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SARAH DE SOUZA ESCUDEIRO

EFEITOS DO ÁCIDO LIPÓICO EM DIFERENTES FASES DE DESENVOLVIMENTO DE CAMUNDONGOS EM MODELO DE PREFERÊNCIA AO ETANOL

FORTALEZA
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Tese de doutorado apresentada ao Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia-RENORBIO-UFC como parte dos requisitos para obtenção do título de Doutor em Biotecnologia. Área de concentração: Biotecnologia em Saúde

Orientadora: Profa. Dra. Silvânia Maria Mendes Vasconcelos

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RESUMO

No Brasil, estima-se que 11,7 milhões de pessoas sejam dependentes de álcool, e embora seja um problema de saúde milenar, os tratamentos disponíveis apresentam eficácia limitada. O ácido lipóico apresenta ação nos mecanismos relacionados ao abuso de drogas. A adolescência é um período de grande vulnerabilidade neuroadaptativa. O presente estudo levanta a hipótese de que o ácido lipóico pode influenciar a preferência ao etanol em diferentes fases do desenvolvimento. Assim, esta pesquisa investigou a ação do ácido lipóico, no período de desenvolvimento da adolescência à fase adulto jovem, sobre os efeitos reforçadores do etanol. Camundongos *Swiss* machos foram divididos em três protocolos de tratamento: agudo-adolescente; agudo-adulto; e crônico, com diferentes animais por grupo. Nos protocolos agudos animais foram tratados com etanol (EtOH) (2g/kg-v.o.), ácido lipóico (AL) (100mg/kg) ou associação (AL+EtOH) e submetidos aos testes comportamentais de campo aberto, Y-maze e preferência condicionada de lugar (PCL). No protocolo crônico animais foram tratados 14 dias com veículo ou AL, e depois com EtOH ou AL+EtOH por 21 dias. Após os testes comportamentais, foi realizada análise neuroquímica (quantificação de BDNF, IL-6, TNFα, TBARS e GSH) nos cérebros dos animais dos protocolos agudo-adolescente e crônico. Na adolescência AL preveniu o aumento da atividade locomotora induzida por etanol, mas na idade adulta apresentou efeito estimulante. A memória foi prejudicada com exposição aguda e crônica de EtOH na adolescência. AL não teve efeito preventivo sobre o prejuízo agudo da memória, mas cronicamente foi capaz de prevenir os déficits de memória induzidos por EtOH. Na adolescência AL induziu PCL quando administrado agudamente antes do EtOH, mas na idade adulta, o mesmo tratamento foi capaz de impedir a aquisição de PCL induzida pelo EtOH. Administração crônica de AL+EtOH induziu forte sensibilização à preferência ao etanol. Administração aguda de EtOH não alterou a concentração de BDNF, mas a exposição crônica reduziu BDNF. Este parâmetro também diminuiu nos grupos tratados com AL+EtOH aguda e cronicamente. As citocinas IL-6 e TNFα se comportaram de forma semelhante nos tratamentos agudo e crônico. Tratamento agudo com EtOH ou AL não alterou suas concentrações, mas AL+EtOH reduziu este parâmetro. EtOH administrado cronicamente diminuiu as concentrações de citocinas, independentemente da associação com AL. Houve aumento de GSH em todos os grupos que apresentaram PCL. Tratamento agudo com EtOH reduziu a peroxidação lipídica, enquanto uso crônico, aumentou. O AL foi capaz de evitar esse aumento de peroxidação lipídica induzido pelo etanol quando co-administrado a ele. Nossos dados demonstram que o etanol age de forma diferente dependendo do tempo de tratamento e fase de

desenvolvimento, tendo em alguns casos seus efeitos prevenidos pelo AL. Os efeitos do AL dependem fortemente do período em que é administrado, e podem interagir de modo diferente com efeitos de reforço de etanol. Além disso, AL não foi capaz de reduzir os efeitos degenerativos de longo prazo induzidos pelo etanol e apresenta efeitos reforçadores sobre as propriedades neuroquímicas de reforço provocadas pela preferência induzida por etanol.

Palavras-chave: ácido tióctico; etanol; desenvolvimento; transtornos relacionados ao uso substâncias.

ABSTRACT

In Brazil, it is estimated that 11.7 million people are dependent on alcohol, and although it is a millenar complication of health, the available treatments have limited effectiveness. Lipoic acid, a powerful antioxidant, has shown to interfere on mechanisms related to drug abuse. Adolescence represents a period of high neuroadaptive vulnerability. The present study raises the hypothesis that lipoic acid may influence the preference to ethanol at different stages of development. Thus, this research investigated the action of lipoic acid during the developmental period since adolescence until young adulthood on the reinforcing effects of ethanol. Male *Swiss* mice were used for the three protocols (acute-adolescent, acute-adult; chronic) with different animals for each group. In acute-adolescent and acute-adult protocols, animals were acutely treated with ethanol (EtOH) (2 g/kg, p.o.), lipoic acid (LA) (100 mg/kg, p.o.) or association (LA+EtOH) and subjected to behavioral tests open field, Y-maze and conditioned place preference (PCL). Chronic protocol was initiated with animals treated for 14 days with vehicle or LA. After this, animals were treated for 21 days with EtOH or LA+EtOH. Behavioral tests were then started. After behavioral tests, the animals brain were used for neurochemical analysis involving dosage of BDNF, cytokines (TNF and IL-6), TBARS and GSH. Lipoic acid behaved in different ways according to the period of life in which was used. On adolescence LA was able to prevent EtOH-induced locomotor activity, but in adulthood it presented stimulant effect. Memory was impaired with acute (in adolescence) and chronic EtOH exposure, and LA had no preventive effect on memory impairment on adolescence but was able to prevent memory deficits induced by chronic EtOH. While in adolescence LA induced CPP when acutely administered prior to EtOH, on adulthood the same treatment was able to prevent the acquisition of EtOH-induced CPP. LA administration chronically with EtOH induced strong sensitization for ethanol preference. No preventive effect of LA during adolescence was observed in chronic EtOH-induced CPP. No significant alteration on BDNF concentration was observed with acute EtOH administration, but chronic exposure to this drug until young adulthood decreased it. BDNF was also reduced in groups treated with the association of LA and EtOH both acutely and chronically over adolescence. Cytokines IL-6 and TNFα behaved in a similar way for both acute and chronic treatment. Acute treatment with EtOH or LA did not change cytokine concentrations, but LA administration prior to EtOH induced a significant decrease of them. When chronically administered over adolescence EtOH curiously decreased cytokine concentrations, independently of the association with LA. The increase of GSH levels was observed when LA and LA+EtOH were acutely administered in adolescence (PFC and ST)

and in all groups that received chronically EtOH with or without association to LA since adolescence over young adulthood (ST). A decrease in lipid peroxidation was shown on EtOH acute treatment, while chronic EtOH administration increased this parameter. LA was able to prevent this raise of EtOH-induced lipid peroxidation when co-administered with it since adolescence until young adulthood. Our data suggest that ethanol acts in different way depending of treatment time and developmental stage, having in some cases its effects prevented by LA. Lipoic acid effects strongly depends of the period in which is administered, and can interact in different way with reinforcing effects of ethanol. Furthermore, LA fail to reduce long-term degenerative effects induced by ethanol and shows reinforcing effects on behavioral and neurochemical rewarding properties elicited by ethanol-induced preference.

Keywords: thioctic acid; ethanol; development; substance-related disorders.

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1 INTRODUÇÃO

O uso do álcool é o terceiro principal fator de risco para problemas de saúde no mundo. Além disso, provoca 2,5 milhões de mortes a cada ano e acarreta custos sociais exorbitantes, sendo um problema global que compromete o desenvolvimento social e individual. Vários países reconhecem os graves problemas de saúde pública causados pelo uso nocivo de álcool, buscando adotar políticas e programas de prevenção (WHO, 2011).

Esta preocupação em torno do uso abusivo do álcool gerou a publicação sobre a estratégia global para reduzir o uso nocivo do álcool, da Organização Mundial de Saúde (OMS) (WORLD HEALTH ORGANIZATION, 2010), e após 1 ano, foi publicado o relatório mundial sobre álcool e saúde (WHO, 2011), abordando o consumo de álcool, suas consequências, e as políticas e intervenções voltadas a minimizar os danos sociais e de saúde causados por seu consumo.

De acordo com o II Levantamento Nacional de Álcool e Drogas (LARANJEIRA, 2013), divulgado em abril de 2013, estima-se que 11,7 milhões de pessoas sejam dependentes de álcool no Brasil. Entre os adultos, um total de 54% revelou consumir álcool regularmente.

O uso do álcool está enquadrado como normas sociais, sendo parte de rituais religiosos para algumas culturas e considerado abuso para outras (FERNANDES, 2002). Dez mil anos atrás a cerveja foi feita a partir de grãos através de um processo descoberto acidentalmente, e oito mil anos atrás o vinho foi consumido. Em 2000 antes de Cristo, Hammurabi, o líder da Babilônia, estabeleceu regras para venda e compra de vinho. Durante o meio século XVIII o álcool assumiu a posição do ingrediente intoxicante de muitas bebidas (RUNDIO, 2013).

Pesquisas também correlacionam o aumento do consumo de álcool com o capitalismo. Devido às transformações atuais provocadas pelo modo de produção capitalista, o ser humano se depara com situações que possam provocar sofrimento psíquico e conseqüentemente levar ao adoecimento e consumo de bebidas alcoólicas (E MOTA, 2014).

Evidências sugerem que as pessoas socialmente desfavorecidas ou aqueles que vivem em zonas socialmente desfavorecidas experimentam mais danos por grama de álcool consumida do que as pessoas com maiores vantagens sociais (OMMA; SANDLUND, 2015).

Além disso, fatores genéticos predispõem determinados tipos de pessoas à vulnerabilidade para desencadear abuso de substâncias e dependência. Nem todas as pessoas são igualmente predispostas a desenvolverem vício de uma droga. Estudos de famílias, gêmeos e filhos adotados sugerem que a herdabilidade de transtornos por uso de substâncias é de

moderado a elevado, com importância também para os fatores ambientais que são compartilhados ou não (WONG; MILL; FERNANDES, 2011).

A literatura descreve que fatores genéticos e ambientais não agem sozinhos para o aumento da susceptibilidade ao vício. Por exemplo, um aumento de aproximadamente cinco vezes na influência genética sobre o consumo de álcool é observado em ambientes urbanos em comparação com ambientes rurais (WONG; MILL; FERNANDES, 2011).

Muitas pesquisas sobre dependência a drogas de abuso, assim como fatores de risco por seu uso, têm focado na adolescência, por ser um período crítico de desenvolvimento que é frequentemente associado com o surgimento do uso de substâncias e riscos comportamentais advindos deste uso (WETHERILL; TAPERT, 2012; WINDLE et al., 2009; WITT, 2010) . A prevalência do uso de substâncias na adolescência e as consequências negativas associadas a isso enfatiza a necessidade para tratamentos e intervenções baseados em evidências nesta população.

Estudos sugerem que características temperamentais e comportamentais observadas na infância predizem resultados subsequentes relacionados ao uso do álcool na posteridade (DICK et al., 2013), portanto, a grande susceptibilidade adaptativa deste período promove uma janela de oportunidade para pesquisas com abordagem preventiva, garantindo a integridade neurodesenvolvimental e minimizando a probabilidade do desenvolvimento da dependência a drogas de abuso na fase adulta.

Visando diminuir a grande incidência do alcoolismo, diversos estudos tem buscado descobrir um tratamento eficaz para esse sério problema de saúde pública (LEE; LEGGIO, 2014; SKINNER et al., 2014). Por se manifestar em diferentes fases do desenvolvimento humano, e a cada geração se apresentar mais cedo à população (WHO, 2011), a prevenção se torna um foco importante a ser destacado. Com abordagens que perpassam fatores psicológicos, sociais, econômicos, dentre outros, mais uma alternativa a se considerar é o uso de substâncias que possam prevenir o desenvolvimento deste grave problema.

Dentre possíveis substâncias que apresentam alguma interferência nos mecanismos relacionados ao abuso de drogas, e já demonstram efeito benéfico ao sistema nervoso central, com grande potencial em prevenir e tratar condições neuropatológicas/neurodegenerativas, está o ácido lipóico, um potente antioxidante natural produzido no corpo humano, já comercializado no Brasil para o tratamento de sintomas da polineuropatia diabética periférica, porém, ainda sem aprovação pela *Food and Drug Administration* (FDA) (MINISTERIO DA SAUDE, 2012).

Os efeitos do ácido lipóico relacionados ao abuso de drogas ainda não estão totalmente elucidados, principalmente se tratando de sua relação com o uso do álcool durante

o período da adolescência. Portanto, se faz necessário buscar elucidar essa complexa relação entre o período desenvolvimental da adolescência e o uso de substâncias que apresentam potencial para desenvolvimento de vício/dependência e neurodegeneração, como o álcool, e potencial neuroprotetor, como o ácido lipóico, uma vez que pesquisas têm associado o aumento de agentes oxidantes no cérebro com os efeitos reforçadores do etanol e, portanto, antioxidantes que diminuam essas espécies reativas de oxigênio seriam capazes de reduzir a aquisição e recondicionamento de reforço da droga (LEDESMA; ARAGON, 2013).

2 REVISÃO DE LITERATURA

2.1 Fisiopatologia da dependência ao álcool

Também conhecido como álcool etílico ou álcool de biomassa, o etanol é produzido através de uma biomassa contendo açúcares, amido ou material celulósico (PAVLAK et al., 2007), podendo ser encontrado em bebidas alcoólicas, produtos de limpeza, perfumes, fitoterápicos e combustíveis (CANCIAM, 2013).

Por ser uma molécula pequena, solúvel em água e lipídios, sua difusão através das membranas biológicas procede-se de modo rápido, permeando todos os tecidos, podendo causar uma ampla variedade de alterações sistêmicas (VEIGA et al., 2007).

A concentração de álcool nos tecidos é proporcional ao conteúdo em água destes, além disso, a velocidade de acumulação é determinada pelo fluxo sanguíneo, processando-se assim rapidamente em órgãos com irrigação abundante, como o encéfalo (GUIMARÃES; MOURA; SILVA, 2014).

A metabolização do etanol ocorre em duas fases. O álcool é convertido a acetaldeído, e o acetaldeído é convertido em acetato. A enzima álcool desidrogenase (ADH) realiza a reação química (catálise) na primeira metade do metabolismo do álcool, e a enzima aldeído desidrogenase (ALDH) catalisa a segunda metade. A coenzima nicotinamida adenosina dinucleotídeo (NAD) desempenha um papel acessório nas reações e aceita átomos de hidrogênio (PONNAPPA; RUBIN, 2000).

Dentre os diversos mecanismos envolvidos na fisiopatologia da dependência ao álcool (figura 1), pode-se incluir alteração nos sistemas de neurotransmissão (SCHILATY et al., 2014), alterações gênicas (ZAKHARI, 2013), alteração nas vias de neuroplasticidade (MOONAT et al., 2010), alteração em vias relacionadas à neuroinflamação (HEBERLEIN et al., 2014) e produção de estresse oxidativo (BOYADJIEVA; SARKAR, 2013).

Essas alterações são observadas principalmente nas vias neurais que perfazem o sistema de recompensa (figura 2), principal mecanismo biológico envolvido com os efeitos reforçadores do etanol, assim como de outras drogas de abuso. O sistema de recompensa envolve neurônios dopaminérgicos originados na área tegumentar ventral (ATV) que se projetam para o núcleo *accumbens* e córtex pré-frontal (CPF). O córtex pré-frontal, por sua vez, projeta neurônios glutamatérgicos para o núcleo *accumbens* e área tegumentar ventral, e o núcleo *accumbens* modula o funcionamento da ATV por meio de neurônios GABAérgicos (ALMEIDA, 2006).

Figura 1 Representação esquemática dos mecanismos envolvidos com o abuso do álcool.

Fonte: Arquivo pessoal do autor.

O CPF medial responde ao resultado da recompensa: ele é ativado se uma recompensa esperada é recebida e desativado quando não é recebida. O córtex frontal orbital (CFO) codifica os resultados esperados e faz uma estimativa do valor motivacional com base no potencial de recompensa. O *accumbens* (região estriatal ventral) responde à intensidade, valência (preferência ou aversão) e a previsibilidade da recompensa (recompensa imprevisível ativa esta região mais que uma recompensa previsível) (BRENHOUSE; ANDERSEN, 2011).

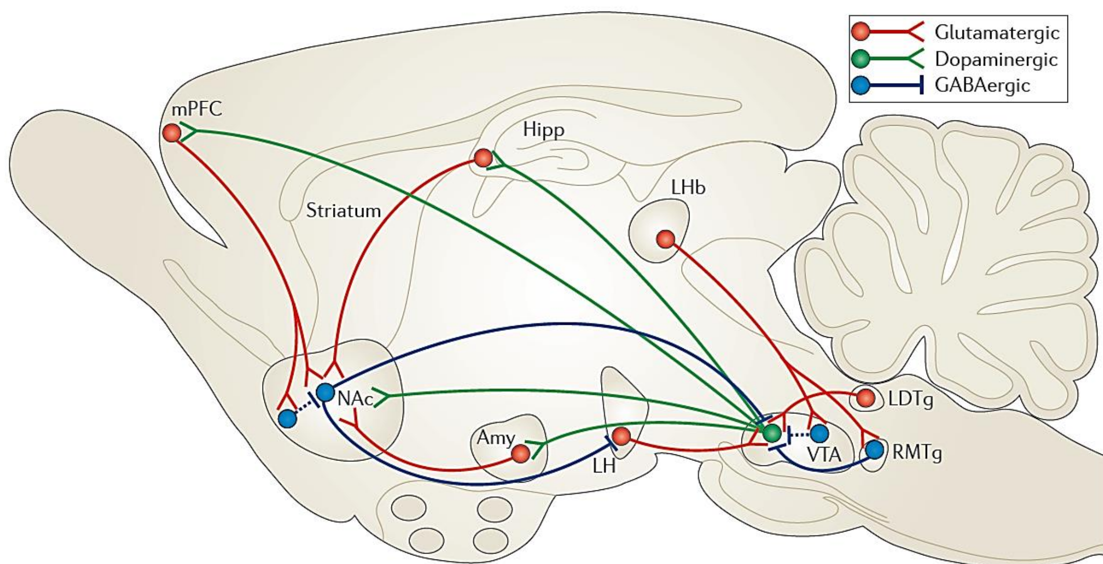
O sistema dopaminérgico tem um papel crucial na dependência de várias substâncias psicoativas, pois influencia o mecanismo de recompensa cerebral (DICK; FOROUD, 2003; TUPALA; TIIHONEN, 2004). Através da estimulação de receptores dopaminérgicos, o etanol promove a liberação de dopamina no corpo estriado, levando a um aumento no consumo dessa substância (KIENAST; HEINZ, 2006).

A exposição ao álcool altera também vários aspectos das vias serotonérgicas, refletindo principalmente em uma intensificação nas sinapses envolvendo a 5-HT, verificada

pelo aumento dos níveis dos metabólitos serotonérgicos na urina e no sangue (LEMARQUAND; PIHL; BENKELFAT, 1994), e pelo aumento de suas concentrações no hipocampo e corpo estriado (VASCONCELOS et al., 2004). Em experimento com ratos, Ding et al. (2009) observaram que os efeitos reforçadores do etanol na área tegumentar ventral são modulados pela ativação de receptores serotonérgicos do tipo 2A.

A administração aguda de etanol produz um efeito bifásico na liberação de norepinefrina (NE): doses baixas levam a um aumento na liberação, tendo uma resposta inversa em doses maiores (ROSSETTI et al., 1992). Além disso, Huttunen (HUTTUNEN, 1991) demonstra em estudos cerebrais que o tratamento crônico do etanol resulta em um aumento na liberação de NE no hipocampo.

Figura 2 Estruturas e vias neurais que integram o circuito de recompensa



Desenho esquemático das principais conexões das vias dopaminérgica, glutamatérgica e GABAérgica de e para a área tegumentar ventral (VTA) e núcleo accumbens (NAc) no cérebro de roedores. mPFC: córtex pré-frontal medial; Hipp: hipocampo; Amy: amigdala; LH: hipotálamo lateral; LHb: habênula lateral; LDTg: tegumento lateral dorsal; RMTg: tegumento rostromedial. Fonte: Russo e Nestler (2013).

O fator neurotrófico derivado do cérebro (BDNF) é uma proteína pertencente a uma família de proteínas homólogas conhecidas como neutrofinas, e tem um papel importante no desenvolvimento, assim como em processos relacionados à plasticidade cerebral como a memória e o aprendizado (YAMADA; MIZUNO; NABESHIMA, 2002). As ações celulares das neutrofinas são mediadas através de dois tipos de receptores: um receptor tirosina quinase (Trk) de alta afinidade e um receptor pan-neurotrófico de baixa afinidade (p75NTR) (HALLBOOK, 1999).

O nível de expressão da proteína BDNF em humanos é regulado durante o desenvolvimento, mas persiste em muitas partes do cérebro adulto, sendo mais abundante no sistema nervoso, em comparação com outros tecidos no período adulto (KATOH-SEMBA et al., 1997). O BDNF exerce efeitos na transmissão sináptica e neurogênese. Em geral, BDNF parece fortalecer sinapses excitatórias (glutamatérgicas) e enfraquecer sinapses inibitórias (GABAergicas) (KANG; SCHUMAN, 1995). O BDNF é capaz de aumentar a neurogênese, e a infusão intraventricular de BDNF é capaz de aumentar o número de neurônios no bulbo olfatório, corpo estriado, septo e tálamos de adultos (BENRAISS et al., 2001).

O BDNF tem sido apontado como tendo ambos papéis positivos e negativos em transtornos psiquiátricos e vício (CARNICELLA; RON; BARAK, 2014; GHITZA et al., 2010), apresentando também participação na regulação de comportamentos associados ao uso do etanol. O aumento da expressão de BDNF está relacionada com uma redução no consumo de etanol (JEANBLANC et al., 2009). Estudos em modelos animais suportam a possibilidade de que o BDNF é parte de uma via homeostática que controla alguns dos efeitos adversos associados à exposição ao etanol (RON; MESSING, 2013).

Além das mudanças nas vias neurotróficas de reparo e regeneração, o abuso de drogas também interfere nas vias de sinalização neuroimune, como ocorre pela ativação do fator nuclear-kappa B (NF-κB), um fator de transcrição envolvido na lesão cerebral e condições neurodegenerativas (VETRENO; CREWS, 2014). O NF-κB regula a transcrição de citocinas como IL-6 e TNFα, dentre outras substâncias. Evidências recentes sugerem que vias de sinalização do NF-κB regulam o circuito de recompensa em modelos de depressão e vício (RUSSO; NESTLER, 2013).

Citocinas são pequenas proteínas que além de fazer parte do sistema imunológico, tem sido visto um papel bem maior na fisiologia do organismo humano. Há evidências que citocinas como IL-6 seja liberada por células da microglia, neuronais, astrócitos e células endoteliais no Sistema Nervoso Central (ERTA; QUINTANA; HIDALGO, 2012). Várias citocinas modulam a funcionalidade neuronal. A IL-6 pode afetar a funcionalidade neuronal, por exemplo, através da indução do fenótipo colinérgico de neurônios simpáticos, apresentando um notório papel na neurogênese no adulto (BAUER; KERR; PATTERSON, 2007; MARZ et al., 1998).

É descrita na literatura a associação de citocinas com a sintomatologia do uso do álcool (FREEMAN et al., 2012; LECLERCQ et al., 2012). Embora os mecanismos pelos quais a liberação de citocinas pode afetar o consumo de álcool sejam pouco esclarecidos, estudos

clínicos oferecem uma explicação reportando uma ligação entre a liberação de TNFα e IL-6 e a expressão de fatores neurotróficos de crescimento, como BDNF (HUANG et al., 2014).

Estudos com animais têm observado que a exposição ao álcool, tanto aguda como crônica, também interfere com a atividade antioxidante, demonstrando que o estresse oxidativo é responsável por diversos efeitos tóxicos associados ao consumo excessivo de álcool, seja agudo ou crônico (MCCARTY, 2013).

O cérebro consome altas taxas de oxigênio, e contêm altos níveis de lipídios peroxidáveis, aminoácidos citotóxicos e baixos níveis de antioxidantes. O tecido neural contém fontes de estresse oxidativo como aminoácidos excitatórios e neurotransmissores cujo metabolismo produz espécies reativas ao oxigênio (PACKER; TRITSCHLER; WESSEL, 1997). Da mesma forma, no cérebro, a oxidação da dopamina pela monoamina oxidase libera peróxido de hidrogênio como um produto metabólico que provoca a lesão tecidual, incluindo a peroxidação lipídica, danos ao DNA, e inativação de enzimas (HALLIWELL, 2006).

A glutationa (GSH) é um tripeptídeo encontrado intracelularmente em altas concentrações. Este tripeptídeo possui um papel central na biotransformação e eliminação de xenobióticos e na defesa das células contra o estresse oxidativo. A glutationa é um antioxidante endógeno primário capaz de neutralizar espécies reativas de oxigênio e nitrogênio nas células tanto direta quanto indiretamente (DRINGEN; HIRRLINGER, 2003).

No Sistema Nervoso Central, células gliais contêm níveis bem maiores de GSH que células neuronais e, também, ajudam na produção neuronal de GSH. Segundo Dringen e Hirrlinger (2003) acredita-se que a produção de GSH neuronal é primariamente mediada pela liberação astrocitária de GSH para o meio extracelular, e a taxa de produção de GSH pelo astrócito é limitada pela concentração de cisteína e pela glutamato-cisteína ligase. Em se tratando do consumo de etanol, enquanto a exposição aguda ao álcool etílico parece não afetar os níveis de GSH ou GSH peroxidase no cérebro e plasma de ratos e etilistas, a exposição crônica reduz consistentemente os níveis de GSH ou GSH peroxidase (SOMMAVILLA et al., 2012). Além da relação com a GSH, pesquisas têm associado o aumento de H_2O_2 no cérebro com os efeitos reforçadores do etanol, e portanto, antioxidantes que diminuam essa espécie reativa de oxigênio seriam capazes de reduzir a aquisição e recondicionamento de reforço da droga (LEDESMA; ARAGON, 2013).

2.2 Dependência alcoólica e neurodesenvolvimento

A tomada de decisão é o arquétipo de comportamentos motivados e implica a seleção de uma opção dentre outras alternativas. O processo completo de tomada de decisão engloba uma série de operações elementares como avaliação discriminativa/comparativa das opções; formação de uma preferência; execução da preferência; antecipação do resultado da ação; resposta ao resultado e atualização do valor das opções. Perturbações em qualquer etapa desta sequência podem prejudicar a qualidade da tomada de decisão (ERNST; ROMEO; ANDERSEN, 2009).

A dependência ao álcool pode ser caracterizada por aspectos como o desejo compulsivo pelo uso da droga ("fissura"), além da perda de controle, e incapacidade de limitar a própria bebida em qualquer ocasião; a tolerância, necessidade de beber cada vez maiores quantidades de álcool para obter o mesmo efeito desejado; e dependência física, que inclui os sintomas de abstinência, tais como náusea, irritabilidade, diaforese, tremores, ansiedade, taquicardia, alucinações e convulsões. Isso ocorre quando o álcool é abruptamente interrompido após um período bebendo intensamente (RUNDIO, 2013).

Para estudar a neurobiologia do reforço de drogas (BARDO; BEVINS, 2000), como também os circuitos neurais envolvidos na recompensa (CARR; WHITE, 1986), o modelo animal de preferência condicionada de lugar (PCL) é amplamente utilizado. Este, é usado para mensurar os efeitos reforçadores de drogas comumente abusadas por humanos e está baseado na capacidade do animal em associar o efeito induzido pela droga com pistas ambientais (CUNNINGHAM; GREMEL; GROBLEWSKI, 2006).

Uma extensa revisão sobre o paradigma da preferência condicionada de lugar abrangendo, dentre outros assuntos, questões metodológicas, estudos usando tratamentos com drogas sistêmicas, injeções intracranianas, modelos genéticos, tolerância e sensibilização aos efeitos de recompensa de drogas, publicados entre o período de 1998-2006, foi realizada por Tzschentke (TZSCHENTKE, 2007).

Pesquisas considerando o sistema de recompensa, reforço de drogas, e suas implicações no desenvolvimento da dependência têm realizado estudos enfocando diferentes faixas etárias. Isso se deve ao fato do período da adolescência ser muito susceptível a mudanças neurais, o que o torna vulnerável, no caso do abuso de drogas, a apresentar como consequência uma maior propensão ao desenvolvimento da dependência na fase adulta, como demonstram estudos nos quais a exposição precoce ao consumo inicial de etanol, especialmente durante o início da adolescência (i.e. pré-puberdade), mostrou-se estar associada com uma

susceptibilidade aumentada ao abuso do etanol (MALDONADO-DEVINCCI et al., 2010; PASCUAL et al., 2009; STRONG et al., 2010).

Em estudo realizado com animais, sobre os efeitos de uma intoxicação semelhante a uma ‘bebedeira’ na faixa etária equivalente à adolescência, sobre a vulnerabilidade ao abuso de álcool, foi demonstrado que repetidas intoxicações com etanol durante a adolescência aumentaram a ingestão diária de etanol e motivação para beber etanol na fase adulta comparado com ratos sem histórico de intoxicação alcoólica (ALAUX-CANTIN et al., 2013). Além disso, a exposição precoce ao etanol também pode contribuir para o desenvolvimento do comportamento de dependência relacionado a outras drogas como cocaína e morfina na fase adulta (MOLET et al., 2013).

As alterações comportamentais que ocorrem durante a adolescência são parcialmente relacionadas à maturação do córtex pré-frontal e suas interconexões com o sistema límbico (o qual regula experiências emocionais, memória e aprendizado). Essas áreas e conexões formam parte do circuito neural que modula o valor motivacional do álcool e outros estímulos reforçadores. A exposição ao etanol durante esse período de rápido desenvolvimento cerebral altera a função neurocognitiva e causa posterior propensão para o uso problemático do álcool (SPEAR, 2002).

2.3 Ácido lipóico

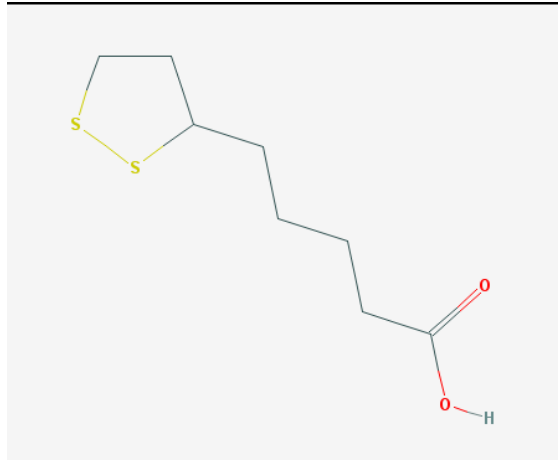
O ácido alfa-lipóico (AL) (ácido 1,2-ditiolano-3-pentanoico) foi originalmente isolado do fígado bovino por Reed et al. (1951). É um antioxidante natural sintetizado no corpo humano, e juntamente com sua forma reduzida, o ácido dihidrolipóico (DHLA), são componentes naturais das membranas biológicas, sendo encontrados nas plantas e nos animais (GORĄCA et al., 2011).

Em sua fórmula estrutural (figura 3) existem dois grupos tióis que podem ser oxidados ou reduzidos, portanto, é um par redox. Tanto na forma oxidada (AL) quanto na reduzida (DHLA), o ácido lipóico aproxima-se muito do ideal como antioxidante, apresentando especificidade na eliminação de radicais (FERREIRA; MILITÃO; FREITAS, 2009). O AL exógeno é rapidamente absorvido pelas células e no espaço intracelular é finalmente reduzido a DHLA com a participação do sistema NADH e NADPH (PACKER; CADENAS, 2011).

Substância anfipática solúvel em água e gordura que, portanto, pode neutralizar os radicais livres nos compartimentos aquosos ou lipídicos de células (BIST; BHATT, 2009), o AL e o DHLA agem como antioxidantes naturais, atuando como cofator para as desidrogenases

do sistema mitocondrial, sendo, portanto, a mitocôndria seu principal local de ação (SMITH et al., 2004).

Figura 3 Representação plana do ácido lipóico.



Fonte: https://pubchem.ncbi.nlm.nih.gov

O ácido dihidrolipóico é também capaz provocar a regeneração de outros antioxidantes de baixo peso molecular, tais como glutationa, coenzima Q10 e vitaminas A e C (BILSKA et al., 2007), bem como diminuição do poder de redução celular e prevenção da destruição de GSH no citoplasma e mitocôndria. Também é atribuída ao ácido lipóico uma atividade anti-inflamatória, por ser capaz de evitar o dano neuronal ocasionado pelas espécies reativas derivadas do oxigênio produzidas durante as doenças neurodegenerativas (SALINTHONE et al., 2008). Além disso, funciona como um quelante de metais, reduzindo a produção de EROs (FERREIRA; MILITÃO; FREITAS, 2009).

Estudos sugerem que o AL produz seus efeitos, também, através da remoção de radicais hidroxilas e da inibição da oxidação de lipídios e proteínas (BIST; BHATT, 2009; NAVARI-IZZO; QUARTACCI; SGHERRI, 2002). Devido ao seu potente efeito antioxidante, seria também capaz de evitar o dano neuronal ocasionado pelas EROs produzidas durante as doenças neurodegenerativas (MACZUREK et al., 2008; SALINTHONE et al., 2008; SILVA et al., 2013). Assim, o ácido lipóico tem sido descrito como um potente antioxidante biológico, agente detoxificante, sendo utilizado no tratamento da neuropatia diabética, melhoramento de déficits cardiovasculares, cognitivos e neuromusculares associados ao envelhecimento, e apontado como modulador de várias vias de sinalização da inflamação (LODGE; TRABER; PACKER, 1998; SMITH et al., 2004; SUH et al., 2004).

Além disso, agentes antioxidantes têm sido utilizados como ferramenta para combater doenças neurodegenerativas, uma vez que o estresse oxidativo é um dos mecanismos envolvidos na patogênese de várias doenças do sistema nervoso central (ALBARRACIN et al., 2012). O ácido lipóico, por ser um potente antioxidante, tem sido investigado como uma nova alternativa terapêutica para doenças como esclerose múltipla (SCHREIBELT et al., 2006), Alzheimer (ROSINI et al., 2011), depressão (SILVA et al., 2013), lesão vascular cerebral (TOMASSONI et al., 2013), isquemia (CONNELL et al., 2012), Parkinson (ARAÚJO et al., 2013), dentre outras. O AL também é eficaz em reverter e prevenir alterações comportamentais e neuroquímicas induzidas por anfetamina, uma droga de abuso, em modelo de mania (MACÊDO et al., 2012). Estudos revelam que o ácido lipóico pode ser uma estratégia de combate aos danos celulares causados pelo uso abusivo de etanol. Além disso, tem apresentado resultados positivos em estudos sobre dependência, diminuindo o poder reforçador do álcool (PEANA et al., 2013; PIRLICH et al., 2002; SHIRPOOR et al., 2008).

Seu uso na clínica, de acordo com o Ministério da Saúde (MINISTERIO DA SAUDE, 2012), tendo como princípio ativo o ácido tióctico, e nome comercial Thioctacid®, possui registro na Agência Nacional de Vigilância Sanitária-ANVISA para tratamento dos sintomas da polineuropatia diabética periférica. Experimentalmente, assemelha-se à insulina, ativando a recaptação de glicose no nervo, no músculo e nas células adiposas via fosfatidilinositol-3-quinase. Sua forma de apresentação é em comprimido revestido com 600mg e solução injetável 25mg/ml.

3 RELEVÂNCIA E JUSTIFICATIVA

Tendo em vista que o alcoolismo é um problema de saúde pública mundial e que, embora seja uma complicação milenar, os tratamentos disponíveis apresentam eficácia limitada (LEE; LEGGIO, 2014), a principal estratégia de combate para esse grande problema de saúde pública tem como foco a prevenção.

Como a formação de radicais livres tem mostrado relação com o mecanismo de reforço de drogas de abuso, e o ácido lipóico é um excelente antioxidante, torna-se de grande valia investigar a ação preventiva do AL na adolescência, período de vulnerabilidade por apresentar grande susceptibilidade adaptativa (ANDERSEN, 2003).

Devido ao fato do desenvolvimento neural estar fortemente presente na adolescência, pesquisas que enfocam a dependência têm buscado compreender quais as consequências comportamentais e moleculares da exposição precoce a drogas de abuso e sua relação com outras substâncias (MATTHEWS, 2010; WETHERILL; TAPERT, 2012).

O ácido lipóico tem apresentado resultados positivos em estudos sobre dependência, diminuindo o poder reforçador do álcool, sendo também uma estratégia de combate aos danos celulares causados pelo uso abusivo de etanol (PEANA et al., 2013; PIRLICH et al., 2002; SHIRPOOR et al., 2008), porém, pesquisas com ácido lipóico em diferentes fases de desenvolvimento não foram conduzidas ainda.

Esse antioxidante já é utilizado na clínica e comercializado no Brasil, mas pouco se sabe sobre a interação do ácido lipóico com drogas de abuso. Portanto é de grande importância se pesquisar os mecanismos que estão envolvidos na relação desse antioxidante com as vias estimuladas por drogas como o álcool. Além disso, a maioria da literatura acerca dessa interação aborda a fase adulta, negligenciando a complexidade neurodesenvolvimental observada na adolescência, carecendo também de estudos enfocando uso crônico durante este período. Assim, este antioxidante, que tem sido investigado como uma nova alternativa terapêutica para doenças do sistema nervoso central, será utilizado no presente estudo com uma perspectiva preventiva sobre os efeitos reforçadores do etanol.

4 OBJETIVOS

4.1 Geral

Investigar as alterações provocadas pelo uso do ácido lipóico, em diferentes períodos desenvolvimentais sobre as propriedades reforçadoras do etanol, avaliando variáveis comportamentais e neurobiológicas.

4.2 Específicos

- Verificar o efeito do ácido lipóico sozinho ou associado ao etanol, em tratamento agudo na adolescência e na fase adulta sobre a atividade exploratória, memória de trabalho e preferência condicionada de lugar;

- Comparar respostas comportamentais de animais com preferência ao etanol, tratados com ácido lipóico na adolescência ou na fase adulta;

- Determinar os níveis de BDNF nas áreas cerebrais envolvidas com o sistema de recompensa (CE) de animais com preferência ao uso de etanol, em tratamento com ácido lipóico;

- Quantificar mediadores inflamatórios (IL-6; TNF-α) em áreas cerebrais envolvidas com o sistema de recompensa (CE) de animais com preferência ao uso de etanol tratados com ácido lipóico;

- Quantificar parâmetros de estresse oxidativo (peroxidação lipídica –TBARS; e o antioxidante GSH), em áreas cerebrais envolvidas com o sistema de recompensa (CPF e CE) de animais com preferência ao uso de etanol tratados com ácido lipóico.

5 MATERIAIS E MÉTODOS

5.1 Animais

Foram utilizados camundongos *Swiss* machos a partir de 35 dias pós-natal até 72 dias pós-natal, provenientes do biotério do Departamento de Fisiologia e Farmacologia da UFC. Os animais foram ambientados em grupos de no máximo 10 animais em caixas de propileno, a 26 ± 2 ºC, com ciclo claro/escuro de 12 h, recebendo ração padrão e água "*ad libitum*".

A presente pesquisa foi submetida ao Comitê de Ética e Pesquisa Animal (CEPA) da Universidade Federal do Ceará-UFC, sendo aprovada sob protocolo nº 18/14.

5.2 Drogas utilizadas

Ácido lipóico (AL) (Sigma Aldrich) foi dissolvido em carboximetilcelulose 5% e administrado na dose de 100 mg/kg via oral (v.o.). A solução de etanol (EtOH) na concentração de 20% v/v, foi dissolvida a partir de um estoque inicial absoluto (Merck) em água destilada e administrado na dose de 2 g/kg v.o. Para os grupos controle, água destilada (v.o.) foi usada como veículo com a proporção de dosagem de 0,1 ml para cada 10 g de peso do animal. A dose de AL foi escolhida com base em estudos anteriores realizados em nosso laboratório (ARAÚJO et al., 2013; MACÊDO et al., 2012; SILVA et al., 2013, 2014; VASCONCELOS et al., 2015). Os animais permaneceram em jejum durante 30 minutos antes da administração das drogas.

5.3 Indução da preferência ao etanol

Para avaliar o tempo de tratamento utilizando administração por via oral, capaz de induzir preferência ao etanol nos animais, uma curva de tempo de tratamento foi realizada. Para isso, o etanol foi administrado na dose de 2g/kg (v.o.) em diferentes grupos de animais durante 1, 7, 14 e 21 dias antes de serem submetidos ao teste de preferência condicionada de lugar. A melhor resposta foi encontrada com 21 dias de tratamento, portanto, este foi o tempo escolhido para induzir preferência ao etanol.

5.4 Desenho experimental

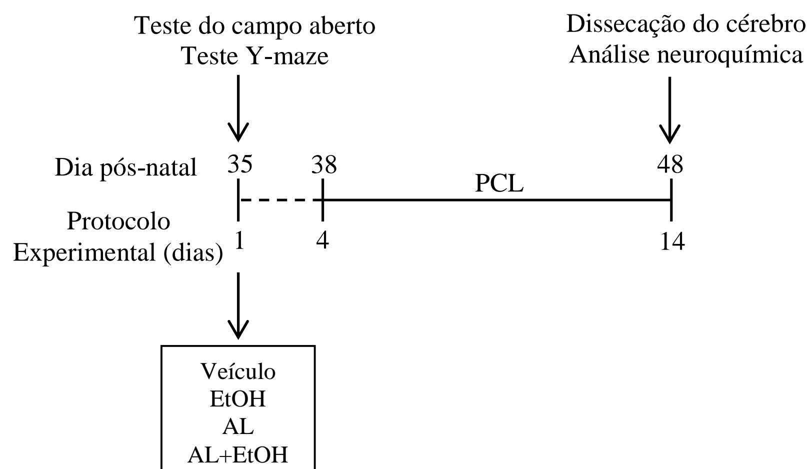
Para a determinação dos efeitos da associação do ácido lipóico com o etanol em diferentes fases do desenvolvimento, o desenho experimental foi composto por três protocolos de tratamento (agudo-adolescente, agudo-adulto, crônico), com diferentes animais para cada grupo, e está descrito abaixo e representado esquematicamente nas figuras 4, 5 e 6:

- Protocolo agudo-adolescente

A figura 4 representa o protocolo agudo-adolescente, que compreende quatro grupos: veículo, EtOH, AL, AL+EtOH. Para cada grupo, diferentes animais foram utilizados.

Animais com 35 dias pós-natal (DPN), período equivalente à periadolescência (BRENHOUSE; ANDERSEN, 2011), foram tratados de forma aguda com etanol (EtOH), ácido lipóico (AL) ou associação (AL 20 min antes do EtOH – AL+EtOH), e após 30 minutos da administração da substância, os animais foram submetidos aos testes comportamentais de campo aberto e Y-maze. Após esses testes, os animais foram recolocados em suas caixas, e 72hs depois, foram submetidos ao teste de preferência condicionada de lugar. Ao final destes testes, os animais foram sacrificados e seus cérebros dissecados e armazenados para posterior análise neuroquímica.

Figura 4 Desenho experimental do protocolo agudo-adolescente.

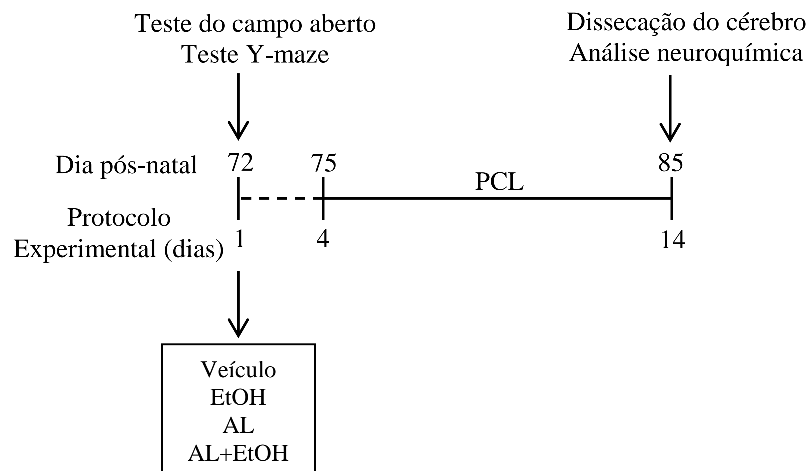


- Protocolo agudo-adulto

O protocolo agudo-adulto abrange quatro grupos: veículo, EtOH, AL, AL+EtOH. Para cada grupo diferentes animais foram utilizados.

Animais com 72 DPN (período equivalente à fase adulta) foram tratados de forma aguda com etanol (EtOH), ácido lipóico (AL) ou associação (AL 20 min antes do EtOH – AL+EtOH), e após 30 minutos da administração da substância, os animais foram submetidos aos testes comportamentais de campo aberto e Y-maze. Após esses testes, os animais foram recolocados em suas caixas, e 72hs depois, foram submetidos ao teste de preferência condicionada de lugar. Ao final destes testes, os animais foram sacrificados e seus cérebros dissecados e armazenados para posterior análise neuroquímica (figura 5).

Figura 5 Desenho experimental do protocolo agudo-adulto.



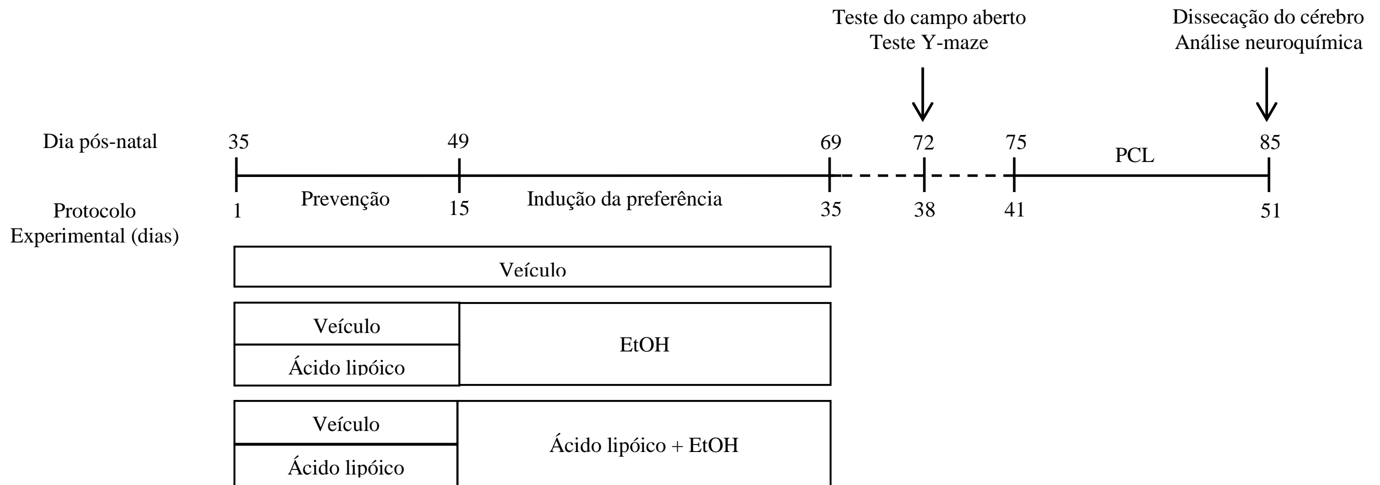
- Protocolo crônico

A figura 6 apresenta o protocolo crônico, que compreende cinco grupos (veículo, Vh-EtOH; AL-EtOH; Vh-AL+EtOH, AL-AL+EtOH) descritos descritos posteriormente. Para cada grupo diferentes animais foram utilizados.

Animais com 35 DPN, foram tratados diariamente por gavagem durante 14 dias com veículo ou AL. Após este tratamento, foi iniciada a indução da preferência ao etanol, onde estes animais foram tratados com etanol ou AL+EtOH (AL 20 minutos antes do EtOH)

diariamente, por gavagem, durante 21 dias. O grupo controle continuou a receber tratamento com veículo.

Figura 6 Desenho experimental do protocolo crônico.



Os testes comportamentais foram então iniciados a partir do 72 DPN (período equivalente à fase adulta), e um dia após o término do modelo comportamental os animais foram sacrificados e as áreas cerebrais retiradas para os testes neuroquímicos.

OBS: Nos grupos crônicos, a preferência condicionada de lugar foi avaliada no período em que os animais tratados durante a adolescência chegaram à fase adulta. No início do procedimento de PCL, os camundongos previamente tratados com ácido lipóico/veículo durante a adolescência estavam com 75 DPN. Utilizando esse protocolo, nós objetivamos avaliar os efeitos reforçadores do etanol em camundongos adultos pré-expostos ao ácido lipóico durante a adolescência.

5.5 Estudo comportamental

5.5.1 Avaliação da atividade exploratória

O campo aberto (figura 7) foi utilizado para avaliar a atividade exploratória do animal (ARCHER, 1973). É feito de acrílico (paredes transparentes e piso preto, 30 x 30 x 15

cm) dividido em nove quadrantes iguais. Os parâmetros observados foram: distância total percorrida, número de cruzamentos (movimentação espontânea), rotações, número de levantamentos do animal na vertical (“rearing”), e grooming, registrados durante 5 minutos, após 1 minuto de habituação. A tendência natural do animal em um ambiente novo é explorá-lo, apesar do conflito com o medo provocado por esse novo ambiente. Além disso, drogas estimulantes aumentam a atividade locomotora do animal e drogas depressoras diminuem a mesma. O experimento foi conduzido em uma sala com som e intensidade luminosa controlados, registrado via câmera de vídeo posicionada acima do aparato.

Figura 7 Campo aberto para avaliação da atividade exploratória de camundongos.

Fonte: Arquivo pessoal do autor.

5.5.2 Avaliação da memória de trabalho (Y-maze)

A memória de trabalho e o aprendizado foram avaliados pela taxa de alternações espontâneas em um labirinto em Y (figura 8), com os três braços (40 x 5 x 16 cm) posicionados em ângulos iguais como descrito anteriormente (SARTER, 1987). Antes do teste, os braços foram numerados, o animal foi então, colocado em um braço e alternou espontaneamente as entradas nos outros durante 8 minutos. A sequência dos braços em que os animais entraram foi anotada, e as informações analisadas de forma a determinar o número de entradas no braço sem repetição. Uma alternação foi considerada correta se o animal visitou um novo braço e não retornou aos dois braços anteriormente visitados. Assim, a percentagem das alternações foi

calculada como a razão entre as alternações corretas (n) e o número de visitas realizadas durante o período de observação (n-2), multiplicado por 100.

$$\% \text{ Alternações espontâneas} = n / n\text{-}2 \times 100$$

Figura 8 Aparato para teste de memória (Y-maze)

Fonte: Arquivo pessoal do autor.

5.5.3 Modelo de preferência condicionada de lugar

O paradigma da preferência condicionada de lugar (figura 9) consistiu de três fases (CUNNINGHAM; GREMEL; GROBLEWSKI, 2006): habituação e pré-condicionamento (dias 1 e 2, respectivamente), condicionamento (dias 3-10) e pós-condicionamento ou teste (dia 11). O aparato, feito de acrílico (45 x 15 x 30 cm) consiste em dois compartimentos (20 x 15 x 30 cm), um com paredes pretas e piso com grades, outro com paredes brancas com listras e piso macio, e um compartimento central (5 x 15 x 30 cm) que conecta os outros dois.

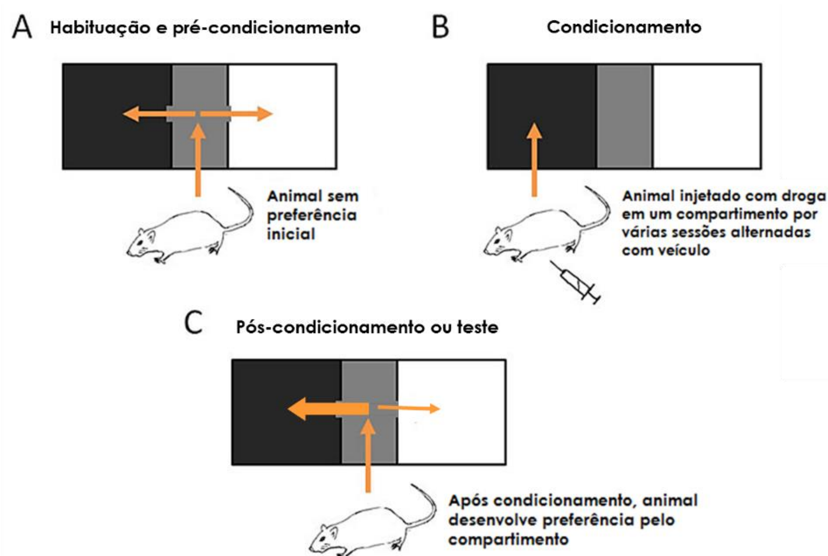
Neste paradigma foi utilizado o aparato sem viés, no qual os animais não mostraram preferência inicial por um compartimento em detrimento do outro. Assim, os animais que permaneceram acima de 85% do tempo total em um dos compartimentos foram excluídos. Além disso, foi adotado o procedimento para atribuição do estímulo com viés, no qual o estímulo pareado com a droga foi escolhido baseado na preferência inicial do animal determinada na avaliação do pré-condicionamento (CUNNINGHAM; GREMEL; GROBLEWSKI, 2006; TZSCHENTKE, 2007).

Nos dois primeiros dias os camundongos exploraram livremente, por 15 min, os dois compartimentos. No segundo dia, o tempo gasto em cada um dos compartimentos foi registrado por 15 min (preferência basal). Nas sessões de condicionamento, que duraram 8 dias, os animais dos grupos agudos foram tratados por via oral com etanol (2 g/kg), AL (100 mg/kg) ou associação (AL+EtOH) e, imediatamente após, confinados no compartimento mais aversivo por 25 min. Em dias alternados, os animais receberam veículo e foram colocados no compartimento oposto. O grupo controle recebeu veículo em ambos os compartimentos. Os animais dos grupos crônicos foram tratados com EtOH nas sessões de condicionamento, e em dias alternados com veículo.

No dia do teste (pós-condicionamento), os animais foram colocados no corredor central que dá acesso aos compartimentos, e o tempo gasto no compartimento pareado com a droga foi registrado por 15 min. Os níveis de preferência (ou aversão) de lugar foram definidos pela comparação do tempo gasto pelos animais no compartimento pareado com a droga antes e depois do tratamento.

Os experimentos foram conduzidos em uma sala com som e intensidade luminosa controlados, registrados via câmera de vídeo posicionada acima do aparato.

Figura 9 Procedimento do teste de preferência condicionada de lugar.



A, B e C representam as fases que compõem o teste. Adaptado de Braida et al. (2007).

5.6 Estudo neuroquímico

Após os testes de comportamento, os animais foram sacrificados por deslocamento cervical e seus cérebros foram removidos. As regiões cerebrais do córtex pré-frontal (CPF) e corpo estriado (CE) foram dissecadas e armazenadas a -80ºC até serem utilizadas para os estudos neuroquímicos.

5.6.1 Dosagem de BDNF

As concentrações de BDNF foram determinados no CE com um ensaio imunoenzimático (ELISA), de acordo com as instruções do fabricante (Sigma Aldrich). Os homogenatos cerebrais e padrões, foram pipetados em poços de uma microplaca (96 poços) pré-revestidos com anticorpo monoclonal específico para BDNF. Qualquer BDNF presente foi ligado pelo anticorpo imobilizado. Uma enzima ligada ao anticorpo monoclonal específico para o BDNF foi adicionada aos poços. Posteriormente foi feita uma lavagem para remover qualquer reagente que não estivesse ligado ao complexo enzima-anticorpo, adicionando uma solução de substrato aos poços; a cor se desenvolveu em proporção à quantidade de BDNF ligada no passo inicial. A reação foi interrompida e a intensidade da cor medida dentro de 30 min, usando um leitor de microplacas com absorbância de 450nm. Os resultados foram expressos em ng/ml.

5.6.2 Atividade de mediadores inflamatórios – IL-6 e TNF-α

As áreas cerebrais dissecadas (CE) foram homogeneizadas em 10 volumes de tampão PBS com inibidores de protease (EMD Biosciences) e fosfatase (Sigma-Aldrich) para posteriormente serem centrifugadas (10.000 *g*, 5 min). O sobrenadante foi usado sem diluição. A concentração das citocinas, em 50 µL de amostra, foi determinada por ELISA (R & D Systems, Minneapolis, MN, EUA) de acordo com o protocolo do fabricante e expressa em pg/g de tecido.

5.6.3 Avaliação do estresse oxidativo

As amostras de tecido cerebral foram homogenizadas (10 vezes [p/v]) com tampão fosfato 0,1 M e pH 7,4. Os homogenatos foram centrifugados a 10.000 *g* por 15 min e alíquotas dos sobrenadantes foram separadas e usadas para os testes de estresse oxidativo.

5.6.3.1 Determinação da concentração de Glutationa Reduzida (GSH)

Foram retirados 400µL desse homogenato e adicionados a 320µL de água destilada. Mais 80µL de ácido tricloroacético a 50% foram adicionados ao meio reacional. O material foi agitado e centrifugado a 956 *g* por 15 minutos. Em seguida foi recolhido 400µL do sobrenadante e acrescidos 800µL de tampão Tris-HCl 0,4M, pH 8,9, juntamente com 20µl de DTNB 0,01M e após 1 minuto da reação foi feita a leitura da coloração em 412nm através de espectrofotômetro. A concentração de glutationa reduzida foi expressa em µg de GSH/g de tecido, tendo por base uma curva padrão (SEDLAK; LINDSAY, 1968).

5.6.3.2 Determinação da peroxidação lipídica

O grau de peroxidação lipídica nas áreas cerebrais foi analisado através da determinação das concentrações de substâncias reativas ao ácido tiobarbitúrico (TBARS) nos homogenatos (DRAPER et al., 1993), como um índice da produção de ROS. Foi misturado ao homogenato 1 mL de solução de ácido tricloroacético a 10% e 1 mL de solução de ácido tiobarbitúrico 0,6%. Após a agitação, essa mistura foi mantida em um banho de água fervente (95-100°C) por 15 min, e a seguir, resfriada em banho de gelo. Posteriormente a amostra foi centrifugada (800 *g*, 5 min). O conteúdo de TBARS foi determinado em espectrofotômetro a 532 nm. Os resultados foram expressos em µg de malonildialdeído (MDA)/g de tecido.

5.7 Análise dos dados

A análise comportamental dos testes de campo aberto, e preferência condicionada de lugar foi realizada pelo programa de análise do comportamento Any-maze®, através das gravações dos vídeos. A análise estatística de todos os dados foi realizada utilizando o programa GraphPad Prism 5.0. Os dados estão expressos como média ± EPM, e o valor de alfa foi fixado em $p<0.05$ para todas as análises.

Experimento 1: Curva do tempo de tratamento capaz de induzir preferência ao etanol

Para o experimento da curva de tempo de tratamento do EtOH foram comparados o tempo gasto no compartimento pareado com a droga antes e depois das sessões de condicionamento. Medidas repetidas foram analisadas através de two-way ANOVA, com os

fatores estágio de condicionamento e dias de tratamento. Para resultados significantes, Bonferroni foi utilizado como teste post hoc.

Experimento 2: Atividade exploratória no campo aberto

Para comparar os parâmetros de atividade exploratória, medidas repetidas foram analisadas por one-way ANOVA, com Tukey como teste post hoc.

Experimento 3: Taxa de alternações espontâneas no labirinto em Y (memória de trabalho)

Para comparar as taxas de alternações espontâneas, medidas repetidas foram analisadas por one-way ANOVA, com Tukey como teste post hoc. A análise comparativa no teste de memória entre os protocolos agudos (adolescente e adulto), foi realizada através de two-way ANOVA, com os seguintes fatores: fase da vida e droga.

Experimento 4: Aquisição da preferência condicionada de lugar (PCL)

Para verificar a aquisição da PCL, foi comparado o tempo gasto no compartimento pareado com a droga antes e depois das sessões de condicionamento. Medidas repetidas foram analisadas através de two-way ANOVA com estágio de condicionamento e droga como fatores. Para resultados significantes, Bonferroni foi utilizado como teste post hoc.

Experimentos 5, 6 e 7: Concentrações de BDNF; citocinas (IL-6 e TNFα); GSH e TBARS

Para comparar as concentrações dos parâmetros neuroquímicos, medidas repetidas foram analisadas por one-way ANOVA, com Tukey como teste post hoc.

6 RESULTADOS

Os resultados serão agrupados em dois capítulos, conforme organização dos artigos derivados da tese. Será apresentado no primeiro capítulo o artigo referente aos dados de comportamento coletados durante o desenvolvimento da tese. No segundo capítulo, serão descritos os resultados da análise neuroquímica, que compõem o segundo artigo desenvolvido através da presente tese de doutorado.

6.1 CAPÍTULO I – Lipoic acid differentially acts on ethanol reinforcing properties according to treatment time and developmental phase

Artigo proveniente dos resultados parciais da tese de doutorado, contendo dados referentes ao estudo comportamental realizado. O presente artigo será submetido à uma revista internacional.

Lipoic acid differentially acts on ethanol reinforcing properties according to treatment time and developmental phase

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ABSTRACT

Although alcohol abuse is a very old health complication, the available treatments have limited effectiveness. Adolescence is a critical period of development with great opportunity for researches with preventive approach. Lipoic acid, an antioxidant, showed positive results in studies about dependence. Thus, the aim of this study was to investigate lipoic acid action during developmental period on ethanol reinforcing effects. For this purpose, male *Swiss* mice covered three protocols (acute-adolescent; acute-adult; chronic). On acute-adolescent protocol, animals during adolescence were treated acutely with ethanol (EtOH – 2 g/kg v.o.) lipoic acid (LA – 100 mg/kg v.o.) or association (LA+EtOH), and submitted to behavioral tests to assess locomotor activity, memory task, and reinforcing effects on conditioned place preference (CPP). Acute-adult protocol followed the same procedures with mice on adulthood. Chronic protocol started with vehicle or LA administration during adolescence. After this, it was started induction of EtOH preference over young adulthood. In two more groups, LA was administered 20 min prior to EtOH. Behavioral tests were then performed. EtOH modulated locomotor activity in opposite manner when administered acutely or chronically. Lipoic acid behaved in different ways according to the period of life in which was used. On adolescence LA was able to prevent EtOH-induced locomotor activity, but in adulthood it presented stimulant effect. Memory was impaired with acute (in adolescence) and chronic EtOH exposure, and LA had no preventive effect on memory impairment on adolescence but was able to prevent memory deficits induced by chronic EtOH. While in adolescence LA induced CPP when acutely administered prior to EtOH, on adulthood the same treatment was able to prevent the acquisition of EtOH-induced CPP.LA administration chronically with EtOH induced strong sensitization for ethanol preference. No preventive effect of LA during adolescence was observed in chronic EtOH-induced CPP. Our data suggest that ethanol acts in different way depending of treatment time and developmental stage, having in some cases its effects prevented by LA. Lipoic acid effects strongly depends of the period in which is administered, and can interact in different way with reinforcing effects of ethanol.

Keywords: Lipoic acid; ethanol; neurodevelopment; dependence.

1 INTRODUCTION

Alcohol use is the third principal risk factor for health problems worldwide. Furthermore, causes 2,5 million deaths each year, and result in exorbitant social costs, being a global problem that undermines social and individual development. Many countries recognize the serious public health problem caused by harmful use of alcohol, looking for adopt policies and prevention programs (Who, 2011).

In order to decrease the high incidence of alcoholism, several studies have sought to find an effective treatment for this serious public health problem (Lee & Leggio, 2014; Skinner, Lahmek, Pham, & Aubin, 2014), which can occur at different stages of human development and to each generation is presented earlier in the population (Who, 2011). As the available treatments still have limited effectiveness (Lee & Leggio, 2014), prevention becomes a major focus to be highlighted.

Childhood and adolescence are critical periods of development that are frequently associated with onset of substance use and behavioral risks due to this use (Wetherill & Tapert, 2012; Windle et al., 2009; Witt, 2010).

Studies suggest that temperamental and behavioral features observed in childhood predict subsequent results related to alcohol in posterity (Dick et al., 2013), therefore the high adaptive susceptibility of this period promotes a window of opportunity for research on preventive approach, ensuring the neurodevelopmental integrity and reducing the probability of development of drug abuse dependence in adulthood.

Lipoic acid, a potent antioxidant, have been investigated as a new therapeutic alternative for diseases such as multiple sclerosis (Schreibelt et al., 2006), Alzheimer (Rosini et al., 2011), depression (Silva et al., 2013, Silva et al., 2014), cerebrovascular injury (Tomassoni, Amenta, Amantini, & Farfariello, 2013), ischemia (Connell, Khan, Rajagopal, & Saleh, 2012), Parkinson (De Araújo et al., 2011,Araújo et al., 2013), Schizophrenia (Vasconcelos et al., 2015), among others. Lipoic acid is also effective in reverse and prevent behavioral and neurochemical alterations induced by amphetamine, a drug abuse, used as mania model (Macêdo et al., 2012).

Furthermore, lipoic acid presented positive results in studies about dependence, reducing alcohol reinforcing properties, being also a combat strategy to cell damage caused by abusive use of alcohol (Peana, Muggironi, Fois, & Diana, 2013; Pirlich, Kiok, Sandig, Lochs, & Grune, 2002; Shirpoor, Minassian, Salami, Khadem-Ansari, & Yeghiazaryan, 2008).

Some researchers showed that lipoic acid affects the reinforcement properties of alcohol (Peana et al., 2013), through study that demonstrates that lipoic acid is capable of reduce ethanol self-administration in rats. However, new investigations are required to evaluate how this lipoic acid effect on the ethanol reinforcement properties is linked with the developmental phase and neuroplasticity of CNS in mice.

2 MATERIALSAND METHODS

2.1 Animals

Male *Swiss* mice, from 35 postnatal days (periadolescence), from the Department of Physiology and Pharmacology-UFC, were acclimated to 26 ± 2 ºC, in a light/dark cycle of 12 h, with free access to standard food and water. Procedures used in the present research were approved by Ethics and Animal Research Committee (CEPA) from Federal University of Ceará-UFC under protocol nº 18/14, and follows the principles of laboratory animal care of the National Institute of Health (NIH).

2.2 Drugs

Lipoic acid (LA) (Sigma Aldrich) was dissolved in cellulose at 5% and administered at 100 mg/kg orally (p.o.). Ethanol (EtOH) solutionat a concentration of 20% v/v, dissolved from an initial stock of ethanol absolute (Merk) in distilled water was administered at 2 g/kg p.o. For the control groups, distilled water (p.o.) was used as vehicle.

2. Ethanol-induced preference

To evaluate the treatment time using intragastric administration that would induce ethanol preference in mice, a treatment time curve was made. For this purpose, ethanol was administered at 2g/kg (p.o.) during 1, 7, 14 and 21 days before being submitted to conditioned place preference (Figure 2).

2.4 Experimental design

Experimental design is composed by three major groups (acute-adolescent; acute-adult; chronic), described posteriorly.

- Acute-adolescent protocol

At 35º postnatal day (PND), period equivalent to periadolescence (Brenhouse & Andersen, 2011), animals were treated acutely with EtOH, LA or association (LA+EtOH; LA administered 20 min prior to EtOH), and after 30 minutes of substance administration, animals were submitted to behavioral tests (open field and y-maze). After this, it were replaced into home cages, and 72hs later, started the conditioned place preference (CPP) model (Figure 1A).

- Acute-adult protocol

Behavioral tests (open field and y-maze) were started at 72º PND (adulthood), being performed 30 minutes after substance administration, comprising the same groups and methodology mentioned above. After this, it were replaced into home cages, and 72hs later, started the conditioned place preference model (Figure 1B).

- Chronic protocol

From 35º PND, animals received vehicle or LA daily, during 14 days. After this treatment, it was started the chronic ethanol exposure, in which animals were treated with ethanol once daily, during 21 days. LA was also administered 20 min before EtOH in two more groups. Control group remained receiving only vehicle. Behavioral tests (open field and y-maze) then started from 72º PND (adulthood). After these tests, animals were replaced into home cages, and 72hs later, started the conditioned place preference model (Figure 1C).

The CPP was evaluated in a period in that the animals, which were treated during adolescence, reached adulthood. Thus, using this protocol, we aimed to evaluate reinforcing effects of ethanol in adult mice pre-exposed to LA during adolescence.

2.5 Behavioral tests

- Evaluation of exploratory activity

Open field was used to evaluate the exploratory activity of mice (Archer, 1973). It is made of acrylic divided into nine quadrants (transparent walls and dark floor, 30 x 30 x

15cm). The parameters observed were total distance, crossing number, rotations, rearing, and grooming, registered during 5 min, after 1 min of habituation. Experiments were conducted in a room with light intensity and sound controlled, recorded via camera positioned above the apparatus.

- Memory task (Y-maze)

Memory task was evaluated by the rate of spontaneous alternation into a Y-maze, with three arms (40 x 5 x 16 cm) positioned in equal angles as described by Sarter (Sarter, 1987) for 8 min. Entry sequence in the arms by the animals was registered to determine the number of entries without repetition, considered a correct alternation. The ratio between correct alternations (n) / total number of visits (n-2) x 100, provided the correct alternations percentage.

- Conditioned place preference model

The CPP paradigm consisted of three phases (Cunningham, Gremel, & Groblewski, 2006): habituation and pre-conditioning (days 1 and 2, respectively), conditioning (days 3-10), and post-conditioning or test (day 11). The apparatus made of acrylic (45 x 15 x 30 cm) consisted of two compartments (20 x 15 x 30 cm), one with black walls and grid floor, other with white walls and stripes and soft floor, and one central compartment(5 x 15 x 30 cm) to connect the other two.

In this paradigm it was used an unbiased apparatus, in which animals shows no initial preference for one compartment over another. Thus, animals that remained above 85% of total time in one of the compartments were excluded. Moreover, abiased stimulus assignment procedure was adopted, in which the stimulus paired with the drug was assigned on the basis of the subject’s initial preference as determined in a pre-conditioning evaluation (Cunningham et al., 2006; Tzschentke, 2007).

In the habituation and pre-conditioning, animals explored freely for 15 min all three compartments, but in the second day (pre-conditioning) the exploration was recorded (basal preference). At the conditioning sections, which consisted of 8 days, animals from acute groups were treated by gavage with EtOH (2 g/kg), LA (100 mg/kg) or association (LA+EtOH), and immediately after, confined into the non-preferred compartment for 25 min. In alternate days, animals received vehicle and were placed into the other compartment. Control group received

vehicle in both compartments. The animals from chronic groups, at the conditioning sections, were treated with EtOH (2 g/kg), and in alternate days with vehicle.

On the test day (post-conditioning), animals were placed on the central (neutral) compartment, and the free exploration was recorded for 15 min. CPP was considered when time spent in drug-paired compartment on post-conditioning significantly exceeded time spent in drug-paired compartment on pre-conditioning. Other parameters registered were the percentage difference of time spent on aversive or preferred compartment before and after conditioning sections. Experiments were conducted in a room with light intensity and sound controlled, recorded via camera positioned above the apparatus.

2.6 Data analysis

The software Any-maze®, through video records, performed behavioral analysis of open field and CPP tests. Statistical analysis of all data was carried out using the software GraphPad Prism 5.0.

For the CPP data, regarding the time spent in drug-paired compartment before and after conditioning, repeated measures were analyzed by two-way analysis of variance (ANOVA) with conditioning stage and drug as factors, and on treatment time curve of EtOH experiment, factors were conditioning stage and treatment days. Indeed, stage of life and drug were used as factor to compare both acute protocols (adolescent and adult) on Y-maze test. For significant results, Bonferroni was used as post hoc test.

To compare the means of three or more groups on the other parameters among CPP, Y-maze and open field, one-way ANOVA was adopted with Tukey as post hoc test, and test *t* used to compare group-to-group. All data are reported as mean ± SEM, and the alpha level was set at $p<0.05$ for all analyses.

3 RESULTS

3.1 Treatment time curve of EtOH

The effects of treatment time with ethanol on place preference are illustrated in Figure 2. Repeated measures two-way ANOVA showed an extremely significant interaction [$F_{3,26}=15.47$; $p<0.0001$] between treatment days and conditioning stage, and significant effect for the conditioning stage factor [$F_{1,26} = 66.90$; $p<0.0001$], but not for treatment days factor

[$F_{3,26}$= 0.22; p=0.88]. Post hoc comparisons revealed that EtOH treatment during 14 and 21 days were effective to induce CPP, since these groups spent significantly more time in drug-paired compartment after conditioning sections (p<0.0001). Furthermore, this place preference was higher in groups treated for 21 days rather than 14 days of treatment, as confirmed by test t[$F_{6,6}$ = 2.81; p=0.02].

3.2 The pattern of locomotor activity and rotational behavior differs according the animals' developmental phases.

Table 1 displays the effects of EtOH, LA and association (LA+EtOH) on locomotor activity of mice exposed to acute and chronic protocols. One-way ANOVA showed increased locomotor activity on EtOH groups, in both acute protocols, as observed by the rise of total distance (Adolescent [$F_{3,24}$ = 3.71; p=0.01] / Adult [$F_{3,33}$ = 4.42; p=0.01]) and crossing number (Adolescent [$F_{3,44}$ = 3.79; p=0.01] / Adult [$F_{3,34}$ = 6.05; p=0.002]), as compared to vehicle groups. This stimulant effect in both parameters was also present on LA group of adult mice, whereas for LA+EtOH group also from adult mice, this rise was only observed regarding crossing number. On the other hand, LA administration before ethanol (LA+EtOH) averted the stimulant effect of ethanol on these parameters in adolescent mice. Furthermore, chronic treatment with ethanol showed a decrease in locomotor activity, demonstrated by a reduction in the crossing number in all groups treated chronically with ethanol [$F_{4,48}$ = 4.72; p=0.002].

Taking into account rotational behavior, EtOH group in acute adolescent protocol [$F_{3,41}$ = 3.15; p=0.03], and LA group in acute adult protocol [$F_{3,36}$ = 3.24; p=0.03] increased it. No alterations in this parameter were seen in chronic groups.

When administered with EtOH (LA+EtOH), LA expressed a reduction on vertical exploration (rearing) compared to LA alone in both acute protocols (Adolescent [$F_{3,42}$ = 5.54; p=0.002] / Adult [$F_{3,35}$ = 8.43; p=0.0002]), bringing values close to EtOH groups. Besides, when compared to control, rearing was diminished on EtOH group from adolescent mice, and enhanced on LA group from adult mice. No significant alterations were observed for grooming parameters in all groups.

3.3Memory task (Y-maze)

Memory task was evaluated through Y-maze test. As demonstrated on table 2, one-way ANOVA expressed significant decrease on the percentage of correct alternations in

adolescent groups treated with EtOH alone or pretreated with LA [$F_{3,44} = 5.41$; p=0.002]. This effect was also observed on the association group (LA+EtOH) when compared to LA group, showing a strong prevalence of ethanol feature. On adulthood, acute LA treatment improved correct alternations compared to vehicle group [$F_{3,32} = 3.36$; p=0.03].As expected, chronic treatment with EtOH reduced the percentage of correct alternations, while the concomitant administration of LA to EtOH, only in young adulthood(Vh-LA+EtOH), was able to improve this deficit observed on EtOH group [$F_{4,48} = 3.32$; p=0.01].The difference between acute protocols was not statistically significant as observed through two-way analysis of variance (ANOVA), with stage of life and drug as factors.

3.4 Conditioned place preference (CPP)

Results from CPP paradigm across different stages of life, with acute and chronic drug treatment are shown through figures 3 and 4, and table 3. Concerning acute treatment in adolescence (Figure 3A), two-way ANOVA indicated an extremely significant interaction [$F_{3,36} = 8.15$; p=0.0003] between drug and conditioning stage, as main significant effect for conditioning stage factor [$F_{1,36} = 13.35$; p=0.0008], but not for drug factor [$F_{3,36} = 0.45$; p=0.71]. Post test revealed that, on adolescent mice, acute treatment with vehicle, ethanol or LA did not produce any conditioning effect by itself, but the association of LA and EtOH produced conditioned place preference ($p<0.001$).

No statistically significant interaction [$F_{3,16} = 2.15$; p=0.1] between drug and conditioning stage, nor variability for drug factor [$F_{3,16} = 0.36$; p=0.77], were observed on adult mice treated acutely (Figure 3B), but there was significant effect for conditioning stage factor [$F_{1,16} = 6.58$; p=0.02]. Bonferroni posttest indicated that acute ethanol administration on adulthood induced CPP ($p<0.001$), which was not observed with LA pretreatment, showing a blockade of ethanol reinforcing properties. As seen in adolescence, vehicle and LA likewise did not produce any conditioning effect by itself on adulthood.

As proposed by chronic protocol, effects of EtOH and LA treatment over a long period are expressed in Figure 3C.It shows by two-way ANOVA a significant interaction [$F_{4,37} = 4.84$; p=0.003]between drug and conditioning stage, with significant effect for conditioning stage factor [$F_{1,37} = 66.53$; $p<0.0001$], but not for drug factor [$F_{4,37} = 2.52$; p=0.057]. Post test revealed that chronic treatment with ethanol during young adulthood induced CPP on adulthood in all groups treated with it, independently of LA pretreatment on adolescence ($p<0.05$),young adulthood($p<0.001$) or during these two stages of life continuously ($p<0.001$). Furthermore, LA

treatment concomitant to ethanol administration over young adulthood intensified the place preference to ethanol-paired compartment on adulthood when compared to place preference induced by EtOH treatment in young adulthood with and without LA pretreatment in adolescence [$F_{4,37} = 4.84$; p=0.003].

Table 3 illustrates the percentage difference of time spent on aversive or preferred compartment before and after conditioning sections. In addition to the percentage increase on time spent in aversive compartment (drug-paired compartment) after conditioning sections [$F_{4,37} = 4.84$; p=0.003], animals also decreased percentage time spent on preferred compartment, suggesting a change of the compartment choice after conditioning sections.

4 DISCUSSION

In the present study, we investigated reinforcing effects of ethanol in adult mice pre-exposed to LA during adolescence, as a preventive perspective for alcohol dependence. The most physiological methods used to study alcohol's effects involve acute or chronic alcohol administration to animals. In general, chronically exposed animals receive alcohol either with their diets (i.e., orally), intragastrically, or by inhalation (Ponnappa & Rubin, 2000).Several studies report chronic ethanol exposure in rodents with a different range of treatment days, according to the main target to be investigated, as well as protocol parameters (Knapp & Breese, 2012), varying since few weeks (Bertola, Mathews, Ki, Wang, & Gao, 2013), until months (Hedlund & Wahlström, 2000; Simon O'Brien et al., 2011; Teixeira et al., 2014). Thus, it is important ensure that the treatment time proposed is suitable to the effects that are aimed to investigate. For this purpose, to evaluate the efficacy and choose the chronic treatment time that could promote ethanol preference in this approach, taking into account the dosage of 2 g/kg [20%] and the intragastric route of administration with a single dose/day, it was performed a treatment time curve, which expressed a better response on 21 days of treatment.

Locomotor activity of mice, as well as memory task were also evaluated in the present research, since alcohol effects can include motor and memory impairment (Teixeira et al., 2014; Zorumski, Mennerick, & Izumi, 2014). Moreover, the acute locomotor effects of ethanol intake in mice are being used as indicators of an animal's propensity to engage in EtOH consumption and/or EtOH seeking behaviors (Linsenbardt & Boehm II, 2012).

In mice, acute administration of low-to-moderate doses of ethanol induce locomotor stimulation (Kruse, Linsenbardt, & Boehm II, 2012), also with an increase in locomotion in a U-shaped dose-related fashion (Viana, Almeida-Santos, Aguiar, & Moreira, 2013). Our results

showed increased locomotor activity on EtOH treated groups, in both acute protocols. In contrast, chronic ethanol use resulted in a decrease in this parameter of all groups treated with it. As observed in other study, chronic EtOH exposure during adolescence affects locomotor activity of rats, as indicated by reduction in the number of squares crossed in the open field (Teixeira et al., 2014).

Further, LA pretreatment averted the stimulant effect on locomotor activity induced by ethanol in adolescent mice, maintaining parameters next to control group, but in adult mice, acute LA showed a stimulant effect, increasing locomotor activity, as well as ethanol and association of both.

In a review about stimulants and the developing brain, Andersen (Andersen, 2005) cite the dose–response relationship of stimulants into an inverted U-shaped function, correlating the ascending limb of the U with increase behavior with increasing dose. When the behavior reaches its plateau, stimulants will cause the behavior to enter the descending limb of the curve with subsequently decreased activity. In our study, ethanol treatment may be reached plateau, and because of this, LA pretreatment prevented the locomotor stimulation.

Alcohol causes deleterious effects in the adolescent brain which are distinct from those observed in adults (Lacaille et al., 2015). Compared with adults, alcohol-exposed adolescent animals are more likely to exhibit cognitive deficits, including learning and memory dysfunctions (Guerri & Pascual, 2010), as shown in our experiments by a significant decrease on the percentage of correct alternations observed in adolescent mice treated acutely with EtOH, but not in adult mice treated with it. Memory impairment induced by ethanol was maintained even when animals were pretreated with LA, i.e. lipoic acid, was not able to prevent this unwholesome effect, since it was significant also when compared to LA treated mice, showing a strong prevalence of ethanol feature.

Significant impairment of spatial memory is described in studies focusing chronic alcohol exposure (injection, voluntary, chronic intermittent exposure, liquid diet) (Matthews & Morrow, 2000). Another extremely important approach, indeed to the drug exposure time, is to consider the stage of development that drugs will be act. Some of the cognitive effects, such as learning impairments, induced by repeated ethanol treatment in adolescent rats continue into adulthood (Pascual, Blanco, Cauli, Minarro, & Guerri, 2007; Sircar & Sircar, 2005), confirming our findings, in which chronic treatment with ethanol during young adulthood impaired memory task evaluated on adulthood.

On the other hand, chronic LA administration prior to ethanol during young adulthood prevented memory deficit induced by ethanol. Besides, improved memory was

shown with acutely LA administration on adulthood. Lipoic acid has been described as a beneficial substance toward improving cognitive deficits in several situations (Farr, Price, Banks, Ercal, & Morley, 2012; Holmquist et al., 2007; Sancheti et al., 2013), but little is known about age-associated alterations in the characteristics of LA, taking into account adolescence stage. It seems that lipoic acid behave in a different manner when occur through adolescence or adulthood, consequently expressing different feature when associated with other drugs.

Because the adolescent brain is still developing, substance use during this stage could adversely affect neural development and potentially have long-term consequences in behavior and neural morphology (Andersen, 2005; Wetherill & Tapert, 2012). Indeed, reinforcement mechanisms do not function identically in late adolescent and young adult animals. Alcohol use during this critical period likely alters the natural transition of these reinforcement mechanisms as adulthood approaches (Philpot, Badanich, & Kirstein, 2003).

An alteration on modulation of reinforcing properties, during different neurodevelopmental stages, occurred in our findings. Considering adolescent mice, acute treatment with ethanol or LA did not produce any conditioning effect by itself, but surprisingly, LA treatment prior to EtOH induced a conditioned place preference.

It has been reported that adolescent male mice require higher doses of ethanol than adults to acquire CPP (Dickinson, Kashawny, Thiebes, & Charles, 2009). As shown by Roger-Sánchez et al.(Roger-Sánchez, Aguilar, Rodríguez-Arias, Aragon, & Miñarro, 2012), even into adolescence it can be observed different effects of ethanol, in which late adolescent mice are less sensitive than early adolescent mice to the acquisition of ethanol induced CPP, without any conditioning effect of ethanol observed on late adolescent mice at similar age adopted in the present study.

On the other hand, acute EtOH administration on adulthood induced CPP, but this effect was not observed with LA pretreatment, thus inhibiting the acquisition of ethanol-induced CPP, showing a blockade of ethanol reinforcing properties. Moreover, as seen in adolescence, LA likewise did not produce any conditioning effect by itself on adulthood. These data are in accordance with previous studies in which acute LA prevented not only the acquisition, but also reconditioning of EtOH-induced CPP (Ledesma & Aragon, 2013).

Reinforcing properties of ethanol, like other drugs of abuse, can be inferred by the acquisition of drug-induced CPP. This conditioning paradigm is based on classical Pavlovian learning, in which a neutral distinctive environmental cue becomes associated with a motivationally significant event (Tzschentke, 2007). Thus, the CPP paradigm measures the reward value of a drug by using drug-related environmental cues and context. The evidence for

a reinforcing drug effect happens when a greater approach and contact with the drug-paired cue occurs. Chronic ethanol treatment during young adulthood induced CPP in all groups treated with it, independently of LA pretreatment on adolescence, young adulthood or during these two stages of life continuously.

During maturation, there are stages which environmental conditions (including drug exposure) influence development by fine-tuning connections and function. Young adult stimulant exposure produces sensitization that increases with time (Andersen, 2005). Interestingly, our findings suggests that lipoic acid when treated acutely with ethanol in adolescence induce CPP, and when treated concomitant to ethanol administration over young adulthood increases sensitization to ethanol, intensifying the place preference to ethanol-paired compartment on adulthood.

Furthermore, in addition to the percentage increase on time spent in aversive compartment (drug-paired compartment) after conditioning sections, animals treated chronically with LA concomitant to ethanol also decreased percentage time spent on preferred compartment, expressing a change on compartment choice after drug conditioning sections.

5 CONCLUSION

Ethanol presents different characteristics, stimulating locomotor activity when administered acutely in both adolescence and adulthood, and decreasing it when administered chronically in young adulthood. Lipoic acid also behave in different ways according to the period of life in which is used. Acute pretreatment with LA was able to prevent increased locomotor activity in adolescence, but in adulthood it presents stimulant effect.

Memory was impaired with acute (in adolescence, but not in adulthood) and chronic ethanol exposure, showing adolescence as a period more sensitive than adulthood for cognitive deficits. Lipoic acid has no preventive effect on memory impairment on adolescence but if chronically administered concomitantly with ethanol, it is able to prevent memory deficits induced by ethanol.

Lipoic acid effects strongly depends of the period in which is administered. While in adolescence it induced CPP when acutely administered prior to ethanol, on adulthood this same treatment is able to prevent the acquisition of ethanol induced CPP. Furthermore, lipoic acid administration chronically with ethanol over young adulthood induces strong sensitization for ethanol preference. Indeed, any preventive effect on chronic ethanol induced CPP is present

with lipoic acid use during adolescence. Additional research is encouraged to better understand the effects of alcohol and lipoic acid use across developmental period.

6 ACKNOWLEDGEMENTS

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7 CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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Figure 1 – Experimental design

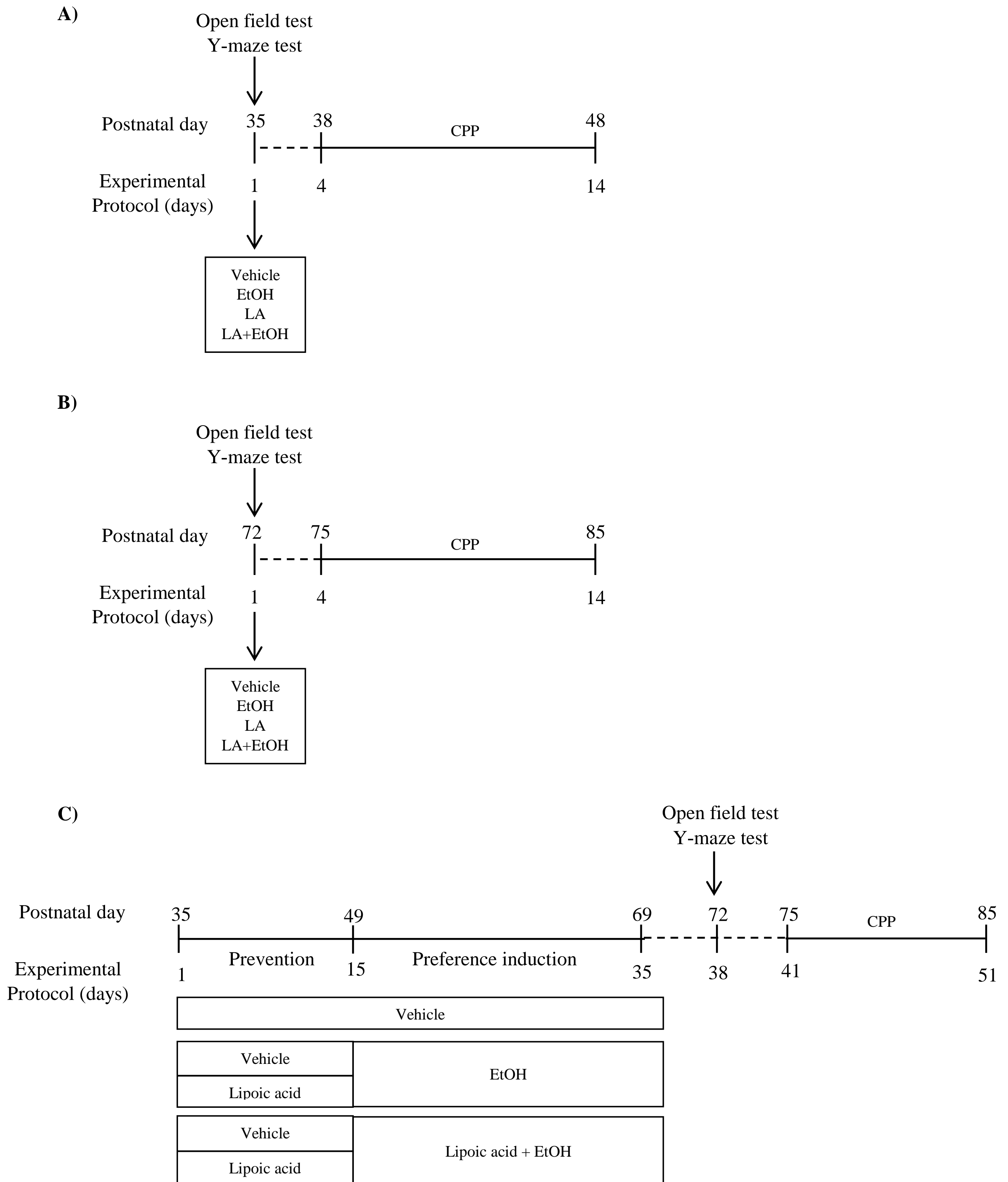


Figure 1 Experimental design of acute adolescent (A) acute adult (B) and chronic (C) protocols of treatment and behavioral tests performed. CPP: conditioned place preference; EtOH: ethanol.

Figure 2 - Treatment time curve of EtOH

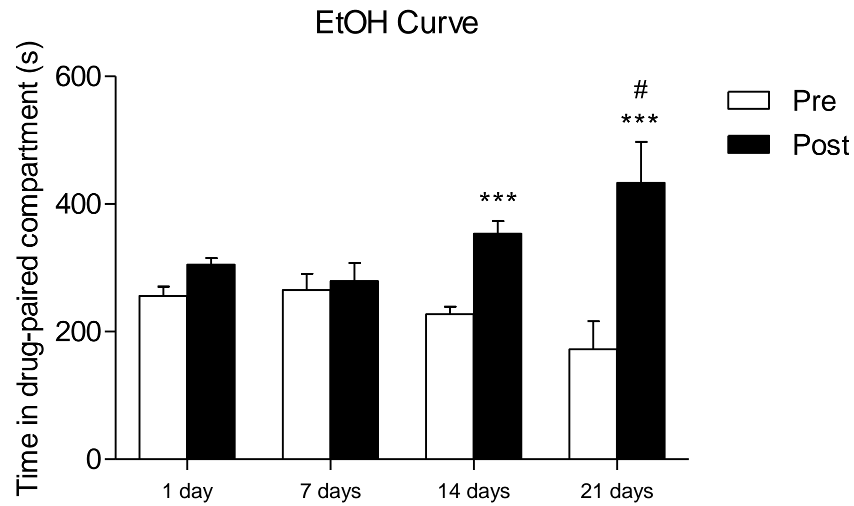


Figure 2 Treatment time curve of EtOH to evaluate the induction of ethanol preference in mice. Animals were treated with ethanol (2g/kg - v.o.) during 1, 7, 14 and 21 days before being submitted to CPP. Bars represent means ± SEM of time spent in drug-paired compartment before (white bars) and after (black bars) conditioning sections. CPP was considered when time spent in drug-paired compartment on post-conditioning significantly exceeded time spent in drug-paired compartment on pre-conditioning (***$p<0.001$, two-way ANOVA, Bonferroni as post hoc test). [#] represents significant difference between CPP on 14 and 21 days ($p=0.02$, unpaired t test).

Table 1 – Open field parameters from acute and chronic groups

Table 1 Open field parameters from acute and chronic groups.

	Total distance (m)	Crossing number	Rotations	Rearing	Grooming
Acute adolescent					
Vehicle	7.2 ± 0.6	68.3 ± 5.6	10.8 ± 0.7	40.6 ± 3.3	3.0 ± 0.5
EtOH	**11.1 ± 1.0**[a]	**107.2 ± 10.8**[a]	**15.9 ± 1.2**[a]	**27.3 ± 2.7**[a]	2.0 ± 0.2
LA	8.0 ± 0.8	80.1 ± 9.0	12.5 ± 1.1	38.2 ± 3.5	3.5 ± 0.4
LA + EtOH	**7.5 ± 0.9**[b]	**69.0 ± 8.8**[b]	11.5 ± 1.2	**24.6 ± 3.4**[a,c]	3.8 ± 0.8
Acute adult					
Vehicle	5.5 ± 0.6	54.9 ± 5.6	9.4 ± 1.1	30.8 ± 2.6	2.6 ± 0.4
EtOH	**8.7 ± 0.8**[a]	**84.6 ± 8.2**[a]	10.2 ± 1.4	22.2 ± 4.4	2.2 ± 0.3
LA	**8.7 ± 0.5**[a]	**89.3 ± 5.6**[a]	**13.9 ± 0.9**[a]	**48.0 ± 4.2**[a]	2.6 ± 0.2
LA + EtOH	8.6 ± 1.4	**91.2 ± 13.1**[a]	12.8 ± 2.2	**26.3 ± 4.9**[c]	1.8 ± 0.4
Chronic					
Vehicle	6.9 ± 0.5	82.2 ± 3.9	11.1 ± 0.8	30.0 ± 2.6	3.5 ± 0.3
Vh – EtOH	5.4 ± 0.6	**56.6 ± 3.9**[a]	9.1 ± 1.0	33.6 ± 2.6	3.1 ± 0.5
LA – EtOH	6.0 ± 0.9	**57.1 ± 8.3**[a]	8.8 ± 1.4	29.3 ± 2.4	2.1 ± 0.3
Vh – LA+EtOH	5.8 ± 0.7	**57.4 ± 7.3**[a]	8.7 ± 0.9	26.7 ± 2.6	3.0 ± 0.5
LA – LA+EtOH	6.0 ± 0.8	**57.0 ± 6.3**[a]	9.7 ± 1.1	36.2 ± 5.0	2.9 ± 0.3

Data are expressed as mean ± SEM. [a,b,c] represent significant difference as compared to control, EtOH and LA groups, respectively. Comparisons were made only between groups from the same protocol. Statistical analysis used one-way ANOVA with Tukey as post hoc test. Alpha level was set at $p<0.05$.

Table 2 – Percentage of correct alternation on Y-maze

Table 2 Percentage of correct alternation on Y-maze of all groups.

Acute adolescent	% alternation
Vehicle	68.9 ± 3.2
EtOH	**57.5 ± 2.8**[a]
LA	68.3 ± 2.2
LA + EtOH	**57.7 ± 2.6**[a,c]

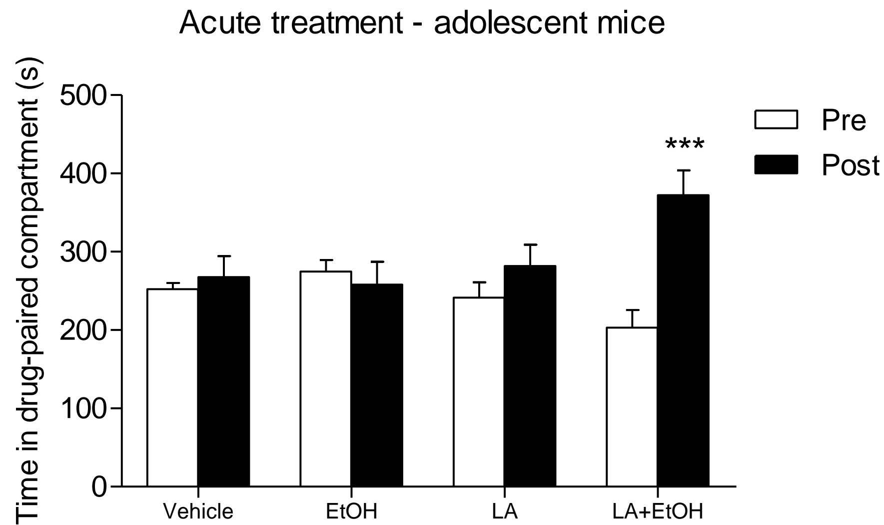
Acute adult	% alternation
Vehicle	61.9 ± 2.1
EtOH	62.7 ± 3.5
LA	**72.7 ± 3.0**[a]
LA + EtOH	62.8 ± 3.8

Chronic	% alternation
Vehicle	69.4 ± 2.1
Vh – EtOH	**58.0 ± 4.7**[a]
LA – EtOH	62.0 ± 2.9
Vh – LA+EtOH	**70.4 ± 2.1**[b]
LA – LA+EtOH	63.4 ± 2.6

Data are expressed as mean ± SEM. In results from acute protocol, [a,c] represent significant difference as compared to vehicle and LA groups, respectively. In chronic protocol, [a,b] represent significant difference as compared to vehicle and Vh-EtOH groups, respectively. Statistical analysis used one-way ANOVA with Tukey as post hoc test. Alpha level was set at $p<0.05$.

Figure 3 – Conditioned place preference from acute protocols

A)



B)

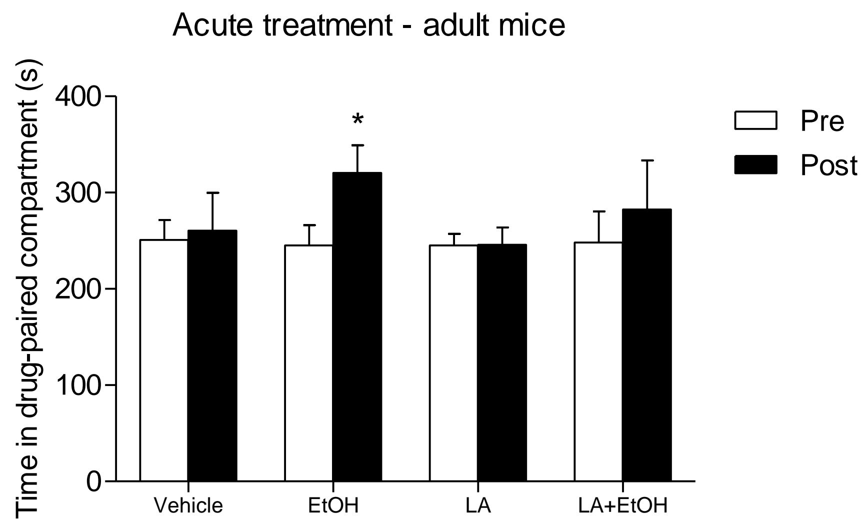


Figure 3 CPP of acute EtOH or LA treatment on different stages of life: (A) periadolescent mice and (B) adult mice. During conditioning sections, on CPP paradigm, animals were treated with vehicle, ethanol (2g/kg - v.o.), LA (100 mg/kg – v.o.) or LA+EtOH. Bars represent means ± SEM of time spent in drug-paired compartment before (white bars) and after (black bars) conditioning sections. CPP was considered when time spent in drug-paired compartment on post-conditioning significantly exceeded time spent in drug-paired compartment on pre-conditioning (***p<0.001, *p<0.05, two-way ANOVA, Bonferroni as post hoc test).

Figure 4 – Conditioned place preference from chronic protocol

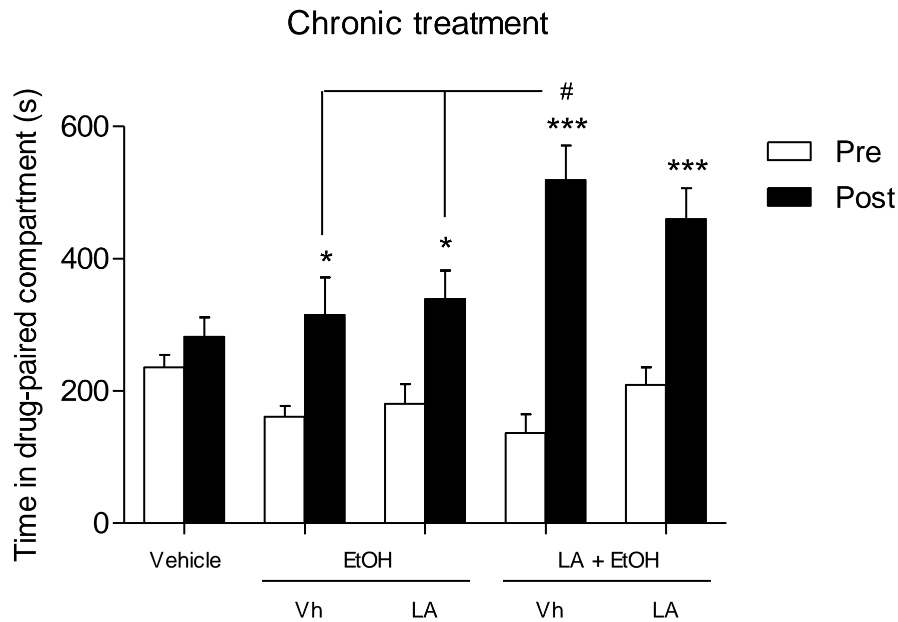


Figure 4 CPP of chronic EtOH or LA treatment. Over periadolescence, mice were treated for 14 days with vehicle or LA (100 mg/kg) daily. After this, animals were treated with EtOH (2 g/kg) or LA+EtOH daily, for 21 days. On adulthood, animals were submitted to CPP paradigm, and during conditioning sections were treated with EtOH (2g/kg - v.o.). Bars represent means ± SEM of time spent in drug-paired compartment before (white bars) and after (black bars) conditioning sections. CPP was considered when time spent in drug-paired compartment on post-conditioning significantly exceeded time spent in drug-paired compartment on pre-conditioning (***$p<0.001$, *$p<0.05$, two-way ANOVA, Bonferroni as post hoc test). # represents significant difference between post and pre-conditioning time spent on drug-paired compartment, as compared to Vh-EtOH and LA-EtOH groups (One-way ANOVA, Tukey as post hoc test).

Table 3 – Percentage difference of time on aversive or preferred compartment before and after conditioning sections

Table 3 Percentage difference of time spent on aversive or preferred compartment before and after conditioning sections.

	% Difference at the time on aversive compartment	% Difference at the time on preferred compartment
Vehicle	5.2 ± 2.9	-4.3 ± 5.7
Vh – EtOH	17.1 ± 6.3	0.0 ± 7.7
LA – EtOH	17.6 ± 4.2	-5.5 ± 6.4
Vh – LA+EtOH	**42.5 ± 8.3** [a,b,c]	-25.5 ± 7.9
LA – LA+EtOH	27.9 ± 5.1	-20.6 ± 6.9

Data were calculated through the difference between the percentage of post and pre-conditioning time spent on aversive or preferred compartment. Positive and negative values indicate an increase and decrease at the time spent in compartment, respectively. [a,b,c] indicate significant difference as compared to Vehicle, Vh-EtOH, and LA-EtOH groups. Alpha level was set at $p<0.05$ (One-way ANOVA with Tukey as post hoc test).

6.2 CAPÍTULO II – Lipoic acid and ethanol reinforcing properties: interaction mechanisms during developmental phase

Artigo proveniente dos resultados da tese de doutorado, contendo dados referentes ao estudo neuroquímico realizado. O presente artigo será submetido à uma revista internacional.

Lipoic acid and ethanol reinforcing properties: interaction mechanisms during developmental phase

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ABSTRACT

Among the processes who interact with rewarding system, could be mentioned the activity of neurotrophic factors, inflammatory mediators and antioxidants, which are altered on drug abuse. Alcohol causes deleterious effects in the adolescent brain which are distinct from those observed in adults. Lipoic acid, an antioxidant, have been implicated on alcohol reinforcing properties and cell damage caused by its abusive use. Thus, the aim of this study was to investigate the activity of neurotrophic factors, pro-inflammatory cytokines and oxidative stress parameters in brain areas of mice exhibiting place preference to ethanol and lipoic acid during adolescence. Brain areas (prefrontal cortex – PFC and striatum – ST) were dissected from animals previously treated acutely and chronically with ethanol (EtOH – 2 g/kg v.o.), lipoic acid (LA – 100 mg/kg v.o.) or association (LA+EtOH) and submitted to behavioral tests (locomotor activity, memory task and conditioned place preference) over adolescence. Concentrations of brain-derived neurotrophic factor (BDNF) (ST), IL-6 and TNFα cytokines (ST) and GSH (PFC and ST) were assessed. No significant alteration on BDNF concentration was observed with acute EtOH administration, but chronic exposure to this drug until young adulthood decreased it. BDNF was also reduced in groups treated with the association of LA and EtOH both acutely and chronically over adolescence. Cytokines IL-6 and TNFα behaved in a similar way for both acute and chronic treatment. Acute treatment with EtOH or LA did not change cytokine concentrations, but LA administration prior to EtOH induced a significant decrease of them. When chronically administered over adolescence EtOH curiously decreased cytokine concentrations, independently of the association with LA. The increase of GSH levels was observed when LA and LA+EtOH were acutely administered in adolescence (PFC and ST) and in all groups that received chronically EtOH with or without association to LA since adolescence over young adulthood (ST). A decrease in lipid peroxidation was shown on EtOH acute treatment, while chronic EtOH administration increased this parameter. LA was able to prevent this raise of EtOH-induced lipid peroxidation when co-administered with it since adolescence until young adulthood. These data demonstrate that LA fail to reduce long-term degenerative effects induced by ethanol and shows reinforcing effects on behavioral and neurochemical rewarding properties elicited by ethanol-induced preference.

Keywords: Lipoic acid; Ethanol; cytokines; BDNF; oxidative stress

1 INTRODUCTION

Childhood and adolescence are critical periods of development that are frequently associated with onset of substance use and behavioral risks due to its use (Wetherill & Tapert, 2012; Windle et al., 2009; Witt, 2010). Alcohol causes deleterious effects in the adolescent brain which are distinct from those observed in adults (Lacaille et al., 2015), and some of the cognitive effects induced by repeated ethanol treatment in adolescence continue into adulthood (Pascual, Blanco, Cauli, Minarro, & Guerri, 2007; Sircar & Sircar, 2005).

Studies suggest that temperamental and behavioral features observed in childhood predict subsequent results related to alcohol in posterity (Dick et al., 2013), therefore the high adaptive susceptibility of this period promotes a window of opportunity for research on preventive approach, ensuring the neurodevelopmental integrity and reducing the probability of development of drug abuse dependence in adulthood.

A complex relationship of diverse factors and pathways interact with rewarding system and interfere on reinforcing mechanisms of drug abuse. In a brain on developmental phase, this relationship becomes even more singular and delicate. In this context, great attention is given to the understanding of molecular and epigenetics mechanisms to try to elucidate the processes involving these relationships (Moonat, Starkman, Sakharkar, & Pandey, 2010; Yang et al., 2014).

Among the processes who interact with rewarding system, could be mentioned the activation/inactivation of transcription factors, the activity of neurotrophic factors, inflammatory mediators and antioxidants, which are altered on drug abuse (Yang et al., 2014). These, play a key role by influencing the rewarding system and being influenced by it. Substances or behaviors that interfere on these processes may therefore have implications for the development of dependence.

Lipoic acid, a potent antioxidant, have been investigated as a new therapeutic alternative for diseases such as Alzheimer (Rosini et al., 2011), depression (Marcia Calheiros Chaves Silva et al., 2014; Márcia Calheiros Chaves Silva et al., 2013) Parkinson (Araújo et al., 2013), mania (Macêdo et al., 2012), as well as schizophrenia-like symptoms (Vasconcelos et al., 2015) among others. Indeed, it have been implicated on alcohol reinforcing properties and cell damage caused by its abusive use (Peana, Muggironi, Fois, & Diana, 2013; Pirlich, Kiok, Sandig, Lochs, & Grune, 2002; Shirpoor, Minassian, Salami, Khadem-Ansari, & Yeghiazaryan, 2008). Nevertheless, little is known about age-associated alterations in the characteristics and mechanisms of LA, taking into account the periadolescence and addiction.

Previous experiments of our laboratory found an interference of lipoic acid on locomotor and cognitive effects elicited by alcohol use, besides increasing ethanol reinforcing effect during adolescence (data not shown). Thus, considering these findings and the body of evidence for the central involvement between ethanol, lipoic acid and rewarding properties (Ledesma, Baliño, & Aragon, 2014; Peana et al., 2013; Shirpoor et al., 2008), we aim to investigate the activity of neurotrophic factors, pro-inflammatory cytokines and oxidative stress parameters on this association in a period of high vulnerability, the adolescence.

2 MATERIALS AND METHODS

2.1 Subjects

Male *Swiss* mice, from 35 postnatal days, from the Department of Physiology and Pharmacology-UFC, were acclimated to 26 ± 2 ºC, in a light/dark cycle of 12 h, with free access to standard food and water. Procedures used in the present research were approved by Ethics and Animal Research Committee (CEPA) from Federal University of Ceará-UFC under protocol nº 18/14, and follows the principles of laboratory animal care of the National Institute of Health (NIH).

2.2 Drugs

Lipoic acid (LA) (Sigma Aldrich) was dissolved in carboxymethylcellulose at 5% and administered at 100 mg/kg intragastrically (i.g.). Ethanol (EtOH) solution at a concentration of 20% v/v, dissolved from an initial stock of ethanol absolute (Merk) in distilled water was administered at 2 g/kg i.g. For the control groups, distilled water (i.g.) was used as vehicle using a dose ratio of 0,1ml for each 10g of body weight.

2.3 Experimental design

For the present study, it was used brain areas from animals that have underwent a treatment protocol and behavioral tests (open field, Y-maze and conditioned place preference) performed in another moment in our laboratory. Data provided by these tests are in process of submission to a scientific journal. Thus, it will be explained the treatment protocol that preceded the obtaining of brain areas. Experimental design consists of two major protocols (acute and

chronic) with different animals for each group, represented in figure 1 and described posteriorly.

- Acute protocol

At 35 postnatal day (PND), period equivalent to periadolescence (Brenhouse & Andersen, 2011), animals were treated acutely with EtOH, LA or association (LA+EtOH; LA administered 20 min prior to EtOH), and after 30 minutes of substance administration animals were submitted to behavioral tests. After this, animals were sacrificed and it brains dissected for neurochemical analysis (Figure 1A).

- Chronic protocol

As expressed in figure 1B, from 35 PND, animals received vehicle or LA daily, during 14 days. After this treatment, it was started the chronic ethanol exposure, in which animals were treated with ethanol or LA+ EtOH (LA administered 20 min prior to EtOH) once daily, during 21 days. Control group remained receiving only vehicle. On 72 PND (adulthood) animals were submitted to behavioral tests, and after this, it were sacrificed and it brains dissected for neurochemical analysis.

Neurochemical tests were evaluated in a period in that the animals, which were treated during adolescence, reached adulthood. Thus, using this protocol, we aimed to evaluate reinforcing effects of ethanol in adult mice pre-exposed to LA during adolescence.

2.4 Quantification of proinflammatory cytokines (IL-6 and TNFα)

Brain areas dissected (ST) were homogenized in 10 vol of PBS buffer with protease (EMD Biosciences) and phosphatase (Sigma-Aldrich) inhibitors to be later centrifuged (10000g, 5 min). Supernatant was used without dilution. Cytokines concentration, in 50 µL of sample, was determinate by ELISA (R & D Systems, Minneapolis, MN, EUA) according to manufacturer’s protocol and expressed in pg/ml.

2.5 BDNF dosage

BDNF concentration was determined in the ST using an immunoenzymatic assay

(ELISA) according to manufacturer's protocol (Sigma Aldrich). Samples were homogenized in 10 vol of PBS buffer with protease (EMD Biosciences) and phosphatase (Sigma-Aldrich) inhibitors to be later centrifuged (10000g, 5 min). It was measured using a spectrophotometer in 450 nm wavelength and results were expressed in ng/ml.

2.6 Evaluation of oxidative stress parameters

The samples of brain tissue from prefrontal cortex (PFC) and striatum (ST) were homogenized using 0,1 M phosphate buffer (pH 7,4). The homogenates of the tissue were subjected to centrifugation (10000g during 15 minutes). Supernatants were isolated and used to measurement tests of oxidative stress.

- Determination of GSH concentration

It was used 400μL of homogenates in which was added 320μL of distilled water and 80μL of 50% trichloroacetic acid. The material was submitted to agitation and centrifugation (956g/15 minutes). Then, a microplate was prepared with 400μL of supernatants, 800μL of 0,4M Tris-HCl buffer (pH 8,9) and 20μl of 0,01M DTNB, and the absorbance was measured in a wavelength of 412nm using a spectrophotometric method. Reduced glutathione level was expressed in μg of GSH/g of tissue using a standardized curve (Sedlak & Lindsay, 1968).

- Lipid peroxidation evaluation

Lipid peroxidation level in brain tissue was analyzed by measurement of the thiobarbituric acid reactive substances (TBARS) (Draper et al., 1993). In order to prepare the analysis solution, homogenate was added to 1 mL of 10% trichloroacetic acid solution and 1 mL of 0,6% thiobarbituric acid solution.. After centrifugation, it was submitted to a boil water bath (95°C during 15 minutes) and posteriorly centrifuged (800g/5min). The concentration of TBARS was measured using a spectrophotometer in 532 nm wavelength. The results were expressed in μg of malondialdehyde (MDA)/g of tissue.

2.7 Data analysis

Statistical analysis was carried out using the software GraphPad Prism 5.0. To compare the means of groups on the parameters among neurochemical tests, one-way ANOVA was adopted with Tukey as post hoc test. All data are reported as mean ± SEM, and the alpha level was set at $p<0.05$.

3 RESULTS

3.1 Changes on BDNF concentration

Figure 2 shows BDNF concentrations on the ST of mice treated acutely or chronically with EtOH and LA during adoelscence. It was observed through one-way analysis a reduction of BDNF concentration in animals treated acutely with LA+EtOH during adolescence when compared to vehicle and ethanol treated groups [$F_{3,30} = 4.44$; $p=0.01$]. All groups chronically treated with EtOH showed a decrease on the concentration of this neurotrophic factor [$F_{4,27} = 37.71$; $p<0.0001$], and LA had no effect on the BDNF reduction induced by chronic use of EtOH independently of its administration on adolescence or concomitant with EtOH during young adulthood.

3.2 Quantification of proinflammatory cytokines

- IL-6

Figure 3 displays the effects of EtOH, LA and association on IL-6 activity on striatum of mice exposed to acute and chronic protocols. Adolescent mice treated acutely with the association of LA and EtOH showed a decrease on IL-6 concentration when compared with EtOH group [$F_{3,21} = 3.16$; $p=0.04$]. Furthermore, chronic treatment with EtOH also decreased this parameter with no alterations caused by LA administration on adolescence or young adulthood [$F_{4,28} = 5.88$; $p=0.001$].

- TNFα

Quantification of TNFα activity on mice treated with EtOH, LA and association is shown in figure 4. Post hoc test revealed, as observed on IL-6 results, a reduction of TNFα concentration on acute treatment with LA+EtOH during adolescence compared to ethanol

group [$F_{3,22}$ = 4.86; p=0.009]. Indeed, a reduction in this parameter was present on groups treated with ethanol chronically, with no alterations in this lowered concentration by LA treatment over different time of treatment [$F_{4,27}$ = 7.41; p=0.0004].

3.3 Alterations on oxidative stress parameters

- Determination of GSH concentration

The GSH concentration of animals treated with EtOH, LA and association with different treatment time is present on table 1. One-way ANOVA revealed that on adolescence, acute LA administration increased GSH concentration in both PFC [$F_{3,42}$ = 5.57; p=0.002] and ST [$F_{3,41}$ = 6.27; p=0.0013], a feature also found in animals treated with the association drugs. Chronic treatment of LA concomitant with EtOH enhanced GSH on PFC compared with both vehicle and Vh-EtOH groups [$F_{4,39}$ = 5.02; p=0.002]. All groups chronically treated with EtOH increased GSH concentration on ST [$F_{4,35}$ = 14.69; p<0.0001]. Moreover, concomitant treatment of LA with EtOH over young adulthood further enhanced GSH concentration as shown by comparison with groups treated in this period only with ethanol or vehicle, independently of LA pretreatment on adolescence.

- Lipid peroxidation

The degree of lipid peroxidation was availed through determination of thiobarbituric acid reactive substances (TBARS) (Table 2). Statistical analysis demonstrated a reduction of TBARS on PFC of adolescent mice treated acutely with EtOH and association (LA+EtOH) [$F_{3,44}$ = 5.38; p=0.003] with no alteration on ST [$F_{3,41}$ = 0.94; p=0.42]. Chronic treatment of LA since adolescence until adulthood associated with ethanol as from young adulthood once again decreased this parameter on PFC [$F_{4,32}$ = 4.50; p=0.005]. Indeed, on ST this association was able to prevent the raise on TBARS levels caused by chronic EtOH treatment with and without LA administration during adolescence, reducing this parameter [$F_{4,40}$ = 8.19; p<0.0001].

4 DISCUSSION

The present study investigated neurochemical mechanisms involved on reinforcing

properties of the ethanol and LA interaction in different age and treatment time. Previous experiments from our laboratory (data not shown) showed a reinforcing effect of LA associated with ethanol in adolescence and over young adulthood on behavioral test assessing conditioned place preference. Now, the focus pointed to neurotrophic factors, pro-inflammatory cytokines and oxidative stress parameters to elucidate this reinforcing modulation.

The involvement of lipoic acid on ethanol reinforcing effects is already suggested comprising modulation of diverse features including ethanol intake, ethanol-induced conditioned place preference, by interacting with brain-EtOH metabolism, among others (Ledesma & Aragon, 2013; Peana et al., 2013; Pirlich et al., 2002). Nevertheless, lipoic acid interacts in a different way on reinforcing effects of ethanol strongly depending of the period in which is administered, as well as the treatment time.

Among a variety of mechanisms and pathways surrounding the motivational/rewarding properties of abused drugs, great attention is given to knowledge of the molecular process and epigenetic background. Alterations in cyclic AMP-responsive element binding protein (CREB) (neurotrophic) and nuclear factor-kappa B (NF-κB) (neuroimmune) signaling contribute to the development and persistence of alcoholism (Vetreno & Crews, 2014).

Transcription factors as CREB controls genic expression of diverse functions from central nervous system in response to hormones, growth factors, synaptic activity and other cellular stimuli, playing an important role in processes as memory, learning, neuroplasticity and dependence (Lonze & Ginty, 2002; Moonat et al., 2010). CREB transcription is one of the mechanisms involved in addiction process, representing a convergence point for the action of many neurotransmitters and immunomodulators in several brain circuits (Dinieri et al., 2009).

One of the target genes of CREB is the brain derived neurotrophic factor (BDNF) gene. BDNF is a neuromodulator present on central nervous system of mammalian that have been implicated in several neuropsychiatric conditions (Carlezon, Duman, & Nestler, 2005; Nestler et al., 2002; Soule, Messaoudi, & Bramham, 2006). Furthermore, it is known that BDNF contributes for neuroadaptive changes caused by drugs of abuse and, in fact, an increasing body of evidence on literature suggest an involvement of BDNF on addiction process (Ghitza et al., 2010).

In the present study, no significant alteration on BDNF concentration was observed with acute ethanol administration during adolescence, but chronic exposure to this drug decreased BDNF concentration. These findings corroborate with data pointing that ethanol exerts effects dependent of the age of the exposed individual as well as the exposure time

(Philpot, Badanich, & Kirstein, 2003; Yang et al., 2014).

BDNF concentrations were also reduced in groups treated with the association of LA and ethanol both acutely and chronically over adolescence. Interestingly, groups that exhibited lower levels of BDNF also showed drug preference (data not shown), providing evidence for the inverse relationship between BDNF concentration and drug preference. Moreover, LA was not able to prevent or minimize the reduction of BDNF content caused by chronic ethanol exposure, showing no effect to restore neurotrophic factor levels in a condition of drug preference as the one adopted in the present study, independently of pretreatment or concomitant treatment during adolescence.

Relationship of ethanol-induced changes in BDNF protein and extracellular concentrations of striatal dopamine was demonstrated in a study that indicates that ethanol-mediated regulation of endogenous BDNF may, in turn, influence the dopaminergic response to ethanol (Bosse & Mathews, 2011). Together, ethanol and LA acute administration aroused BDNF reduction, what was not seen with drugs alone. This shows an effect dependent of this association that also induced place preference, pointing to an influence in the dopaminergic response by these drugs.

Studies have demonstrated the role of BDNF in modulation of the dopaminergic system (Bosse & Mathews, 2011), besides having an important participation on the regulation of behaviors associated to dependence (Bolanos & Nestler, 2004).

A study addressed the relationship between BDNF and behavioral, neurochemical and neuroadaptive responses to drugs of abuse, as well as the relationship with dopaminergic mesolimbic system. Behavioral studies involving cocaine showed that heterozygous animals to BDNF exhibited a decrease in conditioned place preference (Hall, Drgonova, Goeb, & Uhl, 2003).

Furthermore, studies indicate the participation of BDNF on regulation of ethanol associated behaviors. Investigations with heterozygous mice for BDNF showed that these animals presented an increase in consumption, behavioral sensitization and conditioned place preference to ethanol when compared to wild animals (Jeanblanc et al., 2006; McGough et al., 2004). On the other hand, the increase in BDNF expression is related with a decrease in ethanol consumption (Jeanblanc et al., 2009). These effects are, in part, due to BDNF action on dopaminergic receptors and modulation of the plasticity of dopaminergic neurons on mesolimbic pathway of rewarding system (Andressoo & Saarma, 2008; Jeanblanc et al., 2006).

Thus, the results of this study suggest that the loss of BDNF homeostasis can be related to stimulation of rewarding system generated by LA and ethanol consumption. In

accordance with these data, low BDNF concentrations have been associated with increased ethanol intake (Prakash, Zhang, & Pandey, 2008), while high levels of this factor are observed in animals that show decreased intake (McGough et al., 2004), ratifying the participation of this neurotrophin on the control of LA and ethanol consumption.

Besides the change in neurothophic pathways of repair and regeneration, drug abuse also interfere on neuroimmune signaling pathways, as occurs by the activation of NF-κB, a DNA binding protein involved in brain injury and neurodegenerative conditions (Vetreno & Crews, 2014). In the cellular nucleus, NF-κB regulates the transcription of target genes, including cytokines, chemocytokines, adhesion molecules among others (Yang et al., 2014). It acts on cell survival and proliferation, apoptosis and CNS functions.

NF-κB can be activated by stimuli as cytokines, oxidative stress, and others. Its activation kinetics interferes in a dual form on cell survival. A transient activation can lead to expression of anti-apoptotic genes, while sustained activation can induce a set of pro-apoptotic genes (Cone, 2001).

Chronic ethanol exposure leads to an increase of pro-inflammatory gene transcription factors as NF-κB and a reduction of transcription factors pro-survival as CREB, leading to neurodegeneration (Crews & Nixon, 2009). However, NF-κB can have decreased signaling through CREB pro-survival pathways, as well as by activating its own pro-inflammatory and pro-apoptotic cascades (Cho et al., 2001).

On the other hand, studies demonstrate that lipoic acid counteracts NF-κB activation. LA has been shown to suppress the NF-κB-dependent up-regulation of intracellular adhesion molecules (ICAM), TNF- and monocyte chemoattractant protein (MCP-1) in vitro and in vivo, and it also causes a down-regulation of NF-κB in the monocytes of diabetes patients (Anna Bilska & Włodek, 2005).

It is reported in the literature the association of cytokines with the symptomatology of alcohol use (Freeman et al., 2012; Leclercq et al., 2012). Although the mechanisms by which cytokine release may impact alcohol consumption are rather unclear, preclinical study results offer a putative explanation by reporting a linkage between the release of TNFα and IL-6 and the expression of neurotrophic growth factors such as brain-derived neurotrophic factor (BDNF) (Huang et al., 2014). Another preclinical studies found significantly elevated serum levels of IL-6 and TNFα in alcohol-dependent patients compared with healthy controls (Heberlein et al., 2014).

Cytokines IL-6 and TNFα behaved in a similar way on the assays performed for both acute and chronic treatment. Acute treatment in adolescence with ethanol or LA did not

change cytokine concentrations on striatum, but LA administration prior to ethanol induced a significant decrease of them compared to animals that were treated only with ethanol. These findings suggest an influence of LA on neuroinflammatory activity related to ethanol intake.

Corroborating with our data, it is described in the literature that LA can scavenge intracellular free radicals (acting as second messengers), down-regulate pro-inflammatory redox-sensitive signal transduction processes including NF-κB translocation, and thus attenuates the release of more free radicals and cytotoxic cytokines (Maczurek et al., 2008).

Further, a binge ethanol exposure during adolescence was described to sufficiently trigger widespread hippocampal microglial proliferation, without induction of a full microglial-driven neuroinflammatory response. This ethanol exposure in adolescence had no effect on TNF-α expression (McClain et al., 2011), a similar response found in our results for both TNFα and IL-6 concentration after acute ethanol exposure in adolescent mice.

On the other hand, it was found in our results that when chronically administered over adolescence ethanol curiously decreased cytokine concentrations, independently of the association with lipoic acid. Adolescent rats show forebrain degeneration during binge alcohol–induced neurodegeneration that is not found in adults, suggesting complex mechanisms to neurodegeneration related to ethanol exposure considering this vulnerable period of development (Crews et al., 2004). Moreover, it is reported that partial microglial activation triggered by ethanol in the adolescent hippocampus persists through early adulthood (McClain et al., 2011).

Compared with acute ethanol intake, chronic ethanol treatment results in persistent alteration of cytokines (Qin et al., 2008). Indeed, EtOH has been described to down-regulate inflammatory cytokines, providing negative feedback that ameliorates microglial proliferation and intracranial pressure (Chaturvedi, Zhang, & Basson, 2012).

Although lipoic acid is reported to attenuates the release of cytotoxic cytokines, which was also observed in our data, it seems that in chronic treatment prevailed ethanol influence, since even the animals that did not ingest lipoic acid also showed low levels of cytokines. Thus, suggesting an overstimulation of pro-apoptotic pathways leading to decreased ability to release pro-inflammatory cytokines, maybe because an exhaustive and long-term mechanism requested, which generated a reduction of its activity.

Drugs of abuse also lead to imbalance between substances pro-oxidants and antioxidants, causing oxidative stress often harmful to cell. Oxidative stress has been suggested to play a role in neurodegeneration and excitotoxicity as well as activation of the transcription factor NF-κB (Zou & Crews, 2006).

Acute and chronic effects of alcohol on GSH levels have been studied like biomarkers of oxidative-nitrosative stress. GSH is described to have profound relationship with substance abuse (Uys, Mulholland, & Townsend, 2014).

Unlike exposed in previous studies that propose a GSH reduction after ethanol administration (Boyadjieva & Sarkar, 2013), our results demonstrate an increasing of GSH concentration after EtOH treatment capable to induce reinforcing properties, mainly when in association with lipoic acid. A significant correlation of this increasing GSH concentration and ethanol preference was found, also regarding the rise of this preference induced by LA (data not shown).

It is likely that increased GSH in these groups represents a compensatory mechanism to exacerbated rise of dopamine on rewarding pathways, stimulated by the use of abused drugs, apparently occurring to neutralize the damage generated by the dopamine metabolism. Besides, studies suggest strong relationship of lipoic acid with dopaminergic system, and it was shown that LA acute administration (20 mg/kg), increases DA levels in rat hippocampus (Ferreira, Militão, & Freitas, 2009), which maybe potentiated ethanol preference when lipoic acid was co-administered.

The increased DA metabolism results in toxic effects for the cell. Cytoplasmic dopamine can rapidly be auto-oxidized to toxic products, such as superoxide radicals, hydrogen peroxide and DA quinones. In addition to non-enzymatic mechanisms of DA oxidation, enzymatic mechanisms can also contribute to dopamine metabolism and subsequent oxidative stress. The mitochondrial enzyme, monoamine oxidase, which is present on the cytoplasmic side, catalyzes the deamination of dopamine producing 3,4-dihydroxyphenylacetic acid (DOPAC) and hydrogen peroxide (Anna Bilska & Włodek, 2005).

The increase of GSH levels in mice brain areas is observed when the association of EtOH and LA is acutely administered in adolescence (PFC and ST) and when administered chronically since adolescence over young adulthood (ST). These findings are in accordance of studies that showed that treatment with this antioxidant compound increased GSH levels through different mechanisms: reducing cystine to cysteine, the limiting substrate for GSH synthesis (Shay, Moreau, Smith, Smith, & Hagen, 2009), or directly reducing GSSG to GSH (Moini, Packer, & Saris, 2002). It has been also demonstrated that LA and DHLA are capable to promote regeneration of other endogenous antioxidants of low molecular weight as glutathione, coenzyme Q10 and vitamins A and C (A Bilska, Dubiel, Sokołowska-Jezewicz, Lorenc-Koci, & Włodek, 2007).

Although literature reports that drugs that block oxidative stress and NF-κB transcription or increase CREB transcription can block alcohol binge-induced neurodegeneration, inhibition of neurogenesis and proinflammatory enzyme induction (Crews & Nixon, 2009), different data were found in the present study with lipoic acid treatment during ethanol exposure in adolescence. Instead, acute lipoic acid treatment associated with ethanol, aroused mechanisms inherent to alcohol use, and when administered chronically, not only failed to reduce long-term degenerative effects induced by ethanol but also showed reinforcing effects on behavioral and neurochemical rewarding properties elicited by ethanol-induced preference.

5 CONCLUSION

Chemical mediators focused in the present study suffered changes with the association of lipoic acid to ethanol in adolescence in a condition that evoked drug preference. It seems that lipoic acid reinforces the acquisition of ethanol preference when associated with it during adolescence, both acutely and chronically, intensifying modifications arising from the use of ethanol concerning ethanol-reinforcing mechanisms. These modifications are related to brain-derived neurotrophic factor, IL-6 and TNFα cytokines, antioxidant GSH alterations in brain areas of mice, as striatum.

It is worth emphasizing the doubt about the relationship of cause and effect found on the association of lipoic acid and ethanol, to elucidate if the drug preference tested in this study caused alteration in concentrations of biomarkers assessed, or if these altered concentrations induced animals preference to drugs.

Thereby, researches that aim unveil this relationship are encouraged, once lipoic acid is already widespread and several studies point to a promising character of this antioxidant in many neurodegenerative diseases and other pathological conditions. However, this substance behaves in a different ways according to treatment time and developmental phase in which is used, being necessary greater understanding about its relationship with ethanol during adolescence.

6 ACKNOWLEDGEMENTS

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7 CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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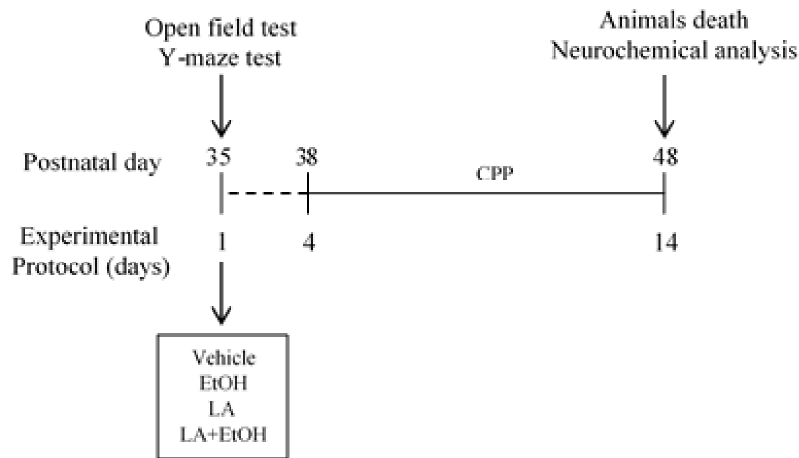
Figure 1 – Experimental design

A)



B)

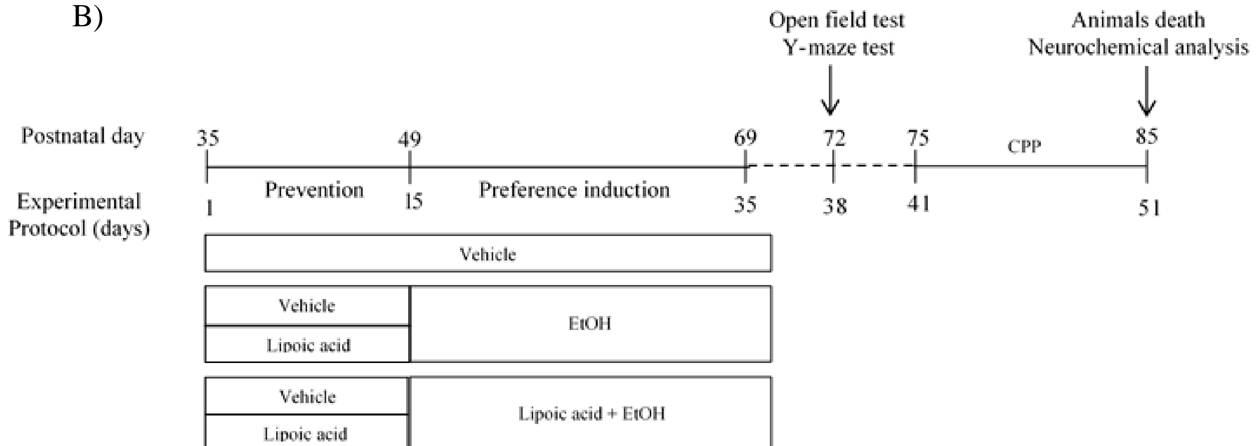
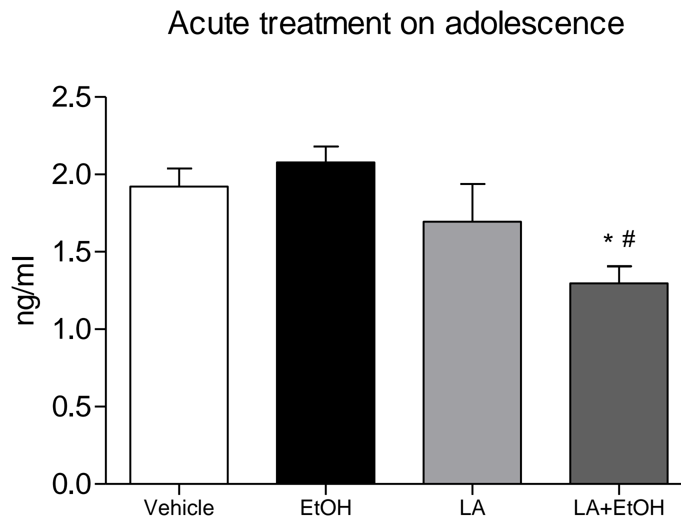


Figure 1 Experimental design of acute (above) and chronic (below) protocols of treatment and behavioral tests performed before the acquisition of brains areas. After these procedures, animals were sacrificed and it brains dissected and used for neurochemical analysis. CPP: conditioned place preference; EtOH: ethanol.

Figure 2 – Changes on BDNF concentration

A)



B)

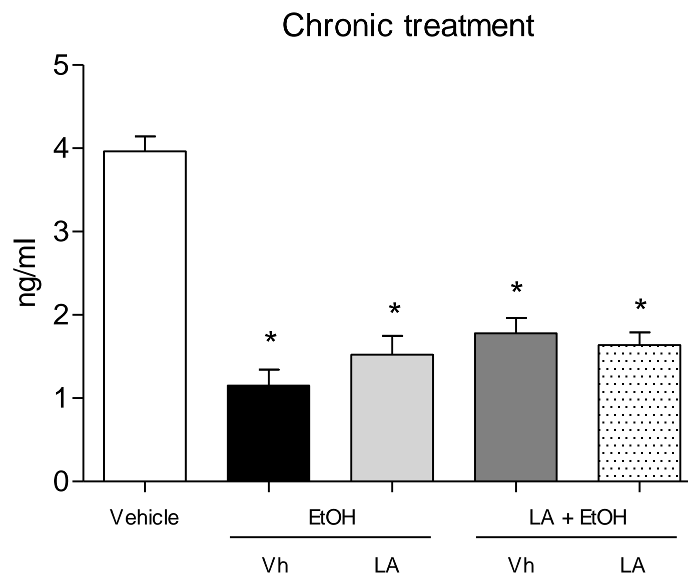
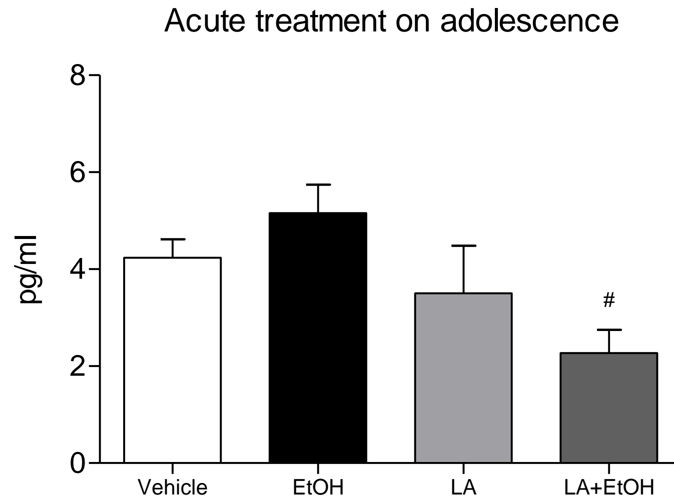


Figure 2 BDNF concentrations on ST of mice treated acutely or chronically with EtOH and LA. (A) Acute treatment on adolescence (B) Chronic treatment since adolescence until adulthood. On acute groups, animals were treated with vehicle, EtOH (2 g/kg), LA (100 mg/kg) or association. Chronic groups were treated during adolescence with vehicle or LA over 14 days. After this, it were treated with EtOH or LA+EtOH for 21 days. Bars represent mean ± SEM of BDNF concentration in ng/ml. $^{*,\#}$ represent significant difference compared to vehicle and EtOH groups (One-way ANOVA, Tukey as post hoc test).

Figure 3 – Quantification of IL-6

A)



B)

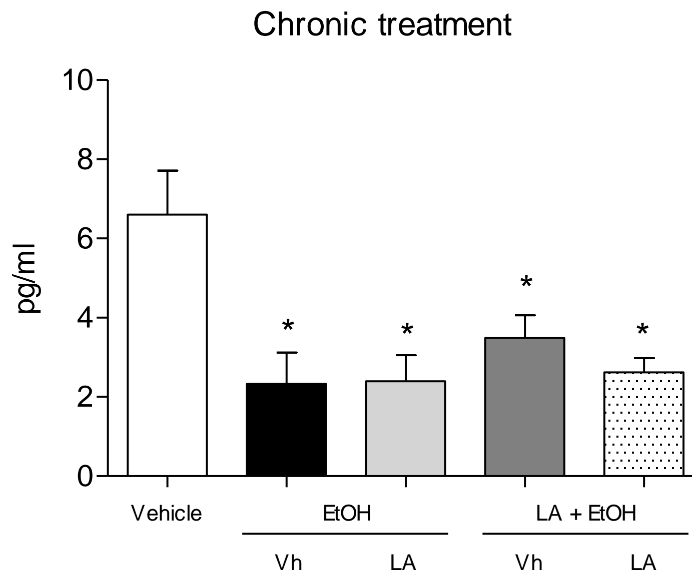
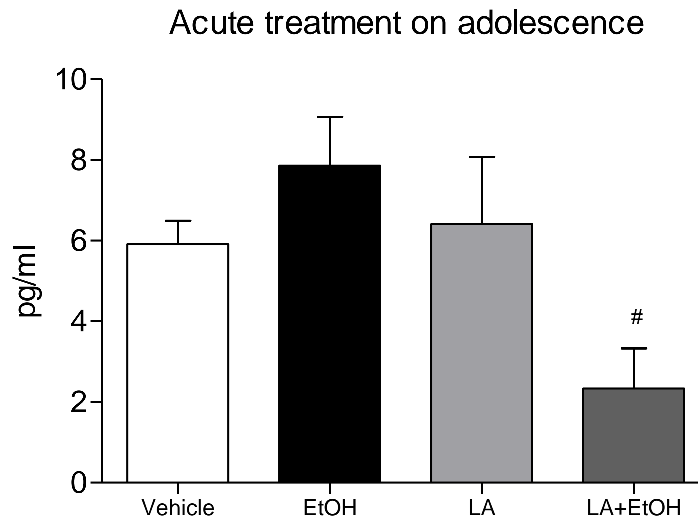


Figure 3 IL-6 quantification on ST of mice treated acutely or chronically with EtOH and LA. (A) Acute treatment on adolescence (B) Chronic treatment since adolescence until adulthood. On acute groups, animals were treated with vehicle, EtOH (2 g/kg), LA (100 mg/kg) or association. Chronic groups were treated during adolescence with vehicle or LA over 14 days. After this, it were treated with EtOH or LA+EtOH for 21 days. Bars represent mean ± SEM of IL-6 concentration in pg/ml. $^{*,\#}$ represent significant difference compared to vehicle and EtOH groups (One-way ANOVA, Tukey as post hoc test).

Figure 4 – Quantification of TNFα

A)



B)

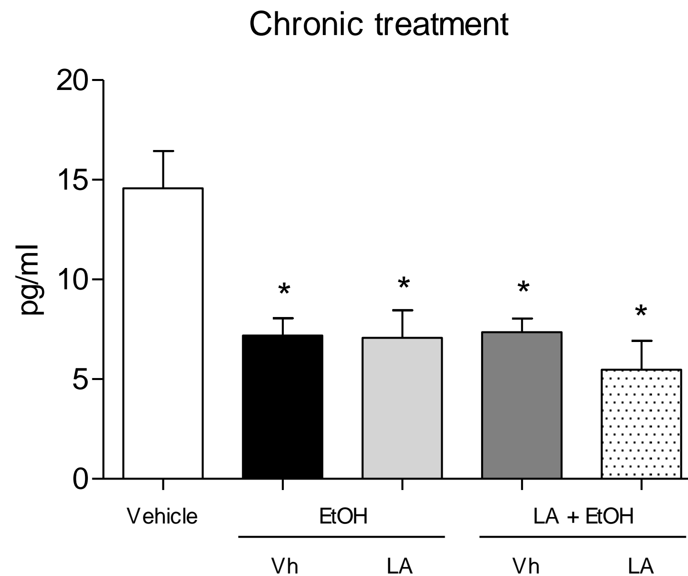


Figure 4 TNFα quantification on ST of mice treated acutely or chronically with EtOH and LA. (A) Acute treatment on adolescence (B) Chronic treatment since adolescence until adulthood. On acute groups, animals were treated with vehicle, EtOH (2 g/kg), LA (100 mg/kg) or association. Chronic groups were treated during adolescence with vehicle or LA over 14 days. After this, it were treated with EtOH or LA+EtOH for 21 days. Bars represent mean ± SEM of TNFα concentration in pg/ml. $^{*,\#}$ represent significant difference compared to vehicle and EtOH groups (One-way ANOVA, Tukey as post hoc test).

Table 1 - GSH levels on PFC and ST of mice treated with different treatment time

Table 1 GSH concentration on PFC and ST of mice treated with different treatment time.

	PFC	ST
Acute		
Vehicle	508.8 ± 24.6	552.4 ± 15.8
EtOH	642.7 ± 37.2	607.6 ± 25.5
LA	**754.0 ± 43.0[a]**	**724.5 ± 29.2[a,b]**
LA + EtOH	**715.5 ± 71.1[a]**	**695.0 ± 59.2[a]**
Chronic		
Vehicle	762.3 ± 100.5	501.2 ± 29.19
Vh – EtOH	779.4 ± 110.3	**970.3 ± 107.50[a]**
LA – EtOH	860.9 ± 109.0	**965.7 ± 91,31[a]**
Vh – LA+EtOH	**1227.0 ± 88.7[a,b]**	**1323.0 ± 93.02[a,b,c]**
LA – LA+EtOH	1058.0 ± 34.8	**1032.0 ± 51.18[a]**

GSH concentrations on PFC and ST of mice treated acutely (on adolescence) or chronically (over adolescence until adulthood) with vehicle, EtOH (2 g/kg), LA (100 mg/kg) or association. Bars represent mean ± SEM of GSH concentration in µg/g of tissue. For acute groups, [a,b] represent significant difference compared to vehicle and ethanol groups. For chronic groups, [a,b,c] represent significant difference compared to vehicle, Vh-EtOH and LA-EtOH groups respectively (One-way ANOVA, Tukey as post hoc test).

Table 2 – Lipid peroxidation on PFC and ST of mice with different treatment time

Table 2 TBARS levels on PFC and ST of mice with different treatment time.

	PFC	ST
Acute		
Vehicle	14.3 ± 0.8	8.5 ± 0.6
EtOH	**9.0 ± 1.1**[a]	7.5 ± 1.3
LA	11.3 ± 0.7	9.7 ± 0.6
LA + EtOH	**9.8 ± 0.6**[a]	8.4 ± 0.6
Chronic		
Vehicle	15.4 ± 1.0	8.0 ± 0.7
Vh – EtOH	14.8 ± 1.3	**12.3 ± 1.2**[a]
LA – EtOH	13.1 ± 1.5	**12.4 ± 1.2**[a]
Vh – LA+EtOH	13.3 ± 1.2	9.5 ± 0.7
LA – LA+EtOH	**9.1 ± 0.8**[a,b]	**5.8 ± 0.4**[b,c]

TBARS levels on PFC and ST of mice treated acutely (on adolescence) or chronically (over adolescence until adulthood) with vehicle, EtOH (2 g/kg), LA (100 mg/kg) or association. Bars represent mean ± SEM of TBARS concentration in µg/g of tissue. For acute groups, [a] represents significant difference compared to vehicle group. For chronic groups, [a,b,c] represent significant difference compared to vehicle, Vh-EtOH and LA-EtOH groups respectively (One-way ANOVA, Tukey as post hoc test).

7 CONSIDERAÇÕES FINAIS

Os efeitos do ácido lipóico dependem fortemente do período em que é administrado. Enquanto na adolescência, quando administrado de forma aguda antes do etanol, o AL induz preferência condicionada de lugar, na idade adulta este mesmo tratamento é capaz de impedir a aquisição de PCL induzida pelo etanol. Além disso, a administração crônica de ácido lipóico com etanol, durante a adolescência até a idade adulto-jovem, induz forte sensibilização para a preferência etanol. Os dados desta pesquisa também demonstram que o uso de ácido lipóico durante a adolescência apresenta nenhum efeito preventivo sobre a preferência induzida pelo uso crônico de etanol.

É possível que o ácido lipóico reforce a aquisição de preferência ao etanol, quando associado a ele durante a adolescência, tanto de forma aguda como crônica, intensificando modificações no que concerne aos mecanismos de reforço do etanol. Estas alterações estão relacionadas com o BDNF, citocinas IL-6 e TNFα, alterações de antioxidantes como GSH, em áreas cerebrais de camundongos, como o corpo estriado.

Vale ressaltar o questionamento sobre a relação de causa e efeito encontrada na associação de ácido lipóico e etanol, para elucidar se a preferência à droga testada neste estudo causou alterações nas concentrações dos biomarcadores avaliados, ou se estas concentrações alteradas foram responsáveis por induzir preferência dos animais às drogas.

Assim, pesquisas que buscam desvendar essa relação devem ser encorajadas, uma vez que o ácido lipóico já é amplamente utilizado, e diversos estudos apontam este antioxidante como promissor em muitas doenças neurodegenerativas e outras condições patológicas. Porém, esta substância apresenta comportamento diferenciado de acordo com o tempo de tratamento e, principalmente, fase de desenvolvimento em que é utilizada, sendo necessário maior compreensão sobre a sua relação com o etanol durante a adolescência. Portanto, pesquisas adicionais são de grande importância para um melhor entendimento acerca dos efeitos do ácido lipóico associado ao álcool durante o período desenvolvimental.

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APÊNDICE A – RESUMO ESQUEMÁTICO DOS RESULTADOS DO ESTUDO COMPORTAMENTAL

	Ativ locomot	Memória	PCL
Adolescência (agudo)			
EtOH	↑	↓	↔
AL	↔	↔	↔
AL+EtOH	↔ ↓*	↓	↑
Adulto (agudo)			
EtOH	↑	↔	↑
AL	↑	↑	↔
AL+EtOH	↑	↔	↔
Crônico			
Vh – EtOH	↓	↓	↑
AL – EtOH	↓	↔	↑
Vh – AL+EtOH	↓	↔ ↑*	↑*
AL – AL+EtOH	↓	↔	↑

Efeitos comparados ao grupo controle. *Comparando com o grupo EtOH.

APÊNDICE B – RESUMO ESQUEMÁTICO DOS RESULTADOS DO ESTUDO NEUROQUÍMICO

	BDNF	Citocinas	GSH
Adolescência (agudo)			
EtOH	↔	↔	↔
AL	↔	↔	↑
AL+EtOH	↓	↓	↑
Crônico			
Vh – EtOH	↓	↓	↑
AL – EtOH	↓	↓	↑
Vh – AL+EtOH	↓	↓	↑*
AL – AL+EtOH	↓	↓	↑

Efeitos comparados ao grupo controle. *Comparando com o grupo EtOH.