN2 ou GluN3 (SANZ-CLEMENTE et al., 2013), formando um canal permeável ao Ca^{2+}, Na^{+} e K^{+}. Sua ativação se dá pela ligação do Glu (via subunidades GluN2) ou glicina (via GluN1), ocasionando a despolarização da membrana e liberação do canal, bloqueado por Mg^{2+} (JANSSENS; LESAGE, 2001).

Os receptores NMDA sinápticos são compostos predominantemente por subunidades NR1 e NR2A (LI et al., 1998), enquanto a subunidade NR2B possui distribuição extra sináptica (MONYER et al., 1994). Estudos sugerem que a ativação de receptores NMDA sinápticos ativa mecanismos de sobrevivência celular, enquanto a estimulação de receptores NMDA extra sinápticos induz a morte neuronal (HARDINGHAM; FUKUNAGA; BADING, 2002; LIU et al., 2007; GASCÓN et al., 2008). Entretanto, há evidências de que o efeito da ativação de receptores NMDA sinápticos não é exclusivamente pró-sobrevivência (BREWER et al., 2007; ALEX et al., 2011; ZHOU et al., 2013).

1.1.2.2 Neurotransmissão Colinégica

A acetilcolina (ACh) é outro importante neurotransmissor do sistema nervoso central (SOLBERG; BELKIN, 1997) e tem sido amplamente estudada devido ao seu papel de destaque na atenção, aprendizado e memória (MICHEAU; MARIGHETTO, 2011).

A ACh está também envolvida na etiologia de doenças neurodegenerativas como doença de Alzheimer (SCHLIEBS; ARENDT, 2011). Alterações de aprendizagem e memória espacial em roedores de diferentes idades foram demonstradas em um estudo realizado por Wyss e outros (2000); e tais disfunções foram associadas a mudanças funcionais e/ou morfológicas nas estruturas colinérgicas no SNC, como o hipocampo (SUGAYA et al., 1998). Da mesma forma, Pires e outros (2005) demonstraram que a diminuição da liberação de ACh tanto no córtex quanto no hipocampo está diretamente relacionada a déficits na memória espacial de referência.

Os receptores de acetilcolina (AChRs) consistem em dois subtipos: receptores muscarínicos metabotrópicos (mAChR) e os receptores nicotínicos ionotrópicos (nAChR). Ambos são expressos por células neuronais e não neuronais em todo organismo (DANI; BERTRAND, 2007; EGLEN, 2005). Os mAChR possuem cinco subtipos (M1-M5), de modo que, M2 e M4 são receptores acoplados a família $G_{i\alpha}$ das proteínas G, enquanto M1, M3, M5 são receptores acoplados a família $G_{q/11\alpha}$ das proteínas G (HIROTA; MCKAY, 2006). Os receptores nAChR neuronais são canais de cátion permeáveis a íons Na^+, K^+ e Ca^{2+} (GOTTI et al., 2009), podendo ser heteroméricos, formados por um arranjo pentamérico de subunidades β (β2-β4) e α (α2-α10) (ALBUQUERQUE et al., 2009), ou canais homoméricos compostos exclusivamente por subunidades α7 (ALEXANDER et al., 2011).

No cérebro, os α7-nAChRs são expressos em células neuronais e não neuronais, como astrócitos, microglia, oligodendrócitos e células endoteliais (SHARMA e VIJAYARAGHAVAN, 2001; SHYTLE et al., 2004; HAWKINS et al., 2005). A inflamação contribui significativamente para a lesão tecidual após o AVE (NEUMANN et al., 2015). Estudos recentes demonstram que a modulação da via anti-inflamatória colinérgica exerce um importante papel na atenuação da resposta inflamatória após AVE (MIYAMOTO et al., 2003; OTTANI et al., 2009; CAI et al., 2014).

Segundo Inestrosa e outros (2013), a ligação da nicotina a α7-nAChR demonstrou prevenir e reverter o acúmulo de β-amilóide no cérebro. Em modelo de isquemia

cerebral, a ativação de receptores α -7 inibe a inflamação e melhora a integridade da barreira hematoencefálica (ZOU et al., 2016) e reduz o estresse oxidativo (HAN et al., 2014).

Receptores muscarínicos M1 são abundantemente expressos no córtex cerebral, hipocampo e estriado (LEVEY et al., 1993) e desempenham papel importante na plasticidade sináptica hipocampal (SHINOE 2005). Os mAChRs presentes no sistema nervoso central estão associados a funções cognitivas, comportamentais, sensoriais, motoras e autonômicas (WESS et al., 2004).

Estudos com camundongos *knockout* demonstram que a ausência de receptores M1 compromete a aprendizagem, memória de trabalho e a plasticidade sináptica (ANAGNOSTARAS et al., 2002; WESS et al., 2004; SHINOE et al.,2005). A utilização da quitosana, um polissacarídeo utilizado na medicina tradicional japonesa, diminuiu o déficit cognitivo induzido pela isquemia cerebral em camundongos, através de mecanismos que envolvem a estimulação dos receptores M1 (ZHAO et al. 2005).

1.1.3 Diagnóstico e tratamento

O diagnóstico do AVE é realizado por meio da avaliação do histórico do paciente e, principalmente, pela avaliação de sinais clínicos como perda de força e sensibilidade, dificuldade visual e de fala, cefaleia intensa, desequilíbrio e tontura (MESCHIA et al., 2014). O diagnóstico diferencial é realizado por meio de tomografia computadorizada e ressonância magnética (WHO, 2005).

A revascularização e restauração do fluxo sanguíneo são a principal abordagem terapêutica para o tratamento da isquemia cerebral na fase aguda (KALOGERIS et al., 2012). De acordo com as recomendações da Food and Drug Administration (FDA), utiliza-se o ativador do plasminogênio tissular recombinante (rt-PA), o Alteplase (KNUTTINEN et al. 2010). A intervenção na fase subaguda do AVE

isquêmico compreende o tratamento de agravos no quadro neurológico, que incluem edema cerebral, ocorrência de hemorragia de lesões isquêmicas e tratamento de convulsões (JAUCH et al., 2013).

O rt-PA é fabricado por meio de tecnologia de DNA recombinante e promove a conversão do plasminogênio (uma pró-enzima inativa) em plasmina (uma enzima ativa), atuando na degradação das moléculas de fibrina presentes no coágulo (COLLEN; LIJNEN, 2005). Segundo Hacke e outros (2004), os melhores prognósticos são observados se a terapia trombolítica é iniciada nos primeiros noventa minutos após o início dos sintomas. Visando minimizar os danos ao SNC, o Ministério da Saúde preconiza que o tratamento trombolítico com rt-PA deva ser iniciado até três horas após o início dos sintomas (MINISTÉRIO DA SAÚDE, 2009). Entretanto, seu uso é contraindicado em pacientes que apresentam lesões vasculares, hipertensão grave e descontrolada, tumor cerebral, sangramento ativo (exceto sangramento menstrual normal) ou que sofreram trauma, cirurgia craniana recente ou AVE isquêmico nos últimos três meses (ALI et al., 2014).

Fármacos inibidores da acetilcolinesterase, antagonistas de receptores N-metil-D-aspartato (NMDA) e antidepressivos, convencionalmente utilizados no tratamento da Doença de Alzheimer, têm demonstrado pequenas melhorias no comprometimento cognitivo pós-AVE (GARDONI; DI LUCA, 2006; MALOUF; BIRKS, 2004). Entretanto, estas drogas não melhoram os resultados clínicos globais, além de possuírem efeitos adversos e custos elevados (LEVINE; LANGA, 2011). Até o momento, não há tratamento eficaz para o comprometimento cognitivo pós-AVE (SUN et al., 2014). Considerando as limitações da terapêutica atualmente empregada, novas estratégias são necessárias para reduzir a incidência de pacientes acometidos e melhorar o prognóstico (MINISTÉRIO DA SAÚDE, 2013). Nesse contexto, o AE emerge como uma potencial intervenção não farmacológica na prevenção e/ou tratamento do AVE isquêmico, uma vez que já foi demonstrada sua eficácia em várias doenças neurodegenerativas como Doença de Parkinson, Doença de Alzheimer, Doença de Huntington, entre outras (NITHIANANTHARAJAH; HANNAN, 2006).

1.2 AMBIENTE ENRIQUECIDO E AVE

A eficácia de terapias não farmacológicas, como estimulação elétrica, reabilitação virtual, massagem tailandesa e acupuntura, vêm sendo estudada na reabilitação pós-AVE (KAFRI; LAUFER, 2014; TOUSIGNANT et al., 2014; THANAKIATPINYO et al., 2014; RIBEIRO et al., 2015; CHEN et al., 2016). Da mesma forma, o ambiente enriquecido (AE) é uma abordagem alternativa para o tratamento do comprometimento cognitivo pós-AVE (VEDOVELLI et al., 2011; HARATI et al., 2013; MESA-GRESA et al., 2013).

O termo “ambiente” abrange uma variedade de fatores, que compreende as condições socioeconômicas, familiares, relacionamentos e todas as experiências de vida pré e pós-natal que podem influenciar o cérebro e as respostas comportamentais do indivíduo (SOLINAS et al., 2010). Nunnari, Bramanti, e Marino (2014), em uma revisão qualitativa, analisaram a influencia da educação sobre o déficit cognitivo pós-AVE, mostrando que mais anos de escolaridade podem estar associados a um menor declínio cognitivo pós-AVE, com melhor desempenho de linguagem, funcionamento executivo e memória, em comparação com pacientes com menos anos de educação formal.

Experimentalmente, um AE fornece uma diversidade de estímulos sensoriais, motores e cognitivos ao animal exposto a ele, em relação ao ambiente convencional de laboratório (VAN PRAAG et al., 2000; NITHIANANTHARAJAH; HANNAN, 2006). Durante a exposição ao AE, o meio habitual em que os animais são mantidos no laboratório é modificado, fornecendo espaço mais amplo e grande variedade de novos objetos, objetivando a promoção de maior atividade física em exploração, além da interação com um novo e complexo ambiente (ZEBUNKE; PUPPE; ANDLANGBEIN, 2013). O AE é uma intervenção comportamental comumente empregada para roedores e primatas não humanos após a lesão cerebral e pode produzir mudanças estruturais, funcionais e bioquímicas no cérebro (NILSSON et al.,

1999). Segundo Kempermann, Gast e Gage (2002), o AE tem demonstrado eficácia em retardar o envelhecimento neuronal, além de provocar uma melhora da cognição, memória, comportamento e coordenação motora em modelos pré-clínicos de demência, depressão, doença de Alzheimer, doença de Parkinson, e doença de Huntington (FAHERTY et al., 2005; JANKOWSKY et al., 2005; KLAISSLE et al., 2012; BLÁZQUEZ et al., 2014; HANNAN, 2014).

Estudos demonstram os efeitos positivos do AE sobre a recuperação cognitiva e motora após evento isquêmico (HICKS et al.; 2002; JADAVJI et al., 2006). A exposição ao AE é capaz de atenuar o dano oxidativo e neurodegeneração em ratos isquemiados (BRIONES; ROGOZINSKA; WOODS, 2011). Especificamente no hipocampo, o AE já se mostrou capaz de reverter danos em vários modelos experimentais de alterações no SNC e em lesões cerebrais induzidas nessa região (NITHIANANTHARAJAH; HANNAN, 2006). O hipocampo exerce papel fundamental na formação e manutenção de funções cognitivas como memória e aprendizado (ROSSATO et al., 2007; IZQUIERDO et al., 2013). A atividade de modulação do hipocampo pode cumprir um papel essencial nos circuitos neurais envolvidos na melhoria da memória por AE, uma vez que, foi observada ativação do hipocampo e do córtex infra-límbico em camundongos expostos ao AE (LEGER et al., 2012).

1.2.1 Tolerância isquêmica

A tolerância isquêmica é definida como resistência transitória à isquemia letal gerada por um estímulo subletal prévio, o pré-condicionamento (DURUKAN; TATLISUMAK, 2010). Estímulos de pré-condicionamento, como isquemia, hipóxia e exercício tem demonstrado capacidade de induzir tolerância contra isquemia (POINSATTE et al., 2015; MA et al., 2016; OTSUKA et al., 2016). Um animal submetido a um estímulo de pré-condicionamento estressante sofre a ativação de mecanismos protetores endógenos, de modo que, quando o insulto isquêmico letal é aplicado um conjunto

de respostas são acionadas, constituindo assim, o fenótipo tolerância à isquemia (DURUKAN; TATLISUMAK, 2010).

O emprego do AE como um estímulo de pré-condicionamento tem demostrado potencial na indução de tolerância contra isquemia. Em trabalho realizado por Xie e outros (2013), o AE promoveu diminuição do déficit motor, melhora do aprendizado e da memoria espacial de ratos, avaliada no labirinto aquático de Morris. Nesse estudo a tolerância à isquemia foi associada ao aumento no nível de atividade física no AE, em comparação ao ambiente padrão (AP). O AE pré-isquemia foi capaz de prevenir danos cognitivos, tais como os associados ao estresse oxidativo e lipoperoxidação no hipocampo, causado por hipoperfusão cerebral crônica em ratos Wistar (CECHETTI et al., 2012). Adicionalmente, foi observada a diminuição da morte neuronal no hipocampo e a prevenção da deficiência cognitiva pós-isquemia, avaliada pelos paradigmas do labirinto em Y e de reconhecimento do novo objeto (KATO et al., 2014).

Deste modo, o pré-condicionamento em AE pode ser uma nova estratégia para o tratamento de distúrbios neurológicos relacionados à isquemia cerebral. No entanto, os mecanismos moleculares envolvidos não são bem compreendidos. Por isso, torna-se relevante a identificação dos mecanismos neuroprotetores desencadeados pelo AE em modelo animal de AVE.

2 JUSTIFICATIVA

O AVE está descrito entre as maiores causas de morte no mundo (MINISTÉRIO DA SAÚDE, 2013) e a incidência de pacientes acometidos tende a aumentar ao longo do tempo (LACKLAND et al., 2012). Estudos demonstram que cerca de 30% dos indivíduos sobreviventes a um AVE apresentam algum tipo comprometimento cognitivo (RIST et al., 2013).

O mecanismo da reabilitação cognitiva no tratamento pós-AVE é em grande parte desconhecido (JACKSON et al., 2012). A terapia farmacológica convencional, com alteplase, tem-se demonstrado efetiva na redução dos danos motores pós-AVE, entretanto, não foi mostrado sua influência na melhora dos resultados cognitivos (NYS et al., 2006). Até o momento, não há tratamentos eficazes em prevenir e/ou melhorar o comprometimento cognitivo pós-AVE (SUN et al., 2014), tornando relevante a identificação de novas estratégias para reduzir a incidência de pacientes acometidos e melhorar o prognóstico (MINISTÉRIO DA SAÚDE, 2013).

Estímulos de pré-condicionamento, como isquemia, hipóxia e exercício tem demonstrado capacidade de induzir tolerância contra isquemia (MA et al., 2016; POINSATTE et al., 2015; OTSUKA et al., 2016). Estudos pré-clínicos em roedores apontam que o AE pode induzir tolerância cerebral contra isquemia, minimizando sequelas motoras e cognitivas (XIE et al., 2013), prevenindo danos cognitivos associados ao estresse oxidativo (CECHETTI et al., 2012) e com diminuição significativa da morte neuronal (KATO et al., 2014).

Deste modo, animais pré-condicionados ao AE podem ser uma nova estratégia para o tratamento de distúrbios neurológicos. No entanto, os mecanismos moleculares envolvidos não são bem compreendidos. Por isso, torna-se relevante a identificação dos mecanismos neuroprotetores desencadeados pelo AE em modelo animal de AVE.

Assim, no presente projeto, propomos identificar os mecanismos envolvidos na neuroproteção e/ou prevenção de danos cognitivos induzidos pelo AE em modelo experimental de isquemia cerebral global, investigando componentes dos sistemas colinérgico e glutamatérgico, uma vez que estão envolvidos na neurotoxicidade e em processos de aprendizagem e memória. Assim, a compreensão dos eventos moleculares promovidos pelo AE na isquemia poderá ampliar também possíveis alvos para o desenvolvimento de novos fármacos no combate ao AVE e, adicionalmente, trazer dados científicos robustos que suportem a utilização dessa estratégia não farmacológica nos sistemas de saúde.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Identificar os mecanismos moleculares envolvidos na neuroproteção cognitiva induzida pelo AE em modelo experimental de AVE isquêmico em modelo experimental, com foco nos sistemas colinérgico e glutamatérgico e em processos inflamatórios e de plasticidade sináptica.

3.2 OBJETIVOS ESPECÍFICOS

- Verificar o efeito do AE na prevenção de déficits na memória de reconhecimento de objetos, de curta e longa duração em camundongos submetidos à isquemia cerebral;
- Verificar o efeito do AE na prevenção de déficits na memória espacial de trabalho em camundongos submetidos à isquemia cerebral;
- Avaliar o perfil de expressão gênica de receptores colinérgicos (M1 e α7) e subunidades dos receptores glutamatérgicos NMDA no hipocampo;
- Avaliar a expressão gênica de GFAP, IL1-β e BDNF no hipocampo;
- Avaliar os níveis do neurotransmissor Glu no hipocampo;
- Correlacionar estatisticamente a eventual melhora cognitiva com alterações bioquímicas e moleculares nos sistemas avaliados.

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Molecular bases of environmental enrichment cognitive neuroprotection in an animal model of cerebral ischemia

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Highlights

- EE prevented short-term memory deficit.
- EE reduced the infarct volume.
- IL1-β expression levels were downregulated by EE after stroke.

Abstract

Stroke is considered a major cause of global morbidity. Currently, there are no effective treatments for post-stroke cognitive impairment. Enriched environment (EE) may be used as a preconditioning method to induce cerebral tolerance in an ischemic event. However, molecular mechanisms involved in this tolerance are not clear yet. Therefore, it becomes relevant to identify the mechanisms of neuroprotection triggered by the EE preconditioning in murine model of AVE. In this study, C57Bl/6 mice were kept for five weeks in EE or standard environment (SC). After that period, they were divided into four groups: sham standard environment (SS), sham enriched environment (ES), ischemic standard environment (SI) and ischemic enriched environment (EI). Animals from SI and EI underwent bilateral occlusion of the common carotid arteries for 30 minutes and, SS and ES, underwent a similar surgical procedure without the occlusion. In a set of behavioral tests, we demonstrated that EE paradigm prevented short-term memory deficit caused by ischemia, significantly reducing the infarct volume. Relative gene expression from NMDA glutamatergic receptor subunits (GluN1, GluN2A, GluN2B and GluN2C), cholinergic receptors muscarinic M1 and ionotropic alpha 7, astrocytic activation marker GFAP, inflammatory marker IL-1β and BDNF were evaluated in the hippocampus. In addition, tissue levels of the neurotransmitter glutamate were analyzed. The study strongly suggests that changes observed in glutamatergic receptors and reduction of the inflammatory cytokine IL1-β expression and increase GFAP in ischemic animals may be contributed to cognitive improvement induced by the AE paradigm.

Abbreviations: EE, enriched environment; SC, standard cages; SS, sham standard environment; ES, sham enriched environment; SI, ischemic standard environment; EI, ischemic enriched environment; NMDA, n-methyl-d-aspartate; M1, muscarinic receptor M1; GFAP, glial fibrillary acidic protein; IL-1 β, interleukin 1 beta; BDNF, brain-derived neurotrophic factor; NMDAR, n-methyl-d-aspartate receptors; rt-PA,

recombinant tissue plasminogen activator; STM, short-term memory; LTM, long-term memory; BCCAO, bilateral common carotid artery occlusion; c.c.a., common carotid; NOR, novel object recognition task; α7, alpha 7 nicotinic receptor; CREB, cAMP response element binding protein; PI3K, phosphoinositide 3-kinase; CaMKIV, calcium/calmodulin-dependent protein kinase type IV; ERK, extracellular signal–regulated kinases; TNF, tumor necrosis factor.

Keywords: environmental enrichment, cerebral ischemia, neuroprotection, learning and memory.

1. Introduction

Stroke is described as the second largest cause of death in the world [1]. Ischemic stroke is caused by vessel obstruction, which can cause tissue damage [2]. Depletion of the blood supply triggers several pathophysiological processes such as inflammation, oxidative stress, excitotoxicity, apoptosis and necrosis of neurons [3], which may result in motor, sensory and cognitive alterations [2]. Aphasia, hemispatial neglect, deficits in work memory, attention, learning, visual perception or executive functions are some of the cognitive impairments that can be observed after a brain ischemic event [4].

Cognitive deficits associated with ischemic stroke may be related to dysfunctions in several neurotransmitter systems, including the glutamatergic and cholinergic systems [5,6]. Glutamate excitotoxicity is a process mediated by n-methyl-d-aspartate receptors (NMDARs) present in post-synaptic neurons [6,7], which, when stimulated excessively, generate the uncontrolled influx of calcium capable of inducing elevation in reactive oxygen species [8], apoptosis [9], necrosis and autophagy [10]. Moreover, regarding the cholinergic system, the stimulation of the hippocampal M1 muscarinic receptors contributes to cerebral ischemia-induced deficits [11].

The main therapeutic approach for stroke in the acute phase is revascularization and re-establishment of blood flow [12], made through the use of the recombinant tissue plasminogen activator (rt-PA) [13]. However, it has a limited time window and numerous side effects [14,15], and so far, there is no effective treatment for cognitive impairment after stroke [16]. Considering the limitations of the currently available therapy, new strategies are necessary to improve patient´s life quality and the prognosis of the disease [17].

The use of non-pharmacological therapies have been studied in post-ischemia rehabilitation [18–24]. Pre-conditioning stimuli, such as ischemia, hypoxia, and exercise have demonstrated the ability to induce tolerance against ischemia [25–27]. In this context, the environmental enrichment (EE) pre-ischemia has been demonstrated as a promising approach capable of generating tolerance against

ischemia in animal models [28,29]. The EE provides a diversity of sensory, motor and cognitive stimuli [30,31], capable of producing structural, functional and biochemical changes in the brain [32]. In EE, animals are kept in larger boxes and with a large variety of new objects, promoting greater physical activity and exploration [33].

The pre-ischemia EE presented a positive effect on the motor function, learning and the spatial memory of rats, being associated with the increase in the level of physical activity [29]. Additionally, EE was also able to prevent cognitive damages associated with oxidative stress and lipoperoxidation in a rat model of chronic cerebral hypoperfusion [28]. Specifically in the hippocampus, EE has been shown to be capable of reversing damage in several experimental models of CNS changes and induced brain lesions in this region [31]. The hippocampus plays a key role in the formation and maintenance of cognitive functions such as memory and learning [34,35] and modulates the activity of neural circuits involved in memory enhancement by EE [36]. EE reduced neuronal death in the hippocampus of rats and prevented post-ischemia cognitive deficiency, assessed through the Y-maze and novel object recognition tests [37].

Thus, EE preconditioning may be a new strategy for the treatment of neurological disorders. However, the molecular mechanisms involved are not well understood. Therefore, it becomes relevant to identify the neuroprotective mechanisms triggered by EE in animal models of stroke.

In the present project, we propose to identify the molecular bases of cognitive neuroprotection induced by EE in an experimental model of cerebral ischemia. Since cholinergic and glutamatergic systems are involved in neurotoxicity and in learning and memory processes, we have chosen to evaluate the gene expression pattern of glutamatergic and cholinergic receptors, as well as the glutamate content in the hippocampus. In addition, GFAP (glial fibrillary acid protein), inflammatory marker IL-1 β (interleukin 1 beta) and the neurotrophin BDNF (brain-derived neurotrophic factor) were also evaluated, aiming to depict possible correlations between alterations in the neurotransmitter systems evaluated and inflammatory processes and synaptic plasticity.

2. Materials and methods

2.1. Animals

A total of 108 C57Bl/6 male mice (21 days of age), weighing 9-10 g, were used to conduct the experiments. Ethical principles of the Brazilian College for Animal Experimentation (COBEA, www.cobea.org.br) were respected, which are in accordance with international standards for research involving animals. The experimental procedures were approved by The Ethics Committee on Animal Use (CEUA) of the Federal University of Espírito Santo (UFES) (protocol number 58/2016).

2.2. Experimental Design

Animals were divided into four independent groups according to housing conditions and surgery, namely sham standard environment (SS) (n=30), sham enriched environment (ES) (n=18), ischemic standard environment (SI) (n=39), and ischemic enriched environment (EI) (n=21). Animals were weaned at 21 days of age and housed in either standard or enriched cages for 5 weeks before surgery procedure. At day 36, ischemia/reperfusion and sham surgery was performed. Behavioral testing initiated at day 38. At day 40 or day 41 (for short-term (STM) or long-term memory (LTM), respectively) animals were euthanized (Figure 1). The hippocampus was dissected and stored at −80 °C for further analysis.

2.3. Housing conditions

Mice were split in two housing conditions: standard cages (SC) or EE cages. For SC, 69 animals were maintained in 30 cm × 19 cm × 13 cm polypropylene cages. In EE housing conditions, 39 animals were kept in 60 cm × 50 cm × 22 cm cages, containing four to five toys- changed weekly a running wheel and a miniature house [38]. The animals were housed under enriched or standard conditions five weeks before the surgery in a temperature and humidity-controlled room, under a 12-h light/dark cycle (lights on at 7:00 a.m.) with food and water ad libitum. All testing was

carried out during the light phase, at the vivarium of the Laboratory of Molecular and Behavioral Neurobiology of UFES.

2.4. Induction of global cerebral ischemia

Animals were anaesthetized by xylazine (20 mg/kg, i.p.; Sedomin, Argentina) and 2,2,2-Tribromoethanol (200 mg/kg, i.p.; Sigma-Aldrich). The global cerebral ischemia protocol used was adapted from Tsuchiya et al. [39]. Global ischemia was induced by the bilateral common carotid artery occlusion (BCCAO). Animals were placed in the dorsal position, the neck area was shaved, both common carotid (c.c.a) were exposed carefully by blunt dissection for temporary occlusion with cotton yarn (right c.c.a.) and permanent occlusion (left c.c.a.) with nylon wire (5-0, 1.5 cm, Shalon). Reperfusion of the right c.c.a. was performed after 30 minutes and was confirmed visually. The skin was sutured with nylon wire. In sham-operated animals, both c.c.a. were exposed, but not occluded. Mice were allowed to recover in individual cages.

2.5. Quantification of infarct area

Four animals from each group were killed by decapitation 48 hours after ischemia. Brains were removed and 2-mm coronal sections were made from the olfactory bulb to the cerebellum. Slices were immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride (Sigma-Aldrich) at room temperature for 25 minutes, and fixed with formaldehyde 4% for 24 hours. The following day, digital images were obtained for further analysis in the Image J software (1.49g/Java 1.6.0, 32-bit). The unstained infarcted areas were measured and calculated as a percentage of the whole coronal sections.

2.6. Novel object recognition task (NOR)

NOR protocol used was adapted from Gusmão et al. [40]. All animals were given a 30 minutes habituation with no objects in a white plastic cage (41x32x16 cm).

Twenty-four hours later they were given a 10 minute habituation and performed the training session. Animals were allowed to freely explore for 10 minutes, and two floor-fixed identical objects (A1 and A2) were placed in the center of the box, and separated by approximately 10 cm. During the training phase, we evaluated the mean speed and the distance traveled by the animals to evaluate possible motor damage caused by ischemia. Animals that had mobility difficulty were excluded from the test. STM and LTM was evaluated 1.5 hours or 24 hours after the training session, respectively. STM and LTM were evaluated in distinct groups of animals. During the five minute test, mice were returned to the arena which contained one of the original objects ("familiar"), and a new object different in shape and color ("novel"). Between animals boxes and objects were cleaned with 70% alcohol. Exploration time was defined as sniffing or touching the object with the nose and was quantified by AnyMaze® software. Rodents' performance was assessed by recording the time spent investigating the familiar and novel objects.

2.7. Y-maze test

Working memory was assessed 96 hours after surgery, by recording the spontaneous alternation behavior in a Y-maze as previously described [41]. The maze was constructed of gray acrylic with three identical arms (31 x 30 x 6 cm) positioned at equal angles (EP 150Y, Insight®). Animals were placed at the end of one arm and allowed to move freely through the maze during an 8-min session. The series of arm entries was recorded visually and an arm entry was considered to be complete when the hind paws of the mouse were completely placed in the arm. Alternations were defined as successive entries into the three arms on overlapping triplet sets. The percent alternation was calculated according to formula: percent alternation = [(number of alternations) / (total number of arm entries - 2)] x 100%.

2.8. Gene expression analysis

The hippocampus was dissected, immediately frozen in dry ice, and stored at −80◦C until RNA extraction. As previously described [42], total RNA was extracted

using TRI Reagent RNA Isolation Reagent (Sigma-Aldrich, MO, USA). cDNA was synthesized using an iScript cDNA synthesis kit (Biorad, CA, USA). Subsequent qPCR was performed using a CFX96 Real Time PCR (Biorad, CA, USA) and iQ SYBR Green Supermix (Biorad). Relative quantification of gene expression was analyzed via the $2^{-\Delta\Delta Ct}$, using GAPDH as the reference gene for normalization. Genes chosen for qPCR analysis were GluN1, GluN2A, GluN2B, GluN2C NMDA glutamate receptor subunits, muscarinic receptor M1 (M1), alpha 7 nicotinic receptor (α7), GFAP, IL-1 β and BDNF. Primer sequences for each gene are described in Table 1.

2.9. Glutamate assay

The levels of glutamate present in the hippocampus of the animals were measured using the glutamate assay kit (Sigma-Aldrich), according to the manufacturer's instructions. The right and left hippocampus, of animals that underwent LTM tasks, was dissected, homogenized and divided into 10 mg samples. The samples were homogenized in 100 µl of Glutamate Assay Buffer and centrifuged 13000 xg for 10 minutes. To the supernatant was added reagent mix, with final reaction volume of 50 µl. The absorbance was measured in 450nm in spectrophotometer. The assays were performed in duplicate. The mean of each duplicate was normalized based on the protein content of the samples, measured by the Bradford method.

2.10. Statistical analyses

Behavioural and molecular data were analyzed via two-way ANOVA with repeated measures followed by Bonferroni post hoc and unpaired Student’s t-tests, when necessary. Glutamate assay, distance traveled and mean speed data were analyzed via one-way ANOVA. Analysis of the infarct area was performed via Student’s t-tests. The software Graph Pad Prism v. 5 was used for statistical analyses. All data are represented as the mean ± SEM with $p < 0.05$ considered statistically significant.

3. Results

3.1. Analysis of the infarct area

In order to examine whether EE could produce ischemic tolerance at the morphological level, measurement of the infarct area after global ischemia was assessed. As expected, mice from the SS and ES groups did not exhibited ischemic area (Fig. 2A). On the other hand, EE was able to induce ischemic tolerance, as revealed by the reduced ischemic area presented by EI animals when compared to those from the SI group ($p= 0.0393$; Fig. 2B).

3.2. Novel object recognition

The STM was accessed through the NOR test. During the training phase, no motor deficit was identified, as the distance traveled ($F (1, 38) = 0.1510$; $p = 0.6995$) and the mean speed ($F (1, 38) = 0.05727$; $p = 0.8121$) did not differ among experimental groups (Supplementary fig. 1A-B). Although animals from the EE had an overall increased exploration time towards either the A1 or the A2 object ($F (3, 40) = 3.072$; $p=0.0385$), animals within each group had no exploratory preference between objects, as revealed by the Bonferroni post-hoc (Supplementary fig. 1C). During the test phase (Fig. 3A), we observed once more an effect of the environment of the performance of the animals ($F (3, 43) = 7.341$; $p=0.0004$). Within animals kept in the EE, those of the sham group had an increased exploration of the novel object ($p=0.0015$), the ones that underwent ischemia had no preference towards the novel object, indicating that ischemia leads to STM impairment. On the other hand, animals from the EE, both from the sham ($p<0.001$) and ischemia ($p<0.01$) groups, had an increased exploration of the novel object. Moreover, animals expended more time exploring the novel objects when exposed to EE when compared to those kept in the SC ($p<0.001$, comparing SS vs. ES groups). Likewise, animals of the EI expended more time exploring the novel objects than SI ($p<0.01$).

In the LTM, once more, during the training phase motor deficits were not identified as the distance traveled ($F (1, 49) = 0.4897$; $p = 0.4874$) and the mean

speed (F (1, 52) = 0.2310; p = 0.6328) did not differ among groups (Supplementary fig. 1D-E). In addition, animals from each group explored the two objects equally (F (1; 50) = 3.542; p = 0.0657; Supplementary fig. 1F). During the test phase (Fig. 3B), there was no significant difference between SC and EE (F (3, 57) = 0.9864; p = 0.4057). The animals from the SS, SI, ES and EI groups showed more interest to explore the new object (F(1, 57)= 64.97; p< 0.0001), indicating that ischemia did not affect LTM in the NOR paradigm.

3.3. Working memory

The evaluation of the short-term spatial working memory performance in the Y-maze task revealed that BCCAO did not decrease the number of spontaneous alternations (F (1; 30) = 0.1478; p=0.7033), as animals that underwent ischemia had similar performance as that from the sham mice. Moreover, when examining the performance of animals exposed to EE in comparison with those kept in SC, we observed that The EE did not alter the performance of the working memory (F (1; 30) = 0.2655; p=0.6101; Supplementary fig. 2).

3.4. Gene expression analysis

Relative Gene expression alterations were assessed by qPCR. Analysis were performed separately for animas who underwent STM and LTM tasks, as different molecular processes may be involved in both types of memory [5,6,34].

Regarding animals that underwent STM tasks, analysis of the gene expression of NMDA glutamatergic system components are presented in Fig. 4. Hippocampal expression of GluN1 (F (1; 11) = 0.01054; p = 0.9201), GluN2A (F (1; 11) = 1.256; p = 0.2862), GluN2B (F (1: 11) = 1.335, p = 0.2724) and GluN2C genes (F (1; 12) = 2,032; p = 0.1845) were not altered (Fig. 4A-D). Gene expression analysis of the cholinergic system components (Fig. 4E-F) revealed that M1 (F (1; 11) = 2.645; p = 0.1322) and α7 (F (1; 12) = 0.2370, p = 0.6359) expression were not altered either. The expression of GFAP (F (1; 10) = 6.493, p = 0.0290) was increased in EI animals

when compared to ES ones ($p < 0.05$; Fig. 4G), and was not affected by the environment ($F (1; 10) = 0.9258$, $p = 0.3586$), whereas IL-1β expression was detected only in the SI group (Fig. 4H). BDNF expression was not significantly affected ($F (1; 9) = 2.682$, $p = 0.1359$; Fig. 4I).

Regarding animals that underwent LTM tasks, the expression of GluN1 ($F (1; 20) = 8.592$; $p = 0.0083$) was increased in EI animals when compared to ES ones ($p < 0.01$; Fig. 5A). However, the expression of this NMDAR subunit was not affected by the environment ($F (1; 20) = 1.433$; $p = 0.2452$). We were able to show that EE promoted increased GluN2A ($F (1; 19) = 8.930$, $p = 0.0076$; Fig. 5B) gene expression in the sham mice, without being affected by the surgery ($F (1; 19) = 0.7036$, $p = 0.4120$), and promoted increased GluN2C gene expression in the ischemic mice ($F (1; 20) = 6,274$, $p = 0.0210$). Furthermore, GluN2C ($F (1; 20) = 8.571$; $p = 0.0083$) gene expression was increased in EI group when compared to ES group ($p<0.01$; Fig. 5C). Gene expression of GluN2B ($F (1; 12) = 0.2712$; $p= 0.6120$; Fig. 5D) was not altered. Gene expression analysis of the cholinergic system components (Fig. 5E-F) revealed that the expression α7 ($F (1; 12) = 0.2700$; $p= 0.6128$) and M1 ($F (1; 20) = 2.030$, $p=0.1696$) expression was not altered. The expression of the GFAP ($F (1; 11) = 17.30$; $p = 0.0016$) was increased in EI when compared to ES group ($p<0.05$; Fig. 5G), and was not affected by EE ($F (1; 11) = 0.3502$, $p=0.5660$). IL-1β (Fig. 5H) gene expression was higher in the group EI when compared to ES group ($F (1; 18) = 5.354$; $p = 0.0327$; $p<0.05$), and was not affected by EE ($F (1; 19) = 0, 01995$; $p = 0.8893$).

3.5. Measurement of glutamate tissue content

In order to verify if the gene expression alteration observed in the NMDARs correlates with the amount of glutamate, we evaluated the content of the neurotransmitter in the hippocampus. There was no difference in glutamate content between SC and EE groups ($F (1, 19) = 0.2078$, $p = 0.6537$; Fig. 6).

4. Discussion

EE is a non-pharmacological intervention that protects the brain against excitotoxic lesions [43]. The use of EE as a preconditioning stimulus has been considered as an alternative in the prevention of cognitive impairment after ischemia [28,29]. Previous studies demonstrate that EE is an experimental paradigm capable of promoting the improvement of STM and LTM in NOR [44,45], including in cerebral ischemia models [46,47]. In the present study, EE prevented the short-term memory deficit, caused by ischemia, assessed in the NOR. In an animal model, recognition memory is based on the instinctive tendency of species to explore novelty [48] and the ability to distinguish new and familiar objects [34]. The formation and storage of long-term object recognition memory requires protein synthesis in the hippocampus, differently than that of short-term object recognition memory, which does not depend on such changes [34]. In a study carried out by Viola et al. [44], ischemic animals kept in SC spent more time in the exploration of objects during the NOR, when compared to the animals kept in EE. In our work, we observed a distinct behavior in the animals underwent STM task, the ES and EI groups explored the new object for a longer time than the animals kept in SC, indicating that, in addition to preventing the STM deficit, EE promoted the increase of exploratory activity in these animals.

Besides preventing short-term object-recognition-memory impairment, EE significantly reduced the infarct area, showing that EE induced tolerance against ischemia at the morphological level. Several studies have reported that post-ischemia EE promotes cognitive improvement with decreasing infarct volume in rodents [29,48]. In contrast, in another study, the pre and post-ischemia EE paradigm used did not reduce the infarct volume [29,49,51]. Xie et al. studied the effects of EE on focal cerebral ischemia in Sprague-Dawley rats, adopting a shorter exposure time of the animals to EE (6 hours / day for 28 days) [29]. The variation of results between studies may be due to the different protocols adopted.

Consistent studies demonstrate the potential of EE to promote increased density of dendritic spines in healthy [52–54] and in ischemic rodents [55,56]. In addition, EE after ischemia may lead to increased neurogenesis in the hippocampus [30,57,58]. Kato et al. [37] suggested that the activation of the cAMP response

element binding protein (CREB) signaling pathway in the hippocampus may be involved in decreased cell death promoted by EE pre-ischemia.

NMDARs are calcium-permeable ion channels, and their activation generates influx of cellular calcium [59], that may triggers the signaling cascade of the CRE - BDNF, favoring neuronal survival and synaptic plasticity [60]. The hippocampus is one of the main areas of action of BDNF [61] and, transcription of BDNF in this area is controlled by calcium-dependent signaling pathways [62]. In addition, BDNF may influences the regulation of NMDAR mRNA expression in hippocampus [63]. Although we observed changes in NMDAR expression, there was no significant variation in Glut levels and BDNF expression between the animals kept in SC and EE. Thus, we suggest that EE promoted the reduction of neuronal death by means that did not involve the CREB-BDNF pathway.

An ischemic stroke may affect a person's working memory [4]. The electrical activity in the prefrontral cortex, hippocampus and amygdala is important for the formation of working memory [35,64–66]. Although the ischemic lesion has reached the hippocampus and part of the cortex, in the present study, the BCCAO did not generate any impairment of spatial working memory in the Y-maze context and the EE did not influence the performance of the animals. In the Y-maze, the spatial working memory is evaluated by means of observation of the spontaneous alternations of behavior. In previous studies, different models of cerebral ischemia caused impairment of spatial working memory [41,67,68].

Park et al. [69], using a BCCAO model in Wistar rats, suggested that post-ischemia EE promoted improvement in cognitive performance in Y-maze by increasing BDNF protein expression, assessed 14 weeks after BCCAO. The expression of neurotrophins, such as BDNF, varies spatially and temporally after ischemia, which may be a factor that influences cognitive functions [52]. In another experiment, the focal cerebral ischemia reduced spontaneous alternation of swiss mice [41]. In addition to the variability generated by the use of different models of ischemia, we must consider that there are genetic and behavioral differences between animal strains [72].

Considering the cognitive impairments in response to ischemia, and the protective effect of EE, we next evaluated the expression of glutamatergic and cholinergic system components, as well glutamate content in hippocampus, as these systems have been show to participate in learning and memory processes [73,74].

The rapid elevation of glutamate levels in an ischemic event is the first step in brain excitotoxicity [75], causing deficiency in Glu reuptake processes, prolonged activation of NMDAR and intracellular calcium overload [7,76,77].

Saad et al. [78] observed increased hippocampal Glu content 60 minutes after global cerebral ischemia in rats. The increased Glu level in the hippocampus was also demonstrated in a study by Badu et al. [79], after 72 hours of reperfusion in a focal ischemia model. However, our work has shown that glutamate content was not altered by either ischemia nor EE exposure. Unlike previous studies, the dissection of samples for Glu dosing was performed with 120 hours of reperfusion, being a relevant factor that possibly contributes to the variability between experiments.

Further, the Glu dosage encompasses the intracellular and extracellular content, which gives an idea about the turnover of glutamate during ischemia. Considering that the increased expression of GFAP in the ischemic animals maintained in EE may be indicative of increased astrocytic recruitment [80], we can infer that, although the mechanisms of reuptake and astrocytic activity are impaired during an ischemic event, the accumulation of these cells in the penumbra zone may contribute to the maintenance of Glu levels in the late phase of ischemia [81,82].

During the first few hours after the initiation of ischemia, mobilization of microglial cells, astrocytes and macrophages contribute to the pathogenesis of the disease through the release of inflammatory mediators, such as IL-1β, IL-6 and nitric oxide [83], which contributes to an acute process of neuronal death [84]. At the later stage, a regenerative response occurs in adjacent tissue [83]. In the first days after the onset of ischemia, the accumulation of astrocytes in the penumbra region contributes to the reduction of cell damage by releasing growth factors and controlling the levels of free radicals and glutamate [80,85].

Although there has been no increase in glutamate levels, it was possible to detect the increase of the expression of some NMDARs. The EI group that underwent LTM tasks showed increased GluN2C expression in the hippocampus. The increase of GluN2C transcript in the hippocampus is reported *in vitro* ischemia models [86–88]. NMDARs containing the GluN2C subunit show low conductivity compared to other subtypes [89]. GluN2C-expressing hippocampal neurons show resistance to NMDA-induced toxicity and decreased calcium influx, compared to knockout animals, preventing neuronal damage after transient cerebral ischemia [88].

It has already been shown that synaptic NMDAR stimulation, composed predominantly of subunits GluN1 and GluN2A [90], is associated with the activation of cellular survival mechanisms [91–93]. On the other hand, there is evidence that the effect of activation of synaptic NMDARs is not exclusively pro-survival [94–96]. Studies suggest that GluN2A subunits-containing NMDAR blockade protects hippocampal neurons from NMDA-induced excitotoxicity [97], in addition to promoting a significant decrease in infarct volume [98].

In the present study, we observed the increased GluN2A expression in EI animals that underwent LTM task, without there being an increase in Glu levels in the late phase after ischemia. The GluN2A-mediated pro-death and pro-survival effects result from distinct pathways. In the initial stage of ischemia, super activation of GluN2A leads to excessive influx of Ca^{2+} and activates cell death signaling pathways. In the late phase, during the recovery of ischemia, GluN2A activity is normalized and becomes C-terminal-dependent pro-survival signaling, and may be associated with several proteins that contribute to neuronal survival, such as PI3K (phosphoinositide 3-kinase), CaMKIV (calcium/calmodulin-dependent protein kinase type IV), ERK (extracellular signal–regulated kinases) and CREB [99]. Thus, we may suggest that increased GluN2A expression may be a mechanism that contributes to neuroprotection generated by EE.

Regarding the components of the cholinergic system, M1 receptors are the subtype of muscarinic receptors most expressed in the hippocampus [100]. M1 receptor deficiency compromises learning, memory, and synaptic plasticity [101–103], so that, stimulation is involved in the reduction of cognitive deficit induced by

cerebral ischemia in mice [104]. The α7 receptors are expressed in neuronal and non-neuronal cells, such as astrocytes, microglia, oligodendrocytes and endothelial cells [105–107]. In middle cerebral artery occlusion model, performed in C57BL/6J mice, the activation of α7 receptors inhibits inflammation, improves the integrity of the blood-brain barrier [108] and reduces oxidative stress [109]. In the present study, the M1 and α7 receptors does not appear to be involved in the cognitive enhancement promoted by EE, since its expression was not altered. Moreover, the expression of these receptors was not affected by BCCAO.

IL-1β expression contributes to ischemic brain damage [110–112] and is induced by pro-inflammatory stimuli, such as bacterial and viral products, TNF (tumor necrosis factor), cell lesions and hypoxia [113]. IL-1β acts on the pathogenesis of various neurological disorders, increasing neuronal excitability and leukocyte infiltration, inducing neurotoxin production, activation of microglial cells and promoting astrogliosis [114]. In animals that underwent STM task, EE prevented the expression of this inflammatory marker in ischemic animals, this being a factor that contributes to STM improvement. In contrast, in animals that underwent LTM task, IL-1β expression was increased in EI group. The expression of inflammatory factors, such as IL-1β, varies spatially and temporally after ischemia [53]. In this way, we can suggest that the difference in sample collection time may have contributed to the different results observed, so that, the dissection of the hippocampus samples to perform the gene expression analyzes of the animals that underwent LTM task were collected 24h later, compared to animals that underwent STM task.

In summary, we observed in this study the effect of EE in the prevention of Short-term object- recognition-memory deficit in mice submitted to BCCAO. In contrast, we did not detect changes in working memory evaluated in Y-maze. In the ischemic animals that underwent STM task, EE promoted reduction of IL1-β expression in the hippocampus. While in the animals that underwent LTM task, influence of EE on the expression of GluN1, GluN2A and GluN2C was observed. Our study strongly suggests that changes observed in glutamatergic receptors and reduction of the inflammatory cytokine IL1-β expression and increase GFAP in

ischemic animals may contributed to cognitive improvement induced by the AE paradigm.

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Figure captions

Fig. 1. Experimental design: Animals were weaned at the age of 21 days and housed in either standard cages or environmental enrichment for 5 weeks. At day 36, ischemia/reperfusion and sham surgery was performed. Behavioral testing was initiated at day 38. At day 40 or 41 (for short-term or long-term memory, respectively) animals were euthanized. The hippocampus was dissected for gene expression analysis and glutamate assay. At the end, statistical analysis of the data was performed.

Fig. 2. EE reduced the infarct area. Representative images (A); Quantitative analysis of the infarct area (B). Sham standard environment (SS), ischemic standard environment (SI), sham enriched environment (ES) and ischemic enriched environment (EI). Data is expressed as mean ± SEM. Unpaired Student's t test. *p<0.05

Fig. 3. Short- and long-term memory assessment in the novel object recognition paradigm. Test phase of animals that underwent STM task (A). Test phase of animals that underwent LTM task (B). Sham standard environment (SS), ischemic standard environment (SI), sham enriched environment (ES) and ischemic enriched environment (EI). Data is expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001 and # p < 0.05 in relation to SC groups.

Fig. 4. Gene expression analyses in the hippocampus in short-term memory. Relative mRNA expression levels of GluN1 (A); GluN2A (B); GluN2B (C); GluN2C (D); α 7 (E); M1 (F); fibrillary acidic protein (GFAP) (G); Interleukin-1β (IL-1β) (H); and brain-derived neurotrophic factor (BDNF) (I). mRNA levels were normalized with GAPDH and data was expressed as mean ± SEM. *p < 0.05.

Fig. 5. Gene expression analyses in the hippocampus in long-term memory. Relative mRNA expression levels of GluN1 (A); GluN2A (B); GluN2B (C); GluN2C (D); α 7 (E); M1 (F); fibrillary acidic protein (GFAP) (G); Interleukin-1β (IL-1β) (H); and brain-derived neurotrophic factor (BDNF) (I). mRNA levels were normalized with GAPDH and data was expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$.

Fig. 6. Glutamate content in the hippocampus. Data was expressed as mean ± SEM.

Table 1: Primers used in qPCR.

Gene	Primer sequence	Amplicon length (bp)	Target sequence
Alpha 7	AAA GAG CCA TAC CCA GAT GTC ATG AGC AGA TTG AGG CCA TAG	77	NM_007390.3
GAPDH	CCTCGTCCCGTAGACAAAATG TTGACTGTGCCGTTGAATTTG	194	NM_001289726.1
GFAP	GAA AAC CGC ATC ACC ATT CC CAT CTC CAC AGT CTT TAC CAC G	126	NM_010277.3
GluN1	TGA CCC AGG AAC CAA GAA TG CTT GCC GTT GAT TAG CTG AGG	235	NM_008169.3
GluN2A	ATG ACT ATT CTC CGC CTT TCC AGT TTA CAG CCT TCA TCC CTC	220	NM_008170.2
GluN2B	GAA CGA GAC TGA CCC AAA GAG CAG AAG CTT GCT GTT CAA TGG	248	NM_008171.3
GluN2C	AGA TGG GGA AGC TGG ACG C CAG ATC CCT GAG AGC CAC AC	238	NM_010350.2
IL-1β	ACGGACCCCAAAAGATGAAG CACGGGAAAGACACAGGTAG	169	NM_008361.4
M1	TGG TTT CCT TCG TTC TCT GG GAG GAA CTG GAT GTA GCA CTG	98	NM_001112697.1

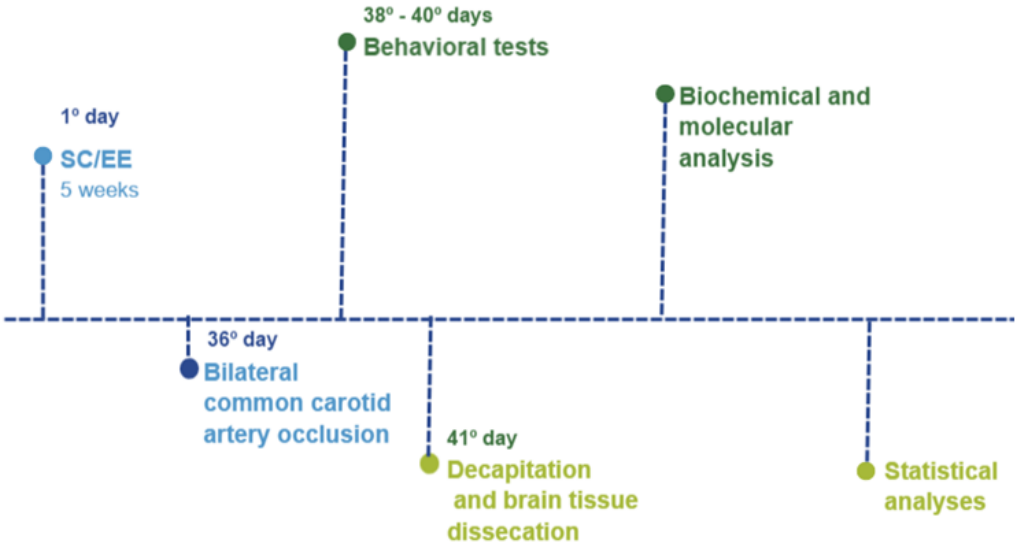

38º - 40º days
Behavioral tests
Biochemical and molecular analysis
1º day
SC/EE
5 weeks
36º day
Bilateral common carotid artery occlusion
41º day
Decapitation and brain tissue dissecation
Statistical analyses

Figure 1

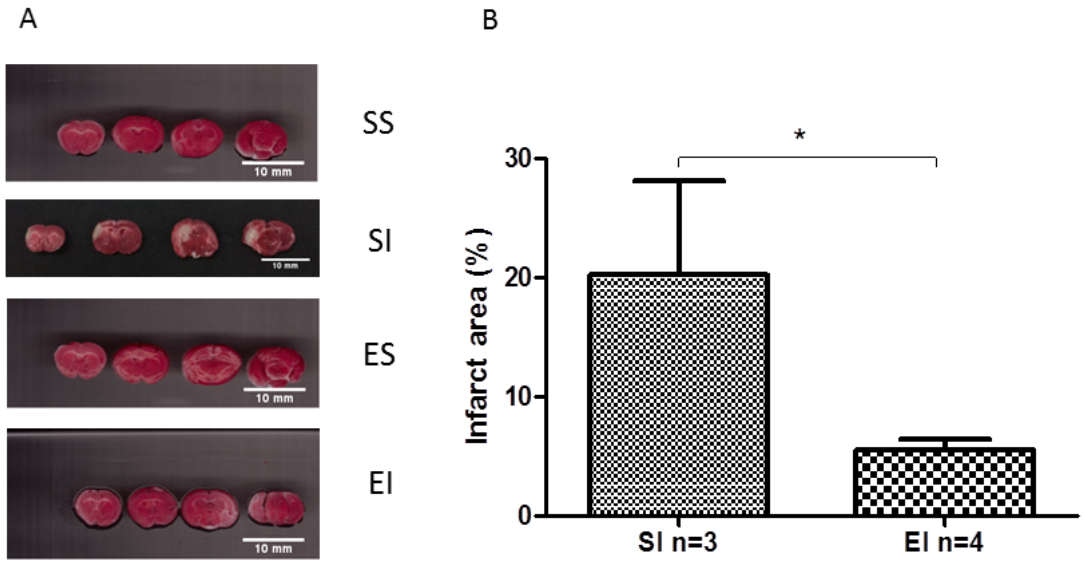
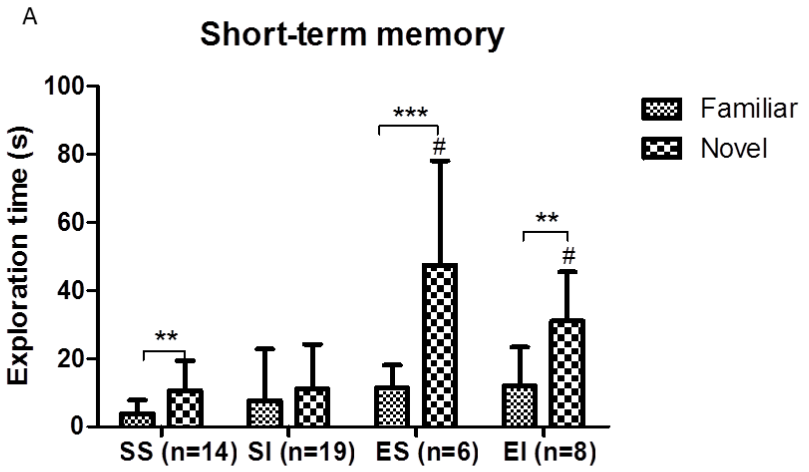


Figure 2


A
Short-term memory
Exploration time (s)
100
80
60
40
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Familiar
Novel
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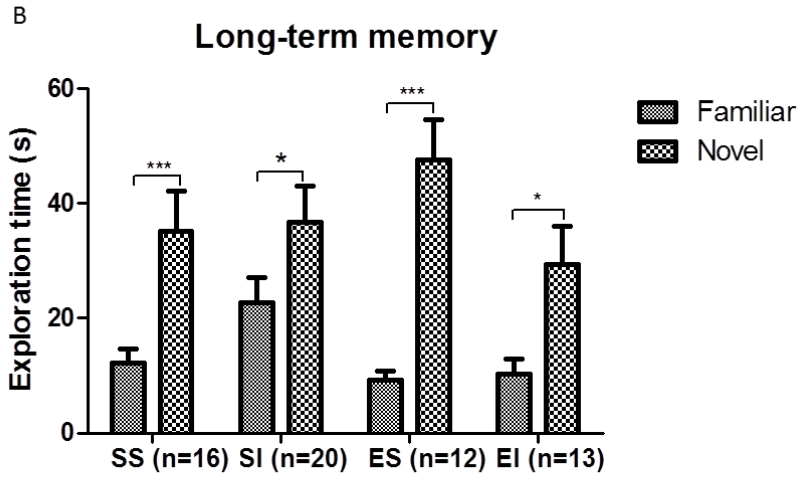
SS (n=14)
SI (n=19)
ES (n=6)
EI (n=8)


B
Long-term memory
Exploration time (s)
60
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SS (n=16)
SI (n=20)
ES (n=12)
EI (n=13)

Figure 3

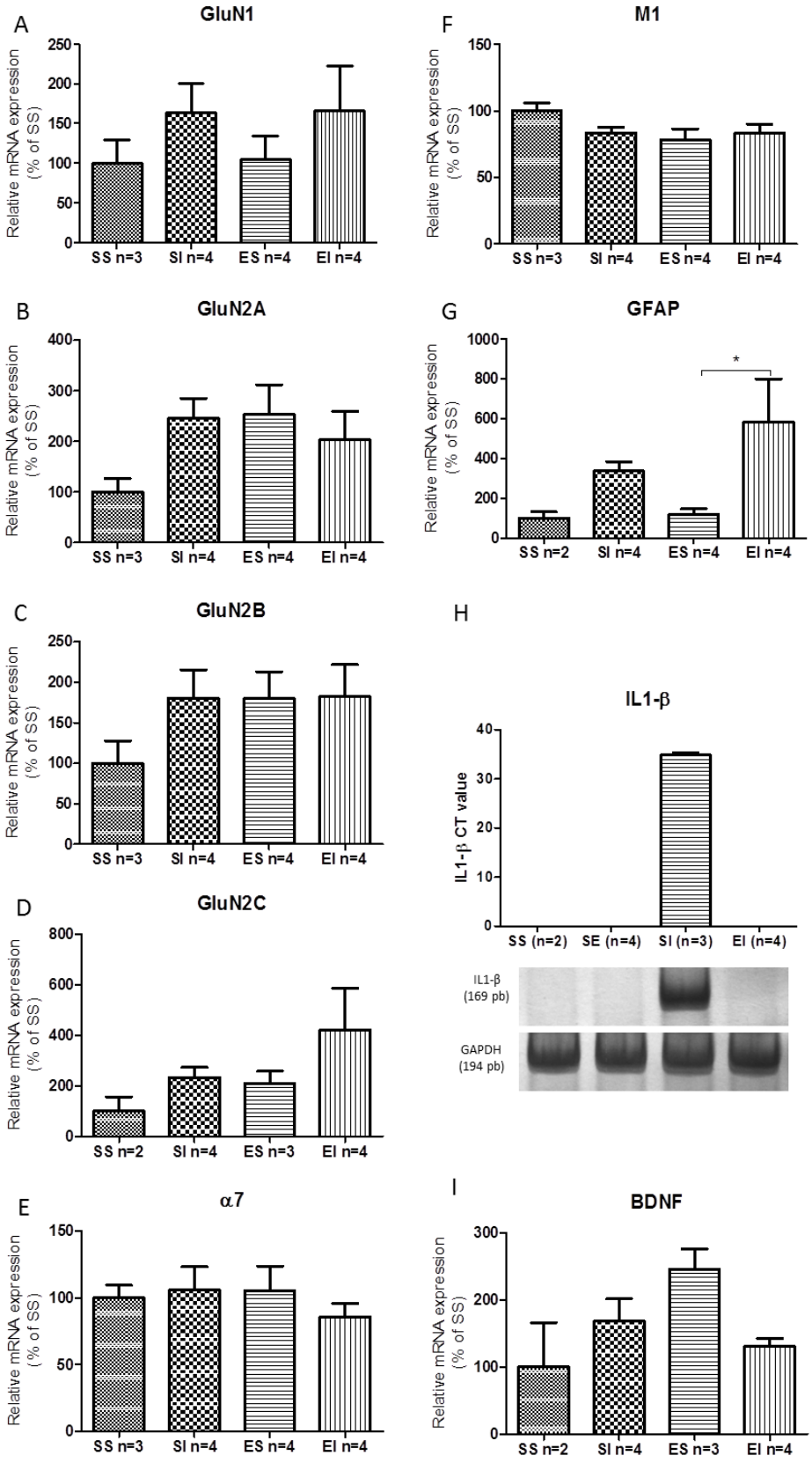
A
GluN1
Relative mRNA expression (% of SS)
SS n=3
SI n=4
ES n=4
EI n=4
F
M1
B
GluN2A
G
GFAP
SS n=2
C
GluN2B
H
IL1-β
IL1-β CT value
SS (n=2)
SE (n=4)
SI (n=3)
EI (n=4)
IL1-β (169 pb)
GAPDH (194 pb)
D
GluN2C
E
α7
I
BDNF

Figure 4

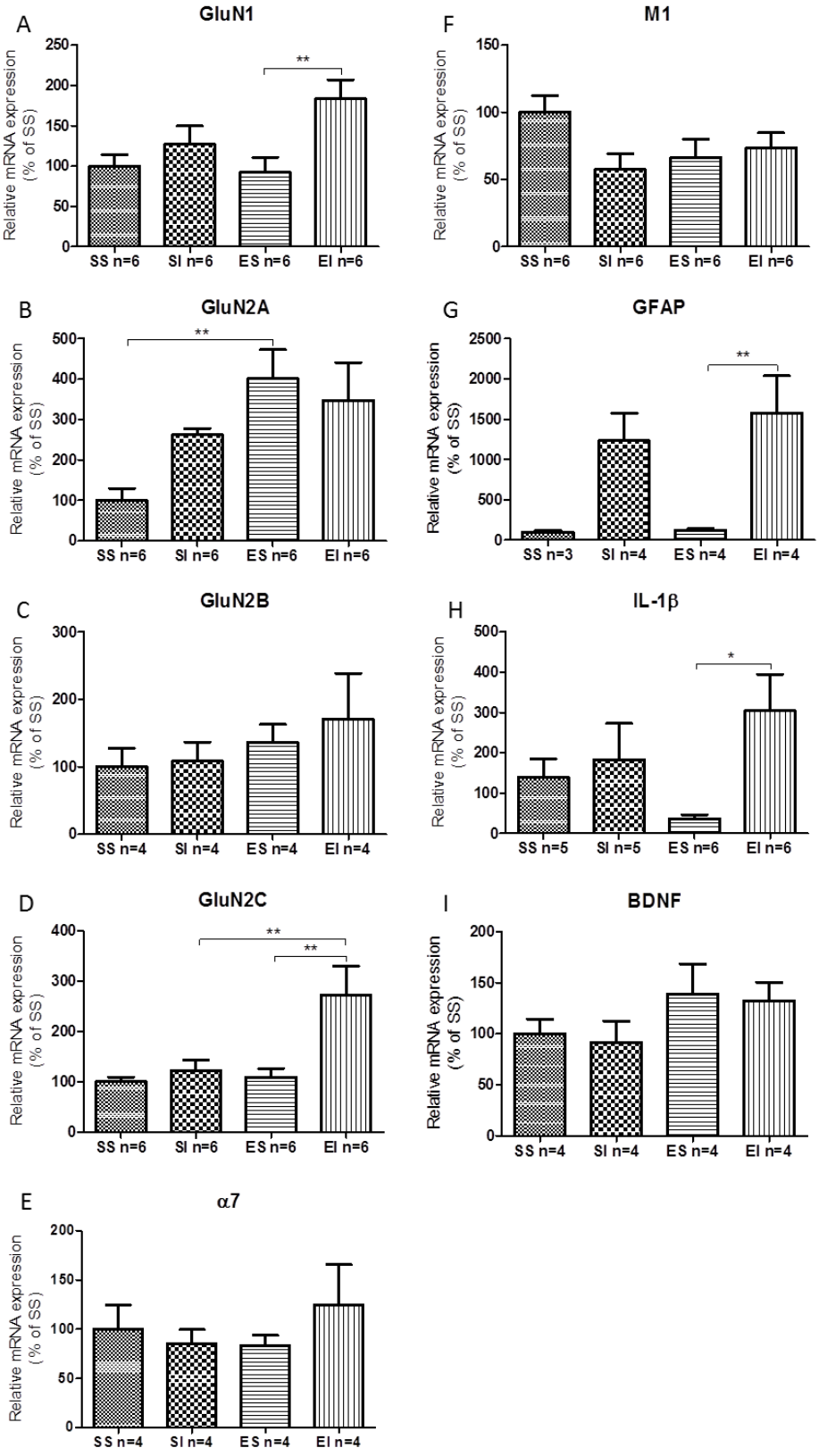
A
GluN1
Relative mRNA expression (% of SS)
SS n=6
SI n=6
ES n=6
EI n=6
B
GluN2A
SS n=6
SI n=6
ES n=6
EI n=6
C
GluN2B
SS n=4
SI n=4
ES n=4
EI n=4
D
GluN2C
SS n=6
SI n=6
ES n=6
EI n=6
E
α7
SS n=4
SI n=4
ES n=4
EI n=4
F
M1
SS n=6
SI n=6
ES n=6
EI n=6
G
GFAP
SS n=3
SI n=4
ES n=4
EI n=4
H
IL-1β
SS n=5
SI n=5
ES n=6
EI n=6
I
BDNF
SS n=4
SI n=4
ES n=4
EI n=4

Figure 5

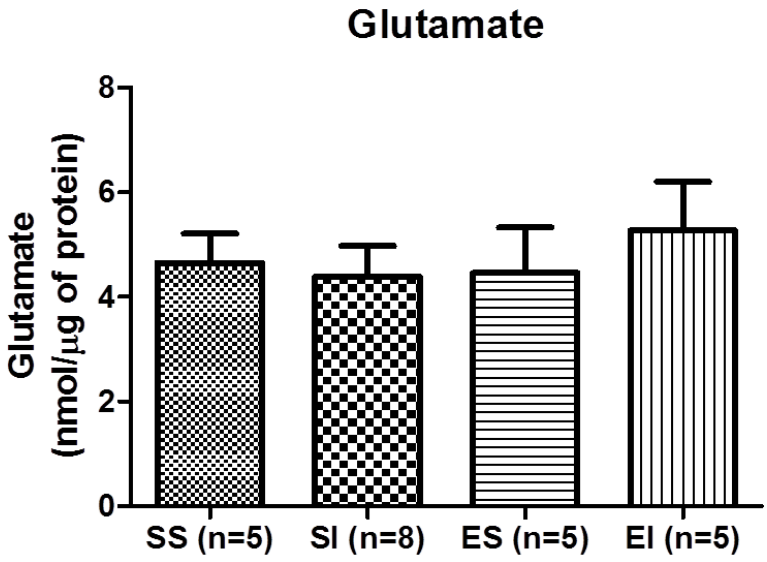
Glutamate
Glutamate (nmol/μg of protein)
8
6
4
2
0
SS (n=5)
SI (n=8)
ES (n=5)
EI (n=5)

Figure 6

Supplementary data

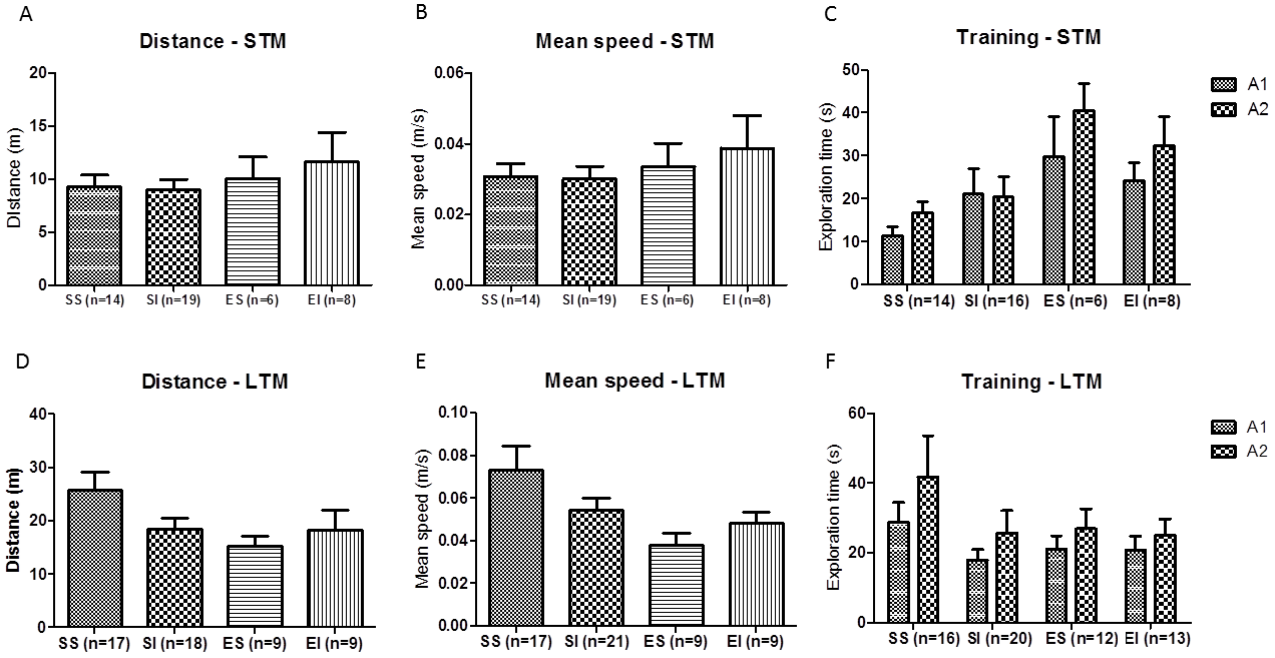

Fig. 1. STM: Distance traveled (A) and mean speed (B) evaluated during the training phase was not statistically different between SS, SI, ES and EI groups. The animals of SS, SI, ES and EI groups demonstrated similar interest for both objects (C). LTM: Distance traveled (D) and mean speed (E) evaluated during the training phase was not statistically different between SS, SI, ES and EI groups. The animals of SS, SI, ES and EI groups demonstrated similar interest for both objects (F). Sham standard environment (SS), ischemic standard environment (SI), sham enriched environment (ES) and ischemic enriched environment (EI). Data is expressed as mean ± SEM.

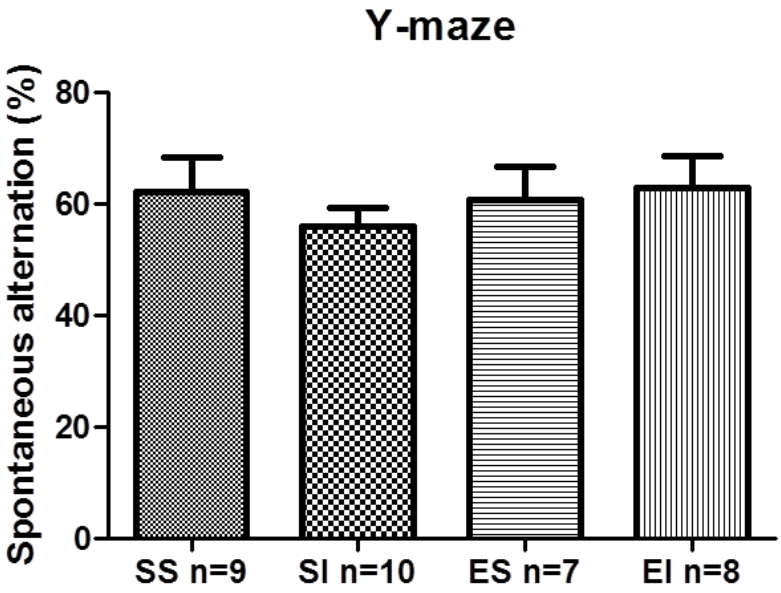

Fig. 2. Working memory. The ischemic animals did not present a working memory deficit. The EE did not influence the performance of the animals. Data is expressed as mean ± SEM.

APÊNDICE A

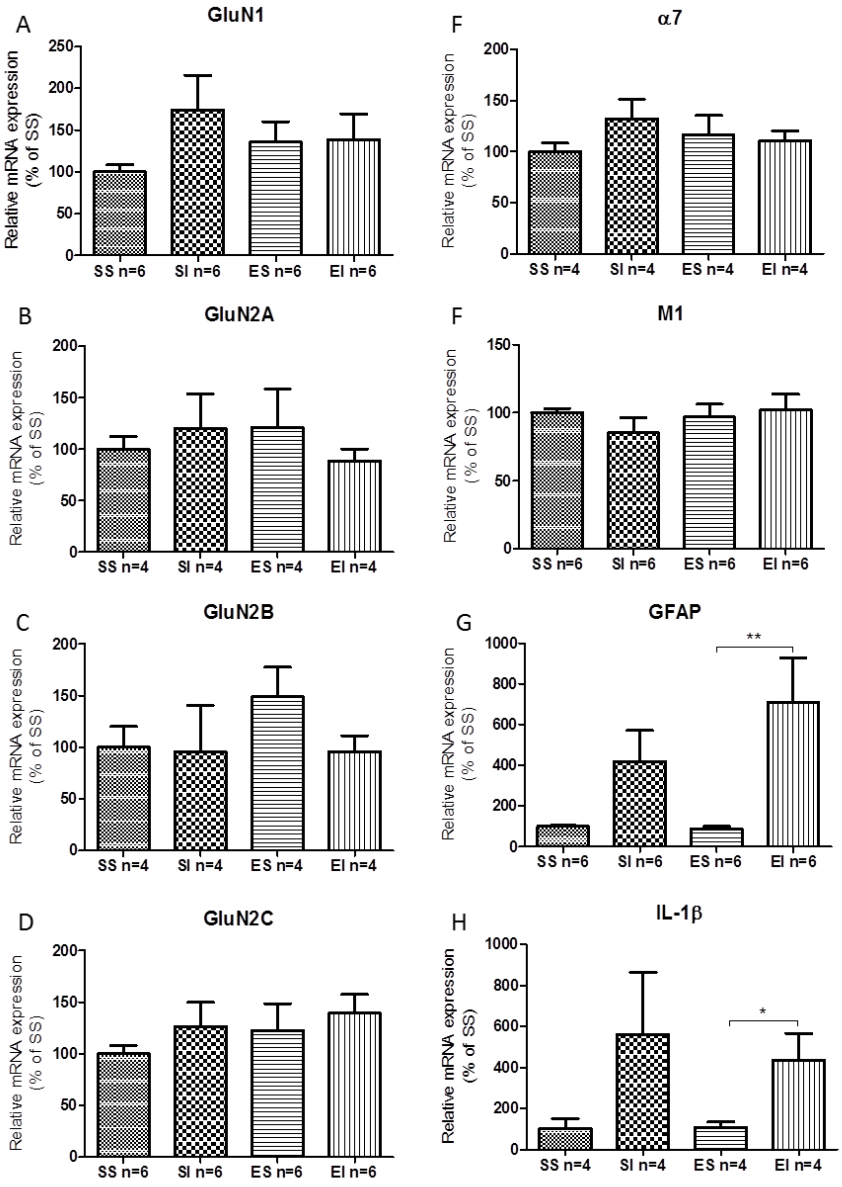

Figura 1. Análise da expressão gênica no córtex total de animais submetidos ao teste de memória de longa duração. A) GluN1 (F(1;20)= 1,760; 1,996); B) GluN2A (F(1;12)= 0,05275; p= 0,8222); C) GluN2B (F(1;12)= 0,9596; p= 0,3466); D) GluN2C (F(1;20)= 1,208; p= 0,2848); E) M1 (F(1;20)= 0,2421; p= 0,6281); F) Alfa7 (F(1;12)= 0,7964; p= 0,3897); G) GFAP (F(1;20)= 12,33; p= 0,0022); H) IL1B (F(1;12)= 5,505; p= 0,0370). Data were expressed as mean ± SEM. *p < 0.05; **p < 0.01.