ADICCIONES

ISSN / 0214-4840
E-ISSN / 2604-6334

2025
VOL. 37 N.º 4

PUBLISHED BY:
SOCIDROGALCOHOL
Sociedad Científica Española de Estudios sobre el Alcohol, el Alcoholismo y las otras Toxicomanías

FUNDED BY:
MINISTERIO DE SANIDAD
SECRETARÍA DE ESTADO DE SANIDAD
DELEGACIÓN DEL GOBIERNO PARA EL PLAN NACIONAL SOBRE DROGAS

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ISSN: 0214-4840 ■ **E-ISSN:** 2604-6334 ■ **SVPF:** 89010R ■ **LEGAL DEP.:** V-1543-1989

INDEXED IN: SOCIAL SCIENCES CITATION INDEX (SSCI-JCR), SCIENCE CITATION INDEX EXPANDED (SCIE-JCR), EMBASE, SCOPUS, MEDLINE, PSICODOC, PSYCINFO, IBECS, ÍNDICE CSIC, LATINDEX, REDALYC, INDEX COPERNICUS, PROQUEST, DIALNET, GOOGLE SCHOLAR, WEB OF SCIENCE (WOS).

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ADICCIONES
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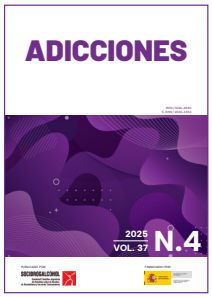

EDITORIAL

Entangled in addiction

Enredados en la adicción

Marta Rodríguez-Arias*; Marta Torrens Melich**.

* Unit of Research Psychobiology of Drug Dependence, Department of Psychobiology, Facultad de Psicología, Universitat de Valencia.

** Institut de Neuropsiquiatria i Addiccions, Parc de Salut Mar and Neurosciences Programme, Addiction Research Group, Hospital del Mar Research Institute, Barcelona, Spain. Falculty of Medicine and life Sciences (MELIS), Universitat Pompeu Fabra, Barcelona, Spain.

Addiction is a highly complex disease that requires a multidisciplinary approach. More than 20 years ago, researchers in the field of addictions, but with different professional profiles, began to establish a collaborative network that obtained funding from the Carlos III Health Institute (ISCIII). This project continues to receive funding and remains active today.

The RIAPAd, or Research Network on Primary Care in Addictions (formerly RTA or Addictive Disorders Network), is currently composed of 19 research groups distributed across 10 autonomous communities in Spain. These include numerous clinical and preclinical research groups, as well as groups dedicated to prevention and epidemiology. The Network is dynamic: although some groups have been involved since the beginning, others have merged or withdrawn, and many have joined over the years.

Through this multidisciplinary approach, the goal of RIAPAd is to improve the health and well-being of individuals and families affected by addiction. From each of our professional areas, all groups contribute to this overarching objective.

This special volume of Adicciones serves as a showcase for some of the work conducted within RIAPAd. We have focused particularly on basic or preclinical studies, as we aim to highlight the lesser-known aspects of the research we undertake. One of the great achievements of the network has been its ability to interconnect and strengthen our research through collaborative work. Most preclinical groups engage in extensive collaborations among themselves, but our efforts go further: the Network also facilitates collaboration among basic, clinical, and prevention-focused groups. The translation of basic research findings to specialists working from different perspectives in addiction, as well as to service users and society at large, is one of the essential goals we seek to achieve within RIAPAd.

The articles included in this volume focus on the main objectives pursued by the network. The article by the group led by Dr. Fernando Rodríguez de Fonseca explores the surprising protective role that exposure to a stressor during adolescence may exert by modifying the neurobiological correlates of alcohol use (*Prior stress history shapes adolescent alcohol-induced transcriptional changes in striatal glutamatergic and*

■ ISSN: 0214-4840 / E-ISSN: 2604-6334

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Another objective of RIAPAd is the monitoring of the evolution of addictive disorders, which is addressed in the collaborative article by the groups of Dr. Laura Orio and Dr. Gonzalo Herradón. Their study examines the pleiotrophin/PTPRZ neurotrophic pathway in the hippocampus of rats exposed to chronic alcohol consumption and/or thiamine deficiency (*Study of the Pleiotrophin/PTPRZ neurotrophic pathway in the hippocampus of rats exposed to chronic alcohol consumption and/or thiamine deficiency*).

Numerous groups are working on the development of new treatments for addiction. The group led by Dr. Marta Torrens presents a preliminary clinical trial evaluating the role of isoflavones in the treatment of cocaine use disorder (*Soy isoflavones for the treatment of cocaine use disorder: an open-label pilot study*). The group of Dr. Marta Rodríguez-Arias addresses the treatment of stress-induced cocaine use through the consumption of small amounts of a high-fat diet (*Blocking the increased reinforcing effects of cocaine induced by social defeat: effects of palatable food*). Finally, the role of omega-3 fatty acids in mitigating alcohol-induced alterations of the endocannabinoid system following binge drinking during adolescence is examined by the group of Dr. Inmaculada Gerrikagoitia (*Omega-3 fatty acids mitigate long-lasting disruption of the endocannabinoid system in the adult mouse hippocampus following adolescent binge drinking*).

One of RIAPAd's most important areas of interest—aligned with the European Drugs Agency—is research with a gender perspective. For this reason, two articles on this essential aspect of addiction are included in this special volume. A review conducted collaboratively by the groups of Dr. Antonio Vidal and Dr. Judit Tirado focuses on vulnerability to addiction from a gender perspective (*The influence of sex and gender factors on the modulation of vulnerability to addictions: a narrative review*). Finally, the group led by Dr. Orio examines how alcohol binge drinking may affect cognitive function and emotional responses depending on the sex of the animals (*Sex differences in emotional and cognitive tasks in adolescent rats exposed to alcohol binges and controls*).

We hope that this volume enables readers of the journal to become familiar with our network and to gain first-hand knowledge of the preclinical studies currently under way. Translating findings into clinical practice is often challenging, and we are fully aware of the difficulty of achieving similar results in our laboratories and in clinical settings—perhaps even more so when dealing with such a multifaceted condition as addiction. Nevertheless, preclinical research, despite its frequent limitations, offers new therapeutic targets, protective factors, or biomarkers that can open new pathways for hope and improved well-being for patients. This volume is also an effort by many basic researchers to open small windows for their colleagues—clinicians, epidemiologists, and prevention specialists—into what we can contribute to their work.

We look forward to welcoming you to RIAPAd:
https://riapad.es/

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ADICCIONES
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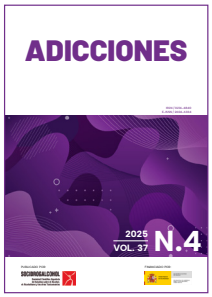

ORIGINAL

The influence of sex and gender factors on the modulation of vulnerability to addictions: A narrative review

La influencia de los factores de sexo y género en la modulación de la vulnerabilidad a las adicciones: Una revisión narrativa

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Abstract

This narrative review identifies the sex- and gender-related factors that influence individual vulnerability to developing addictive disorders. Addiction arises from the complex interaction between neurobiological and psychosocial factors. Sex-based brain dimorphisms, shaped by genetic, hormonal, and epigenetic influences, lead to differences in neural circuits involved in reward, emotional regulation, and executive functioning. Pharmacokinetic differences, such as higher blood alcohol levels, faster nicotine metabolism, and slower μ-opioid receptor internalization in women, contribute to earlier medical complications and faster addiction progression. Gender, understood as a system of socially constructed roles and expectations, further modulates these vulnerabilities. Traditional masculine norms are associated with higher substance use, while certain aspects of femininity may increase risk (e.g., emotional repression or partner dependence) or serve as protective factors through help-seeking behavior. Sexual and gender minorities experience the most significant disparities. Lesbian and bisexual women show the highest rates of substance use disorders; gay and bisexual men report greater illicit drug use; and bisexual individuals consistently display the highest overall risk. Transgender and non- binary populations exhibit increased prevalence of tobacco, stimulant, and chemsex-related substance use, often as a response to minority stress and exclusion from cisnormative care systems. Psychiatric comorbidity affects 50–80% of cases. Women show higher rates of anxiety, trauma histories, and adverse clinical outcomes. Many face "triple stigma" due to their gender, mental health condition, and substance use. Addressing these disparities requires an intersectional, gender-informed, and culturally competent approach to prevention, diagnosis, and treatment.

Keywords: vulnerability, sex differences, gender norms, substance-use disorder, dual diagnosis, sexual and gender minorities

Resumen

En este trabajo se ha realizado una revisión narrativa que identifica los factores asociados al sexo y al género que influyen en la vulnerabilidad frente al desarrollo de un trastorno adictivo. Las adicciones surgen de la interacción entre factores neurobiológicos y psicosociales. Los dimorfismos cerebrales según el sexo, mediados por factores genéticos, hormonales y epigenéticos, generan diferencias en los circuitos implicados en la recompensa, la regulación emocional y las funciones ejecutivas. Las diferencias farmacocinéticas, como mayores niveles sanguíneos de alcohol, metabolismo más rápido de la nicotina y una internalización más lenta de los receptores μ-opioides en mujeres, contribuyen a una progresión más rápida y a la aparición más temprana de complicaciones médicas. El género, entendido como un sistema de roles y normas socialmente construidos, modula estas vulnerabilidades. Las normas masculinas tradicionales se asocian a mayor consumo, mientras que algunos aspectos de la feminidad pueden actuar como factores de riesgo o protección. Las minorías sexuales y de género presentan las mayores disparidades. Las mujeres lesbianas y bisexuales tienen las tasas más elevadas de trastornos por uso de sustancias; los hombres gays y bisexuales informan mayor consumo de drogas ilícitas; las personas bisexuales muestran el mayor riesgo global. Las poblaciones trans y no binarias presentan prevalencias más altas de consumo de tabaco, estimulantes y sustancias asociadas al chemsex, muchas veces como respuesta al estrés de minoría y a sistemas de salud cisnormativos. La comorbilidad psiquiátrica afecta al 50–80 % de los casos. Abordar estas desigualdades requiere un enfoque interseccional, sensible al género y culturalmente competente.

Palabras clave: vulnerabilidad, diferencias según el sexo, normas de género, trastorno por consumo de sustancias, patología dual, minorías sexuales y de género

■ *Received: May 2025; Accepted: September 2025.*

■ ISSN: 0214-4840 / E-ISSN: 2604-6334

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Addiction is a complex phenomenon shaped by biological, psychological, and social factors. Within this framework, vulnerability emerges as a key concept for explaining why some individuals develop pathological dependence while others do not, even with similar patterns. This transition is mediated by individual characteristics such as impulsivity, sensation-seeking, or the presence of affective and anxiety disorders.

These traits can predispose individuals to use substances as a means of regulating negative emotions, coping with stress, or meeting unmet psychological needs.

Biological sex and socially constructed gender profoundly shape the responses of brain and body to addictive stimuli. Incorporating these dimensions into research is therefore essential for accurately pinpointing risk factors and crafting truly effective prevention and treatment strategies. Informed by a targeted search of the scientific literature from the past two decades in Web of Science and Scopus, this article delineates the principal sex/gender related determinants of vulnerability, ranging from biological and social factors to specific concerns, such as abuse, trauma, gender-nonconforming populations and co-occurring disorders (dual diagnosis).

Modulation Of Vulnerability To Addictions By Sex And Gender From A Biological Perspective

Scientific literature has shown sexual dimorphisms in the nervous system (NS) that can influence vulnerability to addictions. Specifically, genetic, epigenetic, and hormonal differences during brain development and organization lead to variations in the neural circuits involved in reward processing, emotion, and executive functions in men and women (McHugh et al., 2018), triggering divergent responses to the same substances of abuse.

At the neuroanatomical and functional level, neuroimaging studies in people with substance-use disorder (SUD) reveal alterations in regions such as the striatum —particularly the nucleus accumbens (NAc), which integrates pleasure and motivation— the amygdala (associates stimuli with reward or punishment), the hippocampus (contextual memory of rewarding experiences), the insula (regulates craving), and the corpus callosum (connects the cerebral hemispheres, integrating cognitive and emotional functions). These alterations differ between sexes, although further research is needed to refine these findings (Cornish & Prasad, 2021; Rando et al., 2013), and they also vary depending on the substance consumed. In alcohol-use disorder (AUD), men show volume reductions in these areas compared with healthy controls, whereas women with AUD exhibit enlarged volumes (Sawyer et al., 2017; Sawyer et al., 2018; Zahr et al., 2020). Studies of heavy alcohol consumption in adolescence and early adulthood likewise reveal sex-specific imaging differences in the prefrontal, striatal, and medial-temporal regions: young women drinkers have greater volumes than healthy women, while men show the opposite pattern with volume loss (Kvamme et al., 2016; Morris et al., 2019; Pfefferbaum et al., 2016). Similarly, Nie et al. (2021) found that male chronic methamphetamine users in abstinence had smaller hippocampi than healthy men, whereas no such structural effects were observed in women. Conversely, significant gray-matter reductions were detected in women with stimulant addiction, cocaine and/or amphetamines, after prolonged abstinence, but not in men (Regner et al., 2015). Collectively, these findings suggest sex-specific neuroplastic adaptations that may underlie the differing clinical manifestations of addiction (Cornish & Prasad, 2021).

Regarding neurochemical sex differences in reward, the mesocorticolimbic dopaminergic system is most prominent, with the ventral tegmental area (VTA) as the main source of dopaminergic neurons. Dopamine (DA) release in the NAc is central to reward; drugs inhibit its reuptake as an artificial reinforcement. Human and rodent studies reveal that men and women activate dopaminergic reward circuits differently, not only in magnitude but also neuroanatomically, with distinct responses in the ventral NAc versus dorsal striatum (Copenhaver & LeGates, 2024; Cosgrove et al., 2014; Gillies et al., 2014). This neural pattern may underlie the sex difference in escalation of use leading to addiction. Cosgrove et al. (2014) showed that men activate the ventral striatum more intensely when smoking, consistent with cigarette reinforcement, whereas women smoke more for emotional regulation and cue-reactivity. PET imaging further demonstrated that men exhibit a rapid, consistent dopaminergic response in the ventral striatum, while women respond more rapidly in the dorsal striatum (putamen). In cocaine addiction, women tend to progress faster to habitual use, partly due to lower DA release in the ventral striatum, prompting higher intake to achieve similar effects. Enhanced dopaminergic activity in the dorsolateral striatum may, in turn, facilitate the transition to compulsive use in women (Becker & Chartoff, 2019).

About the effects of sex on the endocrine system and how this influences vulnerability, there is extensive literature that examines how estradiol interacts with the mesolimbic reward circuit to heighten women's vulnerability to drug addiction. Sex hormones, such as estrogens, progesterone, and androgens, strongly modulate brain and behavior, including reward responses. Estradiol enhances drug reinforcement by increasing drug-induced DA release in mesolimbic and striatal pathways. Consequently, female rats display stronger drug-motivated behaviors when estradiol levels are high (proestrus/estrus, analogous to late follicular/ovulation in women) (Becker, 2016). Hormonal fluctuations across the

estrus/menstrual cycle can alter both the rate of addiction acquisition and the strength of drug reinforcement (Kokane & Perrotti, 2020; Sardari et al., 2024). Estradiol elevates the rewarding, reinforcing properties of opioids, cocaine, and amphetamines, thereby affecting vulnerability (Kokane & Perrotti, 2020). By contrast, progesterone often counters estradiol. In women who use cocaine, high progesterone levels and it derivates such as allopregnanolone (e.g., during the luteal phase or with certain contraceptives) associate with reduced craving and consumption (Peart et al., 2022). These findings have spurred research into progesterone analogues as potential relapse-reduction treatments for female psychostimulant users: progesterone has shown efficacy in lowering cocaine craving and arousal in women, though not in men (Fox et al., 2013).

Hormonal modulation extends to drug metabolism. Estrogens and progestogens partly account for higher peak plasma cocaine levels in women than in men given the same dose (Peart et al., 2022). Conversely, women metabolize nicotine more rapidly, particularly those on oral contraceptives (Allen et al., 2019; Berlin et al., 2007). Such pharmacokinetic variations contribute to sex differences in addiction vulnerability (Thibaut, 2018). Women also reach higher blood-alcohol concentrations than men after equivalent consumption due to lower lean body mass and reduced gastric alcohol-dehydrogenase activity (Komáreková et al., 2013). Consequently, women may suffer alcohol-induced hepatic and cardiac damage at lower doses and shorter durations—a "clinical telescoping" effect (McHugh et al., 2018). Many medical complications of addiction —neuropathy, alcohol-related cognitive decline, cardiomyopathy, cirrhosis, and substance-induced mood disorders— appear earlier and after lower cumulative exposure in women (Trillo et al., 2012). With opioids, morphine shows slower μ-opioid-receptor activation and clearance in women, often necessitating higher doses and making them more prone to hyperalgesia and rapid tolerance, potentially requiring larger opioid doses for equivalent analgesia (Fullerton et al., 2018).

In men, a more stable androgen-dominated hormonal profile exists, and its influence on addictive behavior is less studied than that of estrogens and progesterone. Testosterone and derivatives can modulate dopaminergic and glutamatergic transmission, and some evidence links high androgen levels to greater risk-taking and sensation-seeking, facilitating initiation of use. However, direct testosterone effects on drug reinforcement are less clear and appear subtler than estrogen's impact in women (Bawor et al., 2015). In opioid-use disorder, any opioid intake markedly reduces or suppresses testosterone in men but not in women (Bawor et al., 2015), underscoring sex-specific regulation of the hypothalamic–pituitary–gonadal axis and its hormones GnRH, LH, and FSH (Katz & Mazer, 2009). Resulting mood, stress reactivity, aggression, and libido changes tied to testosterone fluctuations can therefore trigger markedly different responses in male versus female opioid users (Börjesson et al., 2011; Fullerton et al., 2018; Smith & Elliott, 2012).

Understanding the biological determinants of vulnerability to addiction can be very useful in developing psychoeducational programs and interventions aimed at prevention and treatment. Although biological factors help explain some of the differences in addiction vulnerability, a comprehensive understanding of why certain individuals initiate and sustain substance use is possible only when psychosocial determinants are also taken into account.

Sex, Gender, And Social Expectations

Over the past decades, substance-use disorder research has moved toward a more holistic perspective that, in addition to biological determinants, also considers psychological and social factors to understand vulnerability to addiction. Within this framework, sex and gender emerge as essential analytical categories for explaining the differences seen in epidemiological trends, patterns of use, clinical presentations, and access to treatment (Becker & Koob, 2016; Fonseca et al., 2021; Greenfield et al., 2010). Sex differences refer to genetically determined biological characteristics, whereas gender refers to the socially constructed and variable roles, norms, expectations, and behaviors associated with men, women, and non-binary people (Kaufman et al., 2023; World Health Organization, 2011). These norms are deeply embedded in social structures and have evolved over time. In the realm of addiction, gender expectations play a crucial role in vulnerability to substance use and in the development of alcohol-use disorder, influencing both the decision to consume and access to treatment.

According to the World Health Organization (2011), gender norms affect not only people's emotional and social experiences but also their mental and physical health. Social pressures linked to gender roles may therefore lead men and women to experience exposure to substances, the progression of use, and treatment responses differently (Harris et al., 2022). Historically, substance use has been more prevalent among men, which has led to addiction services, research, and policies that are male-centered or "gender-neutral," leaving the specific needs of women and other gender identities unaddressed (Fonseca et al., 2021; Harris et al., 2022; Meyer et al., 2019; Torrens-Melich et al., 2021). Scientific literature indicates that the gender gap in SUDs is narrowing for certain substances and age groups, an alarming trend when psychosocial and clinical settings do not adequately recognize or meet differentiated needs.

Beyond the biological-sex vulnerabilities described in the previous section, social expectations grounded in gender shape consumption behaviors. Social dynamics, especially interpersonal relationships and community

contexts, strongly influence men's and women's patterns of use, because traditional norms assign specific roles to each gender. Men are typically linked to protection and provider roles within the family, characterized by competitiveness, confidence, and persistence; women, to reproductive, caregiving, and emotional-support roles, with traits such as dedication, expressiveness, and empathy (Sánchez-López, 2013; Sánchez-López & Limiñana-Gras, 2017).

Conformity to these norms, however, varies with contextual and individual factors, producing different degrees of adherence or resistance (Sánchez-López et al., 2014).

Femininity and masculinity thus play a significant role in addiction vulnerability. Femininity encompasses the culturally defined traits, behaviors, and roles considered proper for women, shaping expectations about how they should think, feel, and act (Mahalik et al., 2005). In Western societies, normative femininity idealizes, among other qualities, caregiving, emotional expressiveness, and concern with appearance (Levant et al., 2007; Mahalik et al., 2005). Adherence varies across racial and cultural groups: for example, Latin culture values subordination and self-sacrifice; the "strong Black woman" stereotype emphasizes resilience; Asian hyper-femininity prizes docility and reliability (Castillo et al., 2010; Donovan & West, 2014). These norms shape behavior and can create differential risks for substance use (Kulis et al., 2008); understanding vulnerability therefore demands an intersectional approach that integrates gender, ethnicity, and cultural expectations.

A recent systematic review showed that femininity significantly influences within-group variations in women's substance use: 74 % of the studies (n = 17) found that endorsement of feminine norms accounted for a unique portion of variance in consumption (Brady et al., 2016). Although substance use has traditionally been linked to masculinity (Van Gundy et al., 2005), recent research also connects feminine norms to use (Iwamoto et al., 2016). Engaging in stereotypically masculine behaviors such as drinking can be associated, for many women, with empowerment, pleasure, and peer approval (Lyons & Willott, 2008). While femininity often promotes health-seeking behaviors (e.g., getting help) (Shakya et al., 2019), some traits today may increase risk (Kaya et al., 2016). Examining how femininity shapes consumption is essential, as women frequently use substances to manage emotional distress (Dragan, 2015) and are vulnerable to gender-role stress; they often turn to substances to cope with emotional or physical pain and to self-medicate mental-health problems, frequently in response to traumatic experiences (National Institute on Drug Abuse, 2020; Stone et al., 2021).

Additionally, women are at greater risk than men of being introduced to drug use by their partners, suggesting an initiation pattern shaped by intimate relationships and co- dependence (Mburu et al., 2019). Rigid adherence to feminine norms can generate dissatisfaction, devaluation, and shame, raising the likelihood of substance use as a coping strategy (Efthim et al., 2011; Hoffman, 2001). Conversely, a strong identification with traditional femininity may deter some women, as substance use conflicts with expected "proper" behavior (Iwamoto et al., 2018).

Research on substance use often focuses on how femininity or minority identities (e.g., women, LGBTQ+ individuals) shape vulnerability, while the influence of masculinity norms remains underexplored. However, many men engage in substance use to align with ideals of toughness, suppress emotions, or respond to social pressures tied to hegemonic masculinity (Klingemann & Klingemann, 2023). Overlooking this perspective limits the understanding of substance use among men. Integrating gender roles into interventions may enhance both their effectiveness and contextual relevance

Social factors and expectations can also act as protective elements. Women are generally less exposed than men to drug- or alcohol-centered social environments and tend to form close, supportive relationships, which foster psychological resilience (McHugh et al., 2018). Caregiving responsibilities, that sometimes become barriers, can likewise motivate treatment seeking, particularly efforts to maintain or regain child custody (Greenfield et al., 2007; Sword et al., 2009).

Finally, stigma is a key factor that profoundly shapes experiences and outcomes, especially for women. Women with addictions face dual stigma: for their dependence and for violating gender norms of self-control and responsibility (Howard, 2015). This stigma can prompt concealment or delayed help-seeking.

Regarding people whose sexual orientation is non-normative (gay, lesbian, and bisexual individuals), recent evidence indicates that substance-use risk is patterned by sexual identity rather than by sex alone. Lesbian and bisexual women display elevated rates of alcohol- and drug-related disorders, whereas gay and bisexual men are more likely to use illicit drugs and encounter related problems (Green & Feinstein, 2012).

These disparities appear to be shaped by sociocultural factors, such as affiliation with gay culture or HIV status, and are not mitigated by demographic characteristics like female sex or older age (Green & Feinstein, 2012). In fact, bisexual orientation is consistently linked to the highest substance-use risk: bisexual women engage in more forms of use than lesbian or gay women, and no substance-use domain shows lower risk for bisexuals (Schuler & Collins, 2020; Schuler et al., 2018).

As for gender-nonconforming individuals, the determinants are somewhat more complex and will be explained in the next section.

Vulnerability In Gender-Nonconforming Individuals

Among gender-nonconforming people, that is, individuals whose gender identity differs from their sex assigned at birth, sex and gender issues become fundamental and even defining when it comes to vulnerability to addiction. Recent studies show that transgender and non-binary (TNB) individuals have higher prevalence rates of alcohol, tobacco, cannabis, and psychostimulant use, as well as greater involvement in chemsex practices, than their cisgender peers (Cotaina et al., 2022; Scheim et al., 2017). For instance, Scheim et al. (2017) estimated in a Canadian sample that 12 % of TNB people had used at least one high-risk drug in the preceding year, particularly cocaine and amphetamines, a proportion higher than in the general population. Likewise, a recent meta-analysis determined that identifying as TNB roughly doubled the odds of tobacco and drug use compared with cisgender individuals, although no significant differences were found for alcohol use (Cotaina et al., 2022). In addition, analysis of thousands of U.S. medical records revealed higher rates of nicotine, alcohol, and drug-abuse diagnoses in TNB patients than in cis patients (Kidd et al., 2023). Collectively, these data underscore that TNB people bear a disproportionate burden of problematic substance use relative to the cisgender population, making them more vulnerable.

Research has often focused on transgender women, those assigned male at birth, particularly in socially vulnerable contexts and has confirmed high prevalence of methamphetamine and cocaine use (Reback & Fletcher, 2014). In transgender women engaged in sex work, many reported recent methamphetamine or other stimulant use to prolong and enhance sexual encounters (Santos et al., 2014). Continuing the theme of substances used for sexual purposes, chemsex is especially relevant among TNB individuals and reveals differences even within this group; clinical data show stimulant-use rates up to ten times higher in transgender women than in transgender men (Gómez-Gil et al., 2012). However, a recent U.S. study by Kidd et al. (2023) concluded that there were no significant differences between transgender men and women regarding problematic substance use.

Why is this vulnerability higher? Several studies indicate that substance use among TNB people often serves as a coping strategy for chronic stress arising from discrimination, rejection, and trauma (Hendricks & Testa, 2012). TNB individuals frequently face stigma and prejudice in family school, workplace, and healthcare settings, as well as transfobic violence throughout life, all of which contribute to sustained experiences of social exclusion and chronic stress. Empirical evidence shows that transgender people who report higher levels of discrimination also present significantly elevated rates of recent substance use and lifetime substance use disorders, highlighting the cumulative impact of chronic exposure to stigma and exclusion (Wolfe et al., 2021). Faced with insecurity, emotional stress, and perceived lack of support, substance use may emerge as self-medication (Bockting et al., 2013; Kidd et al., 2023). For example, transgender women with a history of sexual trauma or violence have significantly higher odds of using drugs like cocaine to cope with psychological pain (Budhwani et al., 2017). Similarly, in a U.S. study of 600 adult trans people, those who experienced frequent transfobic discrimination showed much higher rates of recent use, lifetime substance-use disorders, and likelihood of having needed treatment (Wolfe et al., 2021). These findings confirm that continual exposure to rejection and violence increases the risk of turning to psychoactive substances to manage stress and emotional distress (Hughto et al., 2021; Klein & Washington, 2024).

Another potentially decisive determinant of TNB vulnerability is the healthcare environment. Living in a cisnormative care system means that mental-health and addiction services were built on the premise that being cisgender is the norm and that being trans was historically classified as a disorder. Consequently, interventions were designed only for cisgender men or women, and even today many programs lack affirmative protocols for TNB people; only a few are specifically geared to the LGBTIQ+ population (Williams & Fish, 2020). A direct outcome of this binary approach is the lack of visibility of the non-binary community: non-binary individuals show poorer mental health than binary trans people, presumably because of the added stress of being invalidated or ignored by the healthcare system, and they report higher anxiety, depression, and risk behaviors than their binary peers (Klein & Washington, 2024; Reisner & Hughto, 2019; Thorne et al., 2019). Moreover, non-binary people have been excluded from many substance-use studies (Connolly & Gilchrist, 2020), further reinforcing their invisibility. A qualitative study in Argentina highlighted that the lack of trans-health education among providers is a main obstacle to appropriate care (Cordero & Saletti-Cuesta, 2025). Likewise, recent work by Jessani et al. (2024) noted insufficient preparation of healthcare teams to recognize and address the needs of transgender and gender-diverse people, especially outside major urban centers. In the face of this training deficit, various authors call for cultural-competence training and affirmative-practice models to improve care quality and reduce biases linked to underdiagnosis and undertreatment (Hughto et al., 2015; Korpaisarn & Safer, 2018), as repeated discrimination is associated with greater psychological distress, risk of depression, and substance abuse, factors that drive many TNB individuals away from formal services and lead to self-medication and self-care as survival strategies (Johnson et al., 2019; Schechner et al., 2025).

All these factors that heighten the vulnerability of TNB people to addiction mean that interventions must be adaptable to this population and attentive to its specificneeds.

Sex, Gender And Dual Diagnosis

Psychiatric comorbidity among individuals with substance-use disorders is remarkably high, with prevalence estimates ranging from 50% to 80%, depending on the population studied (Andersson et al., 2023; Díaz-Fernández et al., 2023; Fernández-Miranda et al., 2024; Szerman et al., 2022). The simultaneous presence of a SUD and another mental disorder is referred to as dual diagnosis (DD) (Fernández-Artamendi et al., 2024; Szerman et al., 2022). The explanation with the strongest scientific backing is that both conditions share common neurobiological bases and genetic risk factors, while the toxic substances consumed alter many neurotransmission systems involved in the pathogenesis of these other mental disorders (Szerman et al., 2022). Determining the primary diagnosis in DD cases is often complex. Most studies have focused on linking substance use to the development of psychiatric disorders; far fewer examine the reverse relationship, and fewer still incorporate a gender perspective.

The limited studies that do consider sex and gender as variables indicate that anxiety and affective disorders are more prevalent among women with DD (Díaz-Fernández et al., 2023; Szerman et al., 2015). Anxiety is a significant risk factor for substance use, especially alcohol and cannabis (Prieto-Arenas et al., 2022). For instance, a study on social anxiety found a stronger association between this condition and alcohol-related problems in women than in men (Buckner et al., 2023). Similarly, panic disorder has been linked to a greater risk of developing alcohol dependence, about twice as high in women as in men (Chang et al., 2020). Gender differences have also been identified in patterns of psychiatric comorbidity and in the use of health services prior to the onset of alcohol dependence. Among young people, cannabis use has been described as a coping strategy for anxiety and low distress tolerance (Morris et al., 2024). Low distress tolerance is significantly associated with heavier cannabis use and related complications. Although women tend to report lower overall consumption, their use is more often motivated by the management of psychiatric symptoms (Arranz et al., 2020; Leadbeater et al., 2019).

Gender disparities are likewise evident in the comorbidity between psychotic disorders and SUDs. Patients with schizophrenia face a three-fold higher risk of developing cannabis-use disorder than the general population (Kozak et al., 2021). Even though women with schizophrenia display a lower substance-use prevalence (Gómez-Sánchez-Lafuente et al., 2022; Novick et al., 2016), substance misuse seems to have more detrimental long-term effects for them (Casanovas et al., 2023; van der Meer et al., 2015). Women with schizophrenia and cannabis-use disorder typically experience more severe symptoms, more hospitalizations, and poorer treatment outcomes (Miquel et al., 2013) and are less likely to reduce cannabis use over time, worsening illness trajectory (Calakos et al., 2017; Casanovas et al., 2023).

The co-occurrence of a psychiatric disorder and a SUD is linked to more severe clinical symptoms and greater psychosocial dysfunction than is usually seen in individuals with a single disorder (Andersson et al., 2023; Mangrum et al., 2006), and this is especially pronounced in women with dual diagnosis. These women face multiple, interconnected vulnerabilities that complicate both clinical and social care. The literature shows that, compared with men with similar diagnoses, they are disproportionately affected by histories of trauma, gender-based violence, and abuse, which increases the risk of relapse, non-adherence, and poorer health outcomes (Fonseca et al., 2021; Meyer et al., 2019; Tirado-Muñoz, 2018; Torrens-Melich et al., 2021). In addition, the interaction between psychiatric symptoms and substance use hinders timely diagnosis and contributes to a higher likelihood of social exclusion, unemployment, stigmatization, and limited access to gender-responsive integrated services (McHugh et al., 2018).

Women with DD also suffer from triple stigma: for being women, for having a mental disorder, and for experiencing a SUD. This compounded stigma may foster shame, guilt, and isolation, decreasing the likelihood of seeking or staying in treatment (Calderón Calvo, 2021). Many also lack strong social and family support networks, key elements in recovery. Both internalized and social stigma not only reinforce negative stereotypes and prejudices but directly affect how these women access, use, and benefit from health services (McCartin et al., 2022).

Although the small number of gender-focused studies hampers analysis of sex differences in dual diagnosis, the lack of data on sexual orientation and gender identity poses an even greater challenge to understanding disparities and designing tailored interventions for LGBTIQ+ populations (Flentje et al., 2015). In this regard, 56 % of LGBTIQ+ youths met criteria for at least one mental-health or substance-use disorder in the past year, compared with 29 % of their cis-heterosexual peers (Kingsbury & Findlay, 2024).

This group not only shows disproportionately high rates of substance use and SUDs but is also more susceptible to severe mental-health problems, such as higher levels of anxiety, depression, suicidal ideation, and greater exposure to trauma and PTSD (Marchi et al., 2023). They also face higher vulnerability to infections such as HIV and HCV, especially in the context of high-risk behaviors related to chemsex. Structural and interpersonal factors, like discrimination, stigmatization, harassment, and internalized homophobia, worsen psychological distress and act as significant barriers to adequate care (Kidd et al., 2019; Marchi et al., 2024; Silveri et al., 2022).

Thus, transgender and gender-diverse individuals face increased vulnerability to substance use disorders due to structural factors such as exclusionary public policies, limited access to inclusive healthcare services, and stigmatizing social norms (Poteat et al., 2023). Recent legislative frameworks specifically target LGBTQ+ youth, increasing their risk of academic underachievement, suicide, and discrimination. Policies that restrict access to gender-affirming care create ethical dilemmas for professionals by limiting their ability to provide safe, evidence-based treatment (Kline et al., 2023). These structural determinants significantly hinder effective prevention and treatment of substance misuse within LGBTQ+ populations (Phillips et al., 2025).

Conclusions

The evidence synthesized in this review indicates that gendered constructs, femininity, masculinity to non-binary identities, shape neurobiological, psychological, and social vulnerability to substance-use disorders (SUDs) and their frequent psychiatric comorbidities (DD). Rigid adherence to normative roles heightens risk behaviors, whereas incongruence between lived identity and societal expectations drives stress, trauma exposure, and maladaptive coping through substance use. These findings demand prevention and treatment strategies that explicitly account for gender identity, expression, and intersectional determinants such as class, race, and sexual orientation.

Only by dismantling binary, cisnormative assumptions in research design, diagnostic criteria, and service delivery, culturally informed interventions that target the mechanisms linking gendered stressors to addiction trajectories can be generated.

Conflict of interest

All authors declare that they have no conflicts of interest.

Financial support

This work was supported by grants: PND2024-I037 and PND2024- I092 funded by Plan Nacional Sobre Drogas (Ministerio de Sanidad); RD24/0003/0004 and RD24/0003/0001 funded by Instituto de Salud Carlos III (ISCIII) and co-funded by the European Union; and Cátedra Género y Salud of the University of Valencia.

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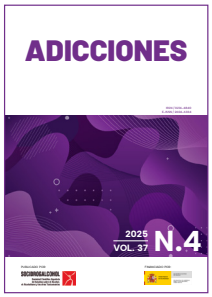

ORIGINAL

Soy isoflavones for the treatment of cocaine use disorder: An open-label pilot study

Isoflavonas de soja para el tratamiento del trastorno por consumo de cocaína: Un estudio piloto abierto

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Abstract

Soybeans contain different isoflavones (mainly daidzein) which work as reversible inhibitors of aldehyde-dehydrogenase-2 enzyme (ALDH2). This activity has been related in animal experiments with a reduction of cocaine use. Our aim was to carry out an open-label pilot study to evaluate the possible efficacy of soy isoflavones as natural inhibitor of ALDH2 in cocaine use disorder. Nine subjects with severe cocaine use disorder participated in a single-center, open, non-controlled trial during 12 weeks of treatment and 4 of follow-up. The Substance Use Report (SUR) showed that three subjects (33.3%) reported a cocaine consumption of less than 20% (80% non-use days) from 10 to 12 weeks of the treatment period, from two (22.2%) at baseline, although non-significant. A finding that could not be confirmed by the detection of urine metabolites of cocaine. Seven participants (77.8%) completed the study at 16 weeks and one (1.11%) at 12 weeks. Urine concentrations of isoflavones, demonstrated that eight participants (88.9%) followed the treatment along the study. The Severity Dependence Scale (SDS) score showed a significant decrease between baseline to 12 weeks, baseline to 16 weeks and 12 to 16 weeks; the Brief Substance Craving Scale (BSCS) and Cocaine Selective Severity Assessment (CSSA) decreased their values but not significantly. Significant improvements in different areas of the SF-36 scale were observed: body pain scores decreased from baseline to 16 weeks statistically significant; social function improved its scores from baseline to 12 weeks and from baseline to 16 weeks significantly; the rest of areas increased their scores but not significantly. These findings show lower ratios of cocaine use days, and high retention and adherence to treatment although the acquisition of complete abstinence was not observed. Soy isoflavones could be considered a potential treatment in future research, to be confirmed by placebo-controlled studies with adequate sample size.
Keywords: cocaine, aldehyde-dehydrogenase-2 enzyme, daidzin, daidzein, soy isoflavone extract, clinical trial

Resumen

La soja y sus habas contienen diferentes isoflavonas (principalmente daidzeína) que actúan como inhibidores reversibles de la enzima aldehído-deshidrogenasa-2 (ALDH2). Esta actividad se ha relacionado en experimentos con animales con una reducción del consumo de cocaína. Nuestro objetivo fue llevar a cabo un estudio clínico piloto abierto para evaluar la posible eficacia de las isoflavonas de soja, como inhibidor natural de ALDH2, en el trastorno por consumo de cocaína. Nueve sujetos con trastorno grave por consumo de cocaína participaron en un ensayo unicéntrico, abierto y no controlado durante 12 semanas de tratamiento y 4 de seguimiento. El Substance Use Report (SUR) mostró que tres sujetos (33,3%) informaron un consumo de cocaína inferior al 20% (80% de días sin consumo) entre las semanas 10 y 12 del periodo de tratamiento, frente a dos (22,2%) al inicio, aunque sin significación estadística. Un hallazgo que no pudo ser confirmado mediante la detección de metabolitos urinarios de cocaína. Siete participantes (77,8%) completaron el estudio en la semana 16 y uno (11,1%) en la semana 12. Las concentraciones urinarias de isoflavonas demostraron que ocho participantes (88,9%) siguieron el tratamiento a lo largo del estudio. La puntuación de la Severity Dependence Scale (SDS) mostró una disminución significativa entre el inicio y las 12 semanas, entre el inicio y las 16 semanas y entre las semanas 12 y 16; la Brief Substance Craving Scale (BSCS) y la Cocaine Selective Severity Assessment (CSSA) disminuyeron sus valores pero no significativamente. Se observaron mejoras significativas en diferentes áreas de la escala Health Survey SF-36 (SF-36): las puntuaciones de dolor corporal disminuyeron desde el inicio hasta 16 semanas de forma estadísticamente significativa; la función social mejoró sus puntuaciones desde el inicio hasta 12 semanas y desde el inicio hasta 16 semanas de manera significativa; el resto de las áreas aumentaron sus puntuaciones pero sin significación. Estos hallazgos muestran menores proporciones de días de consumo de cocaína, y elevada retención y adherencia al tratamiento, aunque no se observó la adquisición de abstinencia completa. Las isoflavonas de soja podrían considerarse un tratamiento potencial en futuras investigaciones, lo cual deberá confirmarse en estudios controlados con placebo y tamaño muestral adecuado.
Palabras clave: cocaína, enzima aldehído-deshidrogenasa-2, daidzina, daidzeína, extracto de isoflavonas de soja, estudio clínico

■ *Received: May 2025; Accepted: September 2025.*

■ ISSN: 0214-4840 / E-ISSN: 2604-6334

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The 2024 World Drug Report, published by the United Nations Office on Drug and Crime (UNODC, 2024), states that in 2022 approximately 23 million individuals (range: 18.5-29.6 million; 0.45%-0.57% of the total population), had used cocaine almost once in the previous year. According to the 2024 European Drug Report from the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (EMCDDA, 2024), cocaine has become the second most frequently consumed illicit drug in Europe. The report also warns about the increasing potential health risks.

Cocaine acts by inhibiting the monoamine transporters of the presynaptic neuronal membrane. Through this inhibition it blocks the reuptake of monoamines resulting an increase of dopamine and the other monoamine concentrations (norepinephrine and serotonin) in the synaptic cleft (Camí et al., 2003; Fernàndez-Castillo et al., 2021). Euphoria, feeling of pleasure, and other positive reinforcing effects of cocaine are related not only with changes in dopamine transporter at the presynaptic level but also with changes in dopamine homo and heteroreceptor complexes at the post-synaptic level and with the maladaptive development of drug memory, in both anti-reward and reward ventral striato-pallidal GABA pathways (Borroto-Escuela et al., 2019; Milton & Everitt, 2012). Continued, chronic administration of cocaine induces changes in several neurotransmitter systems affecting the function of several areas and circuits such as the mesocorticolimbic system (nucleus accumbens, ventral tegmental and prefrontal cortex) (Fernàndez-Castillo et al., 2021). Produces a reduction in the number of dopamine receptors in the central nervous system and, as a result, a decreased sensitivity of the reward system (Ashok et al., 2017). However, recent works demonstrate that other changes at the dopamine receptor structure and function must be considered, for instance, cocaine induced pathological A2AR-D2R, D2R-Sigma1R and A2AR-D2R-Sigma1R complexes which may form a long-term memory with a strong and permanent D2R brake, leading to cocaine addiction (Borroto-Escuela et al., 2017; Borroto-Escuela et al., 2018; Koob & Volkow , 2010). At the clinical level such molecular changes result in an increase in repetitive and compulsive consumption (binge use) (Koob & Volkow, 2010) and loss of behavioral control. Neuroanatomical abnormalities have been described in cocaine users such as a reduction in the grey matter volumes in the prefrontal cortex and consequent dysfunction of this region (Ciccarone & Shoptaw, 2022; Hirsiger et al., 2019; Volkow et al., 2011)

There are as yet no specific drugs or psychotherapy for the treatment of cocaine substance use (Farrel et al., 2019; Kampman, 2019). Data about the therapeutic use of certain drugs, including anticonvulsants (Alvarez et al., 2010; Minozzi et al., 2015), psychostimulants (Castells et al., 2016; Pérez-Mañá et al., 2011) , antidepressants (Pani et al., 2011; Torrens et al., 2011) and antipsychotics (Álvarez et al., 2013; Bentzley et al., 2021; Indave et al., 2016), have been published although lacking sufficient efficacy and evidence. Current lines of research are focused on the production mechanisms of different monoamines (Kohut et al., 2017), and, to date, disulfiram, which interacts with dopamine production, has shown the most promising clinical outcomes (Gaval-Cruz et al., 2009; Kampangkaew et al., 2019; Pani et al., 2010; Schroeder et al., 2010; Weinshenker, 2010;). Findings are, however, still controversial as some studies have reported beneficial results while others have not (Carroll et al., 2016). On the other hand, considering the high rates of concomitant consumption of alcohol and cocaine (60% or more) in the population (Araos et al., 2017) there is a crucial limitation in the use of disulfiram. This compound is contraindicated if alcohol is simultaneous consumed because it is an irreversible inhibitor of aldehyde dehydrogenase-1 (ALDH1) and could induce an adverse reaction.

Several studies have suggested that selective inhibitors of the aldehyde-dehydrogenase-2 enzyme (ALDH2) could be effective in the treatment of cocaine use disorder (Koppaka et al., 2012; Weinshenker, 2010; Yao et al., 2010). Inhibition of ALDH2 blocks the conversion of the substrate 3,4-Dihydroxyphenylacetaldehyde (DOPAL) to 3,4-Dihydroxyphenylacetic acid (DOPAC) increasing its levels and consequently forming with dopamine the product of condensation tetrahydropapaveroline (THP) in the ventral tegmental area. THP decreases dopamine biosynthesis through the inhibition of the tyrosine hydroxylase (TH) and, as a result, the ability to produce positive reinforcement is reduced. Natural selective reversible inhibitors of ALDH2 have been described in soy extract isoflavones (Lowe et al., 2008). The most prominent of which are genistin, glycitin, and especially daidzin. The active metabolites of daidzin, genistin, and glycitin are daidzein, genistein, and glycitein, respectively. In addition, at the intestinal level, bacteria transform daidzein into another active metabolite, equol. Various studies have confirmed the selective action of isoflavones on ALDH2, but not on ALDH1, in contrast to disulfiram. As a consequence, if alcohol is consumed the related adverse effects will not appear (Amigdala Neurosciences, 2025; Martínez-Riera et al., 2019; Penetar et al., 2011). For centuries traditional Chinese medicine has employed isoflavones for the treatment of the alcohol use disorder (Lu et al., 2009; Overstreet et al., 2003), and studies in animals (Arolfo et al., 2009) and humans have demonstrated a reduction in alcohol intake (Lukas et al., 2013; Penetar et al., 2015). There are, however, no data in humans measuring the possible effects of isoflavones or other synthetic inhibitors of ALDH2 on cocaine use. In animal models, the administration of different isoflavones has resulted in specific results, such as the reduction of relapses

conditioned by environmental stimuli and a decrease in repeated consumption (Martin et al., 2021; Yao et al., 2010).

Traditional abstinence-only approaches to substance use disorder treatment often fail to engage many individuals who are not ready or willing to stop using substances entirely (Paquette, 2022). Recognizing that addiction is a chronic condition rather than a binary state, incorporating non-abstinence strategies can increase treatment engagement, retention, and effectiveness by aligning with patients' goals and focusing on alternative endpoints, such as reduced use and improved quality of life, rather than strict abstinence. (Compton & Volkow, 2024; Volkow, 2020)

The objective of this study is to assess the effects of soy isoflavones in patients with cocaine use disorder.

Material and method

Ethics Approval

The study protocol was approved by the local Human Research Ethics Committee (CEIC-Parc de Salut Mar, ref. 2014/5580) and conducted in accordance with the Declaration of Helsinki (Fortaleza, 2013) and local legislation (Biomedical Research Law, 2008).

Study Design

An open-label, single-center, clinical trial was carried out during 12 weeks of treatment and 4 weeks follow-up. All the participants were informed about the study and signed a written consent before taking part. Subjects participated in a financial incentive program to facilitate retention and adherence. All the participants received the same treatment and dose of soy isoflavone extracts (two capsules/12h, see section Soy Isoflavone Extract). The principal study variable was to assess cocaine use days from 10 to 12 weeks of the treatment period. This was measured by the percentage of cocaine use days self-reported by the subjects and confirmed by the detection of urine cocaine metabolites (benzoylecgonine and ecgonine methyl ester). The secondary variables included the mean percentage of negative urine samples for the cocaine metabolites from all the scheduled urine samples from 5 to 12 weeks of the treatment period, retention to treatment, adherence to treatment, reduction in craving and severity of cocaine substance use, and quality of life (see Clinical Evaluation Instruments).

Soy Isoflavone Extract

A commercially available soy extract product (Super-Absorbable Soy Isoflavones®, hard gelatine capsules, Life-Extension, US) was used. Preparation was previously selected after pharmacokinetic (Rodríguez-Morató et al., 2015) and safety studies (Martínez-Riera et al., 2019). Each capsule, according to the manufacturer, was composed of 54mg of total isoflavones (22mg daidzin-daidzein, 28mg genistin-genistein, and 4mg glycitin-glycitein). The dose administered (four capsules/day, two in the morning and two at night), was adjusted to give a total daidzein/daidzein content of approximately 88mg. It was selected so as to be double that recommended as herbal medicine for asthma therapy (Smith et al., 2015), and similar or double doses for menopausal symptoms (according to the technical sheet of the product and others on the market) (Carmignani et al., 2010; Khaodhiar et al., 2008; Rebcack et al., 2004; RxList, 2022; Yang et al., 2012) and knowing the safety of these doses from previous studies (Martínez-Riera et al., 2019).

Subjects

Participants, eight men and one woman, from the Outpatient Treatment Center of Substance Use, Institut de Neuropsiquiatria i Addiccions, Parc de Salut Mar from Barcelona, Spain, with a cocaine use disorder were recruited according to DSM-5 (American Psychiatric Association, 2013) guidelines. They met the following inclusion criteria: (i) aged ≥ 18 years and< 60 years; (ii) seeking treatment for cocaine use disorder; (iii) having at least one positive urine sample in the two weeks prior to commencement of the study sessions; and (iv) women with reproductive potential taking contraceptives. Exclusion criteria were: (i) presenting an active substance use disorder (DSM 5) other than cocaine in the previous year except tobacco, cannabis and alcohol without severe symptoms of physical withdrawal; (ii) having been in treatment with a substitutive opioid (methadone, buprenorphine) in the previous 2 months; (iii) presenting a neurologic or severe psychiatric illness that could interfere with the development of the study; (iv) any serious medical conditions that could interfere with the safety of the subjects or the development of the study; (v) HIV, hepatitis, active syphilis, tuberculosis; (vi) being under a compulsory treatment; (vii) personal history of endometrial or breast cancer or other hormone-dependent cancer; (viii) hypersensitivity to soy derivatives; (ix) under treatment with soy derivatives for another reason; (x) to have been/or be under treatment with drugs that could have adverse symptoms interacting with isofla-vones or could interfere with the study results; and (xi) to be pregnant o lactating.

Clinical Assessment

Sociodemographic data, and previous history of medical and psychiatric illnesses were collected.

Cocaine Use Days

To assess the cocaine use days the Substance Use Report/Inventory (SUR) (Weiss et al., 1995) was used. This is a self-reported questionnaire with a daily calendar that measures recent use of drugs, dose, and route of administration. Less than 20% self-reported cocaine use days was considered a promising result.

Urine samples were collected three times a week to quantify concentrations of urine metabolites of cocaine.

Cocaine non-use values were benzoylecgonine<150ng/ml and ecgonine methyl ester<15ng/ml. Less than 20% positive urine samples were considered a promising result.

Retention and Adherence to Treatment

Retention was measured by the number of subjects who finished the study. Adherence to treatment was assessed by the determination in urine of daidzein, genistein, and the endogenous metabolite equol through liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) using a validated method (Rodríguez-Morató et al., 2015). Samples were collected three times a week.

Clinical Evaluation Instruments

Psychiatric diagnosis was performed with the Spanish Version of the Psychiatric Research Interview for Substance and Mental Disorders IV (PRISM-IV) (Torrens et al., 2004). This is a semi-structured interview designed to evaluate current and life-long disorders of DSM-IV-TR (American Psychiatric Association, 2000).

In addition, the following instruments in their validated Spanish versions were administered:

- Clinical Global Impression (CGI) (Guy, 1976), a hetero-applied scale composed of two sub-scales CGI-S (measures disorder severity) and CGI-I (measures disorder improvement during the consultations). Each is composed of a unique item scored with a Likert-type scale response format. A CGI-S score of 0 represents non-evaluated, and then ranges from 1 "healthy subject" to 7 "extremely ill subject"; a CGI-I score of 0 represents non-evaluated, and then ranges from 1"significant improvement" to 7 "most severe deterioration".
- Addiction Severity Index Lite (ASI-lite) (Cacciola et al., 2007), a semi-structured interview which is the shortened version of the Addiction Severity Index (ASI). It evaluates addiction severity in several areas: medical, psychological, family/social, legal, employment, use of drugs, and use of alcohol. Lifetime information and data within the previous 30 days are obtained. The scores for each area are from 0 to 1, the higher is the score, the greater the severity of use disorder.
- Severity Dependence Scale (SDS) (González-Saiz et al., 2008), a self-applied instrument that measures addiction severity with five items rated with a Likert-type scale from 0 to 3. The global score is the sum of the scores in every item. The higher the punctuation, the greater the severity of use disorder.
- Cocaine Selective Severity Assessment (CSSA) (Kampman et al., 1998, Pérez de los Cobos et al., 2014), a hetero-applied scale that specifically measures severity of cocaine substance use. It is composed of eighteen items about symptoms that usually appear when cocaine consumption suddenly ceases (e.g., craving, depressive symptoms, changes in appetite, sleep disorders, lethargy, and bradycardia). Each item is graded with a Likert-type scale from 0 to 7. The global punctuation is the sum of the scores in every item. The higher the punctuation, the greater the severity of use disorder.
- Brief Substance Craving Scale (BSCS) (Somoza et al., 1995), a self-applied scale that measures the intensity and frequency of the craving for cocaine and other substances in the previous twenty-four hours. There are sixteen items scored with a Likert-type scale from 0 to 4. The global score is the sum of the scores in every item. The higher the punctuation, the greater the severity of use disorder.
- SF-36 Health Survey (SF-36) (Alonso et al., 1995), a self-applied scale that assess quality of life related to health. It measures eight dimensions (physical function, physical role, body pain, general health, vitality, social function, emotional role, and mental health) with a rating from 0 to 100. The higher the score, the better the level of health.
- Revised Clinical Institute Withdrawal Assessment for Alcohol Scale (CIWA-Ar) (Sullivan et al., 1989), a hetero-applied scale that measures the severity of alcohol abstinence syndrome. It has ten items punctuated with a Likert-type scale from 0 to 7 with the exception of one item "orientation and clouding of sensorium", which is scored from 0 to 4. The global punctuation is the sum of the scores in every item. The higher the score, the greater the severity of abstinence.
- HIV Risk-Taking Behaviour Scale (HRBS) (Darke et al., 1991), a hetero-applied scale that evaluates the use of intravenous drugs and sexual risk behaviours. There are eleven items scored with a Likert-type scale from 0 to 5. The higher the score the greater the degree of risk-taking.
- Hamilton Rating Scale for Depression (HAM-D) (Bobes et al., 2003), a hetero-applied scale measuring the patient's subjective level of depression with twenty one symptoms. The global score is the sum of the scores for each item. The higher the punctuation, the greater the severity of depression.
- Columbia Suicide Severity Rating Scale (C-SSRS) (Al-Halabí et al., 2016), a semi-structured scale for the assessment of the intensity of suicide risk. It measures four constructs: severity of ideation, intensity of ideation, behaviour, and lethality.

Procedure

During the screening session, the participants underwent a general physical examination, a 12-lead electrocardiogram, a complete blood and urine analysis (including drugs of abuse), and a pregnancy test in the case of women. In

addition, the following scales were administered: PRISM, SUR, SDS, CSSA, BSCS, CIWA-Ar, HAM-D, and C-SSRS.

The subjects underwent 12 weeks of treatment and 4 weeks of follow-up. Urine samples were collected three times a week during the study. Blood samples at weeks 6, 12, and 16, pregnancy test at baseline, 6, and 12 weeks, and a 12-lead ECG at 12 weeks were carried out. CGI, SUR, SDS, CSSA, BSCS and the assessment of possible adverse effects, were collected at baseline, 2, 4, 6, 8, 10, 12, and 16 weeks. The ASI-Lite scale was performed at baseline and 12 weeks. SF-36 and HRBS were evaluated at baseline, 12, and 16 weeks. C-SSRS and HAM-D scales were administered at baseline, 4, 8, 12, and 16 weeks.

Statistical Analysis

A description of all the variables of interest was carried out using descriptive statistics (percentages, frequencies, and measures of central tendency and dispersion). An intent-to-treat population (ITT) analysis was performed to examine the outcomes. Inferential statistics was carried out to assess the cocaine use days self-reported less or more than 20% from 10 to 12 weeks with the chi-squared test, and to check absolute score changes for the Clinical Evaluation Instruments during the study weeks for ASI-lite from baseline to 12 weeks by the paired sample T-test. A One factor repeated measures ANOVA (factor time) was used for CGI, SDS, CSSA, BSCS, SF-36 Health Survey, HRBS, HAM-D and C-SSRS including baseline, 12 weeks and 16 weeks results. When significant, a Tukey post-hoc multiple comparison analysis was performed to compare baseline-12 weeks, baseline-16 weeks, and 12-16 weeks All the statistical tests were performed using SPSS Statistics 23.0 (SPSS, Chicago, IL, United States). A value of $p<0.05$ was considered statistically significant for chi-squared test, paired sample T-test and for One factor repeated measures ANOVA. Sample size was calculated to evaluate a clinical efficacy equal to or greater than 20% using the Gehan method for phase II clinical trials (9-11 subjects was recommended) (Machin et al., 2009).

Results

Sociodemographic Characteristics

Nine subjects were recruited (eight men and one woman) with a mean age of 48±9.27 years. Mean weight was 84.1±16.19 kg and body mass index 27.3±4.4. Six (66.7%) subjects were married, four (44.4%) had primary school studies, five (55.6%) high school diplomas or higher, and four (44.4%) were employed at the moment of conducting the study (Table 1).

Clinical Characteristics

All the participants suffered severe cocaine use disorder according to DSM-5 criteria. Mean age of onset of the disorder was 30.44±9.28 years. Of the nine participants, six (66.7%) had a previous history of substance use disorder treatment. Four (44.4%) subjects had a prior history of alcohol use disorder before the previous year and one (11.1%) in the previous year. One participant (11.1%) had a prior history of cannabis use disorder before the previous year and one (11.1%) in the previous year. One subject (11.1%) had prior history of opioid use disorder but not in the previous year. Seven of them (77.8%) had familiar history of substance use disorders (Table 1).

The results of the PRISM interview showed that four (44.4%) were diagnosed with an affective disorder in remission.

Table 1
Sociodemographic and Clinical Characteristics of the Sample (N = 9)

Characteristic	Mean (SD) or n (%)
Sociodemographic Characteristics	
Gender (men/women)	8 (88.9%) / 1 (11.1%)
Age, M (SD), years	48 (9.27)
Weight, M (SD), kg	84.1 (16.19)
Body mass index, M (SD), kg/m²	27.3 (4.4)
Marital status (married)	6 (66.7%)
Education — Primary education	4 (44.4%)
Education — High school diploma or higher	5 (55.6%)
Employment status (employed)	4 (44.4%)
Clinical Characteristics	
Cocaine use disorder (DSM-5, severe)	9 (100%)
Age of onset, M (SD), years	30.44 (9.28)
Previous treatment for substance use disorder	6 (66.7%)
Alcohol use disorder — Prior to the previous year	4 (44.4%)
Alcohol use disorder — In the previous year	1 (11.1%)
Cannabis use disorder — Prior to the previous year	1 (11.1%)
Cannabis use disorder — In the previous year	1 (11.1%)
Opioid use disorder — Prior to the previous year	1 (11.1%)
Family history of substance use disorders	7 (77.8%)

Note. M = mean; SD = standard deviation. Percentages are calculated over N = 9.

Cocaine Use Days, Retention and Adherence to treatment

Cocaine Use Days

With respect to self-reported cocaine use, from 10 to 12 weeks three subjects (33.3%) reported a cocaine consumption of less than 20% (80% non-use days), and two (22.2%) from baseline, findings, however, that were non-significant. None of the participants showed less than 20% positive urine analysis from 10 to 12 weeks. The mean percentage of negative urine samples for the cocaine metabolites from all the scheduled urine samples from 5 to 12 weeks of the treatment period was 15.8%.

Retention and Adherence to Treatment

A total of seven (77.8%) subjects completed the 16 weeks of the study. One (11.1%) completed 12 weeks, and another (11.1%) dropped out during the 4 weeks of the study. According to the urine quantitative analysis of the isoflavones, eight (88.9%) participants followed the treatment during the study, and only one (11.1%) did not (isoflavone concentrations in urine were below detection limit).

Other Outcomes

Clinical Global Impression (CGI)

No differences were found throughout the study for each participant regarding score changes in the CGI scale, both in the severity assessment subscale (CGI-S) and in the improvement assessment one (CGI-I) (Table 2).

Addiction Severity Index Lite (ASI-lite)

No differences were observed during the study weeks in the scores of the 7 areas evaluated by the ASI-Lite scale.

Severity Dependence Scale (SDS)

The SDS score showed a significant decrease between baseline to 12 weeks, baseline to 16 weeks and 12 to 16 weeks (Table 2).

Cocaine Selective Severity Assessment (CSSA)

Values in the CSSA decreased but were not significant (Table 2).

Brief Substance Craving Scale (BSCS)

Values in the BSCS scale decreased but were not significant (Table 2).

SF-36 Health Survey (SF-36)

In the case of the SF-36 questionnaire, increases in the scores were observed in some areas. The areas physical function, physical role, general health, vitality, emotional role and mental health increased their scores but not significantly. Body pain scores decreased from baseline to 12 weeks but not significantly, from baseline to 16 weeks the decrease was statistically significant but increased the scores from 12 to

Table 2
Absolute Scores for the Clinical Evaluation Instruments (n = 9)

Scale	Baseline, M (SD)	12 weeks, M (SD)	16 weeks, M (SD)	ANOVA-1F p value	Group differences*
CGI					
CGI-S	2.78 (1.64)	2.89 (1.45)	3.33 (1.50)	0.429	NS
CGI-I	3.78 (0.67)	3.67 (2)	3.33 (1.41)	0.415	NS
SDS	12.11 (3.92)	8.11 (4.99)	6.33 (3.74)	< 0.001	a, B, c
CSSA	26.56 (1.93)	25.56 (25.42)	24.78 (17.23)	0.902	NS
BSCS	9.00 (3.50)	7.11 (4.70)	6.56 (3.64)	0.128	NS
SF-36					
Physical Function	92.78 (8.33)	93.33 (7.91)	96.11 (6.01)	0.132	NS
Physical Role	71.52 (36.39)	72.92 (27.60)	82.64 (22.05)	0.132	NS
Body Pain	80 (19.87)	67.78 (31.77)	70.11 (26.79)	0.001	NS, b, C
General Health	54.44 (20.47)	60.78 (21.02)	69.33 (18.75)	0.220	NS
Vitality	41.66 (18.22)	52.77 (21.45)	57.64 (21.60)	0.266	NS
Social Function	63.89 (15.86)	66.67 (26.52)	70.83 (25)	0.001	A, b, NS
Emotional Role	60.18 (39.48)	60.18 (32.21)	63.89 (31.73)	0.584	NS
Mental Health	47.78 (20.93)	56.11 (22.88)	61.11 (19.65)	0.16	NS
HRBS	3 (5.24)	1.67 (2.45)	1.78 (2.95)	0.696	NS
HAM-D	3.44 (3.61)	5.78 (5.93)	3.11 (3.51)	0.24	NS
C-SSRS	0	1.22 (3.67)	1.22 (3.67)	0.29	NS

Note. M = mean; SD = standard deviation.
Note. CGI = Clinical Global Impression; CGI-S (measures disorder severity) and CGI-I (measures disorder improvement during the consultations); SDS = Severity Dependence Scale; CSSA = Cocaine Selective Severity Assessment; BSCS = Brief Substance Craving Scale; SF-36 = SF-36 Health Survey; HRBS = HIV Risk-Taking Behaviour Scale; C-SSRS = Columbia Suicide Severity Rating Scale.
Note. ANOVA-1F = Repeated measures one factor analysis of variance (time). NS = not-significant differences.
Note. Tukey post-hoc test statistical significant differences ($p < .05$) between conditions are indicated as "a" (baseline–12 weeks), "b" (baseline–16 weeks), "c" (12 weeks–16 weeks). Tukey post-hoc test statistical significant differences ($p < .01$) are indicated as "A" (baseline–12 weeks), "B" (baseline–16 weeks), "C" (12 weeks–16 weeks).

16 weeks significantly. The social function area improved its scores from baseline to 12 weeks and from baseline to 16 weeks significantly, and between 12 to 16 the increased was not statistically significant (Table 2).

Other Psychiatric Sales

During the screening session and throughout the study, none of the participants presented significant changes in the scores for the HRBS, HAM-D, and C-SSRS. During the screening session participants presented values of CIWA-Ar according with the inclusion criteria.

No serious adverse side effects were observed throughout the study.

Discussion

Our findings do not allow a conclusive statement about the possible efficacy of soy isoflavones as a treatment of cocaine use disorder, because the main outcome of self-reported reduction in cocaine use is not significant and not confirmed by urine probes as objective measures and other outcomes are not significant. Nevertheless, our results show that soy isoflavone treatment could produce a reduction of self-reported cocaine use days, although the increase in the number of patients who reported a consumption of cocaine less than 20% was not significant and it was not possible, however, to confirm this decrease by the detection of urine metabolites of cocaine. The intervention improved retention, resulted in adequate adherence, reduced severity of cocaine use, and augmented participants' quality of life.

The percentage of positive urine analyses for cocaine metabolites we observed is similar to other authors regarding the treatment of cocaine use disorder. A recent study in cocaine and opioid co-dependent patients reported a significant reduction of cocaine positive urine during 12 weeks of treatment with disulfiram versus placebo from 79% at study week 1–2 to 63% at study week 11–12 (Kampangkaew et al., 2019). The percentage of positives at cessation of the study was not, however, less than 20% (a value that is considered a positive outcome in our study), as we reported from weeks 10 to 12. Nevertheless, it should be highlighted that, according to the self-registration of daily consumption, in our study three subjects (33.3%) reported a consumption of less than 20% between weeks 10 and 12, and two from baseline, although the difference was not significant. Considering that the participants were patients presenting a serious disorder such an outcome could be an indicator of the reduction of frequency of cocaine use. A possible reason for this not being corroborated by urine analysis could be due to the fact it was performed three times a week. Taking into account the elimination half-life of cocaine metabolites (6-8 hours for benzoylecgonine and 3-8 hours for ecgonine methyl ester) (Farré et al., 1997), some self-reported non-use days could have resulted positive. In addition, missing urine analyses were considered positive.

Considering that all the participants were affected by a serious cocaine use disorder, the retention rates to soy isoflavone treatment were very positive. Seven subjects (77.8%) completed the study at 16 weeks and one (11.1%) dropped out at 12 weeks. A higher figure than that found in the literature which usually cites treatment retention rates for cocaine use disorder at 50% (Stotts et al., 2007). Analysis of urine concentrations of daidzein, genistein, and equol also provided positive results regarding good adherence to soy isoflavone treatment. Eight (88.9%) of the participants followed correct intake. The good tolerance of soy isoflavones throughout the study weeks supports high rates of adherence to treatment.

We observed relevant outcomes indicating that soy isoflavone treatment reduces the severity and craving of cocaine use, as previously indicated in animal studies (Martín et al., 2021; Yao et al., 2010). A significant reduction of the SDS scale scores throughout the study weeks, and a decrease of the scores of the BSCS and CSSA scales, although without achieving significant changes, were found. Previous reports with antipsychotic treatment for cocaine use disorder did not describe a reduction in cocaine use severity with the ASI-lite and CGI scales (Álvarez et al., 2013) as we found in our study. They did not, however, employ other specific scales such as the SDS, CSSA, and BSCS.

Promising data showing an improvement in specific areas of the SF-36 questionnaire (body pain, social function) were observed. Moreover, increases in other areas, even though not statistically significant, reinforce the possible use of soy isoflavone treatment for cocaine use disorder.

Several studies with animals, have suggested that isoflavones have antidepressant-like effects through the regulation of the transcription of BDNF in the brain (Lu et al., 2019; Tantipongpiradet et al., 2019). Other studies in animals suggest that BDNF has an important role for the cocaine use disorder, attenuating relapse for cocaine seeking for a long time (Li & Wolf, 2015; McGinty, 2022). These could be another hypothesis to explain the possible effects of the isoflavones for the cocaine use disorder.

Limitations

Some positive outcomes were found in this trial; nevertheless, there are a few limitations. The most important is the fact that it is an open-label pilot study, and the sample was small, wich limits statistical power and generalizability. The lack of previous studies in humans led us to carry out this pilot study to evaluate possible hypotheses about the use of soy isoflavones for cocaine use disorder. In order to enable a conclusive evaluation of the efficacy of soy isoflavones and considering that in this trial only three

parameters showed significant improvements, a greater sample has to be investigated and compared to a control condition. Although according to the elimination half-life of cocaine metabolites, urine analyses were conducted only three times per week, could be a possibility some missing use days. The isoflavone doses were in accordance with those usually recommended for menopausal climacteric symptoms. We are unaware whether higher ones could provide better results. Neither do we know the possible effect of the isoflavones (daidzein and genistein) when taken separately as we used a compound containing them in a fixed combination. Altough patients were advised to avoid soy products, there was no formal control for soy-based nutrition, otherwise, at the baseline, seven (77.8%) of them referred never taking and two (22.2%) not to taking soy derivatives usually. Also, it was an exclusion criterion to be under treatment with soy derivatives for another reason. As only one woman participated in the study, possible differences between genders could not be assessed.

Conclusions

Our preliminary results suggest the possible action of soy isoflavone treatment in reducing cocaine use days. According to our results, three subjects (33.3%) reported a cocaine consumption less than 20% during the period from 10 to 12 weeks of treatment. This is even though complete abstinence was not observed by urine analysis. Data also suggest that soy isoflavone treatment could improve treatment retention, reduce the severity of cocaine use disorder, and improve patients' quality of life. Soy isoflavones were well tolerated and good adherence was observed. Based on these results, placebo-controlled studies with adequate sample size are needed to evaluate whether soy isoflavones can be effective in treating cocaine use disorder. Finally, based on our results and emerging perspectives in the treatment of substance use disorders, future research should consider non-abstinence-based strategies as a therapeutic goal. These should focus on improving treatment retention, reducing the severity of cocaine use disorder, and enhancing patients' quality of life. This study represents a pioneering step in the exploration of alternative treatments for cocaine use disorder. Given the current lack of effective therapeutic options for this condition, it is particularly relevant to further investigate the potential effects of isoflavones in this context.

Ethics statement

The study protocol was approved by the local Human Research Ethics Committee (CEIC-Parc de Salut Mar, ref. 2014/5580) and conducted in accordance with the Declaration of Helsinki (Fortaleza, 2013) and local laws (Biomedical Research Law, 2008). Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper if applicable.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

Conception and design of the work, M.T., R.M.-R, F.F., and M.F.; validation and investigation, M.T., R.M.-R., F.F., L.G., R.T., J.M., N.P., M.F.; analysis and interpretation of the data, M.T., R.M.-R., F.F., J.M., N.P., R.T., M.F.; draft preparation, R.M.-R., M.T., M.F.; critical review, all authors. All the authors approved the final version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Funding

Supported in part by grants from Ministerio de Sanidad (Investigación Clínica Independiente, grant number: EC-10-166); Instituto de Salud Carlos III (ISCIII, Fondo de Investigación en Salud (FIS)-Fondo Europeo de Desarrollo Regional (FEDER): grants ISCIII-RTA RD16/0017/0003 and RD16/0017/0010, and ISCIII-RICORS-RIAPAd RD21/0009/0004 and RD21/0009/0001, AGAUR Gencat Suport Grups de Recerca (2017 SGR 316 and 2017 SGR 530). L. Galindo was a Rio Hortega fellowship, (CM14/00111).

We would like to acknowledge the initial contributions to the study design made by José Carlos Bouso and Jose Luis Pérez de Heredia.

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Data availability statement

The datasets for this manuscript are not publicly available. Requests to access the datasets should be directed to Magi.Farre@uab.cat.

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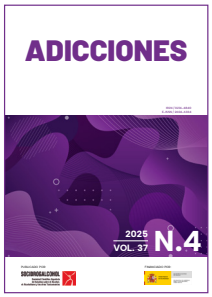
ADICCIONES
2025 ■ VOL. 37 ■ N. 4 ■ PAGES 323–340
www.adicciones.es



ORIGINAL

Blocking the increased reinforcing effects of cocaine induced by social defeat: Effects of palatable food

Bloqueo del incremento en los efectos reforzantes de la cocaína inducidos por la derrota social: Efecto de la comida palatable

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Abstract

Preclinical studies suggest that stimulation of the brain's reward system by high-fat diets (HFD) could act as an alternative reinforcer. The main aim of the present study was to evaluate the effect of a limited and intermittent exposure to an HFD administered during and after exposure to Social Defeat (SD) on a non-effective dose of cocaine-induced Conditioned Place Preference (CPP). Experiment 1 consisted of modulating SD episodes with three different patterns of HFD access: 1h access before each session of SD; 2h access three days a week during the two weeks of SD exposure; and 2h access 4h after each SD. Experiment 2 consisted of modulating the effects of stress on CPP acquisition with three patterns of HFD access: 1h access before each conditioning session; 2h access three days a week throughout the two-week period of the CPP; and 2h access three days a week from the last SD episode to the end of CPP. HFD administered during the period of SD episodes counteracted the increased sensitivity that SD produces on the reinforcing effects of cocaine. Access to HFD before the conditioning session or three days a week (CPP-SD-MWF) during the acquisition of CPP blocked this increased sensitivity. In the striatum, SD induced a decrease in the cannabinoid 1 receptor (*Cb1r)* gene expression, not affected by HFD, and increased corticotrophin releasing hormone receptor 1 (*Crhr1)* gene expression, except for those mice fed on HFD after SD encounters. Our findings indicate that a small intake of HFD may attenuate the social stress–induced increase in the rewarding properties of cocaine.

Key words: social defeat, male mice, cocaine, high-fat diet

Resumen

Los estudios preclínicos sugieren que la estimulación del sistema de recompensa cerebral mediante dietas ricas en grasa (DRG) podría actuar como un reforzador alternativo. El objetivo principal del presente estudio fue evaluar el efecto de una exposición limitada e intermitente a una DRG, administrada durante y después de la exposición a Derrota Social (DS), sobre una dosis no efectiva de la Preferencia de Lugar Condicionado (PLC) inducida por cocaína. El Experimento 1 consistió en modular los episodios de DS con tres patrones diferentes de acceso a la DRG: acceso de 1 hora antes de cada sesión de DS; acceso de 2 horas tres días a la semana durante las dos semanas de exposición a DS; y acceso de 2 horas, 4 horas después de cada DS. El Experimento 2 consistió en modular los efectos del estrés sobre la adquisición de la PLC con tres patrones de acceso a la DRG: acceso de 1 hora antes de cada sesión de condicionamiento; acceso de 2 horas tres días a la semana durante el período de dos semanas de la PLC; y acceso de 2 horas tres días a la semana desde el último episodio de DS hasta el final de la PLC. La DRG administrada durante el período de episodios de DS contrarrestó el aumento de la sensibilidad que la DS produce sobre los efectos reforzadores de la cocaína. El acceso a la DRG antes de la sesión de condicionamiento o tres días a la semana (PLC-DS-LXV) durante la adquisición del PLC bloqueó este aumento de sensibilidad. En el estriado, la DS indujo una disminución en la expresión génica del receptor cannabinoide tipo 1 (*Cb1r*), no afectada por la DRG, y un aumento en la expresión del gen del receptor 1 de la hormona liberadora de corticotropina (*Crhr1*), excepto en los ratones alimentados con DRG después de los encuentros de DS. Nuestros hallazgos indican que una pequeña ingesta de DRG puede atenuar el aumento inducido por el estrés social en las propiedades reforzantes de la cocaína.

Palabras clave: derrota social, ratones machos, cocaína, dieta rica en grasa

■ *Received: July 2025; Accepted: October 2025.*

■ ISSN: 0214-4840 / E-ISSN: 2604-6334

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Stress is widely recognized as a central factor in the onset, progression, and persistence of addictive behaviors (Buchanan & Lovallo, 2019; Burke & Miczek, 2015; Volkow & Blanco, 2023), playing a crucial role in the negative emotional state caused by dependence, leading to both substance withdrawal (Koob, 2009) and relapse episodes (Koob, 2010; Koob & Volkow, 2010). Social stress stands out as a particularly significant stressor in humans, arising from interpersonal relationships and the contextual environment in which individuals develop (Carnevali et al., 2020; Dickerson & Kemeny, 2004). Given the profound physical and psychological impact of social stress in humans, animal models such as the social defeat (SD) paradigm have been established to investigate its neurobiological consequences (Miczek et al., 2008; Shimamoto, 2018). Preclinical studies using the SD model have revealed that exposure to social stress produces lasting effects (Wang et al., 2021), including reduced exploratory behavior and social interaction (Burke et al., 2011; Shimizu et al., 2020), increased anxiety (Weathington & Cooke, 2012), enhanced ethanol consumption (Arenas et al., 2025; Reguilón et al., 2020; Reguilón et al., 2021), and increased sensitivity to the conditioned rewarding properties of psychostimulants like cocaine, in both adolescent (Burke et al., 2016; Burke & Miczek, 2015; Rodríguez-Arias et al., 2018) and adult rodents (Ballestín et al., 2021; Ferrer-Pérez et al., 2018; Giménez-Gómez et al., 2021; Montagud-Romero et al., 2015; Montagud-Romero et al., 2016; Rodríguez-Arias et al., 2017).

Stress also influences nutritional habits. Clinical studies indicate that individuals exposed to stress are more likely to increase their consumption of highly palatable foods (Kim et al., 2013; Konttinen, 2020; Linders et al., 2022), due to their comfort-inducing properties, which help mitigate psychological distress (Dallman et al., 2003; Gemesi et al., 2022). In fact, consumption of such foods in humans reduces plasma cortisol levels and perception of stress (Herhaus et al., 2020; Leigh Gibson, 2006). Parallel findings in animal models show that rodents under chronic stress prefer high-fat diets (HFD) over standard chow (STD) (Packard et al., 2014; Pecoraro et al., 2004), thus attenuating physiological responses to acute stress such as hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis (Kalyani et al., 2016; Linders et al., 2022; Ulrich-Lai et al., 2011;). Notably, a previous study from our group demonstrated that adolescent-isolated mice that were intermittently exposed to HFD exhibited significantly lower corticosterone levels compared to isolated controls receiving only standard diet (Blanco-Gandía et al., 2018), suggesting a stress-buffering effect of palatable food.

Therefore, social stress increases the reinforcing properties of both psychostimulants (Montagud-Romero et al., 2015; Peleg-Raibstein et al., 2016) and palatable diets (Kim et al., 2013), potentially through shared neurobiological mechanisms. Similar to addictive substances, hypercaloric foods rich in fat and/or sugar increase dopamine levels in the nucleus accumbens (NAcc) (DiLeone et al., 2012; Pitman & Borgland, 2015), with the activation of several key structures of the reward system, such as the ventral tegmental area (VTA), the prefrontal cortex, and the amygdala (de Macedo et al., 2016; Volkow et al., 2013). Growing evidence suggests that dietary interventions can influence responses to drugs and addiction vulnerability. For instance, HFD exposure modulates sensitivity to alcohol and cocaine, either enhancing reward responsiveness (Avena et al., 2008; Blanco-Gandía et al., 2017a; Blanco-Gandía et al., 2017b; Puhl et al., 2011) or serving as an alternative reinforcement under drug withdrawal-induced negative emotional states (Blanco-Gandía et al., 2017c). Indeed, both continuous (Blanco-Gandía et al., 2017c) and intermittent (Ródenas-González et al., 2021) HFD administration during cocaine withdrawal facilitates the extinction learning and inhibits reinstatement of cocaine-seeking behaviors, in addition to reducing behavioral withdrawal symptoms (Loebens & Barros, 2003). We have previously observed that EtOH-induced impairment on spatial memory retrieval is absent in mice exposed to continuous or intermittent access to HFD, although the aversive memory deficits persist (Del Olmo et al., 2019). Regarding alcohol use disorders, the pattern of HFD exposure and the stress condition seem to be critical. Prolonged binge-eating or continuous access to HFD during adolescence increases the reinforcing effects of EtOH (Blanco-Gandia et al., 2017b). However, a recent study suggests that intermittent HFD access effectively prevents stress-induced increases in ethanol consumption (Arenas et al., 2025). These results highlight the close relationship between HFD, stress and addiction, which interact not only within the dopaminergic circuitry and the HPA axis, but also with the cannabinoid and the opioid systems (Cristino et al., 2014; Parylak et al., 2012; Sakamoto et al., 2015).

The convergence of these three factors (stress, drugs of abuse intake and nutritional habits) is particularly critical during adolescence, a period when structural changes in many limbic and cortical regions can be disrupted by these factors (Baladi et al., 2012; Daws et al., 2011; Spear, 2000). During this period, individuals display enhanced reward sensitivity, rendering them more vulnerable to the reinforcing effects of drugs (Steinberg, 2010). However, the influence of palatable food consumption on the increased cocaine-reinforcing effects induced by social stress in adolescent animals remains unexplored.

Considering the overlapping neurobiological pathways stimulated by HFD and drugs of abuse, and their modulation by stress-related systems, we hypothesize that the intake of HFD may influence the development of cocaine-induced CPP in adolescent mice, particularly when subthreshold doses of cocaine are used. To test this hypothesis, our study was designed to explore two different approaches. In the

first experiment, we modulated the SD episodes with HFD administration; in the second experiment, after the animals had been exposed to stress, cocaine CPP acquisition was modulated by HFD administration. Mu-opioid receptors are modulated by HFD, contributing to the rewarding effects and hedonic value of palatable food (Mahdavi et al., 2023). Prior findings indicate reduced mu opioid receptor gene expression in the NAcc following binge-like HFD administration (Blanco-Gandia et al., 2017a; Blanco-Gandia et al., 2017b; Martire et al., 2014; Ong et al., 2013), whereas continuous exposure appears to increase its expression (Blanco-Gandia et al., 2017c; Smith et al., 2002). On the other hand, CB1 receptors are also implicated in the rewarding properties of palatable food, particularly HFD (Friuli et al., 2025). Our research group reported reduced CB1r gene expression in the NAcc following exposure to HFD (Blanco-Gandia et al., 2017a; Blanco-Gandia et al., 2017c). Collectively, these findings suggest that food intake can modulate not only dopaminergic but also non-dopaminergic systems, including the cannabinoid and opioid systems. Given the involvement of the opioid and cannabinoid systems, as well as the HPA axis, in stress, addiction, and palatable food reward, we also assessed gene expression of the mu opioid receptor (*Oprm*), cannabinoid receptor 1 (*Cb1r*), and corticotropin-releasing hormone receptor 1 (*Crhr1*) in the striatum at the end of the experiments.

Material and methods

Subjects

A total of 180 male mice of the OF1 outbred strain on PND 42 were acquired commercially from Charles River (France). Of these, 30 animals were housed under standard isolated conditions and were used as aggressive residents during the Social Defeat (SD) procedure. The remaining 150 experimental mice arrived at the laboratory on PND 21 and were housed under standard conditions in groups of four (cage size 28 × 28 × 14.5 cm), at a constant temperature (21 ± 2 °C), with a reversed light schedule (white lights on 19:30–7:30) and food and water available ad libitum (except during behavioral tests). All procedures involving mice and their care complied with national, regional and local laws and regulations, which are in accordance with Directive 2010/63/EU of the European Parliament and the Council of September 22, 2010 on the protection of animals used for scientific purposes. The Animal Use and Care Committee of the University of Valencia approved the present study (2017/VSC/PEA/00224).

Drugs

For CPP, animals were injected i.p. with 1 mg/kg of cocaine hydrochloride (Laboratorios Alcaliber S. A. Madrid, Spain) diluted in physiological saline. The 1 mg/kg dose of cocaine used to induce CPP was based on previous studies (Maldonado et al., 2006; Vidal-Infer et al., 2012), where it was shown to be a subthreshold dose that is not effective in standard animals.

Experimental design

After 5 days of adaptation in the vivarium, at PND 26, mice were exposed to SD, except for the exploration groups (EXP). Following the last SD/EXP session, animals were kept in the vivarium for three weeks, housed in their respective home cages, and then they performed 1 mg/kg cocaine-induced CPP (PND 55). After completing the entire experimental procedure, the animals were euthanized to enable the collection of biological samples.

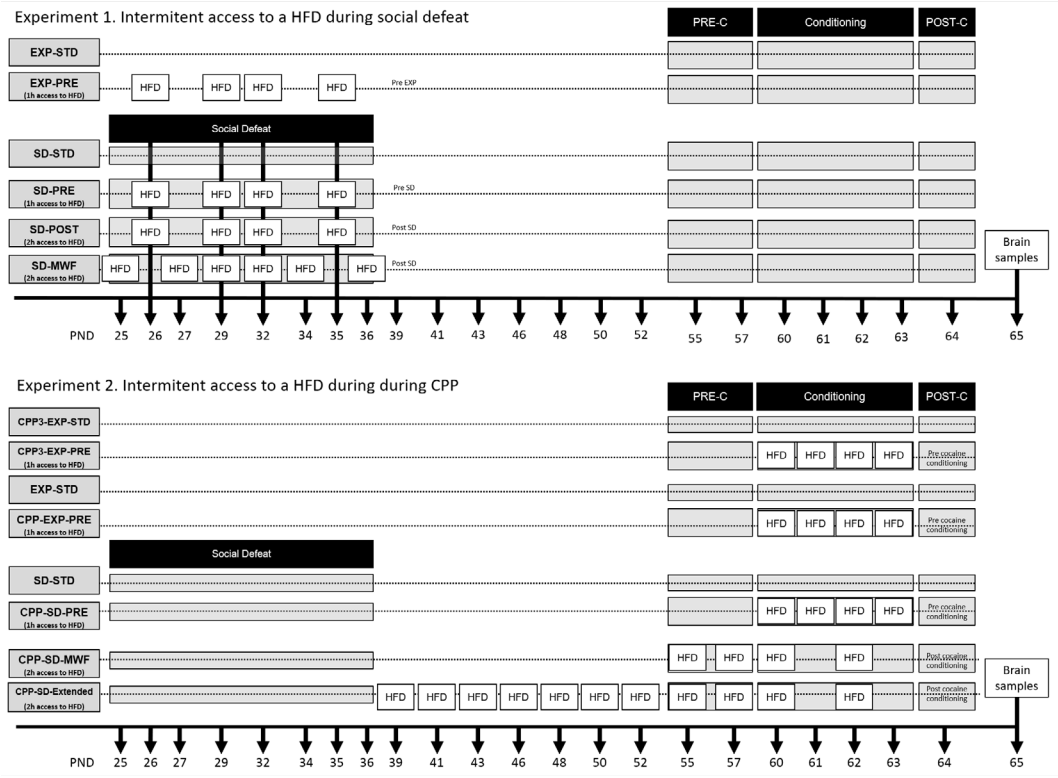
In this study, two different experiments were performed: Experiment 1 consisted of modulating SD episodes with different patterns of HFD access and Experiment 2 tested how different patterns of HFD access modulated the effects of stress on CPP acquisition. An overall and more detailed description of the sets of animals and experimental procedure of each experiment is provided in *Figure 1*.

In Experiment 1, experimental animals (n = 76) were exposed to four episodes of EXP/SD with different HFD administrations during the two weeks of stress exposure. Mice were randomly divided into six groups with similar average body weights (25–26 g) and assigned to the following groups: EXP-STD (non-stressed mice on a standard diet, n = 12), EXP-PRE (non-stressed mice with 1 h access to HFD before each exploration session, n = 12), SD-STD (defeated mice on a standard diet, n = 15), SD-PRE (defeated mice with 1 h access to HFD before each SD, n = 14), SD-MWF (defeated mice with 2 h access to HFD on Monday, Wednesday and Friday during the two weeks of SD, accessing the HFD after SD sessions on overlapping days, n = 13) and SD-POST (defeated mice with 2 h access to the HFD after 4 h of an SD episode, n = 10). Three weeks after the last SD, all groups performed the cocaine-induced CPP.

In Experiment 2 (n = 80), mice were randomly divided into six groups with similar average body weights (25–26 g) and assigned to the following groups: EXP-STD (non-stressed mice on a standard diet, n = 12), CPP-EXP-PRE (non-stressed mice with 1 h access to the HFD before each conditioning session, n = 12), SD-STD (defeated mice on a standard diet, n = 15), CPP-SD-PRE (defeated mice with 1 h access to the HFD before each conditioning session, n = 15), CPP-SD-MWF (defeated mice with 2 h access to the HFD on Monday, Wednesday and Friday during the two weeks of CPP, n = 15) and CPP-SD-Extended (2 h access to the HFD on Monday, Wednesday and Friday, starting from the last SD episode until the end of CPP, n = 11). To minimize the unnecessary use of mice, the groups designated as EXP-STD and SD-STD in Experiment 1 consisted of the same mice in Experiment 2.

Figure 1

Experimental design



Note. In Experiment 1, mice were divided into six groups: EXP-STD (non-stressed mice on a standard diet), EXP-PRE (non-stressed mice with 1 h access to HFD before each exploration session), SD-STD (defeated mice on a standard diet), SD-PRE (defeated mice with 1 h access to HFD before each SD), SD-MWF (defeated mice with 2 h access to HFD on Monday, Wednesday and Friday during the two weeks of SD, accessing the HFD after SD sessions on overlapping days) and SD-POST (defeated mice with 2 h access to the HFD after 4 h of an SD episode). In Experiment 2, mice were divided into eight groups: EXP-STD (non-stressed mice on a standard diet), CPP-EXP-PRE (non-stressed mice with 1 h access to the HFD before each conditioning session), SD-STD (defeated mice on a standard diet), CPP-SD-PRE (defeated mice with 1 h access to the HFD before each conditioning session), CPP-SD-MWF (defeated mice with 2 h access to the HFD on Monday, Wednesday and Friday during the two weeks of CPP) and CPP-SD-Extended (2 h access to the HFD on Monday, Wednesday and Friday, starting from the last SD episode until the end of CPP), CPP3-EXP-STD (non-stressed mice on a standard diet conditioned with 3 mg/kg cocaine) and CPP3-EXP-PRE (non-stressed mice with 1 h access to the HFD before each conditioning session with 3 mg/kg cocaine).

Two more groups of mice (n = 21) were employed to evaluate the effect of HFD on 3 mg/kg cocaine-induced CPP, named as CPP-C3 (non-stressed mice on a standard diet, n = 11) and CPP-C3-PRE (non-stressed mice with 1 h access to the HFD before each conditioning session, n = 10).

Feeding conditions

Two different types of diet were administered in the study. A standard diet (Teklad Global Diet 2014, 13 Kcal % fat, 67 Kcal % carbohydrates and 20 Kcal % protein; 2,9 kcal/g; no sugars added) was given to the control groups, and a high-fat diet (TD.06415, 45 Kcal % fat, 36 Kcal % carbohydrates and 19 Kcal % protein; 4.6 Kcal/g; 20% of carbohydrates are sucrose) was administered in a limited way to the high-fat diet groups. Both diets were supplied by Harlan Laboratories Models, S. L. (Barcelona, Spain) and will be referred to from now on as the standard diet, while the sporadic limited access to the high-fat food will be referred to as the HFD. Ad libitum standard diet and water were always freely available in their home cages. Animals were weighed every week throughout the study, and their daily intake of standard diet in their home cage was also measured.

Repeated social defeat encounters

Animals in the corresponding group were exposed to four episodes of SD, each lasting 25 min. Each episode consisted of three phases, which began by placing the experimental animal or intruder in the home cage of the aggressive opponent or resident for 10 min. During this initial phase, the intruder was protected from attack by a wire mesh wall that permitted social interaction and species-typical threats from the male-aggressive resident (Covington & Miczek, 2001). In the second phase, the wire mesh was removed from the cage and a 5 min confrontation period began. In the third phase, the wire mesh was put back for 10 min to allow social threats from the resident. Mice were exposed to SD on postnatal days (PNDs) 26, 29, 32, and 35. The exploration group (EXP) underwent the same protocol, but without the presence of a Resident mouse in the cage. Following this last phase, animals were kept in the vivarium for three weeks, housed in their respective groups.

Conditioning Place Preference

For place conditioning, we employed 16 identical Plexiglas boxes with two equally sized compartments (30.7 cm length x 31.5 cm width x 34.5 cm height) separated by a gray central area (13.8 cm length x 31.5 cm width x 34.5 cm height). The compartments have different colored walls (black vs white) and distinct floor textures (fine grid in the black compartment and wide grid in the white one). Four infrared light beams in each compartment of the box and six in the central area allowed the recording of the animal's position and crossings between compartments. The equipment was controlled by two IBM PC computers using MONPRE 2Z software (CIBERTEC S.A., Spain).

Acquisition of CPP

The place conditioning procedure, unbiased in terms of initial spontaneous preference, was performed as previously described (Maldonado et al., 2006) and consisted of three phases. Briefly, in the first phase, known as Pre-Conditioning (Pre-C), mice at PND 55 were allowed access to both compartments of the apparatus for 15 min (900 s) per day on 3 days. On day 3, the time spent in each compartment over a 900 s period was recorded, and animals showing a strong unconditioned aversion (less than 33% of the session time) or preference (more than 67%) for any compartment were excluded from the rest of the experiment. Two defeated animals on the standard diet met these criteria and were excluded from SD-STD (n = 13) and SD-STD (n = 13) groups from Experiment 1 and Experiment 2, respectively. Half of the animals in each group received the drug or vehicle in one compartment, and the other half in the other compartment. After assigning the compartments, no significant differences were detected in the time spent in the drug-paired and vehicle-paired compartments during the preconditioning phase. In the second phase (conditioning), which lasted 4 days, animals received an injection of physiological saline immediately before being confined to the vehicle-paired compartment for 30 min. After a 4 h interval, they received an injection of cocaine immediately before being confined to the drug-paired compartment for 30 min. Confinement was carried out in both cases by closing the guillotine door that separated the two compartments, making the central area inaccessible. In the third phase, known as post-conditioning (Post-C), the guillotine door separating the two compartments was removed (day 8) and the time spent by the untreated mice in each compartment was recorded over a 900 s observation period. The difference in seconds between the time spent in the drug-paired compartment during the Post-C test and the Pre-C phase is a measure of the degree of conditioning induced by the drug. If this difference is positive, then the drug has induced a preference for the drug-paired compartment, whereas the opposite indicates that an aversion has developed.

Gene expression analyses: RNA isolation and quantitative RT-PCR

At the end of the experiments, animals were euthanized by cervical dislocation and the brains were immediately removed from the skull and placed on a cold plate. The striatum was dissected, and brain tissue samples were immediately stored at -80ºC until the rt-PCR assay was performed (n = 8/condition).

Total RNA from the striatum was isolated using the Tri Reagent Method (Sigma-Aldrich, St. Louis, MO, USA), as described in the manufacturer's protocol. Reverse transcription of 1 mg of total RNA was performed using the Transcriptor First Strand cDNA synthesis kit (Thermo Fisher Scientific, Madrid, Spain). Amplification of the target and housekeeping (b-glucuronidase) genes was performed using the Taqman Gene Expression Master Mix (Thermo Fisher Scientific, Madrid, Spain) in a LightCycler 480 System (Roche Diagnostics) following the manufacturer's instructions. The assay codes of the primers used were Mm01212171, Mm01188089 and Mm00432670 for cannabinoid receptor 1 (*Cb1r*), opioid receptor μ (*Oprm*) and *Crhr1*, respectively. Data were analyzed using the LightCycler 480 relative quantification software and were normalized to the amplification product of b-glucuronidase or Gusb (Mm00446953).

Statistical analyses

Data related to the percentage of body weight increase were analyzed by a mixed ANOVA, with a between variable -Diet-, with 6 levels (EXP-STD, EXP-PRE, SD-STD, SD-PRE, SD-MWF, SD-POST) for Experiment 1 and 6 levels (CPP-EXP, CPP-EXP-PRE, CPP-SD, CPP-SD-PRE, CPP- SD-MWF, CPP-SD-Extended) for Experiment 2 and a within variable -Week-, with 5 levels. Data for the mean of total Kcal intake were analyzed by a one-way ANOVA with the between variable -Diet- (EXP-PRE, SD-PRE, SD-MWF, SD-POST for Experiment 1 and CPP-EXP-PRE, CPP- SD-PRE, CPP- SD-MWF, CPP-SD-Extended for Experiment 2).

For the CPP procedure, the time spent in the drug-paired compartment was analyzed by two repeated measures ANOVA, with a between variable -Diet-, with 6 levels (EXP-STD, EXP-PRE, SD-STD, SD-PRE, SD-MWF, SD-POST for Experiment 1 and CPP-EXP, CPP-EXP-PRE, CPP-SD, CPP-SD-PRE, CPP- SD-MWF, CPP-SD-Extended for Experiment 2), and a within variable –Days–, with two levels (Pre-C and Post-C).

A one-way ANOVA was conducted to assess the conditioning score (defined as the time spent in the drug-paired side minus the time spent in the saline-paired side) and the gene expression data, with a between variable -Group- (EXP-STD, SD-STD, SD-PRE, SD-MWF, SD-POST for Experiment 1 and CPP-EXP, CPP-SD, CPP-SD-PRE, CPP- SD-MWF, CPP-SD-Extended for Experiment 2). Post-

hoc comparisons were performed by means of Bonferroni tests. All data are presented as mean ± standard error of mean (SEM). A p-value < 0.05 was considered statistically significant. Analyses were performed using SPSS v26.

Results

1. Experiment 1. Modulating SD episodes with palatable food

1.1. Body weight and mean of total Kcal intake in HFD sessions.

The ANOVA for the percentage of weight gain (*Figure 2a*) revealed an effect of the variable *Week* ($F(5, 350) = 847.85$, $p < .001$). Body weight increased from the first week onwards ($p < .001$ in all cases).

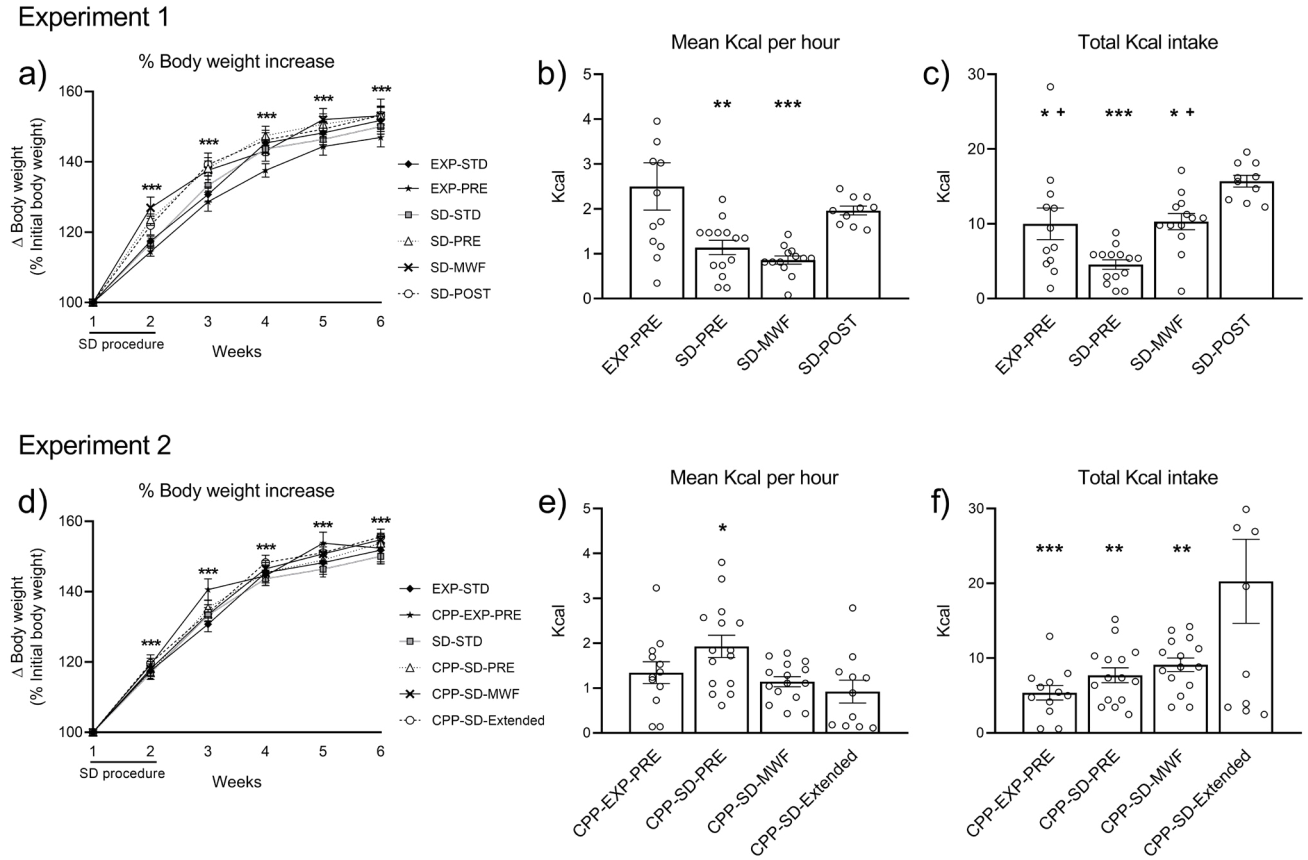
The ANOVA of the mean Kcal intake per hour (*Figure 2b*) revealed significant differences on the variable Diet ($F(3, 45) = 7.50$, $p < .001$). Mice in the SD-PRE and SD-MWF groups consumed less Kcal per HFD session/hour than the EXP-PRE group ($p < .01$ and $p < .001$, respectively). With respect the total kcal intake (*Figure 2c*), the ANOVA also revealed an effect of the variable Diet ($F(3, 45) = 12.28$, $p < .001$). Mice in the SD-POST group consumed more Kcal than the other groups ($p < .05$ for EXP-PRE and SD-MWF and $p < .001$ for SD-PRE). In addition, mice in the SD-PRE group ingested less Kcal than those in the EXP-PRE and SD-MWF groups ($p < .05$).

1.2. Cocaine induced CPP

The results of the 1 mg/kg cocaine-induced CPP in Experiment 1 are presented in *Figure 3*. The ANOVA for the time spent in the drug-paired compartment revealed an effect in the interaction Days × Diet ($F(3, 68) = 3.39$, $p < .023$), Days × Stress ($F(1, 68) = 10.92$, $p < .002$), and Days × Diet × Stress ($F(1, 68) = 4.14$, $p < .05$). Only animals exposed to SD and fed with the standard diet (SD-STD) spent more time in the drug-paired compartment during POST-C compared to PRE-C ($p < .001$). The time spent in the drug-paired compartment during POST-C by the SD-

Figure 2

Body weight and caloric intake changes in mice during Experiments 1 and 2



Note. (a) % Body weight increase of mice over the 6 weeks in Experiment 1. Data are represented as the mean (± SEM) % increase of body weight referred to the initial body weight (week 1). ***p < .001 significant differences within each group between weeks. (b) Kcal per hour Experiment 1. Data are represented as the mean Kcal intake per hour (± SEM) during HFD sessions ***p < .001; ** p < .01 significant difference with respect to the EXP-PRE group (c) Total Kcal intake Experiment 1. Data are represented as the mean total Kcal intake (± SEM) during Experiment 1. *p < .05; **p < .01; ***p < .001 with respect to the SD-POST group; +p < .05 with respect to SD-PRE. (d) % Body weight increase of mice over the procedure Experiment 2. ***p < .001 significant differences within each group between weeks. (e) Kcal per hour Experiment 2. *p < .05 significant difference with respect to the CPP-SD-MWF and CPP-SD-EXTENDED groups; (f) Total Kcal intake Experiment 2. **p < .01; ***p < .001 with respect to CPP-SD-EXTENDED

STD group was significantly higher than that of the rest of the groups ($p < .01$ in all cases).

The ANOVA for the conditioning score revealed an effect of the interaction Diet × Stress ($F(1, 68) = 4.14$, $p < .05$). Among SD mice, those fed with the standard diet (SD-STD) presented a significantly higher conditioning score than those defeated but exposed to HFD on MWF ($p < .01$) or before the PRE-C test ($p < .05$). The SD-STD group also showed higher conditioning score than the EXP-STD group ($p < .001$).

1.3. Gene expression analyses

For the *Cb1r* gene expression (*Figure 4a*), the ANOVA revealed a significant effect of the variable Group ($F(4, 32) = 9.74$, $p < .001$). All mice exposed to SD, regardless of diet, exhibited a significant decrease in *Cb1r* gene expression in comparison with the EXP-STD group ($p < .001$). Regarding the expression of the *Crhr1* (*Figure 4b*) the ANOVA also revealed a significant effect of the variable Group ($F(4, 33) = 10.08$, $p < .001$). Mice in the SD-STD, SD-PRE and SD-MWF groups exhibited a significant increase in *Crhr1* gene expression in comparison with the EXP-STD and SD-POST groups ($p < .01$ in both cases). No significant differences were obtained in the gene expression of the opioid receptor mu (*Figure 4c*).

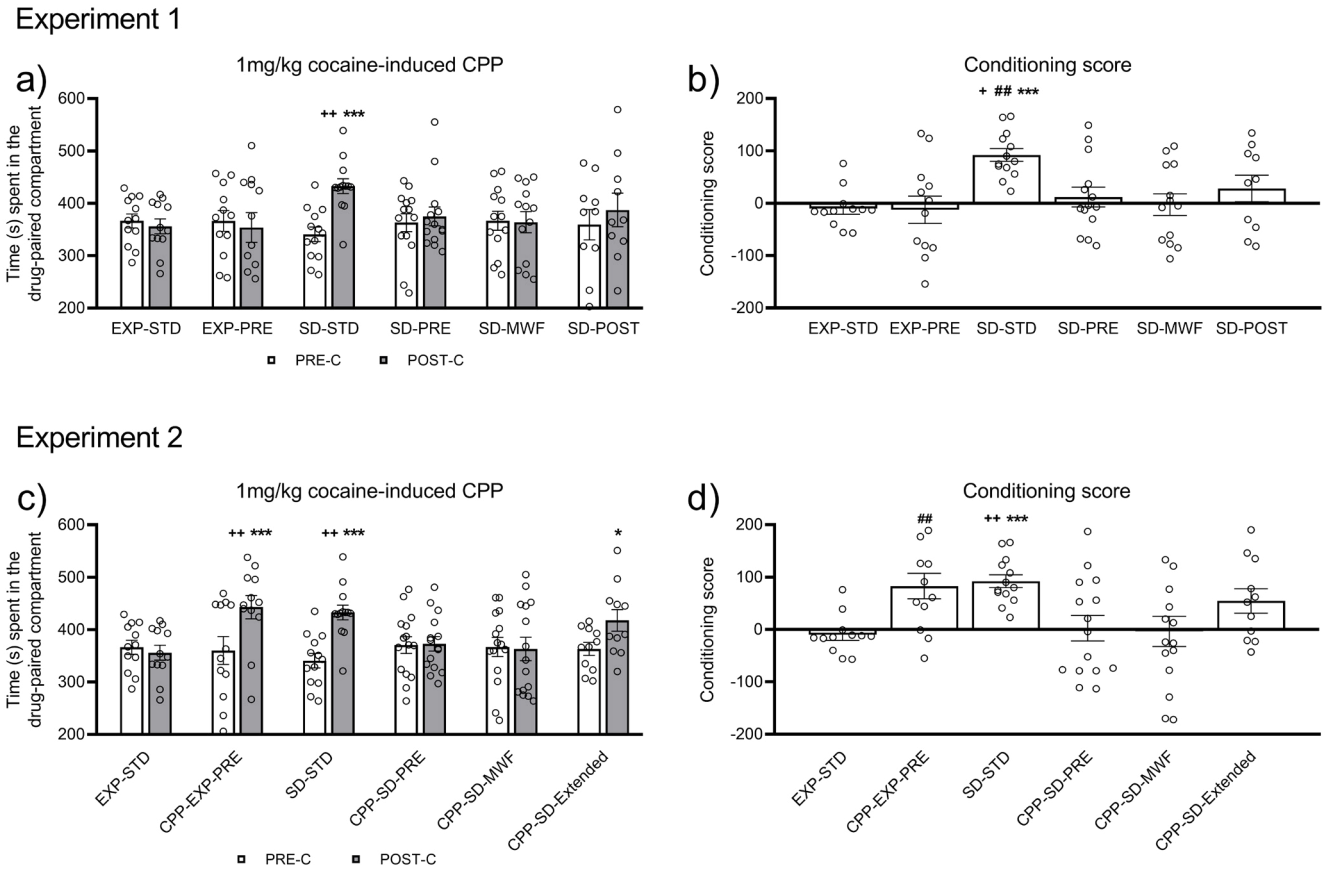
2. Experiment 2. Modulating the increase of cocaine-induced CPP with palatable food

2.1. Body weight and mean of total Kcal intake in HFD sessions.

As in the first experiment, body weight increased from PND 26 (1st week) onwards (*Figure 2d*). The ANOVA for the percentage of weight gain revealed an effect of the variable

Figure 3

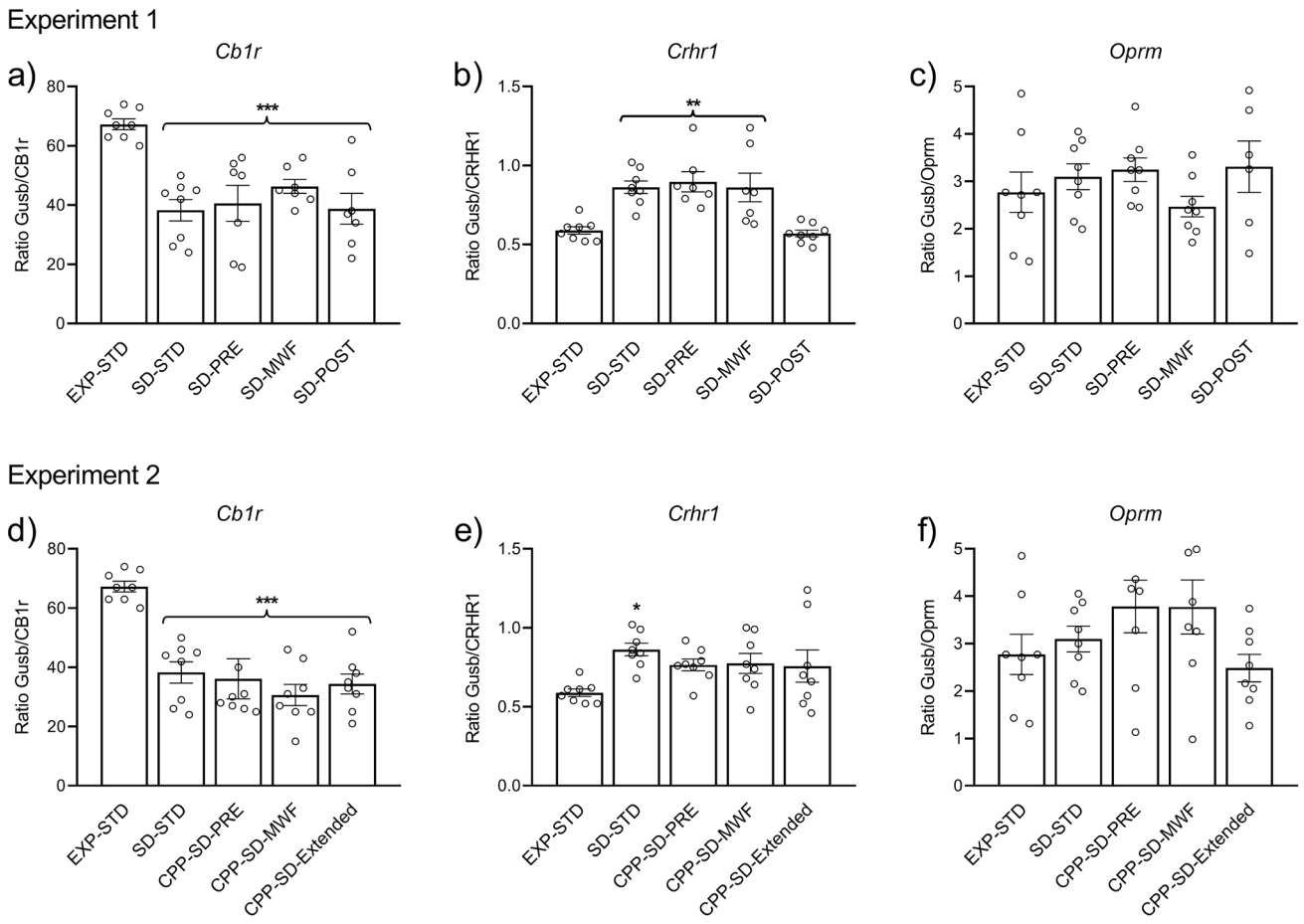
Effects of exposure to palatable food on cocaine-induced CPP in Experiment 1 and 2



Note. (a) Effects of modulating SD episodes with palatable food on cocaine-induced CPP, Experiment 1. Bars represent the mean (± SEM) time in seconds spent in the drug-paired compartment during pre-conditioning (PRE-C, white) and post-conditioning (POST-C, grey). ++p < .01 significant difference with respect to POST-C in the rest of the cases; ***p < .001 significant difference with respect to PRE-C in the SD-STD group. (b) Conditioning score, Experiment 1. Differences in the time spent in the drug-paired compartment versus the saline-paired compartment. Bars represent the mean (± SEM) time in seconds. +p < .05 significant differences with respect to SD-PRE; ##p < .01 significant differences with respect to SD-MWF; and ***p < .001 significant differences with respect to EXP-STD. (c) Effects of exposure to an HFD during CPP on cocaine-induced CPP, Experiment 2. Bars represent the mean (± SEM) time in seconds spent in the drug-paired compartment during pre-conditioning (PRE-C, white) and post-conditioning (POST-C, grey). *p < .05; ***p < .001 significant difference with respect to the corresponding PRE-C test. ++p < .01 significant difference with respect to the Post-C test of the CPP-EXP-STD and CPP-SD-PRE groups. (d) Conditioning score, Experiment 2. Differences between time spent in the drug-paired compartment versus the saline-paired compartment. Bars represent the mean (± SEM) time in seconds. ##p < .01 significant difference with respect to CPP-EXP-STD and CPP-SD-PRE; ++p < .01 significant difference with respect to CPP-SD-PRE and CPP-SD-MWF; ***p < .001 significant difference with respect to CPP-EXP-STD.

Figure 4

Real-time PCR Gene expression in the striatum



Note. (n = 8/condition). (a) Cannabinoid receptor 1 - ***Cb1r***, Experiment 1: ***p < .001 significant differences with respect to the EXP-STD group. (b) Corticotropin-releasing hormone receptor 1 - ***Crhr1***, Experiment 1: **p < .01 significant differences with respect to the EXP-STD and SD-POST groups. (c) Opioid receptor μ - ***Oprm*** Experiment 1: The columns represent means and the vertical lines ± SEM of gene expression in the striatum of OF1 mice. (d) Cannabinoid receptor 1 - ***Cb1r***, Experiment 2: ***p < .001 significant differences with respect to the CPP-EXP-STD group. (e) Corticotropin-releasing hormone receptor 1 - ***Crhr1***, Experiment 2: *p < .05 significant difference with respect to the CPP-EXP-STD group. (f) Opioid receptor μ - ***Oprm***, Experiment 2: The columns represent means and the vertical lines ± SEM of gene expression in the striatum of OF1 mice.

Week ($F(5, 370) = 1123.69$, $p < .001$). Body weight increased from the first week onwards ($p < .001$ in all cases).

The ANOVA of the mean Kcal intake per hour (*Figure 2e*) revealed significant differences on the variable Diet ($F(3, 49) = 4.11$, $p < .01$). Mice in the CPP-SD-PRE group consumed more Kcal per hour of HFD session than the CPP-SD-MWF and CPP-SD-EXTENDED groups ($p < .05$). The ANOVA also revealed an effect of the variable Diet ($F(3, 49) = 6.18$, $p < .001$) with respect the total kcal intake (*Figure 2f*). Mice on the CPP-SD-EXTENDED group consumed more Kcal than the other groups ($p < .001$ for CPP-EXP-PRE and $p < .01$ for CPP-SD-PRE and CPP-SD-MWF).

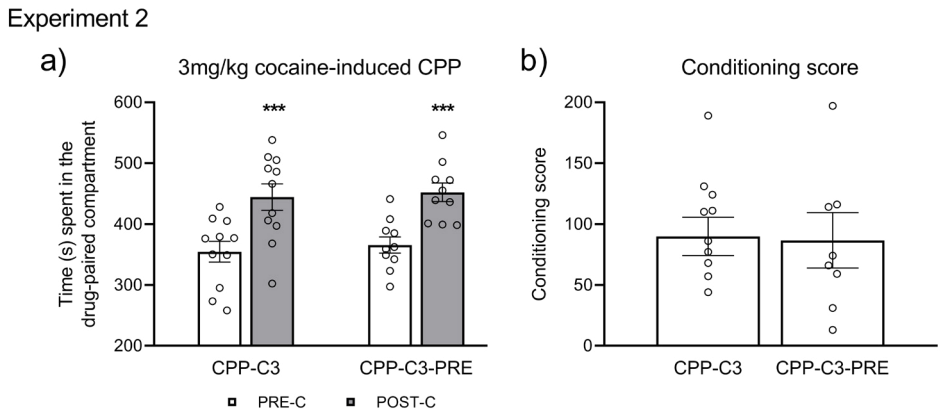
2.2. Cocaine induced CPP

The results of the 1 mg/kg cocaine-induced CPP from Experiment 2 are presented in *Figure 3c*. The ANOVA for the time spent in the drug-paired compartment revealed a significant effect of the variable Days ($F(1, 72) = 11.60$, $p < .001$), and the interaction Days × Diet × Stress ($F(1, 72) = 16.34$, $p < .001$). Preference for 1 mg/kg of cocaine was observed in the CPP-SD-STD ($p < .001$), CPP-SD-Extended ($p < .05$), and in non-stressed mice fed on HFD before conditioning (CPP-EXP-PRE) ($p < .001$). In addition, the SD-STD and CPP-EXP-PRE groups spent more time in the drug-paired compartment during POST-C than the CPP-EXP-STD and CPP-SD-PRE groups ($p < .01$ in all cases).

The ANOVA for the conditioning score revealed an effect of the interaction Diet × Stress ($F(1, 72) = 16.34$, $p < .001$] (*Figure 3d*). Among SD mice, those fed with the standard diet (CPP-SD-STD) presented a significantly higher conditioning score than non-stressed mice (CPP-EXP-STD) and defeated groups exposed to the HFD prior to conditioning (CPP-SD-PRE) and during 3 days a week (CPP-SD-MWF) ($p < .001$ for control and $p < .01$ for the rest of the cases). The non-stressed group fed with the HFD before conditioning (CPP-EXP-PRE) also presented

Figure 5

Effects of exposure to an HFD during CPP on 3 mg/kg cocaine-induced CPP



Note. (a) Effects of exposure to an HFD during CPP on cocaine-induced CPP. Bars represent the mean (± SEM) time in seconds spent in the drug-paired compartment during pre-conditioning (PRE-C, white) and post-conditioning (POST-C, grey). ***p < .001 significant difference with respect to PRE-C. (b) Conditioning score. Differences between time spent in the drug-paired compartment versus the saline-paired compartment. Bars represent the mean (± SEM) time in seconds.

a higher conditioning score than those of the CPP-EXP-STD and CPP-SD-PRE groups (p < .01 in both cases).

To further evaluate the effect of exposure to an HFD before cocaine-induced CPP, we evaluated the development of CPP induced by an effective dose of cocaine (3 mg/kg) (*Figure 5*). The ANOVA revealed an effect of the variable Days ($F(1, 19) = 41.88$, p < .001). As expected, 3 mg/kg of cocaine induced a clear preference (p < .001), which is not affected by HFD administration. The ANOVA of the conditioning score did not reveal differences between either of the groups ($F(1, 19) = 0.01$, p < .907).

2.3. Gene expression analyses

For the *Cb1r* gene expression (*Figure 4d*), the ANOVA revealed a significant effect of the Group variable ($F(4, 35) = 12.65$, p < .001). All groups exposed to an SD, regardless of diet, exhibited a significant decrease in *Cb1r* gene expression in comparison with the CPP-EXP-STD group (p < .001). The ANOVA for the expression of the *Crhr1* (*Figure 4e*) also revealed a significant effect ($F(4, 35) = 2.77$, p < .05). Mice in the SD-STD group exhibited a significant increase in *Crhr1* gene expression in comparison with the CPP-EXP-STD group (p < .05). No significant differences were obtained in the gene expression of the opioid receptor mu (*Figure 4f*).

Discussion

The present work evaluates the modulating effects of HFD on the increase in the conditioned rewarding effects of cocaine induced by social stress at two critical moments: a) during exposure to SD stress and b) long-term after SD during the procedure of cocaine-induced CPP. The present study demonstrated for the first time that intermittent intake of an HFD blocked the long-term increase in the conditioned rewarding effects of cocaine. Access to an HFD during the SD episodes (Experiment 1) efficiently counteracted the development of CPP with a subthreshold dose of cocaine (1 mg/kg). Similarly, we observed that access to an HFD prior to conditioning (CPP-Pre) or three days a week (CPP-MWF) during the acquisition of CPP (Experiment 2) blocked the increased sensitivity to the conditioned reinforcing effects of cocaine induced by SD. However, a longer exposure to HFD (CPP-SD-Extended) did not yield this effect. Despite this consistent result, none of the HFD schedules were able to counteract the decreased expression of the *Cb1r* gene. However, the SD-induced increase in *Crhr1* gene expression was lowered by HFD administration during the CPP or after each SD encounter.

Effects of HFD on body weight and Kcal intake.

A key observation in this research is that HFD consumption did not result in increased body weight. It is well known that prolonged HFD intake is linked to obesity, metabolic issues, and neuroinflammation (Blanco-Gandía et al., 2017c; Li et al., 2022; Tsai et al., 2022). Preclinical studies have observed that *ad libitum* access to an HFD leads to metabolic syndrome, increasing adiposity and leptin levels, and interfering with ghrelin and insulin signaling (Blanco-Gandía et al., 2017c; Davis et al., 2008; Morales et al., 2012). Nevertheless, the specific HFD regimens used in both experiments did not produce any significant alteration in body weight, which is in line with results obtained in previous studies using this administration pattern (Blanco-Gandía et al., 2017b; Hudson et al., 2007; Ródenas-González et al., 2021). We know that after 40 days of intermittent HFD exposure on MWF, leptin levels were not affected, although ghrelin

was significantly reduced (Blanco-Gandía et al., 2017a,b; Blanco-Gandía et al., 2019). In this line, intermittent access to HFD did not modify glucose nor insulin plasmatic levels (Del Olmo et al., 2019). Therefore, our current and previous results allow us to suggest that intermittent and limited exposure to HFD did not induce a deep affectation of 2017a,b2general metabolism.

Regarding HFD kcal intake, it is important to note that, although access to HFD was 1 h in some groups and 2 h in others, the amount of kcal intake remained similar except for the group with access after SD. In Experiment 1, the SD-POST group showed a significant increase in Kcal ingested compared to the rest of the HFD treatment groups. This group had access to HFD after each episode of stress, suggesting that this increased intake may be due to a compensatory response to stress, acting as comfort food. Several studies have reported the same phenomenon, where mice exposed to social stress subsequently increase their intake of HFD (Coccurello et al., 2018; Hassan et al., 2019; Sinha & Jastreboff, 2013). Confirming this effect, only defeated mice of the SD-POST group showed normalized *Crhr1* gene expression. Therefore, the capacity of HFD to block increased cocaine-induced CPP is not related to the amount ingested, highlighting that even a minimal amount of HFD can exert a potent, long-lasting effect.

Palatable food modulates the increase in cocaine-conditioned reward induced by social stress

As expected, and in line with previous studies, defeated mice fed with the standard diet exhibited increased sensitivity to a subthreshold dose of cocaine, developing CPP for the cocaine-paired compartment. This result has been reported in numerous studies, in which socially stressed animals show increased vulnerability to the rewarding effects of cocaine evaluated using the intravenous cocaine self-administration or the cocaine-induced CPP paradigms (Han et al., 2017; Neisewander et al., 2012; Reguilón et al., 2017; Shimamoto, 2018). Similar to the present results, we have also shown that defeated mice developed CPP using a subthreshold dose of cocaine (Ferrer-Pérez et al., 2019; Giménez-Gómez et al., 2021; Montagud-Romero et al., 2021).

In Experiment 1, we observed that the socially defeated groups that were exposed to the different patterns of HFD administration during the two weeks of social encounters did not develop CPP for cocaine. This result suggests that palatable food consumption might be acting as a buffer for stress effects (comfort food), as previous studies corroborate. For example, administering an HFD in socially stressed animals due to isolation decreases cocaine effects, with an attenuated response of cocaine-induced motor hyperactivity (Erhardt et al., 2006), a decrease in the corticosterone response, and a blockade of the acquisition of cocaine-induced CPP (Blanco-Gandía et al., 2018). HFD could reduce HPA activity (Auvinen et al., 2012; Pecoraro et al., 2004), which leads to a long-term reduction in the reinforcing effects of cocaine caused by that stress, especially in adolescence, when sensitivity to reward is enhanced (Blanco-Gandía et al., 2018; Steinberg, 2010).

The results obtained in Experiment 2, in which animals were exposed to different administration patterns of HFD during the CPP procedure, showed that all SD groups exposed to HFD during CPP acquisition did not develop cocaine preference, except for the Extended-CPP group. This group initiated exposure to HFD just after finishing the last episode of SD and continued until the end of the CPP. Despite this long exposure, this group showed increased sensitivity to the rewarding effects of the subthreshold dose of cocaine, similar to the SD animals fed with the standard diet. This suggests that when HFD exposure is prolonged over time and is not contingent with either the stress exposure or the acquisition of cocaine-induced CPP, the protective effect of palatable food is absent. Probably, long exposure to intermittent HFD can even sensitize the reward system, as previous studies suggest (Blanco-Gandía et al., 2017a, 2017b; Puhl et al., 2011). Supporting this hypothesis, we know that six weeks of intermittent HFD administration increases the sensitivity of adolescent mice to a subthreshold dose of cocaine, with the mice also needing more time to extinguish the preference (Blanco-Gandía et al., 2017b). However, we have previously reported that limited and intermittent exposure to HFD after cocaine preference acquisition blocks reinstatement and accelerates extinction in non-stressed mice (Ródenas-González et al., 2021), thus indicating that the timing of HFD exposure is critical in the modulation of the reward system. In line with this, the present results indicate that when intermittent administration of HFD is contingent and limited to the CPP session days, the reinforcing effects of cocaine increased by social stress are blocked, possibly due to a reward competition. These findings are in line with our recent report suggesting that the protective effect of intermittent HFD exposure may extend to various drugs of abuse, such as ethanol, potentially preventing stress-induced susceptibility to different addictive substances (Arenas et al., 2025).

An interesting effect was also observed in this experiment, since non-stressed mice exposed to HFD before CPP acquisition developed cocaine preference. The ability of HFD to induce conditioned preference has been described previously (Jarosz et al., 2007; Mizoguchi et al., 2021). Differently from these studies, our conditioning procedure only required four conditioning sessions and caloric intake was not restricted. In fact, our results did not prove that HFD per se induced conditioned preference, but that, in combination with a non-effective dose of cocaine, it was capable of developing preference. This additive effect has

also been described by Iqbal et al. (2023), who observed that the opiate oxycodone only developed preference in the HFD-associated compartment.

Changes in striatal gene expression after HFD administration of socially defeated mice

Considering the relevance of the cannabinoid and opioid systems in addiction and HFD effects (Barson et al., 2012; Kawahara et al., 2013), and the critical importance of corticotropin-releasing factor in stress (Puhl et al., 2011), we also explored changes in *Cb1r, Crhr1* and *Oprm* gene expression in the striatum at the end of the experimental procedure. Under the standard diet, SD induces a reduction in *Cb1r* and an increase in *Crhr1* gene expression, with no changes in *Oprm* gene expression. The aim of this study was to test the changes in gene expression in stressed mice exposed to intermittent HFD. The effects of HFD administered in non-stressed mice have been previously studied (Blanco-Gandia et al., 2017a,b).

The increase in *Crhr1* gene expression confirms what other studies have reported, namely that *Crhr1* gene expression is usually increased as a response to stress (Logrip et al., 2012). An increase in *Crhr1* gene expression can lead to higher vulnerability to the rewarding effects of cocaine, since some studies have demonstrated that CRHR1 antagonists can block this effect using both the CPP and SA procedures (Boyson et al., 2014; Ferrer-Pérez et al., 2018).

Only when administered after an episode of SD (SD-Post), HFD efficiently decreased *Crhr1* gene expression to levels similar to those observed in the exploration group. SD-Post was the group with the highest Kcal intake per session, suggesting that the rise in fat Kcal intake may play a role in this effect. As previously mentioned, there is a tendency to increase palatable food consumption in rodents exposed to stress (Coccurello et al., 2018; Pecoraro et al., 2004; Zellner et al., 2006). We can suggest that HFD induced a similar effect to CRHR1 antagonists, reducing *Crhr1* expression and consequently HPA activity (Foster et al., 2009; la Fleur et al., 2005; Pecoraro et al., 2004; Ulrich-Lai et al., 2011). Moreover, other studies have demonstrated that an HFD can reduce corticosterone levels in isolated mice (Blanco-Gandía et al., 2018) or in those exposed to restraint stress (Zeeni et al., 2013). HFD also reduced other consequences of social stress, such as social avoidance, anxiety and depression behavior (MacKay et al., 2017; Maniam & Morris, 2010; Otsuka et al., 2019).

On the other hand, HFD interventions during the acquisition of CPP in Experiment 2 only slightly decreased *Crhr1* gene expression. Therefore, although a decrease in the conditioned rewarding effects of cocaine was observed after all HFD administrations during SD, *Crhr1* gene expression was only blocked when access was granted after each stress episode. In Experiment 2, when HFD was administered long-term after stress exposure, although no significant increase in *Crhr1* gene expression was observed after any of the HFD administrations, the expression level of this gene was elevated in all defeated groups, indicating no valuable effect of HFD outside the stress period.

Opioid signaling is closely associated with the rewarding properties of food and plays a key role in regulating palatability (Esch & Stefano, 2024), while the endocannabinoid system is involved in the homeostatic control of intake and provides positive feedback specifically for the intake of fatty foods (Koch, 2001). According to the data available to date, intermittent fat intake could alter reward pathways through the interaction of the opioid and cannabinoid systems.

Regardless of diet, all groups exposed to SD presented a decrease in *Cb1r* gene expression, confirming that SD may have long-term effects on the cannabinoid system. Previous studies have shown that CB1 signaling modulates the stress response (Valverde & Torrens, 2012). For example, chronic stress is associated with a reduction in the *Cb1r* gene expression in the hippocampus (Hill et al., 2005; Hu et al., 2011; Reich et al., 2009), and in the striatum (Rossi et al., 2008; Wang et al., 2010). Furthermore, stimulation of CB1 receptors reduces stress-induced effects such as anhedonia (Rademacher & Hillard, 2007), depressive behaviors (Gobbi et al., 2005) and passive stress-coping behavior (Steiner et al., 2008).

Our results showed that the marked reduction in *Cb1r* gene expression observed in defeated animals was not reversed by any of the HFD administration patterns in either experiment. Consistent whit this, previous studies have also suggested that an HFD decreases *Cb1r* gene expression in the N Acc of adults (Bello et al., 2012; Martire et al., 2014) and adolescent rodents (Blanco-Gandía et al., 2017b). The similar effect of stress and HFD on *Cb1r* gene expression could explain the absence of an observable reversal in our study.

Finally, the results from both Experiment 1 and Experiment 2 suggest that neither social stress nor intermittent HFD administration causes alterations in *Oprm* gene expression. Although the involvement of the endogenous opioid system in stress responses (Komatsu et al., 2011) and fat consumption has been documented (Sakamoto et al., 2015), existing literature shows conflicting results regarding *Oprm* expression, suggesting a complex regulation of the endogenous opioid system. Increases in *Oprm* gene expression have been associated with continuous intake of palatable foods and some sugary or sweetened beverages (Blanco-Gandía et al., 2017a,b; Soto et al., 2015), but a downregulation has also been reported after chronic HFD intake in obese mice models (Vucetic et al., 2011). Moreover, prolonged but limited and intermittent intake of high-fat food seems to reduce *Oprm* expression in the NAcc (Blanco-Gandía et al., 2017a,b). Consequently,

our results could be due to the limited exposure to HFD compared to other studies, indicating that the kcal ingested by mice in our study was not enough to induce any change. These findings are further supported by our previous investigations with non-stressed mice exposed to limited and intermittent HFD after acquiring cocaine preference, which did not present changes in *Oprm* gene expression in the striatum (Ródenas-González et al., 2021).

Conclusion

The results obtained in this study confirmed that palatable food could be a good alternative reinforcer that reduced the acquisition of cocaine-conditioned preference in stressed animals. The specific period of HFD administration appears to be an important factor to be considered, while the duration of exposure was not critical. In fact, our results suggest that long exposure to the HFD may not be effective. Brief administration of a fatty diet after SD or during the acquisition of the preference for cocaine can reduce its conditioned rewarding effects. This effect could be mediated by a reduction in the increased *Crhr1* gene expression. Future studies should address other systems related to stress and reward to provide a broader explanation of the positive effect of HFD on the consequences of social stress.

Based on our previous and present results, we hypothesize that controlled administration of HFD might be a useful strategy to mitigate the effects of social stress on the reinforcing effects of cocaine, especially when this administration occurs during stress or cocaine exposure. Considering the influence of this diet on reward circuits and its effects when administered for a prolonged period of time, the present results highlight the potent effect of minimal exposure to fat. The lack of a suitable and validated model to study social defeat in female mice until recent years has delayed the implementation of this study in females. Additionally, the study of other brain structures, such as the hypothalamus, could shed more light on the mechanisms involved.

From a translational perspective, our results should not encourage prolonged HFD consumption. The value of our findings lies in the potential utility of consuming small amounts of high-fat food during stress experiences or cocaine exposure. It is essential to emphasize that fat intake should be predominantly derived from sources rich in monounsaturated and polyunsaturated fatty acids. It is well established that the consumption of fat-rich foods, such as highly processed and palatable products, stimulates the reward system and increases the risk of developing food addiction (Ulug et al., 2025). Therefore, special caution should be exercised when recommending this type of intake in patients who already present a cocaine use disorder. The consumption of such diets should be carefully controlled, and access to a healthy HFD should be ensured.

Acknowledgements

This work was supported by the following grants: PID-2020-112672RB-I00 by MCIN/AEI/ 10.13039/501100011033 and ERDF A way of making Europe; Instituto de Salud Carlos III, Atención primaria, cronicidad y promoción de la salud, RED DE INVESTIGACIÓN EN ATENCIÓN PRIMARIA DE ADICCIONES (RIAPAd) RD21/0009/0003 and RD23/0003/0004. Generalitat Valenciana, Conselleria de Educación, Dirección General de Universidades, Grupos de Investigación de Excelencia PROMETEO (CIPROM/2021/080).

Conflicts of interest

The authors of this article declare no conflict of interests.

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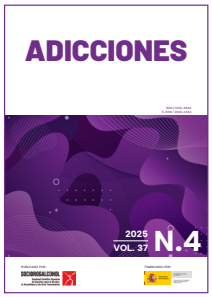

ORIGINAL

Effects of omega-3 fatty acids on CB1 cannabinoid receptor localization in the hippocampal CA1 region following alcohol withdrawal in adolescent male mice

Efectos de los ácidos grasos omega-3 sobre la localización del receptor cannabinoide CB1 en la región CA1 del hipocampo tras la abstinencia de alcohol en ratones machos adolescentes

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Abstract

Adolescent binge drinking has detrimental effects on brain function, leading to long-lasting impairments in synaptic plasticity, cognition, and behavior. These effects are mediated, in part, by disruption of the endocannabinoid system (ECS) and its cannabinoid type-1 (CB1) receptor. Alcohol consumption also depletes omega-3 fatty acids, which are essential for maintaining cell membrane integrity and supporting brain function. This depletion impairs synaptic plasticity by disrupting endocannabinoid signaling and reducing CB1 receptor expression and function. Conversely, enhancement of the ECS can restore brain function and reverse the loss of endocannabinoid-dependent synaptic plasticity associated with omega-3 deficiency. Notably, omega-3 supplementation has been shown to restore CB1 receptor expression in specific brain regions in adult mice following adolescent alcohol exposure. However, despite the established interplay between alcohol, omega-3, and the ECS, the direct impact of omega-3 supplementation on the subcellular localization of CB1 receptors after alcohol exposure remains poorly understood.

In this study, we used immunoelectron microscopy to investigate whether omega-3 supplementation influences CB1 receptor distribution in the hippocampal CA1 region following alcohol withdrawal in adolescent male mice. Our results demonstrate that omega-3 partially restores the excitatory/inhibitory balance disrupted by alcohol, as evidenced by an increased number of excitatory terminals and a significant reduction in inhibitory terminals. However, the distribution and density of CB1 receptors within neuronal and glial compartments remain unchanged following alcohol exposure and omega-3 supplementation.

These findings highlight novel structural effects of omega-3 in mitigating alcohol-induced brain damage.

Keywords: Ethanol, adolescence, endocannabinoid system, neurons, glia, polyunsaturated fatty acids, central nervous system

Resumen

El consumo excesivo de alcohol durante la adolescencia compromete la plasticidad sináptica y las funciones cognitivas, en parte debido a la interferencia con el sistema endocannabinoide (SEC) y su receptor CB1. Asimismo, el alcohol impacta negativamente en los ácidos grasos omega-3, esenciales para la integridad de las membranas celulares y la función cerebral. La deficiencia de omega-3 altera la señalización del SEC y la expresión y funcionalidad del CB1, exacerbando el deterioro inducido por el alcohol. Por el contrario, la activación del SEC contribuye a restaurar las funciones sinápticas dependientes de este sistema, afectadas por la falta de omega-3. De hecho, se ha demostrado que la suplementación con omega-3 normaliza la expresión del CB1 en regiones cerebrales específicas de ratones adultos expuestos al alcohol durante la adolescencia.

A pesar de la estrecha relación entre alcohol, omega-3 y SEC, el efecto directo de los omega-3 sobre la localización subcelular del receptor CB1 tras la exposición al alcohol permanece poco explorado. Para abordar esta cuestión, analizamos la región CA1 del hipocampo en ratones machos adultos en abstinencia después de la ingesta de alcohol durante la adolescencia, utilizando inmunocitoquímica para microscopía electrónica. Nuestros resultados indican que los omega-3 ayudan a restablecer el equilibrio entre excitación e inhibición sináptica, alterado por un aumento en el número de terminales excitadoras y una reducción significativa de las inhibidoras. Sin embargo, ni la distribución ni la densidad del receptor CB1 en compartimentos neuronales y gliales se modificaron tras la exposición al alcohol ni con la suplementación con omega-3.

Estos hallazgos revelan efectos estructurales novedosos de los omega-3 en la protección frente al daño cerebral inducido por el alcohol, aportando nuevas perspectivas sobre los mecanismos neuroprotectores de estos ácidos grasos.

Palabras clave: Etanol, adolescencia, sistema endocannabinoide, neuronas, glía, ácidos grasos poliinsaturados, sistema nervioso central

■ *Received: April 2025; Accepted: July 2025.*

■ ISSN: 0214-4840 / E-ISSN: 2604-6334

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According to the 2025 ESTUDES survey conducted by the Spanish Observatory on Drugs and Addictions (Government Delegation for the National Drug Strategy, Ministry of Health, 2025), adolescents constitute a key demographic in alcohol consumption trends, particularly regarding episodic heavy drinking (binge drinking). Nearly one-quarter of individuals aged 14–18 reported engaging in at least one binge-drinking episode in the 30 days preceding the survey. Prevalence was higher in males (26.1%) than in females (23.3%), although both groups exhibited a significant decline compared with the 2023 assessment, with the reduction being more pronounced among females.

Binge drinking during adolescence has significant implications for brain development. This period is marked by extensive neurobiological remodeling, with changes in neurotransmission, synaptic plasticity, and structural maturation, particularly in brain regions associated with learning, memory, and executive function. Consequently, alcohol consumption during this developmental window can result in long-lasting, potentially irreversible alterations in brain function, affecting synaptic transmission and neuroplasticity, ultimately contributing to cognitive, emotional, and motor deficits (Abrahao et al., 2017; Cservenka & Brumback, 2017; Kang et al., 2024; Keshavan et al., 2014; Lovinger & Abrahao, 2018; Lovinger & Alvarez, 2017; Lovinger & Roberto, 2013; Pava & Woodward, 2012; Vetreno & Crews, 2015).

A growing body of evidence implicates ECS in mediating the effects of alcohol on synaptic function in acute exposure, chronic intake, and withdrawal. The ECS plays a key role in regulating motivation and alcohol consumption and is increasingly recognized as a contributor to the pathophysiology of alcohol use disorder (AUD) (Gonzalez et al., 2002; Kunos, 2020; Navarrete et al., 2022; Peñasco et al., 2020; Sanchez-Marin et al., 2022; Vinod et al., 2006). This system comprises G protein-coupled cannabinoid receptors—primarily CB1 and CB2—endocannabinoids such as 2-arachidonoylglycerol (2-AG) and anandamide (AEA), and the enzymatic machinery responsible for their synthesis, degradation, and transport (Lu & Mackie, 2021).

Multiple studies have demonstrated that chronic alcohol exposure downregulates CB1 receptor mRNA and protein levels, affecting receptor density and function (Basavarajappa et al., 1998; Ortiz et al., 2004; Vinod et al., 2006). In humans, chronic heavy drinking is associated with reduced CB1 receptor availability in several brain regions, often persisting beyond periods of abstinence (Ceccarini et al., 2014; Hirvonen et al., 2013; Wolfe et al., 2022). Conversely, genetic deletion or pharmacological antagonism of CB1 receptors reduces alcohol intake, further underscoring its involvement in alcohol-related behaviors (Maccioni et al., 2010).

While pharmacological interventions hold promise in addressing alcohol-induced cognitive dysfunction, nutritional strategies, such as supplementation with omega-3 polyunsaturated fatty acids (PUFAs), are emerging as effective and accessible alternatives. Prior studies have demonstrated that omega-3 PUFAs can ameliorate the loss of brain plasticity induced by prenatal alcohol exposure, suggesting their potential for preventing and treating alcohol-related neurodevelopmental damage (Joffre et al., 2019; Patten et al., 2013a,b; Serrano et al., 2023). Recent findings indicate that omega-3 restore synaptic plasticity and improve hippocampal-dependent learning and memory following alcohol exposure during critical developmental periods (Haidary et al., 2024; Serrano et al., 2025). Thus, omega-3 supplementation prevents the spatial and associative learning impaired in adolescent male rats exposed to chronic alcohol consumption. The improvement was associated with the reduction in hippocampal oxidative stress and neuroinflammation associated with lower levels of lipid peroxidation and interleukin-6 (Haidary et al., 2024). These findings are in line with previous investigations (Farooqui, 2012; Patten et al., 2013a) and reinforce the potential of omega-3 to exert neuroprotective effects by restoring redox balance and modulating inflammatory pathways. In addition, omega-3 fatty acids, particularly DHA, enhance membrane fluidity ensuring the function of membrane-associated receptors (Bazinet & Layé, 2014; Joffre et al., 2019), which facilitates synaptic transmission and plasticity (Patten et al., 2013a,b). They also influence neurotransmitter systems by modulating receptor expression and signaling cascades involved in long-term potentiation (LTP) and long-term depression (LTD) (Patten et al., 2013b; Serrano et al., 2024, 2025), which are crucial for learning and memory (Peñasco et al., 2020; Serrano et al., 2025).

Adolescent alcohol exposure has been shown to disrupt synaptic plasticity and alter CB1 receptor expression and function in the hippocampus (Peñasco et al., 2020; Risher et al., 2015; Sabeti, 2011; Sanz-Martos et al., 2023), a brain region essential for declarative, spatial, and associative memory. Our previous research has shown that adolescent binge drinking leads to a sustained reduction in CB1 mRNA expression in the mature hippocampus. This downregulation correlates with decreased CB1 receptor density in excitatory terminals of the dentate molecular layer, disrupting endocannabinoid-dependent (LTD) and contributing to memory impairments (Peñasco et al., 2020; Rico-Barrio et al., 2019, 2021). Therefore, understanding the role of the ECS in alcohol-induced synaptic alterations is essential for identifying therapeutic targets aimed at mitigating the long-term effects of adolescent alcohol exposure and treating AUD (Borgonetti et al., 2024; García-Baos et al., 2021; Kunos, 2020; Serrano et al., 2012, 2018; Stopponi et al., 2018; Wolfe et al., 2022). In particular, adolescent binge drinking decreases CB1 receptor expression in the CA1 region of the adult hippocampus after withdrawal, primarily affecting astrocytic localization and, to a lesser extent, excitatory synaptic terminals (Bonilla-Del Río et al., 2019). Similarly, our laboratory has also observed a reduction in CB1

receptors at excitatory synapses in the dentate molecular layer following adolescent alcohol exposure (Peñasco et al., 2020). Interestingly, CB1 receptor density increased at both excitatory and inhibitory synaptic terminals in the dentate molecular layer following omega-3 supplementation (Serrano et al., 2025). However, the effects of an omega-3-enriched diet on CB1 receptor localization in the CA1 hippocampus following alcohol withdrawal remains unexplored. This is important to understand due to the impact of alcohol on CA1 region, which contributes to associative memory formation and play a key role in incremental value learning (Jeong et al., 2018; Takamiya et al., 2021).

In the present study, we investigated whether omega-3 supplementation could modulate alcohol-induced alterations in CB1 receptor distribution in the CA1 region of the hippocampus in male mice. Using immunoelectron microscopy, we provide novel insights into the potential of omega-3 fatty acids to counteract long-term neurobiological consequences of adolescent binge-like alcohol exposure.

Method

Ethics statement

The protocols for animal care and use were approved by the Committee of Ethics for Animal Welfare of the University of the Basque Country (M20-2020-113; date of approval: 09/29/2020). They were also in agreement with the European Communities Council Directive of September 22, 2010 (2010/63/EU) and Spanish regulations (Real Decreto 53/2013, BOE 08-02-2013). The number of animals and suffering were controlled and minimized.

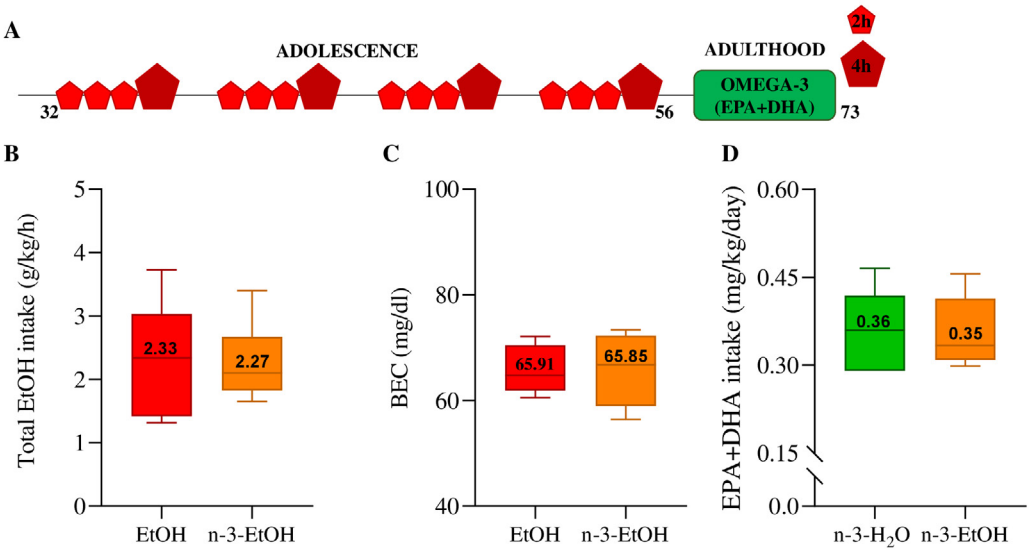
Animal treatment

Four-week-old C57BL/6J male mice (Janvier Labs, Le Genest-Saint-Isle, France) were housed in pairs and randomly distributed in H_2O or alcohol (EtOH) group (n= 12/group). The mice underwent the drinking-in-the-dark (DID) procedure (Rhodes et al., 2005) between postnatal days (PND) 32 and 56 for a period of four weeks, as previously described (Bonilla-Del Río et al., 2019). Briefly, the first four days of each week, mice were individualized with a 10 mL bottle containing tap water or EtOH solution (20% v/v EtOH, prepared from 96% EtOH; Boter S.L., Barcelona, Spain). Mice had access to the bottle for 2 hours on the first three days and the access was extended to 4 hours on the fourth day. During the remaining three days of each week, they were kept resting with food and water *ad libitum* (Figure 1A).

The effectiveness of the DID procedure was evaluated by measuring total EtOH intake (g/kg/h) (Figure 1B). Furthermore, on the last day of DID (PND 56), blood samples were collected from the lateral tail vein 30 minutes after the end of EtOH exposure. Blood ethanol concentration (BEC, mg/dL) was then measured using a commercial ethanol assay kit (Abcam ab65646, Spain). (Figure 1C). In our experimental conditions, BECs were below the threshold associated with adolescent binge drinking (>80 mg/

Figure 1

Schematic representation of the drinking-in-the-dark (DID) model, voluntary ethanol (EtOH) intake, blood ethanol concentration (BEC), and EPA+DHA intake



Note. **A**. C57BL/6J male mice underwent the DID procedure for four weeks (PND 32-56). During this period, they had free access to either H_2O or EtOH for 2 hours on the first three days of each week, and for 4 hours on the fourth day. During the abstinence period (PND 57–73), half of the mice were fed a diet enriched with omega-3 (2% EPA+DHA: 1.2% EPA and 0.8% DHA). **B.** Average EtOH intake per hour (g/kg/h) throughout DID (PND 32-56) (EtOH: 2.33 ± 0.90, n=6; n-3-EtOH: 2.27 ± 0.62, n=6). **C.** BEC (mg/dL) measured after 4 hours of EtOH exposure on the final day of the DID procedure (PND 56) (EtOH: 65.91 ± 4.59, n=6; n-3-EtOH: 65.85 ± 6.96, n=6). **D.** Average daily EPA+DHA intake (mg/kg/day) during the withdrawal period (PND 57–73) (n-3-H_2O: 0.36 ± 0.07, n=6; n-3-EtOH: 0.35 ± 0.06, n=6). All data are expressed as mean ± ***S.E.M.***

dL) (Donovan, 2009). However, these levels have been shown to produce significant neurobiological alterations in adolescent rodents (Bonilla-Del Río et al., 2019; Peñasco et al., 2020; Rico-Barrio et al., 2019; Serrano et al., 2025), thereby supporting the validity of this protocol as binge-like exposure model (Rhodes et al., 2005).

During the withdrawal period (PND 57–73), half of the mice (n = 6/group) were fed a diet enriched with 2% docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (1.2% EPA and 0.8% DHA) (SAFE, Augy, France), corresponding to the n-3-H_2O and n-3-EtOH groups. Mice and food were weighed twice a week to assess EPA and DHA intake (mg/kg/day) (Figure 1D).

Brain tissue processing

On PND 73, three mice from each group were deeply anesthetized using 4% chloral hydrate (10 mL/kg body weight, i.p.) and perfused through the left ventricle with 30 mL of phosphate-buffered saline (PBS, 0.1 M, pH 7.4). This was followed by perfusion with 80 mL of a fixative solution (4% formaldehyde, 0.2% picric acid, and 0.1% glutaraldehyde) prepared in PBS at room temperature (RT). The brains were removed and post-fixed in the same fixative solution at 4°C for one week before being transferred to a 1:10 diluted fixative. Coronal sections of 50 µm thickness were then cut using a vibratome (Leica VT 1000s, Wetzlar, Germany) and collected in phosphate buffer (PB, 0.1 M, pH 7.4) at RT.

Double pre-embedding immunogold and immunoperoxidase method for electron microscopy

The method was conducted based on our previously described protocol (Puente et al., 2015). In brief, 50 µm thick coronal sections of the hippocampus were pre-incubated at RT for 30 minutes in a solution of 10% bovine serum albumin (BSA) and Tris-HCl buffered saline (TBS) (pH 7.4) containing 0.1% sodium azide and 0.02% saponin. Then, the sections were incubated with primary antibodies highly specific against the CB1 receptor and glutamate aspartate transporter (GLAST) (Table 1) in 10% BSA/TBS with 0.1% sodium azide and 0.004% saponin. This step was performed with gentle agitation over two days at 4°C. After extensive washing, sections were incubated with secondary antibodies (Table 1), followed by an incubation in an avidin-biotin complex solution (1:50, PK-7100, Vector Labs) for 1.5 hours at RT. Sections were then washed overnight and post-fixed for 10 minutes in 1% glutaraldehyde prepared in TBS at RT.

Gold particles were then silver-enhanced using an HQ Silver Enhancement Kit (Nanoprobes Inc.) for 12 minutes in the dark. Following this, sections were treated with 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.1 M PB for 3 minutes at RT. After multiple washes, stained sections were post-fixed in 1% osmium tetroxide in 0.1 M PB for 20 minutes, dehydrated in a graded series of alcohols (50–100%) followed by propylene oxide, and embedded in Epon resin 812. Finally, ultrathin sections (50 nm) were cut with a diamond knife (Diatome USA), collected on nickel mesh grids, and stained with 2.5% lead citrate for 20 minutes. These sections were examined using an electron microscope (JEOL JEM 1400 Plus, Tokyo, Japan) and imaged with a digital camera (Morada, sCMOS, Olympus, Tokyo, Japan). Images were captured at 8,000X magnification, covering a total area of approximately 1,100 µm² per mouse. The number of excitatory and inhibitory terminals, as well as GLAST-stained astrocytes, was consistent across experimental groups (Table 2).

Statistical analysis

It was performed using GraphPad Prism 8 (GraphPad Software; RRID: SCR_002798). Data normality was assessed with the Shapiro–Wilk test, followed by a one-way ANOVA and either parametric or nonparametric multiple comparison tests, as appropriate. All values are presented as mean ± S.E.M.

Table 1

Primary and secondary antibodies used in pre-embedding immunocytochemistry for electron microscopy

Antibody	Concentration [µg/mL]	Species and clonality	Immunizing antigen	Source, Cat.
Anti-CB1 receptor	2	Guinea pig polyclonal	mouse CB1, C-terminal 31 aa (NM007726)	Nittobo Medical Co., CB1-GP-Af530
Anti-GLAST	0.3	Rabbit polyclonal	mouse GLAST, C-terminal 41 aa (NM148938)	Nittobo Medical Co., GLAST-Rb-Af660
1.4 nm gold-conjugated anti-guinea pig	0.8	Goat (polyclonal)	-	Nanoprobes, #2055
Biotinylated anti-rabbit	7.5	Goat (polyclonal)	-	Vector Labs, BA-1000

Table 2

Number of excitatory and inhibitory terminals, and GLAST-stained astrocytes in mice from each experimental group

	H_2O				EtOH				n-3-EtOH				n-3-H_2O			
	n 1	n 2	n 3	**Total**	n 1	n 2	n 3	**Total**	n 1	n 2	n 3	**Total**	n 1	n 2	n 3	**Total**
Exc.ter	458	396	283	**1137**	387	467	430	**1284**	228	375	276	**879**^^	356	291	372	**1019**
Inh.ter	35	54	63	**152**	38	38	17	**93***	37	33	45	**115**	45	75	52	**172**^^†
Astros	115	279	238	**632**	102	214	126	**442**	129	135	343	**607**	350	382	179	**911**^^

Note. Statistical significance was analyzed using a one-way ANOVA with either parametric (Bonferroni) or non-parametric (Dunn) multiple comparison tests, as appropriate. *p<0.05 compared to H_2O; ^^p<0.01 compared to EtOH and †p<0.05 compared to n-3-EtOH.

Results

CB1 receptor localization in excitatory and inhibitory synaptic terminals in CA1 *stratum radiatum* two weeks after withdrawal from adolescent binge drinking

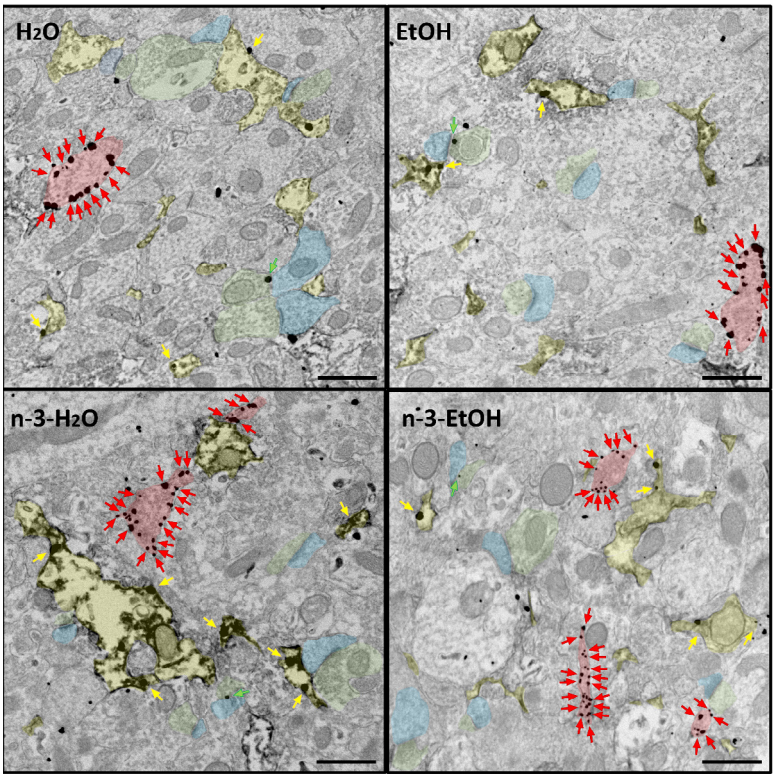
As expected, gold particles were localized in both excitatory and inhibitory axon terminals, forming asymmetric and symmetric synapses with dendritic spines and dendrites, respectively, in all experimental groups. Analyses were performed on three animals per group (Figure 2).

The total number of excitatory terminals in EtOH (~1,100 µm^2 per animal) was significantly decreased in n-3-EtOH mice (EtOH: 1,284; n-3-EtOH: 879; EtOH vs. n-3-EtOH **p<0.01) (Figure 3A) (Table 2). Moreover, the number of inhibitory terminals was significantly lower in EtOH mice compared to both H_2O and n-3-H_2O mice (H_2O: 152; n-3-H_2O: 172; EtOH: 93; EtOH vs. H_2O *p<0.05 and n-3-H_2O **p<0.01). There was no statistically significant difference between EtOH and n-3-EtOH mice; however, n-3-H_2O mice exhibited a significantly higher number of inhibitory terminals compared to n-3-ETOH (n-3-EtOH: 115; n-3-H_2O: 172; n-3-EtOH vs. n-3-H_2O *p<0.05) (Figure 3A) (Table 2). Hence, the ratio of excitatory/inhibitory synaptic terminals was significantly increased in the EtOH mice relative to the other experimental groups (H_2O: 8.36 ± 1.12; n-3-H_2O: 6.83 ± 0.88; EtOH: 25.57 ± 8.11, n-3-EtOH: 8.21 ± 0.88; EtOH vs. H_2O and n-3-EtOH *p<0.05, and vs. n-3-H_2O **p<0.01) (Figure 3B).

Both the percentage of CB1-positive excitatory terminals and the density of CB1 particles were similar across the four experimental conditions (n-3-H_2O, EtOH, n-3-EtOH vs. H_2O p>0.05) (Figure 3C and D) (Table 3). In addition, not significant differences were found in the percentage of CB1-positive inhibitory terminals (n-3-H_2O, EtOH, n-3-EtOH vs. H_2O p>0.05). However, receptor density increased significantly following omega-3 supplementation in H_2O control mice (H_2O: 6.54 ± 0.24, n-3-H_2O: 7.67 ± 0.23; n-3- H_2O vs. H_2O **p<0.01) (Figure 3C and D) (Table 3).

Figure 2

Immunoelectron localization of CB1 receptors in the CA1 stratum radiatum of H_2O, n-3-H_2O, EtOH, and n-3-EtOH male mice



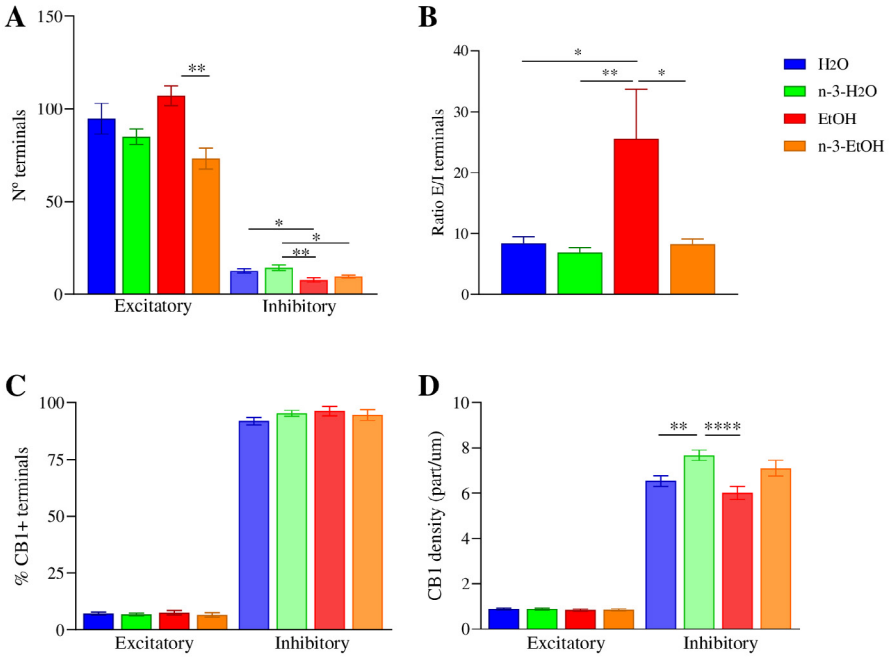
Note. CB1 receptors are localized to axon terminals forming symmetric (red shading and arrows) and asymmetric (green shading and arrows) synapses—the latter with dendritic spines (blue shading)—as well as to astrocytic membranes (yellow shading and arrows). Scale bars = 1 µm.

CB1 receptor localization in GLAST-stained astrocytes in the CA1 stratum radiatum two weeks after withdrawal from adolescent binge drinking

Gold particles were also localized to astrocytic membranes in all four experimental groups (Figure 2). The total number of GLAST-positive astrocytes in the analyzed area (~1,100 µm^2 per animal) was significantly higher in n-3-H_2O mice, in contrast to the trend toward reduction

Figure 3

Ultrastructural localization of the CB1 receptor in neuronal compartments in the CA1 stratum radiatum of H_2O, n-3-H_2O, EtOH and n-3-EtOH adult male mice



Note. **A.** Total number of excitatory (H_2O: 1,137; n-3-H_2O: 1,019; EtOH: 1,284; n-3-EtOH: 879, **p<0.01 vs. EtOH) and inhibitory terminals (H_2O: 152; n-3-H_2O: 172; EtOH: 93, *p<0.05 vs. H_2O, **p<0.01 vs. n-3-H_2O; n-3-EtOH: 115, *p<0.05 vs. n-3-H_2O) in the analyzed area (~1,100 µm²) of the four experimental groups. **B.** Ratio of total excitatory and inhibitory terminals (H_2O: 8.36 ± 1.12; n-3-H_2O: 6.83 ± 0.88; EtOH: 25.57 ± 8.11, *p<0.05 vs. H_2O, **p<0.01 vs. n-3-H_2O; n-3-EtOH: 8.21 ± 0.88, *p<0.05 vs. EtOH). **C.** Percentage of CB1-positive excitatory (H_2O: 7.18 ± 0.58; n-3-H_2O: 6.86 ± 0.54; EtOH: 7.57 ± 0.91; n-3-EtOH: 6.59 ± 0.91) and inhibitory terminals (H_2O: 91.89 ± 1.68; n-3-H_2O: 95.30 ± 1.29; EtOH: 96.30 ± 2.09; n-3-EtOH: 94.64 ± 2.41). **D.** CB1 receptor density (particles/µm) in excitatory (H_2O: 0.89 ± 0.05; n-3-H_2O: 0.89 ± 0.05; EtOH: 0.84 ± 0.05; n-3-EtOH: 0.84 ± 0.05) and inhibitory terminals (H_2O: 6.54 ± 0.24; n-3-H_2O: 7.67 ± 0.23, **p<0.01 vs. H_2O; EtOH: 6.02 ± 0.29, ****p<0.0001 vs. n-3-H_2O; n-3-EtOH: 97.11 ± 0.35). All data are expressed as mean ± S.E.M. The Shapiro–Wilk test was used to assess normality, followed by one-way ANOVA with either parametric (Bonferroni) or non-parametric (Dunn) multiple comparison tests, as appropriate.

Table 3

Percentage of CB1-positive excitatory terminals, inhibitory terminals, GLAST-stained astrocytes, and receptor density in the CA1 stratum radiatum across the four experimental conditions (n = 3 mice per group)

	H_2O	EtOH	n-3-EtOH	n-3-H_2O
Excitatory terminals				
% CB1+	7.18 ± 0.58	7.57 ± 0.91	6.59 ± 0.91	6.86 ± 0.54
Density	0.89 ± 0.05	0.84 ± 0.05	0.84 ± 0.05	0.89 ± 0.05
Inhibitory terminals				
% CB1+	91.89 ± 1.68	96.30 ± 2.09	94.64 ± 2.41	95.30 ± 1.29
Density	6.54 ± 0.24	6.02 ± 0.29	7.11 ± 0.35	7.67 ± 0.23**††††
Astrocytes				
% CB1+	26.92 ± 2.97	19.26 ± 2.67	22.48 ± 2.53	16.65 ± 1.38*
Density	0.64 ± 0.03	0.64 ± 0.05	0.70 ± 0.06	0.66 ± 0.04

Note. Values are expressed as mean ± S.E.M. Statistical significance was assessed using one-way ANOVA with either parametric (Bonferroni) or non-parametric (Dunn) multiple comparison tests, as appropriate.*p<0.05, **p<0.01 compared to H_2O; and ††††p<0.0001 compared to n-3-EtOH.

observed in EtOH mice (n-3-H_2O: 991; EtOH: 442; n-3-H_2O vs. EtOH **p<0.01) (Figure 4A) (Table 2).

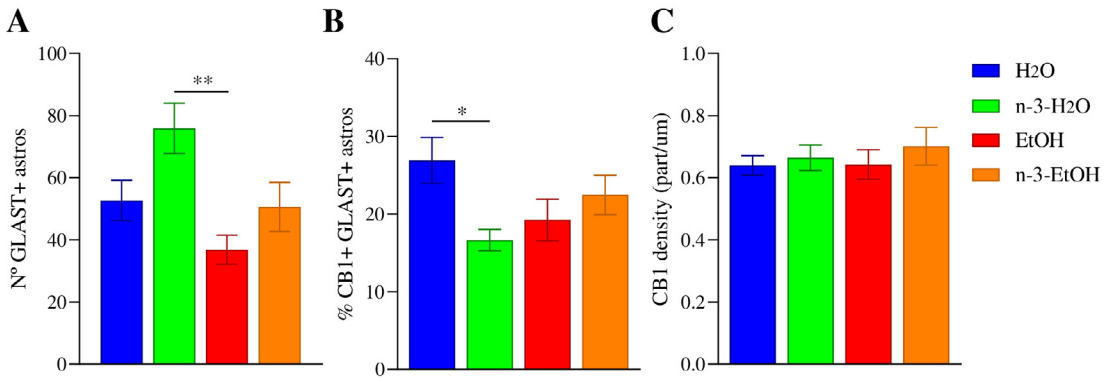
Accordingly, the percentage of CB1-positive, GLAST-stained astrocytes was reduced in n-3-H_2O mice compared to H_2O controls (H_2O: 26.92 ± 2.97; n-3-H_2O: 16.65 ± 1.38; n-3-H_2O vs. H_2O *p<0.05) (Figure 3B). However, CB1 receptor density was similar across the four conditions (n-3-H_2O, EtOH, n-3-EtOH vs. H_2O p>0.05) (Figure 3C) (Table 3).

Discussion

We analyzed the subcellular distribution of the CB1 receptor in excitatory and inhibitory terminals, as well as in astrocytes, in the *stratum radiatum* of the CA1 region of the hippocampus. Using electron microscopy, we achieved high-resolution localization of CB1, allowing detailed insights into the long-term synaptic effects of adolescent binge drinking following withdrawal, and the modulatory influence of an omega-3-supplemented diet. Omega-3 fatty acids, particularly DHA and EPA, are essential for maintaining brain health. As major components of neuronal membranes, omega-3 fatty acids influence cell membrane

Figure 4

Ultrastructural localization of CB1 receptors in astrocytes of the CA1 stratum radiatum in H_2O, n-3-H_2O, EtOH, and n-3-EtOH adult male mice



Note. **A.** Total number of GLAST-stained astrocytes in the analyzed area (~1,100 µm²) for the four experimental groups (H_2O: 632; n-3-H_2O: 991; EtOH: 442, **p<0.01 vs. n-3-H_2O; n-3-EtOH: 607) in the analyzed area (~1,100 µm²). **B.** Percentage of CB1-positive astrocytes (H_2O: 26.92 ± 2.97; n-3-H_2O: 16.65 ± 1.38, *p<0.05 vs. H_2O; EtOH: 19.26 ± 2.67; n-3-EtOH: 22.48 ± 2.53). **C.** CB1 receptor density (particles/µm) in GLAST-stained astrocytes (H_2O: 0.64 ± 0.03; n-3-H_2O: 0.66 ± 0.04; EtOH: 0.64 ± 0.05; n-3-EtOH: 0.70 ± 0.06). All data are expressed as mean ± S.E.M. The Shapiro–Wilk test was used to assess normality, followed by one-way ANOVA with either parametric (Bonferroni) or non-parametric (Dunn) multiple comparison tests, as appropriate.

fluidity, and thus, membrane-associated receptor activity, and synaptic plasticity (Calder, 2016). DHA is especially abundant in the brain and plays a critical role in synapse pruning and neuroprotection (Aguilera García et al., 2010). However, alcohol consumption impairs DHA absorption, leading to reduced brain DHA levels, which exacerbates neural damage, particularly during adolescence (Akbar et al., 2006; Bondi et al., 2014). Conversely, omega-3 supplementation can restore DHA levels in the brain, promoting synaptogenesis, glutamatergic activity, and CB1 receptor expression (Hashimoto et al., 2015; Kim, 2008).

The results of this investigation demonstrate a significant increase in the ratio of excitatory to inhibitory synaptic terminals in alcohol-exposed mice. This imbalance in the stability between excitation and inhibition—critical for normal behavior and cognition—supports previous findings that adolescent alcohol exposure impairs synaptic organization and plasticity, particularly in the hippocampus (Peñasco et al., 2020; Spear, 2018). Such disruption may underlie cognitive deficits observed in adulthood (Serrano et al., 2025; Vetreno & Crews, 2015). In this sense, a persistent excitation/inhibition imbalance in the CA1 region of the mouse hippocampus following a single postnatal binge-like alcohol exposure has been associated with dysregulation of synaptic and mitochondrial gene expression, including Cnr1, which encodes CB1 receptors. This dysregulation contributes to long-lasting synaptic dysfunction, impaired learning and memory, and increased anxiety-like behaviors (Arzua et al., 2024). Remarkably, our data show that omega-3 supplementation significantly reduced the number of excitatory terminals in the adult CA1 hippocampus following adolescent binge alcohol exposure, while having minimal effects on inhibitory terminals. As a result, the dietary intervention effectively restored the balance between excitatory and inhibitory synaptic terminals, highlighting the role of omega-3 fatty acids in maintaining synaptic integrity.

Furthermore, our findings fit with previous reports on the neuroprotective effects of omega-3 against alcohol-induced neurotoxicity (Patten et al., 2013b), and suggest that omega-3 may engage compensatory mechanisms to re-establish homeostatic synaptic plasticity disrupted by binge-like alcohol exposure. Such restoration may involve modulation of membrane composition and fluidity—key factors influencing synaptogenesis, synaptic pruning, and receptor trafficking (Calder, 2016; Kim & Spector, 2018)—as well as the preservation of synaptic networks (Cao et al., 2009; Feltham et al., 2020; Lafourcade et al., 2011).

Given the known role of CB1 receptors in regulating synaptic transmission, functional recovery may also involve changes in CB1 receptor activity or signaling, even in the absence of overt alterations in expression levels. Nevertheless, we observed that omega-3 supplementation increased CB1 receptor density in inhibitory terminals in water-control conditions, while leaving CB1 levels in excitatory terminals unchanged in all conditions examined. Previous studies have shown that omega-3 fatty acids enhance CB1 expression (Kim et al., 2016), and our earlier work demonstrated that omega-3 increased CB1 receptor expression by approximately 30% in hippocampal synaptosomes compared to water-treated controls (Serrano et al., 2024). This upregulation was associated with endocannabinoid-dependent LTP at excitatory medial perforant path–granule cell synapses in the dentate molecular layer, reduced anxiety-like behavior, and significantly improved novel object discrimination

(Serrano et al., 2024). Mechanistically, the potentiation of synaptic plasticity required group I metabotropic glutamate receptors, 2-AG, and CB1 receptors (Serrano et al., 2024).

We have demonstrated recently that omega-3 supplementation during alcohol withdrawal increased CB1 receptor density in presynaptic terminals of the hippocampus in male mice, and restored receptor-stimulated [^{35}S]GTPγS binding to Gαi/o proteins. These changes were also associated with LTP at the medial perforant path excitatory synapses, which depended on anandamide, transient receptor potential vanilloid 1 (TRPV1), and NMDA receptors, and were linked to the partial alleviation of cognitive deficits, as assessed using the Barnes maze (Serrano et al., 2025). Based on our current findings, it is plausible that omega-3 fatty acids also influence inhibitory synaptic transmission and plasticity in both the dentate molecular layer and the CA1 *stratum radiatum*; however, further investigation is needed to confirm this hypothesis.

In male mice exposed to adolescent binge-like alcohol consumption, CB1 receptor density significantly increased in inhibitory terminals of the dentate molecular layer two weeks after withdrawal, with an even greater increase observed following omega-3 supplementation (Serrano et al., 2025). This was not the case in the CA1 *stratum radiatum*, where neither alcohol exposure nor omega-3 supplementation after alcohol intake altered CB1 density in either inhibitory or excitatory terminals. Interestingly, the CB1 density increase observed in inhibitory terminals of the CA1 *stratum radiatum* in omega-3-fed mice was not detected in the dentate molecular layer (Serrano et al., 2025). Together, these findings indicate that the long-term effects of adolescent binge-like alcohol exposure on CB1 receptors and the modulatory influence of omega-3 supplementation vary among hippocampal regions. Therefore, both the pathophysiological consequences of alcohol and the potential therapeutic benefits of omega-3 should be considered region- and subregion-specific within the hippocampus.

Regarding CB1 receptor expression in astrocytes, our laboratory previously demonstrated that adolescent binge drinking alters astrocytic morphology characterized by swollen branches, and reduces the proportion of CB1-positive astrocytic processes by 40% and CB1 receptor density by 30% in astrocytes in the CA1 *stratum radiatum* of adult male mice (Bonilla-Del Río et al., 2019)it is currently unknown how CB 1 receptor expression in astrocytes is affected by long-term exposure to stressors. Here we examined CB 1 receptors in astrocytes of ethanol (EtOH. In contrast, the present study found no changes in CB1 expression in GLAST-labeled astrocytes under similar alcohol exposure conditions. A potential explanation for this discrepancy roots in the different astrocytic markers used in the studies. In our earlier work, we employed antibodies against glial fibrillary acidic protein (GFAP), a cytoskeletal protein primarily localized to astrocytic cell bodies. In contrast, GLAST is more abundantly expressed in astrocytic processes, covering a larger astroglial area and membrane surface than GFAP (Achicallende et al., 2022). Remarkably, CB1 receptors are more accurately localized in GLAST-stained astrocytes, with up to 12% of total CB1 receptor labeling found in GLAST-positive astrocytes—twice the amount observed with GFAP—in the CA1 region of the hippocampus. Given these characteristics, GLAST appears to be a more suitable marker than GFAP for detailed studies of CB1 receptor distribution in astrocytes (Achicallende et al., 2022).

Given the absence of CB1 alterations in GLAST-labeled astrocytes, it seems that the anti-inflammatory signaling via astrocytic CB1 receptors (Metna-Laurent & Marsicano, 2015) remains intact two weeks after withdrawal from adolescent binge drinking. Similarly, CB1 receptor expression in astrocytes in the dentate molecular layer remained unchanged under the same conditions (Serrano et al., 2025), reinforcing the idea that CB1 receptors in GLAST-labeled astrocytes in the hippocampus are not significantly affected in adulthood by adolescent alcohol exposure.

Interestingly, omega-3 supplementation alone led to notable changes in both GLAST-labeled astrocytes and CB1 receptor distribution within astroglial compartments in the CA1 region. Thus, we observed a significant increase in the number of GLAST-positive astrocytic compartments in omega-3-supplemented mice compared to the alcohol group, together with a reduction in the proportion of CB1-positive, GLAST-labeled astrocytes relative to the H_2O control group, without any change in receptor density. These findings suggest that omega-3 may influence astrocytic morphology, potentially increasing the complexity of astrocytic processes in n-3-H_2O mice, as previously reported in other models of neuroinflammation and neuroprotection (Champeil-Potokar et al., 2016). DHA, in particular, is known to promote astrocyte differentiation, enhancing the complexity and ramification of astrocytic processes—features associated with the anti-inflammatory properties of omega-3 PUFAs (Das & Das, 2019; Joffre et al., 2019). Therefore, the reduced proportion of CB1-positive astrocytes in n-3-H_2O mice may result from extensive astrocytic branching. Taken together, these results suggest that omega-3 may modulate astrocyte structure and indirectly influence CB1 receptor localization in astrocytes through morphological remodeling, rather than through direct changes in receptor expression.

In conclusion, our results contribute to the growing body of evidence that dietary interventions can modulate the endocannabinoid system, highlighting a promising non-pharmacological approach to mitigate the adverse effects of adolescent alcohol exposure on brain function. Future

studies are required to clarify the underlying mechanisms, particularly how omega-3 fatty acids influence the excitatory-inhibitory balance through CB1 receptors on inhibitory terminals, and modulate astrocyte function via these receptors. Moreover, future research should include female subjects to determine the extent to which adolescent alcohol intake affects CB1 receptor localization and the impact of omega-3 supplementation.

Acknowledgements

The authors thank Cristina López-Andrés for capturing the EM images. This research was funded by The Basque Government (IT1620-22 to PG); RD21/0009/0006 (to PG) and RD24/0003/0027 (to IG) funded by Instituto de Salud Carlos III (ISCIII); Ministry of Science and Innovation (PID2022-138266NB-I00 supported by MCIN/AEI/10.13039/501100011033 and by ERDF A way of making Europe (to GGdC). Garazi Ocerin is supported by a PhD contract funded through grants RD24/0003/0027 and IT1620-22.

Conflict of interests

The authors declare no conflict of interests.

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2025 ■ VOL. 37 ■ N. 4 ■ PAGES 353–368
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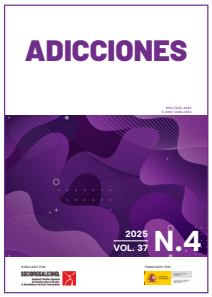

ORIGINAL

Sex differences in emotional and cognitive tasks in rats exposed to alcohol binges and controls during early adulthood

Diferencias sexuales en tareas emocionales y cognitivas en ratas expuestas a atracones de alcohol y en controles durante la adultez temprana

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Abstract

A sex perspective in the behavioural effects induced by alcohol binge exposure during youth or early adulthood remain limited. This study aimed to evaluate the effects of high doses of ethanol on emotional behaviour and cognition in 8-weeks old rats, from a sex perspective. Male and female animals were exposed to ethanol binges (3 g/kg, i.g.; 3 times/day x 4 days) in a 2days on-2days off paradigm and assessed in the elevated plus maze (EPM), forced swimming test (FST), saccharin preference test (SPT), Morris water maze (MWM) and novel object recognition (NOR) test.

Baseline differences between control male and females were observed in emotional, motivational and cognitive tasks. Additionally, Intensive Alcohol Exposure (IAE) exerted sex-specific effects in: a) EPM: males showed anxiety and no effect in females; b) FST: depressive-like symptoms in both sexes but more pronounce immobility time in females; c) NOR test: impairment in a short-term memory in both sexes but females displayed improved performance in a long-term versus their controls. No IAE-related effects were found in the SPT or MWM.

These results suggest inherent sex-differences in rodent performance in behavioural tests assessing emotional, motivational and cognitive behaviours. Additionally, IAE may impact male and females differently during abstinence in early adulthood. These findings underscore the importance of considering sex as a critical variable for preclinical studies.

Key words: Alcohol, youth, behaviour, binge drinking, sex differences

Resumen

Integrar la perspectiva de sexo en estudios preclínicos sobre la exposición excesiva a alcohol durante la juventud o la adultez temprana sigue siendo limitada. Este estudio tuvo como objetivo evaluar los efectos de altas dosis de etanol en el comportamiento emocional y la cognición en ratas de 8 semanas de edad, considerando diferencias de sexo. Machos y hembras fueron expuestos a atracones de etanol (3 g/kg, vía oral; 3 veces al día durante 4 días) en un paradigma de dos días alternos, y evaluados en el laberinto elevado en cruz (EPM), la prueba de natación forzada (FST), la prueba de preferencia de sacarina (SPT), el laberinto acuático de Morris (MWM) y la prueba de reconocimiento de objetos nuevos (NOR).

Se observaron diferencias basales entre machos y hembras controles en tareas emocionales, motivacionales y cognitivas. Además, la Exposición Intensiva al Alcohol (EIA) ejerció efectos específicos por sexo en: a) EPM: los machos mostraron ansiedad y ningún efecto en las hembras; b) FST: síntomas similares a la depresión en ambos sexos, pero un tiempo de inmovilidad más pronunciado en las hembras; c) Prueba NOR: Se observó deterioro de la memoria a corto plazo en ambos sexos, pero las hembras mostraron un mejor rendimiento a largo plazo en comparación con sus controles. No se observaron efectos relacionados con la EIA en las pruebas de conducta SPT ni en MWM.

Estos resultados sugieren diferencias inherentes entre sexos en el rendimiento de los roedores en pruebas conductuales que evalúan las conductas emocionales, motivacionales y cognitivas. Además, la EIA podría afectar de forma diferente a machos y hembras durante la abstinencia en adultez temprana. Estos hallazgos subrayan la importancia de considerar el sexo como una variable crítica en los estudios preclínicos.

Palabras clave: Alcohol, juventud, comportamiento, atracones, diferencias sexuales

■ *Received: July 2025; Accepted: October 2025.*

■ ISSN: 0214-4840 / E-ISSN: 2604-6334

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Traditionally, most of the preclinical studies in animal models have been performed in males and, therefore, there is a significant lack of information and bias by sex in a great deal of biomedical and psychobiological results. Over the last decade, it has been a dizzying turn of the scientific community on this issue, urging the incorporation of the sex variable in all preclinical investigations and also the gender perspective in humans' studies. Indeed, policy changes such as the 2016 mandate by the National Institutes of Health (NIH), emphasize the inclusion of sex as a critical biological variable in preclinical and clinical research (Campbell et al., 2024; Costa-Valle et al., 2022; Kaluve et al., 2022). This emerging urge in understanding sex differences in preclinical investigation also affects studies related with alcohol abuse. Although the efforts to address these methodological biases have been intensified over the last years, exploring sex-based differences in the context of alcohol abuse is still scarce, specifically the impact of alcohol abuse in behavioural responses, including emotional and cognitive subdomains.

Several lines of research studying the impact of alcohol binge drinking (ABD) on youth or early adulthood, including our own experience, have led to important biological and behavioural consequences of such a dangerous pattern of alcohol consumption without specific focus in the sex variable (Antón et al., 2017, 2018) and only more recently we addressed this important issue (López-Valencia et al., 2024; Orio et al., 2018).

ABD consists of an intensive alcohol exposure (IAE). It is defined as the consumption of 4 or 5 standard drink units (SDU) in a short period of time. It is accepted that the acute rise in blood ethanol levels (BELs) achieved during this pattern of alcohol consumption (≥ 80 g/dL) contribute to the neurotoxic effects and alterations in brain plasticity (Patrick et al., 2021; Waszkiewicz et al., 2018) and it is associated with neuroinflammation and emotional and cognitive alterations (Antón et al., 2017; Crews et al., 2016; Orio et al., 2018; Pascual et al., 2007, 2014). Despite its adverse outcomes, ABD is particularly prevalent among adolescents and youths, who are often drawn to this pattern of consumption due to its association with behavioural disinhibition. The WHO and other global monitoring systems have reported the prevalence of heavy episodic drinking across different age groups, emphasizing its impact on public health (World Health Organization, 2024). Young people are more vulnerable to the reinforcing effects of drugs, especially alcohol, which is often easily accessible in many environments. Indeed, a combination of biological vulnerability and a predisposition for seeking new experiences increase the risk of substance use and abuse during adolescence (Chung et al., 2018; Maldonado-Devincci et al., 2022; Sicher et al., 2022; Spear, 2018).

This study aims to investigate the effects of IAE comparatively on young males and females' rats during early abstinence, using a validated animal model and focusing on cognitive and emotional behaviours. Searching for possible sex-specific differences in the performance of several tests will potentially help us to better understand basal differences in normal behaviour and the underlying mechanism of alcohol abuse for each sex in a near future.

Material and methods

Animals

Fifty-seven Wistar rats (Envigo©, Barcelona, Spain) aged seven weeks at arrival were used across all experiments. Females and males were housed in different isolated rooms in groups of 3-4 per cage. They were maintained at constant conditions of temperature (21 ± 1°C) and humidity (59 ± 10%) under a 12 h dark-light inversed cycle (lights on at 8:00 p.m.) with free access to food and water. Animals were habituated to these conditions for one week before the experiments and then they were handled gently to acclimate to the experimenters and gavage procedure.

All procedures were approved and adhered to the guidelines of the Animal Welfare Committee of the Complutense University of Madrid (Ethical approval reference: PROEX 312/19; PROEX 122.7/23) following European legislation (2010/63/EU).

Experimental design

Rats were randomly assigned to control and ethanol groups: male control group (n=9), male ethanol group (n=10), female control group (n=18), and female ethanol group (n=20). Rats received intragastric (i.g.) intermittent doses of ethanol or water three times per day for four days using specific cannulae (16-G needle, Fisher Scientific, Waltham, MA, USA), in a 2-day on/2-day off protocol (Fig. 1A). This is a modified protocol (from Antón et al., 2017; Obernier et al., 2002; Rodríguez-González et al., 2021) previously used by our group (Lopez-Valencia et al., 2024), which introduces a period of abstinence, a relevant factor involved in the harmful effects of binge drinking consumption (Pascual et al., 2007). The experiments in females were conducted in duplicate to increase the internal validity of the results, which are presented as a pool of data from two identical experiments.

Behavioural Assessment

The behavioural assessment was scheduled by sufficient time interval to avoid interferences between tests, and they were performed during the dark phase (see Fig. 1B for experimental timeline). Rats were evaluated in traditional tests used to assess emotional and cognitive alterations, following an alternation of experimental groups in all tests.

The analyses were performed by a double-blind protocol to ensure the truthfulness of the results.

Anxiety-like behaviour: Elevated Plus Maze

To check anxiety-like behaviour, we performed the Elevated Plus Maze (EPM) test 12 h after the last ethanol binge. EPM is based on a balance of fear and curiosity towards novelty, and it is designed to test general anxiety-related behaviours in rodents (Cosquer et al., 2005; Pellow et al., 1985).

The EPM was performed on two open black and grey plastic arms (50 x 10 cm) and two perpendicular enclosed arms of the same size but with opaque walls 50 cm high. The junction of the four arms formed a central square area (10 cm^2). The apparatus was elevated 65 cm above the floor. The light intensity was set up at 20 lux. On the test day, each rat was placed on the central platform facing one closed arm and opposite to the experimenter position. Then, the animal was allowed to freely explore de maze for 5 min. Between animals, the maze was carefully cleaned with ethanol 5% to remove possible odour. One rat fell from the EPM initially and it was excluded from the analysis. The number of entries and the time spent in all the arms were measured by a computer-controlled system (Mazesoft-4) recording the interruptions of infrared photo beams located along each arm. The percentage of each was calculated upon total entries into any arms and upon the total time spent in both arms, respectively. It was considered a visit whenever an animal entered an arm with all four limbs. Anxiety-like behaviour was defined as a decrease in the number of entries and time spent in the opened arms related to the total entries and total time, respectively.

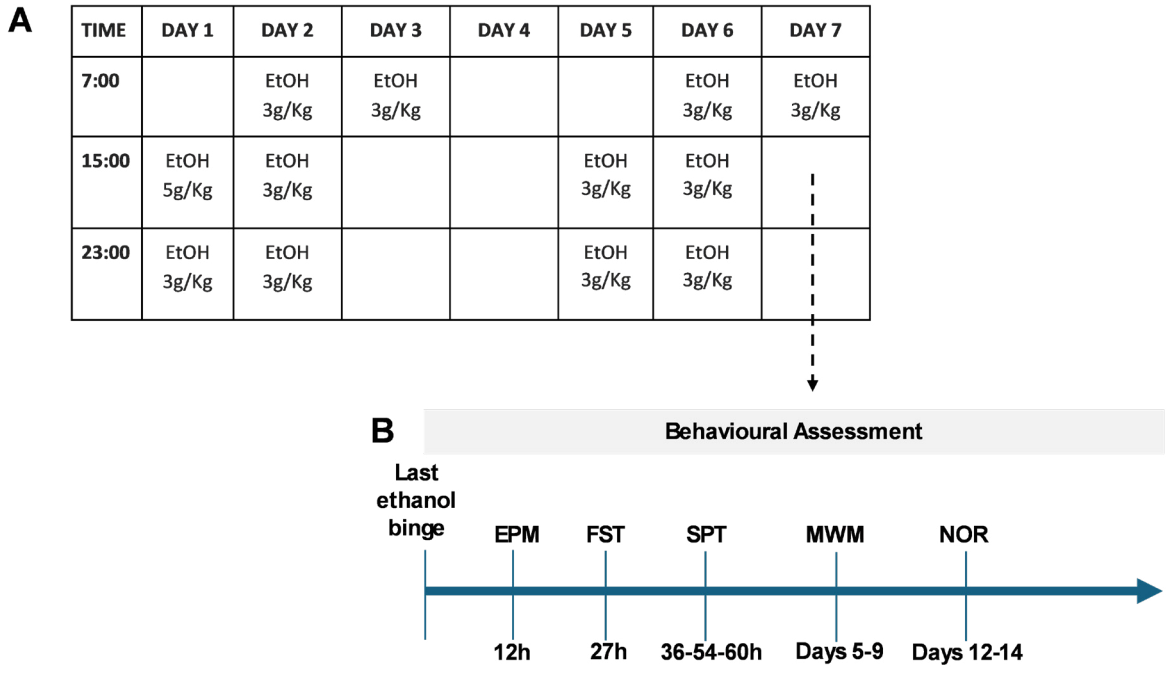
Depressive-like behaviour: Forced Swimming Test

The Forced Swimming Test (FST) is based on the method described by Porsolt (Porsolt et al., 1977) and it is one of the most common assays for the study of depressive-like behaviour in rodents (Slattery & Cryan, 2012; Yankelevitch-Yahav et al., 2015), although there are contradictory opinions (Armario, 2021).

Animals were tested 27 h after the last ethanol administration (see Fig 1 for experimental design). They were placed individually into transparent cylinders (47 x 35 cm) filled with water (25 ± 1ºC) for 5 min. Escape-directed behaviours were analysed, such as swimming, horizontal movements throughout the water tank; climbing, vertical movements of the forepaws; immobility and latency to immobility. All the assays were performed under red light conditions and recorded for subsequent analysis. Either an increase in the immobility or a decrease in the swimming, climbing and latency times were considered depressive-like behaviour, reflecting a failure of persistence in escape (Cryan et al., 2002; Detke et al., 1995).

Figure 1

Schematic representation of the experimental design



Note. A) Alcohol binge drinking treatment; binges were administered intragastrically at 3g/kg, except for the initial loading dose of 5 g/kg every 8h in a 2days on-2days off- 2days on paradigm; B) Timeline of behavioural testing. EtOH: ethanol; ***EPM***: elevated plus maze; ***FST:*** forced swimming test; ***SPT:*** saccharin preference test; ***MWM***: Morris water maze test; ***NOR***: novel object recognition test.

Motivational behaviour: Saccharin Preference Test

The Saccharin Preference Test (SPT) is used to measure the sensitivity to reward in rodents (Scheggi et al., 2018). Once animals were finishing the FST, around 27h after last binge, they were dried with a towel and housed individually, provided with food *ad libitum*. In each individual cage, rats were offered a free choice between 2 bottles, one with 0.1% saccharin (w/v) and another with tap water. Bottles were weighed to determine the liquid consumption and placed into the cage with an alternated position of the water vs. saccharin to avoid place preference. Liquid consumption was measured at specific abstinence times from last binge (36h, 54h, 60h) and accumulated drinking was calculated. The saccharin preference was shown as the percentage of consumed saccharin over the total amount of liquid intake. A decrease in the preference for the sweet solution (natural reward) is considered a reflection of anhedonia, a core symptom of a depressive-like behaviour (Scheggi et al., 2018; Slattery et al., 2007).

Spatial Memory: Morris Water Maze

The Morris Water Maze (MWM) test is used to measure spatial learning and memory in rodents (Rendeiro et al., 2009). This test was carried out during 5 consecutive days in a circular pool (diameter 122 cm). The water in the tank was made opaque with white nontoxic tempera paint (temperature 24 ± 1ºC). The pool was in a room with visible external cues and light intensity controlled. The experimenter worked also as a cue. A platform (diameter 10 cm) was submerged 1-2 cm below the water surface in one of four equal imaginary quadrants. During 4 consecutive days, animals were trained to find the submerged platform in a fixed location of the MWM. Each day consisted of 4 trials in which animals were released facing the wall from different points. Each trial had a maximum latency of 60 s, where animals freely explored the swimming pool to reach the platform. All rats were allowed to stay on the platform for 10 s more before being removed from the water. Latencies to find the platform were recorded in each trial and the average was calculated for each day and animal. After each trial, mice were dried and returned to their home cages.

The fifth day, the test was carried out without the platform for 60 s with a new start position in the pool to ensure that the mice remember the goal location rather than a specific swim path. Here, we measured the latency to reach the previous platform location, the number of platform-site crossovers and the time spent within an imaginary ring (diameter 30 cm) around where the platform had been. All the assays were recorded by a video camera located above the pool for further analysis (Vorhees & Williams, 2006).

Recognition Memory: Novel Object Recognition Test

The Novel Object Recognition (NOR) test was performed to study possible memory impairments based on the tendency of rodents to interact more with a novel object than a familiar one (Bevins & Besheer, 2006).

The test was performed in a square arena (80x80x42cm) with black matte-painted walls and floor. The arena was subdivided into 4 equal sections, allowing the evaluation of 4 rats simultaneously. The NOR was carried out in accordance with previous studies (Marco et al., 2013; Moya et al., 2022) under low-light conditions (20 lux). The test was organized in three phases: habituation (time = 0), a training phase (pre-test) and two test sessions 4h and 24h after the training phase. During the habituation, animals were allowed to freely explore the arena during 5 minutes without objects. In the training phase, 2 identical objects (glass bottles) were in opposite corners of the arena, and animals were allowed to freely explore them for 3 min. During the test session 4h after the training phase, one of the familiar objects (F, glass bottle) was substituted by a novel object (N1, green ashtray), and the rats were allowed to explore both objects for 5 min. In the 24h session, the novel N1 was replaced by another novel object (N2, money box) and the object positions in the arena were alternated in order to avoid possible place preferences. Both the training and test sessions were video recorded (Sony DCRDVD310E, Spain). Exploration of an object was considered whenever animals pointed their nose toward an object at a distance 1 cm, while turning around, climbing or biting the objects was not considered exploration. The latency to first explore the novel object in the test sessions was registered, and the discrimination index (DI) calculated as the difference between the time spent exploring the novel object and the familiar one in relation to the total time spent exploring the objects.

Statistical Analysis

All data are expressed as the mean ± S.E.M and were analysed using a two-way analysis of variance (2-way ANOVA), comparing the factors [alcohol/water] versus sex [male/female], when normality was verified; otherwise, a Kruskal-Wallis test was used. The saccharin preference test was analysed using a three-way ANOVA with time (36h, 54h, 60h), ABD treatment (water versus alcohol) and sex (males vs. females) as independent factors. *Post hoc* comparisons (Bonferroni or Dunn's) were performed in case of significant interaction between factors. Homoscedasticity was checked by Barlett's test, and data were transformed (sqrt, $\log_{10}$) when appropriate. The outliers were excluded using Grubbs' test. A p value <0.05 was set as the threshold for statistical significance in all statistical analyses. All data were analysed using GraphPad Prism version 8.01 (GraphPad Software, Inc., La Jolla, CA, USA).

Ethical aspects

All protocols have been approved by the Animal Welfare Committee of Complutense University of Madrid (reference: PROEX 312/19) following European legislation (2010/63/EU).

Results

Effects on anxiety

In the EPM, we observed an apparent opposite effect of alcohol in males and females versus their controls at 12h of abstinence, regarding the percentage of entries in the open arms (Fig. 2A). The Kruskal-Wallis test was significant (Kruskal-Wallis = 10.66, p=0.0137), and the Dunn's *post hoc* test suggested an anxiogenic behaviour in alcohol-treated males compared to their controls (trend, very close of significance (p=0.055), that was not observed in females. Indeed, data in females appear to follow the opposite pattern and, despite no significant difference between control and ethanol groups in females, the ethanol-treated female rats entered more in the open arms compared to ethanol-treated males (Fig. 2A; p=0.0168).

Regarding the percentage of time spent in the arms (Fig. 2B), the 2-way ANOVA reported a significant interaction between ethanol and sex ($F_{(1,49)}$=14.01, p=0.0005) and an ethanol main effect ($F_{(1,49)}$=7.290, p=0.0095). Bonferroni *post hoc* comparisons revealed a difference between male and female controls, with females spending less time in the open arms (p<0.05). IAE induced a clear anxiogenic effect in males (p<0.01) but not females (p>0.05, n.s.) compared with their respective controls (Fig. 2B), as suggested also in the mentioned data from Fig. A.

Effects on the depressive-like behaviour

In the FST, ethanol binge treatment induced reductions in the latency to first immobility (Fig. 3A; $F_{(1,53)}$=0.01111, p=0.0090), with no sex differences, indicative of a depressive-like behaviour during early alcohol abstinence (~ 27h).

The interaction between factors (treatment and sex) was in the limit of significance for immobility (Fig. 3B; $F_{(1,53)}$=3.964, p=0.0517) and significant for the swimming time (Fig. 3C; $F_{(1,52)}$=4.622, p=0.0362), with Bonferroni *post hoc* tests indicating that ethanol-treated females may have higher immobility versus their female controls (p<0.05). A sex main effect for immobility and swimming ($F_{(1,53)}$=146.1, p<0.0001 and $F_{(1,52)}$=126.5, p<0.0001, respectively) was indicative than females showed higher immobility duration and lower swimming time, an effect also showed after Bonferroni *post hoc* test (Fig. 3B, p<0.0001; Fig, 3C, p<0.0001, respectively).

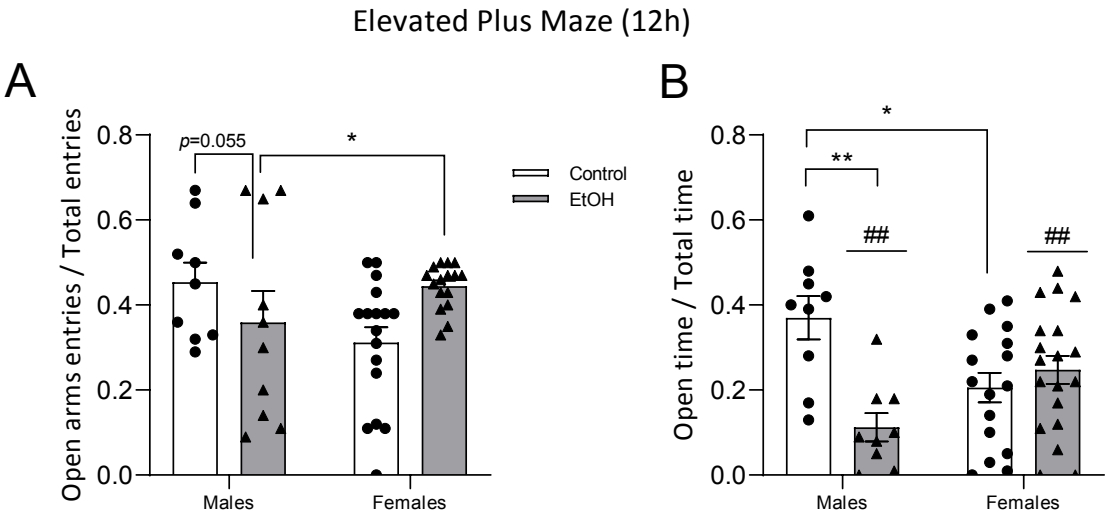
No differences were observed in the climbing times between experimental groups (Fig. 3D; EtOH main effect: $F_{(1,52)}$=1.241, p=0.2705; Sex main effect: $F_{(1,52)}$=0.2063, p=0.6516; Interaction between factors: $F_{(1,52)}$=0.8724, p=0.3546).

Effects on negative motivational state or anhedonia

The SPT started 27h after last binge exposure and we recorded accumulative drinking over the 36h, 54h and 60h of alcohol abstinence (Fig. 4). Repeated-measures 3-way ANOVA, comparing treatment, sex and time, found main effects of abstinence time ($F_{(1.768,85.73)}$=4.034, p=0.0255) and sex ($F_{(1,52)}$=55.90, p<0.0001), being females the sex showing higher saccharin preference over time. Since the

Figure 2

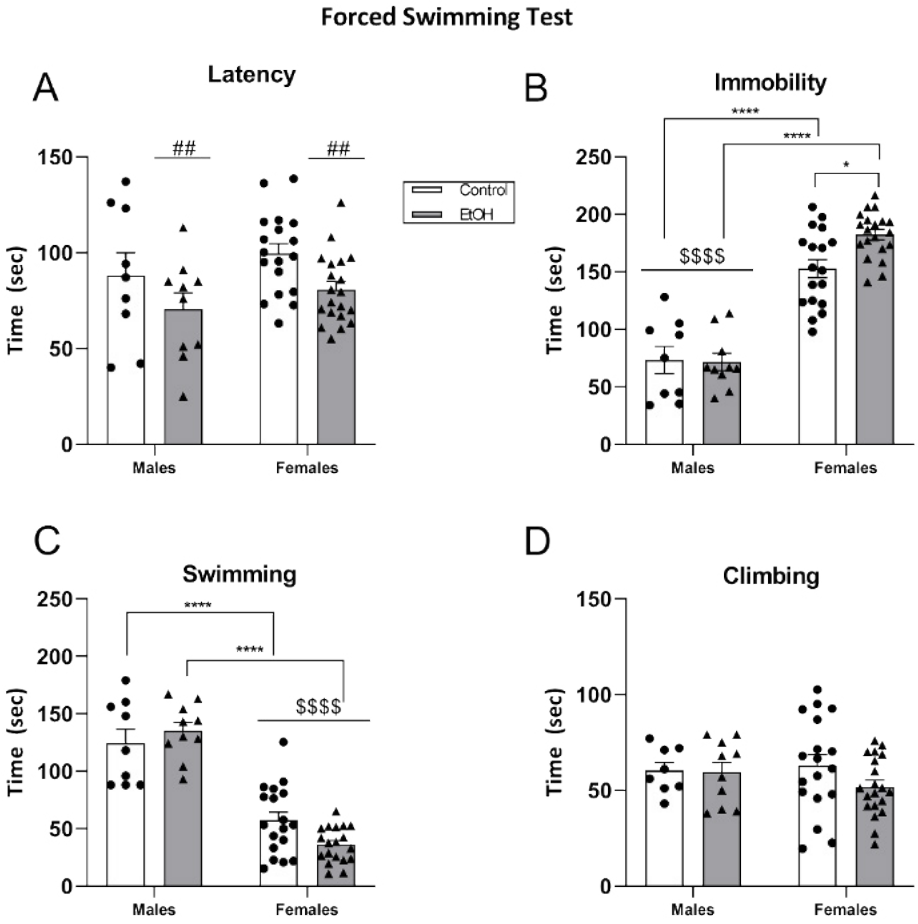
Elevated Plus Maze to assess anxiety-like behaviour in male and females



Note. A) Ratio of entries in the open arms over the total entries; B) Ratio of time spent in the open arms over the total time spent in both arms. Animals were assessed in the test 12h after the last alcohol binge administration. Results represent the mean ± S.E.M. (n=9-10 in males and n=18-20 in females: pool of two experiments). Non-parametric Kruskal-Wallis followed by Dunn's ***post hoc*** test (A): $^{*}p < 0.05$. Two-way ANOVA (B): Interaction followed by Bonferroni ***post hoc*** test (Fig.2A): $^{*}p < 0.05$, $^{**}p < 0.001$. Ethanol (EtOH) main effect: $^{\#\#}p < 0.001$.

Figure 3

Forced Swimming test to assess depressive-like behaviour



Note. A) Latency to immobility; B) Immobility time; C) Swimming time; D) Climbing time. Animals were assessed in the test around 27h after the last ethanol binge. Results represent the mean ± S.E.M. (n=9-10 in males and n=18-20 in females= pool of two identical experiments). Two-way ANOVA: EtOH main effect $^{\#\#}p < 0.001$; sex main effect $^{\$\$\$\$}p < 0.0001$; interaction followed by Bonferroni ***post hoc*** test: ethanol-treated females differ from control females $^{*}p < 0.05$; female groups differ from male groups $^{*}p < 0.0001$.

3-way ANOVA indicated an interaction between time and sex ($F_{(2,97)}$=3.191, p=0.0455) disregarding of ethanol treatment, we performed a 2-way ANOVA (time x sex) and Bonferroni *post hoc* test confirmed that females showed higher preferences at 36h (p<0.0001), 54h (p<0.001), and 60h (p<0.0001) (Figure 3).

Despite of these differences, at the times of evaluation in this experiment the possible effects of IAE on anhedonic behaviour were not evident, since we found no alcohol effects in males or females versus their controls at any time tested ($p > 0.05$, n.s.).

Effects on spatial memory

To study if alcohol consumption affected the spatial learning and memory differentially in males and females, we conducted the MWM test. During the learning curve, there were not significant differences any day between groups (Fig. 5A) and the time needed to find the platform decreased as the sessions progressed for all subjects.

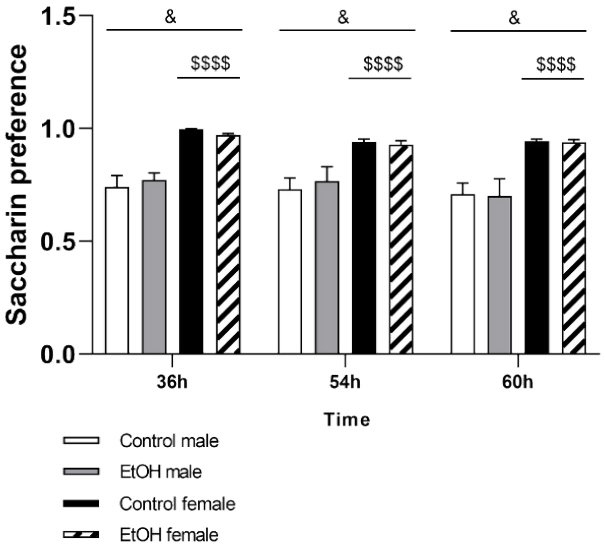
In the probe trial, we did not observe significant effects of alcohol binges in the latency, crossing or quadrant exploration variables of the test (Fig. 5B,C,D; ($F_{(1,51)}$=3.051, p=0.0867; $F_{(1,52)}$=2.535, p=0.1174; $F_{(1.53)}$=0.009591, p=0.9224, respectively), although a trend for increases latency can be observed in ethanol-treated females versus their controls (Fig. 5B, p=0.0867, n.s.). However, it was a main effect of sex on platform crosses and swimming time around the quadrant of platform location (Fig 5C,D; $F_{(1,52)}$=7.144, p=0.0100; $F_{(1,53)}$=14.76, p=0.0003, respectively), with higher scores in females than males.

Effects on recognition memory

In the NOR, we observed a main effect of alcohol in the latency to first approach to the novel object 4h after

Figure 4

Saccharin preference test to assess anhedonia



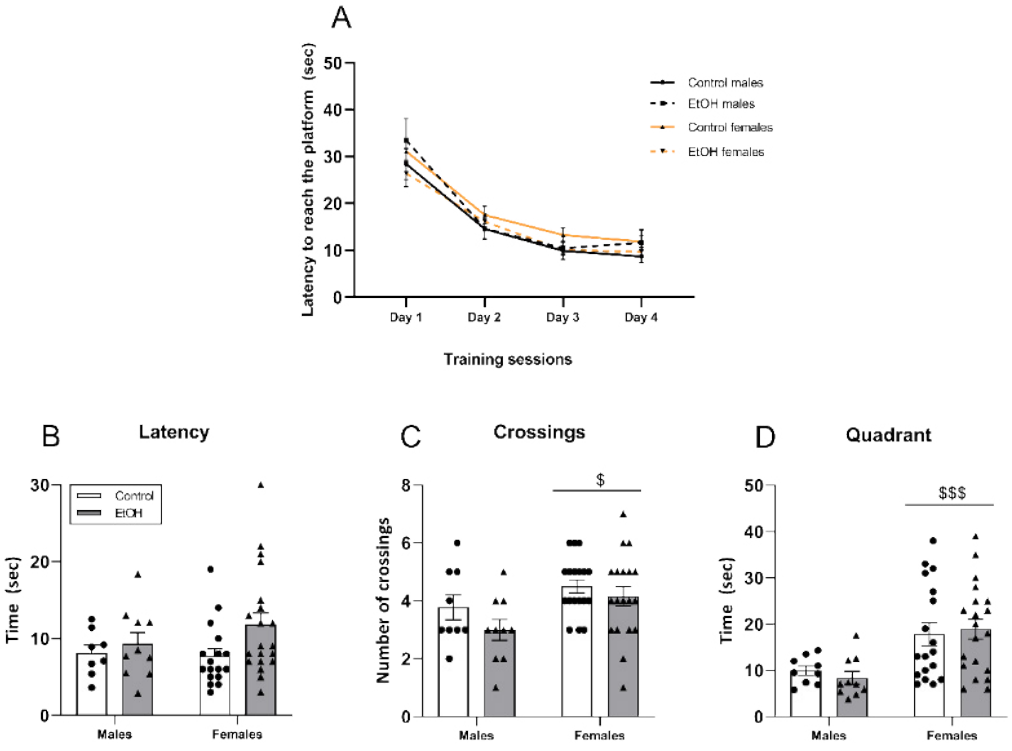
Note. The saccharin preference was calculated as the percentage of consumed saccharin over the total amount of liquid intake. Animals were tested for a cumulative response, starting around 27h after last binge and with measurements at 36h, 54h or 60h of alcohol abstinence. Data represent the mean ± S.E.M. (n=9-10 in males and n=18-20 in females: pool of two experiments). Repeated-measures 3-way ANOVA (sex x treatment x time): sex main effect: effect $^{\$\$\$\$}p < 0.0001$; time main effect $^{\&}p < 0.05$; interaction (time x sex) (not represented in the figure).

the training phase (Fig 6A, $F_{(1,46)}$=4.491, p=0.0395), as both male and female ethanol-treated animals displayed higher latencies than controls, indicative of alterations in the short-term memory. The interaction between factors (sex x treatment) was near of significance ($F_{(1,46)}$=3.551, p=0.065), data suggesting that the effect of alcohol may be stronger for males (Fig. 6A). There was no main effect of sex ($F_{(1,46)}$=0.8515, p=0.3609). Regarding the DI, the interaction was again near of significance (Fig. 6C; $F_{(1,49)}$=3.399, p=0.0713, n.s.) and no alcohol of sex main effects were found ($F_{(1,49)}$=0.1159, p=0.7350, $F_{(1,49)}$=0.5682, p=0.4546, respectively).

In a long-term, 24h after the training phase, the 2-way ANOVA revealed ethanol and sex main effects (Fig 6B $F_{(1,44)}$=5.153, p=0.0282; $F_{(1,44)}$=10.09, p=0.0027, respectively) with an interaction between factors ($F_{(1,44)}$=7.816, p=0.0076). *Post hoc* comparisons revealed than female control animals had higher latencies at 24h to explore the novel object than male controls (p<0.01). In the long-term test, only female ethanol-treated rats showed reduced latency versus their female controls (p<0.001) (Fig. 6B). Regarding the DI, 2-way ANOVA did not show interaction between factors (Fig. 6D; $F_{(1,51)}$=0.04861, p=0.8264), ethanol or sex main effects 24h after the training phase (Fig. 6D; $F_{(1,51)}$=1.037, p=0.3132, $F_{(1,51)}$=1.330, p=0.2541, respectively).

Figure 5

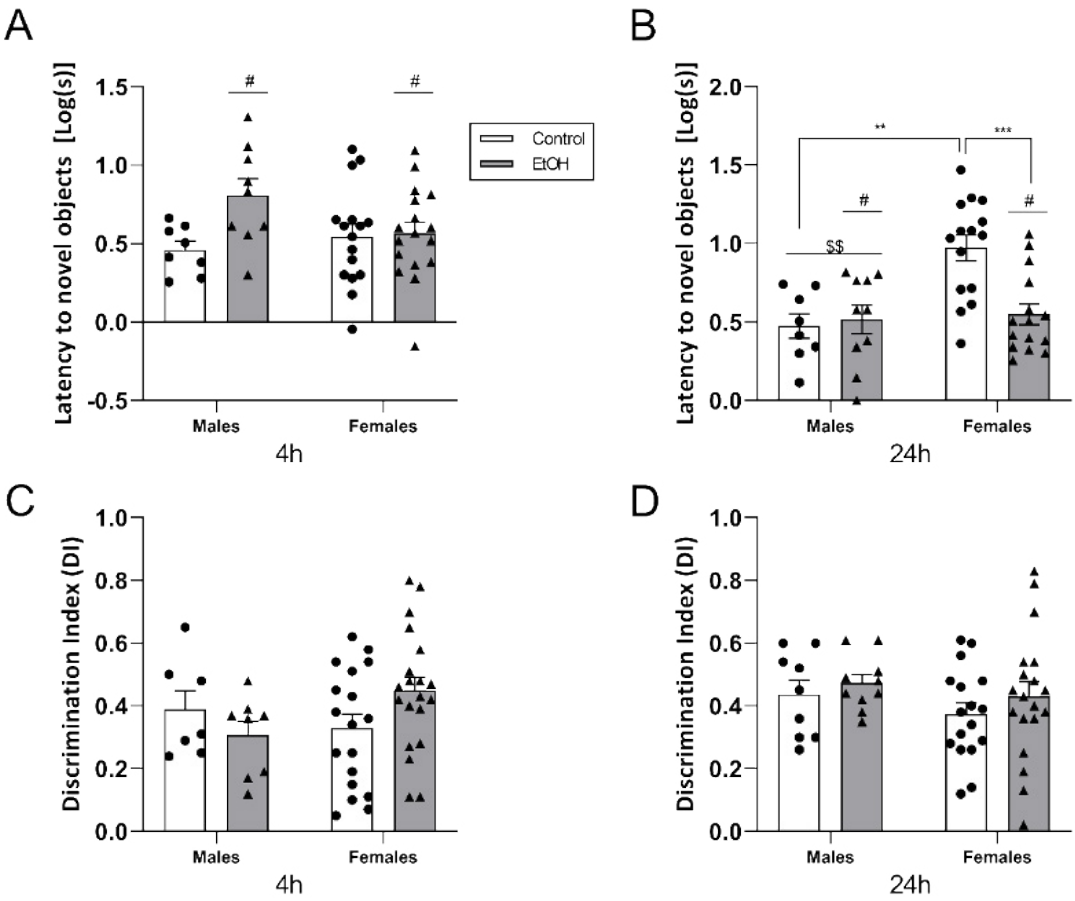
Morris Water Maze test to study spatial memory



Note. A) Learning curve including the average of each experimental group during the four trials. B) Latency to reach the platform location. C) Number of platform-site crossovers. D) Time spent around the platform location. Data is expressed as the mean ± S.E.M. Two-way ANOVA: sex main effect $^{\$}p < 0.05$, $^{\$\$\$}p < 0.0001$.

Figure 6

Novel Object Recognition test to study recognition memory



Note. A) Latency to novel object at 4h session. B) Latency to novel object at 24h session. C) Discrimination index at 4h session. D) Discrimination index at 24h session. Results represent the mean ± S.E.M. Two-way ANOVA: EtOH main effect $^{\#}p < 0.05$; sex main effect $^{\$\$}p < 0.001$; interaction followed by Bonferroni *post hoc* test: $^{**}p < 0.001$, $^{***}p < 0.0001$.

Altogether, the analysis of latency in the NOR reveals a different pattern of behaviour in males and females in the short- and long-term tests. IAE appears to negatively affect the short-term memory in both sexes (Fig. 6A), whereas in the long-term, IAE induce the opposite effect (decrease in latency) in females only (Fig. 6B).

Discussion

This study explores the differential effects of alcohol binge exposure in male and female rats during early adulthood. By focusing on both cognitive and emotional behaviours, it aims to uncover sex-specific differences that shape animal behaviour under physiological (control) conditions in several tests widely used in preclinical studies, and the impact of IAE during early adulthood with this sex perspective.

Our results suggest some sex differences both in control and ethanol conditions depending on the test, highlighting the strong need of including both sexes for any preclinical study evaluating emotional and cognitive behaviour in animals. The implications of these differences are discussed below.

Sex differences in behaviour in physiological (control) conditions

In terms of emotional state, sex-differences were observed in anxiety and depressive-like behaviours under control conditions, as showed by animal performance in the EPM and FST. The EPM is a test widely used to evaluate anxiety-like behaviour in rodents. These tasks exploit the conflict between rodents' exploratory drive and their tendency to avoid open spaces (Campbell et al., 2024; Chen et al., 2024; Donner & Lowry, 2013). Our results indicate that females spend significantly less time in the open arms of the EPM compared to males under normal conditions. While the literature generally establishes that females tend to exhibit less anxious behaviour, showing more interest in open areas than males (Campbell et al., 2024), many authors propose that sexual differences in this behavioural pattern may reflect alternative coping strategies or environmental exploration strategies, rather than true anxiety-related behaviours (Donner & Lowry, 2013).

The FST and the SPT are standard methods for assessing antidepressant effects in rodents (Scheggi et al.,

2018; Slattery et al., 2007), although the usefulness of FST for this purpose has been questioned recently (Armario, 2021). In the FST, swimming and climbing behaviours represent the animals' adaptative response to an adverse situation, suggesting that the absence of these behaviours or an increased tendency for immobility are indicators of behavioural despair or depressive-like behaviour (Dalla et al., 2010). On the other hand, the SPT evaluates motivational behaviour. It assumes that animals will consume more saccharin due to its hedonic properties, and the decrease in the preference for it (a natural reward) represents anhedonia, a core symptom of depression, which is induced by different stressors (Campbell et al., 2024; Sayd et al., 2015). It is common to find sex differences related to basal emotional state in the literature, indicating that females show more depressive-like behaviours compared to males in tasks such as the FST (Dalla et al., 2010), aligning with our results. Nevertheless, the results in tasks related to depressive-like behaviour are inconsistent; as Kokras et al. (2015) point out, in the FST, one-third of studies show greater immobility in females, as we showed here, one-third in males (Pavlova et al., 2020; Xia et al., 2023) and one-third find no sex differences. In contrast, in the SPT, females seem to show a greater preference for saccharin, both in our study and in previous works, exhibiting a different impact of it in the reward system, which calls into question the use of these tests as a measure of depressive behaviour (Dalla et al., 2010; Kokras et al., 2015). This underlines the need to validate representative tasks that correctly describe behavioural differences between male and female animals, in order to translate the results to human behaviour.

Regarding cognitive performance, the MWM provides insights about learning ability and spatial memory through measurements such as the time it takes for the animal to find the platform or the time it spends swimming around it (Zorzo et al., 2024). While most previous work comparing males and females on spatial navigation has found superiority in male performance (Bowman et al., 2022; Gutiérrez-Menéndez et al., 2023; Zorzo et al., 2024), in our study, no sex differences were found during the learning phase, in line with previous research (Qi et al., 2016), although on the day of the test, females showed more crosses and swimming time around the platform than males. Neurobiological studies related to this ability have described different strategies used by the two groups; males tend to rely on global special strategies, while females seem to favour more by spatial cues from the environment (Shansky, 2018; Zorzo et al., 2024). The high number of visual cues arranged in our experimental setup may have favoured the female's performance.

Another cognitive function related to memory is the ability to recognise new objects. The NOR test allows to evaluate this ability obtaining measures on the approach latency and the novel object discrimination index, in a short-term (4h after the training phase) or in a long-term (24h after the training phase) (Marco et al., 2013; Moya et al., 2022). Our results suggest that there are no differences in the performance of control males and females in this test in a short-term, although females perform worst in a long-term. The absence of sexual differences in the recognition memory agrees with most of the studies (Campbell et al., 2024; Van Hees et al., 2022) and only a few studies described a slight superiority of females, in different phases of the estrous cycle (Koszałka et al., 2023; van Goethem et al., 2012).

Table 1

Summary of sex differences in behaviour in physiological (control) conditions

Behavioural test	Sex differences in control animals
EPM	Females spend less time in the open arms than males
FST	Females spend less time swimming and more time immobile than males
SPT	Females showed greater preference for saccharin than males
MWM	No differences during learning phase; females showed more crosses and swimming time during test day than males
NOR	No differences at 4h; worst performance in females at 24h

Sex differences in the effects of intensive alcohol exposure

Most of the studies exploring sex differences on alcohol consumption were performed in C57BL/65 mice, with intermittent and voluntary access paradigms -such as the paradigm of choosing two bottles, or drinking in the dark (DID)-, in which the effects of long-term consumption are studied (Ardinger et al., 2024; Leonardo Jimenez Chavez et al., 2020; Magee et al., 2024; Maldonado-Devincci et al., 2022; Rath et al., 2020; Rivera-Irizarry et al., 2023). In rats, there are studies of voluntary alcohol consumption using the two-bottle paradigm (Abderrahim et al., 2022; Albrechet-Souza et al., 2020; Buján et al., 2024). Only few studies with sex perspective employed intragastric administration of ethanol, usually for long periods, and the results remain mixed (Healey et al., 2023; Jia et al., 2024; Penta et al., 2024; Varlinskaya et al., 2020). To the best of our knowledge, no prior research has examined sex differences in IAE with this specific administration protocol, using high doses of ethanol administration in a short period of time. Our results suggest that forced alcohol binge exposure impact differently in male and female rats only in some tests, which are discussed below.

Our results showed that the effect of IAE on anxiety in the EPM was clear for males, in agreement with our

Table 2

Summary of sex differences in behaviour after IAE

Behavioural test	Sex differences in ethanol-treated animals
EPM	Ethanol-treated males entered the open arms less and spent less time there than their control group whereas this effect was not shown in females.
FST	Ethanol-treated females exhibited more time immobile than female controls and this effect was not observed in males.
NOR	In a long-term phase, ethanol improves performance in females versus their controls and this effect it not observed in males.

previous studies with this animal model (Antón et al., 2017) but this effect is not present in ethanol-treated females, that showed no differences with their female controls. Note that ethanol-treated females showed a tendency to the opposite direction (anxiolytic effect), and this possible sex difference has been highlighted also by other authors using other paradigms of ethanol administration (Albrechet-Souza et al., 2020; Buján et al., 2024; Costa-Valle et al., 2022).

Besides that, our study provide evidence that binge exposure has depressive effects in both sexes, but predominantly in females, who display longer immobility times in the FST, in agreement with other studies. Alcohol-induced depressive-like behaviour during abstinence in males is in agreement with our previous data using this ABD model (Antón et al., 2017) and studies using ethanol vapor exposure (Bach et al., 2021; Walker et al., 2010).

Reductions in saccharin preference in the SPT would be related to this depressive effect. However, in our study, we did not find effects of IAE on this test. We have previously observed that an inflammatory state induced by lipopolysaccharide administration, which is known to be increased after IAE (Antón et al, 2017, 2018), induces an anhedonic state in males (Sayd et al., 2015), but we have never tested the animals under this IAE model before. It is possible that the abstinence period at the start of the test was too late to observe this effect, since the decrease in saccharin preference is highly dependent on the inflammatory status of the animal (Abderrahim et al., 2022; Sayd et al., 2015).

In terms of the cognitive function assessed in the MWM, in our study we did not observe significant effects of alcohol on spatial memory, although a trend (non-significant effect) can be observed in ethanol-treated females, which showed an apparent higher latency in the time taken to locate the platform. Other studies described negative effects of alcohol on spatial memory in males following a protocol of intermittent and voluntary alcohol access (Sanz-Martos et al. 2023), although no ethanol effects were observed in males in our study. In the NOR, we found that the ethanol-treated groups show different behaviours in the two test sessions 4h and 24h after the training phase. At 4h, alcohol shows significant effects on both sexes, increasing the latency of approaching the novel object, with a possible stronger effect in males (trend; interaction non-significant p=0.06). However, at 24h, males treated with alcohol experience an increase in latency as they did in the short-term, but females exhibited lower latency compared to their respective female control group. This effect was surprising, and it could be favoured by the high latency times observed in female controls. Other authors have shown that different paradigms of ethanol administration have negative effects on the ability to recognize the object measured by this task (Lamont et al., 2020; Moya et al., 2022), but no sex differences have been reported (Van Hees et al., 2022). These results in females would need to be confirmed in future studies, and the possible role of IAE in memory consolidation in a long-term.

Taken together, these results suggest that many behavioural tests that have been widely used to date to assess emotional and cognitive tasks in preclinical animal models may be performed differently by male and females in physiological conditions, which has implications for a proper extrapolation of results. Males and females biologically differ in several aspects, including the hormone profile. Numerous studies have demonstrated the critical influence of hormones in regulating behaviour (Hamson et al., 2016; Shirazi et al., 2021), so this could be a contributing factor to the observed variations between male and female outcomes. It is important to note that adolescence and early adulthood are crucial periods for maturation, during which hormone levels fluctuate significantly over time and vary by sex, playing a key role in the neurobiological development (Vijayakumar et al., 2018). Sex hormones such as estradiol (predominant in females) and testosterone (predominant in males) exerts distinct effects on adolescent brain development. Recent evidence suggests that estradiol enhances neurogenesis and plasticity in regions such as the hippocampus, which could be related to the superior performance of females in the MWM. In contrast, elevated testosterone levels appear to be associated with increased reinforcement sensitivity and a greater propensity for risk-taking behaviours, potentially explaining the observed sex differences in saccharin preference and exploratory behaviour in the EPM (Erol & Karpyak, 2015; He et al., 2024).

On the other hand, the effects of IAE may induce differential effects in male and females, as for example, alcohol binges induced a clear anxiogenic effect in males in the EPM and worsen recognition ability in the NOR, consistent with previous literature, whereas IAE appears to have a more disinhibitory effect in the EPM performance at the same time of abstinence and an enhancing of the recognition memory in the NOR, which is intriguing and deserve further investigation. Some authors suggest that hormonal fluctuations across the phases of the estrous

cycle influence both animal behaviour and the effects of alcohol exposure (Corbett et al., 2024; Klein Marcondes et al., 2001; Paiva-Santos et al., 2022; Scholl et al., 2019; Sircar, 2019). Scientific evidence also suggests that alcohol consumption may disrupt circulating levels of sex hormones. For instance, acute alcohol intoxication, as seen in IAE, is linked with a decrease in testosterone levels. Since testosterone has been associated with a propensity for risk-taking behaviours, the ethanol-induced reductions in entries and time in open arms in the EPM could be partially explained by this effect. In our present study we did not explore the estro cycle of females or the hormone levels, so this hypothesis remains speculative and needs further experimentation.

Additionally, some authors suggest that sex-related hormonal differences may interfere with alcohol metabolism, affecting its absorption and distribution throughout the body, ultimately leading to distinct behavioural effects (Erol & Karpyak, 2015). In this regard, some authors suggested that females have a faster metabolism of alcohol than males (Baraona et al., 2001; Desroches et al., 1995; Kishimoto et al., 2002; Thomasson, 2002). The faster elimination of alcohol by females could be due to a higher activity of hepatic enzymes (Kishimoto et al., 2002; Elena Quintanilla et al., 2007; Simon et al., 2002), although other authors found no differences (Livy et al., 2003; Lopez et al., 2003). These sexual differences in alcohol metabolism could explain some of the behavioral discrepancies that IAE induced in male and females in our study. In those experiments, we did not measure BELs or hepatic enzymes, so we cannot confirm this hypothesis, and this is a limitation of the study.

Recently, it has been shown that the pattern of IAE used in this study affects the intestinal microbiota, the integrity of the intestinal barrier, causing the translocation of pathogens into the systemic circulation and triggering a peripheral inflammatory response and disruption of the blood-brain barrier that contributes to alcohol-induced neuroinflammation, which is related to emotional and cognitive alterations (Antón et al., 2018; Orio et al., 2019; Orio, 2020; Rodriguez-Gonzalez & Orio, 2020; Rodriguez-Gonzalez et al., 2021; Rodriguez-Gonzalez et al., 2023). Specific research on the microbiota has shown that sex hormones and diet affect the proliferation of different microbial communities, which in turn impact the immune system, and these differences being more evident during adolescence (Org et al., 2016). Most of the mentioned studies have been done in males and only recently we have some data about the differential impact of this IAE protocol in the gut-brain axis of male and females (López-Valencia et al., 2024). Anyhow, ABD seems to affect cognitive performance differently in young men and women (Lees et al., 2019) and this could be related to inflammatory components (Orio et al., 2018).

Despite providing interesting data about possible sex differences in behaviour, we recognize several limitations of the current study. First, although the behavioural tests used in this study are widely described in the literature and they were administered sequentially according to a design to avoid between-test interferences (Moya et al., 2022, Moya et al., 2021; Marco et al., 2017), it should be noted that most of them were validated in males, so it is possible that their interpretation for females is biased and we cannot discard interferences in female animals. Secondly, this is a study that exclusively focus on behaviour; the absence of biochemical measures such as BELs, hormones or hepatic enzymes limits the interpretation of results. Finally, an intrinsic limitation of using this IAE model is that it does not allow to identify significant differences in the pattern of binge drinking between males and females (higher in females), which compromises the translational character of the study.

Our findings highlight the importance of a rigorous characterization of the test widely used to assess behaviour in preclinical animal models considering the differential performance of females in some of those tests, to enhance the translational relevance of the investigation. In terms of translational value, making sex-specific differences in the effects of IAE during early adulthood visible is essential to questioning the assumption that alcohol exerts identical effects across sexes, breaking the homogeneous perspective that persists regarding the effects of alcohol across sexes in its mechanism of action, toxicity, prevention and treatment. Thus, incorporating a sex-based perspective into future research is vital for the early detection of alcohol-related problems in both sexes, as well as for guiding the design of more effective interventions.

Funding

This work was supported by Ministerio de Ciencia e Innovación (Spain), cofounded by FEDER (European Union) (grant number PID2021-127256OB-I00 to L.O.) and by RICORS – RIAPAd (grant RD24/0003/0010 to L.O.), co-founded by Instituto de Salud Carlos III (ISCIII) and by the European Union (Fondo Europeo de Desarrollo Regional FEDER).

Conflict of interest

The authors declare no conflict of interest.

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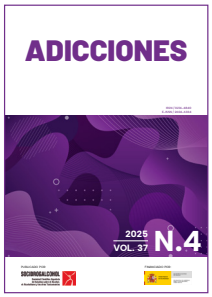

ORIGINAL

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

■ *Received: September 2025; Accepted: November 2025.*

■ ISSN: 0214-4840 / E-ISSN: 2604-6334

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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

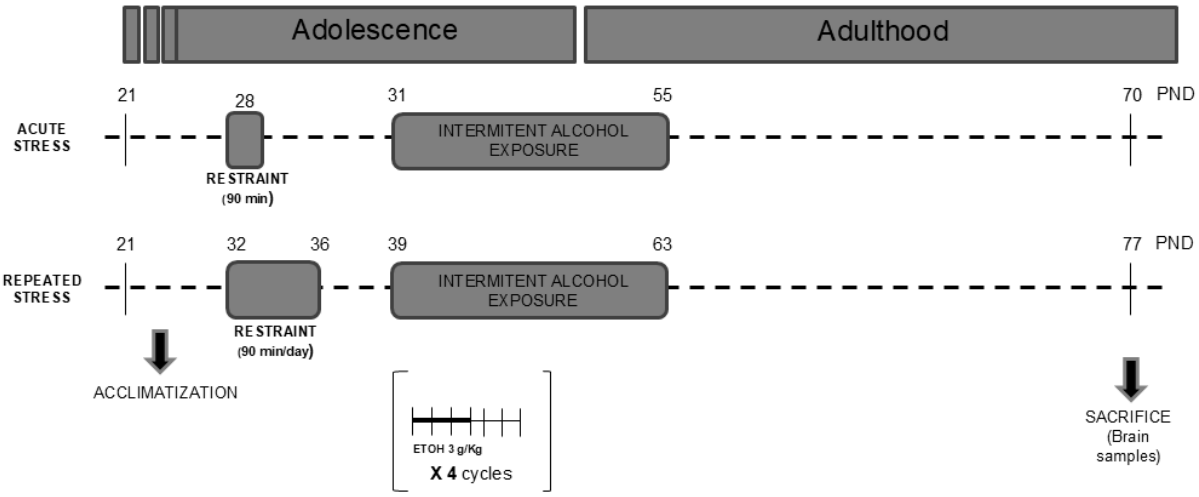
The experimental design has been described previously in two separate studies (Sanchez-Marin et al., 2022a; Sanchez-Marin et al., 2022b). A total of 88 adolescent male Wistar rats were used across two experimental cohorts. In both, animals were randomly assigned to stress or non-stress conditions and 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

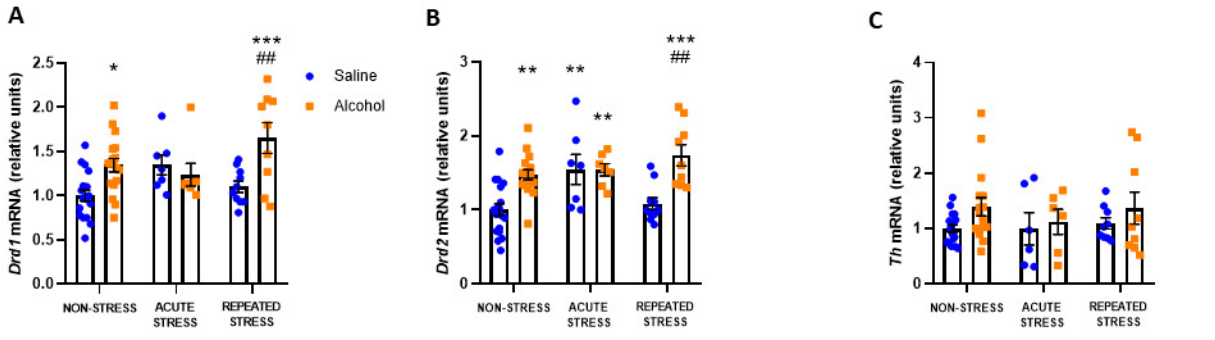
In the present study, we evaluated the expression of selected genes related to dopaminergic signaling, glutamatergic transmission, eCB signaling, neurotrophic support, and reactive glial (neuroinflammatory) markers in the dorsal striatum of young adult male rats (PND 70-77) that had been exposed to restraint 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.

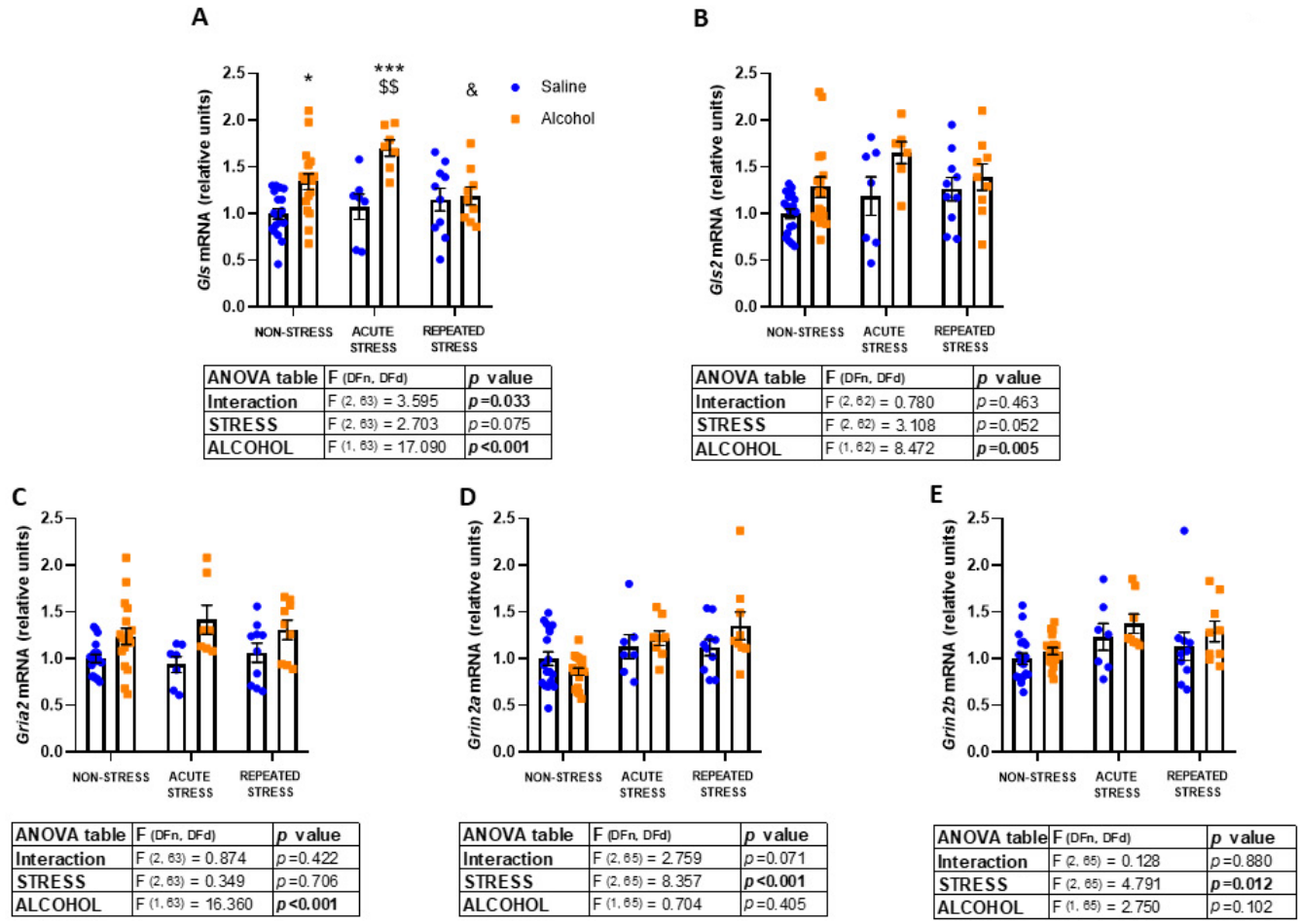
Effects of restraint stress and intermittent alcohol on the mRNA expression of glutamatergic signaling genes in the dorsal striatum

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.

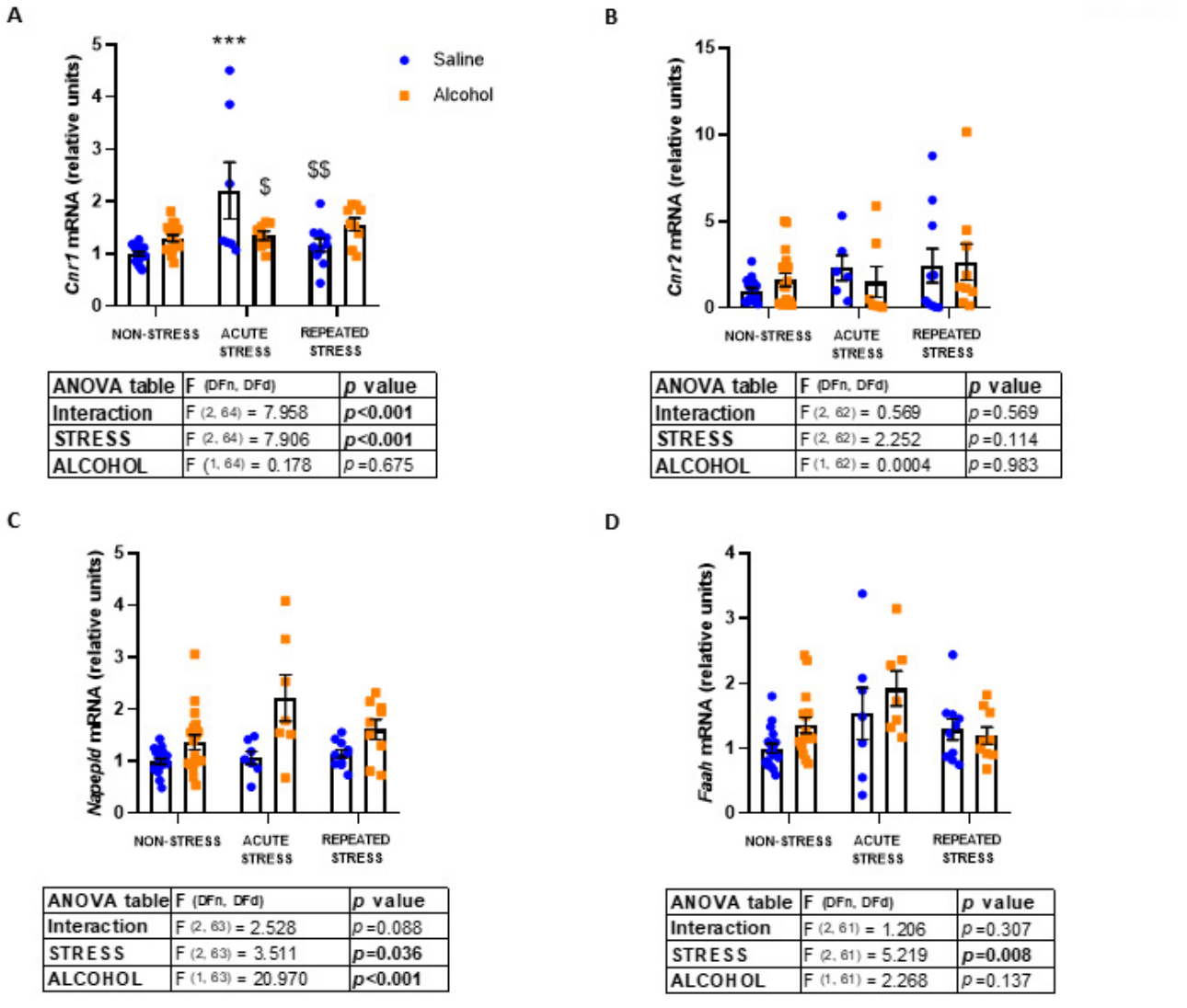
Effects of restraint stress and intermittent alcohol on the mRNA expression of endocannabinoid signaling genes in the dorsal striatum

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

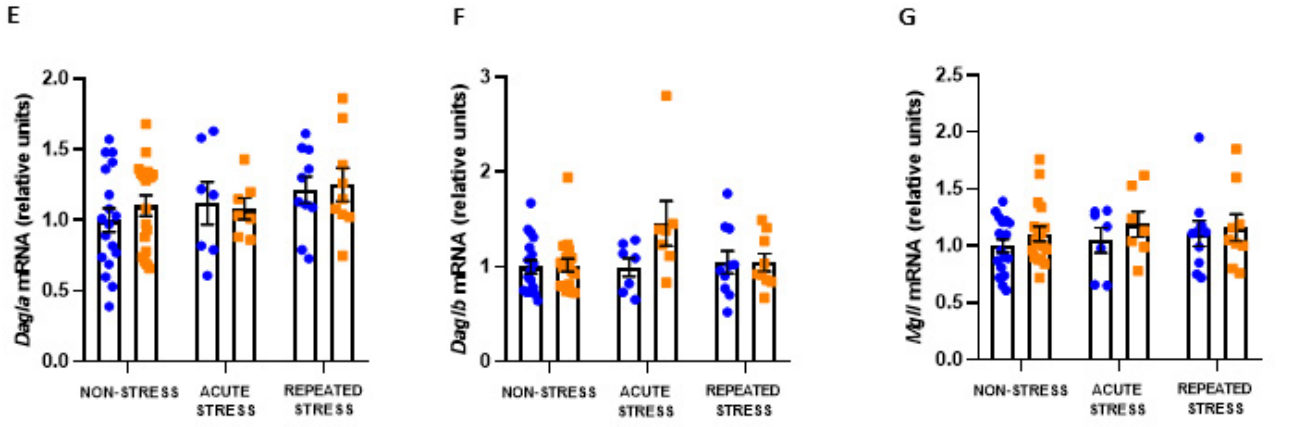


ANOVA table	F (DFn, DFd)	*p* value
Interaction	F (2, 64) = 7.958	*p*<0.001
STRESS	F (2, 64) = 7.906	*p*<0.001
ALCOHOL	F (1, 64) = 0.178	*p* =0.675

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").

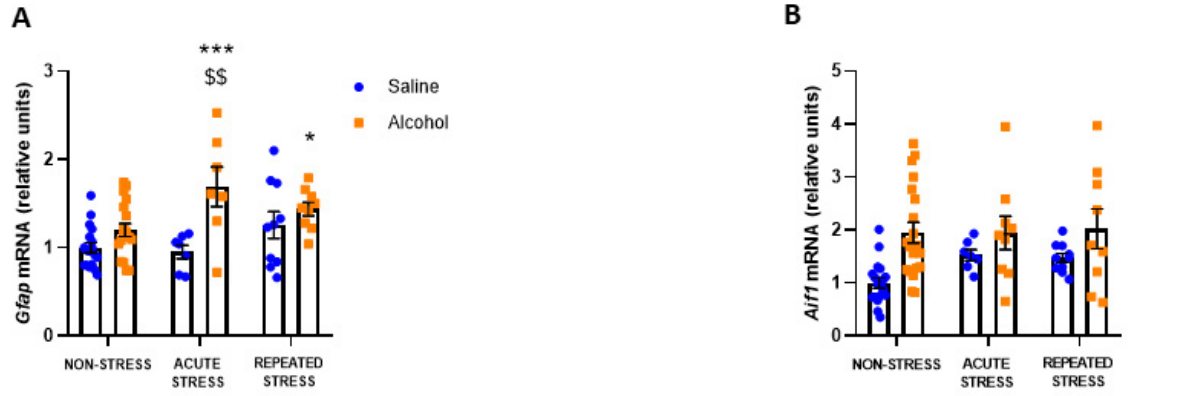
Effects of restraint stress and intermittent alcohol on astrocytic and microglial markers in the dorsal striatum

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.

Acknowledgements

The present study was supported by the following grants: Projects funded by Instituto de Salud Carlos III (ISCIII) and cofounded by the European Union and ERDF-EU (PI20/01399 , PI22/00427 and PI22/01833); Programa RICORS RIAPAD funded by ISCIII and cofounded by the European Union (RD21/0009/0003 and RD24/0003/0012); Project funded by Delegación de Gobierno para el Plan Nacional sobre Drogas, Ministerio de Sanidad y Consumo (PNSD2022/020); Programa Fortalece funded by ISCIII, Ministerio de Ciencia, Innovación y Universidades (FORT23/00013).

Conflicts of interest

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

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2025 ■ VOL. 37 ■ N. 4 ■ PAGES 383–394
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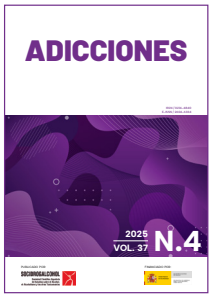

ORIGINAL

Study of the Pleiotrophin/PTPRZ neurotrophic pathway in the hippocampus of rats exposed to chronic alcohol consumption and/or thiamine deficiency

Estudio de la ruta neurotrófica Pleiotrofina/PTPRZ en el hipocampo de ratas expuestas a consumo crónico de alcohol y/o deficiencia de tiamina

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Abstract

Wernicke's encephalopathy (WE) is caused by thiamine deficiency (TD) whose main risk factor is alcohol use disorder. Pathogenic mechanisms associated with WE include mitochondrial dysfunction, oxidative stress and neuroinflammation. This study aims to explore the gene expression signature of certain candidate genes related to neuroinflammation, mitochondrial dysfunction and thiamine metabolism in the hippocampus from animals exposed to chronic alcohol consumption, thiamine deficiency or the combination of both.

Male Wistar rats (n=42) were randomly assigned to 4 experimental groups: control (C) receiving tap water or tap water plus thiamine (0.2 g/L), chronic alcohol (CA) forced ingestion for 36 weeks, TD diet and pyrithiamine for 12 days (TDD) and CA combined with TDD. The relative gene expression of neurotrophic factors (*Ptn*, *Mdk*, *Ptprz*), proinflammatory molecules (*Tlr4*, *Ccl2 and Hmgb1*), mitochondrial homeostatic factors (*Mfn1 and Mfn2*) and thiamine metabolism (*Tpk1*) was analyzed in RNA isolated from the hippocampus across all experimental groups. Differences in gene expression were assessed using non-parametric tests (Kruskal-Wallis).

Ptprz mRNA levels tended to be downregulated in the TDD group compared to controls (p=0.06, non-significant) and levels were significantly decreased related to the CA+TDD group (p<0.05). TDD group showed the lowest expression levels of *Ptn* across all experimental groups, and this decrease was statistically significant compared to the control and CA groups (p<0.05).

Our findings indicate a differential gene expression profile of the PTN-MDK-PTPRZ axis in the hippocampus of rats receiving a TD diet but not in the rest of the WE models analyzed (CA and CA+TDD).

Keywords: Wernicke, Korsakoff, thiamine deficiency, pleiotrophin, Protein Tyrosine Phosphatase Receptor Z, neuroinflammation, hippocampus

Resumen

La encefalopatía de Wernicke (WE) es una enfermedad neurológica causada por la deficiencia de tiamina (TD) cuyo principal factor de riesgo es el trastorno por uso del alcohol. El objetivo de este estudio es explorar el perfil de expresión de genes candidatos relacionados con neuroinflamación, disfunción mitocondrial y metabolismo de la tiamina en el hipocampo de animales expuestos a consumo crónico de alcohol (CA), una dieta deficiente en tiamina (TDD) o la combinación de ambos.

Se analizaron un total de 42 ratas Wistar macho incluidas en 4 grupos experimentales: control (C) que recibieron agua o agua suplementada con tiamina (0,2 g/L), alcohol crónico (CA) durante 36 semanas, dieta TD y piritiamina durante 12 días (TDD) y un grupo que combinaba CA+TDD. La expresión relativa de factores neurotróficos (*Ptn*, *Mdk*, *Ptprz*), factores proinflamatorios (*Tlr4*, *Ccl2 y Hmgb1*), proteínas implicadas en homeostasis mitocondrial (*Mfn1 y Mfn2*) y enzimas del metabolismo de la tiamina (*Tpk1*) se determinó a partir de ARNm obtenido del hipocampo de los distintos grupos experimentales. El análisis estadístico se realizó mediante el test no paramétrico Kruskal-Wallis.

La expresión de *Ptprz* tendía a ser menor en el grupo TDD comparado con el grupo C (no significativo) mientras que la disminución de *Ptprz* observada en el grupo TDD fue estadísticamente significativa cuando se comparaba con el grupo CA+TDD (p<0,05). Además, el grupo TDD mostró los menores niveles de expresión de *Ptn* y esta disminución fue estadísticamente significativa comparada con los grupos C y CA (p<0,05).

Nuestros resultados indican un perfil diferencial de expresión de la ruta PTN-MDK-PTPRZ en el hipocampo de ratas con una dieta TD distinto al observado en el resto de los modelos de encefalopatía WE analizados (CA y CA+TDD).

Palabras clave: Wernicke, Korsakoff, deficiencia de tiamina, pleiotrofina, receptor proteína tirosina Fosfatasa Z, neuroinflamación, hipocampo

■ *Received: July 2025; Accepted: November 2025.*

■ ISSN: 0214-4840 / E-ISSN: 2604-6334

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Thiamine is an essential vitamin (B1) acquired through the diet whose active form, thiamine pyrophosphate, is a required cofactor for crucial enzymes involved in energy metabolism (Jhala & Hazell, 2011; Zhao et al., 2014). Thiamine deficiency (TD) has been associated with mitochondrial dysfunction, increased oxidative and nitrosative stress and activation of inflammatory and cell damage processes (Moya et al., 2021; Moya et al., 2022b). Deregulation of those processes due to TD is associated with different neurological disorders (Abdou & Hazell, 2015; Cassiano et al., 2022; Jhala & Hazell, 2011; Martin et al., 2003; Moya et al., 2022a).

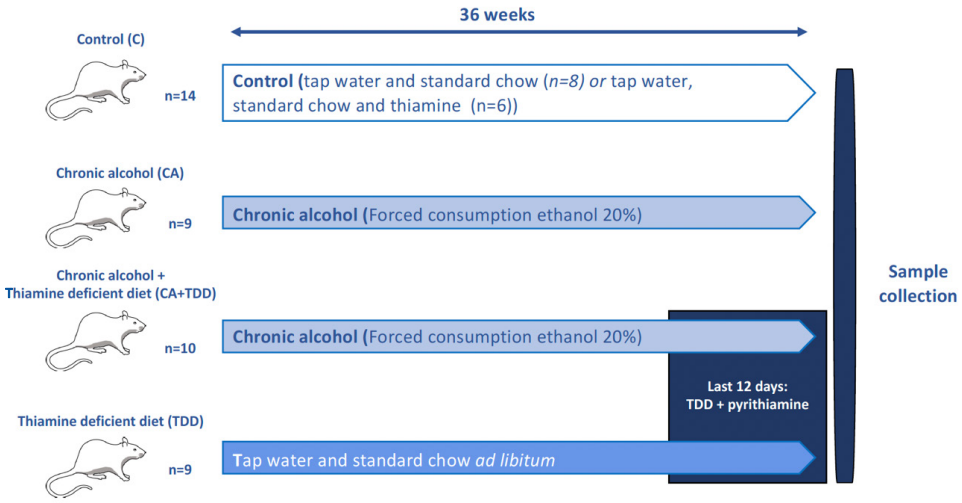
Wernicke's encephalopathy (WE) is an acute severe neurological condition caused by TD. The main cause of WE is alcohol use disorder (AUD), although WE has been also described in patients with non-alcohol related conditions such as inflammatory bowel disease, anorexia nervosa or patients after bariatric surgeries (Eva et al., 2023; Kohnke & Meek, 2021). The diagnosis of the disorder is frequently postmortem by brain imaging studies, with autopsy studies reporting a prevalence of WE between 0.4-2.8% and most of the cases associated with alcohol abuse (Abdou & Hazell, 2015; Li & Xing, 2025). Very few cases (20%) appear to be identified antemortem, and this misdiagnosis rate is alarmingly high for non-alcoholic WE. The disorder is diagnosed by a classic triad of symptoms (encephalopathy, ophthalmoplegia and ataraxia) but the three components are observed in a mere of 16% patients, the majority exhibiting only one or two components and some of them exhibiting a rare condition with no classic symptoms, especially in the early stages of non-alcoholic WE (Li & Xing, 2025). Other WE's main symptoms include cognitive (confusion, disinhibition) and motor (nystagmus, loss of balance, gait alterations, among others) disturbances (Kohnke & Meek, 2021; Oscar-Berman & Maleki, 2019). If left untreated, WE may progress to a more severe neurological condition called Korsakoff's syndrome (WKS), so it can be considered as a continuous, named Wernicke-Korsakoff's syndrome (Hammoud & Jimenez-Shahed, 2019), which is characterized by memory disorders (anterograde and retrograde amnesia) and psychiatric symptoms (confabulation and psychosis) (Arts et al., 2017; Kohnke & Meek, 2021; Oscar-Berman & Maleki, 2019). Neuroimaging studies in WE and WKS patients identified brain damage in different areas, being thalamus, mammillary bodies, hippocampus, frontal lobes or cerebellum, among the most affected areas (Jung et al., 2012). Currently, WE can be treated with thiamine supplementation to avoid progression of the disorder but there is no evidence of a beneficial pharmacological therapy to treat neurological damage in WKS (Arts et al., 2017; Sahu et al., 2025).

Modulation of the immune response in the central nervous system (CNS), especially the innate response mediated by glial cells, helps to restore and minimize the damage caused by pathogenic and toxic insults (Chew et al., 2006; Gomez-Nicola & Perry, 2015; Jung et al., 2019; Kielian, 2016; Lehnardt, 2010). However, chronic neuroinflammatory responses due to persistent insults or imbalance in homeostatic mechanisms contribute to a variety of neurological conditions (Gomez-Nicola & Perry, 2015; Jung et al., 2019; Kielian, 2016). Sustained neuroinflammation is also one of the proposed pathogenic mechanisms involved in WE (Abdou & Hazell, 2015; Cassiano et al., 2022; Moya et al., 2022a; Moya et al., 2022b; Moya et al., 2021; Toledo Nunes et al., 2019; Zahr et al., 2014). In this sense, an upregulation of main proinflammatory cytokines (such as IL1, IL6, TNFa or MCP1) and an increment of microglial activation markers were found in different brain areas (thalamus, inferior colliculus, or hippocampus, among others) in WE models (Toledo Nunes et al., 2019; Zahr et al., 2014). Interestingly, previous findings displayed an upregulation of the TLR4/MyD88 signaling pathway in the same WE model used in this study, specifically in the frontal cortex and cerebellum (Moya et al., 2022a; Moya et al., 2021). Pleiotrophin (PTN) and Midkine (MDK) are neurotrophic factors that act as regulators of neuroinflammation in various neurological conditions (Cañeque-Rufo et al., 2025; del Campo et al., 2021; Fernández-Calle et al., 2018, 2020; Rodríguez-Zapata et al., 2024; Vicente-Rodríguez et al., 2014; Vicente-Rodríguez et al., 2016). While the expression of PTN peaks at birth and readable levels are sustained in adulthood, MDK expression mainly occurs during embryonic development and can be induced in adults by different forms of tissue injury (Ross-Munro et al., 2020). PTN and MDK bind to different receptors, although Protein Tyrosine Phosphatase Receptor Z (PTPRZ, also known as RPTPβ/ζ) seems to be the most implicated in the regulation of neuroinflammation due to its main expression in the CNS (González-Castillo et al., 2015; Herradon et al., 2019; Ross-Munro et al., 2020). PTN and MDK inhibit the intrinsic tyrosine phosphatase activity of PTPRZ increasing the phosphorylation levels of its substrates such as β-catenin, Fyn or ALK (Herradon et al., 2019; Maeda et al., 1999). PTN and MDK have been found upregulated in diverse pathologies with a neuroinflammatory context such as Parkinson's disease, Alzheimer's disease, brain injury or after the administration of drugs of abuse (amphetamine or cocaine) (Herradon et al., 2019). In relation to alcohol consumption (main risk factor of WE and WKS), PTN was also upregulated after an acute ethanol administration in the mouse prefrontal cortex (Vicente-Rodríguez et al., 2014) and an increase of MDK was also observed in the frontal cortex of AUD patients (Flatscher-Bader & Wilce, 2008).

Recent studies have revealed the important role of the PTN-RPTPZ pathway in hippocampal processes.

Figure 1

Experimental design and timeline of the treatments



Note. A total of 42 male Wistar rats were fed with standard chow and tap water ***ad libitum*** for 12 days prior to experimentation. After that, animals were randomly assigned to each experimental group: control [tap water and standard chow] (C, n=14[#]), chronic alcohol [20% w/v for 36 weeks] (CA, n=9), Thiamine deficiency [thiamine deficient diet and a daily pyrithiamine administration (0.25 mg/kg; i.p.) for the last 12 days] (TDD, n=9) and CA combined with TDD (CA+TDD, n=10). [#]Six control animals received oral thiamine (0.2 g/L) in the water. Experimental groups collected from (Moya, López-Valencia, et al., 2022). The rat image was obtained from BioArt Collection NIAID Visual & Medical Arts. 26/06/2025. Black Rat–Grey (BIOART-000054) NIAID BIOART Source: https://bioart.niaid.nih.gov/bioart/54 and its color was modified.

Interestingly, the loss of hippocampal neurogenesis induced by exposure to ethanol during adolescence is regulated by the administration of a selective RPTPZ inhibitor, MY10. Administering MY10 to mice completely prevented the loss of hippocampal neurogenesis caused by acute ethanol exposure during adolescence (Galán-Llario et al., 2023a). Additionally, previous studies have revealed evidence of sex-specific differences in the effects of chronic intermittent alcohol on glial responses and hippocampal neurogenesis (Galán-Llario et al., 2023b). Apart from alcohol-induced neuroinflammation, other studies suggest that endogenous PTN levels play an important role in regulating the acute systemic response to lipopolysaccharide (LPS) and the hippocampal microglial changes in young adult mice, as well as in the regulation of the long-term effects of LPS on the astrocytic response and neurogenesis in the hippocampus (Rodríguez-Zapata et al., 2024). However, the modulatory role of PTN-RPTPZ in hippocampal processes following chronic alcohol consumption and/or thiamine deficiency remains to be studied.

Therefore, this study aims to explore the gene expression signature of the PTN-MDK-PTPRZ axis and other candidate genes related to neuroinflammation, mitochondrial dysfunction and thiamine metabolism in the hippocampus from three different *in vivo* models that potentially may induce WE by chronic alcohol consumption (CA), TD diet and antagonism of thiamine (TDD) or the combination of CA and TDD (CA+TDD).

Materials and methods

Animals

Male Wistar rats (Envigo©, Barcelona, Spain) weighing 100–125 g at arrival were fed with standard food and tap water that were available *ad libitum* for 12 days prior to experimentation. After that (around PD 40), animals (n = 42) were randomly assigned to each experimental group. A detailed description of the animal housing can be found in a previous publication (Moya et al., 2021).

All procedures followed ARRIVAL guidelines and adhered to the guidelines of the Animal Welfare Committee of the Complutense University of Madrid (reference: PROEX 312-19) in compliance with Spanish Royal Decree 53/2013 and following European Directive 2010/63/EU on the protection of animals used for research and other scientific purposes.

Experimental groups

The experimental design is depicted in Figure 1. In this study, 4 experimental groups were employed: chronic alcohol (CA), TD diet + pyrithiamine (TDD), CA combined with TDD (CA+TDD) and control (C) group. A detailed description of the experimental groups can be found in a previous publication (Moya et al., 2022b).

CA group was exposed to forced consumption of an ethanol solution (limited access to a single bottle) based on the protocol described previously (Fernandez et al., 2016). The ethanol solution was prepared from ethanol 96° (Iberalcohol S.L., Madrid, Spain) in tap water. Alcohol was gradually introduced; started at 6% for 5 days, followed by another 5 days at 9%, 5 days at 12%, 2 days at 16% and finally

reaching 20%, which was maintained during the 36-week duration of the experiment. CA rats (n= 9) were provided with standard food *ad libitum* throughout the experiment.

In the TDD group (n= 9), animals were feed with the standard food and had access to a single bottle with tap water. In the last 12 days of the experiment, the chow was substituted by a TD diet (residual thiamine level <0.5 ppm; Teklad Custom Diet, Envigo, Madison, WI, USA), as well as a daily pyrithiamine hydrobromide administration (thiamine pyrophosphokinase inhibitor) (Sigma Aldrich, Madrid, Spain; 0.25 mg/kg; i.p.), as described previously (Moya et al., 2021).

In the CA+TDD group, animals received the same alcohol treatment as the CA group and in the last 12 days of the experiment the standard food was changed to the TD diet plus a daily pyrithiamine hydrobromide injection, as described for the TDD group (n= 10).

The C group had access to a single bottle with tap water and standard chow *ad libitum* for the entire duration of the study (n=8). An additional control group (n=6) supplemented with 0.2 g/L of thiamine in the water throughout the experiment (Moya et al., 2022b) was joined to this group since no significant changes were found in any parameters analyzed (C group, n=14).

During the last 12 days of protocol, C and CA groups received equivalent daily injections of saline (i.p.) to reproduce the same stress conditions in all animals.

Tissue Sample Collection

On day 12 of the TDD protocol, at least 1 h after treatment administration, the animals were decapitated after lethal injection of sodium pentobarbital (320 mg/kg, i.p., Dolethal®, Vétoquinol, Spain). The brains were immediately isolated from the skull, discarding the meninges and blood vessels. Samples of frontal cortex and cerebellum have been used and published in (Moya et al., 2022b). Hippocampus were also excised and frozen at −80 °C until assayed in this study.

Gene expression analysis

Total RNA from hippocampus (left hemisphere) was isolated using the Total RNA Isolation Kit (Nzytech, Lisbon, Portugal) according to manufacturer`s instructions. Then, 1,5 µg of RNA were reverse-transcribed to first-strand cDNA (First-strand cDNA Synthesis Kit, Nzytech, Lisbon, Portugal).

Quantitative real-time PCR was performed in duplicate for the relative quantification of *Ptprz, Ptn, Mdk, Tlr4, Ccl2, Hmgb1, Mfn1, Mfn2 and Tpk1* by using the SsoAdvanced Universal SYBR Green Supermix kit (Bio-Rad, Hercules, CA, USA) in a CFX Opus Real-Time System (Bio-Rad, Hercules, CA, USA). The relative expression of each gene was calculated using *Rpl13* and *β2m* as reference genes, according to Livak method (Livak & Schmittgen, 2001). The primer sequences and experimental conditions are summarized in Supplementary Table 1.

Statistical analysis

Statistical analyses were performed using IBM-SPSS v28 software (IBM Corp., Armonk, N.Y., USA) and data were depicted using Graphpad Prism version 8 (San Diego, CA, United States). Data are presented as mean ± standard error of the mean (SEM). After assessing the non-normality of the data distribution (Kolmogorov-Smirnov test), differences in gene expression between experimental groups were analyzed using non-parametric tests (Kruskal-Wallis) and post-hoc comparisons were applied using the Bonferroni correction. A p value less than 0.05 was considered statistically significant.

Results

PTN-MDK-PTPRZ pathway

First, the relative gene expression of *Ptprz* and its ligands *Ptn* and *Mdk* was analyzed across all experimental groups (C; CA; TDD; CA+TDD) (Figure 2A-C). While the expression levels of *Ptprz* in the group exposed to chronic alcohol with or without TD diet (CA and CA+TDD) were similar to the control group, its gene expression tended to decrease in TDD group compared to controls (p=0.06, non-significant) and significantly decreased in TDD group compared to CA+TDD group (Figure 2A, p<0.05). Moreover, TDD group showed the lowest expression levels of *Ptn* across all experimental groups, and this decrease was statistically significant compared to the control and CA groups (Figure 2B, p<0.05). *Ptn* gene expression was also lower in the group receiving CA and TDD (CA+TDD) compared to the control and CA groups, although these differences did not reach statistical significance after Bonferroni correction. In contrast, no significant differences were observed in the expression of *Mdk* between the analyzed groups (Figure 2C).

Neuroinflammatory molecules

Expression levels of other genes associated with chronic inflammation (*Tlr4, Ccl2 and Hmgb1)* were also assessed in the hippocampus tissues of the different experimental groups (Figure 3). *Tlr4* gene expression was similar across all the experimental groups (Figure 3A). *Ccl2* expression appears to be slightly higher in those experimental groups receiving TDD (CA+TDD and TDD) compared to C and CA groups, despite being a non-significant tendency (Figure 3B). Regarding *Hmgb1,* its relative gene expression was almost stable across all experimental groups, independently of the treatment received (Figure 3C).

Mitochondrial homeostasis and thiamine metabolism

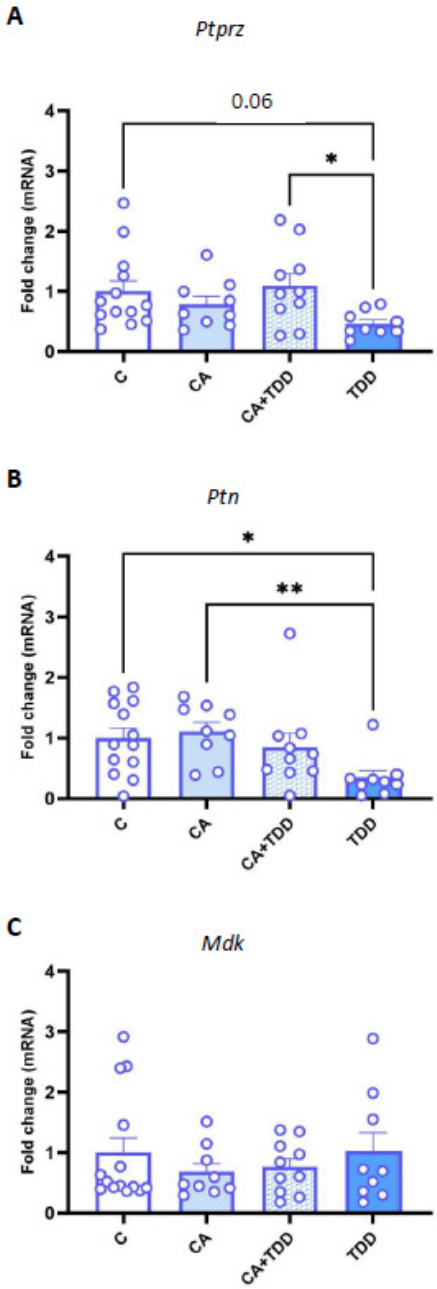
In addition, gene expression levels of *Mfn1* and *Mfn2*, crucial proteins implicated in mitochondrial health and homeostasis, were also analyzed (Figure 4). Interestingly,

Mfn1 expression tended to decrease in the TDD group (non-significant effect) while its expression in the rest of the experimental groups was similar (Figure 4A). Likewise, the lowest levels of *Mfn2* expression were found in the TDD group (non-significant effect), being its expression similar across the rest of the experimental groups (Figure 4B).

Finally, the gene expression levels of *Tpk1*, which encodes the enzyme involved in the conversion of thiamine to its

Figure 2

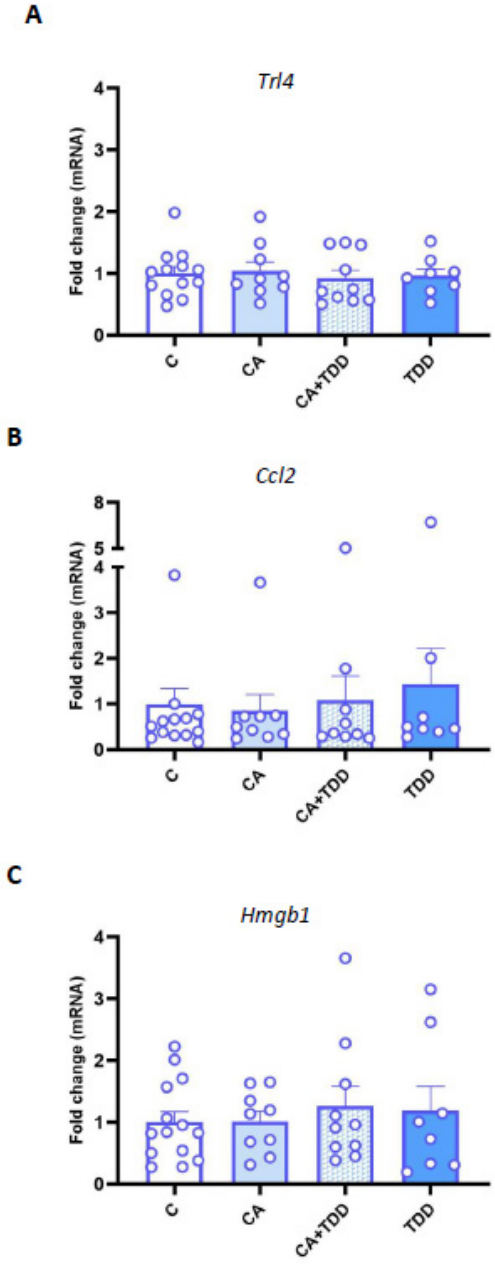
Relative gene expression of Ptprz and its ligands Ptn and Mdk across all experimental groups



Note. Graph represents data (mean ± S.E.M.) from the relative quantification of ***Ptprz*** (A), ***Ptn*** (B) and ***Mdk*** (C) mRNA levels in the hippocampus of male Wistar rats.
Control (C, n=14), chronic alcohol (CA, n=9), thiamine deficient diet and pyrithiamine for 12 days (TDD, n=9) and CA combined with TDD (CA+TDD, n=10). ***p* < 0.05 for significant differences among C ***vs.*** TDD and ****p* < 0.01 for significant differences among CA ***vs.*** TDD. No significant differences were observed in the expression of ***Mdk*** between the analyzed groups. Statistical significance was assessed by non-parametric tests (Kruskal-Wallis) and post-hoc comparisons (Bonferroni correction).

Figure 3

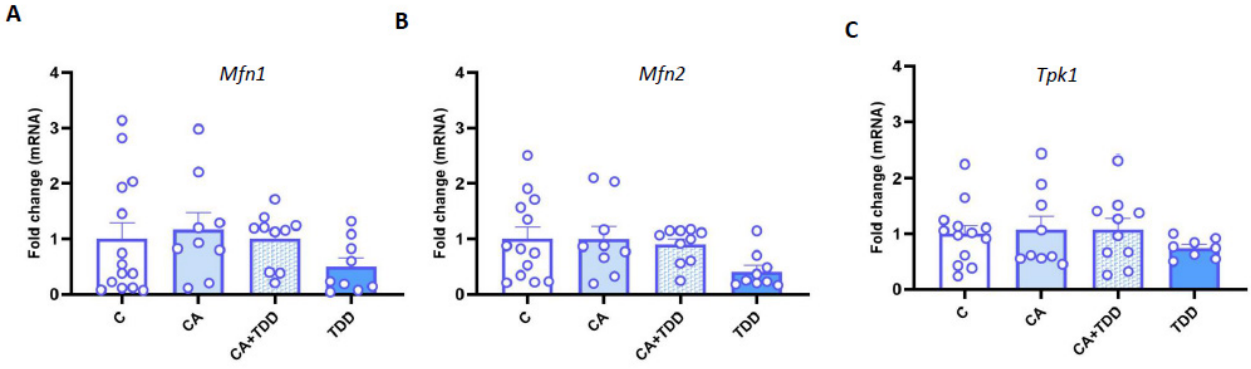
Relative expression of proinflammatory genes in the analyzed in vivo WE models



Note. Graph represents data (mean ± S.E.M.) from the relative quantification of ***Tlr4*** (A)***, Ccl2*** (B) ***and Hmgb1*** (C) mRNA levels in the hippocampus.
Control (C, n=14), chronic alcohol (CA, n=9), thiamine deficient diet and pyrithiamine for 12 days (TDD, n=9) and CA combined with TDD (CA+TDD, n=10). Statistical significance was assessed by non-parametric tests (Kruskal-Wallis) and post-hoc comparisons (Bonferroni correction). No significant differences were observed in the expression of ***Tlr4, Ccl2 and Hmgb1*** between the analyzed groups.

Figure 4

Expression levels of genes related to mitochondrial homeostasis and thiamine metabolism.



Note. Graph represents data (mean ± S.E.M.) from the relative quantification of ***Mfn1*** (A)***, Mfn2*** (B) ***and Tpk1*** (C).
Control (C, n=14), chronic alcohol (CA, n=9), thiamine deficient diet and pyrithiamine for 12 days (TDD, n=9) and CA combined with TDD (CA+TDD, n=10).
Statistical significance was assessed by non-parametric tests (Kruskal-Wallis) and post-hoc comparisons (Bonferroni correction). No significant differences were observed in the expression of ***Mfn1, Mfn2 and Tpk1*** between the analyzed groups.

active form, was measured (Figure 4C). Its expression levels did not differ between the experimental groups, although the lowest levels of *Tpk1* (non-significant) were found in the TDD group (Figure 4C).

Discussion

WE is a major neurological condition caused by TD, being AUD the main risk factor (Eva et al., 2023; Oscar-Berman & Maleki, 2019). Furthermore, WKS can be often developed in those WE patients that do not receive thiamine replacement therapy. Its severe effects, including amnesia and psychiatric disorders, impact significantly the quality of life of these patients (Kohnke & Meek, 2021; Oscar-Berman & Maleki, 2019). In this study, the gene expression signature of a set of candidate genes involved in neuroinflammation, mitochondrial dysfunction and thiamine metabolism was analyzed in three animal models potentially inducing WE (CA, CA+TDD and TDD) to characterize the contribution of alcohol and the nutritional deficiency of thiamine to this condition, specifically in the hippocampus. Our findings indicate a differential gene expression of the PTN-MDK-PTPRZ axis in the group receiving TD diet with no significant contribution of the alcohol groups (CA and CA+TDD).

PTPRZ is the main receptor for PTN and MDK at the CNS, where is found widely expressed. PTN and MDK are two neurotrophic factors involved in the regulation of neuroinflammatory mechanisms underlying different neurological conditions (Herradon et al., 2019). *Ptprz* was downregulated in the hippocampus of the TDD group; however, this decrease was not observed in the experimental groups exposed to chronic alcohol (36 weeks) without or with TD diet (CA and CA+TDD, respectively). In this sense, previous results from our group showed no changes in the expression of *Ptprz* in the mouse PFC after an acute exposure to ethanol (Rodríguez-Zapata et al., 2023). Therefore, our data suggest that *Ptprz* downregulation may be specific to TD diet and is not affected by chronic alcohol exposure as observed in CA and CA+TDD groups. However, further studies are needed to confirm that *Ptprz* expression is not modified by chronic alcohol consumption. Interestingly, *Ptprz* knockout mice exhibit altered social behavior and aggressivity suggestive of some positive symptoms of schizophrenia (also presented in WKS patients) as well as elevated levels of dopamine in PFC, amygdala and hippocampus (Cressant et al., 2017).

Strikingly, TD diet induced a significant decrease on *Ptn* expression while no significant differences were observed in *Mdk* across the groups analyzed. This is interesting because PTN and MDK are the only members of this family of cytokines, and highly overlap in structure and function (Herradon et al., 2005; Herradón & Pérez-García, 2014). However, the data presented here suggest a more prominent role of PTN in situations of TD. In addition, PTN and MDK have been found upregulated in diverse pathologies with a neuroinflammatory component (Herradon et al., 2019). In relation to alcohol consumption, *Ptn* expression was upregulated in the mouse PFC after an acute ethanol administration (Vicente-Rodríguez et al., 2014); however, after a 4-week adolescent intermittent access to ethanol model, we did not observe significant changes of *Ptn* expression in the mouse hippocampus (Galán-Llario et al., 2024). Furthermore, PTN seems to play a protective role against dopaminergic neural loss in different pathological contexts (Gombash et al., 2012; Gramage et al., 2010). In response to a toxic insult, such as amphetamine administrations, *Ptn* knockout mice displayed an intensified

dopaminergic neurotoxicity in the nigrostriatal pathway (Gramage et al., 2010), while *Ptn* overexpression showed protective neurotrophic effects in rodent models of Parkinson's disease (Gombash et al., 2012). Taking together the downregulation of *Ptn* and *Ptprz* in rats with TD diet, it is tempting to hypothesize that deficits in this neurotrophic signaling pathway could be involved in the severity of the brain injury caused by TD diet.

Neuroinflammation is one of the main pathogenic mechanisms underlying brain damage in WE (Cassiano et al., 2022; Eva et al., 2023; Toledo Nunes et al., 2019; Zahr et al., 2014; Zhao et al., 2014). Therefore, the gene expression pattern of *Tlr4, Ccl2 and Hmgb1* were assessed in the animal models of CA consumption and TD different WE models analyzed in the present study. Different studies support the role of the immune TLR4 response in the neuroinflammation observed in WE, particularly in the cortical and cerebellar areas (Moya et al., 2022a; Moya et al., 2021). For example, both TLR4 and HMGB1 protein levels were upregulated in the PFC after 12 days of TDD, whereas alterations of this pathway in the cerebellum were more evident after 16 days of TDD (Moya et al., 2021). In the current study, we did not find significant alterations in the expression of these proinflammatory molecules in the hippocampus after 12 days of TDD, suggesting that the peak of neuroinflammation in this structure may be happening at a different time-point, in accordance with the described different brain regional vulnerabilities to TD over time (Moya et al., 2021). Nevertheless, transcriptome analysis of an *ex vivo* TD model (organotypic hippocampal slice culture) did not show alterations in *Tlr4, Ccl2* or *Hmgb1* among 90 differentially expressed genes, including TNF and FoxO signaling pathways (Cassiano et al., 2022), in agreement with the data in the current study.

Additionally, the gene expression of *Mfn1* and *Mfn2* was determined. Regulation of mitochondrial dynamics is crucial for calcium and energy homeostasis in neurons (McCoy & Cookson, 2012; van Horssen et al., 2019), being MFN1 and MFN2 key for mitochondrial fusion (van Horssen et al., 2019; Wai & Langer, 2016). Both, *Mfn1* and *Mfn2*, tended to decrease in the TDD group (non-significant) while their expression in the rest of the experimental groups was very similar to the control group. Interestingly, *Mfn2* levels have been found to be decreased in the hippocampus of mice subjected to the drinking in the dark procedure (Mira et al., 2020). Furthermore, *Mfn2* inducible knockout mice displayed neurodegeneration through oxidative stress and neuroinflammation at hippocampus and cortex (Han et al., 2020). Therefore, further studies are needed to confirm a possible decrease in *Mfn2* expression in TD and its putative value as an early marker of mitochondrial dysfunction.

Finally, we analyzed *Tpk1,* a crucial protein in thiamine metabolism which encodes the enzyme involved in the conversion of thiamine to its active form (Jhala & Hazell, 2011; Zhao et al., 2014). Low *Tpk1* expression in the brain compared to other tissue seems to contribute to brain vulnerability to thiamine deficiency (Xia et al., 2024). In this sense, we did not find differences in the expression levels of Tpk1, suggesting that thiamine metabolism in the hippocampus may not be crucial in these WE models.

Taking all results together, this study shows a prominent role of the TDD model versus the chronic alcohol consumption model, since any alteration found in this study was present in the TDD experimental group. Similarly, other authors observed a pivotal role for TD in the expression of neuroinflammatory markers compared with models of chronic alcohol, where inflammatory markers displayed only minor modifications (Toledo Nunes et al., 2019). Similarly, regarding parameters of damage and behavioral correlates such as disinhibition, we previously observed that the changes in the CA+TDD group were highly dependent of the TDD (Moya et al., 2022b), confirming the stronger damage potential of this model.

Regarding behavioral correlates, the animals in the TDD group did not show a significant affectation of memory, although a trend in the Novel Object Recognition (NOR) test could be observed, since some TDD animals displayed higher latencies to explore the novel object and a mild decrease in the discrimination index (Moya et al., 2022b). In the present study, we also examined the potential correlation between two parameters of the NOR test (latency to novel object and discrimination index). Our analysis found no significant association between the levels of Ptn and_*Ptprz* expression and these NOR test results (data not shown). Thus, the significant decreases in *Ptn* and *Ptprz* expression observed in the hippocampus of the TDD group may be related to an initial step of hippocampus damage, in which memory and motor functions are not significantly compromised.

We are aware of some limitations of the study, as the inclusion of a combined control group (with and without thiamine supplementation) and the modest changes observed in the expression of certain candidate genes (*Ptn* and *Ptprz*) in the TDD group. In addition, in the animals of the current study we observed a trend toward declining the plasmatic thiamine levels in all CA animals (Moya et al., 2022b) although the active form (TDP) was not analyzed. In this regard, other authors have reported significant decreases in thiamine diphosphate (TDP) levels, the active form of thiamine, in red blood cells of animals that underwent similar CA and TDD protocols, as an objective measurement of thiamine deficiency (Toledo Nunes et al., 2019). Finally, we included only males in this study, which is an important limitation. To our knowledge, females have not been studied with these animal protocols. Considering the current gap in biomedical research regarding the effects on females and giving the growing body of knowledge on

sex differences in the biological impact of CA on brain parameters, including neuroinflammation, it is urgent to complete these investigations using females for comparative analyses. Despite these limitations, these preliminary findings regarding the PTN/PTPRZ axis may provide novel data on the initial steps of brain injury caused by a TD diet.

The data suggest that an overall deficit of the PTN/PTPRZ neurotrophic pathway, together with possible alterations in mitochondrial dynamics, may be events that precede the severe symptomatology of an advanced stage in WE. Further experiments are needed to confirm this hypothesis, which could lead to the development of new therapeutics to prevent neurological affectations and the progression of the disease. In this sense, it has been recently developed a BBB-permeable small molecule, called MY10, that mimics many of the PTN actions in the CNS, such as reducing alcohol consumption in different rodent models (Calleja-Conde et al., 2020; Fernández-Calle et al., 2018; Galán-Llario, Rodríguez-Zapata, Fontán-Baselga, et al., 2023).

Conclusion

Our findings indicate a differential gene expression profile of the PTN-MDK-PTPRZ axis in the hippocampus of rats receiving a thiamine deficient diet and pyrithiamine for 12 days but not in the rest of the WE models analyzed (CA and CA+TDD). The data suggest that deficits of the PTN/PTPRZ neurotrophic pathway may precede the more severe and advanced stages of WE. Further studies elucidating the roles of PTN and its receptor in WE may open new therapeutic avenues to prevent the development and progression of WKS.

Acknowledgments

This work was supported by National Plan on Drug abuse, Ministerio de Sanidad of Spain (grants 2023I018 to G.H. and 2024I044 to. E.G.), by Ministerio de Ciencia e Innovación (Spain), cofounded by FEDER (European Union) (grant number PID2021-127256OB-I00 to L.O.) and by ISCIII Redes de Investigación Cooperativa Orientadas a Resultados en Salud (RICORS), Red de Investigación en Atención Primaria de Adicciones (RIAPAd; grant RD24/0003/0011 to G.H. and RD24/0003/0010 to L.O.).

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary Information

Supplementary Table 1.

List of primers used for gene expression analysis by quantitative real-time PCR.

Gene	Primer Forward/Reverse	T_a (ºC)
Ptn	5′-TTGGGGAGAATGTGACCTCAATAC-3′ 5′-TCTCCTGTTTCTTGCCTTCCTTT-3′	60
Ptprz	5′-ACCACCAACACCCATCTTTCC-3′ 5′-CAGCTCTGCACTTCCTGGTAAA-3′	60
Mdk	5′-CCCGTGAGCGAGATGCAG-3′ 5′-CAGGTCCACTCCGAACACTC-3′	60
Hmgb1	5′-TACAGAGCGGAGAGAGTGAGG-3′ 5′-GACATTTTGCCTCTCGGCTT-3′	60
Mfn1	5′-CTGGGACGGAATGAGTGACC-3′ 5′-CATGTGAGGGGCCCAATCTT-3′	60
Mfn2	5′-AGAGGCGATTTGAGGAGTGC-3′ 5′-CGCTCTTCCCGCATTTCAAG-3′	60
Tpk1	5′-CCCGCTATGGAGCATGTCTT-3′ 5′-GCTTTTCTCCAAAGATGCCGA-3′	60
Ccl2	5′-AGATCTGTGCTGACCCCAAT-3′ 5′-GGTGCTGAAGTCCTTAGGGT-3′	60
Tlr4	5′-GATCTGAGCTTCAACCCCCTG-3′ 5′-GTACCAAGGTTGAGAGCTGGT-3′	60
Rpl13	5′-GAGGCGAAACAAATCCACGG-3′ 5′-GTTAGCTGCGTGGCCAATTT-3′	60
β2m	5′-GAGCCCAAAACCGTCACCT-3′ 5′-GAAGATGGTGTGCTCATTGC-3′	60

Note. Ptn, Pleiotrophin***; Ptprz,*** Protein tyrosine phosphatase receptor type Z1***; Mdk,*** Midkine***; Hmgb1,*** High mobility group box 1***; Mfn1,*** Mitofusin 1***; Mfn2,*** Mitofusin 2***; Tpk1,*** Thiamin pyrophosphokinase 1***; Ccl2***, C-C motif chemokine ligand 2; ***Tlr4,*** Toll-like receptor 4; ***Rpl13,*** Ribosomal protein L13; and ***β2m***, Beta 2 macroglobulin. Ta, annealing temperature.
Primers were designed by using the online tool Primer-BLAST, NIH (https://www.ncbi.nlm.nih.gov/tools/primer-blast).

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2025 ■ VOL. 37 ■ N. 4 ■ PAGES 395–408
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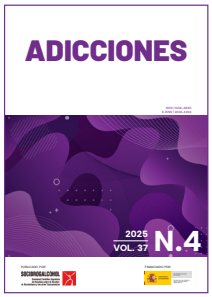

ORIGINAL

Loss of eating control and cognitive flexibility: Involvement of gut microbiota

Pérdida del control de la ingesta alimentaria y flexibilidad cognitiva: Participación de la microbiota intestinal

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Abstract

Loss of eating control is a crucial factor in developing obesity, which has become a global health concern, causing important cardiovascular, metabolic, emotional, and cognitive co-morbidities. A major cognitive alteration associated with loss of eating control and obesity is the impairment of cognitive flexibility and inhibitory control. An increasing number of studies confirm that gut microbiota is a significant contributor to loss of eating control, obesity, and cognitive function. Therefore, we have investigated whether gut microbiota transfer from humans with impaired/not impaired cognitive flexibility could substantially affect this behavioral response in mice in the context of obesogenic versus standard diet. Mice were pretreated with an antibiotic cocktail and later received a gut microbiota transplant from human subjects. The transferred microbiota was maintained in mice for seven weeks. Afterward, behavioral tests were performed to evaluate different cognitive responses, locomotor activity, anxiety-like, and depression-like behaviors. Antibiotic treatment significantly impaired short-term memory in mice, as previously reported. Furthermore, mice that received microbiota from high and low cognitive flexibility subjects modified their short-term and long-term memory performance depending on the diet exposure. Slight changes were observed in the locomotor activity, primarily in the high-fat diet-fed antibiotic-treated mice, and no significant alterations were observed in anxiety-like or depressive-like behaviors. In summary, this study shows that gut microbiota is a major contributor to cognitive flexibility, which may open novel therapeutic strategies for combating loss of eating control and related metabolic co-morbidities.

Keywords: Loss of eating control, obesity, food addiction, gut microbiota, cognitive flexibility, fecal microbiota transplant

Resumen

La pérdida del control de la ingesta alimentaria es un factor crucial en el desarrollo de la obesidad. Una alteración cognitiva importante asociada con la pérdida del control de la ingesta alimentaria y la obesidad es el deterioro de la flexibilidad cognitiva y el control inhibitorio. Un número cada vez mayor de estudios confirman que la microbiota intestinal contribuye significativamente a la pérdida del control de la ingesta alimentaria, la obesidad y la función cognitiva. En base a estos datos, hemos investigado si la transferencia de microbiota intestinal de humanos con obesidad/normopeso y flexibilidad cognitiva deteriorada/no deteriorada podría afectar sustancialmente esta respuesta conductual en ratones expuestos a una dieta obesogénica frente a una dieta estándar. Los ratones fueron tratados previamente con un cóctel de antibióticos y luego recibieron un trasplante de microbiota intestinal de humanos. Posteriormente, se realizaron diferentes test de comportamiento para evaluar la flexibilidad cognitiva como una medida del control inhibitorio. El tratamiento con antibióticos deterioró significativamente la memoria a corto plazo en ratones, como se ha informado previamente. Es importante destacar que los ratones que recibieron microbiota de sujetos con alta y baja flexibilidad cognitiva modificaron su rendimiento de memoria a corto y largo plazo en función de la exposición a la dieta. En resumen, este estudio muestra que la microbiota intestinal es un factor que contribuye de manera importante a la flexibilidad cognitiva, lo que puede abrir nuevas estrategias terapéuticas para combatir la pérdida de control de la ingesta alimentaria y las comorbilidades metabólicas asociadas.

Palabras clave: Adicción a la comida, obesidad, microbiota intestinal, deterioro cognitivo, trasplante de microbiota fecal

■ *Received: March 2025; Accepted: July 2025.*

■ ISSN: 0214-4840 / E-ISSN: 2604-6334

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Loss of eating control is a behavioral alteration closely related to the development of overweight and obesity, metabolic disorders that have reached epidemic levels, with over 890 million people being diagnosed with obesity (WHO. Obesity and Overweight, 2022). Overweight and obesity are serious health concerns associated with an increased risk of cardiovascular disease, type 2 diabetes, and cognitive alterations (Arnoriaga-Rodríguez et al., 2020a; Kloock et al., 2023). Current obesity-related treatments have limited efficacy, and 35 to 50% of obese individuals relapse during the first year, mainly due to a loss of eating control that several authors have even defined as a food addiction behavior with close similarities to the behavioral alterations that define substance use disorders (Hussain & Bloom, 2013).

Food addiction is a compulsive eating disorder arising from a disbalance between homeostatic and hedonic food intake control systems. Individuals with food addiction demonstrate a lack of cognitive flexibility and inhibitory control, leading to high compulsivity, motivation, and impulsivity toward highly palatable food (Domingo-Rodriguez et al., 2020). Despite not being included in the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5), food addiction can be diagnosed by the Yale Food Addiction Scale-2 (YFAS-2). YFAS-2 is a 35-item questionnaire based on the DSM-5 criteria for substance use disorder (Penzenstadler et al., 2019). Interestingly, according to YFAS-2, several studies have demonstrated that not all individuals with obesity have food addiction since around 25-37% of obese and 60% of morbidly obese individuals are diagnosed with food addiction (Gupta et al., 2020), pointing to a rather similar etiology of these eating disorders.

The Western diet is a modern diet high in refined carbohydrates and saturated fats, and is the major factor leading to loss of eating control and obesity (López-Taboada et al., 2020). Indeed, the high palatability of food can induce maladaptive changes in the brain, contributing to the development of these behavioral and metabolic alterations (Moore et al., 2017). Furthermore, cognitive flexibility and inhibitory control play a major role in the regulation of food intake and determining food choices. Several authors have demonstrated that people with obesity have memory alterations (Cheke et al., 2016) and impaired cognitive flexibility (Bocarsly et al., 2015; Song et al., 2022). In addition, high-fat diet consumption has major negative consequences on cognitive performance (Underwood & Thompson, 2016) and cognitive flexibility (Magnusson et al., 2015). On the other hand, memories about past eating experiences determine future eating habits and food choices and guide attention toward food cues, thus shaping our cognitive and inhibitory responses related to food intake control. It is clear that impairments in cognitive flexibility and cognitive performance can exacerbate overeating and contribute to the development of both obesity and food addiction (Higgs, 2016). Indeed, the engagement in secondary sedentary and attention-required activities disrupts memory formation and flexibility, resulting in enhanced food intake (Higgs, 2015). Furthermore, patients with memory impairment show overeating due to the inability to recall recent eating events (Higgs et al., 2008). Thus, a vicious cycle is formed in the case of obesity, where obesity induces cognitive alterations, which in turn further exacerbate the loss of eating control and overeating.

Several studies have highlighted the involvement of gut microbiota in various health conditions, pointing to a new possible tool for disease prevention and treatment. Gut microbiota is involved in gut homeostasis by maintaining the integrity of the gut epithelium and participating in metabolism and immunity. Apart from local effects in the gut, the microbiota is known to play a role in metabolic processes and affect brain functioning through the gut-brain axis (Hou et al., 2022; Megur et al., 2022). Conversely, microbiota alterations due to the consumption of a Western diet or the use of antibiotics often lead to microbial dysbiosis. Gut dysbiosis is associated with an increase in gut permeability and systemic low-grade inflammation, which subsequently contributes to increased blood-brain-barrier permeability and eventual neuroinflammation (Hrncir, 2022; Kearns, 2024). This neuroinflammation can further alter feeding behavior and impair cognitive processes and has been related to various psychiatric disorders (Alboni et al., 2017; DiSabato et al., 2016). Obese individuals display gut dysbiosis represented by a lower gut microbiome diversity and abundance, with a significant decrease in beneficial bacteria that may be related to their impairment in eating control, cognitive performance, and flexibility (Chen et al., 2021). Several of these previous studies have demonstrated that the cognitive alterations revealed in obese individuals can be transferred to mice by the use of fecal microbiota transplantation (FMT) (Arnoriaga-Rodríguez et al., 2020a; Arnoriaga-Rodríguez et al., 2021), further supporting the relationship between gut microbiota and behavioral responses. Indeed, FMT from obese individuals led to a decrease in short-term and working memory (Arnoriaga-Rodríguez et al., 2020a), and cognitive flexibility and inhibitory control impairment in mice (Arnoriaga-Rodríguez et al., 2021), indicating that microbiota modulations can potentially modify cognitive functions. FMT was also sufficient to transfer the depression phenotype from obese humans to mice (Mayneris-Perxachs et al., 2022a). Similarly, a recent study has demonstrated that a specific gut microbiota composition is necessary for the development of food addiction. Thus, food-addicted humans and mice demonstrated a decrease in *Blautia* sp., and the addictive phenotype was prevented by an oral administration of *Blautia wexlerae* in mice (Samulėnaitė et al., 2024). Based on these previous findings, we have now investigated the involvement of gut microbiota in

cognitive flexibility and inhibitory control by evaluating the consequences of FMT from humans with these behavioral disorders in mice exposed to obesogenic or standard diet.

Methods

Human participants

Twenty healthy human adult donors, women and men, were selected based on their cognitive flexibility scores, assessed by the Stroop Color-Word Test (SCWT) as described before (Castells-Nobau et al., 2024). The body mass index (BMI) and socioeducational status among the groups were investigated. SCWT is used to investigate inhibitory control and cognitive flexibility in humans. Cognitive flexibility/inhibitory control was evaluated in three subsequent steps. During the first part, a patient is introduced to 100 cards with printed words (color names) in black ink. During the second part, 100 cards of words are displayed in different colors of ink (green, red, blue). Ultimately, during the test, a patient is given 100 cards of color names, which are printed in a non-matching ink. Each part takes 45 seconds, after which a patient is evaluated. Cognitive flexibility/inhibitory control is evaluated using a formula: $WC - WC'$ ($WC' = W \times C/W + C$). W (word) accounts for part one of the experiment, C (color) for part two, and WC (word-color) for the ultimate test. Those patients who obtained a final score lower than four were considered to have impaired cognitive flexibility/inhibitory control. In contrast, those who had more than six were considered to have optimal inhibitory control/cognitive flexibility.

Donor feces were prepared for oral administration to mice in sterile conditions with L-cysteine to reduce oxygen in an environment to protect anaerobic bacteria and 10% glycerol to protect bacteria from freezing-related damage. Samples were aliquoted and frozen at -80°C.

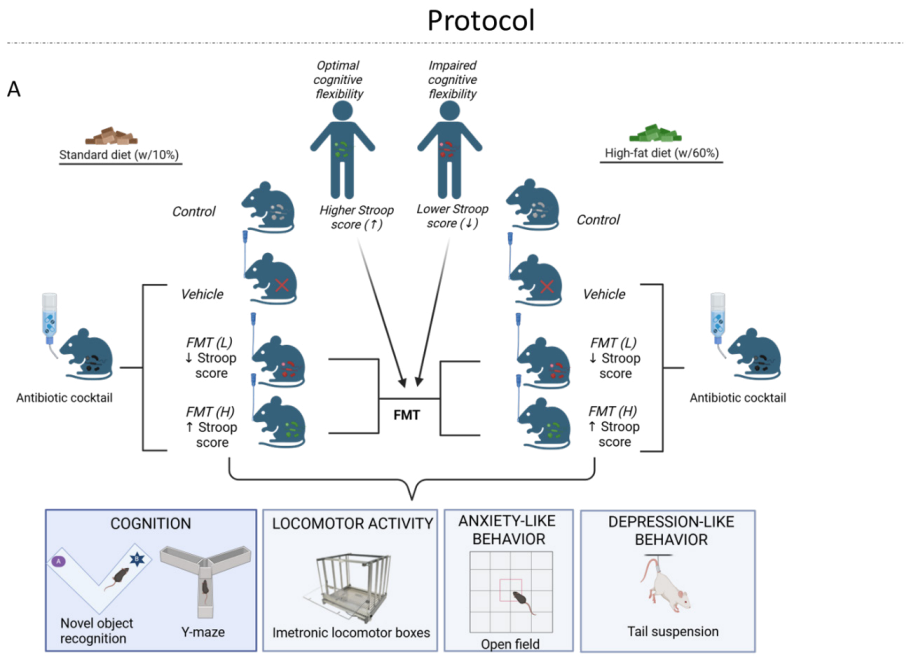
Animals and Experimental Design

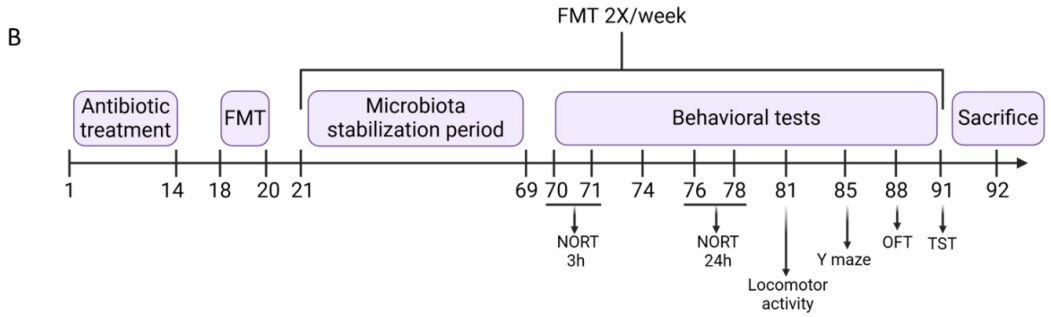
Seventy-seven male C57BL/6J mice (4 weeks of age, 17-21 g weight) were housed individually in controlled laboratory conditions with a temperature of 21 ± 1 ºC and humidity of 55% ± 10%, in an inverted cycle (lights off 7:30 a.m.-7:30 p.m.). Conventional mice, with no initial alterations in their gut microbiota composition, were used. Food and water were available *ad libitum*, and animals` body weight, food, and water intake were monitored during the experimental protocol. All behavioral experiments were approved by the local ethical committee (Comitè Ètic d'Experimentació Animal – Parc de Recerca Biomèdica de Barcelona) and were performed in accordance with the European Communities Council Directive (2010/63/EU).

The information regarding the experimental group assignment and the timeline is represented in Figure 1A and Figure 1B, respectively. Mice were randomly divided into eight groups, four of which were fed on a standard diet (w/10%) (~3.514 kcal/kg) and the other four on a high-fat diet (w/60%) (~5.228 kcal/kg). Irradiated, vacuum-packed diets were bought from Altromin (Germany). Both groups were further divided into 1) control, 2) vehicle, 3) FMT from individuals with low Stroop scores (FMT (L), 4) FMT from individuals with high Stroop scores (FMT (H) (Figure 1A). Each mouse received microbiota from a specific human donor, allowing for individual evaluation of the microbiota's effect on brain function, in the context of high-fat and standard diets.

Figure 1

Experimental protocol. A. Experimental protocol and group assignment. B. Experimental timeline (days)





Note. **A.** Fecal material from humans with impaired (low Stroop scores; FMT (L) or optimal cognitive flexibility (high Stroop scores; FMT (H) was processed for oral administration to mice. All mice, except for the control group, received antibiotic treatment, followed by an oral administration of vehicle or fecal microbiota transplantation (FMT) twice per week for the whole experimental protocol. Mice were fed on a standard or high-fat diet. At the end of the protocol, behavioral tests were performed: novel object recognition test (NORT) for short- and long-term memory, Y-maze for working memory, open field test (OFT), and elevated plus maze test (EPM) for anxiety-like behavior, and tail suspension test (TST) for depression-like behavior. Furthermore, locomotor activity was evaluated. **B.** Mice received antibiotic treatment for two weeks; after a 72-hour window, oral administration of vehicle or fecal microbiota transplantation was performed for three consecutive days and then twice per week for the whole experimental protocol. After seven weeks of stabilization, behavioral tests were performed to assess short-term (days 70-71) and long-term memory (days 76-78) by novel object recognition test, anxiety-like behavior by the elevated plus maze test (day 74), and open field test (day 88), and depression-like behavior (day 91) by tail suspension test.

All groups, except for both controls, received an antibiotic cocktail (500 mg/L ampicillin, 500 mg/L metronidazole, 500 mg/L vancomycin, 250 mg/L imipenem, 1g/l neomycin) daily for two weeks in drinking water, as previously described (Kelly et al., 2016). Seventy-two hours later, mice were orally gavaged with saline (1x PBS+1g/l L-cysteine+10% glycerol) (vehicle group) or fecal material from the donors (FMT groups) via oral gavage (200 mg/ml, 200 µL) for three consecutive days as previously described (Arnoriaga-Rodríguez et al., 2020a), later twice per week throughout the experimental protocol.

After seven weeks, mice were exposed to a series of behavioral tests, including a novel object recognition test (NORT 3h and NORT 24h, on days 70-71 and 76-78, respectively), locomotor activity (on day 81), Y maze (on day 85), open field test (OFT, on day 88) and tail suspension test (TST, on day 91). On the last day of the study (day 92), mice were sacrificed, and several brain areas and blood plasma were collected for further analysis (Figure 1B).

Cognition

Novel object recognition

A novel object recognition test (NORT) was performed to assess long-term and short-term memory as previously described (Burokas et al., 2014). Shortly, it is performed in a V-shaped maze (30 cm long × 4.5 cm wide × 15 cm height of each corridor), illuminated with 2.5 lux on both corners. On the first day, mice were habituated to a maze for 9 minutes. The following day, mice were introduced to a maze with two identical objects on opposite sides of the maze and left to explore for 9 minutes. After 3 hours or 24 hours, the test was performed to assess short-term or long-term memory, respectively. During the test, one of the familiar objects was replaced with a novel one, and mice were left to explore both figures for 9 minutes, and the total time spent exploring the two objects was measured. Mice were verified to ensure that there was no initial preference for the objects used. A discrimination index (DI) was calculated as the difference between the time the animal spent exploring either the novel (Tn) or familiar (Tf) object, divided by the total time exploring both objects: (DI = (Tn − Tf)/(Tn + Tf)). A higher discrimination index reflects high memory retention for the familiar object. Mice that explored for less than 10 seconds were excluded from the analysis. The analysis of the behavioral data was performed manually by the use of calibrated stopwatches.

Y maze

The Y maze test is based on the innate curiosity of rodents to explore previously unvisited areas. This test was performed to assess working memory as previously described (Vijaya et al., 2024). The maze is comprised of three identical arms intersecting at 120° (Y-shaped; 6.5 cm width × 30 cm length × 15 cm height), all three entries were illuminated with 10 lux. Entries into the arms of the maze were counted for 10 minutes (traversing the head and two front paws is considered a valid entry), and the percentage of spontaneous alternation was calculated by the sequential entries in all three arms divided by the total number of possible alternations. The analysis of the behavioral data was performed manually.

Locomotor activity

Locomotor activity was evaluated in the individual locomotor activity boxes (10.8 cm width × 20.3 cm length × 18.6 cm height, Imetronic, Pessac, France). The total activity (number of beam breaks) and the total number of rearings were detected during 1 hour by infrared sensors.

Anxiety-like responses

The open-field test (OFT) was used to assess anxiety-like behavior. This test is based on the conflict of innate fear and avoidance of bright, open areas (that mimic a

situation of predator risk) and an instinct to explore novel environments. Mice were placed in the middle of the box (90 cm x 70 cm, 500 lux), and their movements were recorded over the course of 5 minutes. The preference for being in the periphery of the box indicates higher anxiety-like behavior. The analysis of the behavioral data was performed with Smart 3.0 video tracking software.

Depressive-like responses

The tail suspension test (TST) evaluates depressive-like behavior. During the test, mice were suspended by their tails with tape in a position that prevented them from escaping or holding onto nearby surfaces for 6 minutes. The time of immobility (defined as the time during which the animal is hanging passively and motionless) was measured, and the higher the immobility time, the higher the depressive-like behavior. The analysis of the behavioral data was performed manually by the use of calibrated stopwatches.

Statistical analysis

All data are expressed as mean ± standard error of the mean (S.E.M.). Figures were prepared using GraphPad Prism software (GraphPad Software), illustrations were done with Biorender, and statistical analysis was performed using IBM SPSS 28.0.

The normal distribution of the data was evaluated for all the datasets using the Shapiro-Wilk test to select appropriate statistical tests. Two-way ANOVA or Kruskal-Wallis comparisons were used when appropriate, followed by a Fisher`s post hoc analysis or Mann-Whitney U test, respectively, when necessary. The effect size for the two-way ANOVA was calculated as Eta squared (η^2), using SPSS, while the effect size for Kruskal-Wallis comparisons (η^2) was calculated manually. The values above 0.14 indicate a large effect. The effect size for the Mann-Whitney U test was calculated manually, as the rank biserial correlation (r), with r values above 0.5 indicating a strong effect. In Fisher`s post hoc test, the effect size was evaluated by Cohen`s d, which was calculated manually, and a large effect was considered when d values were above 0.8.

Results

Twenty healthy human donors, men and women with an average age of 48 ± 9, were selected. All individuals were obese, with an average BMI of 32.4 ± 9.8, of similar socioeducational status, with an average of 13 ± 4 years of education. The Stroop scores, evaluated by the SCWT, were lower than 4 in donors with impaired cognitive flexibility and higher than 6 in optimal cognitive flexibility donors. No significant differences between the age, sex, BMI, and socioeducational status were observed between the groups with different cognitive flexibility scores (Table 1).

Table 1
The information on human donors

Characteristic	Overall N = 20[1]	high STROOPI N = 10[1]	low STROOPI N = 10[1]	p-value[2]
age	48 ± 9	47 ± 7	49 ± 11	0.57
sex				0.63
1	6 (30%)	4 (40%)	2 (20%)	
2	14 (70%)	6 (60%)	8 (80%)	
BMI	32.4 ± 9.8	32.5 ± 10.8	32.3 ± 9.4	0.97
education_years	13 ± 4	13 ± 4	13 ± 3	0.81

[1] Mean ± SD; n (%)

[2] One-way analysis of means; Fisher's exact test

Note. The table demonstrates the age, sex, BMI, and years of education in the overall population and between donors with high or low Stroop scores.

A total of 77 male C57BL/6J mice, 4 weeks old, were used in the study. Mice were divided into 8 groups, with 9-10 mice per group (Figure 1A). Half of the mice were fed standard, and the other half were exposed to a high-fat diet. All mice, except for the control group, received an antibiotic cocktail for microbiota depletion and were orally administered a vehicle, as an additional control, or the gut microbiota from a human donor. Each mouse received microbiota from a specific human donor, allowing for individual evaluation of the microbiota's effect on brain function.

The impact of the gut microbiota on metabolic parameters

We monitored the body weight, food, caloric, and water intake in mice during the experimental protocol to examine the impact of diet and microbiota changes on these parameters. Although diet did not primarily affect mice`s body weight (Kruskal-Wallis H Test; H (7) = 37.481, η^2 = 0.442, $p < 0.001$; post-hoc U = 585, n.s.; Figure 2A), microbiota modulations had a significant effect. First, standard diet-fed mice that received microbiota from impaired cognitive flexibility donors showed a significant decrease in body weight (Kruskal-Wallis H Test; H (7) = 37.481, η^2 = 0.442, $p < 0.001$; post-hoc U = 14, r = 0.58, $p < 0.05$; Figure 2A, left), compared to the control group, with no significant changes in other standard diet-fed groups. However, high-fat diet-fed mice that received an antibiotic cocktail, and oral administration of vehicle had significantly decreased body weight compared both to the control group (Kruskal-Wallis H Test; H (7) = 37.481, η^2 = 0.442, $p < 0.001$; post-hoc U = 0, r = 0.84, $p<0.001$; Figure 2A, right) and experimental groups that received FMT from low (Kruskal-Wallis H Test; H (7) = 37.481, η^2 = 0.442, $p < 0.001$; post-hoc U = 11, r = 0.61, $p<0.001$; Figure 2A, right) or high (Kruskal-Wallis H Test; H (7) = 37.481, η^2 = 0.442, $p < 0.001$; post-hoc U = 12, r = 0.62, $p<0.001$; Figure 2A, right) cognitive flexibility donors. In

addition, the experimental high-fat diet-fed FMT groups also demonstrated a decrease in body weight, compared to the control group (Kruskal-Wallis H Test; H (7) = 37.481, η^2 = 0.442, p < 0.001; FMT (L) post-hoc U = 2, r = 0.81, p < 0.001, FMT (H) post-hoc U = 0, r = 0.84, p < 0.001; Figure 2A, right).

Despite changes in body weight, food intake was not affected by diet or microbiota modulations (Kruskal-Wallis H Test; H (7) = 13.230, n.s.; Figure 2B). Due to a higher caloric content of the high-fat diet, mice consumed significantly more calories compared to the standard diet group (Kruskal-Wallis H Test; H (7) = 58.909, η^2 = 0.752, p < 0.001; post-hoc U = 13, r = 0.84, p < 0.001; Figure 2C, right). Furthermore, several changes between the groups of different microbiota compositions were observed. First, standard-diet fed mice that received oral administration of vehicle (Kruskal-Wallis H Test; H (7) = 58.909, η^2 = 0.752, p < 0.001; post-hoc U = 19, r = 0.52, p < 0.05; Figure 2C, left), or microbiota transplantation from impaired (Kruskal-Wallis H; Test H (7) = 58.909, η^2 = 0.752, p < 0.001; post-hoc U = 13, r = 0.84, p <0.01; Figure 2C, left), or optimal cognitive flexibility donors (Kruskal-Wallis H Test; H (7) = 58.909, η^2 = 0.752, p < 0.001; post-hoc U = 22, r = 0.47, p < 0.05; Figure 2C, left) had significantly decreased calorie intake, compared to the control group. Less pronounced effects were observed in the high-fat diet-fed mice, since only the mice that received FMT from donors with high Stroop score values significantly decreased the calorie intake, compared to the control (Kruskal-Wallis H Test; H (7) = 58.909, η^2 = 0.752, p < 0.001; post-hoc U = 21, r = 0.49, p < 0.05; Figure 2C, right),suggesting a potential protective role in the development of obesity.

Interestingly, high-fat diet-fed mice significantly decreased water intake compared to the standard diet group (Kruskal-Wallis H; H (7) = 35.586, η^2 = 0.414, p < 0.001; post-hoc U = 188, r = 0.64, p < 0.001; Figure 2D). Although global analysis has indicated microbiota-related alterations in water intake, it was not confirmed by the subsequent post hoc comparisons neither in the standard diet-fed mice (Kruskal-Wallis H; H (7) = 35.586, η^2 = 0.4143, p < 0.001; control x vehicle, U = 48, n.s., control x FMT (L), U = 28, n.s., control x FMT (H), U = 32, n.s., vehicle x FMT (L) U = 26, n.s.; vehicle x FMT (H) U = 33.5, n.s., Figure 2D, left) nor in the high-fat diet-fed mice (Kruskal-Wallis H; H (7) = 35.586, η^2 = 0.4143, p<0.001; control x vehicle, U = 31, n.s., control x FMT (L), U = 33, n.s., control x FMT (H), U = 47, n.s., vehicle x FMT (L) U = 40, n.s.; vehicle x FMT (H) U = 30, n.s., Figure 2D, right).

Diet also had an impact on locomotor activity, as observed by a significantly reduced activity (two-way ANOVA; diet, F (1, 69) = 26.712, η^2 = 0.279, p < 0.001; Figure 2E, right) and the number of rearings (Kruskal-Wallis H Test; H (7) = 35.963, η^2 = 0.419, p<0.001; post-hoc U = 217.5, r = 0.61, p < 0.001; Figure 2F, right) in high-fat diet-fed mice. These alterations in locomotor activity were modulated by microbiota composition. Thus, mice of the vehicle group that had depleted gut microbiota with antibiotics showed a significant increase in total activity (two-way ANOVA; diet, F (1, 69) = 26.712, η^2 = 0.279, p < 0.001; post-hoc I-J = -978.933, d = 7.49, p < 0.001; Figure 2E, right) and number of rearings (Kruskal-Wallis H Test H (7) = 35.963, η^2 = 0.419, p < 0.001; post-hoc U = 17, r = 0.51, p < 0.05; Figure 2F, right), compared to the high-fat diet control mice. The high-fat diet group that received FMT from impaired cognitive flexibility donors demonstrated a decreased total locomotor activity compared to the vehicle (two-way ANOVA; groups, F (3, 69) = 3.393, η^2 = 0.129, p<0.05; post-hoc I-J = -673.389, d = 1.66, p<0.05; Figure 2E, right), but not the control group (two-way ANOVA; groups, F (3, 69) = 3.393, η^2 = 0.129, p<0.05; post-hoc I-J = -303.544, n.s.; Figure 2E, right). Ultimately, standard diet-fed mice that received a microbiota transplant from optimal cognitive flexibility donors demonstrated a decrease in the number of rearings compared to the vehicle (Kruskal-Wallis H Test; H (7) = 35.963, η^2 = 0.419, p<0.001; post-hoc U = 23, r = 0.46, p < 0.05; Figure 2F, left), but not the control group (Kruskal-Wallis H Test; H (7) = 35.963, η^2 = 0.419, p < 0.001; post-hoc U = 29, n.s.; Figure 2F, left).

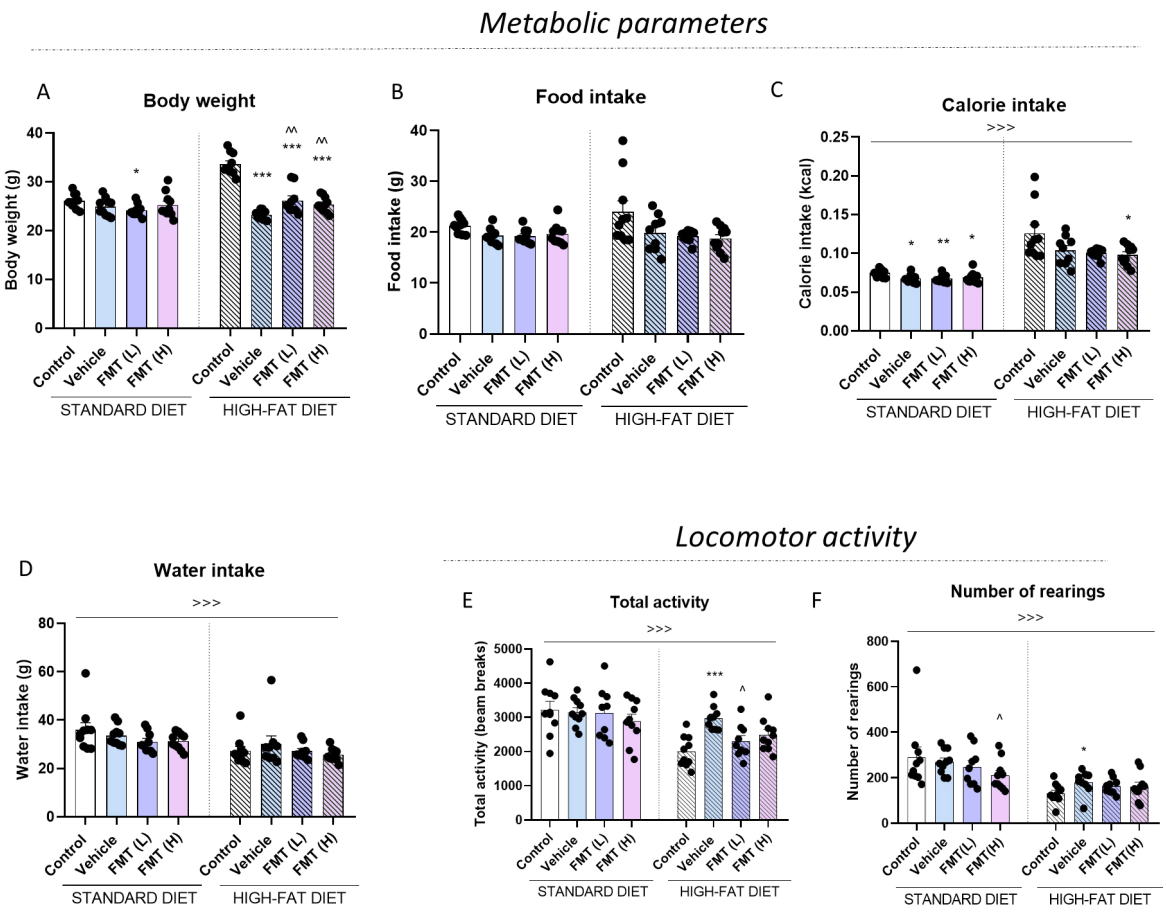
Fecal microbiota transplantation alters memory performance

Short-term, long-term, and working memory were assessed to better understand the relationships between gut microbiota, cognitive flexibility, and cognitive decline. First, no effect of diet was observed on short-term (Kruskal-Wallis H; H (7) = 16.916, η^2 = 0.144; post-hoc U = 683, n.s.; Figure 3A), long-term (two-way ANOVA; diet F (1, 61) = 0.139, n.s.; microbiota F (3, 61) = 6.957, η^2 = 0.255, p < 0.001; interaction F (3, 61) = 0.319, n.s.; Figure 3B) and working memory (two-way ANOVA; diet F (1, 69) = 1.644, n.s.; microbiota F (3, 69) = 1.509, n.s.; interaction F (3, 69) = 0.183, n.s.; Figure 3C).

The standard diet-fed vehicle group with antibiotic-depleted gut microbiota demonstrated a severely impaired short-term memory with a strong effect size (Kruskal-Wallis H Test; H (7) = 16.916, η^2 = 0.144, p < 0.05; post-hoc U = 6, r = 0.74, p < 0.001; Figure 3A). However, the same effect was not observed in the high-fat diet-fed mice (Kruskal-Wallis H Test; H (7) = 16.916, η^2 = 0.144, p < 0.05; post-hoc U = 23, n.s.; Figure 3A). This antibiotic-induced memory impairment was reversed by the microbiota transplantation from the impaired cognitive flexibility donors (Kruskal-Wallis H; H (7) = 16.916, η^2 = 0.144, p < 0.05; post-hoc U = 14, r = 0.58, p < 0.05). Meanwhile, those who received microbiota from donors with optimal cognitive flexibility showed a trend toward improved short-term memory (Kruskal-Wallis H; H (7) = 16.916, η^2 = 0.144, p < 0.05; post-hoc U = 22, p=0.065, n.s., Figure 3A, left). No significant

Figure 2

Metabolic parameters and locomotor activity of mice. **A.** *Average body weight (g).* **B.** *Average food intake (g).* **C.** *Average calorie intake (kcal).* **D.** *Water intake (g).* **E.** *Locomotor activity – total locomotor activity (beam breaks).* **F.** *Locomotor activity – number of rearings*



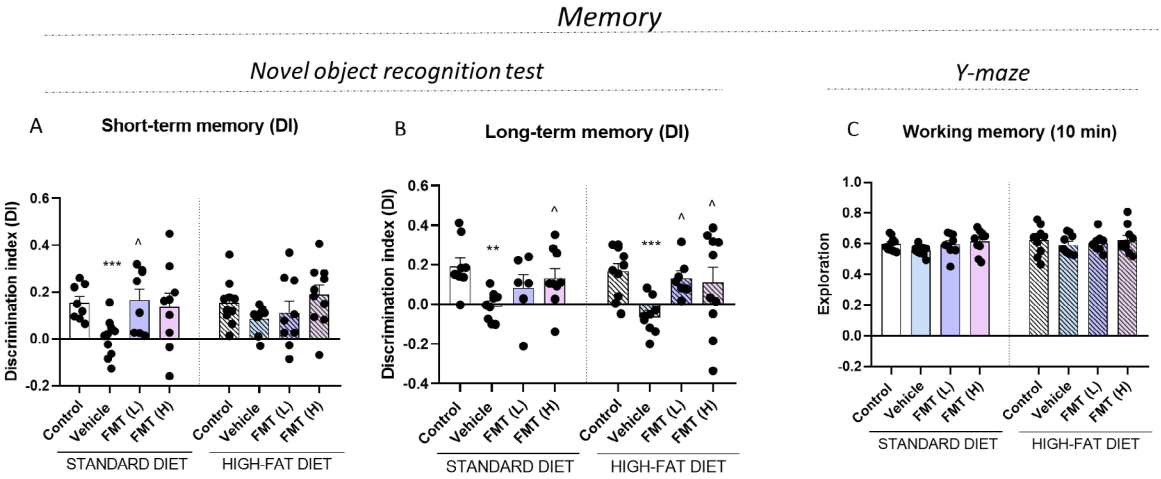
Note. A. Data were analysed using Kruskal-Wallis H Test; H (7) = 37.481, η^2 = 0.442, p<0.001; *sig. STD control x STD FMT (L), post-hoc U = 14, r = 0.58, p < 0.05; ***sig. HFD control x HFD vehicle, post-hoc U = 0, r = 0.84, p < 0.001; ***sig. HFD control x HFD FMT (L), post-hoc U = 2, r = 0.81, p < 0.001; ***sig. HFD control x HFD FMT (H), post-hoc U = 0, r = 0.84, p < 0.001; ^^sig. HFD vehicle x HFD FMT (L), post-hoc U = 11, r = 0.61, p < 0.01; ^^sig. HFD vehicle x HFD FMT (H), post-hoc U = 12, r = 0.62, p < 0.01. C. Data were analysed using Kruskal-Wallis H Test; H (7) = 58.909, η^2 = 0.752, p < 0.001; >>> sig. diet, post-hoc U = 13; r = 0.84, p < 0.001; *sig. STD Control x STD vehicle, post-hoc U = 19, r = 0.52, p < 0.05; **sig. STD Control x STD FMT (L), post-hoc U = 13, r = 0.59, p < 0.01; *sig. STD Control x STD FMT (H), post-hoc U test U = 22, r = 0.47, p < 0.05; *sig. HFD Control x HFD FMT (H), post-hoc U = 21, r = 0.49, p < 0.05). D. Data were analysed using Kruskal-Wallis H Test; H (7) = 35.586, η^2 = 0.414, p < 0.001; >>> sig. diet, post-hoc U = 188, r = 0.64, p < 0.001. E. Data were analysed using two-way ANOVA groups F (3, 69) = 3.393, η^2 = 0.129, p < 0.05; ***sig. HFD control x HFD vehicle, post-hoc I-J = -978.933, d = 7.49, p < 0.001; ^sig. HFD vehicle x HFD FMT (L); post-hoc I-J = -673.389, d = 1.66, p < 0.05); >>> sig. diet, two-way ANOVA diet; F (1, 69) = 26.712, η^2 = 0.279, p < 0.001. F. Data were analysed Kruskal-Wallis H Test; H (7) = 35.963, η^2 = 0.419, p < 0.001; ^sig. STD vehicle x STD FMT (H), post-hoc U = 23, r = 0.46, p < 0.05; *sig. HFD control x HFD vehicle, post-hoc U = 17, r = 0.51, p < 0.05, >>> sig. diet, post-hoc U = 217.5, r = 0.61, p < 0.001. Abbreviations: STD – standard diet; HFD – high-fat diet; FMT – fecal microbiota transplantation; FMT (L) – FMT from low cognitive flexibility donors; FMT (H) – FMT from high cognitive flexibility donors.

differences were observed between the groups fed on a high-fat diet (Kruskal-Wallis H; H (7) = 16.916, η^2 = 0.144, p < 0.05; post-hoc control x vehicle U = 23; control x FMT (L) U = 35; control x FMT (H) U = 38; vehicle x FMT (L) U = 39, n.s.; vehicle x FMT (H) U = 23, n.s, Figure 3A, right).

The effects in long-term memory were more pronounced than in short-term memory. The vehicle group demonstrated a significant impairment in long-term memory independent of diet (STD control x STD vehicle two-way ANOVA; groups F (3, 61) = 6.957, η^2 = 0.255, p < 0.001; post-hoc I-J = -0.199, d = 5.82, p < 0.01); HFD control x HFD vehicle post-hoc I-J = -0.226, d = 6.37, p = 0.001; Figure 3B), with a large effect size. This cognitive impairment was alleviated by FMT from humans with high Stroop scores to standard diet-fed mice (two-way ANOVA; groups F (3, 61) = 6.957, η^2 = 0.255, p < 0.001; post-hoc I-J = -0.139, d = 3.63, p < 0.05; Figure 3B, left) and high-fat diet-fed mice (two-way ANOVA; groups F (3, 61) = 6.957, η^2 = 0.255, p < 0.001; post-hoc I-J = -0.169, d = 2.78, p < 0.05; Figure 3B, right). Furthermore, microbiota transplantation from impaired cognitive flexibility donors also improved long-term memory, compared to the vehicle group, but only in high-fat diet-fed mice (two-way ANOVA; groups F (3, 61) = 6.957, η^2 = 0.255, p<0.001; post-hoc I-J = -0.192, d = 5.97, p < 0.05; Figure 3B, right). These changes in short- and long-term memory reveal the involvement of gut microbiota in cognitive performance.

No significant differences between groups were observed on working memory in the context of different diets and microbiota compositions (two-way ANOVA diet F (1, 69) = 1.644, n.s.; microbiota F (3, 69) = 1.509, n.s.; interaction F (3, 69) = 0.183, n.s., Figure 3C).

Figure 3
Effect of fecal microbiota transplantation on short-, long-, and working memory in mice. **A.** *Discrimination index (DI) during a novel object recognition test for short-term memory (3h).* **B.** *Discrimination index (DI) during a novel object recognition test for long-term memory (24h). C. Exploration of the Y-maze (10 min) for working memory*

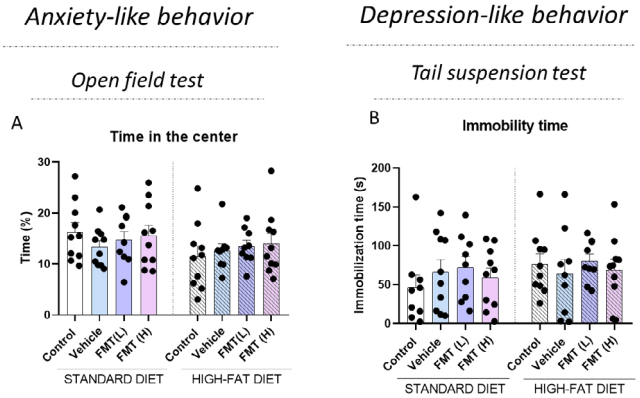


Note. A. Data were analysed using Kruskal-Wallis H Test; H (7) = 16.916, η^2 = 0.144, p < 0.05; ***sig. STD control x STD vehicle, post-hoc U = 6, r = 0.74, p < 0.001; ^sig. STD vehicle x STD FMT (L), post-hoc U = 14, r = 0.58, p < 0.05. B. Data were analysed using two-way ANOVA groups F (3, 61) = 6.957, η^2 = 0.255, p < 0.001; **sig. STD control x STD vehicle, post-hoc I-J = -0.199, d = 5.82, p < 0.01; ^sig. STD vehicle x STD FMT (H), post-hoc I-J = -0.139, d = 3.63, p < 0.05; ***sig. HFD control x HFD vehicle, post-hoc I-J = -0.226, d = 6.37, p = 0.001; ^sig. HFD vehicle x FMT (L), post-hoc I-J = -0.192, d = 5.97, p < 0.05; ^sig. HFD vehicle x FMT (H), post-hoc I-J = -0.169, d = 2.78, p < 0.05.
Abbreviations: STD – standard diet; HFD – high-fat diet; FMT – fecal microbiota transplantation; FMT (L) – FMT from low cognitive flexibility donors; FMT (H) – FMT from high cognitive flexibility donors.

Fecal microbiota transplantation did not affect anxiety and depression

High-fat diet did not have any effect on anxiety-like (Two-way ANOVA; diet F (1, 69) = 3.020, n.s.; microbiota F (3, 69) = 0.403, n.s.; interaction F (3, 69) = 0.586, n.s.; Figure 4A) and depressive-like behaviors (Kruskal-Wallis H Test; H (7) = 5.237, n.s.; Figure 4B). Furthermore, no significant differences between the groups of different microbiota composition were observed in the open-field test, which represents a model of anxiety-like behavior (Two-way ANOVA; diet F (1, 69) = 3.020, n.s.; microbiota F (3, 69) = 0.403, n.s.; interaction F (3, 69) = 0.586, n.s.; Figure 4A), and the tail suspension test, representing depressive-like behavior (Kruskal-Wallis H Test; H (7) = 5.237, n.s.; Figure 4B).

Figure 4
Effect of fecal microbiota transplantation on anxiety-like and depression-like behavior in mice. **A.** *Time (%) in the center of an open field test.* **B.** *Immobility time (s) during the tail suspension test*



Discussion

In this study, we have revealed the relationship between cognitive flexibility and different parameters of cognitive performance, providing new insights to understand the mechanisms involved in the loss of eating control, which has close behavioral similarities with the behavioral alterations underlying substance use disorders. First, we demonstrated antibiotic-induced cognitive impairments, which were alleviated by FMT. Indeed, FMT from individuals with high cognitive flexibility/inhibitory control scores improved long-term memory independent of diet. However, no significant effect was observed in short-term memory. Remarkably, the microbiota transplantation from impaired cognitive flexibility donors improved short-term memory in the standard diet-fed mice and long-term memory in mice fed on a high-fat diet. Overall, our results indicate that diet and microbiota composition influence brain function. No effect of FMT or diet exposure was observed in anxiety- and depression-like behavior was revealed in our experimental conditions.

FMT has been widely investigated as a promising tool to investigate the role of gut microbiota in various pathophysiological conditions. Indeed, taking into account the involvement of gut microbiota in behavioral responses and metabolic functions, it has been observed that FMT can strongly metabolic alterations in mice, including an improvement in fasting blood glucose levels (Qiu et al., 2023), type 2 diabetes (Chen et al., 2023), insulin sensitivity (Vrieze et al., 2012; Wu et al., 2023), lipid profile (Liang et al., 2022), body weight (Arnoriaga-Rodríguez et al., 2020b; Mayneris-Perxachs et al., 2020)

and several centrally mediated behavioral impairments (Arnoriaga-Rodríguez et al., 2021; Arnoriaga-Rodríguez et al., 2020a). Apart from the peripheral effects, the gut microbiota also has a significant impact on brain-related behavioral responses. Several studies have shown that FMT from healthy donors has improved symptoms of various neurological and psychiatric conditions, including autism spectrum disorder, neuropathic pain, epilepsy, multiple sclerosis, Parkinson's disease, Alzheimer's disease, and depression, among others (Vendrik et al., 2020). Some of these studies have also highlighted the impact of gut microbiota on cognitive performance. Thus, humans with poor memory had low microbial diversity, underlying this bidirectional communication between the gut and the brain in cognitive control (El-Sayed et al., 2021). In mice, antibiotic-induced cognitive impairment was reversed by the use of two different bacteria strains through the production of neurotransmitters and antioxidant activity (Yarullina et al., 2024). Exercise, one of the major microbiota modulators, shows the potential to alleviate microbiota-induced hippocampal deficits. Indeed, antibiotic-treated mice display low-grade inflammation and deficits in pattern separation, which were mitigated by exercise (Nicolas et al., 2024). In another study, antibiotic-induced cognitive impairment was reversed by the use of two different *Lactobacillus* strains through the production of neurotransmitters and antioxidant activity (Yarullina et al., 2024). Similarly, in our study, we demonstrated that antibiotic treatment induced cognitive deficits that were improved by microbiota transplantation, independent of the human donors' cognitive flexibility scores, confirming both the detrimental effect of antibiotics and the crucial effect of microbiota recolonization on cognitive performance.

Interestingly, we previously found that gut microbiota from obese donors led to impairments in working and short-term memory. This cognitive impairment was associated with microbiota-related tryptophan metabolism (Arnoriaga-Rodríguez et al., 2020a). Other studies have demonstrated that methanogen archaea were correlated with better cognitive performance, while FMT from humans containing higher amounts of the archaea *M. smithii* to mice led to better inhibitory control (Fumagalli et al., 2025). Furthermore, viruses also play a crucial role in the gut microbiota function since higher levels of Caudovirales in humans were associated with improved executive functions and verbal memory. Interestingly, the FMT from human donors with high levels of Caudovirales improved short-term and emotional memories in mice (Mayneris-Perxachs, et al., 2022b), whereas FMT from *Blastocystis* carriers impaired cognitive function in mice (Mayneris-Perxachs et al., 2022c). Conversely, another study has shown that an eukaryote parasite found in the gut, namely *Blastocystis*, induced executive function deficits together with further alterations in gut microbiota. Obesity-related cognitive decline was linked to adipose tissue gene expression responsible for the alteration of cognitive function (Oliveras-Cañellas et al., 2023). In our study, we have demonstrated that microbiota transplantation from obese subjects (BMI above 30) with high and low cognitive flexibility differentially improved short- and long-term memory depending on the diet. Specifically, FMT from impaired cognitive flexibility donors improved short-term memory only in standard diet-fed mice and long-term memory in high-fat diet-fed mice. In contrast, FMT from high Stroop score donors significantly improved long-term memory independently of diet. No significant effects were observed on working memory, which strongly reflects cognitive flexibility, indicating that memory performance, rather than cognitive flexibility, might be modulated by microbiota modifications. Contrary to our findings, a previous study demonstrates a coherence in the results when FMT from obese humans with impaired inhibitory control led to similar alterations in the reversal test (Arnoriaga-Rodríguez et al., 2021), suggesting that more extensive behavioral tests to assess cognitive flexibility and inhibitory control could be performed in future experiments.

The Western diet is an unhealthy eating pattern with the consumption of highly palatable foods containing sugars and fats. Due to its high palatability, it is the leading cause of loss of eating control, obesity, and the associated microbial imbalance (Burokas et al., 2018; Espinosa-Carrasco et al., 2018; Mancino et al., 2015; Martín-García et al., 2010; Samulėnaitė et al., 2024). In this study, the exposure to a high-fat diet did not induce an obese phenotype in mice. Conversely, a combination of antibiotic treatment and a high-fat diet led to a significant decrease in body weight. Meanwhile, the FMT groups, independently of microbiota content, demonstrated an intermediate body weight, which was significantly higher than in antibiotic-treated mice, but lower when compared to the high-fat diet control. Such an antibiotic-related effect was previously described (Luo et al., 2023), suggesting an explanation to why the obese phenotype was not obtained, despite high-fat diet intake. The length of the protocol and continuous FMT procedures might also have contributed to the lean phenotype. On the other hand, a high-fat diet significantly decreased water intake, number of rearings, and total locomotor activity, which is in agreement with previous studies (Bjursell et al., 2008; Volcko et al., 2020; Yokoyama et al., 2020). Microbiota composition also altered locomotor activity. Indeed, antibiotic-treated, high-fat diet-fed mice demonstrated a substantial increase in total locomotor activity and rearings, which is in agreement with a lower body weight of these mice. This increase in locomotor activity was alleviated by FMT from low cognitive flexibility donors, but not from high cognitive flexibility donors, although a trend towards lower activity was observed. Despite no significant differences observed

in food intake, high-fat diet-fed mice significantly increased caloric intake, indicating obesity-associated changes in eating behavior. However, it was not strong enough to alter body weight. Palatable food exposure can also induce alterations in the brain reward system, contributing to the loss of eating control (Berding et al., 2021; Martín-García et al., 2011; Requena et al., 2018). Indeed, food palatability is a crucial component for the development of food addiction since mice maintained reliable operant conditioning mainly when the response was reinforced by highly palatable chocolate-flavored pellets (García-Blanco et al., 2022). However, despite the overlap between obesity and food addiction, a distinct microbiota profile between obese women with or without food addiction was revealed (Dong et al., 2020). Furthermore, executive functions are frequently impaired in food addiction, which contributes to further overeating. Interestingly, obese individuals also have poor inhibitory control, which positively correlated with gut potentially harmful microbiota changes, while the negative correlations were revealed with beneficial microbiota in both humans and mice (Arnoriaga-Rodríguez et al., 2021; Castells-Nobau et al., 2024; Samulėnaitė et al., 2024).

In summary, this study highlights the interaction between cognitive flexibility impairment related to the loss of eating control and gut microbiota alterations. We have demonstrated that microbiota plays a crucial role in cognitive performance since antibiotic-depleted mice had substantially impaired short-term and long-term memory. Furthermore, microbiota related to the cognitive flexibility phenotype in humans produces important cognitive changes through FMT in mice that depend on their diet exposure. These cognitive responses are closely related to the loss of eating control, which leads to overweight and obesity. Considering the lack of effective therapeutic strategies to fight against loss of eating control, our results could open a new therapeutic perspective for future microbiota-based strategies targeting this behavioral disorder and related comorbidities.

Despite these results, our study has certain limitations. First, the control groups do not fully explain the observed effects. Another control group with antibiotic-pretreated mice that receive autotransplant and an additional experimental group that receives FMT from an intermediate inhibitory control donor could potentially give more explanation for the results obtained. Furthermore, we used only male mice in our study, although human donors of both sexes were used. Ultimately, more behavioral tests to investigate cognitive flexibility in mice could be implemented for a better understanding of how gut microbiota affects cognitive flexibility.

Acknowledgements

This work was supported by the 'Ministerio de Ciencia e Innovación y Agencia Estatal de Investigación' (#PID2020-120029GB-I00/MICIN/AEI/10.13039/501100011033) and (FPU20/05652), 'Generalitat de Catalunya, AGAUR' (#2021 SGR 00912), the European Regional Development Fund (project no. 01.2.2-LMT-K-718-03-0099) under a grant agreement with the Research Council of Lithuania (LMTLT), 'ICREA-Acadèmia' (#2020), Instituto de Salud Carlos III (ISCIII, Madrid, Spain) through the project PI20/01090 (co-funded by the European Union under the European Regional Development Fund (FEDER) "A way to make Europe").

Conflict of interest

The authors declare no conflicts of interest

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   - An abstract in both Spanish and English, of between 120 and 250 words in length. Following the international publication rules set out above, the abstract should specify the objectives of the study or research, the basic methodology employed, the main results, and the most important and/or novel conclusions.The *abstract* should be written in one or more paragraphs according to the APA publication rules, *but not divided into antecedents, method, etc.*
   - The abstract will be followed by a list of around 5 *Key words*, first in Spanish and then in English, in lower case and separated by commas. Where possible, these words should be in line with those normally used in well-known indices (e.g., Mesh terms).

3. The article proper will begin on the third page. Review process will be anonymous, confidential and blinded, it is recommended that the authors delete any information that they consider could help to identify them, such as, for example, place of selection of participants, institution of membership of the Ethics Committee that has approved the study, etc. This information can be concealed by replacing it with the expression "[AUTHORS]" or similar. It is recommended for articles to be written in the passive voice, where appropriate. The text should be clearly divided into sections, following – as far as possible depending on the characteristics of the study – the general scheme: **Introduction** (though the word should not appear as a heading), **Method, Results, Discussion, Acknowledgements, Conflict of interests** and **References**.

Introduction

This section should be relatively brief, but provide the necessary and introductory explanation so that readers can understand the state of the issue prior to the research, and so that they can see the need for such research and how it was approached. This section should not include tables or figures, unless they are essential to the understanding of the text. It should include a final paragraph setting out clearly the objective(s) of the work.

Method

This section should clearly describe the methodology employed (selection of the sample, collection of data, data-collection or assessment instruments, timeframe, etc.). It should also identify the methods, assessment instruments, treatments, drugs used, apparatus, assessment system, statistical methods where they are new, new types of method, and so on. The author should specify the type of study (descriptive, epidemiological, experimental, clinical trial, etc.), system of assignment of participants to groups, randomization, etc. All submissions should indicate the reference of the Ethics Committee or Research Office which has approved the adequacy of this research to the corresponding ethical and legal framework. It is important that experimental studies and clinical trials are registered and the registration number is indicated in the trials database (e.g., **Clinicaltrials.gov**). Authors should state the type of statistical analysis used, describe it where it is new or little known, and indicate the statistics package employed. It is strongly recommended to indicate, when possible, the exact significance data obtained in the results (as opposed to formulas such as $p < .05$ or $p < .01$) as well as to include, also when possible, effect size statistics.

Results

The results should be presented in a logical sequence with regard to text, tables and figures. Use only those tables and figures that are strictly necessary, that clearly express the results of the study. Do not duplicate data in tables and figures. Do not repeat in the text all the data from the tables and figures, only the most important. Emphasize and summarize only the most important observations. ***Adicciones*** adopts the conventional system of 5% as the value for statistical significance, and does not accept the consideration of trends with lower values.

Randomized clinical trials should follow the CONSORT guidelines (**www.consort-statement.org**) and studies with non-experimental designs should follow international guidelines (e.g.,STROBE **https://www.strobe-statement.org/**), to provide greater clarity for readers and reviewers. Likewise, effect size statistics will be presented.

Discussion

It is necessary to start with the general objective of the study. This section will emphasize the novel and important aspects of the study and the conclusions deriving from it. It should not repeat in detail the results presented in either the previous section or the introduction. The discussion should highlight the most important and controversial aspects and relate them to other relevant studies on the matter. Do not make assumptions if they are not supported by the data. Where appropriate, this section can include recommendations. Indicate the implications of the study's findings and its limitations (these will preferably be contained in a paragraph at the end of the article).

Acknowledgements

This section will be situated after the end of the article proper, and just before the **Conflicts of interest**. Where considered necessary, it will include the names of persons, institutions or bodies that have collaborated with or supported the research. It may include mention of those people who have helped in the preparation of the article, but not with the intensity required for being considered as authors. Sources of funding, call for proposals and reference of such funding, indicating whether it is competitive or not, should also be indicated here.

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All articles, editorials, comments, opinions, book reviews and letters published in the journal will be accompanied by a declaration about possible or actual conflicts of interest or a statement to the effect that the authors have no conflicts of interest to declare.

References

These will follow strictly the norms of the American Psychological Association [American Psychological Association (2020). *Publication Manual of the American Psychological Association* (7th ed.). Washington, DC. **http://www.apastyle.org**

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2025 ■ VOL. 37 ■ N. 4
ADICCIONES

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ORIGINALS / ORIGINALES

PUBLISHED BY:
SOCIDROGALCOHOL
Sociedad Científica Española de Estudios sobre el Alcohol, el Alcoholismo y las otras Toxicomanías

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