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Interplay between chronic pain and affective-cognitive alterations: shared neural mechanisms, circuits, and treatment

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Interplay between chronic pain and affective-cognitive alterations: shared neural mechanisms, circuits, and treatment

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Editorial: Interplay between chronic pain and affective-cognitive alterations: shared neural mechanisms, circuits, and treatment

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Editorial on the Research Topic
Interplay between chronic pain and affective-cognitive alterations: shared neural mechanisms, circuits, and treatment

Chronic pain, defined clinically as persisting for at least 3 months, is not merely a sensory phenomenon but a subjective experience shaped by affective and cognitive processing. Patients with persistent pain often suffer from emotional distress, reduced motivation, and cognitive impairments. The elaboration of the emotional dimension of pain requires cognitive processing, engaging multiple cortical and limbic regions involved in perception, modulation, and processing. Moreover, critical cognitive functions such as memory and attention are often disrupted in individuals experiencing chronic pain. In addition, mood disorders (e.g., depression) and anxiety disorders, frequently co-occur in chronic pain populations, highlighting the psychological comorbidity of the condition.

Recent research has shed light on key neural circuits and signaling pathways that contribute to the affective and cognitive components of pain (Corder et al., 2019; Massaly et al., 2019; Talbot et al., 2019; Caputi et al., 2019; Markovic et al., 2021; Choi et al., 2025) leading to the identification of novel therapeutic targets.

This Research Topic aims to describe different signaling pathways implicated in chronic pain and to offer possible novel therapeutic approaches that might pave the way to the management of pain disorders.

By using a spinal nerve ligation (SNL) model, the original research paper proposed by Mazzitelli et al. investigates the role of astrocytes in the amygdala's central nucleus (CeA). They observed increased astrocyte activation at the chronic (4 weeks post-SNL) but not acute (1 week post-SNL) phase. Inhibiting astrocytes with fluorocitric acid (FCA) enhanced neuronal excitability by altering hyperpolarization-activated current without affecting synaptic transmission at the parabrachial nucleus-CeLC synapse. Behavioral tests showed that FCA influenced mechanical withdrawal thresholds and evoked

vocalizations but did not alter facial grimacing or anxiety-like behaviors. These findings indicate astrocytes in the CeA may have protective functions in chronic neuropathic pain.

Cai et al. explored the dorsal medial prefrontal cortex (dmPFC) to basolateral amygdala (BLA) pathway's role in emotional regulation. Optogenetic activation of this circuit in normal rats induced anxiety- and depression-like behaviors, while inhibition reduced anxiety and promoted reward-seeking behaviors. Their findings indicate the dmPFC-BLA circuit plays a critical role in mood regulation, with implications for pain-related emotional dysregulation.

Several key brain areas and neurotransmitters mediate emotional and cognitive aspects of pain. Flores-Garcia et al. reviewed the ventral tegmental area (VTA) as a hub integrating pain and emotional processing. Mounting evidence suggests that the dopaminergic system within the VTA plays a pivotal role in chronic pain's affective components. The review emphasizes the importance of animal models incorporating both pain and mood disorder features to develop effective treatments. A review by Lançon and Seguela examined the anterior cingulate cortex (ACC) in top-down modulation of pain perception, focusing on monoaminergic pathways—dopamine (DA), norepinephrine (NE), and serotonin (5-HT). Chronic pain disrupts these neuromodulators, contributing to hyperexcitability and cognitive impairments. Understanding how these alterations affect pain perception could pave the way for novel, non-opioid-based therapies.

Marino et al. used a machine learning (ML) approach to analyze central μ-opioid (μOR) and dopamine D2/D3 (DOR) receptor profiles in chronic migraine, a debilitating neurovascular pain disorder with a significant impact on cognitive and emotional functions. Using Compressive Big Data Analytics on data from positron emission tomography scans, they distinguished migraine patients from healthy controls with over 90% accuracy by identifying key predictive regions from each receptor system. For μOR, these regions included the anterior insula, thalamic nuclei, and putamen, while DOR dysfunction was primarily identified in the putamen. These findings offer insights into the neurochemical disruptions underlying migraine-related pain and cognitive impairments in humans.

Corasaniti et al. assessed the efficacy of monoclonal antibodies (mAbs) targeting the calcitonin gene-related peptide (CGRP) or its receptor (CGRP-R) in treatment-resistant chronic migraine. Their real-world data suggest that combining anti-CGRP therapy with onabotulinumtoxin A reduces monthly headache days by ≥ 50% in 58.8% of patients. Erenumab, when combined with onabotulinumtoxin A, improved symptoms in 65% of cases, while eptinezumab was notable for its rapid onset. These findings emphasize the potential of combination therapy for improved migraine management.

A review by Alorfi reviewed drugs tested in completed phase IV clinical trials for fibromyalgia. Analyzing 1,263 trials (121 related to fibromyalgia), they identified 10 meeting inclusion criteria. Investigated drugs targeting primary pain and cognitive-emotional pathways included milnacipran, duloxetine, pregabalin, tramadol-acetaminophen, and armodafinil. Despite these trials, current pharmacological treatments remain of limited effectiveness, emphasizing the need for alternative therapeutic strategies.

Liu et al. conducted a Mendelian randomization analysis to explore the causal link between mood instability and chronic low back pain. Their findings demonstrated significant associations, with inverse variance weighting revealing odds ratios above 3.0, reinforcing the role of emotional dysregulation in chronic pain susceptibility.

The issue is concluded with a study by Lopez-Cordoba et al. that investigated spinal α2-adrenoceptor subtypes in nociception. Their results indicated that α2A-adrenoceptor activation contributes to antinociception in acute and tonic pain, while α2C-adrenoceptors may be pronociceptive under tonic nociceptive conditions, possibly by inhibiting GABAergic transmission. These findings highlight spinal adrenergic systems as potential therapeutic targets.

Collectively, these studies underscore the multifaceted nature of chronic pain, integrating neuropathic pain, migraine, fibromyalgia, and low back pain with cognitive and emotional processing. Key brain regions—including the amygdala, VTA, mPFC, and ACC—alongside neurotransmitter-receptor systems such as CGRP-R, D2/D3, μOR, and α2-adrenoceptors, play paramount roles in pain perception, mood regulation, and cognitive function. Advances in understanding these mechanisms may facilitate targeted therapies addressing both sensory and affective pain components. Furthermore, pharmacological innovations, including monoclonal antibodies, botulinum toxin, and novel neuromodulators, hold promise for treatment-resistant conditions. A multidisciplinary approach targeting brain, spinal, and neurochemical systems, could offer more effective and comprehensive strategies for managing chronic pain and its associated cognitive and emotional dysfunctions.

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Pharmacological treatments of fibromyalgia in adults; overview of phase IV clinical trials

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Background: Fibromyalgia is a chronic neurological condition characterized by widespread pain. The effectiveness of current pharmacological treatments is limited. However, several medications have been approved for phase IV trials in order to evaluate them.

Aim: To identify and provide details of drugs that have been tested in completed phase IV clinical trials for fibromyalgia management in adults, including the primary endpoints and treatment outcomes. This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology.

Method: Publicly available and relevant phase IV trials registered at ClinicalTrials.gov were analyzed. The uses of the trialed drugs for fibromyalgia were reviewed.

Results: As of 8 August 2022, a total of 1,263 phase IV clinical trials were identified, of which 121 were related to fibromyalgia. From these, 10 clinical trials met the inclusion criteria for the current study. The drugs used in phase IV trials are milnacipran, duloxetine, pregabalin, a combination of tramadol and acetaminophen, and armodafinil. The effectiveness of the current pharmacological treatments is apparently limited.

Conclusion: Due to its complexity and association with other functional pain syndromes, treatment options for fibromyalgia only are limited and they are designed to alleviate the symptoms rather than to alter the pathological pathway of the condition itself. Pain management specialists have numerous pharmacologic options available for the management of fibromyalgia.

KEYWORDS

fibromyalgia, pain, clinical trials, neuroscience, phase IV

Introduction

Fibromyalgia (FM) is a complex condition affecting a person's sensory processing system and it has a significant morbidity rate (Clauw, 2009; Sumpton and Moulin, 2014). Like other functional pain syndromes, fibromyalgia shows symptoms without pathologic findings. It is characterized by widespread musculoskeletal pain accompanied by anxiety, fatigue, cognitive dysfunction, and sleep disruption (Neumann and Buskila, 2003; Arnold

et al., 2008; Häuser et al., 2015; Andrade et al., 2020). Other symptoms associated with fibromyalgia include tension headaches, temporomandibular joint (TMJ) disorders, irritable bowel syndrome, and depression; these ultimately lead to a decline in patients' quality of life (Silver and Wallace, 2002; Ayouni et al., 2019; Santos et al., 2019). Some patients report morning stiffness and gastrointestinal irritation. Several asymptomatic conditions can also develop in fibromyalgia patients, such as osteoarthritis, hyperparathyroidism, degenerative disk disease, and calcifications (Maria De Freitas Trindade Costa et al., 2016; Maugars et al., 2021). The condition is reported in more women than in men (Yunus, 2001).

Clinically, the presence of fibromyalgia has been linked to an increased response to almost any type of stimulus, including hot, cold, electrical stimulation, photosensitivity, and sometimes the brightness of light or the volume of auditory tones. These all contribute to enhanced pain sensitivity and they induce persistent pain (Geisser et al., 2008; Becker and Schweinhardt, 2012; Martenson et al., 2016). The widespread pain experienced by individuals with fibromyalgia has been attributed to primarily central mechanisms such as central sensitization at the spinal level and abnormal pain processing in the brain (Desmeules et al., 2003; Cagnie et al., 2014). Although the exact cause of fibromyalgia is not fully understood, biological factors, mechanical/physical trauma or injury, genetic factors, and psychosocial stressors have been held responsible for the painful condition (Sommer et al., 2008; Amsterdam and Buskila, 2021). The disease is well known for the abnormal pain sensitivity that it causes, along with abnormal neurotransmitter levels in cerebrospinal fluid (CSF), abnormal activation of cerebral pain-processing areas, and abnormal peripheral pain and sensory thresholds (Larson et al., 2000; Staud and Domingo, 2001; Staud, 2002). These could be treatment targets for some drugs.

Despite its pathophysiological complexity, fibromyalgia may present with several comorbidities that worsen the sensation. Interestingly, the factors contributing to the pathophysiology of fibromyalgia may also exist with other functional pain syndromes such as functional abdominal pain syndrome, irritable bowel syndrome, chronic pelvic pain syndrome, and TMJ disorder (Shaver, 2008; Yunus, 2012; Johnson and Makai, 2018). This co-existence leads to further complexity for clinicians.

Pharmacological targets in fibromyalgia treatment

Research advances are increasingly focusing on the development of analgesics and pain management medications that can be used for fibromyalgia. In this context, selective serotonin reuptake inhibitors, norepinephrine inhibitors, skeletal muscle relaxants, anti-epileptic agents, and anesthetics have been mainly investigated (Sarzi-Puttini et al., 2008; Häuser et al., 2009). Other targets for pain alleviation include the inhibition of excitatory neurotransmitters, substance P, and glutamate (Sarchielli et al., 2007; Owen, 2008; Guymer and Littlejohn, 2021). The aim of treatment for fibromyalgia is often to reduce pain-related symptoms. However, there is no specific pathophysiological therapeutic target.

Pharmacological treatments

Fibromyalgia is a chronic, life-long condition, and it has no known etiology and no single cure. The multidisciplinary approach with medications, physiotherapy, psychologic, and other modalities provides the best chance of improved outcomes without promoting polypharmacy (Menzies et al., 2017; Oliveira Júnior and Ramos, 2019). Caution is advised when designing a treatment plan in fibromyalgia.

A considerable amount of literature has listed different pharmacological treatments used as adjunct medications, such as selective serotonin reuptake inhibitors, e.g., fluoxetine (Lawson, 2002; Häuser et al., 2015; Walitt et al., 2015); skeletal muscle relaxants, e.g., cyclobenzaprine (Tofferi et al., 2004); tramadol (Russell et al., 2000; Goldenberg et al., 2004); clonazepam (Corrigan et al., 2012); lidocaine (Abeles et al., 2007). Moreover, caffeine is used as a non-selective antagonism of adenosine receptors which reduce pain processing (Scott et al., 2017). Most of the above-mentioned drugs are used to induce sleep, reduce the symptoms of depression and anxiety and/or minimize fatigue. However, drugs that are US FDA approved specifically for fibromyalgia include milnacipran, duloxetine, and pregabalin.

This review focuses on medications that have been tested in phase IV clinical trials only. It identifies and summarizes the drugs that have been trialed for fibromyalgia and it provides an overview of phase IV clinical trials that assess a pharmacological treatment of fibromyalgia in adults. The drugs found to be used in phase IV trials on fibromyalgia are milnacipran, duloxetine, pregabalin, the combination of tramadol and acetaminophen, and armodafinil.

Non-pharmacological treatments

Several studies have revealed that not only pharmacological treatments are available for fibromyalgia, but that non-pharmacological options could also help patients with this condition. For patients with chronic pain, a number of non-pharmacological options, including physical and aerobic exercises and cognitive-behavioral therapy (CBT), have shown promising results as standalone, adjunctive treatments (Hassett and Williams, 2011; Nüesch et al., 2013). A study conducted by Bernik et al. (2013) concluded that pharmacotherapy and CBT therapy should preferably be provided to all patients with fibromyalgia.

Moreover, studies suggest that psychological support is essential for patients with fibromyalgia due to the many negative emotions that can accompany the condition. Psychological treatments include mindfulness meditation, stress management and coping mechanisms, e.g., sleep hygiene and relaxation techniques improved pain perception and minimized pain symptoms (Hassett and Gevirtz, 2009; Aman et al., 2018). Indeed, complementary treatments including frequent movement, electroacupuncture, acupuncture, and chiropractor therapy are effective as adjuvant therapies to all patients with fibromyalgia (Sarac and Gur, 2005; Zheng and Faber, 2005; Martin et al., 2006; Ablin et al., 2013).

Study design

ClinicalTrials.gov was searched to identify trials relevant to this review. ClinicalTrials.gov is an online database, effectively a public registry of clinical trials conducted in 221 countries; it contains information about medical studies with human volunteers. This review evaluated drugs used in fibromyalgia and it examined entries related to pharmacological fibromyalgia studies. See the following section for the parameters used in the search. Articles were screened independently by two reviewers and assessed for risk of bias. Data from 1,263 clinical trials were downloaded from ClinicalTrials.gov on 08/08/2022. After excluding trials that involved the treatment of other complications or were not at phase IV, 10 clinical trials remained as eligible for the review. The inclusion criteria were: 1) primary focus on fibromyalgia; 2) completed studies; 3) for patients of adult age (18–64 years old), and 4) results were available.

Data Extraction

The data were extracted manually and downloaded from ClinicalTrials.gov, covering the following:

- Interventions: Details of interventional (clinical trial).
- Conditions: The medical condition treated was selected as "Fibromyalgia."
- Trial design: Phase IV only.

The trials' results were extracted manually from the results reported in the registry. The primary outcomes, number of participants, timeframe and results were collected.

Results

The drugs used in the phase IV trials are milnacipran, duloxetine, pregabalin, a combination of tramadol and acetaminophen, and armodafinil. Interestingly, five out of the ten clinical trials were used to evaluate the role of milnacipran in fibromyalgia. The official title of the trial, the intervention, aims, primary measures (endpoints) and timeframe are shown in Table 1.

Milnacipran

Milnacipran is a non-tricyclic compound that inhibits the re-uptake of both serotonin and norepinephrine (Yoshida et al., 2004; Häuser et al., 2015). As a result, serotonin and norepinephrine levels are increased and disorders resulting from a lack of these neurotransmitters are improved. This leads to a reduction in symptoms, including pain, fatigue, and cognitive deficits. A three-fold greater selectivity for norepinephrine inhibition is found with milnacipran than for serotonin inhibition (Palmer et al., 2010; Raouf et al., 2017). The drug is approved by the US Food and Drug Authority (FDA) exclusively for fibromyalgia. Milnacipran is believed to inhibit some excitatory neurotransmitters such as substance P, which may result in the reduction of pain severity. It is recommended for the treatment of various chronic pain syndromes (Elliott et al., 2009; Kyle et al., 2010). It can also be effective for fibromyalgia patients with coexisting depression (Ormseth et al., 2010). A drug evaluation paper concluded that milnacipran gives modest pain relief in fibromyalgia patients and is best used as part of a multidisciplinary treatment strategy (Bernstein et al., 2013). The results of another study showed that milnacipran significantly reduced pain scores, helped patients to achieve lower mean global impression scores, and significantly increased response rates, regardless of the depressive symptoms at baseline (Arnold et al., 2012). Milnacipran has some major but rare side effects such as worsening suicide risk and causing liver damage (Montgomery and Briley, 2010; Park and Ishino, 2013). Moreover, some common side effects include gastrointestinal upset and urinary disorders (Tignol et al., 1998; Levin, 2016). Interestingly, milnacipran does not inhibit the cytochrome P 450 system, which reduces the chances of possible interactions with other drugs (Pae et al., 2009; Paris et al., 2009). The five studies that have used milnacipran in phase IV trials to treat fibromyalgia are shown in Table 1.

Duloxetine

Duloxetine is a serotonin–norepinephrine reuptake inhibitor (SNRI) (Westanmo et al., 2005; JS et al., 2019). Pre-clinical studies show that duloxetine can alleviate diabetic peripheral neuropathic pain, fatigue, pain and other related symptoms (Wernicke et al., 2006). It has also been found to significantly reduce pain and improve functioning in patients with chronic low back pain (Skljarevski et al., 2010). In patients with fibromyalgia, duloxetine improved average pain severity and self-reported improvement (Chappell et al., 2008). It has also been found to be effective in the long-term treatment of fibromyalgia (Mease et al., 2010). The study by Mease et al.

TABLE 1 (Data from https://clinicaltrials.gov, updated on 08-08-2022).

No.	Study title	Treatment	Aim	*n*	Primary measures (endpoints)	Time frame
1	Effects of a 12-Week milnacipran 200 mg treatment on pain perception and pain processing in fibromyalgia—An open-label study	Milnacipran	To investigate the effects of milnacipran treatment on neurotransmitter release in fibromyalgia	8	The concentration of Substance P in cerebrospinal fluid in response to experimental pain before and after Milnacipran treatment	12 weeks
2	A randomized, double-blind, placebo-controlled, two-way crossover study to evaluate the effect of milnacipran on pain processing and functional magnetic resonance imaging activation patterns in patients with fibromyalgia	Milnacipran	To evaluate the effects of milnacipran on pain processing and functional mri in patients with fibromyalgia	22	1) Concentration of Substance P in cerebrospinal fluid in response to experimental pain before and after milnacipran treatment 2) Measure the sensory threshold for temperature pain and pressure pain 3) Measure pain ratings and fibromyalgia symptoms 5) Measure concentrations of serotonin and norepinephrine cerebrospinal fluid and plasma	16 weeks
3	A multicenter, randomized, double-blind, placebo-controlled switch study to evaluate the safety, tolerability and efficacy of milnacipran in patients with an inadequate response to duloxetine for the treatment of fibromyalgia	Milnacipran	To evaluate the safety, tolerability and efficacy of milnacipran in patients with an inadequate response to duloxetine for the treatment of fibromyalgia	107	1) Responder status based on patient global impression of change (PGIC) Score at Visit 5 (Week 13) 2) Change from baseline to visit 5 (Week 13) in the visual analog scale (VAS) 1-week pain recall score	12 weeks
4	The effects of milnacipran on sleep disturbance in fibromyalgia	Milnacipran	The study aimed at examining the effects of milnacipran on sleep disturbance in patients with fibromyalgia	19	The primary endpoints were the difference in sleep maintenance defined by PSG-recorded wake after sleep onset (WASO), number of awakenings after sleep onset (NAASO), and SE	4 weeks
5	A multicenter, randomized, double-blind, placebo-controlled discontinuation study of the durability of effect of milnacipran for the treatment of fibromyalgia in patients receiving long-term milnacipran treatment	Milnacipran	To evaluate the durability of effect of milnacipran for the treatment of fibromyalgia in patients receiving long-term milnacipran treatment and to characterize the effects of milnacipran on multiple symptoms of fibromyalgia, as demonstrated by changes in symptoms following the discontinuation of milnacipran	340	1) Time to loss of therapeutic response (LTR) 2) Time to worsening in patient global impression of change (PGIC) 3) Time to worsening in multidimensional assessment of fatigue (MAF)	17 weeks
6	Flexible dosed duloxetine versus placebo in the treatment of fibromyalgia	Duloxetine hydrochloride	To confirm the efficacy and safety of duloxetine 60–120 mg once daily in comparison to placebo on symptom improvement in patients meeting criteria for fibromyalgia aged 18 and older	530	1) Patient's global impressions of improvement (PGI-I) at Week 12 2) Change from baseline in brief pain inventory (BPI) (Modified Short Form), Multidimensional fatigue inventory (MFI), Clinical global impressions of severity (CGI-S), Beck anxiety inventory (BAI), 36-Item Short-form Health Survey (SF-36), Massachusetts General Hospital Cognitive and Physical Functioning Questionnaire (MGH-CPFQ), the Mood, Anxiety, Pain, Sleep, and Stiffness Likert Scale, blood pressure and heart rate at 12 Week Endpoint 3) Number of responders: 30% and 50% improvement in brief pain inventory average pain at 12 week endpoint	12 weeks
7	A randomized, double-blind comparison of duloxetine 30 mg QD and placebo in adult patients with fibromyalgia	Duloxetine	To determine if 30 milligrams (mg) of duloxetine is effective in the treatment of fibromyalgia compared to placebo	308	1) Change in weekly average pain intensity 2) Change in evoked pain scores 3) Identification of group assignment	12 weeks

(Continued on following page)

TABLE 1 (*Continued*) (Data from https://clinicaltrials.gov, updated on 08-08-2022).

No.	Study title	Treatment	Aim	*n*	Primary measures (endpoints)	Time frame
8	A 6-month, open-label, safety trial of pregabalin in adolescent patients with fibromyalgia	Pregabalin	To evaluate the long-term safety of pregabalin in adolescent patients who participated in the previous fibromyalgia study (A0081180) and who wish to receive open-label pregabalin	63	Change from baseline in pain numeric rating scale by week	24 weeks
9	Ultracet [Tramadol HCL (37.5 mg)/ acetaminophen (325 mg)] combination tablets in the treatment of the pain of fibromyalgia	Tramadol and acetaminophen	To evaluate the analgesic effect of combination of tramadol hydrochloride and acetaminophen in participants for treatment of fibromyalgia pain (chronic widespread pain and presence of tender points)	80	Pain visual analog scale score at day 14, 28 and 56	8 weeks
10	An 8 week, double-blind efficacy study of armodafinil augmentation to alleviate fibromyalgia fatigue	Armodafinil	To determine if armodafinil is safe and tolerable in the treatment of FM-induced fatigue	55	Brief Fatigue Inventory	8 weeks

(2010) reported that dry mouth and nausea were the most reported side effect, but duloxetine is generally safe and well tolerated, including in older patients and those with concomitant illnesses (Wernicke et al., 2005). It is preferably avoided in patients with hepatic insufficiency.

Pregabalin

Pregabalin, the first of the three medications that have gained US FDA approval for fibromyalgia, is an α2 δ ligand that acts by binding the α2 δ subunit of voltage-gated calcium channels (Micó and Prieto, 2012). This leads to a decrease in calcium influx at nerve terminals, with a consequential modulation in excitatory neurotransmitter release pain-related neurotransmitters, including glutamate and substance P (Tanabe et al., 2008; Alles et al., 2020). Studies have also suggested that pain associated with major depressive disorder can be reduced with pregabalin (Showraki, 2007; Stein et al., 2008; Frampton, 2014). Previous studies concluded that pregabalin was generally well tolerated in the long-term treatment of anxiety disorders (Feltner et al., 2008; Montgomery et al., 2013). Generally, pregabalin is safe and well tolerated (Durkin et al., 2010). However, number of uncomfortable side effects have been reported with pregabalin, although these tend to be transient and dose dependent (Zin et al., 2010; Toth, 2014). Only a single clinical trial was available at phase IV as shown in Table 1.

Tramadol and acetaminophen

Tramadol is a centrally acting, fully synthetic opioid and one of the most commonly used central nervous system analgesics (Leppert, 2009; Duehmke et al., 2017). It is an effective and well tolerated agent that is taken to reduce pain (Minami et al., 2015; Nakhaee et al., 2021). Tramadol can be used as a second-line treatment for more resistant cases in fibromyalgia patients and it has a positive effect on fibromyalgia pain (Maclean and Schwartz, 2015; Pereira Da Rocha et al., 2020). On the other hand, acetaminophen is a central analgesic drug that is mediated through the activation of descending serotonergic pathways (Pickering et al., 2008; Jozwiak-Bebenista and Nowak, 2014). Only one single clinical trial was available at phase IV as shown in Table 1. In fibromyalgia, a combination of tramadol and acetaminophen was effective for the treatment of fibromyalgia pain without any serious adverse effects (Bennett et al., 2003).

Armodafinil

Armodafinil is the R-enantiomer of racemic modafinil, a wakefulness-promoting medication, that primarily affects the areas of the brain involved in controlling wakefulness (Hirshkowitz et al., 2007; Garnock-Jones et al., 2009). The mechanism of action of armodafinil is not completely understood. It is mainly used for the treatment of excessive sleepiness associated with narcolepsy, and obstructive sleep apnea (Lankford, 2008; Schwartz et al., 2010). The potential role of stimulants such as armodafinil is often to manage the fatigue that is a common symptom of fibromyalgia. It has been used to improve menopause-related fatigue (Meyer et al., 2016) and sarcoidosis-associated fatigue (Lower et al., 2013). However, only a single clinical trial was available at phase IV for fibromyalgia management. The study concluded that there was no significant difference in any effectiveness outcome (Thomas et al., 2010).

TABLE 2 FDA-approved drugs for the treatment of fibromyalgia.

Treatment	Mechanism of action	Medication class	Indication	Side effect
Milnacipran	A selective norepinephrine reuptake inhibitor with a three times greater selectivity for norepinephrine reuptake inhibition over serotonin reuptake inhibition	Selective serotonin and norepinephrine reuptake inhibitors (SSNRIs)	Fibromyalgia	GI Upset Palpitations, increased heart rate Dry mouth Increases blood pressure
Duloxetine	Inhibits the reuptake of serotonin and norepinephrine (NE) in the central nervous system	Selective serotonin and norepinephrine reuptake inhibitors (SSNRIs)	Major depressive disorder Generalized anxiety disorder Diabetic peripheral neuropathic pain Fibromyalgia Chronic musculoskeletal pain in adults	Increases blood pressure Hepatitis Cholestatic
Pregabalin	Potentiates GABA activity	Anticonvulsants	Neuropathic pain Postherpetic neuralgia Partial-onset seizures Fibromyalgia	Sedation

Discussion

Fibromyalgia is a chronic, life-long condition with no single cure. There is no one treatment that can treat all the symptoms of fibromyalgia since the condition has many symptoms. Pain severity, global severity and physical functioning are significantly and negatively influenced by fibromyalgia. Also, psychological factors such as anxiety, stress, and depression exacerbate the condition. The treatments being assessed in the 10 trials reviewed here aimed to improve several health parameters, including physical fitness, work and other functional activities, and mental health. This review focused on medication that was tested in phase IV clinical trials only. The drugs used in phase IV trials for fibromyalgia are milnacipran, duloxetine, pregabalin, a combination of tramadol and acetaminophen, and armodafinil. The US FDA has approved three drugs to treat fibromyalgia: milnacipran, duloxetine and pregabalin, as shown in Table 2. Of these milnacipran is exclusively for the management of fibromyalgia. Both milnacipran and duloxetine act as selective serotonin and norepinephrine re-uptake inhibitors. A considerable amount of research concluded that patients with fibromyalgia have abnormally low levels of serotonin, noradrenaline and dopamine in their brains, which leads to serious side effects including the worsening of fibromyalgia status. Serotonin regulates numerous bodily functions. In adult patients with fibromyalgia, milnacipran, duloxetine and pregabalin were associated with significant improvements in pain and other symptoms. One of the considerations that should be included in future investigations is the combined therapy of pharmacological and non-pharmacological treatments to manage fibromyalgia symptoms. Further clinical trials investigating the efficacy and safety of treatments used for fibromyalgia are warranted.

Conclusion

The findings from the review suggest that the drugs most commonly used for fibromyalgia that can be considered first-line options are milnacipran, duloxetine, and pregabalin. The number of trials for fibromyalgia are extremely limited Pharmacological options can be employed to provide patients with a better quality of life. Despite years of investigation of pharmacological treatments for fibromyalgia, non-pharmacological treatments show promise for future investigation.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The differential *in vivo* contribution of spinal α_{2A}- and α_{2C}-adrenoceptors in tonic and acute evoked nociception in the rat

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Spinal α_2-adrenoceptor induces analgesia by neuronal inhibition of primary afferent fibers. This family receptor coupled to $G_{i/o}$ proteins can be subdivided into three functional subtypes: α_{2A}, α_{2B}, and α_{2C}-adrenoceptors, and current evidence on spinal analgesia supports the relevance of α_{2A} and seems to exclude the role of α_{2B}, but the functional contribution of α_{2C}-adrenoceptors remains elusive. The present study was designed to pharmacologically dissect the contribution of spinal α_2-adrenoceptor subtypes modulating tonic or acute peripheral nociception. Using male Wistar rats, we analyzed the effect of spinal clonidine (a non-selective $\alpha_{2A}/\alpha_{2B}/\alpha_{2C}$-adrenoceptor agonist) and/or selective subtype α_2-adrenoceptor antagonists on: 1) tonic nociception induced by subcutaneous formalin (flinching behavior) or 2) acute nociception induced by peripheral electrical stimulus in *in vivo* extracellular recordings of spinal dorsal horn second-order wide dynamic range (WDR) neurons. Clonidine inhibited the nocifensive behavior induced by formalin, an effect blocked by BRL 44408 (α_{2A}-adrenoceptor antagonist) but not by imiloxan (α_{2B}-adrenoceptor antagonist) or JP 1302 (α_{2C}-adrenoceptor antagonist). Similarly, spinal BRL 44408 reversed the clonidine-induced inhibition of nociceptive WDR activity. Interestingly, spinal JP 1302 *per se* produced behavioral antinociception (an effect blocked by bicuculline, a preferent $GABA_A$ channel blocker), but no correlation was found with the electrophysiological experiments. These data imply that, at the spinal level, 1) presynaptic α_{2A}-adrenoceptor activation produces antinociception during acute or tonic nociceptive stimuli; and 2) under tonic nociceptive (inflammatory) input, spinal α_{2C}-adrenoceptors are pronociceptive, probably by the inactivation of GABAergic transmission. This result supports a differential role of α_{2A} and α_{2C}-adrenoceptors modulating nociception.

KEYWORDS

adrenoceptor, analgesia, clonidine, electrophysiology, pain, behavior

1 Introduction

At the spinal dorsal horn level, α_2-adrenoceptor activation has been related to the inhibition of neural transmission and analgesic actions (Pertovaara, 2006; Bahari and Meftahi, 2019). From a mechanistic perspective, activation of this receptor which is canonically coupled to $G_{i/o}$ proteins, inhibits adenylyl cyclase, inactivates Ca^{2+} channels, and enhances inwardly rectifying K^+ channel activity, leading to neuronal hyperpolarization with a diminution of neural transmission (Pan et al., 2008). *In vivo* electrophysiological recordings of second-order wide dynamic range (WDR) cells in the spinal dorsal horn have shown that spinal clonidine (α_2-adrenoceptor agonist) selectively inhibits the neuronal activity of nociceptive primary afferent fibers (Sullivan et al., 1987). As reviewed by Pertovaara (2013), agonists to this receptor consistently produce antinociception in behavioral and electrophysiological experiments. Indeed, intrathecal clonidine has been successfully used in humans as a potent analgesic (Eisenach et al., 1996; Yaksh et al., 2017; Schwartz et al., 2022).

However, we must keep in mind that α_2-adrenoceptors have been divided into three functional subtypes, namely α_{2A}, α_{2B}, and α_{2C} (Bylund et al., 1994), and researchers have attempted to dissect how these receptor subtypes contribute to spinal pain transmission. Briefly, molecular assays suggest that the three receptor subtypes are expressed in the spinal cord and dorsal root ganglion cells (Aoki et al., 1994; Stone et al., 1998; Nicholson et al., 2005), and current functional evidence supports the notion that α_{2A}-adrenoceptor activation plays a key role in spinal antinociception, whereas α_{2B}-adrenoceptors seem not to contribute to spinal antinociception (for refs., see Pertovaara, 2006; Pertovaara, 2013). In contrast, in the case of α_{2C}-adrenoceptors, although Fairbanks et al. (2002) suggest that spinal activation of this subtype receptor plays an antinociceptive role, the evidence offered by Malmberg et al. (2001) refuses these data showing that this receptor does not play any role in nociception. In both cases, the experiments were performed using transgenic mice and non-selective ligands. Hence, the function of this subtype receptor remains obscure.

Most importantly, although current literature supports the notion that the main effect of α_2-adrenoceptors is inhibition of nociceptive transmission, some *in vitro/in vivo* experiments suggest that noradrenergic transmission at the spinal cord level could also be pronociceptive (Hantman and Perl, 2005; Chen et al., 2008; Gassner et al., 2009; Hickey et al., 2014; Kucharczyk et al., 2022). For example, using spinal cord slices and electrophysiological recordings (patch-clamp) at lamina II/III, it has been shown that some GABAergic neurons can be hyperpolarized by noradrenaline via α_2-adrenoceptor activation (Gassner et al., 2009); in this study, the subtype receptor was not analyzed. Furthermore, also in spinal cord slices, but using immunohistochemistry, Chen et al. (2008) showed that α_{2C}-adrenoceptor activation inhibits the veratridine-induced opioid release. These *in vitro* data may imply that α_{2C}-adrenoceptors may play a pronociceptive role. In any case, the functional role of α_{2C}-adrenoceptors modulating spinal nociception has been hampered (contradictory) in part by the lack of selective ligands used.

On this basis, the present study aimed to functionally dissect $\alpha_{2A/2B/2C}$-adrenoceptors in spinal nociception. Here, we analyzed the effect of spinal clonidine (a non-selective $\alpha_{2A}/\alpha_{2B}/\alpha_{2C}$-adrenoceptor agonist) and/or selective subtype $\alpha_{2A}/\alpha_{2B}/\alpha_{2C}$ -adrenoceptor antagonists (see Table 1) on: 1) tonic nociception (flinching behavior) induced by subcutaneous formalin or 2) acute nociception induced by peripheral electrical stimulus in *in vivo* extracellular recordings of spinal dorsal horn second-order wide dynamic range (WDR) neurons. The data showed that the presynaptic α_{2A}-adrenoceptor produces robust antinociception during acute or tonic nociceptive stimuli. In contrast, under a tonic nociceptive stimulus, α_{2C}-adrenoceptors seem to induce nociception by inactivating GABAergic transmission.

2 Material and methods

2.1 Experimental animals and ethical standards

A total of 115 male Wistar rats (295 ± 15 g) from the Neurobiology Institute Animal House were used in the present experiments. Rats were divided into two main sets (80 rats for behavioral tests and 35 rats for electrophysiological recordings; see Figure 1). The rodents were housed in the satellite bioterium of our laboratory on a 12:12 h light and dark cycle (lights on at 07:00 h) at constant temperature (22°C ± 2°C) and humidity (50%) with food and water *ad libitum*. All experimental procedures were performed during the light phase of the cycle (10:00–19:00 h). Furthermore, all animal protocols in this investigation were approved by our Institutional Ethics Committee, following the Guide for the Care and Use of Laboratory Animals in the United States (NIH publication 86-23), IASP ethical guidelines (Zimmermann, 1983), and ARRIVE guidelines for reporting experiments involving animals (McGrath et al., 2010). At the end of the experiments, the animals were halted in a CO_2 chamber (formalin test) or by an overdose of pentobarbital (electrophysiological experiments).

TABLE 1 Binding affinity constants for the α_{2A}-, α_{2B}-, and α_{2A}-adrenoceptors of clonidine and the antagonists used in the present study. The nanomoles (and their equivalents in μg) spinally administered in the current experiments are also shown.

	Affinity values (pK_i or pK_D) for α_2-adrenoceptor subtypes			Nanomoles (nmol) and their equivalents in μg used in the present study
	α_{2A}	α_{2B}	α_{2C}	
Clonidine[a]	7.2	7.2	6.9	0.1 nmol (0.02 μg)
				1.0 nmol (0.2 μg)
				10.0 nmol (2.0 μg)
BRL 44408[b]	7.2	5.4	6.2	0.1 nmol (0.22 μg)
				1.0 nmol (2.2 μg)
Imiloxan[b]	5.9	6.5	6.3	10 nmol (2.44 μg)
JP-1302[b]	5.3	5.1	6.9	1 nmol (0.37 μg)
				10 nmol (3.7 μg)

All data are given as pK_i.
[a](Jasper et al., 1998) or pK_D.
[b](Proudman et al., 2022) values at human receptors.

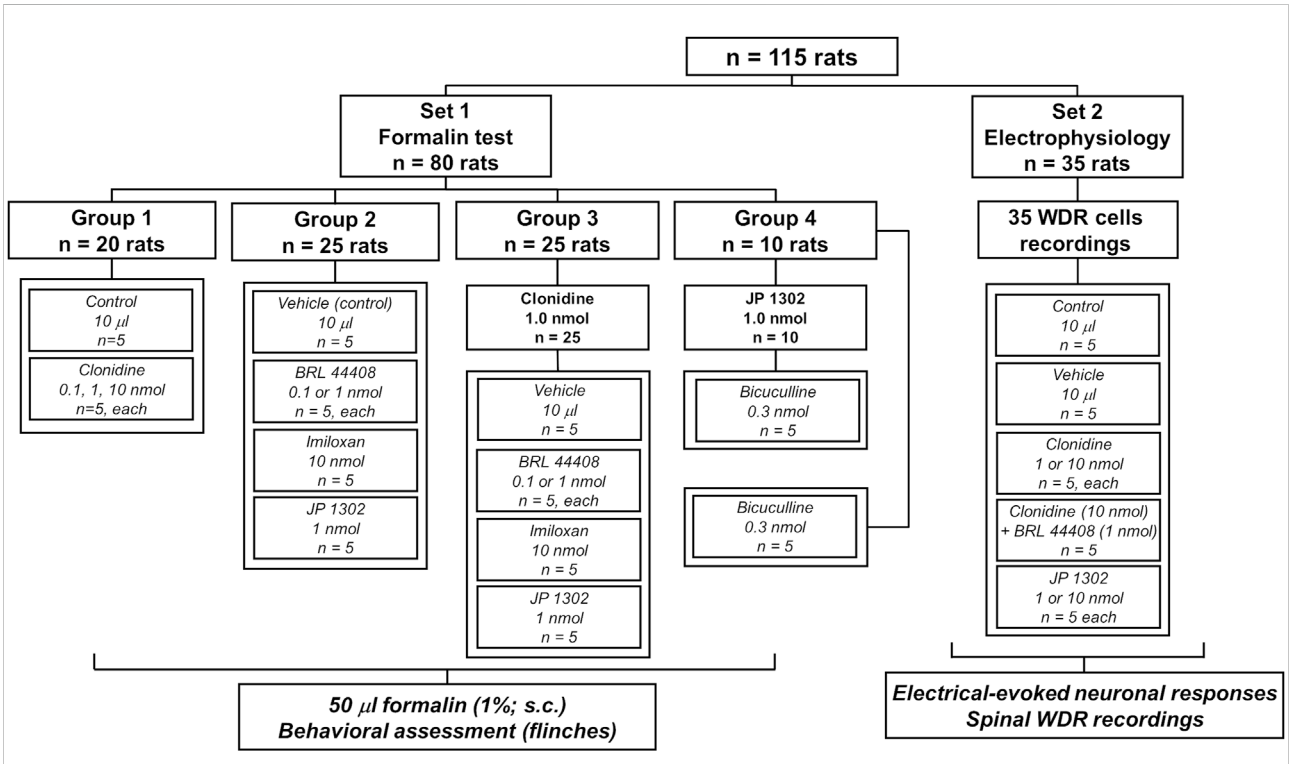

FIGURE 1
Experimental protocols showing the number of animals used in the present study. The animals were divided into two main sets to perform behavioral or *in vivo* electrophysiological recordings.

2.2 General methods

2.2.1 Surgical procedures for behavioral experiments (intrathecal surgery)

Chronic catheterization of the intrathecal (i.t.) subarachnoid space was performed as described by Yaksh and Rudy (1976). The rats were anesthetized with a ketamine-xylazine (75–10 mg/kg, i.p.) and placed in a stereotaxic frame (Kopf Instruments, United States) with the dorsal part of the head and neck previously shaved. The atlanto-occipital membrane was exposed and pierced, and a polyethylene catheter (PE-10, 9.0 cm length) was inserted intrathecally and advanced caudally to the

level of the thoracolumbar junction. The wound was then sutured, and the animals were housed in individual cages to recover from surgery for 5 days before the formalin test.

2.2.2 Formalin-induced tonic nociception and study design

The tonic nociception experiments using the 1% formalin test (Dubuisson and Dennis, 1977; Wheeler-Aceto and Cowan, 1991) were performed by the same tester, blinded to the pharmacological treatment. Rats were placed in open Plexiglass® observation chambers for 30 min for three consecutive days to allow them to become familiar with their surroundings. On the third day, and after 30 min in the Plexiglass® chamber, they were removed for formalin administration. Fifty microliters of diluted formalin (1%) were subcutaneously (s.c.) injected into the dorsal surface of the right hind paw with a 30-gauge needle. The animals were then returned to the chambers, and nocifensive behavior was observed immediately after the formalin injection. Nocifensive behavior was quantified as the number of flinches of the injected paws during 1 min periods every 5 min for up to 1 h after injection. Flinching was characterized as a rapid and brief withdrawal or as a flexing of the injected paw. As previously reported, formalin-induced flinching behavior was biphasic. The initial nociceptive phase (0–10 min) was followed by a prolonged, persistent response (15–60 min). For i.t., administration, the $\alpha_{2A/2B/2C}$-adrenoceptor agonist (clonidine) or selective antagonist (BRL 44408 to α_{2A}-, imiloxan to α_{2B}-, or JP 1302 to α_{2C}-adrenoceptors) were given in a volume of 10 µl using a Hamilton® syringe.

The first group (n = 20 rats) was subdivided into four subgroups and received an i.t., injection of vehicle (saline solution, 0.9% NaCl; n = 5) or clonidine (0.1, 1 or 10 nmol; n = 5 rats each) 10 min before formalin injection. A second group (n = 25 rats) was subdivided into five subgroups to measure the *per se* effect of vehicle (n = 5) or the antagonists, BRL 44408 (0.1 or 1 nmol; n = 5 rats each), imiloxan (10 nmol, n = 5) or JP 1302 (1 nmol; n = 5 rats each) given 20 min before formalin injection.

To determine whether clonidine-induced intrathecal antinociception was mediated by either α_{2A}-, α_{2B}-, or α_{2C}-adrenoceptors, a third group was used (n = 25 rats). In this case, the effect of pretreatment (10 min before 1 nmol clonidine) with vehicle (n = 5), BRL 44408 (0.1 or 1 nmol; n = 5 rats each), imiloxan (10 nmol; n = 5) or JP 1302 (1 nmol; n = 5) on the formalin-induced flinches was assessed.

Since JP 1302 alone inhibited the formalin-induced nociception, the involvement of GABAergic mechanisms was suspected (see discussion section). To test this hypothesis, 0.3 nmol bicuculline was given 10 min before JP 1302 (n = 5 rats; 1 nmol). Also, the *per se* effect of bicuculline on formalin flinches was assessed (n = 5 rats).

Doses and drug administration schedules for clonidine, BRL 44408, and imiloxan were selected based on previous reports (O'Neill and Haigler, 1985; Young et al., 1989; Omote et al., 1991; Uhlén et al., 1994; Erne-Brand et al., 1999; Dwyer et al., 2010) and pilot experiments in our laboratory. Behavioral or motor function changes induced by the different treatments were assessed in catheterized rats from all groups by testing the animals' ability to stand and walk in a normal posture, as proposed elsewhere (Chen and Pan, 2001).

2.2.3 Surgical procedures for electrophysiological experiments

Animals were anesthetized with urethane (2 g/kg), and then an intratracheal cannula was inserted for artificial ventilation (65–75 strokes/min; model Ro Vent®, Kent Scientific Corp. United States). Subsequently, animals were mounted onto a stereotaxic frame (Kopf Instruments, United States) and secured in a spinal cord unit frame. The lumbar vertebrae were fixed to improve stability at the recording site to perform a laminectomy to expose the L2-L4 spinal cord segments. The dura was carefully removed, and the exposed spinal cord was covered with isotonic saline to avoid desiccation. The animals were not paralyzed, and we did not observe any withdrawal response during peripheral electrical stimulation. End-tidal CO_2 was monitored with a Capstar-100 End-tidal CO_2 analyzer (CWE Inc., United States) and kept between 3.0% and 3.5% by adjusting the stroke volume. Core body temperature was maintained at 38°C using a circulating water pad.

2.2.4 Extracellular unitary recordings and study design

Extracellular unitary recordings were made using seven quartz-Pt/W microelectrodes (impedance 4–7 MΩ) mounted in a multichannel microdrive with an integrated preamplifier. This multi-electrode system was manipulated with the 7-channel version of the fiber-electrode manipulator "System Eckhorn" using Eckhorn Matrix multiuser software (Thomas Recording GmbH, Germany). The microelectrodes were lowered (400–900 µm from the surface) in small steps (2–5 µm/s) into the superficial laminae of the left dorsal horn segments to search for single-unit discharges. For each recorded cell, the specific somatic receptive field (RF) was located by gently tapping on the entire ipsilateral glabrous surface of the hind paw. When we found a RF, electrical stimulation using an S88 stimulator (Grass Instruments Co., United States) was then applied. In this case, two needles (27 G) attached to a stimulus isolator unit (PSIU6 model, Grass Instruments Co., United States) were subcutaneously inserted into the RF of the recorded neuron. The electrical stimulation was then conducted and consisted of 20 stimuli at 0.2 Hz with a 1 ms pulse duration at 1.5 times the threshold intensity (0.1–3 mA) required to evoke a C-fiber discharge. It is interesting to note that using this protocol, the spikes associated with activation of Aβ-, Aδ-, C-fibers, and post-

discharge can be observed, but the wind-up was not consistently elicited.

The extracellular neuronal activity induced by electrical stimulation of the RF was amplified ×100 (1700 Differential AC amplifier, A-M Systems, United States), digitalized, and discriminated using CED hardware and Spike 2 software (v5.15; Cambridge Electronic Design, United Kingdom). Raw and discriminated signals were fed through an audio monitor (model 3300, A-M Systems, United States) and displayed on an oscilloscope (TBS1064, Tektronix Inc., United States). Waveforms and recorded spike trains were stored on a hard drive for offline analysis. Evoked activities of the spinal dorsal horn WDR neurons were recorded and analyzed as cumulative frequency and peri-stimulus time histograms (PSTH) to detect the occurrence of neuronal responses. On this basis, the stimulating threshold to evoke action potentials and their frequency of occurrence, resulting from the stimulation of the peripheral RF on the hind paw, were attributed to the recruitment of Aβ-, Aδ-, and C-fibers. Considering the distance between the RF and the recording electrode, the peak latencies observed correspond to peripheral conduction velocities within the Aβ-, (0–20 ms), Aδ- (21–90 ms), C-fibers (90–350 ms), and post-discharge (350–800 ms) (Urch & Dickenson, 2003; González-Hernández et al., 2019; Gamal-Eltrabily et al., 2020). By using this protocol, we exclusively recorded WDR cells, a type of second-order neurons receiving input concomitantly from non-nociceptive (Aβ-type) and nociceptive (Aδ- and C-type) fibers; in addition, post-discharge was also analyzed.

Indeed, during the search of WDR cells responding to peripheral tactile and electrical RF stimulation, some neurons can be classified as only tactile sensitive and, in minor proportion, nociceptive specific; but these types of cells were not further analyzed. Thus, the number of action potentials in response to 20 RF stimuli was compared before (basal, t = 0) and after treatments. In these experiments, a control group without treatment was used (n = 5).

Accordingly, the neuronal evoked responses were evaluated immediately after (basal; t = 0) clonidine (1 or 10 nmol; n = 5 cells, each) or vehicle (isotonic saline, 0.9% NaCl; n = 5 cells) administration and at 10, 20, 30, 40, 50, and 60-min post-treatment. Since in the formalin test, clonidine-induced flinching inhibition was reversed by BRL 44408 but not by imiloxan or JP 1302, the role of the α_{2A}-adrenoceptor was evaluated by spinal administration of BRL 44408 (1 nmol; n = 5 cells; given 10 min prior clonidine). Furthermore, considering that JP 1302 exhibited antinociceptive effects in the formalin test, we tested the effects of this α_{2C}-adrenoceptor antagonist (1 or 10 nmol; n = 5 cells, each) on the electrophysiological responses of WDR cells. The vehicle and compounds were given at the spinal cord level (topical) in a total volume of 10 µl using a Hamilton® syringe.

2.3 Drugs

This study used the following compounds besides the anesthetics (ketamine, xylazine, and urethane). From Sigma Chemical Co., (United States): 1) clonidine hydrochloride (CAS number: 4205-91-8), 2) 2-[2H-(1-methyl-1,3-dihydroisoindole) methyl]-4,5-dihydroimidazole maleate (BRL 44408; CAS number: 118343-19-4), 3) 2-(1-ethyl-2-indazoyl) methyl-1,4-benzodioxan hydrochloride (imiloxan; CAS number: 81167-22-8) and 4) (-)-bicuculline methiodide (CAS number: 40709-69-1). The N-[4-(4-methyl-1-piperazinyl) phenyl]-9-acridinamine dihydrochloride (JP-1302; CAS number: 1259314-65-2) was acquired from Tocris Ltd., (United States). The doses of clonidine, BRL 44408, imiloxan, and JP 1302 refer to their free base, whereas those of the bicuculline refer to their salt (methiodide). Furthermore, in Table 1, the doses in nmol and µg is given. All drugs were dissolved in a physiological saline solution (0.9% NaCl).

2.4 Data presentation and statistical analysis

The data in the figures are presented as mean ± S.E.M., (standard error of the mean). In all cases and before performing a parametric statistical analysis, we checked for normality using the Shapiro-Wilk test ($p > 0.05$). In the formalin test, curves were constructed by plotting the number of flinches as a function of time. The area under the number of flinches against time curves (AUC), an expression of the duration and intensity of the effect, was calculated by the trapezoidal rule, and a one-way analysis of variance (ANOVA) was performed.

For electrophysiological experiments, a baseline neuronal response was established after an identified neuron had a ≤10% variation in the neuronal responses induced by RF stimulation during five consecutive tests (5 min between each trial). The number of basal (t = 0) evoked potential (total spikes and number of Aβ-, Aδ-, C-fibers and post-discharge) in the different experimental groups were analyzed; since the normality test failed, a Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks was performed (see Table 2). On this basis, and to normalize the data, the evoked potentials induced by electrical stimulation of the paw were expressed as a percentage change from the respective baseline. Thus, the baseline value refers to the evoked neuronal response before spinal treatment with clonidine or antagonists. To evaluate the stability of the recorded neurons (only for the control group and vehicle group) across the 60-min time frame, we used a one-way repeated measures ANOVA. The difference in neuronal activity evoked within one group of animals before and after treatments was compared using a two-way repeated-measures ANOVA. In addition, the temporal course was adjusted to obtain global neuronal activity due to the treatment (box and whisker plots); in this case, a one-way ANOVA was performed.

TABLE 2 Mean action potentials elicited (±s.e.m.) by twenty electrical stimuli at the basal time (t = 0) in the different experimental groups. Since the normality test failed (Shapiro-Wilk), a Kruskal-Wallis one-way ANOVA on ranks was performed to compare the action potential elicited by the different treatments.

	Control (n = 5)	Vehicle (n = 5)	Clonidine 1 nmol (n = 5)	Clonidine 10 nmol (n = 5)	Cloni (10) + BRL 44408 (n = 5)	JP 1301 1 nmol (n = 5)	JP 1301 10 nmol (n = 5)	ANOVA on ranks
Total APs	502.4 ± 80.5	724.6 ± 194.2	762.8 ± 143.4	634.2 ± 130.3	709.2 ± 209.1	617.8 ± 93.5	387.5 ± 92.0	$\chi^2 = 4.535$ $p = 0.605$
Aβ-fibers	131.8 ± 8.5	123 ± 17.5	134.8 ± 14.7	120.6 ± 20.3	141 ± 26.8	122 ± 14.31	116.0 ± 26.9	$\chi^2 = 0.872$ $p = 0.990$
Aδ-fibers	38.4 ± 10.6	60 ± 19.0	49.2 ± 14.5	65 ± 7.78	75.6 ± 22.3	70.2 ± 6.8	43.5 ± 10.2	$\chi^2 = 7.024$ $p = 0.319$
C-fibers	226.4 ± 35.6	363.20 ± 115.0	361.2 ± 101.0	316.4 ± 83.4	342 ± 119.5	293.4 ± 65.2	175.6 ± 57.0	$\chi^2 = 1.1$ $p = 0.954$
Post-discharge	105.8 ± 39.9	178.4 ± 60.4	127.60 ± 38.7	132.2 ± 40.6	150.6 ± 61.8	132.2 ± 33.9	42.3 ± 12.7	$\chi^2 = 5.809$ $p = 0.445$

The ordinary one-way ANOVA was followed (if applicable) by the Newman-Keuls *post-hoc* test, whereas in the case of the two-way repeated-measures ANOVA, Holm-Sidak's multiple comparison test was followed. Furthermore, sphericity was not assumed in the case of repeated measures ANOVAs, and corrections to degrees of freedom were made according to the Greenhouse-Geisser method. Differences were considered statistically significant when $p < 0.05$. Graphs and statistical analysis were done using GraphPad Prism V6.0 software (United States). Complete statistical analysis is detailed in Tables 2, 3.

3 Results

3.1 Intrathecal clonidine inhibits flinching behavior induced by formalin

Subcutaneous (s.c.) formalin injection into the right hind paw produced a typical flinching behavior characterized by a biphasic time course (Figure 2A; control curve). Phase I of the nociceptive response began immediately after formalin injection and gradually declined (≈10 min). Then, phase II started about 15 min after formalin injection and lasted 1 h. I.t administration of clonidine (0.1, 1, and 10 nmol) inhibited the formalin-induced flinching behavior during phases I and II (Figures 2A–C).

3.2 Effect of α_{2A}- (BRL 44408), α_{2B}- (imiloxan), and α_{2C}- (JP 1302) adrenoceptor antagonists in the clonidine-induced behavioral antinociception

As shown in Figure 3A, the antinociception induced by 1 nmol clonidine was attenuated by 1 nmol BRL 44408 and remained unaffected by 0.1 nmol BRL 44408, 10 nmol imiloxan, or 1 nmol JP 1302. In particular, 1 nmol BRL 44408 partially reverted the clonidine effect during phase I (Figure 3B) and abolished the clonidine-induced antinociception during phase II (Figure 3C).

When we tested the *per se* effects induced by the antagonists (Figure 3D), we found that i.t. administration of vehicle (0.9% NaCl solution, 10 µl), BRL 44408 (0.1, 1 nmol) or imiloxan (10 nmol) did not have a statistical difference (see Table 2 for details) on the flinching behavior induced by formalin; in contrast, i.t. JP 1302 significantly reduced the number of flinches during phase I and II (Figures 3E,F).

Since the behavioral experiments showed that clonidine inhibits the formalin-induced nociception *via* α_{2A}-adrenoceptor activation, a set of electrophysiological recordings of the second-order WDR dorsal horn spinal cord cells were performed to correlate the behavioral outcome with electrophysiological responses.

3.3 General effects of peripheral electrical stimuli on wide dynamic range cell responses

Figure 4A illustrates the experimental setup used to perform the *in vivo* unitary extracellular recordings of spinal WDR cells. In this figure, the recordings correspond to the baseline neuronal response (t = 0) elicited by twenty electrical pulses given in the paw receptive field. All neurons recorded in the present experiments were found at an average of 700 ± 192 µm from the spinal surface. As illustrated in Figures 4B,C, the peripheral electrical stimulation produces a well-defined and stereotyped WDR cell response. In general, under our parameters of electrical stimulation used (0.2 Hz; 1 ms pulse duration), we observed that although some cells exhibited high post-discharge events (see Cell 1), an inconsistent wind-up was elicited (calculated accordingly to the formula given by Gjerstad et al., 1996). As previously reported, wind-up is considered as a facilitation of neural discharge evoked by repetitive stimulation of primary afferent fibers (Mendell, 1966). This process elicited in WDR cells seems to mainly depend on the frequency stimulation, and in

TABLE 3 Ordinary one-way or two-way repeated-measures analysis of variance (ANOVA) with their respective *post hoc* comparison for each figure.

Fig	Test	*Post hoc* comparison
2	Ordinary one-way ANOVA	Newman–Keuls multiple comparison test
2A	Tx: $F_{(3, 16)} = 19.82$; $p < 0.001$	C vs. Cloni[0.1], $p < 0.001$; C vs. Cloni[1], $p < 0.001$; C vs. Cloni[10], $p < 0.001$
2C	Tx: $F_{(3, 16)} = 21.35$; $p < 0.001$	C vs. Cloni[0.1], $p < 0.001$; C vs. Cloni[1], $p < 0.001$; C vs. Cloni[10], $p < 0.001$
3	Ordinary one-way ANOVA	Newman–Keuls multiple comparison test
3B	Tx: $F_{(5, 24)} = 20.09$; $p < 0.001$	C vs. V + Cloni, $p < 0.001$; C vs. BRL[0.1]+Cloni, $p < 0.001$; C vs. BRL[1]+Cloni, $p = 0.006$; C vs. Imi[10]+Cloni, $p < 0.001$; C vs. JP[1]+Cloni, $p < 0.001$
3C	Tx: $F_{(5, 24)} = 12.4$; $p < 0.001$	C vs. V + Cloni, $p < 0.001$; C vs. BRL[0.1]+Cloni, $p < 0.003$; C vs. BRL[1]+Cloni, $p = 0.402$; C vs. Imi[10]+Cloni, $p = 0.001$; C vs. JP[1]+Cloni, $p < 0.001$
3E	Tx: $F_{(4, 20)} = 4.83$; $p = 0.007$	C vs. BRL[0.1], $p = 0.211$; C vs. BRL[1], $p = 0.205$; C vs. Imi[10], $p = 0.869$; C vs. JP[1], $p = 0.009$
3F	Tx: $F_{(4, 20)} = 3.38$; $p = 0.029$	C vs. BRL[0.1], $p = 0.831$; C vs. BRL[1], $p = 0.328$; C vs. Imi[10], $p = 0.932$; C vs. JP[1], $p = 0.046$
6		
6B	Two-way RM ANOVA	Holm–Sidak's multiple comparison test
	Interaction: $F_{(24, 120)} = 0.85$;$p = 0.664$	Basal: C vs. V, $p = 0.061$; C vs. Cloni[1], $p = 0.061$; C vs. Cloni[10], $p = 0.055$; C vs. Cloni[10]+BRL, $p = 0.093$
		min 10: C vs. V, $p = 0.132$; C vs. Cloni[1], $p = 0.035$; C vs. Cloni[10], $p = 0.096$; C vs. Cloni[10]+BRL, $p = 0.096$
	Time: $F_{(2.53, 50.57)} = 2.79$; $p = 0.059$	min 20: C vs. V, $p = 0.512$; C vs. Cloni[1], $p = 0.271$; C vs. Cloni[10], $p = 0.271$; C vs. Cloni[10]+BRL, $p = 0.422$
	Tx: $F_{(4, 20)} = 2.23$; $p = 0.1023$	min 30: C vs. V, $p = 0.797$; C vs. Cloni[1], $p = 0.302$; C vs. Cloni[10], $p = 0.721$; C vs. Cloni[10]+BRL, $p = 0.721$
		min 40: C vs. V, $p = 0.893$; C vs. Cloni[1], $p = 0.359$; C vs. Cloni[10], $p = 0.595$; C vs. Cloni[10]+BRL, $p = 0.673$
		min 50: C vs. V, $p = 0.626$; C vs. Cloni[1], $p = 0.201$; C vs. Cloni[10], $p = 0.604$; C vs. Cloni[10]+BRL, $p = 0.626$
		min 60: C vs. V, $p = 0.947$; C vs. Cloni[1], $p = 0.829$; C vs. Cloni[10], $p = 0.829$; C vs. Cloni[10]+BRL, $p = 0.837$
6C	Two-way RM ANOVA	Holm–Sidak's multiple comparison test
	Interaction: $F_{(24, 120)} = 2.35$;$p = 0.001$	Basal: C vs. V, $p = 0.282$; C vs. Cloni[1], $p = 0.122$; C vs. Cloni[10], $p = 0.547$; C vs. Cloni[10]+BRL, $p = 0.074$
		min 10: C vs. V, $p = 0.593$; C vs. Cloni[1], $p = 0.431$; C vs. Cloni[10], $p = 0.289$; C vs. Cloni[10]+BRL, $p = 0.445$
		min 20: C vs. V, $p = 0.766$; C vs. Cloni[1], $p = 0.042$; C vs. Cloni[10], $p = 0.022$; C vs. Cloni[10]+BRL, $p = 0.058$
	Time: $F_{(3.698, 73.96)} = 5.25$; $p = 0.001$	min 30: C vs. V, $p = 0.223$; C vs. Cloni[1], $p = 0.033$; C vs. Cloni[10], $p = 0.005$; C vs. Cloni[10]+BRL, $p = 0.096$
	Tx: $F_{(4, 20)} = 12.39$; $p < 0.001$	min 40: C vs. V, $p = 0.525$; C vs. Cloni[1], $p = 0.021$; C vs. Cloni[10], $p = 0.002$; C vs. Cloni[10]+BRL, $p = 0.153$
		min 50: C vs. V, $p = 0.412$; C vs. Cloni[1], $p = 0.006$; C vs. Cloni[10], $p < 0.001$; C vs. Cloni[10]+BRL, $p = 0.032$
		min 60: C vs. V, $p = 0.872$; C vs. Cloni[1], $p = 0.012$; C vs. Cloni[10], $p = 0.012$; C vs. Cloni[10]+BRL, $p = 0.872$
6D	Two-way RM ANOVA	Holm–Sidak's multiple comparison test
	Interaction: $F_{(24, 120)} = 2.79$; $p < 0.001$	Basal: C vs. V, $p = 0.866$; C vs. Cloni[1], $p = 0.866$; C vs. Cloni[10], $p = 0.866$; C vs. Cloni[10]+BRL, $p = 0.866$
		min 10: C vs. V, $p = 0.287$; C vs. Cloni[1], $p = 0.056$; C vs. Cloni[10], $p = 0.033$; C vs. Cloni[10]+BRL, $p = 0.287$
	Time: $F_{(3.3, 66.0)} = 3.52$; $p = 0.017$	min 20: C vs. V, $p = 0.079$; C vs. Cloni[1], $p = 0.165$; C vs. Cloni[10], $p = 0.011$; C vs. Cloni[10]+BRL, $p = 0.165$
	Tx: $F_{(4, 20)} = 6.29$; $p < 0.002$	min 30: C vs. V, $p = 0.153$; C vs. Cloni[1], $p = 0.153$; C vs. Cloni[10], $p = 0.010$; C vs. Cloni[10]+BRL, $p = 0.107$
		min 40: C vs. V, $p = 0.037$; C vs. Cloni[1], $p = 0.126$; C vs. Cloni[10], $p = 0.018$; C vs. Cloni[10]+BRL, $p = 0.008$
		min 50: C vs. V, $p = 0.462$; C vs. Cloni[1], $p = 0.462$; C vs. Cloni[10], $p = 0.003$; C vs. Cloni[10]+BRL, $p = 0.046$
		min 60: C vs. V, $p = 0.991$; C vs. Cloni[1], $p = 0.861$; C vs. Cloni[10], $p = 0.047$; C vs. Cloni[10]+BRL, $p = 0.879$
6E	Two-way RM ANOVA	Holm–Sidak's multiple comparison test
	Interaction: $F_{(24, 104)} = 1.72$; p = 0.03	Basal: C vs. V, p = 0.922; C vs. Cloni[1], p = 0.922; C vs. Cloni[10], p = 0.922; C vs. Cloni[10]+BRL, p = 0.851
		min 10: C vs. V, p = 0.906; C vs. Cloni[1], p = 0.388; C vs. Cloni[10], p = 0.102; C vs. Cloni[10]+BRL, p = 0.962
	Time: $F_{(4.038, 70.0)} = 0.59$; p = 0.67	min 20: C vs. V, p = 0.933; C vs. Cloni[1], p = 0.933; C vs. Cloni[10], p = 0.059; C vs. Cloni[10]+BRL, p = 0.933
	Tx: $F_{(4, 20)} = 4.62$; p < 0.008	min 30: C vs. V, p = 0.819; C vs. Cloni[1], p = 0.819; C vs. Cloni[10], p = 0.019; C vs. Cloni[10]+BRL, p = 0.619
		min 40: C vs. V, p = 0.995; C vs. Cloni[1], p = 0.995; C vs. Cloni[10], p = 0.004; C vs. Cloni[10]+BRL, p = 0.995
		min 50: C vs. V, p = 0.875; C vs. Cloni[1], p = 0.763; C vs. Cloni[10], p = 0.096; C vs. Cloni[10]+BRL, p = 0.763
		min 60: C vs. V, p = 0.966; C vs. Cloni[1], p = 0.966; C vs. Cloni[10], p = 0.148; C vs. Cloni[10]+BRL, p = 0.963
6F	Ordinary one-way ANOVA	
	Tx: $F_{(4, 20)} = 1.385$; $p = 0.275$	
6G	Ordinary one-way ANOVA	Newman–Keuls multiple comparison test

(Continued on following page)

TABLE 3 (*Continued*) Ordinary one-way or two-way repeated-measures analysis of variance (ANOVA) with their respective *post hoc* comparison for each figure.

Fig	Test	*Post hoc* comparison
	Tx: $F_{(4, 20)} = 12.974$; $p < 0.001$	C vs. V, $p = 0.4$; C vs. Cloni[1], $p < 0.001$; C vs. Cloni[10], $p < 0.001$; C vs. Cloni[10]+BRL, $p = 0.1$
6H	Ordinary one-way ANOVA	Newman–Keuls multiple comparison test
	Tx: $F_{(4, 20)} = 6.413$; $p = 0.002$	C vs. V, $p = 0.2$; C vs. Cloni[1], $p = 0.2$; C vs. Cloni[10], $p < 0.001$; C vs. Cloni[10]+BRL, $p = 0.9$
6I	Ordinary one-way ANOVA	Newman–Keuls multiple comparison test
	Tx: $F_{(4, 20)} = 4.169$; $p = 0.013$	C vs. V, $p = 0.9$; C vs. Cloni[1], $p = 0.9$; C vs. Cloni[10], $p = 0.018$; C vs. Cloni[10]+BRL, $p = 0.8$
7		
7C	Two-way RM ANOVA	Holm–Sidak's multiple comparison test
	Interaction: $F_{(18, 96)} = 1.49$; $p = 0.109$	Basal: C vs. V, $p = 0.061$; C vs. JP[1], $p = 0.099$; C vs. JP[10], $p = 0.099$ min 10: C vs. V, $p = 0.246$; C vs. JP[1], $p = 0.246$; C vs. JP[10], $p = 0.620$
	Time: $F_{(2.8, 44.76)} = 2.35$; $p = 0.089$	min 20: C vs. V, $p = 0.526$; C vs. JP[1], $p = 0.526$; C vs. JP[10], $p = 0.526$
	Tx: $F_{(3, 16)} = 0.782$; $p = 0.522$	min 30: C vs. V, $p = 0.941$; C vs. JP[1], $p = 0.744$; C vs. JP[10], $p = 0.941$
		min 40: C vs. V, $p = 0.981$; C vs. JP[1], $p = 0.907$; C vs. JP[10], $p = 0.981$
		min 50: C vs. V, $p = 0.735$; C vs. JP[1], $p = 0.735$; C vs. JP[10], $p = 0.735$
		min 60: C vs. V, $p = 0.947$; C vs. JP[1], $p = 0.615$; C vs. JP[10], $p = 0.799$
7D	Two-way RM ANOVA	Holm–Sidak's multiple comparison test
	Interaction: $F_{(18, 96)} = 1.63$; $p = 0.67$	Basal: C vs. V, $p = 0.213$; C vs. JP[1], $p = 0.213$; C vs. JP[10], $p = 0.136$ min 10: C vs. V, $p = 0.645$; C vs. JP[1], $p = 0.645$; C vs. JP[10], $p = 0.243$
	Time: $F_{(3.39, 54.27)} = 0.99$; $p = 0.409$	min 20: C vs. V, $p = 0.766$; C vs. JP[1], $p = 0.255$; C vs. JP[10], $p = 0.199$
	Tx: $F_{(3, 16)} = 2.351$; $p = 0.111$	min 30: C vs. V, $p = 0.443$; C vs. JP[1], $p = 0.443$; C vs. JP[10], $p = 0.443$
		min 40: C vs. V, $p = 0.665$; C vs. JP[1], $p = 0.223$; C vs. JP[10], $p = 0.665$
		min 50: C vs. V, $p = 0.411$; C vs. JP[1], $p = 0.117$; C vs. JP[10], $p = 0.055$
		min 60: C vs. V, $p = 0.739$; C vs. JP[1], $p = 0.143$; C vs. JP[10], $p = 0.739$
7E	Two-way RM ANOVA	Holm–Sidak's multiple comparison test
	Interaction: $F_{(18, 96)} = 1.60$; $p = 0.074$	Basal: C vs. V, $p = 0.774$; C vs. JP[1], $p = 0.774$; C vs. JP[10], $p = 0.349$ min 10: C vs. V, $p = 0.287$; C vs. JP[1], $p = 0.448$; C vs. JP[10], $p = 0.201$
	Time: $F_{(2.37, 37.95)} = 0.415$; $p = 0.697$	min 20: C vs. V, $p = 0.079$; C vs. JP[1], $p = 0.233$; C vs. JP[10], $p = 0.084$
	Tx: $F_{(3, 16)} = 0.829$; $p = 0.497$	min 30: C vs. V, $p = 0.221$; C vs. JP[1], $p = 0.287$; C vs. JP[10], $p = 0.287$
		min 40: C vs. V, $p = 0.055$; C vs. JP[1], $p = 0.338$; C vs. JP[10], $p = 0.338$
		min 50: C vs. V, $p = 0.462$; C vs. JP[1], $p = 0.464$; C vs. JP[10], $p = 0.464$
		min 60: C vs. V, $p = 0.991$; C vs. JP[1], $p = 0.922$; C vs. JP[10], $p = 0.990$
7F	Two-way RM ANOVA	Holm–Sidak's multiple comparison test
	Interaction: $F_{(18, 91)} = 2.31$; p = 0.005	Basal: C vs. V, p = 0.922; C vs. JP[1], p = 0.946; C vs. JP[10], p = 0.922 min 10: C vs. V, p = 0.972; C vs. JP[1], p = 0.972; C vs. JP[10], p = 0.972
	Time: $F_{(2.011, 30.5)} = 1.31$; p = 0.286	min 20: C vs. V, p = 0.840; C vs. JP[1], p = 0.840; C vs. JP[10], p = 0. 840
	Tx: $F_{(3, 16)} = 0.916$; p = 0.456	min 30: C vs. V, p = 0.666; C vs. JP[1], p = 0.652; C vs. JP[10], p = 0. 652
		min 40: C vs. V, p = 0.824; C vs. JP[1], p = 0.404; C vs. JP[10], p = 0.506
		min 50: C vs. V, p = 0.733; C vs. JP[1], p = 0.743; C vs. JP[10], p = 0.733
		min 60: C vs. V, p = 0.595; C vs. JP[1], p = 0.595; C vs. JP[10], p = 0.421
7G	Ordinary one-way ANOVA	Newman–Keuls multiple comparison test
	Tx: $F_{(3, 16)} = 4.308$; $p = 0.021$	C vs. JP, $p = 0.013$; C vs. JP + Bicu[0.3], $p = 0.218$; C vs. Bicu[0.3], $p = 0.181$
7H	Ordinary one-way ANOVA	Newman–Keuls multiple comparison test
	Tx: $F_{(4, 20)} = 6.413$; $p = 0.002$	C vs. JP, $p = 0.047$; C vs. JP + Bicu[0.3], $p = 0.638$; C vs. Bicu[0.3], $p = 0.9$

Abbreviations: Control (C); Clonidine (Cloni); BRL 44408 (BRL); Imiloxan (Imi); JP 1302 (JP); Bicuculline (Bicu).

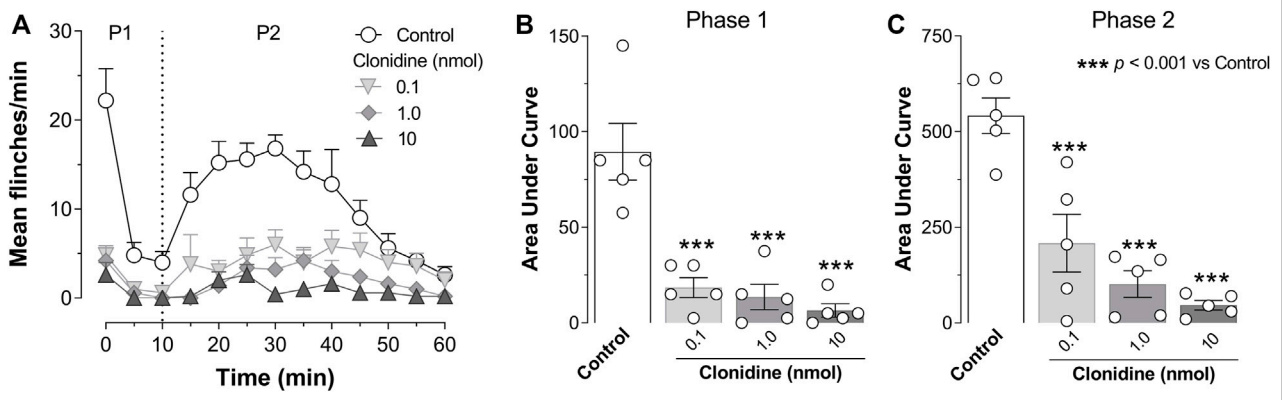

FIGURE 2
Intrathecal (i.t.) clonidine ($\alpha_{2A/2B/2C}$-adrenoceptor agonist) inhibits behavioral nociception. **(A)** Flinching number over time during phase I (P1) and phase II (P2) of the mean number per minute of flinches observed after i.t., clonidine (0.1, 1, and 10 nmol; $n = 5$ each dose) in rats submitted to the 1% formalin test (50 µl, s.c.). **(B,C)** show the time course data expressed as the area under the mean number of flinches against the time curve (AUC). Clonidine reduced AUC values during both phases (P1, P2), indicating an antinociceptive effect. Clonidine was given 10 min prior to formalin injection.

general, frequencies >0.5 Hz are required to consistently produce a wind-up (Mendell, 1966; Dickenson and Sullivan, 1987; Chapman and Dickenson, 1992; Li et al., 1999; You et al., 2003); in this sense, 0.2 Hz seems not to be enough to recruit a facilitatory input leading to wind-up.

Furthermore, at the basal time (t = 0), the mean average evoked potential of all animals tested was 667 ± 29 spikes. Since the evoked potential did not follow a normal distribution along the experimental groups, a non-parametric test was used, and we found no statistically significant difference (see Figure 4G; Table 2 for details). The following results were normalized to perform a parametric statistical analysis.

Since we recorded spinal WDR cells for 60 min, analyzing the effect of time on the neuronal responses was crucial to exclude a time-dependent effect. The one-way ANOVA suggests that no time-dependent changes in neuronal responses occurred during our experimental protocols. Specifically, in the control group, time had no effect on Aδ- ($F_{(2.73,\ 10.90)} = 0.213$; $p = 0.869$) or C-fibers ($F_{(2.09,\ 8.36)} = 1.92$; $p = 0.197$). Similar results were obtained in the case of vehicle administration [Aδ- ($F_{(2.68,\ 10.76)} = 0.204$; $p = 0.869$) and C-fibers ($F_{(2.27,\ 9.09)} = 0.892$; $p = 0.455$)].

3.4 Spinal clonidine-induced electrophysiological antinociception is reversed by BRL 44408

Figure 5 shows the raw tracing of a single WDR neuron cluster response, the raster plots, and the PSTHs before and after different treatments. Figure 5A shows the PSTHs obtained during the control condition, whereas Figures 5B,C exemplify the neural activity of second-order WDR cells before (basal response) and after clonidine treatment. In contrast to the control group (Figure 5A), spinal clonidine, particularly at 10 nmol (Figure 5C), diminished the neuronal firing responses (events) elicited by the RF electrical stimulation. We must clarify that each PSTH represents the neuronal evoked activity of one WDR cell induced by twenty electrical pulses; these evoked neuronal events could be broken down according to the conduction velocities of the primary afferent fibers (i.e., Aβ-, Aδ-, and C-fibers).

In this set of experiments, we found that BRL 44408 reversed the antinociception induced by clonidine. Figure 6A shows an example of a raw tracing of a single WDR neuron cluster response, the raster plots, and PSTH obtained in an animal pre-treated with BRL 44408 before clonidine administration. Note that BRL 44408 blocked the clonidine-induced diminution of WDR neuronal activity.

Upon quantifying the data from the PSTHs, we found that spinal clonidine decreases the firing response elicited by electrical stimuli on the RF (Figures 6B–I). Specifically, clonidine has no substantial effect on the neuronal activity associated with Aβ-fiber activation (Figure 6B), but we observed a potent inhibition of neuronal activity related to the activation of Aδ- (Figure 6C) rather than C-fibers (Figure 6D). This antinociceptive effect started 10 min after clonidine administration, peaked at 40–50 min and lasted up to 60 min as previously reported (Sullivan et al., 1987; Bonnet et al., 1989; Murata et al., 1989). Furthermore, as expected, the clonidine-induced inhibition of nociceptive Aδ- and C-fiber activity was reversed by spinal pre-treatment with BRL 44408 (1 nmol). In addition, as shown in Figure 6E, the post-discharge activity was reduced by 10 nmol clonidine, an

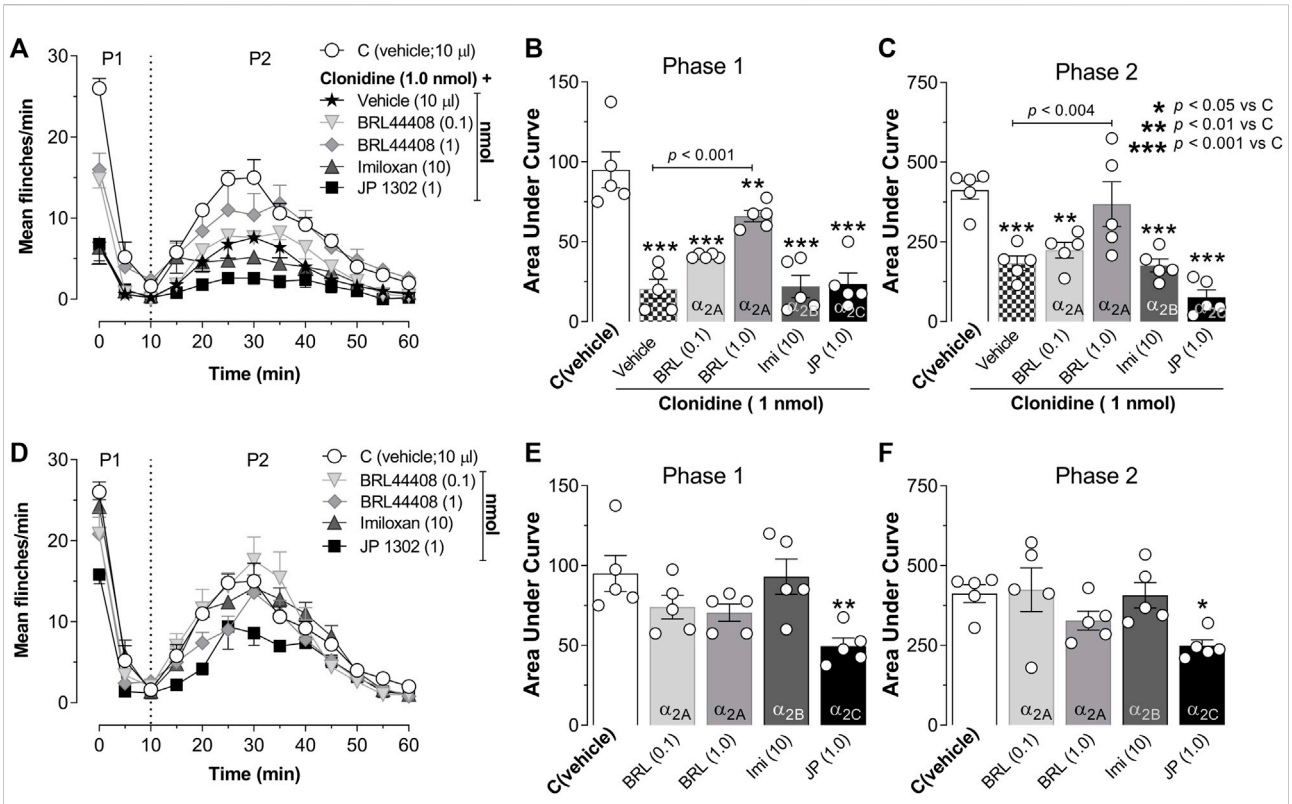

FIGURE 3
Role of spinal α_{2A}-adrenoceptors in the clonidine-induced behavioral antinociception and the antinociceptive effect of JP 1302 (α_{2C}-adrenoceptor antagonist). **(A)** shows the effect of intrathecal (i.t.) injection of vehicle (10 µl; isotonic saline solution) or $\alpha_{2A/2B/2C}$-adrenoceptor antagonists on the clonidine-induced inhibition of the flinches induced by formalin (1%; 50 µl, s.c.) during phase I (P1) and phase II (P2). Vehicle, BRL 44408 (0.1 or 1 nmol; an α_{2A}-adrenoceptor antagonist; n = 5 each dose), imiloxan (10 nmol; an α_{2B}-adrenoceptor antagonist; n = 5), or JP 1302 (1 nmol; an α_{2C}-adrenoceptor antagonist; n = 5) were given i.t. 10 min prior clonidine. **(B,C)** represent the data as the area under the mean number of flinches against the curve (AUC) and show that BRL 44408 (1 nmol) inhibited the clonidine-induced antinociception in both phases (P1, P2). During P2, JP 1302 seems to enhance (statistically non-significant, $p > 0.05$) the antinociception induced by clonidine. **(D)** shows the *per se* effect of BRL 44408 (n = 5 each dose), imiloxan (n = 5), or JP 1302 (n = 5 each dose) in rats submitted to the 1% formalin test. The AUC in **(E,F)** suggest that BRL 44408 and imiloxan do not affect formalin-induced nociception. In contrast, JP 1302 diminishes the AUC values during both phases (P1, P2), suggesting an antinociceptive effect.

effect reversed by BRL44408. Analyzing these results as global neuronal activity (Figures 6F–I), we corroborated that clonidine selectively blocks the neuronal activity associated with the activation of primary nociceptive fibers, and this effect is abolished by a selective α_{2A}-adrenoceptor antagonist (BRL 44408). Together, these results highlight the relevance of primary afferent fibers innervating second-order WDR cells in clonidine-induced antinociception.

3.5 Spinal JP 1302 does not influence the neuronal activity of second-order wide dynamic range cells

Based on the behavioral results, where 1 nmol JP 1302 *per se* inhibited the flinches evoked by formalin (Figures 3D–F), we tested the effect of JP 1302 on WDR neuronal activity (Figures 7A–F). The results showed that spinal JP 1302 (1 or 10 nmol) did not affect the peripheral evoked neuronal activity of Aβ-, Aδ-, C-fibers and post-discharge (Figures 7C–F). These results may imply that the mechanisms involved in the antinociception induced by JP 1302 are not mediated by an action on WDR neurons or primary afferent fibers innervating these second-order cells.

3.6 JP 1302-induced behavioral antinociception is blocked by spinal bicuculline

On this basis and considering that some evidence suggests that α_{2C}-adrenoceptors decrease GABAergic transmission (see discussion section), we hypothesized that the JP 1302-induced behavioral antinociception could be indirectly mediated by a spinal GABAergic mechanism. Accordingly, i.t., administration of bicuculline (0.3 nmol; a

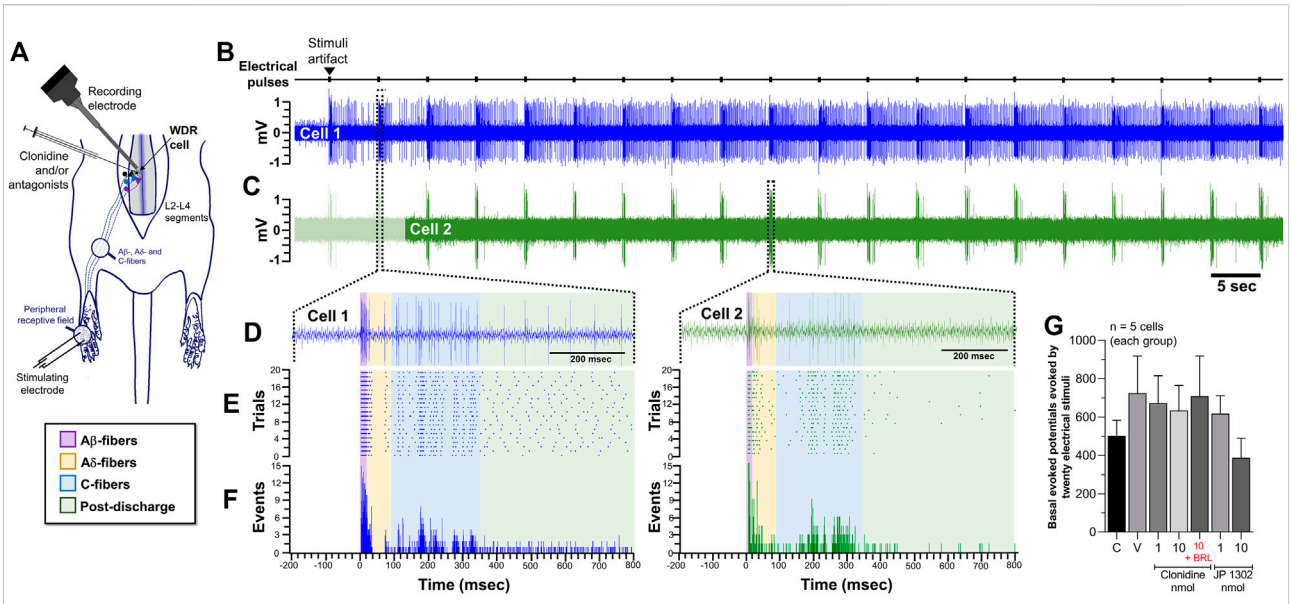

FIGURE 4
Effect of peripheral electrical stimulation on the WDR cell response. **(A)** Experimental set-up design illustrating the site of electrophysiological recording of WDR cells at spinal L2-L4 segments, and the location, in the ipsilateral paw of the electrical stimulation applied to the receptive field (RF). **(B)** Raw data of twenty stimulus artifacts and **(C)** raw data of consecutive WDR responses to RF stimulation in two different cells (Cell 1 and Cell 2). **(D)** shows the raw tracing of a single second-order WDR neuron cluster response induced by one electrical stimulus-evoked in Cell 1 (left) or Cell 2 (right). **(E)** shows the raster plot and **(F)** peri-stimulus time histograms (PSTH) constructed from twenty WDR neuronal responses to RF stimulation depicting the different fiber components (Aβ-, Aδ-, C-fibers, and post-discharge). Note that, under our experimental condition, no clear wind-up is elicited. **(G)** shows that the basal response, i.e., the total of action potentials evoked at t = 0 in the different experimental groups, is not statistically different (χ^2 = 4.535; p = 0.605).

$GABA_A$ receptor blocker) abolished the behavioral antinociception induced by JP 1302 (1 nmol) in phases I and II (Figures 7G–I). In this case, and as previously reported in the formalin test (Dirig and Yaksh, 1995; Peng et al., 2015; Ryu et al., 2021) bicuculline *per se* does not influence flinching behavior.

4 Discussion

4.1 General

This study was designed to pharmacologically dissect the role of different $\alpha_{2A/2B/2C}$-adrenoceptor subtypes in clonidine-induced antinociception. Using tonic (i.e., formalin test) or acute (i.e., peripheral electrical stimulation) nociceptive stimuli, we show that clonidine-induced antinociception is mediated by α_{2A}- but not $\alpha_{2B/2C}$-adrenoceptor activation. Given that in the electrophysiological experiments, clonidine inhibited the neuronal activity associated with electrical activation of Aδ- and C-fibers (but not Aβ-fibers), a presynaptic effect on nociceptive primary afferent fibers (PAFs) is supported. Furthermore, the ongoing firing (post-discharge) was attenuated by clonidine; an effect also reversed by the BRL 44408. Besides, we found that spinal JP 1302 (a selective α_{2C}-adrenoceptor antagonist) induced *per se* behavioral but not electrophysiological antinociception, an effect reversed by bicuculline (a preferential $GABA_A$ receptor blocker). Apart from the implications discussed below, our data support the notion that: 1) antinociception (acute or tonic) induced by clonidine is mediated by α_{2A}-adrenoceptors, whereas 2) pharmacological blockade of α_{2C}-adrenoceptors during tonic nociception elicits antinociception, thus, unmasking a potential pronociceptive action of this receptor (Figure 8).

One important thing in the experimental protocols followed was that the effect of clonidine was analyzed during 1 h. This experimental design was based on the long-lasting antinociception induced by clonidine when given by a spinal route, despite the half-life in the spinal cord being ~30 min (Castro and Eisenach, 1989; Khan et al., 1999). Accordingly, seminal reports have consistently shown that this compound induces a spinal long-lasting (>1 h) analgesia/antinociception peaking after 30 min post-injection (Sullivan et al., 1987; Bonnet et al., 1989; Murata et al., 1989; Khan et al., 1999). At first glance, we could hypothesize that this long-lasting effect could be partly mediated by the engagement of supraspinal mechanisms enhancing a descending inhibitory pathway. Indeed, we must acknowledge that activation of the endogenous noradrenergic descending pathway plays a key role in spinal pain modulation in naïve and neuropathic rodents (Patel et al., 2018). Nevertheless, Murata et al. (1989) showed in WDR cell recordings that antinociception elicited by spinal clonidine was

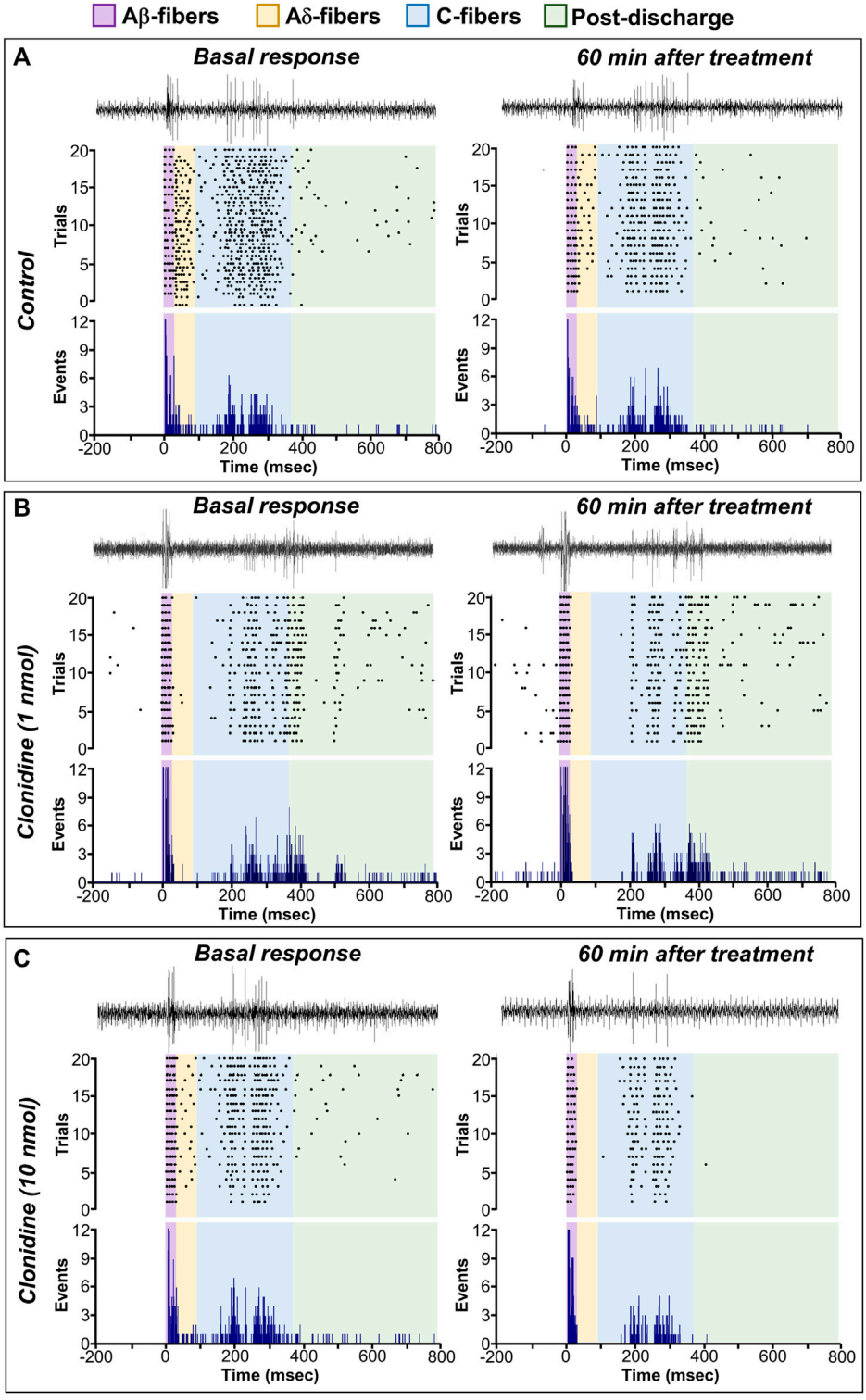

FIGURE 5
Spinal clonidine selectively blocks the nociceptive neuronal firing of WDR cells. **(A–C)** depict the raw tracing of a single second-order WDR neuron cluster response induced by one electrical stimulus (upper tracing), the raster plot (middle), and peri-stimulus time histograms (PSTH) constructed from 20 WDR responses to receptive field (RF) electrical stimulation before (basal) and after treatment. In accordance with the fiber conduction velocities, the panels in the figure depict the different fiber components (Aβ-, Aδ-, C-fibers, and post-discharge) of the spinal WDR cell response. Note that clonidine (1 and 10 nmol) diminished the neuronal activity; this clonidine-induced inhibition (observed as a diminution of events) is mainly associated with neural action on nociceptive fibers (i.e., Aδ- and C-fibers) and ongoing (post-discharge) activity, whereas the firing of Aβ-fibers remains unaltered.

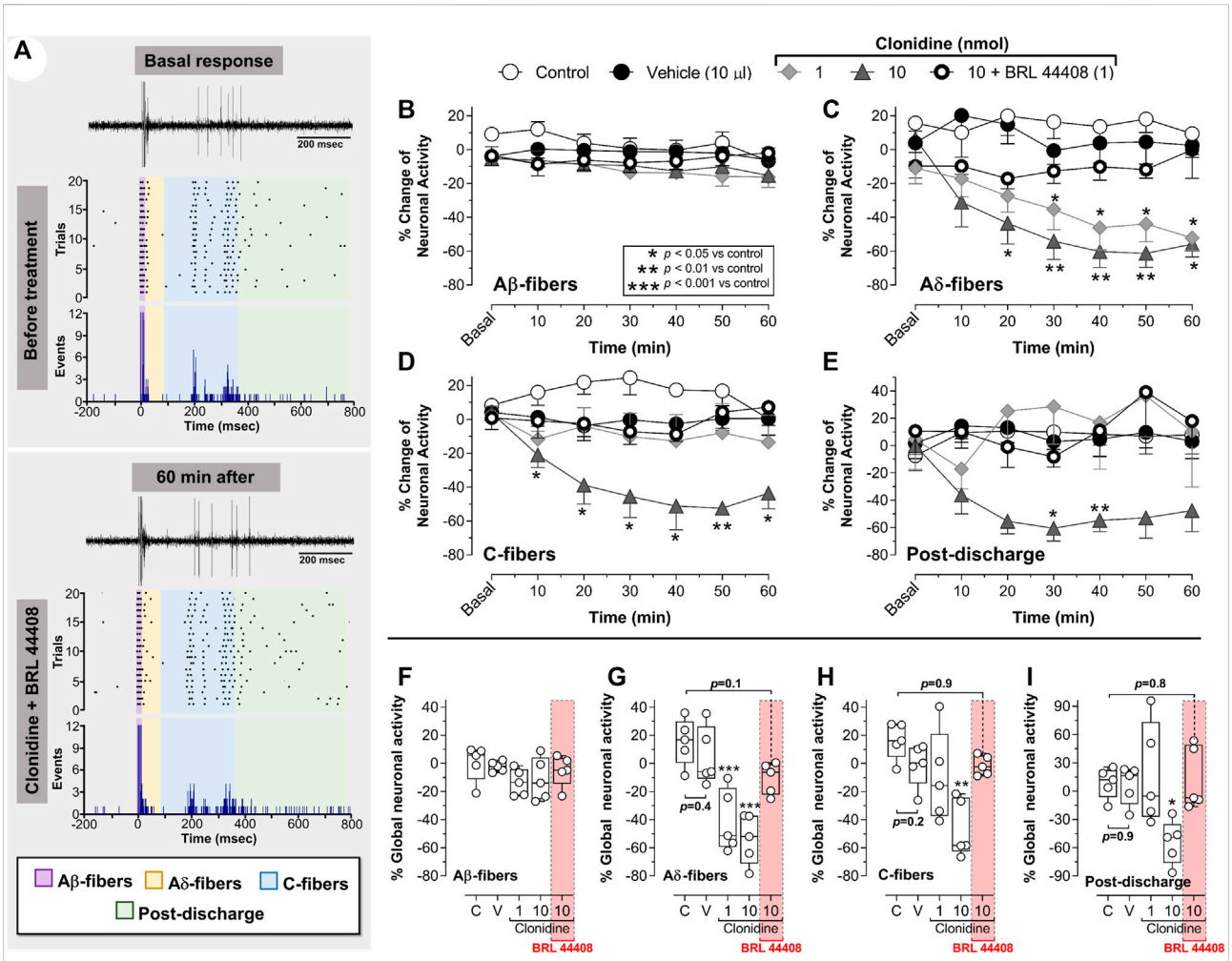

FIGURE 6
The role of spinal α_{2A}-adrenoceptors in clonidine-induced electrophysiological antinociception. In **(A)**, a pair of raw tracings of a single second-order WDR neuron cluster response induced by one electrical stimulus (upper tracing), the raster plot (middle), and peri-stimulus time histogram (PSTH) depicting the effect of BRL 44408 on the clonidine-induced antinociception. Notice that under α_{2A}-adrenoceptors blockade with BRL 44408 (1 nmol), the antinociceptive action of clonidine is not elicited. **(B–E)** show the time-course changes in the percentage average of the different fibers activating WDR cell responses and post-discharge elicited by receptive field (RF) electrical stimulation and the effects of spinal clonidine (1 and 10 nmol/10 µl; *n* = 5 each dose). Notice that 1 nmol clonidine only inhibits the neuronal firing of Aδ-fibers **(C)**, while 10 nmol clonidine inhibits the activity of Aδ-fibers **(C),** C-fibers **(D)**, and post-discharge **(E)**. **(F–I)** show the global neuronal activity (obtained from the respective time course figures) of Aβ-, Aδ-, C-fibers, and post-discharge in response to spinal clonidine. Clonidine preferentially blocks the neuronal activity associated with Aδ-, C-fibers, and post-discharge but not Aβ-fibers. On the other hand, blockade of the α_{2A}-adrenoceptors with BRL 44408 (1 nmol) inhibits the clonidine-induced electrophysiological antinociception.

unaffected in animals spinally transected. Consequently, it seems that the effect of this ligand (when given spinally) mainly relies upon spinal cord level.

4.2 Spinal clonidine inhibits the behavioral and electrophysiological nociception by α_{2A} rather than $\alpha_{2B/2C}$-adrenoceptor activation

Our data showed that i.t., clonidine reduces the number of flinches evoked by 1% formalin (Figure 2). This effect is related to spinal α_2-adrenoceptor activation (Pertovaara, 2013; Llorca-Torralba et al., 2016; Bahari and Meftahi, 2019). Since pretreatment with BRL 44408 (but not imiloxan or JP 1302) reversed the clonidine-induced behavioral antinociception, the role of α_{2A}-adrenoceptors in clonidine's effect is supported (Figures 3A–C). Accordingly, our data agree with the consensus that α_{2A}-adrenoceptor is relevant to the spinal clonidine effect (Pertovaara, 2013).

In this regard, the effect of spinal clonidine on WDR cells was tested to give an electrophysiological correlate. At the spinal level, it is well-known (Urch and Dickenson, 2003) that these second-order neurons receive concomitant input from PAFs, and

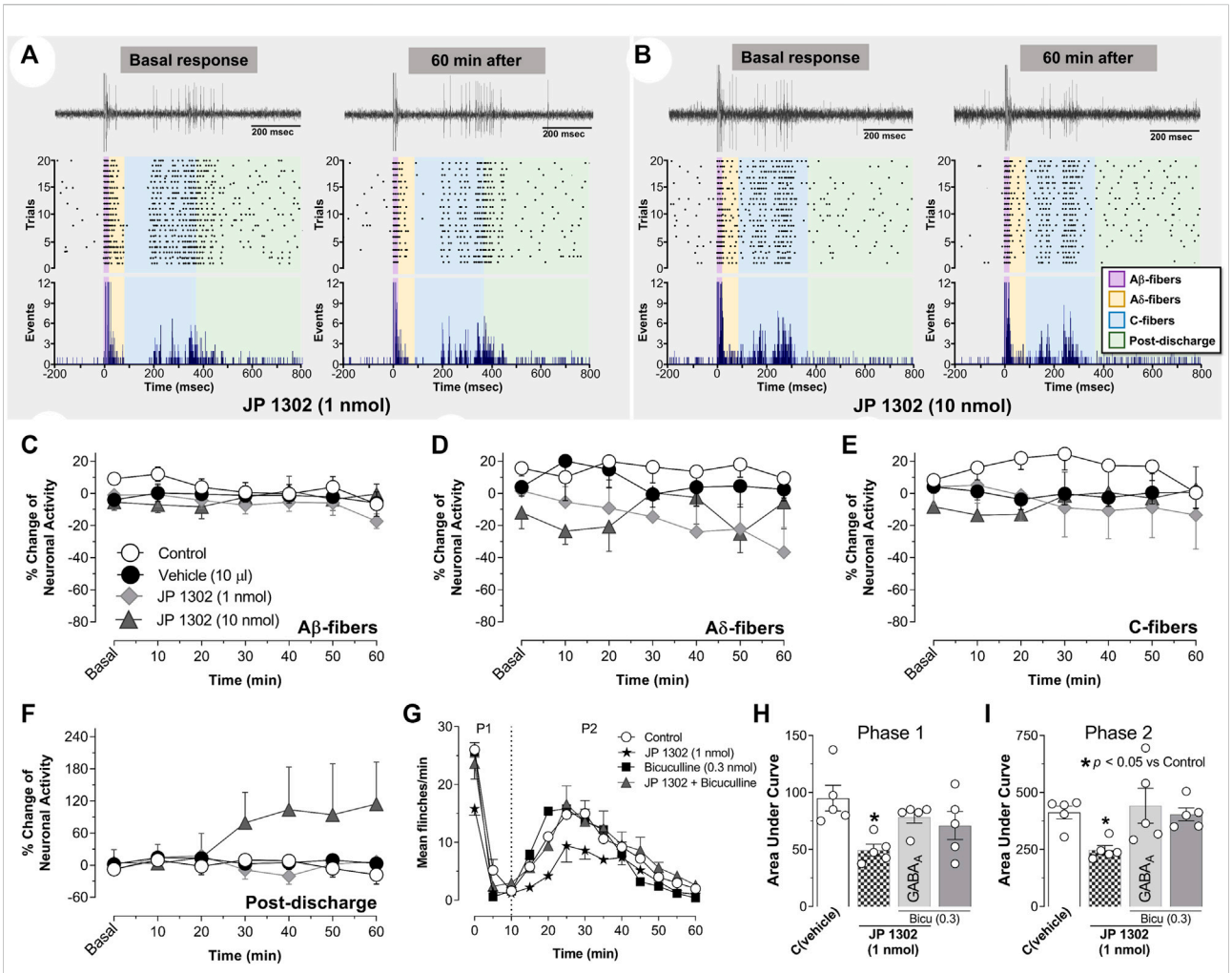

FIGURE 7
Spinal JP-1302 does not affect wide dynamic range (WDR) cell activity, but behaviorally, it inhibits nociception by increasing GABAergic activity. **(A,B)** depict the peri-stimulus time histogram (PSTH) constructed from 20 WDR responses to receptive field (RF) electrical stimulation before (basal) and after treatment (vehicle or JP 1302); also, in these panels, the raster plot and raw tracing of a single second-order WDR neuron cluster response elicited by one electrical stimulus (upper tracing) are illustrated. In accordance with the fiber conduction velocities, the panels in the figure depict the different fiber components (Aβ-, Aδ-, and C-fibers) of the spinal WDR cell response. Note that JP 1302 seems not to exert an effect on the WDR neuronal activity; indeed, when the data are analyzed as a percent of change of the neuronal activity **(C–F)**, JP 1302 does not impact (statistically) the neuronal firing associated with the activation of Aβ-, Aδ-, C-fibers, and post-discharge. **(G)** shows the effect of intrathecal (i.t.) injection of 0.3 nmol bicuculline (a preferent $GABA_A$ receptor blocker) on the JP 1302-induced inhibition of the flinches induced by formalin (1%; 50 µl, s.c.) during phase I (P1) and phase II (P2). Note that bicuculline *per se* did not affect formalin-induced flinching behavior, but this $GABA_A$ channel blocker reversed the effect of JP 1302. **(H,I)** represent the data as the area under the mean number of flinches against the curve (AUC) and show that bicuculline inhibited the JP 1302-induced antinociception in both phases (P1, P2).

electrical stimulation of the peripheral RF produces a triphasic response corresponding to the activation of Aβ-, Aδ- and C-fibers (Figure 4), also a post-discharge can be elicited (Figure 4). This experimental approach let us analyze the impact of clonidine in nociceptive transmission, particularly how this ligand affects the firing of PAFs. As illustrated in the PSTHs of WDR cells (Figure 5) or the temporal course of neuronal activity (Figure 6), 10 nmol clonidine preferentially diminishes the neuronal firing associated with activation of nociceptive Aδ- and C-fibers, supporting the notion that spinal clonidine-induced inhibition via presynaptic action (i.e., on PAFs). In addition, the ongoing activity (i.e., the post-discharge) was also inhibited by 10 nmol clonidine. In direct support of these findings, *in vivo* (Sonohata et al., 2004) and *in vitro* (Pan et al., 2002; Kawasaki et al., 2003) electrophysiological recordings show that α_2-adrenoceptor activation diminished the excitatory postsynaptic current evoked by PAF stimulation. At this point, we need to remember that post-discharge represents a late response of C-fibers to peripheral stimulation that can be endured by a

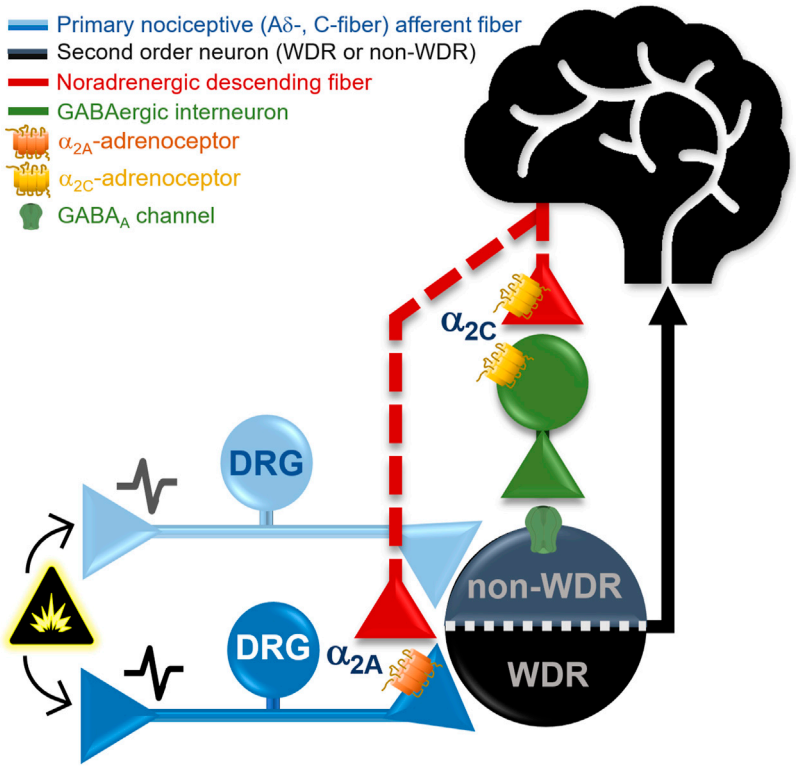

FIGURE 8
Proposed mechanisms implying a differential role of the α_{2A}- and α_{2C}-adrenoceptors subtypes in pain modulation at the spinal cord level. In general, α_2-adrenoceptors are coupled to $G_{i/o}$ proteins; thus, when activated, they could promote the inhibition of neurotransmitter release by inhibiting adenylate cyclase activity and inactivating Ca^{2+} channels and/or facilitation of K^+ currents. Our behavioral and electrophysiological data support the contention that clonidine (a non-selective $\alpha_{2A/2B/2C}$-adrenoceptor agonist) induces antinociception *via* activation of α_{2A}-adrenoceptors, probably located at presynaptic level (in the primary nociceptive Aδ- and C-fibers), causing a diminution of the WDR cell nociceptive firing. Indeed, α_{2A}-adrenoceptors activation inhibits tonic (formalin) and acute (electrical) nociceptive input. On the other hand, the selective α_{2C}-adrenoceptor antagonist, JP 1302, produces *per se* only behavioral but not electrophysiological antinociception (measured on second WDR cells), suggesting an inhibitory effect on tonic nociception. Since JP 1302-induced antinociception was blocked by bicuculline, a GABAergic mechanism has been resembled. The data could be interpreted as follows: (i) presynaptic activation of α_{2A}-adrenoceptors by clonidine inhibits acute and tonic peripheral nociception; (ii) the JP 1302-induced antinociception is not mediated by inhibition of the nociceptive primary afferent fibers activity; and (ii) considering that α_{2C}-adrenoceptors can be found in GABAergic neurons, we could suggest that under a tonic nociceptive stimulus (e.g., inflammatory), activation of α_{2C}-adrenoceptors play a pronociceptive role. Hence (see Discussion for details), the data support the notion that α_{2A}-adrenoceptor are mainly localized in primary nociceptive afferent fibers modulating the activity of WDR cells. In contrast, α_{2C}-adrenoceptors seem to be present in spinal interneurons rather than capsaicin-sensitive fibers modulating second-order nociceptive specific cells.

traumatic injury leading to a neuronal hyperexcitability observed electrophysiologically as a wind-up (Woolf, 1996). However, under our experimental approach, we were unable to induce wind-up. Hence, an interesting question not addressed in the present work relates to how wind-up can be affected by noradrenergic transmission.

Accordingly, to the behavioral experiments, we also showed that presynaptic pharmacological blockade of α_{2A}-adrenoceptors in WDR recordings is relevant to the effect of clonidine. Although these data agree with histological/molecular data showing the presence of α_{2A}-adrenoceptors at the PAF level (i.e., Aδ- and C-fibers) (Stone et al., 1998; Birder and Perl, 1999), no direct *in vivo* evidence about a functional role of the presynaptic α_{2A}-adrenoceptor subtype had been previously reported.

Regarding α_{2B}-adrenoceptors, the behavioral experiments reject the involvement of this adrenoceptor subtype. As shown in Figures 3A–C supramaximal dose of imiloxan (α_{2B}-adrenoceptor antagonist) does not affect clonidine's antinociception. Current literature seems to support the notion that spinal α_{2B}-adrenoceptor does not influence pain modulation (for refs., see Pertovaara, 2013; Llorca-Torralba et al., 2016; Bahari and Meftahi, 2019). For example, in an inflammatory pain model, Zhang et al. (2012) showed that spinal α_{2B}-adrenoceptor blockade with imiloxan does not reverse the antinociception induced by enhancement of the descending noradrenergic tone, suggesting that this receptor subtype is not necessary for the noradrenergic analgesic effect. However, in neuropathic pain models involving ligature of spinal

nerves (not tested in the present experiments) and using selective ligands, it seems that α_{2B}-adrenoceptor acquire a role in the inhibition of nociception (Chu et al., 2015; Rodríguez-Palma et al., 2022) an effect probably related with cortical activation of this receptor subtype rather than spinal mechanism (Chu et al., 2015). In essence, our data suggest that spinal α_{2B}-adrenoceptor does not play a role in tonic inflammatory and acute nociception.

In contrast, current data about the functional role of α_{2C}-adrenoceptors inhibiting nociception is scarce, and as discussed by Pertovaara (2013), the impact of this receptor subtype in spinal nociception is unclear. These inconsistent data are partly due to the lack of selective compounds used to dissect the contribution of different $\alpha_{2A/2B/2C}$-adrenoceptor subtypes. For example, in transgenic mice, Fairbanks et al. (2002) showed that α_{2C}-adrenoceptors have a subtle role in the moxonidine (a mixed I_1 imidazoline/α_{2C}-adrenoceptor agonist)-induced antinociception, whereas Malmberg et al. (2001) suggest that α_{2A}- but not α_{2C}-adrenoceptors are indispensable for the antinociceptive action of i.t., dexmedetomidine (a non-selective $\alpha_{2A/2B/2C}$-adrenoceptor antagonist).

In our behavioral experiments, we used JP 1302, a selective α_{2C}-adrenoceptor antagonist (Sallinen et al., 2007; Proudman et al., 2022), to test the role of this receptor in clonidine-induced antinociception. According to Figures 3A–C i.t., JP 1302 was unable to block clonidine's effect. Similar results were found in a neuropathic pain model (Rodríguez-Palma et al., 2022) or writhes-induced by sleep deprivation (Yaoita et al., 2018), showing that systemic JP 1302 does not preclude the behavioral antinociceptive action of α_2-adrenoceptor agonists (ST-91 or tizanidine given systemically). However, when we analyzed the *per se* effects of the antagonist (Figures 3D–F), we found that in sharp contrast to BRL 44408 or imiloxan, i.t., JP 1302 diminishes the number of flinches induced by formalin (in phase I and II), implying that this antagonist has an antinociceptive effect during the formalin test.

4.3 The potential pronociceptive role of α_{2C}-adrenoceptors in tonic nociception

In contradiction to the behavioral data, when we explored the effect of JP 1302 using an electrophysiological approach (Figures 7A–F), no effect was observed in the neuronal firing of WDR cells. This discrepancy could be attributable to the nature of the noxious stimulus used (i.e., tonic vs. acute) and may imply that JP 1302 exerts its antinociceptive action in a different way than clonidine not involving presynaptic or WDR cell inhibition. Admittedly, an interesting iteration to try to disentangle and give an electrophysiological correlate would have been to analyze the effect of these ligands on wind-up, taking into account that this neuronal process has been related with central sensitization due to recruitment of nociceptive circuits beyond of Aδ- and C-fibers activation. Regardless, if we consider that this compound exerts an antinociceptive effect *via* the blockade of α_{2C}-adrenoceptors, it is interesting to note that α_{2C}-adrenoceptors have been localized in non-noradrenergic brainstem descending fibers and postsynaptic sites in spinal interneurons (Stone et al., 1998; Olave and Maxwell, 2002). To our knowledge, no report about an antinociceptive *per se* action of selective α_{2C}-adrenoceptor antagonists exist. Consequently, the question is: How can α_{2C}-adrenoceptor blockade induce spinal antinociception?

Proudman et al. (2022) suggest that the selectivity of JP 1302 is reliable for dissecting between the $\alpha_{2A/2B/2C}$-adrenoceptor subtypes (see Table 1), but this antagonist also displays some affinity for α_{1A}-adrenoceptors (pK_i:6.2). Hence, we cannot ignore that interaction between JP 1302 and α_{1A}-adrenoceptors could exist. Certainly, using an optogenetic approach, Kucharczyk et al. (2022) showed that activating a discrete noradrenergic descending pathway from locus coeruleus provokes a diminution of WDR activity, an effect blocked by prazosin (a non-selective $\alpha_{1A/1B/1D}$-adrenoceptor antagonist), suggesting a role for α_1-adrenoceptors. However, in the formalin test, i.t., prazosin does not affect nocifensive behavior (Jeong et al., 2011; Park et al., 2011). Similarly, the clonidine-induced inhibition of WDR responses evoked by NMDA was unaffected by prazocin (Zhang et al., 1998). Indeed, subcutaneous formalin injection tends to decrease the [^{3}H]-prazosin binding sites in the spinal cord (Nalepa et al., 2005), suggesting that under an inflammatory stimulus, the probability that this receptor could be exerting a pharmacological effect is minimal. Besides, using an acute (thermal) or neuropathic pain model (L_5 - L_6 nerve ligation), i.t., α_1-adrenoceptor agonist (methoxamine) does not have any impact on the pain threshold (Nagasaka and Yaksh, 1990; Yaksh et al., 1995). Together these data support our contention that under our experimental conditions, the effect of JP-1302 may be mediated by its interaction with the α_{2C}-adrenoceptor.

If spinal α_{2C}-adrenoceptor blockade produces an antinociceptive effect in the formalin test but not in the electrophysiological experiments, the most straightforward interpretation of these findings may suggest (but does not prove) that under tonic nociception α_{2C}-adrenoceptor activation counter-balance the antinociceptive effect of descending noradrenergic system. In this regard, subcutaneous formalin induces not only an increase in the endogenous descending noradrenergic activity (Ma et al., 2001; Sajadeianfard et al., 2005; Martins et al., 2013) but also a rise in the mRNA expression of α_{2C}-adrenoceptor at spinal cord level (Yoon et al., 2011). Coupled with the evidence suggesting that this receptor is expressed in GABAergic interneurons (Holmberg et al., 1999; Olave and Maxwell, 2002), we hypothesize that during spinal tonic nociceptive input, activation of this receptor could, in turn, diminish the GABAergic transmission, favoring a pronociceptive state. This hypothesis gain weight

considering that at the striatum level, it has been suggested that activation of α_{2C}-, but not α_{2A}-adrenoceptors inhibits GABA release (Holmberg et al., 1999; Zhang and Ordway, 2003). Hence, i.t., JP 1302 indirectly favors an antinociceptive effect by improving the activity of GABAergic neurons. To prove this hypothesis, and using the formalin test, we assessed the effect of spinal bicuculline (unspecific antagonist of the $GABA_A$ receptors) on the JP 1302-induced antinociception.

At this point, we must emphasize that 0.3 nmol (0.11 μg) bicuculline *per se* does not influence flinching behavior induced by formalin (Figures 6F–H). This data agrees with previous reports showing that i.t., <0.3 μg bicuculline (<0.8 nmol) does not impact this nocifensive behavior (Dirig and Yaksh, 1995; Peng et al., 2015; Ryu et al., 2021). However, although some reports showed that spinal bicuculline is pronociceptive, this effect depends on the dose of bicuculline and the explored pain model. In the case of the formalin test, Kaneko and Hammond (1997) showed that at formalin concentrations <1%, an increase of flinches is induced by bicuculline, particularly at 0.3 μg (i.e., 0.8 nmol). Therefore, under our experimental conditions (1% formalin), the concentration used of bicuculline (0.3 nmol equivalents to 0.11 μg) seems to be adequate to evaluate the role of GABAergic participation in the JP 1302 effect.

The data show that the antinociception induced by JP 1302 was reversed with 0.3 nmol bicuculline (Figures 7H–J), suggesting that JP 1302 favors GABAergic transmission. *In vitro* evidence indicates that GABAergic neurons in the substantia gelatinosa can be hyperpolarized by noradrenaline (Hantman and Perl, 2005). Specifically, by recording GABAergic neurons at the superficial dorsal horn level using the patch-clamp technique, Gassner et al. (2009) proved that although the main effect of noradrenaline on GABAergic neurons is depolarization, a minor proportion of these cells are hyperpolarized *via* α_2-adrenoceptors. In these studies, the specific receptor subtype was not identified. Together, these data support our contention that by inhibiting GABAergic neurotransmission, α_{2C}-adrenoceptors may play a pronociceptive role (particularly during tonic nociception). The pronociceptive role of this receptor has also been suggested in *in vitro* assays by Chen et al. (2008), where they showed that α_{2C}-adrenoceptor activation inhibits opioid release in the spinal cord; thus, blocking this receptor would induce antinociception.

4.4 A final consideration about the role of spinal α_2-adrenoceptors subtypes in pain modulation

Our data suggest that during tonic pain, both α_{2A}- and α_{2C}-adrenoreceptors are activated, and one question that could arise is: exist a particular situation where α_{2C}- surpass the antinociceptive effect of α_{2A}-adrenoreceptors? Current evidence shows that the net impact of non-selective α_2-adrenoceptor agonists is antinociception in acute and neuropathic pain conditions (for refs. see Bahari and Meftahi, 2019), pointing out the relevance of α_{2A}-over α_{2C}-adrenoreceptors. Certainly, we need to keep in mind that plastic changes in the spinal α_2-adrenoreceptors occur under neuropathic pain and may explain, at least in part, the variability in the efficacy of α_2-adrenoreceptor agonists depending on the stage of neuropathic pain (Yoon et al., 2011). At this point, it is worth mentioning that the expression of the different $\alpha_{2A/2B/2C}$-adrenoceptor subtypes at the spinal cord level under pathological pain has been analyzed. Stone et al. (1999) showed that after spinal nerve ligation, the immunostaining of α_{2A}-adrenoceptor is reduced, whereas α_{2C}-adrenoceptor is enhanced. Hayashida and Eisenach (2010) discussed the physiological relevance of this change, suggesting that under peripheral nerve injury, the function of α_2-adrenoceptors can be altered. Furthermore, the relevance of α_{2C}-adrenoreceptor in spinal nociception will be answered with a selective α_{2C}-adrenoreceptor agonist (not available yet) or the use of more precise approaches (e.g., opto- and chemogenetics). Regardless, at the spinal level, the effect of clonidine in healthy and pathological pain is analgesia. Therefore, our results deserve further investigation.

Finally, at the spinal dorsal horn level, the role of noradrenergic transmission is far more complex than initially conceived. This complexity may depend on the type of adrenergic receptor stimulated, the localization of the receptor (e.g., PAFs, interneurons, second-order neurons), and the type of nociceptive input. For example, at the spinal trigeminal level, both α_{2A}-, and α_{2C}-adrenoceptors seem to inhibit nociceptive transmission (Villalón et al., 2012). More recently, it has been suggested that at the spinal cord level, the noradrenergic system elicits a fine-tunning of nociception by activation of α_1-or α_2-adrenoceptors; indeed, they proposed that spinal α_2-adrenoceptors located presynaptically in the noradrenergic projections from the ventral LC can be modulating the firing of GABAergic interneurons (see Suppl Fig 2 in Kucharczyk et al. (2022)). Furthermore, as illustrated by Hickey et al. (2014), optogenetic experiments demonstrate that stimulation of different regions of locus coeruleus can evoke an antinociceptive or pronociceptive spinal effect; in this case, although i.t., atipamezole (a non-selective $\alpha_{2A/2B/2C}$-adrenoceptor antagonist) blocked the spinal clonidine-induced antinociception, this antagonist also seems to enhance the antinociceptive action of optogenetic stimulation of the noradrenergic system in the LC, but the mechanism/receptor by which atipamezole enhances antinociception was not further explored. This divergent effect of noradrenergic transmission on pain modulation has also been observed in healthy humans, where activation

of α_2-adrenoceptors (by yohimbine) elicits both antinociceptive and pronociceptive actions (Vo and Drummond, 2015).

4.5 Conclusion

Taken together, our data suggest that spinal α_{2A}- and α_{2C}-adrenoceptors exert a differential (opposite) effect on nociceptive transmission. Specifically, α_{2A}-adrenoceptor activation on PAF inhibits the tonic and acute nociceptive input, whereas α_{2C}-adrenoceptor activation appeared to inhibit GABAergic transmission, favoring nociception during a tonic (inflammatory) stimulus.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and the protocol was approved by the Institutional Animal Care and Use Committee at the Instituto de Neurobiología, UNAM.

Author contributions

Conceptualization and resources (JL-C, MC-L, AG-H), investigation (GL-C, GM-L, AG-H), data curation (GL-C, AG-H), formal analysis (GL-C, AG-H), supervision (GM-L, MC-L, AG-H), writing and editing (all authors).

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Classifying migraine using PET compressive big data analytics of brain's μ-opioid and D2/D3 dopamine neurotransmission

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Introduction: Migraine is a common and debilitating pain disorder associated with dysfunction of the central nervous system. Advanced magnetic resonance imaging (MRI) studies have reported relevant pathophysiologic states in migraine. However, its molecular mechanistic processes are still poorly understood *in vivo*. This study examined migraine patients with a novel machine learning (ML) method based on their central μ-opioid and dopamine D2/D3 profiles, the most critical neurotransmitters in the brain for pain perception and its cognitive-motivational interface.

Methods: We employed compressive Big Data Analytics (CBDA) to identify migraineurs and healthy controls (HC) in a large positron emission tomography (PET) dataset. 198 PET volumes were obtained from 38 migraineurs and 23 HC during rest and thermal pain challenge. 61 subjects were scanned with the selective μ-opioid receptor (μOR) radiotracer [^{11}C]Carfentanil, and 22 with the selective dopamine D2/D3 receptor (DOR) radiotracer [^{11}C]Raclopride. PET scans were recast into a 1D array of 510,340 voxels with spatial and intensity filtering of non-displaceable binding potential (BP_{ND}), representing the receptor availability level. We then performed data reduction and CBDA to power rank the predictive brain voxels.

Results: CBDA classified migraineurs from HC with accuracy, sensitivity, and specificity above 90% for whole-brain and region-of-interest (ROI) analyses. The most predictive ROIs for μOR were the insula (anterior), thalamus (pulvinar, medial-dorsal, and ventral lateral/posterior nuclei), and the putamen. The latter, putamen (anterior), was also the most predictive for migraine regarding DOR D2/D3 BP_{ND} levels.

Discussion: CBDA of endogenous μ-opioid and D2/D3 dopamine dysfunctions in the brain can accurately identify a migraine patient based on their receptor availability across key sensory, motor, and motivational processing regions. Our ML-based findings in the migraineur's brain neurotransmission partly explain the

severe impact of migraine suffering and associated neuropsychiatric comorbidities.

KEYWORDS

migraine disease, artificial intelligence, dopamine (raclopride), μ-opioid (carfentanil), computer-aided diagnosis, PET imaging data, CBDA

Introduction

Migraine is a pain disorder with a prevalence of more than one billion people worldwide (Global Burden of Disease GBD, 2017). It dramatically impacts the patients' daily lives with frequent headache attacks, neuropsychiatric comorbidities, and the potential for substance abuse when unremitted, especially opiates (Bigal et al., 2009; Buse et al., 2012; Adams et al., 2015; Lipton et al., 2020). Because of this significant sensory and cognitive-motivational dysregulation in migraine, the endogenous μ-opioid and D2/D3 dopamine (DA) molecular mechanisms have recently been investigated as the potential main culprits of the disorder (Zubieta et al., 2002; De Felice et al., 2013; Martikainen et al., 2013; Jassar et al., 2019). Pharmacologically, they are the targets for the action of the most potent exogenous analgesic and psychotic drugs available. Positron emission tomography (PET) studies with [^{11}C]Carfentanil, a selective μ-opioid receptor (μOR) agonist radiotracer, have demonstrated *in vivo* a decrease in μOR availability (non-displaceable binding potential [BP_{ND}], where BP_{ND} is equal to the distribution volume ratio, DVR, minus one; BP_{ND} = DVR-1) in the brains of episodic migraineurs during headache attacks and allodynia (DaSilva et al., 2014a; Nascimento et al., 2014a). The μOR has a high affinity for the μ-opioid peptides enkephalins and beta-endorphin. The measure of specific uptake of [^{11}C]Carfentanil, BP_{ND}, decreases when there is an increase in endogenous μ-opioid (peptides) release and *vice versa*. The migraine attacks were also accompanied by an increase in DA D2/D3 receptor (DOR) BP_{ND} measured by [^{11}C]Raclopride in the basal ganglia; and the longer the history and recurrence of migraine attacks, the lower the ictal (i.e., during the migraine attacks) endogenous DA release (DaSilva et al., 2017). Interestingly, migraine has been associated with a higher prevalence of DA-deficient disorders, including Restless Legs Syndrome and Parkinson's disease (PD) (Cervenka et al., 2006; Scher et al., 2014).

A growing number of laboratories are attempting to translate migraine and chronic pain neuroimaging data to a more precision-oriented clinical approach by objectively classifying patients via potential brain biomarkers (Zhang et al., 2016; Lamichhane et al., 2021; Dumkrieger et al., 2022; Hsiao et al., 2022; Wang et al., 2022). Machine learning (ML) methods have been firstly implemented in migraine research with electroencephalography (EEG) (Frid et al., 2020) and structural/functional magnetic resonance imaging (MRI) (Chong et al., 2017; Ferroni et al., 2020; Hong et al., 2020).

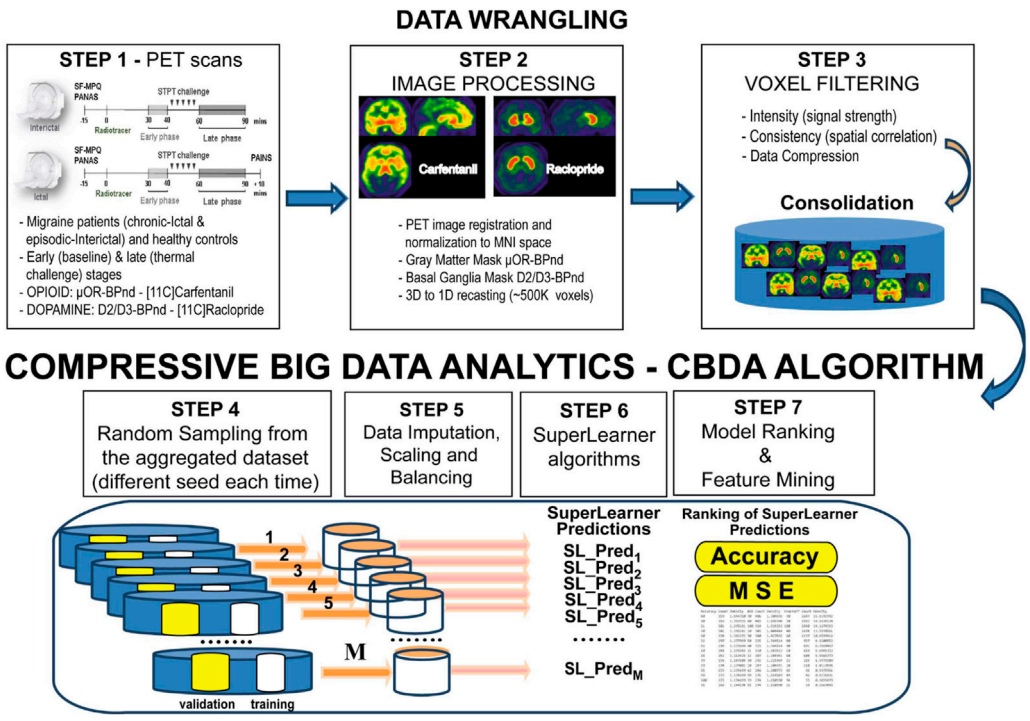

FIGURE 1
End-to-end representation of the CBDA protocol. The initial data wrangling (Steps 1-3) is data-dependent, while the remaining steps (Steps 4-7) of the CBDA protocol are data-independent

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TABLE 1 Data and CBDA specifications.

Carfentanil CFN opioid subjects						Raclopride RCL dopamine subjects	
Healthy		23 (Female: 17, Male: 6)				10 (Female: 5, Male: 5)	
Migraine		38 (Female: 23 [EM(*)], 6 [CM(*)], Male: 8 [EM], 1 [CM])				12 (Female: 7 [EM] Male: 5 [EM])	
Images (Early and Late Stages)		138(**)				60(**)	
	Whole Brain	ROI			Whole Brain?	ROI	
	Gray Matter mask [voxels]	Insula [voxels]	Thalamus [voxels]	Putamen [voxels]	Basal Ganglia mask [voxels]	Putamen [voxels]	
(***)Unfiltered data	510,340	5,318	2,320	2,402	510,340	2,402	
Threshold data	4,675	1,926	1,864	1,547	2,295	2,101	
Threshold value	2	1.1	1.1	1.1	2	1.1	

(*)CM: chronic migraine, EM: episodic migraine(**)8episodic migraineurs underwent both CFN, and RCL, scans during interictal and ictal states. Thus, the images are actually referring to 122 and 44 unique subjects, respectively for CFN, and RCL, scans.(***)The first row here represents the complete # of voxels for each "region" (i.e., WB, insula, Thalamus, Putamen), the second row is the # of voxels left for predictive analytics if we use the threshold in the third row.

However, due to its cost and protocol complexity, molecular pain studies with PET have been scarce and limited to ML approaches specific to a single region of interest or a single radiotracer (Torrado-Carvajal et al., 2021). A multimodal ML technique would be of immense clinical value to characterize migraine based on its broad neurotransmitters-receptor interactions and multiple brain regions associated with it.

This novel study has been conducted following best practices in algorithm development in nuclear medicine and artificial intelligence (AI) (Bradshaw et al., 2021). We use a promising semi-supervised machine learning (ML) technique, called Compressive Big Data Analytics (CBDA) (Marino et al., 2018; Marino et al., 2020), to identify predictive migraine biomarkers spatially at the molecular level *in vivo*. The CBDA protocol exploits the concepts of subsampling and ensemble predictors to investigate the core principles of distribution-free and model-agnostic methods for scientific inference based on Big Data sets. Ensemble predictor algorithms and subsampling or bootstrapping use common approaches for objective function optimization, quantification of noise, bias, prediction error, and variance estimation during the learning process. CBDA combines in a unique way function optimization and statistical inference. An end-to-end representation of the Migraine PET CBDA protocol is shown in Figure 1. The initial data wrangling (Steps 1-3, Figure 1) is data-dependent, while the remaining steps (Steps 4-7, Figure 1) of the CBDA protocol are data-independent. The ensemble predictor embedded in CBDA is SuperLearner (van der Laan et al., 2007), a data-adaptive black-box ML approach to prediction and density estimation. SuperLearner uses cross-validation to estimate the performance of multiple ML models. Currently, the SuperLearner library comprises 60 different classification, regression, and artificial intelligence (AI) algorithms to strengthen its predictive power and sensitivity (see *Material and Methods* section for details on the Migraine CBDA workflow).

A CBDA R package (Marino and Dinov, 2018) can be deployed on a desktop/laptop environment and a high-performance computing platform using the LONI graphical pipeline environment (Rex et al., 2003). Our previous studies (Marino et al., 2018; Marino et al., 2020) showcased the robustness, efficiency, accuracy, and viability of our first-generation CBDA protocol on small-medium-large size data.

This study enhances the protocol to handle PET imaging data specific to migraine and brain double neurotransmission-receptor data. We first perform extensive data wrangling on the 3D imaging data and recast them into 1D vectors of PET intensities. A thresholding protocol has been designed and implemented to reduce the large 1D vector of PET intensities (see *Material and Methods* section for details).

Across the different predictive analytics tasks, CBDA yielded accurate results with robust sensitivity and high specificity metrics. The identified top predictive brain regions of interest (ROIs) confirmed existing results in the migraine literature (DaSilva et al., 2014b; DaSilva et al., 2017; Jassar et al., 2019; Kim et al., 2021). These results suggest new potential avenues for dissecting the molecular brain mechanisms underlying migraine and its treatment. PET imaging and the open-source classifier CBDA point to the importance of using these techniques in synergy for accurate mining *in vivo* of the main biomarkers of

migraine regarding endogenous μ-opioid and dopamine D2/D3 transmitter-receptor dynamics in the migraine brain.

Materials and methods

PET data collection protocol

Thirty-eight migraine subjects, seven chronic migraine (CM), 31 episodic migraine (EM), and 23 healthy control (HC) participants, 20–64 years old, underwent structured clinical phone screening for formal diagnosis as per the International Headache Society Classification (ICHD-3-beta) (Headache Classification. Committee of the International Headache Society, 2004), were studied (see Table 1 for details). The clinical samples (7 chronic migraine, eight episodic migraine and eight healthy controls) used in this study overlaps with the sample utilized in our previous publications (DaSilva et al., 2014a; DaSilva et al., 2017; Jassar et al., 2019). All participants were recruited through local advertisement at the University of Michigan, Internet and flyers on bulletin boards. The exclusion criteria included pregnancy, opioid or hormonal contraceptive use 6 months prior to recruitment, other chronic pain disorders, as well as major systemic medical or psychotic disorders. Additionally, all subjects underwent a urine drug screening to ensure the absence of substance abuse, including cocaine, amphetamine, methamphetamine, marijuana, and opioids. More details on inclusion and inclusion criteria please refer to previous publications (DaSilva et al., 2014a; DaSilva et al., 2017; Jassar et al., 2019).

A total of 198 PET images obtained after scanning 61 subjects, comprising two cohorts of 38 migraines subjects (mean 28.84 ± standard deviation 8.4; nine men and 29 women) and 23 HCs (mean 26.27 ± standard deviation 6.25, six men and 17 women) during both rest and cutaneous thermal pain threshold were used to train and validate the CBDA predictive model.

All 61 subjects were scanned outside (interictal) and/or during (ictal) migraine attacks using PET with the selective μ-opioid receptor (μOR) radiotracer [^{11}C]Carfentanil. Additionally, a subset of 22 subjects were scanned using PET with the selective dopamine D2/D3 receptor (DA) radiotracer [^{11}C]Raclopride. Specifically, seven chronic migraineurs underwent ictal CFN PET (; a total of 14 images). Thirty-one episodic migraineurs underwent interictal CFN PET (62 images total). Twelve out of 31 episodic migraineurs had interictal RCL PET (24 images total) and eight of 31 did ictal CFN/RCL PET (32 images total). In total, there were 132 images for all migraineurs.8 episodic migraineurs underwent both CFN and RCL scans during interictal and ictal states. Thus, the images are actually referring to 122 and 44 unique subjects, respectively for CFN and RCL scans.

PET acquisition protocol

PET scans with a radiotracer [^{11}C]Carfentanil, a selective μ-opioid receptor radiotracer, or [^{11}C]Raclopride, a selective radiotracer for DA D2/3Rs, were acquired with a Siemens HR + scanner (Knoxville, TN) in 3-D mode with septa retracted (DaSilva et al., 2014a; Nascimento et al., 2014b; DaSilva et al., 2017; Jassar et al., 2019). Each image was reconstructed using the full-width at half maximum (FWHM) resolution of ~5.5 mm in-plane and 5.0 in the z-axis. The total dose injected of [^{11}C]Carfentanil was 15 mCi (555 MBq), with a maximum mass of 0.03 μg per kilogram of body weight. Each [^{11}C]Raclopride dose of 15 ± 1 mCi (555 ± 37 MBq), ≤50 μg, was administered. 50% of this dose was administered as a bolus, followed by a continuous and constant infusion of the remainder to quickly achieve the steady-state tracer levels. Twenty-one PET images were acquired over 90 min while increasing duration (30 s up to 10 min). The PET scan with [^{11}C]Carfentanil included a resting early-phase (5–40 min) without stimulation, followed by a sustained thermal pain threshold (STPT) challenge for the late phase (45–90 min) (thermal pain threshold response). Regarding the PET with [^{11}C]Raclopride, participants were resting without challenge during the early phase (30–40 min), followed by the STPT challenge for the late phase (60–90 min). During the STPT challenge, heat stimuli were delivered to the forehead area ipsilateral to the headache using a 16 mm^2 thermal probe (Pathway model; MEDOC, Ramat Yishai, Israel). The temperature was increased from a baseline of 32°C (increasing 1°C/s). Participants were instructed to click a mouse at the first perception of pain to instantly return temperature to baseline level. The heat stimuli recurred every 10 s for 20 min (40–60 min post-radiotracer administration).

The highest temperature allowed by the device during the challenge was 50°C. The time between scans, assuming the use of CFN to RCL, could be up to 10 min to ensure the tracers were optimally delivered. The STPT was applied to the trigeminal nerve region, which is the most common clinical pain location and allodynia in migraineurs. The patients' heads were firmly stabilized with a soft self-adherent compression bandage wrap and headrest before each scan. Nonetheless, images also underwent attenuation correction and quality control before registration. The probe used for the STPT was attached to the headrest via a plastic and sturdy holder adjusted to comfortably rest on the patients' faces. These are the average molar activities for the data collected across the different studies: i) CFN (2011–2014) — 28.57Ci/μmol [1,057 GBq/μmol], ii) Rac (2011–2014) — 20.75Ci/μmol [767.75 GBq/μmol], iii) CFN (2017-2020) — 117.12Ci/μmol [4333.44 GBq/μmol].

PET data preprocessing

PET images were reconstructed using interactive algorithms into a 128 * 128 pixel-matrix in a 24 cm diameter field of view with attenuation and scatter corrections. A patient motion was corrected by performing a linear co-registration of the frames of dynamic PET images. For each participant, PET images were transformed into two sets of parametric maps: 1) K_1, a tracer transport measure usually used for PET-MRI image co-registration and normalization, and 2) BP_{ND} (non-displaceable binding potential), a receptor-related measure estimated by applying the Logan plot (Logan et al., 1996). Both BP_{ND} and K_1 images were then transformed to the Montreal Neurological Institute (MNI) standard space using the DARTEL tool in SPM8 (Ashburner, 2007). The normalized BP_{ND} images were resampled to 2-mm voxels and smoothed with a Gaussian kernel (3 * 3 * 2 mm). The Logan plot output is

distribution volume ratio (DVR), where here the ratio means relative to a reference region, which in our case is the occipital cortex for Carfentanil and cerebellar gray matter for Raclopride, The reference region value is always 1.0. Thus, the normalized BP_{ND} values used by CBDA for predictive analytics are calculated subtracting one to DVR (i.e., BP_{ND} = DVR - 1).

PET data wrangling

After the PET data have been acquired, we developed and implemented the following protocol for our PET data analysis and predictive analytics. We first recast each 3D brain scan into a 1D array of 510,340 voxels and eliminate voxels with DVR (BP_{ND} +1) < 1.1. Two masks have been used, an CFN mask for CFN data (Grey Matter mask) and an RCL mask for RCL data (Basal Ganglia mask). These masks were generated using the Automated Anatomical Labeling (AAL) brain atlas (Tzourio-Mazoyer et al., 2002). Both masks are applied in MNI space. These masks include only the brain tissue where each of the radiotracers most prominently binds to the appropriate neuro-receptors (Baumgartner et al., 2006; Karjalainen et al., 2017). These different masks set voxel intensities outside the ROIs to zero, leaving the 1D array size unchanged. Therefore, CFN and RCL masks are specific to the gray matter and basal ganglia, respectively. Regarding the ROI analysis, ROIs from AAL atlas have been used for *Thalamus*, *Putamen*, and *Insula*. These ROIs are applied in MNI space. We did further analysis for *Thalamus* by mapping each predictive voxel coordinate in MNI space to *Talairach* space to look up the sub-nuclei (Lancaster et al., 2000). Before the data can be used for predictive analytics, we used each PET image data across the different phases (early and late) by thresholding each voxel intensity across the cohorts of subjects using *ad hoc* masks (i.e., Grey Matter and Basal Ganglia) and a newly designed thresholding function (see Table 1 and next section).

The BP_{ND} *thresholding function*

BP_{ND} intensity thresholding is based on two parameters: threshold and consistency. The thresholding function returns a reduced 1D array based on:

- *consistency* (*%*): given a set of subjects, we want the pixel location to be above the
- *intensity threshold* for at least that % of subjects

We apply the *thresholding* function to each set of images from the two experiments, combining the early and late phases. The details on the thresholding protocol and on the datasets used for predictive analytics are given in Table 1. The consistency value used in the thresholding function is 80%. The threshold for the whole brain analysis was set to 2.0, while we used a less stringent threshold for the ROI analyses (i.e., 1.1) since each ROI has a lot less voxels and a 2.0 threshold would eliminate most of the BP_{ND} values from the analysis. We then perform our CBDA protocol on the reduced PET data.

PET data predictive analytics and CBDA implementation

The study design for our CBDA protocol on the PET imaging data comprised multiple experiments to be performed on each dataset. Details on the CBDA protocol are available in our previous publications (Marino et al., 2018; Marino et al., 2020) and Figure 1. Here we highlight the main steps and specifications implemented for analyzing the PET imaging data after data wrangling has been performed (as described in the previous section, in Figure 1 and in Table 1).

The CBDA subsampling module samples and returns subsets of cases/rows and features/columns used to build the smaller training sets. If needed, imputation (Stekhoven and Buhlmann, 2012), normalization (Becker et al., 1988) and balancing (Chawla et al., 2002) are performed on each sample. In this study, there was no need to perform any of these adjustments since each set of voxels with their binding potential values has no missingness, and the data are already normalized. The voxels are then paired to the binary outcome vector for each patient's PET image (i.e., migraine = 1 vs healthy = 0). The CBDA settings for the subsampling module for all the experiments are the following:

i) M (number of samples from the big dataset) = 5000,
ii) We performed internal cross-validation; thus the Case Sampling Rate-CSR (i.e., fraction of cases/rows to sample from the original training/learning set) is set to 138 for the Opioid experiment and to 60 for the Dopamine experiment,
iii) Feature Sampling Rate—FSR (i.e., a fraction of features/columns to sample from the original training/learning set) is set to 20.

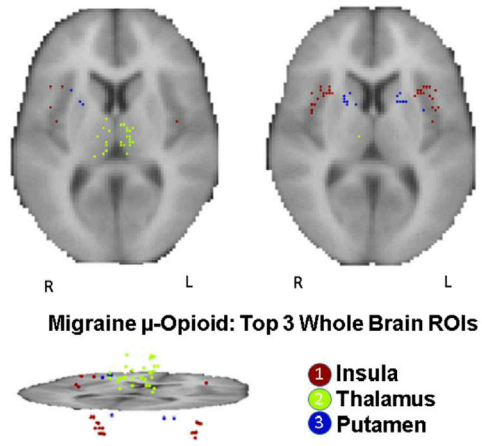
Each of the 5,000 jobs comprises a training sample the size specified in the CSR and FSR. Due to the small number of images/patients, only internal cross-validation is performed.

The sampled pairs are then passed and analyzed by our ensemble predictor (i.e., the *SuperLearner*-SL, see (Van Der Laan and Rubin, 2006; van der Laan et al., 2007; Polley et al., 2017) for details) that combines many different pre-defined algorithms into a single predictive model. The *SuperLearner* algorithm has a large library of classification algorithms that we use to generate our predictions (e.g., Generalized Linear Model (McCullagh and Nelder, 1989), General Additive Model (Hastie and Tibshirani, 1990), Ridge and Lasso Regression (Friedman et al., 2009; Friedman et al., 2016), Random Forest (Breiman, 2001), Support Vector Machine (Hearst et al., 1998), Bayes Auto Regressive Tree (Chipman et al., 2010; Kapelner and BartMachine, 2013)). The SuperLearner ensemble predictor uses a Non-Negative Least Squares loss function to build a weighted linear combination of each algorithm selected to generate the best ensemble prediction (van der Laan et al., 2007). Cross-validation is always performed by default for each algorithm and for the ensemble predictor. We use the default 1 to 10 ratio. A performance metric (accuracy) is then used to rank each prediction. After ranking, we select for features with high frequency (signal) among the top-ranked predictive models and across the M subsamples. The outcome of the CBDA is a set of key features/voxels for prediction/classification, which we then test in a set of nested SuperLearner models for overfitting. We use the top 50 predictive voxels for each CBDA experiment. The

A

	ROI	# Predictive voxel	Rank %
1	**INSULA**	**44**	**19.2982**
	Insula-R	22	9.6491
	Insula-L	22	9.6491
2	**THALAMUS**	**32**	**14.0351**
	Thalamus-L	18	7.8947
	Thalamus-R	14	6.1404
3	**PUTAMEN**	**22**	**9.6491**
	Putamen-R	13	5.7018
	Putamen-L	9	3.5088
4	**ANTERIOR CINGULATE**	**19**	**8.3333**
5	**CAUDATE**	**18**	**7.8947**
	Caudate-L	9	3.9474
	Caudate-R	9	3.9474
6	**AMYGDALA**	**15**	**6.5789**
	Amygdala-L	8	3.5088
	Amygdala-R	7	3.0702
7	**PALLIDUM**	**9**	**3.9474**
	Pallidum-R	6	2.6316
	Pallidum-L	3	1.3158
8	**RECTUS**	**9**	**3.9474**
	Rectus-L	5	2.1930
	Rectus_R	4	1.7544

B



C

	ROI	# Predictive voxel	Rank %
1	**PUTAMEN**	**173**	**70.0405**
	Putamen-L	93	37.6518
	Putamen-R	80	32.3886
2	**CAUDATE**	**47**	**19.0283**
	Caudate-R	24	9.7166
	Caudate-L	23	9.3117
3	**PALLIDUM**	**23**	**9.3117**
	Pallidum-R	12	4.8583
	Pallidum-L	11	4.4534

D

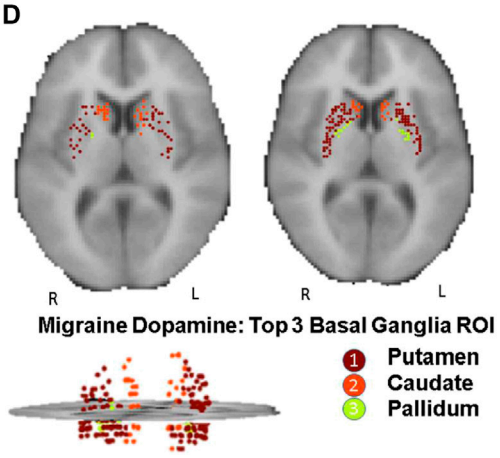


FIGURE 2
CBDA results for the Whole Brain analysis. The binding potentials of the top 50 predictive voxels across 5 replications from the top 3 ROIs are grouped together by their respective ROI descriptions (tables in the figure). Each voxel is then plotted in the 2D rendering of the brain (see the images above the tables). The ranking in the tables is based solely on the fraction of the voxels belonging to their respective ROI. Higher % can be interpreted as a ROI with more predictive power. Panel **(A, B)**: μ-Opioid (Carfentanil) results, based on the Grey Matter mask. Panel **(C, D)**: Dopamine D2/D3 (Raclopride) results based on the Basal Ganglia mask. *Note*: the search for the predictive coordinates for Thalamus and sub-nuclei was performed in Talairach space.

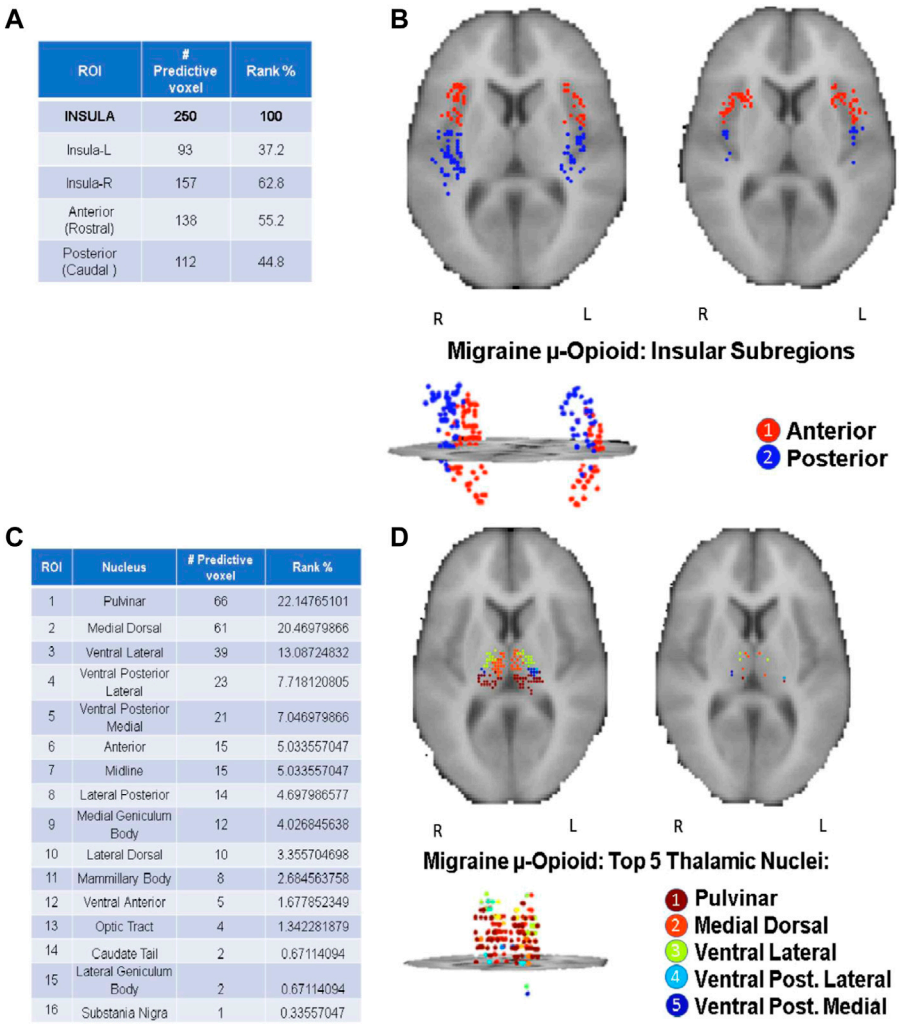
current CBDA R package (Marino and Dinov, 2018) completes each instance/job within 2–7 min. Our CBDA technology becomes feasible and scalable (e.g., thousands of instances/jobs can be run within 1–2 h) by combining *short-burst* completion times for each instance/job with the access to free large scale computational resources through multicore servers, such as the LONI pipeline environment (Dinov et al., 2014).

Features ranking

As highlighted in the Results section, the CBDA protocol has been performed 5 times on each dataset to ensure robustness of the predictive voxels selected. Once a filtered dataset is passed to the CBDA protocol, each time the top 50 predictive voxels from each replication are merged together in a set of 250 non-unique voxels. There is always a 30% up to 40% overlap across the 250 top predictive voxels across the five replications. Usually the maximum accuracy is achieved with less than 50 voxels for each replication, but we list 50 for completeness. We then label each voxel selected based on their ROI (see Figure 2), sub-region or nucleus (see Figures 3, 4), depending on the data used. Each table then rank the voxels by the frequency of occurrence of their respective labels (e.g., ROI, sub-regions, and nuclei). A total of 30 CBDA experiments have been performed, each one generating 5,000 subsamples.

Results

The CBDA results highlight top predictive voxels grouped by region-of-interest (ROI) of non-displaceable binding potential (BP_{ND}) for 198 PET images from migraineurs and healthy controls (HC) (2/1 ratio) during rest and thermal pain challenge. Subjects were scanned with the selective μOR



ROI	# Predictive voxel	Rank %
INSULA	250	100
Insula-L	93	37.2
Insula-R	157	62.8
Anterior (Rostral)	138	55.2
Posterior (Caudal)	112	44.8

ROI	Nucleus	# Predictive voxel	Rank %
1	Pulvinar	66	22.14765101
2	Medial Dorsal	61	20.46979866
3	Ventral Lateral	39	13.08724832
4	Ventral Posterior Lateral	23	7.718120805
5	Ventral Posterior Medial	21	7.046979866
6	Anterior	15	5.033557047
7	Midline	15	5.033557047
8	Lateral Posterior	14	4.697986577
9	Medial Geniculum Body	12	4.026845638
10	Lateral Dorsal	10	3.355704698
11	Mammillary Body	8	2.684563758
12	Ventral Anterior	5	1.677852349
13	Optic Tract	4	1.342281879
14	Caudate Tail	2	0.67114094
15	Lateral Geniculum Body	2	0.67114094
16	Substania Nigra	1	0.33557047

FIGURE 3
CBDA results for the Insula and Thalamus ROI. The binding potentials of the top 50 predictive voxels across 5 replications from the Insula [Panels **(A, B)**] and Thalamus [Panels **(C, D)**]. The binding potentials of the top 50 predictive voxels across 5 replications are grouped together by their respective sub-regions or nuclei, wherever possible (tables in the figure). Each voxel is then plotted in the 2D rendering of the ROI (see the images above the tables). The ranking in the tables is based solely on the fraction of the voxels belonging to their respective subregions/nuclei. Higher % can be interpreted as a subregion/nucleus with more predictive power. Panels **(A, B)**: μ-Opioid (Carfentanil) results for Insula. Panel **(C, D)**: μ-Opioid (Carfentanil) results for Thalamus. *Note*: the search for the predictive coordinates for Thalamus and sub-nuclei was performed in Talairach space.

radiotracer [^{11}C]Carfentanil and the selective DOR D2/D3 radiotracer [^{11}C]Raclopride. The first sets of top predictive voxels for μOR and D2/D3 BP_{ND} were returned after performing CBDA on the whole brain masks filtered datasets (Figure 2). Based on the results described in Figure 2, the voxels of each top predictive ROI are isolated, and CBDA is independently performed on each ROI to identify predictive sub-regions (namely nuclei, see Figures 3, 4). To ensure robustness, the CBDA protocol has been performed five times on each dataset, and the top 50 predictive voxels from each replication have been merged (see *Feature Ranking* in the Methods section for details). There is significant overlap on the top predictive voxels across the five replications. The tables embedded in Figures 2–4 recapitulate most of the 250 top predictive voxels ranked by the frequency of occurrence and grouped by ROIs. The accuracy returned by the internal cross-validation over the set of experiments varied between 80% and 95%, with sensitivity and specificity within 73% and 99% (see Table 2 for detailed results on the Whole Brain Opioid data and Supplementary Text S1 for details on everything else). The following subsections describe the results in detail.

Top predictive ROIs: opioid vs. dopamine whole brain analysis

Figure 2 Illustrates the detailed results returned by CBDA using the Gray Matter and Basal Ganglia masks on the PET images of the entire brain. Figures 2A,C list the top predictive voxels grouped by their respective ROIs and the frequency of each voxel selected as a top predictive one across the five different replications. Due to the binding profile of the

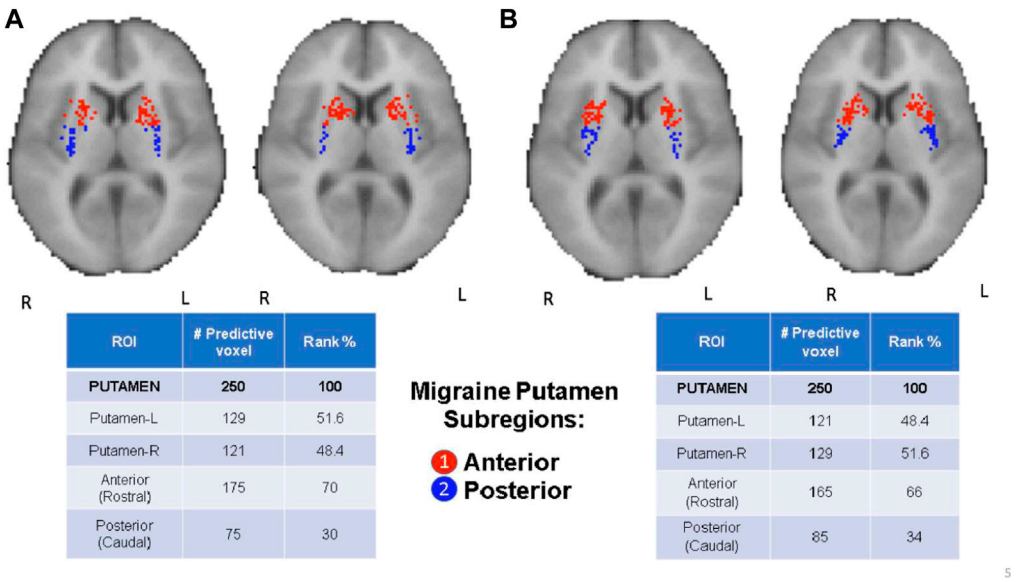


FIGURE 4
CBDA results for the Putamen ROI. The binding potentials of the top 50 predictive voxels across 5 replications are grouped together by their respective sub-regions or nuclei, wherever possible (tables in the figure). Each voxel is then plotted in the 2D rendering of the ROI (see the images above the tables). The ranking in the tables is based solely on the fraction of the voxels belonging to their respective subregions/nuclei. Higher % can be interpreted as a subregion/nucleus with more predictive power. Panel **(A)**: μ-Opioid (Carfentanil) results. Panel **(B)**: Dopamine D2/D3 (Raclopride) results.

TABLE 2 Summary of the CBDA results on the five replications performed on the whole brain for the Carfentanil data (Opioid Grey Matter Mask).

Replication 1	Replication 2	Replication 3	Replication 4	Replication 5
Accuracy: 0.9058	Accuracy: 0.9348	Accuracy: 0.9783	Accuracy: 0.9058	Accuracy: 0.9638
95% CI: (0.8443, 0.9489)	95% CI: (0.8798, 0.9697)	95% CI: (0.9378, 0.9955)	95% CI: (0.9603, 0.9998)	95% CI: (0.9175, 0.9881)
Sensitivity: 0.7391	Sensitivity: 0.8043	Sensitivity: 0.9348	Sensitivity: 0.9783	Sensitivity: 0.8913
Specificity: 0.9891	Specificity: 1	Specificity: 1	Specificity: 1	Specificity: 1

Carfentanil tracer (i.e., more diffused receptors in the brain), the μ-opioid CBDA results are scattered over more ROIs than the Raclopride analysis which mainly binds in the basal ganglia. Figure 2A table lists the top eight predictive ROIs in the μ-opioid analysis, representing ~73% of the top predictive voxels.

The top three ROIs selected are *Insula*, *Thalamus,* and *Putamen*. If we compare these results with the DOR D2/D3 analysis results (shown in Figure 2C table), *putamen* represents alone almost the same percentage of predictive voxels (i.e., ~70%) compared to the top eight ROIs in the μ-opioid analysis. The binding potential levels of the top three predictive ROIs returned by the Dopamine CBDA analysis were able to predict migraine patients from healthy controls with almost 100% accuracy. The detailed results for the μ-opioid analysis are shown in **Supplementary Text S1 (Panel A).** The min-max of the 95% confidence intervals for the accuracy across the five replications are 84% and 99%. Similarly, the min-max values are 0.74 and 0.97 for sensitivity and 0.98 and one for specificity, with positive and negative predicted value rates approaching one in most replications. Although we list and merge the top 50 predictive voxels across the five replications (Figure 2A), the best results can be achieved using as low as the top seven (replication two) up to the top 32 voxels (replication four).

The detailed results for the Dopamine analysis are shown in **Supplementary Text S1 (Panel B).** The min-max of the 95% confidence intervals for the accuracy are 94% and 100%, with 100% sensitivity and specificity. The table in Figure 2C shows the top predictive voxels across the five replications grouped by sub-regions. The best results shown in Figures 2C,D can be achieved using as low as the top three (replication one, two, and five) and up to the top 17 voxels (replication 2).

Figures 2B,D portray the 2D and 3D spatial distributions of the voxels of the top three predictive ROIs for the Opioid and Dopamine analyses, respectively.

Top predictive nuclei: opioid vs. dopamine ROI analysis

CBDA analyses based on the Whole Brain and Basal Ganglia generated separate predictive analytics independently for each of the top predictive ROIs, as illustrated in Figure 2. We focused on the top

three ROIs for the μ-Opioid experiment (i.e., Insula, Thalamus, and Putamen), and only at the Putamen ROI for the Dopamine experiment due to its preeminence in the initial basal ganglia CBDA analysis.

Figure 3 shows the CBDA results on *Insula* and *Thalamus* for the Opioid experiment, while Figure 4 compares the *Putamen* region between the two experiments. The top predictive voxels were then mapped to ROI sub-regions or nuclei.

Detailed results for the Opioid Insular sub-regions, Thalamus nuclei, and Putamen sub-regions analyses (see **Supplementary Text S1—Panel C, D, E, and F)** have similar min-max of the 95% confidence intervals for the accuracy within 97% and 100%, with 100% sensitivity and specificity. The tables in Figures 3, 4 show the top predictive voxels across the five replications grouped by sub-regions and nuclei. The best results shown in the tables can be achieved using as low as the top three up to the top 11 voxel, across the five replications.

Figures 3B,D, and the top row of Figures 4A,B portray the 2D and 3D spatial distributions of the top predictive voxels of the ROIs analyzed, namely Insula, Thalamus, and Putamen for the three μ-opioid data and only putamen for the Dopamine D2/D3 data, respectively.

External cross validation results

Due to the limited number of subjects, the results showcased so far in the study are based on internal cross validation analyses. As a proof of concept, **Supplementary Text S3** showcases a tentative CBDA external cross validation experiment performed on the Grey Matter mask for the whole brain BP_{ND} μ-opioid Carfentanil data. Due to the low number of subjects, it was not feasible to obtain a large enough and balanced subsample for the Dopamine BP_{ND} dataset. In order to achieve meaningful results, we used a 20%–80% split between validation and training set. Based on the demographics (23 healthy, 38 migraine) and since each subject has two PET images (early and late), we selected four healthy (2 males, two females, eight PET images [early/late]) and eight migraine (4 males, four females, 16 PET images [early/late]) subjects. We then performed five CBDA replications on the Grey Matter mask data (Whole Brain), using the trained model to predict the outcomes (0 = healthy, 1-migraine) of the external validation set. The CBDA external cross validation results confirmed our main results (see **Supplementary Text S3** for details).

Predictive analytics results for interictal only patients images

As a proof of concept, similarly to the external cross validation effort, we perform CBDA on the only [11C]Carfentanil BP_{ND} μ-opioid data of episodic migraineurs during the interictal state. Individuals with migraineurs experience various types of discomfort, including sensory hypersensitivities and emotional/cognitive dysfunction, even during headache-free days (Vincent et al., 2022). Previous neuroimaging studies have demonstrated some level of neural abnormalities associated with interictal sensory alterations, susceptibility to migraine attack and severity (Ashina et al., 2021). Thus, we analyzed the data by only including interictal patients/images, which will provide a better insight into the molecular substrate of migraine burden and help guide treatment strategy. Based on Table 1, we included 108 images from 31 patients and 23 HC for the CBDA protocol.

The CBDA results for the interictal patients/images confirmed our main results (see **Supplementary Text S4** for details).

Discussion and conclusions

This work is the result of an interdisciplinary effort across different domain experts. In order to avoid the common pitfalls emerging in AI studies, we focused on reproducibility and transparency. In an effort to promote reproducibility and transparency, we make our code implementations open source and available on public repositories (Marino and Dinov, 2018; Marino and Dinov, 2019). We fully disclose our datasets characteristics and limitations as well as the potential challenges regarding spatial correlation and sample sizes.

There are several limitations when predictive analytics techniques are applied to very high resolution data, such voxel intensities in PET scan measures (like in this study). There is an intrinsic spatial correlation that can be partially eliminated by any data wrangling performed after data has been collected. Of course, this limitation can also be alleviated by a significant progress in PET scan technology. In this study, we do not cluster the voxels before any predictive analytics is performed (like most of the methods in the current literature, see (Peng et al., 2014; Retico et al., 2015; Mete et al., 2016; Klyuzhin et al., 2018) for examples). We apply our CBDA method to the single voxel intensities (upon some thresholding), and then guide the post-optimization clustering by the ROI classification, as well as MNI mapping of the sub-regions (whenever available). Our approach does not bias the predictive analytics by any *a priori* distance-based clustering approaches. We retain the original voxels and ROI coordinates to facilitate any clinical translation of the results.

Another limitation is specific to the data collected in this study, rather than to the methodology implemented here. There is a limited supply of external independent validation datasets due to the very recent use of Carfentanil and Raclopride tracers in PET migraine studies. This scarcity may present some obstacles when it comes to obtaining precise and dependable findings to compare with the present results. However, it is hopeful that future research will broaden the usage of these tracers and offer more data for the purpose of validation.

As a final limitation of this study, we want to stress that results obtained on the Whole Brain Dopamine data (Basal Ganglia Mask), as well as on all the individual ROI predictive analytics, are very similar in terms of accuracy, sensitivity and specificity, likely due to the small number of co-localized voxels which drive the correct classification. We are gathering more subjects to independently validate these results on a larger cohort with external validation settings.

The analysis using the CBDA protocol of our large PET dataset accurately identified migraine patients' brains from those of healthy controls based on their μ-opioid and dopamine D2/D3 receptor availability measure (BP_{ND}). The accuracy, sensitivity, and

specificity were above 90% for both whole-brain and region-of-interest (ROI) analyses. The most predictive ROIs for μ-opioid were insula, Thalamus, and putamen. Putamen led the ranking for dopamine D2/D3, followed by caudate and *pallidum*, respectively. These are critical sensory, motor, and motivational processing regions that are also related to pain and migraine suffering.

The Compressive Big Data Analytics method used i) *subsampling and bootstrapping* for reducing the PET Big Data voxel space into smaller chunks, and ii) *ensemble predictors* for predictive analytics. Given the size of each PET image, we designed and implemented a *thresholding* function that provides significant data compression (up to 60 folds). This functionality improved the clinical predictive power and reduced the computational costs (e.g., CPU time, memory, and storage resources). This method augments the power of machine learning classification and artificial intelligence prediction using MRI and PET in pain and migraine (Chong et al., 2021; Torrado-Carvajal et al., 2021). In addition, the technique provides a robust approach for combining and evaluating heterogeneous data on μ-opioid and dopamine D2/D3 receptor availability (BP_{ND}) in migraine.

The entire CBDA protocol was designed, implemented, and validated as a reproducible, open project using the statistical computing language R. The workflow ran on the LONI pipeline environment, a free platform for high-performance computing, which allowed the simultaneous submission of hundreds of independent components of the CBDA protocol. In addition, our results showcased the scalability, efficiency, and potential of CBDA to compress complex neuroimaging data into structural information leading to derived knowledge and translational action not only the basic diagnostic molecular profile but the ranking of potential treatment targets in the brain for migraine-related μ-opioid and dopamine D2/D3 dysfunction.

Classification of migraine based on its central μ-opioid and dopamine D2/D3

PET studies have recently demonstrated that pain activates endogenous opioid and dopamine receptor-mediated neurotransmission in cortical and brainstem regions. The magnitude of the opioid and dopamine regional activations are related to the individual's capacity to suppress sensory and affective elements of the acute pain experience (Zubieta et al., 2001; Scott et al., 2008), which can lead to maladaptations in the receptor availability in the same regions in chronic pain disorders (Martikainen et al., 2015). CBDA modeling of the data demonstrated a fingerprint of migraine in such pain's μ-opioid and dopamine receptor network. For example, the insula is known to participate in pain perception and chronic pain (Mayr et al., 2021). It is divided into the anterior component, primarily related to limbic regions, including the amygdala, and plays a vital role in cognitive-emotional pain modulation. The posterior insula is associated with sensorimotor integration and receives nociceptive inputs from the Thalamus. This holistic role of the insular cortex in pain processing (Borsook et al., 2016) might explain its leading ranking in our ML classification model for migraine, the anterior insular slightly more than the posterior one. The dysfunctional activity in the insula is commonly linked to migraine, and its moment-to-moment variability of resting-state activity increases with structures on the ascending trigeminal somatosensory system in parallel with the severity of the attacks (Lim et al., 2021). There is also evidence of cortical neuroplasticity that correlates with the history and frequency of the attacks (Maleki et al., 2012; Woldeamanuel et al., 2019). At least in animal models, electric stimulation of the insula effectively reverses mechanical hypersensitivity, which can be abolished by pharmacologically blocking the μ-opioid receptors (MOR) system (Dimov et al., 2018). Such findings indicate the central role in the insular μ-opioidergic malfunction in migraineurs, and further studies can lead to the potential development of new migraine and pain therapies directly targeting the region (Bergeron et al., 2021; Liu et al., 2021).

Our second-ranking structure for μ-opioid dysfunction in migraine was the Thalamus. Thalamus is the relay structure in the brain that participates in multiple phases of migraine pathophysiology outside (interictal) and during (ictal) the attacks (Martinelli et al., 2021). Its highly specialized nuclei dynamically integrate with other regions in the brain associated with patients' suffering. The leading thalamic nuclei in our study were the pulvinar and medial dorsal. They receive inputs from dura-sensitive spinal trigeminal nucleus neurons and play a crucial role in the photophobia and allodynia during migraine (Hodkinson et al., 2016). The other implicated thalamic nuclei were the venteroposterior medial (VPM) and lateral (VPL) that are key in the processing of nociceptive inputs from cranial and extracranial structures during the attacks, respectively, and commonly reported in fMRI studies in migraine (Lim et al., 2021). In a recent study, chronic migraine patients scanned with PET during attacks under thermal challenge showed increased endogenous μ-opioid receptor-neurotransmission in the thalamic venteroposterior nuclei (Lim et al., 2021). Overall, the thalamic fingerprint of migraine confirms its involvement in the broad sensory dysfunction commonly noticed in migraineurs, including inefficient inhibitory pain modulatory responses and sensitization when exposed to multiple external stimuli such as thermal, mechanical, light, and sound (Lim et al., 2021). This broad μ-opioid receptor dysfunction in critical integrative thalamic nuclei explains the long list of symptoms in a migraine attack and the co-existence of multiple pain disorders, such as temporomandibular disorders and fibromyalgia (Onder et al., 2019; Kleykamp et al., 2021).

The comorbidity in migraine goes beyond chronic pain, and in recent years the associations of migraine and motor disorders are becoming more evident in clinical and translational studies, including depression and Parkinson's disease (Cervenka et al., 2006; Scher et al., 2014). This is primarily attributed to the imbalance and deficiency in DA D2/D3 receptor (DOR) BP_{ND} in the basal ganglia in migraineurs (DaSilva et al., 2017). Herein, we found that the putamen was the most predictive region in the basal ganglia for migraine regarding DOR D2/D3 BP_{ND} levels. The third was for μ-opioid BP_{ND} levels, mainly in its anterior portion, which connects with the large associative regions in the cortex. The putamen is one of the major sites of cortical input into basal ganglia and is commonly activated during pain and pain-related motor responses. There is a D2 receptor activity associated with variability in pain suffering and modulation in both acute and chronic pain disorders. For instance, patients with Parkinson's disease have increased thermal pain sensitivity. On the contrary,

patients with lesions in the putamen have this sensitivity impaired (Lorberboym et al., 2004; Starr et al., 2011), which also extends to psychological pain (Husain et al., 1991).

These findings suggest a neural framework for migraine classification based on μ-opioid and D2/D3 dopamine receptor availability dysfunction, two of the most crucial central endogenous receptor-neurotransmitters mechanisms in the brain. It also contributes to our better understanding of the large association of migraine patients' pain and discomfort, their increased sensitivity and aversive reactions to environmental stimuli, and co-existing cognitive-motivational disorders, like Parkinson's disease and depression (Meerwijk et al., 2013).

Data availability statement

The data supporting this study's findings are available from the corresponding author upon reasonable request. Requests to access the datasets should be directed to AD, adasilva@umich.edu.

Ethics statement

The studies involving human participants were reviewed and approved by University of Michigan Medical School Institutional Review Board (IRBMED). The patients/participants provided their written informed consent to participate in the studies.

Author contributions

SM, HJ, DK, ML, TN, ID, RK, AD, HJ, and SM conceived of the presented idea. Data: TN collected the PET imaging data, HJ collected and recasted the PET imaging data into a suitable format for the predictive analytics step. SM filtered and recasted the data to successfully perform the CBDA algorithm. SM, DK, and ML performed the interictal analysis provided in the Supplementary Text. SM and ID developed the CBDA algorithm and SM performed the CBDA computation. All authors verified the analytical methods, both for the data wrangling and the CBDA algorithm. AD encouraged HJ and SM to investigate different masks for the PET data and supervised the findings of this work. AD, HJ, SM, and RK contributed to the interpretation of the results. SM took the lead in writing the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2023.1173596/full#supplementary-material

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Mood instability and low back pain: a mendelian randomization study

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Objective: Low back pain is a prevalent and debilitating condition worldwide, with significant implications for individuals' quality of life and productivity. The aim of this study was to assess the relationship between mood instability and the risk of developing chronic low back pain, using a rigorously designed mendelian randomization methodology.

Method: The study incorporated both univariate and multivariate mendelian randomization to analysis the causal relationship between mood instability and the risk of developing chronic low back pain. The data on mood instability from the Integrative Epidemiology Unit (IEU) opened Genome-Wide Association Studies (GWAS) project (IEU-opened GWAS project). Data on low back pain were collected from two sources: One source is the IEU open GWAS project (discovery data). Another source is a GWAS meta-analysis (replication data). Inverse variance weighted method, weighted median method, MR-Egger regression, and mendelian randomization pleiotropy residual sum and outlier method were used for mendelian randomization analysis.

Result: The univariable mendelian randomization analysis shows a statistically significant correlation between mood instability and the risk of low back pain. Several methods were performed, including inverse variance weighting (discovery data: odds ratio=3.544, 95% confidence interval=1.785–7.039, p=0.000; replication data: odds ratio=3.167, 95% confidence interval=2.476–4.052, p=0.000), MR-Egger (discovery data: odds ratio=7.178, 95% confidence interval=0.057–909.525, p=0.429; replication data: odds ratio=2.262, 95% confidence interval=0.580–8.825, p=0.246), weighted median (discovery data: odds ratio=2.730, 95% confidence interval=1.112–6.702, p=0.028; replication data: odds ratio=3.243, 95% confidence interval=2.378–4.422, p=0.000), MR-PRESSO (discovery data: odds ratio=3.544, 95% confidence interval=1.785–7.039, p=0.001; replication data: odds ratio=3.167, 95% confidence interval=2.476–4.052, p=0.000) methods. The results were consistent across these methods. The results obtained from discovery data are consistent with those obtained from discovery data. In the multivariable mendelian randomization, after adjusting for various covariates such as body mass index, current tobacco smoking, alcohol intake frequency, Total body bone mineral density, and vigorous physical activity, there is a consistent correlation between mood instability and chronic low back pain.

Conclusion: This study provides robust evidence supporting a causal relationship between mood instability and the development of low back pain. Our findings suggest that addressing mood instability may play a crucial role in prevention and management strategies for individuals experiencing low back pain.

KEYWORDS

mendelian randomization, low back pain, mood instability, genome-wide association, summary statistics

1. Introduction

Low back pain (LBP) is a major public health concern worldwide, as it is one of the leading causes of motor dysfunction, pain, and even disability in musculoskeletal diseases. With the global aging process accelerating, LBP's burden on public health will continue to increase (1, 2). LBP patients typically seek medical attention due to pain and functional limitations, but the etiology is often complex and multifactorial, with non-specific risk factors such as aging, muscle and bone decline, unhealthy lifestyle, etc. contributing to recurrent episodes of LBP. These episodes can cause significant physical harm, resulting in frequent medical attention and significant social and economic burdens (3). There are various evidence-based treatment methods available for LBP, including physical therapy, medication, and surgical interventions that can relieve dysfunction and reduce pain. Studies have shown that over 80% of patients can recover within 3 months after treatment (4), but approximately 70% of patients will recur within a year (5). This highlights the importance of prevention strategies in preventing recurrence and exacerbation. Preventive measures can effectively improve patient quality of life and reduce medical burdens (6, 7).

One of the defining features of mood instability (MI) is its rapid and unpredictable changes in emotions and feelings, which can be observed in both healthy individuals and those with mental disorders. MI cannot be used as a standalone diagnostic criterion, so its etiology and treatment plan remain unclear at present. MI is not a single symptom but a multidimensional issue that encompasses various aspects of emotional regulation and expression (8–10). MI can exacerbate other conditions, evidence shows that patients with depression-related LBP experience more severe pain and disability than those with normal emotions, and negative emotional state can lead to noncompliance with treatment and significantly impact therapeutic outcomes (11). On the other hand, LBP-induced pain and disability can also cause significant psychological trauma to patients, resulting in sleep disturbances, anxiety, depression, and fearful behaviors. Limited mobility due to dysfunction can significantly reduce the quality of life of patients. There is a vicious cycle between LBP and MI, greatly affecting patient prognosis and quality of life. A systematic review analyzed the effectiveness of psychological interventions in the treatment of LBP. The study included 2,490 patients and conducted a meta-analysis on the effectiveness of emotion treatment in pain, disability, quality of life, and other MI conditions. The results showed that psychological intervention can effectively reduce pain intensity and improve quality of life. However, due to the lack of appropriate controls, the relationship between MI and LBP has not been systematically analyzed or evaluated, making it uncertain whether MI plays a causal role in the development of LBP (12).

Mendelian randomization (MR) is a technique used to assess the presence of a causal relationship between risk factors and health or disease. It leverages genetic variation as an instrumental variable to mitigate the issue of reverse causation. To ensure validity, MR research requires the identification of genetic variants associated with the exposure under investigation, as well as the testing of associations between these variants and the outcome of interest. To serve as a reliable tool for causal inference in MR studies, a genetic variant must fulfill three fundamental assumptions: Firstly, the instrumental variable SNP should be closely related to the exposure. Secondly, the SNP should not be associated with any confounding factors related to exposure or outcome and should to eliminate the SNPs of linkage disequilibrium. Finally, the SNP should not be a method related to the outcome that is unrelated to the exposure pathway. With MR, the effect of remaining confounding factors on the accuracy of the correlation results is circumvented, making the strength of the correlation result argument reliable.

The use of MR can help establish the causal relationship between exposure and outcomes. By utilizing single nucleotide polymorphisms (SNPs) to predict SNPs associated with MI, we can determine the causal impact of MI on LBP. By using the genetic variant SNP as an instrument, thus effectively avoiding measurement error and reverse causation (genetic factors predate exposure variables in time and are therefore less likely to be associated with confounders), by analyzing genetic variants associated with exposure through MR, and then testing for associations between these genetic variants and outcomes, MR can then be used to estimate the causal relationship between exposure and outcome when the three core assumptions are met (13, 14). Numerous studies have been conducted to explore the relationship between MI and LBP, but many of them are clinical trialls or system reviews with a small sample size (usually <100 participants), and they typically represent single-center studies that may have limited generalizability (15, 16). Moreover, the findings of some of these studies are inconclusive, and there is still no clear consensus. Using large-scale, multicenter, and large-sample GWAS database-based data, MR analysis overcomes the limitations of small sample sizes in previous clinical trials or system reviews, thereby increasing the reliability and generalizability of conclusions. Furthermore, MR analysis is a robust analytical method that avoids confounding, reverse causality, and various forms of bias, thus enabling the inference of a causal relationship between exposure and outcome (17, 18). The application of MR can enhance the identification of potential targets for intervention (e.g., emotional instability), offering an additional rational approach to clinical treatment.

In this study, a two sample MR analysis was employed to uncover the potential causal effect relationship between MI and LBP. Furthermore, the multivariate MR analysis was employed to exclude the influence of confounding factors on LBP. By MR analysis, we avoided causal inversion and also excluded the influence of confounding factors, thus avoiding the bias found in traditional epidemiologic studies in the past and effectively revealing a causal relationship between exposure and outcome rather than simply suggesting an association exposure and outcome.

Our research provides the first evidence of the causal connection between MI and LBP through MR analysis.

2. Methods

2.1. Ethical approval

Our studies are based on publicly available GWAS data, so no additional ethical approval is required.

2.2. Data acquisition

All participants in the GWAS were of European descent and were either male or female. The summary exposure data for MI was obtained from a GWAS study conducted by the UK Biobank (GWAS ID: ukb-b-14,180, N = 451,619).[1] Participants were genotyped using an Affymetrix UK Biobank Axiom array, and extensive quality control was performed on the genetic data. The outcome data for LBP came from the FinnGen study (GWAS ID: finn-b-M13_LOWBACKPAIN),[2] and cases of LBP were identified according to International Classification of Diseases (ICD) coding. To perform replication analysis, we also used the largest genetic study of back pain phenotypes, which is a meta-GWAS dataset include four data bank: the deCODE Genetics (Iceland),the Danish Blood Donor Study (Denmark); DBDS and Copenhagen Hospital Biobank; CHB(Denmark), and the UK Biobank; UKB(United Kingdom) (containing 119,100 cases and 909,847 controls), where the "dorsalgia" code group (M54) was used to identify cases of healthcare-associated back pain, which is primarily considered chronic/recurrent back pain (19). The corrected analysis data for multivariable MR summary (body mass index (BMI), current tobacco smoking, alcohol intake frequency, total body bone mineral density, and vigorous physical activity) were obtained from the IEUOpenGWAS project. The effect alleles of MI and LBP datasets were harmonized to confirm that exposure and the effect outcome correspond to the same allele by using the harmonize data function from the TwoSampleMR package.

2.3. Instrumental variable selection

To ensure that MR analysis meets the three core assumptions, we perform quality control and quality control techniques on all SNPs. Firstly, we set the correlation hypothesis thresholds ($p < 5 \times 10^{-8}$, $F > 10$) and exclusion hypothesis thresholds ($p > 5 \times 10^{-5}$). Secondly, to eliminate the SNPs of linkage disequilibrium, we set the independence hypothesis thresholds (clump $r^2 = 0.001$, clump kb = 10,000). To meet the independence hypothesis, we use the PhenoScanner website to retrieve each SNP and ensure exclusion of confounding factors. We also remove SNPs with palindromic structures using the harmonize data function. For the remaining SNPs, the MR-PRESSO test does not detect potential outliers (20, 21). Finally, we select these SNPs that satisfies the threshold setting as instrumental variables for MI assessment. These instrumental SNPs explain 0.39% of MI variations. In our study, the F statistic value of single SNPs ranges from 29.8 to 63.7, with a power of 100%.

2.4. Mendelian randomization analysis

The inverse variance weighting (IVW) method was utilized as the primary MR analysis to explore the potential causal relationship between MI and LBP. This study employed the IVW method as the main MR approach, which can obtain the most accurate and reliable causal relationship. Additionally, we also utilized MR-Egger regression, Weighted-Median, Mendelian randomization pleiotropy residual sum and outlier (MR-PRESSO) tools for evaluation and comprehensive assessment of consistency evidence (22). Additionally, the heterogeneity analysis, the pleiotropy analysis, the leave-one-out analysis was conducted to evaluate the robustness of the conclusions, whether there is bias in the outcome, and whether there is a SNP that seriously affects the outcome (20, 23–25). These methods have their advantages and disadvantages, such as that the IVW method is based on all core assumptions of MR being valid assumptions, may have potential horizontal polymorphism effects, and the causal estimates of IVW may be biased. The MR-Egger method can provide unbiased assessments even when violating exclusion restriction assumptions, but its statistical capacity is relatively low. Through the integration of multiple MR methods, the bias of confounding and reverse causality is maximized to improve the accuracy of estimating causal relationships.

2.5. Statistical analysis

To conduct the MR analysis, we used the R Studio software (R version 4.2.0) package TwoSampleMR[3] and MendelianRandomization[4] (26). The main analysis of MI and LBP utilized the random effect model using the IVW method, the MR-Egger method, Weighted-Median method, MR-PRESSO method for evaluation and comprehensive assessment of consistency evidence. The Cochran's Q statistical test was used to assay for the presence of pleiotropy. To remove potential confounding factors, the variance backward weighted analysis was conducted. A p value of 0.05 was set to indicate statistical significance for tests of pleiotropy and heterogeneity.

3. Results

3.1. Selection of data sources

The data on MI from the IEU-opened GWAS project (GWAS ID: ukb-b-14,180, a total of 451,619 individuals, including 204,412 cases and 247,207 controls). Data on low back pain were collected from two sources: One source is the IEU open GWAS project (ID:

1 https://gwas.mrcieu.ac.uk/datasets/ukb-b-14180/

2 https://gwas.mrcieu.ac.uk/datasets/finn-b-M13_LOWBACKPAIN/

3 https://github.com/MRCIEU/TwoSampleMR

4 https://github.com/sb452/MendelianRandomization/tree/v0.5.0

TABLE 1 Detailed information on the GWAS datasets used in this MR study.

Dataset type	Item	GWAS ID	Author	Consortium	Year	Population	Sample size	Sex
Exposure	Mood instability	ukb-b-14,180	Ben Elsworth	MRC-IEU	2018	European	451,619. 204,412 cases and 247,207 controls	Males and females
	Body mass index	ukb-b-19,953	Ben Elsworth	MRC-IEU	2018	European	461,460	Males and females
	Current tobacco smoking	ukb-b-223	Ben Elsworth	MRC-IEU	2018	European	462,434	Males and females
	Alcohol intake frequency	ukb-b-5779	Ben Elsworth	MRC-IEU	2018	European	462,346	Males and females
	Total body bone mineral density	ebi-a-GCST005348	Medina-Gomez C	NA	2018	European	56,284	Males and females
	Heavy physical activity	ukb-b-13,184	Ben Elsworth	MRC-IEU	2018	European	460,376. 197,006 cases and 263,370 controls	Males and females
Outcome	Low back pain	finn-b-M13_ LOWBACKPAIN	NA	FinnGen	2021	European	13,178 cases and 164,682 controls	Males and females
	Low back pain	NA	Bjornsdottir G	NA	2022	European	119,100 cases and 909,847 controls	Males and females

finn-b-M13_LOWBACKPAIN), which included 13,178 cases and 164,682 controls (discovery data), 45 independent genetic variants represent MI. Another source is a GWAS meta-analysis, this dataset includes four data banks: the deCODE Genetics (Iceland), the Danish Blood Donor Study (Denmark); DBDS and Copenhagen Hospital Biobank; CHB(Denmark), and the UK Biobank; UKB (United Kingdom), the "dorsalgia" code group (M54) was used to identify cases of healthcare-associated back pain, which is primarily considered chronic/recurrent back pain. The dataset included 119,100 cases and 909,847 controls (replication data) (Table 1). IVW method, weighted median method, MR-Egger regression, and MR-PRESSO method were used for MR analysis.

The thresholds ($(p < 5 \times 10^{-8}$, $F > 10$) was set to satisfy correlation assumptions (Supplementary Table S1). The SNPs related to the ending were excluded ($p > 5 \times 10^{-5}$) to satisfy the exclusionary assumption. The convergence was removed by PhenoScanner website scanning and the chain imbalances was removed by setting thresholds (clump = TRUE, $r^2 < 0.001$, kb = 10,000) to satisfy independence assumptions. We also remove SNPs with palindromic structures using the harmonise_data function (action = 2). After satisfying the above three core assumptions of the MR analysis, 46 SNPs was obtained from the discovery data and 48 SNPs was obtained from the replication data. These SNP all have strong potential to predict MI (Figure 1).

3.2. MR analysis in LBP

The MR analysis was executed to assess whether there was a correlation between MI and LBP. The IVW (discovery data: odds ratio (OR) = 3.544, 95% confidence interval (CI) = 1.785–7.039, $p = 0.000$; replication data: OR = 3.167, 95% CI = 2.476–4.052, p = 0.000), MR-Egger (discovery data: OR = 7.178, 95% CI = 0.057–909.525, $p = 0.429$; replication data: OR = 2.262, 95% CI = 0.580–8.825, $p = 0.246$), weighted median (discovery data: OR = 2.730, 95% CI = 1.112–6.702, $p = 0.028$; replication data: OR = 3.243, 95% CI = 2.378–4.422, p = 0.000), MR-PRESSO (discovery data: OR = 3.544, 95% CI = 1.785–7.039, $p = 0.001$; replication data: OR = 3.167, 95% CI = 2.476–4.052, $p = 0.000$) was executed by TwoSampleMR R package. All these methods demonstrated a robust correlation between MI and LBP. The results of further repeated validation of discovery data and replication data are highly consistent (Figure 2 and Supplementary Table S2).

3.3. The sensitivity analysis

To confirm the reliability of the results of the MR analysis, The Cochran's Q statistical data was calculated to quantify the heterogeneity of individual causal effects, a value of $p > 0.05$ indicating the absence of heterogeneity. Both MR-Egger (discovery data: Cochran's Q = 55.138, $p = 0.121$; replication data: Q = 66.816, $p = 0.024$) and IVW methods (discovery data: Cochran's Q = 55.242, $p = 0.141$; replication data: Q = 67.169, $p = 0.028$) was carried out and the same result is output. The replication data shows the heterogeneity ($p < 0.05$), but the beta values are in the same direction, therefore they are also considered positive results (27). Similarly, the pleiotropy statistical data was calculated by MR-Egger intercept analysis (discovery data: $p = 0.774$; replication data: $p = 0.624$) and MR-PRESSO analysis (discovery data: $p = 0.162$; replication data: $p = 0.055$), there both value of $p > 0.05$ demonstrated the absence of pleiotropy between MI and LBP. The leave-one-out analysis was carried out to calculate the meta effect of remaining SNP and observe whether the results have changed after removing each SNP (Figure 3). After excluding each SNP, all error lines are on the right side of 0 or all error lines are on the left side of 0. The overall error line does not

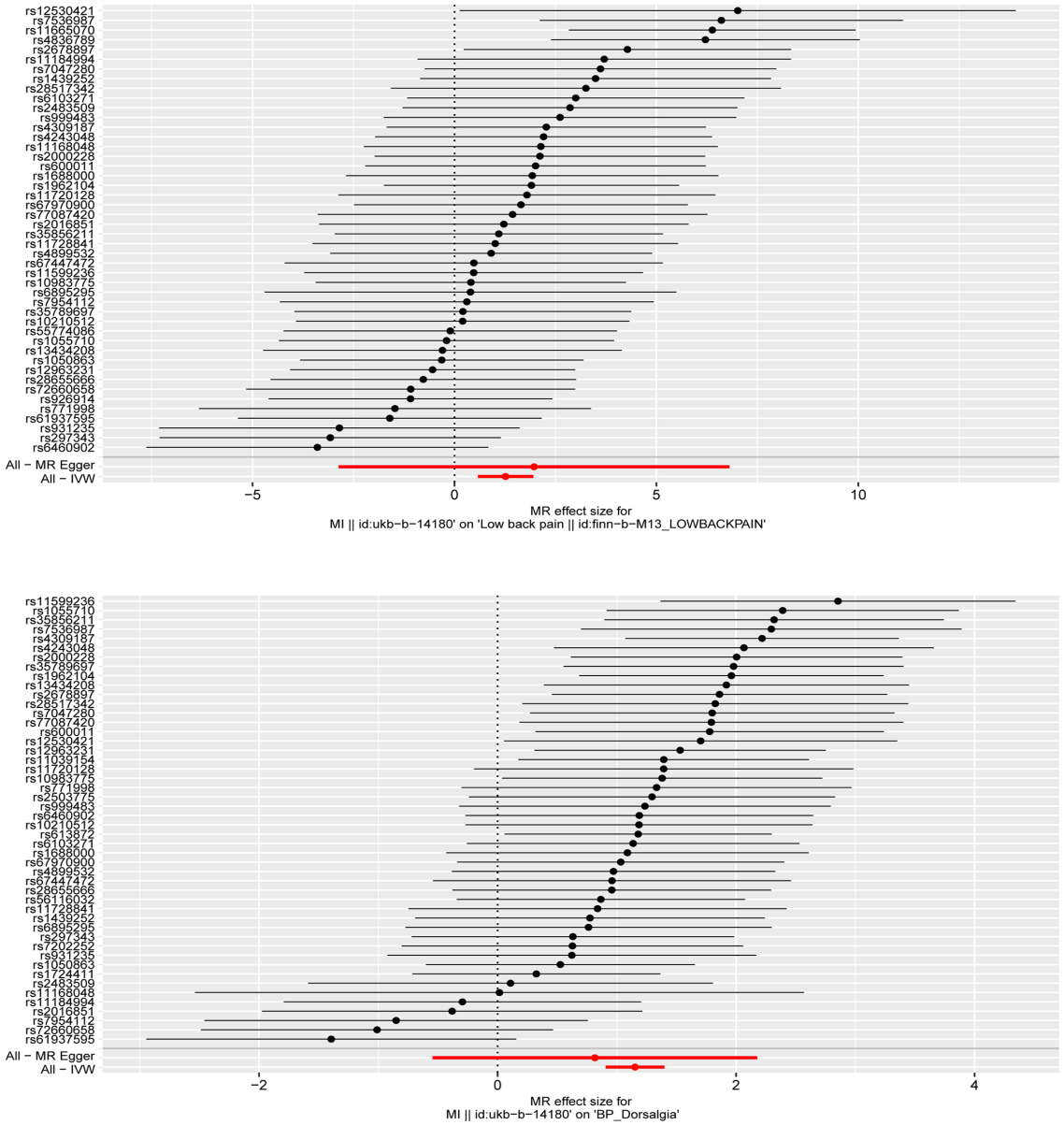

FIGURE 1
The forest plot was used to show the MR estimate and 95% CI values for each SNP, also show the IVW and MR-Egger MR results at the bottom.

change significantly. The scatter plots of the SNP-outcome associations against the SNP-exposure associations (Figure 4). The funnel plot was drawn for observing whether SNPs are symmetrically distributed on both sides of the IVW line (Figure 5). All four sensitivity analyses confirmed the reliability of the MR analysis.

3.4. Multivariate MR

To confirm whether the correlation of MI on LBP is independent of BMI, current tobacco smoking, alcohol intake frequency, total body bone mineral density, and vigorous physical activity, the multivariate MR analysis was conducted. The results of the multivariate MR analysis demonstrate that there is still a correlation between MI and LBP after correcting for other factors (Table 2).

4. Discussion

This is the first study to investigate the relationship between MI and LBP using the multiple validation MR method. Our two sample MR analysis revealed a correlation between MI and LBP, MI increases the likelihood of LBP, and the degree of MI is positively correlated with LBP which was consistent after adjusting for BMI, current tobacco smoking, alcohol intake frequency, total body bone mineral density, and vigorous physical activity.

Until now, the evidence on the relationship between MI and LBP is underdeveloped. Previous studies have suggested a link between emotions and LBP (28, 29). LBP is a chronic recurrent pain condition that often causes fear and depression in patients, MI can exacerbate the pain experience, leading to a vicious cycle. Activities that promote emotional stability, such as yoga, may alleviate LBP by improving

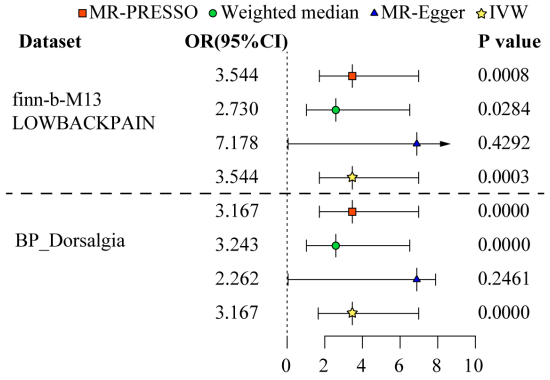

FIGURE 2
Odds ratio plot for mood instability and low back pain. The four methods applied in the current manuscript were all depicted. Four different colors and shapes of logos represent represent IVW, MR-Egger, weighted median, and MR-PRESSO methods.

mood (30). A study utilized breathwork, meditation, and yoga philosophy lectures in LBP patients, resulting in a reduction of anxiety levels by 20.4%, depression levels by 47%, LBP reduction by 49%, improved spinal mobility by 50%, and increased overall well-being (31). Yoga-based physical therapy that enhances mood is more effective in relieving pain than conventional physical therapy (32, 33). Moreover, with continued practice of yoga, the relief of pain and emotional stability persists over time (34). However, some studies have yielded differing results. A systematic review indicated that MI was associated with adverse treatment outcomes in short-term (less than 6 months) low back pain patients but not in long-term LBP (35).

Several potential explanations may account for the heterogeneity observed. Firstly, BMI may serve as a common risk factor for both MI and LBP (36, 37). An increase in BMI can lead to MI and heightened emotional impulses. A MR analysis conducted on patients revealed that an increase of 5 kg/m^2 in BMI can cause MI, such as depression and anxiety (38). Additionally, an increased load on the lower back is associated with an elevated BMI, as demonstrated by another MR analysis

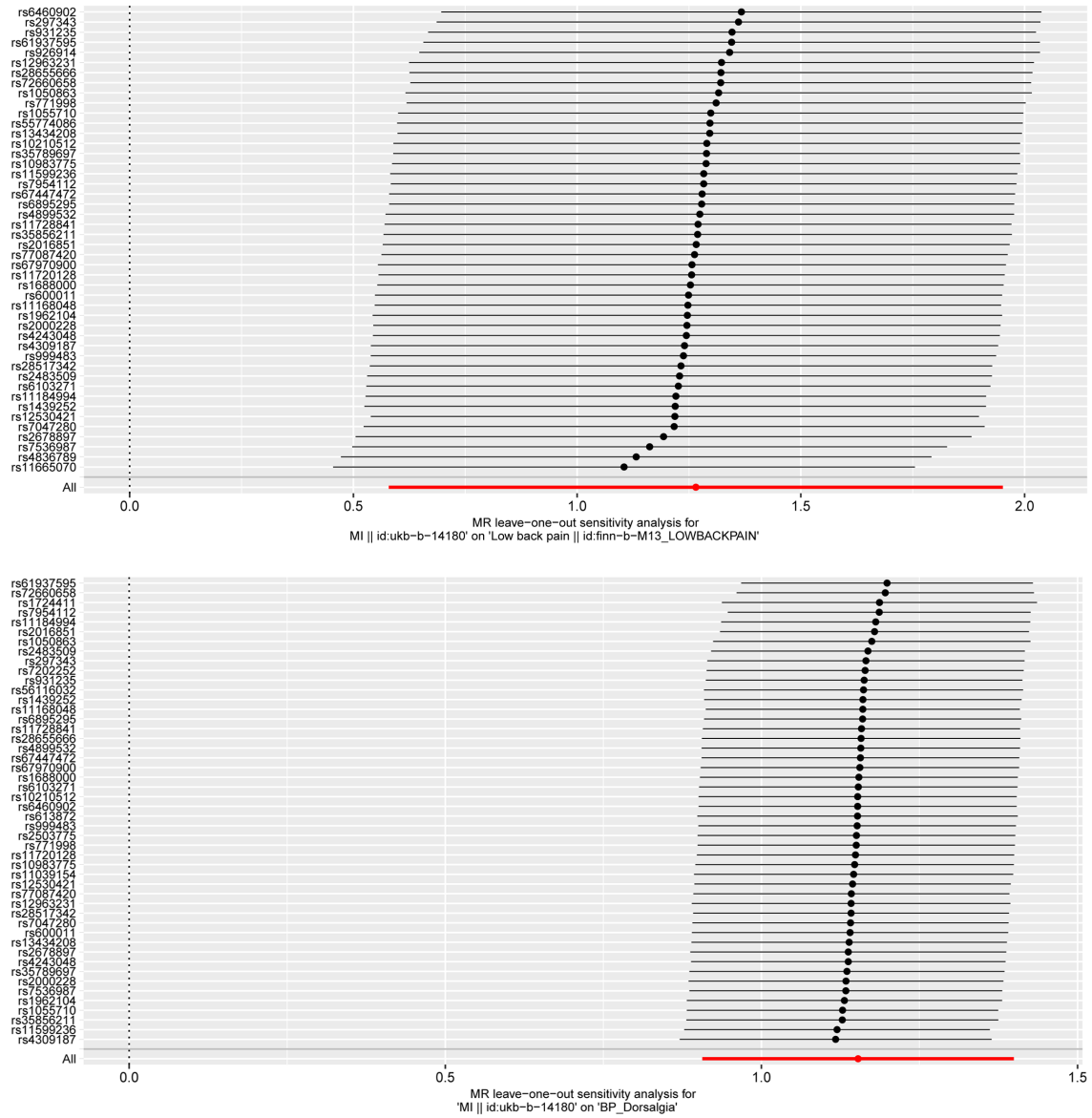

FIGURE 3
Leave-one-out analyses to evaluate whether any single instrumental variable was driving the causal effect.

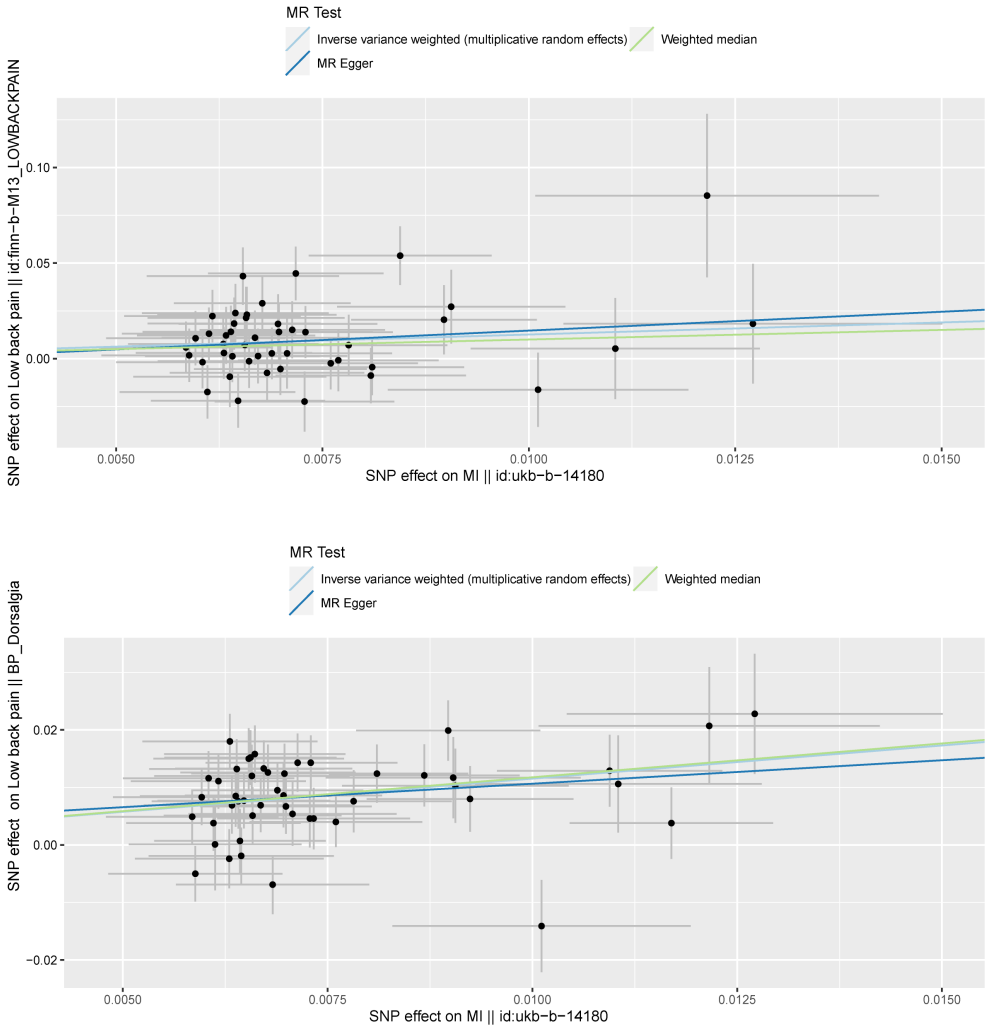

FIGURE 4
Scatter plot showing the genetic correlations of mood instability and low back pain. The slopes of line represent the causal effect of each method (IVW, Weighted median and MR Egger) respectively.

on patients, which revealed that increases in waist circumference, hip circumference, and overall body fat levels can cause degenerative changes in the intervertebral discs and increase the risk of low back pain after adjusting for BMI (39). The correlation between BMI and LBP is significantly weakened when other factors are considered. Secondly, smoking and drinking as risk factors may have contributed to confusion in studying the relationship between MI and LBP (40–42). Furthermore, some genetic variations may be associated with an increased susceptibility to both MI and LBP. Therefore, MI may not be a reliable predictor factor for the development and outcome of LBP. Previous studies have been unable to avoid the influence of these confounding factors. In this study, we effectively eliminated these biases and confounding factors through multi-variate MR analysis and improved research design, thereby providing more credible evidence in support of our findings.

There are several potential pathways that have been widely accepted between MI and LBP at both the biological and behavioral levels. MI can impact endocrine and immune metabolic processes, which can significantly influence the occurrence and development of LBP (43, 44). The endocrine and immune systems are complex networks that are regulated by numerous factors. When emotions are unstable, both the endocrine and immune systems are subject to feedback regulation (45). During periods of emotional instability, hypothalamus neurons release adrenocorticotropin-releasing hormone and arginine vasopressin, which stimulate the release of various hormones from the adrenal glands, leading to changes in the human endocrine system (46, 47). Changes in hormone levels in the endocrine system can also affect the levels of immune cells and inflammatory cytokines, resulting in changes in the immunological microenvironmental homeostasis (48, 49). Additionally, MI can activate central and peripheral immune cells, leading to the release of proinflammatory cytokines, proinflammatory cytokines pass through the blood–brain barrier to reach the brain, creating a vicious cycle, that is consistent with the vicious cycle caused by MI and LBP (50, 51).

In our study, we employed a two sample MR design to assess the causal relationship between MI and LBP. Similar to previous MR studies, we utilized IVW methods in the primary MR analysis. The results of the IVW were statistically significant, indicating that there is no single tool for driving causality estimation using SNPs. Multiple sensitivity analyses were conducted utilizing various methods, and the findings remained consistent and stable. To validate the causal

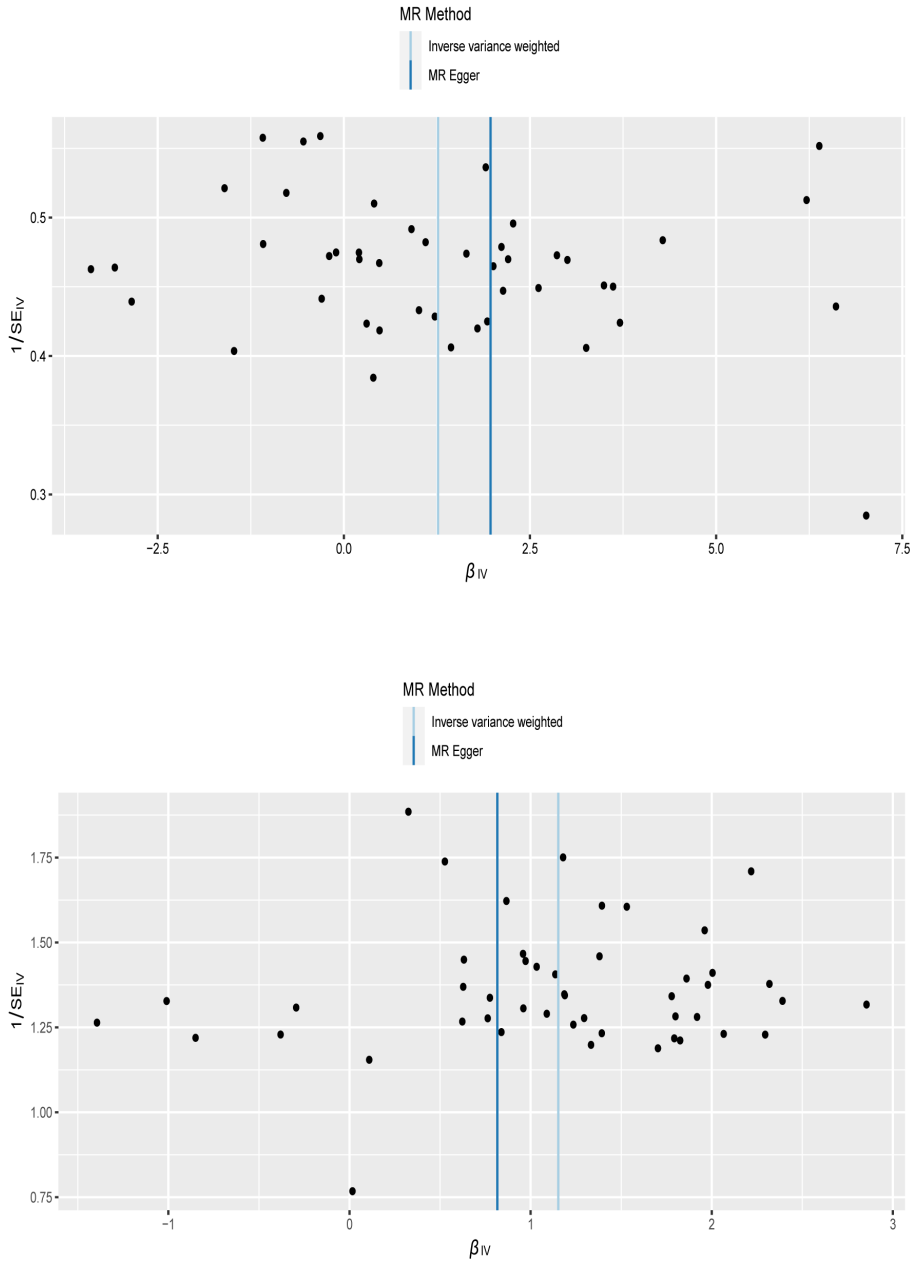

FIGURE 5
A funnel plot was applied to detect whether the observed association was along with obvious heterogeneity.

TABLE 2 Results of multivariable MR.

Exposure	Outcome	Adjusted by factor	SNP	beta	se	*p*-value
Mood instability	Low back pain	Body mass index	400	1.318	0.338	9.72E-05
Mood instability	Low back pain	Current tobacco smoking	77	1.417	0.382	2.09E-04
Mood instability	Low back pain	Alcohol intake frequency	121	1.424	0.353	5.55E-05
Mood instability	Low back pain	Total body bone mineral density	121	1.471	0.348	2.39E-05
Mood instability	Low back pain	Heavy physical activity	68	1.647	0.368	7.57E-06

relationships found in the data set, we employed validation samples of LBP from another meta-GWAS dataset. Repeated analyses yielded similar results, establishing a causal link between MI and LBP. Furthermore, to account for potential confounding factors such as BMI, current tobacco smoking, alcohol intake frequency, total body bone mineral density, and vigorous physical activity, we employed multivariate MR. Overall, these pieces of evidence have bolstered the robustness of our research findings.

Our study has several advantages. Firstly, we conducted bidirectional MR analysis and repeated validation to summarize and calculate the causal relationship between MS and LBP, effectively avoiding confounding bias and reverse causality, which are not available in many observational and prospective studies. By eliminating confounding bias and reverse causality, our study provides more robust evidence for the correlation between MI and LBP. Secondly, our research results offer insights into the prevention and treatment of both MI and LBP. Given the high incidence of both conditions in the general population, uncovering the causal relationship between them will help us make more efforts in early prevention and timely intervention. Additionally, recognizing this causal relationship suggests that providing more mental health treatment during the LBP process can bring more clinical benefits to patients. However, our study also has some limitations. Firstly, all GWAS data come from European populations, and it remains to be studied whether the results described are consistent in other populations. Secondly, Because the MI dataset and the LBP dataset we use both have UK Biobank databases (replication data is a meta-GWAS dataset include four data banks, the UK Biobank was among them). So, there is a small overlap between the samples (<10%), which is usually considered acceptable and does not affect the results significantly (21, 52). Thirdly, the replication data shows the heterogeneity ($p < 0.05$), but the beta values are in the same direction, therefore they are also considered positive results. But these results are all available and it can support our conclusions.

5. Conclusion

This is the first MR analysis to examine the causal relationship between MI and LBP. Our analysis revealed that MI is associated with an increased risk of developing LBP, which remained consistent after adjusting for BMI, current tobacco smoking, alcohol intake frequency, total body bone mineral density, and vigorous physical activity. This study provides further support for the correlation between MI and LBP and offers valuable insights into the prevention and treatment of LBP.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the patients/participants or patients/participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

RL and RM conceived the idea for the study and provided critical revision of the manuscript. QL participated in study design, data acquisition, and data analysis. SX and RM participated in writing and drafting of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2023.1252329/full#supplementary-material

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Converging circuits between pain and depression: the ventral tegmental area as a therapeutic hub

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Chronic pain and depression are highly prevalent pathologies and cause a major socioeconomic burden to society. Chronic pain affects the emotional state of the individuals suffering from it, while depression worsens the prognosis of chronic pain patients and may diminish the effectiveness of pain treatments. There is a high comorbidity rate between both pathologies, which might share overlapping mechanisms. This review explores the evidence pinpointing a role for the ventral tegmental area (VTA) as a hub where both pain and emotional processing might converge. In addition, the feasibility of using the VTA as a possible therapeutic target is discussed. The role of the VTA, and the dopaminergic system in general, is highly studied in mood disorders, especially in deficits in reward-processing and motivation. Conversely, the VTA is less regarded where it concerns the study of central mechanisms of pain and its mood-associated consequences. Here, we first outline the brain circuits involving central processing of pain and mood disorders, focusing on the often-understudied role of the dopaminergic system and the VTA. Next, we highlight the state-of-the-art findings supporting the emergence of the VTA as a link where both pathways converge. Thus, we envision a promising part for the VTA as a putative target for innovative therapeutic approaches to treat chronic pain and its effects on mood. Finally, we emphasize the urge to develop and use animal models where both pain and depression-like symptoms are considered in conjunction.

KEYWORDS

ventral tegmental area, dopamine, pain, depression, nociception, anhedonia, electrical brain stimulation

Introduction

Chronic pain, with a prevalence that ranges from 10% to 40% worldwide, is a major concern in public health. It is the main reason for seeking medical care and represents a major societal burden due to its high socioeconomic impact (Goldberg and McGee, 2011; Breivik et al., 2013; Cohen and Mao, 2014; Cohen et al., 2021; De Ridder et al., 2021). Chronic pain significantly affects the quality of life and psychological wellbeing of patients. It affects not only the physical condition but also the emotional and psychological state of patients,

disturbing mood, sleep, or cognitive processes (Baliki et al., 2006; Timmers et al., 2019). Neuropsychiatric disorders (e.g., depression) are often comorbid to chronic pain (Bassols et al., 1999; Mäntyselkä et al., 2001; Frießem et al., 2009; Goldberg and McGee, 2011; Cherif et al., 2020; Cohen et al., 2021). Several studies support the idea that persistent pain increases the likelihood of depression, together with exacerbating its symptoms (Humo et al., 2019). In addition, there are other studies showing fewer functional benefits for antidepressants in patients with comorbid chronic pain (Roughan et al., 2021). Similarly, depression worsens the prognosis of chronic pain patients and diminishes the effectiveness of analgesic treatments (Fishbain et al., 1997; Ren et al., 2016; Sheng et al., 2017). Altogether, it appears probable that there is an association between chronic pain and depression and that their treatment requires a comprehensive approach addressing both the physical and psychological aspects of both conditions.

Chronic pain and depression might share common or overlapping mechanisms. From a pharmacological point of view, antidepressant drugs can be used as analgesics, especially in neuropathic pain and other types of pain that are not well managed with first in line analgesic drugs (e.g., NSAIDs, opioids) (Bonilla-Jaime et al., 2022). Some of these effects may be explained by the activation of the descending pain modulatory system, which consists of neuronal projections from midbrain areas, including the periaqueductal gray (PAG) and the rostral ventral medulla (RVM), to the spinal cord (Obata, 2017). These projections are primarily formed by monoaminergic neurons (releasing noradrenaline and serotonin) that also release endogenous opioid peptides (Hokfelt et al., 1977). Nevertheless, the processing of pain is a complex mechanism and involves other areas, such as some thalamic nuclei, especially in the mediodorsal thalamus, which receive and process sensory information from the periphery (Willis and Westlund, 1997), and in addition have the highest density of opioid receptors (Tempel and Zukin, 1987; Ko et al., 2003). Other relevant areas are the amygdala or the insula, which are involved in the processing of emotional responses to pain (Corder et al., 2019; Takahashi et al., 2019).

Similarly, some analgesics have been proposed as possible antidepressants based on 1) the presence of endogenous opioid peptides in brain areas playing a major role in affective disorders (Jelen et al., 2022), and 2) the affinity for opioid receptors exhibited by some antidepressant drugs (Berrocoso et al., 2009; McHugh and Kelly, 2018). The recent development and approval of the well-established anesthetic and analgesic drug ketamine as a novel antidepressant is another example sustaining that pain and depression may share overlapping mechanisms. While the anesthetic effects of ketamine (and esketamine) are parsimoniously explained by its action as a glutamate N-methyl-D-aspartate receptor (NMDAR) non-competitive antagonist, the molecular mechanisms for its antidepressant effects are more controverted (Zanos et al., 2018). Indeed, we and others have shown that some of the rewarding and antidepressant effects of these drugs are mediated via opioid receptors (Bonaventura et al., 2021; Levinstein et al., 2023).

Taken from a neurochemical perspective, the interplay between the opioidergic and monoaminergic systems and the crossover effects for some analgesic and antidepressant drugs may explain the bidirectional connection between chronic pain and depression. However, from a circuit perspective, other neurotransmitter systems and brain areas might also be involved. One of these areas is the ventral tegmental area (VTA). The VTA is a midbrain region critical for motivation and reward via the projection of dopaminergic neurons to the nucleus accumbens (NAcc) and the prefrontal cortex (PFC) (Koob, 2009; Russo and Nestler, 2013; Cai and Tong, 2022), However, the VTA also projects to other nuclei including the anterior cingulate cortex (ACC), the olfactory bulb, the amygdala, and the hippocampus (Nair-Roberts et al., 2008; Sesack and Grace, 2010; Qi et al., 2016; Montardy et al., 2019). Accordingly, it could play a role both in the processing of pain and emotion. Here, we will review clinical and preclinical evidence supporting the role of the VTA as a possible hub where both pain and emotional processing converge.

Involvement of the VTA in pain processing

The perception of pain is a highly coordinated and dynamic process that involves interactions between multiple brain areas. As briefly discussed above, some thalamic nuclei are major hubs in the processing of pain (Willis and Westlund, 1997), since they receive information from the periphery and filter and direct the signal to other areas for further processing (Basbaum et al., 2009). The somatosensory cortex plays a crucial role in localizing where pain occurs, while the ACC and the PFC are responsible for processing its emotional and cognitive aspects and for other higher-order cognitive functions, such as decision-making. Other important areas involved in this process are the insula, which integrates sensory, emotional, and cognitive information related to pain; the periaqueductal gray (PAG), modulating the descending pain modulatory system; the hypothalamus, involved in autonomic nervous system responses; and the amygdala, which is part of the limbic system and is mainly associated with its emotional processing (Wiech et al., 2008; Basbaum et al., 2009; Garland, 2012; Moradi et al., 2015; Corder et al., 2019; Huang et al., 2022; Ma et al., 2023).

The VTA is not often considered as a primary area involved in pain processing. However, several studies support a putative role for the VTA in pain perception and processing. For instance, a recent study in rats showed that chronic pain decreases dopaminergic activity due to increased inhibition from the bed nucleus of the stria terminalis (BNST) (Takahashi et al., 2019), a region that mediates aversive experiences (Davis et al., 2010; Minami, 2019) (Figure 1). Conversely, in a rat model of neuropathic pain, VTA neurons showed increased burst firing 2 weeks after peripheral nerve injury, which suggested that the increased dopaminergic activity could be an early result of chronic maladaptation to persistent pain (Sagheddu et al., 2015). Of note, these dopaminergic neurons in the VTA are modulated by GABAergic neurons projected from the rostromedial tegmental nucleus (RMTg). These inhibitory projections have been shown to reduce VTA excitability during inflammatory pain (Markovic et al., 2021). Similarly, optogenetic stimulation of VTA reversed allodynia caused by nerve injury in mice (Watanabe et al., 2018). On the other hand, RMTg GABAergic neurons, which express opioid receptors, are thought to mediate the inhibitory effects of opioid drugs in regulating the VTA dopaminergic neurons (Johnson and North, 1992; Fields and

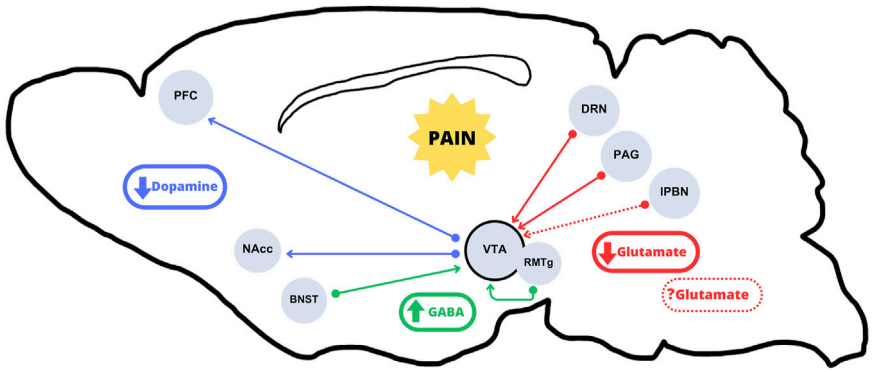

FIGURE 1
Incoming and arising pathways to and from the VTA subject to alterations under chronic pain conditions. The literature mainly suggests an overall hypoactivity of the VTA dopaminergic outputs in the mesolimbic and mesocortical pathways (blue arrows) in chronic pain conditions. Regarding VTA inputs, there would be a decrease in excitatory glutamate release from projections originating in the DRN and the PAG (solid red arrows). The role of lPBN excitatory neurons, which have been shown to be either activated or inhibited (dotted red arrows), is not clear. The resulting hypoglutamatergia, together with increased GABA release from the BNST and the RMTg (green arrows) would result in an overall VTA dopaminergic output inhibition.

Margolis, 2015; Taylor et al., 2019). Indeed, it has been shown that morphine elicits some of its antinociceptive effects through RMTg neurons, thus supporting a critical role of VTA activity not only on the rewarding effects of opioids but also on opioid-mediated analgesia (Taylor et al., 2019).

The activity in the VTA can lead to multifaceted, even contrasting, effects in its projection areas (Lammel et al., 2014) and therefore, the outcomes of its activation are highly context dependent (Belujon and Grace, 2015). The mesolimbic dopaminergic circuit, which consists of the projection of dopaminergic neurons from the VTA to the NAcc, has been shown to be altered in chronic pain conditions (Ren et al., 2016; Campos-Jurado et al., 2019; Yang et al., 2020; Wang et al., 2023). These alterations often involve changes in neurochemistry or neurotransmitter receptors of both the dopaminergic and opioidergic systems [for review see (Taylor et al., 2016; Lin et al., 2023)]. For instance, in different neuropathic pain models, it was observed that an increase in the activity of the VTA dopaminergic neurons had positive effects on pain regulation and its associated mood effects (Abdul et al., 2022; Huang et al., 2022). Similarly, a recent study showed diminished firing and intrinsic excitability of VTA dopaminergic neurons under chronic pain conditions that was due to reduced glutamatergic input from the dorsal raphe nucleus, and that optogenetic modulation of this pathway produced analgesic effects (Wang et al., 2023). Nevertheless, few reports have focused on assessing whether modulating the reward system can exert analgesic effects. For instance, in neuropathic or cancer pain, activation of dopamine neurons projecting to the NAcc restore the allodynia (Watanabe et al., 2018). Similarly, microinjecting dopamine receptor ligands into the NAcc led to reduced injury-induced thermal allodynia (Sato et al., 2022). Particularly, they showed that the specific facilitation of dopamine D1 receptor-expressing medium spiny neurons (MSN) in the NAcc projecting to the VTA led to pain relief, while the inhibition of dopamine D2 receptor-expressing MSNs led to significant antinociceptive effects (Sato et al., 2022). Interestingly, the increased excitability of the D2-containing indirect pathway MSN of the medial shell of the NAcc worsens tactile allodynia following peripheral nerve injury (Ren et al., 2016). Finally, it has been also shown that, in chronic pain conditions, extracellular dopamine release evoked by morphine or cocaine administration is decreased suggesting that a hyporeactive state of the mesolimbic dopaminergic system under chronic pain conditions (Taylor et al., 2015).

Similarly, VTA projections to the PFC are altered upon chronic pain conditions. An example of this, and of the diverse effects of opioid drugs within the brain, is shown in the work by Zhao et al. (2007), in which the injection of morphine in the PFC alleviated mechanical allodynia in a mouse model of neuropathic pain. Another study in mice demonstrated that not necessarily opioid-mediated effects but just increased dopaminergic activity in the PFC, elicited by selective activation of VTA's dopaminergic neurons, reduced mechanical hypersensitivity (Huang et al., 2020). This effect was explained by an increased PFC-mediated activity in the PAG (Huang et al., 2020). In fact, by using optogenetic manipulations, they demonstrated that the PFC-PAG circuit altered pain behavior by reducing the descending noradrenergic and serotoninergic modulation of spinal pain signals (Huang et al., 2019). The capacity of the PFC to receive nociceptive inputs but also to exert control over the pain sensation can be found elsewhere [for example, see (Apkarian et al., 2005; Ong et al., 2019; Kummer et al., 2020)]. Therefore, there is compelling evidence to support that areas other than the thalamus and canonical pain-related areas play a role in the processing of pain. Another area that might suffer alterations during chronic pain is the claustrum (Atilgan et al., 2022; Ntamati et al., 2023), a multimodal node with brain-wide connectivity and involved in several networks (Atilgan et al., 2022). Interestingly, the claustrum also receives dopaminergic inputs from the VTA (Wong et al., 2021), with dopamine causing a mostly inhibitory response of this structure (Wong et al., 2021), although the precise mechanisms of this inhibition are not yet well understood. However, it is interesting to note that an hypodopaminergic state could explain the reduced excitatory drive of the claustrum onto cortical structures like the ACC (Ntamati et al., 2023). In summary, speculatively, the VTA might have a central role as a flow regulator of the information

integrated and processed in areas such as the NAcc, PFC, claustrum, thalamus and PAG. Altogether, these results suggest that the effects elicited by chronic pain through the limbic system could also be viewed as a promising target to reach effective analgesia.

The VTA as an emerging therapeutic target to treat depression

Depression is a complex, multifaceted, and disabling disorder affecting an estimate of 5% of the world's adult population (World Health Organization, 2023). Decades of evidence showed the link between depression and the monoaminergic systems, especially the serotoninergic and noradrenergic (Coppen, 1967; Yohn et al., 2017). Although highly controversial and failing to reconcile the diverse symptomatology of depression (see Moncrieff et al., 2022) and its associated correspondence, the serotonergic hypothesis of depression supports the principal therapeutic treatments. The first-line class of antidepressant drugs are selective serotonin re-uptake inhibitors, which increase extracellular serotonin concentrations by blocking presynaptic serotonin transporters. Similarly, other popular antidepressant drug classes include both serotonin-noradrenaline reuptake inhibitors, tricyclic antidepressants, or monoamine-oxidase inhibitors (Cipriani et al., 2018; Lin et al., 2023), which also lead to increased extracellular levels of monoamines. However, all these medications have a delayed onset of action, and around 30%–40% of patients do not show an adequate response (Rush et al., 2006). More recently, psychedelic drugs, which may also act through the serotonergic system but targeting serotonin receptors instead of serotonin re-uptake or degradation, have been proposed as novel treatments for depression (Carhart-Harris et al., 2016; Griffiths et al., 2016; Reiff et al., 2020; Carhart-Harris et al., 2021; Daws et al., 2022). Of note, while their efficacy and safety are still a matter of debate, the role of the serotonergic system in their antidepressant action is also questioned (Hesselgrave et al., 2021; Moncrieff et al., 2022; Moliner et al., 2023).

Despite being the most studied, serotonin and noradrenaline are not the only monoamines implicated in depression. Several studies have shown that changes in the dopaminergic system are associated with both the pathogenesis and treatment of depression (Pani et al., 2000; Nestler and Carlezon, 2006; Yadid and Friedman, 2008; Tye et al., 2013; Belujon and Grace, 2017). Positron emission tomography (PET) imaging studies in humans reported a decrease in dopamine transporter binding potential in depressed patients, which is usually associated with an hypodopaminergic state (Meyer et al., 2001). Accordingly, pharmacological depletions in dopamine led to an increase of depressive symptoms in depressed patients or in subjects with a family history of depression but not in healthy subjects (Ruhé et al., 2007; Hasler et al., 2008). On the other hand, dopamine agonists such as the Parkinson's disease (PD) medications pramipexole or aripiprazole have antidepressant properties in PD patients with concurrent depression or anhedonia (Lemke et al., 2006).

As a complex disorder, it is not possible to pinpoint a unique brain region or circuit responsible for all the symptoms of depression (Kempton et al., 2011; Spellman and Liston, 2020). Despite this, there are converging reports that observed alterations of the limbic corticostriatal circuitry (precisely, several subregions of the PFC and the NAcc) in anhedonia and reward processing in patients suffering from depression (Johnstone et al., 2007; Siegle et al., 2007; Pizzagalli et al., 2009; Mayberg et al., 1999). The activity of the PFC and the NAcc is heavily modulated by dopaminergic transmission originating in the VTA (Koob, 2009; Russo and Nestler, 2013). Hence, the putative role of the VTA as a therapeutic target for depression has been studied in several preclinical studies in rodents. Interestingly, it has been shown that optogenetic or electrical stimulation of the VTA to NAcc or PFC pathways can ameliorate depressive-like symptoms in animal models of depression (Gersner et al., 2010; Sesia et al., 2010; van der Plasse et al., 2012; Chaudhury et al., 2013; Tye et al., 2013; Bambico et al., 2015; Ferenczi et al., 2016). On the other hand, non-pharmacological treatments of depression (Schlaepfer et al., 2013; Bewernick et al., 2017), such as deep brain stimulation (DBS) or transcranial magnetic stimulation, have also successfully targeted the VTA or the medial forebrain bundle (the main pathway of fibers connecting the limbic midbrain and forebrain) to achieve antidepressant activity in treatment-resistant depressed patients (Schlaepfer et al., 2013; Fenoy et al., 2016; Bewernick et al., 2017). Similar results have been observed with peripheral stimulation: vagus nerve stimulation (VNS) has shown promising results in ameliorating depression scores in clinical trials with patients with mild or moderate symptoms (Fang et al., 2016; Rong et al., 2016). Interestingly, the mechanistic studies of VNS [reviewed in (Carron et al., 2023)] support the idea that its therapeutic effects are due to increased catecholamine release.

Lastly, it is important to highlight that emerging antidepressant therapies, distinct from those directly linked to monoaminergic function -such as ketamine- consistently pinpoint the PFC-NAcc-VTA pathways as crucial anatomical sites of their effects. (Kokkinou et al., 2018; Wu et al., 2021).

Converging mechanisms between pain and depression in the VTA

Taking the above into consideration, it becomes evident that the VTA is a key structure where both pathogenic and therapeutic mechanisms of pain and depression might converge. Therefore, the VTA might arise as a candidate to target the concurrence of both pathologies. However, despite the high comorbidity between both pathologies in humans (Goldberg and McGee, 2011; Cherif et al., 2020; Cohen et al., 2021) and the extensive clinical data that relate them (Lampl et al., 2016; Roughan et al., 2021; Zhou et al., 2021; Voute et al., 2023), only a few preclinical studies have addressed them together. Of note, these studies generally coincide in suggesting that afferents from the PAG or the parabrachial nucleus (PBN), the dorsal raphe nucleus (DRN) or the BNST directly or indirectly control dopaminergic activity in the VTA efferent regions, such as the NAcc and PFC (Waung et al., 2019; Yang et al., 2021; Zhang et al., 2021; Lee et al., 2023). Hence, the VTA seems to be critical in translating painful stimuli into aversive or depressive-like behaviors.

The work by Waung et al. (2019) demonstrated that excitatory neurons from the ventrolateral PAG (vlPAG) mainly project to VTA GABAergic neurons; however, it is important to highlight that they

also detected both excitatory and inhibitory vlPAG inputs into a few dopaminergic neurons (Waung et al., 2019). Similarly, other studies described a parallel glutamatergic input from the lateral PBN (lPBN) to dopaminergic VTA neurons (Yang et al., 2021; Zhang et al., 2021). Therefore, VTA dopaminergic neurons may be relevant for relaying nociceptive signals from the spinal cord to midbrain nuclei. Indeed, when silencing these neurons pain sensation can be blocked. Thus, the ablation of lPBN to substantia nigra pars reticulata glutamatergic neurons was enough to reduce pain-mediated inhibition of dopamine release in mice (Coizet et al., 2010; Yang et al., 2021). Interestingly, one of the reports that addressed chronic pain and depression together (Lee et al., 2023) used a spinal nerve ligation (SNL) mouse model. This model resulted in a dysregulation of the glutamatergic transmission from PAG into the VTA. Then, by using chemogenetic tools to selectively recover the activity of the PAG-VTA pathway, they attenuated the SNL-induced depressive behavior of mice. Altogether, these studies showed that, in mice, pain reduced glutamatergic transmission to the VTA causing a decrease in dopamine release in its efferent areas (NAcc and PFC). The same hypodopaminergic state has been observed in chronic pain conditions, which might be due to the dampening of glutamatergic transmission from the DRN to the VTA, consequently decreasing dopamine release in the NAcc (Wang et al., 2023) (Figure 1). Accordingly, the activation of the DRN-VTA-NAcc pathway would be enough to decrease pain-like hypersensitivity and the concomitant anhedonic state. Another critical area could be the BNST. Several studies (Takahashi et al., 2019; Hara et al., 2020) have revealed the capacity of BNST to modulate the mesolimbic system, not only under chronic pain conditions but also in a depression model. In both cases (pain and depression), it seems likely that the inhibitory inputs to the VTA-projecting BNST neurons would led to neuroplastic changes, which would be a common mechanism between the two diseases (Takahashi et al., 2019; Hara et al., 2020).

As mentioned above, the opioidergic system plays an important role in controlling VTA-mediated dopamine release to the projecting areas (i.e., NAcc), an effect that is critical in regulating opioid reward and reinforcement. Nevertheless, some studies have shown that pain conditions can induce presynaptic MOR desensitization in the VTA, causing an increment in GABA release from RMTg neurons, and resulting in reduced dopamine release to dopaminergic projecting areas (Ozaki et al., 2002; Hipólito et al., 2015; Campos-Jurado et al., 2019). Similarly, chemogenetic activation of the NAcc-projecting VTA dopamine neurons allowed to overcome the pain-reduced motivated behaviors (Markovic et al., 2021). Altogether, pain-induced dysregulation of this circuit affects motivation and reward, supporting that it may act as a trigger to anhedonic-like behaviors. Additionally, apart from the neurochemical changes and the balance shift that leads to a hypodopaminergic state (Figure 1), chronic pain may cause other maladaptive effects in the VTA. For instance, chronic pain induced microglial activation in the VTA, which disrupted the homeostasis in GABAergic neurons and contributed to the decreased extracellular dopamine in the NAcc (Taylor et al., 2015). In conclusion, there are a number of studies supporting the idea that a hypodopaminergic state occurs upon chronic pain conditions, and that the decline in dopamine levels impairs motivated behavior (summarized in Figure 1).

On the other hand, other authors have proposed an alternative scenario. Thus, the development of depression-like behaviors in an animal model of chronic pain correlated with increased firing of the VTA dopaminergic neurons (Zhang et al., 2021). In this study, it was observed that blocking glutamatergic lPBN input to VTA dopamine neurons reversed the depressive-like behavior associated with chronic pain, however it did not affect the induced neuropathic pain sensitivity. In the opposite way, activation of this same circuit in naïve animals resulted in increased depressive-like behaviors, suggesting that the plastic changes induced by pain led to an increased firing neuronal rate that would be responsible for the comorbid emotional impaired state (Zhang et al., 2021).

Taken together, the evidence presented above suggests that the brain pathways incoming and arising from the VTA are part of a key, albeit understudied, circuit to explain the relationship between pain and depression. This hypothesis is further supported by the fact that pharmacological treatments for both pathologies not only overlap but they also directly or indirectly target the VTA. A paradigmatic example of this idea is represented by opioid drugs, which are used to treat pain but have ample actions on mood and have been proposed to treat depression (Browne et al., 2020). However, the abuse liability of opioid drugs makes them far from ideal to treat depression due to the higher vulnerability and comorbidity of addiction in patients with depressive disorders (Swendsen and Merikangas, 2000; Quello et al., 2005; McGrath et al., 2020). Alternatively, non-canonical drugs that target opioid receptors such as ketamine or methadone, which in addition show reduced abuse liability (Cai et al., 2019; Bonaventura et al., 2021), have become depression and pain medications.

Finally, as above-mentioned, a different, non-pharmacological, therapeutic approach to tackle neuropsychiatric disorders is electrical brain stimulation. This technique has been used for treating multiple conditions, such as movement disorders (e.g., Parkinson's disease), epilepsy, pain and psychiatric conditions like addiction, schizophrenia, or depression, focusing on multiple areas (Lozano et al., 2019). For depression, most studies have proposed targeting areas like the lateral habenula, the NAcc, or the ACC [reviewed in (Schlaepfer and Bewernick, 2013)]. More recently, DBS into the VTA and/or the medial forebrain bundle has also emerged as a suitable treatment. Clinical studies have shown rapid and sustained antidepressant effects (Schlaepfer et al., 2013; Fenoy et al., 2018; Coenen et al., 2019), and they also support its safety as a chronic treatment for up to 6 weeks (Thiele et al., 2018). Regarding pain, DBS has been mostly investigated for cluster headache, a highly disabling pain that tremendously affects patients' quality of life. Recent studies (Akram et al., 2016; Cappon et al., 2019) have demonstrated that this kind of treatment reduces the frequency and severity of migraine. Interestingly, Cappon et al. (2019) assessed the effects of DBS into the VTA in patients with cluster headache. They assessed the effects of the treatment on cognition, mood, behavior, and quality of life. Their findings suggest that the treatment induced a significant decrease in anxiety and better coping with pain, and an improvement in depression albeit it was not statistically significant (Akram et al., 2016; Cappon et al., 2019).

Conclusion

About a third of patients suffering from chronic pain also present depressive symptoms (Cherif et al., 2020). In addition, patients with concurrent chronic pain and depression show poorer prognosis than those with chronic pain alone (Fishbain et al., 1997). Hence, individuals with comorbid chronic pain and depression could benefit from a comprehensive approach to address both conditions simultaneously and improve their overall wellbeing and quality of life. However, most of the preclinical research is focused on either the development of novel analgesic treatments or understanding depressive-like behaviors per separate. Here, we highlight the importance of studying pain and depression as coexisting pathologies rather than by themselves to learn more about the converging pathways and mechanisms that can explain their comorbidity and find new effective strategies to treat them in conjunction.

A potential approach could involve targeting, through either pharmacological means or non-pharmacological methods like several forms of brain stimulation, toward brain regions that function as a communication hub connecting both conditions. From the evidence presented in this review it becomes evident that one of such brain regions could be the VTA. Thus, we propose that modulating the activity of the VTA can be regarded as a novel therapeutic opportunity to treat the concurrence of pain and depression. Although further studies will be required to elucidate the precise actions needed to target the VTA with the desirable therapeutic effects, the growing range of neuromodulation technologies, which allow precise and cell-specific control of neural activity, present unprecedented possibilities to tackle these devastating disorders from a newer perspective.

Author contributions

MF-G: Conceptualization, Writing–original draft. AR: Conceptualization, Writing–original draft. MG-P: Conceptualization, Writing–original draft. VF-D: Conceptualization, Funding acquisition, Supervision, Writing–review and editing. JB: Conceptualization, Funding acquisition, Supervision, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Dysregulated neuromodulation in the anterior cingulate cortex in chronic pain

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Chronic pain is a significant global socioeconomic burden with limited long-term treatment options. The intractable nature of chronic pain stems from two primary factors: the multifaceted nature of pain itself and an insufficient understanding of the diverse physiological mechanisms that underlie its initiation and maintenance, in both the peripheral and central nervous systems. The development of novel non-opioidergic analgesic approaches is contingent on our ability to normalize the dysregulated nociceptive pathways involved in pathological pain processing. The anterior cingulate cortex (ACC) stands out due to its involvement in top-down modulation of pain perception, its abnormal activity in chronic pain conditions, and its contribution to cognitive functions frequently impaired in chronic pain states. Here, we review the roles of the monoamines dopamine (DA), norepinephrine (NE), serotonin (5-HT), and other neuromodulators in controlling the activity of the ACC and how chronic pain alters their signaling in ACC circuits to promote pathological hyperexcitability. Additionally, we discuss the potential of targeting these monoaminergic pathways as a therapeutic strategy for treating the cognitive and affective symptoms associated with chronic pain.

KEYWORDS

chronic pain, anterior cingulate cortex, analgesia, dopamine, norepinephrine, serotonin, acetylcholine, monoamines

Introduction

Chronic pain remains a significant global socioeconomic burden, afflicting millions of individuals worldwide, and effective long-term treatment options remain elusive (Holmes, 2016; Dahlhamer et al., 2018; Nahin et al., 2023). The complexity of chronic pain arises from its multifaceted nature, encompassing sensory and emotional components that involve distinct regions of the central and peripheral nervous systems. While there have been notable advancements in identifying neuronal pathways associated with chronic pain, the precise pathophysiological mechanisms inducing their dysfunction are diverse and not well established. As a result, the development of effective non-opioidergic pharmacological treatments to alleviate chronic pain symptoms has been hindered.

Recent technological advances have provided unprecedented opportunities for investigating the cortical networks involved in nociception, as well as those dysregulated by chronic pain. Significant progress has been made in identifying key regions of the "pain matrix," a network of supraspinal regions involved in nociceptive signaling that participate in promoting pathological pain processing (Kuner and Flor, 2017; Kuner and Tan, 2020; Mercer et al., 2021).

Within the pain matrix, the anterior cingulate cortex (ACC), a region of the medial prefrontal cortex (mPFC), is particularly relevant due to its role in top-down pain

modulation and its ability to regulate executive functions commonly disrupted in chronic pain (Rainville et al., 1997; Kummer et al., 2020; Phelps et al., 2021). Several studies have shown that, following neuropathic injury, pyramidal neurons in the ACC become hyperexcitable, and functional imaging studies have identified the ACC as one of the most constitutively active brain regions in chronic pain patients (Cordeiro Matos et al., 2015a; Jensen et al., 2016; Ferraro et al., 2021; Lançon et al., 2021).

Emerging evidence suggest a link between alterations in the cortical function of neuromodulators such as the monoamines dopamine (DA), norepinephrine (NE), serotonin (5-HT), and chronic pain-related cortical hyperexcitability (Ong et a, 2019; Kuner and Tan, 2020; Yang et al., 2020). DA and NE appear particularly important due to their molecularly antagonistic, yet functionally synergistic role in modulating pyramidal activity in the ACC. These neuromodulators and others, such as acetylcholine (ACh) and the neuropeptide oxytocin, are crucial in adjusting the activity of ACC circuits by balancing excitatory and inhibitory inputs on pyramidal neurons and thus play a major role in tuning pain perception. Additionally, chronic pain is linked to a high prevalence of cognitive disabilities also observed in other disorders that impact the release of neuromodulators (Hart et al., 2003; Cools and Arnsten, 2022). Affect, attention, memory, executive planning, spatial awareness, sensory discrimination, and other cognitive functions heavily influenced by cortical monoamines are common issues in chronic pain patients (Jarcho et al., 2012; Bushnell et al., 2013; Markovic et al., 2021). Our current treatments for chronic pain can be viable in the short term but lose effectiveness and create tolerance and addiction in the long term (Varrassi et al., 2010; Speed et al., 2018). Furthermore, current pain treatments do not effectively alleviate the cognitive deficits associated with chronic pain (Chapman et al., 2002; Schiltenwolf et al., 2014).

Here we discuss the involvement of the three major monoamines (DA, NE, and 5-HT), as well as ACh and oxytocin, in promoting hyperexcitability in the ACC, leading to pathological pain processing and cognitive deficits in chronic pain conditions. We will review the complex interplay between neuromodulation of ACC circuits and pain processing, ultimately fostering better understanding of the cortical causes of chronic pain at the cellular and molecular level, to aid the development of novel and more effective interventions for chronic pain management.

Dysregulated ACC major contributor to pathological pain processing

The mPFC, and especially the ACC, is a critical hub for attention, emotional regulation, and pain modulation (Rainville et al., 1997; Stevens et al., 2011; Arnsten and Katya, 2012). Neuroimaging studies consistently report ACC activation following pain stimulation (Casey, 1999; Bastuji et al., 2016). Afferents from the medial thalamus and the somatosensory cortex relay noxious signals to the ACC, which then filters them and relays them back down to other cortical or subcortical regions involved in pain perception. These include, but are not limited to, the periaqueductal grey (PAG), the midbrain, the parabrachial nucleus (PBN), the dorsal horn of the spinal cord (DHSC), and other cortical areas such as the prelimbic, somatosensory, and infralimbic cortices (Kuner et al., 2020).

While the ACC predominantly mediates the affective dimension of nociception, it has also been shown to modulate its sensory component (Rainville et al., 1997; Chen et al., 2018; Singh et al., 2020). If the output of the ACC becomes dysregulated, the downstream signals processed by its target regions will be disrupted and will promote abnormal nociception. For example, experimental *in vivo* stimulation of pyramidal neurons in the ACC through opto- and chemogenetic actuators consistently leads to a decrease in mechanical withdrawal thresholds as well as the induction of conditioned place aversion (CPA), established readouts for the sensory and affective aspects of pain, respectively (Johansen et al., 2001; Kang et al., 2015). The ACC is pathologically hyperactive in the case of patients with nerve injuries or other chronic illnesses known to cause prolonged painful states (Jensen et al., 2016; Tan and Kuner, 2021). Decreasing this hyperactivity in animal models of chronic pain reduces both allodynia (abnormal sensitivity to innocuous stimuli) and hyperalgesia (increased sensitivity to noxious stimuli), thereby demonstrating that dysregulated ACC circuits contribute to abnormal pain processing (Kang et al., 2015; Sellmeiher et al., 2018; Juarez-Salinas et al., 2019).

This increase in excitability is caused by changes in the balance of excitatory and inhibitory inputs (E/I balance) as well as intrinsic changes in the excitability of pyramidal neurons. Following nerve injury, the E/I balance in the ACC is shifted due to enhanced excitatory glutamate release and decreased inhibitory gamma-aminobutyric acid (GABA) release. The increase in glutamate release induces a robust facilitation of long-term potentiation (LTP) that is correlated with increases in NMDA-R currents and an upregulation of the GluN2B subunit (Kuner and Flor, 2017; Zhuo, 2020; Chen et al., 2021). For GABA release, although neuropathic pain does not appear to decrease the excitability of GABAergic interneurons, there is evidence of disinhibition due to loss of GABAergic synapses (Blom et al., 2014). The intrinsic excitability of synaptically isolated pyramidal neurons in the ACC is also potentiated following nerve injury. Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, dendritic cation channels known to tune the excitability of neurons by controlling their input resistance, are dysfunctional in neuropathic states (Cordeiro-Matos et al., 2015b; Santello and Nevian, 2015; Santello et al., 2017; Lançon et al., 2021). Since HCN channel activity is controlled by the ratio of G_s to G_i-linked metabotropic signaling, up and down regulators of adenyl cyclase respectively, this strongly suggests that G protein-coupled receptor activation is altered in the ACC in neuropathic states.

Identifying the factors that promote abnormal GPCR signaling in chronic pain conditions could be key to the development of novel analgesics or treatments that impede the instigation or maintenance of the pathological phenotype following nerve injury.

Dopamine

The role of DA in modulating mPFC function is relatively well studied in cognitive and attentional disorders, and more recently, in pain (Figure 1) (Vijayraghavan et al., 2007; Huang et al., 2019a). A genetically defined subset of VGluT2/DAT+ projection neurons

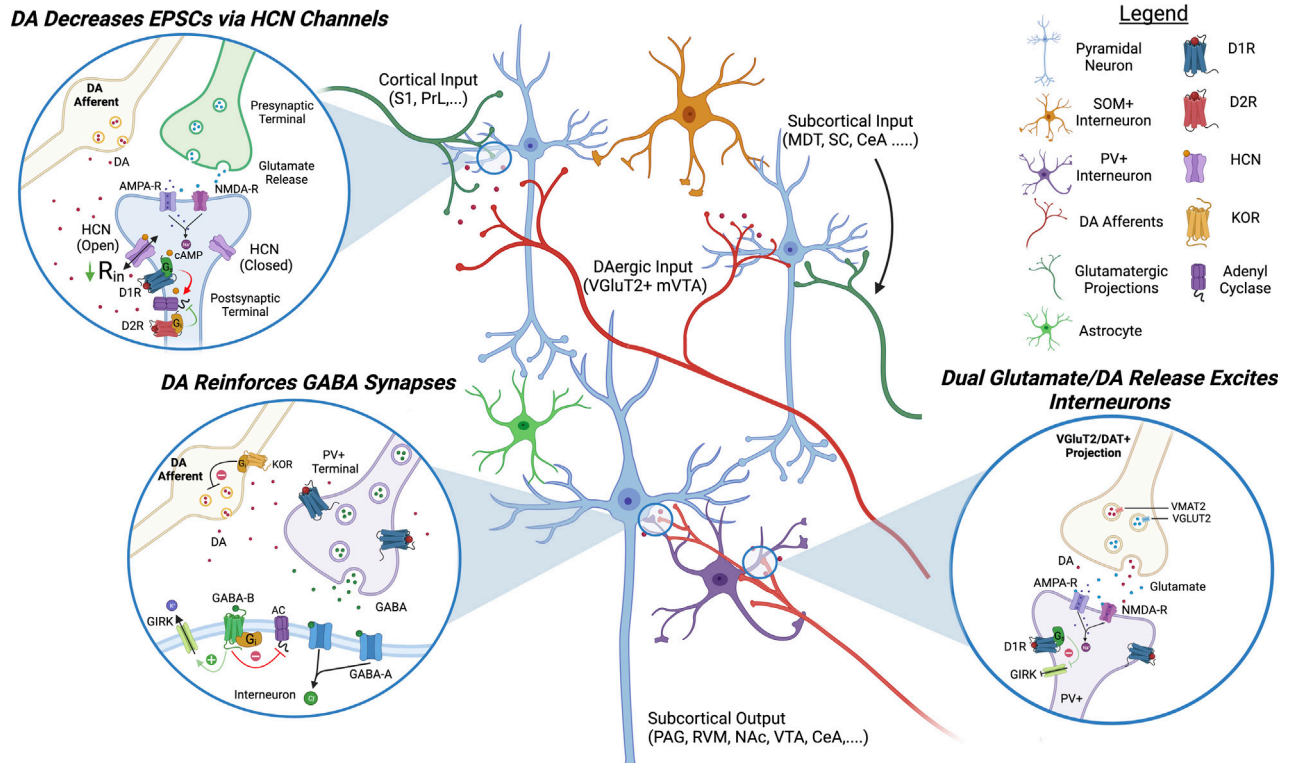

FIGURE 1
Proposed Model of DAergic Modulation of ACC Circuits. DAergic signaling in the ACC inhibits pyramidal neurons and activates inhibitory interneurons. D1R activation decreases EPSCs on pyramidal neurons by increasing the open channel probability of HCN channels and activates GABAergic interneurons via glutamatergic signaling and inhibition of K+ channels. DA, dopamine; HCN, hyperpolarization-activated cyclic nucleotide-gated channels; AC, adenyl cyclase; GIRK, G-protein coupled inward rectifying potassium channels; KOR, kappa opioid receptor; PAG, periaqueductal gray; S1, somatosensory cortex 1; PrL, prelimbic cortex; MDT, medial dorsal thalamus; SC, spinal cord; CeA, central nucleus of amygdala; RVM, rostral ventromedial medulla; NAc, nucleus accumbens; VTA, ventral tegmental area.

located in the medial ventral tegmental area (VTA) has been reported to innervate the ACC and this mesocortical pathway is hypothesized to underlie salience and tune attention (Horvitz, 2000; Arnsten and Pliszka, 2011; Poulin et al., 2018). This pathway is now being studied further in its response to pain states and whether it is dysfunctional in chronic pain conditions. A recent study indicates that acute pain induces an inhibition of VTA neurons indirectly via the PBN and the substantia nigra (SNr) (Yang et al., 2021). In line with this, the VTA is hypoactive in chronic pain conditions and HPLC analysis demonstrates that DA levels are reduced in the ACC 3 days following CFA injection to evoke inflammatory pain (Narita et al., 2003; Ko et al., 2018; Huang et al., 2019b; Darvish-Ghane et al., 2020). Human imaging studies have also indicated a reduced activity of the VTA following salient stimulation in multiple forms of chronic pain (Loggia et al., 2014; Martikainen et al., 2015). Given these findings, it becomes critical to understand the role of DA in modulating ACC circuits and the impact that decreased cortical DAergic signaling would have on cortical output in chronic pain states.

G_s-coupled DA receptor subtype 1 (D1R) is the most studied DA receptor in the ACC and is expressed on pyramidal neurons in layers 2/3 and L5/6 as well as GABAergic interneurons (Muly et al., 1998; Clarkson et al., 2017). In pyramidal neurons, D1Rs are co-localized with HCN channels on dendritic spines and electrophysiological studies have demonstrated that their activation increases the open channel probability of HCN channels, decreasing the input resistance (Paspalas et al., 2012; Lançon et al., 2021). Functionally, activation of D1R expressed on pyramidal ACC inhibits excitatory post synaptic currents (EPSCs), AMPA-R currents, and causes general inhibition by increasing the rheobase of pyramidal neurons (Darvish-Ghane et al., 2016; Lançon et al., 2021). D1Rs are also expressed on parvalbumin (PV+) fast-spiking GABAergic interneurons in the ACC (Le Moine and Gaspar, 1998; Glausier et al., 2009; Santana et al., 2009). Activation of D1Rs expressed on GABAergic interneurons inhibits leak K+ channels and inward-rectifying K+ channels to induce a robust excitation and increase inhibitory post synaptic currents (IPSCs) on pyramidal neurons (Seamans et al., 2001; Gorelova et al., 2002; Kröner et al., 2007; Ren et al., 2018; Satoh et al., 2018). In chronic pain conditions, overall D1R activation in the ACC appears to play a critical role in controlling pain perception and pain relief. Not only is microinjection of D1R agonists in the ACC analgesic but D1R signaling in the ACC is required for effective pain relief in neuropathic mice (Lançon et al., 2021). Conversely, conditional KO of D1Rs in the ACC using a crisper/cas9 construct is hyperalgesic (Darvish-Ghane et al., 2020).

G_i-coupled DA receptor subtype 2 (D2Rs) is also expressed on PV+ interneurons but its expression decreases with age and is

thought to play a role in the development of cortical circuits and in cognitive disorders such as schizophrenia in adulthood (Le Moine and Gaspar, 1998; Porter et al., 1999; Santana et al., 2009; Graham et al., 2015). Activation of D2Rs on interneurons has been shown to decrease IPSCs on pyramidal neurons (Seamans et al., 2001). D2Rs are also expressed on pyramidal neurons and their activation appears to play an excitatory role but the effect appears less robust than D1R activation (Green et al., 2020; Lançon et al., 2021). DA receptor subtype 3 (D3Rs) are also present in L2/3 ACC and although less is known about their direct effect on neuron excitability, they appear to play a role in gating the release of DA, NE, and ACh (Lacroix et al., 2003). The DA receptor subtype 5 (D5R) is expressed on pyramidal and might play a similar role as the D1R (Sarinana and Tonegawa, 2016).

In chronic pain D1R mRNA is decreased whereas D2R mRNA is increased in the ACC (Ortega-Legaspi et al., 2011). In line with this, D1R-mediated inhibition of AMPA-R currents is reduced in inflammatory pain models and other evidence indicates that D1R-evoked IPSCs are reduced in high-stress models (Satoh et al., 2018; Darvish-Ghane et al., 2022). All data converge to conclude that chronic pain is inducing a hypodopaminergic state in the ACC. Given that D2Rs have a higher sensitivity for DA than D1Rs, this hypodopaminergic state likely leads to a decrease in G_s-coupled signaling and an increase in G_i-coupled signaling. Activating DAergic projections in the mPFC, or activating DA neurons in the VTA directly, is analgesic in chronic pain models, thereby demonstrating that increasing cortical DA signaling is sufficient to occlude chronic pain symptoms (López-Avila et al., 2004; Huang et al., 2020).

What is causing a hypoactive VTA in chronic pain remains unclear but could be due to recruitment of the endogenous opioid system, with both the kappa opioid receptors (KORs) and the mu opioid receptors (MORs) playing a role. Dynorphin, the endogenous agonist for the G_i-coupled KOR, is well known for its role in gating DA release (Xi et al., 1998). The KOR is highly expressed on DA terminals within the mesolimbic pathway where its activation inhibits the release of DA. In line with this, selective KOR agonists have been shown to be highly aversive in both humans and rodents and this aversion is dependent on KOR expression in DA neurons (Liu et al., 2019). In the ACC, prodynorphin mRNA is increased following nerve injury, suggesting KOR over-activation could play a role in decreasing mesocortical DA release in chronic pain conditions (Palmisano et al., 2019). Actually, a recent study demonstrates that microinjection of a KOR antagonist in the ACC is analgesic in neuropathic mice while microinjection of a KOR agonist can produce a neuropathic-like phenotype in naïve mice (Navratilova et al., 2023). MORs, other G_i-coupled opioidergic receptors, are expressed on GABAergic neurons in the VTA and their activation inhibits GABA release, causing disinhibition of DAergic projections (Johnson and North, 1992; Narita et al., 2001). In chronic pain, MOR activation is decreased in the VTA, possibly mediated by MOR internalization, causing an elevation of synaptic GABA levels (Ozaki et al., 2002; Zuo, 2005). It is also possible that increased activity of the locus coeruleus (LC), the main source of central NE, is contributing to a hypoactive VTA in chronic pain. There is evidence of LC projections to the VTA and NE application on VTA neurons decreases the firing activity of DA neurons (Guiard et al., 2008).

Interestingly, there is a high comorbidity between several hypodopaminergic disorders, including Parkinson's disease and major depressive disorder, and chronic pain (Miller and Cano, 2009; Ford, 2010). Parkinsonian patients have an exceptionally high incidence of non-myogenic chronic pain and fMRI studies demonstrate their ACC is hyperactive (Schestatsky et al., 2007; Blanchet and Brefel-Courbon, 2018). Supplementing supraspinal DA with L-DOPA decreases the sensory symptoms of chronic pain alongside ACC activity in Parkinsonian patients (Brefel-Courbon et al., 2005). In line with this, supplementing neuropathic mice with L-DOPA decreases chronic pain symptoms and ACC excitability (Lançon et al., 2021). As a proof of concept, it has also recently been shown that low doses of L-DOPA can reduce symptoms of chronic back pain in humans as well (Reckziegal et al., 2019). However, due to the known effects of long-term L-DOPA treatment on fine motor control, a more restrained approach to increasing cortical DA will be needed to reduce side effects.

Norepinephrine

NE is another monoamine with a robust effect on supraspinal pain circuits and it appears dysregulated in chronic pain conditions (Figure 2). Similar to the VTA, the LC has high cellular heterogeneity with projection-specific neuronal subsets. Whereas spinal-projecting LC neurons are antinociceptive (see the analgesic effects of α2 agonist clonidine injected intrathecally), cortically-projecting LC neurons are pro-nociceptive (Filos et al., 1992; De Kock et al., 2005; Hirschberg et al., 2017; Koga et al., 2020). Although the LC has a less defined topographic organization than the VTA, transcriptomic studies have shown that LC projections to the mPFC express high levels of both VMAT2 and the sodium channel accessory subunit $Na_v\beta3$, allowing them to fire at high frequencies for prolonged periods (Kole et al., 2008; Chandler, 2016). In chronic pain, the LC displays increased excitability and seems to play a role in pathological pain processing as stimulation of LC projections to the ACC increases glutamatergic transmission and induces sensitization to pain and itch (Hirschberg et al., 2017; Koga et al., 2020; Camarena-Delgado et al., 2022; Iqbal et al., 2023).

The predominant adrenergic receptors in the ACC are the GPCRs β2, α1, and α2. The G_q-coupled α1 receptor is expressed in L2/3/5/6 on glutamatergic terminals where its activation promotes the release of glutamate, increasing EPSCs (Santana et al., 2013; Zhang et al., 2013). The G_i-coupled α2 receptor is found on pyramidal neurons in L2/3/5/6 of the ACC and its activation has the opposite effect of D1R on HCN channels, i.e., closing them (Wang et al., 2007). α2 agonists inhibit HCN channels, increase input resistance, and increase the excitability of pyramidal neurons in the ACC (Zhang et al., 2013). The synergistic role of α1 and α2 activation leads NE to have a robust excitatory drive on ACC pyramidal and consequently on pain perception (Zhang et al., 2013; Kaushal et al., 2016).

Astrocytes and microglia play a key role in synapse reinforcement and pruning in cortical circuits and these non-neuronal cell types are also modulated by NE in the ACC and have been shown to play a role in tuning pain-induced aversion (Romanos et al., 2020; Iqbal et al., 2023). β2 and α1 receptors are expressed on astrocytes and their activation induces aversion:

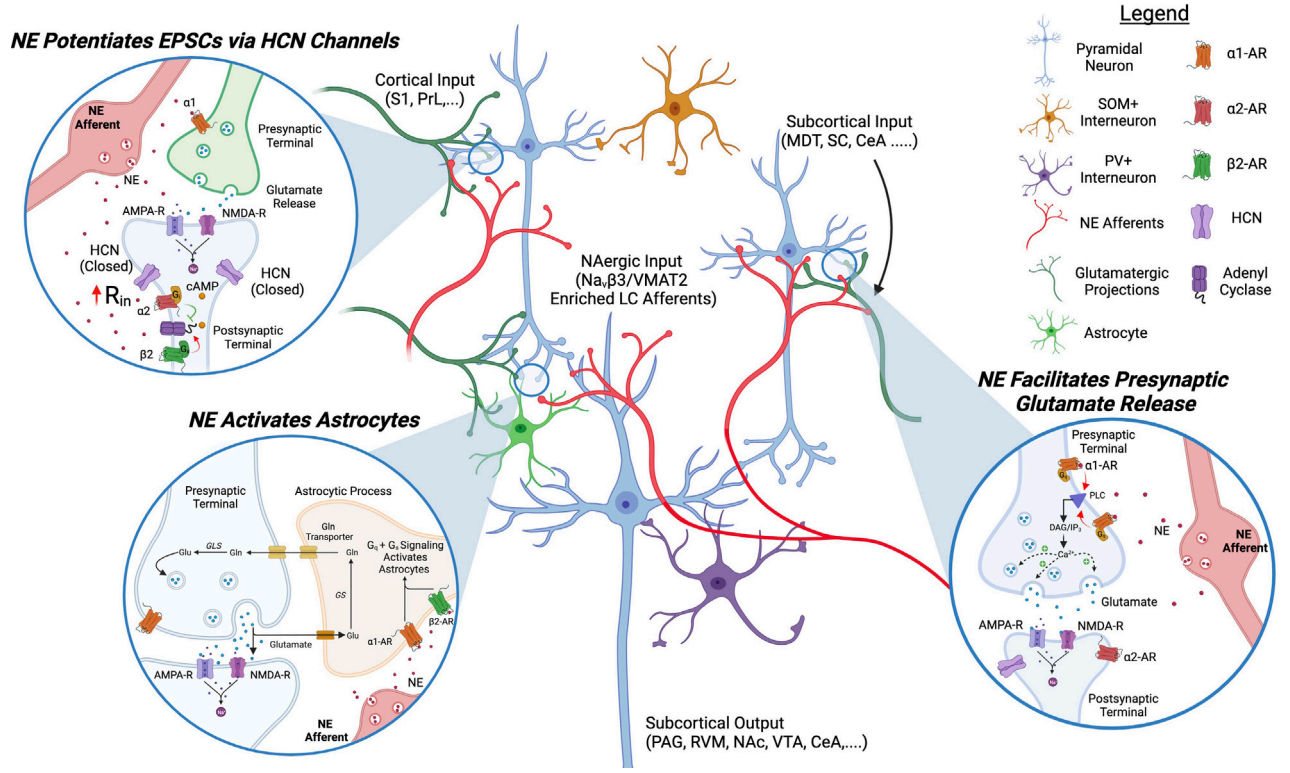

FIGURE 2
Proposed Model of NAergic Modulation of ACC Circuits. NAergic signaling in the ACC excites pyramidal neurons and activates astrocytes. α2 and α1 receptor activation increases EPSCs on pyramidal neurons by decreasing the open channel probability of HCN channels and promoting presynaptic glutamate release, respectively. α1 and β2 signaling activate astrocytes. NE, norepinephrine; HCN, hyperpolarization-activated cyclic nucleotide-gated channels; AC, adenyl cyclase; Gln, glutamine; PLC, phospholipase C; PAG, periaqueductal gray; S1, somatosensory cortex 1; PrL, prelimbic cortex; MDT, medial dorsal thalamus; SC, spinal cord; CeA, central nucleus of amygdala; RVM, rostral ventromedial medulla; NAc, nucleus accumbens; VTA, ventral tegmental area.

conditional KD of β2 receptors using micro-RNA based interference (miRNAi) reduces pain-induced CPA while their opto-activation on astrocytes promotes aversive memory formation (Iqbal et al., 2023). This is particularly interesting as it has been reported that overactivation of microglia in the SC is involved in pruning inhibitory synapses and promoting pain sensitization (Yousefpour et al., 2023). Given the ACC also exhibits a reduction in GABAergic signaling in neuropathic conditions, NAergic overactivation of microglia could play a similar role in cortical circuits (Blom et al., 2014). There is evidence that DA, by chemical similarity with NE, can also bind to α1 noradrenergic receptors expressed on astrocytes in the ACC and this α1-mediated catecholaminergic pathway may play a role in cognition. (Pittolo et al., 2022).

Due to the excitatory effect of NE on pyramidal excitability, activation of PFC-projecting LC fibers causes aversion and exacerbates pain perception whereas microinjection of an α1 antagonist in the ACC is analgesic (Kaushal et al., 2016; Hirschberg et al., 2017; Koga et al., 2020). In chronic pain states, the LC displays hyperexcitable characteristics and there is anatomical evidence of NE fiber sprouting in the ACC in neuropathic rodents (Cordeiro Matos et al., 2018; Camarena-Delgado et al., 2022). What causes the increase in cortical adrenergic activity is not established yet, but similarly to the situation in the VTA, ineffective MOR activation by endogenous opioids in ACC-projecting LC neurons may be causing a disinhibition in chronic pain states (Guajardo et al., 2017). The pathway leading to an increased NEergic excitation of pyramidal neurons in the ACC in chronic pain conditions could become a therapeutic target. However, due to the analgesic effects of spinal NE, designing a clinical approach that exclusively targets NE signaling in the mPFC remains a challenge.

Serotonin

5-HT projections from the medial dorsal raphe nucleus (DRN) to the ACC have also been shown to modulate E/I balance and pain sensitivity. Although DRN projections to the ACC have been identified using retrograde labeling, little is known about their response to both acute pain stimulation and whether they are dysregulated in chronic pain conditions (O'Hearn and Molliver, 1984; Sarter and Markowitsch, 1984; Waselus et al., 2011). Fortunately, due to the established role of 5-HT in psychosis, the impact of 5-HT on ACC circuits is well characterized (Figure 3).

Pyramidal neurons have been shown to express the receptors 5-$HT_{1A/B}$, 5-HT_{2A}, 5-HT_7, and 5-HT_4; and 5-HT application on acute brain slices is generally inhibitory in synaptically isolated pyramidal

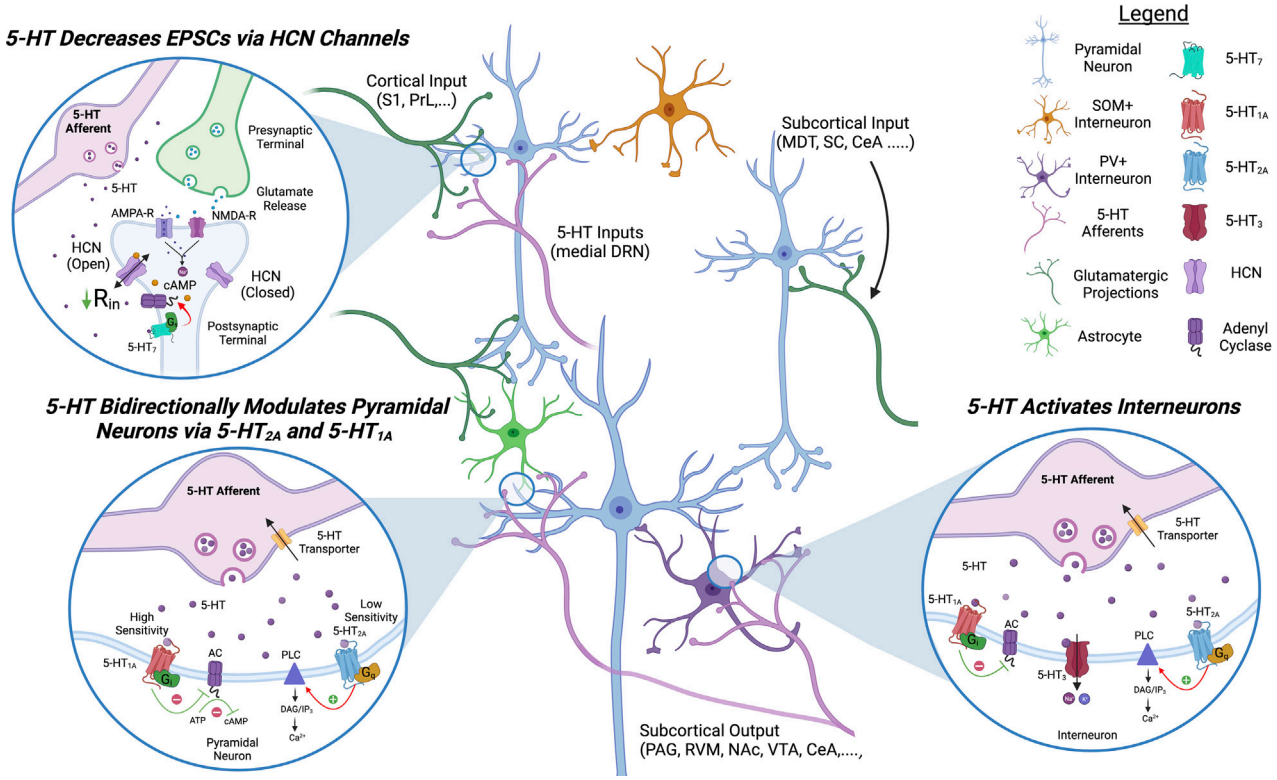

FIGURE 3
Proposed Model of Serotonergic Modulation of ACC Circuits. 5-HT signaling in the ACC inhibits pyramidal neurons and activates inhibitory interneurons. 5-HT_7 decreases EPSCs on pyramidal neurons by increasing the open channel probability of HCN channels. 5-HT_1 and 5-HT_2 bidirectionally modulate pyramidal activity via G_i- and G_q-signaling, respectively. 5-HT_2 and 5-HT_3 signaling activates inhibitory interneurons. 5-HT, serotonin; HCN, hyperpolarization-activated cyclic nucleotide-gated channels; AC, adenyl cyclase; PLC, phospholipaseC; PAG, periaqueductal gray; S1, somatosensory cortex 1; PrL, prelimbic cortex; MDT, medial dorsal thalamus; SC, spinal cord; CeA, central nucleus of amygdala; RVM, rostral ventromedial medulla; NAc, nucleus accumbens; VTA, ventral tegmental area.

neurons (Tanaka and Alan North, 1993; Santana et al., 2004; Santello et al., 2017; Tian et al., 2017). However, selective activation of postsynaptic 5-HT_{1A} or 5-HT_{2A} receptors appears to have opposite effects: agonists of G_i-coupled 5-HT_{1A} hyperpolarize pyramidal cells, reduce EPSCs and NMDA currents, while selective activation of G_q-coupled 5-HT_{2A} depolarizes pyramidal cells (Araneda and Andrade, 1991; Tanaka and Alan North, 1993; Avesar and Gulledge, 2012; Tian et al., 2017). As the 5-HT_{2A} receptor has a lower affinity for 5-HT than 5-HT_{1A}, an increase in 5-HT release could turn an inhibitory response into an excitatory response. The G_s-coupled 5-HT_7 receptor, located on apical dendrites in L5, has also been demonstrated to modulate HCN channels similarly to D1R. Selective 5-HT_7 agonists inhibit pyramidal neurons via opening of HCN channels and treatment with the 5-HT_7 agonist LP-211, permeable to the blood-brain barrier, alleviates symptoms of chronic pain in animal models (Santello and Nevian, 2015; Santello et al., 2017). Less is known about the overall effects of G_s-coupled 5-HT_4 receptor in the ACC circuits as its activation has mixed effects of pyramidal excitability (Yan, 2002).

The 5-HT_{1A}, 5-HT_{2A}, and 5-HT_3 receptors are also expressed on GABAergic interneurons (Zhou and Hablitz, 1999; Yan, 2002; Santana et al., 2004; Puig et al., 2010). Roughly 30% of PV+ interneurons express 5-HT_{1A} or 5-HT_{2A} and the 5-HT_{2A} receptor is expressed on somatostatin (SOM+) interneurons in neighboring cortical regions (Puig et al., 2010; De Filippo et al., 2021). 5-HT_{1A} activation on fast spiking interneurons in the ACC decreases interneuron activity while 5-HT_{2A} activation increases it (Puig et al., 2010). The 5-HT-gated cation channel 5-HT_3 depolarizes GABAergic interneurons and increases IPSCs on pyramidal neurons (Zhou and Hablitz, 1999; Férézou et al., 2002; Puig et al., 2004). The role of cortical 5-HT on pain responses is less characterized than DA or NE but there is evidence the 5-HT_{2A} and 5-HT_7 receptors play a role in sensory discrimination and pain sensitization (Gresch et al., 2007; Santello et al., 2017).

At the source of cortical 5-HT projections, it has been reported that the DRN can be inhibited but also activated by noxious stimulation (Sanders et al., 1980; Porro et al., 1991). A study using microdialysis indicates increased 5-HT concentrations in the PFC of neuropathic mice following DRN stimulation (Ito et al., 2013) Modulating GABAergic interneurons in the DRN can bidirectionally modulate nociception: activation of DRN-GABA+ neurons is anti-nociceptive while inhibition of DRN-GABA+ is pro-nociceptive (Xie et al., 2022). Additionally, there is a high degree of crosstalk between the DRN and VTA, likely affecting cortical DA release (Wang et al., 2023). Recent findings

indicate that VGluT3+ DRN projections to the VTA play a role in tuning pain perception by modulating DA release in the striatum and that this pathway is inhibited in neuropathic conditions, decreasing glutamatergic drive on DAT+ VTA neurons (Wang et al., 2023). Further research is required to determine how the DRN and its projections to the ACC are affected by the onset of chronic pain.

Acetylcholine

ACh, originating from the basal forebrain, is another neuromodulator that has a strong impact on mPFC circuitry and is well studied for its role in sustained attention and working memory (Passetti et al., 2000; Howe et al., 2017). The ACC expresses both nicotinic ACh receptors (nAChRs), consisting mostly of the α7, α4, and β2 subunits, and muscarinic receptors (mAChRs), mainly the subtypes M_1, M_2, and M_4 (Albuquerque et al., 2009). Cholinergic projections to the mPFC can be separated into 4 distinct pathways, each innervating different regions of the mPFC with different modulatory effects (Bloem et al., 2014; Oswald et al., 2022). Due to the expression of a wide range of cholinergic receptors and distinct cholinergic inputs, the effect of ACh on ACC circuitry is situationally dependent as it can both inhibit or excite neurons depending on the situation, and whether ACh is release phasically or tonically (Gulledge and Stuart, 2005; Aracri et al., 2010). nAChRs are ACh-gated cation channels expressed on both GABAergic interneurons and pyramidal neurons in the ACC and their activation impacts ACC circuitry differently depending on the cortical layer. In L2/3 nAChR activation is inhibitory to pyramidal neurons due to the expression of nAChRs on GABAergic somata and terminals whereas in L5/6 nAChR activation induces pyramidal firing due to a higher expression on pyramidal neurons (Araci et al., 2010; Poorthuis et al., 2013). Although nAChRs are also expressed on GABAergic interneurons in L5/6, their effect is dependent on glutamatergic transmission and their activation can be either excitatory or inhibitory on interneurons (Vidal and Changeux, 1989; Gulledge and Stuart, 2005; Aracri et al., 2010).

The mAChRs are GPCRs expressed on pyramidal neurons in L2/3/5/6 and are generally excitatory to L5/6 but can induce inhibition in L2/3 due to dual expression on PV+ GABAergic interneurons (Disney and Aoki, 2008; Shirey et al., 2009). Selective M_1 agonists increase EPSCs in the mPFC L5/6 and can induce persistent firing, the neuronal substrate for working memory (Zhang and Philippe, 2010; Kamiński et al., 2017). Due to this, mAChRs play a major role in modulating sustained memory tasks in rodents and primates (Shirey et al., 2009; Galvin et al., 2020).

In pain, cholinergic projections from the medial septal nucleus (MSN) to the ACC is pronociceptive and stimulation of these cholinergic projections is hyperalgesic in inflammatory models of chronic pain (Jiang et al., 2018). Conversely, chemogenetic inhibition of the MSN to ACC pathway is analgesic and produces conditioned place preference (CPP) in chronic pain (Jiang et al., 2018). Scopolamine, a nonspecific muscarinic antagonist, is commonly used for its anti-depressive effects and microinjection into the ACC produces anti-nociceptive effects, possibly by inhibiting nociception-related attention (Ortega-Legaspi et al., 2003). However, due to M_1 expression on GABAergic interneurons and the differential effect of ACh depending on physiological state, increased ACh release can be analgesic: microinjection of the M_1 agonist McN-A-343 into the ACC increases mechanical nociceptive thresholds via promoting GABA release (Koga et al., 2017).

Although it is unknown if chronic pain states disrupt the release of ACh in the ACC, the monoamines DA and NE play an active role in modulating mAChR-induced persistent firing (Zhang et al., 2013; Lançon et al., 2021). Therefore, we can safely infer that dysregulation of their release affects the functionality of cholinergic signaling in the ACC. In the striatum, ACh has also recently been shown to generate action potentials in distal DA axons via activation of nAChRs, promoting the local release of DA (Liu et al., 2022). Therefore, alterations in the release of ACh could be affecting the local release of DA in the ACC through a similar crosstalk, and this merits further investigation.

Oxytocin

Neuropeptides and other intercellular mediators have also been shown to significantly affect ACC circuitry and are affected by the onset of chronic pain. The neuropeptide oxytocin (OXT) specifically appears to play a key role in modulating ACC outputs as oxytocin microinjection in the ACC increases mechanical withdrawal thresholds (Li Anna et al, 2021). Physiologically, OXT is released in the ACC via projections from the paraventricular nucleus (PVN) in hypothalamus. In agreement with the analgesic effects of OXT microinjection in the ACC, optogenetic stimulation of the PVN to ACC pathway is also anti-nociceptive (Li Xu-Hui et al, 2021). This effect appears mediated by both the modulation of pyramidal neurons and GABAergic interneurons via the G_q-coupled oxytocin receptor (OXTR) and vasopressin 1A receptor (Schorscher-Petcu et al., 2010). OXT application in acute brain slices significantly decreases E/I ratio by decreasing EPSCs and increasing IPSCs on pyramidal neurons (Li Anna et al, 2021). This is mainly mediated by activation of GABAergic interneurons: oxytocin increases the resting membrane potential and decreases the rheobase of interneurons but not pyramidal cells. In chronic pain, OXT levels in the ACC are unchanged but the OXTR expression is significantly increased (Li Xu-Hui et al, 2021).

Connecting chronic pain with attentional dysfunction

Chronic pain patients commonly report deficits in working memory, emotional regulation, attention, and anxiety; all of which have been shown to be processed by the ACC (Rudy et al., 1988; Hart et al., 2003; Apkarian et al., 2004; Grégoire et al., 2012; Guerreiro et al., 2022). There is an overwhelming overlap between the cognitive deficits commonly seen in chronic pain patients and the cognitive roles associated with the ACC, hinting that a dysregulated ACC could be a central hub for chronic pain-induced cognitive deficits (Kummer et al., 2020). This link works in the other direction as well, as patients with pathological deficits in attention control and working memory, such as ADHD and PTSD, display a high prevalence of chronic pain (Brennstuhl et al., 2015;

Kind and Otis, 2019; Kasahara et al., 2020; Battison et al., 2023). Pain, being one crucial survival signal from an evolutionary standpoint, demands our immediate attention; thus, it is understandable why it is so deeply integrated into our cognitive processes, and why chronic pain can exert such a profound influence on our attention.

The Arnsten group has repeatedly shown that the homeostatic balance of DA and NE in the mPFC is key to tuning attention in primates and changes in the cortical release of these neuromodulators is associated with attention disorders such as ADHD and PTSD (Arnsten, 2000; Vijayraghavan et al., 2007). Since chronic pain also influences the ratio of NE/DA release in the ACC, it may explain why chronic pain patients have a high prevalence of attention deficits and why there is a high comorbidity between attention disorders and chronic pain. Both ADHD and chronic pain states could result in a similar shift in the differential release of NE and DA, explaining the significant overlap in their symptoms. Similarly, depressive and Parkinsonian patients have decreased DAergic signaling and commonly report attention deficits as well as chronic pain (Mehler-Wex et al., 2006; Zhou et al., 2012).

ACC pyramidal neurons are capable of glutamatergic and cholinergic persistent firing, a non-synaptic cellular substrate for working memory, and it has been demonstrated that DA and NE bidirectional control the duration of this persistent activity: DA decreases it while NE increases it (Zhang et al., 2013; Kamiński et al., 2017; Lançon et al., 2021). Therefore, in neuropathic conditions, the disrupted homeostatic ratio in DA/NE release leads to a dysfunction in how persistent firing is initiated and maintained, likely contributing to deficits in sustained attention and working memory.

Furthermore, decreased levels of DA in the ACC, together with increased levels of NE, decreases the rheobase of pyramidal neurons, making them easier to fire ectopically. Chronic pain-induced increase in the likelihood of pyramidal activation with any given stimulus leads to disruptions in what we pay attention to. Recent endoscopic imaging studies conclude that the proportion of nociception-sensitive neurons in the ACC is increased in neuropathic conditions (Li Anna et al, 2021; Acuña et al., 2023). Behaviorally, a disruption in the filtering process required for sensory discrimination might explain why a noxious stimulus specifically receives our attention in normal conditions and why an innocuous stimulus can be perceived as painful in neuropathic conditions (Vander Weele et al., 2018). This filtering mechanism in the ACC could underlie a key cortical mechanism sensitive to modulation by monoamines that can underlie allodynia and spontaneous pain in chronic pain patients. Persistent pain pathologically sensitizes our attention, increasing both pain-related and innocuous attention signals, shifting the homeostatic balance of attention to innocuous somatosensory, auditory, and visual stimuli. In this regard, chronic pain can be considered an attentional disorder.

Given this, the analgesic effects of monoamine signaling in the ACC likely stems from their influence not only on the emotional valence of pain, but also through their role in tuning our pain awareness. This might explain the role of the ACC in modulating the sensory aspect of pain since pain thresholds, commonly assayed via withdrawal and threshold tests such as the von Frey and the Hargreaves assays, are a function of both peripheral and central sensitivity to external stimuli. Schizophrenic patients commonly report pain insensitivity and have increased DAergic and serotonergic signaling in the brain (Dworkin, 1994; Stahl, 2018). As their peripheral nociceptive nerves are functional and they do not exhibit any somatosensory deficits apart from abnormal perception, this pain insensitivity is potentially mediated by the PFC (Chang and Lenzenweger, 2005). This is supported by EEG studies, which show that peripheral nerve stimulation in schizophrenic patients leads to reduced response amplitudes in the dlPFC and ACC, but not in somatosensory cortex (Huang et al., 2010; Daskalakis et al., 2020). Therefore, analgesia induced by microinjection of DA or 5-HT in the ACC is plausibly functioning by decreasing pain awareness rather than pain sensation.

In light of this, current treatments aimed at treating chronic pain can be somewhat effective at decreasing the sensory symptoms, but because they are mainly peripherally acting, they do not alleviate the cognitive deficits (Schiltenwolf et al., 2014). Systemic gabapentin, a commonly prescribed analgesic, leads to even greater cognitive dysfunction in chronic pain patients (Grégoire et al., 2012; Shem et al., 2018). Centrally acting non-selective monoamine reuptake inhibitors show some efficacy at treating the cognitive deficits in chronic pain. The non-selective NE and 5-HT reuptake inhibitor duloxetine, a commonly prescribed analgesic for cancer treatments, alleviates chronic pain-induced cognitive, but not emotional, symptoms (Grégoire et al., 2012; Chu et al., 2015).

Next steps and conclusion

Dysregulation of monoamines and other neuromodulators in the ACC in chronic pain as a main contributor to pathological cortical hyperexcitability and chronic pain symptoms is now a widely accepted working hypothesis in the pain research community (Ong et al., 2019; Tan and Kuner, 2021; Kasanetz et al., 2022). The controlled balance of G_s/G_i-signaling in specific ACC neurons, as well as in their astrocytic and microglial partners, is key for the healthy integration of innocuous and nociceptive inputs reaching the pain matrix (Figure 4). Disruptions in this balance in one direction leads to hypersensitivity for both nociceptive and innocuous signals while disruptions in the other direction can induce dissociation and pain insensitivity (Dworkin, 1994). Although key neuromodulatory pathways have been identified as instigators of pathological pain signaling, there are still holes in the literature that need to be addressed.

For instance, how chronic pain influences ACh and 5-HT inputs in the ACC has yet to be investigated and could provide insight into their roles in modulating normal and pathological pain perception. In the LC and VTA, it remains unclear what is prompting the changes in their activity and these midbrain regions should be further studied in neuropathic conditions. Additionally, what causes a loss of inhibitory synapses in the neuropathic ACC has yet to be identified. This could be mediated by overactivation of astrocytes and/or microglia but could also be mediated by abnormal corelease of DA with the classical neurotransmitter glutamate. There is precedence for glutamate originating for DAT+ projections forming asymmetrical synapses on PV+ interneurons and dopamine can form hub synapses with both glutamatergic and GABAergic synapses (Qi et al., 2016; Paget-Blanc et al., 2022). As the D1R was found to be expressed on the presynaptic GABA terminals innervating pyramidal cells, it is possible DA is reinforcing

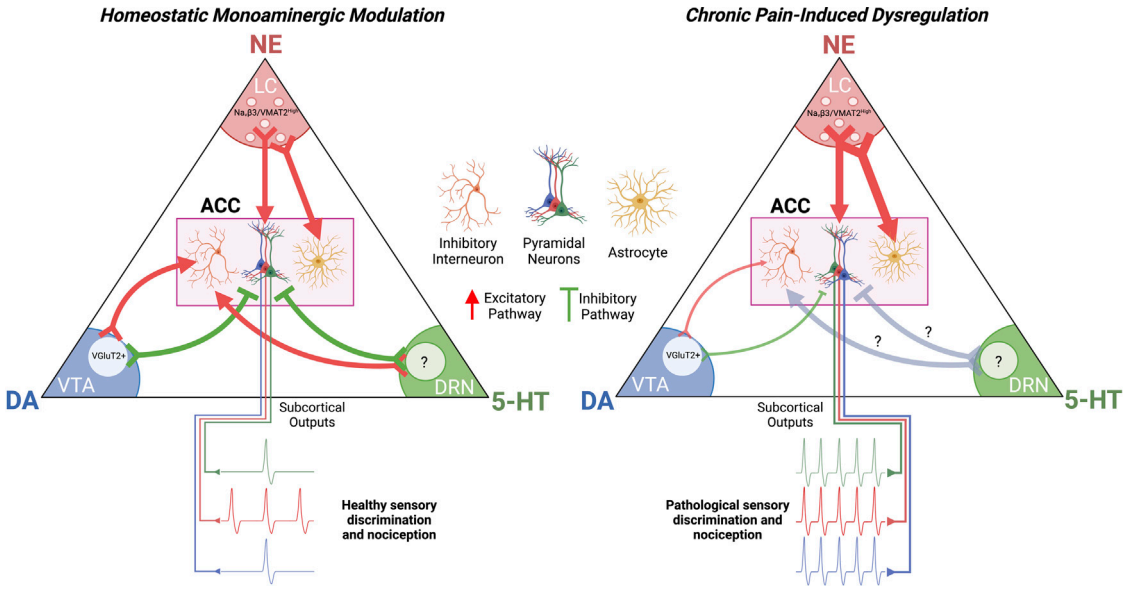

FIGURE 4
Chronic Pain-Induced Dysregulation of Cortical Monoamine Signaling. Chronic pain potentiates NAergic signaling in the ACC while decreasing DAergic signaling. This increases excitatory drive on pyramidal neurons while decreasing excitatory drive on inhibitory interneurons. The effect of chronic pain on 5-HT signaling in the ACC is not established. The resultant effect of dysregulated monoaminergic signaling in the ACC is pathological nociception and a decrease in sensory discrimination. ACC, anterior cingulate cortex; LC, locus coeruleus; VTA, ventral tegmental area; DRN, dorsal raphe nucleus.

these synapses as well as activating PV+ interneurons with glutamate (Muly et al., 1998). The role of KORs in modulating DA release in the ACC also deserves serious investigation.

Moreover, it is intriguing, and apparently contrary to expectation, that G_s activation via DA/NE/5-HT signaling on pyramidal neurons in the ACC results in inhibition, whereas G_i activation leads to excitation. This phenomenon seems to arise from the modulation of HCN channels through cAMP and their consequent impact on input resistance. Nevertheless, further research is required to determine all the downstream effects of PKA activation.

Central monoaminergic pathways also exhibit a high degree of sexual dimorphism and should be studied further in this regard. For example, the female hormone estradiol has a robust excitatory effect on VTA neurons and could explain why L-DOPA is more successful in females than males at decreasing symptoms of lower back pain (Yoest et al., 2018; Reckziegel et al., 2019). The LC also exhibits prominent sex differences: MOR activation on LC neurons causes a dramatic reduction in NE release in males, but not females (Bangasser et al., 2011; Guajardo et al., 2017).

In addition, this review focuses on the ACC but more attention should be given to how monoamines affect other cortical regions of the pain matrix dysregulated in chronic pain states. The neighboring prelimbic (PrL) cortex is particularly intriguing due to the opposite effect chronic pain induces relative to the ACC: PrL neurons are hypoactive in neuropathic conditions and their activation is analgesic (Wang et al., 2015). Since the PrL cortex also receives dense monoaminergic inputs, it would be interesting to understand how alterations in the release of monoamines induces hypoactivity rather than hyperexcitability. Additionally, the insular cortex appears to play a larger role in modulating pain perception in primates than in rodents. It deserves more investigation as it has also been identified as one of the most active brain regions in neuropathic patients (Jensen et al., 2016; Lu et al., 2016; Ferraro et al., 2021; Tan and Kuner, 2021).

Lastly, although ACC activity is positively correlated with pain sensitization, this is not always the case. Recent reports indicate that gabapentin and nitrous oxide can in fact induce an increase in ACC activity alongside analgesia (Acuña et al., 2023; Weinrich et al., 2023). Given this, the role of the ACC in modulating attention and pain perception is more nuanced than it appears and it may be possible to reinstate proper somatosensory filtering by increasing the activity of specific ACC circuits.

Although further studies are warranted to determine the impact of chronic pain on supraspinal regions involved in the processing of nociceptive information, tremendous progress has been made in the past decade. Our current model remains incomplete however and finding a treatment that selectively corrects cortical monoaminergic function in chronic pain might be key to the development of novel therapeutics.

Author contributions

KL: Conceptualization, Writing–original draft, Writing–review and editing. PS: Conceptualization, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Combination of anti-CGRP/CGRP-R mAbs with onabotulinumtoxin A as a novel therapeutic approach for refractory chronic migraine: a retrospective study of real-world clinical evidence and a protocol for a double-blind, randomized clinical trial to establish the efficacy and safety

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Chronic migraine is a disabling neurovascular disorder that ranks amongst the top causes of years lived with disability worldwide. The duration and the frequency of migraine affect cognitive and affective domains, inducing worsening of memory, executive functions, orientation and causing anxiety. Population-based studies report a worrying level of resistance to treatments. Therefore, this study aims: 1) to assess efficacy of monoclonal antibodies (mAbs) directed towards the calcitonin gene-related peptide (CGRP) or its receptor (CGRP-R) for chronic migraine resistant to current preventatives; 2) to design a clinical trial protocol to evaluate the efficacy and safety of combination therapy utilizing anti-CGRP/CGRP-R together with onabotulinumtoxin A in patients suffering from resistant chronic migraine; 3) to provide a molecular rationale for combination therapy. A controlled trial is warranted as pooled analysis of real-world data from our group highlighted that combined treatment provides ≥50% reduction vs. baseline (onabotulinumtoxin A) of monthly headache days (MHDs) in up to 58.8% of patients, but there has been only sparse application of this combined therapy to date. The mAbs chosen are: erenumab, because its combination effect with onabotulinumtoxin A improved symptoms in 65% of patients; eptinezumab, due to its faster action. The results highlight that early diagnosis of migraine improves

therapeutic outcomes with mAbs alone, confirming their effectiveness and the need for an adequately powered clinical trial evaluating the safety and potential superior effectiveness of eptinezumab/erenumab and onabotulinumtoxin A together.

KEYWORDS

onabotulinumtoxin A, chronic migraine, anti-CGRP monoclonal antibodies, anti-CGRP-R monoclonal antibodies, erenumab, eptinezumab

1 Introduction

1.1 Migraine and refractoriness

Migraine is a disabling, primary headache endowed with a serious social impact, ranking as one of the top causes of years lived with disability worldwide, particularly in people under fifty (Steiner et al., 2018), is 3-to-4 times more frequent in females than in males (Rossi et al., 2022). Chronic migraine is a disease characterized by episodic manifestations (Haut et al., 2006), which the International Classification of Headache Disorders (ICHD, third revision) beta diagnostic criteria defines as at least 15 headache days per month, of which 8 days present the features of migraine, for 3 months consecutively (Headache Classification Committee of the International Headache Society, 2013). The social burden of chronic migraine is increased by its remarkable undertreatment and a high prevalence of resistance to current treatments such as the widely used triptans that stimulate serotonin receptors and the anti-epileptic topiramate which suppresses electrical overactivity in the central nervous system (Schulman et al., 2008). The mechanisms causing resistance to treatments have not yet been elucidated, but a role for genetic polymorphisms has been highlighted (Scuteri et al., 2021b). The duration and the frequency of migraine correlates with harm in both the cognitive and affective domains, damage to memory, executive functions and of orientation, as assessed through the Rey–Osterrieth complex figure test (ROCF) and the Montreal Cognitive Assessment (MoCA), and causing anxiety (Huang et al., 2017). Reduction of monthly headache days (MHDs) and monthly migraine days (MMDs) is a main goal of treatments, but the difficulty in treating chronic migraine in a large proportion of patients has prompted the development of novel therapies.

1.2 Game-changing novel therapies

The discovery of the involvement of the calcitonin gene-related peptide (CGRP) in the pathogenesis of migraine (Scuteri et al., 2019a) fostered the advance of novel specific small molecules (Scuteri et al., 2022b; Scuteri et al., 2022c) and biotechnological drugs (Scuteri et al., 2019b; Scuteri and Bagetta, 2021) targeting this peptide (Scuteri et al., 2020). Onabotulinumtoxin A has been approved since 2010 for the treatment of chronic migraine relying on data from the Phase III Research Evaluating Migraine Prophylaxis Therapy (PREEMPT) clinical program (Diener et al., 2010; Aurora et al., 2011; Aurora et al., 2014). This drug inhibits the exocytosis of CGRP from primary sensory neurons, as well as the release of several other neurotransmitters (Dolly, 2003; Aoki, 2005) by cleavage of the 25 kDa synaptosomal-associated protein (SNAP-25) (Welch et al., 2000). It is effective, well-tolerated (Herd et al., 2018) and despite a lack of homogeneous and long-term data, the available results indicate that it is safer than one of the most commonly used preventative drugs, topiramate. In fact, topiramate was reported to be associated to the highest rate of drop-out in comparison with onabotulinumtoxin A and the most novel antibodies (Frank et al., 2021) and with teratogenicity (Rothrock et al., 2019). Hence, more trials are needed to assess the effect of topiramate in chronic migraine with medication overuse (Giri et al., 2023). Side effects of onabotulinumtoxinA are rare, mild, self-limiting and usually resolve within a short time when used as directed by the label, but the potential for unwanted neuromuscular and/or autonomic side-effects precludes increasing the doses used above those specified in the label instructions. The most novel biotechnological drugs (authorized between 2018 and 2020) are the specific monoclonal antibodies (mAbs) directed towards CGRP (known as eptinezumab, galcanezumab and fremanezumab) or its receptor complex (erenumab). However, 38% of patients that failed all the available preventative drugs also did not respond to erenumab after 6 months of treatment (Lambru et al., 2020; Sacco et al., 2021). Such a large refractory group prompted the investigation of a treatment regime combining onabotulinumtoxin A and anti-CGRP mAbs.

1.3 Combined therapy with onabotulinumtoxin A and mAbs targeting the CGRP machinery

This afforded a pooled ≥50% reduction of MHDs with respect to baseline (onabotulinumtoxin A injections (Toni et al., 2021) of ≥ 2 consecutive cycles of (Blumenfeld et al., 2021; Mechtler et al., 2022)) in up to 58.8% of patients, with a decline of 35.5% after the 6th month (Scuteri et al., 2022d). Moreover, the combined therapy was more effective than erenumab, administered alone or with other preventative drugs, with the efficacy being prolonged by an average of 2 weeks, a fundamental improvement for refractory patients (Ailani and Blumenfeld, 2022). Nevertheless, head-to-head comparisons are still needed (Lu et al., 2021). The recent American Headache Society (AHS) consensus statement of 2021 (Ailani et al., 2021) reported the possible efficacy of the combination of CGRP-targeted mAbs and onabotulinumtoxin A for patients suffering from continued migraine and disability on a single preventive treatment, occurring when experiencing ≥4 MMDs with at least moderate disability, assessed as Migraine Disability Assessment ≥11, Headache Impact Test >50, or ≥8 MMDs (Ailani and Blumenfeld, 2022). Therefore, the purposes of the present study are: 1) to assess the resistance of chronic migraine

to current preventative drugs and the consequent use and efficacy of the anti-CGRP/CGRP-R mAbs in a real-world setting; 2) to design a clinical trial protocol to evaluate the effectiveness and tolerability of combination therapy for resistant chronic migraine utilizing anti-CGRP/CGRP-R together with onabotulinumtoxin A; 3) to provide a molecular rationale for such combination therapy.

2 Methods

2.1 Objectives of the retrospective phase

This retrospective study was conducted in collaboration with the Pain Therapy Center of the Provincial Health Authority of Cosenza (Calabria, Italy). Anonymized data were collected concerning the following aspects: demographic characteristics of the patients; diagnosis of chronic migraine according to the ICHD 3 beta; failed preventative treatments; anti-CGRP mAbs administered; baseline MMDs; reduction of MMDs after 1, 3 and 6 months of treatment with mAbs; decrease of pain intensity measured by the numeric rating scale (NRS) after 6 months of treatment. The district consists of 298,000 inhabitants, 213,000 under 60 years of age, i.e., a sample typical for migraine occurrence. The patients enrolled in the study had no concomitant pathologies and were not undergoing concurrent treatments. The need for written informed consent and ethical approval was waived owing to the retrospective use of anonymized data only. The study was conducted in accordance with the Declaration of Helsinki.

2.2 Trial design

The trial assessing the efficacy and safety of the combined treatment with onabotulinumtoxin A and either erenumab or eptinezumab will be a double-blind, randomized single-center trial recruiting patients eligible for the intervention, i.e., those suffering from chronic migraine (according to the ICHD 3 beta) that are refractory to the most commonly used preventative treatments (see below for details). The patients allocated to the combination therapy arm of the study will be assigned randomly to erenumab or eptinezumab subgroups, whereas those allotted to the control arm will continue with their usual treatment. The study protocol included implements the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) Checklist (Chan et al., 2013) and the Consolidated Standards of Reporting Trials (CONSORT) statement (Moher et al., 2010). It has been submitted for approval to the Calabria Region Ethics Committee and a request has been made for a ClinicalTrials.gov ID. According to the D.lgs 196/2003, the Helsinki agreements and subsequent amendments, the Good Clinical Practice and Guidelines for the treatment of personal data in clinical trials of 24 July 2008, and in accordance with European data protection legislation, each participant or his/her legal representative will be required to sign a consent form as acceptance of all aspects of the study contained in the patient information sheet and as a consequent expression of his/her willingness to participate in the study. The information sheet will be explained to the subjects or legal representatives by the study staff and the same staff will ensure that the consent form is properly signed and dated by all the parties involved before any procedure detailed in the protocol is carried out. Information on opt-out will be provided to the subjects or legal representatives by the study staff. The primary endpoint will be the mean change in MMDs from the baseline phase after 1, 3, 6, 9 and 12 months of treatment. The secondary endpoints are as follows: mean MHDs; numeric rating scale (NRS); six-item Headache Impact Test (HIT-6™); migraine disability assessment (MIDAS); MoCA; GAD-7; blood tests; anti-drug antibodies (ADA); safety end points that, according to usual safety assessment in clinical trials for chronic migraine with anti-CGRP mAbs, include patient incidences or exposure-adjusted patient incidences of adverse events (AEs) and serious AEs, identifying cardiovascular and cerebrovascular risk factors, such as diabetes, at baseline (Ashina et al., 2022). A complete CONSORT flow diagram for the study is reported in Figure 1.

2.3 Inclusion criteria

Patients eligible for inclusion in the present clinical trial are:

- adults ≥ 18 years of age;
- diagnosed with chronic migraine according to ICHD 3 beta;
- presenting with at least 4 MMDs and showing either an insufficient response to, or inability to tolerate, at least two previously prescribed prophylactic agents for migraine.

2.4 Data analysis

Data for the retrospective observational study were extracted from anonymized migraine diaries of the patients and case report forms, then collated using Microsoft Office Excel 2010 (Microsoft, Milan, Italy). Statistical analyses on data expressed as percentage of reduction relative to baseline were performed using GraphPad Prism® 6.0 (GraphPad software Incorporated, San Diego, CA, United States). The results were statistically evaluated for differences using $\chi 2$ test for categorical variables considering $p < 0.05$ significant. The prespecified statistical analysis plan (SAP) for the combination therapy clinical trial consists of an assessment of differences for both the primary and the secondary endpoint measures, according to the calculation of the least-squares mean at each timepoint, evaluated through a linear mixed effects model including all the patient-level variables (Tepper et al., 2017).

3 Results

3.1 Effectiveness of anti-CGRP/R mAbs in a small sample in the real-world setting

The present retrospective, observational study identified $n = 10$ patients (9 females and 1 male) aged 38–66 years at the time of data collection (2022) that met the inclusion criteria after referral to the Pain Therapy Center of the Provincial Health Authority of Cosenza (Calabria, Italy) in the preceding decade (2010–2022). The gender distribution in this cohort is compatible with literature reporting that migraine is 3-to-4 times more

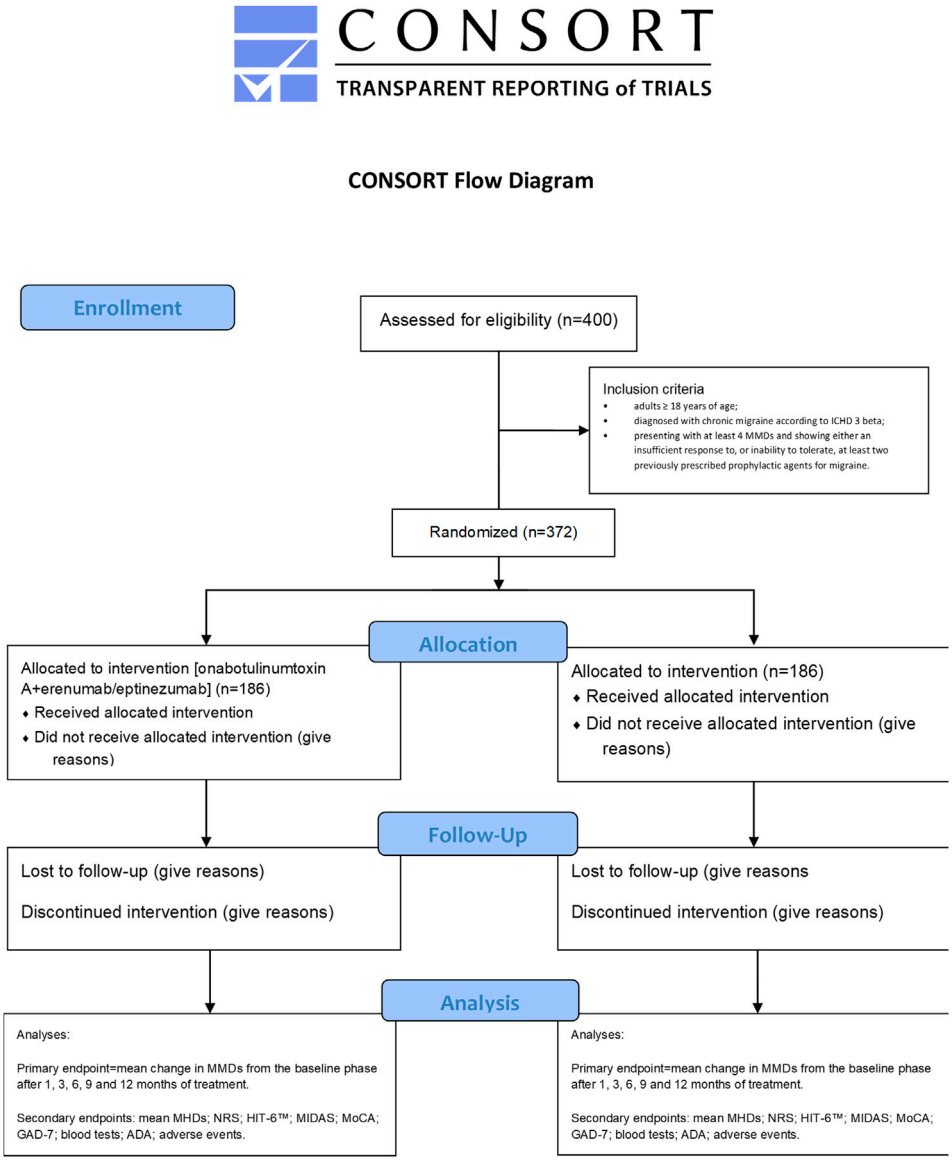

FIGURE 1
Consolidated Standards of Reporting Trials (CONSORT) flow diagram of the proposed clinical trial.

frequent in females than in males (Rossi et al., 2022). The baseline MHDs and MMDs ranged from 15 to 25 and all the patients suffered from chronic migraine without aura (Table 1). Baseline migraine pain intensity was moderate to severe with NRS values from 7 to 10. The patients had all used two or three preventative drugs for an average of 7 years, and failed in the prophylaxis of chronic migraine (patient 017 even showed a worsening of MMDs), before then switching to anti-CGRP mAbs. Although patients 015 and 024 displayed a noteworthy reduction in MMDs with preventative drugs, they elected mAb therapy because their post-treatment MMDs remained high. The drugs used were a selection from the following: topiramate, amitriptyline, flunarizine, verapamil, propranolol, levosulpiride and gabapentin (Table 1). The baseline features of the patients recruited are illustrated in Table 1.

Six out of the 10 patients eligible for anti-CGRP/R mAbs treatment received monthly administrations of erenumab with titration from 70 mg to 140 mg in two cases, while two patients in treatment with 225 mg of fremanezumab monthly and the other 2 patients with monthly galcanezumab titrated from 240 mg loading dose to 120 mg. Data are reported in Table 2. Nine of the ten patients reported a reduction of MMDs after 1 month of treatment and all recorded a reduction upon assessment after 3 and 6 months of treatment, confirming efficacy. Moreover, 8 of the 10 patients scored a reduced pain intensity at 6 months. Interestingly, older patients (017 and 022) showed more resistance to anti-CGRP mAbs in terms of a less pronounced reduction of MMDs with respect to the whole sample, although the possible age-dependency for treatment outcomes will require further investigation in a larger sample because 2 of the younger patients (012 and 023) also exhibited similarly small reductions in MMDs. Eptinezumab and

TABLE 1 Baseline characteristics of patients included in the study. The cohort were of predominantly females, with a late age of first chronic migraine observation that failed to achieve an adequate reduction of monthly migraine days (MMDs) with current preventative treatments.

Patient code	Age at the time of the study	Sex	Baseline MMDs	Preventative treatments	Post-treatment MMDs	Treatment period
011	43	F	20	Topiramate	20	2013–2021
				Flunarizine		
012	40	F	15	Topiramate	15	2011–2022
				Amitriptyline		
				Verapamil		
014	40	F	22	Propranolol	20	2017–2022
				Topiramate		
				Amitriptyline		
015	46	F	25	Flunarizine Topiramate	17	2014–2022
				Amitriptyline		
016	45	F	18	Gabapentin	18	2017–2022
				Amitriptyline		
				Topiramate		
017	66	F	15	Verapamil	20	2015–2022
				Amitriptyline		
				Levosulpiride		
018	38	F	20	Amitriptyline	20	2014–2021
				Topiramate		
				Flunarizine		
022	58	F	18	Topiramate	18	2010–2020
				Amitriptyline		
023	46	M	25	Propranolol	22	2016–2021
				Gabapentin		
				Amitriptyline		
024	47	F	20	Propranolol	15	2019–2022
				Gabapentin		
				Topiramate		

onabotulinumtoxin A were not used in this sample taken from real-world clinical practice. A mean 42% reduction of MMDs was observed with erenumab producing a 41.83% decline, fremanezumab 41.5% and galcanezumab 42.5%. Data are summarized in Table 2.

3.2 Clinical trial protocol for combination therapy of onabotulinumtoxin A and anti-CGRP mAbs

A double-blind, randomized clinical trial will be conducted at the Headache Center of the Regional Hospital "Pugliese-Ciaccio" directed by Dr. Rosario Iannacchero to assess the effectiveness of combination therapy utilizing onabotulinumtoxin A together with an anti-CGRP mAb compared to patients' continuation with their current treatment with regard to the mAbs to be used in the combination therapy, both erenumab and eptinezumab will be assessed separately in individual subgroups of the study. Applying the structure of the exploratory analysis of patient-reported outcomes (PROs) for superiority of the intervention in the primary endpoint, that consists in the decrease of mean MMDs (from baseline), NCT02066415, a sample size of $n = 186$ patients for the intervention group is required assuming a treatment effect of −1.9 days with a standard deviation of 6.1, providing 85% power using a two-sample t-test with a two-sided significance level of 0.04 (Tepper et al., 2017). Eligible patients will be randomly assigned in ratio 1:1 to the combination therapy or

TABLE 2 Reduction of monthly migraine days (MMDs) and of migraine pain intensity (NRS) after 1, 3 and 6 months of treatment with mAbs targeting either the CGRP (fremanezumab or galcanezumab) or the receptor (erenumab).

Patient code	Antibody	Dose	Frequency of administration	Baseline monthly migraine days (MMDs)	Reduction of MMDs after 1 month	Reduction of MMDs after 3 months	Reduction of MMDs after 6 months	Percentage reduction of MMDs (%)	Baseline pain intensity measured through numeric rating scale (NRS)	Pain intensity NRS after 6 months
011	Fremanezumab	225 mg	Monthly	20	(-5)	(-14)	(-15)	75	8	5
012	Erenumab	70 mg	Monthly	15	(-3)	(-3)	Loss to follow-up	20	10	5
		140 mg								
014	Erenumab	70 mg	Monthly	20	(-10)	(-13)	(-13)	65	10	7
015	Erenumab	70 mg	Monthly	17	(-5)	(-7)	(-7)	41	10	7
016	Erenumab	70 mg	Monthly	18	(-3)	(-8)	(-6)	33.3	10	7
		140 mg								
017	Galcanezumab	240 mg (loading dose)	Monthly	20	(-5)	(-5)	(-5)	25	7	7
		120 mg								
018	Galcanezumab	240 mg	Monthly	20	(-10)	(-12)	(-12)	60	10	5
		120 mg								
022	Erenumab	70 mg	Monthly	17	(-2)	(-2)	(-2)	11.7	10	4
023	Fremanezumab	225 mg	Monthly	25	0	(-13)	(-3)	8	10	10
024	Erenumab	70 mg	Monthly	15	(-12)	(-12)	(-12)	80	7	3

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usual treatment group. Patients in the combined therapy group will receive per label administration of onabotulinumtoxin A and one of either erenumab or eptinezumab. In this way it will be possible to cover all the possible mechanisms, responding either to the inhibition of the signaling of the CGRP ligand (eptinezumab) or its receptor (erenumab) and with the fastest onset of action. Patients, administrators, raters and data analysts will be blinded to the assignments to the intervention or usual care groups, although the administration route can differ. In order to obtain long-term efficacy and safety data the trial will last 52 weeks. Demographic characteristics and baseline information of the patients will be collected through a migraine diary to be completed each day with details including incidence of headache; incidence of migraine with or without aura; time of onset of headache; time to resolution of headache; headache intensity assessed as NRS; pain features; migraine symptoms and most bothersome symptom; use of acute drug treatment during aura or headaches. Baseline assessments will be carried out during the first month immediately before allocation to either of the groups detailed in 2.2. The primary endpoint will be the mean change in MMDs from the baseline phase after 1, 3, 6, 9 and 12 months of treatment. MMDs will be identified based on headache duration, symptoms, pain features and use of migraine-specific drugs use. Migraine is defined as follows: headache (with or without aura) lasting for at least 4 h continuously, with two or more pain features (unilateral, throbbing, moderate to severe intensity, or aggravation by exercise or physical activity) or one or more associated non pain features (nausea, vomiting, or photophobia accompanied by phonophobia) (Tepper et al., 2017). Secondary endpoints include: reduction of mean MHDs; decrease of pain severity measured as NRS; improvement in impact of headache and disability evaluated by the HIT-6™ (Ware et al., 2000; Kosinski et al., 2003; Yang et al., 2011) and the MIDAS (Lipton et al., 2001) scores, respectively; decrease of need for rescue medications; assessment of tolerability. Moreover, MoCA and the Generalized Anxiety Disorder Scale 7-item (GAD-7), validated for migraineurs, will be performed to assess the efficacy of treatment on the cognitive and affective domains (Seo and Park, 2015). Blood tests and searches for neutralizing antibodies against the mAbs will be performed at the beginning and at the end of the study. Any adverse events will be recorded on the case report form during the trial. No sponsor will participate in the trial.

4 Discussion

4.1 Real-world data and delay in diagnosis

According to age-standardized data from the Global Burden of Disease Study 2016, Italy exhibits the highest calculated prevalence of migraine in the world at 20,000 to 21,000 patients per 100,000 inhabitants population (Stovner et al., 2018). Chronic migraine is a debilitating condition consequent to the process of transformation and progression of episodic migraine (Bigal et al., 2006). Therefore, the prevention of migraine chronification and of medication-overuse headache (MOH) is fundamental. For a long time triptans have represented the sole specific treatment for migraine attacks, which offer 2-h sustained freedom from pain to some 18%–50% patients and sustained headache relief at 24 h to some 29%–50% of patients (Cameron et al., 2015). However, the discovery of CGRP as a mediator of chronic migraine pathophysiology (Edvinsson et al., 2018) has caused a revolution in the treatment and prophylaxis of this condition. Data presented herein, gathered from current clinical practice in Calabria in Italy, reports the efficacy of antibody therapies targeting CGRP or its receptor for reducing MHDs, MMDs and NRS pain score. Interestingly, our sample included patients arriving to clinical observation at the age of over 50 years. This highlights a serious delay in diagnosis, in accordance with an underestimation of the problem of migraine (Gupta and Gaurkar, 2022). Notably nine out of the ten patients analyzed showed MMDs decrease after 1 month of treatment and a reduction after 3 and 6 months. Older patients (017 and 022) showed more resistance to anti-CGRP mAbs. Moreover, the eldest patient (at 66 years old) not only proved completely refractory to current prophylactic therapies, the treatments failed to prevent the further deterioration in symptoms, so further investigations into a possible relationship between age and refractory migraine is warranted. Pain processing alters during aging (Hamm and Knisely, 1985; Jourdan et al., 2000; Jourdan et al., 2002) and this could foster new studies investigating the role of natural products with analgesic activity as potentially useful and safe add-on therapies (Scuteri et al., 2021a; Scuteri et al., 2022a). The development of migraine in over 50 year old patients is unusual (Herrero et al., 2013) and, consequently, this population is often neglected from clinical trials for painkillers in general (Bayer and Tadd, 2000). Another issue for refractory migraine treatment, also in the population of over 50-year-old, is MOH induced by the overuse of drugs. By contrast to the experience of the older patients in this study, a recent real-life multicentre analysis of 162 over 65 year-old patients, showing that anti-CGRP mAbs provided a reduction of MMDs ≥ 50% and ≥ 75% to some 57% and 33% patients, respectively (Muñoz-Vendrell et al., 2023). The present clinical practice data analyzed herein highlight a preference to switch patients from non-specific, preventative, small molecules to mAbs rather than onabotulinumtoxin A. Interestingly, no patients were transferred to eptinezumab, in spite of its rapid action. The use of mAbs proved to be effective, but to different extents in individual recipients, a finding that corroborates international data, possibly due to differences in hypothalamic modulation (Torres-Ferrús et al., 2021; Basedau et al., 2022; Iannone et al., 2022). The real-world data on anti-CGRP-mAbs suffer from the following limitations: retrospective collection of data, small sample size, short follow-up periods (Pavelic et al., 2023). This is confirmed by a recent systematic search that included randomized controlled trials reporting the outcomes of change in MHDs, MMDs, ≥50% response rates and change in MOH status (Giri et al., 2023).

4.2 Neuropharmacology of resistant, chronic migraine

As an alternative to mAbs targeting CGRP signalling, onabotulinumtoxin A has been approved for use in chronic migraine since 2010 (Simpson et al., 2016). It proteolyses SNAP-25, one of the proteins required for the membrane fusion reaction that mediates the exocytosis of CGRP, as well as other neuropeptides and various neurotransmitters (Meng et al., 2009; Cernuda-Morollón et al., 2015; Belinskaia et al., 2023). Among these neurotransmitters, it is possible to

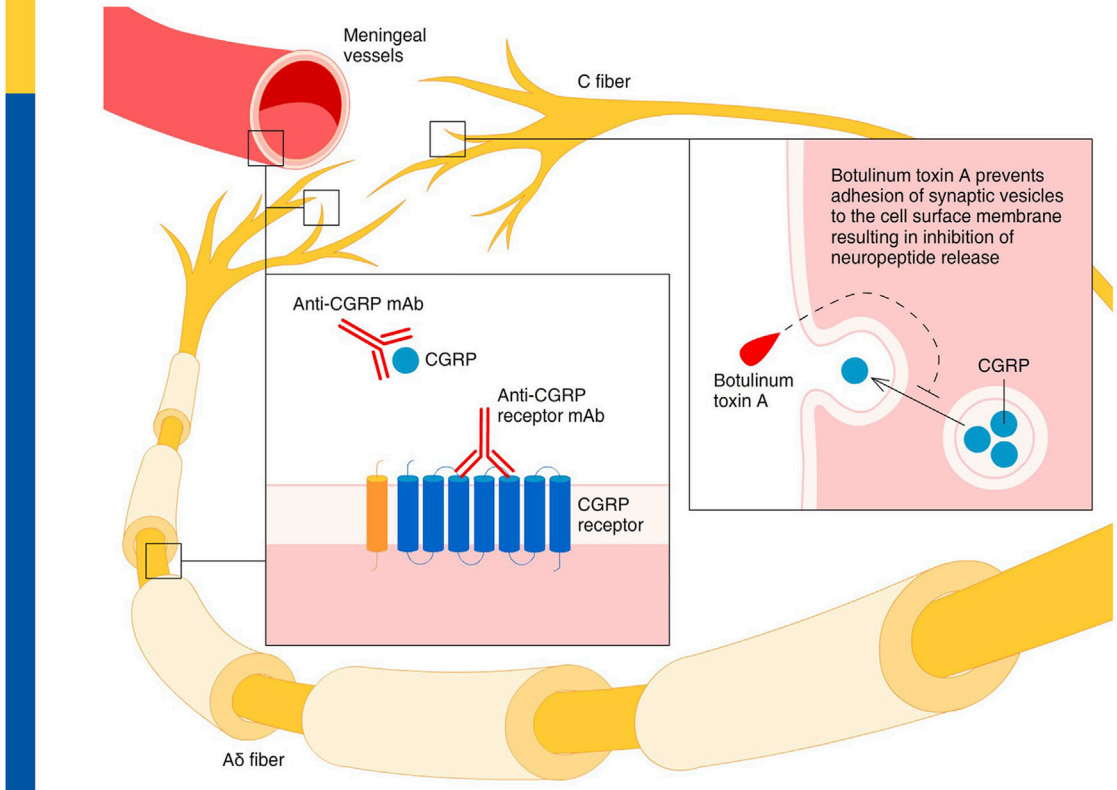

FIGURE 2
Proposed synergic activity of onabotulinumtoxin A and anti-CGRP mAbs in migraine. Onabotulinumtoxin A inhibits the release of CGRP from thin unmyelinated C fiber meningeal nociceptors in the dura, thus preventing a CGRP-dependent activation of meningeal vessels and thick myelinated Aδ nociceptors. At the same time, anti-CGRP mAbs prevent the interaction between the CGRP and its receptor within the meningeal vessel walls, as well as in the extremities and along the fibers in at the nodes of Ranvier of the Aδ nociceptors. Reproduced with permission from (Pellesi et al., 2020).

find acetylcholine, glutamate, pituitary adenylate cyclase activating peptide 38 (PACAP 38) and substance P, inhibition of the trafficking of transient receptor potential cation channel subfamily V member 1 [TRPV1], transient receptor potential cation channel subfamily A member 1 [TRPA1] and purinergic receptor P2X ligand-gated ion channel 3 [P2X3] (Devesa et al., 2014; Meng et al., 2016) is also implicated in the mechanism of action of onabotulinumtoxin A (Burstein et al., 2020). The effect is long-lasting, as supported by the evidence that SNAP-25 is cleaved over 80 days in cultured spinal cord cells (Keller et al., 1999) and a minimum of 3 months is recommended

between clinical injections for chronic migraine (BOTOX® label information). Botulinum toxin can be useful in several other types of pain, as including neuropathic. In fact, a double-blind, crossover, pilot trial investigating the effectiveness of botulinum toxin type A for diabetic neuropathy, demonstrated a significant reduction of pain intensity assessed through visual analog scale (VAS) at 1-4-8 and 12 weeks after administration (Yuan et al., 2009). In relation to its mechanism of action, onabotulinumtoxinA reduced interictal plasma levels of CGRP, determined in samples obtained from the right antecubital vein using ELISA, one month after treatment, in chronic migraineurs who are responders to treatment, but not in nonresponders (Cernuda-Morollón et al., 2015). By contrast, the levels of vasoactive intestinal peptide (VIP), investigated in samples obtained from the right antecubital vein by ELISA, were significantly increased in responders (Cernuda-Morollón et al., 2014). The gathered evidence suggests that measurements of the interictal levels of CGRP may be helpful to predict the response to onbaotulinumtoxin A (Cernuda-Morollón et al., 2015), supporting the importance of an additive/synergic mechanism with antibody therapy. The synergic mechanism of the combination in migraine may be related to the inhibition of CGRP release from thin unmyelinated C fiber dural nociceptors produced by onabotulinumtoxin A, while anti-CGRP mAbs prevent the binding of the ligand to its receptor (Figure 2; reproduced with permission from (Pellesi et al., 2020)). This is corroborated by the finding that fremanezumab can prevent the activation of Aδ- but not C-fibers, whilst onabotulinumtoxin A might selectivley interact with C- but not Aδ-fibers, in agreement with distribution of CGRP in C-fibers and CGRP receptors in Aδ-fibers (Strassman et al., 2004; Eftekhari et al., 2013; Pellesi et al., 2020). It has also been proposed that onabotulinumtoxin A reverses mechanical hypersensitivity of sensitized C-units by interference with the expression of high-threshold mechanosensitive ion channels on the surface of nerve cells (Burstein et al., 2014). Thus, onabotulinumtoxin A can be exploited as a multipurpose drug offering long-lasting relief from several forms of pain including migraine (Sandrini et al., 2017; De Icco et al., 2019) and neuropathic pain in experimental conditions (Wang et al., 2017). Accordingly, clinical use of onabotulinumtoxin A has been shown to decrease the need for rescue medications (Sandrini et al., 2011). Chronic migraine is a form of complex, neurological disorder characterized by sensory, cognitive and affective comorbidities, likely due to network disruption (DeSouza et al., 2020), that needs to be prevented to avoid patients' disability. So to prevent patients' disability, it is fundamental to provide relief from all these associated modalities. Indeed, a prospective cross-sectional study on 165 patients highlighted that some 89.7% of them experienced cognitive symptoms with consequent dysfunction involved in the attack-related disability (Gil-Gouveia et al., 2016), thus representing a pathological outcome of the utmost importance. This can be, at least in part, due to the perturbations of brain areas important for cognition, i.e., amygdala, hypothalamus, periaqueductal gray (PAG), ventral pontine tegmentum, ventral and dorsal medulla and also spinal cord (Kozlowska et al., 2015; Gil-Gouveia and Martins, 2019). Executive functions, working memory, visual-spatial processing and attention are among the most affected cognitive skills (Gil-Gouveia and Martins, 2019). Cognitive symptoms rank second in the symptoms related to the attack of migraine (Vuralli et al., 2018; Gil-Gouveia and Martins, 2019) and these are accompanied by low-rate depression, anxiety and apathy (Santangelo et al., 2016), supporting the need for long-term assessment of cognitive impairment in chronic migraineurs. CGRP is thought to be a trigger factor for migraine because injections of levels of this neuropeptide can induce migraine-like headache symptoms. Also, in some migraineurs, its levels are elevated (compared to healthy controls) in the inter-ictal phase and have been seen to increase even further during the pre-ictal to headache onset phase (Kamm, 2022, Front. Neurol.). Anti-CGRP mAb therapies, or botulinum toxin injections, are thought to reduce migraine incidence and severity by suppressing interictal and ictal levels of this neuropeptide, thereby, halting migraine progression and alleviating the development of cognitive and affective comorbidity. Hence, the inclusion in the clinical study herein of the MoCA and GAD-7 analyses to investigate the impact of combination therapy on these facets.

4.3 Rational basis for co-administration of onabotulinumtoxin A and mAbs directed towards the CGRP ligand or receptor

Notably, retrospective studies indicate that co-administering onabotulinumtoxin A with anti-CGRP/R mAbs can more successfully reduce MMDs and MHDs and prolong the suppression of migraine impact with induced disability than either individual intervention alone. In fact, the combination therapy was associated with statistically significant reductions of 8.1 MHDs ($p < 0.001$) and of 7.4 MMDs [30% ($p < 0.001$)] at 90 days (Armanious et al., 2021). In this regard, an interesting study by Blumenfeld and coworkers of 2021 (Blumenfeld et al., 2021) reported a wide primary analysis cohort (n = 257) and sensitivity analysis cohort (n = 172), including only patients suffering from moderate disability defined by MIDAS score>11 or HIT-6™ score >50. This study demonstrated that, after 6–12 months of combined therapy, one-third (31.5%–36.7%) of patients presented a reduction of MHDs ≥50% and a reduction in migraine-related disability ≥30%. Furthermore, the mean MIDAS score for 27.1%–29.6% of the cohort was significantly reduced from baseline by between 6.1 and 11.1 points. Another retrospective study likewise highlighted the effectiveness of onabotulinumtoxin A as an add-on therapy to mAbs in patients suffering from refractory chronic migraine, who failed two oral migraine preventative drugs, three onabotulinumtoxin A cycles and three sessions with either fremanezumab or erenumab delivered sequentially as monotherapies (Argyriou et al., 2022). Furthermore, addition of an anti-CGRP mAb to the treatment for MOH in chronic migraineurs has been recently suggested to reduce headache frequency and symptomatic medication use (Krymchantowski et al., 2023). Although patient persistence with onabotulinumtoxinA is better than that seen with anti-CGRP mAbs (Schwedt et al., 2023), the doses of botulinum toxin A that can be delivered are strictly limited to restrict the unwanted spread of the toxin beyond the treatment area and to preclude the development of potentially debilitating motor and autonomic side-effects. Consideration of all these data together prompted the design of a prospective clinical trial to evaluate the effectiveness and tolerability of combination treatment using mAbs together with onabotulinumtoxin A (Guerzoni et al., 2022). Therefore, here we propose a study protocol for a 52-week, randomized, adequately powered, clinical trial to provide long-term evidence for effectiveness and tolerability of the combined treatment of onabotulinumtoxin A and anti-CGRP mAbs. The mAbs chosen are erenumab, because its combination beneficial effect was

demonstrated in some 65% patients (Boudreau, 2020) and eptinezumab due to its faster action. The main goal of this prospective randomized clinical trial is to fill the gap between clinical practice and research due to the still unmet need for wide prospective clinical trials assessing long-term follow-up of combined therapies and to offer a new therapeutic approach for refractory patients. This trial falls in the novel field considering chronic migraine management of patients with frequent and disabling attacks as a multimodal strategy including the control of the following milestones: 1) comorbidities; 2) modifiable risk factors involved in the process of progression as the overuse of medications and of caffeine; 3) secondary headaches; 4) tailored acute and preventative therapies with the aim of reducing pain, allodynia, cognitive and affective impairment and consequent disability (Blumenfeld et al., 2023). Moreover, statistical modeling deserves further investigation since it can represent an important aid in the prediction of synergistic or additive effects of treatments combinations also in acute management of the attacks (Blumenfeld et al., 2012).

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The requirement of ethical approval was waived by the need for written informed consent and ethical approval was waived owing to the retrospective use of anonymized data only for the studies involving humans because the studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board also waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because the need for written informed consent and ethical approval was waived owing to the retrospective use of anonymized data only.

Author contributions

MC: Conceptualization. GL: Conceptualization, Language review. GB: Conceptualization. RI: Methodology, Data curation. AT: Methodology, Data curation. AM: Methodology, Data curation. MP: Methodology, Data curation. PT: Methodology, Data curation. GS: Conceptualization. PN: Conceptualization. DS: Conceptualization, Methodology, Data curation. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The projection from dorsal medial prefrontal cortex to basolateral amygdala promotes behaviors of negative emotion in rats

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Brain circuits between medial prefrontal cortex (mPFC) and amygdala have been implicated in cortical control of emotion, especially anxiety. Studies in recent years focus on differential roles of subregions of mPFC and amygdala, and reciprocal pathways between mPFC and amygdala in regulation of emotional behaviors. It has been shown that, while the projection from ventral mPFC to basomedial amygdala has an anxiolytic effect, the reciprocal projections between dorsal mPFC (dmPFC) and basolateral amygdala (BLA) are generally involved in an anxiogenic effect in various conditions with increased anxiety. However, the function of the projection from dmPFC to BLA in regulation of general emotional behaviors under normal conditions remains unclear. In this study, we used optogenetic analysis to identify how this dmPFC–BLA pathway regulates various emotional behaviors in normal rats. We found that optogenetic stimulation of the dmPFC–BLA pathway promoted a behavioral state of negative emotion, increasing anxiety-like and depressive-like behaviors and producing aversive behavior of place avoidance. Conversely, optogenetic inhibition of this pathway produced opposite effects, reducing anxiety-like and depressive-like behaviors, and inducing behaviors of place preference of reward. These findings suggest that activity of the dmPFC–BLA pathway is sufficient to drive a negative emotion state and the mPFC–amygdala circuit is tonically active in cortical regulation of emotional behaviors.

KEYWORDS

optogenetics, emotion, pain, medial prefrontal cortex, basal lateral amygdala

Introduction

The medial prefrontal cortex (mPFC) regulates higher brain functions of emotion, cognition, motivation and working memory and is involved in evaluation and execution of related behaviors (Miller and Cohen, 2001; Davidson, 2002; Dalley et al., 2004). It orchestrates these brain functions through diverse efferent projections to other cortical, subcortical and thalamic regions and among them is the amygdala complex (Anastasiades and Carter, 2021; Kenwood et al., 2022). Amygdala is known as a crucial limbic structure for regulation of emotion-related behaviors including anxiety and depression of negative emotion, drug reward of positive emotion, and pain and fear of aversive behaviors (Baxter and Murray, 2002; Gottfried et al., 2003; Murray, 2007; Etkin et al., 2011; Mahan and Ressler, 2012; Fernando et al., 2013; Janak and Tye, 2015; Cai et al., 2018; Neugebauer et al., 2020). The mPFC sends strong projections to the amygdala complex and particularly to basomedial amygdala (BMA)

and basolateral amygdala (BLA), which also prominently projects back to mPFC, making direct reciprocal connections between mPFC and BLA (Orsini et al., 2011; Knapska et al., 2012; Little and Carter, 2013; Burgos-Robles et al., 2017; Bloodgood et al., 2018). mPFC is mainly divided into dorsal mPFC (dmPFC) in prelimbic cortex and ventral mPFC (vmPFC) in infralimbic cortex and is composed of glutamatergic projection pyramidal neurons (PNs) and local inhibitory GABAergic interneurons (Tremblay et al., 2016; Kim et al., 2017; Ong et al., 2018; Anastasiades and Carter, 2021). The glutamatergic PNs in mPFC project to their target regions in monosynaptic pathways, forming general excitatory mPFC outputs to its projection targets (Anastasiades and Carter, 2021).

Recent studies suggest that these mPFC–amygdala projection pathways are key brain circuits in the top-down control mechanism for emotional behaviors (Shackman et al., 2011; Kenwood et al., 2022; Ressler et al., 2022). Particularly, it has been demonstrated that activation of the vmPFC–BMA pathway suppresses anxiety behavior in mice (Adhikari et al., 2015), but stimulation of the ascending BLA–mPFC pathway has an anxiogenic effect, increasing anxiety behavior (Tejeda et al., 2015; Felix-Ortiz et al., 2016; Marcus et al., 2020). Activities of the projection from dmPFC to BLA have been implicated in the increased anxiety induced by ethanol withdrawal, chronic restraint stress and chronic pain (Liu et al., 2020; McGinnis et al., 2020; Gao et al., 2023). However, stimulation of the mPFC–BLA projection blocked the anxiogenic effect of cholecystokinin infused into mPFC, indicating an anxiolytic effect of this pathway in that condition (Vialou et al., 2014). Therefore, to further characterize the normal function of the dmPFC–BLA pathway in regulation of overall emotion state, we used optogenetic analysis to identify how this specific pathway regulates typical behaviors of negative and positive emotional behaviors in normal rats.

Materials and methods

Animals

All procedures involving the use of animals conformed to the guidelines set by the Institutional Animal Care and Use Committee of MD Anderson Cancer Center. Wistar rats (300–400 g) of both sexes were used in this study. Our sex-based analysis showed that there was no significant sex difference, so rats of both sexes were pooled in analysis. The rats were housed in groups of three with food and water available *ad libitum*, and in a 12 h light/dark cycle. For surgery, implantation of optical fiber cannula and vector injection, a rat was anesthetized by constant inhalation of isoflurane (2%) in a stereotaxic apparatus. All behavioral trainings and tests were performed between 8: 00 am and 18: 00 pm.

Adeno-associated viral vectors and microinjections into dorsal mPFC

Adeno-associated virus (AAV) particles of serotype 5 were obtained from the Vector Core Facility at The University of North Carolina at Chapel Hill. An AAV5-CaMKIIα-hChR2 (H134R)-mCherry vector (AAV-ChR2), an AAV5-CaMKIIα-eNpHR3.0-mCherry (AAV-eNpHR) vector or a control vector AAV5-CaMKIIα-mCherry (AAV-mCherry) was bilaterally injected (1 μL each side) into dmPFC (anteroposterior, 3.2 mm from the bregma; lateral, ±0.5 mm; ventral, −4.0 mm from dura) in rats under anesthesia in a stereotaxic instrument. Behavioral experiments were performed 4 weeks after the vector injection. After the experiments, brain tissues were harvested for anatomical identification of the injection sites. Data from injections that were outside of the targeted area were excluded.

Implantation of optical fiber cannula and optical stimulation in BLA

Two weeks after the viral injection, a mono fiber-optic cannula (0.4 mm in diameter, Doric Lenses Inc., Canada) was stereotaxically implanted on each side of the brain just above BLA (anteroposterior, −2.8 mm from the bregma; lateral, ±5.2 mm; ventral, −7.4 mm from dura) in an anesthetized rat. After the implantation surgery, the animals were single housed and allowed to recover for 14 days before behavioral tests. For optical stimulation, the implanted cannula was connected to a 473 nm (for ChR2) or 590 nm (for eNpHR) DPSS laser (Shanghai Laser & Optic Century Co., China) through a fiber-optic patch cord with a rotary joint for free movement of the animal. For the excitatory ChR2 vector, laser light pulses of 20 Hz, 15 ms and 5 mW were delivered to BLA via the implanted cannula as we described before (Cai et al., 2018). For the inhibitory eNpHR vector, light stimulation at 1 Hz, 999-ms, 10 mW was similarly delivered. Intensity of the fiber-optic light at the end of fiber was verified before and after each experiment by a power meter (PM-100D, Thor Labs). All laser outputs were controlled by a Master-8 pulse stimulator (A.M.P.I).

Open field test

The open field test was conducted in an illuminated chamber (72 × 72 × 50 cm) divided by a central zone and an outer zone as we described before (Cai et al., 2018; Ge et al., 2022). A rat was connected to the light source and was placed in the center of the chamber. The rat was allowed to move freely for 15 min and locomotion activity of the animal in the two zones was video-recorded and analyzed with an automated video-tracking system (EthoVision XT, Noldus Information Technology Inc.). In the test, a total test time of 15 min was divided into three consecutive 5-min periods with the light off in the 1st period (control), light on in the 2nd period and light off in the 3rd period in rats with dmPFC injection of excitatory vector AAV-ChR2 or inhibitory vector AAV-eNpHR, and corresponding control vector AAV-mCherry. Reduced time spent and distance traveled in the unprotected central zone (central time and central distance, respectively) were regarded as anxiety-related indices. The total distance traveled in the entire chamber during the test was recorded and used as a measure of general locomotor activity.

Forced swim test

The forced swim test was conducted in a cylinder (diameter 30.5 × height 45.7 cm) for rats (ENV-590R, Med Associates Inc.) according to the protocol described in previous reports (Slattery and

Cryan, 2012; Cai et al., 2018). On day 1, a rat was placed into the water-filled cylinder for 15-min for pre-test swim. On day 2, the rat was connected with the light source and was allowed to swim for 5 min. Light was delivered for 1-min before and 5-min during the swim test in the AAV-ChR2-, AAV-eNpHR-, or AAV-mCherry-injected rats. The swim activity was videotaped and immobility time was counted manually afterwards. Immobility was defined as cessation of active swimming and escaping activities. Time the animal spent immobile during the test was recorded as a measure of despair-like behavior.

Test of conditioned place preference and conditioned place aversion

Detailed procedures of conditioned place preference (CPP) and conditioned place aversion (CPA) have been described in our previous studies (Zhu et al., 2007; Bie et al., 2009; Hou et al., 2015; Cai et al., 2018). CPP and CPA tests were conducted in a standard 3-chamber CPP apparatus (MED Associates, St. Albans, VT). The rats were subjected to 2 sessions of habituation, one session per day for 2 days before the CPP/CPA test. In a habituation session, a rat was placed in the center connecting chamber and allowed to move freely between the two test chambers for 30 min. After habituation to the test chambers, a rat was placed in the center chamber and was allowed to move freely among the chambers for 15 min in a pre-test. The time the rat spent in each chamber was recorded automatically. Then, the rat received 4 conditioning sessions for 8 days, each two-day session consisting of light stimulation-pairing conditioning for 30 min in a chamber on day 1 and no light stimulation-pairing conditioning for 30 min in the other chamber on day 2. After the four conditioning sessions, the rat underwent a post-test for 15 min with the same procedures as in the pre-test. The AAV-ChR2-, AAV-eNpHR- or AAV-mCherry-injected rats were subject to the same conditioning procedures. The CPP/CPA score was defined as the difference in time the rat spent in the light-paired chamber between the pre-test and the post-test for the same rat.

Tests for thermal and mechanical pain

For thermal pain, a rat was placed in a Plantar Test Instrument (Model 37,370, Ugo Basile, Italy). Paw withdrawal response to an infrared heat stimulus was measured with a Hargreaves apparatus. Latency from the onset of the heat stimulus to the paw-withdrawal response was recorded automatically by the apparatus and was measured twice with a 5-min interval. The data presented were the averaged values of paw-withdrawal latencies of both right and left hind paws measured alternatively. For mechanical pain, a rat was extensively handled and habituated to the test environment and test apparatus for 3 d before the pain test. Then, the rat was placed in a plastic box with mesh floor and allowed to acclimate for 20 min. A series of calibrated von Frey filaments were applied perpendicularly to the plantar surface of a hind paw with sufficient force to bend the filament for 6 s. A brisk movement of the hind paw (withdrawal or flinching) was considered as a positive response. The threshold (g) of the tactile stimulus producing a 50% likelihood of withdrawal was determined by the "up-down" calculating method (Chaplan et al., 1994). The hind paw withdrawal response was measured twice with a 5-min interval. The latency and the threshold were measured before optical stimulation as baseline control and after 5-min optical stimulation administered 2–3 min after completion of baseline measurements in AAV-ChR2-, AAV-eNpHR- or AAV-mCherry-injected rats.

Novel object recognition assay for non-spatial memory

Novel object recognition assay is a well-established method to evaluate non-spatial memory in an open field (Bevins and Besheer, 2006). As we showed previously (Cai et al., 2013), the test includes 3-sessions in 2 days for habituation, training and retention test. On day 1, rats were habituated individually in a grey open arena ($60 \times 60 \times 50$ cm for L × W × H) for approximately 10 min without any object in the field. Two objects placed in the opposite corners of the arena were used in the test: one was a yellow glass cylinder (H = 12 cm, $r = 3$ cm) and the other was a green plastic cuboid (H = 12, L = 6, W = 3.7 cm), and both had similar surface area and the same height. For the training session on day 2, two identical objects were placed in the arena. The rat was allowed to freely explore the objects for 5 min and the amount of time the animal explored on each object was videotaped for analysis afterwards. For the retention test 4 h after the training session, one object from the training session (familiar object) was randomly replaced by a novel object. The rat was allowed to freely explore the objects in the field for 5 min. The time spent exploring on each object was manually counted from the recorded videos. The preference ratio for novel object was determined by the time exploring on the novel object/total test time (5 min).

To minimize the potential influence of the same and different behavioral tests above on the same animals, we conducted the same test only once, reversed the order of different tests (e.g., OFT and FST), and waited at least 3 days between the two different tests in the same animals.

Immunohistochemistry

A rat was deeply anesthetized with pentobarbital and transcardially perfused with heparinized saline and subsequently with ice-cold 4% paraformaldehyde in 1 × PBS (pH 7.4). The brain was removed and post-fixed in 4% paraformaldehyde overnight at 4°C, followed by dehydration with 30% sucrose in 1 × PBS. Tissues were sectioned into 30-μm thick coronal sections with a cryostat at-20°C. Sections were blocked with 5% normal donkey serum in PBS containing 0.3% Triton X-100 and incubated overnight with primary antibodies (mouse or rabbit anti-mCherry antibodies from Abcam, ab167453 or ab125096, 1: 500 dilution). Sections were then rinsed and incubated with the Alexa Fluor-conjugated secondary antibodies (1: 500, Alexa Fluor 488, green color, or 568, red color, Invitrogen), and were mounted on slides, dried and cover-slipped with ProLong Gold anti-fade reagent. The stained sections were examined with an Olympus BX51 fluorescence microscope or a Zeiss 710 confocal microscope.

Statistical analysis

Comparisons of averages of two groups were performed with the unpaired, two-tailed Student's t test. Two-way ANOVA for repeated measures with *post hoc* analysis of the Bonferroni method was used to determine statistical significance in behavioral experiments for effects of treatment and between-group interactions at each time point. Data were tested with the Shapiro–Wilk test for normal distribution. All data sets passed the normality test ($p > 0.05$), suggesting a normal distribution of the data. A $p < 0.05$ was considered statistically significant. All statistical analyses were performed with the Prism software version 9.0 (GraphPad Software). Data are presented as mean ± S.D.

Results

Dorsal mPFC excitatory neurons project to BLA, not to CeA

We used a viral vector and immunomicroscopy to identify axon projections of excitatory PNs in dmPFC to amygdala in rats. The vector AAV-CaMKII-hChR2-mCherry was bilaterally injected into dmPFC of naïve rats to transfect local excitatory (CaMKIIα-expressing) PNs and their axon terminals in their projection areas. Four weeks later, we examined mCherry expression in the dmPFC and in the amygdala. Intense mCherry staining was observed in the vector-infused dmPFC, suggesting successful transfection of local excitatory PNs with the vector through CaMKIIα promoters (Figures 1A,B). Figure 1C shows a representative image of the brain section stained by hematoxylin eosin with a cannula track targeting BLA. In the amygdala, we found robust mCherry expression in BLA, but not in the central nucleus of amygdala (CeA) (Figure 1D). This result illustrates selective and strong excitatory projections from dmPFC to BLA, but not to CeA.

Stimulation of dmPFC–BLA projections induces anxiety- and depressive-like behaviors

We examined the function of this dmPFC–BLA pathway by optogenetic activation of this pathway and real-time behavioral tests of anxiety-like and depressive-like behaviors of negative emotion in naïve rats *in vivo*. Four weeks after bilateral infusion of the AAV-CaMKII-hChR2-mCherry vector (AAV-ChR2, $n = 6$) or a control vector AAV-CaMKII-mCherry (AAV-mCherry, $n = 6$) into the rat dmPFC, we found that the AAV-ChR2- and AAV-mCherry-injected rats displayed a similar level of anxiety-like behavior as measured by the open field test (OFT) during the initial 5-min period without stimulation (light off) (time spent in central zone or central time: control, 13.3 ± 5.4 s, ChR2, 13.2 ± 3.4 s, $t = 0.052$, $p > 0.99$, multiple comparisons of 2-way ANOVA). However, in the 5-min period immediately following the preceding period, with optical stimulation (light on) in the BLA to activate the dmPFC–BLA pathway, the AAV-ChR2-injected rats showed significantly decreased central time indicating increased anxiety behavior when compared to the control rats (central time with light: control, 16.9 ± 8.8 s, ChR2, 5.4 ± 2.9 s, $F_{(2,30)} = 4.0$ and $p = 0.028$, $t = 3.60$ and $p < 0.05$, multiple comparisons of 2-way ANOVA, Figure 1E). During the following 5-min period without stimulation (light off), the central time was no longer statistically different between the ChR2-injected and control animals (central time: control, 10.7 ± 7.3 s, ChR2, 3.2 ± 1.7 s, $t = 2.34$, $p = 0.078$, multiple comparisons of 2-way ANOVA). Similar results were obtained in the distance traveled by the rats in the central zone

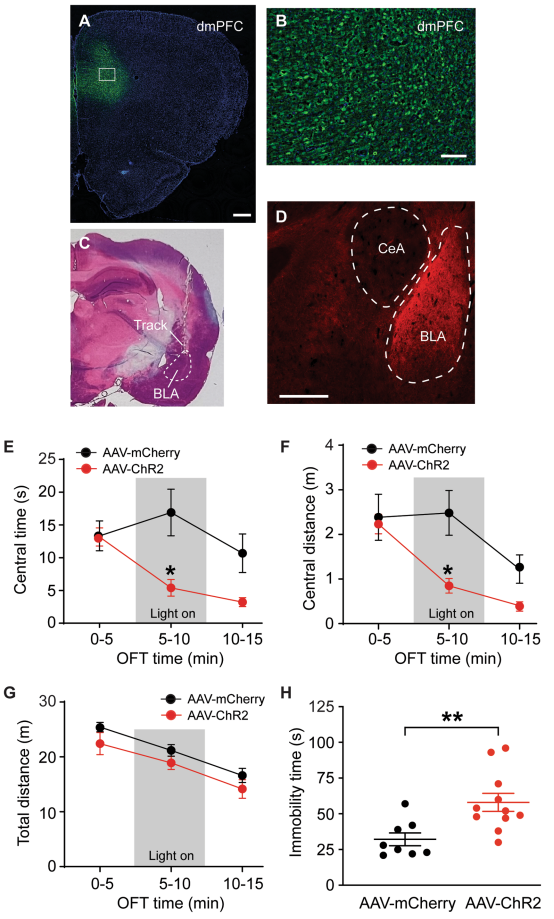

FIGURE 1
Stimulation of dmPFC–BLA projections induces behaviors of negative emotion. **(A,B)** Immunohistochemical images of ChR2-mCherry expression in dorsal medial prefrontal cortex (dmPFC, **A**) and with high magnification in the dmPFC area as marked in A **(B)** 4 weeks after bilateral infusion of the viral vector AAV5-CaMKIIα-ChR2-mCherry (AAV-ChR2-mCherry) into the mPFC of a rat. **(C)** A representative brain section stained by hematoxylin eosin, showing a cannula track targeting the basolateral amygdala (BLA). **(D)** An immunohistochemical image showing ChR2-mCherry expression in BLA, but not in the central nucleus of the amygdala (CeA) 4 weeks after similar infusion of the AAV-ChR2-mCherry vector into the dmPFC in a rat. Secondary antibody Alex-Fluor 488 (green) was used in **A** and **B**, and Alex-Fluor 568 (red) was used in **D**. Scale bars = 500 μM **(A,D)** and 50 μM **(B)**. **(E–G)** Group data of time spent **(E)** and distance traveled **(F)** in central zone and total distance traveled **(G)** in rats with mPFC injection of the control vector AAV-mCherry ($n = 6$) and AAV-ChR2 vector ($n = 6$) in three consecutive 5-min periods in the open field test. The light was on during the 2nd period (grey areas) for optical stimulation of the mPFC–BLA projections. **(H)** Immobility time in rats with mPFC injection of the control vector ($n = 8$) and AAV-ChR2 vector ($n = 11$) during optical stimulation in BLA in the forced swim test. Errors are S.E.M in all figures. *$p < 0.05$, **$p < 0.01$.

(Figure 1F). In contrast, the total distance travelled in the entire test chamber during each of the three periods was not different between the two rat groups, indicating that the optical stimulation did not affect the overall locomotor activity of the rats (Figure 1G). In addition, we determined the effect of activating this dmPFC–BLA pathway on the depressive-like behavior of another form of negative emotion by the forced swim test (FST). In mostly the same two groups of rats injected with AAV-ChR2 ($n = 11$) or AAV-mCherry ($n = 8$), we found that optical stimulation of the dmPFC-BLA pathway significantly increased immobility time indicating depressive-like behavior in the FST (control, 32.1 ± 12.7 s, ChR2, 58.0 ± 20.9 s, $t = 3.09$, $p = 0.006$, Figure 1H). These findings suggests that acute activation of the excitatory dmPFC-BLA pathway is sufficient to induce anxiety-like and depressive-like behaviors of negative emotion in rats under normal condition.

Stimulation of dmPFC-BLA projections induces behaviors of aversion and memory impairment

We then determined whether stimulation of the dmPFC-BLA projection would induce aversive behavior, another form of negative emotion, with the conditioned place aversion (CPA) test. Separate groups of rats with AAV-ChR2 or AAV-mCherry injection into dmPFC were conditioned with the optical stimulation in BLA. After the 4 conditioning sessions for 8 days, we found that optical stimulation of the dmPFC-BLA projection produced strong place aversion in the ChR2-injected rats ($n = 10$), but not in the control rats ($n = 8$), as measured by the CPA score (control, −17.2 ± 63.9 s, ChR2, −138.0 ± 112.9 s, $t = 2.69$, $p = 0.02$, Figure 2A). Thus, it appears that activating the dmPFC-BLA projection can also induce strong place aversion of negative reinforcement in normal rats.

Next, we wondered whether these emotional changes could affect memory. We tested the recognition memory using the novel object recognition test in the same groups of rats. We found that, after optical stimulation in the BLA, the AAV-ChR2-injected rats ($n = 6$) spent significantly less time on the novel object than the control vector-injected rats ($n = 6$), as measured by the preference ratio for novel object (control, 0.62 ± 0.07, ChR2, 0.46 ± 0.12, $t = 2.72$, $p = 0.0216$, Figure 2B). These results show that activation of the excitatory dmPFC-BLA pathway likely attenuates the recognition memory for novel object as measured in this test.

Inhibition of dmPFC–BLA projections reduces anxiety- and depressive-like behaviors

To further validate the functions of this dmPFC–BLA pathway, we optically inhibited the pathway and investigated its effects on the same emotion-related behaviors in naïve rats. The inhibitory vector AAV-CaMKII-eNpHR3.0-mCherry (AAV-eNpHR, $n = 7$) or the control vector AAV-mCherry ($n = 6$) was bilaterally injected into dmPFC and 4 weeks later, optical stimulation was administered during the behavioral tests. We found that, while the two groups of rats had no difference in the central time without light stimulation in the first 5-min period in the OFT (central time: control, 10.9 ± 2.4 s, AAV-eNpHR, 11.1 ± 3.2 s, $t = 0.07$, $p > 0.99$, multiple comparisons of 2-way ANOVA), the AAV-eNpHR-injected animals displayed significantly increased central time indicating decreased anxiety-like behavior during the following 5-min period with optical stimulation (central time with light: control, 13.0 ± 2.0 s, AAV-eNpHR, 20.8 ± 7.4 s, $F_{(1,11)} = 10.56$ and $p = 0.0006$, $t = 3.50$ and $p = 0.004$, multiple comparisons of 2-way ANOVA, Figure 3A). During the following 5-min period without light stimulation, there was no difference in central time between the two rat groups

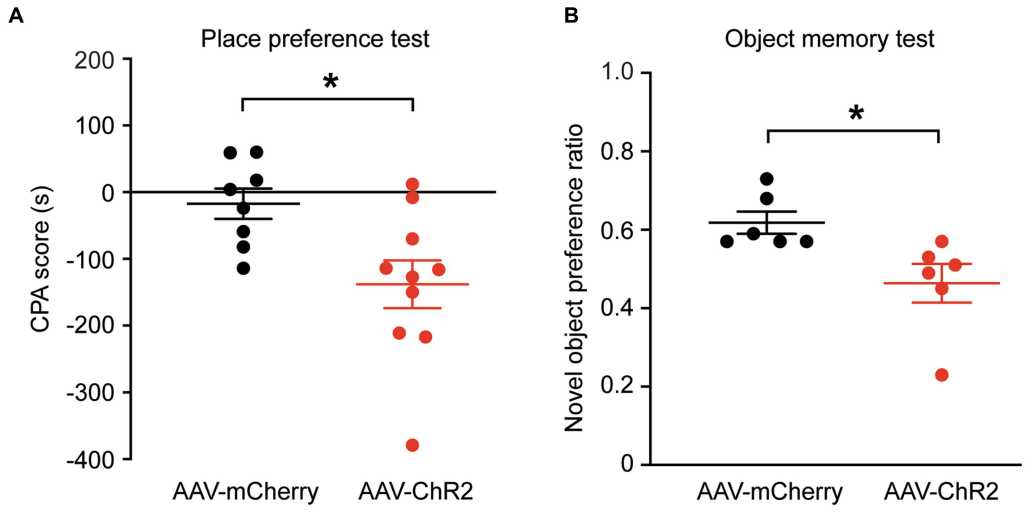

FIGURE 2
Stimulation of dmPFC-BLA projections induces behaviors of aversion and memory impairment. **(A)** Scores of conditioned place aversion (CPA) in rats with dmPFC injection of the control vector ($n = 8$) and AAV-ChR2 vector ($n = 10$) after conditioning sessions paired with the optical stimulation in BLA. **(B)** Preference ratios for novel object in rats ($n = 6$ each group) after dmPFC injection of the AAV-mCherry or AAV-ChR2 vector in the novel object recognition test. *$p < 0.05$.

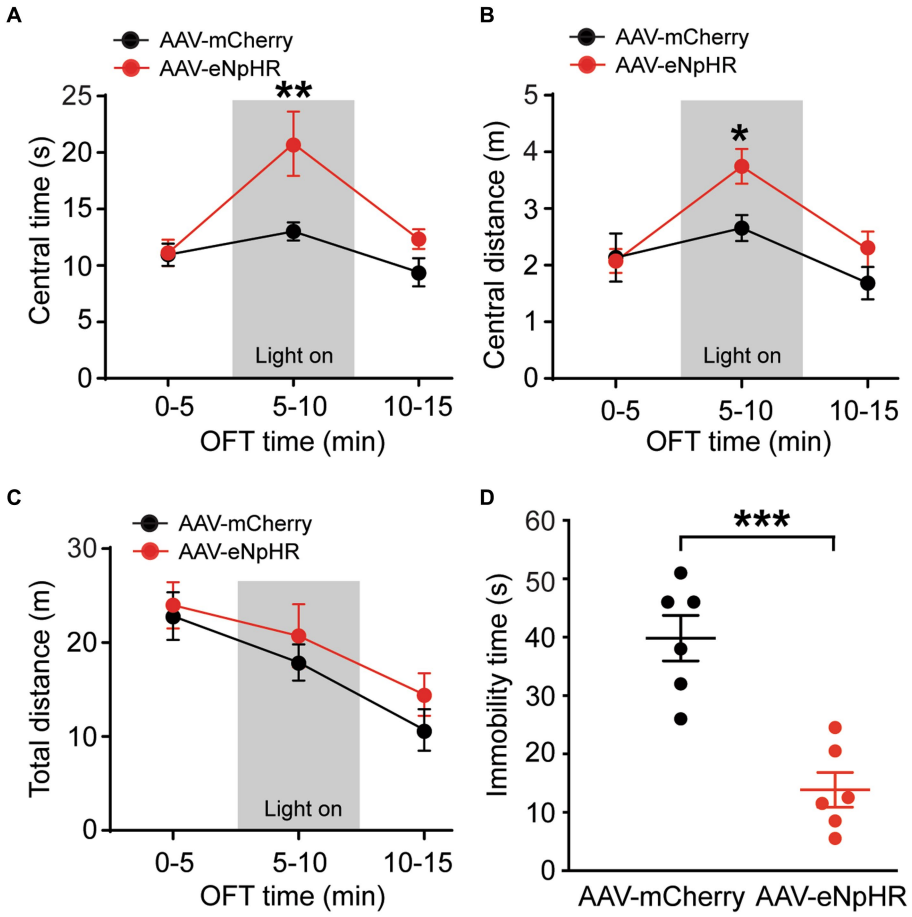

FIGURE 3
Inhibition of dmPFC–BLA projections reduces behaviors of negative emotion. **(A–C)** Group data of time spent **(A)** and distance traveled **(B)** in central zone and total distance traveled **(C)** in rats with mPFC injection of the control vector AAV-mCherry ($n = 6$) and the inhibitory vector AAV-eNpHR ($n = 7$) in three consecutive 5-min periods in the open field test. The light was on during the 2nd period (grey areas) for optical inhibition of the dmPFC–BLA projections. **(D)** Immobility time in rats with dmPFC injection of the control vector ($n = 6$) and AAV-eNpHR vector ($n = 6$) during optical stimulation in BLA in the forced swim test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(central time: control, 9.4 ± 2.9 s, AAV-eNpHR, 12.3 ± 2.4 s, $t = 1.32$, $p = 0.59$, multiple comparisons of 2-way ANOVA). The central distance was also significantly increased in the AAV-eNpHR-injected rats during the optical stimulation period (Figure 3B). The total distance traveled for overall locomotor activity during each of the three 5-min periods had no difference between the two rat groups (Figure 3C). We then examined the depressive-like behavior after the optical inhibition of the dmPFC–BLA pathway in these two groups of rats with the FST. We found that, when compared with the control vector-injected rats ($n = 6$), light stimulation in the AAV-eNpHR-injected rats ($n = 6$) significantly reduced immobility time, consistent with decreased depressive-like behavior in the FST (control, 39.8 ± 9.6 s, AAV-eNpHR, 14.3 ± 7.3 s, $t = 5.205$, $p = 0.0004$, Figure 3D).

These results demonstrate that selective optical inhibition of the excitatory dmPFC-BLA projection attenuates anxiety-like and depressive-like behaviors, just opposite to the effects of activating this pathway shown earlier, further supporting the notion that activation of the excitatory dmPFC-BLA pathway promotes behaviors of negative emotion with anxiogenic and depressive effects in rats under normal conditions.

Inhibition of dmPFC-BLA projections promotes behaviors of place preference and memory

We further tested the effects of inhibiting the dmPFC-BLA projection on reward-and memory-related behaviors. In separate groups of naïve rats injected with AAV-mCherry or AAV-eNpHR in the dmPFC, we first examined behavior of conditioned place preference (CPP) related to a reward effect. After conditioning the rats with optical stimulation in the BLA, we found that light stimulation in the AAV-eNpHR-injected rats ($n = 7$) produced robust CPP behavior of reward when compared with the AAV-mCherry-injected control rats ($n = 6$) (CPP score: control, 53.8 ± 79.1 s, AAV-eNpHR, 233.9 ± 68.0 s, $t = 4.424$, $p = 0.001$, Figure 4A). We then determined how inhibiting the dmPFC-BLA pathway would affect memory-related behavior with the novel object recognition test. In the same two groups of rats, we found that, after the optical stimulation in the BLA, the AAV-eNpHR group ($n = 7$) displayed higher preference for the novel object than the control group ($n = 6$) (preference ratio: control, 0.60 ± 0.05, AAV-eNpHR, 0.69 ± 0.08, $t = 2.37$, $p = 0.037$, Figure 4B).

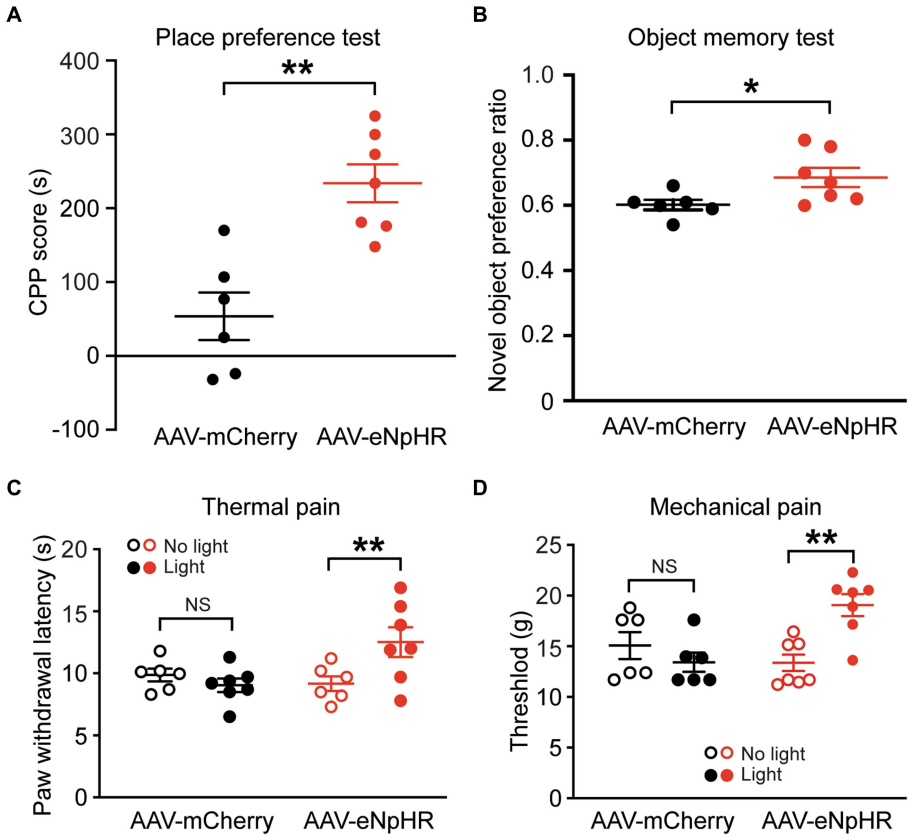

FIGURE 4
Inhibition of dmPFC-BLA projections facilitates behaviors of reward and memory and reduces nociceptive responses. **(A)** Scores of conditioned place preference (CPP) in rats with dmPFC injection of the control vector ($n = 6$) and the inhibitory AAV-eNpHR vector ($n = 7$) after conditioning sessions paired with the optical stimulation in BLA. **(B)** Preference ratios for novel object in rats after dmPFC injection of the AAV-mCherry ($n = 6$) or AAV-ChR2 vector ($n = 7$) in the novel object recognition test. **(C,D)** Paw-withdrawal latencies for thermal pain **(C)** and paw-withdrawal thresholds for mechanical pain **(D)** before (no light) and after (light) optical stimulation in the BLA for inhibition of the dmPFC–BLA projections in rats with dmPFC injection of the control vector AAV-mCherry ($n = 6$) or the inhibitory vector AAV-eNpHR ($n = 7$). NS, not significant. *$p < 0.05$, **$p < 0.01$.

These results suggest that inhibiting the dmPFC-BLA pathway induces a rewarding effect and likely promotes recognition memory, again opposite to the effects of activating this pathway as shown earlier. Thus, it appears that the excitatory dmPFC-BLA projection, once activated, reduces reward-and memory-related behaviors under normal conditions.

Inhibition of dmPFC-BLA projections reduces nociceptive response

Finally, we determined how inhibiting the dmPFC-BLA projection would change pain response, the behavior often associated with negative emotion. In separate groups of naive rats injected with the AAV-eNpHR vector ($n = 7$) or the AAV-mCherry vector ($n = 6$) into the dmPFC, we found that the light stimulation in the BLA significantly reduced nociceptive responses both to thermal stimulus and to mechanical stimulus with increased paw withdrawal latency in the thermal pain test (AAV-mCherry: before stimulation, 9.8 ± 1.2 s, after stimulation, 9.2 ± 1.5 s, $t = 0.818$, $p = 0.862$; AAV-eNpHR: before stimulation, 9.0 ± 1.3 s, after stimulation, 12.5 ± 3.2 s, $F_{(1,11)} = 5.647$ and $p = 0.037$, $t = 4.382$ and $p = 0.002$, multiple comparisons of 2-way ANOVA, Figure 4C), and increased paw-withdrawal threshold in the von Frey test (AAV-mCherry: before stimulation, 15.1 ± 3.2 g, after stimulation, 13.4 ± 2.2 g, $t = 1.108$, $p = 0.583$; AAV-eNpHR: before stimulation, 13.01 ± 2.1 g, after stimulation, 19.1 ± 2.9 g, $F_{(1,11)} = 13.02$ and $p = 0.004$, $t = 4.114$ and $p = 0.003$, multiple comparisons of 2-way ANOVA, Figure 4D). These findings indicate that inhibiting this dmPFC–BLA pathway can also reduce nociceptive responses.

Discussion

In this study, we have shown that activation of the specific pathway from dmPFC to BLA is sufficient to cause a series of behaviors of negative emotion including anxiety-like and depressive-like behaviors, and place aversion with likely impaired recognition memory. Our results of inhibiting this pathway suggest that this pathway is tonically active in cortical regulation of these behaviors of negative emotion under normal conditions. Together, these findings suggest that activity of this dmPFC–BLA pathway can sufficiently drive a behavioral state of negative emotion in rats.

Anxiety has been the research focus in recent studies on the functions of specific pathways for the reciprocal connections between mPFC and amygdala. Using optogenetic stimulation, Adhikari et al. have shown that neurons in vmPFC mainly target BMA and

stimulation of the vmPFC–BMA pathway decreases anxiety in mice (Adhikari et al., 2015). In contrast, studies on the reciprocal projections between dmPFC and BLA generally suggest an anxiogenic effect of the dmPFC–BLA circuits. Particularly for the mPFC–BLA projection, it has been shown that activity of this descending projection contributes to the increased anxiety in various anxiety-related animal models including ethanol withdrawal, stress, chronic pain and fear (Cho et al., 2013; Liu et al., 2020; McGinnis et al., 2020; Gao et al., 2023; Gunduz-Cinar et al., 2023). Our current study shows that the activity of the dmPFC–BLA pathway tonically maintains an emotional status as part of top-down cortical control of emotional behaviors under normal conditions, as its activation is sufficient to drive towards a more negative emotional state and its inhibition causes the shift to a more positive emotional state involving a series of emotion-related behaviors including anxiety-like and depressive-like behaviors, place preference of reward, aversive pain responses, and recognition memory.

Previous studies have shown that dmPFC and vmPFC have differential roles in regulation of other emotional behaviors including fear, drug seeking and reward (Ishikawa et al., 2008; LaLumiere and Kalivas, 2008; Sangha et al., 2014; Gourley and Taylor, 2016; Trask et al., 2017; Caballero et al., 2019). For fear regulation, in particular, activity in dmPFC inputs to lateral amygdala neurons promotes fear expression while the pathway of vmPFC to basal and basolateral amygdala decreases fear (Maren and Quirk, 2004; Herry et al., 2010; Orsini et al., 2011; Sierra-Mercado et al., 2011; Knapska et al., 2012). Thus, the promoting role of the dmPFC–lateral amygdala in fear, another form of negative emotion, is consistent with our current results showing that activating the dmPFC–BLA pathway induces anxiety-like and depressive-like behaviors, and place aversion of negative emotion. Thus, it appears that vmPFC that mainly projects to BMA and dmPFC that selectively projects to BLA have opposing effects in regulation of anxiety and other emotional behaviors, providing a bi-directional cortical control of emotion through amygdala.

Pain is an aversive experience involving several forms of negative emotion such as anxiety and depression (Wilson et al., 2001; McWilliams et al., 2003; Wiech and Tracey, 2009). mPFC has been implicated in regulation of pain, but the detailed neuronal pathways and neural mechanisms involved remain poorly understood (Ong et al., 2018). Our current results show that inhibiting the dmPFC–BLA pathway reduces pain responses, which is consistent with its effects on other emotional behaviors in promoting a positive emotional state. It also indicates that tonic activity of this pathway maintains pain sensitivity under normal conditions.

It is interesting to observe in our results that changing the activity of the dmPFC–BLA pathway alters the memory involved in novel object recognition memory, but not CPA behavior that probably also involves memory of environment. This is likely due to the striking and distinct properties and characteristics in object recognition-involved memory and CPA-involved memory. CPA memory is strongly emotional and aversive, induced by an aversive and external stimulus, and is intense in degree and long lasting (Hou et al., 2015), whereas object recognition memory is emotionally neutral, occurs naturally without manipulating stimulus, and is much more subtle and acute. Given these differences, our results may suggest that the dmPFC–BLA pathway could alter the memory related to novel object learning and memory, but not the memory induced by strong aversive stimulation, or the latter memory is simply so intense that it overwhelms any effect by manipulating activity of the dmPFC–BLA pathway in the behavioral test under our experimental settings.

In summary, findings from this study suggest that activity of the dmPFC–BLA pathway is sufficient to drive and promote a state of negative emotion under normal conditions in a tonically active way in the circuit mechanisms for a bi-directional mPFC control of emotion.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Animal Care and Use Committee of University of Texas MD Anderson Cancer Center. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YQC: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Writing – original draft, Writing – review & editing. JG: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. ZZP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Impaired amygdala astrocytic signaling worsens neuropathic pain-associated neuronal functions and behaviors

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Introduction: Pain is a clinically relevant health care issue with limited therapeutic options, creating the need for new and improved analgesic strategies. The amygdala is a limbic brain region critically involved in the regulation of emotional-affective components of pain and in pain modulation. The central nucleus of amygdala (CeA) serves major output functions and receives nociceptive information via the external lateral parabrachial nucleus (PB). While amygdala neuroplasticity has been linked causally to pain behaviors, non-neuronal pain mechanisms in this region remain to be explored. As an essential part of the neuroimmune system, astrocytes that represent about 40–50% of glia cells within the central nervous system, are required for physiological neuronal functions, but their role in the amygdala remains to be determined for pain conditions. In this study, we measured time-specific astrocyte activation in the CeA in a neuropathic pain model (spinal nerve ligation, SNL) and assessed the effects of astrocyte inhibition on amygdala neuroplasticity and pain-like behaviors in the pain condition.

Methods and Results: Glial fibrillary acidic protein (GFAP, astrocytic marker) immunoreactivity and mRNA expression were increased at the chronic (4 weeks post-SNL), but not acute (1 week post-SNL), stage of neuropathic pain. In order to determine the contribution of astrocytes to amygdala pain-mechanisms, we used fluorocitric acid (FCA), a selective inhibitor of astrocyte metabolism. Whole-cell patch-clamp recordings were performed from neurons in the laterocapsular division of the CeA (CeLC) obtained from chronic neuropathic rats. Pre-incubation of brain slices with FCA (100 μM, 1 h), increased excitability through altered hyperpolarization-activated current (I_h) functions, without significantly affecting synaptic responses at the PB-CeLC synapse. Intra-CeA injection of FCA (100 μM) had facilitatory effects on mechanical withdrawal thresholds (von Frey and paw pressure tests) and emotional-affective behaviors (evoked vocalizations), but not on facial grimace score and anxiety-like behaviors (open field test), in chronic neuropathic rats. Selective inhibition of astrocytes by FCA was confirmed with immunohistochemical analyses showing decreased astrocytic GFAP, but not NeuN, signal in the CeA.

Discussion: Overall, these results suggest a complex modulation of amygdala pain functions by astrocytes and provide evidence for beneficial functions of astrocytes in CeA in chronic neuropathic pain.

KEYWORDS

amygdala, astrocyte, neuroimmune signaling, electrophysiology, behavior, neuronal excitability, neurotransmission, neuropathic pain

1 Introduction

The availability of effective therapeutic options for the treatment of chronic pain remains inadequate and limited, resulting in a major health issue worldwide and the need for a better understanding of pain mechanisms to provide insights into novel treatment approaches (Furlan et al., 2006; Chou et al., 2009; Martel et al., 2015). Evidence from clinical and preclinical studies suggests that the amygdala, a limbic brain structure, is critically involved in the emotional aspect of pain and pain modulation. The central nucleus of amygdala (CeA) serves major pain-related output functions within the amygdala circuitry. The laterocapsular division of CeA (CeLC) receives nociceptive information via the parabrachial (PB) nucleus in the brainstem, and this pathway has been linked to the modulation of pain behaviors (Veinante et al., 2013; Thompson and Neugebauer, 2017; Neugebauer, 2020). Pain-related changes in amygdala activity are now well documented in rodents (Thompson and Neugebauer, 2017; Thompson and Neugebauer, 2019; Neugebauer, 2020) and humans (Simons et al., 2014), but underlying mechanisms are not fully understood. Recent evidence suggests that pain mechanisms cannot be explained solely by neural factors, but alterations of the neuroimmune system, a key player in maintaining homeostasis in the central nervous system (CNS), have been linked to pain induction and maintenance (Grace et al., 2014; Ji et al., 2018; Ji et al., 2019; Donnelly et al., 2020). Astrocytes represent the majority of the glial cells in the CNS and serve critical physiological functions related to neuronal metabolism and synapse processes, but their contribution to the pathophysiology of pain in different brain areas is not clear (Grace et al., 2014; Ji et al., 2014; Ji et al., 2016; Donnelly et al., 2020; Cheng et al., 2022). In pathological conditions, astrocytes become activated ("reactive"), undergoing a series of structural and functional changes that serve to protect the brain (Tian et al., 2012; Ji et al., 2019). A large body of evidence has focused on the contribution of reactive glia to nociceptive processing at the peripheral and spinal cord levels (Li et al., 2019; Miranpuri et al., 2021; Xu et al., 2021; Cheng et al., 2022; Lu and Gao, 2023). Astrocyte activation has also been implicated in several brain regions involved in pain processing (Raghavendra et al., 2004; Narita et al., 2006; Roberts et al., 2009; Chen et al., 2012; Kim et al., 2016; Ni et al., 2019), but the astrocytic contribution to amygdala-related neuroplasticity and behaviors in the context of pain has not been reported. Interestingly, evidence from clinical studies detected higher glial responses at the neuroforamina (dorsal root ganglion and nerve roots) and spinal cord levels (Albrecht et al., 2018) as well as in various brain regions, including thalamus, somatosensory cortex, cingulate cortex and ventromedial prefrontal cortex, in patients suffering from chronic pain (Loggia et al., 2015; Albrecht et al., 2019; Albrecht et al., 2021), supporting the translational relevance of this line of research. Here, we sought to determine the involvement of astrocytes in the right amygdala (CeA) in neuroplasticity and pain behaviors at the chronic (4 weeks) stage of a neuropathic pain model, using a selective astrocytic inhibitor (fluorocitric acid, FCA).

2 Materials and methods

2.1 Animals

Experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC; protocol #21026) at Texas Tech University Health Sciences Center and conform to the guidelines of the International Association for the Study of Pain (IASP) and National Institutes of Health (NIH). For patch-clamp experiments, male heterozygous transgenic Crh-Cre(+)/tdTomato(+) rats (Crh, corticotropin releasing hormone also referred to as CRF) on Sprague Dawley background (bred in house) were used to record from non-CRF neurons, which are the predominant cell type in the capsular CeA where the most prominent GFAP signal changes were found in the pain model (see 3.1). For behavioral, immunohistochemical and qRT-PCR assays, male Sprague Dawley rats were purchased from Envigo (Indianapolis, IN). All animals, 250–350 g (2–3 months old) at time of testing, were housed in a temperature-controlled room under a 12 h day/night cycle with unrestricted access to food and water. Rats were randomly assigned to the different experimental groups, and a researcher blinded to drug treatments, and where applicable condition, carried out the experimentation.

2.2 Neuropathic pain model

The well-established spinal nerve ligation (SNL) model was used in this study to induce a neuropathy in the left hind paw of rats (Kim and Chung, 1992). Rats were anesthetized with isoflurane (2%–3%; precision vaporizer, Harvard Apparatus) and the L5 spinal nerve was tightly ligated using sterile procedures. Sham operated animals undergoing the same surgical procedure without L5 nerve ligation were used as control group.

2.3 Drugs

DL-fluorocitric acid (FCA) barium salt (#F9634) and barium chloride ($BaCl_2$, #217565) were purchased from Sigma-Aldrich. Because FCA was commercially available as Ba^{2+} salt, which may interfere with neuronal physiological properties leading to confounding results, we decided (Chou et al., 2009) to use $BaCl_2$

salt in control patch-clamp experiments and (Furlan et al., 2006) to precipitate Ba^{2+} during drug preparation for *in vivo* stereotaxic microinjections. For electrophysiology experiments (see 2.6), $BaCl_2$ was dissolved in H_2O and FCA in HCl 0.1 M as a stock solutions and diluted (1:100) to the final concentration (100 µM) in artificial cerebrospinal fluid (ACSF, in mM: 117 NaCl, 4.7 KCl, 1.2 NaH_2PO_4, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 25 $NaHCO_3$ and 11 glucose) on the day of the experiment (Khan et al., 2019; Vizuete et al., 2019). For intra-CeA microinjection (see 2.7.1), the compound was dissolved in HCl 0.1 M and the Ba^{2+} was precipitated by the addition of (2–3 drops) Na_2SO_4 0.1 M. This solution was buffered with Na_2HPO_4 0.1 M and centrifuged at 800 *g* for 10 min. The supernatant was removed and diluted in ACSF to the final concentration (100 µM) and pH was adjusted to 7.3 (Paulsen et al., 1987; Hayakawa et al., 2010; Paquette et al., 2021).

2.4 qRT-PCR

At either the acute phase (1 week post-SNL or sham surgery) or chronic phase (4 weeks post-SNL or sham surgery) of neuropathic pain (see 2.2), rats were euthanized by decapitation. Brains were rapidly extracted and oxygenated in an ice-cold sucrose-based physiological solution of the following composition (in mM): 87 NaCl, 75 sucrose, 25 glucose, 5 KCl, 21 $MgCl_2$, 0.5 $CaCl_2$, and 1.25 NaH_2PO_4. Two coronal brain slices (1,000 µM) containing the amygdala were prepared using a Vibratome (VT1200S, Leica Biosystems, Nussloch, Germany) as described previously (Presto and Neugebauer, 2022; Presto et al., 2023). The right CeA was dissected for mRNA analysis. RNA was extracted using the MagMAX-96 Total RNA Isolation Kit (Life Technologies, Carlsbad, CA, United States) and quantified on a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Rockford, IL, United States). Total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Thermo Fisher Scientific). Taqman Fast Advanced Master Mix (Thermo Fisher Scientific) was used to perform quantitative reverse transcription polymerase chain reactions (qRT-PCR). Applied Biosystems Taqman Gene Expression Assays included GFAP (Gfap; Rn01460868_m1), β-actin (Actb; Rn00667869_m1), ribosomal protein L3 (Rpl3; Rn01505100_g1), and ribosomal protein L29 (Rpl29; Rn00820801_g1). Reactions containing 5 ng of cDNA were conducted in duplicate using the CFX384 Real-Time System (BioRad, Hercules, CA, United States). Relative mRNA expression was determined using the $2^{-\Delta\Delta Ct}$ method, standardizing samples to the geometric mean of β -actin, Rpl3, and Rpl29; these reference genes have been shown to exhibit consistent expression in a rat neuropathic pain model (Wan et al., 2010) and serve as reliable internal markers for the analysis of mRNA expression within the CeA tissue of neuropathic rats in our prior studies (Presto and Neugebauer, 2022; Presto et al., 2023).

2.5 Immunohistochemistry

Rats were deeply anesthetized (4% isoflurane; precision vaporizer, Harvard Apparatus) and transcardially perfused with phosphate buffer saline (PBS) (0.01 M phosphate, 0.0027 M KCl and 0.137 M NaCl, pH ~7.4), followed by 4% paraformaldehyde in PBS (PFA). Brains were extracted and fixed overnight in 4% PFA, cryoprotected for 48 h in 30% sucrose in PBS, frozen in OCT and sectioned at 40 µm thickness using a cryostat (Epredia HM525 NX, Epredia, Kalamazoo, MI, United States). Similar brain sections containing the amygdala area were selected for immunostaining experiments. Sections were permeabilized in 0.3% Triton X100 in PBS (PBST) for 10 min, blocked in 5% normal goat serum (NGS) in PBS for 1 h and incubated with primary antibodies overnight at 4°C. The following day sections were washed with PBS, incubated with secondary antibodies for 2 h at room temperature, and finally mounted using ProLong Mountant (Invitrogen, P36961) after washes with PBS. Primary antibodies used: chicken anti-GFAP (Novus, NBP1-05198, 1:1,000), mouse anti GFAP-Alexa488 conjugated (Invitrogen, eBioscience 53-9892-80, 1:500) and guinea pig anti-NeuN (Millipore, ABN90, 1:1,000). Secondary antibodies used: goat anti-chicken Alexa488 (Invitrogen, A11039, 1:1,000) and goat anti-guinea pig Alexa 647 (Invitrogen, A21450, 1:1,000). Sections were imaged at the identical conditions with a confocal microscope (Olympus FV3000), using a 7 × 7 mosaic taken with ×60 oil immersion objective. Image analysis was performed with ImageJ software (ImageJ v1.53f51, NIH). The different amygdala regions were identified according to the Paxinos Watson Rat Brain Atlas (Paxinos and Watson, 2006). After marking amygdala regions in the NeuN channel, capsular and lateral divisions of CeA (CeC and CeL) were filled with sample areas of 250 × 250 px using "Rectangle" tool while avoiding large blood vessels. For GFAP expression, images were thresholded in the GFAP channel, and in each sample area the GFAP positive (+) area was analyzed using Analyze Particles function. In each section, multiple sample areas were analyzed (CeC, 21–39; CeL, 9–20) and averaged. Each group contained three animals with 3-4 sections per animal. For the effect of FCA injections on GFAP and NeuN positive signals, mosaic images taken with ×10 objective in two channels (GFAP and NeuN) were analyzed. Amygdala regions were marked in the NeuN channel. Images were thresholded in the analyzed channels (GFAP or NeuN), new region of interest (ROI) was created while avoiding large blood vessels, and percent of signal positive area or mean of positive signal were measured using Analyze Particles function. Each group had five animals, and one section including same region (CeL and CeC) per animal was evaluated.

2.6 Patch-clamp electrophysiology

Brain slices containing the right amygdala were obtained from neuropathic rats 4 weeks after SNL surgery as described previously (Kiritoshi and Neugebauer, 2018; Hein et al., 2021; Mazzitelli et al., 2022; Yakhnitsa et al., 2022), because significant changes of astrocytic marker GFAP were found only at the chronic neuropathic stage (see 3.1). Brains were quickly removed and immersed in an oxygenated ice-cold sucrose-based physiological solution (see 2.4). Coronal brain slices (400 µm) were obtained using a Vibratome (Leica Biosystems VT Series, Buffalo Grove, IL) and incubated in oxygenated ACSF (see 2.3) at room temperature (21°C). Slices were incubated with FCA barium salt or control solutions ($BaCl_2$, 100 µM, in ACSF; or ACSF alone; see 2.3) for 1 h before

recordings. Then, an individual brain slice was transferred to the recording chamber and submerged in ACSF (31°C ± 1°C) superfusing the slice at ~2 mL/min. Residual Ba^{2+} salt due to the pre-treatment was quickly washed out by ACSF in the recording chamber. Only two brain slices per animal were used, and 2–3 neurons were recorded in each brain slice. Whole-cell patch-clamp recordings were made from visually identified non-CRF neurons in the lateral-capsular division of the CeA (CeLC) using DIC-IR videomicroscopy and fluorescence illumination (BX51, Olympus, Waltham, MA) as described previously (Kiritoshi et al., 2013; Kiritoshi and Neugebauer, 2018; Mazzitelli et al., 2022; Yakhnitsa et al., 2022). Recording pipettes (tip resistance 5–8 MΩ) were made from borosilicate glass and filled with an intracellular solution containing (in mM): 122 K-gluconate, 5 NaCl, 0.3 $CaCl_2$, 2 $MgCl_2$, 1 EGTA, 10 HEPES, 5 Na_2-ATP, and 0.4 Na_3-GTP; pH was adjusted to 7.2–7.3 with KOH and osmolarity to 280 mOsm/kg with sucrose. Data acquisition and analysis were done using a dual four-pole Bessel filter (Warner Instr., Hamden, CT), low-noise Digidata 1550B interface (Axon Instr., Molecular Devices, Sunnyvale, CA), MulitClamp700B amplifier (Axon Instr., Molecular Devices, Sunnyvale, CA), and pClamp11 software (Axon Instr.). If series resistance (monitored with pClamp11 software) changed more than 20%, the neuron was excluded from analysis. To characterize the frequency-current (F–I) relationship as a measure of excitability, depolarizing current pulses (500 ms, 25 pA step) were applied. The total number of action potentials generated was measured at each current magnitude, and the rheobase was calculated as the smallest current step that elicited an action potential. Additionally, the firing threshold was identified by applying a single ramp voltage protocol. The depolarizing voltage sag was measured by the application of hyperpolarizing currents (500 ms, 20 pA steps) and was calculated as the difference between the largest decrease in voltage and the steady state value (ΔV). Current-voltage (I-V) relationship was obtained by voltage clamping the neuron at −60 mV and measuring current amplitudes in response to voltage steps (20 mV, 300 ms). Synaptic responses were evoked in voltage-clamp, using a concentric bipolar stimulating electrode (David Kopf Instruments) to stimulate glutamatergic afferent inputs presumably from the PB as in our previous studies (Kiritoshi and Neugebauer, 2018; Hein et al., 2021; Mazzitelli et al., 2022; Yakhnitsa et al., 2022). Mono-synaptic glutamatergic excitatory (EPSC) and glutamate-driven feedforward inhibitory (IPSC) post-synaptic currents were evoked at −70 mV and 0 mV, respectively. Peak amplitude, area under the curve (AUC), and decay time were analyzed. For paired-pulse ratio (PPR) analysis, two orthodromic synaptic stimuli of equal intensity (50-ms interstimulus interval) were applied and EPSCs were recorded (−70 mV). Peak amplitudes of the first EPSC (EPSC1) and the second EPSC (EPSC2) were measured and PPR was calculated as the ratio of EPSC2 over EPSC1.

2.7 Behavioral assays

Behavioral assays were performed 16–24 h after intra-CeA microinjection of FCA or vehicle (see 2.7.1) and 4 weeks after the neuropathic surgery (see 2.2). The following behavioral assays were performed in shielded temperature- and light-controlled rooms. Each rat underwent all behavioral assays in the following order: nocifensive reflex threshold using von Frey test (see 2.7.2), spontaneous behavior (facial grimace scoring) (see 2.7.3), anxiety-like behavior (see 2.7.4), emotional-affective behavior (evoked vocalizations) (see 2.7.5), and nocifensive reflex threshold using a calibrated forceps (see 2.7.2). The evoked behavior tests (reflexes and vocalizations) were repeated twice in the same animal and then averaged.

2.7.1 Intra-CeA microinjection

To inhibit astrocytes in the CeA, FCA was injected into the right CeA of SNL rats (see 2.2) using a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, United States) as previously described (Hein et al., 2021; Mazzitelli et al., 2022; Presto et al., 2023). Animals were anesthetized with isoflurane (2%–3%; precision vaporizer, Harvard Apparatus, Holliston, MA, United States) and a small unilateral craniotomy was performed. FCA (100 μM) or vehicle (Ba^{2+} was removed by precipitation; see 2.3) was injected (1 μL, 10 min) with a 5 μL Hamilton syringe using the following coordinates: 2.5 mm caudal to bregma, 4.0–4.3 mm lateral to midline, and 7.3–7.6 mm deep.

2.7.2 Mechanosensitivity

Nocifensive reflex thresholds were assessed using a plantar electronic von Frey anesthesiometer (IITC Life Science, Woodland Hills, CA) or a calibrated forceps connected to a force transducer whose output was displayed in grams on an LCD screen to gradually compress the hind paw with continuously increasing intensities (paw compression test); the force required for evoking a reflex response was displayed on an LCD screen and recorded. Von Frey tests were performed before (baseline) and 4 weeks after SNL surgery (see 2.2) to confirm the mechanical allodynia induced by the neuropathic pain model. To determine the effects of the intra-CeA treatment on mechanosensitivity, von Frey or paw pressure tests were performed on both left (injured) and right hind paws 16–24 h after drug delivery in neuropathic rats (see 2.7.1).

2.7.3 Spontaneous behaviors

Facial rodent grimace scale (RGS) was used to assess spontaneous pain behaviors (Sotocinal et al., 2011). The rats were placed in individual plexiglass chambers with home cage bedding in a quiet environment. A video camera (Sony Handycam HDR-CX455 9.2 megapixels with lenses Zeiss Vario-Tessar, Sony Corporation of America, New York, NY, United States) was placed on the outside of the chambers and each rat was video-recorded for a 5-min period. Ten images (at least 30 s apart) of the rat face were randomly selected from the still images using a random number generator. Only those images were selected that showed rats directly facing the camera; for images that did not qualify for analysis, a new random number was generated. Three final images for scoring were then selected using a random number generator and assigned a random number code. Scoring was performed by five treatment-blinded experienced evaluators (Sotocinal et al., 2011). Each image was scored based on four action units: orbital tightening, nose/cheek flattening, ear changes and whisker change. A score from 0 to 2 (0 = not present, 1 = moderate,

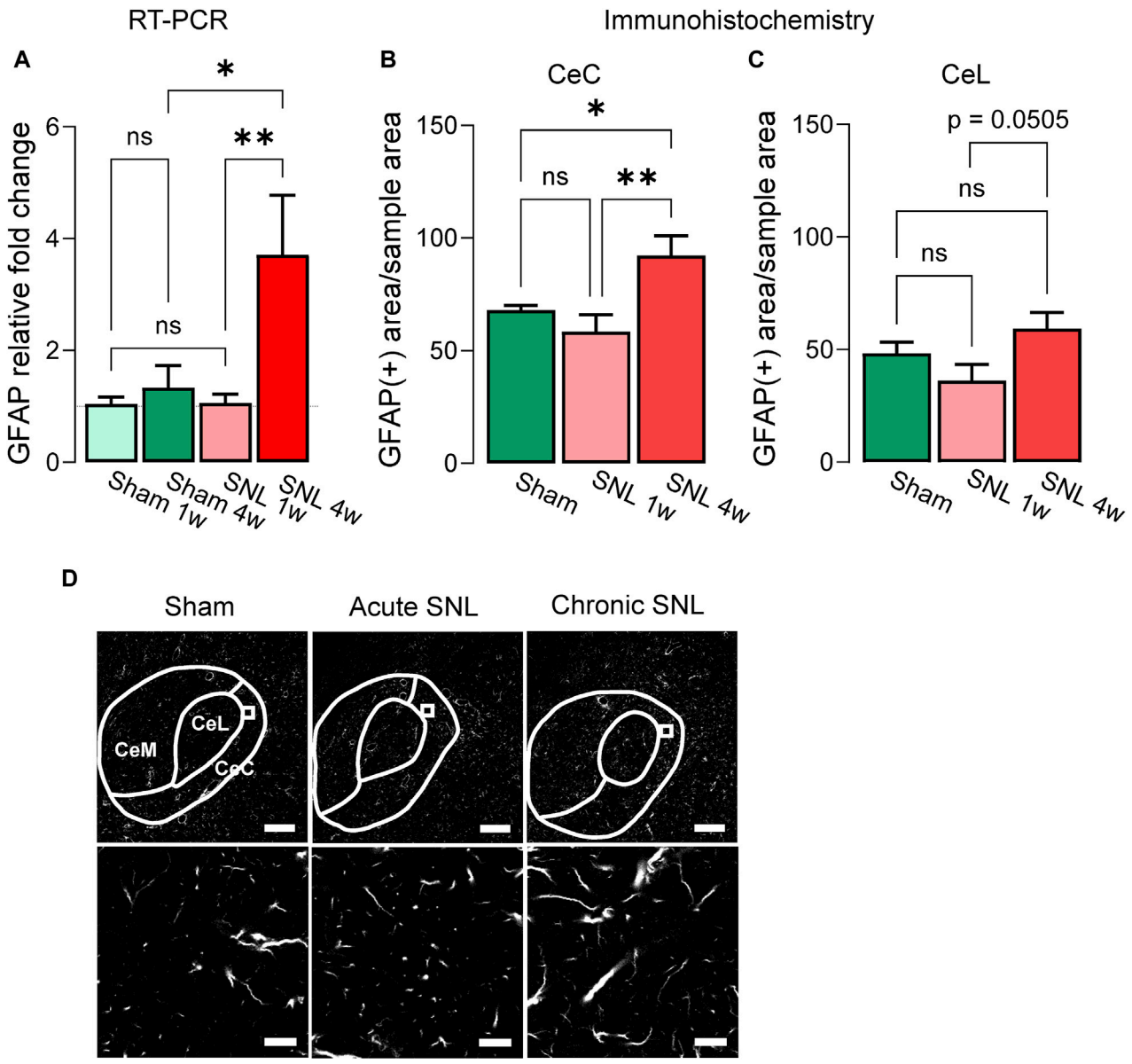

FIGURE 1
Temporal changes of GFAP mRNA and protein in the CeA of neuropathic rats. **(A)** Increased GFAP mRNA expression levels in the right CeA 4 weeks, but not 1 week, post-induction of SNL compared to sham control group. Importantly, no significant differences were observed between the two sham control groups (sham 1w vs. sham 4w). Bar histograms show means ± SEM. *,**$p < 0.05$, 0.01, two-way ANOVA with Bonferroni posthoc tests. Sham 1w, n = 7; sham 4w, n = 4; SNL 1w, n = 7; SNL 4w, n = 4. **(B)** Immunohistochemistry shows significantly increased GFAP (+) signal in CeC of chronic SNL rats compared to acute SNL or sham groups. A similar trend was detected in the CeL **(C)** though no statistical significance was reached ($p = 0.0505$). **(D)** Representative images of GFAP (+) signal in the CeA of different treatment groups. Bottom: representative sample area used for analysis. Scale bars, top, 200 µm; bottom, 10 µm *, **, $p < 0.05$, 0.01, one-way ANOVA Tukey's posthoc tests. CeC and CeL: sham, n = 10, SNL 1w, n = 10, SNL 4w, n = 10 sections from three animals per group.

2 = severe) was assigned to each facial unit (parameter). Scores were entered in an excel spreadsheet. The four action unit scores were summed to produce the total score and a mean of the scores for all three images was obtained.

2.7.4 Anxiety-like behaviors

The open field test (OFT) was used to investigate anxiety-like behavior in SNL rats (see 2.2). Behavioral activity in a square arena (70 cm × 70 cm) was videotracked for 5 min with EthoVision (Noldus Information Technology, Leesburg, VA, United States). Time spent (s) in the center area of the field (35 cm × 35 cm) and locomotor activity as the total distance traveled (cm) were calculated for the first 5 min. Avoidance of the center area of the OFT was interpreted as anxiety-like behavior.

2.7.5 Emotional responses

Vocalizations in the audible (20 Hz–16 kHz) and ultrasonic (25 ± 4 kHz) ranges were measured in neuropathic rats (see 2.2) as described before (Brudzynski, 2007; Neugebauer et al., 2007; Thompson et al., 2015; Mazzitelli and Neugebauer, 2019; Presto

et al., 2021). Rats were briefly anesthetized with isoflurane (2%–3%, precision vaporizer) to minimize stress of handling, and placed in a custom-designed recording chamber (U.S. Patent 7,213,538) to ensure a fixed distance from the sound detectors. A microphone connected to a preamplifier was used to record audible vocalizations, and a bat detector connected to a filter and amplifier measured ultrasonic vocalizations. (UltraVox four-channel system; Noldus Information Technology). After recovery from the brief anesthesia, vocalizations were evoked by brief (10 s) normally innocuous (300–500 g/6 mm^2), and noxious (1,000–1,500 g/6 mm^2) stimuli applied to the left (injured) or right hind paws using a calibrated forceps (see 2.7.2). Vocalizations were recorded for 1 min and analyzed using Ultravox 2.0 software (Noldus Information Technology) as described before (Neugebauer et al., 2007; Presto et al., 2021; Mazzitelli et al., 2022; Yakhnitsa et al., 2022). Innocuous stimulation preceded noxious stimulation.

2.8 Data and statistical analysis

All averaged values are presented as means ± SEM. GraphPad Prism 10.0 software (Graph-Pad Software, San Diego, CA) was used for all statistical analyses. Statistical significance was accepted at the level $p < 0.05$. Two-way ANOVA (repeated measures as appropriate) with Bonferroni posthoc tests or one-way ANOVA with Tukey's posthoc tests were used for multiple comparisons, and unpaired t-tests were used for comparison of two sets of data that had Gaussian distribution and similar variance as indicated.

3 Results

3.1 Increased astrocyte activation at the chronic but not acute stage of neuropathic pain

Levels of GFAP mRNA expression in the right CeA were examined at the acute (1 week post-SNL or sham surgery) or chronic (4 weeks post-SNL or sham surgery) stages of neuropathic pain (Figure 1A). RT-PCR analysis showed significant differences in the GFAP mRNA expression level between the acute and chronic time points and compared to sham control animals (sham 1w, n = 7; sham 4w, n = 4; SNL 1w n = 7; SNL 4w, n = 4; treatment (column) factor, $F_{(3,\ 12)} = 6.618$, $p = 0.0069$; Subject (row) factor, $F_{(6,\ 12)} = 0.6736$, $p = 0.6737$, two-way ANOVA). Specifically, GFAP mRNA expression in the CeA of SNL rats was increased compared to sham rats at the chronic (sham 4w, n = 4; SNL 4w, n = 4) but not acute (sham 1w, n = 7; SNL 1w n = 7) stage of neuropathic pain ($p < 0.05$, two-way ANOVA with Bonferroni's *post hoc* tests). GFAP mRNA expression levels were significantly higher at the chronic compared to the acute stage of SNL ($p < 0.01$, two-way ANOVA with Bonferroni's *post hoc* tests), suggesting a delayed upregulation of astrocytic mRNA in the CeA in neuropathic pain. No significant differences in GFAP mRNA expression levels were found between the acute and chronic sham cohorts; therefore, 4-week sham rats were used as control for the subsequent experiments.

Immunohistochemical analysis showed that the GFAP (+) signal was significantly enhanced in the CeC of chronic SNL rats compared to acute SNL or sham groups (Figures 1B, D, sham = 10, SNL 1w = 10, SNL 4w = 10 total GFAP(+) sample areas per section, 3-4 sections from three animals per group; $F_{(2,27)} = 6.477$; $p = 0.0050$, one-way ANOVA with Tukey's multiple comparison tests). In the CeL, a clear trend ($p = 0.0505$, one-way ANOVA with Tukey's posthoc tests) for increased GFAP (+) signals at chronic SNL compared to acute SNL was observed, but there were no statistically significant differences between the three groups (Figure 1C, sham = 10, SNL 1w = 10, SNL 4w = 10 total GFAP (+) sample areas per section, 3-4 sections from three animals per group; $F_{(2,27)} = 3.065$; $p = 0.0631$, one-way ANOVA with Tukey's posthoc tests).

3.2 Electrophysiological effects of astrocyte inhibition at the chronic stage of neuropathic pain

Considering that significant changes of GFAP astrocytic marker at mRNA and protein level were found only at the chronic phase of the neuropathic pain model (Figure 1), we focused our analysis of astrocyte functions on the chronic neuropathic stage (4-week time point). Whole-cell patch clamp experiments were performed on non-CRF neurons located in the CeLC to evaluate the effects of fluorocitric acid (FCA) on the neuronal properties (Figure 2) and synaptic responses evoked by the electrical stimulation of PB inputs (Figure 3) based on the results obtained from the immunohistochemical analyses showing increased GFAP (+) staining in both CeC and CeL in chronic SNL rats. Brain slices were incubated with FCA barium salt (100 μM, 1 h) and then transferred to the recording chamber which allowed the washout of Ba^{2+}. Neurons in FCA-treated slices showed increased neuronal excitability (Figures 2A, B) ($p < 0.05$, two-way ANOVA with Bonferroni's *post hoc* tests) but no changes of rheobase (Figure 2C) or AP threshold (Figures 2D, E) compared to the control (ACSF) group. In the same neurons, FCA pretreatment decreased the depolarizing voltage sag (Figures 2F, G) without affecting the I-V relationship (Figures 2H, I) compared to vehicle control (ACSF) group, suggesting that astrocytes may modulate neuronal excitability through mechanisms involving I_h. In order to avoid confounding results on neuronal excitability due to the presence of Ba^{2+} salt in the FCA solution, some brain slices were incubated with $BaCl_2$ (100 μM, 1 h) which resulted in no significant effects compared to control (ACSF) group (Figure 2B) (ACSF, n = 7; FCA, n = 8, Ba^{2+}, n = 6; Figure 2B, current injected (row) factor, $F_{(14,\ 285)} = 38.87$, $p < 0.001$; treatment (column) factor, $F_{(2,\ 285)} = 18.88$, $p < 0.001$; interaction, $F_{(28,\ 285)} = 1.178$, $p = 0.2500$; two-way ANOVA; Figure 2C, $p = 0.4293$, t = 0.8158; Figure 2E, $p = 0.5017$, t = 0.6910; Figure 2G, $p < 0.05$, t = 2.198, unpaired t-tests; Figure 2I, voltage (row) factor, $F_{(11,\ 156)} = 27.02$, $p < 0.001$; treatment (column) factor, $F_{(1,\ 156)} = 0.1075$, $p = 0.7434$; interaction, $F_{(11,\ 156)} = 0.0640$; $p > 0.9999$, two-way ANOVA).

In the same neurons, no significant effects of FCA on excitatory or inhibitory synaptic transmission were observed. Peak amplitude, area under the curve (AUC) or decay time of EPSCs (Figures 3A–D) or paired-pulse ratio (PPR; Figures 3E, F) recorded at the PB-CeLC synapse were not significantly different between FCA or ACSF treated groups (ACSF, n = 7; FCA, n = 8; Figure 3B, current

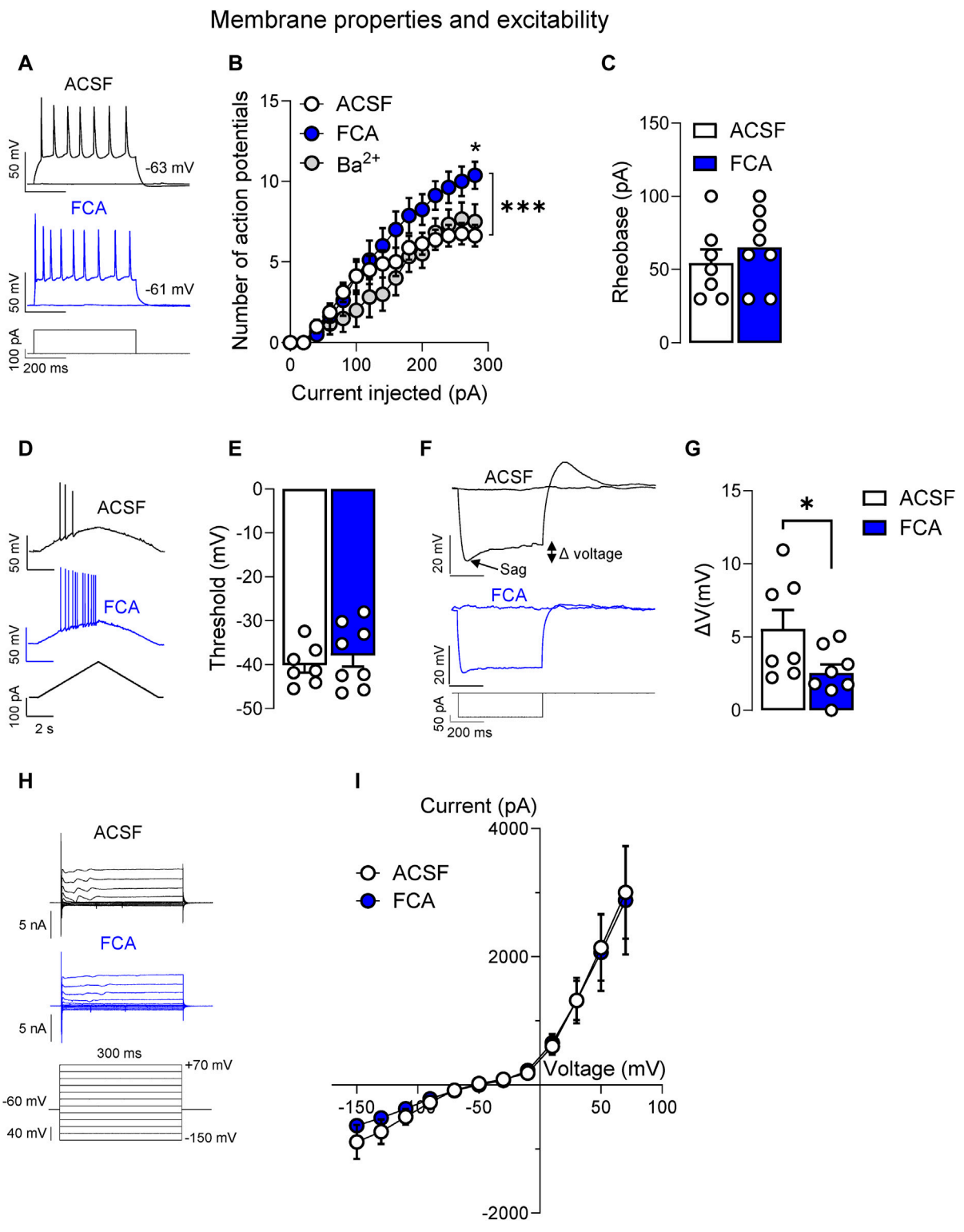

FIGURE 2
Facilitatory effects of astrocyte inhibition by fluorocitric acid (FCA) on CeLC neuronal properties in a neuropathic model. Neurons recorded from FCA-treated slices (100 μM, 1 h) showed increased neuronal excitability **(A,B)** induced by depolarizing current injections, while Ba^{2+} pretreatment had no effects compared to the control (ACSF) group (*, $p < 0.05$, two-way ANOVA with Bonferroni *post hoc* tests). Symbols show means ± SEM. ***, $p < 0.001$, two-way ANOVA. In the same neurons, FCA had no effects on rheobase **(C)** or AP threshold **(D,E)**, but it decreased the depolarizing voltage sag **(F,G)** without affecting the I-V relationship **(H,I)** compared to the control (ACSF) treated cells. Bar histograms show means ± SEM. *, $p < 0.05$, unpaired student t-tests. Symbols show means ± SEM, two-way ANOVA. ACSF, n = 7; FCA, n = 8.

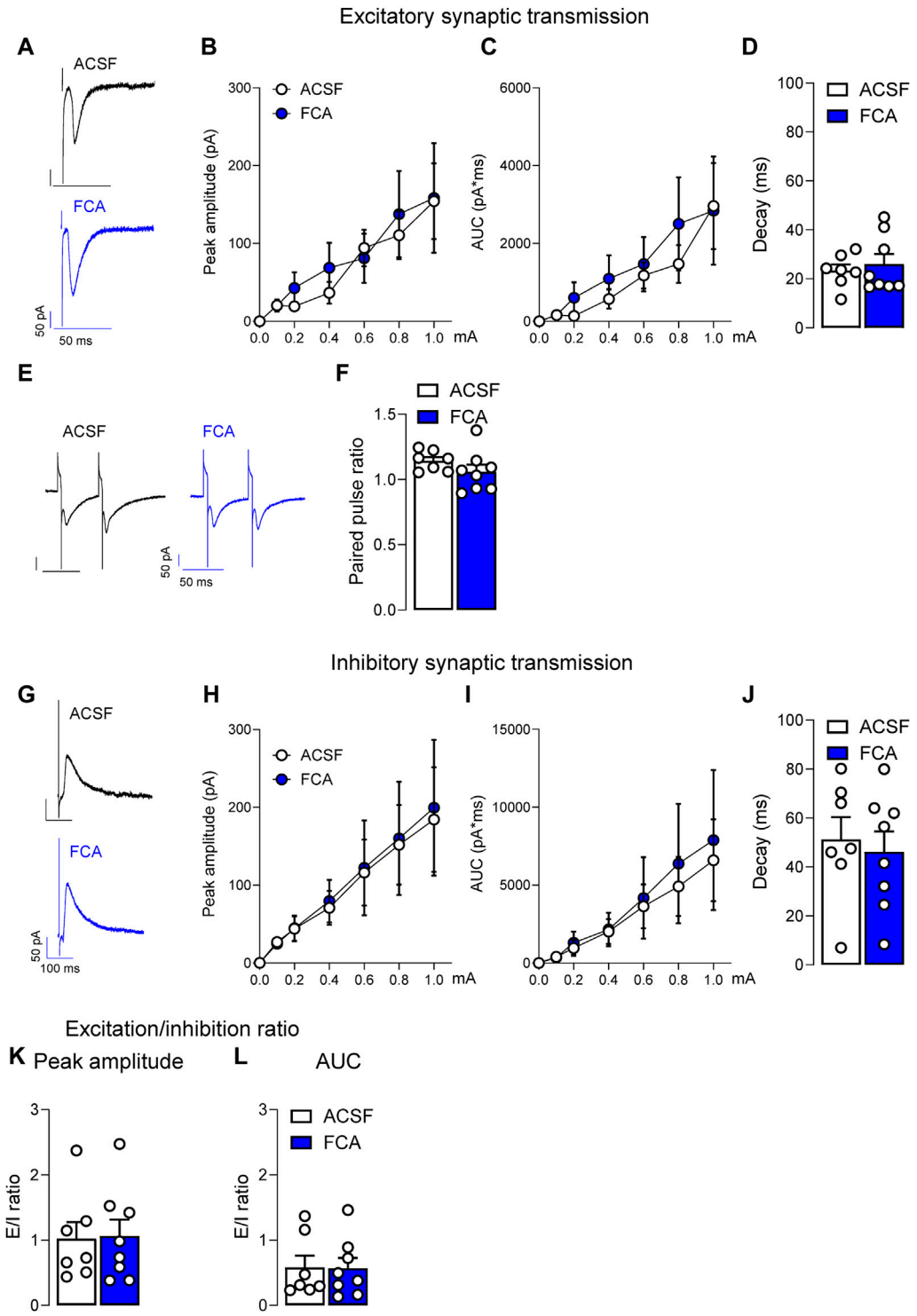

FIGURE 3
Lack of effects of astrocyte inhibition by FCA on evoked synaptic responses in a neuropathic model. Pre-treatment of brain slices with FCA (100 μM, 1 h) had no effect on the peak amplitude, area under the curve (AUC) or decay time of mono-synaptic excitatory post-synaptic currents (EPSCs) evoked by the electrical stimulation of PB input to CeLC neurons **(A–D)** recorded in brain slices obtained from neuropathic animals. Additionally, no change was observed in the paired-pulse ratio at the PB-CeLC synapse **(E,F)**. Similarly, pre-incubation with FCA (100 μM, 1 h) did not affect the peak amplitude, AUC or decay time of glutamate-driven inhibitory post-synaptic currents (IPSCs) evoked by the electrical stimulation of PB afferents onto CeLC neurons **(G–J)** in the neuropathic pain condition. No significant change was observed in the excitation/inhibition (E/I) ratio **(K,L)**. Symbols show means ± SEM, two-way ANOVA. Bar histograms show means ± SEM, unpaired t-tests. ACSF, n = 7; FCA, n = 8.

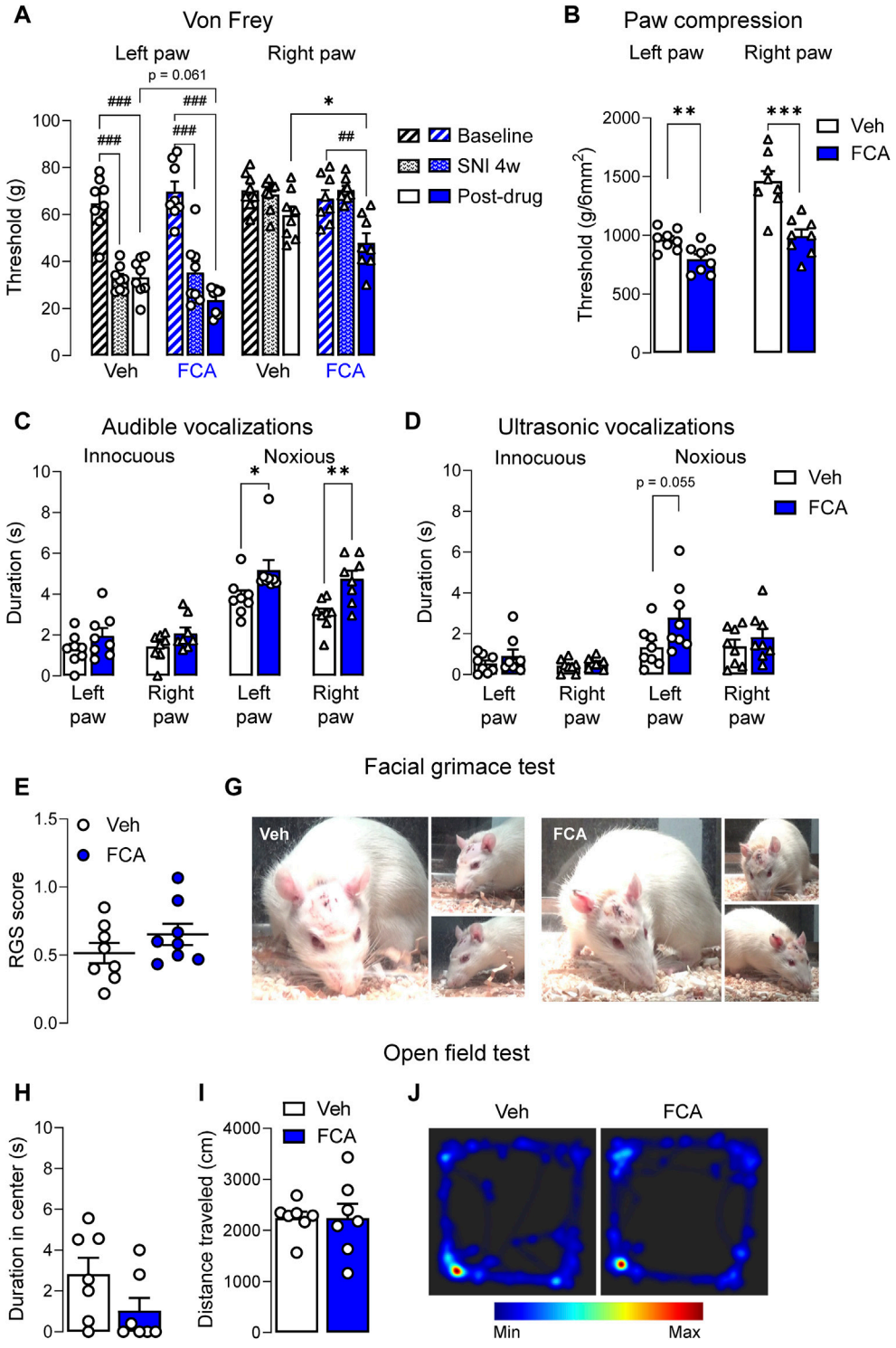

FIGURE 4
Pronociceptive effects of astrocyte inhibition by FCA in CeA on neuropathic pain-like behaviors. **(A)** Mechanical thresholds (measured by electronic von Frey) of the left (injured), but not right, hind paw were significantly decreased 4 weeks after SNL surgery, confirming the neuropathic pain condition. Stereotaxic injection of FCA (100 μM, 1 μL) into the CeA significantly lowered mechanical withdrawal thresholds on the right hind paw and further decreased the thresholds on the left hind paw compared to vehicle (Veh) treatment. Bar histograms show mean ± SEM. $^{\#\#}$, $^{\#\#\#}p < 0.01$, 0.001 compared to baseline (pre-SNL surgery). *, $p < 0.05$, compared to vehicle, two-way ANOVA with Bonferroni's multiple comparisons. Intra-CeA administration of FCA (100 μM, 1 μL) significantly decreased the withdrawal thresholds measured by compression **(B)** of the left (injured) and right paws and increased the audible vocalizations **(C)** evoked by noxious, but not innocuous, stimulation of the left (injured) and right hind paws, but had no significant effect on the ultrasonic vocalizations **(D)** though a trend for a facilitatory effect was observed for the noxious compression of the left (injured) hind paw. Injection of FCA (100 μM, 1 μL) into the CeA showed a non-significant trend to facilitatory effects on the facial grimace scale **(E,G)** and anxiety-like behaviors measured in the open field test **(H–J)** compared to vehicle. Bar histograms show mean ± SEM. *, **, ***$p < 0.05$, 0.01, 0.001 compared to vehicle, unpaired student t-tests. (A-G) Veh, n = 8; FCA, n = 8; (H-L) Veh, n = 7; FCA, n = 7.

(row) factor, $F_{(6, 91)} = 5.886$, $p < 0.001$; treatment (column) factor, $F_{(1, 91)} = 0.3429$, $p = 0.5596$; interaction, $F_{(6, 91)} = 0.1287$, $p = 0.9924$; Figure 3C, current (row) factor, $F_{(6, 91)} = 4.860$, $p < 0.001$; treatment (column) factor, $F_{(1, 91)} = 0.7441$, $p = 0.3906$; interaction, $F_{(6, 91)} = 0.1737$, $p = 0.9833$, two-way ANOVA; Figure 3D, $p = 0.6040$, t = 0.5315; Figure 3F, $p = 0.2161$, t = 1.300, unpaired t-tests). FCA also had no effect on glutamate-driven IPSCs evoked by the electrical stimulation of PB input (Figures 3G–J) onto CeLC neurons, suggesting that astrocytes were not involved in synaptic transmission at the PB-CeLC synapse (ACSF, n = 7; FCA, n = 8; Figure 3H, current (row) factor, $F_{(6, 91)} = 4.883$, $p < 0.001$; treatment (column) factor, $F_{(1, 91)} = 0.0467$, $p = 0.8294$; interaction, $F_{(6, 91)} = 0.0092$, $p > 0.999$; Figure 3I, current (row) factor, $F_{(6, 91)} = 3.475$, $p < 0.01$; treatment (column) factor, $F_{(1, 91)} = 0.2246$, $p = 0.6367$; interaction, $F_{(6, 91)} = 0.0430$, $p = 0.9997$, two-way ANOVA; Figure 3J, $p = 0.6892$, t = 0.4090, unpaired t-tests). No significant change was observed in the excitation/inhibition ratio (Figures 3K, L) (ACSF, n = 7; FCA, n = 8; Figure 3K, $p = 0.9113$, t = 0.1136; Figure 3L, $p = 0.9573$, t = 0.0545, unpaired t-tests).

3.3 Behavioral effects of astrocyte inhibition at the chronic stage of neuropathic pain

To determine the behavioral consequences of astrocyte inhibition in the amygdala, pain-like behaviors were measured after FCA (100 μM, 1 μL) or vehicle injection into the CeA of SNL rats. See 3.2 for the rationale to focus on the chronic stage (4-week time point). SNL surgery significantly increased mechanosensitivity on the left (injured) hind paw in the von Frey assay (Figure 4A) ($p < 0.001$ compared to baseline, two-way ANOVA with Bonferroni's posthoc tests), confirming the neuropathic pain condition. FCA delivered into the CeA (see 2.7.1) significantly decreased the mechanical withdrawal thresholds on the right hind paw and decreased the thresholds on the left hind paw even further compared to the vehicle-treated group in SNL rats (Figure 4A; $p < 0.05$ compared to vehicle, two-way ANOVA with Bonferroni's posthoc tests). Significant effects were observed after SNL induction or FCA treatment (Figure 4A, vehicle = 8, FCA = 8, left paw, treatment (column) factor, $F_{(1, 14)} = 0.04457$, $p = 0.8358$; time (row) factor, $F_{(2, 28)} = 79.23$, $p < 0.001$; interaction, $F_{(2, 28)} = 2.785$, $p = 0.0789$; right paw, treatment (column) factor, $F_{(1, 14)} = 4.016$, $p = 0.0648$, time (row) factor, $F_{(2, 28)} = 13.89$, $p < 0.001$; interaction, $F_{(2, 28)} = 2.166$, $p = 0.1334$, two-way ANOVA with Bonferroni's posthoc tests). FCA administered into the right CeA of neuropathic rats significantly decreased the withdrawal thresholds measured by compression (Figure 4B) of the left (injured) paw ($p < 0.01$, t = 3.176, unpaired t-tests) and right paw ($p < 0.001$, t = 4.571, unpaired t-tests). The data suggest that astrocyte inhibition in the amygdala exacerbates hypersensitivity in chronic pain.

FCA also increased audible vocalizations (Figure 4C) evoked by noxious, but not (normally) innocuous, stimulation of the left (injured) paw (innocuous, $p = 0.1779$, t = 1.419; noxious, $p < 0.05$, t = 2.215, unpaired t-tests) and right paw (innocuous, $p = 0.1056$, t = 1.730; noxious, $p < 0.01$, t = 3.586, unpaired t-tests) paw, but had no effect on ultrasonic vocalizations (Figure 4D, left paw, innocuous, $p = 0.3049$, t = 1.065; noxious, $p = 0.0550$, t = 2.094; right paw, innocuous, $p = 3{,}523$, t = 0.9622; noxious, $p = 0.4122$, t = 0.8453, unpaired t-tests) though a non-significant facilitatory effect of FCA was observed on ultrasonic vocalizations evoked by noxious compression of the left (injured) hind paw compared to vehicle treatment (vehicle, n = 8; FCA, n = 8). A non-significant trend for differences between the FCA or vehicle treated groups was observed on the facial grimace scale score (Figures 4E, G; vehicle, n = 8; FCA, n = 8; $p = 0.2260$, t = 1.267, unpaired t-tests) and on anxiety-like behaviors measured in the OFT (Figures 4H–J; vehicle, n = 7; FCA, n = 7; Figure 4H, $p = 0.1061$, t = 1.747; Figure 4I, $p = 0.9992$, t = 0.0010, unpaired t-tests). The data suggest that inhibition of astrocytes may affect evoked behaviors (reflexes and vocalizations) rather than spontaneous behaviors (grimace and open field tests).

3.4 Validation of astrocyte inhibition by FCA

Immunohistochemical analysis of GFAP and NeuN staining was used to validate the pharmacological approach. After the behavioral experiments, the animals were perfused and tissue collected for immunohistochemical analysis, which revealed that in the CeC and CeL, the area of GFAP (+) marker (Figures 5A, C), but not the mean of positive signal (Figures 5B, D), was significantly decreased after FCA (100 μM, 1 μL) microinjection compared to vehicle group in chronic SNL rats. Importantly, no differences in NeuN marker signal were observed between the two groups (Figures 5E–H), confirming the selective inhibition of astrocytes rather than neurons in the targeted region (vehicle, n = 5; FCA, n = 5 animals; Figure 5A, $p < 0.01$, t = 4.347; Figure 5B, $p = 0.1702$, t = 1.507; Figure 5C, $p < 0.001$, t = 7.074; Figure 5D, $p = 0.1581$, t = 1.557; Figure 5E, $p = 0.9566$, t = 0.05619; Figure 5F, $p = 0.5683$, t = 0.5949; Figure 5G, $p = 0.5247$, t = 0.6651; Figure 5H, $p = 0.5931$, t = 0.5565, unpaired t-tests). Analysis of GFAP and GS markers in the CeL area at high resolution (×60 oil immersion objective) confirmed the selectivity of the pharmacological approach (Supplementary Figure S1).

4 Discussion

This study addressed an important knowledge gap concerning the contribution of astrocyte signaling to amygdala functions in a model of chronic pain. The involvement of neuroimmune elements to pain processing has been shown extensively in preclinical research [for reviews (Tian et al., 2012; Salter and Stevens, 2017; Donnelly et al., 2020; Grace et al., 2021)] and most recently in clinical studies (Loggia et al., 2015; Albrecht et al., 2018; Albrecht et al., 2019; Albrecht et al., 2021), but mechanistic research was largely focused on peripheral and spinal nociceptive processing. Less is known about supraspinal neuroimmune signaling as a pain mechanism, and little if anything about the situation in the amygdala with regard to pain conditions. The key findings of this project are: 1) Increased reactive astrocytes in the amygdala were observed only at the chronic phase (4-week time point) of a neuropathic pain model and 2) selective pharmacological inhibition of the astrocytic population in the amygdala (CeA) had facilitatory electrophysiological and behavioral effects in the chronic pain condition. These results were somewhat unexpected based on existing literature pointing to beneficial effects of interventions aimed to silence glia at peripheral and spinal levels in pain conditions. It should be noted that research has largely focused on the modulation of specific glial factors, while we targeted the astrocytes themselves.

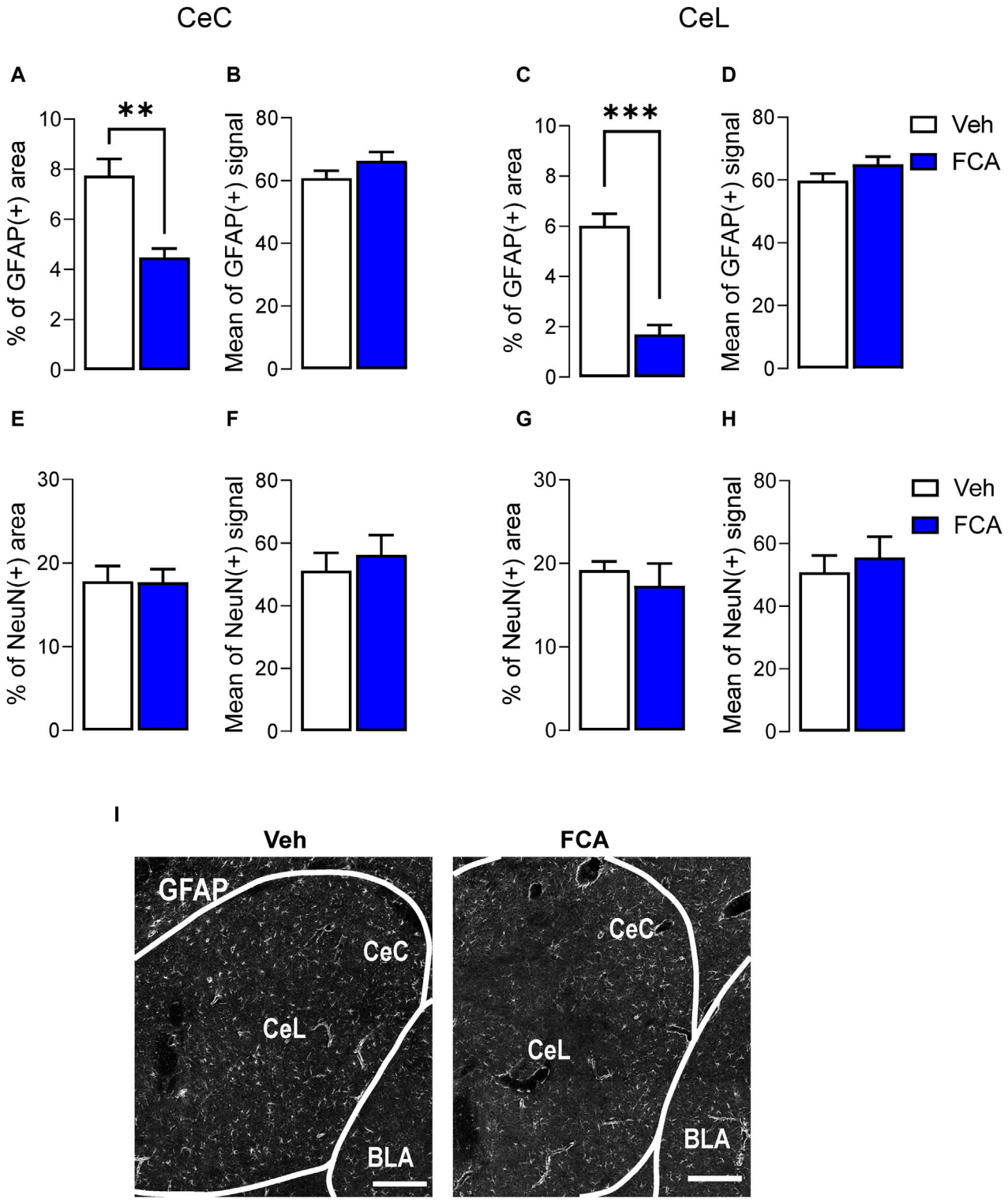

FIGURE 5
Validation of astrocyte inhibition by FCA in CeA in neuropathic rats. Immunohistochemical analysis showed decreased percentage of astrocytic GFAP positive area, but not mean of positive signal, in the CeC **(A,B)** and CeL **(C,D)** after FCA (100 μM, 1 μL) injection into the CeA of SNL (4 weeks) rats compared to vehicle (Veh) group, consistent with astrocyte inhibition in the targeted area. NeuN staining showed no significant differences between the two groups **(E–H)**, confirming the glia-specific effect of the pharmacological approach. Bar histograms show mean ± SEM. **, ***$p < 0.01$, 0.001 compared to vehicle, unpaired student t-tests. Veh, n = 5; FCA, n = 5 **(I)** Representative images of GFAP (+) staining in brain sections from Veh (left) and FCA (right) injected neuropathic rats. Scale bar = 200 μm.

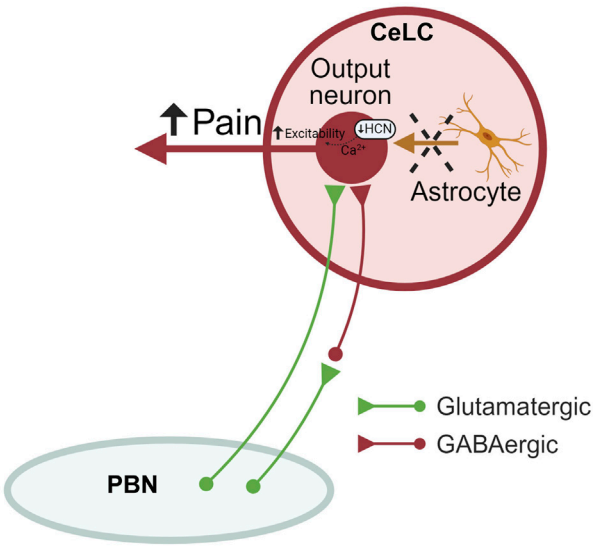

FIGURE 6
Astrocyte signaling in the amygdala (CeA) in neuropathic pain. Astrocytic silencing increases the output of CeLC neurons through mechanisms involving decreased HCN signaling, resulting in increased neuropathic pain-related behaviors without affecting synaptic transmission at the PB-CeLC synapse. Therefore, endogenous astrocyte signaling may sustain protective neuronal functions. CeLC, laterocapsular division of the central nucleus of amygdala; PBN, parabrachial nucleus; HCN, hyperpolarization-activated cyclic nucleotide–gated (HCN) channels. Created with BioRender.com.

Thus, the data suggest differences between amygdala and peripheral and spinal nociception and a unique role of astrocytes.

Evidence is as follows. Astrocyte activation was measured as increased GFAP(+) mRNA and protein levels in the CeA at the chronic (4 weeks) but not acute (1 week) stage (Figure 1), pointing to a delayed astrocytic response in the amygdala in neuropathic pain. Surprisingly, in patch-clamp experiments, selective inhibition of astrocyte metabolism achieved with the incubation of the brain slices with FCA enhanced excitability of CeA neurons through a mechanism that involved I_h (Figure 2) in the absence of synaptic effects (Figure 3), arguing against generalized non-specific inhibitory effects of FCA treatment. As a consequence, intra-CeA microinjection of FCA resulted in increased evoked vocalizations and decreased mechanical withdrawal thresholds (in the von Frey and paw compression tests), and non-significant facilitatory effects in the grimace test and anxiety-like behaviors (OFT) in rats 4 weeks after SNL induction (Figure 4), supporting the idea that amygdala astrocytes may serve beneficial protective functions in chronic pain (Figure 6). It remains to be determined if these differential behavioral effects reflect a beneficial role of astrocytes on sensory *versus* affective aspects or rather on evoked *versus* spontaneous behaviors. Importantly, GFAP (+) and GS (+), but not NeuN (+), staining decreased after FCA administration into CeA (Figure 5 and Supplementary Material), indicating that FCA selectively affected astrocytes but not neuronal functions in the amygdala.

The amygdala plays a critical role in the development and maintenance of pain and pain modulation. Several lines of research have focused on the modulation on neuronal factors to reduce uncontrolled amygdala activity as a desirable strategy to mitigate pain [for reviews (Thompson and Neugebauer, 2017; Thompson and Neugebauer, 2019; Neugebauer, 2020)]. The contribution of neuroinflammation at different levels of the pain neuroaxis, especially in supraspinal regions, is a more recent area of investigation. A recent study explored the effects of exogenous activation of astrocytes in the amygdala in a neuropathic pain model (Wahis et al., 2021), whereas our study is the first study to show the consequences of inhibition of astrocytic signaling in the amygdala, hence the role of endogenous astrocyte function, in pain processing and pain modulation.

Previous studies demonstrated that intrathecal injections of FCA had antinociceptive effects in zymosan- (Clark et al., 2007) or formalin- (Watkins et al., 1997) induced peripheral inflammatory, neuropathic (SNL) (Cao et al., 2014), and chronic post-ischemia (Tian et al., 2017) pain models. Similarly, intra-RVM FCA decreased carrageenan-induced thermal and mechanical hypersensitivity (Roberts et al., 2009) and ameliorated infraorbital tactile allodynia in a model of trigeminal neuropathy (chronic constriction injury to the unilateral infraorbital nerve) (Wei et al., 2008) in rats. Additionally, repeated injections of FCA into the PAG had inhibitory effects on mechanical allodynia in a model of diabetic neuropathic pain induced by systemic streptozotocin (Liu X. et al., 2022), suggesting that astrocyte activation engaged the descending facilitatory pain system. In contrast, our results point to a beneficial role of amygdala astrocytes in a chronic pain condition. This is more in line with data showing beneficial functions of astrocytes in the forebrain. In a model of focal cerebral ischemia (middle cerebral artery occlusion), repeated injections of FCA into the motor cortex reduced vascular remodeling of the ischemic area and induced neurological deficits in rats (Hayakawa et al., 2010) while intracerebral administration of FCA induced seizures events in anesthetized rats (Willoughby et al., 2003; Broberg et al., 2008).

The dual role of astrocytes in neuropathology is only beginning to emerge, and two different phenotypes of reactive astrocytes have been proposed. The A1 subtype seems to be induced by neuroinflammation and promotes cells death, while the A2 subtype, induced by ischemia, promotes tissue healing and repair (Liddelow et al., 2017). Reactive astrocytes have been associated with several disorders in preclinical research, including pain (Li et al., 2019), although the astrocytic subtype involved (A1 vs. A2) remains to be determined. Importantly, A1 astrocytes have been implicated in neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's disease, in clinical studies (Liddelow et al., 2017). If and how reactive astrocytes contribute to chronic pain mechanisms is not clear yet, perhaps because glial cells undergo a series of morphological and molecular changes related to time, region, microenvironment, and type of insult. The relative contribution of A1 and A2 subtypes to the findings of the present study remains to be determined. Additionally, the role of amygdala astrocytes in pain mechanisms is an open question considering that pain-behaviors and neuroplasticity persist in chronic pain condition, suggesting that astrocyte activation, even though "beneficial", is not sufficient to accomplish pain relief, but perhaps other non-neuronal elements such as microglia or oligodendrocytes drive maladaptive pain plasticity.

In support of the idea of a protective role of astrocytes in amygdala pain mechanisms, a recent study found that bilateral pharmacological or optogenetic activation of a subpopulation of astrocytes containing oxytocin receptors decreased anxiety-like behaviors in a neuropathic pain model whereas mechanical hypersensitivity was improved only by the pharmacological treatment; this effect was linked to increased inhibition of output neurons in the medial CeA, but it is unclear if

mechanistic analyses were performed in pain model. Although this study did not explore effects of astrocyte inhibition and did not account for hemispheric lateralization (Wahis et al., 2021), the results with exogenous activation of astrocytes complement our findings with inhibition of endogenous astrocyte signaling. Preclinical [for reviews (Thompson and Neugebauer, 2017; Neugebauer, 2020; Allen et al., 2021)] and most recently clinical evidence (Barroso et al., 2023) points to preferential pain-related lateralization to the right amygdala. The complexity of the neuroimmune system and the intricate bidirectional interactions between neuronal and non-neuronal cells pose challenges to the better understanding of the chronic pain processing and more effective therapeutic options available in the clinics.

Our electrophysiological data suggest inhibitory effects of astrocyte function on neuronal hyperexcitability in chronic neuropathic pain. Under physiological conditions, astrocytes sustain important neuronal functions throughout the central nervous system, including regulation of energy and blood flow, control of extracellular levels of K^+ or neurotransmitters (glutamate and GABA), and support of synaptic properties (Han et al., 2021; Valles et al., 2023). Astrocytes have been extensively implicated in inhibitory neurotransmission (especially GABA-A receptors) and K^+ or I_h currents (Bellot-Saez et al., 2017; Lezmy et al., 2021; Liu et al., 2021; Liu J. et al., 2022). We found effects of FCA on excitability and Ih currents, but not synaptic transmission, suggesting region- and model-specific functions. As a note of caution, the presence of Ba^{2+}, a well-known K^+ channel blocker (Rohaim et al., 2020), in the FCA solution used for incubation of the brain slices before recordings started, did not appear to confound the patch-clamp data, because FCA had significantly different effects on neuronal excitability compared to brain slices pre-treated with $BaCl_2$ (Figure 2B). Additionally, recordings were done in brain slices superfused with ACSF after incubation in FCA Ba^{2+} salt, and Ba^{2+} is known to wash out quickly (Sutor and Hablitz, 1993; Shi et al., 2000; Xiao et al., 2011). Accordingly, evidence from a previous study established that the effects of FCA observed on neuronal functions were not determined by residual Ba^{2+} in the bath (Wenker et al., 2010). The impairment of hyperpolarization-activated cyclic nucleotide-gated (HCN) cation channels could explain the observed effects of FCA on the depolarizing voltage sag, although this was not confirmed by pharmacological blockade in our experiments. Importantly, Ba^{2+} should not interfere with I_h (Ludwig et al., 1998). The lack of effects on synaptic transmission at the PB-CeLC synapse could be explained by the fact that amygdala neuroplasticity may not be sustained by synaptic plasticity in this pathway at the chronic pain stage.

Some limitations of this work should be considered. Although a strong sexual dimorphic influence of the neuroimmune system on pain-related mechanisms has been observed and represents an important line of research (Rosen et al., 2017; Presto et al., 2022), this study does not address sex specific differences in amygdala astrocyte function in pain. Moreover, the involvement of astrocytic signaling in amygdala pain functions was determined at the chronic but not acute stage of neuropathic pain, based on molecular and immunohistochemical evidence for changes in astrocyte activation at the chronic but not acute neuropathic pain stage (Figure 1). The identity of reactive astrocyte subtypes and the influence of astrocytic signaling on specific neuronal ion channels besides I_h currents also remain to be investigated. The current study provides the rationale for future studies into the role of astrocyte function in different brain regions and different stages of different pain models.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee (IACUC; protocol #21026) at Texas Tech University Health Sciences Center. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MM: Conceptualization, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing–original draft. OP: Investigation, Methodology, Visualization, Writing–review and editing. PP: Investigation, Methodology, Writing–review and editing. JJ: Investigation, Methodology, Writing–review and editing. VN: Writing–review and editing, Conceptualization, Funding acquisition, Project administration, Resources, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2024.1368634/full#supplementary-material

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