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“Maladaptive stress-coping behavior in CX$_3$CR1-deficient mice: Impact of adolescent stress and alcohol exposure on neuroimmune responses and inflammation”

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ABSTRACT

The CX$_3$CL1/CX$_3$CR1 chemokine axis regulates synaptic pruning, plasticity, and stress-related behaviors, influencing resilience or vulnerability to psychiatric disorders. Adolescence, a critical period for neuroimmune development, increases susceptibility to stressors. This study investigated how adolescent restraint stress and alcohol exposure affect stress-coping behavior, neuroimmune signaling, and systemic inflammation in adult wild-type (WT) and CX$_3$CR1 knock-out (KO) mice.

Eighty-one male and female WT and KO mice were assigned to control (non-stressed, saline-treated), stress (stressed, saline-treated), alcohol (non-stressed, alcohol-treated), and stress + alcohol (stressed, alcohol-treated) groups. Behavioral responses were evaluated using the tail suspension test. Hypothalamic gene expression of CX$_3$CL1/CX$_3$CR1, corticotropin-releasing hormone (CRH), and neuropeptide Y (NPY) systems was analyzed alongside plasma corticosterone, adrenocorticotropic hormone (ACTH), CX$_3$CL1, and inflammatory mediators.

Adolescent stress—but not alcohol—increased plasma CX$_3$CL1 levels, which inversely correlated with immobility time in WT mice. KO mice displayed higher baseline immobility than WT mice, whereas stress and/or alcohol paradoxically reduced immobility. These behavioral effects were reproduced by pharmacological inhibition of CX$_3$CR1. Additionally, KO mice showed disrupted hypothalamic expression of multiple genes in the CRH pathway and *Npy1r*, attenuated corticosterone responses to stress, and abolished ACTH–corticosterone correlation, suggesting HPA axis dysregulation. KO mice also exhibited exacerbated inflammatory responses to stress and alcohol, including elevated IL-17A/F, IL-11, and IFN-β1 levels.

CX$_3$CR1 deficiency disrupts neuroimmune homeostasis, leading to maladaptive stress-coping behaviors and heightened inflammatory reactivity. These findings underscore the protective role of the CX$_3$CL1/CX$_3$CR1 axis in neuroinflammatory regulation and stress resilience, supporting CX$_3$CR1 as a potential therapeutic target in stress-related disorders.

1. Introduction

Fractalkine (CX$_3$CL1) is a member of the CX$_3$C chemokine family and is constitutively and abundantly expressed in the central nervous system (CNS), where it is primarily released by neurons (Paolicelli et al., 2014). Unlike other chemokines, CX$_3$CL1 can exist in two forms, soluble and

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membrane-bound, each playing distinct roles in the body (Bajetto et al., 2002). This unique chemokine serves as a crucial mediator in neuroimmune interactions, influencing both the immune response and neuronal communication within the CNS. CX_3CL1 interacts with its specific receptor, CX_3CR1, which is expressed on the surfaces of cells within the CNS, predominantly on microglia, the resident immune cell in the brain (Zhao et al., 2023). The interaction between CX_3CL1 and CX_3CR1 is essential for various physiological processes, including neuroinflammation, synaptic plasticity and pruning, and neuronal survival (Inoue et al., 2021; Luo et al., 2019). Specifically, the CX_3CL1/CX_3CR1 axis plays a key role in glial-neuron communication, modulating microglial activity (Lyons et al., 2009). During stress responses, CX_3CL1 signaling can activate microglia, leading to the release of pro-inflammatory cytokines. This neuroinflammatory response has been linked to altered neuronal function and behavioral changes, including increased anxiety and depressive-like symptoms, suggesting that CX_3CL1 may play a role in the pathophysiology of depression (Luo et al., 2019). Furthermore, studies have suggested that CX_3CL1/CX_3CR1 signaling modulates stress-related mood alterations by modulating microglial activity. Thus, CX_3CR1 deficiency has been shown to promote resilience to chronic stress-induced depressive behaviors, highlighting the relevance of this pathway in susceptibility to stress-related disorders and its potential as a therapeutic target (Hellwig et al., 2016; Milior et al., 2016).

The adolescent brain is particularly vulnerable to stress, making it an important period for studying neuroimmune signaling (Tottenham and Galván, 2016). During adolescence, ongoing development and neurobiological changes can make stress exposure have lasting effects on behavioral and physiological responses, persisting into adulthood (Dinan, 2005; Sheth et al., 2017). A key neurodevelopmental process during this period is the removal of excess synapses, known as synaptic pruning, which is regulated by microglia through mechanisms that include the modulatory role of CX_3CL1/CX_3CR1 signaling (Paolicelli et al., 2011, 2014). Adolescent stress serves as a significant modulator of pruning and has been shown to increase the risk of developing mental health disorders in later life (Eiland and Romeo, 2013). This is especially relevant in the context of the CX_3CL1/CX_3CR1 axis, as neuroimmune interactions during stress play a central role in influencing behavioral responses (Koo and Wohleb, 2021). Therefore, understanding how CX_3CR1-mediated neuroimmune communication contributes to stress resilience or vulnerability in adolescence is crucial for developing effective therapies targeting stress-related mental health conditions.

In our group, preclinical studies often employ restraint stress and forced alcohol exposure to model the impact of stress on physiology and behavior (Gobbi et al., 2024; Sánchez-Marín et al., 2022a, 2022b). Restraint stress is commonly used in rodent models as it does not cause physical pain or harm to the animals, making it an effective method for examining stress-related activation of the hypothalamic-pituitary-adrenal (HPA) axis, which leads to changes in stress hormones, behavior, and neuroimmune signaling (Gobbi et al., 2024; Herman and Cullinan, 1997; Sánchez-Marín et al., 2022a, 2022b). Alcohol exposure further complements this model, simulating substance-induced stress that disrupts normal growth, neuroendocrine function, and immune responses (Crews et al., 2015). Both restraint stress and alcohol exposure significantly impact physiological markers, such as body weight, which is typically reduced under these stress conditions (Sánchez-Marín et al., 2022a). These stressors also influence behavioral responses, including anxiety and depressive-like symptoms (Boden and Fergusson, 2011; Phillips et al., 2005; Sánchez-Marín et al., 2022a, 2022b). Additionally, hormonal changes, including elevated corticosterone and adrenocorticotropic hormone (ACTH) levels, reflect activation of the HPA axis under these conditions, providing insight into the physiological underpinnings of stress-related behavior (Gobbi et al., 2024; Sánchez-Marín et al., 2022b).

In this context, CX_3CR1 knock-out (KO) mice may exhibit alterations in both developmental pruning and stress responses compared with wild-type (WT) mice. Previous studies have shown that CX_3CR1 deficiency can affect stress-related behaviors and disrupt neuroimmune signaling, potentially influencing the resilience or susceptibility to stress (Hellwig et al., 2016; Winkler et al., 2017). By investigating the effects of CX_3CR1 deficiency in both restraint stress and forced alcohol exposure models, we aim to elucidate how CX_3CL1/CX_3CR1 signaling contributes to stress and substance use disorders, as this deficiency may lead to persistent behavioral and neuroendocrine traits characteristic of emotional disorders. In this regard, a previous study from our group has reported changes in plasma CX_3CL1 levels in individuals with alcohol use disorder and Wistar rats exposed to alcohol (García-Marchena et al., 2016), supporting the potential of CX_3CL1 as a plasma biomarker for alcohol-related disorders.

The primary objective of this study was to assess long-term effects of adolescent restraint stress compounded with forced alcohol treatment on stress-coping behavior, hormonal response, and neuroimmune signaling in CX_3CR1-deficient animals. Specifically, we focused on the modulation of stress-related systems, including corticotropin-releasing hormone (CRH) and neuropeptide Y (NPY) in the hypothalamus, along with systemic stress-response hormones and inflammatory mediators in the plasma of WT and KO mice. This integrative approach aims to provide a better understanding of how CX_3CL1/CX_3CR1 signaling may contribute to maladaptive stress responses and vulnerability to psychiatric conditions.

2. Materials and methods

2.1. Ethics statement and animals

All procedures were conducted in accordance with ARRIVE guidelines(Kilkenny et al., 2010) and the principles of replacement, reduction, and refinement (the “3Rs”), complying with the European Communities Council Directive 2010/63/EU, Regulation (EC) No. 86/609/ECC (November 24, 1986), as well as Spanish National and Regional Guidelines for Animal Experimentation (Real Decreto 53/2013). The experimental protocols were approved by the Local Ethical Committee for Animal Research at the University of Malaga (CEUMA #59-2020-A). Every effort was made to minimize animal suffering and reduce the number of animals used.

We used the Cx3cr-1^{GFP} knock-in/knock-out mouse line (C57BL/6J genetic background), which expresses an enhanced green fluorescent protein (E*GFP*) sequence replacing the first 390 bp of the coding exon (exon 2) of the chemokine (C-X3-C motif) receptor 1 *Cx3cr1* gene (Jung et al., 2000). The Cx3cr-1^{GFP} knock-in/knock-out (CX_3CR1 KO) mice were maintained by crossing homozygous transgenic KO mice (The Jackson Laboratory, Bar Harbor, ME, USA). Additionally, C57BL/6J mice were used as control non-mutants and maintained by crossing wild-type (WT) mice (The Jackson Laboratory, Bar Harbor, ME, USA). Both WT and KO mice were housed in groups of 3–4 per cage on a 12-h light/dark cycle (lights on at 07:00 h) with water and food provided *ad libitum* and appropriate environmental enrichment.

2.2. Experimental groups and procedures

Male and female WT and KO mice were randomly assigned to one of four experimental groups, each consisting of 6–8 animals (3–4 males and 3–4 females): Control (non-stressed, saline-treated mice), Stress (stressed, saline-treated mice), Alcohol (non-stressed, alcohol-treated mice), and Stress + Alcohol (stressed, alcohol-treated mice). See Fig. 1 for the timeline and details of the experimental schedule. For this study, data from male and female mice were combined for analysis.

2.2.1. Acute restraint stress

Five-week-old mice assigned to the stress and stress + alcohol groups were placed in plexiglass restraint tubes of appropriate size for a continuous 90-min session, after which they were returned to their home cages (Sánchez-Marín et al., 2022b). Non-stressed mice in the control

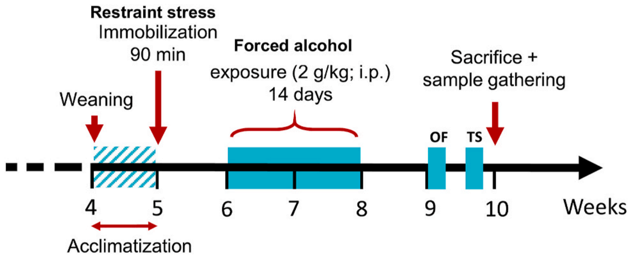

Fig. 1. Timeline and experimental schedule. At four weeks of age, mice were introduced to the testing and treatment environment for a 1-week acclimatization period. Following this, WT and KO mice were randomly assigned to either the experimental stress or non-stress groups. Mice in the stress groups underwent acute restraint stress for 90 min. One week later, WT and KO mice from each group were randomly assigned to receive saline or alcohol treatment for 14 days. Locomotion was assessed using the open field (OF) test, and stress-coping behavior was evaluated using the tail suspension test (TS), conducted 7 and 10 days after the final alcohol administration, respectively. Mice were sacrificed two weeks after the final alcohol administration, with brain and blood samples collected.

and alcohol groups remained undisturbed in their home cages throughout the procedure.

2.2.2. Forced alcohol treatment

For alcohol exposure, mice in the alcohol and stress + alcohol groups received a daily intraperitoneal (i.p.) dose of ethanol (2 g/kg in a volume of 10 mL/kg) for 14 consecutive days (Anderson et al., 2016). Following the final administration, mice were left undisturbed in their home cages until the tail suspension test was conducted. Mice in the control and stress groups received saline via i.p. injection under the same protocol.

2.3. Open field and tail suspension test

One week after the final administration of alcohol or saline, control WT and KO mice (non-stressed, saline-treated) were weighed, and their locomotor activity and anxiety-like behavior were assessed in the open field. Three days later, the tail suspension test was conducted to measure total time spent immobile, serving as an indicator of stress-coping behavior in WT and KO mice, as previously described (Pavón et al., 2021).

Mice were suspended by their tails on a bar positioned 30 cm above the floor, secured with tape strong enough to support their weight. They were suspended for 6 min in a quiet, dimly lit room, and immobility duration, defined as a lack of movement aside from respiration, was recorded. The experiment was conducted according to specific guidelines to ensure consistent, reliable results (Aslam, 2016). Data were analyzed under double-blind conditions by two independent researchers.

Additionally, a separate set of WT mice from the four experimental groups, consisting of 6 animals (3 males and 3 females), received AZD8797 (CAS #911715-90-7, Axon Medchem, Groningen, Netherlands), a potent and selective CX_3CR1 antagonist, or saline (vehicle) prior to behavioral assessment. AZD8797 is an allosteric, non-competitive modulator of the CX_3CR1, with a dissotiation rate constant (koff) of 0.042 min^{-1}, corresponding to a half-life of approximately 17 min for its dissociation from the receptor (Cederblad et al., 2016). We first conducted a pharmacological trial to determine the optimal antagonist dose by testing various concentrations (0.2, 2, and 20 mg/kg, i.p.) with no locomotor effects in the open field. AZD8797 was dissolved in saline and administered intraperitoneally at a dose of 20 mg/kg (in a volume of 10 mL/kg) 30 min prior to the tail suspension test.

2.4. Sample collection

Four days after the tail suspension test (i.e., two weeks after the final administration of alcohol or saline), mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and trunk blood and brain samples were collected. Blood samples were centrifuged at 2000×*g* for 15 min, and plasma from the supernatant was collected and transferred to cryotubes. Brains were rapidly removed and immediately frozen on dry ice. Both plasma and brain samples were stored at −80 °C.

2.4.1. Gene expression of stress-related proteins in the brain

Frozen brains were placed in a stainless steel mouse brain matrix (40–75 g, coronal) (RWD Life Sciences, Sugar Land, TX, USA), and 1-mm thick slices were obtained using brain matrix razor blades. The hypothalamus was bilaterally dissected and collected using a scalpel with surgical blades and a sample corer, guided by a mouse brain atlas.

Total RNA was extracted from 25 mg of brain tissue sections using the TRIzol® method, according to the manufacturer's instructions (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). RNA isolation was further processed using an RNA easy minelute cleanup kit, including DNase I column digestion (Qiagen), and quantified with a spectrophotometer to ensure A260/280 ratios between 1.8 and 2.0. Reverse transcription was performed using 1 μg of RNA with the Transcriptor Reverse Transcriptase kit (Transcriptor RT, Roche Applied Science, Mannheim, Germany) and specific primer probe sets for *Crh, Crhr1, Crhr2, Npy, Npy1r, Npy2r, Cx3cl1, Cx3cr1, and β-actin* (see Table S1) from TaqMan® Gene Expression Assays (Thermo Fisher Scientific, Waltham, MA, USA).

Real-time qPCR reactions were run on a CFX96TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using FAM dye-labeled TaqMan® Gene Expression Assays (Thermo Fisher Scientific, Waltham, MA, USA). A melting curve analysis confirmed the amplification of a single product. Results were normalized to β-actin levels (Thermo Fisher Scientific, Waltham, MA, USA).

2.4.2. Inflammatory and stress-response mediators in plasma

Plasma levels of inflammatory mediators, including interleukins, chemokines, and growth factors, were quantified in both genotypes using a multiplex immunoassay system with two commercial kits: (1) MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel II (CAS #MECY2MAG-73K; Merck Millipore, Merck, Darmstadt, Germany) [Erythropoietin (EPO); Exodus-2/CCL21; Fractalkine (CX_3CL1); Interferon beta (IFN-β1); IFN gamma (IFN-γ); IL-11; IL-16; IL-17A/F; IL-20; Murine monocyte chemoattractant protein-5 (MCP-5/CCL12); Macrophage-derived chemokine (MDC/CCL22); Macrophage inflammatory protein-3 alpha (MIP-3α/CCL20); MIP-3 beta (MIP-3β/CCL19); and Thymus and activation-regulated chemokine (TARC/CCL17)]. (2) ProcartaPlex Mouse Cytokine & Chemokine Panel 1 (CAS #EPX260-26088-901; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) [Granulocyte-macrophage colony-stimulating factor (GM-CSF); IL-1 beta (IL-1β); IL-2; IL-4; IL-5; IL-6; IL-12p70; IL-13; IL-18; Tumour necrosis factor alpha TNF-alpha (TNF-α); IL-9; IL-10; Cytotoxic T-lymphocyte-associated antigen 8 (CTLA-8/IL-17A); IL-22, IL-23, IL-27; Eotaxin-1/CCL11; Growth-regulated oncogene alpha (GROα/CXCL1); Interferon gamma-induced protein 10 (IP-10/CXCL10); MCP-1/CCL2; MCP-3/CCL7; MIP-1 alpha (MIP-1α/CCL3); MIP-1 beta (MIP-1β/CCL4); MIP-2α/CXCL2; and Regulated on activation, normal T-cell expressed and secreted (RANTES/CCL5)]. These 96-well plates were run on a Bio-Plex MAGPIX™ Multiplex Reader with Bio-Plex Manager™ MP Software (Luminex, Austin, TX, USA). Concentrations were expressed as pg/mL.

Plasma levels of corticosterone and adrenocorticotropic hormone (ACTH) were quantified using the Corticosterone ELISA kit (CAS #ab108821; Abcam, Cambridge, UK), and the Mouse/Rat ACTH ELISA Kit (CAS #ab263880; Abcam, Cambridge, UK) according to the manufacturer's instructions. Plates were analyzed on an Accuris

SmartReader™ 96 microplate absorbance reader (Merck, Darmstadt, Germany). Concentrations were expressed as pg/mL.

All samples were run in duplicate. The concentration assigned to samples with absorbance lower than the limit of detection but higher than the background in the immunoassays was set at half of the minimum concentration interpolated from the corresponding standard curve (Jiménez-López et al., 2024; Whitcomb and Schisterman, 2008).

2.5. Statistical analysis

Data are presented as the mean and standard error of the mean (mean ± SEM). To assess genotype-related differences in behavioral and molecular variables, we conducted a two-way analysis of variance (ANOVA) with “genotype” (WT mice vs. CX_3CR1 KO mice) and “experimental group” (control, stress, alcohol, and stress + alcohol) as factors, followed by Sidak’s *post hoc* test for multiple comparisons when an interaction was detected. Specifically, significant main effects of “genotype” and/or an interaction between the two factors were reported.

Subsequently, differences within and between experimental groups for each genotype were evaluated using two-way ANOVA with “stress” (non-stress vs. stress) and “alcohol” (saline vs. alcohol) as factors, followed by the Sidak’s *post hoc* test when an interaction was observed. Analyses were not stratified by sex; instead, the number of male and female mice was balanced across experimental groups to focus on the effect of genotype and minimize potential bias due to sex differences.

Correlation analyses between plasma corticosterone and ACTH, as well as between plasma CX_3CL1 and behavioral variables, were performed using Pearson’s correlation coefficient (r).

Test statistic values and degrees of freedom are indicated in the results, and a *p*-value less than 0.05 was considered statistically significant. Statistical analyses were conducted using GraphPad Prism version 9 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Effects of stress and alcohol on stress-coping behavior following genetic or pharmacological blockade of CX_3CR1

3.1.1. Body weight gain, locomotion, and anxiety-like behavior in WT and CX_3CR1 KO mice

One week after the final alcohol or saline administration, we measured body weight gain from the onset of the forced alcohol/saline exposure and examined both locomotion and the percentage of total time spent in the center of the open field test in WT and KO mice to evaluate their potential as confounding variables. Two-way ANOVA, with genotype and experimental group as factors, revealed no main effect of genotype and no genotype × experimental group interaction for body weight gain, distance traveled, or time spent in the center of the open field. Furthermore, two-way ANOVA conducted with stress and alcohol as factors within each genotype also showed no significant main effects or interactions for these variables (Fig. S1).

3.1.2. Immobility time in WT and CX_3CR1 KO mice

Three days after the open field test, stress-coping behavior was assessed in adult WT and KO mice using the tail suspension test. Two-

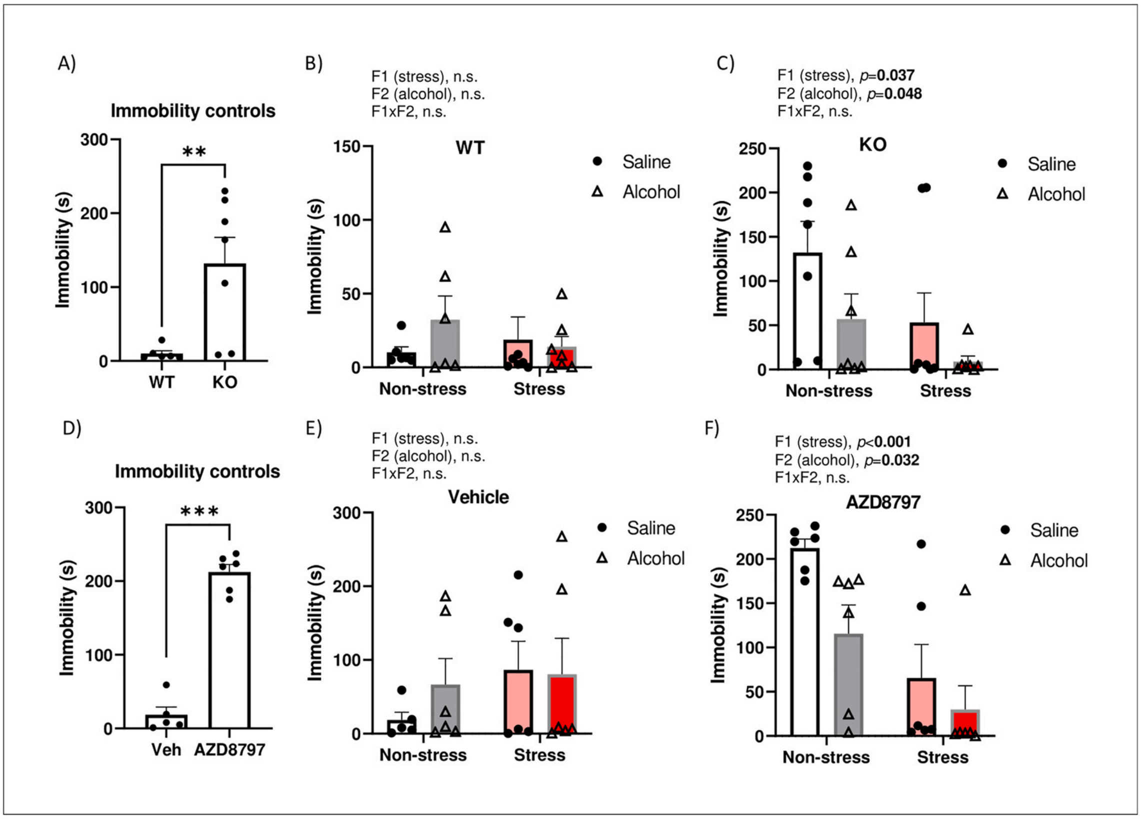

Fig. 2. Effects of adolescent restraint stress and forced alcohol treatment on immobility time in the tail suspension test following genetic and pharmacological inhibition of CX_3CR1 in adult mice. (A) Immobility time (s) in control groups of WT and KO mice. **(B)** Immobility time (s) in WT mice exposed to stress and/or alcohol. **(C)** Immobility time (s) in KO mice exposed to stress and/or alcohol. **(D)** Immobility time (s) in control groups of vehicle and AZD8797 mice. **(E)** Immobility time (s) in WT mice exposed to stress and/or alcohol after saline administration. **(F)** Immobility time (s) in WT mice exposed to stress and/or alcohol after AZD8797 (20 mg/kg, i.p.) administration. Histograms represent the mean ± SEM (6–8 mice per group). Data were analyzed using Sidak’s *post hoc* test (A and D) and two-way ANOVA with stress (F1) and alcohol (F2) as factors (B, C, D and F). (**) denotes $p < 0.01$ and (***) denotes $p < 0.001$. *P*-values in bold indicate significant main effects of factors (F1 and F2) or significant interaction (F1 × F2). n.s. = non-significant.

way ANOVA, with genotype and experimental group as factors, revealed a significant interaction between the two factors for immobility time ($F_{3,48} = 2.859$; $p = 0.046$). Specifically, control KO mice exhibited significantly higher immobility than control WT mice ($p < 0.01$) (Fig. 2A), whereas no differences were observed among the other groups. Furthermore, no sex differences were detected in control mice (Fig. S2A).

Next, two-way ANOVA for immobility time was performed within each genotype, using stress and alcohol as factors. In WT mice, no significant main effects or interactions were observed, although a trend toward increased immobility time was noted in non-stressed, alcohol-treated mice (Fig. 2B). In contrast, analysis in KO mice revealed significant main effects of both stress ($F_{1,25} = 4.837$; $p = 0.037$) and alcohol ($F_{1,25} = 4.307$; $p = 0.048$). Accordingly, stressed mice and alcohol-treated mice exhibited significantly lower immobility time compared with non-stressed mice and saline-treated mice, respectively (Fig. 2C).

3.1.3. *Immobility time in mice treated with vehicle and CX_3CR1 antagonist*

To confirm the decrease in immobility time observed in KO mice, CX_3CR1 was pharmacologically inhibited in WT mice using AZD8797. Consistent with the KO phenotype, two-way ANOVA revealed a significant interaction between pharmacological treatment and experimental group ($F_{3,39} = 5.122$; $p = 0.004$), with AZD8797-treated mice displaying significantly higher immobility compared with vehicle-treated mice in the control group ($p < 0.001$) (Fig. 2D). Notably, this effect was not associated with changes in locomotor activity or stereotyped behaviors. As in previous analyses, no sex differences were detected among control mice treated with either vehicle or the CX_3CR1 antagonist (Fig. S2B).

In the vehicle-treated groups, no significant main effects or interaction of stress and alcohol were found (Fig. 2E). In contrast, the AZD8797-treated groups, two-way ANOVA revealed significant main effects of stress ($F_{1,20} = 16.23$; $p < 0.001$) and alcohol ($F_{1,20} = 5.294$; $p = 0.032$). Specifically, stressed mice and alcohol-treated mice exhibited significantly lower immobility than non-stressed mice and saline-treated mice, respectively (Fig. 2F). These results in stress-coping behavior following AZD8797 treatment closely resembled those observed in KO mice.

3.2. *Effects of stress and alcohol on stress-related hormones in plasma of WT and CX_3CR1 KO mice*

We next investigated the impact of acute stress and/or alcohol exposure on plasma corticosterone and ACTH levels in adult WT and KO mice.

3.2.1. *Corticosterone levels*

Corticosterone levels were measured in WT and KO mice, and two-way ANOVA revealed no main effect of genotype and no genotype × experimental group interaction.

When analyzed separately by genotype, two-way ANOVA revealed a significant main effect of alcohol on corticosterone levels in WT mice ($F_{1,33} = 7.601$; $p = 0.009$), with alcohol-treated mice exhibiting higher corticosterone levels than saline-treated mice (Fig. 3A). In KO mice, alcohol had no main effect; however, a significant main effect of stress was observed ($F_{1,31} = 7.799$; $p = 0.009$), with stressed mice showing lower corticosterone levels compared with non-stressed mice (Fig. 3B).

3.2.2. *ACTH levels*

Consistent with the findings for corticosterone, two-way ANOVA revealed no significant main effect of genotype or genotype ×

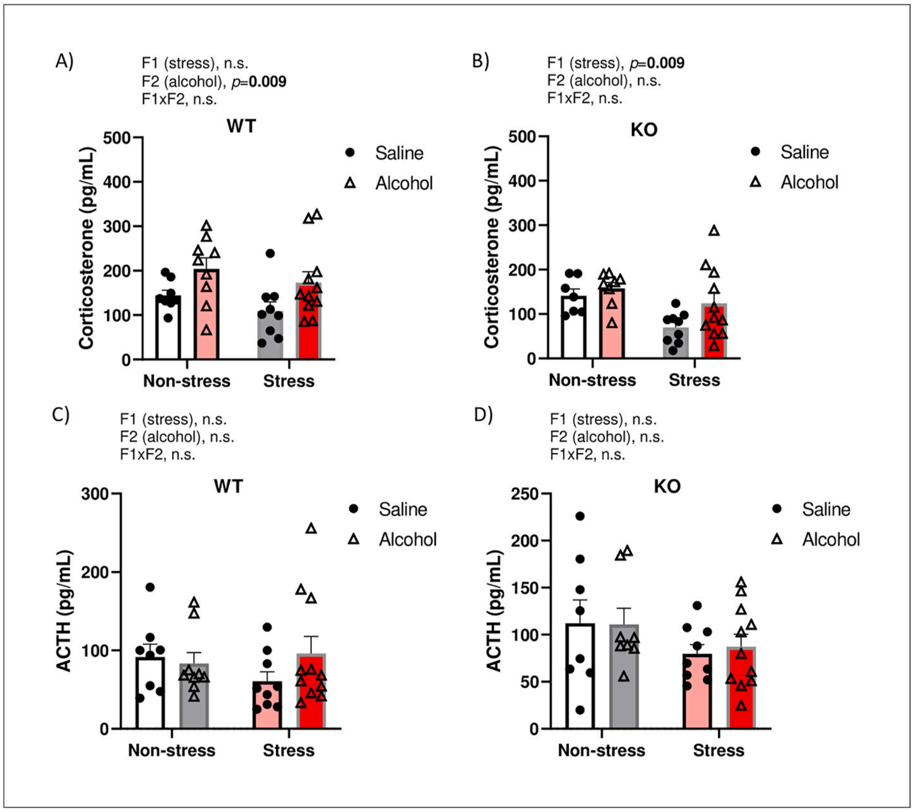

Fig. 3. Effects of adolescent restraint stress and forced alcohol treatment on plasma corticosterone and ACTH levels in adult WT and CX_3CR1 KO mice. (A) Plasma corticosterone levels (pg/mL) in WT mice exposed to stress and/or alcohol. **(B)** Plasma corticosterone levels (pg/mL) in KO mice exposed to stress and/or alcohol. **(C)** Plasma ACTH levels (pg/mL) in WT mice exposed to stress and/or alcohol. **(D)** Plasma ACTH levels (pg/mL) in KO mice exposed to stress and/or alcohol. Histograms represent the mean ± SEM (6–8 mice per group). Data were analyzed using two-way ANOVA with stress (F1) and alcohol (F2) as factors. *P*-values in bold indicate significant main effects of factors (F1 and F2) or significant interaction (F1 × F2). n.s. = non-significant.

experimental group interaction on ACTH levels in WT and KO mice. However, in contrast to corticosterone, ACTH levels were not significantly affected by stress, alcohol, or their interaction in either WT (Fig. 3C) or KO mice (Fig. 3D).

3.2.3. Association between corticosterone and ACTH levels

Given the reported association between these plasma hormones, their correlation was assessed in WT and KO mice. A significant positive correlation between corticosterone and ACTH levels was observed only in WT mice ($r = +0.348$; $p = 0.017$), suggesting a disruption of HPA axis regulation in KO mice.

3.3. Effects of stress and alcohol on anxiety-related genes in the hypothalamus of WT and CX$_3$CR1 KO mice

To assess genotype-related differences and the effects of acute stress and/or alcohol, mRNA levels of genes involved in CRH and NPY signaling systems were analyzed in the hypothalamus of WT and KO mice.

3.3.1. Gene expression of CRH system

The mRNA levels of *Crh* and its receptors, *Crhr1* and *Crhr2*, were measured in the hypothalamus.

3.3.1.1. Crh expression. The analysis of *Crh* expression in WT and KO mice revealed no significant main effect of genotype and no genotype × experimental group interaction. However, significant differences emerged when each genotype was analyzed separately.

In WT mice, two-way ANOVA indicated a main effect of stress and a significant stress × alcohol interaction ($F_{1,31} = 6.076$; $p = 0.019$). *Post hoc* testing indicated that stressed, alcohol-treated mice had significantly higher *Crh* mRNA levels compared with non-stressed, alcohol-treated mice ($p < 0.01$) and stressed, saline-treated mice ($p < 0.05$) (Fig. 4A). In KO mice, a significant main effect of stress was observed ($F_{1,30} = 8.314$; $p = 0.007$), although alcohol did not enhance the effect of stress. Accordingly, stressed mice exhibited higher *Crh* mRNA levels

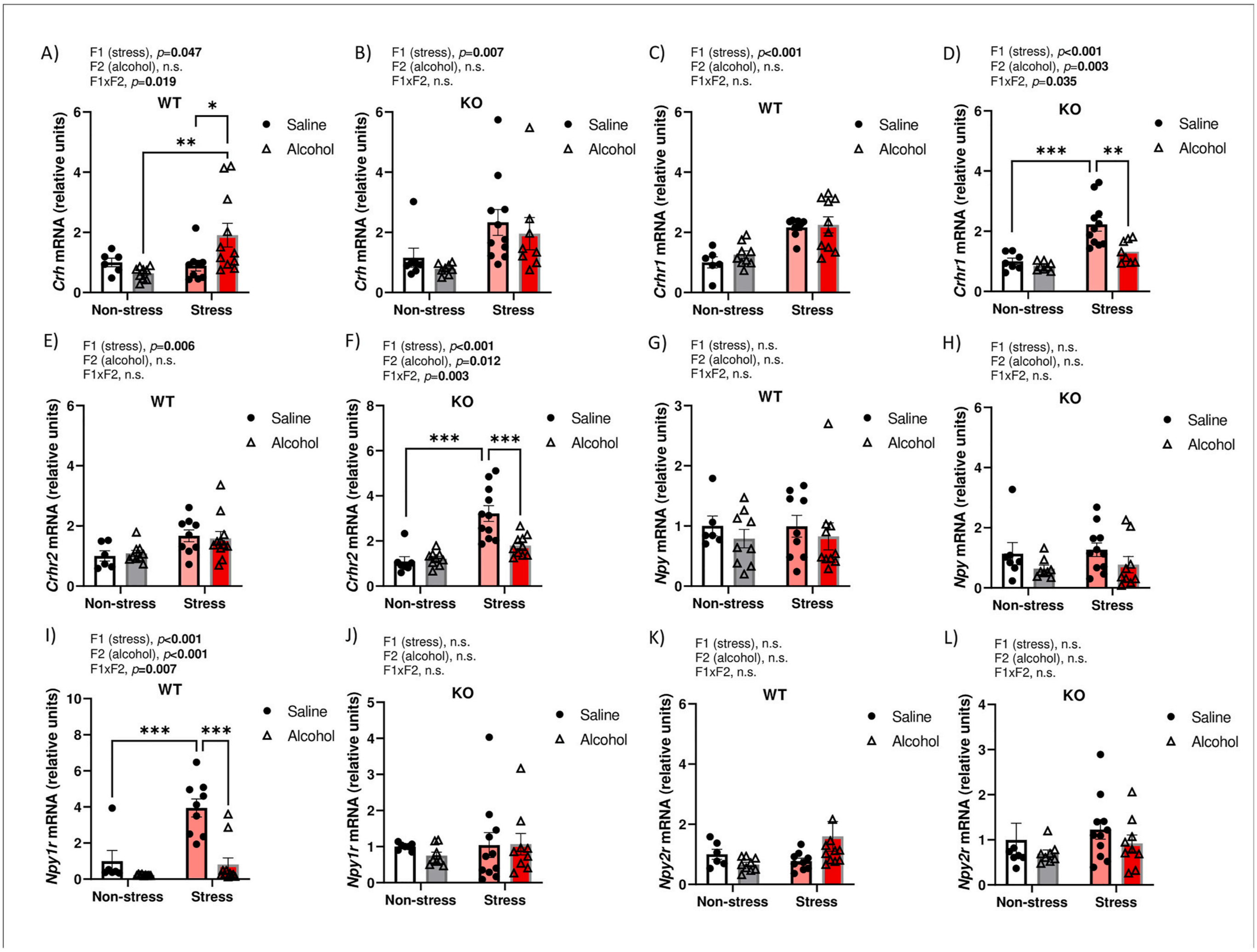

Fig. 4. Effects of adolescent restraint stress and forced alcohol treatment on anxiety-related genes in the hypothalamus of adult WT and CX$_3$CR1 KO mice. **(A)** Relative *Crh* mRNA levels in WT mice exposed to stress and/or alcohol. **(B)** Relative *Crh* mRNA levels in KO mice exposed to stress and/or alcohol. **(C)** Relative *Crhr1* mRNA levels in WT mice exposed to stress and/or alcohol. **(D)** Relative *Crhr1* mRNA levels in KO mice exposed to stress and/or alcohol. **(E)** Relative *Crhr2* mRNA levels in WT mice exposed to stress and/or alcohol. **(F)** Relative *Crhr2* mRNA levels in KO mice exposed to stress and/or alcohol. **(G)** Relative *Npy* mRNA levels in WT mice exposed to stress and/or alcohol. **(H)** Relative *Npy* mRNA levels in KO mice exposed to stress and/or alcohol. **(I)** Relative *Npyr1r* mRNA levels in WT mice exposed to stress and/or alcohol. **(J)** Relative *Npyr1r* mRNA levels in KO mice exposed to stress and/or alcohol. **(K)** Relative *Npyr2r* mRNA levels in WT mice exposed to stress and/or alcohol. **(L)** Relative *Npyr2r* mRNA levels in KO mice exposed to stress and/or alcohol. Histograms represent mean ± SEM (6–8 mice per group). Data were analyzed using two-way ANOVA with stress (F1) and alcohol (F2) as factors. (*) denotes $p < 0.05$, (**) denotes $p < 0.01$, and (***) denotes $p < 0.001$. *P*-values in bold indicate significant main effects of factors (F1 and F2) or significant interaction (F1 × F2). n.s. = non-significant.

than non-stressed mice (Fig. 4B).

3.3.1.2. Crhr1 and Crhr2 expression. The analysis for *Crhr1* expression, with genotype and experimental group as factors, revealed a significant main effect of genotype ($F_{1,61} = 17.39$; $p < 0.001$) and a significant interaction ($F_{3,61} = 4.700$; $p = 0.005$). *Post hoc* comparisons showed that stressed KO mice exhibited significantly higher *Crhr1* mRNA levels than stressed WT mice ($p < 0.001$). Similarly, analysis of *Crhr2* mRNA levels revealed a significant main effect of genotype ($F_{1,61} = 9.176$; $p = 0.004$) and a significant interaction ($F_{3,61} = 5.168$; $p = 0.003$), with stressed KO mice displaying significantly higher *Crhr2* mRNA levels than stressed WT mice ($p < 0.001$).

In WT mice, subsequent analyses showed a significant main effect of stress on both *Crhr1* ($F_{1.30} = 30.41$; $p < 0.001$) and *Crhr2* mRNA levels ($F_{1.31} = 8.740$; $p = 0.006$), with stressed mice exhibiting higher expression than non-stressed mice (Fig. 4C and D). In KO mice, two-way ANOVA revealed significant main effects of stress and alcohol, as well as significant interactions for *Crhr1* ($F_{1,30} = 4.841$; $p = 0.035$) and *Crhr2* ($F_{1,33} = 9.874$; $p = 0.003$). *Post hoc* comparisons indicated that stressed, saline-treated mice had significantly higher expression of both receptors *Crhr1* and *Crhr2* mRNA levels compared with both non-stressed, saline-treated mice ($p < 0.001$) and stressed, alcohol-treated mice ($p < 0.01$ and $p < 0.001$, respectively) (Fig. 4E and F).

3.3.2. Gene expression of NPY system

The mRNA levels of *Npy* and its receptors, *Npy1r* and *Npy2r*, were also measured in the hypothalamus.

3.3.2.1. Npy expression. Two-way ANOVA revealed no significant main effects of genotype and no genotype × experimental group interaction for *Npy* mRNA levels. Furthermore, separate analyses within each genotype showed no significant main effects of stress or alcohol, nor any interactions between these factors (Fig. 4G and H).

3.3.2.2. Npy1r and Npy2r expression. The analysis of *Npy1r* mRNA levels revealed a significant interaction between genotype and experimental group ($F_{3,62} = 11.88$; $p < 0.001$). *Post hoc* comparisons indicated that stressed KO mice exhibited significantly lower *Npy1r* mRNA levels than stressed WT mice ($p < 0.001$). In contrast, no significant main effects or interactions involving genotype were observed for *Npy2r* mRNA levels.

Within-genotype analyses revealed differential effects of stress and alcohol on receptor expression. In WT mice, significant main effects of both factors were observed, along with a significant interaction for *Npy1r* mRNA levels ($F_{1,31} = 8.328$; $p = 0.007$). *Post hoc* comparisons showed that stressed, saline-treated mice exhibited significantly higher *Npy1r* mRNA levels compared with both non-stressed, saline-treated mice ($p < 0.001$) and stressed, alcohol-treated mice ($p < 0.001$) (Fig. 4I). In KO mice, no significant effects or interaction of stress and/or alcohol were found for *Npy1r* expression (Fig. 4J). Similarly, analysis of *Npy2r* mRNA levels revealed no significant main effects or interactions in either genotype (Fig. 4K and L).

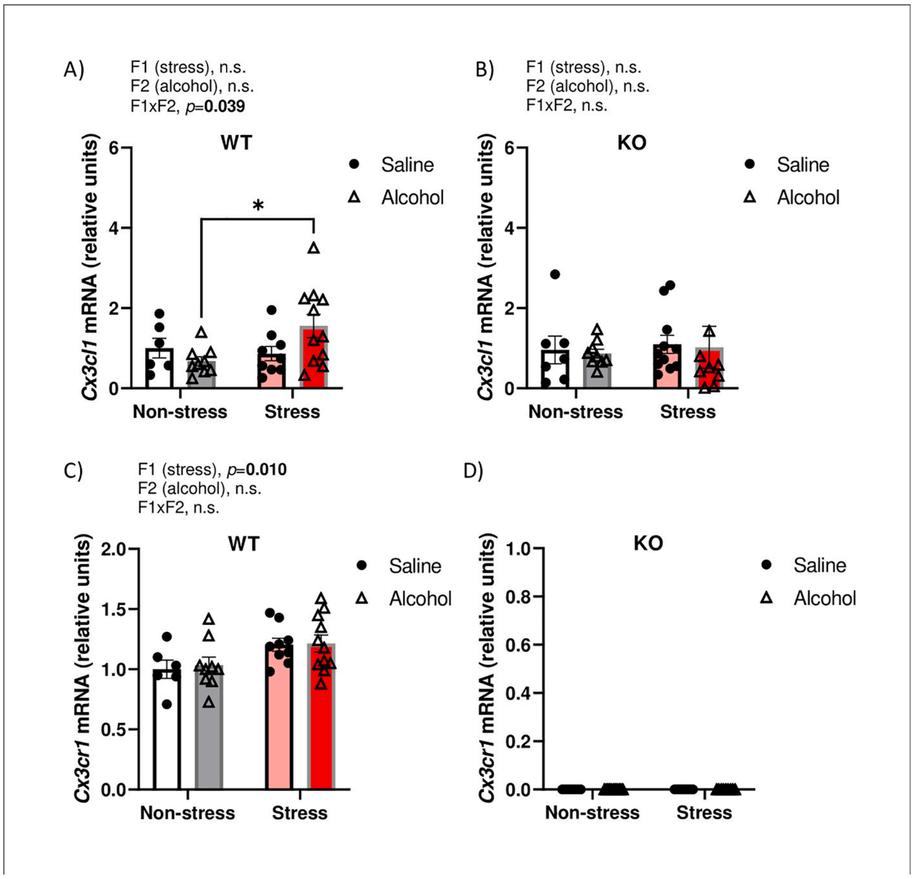

Fig. 5. Effects of adolescent restraint stress and forced alcohol treatment on *Cx3cl1 and Cx3cr1* expression in the hypothalamus of adult WT and CX_3CR1 KO mice. (A) Relative *Cx3cl1* mRNA levels in WT mice exposed to stress and/or alcohol. **(B)** Relative *Cx3cl1* mRNA levels in KO mice exposed to stress and/or alcohol. **(C)** Relative *Cx3cr1* mRNA levels in WT mice exposed to stress and/or alcohol. **(D)** Relative *Cx3cr1* mRNA levels in KO mice exposed to stress and/or alcohol. Histograms represent the mean ± SEM (6–8 mice per group). Data were analyzed using two-way ANOVA with stress (F1) and alcohol (F2) as factors. (*) denotes $p < 0.05$. *P*-values in bold indicate significant main effects of factors (F1 and F2) or significant interaction (F1 × F2). n.s. = non-significant.

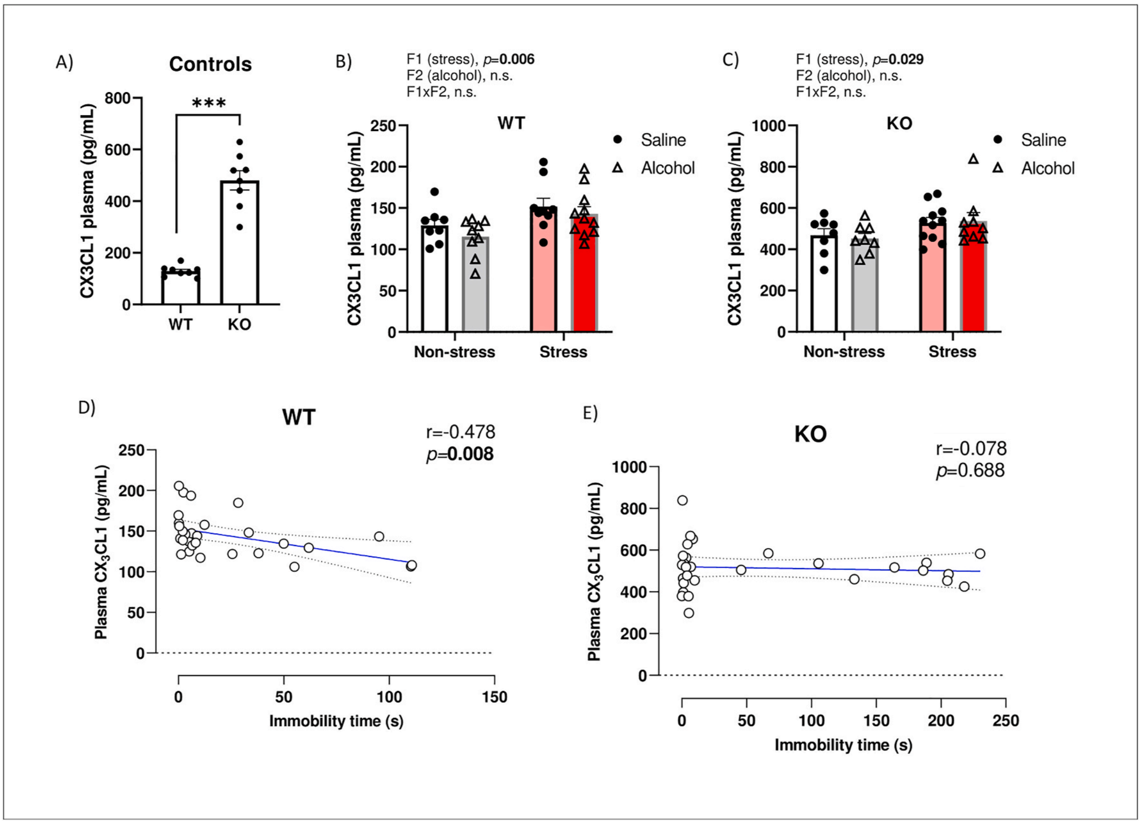

Fig. 6. Effects of adolescent restraint stress and forced alcohol treatment on plasma CX_3CL1 levels of adult WT and CX_3CR1 KO mice. (A) Plasma CX_3CL1 levels (pg/mL) in control groups of WT and KO mice. **(B)** Plasma CX_3CL1 levels (pg/mL) in WT mice exposed to stress and/or alcohol. **(C)** Plasma CX_3CL1 levels (pg/mL) in KO mice exposed to stress and/or alcohol. **(D)** Pearson's correlation analysis (r) between plasma CX_3CL1 levels (pg/mL) and immobility time (s) in the tail suspension test in WT mice **(E)** Pearson's correlation analysis (r) between plasma CX_3CL1 levels (pg/mL) and immobility time (s) in the tail suspension test in KO mice. Histograms (A–C) represent the mean ± SEM (6–8 mice per group). Data were analyzed using Sidak's *post hoc* test (A) and two-way ANOVA with stress (F1) and alcohol (F2) as factors (B and C). (***) denotes $p < 0.001$. *P*-values in bold indicate significant main effects of factors (F1 and F2) or significant interaction (F1 × F2). n.s. = non-significant.

3.4. Effects of stress and alcohol on *Cx3cl1* and *Cx3cr1* expression in the hypothamus of WT and CX_3CR1 KO mice

3.4.1. *Cx3cl1* expression

Two-way ANOVA revealed no significant main effects of genotype and no significant genotype × experimental group interaction for *Cx3cl1* mRNA levels in the hypothalamus.

When analyzed separately in WT mice, two-way ANOVA revealed a significant interaction between stress and alcohol ($F_{1,31} = 4.633$; $p = 0.039$). *Post hoc* testing indicated that stressed, alcohol-treated mice exhibited significantly higher *Cx3cl1* mRNA levels compared with non-stressed, alcohol-treated mice ($p < 0.05$) (Fig. 5A). In contrast, in KO mice, no significant main effects or interaction of stress or alcohol were observed for *Cx3cl1* mRNA levels (Fig. 5B).

3.4.2. *Cx3cr1* expression

As expected, given the deletion of this receptor in KO mice, two-way ANOVA revealed a significant main effect of genotype on *Cx3cr1* mRNA levels ($F_{1,62} = 1024$; $p = 0.010$).

In WT mice, analysis showed a significant main effect of stress ($F_{1,31} = 7.532$; $p = 0.010$), with stressed mice exhibiting higher *Cx3cr1* mRNA levels compared with non-stressed mice (Fig. 5C). As anticipated, *Cx3cr1* mRNA levels were undetectable in KO mice, and further analysis was not conducted (Fig. 5D).

3.5. Effects of stress and alcohol on CX_3CL1 levels in the plasma of WT and CX_3CR1 KO mice

3.5.1. CX_3CL1 levels

Plasma levels of soluble CX_3CL1 were measured, and two-way ANOVA, with genotype and group as factors, revealed a significant main effect of genotype ($F_{1,65} = 493.5$; $p < 0.001$), with KO mice exhibiting significantly higher levels than WT mice across all groups. As an example, data from control mice are presented in Fig. 6A.

Within-genotype analyses indicated a significant main effect of stress on CX_3CL1 levels in both WT ($F_{1,33} = 8.556$; $p = 0.006$, Fig. 6B) and KO mice ($F_{1,32} = 5.172$; $p = 0.029$, Fig. 6C), with stressed mice displaying higher levels than their non-stressed counterparts.

3.5.2. Association between CX_3CL1 levels and immobility time

A correlation analysis was conducted to assess the association between plasma CX_3CL1 levels and immobility time in the tail suspension test. Notably, a significant negative correlation was observed in WT mice ($r = -0.585$; $p < 0.001$) (Fig. 6D), whereas no significant association was found in KO mice (Fig. 6E).

3.6. Effects of stress and alcohol on inflammatory mediator levels in the plasma of WT and CX_3CR1 KO mice

In addition to CX_3CL1, we evaluated the levels of other cytokines and growth factors. Two-way ANOVA revealed significant main effects of genotype and/or genotype × experimental group interactions for several analytes.

Specifically, a significant main effect of genotype was observed for IL-5 ($F_{1,64} = 5.624$; $p = 0.021$), with KO mice displaying significantly lower levels than WT mice across all groups. Significant interactions were detected for CTLA-8/IL-17A ($F_{3,62} = 3.133$; $p = 0.032$) and IL-13 ($F_{3,63} = 2.926$; $p = 0.040$), with KO mice exhibiting significantly lower levels than WT mice in the alcohol group ($p < 0.01$); IL-16 ($F_{3,63} = 4.058$; $p = 0.010$), with KO mice exhibiting significantly higher levels in the control and stress groups ($p < 0.05$); IL-17A/F ($F_{3,64} = 5.508$; $p = 0.002$), MDC/CCL22 ($F_{3,64} = 3.752$; $p = 0.015$), and MIP-3β/CCL19 ($F_{3,62} = 4.226$; $p = 0.009$), with KO mice exhibiting significantly higher levels in the stress + alcohol group ($p < 0.05$); and TARC/CCL17 ($F_{3,64} = 3.474$; $p = 0.021$), where KO mice displayed significantly higher levels in the stress group.

Subsequently, multiple two-way ANOVAs were conducted for each genotype to assess the effects of stress and/or alcohol on the plasma levels of these inflammatory mediators. For clarity, plasma concentrations of inflammatory markers and significant statistical details are presented separately for WT (Table 1) and KO mice (Table 2). In cases where a significant stress × alcohol interaction was identified, main effects were not further interpreted in order to focus on the combined influence of both factors.

3.6.1. *Inflammatory mediators in WT mice*

3.6.1.1. Stress effect. Two-way ANOVA revealed a main effect of stress on IL-20 ($F_{1,33} = 9.301$; $p = 0.004$) and MIP-3α/CCL20 levels ($F_{1,33} = 4.931$; $p = 0.033$). For both cytokines, stressed mice exhibited lower levels compared with non-stressed WT mice.

3.6.1.2. Alcohol effect. A significant main effect of alcohol was observed for IL-17A/F ($F_{1,33} = 5.382$; $p = 0.026$), IFN-γ ($F_{1,33} = 5.090$; $p = 0.030$), and EPO ($F_{1,33} = 6.316$; $p = 0.017$), with alcohol-treated mice exhibiting higher levels of these mediators compared with saline-treated WT mice.

3.6.1.3. Stress and alcohol interaction. IL-1β levels showed a significant stress × alcohol interaction ($F_{1,33} = 6.261$; $p = 0.033$). *Post hoc*

Table 1
Plasma levels of inflammatory mediators in WT mice by experimental group and two-way ANOVA results with stress and alcohol as factors.

Inflammatory Mediator (pg/mL)	GROUPS				STATISTICS *					
	CONTROL	STRESS	ALCOHOL	STRESS + ALCOHOL	Factor 1 (F1) Stress		Factor 2 (F2) Alcohol		F1 × F2 Interaction	
	non-stressed, saline-treated	**stressed, saline-treated**	**non-stressed, alcohol-treated**	**stressed, alcohol-treated**	**F statistic**	***p*-value**	**F statistic**	***p*-value**	**F statistic**	***p*-value**
	Mean ± SEM	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**						
CTLA-8/IL-17A	10.081 ± 1.158	7.722 ± 0.683	11.442 ± 1.894	14.362 ± 4.718	3.257	0.081	1.923	0.175	0.095	0.759
Eotaxin-1/CCL11	1842 ± 309	2746 ± 351	1912 ± 305	2236 ± 263	3.979	0.054	0.512	0.479	0.886	0.353
EPO	75.69 ± 3.74	89.61 ± 16.47	123.80 ± 27.99	143.00 ± 20.90	0.722	0.402	**6.316**	**0.017**	0.012	0.913
Exodus-2/CCL21	8604 ± 678	9222 ± 1008	9695 ± 1001	9002 ± 719	0.002	0.966	0.251	0.620	0.569	0.456
Fractalkine/CX3CL1	128.8 ± 7.61	151.8 ± 10.05	115.3 ± 7.60	143.0 ± 8.51	**8.556**	**0.006**	1.657	0.207	0.074	0.787
GM-CSF	8.625 ± 1.802	8.944 ± 1.617	10.440 ± 1.444	15.051 ± 6.392	0.393	0.536	1.012	0.322	0.298	0.589
GROα/CXCL1	74.44 ± 8.61	74.33 ± 6.52	67.52 ± 7.83	71.41 ± 8.41	0.161	0.691	0.597	0.445	0.172	0.681
IFN-β1	58.13 ± 3.34	74.72 ± 8.63	69.00 ± 5.29	79.09 ± 11.83	2.307	0.138	0.282	0.599	1.812	0.187
IFN-γ	69.13 ± 6.74	58.94 ± 5.65	77.94 ± 4.76	80.86 ± 8.25	0.284	0.598	**5.090**	**0.030**	0.924	0.343
IL-1β	7.12 ± 0.61	5.44 ± 0.29	6.11 ± 0.48	6.63 ± 0.36	1.718	0.199	0.040	0.841	**6.261**	**0.017**
IL-2	14.00 ± 2.23	13.67 ± 2.21	19.89 ± 3.89	15.91 ± 1.60	0.700	0.409	2.487	0.124	0.500	0.484
IL-4	8.625 ± 0.680	35.834 ± 28.154	8.889 ± 0.545	8.091 ± 0.343	3.200	0.083	0.473	0.497	0.021	0.887
IL-5	22.63 ± 6.54	11.22 ± 0.41	19.17 ± 4.46	21.86 ± 5.64	0.778	0.384	0.530	0.472	2.040	0.163
IL-6	11.062 ± 1.371	9.944 ± 1.254	14.221 ± 3.722	13.181 ± 1.571	0.236	0.630	2.072	0.159	0.001	0.986
IL-9	15.81 ± 0.83	16.33 ± 2.87	24.61 ± 9.29	23.55 ± 5.74	0.044	0.835	1.407	0.244	0.089	0.768
IL-10	10.50 ± 0.63	10.78 ± 1.26	10.28 ± 0.82	9.96 ± 0.39	0.001	0.978	0.410	0.526	0.136	0.715
IL-11	31.94 ± 3.08	32.67 ± 4.83	36.17 ± 2.46	38.82 ± 4.80	0.166	0.686	1.565	0.220	0.054	0.818
IL-12p70	27.75 ± 8.73	25.78 ± 3.98	58.83 ± 16.53	85.09 ± 43.65	0.189	0.666	2.623	0.115	0.256	0.616
IL-13	34.00 ± 2.58	35.28 ± 3.91	47.39 ± 9.38	36.80 ± 2.93	0.722	0.402	1.852	0.183	1.173	0.287
IL-16	7570 ± 594	8735 ± 814	9480 ± 1114	9220 ± 467	0.654	0.425	3.089	0.088	1.300	0.263
IL-17A/F	44.94 ± 1.78	49.02 ± 3.70	55.83 ± 2.99	53.32 ± 3.70	0.050	0.815	**5.382**	**0.026**	1.006	0.323
IL-18	45.94 ± 20.70	17.61 ± 4.53	23.50 ± 8.56	21.91 ± 5.08	0.076	0.785	0.413	0.525	1.818	0.187
IL-20	60.88 ± 7.81	31.06 ± 6.70	63.39 ± 13.87	39.64 ± 4.97	**9.301**	**0.004**	0.398	0.532	0.119	0.732
IL-22	13.13 ± 1.25	12.50 ± 0.82	17.56 ± 4.58	20.77 ± 5.19	0.110	0.742	2.639	0.114	0.241	0.626
IL-23	20.25 ± 5.01	10.94 ± 0.97	24.94 ± 6.24	35.73 ± 11.96	0.008	0.928	3.270	0.080	1.519	0.227
IL-27	32.69 ± 4.84	22.78 ± 2.84	33.83 ± 7.47	36.38 ± 8.14	1.193	0.283	0.621	0.437	0.352	0.557
IP-10/CXCL10	556.4 ± 96.9	1098.0 ± 147.2	626.3 ± 106.3	679.3 ± 81.5	**7.326**	**0.011**	2.519	0.122	**4.946**	**0.033**
MCP-1/CCL2	12.25 ± 1.31	11.17 ± 1.14	11.56 ± 1.40	11.09 ± 1.12	0.386	0.539	0.096	0.759	0.062	0.805
MCP-3/CCL7	1380 ± 121	1931 ± 187	1495 ± 385	1279 ± 124	0.540	0.468	1.382	0.248	2.824	0.102
MCP-5/CCL12	328.0 ± 31.29	293.4 ± 45.76	316.7 ± 30.64	263.0 ± 30.87	1.554	0.221	0.348	0.560	0.073	0.789
MDC/CCL22	2076 ± 211	2381 ± 325	3044 ± 468	2531 ± 231	0.102	0.751	2.938	0.096	1.575	0.218
MIP-1α/CCL3	43.94 ± 2.48	49.83 ± 7.07	47.94 ± 9.08	52.91 ± 6.10	0.266	0.609	0.051	0.823	0.154	0.698
MIP-1β/CCL4	79.88 ± 8.27	89.72 ± 7.20	97.67 ± 23.46	156.60 ± 53.78	0.975	0.331	1.478	0.233	0.497	0.486
MIP-2α/CXCL2	39.88 ± 0.92	36.11 ± 2.16	38.11 ± 1.53	39.77 ± 1.38	0.438	0.513	0.357	0.554	2.918	0.097
MIP-3α/CCL20	40.75 ± 5.17	31.44 ± 2.25	45.50 ± 5.92	36.18 ± 2.86	**4.931**	**0.033**	1.280	0.266	<0.001	0.999
MIP-3β/CCL19	31.50 ± 2.62	27.39 ± 2.89	29.56 ± 1.82	32.77 ± 2.65	0.030	0.863	0.446	0.509	2.025	0.164
RANTES/CCL5	172.3 ± 9.64	181.6 ± 27.22	168.7 ± 15.75	227.70 ± 48.45	0.350	0.558	0.011	0.915	0.008	0.931
TARC/CCL17	445.1 ± 27.3	403.6 ± 38.6	524.7 ± 74.6	508.4 ± 58.7	0.271	0.606	2.760	0.106	0.052	0.822
TNF-α	9.500 ± 1.086	10.671 ± 0.799	12.003 ± 1.333	9.545 ± 1.260	0.301	0.587	0.345	0.561	2.382	0.132

(*) Bold values indicate a significant main effect or interaction.

Table 2
Plasma levels of inflammatory mediators in CX_3CR1 KO mice by experimental group and two-way ANOVA results with stress and alcohol as factors.

Inflammatory Mediator (pg/mL)	GROUPS				STATISTICS *					
	CONTROL	STRESS	ALCOHOL	STRESS + ALCOHOL	Factor 1 (F1) Stress		Factor 2 (F2) Alcohol		F1 × F2 Interaction	
	non-stressed, saline-treated	**stressed, saline-treated**	**non-stressed, alcohol-treated**	**stressed, alcohol-treated**	**F statistic**	***p*-value**	**F statistic**	***p*-value**	**F statistic**	***p*-value**
	Mean ± SEM	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**						
CTLA-8/IL-17A	8.143 ± 0.404	8.550 ± 0.337	6.750 ± 0.491	9.000 ± 0.764	2.949	0.096	0.690	0.413	1.834	0.186
Eotaxin-1/CCL11	1835 ± 413	2870 ± 297	1838 ± 392	2159 ± 258	2.852	0.101	1.413	0.243	1.433	0.240
EPO	132.34 ± 25.18	91.80 ± 15.43	123.22 ± 20.81	87.56 ± 32.02	2.681	0.112	0.093	0.762	0.006	0.936
Exodus-2/CCL21	9716 ± 633	11062 ± 547	8863 ± 551	10675 ± 444	**5.634**	**0.024**	0.867	0.359	0.122	0.729
Fractalkine/CX3CL1	480.3 ± 37.3	527.9 ± 26.4	452.0 ± 24.8	537.1 ± 40.6	**5.172**	**0.029**	0.010	0.922	0.148	0.703
GM-CSF	14.75 ± 7.10	26.64 ± 9.77	12.57 ± 5.50	19.22 ± 7.17	1.439	0.240	1.89	0.667	0.033	0.857
GROα/CXCL1	67.14 ± 10.19	72.73 ± 8.16	57.63 ± 7.26	65.78 ± 5.61	0.398	0.533	1.395	0.247	0.002	0.969
IFN-β1	71.07 ± 5.73	76.86 ± 3.23	57.69 ± 3.59	94.50 ± 13.43	**11.28**	**0.002**	0.225	0.638	**4.845**	**0.035**
IFN-γ	71.79 ± 6.99	74.45 ± 2.04	61.31 ± 4.13	81.28 ± 10.76	2.862	0.101	0.074	0.787	1.672	0.206
IL-1β	5.444 ± 0.294	6.455 ± 0.390	7.000 ± 1.206	8.833 ± 2.536	1.428	0.241	2.285	0.141	1.203	0.281
IL-2	17.14 ± 3.25	20.27 ± 4.88	21.06 ± 5.44	11.61 ± 1.65	0.244	0.625	0.098	0.756	1.464	0.235
IL-4	7.571 ± 0.429	7.955 ± 0.418	7.750 ± 0.559	7.944 ± 0.317	0.973	0.331	1.283	0.266	0.394	0.535
IL-5	10.86 ± 0.80	14.05 ± 1.73	11.44 ± 1.32	12.06 ± 0.84	2.542	0.121	0.004	0.953	0.183	0.672
IL-6	12.86 ± 3.53	20.59 ± 9.81	10.00 ± 0.85	9.89 ± 0.75	0.004	0.953	0.249	0.622	0.957	0.336
IL-9	17.43 ± 3.43	14.86 ± 1.20	10.81 ± 0.96	16.17 ± 3.44	0.911	0.347	0.517	0.478	4.057	0.053
IL-10	11.79 ± 1.53	14.59 ± 4.81	10.50 ± 0.91	9.67 ± 0.44	0.104	0.749	0.049	0.826	1.835	0.185
IL-11	30.93 ± 4.59	37.95 ± 3.05	26.31 ± 1.67	50.72 ± 5.04	**16.72**	**<0.001**	1.125	0.297	**5.114**	**0.030**
IL-12p70	63.71 ± 35.06	52.73 ± 21.77	31.88 ± 9.44	79.33 ± 31.54	0.531	0.472	0.005	0.942	1.324	0.259
IL-13	35.71 ± 4.87	32.09 ± 2.20	23.63 ± 1.38	33.39 ± 4.05	0.778	0.384	2.856	0.101	3.933	0.056
IL-16	9526 ± 706	10872 ± 513	7516 ± 782	11099 ± 508	**15.60**	**<0.001**	2.045	0.163	3.213	0.083
IL-17A/F	45.93 ± 3.31	54.18 ± 2.20	42.94 ± 0.61	71.39 ± 7.79	**16.50**	**<0.001**	2.476	0.126	**4.997**	**0.032**
IL-18	15.67 ± 4.59	16.59 ± 2.87	11.63 ± 1.71	17.67 ± 5.61	2.082	0.159	0.031	0.861	0.405	0.529
IL-20	54.93 ± 13.81	50.77 ± 5.73	44.50 ± 5.21	45.61 ± 3.90	0.036	0.850	1.094	0.304	0.114	0.738
IL-22	10.57 ± 0.69	16.85 ± 2.28	13.63 ± 1.58	14.22 ± 1.70	**4.823**	**0.035**	0.266	0.610	1.155	0.291
IL-23	10.50 ± 0.97	13.32 ± 1.47	9.81 ± 1.48	21.28 ± 7.04	2.016	0.165	2.418	0.130	0.566	0.458
IL-27	25.29 ± 4.08	24.00 ± 2.26	24.31 ± 4.36	24.67 ± 5.21	<0.001	0.993	0.008	0.931	0.110	0.743
IP-10/CXCL10	847.0 ± 266.8	810.6 ± 126.0	618.1 ± 82.80	796.9 ± 121.7	1.471	0.235	0.044	0.834	0.037	0.848
MCP-1/CCL2	10.07 ± 1.30	10.36 ± 0.83	14.50 ± 1.30	13.89 ± 2.57	0.226	0.637	**8.397**	**0.006**	0.092	0.763
MCP-3/CCL7	1465 ± 457	1552 ± 151	1164 ± 182	1707 ± 453	0.737	0.397	0.093	0.762	0.357	0.554
MCP-5/CCL12	305.3 ± 23.0	304.7 ± 23.0	277.8 ± 60.3	322.9 ± 34.7	0.351	0.558	0.015	0.903	0.370	0.547
MDC/CCL22	2855 ± 165	3166 ± 223	2278 ± 263	3333 ± 170	**9.783**	**0.003**	0.892	0.352	2.918	0.098
MIP-1α/CCL3	10.07 ± 1.30	10.36 ± 0.83	14.50 ± 1.31	13.89 ± 2.57	**4.575**	**0.040**	0.609	0.441	**4.270**	**0.047**
MIP-1β/CCL4	122.8 ± 49.6	121.5 ± 29.6	100.2 ± 27.4	109.3 ± 26.6	0.002	0.961	0.345	0.562	0.007	0.932
MIP-2α/CXCL2	34.79 ± 2.83	37.00 ± 2.88	37.44 ± 1.59	40.72 ± 1.53	0.559	0.460	0.839	0.367	0.011	0.918
MIP-3α/CCL20	33.71 ± 6.04	38.59 ± 3.34	40.81 ± 8.42	41.22 ± 4.03	0.658	0.423	0.567	0.457	0.042	0.839
MIP-3β/CCL19	22.79 ± 3.19	30.27 ± 2.63	23.75 ± 1.38	42.00 ± 5.66	**14.96**	**<0.001**	3.450	0.073	2.551	0.121
RANTES/CCL5	191.61 ± 34.75	171.10 ± 9.37	144.40 ± 9.39	148.23 ± 9.13	0.587	0.449	**5.384**	**0.026**	0.168	0.684
TARC/CCL17	387.7 ± 49.6	568.8 ± 28.9	397.4 ± 45.8	620.7 ± 68.4	**17.04**	**<0.001**	0.247	0.622	0.093	0.762
TNF-α	16.36 ± 5.97	10.73 ± 0.94	9.19 ± 0.68	9.50 ± 0.87	0.593	0.447	0.077	0.783	0.531	0.472

(*) Bold values indicate a significant main effect or interaction.

comparisons indicated that stressed, saline-treated mice exhibited significantly lower IL-1β levels compared with non-stressed, saline-treated mice ($p < 0.05$). Additionally, a significant interaction between stress and alcohol was also observed for IP10/CXCL10 levels ($F_{1,33} = 4.946$; $p = 0.033$). In this case, stressed, saline-treated mice exhibited significantly higher IP10/CXCL10 levels than non-stressed, saline-treated mice ($p < 0.01$), while stressed, alcohol-treated mice showed significantly lower IP10/CXCL10 levels compared with stressed, saline-treated mice ($p < 0.01$).

3.6.2. *Inflammatory mediators in CX_3CR1 KO mice*

3.6.2.1. Stress effect. A significant main effect of stress was found for IL-16 ($F_{1,31} = 15.60$; $p < 0.001$), IL-22 ($F_{1,30} = 4.823$; $p = 0.035$), TARC/CCL17 ($F_{1,30} = 17.04$; $p < 0.001$), MIP-3β/CCL19 ($F_{1,29} = 14.96$; $p < 0.001$), Exodus-2/CCL21 ($F_{1,31} = 5.634$; $p = 0.024$), and MDC/CCL22 ($F_{1,31} = 9.783$; $p = 0.003$), with stressed KO mice displaying higher levels than non-stressed KO mice.

3.6.2.2. Alcohol effect. Analysis revealed a main effect of alcohol on MCP-1/CCL2 ($F_{1,31} = 8.397$; $p = 0.006$) and RANTES/CCL5 levels ($F_{1,32} = 5.381$; $p = 0.026$). Specifically, alcohol-treated mice exhibited lower levels of RANTES/CCL5 and higher levels of MCP-1/CCL2 compared with saline-treated KO mice.

3.6.2.3. Stress and alcohol interaction. IL-11 levels showed a significant main effect of stress and a significant stress × alcohol interaction ($F_{1,31} = 5.114$; $p = 0.030$). *Post hoc* comparisons indicated that stressed, alcohol-treated mice exhibited significantly higher IL-11 levels compared with non-stressed, saline-treated mice ($p < 0.01$), non-stressed, alcohol-treated mice ($p < 0.001$), and stressed, saline-treated mice ($p < 0.05$). Similarly, IL-17A/F levels showed both a significant main effect of stress and a stress × alcohol interaction ($F_{1,31} = 4.997$; $p = 0.032$). Stressed, alcohol-treated mice exhibited significantly higher IL-17A/F levels compared with non-stressed, saline-treated mice ($p < 0.001$), non-stressed, alcohol-treated mice ($p < 0.001$), and stressed, saline-treated mice ($p < 0.01$). Analysis of IFN-β1 levels also revealed a significant main effect of stress and a significant interaction between the two factors ($F_{1,31} = 4.845$; $p = 0.035$). *Post hoc* testing indicated that stressed, alcohol-treated mice had significantly higher IFN-β1 levels than non-stressed, alcohol-treated mice ($p < 0.001$).

4. Discussion

This study reveals a critical role for the CX_3CL1/CX_3CR1 chemokine system in modulating the behavioral, endocrine, neuropeptidergic, and inflammatory responses to acute stress and alcohol exposure during adolescence. Our results demonstrate that the absence or pharmacological inhibition of CX_3CR1 profoundly alters stress-coping behavior, particularly under conditions of combined stress and alcohol, suggesting a maladaptive phenotype characterized by atypical behavioral, hormonal, and immune responses.

We first confirmed that neither genotype nor experimental condition affected body weight gain, spontaneous locomotor activity, nor anxiety-like behavior, indicating that observed differences in immobility behavior were unlikely to be confounded by general physiological or motor impairments.

Stress-coping behavior, assessed using the tail suspension test, revealed a significant increase in immobility time in control KO mice compared with WT controls, consistent with a passive coping phenotype in the absence of external challenges. In contrast, KO mice exposed to stress and/or alcohol exhibited significantly reduced immobility, suggesting that CX_3CR1 deficiency leads to a paradoxical behavioral shift under adverse conditions. This behavioral profile was mirrored in WT mice treated with AZD8797, a selective CX_3CR1 antagonist, supporting the functional role of the receptor in modulating coping strategies. Notably, in both KO and AZD8797-treated mice, stress and alcohol each significantly reduced immobility, with no sex-related differences observed. These findings imply that CX_3CR1 signaling may normally limit behavioral overactivation under duress, and that its absence unleashes an atypical, potentially maladaptive form of active coping (Hellwig et al., 2016; Liu et al., 2020; Winkler et al., 2017).

At the endocrine level, KO mice exhibited a blunted corticosterone response to acute stress, despite displaying elevated *Crh* expression, particularly under stress conditions. In contrast, WT mice showed increased corticosterone in response to alcohol, and *Crh* upregulation was restricted to the stress + alcohol condition, highlighting genotype-dependent HPA axis dynamics. These findings are consistent with earlier studies indicating genotype-related alterations in corticosterone regulation (Winkler et al., 2017). Importantly, ACTH levels remained unaffected by experimental manipulations, and a positive correlation between ACTH and corticosterone levels was only found in WT mice, indicating that the absence of CX_3CR1 disrupts coordinated HPA axis feedback. These hormonal findings reinforce previous evidence that KO mice may present altered adrenal sensitivity and disrupted regulatory feedback within the HPA axis (Gjerstad et al., 2018; Winkler et al., 2017).

The interplay between CRH and NPY systems is fundamental for regulating the stress response (Thorsell, 2010), and the observed alterations in their expression further support a dysregulated neuropeptidergic profile in KO mice. The CRH system, a central regulator of HPA axis activity (Sukhareva, 2021), was differentially modulated by stress and alcohol across genotypes. KO mice exhibited higher baseline *Crhr1* levels and heightened *Crhr1* and *Crhr2* upregulation in response to stress, which was attenuated by alcohol. In WT mice, stress increased both *Crhr1* and *Crhr2* expression, and this was again blunted by alcohol, suggesting that alcohol interferes with receptor adaptation to stress in both genotypes, although via distinct mechanisms. These findings align with prior work indicating that CX_3CL1 signaling may suppress HPA axis hyperactivity (Winkler et al., 2017). When examining the NPY system, no baseline genotype differences were found in *Npy*, *Npy1r*, or *Npy2r* expression. However, stress induced *Npy1r* upregulation in WT mice, which was prevented by alcohol, indicating a compensatory NPY-mediated buffering response that is disrupted under alcohol exposure. In contrast, KO mice showed no modulation of *Npy1r* by either stress or alcohol, suggesting that the absence of CX_3CR1 impairs the dynamic responsiveness of the NPY system to stressors. The lack of adaptive NPY signaling in KO mice may further contribute to maladaptive coping responses (Ciccocioppo et al., 2009).

In the hypothalamus, *Cx3cl1* expression increased in WT mice only under combined stress and alcohol exposure, and *Cx3cr1* was upregulated following stress alone. These findings are consistent with prior studies linking stress and alcohol to increased chemokine signaling and microglial activation (Pascual et al., 2015; Walter et al., 2017). In KO mice, *Cx3cl1* expression remained unchanged across conditions, reinforcing the notion that CX_3CR1 is required for the dynamic regulation of its ligand in response to stress and alcohol (Rivas-Fuentes et al., 2021).

In the periphery, KO mice exhibited consistently higher plasma CX_3CL1 levels across all conditions, with stress further increasing CX_3CL1 in both genotypes, although only WT mice showed a negative correlation between plasma CX_3CL1 levels and immobility time. These data suggest that CX_3CL1 may contribute to active coping in WT mice, but this association is lost in KO animals, possibly due to impaired receptor-mediated signaling (Inoue, 2022; Rodriguez et al., 2024).

In addition to CX_3CL1, we observed broad alterations in peripheral inflammatory mediators, particularly in KO mice. At baseline, KO mice displayed elevated IL-16 and IL-17A/F levels, consistent with previous evidence of heightened inflammatory tone in CX_3CR1-deficient animals (Mattison et al., 2013). Stress and/or alcohol exposure led to selective increases in IL-17A/F, IL-11, IFN-β1, MDC/CCL22, MIP-3β/CCL19, and TARC/CCL17 in KO mice, highlighting a heightened neuroimmune sensitivity and exaggerated inflammatory profile under adversity. In WT mice, responses were more restricted, with stress and alcohol increasing IL-17A/F, IFN-γ, EPO, and IP10/CXCL10, and reducing IL-1β and IL-20 levels. These differential patterns point to a regulatory role of CX_3CR1 in fine-tuning immune responses to environmental insults. Although sex differences were not analyzed in molecular measures, it is important to consider that neuropeptide expression and stress responses may exhibit sex dimorphisms (Dong et al., 2020), which could be obscured by the combined analysis of males and females.

Taken together, these results indicate that CX_3CR1 plays a multifaceted role in orchestrating the stress response by regulating behavioral coping, HPA axis feedback, neuropeptidergic signaling, and immune activation. Its absence results in behavioral disinhibition, impaired endocrine coordination, dysregulated neuropeptide responses, and exaggerated peripheral inflammation, especially when stress and alcohol are combined. These findings are consistent with previous reports linking CX_3CL1/CX_3CR1 signaling to microglial activation, synaptic pruning, and stress susceptibility (Paolicelli et al., 2011; Zhan et al., 2014), and may have relevance to neurodevelopmental and mood disorders characterized by stress dysregulation and heightened inflammation, such as atypical depression or comorbid substance use disorders (Juruena et al., 2018).

In conclusion, this study highlights the critical role of the CX_3CL1/CX_3CR1 axis in modulating stress-coping behaviors, neuroimmune responses, and HPA axis activity under adolescent stress and alcohol exposure. The absence of CX_3CR1 led to maladaptive behavioral and neuroimmune changes, including disrupted CRH and NPY signaling and heightened inflammatory responses, emphasizing its protective role in maintaining physiological and immune balance. These findings provide insights into the mechanisms linking CX_3CR1 signaling to stress resilience and vulnerability, with relevance to stress-related and neuroinflammatory disorders.

CRediT authorship contribution statement

Dina Medina-Vera: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Laura Martín-Chaves:** Methodology, Investigation. **Laura Sánchez-Marín:**

Methodology, Investigation. **María Díaz-Ottaviano:** Methodology. **Ana L. Gavito:** Methodology. **Olga Popova:** Methodology. **María José Sánchez-Quintero:** Investigation. **Jorge Rodríguez-Capitán:** Supervision, Formal analysis, Data curation. **Fernando Rodríguez de Fonseca:** Writing – original draft, Supervision. **Manuel F. Jiménez-Navarro:** Resources, Project administration, Funding acquisition, Conceptualization. **Antonia Serrano:** Writing – original draft, Resources, Funding acquisition. **Francisco Javier Pavón-Morón:** Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2025.110503.

Data availability

Data will be made available on request.

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