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Excessive alcohol intake produces persistent mechanical allodynia and dysregulates the endocannabinoid system in the lumbar dorsal root ganglia of genetically-selected Marchigian Sardinian alcohol-preferring rats

Vittoria Borgonetti [a,b,1], Valentina Vozella [a,1], Tim Ware [c], Bryan Cruz [a], Ryan Bullard [a], Benjamin F. Cravatt [c], Nicoletta Galeotti [b,*], Marisa Roberto [a,*]

[a] Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA, USA
[b] Department of Neuroscience, Psychology, Drug Research, and Child Health (NEUROFARBA), Section of Pharmacology and Toxicology, University of Florence, Florence, Italy
[c] Department of Chemistry, The Scripps Research Institute, La Jolla, CA, USA

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ABSTRACT

Epidemiological data indicate a strong association between alcohol use disorder (AUD) and neuropathic pain. Genetically-selected Marchigian Sardinian alcohol-preferring (msP) rats exhibit a high preference for alcohol compared with their background strain (Wistar rats), but their sensitivity to mechanical allodynia after chronic alcohol exposure is unknown. The present study compared the development of mechanical allodynia between "low, non-pathological drinker" Wistar rats and "high drinker" msP rats using the two-bottle choice (2BC) free-access procedure. Several studies reported the involvement of endocannabinoids (eCBs) in modulating mechanical allodynia, but there are no data on their role in alcohol-related allodynia. Thus, the present study assessed eCBs and their related lipid species in lumbar dorsal root ganglia (DRG) and correlated them with mechanical allodynia in our model. We found that male and female msP rats developed persistent mechanical allodynia during protracted abstinence from alcohol, presenting no sign of recovery, as opposed to Wistar rats. This effect directly correlated with their total alcohol intake. Notably, we found a correlation between lower lumbar DRG 2-arachidonoylglycerol (2-AG) levels and the development of higher mechanical allodynia during abstinence in msP rats of both sexes but not in Wistar rats. Moreover, alcohol-exposed and abstinent msP and Wistar females but not males exhibited significant alterations of thromboxane B2 and prostaglandin E2/prostaglandin D2 compared with naive rats. These findings demonstrate that DRG 2-AG metabolism is altered in msP rats during prolonged abstinence and represents a potentially interesting pharmacological target for the treatment of mechanical allodynia during alcohol abstinence.

1. Introduction

Epidemiological data indicate a strong association between alcohol use disorder (AUD) and neuropathic pain [1], which usually worsens during alcohol withdrawal [2–6]. Neuropathic pain is a form of chronic pain that is induced by damage to the somatosensory system, in which mechanical allodynia is a main symptom. Mechanical allodynia is defined as an innocuous mechanical stimulus that becomes painful [7].

Abbreviations: 15-PGDH, 15-hydroxyprostaglandin dehydrogenase; 2-AG, 2-arachidonoylglycerol; 2BC, two-bottle choice; AA, arachidonic acid; ABHD6, α,β-hydrolase 6; AEA, N-arachidonoylethanolamide; ANOVA, analysis of variance; AUD, alcohol use disorder; CB, cannabinoid; CeA, central nucleus of the amygdala; COX-2, cyclooxygenase-2; CRF, corticotropin-releasing factor; DAG, diacylglycerol; DAGL, diacylglycerol lipase; DPBS, Dulbecco's phosphate-buffered saline; DRG, dorsal root ganglia; ECB, endocannabinoid; FAAH, fatty acid amide hydrolase; LC-MS/MS, liquid chromatography-mass spectrometry; LSD, Least Significant Difference; MAG, monoacylglycerol; MAGL, monoacylglycerol lipase; MsP, Marchigian Sardinian alcohol-preferring; NAE, N-acylethanolamide; NAPE, N-acylphosphatidylethanolamine; OEA, oleoylethanolamide; PEA, palmitoylethanolamide; PGD2, prostaglandin D2; PGE2, prostaglandin E2; PLD, phospholipase D; SEM, standard error of the mean; TXB2, thromboxane B2.

* Corresponding authors.
E-mail addresses: nicoletta.galeotti@unifi.it (N. Galeotti), mroberto@scripps.edu (M. Roberto).
[1] Equal contribution.

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Preclinical studies in both rats [8,9] and mice [10–12] found an association between chronic alcohol consumption and the development of mechanical allodynia. The current clinical approach to relieving the pain caused by chronic alcohol consumption is based on lifestyle changes (i.e., discontinuation of alcohol consumption; B vitamins diet supplementation) or on a pharmacological approach using drugs to treat symptoms associated with neuropathic pain, such as antidepressants (TCA or SNRIs) or anticonvulsants (gabapentin or pregabalin) [13]. However, these therapeutic interventions are only symptomatic and do not repair the damage caused by alcohol. As a result, there is a need to understand what mechanisms are involved in this form of neuropathy, to seek effective long-term therapies able to reduce symptoms and repair the damage caused by alcohol.

Genetically selected Marchigian Sardinian alcohol-preferring (msP) rats exhibit a high preference for alcohol, characterized by binge-like drinking behavior and leading to pharmacologically relevant blood alcohol levels. The msP rats are also highly sensitive to stress and present an anxiety-like phenotype compared with other rat strains, including their background strain (Wistar rats) [14–19], but their sensitivity to mechanical allodynia after chronic alcohol exposure is unknown. More than half of AUD patients experience significant persistent pain, with a higher prevalence in women (~60 %) than in men (~50 %) [20]. Notably, sex differences in AUD and chronic pain represent an important gap in knowledge that needs to be addressed. Thus, the present study investigated the development of mechanical allodynia that is associated with alcohol consumption in msP and Wistar rats of both sexes to assess potential sex differences. The overall aim of this study was to compare the development of mechanical allodynia between "low, non--pathological-drinker" Wistar rats and "high-drinker" msP rats. We used the two-bottle choice (2BC) free-access procedure, a well-characterized model of voluntary alcohol drinking, in which msP rats exhibit significantly higher alcohol intake and preference compared with Wistar rats [21]. We then assessed the development of allodynia using the von Frey test in three different conditions—before starting drinking (baseline), immediately after alcohol intake (alcohol), and 26 days after the last alcohol intake (protracted abstinence)—in both male and female rats. msP rats develop a higher preference for alcohol because of aberrations in brain stress signaling, indicated by the overexpression of corticotropin-releasing factor 1 (CRF_1) receptors [12,22,23].

Previous studies report that msP rats exhibit disrupted endocannabinoid (eCB) signaling in several brain regions [24]. In addition, pharmacological modulation of the eCB system with cannabinoid 1 (CB_1) and CB_2 receptor-selective agonists [25,26] is largely investigated and employed in preclinical [27] and clinical [28,29] studies for the treatment of different forms of neuropathic pain. Exogenous natural CB receptor agonists (e.g., Δ^9-tetrahydrocannabinol) or synthetic CB receptor agonists and inverse agonists [30] can cause possible side effects. Therefore, the development of pharmacological tools currently focuses on the inhibition of enzymes that are responsible for eCB degradation, such as fatty acid amide hydrolase (FAAH), which metabolizes anandamide (AEA), and monoacylglycerol lipase (MAGL) and α,β-hydrolase 6 (ABHD6), which hydrolyze 2-arachidonoylglycerol (2-AG) [31–39]. Given that 2-AG and AEA are involved in neuropathic pain modulation, along with other non-cannabinoid bioactive N-acylethanolamides (NAEs), such as palmitoylethanolamide (PEA) [39–41] and oleoylethanolamide (OEA) [42], exploring their role in alcohol-related allodynia may represent a novel therapeutic strategy.

Although several studies reported the involvement of eCB and congener lipids in modulating mechanical allodynia in various models of neuropathy [43–47], there are no data on their role in alcohol-related allodynia in msP rats or their role in alcohol-induced toxicity in the peripheral nervous system. Thus, we assessed eCBs and their related lipid species in the lumbar dorsal root ganglia (DRG) in msP and Wistar rats and correlated them with mechanical allodynia in our model. Primary sensory neurons and immune cells (i.e., satellite glia, macrophages) in DRG are key components in the development of neuropathic pain [48].

The goal of the present study was to assess whether alcohol intake produces alterations of lipid mediators in lumbar DRG in genetically-selected high alcohol-drinking msP rats compared with low-drinker Wistar control rats during alcohol intake and prolonged abstinence, and whether there were specific sex differences in the eCBs.

2. Materials and methods

2.1. Animals

We used a total of 100 rats. Adult male (n = 28, ~450 g) and female (n =21, ~250 g) msP rats were bred at The Scripps Research Institute (La Jolla, CA, USA). Adult male (n =26, ~450 g) and female (n =25, ~350 g) Wistar rats (the genetic background for msP rats) were purchased from Charles River Laboratories (Wilmington, MA, USA). Animals of the same age were used. The rats were housed in a temperature- and humidity-controlled vivarium on a 12 h/12 h reverse light/dark cycle (lights off at 8:00 AM), with food and water available ad libitum. The rats were pair-housed, separated by a perforated clear Plexiglas divider to habituate them to the behavioral testing conditions while also reducing isolation stress [49]. The rats were randomly assigned to the different treatment groups. We conducted all procedures according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and The Scripps Research Institute Institutional Animal Care and Use Committee (IACUC) policies (protocol no. 09–0006).

2.2. Two-bottle choice (2BC) alcohol access procedure

The 2BC procedure (free choice between water and 10 % v/v alcohol) was used to measure voluntary alcohol drinking and preference [21,50]. The rats were given free access to water and alcohol (10 % v/v) 24 h/day for 15 days to establish a stable drinking baseline and preference for alcohol (80–90 % preference for alcohol vs. water in msP rats). Once the baseline was reached, access to alcohol was then decreased to a 2 h period, starting from 2 h into the animals' dark (active) phase. Fluids were offered through graduated drinking tubes equipped with metallic spouts, and intake was measured by weighing the tubes at the end of each drinking session. The drinking tubes' positions were switched daily to avoid the development of side preferences. The animals had free access to food. Alcohol intake was calculated as absolute consumption at each time interval and is expressed as grams per kilogram of body weight to control body weight variability [51]. The total alcohol intake (g/kg) of each rat is reported in the supplementary materials (Supplementary Fig. 1S) and was calculated by summing the daily alcohol consumption over the 5 weeks of alcohol exposure. The terminal blood alcohol levels (mg/dL) are also reported in Supplementary Fig.1S.

2.3. Experimental design overview

In the present study, three experimental groups were used for both Wistar and msP rats (Table 1).

- Naive: Rats that did not receive alcohol.
- Alcohol: Rats that received 2 weeks of alcohol (24 h/day, 10 % v/v) + 3 weeks of alcohol (2 h/day) and were sacrificed immediately 2 h after the last drinking session.
- Abstinence: Rats that received 2 weeks of alcohol (24 h/day) + 3 weeks of alcohol (2 h/day) and were sacrificed after 26 days of abstinence.

2.4. Measurement of mechanical allodynia with von Frey filaments

Mechanical allodynia was measured by a blind experimenter before starting alcohol intake (baseline), during alcohol exposure, and after

Table 1
Experimental groups.

Experimental groups	Sex	Strain	N
Naive	Female	Wistar	6
Alcohol	Female	Wistar	19
Abstinence	Female	Wistar	7*
Naive	Male	Wistar	6
Alcohol	Male	Wistar	20
Abstinence	Male	Wistar	12*
Naive	Female	msP	7
Alcohol	Female	msP	14
Abstinence	Female	msP	6*
Naive	Male	msP	8
Alcohol	Male	msP	20
Abstinence	Male	msP	10*

* Subset of rats tested during alcohol and abstinence.

protracted abstinence using von Frey filaments (North Coast Medical, Gilroy, CA, USA) with ascending force as previously reported [11]. Briefly, the rats were first habituated to the testing apparatus by placing them on a metal mesh stand. Von Frey filaments (4, 6, 8, 10, 15, 26, 60, and 100 g) were then manually applied to the plantar surface of one hind paw with increasing force until a withdrawal response was elicited. The von Frey filament force that elicited the withdrawal response was the mechanical withdrawal threshold. A positive value was recorded if the paw was lifted at least three times in five measurements. If the rats did not respond, then we used the next filament with a greater force. The animals were tested under red lights, during their dark (active) phase. The allodynia measures were performed by the same experimenter throughout the study.

2.5. Calculation of the allodynia development index during alcohol and abstinence

The Allodynia Development Index during alcohol exposure and abstinence was calculated using the following formula:

Allodynia Development Index during alcohol exposure (%) = 100 - [(last alcohol exposure mechanical allodynia threshold) / (baseline mechanical allodynia threshold) x 100].

The last mechanical allodynia threshold measured during alcohol exposure is the last 2BC session of the 3rd week of exposure. The index is represented by a purple square for msPs and a green square for Wistars.

Allodynia Development Index during abstinence (%) = 100 - [(last abstinence day mechanical allodynia threshold) / (baseline mechanical allodynia threshold) x 100].

The last abstinence day mechanical allodynia threshold is abstinence day 26 in Wistars and in msPs. The index is represented by a purple circle for msPs and a green circle for Wistars.

The value "0" represents no change in mechanical allodynia. Animals with an index ≤ 0 did not develop allodynia. Animals with an index > 0 developed allodynia.

2.6. Lumbar dorsal root ganglia extraction

A subgroup of rats (n = 60) was used for the liquid chromatography-mass spectrometry (LC-MS/MS) analysis of lumbar DRG (four DRG from each rat) in the three different experimental conditions (naive, alcohol, and abstinence) for both strains and sexes. To collect lumbar DRG, the rats were deeply anesthetized with isoflurane and rapidly decapitated while unconscious using a guillotine. Dissection of DRG was performed by adapting previously published methods to rats [52]. Lumbar DRG were removed, flash-frozen on dry ice, and stored at −80°C.

2.7. Targeted lipidomic analysis of tissue samples

Tissues were Dounce-homogenized with 1 ml of cold Dulbecco's phosphate-buffered saline (DPBS), and the total tissue lipidome was extracted in 4 ml of 2:1:1 $CHCl_3$/MeOH/DPBS (v/v/v) solution that contained the internal standard mix of 100 pmol 2-AG-d5, 1-stearoyl-2-arachidonoyl-sn-glycerol-d8, AEA-d4, PA (17:0/17:0), and 500 pmol AA-d8. The mixture was vigorously vortexed and centrifuged at 2000 g for 5 min at 4°C. The bottom organic phase was collected, and the remaining aqueous phase was acidified with 100 µl of 3 N HCl and re-extracted by the addition of 1 ml of $CHCl_3$. Both organic extracts were pooled, dried down under a N_2 stream, and reconstituted in 150 µl of 2:1 $CHCl_3$/MeOH (v/v) for LC-MS/MS analysis.

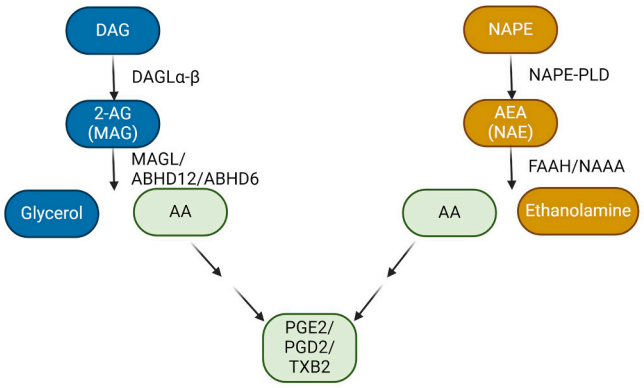
Lipids were quantified using LC-MS/MS-based multiple reaction monitoring (MRM) methods (Agilent Technologies, 6460 or 6470 Triple Quadrupole) [53]. The MS analysis was performed using electrospray ionization with the following parameters: drying gas temperature, 350°C; drying gas flow, 9 L/min; nebulizer pressure, 45 Ψ; sheath gas temperature, 375°C; sheath gas flow, 12 L/min; fragmentor voltage, 100 V; capillary voltage, 3.5 kV. Multiple reaction monitoring transitions for the targeted LC-MS/MS analysis are presented in the compiled lipidomic data spreadsheet (Supplementary Table 1–2). The separation of lipids was achieved using a 50 mm´4.6 mm 5-µm Gemini C18 column (Phenomenex) coupled to a guard column (Gemini: C18: 4 × 3 mm). For negative mode analysis, H_2O:MeOH (95:5, v/v) with 0.1 % NH_4OH (v/v) and iPrOH:MeOH:H_2O (60:35:5, v/v) with 0.1 % NH_4OH (v/v) were used as solvent A and solvent B, respectively. For positive mode analysis, H_2O:MeOH (95:5, v/v) with 0.1 % formic acid (v/v) and iPrOH:MeOH:H_2O (60:35:5, v/v) with 0.1 % formic acid (v/v) were used as solvent A and solvent B, respectively. The LC gradient for negative mode analysis was the following after injection: 20 % B at 0.1 ml/min for 5 min; increase to 85 % B at 0.4 ml/min for 15 min; increase to 100 % B at 0.5 ml/min for 5 min; run at 100 % B at 0.5 ml/min for 2 min; and then back to 20 % B and equilibrate at 0.5 ml/min for 5 min. The LC gradient for positive mode analysis was the following after injection: start from 0 % B and hold at 0.1 ml/min for 5 min; run at 60 % B at 0.4 ml/min and increase to 100 % B at 0.4 ml/min for 15 min; run at 100 % B at 0.5 ml/min for 13 min; and then back to 0 % B and equilibrate for 2 min. Lipid species were quantified by measuring areas under the curve compared with the corresponding internal standards and then normalizing to the tissue protein abundance.

2.8. Nomenclature of eCBs

The monoacylglycerol 2-arachidonoylglycerol (2-AG) and the N-acylethanolamide (NAE) anandamide (or N-arachidonoylethanolamide [AEA]) are bioactive lipids that are the primarily investigated eCBs. 2-Arachidonoylglycerol biosynthesis is catalyzed by diacylglycerol lipase (DAGL) activity, which converts diacylglycerols (DAGs) into MAGs via hydrolysis at the sn-1 position. 2-arachidonoylglycerol is metabolized into glycerol and arachidonic acid [54], which can be further oxidized into prostaglandins (i.e., prostaglandin E2 [PGE2]/prostaglandin D2 [PGD2]) and thromboxane B2 (TXB2). N-arachidonoylethanolamide is biosynthesized from its precursors N-acylphosphatidylethanolamines (NAPEs). N-acylphosphatidylethanolamines are substrates for multiple phospholipases, including a NAPE-specific phospholipase D (NAPE-PLD) [55–57]. N-arachidonoylethanolamide is then metabolized into ethanolamine and AA (see Schematic).

2.9. Statistical analyses

Behavioral experiments (2BC drinking and mechanical allodynia measurements) and LC-MS/MS lipid measurements were analyzed using two-way analysis of variance (ANOVA), with alcohol condition (naive, alcohol, abstinence) and genotype (Wistar, msP) as between-subject factors. Significant interactions in the ANOVA were followed by



Schematic. Simplified biosynthesis and metabolism of eCBs. Diacylglycerol (DAG), 2-arachidonoylglycerol (2-AG), arachidonic acid [54], N-acylphosphatidylethanolamine (NAPE), anandamide (AEA), prostaglandin E2 (PGE2), prostaglandin D2 (PGD2), thromboxane B2 (TXB2), diacylglycerol lipase (DAGLα-β), monoacylglycerol lipase (MAGL), alpha/beta-hydrolase domain containing 12 (ABHD12), alpha/beta-hydrolase domain containing 6 (ABHD6), N-acyl-phosphatidylethanolamine phospholipase D (NAPE-PLD), fatty acid amide hydrolase (FAAH), N-acylethanolamine acid amidase (NAAA).

Tukey's and Sidak's multiple-comparison post hoc test, as specified in the figure legends. The Allodynia Development Index was analyzed using unpaired t-test. Pearson's correlations were calculated to examine relationships between the Allodynia Development Index and total alcohol intake and between the Allodynia Development Index and lipid levels.

The experiments were designed to generate groups of comparable size using randomization and blinded analysis. Group size is the number of independent values (i.e., individual rats), and the statistical analysis was performed using these independent values. Sample sizes were based on experience from our laboratory and the Cravatt laboratory [58] for similar studies. All datasets were derived from $n \geq 6$ rats (behavioral experiments) or $n = 5$ rats (LC-MS/MS experiments) for each sex and genotype. All data are expressed as the mean ± standard error of the mean (SEM). Values of $p < 0.05$ were considered statistically significant. Two male Wistar rats were excluded because they were outliers in the 2BC procedure after applying the ROUT test. All data graphing and statistical analysis were conducted using GraphPad Prism Version 10 (La Jolla, CA, USA).

3. Results

3.1. Baseline mechanical allodynia and lumbar DRG lipids differ between Wistar and msP rats

We first determined whether there were baseline differences in the mechanical threshold between naive msP and Wistar rats (Fig. 1A). A significant strain difference was observed in both male ($^{****}p < 0.0001$) and female ($^{*}p < 0.05$) rats, in which msP rats had a lower mechanical threshold, indicating a higher level of allodynia, compared with Wistar rats. Among the lipid species analyzed, no baseline difference was found in male (Fig. 1B) or female (Fig. 1C) msP rats compared with male Wistar rats. A nonsignificant trend toward a reduction of 2-AG (MAG C20:4) levels was detected in both male (Fig. 1B, $p = 0.95$) and female (Fig. 1C, $p = 0.99$) msP rats compared with their Wistar controls.

3.2. Evaluation of mechanical allodynia during alcohol-exposure in Wistar and msP rats

We used the von Frey test to measure the development and progression of mechanical allodynia during the 2BC drinking procedure in male (Fig. 2A) and female (Fig. 2B) msP and Wistar rats. As previously reported [21], total alcohol intake in male and female Wistar rats was lower than in msP rats (Supplementary Fig.1S). Mechanical allodynia was measured once weekly during alcohol exposure (i.e., immediately after 2BC exposure). The mechanical allodynia threshold remained lower in male msP rats (Fig. 2A) compared with Wistar rats throughout the entire alcohol exposure ($^{****}p < 0.0001$) both after the 2 weeks of 24 h 2BC access and during the 3 weeks of 2 h-restricted 2BC access. Additionally, the male Wistar group exhibited a lower mechanical allodynia threshold after 1 week of 2 h 2BC that persisted for 3 weeks (Fig. 2A). Female msP rats (Fig. 2B) exhibited a persistent significantly ($^{****}p < 0.0001$) lower mechanical allodynia threshold compared with the female Wistar group after the 2 weeks of 24 h continuous alcohol access and after the first week of 2 h 2BC access. The strain difference in female rats disappeared during the second and third weeks of 2 h alcohol access. Female Wistar rats began to develop allodynia after the second week of 2 h 2BC access and reached the same mechanical allodynia threshold as female msP rats after the third week of 2BC exposure (Fig. 2B). Despite the different alcohol intake between msP and Wistar rats (Supplementary Fig.1S), male (Fig. 2A) and female (Fig. 2B) msP rats did not present a further reduction of mechanical allodynia compared with their baseline level.

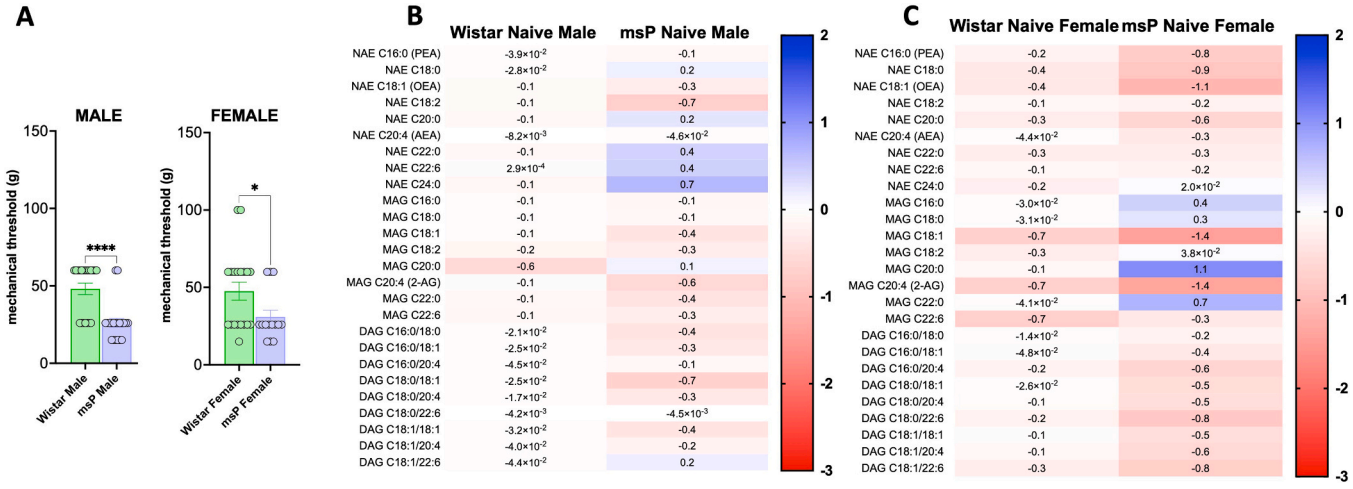


Fig. 1. (A) Differences in baseline mechanical allodynia between male and female naive Wistar (Wst) and msP rats. The data were analyzed using unpaired t-test (Male: $^{****}p < 0.0001$, Two-tailed, t=4.708, df=38; Female: $^{*}p < 0.05$, Two-tailed, t=2.142, df=31). (B, C) Dorsal root ganglia (DRG) endocannabinoid changes in male msP naive rats (B) and female msP naive rats (C) normalized to naive Wistar rats. The data are expressed as log2-based fold change in msP vs. Wistar rats. The data were analyzed using two-way ANOVA followed by Tukey's multiple-comparison post hoc test.

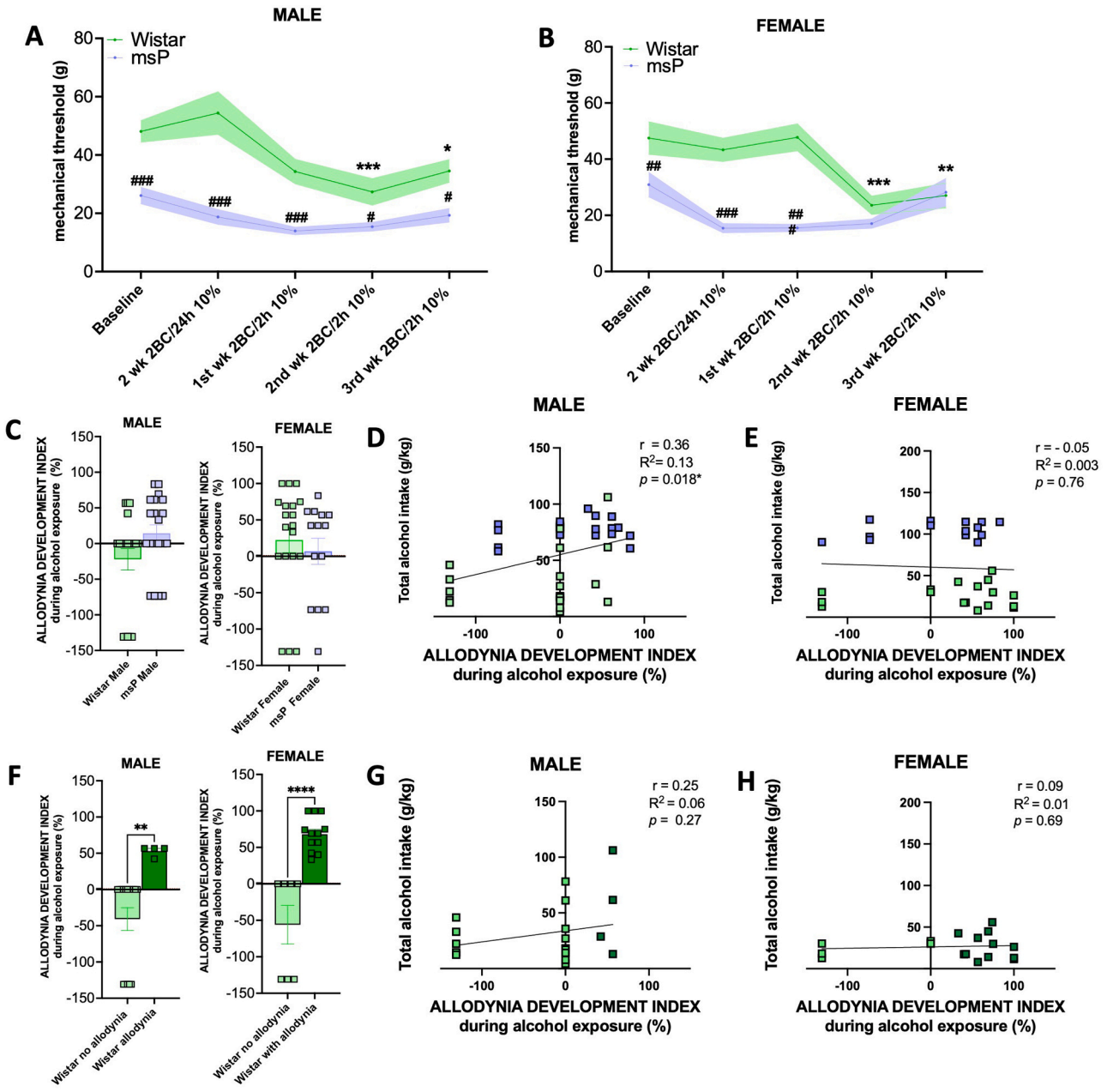


Fig. 2. (A, B) Measurement of mechanical allodynia with von Frey filaments in male (A) and female (B) Wistar and msP rats before (baseline) and during alcohol consumption using the two-bottle choice (2BC) procedure (free choice between water and 10 % v/v alcohol). The data were analyzed using two-way ANOVA followed by Tukey's multiple-comparison post hoc test. (A) male: $F_{4,165} = 3.012$, $^{***}p < 0.001$, $^{*}p < 0.05$, Wistar/msP vs. baseline; $^{\#\#\#}p < 0.001$, msP vs. Wistar; (B) female: $F_{4,146} = 4.981$, $^{***}p < 0.001$, $^{**}p < 0.01$, Wistar/msP vs. baseline; $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, msP vs. Wistar. (C) Allodynia Development Index during alcohol exposure (%) was calculated using the final mechanical allodynia threshold during alcohol exposure and the baseline mechanical allodynia threshold for each rat. The data were analyzed using unpaired t-test (Male: p = 0.09, Two-tailed, t=1.700, df=40; Female: p = 0.54, Two-tailed, t=0.6071, df=31). (D) Correlation between Allodynia Development Index (%) and total alcohol intake (g/kg) in male msP and Wistar rats. Pearson's correlation: r = 0.36, $R^2 = 0.13$, $^{*}p < 0.018$. (E) Correlation between Allodynia Development Index (%) and total alcohol intake (g/kg) in female msP and Wistar rats (E). Pearson's correlation: r = −0.05, $R^2 = 0.003$, p = 0.76. (F) Separation of male and female Wst rats into two sub-populations: allodynia and no allodynia. The data were analyzed using unpaired t-tests (Male: $^{**}p < 0.01$, Two-tailed, t=2.937, df=18; Female: $^{****}p < 0.0001$, Two-tailed, t=5.719, df=17). (G) Correlation between Allodynia Development Index (%) and total alcohol intake (g/kg) in male Wst rats. Pearson's correlation: r = 0.25, $R^2 = 0.06$, p = 0.27. (H) Correlation between Allodynia Development Index (%) and total alcohol intake (g/kg) in female Wistar rats. Pearson's correlation: r = 0.09, $R^2 = 0.01$, p = 0.69.

To assess strain differences in the development of allodynia during alcohol exposure, we calculated the Allodynia Development Index during alcohol exposure (i.e., the ratio between the last measured mechanical allodynia threshold on the third week of 2 h 2BC access and the baseline value), where an index ≤ 0 corresponds to the absence of allodynia. As reported in Fig. 2C, there were no significant differences between msP and Wistar rats of either sex, despite their total alcohol intake being different.

When we correlated the Allodynia Development Index during alcohol exposure and total alcohol intake, we found that the development of allodynia directly correlated with total alcohol intake in males only (Fig. 2D), suggesting that rats that drank more developed more allodynia. In females (Fig. 2E), however, allodynia did not correlate with total alcohol intake.

Only 40–50 % of people who chronically consume moderate amounts of alcohol develop neuropathy. This clinical condition was recapitulated in our model by Wistar rats. Wistar rats did not develop a preference for alcohol; for this reason, they are considered moderate drinkers, but a percentage of Wistar rats developed significant allodynia: 18.18 % of male Wistar rats and 63.15 % of female Wistar rats (index > 0; Fig. 2 F, male: $^{**}p < 0.01$; female: $^{****}p < 0.0001$). These results are also consistent with clinical studies, in which women were more likely to develop alcohol-induced neuropathy. Lastly, in male Wistar rats (Fig. 2G), the mechanical Allodynia Development Index during alcohol exposure tended to correlate with total alcohol intake, whereas females developed allodynia regardless of their total alcohol intake (Fig. 2H).

3.3. msP rats develop long-lasting allodynia during alcohol abstinence

Abstinence-related allodynia belongs to the category of physical withdrawal symptoms. For this reason, we compared the long-lasting effect of alcohol consumption in msP and Wistar rats during protracted abstinence (for 26 days after the last alcohol consumption; Fig. 3). Male msP rats exhibited a significant reduction of the mechanical allodynia threshold compared with Wistar rats after 5 days of abstinence, which remained unchanged until day 26 (Fig. 3A). In contrast, male Wistar rats exhibited the development of allodynia immediately after 2BC, but they completely recovered and returned to baseline levels starting from 7 days into alcohol abstinence (Fig. 3A; $F_{15,353} = 3.195$, $^{*}p < 0.05$, Wistar/msP vs. baseline; $^{###}p < 0.001$, $^{##}p < 0.01$, $^{#}p < 0.05$, msP vs. Wistar).

Like msP males, a long-lasting effect (26 days) of alcohol drinking on mechanical allodynia was observed in female msP rats compared with Wistar rats (Fig. 3B; $F_{15,215} = 4.088$, $^{***}p < 0.001$, Wistar/msP vs. baseline; $^{###}p < 0.001$, $^{##}p < 0.01$, msP vs. Wistar) but with earlier onset (i.e., 3 days after alcohol removal) and no sign of recovery. At the same time, female Wistar rats (Fig. 3B) exhibited slower recovery from allodynia that was induced by alcohol consumption compared with male Wistar rats (Fig. 3A), with a relapse-remitting trend. After improvement at 3 days and worsening at days 5–6, female Wistar rats completely recovered after 15 days of abstinence. To confirm the total recovery of mechanical sensitivity by male and female Wistars before sacrifice, we took an additional measurement after 26 days of abstinence (Supplementary Fig.3S).

The Allodynia Development Index during abstinence (Fig. 3C, male: $^{****}p < 0.0001$) showed that msP rats exhibited a significant increase in abstinence-related allodynia compared with Wistar rats, which directly correlated with total alcohol intake both in male (Fig. 3D,) and in female (Fig. 3E) rats. These results suggest that the main difference in allodynia development between msP and Wistar rats occurred during protracted

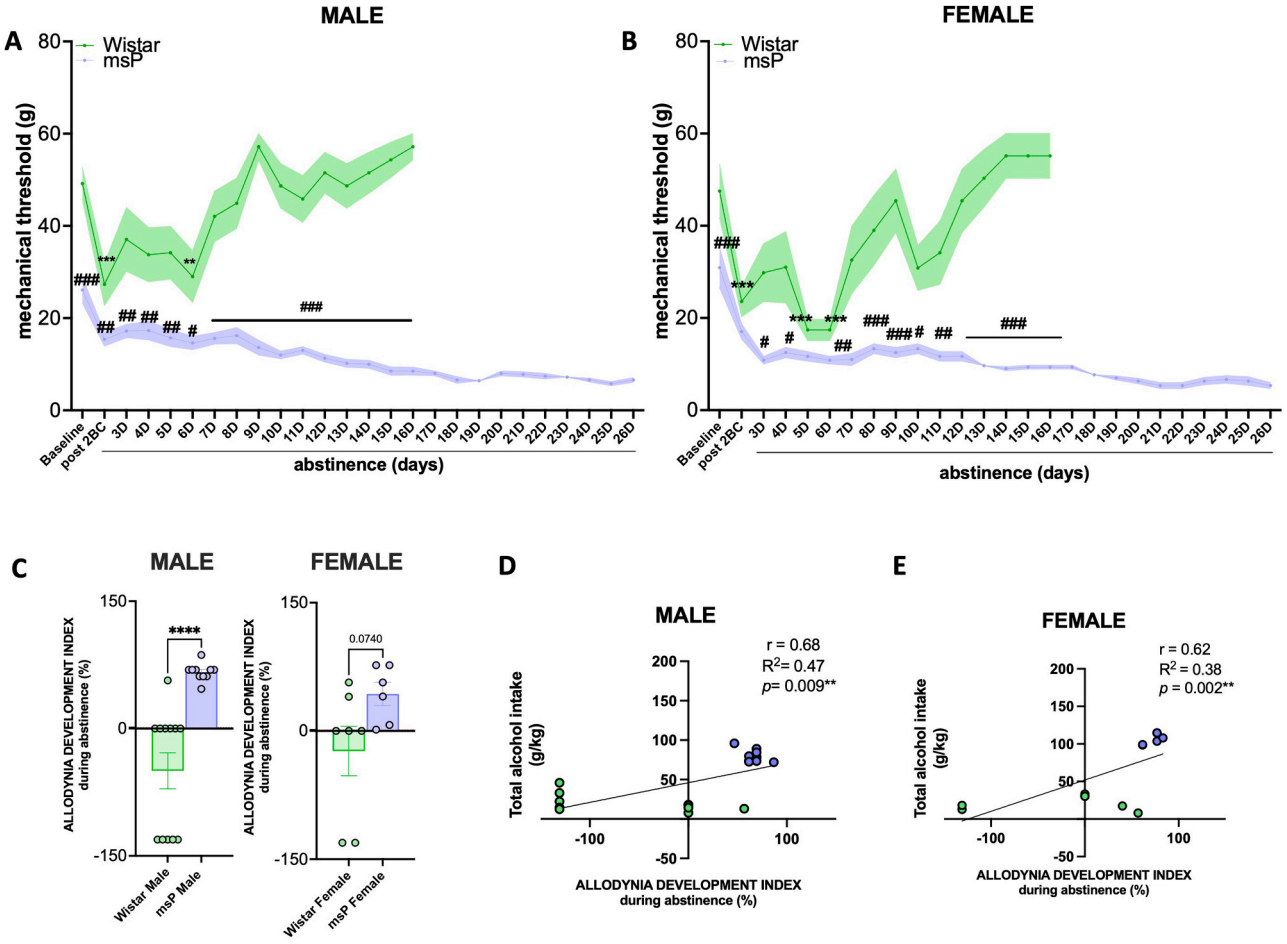


Fig. 3. (A, B) Measurement of mechanical allodynia with von Frey filaments in male (A) and female (B) Wistar and msP rats before (baseline) alcohol intake and during abstinence. The data were analyzed using two-way ANOVA followed by Tukey's multiple-comparison post hoc test. (A) male: $F_{15,353} = 3.195$, $^{*}p < 0.05$, Wistar/msP vs. baseline; $^{###}p < 0.001$, $^{##}p < 0.01$, $^{#}p < 0.05$, msP vs. Wistar; (B) female: $F_{15,215} = 4.088$, $^{***}p < 0.001$, Wistar/msP vs. baseline; $^{###}p < 0.001$, $^{##}p < 0.01$, msP vs. Wistar. (C) Allodynia development index during abstinence (%) was calculated using the final mechanical allodynia threshold during abstinence and the baseline mechanical allodynia threshold for each rat. The data were analyzed using unpaired t-test (Male: $^{****}p < 0.0001$, Two-tailed, t=4.958, df=20; Female: $p = 0.0740$, Two-tailed, t=1.974, df=11). (D) Correlation between Allodynia Development Index (%) and total alcohol intake (g/kg) in male msP and Wistar rats. Pearson's correlation: $r = 0.68$, $R^2 = 0.47$, $^{**}p = 0.009$. (E) Correlation between Allodynia Development Index (%) and total alcohol intake (g/kg) in female msP and Wistar rats. Pearson's correlation: $r = 0.62$, $R^2 = 0.38$, $^{***}p = 0.002$.

abstinence (Fig. 3).

3.4. Effect of chronic alcohol consumption on lipids in lumbar dorsal root ganglia

We next quantified three classes of eCB-related lipids, NAEs, MAGs, and DAGs, in lumbar DRG, which represent an important gateway for transporting peripheral signals to the central nervous system and are involved in the development of mechanical allodynia (Fig. 4). Although male Wistar rats (Fig. 4A) did not develop mechanical allodynia, alcohol consumption changed their lipidome. We found a significant increase of NAE C18:0, NAE C20:0, NAE C22:0, and MAG C18:1 during abstinence compared to the alcohol group ($^{\#}p < 0.05$, abstinent vs. alcohol). We also detected an increase of NAE C22:0, NAE C24:0, and MAG C20:0 in the abstinent rats compared to naive rats ($^{**}p < 0.01$, $^{*}p < 0.05$ abstinent vs. naive). Lastly, we found an increase of MAG C20:0 ($^{**}p < 0.01$ alcohol vs. naive), but a decrease in MAG C22:6 ($^{*}p < 0.05$ alcohol vs. naive) in alcohol compared to naive rats. These lipid alterations could indicate some long-lasting effects that are associated with alcohol consumption that deserve further investigation.

Interestingly, male msP rats (Fig. 4B) that developed mechanical allodynia during protracted abstinence exhibited a significant decrease in 2-AG and MAG 22:6, but not the other lipids compared with alcohol rats ($^{\#\#}p < 0.01$ abstinent vs. alcohol). Thus, 2-AG was the discriminating lipid species between the two conditions. When comparing 2-AG levels between Wistar and msP rats, we observed a significant strain difference during abstinence (Fig. 4C; $^{*}p < 0.05$ msP vs Wistar). None of the other main lipid species showed any significant changes: PEA (Supplementary Fig.4S-A), OEA (Supplementary Fig. 4S-B), and AEA (Supplementary Fig. 4S-C). Moreover, 2-AG levels correlated with the Allodynia Development Index calculated for Naive (empty circle), alcohol exposure (square) and abstinent (full circle) (Fig. 4D), in which higher 2-AG levels corresponded to lower allodynia. Additionally, a correlation trend, although not significant ($p = 0.07$), was observed between low 2-AG levels and high total alcohol intake (Fig. 4E).

Interestingly, male msP rats (Fig. 4B) that developed mechanical

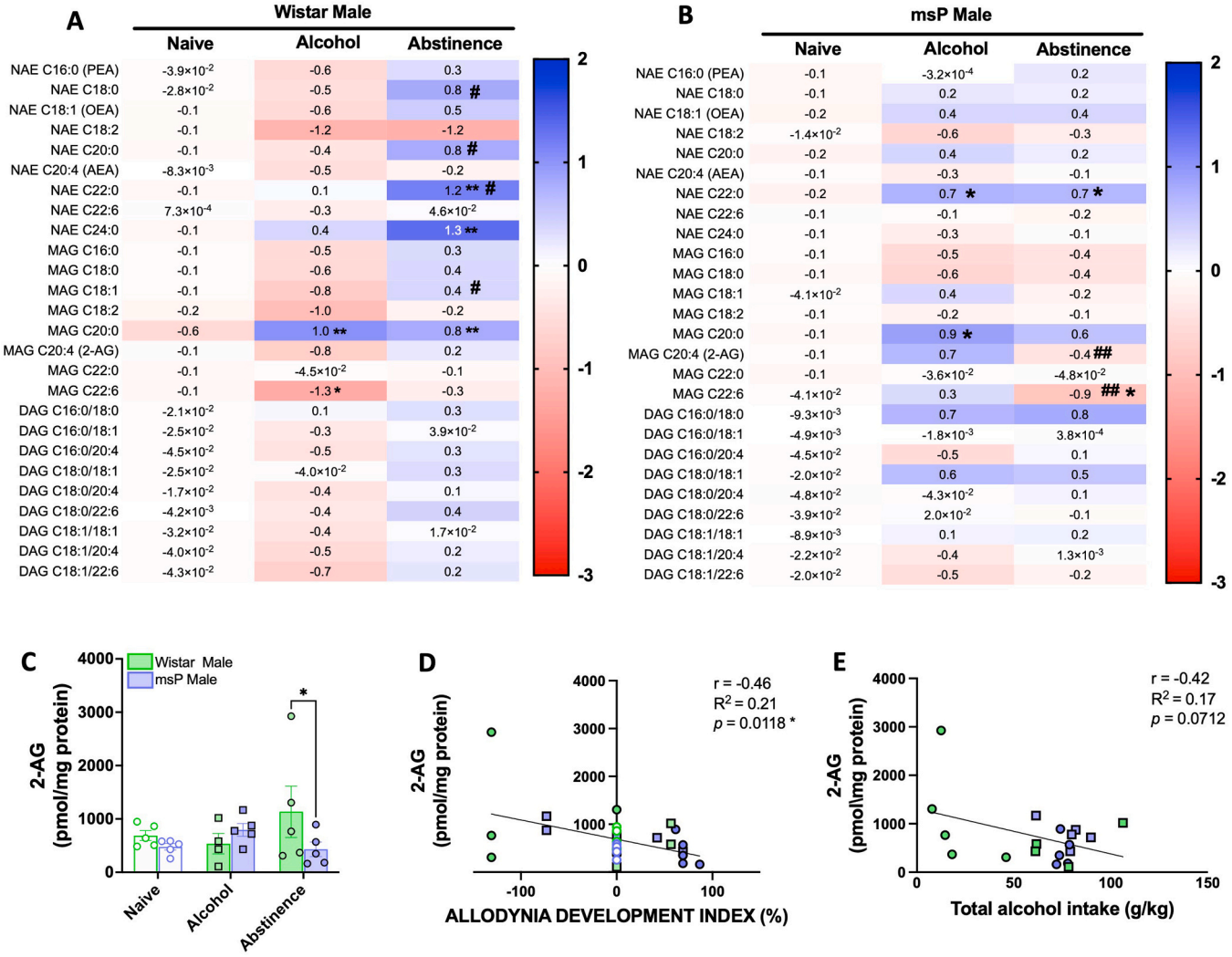


Fig. 4. (A) Endocannabinoid changes in lumbar dorsal root ganglia (DRG) in naive, alcohol, and abstinent male Wistar rats. The values were normalized to naive male Wst rats and are expressed as log2-based fold change vs. naive. The data were analyzed using two-way ANOVA followed by Tukey's multiple-comparison post hoc test ($F_{2,286} = 28.47$, $^{**}p < 0.01$, $^{*}p < 0.05$, alcohol/abstinent vs. naive; $^{\#}p < 0.05$, abstinent vs. alcohol). (B) Endocannabinoid changes in DRG in naive, alcohol, and abstinent male msP rats. The values were normalized to naive male msP rats and are expressed as log2-based fold change vs. naive. The data were analyzed using two-way ANOVA followed by Tukey's multiple-comparison post hoc test ($F_{2,312} = 1.637$, $^{*}p < 0.05$, alcohol/abstinent vs. naive; $^{\#\#}p < 0.01$, $^{\#}p < 0.05$, abstinent vs. alcohol). (C) Dorsal root ganglia 2-AG levels (pmol/mg protein) in naive, alcohol, and abstinent male Wistar and msP rats. The data were analyzed using two-way ANOVA followed by Tukey's multiple-comparison post hoc test ($F_{2,23} = 2.105$, $^{*}p < 0.05$ Wistar male vs msP male during abstinence). (D) Correlation between Allodynia Development Index (%) and 2-AG levels in male msP and Wistar rats. Pearson's correlation: $r = -0.46$, $R^2 = 0.21$, $^{*}p = 0.0118$. (E) Correlation between total alcohol intake (g/kg) and 2-AG levels in male msP and Wst rats. Pearson's correlation: $r = -0.42$, $R^2 = 0.17$, $p = 0.0712$.

allodynia during protracted abstinence exhibited a significant decrease in 2-AG and MAG 22:6, but not the other lipids compared with alcohol rats ($^{\#\#}p < 0.01$ abstinent vs. alcohol). Thus, 2-AG was the discriminating lipid species between the two conditions. When comparing 2-AG levels between Wistar and msP rats, we observed a significant strain difference during abstinence (Fig. 4C; $^{*}p < 0.05$ msP abstinence vs Wistar abstinence). None of the other main lipid species showed any significant changes: PEA (Supplementary Fig. 4S-A), OEA (Supplementary Fig. 4S-B), and AEA (Supplementary Fig. 4S-C). Moreover, 2-AG levels correlated with the Allodynia Development Index calculated for naive (empty circle), alcohol exposure (square) and Abstinent (full circle) (Fig. 4D), in which higher 2-AG levels corresponded to lower allodynia. Additionally, a correlation trend, although not significant ($p = 0.07$), was observed between low 2-AG levels and high total alcohol intake (Fig. 4E).

Female Wistar rats (Fig. 5A) exhibited lower levels of OEA and MAG C20:0 during protracted abstinence compared with the alcohol group ($^{**}p < 0.01$, $^{*}p < 0.05$ alcohol vs. naive). Female msP rats showed higher levels of NAE C18:2 and DAG C16:0/20:4, but lower levels of MAG C18:1, 2-AG, MAG C22:6, DAG C18:0/20:4 in abstinence compared to alcohol rats ($^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$ abstinent vs. alcohol). MAG C16:0 and MAG C18:0 levels were higher, while 2-AG and MAG C22:6 levels were lower in abstinent compared to naive rats ($^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ abstinent vs. naive). Lastly, alcohol-exposed rats presented lower NAE C18:2, AEA, NAE C22:6, MAG C16:0, MAG C18:0, DAG C16:0/20:4, DAG C18:0/20:4, DAG C18:1/20:4 and DAG C18:1/22:6 compared to naive rats ($^{**}p<0.01$, $^{*}p<0.05$ alcohol vs naive).

Moreover, female msP rats developed significant mechanical allodynia during protracted abstinence compared with the alcohol group (Fig. 3) and presented a significantly lower level of 2-AG during abstinence compared with the alcohol group. Similar to males, 2-AG was slightly lower in female msP rats compared with female Wistar rats during abstinence (Fig. 5C), with a correlation trend between 2-AG and

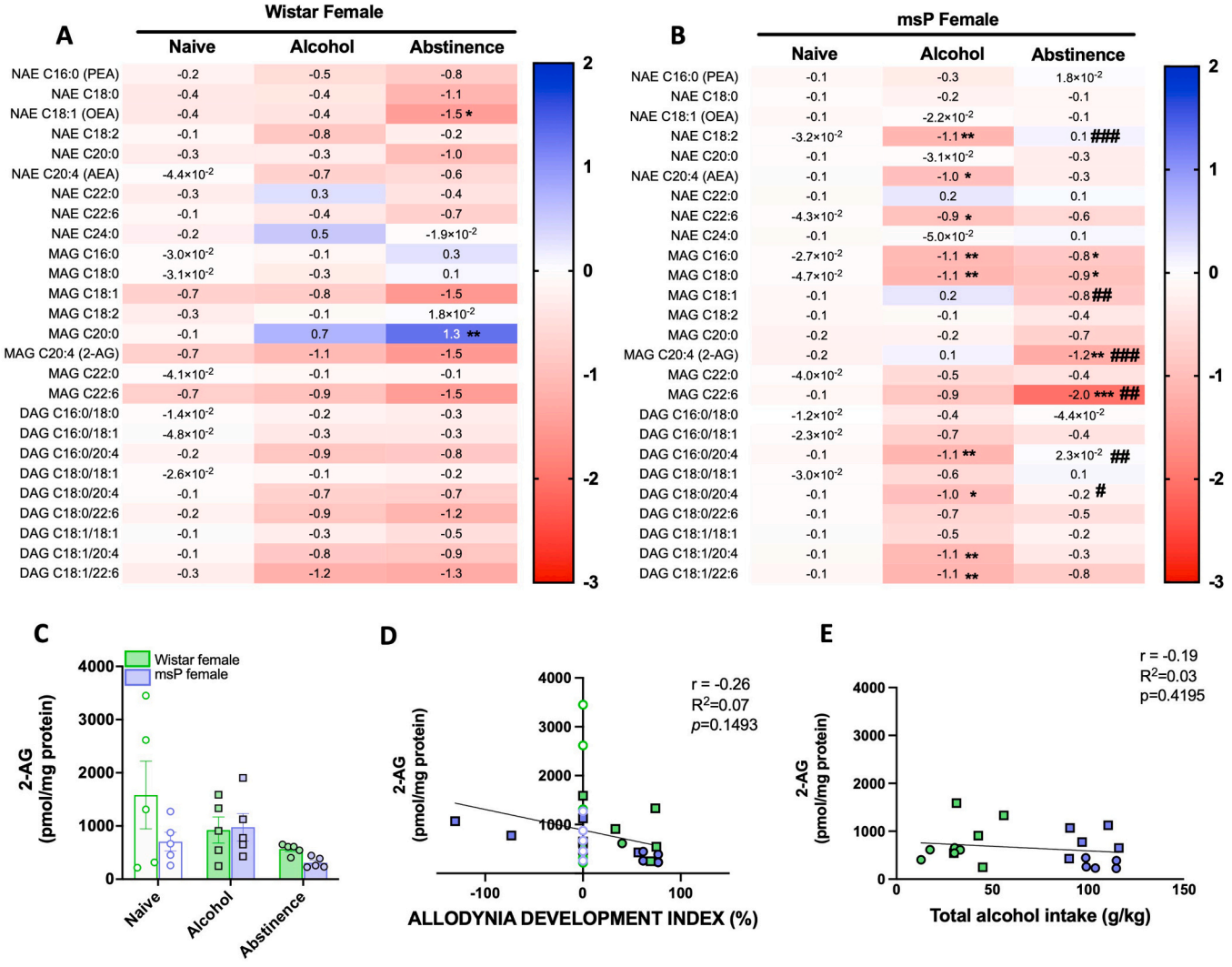


Fig. 5. (A) Endocannabinoid changes in lumbar dorsal root ganglia (DRG) in naive, alcohol, and abstinent female Wistar rats. The values were normalized to naive female Wst rats and are expressed as log2-based fold change vs. naive. The data were analyzed using two-way ANOVA followed by Tukey's multiple-comparison post hoc test ($F_{2,312} = 8.669$, $^{**}p < 0.01$, alcohol/abstinent vs. naive). (B) Endocannabinoid changes in DRG in naive, alcohol, and abstinent female msP rats. The values were normalized to naive female msP rats and are expressed as log2-based fold change vs. naive. The data were analyzed using two-way ANOVA followed by Tukey's multiple-comparison post hoc test ($F_{2,312} = 29.23$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$, alcohol/abstinent vs. naive; $^{\#\#\#}p < 0.001$, $^{\#\#}p < 0.01$, $^{\#}p < 0.05$, abstinent vs. alcohol). (C) Dorsal root ganglia 2-AG levels (pmol/mg protein) in female Wistar and msP rats. The data were analyzed using two-way ANOVA followed by Tukey's multiple-comparison post hoc test ($F_{2,24} = 1.187$). (D) Correlation between Allodynia Development Index (%) and 2-AG levels in female msP and Wistar rats. Pearson's correlation: $r = -0.26$, $R^2 = 0.07$, $p = 0.149$. (E) Correlation between total alcohol intake (g/kg) and 2-AG levels in female msP and Wistar rats. Pearson's correlation: $r = -0.19$, $R^2 = 0.03$, $p = 0.41$.

the Allodynia Development Index (Fig. 5D), but not between 2-AG and total alcohol intake (Fig. 5E). No differences between Wistar and msP rats were observed in PEA, OEA, or AEA levels (Supplementary Fig. 3S-D/E/F).

3.5. Dysregulation of PGE2/PGD2 and TXB2 levels in female msP rats

Arachidonic acid (omega-6) and docosahexaenoic acid (omega-3) represent critical components of neural synaptic membranes and they are byproducts of eCB metabolism. 2-Arachidonoylglycerol is hydrolyzed by MAGL and ABHD6 into AA, which can be oxidized by cyclooxygenase-2 (COX-2) to form prostaglandins (PGE2, PGD2) and TXB2 [59]. Arachidonic acid metabolism is one of the main enzymatic steps that is responsible for the inflammatory cascade [60].

Male Wistar rats exhibited a significant reduction (Fig. 6A; $^{*}p < 0.05$ abstinence vs. naive) of TXB2 during abstinence compared with naive. In contrast, male msP rats showed an elevation of TXB2 during abstinence ($^{*}p < 0.05$ abstinence vs. naive) and a reduction of PGE2/PGD2 during alcohol ($^{*}p < 0.05$ alcohol vs naive) compared with naive rats (Fig. 6B). However, no strain differences were observed (Supplementary Fig. 5S). No significant differences in AA or DHA levels were found among experimental conditions (naive, alcohol, and abstinence).

In female Wistar rats, we found an elevation of TXB2 levels after alcohol exposure and during abstinence compared with naive (Fig. 6C and H, $^{***}p < 0.001$, $^{2}p < 0.05$ alcohol/abstinence vs. naive). During abstinence, they also showed a significant reduction of PGE2/PGD2 compared with alcohol rats (Fig. 6C, $^{\#}p < 0.05$, abstinent vs. alcohol). Similarly, female msP rats exhibited a significant increase in TXB2 levels and a decrease in PGE2/PGD2 levels during protracted abstinence compared with naive ($^{**}p < 0.01$, abstinent vs. naive) and alcohol-exposure females (Fig. 6D, $^{\#}p < 0.05$, abstinent vs. alcohol).

We did not find any difference in PGE2/PGD2 (Fig. 6E) or TXB2 (Fig. 6H) levels between Wistar and msP rats. However, we observed a significant increase of TXB2 levels in abstinent Wistar females compared to naive (Fig. 6H, $^{**}p<0.01$ naive vs. abstinent Wistars). Overall, in female rats, low PGE2/PGD2 levels significantly correlated with the development of allodynia (Fig. 6F) but not with total alcohol intake (Fig. 6G). In contrast, high TXB2 levels correlated with high allodynia (Fig. 6I), and, similarly to PGE2/PGD2, did not correlate with alcohol intake (Fig. 6J).

4. Discussion

Extensive research suggests that chronic pain acts as a primary negative reinforcer of alcohol intake to buffer the symptoms experienced by AUD patients [3,4,61–63], particularly during alcohol abstinence. Although acute alcohol consumption can produce temporary analgesia, prolonged and excessive alcohol use increases pain sensitivity over time [4]. Importantly, pain represents a severe risk factor that prevents patients from recovering from AUD. Although alcohol abstinence-related pain is a highly observed condition in both preclinical and clinical studies, the mechanisms that underlie its occurrence are still unclear. In the present study, we found that msP rats, a genetically-selected alcohol-preferring rat line [64], developed significant mechanical allodynia during protracted abstinence from alcohol, which never returned to baseline levels. The lack of return to baseline suggests important permanent effects of excessive alcohol drinking on pain using this genetically-selected rodent line of inheritable anxiety and high alcohol intake. This effect returned to baseline levels in non-selected Wistar control rats after 5 days into their abstinence period, consistent with prior work [65]. This hypersensitivity to mechanical stimuli difference combined with msPs genetic predisposition implies greater response to external stressors, which may relate to their innate high preference for alcohol consumption as a form of stress-coping behavior [14]. Our laboratory employed voluntary 2BC drinking procedures wherein rats can freely choose between 10 % alcohol or water [16,51], allowing us to compare temporal effects of chronic alcohol intake across measures of allodynia. Chronic alcohol consumption has been shown to elicit peripheral nerve damage and increase neuroinflammation [66–70], regardless of the development of alcohol dependence. We observed this toxic effect in Wistars rats, in which chronic alcohol consumption for 5 consecutive weeks led to an increase in mechanical allodynia, particularly in females (~60 %). This toxic effect in Wistar rats directly correlated with total alcohol intake, in which rats that drank more developed higher allodynia. Consistent with our results, women are generally more sensitive to the side effects of chronic alcohol consumption and develop medical problems more easily [71,72]. Only a small percentage of male Wistar rats (~20 %) developed allodynia after chronic moderate alcohol consumption. Because we could not predict the number of Wistar rats that would develop allodynia, one limitation of the present study was the relatively low number of high-allodynia Wistar rats that we could follow during abstinence (Supplementary Fig. 2S). We did not observe any sex differences in msP rats.

The eCB system has been studied regarding chronic pain [73], and alterations of eCBs and CB receptors in msP rats have been reported. Specifically, msP rats have a higher level of CB_1 receptor mRNA expression than Wistar rats in several brain regions (including the frontoparietal cortex, caudate putamen, and hippocampus) [74], and CB_1 receptor antagonist administration can reduce their alcohol self-administration [24,75]. Moreover, msP rats have a lower baseline level of AEA in the CeA compared with Wistar rats, together with a higher activity of FAAH, the main responsible for AEA catabolism. No differences between msP and Wistar rats were observed in 2-AG levels in either CeA or prefrontal cortex (infralimbic and prelimbic subregions) [76]. The lack of changes in 2-AG levels are consistent with the lack of pharmacological effects of MAGL inhibitors in voluntary alcohol drinking [16].

Our previous work highlighted the important role of impaired eCB system in male and female msP rats [77]. However, there is no data about eCBs in peripheral tissues using the current drinking model and genetically selected line. Alcohol causes damage not only to the brain but also to several peripheral tissues, including DRG, which represent an important point for processing pain stimulus information from the periphery to central regions [78]. Despite this, there is a lack of evidence of how alcohol exposure modifies the physiological functions of DRG [69,79,80]. We quantified eCBs and congener lipids (i.e., NAEs, DAGs, and MAGs) in lumbar DRG in msP rats and assessed potential differences compared with Wistar rats both at baseline and after alcohol consumption. In male and female msP rats, 2-AG levels were decreased during protracted abstinence compared with naive and alcohol rats. Notably, we found a significant correlation between low DRG 2-AG levels and the development of mechanical allodynia that was induced by chronic alcohol consumption. Consistent with our results, a correlation between low plasma levels of 2-AG and pain sensitivity was previously reported in patients with neuromyelitis optica [81]. Moreover, the pharmacological inhibition of MAGL decreased mechanical allodynia in mice that were subjected to chronic constriction injury and paclitaxel-induced peripheral neuropathy [33]. Other studies reported that enhanced levels of 2-AG after 7–14 days from painful stimuli were detected in neuropathic pain models as a rapid response to restore normal activity [82]. To our knowledge, here we are the first to report significant changes in other endocannabinoid-related NAEs, MAGs and DAGs whose biological functions are still understudied and not well understood. Additionally, some of the changes are sex and/or strain-dependent and future studies will investigate their role. Interestingly, C20:4-containing DAGs (i.e., DAG C16:0/20:4 and DAG C18:0/20:4), the precursors of 2-AG, are reduced in msP females, but not males, during alcohol exposure and abstinence compared to naive, pointing to a possible overall downregulation of the 2-AG biosynthesis.

In the present study, we speculated that the downregulation of 2-AG might disrupt the synaptic transmission involved in mechanical allodynia, preventing recovery from alcohol-induced damage. CB_1 and CB_2

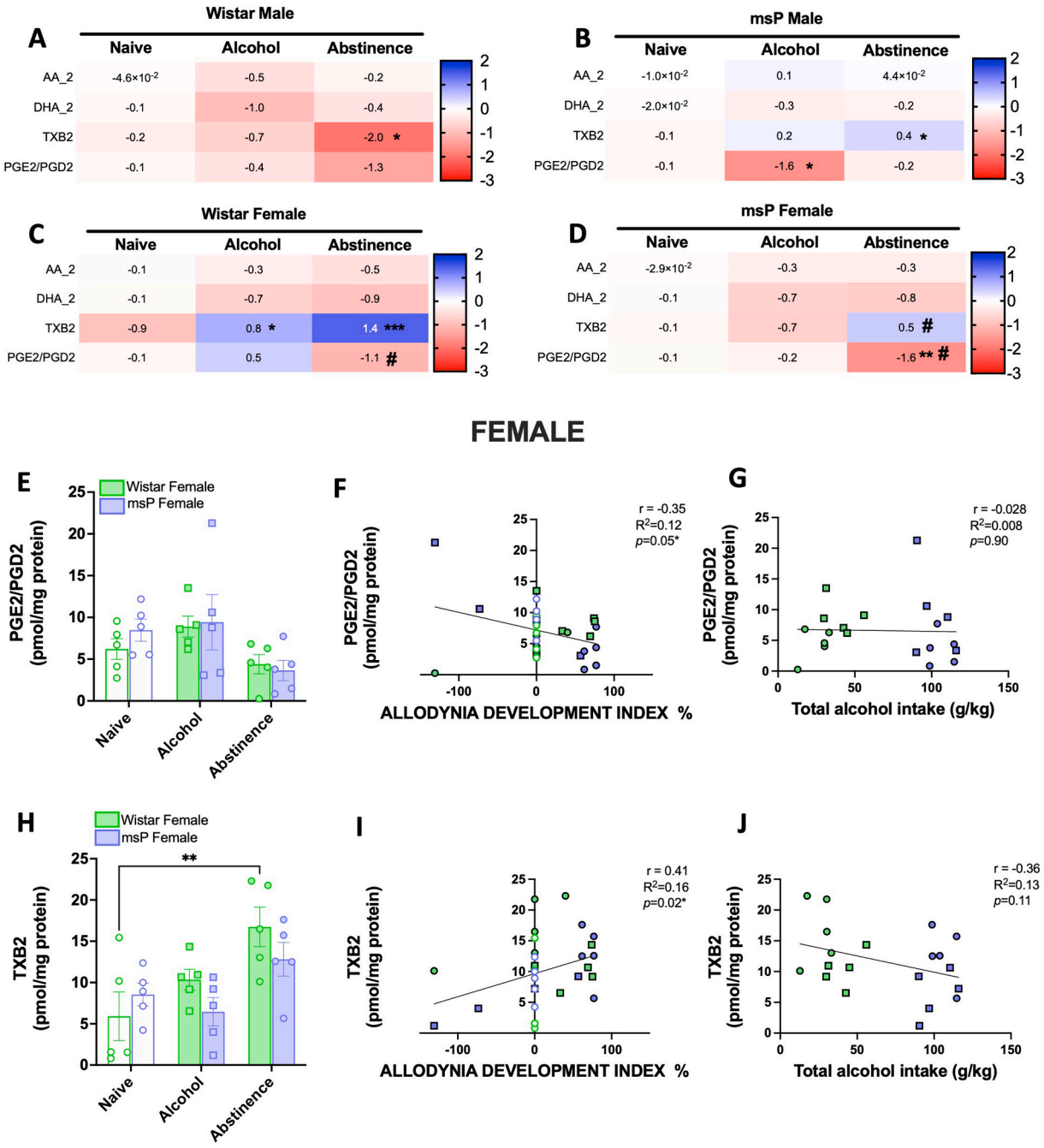


Fig. 6. (A) Arachidonic acid, docosahexaenoic acid (DHA), thromboxane B2 (TXB), and prostaglandin (PGE2/PGD2) changes in DRG in naive, alcohol, and abstinent male Wistar rats. The values were normalized to naive male Wistar rats and are expressed as log2-based fold change vs. naive. The data were analyzed using two-way ANOVA followed by Tukey's multiple-comparison post hoc test ($F_{2,44} = 4.164$, *$p < 0.05$, alcohol/abstinent vs. naive). (B) AA_2, DHA, TXB, and PGE2/PGD2 changes in DRG in naive, alcohol, and abstinent male msP rats. The values were normalized to naive male msP rats and are expressed as log2-based fold change vs. naive. The data were analyzed using two-way ANOVA followed by Tukey's multiple-comparison post hoc test ($F_{2,48} = 1.404$, *$p < 0.05$, alcohol/abstinent vs. naive). (C) AA_2, DHA, TXB, and PGE2/PGD2 levels in DRG in naive, alcohol, and abstinent female Wistar rats. The values were normalized to female naive Wistar rats and are expressed as log2-based fold change vs. naive. The data were analyzed using two-way ANOVA followed by Tukey's multiple-comparison post hoc test ($F_{2,48} = 0.96$, ***$p < 0.001$, *$p < 0.05$, alcohol/abstinent vs. naive; #$p < 0.05$, abstinent vs. alcohol). (D) AA_2, DHA, TXB, and PGE2/PGD2 changes in DRG in naive, alcohol, and abstinent female msP rats. The values were normalized to naive female msP rats and are expressed as log2-based fold change vs. naive. The data were analyzed using two-way ANOVA followed by Tukey's multiple-comparison post hoc test ($F_{2,48} = 2.58$, **$p < 0.01$, alcohol/abstinent vs. naive; #$p < 0.05$, abstinent vs. alcohol). (E) Dorsal root ganglia PGE2/PGD2 levels (pmol/mg protein) in female Wistar and msP rats. The data were analyzed using two-way ANOVA followed by Tukey's multiple-comparison post hoc test. (F) Correlation between Allodynia Development Index (%) and PGE2/PGD2 levels in female msP and Wistar rats. Pearson's correlation: $r = -0.35$, $R^2 = 0.12$, *$p = 0.05$. (G) Correlation between total alcohol intake (g/kg) and PGE2/PGD2 levels in female msP and Wst rats. Pearson's correlation: $r = -0.028$, $R^2 = 0.006$, $p = 0.90$. (H) Dorsal root ganglia TXB2 levels in female Wistar and msP rats. The data were analyzed using two-way ANOVA followed by Tukey's multiple-comparison post hoc test ($F_{2,24} = 1.709$, **$p < 0.01$). (I) Correlation between Allodynia Development Index (%) and TXB2 levels in female msP and Wistar rats. Pearson's correlation: $r = 0.41$, $R^2 = 0.16$, *$p = 0.02$. (J) Correlation between total alcohol intake (g/kg) and TXB2 levels in female msP and Wistar rats. Pearson's correlation: $r = -0.36$, $R^2 = 0.13$, $p = 0.11$.

receptors, along with other key receptors that are involved in neuropathic pain [54], are 2-AG targets, and their modulation could be related to the development of allodynia during abstinence. Thus, we hypothesize that 2-AG metabolism is an interesting pharmacological target for treating alcohol-induced allodynia. Future studies will investigate the cell-specificity (glia vs. neurons) of altered 2-AG levels after alcohol intake to determine differences in cells that synthesize or release 2-AG. Additionally, 2-AG is an important intermediate of lipid mediators' metabolism. Monoacylglycerol lipase, ABHD6, and ABHD12 convert 2-AG into AA [83], making 2-AG a key lipid intermediate in the inflammatory cascade. Arachidonic acid-derived eicosanoids (i.e., prostaglandins G2 and H2) are produced by COX-2 activity. They can then be further metabolized into PGE2, PGD2, prostaglandin I2, and TXB2 by a selective prostaglandin synthase. All these mediators are involved in the inflammatory cascade, and most of them are associated with pain development [82].

Central and peripheral inflammation is a crucial pathway that is involved in the progression of neuropathic pain. In the present study, we found sex-dependent alterations of DRG eicosanoid levels. Alcohol-exposed and abstinent msP and Wistar females but not males exhibited significant alterations of TXB2 and PGE2/PGD2 compared with naive rats. The reduction of PGE2/PGD2 in female msP rats during protracted abstinence compared with alcohol-naive and the alcohol-exposed group is very interesting. Although a general increase in PGE2 has been observed in different pain models [84], Bakooshli and colleagues showed that inhibition of the prostaglandin-degrading enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH) promoted motor and axon regeneration, together with muscle force improvement, in aged mice with chronic muscle denervation [85]. Alcohol causes damage to the sciatic nerve [10], DRG [69], and the spinal cord [11,12,86], which are tissues that are deeply involved in the process of myelination and axonal regeneration. Therefore, we hypothesize that the reduction of PGE2/PGD2 is related to an increase in the activity of 15-PGDH. Future studies will test this hypothesis.

In conclusion, the present study found that excessive alcohol consumption, which is typical of msP rats, induced significant mechanical allodynia that developed rapidly into their abstinence period and persisted without recovering. Wistar rats that consumed alcohol chronically but not excessively exhibited the development of allodynia during their drinking phase. Additionally, alterations of DRG 2-AG levels were found in msP rats during prolonged abstinence, which was directly associated with the development of mechanical allodynia, thus, making 2-AG metabolism an interesting pharmacological target for the treatment of mechanical allodynia associated with alcohol abstinence.

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CRediT authorship contribution statement

Valentina Vozella: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Vittoria Borgonetti:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Bryan Cruz:** Writing – review & editing, Investigation. **Tim Ware:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Data curation. **Benjamin F. Cravatt:** Writing – review & editing, Supervision, Resources. **Ryan Bullard:** Investigation, Data curation. **Marisa Roberto:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Nicoletta Galeotti:** Writing – review & editing, Supervision, Conceptualization.

Declaration of Competing Interest

None

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2024.107462.

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