APPLICATION OF PLANT SECONDARY METABOLITES TO PAIN NEUROMODULATION

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PUBLISHED IN: Frontiers in Pharmacology and Frontiers in Neuroscience

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88966-476-4
DOI 10.3389/978-2-88966-476-4

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APPLICATION OF PLANT SECONDARY METABOLITES TO PAIN NEUROMODULATION

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Citation: Guimarães, A. G., Zengin, G., Singla, R. K., eds. (2021). Application of Plant Secondary Metabolites to Pain Neuromodulation. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88966-476-4

Table of Contents

frontiers in Pharmacology

EDITORIAL
published: 14 January 2021
doi: 10.3389/fphar.2020.623399

Editorial: Application of Plant Secondary Metabolites to Pain Neuromodulation

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Keywords: pain, nociception, natural products, secondary metabolites, phytochemicals, medicinal plants

Editorial on the Research Topic

Application of Plant Secondary Metabolites to Pain Neuromodulation

Pain is a highly unpleasant and intolerable condition, which is associated with multiple diseases and disorders, including but not limited to cancer, diabetes, infectious diseases, neurological and dysfunctional disorders, etc (Li et al., 2019; Yang, 2019; Singla et al., 2020). Further, tissue damage or chronic disease of the somatosensory nervous system cause neuropathic pain, which impacts and disturbs life to a higher extent (Pu et al., 2019; Finnerup et al., 2020). Natural resources especially plants have served and contributed several potential drugs for the alleviation and treatment of pain, either directly or in the derived form (Singla et al., 2018; Santos et al., 2019). For instance, the standard drugs like morphine, cannabidiol, acetylsalicylic acid are some of the key examples gifted by the nature. Even regular and healthy dietary food contains many phenolic compounds that elicit the potential to be anti-inflammatory (Laganà et al., 2019). Thus, in the present research topic, we further emphasized and collected the articles which fill the knowledge gap in this domain.

Brugnatelli et al. (2020) in their article "Irritable Bowel Syndrome: Manipulating the Endocannabinoid System as First-Line Treatment" described the role of the endocannabinoid system (ECS) in irritable bowel syndrome as ECS controls the gut homeostasis and explained how it is an efficient target to do the first-line treatment (Russo, 2016; Zhang et al., 2020). They briefed about the endocannabinoids like anandamide, 2-arachidonoyl glycerol, etc where the former was regulating the appetite and energy balancing while the latter was more involved in the general hunger signal (Di Marzo and Matias, 2005; Acharya et al., 2017). Further, they have also elaborated why menthol, an important phytoconstituent of peppermint oil is effective in IBS treatment.

Uddin et al. (2020) have reviewed the potential of flavonoids for the treatment of neuropathic pain and covered it in their article "Exploring the Promise of Flavonoids to Combat Neuropathic Pain: From Molecular Mechanisms to Therapeutic Implications". They have comprehensively reviewed and documented how various flavonoids carries the potential to decrease and alleviate various neuropathic pain like that of diabetic neuropathy, chemotherapy-induced peripheral neuropathy, spared nerve injury, thermal hyperalgesia, sciatic nerve ligation-induced neuropathic pain, and sciatic nerve chronic constriction injury. They cited that flavonoids are multimodal and act by different mechanisms viz. inhibiting the reduction of antioxidant defense, decreasing oxidative stress, inhibiting PARP over-activation, inhibiting cellular injury and mitochondrial dysfunction processes, and inhibiting glial cells activation and neuroinflammation.

Jin et al. (2020) in their research article "Lipoxin A4 Inhibits NLRP3 Inflammasome Activation in Rats With Non-compressive Disc Herniation Through the JNK1/Beclin-1/PI3KC3 Pathway" have

Edited and reviewed by:
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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *30 October 2020*
Accepted: *07 December 2020*
Published: *14 January 2021*

Citation:
Singla RK, Guimarães AG and Zengin G (2021) Editorial: Application of Plant Secondary Metabolites to Pain Neuromodulation. Front. Pharmacol. 11:623399. doi: 10.3389/fphar.2020.623399

studied the lipoxin modulated molecular mechanisms associated with inflammation in female Sprague-Dawley rats having non-compressive disc herniation. The test analog of lipoxin, LXA4 was compared with standard LY294002 (phosphoinositide-3 kinase (PI3K) inhibitor) alone or in combination with the test drug. Results indicated that LXA4 was a potential agent leading to an increase in the pain threshold, decrease in the proinflammatory cytokines like TNF-α, IL-1β, and IL-18, while increasing the anti-inflammatory mediators like IL-4, IL-10, and TGF-β as well as autophagy-related proteins like MAP1LC3B, Beclin-1, and PI3KC3.

Boccella et al. in their research article "Treatment With 2-Pentadecyl-2-Oxazoline Restores Mild Traumatic Brain Injury-Induced Sensorial and Neuropsychiatric Dysfunctions" evaluated the effect of 2-pentadecyl-2-oxazoline (PEA-OXA) which is a natural product, on the mild traumatic brain injury (mTBI) induced in the male C57BL/6J mice. PEA-OXA was found to be the adrenergic α-2 antagonist, with the potential to restore and reverse all the effects of mTBI i.e. behavioral changes like depression, the cortical GABA levels, and neuronal activity.

Chia et al. in their research article "Zerumbone Modulates α2A-Adrenergic, TRPV1, and NMDA NR2B Receptors Plasticity in CCI-Induced Neuropathic Pain *In Vivo* and LPS-Induced SH-SY5Y Neuroblastoma *In Vitro* Models", have studied the neuropathic pain-alleviating effects of zerumbone which is a bioactive compound obtained from the rhizome of *Zingiber zerumbet* (family Zingiberaceae). They reported that zerumbone is a multimodal molecule that elicits its anti-allodynic and antihyperalgesic effects by acting on various receptors viz. TRPV1, NMDA, α-1 adrenoreceptor, α-2 adrenoreceptor, β-1 adrenoreceptor, β-2 adrenoreceptor, and NR2B. They previously documented and validated the zerumbone involvement in the serotonergic system also.

Argueta et al. in their article "A Balanced Approach for Cannabidiol Use in Chronic Pain" briefly documented the use of cannabidiol in the treatment of chronic pain. Cannabidiol, contrary to the tetrahydrocannabinol which was another major metabolite of Cannabis sativa, is a non-psychostimulant molecule and evidently recorded its potential use in intractable chronic pain. As the cannabidiol also possessed teratogenic effects proven through the preclinical studies as well as devoid of any long-term studies, authors recommended that the public should use a balanced approach while dealing with cannabidiol and should avoid any sort of drug abuse.

Assis et al. in their article "Antinociceptive Activity of Chemical Components of Essential Oils That Involves Docking Studies: A Review", have systemically reviewed the computational studies which had been conducted on essential oil's chemicals for their detailed analysis of antinociceptive potential. The data was extracted from Science Direct and PubMed. They have categorized the various antinociceptive chemicals, software used for evaluation, as well as the molecular targets and their interacting amino acids for eliciting the antinociceptive potential.

Uddin et al. have contributed another review article "Emerging Promise of Cannabinoids for the Management of Pain and Associated Neuropathological Alterations in Alzheimer's Disease" where they have comprehensively reviewed the potential and applications of various cannabinoids for pain management especially in case of Alzheimer's disease (AD). They cited in their article how pain via cascade pathways initiated at the locus coeruleus-noradrenaline system can lead to neuronal death and Alzheimer's disease. Further, they have well elaborated on how cannabinoids are functional in tackling various pathological conditions of AD.

Muñoz-Montesino et al. in their research article "Inhibition of the Glycine Receptor alpha 3 Function by Colchicine" have studied the potential inhibitory effect of colchicine and its mechanism involved while inhibiting the ion channel, α3 subunit based glycine receptor (α3GlyRs) which is primarily involved in the chronic inflammatory pain. They found that the orthosteric site of the α3GlyR's closed state is the main binding site for this colchicine which was found to be the competitive antagonist for the target receptor.

Alberto et al. in the article "Molecular Modeling Applied to the Discovery of New Lead Compounds for P2 Receptors Based on Natural Sources" have comprehensively covered various natural products against P2Y and P2X classes of P2 purinergic receptors as well as elaboratively explained the potential role of various *in silico* tools in achieving the goals.

Singla et al. in their research article "Regulation of Pain Genes-Capsaicin vs Resiniferatoxin: Reassessment of Transcriptomic Data", have bioinformatically reassessed the transcriptomic data covering the gene regulatory information of two natural products, capsaicin and resiniferatoxin which was earlier published by Isensee et al., (2014). They have found that resiniferatoxin was regulating more non-pain associated genes as compared to capsaicin when the filtering of the genes was done by two pain gene databases.

This research topic, thus covered one brief research report, one mini-review, one opinion, four original research, three reviews, and one systematic review article. In conclusion, it is indeed very clear that plant secondary metabolites are highly efficient in neuromodulating pain via multimodal pathways. Exhaustive exploration in the next step can lead to the development of more potent drugs with the least or minimal side effects.

AUTHORS CONTRIBUTIONS

RS, AG, and GZ have collectively conceived and wrote the text. 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.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 25 February 2020
doi: 10.3389/fphar.2020.00091

Treatment With 2-Pentadecyl-2-Oxazoline Restores Mild Traumatic Brain Injury-Induced Sensorial and Neuropsychiatric Dysfunctions

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: 16 September 2019
Accepted: 27 January 2020
Published: 25 February 2020

Citation:
Boccella S, Iannotta M, Cristiano C, Iannotti FA, Bello FD, Guida F, Belardo C, Infantino R, Ricciardi F, Giannella M, Calignano A, Di Marzo V, Maione S and Luongo L (2020) Treatment With 2-Pentadecyl-2-Oxazoline Restores Mild Traumatic Brain Injury-Induced Sensorial and Neuropsychiatric Dysfunctions. Front. Pharmacol. 11:91. doi: 10.3389/fphar.2020.00091

Traumatic brain injury (TBI) represents an important public health problem and is followed by neuroinflammation and neurological dysfunctions. It has been suggested that brain trauma is often associated to deep behavioral alterations and chronic pain-like syndrome. Despite inducing minimal brain damage, mild TBI (mTBI) leads to persistent behavioral changes, including anxiety, depression, social interaction impairment, and aggressiveness. The clinical management of these symptoms is still unsatisfactory and new pharmacological treatments are needed, especially for the aggressiveness and depression. In a mouse model of mTBI, we investigated the effect of 2-Pentadecyl-2-Oxazoline (PEA-OXA), a natural compound, that is a secondary metabolite, found in green and roasted coffee beans, on both the pain perception, and neuropsychiatric dysfunctions. We found that the compound acts as a α2 adrenergic antagonist and this mechanism is here described for the first time. Mild TBI mice, starting from 14-d post-trauma, developed anxious and aggressive behavior, whilst depressive-like behavior and impaired social interactions were observed from the 60th d onward. PEA-OXA normalized all the behavioral changes investigated. We also investigated the memory impairments through Morris Water Maze (MWM) test. Both sham and mTBI mice treated with PEA-OXA showed amelioration in the reversal task of the MWM. Nevertheless, the main symptom of the long-term mTBI is represented by the depressive-like behavior, which was completely reversed by PEA-OXA repeated administration. In humans, mTBI-induced depression precedes the appearance of dementias and is characterized by a massive deficit of GABAergic transmission in the cortices. We found that PEA-OXA normalized the GABA changes in the prefrontal cortex. In order to prove the α2-mediated effect of the PEA-OXA we have performed open field test in naïve animals by microinjecting into the medial prefrontal cortex the dexomedetomidine, a selective α2 agonist with or without PEA-OXA co-injection. We found that PEA-OXA antagonized the α2 agonist effect on the locomotor activity. Moreover, PEA-OXA microinjection into the medial prefrontal cortex

induced an enhancement of dopamine release. Collectively, these data suggest that this natural compound, through its multi-target activity is able to: i) ameliorate behavioral alterations (i.e. depression), ii) selectively normalize cortical GABA levels, iii) rescue the impaired neuronal activity in the prefrontal cortex.

Keywords: traumatic brain injury, behaviour, electrophyiology, pain, plant metabolite

INTRODUCTION

Traumatic brain injury (TBI) is an important public health problem. It may be associated to several neurological dysfunctions, inflammatory processes, and cell death (Arciniegas, 2011). Brain trauma is divided into two phases: an early injury and a secondary late reaction. For every 100,000 people in the population, about 500 people per year present to an emergency department with mild traumatic brain injury (mTBI)/concussion (Bazarian et al., 2005). Several animal models of TBI have been suggested (Shultz et al., 2016). It seems that neuro-inflammatory and pro-apoptotic processes occur in the early phase of mTBI (Zetterberg et al., 2013), whereas plastic phenomena, responsible for the change in neuronal activity, become evident in the late phase. The involvement of peripheral immune cells, such as mast cells and T-cells, has also been reported (Kelso and Gendelman, 2014; Corrigan et al., 2016). The secondary behaviors are associated with changes in brain activity, in particular in the medial prefrontal cortex (*m*PFC). The *m*PFC is also thought to play a key role in forebrain chronic pain-(Giordano et al., 2012; Luongo et al., 2013) and pain-associated behaviors (i.e., anxiety, depression, and cognitive impairments), which are often present as comorbidities of chronic degenerative diseases (Neugebauer et al., 2009). Although knowledge of the mechanisms involved in concussion is improving, the treatments are still unsatisfactory and new pharmacological tools are needed (Loane and Faden, 2010). We previously demonstrated that some of the central *sequelae* of mTBI could be treated, at least in part, with *N*-palmitoylethanolamide (PEA), a natural endogenous compound belonging to the fatty acid ethanolamide (FAE) family. However, in our hands PEA failed to counteract repetitive/anxious behaviours and had no effect on neuronal activity during the late phase of mTBI (Guida et al., 2017).

Several neurotransmitters and neuromodulators are involved in the pathophysiology of chronic conditions such as neuropathic pain (Yang and Chang, 2019), degenerative diseases (Kandimalla et al., 2018), and trauma (Guida et al., 2017). Recent evidence highlights a common soil for these pathologies, especially for the mechanisms responsible for the psychiatric component (Sandu et al., 2015). In the present study, we aimed to characterize the behavioral and electrophysiological phenotype of mTBI mice 60 days after injury, and to investigate the effect of a recently identified natural compound, i.e. 2-pentadecyl-2-oxazoline (PEA-OXA). This compound has been suggested to be anti-inflammatory in preclinical models of inflammation (Impellizzeri et al., 2016a; Petrosino et al., 2017) and to exert neuroprotective effect in an experimental model of Parkinson disease (Cordaro et al., 2018). Moreover, neuroprotective effects of PEA-OXA have been recently demonstrated in different animal models of ischemic brain damage (Impellizzeri et al., 2019; Fusco et al., 2019).

The mechanism through which PEA-OXA exerts its pharmacological effects is still poorly understood and seems to be different from that of its analogue, PEA. It has been suggested that PEA-OXA exerts an indirect effect on the endocannabinoid system as well as a neuroprotective effect through the modulation of the nuclear factor E2-related factor 2 pathways (Cordaro et al., 2018). Based on our previous study on mTBI (Guida et al., 2017), we have investigated the possible beneficial effects of chronic treatment with PEA-OXA on the behavioral, biochemical, and electrophysiological changes associated with concussion. Moreover, we also highlighted in this study a possible new mechanism of action of the compound in view of the possible involvement of neurotransmitters such as catecholamines. Therefore, even though the present study is based on the same experimental model and we performed several matched experimental procedures of our previous paper (Guida et al., 2017), here we tested a different compound, which is also a secondary plant metabolite of the green coffee, with a completely different pharmacodynamic profile that we identify for the first time.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice (Charles River, Italy) weighing 18–20 g were housed three per cage under controlled illumination (12 h light/dark cycle; light on 6:00 A.M.) and standard environmental conditions (ambient temperature 20–22° C, humidity 55%–60%) for at least 1 week before the commencement of experiments. Mice chow and tap water were available ad libitum. The Animal Ethics Committee of the University of Campania "L. Vanvitelli" and University of Naples "Federico II, Naples, approved the experimental procedures. Animal care was in compliance with Italian (D.L. 116/92) and European Commission (O.J. of E.C. L358/1 18/12/86) regulations on the protection of laboratory animals. All efforts were made to reduce both animal numbers and suffering during the experiments.

Mild Traumatic Brain Injury (mTBI)

Experimental mTBI was performed using a weight-drop device developed in our laboratory. Mice were anesthetized with intraperitoneal injection of Tribromoethanol (250 mg/kg) and

placed in a prone position on a spongy support. After a midline longitudinal incision, the skull was exposed to locate the area of impact and placed under a metal tube device where the opening was positioned directly over the animal's head. The injury was induced by dropping a cylindrical metal weight (50 g), through a vertical metal guide tube from a height of 20 cm. The point of impact was between the anterior coronal suture (bregma) and posterior coronal suture (lambda). Immediately following injury, the skin was closed with surgical wound clips and mice were placed back in their cages to allow for recovery from the anesthesia and mTBI. Sham mice were submitted to the same procedure as described for mTBI, but without release of the weight.

Drugs

2-Pentadecyl-2-Oxazoline (PEA-OXA) and ultra-micronized (0.8–6.0 μm) Palmitoylethanolamide (PEA) were kindly provided by EPITECH Group SpA, Saccolongo (PD). PEA-OXA and PEA were dissolved in Kolliphor, used as vehicle, that was purchased by Sigma-Aldrich (Milan, Italy). PEA-OXA (10 mg/Kg i.p.), PEA or vehicle were systemically administered starting from the day after mTBI (day 1) induction until the end of experimental evaluations (day 60). For microdialysis experiments, a single injection of PEA-OXA or vehicle was orally or intraperitoneally performed at 10 and 20 mg/kg. For open field test, single intra-mPFC microinjections of ACSF, PEA-OXA or Dexmedetomidine (DEX) were performed at 6nmol/0.3 ul. Dexmedetomidine was purchased by Vetoquinol Italia S.r.l.

Experimental Design

Time points of evaluations were based on our previous study (Guida et al., 2017). A total number of 80 mice were divided in four experimental groups: SHAM/veh, mTBI/veh, SHAM/PEA-OXA, and mTBI/PEA-OXA. The day after mTBI induction, Behavioral tasks were performed at different time points and scheduled in order to avoid carry-over effects from prior testing experience. At the end of each set of experiments mice were sacrificed for further evaluations. The application of different pain stimuli -mechanical or thermal- was performed in separate groups of animals in order to avoid interferences in the nociceptive response. The timeline of mTBI induction, treatments, and behavioral and biochemical characterization is given in the **Figure 1A**.

Mechanical Allodynia

Mechanical allodynia was evaluated at 14 and 30 days after mTBI or sham surgery by the Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy), as described by Guida et al., 2017. Mice were allowed to move freely in one of the two compartments of the enclosure positioned on the metal mesh surface. Mice were adapted to the testing environment for 30 min before any measurement was taken. After that, the mechanical stimulus was delivered to the plantar surface of the hindpaw of the mouse from below the floor of the test chamber by an automated testing device. A steel rod (2 mm) was pushed with electronical ascending force (0–30 g in 10 s). When the animal withdrawn

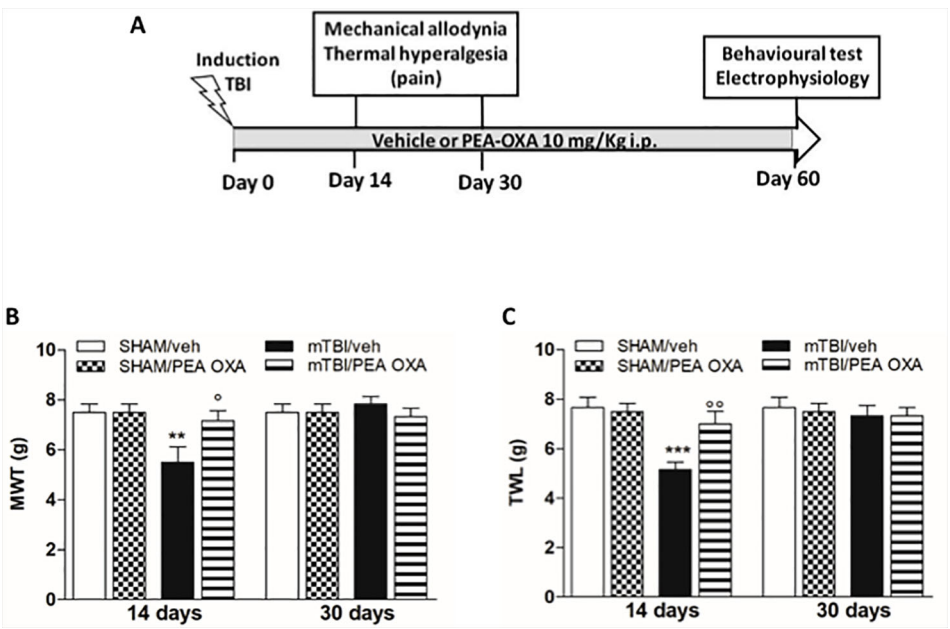

FIGURE 1 | Effects of repeated administration of vehicle (Kolliphor 5%) or PEA-OXA (10 mg/kg, i.p.) on pain behavioral evaluations in sham and mTBI mice. **(A)** Timeline of the experimental procedure of mild Traumatic Brain Injury (mTBI) induction and related behavioral and electrophysiological characterization in presence of vehicle or PEA-OXA treatment. **(B)** Mechanical Withdrawal Thresholds (MWT) measured through Dynamic Plantar Aesthesiometer, **(C)** Thermal Withdrawal Latency (TWL) measured through the plantar test. Data are represented as mean ± SEM of 6–8 mice per group. **P < 0.01 and ***P < 0.001 indicate significant differences compared to SHAM/veh. °P < 0.05 and °°P < 0.01 indicate significant differences compared to mTBI/veh. *P*< 0.05 was considered statistically significant. One-way ANOVA, followed by Bonferroni *post hoc* test was performed.

its hindpaw, the mechanical stimulus was automatically withdrawn and the force recorded to the nearest 0.1 g. Nociceptive responses for mechanical sensitivity were expressed as mechanical withdrawal thresholds (MWT) in grams. Each mouse served as its own control, the responses being measured both before and after surgical procedures. An observer blind to the treatment quantified MWT.

Thermal Hyperalgesia

Thermal hyperalgesia was evaluated at 14 and 30 days after mTBI or sham surgery by the Plantar Test Apparatus (Ugo Basile, Varese, Italy), as described by Guida et al., 2017. On the day of the experiment each mouse was placed in a plastic cage (22 cm × 17 cm × 14 cm; length × width × height) with a glass floor. After 30 min habituation period, the plantar surface of the hind paw was exposed to a beam of radiant heat through the glass floor. The radiant heat source consists of an infrared bulb (Osram halogen-bellaphot bulb; 8 V, 50 W). A photoelectric cell detected light reflected from the paw and turned off the lamp when paw movement interrupted the reflected light. Data were expressed as thermal withdrawal latency (TWL) in seconds and TWL was automatically displayed to the nearest 0.1 s; the cut-off time was 20 s in order to prevent tissue damage. Each mouse served as its own control, the responses being measured both before and after surgical procedures. An observer blind to the treatment quantified TWL.

Marble Burying Test

Obsessive-compulsive behaviour was evaluated through the Marble burying test as previously described by D'Agostino et al., 2015. Mice were individually placed in a cage (42 cm × 24 cm × 12 cm length x width x height) containing 5 cm layer of sawdust bedding and 20 glass marbles (1.5 cm in diameter) arranged in four rows. Mice were left undisturbed for 15 min under dim light. An observer blind to the treatment counted the time spent in digging behaviour and the number of marbles buried (at least two or third buried in the sawdust). At the end of the test the animal was removed to its own cage.

Tail Suspension Test

To assess the depression-like behaviour, tail suspension test was performed as previously described by Belardo et al., 2019. Mice were individually suspended 30 cm above the floor in a visually isolated area by adhesive tape placed approximately 4 cm from the tip of the tail. The duration of immobility, recorded in seconds, was recorded during the last 4 min of the 6-min test. Immobility time was defined as the absence of escape-oriented behaviour. Mice were considered to be immobile when they did not show any body movement, hung passively and completely motionless.

Elevated Plus Maze Test

The elevated plus maze test has been performed as described by Marcinkiewcz et al., 2016. It consisted of two opened and two enclosed arms of the same size with 15-cm-high transparent walls. The arms and central square were made of white plastic plates and were elevated 55 cm above the floor. Arms of the same type were located opposite from each other. Each mouse was placed in the central square of the maze (5 × 5 cm), facing one of the closed arms. Mouse behavior was recorded during a 5-min test period. The number of entries into an arm and the time spent in the open and enclosed arms were recorded using a camera capture and analysed using a video tracking software (Any-maze, Stoelting, Wood Dale, IL, USA). The number of entries into open arms, the number of entries into closed arms and total distance travelled (m) were analysed.

Light-Dark Box

Mice were tested to measure reckless behavior using light/dark box test, as described by Guida et al., 2017. The apparatus (60 cm × 30 cm × 30 cm; length × width × height) is divided into two equally sized compartments: the dark chamber was covered, whereas the light compartment was opened brightly illuminated (∼150 lux). Mice were placed in the light compartment and allowed to move freely between the two chambers for 10 min. The time spent in in each compartment, latency of first entry into the dark compartment and number of transitions were recorded.

Resident-Intruder Test

At day 60 after mTBI or sham surgery, mice were tested for aggressive behavior using a resident intruder test as previously described by by Guida et al., 2017. Mice were individually housed for 1 week in Plexiglas cages to establish a home territory and to increase the aggression of the resident experimental mice. To begin, food containers were removed and an intruder mouse of the same gender was placed in a resident home cage and resident–intruder interactions were analyzed for 10 min. The aggressive behavior of resident socially-isolated mice was characterized by an initial pattern of exploratory activity around the intruder, which was followed by rearing and tail rattle, accompanied in a few seconds by wrestling and/or a violent biting attack. The analysis of aggressive behavior was performed by evaluating the following parameters: 1) number of attacks: each attack is characterized usually by the combination of at least two aggressive behaviors, including biting, chasing, wrestling, or lunging 2) anogenital sniffing: cumulative count for the test mouse to investigate the tail/anogenital area of the companion mouse. 3) social interaction: total count for the test mouse to approach the companion mouse, starting from a body separation distance of at least 1 cm apart (about half of the body width),

Y-Maze Test

Spontaneous alternation is a measure of spatial working memory. Such short-term working memory was assessed by recording spontaneous alternation behavior during a single session in the Y-maze (made with three arms, 40 cm long, 120° C separate) positioned at exactly the same location for all procedures. Each mouse was placed at the end of one arm and allowed to move freely through the maze during a 5 min session. The series of arm entries were visually recorded. An arm choice was considered only when both fore paws and hind paws fully entered the arm. The Y-maze was cleaned after each test with 80% ethanol to minimize odor cues. Alternation was defined as a successive entrance onto the three different arms. The number of correct entrance sequences (e.g., ABC and BCA) was defined as

the number of actual alternations. The number of total possible alternations was therefore the total number of arm entries minus two, and the percentage of alternation was calculated as actual alternations/total alternations × 100 (D'Agostino et al., 2012).

Morris Water Maze

Morris water maze test has been performed as described by D'Agostino et al., 2015. It consists of a circular water tank (diameter 170 cm, height 60cm). The water temperature of 24 ± 1°C, light intensity and external visual cues in the room were rigorously reproduced. A circular clear Plexiglas escape platform (10 cm in diameter), was submerged 1.5 cm below the water surface. Swimming was recorded using a camera capture and analysed using a video tracking software (Any-maze, Stoelting, Wood Dale, IL, USA) that divided the pool into four equal quadrants: NE, SE, SW, and NW. The escape platform was placed in the midpoint of the SW and the position remained stable during 5 days. Mice were trained daily for four trials per day for 5 days, with inter-trial interval of 15 min and the start position was pseudorandomized across trials. Each trial terminated as soon as the animal reach the platform with a cut-off of 60 s. Average of the four trials for each mouse and then the average for each group to give a single path length and escape latency expressed as Mean ± SEM, was calculated for each training day.

A probe test was performed 1 h after the last swim on day 5. The platform was removed from the tank and each animal was allowed a free 60 s swim. The time spent in the quadrant where the platform was previously placed, was determined. A higher percentage of time spent in the platform quadrant is interpreted as a higher level of memory retention. Following the probe trial, reversal training was conducted for a further 3 days. During reversal training, the escape platform was moved to the midpoint of the opposite quadrant (NE) and, as for the training phase, mice were allowed to swim to this new position for four trials per day. A second probe trial was conducted 1 h after the last swim on day 8. All tests were conducted in the morning.

Open Field Test (OFT)

Open field test has been set as described by Belardo et al., 2019. This test has been performed only in naïve mice in order to demonstrate a α2-mediated mechanism. The naïve mice were implanted with a further cannula into the mPFC (AP: +1.42 mm; L: 0.5 mm; V: 3 mm) for microinjecting of Dexmedetomidine (Dex) or PEA-OXA 6 nmol/0.3µl. Behavioral assays were performed 10 min after drugs injection. The apparatus was cleaned before each behavioral session by solution of 70% EtOH. Naïve mice were randomly assigned to a treatment group. Behaviors were recorded, stored, and analyzed using an automated behavioral tracking system (Smart v3.0, Panlab Harvard Apparatus). Mice were placed in an OFT arena (l × w × h: 25 cm × 25 cm), and ambulatory activity (total distance travelled in centimeter), were recorded for 20 min and analyzed.

In Vivo Single Unit Extracellular Recordings

Single unit extracellular recordings have been performed as described by Guida et al., 2017. Mice for electrophysiological recordings were anaesthetized with Tribromoethanol (250 mg/kg) and placed in a stereotaxic device (David Kopf Instruments, Tujunga, CA). Body temperature was maintained at 37° C with a temperature-controlled heating pad. For electrode implantation, the scalp was surgically incised and the hole was drilled in the skull overlying the site of recording, *m*PFC (AP: +1.54–1.78 mm from bregma, L: 0.3–0.5 from midline and V: -1.5–3 mm below dura) according to the coordinates from the atlas of Franklin and Paxinos (1997). Anaesthesia was maintained with a constant continuous infusion of propofol (5–10 mg/kg/h, i.v.). A glass-insulated tungsten filament electrode (3–5 MX) (FHC Frederick Haer & Co., ME) was stereotaxically lowered into the prelimbic cortex (PLC) in *m*PFC. The recorded signals were amplified and displayed on a digital storage oscilloscope to ensure that the unit under study was unambiguously discriminated throughout the experiment. Signals were processed by an interface CED 1401 (Cambridge Electronic Design Ltd., UK) and analysed through Spike2 software (CED, version 4) to create peristimulus rate histograms (PSTHs) online and to store and analyse digital records of single-unit activity off-line. Configuration, shape, and height of the recorded action potentials were monitored and recorded continuously. This study only included neurons with a regular spiking pattern and a spontaneous firing rate between 0.1 and 3.82 Hz that were classified as pyramidal neurons in rodents (Jung et al., 1997; Tierney et al., 2004; Floresco and Tse, 2007). Once a neuron was single out, the position of the microelectrode was adjusted to maximize the spike amplitude relative to background noise and mechanical stimuli were applied. Mechanical stimuli were applied to the hind paw (contralateral to the *m*PFC) by Von Frey filaments with bending force of 97.8 mN (noxious stimulation) for 2 s (Simone et al., 2008). From the PSTHs, we measured neuron spontaneous activity measured in spikes/sec, the duration of excitation (in seconds) as the period of the increased firing activity, which exceeds the average baseline value +2SDs and the frequency of the evoked excitatory responses. Moreover, the extracellular action potentials' (EAP) amplitude, indicating synaptic current was used for the evaluation of the efficacy of synaptic transmission (Fagni et al., 1987).

Microdialysis *In Vivo*

Microdialysis experiments were performed in awake and freely moving mice, as described by Belardo et al., 2019. In brief, mice were anaesthetized with Tribromoethanol (250 mg/kg) and stereotaxically implanted with concentric microdialysis probes into the *m*PFC using the coordinates: AP: +1.42 mm, L: 0.5 mm from bregma, and V: 3 mm below dura. Dialysis probes, were constructed with 25G (0.3 mm inner diameter, 0.5 mm outer diameter) stainless steel tubing (A-M Systems). Inlet and outlet cannulae (0.04 mm inner diameter, 0.14 mm outer diameter) consisted of fused silica tubing (Scientific Glass Engineering). The probe had a tubular dialysis membrane (Enka AG, Wuppertal, Germany) 1.3 mm in length. Following a recovery period of 24 h, dialysis was commenced with artificial cerebrospinal fluid (ACSF: KCl, 2.5; NaCl, 125; $MgCl_2$, 1.18; $CaCl_2$, 1.26, pH 7.2) perfused at a rate of 1 µl/min by a Harvard Apparatus infusion pump. Following a 60-min equilibration period, 12 consecutive

20–30 min dialysate samples were collected. After an initial 60 min equilibration period, dialysate samples were collected every 20–30min for 100–150 min to establish baseline release of L-glutamate (L-Glu), GABA and Dopamine (DA). At the end of the fifth dialysate sample, the drug (PEA-OXA 10-20 mg/kg or vehicle) was orally or intraperitoneally administered, and samples were collected for approximately 3 h. At the end of experiments, mice were anaesthetized and their brains perfused fixed *via* the left cardiac ventricle with heparinized paraformaldehyde saline (4%). Brains were dissected out and fixed in a 10% formaldehyde solution for 2 days. The brain was cut in 40-µm thick slices and observed under a light microscope to identify the probe locations. The concentrations of L-glutamate and GABA contained in the dialysate were analyzed using by HPLC coupled with fluorimetric detection method. The system comprised two Gilson pumps (model no.303), a C-18 reverse-phase column, and a Gilson fluorimetric detector (model no.121). Dialysates were pre-column derivatized with o-pthaldialdehyde-N-acetylcysteine (OPA-NAC) (10 µl dialysate + 5 µl OPA-NAC + 10 µl borate buffer 10%). The mobile phase consisted of two components: (A) 0.2 M Na2HPO4, 0.2 M citric acid and 20% methanol and (B) 90% acetonitrile. Gradient composition was determined using an Apple microcomputer installed with Gilson gradient management software. Mobile phase flow rate was maintained at 1.2 ml/min. Data were collected using a Dell Corporation PC system 310 interfaced to the detector *via* a Drew data-collection unit.

Concentration of dopamine was determined using HPLC equipment fitted with an electrochemical detector. The composition of the mobile phase was 0.15 mM NaH_2PO_4, 0.01 mM octyl sodium sulfate, 0.5 mM EDTA (pH 3.8 adjusted with chloride acid), and 12.5% methanol. The mobile phase was delivered (flow rate: 1 ml/min) by a model 590 pump (Waters) into an Ultrasphere 5 µm ODS column (4.6 mm × 7.5 cm; Beckman Ltd). The electrochemical detector was an BIORAD mod. 1640, set at a potential of 0.55 V versus an Ag/AgCl reference electrode. The limit of detection for dopamine was 2–3 fmol per sample injected with a signal-to-noise ratio of 2. Data were expressed as mean ± SEM of 12 samples for each mouse of the fmol and pmol in 10 µl of perfusate.

Cell Culture, Reagents, and Transfection

Fibroblast-like (COS-7) cells (ATCC CRL-1651) were grown in 24-mm plastic Petri dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, non-essential amino acids (0.1 mM), penicillin (50 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere at 95% O2/5% CO2 at 37°C. After plating, the cells were transfected on the next day with the plasmid encoding for the human adrenergic, α2B receptor (Origene Technologies, MD, USA; cat. RG220659) by use of Lipofectamine 2000 (Thermo Fisher, Milan, IT: cat. 11668027). A negative scramble control vector (Origene Technologies, MD, USA; cat. SKUGE100003) was used as control. The following day, the standard media was replaced with fresh DMEM containing G418 (Thermo Fisher, Milan, IT: cat. 10131027) to set up for the stable cell line selection. The sub-clones were generated using 0.6 ug/ml G418 in the media for one month. Sub-clones with the highest expression of the genes of interest were selected by quantitative PCR using the following primer sequences: a) human adrenergic alpha 2 receptor *forward:* GCCTCAACGACGAGACCTG *and reverse:* CCCAGCCCGTTTTCGGTAG and b) ribosomal protein S16 *forward:* TCGGACGCAAGAAGACAGC and reverse AGCAGCTTGTACTGTAGCGTG.

RNA Purification and Quantitative Real-Time PCR

Total RNA was isolated from cells by use of the Pure Link RNA Mini Kit (Cat. N.: 12183018A; Thermo Fisher Scientific, Milan, Italy) following the manufacturer's instruction, and then quantified by spectrophotometric analysis. The purified mRNA was reverse-transcribed by use of iScript reverse transcriptase enzyme (Cat. N.: 1708840; Biorad, Milan, Italy). Quantitative real-time PCR was carried out in CFX384 real-time PCR detection system (Bio-Rad, Milan, Italy) with specific primers (see **Supplementary Table 1**) using Advance Universal SYBR Green Supermix (Cat. N.: 1725270 Bio-Rad, Milan, Italy). Samples were amplified simultaneously in quadruplicate in one-assay run with a non-template control blank for each primer pair to control for contamination or primer-dimers formation, and the ct (cycle threshold) value for each experimental group was determined. The housekeeping genes (the ribosomal protein S16) have been used as an internal control to normalize the ct values using the $2^{\wedge -\Delta\Delta ct}$ formula (Iannotti et al., 2010).

Measurement of Cyclic AMP

Adenosine-3', 5'-cyclic monophosphate or cyclic AMP (cAMP) was measured in control and α2- COS transfected cells using to detect direct cyclic AMP immunoassay kit (Arbor Assays, Michigan, USA) following the manufacturer's instructions (Gosh et al., 2012).

Statistical Analysis

Data were represented as mean ± SEM. Behavioral data were analyzed by using One-way or Two-way ANOVA, followed by Bonferroni's multiple comparison. Dunnet's *post hoc* test was used as *post hoc* test in microdialysis analysis. P values < 0.05 were considered statistically significant. Statistical analysis was carried out using Prism/Graphpad (GraphPad Software, Inc.) software.

RESULTS

PEA-OXA Effects on Pain Behavior in mTBI Mice

A significant decrease of MWT and TWL was observed in vehicle-treated mTBI mice 14 days after trauma induction [MWT: 5.5 g ± 0.6, $F_{(7,40)}$ = 3.441, P = 0.0056; TWL: 5.2 s ± 0.3, $F_{(7,40)}$ =4.430, P = 0.0010] as compared to the sham group (MWT: 7.5 g ± 0.3; MWT; TWL: 7.7 s ± 0.4) (**Figures 1B, C**). No differences in pain threshold were observed between right and left paw (data not shown). Moreover, a complete physiological re-establishment of normal pain response was observed 30 days

after trauma induction (MWT: 7.8 g ± 0.3; TWL: 7.3 s ± 0.4) as compared to the sham mice (MWT: 7.5 g ± 0.3; TWL: 7.7 s ± 0.4) (**Figures 1B, C**). Repeated administration of PEA-OXA (10 mg/kg, i.p.) significantly reduced both the mechanical allodynia and thermal hyperalgesia in mTBI mice 14 days after trauma (MWT: 7.2 g ± 0.4; TWL: 7.0 s ± 0.5) as compared to the vehicle treated mice (MWT: 5.5 g ± 0.6; TWL: 5.2 s ± 0.3) (**Figures 1B, C**), as revealed by One-way ANOVA followed by Bonferroni *post hoc* test. No difference was observed between right and left paw with PEA-OXA treatment (data not shown). PEA-OXA administration in sham mice did not change the pain response as compared to sham/vehicle mice at 14 and 30 days post trauma (MWT: 7.5 g ± 0.3; TWL: 7.5 s ± 0.3 and MWT: 7.5 g ± 0.3; TWL: 7.5 s ± 0.3, respectively).

PEA-OXA Effects on Compulsive and Depressive-Like Behaviour in mTBI Mice

At day 60 post trauma, mTBI/veh mice showed a significant increase of number of buried marble and digging events [11.8 ± 0.4 and 323.3 ± 50.0, $F_{(3,28)}$ = 11.24, P = 0.0303 and $F_{(3,12)}$ = 12.52, P = 0.0005 respectively], as compared to the sham/veh group (4.1 ± 0.9 and 84.5 ± 13.9 respectively) (**Figures 2A, B**). The treatment with PEA-OXA (10 mg/kg, i.p.) significantly reduced both parameters in mTBI mice (6.5 ± 1.0 and 137.3 ± 30.9 respectively) without inducing any change in sham mice (4.6 ± 1.5 and 95.5 ± 17.4 respectively), as revealed by One-way ANOVA followed by Bonferroni *post hoc* test.

Moreover, mTBI/veh mice showed an increased immobility time in the tail suspension test, measured as the lack of escape-oriented activity (128.5 ± 13.9 s) as compared to the sham/veh mice (60.0 ± 8.2 s) (**Figure 2C**). This depressive-like behaviour was significantly reduced by PEA-OXA treatment [10 mg/kg, i.p.; 78.5 s ± 13.1, $F_{(3,12)}$ = 7.039, P= 0.0055] in mTBI mice, as revealed by One-way ANOVA followed by Bonferroni *post hoc* test. Sham mice treated with PEA-OXA did not show any change in immobility time as compared to vehicle treated mice (82.5 s ± 7.1) (**Figure 2C**).

PEA-OXA Effects on Recklessness and Aggressive-Like Behavior in mTBI Mice

During the elevated plus maze test, although no significant differences were observed in the number of open arms entries (**Figure 3A**), mTBI/veh mice showed a significant lower number of entries in the closed arms (9.7 ± 0.3) as compared to sham/veh group (14.8 ± 1.2), indicating an anxiolytic-like effect [$F_{(3,14)}$ =3.021, P=0.0652] (**Figure 3B**). This effect was not reverted by PEA-OXA in mTBI mice (10 mg/kg, i.p.). Moreover, no differences between groups were observed in the total distance travelled (**Figure 3 C**). In the same way, in the light/dark box test, mTBI mice displayed a significant increase in the latency to enter in the dark box (29.8 s ± 4.9) as compared to sham/vehicle mice (9.5 s ± 2.5), which we identified as recklessness-like behaviour (**Figure 3D**). In this case, PEA-OXA did reduce the latency to enter in the dark box in mTBI mice [9.8 s ± 3.3; $F_{(3,14)}$=6.963, P=0.0042]. Accordingly, mTBI/veh mice spent more time in the illuminated compartment of the light/dark box [196.8 s ± 7.6, $F_{(3,13)}$ = 5.412, P = 0.0123] as compared to sham/veh mice (128.3 s ± 23.1), also showing an increased number of transitions in the two compartments [47.0 ± 1.8; $F_{(3,13)}$ = 4.561, P = 0.0215] (**Figures 3E, F**) as compared to the control mice (28.2 ± 6.2). The treatment with PEA-OXA (10 mg/kg, i.p.) significantly decreased these effects (125.5 s ± 16.8, 27.7 ± 5.1) in mTBI mice (**Figure 3E, F**),

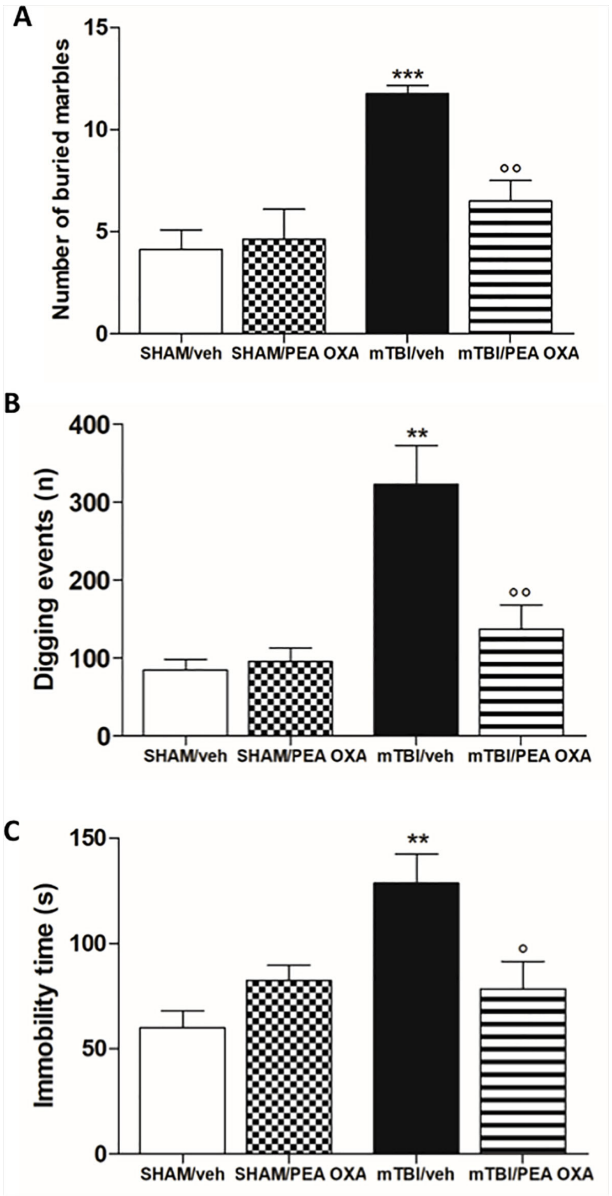

FIGURE 2 | Effects of repeated administration of vehicle (Kolliphor 5%) or PEA-OXA (10 mg/kg, i.p.) on behavioral evaluations in sham and mTBI mice. **(A**, **B)** Number of buried marbles and digging events in marble burying test, respectively **(C)** Duration of immobility in the tail suspension test. Data are represented as mean ± SEM of eight mice per group. **P < 0.01 and ***P < 0.001 indicate significant differences compared to SHAM/veh. °P < 0.05 and °°P < 0.01 indicate significant differences compared to TBI/veh. *P*< 0.05 was considered statistically significant. One-way ANOVA, followed by Bonferroni *post hoc* test was performed.

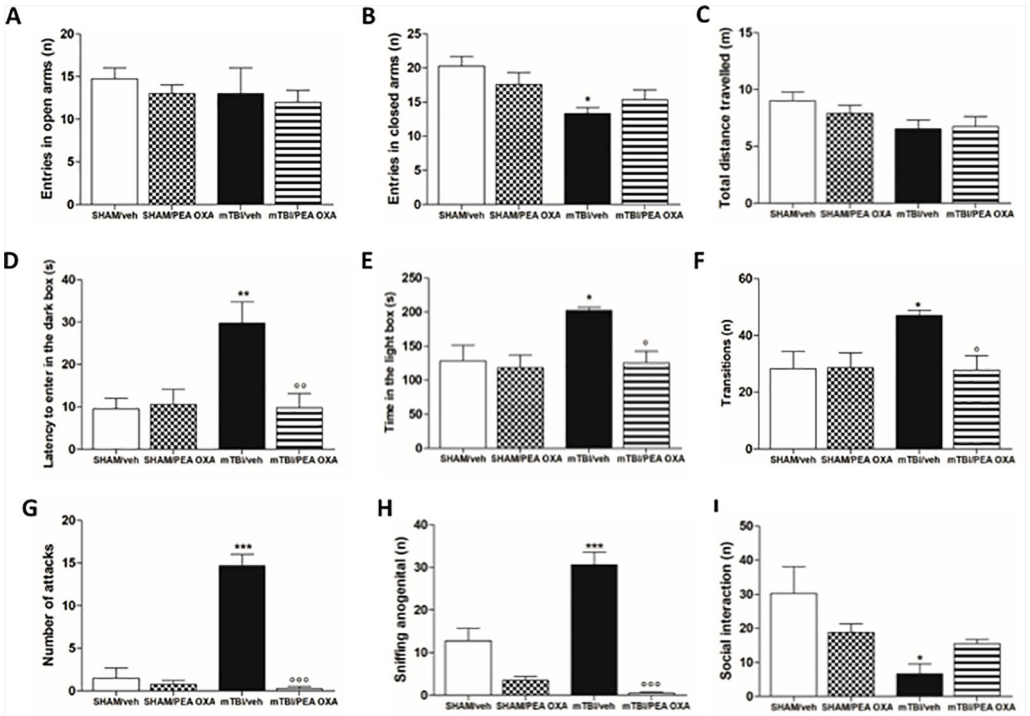

FIGURE 3 | Effects of repeated administration of vehicle (Kolliphor 5%) or PEA-OXA (10 mg/kg, i.p.) on behavioral evaluations in sham and mTBI mice. **(A–C)** Entries in open arms, entries in closed arms and total distance travelled, respectively in elevated plus maze, **(D–F)** latency to enter in the dark box, time in the light box and transition, respectively, in light-dark box test, **(G–I)** number of attacks, sniffing ano-genital and social interaction, respectively, in resident intruder test. Data are represented as mean ± SEM of six to eight mice per group. *P < 0.05, **P < 0.01 and ***P < 0.001 indicate significant differences compared to SHAM/veh. °P < 0.05, °°P < 0.01, and °°°P < 0.001 indicate significant differences compared to TBI/veh. *P*< 0.05 was considered statistically significant. One-way ANOVA, followed by Bonferroni *post hoc* test was performed.

as revealed by One-way ANOVA followed by Bonferroni *post hoc* test. Sham mice treated with PEA-OXA did not show any change in these paradigms as compared to the sham/veh mice (10.5 s ± 3.3; 118.3 s ± 18.7; and 28.5 s ± 5.4 respectively).

Moreover, mTBI/veh mice showed a significant increase in the number of attacks (14.7 ± 1.3) (1.5 ± 1.2) and ano-genital sniffing frequency (30.8 ± 2.9) during the resident intruder test, 60 days post trauma, as compared to the sham animals (**Figures 3G, H**). Additionally, they showed decreased number of social exploration (6.7 ± 2.9) as compared to the sham mice (30.2 ± 7.9) (**Figure 3I**). PEA-OXA administration (10 mg/kg, i.p.) reduced the number of attacks and ano-genital sniffing (0.3 ± 0.3, $F_{(3,11)} = 54.40$, $P < 0.0001$; 0.5 ± 0.3, $F_{(3,11)} = 40.70$, $P < 0.0001$, respectively) in mTBI mice, and increased the number of social interaction in mTBI mice, although One-way ANOVA followed by Bonferroni *post hoc* test analysis did not show a significant difference (15.5 ± 1.3) (**Figures 3G, H**). Finally, sham mice treated with PEA-OXA did not show any change in the number of attacks (3.5 ± 0.9), as compared to SHAM/veh mice (**Figure 3G**).

PEA-OXA Effects on Spatial and Working Memory in mTBI Mice

The effect of mTBI on spatial and working memory was examined by means of the Morris Water Maze and Y-maze tests. During the standard training trials, the escape latency (**Figure 4A**) and path length (**Figure 4B**) were longer in mTBI/veh mice especially on day 2 (37.1 s ± 2.9 and 7.3 ± 0.4, respectively) than that observed in sham/veh mice (17.3 s ± 0.7 and 2.8 s ± 0.1, respectively), and on day 3 in path length (4.9 s ± 0.4) as compared to sham/veh mice (2.3 s ± 0.4).

Conversely, mTBI mice treated with PEA OXA (10 mg/kg, i.p.) showed a significant decrease in the escape latency in mTBI/veh mice on day 2 (21.2 s ± 6.0, $P < 0.01$ and 2.8 ± 0.8, $P < 0.001$, respectively). Two-way ANOVA followed by Bonferroni post-hoc test revealed significant differences for time in both parameters [$F_{(7,105)} = 45.91$, $P < 0.0001$ and $F_{(7,105)} = 53.93$, $P < 0.0001$, respectively], for treatment x time interaction [$F_{(10,105)} = 2.30$, $P = 0.003$], but no significant differences were for treatments [$F_{(3,105)} = 7.51$, $P = 0.0027$ and $F_{(3,105)} = 11.63$, $P = 0.0003$, respectively] (**Figures 4A, B**). On day 6, we moved the platform in the opposite quadrant (NE) to analyse the time that mice spent in learning the new position. During the first day, sham (28.4s ± 1.4 and 3.2 ± 0.2, respectively) and mTBI mice treated with PEA-OXA (16.9 s ± 3.8 and 2.7 ± 0.3, respectively), were able to adapt their spatial strategies to find the new platform position very quickly, whereas mTBI/vehicle mice (42.7 s ± 1.8 and 5.7 ± 0.4, respectively) largely failed to deploy spatial strategies during this initial phase of reversal learning. After

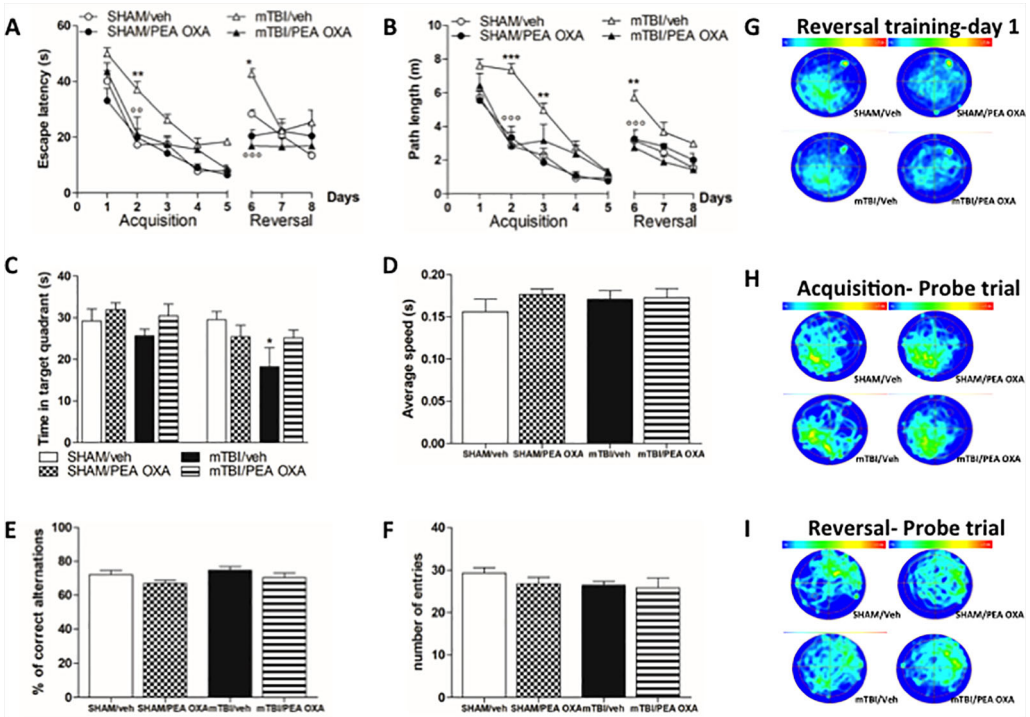

FIGURE 4 | Effects of repeated administration of vehicle (Kolliphor 5%) or PEA-OXA (10 mg/kg, i.p.) on spatial and working memory in sham and mTBI mice. **(A**, **B)** Escape latency and path length, respectively, across 9 days of MWM reference platform task, **(C**, **D)** Time spent in the target quadrant and average speed, respectively, during probe tests of MWM test, **(E**, **F)** % of correct alternations and number of entries, respectively, in Y-maze test, **(G–I)** Occupancy plots during repeated MWM training and probe tests. Data are represented as mean ± SEM of eight mice per group. *P < 0.05, **P < 0.01, and ***P < 0.001 indicate significant differences compared to SHAM/veh. °P < 0.05, °°P < 0.01, and °°°P < 0.001 indicate significant differences compared to TBI/veh. *P*< 0.05 was considered statistically significant. One-way or Two-way ANOVA, followed by Bonferroni *post hoc* test was performed.

3 days of training the mTBI/veh mice (25.3 s ± 4.4 and 2.9 ± 0.2, respectively) still spent more time to get the platform position as compared to sham group (13.4s ± 1.3 and 1.5 ± 0.4, respectively) even though the difference was not statistically significant. PEA-OXA administration (10 mg/kg, i.p.) in sham mice did not reveal any change in both phases (**Figures 4A, B, G**).

Probe trials were held to assess spatial reference memory during the acquisition and reversal phases and the percentage of time spent in the correct quadrant was measured (**Figure 4C**). Although there was a downward trend in the percent of time spent in the correct quadrant for the mTBI/veh mice (25.7 s ± 1.5) as compared to sham/vehicle mice (29.2 s ± 2.9), Bonferroni post-hoc test did not reveal significant differences between groups after the acquisition phase. Conversely, the probe after the reversal phase revealed a significant deficit in mTBI/veh [18.2 ± 4.6, $F_{(7,29)}$=2.477, P=0.0402] as compared to sham/veh mice (29.5 s ± 1.9), which was partially restored by PEA-OXA treatment in mTBI mice (25.2 s ± 1.9, t = 1.855). However, heat maps of probes conducted after acquisition (**Figure 4H**) and reversal (**Figure 4I**) trials illustrated that all groups of mice finally developed a focal search pattern, although sham and treated mice rapidly focused on the target quadrant, whereas mTBI mice displayed a more diffuse and confuse search pattern. Notably, swimming speed was not different between the groups indicating that mTBI did not affect swimming ability [$F_{(3,16)}$= 0.5678, P = 0.6442] (**Figure 4D**).

Finally, to determine whether mTBI led to working memory impairment, we performed Y-maze test. There were no differences in % of correct alternations during the spontaneous alternation in the Y-maze test [$F_{(3,16)}$ = 1.984, P = 0.1571] (**Figure 4E**) and in the number of arms entries [$F_{(3,16)}$ = 0.9421, P = 0.4435] (**Figure 4F**).

PEA-OXA Effect on *m*PFC(+) Neurons in mTBI Mice

As previously demonstrated (Guida et al., 2017), TBI mice, 60 days after induction of trauma (TBI/VEH), showed an important *m*PFC hypoactivation (**Figure 5**). In fact, a significant reduction of frequency [1.32 Hz ± 0.59, $F_{(3,21)}$ = 5.87, P = 0.0045] and of duration of evoked activity (0.77 s ± 0.07, $F_{(3,21)}$ = 3.09, P = 0.037) was observed, as revealed by One-way ANOVA followed by Bonferroni *post hoc* test (**Figures 5F, G**). Also, the spontaneous activity displayed a trend to reduction as compared to sham/Veh group (0.25 spikes/sec ± 0.03, $F_{(3,21)}$ = 1.88, P = 0.17) (**Figure 5E**). Repeated treatments with PEA-OXA (10 mg/kg, i.p.) did not affect either spontaneous (0.36 spikes/sec ± 0.05) or stimulus-evoked activity (5.0 Hz ± 0.24 for frequency and 2.12 s ± 0.37 for duration) of *m*PFC (+) neurons of sham mice

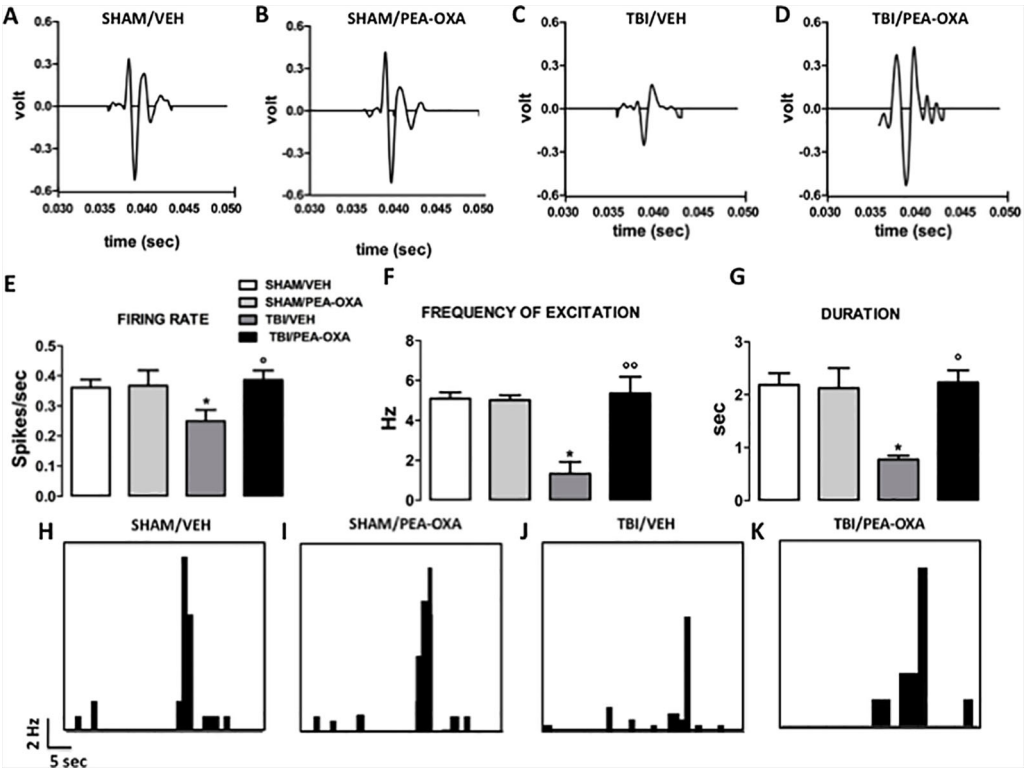

FIGURE 5 | Effects of repeated administration of vehicle (Kolliphor 5%) or PEA-OXA (10 mg/kg, i.p.) on spontaneous and mechanical stimulus-evoked excitation of single *m*PFC (+) neuron in SHAM and TBI mice 60 days after trauma **(A–D)** Representative action potential of single *m*PFC (+) neuron SHAM and TBI mice treated with vehicle or with PEA-OXA, **(E–G)** Mean of spontaneous activity, of the frequency and of the duration of excitation, respectively, in different groups of mice, **(H–K)**. Representative PSTHs of *m*PFC (+) neuron of SHAM and TBI mice 60 days post trauma treated for 60 days with vehicle or PEA-OXA. Data are represented as mean ± SEM of six to eight mice per group. *P < 0.05 indicate significant differences compared to SHAM/veh. °P < 0.05 and °°P < 0.01 indicate significant differences compared to TBI/veh. *P*< 0.05 was considered statistically. One-way ANOVA, followed by Bonferroni *post hoc* test was performed.

(**Figures 5E–G**). Instead, treatment with PEA-OXA (10 mg/Kg, i.p.) normalized the frequency (5.35 Hz ± 0.82) and the duration of excitation (2.23 s ± 0.22) in *m*PFC (+) neurons of mTBI mice. Finally, the same treatment increased spontaneous activity rate (0.38 spikes/sec ± 0.03) as compared to mTBI/veh mice (**Figures 5E, J, K**). These effects are also represented in PSTHs samples of single *m*PFC (+) neurons in SHAM and mTBI mice 60 days post trauma treated for 60 days with vehicle or PEA-OXA (**Figures 5H–K**).

PEA-OXA Effect on Amino Acid Release in *m*PFC of Naïve Mice

The effects of single injection of PEA-OXA (10 or 20 mg/kg *o.s.*) on L-Glu and GABA extracellular concentrations in the *m*PFC are shown in **Figures 6A, B**. Baseline levels of L-Glu and GABA were 7.0 pmol/10µl ± 0.6 and 2.3 pmol/10µl ±0.2 (data not show), respectively, in the *m*PFC of naïve mice. We found that acute administration of PEA-OXA, at either dose, did not significantly change L-Glu extracellular levels in the *mPFC* of Naïve mice [$F_{(7,16)}$=0.6018, p=0.7560 for 10 mg/kg; $F_{(2,7)}$=0.6934, p=0.4981 for 20 mg/kg] (**Figure 6A**). On the other hand, when PEA-OXA was administered at the highest dose (20 mg/kg), GABA levels significant increased in the *m*PFC (time 80 min: 508.4% ± 107.4 *vs* time 0: 100% ± 4.4, p=0,0036, $F_{(3,7)}$=14.52) as revealed by One-way ANOVA followed by Dunnett's *post hoc* test for multiple comparisons within groups. Instead, extracellular levels of GABA in the *m*PFC did not significantly change after a single injection of PEA-OXA at 10 mg/kg o.s. [$F_{(7,16)}$=1.487, p=0.2409] (**Figure 6B**).

In Vitro Results

To investigate for the potential regulation of adrenergic (α2B) receptors by PEA-OXA, we generated human α2B stably transfected COS cells. Next, we measured potential changes in the cyclic adenosine-3', 5'-cyclic monophosphate or cyclic AMP (cAMP), being these subclasses of receptors coupled to G_i protein dependent mechanisms. By means of an immune-enzymatic assay, we found that PEA-OXA (from 0.1 to 3 µm) did not interfere with the elevation of cAMP induced by NKH477, a water-soluble enhancer of cAMP. However, PEA-OXA (from 0.1 to 3 µM) counteracted the inhibitory effect on cyclic AMP of dexmedetomidine, a selective α2 agonist, suggesting that PEA-OXA could be considered as a novel antagonist of α2 adrenergic receptors (**Figure 7**). This was also

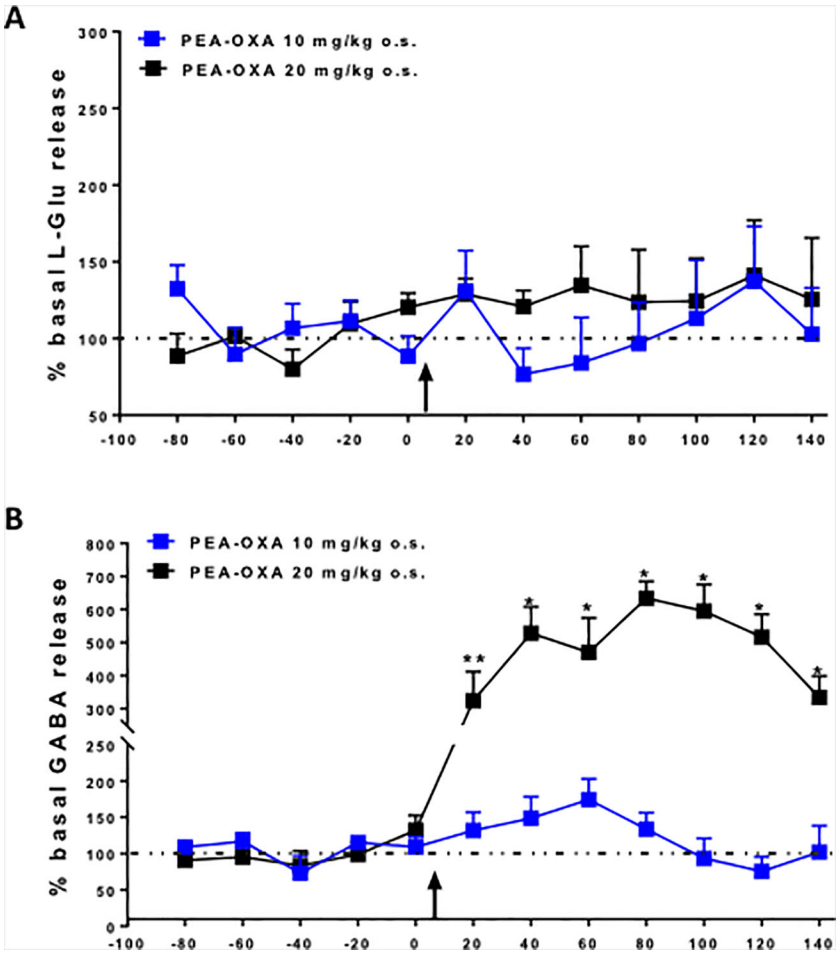

FIGURE 6 | Effects of single injection of PEA-OXA (10 and 20 mg/kg, o.s.) on L-glutamate **(A)** and GABA **(B)** extracellular levels in *m*PFC of Naïve mice. Each point represents the mean ± S.E.M of 3-4 animals per group. *P < 0.05 and **P < 0.01 indicate significant differences versus time 0. The black arrow indicates the administration of the drugs. P values < 0.05 were considered statistically significant. One-Way ANOVA, followed by Dunnett's *post hoc* test for multiple comparisons test was performed.

confirmed by the results of binding assays with PEA-OXA for α2 adrenergic receptors (See **Supplementary Materials**).

Single Administration of PEA-OXA in mPFC Ameliorated Low Motor Activity Deficits in Open-Field Exploration Induced by α_2-Agonist

In order to confirm the *in vitro* results and the binding assay we performed the open field task in freely moving naïve mice by microinjecting into the medial prefrontal cortex (mPFC) the selective α2-agonist dexmedetomidine alone or in combination with PEA-OXA (**Figure 8A**). In the open field test, Naïve mice microinjected with Dex 6nmol/0.3µl in mPFC showed a significant decrease in the travelled distance (1618 ± 330.7 cm, one way-ANOVA followed by Tukey's post-hoc test) compared with Naïve mice treated with ACSF [3237 ± 181.3 cm, $p < 0.0001$, $F_{(3,8)} = 62.24$].

By contrast, Naïve mice that received PEA-OXA 6nmol/0.3µl microinjection in mPFC showed a significant increase in the ambulatory activity (7827 ± 375 cm, one way-ANOVA followed by Tukey's post-hoc test) compared with Naïve mice that received ACSF [3237 ± 181.3 cm, $p < 0.0001$, $F_{(3,8)}=62.24$]. The co-injection of Dex and PEA-OXA, at the same concentration, produced a normalization in the travelled distance (3186 ± 423.2 cm, one way-ANOVA followed by Tukey's post-hoc test) compared with Naïve mice that received Dexmedetomidine alone [1618 ± 330.7 cm, $p < 0.0001$, $F_{(3,8)} = 62.24$], confirming the α_2-mediated effect of PEA-OXA (**Figures 8B, C**).

Effect of Single Administration of PEA-OXA on Dopamine Release in the mPFC of Naïve Mice

To evaluate a possible neurochemical substrate associated with the increased locomotor activity induced by PEA-OXA,

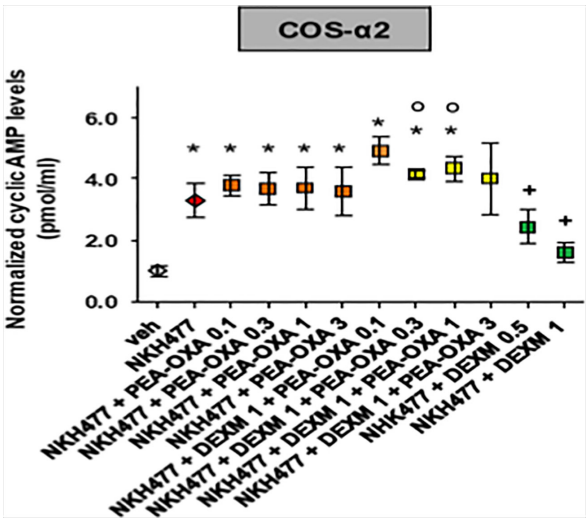

FIGURE 7 | Effect of PEA-OXA in COS cells stably expressing human adrenergic α2 receptors. Scatter plots showing the effect of PEA-OXA in COS cells expressing α2 receptors on intracellular AMPc levels. Data represent the mean ± S.E.M. of four/five separate determinations. Data sets were compared using one-way ANOVA followed by Bonferonni's test. *** p value ≤ 0.001 vs the indicated experimental groups; *p value ≤ 0.05 vs vehicle; ⁺p value ≤ 0.05 vs NKH+PEA-OXA; °p value ≤ 0.05 vs NKH+DEXM.

we have measured dopamine levels after PEA-OXA cortical microinjection, through microdialysis *in vivo*. The effects of single injection of PEA-OXA (20 mg/kg i.p.) on dopamine extracellular concentrations in the mPFC are shown in **Figure 8D**. Baseline levels of dopamine 8.92 pmol/10µl ± 0.58 in the mPFC of naïve mice. Two-way ANOVA revealed that the single injection of PEA-OXA significantly increased the dopamine levels in the mPFC, from 60 min until 140 min, in naïve mice compared to the vehicle treated mice [time 120: 340.87 ± 23.22% vs 108.20 ± 15.50%, $p < 0.0001$, $F_{(1,66)} = 37.16$, time x treatment: $p = 0.0031$, $F_{(10,66)} = 3.047$] (**Figure 8D**).

DISCUSSION

Mild traumatic brain injury (mTBI), or, more simply, concussion, is associated with several neuropsychiatric changes that are still neglected from a therapeutic management point of view. Safe pharmacological tools are needed for some of the concussion features related to the behavioral psychiatric changes such as aggressiveness, anxiety, obsessive compulsive like behaviors and depression, which are also reported in the DSM-V for post traumatic stress disorders (PTSDs). In the present study, we aimed at investigating whether pharmacological treatment with a new natural compound found in green and roasted coffee beans (Impellizzeri et al., 2016b), 2-pentadecyl-2-oxazoline (PEA-OXA), could be of any use for these comorbidities. Structurally related to palmitoylethanolamide (PEA), PEA-OXA shows both PEA-related and unrelated mechanisms of actions. Indeed, it has been demonstrated to produce anti-inflammatory effects mediated by inhibition of N-acylethanolamine hydrolyzing acid amidase (NAAA), the main enzyme responsible for the degradation of PEA, especially in white cell lineages such as macrophages and mast cells (Petrosino et al., 2017). Moreover, treatment with PEA-OXA normalized the otherwise reduced PEA and endocannabinoid levels found in the carrageenan-inflamed mouse paw, suggesting either a role of pro-drug for this molecule and/or indirect actions on the endocannabinoid system and its metabolic machinery (Petrosino et al., 2017). On the other hand, the anti-inflammatory effect of the drug was not abolished, as instead is observed for PEA, in PPARα null mice, suggesting additional molecular mechanisms at the basis of PEA-OXA pharmacological actions (Petrosino et al., 2017). In addition, alternative targets have also been proposed for this compound, involving the nuclear factor E_2-related pathway (Cordaro et al., 2018). Thus, the multi-target nature of PEA-OXA makes it very interesting for chronic multi-factorial diseases, also in light of its good tolerability and safety profile (Impellizzeri et al., 2016b). In the present study we found differences in the pharmacological effect of PEA-OXA, compared to PEA, in a mouse model of mTBI that we have previously characterized in terms of behavioural phenotype using as a pharmacological tool the latter compound (PEA) (Guida et al., 2017). Indeed, while we found many similar pharmacological effects between the two molecules, PEA-OXA reduced the obsessive compulsive/repetitive-like behavior in the late phase of the disease, an endpoint that instead was not affected by PEA (**Supplementary Figure 1**).

We observed here another important difference between the pharmacological profile of PEA and PEA-OXA in terms of electrophysiological activity of the medial prefrontal cortex (*m*PFC) that represents a brain area highly involved in almost all the behavioral features of the concussion. In the previous study (Guida et al., 2017) we reported that PEA reduces the depressive-like behavior without any effect on the depressed neuronal activity 60 days after trauma. By contrast, in this study we found that the repeated administration with PEA-OXA reduced the depressive-like phenotype together with a clear effect at normalizing the neuronal activity in the *m*PFC, suggesting that this compound beneficially affects the impaired neuronal plasticity occurring in this brain area during the late consequences of mTBI injury.

We also demonstrated that mTBI mice showed memory retention impairment. Indeed, mTBI mice spent significant longer time to learn the position (quadrant) of the platform in the acquisition and reversal training and spent significant less time in the targeted quadrant in the probe test as compared to sham mice. Intriguingly, both sham and mTBI mice treated with PEA-OXA significantly spent less time to learn how to get to the targeted quadrant in the reversal training phase as compared to sham and mTBI mice treated with vehicle. Repeated PEA-OXA administration did not significantly prevent the memory impairment observed in the probe test, although it showed a trend in the amelioration of this task. Such a "nootropic" activity of the compound, together with the other beneficial effects, is intriguing and deserves further investigation, also in view of the

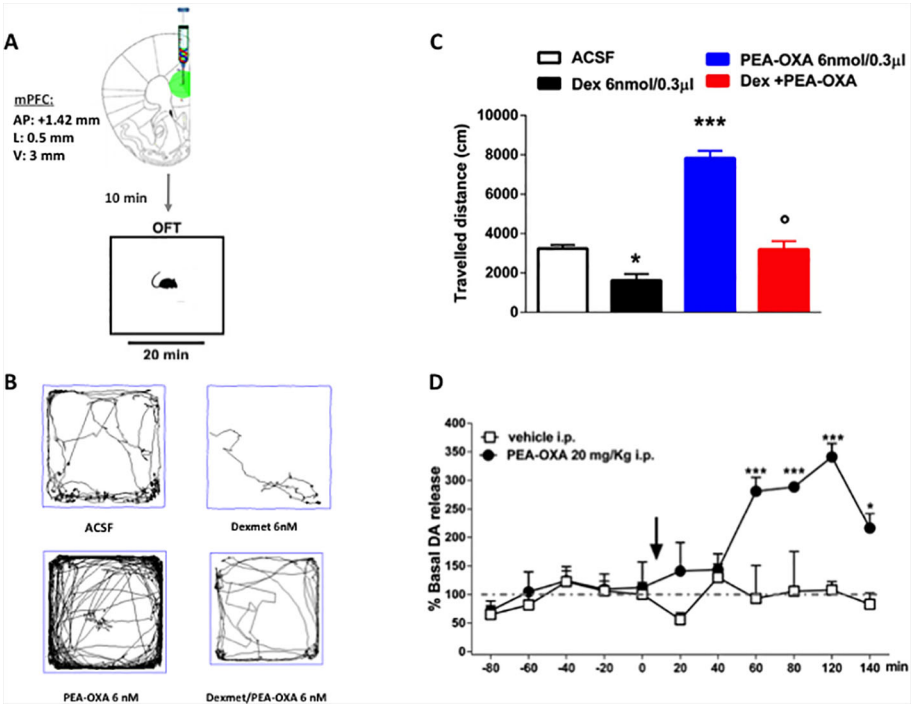

FIGURE 8 | Effects of single injection of PEA-OXA (6 nmol/0.3 µl i.c.v. and 20 mg/kg i.p.) on dexomedetomidine-induced decreased locomotor activity and on Dopamine extracellular levels in *m*PFC of Naïve mice. **(A)** Representation of coronal sections of the mouse brain with the cannula placement in mPFC, **(B)** Representative traces of mouse movement during an open field test, **(C)** Total distance traveled in OFT. Data are represented as mean ± SEM of 3 mice per group. *P < 0.05 and ***P < 0.001 indicate significant differences compared to ACSF. P < 0.05 indicate significant differences compared to PEA-OXA 6 nM. *P* < 0.05 was considered statistically. One-Way ANOVA, followed by Tukey's *post hoc* test for multiple comparisons test was performed. **(D)** Dopamine extracellular levels in mPFC of Naïve mice. Each point represents the mean ± S.E.M of four animals per group. *P < 0.05 and ***P < 0.001 indicate significant differences versus time 0. °P < 0.05 indicates significant differences compared to PEA-OXA 6 nmol/0.3 µl. *P*< 0.05 was considered statistically. Two-Way ANOVA, followed by Bonferroni's *post hoc* test for multiple comparisons test was performed.

possible mechanisms through which PEA-OXA may exert such effects.

In fact, due to its chemical structure, this compound might also act on other receptors such as norepinephrine α2 receptors. Therefore, we attempted to identify the possible activity of PEA-OXA on α2 receptors. We used a cAMP assay in HEK-293 cells overexpressing the α2 receptors. Our results demonstrate that PEA-OXA behaves as a α2 antagonist. Accordingly, we performed a binding assay for the α2 receptor and found that the compound shows indeed a high affinity for the receptor (see **Supplementary Material**). In order to verify the α2-mediated pharmacological effect, we performed open field task by microinjecting into the mPFC the selective α2 agonist dexomedetomidine, alone or in combination with PEA-OXA. Interestingly, we found a reduced locomotion induced by dexomedetomidine, which was prevented by the co-injection with PEA-OXA, confirming a possible α2-mediated mechanism. However, PEA-OXA microinjection alone was associated with increased locomotory activity, therefore we measured the dopamine levels after PEA-OXA microinjection. As suspectable PEA-OXA, increased the levels of Dopamine in the mPFC. This neurobiochemical effect deserves further investigation for a potential role of this secondary plant metabolite in neuropsychiatric disorders associated with dopamine changes.

The present new insights in the mechanism of action of PEA-OXA are potentially relevant to its pharmacological effects *in vivo*. In fact, it is known that several very effective antipsychotic drugs (i.e. clozapine) also show a similar pharmacodynamic profile (Marcus et al., 2010). Also other drugs in psychiatry act on those receptors such as mirtazapine and mianserine (De Boer et al., 1996).

Moreover, α2 blockade has been demonstrated to increase dopamine levels in cortical areas, thereby reducing the worsening effect of classical (also known as "typical") antipsychotic drugs (Uys et al., 2017). We also showed here that the compound administered at the dose of 20 mg/kg per os significantly increased GABA levels in the *m*PFC, without evident effects on glutamate release. This effect is likely to be important in psychosis, in which GABAergic interneurons play a key role in maintaining homeostasis of neural circuitries (in dopamine or serotonin producing neurons) (Kolata et al., 2018). Indeed, it has been recently demonstrated in patients with schizophrenia and depression, that the negative symptoms are associated to GABA level reduction in the *m*PFC and hippocampus (Kolata et al., 2018). The increased levels of GABA by PEA-OXA could be mediated by blockade of the α2 receptor, which is massively expressed in GABAergic interneurons (Manns et al., 2003).

Finally, the catecholaminergic-mediated mechanisms could also result in an anti-neuro-inflammatory effect that is important in the early phase of mTBI and other psychiatric diseases, in which neuroinflammation is being increasingly suggested to play a key role (Schimmel et al., 2017). Intriguingly, it has been also suggested that, although the stimulation of the α2 noradrenergic receptors reduces the proliferation of T-lymphocytes, its blockade, together with a block of the I_2 imidazoline receptors, reduces nitric oxide (NO), interferon γ (IFN-γ) and interleukin 2 (IL-2) release from splenocytes (Priyanka and Thyagarajan, 2013), suggesting an overall anti-inflammatory effect of α2 antagonists.

In summary, these data pave the way to the clinical investigation of the possible treatment of post-TBI-associated behavioral disorders, especially depressive-like behaviors, with PEA-OXA. Moreover, the pharmacodynamic profile of the drug is interesting since it shares several targets with PEA, but the efficacy and spectrum of its effects are strengthened and widened by its capability to modulate other receptors. Further studies are needed to better understand the pharmacodynamics of PEA-OXA and its possible use in other psychiatric diseases such as mood disorders, depression, and schizophrenia.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethical Committee of the University of Campania "L. Vanvitelli" and the University of Naples "Federico II, Naples."

AUTHOR CONTRIBUTIONS

SB, MI, CC, FI, CB, RI, and FR performed experiments. FB, FG, LL, and SB analyzed the data and planned experiments. LL, VD, AC, MG, and SM designed the study, wrote, and sponsored the paper.

FUNDING

The paper is supported by PRIN 2015 of SM and LL. MI and SB are supported by Epitech group.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00091/full#supplementary-material

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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.

The reviewer, DDG, declared a past co-authorship with several of the authors, MI, FI, FG, VD, SM, LL, to the handling editor.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 04 March 2020
doi: 10.3389/fphar.2020.00092

Zerumbone Modulates α_{2A}-Adrenergic, TRPV1, and NMDA NR2B Receptors Plasticity in CCI-Induced Neuropathic Pain *In Vivo* and LPS-Induced SH-SY5Y Neuroblastoma *In Vitro* Models

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OPEN ACCESS

Edited by:
Adriana Gibara Guimarães, Federal University of Sergipe, Brazil

Reviewed by:
Thallita Kelly Rabelo, Federal University of Sergipe, Brazil
Ernest Jennings, James Cook University, Australia

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *24 September 2019*
Accepted: *27 January 2020*
Published: *04 March 2020*

Citation:
Chia JSM, Izham NAM, Farouk AAO, Sulaiman MR, Mustafa S, Hutchinson MR and Perimal EK (2020) Zerumbone Modulates α_{2A}-Adrenergic, TRPV1, and NMDA NR2B Receptors Plasticity in CCI-Induced Neuropathic Pain In Vivo and LPS-Induced SH-SY5Y Neuroblastoma In Vitro Models. Front. Pharmacol. 11:92. doi: 10.3389/fphar.2020.00092

Zerumbone has shown great potential in various pathophysiological models of diseases, particularly in neuropathic pain conditions. Further understanding the mechanisms of action is important to develop zerumbone as a potential anti-nociceptive agent. Numerous receptors and pathways function to inhibit and modulate transmission of pain signals. Previously, we demonstrated involvement of the serotonergic system in zerumbone's anti-neuropathic effects. The present study was conducted to determine zerumbone's modulatory potential involving noradrenergic, transient receptor potential vanilloid type 1 (TRPV1) and *N*-methyl-D-aspartate (NMDA) receptors in chronic constriction injury (CCI)-induced *in vitro* and lipopolysaccharide (LPS)-induced SH-SY5Y *in vitro* neuroinflammatory models. von Frey filament and Hargreaves plantar tests were used to assess allodynia and hyperalgesia in the chronic constriction injury-induced neuropathic pain mouse model. Involvement of specific adrenoceptors were investigated using antagonists—prazosin (α_1-adrenoceptor antagonist), idazoxan (α_2-adrenoceptor antagonist), metoprolol (β_1-adrenoceptor antagonist), ICI 118,551 (β_2-adrenoceptor antagonist), and SR 59230 A (β_3-adrenoceptor antagonist), co-administered with zerumbone (10 mg/kg). Involvement of excitatory receptors; TRPV and NMDA were conducted using antagonists capsazepine (TRPV1 antagonist) and memantine (NMDA antagonist). Western blot was conducted to investigate the effect of zerumbone on the expression of α_{2A}-adrenoceptor, TRPV1 and NMDA NR2B receptors in CCI-induced whole brain samples of mice as well as in LPS-induced SH-SY5Y neuroblastoma cells. Pre-treatment with α_1- and α_2-adrenoceptor antagonists significantly attenuated both anti-allodynic and anti-hyperalgesic effects of zerumbone. For β-adrenoceptors, only β_2-adrenoceptor antagonist significantly reversed

the anti-allodynic and anti-hyperalgesic effects of zerumbone. β_1-adrenoceptor antagonist only reversed the anti-allodynic effect of zerumbone. The anti-allodynic and anti-hyperalgesic effects of zerumbone were both absent when TRPV1 and NMDA receptors were antagonized in both nociceptive assays. Zerumbone treatment markedly decreased the expression of α_{2A}-adrenoceptor, while an up-regulation was observed of NMDA NR2B receptors. Expression of TRPV1 receptors however did not significantly change. The *in vitro* study, representing a peripheral model, demonstrated the reduction of both NMDA NR2B and TRPV1 receptors while significantly increasing α_{2A}-adrenoceptor expression in contrast to the brain samples. Our current findings suggest that the α_1-, α_2-, β_1- and β_2-adrenoceptors, TRPV1 and NMDA NR2B are essential for the anti-allodynic and antihyperalgesic effects of zerumbone. Alternatively, we demonstrated the plasticity of these receptors through their response to zerumbone's administration.

Keywords: zerumbone, neuropathic pain, α_{2A}-adrenoceptor, TRPV1, NMDA NR2B, allodynia and hyperalgesia

INTRODUCTION

Zingiber zerumbet (*Z. zerumbet*) Smith is a wild ginger plant species that has long been used as traditional medicine in Southeast Asia. From rhizomes of *Z. zerumbet*, the main bioactive compound zerumbone has been isolated. Zerumbone has been shown to possess anti-inflammatory (Chien et al., 2008; Sulaiman et al., 2010a), antinociceptive (Sulaiman et al., 2010b), chemopreventive (Murakami et al., 1999), antimicrobial, and anti-oxidative properties (Habsah et al., 2000). Most importantly, we have reported the anti-allodynic and anti-hyperalgesic properties of zerumbone in a neuropathic pain mouse model (Zulazmi et al., 2015; Chia et al., 2016; Zulazmi et al., 2017).

The prevalence of neuropathic pain in the society is unfortunately increasing at a worrying rate. A pain condition due to lesions or diseases that affect the somatosensory nervous system give rise to neuropathic pain (Merskey, 1986). This debilitating chronic pain condition is common to those who suffer from diabetes, tumor nerve compression, viruses (HIV, varicella zoster virus), central nervous system disorders (multiple sclerosis, stroke), and surgical procedures (Baron and Tolle, 2008; Jensen et al., 2009).

The descending pain pathway plays an important role in modulating nociceptive signals, where bidirectional facilitatory or inhibitory control of nociception occurs. The periaqueductal gray (PAG) and rostroventromedial medulla (RVM) have been established as brain structures that provide the most influence on the descending pain pathway (Basbaum and Fields, 1978; Gebhart, 2004; Tracey and Mantyh, 2007). The monoaminergic system mainly utilizes serotonin and noradrenaline neurotransmitters in modulating nociception. These monoamines will act upon their respective subtypes to activate either the descending inhibitory or facilitatory pain pathway (Bannister and Dickenson, 2016).

As we have already shown the involvement of serotonergic system in the anti-neuropathic properties of zerumbone (Chia et al., 2016), this study will further explore the noradrenergic receptors of the monoaminergic system. Projections of noradrenergic neurons to the spinal cord arise from the pontine nuclei, mainly the A5, A6 (locus coeruleus), and A7 (Kölliker-Füse). The PAG and RVM brain structures communicate with these regions to modulate nociceptive transmission (Holden and Proudfit, 1998; Bajic and Proudfit, 1999; Pertovaara, 2006; Bruinstroop et al., 2012). The feedback mechanism of the noradrenergic system in terms of nociceptive modulation occurs following stimulation of sympathetic postganglionic axons, inducing release of noradrenaline neurotransmitters. The neurotransmitter released will then act upon adrenergic receptors to activate downstream effector molecules to inhibit nociceptive transmission (Pertovaara, 2013).

Apart from the descending modulatory controls, other receptors also play a role in inhibiting nociceptive signals. Excitatory receptor; transient receptor potential vanilloid 1 (TRPV1) and *N*-methyl-D-aspartate (NMDA) receptors are known to be involved in transmission of nociception. This is due to their localization on nociceptive neurons and pathophysiological changes in relation to their relative neurotransmitters, altering the activation threshold of action potential (Baron, 2006; Yogeeswari et al., 2009). Targeting of these excitatory receptors through agents that antagonize or agonize have shown promising results. Capsaicin cream, for example, is a TRPV agonist and is clinically used for chronic pain (Anand and Bley, 2011).

Multiple pathways and receptors in our body's physiological system intertwine to modulate pain signaling pathways. The underlying mechanism of zerumbone's anti-allodynic and antihyperalgesic effects should be investigated to further potentiate its effectiveness as an analgesic. Therefore, the main objectives of this study were to (1) determine the involvement of the noradrenergic, TRPV and NMDA receptors in the anti-allodynic and antihyperalgesic effects of zerumbone and (2) observe the change in α_{2A}-adrenoceptor, TRPV1 and NMDA NR2B receptors expression in the brain regions following zerumbone treatment in neuropathic pain conditions as well as complementing our findings with the *in vitro* LPS-induced

SH-SY5Y neuroblastoma neuroinflammation model for peripheral involvement.

MATERIALS AND METHODS

Experimental Animals

Male ICR mice (6–8 weeks, 25–35 g) were used in this study. All mice were housed under a 12 h light/dark cycle at 24 ± 1°C with unlimited access to food and water. Handling of animals and experiments were conducted according to the Ethical Guidelines for Investigation of Experimental Pain in Conscious Animals (Zimmermann, 1983) by the International Association for the Study of Pain (IASP). This study has been approved by the Institutional Animal Care and Use Committee (IACUC) UPM (Ref: UPM/IACUC/AUP- R060/2013).

Chronic Constriction Injury

The surgery to induce neuropathic pain was adapted from (Bennett and Xie, 1988) with some modifications (Gopalsamy et al., 2019). Briefly, mice were anaesthetized with tribromoethanol (250 mg/kg, i.p.). After shaving the fur on the left thigh region, the sciatic nerve was exposed after an incision was made through the biceps femoris. One loose ligature was placed using a 4-0 braided silk suture until a slight twitch of the left limb was observed. Same surgical procedures were conducted in mice from the sham group, except without ligation of the sciatic nerve. Mice were allowed to recover and behavioral tests were conducted on the 14th day after CCI.

Zerumbone

Compound extraction and isolation were conducted as previously reported (Chia et al., 2016). Zerumbone was dissolved in dimethylsulfoxide (DMSO), Tween 20 and normal saline (0.99% NaCl) in a ratio of 5:5:90 (v/v). The final concentration of DMSO did not exceed 5% of the total volume and caused no detectable effect on its own. Zerumbone was administered at 10 mg/kg through the intraperitoneal route based on our previous studies (Zulazmi et al., 2015; Chia et al., 2016; Zulazmi et al., 2017). The dosage of zerumbone (10 mg/kg) was chosen based on previous studies published by our colleagues Zulazmi et al. (2015), where they found zerumbone at 10 mg/kg was sufficient to provide anti-allodynic and antihyperalgesic properties in the CCI-induced neuropathic pain mice model. **Figures S1** and **S2** are included as supplementary to provide clarity. In addition, the ED_{50} of zerumbone in a similar neuropathic pain mice model was reported to be 10 mg/kg (Gopalsamy et al., 2017). For the *in vitro* assays, zerumbone was dissolved in phosphate buffered saline (PBS) at 0.25 mg/ml as stock solution.

Behavioral Tests

von Frey Filament Test

Mechanical allodynia was evaluated using the Electronic von Frey Aesthesiometer (IITC, Woodland Hills, CA, USA), adapted from methods by Chaplan et al. (1994). Mice were individually placed in the set-up of clear Plexiglass boxes placed on a wire-mesh platform. The automatic thin steel von Frey filament was positioned under the midplantar surface of the hindpaw. A gradual increase in force was applied until withdrawal of the paw was observed, measuring the maximum force of a mechanical stimulus to elicit a response. Withdrawal thresholds of force greater than 4.5 g was the cut-off point to avoid paw damage.

Hargreaves Plantar Test

Thermal hyperalgesia was evaluated using the thermal plantar apparatus (Ugo-basile, 37370, Verase, Italy), adapted from methods by Hargreaves et al. (1988). Mice were individually placed in the set-up clear Plexiglass boxes placed on a glass platform. The radiant heat source was positioned under the midplantar surface of the hindpaw, measuring the withdrawal latency for the mice to lift its paw. Cut-off point to avoid tissue damage was set at 20 s.

In Vivo Analysis of the Mechanisms of Action of Zerumbone

Involvement of Noradrenergic System

To firstly investigate the involvement of noradrenergic receptors, non-specific noradrenaline receptor antagonists were used; phentolamine (non-selective α-adrenoceptor antagonist, 5 mg/kg) and propranolol (non-selective β-adrenoceptor antagonist, 5 mg/kg).

Following confirmation of the involvement of α-adrenoceptors, further investigation into the specific noradrenergic receptor subtypes was conducted using selective α-adrenoceptor antagonists; prazosin (α_1-adrenoceptor antagonist, 10 mg/kg), idazoxan (α_2-adrenoceptor antagonist, 2 mg/kg). Specific β-adrenoceptor antagonists metoprolol (β_1-adrenoceptor antagonist, 1 mg/kg), ICI 118,551 (β_2-adrenoceptor antagonist, 2 mg/kg), and SR 59230 A (β_3-adrenoceptor antagonist, 2.5 mg/kg) were used following confirmation of the involvement of β-adrenoceptors.

Vehicle or zerumbone (10 mg/kg) were administered 30 min following antagonists' administration. Following 30 min after last respective treatments, behavioral tests were conducted.

Phentolamine, propranolol, metoprolol, and SR 59230 A were dissolved in 0.9% NaCl, ICI 118,551 was dissolved in 5% DMSO, 95% normal saline (0.9% NaCl) and idazoxan was dissolved in 10% DMSO and 90% normal saline (0.9% NaCl). Phentolamine, propranolol, ICI 118, 551, and SR 59230 A were administered in a volume of 5 ml/kg while idazoxan and metoprolol were administered in a volume of 10 ml/kg. All injections were intraperitoneal, 30 min prior to zerumbone administration. Dosages were chosen based on previous literature (Yalcin et al., 2009a; Yalcin et al., 2009b; Zhao et al., 2012).

Involvement of Excitatory Receptors

To assess the possible involvement of excitatory receptors—TRPV1 and NMDA, in the anti-allodynic and antihyperalgesic effects of zerumbone, mice were pre-administered with antagonists prior to zerumbone. Antagonists used were capsazepine (TRPV1 receptor antagonist, 10 mg/kg) and memantine (NMDA receptor antagonist, 10 mg/kg).

Vehicle or zerumbone (10 mg/kg) were administered 30 min following antagonists' administration. Following 30 min after last respective treatments, behavioral tests were conducted.

Capsazepine and memantine were dissolved in 0.9% NaCl and were administered intraperitoneally, in a volume of 10 ml/kg. Dosages were chosen based on previous studies (Eisenberg et al., 1995; Costa et al., 2008).

Western Blot Analysis

Protein analyses were conducted to evaluate the changes in expression level of α_{2A}-adrenergic, TRPV1 and NMDA NR2B receptors following neuropathic pain induction and zerumbone treatment. Selection of receptor subtypes were based on behavioral test results and the significant roles played by the receptors in neuropathic pain conditions.

Following behavioral tests, whole brain tissue samples were collected from the experimental animals. Tissue samples were homogenized in cold RIPA lysis buffer with protease inhibitors and the supernatants collected after centrifugation (6,000 g 30 min, 4°C) stored at -20°C until further usage. Sample supernatants (80 µg) were resolved on 8–12% sodium dextran sulfate-polyacrylamide gels, followed by protein transfer to a polyvinylidene fluoride (PVDF) membrane (Pall Life Sciences, Port Washington, NY, USA). The blots were then blocked with 5% Bovine Serum Albumin (BSA) in TBST (Tris Buffer Saline with 0.1% Tween 20) for 1 h. After blocking, blots were incubated overnight with anti- α_{2A} adrenergic receptor (1:500, PA1-048, Thermo Fisher Scientific, USA), anti-VR1 (1:1,000, ab31895, Abcam, USA), or anti-NR2B (1:1,000, ab65783, Abcam, USA) primary antibodies. Blots were then incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000, ab97051, Abcam, USA) following sufficient washing with TBST. Blots were washed four times, 20 min each time, with TBST prior to detection using enhanced chemiluminescent (ECL) detection system (Perkin Elmer, USA). Protein bands were analyzed and quantified using ImageJ processing software (National Institutes of Health). Blots were stripped and re-probed with HRP-conjugated anti-β-actin (1:5,000, ab20272, Abcam, USA) for one hour. Bands corresponding to β-actin were normalized to protein bands of samples.

In Vitro Analysis of the Mechanisms of Action of Zerumbone

Cell Culture

Dulbecco's Modified Essential Medium/Ham's Nutrient Mixture (DMEM:F12), Penicillin-Streptomycin solution and 2.5g/l-Trypsin/1mmol/l-EDTA Solution were purchased from Nacalai Tesque (Tokyo, Japan). Fetal bovine serum (FBS) and non-essential amino acids (NEAA) was purchased from Gibco-BRL (Grand Island, NY). Lipopolysaccharide (LPS) from *Escherichia coli* O55:B5 was purchased from Merck (Darmstadt, Germany).

SH-SY5Y neuroblastoma cell line were purchased from ATCC (ATCC® CRL-2266™). The cells were initially grown in Dulbecco's Modified Essential Medium (DMEM:F12) which contains 4.5 g/l glucose with 2mM of L-glutamine and sodium pyruvate, supplemented with 15% FBS, 1% of Penicillin-Streptomycin mixed solution and 1% NEAA at 37°C with 5% carbon dioxide (CO_2). Then, the cells were induced with 10 µM of all-*trans* retinoic acid for 5 days in a differentiation media (DMEM:F12, supplemented with 2.5% fetal bovine serum (FBS) and 1% of Penicillin-Streptomycin mixed solution) (Forster et al., 2016; Izham et al., 2018).

LPS-Induction and Treatment Groups

Following differentiation, the cells were induced with 1 µg/ml of LPS for 12 h at 37°C with 5% CO_2 to induce neuronal sensitization (Das et al., 2012). After LPS induction, 8 µg/ml zerumbone, 16 µg/ml amitriptyline as a positive control and vehicle (PBS) were added to the LPS-induced cell culture and incubated for 24 h at 37°C with 5% CO_2. The whole culture media was not removed and the amount of treatment required were calculated respectively.

Western Blot Analysis of α_{2A}-Adrenergic, TRPV1 and NMDA NR2B Receptors

In order to extract protein from the cell culture, ice-cold PBS was added to rinse the cell culture following 24 h of treatment. Then, 200 µl of RIPA lysis buffer (with protease inhibitor) was added and the cells were scrapped using the cell scrapper. The sample was then centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was collected for protein quantification and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined by using BCA protein assay (Pierce™ BCA Protein Assay Kit). 10 µg of protein sample was prepared by mixing the protein sample and the sample loading buffer (1:1). The protein was separated through SDS-PAGE at 120V for 2 h. Then, the protein was transferred to Polyvinylidene fluoride or polyvinylidene difluoride (PVDF) membrane at 0.35A for 2 h in ice. After transfer, the blot was blocked with 5% skimmed milk in TBST [mixture of tris-buffered saline (TBS) and Tween-20] for 1 h. Then, the blot was incubated overnight at 4°C with polyclonal antibody against β-actin (1: 5,000, #12620, Cell Signalling Technology, Danvers, MA, USA), with monoclonal antibody against GluN2B (NMDA receptor) (1:1,000, #4212, Cell Signalling Technology, Danvers, MA, USA), with polyclonal antibody against Alpha-$_{2A}$ (α_{2A}) adrenoceptor (1:1,000, ab85570, Abcam, Cambridge, MA, USA) and with polyclonal antibody against TRPV1 (1:500, bs-1931R, Bioss, MA, USA). After primary antibody incubation, the blots were incubated in secondary antibody (anti-rabbit IgG HRP-linked, 1:2,000, #7074S, Cell Signalling Technology, Danvers, MA, USA) for 1 h at room temperature with continuous agitation. Following incubation, the blots were developed by using ECL solution (Advansta, USA) and the chemiluminescence were detected by ChemiDoc™ imaging system. The band intensity quantification was carried out through the basis of molecular weight by using NIH ImageJ software.

Data Analysis

All results are expressed as mean ± standard error of mean (S.E.M.). Parametric values were analyzed by one-way ANOVA followed by Tukey's *post hoc* test using Graphpad Prism v6.0 software (Graphpad San Diego, CA). P values of less than 0.05 were considered significant.

Full images of blots with ladders are provided as supplementary materials, **Figures S3–S5**. Blots shown in the manuscript are images from full blots as provided in the supplementary materials, **Figures S6–S9**.

RESULTS

Involvement of Noradrenergic System in the Anti-Allodynic and Antihyperalgesic Effects of Zerumbone

Before investigating specific adrenoceptors involved in the anti-neuropathic effects of zerumbone, α-adrenoceptors and β-adrenoceptors were non-selectively blocked to determine the involvement of α and β noradrenergic receptors. Phentolamine (5 mg/kg, i,p.) and propranolol (5 mg/kg, i.p.), non-specific α- and β-adrenoceptor antagonists respectively, were pre-administered prior to zerumbone (10 mg/kg, i.p.). Administration of antagonists alone did not significantly affect allodynia and hyperalgesia induced by CCI (**Figure 1**, **Figure 2**). As shown in **Figure 1**, pre-treatment with phentolamine significantly ($p < 0.0001$) abolished the anti-allodynic effect of zerumbone. Similarly, the anti-allodynic effect of zerumbone was also abolished in the presence of propranolol ($p < 0.0001$). In **Figure 2**, similarly pre-treatment with both phentolamine and propranolol attenuated the antihyperalgesic effect of zerumbone.

Effects of α-Adrenoceptors Antagonists on Zerumbone-Induced Antineuropathy

As the non-specific α-adrenoceptor antagonist attenuated the anti-neuropathic effects of zerumbone, further investigation into specific adrenoceptor subtypes were conducted. Specific α-adrenoceptor antagonists to α_1 and α_2, prazosin and idazoxan respectively, significantly ($p < 0.0001$) prevented the anti-allodynic effect of zerumbone in the von Frey Filament Test as shown in **Figure 3**. The antihyperalgesic effect of zerumbone was similarly absent ($p < 0.0001$) in the Hargreaves Plantar Test when α_1- and α_2-adrenoceptor antagonists were co-administered with zerumbone as shown in **Figure 4**. Administration of antagonists alone did not significantly affect allodynia and hyperalgesia induced by CCI (**Figures 3**, **4**).

In Vivo Analysis of the Mechanisms of Action of Zerumbone

Effects of β-Adrenoceptors Antagonists on Zerumbone-Induced Anti-Neuropathy

As pre-administration of the non-selective β-adrenoceptor attenuated the anti-neuropathic effects of zerumbone, specific β-adrenoceptors were then investigated. In **Figure 5**, the anti-allodynic effect of zerumbone was investigated in the presence of β_1-, β_2-, and β_3-adrenoceptor antagonists. Metoprolol and ICI 118, 551, antagonists to β_1- and β_2-adrenoceptors respectively, significantly ($p < 0.0001$) attenuated the anti-allodynic effect of zerumbone. However, SR 59230 A, antagonist to β_3-adrenoceptor, did not reverse the anti-allodynic effect of zerumbone. In **Figure 6**, the antihyperalgesic effect of zerumbone was attenuated ($p < 0.0001$) only in the presence of ICI 118, 551, a β_2-adrenoceptor antagonist. When metoprolol and SR 59230 A antagonists were pre-administered prior to zerumbone, the withdrawal latency was not significantly different when compared to zerumbone. Administration of antagonists alone did not significantly affect allodynia and hyperalgesia induced by CCI (**Figures 5** and **6**).

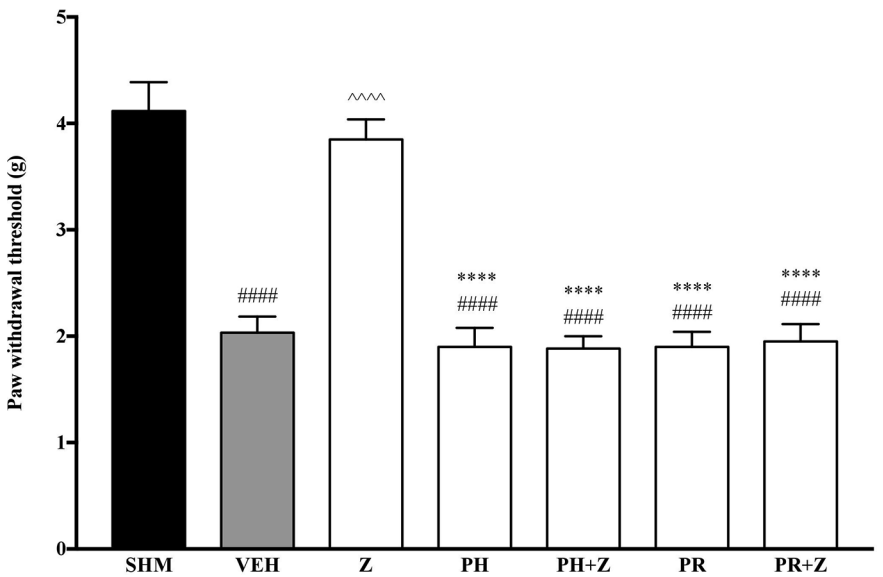

FIGURE 1 | Effect of phentolamine (non-selective α-adrenoceptor antagonist) and propranolol (non-selective β-adrenoceptor antagonist) pre-treatment on zerumbone against mechanical allodynia in CCI-induced neuropathic pain mice. Data are presented as mean ± SEM (n = 6). $^{\#\#\#\#}p < 0.0001$ as compared to sham, $^{\wedge\wedge\wedge\wedge}p < 0.0001$ as compared to vehicle and $^{****}p < 0.0001$ as compared to zerumbone-treated group. SHM (Sham); VEH (Vehicle, 10 mL/kg i.p.); Z (Zerumbone, 10 mg/kg i.p.); PH (Phentolamine, 5 mg/kg i.p.); PR (Propranolol, 5 mg/kg i.p.).

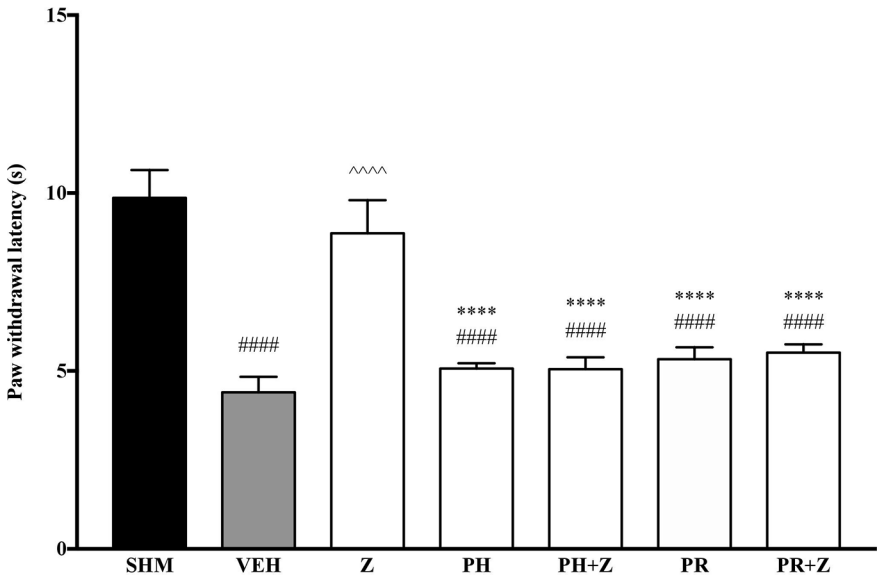

FIGURE 2 | Effect of phentolamine (non-selective α-adrenoceptor antagonist) and propranolol (non-selective β-adrenoceptor antagonist) pre-treatment on zerumbone against thermal hyperalgesia in CCI-induced neuropathic pain mice. Data are presented as mean ± SEM (n = 6) $^{\#\#\#\#}p < 0.0001$ as compared to sham, $^{\wedge\wedge\wedge\wedge}p < 0.0001$ as compared to vehicle and $^{****}p < 0.0001$ as compared to zerumbone-treated group. SHM (Sham); VEH (Vehicle, 10 ml/kg i.p.); Z (Zerumbone, 10 mg/kg i.p.); PH (Phentolamine, 5 mg/kg i.p.); PR (Propranolol, 5 mg/kg i.p.).

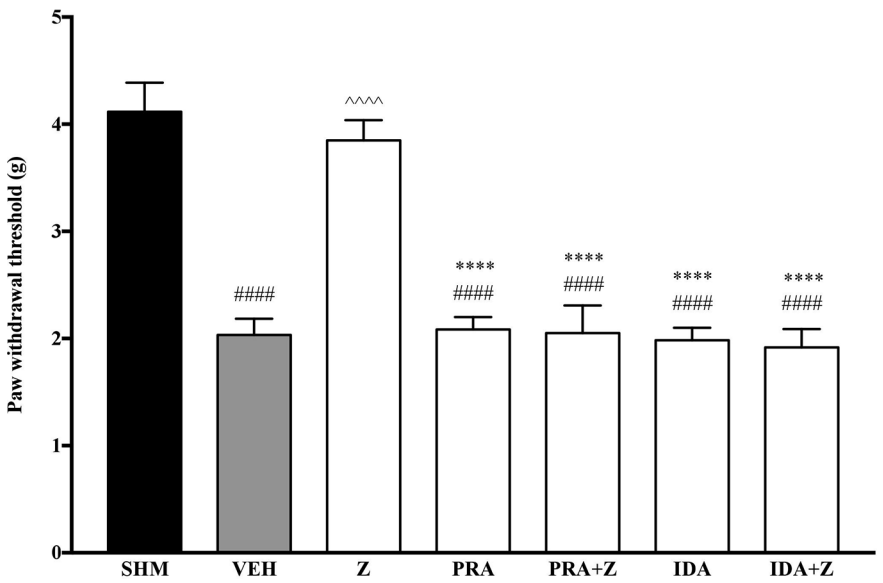

FIGURE 3 | Effect of prazosin (α_1-adrenoceptor antagonist) and idazoxan (α_2-adrenoceptor antagonist) pre-treatment on zerumbone against mechanical allodynia in CCI-induced neuropathic pain mice. Data are presented as mean ± SEM (n = 6). $^{\#\#\#\#}p < 0.0001$ as compared to sham, $^{\wedge\wedge\wedge\wedge}p < 0.0001$ as compared to vehicle and $^{****}p < 0.0001$ as compared to zerumbone-treated group. SHM (Sham); VEH (Vehicle, 10 mL/kg i.p.); Z (Zerumbone, 10 mg/kg i.p.); PRA (Prazosin, 10 mg/kg i.p.); IDA (Idazoxan, 2 mg/kg i.p.).

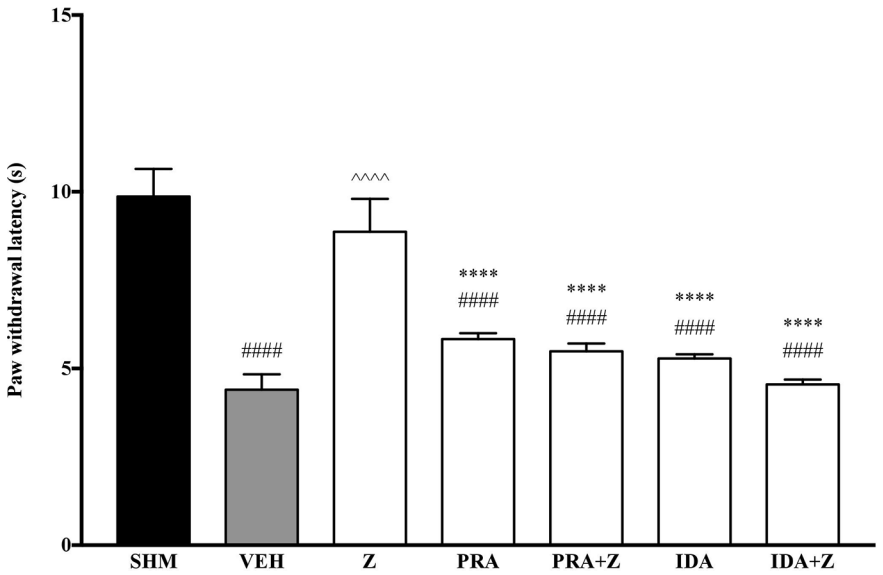

FIGURE 4 | Effect of prazosin (α_1-adrenoceptor antagonist) and idazoxan (α_2-adrenoceptor antagonist) pre-treatment on zerumbone against thermal hyperalgesia in CCI-induced neuropathic pain mice. Data are presented as mean ± SEM (n = 6). $^{\#\#\#\#}p < 0.0001$ as compared to sham, $^{\wedge\wedge\wedge\wedge}p < 0.0001$ as compared to vehicle and $^{****}p < 0.0001$ as compared to zerumbone-treated group. SHM (Sham); VEH (Vehicle, 10 mL/kg i.p.); Z (Zerumbone, 10 mg/kg i.p.); PRA (Prazosin, 10 mg/kg i.p.); IDA (Idazoxan, 2 mg/kg i.p.).

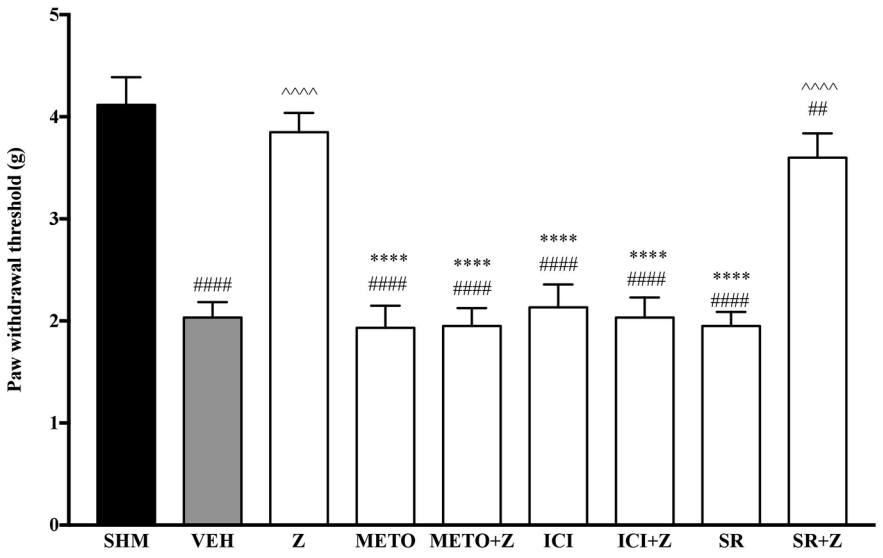

FIGURE 5 | Effect of metoprolol (β_1-adrenoceptor antagonist), ICI 118, 551 (β_2-adrenoceptor antagonist) and SR 59230 A (β_3-adrenoceptor antagonist) pre-treatment on zerumbone against mechanical allodynia in CCI-induced neuropathic pain mice. Data are presented as mean ± SEM (n = 6). $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$ as compared to sham, $^{\wedge\wedge\wedge\wedge}p < 0.0001$ as compared to vehicle and $^{****}p < 0.0001$ as compared to zerumbone-treated group. SHM (Sham); VEH (Vehicle, 10 mL/kg i.p.); Z (Zerumbone, 10 mg/kg i.p.); METO (Metoprolol, 1 mg/kg i.p.); ICI (ICI 118,551, 2 mg/kg i.p.); SR (SR 59230 A, 2.5 mg/kg i.p.).

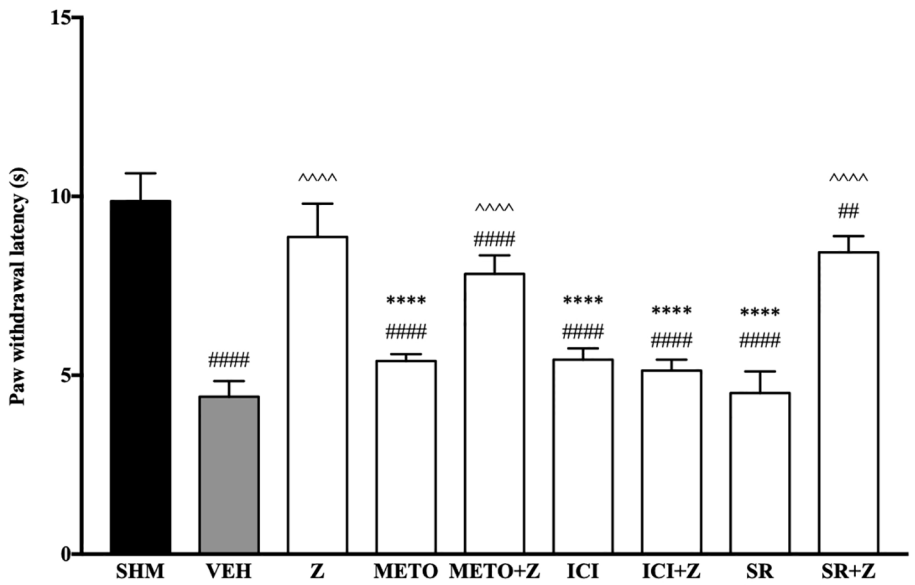

FIGURE 6 | Effect of metoprolol (β_1-adrenoceptor antagonist), ICI 118, 551 (β_2-adrenoceptor antagonist) and SR 59230 A (β_3-adrenoceptor antagonist) pre-treatment on zerumbone against thermal hyperalgesia in CCI-induced neuropathic pain mice. Data are presented as mean ± SEM (n = 6). $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$ as compared to sham, $^{\wedge\wedge\wedge\wedge}p < 0.0001$ as compared to vehicle and $^{****}p < 0.0001$ as compared to zerumbone-treated group. SHM (Sham); VEH (Vehicle, 10 mL/kg i.p.); Z (Zerumbone, 10 mg/kg i.p.); METO (Metoprolol, 1 mg/kg i.p.); ICI (ICI 118,551, 2 mg/kg i.p.); SR (SR 59230 A, 2.5 mg/kg i.p.).

Effect of Zerumbone on the Expression of α_{2A}-Adrenergic Receptor

Changes in the expression of α_{2A}-adrenoceptor following CCI and zerumbone treatment were assessed using Western blot. Samples from mice brain on Day 14 revealed bands corresponding to α_{2A}-AR at ~60 kDa. As shown in **Figure 7**, CCI causes a significant increase in expression of α_{2A}-AR as shown between vehicle and naïve groups ($p < 0.001$). In contrast, expression of α_{2A}-AR significantly ($p < 0.05$) decreased following zerumbone (10 mg/kg) treatment in comparison to vehicle group.

Involvement of TRPV and NMDA Receptors in the Anti-Allodynic and Antihyperalgesic Effects of Zerumbone

In **Figure 8**, the anti-allodynic effect of zerumbone was investigated in the presence of TRPV and NMDA receptor antagonists. Pre-treatment with capsazepine and memantine, antagonists to TRPV1 and NMDA respectively, significantly ($p < 0.0001$) attenuated the anti-allodynic effect of zerumbone. Similarly, the antihyperalgesic effect of zerumbone was also absent when antagonists capsazepine and memantine were pre-administered as shown in **Figure 9**. Administration of antagonists on its own did not affect paw withdrawal responses in both behavioral tests (**Figures 8** and **9**).

Effect of Zerumbone on the Expression of TRPV1 Receptor

Analysis on the expression of TRPV1 receptors were analyzed using brain samples of naïve, sham, vehicle, and zerumbone-treated mice. As shown in **Figure 10**, the bands observed corresponded to the expected molecular weight ~94 kDa. The induction of neuropathic pain caused a significant ($p < 0.05$) up-regulation of TRPV1 receptors, when comparing vehicle against sham group. No significant changes were observed between vehicle and zerumbone-treated groups. However, expression of TRPV1 receptors in zerumbone-treated groups is significantly ($p < 0.01$) higher against naïve and sham groups.

Effect of Zerumbone on the Expression of NMDA NR2B Receptor

The changes on NMDA NR2B receptor expression were analyzed following CCI and zerumbone treatment. As shown in **Figure 11**, the bands observed corresponded to the expected molecular weight ~160 kDa. In vehicle group, no significant changes were observed of the NR2B receptor expression in comparison to naïve and sham groups. However, a significant ($p < 0.05$, $p < 0.01$) up-regulation was observed in zerumbone-treated groups, compared against sham and vehicle groups.

In Vitro Analysis of the Mechanisms of Action of Zerumbone

Western Blot Analysis of α_{2A}-Adrenergic, TRPV1 and NMDA NR2B Receptors

Changes in the expression of α_{2A}-adrenergic, TRPV1, and NMDA NR2B receptors in the LPS-induced SH-SY5Y neuroblastoma cells were analyzed 24 h after the administration of 8 μg/ml zerumbone, 16 μg/ml amitriptyline, and vehicle. As shown in **Figure 12**, zerumbone administration significantly

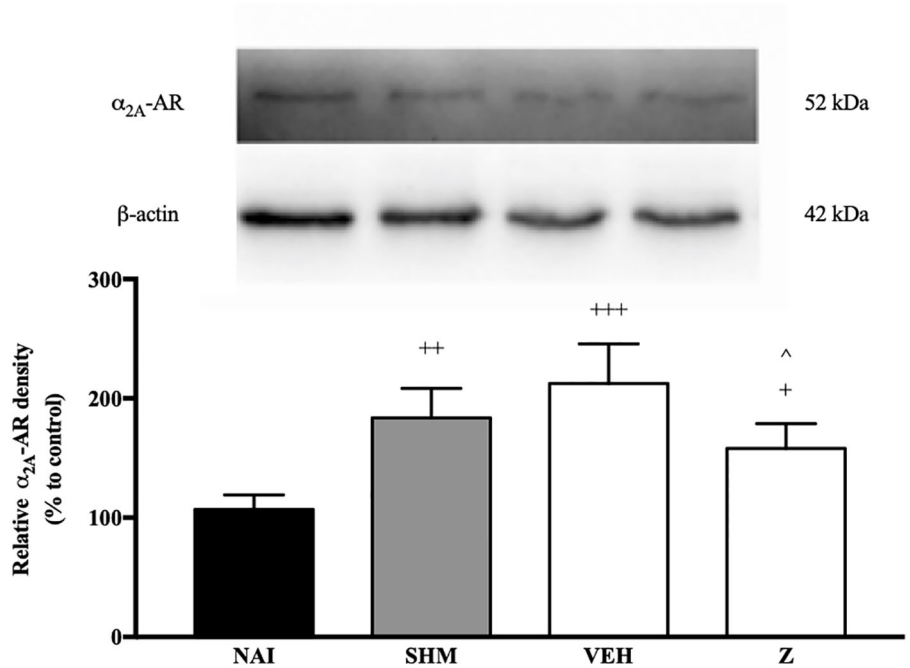

FIGURE 7 | Representative western blots of α_{2A}-adrenergic receptor from brain samples of naïve, sham, vehicle and zerumbone-treated groups. Data presented as mean ± SEM (n = 4), which were normalized to β-actin. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ as compared to naïve and $^{\wedge}p < 0.05$ as compared to vehicle group. NAI (Naïve); SHM (Sham); VEH (Vehicle, 10 mL/kg); Z (Zerumbone, 10 mg/kg).

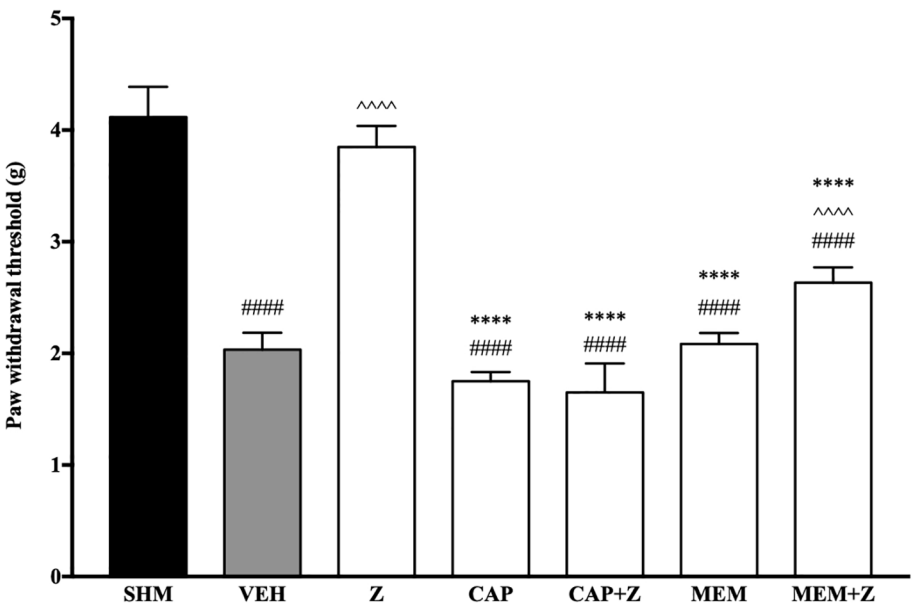

FIGURE 8 | Effect of capsazepine (TRPV1 antagonist) and memantine (NMDA antagonist) pre-treatment on zerumbone against mechanical allodynia in CCI-induced neuropathic pain mice. Data are presented as mean ± SEM (n = 6). $^{\#\#\#\#}p < 0.0001$ as compared to sham, $^{\wedge\wedge\wedge\wedge}p < 0.0001$ as compared to vehicle and $^{****}p < 0.0001$ as compared to zerumbone-treated group. SHM (Sham); VEH (Vehicle, 10 mL/kg i.p.); Z (Zerumbone, 10 mg/kg i.p.); CAP (Capsazepine, 10 mg/kg i.p.); MEM (Memantine, 10 mg/kg i.p.).

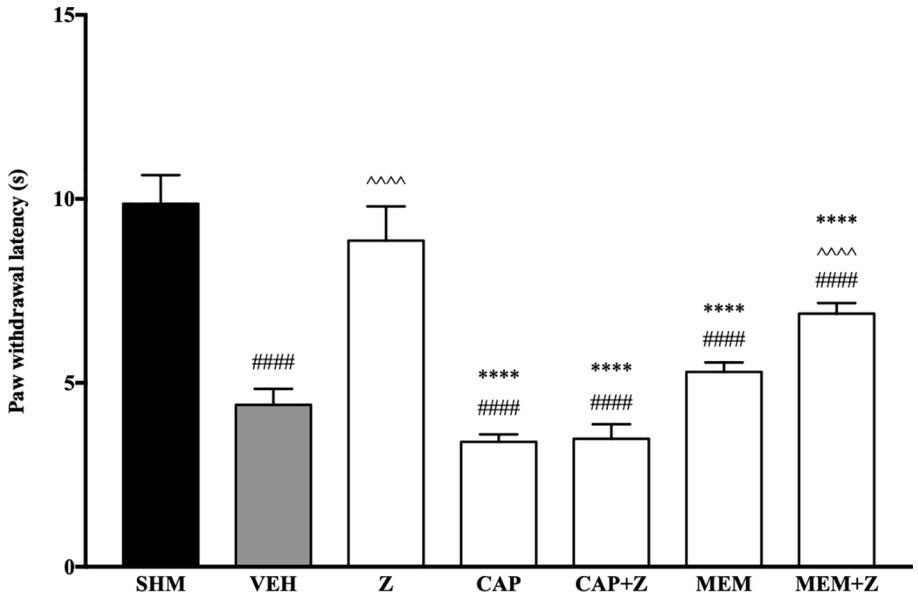

FIGURE 9 | Effect of capsazepine (TRPV1 antagonist) and memantine (NMDA antagonist) pre-treatment on zerumbone against thermal hyperalgesia in CCI-induced neuropathic pain mice. Data are presented as mean ± SEM (n = 6). $^{\#\#\#\#}p < 0.0001$ as compared to sham, $^{\wedge\wedge\wedge\wedge}p < 0.0001$ as compared to vehicle and $^{****}p < 0.0001$ as compared to zerumbone-treated group. SHM (Sham); VEH (Vehicle, 10 mL/kg i.p.); Z (Zerumbone, 10 mg/kg i.p.); CAP (Capsazepine, 10 mg/kg i.p.); MEM (Memantine, 10 mg/kg i.p.).

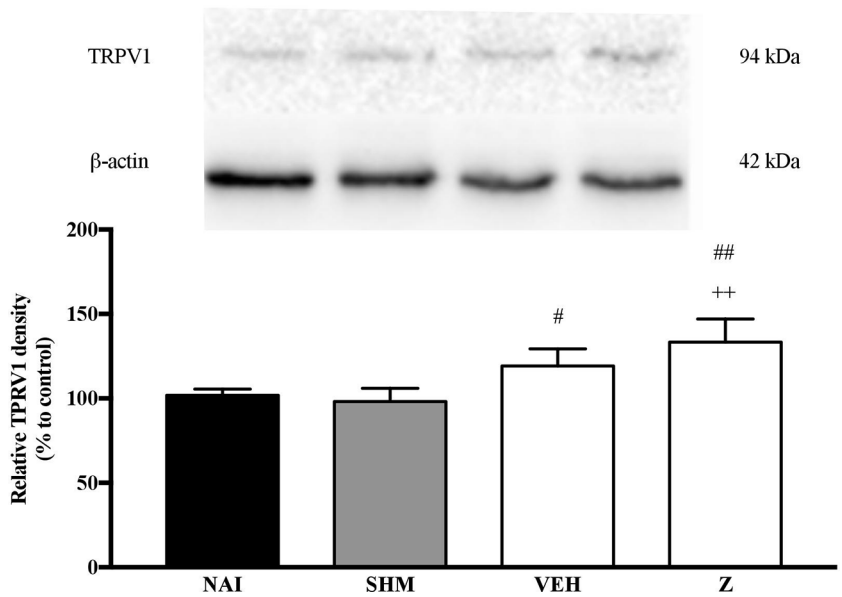

FIGURE 10 | Representative western blots of TRPV1 receptor from brain samples of naïve, sham, vehicle and zerumbone-treated groups. Data presented as mean ± SEM (n = 4), which were normalized to β-actin. $^{++}p < 0.01$ as compared to naïve and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ as compared to sham group. NAI (Naïve); SHM (Sham); VEH (Vehicle, 10 mL/kg); Z (Zerumbone, 10 mg/kg).

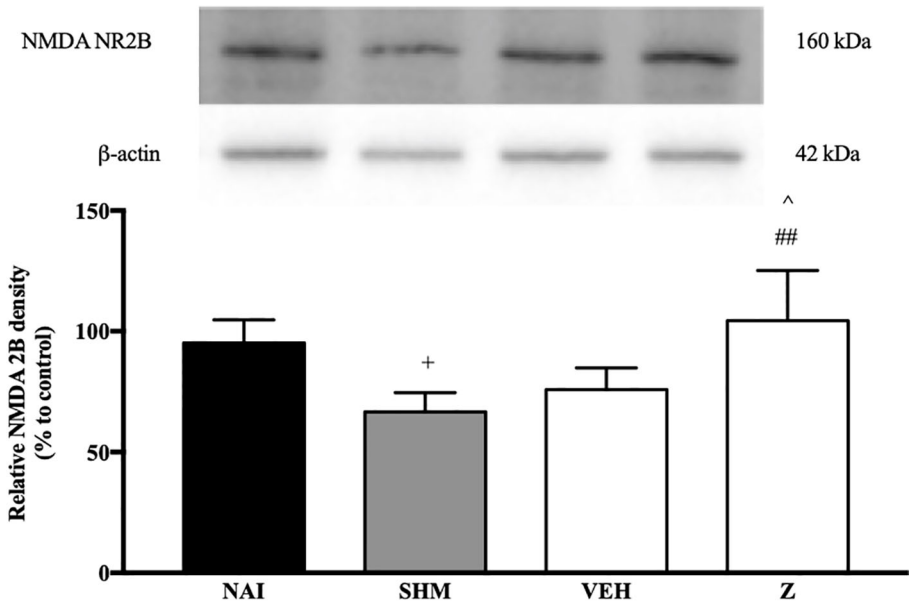

FIGURE 11 | Representative western blots of NMDA NR2B receptor from brain samples of naïve, sham, vehicle and zerumbone-treated groups. Data presented as mean ± SEM (n = 4), which were normalized to β-actin. $^{+}p < 0.05$ as compared to naïve, $^{\#\#}p < 0.01$ as compared to sham group and $^{\wedge}p < 0.05$ as compared to vehicle. NAI (Naïve); SHM (Sham); VEH (Vehicle, 10 mL/kg); Z (Zerumbone, 10 mg/kg).

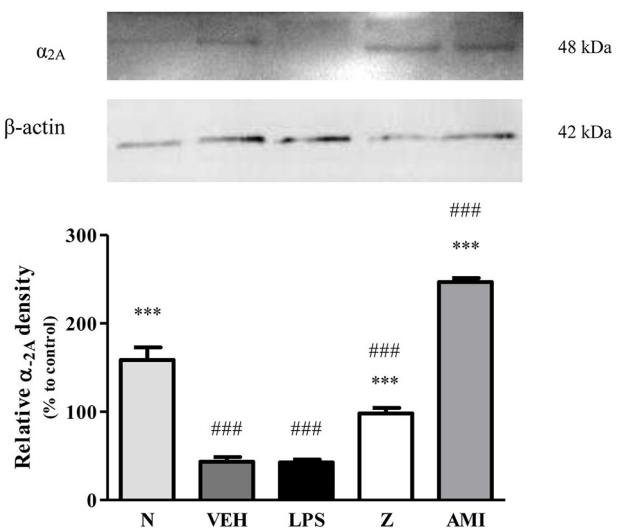

FIGURE 12 | Representative western blots of α_{2A}-adrenergic receptor from LPS-induced SH-SY5Y cells samples of normal, vehicle, LPS only, zerumbone, amitriptyline-treated groups. Data presented as mean ± SEM (n = 4), which were normalized to β-actin. $^{***}p < 0.001$ as compared to LPS only group and $^{\#\#\#}p < 0.001$ as compared to normal group. N (Normal); VEH (Vehicle, PBS); Z (Zerumbone, 8 μg/ml); AMI (Amitriptyline, 16 μg/ml).

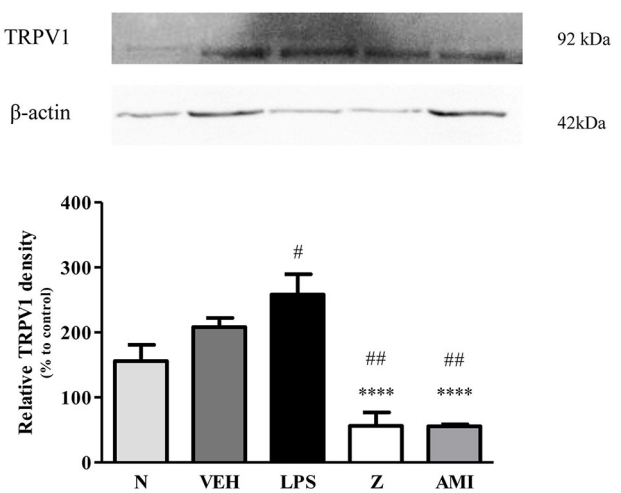

FIGURE 13 | Representative western blots of TRPV1 channel from LPS-induced SH-SY5Y cells samples of normal, vehicle, LPS only, zerumbone, amitriptyline-treated groups. Data presented as mean ± SEM (n = 4), which were normalized to β-actin. $^{****}p < 0.0001$ as compared to LPS only group and $^{\#}p < 0.1$, $^{\#\#}p < 0.01$ as compared to normal group. N (Normal); VEH (Vehicle, PBS); Z (Zerumbone, 8 μg/ml); AMI (Amitriptyline, 16 μg/ml).

increased the expression of α_{2A}-adrenergic receptors by the SH-SY5Y cells. In contrast, both the TRPV1 and NMDA NR2B receptors were down-regulated following the treatment with zerumbone as shown in **Figures 13** and **14**. Additionally, the *in vitro* findings are in contrast to the receptor's expression in the *in vivo* brain regions where, α_{2A}-adrenergic receptors were down-regulated while the TRPV1 and NMDA NR2B receptors were up-regulated.

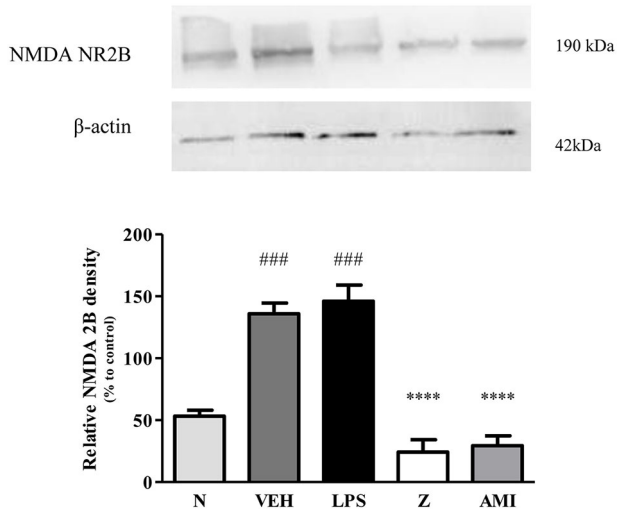

FIGURE 14 | Representative western blots of NMDA NR2B receptor from LPS-induced SH-SY5Y cells samples of normal, vehicle, LPS only, zerumbone, amitriptyline-treated groups. Data presented as mean ± SEM (n = 4), which were normalized to β-actin. ****$p < 0.0001$ as compared to LPS only group and $^{###}p < 0.001$ as compared to normal group. N (Normal); VEH (Vehicle, PBS); Z (Zerumbone, 8 μg/ml); AMI (Amitriptyline, 16 μg/ml).

DISCUSSION AND CONCLUSION

We have previously demonstrated the antinociceptive properties of zerumbone in the chronic constriction injury neuropathic pain mice model and its mechanisms through serotoninergic (Chia et al., 2016) and the L-arginine–nitric oxide (Zulazmi et al., 2017) pathways. With this study, we now show that the noradrenergic system and excitatory receptors are crucial to zerumbone's anti-allodynic and antihyperalgesic properties. Our current study suggests that zerumbone produces its anti-allodynic properties by interacting with α_1-, α_2-, β_1-, and β_2-adrenergic receptors. Meanwhile, α_1-, α_2-, β_2-adrenergic receptors are responsible for zerumbone's antihyperalgesic property. Excitatory receptors TRPV and NMDA are involved in both zerumbone-induced anti-allodynia and antihyperalgesia. Therefore, we hypothesize that a synergistic mechanism between noradrenaline, TRPV, and NMDA is utilized by zerumbone to produce its anti-allodynic and antihyperalgesic properties.

The noradrenergic system is part of the descending monoaminergic pain modulation pathway. The serotonergic system is known to exert both inhibitory as well as excitatory effects in pain modulation, whereas the noradrenergic system predominantly has an inhibitory role in pain modulation (Suzuki et al., 2004b). Two main classes of adrenergic receptors (AR) arise from the noradrenergic projections from the locus coeruleus (LC), which are the α and β ARs (Millan, 2002; Hentall et al., 2003). Both α and β ARs and their subtypes are G protein-coupled receptors (GPCR), thus their main action after binding of noradrenaline differs depending on the sub-class of G proteins each receptor couples to (Llorca-Torralba et al., 2016).

Following nerve injury, the central and peripheral nervous system undergoes physiological changes. Possible alterations to descending monoaminergic pathway influences neurotransmitter metabolism and/or number and affinity of receptor uptake sites lead to neuropathic pain (Suzuki et al., 2004a; Rahman et al., 2008; Leong et al., 2011). The induction of neuropathic pain was measured by the endurance of the animals through behavioral tests on their pain threshold. Firstly, we investigated the involvement of noradrenergic receptors by administering non-selective α- and β-adrenoceptor antagonists, phentolamine and propranolol respectively. In the presence of phentolamine and propranolol, both the anti-allodynic and antihyperalgesic properties of zerumbone we attenuated. Thus, further examinations into specific receptors to the noradrenergic system were conducted.

Administration of prazosin, a selective α_1-AR antagonist prior to zerumbone treatment managed to abolish zerumbone's anti-neuropathic properties. The α_1 adrenoceptors are $G_{q/11}$ protein receptors, which are coupled to phospholipase C (PLC) (Bylund et al., 1994). Binding of noradrenaline to α_1-AR causes increase in intracellular calcium pool as a result of hydrolysis of inositol phosphates, with diacyl glycerol (DAG) and inositol triphosphate (IP_3) as its products (Millan, 2002). The α_1-AR has been implicated to facilitate nociception (Millan, 1999; Fuchs et al., 2001; Hord et al., 2001b), and is said to contribute to the development of chronic pain. However, previous studies have reported antinociceptive activity when α_1-AR agonists were used, possibly acting pre-synaptically on central primary afferent nociceptors (Howe et al., 1983; Kawabata et al., 1994; Hord et al., 2001a). As discussed by Millan (2002), the bidirectional reports on both pro- and anti-nociceptive effects of α_1-AR could be due to co-localization of α_1- and α_2-AR.

As with the α_1-AR antagonist, the anti-allodynic and antihyperalgesic properties of zerumbone were absent when α_2-AR antagonist, idazoxan, was administered prior to zerumbone treatment. Unlike α_1-AR, α_2-adrenoceptors are coupled to $G_{i/o}$ proteins, which alters membrane polarization through K^+ and Ca^{2+} channels (Millan, 2002). Activation of α_2-AR results in intracellular changes whereby cAMP levels are decreased due to inhibition of adenylyl cyclase. The α_2-AR is the most commonly implicated adrenergic receptor to be responsible in inhibiting pain transmission. Pre-synaptically, the α_2-AR plays an important inhibitory feedback mechanism in the release of noradrenaline from adrenergic neurons (Gilsbach and Hein, 2008). There are three subtypes to α_2-AR, which are the 2A, 2B, and 2C receptor subtypes. α_{2A}- and α_{2C}-AR are widely expressed in the central nervous system (CNS) while the α_{2B}-AR can be commonly found in non-neuronal tissues. The α_{2A}-AR is the predominant subtype found in the brainstem.

Based on the results obtained in the present study, zerumbone utilizes the α_2-AR in exhibiting its anti-neuropathic effect. Activation of α_2-AR causes an increase in neuronal firing activity from the LC and studies have found that activation from a α_2-AR agonist to decrease noradrenaline (NA) concentration in the

prefrontal cortex (PFC) (Svensson et al., 1975; Pudovkina et al., 2001; Jedema et al., 2008). However, previous studies have reported that the inhibitory actions of α_2-AR to be absent in neuropathic conditions (Xu et al., 2000; Obata et al., 2005; Omiya et al., 2008; Chen et al., 2011). Thus, the antinociceptive activity due to α_2-AR activation is said to originate from the LC, to compensate in the loss of spinal α_2-adrenergic receptor activity. Alternatively, Alba-Delgado et al. (2012) has proposed that α_2-AR desensitization to occur in the LC that enhances the antinociceptive noradrenergic effects in neuropathic pain conditions.

β-adrenoceptors can be further classified into β_1-, β_2-, and β_3-adrenergic receptors (Bylund et al., 1994). All three β-AR are G_s proteins, coupled to adenylyl cyclase to increase intracellular secondary messenger cAMP synthesis. These receptors are widely distributed in the CNS (Nicholas et al., 1993). Although most of the focus on the noradrenergic system is on the α_2 subtype, studies have shown that β-AR are also involved in pain modulation (Brochet et al., 1986; Choucair-Jaafar et al., 2009).

The zerumbone-induced anti-allodynic and antihyperalgesic effects were significantly reversed by administration of ICI 118,551, a β_2-AR antagonists, but not of SR 59230 A, a β_3-AR antagonist. Metoprolol, a β_1-AR antagonist, only attenuated the anti-allodynic effect of zerumbone. β_1- and β_2-AR are found in both central and peripheral nervous systems, with the β_1-AR densely expressed in cerebral cortex, thalamus, and sympathetic ganglia whereas β_2-AR to localize more in the olfactory bulb, hippocampus, hypothalamus, and spinal cord (Nicholas et al., 1996; Gilsbach and Hein, 2008). As mentioned, the α_2-adrenergic receptors are the predominant adrenergic receptors to inhibit nociceptive transmission. Thus, not many studies have been conducted on β-adrenergic receptor subtypes. However, studies have shown that β_2-AR is necessary for antidepressants to exhibit its anti-neuropathic effects (Yalcin et al., 2009a; Yalcin et al., 2009b). It is possible that the activation of downstream proteins due to β_2-AR facilitates protein kinase A activation by cAMP, which results in enhanced NA release from sympathetic nerves (Boehm and Kubista, 2002; Kubista and Boehm, 2006).

With consideration to our current findings and in line with literature on the more prominent role of α_2-adrenergic receptors, primarily the α_{2A} subtype, we investigated whether the α_{2A}-adrenoceptor is involved in zerumbone's anti-neuropathic effects. Our findings have shown (**Figure 7**) that chronic constriction injury induces an increase in expression of α_{2A}-adrenergic receptors in the brain. Previous studies have also reported similar findings, where nerve lesions causes an up-regulation in α_{2A}-adrenergic receptor expression as early as 7 days following injury induction (Alba-Delgado et al., 2013).

In normal conditions, the noradrenergic system primarily functions to inhibit nociceptive transmission. Following nerve injury, the plastic changes that occur shift the inhibitory tone of the noradrenergic system to facilitate nociceptive transmission instead (Brightwell and Taylor, 2009; Kaushal et al., 2016). Therefore, the increase in expression of α_{2A}-adrenoceptor following CCI in vehicle-treated group may be due to plastic changes that occur, abolishing the inhibitory tone of the noradrenergic system. In support of this hypothesis, zerumbone treatment suppressed the increased expression of α_{2A}-AR as shown in this study. Development of neuropathic pain is the result of cumulative plastic changes that occur throughout the nervous system. Our findings as shown in **Figure 7** indicates that the expression of α_{2A}-AR decreases upon zerumbone administration. It is possible that the primary action of zerumbone in attenuating allodynia and hyperalgesia is through suppression of the α_{2A}-AR up-regulation.

Therefore, the cumulative action of zerumbone against neuropathic pain might not only utilize the descending noradrenergic pathway, but also through the noradrenergic projections to the other brain sites involved in the pain pathway. In particular, the rostroventromedial medulla and periaqueductal gray brain regions are important in modulating nociceptive signals (Pertovaara, 2006). Moreover, adrenoceptors are also localized on the descending serotonergic pathway. The inhibitory α_2-AR especially, has been reported to be highly concentrated in serotonergic neurons (Rosin et al., 1993; Guyenet et al., 1994; Millan et al., 2000; Millan, 2002).

Our current findings implicate the involvement of TRPV1 and NMDA NR2B receptors in zerumbone's anti-allodynic and antihyperalgesic properties. Zerumbone and the essential oil of *Zingiber zerumbet* have been associated with TRPV and glutamatergic (NMDA) system in its mechanistic actions against acute pain (Khalid et al., 2011; Perimal et al., 2011). Due to similarities between acute and chronic pain pathways, zerumbone is therefore implicated to also involve TRPV and NMDA receptors in exerting its anti-neuropathic properties in the CCI model of neuropathic pain.

The families of transient receptor potential (TRP) ion channels are primarily expressed on nociceptive neurons. TRPV1 receptors in particular, were discovered to be involved in nociceptive processing due to capsaicin, an active component from *Capsicum* chili peppers (Caterina et al., 1997). Acidic conditions, high temperatures, and noxious stimuli activate TRPV1 receptors. In the pathogenesis of neuropathic pain, expression of TRPV1 is up-regulated on uninjured A- and C-fibers, as well as injured dorsal root ganglions. These alterations to receptor expression causes an amplification of noxious stimuli resulting in peripheral sensitization (Baron, 2006).

Zerumbone is hypothesized to exert either an antagonist-like effect or desensitize TRPV1 receptors to suppress mechanical allodynia and thermal hyperalgesia. Previous studies have reported that TRPV1 antagonists are able to alleviate nociception. Yamamoto et al. (2008) reported the TRPV1 antagonist, N-(4-Tertiarybutylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropyrazine-1(2H)-carbox-amide (BCTC), suppressed mechanical allodynia in the CCI neuropathic pain model. As a relative comparison to zerumbone, curcumin has also exhibited potent analgesic properties (Sharma et al., 2006; Mittal et al., 2009; Zhao et al., 2014). Curcumin, the active ingredient of turmeric (*Curcuma longa*), with several of its analogues behave as antagonists on TRP channels (Nalli et al., 2017). Similarly, Yeon et al. (2010) observed suppression of TRPV activation by curcumin, thereby exhibiting antihyperalgesic effect.

On the contrary, zerumbone could also act as an agonist to desensitize TRPV receptors. Similar mechanisms can be observed with capsaicin, a TRPV agonist. Many over-the-

counter topical creams contain low concentrations of capsaicin, typically used as an analgesic. Analgesia produced from TRPV activation occurs due to the lasting refractory rate. This desensitization phase causes the excitable receptors to be insensitive to any noxious stimuli (Perry et al., 2007). Among the ligands that are able to activate the TRPV1 channels are vanilloids (e.g.: resineferatoxin, capsaicin), protons, endogenous lipids, polyamines, and noxious heat (Caterina et al., 1997; Zygmunt et al., 1999; Ahern et al., 2005; Ahern et al., 2006).

The noradrenergic system and TRPV receptors have shown their interrelation in the pain pathway. Our findings show zerumbone utilizes noradrenergic receptors as well as TRPV receptors in eliciting its anti-neuropathic properties. Recently, Chakraborty et al. (2017) found that noradrenaline released *via* the descending noradrenergic system inhibits pre-synaptic TRPV1 channels. Therefore, it is possible that the descending noradrenergic system enhances the inhibition on TRPV1 channels in the presence of zerumbone to attenuate neuropathic pain symptoms.

Further analysis into expression of TRPV1 receptors on mice in neuropathic pain conditions shows a significant increase. The present findings observed a slight increase in TRPV1 expression in comparison to neuropathic pain mice. An up-regulation in TRPV receptor expression has been reported. On the basis of their known mechanisms, the increase in expression of the excitatory receptor TRPV1 is therefore implicated in the enhanced excitability state of nociceptive transmission. As a result, the peripheral sensitization that occurs soon develops to neuropathic pain (Mannion et al., 1999; Ji et al., 2002).

Expression of TRPV1 in brain is primarily in microglial cells and in discrete amounts at other brain regions such as anterior cingulate cortex (ACC). TRPV1 activation modulates synaptic neurotransmission and indirectly enhances glutamatergic neuronal transmission, heightening nociceptive transmission leading to pathophysiological persistence of pain (Marrone et al., 2017). However, recent advances by Silva et al. (2016) have also provided new evidences in the inhibitory influence of TRPV1 channels in modulating nociceptive transmission. The modulatory role is reportedly controlled by the expression of TRPV1 on the rostral ventromedial medulla (RVM), in contrast to ACC region mentioned earlier. Therefore, the inhibitory role of TRPV1 up-regulation from zerumbone treatment is possibly implicated by its modulatory role expressed by TRPV1 in the RVM.

NMDA is an ionotropic glutamate receptor. NMDA receptors bind to glutamate, the major excitatory neurotransmitter of the central nervous system. Glutamate and its receptors are the major contributors to the development of neuropathic pain through central sensitization (Bennett, 2000). Activation of this class of excitatory receptors causes an influx of Ca^{2+}, activation of nitric oxide synthases (NOS) and cyclooxygenase-2 (COX-2). The disproportionate availability of nitric oxide and prostaglandin results in prolonged excitation of neural and glial cells (Fundytus, 2001).

The present study shows that zerumbone partially utilizes NMDA receptors to elicit its anti-allodynic and antihyperalgesic effects. These results conform to previous study on zerumbone in an animal model of acute pain where zerumbone dose-dependently inhibited glutamate-induced nociception (Perimal et al., 2011). Considering the excitatory functioning on NMDA receptors, antagonists to these receptors are now considered as clinical analgesics. Zerumbone is therefore postulated to act as an antagonist on NMDA receptors, thus suppressing calcium ions influx to dampen nociceptive transmission. The action of zerumbone on glutamatergic transmission may occur either peripherally or centrally.

Several populations of glutamatergic receptors, including NMDA, are known to localize on noradrenergic terminals. Presynaptic activation these NMDA receptors regulates the release of NA (Forray et al., 2000; Luccini et al., 2007). Increased availability of NA thus enhances the inhibitory tone of the noradrenergic system. A *vice versa* mechanisms have also been reported, where activation of the locus coeruleus noradrenergic system causes a down-regulation of NMDA receptors (Roh et al., 2008; Kang et al., 2012). Therefore, our current findings imply that the mechanistic action of zerumbone is not specific to a single pathway, but rather a summative effect through various inhibitory and excitatory receptors.

The NMDA NR2B subunit was chosen to further analyze the effects of zerumbone. Activation of NR2B subunit contributes to the excitatory role of NMDA receptors as it induces c-Jun N-terminal kinase (JNK) activation and enhances astrocytic-neuronal signaling (Kato et al., 2006). The NR2B subunit of NMDA receptors are regionally distributed, however it is primarily expressed superficially at the dorsal horn and is highly associated with nociceptive transmission (Laurie et al., 1997). In the present study, it was found that expression of NMDA NR2B in CCI mice did not significantly increase in comparison to naïve and sham groups. On the contrary, zerumbone-treated groups presented a significantly increased expression in comparison to sham and vehicle groups.

Over-expression of NMDA NR2B subunit has also been reported in the brain and spinal cord in chronic pain conditions (Tang et al., 1999; Wei et al., 2002). The increased expression of NR2B subunit is linked to inflammation leading up to persistent pain, where its over-expression was observed in the ACC in the Complete Freund's Adjuvant chronic inflammatory animal model (Wu et al., 2005). The use of CCI model as well as whole brain sample could be a possible rationalization to the insignificant change in NMDA NR2B subunit observed in this study. Furthermore, previous studies have reported a down-regulation of the NR2B subunit in analgesic compounds (Hu et al., 2009; Wang et al., 2013). It is reasonable to hypothesize that the up-regulation observed in zerumbone-treated groups is due to the acute administration of treatment— which may not be sufficient to cause any effect of NR2B expression in the brain. Possibility of a modulatory action of NR2B subunit up-regulation should be further explored.

It is interesting to note that the expression of α_{2A}-AR was up-regulated while both the TRPV1 and NMDA NR2B receptors were down-regulated respectively in the *in vitro* SH-SY5Y neuroblastoma cell model. These findings are coherent with

both the behavioral allodynia and hyperalgesia assays. We note that the etiology of neuropathic pain is complex, and in some cases findings are contradictory (Lee et al., 2013) and left unexplained, however in this case, we postulate that zerumbone interacts and possibly triggers modulation differently in the peripheral nervous system compared to the central nervous system. As we have explained the proposed mechanisms in the brain regions explicitly in the preceding paragraphs, we can only conclude that in the peripheral nervous system, zerumbone acts as an agonist for the α_{2A}-adrenoceptor (Kimura et al., 2012), and modulates both the TRPV1 (Perry et al., 2007; Yamamoto et al., 2008) and NMDA NR2B (Hu et al., 2009; Wang et al., 2013) receptors. These receptors and pathways are well established and studied.

Our findings on the expression of α_{2A}-adrenoceptor, TRPV1 and NMDA NR2B receptors at the brain regions complemented by the exact opposite on the *in vitro* model further reiterated the plasticity of neuronal signaling in pain and the nervous system. While we know that the dorsal horn is one of the first point for pain signal transmission, a huge body of literature is now pointing to the relevance of the descending control of the nervous system in further regulating and modulating pain signals both ascending and descending. Interestingly, they can be both facilitatory or inhibitory or both (Heinricher et al., 2009).

In conclusion, our findings indicate the interaction between the noradrenergic system, TRPV1, NMDA receptors, and zerumbone in exhibiting anti-allodynic and antihyperalgesic effects in a neuropathic pain mice model. Moreover, the action of zerumbone on α_{2A}-adrenoceptor, TRPV1 and NMDA NR2B receptor expression provides significant information on the mechanism of action of zerumbone. In support with previous studies on zerumbone against neuropathic pain, zerumbone has high potential as an antinociceptive compound for treatment of neuropathic pain. Research into new and better treatments for neuropathic pain patients are in critical need. A combinatorial therapy approach, consisting of drugs with different mechanisms of action, is currently used to treat neuropathic pain patients. Future research into the effect of zerumbone, in both chronic and acute treatments, on the relationship between various pain modulatory pathways in neuropathic pain models should be conducted.

DATA AVAILABILITY STATEMENT

The datasets used and/or analysed for this manuscript are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) UPM (Ref: UPM/IACUC/AUP- R060/2013).

AUTHOR CONTRIBUTIONS

All authors equally contributed to the study and critically reviewed the final version of the manuscript

FUNDING

This study was supported by the Putra Grant—Putra Graduate Initiative (GP-IPS/2017/9528400), the Putra Grant—High Performance Research Grant (GPB-9659000) from Universiti Putra Malaysia and the Science Fund Scheme (Grant No: 5450778) under the Ministry of Science, Technology & Innovation Malaysia.

ACKNOWLEDGMENTS

The authors would like to thank the Faculty of Medicine and Health Sciences, the Physiology Research Laboratory, Universiti Putra Malaysia, Malaysia and the Australian Research Council Centre of Excellence for Nanoscale BioPhotonics, The University of Adelaide, Australia for providing the necessary support for this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.00092/full#supplementary-material

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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.

frontiers in Neuroscience

OPINION
published: 21 April 2020
doi: 10.3389/fnins.2020.00371

Irritable Bowel Syndrome: Manipulating the Endocannabinoid System as First-Line Treatment

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Keywords: IBS, endocannabinoid, CB1, CB2, TRPV1, THC, CBD, PEA

INTRODUCTION

Irritable Bowel Syndrome (IBS) is a functional disorder characterized by abdominal pain, spasms, and altered bowel movements, either predominantly diarrhea (IBS-D), or predominantly constipation (IBS-C), or alternating between those states (Saha, 2014). In the Western world it affects the 10–15% of the population (Canavan et al., 2014). IBS represents a visceral hypersensitivity, with features of gastrointestinal (GI) allodynia and hyperalgesia. Considered a life-long condition, it is clear that significant gastrointestinal insults, such as food poisoning or antibiotic administration, may generate attacks that persist, often indefinitely. Attacks are associated with anxiety and depression, but controversy carries on to which incites the other (Saha, 2014). It is possible that some patients may develop a vicious cycle of worsening physical and psychological symptoms (Jones et al., 2013, 2017).

Currently, IBS sufferers are prescribed opioids, anticholinergics, and antidepressants, however with quite suboptimal results. Other compounds have been formulated to interact with serotoninergic circuitry, nevertheless these have been withdrawn from certain markets due to association with ischemic colitis (alosetron, cilansetron) and cardiovascular events (tegaserod), leaving, *de facto*, an urgent clinical need (Ford et al., 2014; Lexicomp Online, 2017).

The Endocannabinoid System (ECS) is known to modulate several functions, including mood, anxiety, and memory retrieval of traumatic events and it directly coordinates GI propulsion, secretion, inflammation, and nociception, providing a rationale for agents capable of interacting with the ECS as treatment candidates for IBS (Russo, 2016).

IRRITABLE BOWEL SYNDROME AND THE ENDOCANNABINOID SYSTEM

Endocannabinoid System in the Bowel

The ECS is ubiquitously expressed in the human body and it actively controls gut homeostasis. The best characterized ECS receptors are the cannabinoid receptors 1 (CB1) and 2 (CB2) (Mackie, 2005).

CB1 has been found in intestinal epithelial and in the enteric nervous system (ENS) (Coutts and Izzo, 2004).

Physiologically, the activation of presynaptic CB1 attenuates large and small bowel muscle tone and inhibits GI motility, mainly by reducing the release of acetylcholine from enteric nerves and also by inhibiting all the components of the peristaltic reflex (Wright et al., 2005). Moreover, CB1 activation, via the purinergic system, inhibits spontaneous ileal contractions and modulate the activity of vagal neurotransmission, reducing intestinal peristalsis (DiPatrizio, 2016).

OPEN ACCESS

Edited by:
Rajeev K. Singla,
Sichuan University, China

Reviewed by:
Vipan K. Parihar,
University of California, Irvine, United States
Marcus Scotti,
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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience

Received: *19 December 2019*
Accepted: *26 March 2020*
Published: *21 April 2020*

Citation:
Brugnatelli V, Turco F, Freo U and Zanette G (2020) Irritable Bowel Syndrome: Manipulating the Endocannabinoid System as First-Line Treatment. Front. Neurosci. 14:371. doi: 10.3389/fnins.2020.00371

CB2 has been found on enteric neurons but it is predominantly expressed by intestinal immune cells (Izzo, 2007). Targeting intestinal CB2 decreases inflammation through the reduction of cytokine and chemokine production from activated immune cells (Wright et al., 2008). In pathophysiological conditions, CB2 controls intestinal motility (Wright et al., 2008) and its activation slows down gut transit (Mathison et al., 2004).

Bot1 and CB2 have been identified in the intestinal neuronal circuitry of the transmission of visceral pain and their activation reduce visceral sensation and nociception (Hohmann and Suplita, 2006).

N-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoyl glycerol (2-AG) are the best characterized endocannabinoids; they are synthesized from membrane phospholipids on demand: AEA is synthesized by N-acyl-phosphatidylethanolamine phospholipase D (NAPE-PLD); and 2-AG by diacylglycerol lipase (DAGL), then they are released and induce a local response by activating CB1 and/or CB2 receptors (the latter being involved mainly in pathophysiological conditions) (Izzo and Camilleri, 2008). These compounds are involved in the control of food intake and hunger (DiPatrizio, 2016; Lee et al., 2016). Specifically, AEA seems to regulate appetite and energy balance, while 2-AG may serve as a general hunger signal (Di Marzo and Matias, 2005; DiPatrizio, 2016). AEA, via CB2, plays also a pivotal role in maintaining immunological health in the gut (Acharya et al., 2017).

Subsequent to their activation, endocannabinoids are inactivated by reuptake from the degradative enzymes fatty acid amide hydrolase (FAAH), located in cells of the myenteric plexus and monoacylglycerol lipase (MAGL), present in the nerve cells and fibers throughout the muscle mucosal layers of the intestine (Di Marzo, 2006).

Inhibition of MAGL and FAAH in the gut significantly reduces experimental colitis in mice, through mechanisms that involve a rise in 2-AG or AEA levels, respectively, and the stimulation of both CB1 CB2 signaling (Massa et al., 2004; Sałaga et al., 2014; Vera and Fichna, 2017).

N-palmitoylethanolamine (PEA) and other N-acylethanolamides (NAEs) are also expressed in the gut (Izzo and Sharkey, 2010). NAEs are atypical endocannabionoids: their structures resemble the classical endocannabinoids and they are preferentially metabolized by FAAH, but they do not bind CB receptors (Izzo and Sharkey, 2010; Ahn et al., 2014). NAEs, especially PEA, are involved in the control of various functions, including food intake, neuroprotection, nociception, and inflammation (Suardíaz et al., 2007; Ahn et al., 2014; Lowin et al., 2015).

Other components of the ECS are the transient receptor potential (TRP) channels, such as TRPV1, TRM8, and others (Storozhuk and Zholos, 2018). These receptors, widely expressed throughout the digestive tract, are involved in numerous processes: taste, chemo- and mechanosensation, thermoregulation, pain and hyperalgesia, mucosal function, gut homeostasis, and control of motility, amongst others (Kaneko and Szallasi, 2014).

GPR55, another potential cannabinoid receptor, seems to be also implicated in gut motility. Its inhibition reduce motility in mice and this effect was reversed by cannabidiol (CBD), but not by CB1 or CB2 receptor antagonists (Li et al., 2013).

The ECS is also an important modulator of the gut-brain axis. In the gut, receptors of the ECS (especially TRPs) are involved in sensory transduction of a large number of external and noxious stimuli (Holzer, 2011). In the brain, the ECS controls nausea and vomiting, especially through CB1 receptors in the dorsal vagal complex of the brainstem, and stress-induced alterations of the ECS have been linked to altered visceral sensations (Sharkey and Wiley, 2016).

The main role of ECS in the GI tract is controlling intestinal hyper-contractility. Moreover, it modulates visceral sensations, intestinal inflammation and gut–brain communications, all functions that appear to be dysregulated in IBS.

IBS and Endocannabinoid Deficiency

Clinical Endocannabinoid Deficiency (CED) has been confirmed as a plausible feature in a series of difficult-to-characterize psychosomatic pathologies, which display hyperalgesia, anxiety, and depression (Russo, 2004, 2016); Migraine, fibromyalgia and IBS fall in this category, often showing comorbidity in the three diagnosis (Nicolodi and Sicuteri, 1996; Sperber et al., 1999; Peres et al., 2001). CED occurs either as a congenital disorder, or as a result of epigenetic changes.

IBS subtypes exhibit distinct variations of the ECS tone. IBS-D patients show genetic alterations affecting endocannabinoid metabolism, variants of the CNR1 and FAAH genes, and lower levels of Oleoylethanolamine (OEA) and PEA compared to healthy subjects (Fichna et al., 2013). Specifically, the CNRI rs806378 CT/TT genotype shows a significant association with colonic transit in IBS-D (Camilleri et al., 2013). Conversely, IBS-C patients show levels of OEA higher than healthy volunteers, and reduced levels of FAAH mRNA in intestinal tissues (Fichna et al., 2013).

Some of these changes may occur as the result of chronic stress, which profoundly impacts the ECS: it silences the Cnr1 gene promoter via an increased methylation by DNA (cytosine-5)-methyltransferase 1, but it also activates the Trpv1 promoter via acetylation (Hong et al., 2015). This results in reduced levels of CB1 and increased levels of TRPV1 in the sensory neurons localized in the pelvic organs, including the colon, which is a feature of visceral pain, as later discussed (Fichna et al., 2013).

Stress in the early-life stage is also an important contributor to IBS development and it is associated with epigenetic changes that lead to visceral hypersensitivity (Moloney et al., 2015). Maternal deprivation increases the expression of the endocannabinoid genes Cnr1, Cnr2a, Cnr2b, and GPR55 in the frontal cortex of male rats, whereas in female rats, increased expression was reported only in the hippocampus, a difference that may underline the prevalence of IBS in the female population (Marco et al., 2014). The relevance of pediatric stress in IBS is supported by the fact that infantile colitis, characterized by visceral sensitivity and dysphoria and resistant to most pharmacotherapies, seem to be offset by the endocannabinoids present in maternal milk, reason for it is hypothesized that this condition may also be a CED (Russo, 2004). Taken these data together, genetic polymorphisms and alterations in gene

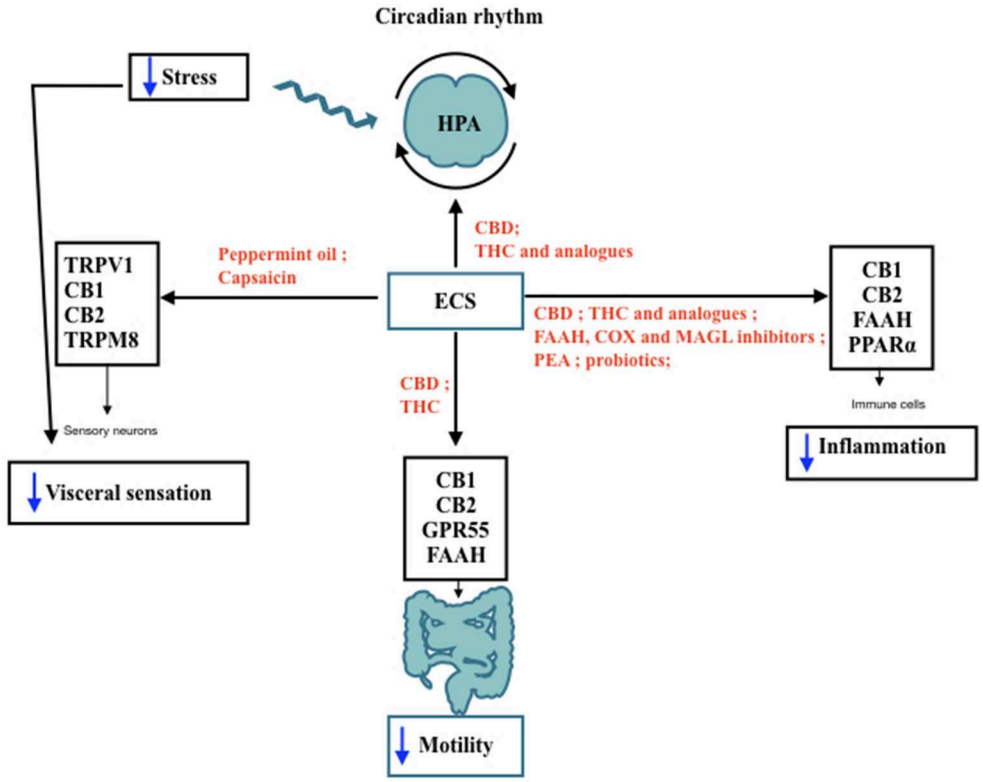

FIGURE 1 | IBS features modulated by ECS. Schematic representation of the Endocannabinoid System (ECS) involvement in IBS features and its interaction with hypothalamic-pituitary-adrenal (HPA) axis throughout day/night. The black arrows indicate the receptors and the target sites controlled by ECS components. In red are shown the agents capable of modulating ECS activities that may be useful to improve IBS symptoms, such as motility, visceral pain, and low-grade inflammation. Blue arrows indicate a decrease in the functions targeted by ECS stimulation. TRPV I, Transient receptor potential vanilloid I; CB I, Cannabinoid Receptor I; CB2: Cannabinoid Receptor 2; TRPM8, Transient receptor potential melastatin 8; CBD, cannabidiol; THC, tetrahydrocannabinol; FAAH, fatty acid amide hydrolase; COX, cyclooxigenase; MAGL, monoacyl glycerol lipase; PEA, palmitoylethanolamide; PPARa: Peroxisome proliferator-activated receptor a.

expression are associated with disturbances in GI motility and sensation, supporting the pathophysiologic significance of alterations in the ECS in the gut (Moloney et al., 2015).

UTILIZING ECS-MODULATING AGENTS FOR IBS

ECS-Modulating Agents

Gut health devoid of pain and maintenance of balanced body weight seems to require a complex interplay between diet, enteric flora, and endocannabinoid balance (Clarke et al., 2012; Russo, 2016). Oral administration of *Lactobacillus acidophilus NCFM* induce a direct increase of the cannabinoid receptors CNR2 mRNA (Rousseaux et al., 2007). This result corresponded with an enhancement of morphine analgesic effect in rats, which was inhibited by administration of the CB2 antagonist, AM-630 (Rousseaux et al., 2007). Cannabinoids may also directly alter the microfloral balance, as underscored by the finding that THC affected the F*irmicutes:Bacteroidetes* ratio in obese mice, preventing their weight gain despite a high-fat diet (Cluny et al., 2015).

The interaction of the microbiome–gut–brain axis is highly dependent on hypothalamic–pituitary–adrenal (HPA) stress modulation, which is dysregulated in IBS patients (Chang et al., 2009). The ECS regulates basal and circadian HPA axis activation (Patel et al., 2004; Liedhegner et al., 2014), and these changes relate to the differences in visceral sensation that feature in IBS (Gschossmann et al., 2001). Linkage of the cannabinoid–vanilloid pathway to the HPA axis has been demonstrated by experiments monitoring rats inoculated with corticosterone, mimicking chronic stress, which developed visceral hyperalgesia (Hong et al., 2011); moreover, as also shown by another stressed rat model (Hong et al., 2009), the levels of AEA and the expression and phosphorylation of TRPV1 increased in the animals, whilst CB1 expression decreased in lumbosacral primary afferent neurons localized in the colon, but not in those innervating the lower extremities (Hong et al., 2009, 2011). AEA is an endogenous agonist at both CB1 and TRPV1 (McPartland et al., 2007), receptors that co-localize in nociceptive primary sensory neurons (Ahluwalia et al., 2000). Activation of CB1 inhibits nociception, whereas agonism at TRPV1 increases pain perception (Malik et al., 2015). Treatment of stressed rats with the CB1 agonist WIN 55,212-2 or the TRPV1 antagonist capsazepine

prevented visceral hyperalgesia (Hong et al., 2009). Similar data have been observed in biopsies from IBS sufferers, which show a 3.5-fold elevation in TRPV1-immunoreactive nerve fibers (Akbar et al., 2008).

Considering this evidences, it has been posited that chronic stress causes down-regulation or loss of CB1, activation of the HPA stress response, anxiety, and induces visceral hyperalgesia that involve region-specific changes in endovanilloid and endocannabinoid pathways in sensory neurons innervating the pelvic viscera (Morena et al., 2016). Thus, a rationale exists for the use of compounds that boost AEA and PEA levels and desensitize TRPV1, to treat hypersensitivity and pain in IBS. While some authors have encouraged the use of the phytocannabinoid cannabidiol (CBD), no clinical trials have tested this hypothesis (Russo, 2004; Pandey et al., 2020). CBD may be an useful therapeutic intervention as it desensitizes TRPV1 and inhibits PEA and AEA hydrolysis and uptake (Bisogno et al., 2001).

Targeting endocannabinoid-degrading enzymes to increase AEA may be an interesting model (Sakin et al., 2015), given their role in the tonic disinhibition of periaqueductal gray region of the brainstem to promote analgesia and chronic stress-induced anxiety (Lau et al., 2014; Sakin et al., 2015). A dual FAAH and COX inhibitor has been shown to increase AEA and PEA levels, reducing features of colitis in mice (Sasso et al., 2015).

Clinical Trials With ECS-Acting Agents

Despite the numerous lines of evidence showing the involvement of ECS in the regulation of IBS features and the promising data from pre-clinical studies, few clinical trials tested the effect of ECS-modulating agents in IBS.

On the other hand, ECS alteration in IBS patients has been clearly documented.

As ECS is known to decrease motility, effects of dronabinol, a non-selective agonist of the cannabinoid receptors, have been tested on IBS patients (Wong et al., 2011). In a 2011 clinical trial, dronabinol reduced fasting colonic motility in all IBS-D patients, particularly those carrying the CB1 receptor polymorphism rs806378 (Wong et al., 2011). Another clinical study carried out a few years later, failed to replicate these results, obtaining only modest delay in motility, maybe for differences in methods (manometry vs. radioscintigraphy) and the lower number of patients enrolled (Wong et al., 2012). Dronabinol can also improve visceral sensitivity and colonic relaxation, as showed in a double-blind, placebo-controlled trial (Esfandyari et al., 2007).

As mentioned before, Fichna et al. showed that lower PEA levels are associated with cramping abdominal pain (Fichna et al., 2013). A randomized placebo-controlled multicenter study assessing the efficacy of PEA in IBS, revealed that PEA may be an useful tool for pain management in this condition (Barbara et al., 2014).

Since visceral hypersensitivity is linked to an increase in Ts regard, a 2011 pilot study found that ingesting capsaicin-containing enteric-coated pills desensitized TRPV1 and decreased the intensity of abdominal pain and bloating in IBS patients vs. placebo (Bortolotti and Porta, 2011). Another study confirmed that TRPV1 desensitization reduced visceral hypersensitivity, symptoms, and abdominal pain (Wouters et al., 2016).

Menthol-induced analgesia and pain relief is mediated mainly by TRPM8 (Liu et al., 2013). This is the rationale for various trials that analyzed the efficacy of peppermint oil (containing menthol) in IBS. Even with some limitations mainly due to the delivery system of peppermint oil in the digestive tract, it turned out an effective treatment capable of improving IBS symptoms, especially abdominal pain, even in children suffering IBS (Kline et al., 2001; Cappello et al., 2007; Merat et al., 2010; Cash et al., 2016).

CONCLUSIONS

Although the pathophysiology of IBS remains unclear, targeting the ECS may represent a promising strategy to modulate gut motility, visceral hyperalgesia, low-grade intestinal inflammation, and gut–brain axis alteration, all features that may improve IBS symptoms onset. It is also evident that both an IBS-diet (Wouters et al., 2016) and a stress-relief practice are required to boost the beneficial effects of any of the agents suggested.

In light of this, agents capable of modulating the ECS may provide a strategy worth attempting even first line treatment for IBS patients (**Figure 1**). This is due to the fact that compounds such as PEA, CBD and peppermint oil display a very large safety profile and have been proving beneficial to improve IBS symptoms (Halford et al., 2018); PEA, peppermint oil, THC and its synthetic analogs may be recommended in IBS patients to improve abdominal spasms, cramps and visceral pain. THC and CBD may alter ECS-driven response to the pathology. However, there is still a wide gap in the current understanding of IBS mechanism and in the use of cannabis containing both CBD and THC as potential therapy, which can only be bridged by randomized clinical trials.

AUTHOR CONTRIBUTIONS

VB and FT contributed to conception and design of the study and wrote sections of the manuscript. VB wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

FUNDING

VB was supported by the grant number is 1973.

ACKNOWLEDGMENTS

The authors would like to acknowledge the contribution of Adrian Devitt-Lee, University College of London.

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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.

frontiers in Pharmacology

MINI REVIEW
published: 30 April 2020
doi: 10.3389/fphar.2020.00561

A Balanced Approach for Cannabidiol Use in Chronic Pain

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Cannabidiol (CBD), the major non-psychoactive constituent of *Cannabis sativa* L., has gained traction as a potential treatment for intractable chronic pain in many conditions. Clinical evidence suggests that CBD provides therapeutic benefit in certain forms of epilepsy and imparts analgesia in certain conditions, and improves quality of life. CBD continues to be Schedule I or V on the list of controlled substances of the Drug Enforcement Agency of the United States. However, preparations labeled CBD are available publicly in stores and on the streets. However, use of CBD does not always resolve pain. CBD purchased freely entails the risk of adulteration by potentially hazardous chemicals. As well, CBD use by pregnant women is rising and poses a major health-hazard for future generations. In this mini-review, we present balanced and unbiased pre-clinical and clinical findings for the beneficial effects of CBD treatment on chronic pain and its deleterious effects on prenatal development.

Keywords: cannabidiol, CBD, cannabis, chronic pain, teratogenicity

OPEN ACCESS

Edited by:
Rajeev K. Singla,
Sichuan University, China

Reviewed by:
Ashok K. Dubey,
University of Delhi, India
Francisco Mendonça Mendonça-Junior,
State University of Paraíba, Brazil

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *27 February 2020*
Accepted: *14 April 2020*
Published: *30 April 2020*

Citation:
Argueta DA, Ventura CM, Kiven S, Sagi V and Gupta K (2020) A Balanced Approach for Cannabidiol Use in Chronic Pain. Front. Pharmacol. 11:561. doi: 10.3389/fphar.2020.00561

INTRODUCTION

Cannabis and its components are being widely used for chronic pain, especially given the multifaceted and persistent nature of chronic pain in many conditions (Kalant, 2001). Cannabidiol (CBD), one of the major phytocannabinoids, has gained significant attraction because it is devoid of the psychoactive effects associated with tetrahydrocannabinol (THC), another major constituent of cannabis (Leweke et al., 2012). With the recent rescheduling (Schedule V) of CBD as Epidiolex for the treatment of Dravet and Lennox-Gastaut syndromes there has been a major shift in the view of these ancient molecules for their medicinal potential (Laux et al., 2019). Preclinical and clinical studies have indicated a potential benefit of CBD use in chronic pain associated with multiple conditions (Wade et al., 2003). However, increasing access to cannabis derived products especially CBD partly because of their approval for recreational and medicinal use in the United States poses risks with inadvertant side-effects from overuse, contamination with adulterants in preparation or harsh chemicals in the plant cultivation, and its teratogenicity in the offspring of users (Bonn-Miller et al., 2017; Young-Wolff et al., 2017; Rubin, 2019). In this mini-review we will evaluate literature discussing CBD use in treating intractable pain and the potential hazards of its overuse and/or misuse (see **Figure 1**).

CHRONIC PAIN IN THE UNITED STATES

Chronic pain affects between 50 and 116 million American adults, a staggering number that surpasses those affected by heart disease, cancer, and diabetes combined (Committee on Advancing Pain Research Care and Education, 2011; Nahin, 2015; NIH, 2020). In addition, these reports conclude that chronic pain costs between $560 and $635 billion annually in both medical expenses and lost productivity. Although there have been some recent therapeutic advances, many patients with chronic pain develop tolerance to conventional medical treatments or suffer adverse effects from widely used prescription medications, such as non-steroidal anti-inflammatory agents or opiates, that have high addictive potential (Labianca et al., 2012). As early as 2003, formulations containing CBD have been used in the clinic to study its efficacy in reducing pain when traditional treatment options have failed.

RELIABILITY AND SAFETY OF CANNABIDIOL LABELED PRODUCTS

Human use of *Cannabis sativa* L. for rituals and medicine dates back millennia, and it has made recent advances in treatment of varied conditions (Kalant, 2001; Whiting et al., 2015; Aviram and Samuelly-Leichtag, 2017; Ren et al., 2019). CBD is the major non-psychoactive constituent of cannabis and is also found in hemp, a subspecies of *Cannabis sativa* that does not produce psychoactive compounds in significant amounts (Pertwee, 2006; Hilderbrand, 2018). With the exception of Epidiolex, a Schedule V preparation, which is a pharmaceutical CBD extract from the plant, cannabis-derived CBD still remains a Schedule I substance according to the United States (US) Drug Enforcement Administration (Drug Enforcement Administration, 2018). However, the US Hemp Farming Act of 2018 legalized the cultivation and refinement of hemp and its constituents, thus beginning a trend of mass marketing for CBD products both legal and illegal (Hemp Production and the 2018 Farm Bill, 2019; Mead, 2019). In states where cannabis has been approved for recreational and/or medical use, there are efforts to equip dispensary staff with scientific knowledge to make evidence-based recommendations, but these efforts are limited and often overshadowed by anecdotal understanding of CBD and other cannabinoids (Haug et al., 2016; Piermarini and Viswanath, 2019). Of major concern, CBD-labeled products have flooded the markets, including, but not limited to, inhalants, bath salts, cookies, ointments, and liquids, for human use. Many forms tout medicinal value for claims that have not been scientifically evaluated. Reports indicate that the cannabinoid content in products purchased online were only accurate in 26 of the 84 products tested (Bonn-Miller et al., 2017). In a more recent report, safety of using unregulated CBD products has been questioned because, of 20 popular CBD products tested by CannaSafe, a cannabis-testing company in California, only 3 contained the contents claimed on the labels (Rubin, 2019). Of these, 2 products had no CBD, and about half of the CBD products had less than 20% of the CBD content claimed. Additionally, toxic gases and solvents were reported in some of these CBD products. Thus, these unregulated products labeled CBD may be a serious health hazard. An urgent need is to regulate CBD products after reliable testing to prevent the inadvertent harmful effects of unidentified constituents of products labeled CBD.

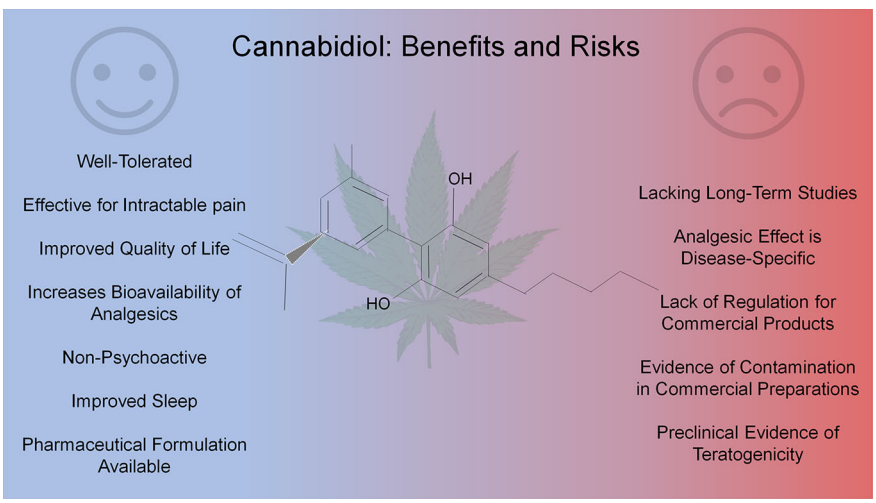

FIGURE 1 | Pros and cons of cannabidiol use in chronic pain.

CLINICAL OUTCOMES FOR CANNABIDIOL IN INTRACTABLE CHRONIC PAIN

Since the early 2000s, clinical trials involving CBD for the treatment of chronic pain have shown effects ranging from placebo-equivalent to highly effective; many of these studies have been well-designed randomized, double-blinded, and placebo-controlled. In a mixed cohort of patients suffering from intractable pain due to multiple sclerosis, spinal cord injury, brachial plexus injury, and limb amputation, CBD treatment significantly reduced pain on a visual analog scale (Wade et al., 2003). However, these studies were often limited by small cohorts, and the varied disease states indicated that the beneficial effects of CBD are context dependent, which was illustrated in a study where treatment did not improve outcomes in patients suffering from Crohn's disease (Naftali et al., 2014). CBD was also seemingly effective in treatment of chronic pain associated with kidney transplantation and when given topically to patients suffering from peripheral neuropathy of their lower extremities (Cuñetti et al., 2018; Xu et al., 2019). As well, in patients with fibromyalgia, CBD treatment decreased pain by more than 30% in significantly more patients than placebo (Van De Donk et al., 2019). In studies of generalized chronic pain, CBD treatment did not significantly reduce measures of pain, however there was consistent improvement in patient-reported quality of life and quality of sleep (Notcutt et al., 2004; Capano et al., 2020). A New Zealand study on the safety of CBD treatment in 400 non-cancer chronic pain patients indicated its safety for prolonged use, which was accompanied by self-reported improvements in pain and quality of life (Gulbransen et al., 2020).

The majority of clinical studies for the treatment of intractable chronic pain with CBD typically utilized a combination of 1:1 CBD : THC, which was often in the form of the well-tolerated oromucosal spray Sativex (Nabiximols in the US) (Johnson et al., 2013; Sellers et al., 2013). Combination of the two often improved upon the deleterious and psychoactive effects of THC-only administration (Ueberall et al., 2019). The CBD : THC formulations were effective at reducing mean pain scores in chronic pain patients with multiple sclerosis, improved neurophysical measurements in response to noxious stimuli, reduced intractable chronic pain in advanced cancer, and improved refractory/neuropathic pain following failed spinal cord surgery (Rog et al., 2005; Conte et al., 2009; Johnson et al., 2010; Portenoy et al., 2012; Mondello et al., 2018). There is contradictory evidence that CBD : THC treatment does not always relieve chronic pain in patients with brachial plexus avulsion or advanced-cancer, as evidenced by studies in two-independent cohorts, thus indicating the heterogeneity in disease contexts for which cannabinoids may be effective; of note, although pain was not significantly improved, patients in these studies indicated an improved quality of life (Berman et al., 2004; Fallon et al., 2017; Lichtman et al., 2018). There is a demonstrated need to further understand the mode of action of CBD, and these results are promising, but efficacy of treatment must also be evaluated in other disease states that produce chronic pain such as diabetic neuropathy, rheumatic diseases, and sickle cell disease (Fitzcharles et al., 2016).

MECHANISTIC INSIGHTS FOR CANNABIDIOL TREATMENT OF CHRONIC PAIN

Few preclinical studies have been performed to evaluate the mechanism of analgesia for CBD treatment of chronic pain. Currently available studies rely on rodent and *in vitro* models but suggest molecular pathways that may be used to enhance CBD use in the clinic, or offer alternative approaches for higher efficacy. Evidence strongly supports that prolonged treatment (i.e. > 7 days) with CBD alleviates chronic pain caused by chronic constriction injury of the sciatic nerve in rats and mice in a cannabinoid receptor-independent manner, and treatment is coincident with decreased hepatic cytochrome p450 and intestinal P-glycoprotein that may increase bioavailable circulating CBD (Costa et al., 2007; Comelli et al., 2008; Casey et al., 2017; Abraham et al., 2019). *In vitro* studies using human embryonic kidney cells reveal that at high doses CBD interacts with and selectively activates α_1- and α_1ß-glycine receptors, but these results have yet to be confirmed *in vivo* (Ahrens et al., 2009; Foadi et al., 2010). Alternatively, there is preliminary evidence that CBD may interact with α_3-glycine receptors to reduce inflammation and hyperalgesia following simulated neuropathic pain by ligation of the L5 spinal nerve in adult Sprague-Dawley rats (Xiong et al., 2012). CBD also attenuates hyperalgesia in a mouse model of diabetic neuropathy with data suggesting that treatment reduced inflammatory milieu (Toth et al., 2010). Mouse models of pain associated with chemotherapy were simulated by Paclitaxel treatment, in which CBD produced an analgesic and anti-inflammatory effect *via* interactions with spinal cord 5-HT(1A) receptors (Ward et al., 2014; King et al., 2017). CBD also exerts analgesia in a 5-HT(1A)-dependent manner in streptozotocin-induced diabetic neuropathy in rats (Jesus et al., 2019). Similar to human studies, CBD did not produce complete analgesia in all models of chronic pain; in a cisplatin-induced mouse model of neuropathy, CBD attenuated but did not prevent hyperalgesia (Harris et al., 2016). Mechanical hyperalgesia was improved by CBD treatment following traumatic brain injury in mice, myofascial pain in rats, and 6-hydroxydopamine-induced mouse model of Parkinson's disease, however these studies require follow-up to inspect potential mechanisms of action (Belardo et al., 2019; Wong and Cairns, 2019; Crivelaro Do Nascimento et al., 2020). The preclinical work being done to disentangle the mechanisms of CBD in providing analgesic support in chronic pain is flourishing, but much remains in the wake of chronic disease and enhancing our understanding of the mechanisms at play.

PUBLIC HEALTH HAZARDS AND TERATOGENICITY OF CANNABIS PRODUCTS

Rising legalization and use of medical and recreational cannabis and CBD products raises significant health concerns with regards to both unregulated sources of these products discussed above as well as the health effects of prolonged usage. A recent multistate outbreak of coagulopathy from synthetic cannabinoids has been

traced to the presence of long-acting anticoagulant rodenticides in "fake weed" (Arepally and Ortel, 2019). Furthermore, the US Centers for Disease Control and Prevention has increased awareness of the risks of severe pulmonary disease associated with use of electronic cigarette devices to "vape" tobacco and cannabis (Centers for Disease Control and Prevention, August 23, 2019). The effects of long-term cannabinoid use are especially unclear in pregnant women, in whom potential teratogenic effects could have implications on future generations. Cannabis and CBD use are rising amongst pregnant women. An estimated 4% of pregnant women use cannabis, and in California, which recently legalized cannabis, about 20% in a cohort of 18- to 24-year-old pregnant women reported using cannabis products in retrospective studies (Young-Wolff et al., 2017). These numbers are likely to rise as legalization continues throughout the US, and pharmaceutical strength preparations become available for several conditions, and because of the availability of CBD through stores and online sources (Millar et al., 2019). While several studies have focused on THC during pregnancy, investigation focused on the effects of CBD usage by pregnant women before, during, and/or after pregnancy are rare. Thus, there is an unmet need to examine the potential effects of CBD on embryonic and fetal development and the postnatal health of children exposed to CBD before birth. We will summarize here conclusions from both animal and human studies on some possible effects of CBD prenatally, perinatally, and postnatally.

CBD use during early gestation could pose a risk to critical pre-pregnancy and early pregnancy events. Successful pregnancy depends on reciprocal interactions between a competent embryo and a receptive endometrium in the mother. In early gestation CBD, THC, and cannabinol are thought to inhibit embryo implantation and placenta development by altering endometrial receptivity (Neradugomma et al., 2019). However, this effect has yet to be seen outside transformed human endometrial cell models. Exposure to CBD in chick embryos decreases the viability of the embryo by 50% to 80% dependent on CBD concentration and can delay embryonic development (Gustafsson and Jacobsson, 2019). Similar delays in embryonic development have been reported in zebrafish embryos exposed to CBD albeit without the decrease in viability (Valim Brigante et al., 2018). Teratogenicity of CBD has been reported in mice where prenatal exposure leads to an increase in craniofacial malformations and eye defects (Fish et al., 2019). Interestingly, these teratogenic effects are similar though milder than those observed for alcohol, THC, and the synthetic cannabinoids HU-210 and CP55,940 countering the popular perception that CBD is an unequivocally safe alternative to THC and other cannabis constituents (Fish et al., 2019). In humans, retrospective meta-analysis has determined that *in utero* exposure to cannabis is associated with a decrease in birth weight and increased need for neonatal intensive care in infants (Gunn et al., 2016). This effect is likely due in part to the effects of CBD as low birth weights in mice offspring have been reported in response to prenatal CBD exposure exclusively (Fish et al., 2019). The observed teratogenic effects of CBD exposure may be due to the compound itself and/or due to CBD working synergistically with other teratogenic compounds perhaps by enhancing permeability of xenobiotics through the human placental barrier thereby increasing fetal exposure (Feinshtein et al., 2013).

Effects on hormonal and reproductive function following maternal exposure to CBD have been reported in male mice. CBD exposed mice had lower testicular weights and lower overall levels of testosterone (Dalterio et al., 1984). These effects are in line with reports of hormonal and reproductive effects due to postnatal exposure to CBD or cannabis in rats and monkeys. Chronic doses of THC or CBD in rat suppress hepatic testosterone oxidation by selective inhibition of male-specific cytochrome p450 in the adult male rat (Narimatsu et al., 1988). Chronic doses of CBD in rat also cause a significant reduction in testosterone formation and a decrease in testicular enzyme activity (List et al., 1977). In both rhesus monkeys and rats, gonadal function is altered due to exposure to THC or CBD which leads to hormonal imbalance including a decrease in testosterone in male rats and an increase in follicle-stimulating hormone in male monkeys (Rosenkrantz and Esber, 1980). Together, these data suggest that CBD may influence spermatogenesis and libido in males.

Maternal exposure to CBD is also likely to cause neurochemical changes in the brain of the offspring. The α1-adrenergic and D2-dopaminergic receptors in the cerebral cortex and striatum of rats exposed prenatally to either CBD or THC exhibited smaller binding affinities for their respective ligands and hypothalamic dopamine levels in mice have been observed to be greatly depleted in CBD-exposed males as well (Dalterio et al., 1984; Walters and Carr, 1988). Overall, these studies suggest that prenatal exposure to CBD is likely to alter the production of testosterone, the function of the male gonads, and the receptor ligand interactions in the brain of offsprings.

CONCLUSION

Rising prevalence of the non-psychoactive cannabinoid CBD presents an opportunity for the treatment of intractable chronic pain for which primary treatments are insufficient or not possible. As depicted by the studies reviewed herein, the use of CBD is context-specific, and it should not be used indiscriminately (see **Table 1**). Preliminary mechanistic studies indicate conservation of function *via* modulation of hepatic cytochrome p450 leading to increased bioavailability of endogenous mediators of pain (i.e. serotonin) and exogenous analgesics (i.e. THC). Therefore, it is important to continue studies into the conditions for which CBD may be effective as a treatment *via* novel actionable targets. Simultaneously, the growing access to unregulated CBD products, which may be adulterated with potentially toxic compounds, requires regulation and education about CBD for its potential benefits and/or adverse effects in health and disease. This is especially the case in pregnant women, which raises the highest possible risk for the developing fetus and future offsprings.

TABLE 1 | Consequence of Cannabidiol treatment in preclinical and clinical settings.

Source	Species	Effect	References
		Beneficial Effects	
CBD ([1]*Sigma*)	HEK cells	Activation of α1 and α1ß -glycine receptors	Ahrens et al., 2009[1]; Foadi et al., 2010[1]
CBD ([1]*Enecta Group;* [2]*Cayman;* [3]*NIH;* [4]*NS*)	Mice	↓inflammation; ↓hyperalgesia	Belardo et al., 2019[1]; [4]Crivelaro Do Nascimento et al., 2020; (Toth et al., 2010)[2]; (Ward et al., 2014; King et al., 2017); [3](Harris et al., 2016);
CBD ([1]NIH; [2]THC Pharm; [3]GW Pharma; [4]Cayman; [5]NS)	Rat	↓inflammation; ↓hyperalgesia; ↓ hepatic cytochrome p450	(Costa et al., 2007; Comelli et al., 2008; Casey et al., 2017; Abraham et al., 2019)[3]; (Xiong et al., 2012)[1]; (Jesus et al., 2019)[5]; Wong and Cairns, 2019[4]
CBD ([1]Stanley Brothers; [2]Bedrocan International; [3]Ananda Professional; [4]Tilray; [5]NS)	Humans	Patient-reported: ↓ chronic pain; ↑ quality of life; ↑ quality of sleep	(Wade et al., 2003)[5]; (Cuñetti et al., 2018; Xu et al., 2019); (Van De Donk et al., 2019)[2]; (Notcutt et al., 2004; Capano et al., 2020)[5]; (Gulbransen et al., 2020)[4]
1:1 CBD : THC (GW Pharma.)	Humans	improved refractory/neuropathic pain; Patient-reported ↓ chronic pain; ↑ quality of life; Improved responses to noxious stimuli	Johnson et al., 2013; Sellers et al., 2013; Ueberall et al., 2019; (Rog et al., 2005; Conte et al., 2009; Johnson et al., 2010; Portenoy et al., 2012; Mondello et al., 2018) (Berman et al., 2004; Fallon et al., 2017; Lichtman et al., 2018); NCT01424566; NCT01361607; NCT01262651; NCT01606189; NCT01337089
		Adverse Effects	
CBD (NS)	HES model cells	Adversely impact embryo implantation; Delay placenta development	Neradugomma et al., 2019
CBD (The Hebrew University)	MCF7/P-gp, BeWo and Jar cells	↑ placental xenobiotic permeability	Feinshtein et al., 2013
CBD (BSPG Pharm.)	Zebrafish embryos	Delay in embryo development; ↑ embryo activity	Valim Brigante et al., 2018
CBD (Tocris)	Chick embryos	50–80% ↓ in embryo viability; Delay in embryo development	Gustafsson and Jacobsson, 2019
CBD (Cayman Chemical)	Mice offspring	↑ Eye defects; ↓ birth weight; Abnormal craniofacies; ↓ testicular weight; ↓ testicular testosterone levels ↓ hypothalamic dopamine levels	Dalterio et al., 1984; Fish et al., 2019
CBD ([1]NIH; [2]Kyushu University)	Rat offspring	↓ hepatic cytochrome p450 ↓ testicular testosterone levels ↓ binding affinities for α1-adrenergic and D2-dopaminergic receptors	List et al., 1977[1]; Rosenkrantz and Esber, 1980[1]; Narimatsu et al., 1988[2]; Walters and Carr, 1988[1]
CBD (NIH)	Rhesus monkeys	↑ follicle-stimulating hormone; hormonal imbalance	Rosenkrantz and Esber, 1980
Cannabis (NS)	Humans	↓ birth weight ↑ need for neonatal intensive care	Gunn et al., 2016

CBD, cannabidiol; THC, tetrahydrocannabinol; HEK cells, human embryonic kidney cells; HES cells, human endometrial stroma cells; JAr cells, human choriocarcinoma cells; BeWo cells, human placental cell line from choriocarcinoma; MCF7/P-gp cells, MCF-7 breast carcinoma cells expressing P-glycoprotein; NIH, National Institutes of Health National Institute on Drug Abuse; NS, not specified. The superscripted numbers corresponds with the vendor in the source and the cited article within that row. The symbols "↓" and "↑" indicate worsening and improvement of outcomes, respectively.

Unfortunately, efforts to discuss the dangers of CBD use have been severely lacking and require immediate attention to prevent the irreparable harm to the masses from the tsunami of CBD products.

AUTHOR CONTRIBUTIONS

DA wrote the manuscript and prepared it for communication. CV co-wrote the manuscript and prepared the table. SK reviewed the contents and prepared the figure. VS contributed to the structure and edited the manuscript. KG defined the content, searched the literature, supervised the writing, and edited the manuscript.

FUNDING

We gratefully acknowledge funding from NIH grants U18 EB029354 and HL147562 to KG. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

ACKNOWLEDGMENTS

We acknowledge creative commons for their licensing of images used in the figure. All images are freely available through pixabay.com under creative commons license.

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Conflict of Interest: KG reports grants from Grifols, Cyclerion, and 1910 Genetics, and honorarium from Novartis, Tautona Group, and CSL Behring, outside the submitted work.

The remaining 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.

frontiers in Pharmacology

SYSTEMATIC REVIEW
published: 29 May 2020
doi: 10.3389/fphar.2020.00777

Antinociceptive Activity of Chemical Components of Essential Oils That Involves Docking Studies: A Review

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Introduction: Pain is considered an unpleasant sensory and emotional experience, being considered as one of the most important causes of human suffering. Computational chemistry associated with bioinformatics has stood out in the process of developing new drugs, through natural products, to manage this condition.

Objective: To analyze, through literature data, recent molecular coupling studies on the antinociceptive activity of essential oils and monoterpenes.

Data source: Systematic search of the literature considering the years of publications between 2005 and December 2019, in the electronic databases PubMed and *Science Direct*.

Eligibility Criteria: Were considered as criteria of 1) Biological activity: non-clinical effects of an OE and/or monoterpenes on antinociceptive activity based on animal models and *in silico* analysis, 2) studies with plant material: chemically characterized essential oils and/or their constituents isolated, 3) clinical and non-clinical studies with *in silico* analysis to assess antinociceptive activity, 4) articles published in English. Exclusion criteria were literature review, report or case series, meta-analysis, theses, dissertations, and book chapter.

Results: Of 16,006 articles, 16 articles fulfilled all the criteria. All selected studies were non-clinical. The most prominent plant families used were Asteraceae, Euphorbiaceae, Verbenaceae, Lamiaceae, and Lauraceae. Among the phytochemicals studied were α-Terpineol, 3-(5-substituted-1,3,4-oxadiazol-2-yl)-N′-[2-oxo-1,2-dihydro-3H-indol-3-ylidene] propane hydrazide, β-cyclodextrin complexed with citronellal, (–)-α-bisabolol, β-cyclodextrin complexed with farnesol, and p-Cymene. The softwares used for docking studies were Molegro Virtual Docker, Sybyl®X, Vlife MDS, AutoDock Vina, Hex Protein Docking, and AutoDock 4.2 in PyRx 0.9. The molecular targets/complexes used were Nitric Oxide Synthase, COX-2, GluR2-S1S2, TRPV1, β-CD complex, CaV_1, $CaV_{2.1}$, $CaV_{2.2}$, and $CaV_{2.3}$, 5-HT receptor, delta receptor, kappa receptor, and MU (μ)

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *24 February 2020*
Accepted: *11 May 2020*
Published: *29 May 2020*

Citation:
Assis DB, Aragão Neto HC, da Fonsêca DV, de Andrade HHN, Braga RM, Badr N, Maia MdS, Castro RD, Scotti L, Scotti MT and de Almeida RN (2020) Antinociceptive Activity of Chemical Components of Essential Oils That Involves Docking Studies: A Review. Front. Pharmacol. 11:777. doi: 10.3389/fphar.2020.00777

receptor, alpha adrenergic, opioid, and serotonergic receptors, muscarinic receptors and $GABA_A$ opioid and serotonin receptors, 5-HT_3 and M_2 receptors. Many of the covered studies used molecular coupling to investigate the mechanism of action of various compounds, as well as molecular dynamics to investigate the stability of protein-ligand complexes.

Conclusions: The studies revealed that through the advancement of more robust computational techniques that complement the experimental studies, they may allow some notes on the identification of a new candidate molecule for therapeutic use.

Keywords: nociception, essential oils, docking, *in silico*, molecular target

INTRODUCTION

Pain was conceptualized for the first time in 1986 by the International Association for the Study of Pain (IASP); being defined as an response of the Central Nervous System to a tissue injury or an emotional change and classifiable as acute or chronic (McKune et al., 2015), adaptive or non-adaptive (Hellyer et al., 2007; de Oliveira Alves et al., 2017), and as physiological or pathological (Klaumann et al., 2008). The neural process of coding and processing noxious stimuli is called nociception (Naidu and Pham, 2015).

Various animal models have been developed with the purpose of understanding the mechanisms involved in nociception, including hot-plate, Hargreaves, von Frey, Randall-Selitto, capsaicin, glutamate, and formalin methods among others. Most of the non-clinical experimental models involve the use of stimuli; of a chemical, thermal, or mechanical nature where characteristic behaviors reflecting the nociceptive response are recorded. Animal models should be able to predict the effects of new drugs in humans, as well as clinical analgesic sensitivity. For this purpose, the animals are usually rodents (mice and rats), but alternative species have been used (Barrot, 2012; Barrett, 2015).

Computational chemistry associated with bioinformatics has led the process of developing new drugs with various activities, especially analgesia. Molecular docking is a computational technique that predicts the positioning (orientation and conformation) of a ligand (drug or molecule of therapeutic interest) in a target interaction site, and aids in the understanding of biological activity, by both explaining and predicting possible interactions, and helping to evaluate pharmacological properties and relations between chemical structure and biological activity (Chen and Zhi, 2001; Kirchmair et al., 2012). Thus, for molecules of therapeutic interest, molecular docking serves as a predictive model that can contribute to *in vivo* evaluations of pharmacological activity.

Since natural products derived from plants have a wide variety of bioactive chemical compounds, they present an important alternative in the search for therapeutic agents. Essential oils and their constituents, monoterpenes and sesquiterpenes, have several pharmacological properties, among them the potential analgesic effect. (Bahmani et al., 2014; Gomathi and Manian, 2015; de Oliveira Júnior et al., 2018). However, these products are often unexplored (Leonardão et al., 2016), and this fact collaborates to promote the scientific hypothesis related to the antinociceptive effect, including performing the molecular anchorage studies.

The objective of this study was to conduct a survey of recent molecular docking studies involving the antinociceptive activity of essential oils and monoterpenes.

MATERIALS AND METHODS

The Question Under Study

This systematic review was carried out to address the specific question: "What are the scientific findings associating non-clinical animal studies and *in silico* analysis when evaluating the antinociceptive activity of essential oils?"

Search Strategy and Selection of Studies

The guidelines of the PRISMA guide of Systematic Reviews and Meta-Analyses) (Liberati et al., 2009) were followed. Two databases were systematically searched for experimental antinociceptive *in vivo* studies and *in silico* analysis of essential oil activity—as published through December 20, 2019 (**Table 1**).

Eligibility Criteria

Systematic screening of the articles was performed by two independent examiners according to the following inclusion criteria:

1. Biological activity: non-clinical effect of essential oil (EO) on nociceptive activity based on animal models and *in silico* analysis.
    a. Primary outcomes of interest: acetic acid-induced abdominal writhing, formalin-induced nociception, orofacial formalin-induced nociception test, chronic muscle pain test, tail-flick test, hot plate test, tail immersion test, and von Frey test.
    b. Secondary outcomes of interest—Studies of the antinociceptive mechanisms of action: Involvement of opioid receptors, Involvement of ATP-sensitive K_{ATP}channels, *in silico* (molecular docking) analyses.
2. Plant material and chemical elucidation: chemically characterized essential oils and/or their isolated constituents from aromatic plants.

3. Study design: non-clinical animal studies, clinical studies, and *in silico* analysis to evaluate the antinociceptive activity of essential oils.
4. Methodological quality: accuracy of methods and outcomes; internal and external validity.
5. Language: for articles written in English, in cases of inconsistency, the examiners would give the final verdict on which articles should be included in this review would be reached by consensus.

Study Selection

To compose the sample of this review, a database was initially searched according to the strategies mentioned in **Table 1**. In this phase of the search, the results were compared, and duplicated articles found between the databases were excluded, and studies that were explicitly different from the criteria and objective of this review were excluded through the evaluation of titles and abstracts. Thus, 16 articles were included in this review, which deals with evaluations of monoterpene and sesquiterpene antinociceptive activity *via in silico* docking studies from 2011 to December 2019.

Data Collection and Analysis

The following variables were collected: plant family, plant species, source, phytochemical, molecular target, route of administration, animal species, antinociceptive test, software, results, country, and reference.

This information is detailed in **Tables 2** and **3**. The research data were analyzed based on the ARRIVE guidelines (Animal Research: Reporting of *In Vivo* Experiments) published by the Animal Center for the Replacement, Refinement & Reduction of Animals in Research (Kilkenny et al., 2010).

TABLE 1 | Search mechanism and bibliographic databases used to choose the articles for this review.

Primary bibliographic sources	Search strategy (descriptors/combinations with Boolean operators)
Science Direct (2005–2019)	• (essential oils) AND (monoterpenes OR sesquiterpenes) AND (antinociceptive)
	• (essential oils) AND (monoterpenes OR sesquiterpenes) AND (antinociceptive) AND (docking)
	• (essential oils) AND (monoterpenes OR sesquiterpenes) AND (*in silico*)
	• (essential oils) AND (monoterpenes OR sesquiterpenes) AND (antinociceptive) AND (*in silico*)
	• (essential oils) AND (monoterpenes) OR (sesquiterpenes) AND (pain) AND (*in silico*)
	• (essential oils) AND (monoterpenes) OR (sesquiterpenes) AND (pain) AND (docking)
Pubmed (2005–2019)	• (essential oils) AND (monoterpenes OR sesquiterpenes) AND (antinociceptive)
	• (essential oils) AND (monoterpenes OR sesquiterpenes) AND (antinociceptive) AND (docking)
	• (essential oils) AND (monoterpenes OR sesquiterpenes) AND (*in silico*)
	• (essential oils) AND (monoterpenes OR sesquiterpenes) AND (antinociceptive) AND (*in silico*)
	• (essential oils) AND (monoterpenes) OR (sesquiterpenes) AND (pain) AND (*in silico*)
	• (essential oils) AND monoterpenes) OR sesquiterpenes) AND (pain) AND (docking)

RESULTS

The initial search of the databases (with the strategies presented in **Table 1**) allowed the identification of 16,006 citations. After filtering the remaining texts included English language articles and various complete free articles; review studies were excluded, leaving 1326 articles, from which a selection based on titles and abstracts for the inclusion criteria mentioned above was performed. At this stage, 1289 articles were excluded, leaving only 37. Upon removal of 13 repeated articles, 24 remained. These studies were subsequently completely read, and finally, 16 articles were selected; 8 articles did not meet all inclusion criteria and were excluded. The selection process can be better visualized in **Figure 1** below, the search flowchart.

The studies identified were concentrated between 2011 and 2019 and are considered current.

There was variability in the study regions for the selected manuscripts, with 75% of the papers coming from the American continents, with 6.25% from North America and 68.75% from South America, 12.5% of the articles originated from the European continent, and 12.5% from the Asian continent.

Of the countries in North America, Mexico represented 6.25% of the publications, in South America, Brazil represented 68.75%, in the European continent, Italy represented 6.25% and Serbia represented 6.25%. In Asia, India and Indonesia stood out, both represented 6.25% of publications.

The most prominent plant families used in the studies identified were *Asteraceae*, (García et al., 2011; Radulović et al., 2015; Leite et al., 2019); *Euphorbiaceae*, (De Oliveira Junior et al., 2017; de Oliveira junior et al., 2018); *Verbenaceae*, (Siqueira-Lima et al., 2017); *Lamiaceae*, (Quintans-Junior et al., 2018), and *Lauraceae* (Sumiwi et al., 2015).

Of the species, *Croton conduplicatus* Kunth prevailed (De Oliveira Junior et al., 2017; de Oliveira junior et al., 2018); with *Vanillosmopsis arborea* Baker (Leite et al., 2019); then *Achillea falcata* L.; (Radulović et al., 2015); *Ageratin glabrata*; (García et al., 2011); *Hyptis pectinata*; (Quintans-Junior et al., 2018); *Cinnamomum; sintoc bl.*; (Sumiwi et al., 2015); and *Grateful Lippia Schauer* (Siqueira-Lima et al., 2017).

Among the phytochemicals studied were α-Terpineol (Oliveira et al., 2016; Gouveia et al., 2018); 3-(5-substituted-1,3,4-oxadiazol-2-yl)-N′-[2-oxo-1,2-dihydro-3H-indol-3-ylidene] propane hydrazide (Kerzare et al., 2016); β-cyclodextrin (CT-βCD) complexed with citronellal (CT) (Santos et al., 2016); (−)-α-bisabolol (Melo et al., 2017; Teixeira, et al., 2017); β-cyclodextrin complexed with farnesol (Silva et al., 2017), and p-Cymene (Santos et al., 2019).

All of the selected studies were non-clinical, the animals used in the studies were Swiss Mice, BALB/c, Wistar, and Sprague Dawley Rats. The tests for evaluation of antinociceptive activity included chemical nociception induction tests: Formalin Test (De Oliveira Junior et al., 2017; de Oliveira Júnior et al., 2018;

TABLE 2 | Information ethnobotanical, molecular, pharmacological, and docking programs used *in vivo* studies involving the antinociceptive activity of essential oils.

Plant Family	Plant Species	Phytochemical	Molecular target	Source	Route of administration	Animal(s) species	Antinociceptive test	Software	Results	Country	Reference
Verbenaceae	*Lippia grata Schauer*	*Bicyclogermacrene*	Alpha Adrenergic, Opioid and serotoninergic receptors.	Northeastern Brazil	Gavage	Swiss	Chronic muscle pain model	Molegro Virtual Docker v. 6.0.1.	Anti-hyperalgesic activity of LG-β-CD seems to involve opioid and serotoninergic receptors	Brazil	Siqueira-Lima et al., 2017.
Lauraceae	*Cinnamomum sintoc bl.*	*Eugenol*	COX-2.	Yogyakarta district	Ip	Swiss albino mice	Acetic acid induced writhing method	–	Eugeunol presented better molecular prediction for naproxen (control ligand-blue carbon) at binding site of COX-2.	Indonesia	Sumiwi et al., 2015.
Euphorbiaceae	*Croton conduplicatus* Kunth	Spathulenol Caryophyllene oxide	Muscarinic receptors and GABAA.	Northeastern Brazil	Ip	Male Swiss mice	Acetic-acid-writhing-induced nociception, Formalin-induced nociception, Hot plate test.	Molegro Virtual Docker, v. 6.0.1.	Majority EO compounds (1,8-cineole, spathulenol, caryophyllene oxide and p-cymene) were dose-dependent and appear to involve muscarinic, opioid, and GABAA receptors.	Brazil	de Oliveira Júnior et al., 2018.
Euphorbiaceae	*Croton conduplicatus Kunth*	*(E)-caryophyllene Caryophyllene oxide*	Muscarinic receptors.	Northeastern Brazil	Ip	Male Swiss mice	Acetic-acid-writhing-induced nociception, Formalin-induced nociception, Hot plate test.	Molegro Virtual Docker.	Majority compound structures of camphor, caryophyllene oxide, and (E)-caryophyllene submitted to molecular docking—EO acts through central and peripheral mechanisms, possibly involving K_{ATP} channels and muscarinic receptors	Brazil	De Oliveira Junior et al., 2017.
Lamiaceae	*Hyptis pectinata*	*Caryophyllene oxide Germacrene D*	Opioid and serotonin receptors.	Malhada dos Bois (Sergipe State), in northeastern Brazil	Subcutaneous	Male Swiss mice	Acid Saline-Induced Chronic Muscle Pain, Mechanical Sensitivity of the Muscle (Primary Hyperalgesia), Mechanical Sensitivity of the Paw (secondary hyperalgesia)	Molegro Virtual Docker v. 6.0.1.	Main components of EOH were β-caryophyllene, caryophyllene oxide, germacrene D, and linalool. Central analgesic activity seems to be evoked by the action of NE-EOH on the opioid and serotonin systems.	Brazil	Quintans-Junior et al., 2018.
Asteraceae	*Ageratina glabrata*	Chromene derivative Meloxicam	COX-2.	Mexico	Ip	Sprague Dawley rats	Hot plate	Sybyl®X software suite.	Antinociceptive effect (COX-2 inhibition) Not affected by hormonal changes	Mexico	García et al. (2011)
Asteraceae	*Achillea falcata L.*	*trans*-Sabinol	–	Syria	Ip	BALB/c mice	Ach writhing Hot plate Tail immersion	–	Antinociceptive effect Toxicity of some derivatives not ruled out	Serbia	Radulović et al. (2015)
Asteraceae	*Vanillosmopsis arborea Baker*	*(–)-α-bisabolol*	5-HT3 and M2 receptors.	Brazil	Gavage	Swiss mice Wistar rats	Formalin Capsaicin Acidic saline Glutamate	Molegro Virtual Docker.	Antinociceptive effect	Brazil	Leite et al. (2019)

This table summarizes the main results obtained in research where essential oils from natural products were used to test for possible antinociceptive activity.

TABLE 3 | Information ethnobotanical, phytochemical, molecular, pharmacological, and docking programs used *in silico* studies involving the antinociceptive activity isolated from essential oils.

Phytochemical	Molecular target	Chemical marker	Route of administration	Animal(s) species	Antinociceptive test	Software	Results	Country	Ref
α-Terpineol	Nitric Oxide Synthase enzyme	TP, amino guanidine, dexamethasone, Nitro-L-arginine methyl ester (L-NAME)	Subcutaneous	Male Swiss	Mechanical hyperalgesia was assessed by means of digital von Frey	Molegro Virtual Docker v. 6.0.1.	Antinociceptive effect of TP probably occurs *via* mechanisms related to modulation of oxidative stress, with maintenance of endogenous antioxidant substances and reduction of iNOS levels.	Brazil	Gouveia et al., 2018.
3-(5-substituted-1,3,4-oxadiazol-2-yl)-*N*′-[2-oxo-1,2-dihydro-3*H*-indol-3-ylidene] propane hydrazides	COX-2	indomethacin	Po	Albino Wistar mice	Hot plate	Vlife MDS.	Antinociceptive effect	India	Kerzare et al., 2016
β-cyclodextrin (CT-βCD) complexed with citronellal (CT)	GluR2-S1S2	1FTJ protein complexed with glutamate	Po	Swiss mice	Digital von Frey Grip strength meter	AutoDock Vina.	CT-βCD has a greater analgesic effect than the free form (CT alone)	Brazil	Santos et al., 2016
(–)-α-bisabolol	TRPV1	–	Intraocular	Swiss mice	Hypertonic saline-induced corneal nociception	Hex Protein Docking (HEX)	Nanoencapsulated BISA is topically active —attenuates 5 M NaCl-induced corneal nociception	Brazil	Teixeira et al., 2017
(–)-α-bisabolol	TRPV1	–	Po and topical	Adult male Swiss albino mice and adult male Wistar rats	Orofacial formalin test Orofacial cinnamaldehyde test Temporomandibular joint formalin test	Hex Protein Docking (HEX)	The study confirmed the anti-nociceptive effect of BISA on orofacial pain. The effect may in part be due to TRPA1 antagonism	Brazil	Melo et al., 2017
B-cyclodextrin complexed with farnesol	β-CD complex	–	Ip	Male Swiss mice	Formalin, Orofacial capsaicin, glutamate	AutoDock 4.2 software in the PyRx 0.9.	Farnesol complexed with β-CD presented best antinociceptive activity, probably *via* 5-HT3 receptor	Brazil	(Silva et al., 2017).
p-Cymene	CaV1, CaV2.1, CaV2.2 and CaV2.3	p-cymene, nicardipine, ω-agatoxin IVA, ω-conotoxin GVIA, and N-Triazole Oxindole	Subcutaneous	Male Albino Wistar mice	Digital von Frey Grip strength meter	Molegro Virtual Docker	p-Cymene was able to reduce calcium current density	Brazil	Santos et al., 2019
α-terpineol	5-HT receptor Delta receptor Kappa receptor MU receptor	–	Ip	Male Swiss mice	Mechanical hyperalgesia induced by acid saline Formalin-induced nociception test	Molegro Virtual Docker 6.0.	β-CD improves the anti-hyperalgesic effect of α-TPN; α-TPN-βCD enhances analgesic profile producing a longer-lasting analgesic profile when compared to α-TPN alone; Docking study demonstrated that anti-hyperalgesic effect produced by α-TPN-βCD implies opioid and serotoninergic receptors	Brazil	Oliveira et al., 2016

The table summarizes the main results obtained in research where isolated molecules of essential oils from natural products were used to test for possible antinociceptive activity.

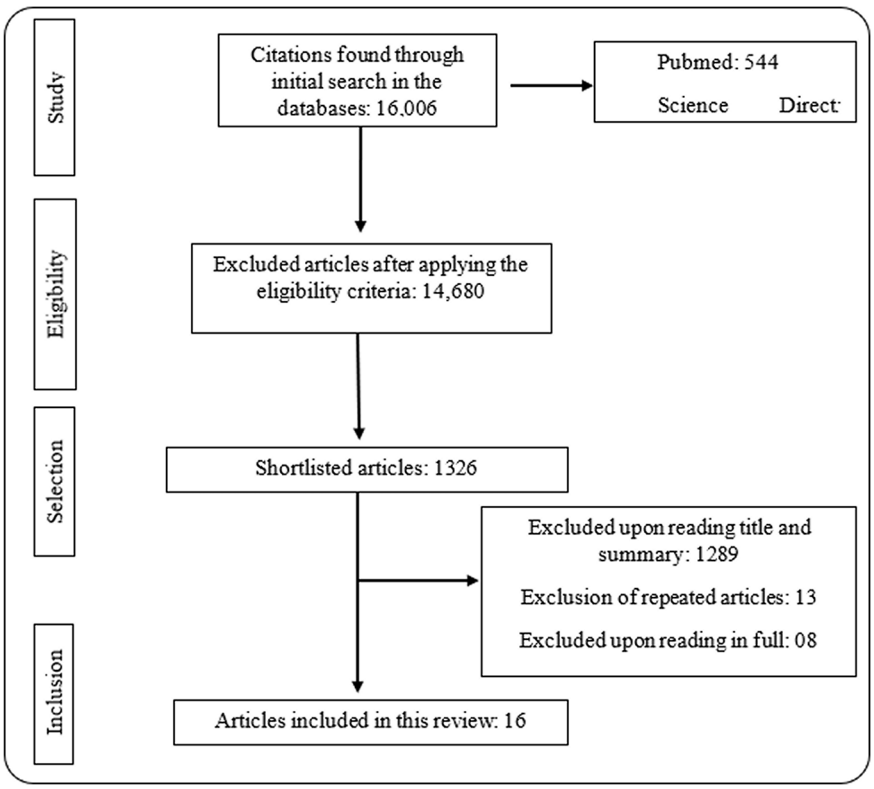


FIGURE 1 | Flowchart of article selection for systematic review. The bibliographic study started with 16,006 articles, which after applying the eligibility criteria, 14,680 remained. Among these, 1326 were selected. A total of 1,289 was excluded after reading the title, 13 were excluded by repetition and 8 were excluded after full reading. In total, 16 articles fit the purpose and were selected for this review.

Leite et al., 2019); Capsaicin (Silva et al., 2017; Leite et al., 2019); Acetic acid (Sumiwi et al., 2015); Glutamate (Silva et al., 2017; Leite et al., 2019); Orofacial formalin (Silva et al., 2017); Saline-induced chronic muscle pain (Siqueira-Lima et al., 2017; Quintans-Junior et al., 2018); Thermal induction tests: Hot Plate Test (Radulović et al., 2015; García et al., 2011; Kerzare et al., 2016; De Oliveira Junior et al., 2017; de Oliveira Júnior et al., 2018); Tail immersion (Radulović et al., 2015); and Mechanical nociceptive induction testing: Von Frey (Santos et al., 2016; Gouveia et al., 2018 Santos et al., 2019).

The *in silico* programs used for docking studies were; Molegro Virtual Docker (Oliveira et al., 2016; De Oliveira Junior et al., 2017; Siqueira-Lima et al., 2017; de Oliveira Júnior et al., 2018; Gouveia et al., 2018; Leite et al., 2019; Santos et al., 2019); Sybyl®X software suite (García et al., 2011), Vlife MDS (Kerzare et al., 2016); AutoDock Vina (Santos et al., 2016); Hex Protein Docking (HEX) (Teixeira et al., 2017; Melo et al., 2017); and AutoDock 4.2 software in PyRx 0.9 (Silva et al., 2017).

The molecular targets/complexes used in the docking studies were Nitric Oxide Synthase (Gouveia et al., 2018), COX-2 (García et al., 2011; Sumiwi et al., 2015; Kerzare et al., 2016); GluR2-S1S2 (Santos et al., 2016); TRPV1 (Teixeira et al., 2017; Melo et al., 2017); β-CD complex (Silva et al., 2017); CaV1, CaV2.1, CaV2.2, and CaV2.3 (Santos et al., 2019); 5-HT receptor, Delta receptor, Kappa receptor, and MU (μ) receptor (Oliveira et al., 2016); Alpha Adrenergic, Opioid, and Serotonergic receptors (Siqueira-Lima et al., 2017); Muscarinic receptors and $GABA_A$ (De Oliveira Junior et al., 2017; de Oliveira Júnior et al., 2018); Opioid and Serotonin receptors (Quintans-Junior et al., 2018); 5-HT 3 and M2 receptors (Leite et al., 2019).

The study results were analyzed separately and can be seen in **Table 2**, along with other information from the articles.

DISCUSSION

With technological development and advances in understanding the pathophysiological bases of pain/nociception, alternative screening methods for naturally occurring compounds have gained specified target approaches. Various models have been developed and tested against natural compounds. Previous studies, including a systematic review, showed the potential antinociceptive effect of essential oils and their phytoconstituents, especially monoterpenes. However, most of the reports of these investigations present obtained by experimental animal assays (Guimarães et al., 2012; Sá et al., 2017). Through this review, although scarce, studies coupling molecular anchoring with EO antinociceptive activity and their constituents (monoterpene - sesquiterpene) (**Table 4**) allow observations

concerning the obtained molecular hits. This increases the chances of finding candidate molecules for therapeutic use. Certain species were studied for possible antinociceptive activity with the aid of molecular coupling. For these, it was possible to predict the preferential orientation (when linked together to form a stable complex) of one molecule to another, and further elucidated molecular interactions.

Vanillosmopsis arborea Baker

Vanillosmopsis arborea Baker (Asteraceae) is a native plant to northeastern Brazil, especially the state of Ceará (Matos et al., 1988). There are insufficient studies reporting the biological effects of the essential oil extracted from this plant, but its leishmanicidal (Colares et al., 2013), gastroprotective (De Oliveira et al., 2009), and antimalarial (Mota et al., 2012) activities have been described. According to De Oliveira et al. (2009), an analysis by gas chromatography coupled to mass spectrometry (GC/MS) of *V. arborea* stem bark essential oil (EOVA) evidenced the existence of α-bisabolol (70%), and also α-cadinol (8.4%), elemicin (6.21%), β-bisabolene (4.46%), δ-guaiene (2.31%), β-cubebene (1.76%), and estragole (1.08%).

To increase the bioavailability and pharmacological properties of essential oils, complexation with β-cyclodextrin is very useful (Santos et al., 2016). Cyclodextrins are cyclic oligosaccharides presenting a hydrophobic center that complexes with molecules yet improves water solubility and reduces toxic effects (Oliveira et al., 2009; Abril-Sánchez et al., 2019). EOVA and its form as complexed with β-cyclodextrin (EOVA-pCD) at a dose of 50 mg/kg, i.p. reduced orofacial nociception induced by various stimuli, as well as in a model of temporomandibular joint dysfunction caused by the administration of formalin. The study also suggested that EOVA modulates type 1 vanilloid transient potential receptors, yet without interacting with the glutamatergic inhibitory pathway. Fos protein expression in the dorsal horn of the spinal cord was decreased by pCDEVA, inferring a reduction in pain-sensitive neuronal activation. In the same study, (through molecular docking) the researchers observed favorable interactions between bisabolol, the major constituent of EOVA, the 5-HT3 receptor, and type 3 muscarinic receptors (Leite et al., 2019).

Citronellal

Citronellal (**Table 5**, ID 01) is a monoterpene isolated from aromatic plants of the genus Cymbopogon (Quintans-Junior et al., 2008). Several pharmacological activities have been described for this compound, which acts as an anti-atherosclerotic (Lu et al., 2019), antifungal (Wu et al., 2016), anti-inflammatory (De Santana et al., 2013), and anticonvulsant (Melo et al., 2011).

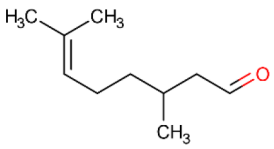
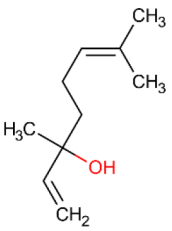
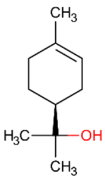
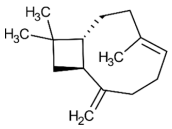
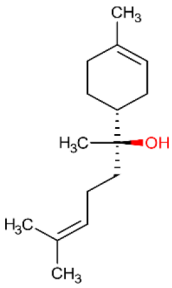
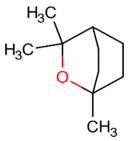
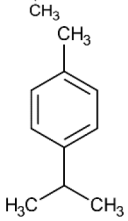
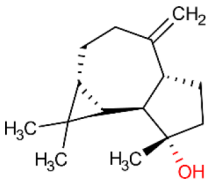
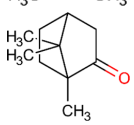
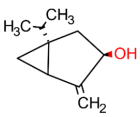
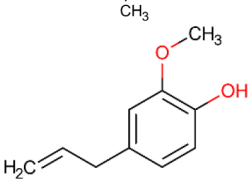
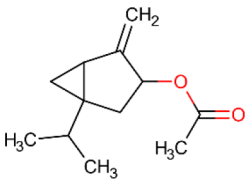
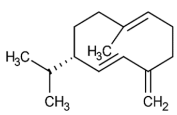
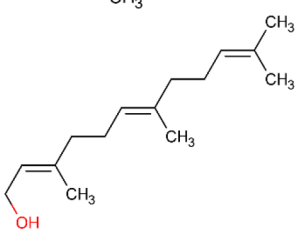
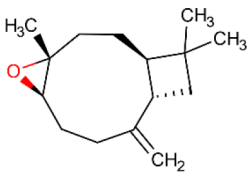
Despite the known effects of isolated monoterpenes, the complexation of monoterpenes with β-cyclodextrin has

TABLE 4 | Interactions observed in docking studies involving antinociceptive activity.

Ligand	Molecular target	Interacting amino acids	Reference
Citronellal	GluR2-S1S2	Arg96, Ser142 e Thr143.	Santos et al., 2016
α-Terpineol	Nitric Oxide Synthase	Thr324, Trp325 e Ile327.	Gouveia et al., 2018.
(–)-α-bisabolol	TRPV1	Ala680, Gly683, Asn687.	Teixeira et al., 2017.
(–)-α-bisabolol	TRPV1	Ile695, Ser972, Leu973 and Lys969.	Melo et al., 2017
p-Cymene	CaV1, CaV2.1, CaV2.2 and CaV2.3	Glu84, Glu87, Ala88, Val91, Met144.	Santos et al., 2019
α-Terpineol	5-HT receptor Delta receptor Kappa receptor MU receptor	Asp129 and Cys133. Asp128. Leu67 and Val63. Asp147.	Oliveira et al., 2016
(–)-α-bisabolol	5-HT3 and M2 receptors.	Tyr64, Arg65, Thr154, Trp156 and Glu209. Asp103, Tyr104, Ala194 and Tyr403.	Leite et al., 2019.
Camphor, transcarophyllene and bicyclogermacrene	Alpha adrenergic, μ Opioid, and 5-HT.	Arg14, Tyr15, Ile18 and Thr19. Asp135, Val136, Thr140, Phe340 and Phe341. Gln124, Tyr148, Val236, His297, Trp318.	Siqueira-Lima et al., 2017.
Eugenol	COX-2	Val116, Arg120, Val349, Leu352, Tyr355, Phe518, Met522.	Sumiwi et al., 2015.
b-FNA, Germacrene D, Caryophyllene oxide, Linalool and β-caryophyllene	Opioid and serotonin receptors.	Thr134 and Val201.	Quintans-Junior et al., 2018.
3-(5-substituted-1,3,4-oxadiazol-2-yl)-N′-[2-oxo-1,2-dihydro-3H-indol-3-ylidene] propane hydrazides derivatives	COX-2	Pro127, Tyr373, Gly536, Gln374, Arg376 and Ser541.	Kerzare et al., 2016.
1,8-Cineole, Caryophyllene oxide, p-Cymene, Spathulenol,	Muscarinic receptors and GABAA.	Not described.	de Oliveira Júnior et al., 2018.
10-benzoiloxi-6,8,9-isobutirato de tri-hidroxi-timol	COX-2	Not described.	García et al., 2011.
trans-sabinol and trans-sabinyl acetate	AChE	Ser200, Glu327, His440, Phe330 and Trp 84.	Radulović et al, 2015.
B-cyclodextrin complexed with Farnesol	β-CD complex	Not described.	Silva et al., 2017.

The table summarizes the ligands and molecular targets used in molecular docking research, as well as the residues that showed interaction with the compounds.

TABLE 5 | Main docking compounds used in the articles included in this review.

ID	Name	Structure	Reference	ID	Name	Structure	Reference
01	Citronellal		Santos et al., 2016	09	Linalool		Quintans-Junior et al., 2018.
02	α-Terpineol		Gouveia et al., 2018. Oliveira et al., 2016	10	β-caryophyllene		Quintans-Junior et al., 2018.
03	(–)-α-bisabolol		Teixeira et al., 2017 Melo et al., 2017. Leite et al., 2019.	11	1,8-Cineole		de Oliveira Júnior et al., 2018.
04	p-Cymene		de Oliveira Júnior et al., 2018.	12	Spathulenol		de Oliveira Júnior et al., 2018.
05	Camphor		Siqueira-Lima et al., 2017.	13	trans-sabinol		Radulović et al, 2015.
06	Eugenol		Sumiwi et al., 2015.	14	sabinyl acetate		Radulović et al, 2015.
07	Germacrene D		Quintans-Junior et al., 2018.	15	Farnesol		Silva et al., 2017.
08	Caryophyllene oxide		Quintans-Junior et al., 2018.				

This table summarizes the structure of compounds that interact with protein targets in docking studies.

produced more promising results for treating pain and inflammation (Quintans-Junior et al., 2013; Nascimento et al., 2014; Silva et al., 2016). In a chronic non-inflammatory muscle pain model, chronic treatment using a citronellal/β-cyclodextrin complex at a dose of 50 mg/kg, p.o. presented longer-lasting antihyperalgesic effects than citronellal alone. The results can be explained by an increase in c-Fos protein expression in the ventrolateral periaqueductal gray and rostroventral medullary areas of the brain and reductions in this activity in the superficial dorsal horn, suggesting inhibitory modulation of descending pain pathways (Santos et al., 2018). In a molecular docking study, a favorable energy bond between citronellal and the structure GluR2-S1S2J, an ionotropic glutamate receptor responsible for painful stimulus propagation was observed (Santos et al., 2016).

α-Terpineol

α-Terpineol (**Table 5**, ID 02) is a monoterpene present in the essential oils of *Ravensara aromatica, Melaleuca qinquenervia,* and mainly, species of the genus Eucalyptus (Elaissi et al., 2010). Studies show the clinical potential of this compound to treat various types of pain (Quintans-Júnior et al., 2011; De Oliveira et al., 2012; Oliveira et al., 2016; Safaripour et al., 2018).

In an experimental model of cancer pain consisting of subcutaneous implantation (in the plantar region) of tumor cells in mice, subcutaneous treatment with α-terpineol (12.5, 25, and 50 mg/kg, sc) promotes an anti-hyperalgesic effect and seems to reduce allodynia in these painful conditions, without promoting myorelaxant effects. The effect may be related to antioxidant capacity and reduced inducible nitric oxide synthase (iNOS) levels in the tumor microenvironment. Molecular docking corroborates these results, since it was observed that the monoterpene binds to iNOS in the same regions as N-Nitroarginine methyl ester (an iNOS inhibitor) (Gouveia et al., 2018).

A complex containing α-terpineol and cyclodextrin (αTPN-pCD) at doses of 25, 50, and 100 mg/kg, p.o. reduced mechanical hyperalgesia caused in an animal model of fibromyalgia. In the same study, the involvement of the opioid and serotonergic system in the analgesic activity was observed both through the use of pharmacological antagonists in animals, and when employing molecular docking (Oliveira et al., 2016).

α-Bisabolol

α-Bisabolol (**Table 5**, ID 03) is a monocyclic sesquiterpene extracted from the essential oils of various plants, mainly the flowers of *Matricaria chamomilla* and the bark of *Vanillosmopsis arborea* Baker (Kamatou and Viljoen, 2010; Inocencio Leite et al., 2014). Several biological effects have been described for α-bisabolol including neuroprotective (Fernandes et al., 2019), antiparasitic (de Menezes et al., 2019), anticancer (Wu et al., 2018), and antibacterial activity (de Sousa Oliveira et al., 2017).

In an orofacial antinociceptive evaluation of α-bisabolol, the non-clinical efficacy of this compound when administered systemically or topically was confirmed. In a temporomandibular joint dysfunction model, α-bisabolol reduced nociceptive orofacial rubbing behavior in rats. The pharmacological effects occur independently of ATP-sensitive nitrergic systems, opioids, and potassium channels. Molecular docking revealed a strong interaction and the absence of significant electrostatic repulsion between α-bisabolol and the TRPA1 receptor, suggesting possible antagonism (Melo et al., 2017). Texeira et al. (2017), observing TRPV1 antagonism in molecular docking studies, revealed that nanocapsules containing α-bisabolol present antinociceptive activity in an orofacial pain model caused by administration of hypertonic saline into the mouse cornea.

Still talking about terpenes and TRPV1 receptors, Jansen et al. (2019), found that myrcene has a significant participation in calcium inflows mediated by the TRPV1 receptor. Myrcene has been identified as an analgesic in previous studies, demonstrating its anti-nociceptive potential in mice (Rao et al., 1990). Responses to myrcene showed total dependence on the presence of TRPV1 and were effectively blocked by capsaicin, a TRPV1 antagonist. Patch Clamp studies showed that myrcene is indeed an effective TRPV1 ligand and in cells that contain high TRPV1 receptor densities, leading to intracellular calcium release. While for many pain applications there is a focus on TRPV1 antagonists, other applications depend on the chronic agonism of TRPV1, leading to the desensitization of TRPV1 at the cellular level and the induction of neuronal cell death by large inflows of calcium and sodium through the channel (Derry et al., 2017). Preliminary molecular coupling studies suggest that myrcene is interacting hydrophobically, non-covalently, and in a way that does not depend heavily on reactive cysteines. Tyr 554 is involved in the binding site, which has also been implicated in capsaicin binding (Elokely et al., 2016) and forms part of the S4-S5 loop between the fourth and fifth transmembrane domains of TRPV1 (Boukalova et al., 2010).

Like myrcene, camphor also desensitizes TRPV1 receptors, but in a more rapidly and completely way than capsaicin, it activates TRPV3 and inhibits TRPA1, correlating to its analgesic properties (Xu et al., 2005).

The endocannabinoid system involves the central and peripheral nervous systems. It is involved in inflammatory and pain processes (Rodriguez de Fonseca et al., 2005). The endocannabinoid system appears to work both independently and synergistically (Mallat et al., 2011; Baron, 2018). Cannabidiol (CBD) is the second major cannabinoid and has much lower affinity for CB1 and CB2 receptors as compared to Δ9-tetrahydrocannabinol (THC), and it acts as a non-competitive CB1 and CB2 receptor antagonist (Thomas et al., 2007). CBD has additional actions that may account for its anti-inflammatory and analgesic effects including TRPA1 agonist, TRPV1 agonist (similar to capsaicin, although without the noxious side effects), TRPM8 antagonist (De Petrocellis et al., 2011; Baron, 2018).

Jansen et al. (2019) show that myrcene and CBD share elements of a binding site and can influence one another physiologically. Their data suggest that several minor cannabinoids discriminate between TRPV1, TRPA1, and TRPM8 (Starkus et al., 2019). In CBD docking studies, a binding pocket partially over-lapping with that of Myrcene was identified and the best scoring pose showed a docking score of

−26.5 kcal/mol. In this site, Thyr 554 and Arg 491 are important, as for Myrcene. The remaining residues implicated in CBD binding show both similarities and differences to the Myrcene site (Jansen et al., 2019).

Croton conduplicatus Kunth

Croton conduplicatus Kunth is a Brazilian medicinal plant, belonging to the Euphorbiaceae family, called as "quebra-faca" by the locals; it is found in South America. In the Brazilian northeast, folk medicine uses its leaves to treat stomach pain (Cartaxo et al., 2010). GC/MS analysis of *Croton conduplicatus* Kunth stem bark (essential oil) revealed a majority presence of the terpenoids (E)-caryophyllene (13.72%), caryophyllene oxide (**Table 5**, ID 08) (13.15%) and camphor (8.25%). The essential oil presents central and peripheral antinociceptive activity *via* possible involvement of K_{ATP} channels and muscarinic receptors, yet without participation of opioid receptors. In the same study, a satisfactory link was observed in molecular docking between the constituent terpenoids and muscarinic receptors (M2, M3, and M4) (De Oliveira Júnior et al., 2017).

Chemical analysis of *Croton conduplicatus* Kunth leaf essential oil revealed 1,8-cineole (**Table 5**, ID 11) (21.42%), p-cymene (12.41%), spathulenol (**Table 5**, ID 12) (15.47%), and caryophyllene oxide (12.15%) as the main constituents. The oil presents antinociceptive activity equal to that of the leaves, yet involvement of the opioid system is observed. In molecular docking, the interactivity of spathulenol and caryophyllene oxide with opioid and muscarinic receptors was verified (de Oliveira Júnior et al., 2018).

Complexed β-cyclodextrin (βCD)—*Lippia grata essential oil*

Lippia grata Schauer is a shrub widely distributed in northeastern Brazil. Folk medicine uses it to treat pain conditions (Viana et al., 1981). Terpene-rich *L. grata* (LG) leaf essential oil is known to promote an orofacial analgesic profile. Since it is known that the therapeutic analgesic effects of certain EO and its constituents can be enhanced by forming a β-cyclodextrin (βCD) inclusion complex (Oliveira et al., 2016), Siqueira-Lima et al., 2017 evaluated the antinociceptive effect of a β-cyclodextrin (βCD) complex with *Lippia grata* essential oil in an animal model of chronic musculoskeletal pain. The model mimicked painful muscle diseases such as fibromyalgia (FM), which is a rheumatic disease characterized by widespread chronic pain and is difficult to treat. The low clinical efficacy and side effects of current treatments make therapeutic adherence more difficult, and the lack of knowledge about the pathophysiology of FM complicates the development of new drugs. Animal models are an alternative for reproducing FM symptoms. In a model of chronic non inflammatory muscle pain, caused by saline acid injection in the mouse gastrocnemius, oral administration of LG-βCD produced excellent antihyperalgesic activity. LG-βCD also did not alter muscle strength, discarding the chance of reduced motor performance, common in some CNS drugs. An assay to evaluate possible antagonist involvement of opioid, serotoninergic, and noradrenergic pathways revealed that the antihyperalgesic effect of LG-βCD may involve serotonergic and opioid receptors, indicating probable participation of the inhibitory modulation system of descending pain. This was partially supported by the *in silico* study. The receptor structures were obtained from Protein Data Bank (PDB). The investigated receptors, by comparing binding energy with ligands; camphor (**Table 5**, ID 05), trans-caryophyllene, bicyclogermacrene (**Table 5**, ID 07) (major LG compounds) were alpha-adrenergic, μOpioid, and 5HT. *In silico* target validation analysis favored the understanding of the drug-receptor interaction, and confirmed the observed result within *in vivo* tests, demonstrating that camphor and E-caryophyllene bind to the alpha-adrenergic receptor, while bicyclogermacrene binds with moderate energy to 5-HT and μOpioid receptors (Siqueira-Lima et al., 2017).

Hyptis pectinata Leaf Essential Oil

Hyptis pectinata belongs to the Lamiaceae family, which is characterized by the presence of strongly aromatic plants, present in North and South America, mainly in tropical areas. In traditional medicine, *H. pectinata* extracts have been used as medicinal teas in pain treatment. The anti-inflammatory and analgesic profile of *H. pectinata* has been reported in many studies in the literature. The analgesic effects of *H. pectinata* EO occur due to the terpenoid compounds, such as β-caryophyllene (**Table 5**, ID 10), caryophyllene oxide, linalool (**Table 5**, ID 09), and limonene (McNeil et al., 2011). The antinociceptive effect of β-caryophyllene occurs due to its cannabimimetic effects (Gertsch et al., 2008). The complex containing β-caryophyllene and β-cyclodextrin has anti-hyperalgesic properties in the model of chronic muscle pain *via* inhibition of c-Fos expression in the lumbar spinal cord (Quintans-Júnior et al., 2016).

The EO of *H. pectinata* complexed with β-cyclodextrin is able to increase the analgesic effect, as well as extend its duration (Menezes et al., 2015). Quintans-Junior et al. (2018) evaluated the capacity of a nanostructured thermoreversible subcutaneous hydrogel aggregated with *H. pectinata* essential oil (NE-EOH) to promote long-term antihyperalgesic effect in a fibromyalgia (FM) animal model. This formulation (containing *H. pectinata* essential oil) produced a lasting antihyperalgesic effect in a non-inflammatory muscle pain model in mice. NE-EOH produces analgesia through CNS inhibitory mechanisms. Through a molecular anchoring study, it was possible to predict that this antihyperalgesic response involves central pain inhibitory pathways and endogenous serotonin and opioid neurotransmitters.

To evaluate the binding ability of NE-EOH to the tested targets (serotonin and opioid receptors), coupling analysis using MolDock was performed, taking into account the binding energy of the major *H. pectinata* EO compounds (β-caryophyllene, caryophyllene oxide, germacrene D, and linalool) with μ-OR β-FNA receptors and 5-HT1B dihydroergotamine. A hydrogen bond was observed between the epoxy portion of caryophyllene oxide and 5-HT1B (Thr 134), an equal interaction could be seen for dihydroergotamine. Caryophyllene oxide and germacrene D presented the lowest binding energies of the studied secondary metabolites, suggesting that germacrene D and caryophyllene

oxide possibly contributes in the antihyperalgesic activity of NE-EOH, *via* the μ-OR pathway (Quintans-Junior et al., 2018).

Cinnamomum sintoc bl Bark Essential Oil (sintoc)

Sintoc (*C. sintoc bl*) is a plant grown in Indonesia, Malaysia, and Thailand and used in folk medicine to treat swelling (inflammation). Sumiwi et al., 2015 investigated the anti-inflammatory and analgesic activity of *Cinnamomum sintoc bl* (sintoc) bark essential oil, (inhibiting the enzyme COX-2), using animal models, together with molecular coupling to predict the interaction of sintoc compounds with COX-2. Eugenol (a constituent of sintoc (**Table 5**, ID 06)) presented a good visual interaction with COX-2. The phenol part of eugenol forms hydrogen bonds with Gly 526 and Met522 of COX-2, as well as the naproxen phenol. However, eugenol presents no electrostatic interaction between carboxyl and ammonium ions, such as naproxen carboxyl groups with Arg120 ammonium ions.

In the *in vivo* tests, the essential oil of sintoc bark presented analgesic activity in the acetic acid-induced abdominal writhing test and anti-inflammatory activity in the carrageenan-induced paw edema test. In a previous molecular anchorage study, the results revealed that isoeugenol can effectively inhibit the enzymatic activity of cyclooxygenase and lipoxygenase. Isoeugenol anchored at the active site with an orientation similar to that of indomethacin (Zarlaha et al., 2014).

Ageratina glabrata

Species of the genus Ageratina belong to the Asteraceae family. In general, this genus is known to have therapeutic activities, among which its analgesic effects stand out (Mandal et al, 2005; Chakravarty et al., 2011).

Ageratina glabrata (Kunth) is commonly known in Mexico as "chamizo blanco" or "hierba del coup". Folk medicine reveals the use of this plant for pain relief. The literature describes the presence of flavones, thymol derivatives, and other phenolic terpenoids in its chemical composition (Vivar et al., 1971; Bohlmann et al., 1977; Guerrero et al., 1978).

Using the hot plate test, García et al. (2011), verified the presence of analgesic effect in a group of animals treated at 100mg/kg with *A. glabrata* leaf extract. The results, because of the duration and pain suppression characteristics, and is similar to those observed in the positive control treated with meloxicam, suggest that the molecular mechanism involved may be *via* cyclooxygenase (COX).

After isolation and purification, Garcića et al. (2011) identified the presence of trihydroxy thymol 10-benzoyloxy-6,8,9-isobutyrate, which became the molecule from *A. glabrata* extract chosen to conduct a docking study using the Sybyl® software. The protein was downloaded from the Protein Data Bank with code 3LN0. Both meloxicam and the derivative was successfully docked in the same position and orientation at the PDB ligand, at the active site of the COX-2 enzyme. The binding and formation of the ligand-enzyme complex were in agreement with the crystallographic structure, showing the potential of this derivative to interact with COX-2 and promote the analgesic effect evidenced in the *in vivo* thermal nociception model.

Achillea falcata L.

Achillea falcata L. is an endemic Mediterranean species widely considered for its pharmacological effects, and traditionally used to treat fever, stomach pain, and hemorrhoids (Aburjai et al., 2007; Alzweiri et al., 2011).

Radulović et al. (2015) have been able to provide clear evidence that *A. falcata* is capable to produce trans-sabinol (**Table 5**, ID 13) and some of its esters. Its constituents are biosynthesized and accumulate in the aerial parts and roots of the plant (Kürkçüoğlu et al., 2003; AburjaiHudaib, 2006). The compounds formate and tiglate have been recently discovered (Radulović et al., 2015).

Achillea falcata essential oil characterization (aerial parts and roots) was performed near the city of Ma'loula, Syria, revealing the presence of two principal constituents, trans-sabinol (19.1%) and trans-sabinyl acetate (**Table 5**, ID 14) (11.4%), as well as their rare esters. Using different models such as abdominal contortions and hot plate, Radulović et al. (2015) screened to investigate the analgesic potential of trans-sabinol and its esters.

Due to the structural similarity of these compounds with rivastigmine, an acetylcholinesterase (AChE) inhibitor, there was an interest in verifying *in silico* (molecular docking), the ability of these esters to interact with AChE. The active site of AChE is known to be deep within the enzyme. The residues of Ser200, His440, and Glu327, located at the bottom depth, form the catalytic triad and participates directly in hydrolysis of acetylcholine (Sussman et al., 1991; Millard et al., 1999).

All of the esters found energetically favorable coupling poses, placing the ligands at the catalytic site, and suggesting that the compounds may indeed approach the amino acid residue triad. The most favorable anchorage position was achieved by trans-sabinyl. This compound was placed with the electrophilic ester/bond group near the residues of Ser200, Glu327, His440, Phe330, and Trp 84, which are relevant for AChE function (Sussman et al., 1991; Millard et al., 1999). The ligand disposal indicates that it might interact with the enzyme allowing covalent modification. The other esters came with a similar outcome, and the calculated binding energies suggests that trans-sabinyl tiglate and senecioate initially bind more strongly to the enzyme (8.5 kcal/mol), while trans-sabinyl formate likely has the lowest affinity for AChE (Radulović et al., 2015).

The mentioned compounds also presented antinociceptive activity in two different animal models. Trans-sabinol promoted a reduction in the animal's response to thermal stimuli in the hot plate test, and a reduction in the abdominal writhing test response as well. When subjected to the hot plate test at the dose of 50 mg/kg, trans-sabinol was able to reach its maximum effect after 15 min. In the same test, time and dose, trans-sabinyl tiglate increased the residence time by 140% (Radulović et al., 2015). These results, associated with molecular anchoring tests, indicate that trans-sabinol and its esters interact with different targets and influence both the periphery and the central nervous system, been capable to promote a considerable antinociceptive effect.

β-Cyclodextrin Complexed With Farnesol

Farnesol (FAR) (**Table 5**, ID 15) is a naturally occurring sesquiterpene alcohol known to exhibit multiple functions including Ca^{2+} channel inhibition (Endo et al., 2011; Khan and Sultana, 2011; Santhanasabapathy et al., 2015), an important target for drugs used in chronic pain (Clark et al., 2016). This sesquiterpene has been shown to possess anti-inflammatory and analgesic properties but without considerable neurotoxicity in the brain of adult mice (De Oliveira Junior et al., 2013).

A molecular docking study was performed to predict the likely interaction between β-cyclodextrin (β-CD) and FAR. It was observed that of the ten conformations generated by FAR with β-CD there were stable fittings (forming complexes) with the lowest energy value being - 3.45 kcal/mol. The presence of hydrogen bonds between farnesol and β-CD was also observed.

In the formalin test, administration of FAR (50 mg/kg) was able to reduce face rubbing time ($p < 0.05$); FAR at 100 mg/kg, and FAR + β-CD in 50 and 100 mg/kg doses reduced this behavior in both phases of the formalin test ($p < 0.001$). From the results, FAR + β-CD was significantly more effective in reducing pain behavior than FAR alone (Silva et al., 2017).

Experiments using a capsaicin-induced orofacial pain model showed that administration of FAR (50 mg/kg) also reduced face rubbing time ($p < 0.05$), yet FAR at 100 mg/kg, and the FAR + β-CD complex at doses of 50 and 100 mg/kg reduced this behavior more effectively ($p < 0.001$). Two targets act in this pain pathway, TRP receptors, and Ca^{2+} channels, thus suggesting their inhibition by the compounds (Silva et al., 2017).

Glutamate tests revealed that FAR, and FAR + β-CD at doses of 50 and 100 mg/kg significantly inhibited nociception ($p < 0.0001$). Statistical differences between FAR + β-CD at 100 mg/kg dose, and doses of isolated FAR at 50 and 100 mg/kg ($p < 0.0001$) indicated once again that the complex is more effective (Silva et al., 2017).

In a study on the mechanism of action, both isolated FAR and FAR + ondansetron presented statistical differences ($p < 0.0001$), demonstrating potential interactions with serotonergic receptors. The 5-HT3 receptor plays a pro-nociceptive role, mediating descending excitatory pathways in the spinal cord dorsal horn (Azimaraghi et al., 2014).

Overall, FAR + β-CD demonstrated a better pharmacological effect than the active compound alone. FAR + β-CD reduced orofacial pain behavior, which according to the investigation of the mechanism of action, was potentially mediated by interaction with 5-HT3 receptors. The inclusion complex that contains FAR + β-CD, therefore, suggest having therapeutic potential in the treatment of some types of dysfunctional pain, such as orofacial pain.

2-Oxoindolin-3-ylidene-3-(5-substituted phenyl-1,3,4-oxadiazol-2-yl) Propanehydrazide Derivatives

The hybrid approach involves the development more effective synergistic molecules by hybrid mixing of two or more already active biomolecules to produce new derivatives that have better pharmacological activity (Gediya and Njar, 2009).

The target portions selected for the formation of 2-oxoindolin-3-ylidene-3-(5-substituted phenyl-1,3,4-oxadiazol-2-yl)-propanehydrazide hybrids were based on studies revealing the analgesic and anti-inflammatory properties of indole and oxadiazole nuclei (Pandeya et al., 1998; Wagle et al., 2008; Chikhale et al., 2009; Jayashankar et al., 2009; Singh et al., 2010; Chaluvaraju et al., 2011).

Fifteen different hybrids of indole and oxadiazole were synthesized. *In silico* determinations of potentials compound–receptor interactions were performed through molecular coupling studies of the ligands at the cyclooxygenase (COX) site (Chikhale et al., 2015). Docking of these derivatives with COX-2 (PDB code 4Z0L) was performed using Vlife MDS Molecular Modeling 4.3.1 software. One of the reasons for choosing this enzyme was that its crystallographic structure already provides complexing with an indole derivative, and it can act as a reference molecule for coupling. As well, the animal model used also favored the choice. Anchoring was performed for all of the synthesized compounds. Three compounds, 50 (p-OH), 51 (p-CH3), and 53 (o-OCH3, m′-OCH3) exhibited the best activity, with 50 and 51 the most active. As energias de 50 (−4,44) e 51 (−4,37) foram as mais altas da série de derivados sintetizados, comparáveis à indometacina, o medicamento padrão, com uma pontuação de 4,47. Compound 50 was shown to bind at the active site of COX-2, forming hydrogen bonds at GLY536 and TYR373. Hydrophobic interactions were found mainly at GLN374 and ARG376. Compound 51 also stood out for interacting at the COX-2 active site, forming two hydrogen bonds at ASN375 (Kerzare et al., 2016). The presence of methyl or hydroxyl groups, as well as a 1,3,4-oxadiazole nitro substitution, increased the activity. The nesting of these engineered molecules demonstrated their entry into a deep region of the enzyme. Thus, the presence of an indole ring and oxadiazole in the molecule are considered beneficial for activity, but the presence of halogens such as chlorine or fluorine reduces activity.

Was evaluated the analgesic activity of the synthesized compounds using the hot plate test. They were tested at an oral dose of 100 $mg{\cdot}kg^{-1}$, and compared to indomethacin at a dose of 100 $mg{\cdot}kg^{-1}$ (v.o.), the tested compound series showed analgesic activity after 90 min ranging from 25.13% to 84.11%. The results revealed compounds 50, 51, and 52 (*m*-NO_2) as presenting good analgesic activity, while compounds 49 and 61 (o-F) presented intermediate activity, and compounds 56 (p-COCH3) and 57 presented lesser activity as compared to the medicinal standard. The results indicate that compounds possessing electron-withdrawing groups with *para* and *meta* substituents can increase analgesic activity, while electron donor groups decrease activity (Kerzare et al., 2016).

p-Cymene

P-cymene (**Table 5**, ID 04) is a monoterpene found in the essential oil of approximately 100 herb species, and also present in over 200 types of food (Selvaraj et al., 2002). Its many biological effects, such as anti-inflammatory and analgesic activity, have been studied and demonstrated in various parts of

the world (Bonjardim et al., 2012; De Souza Siqueira Quintans et al., 2013; Quintans-Junior et al., 2013; De Santana et al., 2015).

As a potential alternative to cancer-associated pain, Santos et al. (2019) evaluated the effects of p-cymene on animal models of Sarcoma 180 (S180) induced nociception and investigated how it may act to promote such effect.

The animals submitted to the sarcoma-causing agent received the treatment with p-cymene at doses of 12.5, 25 or 50 mg/kg subcutaneously for 15 days in a row. The rats were evaluated for sensitivity to mechanical stimulation using the von Frey test on the tumor-bearing paw. Four measurements were taken between 3-min intervals to verify the stimulus intensity. It was seen that p-cymene at a dose of 50 mg/kg was able to reverse the hyperalgesic graph, starting from the 11th to the 15th day, with 60.4% of inhibition, equivalent to morphine, which similarly caused a decrease in animal perception (Santos et al., 2019).

The molecular interactions between p-cymene and the various voltage-dependent calcium channel subtypes were analyzed by docking studies, using p-cymene, nicardipine, ω-conotoxin GVIA, ω-agatoxin IVA, and N-triazole oxindole as ligands. The Protein Data Bank provided the macromolecules for this study, which were CaV1 calcium channel (type L) (PDB ID 5GJV), CaV2.1 calcium channel (type P/Q) (PDB ID 3BXK), CaV2.2 calcium channel (type N) (PDB ID 3DVE) and CaV2.3 calcium channel (type R) (PDB ID 3BXL). All ligands were subjected to molecular anchoring using the MolDock algorithm (Thomsen and Christensen, 2006; Santos et al., 2019).

When p-cymene interacted with CaV1, CaV2.1, CaV2.2, and CaV2.3 calcium channels, the respective negative energy values of −60.118, −59.60, −49.55, and −59.95 kcal/mol suggested that binding between the targets is both favorable and likely occurs since such negative values suggest lower energy expenditure to assume a more stable interaction (Du et al., 2016). These voltage-dependent calcium channels can be found at presynaptic terminals and participate in the release of neurotransmitters, such as substance P, glutamate, and CGRP (Lee, 2013). The density of the calcium stream was significantly reduced by p-cymene. It is known that direct inhibition of calcium channels alone by exogenous ligands may cause antinociception (Freitas et al., 2018). In addition, this mechanism of action is equivalent to certain existing drugs that are currently used to treat chronic pain, such as gabapentin (Santos et al., 2019; Catterall and Swanson, 2015).

We observed that many studies covered in this review used molecular coupling to investigate the mechanism of action of several compounds, one of the objectives of molecular docking studies. Thus, the studies described in this review use docking in the second stage, that is, after the experimental tests. However, there are other docking approaches that are used in a first stage, that is, before biological assessment to avoid spending on reagents and the irrational use of animal models. Therefore, instead of attempts to find potential compounds, other methodologies can be used to select the most promising compounds with the potential therapeutic effect even before biological assays.

With the advancement of more robust computational techniques, in silico studies guarantee greater reliability of results and rational drug planning.

Virtual screening is one of the methods that can be used in the investigation of compounds with antinociceptive activity. This method consists of screening chemical compound libraries using computational models or molecular docking in order to evaluate and/or select compounds with desired properties. Virtual screening is a fast and low-cost alternative for screening and selecting potential compounds for experimental evaluation (Alves et al., 2017). Docking is the main technique used in virtual screening based on structure. In this case, the molecules are coupled to the binding site and classified based on their predicted binding affinity or complementarity.

Pharmacophoric models based on structure are also a good alternative for the investigation of compounds with therapeutic potential. A pharmacophore model consists of a molecular recognition of a biological target shared by a group of compounds. Structure-based pharmacophores (SBPs) can work with either a free structure (apo) or a structure of the macromolecule-ligand complex (holo) (Pirhadi et al., 2013). These methods use the potential interactions observed between the ligand and the protein, while the SBP method, which aims to derive the pharmacophore from the free protein of the ligand, uses only information from the active site of the protein. This type of method also reduces costs and is considered a valuable tool for optimizing hits for leads, virtual screening, scaffolding jumping and design of drugs with multiple targets (Pirhadi et al., 2013).

Consensus docking uses various docking programs or various types of scoring functions to increase docking accuracy. The method helps to increase the classification power and, therefore, the hit rates, but combines information about the predicted connection modes instead of predicted connection affinities (Houston and Walkinshaw, 2013). Ao usar mais um programa de encaixe para visualizar uma pose de ligação, as poses podem atingir um índice de 82% (Houston and Walkinshaw, 2013).

Different Molecular Docking Approaches Applied to Antinociceptive Studies

Most of the studies reported in this review describe the use of docking to investigate the mechanism of action, characterize interactions between targets and ligands, and assess antinociceptive activity at the molecular level. However, there are some Docking approaches that can be used to assess antinociceptive activity for different purposes.

An interesting study addressed by Poli et al. (2019), used virtual screening as a method to filter the compounds with the greatest potential to be tested experimentally. Considered one of the first examples of virtual screening studies focused on the identification of new peptide ligands as opioid modulators, the authors used parallel virtual screening from an internal library. The library containing 198,000 tetrapeptides was filtrated using a pharmacophore coupled to the X-ray structure of the μ-opioid receptor linked to the β-FNA morphine antagonist (PDB 4DKL code) using the LigandScout software. With this first filter,

28,070 compounds were selected and submitted to Docking using the software Glide. The software was able to select 146 compounds that were subjected to a second coupling using the Autodock Vina software. Vina was able to select 15 best compounds with probability of antinociceptive activity in opioid targets that were subjected to molecular dynamics studies to investigate the affinity of interactions in the presence of factors, such as solvent and ions. The three most promising peptide compounds were synthesized and subjected to biological evaluation. The results showed that peptide 1 showed selectivity for MOR and demonstrated an appreciable inverse agonist effect in MOR. The authors concluded that this peptide may represent a promisingly successful new compound to be used as a starting point for the optimization of structure-based ligands, with the aim of discovering potent opioid modulators.

Another study by Khanna et al. (2019) also used virtual screening to identify small molecules that disrupt the CaVα–CaVβ interaction. A commercially available library at ChemBridge was used to couple 50,000 small commercially available drug-like molecules. Of these compounds, 49

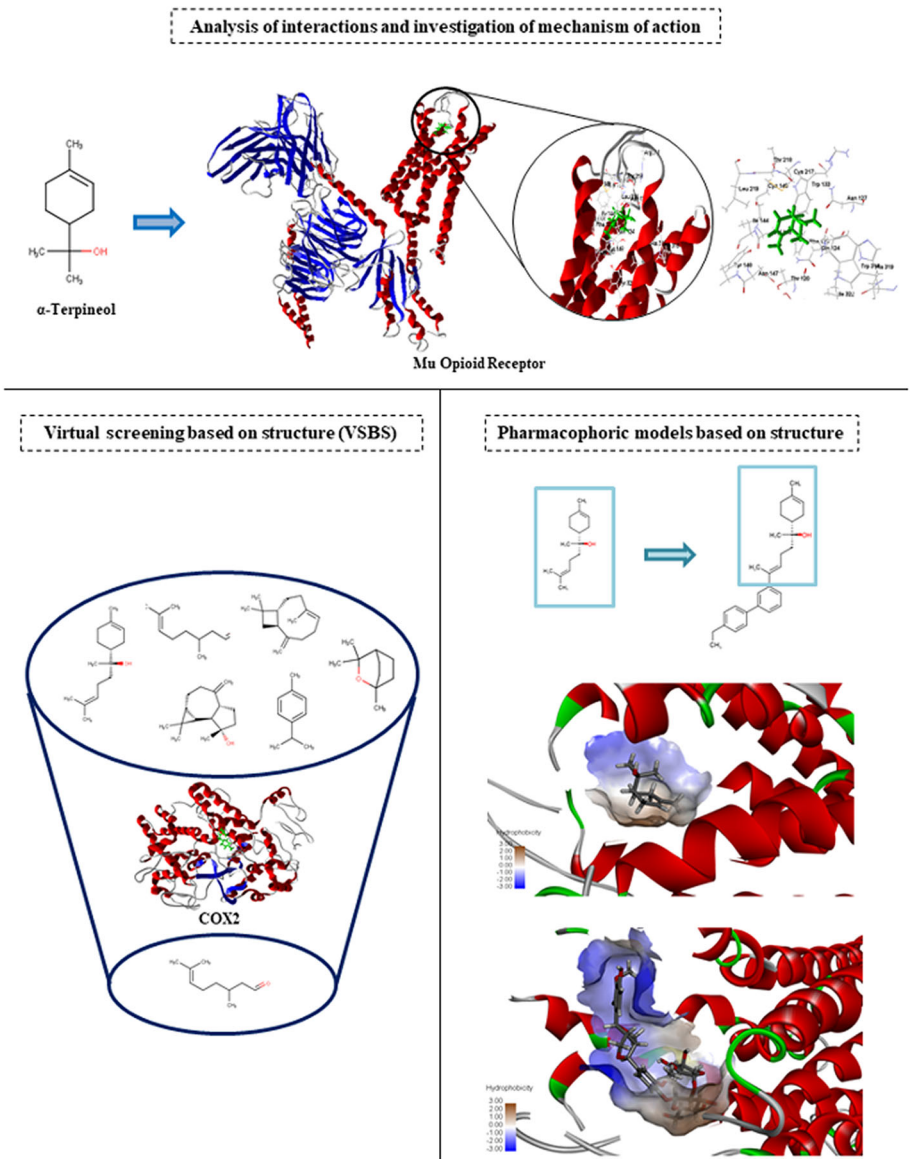


FIGURE 2 | Different molecular docking approaches that can be applied in studies of antinociceptive activity. The interaction analysis allows to evaluate the main connections and interactions observed between compounds and targets before and after experimental tests. The virtual screening based on the structure consists of selecting selective compounds according to the binding affinity with the target protein. Pharmacophoric models based on structure consist of the molecular recognition of a target shared by a group of compounds with a similar structural base.

compounds were screened for their ability to inhibit calcium influx induced by depolarization in rat DRG neurons. These compounds were purchased, 13 were found to be insoluble or killed neurons, and 11 compounds inhibited the influx of Ca by 50%. The anchored compound, 2-(3,5-dimethylisoxazol-4-yl)-N-((4-((3-phenylpropyl)amino)quinazolin-2-yl)methyl) acetamide (IPPQ) was capable to interrupt the CaVα interaction CaVβ and considered as a non-opioid therapy for chronic pain.

An approach based on the design of compounds from docking and virtual screening was used by Lee et al. (2014). The researchers observed through bibliographic research that some compounds with a piperidine portion, such as haloperidol, penfluridol, pimozide, flunazirine, and TTA-P2, were well known as type T calcium channel inhibitors. Thus, inspired by these compounds and other types second generation, new compounds with greater potential for the treatment of neuropathic pain were designed. To predict a binding affinity of the projected compounds to the T-type calcium channel before synthesizing them, the researchers mapped the 3D ligand-based pharmacophore model generated by a common resource generation approach (HipHop) implemented in the CATALYST program (Doddareddy et al., 2007). The results showed that 4-phenyl compounds and 4-(3,4-dichloro) phenyl tetrahydropyridine compounds 7a and 7b showed bonds and interactions with the target similar to that of their tetrahydropyridine analogs 7. These compounds were evaluated for effect antinociceptive in rat models for neuropathic pain and were significantly effective in decreasing pain responses to mechanical mechanisms.

Daga et al. (2014), presented a combined method based on structure and ligand. They used a manual structure-based pharmacophore, which has the advantage of finding the binding conformation of the ligand in the bioactive state of the receptor. The structure of the nociceptin receptor (NOP), of the family of opioid receptors was used to fit with agonist ligands. Given the structure-activity relationships for known NOP ligands, the researchers developed a hybrid method that combines a structure-based and ligand-based approach, using the active state NOP receptor, as well as the pharmacophoric resources of ligands showed greater effectiveness than methods individual. The results showed that the NOP receptor binding affinity of a selected set of high-scoring hits resulted in the identification of several compounds with measurable binding affinity at the NOP receptor, one of which had a new chemotype for binding to the NOP receptor.

A summary of all docking approaches observed in this review is shown in **Figure 2**.

CONCLUSION

The data presented in this review demonstrate the importance of studies with natural products, more specifically involving pharmacology with the help of bioinformatics/cheminformatics techniques, which is currently complementing and facilitating the discovery of new compounds, guiding and orienting studies towards specific molecular targets.

The most cited compounds in docking studies in this review are monoterpenes, especially α-Terpineol, (−)-α-bisabolol, Camphor, and p-Cymene. Many of the research addressed in this review used molecular docking to investigate the mechanism of action of various compounds and molecular dynamics to elucidate the stability of protein–ligand complexes in systems containing water, ions, temperature, and pressure. For new investigations, future perspectives include the advancement of more robust computational techniques and the increase *in silico* studies that complement the experimental studies, the tendency is to use computational resources in the first stage in the investigation of molecules with potential biological activity for certain diseases. Computational methods contribute to the selection of chemical structures with the highest probability of biological activity and the rationalization of these compounds. Several studies use QSAR (Quantitative Structure-Activity Relationship) methods to identify potential molecules with antinociceptive activity.

AUTHOR CONTRIBUTIONS

All authors contributed to the development of the article. DA, HA, DD, and HA held the discussion of the articles. RC, RB, and NB performed the methodology, bibliographic search, and selection of articles. MM and LS were in charge of generating the tables and discussing the content of chemoinformatics. MS and RA responsible for the general review of the content.

FUNDING

This study was supported by funds from the Coordination for the Improvement of Higher Education Personnel (CAPES) and the National Council for Scientific and Technological Development (CNPq) and the Federal University of Paraíba (UFPB) for funding the article invoice.

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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.

frontiers in Neuroscience

REVIEW
published: 09 June 2020
doi: 10.3389/fnins.2020.00478

Exploring the Promise of Flavonoids to Combat Neuropathic Pain: From Molecular Mechanisms to Therapeutic Implications

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience

Received: 15 January 2020
Accepted: 17 April 2020
Published: 09 June 2020

Citation:
Uddin MS, Mamun AA, Rahman MA, Kabir MT, Alkahtani S, Alanazi IS, Perveen A, Ashraf GM, Bin-Jumah MN and Abdel-Daim MM (2020) Exploring the Promise of Flavonoids to Combat Neuropathic Pain: From Molecular Mechanisms to Therapeutic Implications. Front. Neurosci. 14:478. doi: 10.3389/fnins.2020.00478

Neuropathic pain (NP) is the result of irregular processing in the central or peripheral nervous system, which is generally caused by neuronal injury. The management of NP represents a great challenge owing to its heterogeneous profile and the significant undesirable side effects of the frequently prescribed psychoactive agents, including benzodiazepines (BDZ). Currently, several established drugs including antidepressants, anticonvulsants, topical lidocaine, and opioids are used to treat NP, but they exert a wide range of adverse effects. To reduce the burden of adverse effects, we need to investigate alternative therapeutics for the management of NP. Flavonoids are the most common secondary metabolites of plants used in folkloric medicine as tranquilizers, and have been claimed to have a selective affinity to the BDZ binding site. Several studies in animal models have reported that flavonoids can reduce NP. In this paper, we emphasize the potentiality of flavonoids for the management of NP.

Keywords: neuropathic pain, neuronal injury, flavonoids, benzodiazepines, GABA

INTRODUCTION

Neuropathic pain (NP) is caused by damage or disease affecting the somatosensory nervous system (SSNS) (Colloca et al., 2017; Murnion, 2018). NP may be connected with aberrant sensations, known as dysesthesia, or pain from usually non-painful stimuli, called allodynia. The SSNS plays a pivotal role in the transfer of noxious stimuli to the central nervous system (CNS) under normal

Abbreviations: BDZ, benzodiazepines; CNS, central nervous system; CIPN, chemotherapy-induced peripheral neuropathy; CCI, chronic constriction injury, EGCG, epigallocatechin gallate; GABA, γ-amino butyric acid; MDA, malondialdehyde; NMDA, N-methyl-D-aspartate; NP, neuropathic pain; NF-κB, nuclear factor kappa B; Nrf2, nuclear factor erythroid 2-related factor 2; SDH, spinal dorsal horn; SNL, spinal nerve ligation; SSNS, somatosensory nervous system.

circumstances (Myers and Bennett, 2008). Therefore, lesion of the SSNS leads to the innervations of nerve cells stopping and causes pain with or without a sensory hypersensitivity event in the painful region (Jensen and Baron, 2003). Furthermore, an injury in the SSNS could reveal itself as negative sensory symptoms or positive sensory symptoms. The positive sensory symptoms occur because of the regeneration as well as disinhibition of the nerve cells, whereas the negative sensory symptoms occur owing to the partial or complete loss of input to the nervous system (Shehla, 2019; von Hehn et al., 2012). Moreover, positive symptoms can be either spontaneous or stimulus-induced.

Paresthesia (i.e., aberrant sensations of the skin including tingling, chilling, numbness, burning, and pricking), spontaneous or shooting stimulus-independent pain, as well as electric shock-like sensations are involved in spontaneous positive symptoms; whereas, stimulus-induced positive symptoms of neuropathy include allodynic and hyperalgesia pain (Rasmussen et al., 2004; Beran, 2015). On the other hand, hypoesthesia (i.e., decreased sensations to non-painful stimuli), hypoalgesia (i.e., decreased sensations to toxic stimuli), pallhypesthesia (i.e., decreased sensations to vibration), and thermohypoesthesia (i.e., decreased sensations to cold/warm stimuli) are negative symptoms of NP (Jensen and Baron, 2003; Toh et al., 2018).

Many studies have revealed the prospective efficacy of phenytoin, mexiletine, dextromethorphan, tricyclic antidepressants, gabapentin, tramadol, pregabalin, opioids, and lamotrigine for painful sensory neuropathy (Harden, 1999; Attal, 2001). Conversely, these treatments cause a 30–50% decline in pain and are frequently restricted owing to their noticeable adverse effects, with dominant sedative action. Nowadays, natural products like plant secondary metabolites are widely used to treat several chronic diseases due to their limited adverse effects as well as high efficacy (Uddin et al., 2018b, 2020e; Begum et al., 2019; Samsuzzaman et al., 2019; Thangapandiyan et al., 2019; Basu and Basu, 2020). Flavonoids are a broad group of secondary metabolites, extensively found in many fruits, vegetables, wine, cocoa, and tea (Chun et al., 2007; Egert and Rimbach, 2011). Flavonoids are recognized to have antioxidant, analgesic, and anti-inflammatory properties (Uddin and Upaganlawar, 2019). Moreover, these effects are associated with the suppression of nuclear factor kappa B (NF-κB)-dependent pro-inflammatory cytokines (Borghi et al., 2018), vascular endothelial growth factor, intercellular adhesion molecule 1, signal transducer and activator of transcription 3 (Verri et al., 2012), and activation of antioxidant transcription factor including nuclear factor erythroid 2-related factor 2 (Nrf2) (Borghi et al., 2018).

Numerous flavonoids have been demonstrated to be safe natural alternative treatments against neuropathic pain, oxidative stress, and neuroinflammatory diseases (Azevedo et al., 2013; Quintans et al., 2014; Anusha et al., 2017; Carballo-Villalobos et al., 2018; Ginwala et al., 2019). Hence, flavonoids are considered as multi-target drugs, which elucidate their wide range of actions. Here, we have reviewed the recent studies on the promising effects of flavonoids for the treatment of NP.

MECHANISMS OF NEUROPATHIC PAIN

Copious research in animal models have delivered some hint as to the pathophysiological mechanisms that produce NP, which involves both central and peripheral mechanisms (Baron, 2006; Campbell and Meyer, 2006; Gilron et al., 2006) as shown in **Figure 1**. Furthermore, the peripheral sensitization is performed through unmyelinated C- as well as finely myelinated Aδ-primary afferent neurons, which usually produce the sensation of pain in response to noxious stimuli. Conversely, the peripheral nerve injuries sensitize these neurons that develop a spontaneous activity. Moreover, these injuries result in significant alterations on the molecular and cellular levels activating the nerve cells (Baron, 2006).

Overexpression of messenger ribonucleic acid (mRNA) for voltage-gated sodium channels in the primary afferent neurons is accountable for ectopic spontaneous activity after nerve damage. This event might cause the clustering of these channels, which declines the action potential threshold, leading to hypersensitivity. Therefore, sodium channel blockers, including lidocaine, demonstrate pain relief action in NP through this mechanism (Lai et al., 2003).

Peripheral nerve injury is also responsible for the upregulation of various receptor proteins. These receptors are usually found at the membranes of the primary afferents and are partly expressed during physiological conditions. Vanilloid receptors, including the transient receptor potential cation channel subfamily V member 1 (TrpV1), play a crucial role in the sensing of toxic heat exceeding 43°C (Patapoutian et al., 2003), while transient receptor potential cation channel subfamily M (melastatin) member 8 (TRPM8) has been recognized as cold and menthol-sensitive which increase at temperature ranges from 8 to 28°C. Furthermore, the TRPM8 receptor is expressed in neurons that are small in diameter from the dorsal root ganglia (McKemy et al., 2002). Nerve injuries can cause the upregulation of this channel, contributing to peripheral sensitization of C-nociceptors, which results in cold hyperalgesia (Wasner, 2004).

In addition, acid-sensing ion channels are thought to take part in static mechanical hyperalgesia (Price et al., 2001). In contrast, both α_1- and α_2-adrenoceptors situated on the cutaneous afferent fibers play an essential role in hypersensitivity from nerve damage (Baron et al., 1999). Furthermore, adrenergic sensitivity has extensively been expressed in complex regional pain syndromes II, post-traumatic neuralgias, and postherpetic neuralgias; while there is no sensitivity in the primary afferent neurons, which have been claimed in the case of polyneuropathies (Schattschneider et al., 2006). Therefore, sympathetically-induced and temperature-mediated pain can be cured by inhibiting their relevant receptors on nociceptive neurons.

Ectopic activity is mediated by inflammation in both injured and contiguous typical primary afferent nociceptors, which are activated by nerve damage that generates proinflammatory cytokines, particularly tumor necrosis factor-α (TNF-α) (Sommer, 2003). Furthermore, deep proximal, as well as paroxysmal pains are noticeable symptoms in the case of patients who have peripheral neuropathies, including human immunodeficiency virus-neuropathy. Increased concentrations

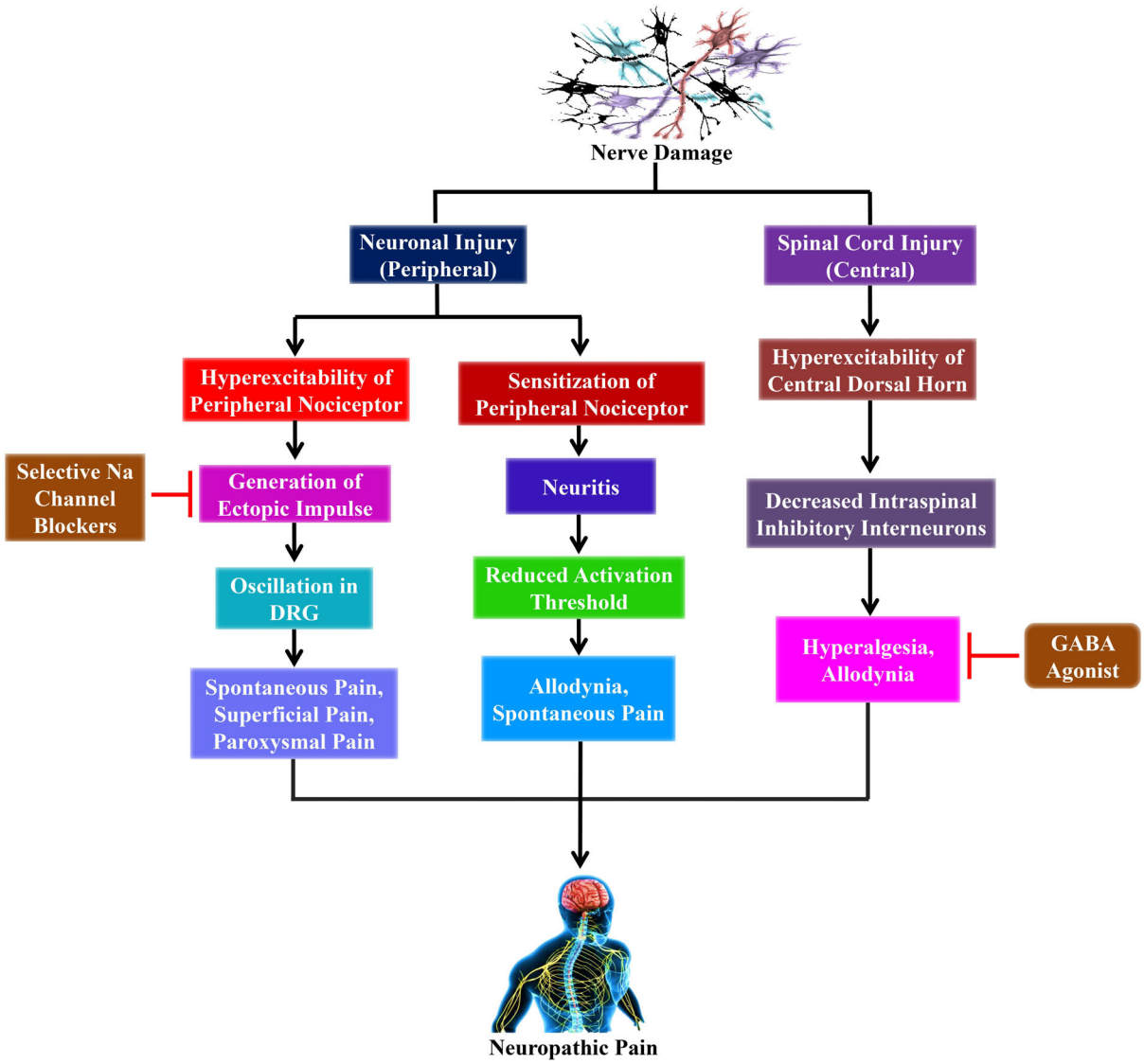

FIGURE 1 | The outlines of the mechanism of neuropathic pain from nerve damage with probable clinical interventions. Nerve damage leads to peripheral nerve injury as well as spinal cord injury (central). The peripheral sensitization and hyperexcitability take place due to the peripheral nerve injury. Furthermore, the hyperexcitability of peripheral nociceptor leads to the generation of ectopic impulses, which plays a crucial role in producing spontaneous pain, superficial pain, and paroxysmal pain that ultimately leads to neuropathic pain. Conversely, selective sodium (Na) channel blockers such as lidocaine and carbamazepine inhibit the generation of ectopic impulses that reduces the sensation of neuropathic pain. On the other hand, the hyperexcitability of the central dorsal horn is caused by spinal cord injury that subsequently decreases intraspinal inhibitory interneurons, which finally leads to neuropathic pain. However, GABA agonists, including baclofen, inhibit the decreased intraspinal inhibitory interneurons that plays an essential role in reducing the sensation of neuropathic pain. DRG, dorsal root ganglion; Na, sodium.

of proinflammatory cytokines and cyclooxygenase-2 (COX-2) have been found in the nerve biopsy specimens of these patients (Lindenlaub and Sommer, 2003).

CNS forms precise anatomical connections with the thalamus, brain stem, cortex, and spinal cord. Furthermore, these relations can connect the sensations that are produced in the high threshold primary afferents with the cortical areas of the CNS, which subsequently processes it into final painful sensations (Woolf, 2011). The constant hyperactivity is produced by damaged nerves that are considered to be a causative factor for central sensitization, as well as triggering activity-dependent synaptic flexibility occurring inside the cortex. Moreover, various factors are involved in central sensitization such as excitatory amino acid, changes in ion channel kinetics, different synaptic modulators, pre- and post-synaptic activation of kinases, and increased bulk of ionotropic receptors.

Most of the patients who have peripheral as well as central neuropathy demonstrate dominant synaptic facilitation leading to hypersensitivity and allodynia (Campbell and Meyer, 2006). Additionally, peripheral nerve damage results in pre-synaptic

changes such as alterations in the synthesis of neuromodulators, neurotransmitters, and modifications in the calcium channels density (Hendrich et al., 2008). In contrast, post-synaptic changes take place due to the increased density of receptors on account of increased synthesis of ion channels and scaffold proteins and the phosphorylation of N-methyl-D-aspartate (NMDA) subunits (Cheng et al., 2008). These alterations also lead to aberrant expression of the mitogen-activated protein kinase system and Nav 1.3 (Hains et al., 2004; Ji and Woolf, 2001). Furthermore, pathologically sensitized C-fibers sensitize neuropeptide substance P as well as a spinal dorsal horn (SDH) through the release of glutamate, which cannot be neglected. Subsequently, glutamate demonstrates an excitatory action by acting upon the postsynaptic NMDA receptor contributing to central sensitization (Ultenius et al., 2006). It has been observed that the involvement of loss of function of tonic γ-amino butyric acid-A ($GABA_A$)-conciliated inhibition and enhanced excitatory neurotransmitters are caused by an induction of central sensitization, leading to peripheral hypersensitivity, specifically hyperalgesia and allodynia (Knabl et al., 2008). When this sensitivity is developed, the generally harmless tactile stimuli can trigger Aβ as well as Aδ low threshold mechanoreceptors (Tal and Bennett, 1994).

GABA AND NEUROPATHIC PAIN

The most abundant inhibitory neurotransmitter in the brain is GABA (Uddin et al., 2018a). GABA regulates diverse physiological functions such as anxiety, sleep, reward, and memory formation (Zeilhofer et al., 2009; Spiering, 2018; Uddin and Amran, 2019). GABA also regulates the excitatory action of neuronal cells of the CNS, assisting and maintaining the neural circuit's homeostasis. Previously, it has been described that the role of inhibitory neurons, especially in SDH, act and monitor transmission of pain via the periphery to greater intensities of the brain (Melzack and Wall, 1965). After this, GABA was established to be the primary inhibitory neurotransmitters in the brain's SDH (Yaksh, 1989).

GABA, releasing from presynaptic neurons, acts postsynaptically with several receptors; G protein-coupled channels, $GABA_A$, $GABA_B$, as well as $GABA_C$, are ligand-gated ion channels (Gavande et al., 2011). Generally, ionotropic $GABA_A$ receptors are comprised of 5 heteropentameric subunits that form transmembrane protein complexes (Uddin and Rashid, 2020). Meanwhile, the $\alpha_1\beta_2\gamma_2$ subunit is thought to be the most dominant one in the human brain (Wafford, 2005). GABA initiation stimulates the membrane penetrability to chloride and carbonate ions that produce a net inner flow of anions as well as resulting in hyperpolarization. Therefore, this hyperpolarizing post-synaptic reaction is known as inhibitory post-synaptic potential (Semyanov et al., 2003).

Physiologically, GABA-liberating interneurons impose a robust inhibitory regulation through dorsal horn neuronal cells. Besides, damage of these neurons might additionally stimulate the dominant sensitization of the models of peripheral partial nerve injury. In rodents, injury caused the reduction of GABA release from the spine, with reduced GABA-producing glutamic acid decarboxylase (Moore et al., 2002). However, in diseased conditions, an improved excitation state arises that is recognized as an enormous GABAergic neuronal loss or deterioration of interneurons. Therefore, an imbalance of this condition could culminate into several neurological as well as psychiatric diseases such as Alzheimer's disease, Parkinson's disease, schizophrenia, epilepsy, NP, and the collective role of inhibitory and excitatory neurons show a dynamic role in regulating many brain activities (Tyson and Anderson, 2014).

It has been found that peripheral and central sensitization causes nerve injury and NP. GABAergic interneuronal loss is considered to be the main contributor to persistent pain states (Bráz et al., 2012). In the spinal cord, pharmacological inhibition of GABAergic neurotransmission causes hyperalgesia and allodynia (Gwak et al., 2006; Jergova et al., 2012). Likewise, $GABA_A$ receptor blockage could prompt a behavioral reaction, which was revealed by electrophysiological studies (Hwang and Yaksh, 1997). Furthermore, the GABAergic system impaired chronic NP in animals (Zeilhofer, 2008). As a result, spinal inhibitory neurotransmission may be appreciated as a pharmacological NP treatment.

Additionally, the crucial function of GABA in opioid-mediated antinociception has long been recognized (Ossipov et al., 2010). Also, agonists of $GABA_A$ receptor-mediated antinociceptive activity have been recognized to stimulate or inhibit additional neurotransmitters (McCarson and Enna, 2014). As a consequence, the agonists of the GABA receptor might play a dynamic role in considering chronic and acute pain (McCarson and Enna, 2014). Incidentally, isoguvacine and muscimol, agonists of $GABA_A$ receptors, are described to oppose nerve injury-stimulated tactile allodynia (Hwang and Yaksh, 1997). These receptors are strictly linked to huge diameter afferents involved in innocuous sensation (Price et al., 1984; Sivilotti and Woolf, 1994; Reeve et al., 1998; Ataka et al., 2000; Riley et al., 2001; Turner, 2003). Pharmacological as well as behavioral examinations have stated that a single or continuous intrathecal GABA response to spinal cord or GABA liberating cells reduce NP (Eaton et al., 1999a,b; Stubley et al., 2001; Malan et al., 2002). In addition, spinal $GABA_A$ receptors inhibition shows annoying peripheral nerve injury connected to hyperalgesia (Yamamoto and Yaksh, 1993).

In contrast, intrathecal administration of benzodiazepines (BDZs) and allosteric positive modulators of $GABA_A$ receptors have been extensively used in sleep complaints, convulsions, anxiety, and analgesic activity (Tucker et al., 2004). Even though it has analgesic properties, its usage in pain relief is limited due to sedation. Therefore, study is urgently needed in to GABAergic modulators which might play a prominent role in the attenuation of NP.

FLAVONOIDS

Flavonoids are polyphenolic compounds found in fruits, flowers, barks, grains, vegetables, roots, tea, stems, and so on (Uddin et al., 2020a). Chemically, flavonoids are 15-carbon skeletons

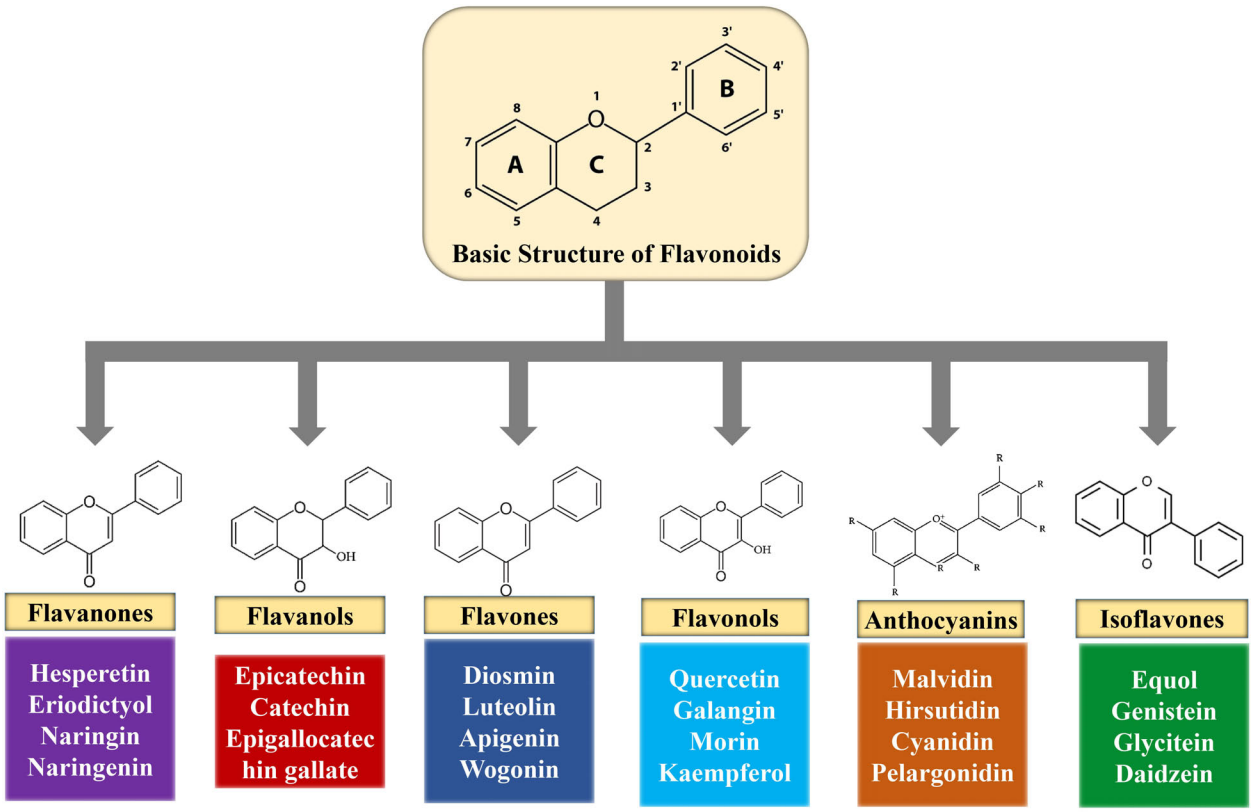

FIGURE 2 | Basic structure of flavonoids and their sub-classes.

comprising of two benzene rings (A and B) linked via a heterocyclic pyrane ring (C) (Kumar and Pandey, 2013) as shown in **Figure 2**. Flavonoids can be divided into diverse subgroups according to the carbon of the C ring whereon the B ring is connected as well as the oxidation and degrees of unsaturation of the C ring (Panche et al., 2016).

In 1930, a novel constituent derived from an orange was believed to be a vitamin and called vitamin P. It was subsequently proved to be a flavonoid, rutin, that played an essential role in the isolation as well as the study of the mechanisms of several individual flavonoids. In fact, several traditional medicines are mainly flavonoids. In past centuries, *Tanacetum parthenium* has been used as a prophylactic drug in the treatment of migraine, while *Matricaria recutita*, chamomile flowers, has been used as a tranquilizer for many decades, with both comprising of the active constituent apigenin (Jäger et al., 2009). Moreover, linden flowers, *Tilia sp. Tiliaceae*, have been used as sedative agents, and *Calluna vulgaris* might serve as a nerve-calming medicine, which has active components of kaempferol and quercetin (Aguirre-Hernández et al., 2010). Apart from the separation of natural flavonoids, several synthetic and semi-synthetic products have been synthesized and separated for their therapeutic potential (Cushnie and Lamb, 2005). Up to now, 6000 diverse flavonoids have been isolated. Flavonoid compounds show different biological effects, such as neuroprotective (Cho et al., 2013; Uddin et al., 2016; Uddin and Kabir, 2019; Zaplatic et al., 2019), antifungal (Ammar et al., 2013), antimicrobial (Cushnie and Lamb, 2005; Górniak et al., 2019), anticancer (Liu et al., 2010; Abotaleb et al., 2019), anti-inflammatory (Wang et al., 2010; Begum et al., 2019), anxiolytic (Ognibene et al., 2008), antioxidant (Heim et al., 2002; Uddin et al., 2017), antiviral (Orhan et al., 2010; Dai et al., 2019), cardioprotective (Yu et al., 2005; Mahmoud et al., 2019), and antinociceptive activities (Wang et al., 2014; Hossain et al., 2017a,b).

ROLE OF FLAVONOID ON IONOTROPIC $GABA_A$ RECEPTORS

Flavonoids are widely targeted for their peripheral events; though, their selective affinity for $GABA_A$ receptors has extensively been demonstrated in studies using bovine and rat brain membrane binding analyses (Hong and Hopfinger, 2003). Numerous behavioral tests have also widely been performed, which confirm the sedative effects of flavonoids in an animal model of anxiety that was devoid of the additional side effects of BDZs (Griebel et al., 1999). Remarkably, negative, positive, and neutral allosteric modulatory flavonoid actions of an extensive variety of ionotropic GABA receptors have been focused on and intensely supported through enormous evidence. In the 1990s, flavonoids had been well-defined as a novel family of BDZ receptor ligands (Medina et al., 1997; Marder and Paladini, 2002).

Typically, they were believed to be acting upon BDZ receptors, as well as many synthetic flavonoids having a remarkable affinity for BDZ binding site (Yao et al., 2007), until they were claimed to be insensitive to the BDZ receptor antagonist, flumazenil, therefore focusing a distinctive site of action (Hanrahan et al., 2011).

It has been found that the replacement at 6- or 3′-positions of flavones with an electronegative functional group improved the affinity toward the receptors of BDZ (Paladini et al., 1999). Moreover, GABA ratios were measured by the impact of ligand binding on the GABA binding site. These ratios displayed that flavones showed substantial biological actions at BDZ receptors (Hanrahan et al., 2011). 6-Bromo-3′-nitroflavone, 6-chloro-3′-nitroflavone, and 6-bromoflavone with a GABA ratio of 1.38, 2.0, and 1.6–2.0 were demonstrated as a partial agonist, an antagonist, and full agonist at these receptors respectively (Marder et al., 1996; Wolfman et al., 1998; Viola et al., 2000).

$GABA_A$ receptors were enhanced by the flux of chloride ion that deliver a robust inhibitory effect via positive ionotropic modulators. As a result, these modulators are the strongest candidates for the management of CNS-associated diseases such as generalized anxiety, seizure disorders, sleep disturbances, panic disorders, muscle spasms, and NP (Rudolph and Möhler, 2006). Furthermore, flavonoids might act upon a new binding site, excluding the classical BDZ binding site, which plays a pivotal role in searching for novel therapeutic candidates with limited adverse effects (Rudolph and Möhler, 2006). Incidentally, 6-methoxyflavonone has been described to act as a positive allosteric modulator at $\alpha1\beta2\gamma2L$ and $\alpha2\beta2\gamma2L$ subunits of $GABA_A$ receptors (Hall et al., 2014).

The substitution at 6-position on flavones is linked to its role in recombinant $GABA_A$ receptors. 6-Hydroxyflavone showed a remarkable effect at the flumazenil-sensitive BDZ site (Ren et al., 2010). Furthermore, 6-methoxyflavone and 6-methoxyflavanone have been claimed to display anti-allodynic effects in cisplatin- and streptozotocin-stimulated NP models (Akbar et al., 2016; Shahid et al., 2017). Therefore, these defensive properties against NP have been recognized to cause allosteric positive modulatory effects on opioid and $GABA_A$ receptors (Akbar et al., 2016).

Additionally, myrcitin and baicalin exerted antiallodynic effects in sciatic nerve ligation models (Cherng et al., 2014; Meotti et al., 2006). Besides, quercetin and rutin have widely been claimed to suppress oxaliplatin-mediated chronic peripheral neuropathic pain (Azevedo et al., 2013). Meanwhile, the antiallodynic potential of streptozotocin-induced painful diabetic neuropathy has been reported by naringin (Kandhare et al., 2012).

ROLE OF FLAVONOIDS IN DIFFERENT NEUROPATHIC PAIN MODELS

Effect of Flavonoids on Diabetic Neuropathy

NP is arduous to treat properly and is related to the remarkable impairment of health conditions as well as economic problems (O'Connor, 2009; Langley et al., 2013). Diabetic neuropathy is one of the most common causes of neuropathy and affects about 382 million people in the world (Boulton et al., 1998). Furthermore, genistein (Valsecchi et al., 2011), luteolin (Li et al., 2015), catechin (Addepalli and Suryavanshi, 2018), rutin (Tian et al., 2016), and pelargonidin (Mirshekar et al., 2010) have been revealed to decrease the levels of malondialdehyde (MDA) in animal models of diabetes. Moreover, MDA serves as a key biomarker for lipid damage as well as oxidative stress that can be caused by free radicals. In diabetic patients, the increased level of MDA has widely been observed in the serum as well as other tissues, which significantly affects the peripheral nerves (Feldman et al., 1994; Perkins et al., 2001). Some flavonoids, such as genistein (Valsecchi et al., 2011), naringenin (Al-Rejaie et al., 2015), luteolin (Li et al., 2015), hesperidin, catechin (Addepalli and Suryavanshi, 2018), kaempferol (Kishore et al., 2018), fisetin (Zhao et al., 2015), rutin (Tian et al., 2016), and morin (Bachewal et al., 2018) have been shown to reduce the ROS level by increasing the level of diverse antioxidative enzymes including glutathione peroxidase, reduced glutathione peroxidase, superoxide dismutase, glutathione reductase, and catalase in various tissues such as the liver, sciatic nerve, and brain of diabetic animals (**Table 1**). In the diabetic animal model, rutin, luteolin, and morin have been demonstrated to raise the expression of Nrf2 as well as its downstream effector's heme oxygenase-1 (HO-1) in nerve tissues. Numerous studies have found that Nrf-2/HO-1 could fight against oxidative stress-mediated neuroinflammation and nerve damage in diabetic animal models (Cardozo et al., 2013; Agca et al., 2014; Kumar and Mittal, 2017). Moreover, kaempferol decreased advanced glycation end products and epigallocatechin gallate (EGCG) causes a reduction of 8-hydroxy-2-deoxyguanosine, which is considered as the major form of free radical-mediated oxidative stress in the nucleus and mitochondria (Valavanidis et al., 2009). It has also been found that in the diabetic animal model, genistein and naringenin raised nerve growth factor (NGF) in sciatic nerves (Basu and Basu, 2020). Therefore, NGF servesas the survival and life maintenance of the neurons.

Diabetic neuropathy in animal models has widely been marked by evaluating behavioral signs, such as chemical, mechanical, thermal hyperalgesia, and tactile allodynia (Pittenger et al., 2005). It has also been observed that flavonoids considerably downregulated thermal, mechanical, chemical hyperalgesia, and tactile allodynia in diabetic animal models (**Figure 3**). A number of flavonoids including fisetin (Zhao et al., 2015), baicalin (Li et al., 2018), naringenin (Al-Rejaie et al., 2015), pelargonidin (Mirshekar et al., 2010), rutin (Tian et al., 2016), naringin (Kandhare et al., 2012), hesperidin (Visnagri et al., 2014), and luteolin (Li et al., 2015) reduced diabetes-mediated thermal hyperalgesia, although kaempferol (Kishore et al., 2018), EGCG (Raposo et al., 2015), rutin (Tian et al., 2016), naringenin (Al-Rejaie et al., 2015), luteolin (Li et al., 2015), morin (Bachewal et al., 2018), and naringin (Kandhare et al., 2012) attenuated mechanical hyperalgesia (**Figure 3**). Furthermore, fisetin (Zhao et al., 2015), baicalein (Li et al., 2018), hesperidin (Visnagri et al., 2014), morin (Bachewal et al., 2018), and puerarin (Liu et al., 2014) ameliorated mechanical allodynia, while naringin (Kandhare et al., 2012) and genistein

TABLE 1 | Promising studies of flavonoids for the management of neuropathic pain.

Flavonoids	Species/studied materials	Experimental model	Dose	Route of administration	Effects	References
Genistein	C57BL/6J male mice	Chronic constriction sciatic nerve injury	1, 3, 7.5, 15, and 30 mg/kg	Subcutaneous injection	Ameliorate painful neuropathy by decreasing the mRNA expressions of IL-1β and IL-6 in sciatic nerve as well as protein expression of IL-1β in dorsal root ganglion and spinal cord	Valsecchi et al., 2008
	Male C57BL/6J mice	Streptozotocin-induced diabetic	3 and 6 mg/kg	Subcutaneous injection	Ameliorates diabetic peripheral neuropathy by inhibiting proinflammatory cytokine and the overproduction of reactive oxygen species, as well as restored the NGF content in diabetic sciatic nerve	Valsecchi et al., 2011
	Male Sprague-Dawley rats	High-fat diet	4 and 8 mg/kg/day	Intragastrical	Decreases the levels of TNF-α and IL-6 in serum that produce anti-inflammatory actions	Ji et al., 2011
Quercetin	Male albino mice	Streptozotocin-induced diabetic	50 and 100 mg/kg	Oral	Antinociceptive activity via the modulation of opioidergic mechanism that attenuates diabetic neuropathic pain	Anjaneyulu and Chopra, 2003
	Male Sprague–Dawley rats	Streptozotocin-induced diabetic	10 mg/kg	Oral	Effective in diabetic neuropathy	Anjaneyulu and Chopra, 2004
	Male Sprague-Dawley rats and mice	Paclitaxel-induced neuropathic pain	20 and 60 mg/kg – *in vivo* and 3, 10, 30 μM/L – *in vitro*	Intraperitoneal injection	Ameliorates neuropathic pain by decreasing the levels of protein kinase C (PKC)ε and TrpV1 in the spinal cord dorsal horns and dorsal root ganglions	Gao et al., 2016
Quercetin and rutin	Male Swiss mice	Oxaliplatin-induced peripheral neuropathy	Rutin and quercetin (25, 50, and 100 mg/kg)	Intraperitoneal injection	Ameliorates peripheral neuropathy	Azevedo et al., 2013
Myricitrin	Adult Swiss mice	Partial Sciatic Nerve Ligation	30 mg/kg	Intraperitoneal injection	Antinociceptive activity via the inhibition of PKC and nitric oxide cell signaling	Meotti et al., 2006
	Adult male Wistar rats	Spinal nerve ligation	0.1, 1 and 10 mg/kg	Intraperitoneal injection	Reduces neuropathic pain that might be related to its PKC-induced decrease of voltage-gated calcium channel currents in dorsal root ganglia neurons	Hagenacker et al., 2010
Epigallocatechin gallate	Adult male Wistar rats	Alcoholic neuropathy	25, 50, 100 mg/kg	Oral	Reduces neuropathic pain through the modulation of oxido-inflammatory pathway	Tiwari et al., 2011
	Male Sprague-Dawley rats	Chronic constriction injury	1 mg/kg	Intrathecal injection	Ameliorates neuropathic pain through the suppression of TLR4 signal pathway that reduces the expressions of NF-κB, IL-1β and TNF-α	Kuang et al., 2012
	Male Wistar rats	Streptozotocin-induced diabetic	2 g/L	Oral gavage	Ameliorates diabetic neuropathy by preventing oxidative stress	Raposo et al., 2015
Puerarin	Male Sprague-Dawley rats	Chronic constriction injury	100 mg/kg/day	Intraperitoneal injection	Reduces neuropathic pain through the P2X3 receptors in dorsal root ganglion neurons	Xu et al., 2012
	Male Sprague-Dawley rats	Chronic constriction injury	4, 20, and 100 nM	Intrathecal injection	Reduces neuropathic pain by the inhibition of spinal NF-κB activation and the upregulation of cytokines	Liu et al., 2014
2′′– O–rhamnosylswertisin	Female Swiss and C57/BL6 mice	Partial Sciatic Nerve Ligation	125, 250 or 500 mg/kg	Oral	Antinociceptive activity by reducing the neutrophil migration and IL-1β levels	Quintão et al., 2012

(Continued)

TABLE 1 | Continued

Flavonoids	Species/studied materials	Experimental model	Dose	Route of administration	Effects	References
Naringin	Adult male Wistar rats	Streptozotocin-induced diabetic	20, 40, and 80 mg/kg	Oral	Reduces neuropathic pain by down−regulation of cytokine including TNF-α	Liu et al., 2017
	Wistar rats.	Cisplatin-Induced Cognitive Dysfunction	25, 50, and 100 mg/kg	Oral gavage	Ameliorates neuropathic pain through the involvement of oxidative-stress-mediated inflammatory signaling	Chtourou et al., 2015
	Male Wistar rats	Streptozotocin-induced diabetic	40 and 80 mg/kg	Intraperitoneal injection	Ameliorates diabetic neuropathy by downregulation of free radical, cytokine mediator including TNF-α	Kandhare et al., 2012
	Male Wister albino rats	Streptozotocin-induced diabetic	25 and 50 mg/kg/day	Intraperitoneal injection	Ameliorates diabetic neuropathy through its antioxidant and anti-inflammatory properties	Al-Rejaie et al., 2015
Icariin	Male Sprague-Dawley rats	Paclitaxel-induced neuroinflammation and peripheral neuropathy	25, 50, and 100 mg/kg	Intrathecal injection	Reduces neuropathic pain by the level of TNF-α, IL-1β, and IL-6, astrocytes, NF-κB (p65) phosphorylation in spinal cord	Gui et al., 2018
6-Methoxyflavone	Male Sprague-Dawley rats	Chemotherapy-induced peripheral neuropathy	25, 50 and 75 mg/kg	Intraperitoneal injection	Reduces neuropathic pain	Shahid et al., 2017
	Female Sprague-Dawley rats and BALB/c mice	Streptozotocin-induced diabetic	10 and 30 mg/kg	Intraperitoneal injection	Attenuates neuropathic pain through interactions with the GABAergic and opioidergic systems	Akbar et al., 2016
Catechin	Male Sprague-Dawley rats	Streptozotocin-induced diabetic	25 mg/kg and 50 mg/kg	Intraperitoneal injection	Attenuation of diabetic autonomic neuropathy through the improvement in antioxidant enzymes in vagus nerves	Addepalli and Suryavanshi, 2018
Morin	Male Sprague-Dawley rats	Streptozotocin-induced diabetic	50 and 100 mg/kg – *in vivo*, 10 and 20 μM – *in vitro*	Oral gavage	Reduces diabetic neuropathy by inhibiting NF−κB−mediated neuroinflammation and increasing Nrf2−mediated antioxidant defenses in high glucose−induced N2A cells	Bachewal et al., 2018
	Male Sprague-Dawley rats	Chronic constriction injury	15 and 30 mg/kg	Oral gavage	Ameliorates neuropathic pain by decreasing the inflammatory markers (PARP, iNOS, COX-2, NF-κB and phospho-NF-κB, TNF-α, and IL-6) in the spinal cord	Komirishetty et al., 2016
Kaempferol	Male Wistar rats	Streptozotocin-induced diabetic	5 and 10 mg/kg		Reduces diabetic neuropathy by attenuating oxidative stress-mediated release of pro-inflammatory cytokines	Kishore et al., 2018
Rutin	Male Sprague-Dawley rats	Streptozotocin-induced diabetic	5, 25, and 50 mg/kg	Intraperitoneal injection	Ameliorates diabetic neuropathy through the up-regulation of the expression of Nrf2	Tian et al., 2016
Baicalin	Male Sprague-Dawley rats	Streptozotocin-induced diabetic	10, 20, and 40 μg/kg	Intraperitoneal injection	Analgesic activity in diabetic neuropathic pain through transient receptor potential vanilloid 1	Li et al., 2018
	C57Bl6/J mice	Streptozotocin-induced diabetic	30 mg/kg	Intraperitoneal injection	Reduces diabetic peripheral neuropathy via the suppression of oxidative-nitrosative stress as well as p38MAPK activation	Stavniichuk et al., 2011

(Continued)

TABLE 1 | Continued

Flavonoids	Species/studied materials	Experimental model	Dose	Route of administration	Effects	References
Luteolin	Male Sprague-Dawley rats	Streptozotocin-induced diabetic	50, 100, and 200 mg/kg	Intraperitoneal injection	Ameliorates diabetic neuropathy through the up-regulation of the expression of Nrf2	Li et al., 2015
	Male Sprague-Dawley rats	Chronic constriction injury	0.1–1.5 mg	Intrathecal or intracerebroventricular injection	Reduces mechanical and cold hyperalgesia by activating $GABA_A$ receptors in a flumazenil-insensitive manner as well as μ-opioid receptors in the spinal cord	Hara et al., 2014
Fisetin	Male C57BL/6J mice	Chronic constriction injury	10 mg/kg	Oral gavage	Ameliorates chronic neuropathic pain	Zhao et al., 2015
	Male C57BL/6J mice	Chronic constriction injury	5, 15 and 45 mg/kg	Oral gavage	Exerts antinociceptive activity through the serotonergic system (coupled with 5-HT7)	Zhao et al., 2015
Diosmin	Male Swiss mice	Chronic constriction injury	1, 10 mg/kg	Intraperitoneal injection	Ameliorates neuropathic pain by activating the NO/cGMP/PKG/KATP channel signaling	Bertozzi et al., 2017
Hesperidin	Sprague Dawley rats	Streptozotocin-induced diabetic	25, 50 and 100 mg/kg	Oral gavage	Reduces diabetic neuropathy by down-regulating the production of free radical, release of cytokines (TNF-α and IL-1β) and elevation in membrane bound enzyme	Visnagri et al., 2014
Diosmin and hesperidin	Male Wistar rats	Chronic constriction injury	Hesperidin (10, 100, 316.2, 562.3, 1000 mg/kg); Diosmin (10, 100 mg/kg)	Intraperitoneal injection	Ameliorates neuropathic pain by the modulation of D2 dopamine, and opioids receptors	Carballo-Villalobos et al., 2016
Pelargonidin	Male Albino Wistar rats	Streptozotocin-induced diabetic	10 mg/kg	Oral gavage	Ameliorates diabetic neuropathic hyperalgesia via attenuation of oxidative stress	Mirshekar et al., 2010
Isoorientin	Male pathogen-free Institute of Cancer Research (ICR) mice	Chronic constriction injury	7.5, 15, and 30 mg/kg	Intragastrical	Ameliorates neuropathic pain by decreasing the expression of IL-6, IL-1β, and TNF-α levels	Zhang et al., 2019
Grape seed proanthocyanidins	Wistar rats	Chronic constriction injury	100 and 200 mg/kg	Oral gavage	Anti-nociceptive and anti-inflammatory effect by inhibiting the inflammatory pathways	Kaur et al., 2016

(Valsecchi et al., 2011) decreased mechano-tactile allodynia, and rutin (Tian et al., 2016) and luteolin (Li et al., 2015) improved cold allodynia. Numerous investigations have demonstrated that short term diabetes mediated mechanical, chemical, and thermal hyperalgesia (Dyck et al., 2000; Freshwater and Calcutt, 2005), however chronic diabetes induces mechanical and thermal hypoalgesia (Calcutt et al., 2004). Besides, baicalein attenuated thermal hypoalgesia (Stavniichuk et al., 2011).

Effect of Flavonoids on Chemotherapy-Induced Peripheral Neuropathy

The use of diverse chemotherapeutic agents and other anticancer drugs leads to the impairment of the peripheral nerves. Chemotherapy-induced peripheral neuropathy (CIPN) is another form of neuropathy caused by anticancer drugs (Hershman et al., 2014). Platinum compounds are extensively used in the management of several solid tumors. Oxaliplatin, a third-generation platinum agent, plays a pivotal role in diminishing antitumoral resistance with noticeable cytotoxicity (Argyriou et al., 2008; Stein and Arnold, 2012). It has been observed that (Azevedo et al., 2013) rutin and quercetin suppressed oxaliplatin-mediated mechanical as well as cold nociceptive thresholds. In a study by Schwingel et al. (2014) it was demonstrated that rutin and nanoemulsion of quercetin ameliorated oxaliplatin-mediated mechanical allodynia. According to the study by Shahid et al. (2017) 6-methoxyflavone showed antinociceptive activity in a rat model of CIPN (**Table 1**). Hence, 6-methoxyflavone considerably reduced cisplatin-mediated mechanical allodynia by raising the paw withdrawal threshold as well as thermal hypoalgesia (**Figure 3**) by improving the paw thermal threshold.

Effect of Flavonoids on Sciatic Nerve Chronic Constriction Injury

Chronic constriction injury (CCI) is considered to be the most extensively studied model for chronic neuropathic

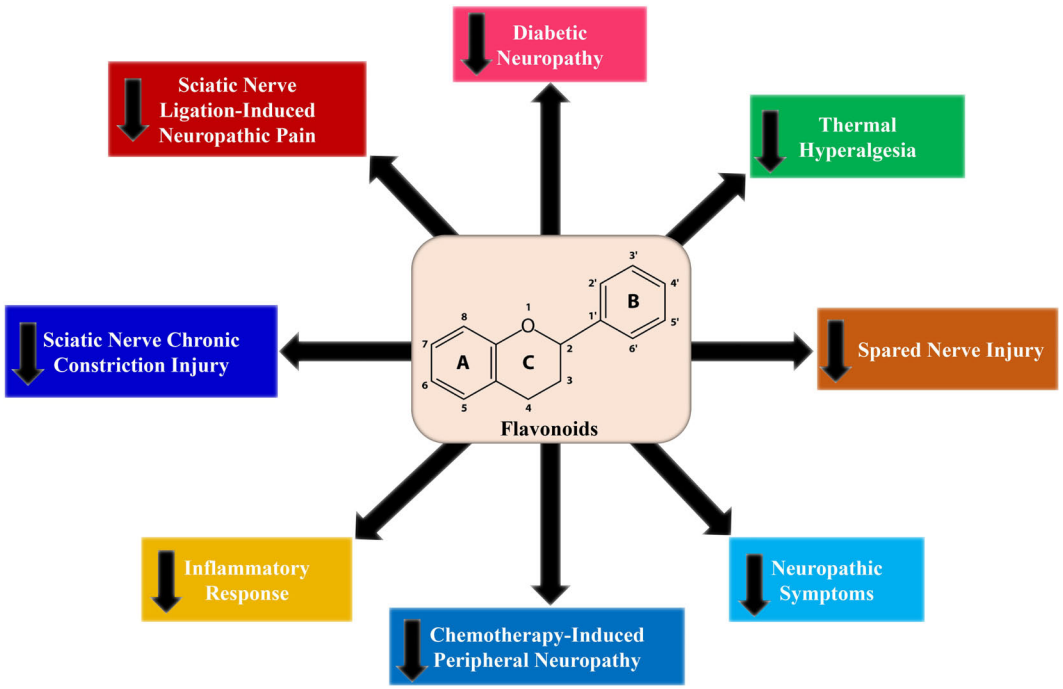

FIGURE 3 | Effects of flavonoids on different neuropathic pain models.

pain. There are various symptoms of CCI-induced pain including hyperalgesia, allodynia, paraesthesia, dysesthesia, and spontaneous pain (Austin et al., 2012). Flavonoids including hesperidin (Carballo-Villalobos et al., 2016), diosmin (Bertozzi et al., 2017), and grape seed proanthocyanidins (Kaur et al., 2016) decreased both mechanical and thermal hyperalgesia (**Figure 3**). In contrast, other flavonoids including genistein (Valsecchi et al., 2008), EGCG (Kuang et al., 2012), EGCG-derived compounds (Xifró et al., 2015), morin (Komirishetty et al., 2016), and isoorientin (Zhang et al., 2019) decreased only thermal hyperalgesia (**Table 1**). As compared to morphine and gabapentin, quercetin decreased the mechanical and thermal hypersensitivities to a greater extent (Çivi et al., 2016). When quercetin was administered in a pre-injury condition, it exerted long term actions on mechanical hypersensitivity, which further suggests the antinociceptive properties of quercetin in the CCI model (Çivi et al., 2016). Additionally, flavonoids including genistein (Valsecchi et al., 2008), EGCG (Kuang et al., 2012), puerarin (Liu et al., 2014), morin (Komirishetty et al., 2016), and isoorientin (Zhang et al., 2019) decreased CCI-mediated mechanical allodynia. On the other hand, cold allodynia was reduced by morin (Komirishetty et al., 2016) and isoorientin (Zhang et al., 2019). Although mechanical and cold hyperalgesia was reduced by luteolin, it did not affect thermal hyperalgesia (Hara et al., 2014). However, thermal hyperalgesia was decreased by fisetin, but did not affect the nociceptive sensitivity to mechanical stimuli (Zhao et al., 2015).

An elevated level of nitro oxidative stress can cause DNA damage, which can cause the activation of poly-ADP ribose polymerase (PARP) (**Figure 4**), which can further lead to PARP-induced DNA repair by transferring ADP-ribose units to the nuclear proteins. Nevertheless, activation of PARP can cause NF-κB activation, which can subsequently activate various inflammatory markers including interleukin (IL)-6, TNF-α, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) (Obrosova et al., 2004; Sommer and Kress, 2004) that lead to neuroinflammation (**Figure 4**). Studies involving the CCI-induced neuropathic pain model revealed that flavonoids exert effects on various pro-inflammatory biomarkers (Bertozzi et al., 2017; **Figure 3**). A single administration of diosmin decreased the levels of mRNA expressions of IL-33/ST2 and IL-1β, while chronic administration decreased the mRNA expression level of TNF-α along with ST2, IL-33, and IL-1β. Furthermore, a single administration also decreased the expression levels of oligodendrocytes and microglia, whereas chronic treatment decreased astrocytes together with oligodendrocytes and microglia (Bertozzi et al., 2017). Puerarin decreased the elevated immunoreactivity of glial fibrillary acidic protein and ionized calcium-binding adaptor protein-1, which are astroglia and microglial activation markers, successively (Liu et al., 2014). In the CCI-induced neuropathic pain model, morin decreased various inflammatory biomarkers including IL-6, TNF-α, phospho-NF-κB, NF-κB, COX-2, iNOS, and PARP (Komirishetty et al., 2016). Deoxyribonucleic acid (DNA) damage was found to be increased due to the CCI-induced nerve injury, which resulted in PARP overactivation (Jagtap and Szabo, 2005). It was found that overactivation of PARP caused bioenergetic failure because overactivity of PARP requires a high amount of nicotinamide adenine dinucleotide (NAD) during DNA repair, and finally, NAD synthesis also requires

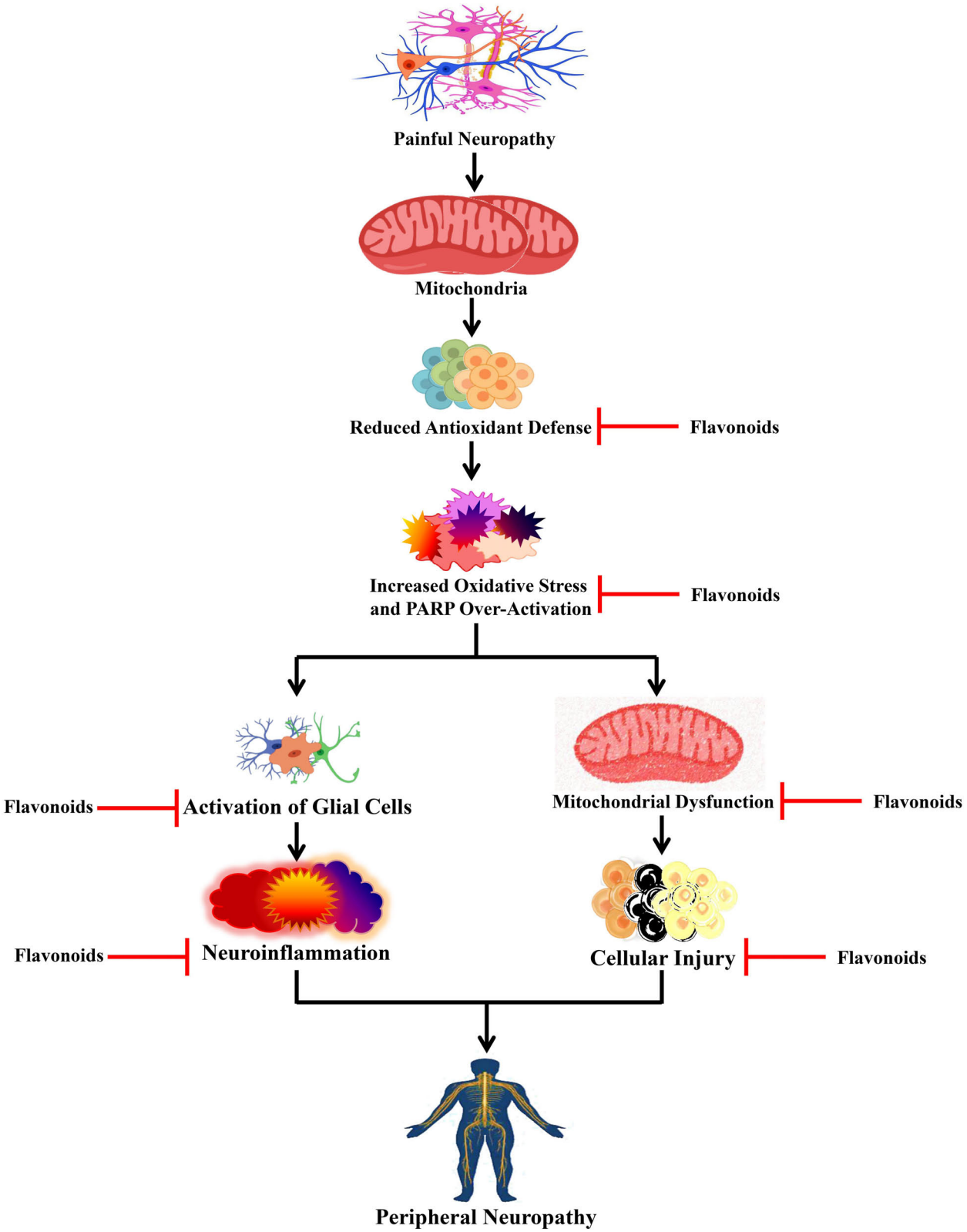

FIGURE 4 | Effects of flavonoids on peripheral neuropathy. Flavonoids act on different peripheral neuropathic pain conditions by blocking oxidative stress, activation of glial cells, and mitochondrial dysfunction. PARP, poly-ADP ribose polymerase.

adenosine triphosphate (ATP), which can eventually lead to the disruption of biochemical processes that are dependent on ATP (Hyo and Snyder, 1999).

Treatment with morin caused marked restoration of CCI-mediated reduction in the ATP levels and also restored the neuronal cells from the bioenergetic crisis (Komirishetty et al., 2016). In a study, Kuang et al. (2012) revealed that treatment with EGCG reduced the mRNA and protein expressions of the toll-like receptor (TLR4) and its endogenous ligand HMGB1. It is known that TLR4 is a pattern recognition receptor and plays roles in the immune system and inflammatory diseases. When endogenous ligands bind with TLR4, it gets activated and stimulates the generation of pro-inflammatory cytokines by causing NF-κB activation (Janeway and Medzhitov, 2002; Akira et al., 2006). Furthermore, EGCG elevated the level of IL-10, reduced the downstream pro-inflammatory cytokines (i.e., TNF-α and IL-1β) of the TLR4 signaling pathway, and reduced the expression of NF-κB in the lumbar SDH of CCI rats (Kuang et al., 2012). In the dorsal horn of the spinal cord, an EGCG-derived compound decreased the levels of mRNA and protein expressions of IL-6, NF-κB, IL-1β, and TNF-α (Xifró et al., 2015). Administration of isoorientin and puerarin also decreased the level of CCI-induced pro-inflammatory cytokines including IL-6, IL-1β, and TNF-α (Liu et al., 2014; Zhang et al., 2019). Interestingly, genistein reduced the level of IL-1β expression in the spinal cord and dorsal root ganglion, while genistein also decreased mRNA expressions of both IL-6 and IL-1β in the sciatic nerve (Valsecchi et al., 2008).

Effect of Flavonoids on Other Neuropathic Pain Signaling Pathways

Flavonoids show anti-inflammatory as well as antioxidant effects due to their action on $GABA_A$ receptors (Hanrahan et al., 2011). Maximum metabolic disorders are the result of oxidative stress. Along with exogenous factors, regular metabolism of oxygen inside the tissues and cells produce reactive oxygen species (ROS) and free radicals that steadily endanger them (Forrester et al., 2018; Uddin et al., 2019). Flavonoids are well-recognized for their antioxidant properties and are also confirmed to show beneficial effects in several chronic diseases, including neurodegenerative disease, diabetes, atherosclerosis, and cancer (de Teles et al., 2018; Kozłowska and Szostak-Węgierek, 2019; Uddin and Kabir, 2019; Uddin et al., 2020c,d). Moreover, certain flavonoids play a crucial role in the iron chelation thus stopping the development of free radicals (Nelson et al., 1992; Ferrali et al., 1997). Rutin and epicatechin are shown to have the capability to be oxidized themselves through free radicals, producing a less reactive and stable species (Hanasaki et al., 1994). Correspondingly, quercetin, a plant pigment flavonoid, prevents nitric oxide (NO)-mediated cell injury. A combination of NO and free radicals generates the enormously injurious peroxynitrite, which directly oxidizes low-density lipoprotein and plays a crucial role in the permanent damage of the cell membrane. Therefore, free radicals are scavenged by quercetin and restrained from reacting with NO, whereas, silibin reacts directly with NO (Dehmlow et al., 1996; Shutenko et al., 1999). Mechanical allodynia induced by spinal nerve ligation (SNL) was found to be decreased by various flavonoids including myricetin (Hagenacker et al., 2010), EGCG (Choi et al., 2012), and baicalein (Cherng et al., 2014). SNL-induced thermal hyperalgesia was reduced by myricetin (Hagenacker et al., 2010) and baicalein (Cherng et al., 2014), while quercetin decreased both cold and thermal hyperalgesia in SNL rats (Ji et al., 2017). In addition to this, hesperetin and quercetin decreased partial sciatic nerve ligation-stimulated neuropathic pain and spared nerve injury (**Figure 3**; Aswar et al., 2014; Muto et al., 2018).

Physiologically, xanthine dehydrogenase plays an important role in the metabolism of xanthine to uric acid, however, this enzyme alters into xanthine oxidase in the case of ischemic-reperfusion, which works as a precursor of free radicals. There are various flavonoids, such as quercetin, silibinin, and luteolin, that are recognized to work as antioxidants through stopping xanthine oxidase (Chang et al., 1993; Shoskes, 1998). Similarly, reperfusion is also caused by the mobilization of leucocytes producing the subsequent release of inflammatory mediators as well as cytotoxic oxidants, which provokes the complement system. Many flavonoids play a key role in the immobilization of leucocytes, eventually resulting in a decline in the serum complement system as well as inflammation (Friesenecker et al., 1995; Ferrándiz et al., 1996). It has been observed that the connection of the same pathophysiological mechanisms takes place with both NP of peripheral origin and inflammation. Both kinds of pathologies express as hyperalgesia and allodynia (Clatworthy et al., 1995; DeLeo and Yezierski, 2001; Jin et al., 2003). Moreover, inflammatory cells infiltration and their main secretory products, including cytokines and arachidonic acid, affect peripheral nerve damage, which is accountable for the production and maintenance of the constant pain (Tracey and Walker, 1995; Cui et al., 2000; Ma and Eisenach, 2003). When cytokines such as IL-1, IL-6, and TNF-α were injected into a rat paw, it would result in the initiation of thermal and mechanical hyperalgesia (Cunha et al., 1992; Ferreira et al., 1993). On the other hand, the inhibition of TNF-α in the animal models with painful neuropathy led to the reduction of hyperalgesia (Sommer et al., 1998). The release of cytokines also activates COX-2 dependent prostanoid releases. Furthermore, prostaglandins (PGs) also play a pivotal role in triggering inflammation that increases sensitivity to pain (Uddin et al., 2020b). It had been found that intrathecal injection of PGs such as PGE_2 and $PGF_{2\alpha}$ triggered allodynia in conscious mice (Minami et al., 1992, 1994), while intrathecal administration of PGD_2 and PGE_2 led to the initiation of hyperalgesia (Uda et al., 1990). Additionally, synthesis of NO and PG through COX-2 as well as iNOS is increased in the microglia on account of peripheral nerve damage, leading to hypersensitization (Hanisch, 2002). It is evident that flavonoids show anti-inflammatory activity both *in vitro* and *in vivo*. One of the imperative mechanisms

of anti-inflammatory action is recognized by inhibiting eicosanoid producing enzymes such as phospholipase A2, lipoxygenases, and COX (Kim et al., 2004). Along with anti-inflammatory activity, flavonoids also block arachidonic acid metabolism (Ferrándiz and Alcaraz, 1991).

CONCLUSION

In this review, we discuss the effects of flavonoids in improving different NP conditions and how flavonoids control diverse pain biomarkers in animal models of NP. Allosteric modulators at $GABA_A$ receptors can alter either the affinity or efficacy of agonists including GABA, subsequently controlling their activity. Flavonoids are strong allosteric modulators and may serve as valuable candidates in the management of NP. Hence, it can be said that there is huge potentiality in flavonoids for the development of novel therapeutics agents for NP, however, further studies are needed.

AUTHOR CONTRIBUTIONS

MU conceived the original idea and designed the outlines of the study. MU, AM, MR, and MK wrote the draft of the manuscript. MU and AM prepared the figures for the manuscript. SA, IA, AP, GA, MB-J, and MA-D revised and improved the draft. All authors have read and approved the final manuscript.

ACKNOWLEDGMENTS

This work was funded by the Deanship of Scientific Research at Princess Nourah bint Abdulrahman University, through the Fast-track Research Funding Program.

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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.

frontiers in Pharmacology

REVIEW
published: 22 July 2020
doi: 10.3389/fphar.2020.01097

Emerging Promise of Cannabinoids for the Management of Pain and Associated Neuropathological Alterations in Alzheimer's Disease

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Alzheimer's disease (AD) is an irreversible chronic neurodegenerative disorder that occurs when neurons in the brain degenerate and die. Pain frequently arises in older patients with neurodegenerative diseases including AD. However, the presence of pain in older people is usually overlooked with cognitive dysfunctions. Most of the times dementia patients experience moderate to severe pain but the development of severe cognitive dysfunctions tremendously affects their capability to express the presence of pain. Currently, there are no effective treatments against AD that emphasize the necessity for increasing research to develop novel drugs for treating or preventing the disease process. Furthermore, the prospective therapeutic use of cannabinoids in AD has been studied for the past few years. In this regard, targeting the endocannabinoid system has considered as a probable therapeutic strategy to control several associated pathological pathways, such as mitochondrial dysfunction, excitotoxicity, oxidative stress, and neuroinflammation for the management of AD. In this review, we focus on recent studies about the role of cannabinoids for the treatment of pain and related neuropathological changes in AD.

Keywords: cannabinoids, marijuana, endocannabinoid system, pain, Alzheimer's disease

OPEN ACCESS

Edited by:
Rajeev K. Singla, Sichuan University, China

Reviewed by:
Rohit Gundamaraju, University of Tasmania, Australia
Ernest Jennings, James Cook University, Australia

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *14 April 2020*
Accepted: *06 July 2020*
Published: *22 July 2020*

Citation:
Uddin MS, Mamun AA, Sumsuzzman DM, Ashraf GM, Perveen A, Bungau SG, Mousa SA, El-Seedi HR, Bin-Jumah MN and Abdel-Daim MM (2020) Emerging Promise of Cannabinoids for the Management of Pain and Associated Neuropathological Alterations in Alzheimer's Disease. Front. Pharmacol. 11:1097. doi: 10.3389/fphar.2020.01097

INTRODUCTION

Pain is a complex emotional and perceptual experience, which has sensory, cognitive, and affective dimensions (Bushnell et al., 2013; Talbot et al., 2019). There is a cortical response to nociceptive stimuli under vegetative as well as minimal conscious state, hence the pain perception appears crucial for survival and needs assessment in the absence of people with severe cognitive dysfunctions (De Tommaso et al., 2013). Neuropathological alterations that take place in dementia patients are accountable for changes in the perception of pain (Van Kooten et al., 2016). Though these changes can be common in different forms of dementia, however, scientists are trying to investigate the pain perception and processing in the patients of Alzheimer's disease (AD), which is characterized by behavioral and cognitive impairments (Al Mamun et al., 2020b; Uddin et al., 2020a; Uddin et al., 2020l). Neuropathological hallmarks of AD are extracellular accumulations of amyloid beta (Aβ) as well as intracellular accumulations of neurofibrillary tangles (NFTs) that are comprised of hyperphosphorylation of tau (Uddin et al., 2019; Mamun et al., 2020; Uddin et al., 2020d). Furthermore, the development of Aβ plaques occurs primarily in the basal, orbitofrontal neocortex, and temporal areas of the brain and subsequently develops all over the hippocampus, diencephalon, neocortex, basal ganglia, and amygdala (Tiwari et al., 2019). Some events such as increased generation, oligomerization as well as accumulation of Aβ are the key factors at the beginning stage of AD. The noxious Aβ peptides including $A\beta_{40}$ and $A\beta_{42}$ are formed by the amyloid precursor protein (APP) through the cleavage by β- and γ-secretases (Kabir et al., 2020a; Uddin et al., 2020e; Uddin et al., 2020j). Moreover, APP is one of the transmembrane proteins that is folded and altered in the endoplasmic reticulum (ER) as well as transferred *via* the Golgi complex to the external membrane. It is evident that ER stress plays a crucial role in AD pathology. Various pathological events of AD such as accumulation of Aβ and tau proteins, disturbances in calcium (Ca^{2+}) homeostasis, and oxidative stress might be triggered by ER stress in brains (Salminen et al., 2009; Uddin et al., 2020m). In contrast, this type of pathology could also produce ER stress and therefore exacerbate the pathogenesis of AD (Salminen et al., 2009; Uddin et al., 2020m).

The occurrence of chronic pain in AD patients was 45.8% (Van Kooten et al., 2016). Perception of pain might be neglected in AD patients as they might be unable to express their pain as well as seek attention as efficiently as their cognitively healthy peers (Cravello et al., 2019). Remarkably, pain is found more prevalently in severe dementia patients (van Kooten et al., 2017), and pain intensity is also connected positively with the severity of dementia (Scherder et al., 2008; Rajkumar et al., 2017; Whitlock et al., 2017). Although a bidirectional relationship exists between AD and chronic pain, however, the exact mechanism remains unclear. In a study by Hayashida and Obata, (2019) observed several common pathologies, such as aberrations of the noradrenergic system in the locus coeruleus (LC), microglial activation in brain regions including the frontal cortex, and raised central neuroinflammation in these areas in AD patients or the patients with chronic pain (Salter and Stevens, 2017). The neuropathological alterations that take place in the patients with AD selectively affect vital regions, which involved in the medial pain pathway, particularly the medial nuclei of the hypothalamus, cingulate, insula, and thalamus, while the brain regions involved in the lateral pain pathway are comparatively well conserved (Braak et al., 1993).

Cannabis, also called marijuana, has widely been used for therapeutic purposes throughout human history (Bridgeman and Abazia, 2017). The first use of this plant had been recorded about 5000 years ago in ancient China, where plant extracts were used for the treatment of pain and cramps (Zou and Kumar, 2018). Furthermore, the uses of cannabis have been recognized for medical purposes such as anti-inflammatory, anticonvulsant, anti-nociception, anti-emetic, and recreational use, which has mostly restricted its medical uses (Uddin et al., 2018; Vučkovic et al., 2018; Zou and Kumar, 2018). Cannabis comprises over 500 constituents, among them about 104 cannabinoids have currently been detected (Lafaye et al., 2017). Moreover, two constituents of cannabinoids including cannabidiol (CBD) and delta-9-tetrahydrocannabinol (Δ9-THC) has widely been studied for investigating their pharmacological properties (Lafaye et al., 2017). Medical cannabis has extensively been considered as one of the prospective alternative approaches for the treatments of dementia (Liu et al., 2015; Broers et al., 2019).

Numerous research suggested promising effects of cannabis for decreasing pain and noxious protein from the brain as well as restore cognitive dysfunctions of AD (Esposito et al., 2006a; Russo, 2008; Cheng et al., 2014). Moreover, endocannabinoid signaling has broadly been revealed to control the foremost pathological processes in neurodegenerative disorders, such as misfolding of protein, mitochondrial dysfunction, oxidative stress, excitotoxicity, and neuroinflammation. In this review, we highlight the emerging studies regarding the effect of cannabinoid compounds for treating pain and related neuropathological changes in AD.

CANNABIS PLANT

Although cannabis has widely been cultivated and used by mankind for at least 6000 years, (Li, 1973) however, our insight into its pharmacological properties is based on researches that have occurred merely since the end of the 19th century. Cannabinol was the first compound that had been separated in pure form from the cannabis plant (Wood et al., 1899). Primarily, it was mistakenly supposed to be the chief active compound of the cannabis plant that was accountable for its psychoactive actions (Mechoulam and Hanuš, 2000). Furthermore, CBD (**Figure 1**) was the second compound that had been observed by Mechoulam and Shvo (Mechoulam and Shvo, 1963). Subsequently, Gaoni and Mechoulam separated the chief active compound, Δ9-THC (**Figure 1**) in 1964 (Gaoni and Mechoulam, 1964).

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid beta; CBD, cannabidiol; ECS, endocannabinoid system; LC, locus coeruleus; NE, norepinephrine; NFTs, neurofibrillary tangles; Δ9-THC, delta-9-tetrahydrocannabinol.

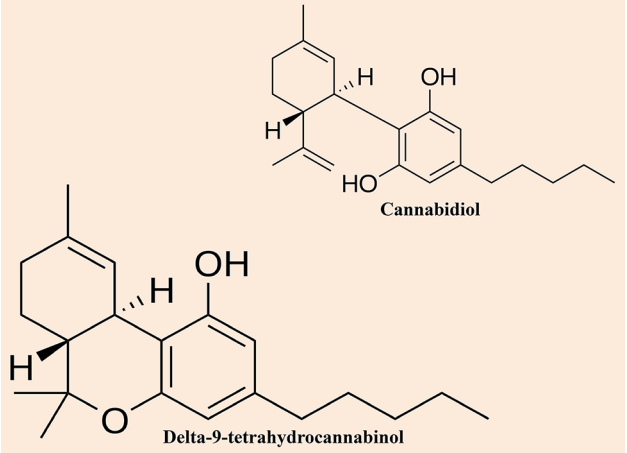

FIGURE 1 | Chemical structures of the most notable cannabinoids (i.e. cannabidiol and delta-9-tetrahydrocannabinol) found in cannabis.

There are two main subspecies of the cannabis plant, including *Cannabis sativa* and *Cannabis indica*, and they could be distinguished by their diverse physical properties. Moreover, *indica*-dominant species are small plants with wide, dark green leaves as well as have a higher concentration of CBD than the *sativa* plants where THC content is higher. Conversely, *sativa*-dominant species are usually tall plants and thin with finger-like leaves with a pale green color. *Cannabis sativa* is the favorite choice by consumers because of its higher THC content. The four major compounds are CBD, cannabinol, Δ-8-THC, and Δ-9-THC, which have extensively been studied (Pertwee, 1997; Pertwee, 2008; Pamplona and Takahashi, 2012). Initially, it was believed that CBD was the metabolic parent to Δ-9-THC, however, later it was observed that its biosynthesis occurred by a genetically determined ratio (Russo and Guy, 2006). Although, all four compounds have similar chemical structures, however, their pharmacological effects are different. CBD and Δ-9-THC are the most studied compounds of the cannabis plant.

PAIN, COGNITIVE IMPAIRMENT, AND AD

The pain sensation is connected with the triggering of the receptors in the primary afferent fibers including myelinated Aσ-fiber and unmyelinated C-fiber (Yam et al., 2018; Uddin et al., 2020k). Furthermore, both nociceptors are initiated when there is a probable toxic stimulus as well as stay silent during homeostasis where the pain is absent. The perception of a sequence of sensory actions is needed for the brain with the purpose of detecting pain as well as generate a response on the way to the threat. Moreover, the perception of pain usually contains three main phases. The first phase is the sensitivity of pain, after that the second phase in which the signals are transferred to the dorsal horn in the spinal cord from the periphery. Finally, the third phase is to execute the transference of the signals to the higher brain through the central nervous system (CNS) (Yam et al., 2018). Numerous research found that chronic pain is connected with the raised objective as well as self-reported cognitive dysfunctions (Cha et al., 2017; Whitlock et al., 2017). These cognitive dysfunctions are not precise to a specific pain modality and could be found in postherpetic neuralgia (Pickering et al., 2014), chronic back pain (Baker et al., 2018) and fibromyalgia (Leavitt and Katz, 2015).

Based on the Einstein Aging Study, Ezzati et al. (2019) assessed the connection of pain intensity, as well as pain interference with incident dementia in 1,114 participants who were 70 years of age or older and 10% of the participants, developed dementia over 4.4 years. In this study, it has been observed that higher levels of pain interference are directly connected with a higher possibility of developing dementia (Ezzati et al., 2019). In a study by Ikram et al. (2019) also advocated that pain interference was considerably linked with AD and related dementia (ADRD). Furthermore, according to the study of Malfliet et al. (2017) chronic pain and AD demonstrated aberrations of the volume of gray matter, and neuroimaging recommended that the patients of cognitive dysfunctions with chronic pain might be connected with alterations of the volume of gray matter in the brain. Importantly, many of these changed brain regions are playing a crucial role in sensory perception, the affective element of cognition and pain (Baliki and Apkarian, 2015; Ng et al., 2017). For example, gray matter volume loss has extensively been observed in the thalamus, parahippocampal gyrus, amygdala, entorhinal cortex, insula, and anterior cingulate cortex (Busatto et al., 2008; Yi et al., 2016; Kang et al., 2019). Numerous studies found that the dysfunction of LC-norepinephrine (NE) system was connected with chronic pain (Cao et al., 2019) as shown in **Figure 2**.

In the CNS, microglia is the main innate immune cells (Ibrahim et al., 2020; Uddin et al., 2020f). Neuroinflammation mediated by microglia is a typical feature in chronic pain (Chen et al., 2018). Proinflammatory microglia release chemokines and cytokines related to inflammation, including umor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β in chronic pain states (Liu et al., 2017; Barcelon et al., 2019). Subsequently, this proinflammatory (**Figure 2**) state contributes to altering the connection of the brain, network function, and synaptic remodeling (Inoue and Tsuda, 2018). Copious studies reported that activation of microglia by persistent exposure to Aβ causes proinflammatory response (Meda et al., 1995; Gold and El Khoury, 2015) that leads to secretion of cytokines, and chemokines, as well as reactive oxygen/nitrogen species (Wyss-Coray, 2006; Rivest, 2009; Balducci and Forloni, 2018). On the other hand, misfolded tau, and truncated tau as well as hyperphosphorylated tau accompany with the proliferation of microglia and amplified the expression of the inflammatory genes (Andrés-Benito et al., 2017). Furthermore, in the brain, reactive microglia causes tau pathology and contribute to the spreading of pathological tau (Nicole et al., 2015). AD brains and chronic pain both show aberrant LC structure and function as well as dynamic alterations in the turnover of NE in LC-projecting regions (Gannon et al., 2015; Llorca-Torralba et al., 2016). In these two disease states, the alterations of NE content might not completely overlap in all brain regions, however, pathological alterations in LC-NE in selective areas can be one of the initiators that are responsible for the dysfunction of the neuron and proinflammatory activation of microglia. Additionally, chronic pain

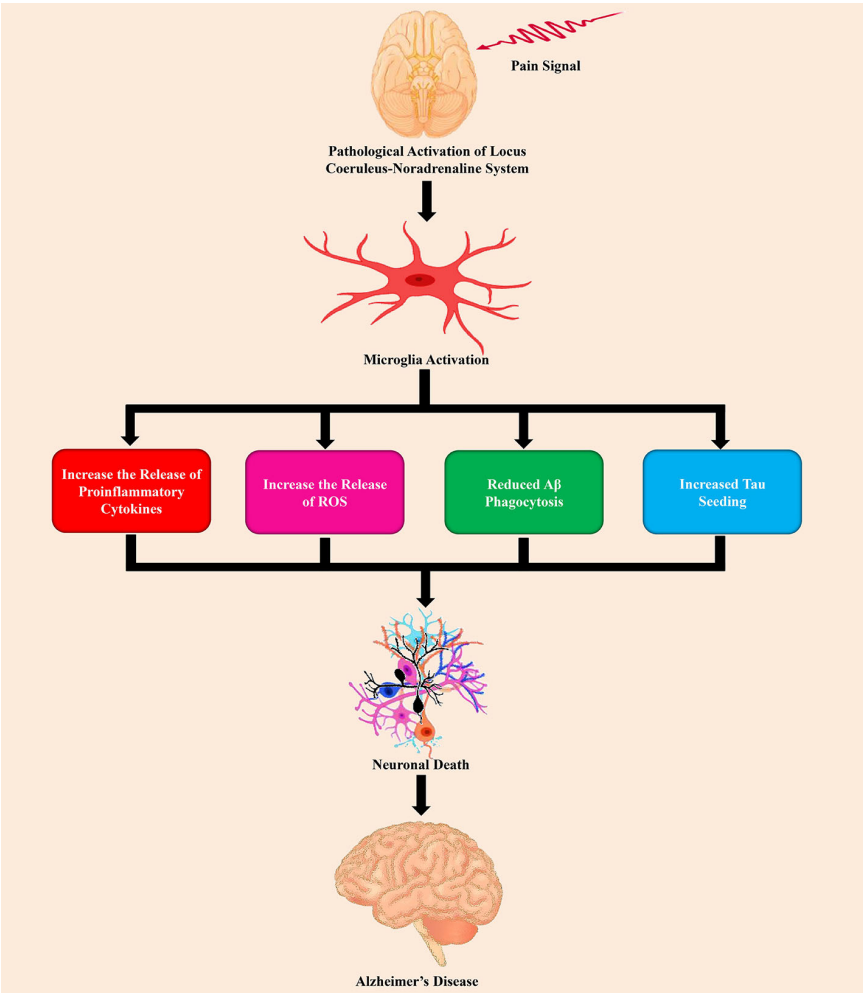

FIGURE 2 | The role of pain stimuli in the pathogenesis of Alzheimer's disease by the dysfunction of the LC-NE system.

might exacerbate the neuropathogenesis of AD *via* LC-NE-mediated microglial neuroinflammation (Cao et al., 2019).

CANNABINOIDS AND PAIN REGULATION IN AD

Numerous studies in AD patients have reported reduced, raised, or typical sensory, affective, as well as behavioral reactions to painful stimuli (Benedetti et al., 2004; Pickering et al., 2006; Kunz et al., 2007; Kunz et al., 2009). The duration of impairment to the medial (affective) and lateral (sensory) pain network is recognized in AD. Besides, the locus, intensity, as well as pain quality are controlled by the lateral pain system that intervenes acute or quick sensations of pain. Some studies have recommended that the lateral system is less affected during AD (Scherder et al., 2003; Scherder et al., 2005). On the other hand, the medial pain system interferes the unfavorable, affective reaction to toxic stimuli as well as the neurodegenerative alterations in AD influence the medial pain system during disease (Vogt et al., 1990; Scherder and Bouma, 1997; Scherder and Bouma, 2000).

Cannabinoid receptor 1 (*CNR1*) gene is responsible for encoding the cannabinoid receptor type 1 (CB1R) and this receptor comprises of 472 amino acids in humans as well as 473 amino acids in mouse and rat, with the identification of 97%–99% amino acid sequence amid these species. Numerous research found that some variations of *CNR1* had been connected with the dependence of *Cannabis* (Agrawal and Lynskey, 2009; Hartman et al., 2009; Schacht et al., 2012). Conversely, the cannabinoid receptor 2 (*CNR2*) gene is accountable for encoding the cannabinoid receptor type 2 (CB2R) and this receptor comprises of 360 amino acids in humans. At the protein level, CB2R shares merely 44% sequence homology in comparison with CB1R. Furthermore, the CB2R has also bigger species variations among rodents and humans when compared to CB1R, as the homology of the amino acid sequence is slightly more than 80% between rodents and humans (Liu et al., 2009; Zhang et al., 2015). Two polymorphisms of the CB2R have also been identified in humans

(Liu et al., 2009). CB1Rs are not only expressed in the brain, predominantly in the limbic system, cerebellum, substantia nigra, hippocampus, and basal ganglia, but also they are expressed in the peripheral nervous system (PNS), including uterus, bones, liver, testicular tissue, and thyroid (Pagotto et al., 2006; Pertwee, 2006; Russo and Guy, 2006). On the other hand, CB2Rs are mainly expressed in the gastrointestinal system spleen, and immune cells, and slightly in the brain and PNS (Izzo, 2004; Pertwee, 2006). Remarkably, both CB1Rs and CB2Rs are observed in the human placenta and play a pivotal role in controlling the serotonin transporter activity (Kenney et al., 1999).

The characterization of CB_1 and CB_2 receptors endorsed the revealing of endocannabinoids (Aso and Ferrer, 2014). In preclinical and clinical studies found that central as well as peripherally situated CB1R had widely been connected with nociception (Manzanares et al., 2006). CB2R are mostly found in the cells of the immune system might play an essential role in reducing pain, since they have extensively been linked with the inhibition of pain and inflammatory processes (Ashton and Glass, 2007). Furthermore, the endocannabinoid system (ECS) has widely been connected with central stress-mediated analgesia (Hohmann et al., 2005). THC has also revealed anti-inflammatory effects in many preclinical and clinical studies. THC has also a great effect on serotonergic, glutamatergic, and opioid receptors that have played a pivotal role in the development as well as regulation of neuropathic pain (Manzanares et al., 2006). Therefore, these results recommend that although endogenous cannabinoids might be necessary for the homeostatic regulation of pain, however, exogenous cannabinoids, including synthetic cannabinoids and THC might be a promising adjunct therapy for the treatment of clinical pain (Fine and Rosenfeld, 2013).

Pain regulation is the initial medical uses of cannabinoids. Many studies have reported that the analgesic effects of cannabinoids in diverse kinds of pain, such as mechanical, heat, and chemical pain, and they also reduced inflammation as well as neuropathic pain (Fine and Rosenfeld, 2013; Donvito et al., 2018). The ECS is also played a crucial role in the control of nociception (Pacher et al., 2006). Similarly, endocannabinoids have also a great effect on the regulation of inflammation as well as neuropathic pain (Donvito et al., 2018). Apart from the CB1R, there is also positive evidence for advocating the involvement of the transient receptor potential vanilloid-1 (TRPV1) and CB2R in cannabinoid-induced control of pain (Jhaveri et al., 2007; Akopian et al., 2009). Moreover, scientists are now focusing the phytocannabinoids for the management of nociception as well as other neurological complications. For example, CBD has widely been reported to control chronic pain in many studies (Donvito et al., 2018).

In a clinical setting, pain could be a highly subjective measure. It is very challenging to measure and manage pain in dementia patients because the communication is frequently reduced as well as many other symptoms present concurrently (Chow et al., 2016). Nowadays various analgesics including nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, and acetaminophen are used for treating pain in dementia (Sherman et al., 2018). Conversely, as NSAIDs must use carefully as they exert gastrointestinal side effects and the use of opioids should be carefully monitored because of common adverse effects including nausea, vomiting, and sedation (Sherman et al., 2018). Furthermore, targeting the ECS has demonstrated favorable effects for the management of pain in diseases including multiple sclerosis and fibromyalgia (Svendsen et al., 2004; Tsang and Giudice, 2016). Recently, nabilone, a synthetic cannabinoid has used in AD patients for examining pain as an exploratory finding of the clinical trial (NCT02351882) by using the pain assessment in advanced dementia scale (ClinicalTrials.gov, 2020). Based on the cautious assessment of the effects of the ECS as well as preclinical studies, it can be said that cannabinoids might show a positive effect on pain in AD (ClinicalTrials.gov, 2020). Therefore, the use of randomized controlled trials is needed to assess the safety as well as the efficacy of cannabinoid for treating pain as a primary finding in the dementia patients.

ENDOGENOUS CANNABINOID SYSTEM IN ALZHEIMER'S BRAINS

The investigation of human post-mortem samples exposed several changes in the composition of ECS as well as signaling in AD brains, though the alterations in the pathophysiology of the disease remain unclear until now. Likewise, the alterations in the expression of CB1R in AD are unknown. Although, some studies found that a considerable decrease in the levels of CB1R in cortical areas as well as in neurons faraway from senile plaques (Ramírez et al., 2005; Solas et al., 2013), however, many investigations have reported no changes in the distribution and expression of CB1R in hippocampus and cortex in AD (Benito et al., 2003; Ahmad et al., 2014; Lee et al., 2010; Mulder et al., 2011). Moreover, no relationship between the levels of CB1R and any pathological marker of AD has been observed (Solas et al., 2013). On the other hand, the significant levels of CB2R have found in AD brains because of the expression of CB2R on microglia nearby senile plaques (Ramírez et al., 2005; Solas et al., 2013). Importantly, the expression of CB2R levels connects with the levels of $A\beta_{42}$ and the accumulation of plaque, even though not with cognitive impairment (Solas et al., 2013), recommending that these pathogenic events trigger the expression of CB2R. Furthermore, both CB1R and CB2R in the brain of AD are nitrosylated, as well as this can lead to the impaired connection of these receptors to downstream effector signaling molecules (Ramírez et al., 2005).

Some investigations demonstrated other elements of ECS in AD human samples. The initial study examining the levels of endocannabinoid showed no changes between healthy controls and AD patients in the plasma concentrations of 2-arachidonoyl-sn-glycerol (2-AG) and anandamide (AEA) (Koppel et al., 2009). However, in a study by Jung et al. (2012) reported that the lower levels of AEA in temporal cortices and midfrontal in AD when compared to control subjects in post-mortem brain samples, which reciprocally connected with the levels of $A\beta_{42}$ in the neurotoxic brain as well as cognitive deficiencies documented in these patients, recommending an involvement in the $A\beta_{42}$-dependent impairment of AEA to cognitive decline. Besides, several changes have widely been observed in the activity of the

enzymes associated with the synthesis of endocannabinoid as well as degradation in the brains of AD. Therefore, fatty acid amide hydrolase (FAAH), the endocannabinoid metabolizing enzyme, is up-regulated in AD brains both in peripheral blood mononuclear cells (D'Addario et al., 2012) and neuritic plaque-related glia (Benito et al., 2003), and this can lead to the increase of the degradation of AEA in the surrounding area of the senile plaque. Furthermore, the increased expression of FAAH might have two detrimental outcomes in the progression of disease such as limitation of the availability of neuronal AEA and increases proinflammatory molecules mediated by the metabolites of AEA including arachidonic acid (Calder, 2005). In a study by Mulder et al. (2011) reported that changed 2-AG signaling throughout late phases of AD because of the combination of impaired recruitment of monoacylglycerol lipase (MAGL) as well as raised levels of diacylglycerol lipase that promote synapse silencing in AD.

EFFECT OF CANNABINOIDS ON ALZHEIMER'S HALLMARKS

Aβ Pathology

As stated earlier, atypical production and accumulation of Aβ peptides in the brain are considered as the hallmark of AD (Al Mamun et al., 2020a; Sharma et al., 2020; Uddin et al., 2020i). Exogenous cannabinoids, a neuroprotective agent, have persistently been disclosed to restrain memory deficits in Aβ-treated animal models for both synthetic selective cannabinoid receptors agonists (Haghani et al., 2012; Wang et al., 2013), as well as mixed cannabinoid receptors agonists (Ramírez et al., 2005; Martín-Moreno et al., 2011; Fakhfouri et al., 2012) and natural CBD (Martín-Moreno et al., 2011). For example, prolonged treatment of two separate transgenic (Tg) mice models of brain amyloidosis with CB1R agonist arachidonyl-2-chloroethylamide (ACEA) (Aso et al., 2012), CB2R agonist JWH-133 (Martín-Moreno et al., 2012; Aso et al., 2013), or non-selective agonist WIN55,212-2 (Martín-Moreno et al., 2012) resulted in the improvement of cognitive parameters. Interestingly, the protective effects of cannabinoid compounds in Tg animals against cognitive deterioration had declined with disease advancement (Aso et al., 2012; Aso et al., 2013).

The neuroprotective mechanisms of cannabinoid that eventually responsible for memory improvement in Aβ are multiplex and are presumed to take action in parallel or interacting within them. Even if most of the suggested defensive mechanisms of action depending on the magnitude of cannabinoids to indirectly alleviate the devastating effects of Aβ, the direct consequences of cannabinoids on Aβ processing also proposed. For that reason, activation of CB2R provoked Aβ clearance by human macrophages (Gangaidzo et al., 1997; Wu et al., 2013) and supported Aβ transfer through the choroid plexus (Martín-Moreno et al., 2012). This supports the Aβ-clearance across the blood-brain barrier (BBB) had also revealed for the 2-AG, a synthetic endocannabinoid that CB1R/CB2R agonist, in *in vitro* and *in vivo* BBB clearance models (Bachmeier et al., 2013). In the Tg AD mice model, these results could interpret that prolonged administered with CB2R or CB1R/CB2R agonists markedly reduced Aβ levels (Martín-Moreno et al., 2012). Conversely, chronically treated with ACEA (Aso et al., 2012) or HU-210 (Chen et al., 2010) were observed no significant effects on Aβ generation, accumulation, or clearance in Tg AD animal models. Nevertheless, the Stumm et al. (2013) demonstrated that APP23/CB1(-/-) mice decreased the formation of Aβ plaque and APP levels, probably caused by a shift in intracellular APP transport, even though the experimental mice showed elevated cognitive deficits. Furthermore, Chen et al. (2013) study disclosed that Δ-9-THC, one of the cannabinoid isomers, markedly elevated the neprilysin (an enzyme that can degrade Aβ) expression, but not β-secretase (BACE1), as a result to a significant decreased of Aβ plaques in 5xFAD APP Tg animal model. This study fizzled to elucidate, the distinctive CB1R or CB2R function in such a Δ-9-THC effect on Aβ-clearance (**Figure 3**).

Tau Pathology

Hyperphosphorylation and aggregation of tau is another major hallmarks of AD (Uddin et al., 2020h: Uddin et al., 2020g). Accumulating evidence suggested that cannabinoids also play a significant role in tau pathology. Previously, Aβ-induced PC12 neuronal cell culture study revealed that CBD, ACEA, and WIN55,212-2 impede hyperphosphorylation of tau protein (Esposito et al., 2006a). For CBD, this effect might mediate by reducing of phosphorylated glycogen synthase kinase-3β (GSK-3β) (**Figure 3**), as a consequence activation of the Wnt/β-catenin signaling pathway that eventually responsible for the reduction of neuronal apoptosis (Ferrer et al., 2005; Esposito et al., 2006a). On the other side, the effect of both the selective (ACEA) and non-selective (WIN55,212-2) CB1R agonist on tau hyper-phosphorylation was selectively induced by the CB1R in Aβ-induced C6 glioma cells co-cultured with PC12 neuronal cells through down-regulating inducible nitric oxide synthase (iNOS) and nitric oxide (NO) generation (Esposito et al., 2006b). In line with the molecular mechanism of the CB1 receptor on tau hyper-phosphorylation, chronically treated APP/PS1 mice with ACEA decreased the proportion of phosphorylated tau at Thr181 area (a site nearby Aβ plaques), perhaps ACEA-mediated reduction in GSK-3β detrimental effects (Aso et al., 2012). Additionally, a particular mechanism for the CB2R in the regulation of tau phosphorylation has also proposed. For example, in double Tg mice, chronically administered specific CB2R agonist (JWH-133) lowered tau hyper-phosphorylation in the surrounding of Aβ plaques that may be achievable by reducing the action of GSK-3β, p38, and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (Aso et al., 2013).

To validate these trials, recently, one study reported that long-term treatment with Sativex®, an approved medicine that made by mixed Δ-9-THC and CBD natural extracts, significantly decreased NFTs in PK(-/-)/Tau(VLW) (parkin-null, human tau overexpression) mice model (Casarejos et al., 2013). The investigators of this study proposed cannabinoid strengthen of autophagy improving redox status as likely mechanisms responsible for the lowering of tau accumulation.

EFFECT OF CANNABINOIDS AGAINST NEUROINFLAMMATION IN AD

Neuroinflammation, primarily expressed as microglial activation, is an important characteristic in AD that accelerates cell damage and neuronal loss as well (Akiyama et al., 2000; Hensley, 2010; Sardi et al., 2011; Uddin et al., 2020c; Uddin et al., 2020f). Accumulating data indicating that CB2R is fundamentally responsible for several immune reactions, where they are capable of suppressing microglia-induced neurotoxicity, eventually, cannabinoids compounds that act on the CB2R can serve as an anti-inflammatory agent (**Figure 3**) in neuroinflammation (Cabral and Griffin-Thomas, 2009). Previously, it has demonstrated that by activating CB2R notably decreased Aβ-mediated neuroinflammatory response in several AD animal models. For instance, several studies reported that the microglial response and production of proinflammatory mediators were significantly reduced by both the selective or mixed CB2R agonists in Aβ-induced animal brains (Ramírez et al., 2005; van der Stelt et al., 2006; Esposito et al., 2007; Fakhfouri et al., 2012; Wu et al., 2013). Likewise, in APP Tg models, the proportion of reactive microglial cells adjacent to the Aβ deposition area and concentration of the proinflammatory cytokines were depleted by selective CB2R agonists (Martín-Moreno et al., 2012; Aso et al., 2013). Furthermore, in a tauopathy animal model, Sativex® can be able to dampen the microglial activation (Casarejos et al., 2013), though no directly implicating proof of CB2R or other receptors in such effects provided. Moreover, recently one study reported that chronically treated with CB1R agonist ACEA decreased astrocytic reactivation and lower expression of interferon-γ in AβPP/PS1 Tg mice (Aso et al., 2012). Surprisingly, CBD had not shown an affinity to CB1 or CB2 receptors but manifested anti-inflammatory characteristics in the AD animal model (Esposito et al., 2006a; Martín-Moreno et al., 2011). The exact location where CBD expresses its neuroprotective effects is yet to confirm, but few findings indicate that CBD may have selective interaction with peroxisome proliferator-activated receptors-γ (PPARγ) (Esposito et al., 2011).

In AD, the enzymes that are related to AEA and 2-AG deterioration may also responsible for regulating the inflammation. FAAH is an enzyme that manifested not only in the neurons but also astrocytes, where it can contribute to a role in response to the inflammatory process. A study reported that FAAH overexpressed in astrocyte and notably sustained in the neuroinflammatory process, which was possessed to assist the detrimental process mediated by toxic insults due to the lowering of endocannabinoid tone (Benito et al., 2003). However, FAAH-knockout mice expressed more responsive to Aβ than wildtype astrocytes and exhibited higher proinflammatory phenotype, distinguished by an elevation in cytokine production as well as cell death likely due to the alternation of signaling pathways involved in cell survival and inflammation, including, extracellular signal-regulated protein kinases 1/2 (ERK1/2), p38 mitogen-activated protein kinase (p38MAPK), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), as well as to the escalation in inflammatory molecules such as iNOS and cyclooxygenase (Benito et al., 2012). The researchers of this study disclosed that these processes not related to CB1 or CB2 receptors but PPAR-α, PPAR-γ, and TRPV1. So far, in astrocytes, the proinflammatory phenotype could not be initiated by the pharmacological blockade of FAAH, suggesting that the audited effects in astrocytes absent FAAH could be due to compensative shifts that result from the probably prolonged augment of N-acylethanolamines. The result of this study indicates that an exceedingly long-term prolongation of endocannabinoid tone may have detrimental effects. On the contrary, the inhibition of MAGL, an enzyme that role in hydrolyzing endocannabinoids (Nomura et al., 2011), and regulate the arachidonic acid release, reduced the Aβ levels and diminished neuroinflammation in AD animal model (Piro et al., 2012). These findings were verified by the pharmacological MAGL inhibitor, which reiterated the proinflammatory cytokine-reducing effects through lowering prostaglandin generation, rather than intensified endocannabinoid signaling pathway.

EFFECT OF CANNABINOIDS AGAINST OXIDATIVE STRESS IN AD

Overwhelming evidence demonstrated that mitochondrial dysfunction as a causative factor to neurodegenerative diseases, including AD (Ferrer, 2009; Ankarcrona et al., 2010; Burchell et al., 2010; Uddin and Kabir, 2019). Impaired mitochondrial function emerges at the early stage in AD, which eventually causative factor for neurons exhaustion as a consequence of converging in the reduction of energy generation, elevated energy demand, and uncontrolled oxidative stress (Ferrer, 2009; Uddin et al., 2020b). The cannabis derivatives also have a potent antioxidant property, particularly CBD was shown more protective than α-tocopherol against glutamate neurotoxicity (Hampson et al., 1998). Besides, CBD not only suppressed ROS generation and lipid peroxidation but also reduced caspase 3, (**Figure 3**) and intracellular calcium levels in Aβ-induced PC12 neuronal cells (Iuvone et al., 2004). Furthermore, it also lowered iNOS and NO in similar conditions (Esposito et al., 2006b). Moreover, other cannabinoids, for example, selective CB2R agonists exerted antioxidant properties in AD animal models. Therefore, JWH-133, a selective CB2R agonist, lowered hydroxynonenal adducts (produced from lipid peroxidation), elevated superoxide dismutases-1 (SOD-1) and superoxide dismutases-2 (SOD-2) in surrounding of plaques in APP/PS1 mice, suggesting the role of CB2R in lowering harmful effects against oxidative stress (Aso et al., 2013). Chronically administered with Sativex® in the tauopathy animal model was also proposed to lower the free radicals and mitochondrial activity in the tauopathy animal model.

EFFECT OF CANNABINOIDS ON ENERGY METABOLISM IN AD

The functional role of cannabinoid receptors in regulating neuronal energy metabolism has been becoming great attention in the scientific community. However, only a few studies available

so far to confirm the direct possession of CB1R over neuronal respiration and energy generation. For instance, Bénard et al. (2012) used anti-CB1R antibodies, disclosed the protein localization of CB1R nearly 30 percent of neuronal mitochondria, which when triggered by exogenous/endogenous cannabinoids lowers the respiratory chain complex-I activity and oxygen consumption, probably *via* cyclic adenosine monophosphate (cAMP) and protein kinase-A (PKA) signaling. These results are supported by Athanasiou et al. (2007) findings, which reveal that all of the partial CB1R agonists including AEA, Δ-9-THC, and HU-210 markedly reduced oxygen consumption (**Figure 3**) and mitochondrial membrane potential. However, care must be taken to interpret these findings due to using commercial anti-CB1R antibodies (Morozov et al., 2013).

EFFECT OF CANNABINOIDS ON THE MODULATION OF NEUROTRANSMISSION IN AD

In recent years, acetylcholine esterase (AChE) inhibitors mostly approved by drug administration to treat AD, which rises the acetylcholine (ACh) availability to some extent alleviating this neurotransmitter insufficiency in AD patients, or they are non-competitive N-methyl-D-aspartate (NMDA) receptor antagonists, which blocks the NMDA-associated ion channel consequently lower calcium influx and restrain excitotoxicity (Kabir et al., 2019b; Kabir et al., 2019a; Kabir et al., 2020b). Surprisingly, particular cannabinoid molecules play a role in the same target compared to contemporary medicaments, resulting in analogous or increased favorable effects. For example, Δ-9-THC competitively impedes AChE, (**Figure 3**) consequently elevating ACh levels, as well as hindering AChE-mediated Aβ deposition by binding in the peripheral-anionic-site of AChE, the dreadful area engaged with amyloidogenesis (Eubanks et al., 2006). Some synthetic cannabinoids can act as stereoselective NMDA receptors blockers (Feigenbaum et al., 1989). For example, HU-211, which can protect cells against NMDA-inducing neurotoxicity (Feigenbaum et al., 1989; Eshhar et al., 1993; Nadler et al., 1993). The neuroprotective activity of HU-211 caused by direct binding to NMDA receptors, unfortunately not to cannabinoid receptors, however; the widely considered cannabinoid-induced neuroprotective effects against excitotoxicity may be accomplished through several mechanisms, including suppression of presynaptic glutamate release (Marsicano et al., 2003), interruption of voltage-dependent calcium channels (Mackie and Hille, 1992), and the prohibition of calcium release

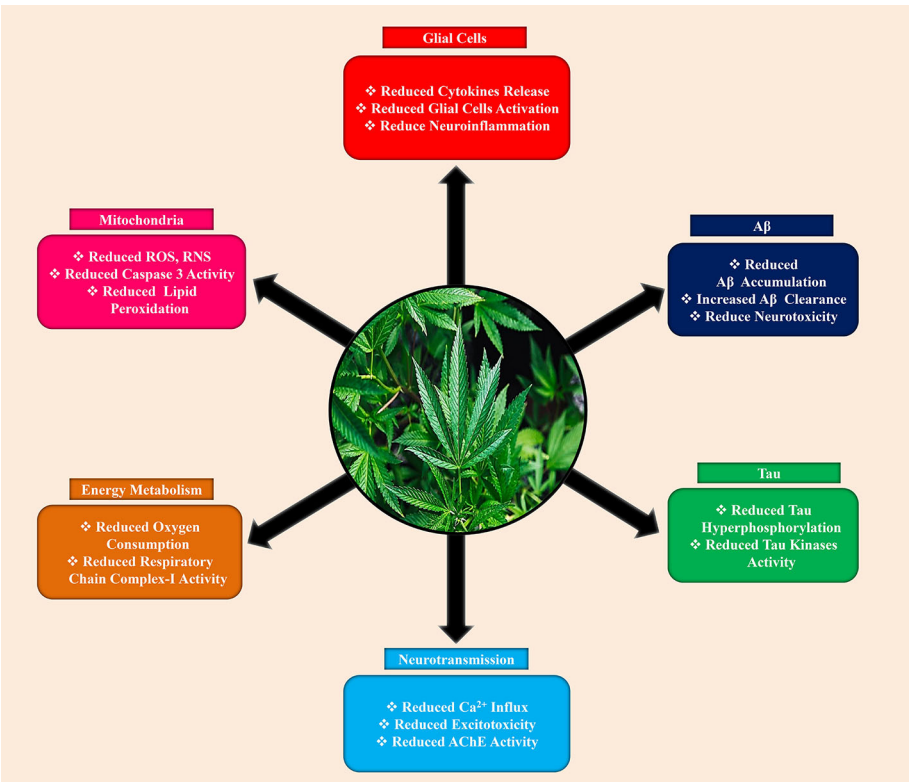

FIGURE 3 | Neuroprotective effects of cannabinoids against Alzheimer's disease.

(Zhuang et al., 2005), which predominantly indicates the direct or indirect involvement of CB1R.

CONCLUSION

Cannabinoids act by targeting several signaling processes, such as pain, abnormal processing of Aβ and tau, neuroinflammation, excitotoxicity, oxidative stress, and mitochondrial dysfunction, which play a pivotal role in the management of AD. Cannabinoids also ameliorate behavioral and cognitive dysfunctions. Therefore, due to these extensive medical uses of cannabinoid compounds, it can be said that targeting the endocannabinoid system can be a promising strategy to develop an effective therapy for the management of AD. Furthermore, cannabinoids may demonstrate a safe and reliable low-cost therapy, with limited side effects. Future research is needed to investigate the use of cannabinoids for the treatment of AD in a clinical trial setting.

AUTHOR CONTRIBUTIONS

MU conceived the original idea and designed the outlines of the study. MU, AM, and DS wrote the draft of the manuscript. MU prepared the figures for the manuscript. GA, AP, SB, SM, HE-S, MB-J, and MA-D revised and improved the draft. All authors contributed to the article and approved the submitted version.

ACKNOWLEDGMENTS

This work was funded by the Deanship of Scientific Research at Princess Nourah bint Abdulrahman University through the Fast-Track Research Funding Program. This work was supported by King Saud University, Deanship of Scientific Research, College of Science Research Center.

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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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BRIEF RESEARCH REPORT
published: 30 July 2020
doi: 10.3389/fphar.2020.01143

Inhibition of the Glycine Receptor alpha 3 Function by Colchicine

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Colchicine is a plant alkaloid that is widely used as a therapeutic agent. It is widely accepted that colchicine reduces the production of inflammatory mediators mainly by altering cytoskeleton dynamics due to its microtubule polymerization inhibitory activity. However, other lines of evidence have shown that colchicine exerts direct actions on the function of ion channels, which are independent of cytoskeleton alterations. Colchicine is able to modify the function of several pentameric ligand-gated ion channels, including glycine receptors (GlyRs). Previous electrophysiological studies have shown that colchicine act as an antagonist of GlyRs composed by the α_1 subunit. In addition, it was recently demonstrated that colchicine directly bind to the α_3 subunit of GlyRs. Interestingly, other studies have shown a main role of α_3GlyRs on chronic inflammatory pain. Nevertheless, the functional effects of colchicine on the α_3GlyR function are still unknown. Here, by using electrophysiological techniques and bioinformatics, we show that colchicine inhibited the function of the α_3GlyRs. Colchicine elicited concentration-dependent inhibitory effects on α_3GlyRs at micromolar range and decreased the apparent affinity for glycine. Single-channel recordings show that the colchicine inhibition is associated with a decrease in the open probability of the ion channel. Molecular docking assays suggest that colchicine preferentially bind to the orthosteric site in the closed state of the ion channel. Altogether, our results suggest that colchicine is a competitive antagonist of the α_3GlyRs.

Keywords: glycine receptor, antagonist, colchicine, pentameric ligand-gated ion channel, pain

OPEN ACCESS

Edited by:
Gokhan Zengin, Selçuk University, Turkey

Reviewed by:
Gillian Grafton, University of Birmingham, United Kingdom
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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *29 November 2019*
Accepted: *13 July 2020*
Published: *30 July 2020*

Citation:
Muñoz-Montesino C, Burgos CF, Lara CO, Riquelme CR, Flaig D, San Martin VP, Aguayo LG, Fuentealba J, Castro PA, Guzmán L, Yévenes GE and Moraga-Cid G (2020) Inhibition of the Glycine Receptor alpha 3 Function by Colchicine. Front. Pharmacol. 11:1143. doi: 10.3389/fphar.2020.01143

INTRODUCTION

Colchicine is a tricyclic alkaloid known for their effects over cytoskeleton, acting as a microtubule depolymerizing agent (Slobodnick et al., 2015). Colchicine has been used in a variety of illnesses, including rheumatic and cardiovascular diseases (Campbell et al., 2015; Slobodnick et al., 2015). By changing cytoskeleton dynamics through microtubule disruption, colchicine affects cellular processes including neutrophil extravasation, cytokine secretion among others events associated with inflammation (Dalbeth et al., 2014). Based on these properties, it has been approved by the FDA for the treatment of inflammatory diseases in 2009 (Center for Drug Evaluation and Research).

Several electrophysiological studies have shown that colchicine modulates neurotransmission and the function of pentameric ligand-gated ion channels (pLGICs) by altering the microtubule

organization (Whatley et al., 1994; van Zundert et al., 2002; Whatley et al., 2002; Van Zundert et al., 2004). For example, alteration of microtubule stability by colchicine selectively impaired the function of the synaptic and extrasynaptic glycine receptors (GlyRs) in spinal neurons (van Zundert et al., 2002; van Zundert et al., 2004) and reduced the function and allosteric modulation of the γ-aminobutyric acid receptor type A ($GABA_A$Rs) (Whatley et al., 1994; Whatley et al., 2002). Nonetheless, several works have reported the direct effects of colchicine on the function of inhibitory and excitatory pLGICs. Electrophysiological experiments performed in *Xenopus* oocytes have shown that colchicine acts as a competitive antagonist of GlyRs (Machu, 1998) and of $GABA_A$Rs (Bueno and Leidenheimer, 1998). The inhibition of the glycine and GABA-activated currents was observed instantly and was concentration-dependent. Since the depolymerization of microtubules with colchicine takes at least 1.5 h to reach equilibrium at 30°C (Owellen et al., 1972), the inhibitory actions of colchicine on GlyRs and $GABA_A$Rs were proposed to be independent of microtubule alterations. In addition, the effects of colchicine on GABA responses required no pre-incubation with colchicine (Whatley et al., 1994), and pre-incubation with the drug failed to enhanced its effect on GlyRs (Machu, 1998). Moreover, other microtubule-depolarizing agents failed to have similar effects over the ion channel function (Machu, 1998). On the other hand, further reports have shown that colchicine is able to modulate the 5-Hydroxytryptamine 3A receptor ($5\text{-}HT_{3A}R$). Similar to GlyRs and $GABA_A$Rs, the colchicine inhibition of $5\text{-}HT_{3A}R$ function occurred in the absence of pre-incubation through microtubule-independent mechanisms (de Oliveira-Pierce et al., 2009).

Using biochemical techniques, a recent report has demonstrated that colchicine is able to bind homo-pentameric α_3GlyRs (Zhou et al., 2018). This report is particularly interesting because colchicine is an effective treatment for the inflammatory pain in gout flares (Dalbeth et al., 2014). On the other hand, recent reports have shown that positive allosteric modulators of α_3-containing GlyRs displayed analgesic effects in behavioral models of chronic pain (Acuña et al., 2016; Zeilhofer et al., 2018). These pieces of evidence suggest that colchicine may exert part of its therapeutic effects through the modulation of α_3GlyRs. However, the effects of colchicine in the function of α_3-containing GlyRs are still unknown. In this work, we combine *in silico* docking assays and electrophysiological recordings to evaluate the effects of colchicine in the function of homo-pentameric α_3GlyRs.

MATERIAL AND METHODS

Cell Culture and Transfection

HEK 293 cells (CRL-1573; American Type Culture Collection, Manassas, VA, USA) were cultured using standard methods (Lara et al., 2019). The cells were transfected using XfectTM Transfection Reagent (Clontech, USA) using 1.0 μg of cDNA plasmid encoding the rat GlyR α_3 subunit. Cell were co-transfected with a plasmid encoding EGFP (0.5 μg) to identify the transfected cells (Lara et al., 2019). All recordings were made 24–36 h after transfection.

Electrophysiology

Glycine-evoked currents were recorded from EGFP-positive transfected HEK 293 cells in the whole-cell voltage-clamp configuration at room temperature (20–24°C) at a holding potential of −60 mV (Lara et al., 2019). Patch electrodes (3–4 mΩ) were pulled from borosilicate glass and were filled with (in mM): 120 CsCl, 8 EGTA, 10 HEPES (pH 7.4), 4 $MgCl_2$, 0.5 GTP, and 2 ATP. The external solution contained (in mM) 140 NaCl, 5.4 KCl, 2.0 $CaCl_2$, 1.0 $MgCl_2$, 10 HEPES (pH 7.4), and 10 glucose. Whole-cell recordings were performed with an Axoclamp 200B amplifier (Molecular Devices, USA) and acquired using Clampex 10.1 or Axopatch 10.0 software. Data analysis was performed off-line using Clampfit 10.1 (Axon Instruments, Sunnyvale, CA, USA). Exogenous glycine-evoked currents were obtained using a manually applied pulse (3–4 s) of the agonist and an outlet tube (200 μm ID) of a custom-designed gravity-fed microperfusion system positioned 50–100 μm from the recorded cell. The methodologies employed for the single channel recordings of α_3GlyRs in the cell-attached configuration have been previously published (Marabelli et al., 2013; Lara et al., 2019). The patch pipettes had tip resistances of 10–20 mΩ and were manually fire polished in a microforge (Narishige, Japan). The data were filtered (1-kHz low-pass 8-pole Butterworth) and acquired at 5–20 kHz using an Axopatch 200B amplifier and a 1322A Digidata (Axon Instruments, Union City, CA). Data was acquired using pClamp software and analyzed off-line with Clampfit 10.1 (Axon Instruments, Union City, CA). Colchicine stock was prepared in high purity distilled water and subsequently diluted into the recording solution on the day of the experiment. In whole-cell experiments colchicine was co-applied with glycine using a manually applied pulse (1–2 s). In cell-attached recordings, colchicine was applied to the intra-pipette solution together with glycine. Colchicine was obtained from AK Science (CA, USA). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

Molecular Docking Simulations

Protein-ligand docking was performed using the structures of α_3GlyRs in open/closed conformations from the Protein DataBank (PDB ID:5CFB, 5TIO) (Huang et al., 2007; Huang et al., 2017). The structures of colchicine, strychnine, and glycine were obtained from the PubChem database (CID: 26719, 441071, 750) and prepared using LigPrep (Schrödinger, LLC, New York, NY, 2016) before docking simulations (Irwin et al., 2012). Initially, free docking protein-ligands were performed using Autodock Vina (Trott and Olson, 2010) in which the extracelullar domain (ECD) of the α_3GlyRs was used as the protein target. The generated complexes were ranked based on the affinity constants calculated by the same program. Subsequently, site-directed docking was created with Glide (Schrödinger, LLC, New York, NY, 2016) using a receptor grid centered on the amino acids that form the binding site defined in the previous step and an extra-precision (XP) configuration. Analysis of the interface GlyR-molecules included structural

and energetic parameters performed by the same software. Additionally, a theoretical ΔG bind was calculated by an energy calculation MM-GBSA using Prime (Schrödinger, LLC, New York, NY, 2016). All images were created using PyMol (version 1.5, DeLano Scientific LLC).

Data Analysis

All values were expressed as mean ± s.e.m. of normalized agonist-activated currents. $P < 0.05$ was considered statistically significant. For statistical analyses, at least six cells were analyzed per condition. All the statistical analyses and plots were performed with MicroCal Origin 8.0 (Northampton, MA, USA).

RESULTS

To test the hypothesis that colchicine modulates directly the function of GlyRs, concentration response curves were generated from HEK293 expressing α_3GlyRs. All these experiments were performed in the absence of any pre-incubation with colchicine. The co-application of colchicine produced a rapid inhibition of glycine-activated currents (tested at an EC_{10-15} concentration; 30–50 μM). The alkaloid exerted inhibitory effects in concentrations ranging from 1 to 200 μM (**Figure 1A**). The concentration producing the 50% inhibition (IC_{50}) was 24 ± 7 μM, with a maximal inhibition of 39 ± 2% at 200 μM (n = 8). Interestingly, the colchicine potency observed in the α_3GlyRs was not different from the IC_{50} obtained in the α_1GlyRs (25 ± 6 μM, **Supplementary Figure S1**). To obtain additional insights on the nature of this inhibition, glycine concentration–response curves were generated in the absence or presence of 100 μM of colchicine (**Figure 1B**). A right-shift in the glycine concentration curve was observed in presence of colchicine, changing the apparent affinity from 56 ± 4 μM to 253 ± 29 μM (n = 8; $p < 0.01$). An estimated K_i value of 15 ± 3 μM in the presence of 200 μM of colchicine was calculated using the Cheng–Prusoff equation (Cer et al., 2009). To investigate further the mechanisms underlying the ion channel modulation by

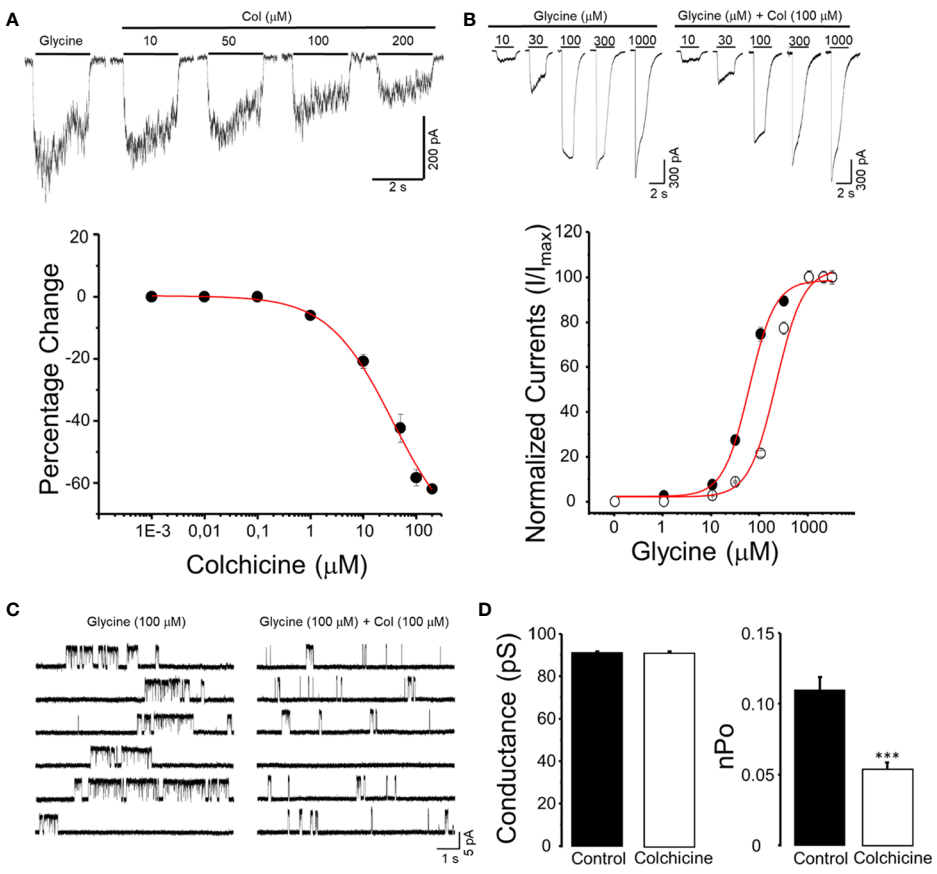

FIGURE 1 | Functional modulation of α_3GlyRs by colchicine. **(A)** The panel shows typical whole-cell current traces recorded in HEK293 cells expressing α_3GlyRs activated by glycine 30 μM before and during the application of colchicine (Col) (10, 50, 100, and 200 μM). The graph summarizes the percentage of inhibition of the glycine-evoked currents in a concentration-response fashion. **(B)** The panel shows current traces recorded in HEK293 cells expressing α_3GlyRs activated by glycine (1–1,000 μM) in the absence (left) or presence (right) of colchicine (Col 100 μM). The plot summarizes the glycine concentration-response curve obtained in the absence (black circles) or presence (white circles) of colchicine (100 μM). **(C)** Single-channel activity recorded in cell-attached configuration from cells expressing α_3GlyR before and in the presence of 100 μM of colchicine. **(D)** The graphs show that colchicine did not modified the main unitary conductance but significantly decreased the open probability (nPo) of the α_3GlyRs (***$P < 0.001$, paired Student t-Test).

colchicine, we performed single-channel recordings in the cell-attached configuration (**Figures 1C, D**). No spontaneous activity was observed when patches were perfused with glycine-free solution (>1 min). However, glycine 100 μM triggered clusters of active periods (**Figure 1C**). The mean current amplitude at +60 mV calculated from the amplitude histogram was 5.5 ± 0.08 pA. The main single conductance (92 ± 2 pS) was determined by current–voltage relationship over a range of voltages from −80 mV to +80 mV as an average from three to five patches (**Figure 1D**). The application of colchicine (100 μM) to membrane patches obtained in the same control cells did not change the main current amplitude (5.4 ± 0.06 pA) and main conductance (91 ± 2 pS (n = 6; p = 0.66). In agreement with our data obtained from whole-cell experiments, colchicine significantly reduced the normalized open probability (nPo) by 47.3% (0.11 ± 0.09 in control conditions *v/s* 0.056 ± 0.04 in colchicine conditions ($p < 0.01$, paired t-test). Altogether, these results support a direct inhibition of the channel function by colchicine. Moreover, these data are consistent with a competitive antagonism of colchicine on the α_3GlyRs.

We next performed *in silico* analysis of the interaction of α_3GlyRs with colchicine using the crystal structure of α_3GlyRs (PDB: 5CFB) as a template (**Figure 2**). We first performed a free docking protein-ligand using the ECD of α_3GlyRs as a target region. Under this condition, colchicine showed a preference for the closed state of the α_3GlyR, generating a series of complexes centered at the orthosteric binding site with slight differences between the orientations of the molecule (**Figure 2A**). We next restricted the docking analyses to the orthosteric site. The analyses revealed that colchicine binds to the closed state of α_3GlyRs (**Figure 2B**), reaching a docking score of −5.05 and a predicted ΔG bind of −39.48 kcal/mol. The values were in the range to those showed by glycine and strychnine, suggesting a functional interaction (**Figure 2C**). The α_3GlyRs–colchicine interface showed the formation of two H-bonds from amino acids Q177 (+) and T204 (−), together with a π-cation interaction

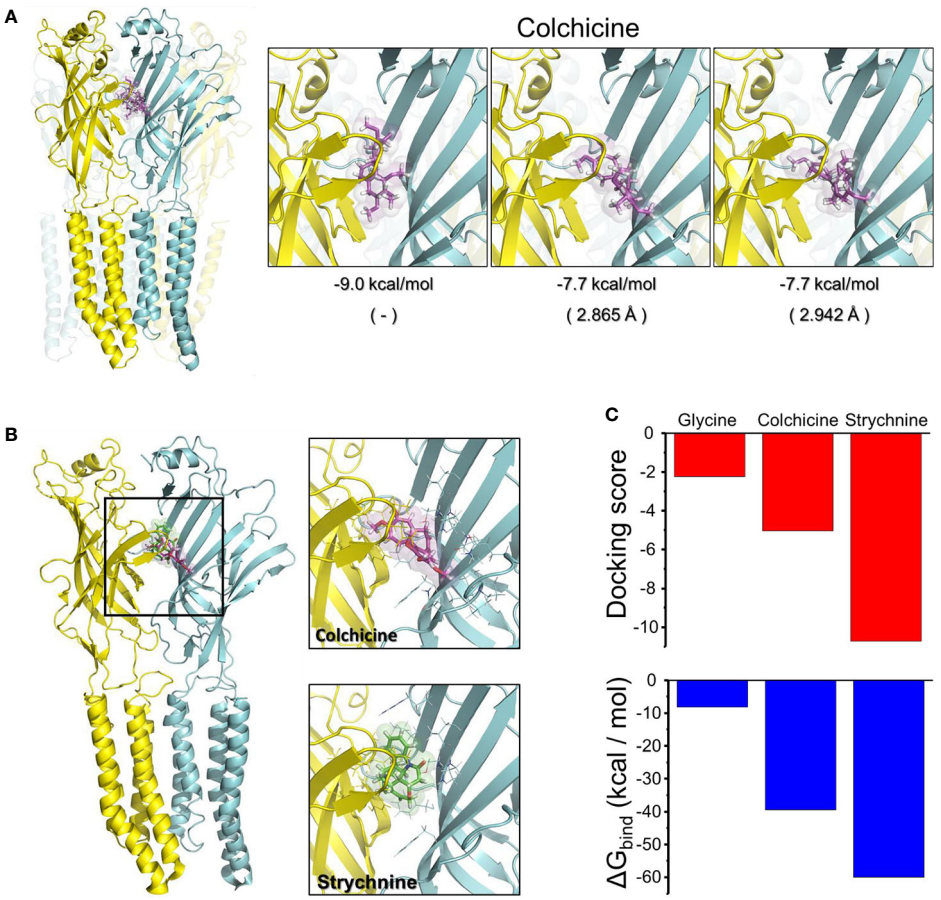

FIGURE 2 | Binding prediction of colchicine on the α_3GlyR structure. **(A)** Representative interaction modes of the free docking between the ECD of α_3GlyR and colchicine. All chains are identical and were colored in cyan and yellow to facilitate the identification of intersubunit regions. **(B)** Binding of colchicine to the orthosteric site of α_3GlyR predicted by Glide. For comparison, the binding of strychnine is also shown. **(C)** The graphs present the docking scores and the theoretical ΔG bind of interaction of colchicine and strychnine with α_3GlyRs in the closed state. The value obtained from the docking with glycine under similar conditions has been added as reference.

with R65 (+) that contributes to the stabilization of the complex (**Supplementary Figure S2**). No α_3GlyRs–colchicine complexes were observed when the open conformation of α_3GlyRs was tested. Thus, our *in silico* studies match well with the functional data obtained in our electrophysiological recordings, suggesting that colchicine modulate the α_3GlyRs function by direct binding to the orthosteric site, in a competitive manner.

DISCUSSION

Colchicine has been used for many years as a therapeutic agent. Its uses also include undesired effects, such as gastrointestinal disturbances and neutropenia. Traditionally, the mechanism by which colchicine affects the cell function has been linked to the inhibition of microtubule polymerization. Since the cytoskeleton controls many aspects of the cell physiology, such as migration and intracellular signaling, the capacity of colchicine as an anti-inflammatory agent has been associated with this specific action (Angelidis et al., 2018). Nevertheless, other studies have shown that colchicine exerts direct actions on ion channels, including GlyRs composed of α1 and α2 (Bueno and Leidenheimer, 1998; Machu, 1998; de Oliveira-Pierce et al., 2009). Interestingly, using a biochemical approach, a recent report demonstrated that colchicine in complex with biotin binds directly to α_3GlyRs (Zhou et al., 2018). The authors thus proposed that the colchicine–α_3GlyRs interaction may explain the mitigating effects of colchicine on inflammatory pain, especially the one caused by the deposition of monosodium urate crystal in gouty arthritis disease. Interestingly, these pieces of evidence are in line with experimental data showing the relevance of the α_3GlyRs on chronic pain of inflammatory origin and the ability of positive allosteric modulators to exert analgesic effects in behavioral models of chronic pain (Ahmadi et al., 2002; Harvey et al., 2004; Acuña et al., 2016; Zeilhofer et al., 2018). However, the effects of colchicine on the α_3GlyRs function were not described. The present work characterized the α_3GlyRs–colchicine functional interaction by electrophysiology and bioinformatics. Our electrophysiological studies show that the function of the α_3GlyRs is inhibited by colchicine at micromolar range (1–200 μM). The glycine-activated current inhibition was not significantly different to those obtained using α_1GlyRs, in which colchicine was described as a competitive antagonist in a previous study (Machu, 1998). Single-channel analysis showed that colchicine reduced the ion channel open probability without changes in the conductance. Moreover, molecular modeling and *in silico* docking simulations based on the crystal structure of α_3GlyRs (Huang et al., 2017) showed a favorable binding of colchicine to the orthosteric site. Interestingly, colchicine has a higher preference for the orthosteric site in the closed conformation. However, since our data suggest that colchicine is a competitive antagonist of the α_3GlyRs, our results suggest that the analgesic effects of colchicine in inflammatory pain are possibly not linked to an enhanced α_3GlyRs activity. In addition, the comparison between the plasmatic concentrations of colchicine from patients (0.6–9.5 ng/ml) (Berkun et al., 2012) and the concentrations required to inhibit the GlyR function reported here makes it difficult to suggest that the modulation of GlyRs is a relevant colchicine target in humans.

CONCLUSION

The present work defines the modulatory effects of colchicine on homomeric α_3GlyRs. Our results provide novel functional information regarding the direct interaction of colchicine with GlyRs, suggesting a molecular mechanism associated with a competitive inhibition at the orthosteric site. Although our experiments does not rule out a possible relevance of α_3GlyRs on the analgesic actions of colchicine in inflammatory pain (especially in the context of gouty arthritis), our results at least confirm that GlyRs are targets of the alkaloid colchicine at the functional level. Further behavioral and functional experiments are necessary to clarify whether the effects of colchicine on GlyRs are relevant players on the beneficial effects of the alkaloid in inflammatory pain conditions.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

CM-M, COL, CRR,DF and VPSM performed the experiments and data analysis. LGA, JF, PAC, LG, GEY and GM-C designed the research and contribute with analytical tools. CFB and CRR performed in silico analysis. CM-M, GEY and GM-C wrote the paper. All authors read and approved the submitted version.

FUNDING

This work was supported by grant FONDECYT 1160851 and VRID 219.033.111-INV. (to GM-C), FONDECYT 1170252 (to GEY) and FONDECYT 3170108 (to CFB). COL and VPSM were supported by CONICYT doctoral fellowship 21171549 and 21201722 respectively. CRR was supported by CONICYT master fellowship 22200510.

ACKNOWLEDGMENTS

The authors thank L. Aguayo and I. Cid for their outstanding technical assistance. We thank the Graduate School Fellowship of the University of Concepcion (Master Program in Neurobiology) for supporting DF.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.01143/full#supplementary-material

FIGURE S1 | Functional modulation of α_1GlyRs by colchicine. **(A)** The panel shows typical whole-cell current traces recorded in HEK293 cells expressing α1GlyRs activated by glycine 20 μM before and during the application of colchicine (Col) (10, 50, 100, and 200 μM). **(B)** The graph summarizes the percentage of inhibition of the glycine-evoked currents in a dose–response fashion (IC_{50} = 25 ± 6 μM, n = 6).

FIGURE S2 | Ligand interaction diagrams of colchicine with the ECD of α_3GlyRs. Schematic representation of the amino acids involved in the binding of colchicine with the ECD of α_3GlyR in closed-state conformation. All interactions detected in the α_3GlyR-colchicine interface are located in the inner box with a cutoff of 4 Å from the receptor. In the two H-bonds formed, T204 and Q177 act as hydrogen-bond donors. For its part, R65 interacts with an aromatic ring of colchicine, an electron-rich π group, to create a pi–cation interaction. The analysis was performed using Maestro (Schrödinger, LLC, NY, 2018).

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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.

frontiers in Neuroscience

ORIGINAL RESEARCH
published: 17 September 2020
doi: 10.3389/fnins.2020.00799

Lipoxin A4 Inhibits NLRP3 Inflammasome Activation in Rats With Non-compressive Disc Herniation Through the JNK1/Beclin-1/PI3KC3 Pathway

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Neuroscience

Received: *16 February 2020*
Accepted: *07 July 2020*
Published: *17 September 2020*

Citation:
Jin J, Xie Y, Shi C, Ma J, Wang Y, Qiao L, Li K and Sun T (2020) Lipoxin A4 Inhibits NLRP3 Inflammasome Activation in Rats With Non-compressive Disc Herniation Through the JNK1/Beclin-1/PI3KC3 Pathway. Front. Neurosci. 14:799. doi: 10.3389/fnins.2020.00799

Background: Non-compressive disc herniation is induced by an inflammatory response from the nucleus pulposus tissue and nerve roots. Lipoxins (LXs) are important endogenous anti-inflammatory mediators in the body, helping to inhibit neutrophil recruitment and stimulate autophagy in monocytes and macrophages. Here, we investigated the molecular mechanisms underlying the effects of exogenous lipoxin administration on rats with non-compressive disc herniation.

Method: A non-compressive disc herniation model was established in rats. Fifty rats were randomly divided into: sham group, model group, PI3K inhibitor (LY294002) group, lipoxin A4 group (LXA4), and PI3K inhibitor and lipoxin A4 group (LY294002 + LXA4). Similar groupings were established for rat spinal neurons. Changes in the mechanical pain threshold and thermal pain threshold were monitored at different times. The expression of proinflammatory and anti-inflammatory mediators was assessed by ELISA, while immunohistochemistry was employed to measure the expression levels of NLRP3 and p-JNK1. The expression levels of autophagy-related proteins were measured by western blot.

Results: *In vivo*, the pain threshold was markedly decreased in the model group at each time point examined compared with that in sham group. LY294002 treatment further reduced the pain threshold. After LXA4 injection, the pain threshold was significantly increased, and the effect of LY294002 was significantly weakened ($p < 0.05$). The levels of proinflammatory cytokines were increased in rats with non-compressive disc herniation, and these levels were further increased by LY294002 treatment ($p < 0.05$). However, treatment with LXA4 significantly reduced the levels of these proinflammatory cytokines in the model group ($p < 0.05$). The opposite effect was observed for anti-inflammatory mediators. The expression of NLRP3 was largely increased in the model group compared with that in the sham group ($p < 0.05$). Treatment with LY294002 also increased the NLRP3 expression level, while the administration of LXA4 elicited

the opposite effect. Furthermore, western blot analysis showed that the expression of autophagy-related proteins was greatly decreased in the model group, whereas it was significantly increased in the LXA4 group ($p < 0.05$). The *in vitro* results were consistent with the outcomes observed *in vivo*.

Conclusions: These data suggested that LXA4 inhibited NLRP3 activation in rats with non-compressive disc herniation by regulating the JNK1/beclin-1/PI3KC3 pathway.

Keywords: lipoxin A4, non-compressive disc herniation, NLRP3, JNK1, beclin-1, PI3KC3

INTRODUCTION

Root nerve pain, a painful and protracted condition, greatly affects the quality of life in patients. Disc herniation is the major cause of root nerve pain, and is induced by either mechanical compression or an inflammatory response originating from the nucleus pulposus and nerve roots (non-compressive disc herniation) (Li et al., 2015; Choi et al., 2016). Studies have shown that non-compressive disc herniation plays an important role in root neuralgia, and has attracted significant research attention in recent years (Miao et al., 2015; Liu et al., 2016).

The nucleus pulposus, the cause of non-compressive disk herniation, can be regarded as an "isolated antigen." It can stimulate immune responses and cause inflammatory reactions once the "antigen" is exposed to the immune system (Olmarker et al., 1995). Neutrophils, mononuclear macrophages, and other inflammatory cells will be recruited and proliferate. The released enzymes and inflammatory cytokines, such as interleukin-1β (IL-1β), IL-6, tumor necrosis factor (TNF), and prostaglandins, will lead to swelling of the nerve roots, vacuolar degeneration, finally resulting in root pain (Zhang and An, 2007). The NLRP3 (nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3) inflammasome is recruited following pathogenic or endogenous signals mediated by pattern-recognition receptors (Martinez et al., 2018). Caspase-1 is the main component of the NLRP3 inflammasome, and stimulates the expression of IL-1β and IL-18 (Doyle et al., 2019). When caspase-1 is activated, pro-IL-1β and pro-IL-18 are cleaved, thereby promoting the maturation and secretion of IL-1β and IL-18.

Lipoxins (LXs) are important endogenous anti-inflammatory mediators (Chandrasekharan and Sharma-Walia, 2015) synthesized from arachidonic acid through the activity of lipoxygenases (English et al., 2017). LXs are important "brake signals" for relieving and inhibiting neutrophil recruitment, and stimulate autophagy in monocytes and macrophages (Hughes et al., 2017). They can also regulate cytokine secretion and expression, which greatly contributes to the pathophysiology of chronic pain (Brennan et al., 2018).

For many diseases associated with chronic pain, LXs exert significant regulatory effects on a variety of inflammatory cell types and factors. The beneficial effects of LXs reported to date include the promotion of inflammation regression in acute lung injury (Fang et al., 2015), asthma (Lu et al., 2018), and renal fibrosis (Roach et al., 2015). LXA4, one of the most widely investigated lipoxins, has been reported to effectively inhibit inflammation-related and neuropathic pain (Kim et al., 2011; Martini et al., 2016), with one study showing that intrathecal injection of LXA4 could alleviate neuropathic pain in a rat model of non-compressive lumbar disc herniation (Miao et al., 2015).

However, relatively few studies have investigated the role of LXA4 in non-compressive disc herniation-induced root neuralgia. Consequently, the aim of this study was to investigate the molecular mechanisms underlying the effects of exogenous LXA4 administration on rats with non-compressive disc herniation.

MATERIALS AND METHODS

Experimental Animals

Fifty specific-pathogen-free (SPF)-grade, healthy, female Sprague–Dawley (SD) rats, 8–10 weeks old, weighing 240–320 g, were purchased from Beijing Wei Tong Lihua Experimental Animal Technology Co., Ltd (Beijing, China; license number: SCXK [Beijing] 20160006). The rats were maintained in a controlled environment at a temperature of 23 ± 2°C, humidity of 55 ± 5%, 12-h light–dark cycle, and with *ad libitum* access to food and water. All animal experiments were carried out following NIH guidelines (NIH Pub. No. 85–23, revised 1996), and were reviewed and approved by the Animal Protection and Use Committee of Shandong University.

Establishment of a Rat Model of Non-compressive Disc Herniation

Rats were first anesthetized by intraperitoneal injection of 10% chloral hydrate (300 mg/kg). Under a sterile environment, a 25–30-mm medial longitudinal incision was made at the midpoint between the two iliac crests, then subcutaneous tissue and paravertebral muscles were incised through blunt separation from the right L4–L5 spinal. It exposes L4/5 facet joints, removes right L4 lamina and articular processes, and exposes L5 nerve roots and dorsal root ganglion (Kim et al., 2011; Miao et al., 2015). The nucleus pulposus (0.5 mg) of the rat's autologous caudal vertebrae was removed and placed gently at the proximal end of the L5 dorsal root ganglia, and placed in contact with the nerve roots.

Drug Injection Through an Intrathecal Catheter

Following anesthesia by intraperitoneal injection of 10% chloral hydrate (300 mg/kg), a 2–3 cm incision was made in the

middle of the back of the rats to expose the L4–5 intervertebral foramen. A PE-10 catheter was inserted into the L6 spinous process through the exposed intervertebral foramen, and the cerebrospinal fluid flowed out along the catheter, which indicated that implantation was successful. The catheter was fixed and the wound stitched. After recovery from general anesthesia, 3 μL of 2% lidocaine was injected through the catheter, and reversible side limb paralysis after injection was indicative of successful catheter placement.

Animal Grouping and Medication

Fifty rats were randomly divided into 5 groups, with 10 rats per group: (i) Sham operation group (sham), which included a sham operation and the injection of 10 μL of saline solution; (ii) model group, which included modeling surgery and the injection of 10 μL of saline solution; (iii) lipoxin group (LXA4) (Miao et al., 2015), which consisted of model group rats injected with LXA4 (10 μL, 100 ng); (iv) PI3K inhibitor (LY294002) group, which comprised modeling surgery and injection of LY294002 (10 μL, 25 mmol/L); and (v) PI3K inhibitor and LXA4 combination group (LY294002 + LXA4), comprising model surgery, followed by injection of LY294002 and then that of LXA4. Each group of rats was injected with the corresponding drugs once a day for 28 consecutive days. LY294002 was purchased from Sigma–Aldrich (St. Louis, MO, United States). LXA4 was purchased from Cayman Chemical Co., United States. The complete stereochemistry of LXA4 is shown in **Figure 1A**.

Determination of the Mechanical and Thermal Pain Thresholds

Changes in the mechanical pain threshold (paw withdrawal threshold, PWT) and thermal pain threshold (thermal withdrawal latency, TWL) before and after drug injection were detected before surgery and 1, 3, 5, 7, 14, 21, and 28 days after surgery. In each group, the "up–down" method was used to stimulate the plantar side of the rat's paw using von Frey hairs with different bending forces. A PWT of 50% was assumed if the rat showed a rapid withdrawal and a cringe in the hind limbs, which was also called a positive reaction. To determine the TWL, the rats were first placed on a 6-mm thick plexiglass plate. The palm of the rat's hindlimb was irradiated through the plexiglass plate using a thermal pain stimulator. The incubation period from the start of irradiation to hind limb retraction, acting as an indicator of thermal pain, was recorded using an electronic stopwatch. The analgesic effects of the treatments and their duration were also analyzed.

Specimen Collection

The TWL and 50% PWT were measured on postoperative day 28. The rats were then anesthetized by intraperitoneal injection of 3% sodium pentobarbital (50 mg/kg) and euthanized by cervical dislocation. On ice, an incision was made in the skin of the back of the rats and the L4–L6 spinal dorsal horn was removed. Part of the spinal dorsal horn and dorsal root ganglion was stored in 4% paraformaldehyde, and the remaining tissue was stored in a freezer at -80°C.

Cell Culture

Rat spinal neurons were purchased from Procell Life Science & Technology Co., Ltd (CP-R144, Wuhai, China) and cultured in DMEM (Gibco, Rockville, MD, United States) supplemented with 10% fetal bovine serum (FBS) (Sigma–Aldrich) and 100 U/mL penicillin/100 mg/mL streptomycin. All cells were incubated at 37°C with 5% CO_2. Cells were collected and used for experiments in the logarithmic growth phase.

Cell Model Grouping

Spinal neurons were stimulated with 0.01 μg/mL TNF-α (Chen et al., 2019) and then treated with 100 nM LXA4. These cells were randomly divided into 5 groups: (1) Control (untreated) group; (2) model group: treatment with 0.01 μg/mL TNF-α; (3) LXA4 group: treatment with 100 nM LXA4 and 0.01 μg/mL TNF-α; (4) LY294002 group: treatment with 0.01 μg/mL TNF-α, and then with 20 μM of LY294002 (Xu et al., 2018); and (5) LY294002 + LXA4 group: as for the LY294002 group, plus treatment with 100 nM LXA4. After 48 h, the treated cells were collected for subsequent experiments.

ELISA

The levels of TNF-α (orb452907), IL-1β (orb453587), IL-18 (orb107403), IL-4 (orb303658), IL-10 (orb76364), and TGF-β1 (orb7087) (all from Biorbyt, Cambridge, United Kingdom) in the spinal dorsal horn and dorsal root ganglion were measured following the instructions of the respective ELISA kits.

Immunohistochemistry

Tissues (5 μm) were treated with xylene, and hydrated via an ethanol gradient, followed by incubation in 3% H_2O_2 at room temperature for 10 min to block endogenous peroxidase activity. The slices were subsequently blocked with 5% goat serum (Gibco) for 20 min, and then incubated with rabbit anti-rat NLRP3 (1:500, DF7438, Affinity Biosciences, Beijing, China) or p-JNK1 (1:500, orb312293, Biorbyt) overnight at 4°C. Samples were then incubated with a horseradish peroxidase-labeled goat anti-rabbit IgG antibody (1:1000, ABIN101988, antibodies-online, Aachen, Germany) at 37°C for 40 min.

Cells grown on glass coverslips were fixed in paraformaldehyde for 30 min and added into 3% H_2O_2 for 15–20 min, then followed by 2–3 PBS washes. After blocking (AbDil-Tx; TBS containing 0.1% Triton X-100, 2% BSA, and 0.1% sodium azide) at 37°C for 30 min, cells were incubated with anti-NSE (1:200, ab53025, Abcam, United Kingdom) and anti-p-JNK1 (1:200, orb312293, Biorbyt) antibodies at 37°C for 30 min, washed 2–3 times with PBS, and then incubated with rabbit anti-Goat-IgG (1:800, SA0004-4, Proteintech, Wuhan, China) at 37°C for 30 min.

After washing, the cells were incubated with SABC for 30 min and washed 3 times with PBS, stained with DAB, slightly restained with hematoxylin, dehydrated in an ethanol gradient, made transparent, and then mounted in neutral tree lipid. Finally, the slides were observed under a microscope (IX83, Olympus, Japan) at × 400 magnification.

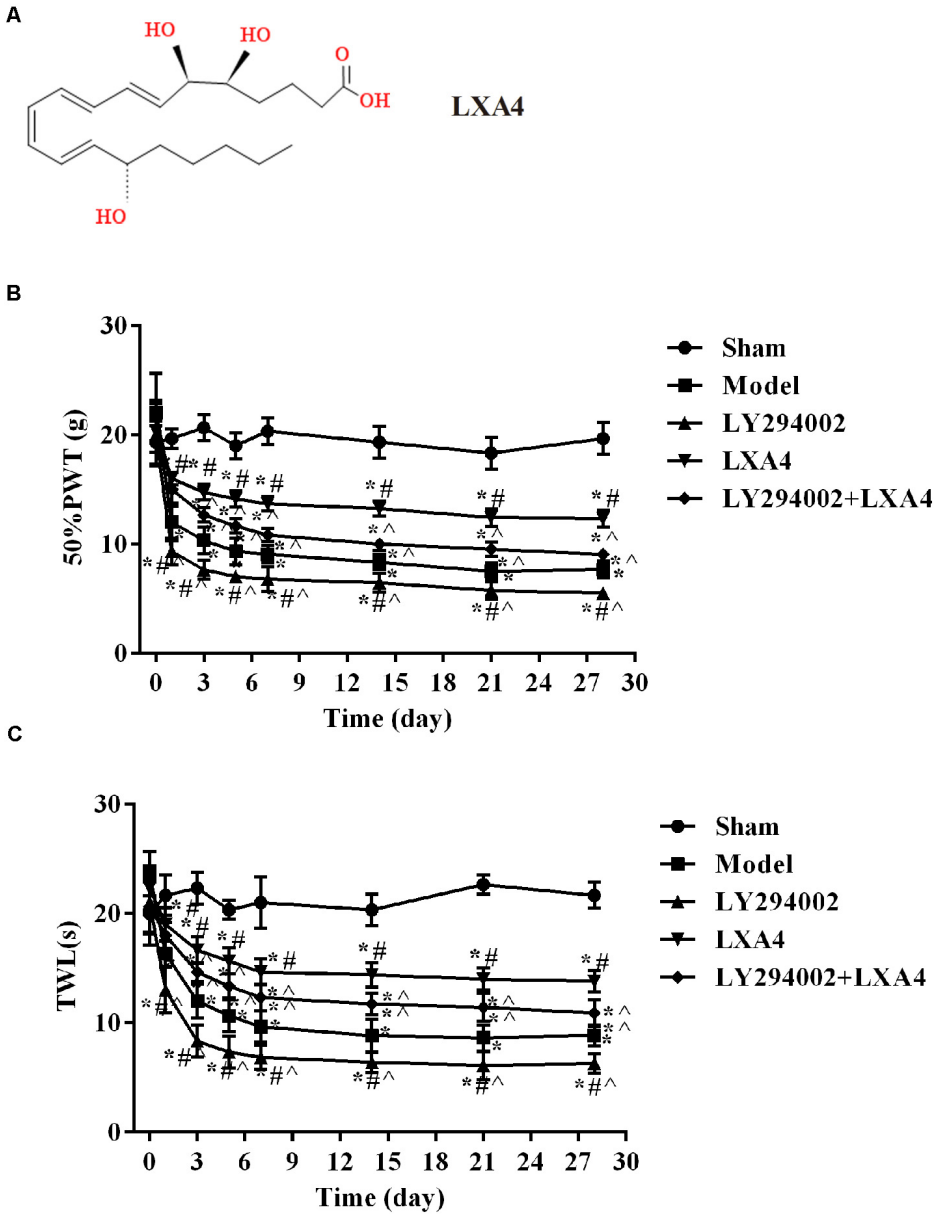

FIGURE 1 | Comparison of 50% mechanical pain threshold (paw withdrawal threshold, PWT) and thermal pain threshold (thermal withdrawal latency, TWL) at different time points in each treatment group. **(A)** The complete stereochemistry of LXA4; **(B)** 50% PWT; **(C)** TWL. $^{*}p < 0.05$ compared with the sham group; $^{\#}p < 0.05$ compared with the model group; $^{\wedge}p < 0.05$ compared with the LXA4 group. Experiments were repeated three times for each group. Data are presented as means ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for comparisons among groups followed by Dunnett's *t*-test.

Western Blot

Total protein was extracted from both tissues and cells using a total protein extraction kit (cat. no. BC3640-50T; Beijing Solarbio Science & Technology Co., Ltd). Protein concentration was measured using a BCA kit (Solarbio, Beijing, China). A total of 40 μg of each protein sample was mixed with 10% SDS gel buffer at a ratio of 1:1 and the protein denatured by heating at 95°C for 5 min. The PVDF membrane (Merck, Darmstadt, Germany) was rotated at 80 V for 30 min, and then blocked with 5% skimmed milk powder in TBST for 1 h at 4°C. Next, the membranes were incubated overnight at 4°C with rabbit anti-rat NLRP3 (1:500, DF7438, Affinity Biosciences), OX42 (1:500, orb176288), and p-JNK1 (1:500, orb312293); polyclonal rabbit anti-rat MAP1LC3A (1:500, orb378164), MAP1LC3B (1:500, orb382715), beclin1 (1:500, orb227780), PI3KC3 (1:500, orb382585), caspase-1 (1:200, orb213639), and β-actin (1:2000, orb178392) (all from Biorbyt) antibodies diluted in a TBST solution containing 3% BSA. Before testing, the membranes

were rewarmed, and then incubated with horseradish peroxidase-labeled goat anti-rabbit IgG (1:1000, ABIN101988, antibodies-online) for 1 h. Then, the membranes were washed with an ECL substrate for 3–5 min. Protein expression levels were normalized to those of β-actin, measured through grayscale scanning, and quantified using ImageJ.

Statistical Analysis

Data were analyzed using SPSS 19.0, and the results presented as means ± standard deviation (SD). Comparisons between groups were performed by one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test. A p-value < 0.05 was considered to be statistically significant.

RESULTS

LXA4 Ameliorated the Pain Threshold in Rats With Non-compressive Disc Herniation

The changes in the PWT (**Figure 1B**) and TWL (**Figure 1C**) are shown in **Tables 1**, **2**. Before surgery, there was no significant difference in the 50% PWT and TWL values among all the groups ($p > 0.05$). After surgery, the 50% PWT and TWL were differentially decreased in the spinal surgery groups, with the lowest levels being observed in the LY294002 group ($p < 0.05$). Compared with the sham group, the 50% PWT and TWL were significantly lower in all the surgery groups at each time point evaluated ($p < 0.05$). Notably, the 50% PWT and TWL were significantly higher in the LXA4 group than in the other surgery groups at each time point ($p < 0.05$). These data suggested that LXA4 could improve the pain threshold.

LXA4 Decreased the Levels of Proinflammatory Factors and Increased Those of Anti-inflammatory Mediators

We investigated the levels of proinflammatory and anti-inflammatory mediators in the spinal dorsal horn (**Figure 2A**) and dorsal root ganglion (**Figure 2B**) by ELISA. In the spinal dorsal horn (**Figure 2A**), the expression levels of TNF-α, IL-1β, and IL-18 were significantly higher in the model group than in the sham group ($p < 0.05$). After LXA4 treatment, the levels of these proinflammatory factors were significantly reduced in the spinal dorsal horn of surgery rats ($p < 0.05$). We also found that injection of LY294002 led to an increase in the secretion of proinflammatory factors, while LXA4 injection weakened the effect of LY294002 ($p < 0.05$). Similar results were also recorded for the dorsal root ganglion (**Figure 2B**). For anti-inflammatory mediators in the spinal dorsal horn (**Figure 2A**), the expression of IL-4, IL-10, and TGF-β was significantly decreased in the surgery groups when compared with the sham group ($p < 0.05$). Among the surgery groups, the levels of the anti-inflammatory mediators were highest in the LXA4-treated group and lowest in the LY294002-injected group. Similarly, LAX4 treatment led to a significant increase in the contents of anti-inflammatory factors and weakened the effect of LY294002 in the dorsal root ganglion (**Figure 2B**, $p < 0.05$). Taken together, these results suggested that treatment with LXA4 could effectively regulate

TABLE 1 | Data for 50% mechanical pain threshold (paw withdrawal threshold, PWT) at different time points in each treatment group.

Time (Day)	Sham group	Model group	LXA4 group	LY294002 group	LY294002+LXA4 group
0	19.33 ± 3.51	22.00 ± 6.25	20.33 ± 1.53	20.00 ± 5.00	22.00 ± 5.00
1	19.67 ± 1.53	12.00 ± 3.00*	16.00 ± 1.00*#	9.33 ± 2.08*#^	15.00 ± 2.64*^
3	20.67 ± 2.08	10.33 ± 2.08*	14.73 ± 1.27*#	7.67 ± 1.53*#^	12.67 ± 1.15*^
5	19.00 ± 2.00	9.33 ± 2.08*	14.13 ± 1.27*#	7.03 ± 0.95*#^	11.67 ± 1.15*^
7	20.33 ± 2.08	9.07 ± 1.41*	13.70 ± 1.13*#	6.80 ± 1.93*#^	10.83 ± 1.05*^
14	19.33 ± 2.52	8.33 ± 1.15*	13.27 ± 1.16*#	6.47 ± 1.50*#^	10.00 ± 1.00*^
21	18.33 ± 2.52	7.60 ± 1.44*	12.43 ± 1.40*#	5.77 ± 1.36*#^	9.53 ± 1.10*^
28	19.67 ± 2.52	7.70 ± 1.37*	12.33 ± 1.40*#	5.50 ± 0.50*#^	9.07 ± 0.81*^

*Compared with sham group, *$p < 0.05$; compared with model group, #$p < 0.05$; compared with LXA4 group, ^$p < 0.05$.*

TABLE 2 | Data for thermal pain threshold (thermal withdrawal latency, TWL) at different time points in each treatment group.

Time (Day)	Sham group	Model group	LXA4 group	LY294002 group	LY294002+LXA4 group
0	21.00 ± 3.61	23.67 ± 3.52	22.33 ± 3.51	21.00 ± 5.00	20.67 ± 4.04
1	21.67 ± 3.21	16.33 ± 2.08*	19.00 ± 2.65*#	13.00 ± 3.60*#^	18.00 ± 2.00*^
3	22.33 ± 2.52	12.00 ± 2.65*	16.67 ± 2.08*#	8.33 ± 2.52*#^	14.67 ± 1.53*^
5	20.33 ± 1.53	10.67 ± 2.52*	15.67 ± 2.08*#	7.33 ± 2.52*#^	13.37 ± 1.93*^
7	21.00 ± 4.00	9.67 ± 2.52*	14.67 ± 2.08*#	6.87 ± 1.89*#^	12.33 ± 2.08*^
14	20.33 ± 2.52	8.83 ± 2.57*	14.40 ± 1.91*#	6.40 ± 1.64*#^	11.73 ± 1.70*^
21	22.67 ± 1.53	8.63 ± 2.07*	14.00 ± 1.73*#	6.10 ± 2.23*#^	11.43 ± 2.21*^
28	21.67 ± 2.08	8.87 ± 1.67*	13.80 ± 1.65*#	6.30 ± 1.54*#^	10.90 ± 2.15*^

*Compared with sham group, *$p < 0.05$; compared with model group, #$p < 0.05$; compared with LXA4 group, ^$p < 0.05$.*

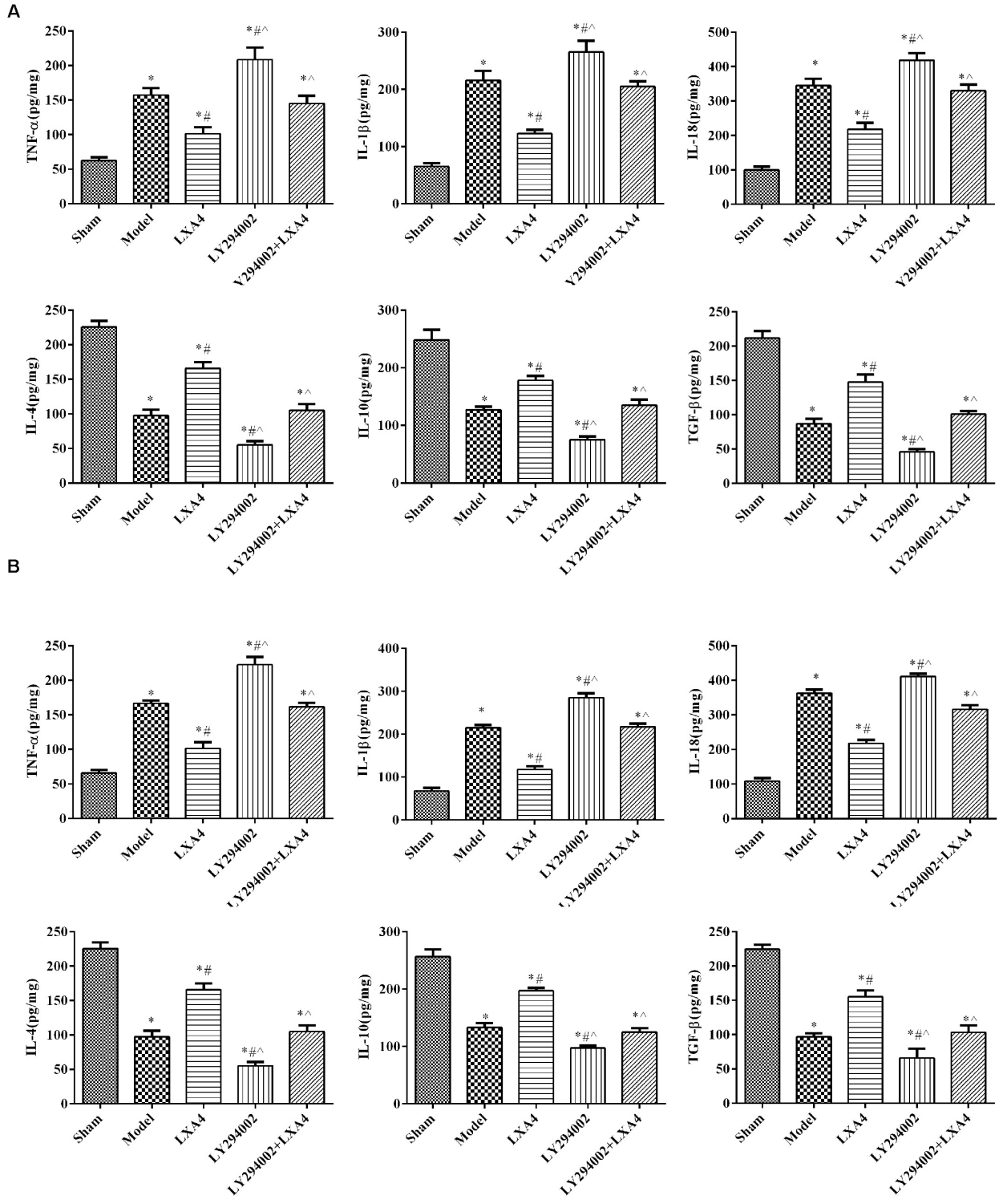

FIGURE 2 | The expression of proinflammatory and anti-inflammatory mediators in the spinal dorsal horn **(A)** and dorsal root ganglion **(B)** by ELISA. $^{*}p < 0.05$ compared with the sham group; #p < 0.05 compared with the model group; $^{\wedge}p < 0.05$ compared with the LXA4 group. Experiments were repeated three times for each group. Data are presented as means ± SD. One-way analysis of variance (ANOVA) was used for comparisons among groups followed by Dunnett's *t*-test.

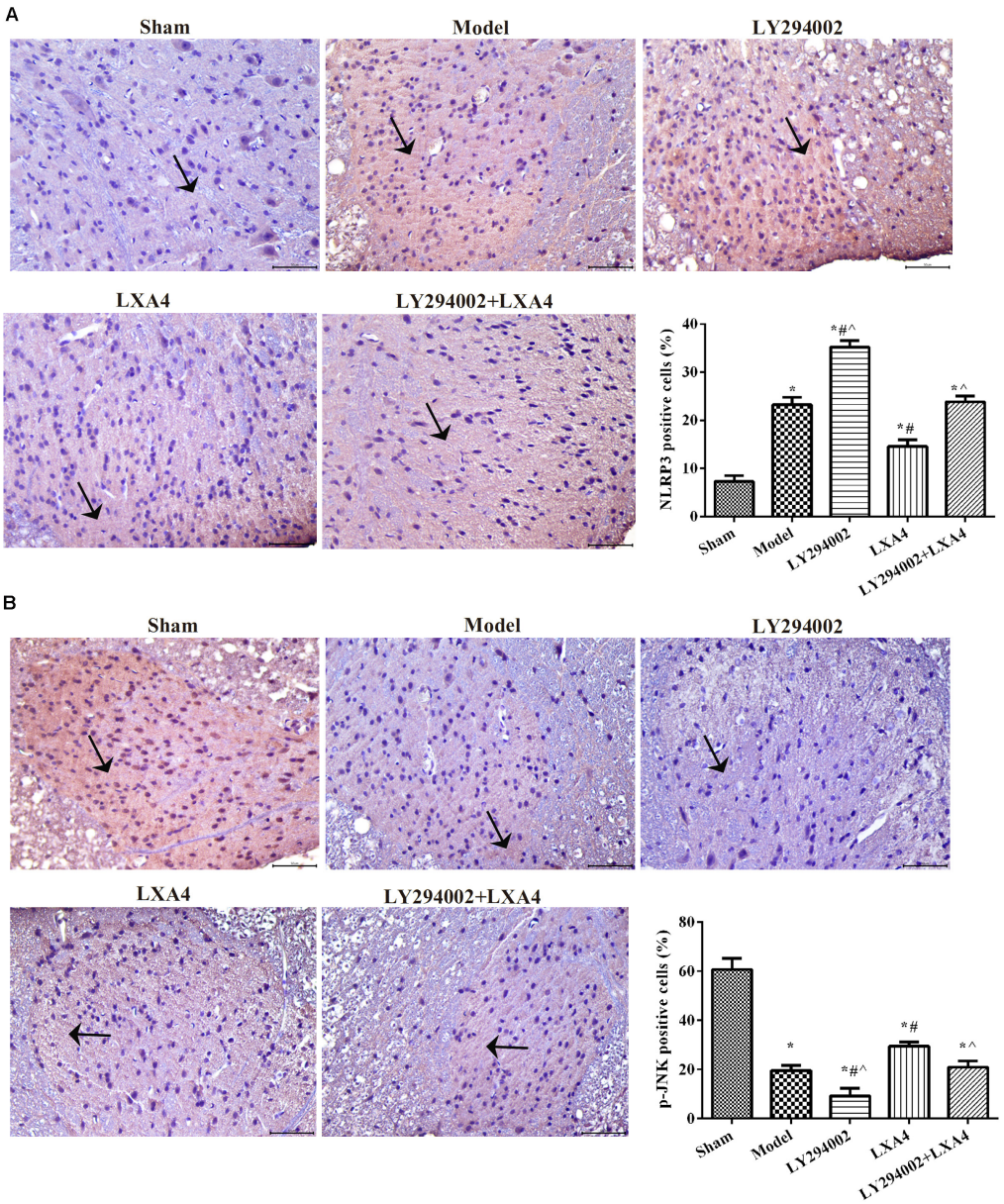

FIGURE 3 | Immunohistochemical analysis of NLRP3 **(A)** and p-JNK1 **(B)** expression in the spinal dorsal horn. Magnification: × 400. Arrows indicate positive expression. *$p < 0.05$ compared with the sham group; #$p < 0.05$ compared with the model group; ^$p < 0.05$ compared with the LXA4 group. Experiments were repeated three times for each group. Data are presented as means ± SD. One-way analysis of variance (ANOVA) was used for comparisons among groups followed by Dunnett's *t*-test.

the levels of inflammatory factors in rats with non-compressive disc herniation.

LXA4 Reduced the Expression of NLRP3 via the JNK1 Pathway in Rats With Non-compressive Disc Herniation

We also analyzed the expression of NLRP3 and p-JNK1 in the spinal dorsal horn (**Figure 3**) and dorsal root ganglion (**Figure 4**) of rats by immunohistochemistry. As shown in **Figures 3A, 4A**, the expression of NLRP3 was greatly increased in the surgery groups compared with the sham group ($p < 0.05$). Injection of LY294002 further upregulated the expression of NLRP3 in the spinal dorsal horn of surgery rats. However, LXA4 administration decreased the expression level of NLRP3 and blocked the effect of LY294002 ($p < 0.05$). The expression of p-JNK1 was highest in the sham group and lowest in the LY294002 treatment group ($p < 0.05$). After surgery, the expression of p-JNK1 was significantly reduced, while LXA4 injection significantly increased its expression ($p < 0.05$). These

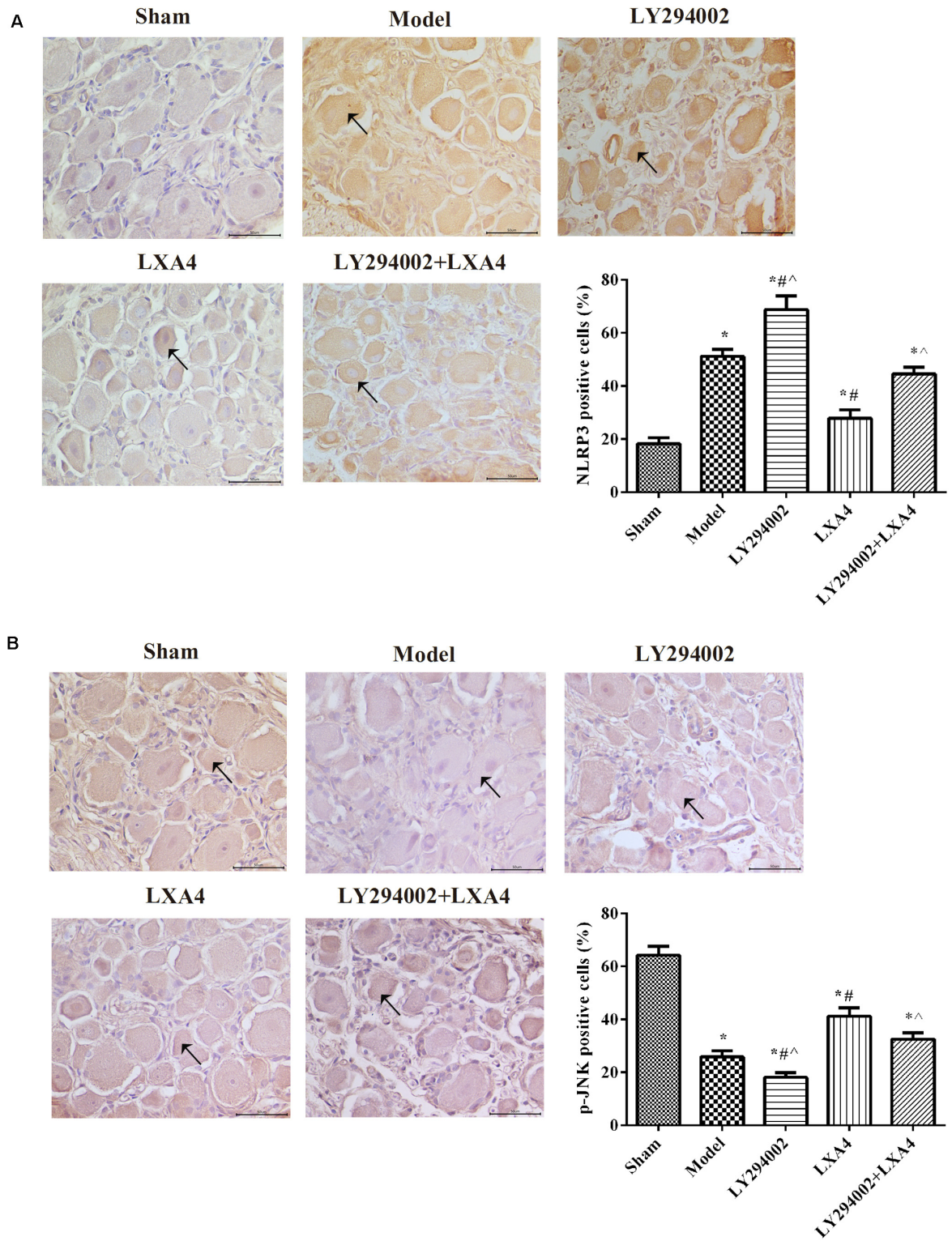

FIGURE 4 | Immunohistochemical analysis of NLRP3 **(A)** and p-JNK1 **(B)** expression in the dorsal root ganglion. Magnification: × 400. Arrows indicate positive expression. $^{*}p < 0.05$ compared with the sham group; $^{\#}p < 0.05$ compared with the model group; $^{\wedge}p < 0.05$ compared with the LXA4 group. Experiments were repeated three times for each group. Data are presented as means ± SD. One-way analysis of variance (ANOVA) was used for comparisons among groups followed by Dunnett's *t*-test.

results suggested that LXA4 suppresses NLRP3 activation and promotes p-JNK1 expression in rats with non-compressive disc herniation (**Figures 3B**, **4B**).

The Effect of LXA4 on the NLRP3 Inflammasome and Autophagy-Related Protein Expression *in vivo*

The expression of the NLRP3 inflammasome and that of autophagy-related proteins in the spinal dorsal horn (**Figure 5A**) and dorsal root ganglion (**Figure 5B**) were measured by western blot. For caspase-1 expression, compared with the sham group, the expression of caspase-1 was significantly increased in the model group ($p < 0.05$), and adding LY294002 further increased caspase-1 expression. Injection of LXA4 significantly reduced the expression of caspase-1 and weakened the effect of LY294002 ($p < 0.05$). For autophagy-related proteins, compared with the sham group, the expression of MAP1LC3B/MAP1LC3A, beclin-1, and PI3KC3 was significantly decreased in the model group ($p < 0.05$). LY294002 administration further decreased the

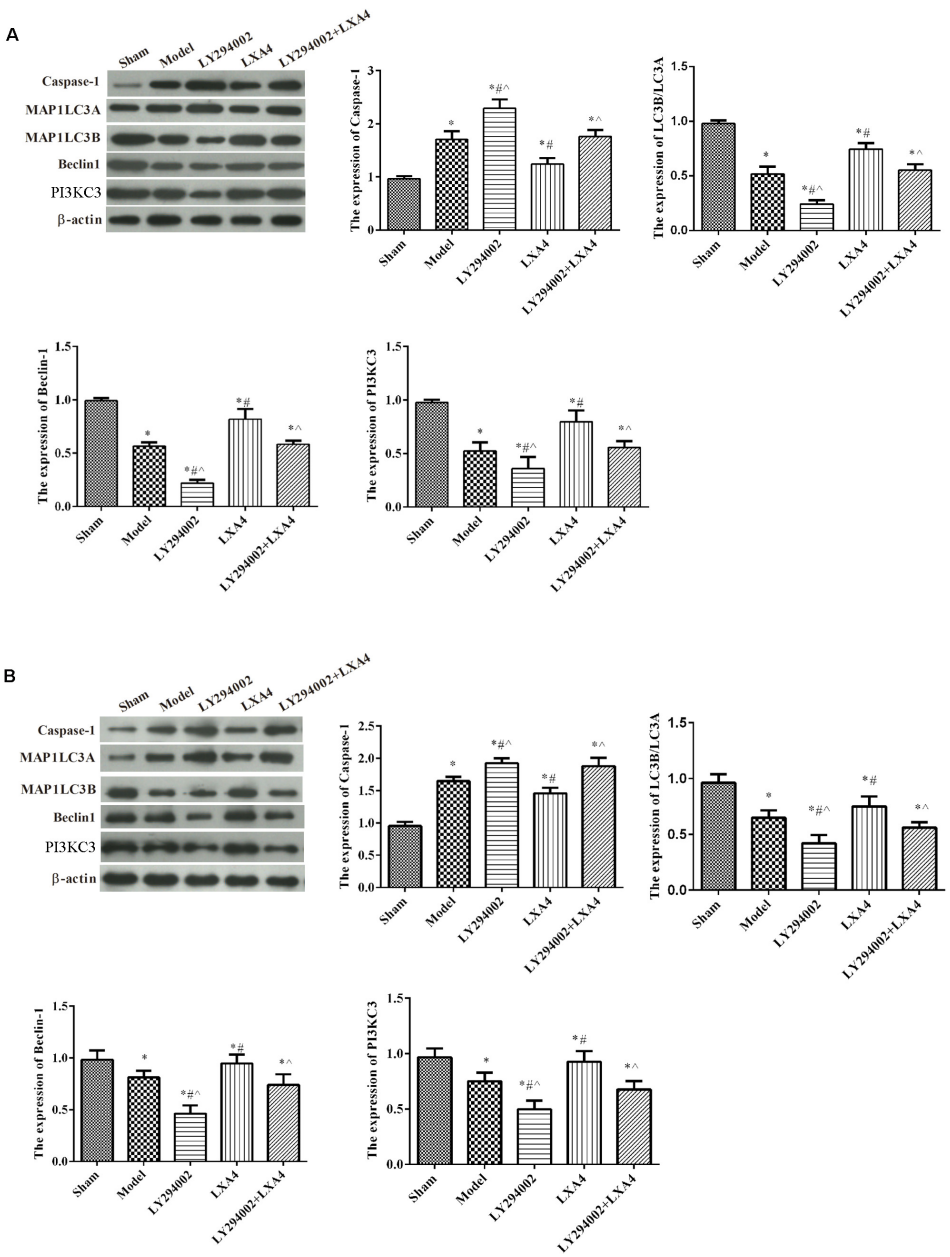

FIGURE 5 | Western blot analysis of autophagy- and apoptosis-related protein expression in the spinal dorsal horn **(A)** and dorsal root ganglion **(B)**. $^{*}p < 0.05$ compared with the sham group; $^{\#}p < 0.05$ compared with the model group; $^{\wedge}p < 0.05$ compared with the LXA4 group. Experiments were repeated three times for each group. Data are presented as means ± SD. One-way analysis of variance (ANOVA) was used for comparisons among groups followed by Dunnett's *t*-test.

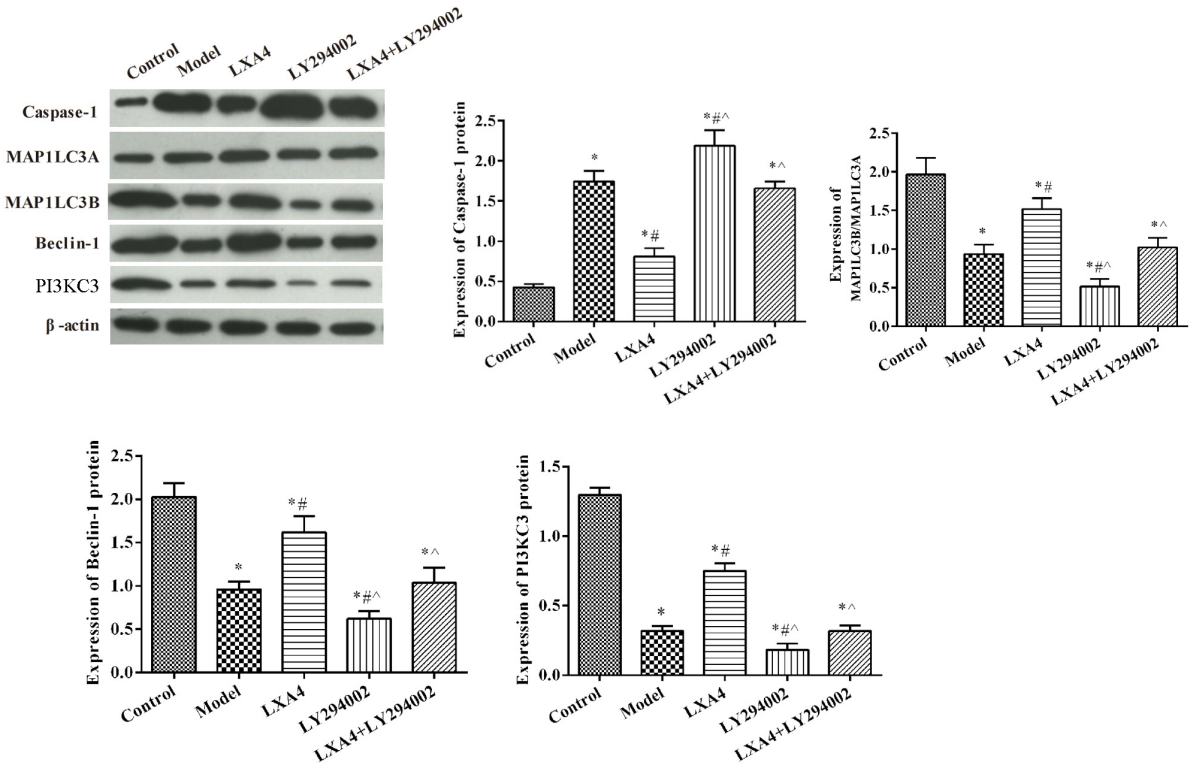

FIGURE 6 | The expression of autophagy- and apoptosis-related protein expression in spinal neuron cells by western blot. $^{*}p < 0.05$ compared with the control group; $^{\#}p < 0.05$ compared with the model group; $^{\wedge}p < 0.05$ compared with the LXA4 group. Experiments were repeated three times for each group. Data are presented as means ± SD. One-way analysis of variance (ANOVA) was used for comparisons among groups followed by Dunnett's *t*-test.

expression levels of these proteins. Meanwhile, treatment with LXA4 significantly increased the expression of autophagy-related proteins and diminished the effect of LY294002 ($p < 0.05$). These data suggested that LXA4 inhibited NLRP3 activation in spinal neuronal injury through the beclin-1/PI3KC3 pathway.

The Effect of LXA4 on the Expression of Autophagy-Related Proteins in TNF-α-Induced Neuronal Cells *in vitro*

The expression of autophagy-related proteins were also measured *in vitro* (**Figure 6**). Compared with the control group, the expression of MAP1LC3B/MAP1LC3A, Beclin-1, and PI3KC3 was significantly decreased in other groups ($p < 0.05$). The expression of caspase-1 was significantly increased after TNF-α stimulated, compared with control group ($p < 0.05$). LY294002 administration further decreased the expression levels of these proteins. Meanwhile, treatment with LXA4 significantly increased the expression of autophagy-related proteins and weakened the effect of LY294002 ($p < 0.05$).

LXA4 Enhanced NSE and p-JNK1 Expression in TNF-α-Induced Neuronal Cells *in vitro*

The expression of NSE and p-JNK1 in neuronal cells was measured by immunohistochemistry (**Figure 7**). NSE expression was notably decreased in TNF-α-induced spinal neurons when compared with control cells ($p < 0.05$) (**Figure 7A**). Following LXA4 treatment, NSE expression showed a significant increase ($p < 0.05$). When added alone, LY294002 elicited the opposite effect. Interestingly, however, in co-treated neuronal cells, LXA4 administration weakened the effect of LY294002. Additionally, the levels of p-JNK1 in spinal neuronal cells were highest in the control group and lowest in the LY294002 treatment group ($p < 0.05$) (**Figure 7B**). LXA4 administration increased the expression of p-JNK1 and suppressed the effect of LY294002 in TNF-induced cells. These data further indicated that LXA4 might improve spinal neuronal injuries through promoting p-JNK1 expression.

DISCUSSION

In this study, we found that intrathecal LXA4 injection could greatly improve the PWT in rats with non-compressive disc herniation. We also showed that LXA4 treatment effectively modulated NLRP3 inflammasome and autophagy-related protein levels via the JNK1 pathway both *in vivo* and *in vitro*.

Studies have shown that LXA4 inhibits the expression of proinflammatory factors, while increasing that of anti-inflammatory mediators (Luo et al., 2013). In addition, injection of exogenous LXA4 has been reported to help block TNF

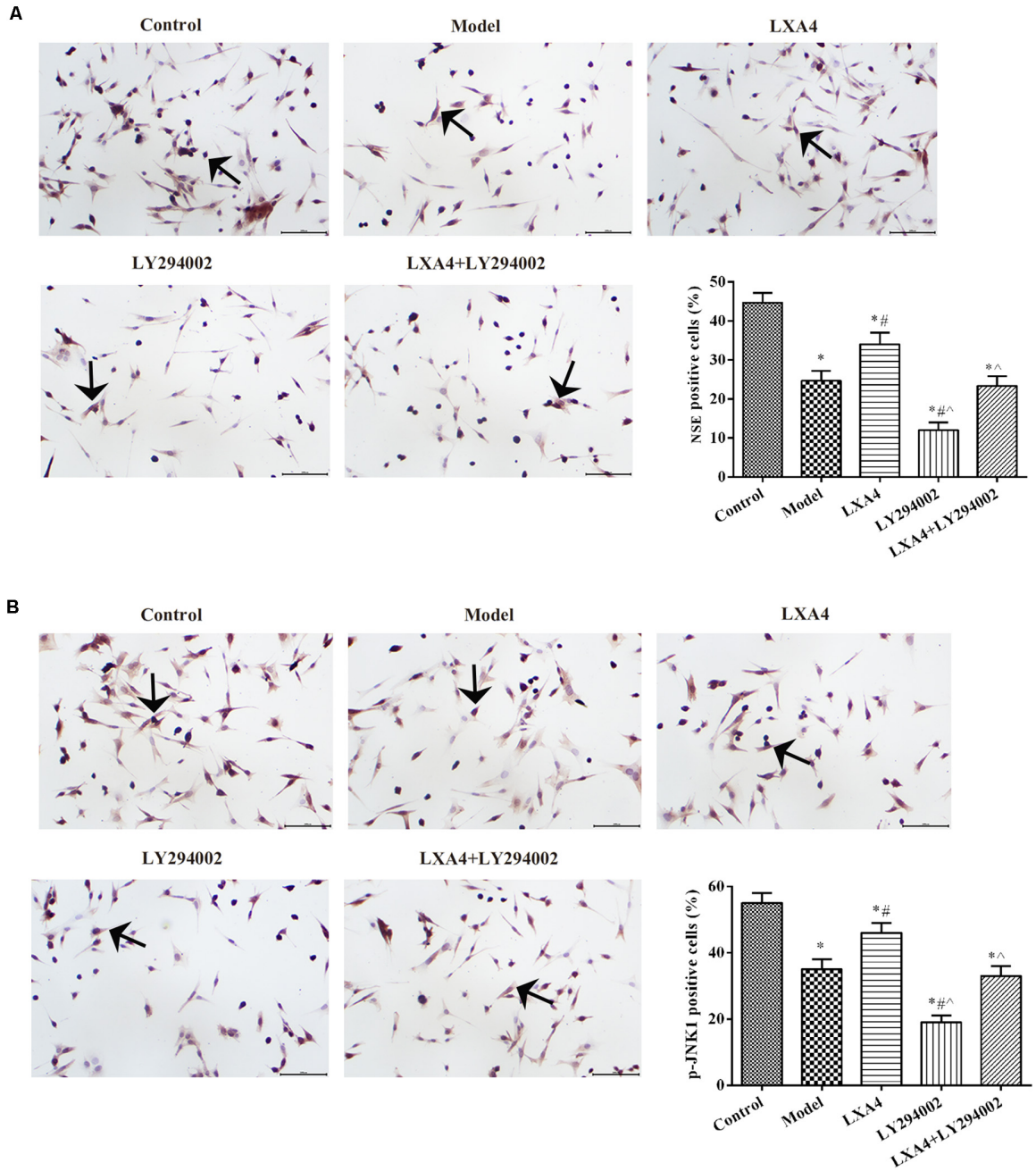

FIGURE 7 | The expression of NSE **(A)** and p-JNK1 **(B)** in spinal neuron cells by immunohistochemistry. Magnification: × 400. Arrows indicate positive expression. $^{*}p < 0.05$ compared with the control group; $^{\#}p < 0.05$ compared with the model group; $^{\wedge}p < 0.05$ compared with the LXA4 group. Experiments were repeated three times for each group. Data are presented as means ± SD. One-way analysis of variance (ANOVA) was used for comparisons among groups followed by Dunnett's *t*-test.

secretion, as well as significantly lower rat sensitivity to pain (Ariel et al., 2003). In this study, the data also showed that LXA4 can alleviate hyperalgesia in the spinal dorsal horn of rats. LXA4 treatment reduced the expression level of the inflammatory cytokines TNF, IL-1β, and IL-18. TNF is a proinflammatory cytokine known to increase the level of hyperalgesia, while IL-1β plays an important role in inducing neuropathic pain (Pociot et al., 1992). TGF-β is an important anti-inflammatory mediator (Shi and Massagué, 2003). In our study, the data were consistent with those of previous studies, and further confirmed the role and functions of LXA4 in regulating disc herniation through the downregulation of proinflammatory mediators and upregulation of anti-inflammatory factors.

In the current study, we also investigated the role of NLRP3 inflammasome activation in the dorsal horn of the spinal cord. Caspase-1 is the effector of the NLRP3 inflammasome. Formerly known as IL-1-converting enzyme, caspase-1 mediates IL-1β maturation and is critical for the regulation of the dorsal horn of the spinal cord (Doyle et al., 2019). In this study, we found that, compared with the model group, the expression of caspase-1 was decreased following LXA4 treatment, both *in vivo* and *in vitro*. LXA4 treatment was recently reported to reduce NLRP3 inflammasome-mediated IL-1β and IL-18 production in osteoclast-mediated diabetic osteoporosis (An et al., 2019). Abnormal activation of caspase-1 is associated with the aberrant activation of inflammasomes caused by ligands and IL-1β release. Here, we observed NLRP3 immune-reactivity in neurons of the spinal cord dorsal horn after the induction of non-compressive disc herniation in rats. Furthermore, the addition of the PI3K inhibitor supported that LXA4 regulates the NLRP3 inflammasome and JNK1 pathway, both *in vivo* and *in vitro*.

To further elucidate how LXA4 signaling affects radicular pain in rats, we assessed the changes in JNK phosphorylation status and expression levels of autophagy-related proteins. The expression of autophagy-related genes was shown to be markedly altered after LXA4 administration, and involved the conversion of cytosolic LC3 I to LC3 II and reduction of beclin-1 (Jia et al., 2015). In 2012, Manassero and colleagues reported that p-JNK1/JNK1 are significant regulators of tissue- and nerve injury-induced pain (Manassero et al., 2012). More recently, several studies have shown that autophagy is required for the precise regulation of the inflammatory response through suppressing the overstimulation of inflammatory responses, thereby avoiding damage to the body. A close relationship exists between autophagy and inflammasome activation (An et al., 2019; Doyle et al., 2019). In early autophagy, the PI3KC3 complex phosphorylates phosphatidylinositol to generate PI3P. In this complex, beclin-1, as a platform molecule, binds to PI3KC3, which can mediate the localization of autophagy-related proteins to phagocytic vesicles, thereby promoting the autophagic process (Fimia et al., 2007). Normally, Bcl-2 and beclin-1 interact and inhibit autophagy, and phosphorylation of Bcl-2 in autophagy has been proposed to be primarily mediated by JNK1 activation (Wei et al., 2008). Here, we found that intrathecal drug delivery of LXA4 promoted the expression of the autophagy-related proteins MAP1LC3B/MAP1LC3A, beclin-1, and PI3KC3. Our results further confirmed that LXA4 treatment increased the levels of p-JNK1/JNK1 and alleviated root pain in rats with non-compressive disc herniation. These results suggested that LXA4 may inhibit NLRP3 inflammasome activity *via* the JNK1/beclin1/PI3KC3 axis. Overall, our results support those of previous studies on the functions of LXA4 in non-compressive disc herniation.

Although the above results suggested that LXA4 clearly improved radicular pain in rats with non-compressive disc herniation via the JNK1/beclin1/PI3KC3 axis, this study has many limitations. The PI3KC3 may be the upstream of the JNK signaling pathway, and the relationship between JNK and PI3KC3 should be further identified. The autophagy-related protein levels are associated with the JNK1 pathway, and further mechanisms need to be researched.

In summary, delivery of exogenous LXA4 significantly alleviated radicular pain in rats with non-compressive disc herniation. LXA4 may exert its effects through reducing NLRP3 inflammasome activation, promoting the production of anti-inflammatory factors, and increasing the activity of JNK1, beclin-1, and PI3KC3.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal experiments were carried following the NIH guidelines (NIH Pub. No. 85–23, revised 1996), and have been reviewed and approved by the Animal Protection and Use Committee of Shandong University.

AUTHOR CONTRIBUTIONS

JJ, YX, and CS designed the study and drafted the manuscript. YX, TS, and JM performed the experiments. YW, LQ, and KL performed the statistical analysis. All authors read and approved the final manuscript.

FUNDING

This work was supported by National Natural Science Foundation of China (Nos. 81972145 and 81772443), Natural Science Foundation of Shandong Province (Nos. ZR2019PH119 and ZR2019MH124), and Key R&D project of Shandong Province (No. 2016GSF201071).

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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.

frontiers in Pharmacology

REVIEW
published: 29 September 2020
doi: 10.3389/fphar.2020.01221

Molecular Modeling Applied to the Discovery of New Lead Compounds for P2 Receptors Based on Natural Sources

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P2 receptors are a family of transmembrane receptors activated by nucleotides and nucleosides. Two classes have been described in mammals, P2X and P2Y, which are implicated in various diseases. Currently, only P2Y12 has medicines approved for clinical use as antiplatelet agents and natural products have emerged as a source of new drugs with action on P2 receptors due to the diversity of chemical structures. In drug discovery, *in silico* virtual screening (VS) techniques have become popular because they have numerous advantages, which include the evaluation of thousands of molecules against a target, usually proteins, faster and cheaper than classical high throughput screening (HTS). The number of studies using VS techniques has been growing in recent years and has led to the discovery of new molecules of natural origin with action on different P2X and P2Y receptors. Using different algorithms it is possible to obtain information on absorption, distribution, metabolism, toxicity, as well as predictions on biological activity and the lead-likeness of the selected hits. Selected biomolecules may then be tested by molecular dynamics and, if necessary, rationally designed or modified to improve their interaction for the target. The algorithms of these *in silico* tools are being improved to permit the precision development of new drugs and, in the future, this process will take the front of drug development against some central nervous system (CNS) disorders. Therefore, this review discusses the methodologies of *in silico* tools concerning P2 receptors, as well as future perspectives and discoveries, such as the employment of artificial intelligence in drug discovery.

Keywords: natural products, P2 receptors, virtual screening, molecular dynamics, homology modeling, drug discovery, molecular modelling

OPEN ACCESS

Edited by:
Adriana Gibara Guimarães, Federal University of Sergipe, Brazil

Reviewed by:
Lucka Bibic, University of East Anglia, United Kingdom
Rajeev K. Singla, Sichuan University, China

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *17 February 2020*
Accepted: *27 July 2020*
Published: *29 September 2020*

Citation:
Alberto AVP, da Silva Ferreira NC, Soares RF and Alves LA (2020) Molecular Modeling Applied to the Discovery of New Lead Compounds for P2 Receptors Based on Natural Sources. Front. Pharmacol. 11:01221. doi: 10.3389/fphar.2020.01221

INTRODUCTION

Plants have been used as medicine for over 60,000 years and form the basis of traditional medicines worldwide, including Chinese Medicine, Korean Medicine, Kampo (Japan), Ayurveda and Unani (India) (Yuan et al., 2016). Currently, about 20,000 medicinal plants are used in 91 countries worldwide, including Brazil, China, France, Germany, and the United Kingdom (Sasidharan et al., 2011).

Natural products have been explored in drug development since the beginning of the 19th century. The first isolated compound from natural products was morphine, isolated from the opium plant by Friedrich Sertürner in 1805 and commercialized by Merck in 1826 (Ji et al., 2009; Yuan et al., 2016). Currently, several synthetic compounds whose original structures are based on natural products are used in the treatment of numerous diseases, including hypercholesterolemia (e.g. simvastatin and lovastatin), hypertension (e.g. captopril and enalapril), cancer (e.g. taxol and docetaxel), and infection (e.g. penicillin and amphotericin B) (Calixto, 2019). Furthermore, approximately 35% of global medicines directly or indirectly originate from natural products, including plants, animals, and microorganisms. In the field of cancer and infectious diseases, up to 60 to 75% of drugs originate from natural products, respectively (Gullo et al., 2006; Calixto, 2019).

Newman and Cragg (2016) conducted a search of the FDA database to investigate the amount of new chemical entities (NCEs) based on natural products that emerged between 1981 and 2014. Among 1,562 NCEs, 16% have a biological origin, 4% were unaltered natural products, 1% comprised botanical drugs, 21% suffered semisynthetic modification, and 4% were synthetic drugs with a pharmacophore similar to that of a natural product. These drugs display wide applications in therapy, including in the treatment of neurodegenerative, cardiac, metabolic, infectious, and inflammatory diseases (Newman and Cragg, 2016). In addition, in 2007 at least 91 plant-derived molecules were used in clinical trials worldwide for the treatment of several diseases (Saklani and Kutty, 2008).

The use of natural products in the process of drug discovery has immeasurable value. First, natural products display a great diversity of chemical structures, acquired over thousands of years as a result of a co-evolution within communities. Second, many of these structures have not yet been reported and may constitute a model for the synthesis of novel drugs, which could be modified by chemists to improve characteristics including efficacy, solubility, and stability in the human body (Ji et al., 2009; Calixto, 2019). These modifications and many other features are included in a field of cheminformatics, discussed later in this paper.

The process of drug discovery using natural products exhibits some obstacles. One is the need to perform various processes until the determination of the active molecule since test samples often consist of extracts or fractions (Siddiqui et al., 2014; Chen et al., 2017). Several studies have ended before they were able to conduct active molecule purification, possibly due to the high complexity of such mixtures. The therapeutic activity found in extracts may be in some cases due to the synergistic and simultaneous action of several molecules (Shen, 2015; Thomford et al., 2018). Isolated molecules are often not available in sufficient quantities for use during high throughput screening campaigns (Siddiqui et al., 2014). Lack of selectivity might also limit research, because the different molecules that are present in extracts can bind to several cellular targets (Shen, 2015). Finally, legal regulations may impact natural product research, i.e. the certification required to use biodiversity information in research (Siddiqui et al., 2014).

The evolution of bioinformatics and cheminformatics in conjunction with analytical technologies has revolutionized the field of natural product research by enabling the rapid detection of hits through virtual screening (VS) and facilitating the isolation and structural elucidation of active molecules (Shen, 2015). Virtual databases can be tested using the molecular docking technique, allowing for the selective analysis of test molecule and target pharmacological interactions, saving time and the expense of reagents and lab consumables (Fischer et al., 2014). Several virtual libraries in which thousands of molecules of synthetic and natural origin are already registered and available. Some databases even offer plant-derived molecules that are used in Traditional Chinese or African Medicine, such as the Traditional Chinese Medicine Integrated Database (TCMID) and the African Medicinal Plants Database (AfroDb) (Chen et al., 2017). These techniques and approaches to virtual databases will be discussed in further detail in this study.

PURINERGIC RECEPTORS AS TARGETS FOR DRUG DEVELOPMENT

Extracellular nucleotides activate plasma membrane receptors in mammalian species, termed P2 purinergic receptors. P2 receptors are categorized into two classes: P2Y, which comprises G protein-coupled receptors; and P2X, which consist of ionotropic receptors (Burnstock and Kennedy, 1985). The P2Y class contains eight members described in humans: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14 (Abbracchio et al., 2006). P2X includes seven members (P2X1-P2X7) (Fountain, 2013). As ionotropic receptors, P2X members open an ion channel permissive to cations when activated. The P2X5 receptor is the only exception since it is more permeable to anions than cations. The P2X7 receptor possesses the unique characteristic of membrane pore formation, which is activated at high ATP concentrations (above 100 μM). This pore can transport molecules of up to 900 Da to the intra or extracellular medium, according to an electrochemical gradient. These molecules include the fluorodyes propidium iodide, lucifer yellow, ethidium bromide, and YO-PRO-1 (Coutinho-Silva et al., 1996; Coutinho-Silva and Persechini, 1997; Persechini et al., 1998; Alves et al., 2014; Pacheco et al., 2016).

P2 receptors are broadly expressed in humans, including in the immune, respiratory, cardiovascular, and central nervous systems, as well as gastrointestinal and urinary tracts (Burnstock and Knight, 2004). The P2 receptors in these tissues have important physiological functions. In airways, for example, P2 receptors promote surface lubrification, mucus hydration and secretion, and ciliary beat (Jacobson and Boeynaems, 2010). The ATP released to the extracellular medium alerts the immune system to danger and generates a chemotaxis gradient for immune cells to the injury site. Activation of these receptors results in cytokine release, reactive oxygen species (ROS) formation, phagocytosis, and antigen presentation, which may contribute to chronic inflammation (Rayah et al., 2012). The

upregulation of P2 receptors in neurons and glial cells has been associated with pain development (da Silva Ferreira et al., 2019). The P2 receptors expressed on platelets are also associated with platelet aggregation and are important targets for anti-thrombotic drugs in recent decades (Zhang et al., 2017; da Silva Ferreira et al., 2019).

In terms of structure, P2Y presents seven transmembrane domains, a carboxyl terminus into the intracellular milieu and an amino terminus facing the extracellular compartment (Zhang et al., 2014a; Zhang et al., 2014b; Zhang et al., 2015). P2X receptors are structurally much more simple, presenting a monomer with two transmembrane domains, an extracellular loop, and both amino and carboxy termini facing the intracellular compartment (Hattori and Gouaux, 2012; Habermacher et al., 2016; Kasuya et al., 2017). The functional protein works as a trimer that can be formed by equal subtypes (homotrimers) or different subtypes (heterotrimers). Only a few functional heterotrimers have been described in the literature, namely P2X1/2, P2X1/5, P2X2/3, P2X2/5, P2X2/6, P2X4/6, and P2X4/7 (Slater et al., 2000; Saul et al., 2013). It is important to note that P2X6 is the only subtype that does not form functional homotrimers (Collo et al., 1996; King et al., 2000; Jones et al., 2004).

The studies discussed above were reconfirmed in recent years by the crystallization of some P2 receptors. The first one resolved by x-ray crystallography was the zebrafish P2X4 (zfP2X4) in 2009 (Kawate et al., 2009). Next, other P2 receptors were resolved both by x-ray crystallography and cryo-electron microscopy, including the human P2Y12 (hP2Y12) (Kiselev et al., 2014), human P2Y1 (hP2Y1), human P2X3 (hP2X3), chicken P2X7 (ckP2X7), giant panda P2X7 (gpP2X7), and rat P2X7 (rP2X7) (Hattori and Gouaux, 2012; Zhang et al., 2014a; Zhang et al., 2014b; Zhang et al., 2015; Habermacher et al., 2016; Karasawa and Kawate, 2016; Mansoor et al., 2016; Kasuya et al., 2017; McCarthy et al., 2019).

In recent decades there have been important breakthroughs in research on purinergic receptors, resolved by the elucidation of the structure of zfP2X4 (**Figure 1**) (Kawate et al., 2009). This allowed for the clarification of the agonist pocket, the protein organization in the trimeric assembly, and the folding of its subunits since crystallization was performed in both open and closed states (Kawate et al., 2009; Kawate et al., 2011). Moreover, it was possible to postulate that the passage of ions could occur through an adjacent region close to the membrane (**Figure 1**), i.e., fenestrations, and not by the central receptor pathway (Habermacher et al., 2016). The zfP2X4 structure was compared to a dolphin and some papers mention the left flipper, right flipper, tail, body, and head when indicating the studied portion of the protein (Kawate et al., 2009; Kawate et al., 2011). These findings enabled research into new drugs to treat P2X-related diseases such as chronic inflammation and pain (Cockayne et al., 2005; Honore et al., 2006; Donnelly-Roberts and Jarvis, 2007).

The resolution of the P2Y12 receptor by x-ray crystallography facilitated new insights into its structure, including the presence of two binding pockets, one for nucleotide ligands and another for non-nucleotide ligands (**Figure 2**) (Zhang et al., 2014a; Zhang et al., 2014b). Although the binding mode is different, other studies have had similar findings for the P2Y1 receptor (Zhang et al., 2015). Currently, four P2Y12 drugs are being used in clinical therapy for thrombosis prevention: clopidogrel (Plavix®), prasugrel (Effient®), ticagrelor (Brilinta®), and cangrelor (Kengreal®) (Savi et al., 2006; Cattaneo, 2007; Deflorian and Jacobson, 2011; Jacobson et al., 2011; Paoletta et al., 2015). These data are crucial in the context of antithrombotic drugs facilitating the exploration of new targeted therapies based on the ligand pocket.

Recent advances in research on the P2 receptor structure are particularly significant for bioinformatics, a field of science that is growing exponentially (Hillisch et al., 2015; Ferreira and Andricopulo, 2018), and 3D resolved structures, alongside advances in the development of algorithms have allowed for more accurate predictions.

3D STRUCTURES AND MOLECULAR MODELING TECHNIQUES APPLIED TO DRUG DISCOVERY

In recent years, computer programs and algorithms have become more efficient at processing complex data. Artificial intelligence (AI) regularly outperforms humans, for example, an AI recently beat the best player at Go, a Chinese game considered more difficult than chess (Silver et al., 2018). Today, many algorithms function in the Windows operating system, although it is more common to operate in linux kernel base of several open source system, an open-source operating system.

Programs are often used to simulate the steps in classical approaches to high throughput screening (HTS) assays, by downloading molecule databases such as plant metabolites and secondary natural products and adding them to a list that enables them to conduct a virtual screening. This is followed by assessments of toxicity, absorption, solubility, lead-likeness, and other clinical parameters, displayed in **Figure 3**. The entire flow is low cost and faster than the HTS used by the industry to discover new drugs and classic HTS is starting to be exchanged for VS in the search for new drugs (Ekins et al., 2007; Biggin and Bond, 2008; Morris and Lim-Wilby, 2008). In the future, molecular modeling will take the place of HTS in research groups and the pharmaceutical industry. The next sections detail each form of molecules and other ligand investigations concerning specific proteins.

COMPARATIVE PROTEIN STRUCTURE MODELING

In protein structure determination, the cloning, expression, and purification steps often exhibit problems that slow progress. Similarly, crystallization methods also display methodological

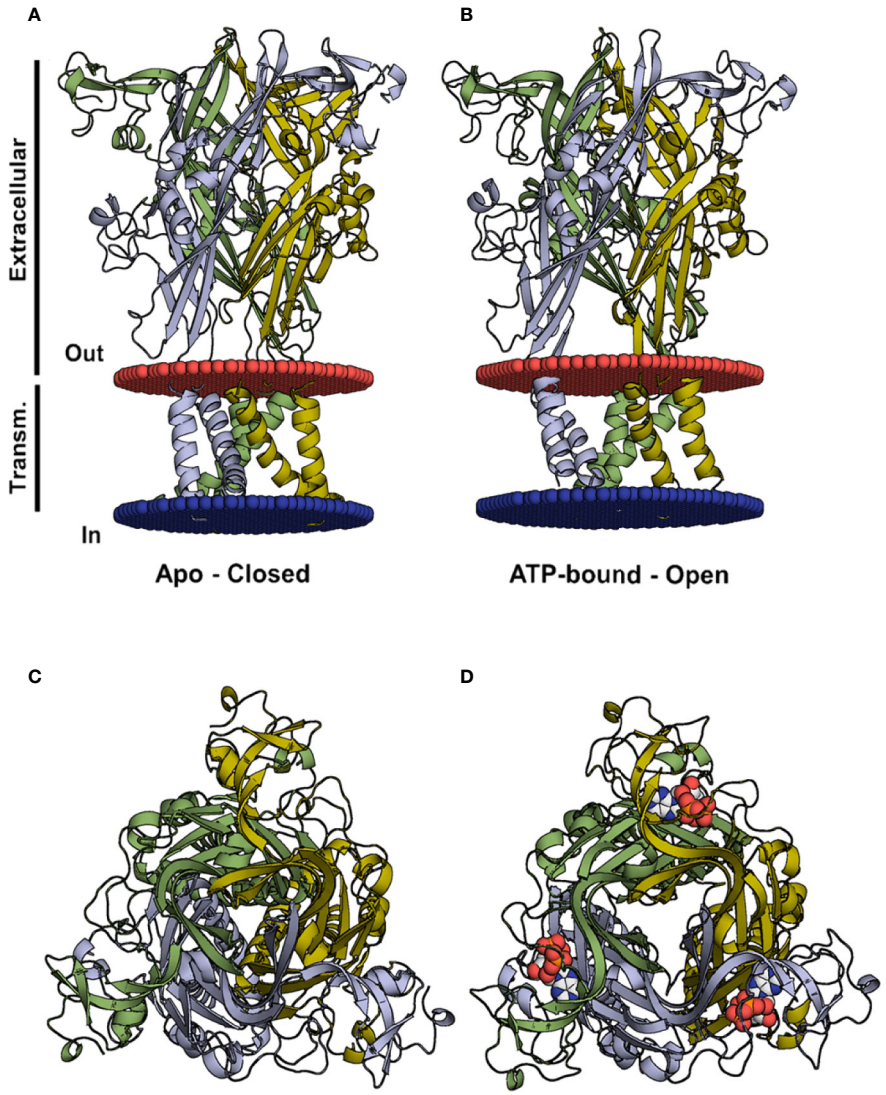

FIGURE 1 | Three-dimensional structures of the closed and open (ATP-bound) P2X4 states. **(A, B)** Crystal structure of the zfP2X4 (PDB code 4DW0) in different views, apo and open, with the transmembrane helices colored according to the subunit colors in blue, yellow, and green. ATP molecules are displayed as a van der Waals representation. **(C)** The same structure as in **(A)**, viewed from the top along the axis of the central channel. **(D)** The same structure as in **(B)**, seen from the top along the axis of the central channel, with ATP bound to the pharmacophore site.

and technical difficulties that can delay or hinder the obtaining of a crystal. In this context, predictive methods such as homology modeling, also known as comparative modeling, save time and reduce costs.

Comparative modeling is a technique that generates a 3D model of a protein from an amino acid sequence (target sequence) using one or more related, known structures (templates). Since this method is based on similarities in the amino acid sequence from two proteins that belong to the same family, both are expected to show some degree of similarity in 3D structure (Mosimann et al., 1995). The protein structure of the same family is more highly conserved than their amino acid sequences (Forrest et al., 2006).

The accuracy, applicability, and success of comparative modeling depends on structural divergence during the evolutionary time between template and target and also on the extent of sequence similarity. Usually, the sequence identity requires 70% or higher similarity, for it to be considered a reliable prediction. Inaccurate models generally display sequence identities lower than 30% (Forrest et al., 2006).

The prediction of membrane proteins has fewer restrictions in terms of sequence identity, i.e., approximately 30% or higher similarity. This also occurs even if the extracellular domain prediction has low accuracy (Forrest et al., 2006). The inaccuracy of transmembrane domains from the model can be related to problems inherent to the technique and structural

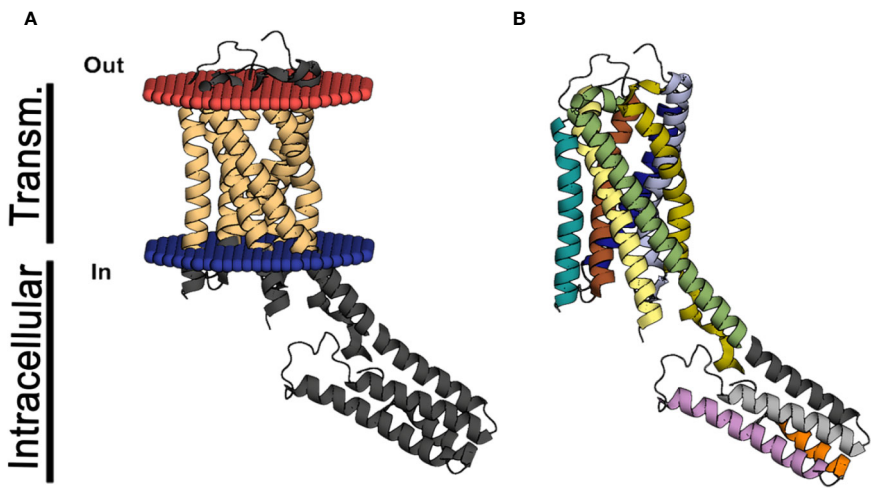

FIGURE 2 | 3D structure of P2Y12. The structure in **(A**, **B)** represents the P2Y12 (PDB code 4PXZ) crystal in the lateral view. **(A)** indicates the helix in light brown represents the transmembrane domain of P2Y12 and **(B)** exhibits the same segments represented in dark blue, light blue, red, yellow, brown, light brown, and green. The intracellular helices are represented in **(A)** in dark gray and in **(B)**, in gray, light gray, orange and purple. The part of the transmembrane domain that extends to the intracellular space is displayed in yellow and green.

deficiencies such as the presence of detergents to solubilize the template structure.

Despite its limitations, the comparative modeling approach comprises some solutions to minimize occurrences that lead to inaccuracies. Many user-friendly servers with automated web interfaces currently provide comparative modeling for non-specialist users, meaning that results can be analyzed with no software installation. Common comparative modeling programs include SWISS-MODEL (pioneered automated server) and ROSETTA (Simons et al., 1999; Webb and Sali, 2016; Waterhouse et al., 2018).

Comparative modeling consists of four steps: a) a comparison between the sequences of the known structure and the homologous sequence to maximize template reliability; b) the alignment of the target sequence with one or more selected templates; c) building 3D models based on these alignments; and d) quality evaluation of structure models to perform physicochemical refinements (Kryshtafovych et al., 2005; Waterhouse et al., 2018).

Notwithstanding the difficulties encountered in transmembrane protein studies, several studies have presented models of predicted structures for P2X receptors. This has aided understanding of critical amino acid residues and important domains not completely verified by experimental assessments. In this sense, P2X receptors can be studied from a mutation standpoint to discover critical amino acid residues. These mutations allow studies on the mobility of ion channels (i.e., opening and closing) as well as analyses regarding ATP and protein interactions (Yan et al., 2006). For example, the substitution of glycine for alanine in the lower body of the P2X4 receptor resulted in a more rigid structure, decreased ATP sensitivity, slower activation, and desensitization (Habermacher et al., 2016). The comparative protein structure modeling method is useful in predicting the 3D structures of P2 receptors that do not have crystallographic data yet. These 3D structures are used in molecular docking assays to discover new ligands for the receptors, and this strategy has been adopted by several research groups. In order to understand the interactions between P2X receptors and drug-like compounds, Dal Ben et al. (2015) studied the interactions of this complex using comparative model structures of human and rat P2X receptors based on a zfP2X4 crystallography structure template. Molecular docking of ATP and P2X agonists were performed in the ATP-binding site (Dal Ben et al., 2015). Chen et al. (2011) constructed a human P2Y12 model based on the β1 adrenergic receptor from *Meleagris gallopavo*. Using this structure, they performed a virtual screening campaign from the ZINC database and found nine potential P2Y12 receptor antagonists (Chen et al., 2011). Rafehi et al. (2017a) developed a P2Y4 structure based on the P2Y1 receptor and selected some anthraquinone derivatives compounds to perform molecular docking. The authors demonstrated that compound 61 (sodium 1-amino-4-[4-(2,4-dimethylphenylthio)-phenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate) presented the lowest IC_{50} for P2Y4, therefore constituting a potential antagonist for this receptor (Rafehi et al., 2017a). **Table 1** summarizes studies that apply the homology strategy.

MOLECULAR DOCKING AND VIRTUAL SCREENING

The binding molecule (ligand) has its rotational or translational space fathomed while the receptor remains rigid, usually to save computational time. This fact has guided several studies, enabling them to produce protein structures through experiments that apply crystallography or comparative modeling. The basis of the

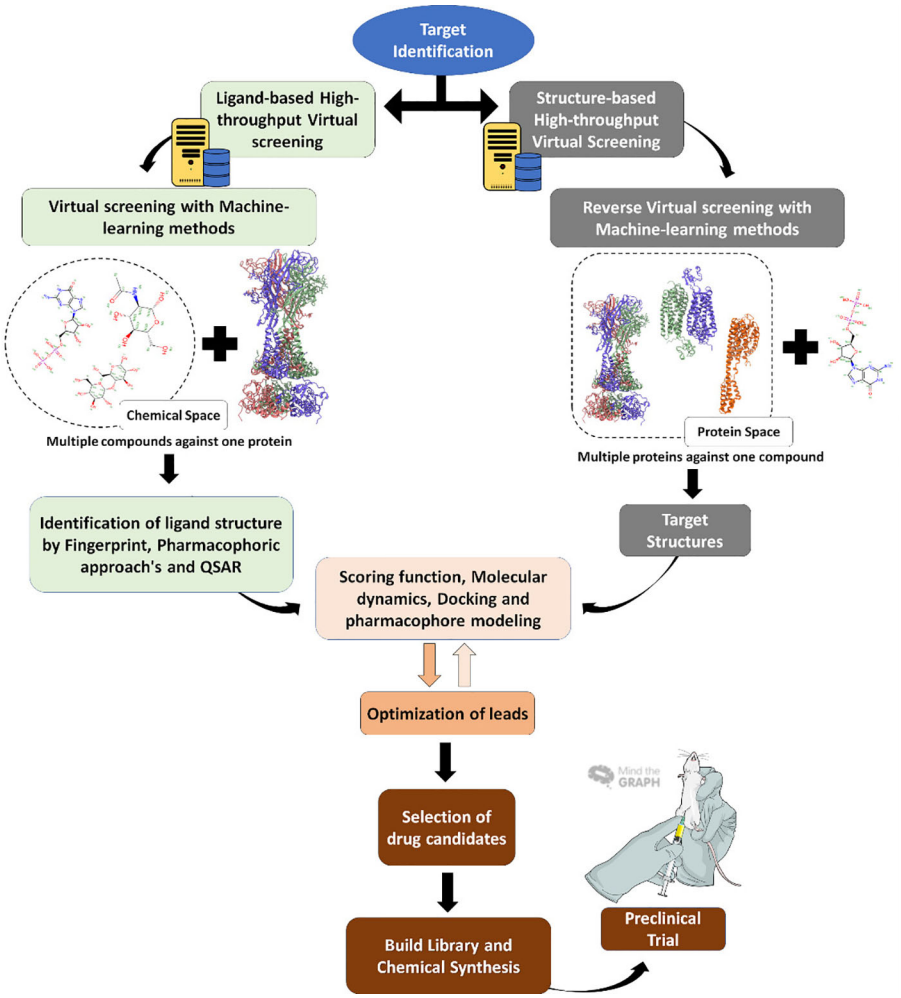

FIGURE 3 | Virtual screening workflow. Virtual screening steps since target identification, usually a protein (right) or the reverse, with the target being the molecule, until the final step of *in vitro* and *in vivo* tests.

molecular docking method is the use of a search algorithm and score function that generates the ligand pose. Currently, it is possible to use various programs with different algorithms to compare the ligands' pose. These programs include AutoDock (Morris et al., 2009), DOCK (Allen et al., 2015), Glide (Friesner et al., 2004), and GOLD (Jones et al., 1997). A comparison of results from different software programs may provide new questions and information concerning the assessed molecule.

Aiming to minimally converge the energy of the ligand, the algorithm evaluates its conformation recursively. A scoring function is applied to estimate the energy related to a specific conformation for a posterior rank (Yan et al., 2006). Docking programs generally sum the electrostatic potential and van der Walls energies to rank conformations.

Molecular docking has proven particularly important to research on the interaction between different molecules and pharmacological targets such as receptors. However, this methodology can be applied to screen large chemical libraries concerning a specific therapeutic target to find new drugs. This broad search, using billions of compounds by the computational approach, is termed virtual screening or *in silico* screening (Spyrakis and Cavasotto, 2015). A structure-based virtual screening can be performed using the molecular docking method, allowing for the evaluation of millions of similar compounds. Despite this, only a small fraction of compounds from the top-ranking conformations can be examined for interaction patterns and prioritized for purchase or synthesis (Spyrakis and Cavasotto, 2015).

One of the benefits of using this approach is the low computational power needed to perform a run and fast data acquisition, i.e., some conformations can be detected and ranked in a few minutes (Chen, 2015). However, the analyzed receptor is inflexible, which can produce inaccurate data, and may not indicate the evaluated molecule as a drug and may instead be a

TABLE 1 | Comparative protein structure modeling methods in the structure prediction of P2 receptors.

P2 receptors	Homologous protein	Reference
Human P2Y1 and P2Y12	Bovine rhodopsin	(Costanzi et al., 2004)
Human P2Y1	Bovine rhodopsin	(Major and Fischer, 2004)
Human P2Y11	Bovine rhodopsin and human P2Y1	(Zylberg et al., 2007)
Human P2Y2	Bovine rhodopsin	(Hillmann et al., 2009)
Human P2Y12	β1 adrenergic receptor from *Meleagris gallopavo*	(Chen et al., 2011)
Rat P2X2 and human P2X5	Zebrafish P2X4	(Kawate et al., 2011)
Human P2Y12	Bovine rhodopsin, human A2A adenosine receptor and human C-X-C chemokine receptor type 4	(Deflorian and Jacobson, 2011)
Rat P2X2	Zebrafish P2X4	(Hausmann et al., 2013)
Human P2Y14	Human P2Y12	(Kiselev et al., 2014)
Human and rat P2X1, P2X2, P2X3, P2X4, P2X5 and P2X7	Zebrafish P2X4	(Dal Ben et al., 2015)
Human P2Y14	Human P2Y12	(Trujillo et al., 2015)
Human P2Y14	Human P2Y12	(Kiselev et al., 2015)
Human P2Y14	Human P2Y12	(Junker et al., 2016)
Human and rat P2X7	Zebrafish P2X4	(Caseley et al., 2016)
Human P2X7	Zebrafish P2X4	(Pippel et al., 2017)
Human P2Y2	Human P2Y1	(Rafehi et al., 2017a)
Human P2Y2	Human P2Y1	(Rafehi et al., 2017b)
Mouse P2X7	Giant panda P2X7	(Pasqualetto et al., 2018)
Rat P2X4	Zebrafish P2X4	(Pasqualetto et al., 2018)
Human P2Y6	Human P2Y1 and P2Y12	(Jacob et al., 2018)
Human P2X4	Zebrafish P2X4	(Dhuna et al., 2019)
Human P2X7	Zebrafish P2X4	(Bidula et al., 2019)
Human P2Y14	Human P2Y12	(Wang et al., 2020)
Human, mouse, rat and zebrafish P2X4	Zebrafish P2X4	(Reyes-Espinosa et al., 2020)

candidate that requires re-evaluation through other experimental methodologies (Chen, 2015).

Using the 3D structures of P2X receptors, the molecular docking approach has been applied to search for the best drug candidates for clinical trials, which could be applied in the treatment of several diseases, including cancer, rheumatoid arthritis and endocrine conditions (Dal Ben et al., 2015). ATP and other ligands are described in research involving P2X receptors, implementing protocols that include ATP stabilization and reduction of its degradation by ectonucleotidases (Adelman, 1976; Evans et al., 1995). Nucleotide-derived molecules, suramin-like analogs, and irreversible antagonists have been used in molecular docking approaches, aiding in the prediction of a druggable ligand in drug research (Dal Ben et al., 2015).

Research on P2 receptors through virtual screening has provided interesting information about the structure and molecular interactions of these receptors. The molecular docking approach is also applied in the testing of ligands from P2 receptors in order to evaluate selectivity and affinity, revealing novel potential drugs. Recently, molecular modeling and mutagenesis have advanced the search for novel P2Y ligands (Jacobson et al., 2012). Costanzi et al. (2012) selected 110 hits among 250,000 compounds tested for the P2Y1 receptor. As they describe, these molecules appear to be present an antagonist behavior even in a low molar range but require optimization to improve physicochemical characteristics (Costanzi et al., 2012). Nofianti and Ekowati (2019) performed a screening campaign of 22 o-hydroxycinnamic derivatives aiming to discover novel antiplatelet candidates. These compounds demonstrated the ability to inhibit both P2Y12 and COX-1 receptors and presented pharmacokinetic characteristics that allow oral administration (Nofianti and Ekowati, 2019). Recently, Wang et al. (2020) performed a virtual screening, which intended to discover novel P2Y14 antagonists. They selected a total of 19 compounds with different structures to conduct *in vitro* tests and found that 10 molecules presented an IC_{50} lower than 50 nM. They even found that compound 8 inhibited caspase-1 activation and IL-1β release (Wang et al., 2020).

Reyes-Espinosa et al. (2020) conducted a screening campaign to evaluate the potential positive allosteric modulator (PAM) activity on P2X4 of 1,657 drugs approved by the Food and Drug Administration (FDA). They evaluated the activity of these drugs in four different species (human, mouse, rat, and zebrafish) and identified nine molecules with PAM activity and eight as potential negative allosteric modulators (NAM) (Reyes-Espinosa et al., 2020). Caseley et al. (2016) have also described three hP2X7 antagonists with micromolar potency ($IC_{50} < 6\ \mu M$) in a screening of over 100,000 compounds concerning the hP2X7 ATP-binding site. These compounds significantly inhibited calcium mobilization, dye uptake, and cell death induced by P2X7 activation, demonstrating that computational analyses can corroborate experimental data (Caseley et al., 2016).

The molecular docking technique has also been used to assess the antagonistic or modulating activity of molecules from natural products, although relatively few studies have to date been carried out. Yi et al. (2017) performed an *in silico* docking analysis from compounds deposited in the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP), which contains structure information from herbs and natural ingredients used to discover novel antithrombotic drugs from medicinal plants. After the exclusion of compounds that were not in accordance with Lipinski's rule of five, the authors evaluated 1,656 compounds from 443 herbs. They focused on compounds from three herbs: cimicifugae (*Cimicifuga foetida* L.), ganoderma (*Ganoderma lucidum* Karst), and licorice (*Glycyrrhiza uralensis* Fisch), as some studies have suggested that they demonstrate anti-thrombosis activity (Yi et al., 2017). Liu et al. (2018) performed a similar screening campaign to find novel ligands for P2Y1 and P2Y12 receptors that could be used as antithrombotic drugs. They evaluated 253 compounds from Traditional Chinese Medicines and tested 11 hits through *in vitro* assays, including salvianolic acids from *Salvia militorrhiza* (Liu et al., 2018). **Table 2** demonstrates some studies that applied virtual screening, using the molecular docking strategy to discover novel ligands for P2 receptors.

TABLE 2 | Virtual screening campaigns targeting P2 receptors by applying the molecular docking strategy.

P2 receptor	Software	Sample	Best hits	References
Human P2Y12	DOCK 6.0	ZINC database	Compounds 1(a-c)[1], 2(a-c)[2] and 3(a-c)[3]	(Chen et al., 2011)
Human P2Y1	MOE	Compounds from Life Chemicals catalog	Compound 2a	(Costanzi et al., 2012)
Human and rat P2X7	eHiTS version 12	ZINC12 database	Compounds C23, C40 and C60	(Caseley et al., 2016)
Human P2Y1	Discovery Studio Client V4.5	Herbs and compounds from TCM	Compounds from herbs *G. uralensis* Fisch, *C. foetida* L., and *G. lucidum* Karst	(Yi et al., 2017)
Human P2Y4	Glide	Anthraquinone derivatives	Compounds 61 (PSB-16133) and 64 (PSB-1699)	(Rafehi et al., 2017a)
Human P2Y1 and P2Y12	Glide XP	TCM compounds with antiplatelet aggregation activity	Salvianolic acids A, B and C from *Salvia militorrhiza*	(Liu et al., 2018)
Human P2Y12	Molegro Virtual Docker	o-hydroxycinnamic acid derivatives	o-hydroxycinnamic acid derivatives (OCA1a–22a)	(Nofianti and Ekowati, 2019)
Human P2Y14	Glide	ChemDiv database	Compound 8	(Wang et al., 2020)
Human, mouse, rat and zebrafish P2X4	Autodock 4.2 and Autodock Vina	FDA-approved drugs deposited on ZINC15 database	Compounds A(1-13)[4], B(1-8)[5], and C(1-9)[6]	(Reyes-Espinosa et al., 2020)

[1]Classified as lead-like compounds, [2]classified as fragment-like compounds, [3]classified as drug-like, [4]classified as allosteric modulators, [5]classified as negative allosteric modulators, and [6]classified as positive allosteric modulators.

Molecular docking has also been applied to evaluate the effect of two diterpenoids (tanshinone II-A and cryptotanshinone) from *Salvia milthiorriza* Bunge on human P2Y12. The analyses revealed that they interact with the binding site of this receptor and can inhibit *in vitro* platelet aggregation (Maione et al., 2015). Dhuna et al. (2019) have examined the activity of ginsenosides from *Panax ginseng*, a traditional Chinese medicinal plant, as positive allosteric modulators of the P2X4 receptor. These compounds enhanced Ca^{2+} influx and ATP-induced currents in HEK-hP2X4 cells, and docking data indicates that they bind to the central vestibule region of P2X4 (Dhuna et al., 2019). Bidula et al. (2019) also evaluate the activity of these ginsenosides on P2X7 using the molecular docking strategy, since previous studies demonstrated that these compounds act as positive allosteric modulators for this receptor. Docking data has demonstrated that the ginsenoside binding site is located within the central vestibule of P2X7 and some mutations in the amino acids from this region have resulted in the loss of dye uptake potentiation, calcium mobilization, ATP-induced current responses, and cell death (Bidula et al., 2019).

MOLECULAR DYNAMICS

Structure-based methods rely on a single pose of the target protein. The utilization of a single structure of a target protein is a major limitation for a detailed analysis of a given protein (Ivetac and Andrew McCammon, 2011). However, with advances in computational power, structural flexibility can be added to several methods that were impossible before. The classical molecular dynamics simulation is one of the most applied approaches to analyzing protein and ligand motion in the complex.

Molecular dynamics (MD) use Newton's motion equation to progressively determine the energy states and conformational in the function of a feasible time scale (picoseconds, nanoseconds, or microseconds) (Spyrakis and Cavasotto, 2015). By obtaining information at the molecular level, the addition of temperature and pressure parameters to classical MD has provided new ways of carrying out studies and interpreting experiments (Rapaport and Rapaport, 2004; Durrant and McCammon, 2011).

MD is significantly cheaper in comparison to current computational methods and techniques, which tend to involve more detail, for example, quantum mechanics, molecular mechanics (QM/MM), or quantum chemistry (MD/QC). One explanation for this is that the Schrödinger equation is used in quantum methods. This equation represents the electron-nuclear in relation to static nuclei, while the classical MD uses an average field surrounding the atom nuclei to describe the electrons (Armunanto et al., 2003; Walker et al., 2008; Brunk and Rothlisberger, 2015).

Although computational power has greatly increased over the years, time scales were still a limitation. Some biological events require hundreds of microseconds to manifest, making classical MD unable to follow the event (depending on the system), particularly when simulations occur in a complex environment such as ion transport through a transmembrane protein. This system comprises a significant number of atoms (over 200,000) including the receptor, lipids in a bilayer configuration, neutralizing ions, a possible ligand, and mostly water molecules. In addition, the conformation of the ion channels tends to the closed state, due to their lower energy configuration (Bernardi et al., 2015).

Concerning these limitations of time, some studies have demonstrated interesting structural information, for example, interactions in the ATP binding site of the zfP2X4, which can determine some hydrophobic interactions between the left flipper and the dorsal fin, producing a downward movement of the left flipper and upward motion of the dorsal fin (Zhao et al., 2014). Lateral fenestrations have also been described as a gateway to ion passage through the channel through MD, later confirmed in cysteine accessibility assay experiments (Kawate et al., 2009; Hattori and Gouaux, 2012).

Due to the limitations of time, relatively few studies have applied or implemented classical MD. Coarse-grained simulation methods and enhanced sampling methods, such as metadynamics, simulated annealing, and replica-exchange

molecular dynamics, are also available. Metadynamics can solve time scale problems depending on the analysis proposal. These methods are an alternative for simulating ion channels and study movements that occur in less than microseconds. The cheaper computational costs of coarse-grained methods are a consequence of the reduction of the number of degrees of freedom of the system, as some interactions can be removed to eliminate resources that are otherwise used to represent all atoms of the system. Additionally, an enhanced sampling method can be implemented to separate high and low-energy conformations to cross the high-energy barriers imposed in some biological systems. As an example of this technique, a coarse-grained simulation of an rP2X2 within a lipid bilayer is indicated by the interposition between lipids and alpha helices of the transmembrane region, which is representative of the stabilization function of these molecules in maintaining the open state of the receptor (Grimes and Young, 2015; Caseley et al., 2016).

In the field of drug discovery and development, MD has been used extensively for the refinement and optimization of constructed P2 receptor homology models to build templates for molecular docking assays (Zylberg et al., 2007; Trujillo et al., 2015; Junker et al., 2016; Liu et al., 2018). MD simulations have also been used in the evaluation of structure-function relationships between the binding pocket of the P2 receptor of interest and the candidate hits obtained through docking assays (Zhou et al., 2017).

ARTIFICIAL INTELLIGENCE

Cheminformatics is an area that applies several different computational methodologies to solve problems related to chemical information (Gasteiger, 2016). One of these methods, Artificial intelligence (AI), is believed by several researchers to be a breakthrough, representing a Fourth Industrial Revolution (Xu et al., 2018). The definition of AI is an area of intense debate, as also observed regarding the definition of human intelligence (Dobrev, 2005; Kok et al., 2009). Nevertheless, AI exhibits some striking features that are, in general, attributed to human intelligence, as measured by the well-known Turing test (Kok et al., 2009). These include automated reasoning, knowledge representation, natural language processing, and machine learning (ML). As in other areas, AI is applied with ML algorithms, an operational branch of AI.

The great advantage of ML algorithms is the capacity to rapidly make a decision based on a dataset with real examples. This is due to a large increase in computation processing in the last years, with the new graphics processing unit (GPU) heightening the capacity of parallel processing, and tensor processing unit (TPU), made to function with ML algorithms. These allow for the identification of several new molecules exhibiting activity in human systems, and thus decreasing the cost of new drugs placed on the market (Lavecchia, 2015; Esteva et al., 2019; Rifaioglu et al., 2019). Diverse ML algorithms have been used to discover new drugs (Carpenter et al., 2018), including Support Vector Machines (SVM), Random forest, k-nearest neighbors, Naïve Bayesian, decision trees, and deep neural networks. Most studies have applied SVM and deep neural networks.

SVM was first established to study chemical compounds in 2001 by two different groups, namely Burdidge and collaborators and Czerminski and collaborators, based on theories by Cortes and Vapnik (1995). The principles of SVM and its applications are explained by Maltarollo et al. (2019). It is important to note that SVM can be used to predict interactions between ligand and receptors, using physicochemical features, protein and compound descriptors, irrespective of structural information.

Deep neural networks are a subtype of artificial neural networks inspired by how neurons communicate with each other. Despite the complexity of the human brain, this algorithm is the only one that learns, using backpropagation to detects results "equal" or similar to the training dataset. This type of network is constructed with several neurons in hidden layers, and the weight of the networks can simulate inhibitory and excitatory synapses, thus leading to algorithm "plasticity" (Carpenter et al., 2018).

ML algorithms in drug discovery have been applied for over ten years now, as reviewed by Melville and collaborators (2009). Recently, Stokes et al. (2020) were the first to discover a new drug using the ML technique. Halicin, an inhibitor of c-Jun N-terminal kinase, was able to inhibit the growth of a broad spectrum of bacteria, both *in vitro* and *in vivo*. This drug was discovered from a screening campaign of over 6,000 molecules deposited in a drug repositioning bank, and has a structure that is considerably different from other antibiotics, acting on the dissipation of the potential of transmembrane pH in bacteria (Stokes et al., 2020).

As expected, no papers have been published on P2 receptors and drug discovery, but it is only a matter of time before new research emerges, as it is a promising area of research, and ML algorithms are already optimized and capable of learning. This is exemplified by Google Alphazero learning to play Go, which is considered to be one of the most complex games invented by humans (Silver et al., 2018; Rifaioglu et al., 2019).

Finally, ML algorithms have been applied in the identification of plant salinity stress (Feng et al., 2020), prediction of biological function based on structure (Liu et al., 2019), and regarding biological targets for natural molecules, like celastrol (Rodrigues et al., 2019). These studies revealed that ML can act as an important partner of MD. Rupp et al. (2014), for example, used ML to estimate the potential energy surfaces of natural molecules to speed up MD simulations (Rupp et al., 2014).

CONCLUSIONS

Nature is a potential source for an almost infinite number of molecules. Natural products play an important role in drug discovery, even when we consider the obstacles presented by extracting, purifying, and separating active compounds. The high throughput screening that is usually applied by the pharmaceutical industry costs millions of dollars, and

bioinformatics can test far more molecules in a faster and cheaper manner. In recent decades, this process has been used to search for and test natural products by *in silico* approaches. As a result, several naturally occurring molecules with action on P2 receptors have been discovered, which can be used as anti-inflammatory and antiplatelet agents. Moreover, several algorithms can also predict physicochemical, pharmacokinetic, and toxicity parameters. Therefore, it is expected that the introduction of artificial intelligence will lead to a more accurate selection of molecular hits and that, in the near future, machines will take the place of humans in the discovery of drugs concerning P2 receptors.

AUTHOR CONTRIBUTIONS

AA and LA formulated the manuscript. AA, RS, NF and LA wrote the manuscript.

FUNDING

This work was supported by the Instituto Oswaldo Cruz (Fiocruz), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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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.

frontiers in Pharmacology

ORIGINAL RESEARCH
published: 29 October 2020
doi: 10.3389/fphar.2020.551786

Regulation of Pain Genes—Capsaicin vs Resiniferatoxin: Reassessment of Transcriptomic Data

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Emerging evidence has shown a strong association between neuropathic pain and chronic diseases. In recent years, the treatment of neuropathic pain has attracted more attention. Natural products, such as capsaicin and resiniferatoxin, have been well utilized to treat this disease. In this study, we aim to compare the regulatory effects of capsaicin and resiniferatoxin on pain-related genes as well as on genes with no direct association with pain. Public transcriptomic and microarray data on gene expression in the dorsal root ganglia and genes associated with TRPV1 (+) neurons were obtained from the GEO database and then analyzed. Differentially expressed genes were selected for further functional analysis, including pathway enrichment, protein-protein interaction, and regulatory network analysis. Pain-associated genes were extracted with the reference of two pain gene databases and the effects of these two natural drugs on the pain-associated genes were measured. The results of our research indicate that as compared to capsaicin, resiniferatoxin (RTX) regulates more non pain-associated genes and has a negative impact on beneficial genes (off-targets) which are supposed to alleviate nociception and hypersensitivity by themselves. So, based on this study, we may conclude that capsaicin may be less potent when compared to RTX, but it will elicit considerably less adverse effects too. Thereby confirming that capsaicin could be used for the efficient alleviation of neuropathic pain with possibly fewer side effects.

Keywords: neuromodulation, resiniferatoxin, capsaicin, DEGs (differentially expressed genes), TRPV1, neuropathic pain (NP)

OPEN ACCESS

Edited by:
Heike Wulff,
University of California, Davis, United States

Reviewed by:
Francesca Luisa Conforti, University of Calabria, Italy
Giacinto Bagetta, University of Calabria, Italy

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Specialty section:
This article was submitted to Neuropharmacology, a section of the journal Frontiers in Pharmacology

Received: *14 April 2020*
Accepted: *11 September 2020*
Published: *29 October 2020*

Citation:
Singla RK, Sultana A, Alam MS and Shen B (2020) Regulation of Pain Genes—Capsaicin vs Resiniferatoxin: Reassessment of Transcriptomic Data. Front. Pharmacol. 11:551786. doi: 10.3389/fphar.2020.551786

INTRODUCTION

Neuropathic pain is one of the most critical neurological diseases and disorders. It affects approximately 3.3 to 17.9% of the population (Cohen and Mao, 2014; He et al., 2020). A total of 14.7% of Chinese patients suffering from chronic pain in Hong Kong had neuropathic characteristics (Cheung et al., 2017). For the neuropathic and ischemic pain, neuromodulative treatment is a modern and effective mode of treatment (Sokal et al., 2011). Neuromodulation can be achieved by electrical or chemical methods, it aims to alter communication between nerves which

can lead to an increase in pain threshold by modifying nociception, hypersensitivity, and analgesia (Richards et al., 2012). Traditionally, natural products were used for the treatment of various ailments, and now due to advancements in scientific research fields, natural products are being validated by scientific methods and are being used in the scientific treatment of various diseases and disorders (Scotti et al., 2016; Singla and Dubey, 2019; Singla et al., 2019). Researchers have also validated the role of natural products from terrestrial plants and marine sources, for the treatment of neuropathic pain (Alonso et al., 2003; Quintans et al., 2014).

Capsaicin, a well-known component obtained from various species of the Capsicum genus (chili peppers), is a very potent agent for the treatment of neuropathic pain. Apart for its potential in the treatment of neuropathic pain, capsaicin also acts as an anti-oxidant, anti-obesity, and is cardioprotective (Lu et al., 2020; Qiao et al., 2020). Its neuropathic mechanism is widely thought to be modulated through the TRPV1 channel (Caterina et al., 1997; Bais and Greenberg, 2020). After clinical level validation, the topical formulation of capsaicin is widely used and quite popular, specifically in the form of an 8% topical cutaneous patch (Baron et al., 2017; Anand et al., 2019), 0.075% topical cream (Derry et al., 2009), and 0.075% lotion (Kulkantrakorn et al., 2019).

Resiniferatoxin (RTX), a capsaicin analogue obtained from the cactus-like plant, *Euphorbia resinifera* (Hergenhahn et al., 1984), is 1,000 times more potent than capsaicin for the alleviation of neuropathic pain (Maggi et al., 1990). The mode of neuropathic pain alleviation in the case of RTX is also *via* the TRPV1 channel (Kissin and Szallasi, 2011). Sorreto Therapeutics, along with other organizations, are conducting clinical trials to establish the clinical efficacy of RTX in treating severe pain in cancer and knee osteoarthritis (Heiss et al., 2014; Hockman et al., 2018; Unger et al., 2019). The treatment of sensory neurons with capsaicin or RTX causes calcium cytotoxicity that rapidly sutures and selectively deletes TRPV1(+) neurons (Isensee et al., 2014).

Isensee and co-workers had studied the effect of capsaicin and RTX in the dorsal root ganglion of rats and submitted the gene expression data in the NCBI (GSE59727). In their research, though they had used and collected data on both capsaicin and RTX, in their manuscript, they mainly focused on the characterization of genes associated with TRPV1(+) neurons. In their published manuscript, they had not correlated and compared the gene regulation of capsaicin and RTX in detail. Very little information has been made available for readers on capsaicin. Further, to the best of our knowledge, there has been no detailed comparison on the effect of capsaicin and RTX on pain genes and non pain-associated genes (Isensee et al., 2014). As RTX is proposed to be highly potent when compared to the already established capsaicin, and the evidence collection for its use in cancer and knee osteoarthritis is in process, it is extremely important to look at both of the drugs in terms of proteomics and gene regulations. This will help in the assessment of the possible adverse effects on the long-term usage of the drugs.

So, in our present study, we have reassessed the transcriptomic data submitted by the team of Isensee and analyzed it in the perspective as mentioned.

MATERIALS AND METHODS

Microarray Data

The raw data of GSE59727, including 12 tissues (4 tissues for DMSO, 4 tissues for capsaicin, and 4 tissues for RTX), were obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo/), using the key words "capsaicin" AND "Rattus norvegicus" [porgn::txid10116] AND "neuron" and "tissues" (attribute name). These data were based on the GPL6101 platforms (Illumina ratRef-12 v1.0 expression beadchip) contributed to by Isensee and co-workers (Isensee et al., 2014). Four replicate experiments with RNA from the dorsal root ganglia (DRG) neurons of one rat per experiment were performed. Segregated neurons were split up into three parts, treated with solvent DMSO (0.1%), capsaicin (10 μM), or RTX (100 nM), and were followed by gradient centrifugation for 30 min (Isensee et al., 2014).

Data Preprocessing and Differentially Expressed Gene Identification

The R package "limma V3.40.6" was used to identify DEGs in rat tissues compared with corresponding tissues treated with DMSO (0.1%) and capsaicin (10μM) or DMSO (0.1%) and RTX (100nM). We used log2-transformation to normalized the data, after that we used the normalized data for further analysis. We used the *t*-test and Benjamini–Hochberg methods to calculate the *p*-value and adjusted *the p*-value and logFC accordingly. To get the differentially expressed genes we set the threshold value as; p-value < 0.01 and $|\ \text{logFC}\ | > 0.5$, in both cases.

Enrichment Analysis

ShinyGO is a graphical web application that displays the functional acuteness from a set of genes; which was developed under different R/Bioconductor packages. In this study, we use ShinyGO (http://bioinformatics.sdstate.edu/go/) to perform enrichment analysis (Ge et al., 2020). For the enrichment analysis we set the cutoff p-value = 0.05.

Analysis of the Protein–Protein Interaction (PPI) Network of the DEGs

We used the Search Tool for the Retrieval of Interacting Genes (STRING v11.0) database (http://string-db.org) for constructing the PPI network of DEGs both from capsaicin and RTX-treated conditions. Then, the Cytoscape software (version 3.8.0) with the MCODE and Networkanalyzer app was used to display the PPI networks between the DEGs.

Differentiation Between Pain-Associated Genes and Non-Pain Genes

The DEGs for capsaicin and RTX were then subjected to the Pain Networks database (http://www.painnetworks.org/rat/) for filtering of the pain-related genes with categorization with the Pain Gene Enrichment (PGE) score (Perkins et al., 2013).

Further, DEGs for capsaicin and RTX were then subjected to the pain gene database (http://www.jbldesign.com/jmogil/PainGenedb_content.html) for more precise filtering of pain-related genes as the mentioned database also specifies the default function of the pain-related genes (Lacroix-Fralish et al., 2007).

RESULTS AND DISCUSSION

Identification of Differentially Expressed Genes

We separated the datasets into two parts, one (capsaicin treatment group) is, DMSO (0.1%) and capsaicin (10μM); the other (RTX treatment group) is, DMSO (0.1%) and RTX (100nM). A total of 274 DEGs were identified, including 84 upregulated and 190 downregulated genes in the case of the capsaicin treatment group. But in the case of the RTX treatment group, a total of 444 DEGs were identified, including 171 upregulated and 273 downregulated genes (**Figure 1**). The top 30 upregulated and downregulated DEGs for capsaicin and RTX are displayed in **Tables S1** and **S2**, respectively. Further on the basis of the filters adopted, we found 102 genes which were common in both RTX and capsaicin (**Figure 2**).

Enrichment Analysis

Gene ontology (GO) enrichment analysis showed that DEGs for both RTX and capsaicin-treated conditions were significantly enriched in the biological process (BP), cellular component (CC),

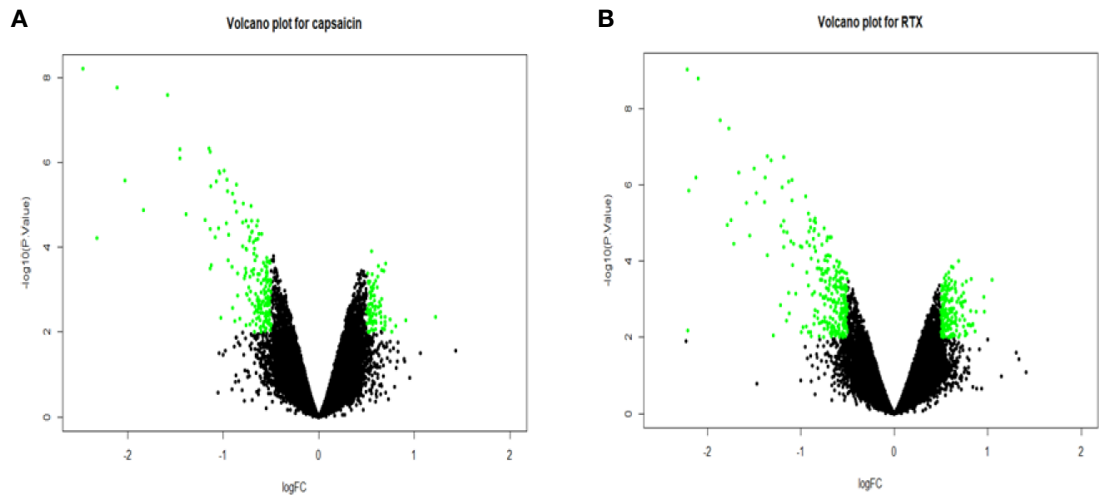

FIGURE 1 | Volcano plot for representing the upregulated and downregulated genes in the capsaicin treatment group **(A)** and the RTX treatment group **(B)**.

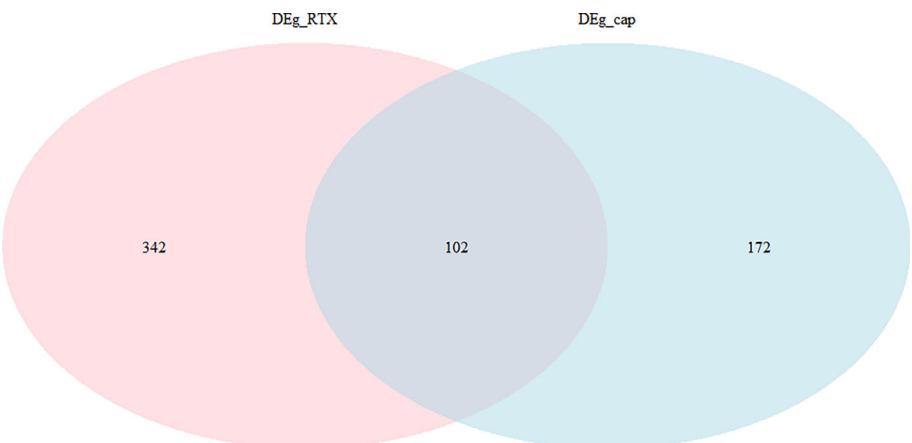

FIGURE 2 | Overlapped genes in the capsaicin treatment group and the RTX treatment group.

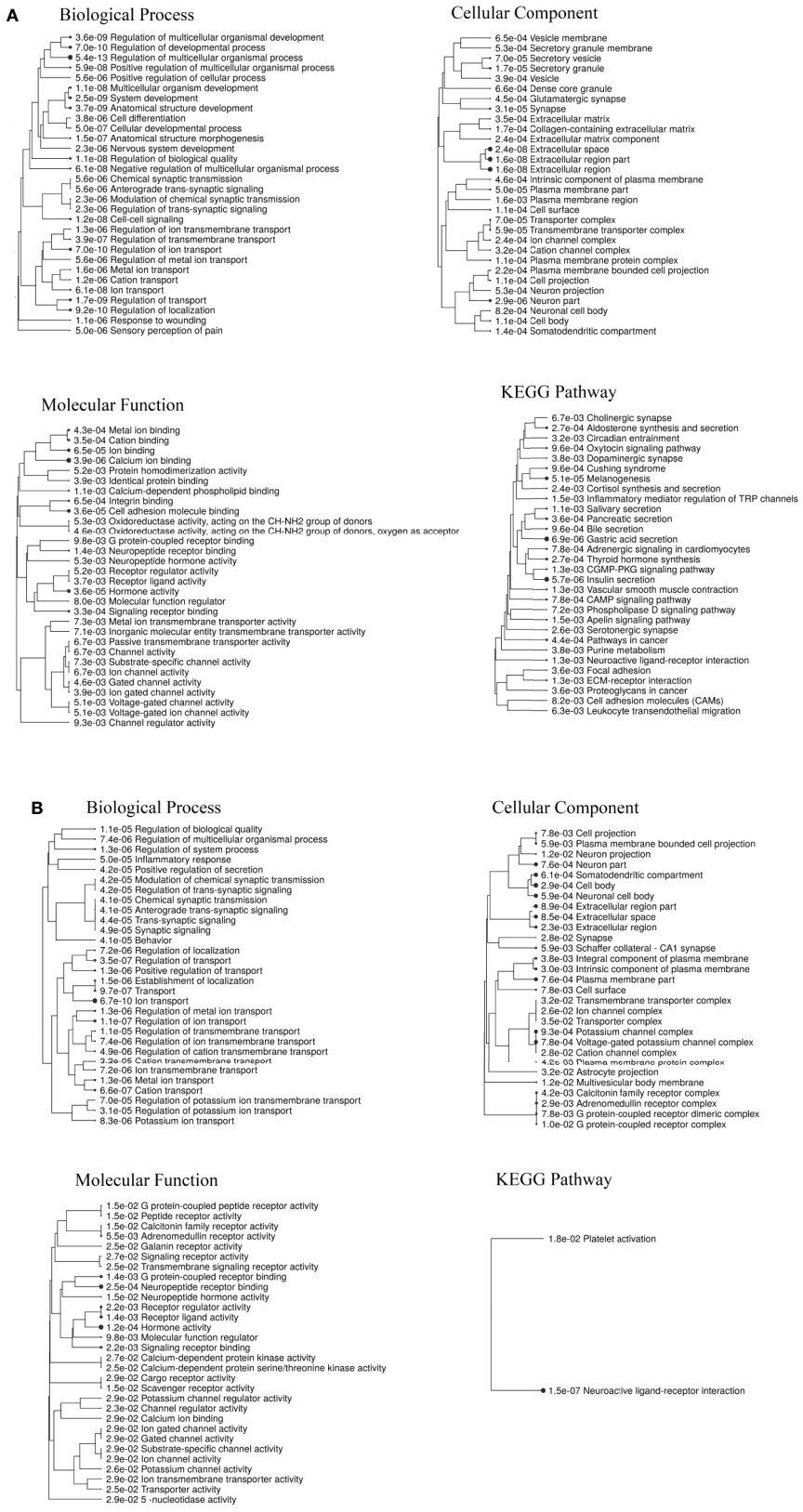

FIGURE 3 | **(A)** Enrichment analysis of the DEGs for RTX. **(B)** Enrichment analysis of the DEGs for capsaicin.

and molecular function (MF). Furthermore, KEGG pathway enrichment analysis indicated that DEGs were significantly enriched in 30 pathways (**Figure 3**). Both the RTX and capsaicin-treated genes share some GO (biological process) like ion transport, the regulation of ion transport, the regulation of transport, cation transport and the regulation of the multicellular organismal process. In the case of GO (cellular component), they share common cellular components like cell body, neuronal cell body, plasma membrane parts, neuron parts, and synapses. In GO (molecular function) enrichment, they share hormone activity, neuropeptide receptor binding, G protein-coupled receptor binding, molecular function regulators, and neuropeptide hormone activity. The KEGG pathways show that genes after treatment with both RTX and capsaicin are involved in neuronal functions, as well as other functions which are closely related with pain (**Figures 3A, B**). There is a KEGG pathway which is common to both the RTX and capsaicin-treated genes, like the neuroactive ligand-receptor interaction (**Figures 3A, B**). These genes appear in several signaling pathways that are specific to neurons, pain, and also to cellular machinery/organelles that are found in most cell types.

Protein–Protein Interaction (PPI) Network of the DEGs

Based on the STRING online database for *Rattus norvegicus*, a total of 274 DEGs (84 upregulated and 190 downregulated genes) from capsaicin-treated conditions and 444 DEGs (171 upregulated and 273 downregulated genes) were filtered into the DEGs PPI network complex from RTX-treated conditions. Initially, in the case of capsaicin, the original number of nodes and edges was 183 and 164, respectively, where the expected number of edges was 59 (**Figure S1**). Hence the network had significantly more interactions than expected. Here the average node degree was 1.79 and the average local clustering coefficient was 0.363. But in order to have better protein-protein interaction analysis, this was further expanded to a level where the number of nodes and edges were 243 and 559, respectively, with the expected number of edges at 221. Average node degree was improved to 4.6 with an average local clustering coefficient of 0.484 (**Figure S2**). Similarly, in case of RTX, the original number of nodes and edges was 325 and 531, respectively, with an average node degree of 3.27 where the expected number of edges was 192, and the average local clustering coefficient was 0.36 (**Figure S3**). It was further expanded to a level where number of nodes and edges were 375 and 1,101, respectively, with an expected number of edges of 438. The average node degree was improved to 5.87 with an average local clustering coefficient of 0.425 (**Figure S4**). In both cases, the PPI enrichment *p*-value was less than 1.0e-16.

After subjecting the STRING dataset to Cytoscape 3.8.0, the protein-protein interactions were analyzed using the Networkanalyzer and MCODE application and different genes were categorized on the basis of degree of centrality (DoC). The genes having higher DoC have been represented both by bigger size as well as by different color for the ease of readers. In case of capsaicin, the color gradient is as follows: red for 15-27 DoC; green for 9.99-15 DoC; yellow for 3-9.99 DoC, and grey for below 3 DoC. The genes in the capsaicin-treated group with a higher DoC were Sst (upregulated), Crh (downregulated), and Tac1 (downregulated) (**Figure 4**). There were some more genes which were shown to have a higher DoC like Calca, Cxcl12, Gnb1, and Epha2, but they were not originally expressed by capsaicin. They were in fact the extended network genes by STRING. Other genes with a good DoC after capsaicin treatment were Gal (downregulated), Nmu (upregulated), RT1-Da (upregulated), Ccl4 (upregulated), Npy5R (downregulated), Lpar3 (downregulated), and Fancl (upregulated). Three main clusters of hub genes have also been identified using the MCODE app in the capsaicin-treated group (**Figure 5**). The fact that cluster B had 11 genes is of importance and some of the significant genes will be briefly discussed later.

In the case of RTX, the color gradient is as follows: red for 29.78-61 DoC; green for 20-29.78 DoC; blue for 15.5-20 DoC; yellow to blue for 4.3-15.5 DoC; and grey for below 4.3 DoC. The genes in the RTX-treated group with a higher DoC were Fn1 (upregulated), Edn1 (upregulated), Spp1 (upregulated), and Cd44 (downregulated) (**Figure 6**). There were some more genes which were shown to have a higher DoC like Src, Ngf, Gnb1, and F2, but they were not the originally expressed by RTX. They were in fact extended network genes by STRING. Other genes with a good DoC after RTX treatment were Ins2 (downregulated), Col1a1 (upregulated), Vcam1 (upregulated), Ptgs2 (upregulated), Tac1 (downregulated), and Ntrk1 (downregulated). Three main clusters of hub genes have also been identified using the MCODE app in RTX-treated group (**Figure 7**). All these three clusters A-C are of importance and some of the significant genes from them will be briefly discussed later.

After the filtering of DEGs for capsaicin and RTX through the pain networks database, the DEGs were categorized by their PGE score. In the case of the capsaicin-treated group, out of 190 downregulated genes, there were only 42 genes with a PGE score ranging from 1-100. The genes with a PGE>20 were Gnaq, Tnr, Tac1, Gfra2, Acpp, Ptgir, Nt5e, Amigo2, Kcnip1, Adcyap1, Rgs4, Lpar3, Grik1, Kcnip2, Trpv1, Hrh2, Kcnj5, Gal, Plcl1, Kcnk2, and Iapp (*arranged in increasing order*). Out of 84 upregulated genes in the capsaicin-treated group, only 15 genes had a PGE score ranging from 1-100. The genes with a PGE>20 were Btk, Adrb1, Fcer1g, and Nmu (*arranged in increasing order*).

In the case of the RTX-treated group, out of 273 downregulated genes, there were only 75 genes with a PGE score ranging from 1-100. The genes with a PGE>20 were Camk2a, Gnaq, Plcb3, Comt, Tnr, Inpp5d, Ucp2, Tac1, Gfra2, Acpp, Adcy5, Ntrk1, Fgf13, Arhgap22, Nfe2l3, Ticam2, Nt5e, Amigo2, Adcyap1, Kcnip1, Cd22, Rgs4, Lpar3, Grik1, Kcnip2, Scn10a, Trpv1, Plcl1, Ece2, Scn11a, Hrh2, Kcnj5, Gal, Kcnk2, and Iapp (*arranged in increasing order*). Out of 171 upregulated genes in the RTX-treated group, only 43 genes had a PGE score ranging from 1-100. The genes with a PGE>20 were Sparc, Edn1, S100b, Itga5, Ptgs2, Sparcl1, Tyrp1, Fzd1, and Myl9 (*arranged in increasing order*). It is quite evident from the above data, that even at a low concentration, RTX is capable of regulating more pain-associated genes than capsaicin. But closely looking at the data, it also revealed that the effect of

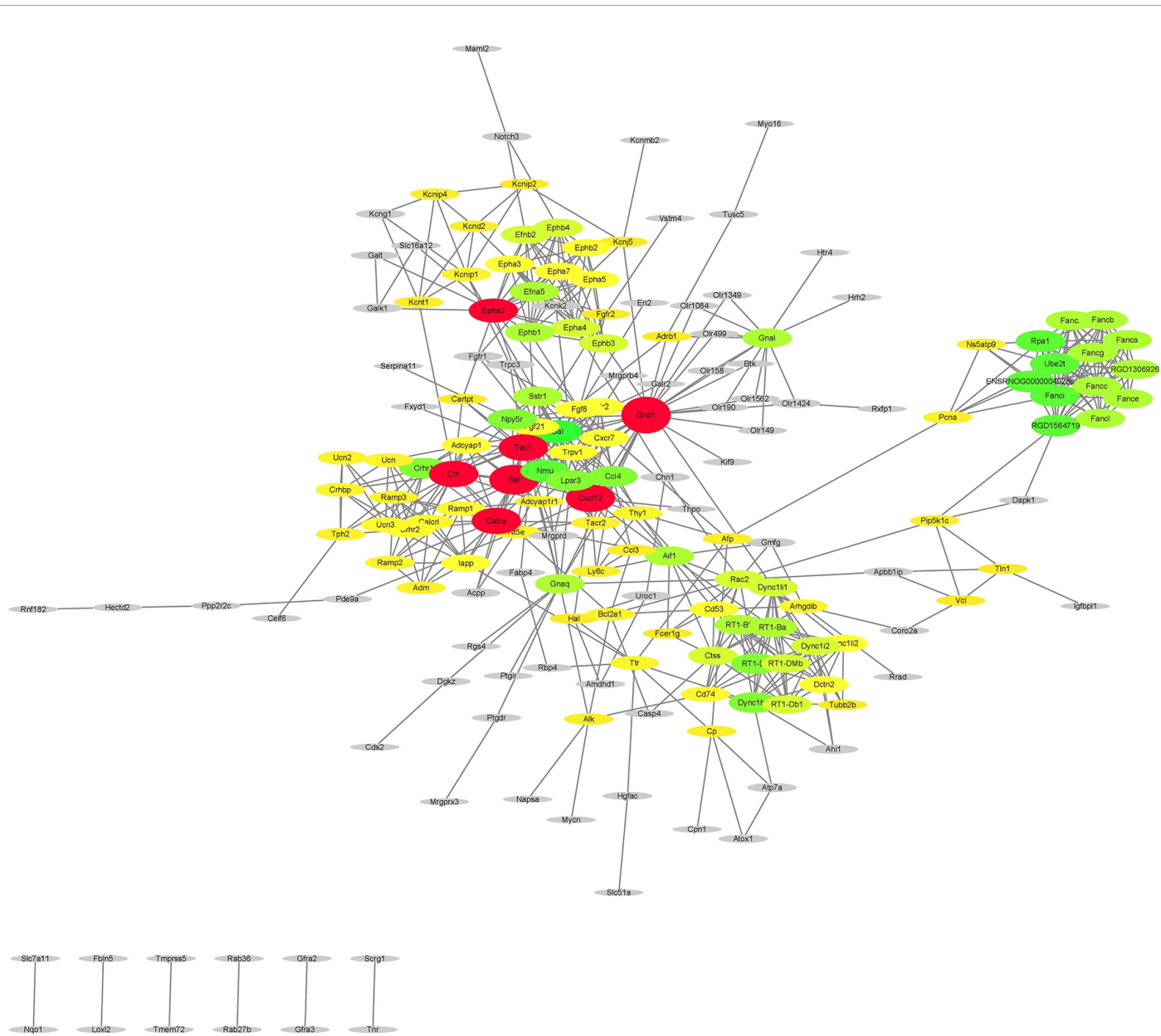

FIGURE 4 | PPI network of the genes regulated after capsaicin treatment.

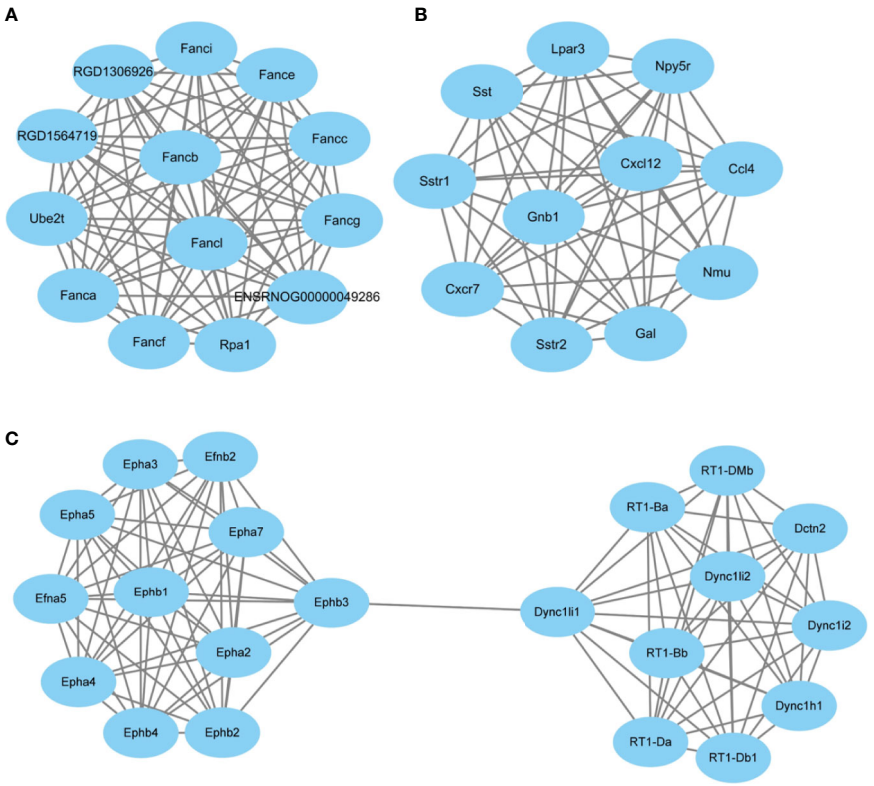

FIGURE 5 | Clusters **(A–C)** of hub genes regulated after capsaicin treatment.

RTX is more non-specific when compared to capsaicin, because in the case of RTX, there are indeed more DEGs with no PGE score.

Further, filtering the DEGs through the Pain Genes database has its own significance as there are inherently few genes in our system which have been designed to alleviate pain, so the downregulation of such genes are actually not appropriate. As per filtering through this database, it has come to our notice that out of 274 DEGs in the capsaicin-treated group, only two upregulated and 21 downregulated DEGs had a direct influence on nociception, hypersensitivity, and analgesia (**Table 1**). On the other hand, in the case of the RTX-treated group, six upregulated and 31 downregulated DEGs had a direct influence on nociception, hypersensitivity, and analgesia (**Table 2**).

After overlapping the data obtained from both the databases, there were some interesting observations. For instance, there are a few pain-related genes like Ctss, Galr2, Kcnt1, and Ptgdr, which have a zero PGE score, but from the pain gene database, it is very clear that these genes have a pain-related function. Further, after cross-validation with the original source manuscript of Isensee and co-workers (Isensee et al., 2014) whose transcriptomic data have been reprocessed by our team, it is also observable that there are a few pain-associated genes like S100b, Spp1, Sparc, Tyrp1, Edn1, Gfra2, Hrh2, Ucp2, Ntrk1, Ece2, Plcl1, Runx1, Comt, and Scn10a which have not been discussed by them in their published manuscript, or been provided as information in form of the supplementary data.

There are few indirect genes associated with pain. For instance, the Ina gene, which is downregulated by RTX (LogFC: -0.905) and capsaicin (LogFC: -0.795), is not directly a pain gene, but is associated with multiple genes which are associated with an increase in nociception like Cdk5, CdK5r1, Csk, Camk2a, Prkce, Pmp22, and Tyrp1 and decreases analgesia like Grk5, Prkce, and Prkcc. So, the downregulation of the Ina gene somehow affects these genes as well (Lacroix-Fralish et al., 2007).

The Hal gene, which is downregulated by capsaicin (LogFC: -0.665), is not a direct pain gene, but is associated with Kcnd2 which is linked with increased hypersensitivity, thus the downregulation of Hal gene somehow influences the effect of Kcnd2 (Lacroix-Fralish et al., 2007).

The Gnal gene, which is downregulated by capsaicin (LogFC: -0.500), is linked with genes like Ccr5, Slc12a5, Slc12a2, and Trpv1 which can increase nociception (Lacroix-Fralish et al., 2007).

The Plat gene, which is upregulated by RTX (LogFC: 0.536), is not directly a pain gene either, but it is associated with the Ptafr gene which is responsible for increased nociception and hypersensitivity. So upregulating the Plat gene will indirectly affects the Ptafr gene as well (Lacroix-Fralish et al., 2007).

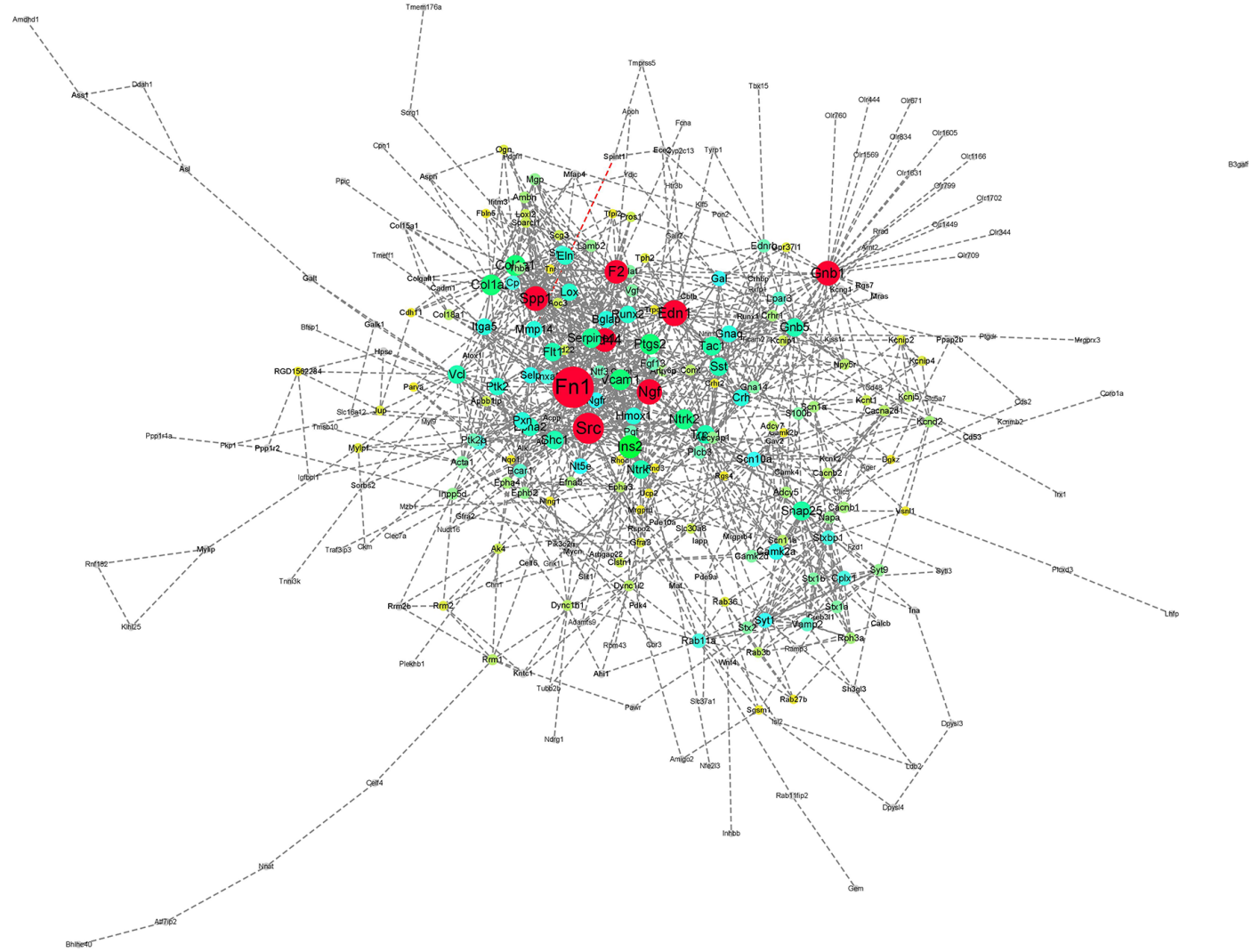

FIGURE 6 | PPI network of the genes regulated after RTX treatment.

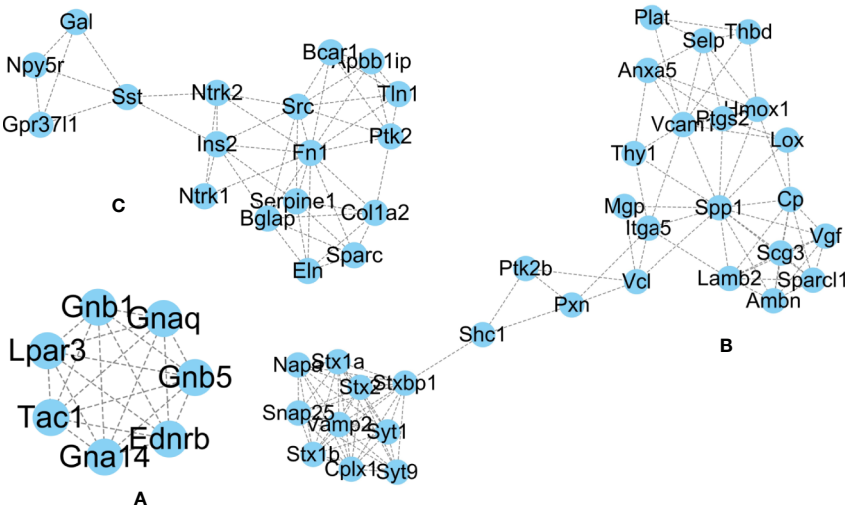

FIGURE 7 | Clusters **(A–C)** of hub genes regulated after RTX treatment.

TABLE 1 | Pain related genes regulated by capsaicin.

S.No.	Gene symbol	LogFC	Regulation	Nociception[1]	Hypersensitivity[1]	Analgesia[1]
1.	Ctss	0.646	Up	No effect	Increase	N.T
2.	Nmu	0.509	Up	Increase	N.T	N.T
3.	Iapp	-2.469	Down	Increase	N.T	N.T
4.	Lpar3	-1.834	Down	No effect	Increase	N.T
5.	Trpv1	-1.581	Down	Increase	Increase	N.T
6.	Kcnk2	-1.139	Down	Decrease	Decrease	Increase
7.	Acpp	-1.041	Down	No effect	Decrease	N.T
8.	Adcyap1	-0.991	Down	Increase	Increase	N.T
9.	Grik1	-0.903	Down	Increase	No Effect	N.T
10.	Rgs4	-0.861	Down	No effect	N.T	Increase
11.	Galr2	-0.740	Down	No effect	Contradictory data	N.T
12.	Kcnj5	-0.732	Down	Decrease	N.T	No effect
13.	Plcl1	-0.696	Down	Decrease	Decrease	N.T
14.	Gal	-0.684	Down	Decrease	Increase	N.T
15.	Nt5e	-0.680	Down	Decrease	Decrease	Increase
16.	Tac1	-0.648	Down	Increase	Contradictory data	Contradictory data
17.	Kcnt1	-0.639	Down	No effect	Decrease	N.T
18.	Hrh2	-0.606	Down	Increase	N.T	Increase
19.	Gnaq	-0.605	Down	Increase	Increase	N.T
20.	Gfra2	-0.595	Down	Decrease	N.T	N.T
21.	Ptgdr	-0.549	Down	Decrease	N.T	N.T
22.	Camk4	-0.536	Down	No effect	N.T	Increase
23.	Ptgir	-0.509	Down	Increase	Increase	N.T

[1]*These are the default functions of the specified genes. The information is gathered from the pain gene database (Lacroix-Fralish et al., 2007). N.T, not tested. Red: undesired gene response; green: desired gene response; orange: off-targets for capsaicin.*

Similarly, Lox is not a pain gene but it is upregulated by RTX (LogFC: 0.733), and is associated with many genes which are responsible for a decrease in nociception like Fstl1, Nedd4l, Crip2, Ret, Kcnq2, and Edn1 as well as a decrease in hypersensitivity like Nedd4l, Crip2, and Edn1 (Lacroix-Fralish et al., 2007).

While analyzing the data tabulated in **Tables 1** and **2**, we have segregated downregulated pain genes into desired/targets and undesired/off-targets. Here, desired targets means that their downregulation will decrease either nociception or hypersensitivity while undesired/off-targets means that those genes are already

TABLE 2 | Pain related genes regulated by RTX.

S.No.	Gene symbol	LogFC	Regulation	Nociception[1]	Hypersensitivity[1]	Analgesia[1]
1.	Ptgs2	0.685	Up	Increase	Increase	N.T
2.	S100b	0.613	Up	N.T	Increase	N.T
3.	Spp1	0.550	Up	Increase	No effect	N.T
4.	Sparc	0.545	Up	Decrease	N.T	N.T
5.	Tyrp1	0.543	Up	Increase	N.T	N.T
6.	Edn1	0.534	Up	Decrease	Decrease	N.T
7.	Iapp	-3.536	Down	Increase	N.T	N.T
8.	Trpv1	-2.215	Down	Increase	Increase	N.T
9.	Lpar3	-2.198	Down	No effect	Increase	N.T
10.	Adcyap1	-1.864	Down	Increase	Increase	N.T
11.	Kcnk2	-1.663	Down	Decrease	Decrease	Increase
12.	Gal	-1.581	Down	Decrease	Increase	N.T
13.	Tac1	-1.503	Down	Increase	Contradictory data	Contradictory data
14.	Acpp	-1.386	Down	No effect	Decrease#009966	N.T
15.	Grik1	-1.318	Down	Increase	No effect	N.T
16.	Galr2	-1.202	Down	No effect	Contradictory data	N.T
17.	Rgs4	-1.187	Down	No effect	N.T	Increase
18.	Nt5e	-0.940	Down	Decrease	Decrease	Increase
19.	Kcnj5	-0.919	Down	Decrease#009966	N.T	No effect
20.	Kcnt1	-0.904	Down	No effect	Decrease	N.T
21.	Gfra2	-0.863	Down	Decrease	N.T	N.T
22.	Hrh2	-0.801	Down	Increase	N.T	Increase
23.	Camk2a	-0.790	Down	Increase	Increase	N.T
24.	Scn11a	-0.712	Down	Increase	Contradictory data	N.T
25.	Adcy5	-0.693	Down	Increase	Increase	Increase
26.	Cacna2d1	-0.669	Down	Increase	Increase	N.T
27.	Ptgdr	-0.647	Down	Decrease	N.T	N.T
28.	Gnaq	-0.635	Down	Increase	Increase	N.T
29.	Camk4	-0.628	Down	No effect	N.T	Increase
30.	Ucp2	-0.617	Down	No effect	N.T	Decrease
31.	Ntrk1	-0.567	Down	Increase	N.T	N.T
32.	Ece2	-0.545	Down	No effect	N.T	Increase
33.	Plcl1	-0.542	Down	Decrease	Decrease	N.T
34.	Runx1	-0.536	Down	Increase	Increase	N.T
35.	Comt	-0.533	Down	Decrease	N.T	Contradictory data
36.	Plcb3	-0.515	Down	No effect	N.T	Decrease
37.	Scn10a	-0.500	Down	Decrease	Contradictory data	Decrease

[1]*These are the default functions of the specified genes. The information is gathered from pain gene database (Lacroix-Fralish et al., 2007). N.T, not tested. Red: undesired gene response; green: desired gene response; orange: off-targets for RTX.*

beneficial genes in alleviating nociception or hypersensitivity. Thus, the downregulation of off-targets here is somehow decreasing the innate capability of the host system to alleviate pain.

Capsaicin Induced Upregulated Genes With No Direct Association With Pain Nociception

We will discuss here the biological functions of the genes which fall under the top 10 upregulated DEGs after capsaicin treatment (**Table S1** lists the top 30 upregulated and downregulated genes), without having any direct association with pain nociception. Olr1349 and Olr190 are genes that encode the olfactory receptor which is primarily responsible for the perception of smell (Gibbs et al., 2004; Antunes and Simoes De Souza, 2016). Tmprss5 which is a transmembrane serine protease 5 (also known as spinesin) is reported to be involved in the function of astrocytes in the spinal cord (Yamaguchi et al., 2008). Notch3 is regarded as the key gene for the maintenance and function of vascular smooth muscle cells, including those which are involved in the blood supply to the brain (Lin et al., 2019). But some of recent studies also suggest that Notch3 plays a vital role in oncogenesis, the maintenance of tumors, and chemotherapy resistance (Aburjania et al., 2018). Ccl4 is a monokine possessing chemokinetic and inflammatory properties and is suggested to be involved in neutrophil recruitment in the intrapulmonary region. It is also recorded as one of the major suppressive factors

for HIV (Farquhar et al., 2005; Lee et al., 2007). The Car13 gene encodes carbonic anhydrases 13 (CA XIII) which is a cytosolic isoform of carbonic anhydrases. CA XIII, though it has a moderate catalytic activity, may contribute in maintaining the acid-base homeostasis in the kidneys, GI tract, and in the reproductive system (Hilvo et al., 2008). The Napsa gene encodes napsin A aspartic peptidase which is preferentially expressed in the kidneys, lungs, and spleen (Schauer-Vukasinovic et al., 2000). Its expression is commonly reported to be associated with pulmonary adenocarcinomas and renal cell carcinomas (Nam et al., 2016). The Colec12 gene is a collectin sub-family member 12, which encodes the scavenger receptor with the C-type lectin/carbohydrate recognition domain. These receptors are considered as an important member contributing to innate immunity (Nakamura et al., 2001). The Eif2a gene encodes eukaryotic translation initiation factor 2-alpha, which is generally observed as an environmental stress-induced gene, and phosphorylation of which results in the reduction of global protein synthesis (Hong et al., 2016). Though these genes may not have a direct association with pain nociception, most of these genes are found to influence nervous system functionality including inflammation.

Capsaicin Induced Downregulated Genes With No Direct Association With Pain Nociception

We will discuss here the biological functions of the genes which fall under the top 10 downregulated DEGs after capsaicin treatment (**Table S1** lists the top 30 upregulated and downregulated genes), without having any direct association with pain nociception. The Cartpt gene encodes to the cocaine and amphetamine-regulated transcript (CART) peptide, which is evidently involved in the negative regulation of estradiol production (Lv et al., 2009), and is also involved in various stages of opioid addiction (Bakhtazad et al., 2016). The Mrgprx3 and Mrgprd genes encode for a G-protein coupled receptor (GPCR) for which adenine acts as the endogenous ligand and which plays a role in nociception, although its clear and specific role has not yet been defined (Bender et al., 2002). The Kcnip1 gene encodes for the potassium voltage gated ion channel, Kcnip1 which is primarily expressed in the brain and testes. Potassium channel blockers are used for the treatment of neuromuscular disorders, thus the downregulation of this gene seems to play an important role in the improvement of the nervous system (Del Pino et al., 2015). The Slc51a gene encodes for the organic solute transporter, and is involved in the transportation of bile acid as well as steroids in the intestine, renal, and biliary epithelia (Ballatori et al., 2005; Christian et al., 2012). The Amdhd1 gene encodes for the aminohydrolase domain which is involved in the formation of intestinal stem cells in adults (Okada et al., 2015). The Trpc3 gene encodes for member 3 of the subfamily C of the transient receptor potential cation channel which is a calcium-activated cation channel, and it is involved in the development of febrile seizures. Thus downregulation of the Trpc3 gene or inhibition of this calcium-activated cation channel significantly attenuates the susceptibility of seizures, neuronal cell death, and neuroinflammation (Sun et al., 2018).

RTX Induced Upregulated Genes With No Direct Association With Pain Nociception

We will discuss here the biological functions of the genes which fall under the top 10 upregulated DEGs after RTX treatment (**Table S2** lists the top 30 upregulated and downregulated genes), without any direct association with pain nociception. As discussed above in the capsaicin section, the Notch3 gene is involved in the maintenance of vascular smooth muscle cells (Lin et al., 2019). Itga5, though it has a PGE score when filtered through the pain network database, no direct association was found when screened through the pain gene database. Itga5 encodes for the integrin subunit alpha 5, and is involved in the regulation of spine morphogenesis as well as the formation of synapses in hippocampal neurons (Webb et al., 2007). The Traf3ip3 gene encodes TRAF3 interacting protein 3 which modulates the pathway associated with c-Jun N-terminal kinase signal transduction, and thereby mediates cell growth (Dadgostar et al., 2003). Further, its upregulation has also been observed in various types of cancers (Nasarre et al., 2018). The Hpse gene is encodes for heparanase which is a endo-beta-glucouronidase and catalyzes the cleavage of heparan sulfate. Heparanase is evidently involved in neuroinflammatory responses (Changyaleket et al., 2017). The Mylip gene encodes the myosin regulatory light chain interacting protein which is involved in the Reelin-induced decrease of very low density lipoprotein receptors in neuronal systems (Do et al., 2013). The Pxn gene encodes to paxillin which is involved in the focal adhesion of cells with an extracellular matrix (Torres et al., 2008). Further, paxillin was reported to be involved in the regulation of cytoskeleton proteins like α-actin, α-tubulin, and destrin (Chen et al., 2014). The Aoc3 gene encodes for amine oxidase, copper containing 3, which is also recorded as vascular adhesion protein-1 and found to be involved in leucocyte recruitment (Noda et al., 2008). The Plpp3 gene encodes for the phospholipid phosphatase 3 enzyme and it is involved in the transport carrier's formation in Golgi bodies (Gutierrez-Martinez et al., 2013). The Bace2 gene encodes for the beta-secretase 2 enzyme which is also recorded as aspartic protease and is involved in the cleavage of the precursor protein of amyloid-β into amyloid-β. Amyloid-β is well known to trigger neurodegenerative diseases like Alzheimer's disease (Bettegazzi et al., 2011). The S100a16 gene encodes for S100 calcium-binding protein A16 which is involved in adipogenesis and thus weight gain (Zhang et al., 2014).

RTX Induced Downregulated Genes With No Direct Association With Pain Nociception

We will discuss here the biological functions of the genes which fall under the top 10 downregulated DEGs after RTX treatment (**Table S2** lists the top 30 upregulated and downregulated genes), without any direct association with pain nociception. As already described in the relevant capsaicin section, the Cartpt gene is involved in various stages of opioid addiction (Bakhtazad et al., 2016). Though, the Crh gene does not have any association with pain, it possesses a good DoC (**Figure 6**) which indicates that it is closely associated with many other genes. Further, **Figure 4**

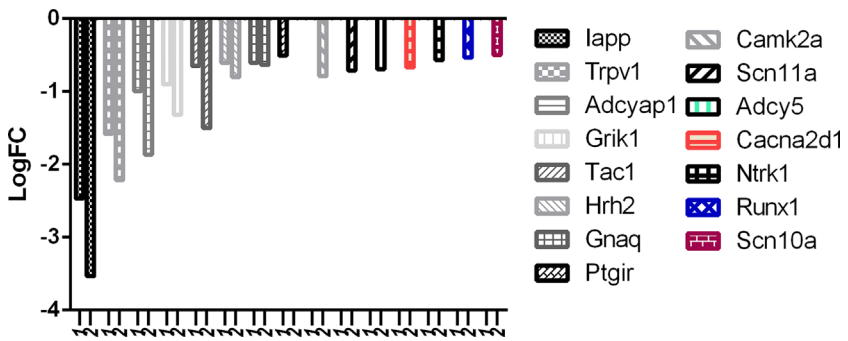

FIGURE 8 | Downregulation of pain genes responsible for increased nociception. 1: capsaicin; 2: RTX.

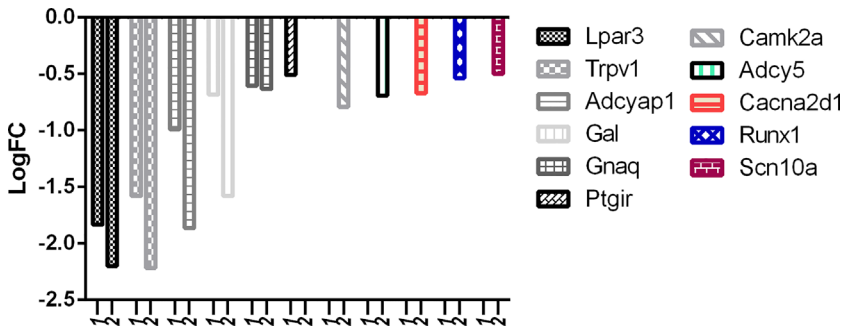

FIGURE 9 | Downregulation of pain genes responsible for increased hypersensitivity. 1: capsaicin; 2: RTX.

represents the Crh gene in the capsaicin-treated protein-protein interaction, but in capsaicin, the downregulation of the Crh gene did not fall into the top 30 downregulated genes (**Table S1**). The Crh gene encodes the corticotrophin releasing hormone which is well known for its mediatory effect in the neuroendocrine stress response (Kozakai et al., 2019). The Mrgprx3 and Mrgprd genes which encode GPCR have recorded significance in nociception, though a specific role is as yet unclear (Bender et al., 2002). The Gfra3 gene encodes for the alpha 3 isoform of GDNF family receptor and is critically involved in neuronal cell protection (Hoke et al., 2000). The functionality of the Slc51a gene which encodes for the organic solute transporter has already been discussed in the capsaicin section (Ballatori et al., 2005; Christian et al., 2012).

There is no question about the potency of RTX, but selectivity should also be considered and a balance of both is what required at the clinical level. With reference to **Figure 8**, it can be easily observed that the downregulation of desired pain genes affecting nociception like Iapp, Trpv1, Adcyap1, Grik1, Tac1, Hrh2, and Gnaq was more evident in the RTX-treated group when

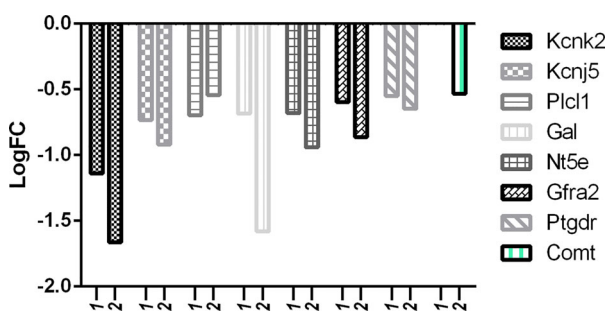

FIGURE 10 | Downregulation of pain genes responsible for decreased nociception. 1: capsaicin; 2: RTX.

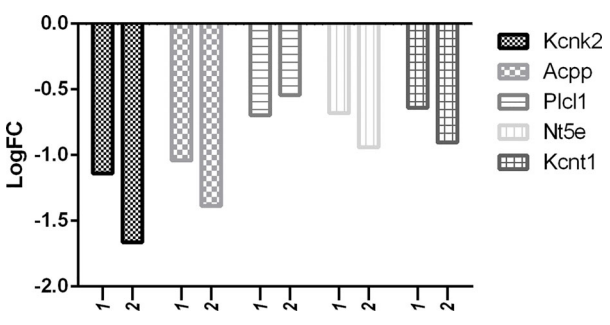

FIGURE 11 | Downregulation of pain genes responsible for decreased hypersensitivity. 1: capsaicin; 2: RTX.

compared to the capsaicin-treated group. Interestingly, only capsaicin was able to downregulate Ptgir, while only RTX was able to downregulate some other desired pain genes like Camk2a, Scn11a, Adcy5, Cacna2d1, Ntrk1, Runx1, and Scn10a.

With reference to **Figure 9**, it is evident that the downregulation of desired pain genes affecting hypersensitivity like Lpar3, Trpv1, Adcyap1, Gal, and Gnaq was observed more in the RTX-treated group when compared to the capsaicin-treated group. Similarly, only capsaicin was able to downregulate Ptgir while only RTX was able to downregulate Camk2a, Adcy5, Cacna2d1, Runx1, and Scn10a.

But in comparison with capsaicin, RTX was not only able to downregulate more of the target pain genes, but also more of the off-target pain genes. With reference to **Figure 10**, it is clear that the downregulation of off-target pain genes associated with nociception like Kcnk2, Kcnj5, Gal, Nt5e, Gfra2, and Ptgdr was higher in RTX when compared to capsaicin, except Plcl1 which was downregulated more by capsaicin. Further, only RTX could downregulate the Comt gene. With reference to **Figure 11**, it can be seen that the downregulation of off-target pain genes associated with hypersensitivity like Kcnk2, Acpp, Nt5e, and Kcnt1 was more evident RTX when compared to capsaicin, except Plcl1 which was downregulated more by capsaicin.

So, after reassessing the transcriptomic data, we have found that when compared to capsaicin, RTX not only upregulates more non-pain related genes, but also downregulates more undesired/off-targets pain genes. This proposes that RTX is a more potent drug compared to capsaicin for its clinical implications.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, GSE59727.

AUTHOR CONTRIBUTIONS

RS and BS designed the experiment. RS, AS, and MS participated in data analysis, All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Key Research and Development Program of China (2016YFC1306605) and the National Natural Science Foundation of China (Grant Nos. 31670851).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2020.551786/full#supplementary-material

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