Cannabidiol in the Anterior Insular Cortex Attenuates Chronic Neuropathic Pain and Comorbid Anxiety- and Depression-Like Behaviors: Involvement of CB1 and 5-HT1A Receptor Signaling

Renata Moreira Acunha
Ribeirão Preto Medical School of the University of São Paulo (FMRP-USP)
Bruna Rabelo Ribeiro Domingues
Ribeirão Preto Medical School of the University of São Paulo (FMRP-USP)
Nubia Barros
Ribeirão Preto Medical School of the University of São Paulo (FMRP-USP)
Priscila Medeiros
Ribeirão Preto Medical School of the University of São Paulo (FMRP-USP)
Jaime Eduardo Cecilio Hallak
Ribeirão Preto Medical School of the University of São Paulo (FMRP-USP)
Norberto Cysne Coimbra
Ribeirão Preto Medical School of the University of São Paulo (FMRP-USP)
Sabatino Maione
University of Campania "Luigi Vanvitelli"
Renato Leonardo Freitas
rlfreitas@usp.br

Ribeirão Preto Medical School of the University of São Paulo (FMRP-USP)

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Abstract

Background

Chronic neuropathic pain (NP) is frequently accompanied by anxiety- and depression-like symptoms, reflecting maladaptive interactions between nociceptive and affective brain networks. The anterior insular cortex (AIC) integrates sensory and emotional dimensions of pain and represents a potential target for pharmacological modulation. Cannabidiol (CBD) exhibits analgesic and anxiolytic/antidepressant-like properties through interactions with endocannabinoid and serotonergic systems.

Objectives

We investigated whether CBD microinjection into the AIC modulates NP and its affective comorbidities, and whether these effects depend on CB_1 and 5-HT_{1A} receptors.

Methods

Male Wistar rats were subjected to chronic constriction injury (CCI) of the sciatic nerve. Fourteen days later, guide cannulae were implanted into the AIC. On day 21 post-CCI, animals received intra-AIC microinjections of CBD (15, 30, or 60 nmol/200 nL) or vehicle. Mechanical (von Frey test) and cold (acetone test) allodynia, anxiety-like behavior (open field and elevated plus maze tests), and depression-like behavior (forced swim and sucrose spray tests) were assessed by different psychobiological tests. The role of cannabinoid and serotonergic receptors was addressed by intra-AIC pretreatment with either the CB_1 receptor antagonist AM251 or the 5-HT_{1A} receptor antagonist WAY-100635 in independent groups.

Results

AIC pretreatment with CBD dose-dependently reduced mechanical and cold allodynia and anxiety- and depression-like behaviors, with the most robust effects observed at 60 nmol. AIC Pretreatment with either AM251 or WAY-100635 abolished the antinociceptive and affective effects of CBD.

Conclusion

CBD administration within the AIC produces integrated analgesic, anxiolytic, and antidepressant-like effects in a model of neuropathic pain. These effects are consistent with the involvement of CB_1 and 5-HT_{1A} receptor signaling. The findings identify the AIC as a relevant cortical substrate linking nociceptive

and affective processes and support CBD as a promising psychopharmacological strategy for neuropathic pain associated with emotional comorbidities.

Highlights

● Cannabidiol in the anterior insular cortex (AIC) reduces neuropathic pain–like behavior.

● Intra-insular CBD attenuates anxiety- and depression-like behaviors.

● The AIC is implicated as a cortical hub linking nociceptive and affective processes.

● CB and 5-HT receptor signaling contribute to the effects of CBD in the AIC.

● The AIC represents a potential pharmacological target for neuropathic pain and associated psychiatric comorbidities.

1 Introduction

Neuropathic pain (NP) is a chronic condition resulting from injury or dysfunction of the somatosensory system and represents one of the most disabling pain disorders worldwide. It profoundly compromises quality of life and imposes a substantial socioeconomic burden worldwide (IASP, 2020; Cohen et al., 2018; Fayaz, 2016; Santiago et al., 2023). Epidemiological data indicate that chronic pain affects approximately 39% of the Brazilian population, with a higher prevalence in women, underscoring its public health relevance in both global and regional contexts (Souza et al., 2017).

From a clinical perspective, NP remains particularly challenging to manage due to its complex and multifactorial pathophysiology. Pharmacological treatments such as alpha2-denta1 blockers, anticonvulsants, and antidepressants are commonly prescribed; however, they provide only partial pain relief in approximately 40–60% of patients and are frequently associated with adverse effects that limit long-term adherence (van Hecke et al., 2014; Cruccu & Truini, 2017; Finnerup et al., 2021; Zambelli et al., 2021). These limitations highlight an urgent need for more effective and better-tolerated therapeutic strategies.

The pathophysiology of NP involves both peripheral and central mechanisms, including nociceptor sensitization and increased excitability within spinal nociceptive circuits, leading to clinical manifestations such as allodynia and hyperalgesia (Apkarian et al., 2005; Jensen et al., 2021). In parallel, immune system interactions with the central nervous system play a critical role in the chronification of pain. Activation of microglia and the release of pro-inflammatory cytokines contribute to central sensitization and sustained nociceptive signaling (Tracey & Mantyh, 2007; Borsook, 2011; Ahmadi et al., 2024).

These maladaptive processes extend beyond the spinal cord and engage supraspinal structures involved in integrating sensory, emotional, and cognitive aspects of pain, including the dorsal thalamus, prefrontal

cortex, amygdala, and insular cortex (IC) (Shi & Wu, 2023; Karcz et al., 2024).

A growing body of evidence identifies the insular cortex (IC) as a pivotal cortical hub for integrating nociceptive and affective information. Human neuroimaging and intracranial electrophysiological studies consistently demonstrate robust IC activation in response to acute and chronic painful stimuli, with activity levels correlating with both pain intensity and subjective unpleasantness (Frot et al., 2014; Garcia-Larrea & Peyron, 2013). Dysregulation of IC activity has been associated with heightened emotional reactivity and impaired affective control, reinforcing its contribution to pain-related psychiatric comorbidities.

Cannabidiol (CBD), a phytocannabinoid without the negative psychoactive effects, has attracted considerable interest due to its analgesic, anxiolytic, and antidepressant-like properties. Despite its name, which suggests selective cannabimimetic action, it binds to several targets (i.e., proteins and receptors), including 5-HT_{1AR}, transient receptor potential vanilloid 1 ($TRPV_1$), the adenosine transporter, and cannabinoid receptors. In fact, previous in vivo and in vitro studies have shown that CBD acts as a negative allosteric modulator of CB_1 receptor and an agonist of CB_2 (Campos et al., 2012; de Gregorio et al., 2019; Mlost et al., 2020; Ewa Galaj and Zheng-Xiong Xi, 2020; Maccarrone et al., 2023).

Preclinical evidence demonstrates that CBD reduces neuropathic pain and associated emotional impairments through receptor-specific mechanisms, notably involving CB_1 and 5-HT_{1A} receptors within corticolimbic circuits (Russo et al., 2005; Rock et al., 2012). Site-specific studies have shown that activation of these receptors in the prelimbic prefrontal cortex and hippocampal networks is sufficient to produce robust antinociceptive and affective effects (Malvestio et al., 2021; Medeiros et al., 2023). Furthermore, CBD, in addition to enhancing neuroplasticity, increased AEA (but not 2-AG) levels in the dentate gyrus of the hippocampus of SNI rats. However, these CBD-induced improvements were reversed by acute blockade of the KOR opioid receptor (Boccella et al., 2025). This indicates a critical relationship between CBD-induced adaptive changes and the need to maintain physiological dynorphin tone in neuropathic rats. Together, these findings provide a strong rationale for investigating receptor-dependent mechanisms of CBD action using selective CB_1 and 5-HT_{1A} antagonists, as employed in the current study.

In the present study, we tested the hypothesis that microinjection of CBD into the AIC attenuates mechanical and cold allodynia as well as anxiety- and depression-like behaviors in an experimental model of chronic constriction injury (CCI) in rats. Furthermore, we investigated whether these effects depend on local activation of CB_1 and 5-HT_{1A} receptors. By combining site-specific pharmacological manipulation with behavioral assessment, this study aims to advance understanding of cortical neurochemistry in NP and to identify the AIC as a potential brain region where drugs may act to provide integrated treatment of pain and affective comorbidities.

2 Material and Methods

2.1 Animals

Adult male Wistar rats (200 g at the beginning of the experiments) were obtained from the institutional animal facility of the University of São Paulo (USP), at Ribeirão Preto campus. Animals were housed 3–4 per cage under controlled environmental conditions (22 ± 2°C; 12 h light/dark cycle, lights on at 07:00 h), with food and water available *ad libitum*, and were allowed to acclimate to the facility for at least 7 days before any experimental manipulation. A total of 88 animals were used in this study (see group sizes specified in the corresponding figure legends). All experimental procedures were conducted in accordance with the ARRIVE 2.0 guidelines and were approved by the Institutional Animal Care and Use Committee of the Ribeirão Preto School of Medicine of the University of São Paulo (FMRP-USP; protocol number 1029/2021), in compliance with national and international regulations for animal research.

2.2 Experimental design, randomization, and blinding

The experimental timeline is summarized in Fig. 1. Animals underwent baseline nociceptive assessment (LB1), followed by CCI or SHAM surgery (Arrow A). Twenty-one days after surgery, a second baseline assessment (LB2) was performed to confirm the NP condition starting. Subsequently, animals received intra-AIC microinjections of CBD, selective antagonists, or vehicle (Arrow B), followed by nociceptive and behavioral testing conducted within predefined time windows, as detailed below and illustrated in Figs. 3–9. Animals were randomly assigned to experimental groups using a computer-generated randomization sequence. All surgical procedures, drug administrations, behavioral testing, and data analyses were performed by experimenters blinded to treatment allocation. Group sizes were determined based on previous studies from our research group using similar experimental designs and behavioral endpoints.

2.3 Chronic constriction injury of the sciatic nerve

Peripheral neuropathy was induced by unilateral *ischiadicus nervus* (sciatic nerve) chronic constriction injury (CCI), as initially described by Bennett and Xie (1988), with subsequent modifications (Sommer & Myers, 1995) and adaptations previously validated by our team (Medeiros et al., 2019, 2020; Malvestio et al., 2021; Negrini-Ferrari et al., 2021; Martins-Pereira et al., 2022; Brito et al., 2025). Animals were anesthetized with ketamine (92 mg/kg, i.m.) and xylazine (9.2 mg/kg, i.m.), and a 15-mm incision was made in the dorsolateral thigh to expose the sciatic nerve of the right hind limb. A single 4 − 0 catgut ligature was placed proximal to the nerve trifurcation with mild tension, sufficient to induce partial ischemia without interrupting epineural blood flow. The incision was closed with 5 − 0 nylon sutures. SHAM-operated animals underwent identical surgical procedures without nerve ligation.

2.4 Stereotactic surgery for guide cannula implantation in the anterior insular cortex

Fourteen days after CCI or SHAM surgery, animals were anesthetized and placed in a stereotaxic apparatus (Insight, Ribeirão Preto, São Paulo, Brazil), with the skull positioned using ear bars and the

upper incisors secured. Before skull exposure, local anesthesia was administered to the skin and subcutaneous tissue (2% lidocaine, 0.1 mL, s.c.). The periosteum was removed, and the skull surface was cleaned and dried using 10% hydrogen peroxide. A single stainless-steel guide cannula was implanted into the left hemisphere, contralateral to the CCI surgery performed on the right hind limb, to enable drug microinjection into the anterior insular cortex (AIC), targeting the region functionally associated with hind limb representation. Stereotaxic coordinates were anteroposterior (AP) -1.0 mm, mediolateral (ML) 7.2 mm, and dorsoventral (DV) -7.0 mm, according to the rat brain atlas of Paxinos and Watson (2005). A 15-mm guide cannula coupled to a 16-mm injection needle was secured to the skull using self-curing acrylic dental cement and anchored with two stainless-steel screws.

2.5 Cannabidiol and antagonist microinjection

Either cannabidiol (CBD), the 5-HT_{1A} receptor antagonist WAY-100635, or the CB_1 receptor antagonist AM251 were freshly prepared and administered as previously described (Malvestio et al., 2021; Medeiros et al., 2023). Independent groups of animals received intra-AIC microinjections of CBD (15, 30, or 60 nmol in 200 nL) or vehicle (100% grape seed oil). The solutions were prepared immediately before the tests and protected from the light during the experimental sessions. Experimental rats were pretreated with either an intra-AIC microinjection of WAY-100635 (0.37 pmol) or AM251 (100 pmol), or with vehicle, followed by either vehicle or CBD intracortical administration. Microinjections were delivered unilaterally into the left hemisphere, contralateral to the CCI surgery, using a 16-mm injection needle extending 1 mm beyond the tip of the guide cannula and connected to a Hamilton microsyringe (Hamilton, Reno, Nevada, USA) mounted on an infusion pump (Stoelting, Kiel, Wisconsin, USA). A fixed volume (200 nL) was infused at a controlled rate to minimize tissue damage and reflux. The injection needle was kept in place for an additional period to allow diffusion of the drug before removal. Control animals received equivalent volumes of vehicle. Behavioral and nociceptive assessments were conducted 5 min after microinjection.

2.6 Histological verification of cannula placement

At the end of the experiments, animals were deeply anesthetized with urethane (25%) and perfused transcardially with phosphate-buffered saline (PBS), followed by 4% paraformaldehyde. Brains were removed, post-fixed, cryoprotected in 30% sucrose, frozen, and sectioned coronally into 40 µm slices using a cryostat (Leica). Tissue sections were stained with hematoxylin and eosin and examined under light microscopy (AxioImager Z1, Zeiss, Oberkochen, Germany) to verify electrode and guide cannula placements within the AIC. Only animals with correct bilateral cannula placements were included in the final analysis. Animals with misplaced cannulas were excluded according to predefined criteria.

2.7 Behavioral assessment and experimental timeline

The experimental timeline for intra-AIC microinjections and subsequent nociceptive and behavioral assessments is illustrated in Fig. 1. Behavioral and nociceptive tests were conducted in a fixed order to minimize stress and carryover effects and to ensure consistency across experimental groups.

At baseline (Day 0; Baseline 1), mechanical and cold sensitivity were assessed using the von Frey and acetone tests, respectively. One hour later, animals underwent chronic constriction injury (CCI) or SHAM surgery. Fourteen days after surgery (Day 14), rats were subjected to stereotaxic implantation of guide cannulas into the AIC.

On Day 21, a second baseline assessment (Baseline 2) was performed using von Frey and acetone tests to confirm the establishment of neuropathic pain. Immediately thereafter, animals received intra-AIC microinjections of vehicle or cannabidiol (CBD; 15, 30, or 60 nmol) in rats with or without chronic neuropathic pain, or intra-AIC microinjections of CBD (60 nmol) following pretreatment with WAY-100635 or AM251 in antagonist experiments. Nociceptive assessments were initiated 5 min after microinjection and repeated at 0, 10, 20, and 30 min, as shown in Fig. 3.

Emotional-like behaviors were evaluated in separate cohorts of animals beginning after nociceptive testing, using the sucrose splash test (Fig. 6), forced swim test (Figs. 7–8), open field test (Fig. 4), and elevated plus-maze (Fig. 5). All behavioral tests were conducted during the light phase of the cycle in a dedicated experimental room with controlled lighting and minimal noise. Animals were habituated to the testing environment before data collection. On Day 22, animals were perfused, and brains were processed for histological verification of cannula placement.

2.7.1 Von Frey test (mechanical allodynia)

Mechanical sensitivity was assessed using calibrated von Frey filaments applied to the plantar surface of both the right and left hind paws, following the up–down method described by Chaplan et al. (1994). The withdrawal threshold was determined using the up–down method. Measurements were obtained at baseline, 21 days after surgery, and following intra-AIC drug administration. After drug injection, a 5-minute interval was allowed before testing, and the 0 min time point corresponds to 5 minutes after microinjection. Subsequent measurements were taken at 10, 20, and 30 minutes after treatments. Mechanical and cold sensitivity tests were performed in an alternating manner to avoid interference between stimuli.

2.7.2 Acetone test (cold allodynia)

Cold sensitivity was evaluated by applying 20 µl of acetone to the plantar surface of both the right and left hind paws (Eliav et al., 1999; Paszcuk et al., 2007; Medeiros et al., 2019, 2024). The time spent licking, shaking, or biting the paw was recorded. Assessments were performed at baseline and on day 21 post-surgery, followed by intra-AIC drug administration. After injection, a 5-minute interval was allowed before testing; the 5 min time point corresponds to 10 minutes after microinjection. Subsequent measurements were taken at 15 and 25 minutes after treatments. Cold and mechanical sensitivity tests were conducted in an alternating sequence, with sufficient intervals between tests to minimize cross-modality interference.

2.7.3 Open field test

Locomotor activity and anxiety-like behavior were evaluated using the open field test, as previously described (Prut & Belzung, 2003). Animals were individually placed in the center of a circular open-field arena and allowed to explore freely for 5 min. Exploratory behaviors were recorded, including total locomotion (crossings), time spent in the central and peripheral zones, grooming, and rearing. The apparatus was cleaned between trials to eliminate olfactory cues.

2.7.4 The Elevated Plus-Maze Test

Anxiety-like behavior was assessed using the elevated plus-maze, as previously described (Walf et al., 1998). The apparatus consisted of a wooden T-shaped maze elevated above the floor, with one closed arm and two open arms. Each animal was placed individually in the maze and allowed to explore for 5 min. The number of entries and the percentage of time spent in the open and closed arms were recorded as indices of anxiety-related behavior.

2.7.5 Sucrose splash test

Anhedonia- and apathy-like behaviors were evaluated using the sucrose splash test, as previously described (Kalueff & Tuohimaa, 2004). Animals were individually placed in their home cage, and a 10% (w/v) sucrose solution was sprayed onto the dorsal coat. Grooming behavior was recorded for 5 min, and both grooming frequency and total grooming duration were quantified as indices of motivational and self-care behaviors.

2.7.6 Forced swimming test (FST)

Depressive-like behavior was assessed using the forced swimming test, as initially described by Porsolt et al. (1997). Animals were individually placed in a transparent cylindrical tank (20 cm diameter, 40 cm height) filled with water (25 ± 1°C; 20 cm depth), from which escape was not possible. The protocol consisted of a 15-minute pre-test session, followed 24 hours later by a 5-minute test session. All sessions were video recorded (Sony Handycam, HDR-SR10, Tokyo, Japan) and analyzed offline using X-Plo-Rat software. The frequencies and total durations of immobility-related behaviors (floating and freezing) and mobility-related behaviors (swimming, climbing, and diving) were quantified, as detailed in Figs. 7 and 8.

2.8 Drugs

Cannabidiol (CBD; ~99.9% purity; FarmaUSA, Volta Redonda, RJ, Brazil) was dissolved in grape seed oil and administered intra-AIC at doses of 15, 30, or 60 nmol in a final volume of 200 nL. The CB_1 receptor antagonist N-(piperidine-1-yl)-5-(4-iodophenyl)-1-(2,4-chlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (AM251; Tocris Bioscience, Bristol, UK) was diluted in 10% dimethyl sulfoxide (DMSO) and administered at a dose of 100 pmol (Medeiros et al., 2021, 2023; Malvestio et al., 2021). The selective 5-HT_{1A} receptor antagonist WAY-100635 (Sigma-Aldrich, USA) was dissolved in physiological saline (0.9% NaCl) and administered at a dose of 0.37 pmol (Roncon et al., 2017; Medeiros et al., 2023).

2.9 Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). The exact number of animals per group (n = 8) is indicated in each figure legend and corresponds to the final number of animals included after histological verification of cannula placement. Normality of the data distribution was assessed using the Shapiro-Wilk test. When data met the assumptions of normality, parametric analyses were performed. Mechanical allodynia and cold sensitivity were analyzed using repeated-measures split-plot or two-way analysis of variance (ANOVA), with surgery and treatment (microinjection of different drugs) as independent factors and time as the repeated measure, when appropriate. Significant main effects or interactions were further examined using Tukey's post hoc test. Behavioral data related to locomotor activity, anxiety-like behavior, and depression-like behavior were analyzed using one-way ANOVA followed by Tukey's post hoc test. Statistical significance was set at $p < 0.05$. All statistical analyses and graphical representations were performed using GraphPad Prism 8. Data supporting the findings of this study are available from the corresponding author upon reasonable request.

2.10 Ethical compliance and sample size transparency

All exclusions were predefined and restricted to technical criteria, including incorrect cannula placement or loss of implant integrity. No animals were excluded based on behavioral or nociceptive outcomes. The total number of animals used in this study (n = 88) and the number of animals per experimental group are consistently reported throughout the Methods, Results, and corresponding figure legends, in accordance with ARRIVE 2.0 recommendations.

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

3 Results

3.1 Effects of Cannabidiol Microinjection into the Anterior Insular Cortex on Mechanical and Cold Allodynia

Histological analysis confirmed that all microinjection sites were located within the anterior insular cortex (AIC) in both chronic constriction injury (CCI) and SHAM animals (Fig. 2).

Cannabidiol (CBD) was microinjected into the AIC at doses of 15, 30, or 60 nmol/200 nl, contralateral to the injured hind paw. Behavioral assessments of mechanical and cold allodynia were conducted at defined time points following intra-AIC administration.

Figure 3 presents the time-course effects of intra-AIC CBD on mechanical and cold withdrawal thresholds in CCI and SHAM animals, as well as the effects of intra-AIC pretreatment with the CB_1 receptor antagonist AM251 or the 5-HT_{1A} receptor antagonist WAY-100635. Based on the dose-response analysis, CBD in a dose of 60 nmol produced the most robust and consistent effects and was therefore selected for subsequent pharmacological interaction experiments.

3.1.1 Effect of Intra-AIC CBD on Mechanical Allodynia

Intra-AIC administration of CBD significantly altered mechanical withdrawal thresholds in CCI animals at day 21 post-surgery. Two-way ANOVA revealed significant main effects of treatment ($F_{5,42}$ = 109.9, p < 0.001) and time ($F_{3595,151}$ = 225.4, p < 0.001), as well as a significant treatment × time interaction ($F_{20,168}$ = 26.53, p < 0.001), on the mechanical withdrawal threshold of the ipsilateral (right) hind paw in relation to CCI. Tukey post hoc analysis demonstrated reduced mechanical withdrawal thresholds in Vehicle/CCI animals compared with Vehicle/SHAM controls (p < 0.001). In CCI animals, intra-AIC CBD at 60 nmolnmol significantly increased mechanical withdrawal thresholds relative to Vehicle/CCI animals from 0 to 20 min post-injection (p < 0.001). A comparable effect was observed following administration of CBD at 30 nmol; however, this effect was time-dependent and was significant only at 10 and 20 min post-injection (p < 0.01), whereas the 15 nmol dose did not significantly modify mechanical thresholds (p > 0.05). In SHAM animals, intra-AIC CBD at 60 nmol did not significantly alter mechanical sensitivity relative to Vehicle/SHAM controls (p > 0.05). A significant increase in mechanical withdrawal thresholds was observed between CBD doses, with the 60 nmol dose differing from the 15 nmol dose throughout the 0–20 min post-microinjection interval (p < 0.01). CBD at 30 nmol increased the threshold compared with CBD at 15 nmol at 10 min post-microinjection (p < 0.05) (Fig. 3A).

Two-way ANOVA revealed no significant main effects of treatment ($F_{5,42}$ = 0.69, p > 0.05) or time ($F_{3417,143.5}$ = 1.27, p > 0.05), nor a significant treatment × time interaction ($F_{20,168}$ = 0.71, p > 0.05), on the mechanical withdrawal threshold of the contralateral (left) hind paw in relation to CCI (Fig. 3B).

3.1.2 Effect of Intra-AIC CBD on Cold Allodynia

Intra-AIC administration of CBD also significantly modified cold score responses in CCI animals. Two-way ANOVA revealed significant main effects of treatment ($F_{5,42}$ = 96.70, p < 0.001) and time ($F_{3.326,139.7}$ = 316.1, p < 0.001), as well as a significant treatment × time interaction ($F_{20,168}$ = 50.96, p < 0.001), on the score of the ipsilateral (right) hind paw in relation to CCI. Post hoc Tukey analysis revealed that Vehicle/CCI animals exhibited increased cold responsiveness compared with the Vehicle/SHAM group at 5, 15, and 30 min after microinjection (p < 0.001). A consistent effect was observed, as CBD at 60 and 15 nmol increased cold score responses relative to Vehicle in CCI animals across all time points (p < 0.001). In CCI animals, intra-AIC CBD at 30 nmol significantly decreased the cold score responses only 30 min post-injection compared with Vehicle/CCI (p < 0.001). CBD at 60 nmol significantly decreased cold response scores compared with CBD at 15 nmol across all time points evaluated (p < 0.001). In contrast, CBD at 30 nmol reduced cold responsiveness only at 30 min post-microinjection (p < 0.01). No significant effects were observed in SHAM animals (p > 0.05) (Fig. 3C).

Two-way ANOVA revealed no significant main effects of treatment ($F_{5,42}$ = 3.89, p > 0.05) or time ($F_{3384,142.1}$ = 0.68, p > 0.05), nor a significant treatment × time interaction ($F_{20,168}$ = 0.84, p > 0.05), on cold responsiveness of the contralateral (left) hind paw in relation to CCI (Fig. 3D).

3.1.3 Effects of CB_1 and 5-HT_{1A} Receptor Blockade in the AIC on CBD-Induced Mechanical Allodynia Modulation

Intra-AIC pretreatment with either the CB_1 receptor antagonist AM251 or the 5-HT_{1A} receptor antagonist WAY-100635 significantly altered the effects of CBD on mechanical withdrawal thresholds in CCI animals.

Two-way ANOVA revealed significant main effects of treatment ($F_{6,49} = 119.4$, $p < 0.001$) and time ($F_{3.730,182.7} = 650.9$, $p < 0.001$), as well as a significant treatment × time interaction ($F_{24,196} = 26.34$, $p < 0.001$), on the mechanical withdrawal threshold of the ipsilateral (right) hind paw in relation to CCI. Tukey post hoc analysis demonstrated reduced mechanical withdrawal thresholds in Vehicle + Vehicle/CCI animals compared with Vehicle + Vehicle/SHAM controls at all times measured after microinjection ($p < 0.001$). Tukey post hoc analysis indicated that Vehicle + CBD at 60 nmol (AIC)/CCI animals displayed increased mechanical withdrawal thresholds compared with AIC Vehicle + Vehicle/CCI animals at all time points evaluated ($p < 0.001$). Pretreatment with either WAY-100635 (0.37 nmol) or AM251 (100 nmol) abolished the effect of CBD, leading to significantly reduced mechanical withdrawal thresholds relative to Vehicle + CBD (60 nmol)/CCI at 10 and 20 min after microinjection ($p < 0.001$). Antagonists administered alone did not significantly affect mechanical thresholds relative to Vehicle + Vehicle/CCI controls ($p > 0.05$) (Fig. 3E).

In the study evaluating intra-AIC pretreatment with either the CB_1 receptor antagonist AM251 or the 5-HT_{1A} receptor antagonist WAY-100635, two-way ANOVA revealed no significant main effects of treatment ($F_{6,49} = 0.86$, $p > 0.05$) or time ($F_{3497,171.4} = 1.71$, $p > 0.05$), nor a significant treatment × time interaction ($F_{24,196} = 0.64$, $p > 0.05$), on the mechanical withdrawal threshold of the contralateral (left) hind paw in relation to CCI (Fig. 3F).

3.1.4 Effects of CB_1 and 5-HT_{1A} Receptor Blockade in the AIC on CBD-Induced Cold Allodynia Modulation

A similar pattern was observed for cold allodynia. Two-way ANOVA revealed significant main effects of treatment ($F_{6,49} = 138.7$, $p < 0.001$) and time ($F_{2768,135.6} = 633.5$, $p < 0.001$), as well as a significant treatment × time interaction ($F_{24,196} = 38.04$, $p < 0.001$), on the cold score responses of the ipsilateral (right) hind paw in relation to CCI.

Tukey Post hoc analysis revealed that Vehicle + Vehicle/CCI animals exhibited increased cold responsiveness compared with the Vehicle + Vehicle/SHAM group at 5, 15, and 30 min after microinjection ($p < 0.001$). Vehicle + CBD 60 nmol/CCI animals significantly decreased the score compared with Vehicle + Vehicle/CCI animals at 5, 15, and 30 min after microinjection (Tukey's post hoc test; $p < 0.001$). Pretreatment with either WAY-100635 or AM251 abolished the CBD (60 nmol)-induced effect, increasing the cold score responses relative to Vehicle + CBD (60 nmol)/CCI ($p < 0.01$). No

significant differences were observed between AIC Vehicle + Vehicle/CCI and a given antagonist + Vehicle in AIC/CCI groups ($p > 0.05$) (Fig. 3G).

In the study assessing the effects of intra-AIC pretreatment with WAY-100635 and AM251, two-way ANOVA revealed no significant main effects of treatment ($F_{6,49} = 1.14$, $p > 0.05$) or time ($F_{2797,137.1} = 3.65$, $p > 0.05$), nor a significant treatment × time interaction ($F_{24,196} = 0.82$, $p > 0.05$), on cold responsiveness of the contralateral (left) hind paw in relation to CCI (Fig. 3H).

3.1.5 Effect of Intra-AIC CBD on Locomotor Activity, Exploratory Behavior, and Anxiety-Related Measures

Intra-AIC microinjection of CBD did not produce changes in general locomotor activity in the open-field test. However, CBD administration significantly modified behavioral parameters related to exploration and anxiety-like behavior in CCI animals.

One-way ANOVA revealed a significant effect of treatment on time spent in the periphery of the arena ($F_{5,42} = 331.3$, $p < 0.001$). Tukey's post hoc analysis showed that Vehicle/CCI animals spent significantly more time in the periphery compared with Vehicle/SHAM animals ($p < 0.001$). In contrast, CCI animals treated with CBD (15, 30, or 60 nmol) spent significantly less time in the periphery compared with Vehicle/CCI animals ($p < 0.001$). Moreover, CBD at 30 ($p < 0.01$) and 60 nmol ($p < 0.001$) significantly reduced the time spent in the periphery compared with CBD at 15 nmol (Fig. 4A). Analysis (one-way ANOVA) of time spent in the center of the arena also revealed significant group differences ($F_{5,42} = 63.81$, $p < 0.001$). According to Tukey's post hoc test, Vehicle (AIC)/CCI-treated animals spent significantly less time in the center of the open-field test circular arena compared with Vehicle/SHAM treatment ($p < 0.001$). In contrast, AIC treatment with CBD at 60 nmol significantly increased the time spent in the center compared with Vehicle (AIC)/CCI animals ($p < 0.001$). CBD at 60 nmol significantly increased the time spent in the center compared with CBD at 15 and 30 nmol ($p < 0.001$) (Fig. 4B).

Concerning grooming behavior, one-way ANOVA indicated significant effects on both frequency ($F_{5,42} = 8.57$, $p < 0.001$) and duration ($F_{5,42} = 16.24$, $p < 0.001$). Tukey post hoc analysis revealed that Vehicle/CCI animals displayed a lower frequency of grooming compared with Vehicle (AIC)/SHAM ($p < 0.001$). AIC CBD at 60 nmol/CCI-treated animals increased the frequency of grooming compared with AIC Vehicle/CCI ($p < 0.01$) (Fig. 4C). AIC CBD at 60 nmol significantly increased grooming frequency compared with CBD at 15 ($p < 0.01$) and 30 nmol ($p < 0.5$) in CCI animals (Fig. 4C). CBD 30 ($p < 0.05$) and 60 nmol ($p < 0.001$) increased the duration of grooming compared with Vehicle (AIC)/CCI (Fig. 4D).

No significant differences were observed among groups for frequency ($F_{5,42} = 1.19$, $p > 0.05$) of rearing, but it was observed significant differences were observed in duration ($F_{5,42} = 5.33$, $p < 0.001$) of rearing. Tukey's post hoc analysis showed that Vehicle (AIC)/CCI animals decreased the duration of rearing compared with Vehicle (AIC)/SHAM animals ($p < 0.05$) (Fig. 4E–F).

Concerning crossing behavior in the arena, one-way ANOVA revealed a significant effect of treatment ($F_{5,42}$ = 11.02, p < 0.001). Tukey's post hoc analysis showed that Vehicle-treated CCI animals displayed a reduced number of arena crossings compared with the Vehicle/SHAM group (p < 0.001). In contrast, AIC treatment with the highest dose of CBD (60 nmol)/CCI significantly increased the number of crossings relative to the AIC Vehicle/CCI group (p < 0.01) (Fig. 4G).

3.1.6 Effects of CB_1 and 5-HT_{1A} Receptor Antagonism in the AIC on CBD-Induced Modulation of Exploratory and Anxiety-Related Behaviors

The effects of CB_1 and 5-HT_{1A} receptor antagonism on CBD-induced behavioral changes were evaluated using the open-field test. One-way ANOVA revealed a significant effect of treatment on time spent in the periphery of the arena ($F_{6,49}$= 28.03, p < 0.001). Tukey's post hoc test showed that (Vehicle + Vehicle) (AIC)/CCI animals spent more time in the periphery than Vehicle + Vehicle/SHAM animals (p < 0.001). In contrast, (Vehicle + CBD 60 nmol) (AIC)/CCI animals spent significantly less time in the periphery compared with (Vehicle + Vehicle) (AIC)/CCI animals (p < 0.001). This effect was abolished by AIC pretreatment with WAY-100635 or AM251, as rats submitted to AIC blockade of either 5-HT_{1A} or CB1 receptors + CBD spent significantly more time in the periphery of the open-field test arena than those treated with Vehicle + CBD at 60 nmol (AIC)/CCI group (p < 0.001) (Fig. 4H).

One-way ANOVA also revealed a significant effect of treatment on time spent in the center of the arena ($F_{6,49}$ = 79.51, p < 0.001). Tukey post hoc analysis of time spent in the center of the arena showed that AIC Vehicle + Vehicle/CCI animals spent less time in the center compared with AIC Vehicle + Vehicle/SHAM animals (p < 0.001). No significant differences were detected between Vehicle + CBD 60 nmol/CCI in comparison with Vehicle + Vehicle/CCI (p > 0.05); however, both AM251 (p < 0,01) and WAY-100635 (p < 0.05) antagonists decreased the duration in the center of the arena in comparison with Vehicle + CBD 60 nmol/CCI (Fig. 4I).

Regarding grooming behavior, one-way ANOVA revealed significant effects on frequency ($F_{6,49}$ = 28.14, p < 0.001) and duration ($F_{6,49}$ = 53.34, p < 0.001). AIC Vehicle + Vehicle/CCI animals displayed a lower frequency and shorter duration of grooming behavior compared with AIC Vehicle + Vehicle/SHAM animals (p < 0.001). AIC Vehicle + CBD 60 nmol/CCI animals showed increased frequency and duration of grooming relative to AIC Vehicle + Vehicle/CCI (p < 0.001). AIC pretreatment with either WAY-100635 or AM251 significantly reduced incidence and duration of self-cleaning compared with the AIC Vehicle + CBD 60 nmol/CCI group (p < 0.001) (Fig. 4J–K).

Regarding rearing behavior, one-way ANOVA revealed significant effects on frequency ($F_{6,49}$ = 12.49, p < 0.001) and duration ($F_{6,49}$ = 11.10, p < 0.001). AIC Vehicle + Vehicle/CCI animals showed reduced rearing frequency compared with AIC Vehicle + Vehicle/SHAM animals (p < 0.05). In addition, AIC Vehicle + WAY-100635/CCI and AIC Vehicle + AM251/CCI animals also exhibited reduced rearing frequency compared with Vehicle + Vehicle/CCI animals (p < 0.05). Moreover, AIC pretreatment with either WAY-100635 (p < 0.01) or AM251 (p < 0.001) further reduced the incidence of rearing in comparison to the AIC Vehicle +

CBD (60 nmol)/CCI-treated group (Fig. 4L). Rearing duration was also reduced in the AIC WAY-100635 + CBD group compared with AIC Vehicle + CBD (60 nmol)/CCI ($p < 0.05$) (Fig. 4M).

Concerning crossing behavior in the arena, one-way ANOVA revealed a significant effect of treatment ($F_{6,49} = 13.94$, $p < 0.001$). Tukey's post hoc analysis showed that AIC Vehicle-treated CCI animals displayed a reduced number of crossings compared with the AIC Vehicle/SHAM group ($p < 0.01$). In contrast, AIC Vehicle + CBD (60 nmol)/CCI-treated animals exhibited an increased number of crossings relative to the AIC Vehicle + Vehicle/CCI-treated group ($p < 0.05$). This effect was abolished by AIC pretreatment with either WAY-100635 or AM251, which significantly reduced the number of crossings compared with the AIC Vehicle + CBD (60 nmol)/CCI-treated group ($P < 0.001$) (Fig. 4N).

3.1.7 Effect of Intra-AIC CBD on Anxiety-Related Behavioral Comorbidity

Intra-AIC microinjection of cannabidiol (CBD; 15, 30, or 60 nmol) significantly altered anxiety-related behavioral parameters in chronic constriction injury (CCI) animals, as assessed using the spontaneous alternation elevated plus-maze test.

One-way ANOVA revealed significant differences between groups in both the frequency of entries into the open arms ($F_{5,42} = 27.57$, $p < 0.001$) and the duration spent in the open arms ($F_{5,42} = 112.6$, $p < 0.001$). Tukey's post hoc analysis showed that Vehicle (AIC)/CCI animals displayed a significantly lower frequency of open-arm entries compared with Vehicle (AIC)/SHAM animals ($p < 0.001$). CCI animals treated in the AIC with CBD at 15 ($p < 0.05$), 30 ($p < 0.05$), and 60 nmol ($p < 0.001$) showed increased open-arm entry frequency relative to Vehicle/CCI animals. Moreover, intra-AIC CBD at 60 nmol significantly increased open-arm entry frequency compared with the 15 ($p < 0.05$) and 30 nmol ($p < 0.01$) doses (Fig. 5A). Regarding duration spent in the open arms, Vehicle (AIC)/CCI animals spent significantly less time compared with Vehicle (AIC)/SHAM animals ($p < 0.001$). AIC treatment with CBD at 30 and 60 nmol in CCI animals nmolinduced significantly much time spent in the open arms compared with AIC Vehicle/CCI animals ($p < 0.001$) (Fig. 5B). Intra-AIC CBD at 60 nmol/CCI significantly increased the time spent in the open arms compared with CBD at 15 ($p < 0.001$) and 30 nmol/CCI ($p < 0.05$). In addition, CBD at 30 nmol microinjected in the AIC also increased time spent in the open-arms duration relative to intra-AIC CBD at 15 nmol treatment ($p < 0.001$) (Fig. 5B).

No significant differences were observed among groups in frequency of the flat-back approach ($F_{5,42} = 1.23$, $p > 0.05$) in the elevated T-maze test. However, there was a significant difference in the duration of flat-back approach ($F_{5,42} = 4.75$, $p < 0.01$), according to the one-way ANOVA (Fig. 5C–D). According to Tukey's post hoc test, AIC Vehicle/CCI-treated animals did not differ in flat-back approaches duration in comparison to AIC Vehicle/SHAM animals ($p > 0.05$). In contrast, CCI animals treated with CBD at 60 nmol (AIC)/CCI exhibited a significant increase in duration of flat-back approaches compared with Vehicle (AIC)/CCI-treated animals ($p < 0.01$) (Fig. 5D).

Considering closed-arm parameters, one-way ANOVA revealed significant effects on both incidence of entries ($F_{5,42}$ = 34.35, $p < 0.001$) and time spent in the closed arms ($F_{5,42}$ = 282.7, $p < 0.001$). Vehicle (AIC)/CCI-treated animals exhibited higher frequency and duration of permanence in the closed arms compared with Vehicle (AIC)/SHAM animals ($p < 0.001$). In contrast, CCI animals treated with CBD at 60 nmol microinjections in the AIC showed a significant decrease in both closed-arm entry frequency ($p < 0.01$) and duration of permanence ($p < 0.001$) in comparison to Vehicle (AIC)/CCI animals. Notably, CBD at 30 nmol, when microinjected into the AIC, also significantly reduced time spent in closed-arms compared with Vehicle(AIC)/CCI animals ($p < 0.05$). AIC CBD at 60 nmol significantly reduced both closed-arm frequency of entries and time spent compared with the treatment of the AIC with CBD at 15 and 30 nmol ($p < 0.001$) (Fig. 5E–F).

3.1.8 Effects of CB_1 and 5-HT_{1A} Receptor Antagonism in the AIC on CBD-Induced Modulation of Anxiety-Related Behavior

The involvement of CB_1 and 5-HT_{1A} receptors in CBD-induced behavioral changes was examined using intra-AIC pretreatment with either AM251 or WAY-100635 in the elevated plus-maze test.

One-way ANOVA revealed significant differences among groups in the frequency of entries into the open arms ($F_{6,49}$ = 752.46, $p < 0.001$) and in the time spent in the open arms ($F_{6,49}$ = 297.9, $p < 0.001$). Tukey's post hoc analysis showed that (Vehicle + Vehicle) (AIC)/CCI animals entered the open arms less frequently into the open arms than (Vehicle + Vehicle) (AIC)/SHAM animals ($p < 0.001$). Vehicle + CBD 60 nmol (AIC)/CCI animals displayed a significantly higher frequency of open-arm entries compared with (Vehicle + Vehicle) (AIC)/CCI animals ($p < 0.001$). This increase was no longer observed following AIC pretreatment with either WAY-100635 or AM251, as both antagonist + CBD-treated groups showed reduced open-arm entry frequency in comparison with Vehicle + CBD at 60 nmol (AIC)/CCI-treated group ($p < 0.001$) (Fig. 5G). Similarly, (Vehicle + Vehicle) (AIC)/CCI-treated animals spent less time in the open arms compared with (Vehicle + Vehicle) (AIC)/SHAM-treated animals ($p < 0.001$). (Vehicle + CBD at 60 nmol) (AIC)/CCI animals spent significantly more time in the open arms in comparison to (Vehicle + Vehicle) (AIC)/CCI-treated animals ($p < 0.001$). AIC pretreatment with either WAY-100635 ($p < 0.05$) or AM251 ($p < 0.001$) significantly reduced time spent in the open arms compared with the (Vehicle + CBD at 60 nmol) (AIC)/CCI-treated group (Fig. 5H).

In the study assessing the effects of intra-AIC pretreatment with either WAY-100635 or AM251 followed by CBD on anxiety-related responses displayed by rats in the elevated plus-maze test, no significant differences were observed among groups in the frequency of the flat-back approach ($F_{6,49}$ = 1.43, $p > 0.05$). In addition, although one-way ANOVA revealed a significant effect of treatment regarding the duration of the flat-back approach ($F_{6,49}$ = 6.25, $p < 0.01$) (Fig. 5), Tukey's post hoc analysis indicated that neither CBD nor treatment with each selective antagonist differed from their respective vehicle-treated CCI groups, indicating no effect of CBD or the antagonists on this parameter. Vehicle (AIC)/CCI-treated animals differed only from Vehicle (AIC)/SHAM animals in flat-back approach duration ($p < 0.05$) (Fig. 5I-J).

Analysis of closed-arm behavior revealed significant differences in both frequency of entries ($F_{6,49}$ = 16.66, $p < 0.001$) and time spent in the closed arms ($F_{6,49}$ = 362.3, $p < 0.001$). (Vehicle + Vehicle) (AIC)/CCI-treated animals exhibited higher closed-arm frequency of entries compared with (Vehicle + Vehicle) (AIC)/SHAM-treated animals ($p < 0.01$). (Vehicle + CBD at 60 nmol) (AIC)/CCI-treated animals showed a significant decrease in closed-arm entries in comparison to (Vehicle + Vehicle) (AIC)/CCI animals ($p < 0.01$). This effect was attenuated by AIC pretreatment with WAY-100635 ($p < 0.01$) (Fig. 5K). For the time spent in the closed arms, (Vehicle + Vehicle) (AIC)/CCI-treated animals displayed higher values than (Vehicle + Vehicle) (AIC)/SHAM-treated rats ($p < 0.001$), whereas (Vehicle + CBD at 60 nmol) (AIC)/CCI-treated animals showed a significantly reduced closed-arm duration of permanence compared with (Vehicle + Vehicle) (AIC)/CCI animals ($p < 0.001$). Both selective antagonist + CBD (AIC)-treated groups exhibited increased time spent in closed-arms in comparison to (Vehicle + CBD at 60 nmol) (AIC)/CCI animals ($p < 0.001$) (Fig. 5L).

3.1.9 Effect of Intra-AIC CBD on Depression-Like Behaviors (anhedonia)

Intra-AIC microinjection of cannabidiol at different doses (15, 30, or 60 nmol) significantly attenuated anhedonia-like behavior in CCI animals, as assessed by the sucrose spray test.

One-way ANOVA revealed significant effects of the treatment on the latency of grooming behavior ($F_{5,42}$ = 74.51, $p < 0.001$). Tukey's post hoc analysis indicated that Vehicle (AIC)/CCI-treated animals exhibited a significantly longer latency of grooming compared to Vehicle (AIC)/SHAM treatment ($p < 0.05$). All CBD (15, 30, and 60 nmol) (AIC)/CCI-treated groups showed a significant decrease in latency of grooming when compared to Vehicle(AIC)/CCI treatment ($p < 0.01$, $p < 0.001$, and $p < 0.001$, respectively). Moreover, CBD at 30 and 60 nmol significantly decreased grooming latency compared with the treatment with CBD in a dose of 15 nmol ($p < 0.01$) (Fig. 6A).

Similarly, the frequency ($F_{5,42}$ = 34.36, $p < 0.001$) and duration ($F_{5,42}$ = 112.0, $p < 0.001$) of nose-grooming differed significantly among groups. According to Tukey's post hoc test, Vehicle (AIC)/CCI treatment decreased both frequency and duration of nose-grooming behavior compared with Vehicle (AIC)/SHAM treatment ($p < 0.001$). AIC CBD at 60 nmol/CCI treatment significantly increased grooming frequency compared with Vehicle (AIC)/CCI-treated animals ($p < 0.001$). In contrast, Microinjections of CBD at 15, 30, and 60 nmol in AIC significantly increased the duration of nose grooming as compared to Vehicle (AIC)/CCI-treated animals ($p < 0.05$) (Fig. 6B–C). Regarding nose grooming, treatment of AIC with CBD at 60 nmol significantly increased grooming frequency compared with the CBD at 15 and 30 nmol ($p < 0.001$). In addition, AIC CBD at 60 nmol also increased the duration of nose grooming in comparison to CBD at 15 and 30 nmol ($p < 0.001$). Notably, CBD at 15 nmol when microinjected into the AIC significantly increased nose grooming duration compared with the intermediate (30 nmol) dose ($p < 0.001$) (Fig. 6B–C).

According to a one-way ANOVA, the frequency ($F_{5,42}$ = 10.82, p < 0.001) and duration ($F_{5,42}$ = 15.31, p < 0.001) of head-grooming differed significantly among groups. Vehicle (AIC)/CCI-treated animals showed reduced frequency and duration of the head grooming behavior compared to Vehicle (AIC)/SHAM treatment (p < 0.001). AIC CBD at 60 nmol treatment increased head grooming frequency (p < 0.001), while all CBD doses (15, 30, and 60 nmol) when microinjected into the AIC significantly increased head grooming duration compared to Vehicle (AIC)/CCI treatment (Tukey's post hoc test; p < 0.001, p < 0.01, and p < 0.001, respectively) (Fig. 6D–E).

According to a one-way ANOVA, both frequency ($F_{5,42}$ = 33.37, p < 0.001) and duration ($F_{5,42}$ = 12.33, p < 0.001) of body grooming differed significantly among groups. Tukey's post hoc analysis revealed that Vehicle (AIC)/ /CCI-treated animals exhibited reduced body-grooming frequency and duration compared with Vehicle (AIC)/SHAM-treated animals (p < 0.001 and p < 0.01, respectively). In contrast, CBD at 60 nmol microinjections into the AIC significantly increased both body-grooming frequency and duration compared with Vehicle (AIC)/CCI treatment (p < 0.001). Moreover, AIC treatment with CBD at 60 nmol increased body-grooming frequency compared with the CBD 30 nmol (AIC)/CCI-treated group (p < 0.001) and increased body-grooming duration compared with the AIC treatment with CBD at 15 nmol (p < 0.01) and 30 nmol/CCI (p < 0.001) (Fig. 6F–G).

Consistently with these findings, total grooming behavior differed significantly among groups in both frequency ($F_{5,42}$ = 43.54, p < 0.001) and duration ($F_{5,42}$ = 36.10, p < 0.001) in accordance with one-way ANOVA. Tukey's post hoc analysis revealed that Vehicle (AIC)/CCI-treated animals exhibited reduced total grooming frequency and duration compared with Vehicle (AIC)/SHAM-treated animals (p < 0.001). Treatment of the AIC with CBD at 30 and 60 nmol significantly increased total grooming frequency compared with Vehicle (AIC)/CCI-treated animals (p < 0.001). In addition, AIC treatment with CBD at all doses in rats submitted to the CCI procedure caused a significant increase in total grooming duration in comparison to Vehicle (AIC)/CCI-treated animals (p < 0.001). Microinjections of CBD at 30 and 60 nmol in AIC significantly increased total grooming frequency compared with CBD at 15 nmol in CCI animals (p < 0.001). In addition, AIC CBD at 60 nmol significantly increased total grooming duration relative to the lower (15 nmol) dose in CCI animals (p < 0.001) (Fig. 6H-I).

3.1.10 Effects of CB_1 and 5-HT_{1A} Receptor Blockade in the AIC on CBD-Induced Antidepressant-Like Effects (anhedonia)

Pretreatment of AIC with either the CB_1 receptor antagonist AM251 or the 5-HT_{1A} receptor antagonist WAY-100635 prevented the AIC CBD-induced attenuation of anhedonia-like behavior in the sucrose spray test.

One-way ANOVA revealed significant group differences in latency of grooming ($F_{6,49}$ = 69.41, p < 0.001). (Vehicle + Vehicle) (AIC)/CCI-treated animals showed longer latencies than (Vehicle + Vehicle) (AIC)/SHAM-treated animals (p < 0.001), whereas Vehicle + CBD at 60 nmol (AIC)/CCI-treated animals

showed reduced latency compared to (Vehicle + Vehicle) (AIC)/CCI ($p < 0.001$). This effect was abolished by AIC pretreatment with AM251, which significantly increased latency relative to Vehicle + CBD 60 nmol (AIC)/CCI treatment ($p < 0.001$) (Fig. 6J).

One-way ANOVA showed significant differences in the frequency ($F_{6,49} = 21.76$, $p < 0.001$) and duration ($F_{6,49} = 248.9$, $p < 0.001$) of the nose-grooming behavior. Tukey's post hoc analysis showed that (Vehicle + Vehicle) (AIC)/CCI-treated animals decreased the frequency and duration of the nose grooming behavior compared to (Vehicle + Vehicle) (AIC)/SHAM treatment ($p < 0.001$). CBD at 60 nmol microinjections into the AIC increased both measures ($p < 0.001$), whereas AIC pretreatment with either WAY-100635 or AM251 significantly reduced these effects compared to Vehicle + CBD 60 nmol (AIC)/CCI treatment ($p < 0.001$) (Fig. 6L-K).

Head grooming behavior frequency ($F_{6,49} = 30.54$, $p < 0.001$) and duration ($F_{6,49} = 199.3$, $p < 0.001$) also differed significantly between groups, as determined by a one-way ANOVA. Tukey's post hoc analysis revealed that (Vehicle + Vehicle) (AIC)/CCI –treated animals decreased the frequency and duration of the head grooming behavior compared to (Vehicle + Vehicle) (AIC)/SHAM treatment ($p < 0.001$). AIC treatment with Vehicle + CBD at 60 nmol followed by CCI procedure increased both measures as compared to (Vehicle + Vehicle) (AIC)/CCI treatment ($p < 0.001$), whereas pretreatment with either WAY-100635 or AM251 reduced these parameters compared to Vehicle + CBD 60 nmol (AIC)/CCI treatment ($p < 0.001$) (Fig. 6L-M).

Similarly, body grooming behavior frequency ($F_{6,49} = 50.66$, $p < 0.001$) and duration ($F_{6,49} = 322.4$, $p < 0.001$) showed significant group effects in accordance with one-way ANOVA. Tukey's post hoc analysis revealed that (Vehicle + Vehicle) (AIC)/CCI-treated animals decreased the frequency and duration of the body grooming behavior compared to (Vehicle + Vehicle) (AIC)/SHAM treatment ($p < 0.001$). Microinjections of CBD into the AIC increased both parameters in CCI animals ($p < 0.001$), and these effects were abolished by AIC pretreatment with either antagonist ($p < 0.001$) (Fig. 6N-O).

According to a one-way ANOVA, the frequency ($F_{6,49} = 113.7$, $p < 0.001$) and duration ($F_{6,49} = 181.9$, $p < 0.001$) of total grooming were significantly different among groups. (Vehicle + Vehicle) (AIC)/CCI-treated animals displayed a significantly reduced total grooming duration compared to (Vehicle + Vehicle) (AIC)/SHAM treatment ($p < 0.001$). Treatment of AIC with Vehicle + CBD at 60 nmol increased total grooming relative to (Vehicle + Vehicle) (AIC)/CCI treatment ($p < 0.001$). AIC pretreatment with either WAY-100635 or AM251, followed by local administration of CBD, significantly reduced the frequency and duration of total grooming compared to Vehicle + CBD 60 nmol (AIC)/CCI ($p < 0.001$) (Fig. 6P-Q).

3.1.11 Effect of Intra-AIC CBD on Depression-Like Behaviors (immobility and mobility)

Intra-AIC microinjection of CBD significantly reduced immobility and increased mobility in the forced swim test. Regarding the behavior in which the animal remains floating on the water, according to the

one-way ANOVA, there was a significant effect of the treatment on frequency ($F_{5,42}$ = 43.44, $p < 0.001$) and duration ($F_{5,42}$= 17.32, $p < 0.001$) of floating behavior. Tukey's post hoc analysis showed that, regarding the frequency of floating behavior, Vehicle (AIC)/CCI-treated animals exhibited a significantly higher frequency compared with Vehicle (AIC)/SHAM treatment ($p < 0.001$). In contrast, Vehicle (AIC)/CCI animals displayed a significantly reduced duration of floating behavior relative to Vehicle (AIC)/SHAM animals ($p < 0.05$). The AIC treatment with ABD at different doses (15, 30, and 60 nmol) exhibited a lower frequency of floating compared to the Vehicle (AIC)/CCI-treated group ($p < 0.001$) (Fig. 7A). The CBD 60 nmol (AIC)/CCI-treated group showed an increased duration of floating time when compared to the Vehicle(AIC)/CCI treatment ($p < 0.001$) (Fig. 7B). AIC microinjections of CBD at 60 nmol in CCI animals significantly decreased the frequency of floating behavior compared with CBD at 30 nmol ($p < 0.001$). AIC treatment with CBD at 60 nmol significantly increased the duration of floating behavior compared with CBD at 15 and 30 nmol ($p < 0.001$) (Fig. 7B).

Regarding freezing behavior, according to a one-way ANOVA, there was a significant effect of the treatment on frequency ($F_{5,42}$ = 7.0, $p < 0.001$) and duration ($F_{5,42}$= 17.32, $p < 0.001$) of freezing. Tukey's post hoc test showed that, in terms of frequency and duration of freezing, the Vehicle (AIC)/CCI-treated group had a higher value than the Vehicle (AIC)/SHAM group ($p < 0.001$). The CBD 60 nmol (AIC)/CCI group had a decrease in the frequency when compared to the Vehicle (AIC)/CCI group ($p < 0.01$) (Fig. 7C). Freezing duration in the CBD 60 nmol (AIC)/CCI group was increased when compared to the Vehicle (AIC)/CCI group ($p < 0.001$) (Fig. 7D).

Regarding swimming behavior, a one-way analysis of variance (ANOVA) revealed significant differences in frequency ($F_{5,42}$ = 38.28, $p < 0.001$) and duration ($F_{5,42}$= 51.50, $p < 0.001$). Concerning swimming frequency and duration, Tukey's post hoc test showed that the Vehicle (AIC)/CCI group showed a decreasing response as compared to the Vehicle (AIC)/SHAM-treated group ($p < 0.001$). The groups treated in the AIC of CCI animals with CBD at 30 nmol ($p < 0.05$) and at 60 nmol ($p < 0.001$) showed a higher frequency and duration of swimming when compared to the Vehicle (AIC)/CCI-treated group (Fig. 7G). Rats treated in the AIC with CBD at 60 nmol submitted to the CCI procedure showed a significant increase in swimming when compared to the AIC treatment with CBD at 15 and 30 nmol in the CCI group ($p < 0.001$) (Fig. 7H). AIC treatment with CBD at 30 nmol significantly increased both the frequency ($p < 0.05$) and duration ($p < 0.001$) of swimming behavior compared with AIC treatment with CBD at 15 nmol (Fig. 7H).

In addition, regarding the climbing behavior, one-way ANOVA revealed significant differences in frequency ($F_{5,42}$ = 17.32, $p < 0.001$) and duration ($F_{5,42}$ = 23.69, $p < 0.001$) of that response. Tukey's post hoc analysis showed that Vehicle (AIC)/CCI-treated animals exhibited a significant reduction in both climbing behavior frequency and duration compared with Vehicle (AIC)/SHAM treatment ($p < 0.001$). CCI animals treated in the AIC with CBD at the highest dose (60 nmol) showed significantly increased climbing behavioral response frequency and duration compared with Vehicle (AIC)/CCI animals ($p < 0.001$) (Fig. 7I-J). In addition, CBD at 30 nmol when microinjected in the IAC of CCI procedure-treated rats significantly increased climbing behavior duration as compared to Vehicle (AIC)/CCI-treated animals

($p < 0.001$) (Fig. 8D). Moreover, AIC treatment with CBD at 60 nmol significantly increased both climbing behavior frequency and duration compared with the AIC treatment with CBD at lower doses (15 and 30 nmol) in CCI animals ($p < 0.001$). Finally, AIC microinjection of CBD at 30 nmol in CCI procedure-treated rats significantly increased both climbing behavior frequency ($p < 0.05$) and duration ($p < 0.001$) compared with the CBD at the lowest dose (15 nmol) (Fig. 7I-J).

In the assessment of total mobility in this test, one-way ANOVA revealed significant group differences in both frequency ($F_{5,42} = 35.62$, $p < 0.001$) and duration ($F_{5,42} = 157.2$, $p < 0.001$). Tukey's post hoc analysis showed that Vehicle (AIC)/CCI animals exhibited reduced total mobility frequency and duration compared with Vehicle (AIC)/SHAM rats ($p < 0.001$). In contrast, CCI animals treated in the AIC with CBD at 60 nmol showed a significantly higher frequency of total mobility compared with Vehicle (AIC)/CCI animals ($p < 0.001$) (Fig. 7K-L). Moreover, treatment of the AIC with ABD at 15, 30, and 60 nmol significantly increased the duration of total mobility relative to Vehicle (AIC)/CCI-treated animals ($p < 0.001$) (Fig. 7E–F). Regarding dose-dependent effects, CBD at 60 nmol when microinjected into the AIC significantly increased total mobility frequency compared with AIC treatment with CBD at lower (15 and 30 nmol) doses ($p < 0.001$). In addition, AIC treatment with CBD at 30 and 60 nmol significantly increased total mobility duration compared with CBD at the lowest (15 nmol) dose ($p < 0.001$) (Fig. 7K-L).

3.1.12 Effects of CB_1 and 5-HT_{1A} Receptor Blockade in the AIC on CBD-Induced Antidepressant-Like Effects (immobility and mobility)

Intra-AIC microinjection of CBD significantly reduced immobility and increased mobility in the forced swim test, and these effects were assessed under pharmacological blockade of CB_1 and 5-HT_{1A} receptors.

Concerning floating behavior, according to a one-way ANOVA, there were significant effects of treatments on both frequency ($F_{6,49} = 16.26$, $p < 0.001$) and duration ($F_{6,49} = 7.52$, $p < 0.001$) of floating responses. Tukey's post hoc analysis showed that Vehicle (AIC)/CCI-treated animals exhibited a significantly higher frequency and longer duration of floating behavior compared with Vehicle (AIC)/SHAM-treated animals ($p < 0.001$). Intra-AIC administration of CBD at 60 nmol in CCI animals significantly reduced both the frequency and duration of floating behavior compared with the Vehicle (AIC)/CCI-treated group ($p < 0.05$). AIC pretreatment with either antagonist reversed the CBD-induced reduction in floating frequency, increasing this response in comparison to Vehicle (AIC)/CCI-treated group ($p < 0.001$). However, regarding the duration of floating behavior, only AIC pretreatment with AM251 significantly reversed the effect of intracerebral microinjections of CBD, increasing the duration compared with the Vehicle (AIC)/CCI-treated group ($p < 0.05$) (Fig. 7M-N).

Regarding the immobility displayed by rats in the forced swimming test, one-way ANOVA revealed no significant differences in the frequency ($F_{6,49} = 2.62$, $p > 0.05$) and duration ($F_{6,49} = 1.96$, $p > 0.05$) of immobility between groups (Fig. 7O-P).

In addition, total immobility behaviors were analyzed, and one-way ANOVA revealed significant group differences in both frequency ($F_{6,49}$ = 15.41, $p < 0.001$) and duration ($F_{6,49}$ = 24.99, $p < 0.001$) of that response. Tukey's post hoc analysis showed that the (Vehicle + Vehicle) (AIC)/CCI-treated group exhibited a significantly higher frequency and duration of total immobility compared with the (Vehicle + Vehicle) (AIC)/SHAM-treated group ($p < 0.001$). In contrast, intra-AIC administration of CBD at 60 nmol significantly reduced both the frequency and duration of total immobility as compared to the (Vehicle + Vehicle) (AIC)/CCI-treated group ($p < 0.001$). Notably, AIC pretreatment with either WAY-100635 or AM251 completely reversed the CBD (AIC)-induced effects, as the (WAY-100635 + CBD 60 nmol) (AIC)/CCI- and (AM251 + CBD 60 nmol) (AIC)/CCI-treated groups showed significantly higher frequency and duration of total immobility compared with the (Vehicle + CBD 60 nmol) (AIC)/CCI treatment ($p < 0.001$) (Fig. 7Q-R).

Regarding swimming behavior, one-way ANOVA revealed significant group differences in both frequency ($F_{6,49}$ = 60.98, $p < 0.001$) and duration ($F_{6,49}$ = 61.00, $p < 0.001$) of that response. Tukey's post hoc analysis showed that the (Vehicle + Vehicle) (AIC)/CCI-treated group exhibited significantly lower swimming frequency and duration compared with the (Vehicle + Vehicle) (AIC)/SHAM-treated group ($p < 0.001$). In contrast, intra-AIC administration of CBD at 60 nmol significantly increased both the frequency and duration of swimming behavior in comparison with (Vehicle + Vehicle) (AIC)/CCI-treated group ($p < 0.001$). Notably, AIC pretreatment with either WAY-100635 or AM251 significantly reversed the CBD (AIC)-induced effects, as the (WAY-100635 + CBD 60 nmol) (AIC)/CCI- and (AM251 + CBD 60 nmol) (AIC)/CCI-treated groups showed a significant reduction in swimming frequency and duration compared with the (Vehicle + CBD 60 nmol) (AIC)/CCI-treatment (Tukey's post hoc test; $p < 0.01$ and 0.001, respectively), as shown in Fig. 7S-T.

With respect to climbing behavior, one-way ANOVA revealed significant treatment effects on both frequency ($F_{6,49}$ = 82.92, $p < 0.001$) and duration ($F_{6,49}$ = 54.11, $p < 0.001$) of climbing. Post hoc comparisons using Tukey's test indicated that CCI animals receiving vehicle into the AIC exhibited a marked reduction in climbing frequency and duration compared with the (Vehicle + Vehicle) (AIC)/SHAM-treated group ($p < 0.001$). Administration of CBD (60 nmol) into the AIC effectively counteracted this impairment, significantly increasing both parameters relative to (Vehicle + Vehicle) (AIC)/CCI-treated animals ($p < 0.001$). Importantly, blockade of either CB_1 or 5-HT_{1A} receptors into the AIC abolished the facilitatory effects of CBD administered in the same cortical structure, as the AIC pretreatment with either WAY-100635 ($p < 0.01$) or AM251 ($p < 0.001$) significantly reduced climbing frequency and duration compared with the (Vehicle + CBD at 60 nmol) (AIC)/CCI-treated group (Fig. 7U-V).

Regarding total mobility in the forced swim test, one-way ANOVA demonstrated a significant effect of treatment on both the frequency ($F_{6,49}$ = 112.9, $p < 0.001$) and duration ($F_{6,49}$ = 244.7, $p < 0.001$) of this behavior. Tukey's post hoc analysis revealed that CCI animals treated with vehicle into the AIC showed a pronounced reduction in total mobility compared with (Vehicle + Vehicle) (AIC)/SHAM-treated animals ($p < 0.001$). Intra-AIC administration of CBD at the highest dose (60 nmol) significantly restored total mobility, increasing both frequency and duration in comparison to the (Vehicle + Vehicle) (AIC)/CCI-

treated group ($p < 0.001$). Notably, pharmacological blockade of CB_1 or 5-HT_{1A} receptors in the AIC prevented the CBD (AIC)-induced enhancement of total mobility, as the AIC pretreatment with either WAY-100635 ($p < 0.01$) or AM251 ($p < 0.001$) resulted in significantly lower values compared with the (Vehicle + CBD at 60 nmol) (AIC)/CCI-treated group (Fig. 7W-X).

4 Discussion

This study demonstrates that site-specific administration of cannabidiol into the AIC produces robust antinociceptive effects and attenuates anxiety- and depression-like behaviors in a rat model of NP. Importantly, these effects were abolished by local blockade of CB_1 and 5-HT_{1A} receptors, indicating that CBD actions within the AIC depend on cannabinoid and serotonergic receptor-specific mechanisms. Together, these findings identify the AIC as a critical neocortical substrate through which CBD exerts integrated modulation of nociceptive and affective dimensions of chronic and NP conditions.

The AIC has emerged as a central hub for encoding pain salience and emotional relevance, rather than merely processing nociceptive input (Craig, 2009; Garcia-Larrea & Peyron, 2013). Our findings extend this framework by providing direct causal evidence that pharmacological modulation of the AIC is sufficient to alleviate both pain hypersensitivity and emotional disturbances in NP.

The AIC is anatomically and functionally positioned to integrate nociceptive information with affective and motivational states through its extensive connectivity with the prefrontal cortex, amygdala, anterior cingulate cortex, and brainstem nuclei involved in autonomic and emotional regulation (Frot et al., 2014; Klein et al., 2021). Dysregulation of AIC activity has been associated with heightened emotional reactivity, anxiety, and depressive symptoms (Paulus & Stein, 2010; Kroemer et al., 2022; Nicolas et al., 2023), all of which commonly accompany chronic pain conditions. By demonstrating that intra-AIC CBD simultaneously reduces pain and affective-like behaviors, our data support the view that maladaptive AIC plasticity constitutes a key neurobiological link between persistent nociception and emotional dysfunction.

CBD has been shown to exert antinociceptive, anxiolytic, and antidepressant-like effects through interactions with multiple molecular targets, including CB_1 and 5-HT_{1A} receptors (de Gregorio et al., 2019; Campos et al., 2012; Mlost et al., 2020; Maccarrone et al., 2023). Preclinical studies have shown that, although CBD is an inverse agonist of CB_1 receptors (Ledgerwood et al., British Journal of Pharmacology 2010), CBD-induced analgesia is reversed or reduced by CB_1 receptor antagonists. Indeed, the most studied CB_1 receptor antagonists, such as AM251 or SR141716A, have been shown to block the analgesic effects of CBD. This apparent mechanistic discrepancy is explained by the fact that CBD can produce analgesia indirectly by increasing the levels of endocannabinoids such as AEA, PEA, OEA, and other acylethanolamines, blocking FAAH metabolism and thus triggering greater endogenous activation of CB1 receptors (Nicoara et al, Cells 2025). This mechanism explains why CB_1 receptor antagonists can block the effects of CBD, a finding also confirmed by the present study.

Therefore, although CBD is not a direct agonist of the CB_1 receptor, its analgesic action is often based on modulation of the CB_1 receptor system, making it susceptible to being reversed by CB_1 receptor antagonists. Accordingly, some of our previous studies have shown that CBD modulates chronic neuropathic pain and associated emotional behaviors by also indirectly involving CB_1 receptors in the prelimbic cortex and hippocampus (Malvestio et al., 2021; Medeiros et al., 2023). This study extends these findings by identifying the AIC as an additional cortical site where CBD exerts both CB_1 and 5-HT_{1A} receptor-dependent effects, highlighting a broader role for cortical psychopharmacology in chronic pain.

The involvement of CB_1 receptors in the effects observed here is consistent with their established role in modulating synaptic transmission, neuronal excitability, and pain processing within cortical circuits (Russo et al., 2005). In the present study, indirect activation of CB_1 receptors, which we hypothesize is due to an endogenous enhancement of endocannabinoid tone via FAAH blockade, could reduce excitatory neurotransmission in the AIC, thereby attenuating altered pain salience and emotional reactivity. Furthermore, the role of 5-HT_{1A} receptors is consistent with evidence demonstrating that CBD can act as a modulator of serotonergic transmission in the CNS, contributing to CBD's anxiolytic, antipanic, and antidepressant effects (Twardowschy et al., 2013; Rock et al., 2012; Campos & Guimarães, 2008; dos Santos Sampaio et al., 2024). The convergence of CB_1- and 5-HT_{1A}-dependent mechanisms within the AIC suggests that CBD engages complementary neuromodulatory systems to restore functional balance in pain-related cortical networks.

The ability of CBD to simultaneously attenuate nociceptive hypersensitivity and affective-like behaviors is particularly relevant given the bidirectional relationship between chronic pain and emotional disorders. Anxiety and depression not only emerge as consequences of persistent pain but also exacerbate pain perception and promote chronification (Michaelides & Zis, 2019; Wong et al., 2024). By targeting a cortical region that integrates these dimensions, CBD may interrupt this vicious cycle, offering a mechanistically grounded approach to the treatment of neuropathic pain with psychiatric comorbidities.

Some limitations of the present study should be acknowledged. First, the use of local microinjection restricts conclusions to region-specific mechanisms and does not directly address the systemic pharmacokinetics of CBD. Second, only male animals were evaluated, and sex-dependent differences in insular function and cannabinoid signaling warrant further investigation. Furthermore, the lack of analysis of endocannabinoid levels in the presence of CBD in the AIC allows us only to hypothesize an increase in endocannabinoid levels, although this possibility has already been previously demonstrated by others in other areas of the CNS. Finally, although the behavioral outcomes strongly support an integrated modulation of pain and affect, future studies combining electrophysiological or neuroimaging approaches would further elucidate how CBD reshapes AIC network activity.

Despite these limitations, our findings provide compelling evidence that the AIC represents a critical cortical target for the psychopharmacological actions of CBD in chronic NP. By elucidating receptor-dependent mechanisms within this region, this study advances the understanding of cortical

contributions to pain versus emotion interactions and opens new avenues for region-specific therapeutic strategies.

5 Conclusion

In conclusion, this study demonstrates that CBD microinjection into the AIC attenuates mechanical and cold allodynia while also reducing anxiety- and depression-like behaviors in an experimental model of neuropathic pain. These effects are consistent with the involvement of intracortical CB_1 and 5-HT_{1A} receptor signaling, supporting a receptor-related neocortical mechanism underlying the integrated modulation of nociceptive and affective processes. However, the present findings do not establish whether CBD acts through increased endocannabinoids and, therefore, indirectly enhances the stimulation of CB1 receptors in the AIC, nor do they distinguish between the potential contributions of pyramidal neurons, interneurons, or afferent inputs. Overall, these findings identify the AIC as a relevant neurobiological substrate linking chronic pain and emotional comorbidities and support CBD as a promising psychopharmacological candidate for neuropathic pain conditions associated with affective dysfunction.

Declarations

Graphical Abstract

Schematic representation of the experimental design and main findings demonstrating that intra-anterior insular cortex (AIC) administration of cannabidiol (CBD) alleviates chronic neuropathic pain (NP) and attenuates anxiety- and depression-like behaviors. Microinjection of CBD into the AIC reversed mechanical and cold allodynia and improved affective outcomes in rats subjected to chronic constriction injury (CCI) of the ischiadicus nervus. These effects were abolished by pretreatment with CB_1 or 5-HT_{1A} receptor antagonists, indicating that the antinociceptive and anxiolytic/antidepressant-like actions of CBD depend on CB_1 and 5-HT_{1A} receptor signaling within the AIC.

Author Contribution

R.M.A., P.M., and R.L.F. conceptualized and designed the study. R.M.A. performed the experiments and collected the data. R.M.A., NB, and B.R.R.D. conducted the behavioral analyses. R.L.F. supervised the project and provided critical intellectual input. R.M.A., B.R.R.D., NB, PM, and R.L.F. analyzed and interpreted the data. R.M.A., NB, and B.R.R.D. and R.L.F. wrote the main manuscript text. J.E.C.H., N.C.C., and S.M. contributed to the interpretation of the data and provided critical revisions of the manuscript. All authors reviewed, edited, and approved the final version of the manuscript.

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Data Availability

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

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Figures

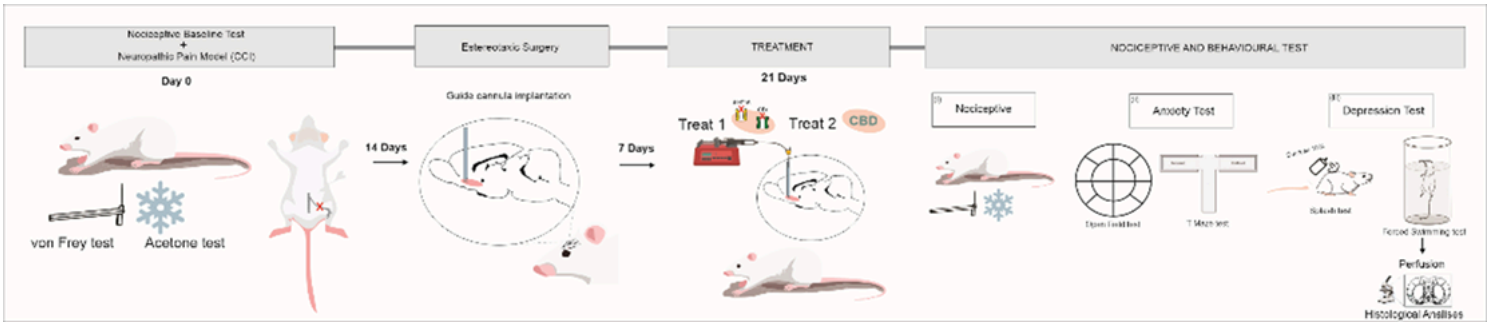

Figure 1

Experimental design and timeline: Schematic representation of the experimental design illustrating the timeline of the study. Rats underwent stereotaxic implantation of guide cannulas targeting the anterior insular cortex (AIC), followed by a post-surgical recovery period. Chronic neuropathic pain was induced by of the ischiadicus nervus chronic constriction injury (CCI) or SHAM surgery. On the experimental day (21 days post-surgery), animals received intra-AIC microinjections of cannabidiol (CBD), alone or preceded by local administration of CB_1 (AM251) or 5-HT_{1A} (WAY-100635) receptor antagonists. Behavioral and nociceptive assessments were conducted at defined time points following microinjection, and brains were subsequently collected for histological verification of injection sites.

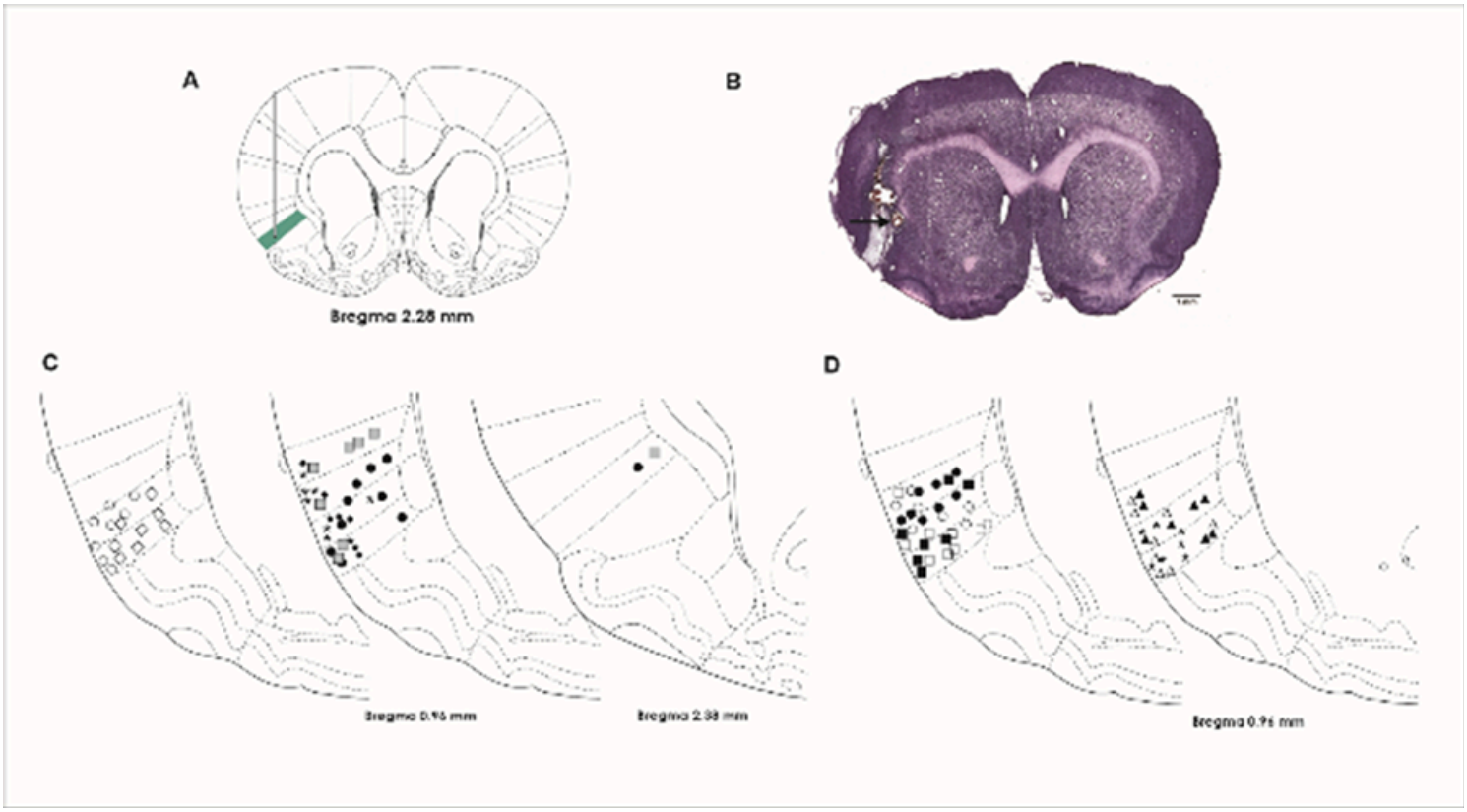

Figure 2

Histological verification of intra-AIC microinjection sites: (A) Schematic representation of microinjection sites for cannabidiol (CBD) and the selective antagonists WAY-100635 100635 and

AM251 in the anterior insular cortex (AIC). (B) Representative photomicrograph (H&E staining) showing cannula placement within the AIC. (C–D) Distribution of histologically verified microinjection sites according to the Paxinos and Watson atlas (2017): (○) SHAM/Vehicle, (●) SHAM/CBD 60 nmol, (□) CCI/Vehicle, (■) CCI/CBD 15 pmol, (◊) CCI/CBD 30 nmol, (*) CCI/CBD 60 nmol, (△) WAY-100635 /Vehicle (CCI), (▽) AM251/Vehicle (CCI), (⊠) Vehicle/CBD 60 nmol (CCI), (▲) WAY-100635 /CBD 60 nmol (CCI), (▼) AM251/CBD 60 nmol (CCI).

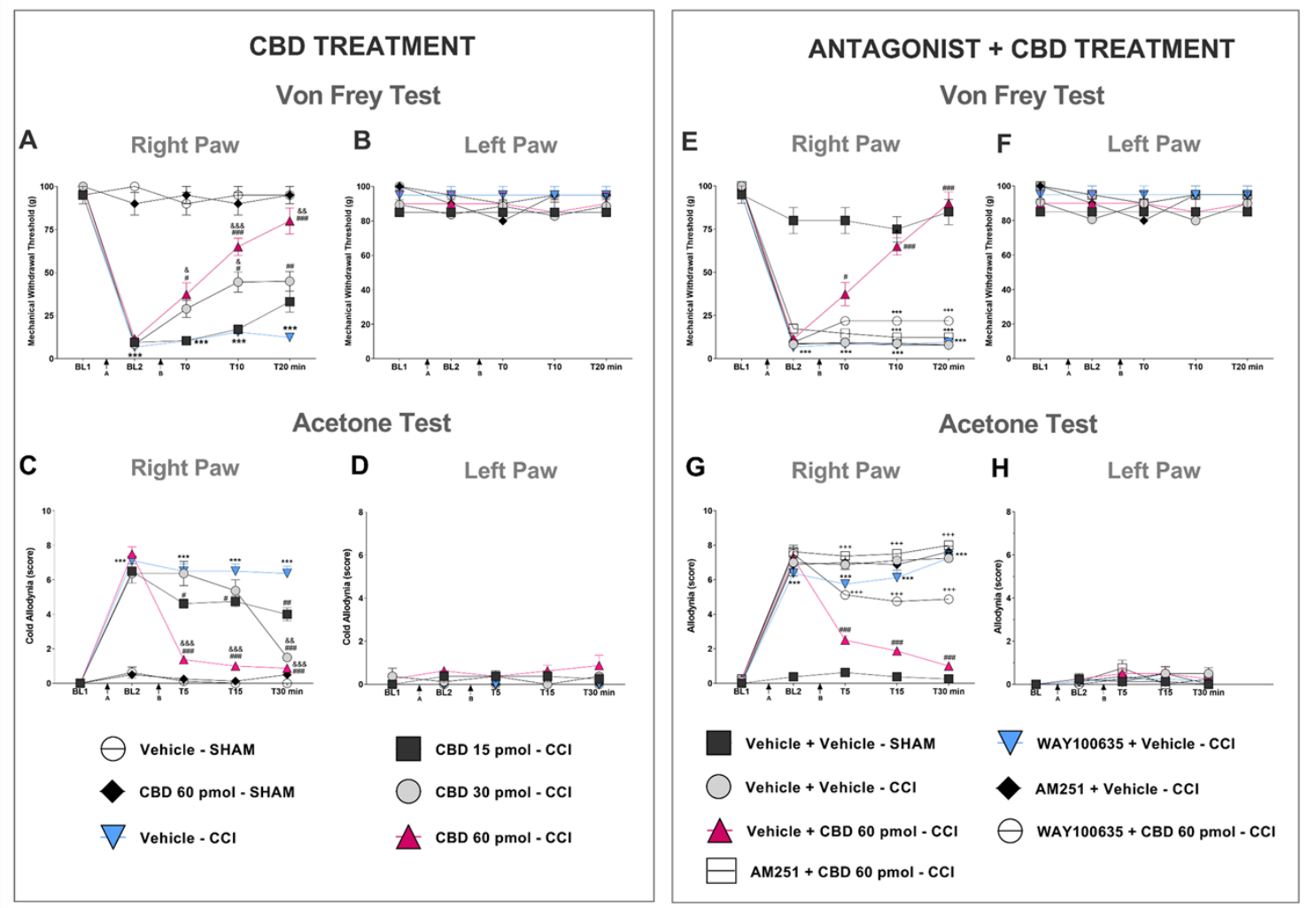

Figure 3

Effects of intra-anterior insular cortex cannabidiol microinjection on mechanical and cold allodynia and the role of CB_1 and 5-HT_1A receptors: Effects of intra-anterior insular cortex (AIC) microinjection of cannabidiol (CBD) on mechanical and cold allodynia in the sciatic nerve chronic constriction injury (CCI) and SHAM animals. Panels A–B show the effects of intra-AIC CBD (15, 30, or 60 nmol/200 nl) on mechanical withdrawal thresholds assessed by the von Frey test. Panels C–D illustrate the effects of intra-AIC CBD on cold allodynia. Panels E–F show the effects of CB_1 receptor antagonism with AM251 (100 pmol) or 5-HT_{1A} receptor blockade with WAY-100635 (0.37 pmol) on CBD-induced modulation of mechanical allodynia. Panels G–H depict the effects of CB_1 or 5-HT_{1A} receptor blockade on CBD-induced modulation of cold allodynia. Data are expressed as mean ± SEM. Statistical analyses were performed

using two-way ANOVA followed by Tukey's post hoc test; *p < 0.05, **p < 0.01, ***p < 0.001 compared with Vehicle (AIC)/CCI-treated group; #p < 0.05 compared with Vehicle + CBD 60 nmol (AIC)/CCI treatment. BL1: baseline before surgery; BL2: baseline 21 days after surgery; Arrow A: CCI or SHAM surgery; Arrow B: intra-AIC pharmacological administration followed by behavioral testing.

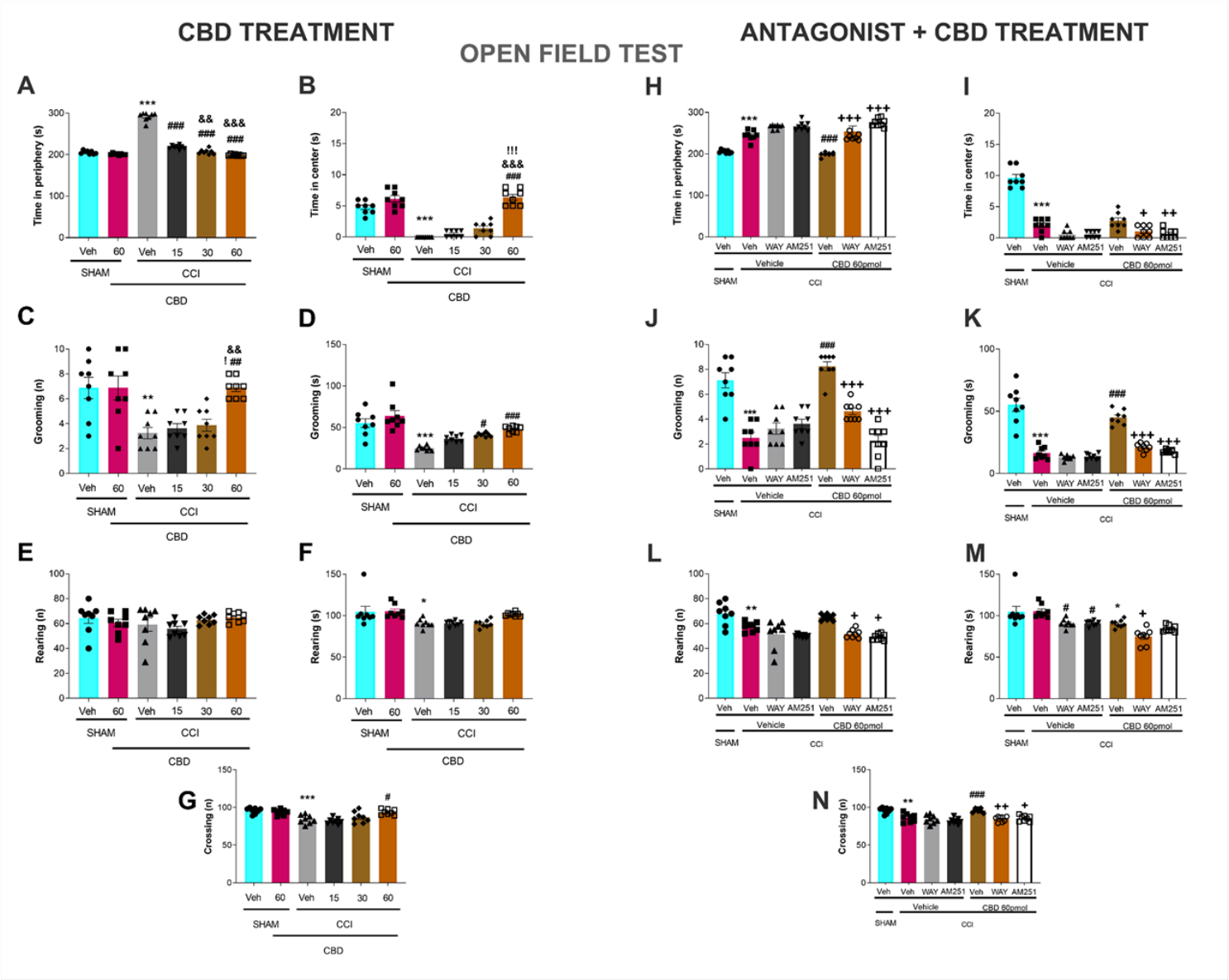

Figure 4

Effects of intra-anterior insular cortex cannabidiol microinjection on locomotor, exploratory, and anxiety-related behaviors in the open-field test: Behavioral effects of intra-anterior insular cortex (AIC) microinjection of cannabidiol (CBD) in the sciatic nerve chronic constriction injury (CCI)- and SHAM procedure-treated animals assessed in the open-field test. Panels A and B show the time spent in the periphery and center of the arena, respectively. Panels C and D depict the frequency and duration of grooming behavior. Panels E and F show the frequency and duration of rearing behavior. Panel G represents the number of arena crossings. Panels H and I illustrate the effects of CB_1 receptor antagonism with AM251 (100 pmol) and 5-HT_{1A} receptor antagonism with WAY-100635 (0.37 pmol) on

CBD (AIC)-induced changes in time spent in the periphery and center of the arena, respectively. Panels J and K show the effects of receptor antagonism on the frequency and duration of grooming behavior. Panels L and M depict the effects of antagonists on grooming frequency and duration. Panel N shows the number of arena crossings following antagonist pretreatment. Data are expressed as mean ± SEM. Statistical analyses were performed using two-way ANOVA followed by Tukey's post hoc test; *p < 0.05, **p < 0.01, ***p < 0.001 compared with Vehicle (AIC)/CCI treatment; #p < 0.05 compared with Vehicle + CBD 60 nmol (AIC)/CCI treatment.

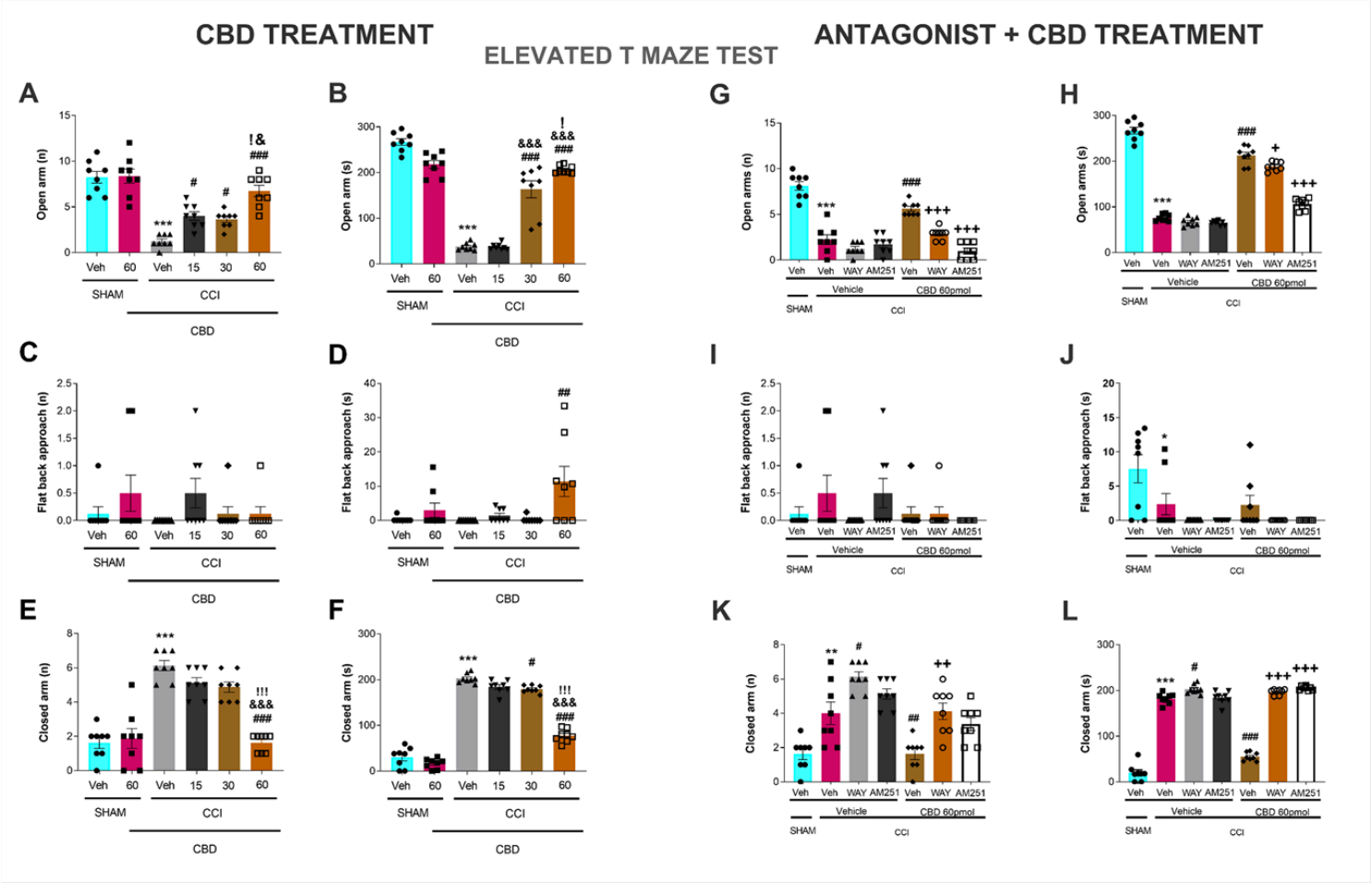

Figure 5

Effects of intra-anterior insular cortex cannabidiol microinjection on anxiety-related behavior in the elevated plus-maze test: Elevated T-maze assessment of anxiety-related behavior in rats subjected to the sciatic nerve chronic constriction injury (CCI) or SHAM surgery. Effects of intra-anterior insular cortex (AIC) microinjection of cannabidiol (CBD) at different doses (15, 30, and 60 nmol) preceded by either vehicle or 5-HT_{1A} and CB1 receptor selective antagonists (WAY-100635, 0.37 pmol; AM251, 100 pmol, respectively) on the frequency of entries into the open arms (A), time spent in the open arms (B), risk assessment behavior (flat-back approach) frequency (C) and duration (D), frequency of entries into the closed arms (E), and time spent in the closed arms (F). Panels G–L depict the effects of CB_1 and 5-HT_{1A} receptor antagonism on CBD at 60 nmol (AIC)-induced behavioral changes. Data are expressed as mean ± SEM. Statistical analyses were performed using two-way ANOVA followed by Tukey's post hoc test; * p

< 0.05 in comparison to Vehicle (AIC)/SHAM-treated group; #p < 0.05 in comparison with Vehicle (AIC)/CCI-treated group; +p < 0.05 in comparison with Vehicle + CBD at 60 nmol (AIC)/CCI treatment.

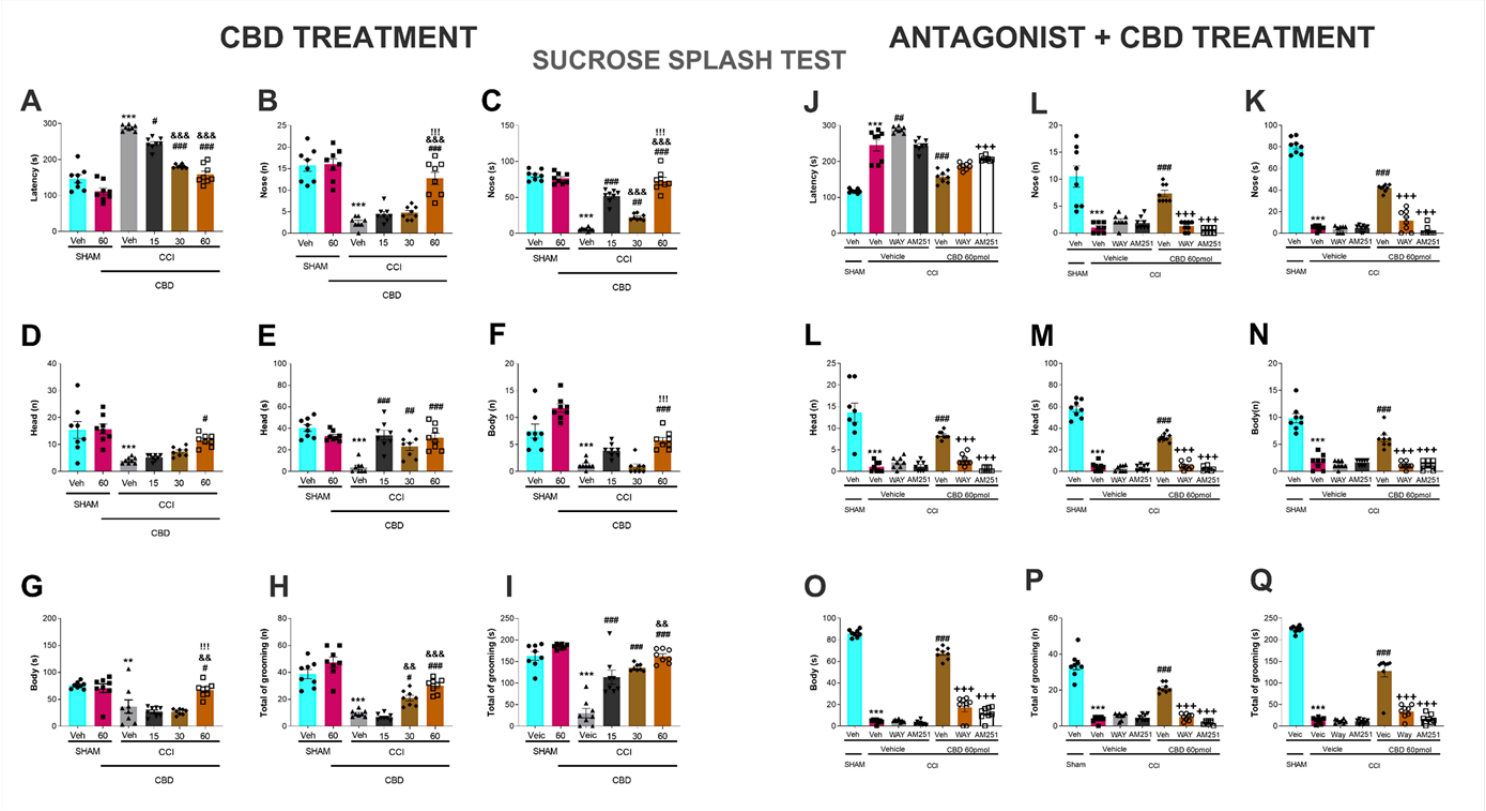

Figure 6

Effects of intra-anterior insular cortex cannabidiol microinjection on anhedonia-like behavior assessed by the sucrose splash test:

Sucrose splash test assessing depression-predictive (anhedonia-like) behavior in rats subjected to the sciatic nerve chronic constriction injury (CCI) or SHAM surgery following intra-anterior insular cortex (AIC) microinjection of cannabidiol (CBD) at different doses (15, 30, and 60 nmol) preceded by AIC pretreatment with either 5-HT_{1A} or CB1 receptor selective antagonists. Latency to initiate grooming and total grooming time (A–B); head grooming frequency and duration (C–D); snout grooming frequency and duration (E–F); and whole-body grooming frequency and duration (G–H). Panels I–P illustrate the effects of CB_1 (AM251) and 5-HT_{1A} (WAY-100635) receptors blockade on CBD at 60 nmol (AIC-induced modulation of grooming-related behaviors. Data are expressed as mean ± SEM. Statistical analyses were performed using two-way ANOVA followed by Tukey's post hoc test; * p < 0.05 as compared to Vehicle (AIC)/SHAM-treated group; #p < 0.05 in comparison to Vehicle (AIC)/CCI-treated group; +p < 0.05 in comparison with Vehicle + CBD at 60 nmol (AIC)/CCI-treated group.

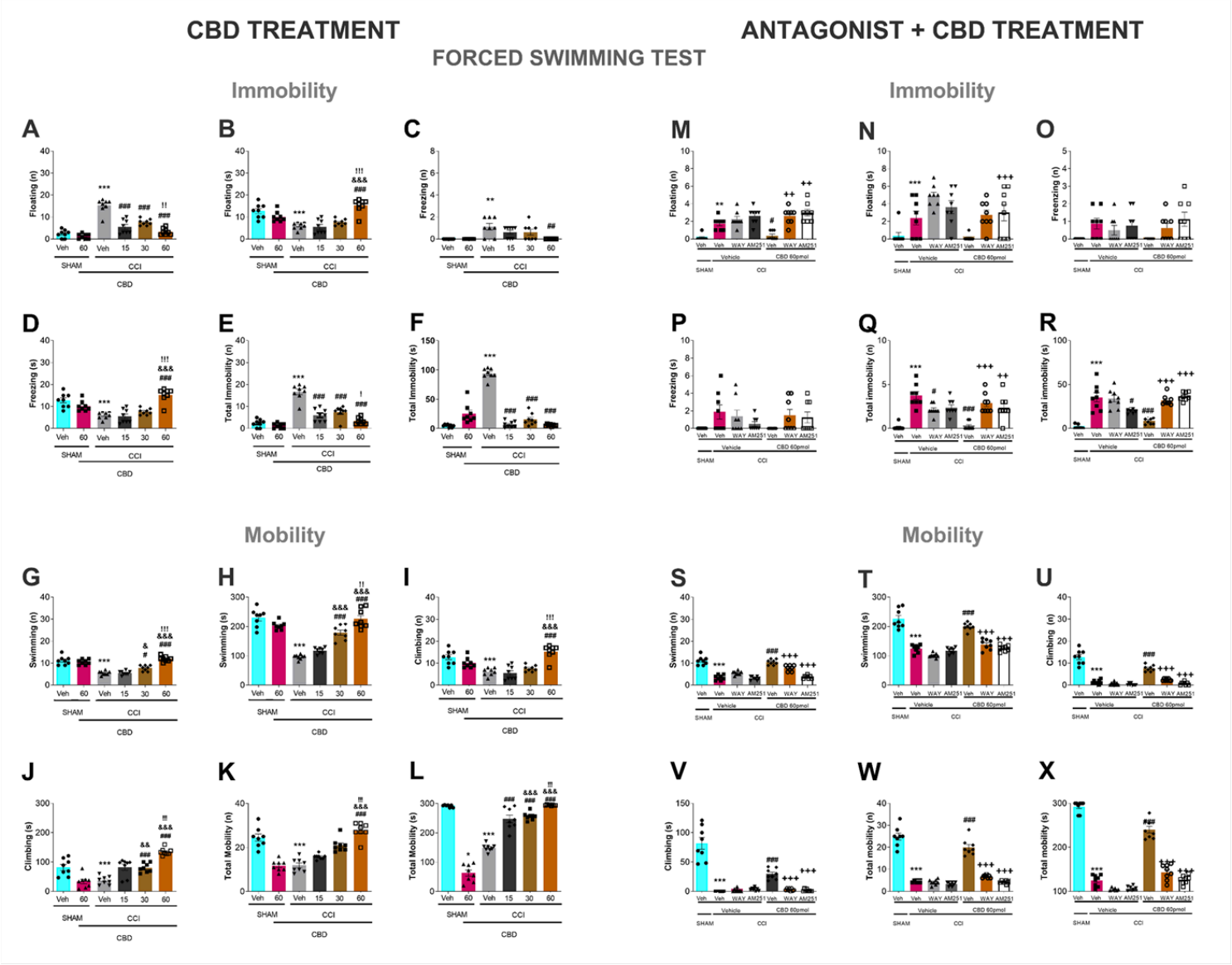

Figure 7

Effects of intra-anterior insular cortex cannabidiol microinjection on depression-like behavior assessed by the forced swim test:

Forced swim test assessing depression-predictive immobility- and mobility-related behaviors in rats subjected to the sciatic nerve chronic constriction injury (CCI) or SHAM surgery following intra-anterior insular cortex (AIC) microinjection of cannabidiol (CBD) at different doses (15, 30, and 60 nmol) preceded by AIC pretreatment with either 5-HT_{1A} or CB1 receptor selective antagonists (WAY-100635 and AM251, respectively). Floating frequency (A) and duration (B); immobility frequency (C) and duration (D); and total immobility frequency (E) and duration (F). Swimming frequency (G) and duration (H); climbing frequency (I) and duration (J); and total mobility frequency (K) and duration (L). Panels M–X illustrate the effects of CB_1 and 5-HT_{1A} receptors antagonism on CBD at 60 nmol (AIC)-induced modulation of immobility- and mobility-related behaviors. Data are expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA followed by Tukey's post hoc test; *p < 0.05 in

comparison to Vehicle (AIC)/SHAM-treated group; #p < 0.05 in comparison to Vehicle (AIC)/CCI-treated group; +p < 0.05 in comparison to Vehicle + CBD at 60 nmol (AIC)/CCI-treated group; $^{\&}$p < 0.05 in comparison to Vehicle + CBD at 15 nmol (AIC)/CCI-treated group; $^{!}$p < 0.05 in comparison to Vehicle + CBD at 30 nmol (AIC)/CCI-treated group.

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