UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS:

NEUROCIÊNCIAS

EFEITOS DA N-ACETILCISTEÍNA SOBRE PARÂMETROS COMPORTAMENTAIS, NEUROINFLAMATÓRIOS E BIOQUÍMICOS DURANTE ABSTINÊNCIA DE ÁLCOOL EM RATOS

Ricardo Schneider Junior

Porto Alegre
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Ricardo Schneider Junior

Orientador

Profª. Drª. Rosane Gomez

Co-orientador

Profª. Drª. Elaine Elisabetsky

Tese apresentada ao curso de Pós-Graduação em Ciências Biológicas: Neurociências da UFRGS como requisito parcial para a obtenção do grau de doutor em neurociências.

Porto Alegre, Novembro de 2015

“La utopía está en el horizonte. Camino dos pasos, ella se aleja dos pasos y el horizonte se corre diez pasos más allá. Entonces para que sirve la utopía? Para eso, sirve para caminar”

Eduardo Galeano

AGRADECIMENTOS

Ao meu pai, mãe e irmã pelo apoio incondicional e contínuo incentivo à busca pelo conhecimento. Vocês foram extremamente importantes.

Aos colegas do LAT pelas risadas, ajuda intensa nos experimentos (inclusive aos finais de semana e feriados) e conhecimentos compartilhados durante nossos seminários. Sem vocês este trabalho não seria possível.

À professora Rosane Gomez por ter me acolhido como orientando, por ser um excelente exemplo de educadora e por toda a paciência e serenidade nas horas difíceis do desenvolvimento da tese.

À professora Elaine Elisabetsky pelos ensinamentos e pela co-orientação.

À Daniela por estar comigo em todas as horas difíceis nestes 4 anos.

Aos meus amigos Eduardo e Kleber pelo constante discurso motivacional ao longo deste trabalho.

Aos docentes do Programa de Pós-Graduação em Neurociências da UFRGS por todo o conhecimento passado com muita qualidade mesmo com todas as dificuldades estruturais de nossa universidade.

A CAPES pelo auxílio financeiro ao longo de toda a tese.

SUMÁRIO

APRESENTAÇÃO

A presente tese teve como objetivo avaliar os efeitos da N-acetilcisteína sobre diversos aspectos da abstinência de álcool em ratos. Tendo em vista de que os sinais e sintomas da abstinência de álcool são críticos para a recaída e a manutenção do transtorno por uso de desta substância, alternativas para o tratamento dessa síndrome poderiam contribuir para a prevenção da recaída em indivíduos dependentes de álcool.

Os resultados desta tese de doutorado estão apresentados sob a forma de três artigos científicos submetidos a diferentes periódicos científicos, sendo um deles publicado, um submetido e um a ser submetido para publicação.

Na **parte I** da tese encontra-se a introdução do trabalho contendo as bases teóricas para o seu entendimento. Na **parte II** encontram-se os artigos científicos oriundos da tese: o primeiro artigo intitulado *"N-acetylcysteine prevents behavioral and biochemical changes induced by alcohol cessation in rats"* foi publicado no periódico *Alcohol.* O segundo artigo intitulado *"N-acetylcysteine prevents the alcohol withdrawal-induced inflammatory response in the rat brain"* foi submetido ao periódico *Alcohol and Alcoholism*. O terceiro artigo intitulado *"Pretreatment with N-acetylcysteine prevents alcohol withdrawal-induced anxiety and increasing on corticosterone and leptin levels in rats."* será submetido ao periódico *Alcoholism: Clinical and Experimental Research*.

Na **parte III** encontram-se discussão, conclusões, limitações e perspectivas sobre os resultados obtidos. Segue-se a esses, o item Referências Bibliográficas que apresenta apenas os artigos utilizados nos tópicos Introdução, Discussão e Conclusões. Como anexo, também é apresentado documento de aprovação deste estudo pela CEUA-UFRGS.

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Artigo 1

saline = NAC 0) for 4 days starting 24 h after alcohol cessation. Data expressed as mean ± S.E.M. n/group: 12-13; NAC = N-acetylcysteine *$P < 0.05$, ** $P < 0.01$ compared to control; two-way ANOVA/Tukey.

Figure 2. Effects of N-acetylcysteine (NAC) on corticosterone (A) and leptin (B) serum levels after 5 days of alcohol cessation. Rats were treated with alcohol for 30 days, followed by NAC (or saline = NAC 0) for 4 days starting 24 h after alcohol cessation. Data expressed as mean ± S.E.M. n/group: 5-8; NAC = N-acetylcysteine. *$P < 0.05$ compared to control; # $P < 0.01$ compared to NAC 0 mg/kg (saline) alcohol; two-way ANOVA/Tukey.

Artigo 2

N-acetylcysteine prevents the alcohol withdrawal-induced inflammatory response in the rat brain

Fig 1. Effects of N-acetylcysteine (NAC) on the TNF-α (A), IL-1β (B), IL-6 (C), IL-18 (D), and IL-10 (E) levels in the rat hippocampus. Rats were treated with alcohol for 30 days, followed by NAC (or saline = NAC 0) for 4 days starting 24 h after alcohol cessation. The data are expressed as the percent of the control ± S.E.M. n= 6; NAC = N-acetylcysteine *$P < 0.05$, ** $P < 0.01$ compared to the control, $^{\#\#} P < 0.01$ compared to alcohol-treated rats; two-way ANOVA/Tukey.

Fig. 2. Effects of N-acetylcysteine (NAC) on the TNF-α (A), IL-1β (B), IL-6 (C), IL-18 (D), and IL-10 (E) levels in the rat frontal cortex. Rats were treated with alcohol for 30 days, followed by NAC (or saline = NAC 0) for 4 days starting 24 h after alcohol cessation. The data are expressed as the percent of the control ± S.E.M. n=6; NAC = N-

acetylcysteine *$P < 0.05$, ** $P < 0.01$ compared to the control, $^{\#\#} P < 0.01$ compared to alcohol-treated rats; two-way ANOVA/Tukey.

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Fig. 4. Effects of N-acetylcysteine (NAC) on the GSH levels in the rat hippocampus (HIP) and frontal cortex (FC). Rats were treated with alcohol for 30 days, followed by NAC (or saline = NAC 0) for 4 days starting 24 h after alcohol cessation. The data are expressed as the percent of the control ± S.E.M. n/group: 8-10; NAC = N-acetylcysteine; two-way ANOVA/Tukey.

Artigo 3

Pretreatment with N-acetylcysteine prevents alcohol withdrawal-induced anxiety and increasing on corticosterone and leptin levels in rats.

Figure 1. Experimental design. Rats were submitted to tree cycles of alcohol treatment (twice daily/5 days) set apart by 2 alcohol-free days. Four days before alcohol cessation rats were treated with saline or N-acetylcisteine (NAC) before subjected to the open field (OF) test.and sample collection (SC). The model was adapted from Overstreet et al. (2002).

Figure 2. Effects of N-acetylcysteine (NAC) on the open field in abstinent rats. (A) Time spent in central zone; (B) Total crossings. Data expressed as mean ± S.E.M. n= 8-10. *$P < 0.05$; two-way ANOVA/Tukey.

Figure 3. Effects of N-acetylcysteine (NAC) on corticosterone serum levels (A) and its correlation with the time spent in the central zone of the open field (B) in abstinent rats. Data expressed as mean ± S.E.M. n = 7-8. *$P < 0.05$, Two-way ANOVA/Tukey.

Figure 4. Effects of N-acetylcysteine (NAC) on leptin serum levels (A) and its correlation with the time spent in the central zone of the open field (B) in abstinent rats. Data expressed as mean ± S.E.M. n = 7-8. *$P < 0.05$, Two-way ANOVA/Tukey.

PARTE I

RESUMO

O transtorno por uso de álcool (TUA) é um transtorno crônico e recorrente, caracterizado pelo uso frequente, perda do controle sobre o uso e sintomas de abstinência quando ocorre a retirada, sendo este processo crítico para a recaída. Durante a síndrome de abstinência de álcool se observam, além de alterações comportamentais, alterações no sistema glutamatérgico, hormônios, neuroinflamação e estresse oxidativo. A N-acetilcisteína (NAC), um modulador do sistema glutamatérgico com propriedades antioxidantes e anti-inflamatórias, vem sendo considerado um fármaco com potencial efeito sobre a adição a algumas drogas de abuso. Entretanto, seus efeitos sobre a abstinência de álcool são pouco explorados. Desta forma, o objetivo principal desta tese foi avaliar os efeitos do tratamento de curto prazo com NAC sobre o comportamento de ratos abstinentes, bem como sobre alterações na concentração de corticosterona e leptina sérica, citocinas inflamatórias e glutationa além da atividade da enzima glutamina sintetase no tecido encefálico durante a abstinência de álcool em ratos. Para isso, se utilizou dois modelos de exposição crônica ao álcool, com diferentes tempos de abstinência e regimes de administração com NAC. No primeiro experimento, ratos Wistar machos adultos foram administrados com 2 g/kg de álcool ou solução glicosada durante 30 dias, 2 vezes ao dia, via gavagem e tratados com NAC (60 e 90 mg/kg), via intraperitoneal (i.p) ou solução salina (n=10/grupo) durante 4 dias após a cessação das administrações de álcool. Após 24 horas da última administração de NAC ou salina (5 dias de abstinência) os animais foram testados no campo aberto por 5 min, logo após eutanasiados e o sangue coletado para a análise de corticosterona e leptina. Em um segundo experimento, foi executado o mesmo protocolo de tratamento do primeiro experimento, entretanto, após a eutanásia foram coletadas estruturas encefálicas para a análise de citocinas inflamatórias, glutationa e atividade da glutamina sintetase. No terceiro experimento, utilizou-se modelo de administração crônica intermitente, sendo os ratos administrados com 2 g/kg de álcool ou salina, via gavagem, 2 vezes ao dia, 5 dias por semana, durante 3 semanas. Quatro dias antes da retirada do álcool os ratos foram tratados com NAC (60 e 90 mg/kg, i.p) ou salina concomitantemente com álcool ou solução glicosada (n=10/grupo). Vinte quatro horas após a última administração de NAC (1 dia de abstinência) os animais foram testados no campo aberto e após eutanasiados para a análise de corticosterona e leptina. Nossos resultados mostraram que o tratamento com NAC instituído após ou prévio ao início da abstinência preveniu hipolocomoção (5 dias de abstinência) ou ansiedade (24 horas de abstinência) em ratos. NAC também preveniu aumento de costicosterona e leptina após abstinência em ratos independentemente do modelo de exposição crônica utilizado. Adicionalmente, NAC preveniu aumento de citocinas inflamatórias no hipocampo e no córtex frontal de ratos abstinentes, além de prevenir a diminuição da atividade da glutamina sintetase no hipocampo durante a abstinência. O presente estudo mostrou que o os modelos de administração crônico-moderados de álcool induziram sinais e sintomas de abstinência em ratos compatíveis com os observados em humanos e, ambos os esquemas de tratamento com NAC, foram eficazes na prevenção de alterações produzidas pela abstinência de álcool. Tais resultados sugerem a indicação da NAC como um adjuvante na terapia para retirada do álcool e consequente prevenção de recaída.

ABSTRACT

Alcohol use disorder (AUD) is a chronic and recurrent disorder characterized by frequent use, loss of control of drug intake and the withdrawal symptoms when alcohol is withdrawn, being this process critical for relapse. During alcohol withdrawal syndrome behavioral changes, as well as changes in the glutamatergic system, hormones levels, neuroinflammation and oxidative stress are observed. N-acetylcysteine (NAC), a glutamate-modulating agent, with antioxidant and anti-inflammatory properties has been considered a putative anti-addictive drug. However, its effects on alcohol withdrawal are poorly understood. Thus, the aim of this thesis was to evaluate the short-term effects of NAC treatment on behaviors, leptin and corticosterone serum levels, as well as brain levels of pro-inflammatory cytokines, glutathione and glutamine synthetase activity during alcohol withdrawal in rats. Two different models of chronic alcohol exposure were used. In the first experiment, male Wistar rats were administered with 2 g/kg of alcohol, or glucose solution, during 30 days, twice daily, by gavage and treated with NAC (60 and 90 mg/kg, ip) or saline for 4 days after alcohol cessation. Twenty-four hours after the last NAC administration animals were tested in the open field test and euthanized for later corticosterone and leptin. In the second experiment, we used the same protocol of treatment as the first experiment, however, after euthanasia, brain structures were dissected for inflammatory cytokines, glutathione and glutamine synthetase activity analysis. In the third experiment, we used a model of chronic intermittent administration. Rats were administered with 2 g/kg of alcohol, by gavage, twice daily, 5 days/week, for 3 weeks. Four days before alcohol cessation rats were concomitantly treated with NAC (60 and 90 mg/kg, ip) or saline and alcohol or glucose solution. Twenty four hours after the last administration with NAC rats were tested in the open field test and euthanized for serum corticosterone and leptin analyses. Treatment with NAC during alcohol withdrawal prevented hypolocomotion and the increases of leptin and corticosterone serum levels in abstinent rats. The same treatment also prevented changes of frontal and hippocampal levels of inflammatory cytokines and hipocampal glutamine synthetase activity during withdrawal. When administered prior to alcohol withdrawal, treatment with NAC prevented anxiety and again prevented increases in leptin and corticosterone serum levels during early withdrawal. The present thesis showed that the model of moderate chronic alcohol administration induced alcohol withdrawal signs and symptoms in rats consistent with those observed in humans. and NAC treatment, in two different regimens, was effective in preventing these alcohol withdrawal-related alterations. These results suggest the indication of NAC as an adjuvant therapy for alcohol withdrawal and relapse.

LISTA DE ABREVIATURAS

ACTH Hormônio adrenocorticotrófico
AMPA ácido α-amino-3-hydroxy-5-metil-4-isoxazolepropionico
ATV Área tegmental ventral
CAT Catalase
CPF Córtex pré-frontal
CRF Fator liberador de corticotrofina
DAMPs padrões moleculares associados ao dano
EAAT1 Transportador de aminoácido excitatório 1
EAAT2 Transportador de aminoácido excitatório 2
ELISA Imuno-ensaio enzimático (*Enzyme Linked Immuno Sorbent Assay)*
EO Estresse oxidativo
ERN Espécies reativas ao nitrogênio
EROs Espécies reativas ao oxigênio
FDA Food and drug andministration
GPx Glutationa peroxidase
GS Glutamina sintetase
GSH Glutationa
HGMB1 grupo de alta mobilidade box-1
HPA Hipotálamo-hipófise-adrenal
IL-10 Interleucina 10
IL-12 Interleucina 12
IL-18 Interleucina 18
IL-1β Interleucina 1 beta
IL-6 Interleucina 6
LepRs Receptores de leptina
LPS Lipopolissacarídeo
MCP1 Proteína quimioatraente de monócitos
mGluR$_{2/3}$ Receptor metabotrópico de glutamato 2 e 3
NAC N-acetilcisteína
NAcc Núcleo acumbens
NFκβ Fator nuclear kβ
NMDA N-metil-D-aspartato
PCR Proteína C reativa
SNC Sistema nervoso central
SOD Superóxido desmutase
TLR4 Receptor tipo toll-4
TNF-α Fator de necrose tumoral alfa
TUA Transtorno por uso de álcool

1. INTRODUÇÃO

1.1. Uso de álcool e suas implicações

O consumo de bebidas alcoólicas faz parte de processos culturais de diversos países há séculos (McGovern, 2009). No mundo, os continentes com maior consumo de bebidas alcoólicas são a Europa e as Américas, sendo as diferenças de consumo total entre as regiões, resultado de complexas interações entre fatores sociodemográficos, econômicos e culturais (Organização Mundial da Saúde, 2014). O uso de álcool está entre os 5 principais fatores de risco para doenças, incapacitação e morte no mundo. É considerado o fator causal de doenças, danos com intenção (agressões físicas e de outros tipos) ou sem intenção (como acidentes de trânsito) (**Figura 1)**, além de estar associado à cerca de 6% das mortes no ano de 2012. Entretanto, permanece ainda com pouca prioridade nas pautas políticas, incluindo as direcionadas para a saúde pública (Organização Mundial da Saúde, 2014).

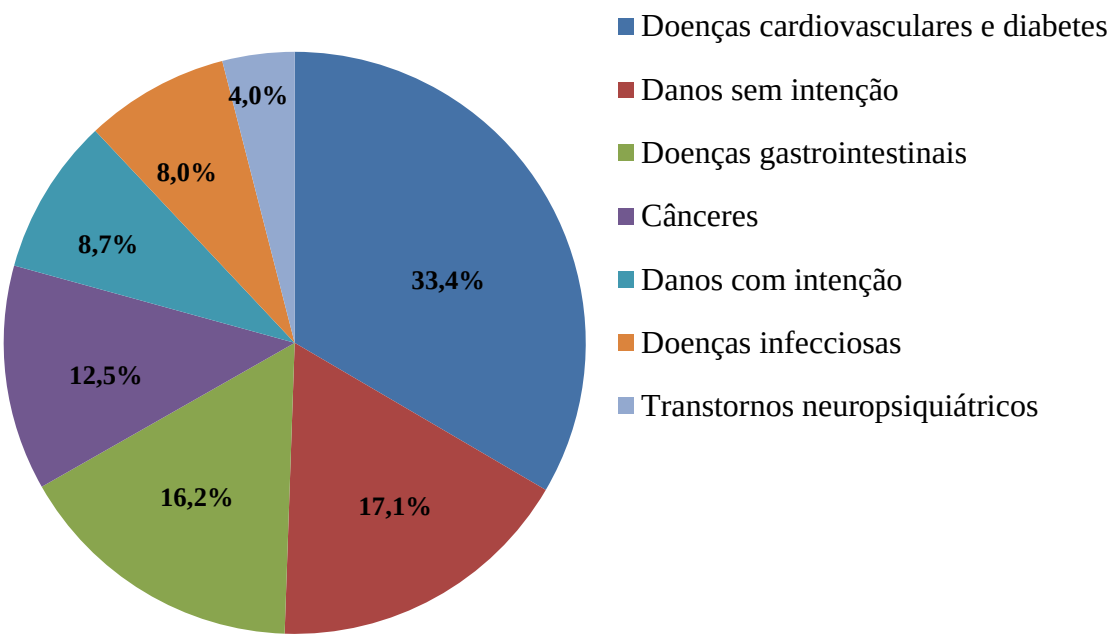

Figura 1. Distribuição de danos e doenças com morte associadas ao uso de álcool (adaptado de Organização Mundial da Saúde, 2014).

O álcool é um depressor do sistema nervoso central, possuindo efeito ansiolítico e sedativo, provocando incoordenação motora, desinibição, relaxamento, euforia, agitação, déficit cognitivo e de memória e quando usado de forma crônica, leva à dependência de álcool (Mckeon, Frye e Delanty, 2008). Condição esta, é reconhecida atualmente pela associação americana de psiquiatria como transtorno por uso de álcool (TUA) (American Psychiatric Association, 2013). Conceitualmente, este transtorno é caracterizado como sendo crônico e recorrente definido por diversas características, dentre elas: frequentes episódios de intoxicação, uso de álcool apesar de consequências adversas, compulsão pelo uso e o aparecimento de sintomas físicos e emocionais na abstinência (American Psychiatric Association, 2013).

1.2. Neurobiologia do TUA

Os traços compulsivos do TUA são, em parte, mediados pelos efeitos reforçadores do álcool (Gilpin e Koob, 2008). Reforço é um processo no qual uma resposta ou um comportamento é consolidado, baseado em experiências prévias. O reforço positivo é descrito por uma situação na qual um estímulo ou uma experiência (como a euforia induzida pelo álcool) aumenta a probabilidade do indivíduo exibir determinada resposta (como a busca por álcool). O reforço negativo ocorre quando uma resposta (como a busca por álcool) aumenta mediante a possibilidade de evitação ou alívio de um estímulo aversivo (ansiedade, por exemplo) (Gilpin e Koob, 2008). Mudanças nos valores de reforço durante a transição do uso para a dependência de álcool refletem processos neurais contra adaptativos resultantes da exposição a altas

doses de álcool. No início do uso, o comportamento de beber é amplamente motivado pelo reforço positivo, enquanto no estado de dependência este comportamento torna-se guiado tanto pelo reforço positivo quanto pelo reforço negativo (Koob e Le Moal, 2008).

O álcool, assim como outras drogas de abuso, possuem diferentes mecanismos de ação que convergem nas vias neurais de recompensa, produzindo efeitos na função celular quando administradas aguda ou cronicamente (Nestler, 2005). A via mais estudada neste contexto tem sido a via mesolímbica dopaminérgica. Esta via, que projeta neurônios dopaminérgicos da área tegmental ventral (ATV) até o núcleo acumbens (NAcc) e córtex pré-frontal (CPF) é um dos mais importantes substratos neurais para a geração de estímulos recompensatórios provocado por drogas de abuso, inclusive o álcool (Nestler, 2005). Cada droga, apesar de mecanismos de ação distintos, parece aumentar direta ou indiretamente a transmissão dopaminérgica nesta via. Com o uso contínuo do álcool, observa-se então uma série de processos adaptativos nesta via levando à sensibilização e tolerância aos efeitos prazerosos do álcool (Gilpin e Koob, 2008). A sensibilização refere-se ao aumento do valor reforçador do álcool após a exposição repetida enquanto que a tolerância se refere à redução do valor reforçador do álcool após a repetição das administrações (Gilpin e Koob, 2008). Uma vez ocorridos estes processos adaptativos, a retirada do álcool levará a síndrome de abstinência.

A síndrome de abstinência de álcool se constitui em um conjunto de sinais e sintomas que se iniciam em indivíduos dependentes de álcool tipicamente nas primeiras 24-48 horas após a última ingestão. Entre os sintomas mais clássicos em humanos estão ansiedade, anedonia, delírio, agitação, irritabilidade e convulsões (Mckeon, Frye e Delanty, 2008). Em roedores, esta síndrome se manifesta através de ansiedade, diminuição da interação social, hipolocomoção e perda da busca por novidades

(Overstreet, Knapp e Breese, 2002; Slawecki e Roth, 2004; Kliethermes, 2005; Fukushiro *et al.*, 2012). Esta fase do TUA é considerada uma fase crítica para a recaída e para a manutenção do ciclo da adição devido à geração de sintomas físicos e psicológicos (Koob e Le Moal, 2008). O processo de abstinência de álcool além de alterações comportamentais acaba induzindo uma série de alterações neuroquímicas no sistema nervoso central (SNC) como nos sistemas dopaminérgico (Koob e Le Moal, 2008), GABAérgico (Tsai e Coyle, 1998) e glutamatérgico (Tsai e Coyle, 1998; Kalivas, 2009), eixo hipotálamo-hipófise-adrenal (HPA) e mudanças dos níveis de peptídeos relacionados ao apetite (Aguiar-Nemer *et al.*, 2013). Além disso, o uso crônico de álcool bem como sua abstinência promove a indução de estresse oxidativo e processos neuroinflamatórios através da liberação de proteínas pró-inflamatórias em diversas áreas do encéfalo (Kelley e Dantzer, 2011). Todos estes processos irão contribuir para o dano e morte neuronal com consequências graves à cognição e a qualidade de vida dos usuários.

1.3. Uso de álcool e estresse oxidativo

Organismos aeróbios necessitam de oxigênio para metabolismo e obtenção de energia. Entretanto, durante o processo do consumo de oxigênio ocorre a formação de radicais livres como espécies reativas ao oxigênio (ERO) ou ao nitrogênio (ERN). Tais moléculas são essenciais para a manutenção e regeneração celular em todos os tecidos, incluindo o SNC. Entre estas moléculas estão o superóxido (O_2^-), o oxido nítrico (NO), radicais hidroxil (OH) e peroxil (ROH) além do peróxido de hidrogênio (H_2O_2) (Cobb e Cole, 2015). Em contraponto, o equilíbrio oxidativo é garantido pela presença de substâncias antioxidantes (Cobb e Cole, 2015). O desequilíbrio entre a produção dos radicais supracitados e os antioxidantes endógenos gera um processo conhecido como

estresse oxidativo (EO), que pode ser deletério para os tecidos e está relacionado à morte celular e diversas doenças (Cobb e Cole, 2015).

Apesar da intensa atividade metabólica, a concentração de antioxidantes no encéfalo é limitada se comparada a outros tecidos, estando ele menos apto a compensar a produção de ERO e ERN, tornando-o mais susceptível ao dano por estresse oxidativo (Cobb e Cole, 2015). Para preservar o equilíbrio redox, à semelhança de outras células, as células nervosas possuem enzimas antioxidantes como a superóxido desmutase (SOD), a catalase (CAT) e a glutationa peroxidase (GPx), além de antioxidantes não enzimáticos como a glutationa (GSH). A GSH é um composto tiólico sintetizado a partir dos aminoácidos cistina, glicina e glutamato que neutraliza uma das mais potentes espécies reativas como o H_2O_2, além de xenobióticos (Aoyama e Nakaki, 2015).

Pelas suas propriedades químicas, o álcool exerce seus efeitos deletérios também por meio de vias oxidativas produzindo EROs e peroxidação lipídica ou ainda pela depleção dos níveis de GSH (Bondy e Guo, 1995; Ramachandran *et al.*, 2003). Tal processo leva a disfunção mitocondrial e consequentes lesões hepáticas (Sastre *et al.*, 2007). Estudos mostram que o uso crônico moderado de álcool diminui os níveis encefálicos de GSH em ratos (Calabrese *et al.*, 2002) especialmente no cerebelo, estriado e córtex (Augustyniak, Michalak e Skrzydlewska, 2005). Ainda em ratos, se verificou o aumento do estresse oxidativo no encéfalo após a administração crônica de álcool nas doses de 1,2 g/kg/dia ou 1,6 g/kg/dia (Das *et al.*, 2007). Este fenômeno pode ser explicado pela concentração de acetaldeído, um metabólito tóxico do álcool, que na medida em que é removido das células através da ação antioxidante da GSH, acaba reduzindo os estoques celulares deste antioxidante (Augustyniak, Michalak e Skrzydlewska, 2005).

Além disso, se sabe que a indução do estresse oxidativo é neurotóxico (Aoyama e Nakaki, 2015) sendo o efeito oxidativo do álcool um fator importante na neurodegeneração causada por este composto. De fato, o uso de álcool leva ao estresse oxidativo induzindo alterações no influxo de cálcio nos neurônios e vias de sinalização de morte e proliferação celular como a via das proteíno-quinases ativadas por mitógenos (MAPK) levando à morte neuronal (Luo, 2014).

1.4. Uso de álcool e inflamação

A inflamação é um processo adaptativo muito importante para a manutenção e reparo tecidual. Entretanto, a persistência deste processo promove uma alteração significativa das funções dos tecidos, com desfechos graves para a homeostase sistêmica (Lugrin *et al.*, 2014). Os sinais inflamatórios são gerados primariamente por células do sistema imune como macrófagos, monócitos e linfócitos. No SNC, os sinais inflamatórios são liberados primariamente pelas células gliais como micróglia e astrócitos. A neuroinflamação induz ativação metabólica destas células, promovendo um acréscimo do consumo de oxigênio, produção de EROs e liberação de citocinas pró-inflamatórias como o fator de necrose tumoral alfa (TNF-α), interleucina 1-β (IL-1β), interleucina 6 (IL-6), interleucina 18 (IL-18) ou ainda citocinas anti-inflamatórias como a interleucina 10 (IL-10) (Maes, 2013). Essas citocinas, por sua vez, induzem vias de sinalização intracelular pela estimulação de receptores próprios (Pandey e Agrawal, 2006), regulando morte e proliferação celular. Recentemente, estudos têm observado uma forte relação entre o uso de álcool e neuroinflamação (Choi *et al.*, 2009; Kelley e Dantzer, 2011; Camacho, 2013; Crews e Vetreno, 2014; González-Reimers *et al.*, 2014).

A dependência de álcool tem sido reconhecida como uma doença inflamatória sistêmica (Kelley e Dantzer, 2011; González-Reimers *et al.*, 2014). O uso crônico de álcool aumenta os níveis séricos de citocinas pró-inflamatórias como IL-1, IL-6, IL-8, IL-10, IL-12, interferon γ, e TNF-α em humanos e roedores (González-Quintela *et al.*, 2000; Crews e Vetreno, 2014; González-Reimers *et al.*, 2014). Sugere-se que os níveis sistêmicos de citocinas inflamatórias podem refletir a severidade da síndrome de abstinência de álcool na medida em que a alteração dos níveis séricos destes mediadores inflamatórios têm sido correlacionada com a fissura e com sintomas de ansiedade durante a abstinência de álcool em humanos (Leclercq *et al.*, 2014) e roedores (Breese *et al.*, 2008).

Além disso, a administração crônica de álcool aumenta os níveis de TNF-α, IL-1 β, IL-6 e da proteína quimioatraente de monócitos (MCP-1) no soro, fígado, córtex e hipocampo de camundongos (Qin *et al.*, 2008). Tal achado também foi verificado no encéfalo de dependentes de álcool em um estudo *post-mortem* (He e Crews, 2008). Ainda, estudos mostram alteração de outros marcadores inflamatórios convencionais em homens com histórico de uso excessivo de álcool, como o aumento da proteína-C reativa sérica (PCR) (Alho *et al.*, 2004; Liukkonen *et al.*, 2006). Além do desencadeamento de processos inflamatórios, o uso e abuso de álcool parecem induzir mudanças significativas no sistema imunológico (Irwin e Miller, 2007) e o consequente aumento da susceptibilidade a diversas infecções (Zhang *et al.*, 2008).

1.5. Estresse e o eixo hipotálamo-hipófise-adrenal (HPA) na síndrome de abstinência de álcool.

Estresse, geralmente definido como um estímulo que perturba o equilíbrio interno do corpo (como a homeostase fisiológica) tem sido postulado como um importante correlato de consumo e recaída por álcool após um período de abstinência em humanos (Stephens e Wand, 2012). Estudos epidemiológicos mostram que diversos tipos de estressores sociais como estresse laboral e familiar levam ao consumo excessivo de álcool (Richman, Flaherty e Rospenda, 1996; Vasse, Nijhuis e Kok, 1998; San José *et al.*, 2000).

O componente de resposta ao estresse mais estudado em humanos e animais é o eixo HPA. Primeiramente, neurônios do núcleo paraventricular liberam fator liberador de corticotrofina (CRF) no sistema porta-hipofisário e, por sua vez, este hormônio estimula a glândula hipófise anterior a produzir hormônio aderenocorticotrófico (ACTH) que é liberado na circulação sistêmica. O ACTH, por sua vez, induz a síntese e liberação de glicocorticódes como o cortisol (em humanos) e a corticosterona (em roedores) nas glândulas adrenais (Stephens e Wand, 2012) (**Figura 2**). Os glicocorticoides têm sido usados como biomarcadores de estresse, sendo úteis para estudar as relações entre estresse, uso e dependência de álcool (Sinha, 2012).

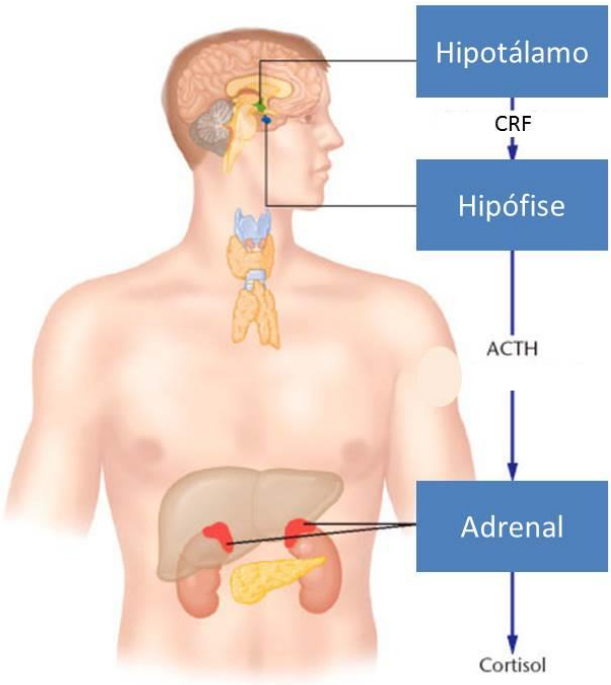

Figura 2. Representação do eixo HPA.

Assim como o estresse, o consumo de álcool ativa o eixo HPA elevando os níveis de glicocorticoides (Richardson *et al.*, 2008). No início da dependência de álcool se observa hipercortisolismo (Besemer, Pereira e Smit, 2011), principalmente quando acompanhado por frequentes episódios de intoxicação seguidos de abstinência (Wand e Dobs, 1991; Adinoff *et al.*, 1998). Além disso, a transição do abuso para a dependência é acompanhada por uma mudança alostática que resulta em uma redução da responsividade ao cortisol em humanos (Koob e Le Moal, 2001).

De fato, alterações da atividade do eixo HPA vêm sendo documentadas em vários estágios do TUA, incluindo intoxicação, abstinência e recaída (Stephens e Wand, 2012). Na abstinência aguda, uma grande quantidade de glicocorticoides é produzida e liberada sistemicamente (Sinha, 2012). Em indivíduos alcoolistas, durante abstinência, os níveis séricos de cortisol aumentam durante a primeira semana após a cessação, retornando aos níveis basais do grupo controle durante a quarta semana, coincidindo com a melhora dos sintomas de abstinência (Esel *et al.*, 2001). Em roedores, altos níveis

de corticosterona são observados durante a abstinência após 4 dias de intoxicação aguda por álcool (Sharrett-Field *et al.*, 2013).

Altos níveis séricos de glicocorticoides vêm sendo associados à fissura durante a abstinência ao álcool entre dependentes (Higley *et al.*, 2011) e ao agravamento dos sintomas de abstinência em ratos (Ara e Bano, 2015). Além disso, se observa uma associação entre o aumento dos níveis séricos destes hormônios com uma maior propensão à recaída em indivíduos alcoolistas (Higley *et al.*, 2011).

Uma das importantes ações do cortisol no contexto do TUA também é seu papel na modulação da memória. Tanto situações de estresse quanto durante exposição ao cortisol exógeno se observa modulação da aquisição e evocação de memória, ativando áreas encefálicas como o hipocampo e o córtex pré-frontal (Van Stegeren, 2009). Sabe-se que tais estruturas são muito importantes para a formação de hábitos, análise de risco e tomada de decisão frente a situações adversas. A alteração de suas funções está associada com o comportamento observado em usuários de diversas drogas, incluindo o álcool (Goldstein e Volkow, 2011).

1.6. Leptina e uso de álcool

A leptina vem ganhando notabilidade nos estudos sobre transtornos por uso de substâncias. Esta adipocina, sintetizada e liberada pelos adipócitos, é um peptídeo composto por 167 aminoácidos, sendo primariamente expresso no tecido adiposo branco, mas também encontrado em uma variedade de outros tecidos como a placenta, glândulas mamárias, ovários, músculo esquelético, sistema nervoso central e tecido linfoide (Park e Ahima, 2015). Embora seja mais conhecida por atuar como sinalizador endócrino envolvido na regulação do apetite e do gasto energético (Ahima *et al.*, 1996) também exerce efeitos centrais por ligação a receptores de leptina (LepRs) específicos,

localizados em diversas áreas encefálicas (Park e Ahima, 2015). Quatro isoformas deste receptor foram identificadas em humanos. Entretanto, a isoforma b (LepRb) é altamente expressa no hipotálamo e outras regiões encefálicas, regulando não apenas a homeostase energética, mas também funções neuroendócrinas (Park e Ahima, 2015).

A ingestão de alimentos também é modulada por estímulos neurais recompensatórios. De fato, vários estudos mostram que a leptina age na via mesolímbica dopaminérgica, modulando a transmissão de dopamina nesta via, à semelhança de drogas de abuso (Opland, Leinninger e Myers, 2010). A leptina também parece desempenhar um papel importante na indução de fissura em usuários de álcool. Diversos estudos em humanos mostram aumento dos níveis séricos de leptina durante a abstinência de álcool em indivíduos alcoolistas, sendo que estes níveis parecem estar fortemente correlacionados com a intensidade da fissura por álcool (Kiefer, Jahn, Jaschinski, *et al.*, 2001; Kiefer, Jahn, Kellner, *et al.*, 2001; Kraus *et al.*, 2004; Kiefer *et al.*, 2005) e com uma maior propensão à recaída durante a abstinência (Kiefer *et al.*, 2005)

Outro aspecto importante deste peptídeo durante o uso e abuso de substâncias é sua ação recíproca com o eixo HPA e a sua influência sobre as respostas neurofisiológicas mediante situações de estresse. A administração de glicocorticoides em roedores induz a síntese (De Vos *et al.*, 1995) e a secreção de leptina (Slieker *et al.*, 1996). A leptina parece modular negativamente a atividade do eixo HPA, inibindo o mesmo mediante situações de estresse. Estudos em camundongos mostram que a injeção intraperitoneal de leptina é capaz de reduzir a resposta a estímulos estressores como o estresse induzido por contenção, reduzindo os níveis séricos de ACTH e corticosterona (Heiman *et al.*, 1997).

A leptina também parece influenciar a hiperexcitabilidade neuronal e a consequente geração de convulsões, outro sintoma clássico da síndrome de abstinência de álcool. Estudos mostram uma associação entre o aumento dos níveis plasmáticos de leptina e a ocorrência de convulsões febris em crianças (Khoshdel, Parvin e Abbasi, 2013; Güven *et al.*, 2014). Em camundongos, se observa efeito pró-convulsivante após injeções intraperitoneais de leptina (Lynch *et al.*, 2010).

Desta forma, esta adipocina parece estar associada com fatores determinantes para o agravamento dos sintomas de abstinência de álcool, como o estresse, a fissura e a geração de convulsões.

1.7. Glutamato e abstinência de álcool

Um sistema neurotransmissor que parece exercer papel importante na recaída por álcool é o sistema glutamatérgico. O glutamato é o principal e mais abundante neurotransmissor excitatório do SNC, sendo crucial para plasticidade neural e, consequentemente, para processos de aprendizado e memória (Peng *et al.*, 2011). No SNC, os astrócitos são os maiores responsáveis por reciclar o glutamato sináptico através de sua captação da fenda sináptica por proteínas transportadoras como os transportadores de aminoácidos excitatórios EAAT1 e EAAT2 (Anderson e Swanson, 2000) e, ainda, convertê-lo em glutamina através da enzima glutamina sintetase (GS) (Hertz *et al.*, 1999).

O glutamato possui diversos receptores específicos amplamente distribuídos pelo encéfalo como receptores ionotrópicos N-metil-D-aspartato (NMDA), ácido α-amino-3-hydroxy-5-metil-4-isoxazolepropionico (AMPA) e cainato e ainda receptores metabotrópicos como o $mGlu_{2/3}$ (Peng *et al.*, 2011). Os receptores NMDA são considerados alvos de ação do álcool sobre o sistema glutamatérgico no encéfalo. Em

roedores se observa inibição destes receptores pelo uso crônico de álcool, com aumento da expressão de diversas subunidades do receptor NMDA como NR1, NR2A e NR2B no córtex e no hipocampo (Trevisan *et al.*, 1994; Follesa e Ticku, 1995). Como resultado, o SNC entra em um estado de hiperexcitabilidade durante a abstinência de álcool levando a episódios convulsivos e morte neuronal (Gass e Olive, 2008)

Quando a atividade sináptica glutamatérgica encontra-se demasiadamente aumentada, pode ocorrer morte celular por excessiva entrada de cálcio nos neurônios, liberação de radicais livres e oxido nítrico (Choi, 1988; Meldrum e Garthwaite, 1990; Mcentee e Crook, 1993). Durante a abstinência de álcool, se verificou o aumento dos níveis de glutamato encefálico tanto em humanos quanto em ratos utilizando-se espectroscopia de ressonância magnética (Hermann *et al.*, 2012). De acordo com a hipótese homeostática glutamatérgica, no TUA, os níveis sinápticos de glutamato em estruturas como o CPF e NAcc encontram-se aumentados enquanto os níveis extra-sinápticos de glutamato encontram-se diminuídos (Kalivas, 2009).

Um dos primeiros indícios sobre a perda da homeostase glutamatérgica no contexto do uso e abuso de substâncias foi observado por Baker e colaboradores (2003) que, em um modelo de autoadministração de cocaína em ratos, verificaram a associação entre a diminuição de glutamato extra-sináptico e o reestabelecimento do comportamento de busca pela droga após abstinência (Baker *et al.*, 2003). Além disso, estudos em animais mostram que o uso crônico de drogas, inclusive o álcool, leva a uma diminuição da expressão do EAAT2, diminuindo a captação do glutamato sináptico (Scofield e Kalivas, 2014). Ainda, se postula que a diminuição do glutamato extracelular acarretaria numa diminuição da ativação de receptores glutamatérgicos metabotrópicos do tipo 2 e 3 ($mGluR_{2/3}$) que normalmente inibem a liberação pré-sináptica de glutamato. O aumento dos níveis sinápticos de glutamato no CPF e no

NAcc, por sua vez levaria à recaída e ao aumento dos efeitos recompensatórios primários do álcool (Gass e Olive, 2008). (**Figura 3**)

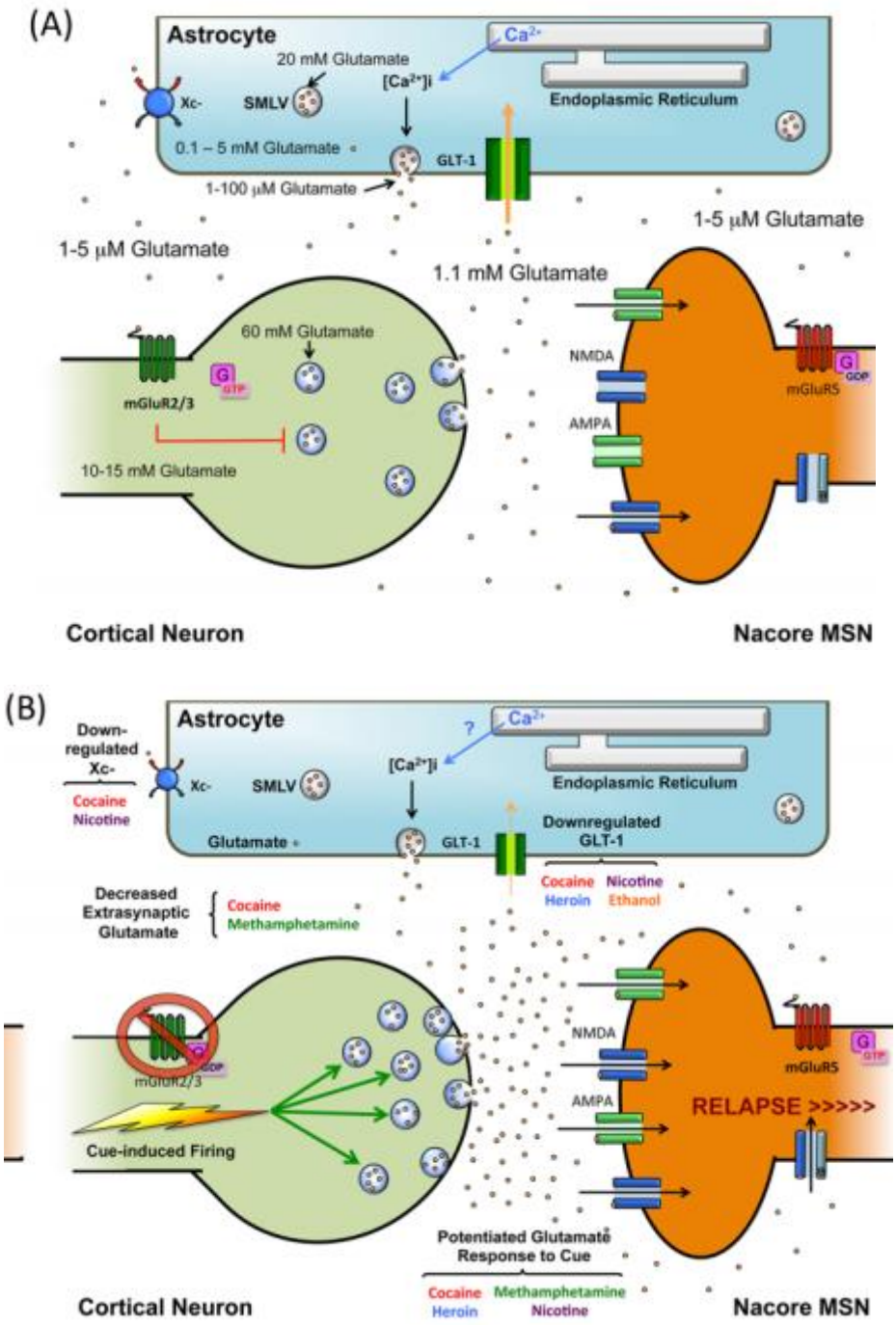

Figura 3. Sinapse glutamatérgica de neurônios corticais e do núcleo acumbens antes (A) ou após (B) a exposição a drogas de abuso (Scofield e Kalivas, 2014). É observado o aumento da concentração de glutamato sináptico devido à redução da expressão do

transportador GLT-1, redução da expressão do receptor $mGlu_{2/3}$ e do antiporter cistina/glutamato nos astrócitos. **Cortical Neuron** = neurônio cortical; **Nacore MSN** = neurônio da zona central (core) do núcleo acumbens; **GLT-1** = transportador de glutamato 1; **$mGlu_{2/3}$** = receptor metabotrópico de glutamato 2/3; **Xc^-** = antiporter cistina/glutamato; **NMDA** = receptor n-metil-D-aspartato; **AMPA** = receptor alfa-amino-3-hidroxi-metil-5-4-isoxazolpropiónico.

Uma interação entre o eixo extra-hipotalâmico do estresse e o sistema glutamatérgico também parece influenciar na hiperatividade glutamatérgica, na medida em que glicocorticoides são secretados durante situações de estresse, aumentando a liberação de glutamato em diversas estruturas límbicas e áreas corticais envolvidas com o comportamento aditivo por álcool como hipocampo, amígdala e CPF de roedores (Lowy, Gault e Yamamoto, 1993; Moghaddam, 1993; Venero e Borrell, 1999). Ainda em roedores, estudos in vivo utilizando microdiálise mostram que estímulos estressores repetidos como pressão sobre a cauda, nado forçado ou contenção podem aumentar transitoriamente nos níveis de glutamato no CPF (Moghaddam, 1993; Bagley e Moghaddam, 1997). Visto que durante uso crônico ou abstinência de álcool são liberadas altas concentrações de glicocorticoides (Stephens e Wand, 2012), está interação entre os dois sistemas supracitados poderia levar ao agravamento dos sinais de abstinência de álcool. Portanto, a modulação (direta ou indireta) do sistema glutamatérgico poder ser útil para o tratamento do TUA.

1.8. Tratamento do TUA

Verifica-se que quando um indivíduo dependente de álcool passa de um beber impulsivo para um beber compulsivo, ocorre uma mudança de ações mediadas por

reforço positivo para ações mediadas por reforço negativo. Tal mudança pode contribuir para a formação do ciclo do alcoolismo (assim como o transtorno por uso de outras substâncias) usualmente composto por três estágios: intoxicação, abstinência e preocupação/antecipação (Koob e Le Moal, 2001). Atualmente, existem alguns fármacos desenvolvidos para tratar cada um destes estágios, variando de acordo com a droga de abuso usada pelo indivíduo (Koob e Le Moal, 2009) (Tabela 1).

Embora fármacos aprovados pelo *Food and Drug Administration* (FDA) estejam disponíveis para a prevenção da recaída (**Tabela 1**), menos de 10% dos pacientes são tratados com estes medicamentos (Jonas *et al.*, 2014). Além disso, estudos testando outros fármacos ainda não aprovados pelo FDA para o tratamento do TUA (como baclofeno e topiramato) falham na hora de replicar os dados entre si (Thompson *et al.*, 2015). Como alternativa, se desenvolvem programas de ambientoterapia (ou comunidades terapêuticas), terapia de grupo (como alcoólicos anônimos) e/ou psicoterapia. Nas comunidades terapêuticas se aplica uma mudança do ambiente do usuário destacando-se este aspecto como fator terapêutico. Neste novo ambiente, livre do álcool, indivíduos alcoolistas vivem juntos de uma forma estruturada e organizada objetivando sua recuperação e reinserção na sociedade (Vanderplasschen *et al.*, 2013). Na terapia de grupo, como alcoólicos anônimos, os indivíduos em reuniões periódicas seguem 12 passos específicos (baseados em princípios teístas) de forma a permanecerem abstinentes (Suire e Bothwell, 2006). Tal estratégia vem mostrando eficácia na redução da impulsividade, consumo de álcool e dos problemas psicológicos relacionados ao consumo (Blonigen *et al.*, 2011). Abordagens psicoterápicas, como a terapia cognitivo-comportamental ou entrevista motivacional também são usadas, entretanto, apresentam pouca eficácia para a redução do consumo de álcool (Helstrom, Hutchison e Bryan, 2007).

Tabela 1. Fármacos disponíveis no mercado para o tratamento do TUA

Nome	*Ano de aprovação pelo FDA*
Dissulfiram	1954
Naltrexona	1994 e 2005 (formulação de liberação estendida)
Acamprosato	2004

Adaptado de Koob e Le Moal, 2009.

Recentemente, estudos clínicos vêm explorando diferentes regimes de tratamento para o transtorno por uso de substâncias. Por exemplo, o tratamento farmacológico aplicado antes da cessação do uso vem mostrando eficácia na redução do número de cigarros em no aumento da taxa de cessação em tabagistas (Ebbert *et al.*, 2015). Em indivíduos com TUA, o pré-tratamento com acamprosato possui um efeito protetor sobre a hiperexcitabilidade durante a abstinência quando aplicado 8 dias antes do início da abstinência (Boeijinga *et al.*, 2004). Desta forma, o tratamento iniciado antes da abstinência se torna uma alternativa para o tratamento do TUA.

1.9. N-acetilcisteína como alternativa terapêutica no tratamento do TUA

N-acetilcisteína (NAC) é precursora da cisteína, conhecida por sua ação antioxidante e utilizada na prática clínica para o tratamento da intoxicação por paracetamol e como expectorante (Cotgreave, 1997; Jones, 1998). Recentemente, estudos em humanos têm explorando a atividade central da NAC mostrando a efetividade deste fármaco como adjuvante no tratamento da Doença de Parkinson (Martínez *et al.*, 1999), esquizofrenia (Berk *et al.*, 2008; Lavoie *et al.*, 2008) e dos

sintomas depressivos do transtorno bipolar (Magalhães *et al.*, 2011). Tais observações clínicas dos efeitos psicofarmacológicos de NAC levaram a várias hipóteses para explicar sua ação sobre o SNC (Berk *et al.*, 2013). Em virtude de sua ação antioxidante, a NAC pode modular o sítio sensível ao redox do receptor NMDA, assim como quelar o íon Zn^{2+} (Hashimoto, 2009). Além disso, NAC é capaz de modular o sistema glutamatérgico de forma indireta através da modulação do antiporter cistina/glutamato presente nos astrócitos (Baker, Shen e Kalivas, 2002), além de possuir propriedades anti-inflamatórias, sendo entendida hoje como uma substância de vários alvos farmacológicos (**Figura 4**)

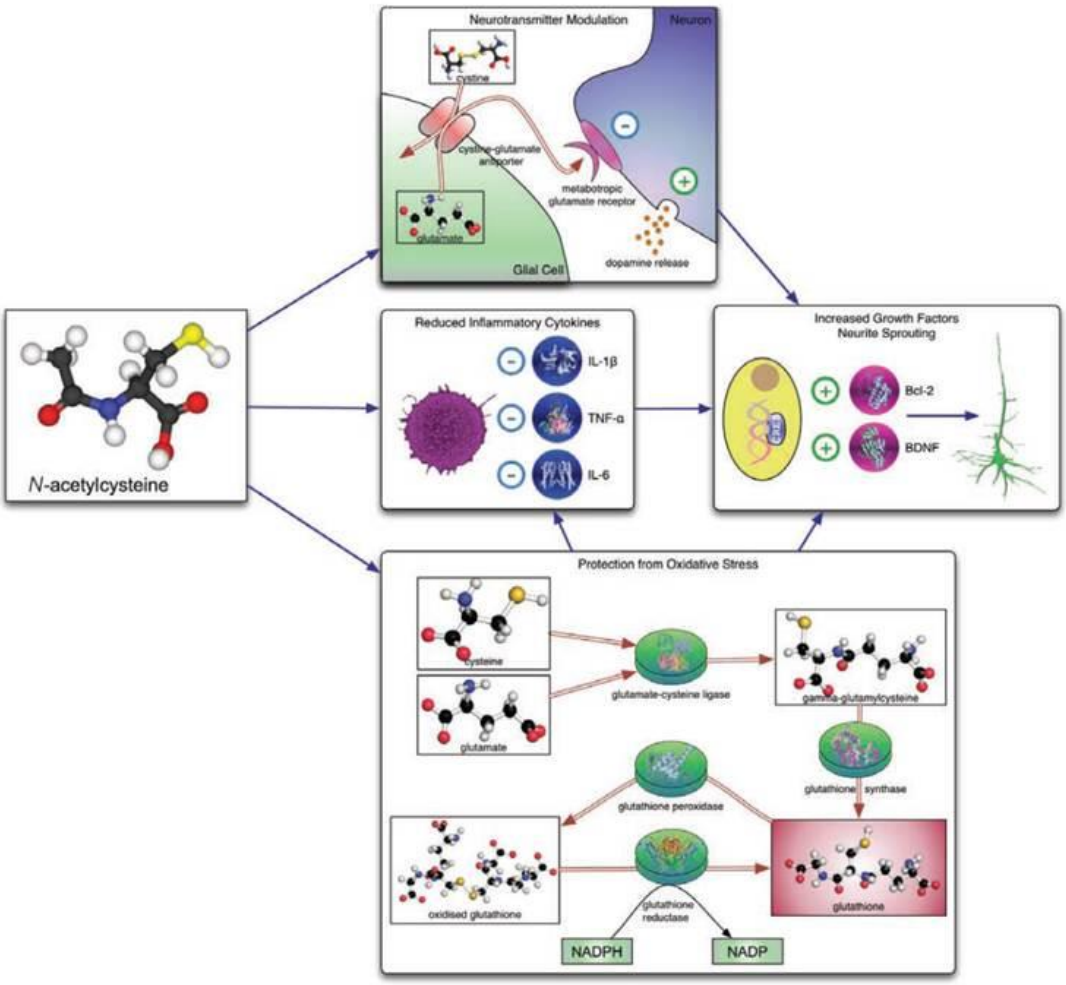

Figura 4. Mecanismos de ação da N-acetilcisteína (Dean, Giorlando e Berk, 2011).

A modulação do sistema glutamatérgico pela NAC pode ser a hipótese mais plausível para explicar os efeitos centrais da NAC. A alteração da homeostase glutamatérgica é responsável por diversas patologias do SNC (Caraci *et al.*, 2012). No encéfalo, os níveis basais extracelulares de glutamato são mantidos primariamente pelos astrócitos através da troca de cistina extracelular por glutamato intracelular (Baker, Shen e Kalivas, 2002). Este glutamato, quando no espaço extracelular, estimula receptores $mGluR_{2/3}$ os quais são muito importantes para a regulação da liberação de glutamato sináptico. Ativando o antiporter cistina/glutamato presente nos astrócitos, NAC aumenta os níveis extrasinápticos de glutamato e reestabelece a atividade de EAAT2 (Berk *et al.*, 2013). Por sua vez, o glutamato extrasináptico ativa receptores mGluR2, inibindo a liberação sináptica de glutamato (Berk *et al.*, 2013).

Os efeitos modulatórios de NAC sobre a atividade glutamatérgica motivaram estudos utilizando a NAC para o tratamento de transtornos por uso de substâncias. Em ratos, trabalhos mostram que o tratamento com NAC diminui o comportamento de busca por drogas como cocaína e heroína (Baker *et al.*, 2003; Zhou e Kalivas, 2008). Em humanos, mostrou-se que a NAC é capaz de diminuir a compulsão por cocaína (Larowe *et al.*, 2013), o número de cigarros fumados (Knackstedt *et al.*, 2009), e o uso de maconha (Gray *et al.*, 2010). Além disto, a NAC parece reduzir a sensação de recompensa pelo cigarro após um período de abstinência (Schmaal *et al.*, 2011). No contexto do uso de álcool, o uso da NAC vem sendo praticado somente para a prevenção de falência hepática em usuários (Hu *et al.*, 2015) ou do estresse oxidativo hepático causado pela intoxicação ou uso crônico de álcool em roedores (Ferreira Seiva

et al., 2009; Caro *et al.*, 2014). Entretanto, os efeitos centrais da NAC sobre aspectos que envolvem a dependência e a abstinência de álcool ainda não estão elucidados.

Considerando a necessidade premente de novas estratégias para o tratamento do transtorno por uso de substâncias e os aparentes benefícios da NAC neste contexto, o presente trabalho visou avaliar o efeito da NAC sobre aspectos comportamentais, neuroinflamatórios e endócrinos em ratos abstinentes de álcool.

2. OBJETIVOS

2.1. Objetivo geral

Avaliar o efeito do tratamento com NAC, em diferentes regimes de tratamento, sobre aspectos comportamentais, neuroinflamatórios e endócrinos da abstinência de álcool em ratos machos.

2.2. Objetivos específicos

Avaliar o efeito do tratamento com NAC durante a abstinência nas doses de 60 e 90 mg/kg sobre a ansiedade após a administração crônica de álcool em ratos no campo aberto;

Avaliar o efeito da NAC nas mesmas doses acima sobre os níveis séricos de corticosterona e leptina durante a abstinência de álcool;

Verificar o efeito anti-neuroinflamatório da NAC sobre as citocinas TNF-α, IL-1β, IL-6, IL-18 e IL-10 no hipocampo e no córtex frontal de ratos submetidos às mesmas condições acima;

Avaliar o efeito do tratamento com NAC, iniciado antes da abstinência, nas mesmas doses, sobre a ansiedade, corticosterona e leptina sérica após a abstinência.

PARTE II

Artigo 1

O primeiro artigo da tese foi publicado no periódico Alcohol. V. 49, 259-63. 2015 sob a forma de artigo intitulado:

"*N-acetylcysteine prevents behavioral and biochemical changes induced by alcohol cessation in rats*"

O referido artigo segue abaixo.

Alcohol 49 (2015) 259–263

Contents lists available at ScienceDirect

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N-acetylcysteine prevents behavioral and biochemical changes induced by alcohol cessation in rats

Ricardo Schneider Jr. [c,*], Carolina Ferreira Santos [a], Vanessa Clarimundo [a], Carla Dalmaz [b,c], Elaine Elisabetsky [a,c], Rosane Gomez [a,c]

[a] Departamento de Farmacologia, Universidade Federal do Rio Grande do Sul (UFRGS), Rua Sarmento Leite, 500/202, Porto Alegre, RS 90050170, Brazil
[b] Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul (UFRGS), Rua Sarmento Leite, 500/202, Porto Alegre, RS 90050170, Brazil
[c] Programa de Pós-Graduação em Neurociências, Universidade Federal do Rio Grande do Sul (UFRGS), Rua Sarmento Leite, 500/202, Porto Alegre, RS 90050170, Brazil

ARTICLE INFO

Article history:
Received 1 August 2014
Received in revised form
20 January 2015
Accepted 23 January 2015

Keywords:
Addiction
Corticosterone
Ethanol
Leptin
N-acetylcysteine (NAC)
Open field

ABSTRACT

N-acetylcysteine (NAC), a glutamate-modulating agent with antioxidant and anti-inflammatory properties, has been considered as a potential anti-addictive drug. Beneficial effects were reported for cocaine, cannabis, and tobacco addicts, but the effect of NAC in alcoholics or in alcohol animal models is unknown. The aggravation of alcohol withdrawal symptoms, such as anxiety, has been associated with increased levels of serum corticosterone and leptin. Thus, the aim of this study was to assess the effects of NAC on anxiety, as well as corticosterone and leptin serum levels, after cessation of chronic alcohol treatment in rats. Male Wistar rats were treated with 2 g/kg ethanol, twice daily, by gavage for 30 days; control animals received an appropriate dose of glucose to balance caloric intake. Rats were treated for 4 days with NAC (60 and 90 mg/kg, intra-peritoneally [i.p.]) or saline after alcohol cessation. Twenty-four hours after the last treatment, rats were exposed to a 5-min session in the open-field test (OF). Corticosterone and leptin serum levels were determined by ELISA in samples collected within 30 min after the OF. Results showed that rats were hypoactive (decreased rearing, peripheral, and total crossings), and that corticosterone and leptin levels were increased 5 days after alcohol cessation. Four days of NAC prevented the behavioral and biochemical changes brought about by alcohol cessation. We suggest that, in addition to the anti-addictive properties reported for other drugs of abuse, NAC is potentially useful in the management of alcohol withdrawal.

Introduction

Alcohol dependence is a relapsing disorder, which shares three distinct phases with other drug addictions: anticipation-preoccupation (including craving), intoxication, and withdrawal-negative affect (Koob & Le Moal, 2001). Alcohol dependence deregulates responses to stress and appetite, contributing to the negative emotional state associated with withdrawal (Engel & Jerlhag, 2014; Gilpin & Koob, 2008). The withdrawal syndrome in alcohol-dependent individuals is a well-characterized set of signs and symptoms, usually manifested 24–48 h after the last drink, and considered to be a key factor in the maintenance of the addiction cycle as well as for relapse (Koob & Le Moal, 2001; McKeon, Frye, & Delanty, 2008). These manifestations of alcohol withdrawal have been especially related to decreases in dopamine and GABA (Gilpin & Koob, 2008; Sanna et al., 2003), along with increases in glutamate activity (Gass & Olive, 2008; Kalivas, 2008). The syndrome frequently includes anxiety, tremors, agitation, delirium, and eventually seizures (McKeon et al., 2008). In rodents, it may be manifested as anxiety, decrease of exploratory behavior, and hypoactivity (Fukushiro et al., 2012; Kliethermes, 2005; Slawecki & Roth, 2004).

A growing body of evidence relates stress, anxiety, and glutamate (Popoli, Yan, McEwen, & Sanacora, 2011). The role of anxiety and stress in withdrawal is well documented (Sinha, 2012). Increased glutamatergic activity on cortico-striatal circuitry has been related to drug-seeking behavior and relapse (Gass & Olive, 2008; Kalivas, 2008). N-acetylcysteine (NAC), a cysteine pro-drug with glutamatergic properties, acts through the cystine/glutamate antiporter located in astrocytes, ultimately restoring extracellular glutamate concentrations and synaptic glutamate activity, via tonic activation of $mGluR_{2/3}$ receptors (Berk, Malhi, Gray, & Dean, 2013). Of specific relevance to drug-seeking behavior and relapse, it has

* Corresponding author. Departamento de Farmacologia, ICBS, UFRGS, Rua Sarmento Leite, 500/202, Porto Alegre, RS 90050170, Brazil. Tel./fax: +55 51 3308 3121.
E-mail address: quimtox@gmail.com (R. Schneider).

http://dx.doi.org/10.1016/j.alcohol.2015.01.009
0741-8329/

been reported that a single administration of NAC normalized glutamate levels in the anterior cingulate cortex in cocaine-dependent patients (Schmaal, Veltman, Nederveen, van den Brink, & Goudriaan, 2012).

In addition to its actions on brain glutamate, NAC exhibits antioxidant properties by stimulating the cysteine/cystine cycle, promoting glutathione (GSH) synthesis, and preventing oxidative stress and cellular damage (Berk et al., 2013). Additionally, NAC has anti-inflammatory properties, either by inhibiting the inflammatory response of microglia (Tsai et al., 2009) and/or by affecting the synthesis of pro-inflammatory cytokines of inflammatory pathways associated with psychiatric disorders, including drug addiction (Berk et al., 2013).

Anti-addictive properties have been documented for NAC. In rodents, NAC reduces self-administration of cocaine (Reichel, Moussawi, Do, Kalivas, & See, 2011), nicotine (Ramirez-Niño, D'Souza, & Markou, 2013), and heroin (Zhou & Kalivas, 2008). Moreover, short-term treatment with NAC reduces cocaine reinstatement in rats (Amen et al., 2011) and craving in humans (LaRowe et al., 2013). NAC decreases marijuana (Gray, Watson, Carpenter, & Larowe, 2010) and cigarette use (Knackstedt et al., 2009), craving for cannabis (Gray et al., 2010) and cocaine (LaRowe et al., 2013), and the positive rewarding effects from cigarette smoking after short-term abstinence (Schmaal et al., 2011). Moreover, NAC treatment reduces cocaine craving (Amen et al., 2011) in abstinent subjects, but not in active users (LaRowe et al., 2013), suggesting a specific effect of NAC during withdrawal. However, there are no studies exploring the effects of NAC during alcohol withdrawal.

During alcohol withdrawal, corticotrophin-releasing factor (CRF) is excessively produced and released from the hypothalamus, activating the hypothalamic-pituitary-adrenal axis (HPA) and increasing cortisol/corticosterone secretion and anxiety in humans and animals (Stephens & Wand, 2012). Leptin is a hormone secreted primarily by the adipose tissue, classically associated with satiety (Ahima et al., 1996), as well as having been shown to play an important role in controlling the HPA (Ahima et al., 1996; Roubos, Dahmen, Kozicz, & Xu, 2012). Interestingly, there seems to be a correlation between serum leptin levels and craving in alcoholics and smokers during withdrawal (Aguiar-Nemer, Toffolo, da Silva, Laranjeira, & Silva-Fonseca, 2013; Kiefer, Jahn, Jaschinksi, et al., 2001). It has been suggested that increased leptin levels during withdrawal, by reducing the dopaminergic transmission in the mesolimbic system, may lead to intensified craving and maintenance of addictive behavior (Fulton et al., 2006).

The aim of this study was to investigate the effects of NAC in the behavioral and biochemical changes induced by alcohol cessation in rats.

Methods

Animals

Seventy-eight adult Wistar rats (~300 g) obtained from the university's own colony (CREAL-UFRGS) were housed in the Pharmacology Department animal facility, in polypropylene cages (5 rats/cage, 33 × 40 × 17 cm), under controlled environmental conditions (22 ± 2 °C, 12-h light/dark, lights on at 7:00 AM), with free access to water and food (Nuvilab, Colombo, Brazil). All procedures were performed according to international and local policies for experimental animal handling, and the study was approved by the Ethics Committee for Animal Experimentation (CEUA-UFRGS #23069).

Drugs and reagents

Ethanol (98%) (Nuclear, São Paulo, Brazil) was diluted to 20% (w/v) in a 3% glucose (D-Glucose, Nuclear, São Paulo, Brazil) solution. Control rats received an 8% glucose solution, matching the caloric intake of the alcohol groups. N-acetylcysteine (Sigma–Aldrich, St. Louis, USA) was diluted in saline. All solutions were prepared fresh daily.

Experimental design

Rats were assigned to two treatment groups ($n = 39$): 2 g/kg of alcohol or glucose (gavage, twice daily at 9:00 AM and 2:00 PM) for 30 days. Twenty-four hours after the last gavage, rats were further divided ($n = 13$) to be treated for 4 days (i.p., 9:00 AM–11:00 AM) with saline (NACD) or 60 and 90 mg/kg NAC. Doses were chosen based on literature (Ramirez-Niño et al., 2013), but the treatment was repeated daily for 4 consecutive days because pilot experiments detected significant behavioral changes 5 days after alcohol cessation. Behavior in the open field was analyzed 24 h after the last NAC administration. The trunk blood was collected within 30 min after the open-field test.

Open-field test (OF)

Rats were habituated to the dimly lighted experimental room in their home cages for at least 30 min before the experiment. The open field consisted of a white wooden arena (100 × 100 × 50 cm) with the floor divided by black lines into 16 equal squares. Rats were individually placed in the center of the arena and the behavior was video-recorded for 5 min. After each session, the floor was cleaned with a wet paper towel. Videos were analyzed by a trained observer blinded to treatments, using a BASIC written software (Kevin Willioma, KD Ware Computer, Boston, MA). The frequency of peripheral, central, and total crossings, as well as rearing and grooming episodes were scored. Total activity was calculated by adding the total crossing with the frequency of rearing (Pähkla, Harro, & Rägo, 1996).

Corticosterone and leptin levels

The rats were euthanized by decapitation within 30 min after the open-field session. The trunk blood was collected in plastic tubes (Vacutainer, NC, USA), centrifuged at 1.4 × g, and the serum was stored at −80 °C until further processing. Serum corticosterone was extracted with ethyl acetate (3 times in 100 μL), diluted to 1:133, and measured with a commercial ELISA kit (Enzo Life Sciences International Inc., Plymouth Meeting, PA, USA).

The intra-assay variation was 4.3%, which is compatible with the manufacturer-expected percentage (6.6%) for the applicable concentration range. In order to minimize the inter-assay variation, all samples were processed at the same day. According to the manufacturer, the minimum detectable concentration is less than 20 pg/mL. For leptin, the serum was diluted 1:5 and measured with a commercial ELISA kit (Invitrogen, Grand Island, NY, USA). The intra-assay variation was 4.4%, which is compatible with the manufacturer-expected percentage (5.6%) for the concentration range used. The minimum detectable dose is 26.99 pg/mL. The corticosterone and leptin levels were measured in a microplate reader (PerkinElmer, Waltham, MA, USA) at 405 nm and 450 nm, respectively, according to the manufacturer's instructions.

Statistical analysis

The results were tested for normal distribution using the Shapiro–Wilks test, and analyzed by two-way ANOVA and Tukey *post hoc* test, with the condition (control × alcohol cessation) and NAC treatment as independent variables. One-way ANOVA was used to identify differences among controls. Pearson's correlation test was used to assess dose–response effect and potential correlations between behaviors (central, peripheral, and total crossing; rearing, grooming, and total activity) and hormone levels. The results were presented as the means ± standard error (S.E.M.). The significance was set at $p < 0.05$. The Sigma Stat program (Jandel Scientific Co., v. 11.0, San Jose, CA, USA) was used.

Results

Regarding the behavior in the open field, two-way ANOVA revealed significant interaction between condition and treatment for peripheral ($F[2,74] = 6.15$, $p = 0.004$) and total crossings ($F[2,74] = 4.84$, $p = 0.011$), rearing ($F[2,74] = 3.78$, $p = 0.028$), and total activity ($F[2,74] = 4.99$, $p = 0.01$). Tukey *post hoc* test shows that 5 days after the last alcohol administration, alcohol-treated rats presented decreased peripheral ($p = 0.002$, Fig. 1A) and total crossings ($p = 0.008$, Fig. 1C), rearing ($p = 0.017$, Fig. 1D), and total activity ($p = 0.005$, Fig. 1F). Four days' treatment with NAC (60 and 90 mg/kg) prevented the alcohol cessation-induced decrease of all these parameters ($p > 0.05$ for all comparisons with alcohol/saline group).

For corticosterone serum levels, two-way ANOVA detected main effects of condition ($F[1,29] = 7.87$; $p = 0.010$), treatment ($F[2,29] = 7.14$; $p = 0.004$), and a significant condition/treatment interaction ($F[2,29] = 12.45$; $p < 0.001$) (Fig. 2A). Tukey test indicated that alcohol cessation significantly increased corticosterone levels ($p < 0.001$, control 0 × alcohol 0), and that the NAC treatment prevented this alcohol-induced corticosterone increase ($p = 0.030$ and $p < 0.001$, for alcohol 60 and 90 × alcohol 0, respectively). The preventive effect of NAC was dose-dependent as shown by Pearson's test ($r = -0.78$, $p = 0.0009$, $n = 14$). One-way ANOVA used in order to check NAC effects on control (non-alcohol) groups did not detect significant differences ($F[2,18] = 2.57$; $p = 0.112$).

Regarding leptin, two-way ANOVA showed an interaction between condition × treatment ($F[2,34] = 3.91$; $p = 0.031$), with alcohol cessation significantly increasing leptin levels ($p = 0.036$), whereas NAC treatment did not modify leptin levels in control rats ($p > 0.05$). 90 mg/kg NAC prevented the alcohol cessation-induced leptin increase ($p = 0.002$, Fig. 2B). Pearson's test showed a negative correlation between doses of NAC and leptin levels ($r = -0.64$, $p = 0.002$, $n = 20$).

Pearson's test was used to check potential correlations between behaviors (central, peripheral, and total crossing; rearing, grooming, and total activity) and hormone levels (corticosterone and leptin) for each treatment and condition group. There were only two significant correlations: higher corticosterone levels were negatively correlated with central crossings in the control group not treated with NAC ($r = -0.75$, $p = 0.019$, $n = 9$), and higher levels of leptin were negatively related to rearing in the alcohol cessation group not treated with NAC ($r = -0.73$, $p = 0.026$, $n = 9$).

There were no significant differences in weight between the groups throughout the experiment ($p > 0.05$).

Discussion

The main finding of this study is that the short-term treatment with NAC was effective in preventing the behavioral and biochemical changes observed after 5 days of alcohol cessation.

Locomotion and rearing can be considered forms of exploratory behavior, and diminished exploration can be understood as decreased motivation (Fukushiro et al., 2012) or enhanced anxiety

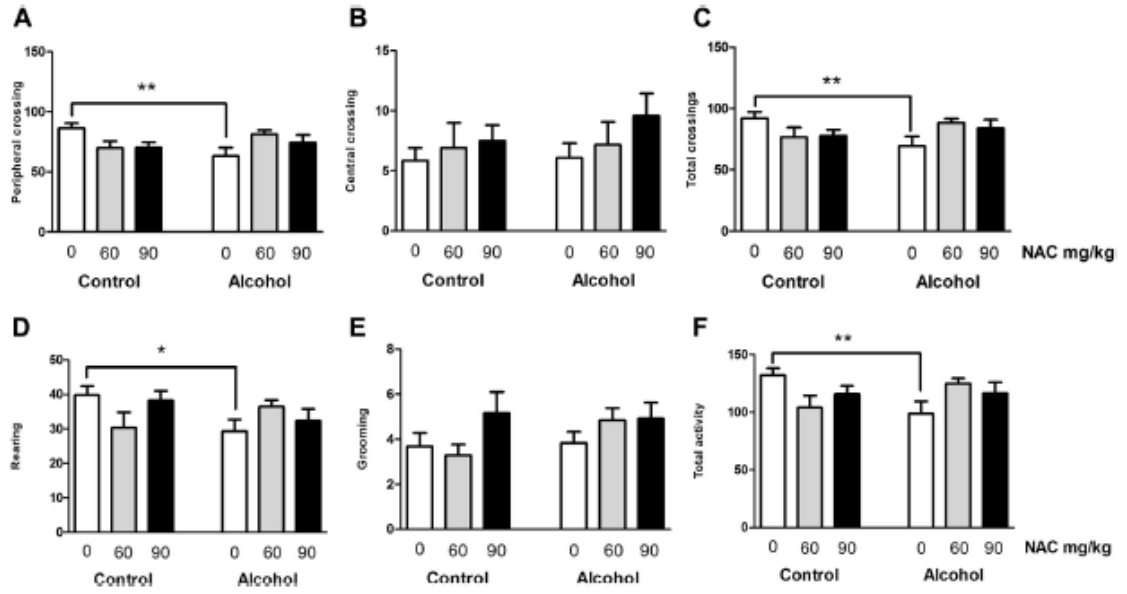

Fig. 1. Effects of N-acetylcysteine (NAC) in the open field 5 days after alcohol cessation. Peripheral (A), central (B), and total crossings (C); rearing (D), grooming (E), and overall activity (F). Rats were treated with alcohol for 30 days, followed by NAC (or saline – NAC 0) for 4 days starting 24 h after alcohol cessation. Data expressed as mean ± S.E.M. $n = 10–13$. NAC – N-acetylcysteine. $^{*}p < 0.05$, $^{**}p < 0.01$ compared to control; two-way ANOVA/Tukey.

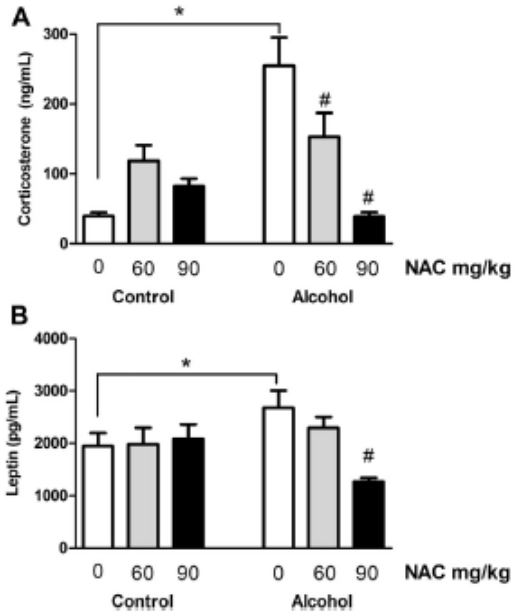

Fig. 2. Effects of N-acetylcysteine (NAC) on corticosterone (A) and leptin (B) serum levels after 5 days of alcohol cessation. Rats were treated with alcohol for 30 days, followed by NAC (or saline – NAC 0) for 4 days starting 24 h after alcohol cessation. Data expressed as mean ± S.E.M. $n = 4–9$. NAC – N-acetylcysteine. *$p < 0.05$ compared to control; #$p < 0.01$ compared to NAC 0 mg/kg (saline) alcohol; two–way ANOVA/Tukey.

(Kliethermes, 2005). As often observed during alcohol withdrawal in rats (Fukushiro et al., 2012; Kliethermes, 2005; Slawecki & Roth, 2004), we here show decreased exploratory behavior after alcohol cessation. There was no significant reduction in the frequency of central crossing, the most widely accepted anxiety parameter in the OF (Prut & Belzung, 2003), which may be related to the impaired risk assessment associated with drugs of abuse in humans (Goldstein & Volkow, 2011). NAC treatment prevented the diminished exploratory behavior after alcohol cessation. Although not reaching significance, NAC seems to decrease peripheral crossing and rearing behaviors in control animals, suggestive of differential effects of NAC during alcohol withdrawal when the glutamate system is expected to be hyperactive.

Changes in HPA activity have been documented in various stages of alcoholism, including intoxication, withdrawal, and relapse (Stephens & Wand, 2012). In early withdrawal, large amounts of glucocorticoids are produced, including cortisol, which is a well-established stress indicator (Sinha, 2012; Stephens & Wand, 2012). In male patients diagnosed with alcohol withdrawal, cortisol levels were increased during the first withdrawal week, returning to control levels at week 4, along with the diminishing of withdrawal symptoms (Esel et al., 2001). In rodents, high levels of corticosterone observed during withdrawal after a 4-day binge-drinking regime are thought to contribute to the development of alcohol dependence (Sharrett-Field, Butler, Berry, Reynolds, & Prendergast, 2013).

In patients experiencing alcohol withdrawal, leptin levels are found to be highly correlated with alcohol craving (Kiefer, Jahn, Jaschinski, et al., 2001; Kiefer, Jahn, Kellner, Naber, & Wiedemann, 2001; Kraus et al., 2004). The fact that drugs used to treat alcohol dependence (such as naltrexone and acamprosate) decrease serum leptin in abstinent alcohol addicts suggests that the correlation is meaningful (Kiefer et al., 2005). In mice, the administration of leptin increases alcohol ingestion, suggesting that this hormone may enhance the motivation for alcohol consumption (Kiefer, Jahn, Wolf, et al., 2001). Adding results from Aguiar-Nemer et al. (2013), we here show, to the best of our knowledge, for the first time in rats, that leptin serum levels are increased after alcohol cessation. This alcohol cessation-induced increase in leptin was prevented by the highest dose (90 mg/kg) of NAC, while NAC did not modify control leptin levels. The mechanism underlying this effect is still unknown and remains to be explored. However, it is possible that in pathological situations, such as alcohol withdrawal, NAC has more efficacy. In sum, this study shows that corticosterone and leptin levels were increased after alcohol cessation in rats and that NAC dose-dependently prevented these alcohol-induced changes. Further investigation on the effects of NAC in rats not treated with alcohol is desirable, given that potential effects may have been overlooked due to the small sample size used for biochemical analysis in our study.

A desirable control for the potential effects of the exposure to the open field, for both control and alcohol-withdrawn rats, was precluded by the ethical mandate to minimize the number of experimental subjects. In the absence of such control, the analysis of correlations between behavior and hormone levels can be informative. Regarding corticosterone levels, data show that though all groups were exposed to the open field, the only significant correlation was found for the control group not treated with NAC, where corticosterone levels were negatively correlated with central crossings. In the case of leptin, the only significant correlation was found for the withdrawn control rats, where leptin levels were negatively related to rearing. The lack of a consistent correlation between hormone levels and behaviors in any of the groups argues against a meaningful effect of merely exposing animals to the open field. As expected, there is a clear increase in corticosterone levels in the alcohol-withdrawal control group; the lack of a positive correlation with specific behaviors in this group can be attributed to the significant decrease in total activity. The correlation between increased leptin levels and decreased rearing in the alcohol cessation group is consistent with increased anxiety. The fact that leptin levels are significantly increased only in the alcohol-cessation vehicle group, supports the proposal that leptin can be useful as a biomarker for withdrawal.

In humans (Gass & Olive, 2008; Hermann et al., 2012) and rodents (Hermann et al., 2012), enhanced glutamate activity is observed during alcohol withdrawal. Leptin enhances the function of the N-methyl-D-aspartate (NMDA) glutamate receptors, particularly those expressing the N2B subunit (Harvey, 2007; Shanley, Irving, & Harvey, 2001). Moreover, leptin binds to dopaminergic neurons in the ventral tegmental area, inhibiting dopaminergic signaling in the nucleus accumbens (Fulton et al., 2006). Thus, it is reasonable to suggest that the NAC mechanism responsible for preventing the alcohol cessation-induced changes in behavior and hormones reported in this study is related to its ability to modulate the glutamate system. Indeed, long-term administration of addictive drugs is associated with the downregulation of the mGluR2 autoreceptors and the glial glutamate transporter 1 (GLT1) (Brown, Kupchik, & Kalivas, 2013). Under this condition, glutamate spills out to the synaptic cleft and activates extrasynaptic receptors, such as mGluR5 and NMDA receptors expressing the N2B subunit, resulting in drug relapse (Brown et al., 2013; Kalivas & Volkow, 2011). Interestingly, NAC activates the cystine/glutamate antiporter, thereby increasing the non-vesicular glutamate release and restoring the GLT1 activity (Berk et al., 2013; Brown et al., 2013). Additionally, by increasing extrasynaptic glutamate, NAC activates presynaptic mGluR2 that inhibits its synaptic release, diminishing synaptic glutamate activation (Berk et al., 2013).

As a glutamate-modulating agent, NAC can quickly stabilize a hyperactive glutamate system in a critical timing for withdrawal and relapse. Based on the preliminary data here reported, and because NAC is a safe, well-tolerated and inexpensive drug with antioxidant, anti-inflammatory, and glutamate-modulating properties (all relevant to abstinence), we suggest further studies to evaluate the potential efficacy of NAC in the management of alcohol withdrawal.

Acknowledgments

The authors are grateful for CNPq (EE, CF, and VC) and CAPES (RSJ) fellowships. The authors acknowledge the expert suggestions of the editor and reviewers.

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Artigo 2

O segundo artigo da tese foi submetido ao periódico *Alcohol and Alcoholism*, o artigo é intitulado:

*"**N-acetylcysteine prevents the alcohol withdrawal-induced inflammatory response in the rat brain"***

O referido artigo segue abaixo.

N-acetylcysteine prevents the alcohol withdrawal-induced inflammatory response in the rat brain

Ricardo Schneider Jr[1,2], Solange Bandiera[2], Greice Caletti[2,3], Débora Guerini Souza[4], Bruna Bellaver[4], André Quincozes-Santos[4,5], Elaine Elisabetsky[1,2,4], Rosane Gomez[1,2,4].

[1]Programa de Pós-Graduação em Neurociências, Universidade Federal do Rio Grande do Sul (UFRGS), Rua Sarmento Leite, 500/313 - Porto Alegre, 90050170, RS, Brazil

[2]Departamento de Farmacologia, UFRGS, Rua Sarmento Leite, 500/305, Porto Alegre, 90050170, RS, Brazil

[3]Programa de Pós-Graduação em Ciências da Saúde, Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA), Rua Sarmento Leite, 450, Porto Alegre, 90050170, RS, Brazil

[4]Programa de Pós-Graduação em Bioquímica, UFRGS, Rua Ramiro Barcelos, 2600, Porto Alegre, RS, 90035003, Brazil

[5]Departamento de Bioquímica, UFRGS, Rua Ramiro Barcelos, 2600, Porto Alegre, RS, 90035003, Brazil

Corresponding Author

Rosane Gomez
Departamento de Farmacologia, ICBS - UFRGS
Rua Sarmento Leite, 500 room 305
90050170 - Porto Alegre, RS, Brazil
Phone/FAX +55 51 3308 3121
E-mail: rosane.gomez@ufrgs.br

Running Title: NAC prevents alcohol-withdrawal induced neuroinflammation

Abstract

Aims: Alcohol withdrawal has been linked to changes in inflammatory cytokines, glutamate homeostasis, and oxidative status in the human and rodent brain. N-acetylcysteine (NAC) is known to modulate inflammation, the glutamate system, and oxidative stress, all of which are relevant to the effects of alcohol abuse, withdrawal, and relapse. Here we assessed the effects of NAC on the brain inflammatory cytokines, glutamine synthetase (GS) activity, and glutathione (GSH) levels after cessation of chronic alcohol treatment in rats. **Methods:** Wistar rats received 2 g/kg alcohol twice daily by gavage for 30 days. At day 31, rats were treated (once daily) with NAC (60 or 90 mg/kg, i.p.) or saline for 4 consecutive days. Twenty-four hours after the last NAC administration, the rats were euthanized and their serum, frontal cortex and hippocampus were analyzed for TNF-α, IL-1β, IL-6, IL-10, IL-18, GS and GSH. **Results:** Alcohol withdrawal increased the levels of all pro-inflammatory cytokines and decreased the IL-10 levels, an anti-inflammatory cytokine; NAC prevented these inflammatory responses in the hippocampus and in the frontal cortex of rats. GS activity was decreased after alcohol withdrawal and the NAC treatment did not prevent this change in the hippocampus. **Conclusion:** Given the association of inflammation with alcohol craving and relapse, the consistent protection afforded by NAC in relevant brain areas may have clinical implications. This study suggests that NAC may be useful to reduce neuroinflammation associated with alcohol withdrawal, which may be pertinent to reduce the risk of relapse in alcoholics.

Keywords: Addiction, Cytokines, Ethanol, Glutamine, Neuroinflammation, GSH.

INTRODUCTION

Alcohol dependence is a chronic and relapsing disorder that shares three distinct phases with other types of drug addiction: anticipation-preoccupation (including cravings), intoxication, and withdrawal-negative effects (Koob e Le Moal, 2001). The withdrawal syndrome in alcoholics is a well-characterized set of signs and symptoms and is considered a key factor for relapse and the maintenance of the addiction cycle (Koob e Le Moal, 2001; Mckeon, Frye e Delanty, 2008). In addition to changes in the dopaminergic and GABAergic systems (Gilpin e Koob, 2008), alcohol withdrawal has also been reported to lead to neuroinflammation (González-Reimers *et al.*, 2014), impairment of glutamate homeostasis by astrocytic dysfunctions, and oxidative stress (Gonzaga *et al.*, 2014; Scofield e Kalivas, 2014).

Cytokines are proteins primarily produced by immune cells that regulate the inflammatory response, cell proliferation and cell death (Peters, 1996). These proteins are highly expressed in the central nervous system (CNS) in neurons and glial cells in response to non-immune stimuli such as stress (O'connor *et al.*, 2003), mood disorders (Camacho, 2013) and alcoholism (González-Reimers *et al.*, 2014). The serum levels of interleukins, such as IL-1β, IL-6, IL-8, IL-10, and IL-18, are higher in alcoholics in comparison to non-alcoholic volunteers (González-Quintela *et al.*, 2000) and decrease after withdrawal. In rodents, the levels of central and peripheral pro-inflammatory cytokines are also increased during the early stages of withdrawal (Doremus-Fitzwater *et al.*, 2014). It has been suggested that the cytokine levels may reflect the severity of alcohol withdrawal, because changes in the cytokine levels have been correlated with craving and anxiety in both humans (Leclercq *et al.*, 2014) and rodents (Breese *et al.*, 2008).

N-acetylcysteine (NAC), a cysteine pro-drug, has been identified as a multi-target centrally acting drug, with glutamatergic, anti-inflammatory, and antioxidant properties (Berk *et al.*, 2013). NAC modulates the cysteine/glutamate antiporter in astrocytes, restoring the extracellular glutamate concentrations and synaptic glutamate activity via tonic activation of $mGluR_{2/3}$ receptors (Berk *et al.*, 2013). NAC possesses anti-inflammatory properties, which are mediated by reductions in the levels of serum inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, after severe burns, abdominal aortic aneurysm repair, or in peritoneal dialysis patients (Mahmoud e Ammar, 2011; Csontos *et al.*, 2012; Purwanto e Prasetyo, 2012). The antioxidant properties of NAC are related to increased glutathione (GSH) synthesis in glial cells (Berk *et al.*, 2013).

Anti-addictive properties for NAC have been explored for drugs of abuse such as marijuana, nicotine, cocaine, and heroin in human and rodents (Zhou e Kalivas, 2008; Knackstedt *et al.*, 2009; Gray *et al.*, 2010; Larowe *et al.*, 2013; Ramirez-Niño, D'souza e Markou, 2013). However, the central antioxidant and anti-inflammatory properties of NAC during alcohol withdrawal are still poorly understood.

We recently reported that four days of NAC treatment initiated 24 h after alcohol cessation prevented changes in behaviors, corticosterone and leptin levels in rats (Schneider *et al.*, 2015a). Considering the gaps in understanding the effects of NAC on chronic alcohol intake and/or alcohol withdrawal, this study explored the effects of NAC on inflammatory cytokines, glutamine synthetase (GS) activity, and the GSH levels in the rat hippocampus and frontal cortex in a rat model of moderate chronic intake.

MATERIALS AND METHODS

Animals

Sixty adult male Wistar rats (~280 g, 70 days old) obtained from our university's colony (CREAL-UFRGS) were housed in the Pharmacology Department animal facility in polypropylene cages (5 rats/cage, 33 × 40 × 17 cm) under controlled environmental conditions (22 ± 2°C, 12 h light/dark, lights on at 7 AM), with free access to water and food (Nuvilab, Colombo, Brazil). All procedures were performed according to international and local policies for experimental animal handling; this study was approved by the Ethics Committee for Animal Experimentation (CEUA-UFRGS # 23069).

Drugs and reagents

Ethanol (98%) (Nuclear, São Paulo, Brazil) was diluted to 20% (w/v) in a 3% glucose (D-Glucose, Nuclear, São Paulo, Brazil) solution. Control rats received an 8% glucose solution to match the caloric intake of the alcohol groups. N-acetylcysteine (Sigma-Aldrich, St. Louis, MO, USA) was diluted in saline. All solutions were prepared fresh daily.

Experimental design

Rats were allocated to two treatment groups (n = 30) to receive 2 g/kg of alcohol or the 8% glucose solution by intragastric gavage (i.g.), twice daily (9 AM and 2 PM), for 30 days. Twenty-four hours after the last gavage, the rats were further divided into groups (n = 10) treated via intraperitoneal (i.p.) injection for 4 days (9 to 11 AM) with saline, 60 mg/kg NAC, or 90 mg/kg NAC. The doses were chosen based on the literature (Ramirez-Niño, D'souza e Markou, 2013) and a previous study (Schneider et al., 2015). Rats were euthanized on the fifth day after alcohol cessation and 24 h after the last treatment. Four (out of 60) rats died due to the gavage procedure. Immediately

after euthanasia, the trunk blood was collected, and the frontal cortex and hippocampus were dissected out and frozen (-80 °C) for further analysis.

Inflammatory cytokines

The TNF-α levels were assayed using a rat TNF-α ELISA kit from PeproTech (Rocky Hill, NJ, USA). The levels of IL-1β, IL-6 IL-18, and IL-10 were assayed using specific rat ELISA kits from eBioscience (San Diego, CA, USA), following the manufacturer's instructions and according to Zheng et al. (2015). The results are expressed as ng/mg of protein (Table 1) and the percentage of the blank control (non-alcohol/saline) levels (Figure 1 and 2). Protein content was measured using Lowry's method with bovine serum albumin as a standard (Lowry *et al.*, 1951).

GS activity

Briefly, 0.1 mL homogenates solubilized in 140 mM KCl was added to 0.1 mL of the reaction mixture containing 10 mM $MgCl_2$, 50 mM L-glutamate, 100 mM imidazole-HCl buffer (pH 7.4), 10 mM 2-mercaptoethanol, 50 mM hydroxylamine-HCl and 10 ATP, and incubated for 15 min (37 °C). The reaction was stopped by the addition of 0.4 mL of a solution containing (in mM): 370 ferric chloride, 670 HCl, and 200 TCA. After centrifugation (1.4 ×*g*), the absorbance of the supernatant was measured at 530 nm and compared to the absorbance generated using standard quantities of γ-glutamylhydroxamate treated with a ferric chloride reagent. Results are expressed as percentage of the blank control (non-alcohol saline) levels.

GSH content assay

Hippocampal homogenates were diluted in 10 volumes of 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA, and protein was precipitated with 1.7% meta-phosphoric acid. The supernatant was assayed with 0-phthaldialdeyde (1 mg/mL methanol) at room temperature for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. A calibration curve was performed with standard GSH solutions (0-500 µM). The results were expressed as percentages relative to the control levels.

Statistical analysis

The results were tested for normal distribution using the Shapiro-Wilks test and were analyzed by a two-way ANOVA followed by the Tukey *post-hoc* test when appropriate, with the condition (non-alcohol × alcohol withdrawal) and treatments as factors. The results are presented as the mean ± standard error (S.E.M.). Significance was set at $P < 0.05$, and the statistical analysis was performed with the Sigma Stat Program (Jandel Scientific Co., v. 11.0, San Jose, USA).

RESULTS

A two-way ANOVA showed that, in the hippocampus, alcohol treated animals subjected to 5 days of withdrawal significantly increased the TNF-α ($F_{1,36} = 18.95$, $P < 0.001$), IL-1β ($F_{1,36} = 47.75$, $P < 0.001$), IL-6 ($F_{1,36} = 20.00$, $P < 0.001$), and IL-18 ($F_{1,36} = 49.19$, $P < 0.001$) levels and decreased the IL-10 ($F_{1,36} = 26.80$, $P < 0.001$) level (Figs. 1A-E). The same pattern of inflammatory mediator responses was observed in the frontal cortex, with increased TNF-α ($F_{1,36} = 6.23$, $P = 0.036$), IL-1β ($F_{1,36} = 18.17$, $P < 0.001$), IL-6 ($F_{1,36} = 12.05$, $P < 0.001$), and IL-18 ($F_{1,36} = 18.66$, $P < 0.001$) levels and

decreased IL-10 ($F_{1,36}$ = 10.70, P < 0.001) levels (Figs. 2A-E). Absolute cytokines concentrations (ng/mg of protein) for control group are shown in Table 1.

Four days treatment with NAC prevented the inflammatory response in the hippocampus, resulting in anti- and pro- inflammatory cytokine levels similar to those in the non-alcohol control group. The levels of TNF-α ($F_{2,36}$ = 7.18, P = 0.003), IL-1β ($F_{2,36}$ = 19.77, P < 0.001), IL-6 ($F_{2,36}$ = 12.79, P < 0.001), and IL-18 ($F_{2,36}$ = 49.19, P < 0.001) were significantly decreased in the NAC-treated rats experiencing withdrawal than alcohol-withdrawal control rats and similar to non-alcohol treated control rats. Likewise, the level of IL-10 ($F_{2,36}$ = 12.80, P < 0.001) was significantly increased (Figs. 1A-E) in the NAC treated group as compared with the untreated rats experiencing withdrawal, and comparable no non-alcohol treated animals. Of note, a two-way ANOVA showed a significant interaction between the condition (alcohol withdrawal x controls) and treatment (saline x NAC) for the cytokine levels (except for the TNF-α level), suggesting that the effect of NAC is only relevant in the context of withdrawal (F for the interactions: TNF-α: $F_{2,36}$ = 1.57, P = 0.224; IL-1β: $F_{2,36}$ = 30.29, P < 0.001; IL-6: $F_{2,36}$ = 19.38, P < 0.001; IL-10: $F_{2,36}$ = 8.94, P < 0.001; IL-18: $F_{2,36}$ = 27.63, P < 0.001). The anti-inflammatory effects of NAC in the hippocampus did not show a dose-dependent pattern, except for IL-18, where Pearson´s test showed a strong inverse correlation (r = - 0.90, P < 0.001) between IL-18 levels and NAC dose (Figs. 1A-E).

The effects of NAC treatment in the frontal cortex mirrored those found in the hippocampus. The TNF-α ($F_{2,36}$ = 11.40, P < 0.001), IL-1β ($F_{2,36}$ = 12.30, P < 0.001), IL-6 ($F_{2,36}$ = 13.93, P < 0.001), and IL-18 ($F_{2,36}$ = 26.83, P < 0.001) levels were significantly lower than those of untreated rats and were comparable to those in the control groups. The IL-10 ($F_{2,36}$ = 29.96, P < 0.001) levels were significantly higher than those of the saline treated rats experiencing withdrawal and were comparable to

those of the non-alcohol controls (Figs. 2A-E). Likewise, a two-way ANOVA revealed a significant interaction between the condition (alcohol withdrawal x controls) and treatment (saline x NAC) for all inflammatory biomarkers (F values for the interactions: TNF-α: $F_{2,36} = 5.60$, $P = 0.008$; IL-1β: $F_{2,36} = 10.02$, $P < 0.001$; IL-6: $F_{2,36} = 14.93$, $P < 0.001$; IL-10: $F_{2,36} = 15.24$, $P < 0.001$; IL-18: $F_{2,36} = 20.63$, $P < 0.001$). Similar to the findings in the hippocampus, the anti-inflammatory effects of NAC in the frontal cortex were dose-dependent only for IL-18 ($r = - 0.91$, $P < 0.001$).

The only effect of NAC detected in non-alcohol-treated rats was a decrease in the IL-18 levels in both hippocampus and the frontal cortex, which were induced by the lower (60 mg/kg) NAC dose ($F_{2,36} = 10.42$, $P = 0.001$, and $F_{2,36} = 6.42$, $P = 0.010$, respectively). No significant differences in the serum cytokine levels were observed among the different groups (data not shown).

A two-way ANOVA showed a significant decrease in GS activity in the alcohol withdrawal group at the hippocampus ($F_{2,53} = 8.79$, $P = 0.005$), but not frontal cortex ($F_{2,51} = 0.02$, $P = 0.890$) (Fig. 3). NAC was devoid of effect on GS activity ($P > 0.05$). There were no significant differences in the GSH levels in the hippocampus ($F_{2,57} = 1.85$, $P = 0.180$) or frontal cortex ($F_{2,55} = 0.45$, $P = 0.503$) (Fig. 4).

DISCUSSION

N-acetylcysteine (NAC) has been suggested as a putative anti-addictive drug in cocaine, nicotine, and marijuana dependence. It possesses anti-inflammatory, glutamate modulating, and antioxidant properties (Berk *et al.*, 2013). This study explored the effects of NAC on the inflammatory response in the rat brain after 30 days of alcohol ingestion followed by 5 days of withdrawal. We show that withdrawal in this moderate chronic alcohol intake model increased the pro-inflammatory and decreased the anti-

inflammatory cytokine levels in the hippocampus and frontal cortex. These areas are implicated in drug addiction and withdrawal (Atkins, Mashhoon e Kantak, 2008; Goldstein e Volkow, 2011). The data also indicate that 4 days of treatment with NAC prevented the alcohol-withdrawal induced changes in the neuroinflammatory responses in these two brain areas.

Alcoholism has recently been acknowledged as a systemic inflammatory condition (Kelley e Dantzer, 2011; González-Reimers *et al.*, 2014). Chronic alcohol consumption increases the serum levels of inflammatory cytokines such as IL-1, IL-6, IL-8, IL-10, IL-12, interferon γ, and TNF-α in humans and rodents (González-Quintela *et al.*, 2000; Crews e Vetreno, 2014; González-Reimers *et al.*, 2014). Chronic, but not acute, alcohol administration increases the levels of TNF-α, IL-1 β, IL-6, and monocyte chemotactic protein-1 (MCP-1) in mouse serum, liver, cortex, and hippocampus (Qin *et al.*, 2008). We herein demonstrated that the rat hippocampus and frontal cortex levels of the pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-18) were increased 5 days after alcohol cessation; no changes were detected in the serum cytokine levels. Although there are limited data available on the cytokine levels in the context of alcohol withdrawal, our results are in agreement with a report from Qin and colleagues (Qin *et al.*, 2008), indicating that the brain TNF-α levels remain elevated after 3 days of alcohol cessation, although the serum levels return to the control level after one day. Long-term (up to 10 months) increases in brain, but not serum, TNF-α levels were also observed in response to the pro-inflammatory endotoxin lipopolysaccharide (LPS) (Qin *et al.*, 2008), indicating that this cytokine may represent a potential biomarker for chronic neuronal damage.

Increased serum levels of IL-10 have been reported in humans during chronic alcoholism, returning to control levels after withdrawal (González-Quintela *et al.*,

2000). We here showed that the IL-10 levels were decreased in the hippocampus and frontal cortex (but not the serum) after 5 days of alcohol withdrawal in rats. The brain changes in the pro and anti-inflammatory cytokines observed in this study may confirm the view that neuroinflammation is also present during alcohol withdrawal. NAC was reported to reduce the cortical levels of pro-inflammatory cytokines after traumatic brain injury and focal brain ischemia in rats (Khan *et al.*, 2004; Chen *et al.*, 2008), while long-term dietary supplementation with NAC decreased the TNF-α, IL-1β, and IL-6 levels in the brains of aged rats (Thakurta *et al.*, 2012). In humans, NAC administration decreased the plasma pro-inflammatory cytokine levels and oxidative stress after surgery or in burned patients (Mahmoud e Ammar, 2011; Csontos *et al.*, 2012). The effects of NAC in the present study were withdrawal (condition)-dependent, except for IL-18, which was consistently altered in both the hippocampus and frontal cortex by the 60 mg/kg NAC treatment in the non-alcohol-treated controls. Higher IL-18 levels are related to sickness behaviors, learning, and memory deficits in rodents (Yaguchi *et al.*, 2010) and humans (Salani *et al.*, 2013).

In rodents, the systemic administration of LPS or interferon-α induces sickness behavior as hypolocomotion, loss of appetite, and depressive-like behavior (Dantzer, 2006), directly correlated with increased cytokine levels in different brain areas (O'connor *et al.*, 2003). We recently reported that rats subjected to the same protocol used in this study present decreased ambulation and rearing (Schneider et al., 2015), consistent with the changes in cytokines levels here reported.

Hyperglutamatergic activity during withdrawal is implicated in neuronal death, relapse, and the maintenance of the addiction cycle (Scofield e Kalivas, 2014). Downregulation of the excitatory amino acid transporters 1 and 2 (EAAT1 and EAAT2) has also been reported to be related to drug-seeking behavior and relapse. GS is the key

astrocyte enzyme responsible for recycling released glutamate; though certainly only an indirect measure of glutamate status, the results show that GS activity is significantly lower in the hippocampus (but not the cortex) of rats experiencing withdrawal compared to non-alcohol-treated controls. Though no transporter activity assessment was performed in the present study, our result is consistent with the reported downregulation of EAAT transporters during withdrawal (Scofield and Kalivas, 2014). Given the report that NAC restored the EAAT2 activity in a cocaine self-administration rat model (Reissner *et al.*, 2014), a comprehensive study on the effects of NAC in glutamatergic transmission during alcohol intake and withdrawal is warranted.

No significant changes were found in the brain GSH levels after alcohol withdrawal or short-term NAC treatment. Although the GSH levels are lower in alcoholics, it has been shown that withdrawal quickly restored the GSH levels in rats (Zhao, Kalhorn e Slattery, 2002), a possible explanation why GHS levels were comparable in our alcohol-withdrawal and control rats. Though the antioxidant properties of NAC are thought to be related to increased GSH (Berk et al., 2013), the lack of such increase in our samples is not surprising, given that larger doses and longer periods of treatment are required for a significant effect of NAC in GSH (Farr *et al.*, 2003).

This study presents limitations. Anti-addictive properties of NAC, suggested to other drugs of abuse (Gray et al., 2010; Knackstedt et al., 2009; LaRowe et al., 2013; Ramirez-Niño et al., 2013; Zhou and Kalivas, 2008), cannot be inferred to alcohol from this study given that our model is based on forced, rather than self, administration. Also, it must be taken into account that the forced alcohol intake by gavage could further increase inflammatory cytokines, given that stress activates the hypothalamic-pituitary-adrenal axis. Nevertheless, gavage was a systematic factor and does not invalidate the

differences between NAC-treated (alcoholics or not) and non NAC-treated rats. Our experimental design does not allow distinguishing the inflammatory response that might result from the long-lasting effect of the chronic alcohol intoxication or from withdrawal. Although the inclusion of a non-alcohol withdrawal group would have been ideal, considering the reports of the specific effect of alcohol withdrawal in increasing brain cytokines in rats (Doremus-Fitzwater et al., 2014) and humans (Gonzáles-Quintela, 2000), we believe our findings do suggest a potential effect of NAC in the context of alcohol relapse.

Chronic alcoholism and/or alcohol withdrawal have previously been reported to lead to hyperactivated glutamate and neurodegeneration (Gilpin and Koob, 2008). More recently, a growing body of evidence supports the relationship between brain hyperactivity and damage with elevated brain inflammatory cytokines (Kelley and Dantzer, 2011). We show that N-acetylcysteine consistently prevented the production of neuroinflammatory mediators in the hippocampus and frontal cortex of rats experiencing withdrawal after chronic moderate alcohol intake. Given the association of inflammation with cravings and relapse (Gonzáles-Quintela, 2000), our data suggest that NAC may be useful to reduce the alcohol-induced neuroinflammation and, therefore, the risk of relapse in alcoholics. Investigating the effects here reported in a self-administration model is desirable.

CONFLICT OF INTEREST

Authors declare that they have no conflicts of interest

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FIGURE LEGENDS

Fig. 1. Effects of N-acetylcysteine (NAC) on the TNF-α (A), IL-1β (B), IL-6 (C), IL-18 (D), and IL-10 (E) levels in the rat hippocampus. Rats were treated with alcohol for 30 days, followed by NAC (or saline = NAC 0) for 4 days starting 24 h after alcohol cessation. The data are expressed as the percent of the control ± S.E.M. n= 6; NAC = N-acetylcysteine *$P < 0.05$, ** $P < 0.01$ compared to the control, $^{\#\#} P < 0.01$ compared to alcohol-treated rats; two-way ANOVA/Tukey.

Fig. 2. Effects of N-acetylcysteine (NAC) on the TNF-α (A), IL-1β (B), IL-6 (C), IL-18 (D), and IL-10 (E) levels in the rat frontal cortex. Rats were treated with alcohol for 30 days, followed by NAC (or saline = NAC 0) for 4 days starting 24 h after alcohol cessation. The data are expressed as the percent of the control ± S.E.M. n=6; NAC = N-acetylcysteine *$P < 0.05$, ** $P < 0.01$ compared to the control, $^{\#\#} P < 0.01$ compared to alcohol-treated rats; two-way ANOVA/Tukey.

Fig. 3. Effects of N-acetylcysteine (NAC) on the GS activity in the rat hippocampus (HIP) and frontal cortex (FC). Rats were treated with alcohol for 30 days, followed by NAC (or saline = NAC 0) for 4 days starting 24 h after alcohol cessation. The data are expressed as the percent of the control ± S.E.M. n/group: 8-10; NAC = N-acetylcysteine; ** $P < 0.01$ compared to the control; two-way ANOVA/Tukey two-way ANOVA/Tukey.

Fig. 4. Effects of N-acetylcysteine (NAC) on the GSH levels in the rat hippocampus (HIP) and frontal cortex (FC). Rats were treated with alcohol for 30 days, followed by NAC (or saline = NAC 0) for 4 days starting 24 h after alcohol cessation. The data are

expressed as the percent of the control ± S.E.M. n/group: 8-10; NAC = N-acetylcysteine; two-way ANOVA/Tukey.

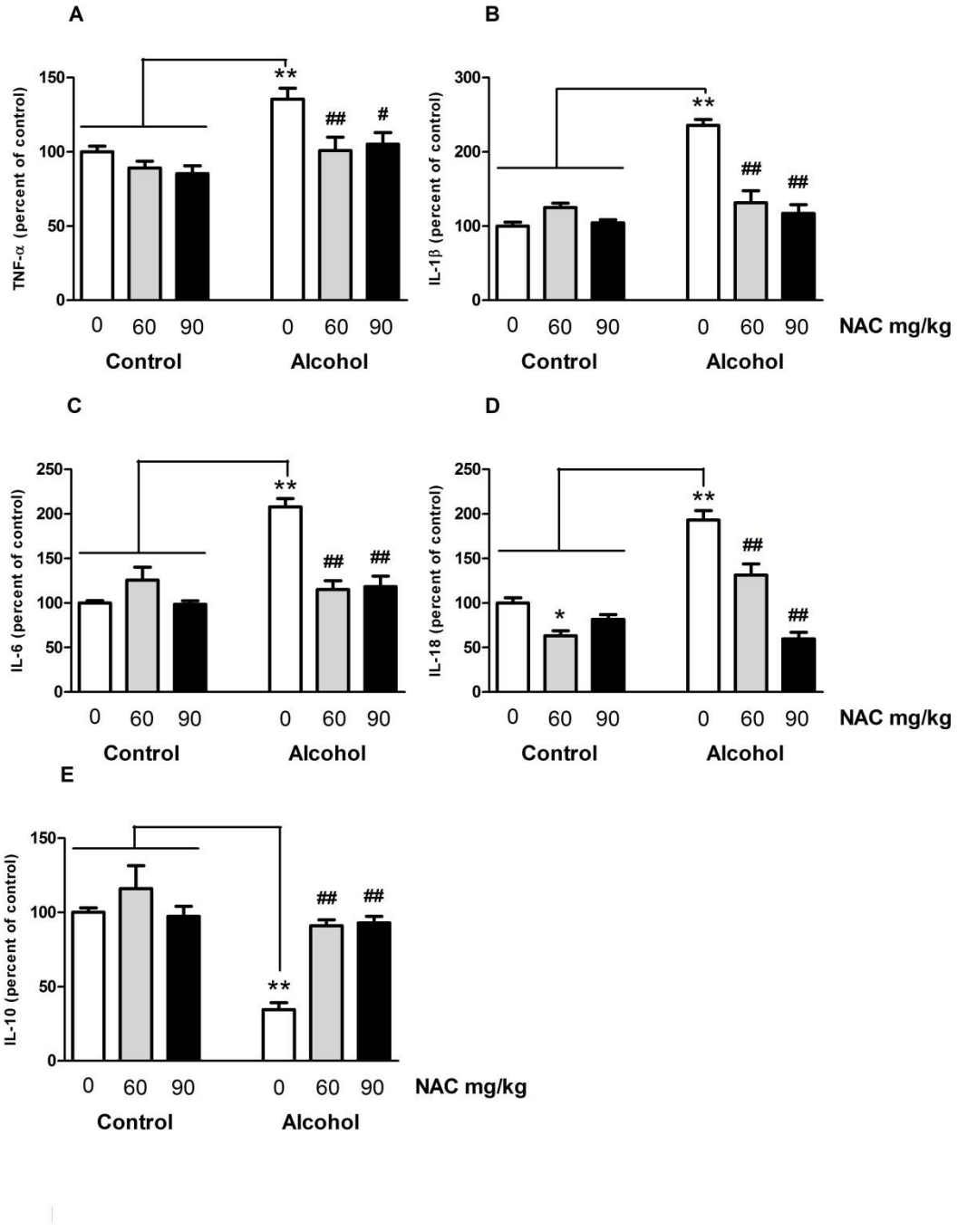
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Figure 2 Schneider et al., 2015

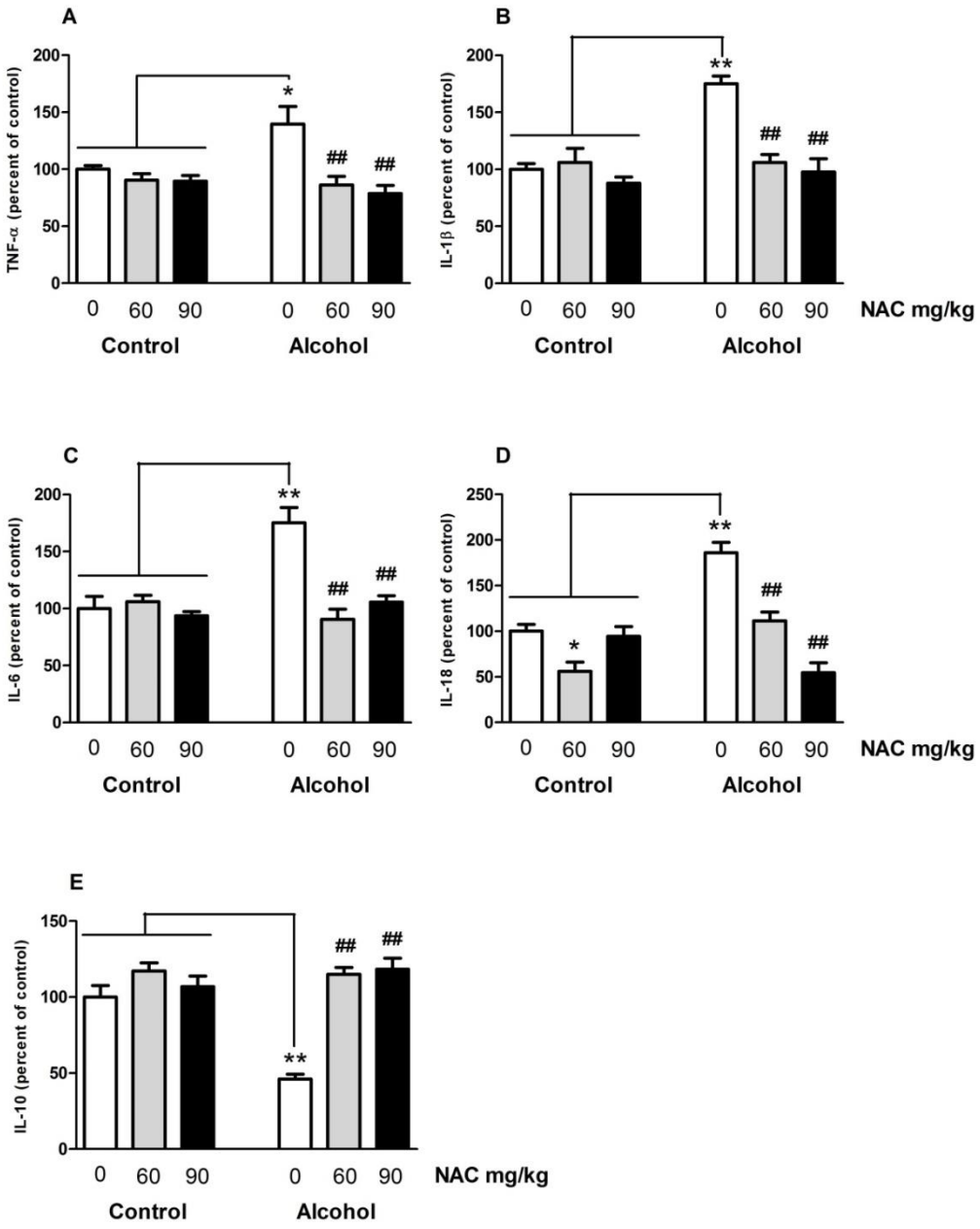

Figure 3

Schneider et al., 2015

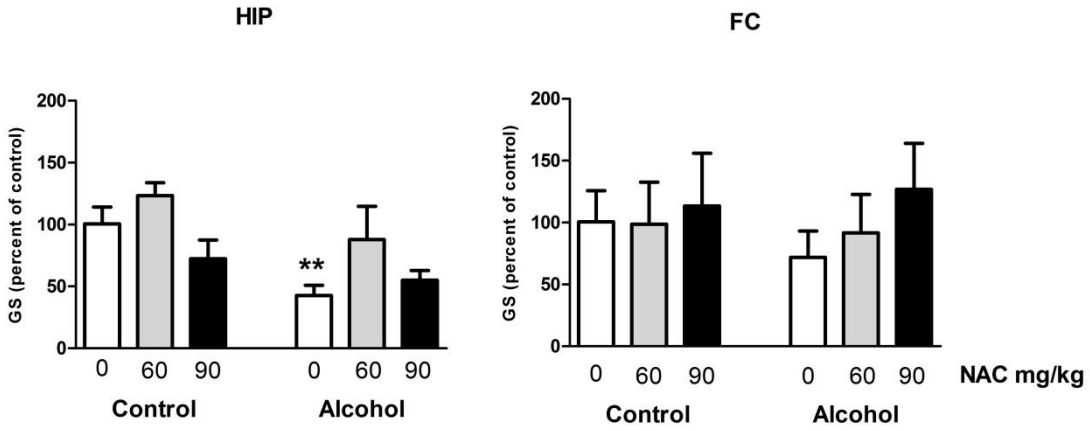

Figure 4

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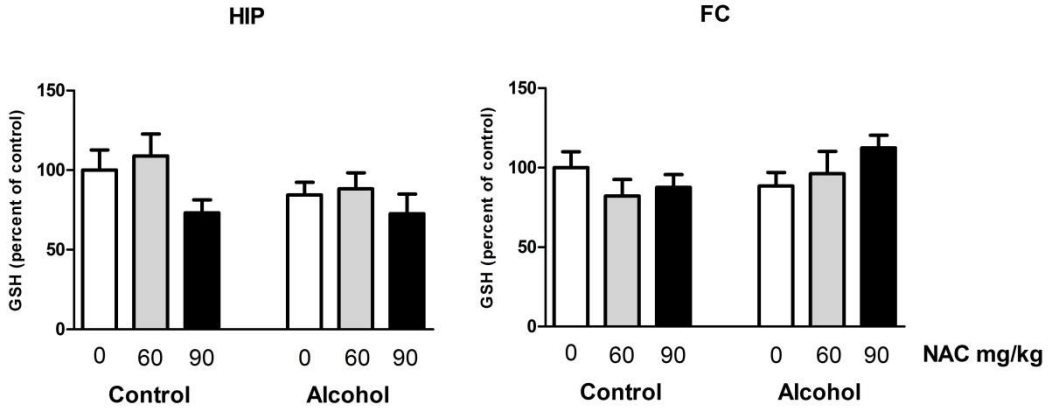

Table 2. Absolute values for cytokines concentrations of control 0 (saline) group (means ± S.E.M).

Cytokines (ng/mg of protein)	**TNF-α**	**IL-1β**	**IL-6**	**IL-18**	**IL-10**
Hippocampus	78.3 ± 3.0	27.6 ± 1.4	29.8 ± 0.8	24.5 ± 1.1	36.6 ± 1.5
Frontal Cortex	102.3 ± 3.3	35.8 ± 1.7	33.3 ± 3.5	25.6 ± 2.1	29.3 ± 1.8

Artigo 3

O terceiro artigo da tese será submetido ao periódico *Alcoholism: Clinical and Experimental Research*, o artigo é intitulado:

"N-acetylcysteine treatment installed before alcohol cessation prevents withdrawal-induced anxiety and hormone changes in rats"

O referido artigo segue abaixo.

N-acetylcysteine treatment installed before alcohol cessation prevents withdrawal-induced anxiety and hormone changes in rats

Ricardo Schneider Jr[a], Rianne Pulcinelli[b], Solange Bandiera[c], Alana Witt Hansen[c], Greice Caletti[b], Elaine Elisabetsky[a,b,c], Rosane Gomez[a,b,c].

[a]Programa de Pós-Graduação em Neurociências, [b]Departamento de Farmacologia, [c]Programa de Pós-Graduação em Farmacologia e Terapêutica, Universidade Federal do Rio Grande do Sul (UFRGS), Rua Sarmento Leite, 500/202 - Porto Alegre, RS, Brazil, 90050170

Corresponding Author

Ricardo Schneider Jr
Departamento de Farmacologia, ICBS - UFRGS
Rua Sarmento Leite, 500/202
90050170 - Porto Alegre, RS, Brazil
Phone/FAX +55 51 3308 3121
E-mail: quimtox@gmail.com

Abstract

Background: N-acetylcysteine (NAC), a glutamate-modulating agent with antioxidant and anti-inflammatory properties, has been considered as a potential anti-addictive drug with beneficial effects for drug addiction. Alcohol withdrawal symptoms, such as anxiety, have been associated with increased levels of serum corticosterone, leptin, and brain glutamate levels. We recently showed that short-term NAC treatment after alcohol cessation prevented withdrawal-induced hypolocomotion and increases in corticosterone and leptin serum levels, however, the effects of pretreatment with NAC before alcohol cessation are unknown. Thus, the aim of this study was to assess the effects of pretreatment with NAC on anxiety, as well as serum corticosterone and leptin during withdrawal from an intermittent model of chronic alcohol exposure. **Methods:** Male adult Wistar rats were treated with 2 g/kg ethanol, twice daily, by gavage in intermittent cycles of intoxication (5 days/week) during 3 weeks. NAC treatment (60 and 90 mg/kg, i.p., once a day) or saline started 4 days before alcohol cessation. Twenty-four hours after the last alcohol and NAC administration, rats were exposed for 5 min in the open field test (OF). Twenty-four hours later, they were euthanized and blood samples were collected for corticosterone and leptin levels analysis (ELISA). **Results:** Results showed that alcohol cessation increased anxiety in rats, evidenced by the decreased time spent in central zone in the OF, and increased serum corticosterone and leptin. Four days of NAC pretreatment prevented alcohol cessation-induced anxiety and increases in hormone levels. **Conclusions:** We suggest that, in addition to the anti-addictive properties reported for other drugs of abuse and previous data reported by our group, NAC is potentially useful in the management of alcohol withdrawal, independently if NAC treatment starts before or after alcohol cessation.

Keywords: Addiction, abstinence, corticosterone, ethanol, leptin.

Introduction

According to World Health Organization (WHO), in 2012, about 3.3 million deaths were attributable to alcohol consumption (WHO, 2015). Alcohol dependence is a relapsing disorder that deregulates responses to stress and reward, contributing to the negative emotional state associated with withdrawal (Gilpin e Koob, 2008; Engel e Jerlhag, 2014). Because withdrawal symptoms are key factor for relapse and the maintenance of the addiction cycle, new drugs, which facilitates and/or prolong abstinence could represent an important contribution for the management of alcohol addiction.

In addition to alterations on the dopaminergic and GABAergic systems (Sanna *et al.*, 2003; Gilpin e Koob, 2008), manifestations of alcohol withdrawal have been related to impairments on glutamate homeostasis (Gass e Olive, 2008; Kalivas, 2009; Scofield e Kalivas, 2014), neuroinflammation (González-Reimers *et al.*, 2014) and oxidative stress (Gonzaga *et al.*, 2014). Additionally, during alcohol withdrawal, corticotrophin-releasing factor (CRF) is excessively released from the hypothalamus, activating the hypothalamic-pituitary-adrenal (HPA) axis, increasing cortisol/corticosterone secretion in humans and animals (Stephens e Wand, 2012). The HPA can also be modulated by leptin, an adipokine usually associated with satiety (Ahima *et al.*, 1996) and recently associated to alcohol craving during withdrawal in humans (Kiefer, Jahn, Jaschinski, *et al.*, 2001; Aguiar-Nemer *et al.*, 2013). In humans, the alcohol withdrawal syndrome is characterized by anxiety, tremors, agitation, delirium, and eventually seizures (Mckeon, Frye e Delanty, 2008). In rodents, alcohol withdrawal is manifested as anxiety, decreased exploratory behavior and hypoactivity (Slawecki e Roth, 2004; Kliethermes, 2005; Fukushiro *et al.*, 2012; Schneider *et al.*, 2015b). It is already known that

withdrawal symptoms are enhanced when humans or rodents are submitted to repeated withdrawals (Brown *et al.*, 1988; Malcolm, Roberts, *et al.*, 2000).

A considerable body of pre-clinical and clinical data is now available to suggest that N-acetylcysteine (NAC) presents an innovative psychopharmacological profile (Berk *et al.*, 2013). NAC anti-addictive properties reported for rodents include reduced self-administration of cocaine (Reichel *et al.*, 2011), nicotine (Ramirez-Niño, D'souza e Markou, 2013), and heroin (Zhou e Kalivas, 2008). In humans NAC has been shown to decrease marijuana (Gray *et al.*, 2010) and cigarette (Knackstedt *et al.*, 2009) use, and reduce cocaine craving (Larowe *et al.*, 2013). However, data on the effects of NAC in alcohol addiction are meager.

We recently showed that a four day treatment with NAC after alcohol cessation prevented the withdrawal-induced behavioral and hormonal changes in rats (Schneider et al., 2015). Given the suggestions that anti-addictive pharmacotherapy installed before withdrawal may be more effective, as shown for smoking (Ebbert et al., 2015) and alcohol (Boeijinga et al., 2004), the purpose of this study was to verify the effect of pre-treatment (prior to alcohol cessation) with NAC on anxiety, corticosterone and leptin serum levels in rats.

Methods

Animals

Sixty adult Wistar rats (~280 g) obtained from the university's own colony (CREAL-UFRGS) were housed in the Pharmacology Department animal facility, in polypropylene cages (5 rats/cage, 33 × 40 × 17 cm), under controlled environmental conditions (22° ± 2°C, 12 h light/dark, lights on at 7 AM), with free access to water and food (Nuvilab, Colombo, Brazil). All procedures were performed according to

international and local policies for experimental animal handling, and the study was approved by the Ethics Committee for Animal Experimentation (CEUA-UFRGS # 23069).

Drugs and reagents

Ethanol (98%) (Nuclear, São Paulo, Brazil) was diluted to 20% (wt/v) in a 3% glucose (D-Glucose, Nuclear, São Paulo, Brazil) solution. Control rats received an 8% glucose solution, matching the caloric intake of the alcohol groups. N-acetylcysteine (Sigma-Aldrich, St. Louis, USA) was diluted in saline. All solutions were prepared fresh daily.

Experimental design

The treatment schedule was adapted from Overstreet et al. (2002) (Overstreet, Knapp e Breese, 2002). Sixty male Wistar rats were allocated in two treatment groups (n = 30): 2 g/kg of alcohol or glucose-control solution, administered by oral gavage, twice daily (9 AM and 2 PM) for 3 weeks, from Monday to Friday, with 2 days of withdrawal between periods (Fig. 1). Four days before the cessation of alcohol administration, rats were divided in six groups (n = 10) to be simultaneously treated for 4 days, via intraperitoneal (at 7 PM) with saline (NAC0) or 60 and 90 mg/kg NAC. Twenty-four hours after the last alcohol administration, rats were tested in the open field (OF) test. Rats were euthanized by decapitation 24 h from the OF and the trunk blood was collected in plastic tubes (Vacutainer, NC, USA), centrifuged at 3500 RPM, and serum stored for later hormonal analysis. Samples were stored in a freezer at -80 ^{o}C for further analysis. Rats were weighted twice a week for adjusting the alcohol dose.

The model of repeated withdrawals (Overstreet, Knapp e Breese, 2002) was elected and adapted based on several evidences supporting a sensitization process in human and rodents subjected to multiple withdrawals from alcohol (Brown *et al.*, 1988; Maier e Pohorecky, 1989; Hölter *et al.*, 1998; Malcolm, Herron, *et al.*, 2000; Malcolm, Roberts, *et al.*, 2000). The interval between OF test and euthanasia was chosen to avoid a bias promoted by the exposition to the apparatus on corticosterone levels. Doses and interval of NAC treatment were chosen based on literature and previous studies conducted in our laboratory (Ramirez-Niño, D'souza e Markou, 2013; Schneider *et al.*, 2015b).

Open Field Test (OF)

Rats were habituated to the dimly lighted experimental room in their home cages for at least 30 min before the experiment. The open field consisted of a white wooden arena (100 × 100 × 50 cm) with the floor divided by black lines into sixteen equal squares. Rats were individually placed in the center of the arena and their behaviors were video recorded for 5 min. After each session, the floor was cleaned with a wet paper towel. Videos were analyzed by a trained observer, blinded to treatments, using a BASIC written software (Kevin Willioma, KD Ware Computer, Boston, MA, modified by Thomas Vatne, 1987). The frequency of crossings, rearing, and grooming, as well as the time spent in the central zone of the apparatus were counted.

Corticosterone and leptin levels

For corticosterone, the serum was diluted as 1:10 and measured using a commercial ELISA kit (IBL International, Hamburg, DE). For leptin, the serum was diluted as 1:5 and also measured using a commercial ELISA kit (Invitrogen, NY, USA).

A microplate reader (PerkinElmer, MA, USA) at 450 nm was used to measure corticosterone and leptin.

Statistical Analysis

The results were tested for normal distribution using the Shapiro-Wilks test, and analyzed by a two-way ANOVA followed by the Tukey *post hoc*, with the condition (control × alcohol) and NAC treatment as factors. Pearson´s correlation test was used to assess correlations between behaviors (crossings and time spent in central zone), corticosterone and leptin serum levels. The results were presented as the means ± standard error (S.E.M.). The significance was set at $P < 0.05$. The Sigma Stat program (Jandel Scientific Co., v. 11.0, San Jose, USA) was used.

Results

Figure 2 shows results from the open field. Two way ANOVA showed that alcohol cessation decreased the time spent in the central area ($F_{2,53} = 4.06$, $P = 0.002$) suggesting increased anxiety in abstinent rats; four days treatment with NAC (60 and 90 mg/kg) established prior to alcohol cessation prevented the alcohol cessation-induced anxiety. There was a significant interaction between condition (abstinence) and treatment (NAC) for the time spent in the central area of the OF ($P = 0.023$, Fig. 2A). No differences were found regarding the total crossings (Fig. 2B), rearing or grooming (data not shown).

Figure 3 and 4 illustrates the results with hormones. Two way ANOVA showed that alcohol cessation significantly increased the corticosterone levels ($F_{2,37} = 2.34$, $P = 0.019$) (Fig. 3A) while NAC prevented this increase ($P < 0.005$). A significant interaction was identified between abstinence and NAC treatment ($F_{2,37} = 4.65$; $P =$

0.017). Two-way ANOVA showed that leptin levels were higher after alcohol cessation ($F_{1,43} = 4.51$; $P = 0.04$) (Fig. 4A); NAC prevented the leptin increase after alcohol cessation ($P > 0.05$). Pearson's correlation analysis indicates that leptin levels were significantly and negatively correlated with the time spent in the OF central zone (r = -0.40, $P = 0.008$, Fig. 4B).

There were no significant differences in the body weight between groups throughout the experiment ($P > 0.05$).

Discussion

We recently reported that four consecutive daily treatments with NAC initiated after alcohol cessation prevented withdrawal-induced changes in a chronic moderate intake rat model (Schneider et al, 2015). Here we showed that four consecutive daily treatments with NAC initiated before alcohol cessation prevented anxiety-like behaviors and increased corticosterone and leptin levels in a repeated withdrawal model.

Time spent in the central zone is a generally accepted measure of anxiety in the OF (Prut e Belzung, 2003). As previously reported (Meert, 1994; Perez e De Biasi, 2015), in this study rats under withdrawal (24h) spent significantly less time in the central zone of the OF compared to controls, indicating withdrawal-induced anxiety. NAC prevented the withdrawal-induced anxiety equally in the two doses tested. Though there is some decrease in total activity in rats treated with NAC and alcohol, differences are not statistically significant. Despite specificities of the two rat models, this result corroborates the ability of NAC in counteracting alcohol withdrawal-induced anxiety in the OF.

Changes in the HPA activity have been documented in various stages of alcoholism, including intoxication, withdrawal and relapse (Stephens e Wand, 2012). In early withdrawal, large amounts of glucocorticoids are produced including cortisol

(Sinha, 2012; Stephens e Wand, 2012). In alcoholics cortisol levels increase during the first withdrawal week and return to control levels within 4 weeks, in a time manner that parallels improvements of withdrawal symptoms (Esel *et al.*, 2001; Sharrett-Field *et al.*, 2013). In rodents, high levels of corticosterone are observed during withdrawal (Sharrett-Field *et al.*, 2013). As reported for NAC and the high corticosterone levels observed after 5 days of alcohol cessation in a different alcohol intake model (Schneider *et al.*, 2015b), we here show that corticosterone serum levels are increased after repeated alcohol withdrawal and that NAC administered before alcohol cessation is effective in preventing the corticosterone increase seen 24 hours after withdrawal.

The hypothalamic release of CRF can be limited by leptin (Roubos *et al.*, 2012). Leptin serum levels have been correlated with craving in alcoholics during withdrawal (Kiefer, Jahn, Jaschinski, *et al.*, 2001; Aguiar-Nemer *et al.*, 2013) and showed to be increased after alcohol cessation in rats (Schneider *et al.*, 2015b). It has been suggested that increased leptin levels during withdrawal, by reducing the dopaminergic transmission in the mesolimbic system, may intensify craving and, consequently, the maintenance of the addictive behavior (Fulton *et al.*, 2006). In alcoholics under withdrawal, leptin levels can be reduced by anti-addictive drugs such as naltrexone and acamprosate (Kiefer *et al.*, 2005). This study shows that, in the rat repeated withdrawal model, leptin serum levels are increased after alcohol cessation and correlated with anxiety related behavior during this period. The alcohol cessation-induced increase in leptin was prevented by NAC treatment initiated before withdrawal. The lack of effect on control animals suggests that NAC seems to affect leptin levels only under abnormal levels.

NAC affects least three pharmacological target areas relevant to counteract withdrawal: it modulates glutamate (especially when hyperactivated), inflammatory

cytokines and oxidative stress (Berk *et al.*, 2013). In humans (Gass e Olive, 2008; Hermann *et al.*, 2012) and rodents (Hermann *et al.*, 2012) enhanced glutamate activity is observed during alcohol withdrawal. Leptin enhances the function of the N-methyl-D-aspartate (NMDA) glutamate receptors (Shanley, Irving e Harvey, 2001; Harvey, 2007), preventing decrease of glutamate release mediated by mGlu extrasynaptic receptors activation and ultimately decreasing glutamate NMDA receptor activation.

The translational value of this study is the suggestion that NAC, as other anti-addictive drugs (Boeijinga *et al.*, 2004; Ebbert *et al.*, 2015), can be useful to enhance the chances to prevent alcohol relapse when administered before alcohol withdrawal. Certainly, studies such as this and others recently reported (Schneider et al, 2015) are needed to investigate optimal posology and the neurobiological basis of NAC properties relevant to alcohol withdrawal. Nevertheless, considering that NAC has been proven to be amply safe, is available in various pharmaceutical formulations (including low cost generics), and the high social costs related to alcoholism, we call for the clinical assessments that could established the value of NAC in the context of alcoholism.

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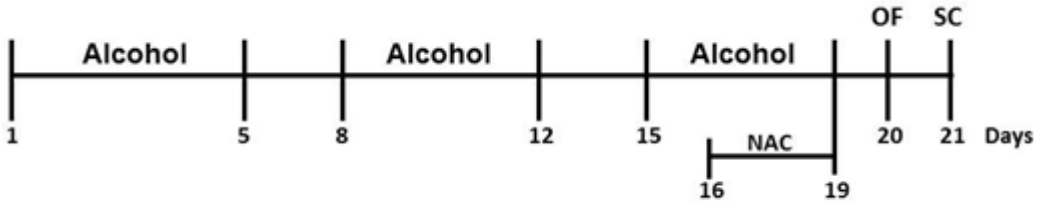
Figure Legends

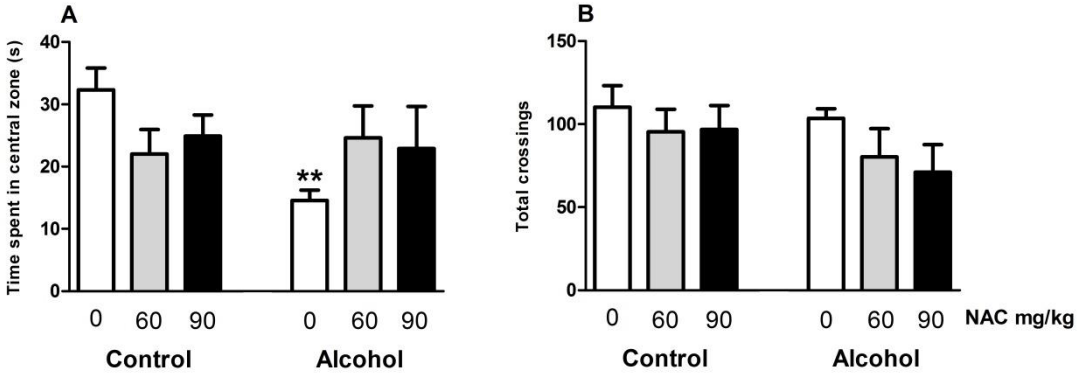
Figure 1. Experimental design. Rats were submitted to tree cycles of alcohol treatment (twice daily/5 days) set apart by 2 alcohol-free days. Four days before alcohol cessation rats were treated with saline or N-acetylcisteine (NAC) before subjected to the open field (OF) test.and sample collection (SC). The model was adapted from Overstreet et al. (2002).

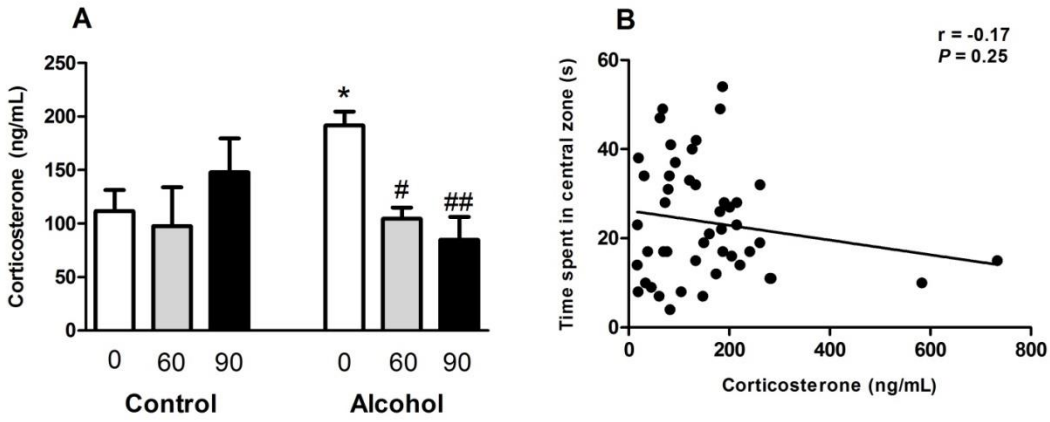
Figure 2. Effects of N-acetylcysteine (NAC) on the open field in abstinent rats. (A) Time spent in central zone; (B) Total crossings. Data expressed as mean ± S.E.M. n= 8-10. *$P < 0.05$; two-way ANOVA/Tukey.

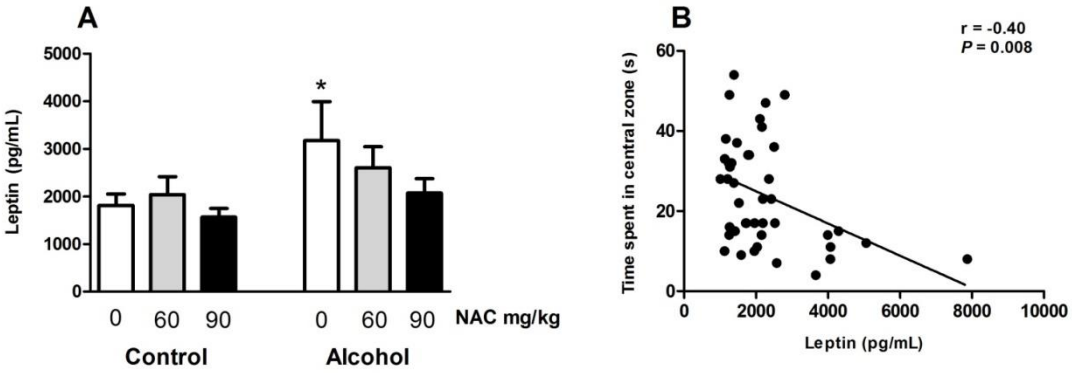
Figure 3. Effects of N-acetylcysteine (NAC) on corticosterone serum levels (A) and its correlation with the time spent in the central zone of the open field (B) in abstinent rats. Data expressed as mean ± S.E.M. n = 7-8. *$P < 0.05$, Two-way ANOVA/Tukey.

Figure 4. Effects of N-acetylcysteine (NAC) on leptin serum levels (A) and its correlation with the time spent in the central zone of the open field (B) in abstinent rats. Data expressed as mean ± S.E.M. n = 7-8. *$P < 0.05$, Two-way ANOVA/Tukey.


OF
SC
Alcohol
Alcohol
Alcohol
1
5
8
12
15
NAC
16
19
20
21
Days


A
Time spent in central zone (s)
40
30
20
10
0
**
0 60 90 0 60 90
Control
Alcohol
B
Total crossings
150
100
50
0
0 60 90 0 60 90
NAC mg/kg
Control
Alcohol


A
Corticosterone (ng/mL)
250
200
150
100
50
0
*
#
##
0
60
90
0
60
90
Control
Alcohol
B
r = -0.17
P = 0.25
Time spent in central zone (s)
60
40
20
0
0
200
400
600
800
Corticosterone (ng/mL)


A
Leptin (pg/mL)
5000
4000
3000
2000
1000
0
*
0 60 90 0 60 90 NAC mg/kg
Control
Alcohol
B
r = -0.40
P = 0.008
Time spent in central zone (s)
60
40
20
0
0 2000 4000 6000 8000 10000
Leptin (pg/mL)

PARTE III

3. DISCUSSÃO

Dentre os principais achados desta tese destacamos o efeito do tratamento com a NAC na prevenção da hipolocomoção e da ansiedade induzida pela abstinência de álcool em ratos. Além disso, o tratamento de curto prazo com NAC preveniu aumento nos níveis séricos de corticosterona e leptina observados após 5 dias ou 24 horas de abstinência. O tratamento com NAC também preveniu o aumento de citocinas inflamatórias em áreas encefálicas após 5 dias de abstinência ao álcool.

Como apresentado no primeiro artigo, após 5 dias de abstinência, precedido por 30 dias de tratamento crônico com álcool (4 g/dia), observamos alteração do comportamento de ratos abstinentes, que apresentaram uma redução significativa de sua locomoção e comportamento de levantar. Em roedores, a ambulação e o comportamento de levantar são considerados formas de comportamentos exploratórios e sua diminuição durante a abstinência de álcool pode representar um estado de intensa ansiedade (Kliethermes, 2005) ou simplesmente diminuição da motivação ou busca pelo novo (Fukushiro *et al.*, 2012). Tal sinal comportamental vem sendo também correlacionado à anedonia (Fukushiro *et al.*, 2012), sintoma comumente observado em alcoolistas (Martinotti *et al.*, 2008). Interessantemente, o tratamento com NAC preveniu a redução do comportamento exploratório quando administrado durante a abstinência de álcool.

Em outro experimento, utilizando um modelo de exposição crônica intermitente de álcool, também observamos alterações de comportamento nos ratos (modificado de Overstreet, Knapp e Breese, 2002). Nesse modelo avaliamos sinais da síndrome de abstinência nas 24 horas seguintes a retirada do álcool e identificamos redução no tempo de permanência na área central do campo aberto entre os ratos abstinentes não tratados com NAC. Esse comportamento, em roedores, é identificado classicamente como sinal de ansiedade (Prut e Belzung, 2003). Não podemos confirmar que o segundo

modelo testado é mais eficaz para a indução de ansiedade comparado ao primeiro modelo, pois não executamos o teste de campo aberto, no primeiro modelo, nas 24 h após o inicio da abstinência mas sim em 5 dias de abstinência.

Em ambos os modelos utilizados, o tratamento com NAC preveniu as alterações de comportamento induzidos pela abstinência de álcool nos animais. Embora os mecanismos pelos quais isso ocorreu precisam ser melhor explorados, é possível sugerir que quando indicada para alcoolistas a NAC possa reduzir o risco de recaída, uma vez que a prevenção dos sintomas de abstinência é crítica para a descontinuação do ciclo do alcoolismo.

Adicionalmente, buscamos correlacionar essas alterações comportamentais com variações nas concentrações séricas de biomarcadores como corticosterona e leptina que, sabidamente, estão alterados pelo uso e abstinência de álcool (Kiefer, Jahn, Kellner, *et al.*, 2001; Sinha, 2012). O primeiro, classicamente relacionado com estresse e o segundo, classicamente relacionado com comportamento alimentar, porém também relacionado com a fissura durante a abstinência de álcool em humanos (Kiefer, Jahn, Jaschinski, *et al.*, 2001; Kiefer, Jahn, Kellner, *et al.*, 2001; Kraus *et al.*, 2004; Kiefer *et al.*, 2005).

Com respeito ao primeiro hormônio, há evidências de alterações na regulação do eixo HPA, oscilando de acordo com as fases de intoxicação, abstinência, antecipação/preocupação ou durante a recaída (Stephens e Wand, 2012). Em humanos, os níveis de cortisol aumentam no sangue durante a abstinência de álcool (Sinha, 2012). Em ratos, os níveis de corticosterona aumentam após intoxicação aguda álcool, sendo este aumento associado a alterações comportamentais durante a abstinência (Sharrett-Field *et al.*, 2013). No presente trabalho, a concentração de corticosterona sérica estava aumentada tanto após 24 h quanto após 5 dias de abstinência, evidenciando que os

modelos foram capazes de modificar esse biomarcador de estresse, indicando que apresentam validade de construto (Nunes e Hallak, 2014).

Replicando resultados encontrados no primeiro modelo de administração de NAC durante a abstinência, o tratamento iniciado previamente à abstinência, mostrou que mesmo em um curto período (4 doses) a NAC foi efetiva na prevenção do aumento dos níveis séricos de corticosterona. Essas alterações ocorreram apenas nos animais abstinentes, sugerindo que a NAC em indivíduos não dependentes, é inócua, reduzindo biomarcadores de ansiedade apenas quando há desregulação do eixo HPA.

Embora especulativo, uma vez que não foram determinadas variações na concentração de glutamato encefálico nos animais, podemos inferir que esse sistema neurotransmissor, implicado nos sinais e sintomas de abstinência, seja um dos responsáveis por essa resposta específica nos animais abstinentes tratados com NAC. Tendo em vista que glicocorticoides modulam positivamente a liberação de glutamato sináptico (Popoli *et al.*, 2012), por meio de sua ação sobre o sistema glutamatérgico, a NAC preveniria liberação de corticosterona por mecanismos de retroalimentação.

O segundo biomarcador investigado, a leptina, como já indicado, está aumentada durante a abstinência de álcool em humanos e esse aumento parece ter uma relação direta com a fissura por álcool e com o risco de recaída (Kiefer, Jahn, Jaschinski, *et al.*, 2001; Kiefer, Jahn, Kellner, *et al.*, 2001; Kraus *et al.*, 2004; Kiefer *et al.*, 2005). O presente trabalho, pela primeira vez em ratos, observou que os níveis séricos de leptina também se encontram aumentados durante a abstinência de álcool. Embora os mecanismos envolvidos na regulação de sua expressão durante a abstinência ainda sejam desconhecidos, se sabe que a leptina exerce efeito sobre a atividade da via mesolímbica dopaminérgica (Opland, Leinninger e Myers, 2010), via esta, classicamente relacionada ao desenvolvimento de comportamentos aditivos (Nestler,

2005). Do mesmo modo que a corticosterona, o aumento da leptina em ratos abstinentes foi prevenido pela administração de NAC, sendo necessários mais estudos avaliando se a redução dos níveis séricos de leptina se dá diretamente pela modulação da via mesolímbica dopaminérgica ou indiretamente pela modulação glutamatérgica.

Analisando a correlação entre os parâmetros bioquímicos e os comportamentos, observamos que apenas leptina apresentou correlação negativa com os comportamentos de ansiedade, indicando que a redução na concentração de leptina pela NAC pode ter sido determinante para retomada do comportamento de exploração e permanência na área central do campo aberto.

Além desses achados, o presente trabalho mostrou que a abstinência de álcool entre ratos está associada a alterações no sistema imune, mesmo depois de 5 dias de abstinência. De fato, tanto humanos quanto em roedores, o uso crônico de álcool aumenta as concentrações séricas de diversas citocinas inflamatórias como IL-1, IL-6, IL-8, IL-10,IL-12, interferon γ e TNF-α (González-Quintela *et al.*, 2000; Crews e Vetreno, 2014; González-Reimers *et al.*, 2014), sugerindo que o alcoolismo seja uma doença inflamatória sistêmica (Kelley e Dantzer, 2011; González-Reimers *et al.*, 2014). Os mecanismos pelos quais o uso de álcool aumenta os níveis de citocinas pró-inflamatórias ainda não foram completamente elucidados, entretanto, parece requerer a estimulação do receptor tipo toll-4 (TLR4), participante da cascata de síntese de citocinas (Kelley e Dantzer, 2011). No encéfalo, o álcool promove a liberação passiva de padrões moleculares associados ao dano (DAMPs), incluindo o grupo de alta mobilidade box-1 (HMGB1) de células lesadas ou necróticas (Yanai *et al.*, 2009). Sabe-se que o HMGB1 se liga a receptores TLR4 ativando o fator nuclear κB (NFκB) e como consequência aumenta a transcrição de citocinas pró-inflamatórias (Kelley e Dantzer, 2011; Crews e Vetreno, 2014).

Explorando os efeitos anti-inflamatórios da NAC em outros contextos, estudos mostram que a administração de NAC reduz citocinas pro-inflamatórias tanto em roedores (Khan *et al.*, 2004; Chen *et al.*, 2008; Thakurta *et al.*, 2012) quanto em humanos (Mahmoud e Ammar, 2011; Csontos *et al.*, 2012). Entretanto, nenhum estudo anterior havia mostrado as propriedades anti-neuroinflamatórias da NAC no contexto do alcoolismo. Uma possível explicação para o efeito anti-neuroinflamatório da NAC seria sua ação sobre a liberação de fatores indutores da inflamação. Neste sentido, estudos mostram que o tratamento com NAC reduz a liberação de HMGB1 em hepatócitos (Tsung *et al.*, 2007; Tang *et al.*, 2011) e reduz a ativação de NFkB durante reperfusão encefálica em ratos (Carroll *et al.*, 1998; Thakurta *et al.*, 2012), dois fatores que sabidamente estão envolvidos com a resposta inflamatória.

Em roedores, a administração de lipopolissacarídeo (LPS) ou interferon-α induz comportamentos relacionados com doença como, hipolocomoção, perda do apetite e comportamentos tipo depressivo (Dantzer, 2006). Tal síndrome é acompanhada pelo aumento de citocinas pró-inflamatórias em diversas áreas encefálicas (O'connor *et al.*, 2003). Corroborando estas evidências, a presente tese em um primeiro experimento demonstrou que a administração crônica de álcool induziu hipolocomoção em ratos sendo este resultado compatível com o aumento das citocinas no hipocampo e no córtex frontal do segundo experimento.

A hiperatividade glutamatérgica durante a abstinência resulta em morte neuronal, aumento dos sintomas de abstinência e a consequente manutenção do ciclo do alcoolismo (Tsai e Coyle, 1998). A GS é uma enzima responsável por reciclar o glutamato captado do espaço extracelular pelos astrócitos sendo muito importante para a homeostase glutamatérgica (Hertz *et al.*, 1999). Os resultados mostram que a atividade desta enzima foi significativamente menor apenas no hipocampo de animais abstinentes

de álcool. Este resultado vai de encontro a achados prévios sobre a redução da expressão de EAAT2 após o uso crônico de álcool (Scofield e Kalivas, 2014). Mediante esta condição, ocorreria uma redução da captação de glutamato resultando na diminuição do substrato intracelular para GS. Embora não tenha sido avaliada a expressão de EAAT2 no presente trabalho, indícios mostram que a NAC aumenta a expressão deste transportador em modelos de autoadministração de cocaína (Reissner *et al.*, 2014) o que pode estar relacionado o aumento da atividade da GS nos animais abstinentes tratados com NAC.

Classicamente, a NAC tem sido testada na prevenção de diversos efeitos da intoxicação por álcool *in vivo* como falência hepática induzida por álcool (Hu *et al.*, 2015) ou estresse oxidativo hepático em ratos (Ferreira Seiva *et al.*, 2009; Caro *et al.*, 2014) e *in vitro* (Wu *et al.*, 2012). A maioria destes estudos tem explorado a atividade antioxidante da NAC devido à habilidade deste fármaco de induzir a síntese de GSH e possuir efeito hepatoprotetor (Berk *et al.*, 2013). Entretanto, não foram encontradas diferenças significativas nos níveis encefálicos de GSH após 5 dias de abstinência de álcool. De fato, se sabe que um curto período de abstinência é capaz de reestabelecer os níveis de GSH (Zhao, Kalhorn e Slattery, 2002) explicando a falta de alterações nos níveis desta molécula nos ratos abstinentes de álcool. Ainda, não se observou um efeito significativo da NAC sobre os níveis de GSH tendo em vista que se observa a indução de sua síntese com tratamentos mais longos e doses maiores (Farr *et al.*, 2003).

4. LIMITAÇÕES

A presente tese buscou avaliar os efeitos da N-acetilcisteína sobre sinais e sintomas de abstinência de álcool em ratos. Entretanto, o estudo apresenta algumas limitações a respeito do modelo animal utilizado.

Embora se tente mimetizar a condição da via de administração humana (via oral), a administração forçada faz com que o modelo perca validade de face, tendo em vista de que em humanos, o uso de álcool é feito de forma voluntária. Entretanto, com a utilização da ingestão forçada é possível equalizar as doses entre os animais e evitar o isolamento social normalmente utilizado nos modelos mais tradicionais de autoadministração.

Há também a impossibilidade de discernir se alguns dos efeitos comportamentais, hormonais e inflamatórios induzidos pelo modelo foram provocados pela ingestão crônica de álcool ou pela abstinência *per se* na medida em que há ausência de um grupo ainda durante a intoxicação por álcool.

5. CONCLUSÕES

Primeiramente, foi demonstrado que o uso crônico de álcool seguido de abstinência leva a alterações comportamentais e bioquímicas em ratos. Desta forma, os dois modelos de administração crônica utilizados foram capazes de reproduzir sinais e sintomas da abstinência de álcool normalmente observados em humanos.

A observação de ansiedade através de parâmetros clássicos no teste de campo aberto foi identificada no terceiro experimento da tese, sugerindo que o modelo crônico intermitente foi capaz de causar um processo de sensibilização aos sintomas de abstinência como o frequentemente observado na literatura em humanos e em animais (Brown *et al.*, 1988; Maier e Pohorecky, 1989; Hölter *et al.*, 1998; Malcolm, Herron, *et al.*, 2000; Malcolm, Roberts, *et al.*, 2000).

O tratamento com NAC preveniu a maioria destas alterações tanto quando administrado durante a abstinência quando antes do início da abstinência (concomitante à intoxicação por álcool) sugerindo que o tratamento é eficaz em ambos os esquemas. Isto configura um possível avanço do ponto de vista clínico tendo em vista de que, em uma perspectiva translacional, o indivíduo dependente de álcool poderia iniciar o tratamento antes da cessação do uso. Outro ponto importante é o tempo de tratamento à curto prazo. Isto representaria um menor tempo de tratamento necessário para a obtenção de efeitos benéficos sobre os sintomas de abstinência e uma recuperação mais rápida.

De acordo com a Organização Mundial da Saúde (OMS), até 2012, cerca de 3,3 milhões de mortes foram associadas ao uso de álcool (OMS, 2014). No Brasil, segundo o ultimo levantamento domiciliar sobre drogas, cerca de 12,3% das pessoas foram diagnosticadas como dependentes de álcool (OBID, 2007). Tendo em vista de que o

TUA é um transtorno crônico, recorrente e gerador de um grande problema de saúde pública, a descoberta de novas drogas que reduzam os sintomas de abstinência de álcool e previnam a recaída são de grande importância social. Portanto, estudos clínicos devem ser feitos, de forma a esclarecer os efeitos da NAC sobre os sintomas de abstinência de álcool humanos.

6. PERSPECTIVAS

O presente trabalho reiterou a importância do estudo dos efeitos da NAC sobre aspectos comportamentais, neuroinflamatórios e bioquímicos da abstinência de álcool. Tendo em vista os resultados encontrados e o crescente número de estudos mostrando os efeitos da NAC no contexto dos transtornos aditivos em humanos e em modelos animais, a presente tese tem ainda como perspectivas:

- Avaliar os efeitos das NAC sobre o consumo voluntário de álcool em modelos de autoadministração;

- Verificar a influência da abstinência de álcool sobre outros peptídeos relacionados ao apetite como neuropeptídeo-Y, grelina, orexínas e colecistocinina em ratos;

- Avaliar os efeitos da NAC sobre parâmetros do sistema glutamatérgico como os transportadores EAAT1 e EAAT2 bem como sobre a captação de glutamato durante a abstinência de álcool;

- Verificar a liberação de glutamato extracelular mediante a administração de NAC durante a abstinência de álcool por meio da técnica de microdiálise;

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ANEXO I

U F R G S
UNIVERSIDADE FEDERAL
DO RIO GRANDE DO SUL

PRÓ-REITORIA DE PESQUISA

Comissão De Ética No Uso De Animais

PROPESQ

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 23069
Título: EFEITOS DA N-ACETILCISTEÍNA SOBRE A ABSTINÊNCIA DE ÁLCOOL EM RATOS

Pesquisadores:

Equipe UFRGS:

ELAINE ELISABETSKY - coordenador desde 01/07/2012
ROSANE GOMEZ - coordenador desde 01/07/2012
DAYANE ALVES QUINTEROS - pesquisador desde 01/07/2012
CAROLINA FERREIRA SANTOS - pesquisador desde 01/07/2012
Ricardo Schneider Junior - pesquisador desde 01/07/2012

Comissão De Ética No Uso De Animais aprovou o mesmo em seus aspectos éticos e metolodológicos, para a utilização de 360 ratos Wistar machos, de acordo com as Diretrizes e Normas Nacionais e Internacionais, especialmente a Lei 11.794 de 08 de novembro de 2008 que disciplina a criação e utilização de animais em atividades de ensino e pesquisa.

Porto Alegre, Quarta-Feira, 4 de Julho de 2012

FLAVIO ANTONIO PACHECO DE ARAUJO
Coordenador da comissão de ética