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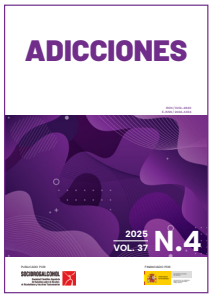


ORIGINAL

# Prior stress history shapes adolescent alcohol-induced transcriptional changes in striatal glutamatergic and endocannabinoid pathways

## *El historial previo de estrés modula los cambios transcripcionales inducidos por alcohol en la adolescencia en las vías glutamatérgicas y endocannabinoides del estriado*

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## Abstract

Adolescence is a critical developmental window during which exposure to stress and alcohol can induce long-lasting neurobiological alterations. Binge-like alcohol consumption is particularly disruptive to corticostriatal circuits, but the extent to which prior stress history modulates these effects remains poorly understood. Here, we investigated how acute versus repeated restraint stress before intermittent alcohol exposure during adolescence shapes transcriptional changes in the dorsal striatum of male rats. Animals were exposed either to a single (acute) or five-day (repeated) restraint stress at postnatal day (PND) 32–36, followed by four weeks of intermittent intragastric ethanol (3 g/kg) or saline administration. At adult age, striatal mRNA expression of dopaminergic (*Drd1*, *Drd2*, *Th*), glutamatergic (*Gls*, *Gls2*, *Gria2*, *Grin2a*, *Grin2b*), endocannabinoid (*Cnr1*, *Cnr2*, *Napepld*, *Faah*, *Dagla*, *Daglb*, *Mgll*), neurotrophic (*Bdnf*, *Ntrk2*), and glial (*Gfap*, *Aif1*) genes was quantified. Alcohol exposure upregulated genes associated with glutamate synthesis and receptor signaling, endocannabinoid metabolism, and astrocytic activation. Acute stress amplified alcohol-induced expression of *Gls*, *Gls2*, *Gria2*, *Napepld*, *Faah*, *Daglb*, *Ntrk2*, and *Gfap*, while repeated stress blunted these effects and selectively enhanced *Drd1*, *Drd2*, *Grin2a*, and *Bdnf* expression. Microglial activation (*Aif1*) was increased by alcohol independently of stress. These results suggest that acute stress sensitizes glutamatergic and endocannabinoid pathways to alcohol, whereas repeated stress engages adaptive mechanisms consistent with the stress inoculation hypothesis. Overall, stress history critically determines the neurobiological outcomes of adolescent alcohol exposure, with implications for resilience and vulnerability to alcohol-induced psychopathology.
***Keywords:*** alcohol, stress, adolescence, striatum, glutamatergic, endocannabinoid

## Resumen

La adolescencia es una etapa de desarrollo crítica durante la cual la exposición al estrés y al alcohol puede inducir alteraciones neurobiológicas de larga duración. El consumo de alcohol en atracón es especialmente disruptivo para los circuitos corticoestriatales, sin embargo, aún se conoce poco sobre hasta qué punto la historia previa de estrés modula estos efectos. En este estudio investigamos cómo el estrés por inmovilización, ya sea agudo o repetido, antes de la exposición intermitente al alcohol durante la adolescencia modula los cambios transcripcionales en el estriado dorsal de ratas macho. Los animales fueron expuestos a una sesión única (agudo) o a cinco días consecutivos (repetido) de estrés por inmovilización entre los días posnatales (DPN) 32–36, seguido de cuatro semanas de administración intermitente intragástrica de etanol (3 g/kg) o solución salina. En la edad adulta se cuantificó la expresión de ARNm estriatal de genes dopaminérgicos *(Drd1, Drd2, Th)*, glutamatérgicos *(Gls, Gls2, Gria2, Grin2a, Grin2b)*, endocannabinoides *(Cnr1, Cnr2, Napepld, Faah, Dagla, Daglb, Mgll)*, neurotróficos *(Bdnf, Ntrk2)* y gliales *(Gfap, Aif1)*. La exposición al alcohol incrementó la expresión de genes asociados con la síntesis de glutamato y la señalización de receptores, el metabolismo endocannabinoide y la activación astrocítica. El estrés agudo amplificó la expresión inducida por alcohol de *Gls, Gls2, Gria2, Napepld, Faah, Daglb, Ntrk2 y Gfap*, mientras que el estrés repetido atenuó estos efectos y aumentó selectivamente la expresión de *Drd1, Drd2, Grin2a y Bdnf*. La activación microglial *(Aif1)* se incrementó por el alcohol independientemente del estrés. Estos resultados sugieren que el estrés agudo sensibiliza las vías glutamatérgicas y endocannabinoides al alcohol, mientras que el estrés repetido activa mecanismos adaptativos consistentes con la hipótesis de la inoculación de estrés. En conjunto, el historial de estrés determina de manera crítica los resultados neurobiológicos de la exposición al alcohol durante la adolescencia, con implicaciones para la resiliencia y la vulnerabilidad a la psicopatología inducida por alcohol.
***Palabras clave:*** alcohol, estrés, adolescencia, estriado, glutamatérgico, endocannabinoide

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Adolescence is a critical developmental window where external insults, such as stress, drugs, infections, or traumas, can induce long-term consequences on emotional and cognitive function. The intensity and temporal sequence of these influences are crucial, as they may lead to outcomes ranging from severe cognitive and emotional disruption to resilient phenotypes. Mechanisms involved in the final developmental phenotype are not totally understood. In the case of alcohol, the extended pattern of binge drinking (repeated, high-intensity alcohol consumption) has been shown to be particularly harmful. Rodent models have demonstrated that adolescent alcohol exposure produces deficits in recognition memory, cognitive flexibility, increased disinhibition, and resistance to extinction learning in adulthood (Sanchez-Marin et al., 2022a; Sanchez-Marin et al., 2017), often associated with anxiety- and depression-like behaviors. Human imaging studies link adolescent heavy drinking with alterations in cortical–subcortical microstructure and disrupted functional connectivity in circuits critical for memory and reward, changes that predict poorer cognitive control and higher risk for substance-use disorders later in life (Huntley et al., 2020; Morris et al., 2018). Findings from both humans and rodents converge: adolescent intermittent ethanol produces persistent deficits in extinction learning of alcohol-seeking behavior that last into adulthood, behaviors that map onto real-world decision-making problems in individuals with early heavy alcohol exposure (Gass et al., 2014). These data underscore the public-health relevance of adolescent binge drinking and its capacity to shape trajectories toward adult psychopathology.

Multiple studies place the dorsal striatum, a hub for action selection and habit learning, at the center of these long-lasting effects of alcohol (Clabough et al., 2021; O'Tousa & Grahame, 2014; Salinas et al., 2022; Vrettou et al., 2017; Wilcox et al., 2014). Adolescent alcohol exposure alters striatal plasticity rules that support flexible goal-directed control, consistent with the adult bias toward habitual responding after adolescent alcohol exposure (Gass et al., 2014). At the synaptic level, a previous study has shown that chronic ethanol impairs mGlu2-dependent long-term depression (LTD) in the mouse striatum in an age-dependent manner, providing a mechanistic precedent for how adolescent exposure can derail corticostriatal maturation critical for adaptive learning (Johnson et al., 2020). Prior work has also shown that adolescent ethanol can remodel striatal interneuron networks and perineuronal nets in adulthood (Dannenhoffer et al., 2022), highlighting cell-type–specific substrates through which dorsal striatal computations may be durably re-tuned. These findings show that adolescent alcohol exposure produces enduring changes in striatal circuitry, which can serve as a mechanistic framework for studying stress–alcohol interactions.

Mechanistically, adolescent alcohol reshapes multiple neuromodulatory systems in the striatum. Among these, dopaminergic, glutamatergic, and endocannabinoid (eCB) signaling are particularly relevant because of their central role in striatal function and their sensitivity to both alcohol and stress. The dopaminergic system undergoes profound maturation during adolescence, shaping reinforcement learning, motivation, and reward sensitivity (Hoops & Flores, 2017). Alcohol exposure during this period persistently alters dopamine transmission, with reports of enhanced ethanol-evoked responses and blunted baseline signaling depending on subregion and exposure pattern, indicating a reinforcement signal that is sensitized yet dysregulated (Carrara-Nascimento et al., 2020; Shnitko et al., 2016; Zandy et al., 2015). Complementing these functional effects, alcohol exposure along adolescence changes the expression of cholinergic and dopaminergic genes (Hauser et al., 2021) and leaves cholinergic interneurons aberrant, with associated cognitive deficits (Galaj et al., 2019). In parallel, glutamatergic inputs from the cortex provide the principal excitatory drive to striatal neurons. Adolescent alcohol disrupts this system at multiple levels, including elevated extracellular glutamate during sensitization and impaired presynaptic and metabotropic regulation, together undermining corticostriatal plasticity and behavioral flexibility (Carrara-Nascimento et al., 2011; Johnson et al., 2020; Pascual et al., 2009). Finally, the eCB system acts as a key regulator of corticostriatal excitation/inhibition and is affected both directly by adolescent intermittent ethanol and indirectly via stress–alcohol interactions (Sanchez-Marin et al., 2022a; Sanchez-Marin et al., 2022b; Sanchez-Marin et al., 2020; Sanchez-Marin et al., 2017). Together, converging alterations in these neurotransmitter systems provide a coherent framework by which adolescent binge drinking can bias dorsal striatal circuits toward habitual, inflexible behavior in adulthood. Because these systems are still maturing during adolescence, stress and alcohol may interact to produce long-lasting neuroadaptations that alter reward sensitivity and increase vulnerability to substance use disorders.

Stress and trauma during adolescence may further influence alcohol's effects on striatal function and adult behavior. Preclinical studies indicate that the impact of early stress on adult behavior depends on its intensity, duration, timing, and predictability, with adolescent exposure often producing both beneficial and detrimental outcomes in a bell-shaped dose–response pattern (Sandi & Pinelo-Nava, 2007). Consistent with the concept of stress inoculation, repeated mild-to-moderate stress during adolescence can buffer against cognitive impairments induced by trauma or alcohol exposure (Chaby et al., 2020; Sircar, 2020).

Originally described by Lyons and Parker (Lyons & Parker, 2007), stress inoculation refers to an evolutionarily conserved mechanism by which moderate stress enhances resilience, likely through adaptive neuroplasticity (Lotan et al., 2018).

Illustrating these dual effects, early-life and adolescent stress increase alcohol intake and alter eCB signaling. For example, maternal separation elevates drinking while reducing eCB levels in the striatum and prefrontal cortex (Favoretto et al., 2025; Portero-Tresserra et al., 2018). In animals exposed to adolescent alcohol, stress exacerbates negative affect and reward-processing deficits, together with alterations in CRF, monoamines, and glutamate, highlighting a stress–alcohol synergy that disrupts motivational circuitry (Boutros et al., 2018; Van Waes et al., 2011). At the same time, stress inoculation through repeated, controllable, or moderate stress can promote resilience. Thus, previous studies have shown that repeated stress in mid-adolescence attenuates later behavioral, noradrenergic, and epigenetic consequences of severe stress in early adulthood (Chaby et al., 2020), while environmental manipulations, such as adolescent wheel running, prevent stress-induced escalation of ethanol intake (Reguilon et al., 2025).

Together, these findings suggest that alcohol research must explicitly compare acute and repeated stress during adolescence to disentangle vulnerability from inoculation mechanisms, particularly within dorsal-striatal circuits where stress, eCB, cholinergic, dopaminergic, and glutamatergic systems converge to shape long-term action selection and addiction risk. While multiple brain regions, such as the prefrontal cortex, hippocampus, amygdala, and nucleus accumbens, are critically involved in stress responsivity and addiction-related behaviors (Juliano et al., 2025), we focused on the dorsal striatum because adolescent alcohol exposure produces robust and long-lasting adaptations in this region. Moreover, the dorsal striatum integrates multiple neuromodulatory systems, providing a mechanistically suitable framework for transcriptional analyses of stress–alcohol interactions. The present study was therefore designed to compare the effects of single versus repeated stress prior to alcohol bingeing on a targeted set of representative genes within dopaminergic, glutamatergic, and eCB signaling pathways, as well as neurotrophic and glial markers, selected for their established relevance to adolescent alcohol- and stress-induced striatal plasticity. Neurotrophic markers, specifically the brain-derived neurotrophic factor BDNF and its receptor TrkB, were included given their central role in activity-dependent synaptic plasticity and adaptive responses to stress and alcohol during adolescence (Binder & Scharfman, 2004; Logrip et al., 2015; Murakami et al., 2005). Alterations in this pathway can influence long-term striatal circuit function and behavioral outcomes, providing a mechanistic substrate for alcohol- and stress-induced changes. We selected only male animals in this study to reduce variability related to sex-specific neuroprotective effects of ovarian hormones, which could confound the interpretation of stress- and alcohol-induced transcriptional changes. While female adolescents also engage in binge drinking, including them in this initial study could obscure mechanistic insights; future work should incorporate both sexes to assess potential sex differences in vulnerability and resilience.

# Methods

## Animals and ethical statement

A total of 88 male Wistar rats (Charles River Laboratories, France), weighing 75–100 g upon arrival, were used across two experimental protocols included in this study. Rats were received at postnatal day (PND) 21 and were pair-housed in a humidity- and temperature-controlled vivarium under a 12 h light/dark cycle (lights off at 7:00 PM). Standard chow pellets and water were available ad libitum throughout the study. All animals were allowed to acclimate to the housing conditions for several days before any experimental procedure was performed.

The study was designed and conducted in accordance with the European Directive 2010/63/EU for the protection of animals used for scientific purposes, as well as relevant Spanish regulations (Real Decreto 53/2013 and 178/2004; Ley 32/2007 and 9/2003; Decreto 320/2010). All procedures were approved by the Ethics and Research Committee of the University of Málaga (CEUMA) and adhered to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. All efforts were made to minimize animal suffering and to use the minimum number of animals necessary.

## Experimental design

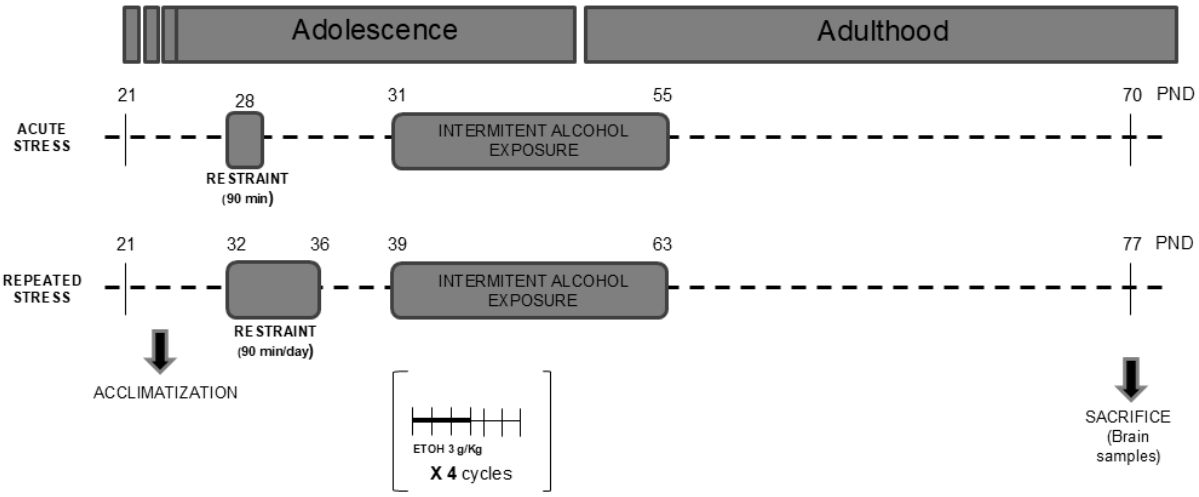
The experimental design has been described previously in two separate studies (Sanchez-Marin et al., 2022a; Sanchez-Marin et al., 2022b). A total of 88 adolescent male Wistar rats were used across two experimental cohorts. In both, animals were randomly assigned to stress or non-stress conditions and further subdivided into alcohol or saline treatment groups, resulting in six main experimental subgroups: non-stress+saline, acute stress+saline, repeated stress+saline, non-stress+alcohol, acute stress+alcohol, and repeated stress+alcohol.

### *Stress exposure*

In the first cohort (n = 48), rats underwent a single acute restraint stress session (90 min on PND28), whereas in the second cohort (n = 40), stress consisted of 5 daily sessions of 90 min each (PND32–36). Non-stressed rats in both cohorts remained undisturbed.

**Figure 1**

*Experimental design*




*Note.* For single stress exposure, half of the adolescent male rats (n=24) were exposed to 90 min of restraint stress at postnatal day (PND) 28, while the other half remained undisturbed in their home cage. For repeated stress, half of the adolescent male rats (n=20) were exposed to restraint stress for 90 min daily over 5 consecutive days (PND 32–36), and the other half remained undisturbed. Following the stress exposure, half of the previously undisturbed animals and half of the stressed animals in each stress condition (single vs. repeated stress) were exposed to 4 cycles of intragastric alcohol administration (3 g/kg). Each cycle consisted of 4 consecutive days of alcohol treatment followed by 3 days of washout. The remaining animals received an isovolumetric saline solution following the same schedule. Animals were sacrificed 2 weeks after the last alcohol/saline administration, and dorsal striatum samples were collected for mRNA expression analysis. This protocol resulted in 6 experimental subgroups: non-stress+saline, acute stress+saline, repeated stress+saline, non-stress+alcohol, acute stress+alcohol, and repeated stress+alcohol.

### *Intermittent alcohol procedure*

As described previously (Sanchez-Marin et al., 2022a; Sanchez-Marin et al., 2022b), rats in alcohol groups received 3 g/kg ethanol (25% v/v in saline) via intragastric gavage on 4 consecutive days followed by a 3-day alcohol-free period. This cycle was repeated for 4 weeks. Saline groups received an isovolumetric saline solution on the same schedule. All administrations were performed by a trained researcher.

## Sample collection and brain dissection

Two weeks after the final alcohol administration (PND70-77), rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) for brain tissue collection. Brains were rapidly extracted, immediately frozen on dry ice, and stored at −80 °C until molecular analyses. For dissection, frozen brains were placed in stainless steel rat brain matrices, and 2-mm-thick coronal sections were obtained using razor blades. The dorsal striatum was bilaterally dissected using a sample corer, guided by anatomical landmarks identified in the rat brain atlas by Paxinos and Watson (Paxinos & Watson, 1998).

## RNA isolation and RT-qPCR analysis

Quantitative real-time PCR (RT-qPCR) was used to measure relative mRNA expression levels of selected genes involved in dopaminergic signaling, glutamatergic transmission, endocannabinoid metabolism, neurotrophic support, and neuroinflammatory markers. The following genes were analyzed: dopamine receptors D1 (*Drd1*) and D2 (*Drd2*); tyrosine hydroxylase (*Th*); glutamate receptor subunits *Gria2* (AMPA), *Grin2a* and *Grin2b* (NMDA); glutaminase isoforms *Gls* and *Gls2*; endocannabinoid-related genes including cannabinoid receptors $CB_1$ (*Cnr1*) and $CB_2$ (*Cnr2*), monoacylglycerol lipase (*Mgll*), diacylglycerol lipase alpha (*Dagla*) and beta (*Daglb*), N-acyl phosphatidylethanolamine phospholipase D (*Napepld*), and fatty acid amide hydrolase (*Faah*); as well as brain-derived neurotrophic factor (*Bdnf*), its receptor TrkB (*Ntrk2*), glial fibrillary acidic protein (*Gfap*), and allograft inflammatory factor 1 (*Aif1*).

Total RNA was extracted from dissected brain tissue using TRIzol Reagent (Gibco BRL Life Technologies, Baltimore, MD, USA) as previously described (Sanchez-Marin et al., 2022a). RT-qPCR reactions were conducted using a CFX Duet Real-Time PCR System (Bio-Rad Laboratories, Hercules City, CA, USA) and the FAM dye label format for the TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). All expression values were normalized to the housekeeping gene β-actin (*Actb*), which showed stable expression across experimental groups. Relative gene expression was calculated using the 2^−ΔΔCt method, with expression levels expressed relative to the control group. TaqMan primers and probes were selected based on validated rat mRNA sequences from the Applied Biosystems genome database (https://www.thermofisher.com/order/genome-database/), and detailed primer information is provided in Supplementary Table S1.

## Statistical analysis

All data for graphs are expressed as the mean ± SEM. The normal distribution of data was evaluated by Kolmogorov-

Smirnov normality test. The significance of differences within and between groups was evaluated using two-way analysis of variance (ANOVA) [factors: f1 "stress" (non-stress/single stress/repeated stress) and f2 "alcohol" (saline/alcohol)]. The Tukey test was used as post-hoc analysis for multiple pairwise comparisons of the subgroups when an interaction (f1 x f2) was revealed by two-way ANOVA.

Test statistic values and degrees of freedom are indicated in the results description and placed as tables in the figures for better understanding of the analysis. A *p*-value less than 0.05 was considered statistically significant. All statistical analyses were performed using the Graph-Pad Prism version 5.04 software (GraphPad Software, San Diego, CA, USA).

## Results

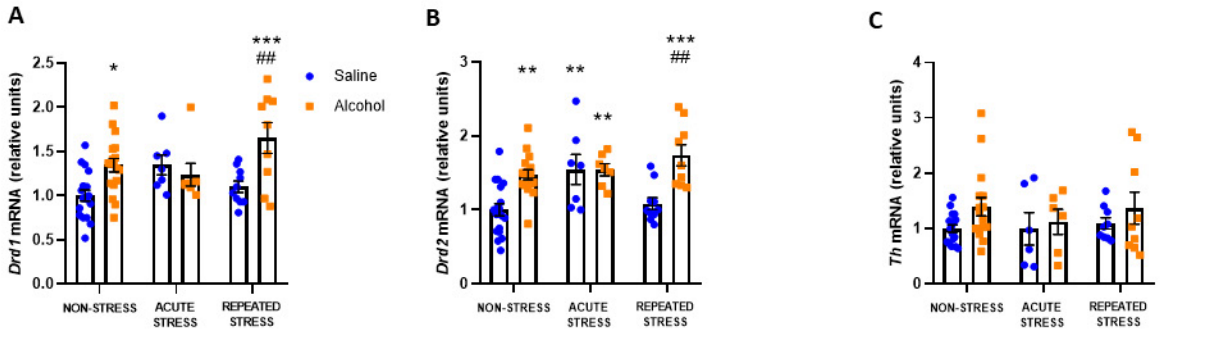
In the present study, we evaluated the expression of selected genes related to dopaminergic signaling, glutamatergic transmission, eCB signaling, neurotrophic support, and reactive glial (neuroinflammatory) markers in the dorsal striatum of young adult male rats (PND 70-77) that had been exposed to restraint stress (acute or repeated) prior to four weeks of intermittent alcohol exposure during adolescence.

### Effects of restraint stress and intermittent alcohol on the mRNA expression of dopaminergic signaling genes in the dorsal striatum

First, we evaluated the effects of restraint stress and/or intermittent alcohol exposure during adolescence on the mRNA expression of dopamine receptors (*Drd1* and *Drd2*), and *Th*, the rate-limiting enzyme in dopamine synthesis. A two-way ANOVA revealed a significant main effect of "alcohol" and a significant "stress" × "alcohol" interaction on the mRNA levels of *Drd1*. The post hoc test for multiple comparisons showed that alcohol increased the mRNA levels of this receptor in the non-stress+alcohol subgroup compared with non-stress+saline (**p*<0.05), and in the repeated stress+alcohol rats compared with non-stress+saline (****p*<0.001) and repeated stress+saline ($^{\#\#}p$<0.01) subgroups. Notably, this effect was absent in animals exposed to an acute stress, where both saline- and alcohol-treated subgroups showed similar values (Figure 2A). These results indicated that acute stress blunted the alcohol-induced increase in *Drd1*, whereas repeated stress potentiated it. For *Drd2* expression (Figure 2B), a two-way ANOVA revealed significant main effects of "alcohol" and "stress", as well as a significant "stress" × "alcohol" interaction. The post hoc test for multiple comparisons showed that *Drd2* mRNA levels were significantly increased in all alcohol-treated subgroups compared with non-stress+saline animals (***p*<0.01 and ****p*<0.001), except in the repeated stress+saline subgroup, suggesting that the effect of acute stress was blunted in repeated-stress animals receiving vehicle. Moreover, repeated stress+alcohol animals showed a significant increase in the mRNA expression of this receptor compared with repeated stress+saline subgroup ($^{\#\#}p$<0.01). In contrast, *Th* expression was not significantly affected by "stress" or "alcohol", although a non-significant trend toward increased expression in alcohol-exposed animals was observed, likely due to substantial inter-individual variability (Figure 2C).

**Figure 2**

*Differential effects of single vs. repeated prior stress on the impact of intensive alcohol exposure during adolescence on the mRNA expression of dopaminergic signaling genes in the dorsal striatum of adult rats*




| ANOVA table | F (DFn, DFd) | *p* value |
|---|---|---|
| Interaction | F (2, 63) = 4.246 | ***p*=0.019** |
| STRESS | F (2, 63) = 2.530 | *p*=0.088 |
| ALCOHOL | F (1, 63) = 9.483 | ***p*=0.003** |

| ANOVA table | F (DFn, DFd) | *p* value |
|---|---|---|
| Interaction | F (2, 62) = 3.826 | ***p*=0.027** |
| STRESS | F (2, 62) = 4.370 | ***p*=0.017** |
| ALCOHOL | F (1, 62) = 17.430 | ***p*<0.001** |

| ANOVA table | F (DFn, DFd) | *p* value |
|---|---|---|
| Interaction | F (2, 57) = 0.254 | *p*=0.776 |
| STRESS | F (2, 57) = 0.354 | *p*=0.703 |
| ALCOHOL | F (1, 57) = 2.848 | *p*=0.097 |

*Note.* Relative mRNA expression of *Drd1* **(A)**; *Drd2* **(B)**; and *Th* **(C)**. Columns represent mean ± SEM (7-10 rats/subgroups). Tables below each panel show the results of the two-way ANOVA. ***p***-values in bold denote significant main effects of factors ("stress" and "alcohol") or significant interaction ("stress" x "alcohol"). ***p**<0.05, ****p**<0.01, and *****p**<0.001 denote significant differences compared with the non-stress+saline subgroup, and ##***p***<0.01 denotes significant differences compared with the repeated stress+saline subgroup using post hoc tests for multiple comparisons when an interaction between factors is found.

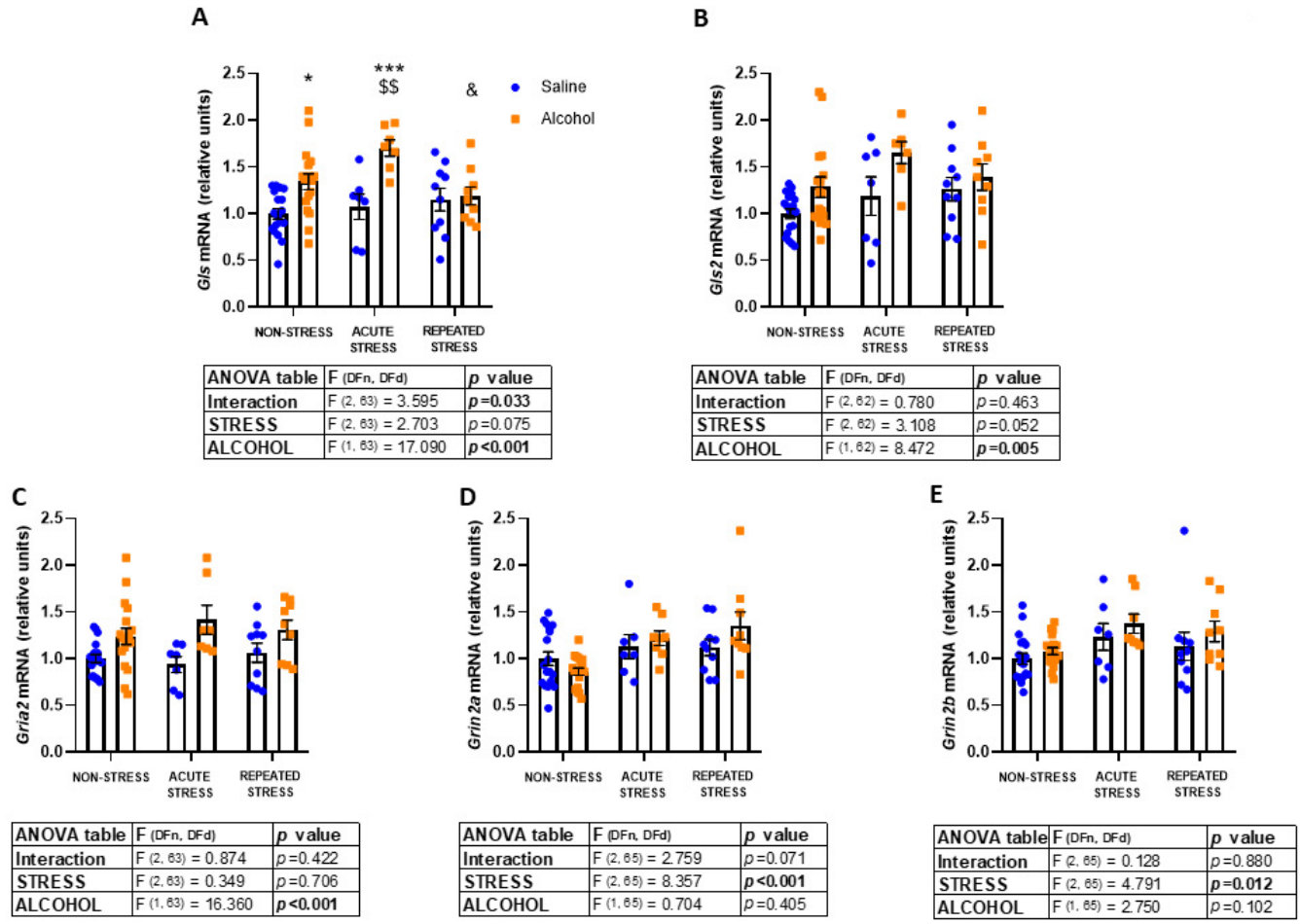
### Effects of restraint stress and intermittent alcohol on the mRNA expression of glutamatergic signaling genes in the dorsal striatum

Next, we evaluated the effects of restraint stress and/or intermittent alcohol exposure during adolescence on the mRNA expression of the glutaminase isoforms *Gls* and *Gls2*, which encode enzymes responsible for converting glutamine to glutamate, a key step in excitatory neurotransmission, as well as on the expression of glutamate receptor subunits *Gria2* (AMPA), *Grin2a*, and *Grin2b* (NMDA).

As shown in Figure 3A, statistical analysis revealed a significant main effect of "alcohol" on the mRNA expression of *Gls*, as well as a significant "stress" × "alcohol" interaction. The post hoc test for multiple comparisons showed a significant increase in *Gls* mRNA levels in non-stress+alcohol and acute stress+alcohol subgroups compared with non-stress+saline animals (**p*<0.05 and ****p*<0.001, respectively). Moreover, acute stress+alcohol rats displayed significantly higher mRNA levels of this enzyme compared with both acute stress+saline ($^{\$\$}p$<0.01) and repeated stress+alcohol ($^{\&}p$<0.05) subgroups. These results suggest that while alcohol increased glutamate availability, acute stress amplified this effect, whereas repeated stress attenuated it. For *Gls2* expression (Figure 3B), statistical analysis also revealed a significant main effect of "alcohol", indicating an alcohol-induced increase in glutamate availability. Stress alone did not alter the expression of either *Gls* or *Gls2*. For AMPA receptors (Figure 3C), alcohol significantly increased the mRNA expression of the *Gria2* subunit. Finally, for NMDA receptors (Figures 3D and 3E), two-way ANOVA showed significant main effects of "stress" on the mRNA expression of both *Grin2a* and *Grin2b*. Stressed animals displayed higher mRNA levels of these NMDA receptor subunits compared with non-stressed controls.

**Figure 3**

*Differential effects of single vs. repeated prior stress on the impact of intensive alcohol exposure during adolescence on the mRNA expression of glutamatergic signaling genes in the dorsal striatum of adult rats*




*Note.* Relative mRNA expression of *Gls* **(A)**; *Gls2* **(B)**; *Gria2* **(C)**; *Grin2A* **(D)**; and *Grin2B* **(E)**. Columns represent mean ± SEM (7-10 rats/subgroups). Tables below each panel show the results of the two-way ANOVA. *p*-values in bold denote significant main effects of factors ("stress" and "alcohol") or significant interaction ("stress" x "alcohol"). **p*<0.05 and ****p*<0.001 denote significant differences compared with the non-stress+saline subgroup, $^{\$\$}p$<0.01 denotes significant differences compared with the acute stress+saline subgroup, and $^{\&}p$<0.05 denotes significant differences compared with the acute stress+alcohol subgroup using post hoc tests for multiple comparisons when an interaction between factors is found.

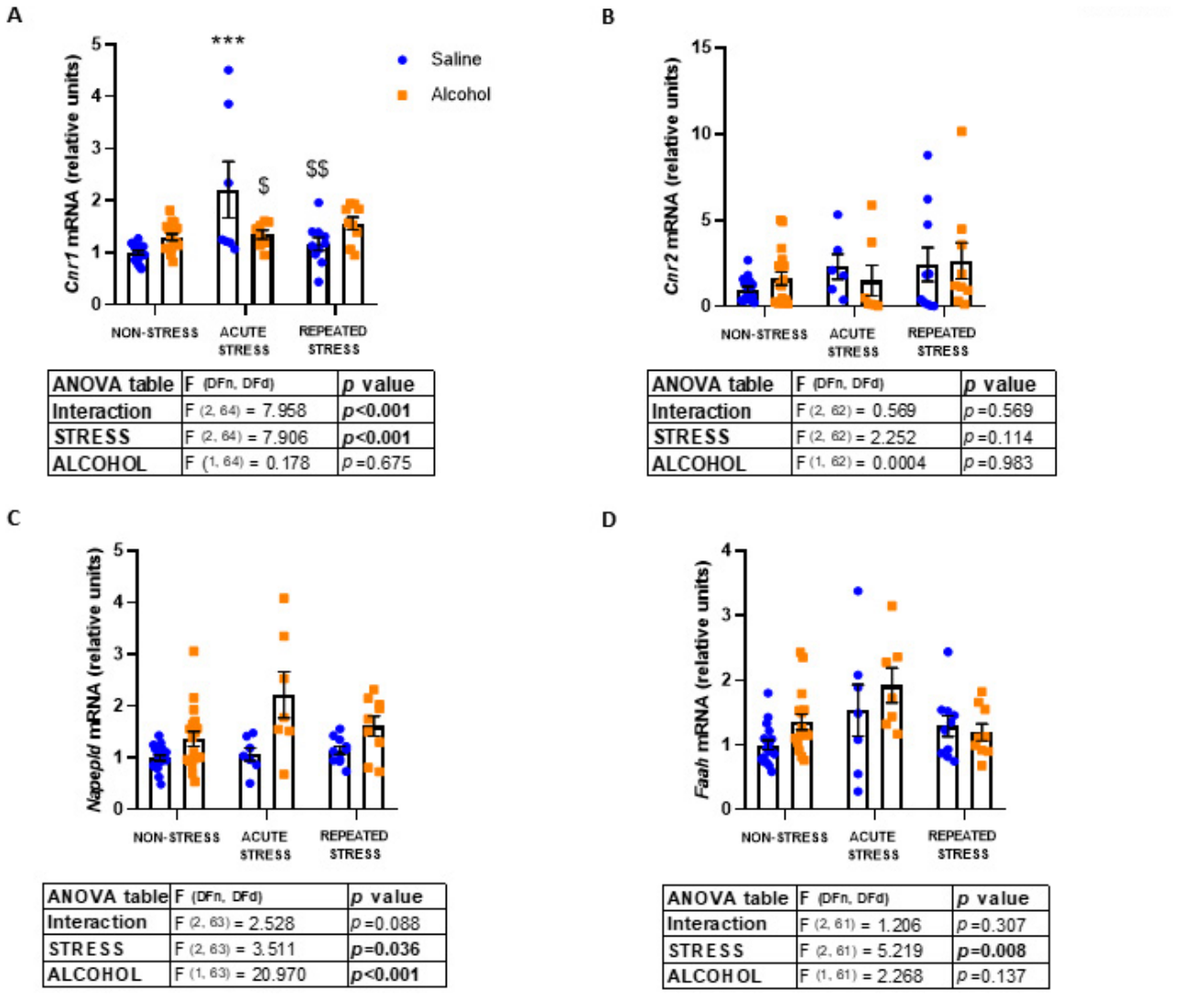
### Effects of restraint stress and intermittent alcohol on the mRNA expression of endocannabinoid signaling genes in the dorsal striatum

Since glutamate activity is regulated by the eCB system, the main retrograde signaling system at glutamatergic synapses, we evaluated the effects of restraint stress and/or intermittent alcohol exposure during adolescence on the mRNA expression of genes encoding receptors and enzymes involved in eCB synthesis, signaling, and degradation.

For *Cnr1* expression, a two-way ANOVA revealed a significant main effect of "stress", as well as a significant "stress" × "alcohol" interaction (Figure 4A). The post hoc test for multiple comparisons showed a marked increase in *Cnr1* mRNA levels in acute stress+saline rats compared with non-stress+saline (***$p<0.001$), acute stress+alcohol ($^{\$}p<0.05$), and repeated stress+saline ($^{\$\$}p<0.01$) subgroups, suggesting that repeated stress counteracted the increase observed after acute stress. In contrast, no significant effects of "stress" or "alcohol" were found on *Cnr2* expression (Figure 4B). Regarding enzymes related to acylethanolamines, a two-way ANOVA revealed significant main effects of "alcohol" and "stress" on *Napepld* expression (Figure 4C). This effect was attributable to alcohol-induced enhancement of the expression of this enzyme in stressed animals. Specifically, alcohol-exposed rats showed higher *Napepld* mRNA levels than saline-treated animals, with the effect being most pronounced in acutely stressed animals. For *Faah* expression (Figure 4D), a two-way ANOVA revealed a significant main effect of "stress", with stressed rats showed higher *Faah* mRNA levels than non-stressed animals. Finally, for the enzymes involved in acylglycerol metabolism, *Dagla* (Figure 4E), *Daglb* (Figure 4F) and *Mgll* (Figure 4G), no significant main effects of "stress" or "alcohol" were observed.

**Figure 4**

*Differential effects of single vs. repeated prior stress on the impact of intensive alcohol exposure during adolescence on the mRNA expression of endocannabinoid signaling genes in the dorsal striatum of adult rats*

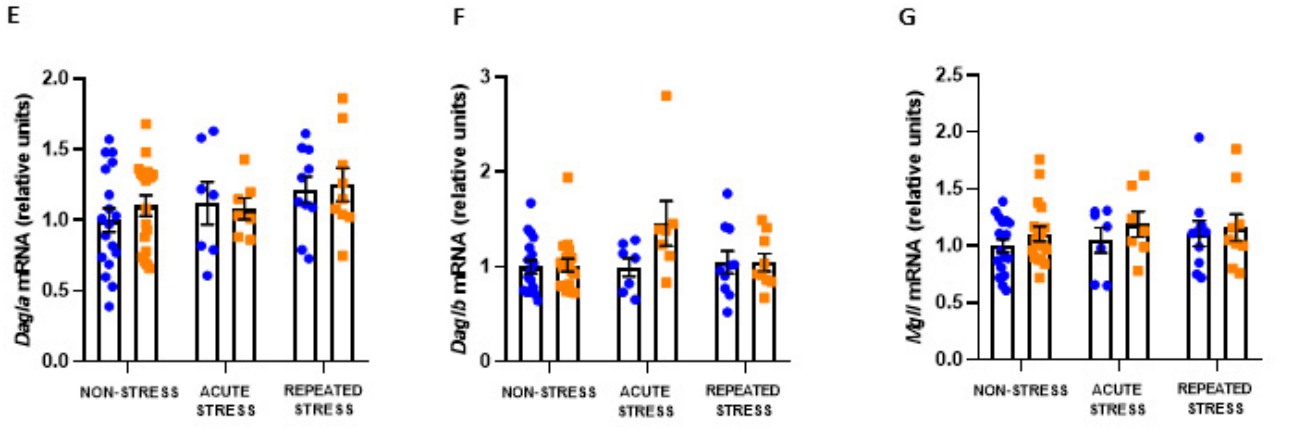



| ANOVA table | F (DFn, DFd) | p value |
|---|---|---|
| Interaction | F (2, 64) = 7.958 | $p<0.001$ |
| STRESS | F (2, 64) = 7.906 | $p<0.001$ |
| ALCOHOL | F (1, 64) = 0.178 | $p=0.675$ |

| ANOVA table | F (DFn, DFd) | p value |
|---|---|---|
| Interaction | F (2, 62) = 0.569 | $p=0.569$ |
| STRESS | F (2, 62) = 2.252 | $p=0.114$ |
| ALCOHOL | F (1, 62) = 0.0004 | $p=0.983$ |

| ANOVA table | F (DFn, DFd) | p value |
|---|---|---|
| Interaction | F (2, 63) = 2.528 | $p=0.088$ |
| STRESS | F (2, 63) = 3.511 | $p=0.036$ |
| ALCOHOL | F (1, 63) = 20.970 | $p<0.001$ |

| ANOVA table | F (DFn, DFd) | p value |
|---|---|---|
| Interaction | F (2, 61) = 1.206 | $p=0.307$ |
| STRESS | F (2, 61) = 5.219 | $p=0.008$ |
| ALCOHOL | F (1, 61) = 2.268 | $p=0.137$ |




| ANOVA table | F (DFn, DFd) | p value |
|---|---|---|
| Interaction | F (2, 63) = 0.241 | p=0.787 |
| STRESS | F (2, 63) = 1.909 | p=0.157 |
| ALCOHOL | F (1, 63) = 0.162 | p=0.689 |

| ANOVA table | F (DFn, DFd) | p value |
|---|---|---|
| Interaction | F (2, 63) = 2.395 | p=0.099 |
| STRESS | F (2, 63) = 2.021 | p=0.141 |
| ALCOHOL | F (1, 63) = 3.140 | p=0.081 |

| ANOVA table | F (DFn, DFd) | p value |
|---|---|---|
| Interaction | F (2, 63) = 0.103 | p=0.902 |
| STRESS | F (2, 63) = 0.560 | p=0.574 |
| ALCOHOL | F (1, 63) = 1.703 | p=0.197 |

*Note.* Relative mRNA expression of *Cnr1* **(A)**; *Cnr2* **(B)**; *Napepld* **(C)**; *Faah* **(D)**; *Dagla* **(E)**; *Daglb* **(F)**; and *Mgll* **(G)**. Columns represent mean ± SEM (7-10 rats/subgroups). Tables below each panel show the results of the two-way ANOVA. ***p*-values in bold denote significant main effects of factors ("stress" and "alcohol") or significant interaction ("stress" x "alcohol"). ***$p<0.001$ denotes significant differences compared with the non-stress+saline subgroup, $^{\$}p<0.05$ and $^{\$\$}p<0.01$ denote significant differences compared with the acute stress+saline subgroup using post hoc tests for multiple comparisons when an interaction between factors is found.

## Effects of restraint stress and intermittent alcohol on the mRNA expression of neurotrophic-related genes in the dorsal striatum

Next, we evaluated the effects of restraint stress and/or intermittent alcohol exposure during adolescence on the mRNA expression of *Bdnf* and its receptor *Ntrk2*. This neurotrophic pathway represents a major adaptive molecular signal in response to both stress and alcohol.

As shown in Figure 5A, statistical analysis revealed significant main effects of "stress" on *Bdnf* expression. Stressed rats displayed higher mRNA levels of *Bdnf* than non-stressed animals. Regarding its receptor (Figure 5B), a two-way ANOVA revealed significant main effect of "alcohol" and "stress". Specifically, alcohol-exposed rats showed higher *Ntrk2* mRNA levels than saline-treated animals. Although ANOVA also indicated a main effect of stress, this effect was less visually apparent, likely due to inter-individual variability.

**Figure 5**

*Differential effects of single vs. repeated prior stress on the impact of intensive alcohol exposure during adolescence on the mRNA expression of neurotrophic-related genes in the dorsal striatum of adult rats*


| ANOVA table | F (DFn, DFd) | p value |
|---|---|---|
| Interaction | F (2, 65) = 0.356 | p=0.702 |
| STRESS | F (2, 65) = 7.520 | **p=0.001** |
| ALCOHOL | F (1, 65) = 0.949 | p=0.334 |

| ANOVA table | F (DFn, DFd) | p value |
|---|---|---|
| Interaction | F (2, 65) = 0.298 | p=0.744 |
| STRESS | F (2, 65) = 3.537 | **p=0.035** |
| ALCOHOL | F (1, 65) = 8.288 | **p=0.005** |

*Note.* Relative mRNA expression of ***Bdnf* (A)**; and ***Ntrk2* (B)**. Columns represent mean ± SEM (7-10 rats/subgroups). Tables below each panel show the results of the two-way ANOVA. ***p*-values in bold denote significant main effects of factors ("stress" and "alcohol") or significant interaction ("stress" x "alcohol").

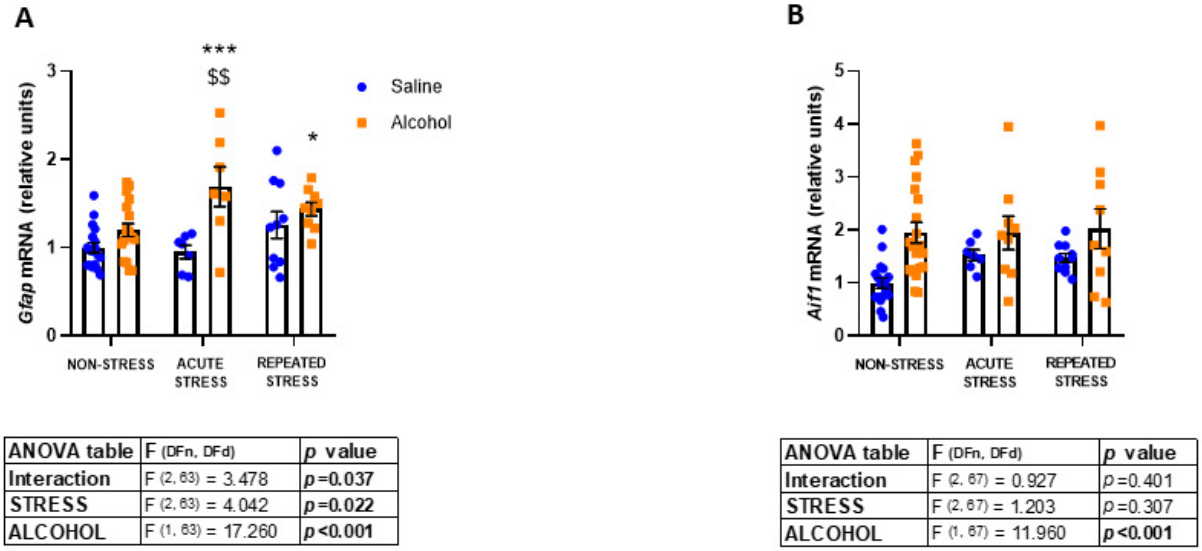
### Effects of restraint stress and intermittent alcohol on astrocytic and microglial markers in the dorsal striatum

Finally, we evaluated the effects of restraint stress and/or intermittent alcohol exposure during adolescence on the mRNA expression of *Gfap* and *Aif1*, markers of astrocytes and activated microglia, respectively.

A two-way ANOVA revealed significant main effects of "stress" and "alcohol", as well as a significant "stress" × "alcohol" interaction on *Gfap* expression (Figure 6A). The post hoc test for multiple comparisons showed a marked increase in *Gfap* mRNA levels in acute stress+alcohol and repeated stress+alcohol subgroups compared with non-stress+saline animals (***$p$<0.001 and *$p$<0.05, respectively). Moreover, acute stress+alcohol rats displayed significantly higher *Gfap* mRNA levels compared with acute stress+saline ($^{\$\$}p$<0.01). For microglia, analysis revealed a significant main effect of "alcohol" on *Aif1* expression, independent of stress (Figure 6B). Across all groups, alcohol-exposed rats displayed higher *Aif1* mRNA levels than saline-treated animal.

**Figure 6**

*Differential effects of single vs. repeated prior stress on the impact of intensive alcohol exposure during adolescence on the mRNA expression of astrocytic and microglial marker genes in the dorsal striatum of adult rats*




| ANOVA table | F (DFn, DFd) | $p$ value |
|---|---|---|
| Interaction | F (2, 63) = 3.478 | $p$=0.037 |
| STRESS | F (2, 63) = 4.042 | $p$=0.022 |
| ALCOHOL | F (1, 63) = 17.260 | $p$<0.001 |

| ANOVA table | F (DFn, DFd) | $p$ value |
|---|---|---|
| Interaction | F (2, 67) = 0.927 | $p$=0.401 |
| STRESS | F (2, 67) = 1.203 | $p$=0.307 |
| ALCOHOL | F (1, 67) = 11.960 | $p$<0.001 |

*Note.* Relative mRNA expression of ***Gfap* (A)**; and ***Aif1* (B)**. Columns represent mean ± SEM (7-10 rats/subgroups). Tables below each panel show the results of the two-way ANOVA. $p$-values in bold denote significant main effects of factors ("stress" and "alcohol") or significant interaction ("stress" x "alcohol"). *$p$<0.05 and ***$p$<0.001 denote significant differences compared with the non-stress+saline subgroup, and $^{\$\$}p$<0.01 denotes significant differences compared with the acute stress+saline subgroup using post hoc tests for multiple comparisons when an interaction between factors is found.

## Discussion

The present study confirms that prior stress during adolescence modulates the neurobiological consequences of alcohol exposure on the expression of dopaminergic, glutamatergic, eCB, neurotrophic, and glial genes in the dorsal striatum. These pathways were selected due to their central roles in striatal synaptic plasticity, stress regulation, and alcohol-induced neuroadaptations. Together, they provide a comprehensive mechanistic framework for understanding how stress history shapes adolescent alcohol outcomes. These findings build on prior evidence that the effects of adolescent stress are highly context-dependent, varying according to timing, intensity, and chronicity (Sandi & Pinelo-Nava, 2007). Importantly, the outcomes observed here were strongly dependent on the type of stress exposure. Acute stress enhanced alcohol-induced upregulation of *Gls, Gls2, Gria2, Napepld, Faah, Daglb, Ntrk2*, and *Gfap*, while repeated stress attenuated these alcohol-driven increases, selectively enhancing the expression of *Drd1, Drd2, Grin2a,* and *Bdnf*. These findings support that stress inoculation during adolescence might result in protective effects against early alcohol abuse-induced harms.

Alcohol exposure robustly increased the expression of both *Drd1* and *Drd2* in the dorsal striatum under non-stress conditions, but this effect was abolished in animals exposed to a single acute stress session. In contrast, repeated stress did not blunt this response; instead, it enhanced alcohol-related upregulation of dopamine receptor genes. In addition to the interaction with alcohol, it is noteworthy that acute stress alone increased *Drd1* and *Drd2* expression, whereas repeated stress did not. This pattern suggests that acute stress transiently enhances dopaminergic receptor availability that may facilitate adaptive behavioral responses to acute challenges, but, if repeatedly triggered by stress or alcohol exposure, could contribute to maladaptive reinforcement processes. The absence of this effect after

repeated stress exposure may reflect adaptive regulatory mechanisms that limit dopaminergic overactivation following repeated stress exposure, consistent with evidence that chronic or repeated stress can blunt dopaminergic responsivity (Baik, 2020). Overall, these findings suggest that acute stress transiently disrupts alcohol-related dopaminergic adaptations, possibly by engaging short-term inhibitory or homeostatic mechanisms, whereas repeated stress facilitates a compensatory upregulation of dopamine receptors. Such a pattern is consistent with evidence of cross-sensitization between stress and alcohol at the level of mesostriatal dopamine neurons (Cheng et al., 2018; Wilcox et al., 2014). Increased receptor availability under repeated stress may represent a counter-regulatory adaptation aimed at maintaining motivational drive, which could, in turn, enhance vulnerability to subsequent alcohol or stress exposure by reinforcing dopaminergic sensitization.

Regarding alcohol-glutamate interactions, it is well known that alcohol robustly perturbs glutamatergic signaling in corticostriatal circuits, increasing presynaptic glutamate release and altering ionotropic receptor subunit expression, including AMPAR and NMDAR components (Abrahao et al., 2017). These changes drive long-term adaptations linked to reinforcement and alcohol seeking. The alcohol-induced upregulation in *Gls* and *Gls2* expression observed in our study is consistent with increased glutamate synthesis through the glutamine–glutamate cycle, providing the excitatory supply necessary to sustain binge-like alcohol intake. Acute stress further amplified this effect, likely reflecting glucocorticoid-mediated facilitation of presynaptic glutamate release and increased receptor trafficking (Popoli et al., 2011; Yuen et al., 2009). Conversely, repeated stress blunted alcohol's induction of *Gls/Gls2*, a pattern consistent with adaptive dampening of glutamatergic tone that may represent a form of metaplasticity (Franklin et al., 2012). This pattern extended to ionotropic receptors, since alcohol significantly increased *Gria2* mRNA levels, and acute stress enhanced alcohol-induced *Gria2* expression. However, we observed higher expression of *Grin2a* and *Grin2b* in stressed animals compared with non-stressed controls, suggesting that stress, rather than alcohol, modulates NMDA receptor subunit expression in this paradigm. Taken together, these results suggest that alcohol-induced increases in glutamate synthesis and AMPA receptor expression are potentiated by acute stress but are attenuated by repeated stress, whereas stress itself selectively enhances NMDA receptor subunit expression. This pattern may reflect adaptive modulation of corticostriatal excitatory transmission depending on stress history.

Because glutamate activity is tightly regulated by the eCB system, we also examined genes encoding cannabinoid receptors and metabolic enzymes. $CB_1$ receptors are densely expressed on striatal glutamatergic terminals, where eCBs such as anandamide (AEA) and 2-AG act retrogradely to reduce presynaptic release probability and support both short- and long-term plasticity (Katona & Freund, 2012; Zou & Kumar, 2018). Our data showed increased *Cnr1* expression in acute stress+saline animals compared with non-stressed controls, whereas this effect was attenuated by either alcohol exposure or repeated stress. The pronounced upregulation of *Cnr1* mRNA observed after acute stress likely reflects a transient compensatory mechanism aimed at restoring homeostatic balance in glutamatergic transmission. Acute stress increases corticosterone release, which enhances glutamate activity in corticostriatal and limbic circuits (Caudal et al., 2010; Yuen et al., 2009). The elevated *Cnr1* expression may therefore represent an adaptive response to counteract stress-induced excitatory drive by facilitating eCB-mediated inhibition of presynaptic glutamate release. This interpretation aligns with previous evidence showing that acute stress can reduce AEA levels through increased FAAH activity (Morena et al., 2019), while repeated stress often downregulates $CB_1$ receptor function (Morena et al., 2016). Thus, the current findings reinforce the idea that the eCB system exhibits a biphasic adaptation to stress, activation following acute exposure and suppression under chronic or repeated conditions, which may critically influence resilience and vulnerability trajectories during adolescence. In contrast, *Cnr2* expression remained unchanged across conditions, consistent with the predominant role of $CB_1$ receptors in regulating striatal synaptic transmission (Lovinger & Mathur, 2012). Interestingly, in the present study, adolescent alcohol exposure via gavage did not significantly alter mRNA expression of cannabinoid receptors in the dorsal striatum under non-stress conditions. This contrasts with our previous report (Sanchez-Marin et al., 2017), where intraperitoneal alcohol administration at higher peak blood alcohol concentrations (BACs) (~200 mg/dL) led to decreased *Cnr1* mRNA in the striatum. The current gavage protocol produced lower peak BACs (~133–140 mg/dL) and slower systemic absorption, which may explain the absence of detectable changes. In addition, the current study specifically analyzed the dorsal striatum, whereas the prior study assessed the entire striatum. Given the heterogeneity of eCB receptor expression across striatal subregions and alcohol sensitivity, these methodological differences likely contribute to the divergent outcomes. Regarding eCB metabolic enzymes, both alcohol and stress significantly influenced *Napepld* expression. Alcohol exposure increased *Napepld* mRNA levels in stressed animals, particularly in those subjected to acute stress, indicating enhanced AEA synthesis under these conditions. In parallel, *Faah* expression was elevated by stress, suggesting increased AEA degradation. The concurrent upregulation of *Napepld* and *Faah* implies a high turnover rate of AEA, potentially generating a dynamic but unstable eCB signaling state

during acute stress and alcohol exposure. By contrast, genes involved in 2-AG metabolism (*Dagla*, *Daglb*, *Mgll*) were not significantly affected by either stress or alcohol. The absence of major changes in these enzymes indicates that stress- and alcohol-related modulation of the eCB system in the dorsal striatum primarily targets the AEA pathway rather than 2-AG metabolism. These molecular adaptations may represent early events influencing long-term regulation of stress and reward circuits. Targeting specific components of the eCB system, such as FAAH or NAPE-PLD, could provide strategies to restore homeostasis and enhance resilience to stress- and alcohol-induced neuroplastic changes.

Both acute and repeated stress increased *Bdnf* expression in the dorsal striatum, with repeated stress showing a slightly higher level than acute stress. Moreover, the repeated stress+alcohol group appears visually higher than repeated stress alone, suggesting a possible additive trend. These observations indicate that stress exposure may drive adaptive increases in *Bdnf*, highlights a potential resilience mechanism. BDNF–TrkB signaling supports synaptic plasticity and structural remodeling, facilitating adaptive responses to environmental challenges (Lotan et al., 2018). Increased BDNF expression has been linked to stress inoculation in preclinical models (Chaby et al., 2020; Sircar, 2020), and in this context may buffer against alcohol-induced neurotoxicity.

Our results also distinguish between microglial and astrocytic responses to alcohol. *Aif1* expression was robustly increased by alcohol across groups, consistent with evidence that alcohol directly activates microglia through TLR4 signaling pathways (Alfonso-Loeches et al., 2010). These results indicate that microglial activation in the dorsal striatum was mainly associated with alcohol exposure rather than stress, suggesting that stress alone was not sufficient to alter microglial gene expression under these conditions. By contrast, *Gfap* expression was enhanced in both acute stress+alcohol and repeated stress+alcohol groups compared with non-stress controls, with the greatest increase in the acute stress+alcohol group, indicating that alcohol-induced astrocytic reactivity is more sensitive to stress history and may be attenuated by stress inoculation.

Taken together, these findings suggest a unified model of stress–alcohol interactions in adolescence. Under acute stress+alcohol, glucocorticoid-driven glutamate release synergizes with alcohol's excitatory effects, increasing *Gls/Gls2* expression and enhancing *Gria2*. In parallel, *Napepld* and *Faah* upregulation attempts to constrain excitation via $CB_1$ signaling, while astrocytic and microglial responses contribute to neuroinflammatory cascades. By contrast, repeated stress+alcohol engages protective "metaplastic" mechanisms: dampening alcohol's induction of glutamate metabolism genes and *Napepld*, shifting NMDA receptor composition toward *Grin2a*, and upregulating *Bdnf*. These adaptations reflect stress inoculation, where prior repeated stress reconfigures striatal circuits to buffer against subsequent perturbations (Franklin et al., 2012). While multiple signaling systems were examined in this study, our aim was to capture the coordinated molecular adaptations that emerge from the interaction between stress and alcohol, rather than to isolate a single pathway. The dopaminergic, glutamatergic, and eCB systems, together with neurotrophic and glial mechanisms, form an integrated network that regulates stress responsivity, reward processing, and synaptic plasticity. Identifying how these pathways interact provides a systems-level understanding of resilience and vulnerability. Nevertheless, our results point to specific therapeutic targets worth further exploration, particularly the modulation of eCB metabolism and BDNF–TrkB signaling, both of which appear to buffer the neurotoxic impact of adolescent alcohol exposure. Pharmacological interventions targeting these mechanisms could represent promising strategies to restore corticostriatal balance and promote stress resilience.

To place these findings in a broader context, the present results in the dorsal striatum should also be interpreted alongside our previous studies examining the effects of stress and adolescent alcohol exposure in other stress-related brain regions such as the hippocampus, amygdala, and medial prefrontal cortex (Sanchez-Marin et al., 2022a; Sanchez-Marin et al., 2022b; Verheul-Campos et al., 2025). In particular, we have reported that acute stress during adolescence produces enduring alterations in glutamatergic and endocannabinoid signaling within the amygdala, which are further modulated by concurrent alcohol exposure (Sanchez-Marin et al., 2022b). Similarly, repeated stress and adolescent alcohol exposure induced long-lasting transcriptional and behavioral changes in the hippocampus and medial prefrontal cortex (Sanchez-Marin et al., 2022; Verheul-Campos et al., 2025). Together with the present findings, these results suggest that stress history modulates alcohol-induced neuroplasticity across multiple corticolimbic and striatal nodes, shaping either vulnerability or resilience depending on the temporal pattern and intensity of stress exposure. Future studies should extend these investigations to determine whether comparable mechanisms operate in other stress-responsive regions, such as the hypothalamus, and to further characterize the impact of acute stress across these interconnected structures. This integrative approach will help clarify how stress experience reorganizes the neural networks that mediate alcohol-related psychopathology.

## Conclusion

This study demonstrates that the neurobiological consequences of adolescent alcohol exposure are critically shaped by prior stress history. Acute stress sensitizes

excitatory and endocannabinoid systems, exacerbating alcohol's impact on glutamatergic and astrocytic pathways, whereas repeated stress attenuates these effects and engages dopaminergic and neurotrophic adaptations consistent with resilience. These findings provide molecular support for the stress inoculation hypothesis and highlight the importance of considering stress history when assessing vulnerability versus resilience to alcohol during development.

## Limitations

A key limitation is that only male animals were studied. Given evidence that females display relative resilience to alcohol-induced neurotoxicity due to sex steroid–dependent neuroprotection, the generalizability of these findings is restricted. Future studies should incorporate both sexes and examine behavioral correlates, such as reward sensitivity and cognitive flexibility, to better link transcriptional changes to functional outcomes. Additionally, while gene expression was quantified, protein levels and functional assays are necessary to confirm downstream effects on synaptic physiology.

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## Conflicts of interest

Authors report no biomedical financial interests or potential conflicts of interest.

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