Received: 5 December 2022 | Revised: 5 April 2023 | Accepted: 6 April 2023

DOI: 10.1111/bph.16091

RESEARCH ARTICLE

Chronic alcohol induced mechanical allodynia by promoting neuroinflammation: A mouse model of alcohol-evoked neuropathic pain

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Funding information

The Scripps Research Institute; National Institutes of Health grants, Grant/Award Numbers: AA006420, AA013498, AA017447, AA021491, AA027700, AA029841

Abstract

Background and Purpose: Chronic pain is considered a key factor contributing to alcohol use disorder (AUD). The mechanisms responsible for chronic pain associated with chronic alcohol consumption are unknown. We evaluated the development of chronic pain in a mouse model of alcohol dependence and investigate the role of neuroinflammation.

Experimental Approach: The chronic-intermittent ethanol two-bottle choice CIE-2BC paradigm generates three groups: alcohol-dependent with escalating alcohol intake, nondependent (moderate drinking) and alcohol-naïve control male and female mice. We measured mechanical allodynia during withdrawal and after the last voluntary drinking. Immunoblotting was used to evaluate the protein levels of IBA-1, CSFR, IL-6, p38 and ERK2/1 in spinal cord tissue of dependent and non-dependent animals.

Key Results: We found significant escalation of drinking in the dependent group in male and female compared with the non-dependent group. The dependent group developed mechanical allodynia during 72 h of withdrawal, which was completely reversed after voluntary drinking. We observed an increased pain hypersensitivity compared with the naïve in 50% of non-dependent group. Increased IBA-1 and CSFR expression was observed in spinal cord tissue of both hypersensitivity-abstinence related and neuropathy-alcohol mice, and increased IL-6 expression and ERK1/2 activation in mice with hypersensitivity-related to abstinence, but not in mice with alcohol-evoked neuropathic pain.

Conclusions and Implications: The CIE-2BC model induces two distinct pain conditions specific to the type of ethanol exposure: abstinence-related hypersensitivity in dependent mice and alcohol-evoked neuropathic pain in about a half of the non-dependent mice.

Abbreviations: 2BC, two-bottle choice; 2BC-N, two-bottle choice with neuropathic pain; CIE-2BC, chronic-intermittent ethanol two-bottle choice; IBA-1, ionized calcium-binding adapter molecule-1; MAPKs, mitogen-activated protein kinases.

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KEYWORDS
alcohol, alcohol use disorder, alcohol-evoked neuropathic pain, microglia, neuroinflammation

1 | INTRODUCTION

Alcohol use disorder (AUD) is a serious public health concern. It is a multifactorial condition characterized by genetic, environmental and neurobiological components (Cui et al., 2015). Epidemiological data indicate a strong association between chronic pain and alcohol use disorder (Beasley et al., 2016). More than half of alcohol use disorder patients experience significant persistent pain, with a higher prevalence in women (about 60%) than in men (about 50%) (Boissoneault et al., 2019). The increase of pain in alcohol use disorders is likely due to multiple factors that occur at different times from the first exposure of **alcohol** (Robins et al., 2019).

Clinical investigations show that alcohol use can cause increased pain both acutely, during alcohol withdrawal (Jochum et al., 2010) and chronically through alcoholic neuropathy (Monforte et al., 1995). During withdrawal in dependent individuals, allodynia, defined as harmless stimuli being perceived as painful (Jensen & Finnerup, 2014), is considered as an abstinence-related pain hypersensitivity and is a negative emotional state caused by alcohol dependence (Egli et al., 2012). Generally, patients suffering from allodynia drink alcohol to reduce pain (Brennan et al., 2005; Riley & King, 2009). On the other hand, an alcoholic neuropathy may not be associated with the development of dependence, but results from chronic consumption of alcohol (Beasley et al., 2016; Macfarlane et al., 2015). Alcoholic peripheral neuropathy is associated with a significant decrease in quality of life and a decrease in nociceptive threshold (Chopra & Tiwari, 2012; Dina et al., 2006). The mechanisms mediating chronic alcohol-induced allodynia and neuropathy are controversial (Edwards et al., 2020). The rat models of the chronic ethanol exposure reported mechanical allodynia (Edwards et al., 2012) and hyperalgesia (Avegno et al., 2018; Roltsch Hellard et al., 2017) in the dependent male rats undergoing withdrawal. Recent mouse studies have reported sex- and time course-specific mechanical and heat hyperalgesia during the alcohol withdrawal (Brandner et al., 2023).

Regardless of ethanol exposure (e.g. liquid diet, oral gavage and ethanol vapour), ethanol dependence in rodents is induced by prolonged repeated cycles of ethanol exposure and interspersed withdrawals that lead to the emergence of negative emotional state (Becker & Lopez, 2004; Patel et al., 2021, 2022; Vendruscolo & Roberts, 2014). Our group has extensively employed the chronic intermittent ethanol vapour paradigm (Rogers et al., 1979) of alcohol dependence in both rats (Khom, Steinkellner, et al., 2020; Khom, Wolfe, et al., 2020; Varodayan et al., 2017; Varodayan et al., 2022) and mice (Bajo et al., 2014, 2019; Patel et al., 2019, 2021, 2022; Warden et al., 2020). In this study, we used the chronic intermittent ethanol vapour–two-bottle choice (CIE-2BC) (Becker & Lopez, 2004) paradigm that mimics different phenotypes in alcohol use disorder including alcohol dependence. A strength of this established CIE-2BC paradigm is that it generates three experimental groups: - (1) alcohol-dependent mice, characterized by escalation of their ethanol drinking, a hallmark of dependence, (2) non-dependent mice, mimicking moderate drinking and (3), alcohol-naïve control mice with no alcohol exposure (Bajo et al., 2014, 2019). This paradigm enables us to study mechanical allodynia (Bosch et al., 1978; Cobos et al., 2018; Tiwari et al., 2009, 2011), alcohol-evoked neuropathic pain and activation of the immune response in the spinal cord (Cobos et al., 2018; Grace et al., 2021), not only in the mice that develop alcohol dependence, but also in those mice consuming chronically alcohol without development of dependence in both sexes.

Although the spinal cord represents an important gateway for transporting peripheral signals to brain level areas (Todd, 2010), much work on roles of microglia in alcohol induced pain pathology has focused on supraspinal CNS area (Robins et al., 2019). Microglia is deeply involved in the pathogenesis of neuropathic pain (Inoue & Tsuda, 2018; Tsuda et al., 2005) and microgliosis participates in the communication between spinal and supraspinal tissue in the development of chronic alcohol pain (Kohno et al., 2022), as observed in other chronic pain conditions (Borgonetti & Galeotti, 2023; Guan

What is already known?

- Unrelieved chronic pain is considered a key factor to the maintenance of alcohol use disorder.
- Urgent need for predictive preclinical model of produced by excessive alcohol consumption.

What does this study add?

- Mouse model of two pathological pain conditions associated with alcohol use disorder was used.
- Abstinence-related allodynia and alcohol-evoked neuropathic pain in both male and female animals.

What is the clinical significance?

- Development of a preclinical model that is predictive of alcohol-related chronic pain
- Targeting IL-6 and microglial activation might be used to alleviate alcohol-evoked neuropathic pain and hyperalgesia.

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et al., 2015; Lee & Kim, 2020; Liu & Quan, 2018). Notably, we recently used the CIE-2BC model to demonstrate that microglia in the central nucleus of the amygdala is critical for the development and progression of alcohol use disorder. Hence, we investigated the role of microglia on the development of chronic alcohol-induced allodynia and neuropathy at the level of the spinal cord tissue.

2 | METHODS

2.1 | Animals

We used adult male and female C57BL/6J mice (10–11 weeks old, male 27.3 g–female 19.8 g at the start of the paradigm; Jackson Laboratories, n = 92). All mice were housed 3–4 per cage in individually ventilated caging (IVC) under sanitary conditions in light (12 h on, 12 h off) and temperature (21 ± 2°C) controlled vivarium rooms with food (Bedding: #12000 Newco Virgin Aspen Hardwood Shavings) and water available *ad libitum*. Lights on 8:00 PM, off 8:00 AM. All procedures involving the housing and use of experimental animals were approved by the Scripps Research Institutional Animal Care and Use Committee (IACUC) and National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

2.2 | Alcohol exposure

Three groups of C57BL/6J mice were produced with differing alcohol experience: - alcohol naïve, alcohol experienced but not dependent (two-bottle choice, 2BC) and alcohol dependent (chronic intermittent ethanol two-bottle choice, CIE-2BC). Previous studies have shown that CIE-2BC mice present signs of dependence relative to the other groups, including increased alcohol drinking and effects on behaviour and sleep (Bajo et al., 2019; Bloch et al., 2022; Huitron-Resendiz et al., 2018; Okhuarobo et al., 2022; Patel et al., 2021; Sidhu et al., 2018; Warden et al., 2020). For this study we used two cohorts of animals: - (*i*) both males and females (Cohort 1, n = 56) and (*ii*) only male C57BL/6J mice (Cohort 2, n = 36). 2BC and CIE-2BC mice were exposed to a limited-access alcohol (15% w/v) 2BC paradigm, followed by either CIE exposure in vapour chambers to induce alcohol dependence or air exposure in identical chambers. To establish baseline drinking, 2BC testing was performed 5 days per week for four consecutive weeks. The mice were singly housed 30 min before lights off and given 2-h access to two drinking tubes (15% alcohol and water). Following this baseline phase, the mice were divided into two balanced groups with equal alcohol and water consumption. Mice in the CIE-2BC group were injected i.p. with 1.75-g·kg^{-1} alcohol + 68.1-mg·kg^{-1} pyrazole (alcohol dehydrogenase inhibitor) and placed in alcohol vapour chambers for 4 days (16 h vapour on, 8 h off). These pyrazole + alcohol injections prior to each exposure to the vapour chambers stabilizes blood alcohol levels in the target range and decreases variability across exposure and between mice (Griffin, 2014). Blood alcohol levels (BALs) were determined immediately upon vapour chamber removal on the third day of exposure. Alcohol drip rates were manipulated to maintain blood alcohol levels of 150–200 mg·dl^{-1}. Naïve and non-dependent (2BC) mice were injected with 68.1 mg·kg^{-1} pyrazole in saline and placed into identical caging and exposed to air. No analgesia was used for the ethanol + pyrazole or pyrazole only injections as this would interfere with the response to alcohol.

Vapour/air exposure was followed by 72 h of abstinence and 5 days of 2BC testing. This regimen was repeated four additional times for a total of five full rounds. Before killing, CIE-2BC mice were exposed to a single alcohol vapour exposure (16 h) and tail blood was collected to determine terminal blood alcohol levels (Figure 1a). Euthanasia of the mice was performed according to an approved animal protocol, with the being mice deeply anaesthetised with 3%–5% isoflurane and rapidly decapitated.

2.3 | von Frey test

Mechanical allodynia was measured using von Frey filaments with ascending force as previously reported (Borgonetti & Galeotti, 2021). This method is based on the application of monofilaments (0.04, 0.07, 0.16, 0.4, 0.6, 1.0, 1.4 and 2.0 g) to the plantar surface of both hind paws, with increasing force until a withdrawal response is elicited. The force of von Frey filaments that elicits this positive response is designated as the mechanical withdrawal threshold. If the paw was not lifted after 5 s, we used the next filament with an increased force. Four measurements were collected for each mouse.

The measurements were conducted 8, 24, 48 and 72 h after the last vapour session and immediately after 2BC test. We chose a threshold value of 1, which was calculated by taking the averages of the naïve group's mechanical threshold during the entire time course of withdrawal. Mice with lower mechanical thresholds at three of four different timepoints were assigned to the neuropathy group (2BC-N).

2.4 | Tissue lysate

The mice were anaesthetised with 3%–5% isoflurane and rapidly decapitated, and the spinal cord and both sciatic nerves were rapidly removed, snap-frozen in isopentane, and stored at −80°C (Borgonetti & Galeotti, 2021). Samples were homogenized in RIPA buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% sodium deoxycolate, 1% Triton X-100, and 2 mM phenylmethylsulfonyl fluoride) with 5-mM ethylene glycol tetra-acetic acid and protease inhibitors (1:100). The homogenate was centrifuged at 12,000 × *g* for 30 min at 4°C and the pellet was discarded. Tissue homogenates were heated at 95°C for 5 min and the total protein concentration was measured using the DC protein assay (Bio-Rad, Hercules, CA, USA). The samples were aliquoted and stored at −80°C until use.

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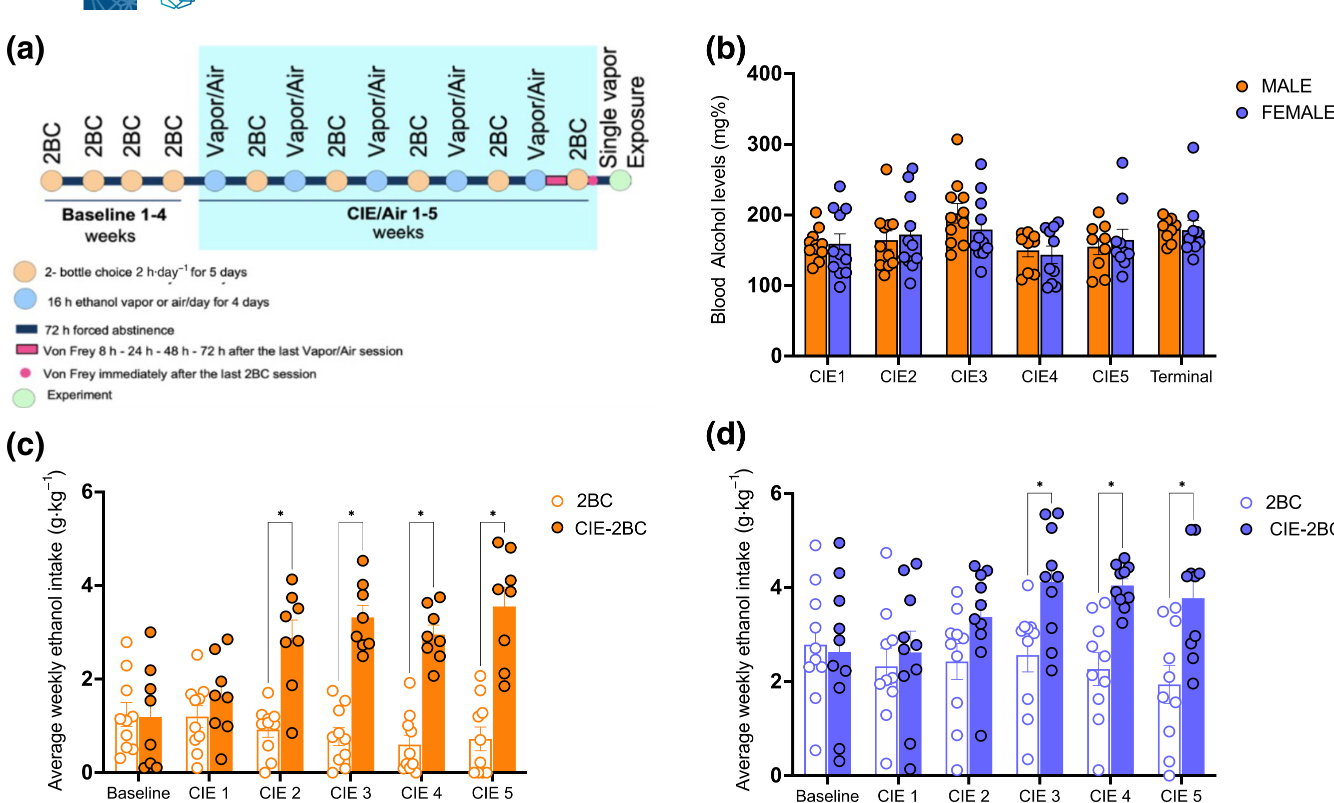

FIGURE 1 (a) Schematic representation of chronic intermittent ethanol two-bottle choice (CIE-2BC) paradigm. (b) Average blood alcohol levels achieved during weeks of CIE vapour exposure in dependent female and male mice (CIE-2BC). (c, d) Average alcohol intake during two-bottle choice (2BC) sessions in the final baseline week and following rounds of air or CIE vapour exposure. (c) Male 2BC and CIE-2BC mice (two-way ANOVA followed by Sidak's multiple-comparison post hoc test, $F_{5,96} = 9.72$, $P < 0.05$). (d) Female 2BC and CIE-2BC mice (two-way ANOVA followed by Sidak's multiple-comparison post hoc test, $F_{5,108} = 2.422$, $^*P < 0.05$).

2.5 | Western blot analysis

Western blot analyses were conducted to evaluate changes in protein levels in the spinal cord and sciatic nerve in mice as previously described (Borgonetti & Galeotti, 2021). Briefly, protein samples (40 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 12% acrylamide gels using a Tris/Tricine/SDS buffer system (ThermoFisher). The gels were electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were blocked for 2 h in 5% non-fat milk at room temperature and incubated overnight in 5% non-fat milk with primary antibody at 4°C. The primary antibodies included mouse macrophage-colony stimulation factor, a receptor involved in signalling necessary for microglia viability (Elmore et al., 2014) (colony stimulating factor 1 receptor [CSFR]; 1:1000; Abcam, catalogue no. 1876-1, RRID:AB_765017), ionized calcium-binding adapter molecule-1 (IBA-1) also known as allograft inflammatory factor 1 (AIF-1), microglia-marker calcium-binding protein (Lituma et al., 2021) (IBA-1; 1:500; Abcam, catalogue no. ab153696, RRID:AB_2889406), the mitogen-activated protein kinases (MAPKs) phosphorylated **p38 mitogen-activated protein kinase**, (p-p38; 1:1000; Cell Signaling Technology, catalogue no.4511, RRID:AB_2139682), total p38 (t-p38; 1:1000; Cell Signaling Technology, catalogue no.9212, RRID:AB_330713), phosphorylated extracellular signal regulated kinase 44/42, **mitogen-activated protein kinase** 44/42 (p-ERK44/42; 1:1000; Cell Signaling Technology, catalogue no.9101, RRID:AB_2315112), total ERK44/42 (t-ERK2/3; 1:1000; Cell Signaling Technology, catalogue no.5013, RRID:AB_10693607) that regulated diverse cellular programs by relaying extracellular signals to intracellular responses (Ji et al., 2009), interleukin 6, a pleiotropic cytokine described in a wide range of pathological conditions as a potent inducer and modulator of microglial activation (Zhou et al., 2016) (IL-6; 1:500, Abcam, catalogue no.ab6672, RRID:AB_2127460) and S100, the most widely used marker of myelination state of peripheral nerves (de Logu et al., 2019) (1:500, Abcam, catalogue no.ab109494, RRID:AB_10859000). Membranes were washed and incubated with species-specific peroxidase-conjugated secondary antibody (1:10,000; Jackson ImmunoResearch) for 2 h at room temperature. Membranes were washed, incubated in chemiluminescent reagent and exposed to film. Following film development, membranes were stripped for 30 min at room temperature to reprobe the blot with another antibody (Restore, Thermo Scientific Fisher**)**. The signal intensity (pixels·mm^{-2}) was quantified using ImageJ software (National Institute of Health, Bethesda, MD, USA). The p38 activation state (a-p38) and activated ERK (a-ERK) was calculated making the ratio between phosphorylated (p)-form/total (t)-form. The signal intensity was normalized to GAPDH (1:5000, Cell Signaling Technology). The Immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

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2.6 | Statistical analysis

The data and statistical analysis comply with the recommendations and requirements of the *British Journal of Pharmacology* on experimental design and analysis (Curtis et al., 2018).

We used Prism 9.0 software (GraphPad, San Diego, CA, USA) for the statistical analyses. Sample sizes subjected to statistical analysis was at least 7 animals per group. The group size (n), where n = number of independent values, in different experiment for each group *in vivo* and *in vitro*, is reported in the figure legends and in the results section. All experimental results are expressed as the mean ± SEM.

Student's *t* test and one- or two-way analyses of variance (ANOVAs) followed by appropriate post hoc tests (Sidak's multiple-comparison test or Tukey's multiple-comparison test, respectively) were used and marked in the figure legend for each experiment. The statistical significance criterion was $^{*}P < 0.05$.

2.7 | Materials

Animal bedding and food (#7090A) were obtained from Harlan Teklad Aspen Sani-Chip Bedding (West Lafayette, IN, USA). Vapour chambers were obtained from La Jolla Alcohol Research (La Jolla, CA, USA). RIPA buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 1% sodium deoxycolate, 1% Triton X-100 and 2 mM phenylmethylsulfonyl fluoride) with 5-mM ethylene glycol tetra-acetic acid were obtained from Sigma-Aldrich (St. Louis, MO, USA) along with protease inhibitors, ethanol, isoflurane and pyrazole (alcohol dehydrogenase inhibitor). Polyvinylidene difluoride membranes were obtained from GE Healthcare (Piscataway, NJ, USA), while chemiluminescent reagent was obtained from Immobilon Crescendo Western HRP Substrate (Millipore, Billerica, MA, USA). Details of other materials and suppliers were provided in the specific sections.

2.8 | Nomenclature of targets and ligand

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY https://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander et al., 2021).

3 | RESULTS

3.1 | Chronic intermittent ethanol two-bottle choice (CIE-2BC) model generated alcohol-dependent mice

We used the CIE-2BC paradigm to generate alcohol dependence in C57BL/6J mice (Becker & Lopez, 2004) with the timeline depicted in Figure 1a. In this first study, Cohort 1 generated three different groups of animals: - (1) naïve mice (n = 7 males, seven females) that did not receive alcohol, (2) non-dependent mice (2BC; n = 10 males, 10 females) that received only 2BC and (3) dependent mice (CIE-2BC; n = 9 males, 10 females) that received CIE-2BC. CIE-2BC mice achieved average blood alcohol levels across the four rounds of CIE of 179.82 ± 5.52 and 178.505 ± 13.97 $mg{\cdot}dl^{-1}$ for males and females, respectively (Figure 1b). A significant escalation of drinking, a marker of a dependence-like phenotype (Bajo et al., 2014, 2019; Becker & Lopez, 2004; Patel et al., 2019), was observed in the CIE-2BC group compared to the 2BC group of both sexes, with an average daily ethanol intake during the last round of 2BC in CIE-2BC mice of 3.412 ± 0.39 $g{\cdot}kg^{-1}$ for males (Figure 1c) and 3.716 ± 0.42 $g{\cdot}kg^{-1}$ for females (Figure 1d) compared with 0.724 ± 0.25 $g{\cdot}kg^{-1}$ (Figure 1c) for male and 1.942 ± 0.407 $g{\cdot}kg^{-1}$ for female (Figure 1d) 2BC mice.

3.2 | Chronic intermittent ethanol two-bottle choice model induced mechanical allodynia-related to abstinence in dependent male and female mice

We used von Frey filaments to measure the development of mechanical allodynia in this model. Male dependent (CIE-2BC) mice lowered mechanical thresholds after 8 h (0.404 ± 0.29 g) of abstinence and remained stable with a non-significant trend of further reducing the threshold hitch at 24 h (0.364 ± 0.17 g), 48 h (0.310 ± 0.14 g) and 72 h (0.281 ± 0.14 g; Figure 2a) of the abstinence compared to naïve mice (8 h: 1.34 ± 0.47 g; 24 h: 1.6 ± 0.40 g; 48 h: 1.11 ± 0.30 g; 72 h: 1.46 ± 0.45 g; Figure 2a).

When we tested the mechanical thresholds following the 2BC (voluntary drinking), the dependent male mice drinking alcohol during the 2BC period and the mechanical threshold increased to the levels found in the non-dependent mice (0.756 ± 0.41 g), but still lower compared to the naïve males (Figure 2a).

Dependent female mice showed significant reduction in the mechanical thresholds as early as 8 h (CIE-2BC: 0.276 ± 0.051 g Figure 2b) of abstinence that persisted at 24 h (CIE-2BC: 0.295 ± 0.044 g Figure 2b), 48 h (CIE-2BC: 0.295 ± 0.044 g Figure 2b) and 72 h (CIE-2BC: 0.282 ± 0.057 g; Figure 2b). After the last session of 2BC, acute alcohol drinking (2BC) increased the mechanical thresholds produced by abstinence in dependent females, with values comparable to the 2BC group (1.040 ± 0.15 g; Figure 2b). Notably, although there were no significant differences in mechanical allodynia between dependent males and females during abstinence (Figure 2c), dependent females showed a stronger significance ($P = 0.0211$) in the reversal of mechanical allodynia compared to males immediately after ethanol drinking (Figure 2c). Interestingly, the ethanol intake of female mice, measured immediately before von Frey measurement, was significantly higher than in males ($P = 0.0489$; *male:* 2.77 ± 0.25 $g{\cdot}kg^{-1}$ *female:* 4.03 ± 0.50; Figure 2d), suggesting that the ethanol drinking effects could correlate with the amount of alcohol ingested.

From these results, male and female dependent mice developed a hypersensitivity to mechanical stimulus-related to abstinence, which is reversed by alcohol drinking.

FIGURE 2 (a–c) Measurement of mechanical allodynia with von Frey filaments in naïve, two-bottle choice (2BC) and chronic intermittent ethanol vapour–two-bottle choice (CIE-2BC mice). (a) Male mice (two-way ANOVA followed by Tukey's multiple-comparison post hoc test, $F_{8,113} = 1.837$, $^*P < 0.05$). (b) Female mice (two-way ANOVA followed by Tukey's multiple-comparison post hoc test, $F_{8,120} = 3.195$, $^*P < 0.05$). (c) Comparison of mechanical allodynia between male and female CIE-2BC mice during withdrawal and after the final 2BC test (two-way ANOVA followed by Sidak's multiple-comparison post hoc test, $F_{4,84} = 2.632$, $^*P < 0.05$). (d) Ethanol intake during the 2BC performed before von Frey measurement in female and male CIE-2BC (Student's unpaired *t* test, $^*P < 0.05$).

3.3 | Dependent male mice developed a long-lasting mechanical allodynia during abstinence

To confirm the results related to allodynia from Cohort 1, we repeated the CIE-2BC model in a second cohort of male mice (Cohort 2) and replicated the escalated drinking in dependent (CIE-2BC) mice (n = 15) compared with non-dependent (2BC) mice (n = 21). In Cohort 2, we used only male mice because in Cohort 1 no significant differences in mechanical allodynia were observed during the withdrawal phase between male and female mice. CIE-2BC mice achieved an average blood alcohol levels across the four rounds of vapour of 175 ± 7.87 $mg \cdot dl^{-1}$ (Figure 3a). CIE-2BC mice increased weekly alcohol intake across weeks, reaching levels of 2.30 ± 0.30 $g \cdot kg^{-1}$ (Figure 3b). In Cohort 2, we wanted to recapitulate the mechanical hypersensitivity produced by this model in dependent mice. Similarly, to Cohort 1 male dependent (CIE-2BC) mice developed mechanical allodynia after 8 h (0.304 ± 0.03 g, Figure 3c) of abstinence, which remained stable until the 72 h (0.220 ± 0.03 g; Figure 3c) of abstinence.

3.4 | CIE-2BC induces microgliosis and mitogen-activated protein kinase activation in spinal cord of male and female mice

After the *in vivo* experiments, we tested our hypothesis that CIE-2BC-induced mechanical hypersensitivity (allodynia) is mediated by the neuroimmune response, particularly by activated microglia in the spinal cord. Therefore, we examined protein levels of IBA1 and **colony stimulating factor 1 receptor (CSFR)** markers of microglial cells and microglial intracellular signalling (Guan et al., 2015; Lituma et al., 2021), respectively, in the spinal cord in mice from both Cohorts 1 and 2. Our results showed that dependent mice (CIE-2BC) exhibited significantly increased expression of IBA1 (Figure 4a) and CSFR (Figure 4b) compared with naïve mice. Moreover, protein levels of pro-inflammatory **interleukin 6 (IL-6**; Figure 4c) were increased in dependent mice compared with naïve and non-dependent mice.

Focusing on microglial mitogen-activated protein kinase (MAPK), we did not observe differences between the 2BC and CIE-2BC groups in phosphorylated-p38 (p-p38) expression, but we detected an

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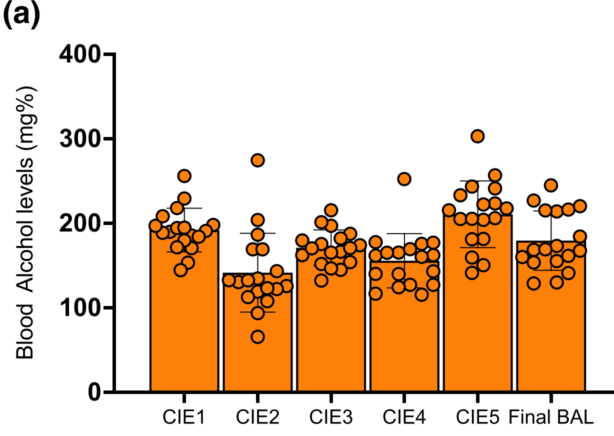

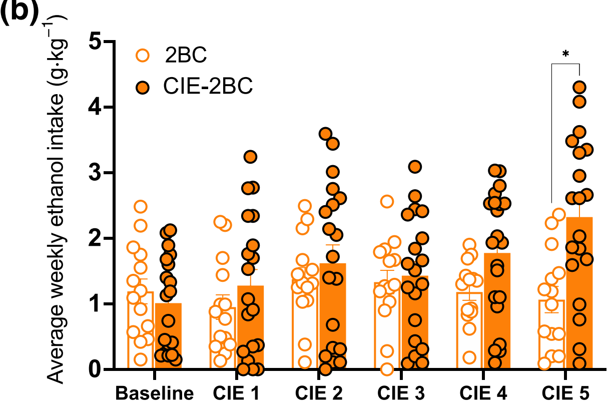

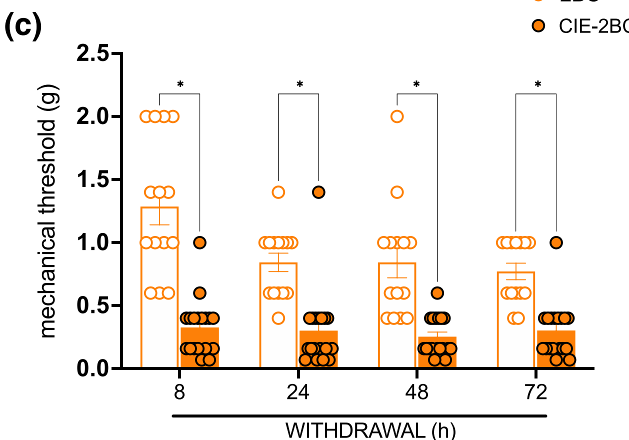

FIGURE 3 Second cohort of male mice developed mechanical allodynia in chronic intermittent ethanol vapour–two-bottle choice (CIE-2BC) model. (a) Blood alcohol levels in a second cohort of male mice in the CIE-2BC exposure paradigm. (b) Average alcohol intake in two bottle choice (2BC) sessions during the final baseline week and following air or CIE vapour exposure weeks in 2BC and CIE-2BC male mice (two-way ANOVA followed by Sidak's multiple-comparison post hoc test, $F_{5,188} = 2.441$, *$P < 0.05$). (c) Measurement of mechanical allodynia with von Frey filaments in male 2BC and CIE-2BC mice (two-way ANOVA followed by Tukey's multiple-comparison post hoc test, $F_{6,148} = 2.824$, *$P < 0.05$).

increase in total-p38 (t-p38) expression in both non-dependent and dependent mice compared with naïve mice (Figure 4d). Similarly, we found no significant changes in phosphorylated-ERK42 or phosphorylated-ERK44 expression in either dependent or non-dependent mice (Figure 4e,f). We observed a significant increase in total-ERK42 and total-ERK44 protein levels in the dependent mice compared with the non-dependent and naïve mice (Figure 4e,f).

3.5 | Chronic ethanol consumption induced neuropathy in 40–50% of 2BC mice

During alcohol withdrawal, the non-dependent (2BC) males reduced mechanical threshold 8 h after the last exposure to air that lasted up to 72 h (8 h: 0.760 ± 0.32 g; 24 h: 0.820 ± 0.36 g; 48 h: 0.740 ± 0.30 g; 72 h: 0.88 ± 0.33 g; Figure 2a) compared to the naïve group. In females, non-dependent mice did not show significant ($P = 0.2241$) changes in the mechanical thresholds at the 8 h time point (naïve: 1.25 ± 0.44 g; 2BC: 0.980 ± 0.113 g Figure 2b) but showed a significant ($P = 0.0001$) decrease at 24 h (naïve: 1.60 ± 0.40 g; 2BC: 0.760 ± 0.102 g Figure 2b) and 48 h (naïve: 1.48 ± 0.22 g; 2BC: 0.880 ± 0.116 g Figure 2b), which disappeared at 72 h (naïve: 1.20 ± 0.50 g; 2BC: 0.880 ± 0.116 g; Figure 2b). When we tested the mechanical thresholds following the 2BC (voluntary drinking), we found no significant effects of the drinking on the mechanical threshold both in the non-dependent males (0.900 ± 0.34 g; Figure 2a) and non-dependent females (0.960 ± 0.16 g; Figure 2b) compared with the previous measurements.

Given that the non-dependent group showed a long-lasting increase in pain sensitivity compared with the naïve group, to identify potential heterogeneity in mechanical thresholds, we combined 2BC female (n = 10) and male (n = 25) mice from the two different cohorts in Figure 5a. In the non-dependent group, we did not expect changes in mechanical hypersensitivity after acute alcohol consumption, as acute alcohol consumption-induced reduction in allodynia in dependent mice (Figure 2c) is typical for withdrawal-related hypersensitivity. Indeed, alcohol drinking did not change mechanical thresholds in the non-dependent mice (Figure 2a,b), which led us to hypothesize that some of the non-dependent mice developed a chronic-neuropathic pain represented by baseline allodynia irreversible by acute alcohol intake. We chose a threshold value of 1, which was calculated by taking the averages of the naïve group's mechanical threshold during the entire time course of withdrawal. Based on this criterion, we stratified the male (Figure 5b) and female (Figure 5c) non-dependent mice (2BC) in two different subunits: mice with alcohol-evoked neuropathic pain (2BC-N; mechanical threshold <1) and mice alcohol-evoked neuropathic pain (2BC; mechanical threshold >1). In approximately half of the non-dependent mice, we observed the development of a chronic neuropathic pain.

Non-dependent male mice with neuropathic pain (2BC-N) possessed a mechanical allodynia significantly higher ($P = 0.0012$, Figure 5b) than non-dependent mice without neuropathic pain (2BC). Similarly, non-dependent female mice with neuropathic pain (2BC-N)

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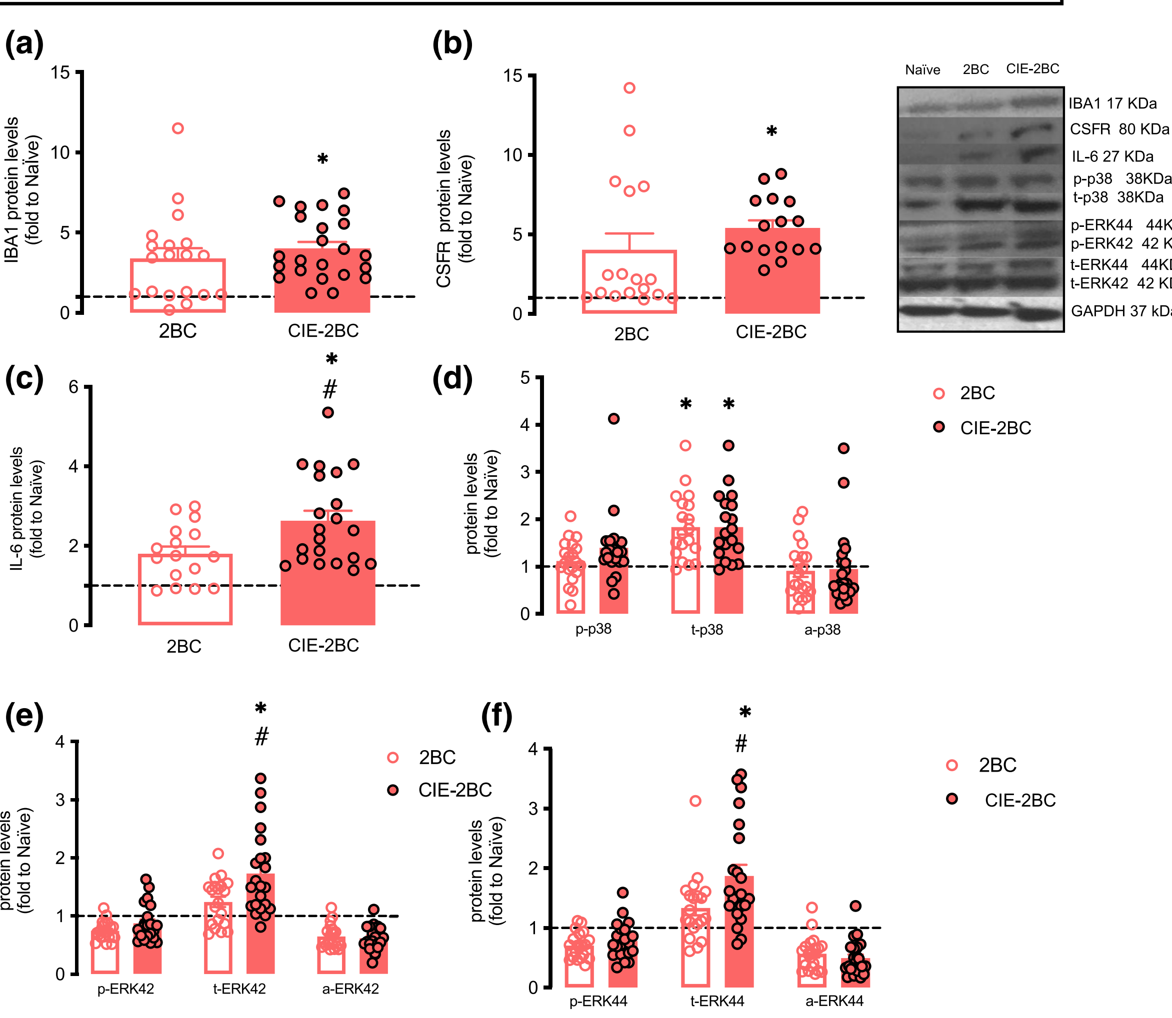

FIGURE 4 Microgliosis and MAPK activation in spinal cord tissue in chronic intermittent ethanol vapour–two-bottle choice (CIE-2BC) mice. CIE-2BC mice showed an increase in (a) ionized calcium-binding adapter molecule-1 (IBA-1) also known as allograft inflammatory factor 1 (AIF-1) (one-way ANOVA followed by Tukey's multiple-comparison post hoc test; $^*P < 0.05$, vs. naïve; $F_{2,43} = 3.040$), (b) colony stimulating factor 1 receptor (CSFR; one-way ANOVA followed by Tukey's multiple-comparison post hoc test; $^*P < 0.05$, vs. naïve; $F_{2,34} = 3.278$) and (c) IL-6 (one-way ANOVA followed by Tukey's multiple-comparison post hoc test, $F_{2,40} = 8.712$; $^*P < 0.05$, vs. naïve; $^{\#}P < 0.05$, vs. 2BC) protein expression. (d) Protein expression of phosphorylated (p)-p38, total (t)-p38 and activated (a)-p38 in naïve, two-bottle choice (2BC) and CIE-2BC mice (two-way ANOVA followed by Sidak's multiple-comparison post hoc test, $F_{2,81} = 1.256$; $^*P < 0.05$, vs. naïve). (e) Protein expression of phosphorylated p-ERK42, total t-ERK42 and activated a-ERK2 (two-way ANOVA followed by Sidak's multiple-comparison post hoc test, $F_{2,86} = 4.039$; $^*P < 0.05$, vs. naïve; $^{\#}P < 0.05$, vs. 2BC) and (f) phosphorylated p-ERK44, total t-ERK44 and activated (a)-ERK44 (two-way ANOVA followed by Sidak's multiple-comparison post hoc test, $F_{2,85} = 4.285$; $^*P < 0.05$, vs. naïve; $^{\#}P < 0.05$, vs. 2BC) in spinal cord tissue in naïve, 2BC and CIE-2BC mice. The naïve group is represented by the black dashed line. Abbreviation: ERK2/1 - mitogen-activated protein kinases 2 and 3, previously known as extracellular signal regulated kinases 44/42.

developed a significantly ($P = 0.002$; Figure 5c) higher allodynia than 2BC without neuropathic pain (2BC). It has been reported that 46% of human chronic alcohol consumers develop peripheral neuropathy (Chopra & Tiwari, 2012). This is consistent with our data showing that 42.85% (n = 15) of the non-dependent mice developed mechanical allodynia (2BC-N group; Figure 5b,c), which was not reversed by alcohol consumption and a chronic-neuropathic pain.

Moreover, there was an inverse correlation (Pearson *r* correlation, Figure 5d) for both male ($r = -0.11$) and female ($r = -0.33$), between level of alcohol drinking and mechanical threshold value: the mice that drank the most were those that showed a lower threshold value and therefore had more pain.

To examine whether the observed chronic ethanol-induced mechanical hypersensitivity is associated with the peripheral neuropathy, we assessed demyelination processes in the sciatic nerve represented by the loss of Schwan cells, frequently associated with the peripheral neuropathies (De Logu et al., 2019; Placheta-Györi et al., 2021). The 2BC-N group showed a significant ($P = 0.0001$;

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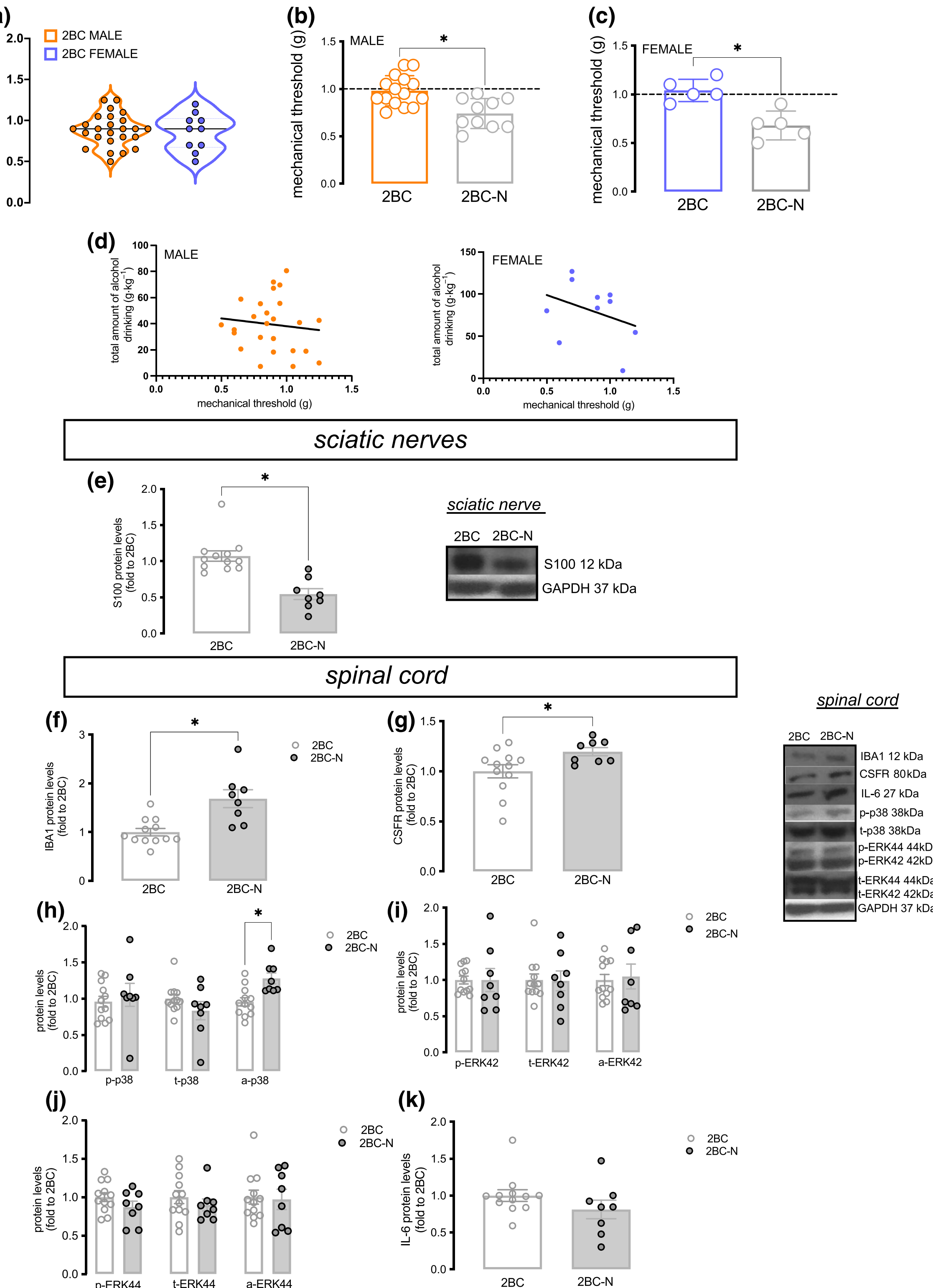

FIGURE 5 Legend on next page.

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2BC: 1.072 ± 0.07 vs. 2BC-N:0.546 ± 0.0749 Figure 5e) decrease in the S100 levels (marker of myelinated Schwann cells) in the sciatic nerve compared to the 2BC group.

Because the activation of the immune system has been observed in the peripheral neuropathies, we also examine activation of the immune response in the spinal cord in the 2BC-N mice. The cytokine colony-stimulating factor 1 (CSF1) is *de novo* expressed in injured sensory neurons and is transported to the spinal cord where it activates the microglia and leads up-regulation of the genes critical to development of allodynia (Guan et al., 2015). We observed an increase in CSFR (Figure 5g) and ionized calcium-binding adapter molecule-1 (IBA-1) also known as allograft inflammatory factor 1 (AIF-1) (Figure 5f), a marker of microglial cells, in 2BC-N mice, indicating activation of microglia and the development of alcohol-induced immune response and alcohol-evoked neuropathy. Moreover, 2BC-N mice showed an increase in activation of p38 (a-p38) compared to 2BC mice (Figure 5h), but there were no significant differences in p-p38 or t-p38 levels between these two groups. We did not detect differences in ERK44/42 phosphorylation or activation (Figure 5i,j) and in IL-6 protein levels between 2BC and 2BC-N group (Figure 5k).

4 | DISCUSSION

In this study, we used the CIE-2BC animal model of alcohol dependence and showed that it modelled two different pathological conditions *in vivo* with different characteristic:- (1) abstinence-related hypersensitivity in dependent mice, which was developed in 100% of dependent animal (both female and male groups) and reduced by acute alcohol administration and (2), alcohol-evoked neuropathic pain, that was developed in 50% of non-dependent mice in both sexes and it was not reverted by acute alcohol administration. These two conditions were accompanied by strong spinal microgliosis, which further confirmed the role of glia in alcohol addiction and the development of comorbidities. The dysregulation of pain neurocircuitry and neurochemistry is critical in a diverse spectrum of diseases, including alcohol dependence and the associated negative emotional affect (Egli et al., 2012). Unrelieved chronic pain is considered a key factor contributing to the maintenance of alcohol use disorder (Karimi et al., 2022). Alcoholic neuropathy is one of the most common adverse effects of chronic alcohol consumption. It is estimated that about half of alcohol use disorder patients in the United States are affected by some form of neuropathy (Julian et al., 2019), but the precise incidence among the population is unknown. Patients with comorbid alcohol use disorder and neuropathy suffer from pain, ataxia and paraesthesia in the lower limbs. This nerve damage is due to the direct toxic impact of alcohol, but the precise causes are still unclear (Zeng et al., 2017). The lack of models of alcoholic neuropathy further limits investigations of pathological mechanisms (Julian et al., 2019). Thus, in this study, we chose the CIE-2BC paradigm, an established model to the study of alcohol dependence, because it is well-controlled to account for internal variables including stress and alcohol exposure, allows for precise control of blood alcohol concentration and reliable measures excessive escalated-drinking (Koob & Volkow, 2016; Vendruscolo & Roberts, 2014) and the negative affect manifested in altered emotional responsivity, such as anxiety-like behaviours and irritability (Becker & Lopez, 2004; Bloch et al., 2022; Okhuarobo et al., 2022; Patel et al., 2022; Sidhu et al., 2018; Warden et al., 2020). We found that chronic alcohol exposure produced stronger mechanical allodynia during withdrawal in dependent (CIE-2BC) mice compared with non-dependent (2BC) and naïve mice, an effect that was completely reversed by subsequent acute voluntary drinking. This effect could be considered abstinence-related pain hypersensitivity, which is a phenomenon that occurs when the chronic administration of abused substances is abruptly stopped. Indeed, this phenomenon has been reported in clinical studies with **nicotine** (Bagdas et al., 2018; Baiamonte et al., 2014; Zhang, Yang, et al., 2020) and opioids (Manning et al., 1996; Mercadante et al., 2019; Roeckel et al., 2016). Moreover, alcohol use disorder patients report pain during the cessation of alcohol consumption and usually drink alcohol to reduce it (Robins et al., 2019). Our data in mice are consistent with findings of alcohol-dependent rats developing mechanical hypersensitivity after 8 weeks of CIE vapour exposure (Edwards et al., 2012) and thermal hyperalgesia following 28 days of alcohol vapour exposure (Roltsch Hellard et al., 2017).

It is hypothesized that the adaptation of neuronal pathways after developing dependence may alter pain perception, due to the strong connection between addiction and pain (Egli et al., 2012; Robins et al., 2019). Several systems have been investigated for their role in the development of this condition, such as **potassium channels** (Kang

FIGURE 5 The two-bottle choice (2BC) group developed alcohol-evoked neuropathic pain (2BC-N). (a) Mechanical threshold in 2BC male and female mice. (b, c) Separation of 2BC male (b) (*P < 0.05, Student's unpaired t-test) and female (c) (*P < 0.05, Student's unpaired t test) in two different groups with (2BC-N) and without (2BC) neuropathy. (d) Correlation between total amount of ethanol drinking during and the mechanical threshold (Pearson r correlation, two-tailed: male $r = -0.11$ R squared $= 0.012$ $P = 0.58$; female $r = -0.33$ R squared $= 0.11$ $P > 0.05$). (e) S100 protein expression in sciatic nerve tissue (*P < 0.05, Student's unpaired t test). Evaluation of microglia markers (f) ionized calcium-binding adapter molecule-1 (IBA-1) also known as allograft inflammatory factor 1 (AIF-1) and (g) colony stimulating factor 1 receptor (CSFR; *P < 0.05, Student's unpaired t test) in spinal cord. Detection of phosphorylated p-p38, total t-p38 and activated a-p38 (h) (two-way ANOVA followed by Sidak's multiple-comparison post hoc test, $F_{2,54} = 3.726$, *P < 0.05), phosphorylated p-ERK42, total t-ERK42 and activated (a)-ERK2 (i) (two-way ANOVA followed by Sidak's multiple-comparison post hoc test, $F_{2,54} = 0.04271$) and phosphorylated p-ERK44, total t-ERK44 and activated a-ERK44 (j) (two-way ANOVA followed by Sidak's multiple-comparison post hoc test, $F_{2,54} = 0.1678$). (k) IL-6 in spinal cord tissue in the 2BC and 2BC-N groups (*P < 0.05, Student's unpaired t test). Abbreviation: ERK2/1 - mitogen-activated protein kinases 2 and 3, previously known as extracellular signal regulated kinases 44/42.

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et al., 2019), **histone deacetylases (HDACs)**, epigenetic modulators (Pradhan et al., 2019) and **cannabinoid receptors** (Fu et al., 2021). However, the exact molecular mechanisms mediating the development of abstinence-related hypersensitivity are still unclear.

To our knowledge, our study is the first to determine mechanical allodynia using von Frey filaments with the mouse CIE-2BC paradigm in both sexes. Previous studies have mainly determined hyperalgesia in rats (Avegno et al., 2018; Dina et al., 2006; Edwards et al., 2020; Fu et al., 2021; Navarro et al., 2008; Pradhan et al., 2019; Roltsch Hellard et al., 2017; Tessitore et al., 2022). We found no sex differences between dependent male and female mice in the abstinence-induced pain hypersensitivity. However, the females exhibited a significantly more robust reduction of hypersensitivity to the mechanical stimulus after acute alcohol drinking. This difference may be caused by the higher alcohol intake in females observed in our study, which is in accord with previously reported findings (Yoneyama et al., 2008). Moreover, Brandner et al. (2023) demonstrated a sex difference in hypersensitivity-state during the 4 weeks of CIE model, in which vapour exposure produced heat hyperalgesia only in female and not in male mice (Brandner et al., 2023), and the dependent mice developed a strong hypersensitivity as early as the first hours of abstinence (8–72 h). A limitation of the current work is the lack of a longer time-course, but future studies will assess additional time points to clarify the trend over time and the resolution of hypersensitivity.

Another strength of our work is that we took advantage of non-dependent group mimicking moderate drinking (but not dependence), to measure alcohol-evoked neuropathic pain. We found that increased hypersensitivity occurred in 40% and 50% of 2BC male and female mice, respectively, compared with the alcohol naïve group. However, contrary to the dependent mice, the hypersensitivity was not reversed by the subsequent acute consumption of alcohol and correlated to the amount of alcohol drinking. Thus, taken together our findings suggest that the CIE-2BC paradigm is a reproducible mouse model of ethanol-evoked neuropathic pain. Chronic alcohol consumption induces a painful peripheral neuropathy, which is generally characterized by small-fibre polyneuropathy, that can be diagnosed by skin biopsy (Chopra & Tiwari, 2012). Bosch et al. (1978) observed the development of no thiamine-deficiency related alcoholic neuropathy in male rats after 16–18 weeks of polydipsia and liquid diet paradigms. In these two models, the animals showed a morphological change in the distal ventral caudal nerve. Indeed, myelinated fibres had changes in axonal degeneration, degeneration of mitochondria, loss of neurotubules, and myelin debris within Schwann-cell cytoplasm and macrophages (Bosch et al., 1978). The loss of Schwann cells represents a key point in the development of neuropathy symptoms, as Schwann cells actively participate in the development of neuropathic pain (de Logu et al., 2019; Placheta-Györi et al., 2021). In line with this evidence, our data showing a reduction of the protein expression of S100, a marker of Schwann cells, in sciatic nerve tissue in 2BC-N mice compared with 2BC mice indicates neuropathy that developed in these animals. Small-fibre axons are enclosed within invaginations of the Schwann-cell membranes and C-fibres represents an important marker of neuropathy development (Chen & Levine, 2007; Zhang, Niu, et al., 2020). The decreased S100 protein expression observed in our work, support a lack of proper communication between the peripheral tissues and the CNS results in altered perception of the mechanical stimulus. These results are consistent with the small-fibre degeneration associated with alcohol induced neuropathic pain that can be diagnosed with skin biopsy in heavy alcohol drinking subjects (Mellion et al., 2014). While our study provides pathological evidence, we did not conduct an electrophysiological evaluation of neuropathy. Therefore, we cannot rule out the possible association this pathological state might have with changes in the electrophysiological properties of the sciatic nerve, as reported in the rats following polydipsia and liquid diet paradigms (Bosch et al., 1978). Future electrophysiological and morphological studies will be conducted to better elucidated the damage at the mechanistic level of the sciatic nerve to characterize the alcohol-evoked neuropathic pain model.

Therapy for the management of alcoholic neuropathic pain is based on analgesics and opioid alternatives that generally are ineffective and rarely cure the actual causes. For this reason, research on alternative targets to be exploited for the management of this chronic condition is important, particularly studies of pathways that are involved in both dependence and pain development.

The cause of alcoholic neuropathy was initially attributable to thiamine deficiency and malnutrition (de la Monte & Kril, 2014), but this is not the only reason. Neuropathy depends more on damage to the central nervous system and microglial activation (Narita et al., 2007). These observations suggest that damage at the axonal level and functional changes related to prolonged excessive alcohol consumption are linked to a neuroinflammatory process. The administration of **rolipram**, a selective inhibitor of phosphodiesterate-4 that can reduce levels of proinflammatory cytokines, decreased mechanical allodynia in male rats exposed to alcohol (Han et al., 2012). Neuroinflammation is a key contributor to neuropathic pain development and progression, and to the pathogenesis of alcohol addiction (Streit et al., 2004). As reported in our recent work, microglia depletion prevented the escalation of voluntary alcohol intake and decreased anxiety-like behaviour associated with alcohol dependence (Warden et al., 2020). For these reasons, we are the first to investigate possible microglial activation in spinal cord tissue in non-dependent 2BC and dependent CIE-2BC male and female mice. Previously, we reported changes in microglial and neuroimmune-related gene expression have been identified in murine and human brains after chronic alcohol consumption (Warden et al., 2020). Here, we found upregulation of the microglia marker IBA-1 in the spinal cord of CIE-2BC and 2BC-N groups, as well as increases in colony stimulating factor 1 receptor (CSFR). Spinal microglia activation has long been considered a significant contributor to neuropathic pain and growing evidence demonstrates the pivotal role of the interaction among spinal neurons and microglia in the induction and maintenance of neuropathic pain (Inoue & Tsuda, 2018; Traiffort et al., 2020; Tsuda et al., 2005). Indeed, sensory neuron central terminals communicate with microglia via release of the CSFR, which is de novo expressed in injured neurons. CSFR binds its receptor expressed by microglia, contributing to activation and up-regulation of genes

critically involved in the development of allodynia (Guan et al., 2015). An emerging role in the induction of mechanical allodynia has been postulated for interactions between macrophages and nociceptive sensory neurons in the dorsal root ganglia (DRG) through the release of CFS1. Specifically, CSF1 binds the CSF1 receptor expressed by both microglia and macrophages, inducing expansion of both resident microglia in the spinal cord and macrophages in the dorsal root ganglia and contributing to the neuropathic pain phenotype (Yu et al., 2021). Notably, we observed a selective increase of IL-6 in the dependent mice, which was not found in the non-dependent mice. IL-6 is implicated in pain (Serizawa et al., 2021) and is also involved in processes related to the development of addiction (Dennis et al., 2014; Doremus-Fitzwater et al., 2014; Roberts et al., 2019). Further functional and morphological experiments will establish specific markers for alcohol-induced neuropathy including IL-6 pathway that may represent a specific linkage of the allodynia associated to alcohol dependence.

Finally, given the role of MAPKs in the spinal mechanisms of neuropathic pain and neuroinflammation (Edelmayer et al., 2014), we investigated spinal cord expression of the MAPKs p38 and ERK44/42 (mitogen-activated protein kinase 3 and 2), in mice with neuropathy (Ji et al., 2009). Different MAPK subtypes play assorted roles in the development of neuropathic pain and colocalize in different cell subtypes: - ERK and p38 are mainly expressed in spinal microglia cells. We found that total-p38 was more highly expressed in dependent (CIE-2BC) mice than in 2BC mice. This could be related to the duration of the model, which might have made phosphorylated forms of these proteins less detectable. Inversely, in mice that developed neuropathy there is a continuous activation of p38. These increases in p38 pathway activation are consistent with chronic alcohol-induced neurodegeneration via upregulation of the adenosine monophosphate-activated protein kinase/sirtuin-1/p38 pathway in rats and human neuroblastoma cells (Gu et al., 2018). Likewise, p38 and total-ERK44/42 increased in dependent mice compared with naïve mice, support a possible involvement in the mechanism of hypersensitivity (Sun et al., 2016). Chronic alcohol exposure induced an increase of ERK phosphorylation, that appears to be maintained for a longer period in the central nervous system mediating the anxiolytic and discriminatory stimulating properties of alcohol exposure (Besheer et al., 2012). Like IL-6 protein expression, there is no differences in ERK activation between group with neuropathy and group without neuropathy for ERK activation. Future studies of IL-6 and ERK will provide probably a better understanding of differences between hypersensitivity-abstinence related and alcoholic neuropathy.

5 | CONCLUSION

Our findings show, for the first time, that the CIE-2BC model in male and female mice generates two different pain conditions *in vivo*: abstinence-related hypersensitivity in dependent mice and alcohol-evoked neuropathic pain in 50% of non-dependent mice. These two conditions shared intense microglial activation in spinal cord tissue, which involves different downstream pathways: dependent mice that developed abstinence-related allodynia showed an increase in IL-6 expression and ERK44/42 activation, which was not observed in mice with alcohol-evoked neuropathic pain. This different IL-6 and ERK44/42 protein expression between hypersensitive dependent mice and alcohol-evoked neuropathic pain will prompt further investigations of the involvement of these two microglial pathways in these forms of pain, which could pave the way for developing targeted therapies.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for for Design & Analysis, Immunoblotting and Immunochemistry and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research. Open Access Funding provided by Universita degli Studi di Firenze within the CRUI-CARE Agreement.

AUTHOR CONTRIBUTIONS

Vittoria Borgonetti: Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); writing—original draft. **Amanda J. Roberts:** Data curation (equal); formal analysis (equal); investigation (equal); writing—review and editing (equal). **Michal Bajo:** Conceptualization (equal); data curation (equal); formal analysis (equal); writing—review and editing (equal). **Nicoletta Galeotti:** Conceptualization (equal); writing—review and editing (equal). **Marisa Roberto:** Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (equal); investigation (equal); writing—review and editing (equal).

ACKNOWLEDGEMENTS

This is manuscript number 30209 from The Scripps Research Institute. This study was supported by National Institutes of Health grants AA013498 (MR), AA027700 (MR), AA021491 (MR), AA017447 (MR), AA006420 (MR and AJR) and AA029841 (MR), The Schimmel Family Chair, The Pearson Center for Alcoholism and Addiction Research and The Scripps Research Institutes Animal Models Core Facility. The authors declare no competing financial interests. The authors thank Michael Arends for English editing the manuscript. Open Access Funding provided by Universita degli Studi di Firenze within the CRUI-CARE Agreement.

CONFLICT OF INTEREST STATEMENT

None.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES

Alexander, S. P., Fabbro, D., Kelly, E., Mathie, A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Pawson, A. J., Southan, C., Davies, J. A., Boison, D., Burns, K. E., Dessauer, C., Gertsch, J., Helsby, N. A., Izzo, A. A., Koesling, D., … Wong, S. S. (2021). THE CONCISE GUIDE TO PHARMACOLOGY 2021/22: Enzymes. *British Journal of Pharmacology*, *178*(S1), S313–S411. https://doi.org/10.1111/bph.15542

Alexander, S. P. H., Roberts, R. E., Broughton, B. R. S., Sobey, C. G., George, C. H., Stanford, S. C., Cirino, G., Docherty, J. R., Giembycz, M. A., Hoyer, D., Insel, P. A., Izzo, A. A., Ji, Y., MacEwan, D. J., Mangum, J., Wonnacott, S., & Ahluwalia, A. (2018). Goals and practicalities of immunoblotting and immunohistochemistry: A guide for submission to the *British Journal of Pharmacology*. *British Journal of Pharmacology*, *175*, 407–411. https://doi.org/10.1111/bph.14112

Avegno, E. M., Lobell, T. D., Itoga, C. A., Baynes, B. B., Whitaker, A. M., Weera, M. M., Edwards, S., Middleton, J. W., & Gilpin, N. W. (2018). Central amygdala circuits mediate hyperalgesia in alcohol-dependent rats. *Journal of Neuroscience*, *38*(36), 7761–7773. https://doi.org/10.1523/JNEUROSCI.0483-18.2018

Bagdas, D., Alkhlaif, Y., Jackson, A., Carroll, F. I., Ditre, J. W., & Damaj, M. I. (2018). New insights on the effects of varenicline on nicotine reward, withdrawal and hyperalgesia in mice. *Neuropharmacology*, *138*, 72–79. https://doi.org/10.1016/j.neuropharm.2018.05.025

Baiamonte, B. A., Valenza, M., Roltsch, E. A., Whitaker, A. M., Baynes, B. B., Sabino, V., & Gilpin, N. W. (2014). Nicotine dependence produces hyperalgesia: Role of corticotropin-releasing factor-1 receptors (CRF1Rs) in the central amygdala (CeA). *Neuropharmacology*, *77*, 217–223. https://doi.org/10.1016/j.neuropharm.2013.09.025

Bajo, M., Madamba, S. G., Roberto, M., Blednov, Y. A., Sagi, V. N., Roberts, E., Rice, K. C., Harris, R. A., & Siggins, G. R. (2014). Innate immune factors modulate ethanol interaction with GABAergic transmission in mouse central amygdala. *Brain, Behavior, and Immunity*, *40*, 191–202. https://doi.org/10.1016/j.bbi.2014.03.007

Bajo, M., Patel, R. R., Hedges, D. M., Varodayan, F. P., Vlkolinsky, R., Davis, T. D., Burkart, M. D., Blednov, Y. A., & Roberto, M. (2019). Role of MyD88 in IL-1β and ethanol modulation of GABaergic transmission in the central amygdala. *Brain Sciences*, *9*(12), 361. https://doi.org/10.3390/brainsci9120361

Beasley, M. J., Macfarlane, T. V., & Macfarlane, G. J. (2016). Is alcohol consumption related to likelihood of reporting chronic widespread pain in people with stable consumption? Results from UK biobank. *Pain*, *157*(11), 2552–2560. https://doi.org/10.1097/j.pain.0000000000000675

Becker, H. C., & Lopez, M. F. (2004). Increased ethanol drinking after repeated chronic ethanol exposure and withdrawal experience in C57BL/6 mice. *Alcoholism: Clinical and Experimental Research*, *28*(12), 1829–1838. https://doi.org/10.1097/01.ALC.0000149977.95306.3A

Besheer, J., Fisher, K. R., Cannady, R., Grondin, J. J. M., & Hodge, C. W. (2012). Intra-amygdala inhibition of ERK1/2 potentiates the discriminative stimulus effects of alcohol. *Behavioural Brain Research*, *228*(2), 398–405. https://doi.org/10.1016/j.bbr.2011.12.023

Bloch, S., Holleran, K. M., Kash, T. L., Vazey, E. M., Rinker, J. A., Lebonville, C. L., O'Hara, K., Lopez, M. F., Jones, S. R., Grant, K. A., Becker, H. C., & Mulholland, P. J. (2022). Assessing negative affect in mice during abstinence from alcohol drinking: Limitations and future challenges. *Alcohol*, *100*, 41–56. https://doi.org/10.1016/j.alcohol.2022.02.001

Boissoneault, J., Lewis, B., & Nixon, S. J. (2019). Characterizing chronic pain and alcohol use trajectory among treatment-seeking alcoholics. *Alcohol*, *75*, 47–54. https://doi.org/10.1016/j.alcohol.2018.05.009

Borgonetti, V., & Galeotti, N. (2021). Intranasal delivery of an antisense oligonucleotide to the RNA-binding protein HuR relieves nerve injury-induced neuropathic pain. *Pain*, *162*(5), 1500–1510. https://doi.org/10.1097/j.pain.0000000000002154

Borgonetti, V., & Galeotti, N. (2023). Microglia senescence is related to neuropathic pain–associated comorbidities in the spared nerve injury model. *Pain*, *164*(5), 1106–1117. https://doi.org/10.1097/j.pain.0000000000002807

Bosch, E. P., Pelham, R. W., Rasool, C. G., Chatterjee, A., Lash, R. W., Brown, L., Munsat, T. L., & Bradley, W. G. (1978). Animal models of alcoholic neuropathy: Morphologic, electrophysiologic, and biochemical findings. *Muscle & Nerve*, *2*, 133–144. https://doi.org/10.1002/mus.880020208

Brandner, A., Baratta, A., Rathod, R., Ferguson, C., Taylor, B., & Farris, S. P. (2023). Mechanical and heat hyperalgesia upon withdrawal from chronic intermittent ethanol vapor depends on sex, exposure duration and blood alcohol concentration in mice. *The Journal of Pain*. https://doi.org/10.1101/2022.08.19.504595

Brennan, P. L., Schutte, K. K., & Moos, R. H. (2005). Pain and use of alcohol to manage pain: Prevalence and 3-year outcomes among older problem and non-problem drinkers. *Addiction*, *100*(6), 777–786. https://doi.org/10.1111/j.1360-0443.2005.01074.x

Chen, X., & Levine, J. D. (2007). Mechanically-evoked C-fiber activity in painful alcohol and AIDS therapy neuropathy in the rat. *Molecular Pain*, *3*, 1744-8069-3-5, 5. https://doi.org/10.1186/1744-8069-3-5

Chopra, K., & Tiwari, V. (2012). Alcoholic neuropathy: Possible mechanisms and future treatment possibilities. *British Journal of Clinical Pharmacology*, *73*(3), 348–362. https://doi.org/10.1111/j.1365-2125.2011.04111.x

Cobos, E. J., Nickerson, C. A., Gao, F., Chandran, V., Bravo-Caparrós, I., González-Cano, R., Riva, P., Andrews, N. A., Latremoliere, A., Seehus, C. R., Perazzoli, G., Nieto, F. R., Joller, N., Painter, M. W., Ma, C. H. E., Omura, T., Chesler, E. J., Geschwind, D. H., Coppola, G., & Costigan, M. (2018). Mechanistic differences in neuropathic pain modalities revealed by correlating behavior with global expression profiling. *Cell Reports*, *22*(5), 1301–1312. https://doi.org/10.1016/j.celrep.2018.01.006

Cui, C., Noronha, A., Warren, K. R., Koob, G. F., Sinha, R., Thakkar, M., Matochik, J., Crews, F. T., Chandler, L. J., Pfefferbaum, A., Becker, H. C., Lovinger, D., Everitt, B. J., Egli, M., Mandyam, C. D., Fein, G., Potenza, M. N., Harris, R. A., Grant, K. A., … Sullivan, E. v. (2015). Brain pathways to recovery from alcohol dependence. *Alcohol*, *49*(5), 435–452. https://doi.org/10.1016/j.alcohol.2015.04.006

Curtis, M. J., Alexander, S., Cirino, G., Docherty, J. R., George, C. H., Giembycz, M. A., Hoyer, D., Insel, P. A., Izzo, A. A., Ji, Y., MacEwan, D. J., Sobey, C. G., Stanford, S. C., Teixeira, M. M., Wonnacott, S., & Ahluwalia, A. (2018). Experimental design and analysis and their reporting II: Updated and simplified guidance for authors and peer reviewers. *British Journal of Pharmacology*, *175*(7), 987–993. https://doi.org/10.1111/bph.14153

de la Monte, S. M., & Kril, J. J. (2014). Human alcohol-related neuropathology. *Acta Neuropathologica*, *127*(1), 71–90. https://doi.org/10.1007/s00401-013-1233-3

de Logu, F., Puma, S. L., Landini, L., Portelli, F., Innocenti, A., de Araujo, D. S. M., Janal, M. N., Patacchini, R., Bunnett, N. W., Geppetti, P., & Nassini, R. (2019). Schwann cells expressing nociceptive channel TRPA1 orchestrate ethanol-evoked neuropathic pain in mice. *Journal of Clinical Investigation*, *129*(12), 5424–5441. https://doi.org/10.1172/JCI128022

Dennis, C. V., Sheahan, P. J., Graeber, M. B., Sheedy, D. L., Kril, J. J., & Sutherland, G. T. (2014). Microglial proliferation in the brain of chronic alcoholics with hepatic encephalopathy. *Metabolic Brain Disease*, *29*(4), 1027–1039. https://doi.org/10.1007/s11011-013-9469-0

Dina, O. A., Messing, R. O., & Levine, J. D. (2006). Ethanol withdrawal induces hyperalgesia mediated by PKCε. *European Journal of Neuroscience*, *24*(1), 197–204. https://doi.org/10.1111/j.1460-9568.2006.04886.x

Doremus-Fitzwater, T. L., Buck, H. M., Bordner, K., Richey, L., Jones, M. E., & Deak, T. (2014). Intoxication- and withdrawal-

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.16091 by CochraneItalia, Wiley Online Library on [12/05/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

dependent expression of central and peripheral cytokines following initial ethanol exposure. *Alcoholism: Clinical and Experimental Research*, *38*(8), 2186–2198. https://doi.org/10.1111/acer.12481

Edelmayer, R. M., Brederson, J.-D., Jarvis, M. F., & Bitner, R. S. (2014). Biochemical and pharmacological assessment of MAP-kinase signaling along pain pathways in experimental rodent models: A potential tool for the discovery of novel antinociceptive therapeutics. *Biochemical Pharmacology*, *87*(3), 390–398. https://doi.org/10.1016/j.bcp.2013.11.019

Edwards, S., Vendruscolo, L. F., Gilpin, N. W., Wojnar, M., & Witkiewitz, K. (2020). Alcohol and pain: A translational review of preclinical and clinical findings to inform future treatment strategies. *Alcoholism: Clinical and Experimental Research*, *44*(2), 368–383. https://doi.org/10.1111/acer.14260

Edwards, S., Vendruscolo, L. F., Schlosburg, J. E., Misra, K. K., Wee, S., Park, P. E., Schulteis, G., & Koob, G. F. (2012). Development of mechanical hypersensitivity in rats during heroin and ethanol dependence: Alleviation by CRF1 receptor antagonism. *Neuropharmacology*, *62*(2), 1142–1151. https://doi.org/10.1016/j.neuropharm.2011.11.006

Egli, M., Koob, G. F., & Edwards, S. (2012). Alcohol dependence as a chronic pain disorder. *Neuroscience and Biobehavioral Reviews*, *36*(10), 2179–2192. https://doi.org/10.1016/j.neubiorev.2012.07.010

Elmore, M. R. P., Najafi, A. R., Koike, M. A., Dagher, N. N., Spangenberg, E. E., Rice, R. A., Kitazawa, M., Matusow, B., Nguyen, H., West, B. L., & Green, K. N. (2014). Colony-stimulating factor 1 receptor signaling is necessary for microglia viability, unmasking a microglia progenitor cell in the adult brain. *Neuron*, *82*(2), 380–397. https://doi.org/10.1016/j.neuron.2014.02.040

Fu, R., Tang, Y., Li, W., Ren, Z., Li, D., Zheng, J., Zuo, W., Chen, X., Zuo, Q. K., Tam, K. L., Zou, Y., Bachmann, T., Bekker, A., & Ye, J. H. (2021). Endocannabinoid signaling in the lateral habenula regulates pain and alcohol consumption. *Translational Psychiatry*, *11*(1), 220. https://doi.org/10.1038/s41398-021-01337-3

Grace, P. M., Tawfik, V. L., Svensson, C. I., Burton, M. D., Loggia, M. L., & Hutchinson, M. R. (2021). The neuroimmunology of chronic pain: From rodents to humans. *Journal of Neuroscience*, *41*(5), 855–865. https://doi.org/10.1523/JNEUROSCI.1650-20.2020

Griffin, W. C. (2014). Alcohol dependence and free-choice drinking in mice. *Alcohol*, *48*(3), 287–293. https://doi.org/10.1016/j.alcohol.2013.11.006

Gu, X., Cai, Z., Cai, M., Liu, K., Liu, D., Zhang, Q., Tan, J., & Ma, Q. (2018). AMPK/SIRT1/p38 MAPK signaling pathway regulates alcohol-induced neurodegeneration by resveratrol. *Molecular Medicine Reports*, *17*(4), 5402–5408. https://doi.org/10.3892/mmr.2018.8482

Guan, Z., Kuhn, J. A., Wang, X., Colquitt, B., Solorzano, C., Vaman, S., Guan, A. K., Evans-Reinsch, Z., Braz, J., Devor, M., Abboud-Werner, S. L., Lanier, L. L., Lomvardas, S., & Basbaum, A. I. (2015). Injured sensory neuron-derived CSF1 induces microglial proliferation and DAP12-dependent pain. *Nature Neuroscience*, *19*(1), 94–101. https://doi.org/10.1038/nn.4189

Han, K. H., Kim, S. H., Jeong, I. C., Lee, Y. H., Chang, S. J., & Kim, S. W. (2012). Electrophysiological and behavioral changes by phosphodiesterase 4 inhibitor in a rat model of alcoholic neuropathy. *Journal of Korean Neurosurgical Association*, *52*(1), 32–36. https://doi.org/10.3340/jkns.2012.52.1.32

Huitron-Resendiz, S., Nadav, T., Krause, S., Cates-Gatto, C., Polis, I., & Roberts, A. J. (2018). Effects of withdrawal from chronic intermittent ethanol exposure on sleep characteristics of female and male mice. *Alcoholism: Clinical and Experimental Research*, *42*(3), 540–550. https://doi.org/10.1111/acer.13584

Inoue, K., & Tsuda, M. (2018). Microglia in neuropathic pain: Cellular and molecular mechanisms and therapeutic potential. *Nature Reviews Neuroscience*, *19*(3), 138–152. https://doi.org/10.1038/nrn.2018.2

Jensen, T. S., & Finnerup, N. B. (2014). Allodynia and hyperalgesia in neuropathic pain: Clinical manifestations and mechanisms. *The Lancet Neurology*, *13*(9), 924–935. https://doi.org/10.1016/S1474-4422(14)70102-4

Ji, R. R., Gereau, R. W., Malcangio, M., & Strichartz, G. R. (2009). MAP kinase and pain. *Brain Research Reviews*, *60*(1), 135–148. https://doi.org/10.1016/j.brainresrev.2008.12.011

Jochum, T., Boettger, M. K., Burkhardt, C., Juckel, G., & Bär, K. J. (2010). Increased pain sensitivity in alcohol withdrawal syndrome. *European Journal of Pain*, *14*(7), 713–718. https://doi.org/10.1016/j.ejpain.2009.11.008

Julian, T., Glascow, N., Syeed, R., & Zis, P. (2019). Alcohol-related peripheral neuropathy: A systematic review and meta-analysis. *Journal of Neurology*, *266*(12), 2907–2919. https://doi.org/10.1007/s00415-018-9123-1

Kang, S., Li, J., Zuo, W., Chen, P., Gregor, D., Fu, R., Han, X., Bekker, A., & Ye, J. H. (2019). Downregulation of M-channels in lateral habenula mediates hyperalgesia during alcohol withdrawal in rats. *Scientific Reports*, *9*(1), 2714. https://doi.org/10.1038/s41598-018-38393-7

Karimi, R., Mallah, N., Nedjat, S., Beasley, M. J., & Takkouche, B. (2022). Association between alcohol consumption and chronic pain: A systematic review and meta-analysis. *British Journal of Anaesthesia*, *129*(3), 355–365. https://doi.org/10.1016/j.bja.2022.03.010

Khom, S., Steinkellner, T., Hnasko, T. S., & Roberto, M. (2020). Alcohol dependence potentiates substance P/neurokinin-1 receptor signaling in the rat central nucleus of amygdala. *Science Advances*, *6* Retrieved from https://www.science.org, eaaz1050. https://doi.org/10.1126/sciadv.aaz1050

Khom, S., Wolfe, S. A., Patel, R. R., Kirson, D., Hedges, D. M., Varodayan, F. P., Bajo, M., & Roberto, M. (2020). Alcohol dependence and withdrawal impair serotonergic regulation of GABA transmission in the rat central nucleus of the amygdala. *Journal of Neuroscience*, *40*(36), 6842–6853. https://doi.org/10.1523/JNEUROSCI.0733-20.2020

Kohno, K., Shirasaka, R., Yoshihara, K., Mikuriya, S., Tanaka, K., Takanami, K., Inoue, K., Sakamoto, H., Ohkawa, Y., Masuda, T., & Tsuda, M. (2022). A spinal microglia population involved in remitting and relapsing neuropathic pain. *Science*, *376*, 86–90. https://doi.org/10.1126/science.abf6805

Koob, G. F., & Volkow, N. D. (2016). Neurobiology of addiction: A neurocircuitry analysis. *The Lancet Psychiatry*, *3*(8), 760–773. https://doi.org/10.1016/S2215-0366(16)00104-8

Lee, J. H., & Kim, W. (2020). The role of satellite glial cells, astrocytes, and microglia in oxaliplatin-induced neuropathic pain. *Biomedicine*, *8*, 324. https://doi.org/10.3390/biomedicines8090324

Lilley, E., Stanford, S. C., Kendall, D. E., Alexander, S. P. H., Cirino, G., Docherty, J. R., George, C. H., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., Sobey, C. G., Stefanska, B., Stephens, G., Teixeira, M., & Ahluwalia, A. (2020). ARRIVE 2.0 and the British Journal of pharmacology: Updated guidance for 2020. *British Journal of Pharmacology*, *177*, 3611–3616. https://doi.org/10.1111/bph.15178

Lituma, P. J., Woo, E., O'hara, B. F., Castillo, P. E., Sibinga, N. E. S., Nandi, S., & Purpura, D. P. (2021). Altered synaptic connectivity and brain function in mice lacking microglial adapter protein Iba1. *PNAS*, *118*(46), E2115539118. https://doi.org/10.1073/pnas.2115539118/-/DCSupplemental

Liu, X., & Quan, N. (2018). Microglia and CNS interleukin-1: Beyond immunological concepts. *Frontiers in Neurology*, Frontiers Media, *9*, 8. https://doi.org/10.3389/fneur.2018.00008

Macfarlane, G. J., Beasley, M., Prescott, G. J., McNamee, P., Hannaford, P. C., McBeth, J., Lovell, K., Keeley, P., Symmons, D. P. M., Woby, S., & Norrie, J. (2015). Alcohol consumption in relation to risk and severity of chronic widespread pain: Results from a UK population-based study. *Arthritis Care & Research*, *67*(9), 1297–1303. https://doi.org/10.1002/acr.22604

Manning, B. H., Mao, J., Frenk, H., Price, D. D., & Mayer, D. J. (1996). Continuous co-administration of dextromethorphan or MK-801 with

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.16091 by CochraneItalia, Wiley Online Library on [12/05/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

morphine: Attenuation of morphine dependence and naloxone-reversible attenuation of morphine tolerance. *Pain*, *67*(1), 79–88. https://doi.org/10.1016/0304-3959(96)81972-5

Mellion, M. L., Silbermann, E., Gilchrist, J. M., Machan, J. T., Leggio, L., & de la Monte, S. (2014). Small-fiber degeneration in alcohol-related peripheral neuropathy. *Alcoholism: Clinical and Experimental Research*, *38*(7), 1965–1972. https://doi.org/10.1111/acer.12470

Mercadante, S., Arcuri, E., & Santoni, A. (2019). Opioid-induced tolerance and hyperalgesia. *CNS Drugs*, *33*(10), 943–955. https://doi.org/10.1007/s40263-019-00660-0

Monforte, R., Estruch, R., Valls-Solé, J., Nicolás, J., Villalta, J., & Urbano-Marquez, A. (1995). Autonomic and peripheral neuropathies in patients with chronic alcoholism. A dose-related toxic effect of alcohol. *Archives of Neurology*, *1*(52), 45–51. https://doi.org/10.1001/archneur.1995.00540250049012

Narita, M., Miyoshi, K., Narita, M., & Suzuki, T. (2007). Involvement of microglia in the ethanol-induced neuropathic pain-like state in the rat. *Neuroscience Letters*, *414*(1), 21–25. https://doi.org/10.1016/j.neulet.2006.10.062

Navarro, M., Cubero, I., Knapp, D. J., Breese, G. R., & Thiele, T. E. (2008). Decreased immunoreactivity of the melanocortin neuropeptide α-melanocyte-stimulating hormone (α-MSH) after chronic ethanol exposure in Sprague-Dawley rats. *Alcoholism: Clinical and Experimental Research*, *32*(2), 266–276. https://doi.org/10.1111/j.1530-0277.2007.00578.x

Okhuarobo, A., Angelo, M., Bolton, J. L., Lopez, C., Igbe, I., Baram, T. Z., & Contet, C. (2022). Influence of early-life adversity on responses to acute and chronic ethanol in female mice. *Alcoholism, Clinical and Experimental Research*, *47*, 336–347. https://doi.org/10.1111/acer.14988

Patel, R. R., Khom, S., Steinman, M. Q., Varodayan, F. P., Kiosses, W. B., Hedges, D. M., Vlkolinsky, R., Nadav, T., Polis, I., Bajo, M., Roberts, A. J., & Roberto, M. (2019). IL-1β expression is increased and regulates GABA transmission following chronic ethanol in mouse central amygdala. *Brain, Behavior, and Immunity*, *75*, 208–219. https://doi.org/10.1016/j.bbi.2018.10.009

Patel, R. R., Wolfe, S. A., Bajo, M., Abeynaike, S., Pahng, A., Borgonetti, V., D'Ambrosio, S., Nikzad, R., Edwards, S., Paust, S., Roberts, A. J., & Roberto, M. (2021). IL-10 normalizes aberrant amygdala GABA transmission and reverses anxiety-like behavior and dependence-induced escalation of alcohol intake. *Progress in Neurobiology*, *199*, 101952. https://doi.org/10.1016/j.pneurobio.2020.101952

Patel, R. R., Wolfe, S. A., Borgonetti, V., Gandhi, P. J., Rodriguez, L., Snyder, A. E., D'Ambrosio, S., Bajo, M., Domissy, A., Head, S., Contet, C., Dayne Mayfield, R., Roberts, A. J., & Roberto, M. (2022). Ethanol withdrawal-induced adaptations in prefrontal corticotropin releasing factor receptor 1-expressing neurons regulate anxiety and conditioned rewarding effects of ethanol. *Molecular Psychiatry*, *27*(8), 3441–3451. https://doi.org/10.1038/s41380-022-01642-3

Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S. T., Howells, D. W., Karp, N. A., Lazic, S. E., Lidster, K., MacCallum, C. J., Macleod, M., … Würbel, H. (2020). The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. *PLoS Biology*, *18*(7), e3000410. https://doi.org/10.1371/journal.pbio.3000410

Placheta-Györi, E., Brandstetter, L. M., Zemann-Schälss, J., Wolf, S., & Radtke, C. (2021). Myelination, axonal loss and Schwann cell characteristics in axonal polyneuropathy compared to controls. *PLoS ONE*, *16*(11), 1–12. https://doi.org/10.1371/journal.pone.0259654

Pradhan, A. A., Tipton, A. F., Zhang, H., Akbari, A., & Pandey, S. C. (2019). Effect of histone deacetylase inhibitor on ethanol withdrawal-induced hyperalgesia in rats. *International Journal of Neuropsychopharmacology*, *22*(8), 523–527. https://doi.org/10.1093/IJNP/PYZ031

Riley, J. L., & King, C. (2009). Self-report of alcohol use for pain in a multi-ethnic community sample. *The Journal of Pain*, *10*(9), 944–952. https://doi.org/10.1016/j.jpain.2009.03.005

Roberts, A. J., Khom, S., Bajo, M., Vlkolinsky, R., Polis, I., Cates-Gatto, C., Roberto, M., & Gruol, D. L. (2019). Increased IL-6 expression in astrocytes is associated with emotionality, alterations in central amygdala GABAergic transmission, and excitability during alcohol withdrawal. *Brain, Behavior, and Immunity*, *82*, 188–202. https://doi.org/10.1016/j.bbi.2019.08.185

Robins, M. T., Heinricher, M. M., & Ryabinin, A. E. (2019). From pleasure to pain, and Back again: The intricate relationship between alcohol and nociception. *Alcohol and Alcoholism*, *54*(6), 625–638. https://doi.org/10.1093/alcalc/agz067

Roeckel, L.-A., Le Coz, G.-M., Gavériaux-Ruff, C., & Simonin, F. (2016). Opioid-induced hyperalgesia: Cellular and molecular mechanisms. *Neuroscience*, *338*, 160–182. https://doi.org/10.1016/j.neuroscience.2016.06.029

Rogers, J., Wiener, S. G., & Bloom, F. E. (1979). Long-term ethanol administration methods for rAts: Advantages of inhalation over intubation or liquid diets. *Behavioral and Neural Biology*, *27*, 466–486. https://doi.org/10.1016/s0163-1047(79)92061-2

Roltsch Hellard, E. A., Impastato, R. A., & Gilpin, N. W. (2017). Intra-cerebral and intra-nasal melanocortin-4 receptor antagonist blocks withdrawal hyperalgesia in alcohol-dependent rats. *Addiction Biology*, *22*(3), 692–701. https://doi.org/10.1111/adb.12360

Serizawa, K., Tomizawa-Shinohara, H., Miyake, S., Yogo, K., & Matsumoto, Y. (2021). Interleukin-6: Evolving role in the management of neuropathic pain in neuroimmunological disorders. *Inflammation and Regeneration*, *41*, 34. https://doi.org/10.1186/s41232-021-00184-5

Sidhu, H., Kreifeldt, M., & Contet, C. (2018). Affective disturbances during withdrawal from chronic intermittent ethanol inhalation in C57BL/6J and DBA/2J male mice. *Alcoholism: Clinical and Experimental Research*, *42*(7), 1281–1290. https://doi.org/10.1111/acer.13760

Streit, W. J., Mrak, R. E., & Griffin, W. S. T. (2004). Microglia and neuroinflammation: A pathological perspective. *Journal of Neuroinflammation*, *1*, 14. https://doi.org/10.1186/1742-2094-1-14

Sun, W.-L., Quizon, P. M., & Zhu, J. (2016). Molecular Mechanism: ERK Signaling, Drug Addiction, and Behavioral Effects. In S. Rahman (Ed.), *Progress in molecular biology and translational science* (Vol. 137) (pp. 1–40). Academic Press. https://doi.org/10.1016/bs.pmbts.2015.10.017

Tessitore, M. E., da Silva Pereira-Rufino, L., Panfilio, C. E., de Cassia Sinigaglia, R., Júnior, O. A., Maluf, L. L., Conte, R., Ladd, F. V., & Céspedes, I. C. (2022). Alcoholic neuropathy associated with chronic alcohol intake. *IBRO Neuroscience Reports*, *13*, 177–186. https://doi.org/10.1016/j.ibneur.2022.08.004

Tiwari, V., Kuhad, A., & Chopra, K. (2009). Tocotrienol ameliorates behavioral and biochemical alterations in the rat model of alcoholic neuropathy. *Pain*, *145*(1–2), 129–135. https://doi.org/10.1016/j.pain.2009.05.028

Tiwari, V., Kuhad, A., & Chopra, K. (2011). Amelioration of functional, biochemical and molecular deficits by epigallocatechin gallate in experimental model of alcoholic neuropathy. *European Journal of Pain*, *15*(3), 286–292. https://doi.org/10.1016/j.ejpain.2010.07.005

Todd, A. J. (2010). Neuronal circuitry for pain processing in the dorsal horn. *Nature Reviews Neuroscience*, *11*(12), 823–836. https://doi.org/10.1038/nrn2947

Traiffort, E., Kassoussi, A., Zahaf, A., & Laouarem, Y. (2020). Astrocytes and microglia as major players of myelin production in Normal and pathological conditions. *Frontiers in Cellular Neuroscience*, *14*, 79. https://doi.org/10.3389/fncel.2020.00079

Tsuda, M., Inoue, K., & Salter, M. W. (2005). Neuropathic pain and spinal microglia: A big problem from molecules in 'small' glia. *Trends in Neurosciences*, *28*(2), 101–107. https://doi.org/10.1016/j.tins.2004.12.002

Varodayan, F. P., Logrip, M. L., & Roberto, M. (2017). P/Q-type voltage-gated calcium channels mediate the ethanol and CRF sensitivity of central amygdala GABAergic synapses. *Neuropharmacology*, *125*, 197–206. https://doi.org/10.1016/j.neuropharm.2017.07.017

Varodayan, F. P., Patel, R. R., Matzeu, A., Wolfe, S. A., Curley, D. E., Khom, S., Gandhi, P. J., Rodriguez, L., Bajo, M., D'Ambrosio, S., Sun, H.,

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.16091 by CochraneItalia, Wiley Online Library on [12/05/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Kerr, T. M., Gonzales, R. A., Leggio, L., Natividad, L. A., Haass-Koffler, C. L., Martin-Fardon, R., & Roberto, M. (2022). The amygdala noradrenergic system is compromised with alcohol use disorder. *Biological Psychiatry*, *91*(12), 1008–1018. https://doi.org/10.1016/j.biopsych.2022.02.006

Vendruscolo, L. F., & Roberts, A. J. (2014). Operant alcohol self-administration in dependent rats: Focus on the vapor model. *Alcohol*, *48*, 277–286. https://doi.org/10.1016/j.alcohol.2013.08.006

Warden, A. S., Wolfe, S. A., Khom, S., Varodayan, F. P., Patel, R. R., Steinman, M. Q., Bajo, M., Montgomery, S. E., Vlkolinsky, R., Nadav, T., Polis, I., Roberts, A. J., Mayfield, R. D., Harris, R. A., & Roberto, M. (2020). Microglia control escalation of drinking in alcohol-dependent mice: Genomic and synaptic drivers. *Biological Psychiatry*, *88*(12), 910–921. https://doi.org/10.1016/j.biopsych.2020.05.011

Yoneyama, N., Crabbe, J. C., Ford, M. M., Murillo, A., & Finn, D. A. (2008). Voluntary ethanol consumption in 22 inbred mouse strains. *Alcohol*, *42*(3), 149–160. https://doi.org/10.1016/j.alcohol.2007.12.006

Yu, X., Basbaum, A., & Guan, Z. (2021). Contribution of colony-stimulating factor 1 to neuropathic pain. *PAIN Reports*, *6*(1), e883. https://doi.org/10.1097/pr9.0000000000000883

Zeng, L., Alongkronrusmee, D., & van Rijn, R. M. (2017). An integrated perspective on diabetic, alcoholic, and drug-induced neuropathy, etiology, and treatment in the US. *Journal of Pain Research*, *10*, 219–228. https://doi.org/10.2147/JPR.S125987

Zhang, T., Niu, J., Wang, Y., Yan, J., Hu, W., & Mi, D. (2020). The role of C-afferents in mediating neurogenic vasodilatation in plantar skin after acute sciatic nerve injury in rats. *BMC Neuroscience*, *21*(1), 15. https://doi.org/10.1186/s12868-020-00564-6

Zhang, Y., Yang, J., Sevilla, A., Weller, R., Wu, J., Su, C., Zheng, C., Rodriguez-Blanco, Y. F., Gitlin, M., & Candiotti, K. A. (2020). The mechanism of chronic nicotine exposure and nicotine withdrawal on pain perception in an animal model. *Neuroscience Letters*, *715*, 134627. https://doi.org/10.1016/j.neulet.2019.134627

Zhou, Y. Q., Liu, Z., Liu, Z. H., Chen, S. P., Li, M., Shahveranov, A., Ye, D. W., & Tian, Y. K. (2016). Interleukin-6: An emerging regulator of pathological pain. *Journal of Neuroinflammation*, *13*, 141. https://doi.org/10.1186/s12974-016-0607-6

How to cite this article: Borgonetti, V., Roberts, A. J., Bajo, M., Galeotti, N., & Roberto, M. (2023). Chronic alcohol induced mechanical allodynia by promoting neuroinflammation: A mouse model of alcohol-evoked neuropathic pain. *British Journal of Pharmacology*, 1–16. https://doi.org/10.1111/bph.16091

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