Neuropharmacology 292 (2026) 110924

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

Aggression after intermittent ethanol intoxication in mice: Sex differences and modulation via medial amygdala relaxin-3/RXFP3 signaling

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ARTICLE INFO

Keywords:
Aggression
Dominance
Nucleus incertus
Relaxin-3
Medial amygdala
Alcohol intoxication

ABSTRACT

Alcohol consumption is strongly associated with aggression and violence in humans, yet the underlying neurobiological mechanisms within key aggression circuits remain poorly understood. In this preclinical study, we examined the effects of intermittent alcohol intoxication on dominance and aggressive behaviors in mice, focusing on sex differences and the potential involvement of the nucleus incertus relaxin-3/relaxin-family peptide receptor 3 (RXFP3) signaling pathway. Using an intermittent ethanol-intoxication protocol, we observed that male mice displayed a transient increase in dominance and aggressive behaviors during acute abstinence, as measured by the tube-dominance and resident-intruder tests, whereas female mice displayed heightened defensive responses. Distinct patterns of neural activation across brain regions, reflected by c-Fos protein expression, were associated with aggression in males, including decreased expression in the medial amygdala (MeA) and increased expression in the ventromedial hypothalamus (VMH), consistent with an established MeA-VMH based aggression circuit. Additionally, the levels of relaxin-3 immunoreactivity in MeA nerve fibers increased in parallel with behavioral recovery, suggesting a modulatory role of relaxin-3/RXFP3 signaling. To test this hypothesis, we bilaterally injected an adeno-associated viral (AAV) vector expressing the selective RXFP3 agonist, R3/I5, into the MeA of male mice. Notably, this chronic localized R3/I5 treatment significantly reduced dominance and aggressive behaviors both before and after alcohol intoxication. Together, these data demonstrate that relaxin-3/RXFP3 signaling in the MeA counteracts alcohol-related aggression in male mice, pointing to this pathway as a potential target for treating impulsive violence associated with alcohol intoxication in humans.

1. Introduction

Alcohol abuse has long been linked to violence and aggressive behavior in humans. About 50% of all violent crimes and sexual assaults worldwide are committed under the influence of alcohol (Foran and O'Leary, 2008; Heinz et al., 2011; Pernanen, 1976; Rossow and Bye, 2013). While both acute and chronic alcohol exposure are associated with heightened aggression (Arseneault et al., 2000; Heinz et al., 2011; Jaffe et al., 1988; Schuckit and Russell, 1984), episodic heavy drinking, characterized by high blood alcohol concentrations followed by periods of abstinence, is a recognized risk factor for alcohol-related aggression (Chermack and Blow, 2002; Hwa et al., 2011, 2015; Lipsey et al., 1997). Notably, gender differences are reported in alcohol use; women are less likely to engage in such episodic heavy drinking than men (Bobrova

Abbreviations: AAV, adeno-associated virus; CRF, corticotrophin-releasing factor; eGFP, enhanced green fluorescent protein; MeA, medial amygdala; NI, nucleus incertus; ACC, anterior cingulate cortex; LS, lateral septum; MS, medial septum; CeA, central amygdala; BNST, bed nucleus of the stria terminalis; VMH, ventromedial hypothalamic nucleus; dPAG, dorsal periaqueductal grey; RI, resident intruder; RLN3, relaxin-3; RXFP3, relaxin-family peptide receptor 3; TDT, tube dominance test; PFA, paraformaldehyde; PBS, phosphate buffer saline; FSTTC, forward spike time tiling coefficients.

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https://doi.org/10.1016/j.neuropharm.2026.110924
Received 10 October 2025; Received in revised form 9 March 2026; Accepted 9 March 2026
Available online 17 March 2026

et al., 2010), while men are more involved in alcohol-related aggression than women (Bushman, 2002; Ito et al., 1997).

Preclinical studies in rodents have demonstrated that alcohol intoxication is linked to impaired sociability and increased anxiety-like behavior (Kampov-Polevoy et al., 2000; Zahran et al., 2024), and acute alcohol exposure, even at low doses, increases aggressive behaviors such as territorial attacks and dominance displays (Miczek et al., 2015; Takahashi et al., 2010; Weerts et al., 1993), mirroring behavioral patterns observed in humans. Despite these findings, the precise neural mechanisms underlying alcohol-related aggression remain poorly understood, particularly in the context of sex-specific responses in which males and females exhibit divergent neuroadaptations to alcohol (Flores-Bonilla and Richardson, 2020; Logrip et al., 2018; Seo et al., 2010; Sharrett-Field et al., 2013), although recent evidence suggests that hormonal and neurochemical factors may modulate this effect (Magee et al., 2024; Peltier et al., 2019, 2021; Towner et al., 2024). Several neuropeptide systems including vasopressin, neuropeptide Y, and corticotrophin-releasing factor (CRF) have been implicated in alcohol-related aggression (Miczek et al., 2015). However, less is known about the role of many other neuromodulatory systems including the relaxin-3 (RLN3)/RXFP3 system (Ma et al., 2017).

The nucleus incertus (NI), a pontine tegmental region enriched in GABAergic and RLN3-expressing neurons in rats (Ma et al., 2007), mice (Smith et al., 2010) and humans (De Ávila et al., 2024) has emerged as a potentially key locus in alcohol-related behaviors (Ryan et al., 2013; Ryan et al., 2013a,b; Shirahase et al., 2016; Walker et al., 2015). In rodents, these NI neurons project strongly to centers that control motivation and emotions, including the amygdala and septum (Olucha-Bordonau et al., 2003, 2012; Smith et al., 2010), where RLN3 signals via the $G_{i/o}$-protein-coupled receptor, relaxin-family peptide receptor 3 (RXFP3), to modulate neural circuits involved in stress, reward, and emotional regulation (Ganella et al., 2013a,b; Gil-Miravet et al., 2021; Ma et al., 2017; Navarro-Sánchez et al., 2024; Smith et al., 2014; Watanabe et al., 2011). Recent evidence suggests that RLN3/RXFP3 signaling influences alcohol intake and relapse (Ryan et al., 2013; Ryan et al., 2013a,b; Shirahase et al., 2016; Walker et al., 2015), but its role in alcohol-related aggression remains unexplored. Furthermore, sex differences in RLN3/RXFP3 system function during alcohol consumption have not been systematically investigated, despite evidence that males and females exhibit divergent responses to modulation of RLN3/RXFP3 signaling (Calvez et al., 2015, 2017; De Ávila et al., 2018, 2021; Lenglos et al., 2015). Accordingly, the present study used an intermittent, ethanol-intoxication protocol—intraperitoneal injections every 48 h that produce cycles of intoxication and acute abstinence—to examine aggression and dominance behaviors in male and female mice. We combined deep-learning-based analysis of resident intruder and tube dominance tests, mapped c-Fos expression as a marker of neuronal activation in aggression-related regions innervated by RLN3 fibers (Lischinsky and Lin, 2020), and tested the causal contribution of MeA RLN3/RXFP3 signaling via bilateral AAV-mediated delivery of the selective chimeric RXFP3 agonist, R3/I5 (Ganella et al., 2013a,b; Rytova et al., 2019).

2. Experimental procedures

2.1. Mice

Male and female C57BL/6 mice aged 3-4 months and weighting 28-34 g were used in all studies. Mice were maintained under standard conditions (12:12 h light/dark cycle, 21 ± 2 °C, and food and water *ad libitum*). All mice were group-housed according to sex after weaning (2-3 mice per cage suitable for the RI test (50 cm × 38 cm × 18 cm)) and then were single-housed one week prior to baseline behavioral testing. All procedures with laboratory mice (*Mus musculus*) were approved by the Committee of Ethics and Animal Welfare of the Universitat Jaume I and the Conselleria de Agricultura de la Generalitat Valenciana (Spain) (procedure 2023-VSC-PEA-0227) and were consistent with directive 2010/63/EU of the European Community.

2.2. Experimental design

For Experiment 1, mice were randomly distributed into four groups, control saline-treated males (CTR-M), ethanol-treated males (ETH-M), control saline-treated females (CTR-F), and ethanol-treated females (ETH-F). Mice received intraperitoneal injections of 3 g/kg ethanol (20% v/v in saline) or an equivalent volume of saline (0.9%) every other day for 3 weeks (Fig. 1A). The dose was adapted from the binge drinking model described by Monleón et al. (2019) to simulate episodic heavy drinking, a risk factor strongly associated with aggression in humans (Beck and Heinz, 2013) while the intermittent administration schedule, every other day for 21 days, was selected to achieve optimal behavioral sensitization (Didone et al., 2019; Reed and Phillips, 2024). All injections were administered during the light phase between 10:00-11:30 h. For female mice, stages of the estrus cycle were determined using vaginal cytology (McLean et al., 2012) and mice were distributed to an experimental group to balance different stages of estrus across the saline and ethanol groups (see Supplementary materials and methods).

For Experiment 2, male mice were injected bilaterally in the MeA with either an AAV vector, expressing the chimeric RXFP3 agonist, R3/I5 and eGFP (AAV1/2-sCAG:R3/I5-IRES-eGFP) or a control AAV expressing eGFP (AAV1/2-sCAG-IRES-eGFP particles) (Fig. 6A–B) (Ganella et al., 2013a,b; Rytova et al., 2019). Details of surgery procedures and AAV titers are provided in the Supplementary materials and methods. After two weeks of recovery and viral expression, mice were subjected to the ethanol intoxication protocol (Fig. 6A).

2.3. Behavioral assays

Behavioral tests were performed at three time points: (i) baseline, before any treatment; (ii) 2 days after the last injection to assess acute abstinence; and (iii) 7 days after the last injection to assess protracted abstinence. Body weights of the mice were recorded at the three time points. Mice were sacrificed one day after the last behavioral test to avoid test effects on the levels of neural activation observed using c-Fos protein staining (Aparicio et al., 2022; Lin et al., 2018). For detailed procedures, see Supplementary materials and methods.

2.3.1. Tube dominance test (TDT)

Social dominance was assessed by performing the TDT. Two mice were introduced into opposite ends of a tube and were left to interact inside the tube (each ethanol-treated mouse was paired against three different opponent mice from the saline-treated group across three trials). A dominance score and dominance-related behaviors were determined (see Supplementary materials and methods).

2.3.2. Resident intruder test (RI)

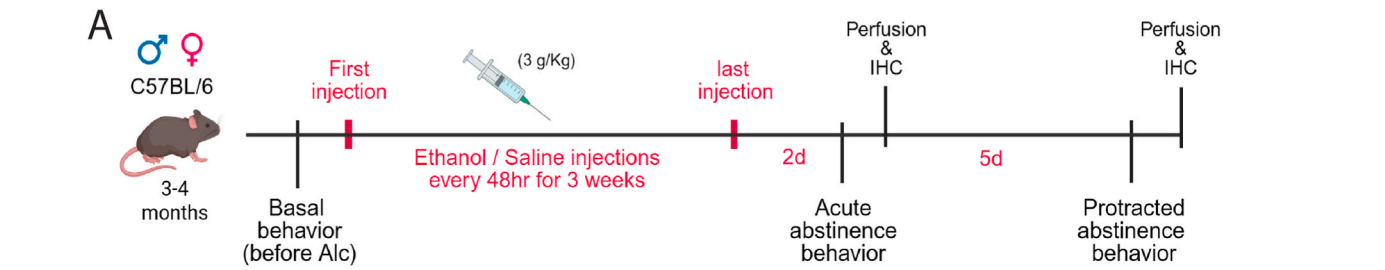
Both male and female mice were tested for aggression for 15 min with an unfamiliar same-sex conspecific intruder in the resident's home cage (see Supplementary materials and methods).

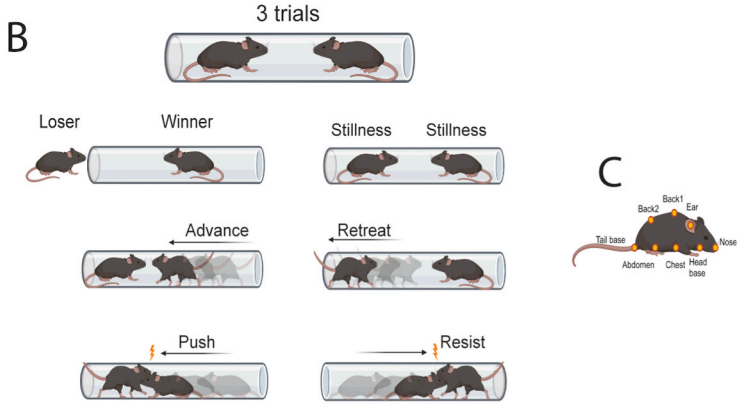
2.4. Digital workflow

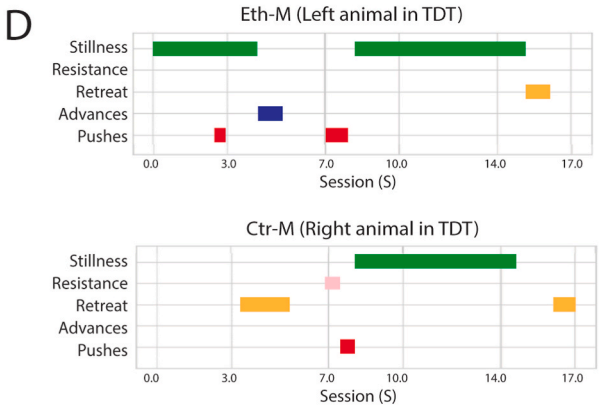
We developed a step-by-step workflow to reproduce the automated analysis of new data (see Supplementary videos). Detailed procedures are provided in the Supplementary materials and methods.

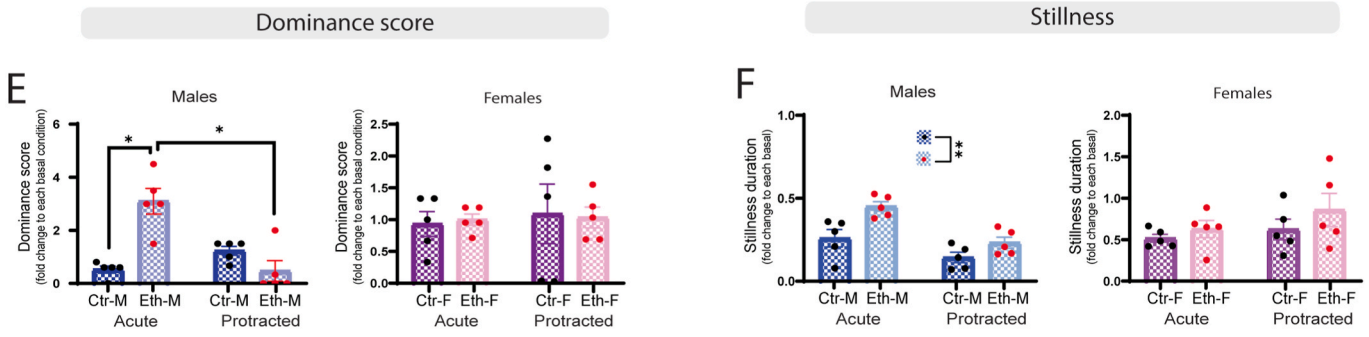
2.5. Histological and microscopic analysis

Mice were euthanized and perfused with saline followed by fixative (PFA 4% in 0.1M PBS). Thereafter brains were removed, cryoprotected, frozen and sectioned at 40 μm with a freezing-sliding microtome. Sections were prepared for chromogenic immunohistochemistry (c-Fos









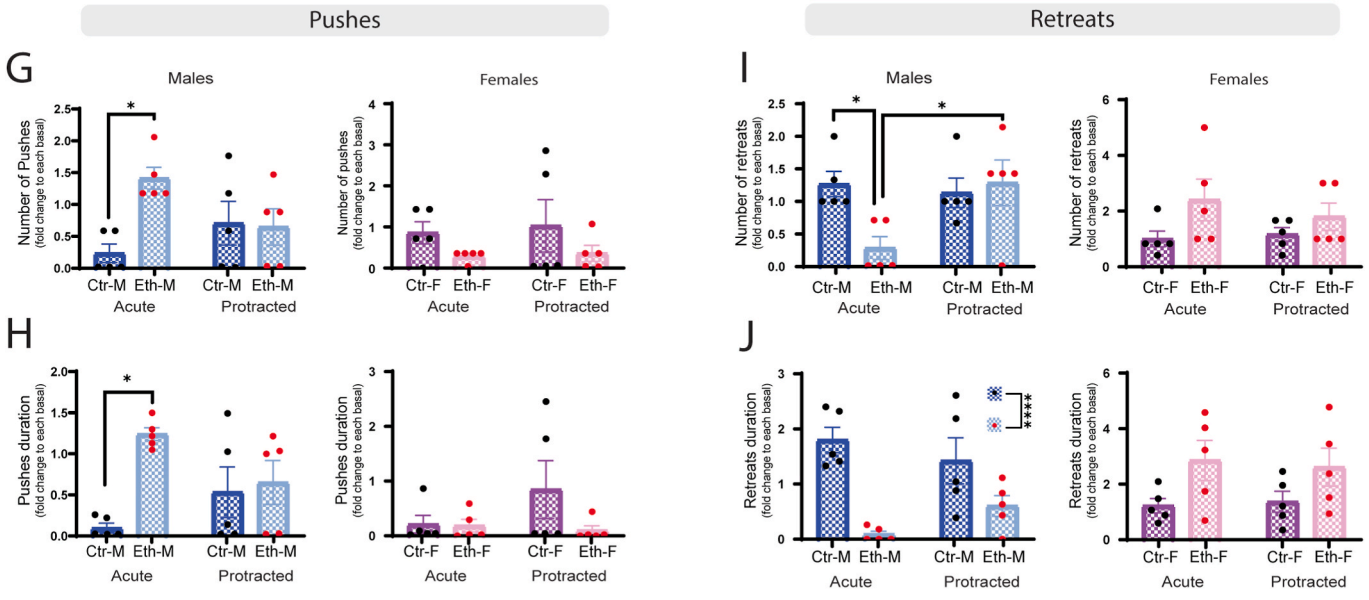


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Fig. 1. Intermittent ethanol intoxication led to increased dominance in male mice. (A). Experimental timeline: Male and female mice were tested for dominance and aggressive behaviors. Thereafter, the mice were injected intraperitoneally with a 20% ethanol solution (3 g/kg) or the same volume of normal 0.9% saline every other day for 21 days. Dominance and aggressive behaviors were then re-tested after 2 days (acute) and 7 days (protracted) of abstinence. Brain tissues were collected at these times and processed for immunohistochemistry. **(B)**. Illustration of the TDT behaviors. Each ethanol-treated mouse was paired against three control saline mice across three trials; and average dominance scores were recorded. Dominance related behaviors were also monitored including stillness. **(C)**. Mouse body-part annotations used in TDT DLC model. **(D)**. Gantt chart displaying quantified behaviors of left/right mice during the TDT in acute abstinence. **(E)**. Ethanol-treated male mice displayed a transient increase in dominance score while ethanol-treated females did not display any difference. **(F)**. Ethanol-treated male mice displayed a transient longer stillness time compared to the control saline-treated group, while ethanol-treated females did not display any difference. **(G–H)**. Ethanol-treated male mice displayed a transient increase in the number (G) and duration (H) of pushes compared to the control group during acute abstinence. Ethanol-treated female mice displayed no changes at either timepoint. **(I–J)**. Ethanol-treated male mice displayed a significant decrease in the number (I) and duration (J) of retreats compared to the control group after acute abstinence. In all graphs, each dot represents an individual mouse, and data are expressed as fold-change of basal behavior ± SEM (n = 5/group). Two-factor ART ANOVA followed by Multiple Mann Whitney test adjusted by Bonferroni-Dunn correction, statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

staining) or immunofluorescence (RLN3 staining). Labeled sections were imaged with an Olympus BX61 microscope. Full details of tissue preparation, histological and image analysis are provided in the Supplementary material and methods.

2.6. Statistics

Statistical analyses were performed using GraphPad Prism version 10.4** (GraphPad Software Inc., La Jolla, CA, USA) and the Real Statistics Resource Pack software (Release 7.6). Prior to analysis, outliers were identified and removed using the Robust Regression and Outlier Removal (ROUT) method with Q = 1%. Shapiro-Wilk tests were performed to test normality, and Levene's tests were conducted to check for homogeneity of variances. For multi-factorial analysis, if the data satisfied both the normality assumption and homogeneity of variances, a two-way ANOVA was conducted, followed by a Bonferroni's multiple comparisons test. Conversely, if the data did not satisfy the normality assumption or if homogeneity of variances was violated, a two-factor ART ANOVA was employed, followed by a Multiple Mann Whitney test adjusted by Bonferroni-Dunn correction. For comparisons between two independent groups, a Welch's *t*-test was performed for normally distributed data (to account for potential unequal variances), and a Mann Whitney test for non-normally distributed data. For FSTTC descriptive analysis, the reported statistical comparisons were driven by pre-defined, *a priori* hypotheses focusing on specific, biologically critical pairwise comparisons, testing the direct effect of ethanol (Ethanol vs. Control) at each distinct abstinence phase (Acute or Protracted). In Experiment 1, each data set was normalized to its control group for microscopic analysis, and to basal conditions for the behavioral tests, with results expressed as fold-change. In Experiment 2, results were expressed as raw numbers because there was no basal behavior. The Spearman correlation coefficient and simple linear regression were used to indicate statistical correlations between mouse behaviors, and c-Fos and relaxin-3 immunoreactivity (RLN3-ir) in different brain regions, and to compare the results of manual scoring and digital workflow scoring. Benjamini, Krieger, and Yekutieli correction was employed to control the False Discovery Rate (FDR) in correlations p values. The statistical level of significance was set at * p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. For detailed statistics see Tables S3–4.

3. Results

3.1. Intermittent ethanol intoxication produced increased dominance in male mice

Dominance related behaviors were assessed using the TDT (Fig. 1B). Ethanol-treated males displayed enhanced social dominance during acute abstinence, as revealed by the significant treatment × period effect (2 factor ART ANOVA, F (1, 16) = 20.95, P = 0.0003). Using multiple comparisons analysis, ethanol-treated males displayed a significant increase in dominance score (P = 0.0158) vs the control saline group during the acute abstinence (Fig. 1E). After protracted abstinence, enhanced social dominance was restored to basal levels (P = 0.031; Fig. 1E). Interestingly, no such alteration in social dominance was observed in female mice after the different abstinence periods, and this sex specific effect was confirmed by a sex × treatment effect (3way ANOVA, F (1, 32) = 4.470, P = 0.0424) (Fig. 1E). Dominance related behaviors were scored by quantifying voluntary behaviors (stillness, pushes and advances) and coping responses (retreats and resistance) (Fig. 1B) (Zhou et al., 2018). Significant period and treatment effects on male mice stillness duration were observed (2 factor ART ANOVA, F (1, 16) = 22.38, P = 0.0002; F (1, 16) = 13.87, P = 0.001, respectively). Significant treatment × period effects were observed for the number and duration of pushes in male mice (2 factor ART ANOVA, F (1, 16) = 9.6, P = 0.006; F (1, 16) = 6.50, P = 0.02, respectively). Ethanol-treated males displayed a significant increase in the number and duration of pushes (P = 0.0158, P = 0.0158, respectively) after acute abstinence (Fig. 1G–H). There was a significant period × treatment effect on the number of retreats in male mice (2 factor ART ANOVA, F (1, 16) = 4.61, P = 0.047) and treatment effects on retreat duration in male mice (F (1, 16) = 36.79, P < 0.0001). Ethanol-treated males displayed a significant decrease in the number of retreats (P = 0.016) compared to control after acute abstinence (Fig. 1I–J). These results suggest that social dominance is enhanced in male mice during acute abstinence after ethanol intoxication. In contrast, female mice did not display such dominant behavior.

3.2. Intermittent ethanol intoxication produced increased aggressiveness in male but not female mice

To determine whether intermittent ethanol intoxication followed by abstinence induced aggressive behavior, male and female mice were subjected to the RI test. An unfamiliar conspecific intruder was introduced into the home cage of the resident test mouse. Seventeen different behaviors were quantified and categorized into five main categories, offensive behavior (attack, chasing, boxing and lateral threat), defensive behavior (defence, escape and freezing), social behavior (allogrooming, approach, anogenital sniffing, and nosing), non-social behavior (immobility, digging, rearing and self-grooming) and miscellaneous behaviors (tail rattle, jump) (Fig. 2A). In male mice, there were significant treatment × period effects on offensive behavior bouts (2-way ANOVA, F (1, 16) = 13.28, P = 0.002) and durations (F (1, 16) = 13.46, P = 0.0021) and treatment effect on latency to first attack (2 factor ART ANOVA, F (1, 16) = 4.72, P = 0.044). Multiple comparisons analysis revealed that ethanol-treated male mice displayed significant increases in offensive behavior bouts (P = 0.0006) and duration (P = 0.0019) after acute abstinence, compared to levels observed in control saline-treated mice (Fig. 2C–D). These increases in offensive behavior bouts and their duration were no longer observed after protracted abstinence (P = 0.004, P = 0.0024, respectively). In contrast, no such increase in offensive behaviors was observed in female mice after acute or protracted abstinence confirmed by sex × treatment effect on duration (3 way ANOVA, F (1, 32) = 9.750, P = 0.004) (Fig. 2D).

Ethanol-treated males displayed fewer defensive behaviors as revealed by the significant treatment effect on bouts (2-way ANOVA, F

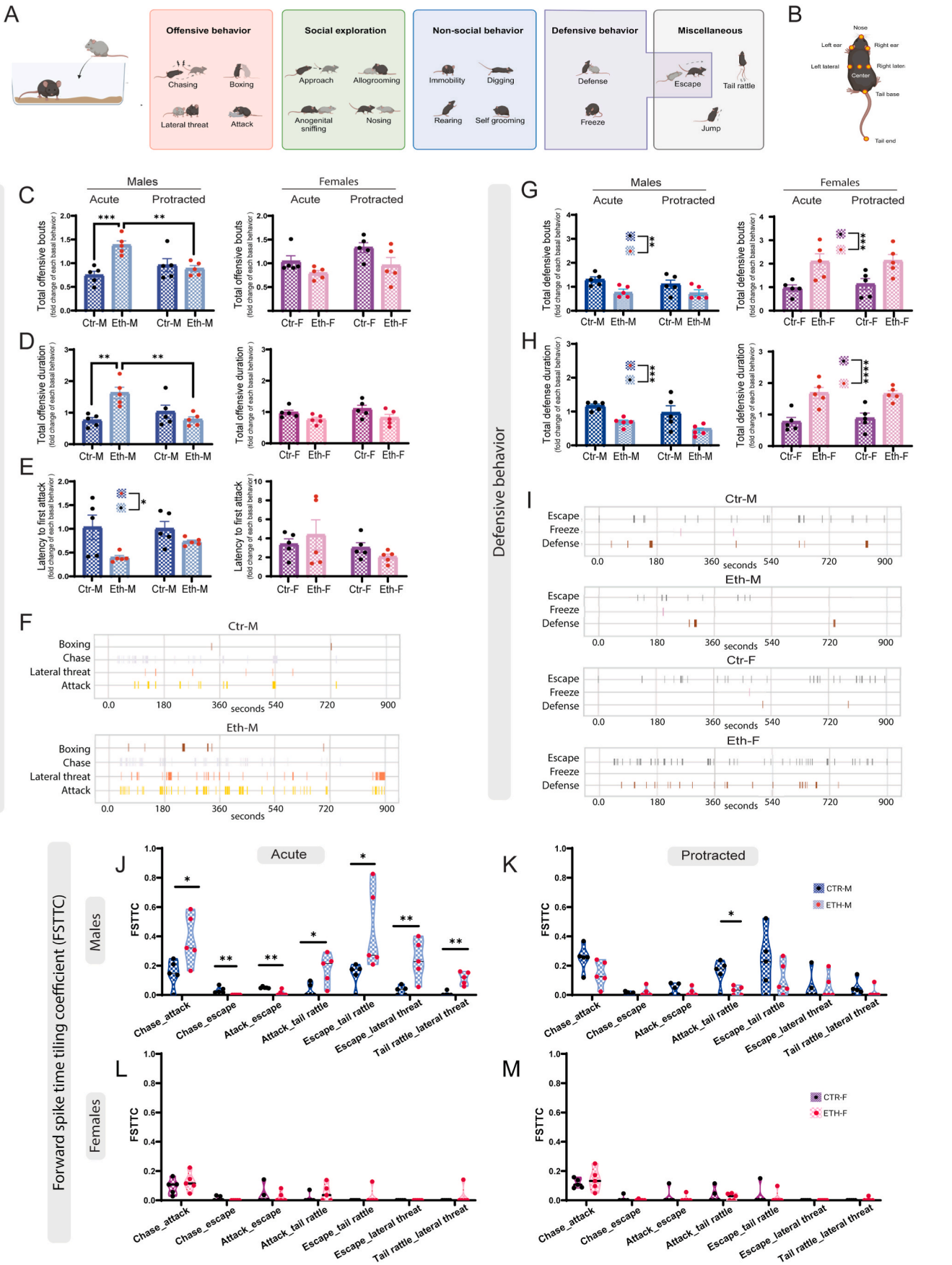

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Fig. 2. Intermittent ethanol intoxication led to increased aggressiveness in male mice. (A). Illustration of the RI test procedures in which 17 behaviours were scored and grouped into five major groups comprising offensive, defensive, social, non-social and miscellaneous behaviors. **(B)**. Mouse body-part annotations used in for the RI DLC model. **(C–D)**. Ethanol-treated male mice displayed a significant increase in the number (C) and duration (D) of offensive behaviors compared to the control group after acute abstinence. Ethanol-treated female displayed no such effects. **(E)**. Ethanol-treated male mice displayed a significant decrease in latency to first attack compared to the control group. Ethanol-treated female displayed no such effects. **(F)**. Gantt chart of offensive behaviors (boxing, chasing, lateral threat, attack) in individual resident male mice after acute abstinence. **(G–H)**. Ethanol-treated male mice displayed a significant decrease in the number (G) and duration (H) of defensive behaviors, while ethanol-treated female mice displayed a significant increase in defensive behaviour. **(I)**. Gantt chart of defensive behaviors (escape, freeze, defense) in individual resident male and female mice after acute abstinence. **(J–M)**. Violin plot of forward-spike time tiling coefficients (FSTTC; $\Delta t = 2000$ ms) for RI behavior sequences. On the X axis, selected sets of sequenced pairwise behaviors in which the first behavior is followed by the second behavior within the time delta. **(J)**. Ethanol-treated male mice after acute abstinence displayed a significantly higher FSTTC coefficient of attacks following chasing, a lower FSTTC coefficient of escaping following chasing and attack, a higher FSTTC coefficient of tail rattle following attack and escape, and a higher FSTTC coefficient of lateral threat following escape and tail rattle. **(K)**. After protracted abstinence ethanol-treated male mice displayed a lower FSTTC coefficient of tail rattle following attack. **(L–M)**. Ethanol-treated female mice displayed no FSTTC differences at either timepoint. In all graphs, each dot represents an individual mouse. In graphs C-H, data are expressed as fold-change of basal behavior ± SEM (n = 5/group) Two-way ANOVA followed by a Bonferroni's multiple comparisons test. In graphs J-M, data are expressed as FSTTC ± SEM (n = 5/group) Welch's *t*-test/Mann Whitney test. In graph E, different scales are used for better visualization. Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

(1, 16) = 10.25, P = 0.0056) and durations (2-way ANOVA, F (1, 16) = 17.90, P = 0.0006). Conversely, ethanol-treated females displayed higher levels of defensive behaviors, reflected by the significant treatment effect on bouts (2-way ANOVA, F (1, 16) = 17.45, P = 0.0007) and durations (2-way ANOVA, F (1, 16) = 32.46, P < 0.0001) (Fig. 2G–H). This sex-specific, opposing defensive behavior was confirmed by the significant sex effect (3-way ANOVA, F (1, 32) = 21.10, P < 0.0001) (Fig. 2H).

In order to detect how behaviors initiated by the resident mouse can trigger another behavior, forward-spike time tiling coefficients (FSTTCs) were measured using a time window (time delta) of 2000 ms (Lee et al., 2019). During the acute abstinence period, ethanol-treated male mice displayed a significantly higher FSTTC coefficient of attacks following chasing (Welch's *t*-test, t = 2.642, df = 8, P = 0.0296), lower FSTTC coefficients of escaping following chasing and attack (Mann Whitney test, U = 0, P = 0.0079; Mann Whitney test, U = 0, P = 0.0079, respectively) which indicated that ethanol-treated males usually attack after chasing and are unlikely to escape after chasing or attacking compared to control, saline-treated mice. There were higher FSTTC coefficients of tail rattle following attack and escape (Mann Whitney test, U = 2, P = 0.0317; Welch's *t*-test, t = 2.337, df = 8, P = 0.0476, respectively), and higher FSTTC coefficients of lateral threat following escape and tail rattle (Welch's *t*-test, t = 3.358, df = 8, P = 0.01; Welch's *t*-test, t = 5.075, df = 8, P = 0.001, respectively) (Fig. 2J), which indicated that ethanol-treated males displayed more tail rattling after attacking and escaping behaviors, and lateral threats were more usually followed by tail rattling and escaping than in control mice. After protracted abstinence, ethanol-treated male mice displayed a lower FSTTC coefficient of tail rattle following attack (Welch's *t*-test, t = 2.829, df = 8, P = 0.02) (Fig. 2K). In contrast, ethanol-treated female mice did not display any differences in FSTTC coefficients at either timepoint (Fig. 2L–M). These results identify a transient increase in aggressiveness in male mice after ethanol intoxication and abstinence and an opposite effect on defensive behaviors in male and female mice.

3.3. Altered neuronal activation in brain regions related to aggressive behaviors after intermittent ethanol intoxication

Neuronal levels of the immediate early gene protein, c-Fos, were assessed in brains collected after acute and protracted abstinence from ethanol treatment (at 2 and 7 days) as a marker of the neuronal activation in different brain regions that contribute to aggressive behaviors, including the ACC, LS, MS, CeA, MeA, BNST, VMH, and dPAG (Lischinsky and Lin, 2020). Notably, all of these areas are known to receive projections from the NI in rodents (Goto et al., 2001; Nasirova et al., 2020; Olucha-Bordonau et al., 2003; Smith et al., 2010).

There were significant treatment × period effects on the density of c-Fos positive neurons in male mice in ACC, LS, CeA, MeA and VMH (F (2-factor ART ANOVA, F (1, 16) = 8.64, P = 0.01; F (1, 16) = 5.634, P = 0.03; F (1, 16) = 5.53, P = 0.03; F (1, 16) = 3.121, P = 0.0096; F (1, 16) = 8.358, P = 0.01; respectively) (Fig. 3A and 3C-D, 3F-G), significant treatment effects on the density of c-Fos positive neurons in male mice in MS (2-factor ART ANOVA, F (1, 16) = 36.1, P = 0.001, Fig. 3B), and dPAG (2-way ANOVA, F (1, 16) = 10.6, P = 0.005, Fig. 3H). Significant treatment effects were also observed in female mice in CeA (2-way ANOVA, F (1, 16) = 5.47, P = 0.033, Fig. 3D) and dPAG (2-way ANOVA, F (1, 16) = 8.002, P = 0.012, Fig. 3H) (Table S3). Multiple comparisons analysis revealed that ethanol-treated males displayed significantly lower levels of c-Fos during acute abstinence in ACC (P = 0.008, Fig. 3A), LS (P = 0.031, Fig. 3C) and MeA (P < 0.001, Fig. 3F), while the VMH displayed a higher density of c-Fos positive neurons (P = 0.0096) (Fig. 3G). Following protracted abstinence, the density of c-Fos positive neurons was comparable to those in control mice receiving saline, suggesting restoration of basal levels of neuronal activity in these brain areas.

3.4. Relaxin-3 immunoreactivity in brain regions related to aggressive behaviors after intermittent ethanol intoxication

The integrated density of relaxin-3 immunoreactivity (RLN3-ir) in nerve fibers was measured after acute and protracted abstinence in different brain regions that contribute to aggressive behaviors (Tanaka et al., 2005; Olucha-Bordonau et al., 2012; Lischinsky and Lin, 2020) There were significant treatment × period effects on the density of relaxin-3 positive fibers in male mice in LS (2-way ANOVA, F (1, 16) = 20.57, P = 0.0003, Fig. 4A), MS (2-factor ART ANOVA, F (1, 16) = 26.36, P = 0.0001, Fig. 4B), BNST (2-factor ART ANOVA, F (1, 16) = 12.80, P = 0.0025, Fig. 4C), MeA (2-way ANOVA, F (1, 16) = 49.59, P < 0.0001, Fig. 4D), ACC (2-factor ART ANOVA, F (1, 16) = 31.9, P = 0.0001, Fig. 4E), and dPAG (2-way ANOVA, F (1, 16) = 7.6, P = 0.014, Fig. 4F). Similarly, significant treatment × period effects were observed in female mice in dPAG (2-way ANOVA, F (1, 16) = 14.83, P = 0.001), and significant treatment effects were observed in female mice in LS (2-factor ART ANOVA, F (1, 16) = 10.3, P = 0.005, Fig. 4A), MS (2-factor ART ANOVA, F (1, 16) = 7.92, P = 0.012, Fig. 4B), BNST (2-way ANOVA, F (1, 16) = 7.1, P = 0.017, Fig. 4C), MeA (2-way ANOVA, F (1, 16) = 12.5, P = 0.002, Fig. 4D), and ACC (2-factor ART ANOVA, F (1, 16) = 4.36, P = 0.05, Fig. 4E). Multiple comparisons analysis revealed that ethanol-treated male mice displayed an increased density of RLN3-ir after protracted abstinence in LS (P < 0.0001, Fig. 4A), MS (P = 0.016, Fig. 4B), BNST (P = 0.05, Fig. 4C), and MeA (P < 0.0001, Fig. 4D) relative to levels in time-matched, saline-treated mice, while in BNST and ACC the density of RLN3-ir was decreased after acute abstinence, relative to control levels (P = 0.016, P = 0.016, respectively) (Fig. 4C–E). Ethanol-treated female mice displayed an increase in the density of RLN3-ir after acute abstinence in the dPAG (P = 0.001) (Fig. 4F).

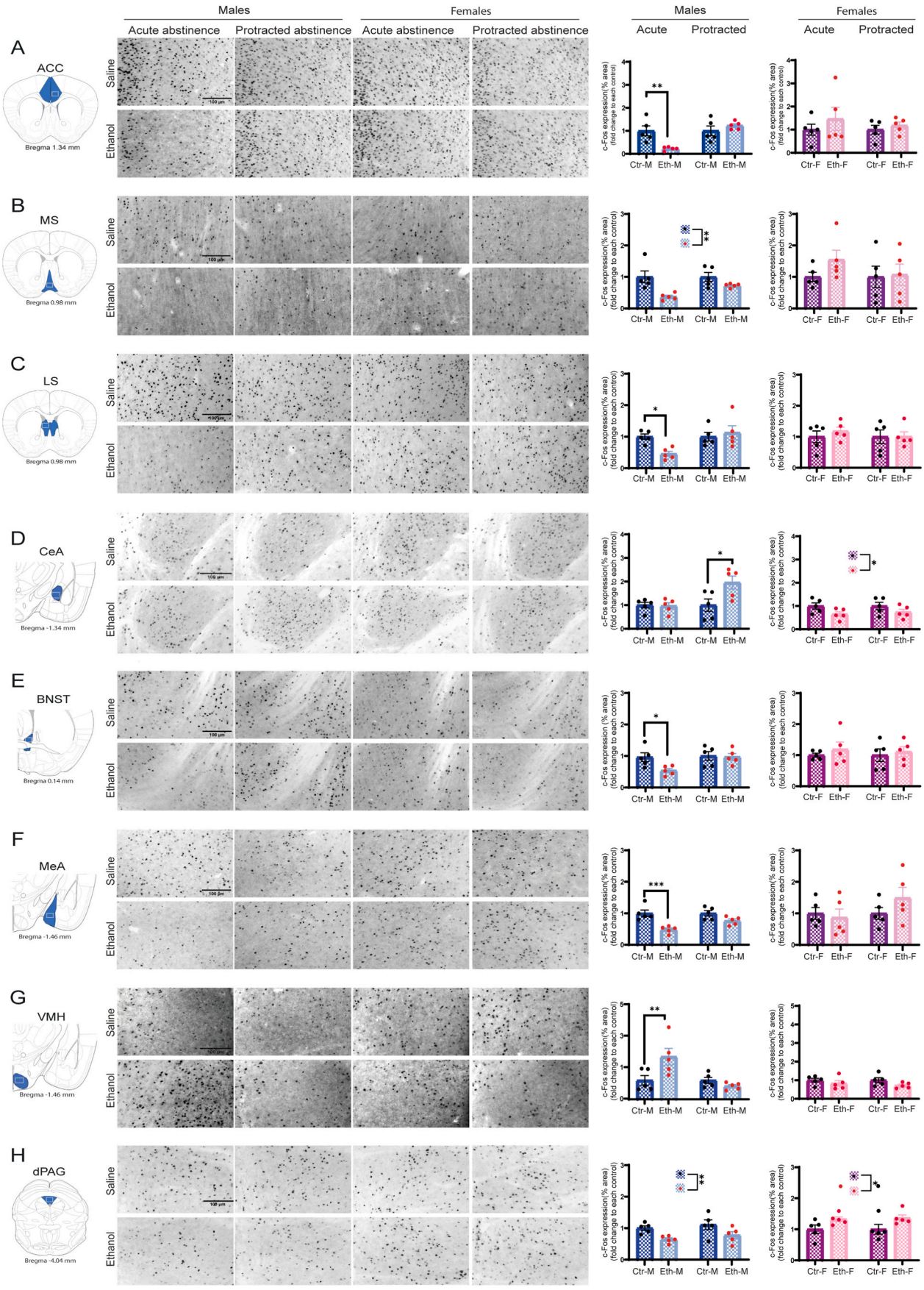

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Fig. 3. Intermittent ethanol intoxication altered neuronal activation, as reflected by c-Fos protein, in aggression-related brain regions. **(A-C)**. Ethanol-treated male mice displayed a significantly lower density of c-Fos protein in anterior cingulate cortex (A), medial septum (B), and lateral septum (C) compared to control, saline-treated male mice after acute abstinence. c-Fos protein levels were similar to control levels after protracted abstinence. Ethanol-treated female mice displayed no such c-Fos changes. **(D)**. Ethanol-treated male mice displayed a significantly higher density of c-Fos protein in central amygdala compared to control after protracted abstinence, while ethanol-treated female mice displayed no differences. **(E–F)**. Ethanol-treated male mice displayed a significantly lower density of c-Fos protein in the bed nucleus of stria terminalis and medial amygdala compared to control after acute abstinence. Ethanol-treated female mice displayed no changes. **(G)**. Ethanol-treated male mice displayed significantly higher c-Fos density in ventromedial hypothalamus compared to control after acute abstinence, and these levels were similar to after protracted abstinence. Ethanol-treated female displayed no differences. **(H)**. No c-Fos densities changes were observed in dorsal peri-aqueductal grey in either sex at either timepoint. Schematics on the left indicate the anatomical location of each targeted nucleus (Bregma coordinates provided); blue shaded areas represent the target region of interest (ROI) used for analysis, and white inset boxes delineate the specific fields of view in the corresponding high-magnification images. All images presented were generated by digital magnification and cropping of the raw images to better visualize the specific structures quantified. Scale bars, 100 μm. In all graphs, each dot represents an individual mouse, and data are expressed as fold-change of each control ± SEM (n = 5/group). Two-way ANOVA followed by a Bonferroni's multiple comparisons test, statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.5. Correlations between TDT/RI behaviors and c-Fos/relaxin-3 immunoreactivity

In an effort to identify neurobiological correlates of behavior, we explored the statistical correlation between specific behavioral outcomes and the regional expression of c-Fos protein and relaxin-3 fiber density across different brain areas. This analysis included 40 male and female mice that were sacrificed at acute and protracted abstinence time points. Spearman correlation revealed that offensive behavior had a direct correlation with c-Fos protein levels in VMH ($r = 0.559$ $q = 0.0004$, Fig. 5B) and had an inverse correlation with c-Fos protein levels in the MeA ($r = -0.684$, $q < 0.0001$, Fig. 5C). Offensive behavior also positively correlated with relaxin-3 fiber density in MeA ($r = -0.708$, $q < 0.0001$, Fig. 5D). Defensive behavior displayed a direct correlation with c-Fos protein levels in the dPAG ($r = 0.533$ $q = 0.0005$, Fig. 5F) and an inverse correlation with c-Fos protein levels in CeA ($r = -0.596$, $q = 0.0001$, Fig. 5G). The dominance score displayed inverse correlations with c-Fos and relaxin-3 immunoreactivity in the MeA ($r = -0.532$ $q = 0.0006$, $r = -0.466$, $q = 0.002$, respectively; Fig. 5H–I). Relaxin-3 fiber density in the MeA displayed an inverse correlation with c-Fos protein levels in the VMH ($r = -0.521$, $q = 0.0007$, Fig. 5E; see Table S4).

3.6. Effect of chronic RXFP3 activation in medial amygdala on dominance and aggressive behavior in male mice

In a study to investigate whether relaxin-3/RXFP3 signaling could influence dominance and offensive behaviors via the MeA, bilateral injections of an AAV vector expressing the chimeric RXFP3 agonist, R3/I5 or a control AAV vector were made into the MeA of male mice and after 2 weeks, the TDT and RI behavioral tests were performed prior to and after ethanol intoxication (Fig. 6). In the TDT performed prior to ethanol intoxication, R3/I5-treated mice displayed a significant decrease in dominance score (Mann Whitney test, $U = 7$, $P = 0.025$; Fig. 6G), and in the number (Mann Whitney test, $U = 6$, $P = 0.015$; Fig. 6J) and duration (Welch's *t*-test, $t = 2.234$, $df = 11.34$, $P = 0.046$, Fig. 6K) of pushes. These effects persisted after ethanol intoxication, as revealed by significant treatment effect on dominance score (2-way ANOVA, $F(1, 22) = 18.4$, $P = 0.0003$) (Fig. 6R) and treatment × period effects on the number and duration of pushes (2-factor ART ANOVA, $F(1, 20) = 5.063$, $P = 0.035$; $F(1, 20) = 6.14$, $P = 0.022$, respectively). Multiple comparisons revealed significant decreases in pushes number ($P = 0.01$) and duration ($P = 0.001$) of R3/I5-treated mice during acute abstinence (Fig. 6T–U).

In the RI test prior to ethanol intoxication, R3/I5-treated mice displayed a longer latency to first attack (Welch's *t*-test, $t = 2.826$, $df = 8.749$, $P = 0.02$; Fig. 6N) and a marked decrease in the number (Welch's *t*-test, $t = 2.262$, $df = 9.122$, $P = 0.049$; Fig. 6L) and duration (Welch's *t*-test, $t = 2.974$, $df = 7.688$, $P = 0.018$; Fig. 6M) of offensive behaviors. Additionally, R3/I5-treated mice displayed a higher number (Mann Whitney test, $U = 6$, $P = 0.017$) and duration (Welch's *t*-test, $t = 2.383$, $df = 9.001$, $P = 0.04$) of defensive behaviors (Fig. 6P–Q). After ethanol intoxication, R3/I5-treated mice continued to display decreased offensive behaviors as reflected by a significant treatment effect on offensive behaviors number (2-way ANOVA, $F(1, 22) = 42$, $P < 0.0001$) and treatment × period effect on duration (2-way ANOVA, $F(1, 22) = 6.177$, $P = 0.02$). Subsequent multiple comparisons indicated reductions in offensive behavior duration after acute and protracted abstinence ($P < 0.0001$, $P = 0.025$, respectively; Fig. 6V–W). A significant treatment effect was observed on defensive behavior duration ($F(1, 22) = 5.018$, $P = 0.035$) (Fig. 6X–Y).

In order to link pharmacological intervention (R3/I5 treatment) with the underlying neural circuit activity, we explored c-Fos protein levels in the MeA and VMH of R3/I5- and sham-treated mice. R3/I5 treatment resulted in a significant increase in c-Fos positive cells in the MeA compared to sham-treated controls (Welch's *t*-test, $t = 3.527$, $df = 11.00$, $P = 0.0047$, Fig. 6E). Conversely, c-Fos protein levels were significantly decreased in the VMH of R3/I5-treated mice (Welch's *t*-test, $t = 5.412$, $df = 8.175$, $P = 0.0006$, Fig. 6F).

4. Discussion

This study addressed a major gap in our understanding of the neural circuits and neuropeptide systems that link alcohol use/abuse to impulsive aggression, an important topic for the field of neuropsychopharmacology. We focused on sex-specific mechanisms and relaxin-3/RXFP3 signaling within the NI-MeA pathway. Our findings demonstrated that: (*i*) after intermittent ethanol intoxication for three weeks, male mice displayed a transient increase in dominance and aggressive behaviors relative to control (saline-treated) mice after acute abstinence; (*ii*) this behavioral phenotype in male mice was associated with distinct neural activation patterns in brain areas that contribute to aggression, as reflected by decreased c-Fos protein expression in the MeA and increased c-Fos expression in the VMH; and (*iii*) relaxin-3/RXFP3 signaling within the MeA modulated alcohol-related dominance and aggressive behaviors, with evidence suggesting increased RXFP3 activation can mitigate these behaviors through a mechanism involving regulation of the MeA-VMH aggression circuit.

4.1. Behavioral dynamics after intermittent ethanol intoxication

Our behavioral data demonstrated that acute abstinence from intermittent ethanol intoxication increased both dominance and aggressive behaviors in male mice. In the TDT, ethanol-treated males displayed increased dominance scores, prolonged stillness, increased pushing, and reduced retreats, which collectively indicated heightened competitive drive. These changes normalized after protracted abstinence, suggesting a transient neuroadaptation rather than a permanent shift in the social hierarchy (Fig. 1E–H). In the RI test, ethanol-treated males displayed increased offensive behavior bouts and durations (Fig. 2C), confirming elevated aggression, and aligning with previous studies (Cardoso et al., 2006; Hwa et al., 2015). Interestingly, behavioral sequence analysis using FSTTC revealed that attacks were more likely

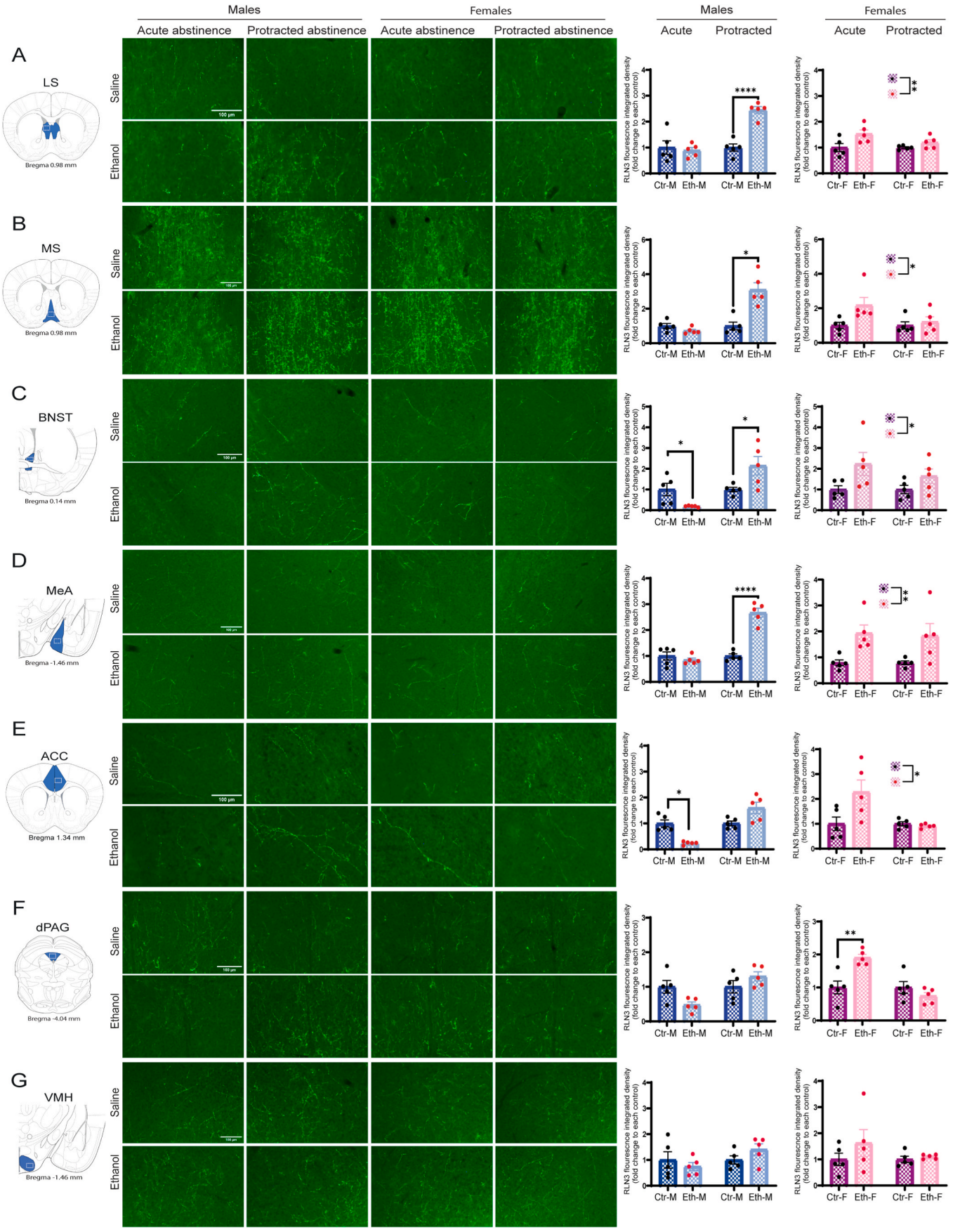

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Fig. 4. Intermittent ethanol intoxication modulated relaxin-3 immunoreactivity (RLN3-ir) levels in aggression-related brain regions. (A–C). Ethanol-treated male mice displayed a significantly higher level of RLN3-ir in lateral septum, medial septum, and bed nucleus of stria terminalis after protracted abstinence, while ethanol-treated female mice displayed a significantly higher level in these same regions after acute abstinence. **(D).** Ethanol-treated male mice displayed a significantly higher level of RLN3-ir in medial amygdala after protracted abstinence, while ethanol-treated female mice displayed a higher level of RLN3-ir after acute and protracted abstinence. **(E).** Ethanol-treated male mice displayed a lower level of RLN3-ir in anterior cingulate cortex after acute abstinence and higher levels after protracted abstinence. Ethanol-treated female mice displayed higher RLN3-ir after acute abstinence. **(F).** Ethanol-treated male mice displayed a non-significant increase in RLN3-ir in dorsal periaqueductal grey after protracted abstinence, while ethanol-treated female mice displayed significantly higher RLN3-ir after acute abstinence **(G).** RLN3-ir in VMH remained unchanged in both sexes at both timepoints. Schematics on the left indicate the anatomical location of each targeted nucleus (Bregma coordinates provided); blue shaded areas represent the target region of interest (ROI) used for analysis, and white inset boxes delineate the specific fields of view in the corresponding high-magnification images. All images presented were generated by digital magnification and cropping of the raw images to better visualize the specific structures quantified. Scale bars, 100 μm. In all graphs, each dot represents an individual mouse, and data are expressed as fold-change of each control ± SEM (n = 5/group). Two-way ANOVA followed by a Bonferroni's multiple comparisons test, statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

preceded by chasing and followed by tail rattling, while escape behaviors were less likely after aggression (Fig. 2G). This pattern suggests proactive rather than reactive aggression, consistent with clinical observations of alcohol-related impulsive violence (Bushman, 2002; Heinz et al., 2011). In contrast, female mice displayed no such increase in offensive behaviors. Instead, they displayed elevated defensive and non-social behaviors (Fig. 2D). This behavioral sex difference aligns with previous studies indicating that males are more susceptible to alcohol-related aggression and that females exhibit different neurobehavioral responses to alcohol (Bobrova et al., 2010; Bushman, 2002; Ito et al., 1997; Quadir et al., 2023). They are also consistent with our previous findings of impaired sociability in female mice during alcohol abstinence (Zahran et al., 2024), underscoring the importance of examining sex as a biological variable when studying alcohol-related aggression.

These behavioral phenotypes mirror key clinical features of alcohol-related impulsive aggression, supporting the face validity of our model for neuropsychopharmacological studies. The transient nature of the male aggression further suggests that pharmacological interventions targeting early abstinence could be strategically timed to prevent escalation of violent behaviors in humans.

4.2. Neuronal activation reflected by c-Fos protein levels

Previous studies have demonstrated that c-Fos expression after ethanol intoxication is very dynamic. During ethanol consumption, c-Fos is generally reduced in numerous brain regions, but by the beginning of the abstinence period at 24-26 h, it has increased in most brain regions; and these increases are followed by widespread reductions at 74 h, with levels returning to baseline by 7 days (de Guglielmo et al., 2023; Roland et al., 2023; Smith et al., 2020). Additionally, sex differences are also reported in the c-Fos expression pattern in different brain regions after ethanol intoxication (Magee et al., 2024; Towner et al., 2024). In our c-Fos analysis, we observed distinct neural activation patterns in aggression-related brain regions; and during acute abstinence, males displayed increased neuronal activation in the VMH, a well-established hub for offensive aggression (Lin et al., 2011; Lischinsky and Lin, 2020) (Fig. 3G), alongside decreased activation in regions such as the LS and MeA that are part of aggression circuitry (Lin et al., 2011; Hong et al., 2014; Wong et al., 2016; Lischinsky and Lin, 2020) (Fig. 3C–F). These patterns displayed significant correlations with offensive behaviors (Fig. 5B–C), which support a neural imbalance facilitating aggression, consistent with findings in aggression circuitry studies (Lin et al., 2011; Lischinsky and Lin, 2020; Mañas-Ojeda et al., 2024; McDonald et al., 2011; Menon et al., 2022; Tonnaer et al., 2023; Wong et al., 2016; Zhang, 2021). In contrast, females displayed distinct c-Fos expression patterns, particularly in the CeA and dPAG that displayed significant correlations with defensive behaviors (Fig. 5F–G), consistent with data on defensive circuitry (Fadok et al., 2018; Moscarello and Penzo, 2022; Tovote et al., 2018), and suggesting activation of a threat-avoidance rather than an aggression-promoting circuit. These differences may reflect sex-specific modulation of aggression and defensive behaviors after intermittent ethanol intoxication (Bobrova et al., 2010; Bushman, 2002; Magee et al., 2024; Towner et al., 2024).

4.3. Relaxin-3/RXFP3 signaling and aggression regulation

Previous studies have demonstrated a role for relaxin-3/RXFP3 signaling in the regulation of stress and behaviors associated with alcohol consumption, as reflected by its influence on alcohol intake (Shirahase et al., 2016) and its anxiolytic properties (Nakazawa et al., 2013; Ryan et al., 2013a,b; Smith et al., 2014; Zhang et al., 2015).

In this study, we identified the potential role of relaxin-3/RXFP3 signaling as a modulator of aggression after intermittent ethanol intoxication. The increased relaxin-3 immunoreactivity observed in males during protracted abstinence suggests its involvement in mitigating alcohol-related aggression and restoring behavioral homeostasis. This increase is further supported by our finding of a higher percentage of c-Fos-positive, relaxin-3-expressing neurons within the nucleus incertus (NI) of ethanol-treated males during protracted abstinence (Welch's *t*-test, $t = 2.8$, $df = 4.45$, $P = 0.04$; Supplementary Fig. 10). This increased activation of the primary source of relaxin-3 provides a clear functional correlate for the heightened fiber immunoreactivity observed in distal projection sites. The observed correlations between relaxin-3, c-Fos, and offensive behaviors (Fig. 5D–E) imply that these effects may be mediated by interactions within the MeA, which is characterized by a high density of RXFP3 (Ma et al., 2007) and is known to play a role in the modulation of aggressive behavior mainly through the alteration of GABAergic transmission (Hong et al., 2014; Mañas-Ojeda et al., 2024; Tonnaer et al., 2023; Zhang, 2021). It is important to acknowledge that our quantification of relaxin-3 immunoreactivity (RLN3-ir) using integrated density (ID) represents a semi-quantitative measure of peptide fiber labelling which should be interpreted as a relative index of signal abundance rather than an absolute linear measure of peptide concentration (Waters, 2009). Critically, to ensure our findings were not dependent on the choice of metric, we re-analyzed two representative regions (ACC and MeA) using area fraction (the percentage of ROI occupied by fibers). This structural analysis yielded results highly comparable to our original ID measurements, maintaining all significant group differences. Specifically, in the ACC, the area fraction analysis confirmed significant differences between groups ($t = 4.66$, $df = 6.04$, $P = 0.003$), mirroring the results obtained with ID ($t = 2.53$, $df = 5.26$, $P = 0.049$). Similarly, in the MeA, the area fraction analysis remained highly significant ($t = 4.53$, $df = 5.22$, $P = 0.005$), consistent with the ID findings for that region ($t = 8.68$, $df = 5.62$, $P = 0.0002$) (Supplementary Fig. 11A–D). These analyses demonstrated a strong positive correlation between the two metrics ($r = 0.95$, $p < 0.0001$) (Supplementary Fig. 11E), suggesting that in this specific biological context, ID serves as a robust proxy for overall innervation density, reflecting both structural occupancy and peptide signal.

To directly test the potential effect of RXFP3 activation in MeA on aggression, we conducted an experiment in which male mice received bilateral injections of a previously characterized AAV expressing the chimeric RXFP3 agonist, R3/I5 (Ganella et al., 2013a,b; Rytova et al.,

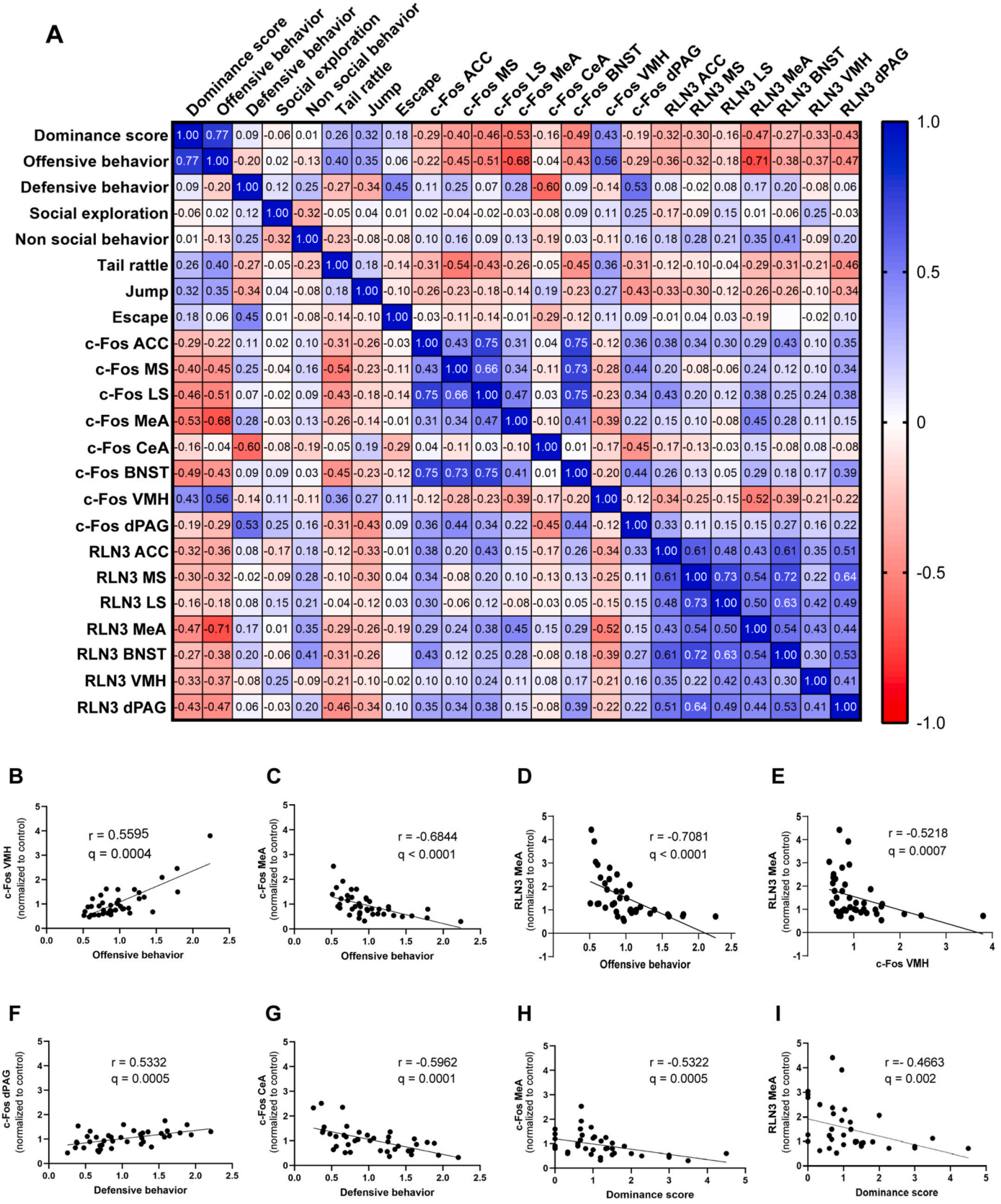

Fig. 5. Behavioral correlations with c-Fos protein and relaxin-3 immunoreactivity in key brain areas. (A). Correlation matrix plot of dominance and RI behaviors, c-Fos and relaxin-3 immunoreactivity in brain regions that contribute to aggression. Spearman correlation coefficients are reported in the color-coded table cells (from −1 "red" to 1 "blue"). **(B–J).** Selected significant correlations between dominance, offensive and defensive behaviors and c-Fos and relaxin-3 levels. The correlation coefficients and q-values are provided on the graphs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

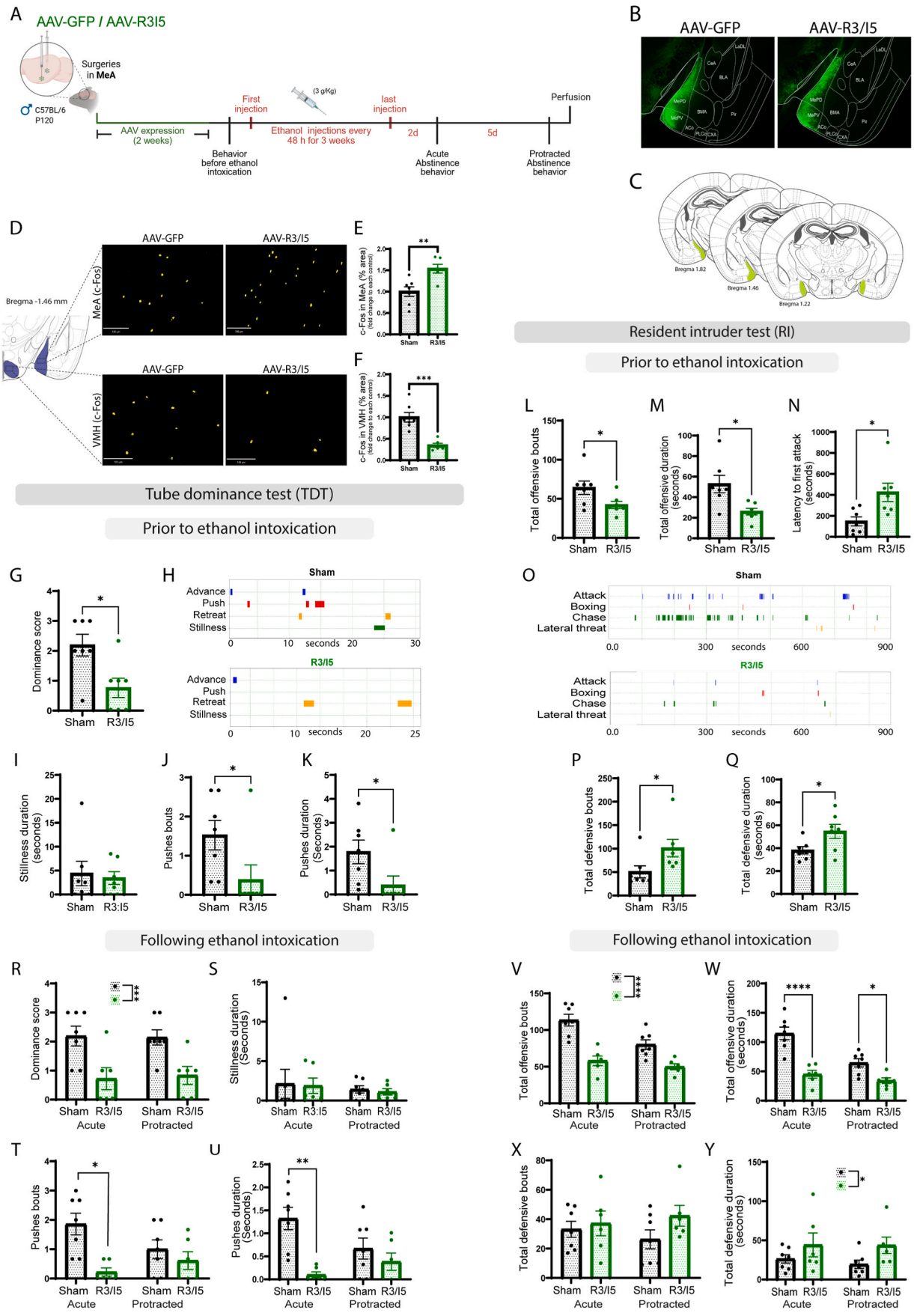

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Fig. 6. Production and localized secretion of the RXFP3 agonist R3/I5 within the MeA reduced aggression in male mice prior to and after intermittent ethanol intoxication. (A). Experimental timeline: Male mice were injected bilaterally in the medial amygdala with either adeno-associated-virus (AAV), expressing the RXFP3 agonist, R3/I5 and eGFP or a control AAV expressing GFP; and after 2 weeks of recovery and time for viral expression, mice were tested for dominance and aggressive behaviors using the TDT and RI test. Mice were subjected to intermittent ethanol intoxication followed by behavioral testing at 2 and 7 days of abstinence. **(B).** Fluorescence images illustrating the AAV injection sites as manifested by GFP + cells in the MeA. **(C).** Schematic of a coronal brain slice illustrating the site of AAV injections. **(D).** Fluorescence images illustrating c-Fos protein expression in MeA and VMH of R3/I5- and sham-treated mice. **(E).** R3/I5-treated mice displayed a significant increase in c-Fos expression in MeA **(F).** R3/I5-treated mice displayed a significant decrease in c-Fos expression in VMH. **(G).** R3/I5-treated mice displayed a significant decrease in dominance score compared to the control group prior to ethanol intoxication. **(H).** Gantt chart illustrating quantified behaviors of confronted mice in the TDT before ethanol intoxication. **(I).** No significant difference was observed in stillness time. **(J,K).** R3/I5-treated mice displayed a significant decrease in the number (J) and duration (K) of pushes compared to the control AAV group prior to ethanol intoxication. **(L–N).** R3/I5-treated mice displayed a significant increase in latency to first attack (L) and a significant decrease in the number (M) and duration (N) of offensive behaviors compared to control prior to ethanol intoxication. **(O).** Gantt chart of offensive behaviors (attack, boxing, chasing, lateral threat) in individual mice treated with the R3/I5 and control AAV prior to ethanol intoxication. **(P,Q).** R3/I5-treated mice displayed a significant increase in the number (P) and duration (Q) of defensive behaviors prior to ethanol intoxication. **(R).** R3/I5-treated mice displayed a significant decrease in dominance score compared to the control AAV group after acute and protracted abstinence. **(S).** No significant difference was observed in stillness time. **(T,U).** R3/I5-treated mice displayed a significant decrease in the number (T) and duration (U) of pushes compared to the control group after acute but not protracted abstinence. **(V,W).** R3/I5-treated mice displayed a significant decrease in the number (V) and duration (W) of offensive behaviors compared to control after both acute and protracted abstinence. **(X,Y).** No differences in defensive behaviors of females were observed after ethanol intoxication. In all graphs, each dot represents an individual mouse, and data are expressed as mean ± SEM (n = 6-7/group). In graphs G-K, a Mann Whitney test was performed. In graphs E-F and L-Q, Welch's *t*-test was performed. In graphs R-U, a two-factor ART ANOVA followed by Multiple Mann Whitney test adjusted by Bonferroni-Dunn correction was performed. In graphs V-Y: Two-way ANOVA followed by a Bonferroni's multiple comparisons test was performed. Statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

2019) into the MeA. Chronic localized activation of RXFP3 in MeA significantly reduced both dominance and aggressive behaviors. Prior to ethanol intoxication, R3/I5-treated mice displayed lower dominance scores and pushes in the TDT, and reduced offensive behavior in the RI test, indicating a baseline suppression of dominance and aggressive behaviors, while defensive behaviors increased in R3/I5-treated mice, suggesting a shift from offensive to defensive coping strategies. Importantly, these effects persisted after ethanol intoxication. R3/I5-treated mice displayed markedly reduced offensive behavior during both acute and protracted abstinence, with no escalation typically observed in controls. While the overall duration of defensive behavior displayed a significant treatment effect, post-hoc comparisons did not reach significance at individual time points. These findings provide evidence that enhancing relaxin-3/RXFP3 signaling in the MeA is sufficient to counteract alcohol-related aggression. Furthermore, an investigation of the neural mechanisms underlying the R3/I5 intervention demonstrated that the anti-aggressive effect was paralleled by altered neuronal activation in the aggression circuit. Specifically, we explored c-Fos expression in the MeA and VMH in R3/I5- and sham-treated mice, revealing that R3/I5 treatment resulted in a significant increase in c-Fos levels in the MeA and a significant decrease in c-Fos expression in the VMH, compared to sham-treated mice. This pattern of neural activation was the inverse of the pattern observed during acute ethanol-induced aggression, providing a clear neurobiological correlate for the observed reduction in dominance and aggressive behavior. This finding is consistent with the established connectivity of the aggression circuit, as the VMH is recognized as a crucial hub for generating attack behavior in male mice (Falkner et al., 2016; Lin et al., 2011). The MeA provides a major projection to the VMH, mediating top-down control over this aggression switch (Nordman et al., 2020). Importantly, a GABAergic neuron population within the MeA is known to promote aggression (Hong et al., 2014). Given this inhibitory control, and data that RXFP3 is predominantly expressed on GABAergic neurons (Albert-Gascó et al., 2018; Albert-Gasco et al., 2019; Haidar et al., 2019), we propose that the R3/I5 agonist acted locally to inhibit a population of GABAergic neurons within the MeA. In turn, this action resulted in the disinhibition of the main MeA projection neurons, likely the population that provides inhibitory input to the VMH, which was reflected by the elevated c-Fos expression observed in the MeA. This heightened MeA output subsequently exerted a stronger inhibitory drive on the central aggression hub, leading to the significant suppression of neuronal activation in the VMH, as reflected by decreased c-Fos. These proposed actions represent a coherent circuit-level explanation for the observed anti-aggressive effects of the sustained R3/I5 treatment.

These findings position relaxin-3/RXFP3 signaling within a broader neurochemical signaling network controlling alcohol-related aggression, complementing established roles for CRF, vasopressin, serotonin, and somatostatin systems (Mañas-Ojeda et al., 2024; Miczek et al., 2015), which also interact with NI- and relaxin-3 related circuits (Ma et al., 2013; Miyamoto et al., 2008). Therefore, pharmacological or gene-therapy approaches that modulate this pathway could represent a future novel strategy to reduce alcohol-related, impulsive violence in humans, which remains a major public-health concern.

4.4. Implications of sex differences and therapeutic potential for reduction of aggressiveness associated with ethanol intoxication

The sex differences observed in brain relaxin-3 immunoreactivity after ethanol intoxication (i.e., delayed upregulation in males and early increases in females) suggest different neuroadaptive mechanisms, in which males require relaxin-3 upregulation to restore behavioral homeostasis, whereas females engage this system earlier, possibly as part of the stress response. These variations may be due to differential hormonal regulation of the relaxin-3/RXFP3 system which has been reported in females (De Ávila et al., 2021), and/or sex differences in NI connectivity. Given the increasing emphasis on sex as a biological variable in clinical trials and neuropharmacological research, our data underscore the need to stratify patients by sex when assessing RXFP3-based interventions or other treatments for alcohol-related aggression.

Moreover, the effect of chronic localized MeA R3/I5 treatment in mitigating alcohol-related aggression identify RXFP3 agonists as a potential therapeutic for alcohol-related impulsive violence, especially in individuals with a history of intermittent binge drinking. While our data demonstrate that RXFP3 activation in the MeA is sufficient to blunt alcohol-related aggression, clinical translation will require careful consideration of potential off-target effects on alcohol-seeking behavior. The RLN3/RXFP3 system is reported to be a significant regulator of alcohol consumption, with its effects varying depending on the context, sex, and anatomical location of targeted modulation. While direct delivery of an RXFP3 antagonist into the BNST reduced alcohol self-administration in rats (Ryan et al., 2013a,b) and relaxin-3 mRNA levels in the NI correlate positively with alcohol intake (Ryan et al., 2013), genetic models provide contradictory data. For example, male RXFP3 deficient (knockout) mice exhibited a stress-induced reduction in alcohol consumption, whereas male relaxin-3 deficient (knockout) mice drank significantly more alcohol than their wildtype control (Shirahase et al., 2016). Collectively however, these findings underscore the likely involvement of the RLN3/RXFP3 system in the neurobiology of alcohol-use disorder. As the present study employed a forced

intermittent intoxication model rather than assessing voluntary alcohol consumption, the influence of MeA RXFP3 modulation on these addiction phenotypes remains unknown. Moreover, the translational viability of RLN3/RXFP3 signaling might depends on the development of highly circuit-specific interventions that can selectively target the MeA without affecting regions such as the CeA and BNST, which could otherwise exacerbate or alter alcohol intake, craving or relapse vulnerability (Ryan et al., 2013a,b).

CRediT authorship contribution statement

Mohamed Aly Zahran: Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Aroa Mañas-Ojeda:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Mónica Navarro Sánchez:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Andrew L. Gundlach:** Writing – review & editing, Writing – original draft, Validation, Conceptualization. **Francisco E. Olucha-Bordonau:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Esther Castillo-Gómez:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Funding

This study was supported by grants from the Spanish Ministry of Science, Innovation, and Universities (Grant: PID2023-153074OB-I00), National Plan on Drugs-Spanish Ministry of Health (Grant: 2020I012), Generalitat Valenciana (CIAICO/2023/244), Universitat Jaume I (GACUJIMB/2024/30) and European Commission (Grant MSCA-SE101086247). MAZ is the recipient of a PhD Scholarship financed by Generalitat Valenciana (GRISOLIA/2021/131). The funding agencies had no role in study design; in the collection, analysis, and interpretation of data; in the writing of the manuscript; and in the decision to submit the article for publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank Prof Ross Bathgate (The Florey) for providing the AAV vectors, Prof Marc Landry (University of Bordeaux) for helpful advice and assistance and Servei Central d'Instrumentació Científica (SCIC) and Servei d'Experimentació Animal (SEA) at Universitat Jaume I for their technical support. The authors (MAZ, RMO, MNS, FEOB, ECG) belong to Red Española de Investigación en Estrés (REIS) financed by MCIN/AEI/10.13039/501100011033 and FEDER (RED2022-134191-T).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2026.110924.

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