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EFEITO DA TAURINA SOBRE OS SISTEMAS GABAÉRGICO E GLUTAMATÉRGICO E SOBRE PARÂMETROS COMPORTAMENTAIS DE RATOS CRONICAMENTE TRATADOS E ABSTINENTES AO ÁLCOOL

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Orientadora: Prof. Dra. Rosane Gomez

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Dedico este trabalho ao
meu pequeno Matheus
que me inspira a sempre
buscar ser a minha
melhor versão.

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RESUMO

A dependência ao álcool é um problema de saúde pública e está relacionada a milhares de mortes por ano. Indivíduos dependentes, quando em abstinência ao uso de álcool, apresentam sintomas negativos que contribuem para a recaída e a manutenção do ciclo da adição. Muitos dos sintomas da abstinência são resultantes do desequilíbrio entre os sistemas GABAérgico e glutamatérgico. A neuroplasticidade induzida pelo álcool afeta principalmente os receptores do ácido gama-aminobutírico A ($GABA_A$) e os receptores N-metil-D-aspartato (NMDA) de glutamato. A taurina, um aminoácido sulfonado, atua como modulador inibitório e diminui a excitotoxicidade induzida por glutamato por interação com os receptores NMDA, diminuindo o influxo de cálcio na célula. Portanto, no primeiro artigo apresentado nesta tese foram avaliados os efeitos da taurina (100 mg/kg/dia) no comportamento de ratos cronicamente tratados (2 g/kg, 2x ao dia) ou após cinco dias de abstinência ao álcool. Também foi verificada a expressão de RNAm da subunidade α2 do receptor de $GABA_A$ e de BDNF no córtex frontal destes animais. Os resultados obtidos mostraram que mesmo após cinco dias de abstinência os ratos ainda apresentam diminuição no comportamento exploratório no campo aberto quando comparados ao grupo controle. O tratamento com taurina foi capaz de restaurar o comportamento dos animais abstinentes. A taurina reduziu a expressão de BDNF no grupo cronicamente tratado com álcool, demonstrando um efeito neuroprotetor e diminuiu a expressão de α2 apenas nos ratos controle. No segundo artigo avaliou-se a expressão de RNAm das subunidades α1, α4, δ e γ2 de $GABA_A$ e GluN2A e GluN2B de NMDA no hipocampo de ratos submetidos ao mesmo protocolo de exposição crônica e abstinência ao álcool. Não foram observadas diferenças significativas de expressão de subunidades entre os grupos estudados. Porém, análise de correlação mostrou que o uso crônico de álcool e a abstinência alteram as correlações entre as subunidades observadas no grupo controle, e a taurina não foi capaz de reverter este efeito. Apesar de sua interação com os receptores $GABA_A$ e NMDA a taurina não é eficaz para equilibrar estes dois sistemas que são afetados pelo uso crônico e abstinência ao álcool. Os resultados obtidos nesta tese contribuem para a elucidação das alterações decorrentes do uso e abstinência ao álcool e do tratamento com taurina sobre

sistemas neurotransmissores e comportamentos. Tais resultados podem contribuir para o desenvolvimento de novos fármacos para o tratamento da dependência.

Palavras-chave: Etanol. Taurina. Campo Aberto. PCR em tempo real. Neuroplasticidade.

ABSTRACT

Alcohol dependency is a public health problem and is related to millions of deaths per year. Alcohol dependent people presents negative symptoms during withdrawal and theses symptoms contribute to relapse and maintenance of addiction cycle. Many of the withdrawal symptoms are due to unbalance of GABAergic and glutamatergic systems. Neuroplasticity induced by alcohol mainly affects gamma-aminobutiric acid A ($GABA_A$) and glutamate N-methil-D-aspartate (NMDA) receptors. Taurine, a sulfonated amino acid, acts as an inhibitory neurotransmitter and decreases glutamate induced excitotoxicity through direct interaction with NMDA receptors, decreasing cellular Ca^{2+} influx. Therefore, in the first presented article we evaluated taurine (100 mg/kg/day) in rats chronically treated (2 g/kg, twice a day) or alcohol five days withdrawal. $GABA_A$ α2 subunit receptor and BDNF mRNA expression was also verified in the frontal cortex of these animals. Results showed that even after five days of withdrawal, the rats still showed difference in exploratory behavior in the open field when compared to the control group. Taurine treatment restored this behavior in withdrawal animals. Taurine also reduced BDNF expression in the chronically alcohol-treated group, demonstrating a neuroprotective effect, and decreased α2 expression only in control rats. In the second article $GABA_A$ α1, α4, δ and γ2 subunits and NMDA GluN2A and GluN2B subunits were evaluated in the hippocampus of rats exposed to the same protocol of chronic alcohol and withdrawal. No significant differences in subunit expression were observed between studied groups. Correlation analysis showed that the chronic alcohol treatment and withdrawal alter the correlations between subunits observed in control group. Taurine was not able to reverse this effect. Besides taurine interaction with $GABA_A$ and NMDA receptors, it is not effective balancing these two systems that are affected by alcohol chronic use and withdrawal. Our results contribute to the elucidation of changes related to alcohol use and withdrawal and taurine effect in different brain areas. They may contributes to the development of new drugs for the treatment of addiction.

Keywords: Ethanol. Taurine. Open field. Real-time PCR. Neuroplasticity.

APRESENTAÇÃO

Os resultados desta tese de doutorado estão apresentados sob a forma de dois artigos científicos, o primeiro já publicado no periódico Pharmacology, Biochemistry & Behavior, volume 161, páginas 6-12, ano 2017, e o segundo a ser submetido ao periódico Neuropharmacology.

A tese está organizada como uma introdução geral, que apresenta as bases teóricas e justificativa deste trabalho, seguida pelos dois artigos citados acima, ambos na língua inglesa e uma discussão geral, englobando os resultados dos dois artigos. Segue-se a ela perspectivas e as referências utilizadas na introdução geral.

SUMÁRIO

1 INTRODUÇÃO

A dependência às drogas, também denominada drogadição é reconhecida como um grande e complexo problema de saúde pública. É considerada uma doença crônica recorrente, caracterizada por compulsão para usar determinada droga e perda do controle sobre o seu uso (American Psychiatry Association, 2014). A dependência de álcool e seu uso abusivo é responsável por 3,3 milhões de mortes ao ano, representando 5,9% do total de mortes no mundo (World Health Organization, 2014). Essas mortes estão relacionadas não apenas com acidentes de trânsito e violência, mas também com diversos problemas de saúde incluindo doenças cardiovasculares, hepáticas, renais, gastrintestinais e psiquiátricas (Zernig et al., 2000; Wallner e Olsen, 2008).

O ciclo de dependência às drogas é caracterizado pela compulsão pelo uso de determinada droga, perda do controle na quantidade administrada e um estado emocional negativo durante sua privação (abstinência) (Koob e Volkow, 2010). A compulsão pelo uso de determinada droga se refere à busca continuada para obtenção de prazer, apesar das consequências negativas associadas ao seu uso (Koob e Volkow, 2010). Já o aumento da dose administrada está relacionada ao fenômeno da tolerância, pois devido à neuroadaptações que ocorrem com o uso crônico da droga, o indivíduo necessita de uma dose cada vez maior para a obtenção do mesmo efeito (Ghezzi et al., 2013). Os sintomas de abstinência, no caso do abuso de álcool, envolvem a ativação da amígdala estendida e incluem ansiedade, disforia, maior susceptibilidade à convulsões, hiperalgesia e distúrbios do sono (Koob e Volkow, 2010; Ron e Barak, 2016). Nesta fase ocorrem neuroadaptações para que o sistema, que estava sofrendo ação constante do uso da droga, volte a sua função normal. Essas alterações graduais são acompanhadas por sinais como preocupação/antecipação e fissura (*craving*), envolvendo o processamento de reforço condicionado na amígdala basolateral e o processamento de informações contextuais pelo hipocampo (Koob e Volkow, 2010). Fissura e síndrome de abstinência são os principais deflagradores para retomada do uso/abuso de drogas e fracasso das terapias para interrupção do ciclo de dependência.

Álcool e outras drogas de abuso possuem diferentes mecanismos de ação que, no entanto, parecem convergir em uma mesma via neural de recompensa, afetando a

função celular de modo diverso quando administradas aguda ou cronicamente. A via de recompensa mais conhecida é a via dopaminérgica mesolímbica. Essa via, que projeta neurônios dopaminérgicos da área tegmental ventral (VTA) até o núcleo accumbens (NAcc) e córtex pré-frontal (CPF) é um dos mais importantes substratos neurais para a geração de estímulos de recompensa provocados por drogas de abuso (Nestler, 2005). Cada droga, apesar de mecanismo de ação distinto, parece levar diretamente ou indiretamente ao aumento da transmissão dopaminérgica nesta via (Koob e Le Moal, 2001) (Figura 1).

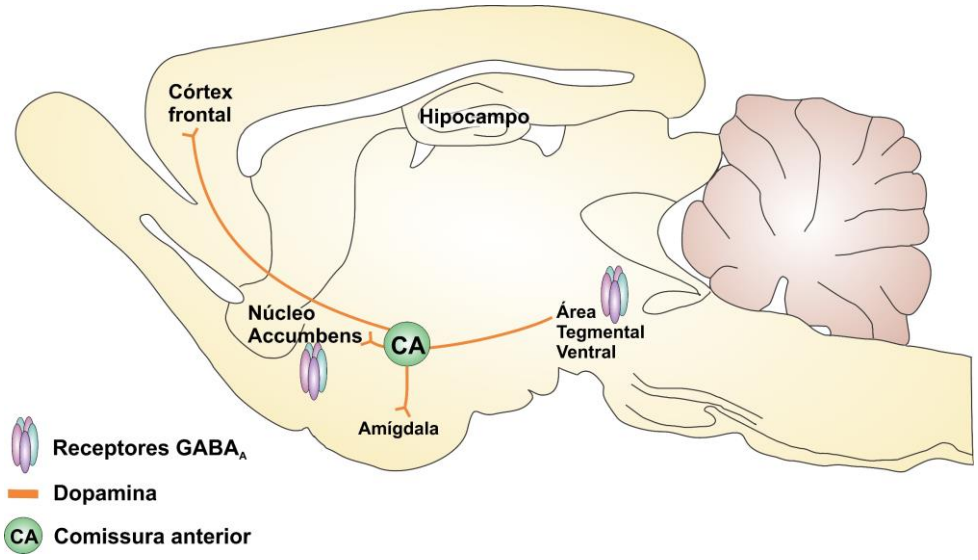

Figura 1 – Vias dopaminérgicas relacionadas com a drogadição representadas no cérebro de um rato. Fonte: elaborada pela autora.

O álcool aumenta a liberação de dopamina através da interação com os neurônios GABAérgicos, tanto pelo aumento direto do neurotransmissor inibitório ácido γ-aminobutírico (GABA) na VTA, NAcc e hipocampo, quanto pela ativação dos receptores do tipo A do ácido γ-aminobutírico ($GABA_ARs$) (Koob e Volkow, 2010). A ativação dos $GABA_ARs$ faz com que a inibição da liberação da dopamina seja inibida (Nestler, 2005). Além da ação sobre o sistema GABAérgico, o álcool também promove liberação de peptídeos opioides na VTA, NAcc e núcleo central da amígdala (Tomkins e Sellers, 2001; Koob e Volkow, 2010). O uso crônico de álcool promove adaptações no sistema GABAérgico, incluindo dessensibilização e, consequentemente, tolerância aos efeitos e dependência ao álcool (Koob e Le Moal, 2001).

Receptores $GABA_A$ são ionotrópicos, constituídos por canais iônicos da família *cys-loop* (Olsen et al., 2018). A inibição do sistema nervoso central (SNC) mediada

por $GABA_AR$ ocorre quando duas unidades do neurotransmissor GABA se ligam ao receptor, modificando sua conformação e desta forma abrindo um poro que permite a entrada de cloreto (Cl^-), resultando na hiperpolarização da célula pós-sináptica (Baumann et al., 2003).

Os $GABA_ARs$ são pentâmeros formados por subunidades que estão divididas em 8 classes: α (1-6), β (1-3), γ (1-3), δ, ε, θ, π e ρ (1-3) (Figura 2). Cada subunidade é formada por quatro domínios transmembrana (TM1-4) hidrofóbicos e um grande domínio extracelular amino-terminal (Jacob et al., 2008). A maioria dos $GABA_ARs$ são constituídos por duas subunidades do tipo α, duas β e uma γ, sendo que as isoformas mais comuns no SNC são α1β2γ2, α2β3γ2 e α3β3γ2. Os receptores que contém subunidades α4-6, β1, γ1, γ3, δ, ε e θ são menos numerosos, mas também apresentam importantes funções. As subunidades ρ formam homo ou heteroligômeros que possuem farmacologia distinta dos receptores formados por outras subunidades, sendo insensíveis a bicuculina, benzodiazepínicos (BZs) e barbitúricos (Olsen et al., 2018).

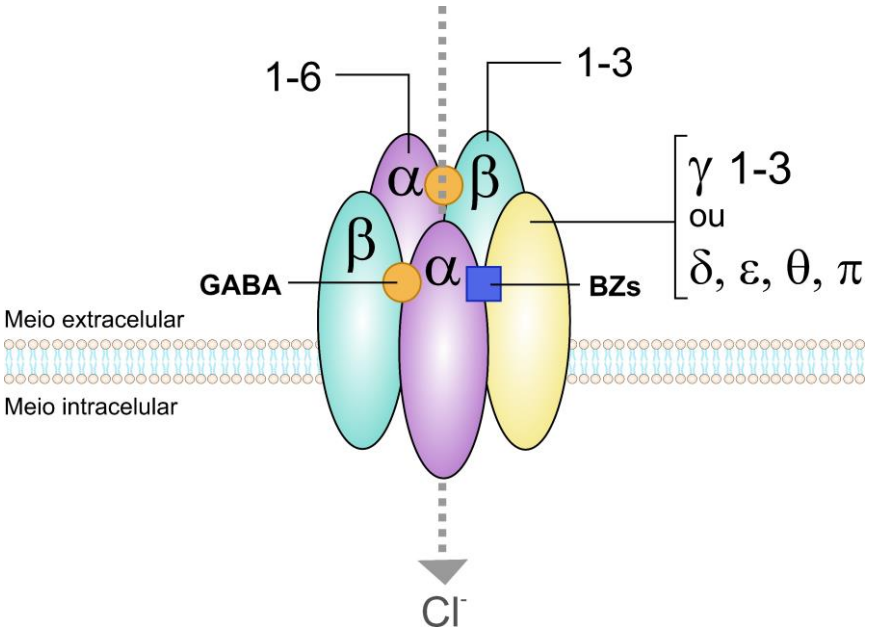

Figura 2 – Estrutura dos receptores $GABA_A$. Estes receptores possuem 5 subunidades, que podem ser de 8 subfamílias (α, β, δ, γ, ε, θ,, π e ρ), formando um canal permeável ao Cl^-. Os receptores formados pelas subunidades ρ não foram representados devido às suas diferentes propriedades farmacológicas. Fonte: elaborada pela autora.

As diferentes combinações de subunidades que formam o $GABA_AR$ determinam a afinidade de ligação do ligante e as propriedades do acoplamento, além

da localização do receptor (Roberto e Varodayan, 2017). Por exemplo, receptores contendo a subunidade α4 ou α6 combinados com β ou δ são predominantemente extra ou não sinápticos, não são sensíveis a benzodiazepínicos e medeiam uma inibição tônica (Jacob et al., 2008). $GABA_AR$ formados por α1, α2, α3 ou α5 em conjunto com β e γ são predominantemente sinápticos e sensíveis a benzodiazepínicos (Jacob et al., 2008) (Figura 3).

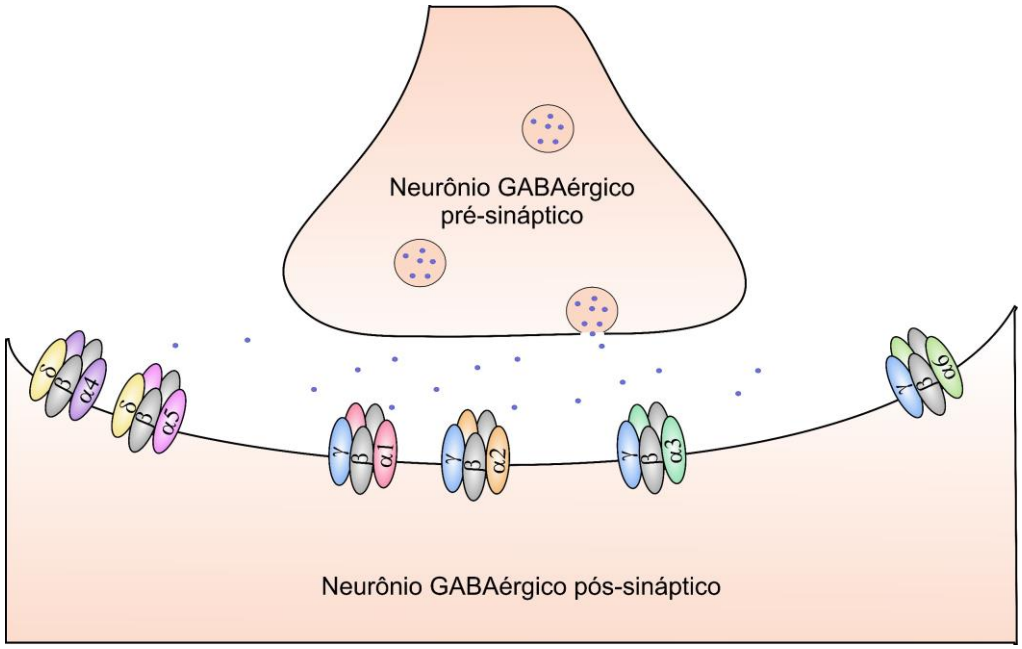

Figura 3 – Localização dos receptores $GABA_A$, formados por diferentes subunidades Receptores formados por α1-3 são predominantemente sinápticos, enquanto aqueles contendo α4-6 são predominantemente extrassinápticos. Fonte: elaborada pela autora.

O uso crônico e a abstinência ao álcool induzem neuroadaptação do sistema GABAérgico. Esse fenômeno não afeta de maneira significativa a quantidade de receptores expostos na fenda sináptica, mas sim a composição das subunidades dos receptores (Jacob et al., 2008). Dados de expressão de RNA mensageiro (RNAm) e peptídeos mostram que animais cronicamente tratados com álcool apresentam diminuição na expressão de α4 e aumento de α1 (Devaud et al., 1995, 2002). No entanto a plasticidade induzida pelo uso crônico do álcool parece apresentar diferenças dependendo da região encefálica e do tempo de exposição. No hipocampo de animais cronicamente tratados com álcool, por 40 dias, observou-se um aumento

significativo de α4, sem mudanças na expressão de α1. No mesmo estudo, mostrou-se que o tratamento com álcool por 14 dias não é capaz de alterar estas subunidades (Matthews et al., 2002).

A plasticidade induzida pelo álcool envolve, portanto, mudanças nas populações sinápticas e extrassinápticas de $GABA_AR$ (Jacob et al., 2008). Pelo uso crônico de álcool, os receptores extrassinápticos contendo as subunidades δ e α4, e os receptores sinápticos contendo α1, β e γ, que formam um receptor mais sensível ao GABA, são internalizados (Jacob et al., 2008). Como um mecanismo compensatório para a sinapse inibitória, receptores contendo α4, β e γ2, que possuem funções fisiológicas distintas, são inseridos na fenda sináptica (Jacob et al., 2008). Esses receptores sinápticos apresentam menor afinidade pelo GABA e parecem justificar a tolerância aos efeitos do álcool observadas pelo uso crônico.

O principal mecanismo de internalização dos $GABA_ARs$ ocorre por endocitose dependente de clatrina, uma proteína formadora de vesícula (Kittler et al., 2000; Jacob et al., 2008). Neste processo, uma proteína adaptadora tipo 2 (AP2) se liga diretamente a subunidades β1-3 e γ2 do $GABA_AR$ e engloba o receptor em uma vesícula revestida por clatrina (Kittler et al., 2000). Já a inserção de novo $GABA_AR$ na membrana plasmática depende do transporte a partir do retículo endoplasmático, onde são formados. Dali são transportados para o complexo de Golgi e segregados para vesículas que transportam o novo receptor e o inserem na membrana da célula (Jacob et al., 2008).

Adicionalmente, o consumo de álcool também é capaz de afetar o sistema glutamatérgico, principal neurotransmissor excitatório do SNC. O consumo agudo de álcool geralmente inibe a neurotransmissão glutamatérgica, enquanto a exposição crônica e a abstinência tendem a aumentá-la (Roberto e Varodayan, 2017). Os efeitos do uso crônico de álcool ocorrem principalmente nos receptores glutamatérgicos pós-sinápticos (Roberto e Varodayan, 2017). Esses receptores são acoplados a proteína G, denominados receptores metabotrópicos como mGluR, ou formam canais, denominados receptores ionotrópicos, como o AMPAR (ácido α-amino-3-hidroxi-5-metil-4-isoxazole-propiônico), o NMDAR (N-metil-D-aspartato) e o kainato (Goudet et al.,; Peters et al.,). As mudanças na ativação dos NMDARs resultantes da exposição crônica ao álcool são muito mais expressivas que sobre outros receptores (Roberto e

Varodayan, 2017). Esta exposição tende a aumentar tanto a função dos NMDARs quanto a sinapse glutamatérgica mediada pelo NMDAR (Roberto e Varodayan, 2017).

Os NMDARs são heterômeros que divergem em sua composição molecular (subunidades), propriedades biofísicas e farmacológicas, interações e localização subcelular (Paoletti et al., 2013). Existem 7 diferentes subunidades descritas: GluN1, GluN2A-D e GluN3A-B (Paoletti et al., 2013). Receptores di-heteroméricos contendo GluN1/GluN2A e GluN1/GluN2B representam uma importante fração dos NMDARs (Paoletti et al., 2013). Receptores tri-heteroméricos contendo GluN1/GluN2A/GluN2B também estão presentes no encéfalo adulto, particularmente no hipocampo e no córtex (Paoletti et al., 2013). O papel dos NMDARs contendo GluN3 ainda não está bem esclarecido.

A ativação dos NMDARs depende da ligação de dois agonistas: o glutamato, que se liga na subunidade GluN2, e a glicina ou D-serina, que se liga na subunidade GluN1 (Wyllie et al., 2013) (Figura 4). O que determina se o agonista será glicina ou D-serina é o subtipo da subunidade GluN1, que pode ser 1-4a ou 1-4b, todas codificadas pelo mesmo gene (Wyllie et al., 2013). Os NMDARs são sensíveis ao bloqueio voltagem dependente por íons Mg^{2+}, por isso, para que sejam ativados, também é necessária a despolarização da célula pós-sináptica (Zhou e Sheng, 2013). Uma vez ativado, o NMDAR permite a entrada de Ca^{2+} e Na^{+} potencializando a despolarização da célula pós-sináptica (Zhou e Sheng, 2013). Receptores contendo GluN2A e GluN2B são mais sensíveis ao bloqueio por Mg^{2+}, tem maior permeabilidade ao Ca^{2+} e apresentam maior condutância em comparação àqueles contendo GluN2C e GluN2D (Wyllie et al., 2013). O distúrbio da função dos NMDARs, que podem ser decorrentes da alteração da atividade dos receptores, expressão de subunidades tráfego ou localização, podem resultar em efeitos deletérios ao SNC (Zhou e Sheng, 2013).

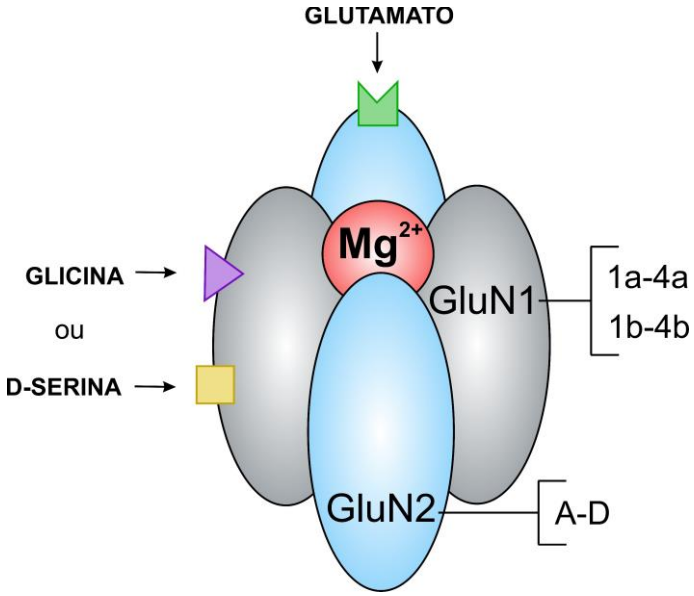

Figura 4 – Estrutura dos receptores NMDA, heterômeros, em sua maioria formados por duas subunidades GluN1, que possuem sítio de ligação para glicina ou D-serina, e duas subunidades GluN2, onde está localizado o sítio de ligação do glutamato. Fonte: elaborada pela autora

Os receptores contendo GluN2A são predominantemente sinápticos enquanto aqueles contendo GluN2B podem ser sinápticos ou extrassinápticos (Pál, 2018). A localização subcelular específica dos NMDARs contendo GluN2A e GluN2B é de grande interesse devido a diferença do papel desempenhado pelos NMDARs sinápticos e extrassinápticos na sobrevivência e morte neuronal (Wyllie et al., 2013). Estudos em cultura de células demonstraram que os NMDARs podem migrar da região sináptica para outras regiões da membrana neuronal em resposta a estímulos (Tovar e Westbrook, 2002; Groc et al., 2006; Paoletti et al., 2013). A mobilidade dos NMDARs depende do subtipo da subunidade GluN2, sendo que os que contém GluN2A são mais estáveis que os que contém GluN2B (Groc et al., 2006). Além disto, a super expressão de GluN2A é capaz de estabilizar os NMDARs de superfície contendo GluN2B (Groc et al., 2006).

Apesar de inibir todos os subtipos de NMDARs, do mesmo modo que para os receptores $GABA_A$, a sensibilidade do receptor ao álcool varia de acordo com a composição de subunidades, sendo que receptores formados por GluN1/GluN2A ou GluN1/GluN2B são os mais sensíveis a ação do álcool (Roberto e Varodayan, 2017). Quando o indivíduo entra em abstinência, a regulação positiva de NMDARs causada pela exposição crônica ao álcool resulta num aumento da vulnerabilidade para a resposta citotóxica induzida por glutamato (excitotoxicidade) (Figura 5) (Dodd et al.,

2000). A síndrome hiperglutamatérgica que ocorre durante a abstinência do etanol é associada não somente com o *craving* e com a recaída ao consumo de álcool, mas também com uma toxicidade induzida pelo glutamato mediada pela entrada de íons Ca^{2+} na célula pós-sináptica (Al Qatari et al., 2001).

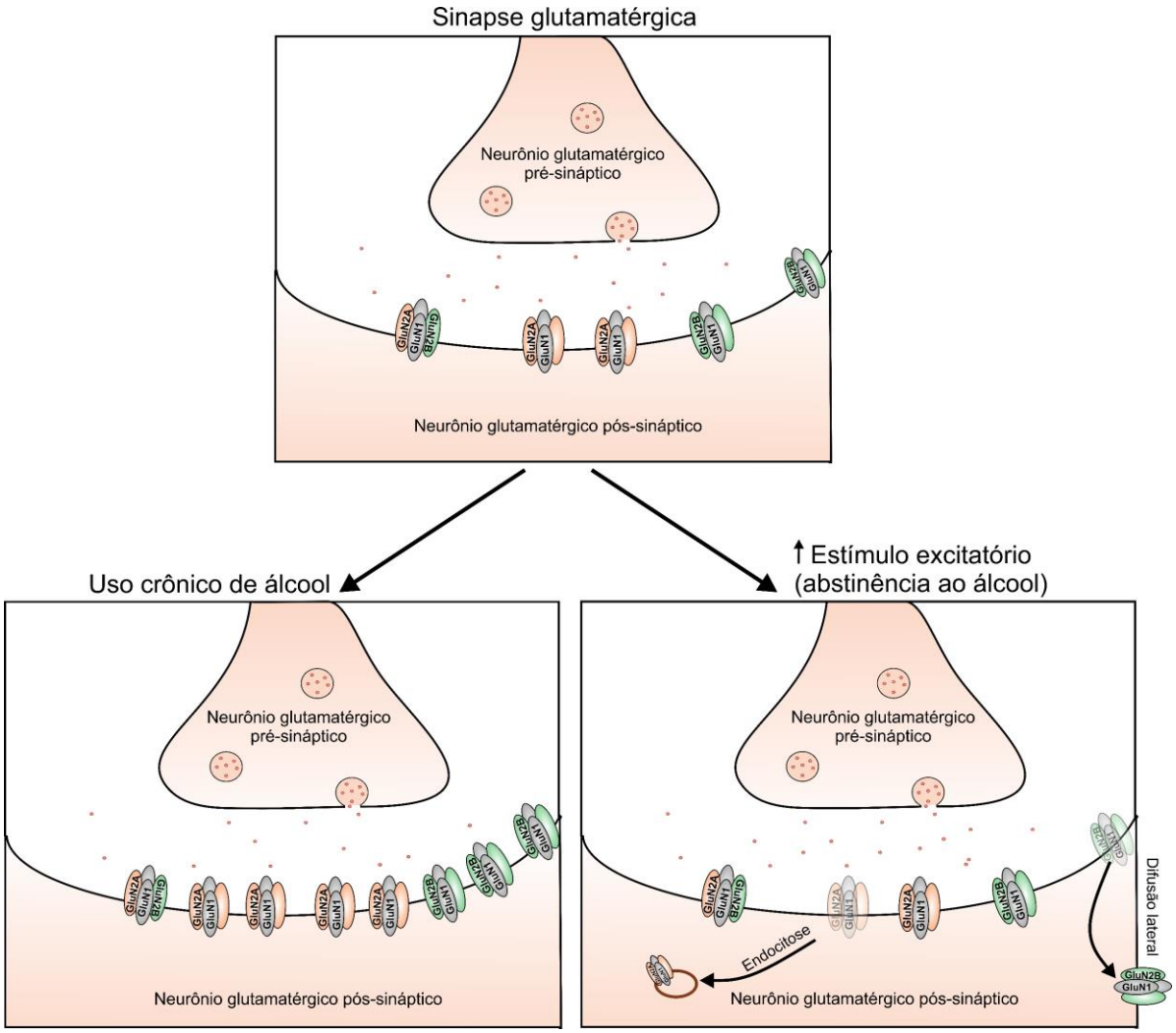

Figura 5 – Representação esquemática do efeito do uso crônico de álcool na região mesolímbica e do aumento do estímulo excitatório na expressão dos receptores NMDA contendo as subunidades GluN2A e GluN2B. Fonte: elaborada pela autora

A taurina, ou ácido 2-aminoetanosulfonico (Figura 6), é o principal β-amino ácido intracelular presente na maioria dos tecidos de mamíferos (Gu et al., 2014). Altas concentrações de taurina são detectadas em tecidos eletricamente excitáveis como cérebro, retina, coração e músculos esqueléticos (Saransaari e Oja, 2000; Rosemberg et al., 2010). A taurina possui diversas propriedades citoprotetoras,

atuando como neuromodulador inibitório, osmorregulador, modulador da homeostase do cálcio intracelular, antioxidante, estabilizador de membrana, além de apresentar efeito anti-inflamatório (Rosemberg et al., 2010; Gu et al., 2014). Existem também evidências de que a taurina tem atividade protetora em várias condições patológicas incluindo hipóxia, neurotoxicidade induzida por glutamato e inflamação (Louzada et al., 2004; Schuller-Levis e Park, 2004).

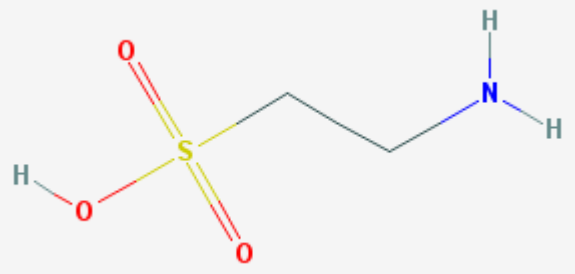

Figura 6 – Representação da estrutura 2D da taurina. Fonte: (PubChem Compound Database, 2018)

Quando em condições que danificam as células, a liberação de taurina endógena é aumentada, enquanto sua captação é inibida (Gu et al., 2014). Este aumento extracelular de taurina parece ser um importante mecanismo de proteção endógeno (Saransaari e Oja, 2000). Estas funções sugerem que a taurina pode restaurar o funcionamento de sistemas após os efeitos induzidos pelo álcool (Quertemont et al., 2000). Quertemont e colaboradores (1998) demonstraram que ratos suplementados com taurina por via oral tem uma diminuição na aversão causada por altas doses de álcool (Quertemont et al., 1998). No entanto, os mecanismos envolvendo as ações da taurina ainda não são suficientemente entendidos, acredita-se que seus efeitos extracelulares são mediados por sua interação com $GABA_AR$ e receptores de glicina e por interação direta com os NMDARs por múltiplos mecanismos (Albrecht e Schousboe, 2005; Chan et al., 2013).

Apesar de indícios de que a taurina possa equilibrar as concentrações destes neurotransmissores GABA e glutamato no SNC durante o período de abstinência ao álcool, não há estudos que avaliem, em indivíduos abstinentes e tratados com taurina, o nível desses neurotransmissores nas regiões encefálicas relacionadas com o processo de adição. Como o mecanismo de ação da taurina no SNC ainda não foi completamente elucidado, estudos exploratórios como esse, relacionando abstinência e sua interferência sobre diferentes sistemas neurotransmissores são elucidativos.

Nossa hipótese é de que a taurina, por sua atividade multialvo e antioxidante pode ser utilizada como adjuvante na terapia de retirada do álcool, reduzindo sinais de fissura e atenuando sinais de abstinência. Adicionalmente, acreditamos que esse efeito da taurina se dá, especificamente, por restabelecimento do equilíbrio entre a atividade inibitória, via sistema GABAérgico, e excitatória, via sistema glutamatérgico. O restabelecimento desse equilíbrio pode interferir tanto sobre comportamentos relacionados com uso crônico ou abstinência ao álcool, como sobre a expressão de diferentes subunidades desses receptores, expressos no terminal sináptico e extrassináptico.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar os efeitos da taurina sobre os sistemas GABAérgico e glutamatérgico e sobre parâmetros comportamentais de ratos cronicamente tratados ou abstinentes ao álcool.

2.2 OBJETIVOS ESPECÍFICOS

a. Determinar o efeito do tratamento com taurina sobre o comportamento de ratos cronicamente tratados ou abstinentes ao álcool, pelo emprego do teste de campo aberto e labirinto em cruz elevado (Artigo 1).

b. Avaliar a expressão de RNA mensageiro (RNAm) da subunidade α2 do receptor $GABA_A$ no córtex frontal de ratos com exposição crônica ou abstinentes ao álcool, tratados com taurina, pela técnica de PCR em tempo real (Artigo 1).

c. Avaliar a expressão de RNAm das subunidades α1, α4, δ e γ2 do receptor $GABA_A$ e GluN2A e GluN2B de NMDA pela técnica de PCR em tempo real no hipocampo de ratos com exposição crônica ou abstinentes ao álcool, tratados com taurina (Artigo 2).

d. Avaliar a correlação da expressão das subunidades α1, α4, δ e γ2 do receptor $GABA_A$ e GluN2A e GluN2B de NMDA em diferentes condições (controle, uso crônico e abstinência ao álcool) e o efeito da taurina, no hipocampo, nos diferentes grupos experimentais (Artigo 2).

3 ARTIGO PUBLICADO

A seguir está apresentado o primeiro artigo referente a este estudo, que foi publicado no periódico *Pharmacology, Biochemistry and Behavior* 161 (2017) 6-12. Este artigo esclarece que o efeito da taurina depende da condição de tratamento, visto que ela reverte o comportamento exploratório de ratos abstinentes, porém não tem efeito nos ratos cronicamente administrados com álcool. O efeito da taurina sobre a subunidade α2 e sobre o BDNF também foi dependente da condição, sendo que apenas o grupo controle e o tratado com álcool tiveram diminuição de α2 e BDNF, respectivamente, confirmando que a abstinência altera funções encefálicas e a resposta à taurina.

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Taurine restores the exploratory behavior following alcohol withdrawal and decreases BDNF mRNA expression in the frontal cortex of chronic alcohol-treated rats

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ABSTRACT

Alcohol use disorder is an alarming health problem, and the withdrawal symptoms increase the risk of relapse. We have hypothesized that taurine, a multitarget substance acting as a gamma-aminobutyric acid A receptor ($GABA_AR$) positive modulator and a partial inhibitor of *N*-methyl-D-aspartate (NMDA) glutamate receptors, may reduce the withdrawal symptoms or modify behaviors when combined with alcohol. Therefore, we investigated the effects of taurine on behavior in the open field test (OFT), the $GABA_AR$ α_2 subunit and BDNF mRNA expression in the frontal cortex of rats after chronic alcohol treatment or upon withdrawal. Rats received alcohol 2 g/kg (alcohol and withdrawal groups) or water (control group) twice daily by oral gavage for 28 days. On day 29, the withdrawal rats received water instead of alcohol, and all groups were reallocated to receive 100 mg/kg taurine or vehicle intraperitoneally, once a day for 5 days. On day 33, the rats were exposed to OFT; 18 h later, they were euthanized, and the frontal cortex was dissected for $GABA_AR$ α_2 subunit detection and BDNF mRNA expression determination by real-time quantitative PCR. Taurine administration restored rearing behavior to the control levels in the withdrawal rats. Taurine also showed anxiolytic-like effects in control rats and did not change the behaviors in the chronic alcohol group. Chronic alcohol treatment or withdrawal did not change the $GABA_AR$ α_2 subunit or BDNF mRNA expression in the frontal cortex, but taurine decreased the α_2 subunit level in control rats and to the BDNF levels in the alcohol rat group. We conclude that taurine restored exploratory behavior after alcohol withdrawal but that this effect was not related to the $GABA_AR$ α_2 subunit or BDNF mRNA expression in the frontal cortex of the rats.

1. Introduction

Alcohol use disorder is a public health problem that is associated with millions of deaths each year worldwide (World Health Organization, 2014). Massive campaigns and policies to decrease alcohol dependence have resulted in little success, probably because the positive reward of alcohol use and negative symptoms of its withdrawal maintain its continuous use and increases the risk of relapse (Gilpin and Koob, 2008).

Alcohol is mainly known as a depressor of the central nervous system (CNS), acting as a positive modulator of the gamma-aminobutyric acid A receptor ($GABA_AR$). $GABA_AR$ is a ligand-gated ion channel with five subunits that are combined according to the function and brain area (Jacob et al., 2008). Alcohol dependence is associated with changes in the α_2 subunit in the limbic brain areas, such as the frontal cortex, hippocampus, and amygdala (Kumar et al., 2009).

Reinstatement after alcohol withdrawal has been linked to the glutamatergic pathway from the prefrontal cortex to NAcc overactivity, lower activity of the dopamine projection from the VTA to the medial prefrontal cortex, as well as lower activity of the GABA projection from the NAcc to the ventral pallidum (Koob and Volkow, 2010). Craving, which is a key component of relapse, is related to overactivity in the glutamatergic pathway, from the frontal cortex to the basolateral amygdala, which projects itself to the ventral striatum (Koob and

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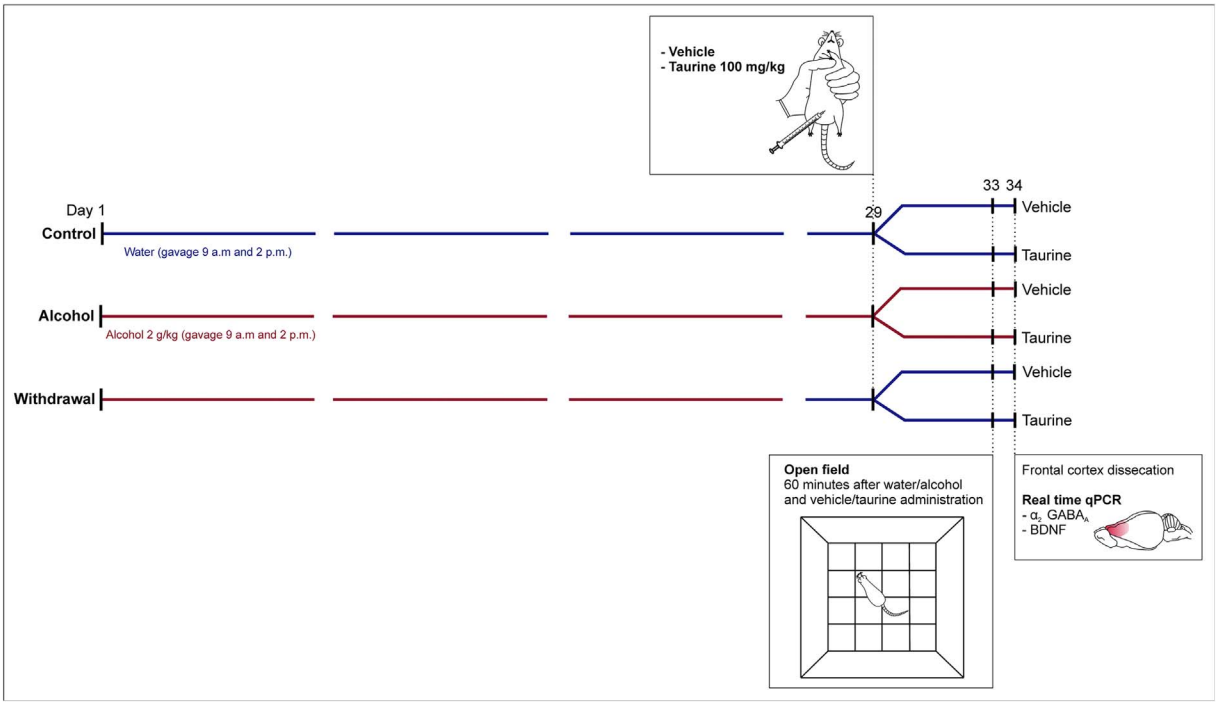

Fig. 1. Experimental Timeline. Wistar rats were treated with 2 g/kg alcohol or distilled water, via oral gavage, twice a day (9 AM and 2 PM), 5 days/week, for 28 days. On day 29, alcohol was interrupted in the withdrawal group, and rats received 100 mg/kg taurine or vehicle, via i.p., once/day. On day 33, they were exposed to the open field test, after 1 h from the last alcohol/water or taurine/vehicle administration. Rats were euthanatized 18 h later under the same treatment, and the frontal cortex was dissected.

Volkow, 2010). Indeed, alcohol withdrawal decreases the inhibitory function of GABA and increases the excitatory function of glutamate, leading to rebound hyper-neuronal excitability. This is related to behavioral changes such as seizures and anxiety, which increase the risk of relapse (Modesto-Lowe et al., 2005). Therefore, novel pharmacological strategies to restore dopamine, GABA, and the glutamate balance during alcohol withdrawal may decrease craving episodes and the risk of relapse in alcoholics.

Alcohol and other drug abuse have been described to regulate brain-derived neurotrophic factor (BDNF), known for its role in the growth, survival, and differentiation of developing neurons (Logrip et al., 2015; Russo et al., 2009). Indeed, the chronic effects of moderate alcohol consumption increases the BDNF levels in the dorsal striatum of rats by activating the genomic mechanisms (Logrip et al., 2015). Until now, little is known about the meaning of changes in BDNF expression during alcohol dependence and withdrawal.

Taurine, a sulfonated amino acid, acts as an agonist of $GABA_AR$, hyperpolarizing the cells by increasing the Cl^- influx (Albrecht and Schousboe, 2005). Additionally, taurine presents osmoregulatory, antioxidant, and neuroprotective properties (Jong et al., 2012; Lambert et al., 2015). Acute taurine administration shows an anxiolytic-like effect in mice and zebrafish (El Idrissi et al., 2009; Mezzomo et al., 2016). Chronic treatment induces an antidepressant-like effect in diabetic rats (Caletti et al., 2015). Taurine also prevents glutamate-induced neurotoxicity and inflammation in animal models of hypoxia (Albrecht and Schousboe, 2005; Schuller-Levis and Park, 2004). Alcohol administration increases the endogenous taurine levels in the NAcc of both alcohol-preferring and non-preferring rats (Quertemont et al., 2000). However, the taurine levels were higher in alcohol-non-preferring rats in this brain area, suggesting that taurine may attenuate the neurotoxic effects of alcohol in rats (Quertemont et al., 2000). However, the multi-target mechanisms of taurine are not completely understood. Few studies have explored taurine's effects in combination with alcohol drinking on the withdrawal symptoms in rats.

Therefore, the aim of this study was to investigate the effects of taurine on behavior and the mRNA expression of the $GABA_AR$ α_2 subunit and BDNF in the frontal cortex after chronic alcohol treatment or withdrawal in rats.

2. Methods

2.1. Animals

Adult male Wistar rats (n = 72), with a body weight of 250–280 g, were born and reared at the animal facility of Universidade Federal do Rio Grande do Sul (UFRGS), Brazil and were housed in polypropylene cages (3 rats/cage, 33 × 40 × 17.8 cm) under controlled environmental conditions (22 ± 2 °C, 12 h light/dark (lights on at 7 a.m.), with free access to water and food (Nuvilab, Colombo, Brazil). All the procedures were performed according to international and local policies for experimental animal handling and had been approved by the Ethics Committee for Animal Experimentation (CEUA-UFRGS #28722).

2.2. Saline, ethanol, and taurine solutions

Ethanol (98%) (Nuclear, São Paulo, Brazil) was diluted to 20% (w/v) in distillated water. Alcohol solution was prepared daily and was administered as 10 mL/kg via oral (gavage). The alcohol dose (2 g/kg twice a day) was chosen based on the literature and from a previous study in our group (Schneider et al., 2015; Gilpin et al., 2009; Gomez and Luine, 2014). This 2 g/kg dose increases blood alcohol concentration (BAC) up to 120 mg/dL at 60 min from the administration (Gilpin et al., 2009; Gomez and Luine, 2014). BAC levels of 0.08 g/dL are considered binge drinking and typically occurs after 4–5 drinks for humans (Drinking Levels Defined, n.d.). Control rats received the same volume of distilled water. Taurine (100 mg/kg; Biofarma, Porto Alegre, Brazil) was diluted in saline 0.9% and was administered intraperitoneally (i.p.). The control group received the same volume of saline 0.9% (vehicle), i.p. This dose of taurine was chosen based on the antidepressant and neuroprotective effects in rats (Caletti et al., 2015) and is the same dose that reduces voluntary alcohol consumption after acute administration (Olive and Hodge, 2001). Taurine treatment

started after withdrawal to investigate its effects to relieve alcohol withdrawal symptoms.

2.3. Experimental procedure

Over 28 days, rats were treated for 5 consecutive days (Monday to Friday) with 2 g/kg alcohol (alcohol and withdrawal groups) or distilled water (control group) twice daily, at 9 a.m. and 2 p.m., by oral gavage (n = 24) (Fig. 1). Rats were weekly weighed for dose adjustment and control of weight gain. At day 29 to 33, rats from the withdrawal group received distilled water by gavage, instead of alcohol, and the alcohol and control groups received oral alcohol or water, respectively. Immediately after the gavage, all groups (n = 12/group) were administered with vehicle or taurine (100 mg/kg) via i.p. At day 33, after 5 days of taurine/vehicle treatment, rats were exposed to the open field test (OFT) for 5 min, 1 h after alcohol/water and taurine/vehicle administration. All behaviors were recorded for posterior analyses with the Kevin Willioma, KD Ware Computer software (Boston, MA, USA). On day 34, 18 h after the last administration of alcohol/water and taurine/vehicle, animals were euthanized, and the frontal cortex was dissected (bregma, 2.16–5.16 mm) (Paxinos and Watson, 2004). The frontal cortex samples were used for analysis of the $GABA_AR$ α_2 subunit and BDNF mRNA expression by real-time quantitative PCR (qPCR).

2.4. Open field test

For locomotor activity and anxiety behavior observation, rats were individually placed in the center of an open box (80 × 80 × 40 cm) with the floor divided into 16 squares. A dim red light (25 W) illuminated the testing room. The behaviors were video recorded, and the number of squares crossed with all four paws (ambulation) in the center zone or near to the walls, and the latency to the first exit from the central zone were registered. Moreover, the frequency of and time spent in rearing and grooming behaviors, and number of fecal boli, were analyzed. The sum of ambulation in the central and peripheral zones were considered as the total ambulation, and the sum of the frequency of all active behaviors (ambulation, rearing, and grooming) were presented as total activity behaviors. All rats were tested both in the OFT between 1 and 4 p.m. and after the test rats were returned to their home cages, and the open field was cleaned with paper towels and water to remove any trace of odor to prevent interference with the behaviors of subsequent rats. The behaviors were analyzed by a trained researcher, blinded for treatments.

2.5. Real-time quantitative PCR

The relative gene expression of the $GABA_AR$ α_2 subunit and BDNF in the frontal cortex was determined using reverse transcription combined with real-time quantitative PCR (qPCR) and the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). For gene expression, 5–6 samples were randomly chosen from the animals subjected to the behavioral protocol. Total RNA was extracted from the frontal cortex using the Trizol™ Isolation Reagent Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, and samples were stored at − 80 °C. cDNA was synthesized using SuperScript III (Life Technologies, Carlsbad, CA, USA). DNA was quantified using a BioSpec-nano™ (Shimadzu, Kyoto, Japan). qPCR analysis was performed at least in duplicate using the StepOnePlus Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). The set of primers for the $GABA_AR$ α_2 subunit (sense: 5′-GACAATGACCACATTAAGCATCAG-3′ and antisense: 5′-TCTTG GCTTCGGCTGGCTTGTTCTC-3′), BDNF (sense: 5′-GATGAGGACCAGAAGGTTC G-3′ and antisense: 5′-GATTGGGTAGTTCGGCATTG-3′), β-actin (endogenous control) (sense: 5′-TGTGATGGTGGGAATGGGTCAG-3′ and antisense: 5′-TTGATGT CACGCACGATTTCC-3′), and GAPDH (endogenous control) (Glyceraldehyde-3-Phosphate Dehydrogenase) (sense: 5′-AACGACCCCTTCATTG ACCTC-3′ and antisense: 5′-CCTTGACTGTGCCGTTGAACT-3′) was chosen from the *Rattus norvegicus* data from the National Center for Biotechnology Information. The reaction contained 15 ng of cDNA and 2 × Power SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA), and the primer quantities were 0.32 μM of the $GABA_AR$ α_2 subunit and BDNF, 0.16 μM β-actin, and 0.64 μM GAPDH.

2.6. Statistical analysis

The data were tested for normal distribution using the Shapiro-Wilks test and were analyzed by two-way ANOVA with the condition (non-alcohol × alcohol × alcohol withdrawal) and treatments (vehicle x taurine) as factors, followed by Turkey's post hoc test when appropriate. The results are presented as means ± standard error of mean (SEM). Significance was set at $P < 0.05$, and statistical analysis was performed using the Sigma Stat Program (Jandel Scientific Co., v. 11.0, San Jose, CA, USA).

3. Results

A summary of the statistical analyses is presented in Supplementary Table S1. We showed that 5 days of alcohol withdrawal decreased the rearing frequencies in the rats ($P = 0.011$; Fig. 2A). Taurine treatment prevented this effect in the withdrawal rats and restored the rearing frequencies to the control levels ($P = 0.021$). An interaction between the alcohol regimen and taurine treatment was detected: taurine decreased the rearing frequencies in the control rats ($P = 0.024$) and increased the rearing frequencies in the withdrawal rats ($P = 0.005$; Fig. 2A). Similarly, taurine increased the rearing durations in the withdrawal rats ($P = 0.035$) and decreased them in the control rats ($P = 0.030$), showing again, an interaction between the condition and treatment ($P = 0.011$; Fig. 2B). Although the alcohol treatment did not significantly decrease the rearing frequencies, it decreased the time spent in rearing, despite the taurine treatment ($P < 0.001$). The rearing durations were almost 60% higher in the taurine-treated withdrawal rats than in the control rats ($P = 0.024$).

The only group with decreased latency to exit from the central area was chronic alcohol treatment, denoting the anxiolytic effects of alcohol ($P < 0.001$; Fig. 2C). Taurine administration did not interfere with chronic alcohol. Chronic alcohol treatment also increased grooming ($P = 0.048$, Fig. 2D), decreased the numbers of fecal boli ($P = 0.039$, Fig. 2E), and increased the total ($P < 0.001$; Fig. 2F) and central ambulations ($P < 0.001$; Fig. 2G). None of these behaviors were affected by the taurine administration. On the other hand, taurine increased the central ambulation in the control ($P = 0.017$) but not in the withdrawal rats ($P = 0.067$, Fig. 2G). Grooming durations were not affected by the chronic alcohol regimen or taurine treatment (data not shown). Concerning the total active behavior frequencies, the chronic alcohol rats were more active (154.00 ± 11.02) than were the control (98.83 ± 10.55) or withdrawal rats (80.40 ± 11.55) ($P < 0.001$). Taurine did not change this parameter in any group (control = 80.70 ± 11.55; alcohol = 136.36 ± 11.02; withdrawal = 100.90 ± 11.55).

The alcohol regimen did not change the $GABA_AR$ α_2 subunit (Fig. 3) or BDNF mRNA expression in the frontal cortex of the rats (Figs. 3 and 4). Taurine administration, however, significantly decreased the $GABA_AR$ α_2 subunit expression in control rats ($P = 0.032$), an effect not seen in chronic alcoholic or withdrawal rats. This was confirmed by interactions between the condition and treatment (Fig. 3). Taurine also decreased the BDNF expression, specifically in the alcohol group ($P = 0.009$; Fig. 4).

Finally, we detected no changes in the weight gain related to the alcohol regimen or taurine treatment in the experiment (Supplementary Fig. S2).

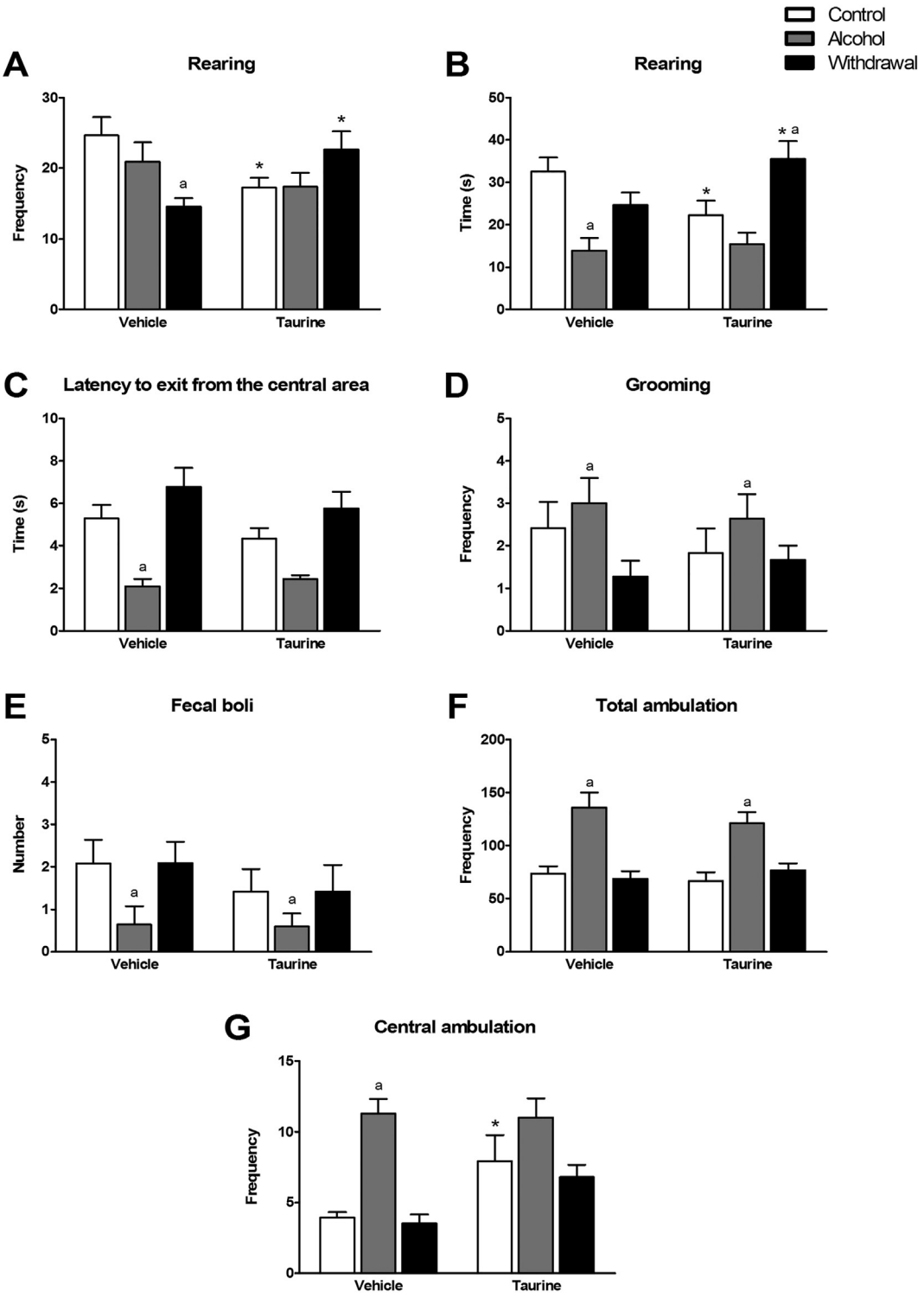

Fig. 2. Effect of 100 mg/kg taurine on different behaviors in the open field test. Rearing frequency (A) and duration (B), latency to exit from the central area (C), frequency of grooming (D), fecal boli (E), total (F) and central (G) ambulation. Control; alcohol-treated rats (4 g/kg/day, oral administration); withdrawal: 5 days alcohol withdrawal rats. Two-way ANOVA + Tukey test; n = 12/group. * difference between treatment (vehicle or taurine 100 mg/kg); [a] difference between condition (control, alcohol or withdrawal).

4. Discussion

Health problems related to alcohol dependence are alarming, and different strategies should be adopted for the management of alcohol-use disorders (World Health Organization, 2014). In line with this thought, we have explored the effect of taurine, a non-essential amino acid that is used as a food supplement frequently added to energetic drinks (Higgins et al., 2010). We have hypothesized that taurine may relieve withdrawal symptoms in an animal model of alcohol abstinence, by restoring the imbalance between GABA and glutamate systems, which are known to be related to different stages of the addiction cycle (Koob and Volkow, 2010; Gass and Olive, 2008).

Here, we have explored the effects of short-term treatment with taurine on behavior and on the $GABA_AR$ α_2 subunit and BDNF mRNA expression in the frontal cortex of rats, after 5 days of abstinence, or with continuous alcohol treatment. We showed a lower frequency of

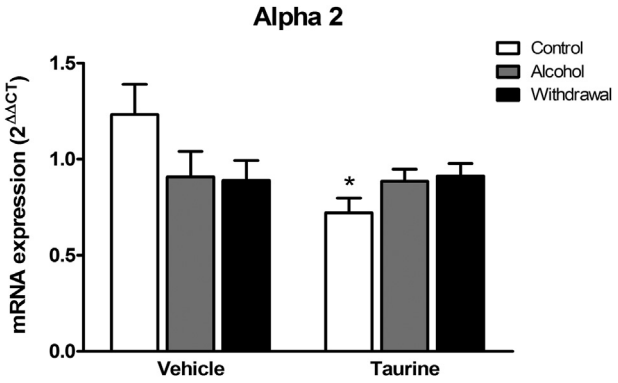

Fig. 3. Effects of taurine (100 mg/kg, i.p.) on the expression of α_2 $GABA_A$ subunit mRNA expression in the frontal cortex of control; chronic alcohol (2 g/kg, vo)-treated rats, or 5 days alcohol withdrawal rats. Two-way ANOVA + Tukey test; n = 6–7/group. * Difference between treatment (vehicle or taurine 100 mg/kg).

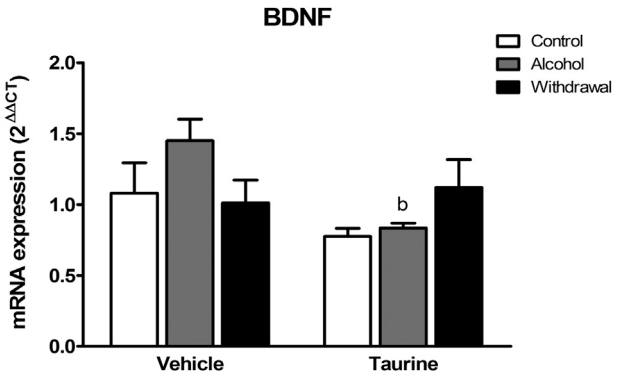

Fig. 4. Effects of taurine (100 mg/kg, i.p.) on the expression of α_2 $GABA_A$ subunit mRNA expression in the frontal cortex of control; chronic alcohol (2 g/kg, vo)-treated rats, or 5 days alcohol withdrawal rats (ABS). Two-way ANOVA + Tukey test; n = 6–7/group. [b] Difference between treatment (vehicle or taurine 100 mg/kg).

rearing during alcohol withdrawal than in the control rats in the OFT. No other behavior was altered in animals during the alcohol withdrawal. The OFT is a common test that is used to explore alcohol withdrawal-induced anxiety in rodents, and usually, alcohol withdrawal decreases behaviors in the OFT in rats (Kliethermes, 2005). We showed that rearing, which is an exploratory behavior, was lower after 5 days of abstinence in chronically treated rats (4 g/day, during 28 days). Other demonstrations of decrease rearing as an anxiety-like behavior at 24–48 h after alcohol withdrawal in the rats have been described (Karadayian et al., 2013). However, studies exploring anxiety-like behaviors in rodents following chronic alcohol exposure have not been consistent (Kliethermes, 2005). Some authors have found that alcohol withdrawal does not change behavior (Manley and Little, 1997; Poelchen et al., 2001) or that it inhibits locomotive behavior, such as total locomotion, time spent in the central area, and rearing, (Meert, 1994) or even that it promotes hyperactivity in rodents.(Uzbay and Erden, 2003; Mehta and Ticku, 1993). A previous study by our group has also found hypoactivity in rats after 5 days of withdrawal, as evidenced by a reduction in the peripheral and total ambulation, rearing, and total activities (Schneider et al., 2015). A reduction in total locomotion was also observed in the mice, seven days after withdrawal, in the free-choice novelty apparatus (Fukushiro et al., 2012). These differences may be related to the dosage or intervals from the last alcohol administration, as seen by the temporal changes on locomotion after 1 h (decreased), 24 h (increased), and 120 h (restored to the control rats) after alcohol administrations (6–10 g/kg/day/v.o., 5 days) that were observed in the OFTs (Bomfim et al., 2014).

Short-term taurine treatment increased the rearing behavior in the withdrawal rats, preventing the anxiety-like behavior that was induced by the alcohol withdrawal. In vitro and in vivo studies have shown that taurine acts as a $GABA_A$ positive modulator, increasing the GABA levels and expression of glutamic acid decarboxylase (GAD), as well as the expression of the GABA synthesis enzyme, in different brain areas (El Idrissi and Trenkner, 2004). Additionally, taurine acts directly as a partial inhibitor of the GluN2b-containing NMDA receptor subtype (Chan et al., 2015) and, indirectly, as a regulator of cytoplasmic and mitochondrial calcium homeostasis, both effects that are known to attenuate glutamate neurotransmission and excitotoxicity (El Idrissi and Trenkner, 2004; Chan et al., 2015). These multitarget mechanisms may be useful for restoring GABA and glutamate imbalance by decreasing the risk of relapse in alcoholics. Additionally, while replicating the results of other studies, we showed that taurine presents an anxiolytic-like effect in the control rats, which was identified by increasing frequencies in the central area, the main behavior that is related to the anxiolytic-like effect of drugs in this particular animal model. Indeed, taurine supplementation increased the time spent in the OFT central area with mice, (El Idrissi et al., 2009) and it increased the time spent in the lit area in a light-dark apparatus by zebrafish (Mezzomo et al., 2016). Benzodiazepines and other $GABA_AR$ agonist drugs, such as alcohol, usually increases the frequencies in the OFT central area (Prut and Belzung, 2003). Beside its anxiolytic effects, taurine also decreased the frequencies and times of rearing in the control rats. The same pattern of behavior was found in the alcohol group, suggesting that taurine administration may replicate some alcohol effects, such as impaired motor coordination and balance in rats. Chen et al. (2004) found that a single administration of taurine (60 mg/kg) decreased the rearing behavior of mice, but not after 7 days of treatment when they were exposed to the OFT (Chen et al., 2004). Moreover, benzodiazepines and related anxiolytic compounds decreased the rearing of mice and rats in the OFT, and this was not related to total ambulations (Kliethermes, 2005). Although taurine presented anxiolytic properties, its effects were not consistent with those that are presented by classic anxiolytics (Chen et al., 2004). Therefore, it is possible to assume that taurine acted as a GABA agonist in the control rats; however, in the withdrawal rats, taurine restored the balance of the glutamatergic and GABAergic systems that were lost during alcohol withdrawal.

Behavioral changes in the OFT were more evident in the alcohol group, probably due to the acute effects of the alcohol. Rats in this group decreased the latency to exit from the central area and decreased the time that was spent in rearing. Moreover, the alcohol rats increased the frequencies in the central area and their total ambulations. All of these behaviors are in alignment with the anxiolytic and stimulant effects of alcohol in rodents after moderate alcohol dosages (Acevedo et al., 2014; Wscieklica et al., 2016). Low to intermediate doses of alcohol increased the locomotion of the rats, while higher doses caused hypoactivity, due to the depressant/sedative effects of alcohol (Smoothy and Berry, 1984). Agreeing with our results, others have also shown increased central locomotion, (Breier and Paul, 1990) together with the motor-stimulating effects of alcohol in rats (Acevedo et al., 2014).

Interestingly, taurine administrations did not change the behaviors in the alcohol group. Because taurine is a GABAergic substance, one could expect synergistic effects due to its combined use with alcohol. However, taurine did not increase the stimulant or anxiolytic effects of alcohol, with no apparent drug interactions. This unresponsiveness may be related to neuroplasticity from chronic alcohol use and down-regulation of $GABA_AR$ in other brain areas than the frontal cortex because chronic use did not significantly decrease the $GABA_AR$ α_2 subunit expression in our study. In contrast, these lacks of drug interactions may allow alcoholics to take taurine prior to quitting drinking. Thus, relieving the negative reinforcement that is associated with the withdrawal syndrome and complying with guidelines that recommend reducing the doses and frequency of drinking. Additional studies will clarify whether taurine decreases the alcohol self-administration in rats

or changes the $GABA_AR$ expression in other brain areas, such as in the NAcc or amygdala.

As stated above, a different alcohol regimen (chronic use or abstinence) did not change the $GABA_AR$ α_2 subunit expression in the frontal cortex of the rats, and taurine treatment did not affect these parameters. Consistent with our study, Chen et al. (1998) did not find significant changes in $GABA_AR$ α_2 expression in the NAcc, amygdala, hippocampus, or cortical areas of alcohol-preferring rats after the chronic consumption of alcohol (Chen et al., 1998). However, others have shown a downregulation of the $GABA_AR$ α_2 subunits without affecting the GABA binding sites in the cortical area of rats that were chronically administered with alcohol (Mehta and Ticku, 2005; Mhatre and Ticku, 1992; Mhatre et al., 1993; Montpied et al., 1991). Indeed, we found a non-significant 26% decrease in the α_2 subunit expression in our alcohol-treated and withdrawal rats, while Montpied et al., 1991 found a very similar and significant 29% decrease in the α_2 expression in the cortex of rats after 14 days of alcohol inhalation, (Montpied et al., 1991) suggesting a downregulation of the $GABA_AR$ containing α_2 subunit.

Short-term alcohol consumption increases $GABA_AR$ function, and prolonged drinking decreases the number of $GABA_AR$ subunits or changes its subunit composition (Vengeliene et al., 2008). Downregulation of $GABA_AR$ may cause hyper-excitability, anxiety, seizure, or other withdrawal syndrome symptoms (Vengeliene et al., 2008). Here, we have shown that taurine decreased the $GABA_AR$ α_2 subunit expression by 42%, but only in the control rats. In vitro studies have shown that chronic exposure of cultured cortical neurons with $GABA_AR$ agonists downregulates the expression of the α_2 subunit, a function that may be related to a decrement in the number of binding sites (Mhatre and Ticku, 1994). We cannot exclude that repeated treatment (5 days) with taurine decreased the α_2 mRNA expression in the control rats when related to increasing GABA and decreasing the glutamate neurotransmission in the frontal cortex of the rats. Regarding the withdrawal and alcohol groups, we have suggested that neuroplasticity by chronic alcohol administrations affected the responses to taurine. Further studies would be necessary to analyze whether longer times of abstinence or taurine treatments could interfere with these results.

Although consistent, our results possess some limitations. First, we cannot exclude that the daily manipulations and invasive procedures interfered with the results. It is already known that intragastric intubation enhances hypothalamic-pituitary-adrenal (HPA) axis activities, as well as the alcohol withdrawal itself (Buck et al., 2011). Thus, the changes in behavior in our withdrawal rats may be related, not uniquely, to alcohol withdrawal but also to prolonged stress imposed by our protocol. However, we chose the method of oral administration because the voluntary alcohol consumption model also presents stress that is related to social isolation, in addition to an uncertainty about the daily alcohol intake. Moreover, body weight, a physiological marker of chronic stress, (Gomez and Luine, 2014) was not affected by our treatments (Supplementary Fig. S2). A previous study has shown lower weight gains in alcohol rats than in the control rats, after being treated daily with 2 g/kg alcohol, once a day, for 7 days (Gomez and Luine, 2014). We are confident that twice a day gavage, for > 4 weeks, habituated the rats more easily than once a day and a shorter time and, consequently, produced decreased stress related to the administrations. Indeed, after five or six days from the beginning of the experiments, we observed a more collaborative behavior for gavage administrations in our rats. Another limitation of our study is that we cannot confirm that our behavioral results are related to repeated (100 mg/kg, for 5 days) or acute (OFT after 60 min) taurine administrations. However, in a previous study, the authors have shown anxiolytic-like effects of taurine (42 mg/kg) after acute administration, but not after repeated taurine administrations, although the mice were tested 40 min after the last taurine administration (Chen et al., 2004; Kong et al., 2006).

Finally, the BDNF expression did not change in the frontal cortex of the alcohol or withdrawal groups under our experimental design. The results from other studies are contradictory, with the BDNF changing according to the brain area and alcohol treatment (McGough et al., 2004; Pandey et al., 1999; Schunck et al., 2015). While 2 weeks of an alcohol liquid diet, followed by 24 h of withdrawal, decreased the BDNF expression, 3 weeks of alcohol gavage administration, followed by 12 days of withdrawal, increased it in the frontal cortex of rodents (Pandey et al., 1999; Schunck et al., 2015). On the other hand, 10 days of voluntary alcohol consumption, or alcohol vapor exposure, did not change the BDNF mRNA expression in the frontal cortex of rats (McGough et al., 2004). Indeed, most studies have shown that chronic alcohol administrations decrease the BDNF mRNA expression focus in the hippocampal area (Hauser et al., 2011; MacLennan et al., 1995). In our result with the frontal cortex, the ALC group showed a tendency to increase (34%) the BDNF levels. This result may not have statistical significance due to the long interval (18 h) between the last dose of alcohol and brain dissections. Cocaine self-administration elevates the BDNF mRNA levels in the NAcc of rats (Graham et al., 2007). Interestingly, BDNF infusion in the NAcc area increases drug intake and drug-seeking behavior, enhancing the drug craving after cocaine withdrawal (Graham et al., 2007). We suggest that neurotoxicity by alcohol, cocaine, and other drugs of abuse may stimulate neurotrophic factors, such as BDNF, according to the dose and regimen. This hypothesis was supported by the taurine administrations in the alcohol group. In these animals, taurine decreased the BDNF mRNA levels. Taurine is a neuroprotective amino acid that acts as a $GABA_A$ agonist and is a partial inhibitor of the NMDA glutamate receptor (El Idrissi and Trenkner, 2004; Chan et al., 2015). Studies have shown that blockade of the stimulation of the GABAergic system and/or the glutamate receptors reduced the BDNF mRNA levels in the hippocampus of rats (Licata et al., 2013; Zafra et al., 1991). Despite the neurotoxicity related to abstinence, we did not find changes in the BDNF levels in the withdrawal group, possibly related to a longer interval of abstinence (5 days).

5. Conclusions

According to our results, taurine prevented a decrease in the exploratory behavior after alcohol abstinence and it showed anxiolytic-like effects in the control rats. Taurine also decreased the BDNF mRNA expression in the frontal cortex of the alcohol rats, denoting neuroprotective effects of this amino acid. Finally, taurine decreased the $GABA_AR$ α_2 subunit, but only in the non-alcoholic control rats. Taurine treatment only reversed the behavior in the withdrawal rats and the BDNF levels in the alcohol rats. Taken together, these results provide evidence that chronic alcohol consumption or alcohol abstinence disturbs the behavioral responses of taurine, as well as the $GABA_AR$ α_2 subunits and BDNF mRNA expression in the frontal cortex of rats. Additional studies need to be conducted to explore other taurine dosages, longer treatments, or animal models with an alcohol dependence. Because taurine is safe and cheap, and presents multiple mechanisms, it may be an important adjuvant in the treatment of alcohol withdrawal symptoms in humans.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.pbb.2017.09.001.

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The authors declare no competing financial interest.

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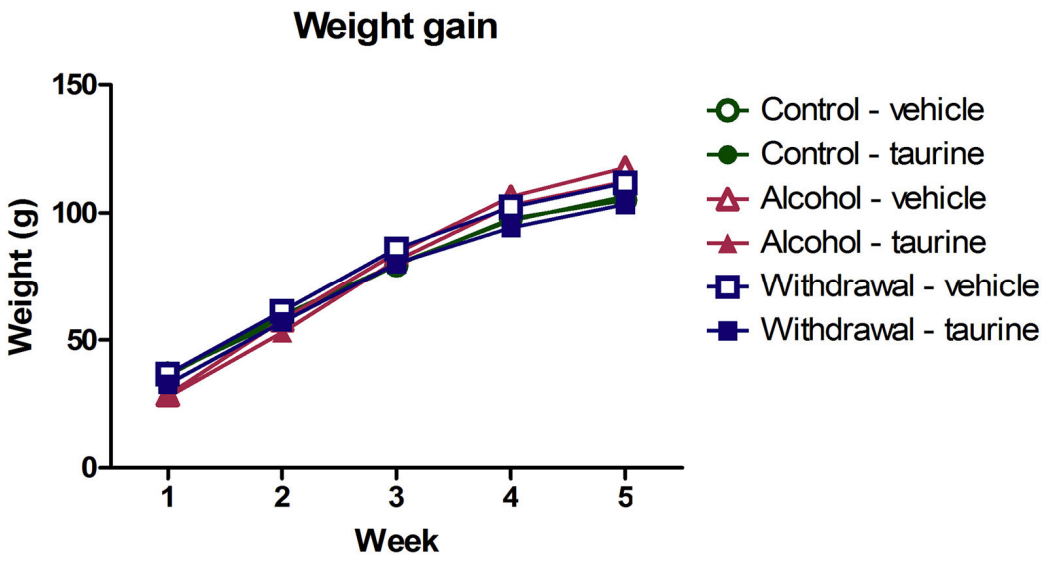
Supplementary information

Table S1. Main effects of and the interaction between condition (control x alcohol x withdrawal) and treatment (vehicle x taurine). Results of Analysis of Variance (ANOVA-two way) from behaviors in the OFT of rats and $GABA_AR$ α_2 subunit and BDNF mRNA expression in the frontal cortex of rats. Significant effects ($P < 0.05$) are given in bold font.

Behaviors	**Source of variation**	**F-value**	**P-value**
Rearing (Frequency)	Condition	0.579	0.565
Figure 2A	Treatment	0.247	0.621
	Interaction	5.888	**0.005**
Rearing (Time)	Condition	13.005	< **0.001**
Figure 2B	Treatment	0.066	0.797
	Interaction	4.906	**0.011**
Freezing (Time)	Condition	0.569	0.569
	Treatment	0.499	0.482
	Interaction	2.811	0.068
Latency do exit from the central area	Condition	23.421	< **0.001**
Figure 2C	Treatment	0.499	0.482
	Interaction	2.811	0.068
Grooming (Frequency)	Condition	3.186	**0.048**
Figure 2D	Treatment	0.182	0.671
	Interaction	0.476	0.624
Gromming (Time)	Condition	1.873	0.162
	Treatment	0.468	0.497
	Interaction	0.262	0.262
Fecal Boli	Condition	3.402	**0.039**
Figure 2E	Treatment	1.276	0.263
	Interaction	0.249	0.781
Total Ambulation (Frequency)	Condition	25.152	< **0.001**
Figure 2F	Treatment	0.369	0.546
	Interaction	0.719	0.492
Central ambulation (Frequency)	Condition	13.857	< **0.001**
Figure 2G	Treatment	5.549	**0.022**
	Interaction	1.751	0.183
Peripheral ambulation (Frequency)	Condition	19.691	< **0.001**
	Treatment	0.265	0.609

	Interaction	0.399	0.673
Total active behaviors	Condition	16.331	< **0.001**
	Treatment	0.309	0.580
	Interaction	1.895	0.160
mRNA expression			
$GABA_AR$ α_2 subunit	Condition	0.361	0.700
Figure 3	Treatment	3.754	0.062
	Interaction	3.885	**0.032**
BDNF	Condition	0.979	0.388
Figure 4	Treatment	4.745	**0.038**
	Interaction	2.892	0.072

Degrees of freedom (DF) of behaviors = (2,68) for condition and interaction, and (1,68) for treatment. DF of mRNA expression = (2,34) for condition and interaction, and (1,34) for treatment. Differences between number of animals used in statistic and total animals are due to outliers or technical errors.



Supplementary Fig. S2. Graphic showing the body weight gain of all groups during the five weeks of experiment.

4 ARTIGO EM PREPARAÇÃO

A seguir está apresentado o segundo artigo referente a este estudo, que está sendo preparado para a submissão no periódico *Neuropharmacology*. Neste artigo ficou evidente que, sob nosso protocolo experimental, a taurina não é capaz de reverter os efeitos do uso crônico e da abstinência ao álcool sobre as correlações entre as subunidades dos receptores $GABA_A$ e NMDA no hipocampo de ratos.

Correlations between subunits of $GABA_A$ and NMDA receptors in chronic alcohol treated or withdrawal and the effect of taurine in the hippocampus of rats

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Abstract

Chronic use of alcohol impairs the delicate balance between GABAergic and glutamatergic systems. A better comprehension of different roles of $GABA_A$ and NMDA receptors subunits could be helpful to define new strategies to counteract the deleterious effects observed during alcohol withdrawal. Taurine, a sulfonated amino acid, have been proposed to attenuate alcohol withdrawal symptoms, due to its neuromodulatory properties. In this work, we evaluated the correlation between $GABA_A$ and NMDA subunits in hippocampus of rats chronically treated or alcohol withdrawal, and the effects of taurine treatment. Male Wistar rats received alcohol (2g/kg) or tap water by oral gavage (control group), twice a day, for 28 days. From day 29 to 33 withdrawal rats received tap water instead of alcohol and all groups were reallocated to receive 100 mg/kg taurine or saline (vehicle) intraperitoneally, once a day. On day 34 the rats were euthanized, and the hippocampus was dissected for $GABA_AR$ α1, α4, δ and γ2 and NMDAR GluN2A and GluN2B subunits mRNA expression determination by real-time quantitative PCR. No differences were observed in two-way ANOVA for the expression of subunits among groups. Taurine induces a correlation of α1 and γ2 subunits, while alcohol results in a correlation between α4 and GluN2A. Combination of alcohol and taurine treatment made an extra correlation, between α1 and GluN2A. After 5 days of withdrawal a correlation of control group, between δ and GluN2A is reestablished. Although there were no significant differences in mRNA expression, the correlation established between subunits show us a neuroadaptation of GABAergic and glutamatergic systems. This article aims to helps elucidate the mechanisms beyond synergic neuroadaptations observed in alcohol use and withdrawal.

Keywords: ethanol, drug dependence, abstinence, ionotropic channels.

1 Introduction

Alcohol abuse is a chronic disease, and is considered a big and complex problem of public health (American Psychiatric Association, 2000). Abusive alcohol consumption and dependency causes 3.3 million of deaths each year in the world (World Health Organization, 2014). Dependency is characterized by anxiety, dysphoria, increased seizure susceptibility, hyperalgesia and sleep disturbances, observed during and following alcohol withdrawal (Koob e Volkow, 2010). Chronic use of alcohol can disrupt the delicate balance between γ-aminobutiric acid (GABA) and glutamate and induce neuroadaptations (Liang e Olsen, 2014). These neuroadaptations of GABAergic and glutamatergic systems are determinants to understand and treat the pathophysiology of alcohol dependency (Roberto e Varodayan, 2017).

Considerable evidence indicates that $GABA_A$ receptors ($GABA_ARs$) are an important target of alcohol in the central nervous system (Koob, 2004). The differential subunit composition of $GABA_ARs$ determines its ligand binding affinity, gating properties, and location (Jacob et al., 2008). Receptors containing the γ subunit are expressed within the synapse where they mediate phasic inhibition, while receptors containing α4 and δ subunits are largely extra- or non-synaptic (Roberto e Varodayan, 2017).

Disfunction of ionotropic glutamate NMDA (N-methil-D-aspartic acid) receptors (NMDARs), expressed as alteration of subunits expression, traffic, location and activity, can contribute to neurological conditions (Paoletti et al., 2013). Chronic alcohol use affects glutamatergic transmission by increasing presynaptic glutamate release and increasing NMDAR function postsynaptically, likely due an increase in receptor containing GluN2A and GluN2B subunits (Jin Chun e Woodward John J., 2006; Roberto e Varodayan, 2017).

Taurine, a sulfonated amino-acid highly abundant in the brain, has been shown to be involved in many important physiological functions, such as regulation of calcium binding

and transport, neuromodulation and neuroprotection against L-glutamate induced neurotoxicity (Wu e Prentice, 2010). Taurine decreases the calcium influx interacting directly with NMDARs by multiple mechanisms (Chan et al., 2013). Additionally, taurine acts as an agonist of $GABA_AR$ (Albrecht e Schousboe, 2005).

In the hippocampus important neuroadaptations induced by alcohol chronic consumption are described (Marszalek-Grabska et al., 2018; McClintick et al., 2018; Meda et al., 2018) . Moreover, hippocampus plays an important role in preoccupation/anticipation stage of addiction cycle (Koob e Volkow, 2010).

Therefore, in this work we investigated the effects of taurine in rats chronically treated or alcohol withdrawal in $GABA_AR$ and NMDAR subunits in the hippocampus.

2 Methods

2.1 Animals

Adult male Wistar rats (n = 72), body weight 250-280 g, born and reared in the animal facility of Universidade Federal do Rio Grande do Sul (UFRGS), Brazil were housed in polypropylene cages (3 rats/cage, 33 × 40 × 17.8 cm) under controlled environmental conditions (22 ± 2 °C, 12 h light/dark (lights on at 7 a.m.), 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 had been approved by the Ethics Committee for Animal Experimentation (CEUA-UFRGS #28722).

2.2 Experimental procedure

During 28 days, rats were treated for 5 consecutive days (Monday to Friday) with 2 g/kg alcohol (alcohol and withdrawal groups) or distilled water (control group) twice daily, at 9 a.m and 2 p.m., by oral gavage (n = 24), as described in our previous article (Hansen et al., 2017). Rats were weekly weighted for dose adjustment and control of weight gain. At day 29

to 33, rats from the withdrawal group received distillated water by gavage, instead of alcohol, and the alcohol and control groups received oral alcohol or water, respectively. Immediately after the gavage, all groups (n = 12/group) were administered with vehicle or taurine (100 mg/kg) via i.p. On day 34, 18 h after the last administration of alcohol/water and taurine/vehicle, animals were euthanized, and the hippocampus was dissected for analysis of $GABA_AR$ α_1, α_4, δ and γ_2 and NMDA GluN2A and GluN2B subunits mRNA expression by real-time quantitative PCR (qPCR).

2.3 Real-time quantitative PCR

Relative gene expression of $GABA_AR$ α_1, α_4, δ and γ_2 and NMDA GluN2A and GluN2B subunits mRNA in the hippocampus was determined using reverse transcription combined with real-time quantitative PCR (qPCR) and the $2^{-\Delta\Delta CT}$ method (Livak e Schmittgen, 2001). For the gene expression, 8 samples were used. Total RNA was extracted from the frontal cortex using the Trizol™ Isolation Reagent Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, and samples were stored at −80 °C. cDNA was synthesized using SuperScript III (Life Technologies, Carlsbad, CA, USA). DNA was quantified by using a BioSpec-nano™ (Shimadzu, Kyoto, Japan). qPCR analysis was performed at least in duplicate using the StepOnePlus Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). The set of primers was chosen from *Rattus norvegicus* data from the National Center for Biotechnology Information and it is listed in Table 1. The reaction contained 20 ng cDNA, 2× Power SYBR Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA), and the primers quantities were 0.32 μM of $GABA_AR$ and NMDA subunits, 0.16 μM of β-actin, 0.64 μM of GAPDH.

2.4 Statistical analysis

The data were tested for normal distribution using the Shapiro-Wilks test and were analyzed by a two-way ANOVA with the condition (non-alcohol × alcohol x alcohol

withdrawal) and treatments (vehicle x taurine) as factors. The results are presented as the mean ± standard error of mean (SEM). Pearson's correlation coefficient followed by Student's t-test was used to test the correlation significance. 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).

3 Results and discussion

$GABA_AR$ subunits

No differences between groups was detected by two-way ANOVA (Supplementary table S1; Fig. 1). To explore the relationship of subunits of $GABA_AR$ a correlational analysis was performed for all rats and the results indicated a remarkable correlation between some subunits. Rats from control group treated with vehicle (saline) did not present any correlation between studied subunits (Fig. 2). However, for those treated with taurine a strong correlation between α1 and γ2 was established (r = 0.765; P = 0.0451; Fig. 3C). $GABA_AR$ containing α1 and γ2 subunits are largely synaptically located and are benzodiazepine-sensitive (Jacob et al., 2008). Specifically, this response can be a neuroadaptation related to the partial agonism that taurine exert in the α1β3γ2 $GABA_AR$ (Dominguez-Perrot et al., 1996).

Rats chronically treated with alcohol demonstrated a strong correlation between α4 and δ subunits (r = 0.884; P = 0.0036; Fig. 4D). Alcohol acts as a positive allosteric modulator in $GABA_AR$. Though, the chronic exposition to this drug of abuse induces some neuroadaptations to balance the excess of inhibitory stimulus. Receptors containing α4 paired with δ subunit are largely extra or non-synaptic (Roberto e Varodayan, 2017) and they are internalized as an effect of alcohol-induced plasticity in $GABA_ARs$ (Jacob et al., 2008). Interestingly, in rats that received alcohol combined with taurine treatment, this correlation

between α4 and δ subunits disappear and prevails the effect of taurine, that is, the strong correlation between α1 and γ2($r = 0.858$; $P = 0.0288$; Fig. 5).

Five days of withdrawal seems to be enough to reestablish the pattern of no correlations between $GABA_AR$ subunits observed in control group (Fig. 6). But differently from control group, the withdrawal group when treated with taurine didn't showed the correlation between α1 and γ2 here attributed to taurine (Fig. 7).

NMDAR subunits

For NMDAR subunits no differences between the studied groups in two-way ANOVA were detected (Supplementary table S2; Fig. 8). However, in the control group there was a correlation between GluN2A and GluN2B subunits ($r = 0.764$; $P = 0.0272$; Fig. 9) that was lost by the treatment with taurine and by chronic alcohol and withdrawal conditions. From these results it is possible to infer that the treatment with taurine, alcohol or withdrawal induces a neuroadaptation of NMDARs containing GluN2B in a different way that those containing GluN2A. It is known that even in mature synapses the NMDAR subunits content changes depending on neuronal activity, but little is known about the regulation of subunit composition at mature synapses (Paoletti et al., 2013). The loss of correlation induced by alcohol treatment corroborates with an in vitro study of Carpenter-Hyland and colleagues indicating that GluN2B-containing receptors are most strongly affected by chronic alcohol exposure and acute withdrawal (Carpenter-Hyland et al., 2004).

Synaptic NMDARs are predominantly di-heteromeric GluN1/GluN2A and tri-heteromeric GluN1/GluN2A/GluN2B, while peri- and extra synaptic sites are enriched in GluN2B containing receptors (Paoletti et al., 2013). Extra synaptic GluN1/GluN2B receptors constitute a major hub for signaling pathways that lead to neuronal death (Hardingham e Bading, 2010).

Correlation between $GABA_AR$ and NMDAR subunits

Control group showed a correlation between δ GABAAR subunit and GluN2A (r = 0.837; P = 0.0096; Fig. 10E) and GluN2B (r = 0.918; P = 0.0013; Fig. 10F) NMDAR subunits. Rats treated with taurine showed this same pattern of correlations (Fig. 11). A distinct effect is observed after chronic use of alcohol, the correlation between δ GABAAR and NMDAR subunits is completely lost, and a correlation between α4 and GluN2A is established (r = 0.853; P = 0.0307; Fig. 12A). When alcohol treated rats receive taurine its also possible to observe the correlation caused by alcohol between α4 and GluN2A (r = 0.844; P = 0.0345; Fig. 13D), besides another correlation between α1 and GluN2A (r = 0.910; P = 0.0118; Fig. 13A). After five days of withdrawal, one correlation, between δ and GluN2A, observed in control group is reestablished (r = 0.807; P = 0.0284; Fig. 14E), but not that between δ and GluN2B. Curiously, when treated with taurine, withdrawal rats lost both correlations (Fig. 15).

NMDAR-dependent plasticity has been convincingly documented in some types of interneurons (Moreau e Kullmann, 2013). NMDAR subunits GluN1, GluN2A, as well as GluN2B are located in most somatic GABAergic synapses postsynaptically, although at a lower density than in excitatory synapses (Szabadits et al., 2011). There is also evidence that NMDARs are expressed presynaptically where they can modulate synapses function and plasticity in GABAergic interneurons (Moreau e Kullmann, 2013). From the results of this study (Fig. 16) it is possible to infer that taurine alone does not induces neuroadaptation capable to interfere in $GABA_AR$ and NMDAR, formed by studied subunits, normal balance. In the other hand, alcohol chronic treatment affects, more significantly, the α4-containing GABAARs and the GluN2A-containing NMDARs. When alcohol and taurine are combined, besides the alcohol effect, there is an effect in α1-containing $GABA_ARs$. α1 subunits are known to form receptors primarily synaptic localized and benzodiazepine-sensitive (Jacob et al., 2008). Five days of withdrawal seems to be enough to restore the balance between δ-

containing GABAARs and GluN2A, but not GluN2B, containing NMDARs. In those withdrawal rats, treated with taurine none of these correlations occurs, reinforcing the observation that these animals, even after 5 days of withdrawal, are still different from the control ones. Results from our previous work showed that taurine decreases the α2 GABAAR subunit only in the frontal cortex of control rats, with no effects in 5 days withdrawal rats. Moreover, withdrawal rats showed a decrease in exploratory behavior (rearing) in the open field test which was restored by taurine treatment, while in control rats taurine decreased this behavior (Hansen et al., 2017). These evidences reinforce that even though in our protocol of 5 days of withdrawal we lose the effects of acute 24-48h withdrawal, there are many important neuroadaptations remaining. A better comprehension of all stages of withdrawal is central to elucidate the mechanisms involved in neurocircuitry of addiction.

Taken together, the results obtained by this work could help to define new strategies to counteract the deleterious effects resulting from NMDAR and $GABA_AR$ deregulated function induced by alcohol dependency.

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Table 1 Set of primers used for real-time quantitative PCR

Primer set	Sequence (5' to 3')	Product size (bp)
β-actina	For - TATGCCAACACAGTGCTGTCTGG	206
	Rev - TACACCTAGTCGTTCGTCCTCAT	
GAPDH	For - AACGACCCCTTCATTGACCTC	85
	Rev - CCTTGACTGTGCCGTTGAACT	
α_1	For - AAGGACCCATGACAGTGCTC	292
	Rev - GGCTCCCTTGTCCACTCATA	
α_4	For - GGTGAAAACTGATATATATGTCAC	331
	Rev - ACCGTCCATAGGAAAATCCACC	
δ	For - GCCATGTCCTGGGTCTCCTT	100
	Rev - TAACCATGAGTGTGGTCATTGTCA	
γ_2	For - TTTGTGAGCAACCGGAAACC	100
	Rev - TCATTTGGATCGTTGCTGATCT	
GluN2A	For - TCCATTCTTCTGTCATCCTGC	224
	Rev - AAGACCGTCTCTCACTCTTGC	
GluN2B	For - TGCACAATTACTCCTCGACG	222
	Rev - TCCGATTCTTCTTCTGAGCC	

Legends of figures

Figure 1. Effects of taurine (100 mg/kg) on the expression of α1, α4, δ and γ2 $GABA_AR$ subunits mRNA expression in the hippocampus; chronically alcohol (2 g/kg, vo) treated rats, or 5 days alcohol withdrawal rats. Two-way ANOVA; n = 6-8/group.

Figure 2. Linear regression between α1, α4, δ and γ2 GABAAR subunits mRNA expression in the hippocampus of control rats treated with saline (vehicle). Pearson's correlation coefficient followed by Student's t-test was used to test the correlation significance ($P < 0.05$).

Figure 3. Linear regression between α1, α4, δ and γ2 GABAAR subunits mRNA expression in the hippocampus of control rats treated with taurine (100 mg/kg). Pearson's correlation coefficient followed by Student's t-test was used to test the correlation significance ($P < 0.05$).

Figure 4. Linear regression between α1, α4, δ and γ2 GABAAR subunits mRNA expression in the hippocampus of rats chronically treated with alcohol (4 g/kg/day) and saline (vehicle). Pearson's correlation coefficient followed by Student's t-test was used to test the correlation significance ($P < 0.05$).

Figure 5. Linear regression between α1, α4, δ and γ2 GABAAR subunits mRNA expression in the hippocampus of rats chronically treated with alcohol (4 g/kg/day) and taurine (100 mg/kg). Pearson's correlation coefficient followed by Student's t-test was used to test the correlation significance ($P < 0.05$).

Figure 6. Linear regression between α1, α4, δ and γ2 GABAAR subunits mRNA expression in the hippocampus of alcohol withdrawal rats (5 days) treated with saline (vehicle). Pearson's correlation coefficient followed by Student's t-test was used to test the correlation significance ($P < 0.05$).

Figure 7. Linear regression between α1, α4, δ and γ2 GABAAR subunits mRNA expression in the hippocampus of alcohol withdrawal rats (5 days) treated with taurine (100 mg/kg). Pearson's correlation coefficient followed by Student's t-test was used to test the correlation significance ($P < 0.05$).

Figure 8. Effects of taurine (100 mg/kg) on the expression of GluN2A and GluN2B NMDAR subunits mRNA expression in the hippocampus; chronically alcohol (2 g/kg, vo) treated rats, or 5 days alcohol withdrawal rats. Two-way ANOVA; n = 6-8/group.

Figure 9. Linear regression between GluN2A and GluN2B NMDAR subunits mRNA expression in the hippocampus of control rats treated with saline (A) or 100 mg/kg taurine (B); chronic alcohol rats treated with saline (C) or 100 mg/kg taurine (D); 5 days withdrawal rats treated with saline (E) or 100 mg/kg taurine (F). Pearson's correlation coefficient followed by Student's t-test was used to test the correlation significance ($P < 0.05$).

Figure 10. Linear regression between α1, α4, δ and γ2 GABAAR and GluN2A and GluN2B NMDAR subunits mRNA expression in the hippocampus of control rats treated with saline (vehicle). Pearson's correlation coefficient followed by Student's t-test was used to test the correlation significance ($P < 0.05$).

Figure 11. Linear regression between α1, α4, δ and γ2 GABAAR and GluN2A and GluN2B NMDAR subunits mRNA expression in the hippocampus of control rats treated with taurine (100 mg/kg). Pearson's correlation coefficient followed by Student's t-test was used to test the correlation significance ($P < 0.05$).

Figure 12. Linear regression between α1, α4, δ and γ2 GABAAR and GluN2A and GluN2B NMDAR subunits mRNA expression in the hippocampus of rats chronically treated with alcohol (4 g/kg/day) and saline (vehicle). Pearson's correlation coefficient followed by Student's t-test was used to test the correlation significance ($P < 0.05$).

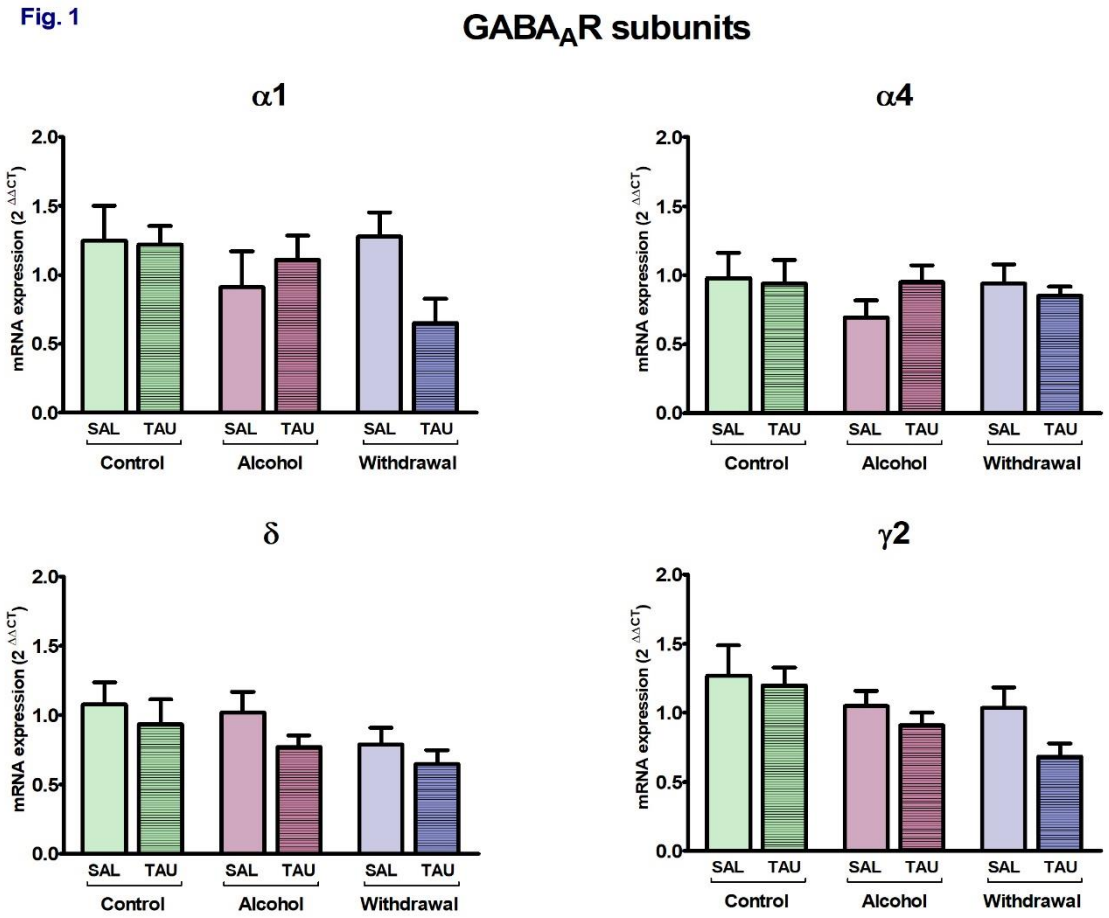
Figure 13. Linear regression between α1, α4, δ and γ2 GABAAR and GluN2A and GluN2B NMDAR subunits mRNA expression in the hippocampus of rats chronically treated with alcohol (4 g/kg/day) and taurine (100 mg/kg). Pearson's correlation coefficient followed by Student's t-test was used to test the correlation significance ($P < 0.05$).

Figure 14. Linear regression between α1, α4, δ and γ2 GABAAR and GluN2A and GluN2B NMDAR subunits mRNA expression in the hippocampus of alcohol withdrawal rats (5 days) treated with saline (vehicle). Pearson's correlation coefficient followed by Student's t-test was used to test the correlation significance ($P < 0.05$).

Figure 15. Linear regression between α1, α4, δ and γ2 GABAAR and GluN2A and GluN2B NMDAR subunits mRNA expression in the hippocampus of alcohol withdrawal rats (5 days) treated with taurine (100 mg/kg). Pearson's correlation coefficient followed by Student's t-test was used to test the correlation significance ($P < 0.05$).

Figure 16. Summary of the results of the effects of taurine (100 mg/kg/day), chronic alcohol (4 g/kg/day) treatment or withdrawal (5 days) in hippocampus of rats. Colorful connections represent a correlation between subunits. Correlations between $GABA_AR$ subunits are presented in the superior line of each group. Correlations between NMDAR subunits are presented in the inferior line of each group. Correlations between $GABA_A$ and NMDA receptors subunits are represented by a diagonal connection.

Fig. 1


GABA$_A$R subunits
α1
α4
δ
γ2
mRNA expression (2 ΔΔCT)
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0.5
1.0
1.5
2.0
SAL
TAU
Control
Alcohol
Withdrawal

-

Fig. 2

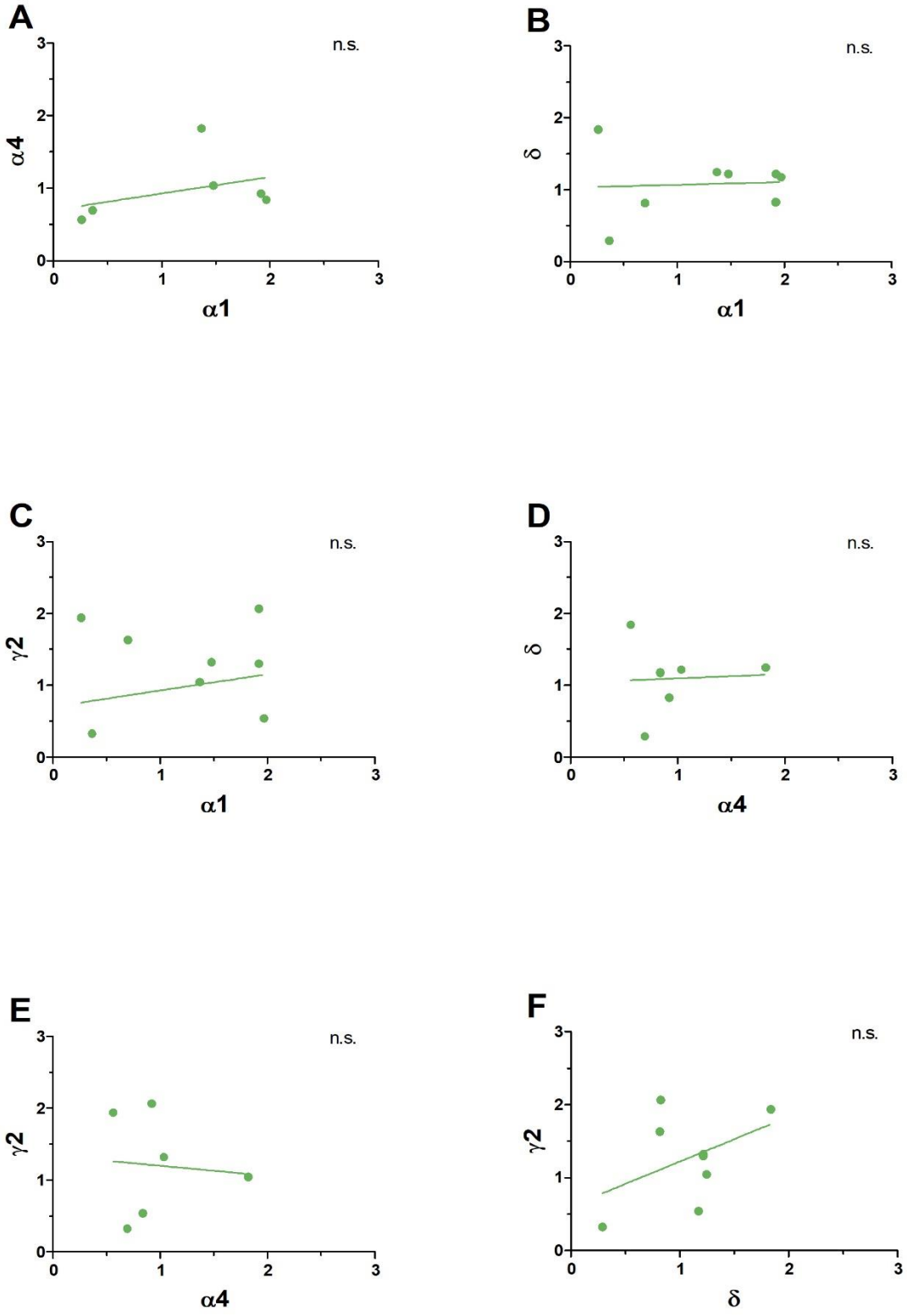


Fig. 3

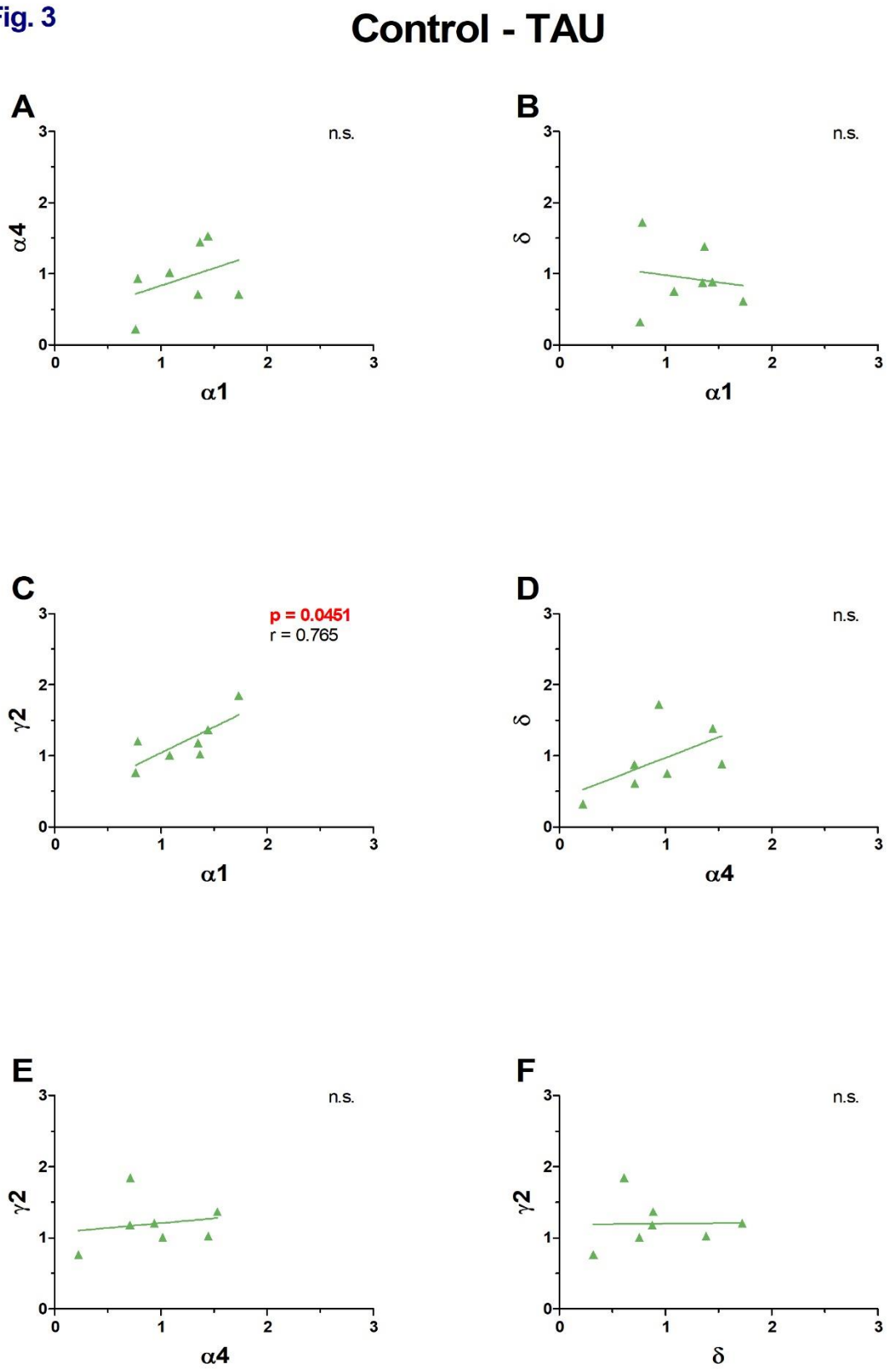
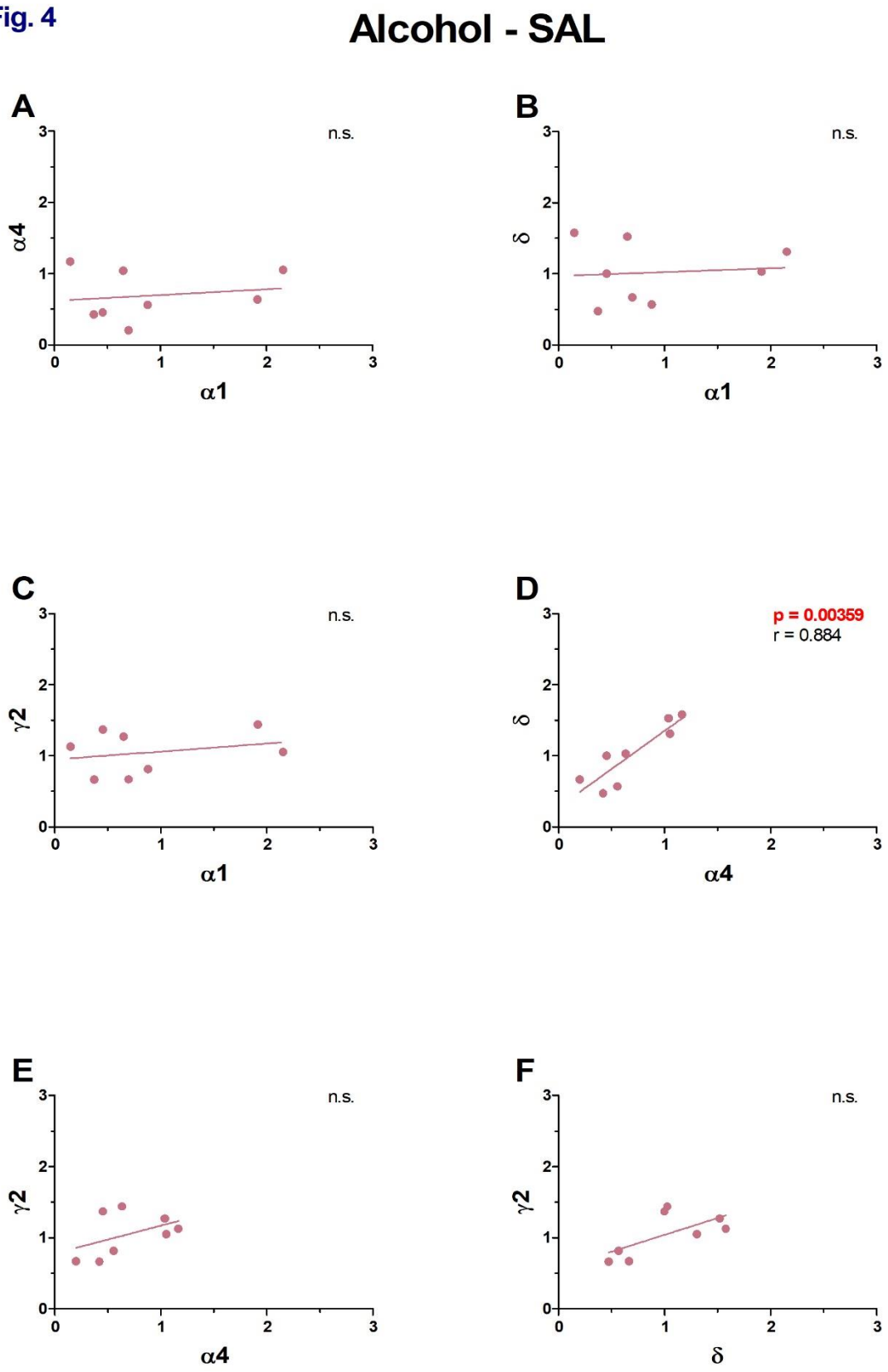

Control - TAU
A
n.s.
α4
α1
B
n.s.
δ
α1
C
p = 0.0451
r = 0.765
γ2
α1
D
n.s.
δ
α4
E
n.s.
γ2
α4
F
n.s.
γ2
δ

Fig. 4


Alcohol - SAL
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n.s.
α4
α1
B
n.s.
δ
α1
C
n.s.
γ2
α1
D
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r = 0.884
δ
α4
E
n.s.
γ2
α4
F
n.s.
γ2
δ

Fig. 5

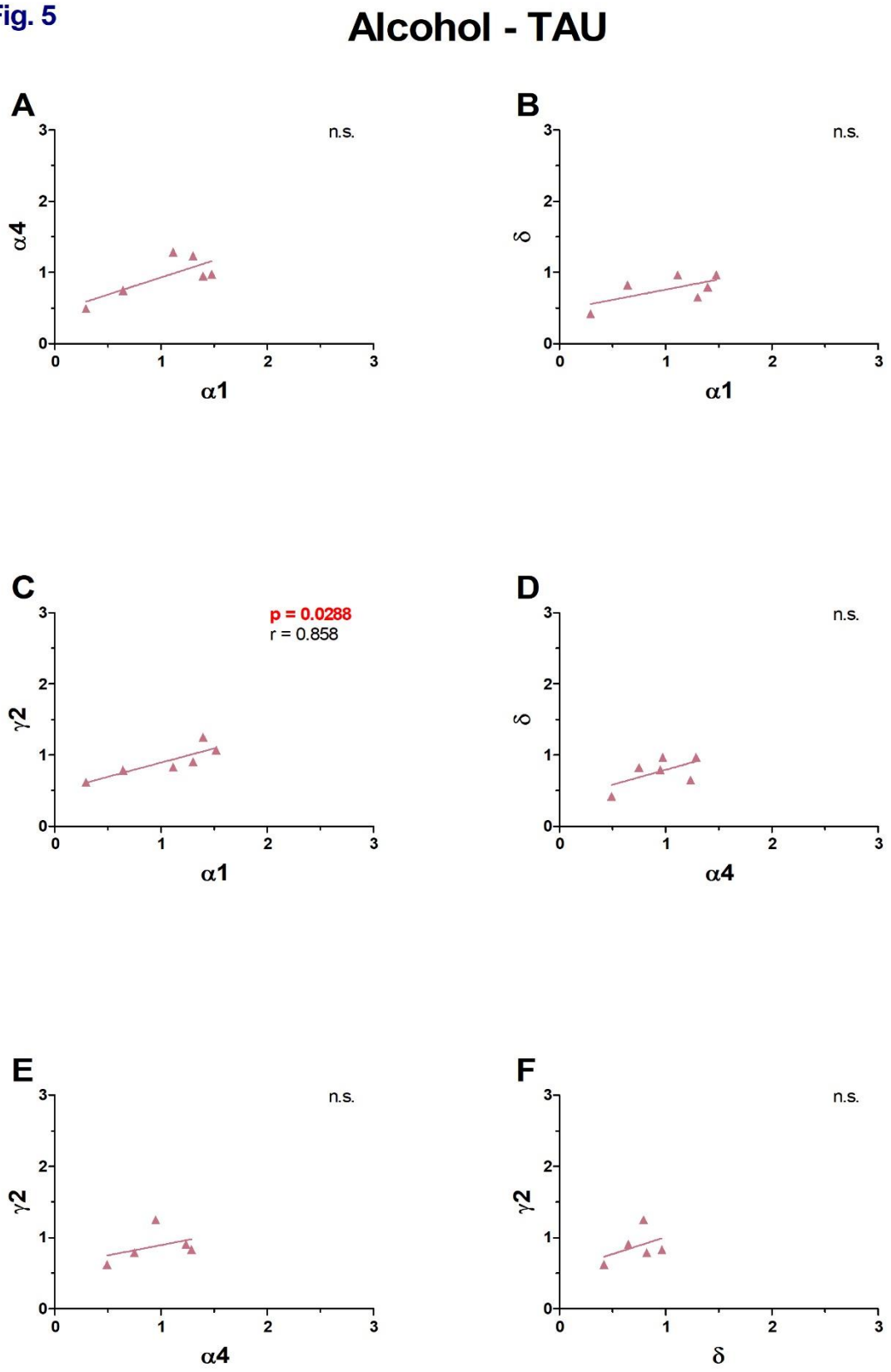

Alcohol - TAU
A
n.s.
α4
α1
B
n.s.
δ
α1
C
p = 0.0288
r = 0.858
γ2
α1
D
n.s.
δ
α4
E
n.s.
γ2
α4
F
n.s.
γ2
δ

Fig. 6

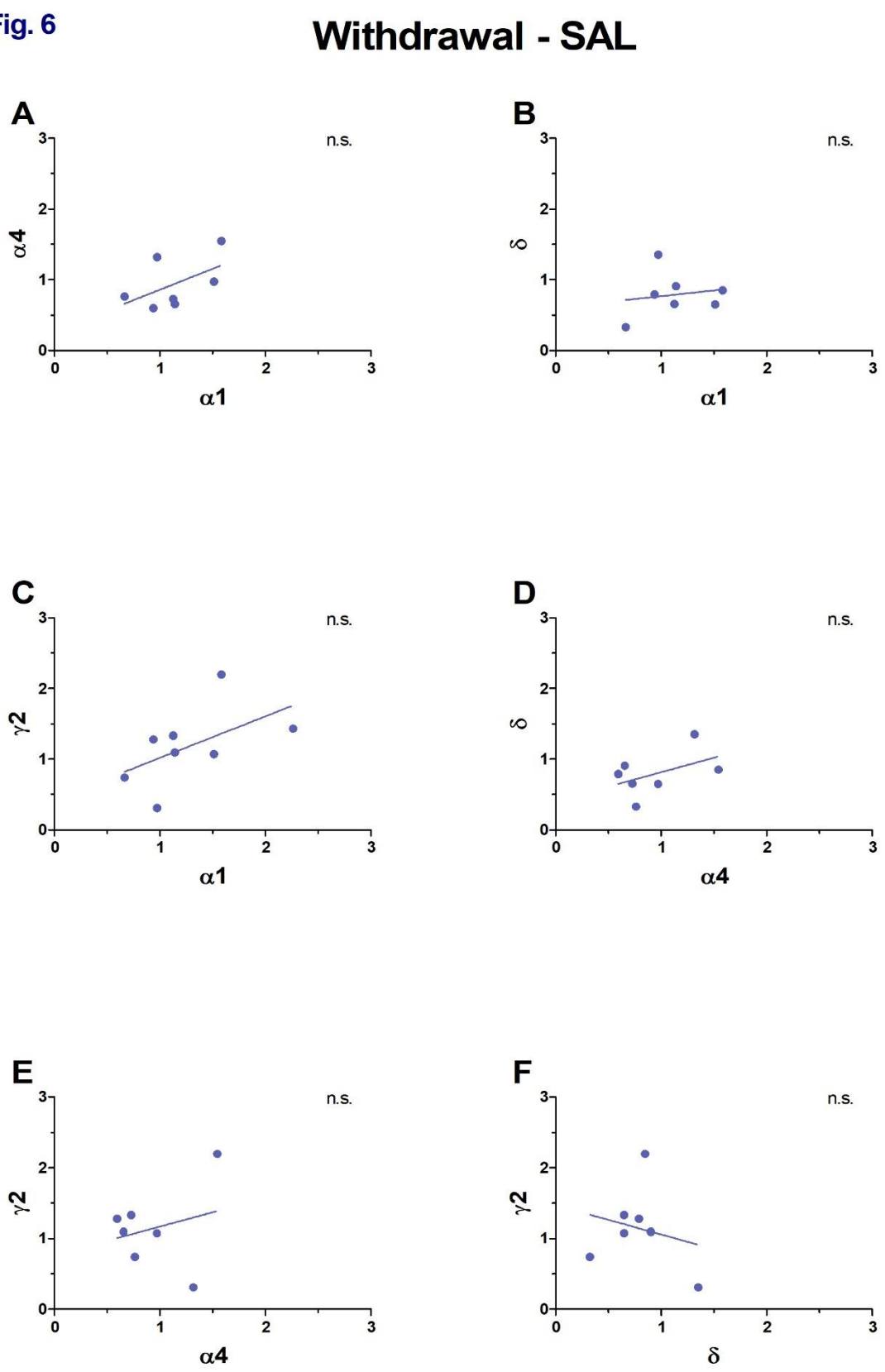

Withdrawal - SAL
A
n.s.
α4
α1
B
n.s.
δ
α1
C
n.s.
γ2
α1
D
n.s.
δ
α4
E
n.s.
γ2
α4
F
n.s.
γ2
δ

Fig. 7

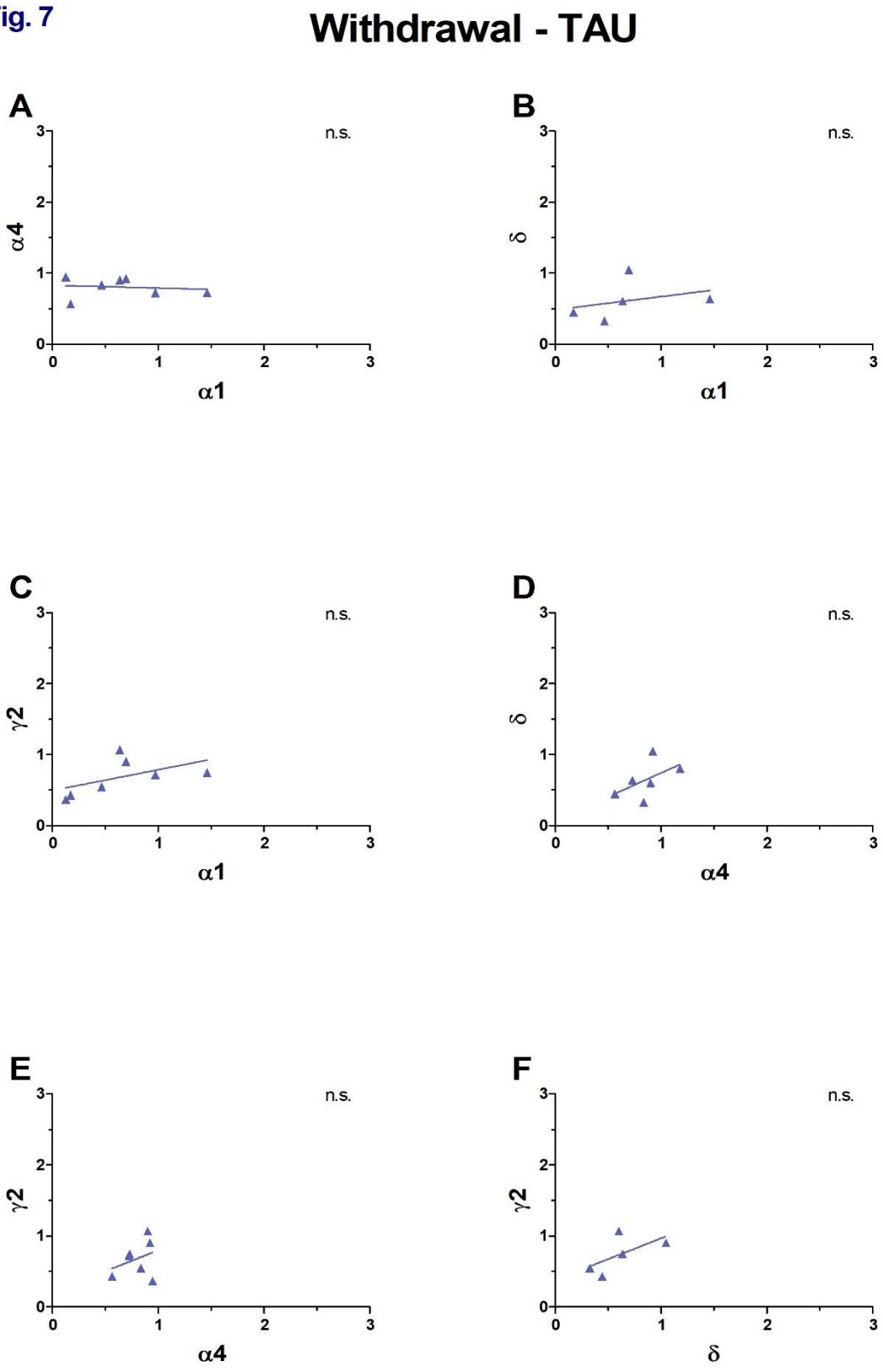

Withdrawal - TAU
A
n.s.
α4
α1
B
n.s.
δ
α1
C
n.s.
γ2
α1
D
n.s.
δ
α4
E
n.s.
γ2
α4
F
n.s.
γ2
δ

Fig. 8

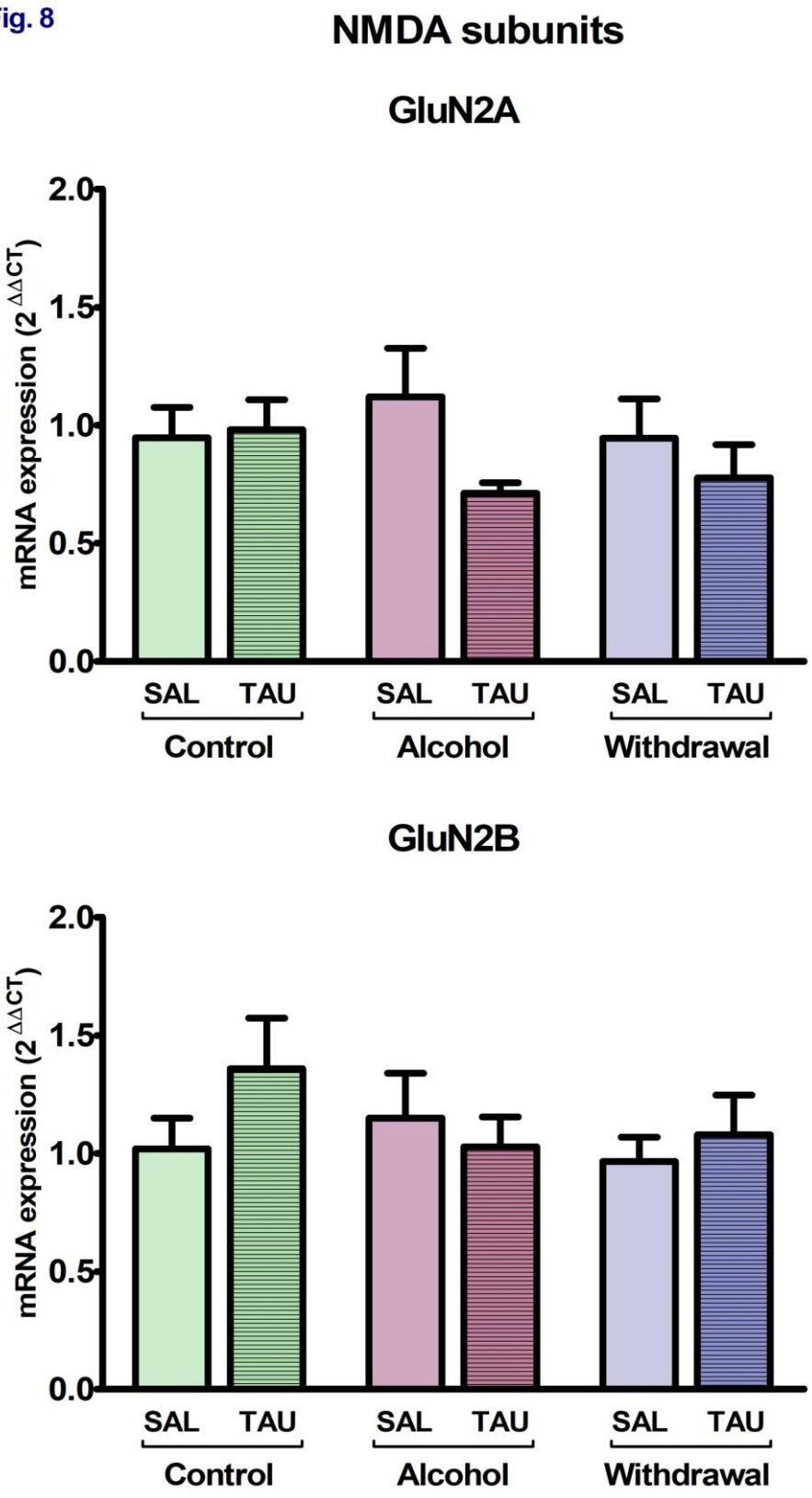

NMDA subunits
GluN2A
mRNA expression (2 ΔΔCT)
2.0
1.5
1.0
0.5
0.0
SAL
TAU
SAL
TAU
SAL
TAU
Control
Alcohol
Withdrawal
GluN2B
mRNA expression (2 ΔΔCT)
2.0
1.5
1.0
0.5
0.0
SAL
TAU
SAL
TAU
SAL
TAU
Control
Alcohol
Withdrawal

Fig. 9

NMDA subunits correlation

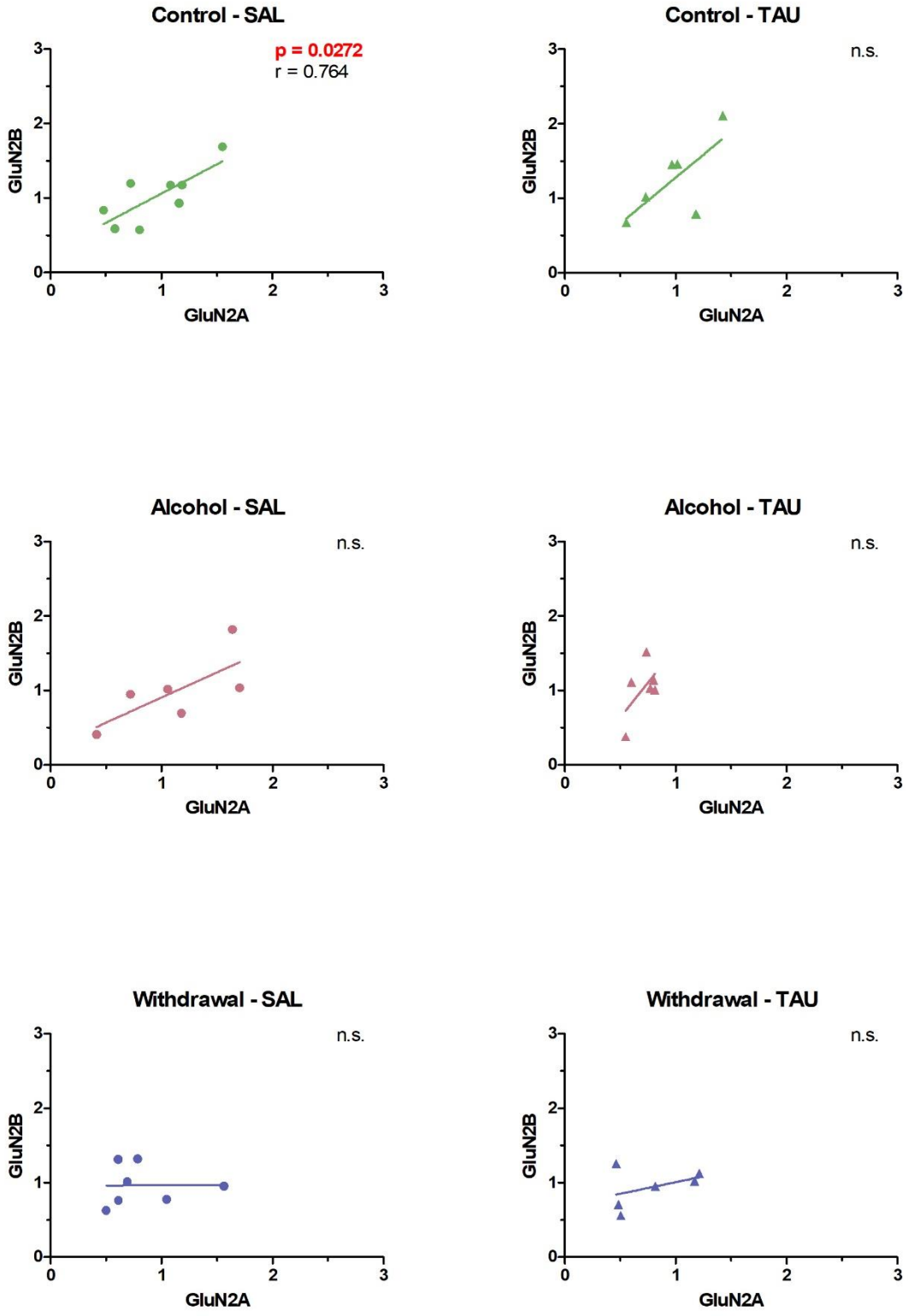


Fig. 10

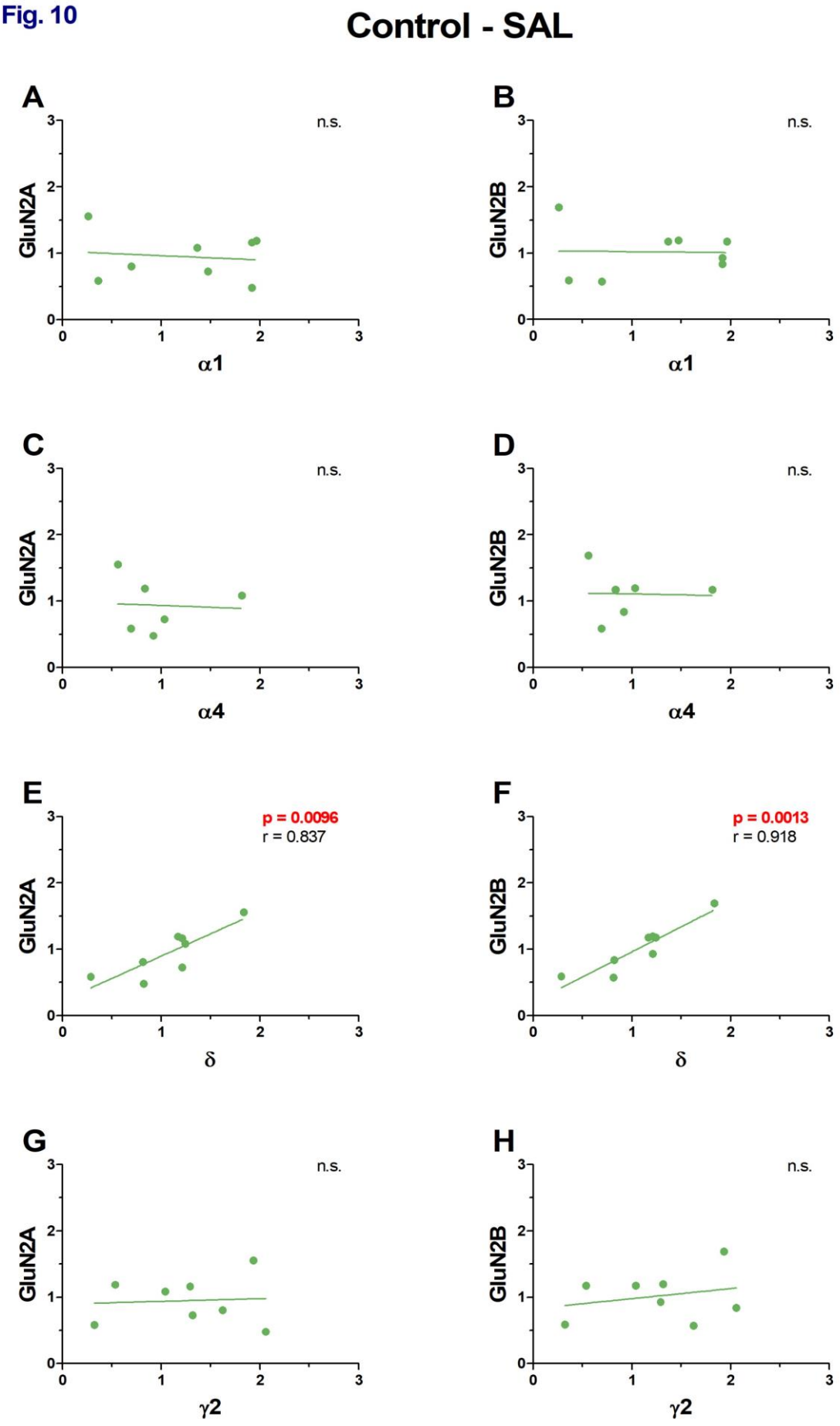

Control - SAL
A
n.s.
GluN2A
α1
B
n.s.
GluN2B
α1
C
n.s.
GluN2A
α4
D
n.s.
GluN2B
α4
E
p = 0.0096
r = 0.837
GluN2A
δ
F
p = 0.0013
r = 0.918
GluN2B
δ
G
n.s.
GluN2A
γ2
H
n.s.
GluN2B
γ2

Fig. 11

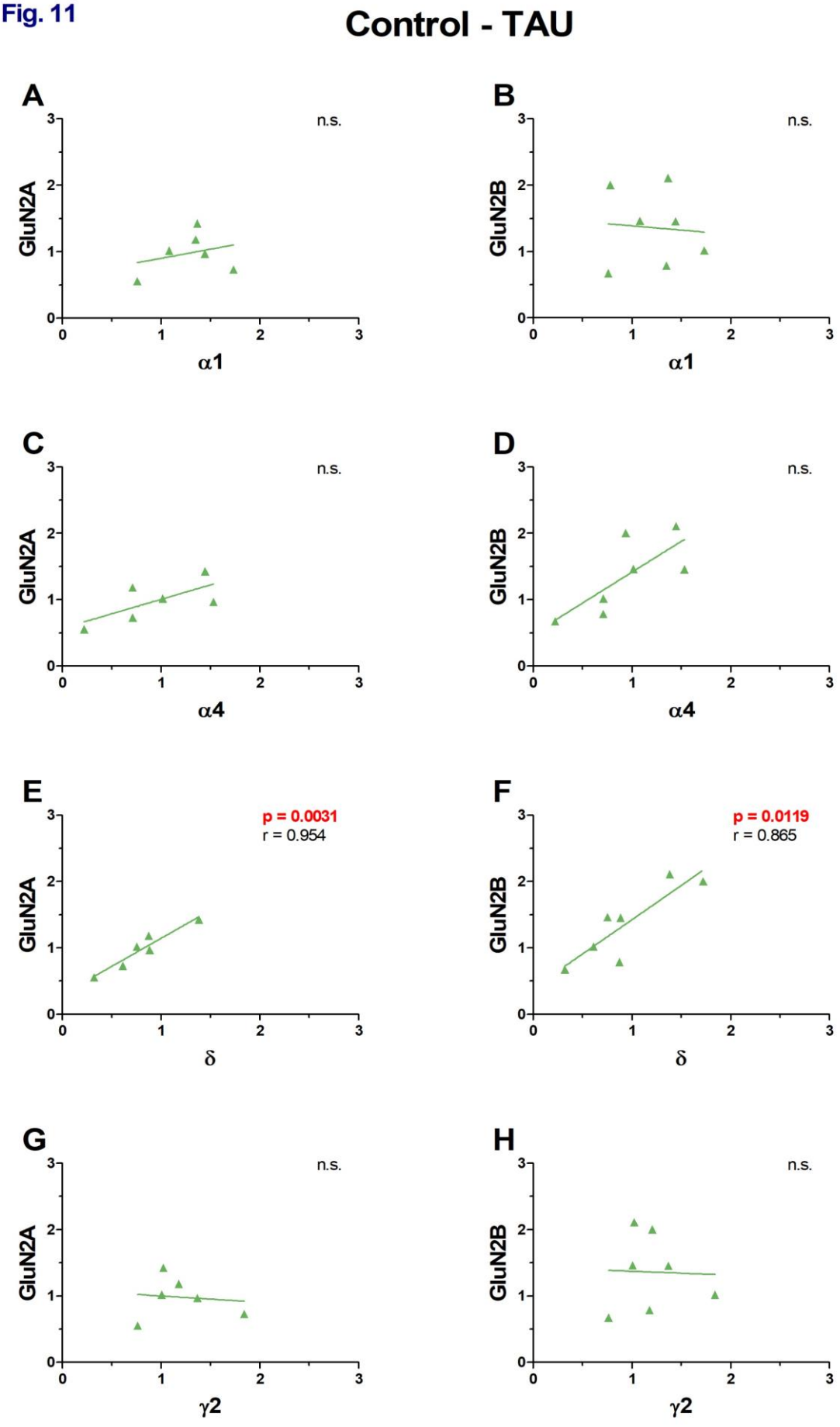

Control - TAU
A
n.s.
GluN2A
α1
B
n.s.
GluN2B
α1
C
n.s.
GluN2A
α4
D
n.s.
GluN2B
α4
E
p = 0.0031
r = 0.954
GluN2A
δ
F
p = 0.0119
r = 0.865
GluN2B
δ
G
n.s.
GluN2A
γ2
H
n.s.
GluN2B
γ2

Fig. 12

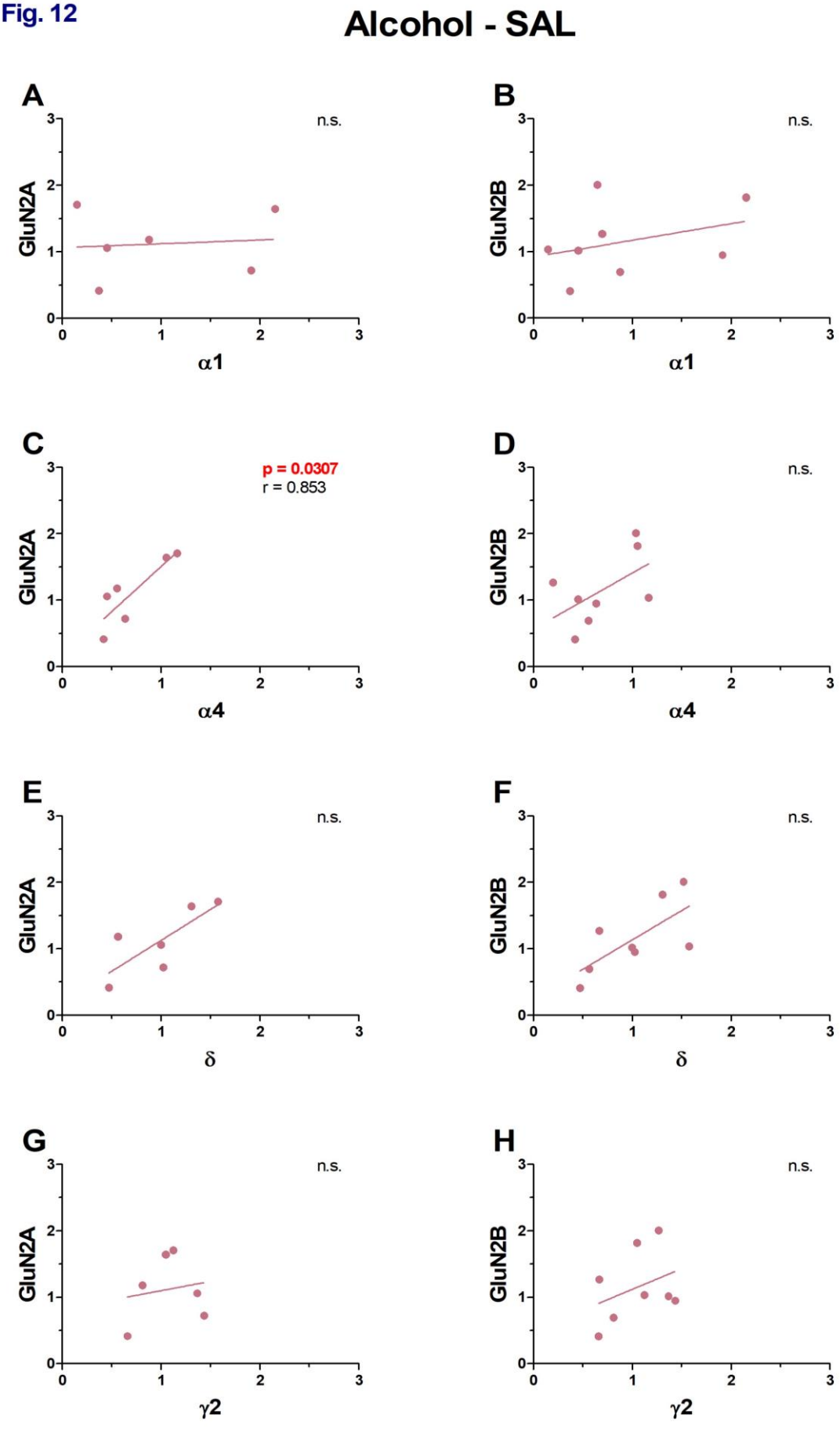

Alcohol - SAL
A
n.s.
GluN2A
α1
B
n.s.
GluN2B
α1
C
p = 0.0307
r = 0.853
GluN2A
α4
D
n.s.
GluN2B
α4
E
n.s.
GluN2A
δ
F
n.s.
GluN2B
δ
G
n.s.
GluN2A
γ2
H
n.s.
GluN2B
γ2

Fig. 13

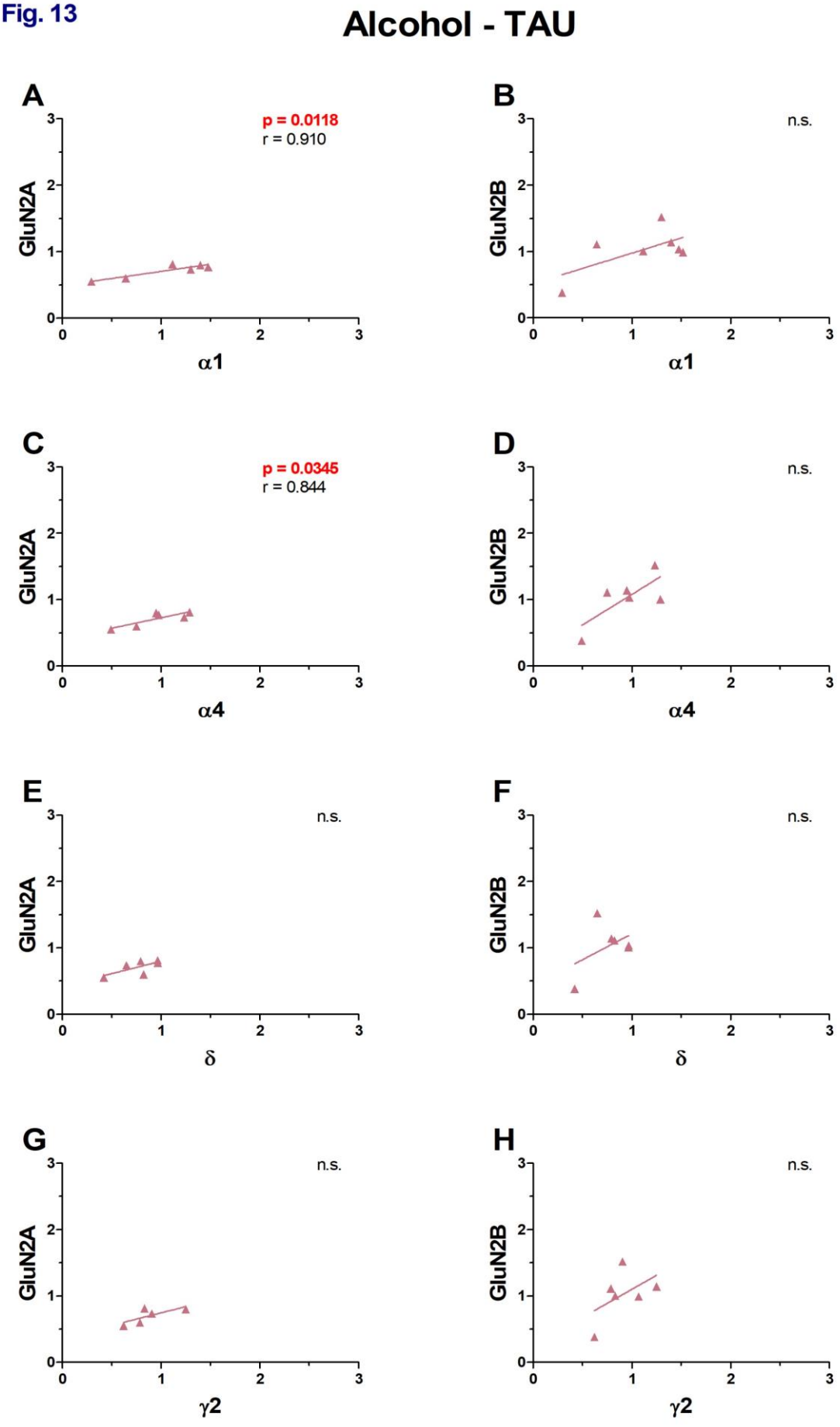

Alcohol - TAU
A
p = 0.0118
r = 0.910
GluN2A
α1
B
n.s.
GluN2B
α1
C
p = 0.0345
r = 0.844
GluN2A
α4
D
n.s.
GluN2B
α4
E
n.s.
GluN2A
δ
F
n.s.
GluN2B
δ
G
n.s.
GluN2A
γ2
H
n.s.
GluN2B
γ2

Fig. 14

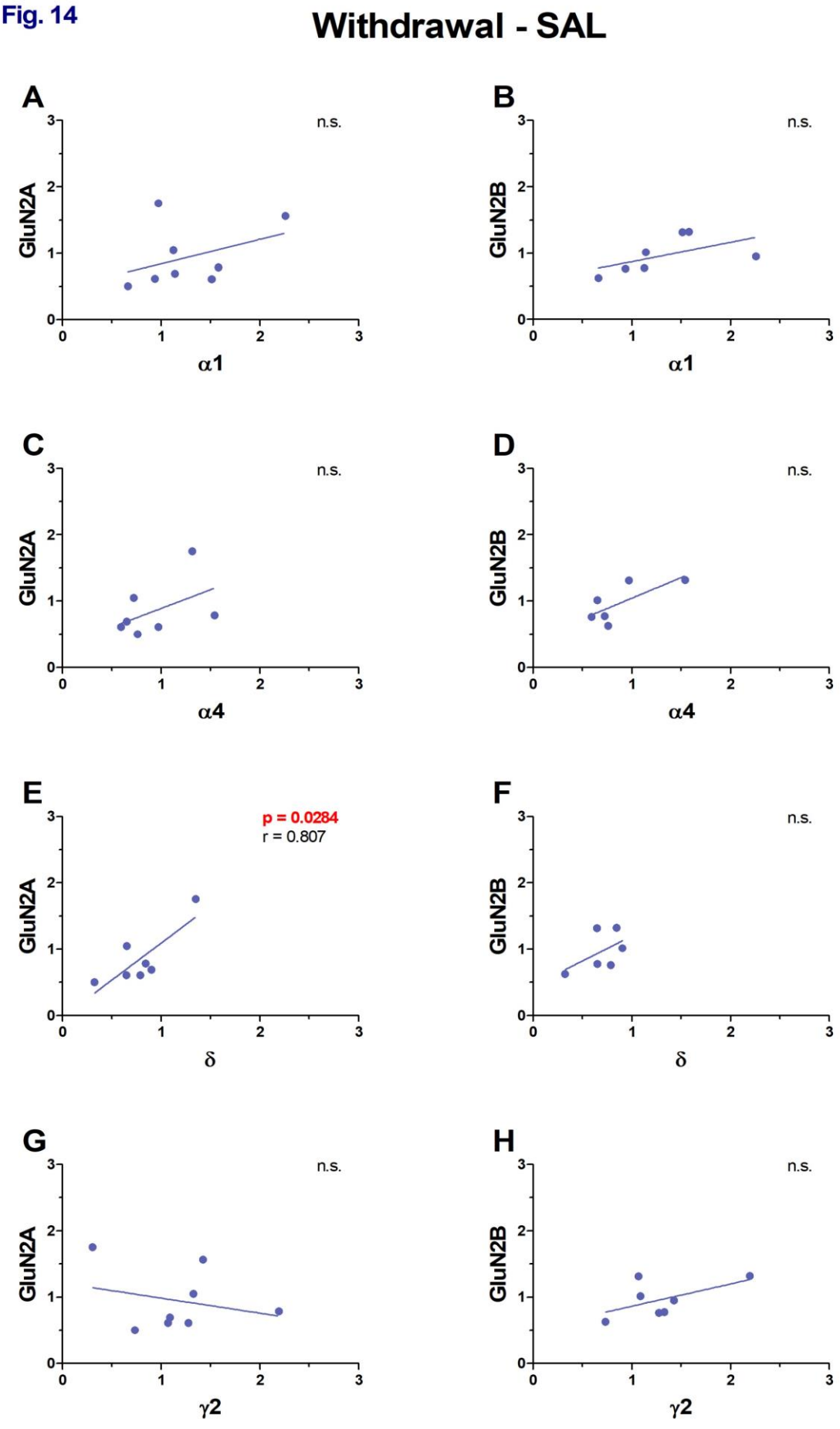

Withdrawal - SAL
A
n.s.
GluN2A
α1
B
n.s.
GluN2B
α1
C
n.s.
GluN2A
α4
D
n.s.
GluN2B
α4
E
p = 0.0284
r = 0.807
GluN2A
δ
F
n.s.
GluN2B
δ
G
n.s.
GluN2A
γ2
H
n.s.
GluN2B
γ2

Fig. 15

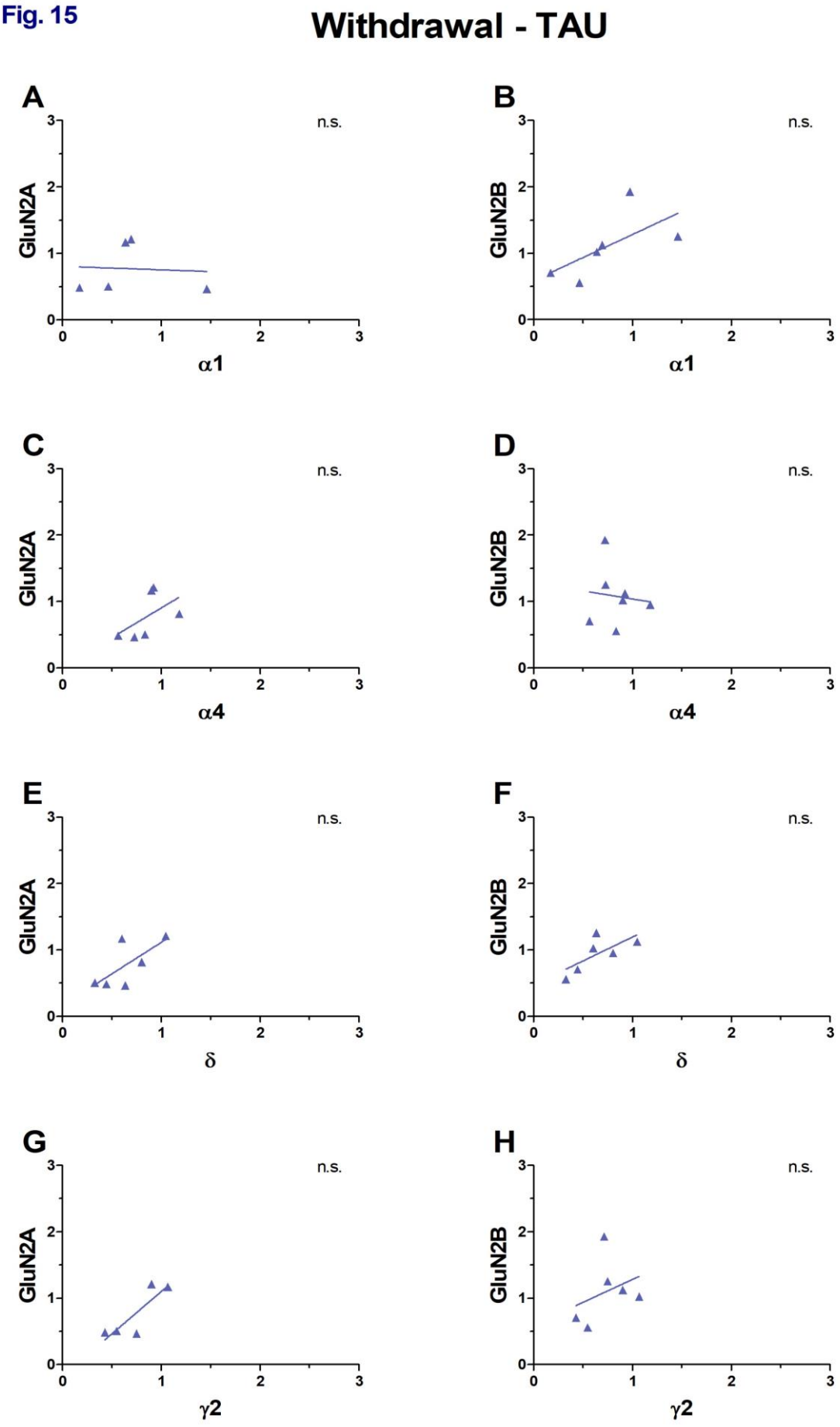
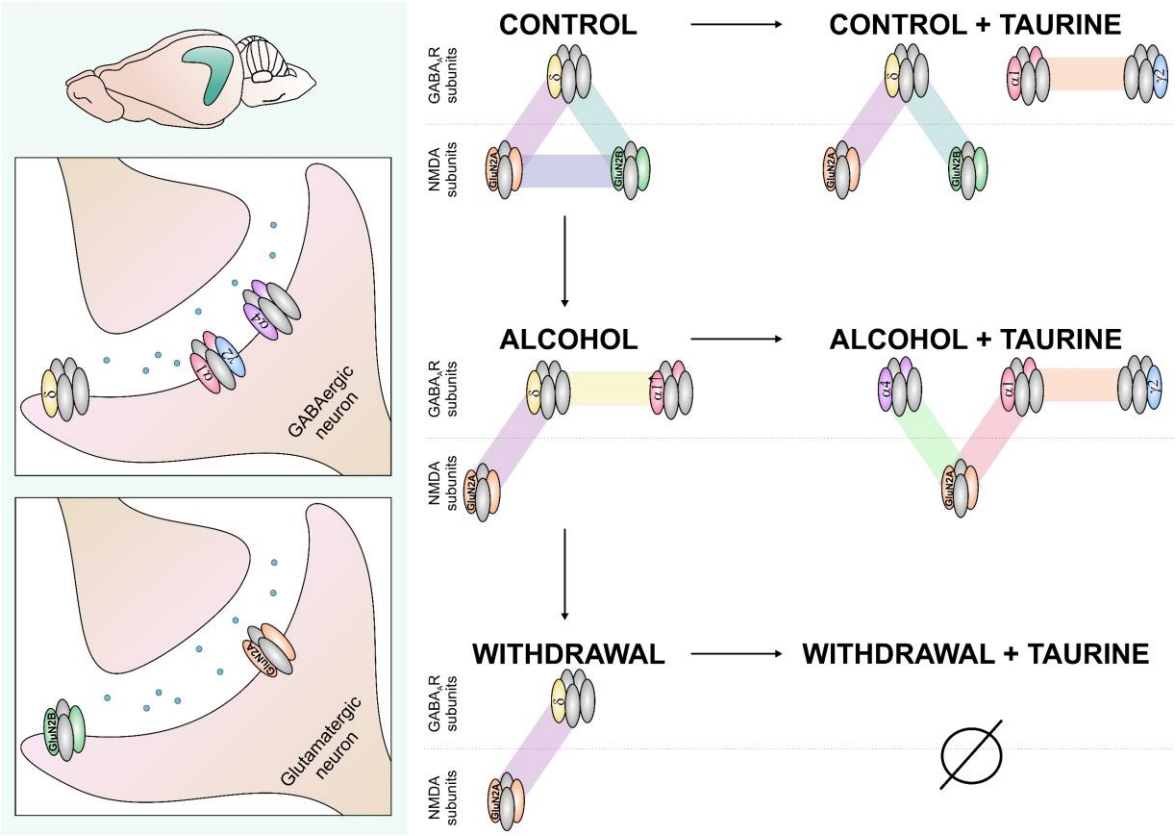

Withdrawal - TAU
A
n.s.
GluN2A
α1
B
n.s.
GluN2B
α1
C
n.s.
GluN2A
α4
D
n.s.
GluN2B
α4
E
n.s.
GluN2A
δ
F
n.s.
GluN2B
δ
G
n.s.
GluN2A
γ2
H
n.s.
GluN2B
γ2

Fig. 16


CONTROL
CONTROL + TAURINE
GABA$_A$R subunits
NMDA subunits
ALCOHOL
ALCOHOL + TAURINE
WITHDRAWAL
WITHDRAWAL + TAURINE
GABAergic neuron
Glutamatergic neuron

Supplementary information

Table S1. Results of Analysis of Variance (ANOVA-two way) from $GABA_AR$ α1, α4, δ and γ2 subunits in the hippocampus of rats. The table summarizes the main effects of and the interaction between condition (control x alcohol x withdrawal) and treatment (saline x taurine).

Subunit	Treatment	Degrees of freedom	F-value	P-value
α1	Condition	2,44	0.985	0.383
	Treatment	1,44	0.837	0.366
	Interaction	2,44	2.130	0.132
α4	Condition	2,41	0.516	0.601
	Treatment	1,41	0.149	0.701
	Interaction	2,41	0.960	0.392
δ	Condition	2,41	2.052	0.143
	Treatment	1,41	2.294	0.139
	Interaction	2,41	0.089	0.915
γ2	Condition	2,43	2.191	0.126
	Treatment	1,43	3.368	0.074
	Interaction	2,43	1.096	0.345

Table S2. Results of Analysis of Variance (ANOVA-two way) from NDMA GluN2A and GluN2B subunits in the hippocampus of rats. The table summarizes the main effects of and the interaction between condition (control x alcohol x withdrawal) and treatment (saline x taurine).

Subunit	Treatment	Degrees of freedom	F-value	P-value
GluN2A	Condition	2,39	0.254	0.777
	Treatment	1,39	2.233	0.144
	Interaction	2,39	1.078	0.352
GluN2B	Condition	2,43	0.541	0.587
	Treatment	1,43	0.707	0.406
	Interaction	2,43	1.063	0.356

Differences between degrees of freedom are due outliers and practical execution errors.

5 CONCLUSÕES

O desequilíbrio entre os sistemas GABAérgico e glutamatérgico decorrente do uso crônico de álcool é responsável por vários sintomas observados durante a abstinência ao uso da droga. Os sintomas negativos da síndrome de abstinência contribuem fortemente para a recaída e a manutenção do ciclo da adição.

O protocolo de tratamento crônico e abstinência ao álcool por gavagem oral utilizado neste estudo apresenta como vantagem o controle da alcoolemia dos animais. Considerando que se tinha como objetivo verificar a expressão de subunidades de $GABA_AR$ e NMDAR, o conhecimento da exata ingestão de álcool era muito relevante.

Os resultados dos estudos aqui apresentados mostram que mesmo após 5 dias de abstinência, os ratos cronicamente tratados com álcool ainda apresentam importantes diferenças comportamentais e de resposta ao tratamento com taurina. Essas evidências reforçam que a neuroplasticidade induzida pelo uso de álcool é duradoura, e a elucidação das diferenças existentes nos sistemas GABA e glutamato desses indivíduos é essencial para o desenvolvimento de novos fármacos para o tratamento da adição.

Os resultados de expressão de RNAm de subunidades tanto de $GABA_AR$ quanto de NMDAR não tiveram diferença estatística significativa e isto provavelmente pode ser atribuído aos valores de baixa grandeza, e a sutileza na diferença entre eles. Visto que o aumento do n amostral possivelmente não solucionaria esta questão de forma eficaz, a associação com a medida da expressão das proteínas relacionados poderia ser uma opção.

O estudo de correlação das subunidades mostrou ser capaz de detectar de maneira mais eficaz a neuroadaptação causada pelo álcool e o efeito da taurina. Essas informações inspiram o desenvolvimento de novos protocolos *in vitro* que possam avaliar essa dinâmica na plasticidade que relaciona de maneira direta os receptores GABAérgicos e glutamatérgicos.

Nossa hipótese de trabalho foi parcialmente refutada, visto que a taurina parecia ser uma forte candidata ao tratamento da dependência ao álcool devido a sua capacidade de interagir tanto com o sistema GABAérgico quanto com o

glutamatérgico. Porém observou-se que, apesar de apresentar um efeito ansiolítico, restaurando o comportamento exploratório de animais abstinentes, a taurina não foi capaz de reestabelecer o padrão de correlações entre as subunidades dos receptores $GABA_A$ e NMDA observadas nos animais controle. Propõe-se então que a taurina possa inspirar o desenvolvimento de novos fármacos, que apresentem um efeito de maior eficácia para o tratamento da dependência ao álcool.

6 PERSPECTIVAS

Considerando que a resposta ao álcool é região específica, propõe-se analisar as subunidades α1, α4, δ e γ do receptor $GABA_A$ e GluN2A e GluN2B do receptor NDMA de glutamato no córtex frontal e estriado, áreas encefálicas também envolvidas com o ciclo da adição. Além de avaliar o efeito da taurina nas subunidades dos receptores desses animais.

Outra perspectiva para a continuação deste trabalho é estudar a correlação das subunidades de $GABA_A$ e NMDA com os comportamentos apresentados pelos animais no campo aberto. Esta avaliação poderia colaborar com a elucidação da influência dos diferentes subtipos de receptores na resposta comportamental da abstinência.

Adicionalmente, seria de grande interesse avaliar, após submetidos ao mesmo protocolo de exposição crônica ao álcool, a expressão das subunidades de $GABA_A$ e NMDA temporalmente durante a abstinência.

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ANEXO A – TRABALHOS DESENVOLVIDOS DURANTE O DOUTORADO

1- Artigos publicados

FREESE, LUANA ; ALMEIDA, FELIPE BORGES ; HEIDRICH, NUBIA ; **HANSEN, Alana Witt** ; STEFFENS, LUIZA ; STEINMETZ, ALINE ; MOURA, DINARA JAQUELINE ; GOMEZ, ROSANE ; BARROS, HELENA MARIA TANNHAUSER . Environmental enrichment reduces cocaine neurotoxicity during cocaine-conditioned place preference in male rats. PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR, v. 169, p. 10-15, 2018.

GOMEZ, ROSANE ; CALETTI, GREICE ; ARBO, BRUNO DUTRA ; HOEFEL, ANA LÚCIA ; SCHNEIDER, RICARDO ; **HANSEN, Alana Witt** ; PULCINELLI, RIANNE REMUS ; FREESE, LUANA ; BANDIERA, SOLANGE ; KUCHARSKI, LUIZ CARLOS ; BARROS, HELENA MARIA TANHAUSER . Acute intraperitoneal administration of taurine decreases the glycemia and reduces food intake in type 1 diabetic rats. BIOMEDICINE & PHARMACOTHERAPY, v. 103, p. 1028-1034, 2018.

HANSEN, Alana Witt; ALMEIDA, FELIPE BORGES ; BANDIERA, SOLANGE ; PULCINELLI, RIANNE REMUS ; FRAGOSO, ANA LUIZA RODRIGUES ; SCHNEIDER, RICARDO ; BARROS, HELENA MARIA TANNHAUSER ; GOMEZ, ROSANE . Taurine restores the exploratory behavior following alcohol withdrawal and decreases BDNF mRNA expression in the frontal cortex of chronic alcohol-treated rats. PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR, p. 6-12, 2017.

2- Capítulo de livro

SOUZA, M. V. ; GOMEZ, R. ; HANSEN, A. W. . Farmacologia de Náuseas e Vômitos. In: Rosane Gomez; Iraci L. S. Torres. (Org.). Farmacologia Clínica. 1ed.Rio de Janeiro: Elsevier, 2017, v. , p. 385-397.

3- Resumos publicados e anais de eventos

GOMEZ, R. ; QUINTEIROS, D. A. ; BELLAVER, B. ; PULCINELLI, RIANNE REMUS ; S Bandiera ; **HANSEN, A. W**. ; QUINCOZES-SANTOS, A. . Combined use of alcohol and cigarette is more deleterius than either drug alone in the rat brain. In: Neuroscience, 2017, Washington. Session 793 - Addiction and Behavior, 2017.

F B Almeida ; **HANSEN, Alana Witt** ; G Caletti ; S Bandiera ; L Freese ; H M T Barros ; GOMEZ, R. . Taurine effects on behaviors and in α 2 GABA A receptor subunit or BDNF mRNA expression in the frontal cortex of chronically treated or alcohol-abstinent rats. In: Neuroscience, 2016, San Diego. Neuroscience 2016, 2016.

4- Participação em eventos

II Latin American Congress of Clinical and Laboratorial Toxicology. Taurine enhances alcohol intake and anxiolytic-like behaviors in alcoholic rats. 2018. (Congresso).

II Latin American Congress of Clinical and Laboratorial Toxicology. Combined use of alcohol and tobacco increases anxiety-like behaviors and glutamate levels in the liquor of rats. 2018. (Congresso).

IX Congresso Internacional de Bioanálises, Xii Congresso Sulbrasileiro de Biomedicina, XVIII Semana Gaúcha de Biomedicina e II Encontro Brasileiro de Monitoramento Terapêutico de Fármacos e Toxicologia Clínica. 2017. (Congresso).

SBFET - 49th Brazilian Congress of Pharmacology and Experimental Therapeutics. Taurine counteracts the neurotoxic effects of chronic hyperglycemia in diabetic rats. 2017. (Congresso).

SBFTE - 49th Brazilian Congress of Pharmacology and Experimental Therapeutics. Effect Of The Combined Use Of Alcohol And Cigarette Smoke On Oxidative Stress Parameters In Different Brain Areas Of Rats. 2017. (Congresso).

SBFTE - 49th Brazilian Congress of Pharmacology and Experimental Therapeutics. Effect Of Repeated Taurine Administration On Voluntary Alcohol Consumption And On Behaviors In Rats. 2017. (Congresso).

SBFTE - 49th Brazilian Congress of Pharmacology and Experimental Therpeutics. Combined Use Of Alcohol And Cigarette Smoke Increases Pro- Inflammatory Cytokines And Decreases BDNF Levels In The Frontal Cortex Of Rats. 2017. (Congresso).

VI Simpósio Internacional de Estresse Oxidativo e Doenças Cardiovasculares. Efeito da associação de álcool e tabaco sobre parâmetros de estresse oxidativo em diferentes áreas encefálicas de ratos. 2017. (Simpósio).

48th Brazilian Congress of Pharmacology and Experimental Therapeutics and 21th Latin American Congress of Pharmachology (LATINFARMA). Combined Use of Alcohol and Tobacco on Behavioral and Neuroinflammatory Parameters in Rats. 2016. (Congresso).

I Simpósio Gaúcho de Farmacologia. Efeito do uso combinado de álcool e tabaco sobre parâmetros comportamentais e neuroinflamatórios em ratos. 2016. (Simpósio).

9th IBRO World Congress on Neuroscience. N-acetylcysteine prevents alcohol cessation induced increases on TNF-α in rat brain. 2015. (Congresso).

XXX Reunião Anual da Federação de Sociedades de Biologia Experimental- FeSBE. N-acetilcisteína previne ansiedade induzida por abstinência de álcool em ratos. 2015. (Congresso).

5- Prêmios

Pôster destaque - Efeito da associação de álcool e tabaco sobre parâmetros de estresse oxidativo em diferentes áreas encefálicas de ratos, Laboratório de Fisiologia Cardiovascular – UFRGS (2017).

Menção Honrosa - Álcool, fumaça de cigarro ou seu uso combinado provocam dano hepático e renal em ratos, FeSBE (2015).

ANEXO B – PARECER DE PROJETO DE PESQUISA ENCAMINHADO AO COMITÊ DE ÉTICA EM PESQUISA COM ANIMAIS

CARTA DE APROVAÇÃO

Comissão De Ética No Uso De Animais analisou o projeto:

Número: 28722
Título: EFEITO DA TAURINA NO SISTEMA GABAERGICO E GLUTAMATERGICO E PARAMETROS NEUROTOXICOS DE RATOS ABSTINENTES AO ALCOOL

Pesquisadores:

Equipe UFRGS:

ROSANE GOMEZ - coordenador desde 05/06/2015
MARIA FLAVIA MARQUES RIBEIRO - pesquisador desde 05/06/2015
ANA PAULA GUEDES FRAZZON - pesquisador desde 05/06/2015
PATRÍCIA PEREIRA - pesquisador desde 05/06/2015
CAROLINA FERREIRA SANTOS - Aluno de Especialização desde 05/06/2015
Ricardo Schneider Junior - Aluno de Doutorado desde 05/06/2015
RIANNE REMUS PULCINELLI - Aluno de Especialização desde 05/06/2015
Alana Witt Hansen - Aluno de Doutorado desde 05/06/2015
Solange Bandiera - Aluno de Doutorado desde 05/06/2015

Equipe Externa:

Greice Caletti - pesquisador desde 05/06/2015

Comissão De Ética No Uso De Animais aprovou o mesmo , em reunião realizada em 01/06/2015 - Sala 330 do Anexo I do Prédio da Reitoria - Campus Centro- Universidade Federal do Rio Grande do Sul - Porto Alegre, em seus aspectos éticos e metolodológicos, para a utilização de 288 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, Quinta-Feira, 11 de Junho de 2015

Cristiane Matte

CRISTIANE MATTE
Coordenador da comissão de ética