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Neuropharmacological intervention for severe mental illness and suicide prevention

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Neuropharmacological intervention for severe mental illness and suicide prevention

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Editorial: Neuropharmacological intervention for severe mental illness and suicide prevention

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Editorial on the Research Topic
Neuropharmacological intervention for severe mental illness and suicide prevention

Severe mental illnesses (SMI), including major depressive disorder, bipolar disorder, and schizophrenia, are leading contributors to disability worldwide and are associated with an elevated risk of suicide. Despite advancements in pharmacological and non-pharmacological interventions, a substantial proportion of individuals with SMI continue to experience persistent symptoms, treatment resistance, and significant comorbidities. These challenges underscore the critical need for novel and more effective therapeutic strategies. Understanding how different medications affect both core psychiatric symptoms and suicide risk remains a key area of research.

The collected publications present data derived from human subjects with mental illnesses, including post-mortem analyses, as well as from preclinical animal models. These translational studies investigate potential pathophysiological mechanisms and novel pharmacological interventions for SMI, effectively bridging the gap between preclinical findings and clinical applications. Additionally, the Research Topic also includes comprehensive reviews that synthesize current knowledge and outline future directions for innovative pharmacological strategies in treating SMI.

Among preclinical findings, Liu et al. employed a single prolonged stress model in rats to investigate the effects of artemisinin, a natural compound derived from *Artemisia annua*, on PTSD-related cognitive and social deficits. The study demonstrated that systemic administration of artemisinin alleviated behavioral impairments, with ultrastructural and molecular imaging data suggesting that its therapeutic effects are mediated by the restoration of synaptic plasticity and the inhibition of neuronal apoptosis in the hippocampus. Zelek-Molik et al. utilized a restraint stress (RS) model in rats to examine the impact of betaxolol, a selective β1-adrenergic receptor antagonist, on stress-induced alterations in glutamatergic signaling. While chronic RS was associated with GluN2B downregulation in the frontal cortex, pharmacological experiments did not support a direct role of β1-adrenergic receptor modulation in this process, implicating alternative pathways in stress-induced neurobiological changes. Further, Espinosa et al. explored the role of purinergic receptor modulation in temporal lobe epilepsy using *in vivo*

electrophysiology in a mouse model. Although ATP receptors, particularly P2XRs, have been implicated in epileptic processes, their precise role in seizure pathophysiology remains unclear. The study documented that systemic administration of TNP-ATP reduced the amplitude and frequency of interictal discharges in the hippocampus. Notably, blocking P2XRs also improved cognitive performance and enhanced phase coherence for both slow (theta) and fast (gamma) oscillations recorded in the hippocampal CA1 region and prefrontal cortex, suggesting potential cognitive benefits of purinergic modulation in epilepsy-related neuropsychiatric symptoms.

Among human studies, Aldossary et al. investigated the adjunctive use of high-dose atorvastatin with fluoxetine in major depressive disorder, revealing modulation of the AMPK/NLRP3 and IL-6/STAT3 pathways, key regulators of neuroinflammation increasingly associated with mood regulation and suicidality. Qi et al. examined the pharmacokinetic consequences of smoking cessation in patients treated with clozapine, reinforcing the necessity of therapeutic drug monitoring to optimize treatment efficacy and minimize adverse effects in this vulnerable population. Wang et al. reported rapid and sustained antidepressant effects of dezocine, an opioid analgesic, in a patient with treatment-resistant depression, highlighting opioid receptor modulation as a potential therapeutic avenue for mood disorders and suicide prevention. Additionally, post-mortem studies by De Simone et al. analyzed the expression of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) in various brain regions of suicide victims with a clinical history of depression. Their findings indicated decreased BDNF and increased GDNF levels in multiple brain regions of suicide victims compared to individuals who died from natural causes, suggesting that these neurotrophic factors could serve as biomarkers for suicide risk and potential targets for specific pharmacological treatments of depression.

In a review article, Liu et al. explored pharmacological agents that mimic the antidepressant effects of exercise, emphasizing the role of irisin, a newly discovered exercise-induced myokine, and its precursor protein, fibronectin type III domain-containing protein 5 (FNDC5). Given the well-documented benefits of physical activity in alleviating depressive symptoms without the adverse effects associated with pharmacotherapy, the review underscores the potential of targeting exercise-related molecular pathways in depression treatment. Zhao et al. examined ferroptosis and its relevance to cardio-cerebrovascular diseases associated with depression. The authors highlight the multi-targeted therapeutic potential of traditional Chinese medicine and proposed GPX4 and Nrf2 as converging molecular targets implicated in both psychiatric and inflammatory diseases. Zelek-Molik and Litwa reviewed recent advances in antidepressant mechanism research, focusing on treatment-resistant depression, which is characterized by poorer outcomes, increased suicide risk, and heightened psychiatric comorbidities. The authors emphasized the necessity of multidimensional therapeutic strategies that account for the heterogeneity of mood disorders and the complex interplay of neurobiological systems. Despite significant advances, the pathophysiology of SMI remains idiopathic and incompletely understood, complicating the development of universally effective treatments.

As illustrated by the contributions in this Research Topic, mood disorders comorbidities are highly complex, and extend beyond the nervous system, involving intricate interactions among neural, immune, endocrine, metabolic, and microbiome-related processes. Ongoing research into novel pharmacological agents, multimodal treatment strategies, and precision medicine approaches represents the most promising pathway toward advancing the treatment of depression and prevent risky behaviors. These findings underscore the importance of personalized interventions tailored to address both core psychiatric symptoms and associated risk factors. Future studies should further integrate neurobiological, genetic, and environmental perspectives to refine pharmacological treatment paradigms and enhance patient outcomes in SMI and comorbidities.

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Artemisinin reduces PTSD-like symptoms, improves synaptic plasticity, and inhibits apoptosis in rats subjected to single prolonged stress

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Post-Traumatic Stress Disorder (PTSD) is a chronic mental disorder characterized by symptoms of panic and anxiety, depression, impaired cognitive functioning, and difficulty in social interactions. While the effect of the traditional Chinese medicine artemisinin (AR) on PTSD is unknown, its therapeutic benefits have been demonstrated by studies on models of multiple neurological disorders. This study aimed to extend such findings by investigating the effects of AR administration on a rat model of PTSD induced by a regimen of single prolonged stress (SPS). After rats were subjected to the SPS protocol, AR was administered and its impact on PTSD-like behaviors was evaluated. In the present study, rats were subjected to a multitude of behavioral tests to evaluate behaviors related to anxiety, memory function, and social interactions. The expression of hippocampal synaptic plasticity-related proteins was detected using Western blot and immunofluorescence. The ultrastructure of synapses was observed under transmission electron microscopy. The apoptosis of hippocampal neurons was examined with Western blot, TUNEL staining, and HE staining. The results showed that AR administration alleviated the PTSD-like phenotypes in SPS rats, including behavior indicative of anxiety, cognitive deficits, and diminished sociability. AR administration was further observed to improve synaptic plasticity and inhibit neuronal apoptosis in SPS rats. These findings suggest that administering AR after the onset of severe traumatic events may alleviate anxiety, cognitive deficits, and impaired social interaction, improve synaptic plasticity, and diminish neuronal apoptosis. Hence, the present study provides evidence for AR's potential as a multi-target agent in the treatment of PTSD.

KEYWORDS

post-traumatic stress disorder, artemisinin, single prolonged stress, synaptic plasticity, apoptosis

1 Introduction

Induced by traumatic experiences, post-traumatic stress disorder (PTSD) is a devastating psychiatric condition characterized by heightened arousal and the continual recollection of stressful memories linked to the trauma (Compean and Hamner, 2019). While approximately 6%–7% of the population will experience PTSD at some point in their lives, the prevalence of PTSD is notably higher among individuals who have undergone

particularly traumatic events, such as war and violent crime (Schrader and Ross, 2021). PTSD is associated with anxiety and deficits in social interaction, cognition, and memory (Wilkins et al., 2021). Among them, anxiety is a common symptom in patients with PTSD (Sadeghi et al., 2024). PTSD-related anxiety symptoms stem from the generalization of individual's initial fear memory, which can trigger anxiety reactions even when exposed to backgrounds similar to the original trauma background (Dunsmoor and Paz, 2015). PTSD is also often associated with significant cognitive impairment. Current PTSD theory suggests that cognitive abnormalities are the core of the occurrence and persistence of PTSD symptoms (Pitts et al., 2022). Social deficits are another typical symptom of PTSD. Patients avoid social situations and have difficulty maintaining positive interpersonal relationships (Mittal et al., 2013; Gou et al., 2023). Preclinical studies have identified synaptic plasticity changes and increased rates of apoptosis as significant pathophysiological mechanisms underlying PTSD (Gu et al., 2023; Xie et al., 2024). While various treatment options, such as psychotherapy and pharmacotherapy, are available for patients with PTSD, much work remains to improve the effectiveness and accessibility of these treatments, as well as clarify the causes that underlie the neuropathological consequences of trauma (Quinones et al., 2020; Astill et al., 2021).

The relationship between the dysfunction of the prefrontal cortex-hippocampus-amygdala circuit related to learning and memory, and the pathological changes of PTSD has long been a focus of attention (Alexandra et al., 2022). The amygdala is the emotional control center of the brain, especially emotions related to fear and anxiety (Simic et al., 2021). The prefrontal cortex is involved in emotional regulation and cognitive control in PTSD (Alexandra et al., 2022). The hippocampus is an important brain region in this circuit, responsible for the key structures of memory storage and retrieval, especially memories related to emotions and trauma (Bremner, 2006; Fenster et al., 2018; Logue et al., 2018; Wang et al., 2022). In PTSD patients, structural and functional changes occur in the hippocampal region, leading to abnormal storage and recall of traumatic memories. This, in turn, results in individuals repeatedly recalling the traumatic event and being unable to shake off negative emotions (Shin et al., 2006; Tural et al., 2018; Bremner et al., 2021). Therefore, studying the role of the hippocampal region in PTSD is conducive to understanding the mechanism of the disease and developing potential therapeutic strategies.

Artemisinin (AR) is a natural compound obtained from the Artemisia annua plant. Although AR is most renowned for its potent anti-malarial properties (Ma et al., 2020), recent findings suggest that it exerts neuroprotective effects (Kshirsagar and Rao, 2021; Arthur et al., 2023). Specifically, it may alleviate oxidative stress, inhibit neuroapoptosis, suppress neuroinflammation, and promote synaptic plasticity in the contexts of multiple neurological conditions, including stroke, Alzheimer's disease, Parkinson's disease, and depression (He et al., 2021; Yan et al., 2021; Peng et al., 2022). However, the potential of AR to act as a neuroprotective agent in patients with PTSD, particularly to improve synaptic plasticity and neuronal apoptosis in affected individuals, remains largely unexplored. To resolve this dearth in the literature and inform future directions in the treatment of PTSD, our study explores the neuroprotective effects of AR on PTSD-like phenotypes in rats exposed to prolonged stress.

The single prolonged stress (SPS) procedure is a widely accepted preclinical paradigm commonly used to simulate PTSD-like behavior in rodents. By subjecting them to a combination of severe and inescapable stressors such as immobilization, forced swimming, and ether exposure, it aims to cause sufficient trauma to the animals to induce enhanced and long-lasting PTSD-like symptoms (Souza et al., 2017; Lisieski et al., 2018; Richter-Levin et al., 2019). Our central hypothesis posits that AR can induce the enhancement of synaptic plasticity and inhibit neuronal apoptosis, thereby attenuating the symptoms of PTSD. The current study used the SPS procedure to simulate PTSD in rodents. A series of tests were then conducted to assess the impact of AR on the rodents' behaviors: the Morris water maze (MWM) test to investigate spatial exploration and memory function, the elevated plus-maze (EPM) to evaluate anxiety-like behaviors, the open field test (OFT) to gauge exploratory behaviors, and the three-chamber social interaction test (SIT) to assess social communication. The present study then measured the effect of AR on the synaptic morphology, synaptic-associated proteins, and neuronal apoptosis in the hippocampal region of SPS-induced rats. The findings obtained from this comprehensive investigation may offer insight into the potential therapeutic impact of AR on PTSD, as well as novel, effective treatments for the disorder.

2 Materials and methods

2.1 Animals

Due to the neuroprotective effects of estrogen, only male SD rats were used in this study (Wang et al., 2019). Specific pathogen-free male SD rats (weighing 200–250 g) were habituated to a 12-h light/dark cycle and allowed to eat and drink *ad libitum*. Room temperature was maintained at 20°C–25°C. All behavioral tests were conducted between 8:00 a.m. and 11:00 a.m. The experimental protocol was approved by the Animal Ethics Committee of Weifang Medical University on 1 January 2021 (approval number: 2021SDL577).

2.2 Experimental animal grouping

In this study, artemisinin was administered to rats at a concentration of 18 mg/kg via intraperitoneal injection (i.p.), as described in previous literature (Lv et al., 2023). As shown in Figure 1, eighty rats were randomly divided into four groups of 20: the SPS + AR group underwent SPS followed by AR administration once a day for 14 consecutive days, the vehicle (Veh) group did not undergo SPS and received saline once a day for 14 consecutive days, the AR group did not undergo SPS and received AR once a day for 14 consecutive days, and the SPS group underwent SPS stimulation followed by saline administration once a day for 14 consecutive days. The animals were housed separately

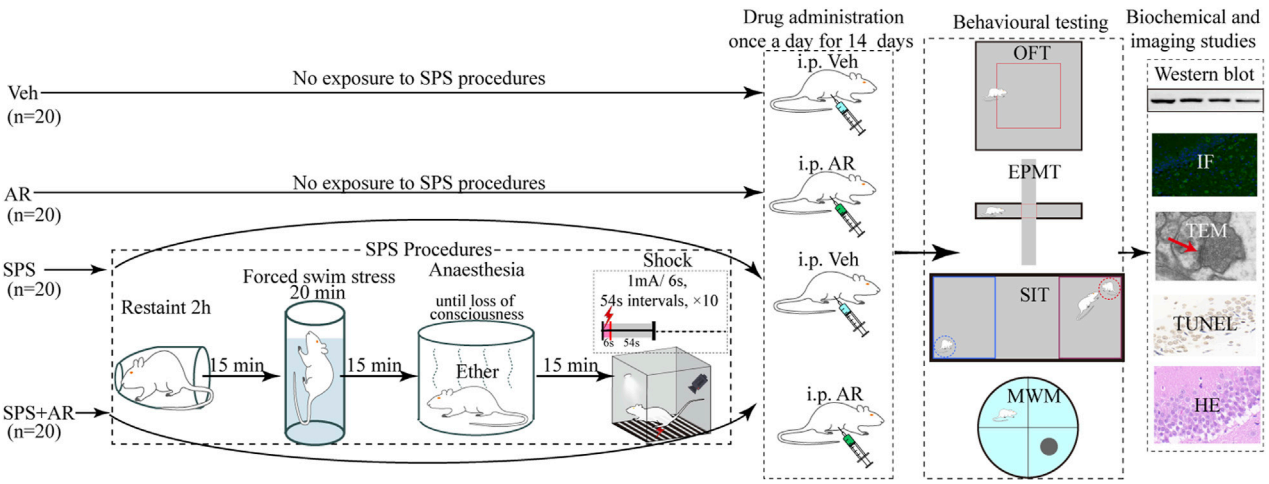

FIGURE 1
In this study, we constructed a schematic of the PTSD model through the SPS procedure in rats, AR or saline administration modality and the four behavioral paradigms of MWM, EPM, OFT, and SIT. Specifically, following the completion of the aforementioned four behavioral tests by all animals, biochemical and imaging studies were conducted. For these studies, five animals were randomly selected from each group for Western blot analysis, five for imaging studies, and five for TEM studies.

according to their respective groups. The AR used in this study was obtained from Sigma-Aldrich (St. Louis, MO, United States of America; CAS 63968-64-9; molecular weight, 282.33 g/mol; purity, ≥98.0%).

2.3 SPS procedure

This study used a typical SPS rat model to simulate trauma experienced by humans. As previously reported (Torok et al., 2019), SPS begins with restraint stress, where the animal is restrained for 2 h to simulate helplessness and immobilization. Fifteen minutes afterward, the animal is placed in a tank filled with water and forced to swim for 20 min to stay afloat (water temperature: 25°C ± 2°C). Fifteen minutes later, the animal is exposed to ether vapor for 15 min to induce anesthesia. Thirty minutes afterward, the animal is exposed to a tone followed by a mild electric foot shock (1 mA for 6 s, 10 times) to induce a fear response (Figure 1).

2.4 Behavioral tests

As shown in Figure 1, all 80 rats in this study were subjected to OFT, EPMT, three-chamber SIT, or MWM test 1 day after administration of AR or saline to monitor the PTSD-associated behavioral and physiological changes, including anxiety, social interaction skills, and spatial learning memory abilities. Throughout the testing, the investigators remained at a minimum distance of 1 m from the equipment while they recorded data for each group. Once each test was completed, the investigator returned the rats to their feeding cages and sanitized the equipment with 75% alcohol to prevent the transmission of any residual information (e.g., urine, feces, and odor) to subsequently tested rats.

2.5 OFT

The OFT was performed in a large, brightly lit open arena (100 × 100 × 50 cm) whose walls were devoid of any visual or tactile cues. The procedure consisted of the following steps. The camera was positioned directly above the arena to capture the rats' paths of movement. The rats were allowed 10 min of free exploration in the center of the arena. The behaviors of the rats were then observed and recorded for 5 min. Data analysis was performed with Smart 3.0 (Barcelona, Spain).

2.6 EPMT

The elevated plus-maze was elevated to a height of 70 cm above the ground. The apparatus contained four closed arms of 50 cm in length and 10 cm in width. Two of the arms were enclosed at a height of 40, and two were left uncovered. Before the experiment, each rat was allowed to acclimatize to the test chamber for ≥20 min. The experiment began with the rat placed at the center of the maze and facing the closed arm. Its subsequent movements were then recorded for 5 min, during which time the rat's number of entries into and durations of stay in the open and closed arms were recorded. After the test, the rats were removed from the maze and returned to their respective cages. The open-arm entries and open-arm dwell time percentages were computed to evaluate each tested rat's level of anxiety.

2.7 Three-chamber SIT

As described previously (Xiao et al., 2022), the experimental arena comprised a topless rectangular box (120 cm × 60 cm × 40 cm) divided into three chambers of equal volumes (left, middle, and right). The chambers were connected by two doors

TABLE 1 Antibody information about the method of Western blot.

Primary antibody	Place of origin	Item no.	Molecular weight (KDa)	Source of antibody	Dilution ratio of primary antibody	Secondary antibody	Place of origin	Item no.	Dilution ratio of primary antibody
GAPDH	Servicebio	GB15002	37	Mouse	1: 2000	HRP-goat anti-mouse	Servicebio	GB23301	1: 5000
β-tubulin	Servicebio	GB122667	55	Mouse	1: 2000	HRP-goat anti-mouse	Servicebio	GB23301	1: 5000
BDNF	ABCAM	ab205067	15	Mouse	1: 2000	HRP-goat anti-mouse	Servicebio	GB23301	1: 5000
Synapsin I	ABCAM	ab254349	75	Rabbit	1: 1000	HRP-goat anti-rabbit	Servicebio	GB23303	1: 5000
PSD95	ABCAM	ab18258	80	Rabbit	1: 1000	HRP-goat anti-rabbit	Servicebio	GB23303	1: 5000
β-actin	Servicebio	GB12001	42	Mouse	1: 2000	HRP-goat anti-mouse	Servicebio	GB23301	1: 5000
BAX	ABCAM	AB32503	20	Rabbit	1: 1000	HRP-goat anti-rabbit	Servicebio	GB23303	1: 5000
BCL2	ABCAM	AB196495	26	Rabbit	1: 1000	HRP-goat anti-rabbit	Servicebio	GB23303	1: 5000
CASPASE3	ABCAM	AB184787	35	Rabbit	1: 1000	HRP-goat anti-rabbit	Servicebio	GB23303	1: 5000

(20 cm × 10 cm) on either wall of the middle volume. The rats were allowed to freely pass between the three parts. After being placed in the empty middle chamber of the device, the tested rats were allowed to freely explore each chamber for 10 min. After this habituation period, a new rat (referred to as unfamiliar 1 [U1]) was placed in a confinement cage located in the right chamber (referred to as chamber U1), while the confinement cage in the left chamber (referred to as chamber E) was left empty. The tested rat was placed in the central chamber and was allowed to wander freely for 10 min (the sociability phase). Following that, a new and unfamiliar rat (unfamiliar 2 [U2]) was placed in the confinement cage in the left chamber (referred to as chamber U2). The U1 rat, which had previously been used in the sociability phase, was left in its original position as a familiar animal. Subsequently, the rats were permitted to explore the testing environment without any limitations for 10 min (the social novelty preference phase). Behavioral data such as rat movement trajectories, time spent in each of the chambers, and time spent interacting with unfamiliar rats or empty chambers were analyzed to assess sociability and social novelty preference during both phases of the test.

2.8 MWM test

The MWM experiment was conducted in a circular pool divided into four quadrants, one of which (the target quadrant) contained a hidden platform submerged to a depth of approximately 2 cm beneath the water. The escape latency—i.e., the time the animal takes to find the hidden platform within 1 min—was automatically recorded by the software of Smart 3.0 during the 5-day locomotor memory learning process. If the platform was not found within 1 min, the observer would direct the animal to climb onto it and remain there for 20 s; in this event, the escape latency was recorded as 60 s. During the 5-day training period, each rat received three training sessions per day. The spatial exploration test was conducted on the sixth day following the removal of the platform. Automated video tracking was used to record the length of time the rats spent in the target quadrant, the number of times they crossed the target quadrant, and the patterns of their movements.

2.9 Western blot

Rat hippocampal tissues were extracted from freshly collected rat brains and homogenized on ice in lysis buffer using the Total Protein Extraction Kit (KGP2100, KeyGEN Biotechnology Co., Ltd., Jiangsu, China), which contains phosphatase inhibitors, protease inhibitors, and phenylmethanesulphonyl fluoride. The homogenized tissues were then centrifuged at −4°C. After the total protein concentration was measured with a BCA assay kit, the protein extract was denatured with a loading buffer containing SDS and boiled. Samples were laid on 10% acrylamide gels and subjected to electrophoresis. Separated proteins were transferred onto polyvinylidene difluoride membranes, incubated with primary and secondary antibodies (Table 1), and visualized using ECL reagent. Membranes were imaged in a chemiluminescence apparatus and saved as TIFF images. The band optical density was then quantified and standardized using ImageJ software. The above Western blot method was based on previous literature with minor modifications (Gao et al., 2019).

2.10 Experimental analysis of morphology

In this study, we performed three molecular imaging studies: immunofluorescence (IF), hematoxylin and eosin staining (HE), and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Five rats were randomly chosen from each group and anesthetized with pentobarbital sodium. After perfusing the heart of each rat with saline, the brain was carefully extracted and fixed with a 4% paraformaldehyde solution. A precision cryostat was used to slice brain sections of 4 μm in thickness. Three consecutive sections were taken from the hippocampal region of the rat brain and subsequently used for IF, HE, and TUNEL staining, respectively. After the sections were made (the specific sectioning process will be described in detail below), images were captured with a Leica DM6 microscope (Leica, Germany) and analyzed for fluorescence intensity or the number of positive cells compared with ImageJ software according to the specific instructions listed in Ref(Jensen, 2013). All rat hippocampal sections were analyzed by a technician (ML), who performed a blinded analysis on the data for each experimental group.

Before IF staining was performed, sections were washed with PBS, permeabilized with 0.2% Triton X-100 solution, and incubated in 5% bovine serum albumin to block non-specific binding. They were then incubated with primary antibody against BDNF (Abcam, ab205067, 1:500) overnight at 4°C, followed by incubation with goat anti-mouse 488-conjugate secondary antibody (Abcam, ab150113, 1:500) at 37°C for 1 h. Cell nuclei were then subjected to DAPI (Solarbio, S2110) staining. The sample slides were covered with Antifade Mounting Medium before images of them were obtained using a fluorescence microscope (Mirchandani-Duque et al., 2022).

HE staining was performed by staining the sections with hematoxylin for 3 min and subsequently with eosin for 1 min before dehydration. Images of the hippocampal region were captured under the DM6 microscope (Yu et al., 2022).

TUNEL staining began by incubating the sections in TUNEL reaction mixture in the dark for 60 min at a temperature of 37°C. Apoptotic cells that stained positively for TUNEL appeared brown and were counted under the DM6 microscope (Yu et al., 2022).

2.11 Transmission electron microscopy (TEM)

After anesthetizing the rats with pentobarbital sodium, their brains were promptly excised and the hippocampus was sectioned into 1 mm blocks. These tissues were immobilized in a TEM-specific solution (Cat#G1102, Servicebio) for 2 hours and subsequently washed twice with PBS. To enhance contrast, the tissues were stained with osmium acid, dehydrated using an alcohol-acetone mixture, and then embedded in resin. Further staining with uranyl acetate and lead prepared the samples for examination under a TEM using a Hitachi device. Quantitative analysis of the synaptic parameters, including synaptic length of active zone, synaptic interface curvature, synaptic cleft width, and post-synaptic density, was performed using ImageJ software (Cui et al., 2023).

2.12 Statistical analysis

The data were analyzed with SPSS 22.0 and GraphPad Prism 8.0. The mean value ± the standard error (SE) was used to express the data. Statistical analysis of the data was performed using one-way analysis of variance (ANOVA), after confirming the normality of the relevant datasets. We then performed *post hoc* tests using least significant difference (LSD) for pairwise comparisons. The statistical analysis was deemed significant at a level of $p < 0.05$.

3 Results

3.1 Effects of AR administration on SPS-induced anxiety-like behaviors, social interaction behaviors, and learning and memory behaviors

Rats that underwent SPS exhibited PTSD-like behaviors manifested as reduced activity in the central area of the OFT, reduced activity in the open arm of the EPMT, decreased sociability and preference for social novelties in the SIT, and impaired spatial learning and memory in the MWM test (Figure 2) (Zhao et al., 2020; Alzoubi et al., 2022; Sun et al., 2022). All of these behaviors suggestive of anxiety, avoidance, fear, and memory impairment were used as indicators to validate the establishment of the PTSD animal model. Administration of AR alleviated the manifestations of anxiety, depression, and memory impairment.

3.2 Effects of AR administration on SPS-induced anxiety-like behaviors

OFT is often used to assess anxiety in laboratory animals (Sadeghi et al., 2024); anxiety in the tested animals is reflected by their total entries into the field and the amount of time they spend in the center of the arena (Zhao et al., 2022), where higher degrees of anxiety are associated with less open-field exploration and more cautious behaviors (Zhang et al., 2021). The number of entries into the center and percentage of total movement spent in the center for the rats in four groups differed statistically significantly [($F(3, 76) = 3.481$, $p = 0.02$) and ($F(3, 76) = 4.249$, $p = 0.008$), respectively]. The data underwent a detailed analysis using the LSD *post hoc* test. In comparison rats in the Veh group, the SPS rats entered the center region significantly less often (LSD *post hoc* test, $p = 0.004$, Figure 2E) and traveled a significantly shorter distance in the central region during the OFT (LSD *post hoc* test, $p = 0.04$, Figure 2F). These two indicators significantly improved with the administration of AR—i.e., in the SPS + AR group (LSD *post hoc* test, $p = 0.027$ and $p = 0.002$, respectively).

Like the OFT, the EPMT is commonly used to monitor anxiety in laboratory animals. Specifically, the amount of time the rats spend in the two closed arms of the apparatus *versus* its two open arms, as well as the frequency of entries into the latter, can be used to assess their degree of anxiety (Qu et al., 2021; Sun et al., 2022). Higher degrees of anxiety are most often associated with less time spent in the open arms and more time spent in the closed arms (Berardi et al.,

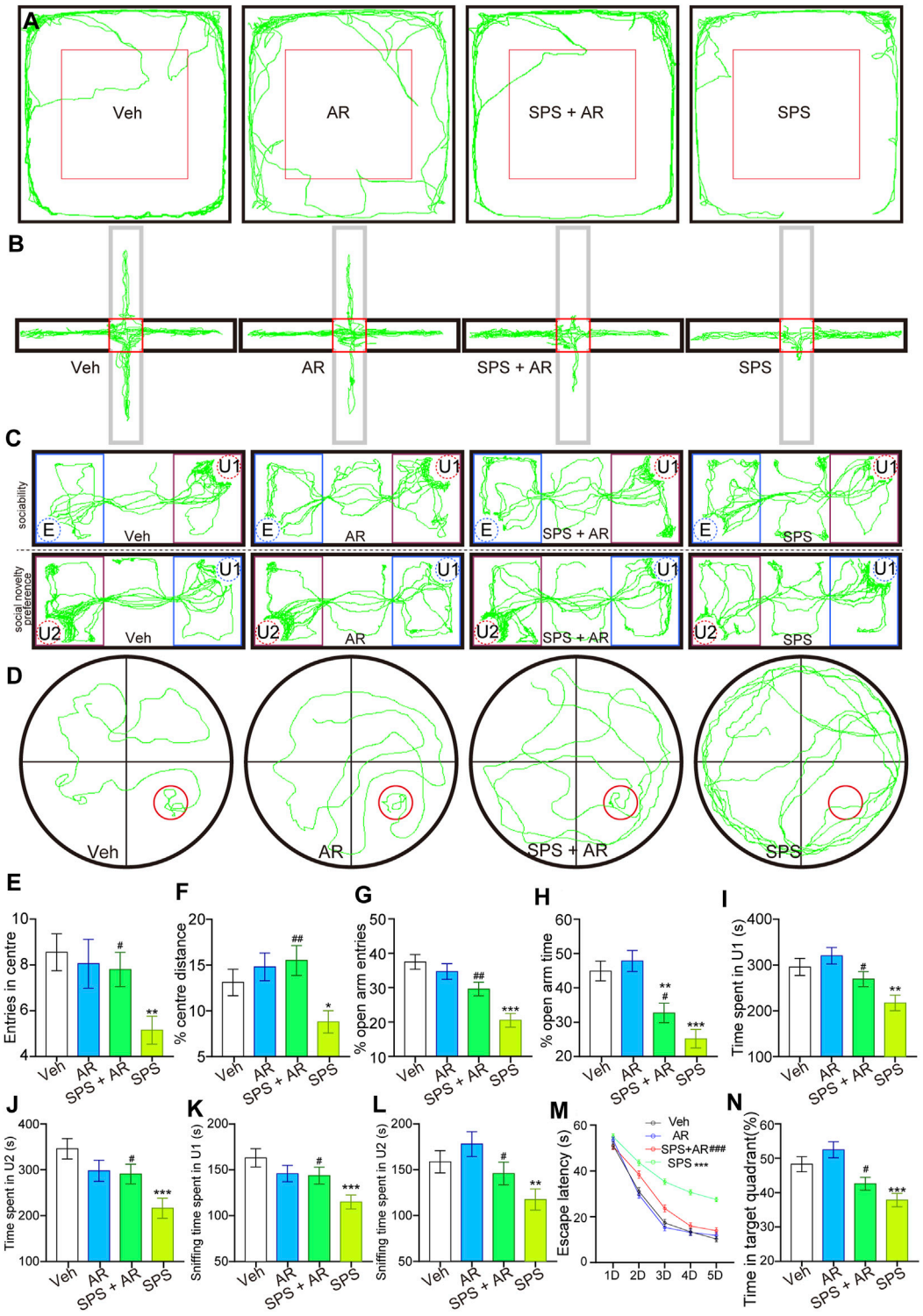

FIGURE 2
AR administration mitigated anxiety-like behaviors, social aversion, and learning and memory impairments mimicking PTSD symptoms in SPS rats. **(A)** Representative travel trajectories of rats in the OFT, **(B)** EPMT, **(C)** three-chamber SIT, and **(D)** MWM. **(E)** Number of entries into the center and **(F)** percentage of total movement spent in the center for the rats in each group during the OFT. **(G)** Percentage of entries into the open arm and **(H)** percentage of time spent in the open arm for the rats in each group in the EPMT. **(I)** Time spent by the tested rats in chamber U1. **(J)** Time spent by the tested rats in chamber U2. **(K)** Time the tested rats spent sniffing U1 rats. **(L)** Time the tested rats spent sniffing U2 rats. **(M)** Escape latency of the rats in each group on different test days. **(N)** Percentage of time the rats stayed in the target quadrant. The study used twenty rats per group. The data are represented as the mean ± SE. The data were analyzed using one-way ANOVAs followed by LSD post hoc tests; * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$, compared to the Veh group. # indicates $p < 0.05$, ## indicates $p < 0.01$, and ### indicates $p < 0.001$, the SPS + AR group vs. SPS group.

2014). Both the percentage of entries into the open arm and the percentage of time spent in it are statistically significant [(F (3, 76) = 3.481, $p = 0.02$, Figure 2G); (F (3, 76) = 13.504, $p < 0.001$, Figure 2H); respectively]. Detailed analysis is conducted on each group using the LSD *post hoc* test. The SPS rats made fewer entries into the open arms and spent notably less time in the open arms relative to the Veh group (LSD *post hoc* test, $p < 0.001$ and $p < 0.001$, respectively). However, AR administration in the SPS + AR group dampened this trend when compared to the SPS group for both indicators mentioned above (*post hoc* test, SPS + AR vs. SPS, $p = 0.003$ and $p = 0.049$, respectively).

3.3 Effects of AR administration on social interaction behaviors

The social behaviors and sociability of the rat models of PTSD were evaluated using the three-chamber SIT (Zhao et al., 2020; Zhao et al., 2022): a common assessment of the severity of PTSD-like symptoms such as social withdrawal and avoidance (Zhao et al., 2020). This test is also used to evaluate the efficacy of potential PTSD treatments by measuring changes in social behaviors and sociability following intervention (Mizrachi et al., 2022; Zhao et al., 2022). As shown in Figures 2C, I–L, significant differences are observed in all four indices of the three-chamber SIT across all four groups [Time spent by the tested rats in chamber U1: (F (3, 76) = 6.413, $p < 0.001$); Time spent by the tested rats in chamber U2: (F (3, 76) = 5.929, $p = 0.001$); Time the tested rats spent sniffing U1 rats: (F (3, 76) = 4.491, $p = 0.006$); Time the tested rats spent sniffing U2 rats: (F (3, 76) = 5.774, $p = 0.001$)]. Following this, detailed analyses are conducted using LSD *post hoc* tests. SPS rats spent significantly less time in chambers U1 or U2 relative to rats in the Veh group ($p = 0.002$ and $p < 0.001$, respectively). The time spent in chambers U1 and U2 increased significantly following the administration of AR (SPS + AR vs. SPS, $p = 0.04$ and $p = 0.027$, respectively). Consistently, as shown in Figures 2C, K, L, SPS rats spent significantly less time sniffing in chambers U1 or U2 than rats in the Veh group (LSD *post hoc* test, $p < 0.001$ and $p = 0.007$, respectively). AR administration significantly increased the sniffing time spent in chambers U1 and U2 (LSD *post hoc* test, in the SPS + AR vs. SPS, $p = 0.045$ and $p = 0.05$, respectively).

3.4 Effects of AR administration on learning and memory behaviors

The MWM test can help to detect deficits in learning, memory, and spatial navigation in murine models of PTSD and evaluate the efficacy of potential PTSD treatments (Wen et al., 2016; Liu et al., 2020; Niu et al., 2022). Specifically, the MWM test assesses cognitive impairment based on the animal's ability to navigate to a submerged platform using memory and spatial cues (Mizrachi et al., 2022). Hence, if a drug or therapy alleviates the spatial learning and memory deficits in animals with PTSD-like symptoms as demonstrated by the MWM test, the intervention shows promise as a treatment for humans with PTSD (Mizrachi et al., 2022). The results presented in Figures 2D, M, N indicate that AR administration achieved a significant improvement in SPS-induced cognitive impairment. With grouping (Veh, AR, SPS + AR, SPS) as the between-subjects factor and test point (D1-D5) as the within-subjects factor, a two-way repeated measures ANOVA determined a significant effect of both grouping and test sessions (D1-D5) on the performance in the MWM [F (3, 76) = 28.909, $p < 0.001$ for between-subjects effects, F (4, 76) = 1654.42, $p < 0.001$ for within-subjects effects]. An interaction between grouping and test session was also observed [F (4, 76) = 20.65, $p < 0.001$]. Furthermore, when employing the LSD *post hoc* test, it was observed that the escape latencies were significantly prolonged in the SPS rats in comparison to the Veh rats ($p < 0.001$). As anticipated, the administration of AR for the SPS + AR rats mitigated this behavior in comparison to the SPS rats ($p < 0.001$). Afterwards, the percentage of time the rats stayed in the target quadrant differed significantly among the four groups (F (3, 76) = 11.416, $p < 0.001$, Figure 2N). Subsequently, the SPS group spent significantly less time exploring the quadrant containing the target platform than did the Veh group (LSD *post hoc* test, $p < 0.001$). Importantly, AR administration significantly increased the amount of time spent staying in the target quadrant (LSD *post hoc* test, SPS + AR vs. SPS, $p = 0.045$).

3.5 Effects of AR administration on the expression of synaptic plasticity-related proteins

As shown in Figures 3A–D, Western blotting was performed to evaluate the levels of synaptic plasticity-associated proteins PSD95, BDNF, and Synapsin I in each group of rats. The analysis revealed significant variations in the levels of these proteins between the groups ($p < 0.001$, one-way ANOVA). LSD *post hoc* pairwise comparisons showed that the SPS group exhibited notably lower levels of these proteins when compared to the other groups. Importantly, relative to the SPS group, the SPS + AR group exhibited a significant elevation in these protein levels (PSD95: $p = 0.005$; BDNF: $p = 0.006$; Synapsin I: $p = 0.014$; LSD *post hoc*). Consistently, as shown in Figures 3E–H, the results of the immunofluorescence assay showed significant differences in the fluorescence intensity of BDNF in the four groups (DG: F (3, 16) = 4.819, $p = 0.014$; CA1: F (3, 16) = 7.144, $p = 0.003$; CA3: F (3, 16) = 5.842, $p = 0.007$). The fluorescence intensity of BDNF protein was significantly lower in SPS rats compared to rats in the Veh group, as indicated by the LSD *post hoc* test (DG: $p = 0.003$; CA1: $p = 0.002$; CA3: $p = 0.003$). Administration of AR significantly reversed this trend, with p values of 0.026, 0.049, and 0.049 for DG, CA1, and CA3, respectively, in the SPS + AR group compared to the SPS group (LSD *post hoc* test).

3.6 Effects of AR admin istration on the ultrastructure of synapses in the CA1 following SPS

As shown in Figures 4A–E, ultrastructural observations observed under TEM revealed significant differences in the synaptic cleft width, postsynaptic density, synaptic interface curvature, and synaptic length of the active zone in CA1 between

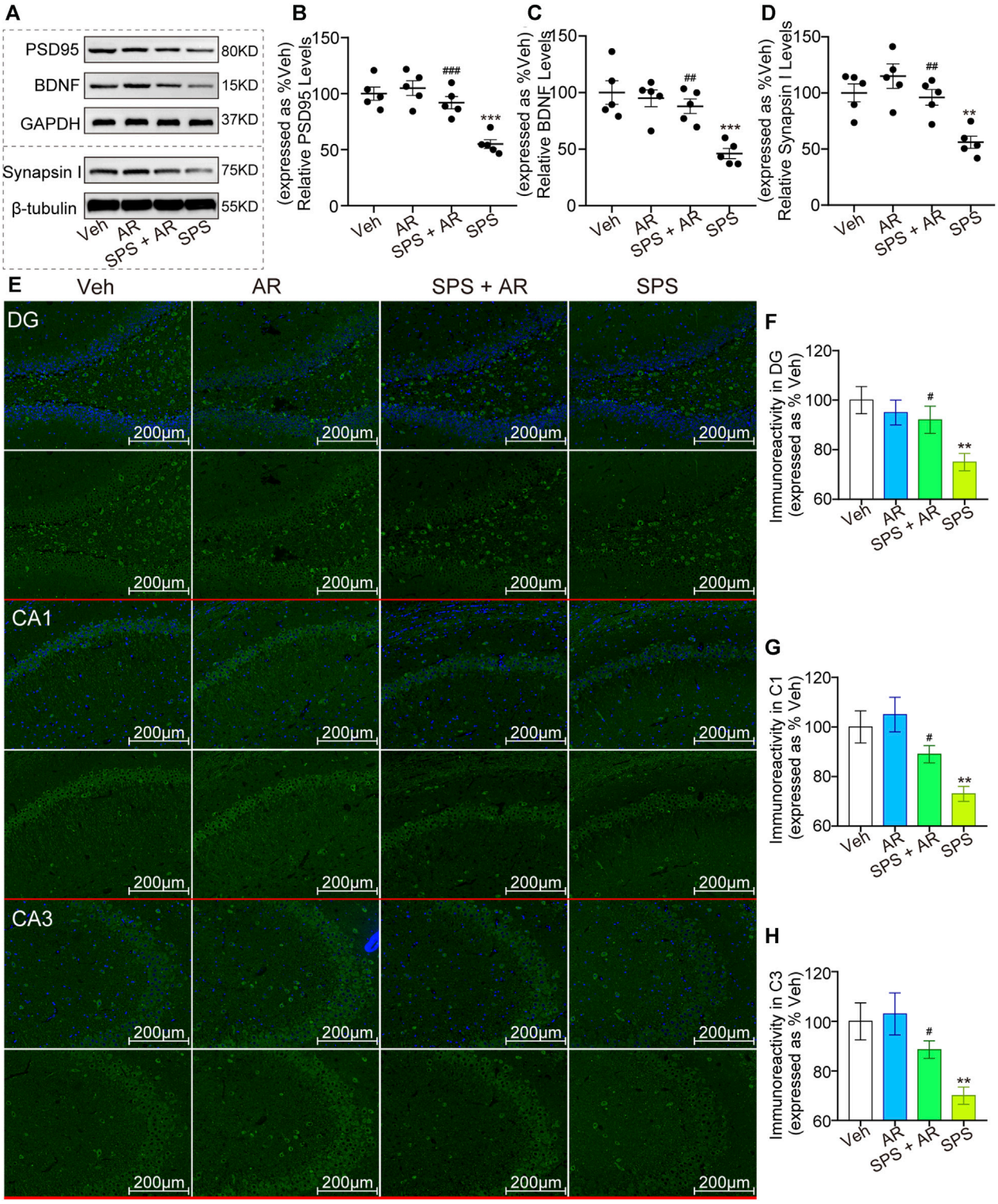

FIGURE 3
AR administration increased the levels of synaptic plasticity-related proteins PSD95, BDNF, and Synapsin I in rats that underwent SPS. **(A)** Representative immunoblots of hippocampal BDNF, PSD95, and Synapsin I for the four groups of rats. **(B–D)** Semi-quantitative analysis of expressions of PSD95, BDNF, and Synapsin I. **(E)** Representative immunofluorescence images of BDNF in the DG, C1, and C3 regions of the hippocampus. **(F–H)** Immunoreactivity analysis of BDNF in DG, CA1, CA3, respectively. Scale bars: 200 μm. The study used five rats per group. The data are represented as the mean ± SE. The data were analyzed using one-way ANOVA followed by an LSD *post hoc* test; * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$, compared to the Veh group. # indicates $p < 0.05$, ## indicates $p < 0.01$, and ### indicates $p < 0.001$, the SPS + AR group vs. SPS group.

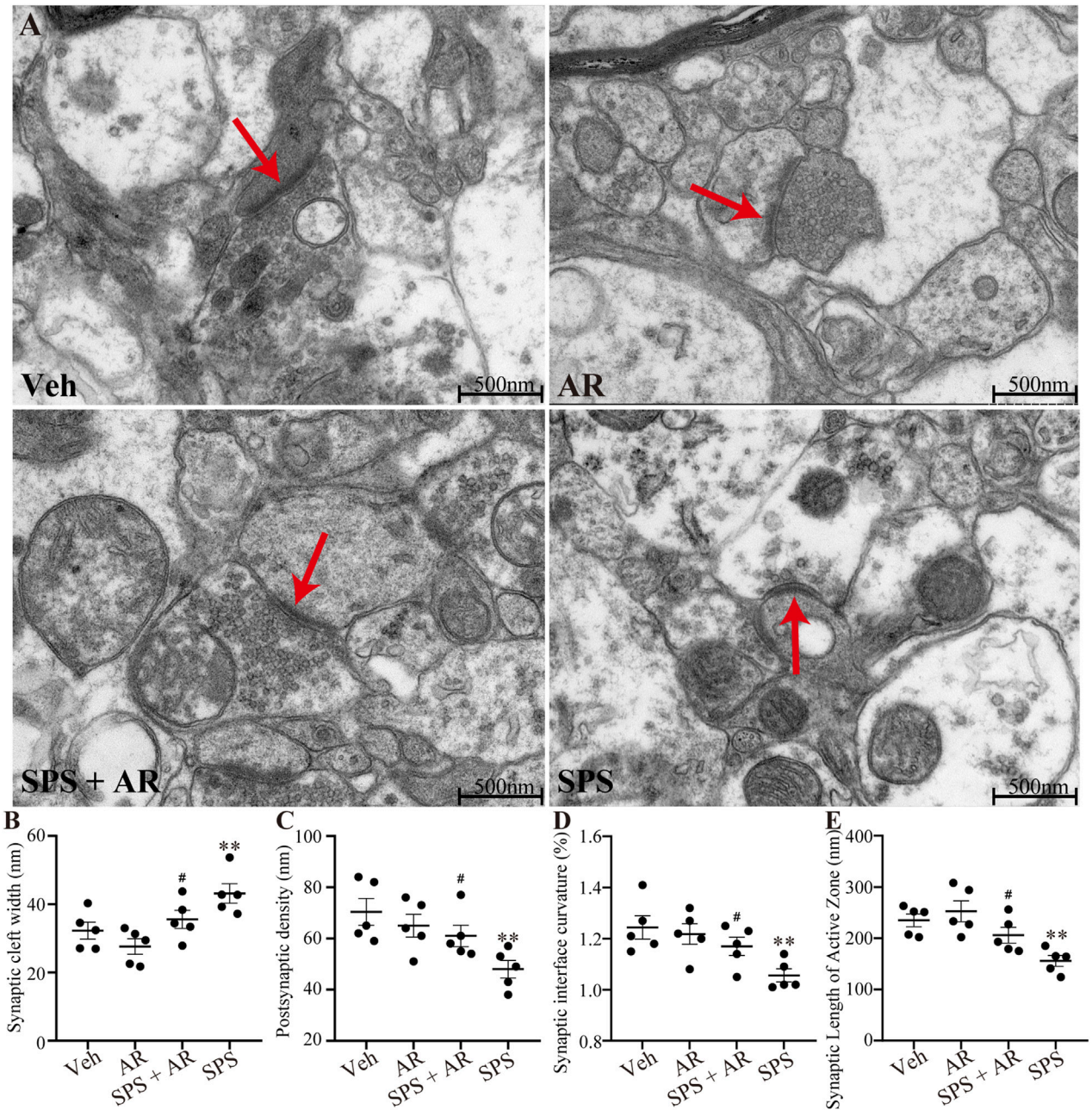

FIGURE 4
Changes in the ultrastructure of synapses in the CA1 region in five images randomly selected from each group for analysis with TEM. **(A)** Representative images of synapses in the hippocampal CA1 region for the four groups of rats. **(B–E)** The synaptic cleft width, postsynaptic density, synaptic interface curvature, and synaptic length of the active zone in CA1. The study used five rats per group. Red arrows indicate presynaptic membranes. The data are represented as the mean ± SE. The data were analyzed using one-way ANOVAs followed by LSD *post hoc* tests; * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$, compared to the Veh group. # indicates $p < 0.05$, the SPS + AR group vs. SPS group.

the different groups of rats (F (3, 16) = 6.408, $p = 0.005$; F (3, 16) = 4.766, $p = 0.014$; F (3, 16) = 4.894, $p = 0.013$; F (3, 16) = 7.716, $p = 0.002$, respectively, one-way ANOVA). Further analysis with LSD *post hoc* tests identified significant differences in the aforementioned four indicators between the SPS and the SPS + AR groups (synaptic cleft width, $p = 0.049$; postsynaptic density, $p = 0.045$; synaptic interface curvature, $p = 0.048$; and synaptic length of the active zone, $p = 0.033$).

3.7 Effect of AR administration on apoptosis of HIP neurons following SPS

As shown in Figures 5A–D, Western blotting was used to evaluate the expression of apoptosis regulators Caspase 3, Bcl 2, and Bax in the hippocampal neurons of rats in each of the four groups. The analysis revealed significant variations in protein levels between the groups (Caspase 3, F (3, 16) = 8.894, $p = 0.001$; Bcl 2, F

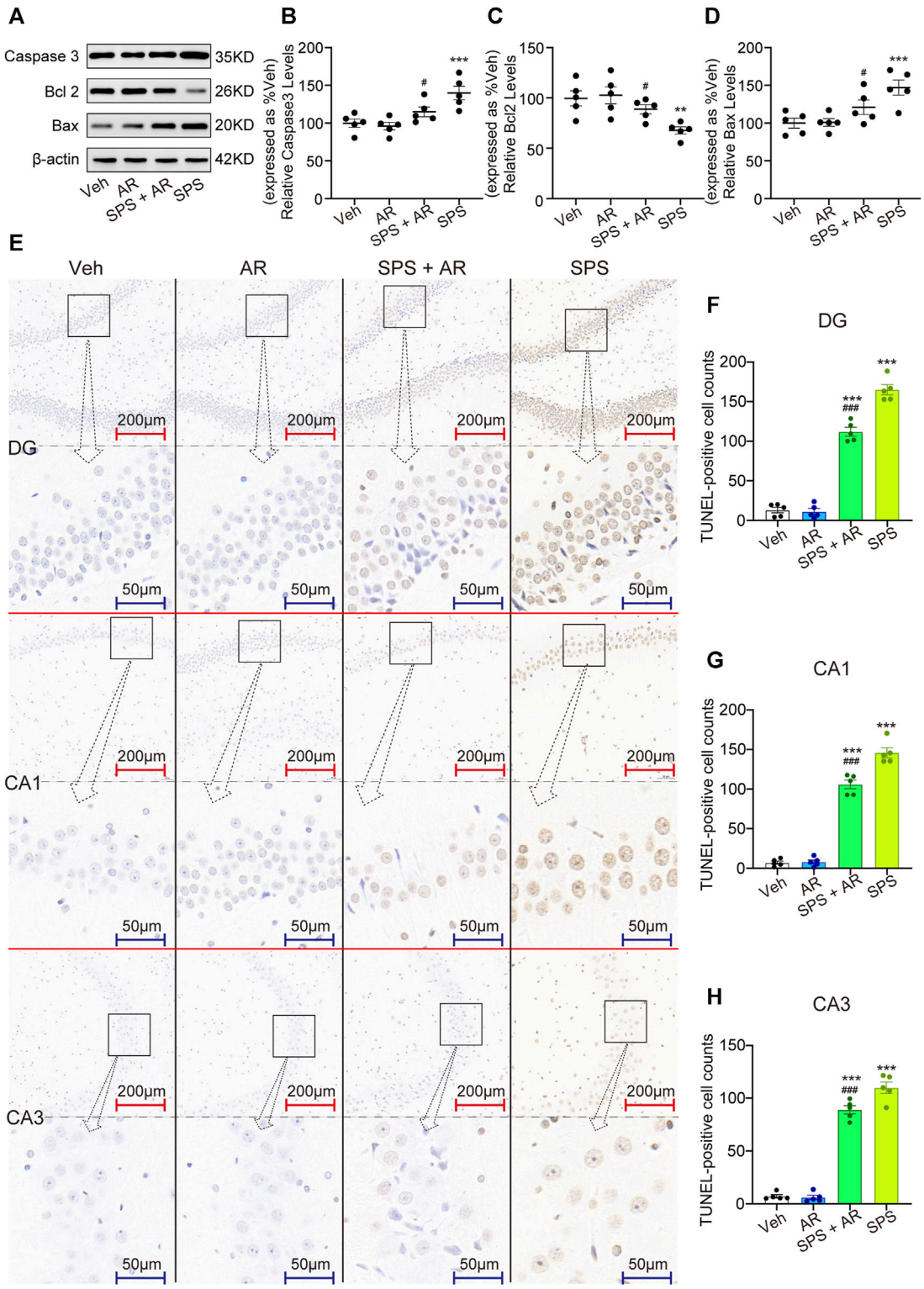

FIGURE 5
AR administration attenuates neuroapoptosis in rats that underwent SPS. **(A)** Representative immunoblots of hippocampal Caspase 3, Bcl 2, and Bax for the four groups of rats. **(B–D)** Semi-quantitative analysis of Caspase 3, Bcl 2, and Bax expression. **(E)** Representative images of TUNEL staining of the hippocampal DG, C1, and C3 regions for each group. **(F–H)** Changes in apoptotic cells for each group as revealed by TUNEL staining of the hippocampus. The study used five rats per group. The data are represented as the mean ± SE. The data were analyzed using one-way ANOVAs followed by an LSD *post hoc* test; * indicates $p < 0.05$, ** indicates $p < 0.01$, and *** indicates $p < 0.001$, compared to the Veh group. # indicates $p < 0.05$, ## indicates $p < 0.01$, and ### indicates $p < 0.001$, the SPS + AR group vs. SPS group.

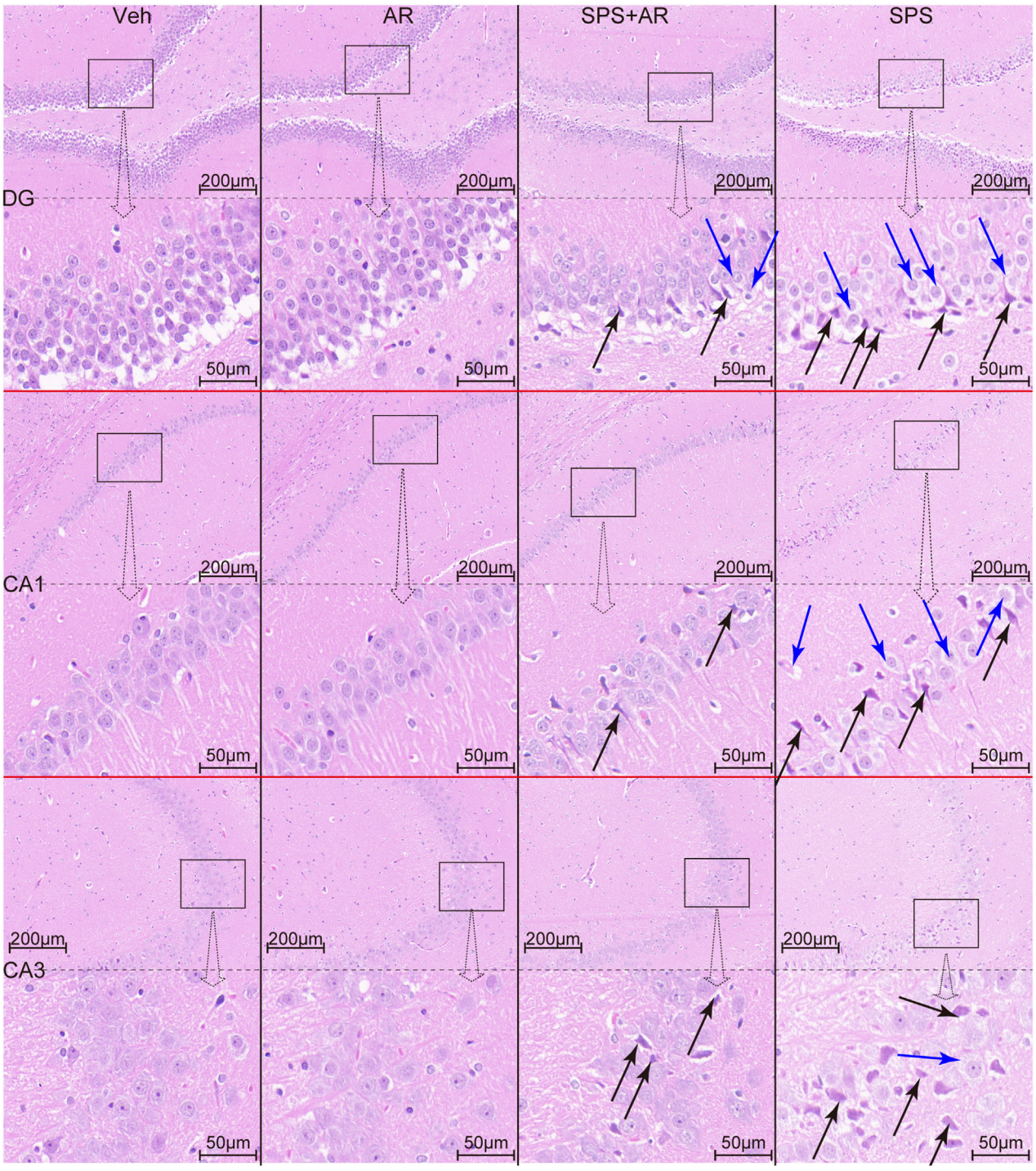

FIGURE 6
HE staining reveals the histopathological changes in hippocampal neurons of rats in each group. Black arrows indicate neuronal consolidation, deep staining, and increased basophilia. Blue arrows indicate neuronal hydropic degeneration and cytoplasmic vacuolization.

(3, 16) = 6.244, $p = 0.005$; and Bax, F (3, 16) = 7.563, $p = 0.002$; one-way ANOVA). LSD *post hoc* pairwise comparisons showed that the SPS group exhibited notably lower levels of these proteins when compared to the Veh group (Caspase 3, $p = 0.001$; Bcl 2, $p = 0.005$, Bax, $p = 0.002$). Administration of AR significantly reversed this trend, with p values of 0.018, 0.033, and 0.036 for Caspase 3, Bcl 2, Bax, respectively, in the SPS + AR group compared to the SPS group (LSD *post hoc* test). As shown in Figures 5E–H, the results TUNEL staining experiment agreed with those obtained from the Western blots: significant differences were found in the number of TUNEL-positive cells in the three subregions of the hippocampus between different groups (DG: F (3, 16) = 230.36, $p < 0.001$; CA1: F (3, 16) = 239.253, $p < 0.001$; CA3: F (3, 16) = 225.306, $p < 0.001$). The fluorescence intensity of BDNF protein was significantly lower in

SPS rats compared to rats in the Veh group, as indicated by the LSD *post hoc* test (DG: $p = 0.003$; CA1: $p = 0.002$; CA3: $p = 0.003$). LSD *post hoc* tests showed that the number of TUNEL-positive cells was significantly increased in three subregions of the hippocampus of rats in the SPS group compared with rats in the Veh group (three hippocampal subregions: $p < 0.001$). Administration of AR significantly reversed this trend, with *p*-values less than 0.001 for DG, CA1, and CA3 in the SPS + AR group compared to the SPS group.

Finally, HE staining was performed to identify any pathological changes in the hippocampal brain tissue samples obtained from each group. We observed shrinkage, deep staining, unclear demarcation between the nucleus and cytoplasm, enhanced basophilia, and sparse cell arrangement (indicated by black arrows in Figure 6) in neurons in the hippocampal DG region, CA1 region, and CA3 region of rats in the SPS group. We also found significant neuronal hydropic degeneration and cytoplasmic vacuolization (indicated by blue arrows in Figure 6). AR administration significantly attenuated the histopathological damage and improved vacuolization.

4 Discussion

The present study evaluated the effects of AR on PTSD-like behavioral phenotypes, hippocampal synaptic plasticity, and hippocampal neuronal apoptosis in rats subjected to SPS. The administration of AR to SPS rats is herein demonstrated to enhance OFT central area activity and EPMT open arm activity, increase sociability and preference for social novelty in the SIT, and alleviate impaired spatial learning and memory in the MWM test. This evidence suggests that AR may improve symptoms related to PTSD—particularly anxiety, social functional impairment, and cognitive dysfunction. The present study further observed that administering AR to SPS rats improved synaptic plasticity by upregulating synaptic plasticity-related proteins PSD95, BDNF, and Synapsin I. The reduction of SPS-induced neuronal apoptosis is consistent with AR's known anti-apoptotic properties. A growing body of evidence demonstrates that AR is a multifunctional neuroprotective agent that may be used to treat PTSD.

The present study observed significant anxiety-like behavior, social interaction deficits, and impaired learning and memory in rats subjected to SPS. Consistent with the hypothesis of this study, AR administration to rats that underwent SPS was found to attenuate the behavioral consequences of SPS. While the "SPS + intervention" behavioral pharmacology paradigm adopted in this investigation has featured broad use in the literature in many studies, our use of AR as the intervention is relatively unique: e.g., Chen YL et al. (PMID: 35259352)found that MiR-153 downregulation alleviates PTSD-like behaviors in SPS rats (Chen et al., 2022), while Chen Y et al. (PMID: 35753367) observed that MicroRNA-124 attenuates PTSD-like behaviors in SPS rats (Chen et al., 2022). These reports fully demonstrate that the behavioral pharmacology paradigm of "SPS + intervention" is well-established in the study of the pathogenesis and possible treatment of PTSD. Hence, the successful construction of a rat model of simulated PTSD with SPS in the present study helps to validate our findings concerning the therapeutic benefits of AR.

While maladaptive social interactions are common among patients with PTSD (Collimore et al., 2010; Stevens and Jovanovic, 2019; Bjornsson et al., 2020; Sharma et al., 2020; Laban et al., 2021), most research on the behavioral characterization of SPS rats has focused on anxiety-like behaviors and learning memory deficits (Schoner et al., 2017; Hou et al., 2021; Lo et al., 2023). Hence, the present investigation was further innovative in its focus on impaired social interactions in SPS rats. We found that SPS intervention induced social impairment and social novelty preference disorder. AR administration attenuated the social impairment and social novelty preference disorder induced by SPS intervention.

PTSD usually results in social impairment due to several interrelated factors. First, patients with PTSD experience strong anxiety and fear responses in social situations (Collimore et al., 2010; Bjornsson et al., 2020). Our observation that SPS rats avoided social interactions and spent less time in the central area of the OFT and the open arm in of the EPMT reflects the above-mentioned symptoms in patients with PTSD. Second, the cognitive deficits associated with PTSD—which leads to diminished attention and focus that exacerbates difficulties in social interactions—are paralleled by the impaired spatial navigational learning memory demonstrated by the SPS rats in the MWM (Stevens and Jovanovic, 2019). The methodology of the present study underscores the need to standardize investigations of animal sociability in basic research on PTSD. Furthermore, as demonstrated by our findings, studying animal sociability helps to assess the effect of interventions (in this study, AR administration) on the integrated treatment of PTSD.

The present investigation observed that administering AR to rats that underwent SPS significantly increases the expression of synaptic plasticity-related proteins PSD95, BDNF, and Synapsin I in the hippocampus, positively affects synaptic ultrastructure, and inhibits neuronal apoptosis. In the context of our behavioral experiments that confirm that AR significantly alleviates PTSD-like phenotype in SPS rats, these molecular studies suggest that AR's neuroprotective effects in SPS rats may be ascribed to its positive effects on synaptic plasticity and inhibition of neuronal apoptosis. A large number of preclinical studies have investigated the therapeutic effects of drugs or other therapeutic strategies on PTSD symptoms by studying changes in synaptic plasticity and neuronal apoptosis (Li et al., 2013; Ji et al., 2019; Feng et al., 2020; Jiang et al., 2022; Li et al., 2022): e.g., Sevoflurane has been shown to attenuate apoptosis and improve synaptic plasticity in the hippocampus of rat models of PTSD (Gu et al., 2023), and down-regulating MiR-153 has been observed to alleviate PTSD-like behaviors by affecting synaptic plasticity and apoptosis (Chen et al., 2022). Hence, the present findings that AR enhances synaptic plasticity and inhibits neuronal apoptosis suggest that it has potential as an effective therapeutic agent in the treatment of PTSD.

Although this study is, to the best of our knowledge, the first to investigate whether AR has a therapeutic effect on PTSD-like symptoms, a large body of literature has reported that AR improves synaptic plasticity and attenuates neuronal apoptosis in models of multiple neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease, Huntington's disease, and multiple sclerosis (Zhao et al., 2020; Arthur et al., 2023). Poorgholam et al. found that artemisinin improved synaptic plasticity in rat models of Alzheimer's disease and diabetes (Poorgholam et al., 2023), while Xia et al. observed that administering Dihydroartemisinin could have a therapeutic effect

on impaired synaptic plasticity caused by tauopathies (Xia et al., 2021). This study expands upon this understanding of the use of AR in promoting neuroprotection by expanding its potential clinical application to the treatment of PTSD.

The present study is subject to two limitations. First, we only considered the neurobiological changes in the rat hippocampus. While this was justified by our focus on investigating the mechanism by which AR affects the behavior of SPS rats, other brain regions (e.g., prefrontal cortex and amygdala) are also involved in the acquisition of fear memories (Shin et al., 2006; Alexandra et al., 2022; Ressler et al., 2022). Research on SPS-induced molecular and biological changes in other neural regions, as well as the effect of AR on these changes in those regions, should also garner our necessary attention. Second, we prioritized the study of AR's effects on synaptic plasticity and neuronal apoptosis without sufficiently investigating other factors contributing to the pathogenesis of PTSD, such as neuroinflammation or epigenetic changes (Al et al., 2021; Lv et al., 2023). A comprehensive understanding of the effects of AR on these factors could bolster support for its potential as a therapeutic agent.

5 Conclusion

By confirming that AR improves synaptic plasticity and inhibits neuronal apoptosis in a rat model of PTSD, the present report provides evidence for the therapeutic potential of AR in the treatment of PTSD. Our findings further contribute to a greater understanding of the multifaceted neuroprotective effects of AR and provide the necessary preclinical data to support future investigation of its application to the treatment of PTSD.

Data availability statement

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

Ethics statement

The animal study was approved by The Animal Ethics Committee of Weifang Medical University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

QL: Writing–original draft. XD: Writing–review and editing. YW: Writing–original draft. HC: Writing–original draft. YG: Writing–original draft. ML: Writing–review and editing. KS: Writing–original draft, Writing–review and editing.

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Conflict of interest

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

Publisher's note

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Effect of a high dose atorvastatin as added-on therapy on symptoms and serum AMPK/NLRP3 inflammasome and IL-6/STAT3 axes in patients with major depressive disorder: randomized controlled clinical study

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Background: Neuroinflammation pathways have been associated with the development of major depressive disorders (MDD). The anti-inflammatory characteristics of statins have been demonstrated to have significance in the pathophysiology of depression.

Aim: To investigate the mechanistic pathways of high dose atorvastatin in MDD.

Patients and methods: This trial included 60 patients with MDD who met the eligibility requirements. Two groups of patients (n = 30) were recruited by selecting patients from the Psychiatry Department. Group 1 received 20 mg of fluoxetine plus a placebo once daily. Group 2 received fluoxetine and atorvastatin (80 mg) once daily. All patients were assessed by a psychiatrist using the Hamilton Depression Rating Scale (HDRS). A HDRS score of ≤7 indicates remission or partial remission [HDRS<17 and>7]. Response was defined as ≥50% drop in the HDRS

score. The serum concentrations of nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP-3), interleukin-6 (IL-6), adenosine monophosphate activated protein kinase (AMPK), and signal transducer and activator of transcription factor-3 (STAT-3) were measured.

Results: The atorvastatin group showed a significant reduction in the levels of all measured markers along with a statistical increase in the levels of AMPK when compared to the fluoxetine group. The atorvastatin group displayed a significant decrease in HDRS when compared to its baseline and the fluoxetine group. The response rate and partial remission were higher in the atorvastatin group than fluoxetine (p = 0.03, and p = 0.005), respectively.

Conclusion: These results imply that atorvastatin at high doses may be a promising adjuvant therapy for MDD patients by altering the signaling pathways for AMPK/NLRP3 and IL-6/STAT-3.

Clinical Trial Registration: clinicaltrials.gov, identifier NCT05792540.

KEYWORDS

atorvastatin, major depressive disorder, neuroinflammation, NLRP-3, STAT-3

1 Introduction

Major depressive disorder (MDD) affects approximately 16% of people worldwide, a major cause of disability that has been associated with ongoing inflammatory conditions (Wittenberg et al., 2020). Though the existing antidepressants are generally successful and focus on the monoamine and serotonin pathways, more than 30% of patients rarely fully recover (Wittenberg et al., 2020). There is evidence that the immunological and neurological systems are strongly correlated (Leonard, 2018). Inflammations, whether acute or chronic, can have a direct or indirect impact on how the central nervous system (CNS) works. Tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6 are examples of proinflammatory mediators that can directly activate neutrophils and macrophages, leading to the generation of oxygen and nitrogen free radicals (Jo et al., 2015). Alternatively, cytokines have an indirect effect on the CNS by altering the monoaminergic systems that are the main target of FDA-approved antidepressant drugs. Thus, a rise in cytokine levels causes a reduction in serotonin levels by activating the metabolic pathways for competing tryptophan and by increasing its uptake (Jo et al., 2015). Additionally, it has been noted that MDD is mostly associated with an increase in these proinflammatory cytokines (Jo et al., 2015).

Although the causes of MDD are still unclear, the NLRP3 inflammasome has been linked to the development of depression (4). First, it has been discovered that rodent depression models and people with MDD both have active NLRP3 inflammasomes (Taene et al., 2020). Second, a functional NLRP3 inflammasome is necessary for depressed behaviors brought on by stress, while NLRP3 inhibition prevents these stress-like effects (Du and Bu, 2020). Third, antidepressant therapy may prevent the activation of the NLRP3 inflammasome (Du and Bu, 2020). These results postulated that the NLRP3 inflammasome might be a major target for novel depression therapeutic approaches. AMP-activated protein kinase (AMPK) is a ubiquitous fuel-sensing enzyme that has a vital role in the control of the metabolism in cells by increasing glucose and lipid absorption and activating the rate of oxidation to optimize cellular energy consumption (Monophosphate, 2017). Additionally, AMPK activation is believed to counter many cellular disturbances, such as inflammation, insulin resistance, and abnormal fat deposition (Ruderman et al., 2013). Crosstalk between AMPK and NLRP3 inflammasome signaling is thought to exist. NLRP3 activity is decreased by AMPK activation (Yang et al., 2019).

Converging evidence from research in human patients and corresponding animal models of the disease suggests that the proinflammatory cytokine interleukin 6 (IL6) plays a role in the pathophysiology of depression (Mohamed et al., 2018; Hammad et al., 2021). The most widely prescribed antidepressant medications, selective serotonin reuptake inhibitors, or SSRIs, primarily act through the serotonin transporter (SERT, SLC6A4). SERT activity shapes serotonergic neurotransmission, which is linked to the pathophysiology and behavioral traits of depression (Blier and El Mansari, 2013; Thorne, 2023). The binding of IL-6 to its receptor (IL-6R) commences the IL-6 trans-signaling mechanism in MDD. The resultant IL-6/IL-6R complex interacts with the gp130 component (Ting et al., 2020). When gp130 interacts with this complex, it activates Janus kinases (JAK) and the signal transducer and activator of transcription factor 3 (STAT-3), a downstream effector signal (Guan et al., 2021). STAT-3 was discovered to be increased in MDD patients (Guan et al., 2021). The inhibition of IL-6-stimulated STAT3 activation reduced the severity of depressive-like behavior (Kong et al., 2015). These findings emphasize the role of IL-6 and STAT3 molecular signaling in the evolution of MDD.

The monoaminergic, cholinergic, and glutamatergic systems, which have been linked to a number of neuropsychiatric illnesses, are all affected by statins in a broad range of neurotransmission processes. Such changes in neurotransmitter levels can be explained by both cholesterol-dependent and independent (such as anti-inflammatory and antioxidant) mechanisms (Giorgi Riccardo et al., 2023). Peroxisome proliferator-activated receptor (PPAR) is a receptor that is liganded by statins and regulates the expression of neurotrophins such as brain-derived neurotrophic factor (BDNF) (Giorgi Riccardo et al., 2023). Drug repositioning, or drug repurposing, is an effective strategy to find new indications for existing drugs. This strategy has been used

with success across multiple diseases. Numerous studies support the idea that taking statins may have a favorable effect on mood, and several preclinical studies investigated the neuroprotective effect of statins on depressive behavior and reduced inflammation among MDD patients (Taniguti et al., 2019; Hai-Na et al., 2020; Massardo et al., 2022; Xiao et al., 2023). Haghighi et al. investigated the role of atorvastatin in alleviating MDD symptoms, but they did not measure any serum biomarkers (Haghighi et al., 2014). Therefore, the current research aimed at discovering the mechanistic role of atorvastatin in patients with MDD by modulation of AMPK/NLRP3 and IL-6/STAT-3 Signaling Pathways.

2 Patients and methods

From June 2023 to December 2023, the research was conducted at Tanta University's Faculty of Medicine's Psychiatry Department. The outpatient clinic recruited 60 eligible participants who fulfilled the inclusion criteria for the study. The Institutional Review Board at Tanta University Faculty of Medicine gave its acceptance for this work with the approval code (36264PR197/5/23). The study's methodology and design adhered to the Helsinki Declaration and its 1964 revisions. Patients were told they could withdraw from the trial at any moment. The exposure type and randomization were blinded by patients and doctors. An unblinded pharmacist provided study drugs to participants to guarantee accurate treatment assignment; however, the pharmacist was not included in outcome evaluations.

2.1 Inclusion criteria

HAM-D score >18, with item 1 (depressed mood) scored two or above, and patients aged ≥18 years with a diagnosis of MDD based on the DSM-IV Mini-International Neuropsychiatric Interview (MINI) (Sheehan et al., 1998; Svenaeus, 2013) were eligible for the study. This study included only newly diagnosed cases of MDD.

2.2 Exclusion criteria

Patients suffering from personality disorders, eating disorders, drug abuse, or bipolar I or II disorders.

Patients having an active medical condition concurrently.

Patients who have previously experienced seizures or are undergoing electroconvulsive therapy (ECT).

Patients with known drug allergies or other contraindications.

Pregnant or lactating females.

Patients with hyperlipidemia and/or statin usage (defined as TC more than 200 mg/dL, or LDL more than 140 mg/dL, or TG more than 150 mg/dL).

Patients with diabetes, and hyperthyroidism.

2.3 Study design

This clinical trial was prospective, parallel, randomized, and double-blinded. ClinicalTrials.gov received this trial's registration as NCT05792540 in March 2023. This study was planned with three arms and registered on clinicaltrials.gov. Here we have data for only two arms. The participants were randomly divided into two groups (n = 30), as shown by the CONSORT flow diagram in Figure 1.

Atorvastatin dose selection was based on a network meta-analysis that recommended high-intensity atorvastatin in a high dose as the best choice for treating depression (Sparks et al., 2005; Lee et al., 2021). The patients were monitored for any adverse effects, including muscle pain, joint pain, or any other adverse effects. Blood-borne markers of liver dysfunction were also monitored as indicators of possible adverse events known to accompany the administration of atorvastatin 80. In addition, the fixed dose was chosen to preserve external validity, prevent blinding, and lessen the possibility of post-randomization biases.

Randomly permuted blocks were chosen for randomization using a computer random number generator. Before taking part in the study, patients had to be off all anti-inflammatory and psychiatric drugs that were used on demand, such as anxiolytics, hypnotics, and psychotropic drugs, for at least 4 weeks. Group 1: control group (n = 30) who received fluoxetine (20 mg) plus placebo once daily for 3 months (Philozac® 20 mg capsule, Amoun, Egypt). Group 2: atorvastatin group (n = 30) who received fluoxetine (20 mg) once daily plus atorvastatin 80 mg once daily for 3 months (Ator® 80 mg tablets, Epico, Egypt). Placebo tablets were manufactured by Zeta Pharma Company and had the same look as atorvastatin tablets.

2.4 Sample size calculation

Based on HDRS data as the primary dependent variable from prior trials using statin adjuvant therapies in MDD (Ghanizadeh and Hedayati, 2013; Haghighi et al., 2014; Gougol et al., 2015), with an average effect size of 0.8, a type I error of 0.05, and a study power of 80%. The sample size was calculated to be 30 patients per group, taking into consideration a 20% dropout rate.

2.5 Study protocol

A psychiatrist evaluated the patients at baseline and 3 months after they received the medication. Patients were also questioned regarding drug adherence and potential adverse effects. Patients were contacted every week by phone to check on their compliance with the study drug, any negative side effects, and any indications of infection or inflammation. All medications were taken orally. To assess patient adherence to therapy, the quantity of tablets left in each supply delivered to the patients was counted. Only in cases of an emergency that called for knowledge of the current course of therapy should the responsible psychiatrist break the blinding. The participant would be removed from the trial once the blinding was broken. If a participant stopped taking the study drug for 7 days in a row, they were also removed from the research.

2.6 Sample collection

10 mL of venous blood was taken from the antecubital vein before the trial began and 3 months after the intervention. To

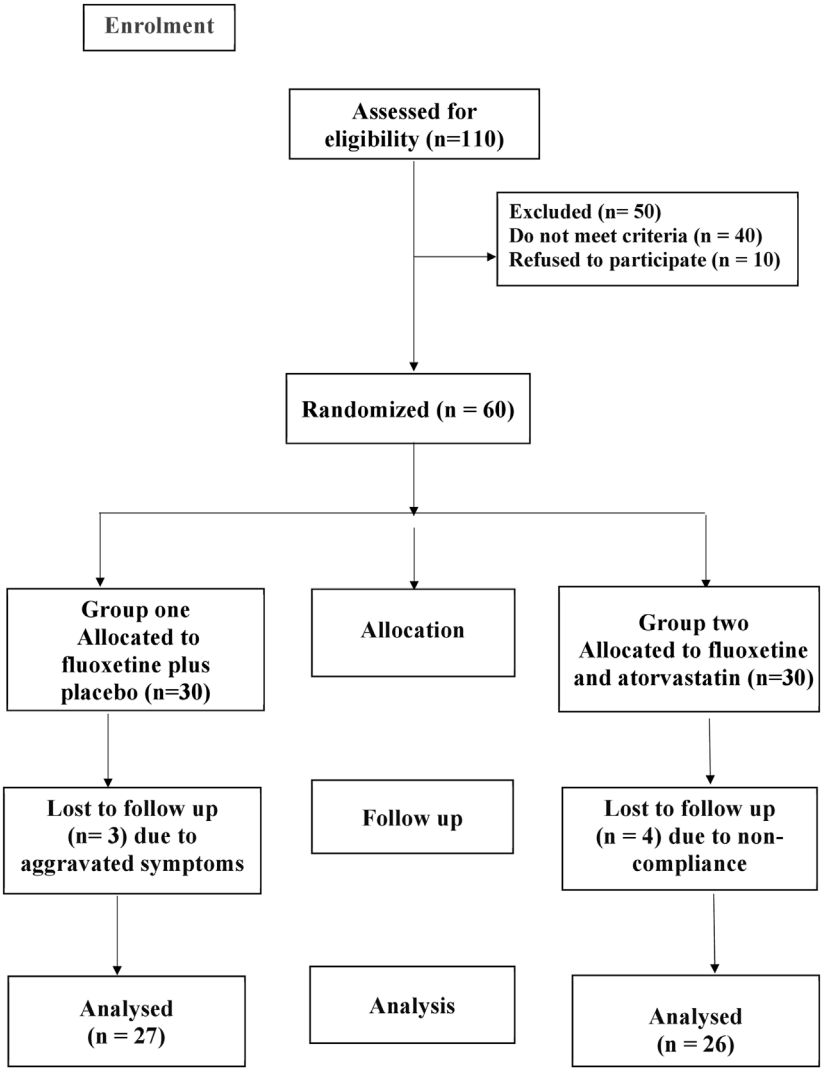

FIGURE 1
CONSORT diagram showing flow chart of the patient participation.

centrifuge the blood for 10 minutes at a speed of 4500 g (Hettich Zentrifugen EBA 20), the blood was slowly placed into plain test tubes, allowed to coagulate, and then centrifuged. One portion of the serum was used for standard hepatic and renal function testing, and the other portion was kept frozen at −80 °C for cytokine analysis.

2.7 Biochemical analysis

Using a spectrophotometric kinetic technique, the liver enzymes aspartate transaminase (AST) and alanine aminotransferase (ALT) were measured. Serum creatinine levels, a marker of renal function, were measured using the Jaffé reaction. Using the manufacturer's instructions (Sunredio, Shanghai), commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to measure serum levels of STAT-3 (catalog no. 201-12-0651), IL-6 (catalog no. 201-12-0091), NLRP-3 (catalog no. 201-12-5748), and AMPK (catalog no. 201-12-0747).

2.8 Outcomes assessment

The 17-item HDRS was the main outcome measure. A HDRS total score of ≤7 indicates remission, partial remission [HDRS<17 and>7], and no remission [HDRS>18]. Response was defined as ≥ 50% drop in the HDRS total score. Serum levels of IL-6, STAT-3, NLRP-3, and AMPK were assessed at baseline and 3 months later as a secondary end measure to assess the treatment's biological effects.

2.9 Statistical analysis

GraphPad Prism v9 (GraphPad Software, Inc., San Diego, CA, USA), a statistical analysis program, was used for the analyses. The normal distribution of continuous variables has been analyzed using the Shapiro-Wilk test. Significant differences within the group before and after therapy were determined using paired Student's t-tests. To find significant variations between groups before and after

therapy, unpaired Student's t-tests were performed. In terms of numbers, qualitative variables were provided, while quantitative values were expressed as mean and SD. Using Pearson's correlation coefficient, parameters were correlated. On categorical data, the Chi-square test or Fisher's exact test was applied, as appropriate. All p values were two-tailed, with $p < 0.05$ regarded as statistically significant.

3 Results

3.1 Clinical and demographic characteristics

Regarding the demographic baseline data, there were no statistically significant differences between the control and atorvastatin groups as follows: age ($p = 0.264$, t = 1.127, df = 58), sex ($p = 0.438$, $\chi^2 = 0.6007$), weight ($p = 0.868$, t = 0.1669, df = 58), height ($p = 0.542$, t = 0.6132, df = 58), ALT ($p = 0.211$, t = 1.263, df = 58), BMI ($p = 0.471$, t = 0.7244, df = 58), AST ($p = 0.209$, t = 1.270, df = 58), and SrCr ($p = 0.609$) (Table 1). After 2 weeks of starting the trial, four patients were withdrawn from the atorvastatin group due to non-compliance with the study medications. Three patients were withdrawn from the control group because they developed severe depressive episodes and shifted to combined therapy with antidepressants. Fifty-three patients completed the study; accordingly, the statistical analysis was performed per protocol analysis for all measured parameters except for HDRS, which was performed per protocol analysis and intention to treat analysis. A protocol analysis was used to assess the treatment's biological and causative effects.

3.2 Effect of study medications on Hamilton Depression Rating Scale

Table 2 demonstrated no significant changes in baseline values between the two study groups using an unpaired t-test ($p > 0.05$). Compared with the baseline data, a paired t-test showed that there was a significant decrease in HDRS total score in the placebo group (25.33 ± 1.90 *versus* 15.33 ± 2.370, $p > 0.0001$, t = 15.07, df = 26) and the atorvastatin group (25.43 ± 1.794 *versus* 12.85 ± 2.11, $p > 0.0001$, t = 40.97, df = 25). The atorvastatin group showed a statistically significant decrease in HDRS total score compared with the control group after treatment [($p = 0.0002$, t = 4.959, df = 51) and ($p = 0.04$, t = 2.052, df = 58)] per protocol analysis and intention to treat analysis, respectively. The response rate was 73.07% (n = 19/26) in the atorvastatin group *versus* 44.44% (n = 12/27) in the placebo group ($p = 0.03$, $\chi^2 = 4.472$).

No patient achieved full remission in both groups. Partial remission was (n = 24/26, 92.3%) for the atorvastatin group *versus* (n = 16/27, 59.25%) for the placebo group ($p = 0.005$, $\chi^2 = 7.815$).

3.3 Analysis of serum biomarkers

An unpaired t-test revealed no discernible differences in baseline values between the two groups ($p > 0.05$). In comparison to the control group, the atorvastatin group had a significantly elevated level of AMPK ($p = 0.015$, t = 2.574, df = 51) and a statistically significant decrease in the levels of IL-6 ($p = 0.018$, t = 2.429, df = 51), NLRP-3 ($p = 0.004$, t = 2.998, df = 51), and STAT-3 ($p > 0.0001$, t = 6.042, df = 51) following therapy. Furthermore, after medication, both groups' serum levels of NLRP-3, STAT-3, and IL-6 reduced significantly from their initial values. However, when compared to baseline values, Table 2 shows a statistically significant increase in AMPK serum levels after medication in both groups.

3.4 Correlation analysis between measured parameters

There was a significant positive correlation between HDRS and NLRP3 (r = 0.431, $p > 0.0001$), HDRS and IL-6 (r = 0.3, $p = 0.002$), HDRS and STAT3 (r = 0.841, $p > 0.0001$), and AMPK and STAT3 (r = 0.8, $p > 0.0001$). On the contrary, there was a significant negative correlation between HDRS and AMPK (r = −0.918, $p > 0.0001$), IL-6

TABLE 1 Clinical and demographic data in the two study groups.

Parameter	Group (1) control group (n = 30)	Group (2) atorvastatin group (n = 30)	p-value
Age (year)	33.43 ± 5.93	35.13 ± 5.75	0.264 (t = 1.127, df = 58)
Sex (M/F)	17/13	14/16	0.438 ($\chi^2 = 0.6007$)
Height (m^2)	1.661 ± 0.077	1.650 ± 0.064	0.542 (t = 0.6132, df = 58)
Weight (kg)	65.27 ± 5.607	65.03 ± 5.216	0.868 (t = 0.1669, df = 58
BMI (Kg/m^2)	23.65 ± 1.33	23.87 ± 0.957	0.471 (t = 0.7244, df = 58)
ALT (U/L)	25.97 ± 4.664	27.47 ± 4.531	0.211 (t = 1.263, df = 58)
AST (U/L)	29.43 ± 4.840	27.93 ± 4.291	0.209 (t = 1.270, df = 58)
SrCr (mg/dL)	0.946 ± 0.134	0.963 ± 0.116	0.609 (t = 0.5132, df = 58)
Moderate depression	12 (40%)	10 (33.33%)	0.715 ($\chi^2 = 0.133$)
Severe depression	18 (60%)	20 (66.66%)	0.799 ($\chi^2 = 0.064$)

Data are expressed as mean ± SD, numbers, and percentage, M: male, F: female, ALT: Alanine amino-transferase, AST: Aspartate amino-transferase, SrCr: Serum creatinine, BMI: body mass index, Significance at ($p < 0.05$).

TABLE 2 Comparison of Hamilton depression rating score and serum biomarkers in the two study groups.

Character	Group (1) control group (n = 27)			Group (2) atorvastatin group (n = 26)			[b] *p*-value
	Before treatment	After treatment	[a] *p*-value	Before treatment	After treatment	[a] *p*-value	After treatment
HDRS (ITT)	25.33 ± 1.90	16.67 ± 3.754	>0.0001 (t = 10.20, df = 29)	25.43 ± 1.794	14.30 ± 5.08	>0.0001 (t = 12.95, df = 29)	0.04 (t = 2.052, df = 58)
HDRS (PPA)	25.33 ± 1.90	15.78 ± 2.722	>0.0001 (t = 15.07, df = 26)	25.43 ± 1.794	12.50 ± 2.025	>0.0001 (t = 40.97, df = 25)	0.0002 (t = 4.959, df = 51)
IL-6 (pg/mL)	191.4 ± 19.1	186.1 ± 20.34	0.016 (t = 2.568, df = 26)	193.3 ± 17.89	173.5 ± 17.14	0.0006 (t = 3.849, df = 25)	0.018 (t = 2.429, df = 51)
STAT-3 (pg/mL)	279 ± 11.44	222.4 ± 7.623	>0.0001 (t = 53.79, df = 26)	277.2 ± 10.57	179.4 ± 36.19	>0.0001 (t = 12.55, df = 25)	>0.0001 (t = 6.042, df = 51)
NLRP-3 (pg/mL)	164.3 ± 12.43	156.6 ± 12.19	0.002	162.7 ± 11.71 (t = 3.412, df = 26)	147.0 ± 10.84	>0.0001 (t = 6.215, df = 25)	0.004 (t = 2.998, df = 51)
AMPK (ng/mL)	65.81 ± 5.135	146.1 ± 15.06	>0.0001 (t = 28.26, df = 26)	64.29 ± 3.255	157.1 ± 15.85	>0.0001 (t = 31.27, df = 25)	0.015 (t = 2.574, df = 51)

Data are expressed as mean ± SD, Significance at ($p < 0.05$). per protocol analysis (PPA), intension to treat analysis (ITT), Interleukin-6 (IL-6), adenosine monophosphate activated protein kinase (AMPK), nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3 (NLRP-3), signal transducer and activator of transcription factor-3 (STAT-3), Hamilton Depression Rating Scale (HDRS), (a) within group comparison, (b) between group comparison.

TABLE 3 Analysis of lipid profile in the two study groups.

Character	Group (1) control group (n = 27)			Group (2) atorvastatin group (n = 26)			[b] *p*-value
	Before treatment	After treatment	[a] *p*-value	Before treatment	After treatment	[a] *p*-value	After treatment
TC (mg/dL)	162.8 ± 16.94	164.7 ± 14.17	0.276 (t = 1.111, df = 26)	163.1 ± 17	156.3 ± 14.43	0.0003 (t = 4.161, df = 25)	0.037 (t = 2.137, df = 51)
TG (mg/dL)	135.1 ± 15.34	132.3 ± 10.28	0.311 (t = 1.033, df = 26)	128.3 ± 12.2	124.8 ± 13.87	0.013 (t = 2.670, df = 25)	0.030 (t = 2.227, df = 51)
HDL (mg/dL)	45.19 ± 8.13	43.85 ± 6.65	0.110 (t = 1.651, df = 26)	45.23 ± 8.83	48.42 ± 7.19	0.0004 (t = 4.049, df = 25)	0.019 (t = 2.403, df = 51)
LDL (mg/dL)	90.61 ± 17.78	94.43 ± 14.87	0.069 (t = 1.896, df = 26)	92.88 ± 18.74	82.95 ± 14.84	>0.0001 (t = 5.164, df = 25)	0.007 (t = 2.812, df = 51)

Data are expressed as mean ± SD, Significance at ($p < 0.05$). Total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein (LDL), (a) within group comparison, (b) between group comparison.

and STAT3 (r = −0.296, $p = 0.002$), and AMPK and NLRP3 (r = −0.386, $p = 0.001$).

3.5 Analysis of lipid profile in the two study groups

No significant changes in baseline values were recorded between the two study groups using an unpaired t-test ($p > 0.05$) (Table 3). After treatment, the atorvastatin group showed a statistically significant reduction in the levels of TC ($p = 0.037$, t = 2.137, df = 51), TG ($p = 0.030$, t = 2.227, df = 51), LDL ($p = 0.007$, t = 2.812, df = 51), and a statistically significant increase in HDL ($p = 0.019$, t = 2.403, df = 51) when compared to the control group. Additionally, after therapy, serum levels of TC, LDL, and TG in the atorvastatin group decreased statistically, and serum levels of HDL increased significantly when compared to their baseline values. On the other hand, Table 2 demonstrates no significant changes in serum levels of all lipid markers following therapy in the fluoxetine group when compared to baseline values.

3.6 Analysis of drug-related adverse effects between the groups

Table 4 showed that there were no significant differences between the studied groups in terms of side effects as followed: nausea ($p = 0.480$, $\chi^2 = 0.497$), stuffy nose ($p = 0.111$), fatigue ($p = 0.300$, $\chi^2 = 1.074$), muscle pain ($p = 0.111$), sexual dysfunction ($p = 0.704$), loss of appetite ($p = 0.464$, $\chi^2 = 0.534$), and insomnia ($p = 0.690$, $\chi^2 = 0.158$).

4 Discussion

Although some clinical studies were conducted to assess the effect of atorvastatin, lovastatin, and simvastatin as adjunctive

TABLE 4 Comparison of drug-related adverse effects between the groups.

Side effect	Group (1) control group (n = 27)	Group (2) atorvastatin group (n = 26)	*p*-Value
Nausea	6	8	0.480 (χ^2 = 0.497)
Stuffy nose	0	3	0.111
Fatigue	5	8	0.300 (χ^2 = 1.074)
Muscle pain	0	3	0.111
Insomnia	6	7	0.690 (χ^2 = 0.158)
Sexual dysfunction	3	4	0.704
Loss of appetite	5	7	0.464 (χ^2 = 0.534)

Data were presented as numbers. Significance at ($p < 0.05$) using fisher exact test and chi-square test as appropriate.

treatments in major depressive disorders, none of them measured any biomarkers that were related to inflammatory pathways in depression (Haghighi et al., 2014; Gougol et al., 2015; Massardo et al., 2022). To our knowledge, this is the first clinical research to assess the anti-inflammatory effect of high-dose atorvastatin in major depressive disorders through AMPK/NLRP3 and IL-6/STAT-3 signaling pathways. The safety of high-dose atorvastatin has been evaluated in patients for periods ranging from 2 weeks to 5 years, and rates of clinically significant myopathy and elevated hepatic enzymes were extremely low (Waters, 2005). The frequency and characteristics of adverse effects were anticipated and could be linked to the medications administered.

The current study demonstrated that atorvastatin treatment in combination with fluoxetine significantly reduced HDRS total score and produced a higher response rate than fluoxetine alone. This was demonstrated by a decrease in depression symptoms, as indicated by the HDRS score. As a result, this study supports the notion that atorvastatin could be an effective adjuvant treatment for MDD. This finding is consistent with the results of a previous study by Haghighi M. et al., which showed that atorvastatin potentiated the efficacy of citalopram (Haghighi et al., 2014). The higher dose of atorvastatin in our trial may explain the superior response and partial remission compared to Haghighi M. et al. Since it was reported that high-intensity atorvastatin at a high dose may enhance its antidepressant effects (Lee et al., 2021). Furthermore, our findings were consistent with previous research on the use of statins as adjuvant treatments for MDD, which showed that lovastatin and simvastatin had an antidepressant effect that increased the effectiveness of fluoxetine (Ghanizadeh and Hedayati, 2013; Gougol et al., 2015).

The current investigation showed that the atorvastatin group had significantly lower serum levels of IL-6 and STAT-3. These findings are consistent with and associated with earlier research in the same field (El-Mahdy et al., 2021). As STAT3 regulates IL6-dependent modulation of serotonin transporter activity and depressive-like behavior, targeting the IL-6/STAT-3 axis becomes a crucial approach in treating depressive-like behavior (Kong et al., 2015). Drug-induced STAT3 inhibition decreased depressive-like behavior and increased serotonin expression (Kong et al., 2015). According to studies, atorvastatin inhibits the IL-6/STAT3/endothelin-1 pathway in patients with spontaneous hypertension (Fang et al., 2019). Also, atorvastatin suppressed the IL-6/STAT3 pathway, resulting in the downregulation of human telomerase reverse transcriptase, which in turn causes atorvastatin-induced senescence of inflammation (Wang et al., 2020). In accordance with our study, celecoxib, an anti-inflammatory drug, exhibited antidepressant activity by suppressing serum IL-6 and reducing HDRS when compared to the placebo group (Gędek et al., 2023). The authors also found a significant correlation between a reduction in HDRS and a reduction in serum IL-6 levels (Gędek et al., 2023). These observations support the idea that atorvastatin could modulate depressive behaviors by modulating the IL-6/STAT-3 axis.

Furthermore, our study demonstrated a significant reduction in serum levels of NLRP3 and a significant increase in AMPK in the atorvastatin group compared to the baseline and fluoxetine group. These results are matched and in accordance with other reports in the same field (Wang et al., 2017; Yang et al., 2019). It is well known that NLRP3 contributes to the chance of acquiring MDD in several studies (Alcocer-Gómez et al., 2014; Pandey et al., 2021; Han et al., 2022). The methylation scores of cg18793688 and cg09418290 in the NLRP3 domain were substantially associated with cortical thickness in MDD patients (Han et al., 2022). Thus, targeting the NLRP3 axis could be a therapeutic strategy for treating MDD. The inhibitory activity of atorvastatin on NLRP3 and the activation of AMPK were explained by several mechanisms. Atorvastatin inhibits NLRP3 inflammasome activation via TLR4/MyD88/NF-κB signaling (Kong et al., 2016). Statins inhibit pregnane X receptor (PXR)-dependent NLRP3 inflammasome activation in vascular endothelial cells by oxidizing LDL cholesterol or TNFα (Wang et al., 2017). Statins also suppress NLRP3 by increasing the expression of aqua porin proteins (AQP2) (Kong et al., 2020) and promoting autophagy through the Akt/mTOR signaling pathway (Han et al., 2018). Atorvastatin also stimulates autophagy by stimulating AMPK production (Dehnavi et al., 2021) by directly activating the enzyme unc-51-like kinase 1 (ULK1) and blocking the mTORC1 complex's inhibitory action on ULK1 (Mihaylova and Shaw, 2011).

Regarding the control group, fluoxetine showed a significant reduction in the levels of STAT-3, NLRP-3, and IL-6, along with a statistically significant elevation in the levels of AMPK compared to their baseline values. Certainly, fluoxetine's role as an SSRI might be used to explain these results. Antidepressants may have a significant anti-inflammatory impact due to serotonin uptake inhibition (Ohgi et al., 2013). Moreover, fluoxetine can upregulate the serum neurotrophic factors and decrease inflammatory biomarkers in depressed patients, as reported previously (García et al., 2022). In

depressed people, fluoxetine reduces levels of IL-6, IL-1β, and TNF-α in the pro-inflammatory pathway (García et al., 2022). In a prior study, fluoxetine therapy reversed an increase in TNF-α and a decrease in BDNF caused by lipopolysaccharides (LPS) in the hippocampus and prefrontal cortex (Taniguti et al., 2019). The effects of fluoxetine on interleukin levels, however, vary among studies. Some authors have noted a decline in serum levels of IL-6 (Lu et al., 2017), whereas others have documented no changes (Amitai et al., 2020). The therapeutic advantages of the combined therapy over the control group are therefore most likely attributable to high dose atorvastatin by altering the signaling pathways for AMP/NLRP3 and IL-6/STAT-3.

Regarding the statistically significant correlation between HDRS and measured parameters, reducing HDRS and relieving depression symptoms might lead to a reduction in IL-6, STAT3, NLRP3, and an elevation in AMPK. Also, there was a positive significant correlation between IL-6 and STAT3, and a negative significant correlation between NLRP3 and AMPK. It is well known that IL-6 is a potent activator and regulator of STAT3 signaling, and they are highly correlated (Guan et al., 2021). Furthermore, there was a significant positive correlation between AMPK and STAT-3. These findings are matched and correlated with previous studies (Mohamed et al., 2018; Taniguti et al., 2019). There is a crosstalk between AMPK and STAT3, as reported by several studies (Li et al., 2014; Speirs et al., 2018). AMPK and its activators lead to a reduction in STAT3 and its activity in various inflammations (Kim et al., 2020). In mouse liver and human hepatocytes, pharmacological stimulation of AMPK reduces the inflammatory reactions triggered by IL-6 and STAT3 signaling (Nerstedt et al., 2013). All of these observations might explain this correlation, and there was a crosstalk between the IL-6/STAT3 and AMPK/NLRP3 axes.

Our study demonstrated that the serum lipid profile was significantly improved in the atorvastatin group compared to the fluoxetine group and their baseline values. These results were in line with previous reports (Avisar et al., 2008; Jose et al., 2012). Atorvastatin efficacy in managing hyperlipidemia was widely investigated (Jose et al., 2012). The advantages of statins stem from their ability to decrease the biosynthesis of cholesterol, primarily in the liver, where they are specifically distributed. Additionally, they influence lipid metabolism through their inhibition of HMG-CoA reductase (Hu et al., 2021).

Inflammation is unlikely to be relevant for all patients with depression. However, it is established that mean concentrations of peripheral inflammatory markers are higher in depressed patients compared with controls (Osimo et al., 2020). Depressed patients are about 50% more likely to have evidence of inflammation as compared to matched non-depressed controls. Furthermore, it was reported that acute depression is a pro-inflammatory state, which lends support to the hypothesis that inflammatory marker elevations in depression are not due to an inflamed sub-group but rather to a right shift of the immune marker distribution (Osimo et al., 2019; Osimo et al., 2020).

Given that each drug is metabolized by a separate isoenzyme, it is expected that there were no pharmacokinetic interactions between atorvastatin and fluoxetine recorded (Mandrioli et al., 2006; Park et al., 2008). Additionally, there were no reported clinically important adverse effects and no notable variations in the clinical parameters of the patients, such as their age, gender, liver function, or renal function. The therapeutic advantages of the combined treatment are therefore most likely attributable to high-dose atorvastatin by altering the signaling pathways for AMP/NLRP3 and IL-6/STAT-3.

The present study had a number of limitations, including its short duration, its small sample size, and its use of specific atorvastatin dosages, despite its optimistic results. The Middle Eastern population is the exclusive focus of the current investigation. Therefore, the advantage seen in this trial should be confirmed in multicenter investigations and in other ethnic groups, especially in western countries where depression is expected to be more prevalent and may be different from a clinical standpoint. Furthermore, the research ethical committee denied using the placebo alone in patients with severe depression. Suicide attempts or suicidal ideation were not included in the secondary outcomes. In addition, there were no self-assessment scales or cognitive tests. Furthermore, creatine kinase (CK) should be monitored before and after treatment. Therefore, future trials may include a placebo and an atorvastatin-only group to assess the statin's and placebo's impact on depression severity.

5 Conclusion

This double-blinded, randomized trial led us to the conclusion that, in terms of decreasing inflammatory markers, atorvastatin combination therapy with fluoxetine is preferable to fluoxetine monotherapy in the treatment of major depressive disorder. By altering the signaling pathways for AMP/NLRP3 and IL-6/STAT-3, atorvastatin may reduce the levels of inflammatory biomarkers. While the results, along with previous research, hint at potential benefits, data on statin usage in MDD is still limited, and there is no specific data identifying which MDD patient subpopulation would gain most from this combination. Recommendations for such therapy should await further, larger, and well-designed studies to evaluate these effects.

Data availability statement

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

Ethics statement

The studies involving humans were approved by The Institutional Review Board at Tanta University Faculty of Medicine. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

KA: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project

administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. MB: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. TE Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. EE: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. FK: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. RE: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. YE: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. MK: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. WN: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. AE: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. AH: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. HA: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. MS: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. MA: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. MH: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing.

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Conflict of interest

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

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Case report: Time response of plasma clozapine concentrations on cessation of heavy smoking

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Smoking cessation in patients treated with clozapine might lead to elevated plasma concentrations and severe side effects. This case report investigated the trajectory of clozapine plasma concentrations over time after smoking cessation in a Chinese inpatient with schizophrenia. This case report delineates the temporal response of plasma clozapine concentrations and dose-corrected clozapine plasma concentrations in a 33-year-old inpatient with schizophrenia who had a substantial smoking history and ceased smoking abruptly during dose titration. This case report presents a sudden increase in plasma clozapine concentrations and dose-corrected plasma clozapine concentrations after smoking cessation, followed by a rapid decline in dose-corrected plasma clozapine concentrations during the initial 2 weeks and a return to pre-cessation levels approximately 1 month later. The findings suggest that clinicians and pharmacists should adjust clozapine dosage in accordance with changes in smoking status, taking into consideration the temporal effects. Post-smoking cessation adjustments to clozapine dosage should be coupled with therapeutic drug monitoring, especially for patients with heavy smoking habits. Moreover, the advice of the clinical pharmacist should be considered in complex cases to ensure safe use of clozapine.

KEYWORDS

schizophrenia, clozapine, smoking cessation, therapeutic drug monitoring, plasma concentrations

Background

Clozapine (CLO) is a distinctive antipsychotic medication extensively utilized for the management of refractory schizophrenia. Despite its efficacy, the drug is linked to a spectrum of severe adverse effects, including granulocytopenia, gastrointestinal hypofunction, myocarditis, and neurological symptoms. The metabolic pathway of CLO primarily involves the CYP 1A2 enzyme, with additional contributions from various CYP isoforms, namely, CYP 2C9, CYP 2C19, CYP 2D6, and CYP 3A4, all contributing to its biotransformation (Pirmohamed et al., 1995; Olesen and Linnet, 2001; Dragovic et al., 2013). Smoking, a source of polycyclic aromatic hydrocarbons, has the potential to induce CYP1A2 enzyme activity (Zevin and Benowitz, 1999), thereby influencing CLO metabolism. Alterations in smoking habits may consequently impact the pharmacokinetics of CLO. Smoking cessation has been shown to normalize CYP1A2 enzyme levels, resulting in a significant elevation in plasma clozapine concentrations and an associated augmented risk of adverse effects (MacLeod et al., 1997). Given the narrow therapeutic range of clozapine, post-smoking cessation elevation

in plasma drug concentrations can lead to clinically significant outcomes (Burns, 1999). In accordance with the Arbeitsgemeinschaft für Neuropsychopharmakologie und Pharmakopsychiatrie (AGNP) consensus guidelines (Hiemke et al., 2018), therapeutic drug monitoring (TDM) for CLO is strongly recommended. Despite this, there is a paucity of studies reporting changes in CLO concentrations following smoking cessation, particularly in Chinese Han patients. The current investigation addresses this gap, presenting the temporal response of plasma CLO concentrations following the cessation of heavy smoking in an inpatient with schizophrenia treated with CLO during the COVID-19 pandemic. The study involving humans was approved by the Review Board of the Beijing Hui-Long-Guan Hospital on 24 October 2019 under registration number 2019-41. The study was conducted in accordance with the local legislation and institutional requirements. The participant provided the written informed consent to participate in this study.

Case description

The patient under consideration was a 33-year-old unmarried Chinese man with an 8-year history of schizophrenia, displaying poor responsiveness to multiple medication trials. He had no reported family history of psychiatric disorders. Upon hospitalization, the patient presented with hyperlipidemia, hyperuricemia, homocysteinemia, and symptoms indicative of a cold. Additionally, he had a decade-long history of smoking one pack of cigarettes per day. Initial manifestations of the patient's condition emerged at the age of 25, marked by frequent tantrums, social reluctance, soliloquy, and reduced sleep requirements. The formal diagnosis of schizophrenia occurred at 27 years of age, following the development of delusions, hallucinations, phantom smells, impulsive behaviors, and other psychotic symptoms. Subsequently, over 5 years, the patient underwent six hospitalizations due to severe psychotic symptoms or relapses. Detailed medication information is shown in Table 1. However, satisfactory alleviation of persistent psychiatric symptoms was not achieved.

At the age of 32, the patient was rehospitalized and subjected to CLO treatment. The daily CLO dosage was incrementally elevated from 25 to 225 mg/day, resulting in a corresponding plasma CLO concentrations of 572 ng/mL. Following 3 months of this treatment, the psychotic symptoms exhibited improvement, leading to his discharge from the hospital. No significant adverse reactions were documented during the hospitalization for the first CLO treatment. After discharge, the daily CLO dosage was reduced to 100 mg/mL in the absence of physician consent.

Six months after discharge, the patient was readmitted due to a deterioration in psychotic symptoms. Physical examination revealed no abnormalities. Initial laboratory assessments, including a complete blood count and a basic biochemistry panel, indicated a white blood cell count of 15.31×10^9/L (reference range 3.5–9.5× 10^9/L), an absolute neutrophil count of 11.75×10^9/L (reference range 1.8–6.3× 10^9/L), an absolute monocyte value of 0.85×10^9/L (reference range 0.1–0.6×10^9/L), serum glutamine transaminase of 74 U/L (reference range 9–50 U/L, triglycerides of 2.30 mmol/L (reference range 0–1.7 mmol/L), lipoprotein(a) of 32.7 mg/dL (reference range 0–30 mg/dL), uric acid of 509 μmol/L (reference range 150–440 μmol/L), homocysteine of 47.9 μmol/L (reference range 0–15 μmol/L), and ultrasensitive C-reactive protein (CRP) of 9.35 mg/L (reference range 0.11–3.11 mg/L). After 10 days of hospitalisation, his cold symptoms he had on admission disappeared, and his ultrasensitive C-reactive protein value decreased to 2.9 mg/L, which was within the normal reference range.

TABLE 1 Medication information for patient from 27 to 32 years of age.

No. of hospitalizations	Age	Medication
1	27 years	Amisulpride, 0.8 g/day
2	28 years	Risperidone, 5 mg/day
3	29 years	Olanzapine, 15 mg/day
4	30 years	Olanzapine, 20 mg/day
5	31 years	Olanzapine, 20 mg/day
6	32 years	Clozapine, 225 mg/day

After admission, the patient received continuous CLO treatment for his psychotic symptoms. The initial daily dosage was 100 mg/day, escalating to 150 mg/day on day 8, resulting in a plasma CLO concentrations of 342 ng/mL on day 14. Subsequently, the daily dosage was further increased to 200 mg/day on day 15; however, specific information regarding the corresponding plasma CLO concentrations was unavailable. On day 19, the daily dosage was raised to 250 mg/day, leading to a plasma CLO concentrations of 1174 ng/mL on day 23, surpassing the laboratory alert threshold of 1000 ng/mL. On the same day (day 23), smoking were prohibited for the patient due to the closed management policy in response to the COVID-19 outbreak, implemented to prevent cross-infection within the ward. On day 30, the plasma concentrations of CLO was remeasured, yielding a value of 1124 ng/mL, still surpassing the laboratory alert threshold. Consequently, the daily dose was adjusted to 225 mg/day on day 33. On day 37, the plasma CLO concentrations decreased to 821 ng/mL, and no exacerbation of psychosis was observed following the dosage reduction. Subsequent measurements on day 49 and day 57, while maintaining a stable dose of 225 mg/day, recorded plasma CLO concentrations of 721 ng/mL and 590 ng/mL, respectively. The patient was then hospitalized with a consistent CLO dosage of 225 mg/day. No exacerbated psychiatric symptoms or serious adverse events were observed during the month of follow-up after smoking cessation. The patient was discharged after 1 month with significant improvement.

During the first week of hospitalization, the patient received haloperidol and chlorpromazine hydrochloride intramuscularly for agitation. During the first 10 days of the hospitalization, he took Shuanghuanglian oral liquid, an herbal medicine for colds. During his hospitalization, he also took Zhibituo capsules, a Chinese herbal extract for hyperlipidemia. The patient's medication compliance was checked by the nurses. The CLO was administered twice daily (08:00 and 20:00). No antibiotics were prescribed.

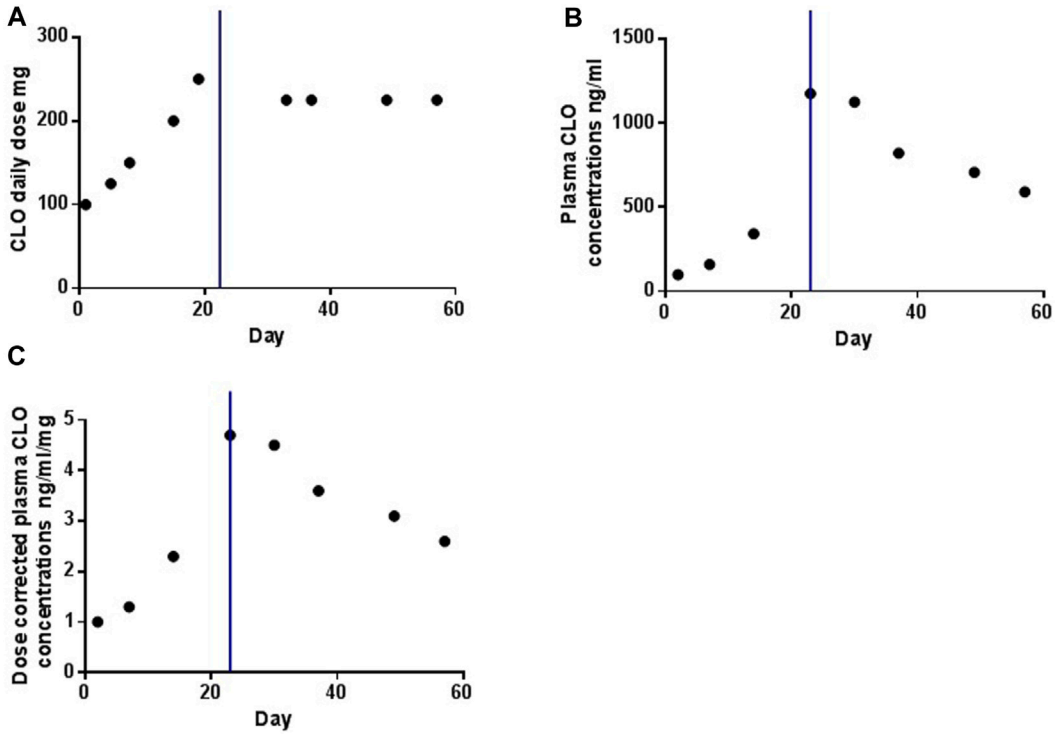

FIGURE 1
The profiles of daily dose **(A)**, plasma concentrations **(B)** and dose-corrected of plasma concentrations **(C)** of CLO over time. The blue line indicates the time point at which the smoking was ceased.

Figure 1 illustrates the temporal profiles of the daily dose, plasma concentrations, and dose-corrected plasma concentrations of CLO. Following smoking cessation, the plasma CLO concentrations abruptly exceeded the laboratory alert threshold of 1000 ng/mL. Dose-corrected CLO plasma concentrations increased from 2.3 to 4.7 ng/ml/day. In the initial 2 weeks post-smoking cessation, dose-corrected CLO plasma concentrations exhibited a decline from 4.7 to 3.6 ng/ml/day, indicating a reduction rate of approximately 1.67% per day. After 33 days without smoking, the dose-corrected CLO plasma concentrations further decreased to 2.6 ng/ml/day, reflecting a decline rate of approximately 1.38% per day. This value was comparable to the dose-corrected CLO plasma concentrations of 2.5 ng/ml/day observed prior to smoking cessation.

Discussion

As several previous studies reported, this case report presents that plasma CLO concentrations, as well as dose-corrected plasma CLO concentrations, exhibit a significant increase in patients with schizophrenia who are on stabilized doses after discontinuation of smoking (Derenne and Baldessarini, 2005; de Leon et al., 2020; Tsukahara et al., 2022; Flanagan et al., 2023). For instance, Derenne et al. documented the case of a white female patient, observing an escalation in dose-corrected CLO concentrations from 2.25 to 4.65 ng/mL/day. Following the abrupt cessation of smoking, the patient experienced adverse effects, including sedation, confusion, muscle spasms, dry mouth, dizziness, sluggish pupils, and mild delirium (Derenne and Baldessarini, 2005). Additionally, the case report notes a more rapid decline in dose-corrected plasma CLO concentrations during the initial 2 weeks after smoking cessation, returning to pre-cessation levels approximately 1 month later. This finding could be explained by De Leon's views, which suggested that the de-induction effect after smoking cessation takes approximately 2–4 weeks due to the need to synthesize new CYP1A2 (de Leon, 2004). Similarly, Faber et al. observed a decrease in CYP1A2 activity by 20% and 36% on days 2 and 7, respectively, after smoking cessation, stabilizing at a new steady state after 1 week (Faber and Fuhr, 2004). Similarly, previous case reports stated that patients with schizophrenia treated with CLO experienced serious adverse events or a sudden increase in plasma CLO concentrations at 2 weeks to 1 month after smoking cessation. For example, Derenne et al. reported the case of a 28-year-old woman with severe psychiatric illness who abruptly stopped her habit of heavy smoking while continuing to take 450 mg of CLO daily. After approximately 4 weeks, she developed serious adverse events. Her serum CLO concentrations exceeded 1000 ng/mL, and these symptoms alleviated rapidly when the CLO dose was reduced (Derenne and Baldessarini, 2005). Bondolfi et al. also reported the case of a 51-year-old man with paranoid schizophrenia who abruptly stopped smoking; 2 weeks later, he started complaining of severe

sedation and fatigue with an approximately threefold increase in plasma CLO concentrations (Bondolfi et al., 2005). Based on these studies, it can be speculated that the impact of smoking cessation on CLO metabolism may persist for at least a month, with the most pronounced effects observed in the initial 2 weeks and a gradual diminishment thereafter.

There are some points that need to be mentioned. First, as we all know, infection or inflammation, co-medication with CYP450 inhibitors, and change in dietary or lifestyle can increase plasma CLO concentrations (Clark et al., 2018; Ruan et al., 2018; de Leon et al., 2020). However, in this case, the patient was not taking CYP450 inhibitors, his dietary or lifestyle remained essentially unchanged during hospitalization except for smoking behavior, only the CRP was higher than the normal range during the first 10 days of admission, after which the cold symptoms disappeared and the CRP values dropped to within the normal reference range, therefore, smoking cessation was the main contributor to the increase in CLO concentrations. Second, the patient's psychiatric symptoms did not worsen after the CLO dose reduction. However, if lower CLO dose was ineffective, several augmentation strategies should be considered, such as the combination of amisulpride, aripiprazole, mirtazapine, omega-3 fatty acids, and electroconvulsive therapy (Porcelli et al., 2012; Grover et al., 2023). Third, CLO is strongly recommended for TDM according to the AGNP consensus guidelines, with a therapeutic reference range of 350–600 ng/mL and a laboratory alert concentration of 1000 ng/mL (Hiemke et al., 2018). In addition, several previous studies have suggested that clinical pharmacist interventions can reduce antipsychotic polypharmacy use and improve treatment guidelines adherence (Stuhec, 2014; Stuhec and Zorjan, 2022; Stuhec et al., 2023). Therefore, in order to improve the safety of CLO administration in clinical practice, dose adjustments should be made in conjunction with TDM data and recommendations from clinical pharmacists.

Several limitations should be considered when interpreting the results of this case report. First, the plasma norclozapine concentrations was not examined. Second, poor CLO metabolism also increased CLO concentrations. However, CYP1A2 genotyping was not performed in this case. Third, no major adverse events were identified in this case. We plan to use the adverse drug reactions probability scale (Naranjo et al., 1981) in future studies to more accurately assess the probability of adverse drug reactions.

This case report delineates the temporal response of plasma CLO concentrations and dose-corrected CLO plasma concentrations in a Chinese Han patient with schizophrenia who had a substantial smoking history and ceased smoking abruptly during dose titration. The findings underscore the importance for clinicians and pharmacists to adjust CLO dosage in accordance with changes in smoking status, taking into consideration the temporal effects. Post-smoking cessation adjustments to CLO dosage should be coupled with TDM, especially for patients with heavy smoking habits. Moreover, the advice of the clinical pharmacist should be emphasized in complex cases to ensure safe CLO use.

Data availability statement

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

Ethics statement

The studies involving humans were approved by the Review Board of the Beijing Hui-Long-Guan Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

LQ: Investigation, Writing–original draft. BM: Data curation, Writing–review and editing. HF: Conceptualization, Writing–review and editing. SQ: Data curation, Writing–review and editing. FY: Resources, Supervision, Writing–review and editing. HA: Conceptualization, Funding acquisition, Writing–review and editing.

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Conflict of interest

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

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Case report: Dezocine's rapid and sustained antidepressant effects

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Anhedonia and motivational impairments are cardinal features of depression, against which conventional antidepressants demonstrate limited efficacy. Preclinical investigations and extant clinical trial data substantiate the promise of opioid receptor modulators in addressing anhedonia, depression, and anxiety. While synthetic opioid agents like dezocine are conventionally employed for analgesia, their distinctive pharmacological profile has engendered interest in their potential antidepressant properties and translational applications. Herein, we present a case in which persistent bupropion treatment was ineffective. However, the incidental administration of a single low-dose intravenous injection of dezocine resulted in a rapid and sustained amelioration of depressive symptoms, particularly anhedonia and motivational deficits. Our findings posit a potentially novel role for the "legacy drug" dezocine.

KEYWORDS

major depressive disorder, opioid, dezocine, anhedonia, case

Introduction

Major depressive disorder (MDD) is a frequently encountered mood disorder characterized by a high prevalence, diagnostic challenges, and significant disability rates. Anhedonia, a core symptom of MDD, serves as a poor prognostic indicator of antidepressant response (Klein et al., 2022). Research indicates that anhedonia affects 37%–72% of individuals with MDD, contributing to heightened morbidity and increased suicidality (Tang et al., 2021). Although monoaminergic antidepressants are the standard first-line treatment for MDD, their effectiveness in alleviating anhedonia remains limited (Shawn et al., 2011). Currently, no medication has been approved for the treatment of anhedonia. This highlights the need for effective and expedited interventions that specifically target this symptom.

Emerging evidence has elucidated the pivotal involvement of endogenous opioid receptors, including μ-opioid receptor (MOR), κ-opioid receptor (KOR), δ-opioid receptor (DOR), and nociceptin/orphanin FQ peptide receptor, in the pathophysiology of MDD (Puryear et al., 2020). These receptors are widely distributed in regions associated with emotional processing and reward systems, notably the mesocorticolimbic system, and play a significant role in modulating pleasurable experiences (Puryear et al., 2020). Imaging modalities have revealed diminished activity in the endogenous opioid system among individuals with MDD, which is correlated with impaired emotional regulation and

Abbreviations: MDD, major depressive disorder; rTMS, repetitive transcranial magnetic stimulation; QD, quaque die; QN, quaque night; PRN, pro re nata.

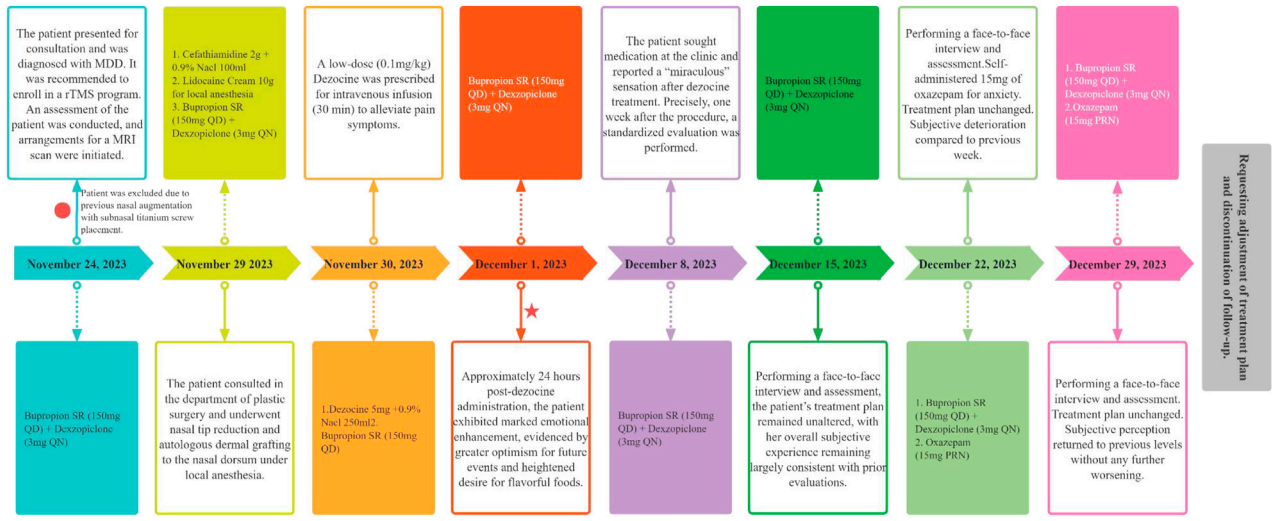

FIGURE 1
Timeline of event onset and evaluation of symptomatic presentation with an outline of therapeutic intervention.

decreased pleasurable experiences (Nummenmaa et al., 2020). Notably, the evidence from several clinical investigations has substantiated that the administration of multimodal opioid agonists, such as ALKS–5461 (Fava et al., 2020), JNJ–67953964 (Krystal et al., 2020), and BTRX–246040 (Post et al., 2016), either as monotherapy or in conjunction with other treatments, is associated with substantial antidepressant efficacy and a beneficial effect on the mitigation of anhedonia. Preclinical investigations have shown that MOR activation enhances rewarding experiences, whereas KOR activation is generally linked to diminished pleasure. DOR activation indirectly promotes dopamine release from the nucleus accumbens, thereby regulating reward-related behaviors (Browne, 2019). Strong clinical and preclinical evidence supports the potential of opioid-based pharmacotherapy in the management of MDD, particularly for the amelioration of anhedonic manifestations.

Dezocine, a synthetic opioid with structural similarities to benzomorphan opioids, is primarily used for the management of moderate-to-severe pain in clinical settings (Ye et al., 2022). Functionally, it acts as a partial agonist/antagonist at MOR, demonstrates partial agonism at DOR, and modulates KOR to varying degrees (Liu et al., 2014; Wang et al., 2018). Additionally, dezocine inhibits the norepinephrine transporter (NET) and serotonin transporter (SERT), thereby impeding the reuptake of the respective neurotransmitters (Liu et al., 2014). The clinical efficacy of dezocine is comparable to that of morphine and other opioids, while displaying a lower potential for addiction and a reduced incidence of side effects (Wang et al., 2017). Despite its established analgesic use, limited research has been conducted on the antidepressant properties of dezocine, despite its interaction with key targets such as MOR, KOR, NET, and SERT. Initial preclinical research indicates that dezocine may alleviate depression-like behavior in rodents in a dose-dependent manner (Shang et al., 2021) and has shown promise in the clinical attenuation of postoperative depression following cancer resection (Zhao et al., 2020). Despite the limited evidence regarding the antidepressant properties of dezocine, its distinct pharmacodynamic profile necessitates a comprehensive investigation into its potential efficacy and mechanism of action in the treatment of depression. Herein, we present a recent case of MDD in which rapid and sustained improvement in depressive mood and anhedonia was observed following dezocine administration (Figure 1).

The case report

A young woman who initially presented with MDD in June 2014 was diagnosed therewith at multiple psychiatric hospitals in China. She was previously prescribed various antidepressants, including escitalopram, paroxetine, and venlafaxine. Treatment adherence was satisfactory, with moderate efficacy observed. The predominant rationale for altering pharmacotherapy was an unwillingness to tolerate medication-induced weight gain. Prior to this depressive episode, she had experienced a remission period of up to 2 years. However, following her mother's death a year ago, the patient experienced a recurrence of symptoms. The outpatient physician initially prescribed venlafaxine, which had previously been effective for the patient. However, with ongoing treatment, the patient's anhedonia symptoms worsened. Consequently, the treatment regimen was gradually switched to bupropion. And so far, she was prescribed sustained-release bupropion (150 mg) for nearly 3 months.

The patient was in good physical condition. Underwent rhinoplasty in April 2022 with titanium screw placement at the nasion. Primogeniture among three siblings with normative prenatal exposure and developmental progression. The individual had attained an undergraduate education and was engaged in a profession within the social media sector. There was no history of matrimony or parenthood. Notably, there existed a positive familial history, with a diagnosis of a mood disorder in a sibling, confirmed by a specialized psychiatric facility.

TABLE 1 Quantitative assessment results of the Patient's depressive symptoms and anhedonia.

	Baseline	1 week*	2 weeks*	3 weeks*	4 weeks*
Depressive assessment					
HAMD-17	18	2	5	10	17
MADRS	23	4	9	15	22
PHQ-9	14	1	2	8	12
Anhedonic assessment					
DARS (Total)	17	52	49	35	28
Hobbies	3	15	15	9	9
Food and drink	4	9	5	8	7
Social activities	4	10	10	4	1
Sensory experience	6	18	19	14	11
Item 7 of HAMD-17	4	0	0	2	3
Item 8 of MADRS	4	0	0	2	4
Item 1 of PHQ-9	3	0	0	1	3

* Represents the time after dezocine was administered intravenously.
Abbreviations: HAMD-17, Hamilton Depression Rating Scale-17; MADRS, Montgomery-Asberg Depression Rating Scale; PHQ-9, Patient Health Questionnaire-9; DARS, Dimensional Anhedonia Rating Scale.

On 24 November 2023, the patient was enrolled in a clinical trial involving repetitive transcranial magnetic stimulation (rTMS) and was diagnosed with MDD using the Mini-International Neuropsychiatric Interview. During the baseline phase, the participants' symptoms were quantitatively assessed using standard psychometric scales, including the Hamilton Depression Rating Scale (HAMD-17), Montgomery–Asberg Depression Rating Scale, Patient Health Questionnaire-9, and Dimensional Anhedonia Rating Scale (DARS). The results indicated moderate depressive symptoms and severe anhedonia (Table 1). Prior to the MRI scan, the subject recalled having undergone nasal augmentation with subnasal titanium screw placement. Given the potential risks associated with metal objects and magnetic fields in rTMS, the investigative team recommended extraction of the titanium nasal screw prior to the initiation of the study protocol to ensure patient safety.

From the November 29 to 1 December 2023, the patient underwent elective removal of a titanium nasal screw and concurrent rhinoplasty (specifically, nasal tip refinement) in the plastic surgery department. Postoperatively, on 30 November 2023, the patient was prescribed an analgesic regimen consisting of a low-dose intravenous dezocine injection at 0.1 mg/kg. The prescribed dezocine (5 mg in total) was diluted in 250 mL of 0.9% sodium chloride solution and administered via infusion over 30 min. Apart from the reported tolerable nausea, no additional adverse effects were noted by the patient and caregivers.

Approximately 24 h after dezocine administration, the patient reported a remarkable and spontaneous enhancement in emotional wellbeing, characterized by a reinvigorated zest for life and cravings for gourmet food. The patient's subjective experience was described as a transition from feeling emotionally "empty" to a sensation of embodying their authentic self. Regrettably, no formal quantitative evaluations were conducted. During the trial, the patient consistently received 150 mg of sustained-release bupropion and 3 mg of dexzopiclone. One week after the initiation of dezocine treatment, the patient returned to the hospital to receive antidepressants and declined further participation in the rTMS program. The patient recounted her "miraculous" experience, prompting an immediate comprehensive evaluation and subsequent weekly face-to-face follow-ups. The results demonstrated a significant amelioration of depressive symptoms and anhedonia by the end of the first and second weeks, with 88.89% and 72.22% reductions in the HAMD-17 scores, respectively, as outlined in Table 1. Additionally, there was a notable improvement in the patient's anhedonia, with weekly increase in DARS scores by 35 and 32 points above the baseline, respectively. By the third week, the patient's reduction rate on HAMD-17 subsided to 44.44%, and the DARS scores decreased to 35 points. At the end of the fourth week, the patient's symptoms had exacerbated, described as "almost as before," and assessments were essentially congruent with pre-dezocine treatment baselines, prompting the patient to express a desire for augmented therapeutic intervention. Consequently, follow-up was discontinued, and the treatment plan was amended. During prolonged follow-up, the patient notably expressed appreciation for recent experiences via social media, without indicating any adverse progression or augmented dependency post-dezocine administration, advocating for additional clinical research.

Discussion

To our knowledge, this is the first clinical report of dezocine ameliorating depressive experiences and anhedonic symptoms in MDD, characterized by both a rapid onset of action and sustained effects, without evidence of rebound phenomena. This can be attributed to the distinct pharmacological properties of dezocine. Given the clinical observation that the efficacy of serotonin-norepinephrine reuptake inhibitors, blocking the activity of NET and SERT, is often delayed, the rapid antidepressant effect of dezocine may be associated with opioidergic modulation rather than monoaminergic systems alone. Synergistic potentiation between the opioidergic and monoaminergic systems cannot be excluded, especially given the evidence that ketamine can expedite

the antidepressant onset of escitalopram and boost its efficacy (Xiao et al., 2023). The sustained antidepressant effects of dezocine, with an average terminal half-life of approximately 2.4 h, raise questions as to whether this reflects placebo effects, reactivation of antidepressants, slow metabolism subsequent to receptor occupancy similar to ketamine (Ma et al., 2023), warranting further investigation.

Furthermore, it is noteworthy that the patient's background antidepressant is sustained-release bupropion. Given the recent FDA approval of the novel antidepressant dextromethorphan HBr-bupropion HCl (Auvelity) (Tabuteau et al., 2022), it is conceivable that the sustained therapeutic effect of dezocine might be attributed to prolonged exposure by competitive inhibition of cytochrome P450 2D6 (CYP 2D6) by bupropion, especially since studies have documented the impact of the CYP 2D6 genotype on the pharmacodynamics and pharmacokinetics of opioid medications (Ballester et al., 2022).

Addiction is the most politically charged adverse effect of opioid pharmacotherapy. However, the extant evidence for its addictive liability remains scant. In animal models, dezocine has even been observed to antagonize addiction-like behaviors induced by opioids such as morphine (Wu et al., 2019). Although it has the low potential to induce tolerance and dependence compared to more potent opioids, caution is warranted considering the protracted nature of antidepressant therapy. Alternatively, future research might actively investigate derivatives that circumvent the risk of addiction, along with rational dosing regimens.

While the current evidence is limited to case reports, the pharmacological profile of dezocine suggests that it has significant potential for the treatment of MDD, particularly in patients manifesting anhedonic symptoms. This has been indirectly validated in studies on buprenorphine, a compound with similar mixed opioid receptor activity (Namchuk et al., 2022). Compared to buprenorphine, dezocine's blockade of SERT and NET appears to more robustly support its antidepressant effects. However, the role of dezocine on KOR remains uncertain, with its agonist or antagonist activity at different doses yet to be clarified (Ye et al., 2022). This highlights the need for high-quality clinical and preclinical research to corroborate these findings and develop viable therapeutic protocols. Moreover, the opioidergic system is necessary but insufficient component for the antidepressant effects of ketamine (Klein et al., 2020). In conjunction with this case report, dezocine exhibited rapid and sustained antidepressant clinical effects, similar to those of ketamine (Grunebaum et al., 2018). However, dezocine is devoid of dissociative manifestations, whether dezocine possesses the potential to emerge as next ketamine in clinical applications still requires validation through large-scale, high-quality clinical studies.

Data availability statement

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

Ethics statement

The studies involving humans were approved by the institutional review board of the Beijing Anding Hospital, Capital Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

HW: Writing–original draft, Writing–review and editing. NL: Data curation, Writing–review and editing. QZ: Writing–review and editing.

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The forensic aspects of suicide and neurotrophin factors: a research study

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Introduction: Suicide represents a significant public health problem whose neurobiology is not yet fully understood. In many cases, suicidal behavior and psychiatric spectrum disorders are linked, in particular, to major depression. An emerging pathophysiological hypothesis underlines the role of neurotrophic factors, proteins involved in neurogenesis, in synaptic plasticity in response to stressors. Our research aims to evaluate the degree of expression of brain neurotrophic factor (BDNF) in brain areas involved in depressive disorder in suicidal subjects. Furthermore, we want to evaluate the expression of glial cell line-derived neurotrophic factor (GDNF) in suicidal subjects.

Methods: We selected twenty confirmed cases of suicide among subjects with a clinical history of depressive pathology and possible psychopharmacological treatment, compared to ten controls of individuals who died of non-suicidal causes. For all selected cases and controls, immunohistochemical investigations were performed using a panel of antibodies against the BDNF and GDNF antigens on samples from the various brain areas.

Results and discussion: The results show that BDNF was under-expressed in the cerebral parenchyma of subjects who died by suicide compared to controls, while there was an overexpression of GDNF in suicide victims, these data could be useful for a clinical application as potential markers for suicidal risk, to assess the severity of depression and development of specific pharmacological therapies for depression.

KEYWORDS

suicide, neurotrophic factor, immunohistochemistry, BDNF, GDNF

1 Introduction

According to the WHO, every year, about 700,000 people end their lives through suicide, making it the fourth leading cause of death in young people (datadot, 2024). According to estimates, for every suicide carried out, there are between 10 and 40 suicide attempts (Krug et al., 2002; Chang et al., 2011). Suicide turns out to be a real social problem, as it cannot leave public opinion indifferent. According to ISTAT (Italian National Institute of Statistics) data from 2016, 3780 committed suicides in Italy, 78.8% of whom were male. The death rate from suicide is 11.8 per 100,000 population for men and 3.0 per 100,000 population for women (Vichi et al., 2019). In Western countries, the suicide rate among men is three to four times higher than among women. This difference is likely to

be the case because men use reliably more lethal means of taking their own lives. This difference is even more marked in men aged 65 years and older, who are likely to be ten times more than women (Sue et al., 2016). In the Eastern Mediterranean countries, on the other hand, female and male suicide rates are almost identical. In many countries, suicide rates appear to be higher in middle age or old age. The absolute number of suicides, however, is highest in the age group between 15 and 29 years old (Pitman et al., 2012; Yip et al., 2012). Suicide is also related to age, with young people under 25 and older adults interested the most in the risk of suicide, although it proportionally increases with age (OECD, 2012). In 2018, the ISTAT estimated that 3,820 people committed suicide, with the highest incidence in males between 35 and 64 years old. The main methods used to commit suicide are hanging, pesticide poisoning, and the use of firearms. In a survey (Ajdacic-Gross et al., 2008) carried out in 56 countries, hanging was by far the most common method used by 53% of male suicides and 39% of female suicides (O Connor et al., 2011; Suicide in the Province, 2022). Suicide presents numerous risk factors (Beghi et al., 2021; Risk and Protective Factors, 2023), such as individuals (previous suicide attempts, chronic psychiatric and physical pathologies, drug addiction), relationships (history of family suicide, isolation, bullying), community (violence, discrimination), and social-linked (spectacularization by the media, access to lethal means). One of the most important risk factors is the presence of psychiatric pathology, primarily major depression, followed by anxiety disorder, bipolar disorder, and drug addiction (Beghi et al., 2021). Also, genetics can influence the risk of committing a self-suppressive act (Brent and Melhem, 2008). Among psychiatric pathologies, it is clear that patients suffering from major depression present a significant risk of suicide or attempted suicide (Conejero et al., 2018; Fusar-Poli et al., 2021). Identifying risk factors can recognize a part of the population at high risk of committing extreme acts (Granier and Boulenger, 2002).

The neurobiology underlying suicide is exceptionally complex, not being the result of a single pathophysiological entity but of a multiplicity of behavioral, socio-environmental, and psychological causal factors that act together simultaneously. According to the literature, suicide follows the "diathesis-stress" model: a behavioral disorder derives from a genetic predisposition that makes one more vulnerable to stress, usually caused by life events (Mann, 2003; Van Heeringen and Mann, 2014; Zeigler-Hill and Shackelford, 2019). In particular, traumatic life events and stress acts as triggers for suicidal behavior (Dwivedi, 2012). Furthermore, the interaction of numerous biological systems is involved (Girardi and Pompili, 2015), including the neurotrophic system. Indeed, the pathogenesis of depression and suicidal behavior involves changes in neuronal plasticity (Garcia, 2002), reducing the brain's ability to adapt.

Neurotrophins are growth factors that play a crucial role in regulating structural and synaptic plasticity, maintaining neuronal functions, and modulating neurotransmission (Thoenen, 1995). Neurotrophins are essential for the survival, growth, and regeneration of different neuronal populations belonging to the central and peripheral nervous systems in the fetal and adult brain (Allen et al., 2013). The first neurotrophin discovered in the 1950s was Nerve Growth Factor (NGF) (Levi-Montalcini, 1987). Neurotrophin is transported from the production site to the nerve terminal via axonal retrograde flow during development. Neurons that present this retrograde flow survive; otherwise, they degenerate ("neurotrophic hypothesis") (Hamburger et al., 1981; Ginty and Segal, 2002). There are five neurotrophic factors expressed in mammals: Brain-Derived Neurotrophic Factor (BDNF), Neurotrophins 3 and 4 (NT-3 and NT-4), Glial Derived Neurotrophic Factor (GDNF) and Ciliar Neurotrophic Factor (CNTF). Neurotrophins are initially synthesized as pro-neurotrophins, and then through proteolytic processes, they are converted into mature neurotrophins that interact with two classes of receptors (p75NRT and Trk) to act (Ibáñez and Simi, 2012; Neurotrophic, 2017). Neurotrophins also have numerous actions outside the nervous system, such as immune (Thorpe and Perez Polo, 1987; Otten et al., 1989; Donovan et al., 2000) and hematopoietic function (Thorpe et al., 1987). They also control some neuroendocrine functions (for example, the development of the female rat's hypothalamus) (Ojeda et al., 1991) and are involved in the transmission of pain (Skaper, 2017). Furthermore, these molecules can increase cholinergic function and protect against neurodegeneration (Hock et al., 2000; Hoxha et al., 2018), hypothesizing a role in Alzheimer's disease (Hefti, 1986). Similarly, the GDNF can be involved in Parkinson's disease, as it acts on dopaminergic neurons (Yasuda and Mochizuki, 2010). Therefore, a pathological alteration of neurotrophic factors could determine defects in neural maintenance and regeneration, possible structural anomalies in the brain, and a reduction in neural plasticity, thus compromising the individual's ability to adapt in critical situations.

BDNF ("Brain-Derived Neurotrophic Factor") is the most widespread neurotrophin in the brain of mammals, both in adults and in the developing phase (Anisman et al., 2012). It is involved in neurogenesis, the development of neurons, neuronal homeostasis, the structural integrity and maintenance of neuronal plasticity in the adult brain (Altar et al., 1997; Bartrup et al., 1997; Edelmann et al., 2014; Panja and Bramham, 2014), synaptic connection, in the mechanisms that regulate learning and memory (Kang and Schuman, 1995), drug addiction, and in stress adaptation mechanisms. Numerous exogenous and endogenous stimuli (such as stress, physical activity, diet, and brain injury) regulate BDNF expression (Aid et al., 2007; Pruunsild et al., 2007). Several neuropathologies cause a reduction in BDNF protein levels and serum in patients' brains (Autry and Monteggia, 2012; Borba et al., 2016). Unfortunately, it is unclear whether serum BDNF levels reflect brain levels, as studies contradict each other (Sartorius et al., 2009; Klein et al., 2011). However, serum BDNF levels should be higher than those in plasma and cerebrospinal fluid (Radka et al., 1996; Serra-Millàs, 2016). According to some clinical studies that examined the blood levels of BDNF *in vivo*, the BDNF can cross the blood-brain barrier in both directions via a high-capacity saturable transport system, which determines an early influx and rapid in the brain (Pan et al., 1998). BDNF concentrations can be measured in serum, plasma, or whole blood and appear highly correlated to those in cerebrospinal fluid. Therefore, BDNF levels in the blood could be related to the concentration of the same neurotrophin at the level of the cerebral cortex (Klein et al., 2011). The blood quantification of BDNF *in vivo* could be a marker of neuronal plasticity (Shimizu et al., 2003). Furthermore, BDNF also plays an essential role in neuroinflammation and aging, identifying a role in degenerative diseases such as Parkinson's and Alzheimer's (Lima et al., 2019). In

humans, BDNF is considered a promising biomarker for various psychiatric pathologies (Chen et al., 2017). In some animal models, mice heterozygous for the BDNF gene showed increased aggression and anxiety, significant weight gain, and memory impairment, suggesting that its depletion could be associated with the development of some psychiatric symptoms (Lindholm and Castracn, 2014). This hypothesis also seems supported by the fact that there is an increase in BDNF levels after treatment of psychiatric pathologies (Nuernberg et al., 2016; Castrén and Monteggia, 2021). Patients with major depression show an appreciable reduction in serum levels of BDNF (Shimizu et al., 2003), probably due to a reduction of the protein in the brain (Karege et al., 2005a). There is also a single nucleotide polymorphism of the BDNF gene (Val66Met) that is associated with the severity of depressive symptoms and memory deficits (Youssef et al., 2018; Zhao et al., 2018), as well as a higher risk of suicide attempts (Zai et al., 2012). Typically, treatment with antidepressant drugs increases BDNF levels in serum and plasma (Molendijk et al., 2011). Some post-mortem studies have shown that treatment-resistant patients had significantly low levels of BDNF, especially in areas such as the hippocampus, which otherwise is generally rich (Colla et al., 2007; Brunoni et al., 2014). Other studies have demonstrated reductions in levels of BDNF mRNA and its protein from post-mortem brain samples of depressed patients, in particular in the hippocampus and amygdala (Guilloux et al., 2012; Ray et al., 2014). The reduction of serum BDNF levels has also been ascertained in other psychiatric pathologies, such as schizophrenia (Ray et al., 2014), autism (Katoh Semba et al., 2007), and bipolar disorder (Fernandes et al., 2015). Furthermore, in animal models, BDNF levels in the prefrontal cortex and hippocampus were increased after pharmacological treatment with mood stabilizers (Yasuda et al., 2009; Jornada et al., 2010). In the literature, there are numerous studies carried out on the brains of suicidal victims, which observe a reduction in the levels of BDNF protein and its receptor in the hippocampus (Karege et al., 2005a; University, 2021) and the prefrontal cortex (Zheng et al., 2016; Misztak et al., 2020), as well as in its mRNA (Hamburger et al., 1981). Thus, BDNF levels could represent an interesting biological marker of suicidal behavior.

Glial cell line-derived neurotrophic factor (GDNF) is a neurotrophin expressed primarily during neuronal development and differentiation. In the adult, its expression decreases and remains limited to some regions, such as the cortex, the hippocampus, the substantia nigra, and the striatum nucleus (Duarte Azevedo et al., 2020). GDNF promotes the differentiation of dopaminergic (Christophersen et al., 2007) and serotonergic (Ducray et al., 2006) neurons. Furthermore, one of its most important roles is the protection of neurons (Hochstrasser et al., 2011; Uzdensky et al., 2013; Jaumotte and Zigmond, 2014) from oxidative stress and inflammation (Pascual et al., 2008). GDNF levels appear to be reduced in patients who have Alzheimer's disease, leading to the hypothesis of its use as a biomarker for the pathology [(Sharif et al., 2021; Kimhy et al., 2015)]. Although there are not many studies in the literature relating to the involvement of GDNF in the suicidal phenomenon, there is evidence linking this neurotrophin to the onset of mood disorders. In fact, according to some studies, there are lower serum GDNF levels in patients suffering from depression, with a subsequent increase after pharmacological treatment (Lin and Tseng, 2015; Wang et al., 2023). In a post-mortem study, an increase in GDNF was observed in the parietal cortex (site of emotion regulation (Anderson et al., 2004)) of patients with depressive disorder (Michel et al., 2008), while in other studies, a reduction in the expression of its mRNA was measured (Otsuki et al., 2008). In the literature, GDNF levels increase following ischemic or inflammatory damage (Wei et al., 2000; Michel et al., 2007) as neuronal resilience and plasticity compromise, hypothesizing that the increase in GDNF is an adaptive and compensatory response to neuronal damage (Chao and Lee, 1999; Tokumine et al., 2003).

Currently, many studies on the possible link between neurotrophins and suicide are carried out *in vivo* or plasma (Kim et al., 2007; Salas Magana et al., 2017; Sonal and Raghavan, 2018). Further interesting data could come from post-mortem studies carried out directly on the brains of suicidal subjects. An essential element is that many of these studies focus on the link between neurotrophins and neuro-psychiatric pathology. However, not all subjects who commit suicide suffer from a diagnosed psychiatric pathology. A recent literature review (De Simone et al., 2022) shows a paucity of experimental work on BDNF and GDNF. It shows that the altered regulation of BDNF, such as the Val66Met polymorphism, can favor the onset of psychiatric disorders linked to stress (Felmingham et al., 2013; Zhao et al., 2018; Miao et al., 2020), leading to an increase in suicidal risk according to some works (Zai et al., 2012; Paska et al., 2013), or no correlation with suicide, according to another study (Ratta-apha et al., 2013). Some of the studies examining the level of BDNF in post-mortem brain samples showed reduced values compared to controls, regardless of psychiatric diagnosis, in the prefrontal cortex and hippocampus (Dwivedi et al., 2003; Karege et al., 2005b; Ducray et al., 2006; Hochstrasser et al., 2011), as well as in the amygdala (Ray et al., 2014) and the anterior cingulate cortex (Tripp et al., 2012) of subjects suffering from major depressive disorder. Schneider et al. (2015) also evaluated subjects suffering from depression, detecting increased methylation of the BDNF promoter in the frontal cortex, in agreement with other studies (Keller et al., 2010; Kang et al., 2013). Another interesting finding, supported by further evidence (Pan et al., 1998; Klein et al., 2011), is that the brain level of BDNF was higher in suicidal subjects suffering from major depression on pharmacological therapy compared to non-suicidal controls (Gadad et al., 2021). As regards GDNF, in the study by Michel et al. (Michel et al., 2007), it was not possible to demonstrate a statistically significant increase in the levels of this neurotrophin in different brain areas of depressed patients taking antidepressant drug therapy.

The results of this systematic review partially support the hypothesis that a lower level of neurotrophins is connected to an increased risk of suicide. The identification of a suicide biomarker remains a challenge for the scientific and forensic community. The objective of the present study is to analyze the correlation between suicide and the degree of expression of BDNF and GDNF on autopsy samples in specific brain areas. These neurotrophic factors could have an important role both in the prevention of suicidal events in the population at high risk for anti-conservative behavior, allowing early action to limit this risk preventively, and in the search for a new potential pharmacological target. In the literature, there are no valuable markers for the identification of suicidal risk, so BDNF and GDNF could be promising for identifying suicidal risk in people with well-defined risk factors.

TABLE 1 The table shows the main information on the selected cases.

N°	Sex	Age	Manner	Psychiatric history	Previous suicide attempts	Pharmacological therapy
1	M	40	Gunshot injury	Pathological gambling	No	No
2	M	36	Hanging	Reported job loss	No	No
3	F	39	Precipitation	Anorexia nervosa and bipolar disorder	No	No[a]
4	M	40	Precipitation	Substance use disorder	No	No
5	M	21	Precipitation	Alcoholism	No	
6	M	56	Hanging	Major depressive disorder	No	No
7	M	37	Hanging	Substance use disorder	No	No
8	M	24	Precipitation	Autism spectrum disorder	No	No
9	M	65	Hanging	Major depressive disorder	No	No
10	M	35	Hanging	Substance use disorder Convicted	No	No[a]
11	M	42	Hanging	Not diagnosed Convicted	No	No
12	M	46	Hanging	Convicted	Yes	No
13	M	35	Hanging	Adjustment disorder Convicted	Yes	No[a]
14	M	89	Use of a sharp weapon	Not diagnosed	Yes	No
15	M	36	Hanging	Convicted	No	No
16	F	22	Hanging	Self-harm History of childhood abuse	No	No
17	M	58	Hanging	Major depressive disorder	No	No
18	M	85	Use of a sharp weapon	Previous mournful events reported	No	No
19	M	62	Hanging	Major depressive disorder	No	No
20	M	84	Hanging	Major depressive disorder	No	No

[a]Indicates subjects on pharmacological therapy with drugs (benzodiazepines) in doses within the therapeutic range.

2 Materials and methods

2.1 Sample selection

We conducted a retrospective study based on a series of judicial autopsies performed at the Forensic Medicine of the University of Foggia and the University of Ferrara.

The sample consists of twenty subjects who died following suicide between November 2020 and March 2023 (Table 1). The autopsies were performed 36–48 after the death and we excluded from the study corpses in an advanced stage of decomposition, corpses testing positive for common substances of abuse, and subjects suffering from neurodegenerative diseases, Alzheimer's, and Parkinson's disease. For subjects who used drugs, only benzodiazepines in three cases, we dosed the active ingredients, ensuring that they were at therapeutic doses (between 2 and 5 mg/day). We selected ten case controls, chosen among subjects who died of natural or only chest traumatic causes, without a history of psychiatric pathology or drug addiction, and who were negative for toxicological analyses.

In our sample, six individuals did not have a psychiatric diagnosis. However, they presented particularly critical and stressful recent events (e.g., bereavement, loss of job, imprisonment). The remaining fourteen cases, however, had a known psychiatric pathology; of these, six were affected by mood disorders (five from major depressive disorder, one from bipolar disorder), eight from other types of diagnosed psychiatric pathology (pathological gambling, substance use disorder, problematic use of alcohol, autism spectrum disorder, self-harm, adjustment disorder). The characteristics of these disorders are summarized in Table 2 based on the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5). In all cases, we performed toxicological analyses on biological fluids to test the primary substances of abuse and the most common pharmacological active ingredients.

To exclude a possible influence of psychopharmacological active ingredients on the expression of BDNF and GDNF, we excluded subjects treated with antidepressant and antipsychotic drugs or positive for narcotic substances. Three of the twenty subjects studied regularly took only a mild therapy with sedative drugs (benzodiazepines), whose active ingredients, based on the results

TABLE 2 The table shows the most relevant features of psychiatric disorders diagnosed in the selected cases, based on the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-5).

Disorder	No of subject affected	Definition based on DSM-V
Major depressive disorder	5	An individual must have five of the following-mentioned symptoms: ersistently low or depressed mood, anhedonia or decreased interest in pleasurable activities, feelings of guilt or worthlessness, lack of energy, poor concentration, appetite changes, psychomotor retardation or agitation, sleep disturbances, or suicidal thoughts. One must be a depressed mood or anhedonia causing social or occupational impairment
Bipolar disorder	1	Chronically occurring episodes of mania or hypomania alternating with depression. A manic episode is defined as a distinct period of persistently elevated or irritable mood with increased activity or energy lasting for at least seven consecutive days or requiring hospitalization. A hypomanic episode is defined as a distinct period of persistently elevated or irritable mood with increased activity or energy lasting for at least four consecutive days
Pathological gambling	1	Persistent, recurrent maladaptive patterns of gambling behavior, associated with impaired functioning, reduced quality of life, and high rates of bankruptcy, divorce, and incarceration
Substance use disorder	3	Patterns of symptoms caused by using a substance that an individual continues taking despite its negative effects. There are 11 diagnostic criteria fall under four basic categories — impaired control, physical dependence, social problems and risky use: using more of a substance than intended or using it for longer than you're meant to; trying to cut down or stop using the substance but being unable to; experiencing intense cravings or urges to use the substance; needing more of the substance to get the desired effect; developing withdrawal symptoms when not using the substance; spending more time getting and using drugs and recovering from substance use; neglecting responsibilities at home, work or school because of substance use; continuing to use even when it causes relationship problems; giving up important or desirable social and recreational activities due to substance use; using substances in risky settings that put you in danger; continuing to use despite the substance causing problems to your physical and mental health
Problematic use of alcohol	1	Persistent and problematic use of alcohol despite significant distress and impairment, with associated behavioral and physical symptoms, including withdrawal, tolerance, and cravings. Alcohol use disorder is characterized by failure to fullfill daily responsibilites and role obligations, alcohol-seeking behavior, unsuccesful efforts to control alcohol use, drinking despite potential hazards (e.g., drinking while driving), the need for increased amounts of alcohol to achieve its effects (tolerance), and withdrawal symptoms when one stops or reduces alcohol intake (e.g., hand tremors, nausea, agitation, hallucinations)
Autism spectrum disorder	1	DSM-V recognizes two main symptom areas: deficits in social communication and interaction AND restricted, repetitive behaviors, interests, or activities. These symptoms appear early in a child's development—although diagnosis may occur later. Autism is diagnosed when symptoms cause developmental challenges that are not better explained by other conditions
Self-harm	1	Deliberate, self-inflicted destruction of body tissue without suicidal intent and for purposes not socially sanctioned, includes behaviors such as cutting, burning, biting and scratching skin, especially present during adolescence
Adjustment disorder	1	Presence of emotional or behavioral symptoms in response to an identifiable stressor(s) occurring within 3 months of the onset of the stressor(s). It could be present with anxiety, depressed mood or both, or disturbance of conduct

of toxicological analyses, still fell within the therapeutic prescribed dosage range.

2.2 Methods

For each case, the entire brain was removed during the autopsy examination, with appropriate preservation in formol solution for 3 weeks to perform a correct fixation of the tissues. The brain was dissected using sagittal cuts, identifying and taking four areas of interest from the right hemisphere: the prefrontal cortex, cingulate gyrus, basal nuclei, and hippocampus.

All the samples obtained were embedded in paraffin and treated by immunohistochemical staining for anti-BDNF and anti-GDNF antibodies to proceed with the search for neurotrophins. The two antibodies used are BDNF (monoclonal mouse antibody Catalog Number: 66292-1-ig, Proteintech Group, Chicago, United States), and GDNF (monoclonal rabbit antibody, Catalog Number: orb572592, Biorbyt Ltd., Cambridge, United Kingdom). For both antibodies, it was used the Protein Atlas website for the selection of positive and negative controls, and then it was performed various tests for the individuation of the correct pretreatment and dilution, according to the indications of the Production Company, published papers, and the suggestions of www.atlasantibodies.com.

For each case, sections of approximately 4 μm were made on the microtome. The sections, mounted on a slide, were hydrated in decreasing alcohol solutions. After inhibiting endogenous peroxidase, the sections were subjected to antigen retrieval in Citric acid 0.1 M and subsequently incubated with the primary antibody (dilution 1:200 for BDNF and 1:500 for GDNF). The formation of the immune complex was highlighted by applying a Streptavidin-Biotin system (HRP-DAB system research and development kit CTS005, R&D Systems, Inc., Minneapolis, MN, United States). The reaction was visualized by peroxidation of 3,3′-diaminobenzidine (DAB). Once the reaction was verified, the nuclei were counter-stained with hematoxylin, and subsequently slides were subjected to dehydration. The slide was then mounted and observed using a Nikon Eclipse E90i optical microscope, using the NIS – Elements F program. BDNF and GDNF immunopositivity in the right frontal cortex, hippocampus (dentate gyrus (DG) -Lacunar-molecular, Radiate, Granular

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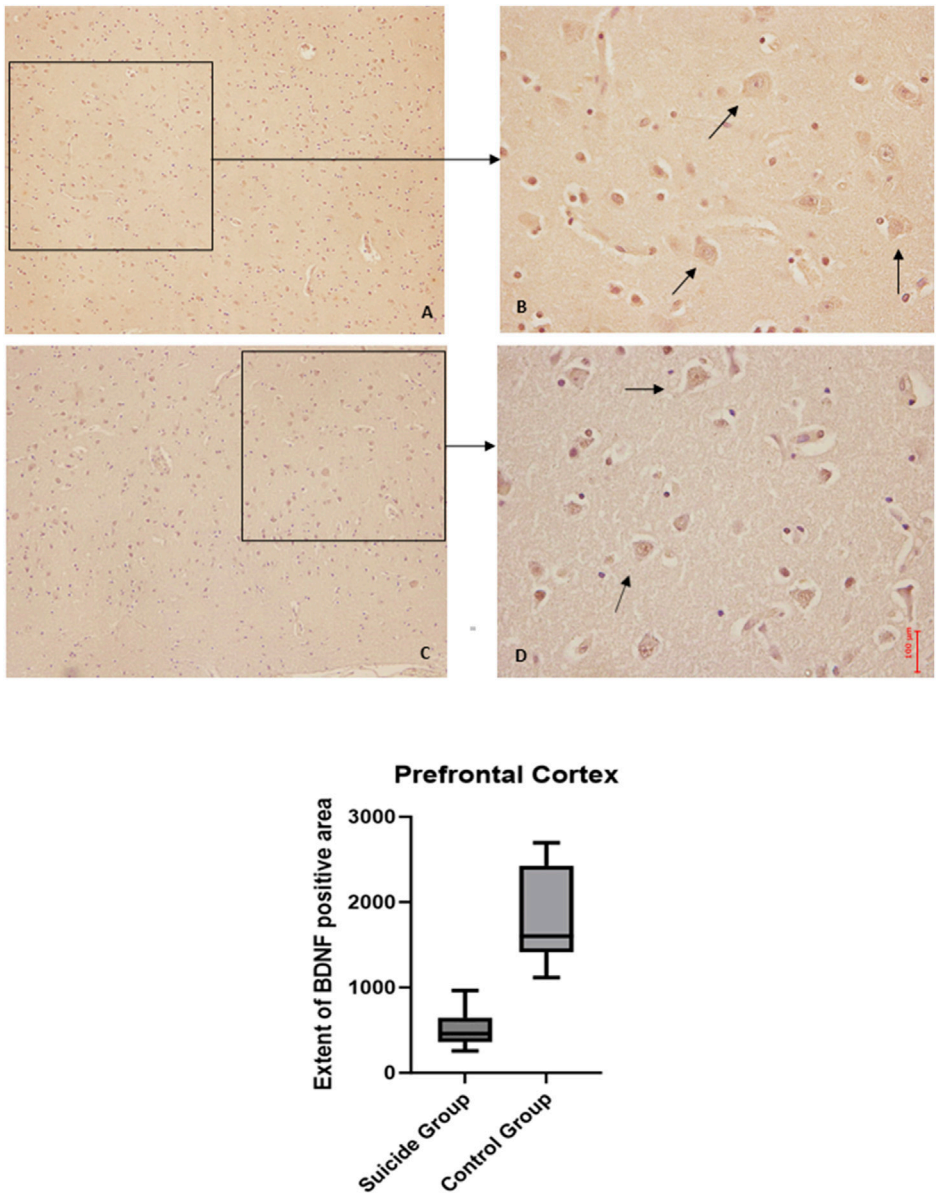

FIGURE 1
BDNF immunohistochemical expression in the prefrontal cortex. Prefrontal cortex immunohistochemistry results: normal immunohistochemistry reaction of BDNF in the control case, see arrows **(A)** 10x magnification **(B)** 40x magnification; reduced expression of BDNF in a sample of suicide cases, see arrows **(C)** 10x magnification **(D)** 40x magnification. In the lower part of the figures, the graphical representation of the statistical analysis, the percentage decrease of BDNF in the suicide cases group is 24%.

layers and Hilus:.), girus of the cingulum, and basal nuclei. For the hippocampus, the granular layer and hilus are the selected areas for the statistical analysis because they are more significant. Four visual fields of approximately 350 μm × 350 μm were randomly selected in interest of each area for each sample of cases and controls. Images for each slide were acquired with a digital camera (Nikon) connected to the microscope at 10x for an exhaustive semiquantitative, preliminary evaluation of the reaction and after at 40 × magnification in the more significant area for quantification. The parameters for image acquisition were established at the beginning of the observation and kept constant for all images. Quantifying cells positive for DAB staining was performed using ImageJ software (imagej.nih.gov/ij/) and expressed as the number of positively stained cells per analyzed area. Quantifications were expressed as the number of positive-stained cells/analyzed area. For hippocampus we quantized the radiate layer and hilus because are the areas. Blind researchers performed histological analyses concerning the information about the cases. The blinding of the data was maintained until the analysis was terminated.

2.3 Statistic analysis

Data were analyzed using Windows GraphPad Prism 10 software for Windows (La Jolla, CA, United States). The data

were analyzed through the two-way ANOVA analytical system (two-way ANOVA) for two independent factors. A *P*-value < 0.05 was considered statistically significant. Results are expressed as means ± standard deviation.

3 Results

3.1 Prefrontal cortex

The two-way ANOVA highlighted a statistically significant difference in both cases ($p < 0.0001$). In particular, a reduced expression of BDNF was noted in suicide cases compared to controls, while the expression of GDNF was increased in cases compared to controls (Figures 1, 2).

3.2 Hippocampus

The application of the two-way ANOVA test returned a statistically significant difference between the expression of BDNF ($p < 0.001$) and GDNF (<0.0001). In particular, it was possible to highlight a reduced expression of BDNF in suicide cases compared to controls, while the expression of GDNF was

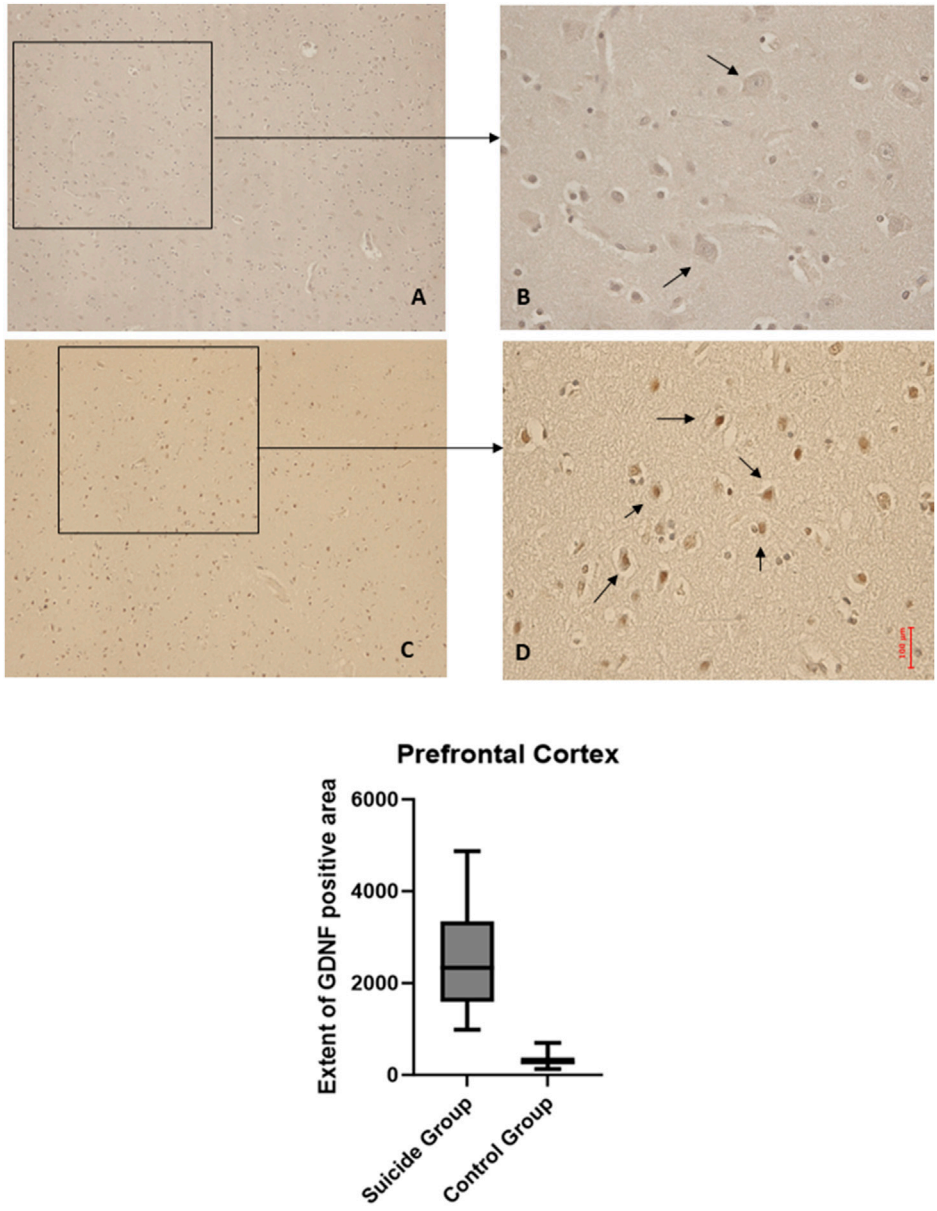

FIGURE 2
GDNF immunohistochemical expression in the prefrontal cortex. Prefrontal cortex immunohistochemistry results: basal immunohistochemistry reaction of GDNF in thecontrol case (the control case used is the same as Figure 1, you can see in consecutive slice section the difference of reaction between the two markers), see arrows **(A)** 10x magnification **(B)** 40x magnification; over-expression of GDNF in a sample of suicide case, see arrows **(C)** 10x magnification **(D)** 40x magnification. The graphical representation of the statistical analysis is in the lower part of the figures.

found to be increased in cases compared to controls (Figures 3, 4).

3.3 Gyrus of the cingulum

The two-way ANOVA analysis highlighted a statistically significant difference in both cases ($p < 0.0001$). In particular, a reduced expression of BDNF was noted in suicide cases compared to controls, although less marked than in the other areas examined. Also, in this case, the expression of GDNF is increased in cases compared to controls (Figures 5, 6).

3.4 Basal nuclei

The two-way ANOVA showed a statistically significant difference in the expression of BDNF ($p < 0.001$), while no statistically significant differences were highlighted in GDNF ($p < 0.1$). In particular, a reduced expression of BDNF was noted in suicide cases compared to controls, while a minimal increase in

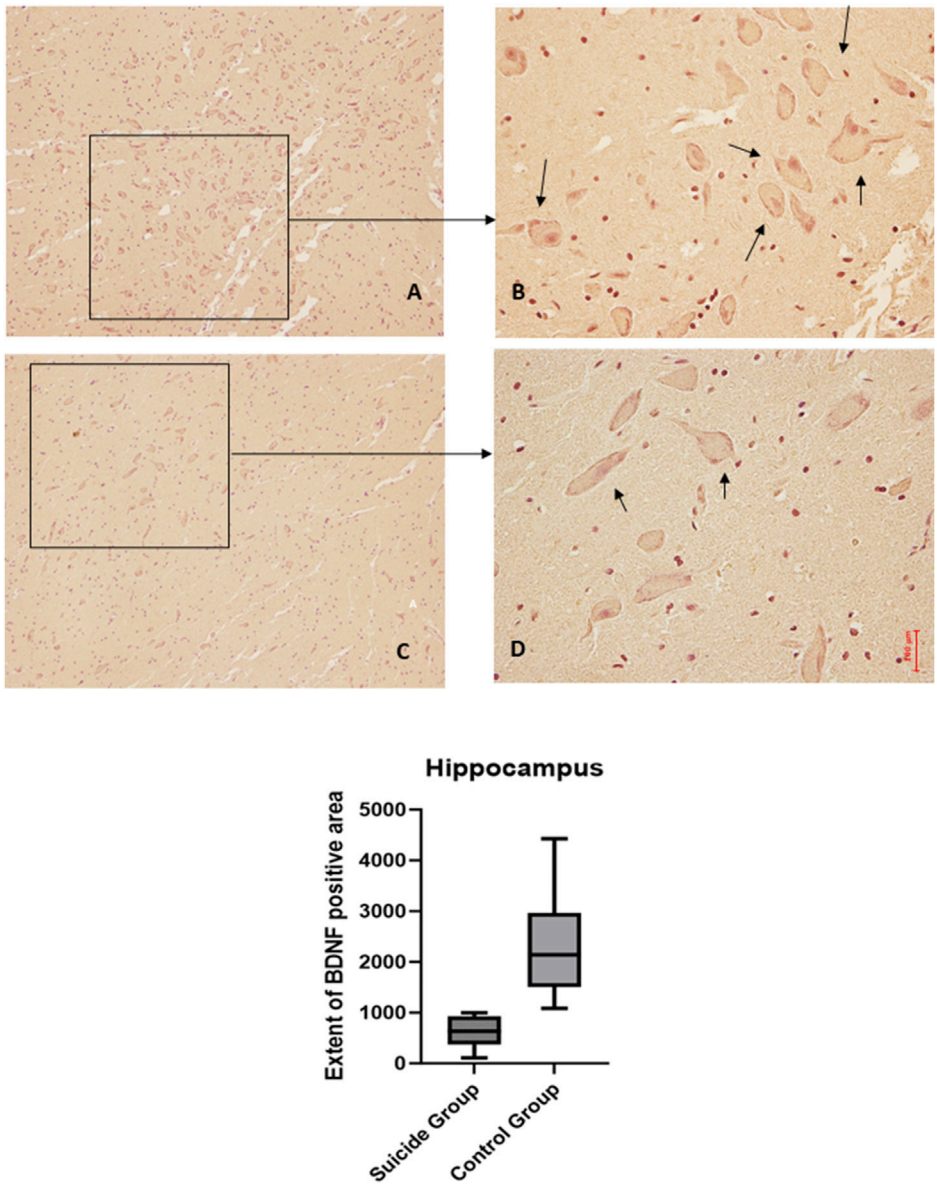

FIGURE 3
BDNF immunohistochemical expression in the hippocampus. Hippocampus (dentate gyrus) immunohistochemistry results: normal immunohistochemistry reaction of BDNF in the control case (granular layer of dentate gyrus), see arrows **(A)** 10x magnification **(B)** 40x magnification; reduced expression of BDNF in a sample of suicide case (granular layer of dentate gyrus), see arrows **(C)** 10x magnification **(D)** 40x magnification. In the lower part of the figures, the graphical representation of the statistical analysis, the percentage decrease of BDNF in the suicide cases group is 27%.

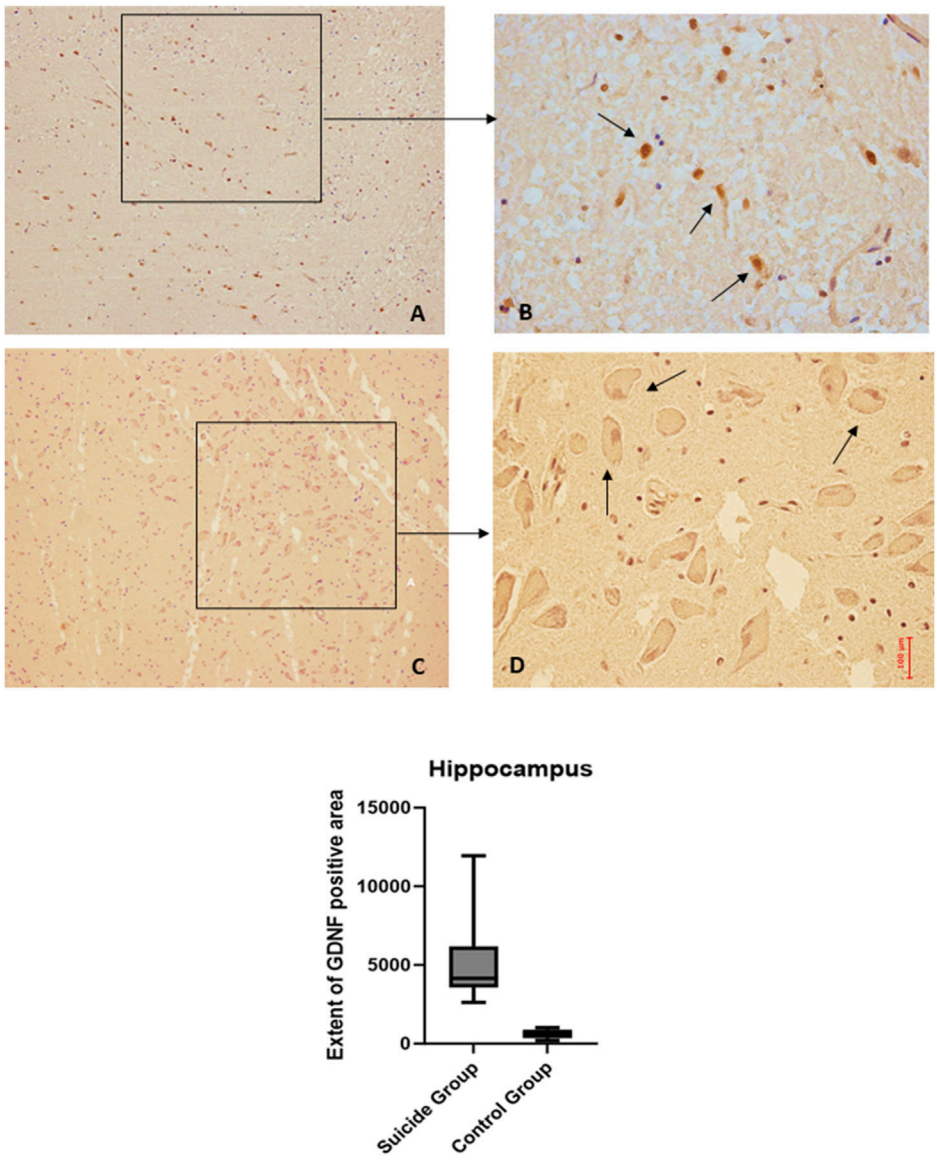

FIGURE 4
GDNF immunohistochemical expression in the hippocampus. Hippocampus (dentate gyrus) immunohistochemistry results: basal immunohistochemistry reaction of GDNF in the control case (hilus of dentate gyrus), see arrows **(A)** 10x magnification **(B)** 40x magnification; over-expression of GDNF in a sample of suicide case (granular layer of dentate gyrus), see arrows **(C)** 10x magnification **(D)** 40x magnification. In the lower part of the figures is the graphical representation of the statistical analysis.

GDNF expression was observed in cases compared to controls (Figures 7, 8).

4 Discussion

There are few studies in the literature relating to the measure of BDNF and GDNF on post-mortem samples of suicidal subjects, especially those without psychiatric pathologies. Our work aims to contribute to science to identify molecules useful as markers of suicide risk. These neurotrophins could also be potential pharmacological targets for researching and developing new targeted therapies.

The sample examined in our study was composed of twenty subjects who died by suicide and ten controls who died of non-suicidal causes, matched for sex and age, who underwent autopsy examination. 90% of subjects were male (18 of 20), 10% were female (2 of 20), 13 of them (65%) were under 50 years old, and 7 (35%) were over 50 years old. Furthermore, 6 out of 20 individuals (30%) did not have any diagnosis of psychiatric disorder. However, they had been involved in recent stressful events, for example, bereavement, job loss, and imprisonment. The remaining 14 cases (70%), however, presented a psychiatric pathology; of these, six were affected by mood disorders (five from major depressive disorder, one from bipolar disorder), eight from other types of diagnosed psychiatric pathology (pathological gambling, substance use disorder, problematic use of alcohol, autism spectrum disorder, self-harm, adjustment disorder). Five of the 20 cases analyzed (25%) were subjects subjected to a prison regime, another risk factor.

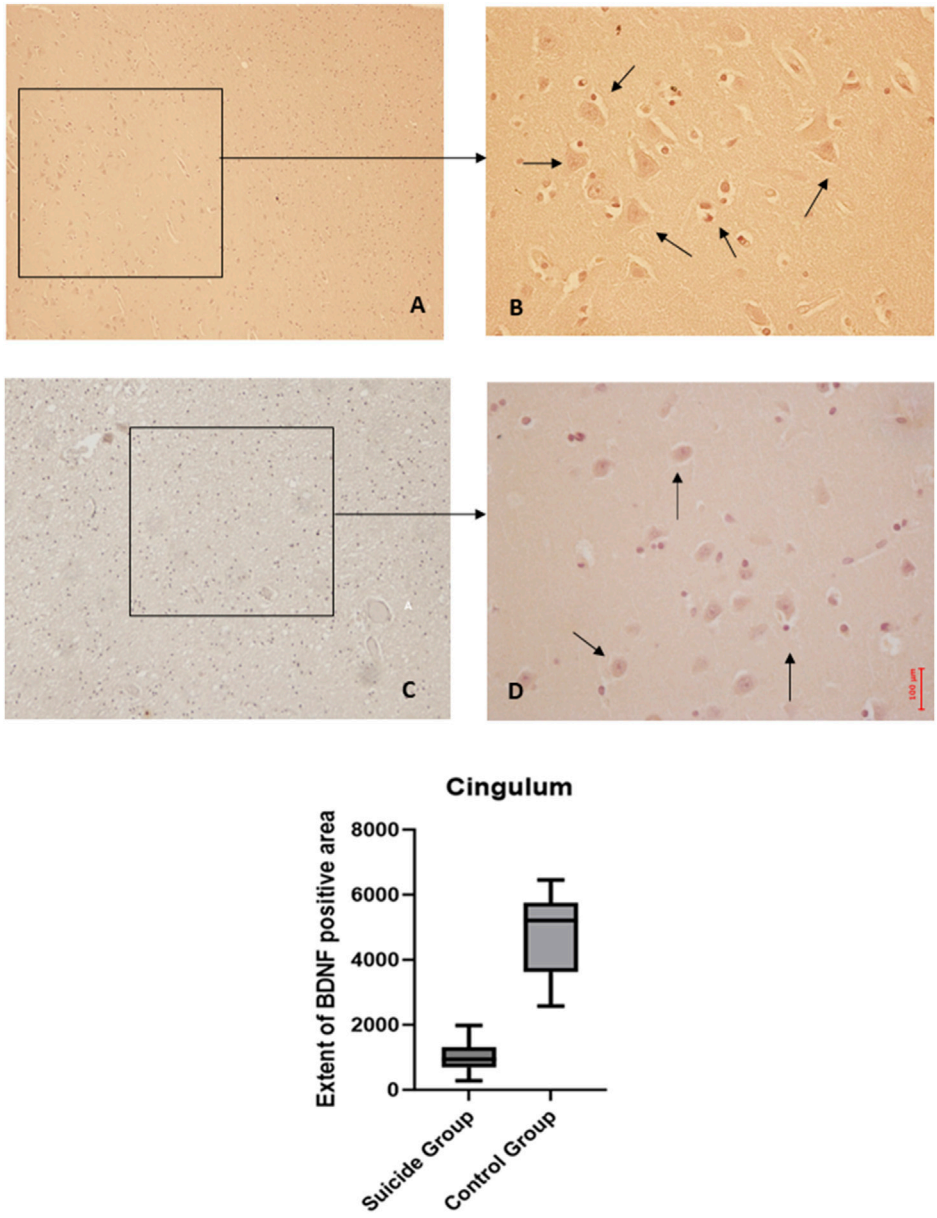

FIGURE 5
BDNF immunohistochemical expression in cingulum. Cingulum immunohistochemistry results: normal immunohistochemistry reaction of BDNF in the control case, see arrows **(A)** 10x magnification **(B)** 40x magnification; reduced expression of BDNF in a sample of suicide cases, see arrows **(C)** 10x magnification **(D)** 40x magnification. In the lower part of the figures, the graphical representation of the statistical analysis shows that the percentage decrease of BDNF in the suicide cases group is 21%.

To exclude the possible influence of psychopharmacological active ingredients on the expression of BDNF and GDNF, the authors excluded patients on therapy with antidepressant and antipsychotic drugs and those who tested positive for narcotic and psychoactive substances in the toxicological analyses. Therefore, subjects who committed suicide due to overdose were also excluded. Only three of the 20 subjects studied were taking mild therapy based on non-neuroleptic or antidepressant drugs (mainly benzodiazepines) whose active ingredients, based on the results of toxicological analyses, were still within the therapeutic dosage range.

11 out of 20 subjects (55%) died from hanging [the most used method, according to literature data (Baldari et al., 2021)], 4 out of 20 (20%) from precipitation, 2 out of 20 (10%) from stab wounds, 2 in 20 (10%) by drowning and 1 in 20 (5%) by gunshot wounds.

The results of our work, which used an immunohistochemical method widely used in the field of forensic pathology (Baldari et al., 2021), highlighted a statistically significant reduction in BDNF levels in the various brain regions examined in all twenty suicidal subjects compared to the ten controls. In particular, the areas most affected are the prefrontal cortex and cingulate gyrus ($p < 0.0001$), to a

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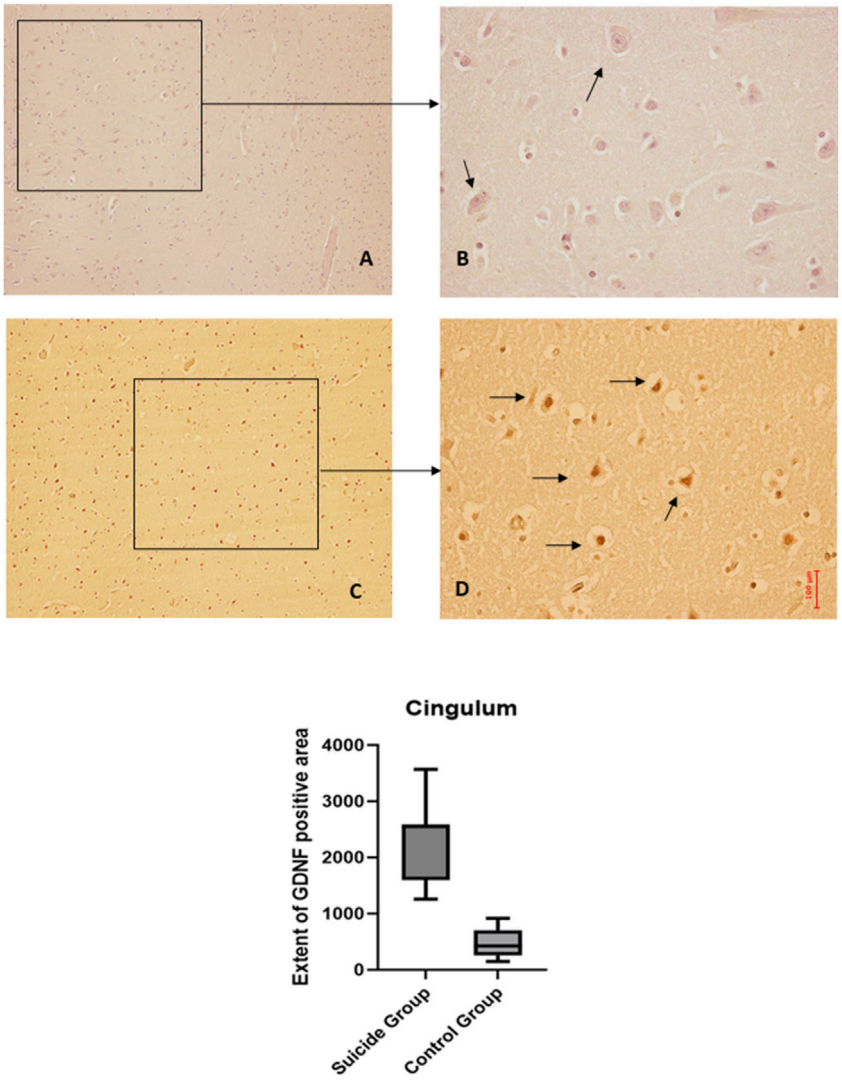

FIGURE 6
GDNF immunohistochemical expression in cingulum. Cingulum immunohistochemistry results: basal immunohistochemistry reaction of GDNF in the control case, see arrows **(A)** 10x magnification **(B)** 40x magnification; over-expression of GDNF in a sample of suicide case, see arrows **(C)** 10x magnification **(D)** 40x magnification. The graphical representation of the statistical analysis is in the lower part of the figures.

slightly lesser extent, hippocampus and basal nuclei ($p < 0.001$). These data agree with the literature (Middlemas et al., 1991; Karege et al., 2002; Dwivedi et al., 2003; Karege et al., 2005b; Tripp et al., 2012), which reports significant decreases in BDNF levels in the prefrontal cortex, hippocampus, and cingulate gyrus. Furthermore, it is interesting to consider that the global examination of the different brain areas highlighted a statistically significant reduction in BDNF in suicidal subjects compared to controls ($p < 0.0001$).

The encephalic expression of GDNF was increased in all 20 study subjects. In particular, the authors observed a statistically significant ($p < 0.0001$) increase in GDNF in suicidal subjects compared to controls at the prefrontal cortex, hippocampus, and cingulate gyrus. In the basal nuclei of suicidal subjects, however, there was a slight increase in GDNF levels compared to controls, which was not statistically significant. Interestingly, the global examination of the different brain areas highlighted an increase in GDNF in suicidal subjects, compared to controls, which was statistically significant ($p < 0.0001$).

Our research is unique, as we analyzed the expression of GDNF on the brain tissue of suicidal subjects without therapy. The few studies in the literature on GDNF have evaluated its concentration in the peripheral blood of patients with depressive or, more generally, mood disorders.

The results obtained agree with the data of another work (Rosa et al., 2006), which documented an increase in GDNF in the peripheral blood of patients who have bipolar disorder in the depressive phase. Thus, increased BDNF synthesis could be a characteristic of acute episodes of mood disorders, although the literature is unclear (Takebayashi et al., 2006; Otsuki et al., 2008).

An important point of the study is that we have only selected autoptic cases of subjects who have not undergone drug therapy. In literature, most of the studies concern subjects on antidepressant drug therapy, finding a reduction in the expression of GDNF in

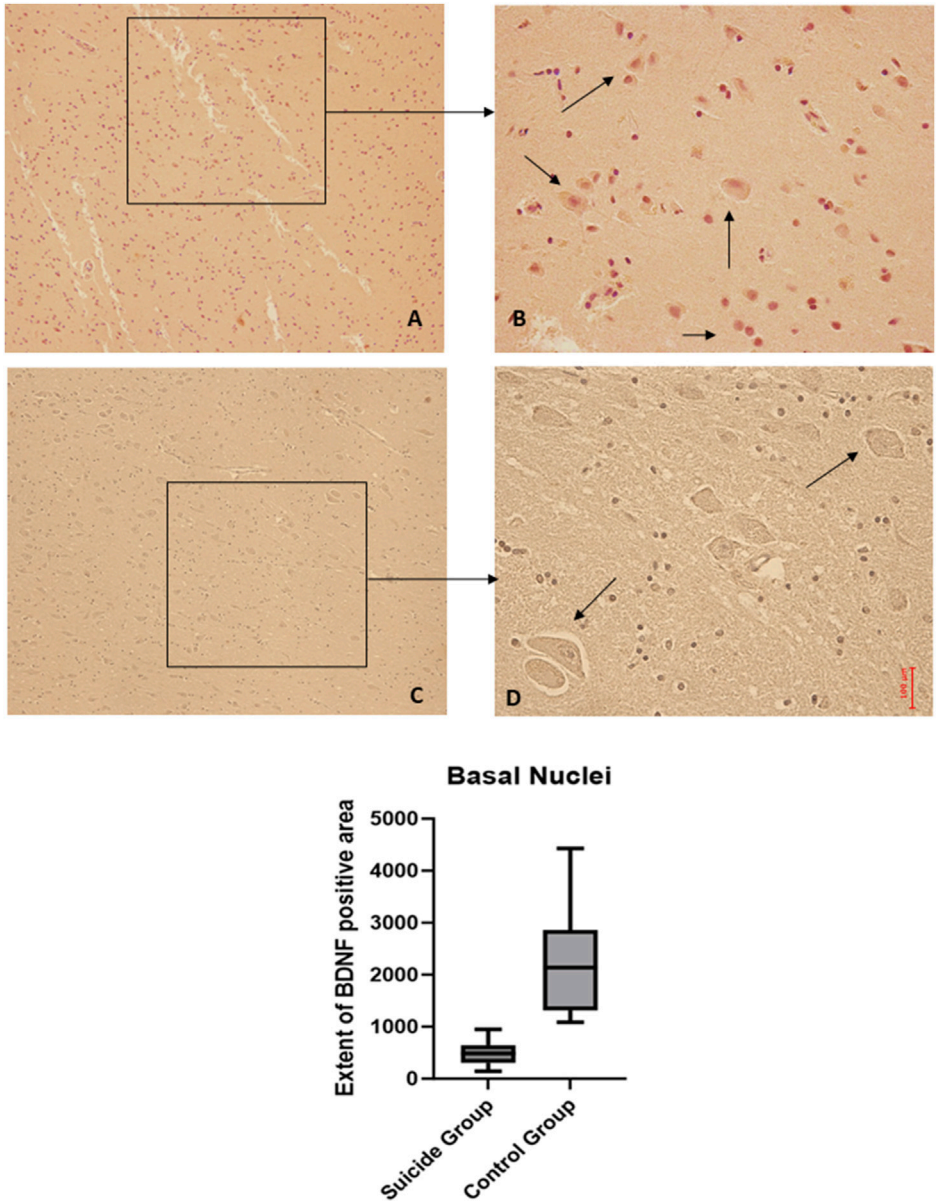

FIGURE 7
BDNF immunohistochemical expression in basal nuclei. Basal nuclei immunohistochemistry results: normal immunohistochemistry reaction of BDNF in the control case, see arrows **(A)** 10x magnification **(B)** 40x magnification; reduced expression of BDNF in a sample of suicide case, see arrows **(C)** 10x magnification **(D)** 40x magnification. In the lower part of the figures, the graphical representation of the statistical analysis shows that the percentage decrease of BDNF in the suicide cases group is 21%.

plasma or blood (Maheu et al., 2015). On the contrary, in our study, the GDNF molecule in the brain is increased. It suggests that central GDNF signaling may represent a novel target for antidepressant treatment (Maheu et al., 2015).

In our study, we evaluate neurotrophic expression at the brain tissue, while other studies evaluate it at the plasma peripheral level (Sun et al., 2019). It would be interesting to conduct a multidisciplinary clinic-based assessment to observe whether the reduction in GDNF correlates with an improvement in symptoms.

The authors analyzed three further subgroups of the 20 cases: six subjects suffering from psychiatric pathology, eight subjects suffering from mood disorders, and six normal subjects. This analysis revealed a statistically significant reduction ($p < 0.0001$) in the expression of BDNF in all psychiatric subjects compared to the control group in all encephalic areas. In contrast, no statistically significant variations appeared in comparison between depressed individuals and those with other mental disorders. The same result was obtained with the GNDF, with a statistically significant increase ($p < 0.0001$) in all brain areas of psychiatric subjects compared to the control group. In contrast, there were no statistically significant variations in the comparison between depressed individuals and those with other psychic disorders.

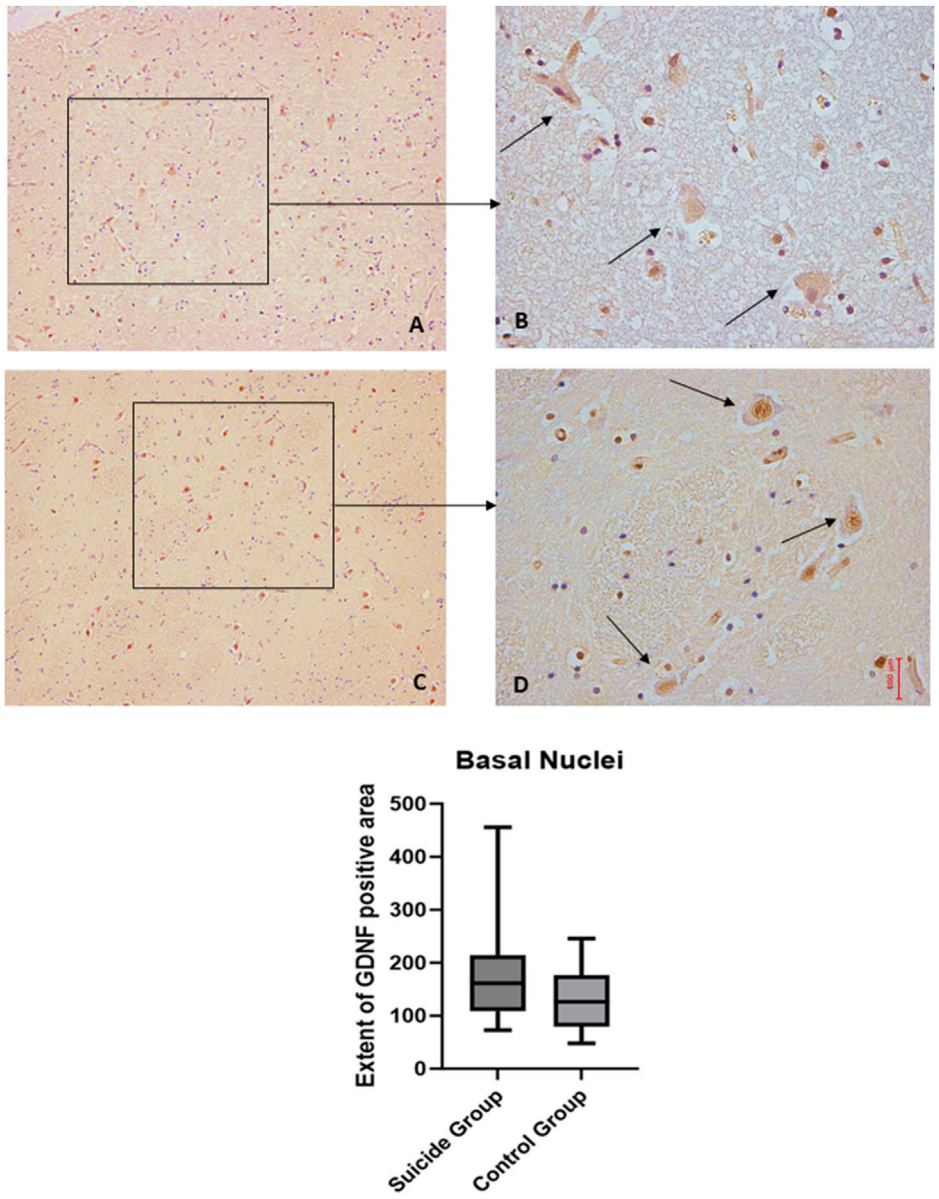

FIGURE 8
GDNF immunohistochemical expression in basal nuclei. Basal nuclei immunohistochemistry results: basal immunohistochemistry reaction of GDNF in the control case, see arrows **(A)** 10x magnification **(B)** 40x magnification; similar basal expression of GDNF in a sample of suicide case, see arrows **(C)** 10x magnification **(D)** 40x magnification. In the lower part of the figures is the graphical representation of the statistical analysis.

5 Conclusions

The results we obtained on the expression of BDNF and GDNF, in agreement with data from works published in the literature, support the hypothesis that lower levels of BDNF and higher levels of GDNF correlate with an increased risk of suicide. The use of these markers could have several clinical implications. BDNF and GDNF testing could be valuable markers for screening patients most at risk of suicide based on demographic and clinical data to estimate suicidal risk. These markers measured *in vivo* and correlated with clinical symptomatology could help assess the severity of depressive symptoms, such as the presence of polymorphism of the BDNF gene (Val66Met). Further studies could have important clinical implications using BDNF and GDNF as targets for specific pharmacological therapies for depression.

This study could be a pilot study to continue the research on a larger sample of subjects who died as a result of suicide. It would, therefore, be necessary to increase the sample size, standardize the data collection methods, and group the samples according to their characteristics. In our study, there is a wide heterogeneity regarding the method of suicide and psychiatric pathology. In addition, the exclusion of psychoactive substance users may have led to a selection bias, although it was necessary to identify the modification in BDNF and GDNF correctly.

Data availability statement

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

Ethics statement

The studies involving humans were approved by Ethics Committee N 57/CE/ 2024 of the "Riuniti" Hospital of Foggia, Italy. The studies were conducted in accordance with the local legislation and institutional requirements. The study was performed in accordance with the guidelines of the Declaration of Helsinki. This study was performed by using human post-mortem brain samples collected during autopsies ordered by the prosecutor and used at the end of the investigations; It is a retrospective study on samples collected during the autopsy, and the acquisition of informed consent is not possible, because the heirs cannot be traced.

Author contributions

SD: Writing–review and editing, Writing–original draft, Resources, Validation, Conceptualization, Project administration. LA: Software, Writing–review and editing, Visualization, Writing–original draft, Resources. MAB: Writing–original draft, Formal Analysis. SC: Writing–original draft, Investigation, Supervision. MC: Writing–review and editing, Writing–original draft. LC: Methodology, Project administration, Writing–review and editing. MN: Conceptualization, Writing–original draft, Investigation, Project administration, Validation, Writing–review and editing, Supervision.

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Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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The role of exercise-related FNDC5/irisin in depression

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The complexity of depression presents a significant challenge to traditional treatment methods, such as medication and psychotherapy. Recent studies have shown that exercise can effectively reduce depressive symptoms, offering a new alternative for treating depression. However, some depressed patients are unable to engage in regular physical activity due to age, physical limitations, and other factors. Therefore, pharmacological agents that mimic the effects of exercise become a potential treatment option. A newly discovered myokine, irisin, which is produced during exercise via cleavage of its precursor protein fibronectin type III domain-containing protein 5 (FNDC5), plays a key role in regulating energy metabolism, promoting adipose tissue browning, and improving insulin resistance. Importantly, FNDC5 can promote neural stem cell differentiation, enhance neuroplasticity, and improve mood and cognitive function. This review systematically reviews the mechanisms of action of exercise in the treatment of depression, outlines the physiology of exercise-related irisin, explores possible mechanisms of irisin's antidepressant effects. The aim of this review is to encourage future research and clinical applications of irisin in the prevention and treatment of depression.

KEYWORDS

exercise, FNDC5/irisin, depression, metabolism, BDNF, neurogenesis, inflammation, oxidative stress

1 Introduction

Depression is a common and serious mental illness that affects approximately 300 million people worldwide and is the third leading cause of the global illness burden (Herrman et al., 2019). It is also one of the leading disabling conditions globally, accounting for nearly 47 million disability cases in 2019, and is expected to become the top global health problem by 2030 (17 Oct 2020, 2020; Friedrich, 2017). Depression is a disease involving genetic, psychological, biochemical, and socio-environmental factors, resulting in severe physical, psychological, and economic burdens for patients and their families (Hammen, 2018). Consequently, identifying effective measures for the prevention and treatment of depression has significant implications for improving population health. Clinical data indicate that current treatments for depression have various limitations regarding their onset of action time and overall efficacy (Wang et al., 2015). Traditional antidepressants often takes weeks or even months to alleviate depressive symptoms, has multiple side effects, and is ineffective for approximately one-third of patients, limiting treatment efficacy (Cipriani et al., 2018). Therefore, further research into the pathophysiology of depression is essential for exploring new therapeutic targets.

Regular exercise not only helps with skeletal muscle growth and development, but also improves brain function and broadly enhances physical and mental health throughout the lifespan (Schulkin, 2016). Several studies have demonstrated that exercise positively impacts cognitive function, memory, and mental health in both human and animal models (Wong-Goodrich et al., 2010; Pietropaolo et al., 2008). Exercise has been recommended as an enhancement strategy for the treatment of depression (Lee et al., 2021; Trivedi et al., 2011). More than 40 meta-analyses and other systematic reviews have confirmed the effectiveness of exercise in alleviating depressive symptoms. Research has identified exerkines as key contributors to the positive effects of exercise (Safdar et al., 2016). Coined by Tarnopolsky et al., this term refers to cytokines, humoral factors, and metabolites produced during exercise that act in a paracrine or endocrine manner, contributing to the systemic benefits of exercise (Safdar and Tarnopolsky, 2018).

During physical activity, skeletal muscle releases different myokines, some of which can cross the blood-brain barrier (BBB) and mediate various beneficial effects associated with exercise. Irisin, a myokine produced during exercise through the cleavage of its precursor protein, fibronectin type III domain-containing protein 5 (FNDC5). Irisin promotes energy metabolism, improves insulin resistance, regulates disorders of glucose and lipid metabolism, and is associated with browning of adipose tissue for thermogenesis (Boström et al., 2012; Lee et al., 2014). Recent research suggests that irisin plays a beneficial role in neurological disorders by regulating energy metabolism, enhancing synaptic plasticity, fostering neurogenesis, reducing neuroinflammation, and inhibiting oxidative stress (Islam et al., 2021; Lourenco et al., 2019; Wang et al., 2022; Zhang et al., 2016). As a novel exercise-induced myokine, irisin serves as a crucial communication link between the skeletal muscle and the brain.

Preliminary studies indicate that irisin may exert antidepressant effects and could be a potential target for depression treatment (Wang and Pan, 2016; Siteneski et al., 2018; Pignataro et al., 2022). We conducted a thorough search of PubMed publications from 2012 to 2024 using the following keywords: exercise, FNDC5, irisin, depression, central nervous system, and neuroprotection. On this basis, we describe the positive effects of exercise on the brain and its preventive and therapeutic potential for depression, highlight the relationship between exercise-associated irisin and depression, and further explore the potential therapeutic role of FNDC5/irisin in depression and its possible mechanisms.

2 The role of exercise in the prevention and treatment of depression

Exercise has long been recognized for its benefits to overall health, and regular physical activity has been associated with better physical health outcomes (Warburton et al., 2006). Consistent evidence supports the effectiveness of exercise-based interventions in reducing the incidence of both physical and mental illnesses and in lowering all-cause mortality rates (Chekroud et al., 2018). Exercise has been shown to be an effective treatment for mild to severe depression, with response rates comparable to those of commonly used treatments, such as antidepressant medications and cognitive behavioral therapy (Babyak et al., 2000; Blumenthal et al., 2007). Moreover, exercise as an adjunct to standard antidepressant therapy has demonstrated significant potential for treating severe depression (Gourgouvelis et al., 2017). In fact, the combination of exercise with standard treatment has been found to produce significantly greater antidepressant effects than standard treatment alone (Lee et al., 2021). Exercise also plays a preventive role, reducing the risk of depression onset (Mammen and Faulkner, 2013). Numerous clinical studies have explored the link between depression and exercise. A recent systematic review and meta-analysis of 15 prospective studies, encompassing over two million person-years, found that physical activity was inversely associated with the development of depression, with the most significant risk reduction seen at lower levels of physical activity (Pearce et al., 2022). Another meta-analysis of 49 prospective cohort studies, including 1,837,794 person-years, conducted by Schuch et al., showed that individuals engaging in high - intensity exercise were less likely to develop depression compared with those leading a sedentary lifestyle. This protective effect of physical activity against depression was observed regardless of age, gender, or geographic region (Schuch et al., 2018).

We have synthesized findings from rodent and human studies to summarize the neurobiological mechanisms underlying the antidepressant effects of exercise. Exercise has been shown to influence neurotransmitters such as dopamine (DA), norepinephrine (NE), and 5-hydroxytryptamine (5-HT) (Lin and Kuo, 2013; Cunha et al., 2013). In a randomized controlled trial, patients with depression who engaged in 16 weeks of physical activity exhibited increased plasma levels of the anti-inflammatory cytokine interleukin-10 (IL-10), along with decreased levels of pro-inflammatory cytokines and pro-inflammatory markers, such as C-reactive protein and interleukin-6 (IL-6), and reduced neutrophil and monocyte counts (Euteneuer et al., 2017). Animal study has also shown that exercise reduces depression-like behaviors, which are associated with changes in systemic inflammation, such as increased IL-10 levels (Sigwalt et al., 2011). These findings suggest that regular physical activity have anti-inflammatory effects. Oxidative stress-related endothelium damage has been linked to the onset and progression of several disorders, including vascular depression and late-life depression (Luca and Luca, 2019). Brocardo et al. found significant increases in lipid peroxidation and protein oxidation in the hippocampus and cerebellum of rats exposed to ethanol; in contrast, voluntary running wheel exercise raised endogenous levels of the antioxidant glutathione and alleviated depression-like behaviors in male rats (Brocardo et al., 2012). Recent research suggests that the antidepressant effects of exercise are associated with improvements in synaptic plasticity, neuronal growth, and neurogenesis in the adult hippocampus (Liang et al., 2022; Liang et al., 2019; Micheli et al., 2018; Steib et al., 2014). In a mouse model of chronic unpredictable stress (CUS)-induced depression, Liang et al. discovered that exercise increased the number of mature neurons and dendritic spines in the hippocampal CA1, CA3, and dentate gyrus (DG) regions and was more effective than fluoxetine in promoting neuronal maturation and modulating synaptic plasticity (Liang et al., 2022). A growing body of evidence suggests that brain-derived neurotrophic factor (BDNF) mediates the antidepressant effects of exercise. Clinical trials have shown that

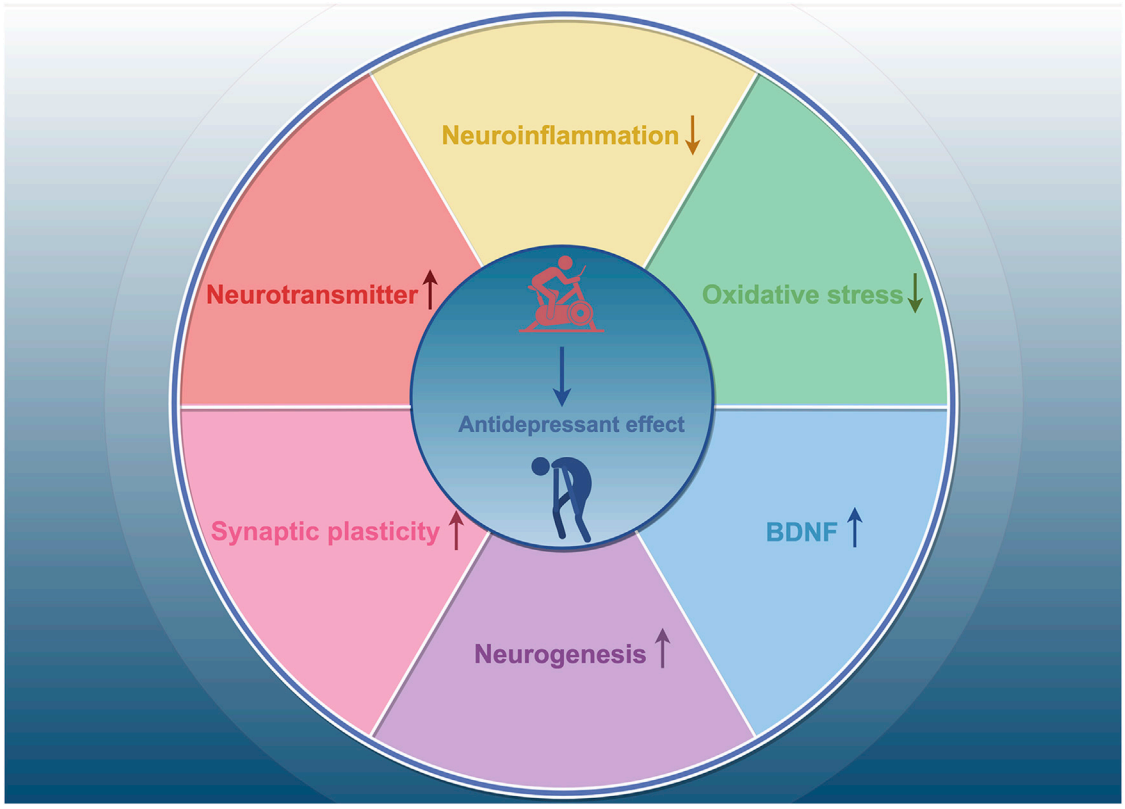

FIGURE 1
The mechanisms of exercise's antidepressant effects. Exercise exerts antidepressant effects by increasing neurotransmitter levels, decreasing neuroinflammation, inhibiting oxidative stress, and enhancing synaptic plasticity, neurogenesis, and BDNF levels.

acute exercise increases BDNF levels in an intensity-dependent manner, and changes in BDNF are strongly associated with improvements in depressed mood following exercise (Meyer et al., 2016). In animal studies, 28 consecutive days of physical activity elevated BDNF mRNA and protein expression in hippocampal neurons and specifically increased BDNF levels in the dendrites of CA3 neurons (Baj et al., 2012). Collectively, these findings suggest that neurotransmitters, neurotrophic factors, neuroinflammation, oxidative stress, neurogenesis, and neuroplasticity are involved in the antidepressant mechanisms of exercise (Figure 1).

3 Source and biological function of irisin

Irisin, a myokine released into the circulation during exercise, was first identified by Boström and colleagues in 2012 (Boström et al., 2012). It is cleaved from FNDC5, a transmembrane precursor protein whose transcription is regulated by peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), a key transcriptional cofactor involved in energy metabolism (Boström et al., 2012). Exercise-induced skeletal muscle contraction stimulates, which directly upregulates FNDC5 expression, leading to irisin synthesis and secretion. FDNC5 consists primarily of a signal peptide (amino acids 1–31) and a fibronectin III structural domain (amino acids 32–212) that contains irisin (amino acids 32–143), with the remaining amino acids forming transmembrane and cytoplasmic domain (Figure 2) (Boström et al., 2012; Nie et al., 2020; Schumacher et al., 2013).

Irisin is predominantly produced by skeletal and cardiac muscles, with the latter producing higher levels (Boström et al., 2012; Huh et al., 2012). However, its production is not confined to muscle tissue; other organs, including the liver, thyroid, adrenal glands, bladder, ovaries, and CNS, also express FNDC5 and release irisin (Huh et al., 2012; Dun et al., 2013; Aydin et al., 2014; Ruan et al., 2019). Emerging research shows that irisin is widely expressed in the brain, particularly in Purkinje cells of the cerebellum and in the cerebral cortex, hippocampus, putamen, and hypothalamus. It plays a crucial role in normal physiological processes in the brain, and is linked to neurodegenerative diseases (Dun et al., 2013). It is unclear, whether cerebrospinal fluid irisin originates from peripheral irisin or central FNDC5. Ruan et al. proposed that irisin can cross the BBB through a saturable transport system (Ruan et al., 2019). Supporting this, peripheral FNDC5 gene overexpression has been shown to influence hippocampal gene expression and mitigate memory impairment (Islam et al., 2021; Lourenco et al., 2019). Irisin's receptors are the αV integrin family, specifically the αV/β5 integrin complex. Inhibition of αV integrins in osteocytes and adipocytes suppresses irisin's signaling and activity (Kim et al., 2018). Notably, integrin αVβ5 is highly expressed in microglia (Welser-Alves et al., 2011). Wang et al. demonstrated that

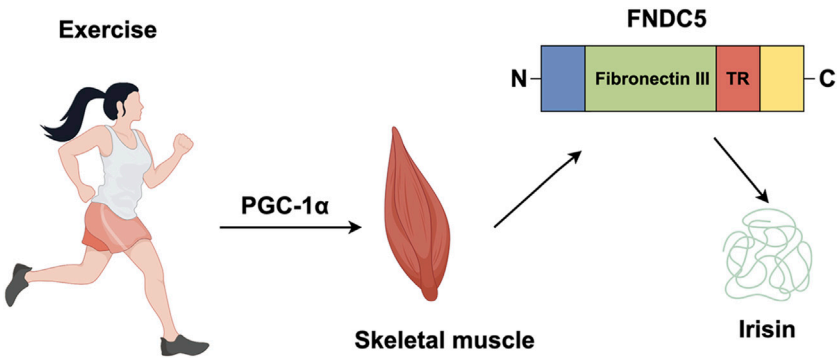

FIGURE 2
Irisin is produced by exercise. In response to exercise peroxisome proliferator, PGC-1α, leads to the production of FNDC5, a protein which contains an N-terminal signal sequence (blue); fibronectin III (green); a transmembrane region (TR) (red); a cytosolic region with a C-terminal tail (yellow). The proteolytic cleavage of mature FNDC5 results in the release of irisin.

TABLE 1 Changes in FNDC5 and/or irisin levels after exercise.

Species	Motion type	Level change	References
C57BL/6J mice	Treadmill exercise	Irisin (plasma) ↑ FNDC5 mRNA and protein (muscle) ↑	Pang et al. (2018)
Wistar rats	Treadmill exercise	FNDC5 protein (hippocampal) ↑	Babaei et al. (2021)
Outbred mouse line DUhTP	Running-wheel exercise Treadmill exercise	Irisin (plasma and muscle) ↑ FNDC5 protein (muscle) —	Brenmoehl et al. (2014)
Mice	Running-wheel exercise	FNDC5 mRNA (hippocampal) ↑	Wrann et al. (2013)
C57BL/6 J mice	Treadmill exercise	Irisin (plasma) ↑ FNDC5 mRNA and protein (muscle) ↑	Park et al. (2022)
Homo sapiens	Acute exercise Chronic exercise	Serum irisin decreased after chronic exercise and increased after acute exercise	Norheim et al. (2014)
Homo sapiens	Aerobic exercise	Irisin (plasma) ↑	Kraemer et al. (2014)
Homo sapiens	Acute exercise Chronic exercise	Serum irisin was upregulated after acute exercise	Huh et al. (2012)
Homo sapiens	Endurance exercise Resistance exercise	Increased plasma irisin levels after resistance exercise	Tsuchiya et al. (2015)

irisin can reduce neuroinflammation after cerebral hemorrhage by binding to integrin αVβ5 expressed in microglia (Wang et al., 2022).

Irisin is considered a critical mediator between exercise and metabolic homeostasis. It enhances the expression of uncoupling protein 1 (UCP1), which promotes the conversion of white adipose tissue (WAT) to brown adipose tissue (BAT), generating heat. Additionally, irisin regulates glucose uptake and mitochondrial biogenesis (Boström et al., 2012; Lee et al., 2014). Numerous studies have confirmed its beneficial effects in the development of various diseases, including obesity, type 2 diabetes (T2D), osteoarthritis, cardiovascular diseases, ischemic stroke, intracerebral hemorrhage, and neurodegenerative diseases like Alzheimer's and Parkinson's disease (Islam et al., 2021; Wang et al., 2022; Li et al., 2017; Askari et al., 2018; Kam et al., 2022; Yan et al., 2022). FNDC5/irisin activates several intracellular signaling pathways to exert its biological effects. The mitogen-activated protein kinase (MAPK) pathway is a key mechanism in white adipocyte browning, neural differentiation, and osteoblast proliferation. Other pathways, such as AMP-activated protein kinase (AMPK), phosphoinositide 3-kinase/protein kinase B (AKT), cAMP/protein kinase A (PKA)/cAMP response element-binding protein (CREB), and signal transducer and activator of transcription 3 (STAT3)/Snail, are involved in other crucial FNDC5/irisin functions (Lourenco et al., 2019; Wang et al., 2022; Rabiee et al., 2020).

4 FNDC5/irisin and exercise

Exercise is an essential factor in inducing irisin secretion from skeletal muscle. Tandem mass spectrometry can be used to identify and quantify circulating irisin in humans. Sedentary individuals have irisin levels around 3.6 ng/mL, which rise significantly to 4.3 ng/mL after aerobic interval training (Jedrychowski et al., 2015). Flori et al. reveal that other factors such as temperature, diet, and certain medicines like fenofibrate and metformin also affect

TABLE 2 Experimental models of the effects of irisin on depression.

Model	Dose (route)	Behavioral test	Main findings	References
C57BL/6mice	100 μg/kg (I.P)	TST, FST, OFT	Irisin treatment decreased the immobility time in the TST and FST; BDNF and IGF-1gene expressions were significantly increased	Pignataro et al. (2022)
C57BL/6 mice	0.5–1 ng/mouse (I.C.V)	TST, FST, OFT	Irisin reduced the immobility time in the TST and FST; Hippocampal PGC-1αand BDNF mRNA levels increased after 6 h of irisin treatment	Siteneski et al. (2018)
propofol-treated mice	0.5 mg/kg (I.P)	TST, FST	Irisin decreased immobility time in the TST and FST in propofol-treated mice; Irisin could protect neurons, inhibit cytokine increase in astrocyte cultures exposed to propofol, and reduce epidermal growth factor receptor expression on cell surfaces	Hou et al. (2020)
CUS rats	100 ng/mL or higher	FST, SPT	Irisin increased the activity of mitochondrial complexes I, II, and IV, as well as the phosphorylation level of creatine kinase and glucose transport	Wang and Pan (2016)

Abbreviations: CUS, chronic unpredictable stress; ARS, acute restraint stress; TST, tail suspension test; OFT, open-field test; FST, forced swim test; SPT, saccharin preference test; I.P, intraperitoneal injection; I.C.V., intracerebroventricular.

irisin levels (Flori et al., 2021). As summarized in Table 1, numerous human and animal studies have demonstrated that exercise increases irisin levels in the bloodstream and hippocampus and elevates FNDC5 mRNA and protein expression in muscle tissue (Boström et al., 2012; Brenmoehl et al., 2014; Pang et al., 2018; Norheim et al., 2014; Babaei et al., 2021). Resistance, aerobic or combined exercise seems to play a positive role. Notably, the level of irisin produced corresponds to an increase in exercise intensity (Tsou et al., 2019). However, in several studies, exercise did not lead to changes in serum irisin (Pardo et al., 2014). Hecksteden et al. (2013) found that neither chronic endurance nor resistance exercise could increase circulating irisin. Several factors need to be considered in clinical trials, such as the age, gender, weight, and fitness level of the subjects. The most critical factors are the timing of sample collection and the type of exercise regimen used, which may lead to the failure of some studies to find an association between exercise and irisin secretion (Tsuchiya et al., 2015; He et al., 2018; Ruas et al., 2012). Since the half-life of irisin in the body is less than 1 h, it is necessary to pay attention to the time of blood collection after a single exercise intervention (Jedrychowski et al., 2015). Therefore, precision studies of irisin following exercise intervention needs to be further clarified.

5 Exercise-related irisin and depression

Several clinical trials have investigated the relationship between depression and exercise-produced irisin. A randomized trial by Bilek et al. showed that aerobic exercise elevated irisin serum levels and reduced depression and fatigue in patients with relapsing-remitting multiple sclerosis (Bilek et al., 2022). Tu et al. tracked patients with acute ischemic stroke for 6 months, finding that serum irisin levels were lower in patients with post-stroke depression (PSD) compared with patients who are not depressed. Irisin also outperformed other biomarkers, such as age and serotonin, in predicting PSD (Tu et al., 2018). Animal studies have found that irisin released during exercise improves depressive-like behaviors by increasing dopamine and norepinephrine levels (Yardimci et al., 2023). The antidepressant-like, pro-neurogenic and neuroprotective effects of exercise were associated with the FNDC5/Irisin pathway (Gruhn et al., 2021). Notably, aerobic exercise enhanced the activity of the PGC-1α/FNDC5/Irisin pathway and further increased BDNF levels in the hippocampus and prefrontal cortex (PFC) (Jo and Song, 2021). Therefore, exercise-induced irisin can exert antidepressant effects. Additionally, exogenous supplementation irisin exerts its antidepressant effects by regulating brain energy metabolism (Wang and Pan, 2016), promoting the expression of BDNF in various brain regions (Pignataro et al., 2022), reducing neuroinflammation, and decreasing the expression of epidermal growth factor receptors (EGFR) in mice (Hou et al., 2020), as shown in Table 2. Although studies suggest irisin has antidepressant effects, the exact mechanisms remain unclear. Understanding how irisin regulates depressive symptoms is crucial for developing therapeutic approaches to depression. Irisin has been shown to play key roles in regulating metabolism, reducing oxidative stress, decreasing inflammation, preventing apoptosis, and improving mitochondrial dysfunction (Askari et al., 2018; Jiang et al., 2021). Considering the involvement of irisin in multiple pathological processes, its role in depression may be complex. Therefore, irisin has great potential as an "exercise-mimicking" intervention in the treatment of depression.

6 The potential role of exercise-related irisin in depression

6.1 Irisin regulates energy metabolism

Impaired brain energy metabolism has gained increasing attention as a key factor in the pathogenesis of depression (Wu et al., 2016; Chan et al., 2019; Cao et al., 2013; Ernst et al., 2017). Combined proteomic and metabolomic analyses provide compelling evidence that patients with mood disorders exhibit abnormalities in energy and substance metabolism, which are linked to an elevated risk of developing depression (Wu et al., 2016; Qin et al., 2019; Liu et al., 2018). Disruptions in glucose, lipid, and amino acid metabolism have been widely reported in patients with major depressive disorder (MDD) (Qin et al., 2019; Liu et al., 2018; Chen et al., 2015).

Research increasingly shows that irisin plays a role in regulating lipid and glucose metabolism in skeletal muscle and adipose tissue (Boström et al., 2012; Luo et al., 2020; Xiong et al., 2015; Moreno-Navarrete et al., 2013). In obese mice, FNDC5/irisin improves insulin resistance, corrects glucose and lipid metabolic abnormalities, and enhances lipolysis via the cAMP-PKA-HSL/

perilipin pathway (Xiong et al., 2015). Irisin promotes the expression of UCP1 and the conversion of WAT to BAT, which increases thermogenesis and energy expenditure to improve metabolism (Boström et al., 2012; Zhang et al., 2014). This effect may be mediated by irisin-induced activation of the p38 MAPK and extracellular signal-related kinase (ERK) signaling pathways (Zhang et al., 2014). Additionally, central irisin injection has been shown to considerably increase oxygen consumption and carbon dioxide production in rats, along with heat generation (Zhang et al., 2015), suggesting that irisin can markedly enhance metabolic activity and may be an effective treatment for metabolic diseases. Wang et al. found that irisin treatment improved prefrontal cortical energy metabolism in CUS rats, as shown by increased activity of mitochondrial complexes I, II, and IV, resulting in improved depression-like behaviors (Wang and Pan, 2016). Impaired brain glucose metabolism and insulin signaling are key pathological features of depression and contribute to its onset and progression (Ernst et al., 2017; Kleinridders et al., 2015; Kimbrell et al., 2002). Some studies have reported that patients with depression exhibit higher-than-normal blood glucose levels, and elevated glucose levels have been associated with symptoms of fatigue in patients with mild depression. Kimbrell et al. (2002) discovered that patients with monophasic depression had lower regional cerebral glucose metabolism in the dorsal prefrontal and anterior cingulate cortices compared with healthy controls, with glucose metabolism negatively correlated with depression severity. Numerous studies have demonstrated that irisin's positive effects on insulin sensitivity and glucose metabolism, suggesting that maintaining glucose homeostasis may be another mechanism through which irisin regulates metabolism. Plasma levels of irisin are linked to T2D and obesity, with circulating irisin levels inversely correlated negatively with insulin resistance (Moreno-Navarrete et al., 2013). Serum irisin levels are reduced in patients with T2D (Choi et al., 2013). Irisin enhances fatty acid oxidation and glucose consumption in T2D by modulating the AMPK signaling pathway and reducing the expression of the gluconeogenic enzymes PEPCK and G6Pase in the liver (Xin et al., 2016). Furthermore, irisin activates p38 MAPK in an AMPK-dependent manner, inhibiting or knocking p38 MAPK blocks irisin-induced glucose uptake (Lee et al., 2015). Irisin also increases the expression of β-trophin, a hormone recently discovered to promote pancreatic β-cell proliferation and improve glucose tolerance (Zhang et al., 2014). Interestingly, increased endogenous ATP release from astrocytes has been shown to have antidepressant-like effects in mouse models of depression, Suggesting a physiologic link between ATP release from astrocytes and MDD (Cao et al., 2013). Irisin treatment markedly boosts levels of key glucose metabolism enzymes, including type I and type II hexokinase, and glucose transporters GLUT-4 in astrocyte membranes (Wang and Pan, 2016). Based on these findings, we hypothesize that irisin may serve as a regulator of energy metabolism, particularly glucose metabolism and insulin activity, in patients with depression.

6.2 Irisin regulates the expression of BDNF

BDNF is highly expressed in the central nervous system and plays a critical role in synaptic function, neurotransmission, and neurogenesis (Numakawa et al., 2018). A substantial amount of clinical and experimental data suggests that BDNF is integral to the pathophysiology of depression. Reduced BDNF production and signaling have been observed in *postmortem* brain tissue of patients with depression (Sheldrick et al., 2017). Antidepressant treatments have been shown to increase BDNF synthesis and signaling, with direct BDNF infusion into the hippocampus potentially providing antidepressant effects (Zavvari and Nahavandi, 2020). However, selective loss of BDNF in the DG has been found to reduce antidepressant efficacy (Björkholm and Monteggia, 2016).

Exercise induces hippocampal BDNF expression via the PGC-1α/FNDC5 pathway (Wrann et al., 2013). Moreover, intraperitoneal administration of recombinant irisin increases PGC-1α, FNDC5, and BDNF mRNA levels in the hippocampus (Kim and Leem, 2019). Peripheral delivery of FNDC5 to the liver via adenoviral vectors also elevates blood irisin levels, promoting BDNF and other neuroprotective gene expressions in the hippocampus (Wrann et al., 2013). Further research by Islam et al. demonstrated that exercise-induced irisin can cross the BBB via peripheral transport, inducing BDNF expression in the CNS (Islam et al., 2021). These findings suggest that FNDC5/irisin can cross the BBB and regulate gene expression in the brain. Huang et al. conducted a study on diabetic rats, dividing them into four groups: control, model, irisin, and irisin-shRNA. They found that irisin and BDNF levels were significantly lower in the model and irisin-shRNA groups but significantly higher in the irisin group, indicating that irisin promotes BDNF expression (Huang et al., 2019). Wrann et al. confirmed similar results when they inhibited FNDC5/irisin expression in cortical neurons using siRNA, resulting in decreased BDNF expression (Wrann et al., 2013). Further studies revealed that recombinant irisin activated the cAMP/PKA/CREB pathway in human cortical slices and increased cAMP and pCREB in mouse hippocampal slices (Lourenco et al., 2019), a pathway crucial for BDNF synthesis. It is well-established that activating the cAMP/PKA/CREB/BDNF signaling pathway and upregulating BDNF expression have antidepressant effects (Wu et al., 2021; Cai et al., 2022). Siteneski et al. investigated the impact of central irisin administration on BDNF mRNA expression and protein levels, finding that irisin reduced BDNF mRNA expression in the hippocampus after 1 h but increased it after 6 h (Siteneski et al., 2018). BDNF mRNA expression paralleled FNDC5 mRNA expression (Siteneski et al., 2018), consistent with FNDC5 being a positive regulator of BDNF expression (Wrann et al., 2013). Additionally, genetic polymorphisms in BDNF (BDNF Met/Met) prevented exercise from producing antidepressant effects and elevating BDNF and FNDC5 mRNA levels in the DG of the hippocampus (Ieraci et al., 2016). Given that BDNF expression may help mitigate the onset and progression of depression, activating the FNDC5/irisin-BDNF axis in the brain may offer a promising therapeutic approach for depression.

6.3 Irisin promotes neurogenesis and synaptic plasticity

Depression is associated with reduced adult neurogenesis in the hippocampus, a reduction that can be alleviated or restored

through chronic antidepressant treatment (Wang et al., 2008; Tunc-Ozcan et al., 2019). Brain imaging studies have shown decreased volume in cortical and limbic regions, particularly in the PFC and hippocampus, in patients with MDD. This volume reduction is more pronounced in patients with multiple episodes, recurrent relapses, and longer disease duration (Lorenzetti et al., 2009). Cotter et al. (2002) analyzed *postmortem* brain tissue from patients with MDD and found diminished glial cell density and neuronal volume in the dorsolateral PFC. Furthermore, reduced synaptic plasticity may contribute to impaired functioning and the loss of the ability to regulate mood and cognition in patients with depression (Duman et al., 2019). Kang et al. discovered that synaptic function-related genes (CALM2, SYN1, RAB3A, RAB4B, and TUBB4) were less expressed in the PFC of patients with MDD PFC, leading to a decrease in the number of synapses (Kang et al., 2012). In a repeated restraint stress model, stress decreased the number of dendritic spines in the medial PFC of rats, indicating not only a reduction in the total number of axons but also potential impairment of neuroplasticity in these regions (Radley et al., 2006).

FNDC5 may play a role in neuronal differentiation and genesis (Hashemi et al., 2013; Forouzanfar et al., 2015; Ebadi et al., 2021). A previous study by Hashemi et al. found that FDNC5 expression increased during neurogenesis in retinoic acid-treated mouse embryonic stem cells (mESCs) (Ostadsharif et al., 2011). Subsequent experiments revealed that knockdown of FNDC5 in neuronal precursor cells inhibited the differentiation of mouse mESCs into neurons and the maturation of astrocytes (Hashemi et al., 2013). Ebadi et al. further demonstrated that mRNA and protein levels of BDNF and its receptors, tyrosine kinase (Trk) and p75, decreased following FDNC5 knockdown, concluding that FNDC5 knockdown inhibits neuronal differentiation by affecting neurotrophic factor expression (Ebadi et al., 2021). In contrast, overexpression of FNDC5 promotes the expression of neuronal precursor markers Sox1 and Pax6, mature neuronal markers such as Neurocan, and the astrocytic marker GFAP, likely due to enhanced BDNF expression following FNDC5 overexpression (Forouzanfar et al., 2015). This underscores the significance of FNDC5 in neuronal differentiation.

The motor-FNDC5/irisin-BDNF axis has been shown to enhance neuroplasticity, including neuronal growth, survival, and synaptic stabilization. Exercise increases plasma irisin levels, elevates hippocampal BDNF expression and BrdU-positive cells, and alleviates cognitive decline and depressive states associated with physical inactivity (Park et al., 2022). Low to moderate-intensity exercise lasting 4 weeks improved depression-like behavior in mice and increased neuronal proliferation, differentiation, and survival in the hippocampus, correlated with elevated hippocampal FNDC5/irisin levels post-exercise (Siteneski et al., 2020). Intraperitoneal administration of recombinant irisin activates the hippocampal PGC-1α/FNDC5/BDNF signaling pathway, enhancing dendritic length and spine density in the CA1 and CA3 regions but not in the DG (Kim and Leem, 2019). Exercise-mediated irisin also plays a crucial role in improving cognitive function, with plasma irisin levels positively correlated with hippocampal BDNF concentrations and cell proliferation (Park et al., 2022). In Alzheimer's disease models, irisin can activate the cAMP/PKA/CREB signaling pathway in the brain, increasing BDNF expression, promoting neurogenesis and synaptogenesis, and enhancing synaptic plasticity, thereby improving cognitive function (Lourenco et al., 2019). Thus far, both exercise and BDNF have been associated with increased neurogenesis. Interestingly, BDNF is not the only factor influencing hippocampal neurogenesis; neurogenesis-related STAT3 signaling has also been shown to affect proliferation (Moon et al., 2013a; Yang et al., 2015). Moon et al. explored whether irisin could activate STAT3/AMPK/ERK signaling in mouse HT19-7 HN cells to influence hippocampal neurogenesis. They found that pharmacological concentrations of irisin (50–100 nmol/L), but not physiological concentrations (5–10 nmol/L), increased the proliferation of mouse hippocampal neuronal cells via the STAT3 signaling pathway. Notably, neither concentration affected neurogenesis in hippocampal neuronal cells nor activated AMPK or ERK signaling pathways (Moon et al., 2013b). There may be other signaling pathways activated by irisin that have yet to be reported, indicating a need for further research to elucidate the regulation of irisin levels and its physiological effects on hippocampal neurogenesis. Taken together, this discussion suggests that FNDC5/irisin plays a role in promoting neurogenesis and enhancing synaptic plasticity, providing a novel approach to improving depression-like behavior.

6.4 Irisin inhibits neuroinflammation

Neuroinflammation is known to play a crucial role in the pathogenesis of depression. Pro-inflammatory cytokine levels, such as IL-6 and tumor necrosis factor α (TNF-α), are elevated in the serum and cerebrospinal fluid of patients with depression (Haapakoski et al., 2015). Elevated mRNA and protein levels of interleukin-1β (IL-1β), IL-6, and TNF-α have also been observed in the PFC of patients with depression who died by suicide (Pandey et al., 2018). Studies involving animal models indicate that depressive-like behavior is linked to increase inflammation markers in peripheral and MDD-associated brain regions (Lu et al., 2017). Administration of lipopolysaccharide (LPS), which triggers an immune and inflammatory response, has been shown to induce depressive-like behaviors in rodents (Franklin et al., 2018).

In recent years, the anti-inflammatory, anti-apoptotic, and antioxidant properties of irisin in neurological diseases have garnered considerable attention (Askari et al., 2018). Although no definitive mechanism has been established regarding how irisin suppresses inflammation, it is hypothesized that it may inhibit inflammatory signal transduction systems and/or the nucleotide-binding oligomerization domain-like receptor containing pyrin domain 3 (NLRP3) inflammasome. The toll-like receptor 4 (TLR4)/Myeloid differentiation primary response gene 8 (MyD88)/NF-κB pathway is a classical inflammatory signaling pathway involved in the development of depression (Xiao et al., 2022). A study by Mazur-Bialy et al. found that high concentrations of irisin (50, 100 nM) attenuated LPS-stimulated inflammatory activation of macrophages and reduced the release of pro-inflammatory cytokines by inhibiting the downstream TLR4/MyD88 pathway and NF-κB phosphorylation, which is

associated with irisin's effects on MAPK phosphorylation (Mazur-Bialy et al., 2017). Yu et al. (2020) constructed a middle cerebral artery occlusion (MCAO) model to study the role of irisin in cerebral ischemia-reperfusion injury, obtaining similar results; irisin inhibited neuroinflammatory responses and reduced neuronal injury by downregulating TLR4/MyD88 and inhibiting NF-κB activation. In another study, irisin treatment inhibited the activation of Iba-1 microglia, infiltration of monocytes, oxidative stress, and the expression of inflammatory factors (TNF-α and IL-6) in mice with MCAO (Li et al., 2017), a finding confirmed in P12 neuronal cells. Moreover, pretreatment with neutralizing antibodies to irisin significantly attenuated the reduction in neuroinflammation observed following physical exercise (Li et al., 2017). Irisin suppressed the release of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and COX-2 in a rat spinal cord injury model through the AMPK-NF-κB pathway (Jiang et al., 2020), with these findings further verified in an LPS-induced P12 cell injury model (Jiang et al., 2020). In a brain hemorrhage model, irisin inhibited microglia/macrophage pro-inflammatory polarization, reduced neutrophil infiltration, and downregulated the expression of pro-inflammatory cytokines TNF-α and IL-1β by upregulating the integrin αVβ5/AMPK signaling pathway (Wang et al., 2022). An *in vitro* assay investigating whether irisin protects neurons from Aβ-induced cellular damage showed that irisin attenuates the release of IL-6 and IL-1β and reduces COX-2 expression levels in astrocytes. Finally, irisin can reduce NF-κB activation in astrocytes exposed to Aβ by blocking phosphorylation and loss of I κ Bα (Wang et al., 2018). Another study indicated that irisin could attenuate inflammation induced by oxygen-glucose deprivation by inhibiting reactive oxygen species (ROS) and the NLRP3 inflammatory signaling pathway (Peng et al., 2017).

Irisin plays a significant role in macrophage polarization and its anti-inflammatory function. Macrophages are classified as either traditionally activated (M1-type) or alternatively activated (M2-type), which have opposing roles in inflammation (Duan et al., 2022; Kawanishi et al., 2010; Van den Bossche et al., 2016). M1-type macrophages produce pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β; in contrast, M2-type macrophages secrete anti-inflammatory cytokines, such as IL-10 (Van den Bossche et al., 2016). Xiong et al. (2018) demonstrated that exogenous FNDC5 and FNDC5 overexpression inhibited LPS-induced M1 macrophage polarization and inflammatory cytokine production via the AMPK pathway. Previous studies have shown that irisin treatment can inhibit the expression of pro-inflammatory cytokines, reduce macrophage migration, and induce a phenotypic switch in macrophages from an M1 to an M2 state (Dong et al., 2016). Notably, irisin-mediated BDNF upregulation has the potential to reduce neuroinflammation by inhibiting the synthesis of NF-κB and pro-inflammatory cytokines IL-6 and IL-1β via activation of the ERK-CREB pathway through its receptor TrkB (Madhu et al., 2022). In conclusion, irisin reduces neuroinflammation and decreases the production of inflammatory factors in the brain. These effects of irisin in inflammatory conditions suggest potential therapeutic applications for irisin in the context of depression-related neuroinflammation.

6.5 Irisin blocks oxidative stress to prevent neuronal damage

Dysregulated redox homeostasis is implicated in the pathophysiology of depression (Pandya et al., 2013; Zuo et al., 2022; Bhatt et al., 2020). Oxidative stress can initiate or exacerbate several pathogenic processes associated with depression, including iron death, neuroinflammation, impaired autophagy, and mitochondrial dysfunction (Zuo et al., 2022). Meta-analyses have shown impaired antioxidant capacity and increased levels of oxidative damage products in patients with depression. Notably, antioxidant levels have been found to increase with the use of antidepressants (Liu et al., 2015; Palta et al., 2014). Therefore, inhibiting oxidative stress may improve depressive symptoms, as some antioxidants exhibit potential antidepressant efficacy (Eren et al., 2007). Oxidative stress refers to an imbalance between the generation of ROS and antioxidant defenses (Betteridge, 2000). High levels of ROS can damage proteins and DNA while promoting the release of inflammatory mediators, ultimately leading to cell death and apoptosis (Bhatt et al., 2020; Juszczyk et al., 2021). Furthermore, uncoupling protein 2 (UCP2), expressed in the central nervous system, has demonstrated strong neuroprotective effects (Wang et al., 2014; Hoang et al., 2012). UCP2 reduces mitochondria-mediated ROS production through uncoupling, increases ATP levels, mitigates mitochondrial damage caused by free radicals, and assists neural cells in utilizing energy derived from free radicals. UCP2 deficiency has been shown to exacerbate depression-like behavior and promote mitochondrial damage and ROS production in astrocytes in a chronic mild stress model (Du et al., 2016).

Irisin has been shown to protect against neuronal damage caused by oxidative stress in various neurological disease models. In the MCAO model, irisin considerably reduced levels of nitrotyrosine, superoxide anion, and 4-hydroxynonenal in peri-infarct brain tissue by activating AKT and ERK1/2 signaling pathways. It also inhibited the secretion of pro-inflammatory factors and alleviated ischemia-induced neuronal damage (Li et al., 2017). In a mouse model of oxygen-glucose deprivation (OGD), irisin mitigated OGD-induced neuronal damage by blocking the ROS-NLRP3 inflammatory signaling pathway and reducing ROS and malondialdehyde production to inhibit oxidative stress (Peng et al., 2017). In rat models of kainic acid-induced status epilepticus and chronic spontaneous epilepsy, exogenous irisin considerably enhanced the expression of BDNF and UCP2 while reducing levels of neuronal damage and mitochondrial oxidative stress (Yu et al., 2022; Cheng et al., 2021). In a mouse model of traumatic brain injury, exogenous irisin alleviated inflammatory responses and oxidative stress by inducing UCP2 expression in neuronal mitochondrial membranes, leading to reduced mitochondrial damage and decreased ROS production and malondialdehyde content (Guo et al., 2021). Consequently, irisin is a critical regulator of oxidative stress and a potential therapeutic agent for depression.

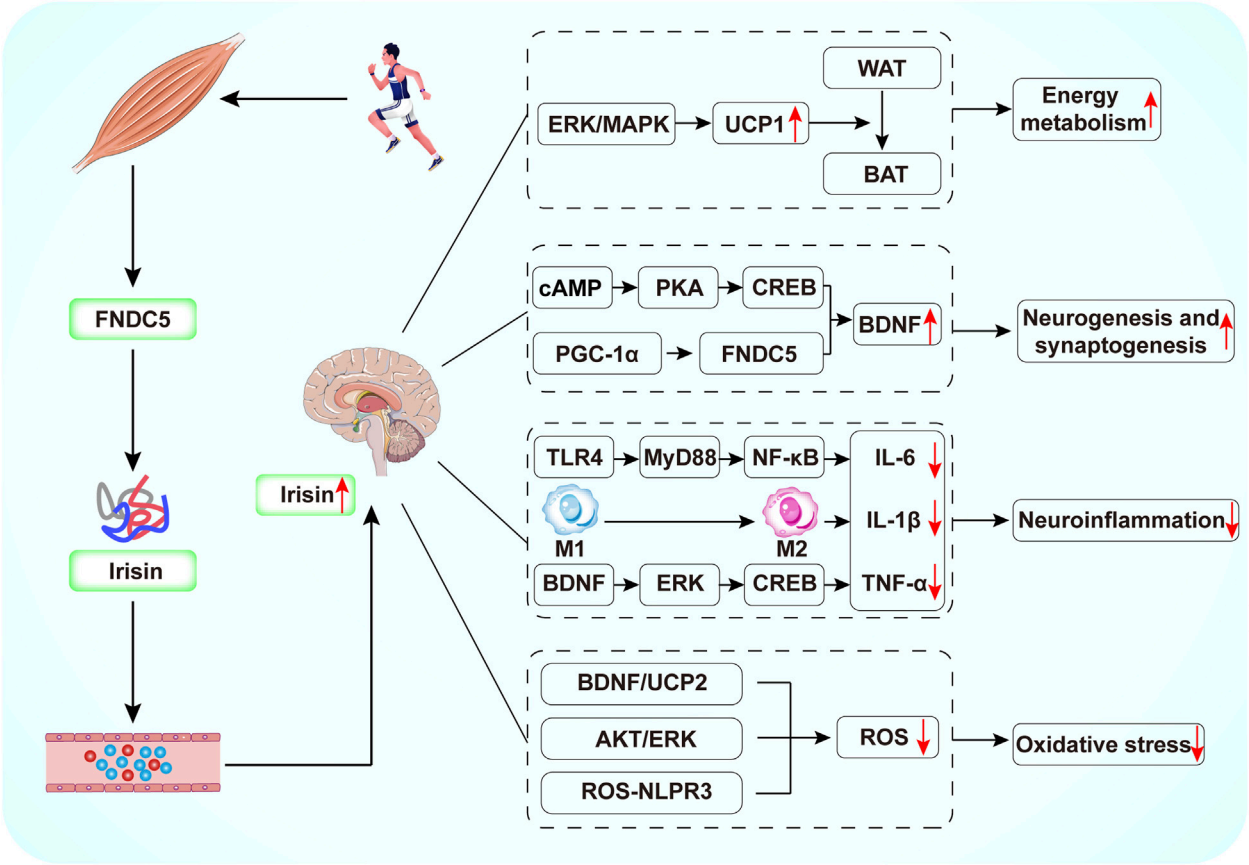

FIGURE 3
Schematic diagram of the potential mechanisms by which exercise-mediated release of irisin exerts antidepressant effects. Exercise induces FNDC5 expression in skeletal muscle. The FNDC5 protein is subsequently cleaved to irisin, which enters the brain by crossing the BBB. Irisin can increase BDNF production by activating cAMP/PKA/CREB, PGC-1/FNDC5 signaling. Irisin inhibits NF-κB phosphorylation through downregulation of TLR4/MyD88 pathway, induces the conversion of M1-type macrophages to M2-type macrophages, and activates the BDNF/ERK/CREB pathway, thereby reducing the production of pro-inflammatory cytokines. In addition, irisin reduces ROS production by activating the BDNF/UCP2 and AKT/ERK pathways and by inhibiting the ROS-NLPR3 inflammatory signaling pathway.

7 Conclusion

Compared to antidepressants, exercise is effective in improving depressive symptoms without the toxic side effects associated with medications. However, for patients with depression who are more severely ill and unable to engage in regular physical activity, irisin, as an exercise mimetic, has great potential in the treatment of depression. Research suggests that exercise-induced FNDC5/irisin plays a key role in connecting muscle and brain function. This review highlights the potential benefits of irisin in depression, presenting five key theories regarding its role in the depressed brain (Figure 3). First, irisin can improve metabolic dysfunction in the brain by promoting energy expenditure and regulating glucose and lipid metabolism. Second, FNDC5/irisin can increase hippocampal BDNF expression, promoting neurogenesis and synaptic plasticity. Third, irisin can attenuate neuroinflammatory responses. Fourth, it inhibits oxidative stress, thus preventing neuronal damage. Additionally, irisin levels may serve as an important biomarker for the diagnosis or treatment of depression. Therefore, further in-depth animal and clinical studies on the mechanisms by which irisin alleviates depression could facilitate the development and testing of new treatments, reduce the incidence of depression, and provide benefits for patients unable to exercise due to physical limitations.

Prospectively, several unexplored areas deserve more intensive research. Exercise can significantly alter the structure, composition, and abundance of the intestinal microbiota, thereby affecting overall health. In recent years, the complex interrelationship between exercise, intestinal microbiota and depression has received much attention. However, conclusive studies on whether irisin can treat depression by modulating the intestinal microbiota have not yet been conducted. Therefore, this area of research deserves more studies. Furthermore, the application of irisin in the clinical setting is challenging due to its short half-life *in vivo*. Fortunately, there are several approaches to extend the lifespan of protein drugs, such as Fc fusion proteins and coupling with albumin (Glaesner et al., 2010; Sleep et al., 2013). Additionally, the emergence of nanotechnology-based drug delivery strategies, like hydrogels, offers potential solutions, promising to prolong the therapeutic effect and reduce the frequency of administration (Tsou et al., 2019). Future research should aim to enhance the stability of irisin using these innovative modifications to optimize therapeutic outcomes and expand its

clinical potential. It should be emphasized that irisin is still in its infancy as a potential therapeutic strategy for depression. There is a lack of data from large-scale clinical trials to validate its safety and efficacy, as well as to clarify its dosage range and time window for clinical application. Therefore, further research and validation are needed in the future to deeply explore the potential value of irisin in the treatment of depression.

Author contributions

YL: Writing–original draft, Writing–review and editing, Conceptualization. XF: Writing–review and editing, Funding acquisition. XZ: Writing–review and editing. RC: Writing–review and editing. WY: Writing–review and editing, Funding acquisition, Conceptualization.

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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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Restraint stress effects on glutamate signaling protein levels in the rats' frontal cortex: Does β1 adrenoceptor activity matter?

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Introduction: Stress-evoked dysfunctions of the frontal cortex (FC) are correlated with changes in the functioning of the glutamatergic system, and evidence demonstrates that noradrenergic transmission is an important regulator of this process. In the current study, we adopted a restraint stress (RS) model in male Wistar rats to investigate whether the blockade of β1 adrenergic receptors (β1AR) with betaxolol (BET) in stressed animals influences the body's stress response and the expression of selected signaling proteins in the medial prefrontal cortex (mPFC).

Methods: The study was divided into two parts. In the first part, rats were exposed to RS for 3, 7, or 14 days, and the expression of glutamate signaling proteins (p(S845)/t GluA1, p(Y1472)/t GluN2B, VGLUT1, and VGLUT2) in the FC was analyzed to determine the optimal RS duration for studying the mechanisms of hypofrontality. In the second part, rats were exposed to RS for 14 days, and BET (5 mg/kg, p. o.) was administered during the last 8 days immediately after RS. The body's stress reaction was assessed by analyzing body weight and blood levels of adrenocorticotropic hormone (ACTH) and corticosterone (CORT). Behavioral responses were evaluated using the novel object recognition (NOR) and elevated plus maze (EPM) tests. The impact of RS and BET on the expression of p(Y530)/t Fyn and p (S133)/t CREB in the mPFC was measured via Western blotting.

Results and Discussion: The first part of the study demonstrated a decreased level of glutamate receptors in rats exposed to 14 days of RS, following an initial increase observed after 7 days of RS. Results from the second part revealed that chronic RS reduced body weight, impaired recognition memory in the NOR test, augmented blood levels of ACTH, and increased the expression of p(Y530) Fyn in the mPFC. However, β1AR blockade did not alter the effects of RS on weight gain, cognitive function, or the expression of p(Y530) Fyn. β1AR blockade normalized only the blood concentration of ACTH. These results suggest that decreased Fyn kinase activity, indicated by phosphorylation at Y530, underlies the stress-evoked downregulation of GluN2B in the FC in a manner independent of β1AR activity.

KEYWORDS

adrenocorticotropic hormone, betaxolol, elevated plus maze, frontal cortex, Fyn kinase, restraint stress, glutamate receptors, novel object recognition

1 Introduction

During stress reactions, two main body–brain systems are activated sequentially, namely, the autonomic nervous system and the hypothalamic–pituitary–adrenal axis (HPA), leading to an increase in the blood level of glucocorticoids (for details, see McEwen, 2007; Joëls et al., 2012). Through circulating glucocorticoids, the HPA axis mobilizes energy reserves necessary to deal with the extended presence of a stressor or its anticipation (Herman et al., 2016), which is effectively regulated via a negative feedback mechanism (Flak et al., 2012; Myers et al., 2012). Repeated exposure to stress may lead to an unadaptable stress response and the development of psychiatric disorders. A feature of the pathological stress response is reduced frontal cortex (FC) activity (hypofrontality), which has been observed in both clinical (Mayberg et al., 2005; Kamp et al., 2016) and preclinical studies (Bobula et al., 2011; Chen et al., 2013).

The function of the FC highly depends on the effectiveness of glutamate intracellular signaling (McGinty et al., 2015; Musazzi et al., 2015; Zelek-Molik et al., 2021), which is generally upregulated after acute and short-term exposure to stress but downregulated after chronic stress (see Musazzi et al., 2015). However, the dynamics of these changes and the underlying mechanisms remain elusive.

The activity of glutamate receptors depends on their presence at the neuronal membrane, which is stabilized by the phosphorylation of tyrosine residues at the intracellular C-terminal tail of GluN2B, GluA1 subunits, and the mGluR1/5 dimer. Tyrosine phosphorylation of glutamate receptors in the FC is catalyzed mainly by a family of non-receptor tyrosine kinases, including kinase Fyn (for details, see Mao and Wang, 2016). Interestingly, pharmacologically decreased activity of cortical Fyn in mice is accompanied by BDNF downregulation and depressive-like behavior (Kulikova and Kulikov, 2017).

Noradrenaline (NE) is an important regulator of glutamate receptor activity and cortical function. The noradrenergic system is known to be overactivated in stress pathologies (Birnbaum et al., 1999; Ramos et al., 2005; Joyce et al., 2024). NE signaling is mediated by three types of metabotropic receptors: α2 adrenergic receptors (α2AR), α1 adrenergic receptors (α1AR), and β adrenergic receptors (βAR). It has been shown that NE has the lowest affinity for βAR (740 nM) (Ramos and Arnsten, 2007). Among all βAR, the β1AR is the densest subtype present in the FC (Ramos et al., 2005; Paschalis et al., 2009). We hypothesized that the high NE concentration released during stress reactions in the medial prefrontal cortex (mPFC) through β1AR stimulation may affect the activity of glutamatergic receptors.

We tested our hypothesis using the procedure of restraint stress (RS) in rats, which is commonly used to model stress-related psychiatric disorders (Musazzi et al., 2015). However, during the repeated exposure of animals to the same (homotypic) stressor, including RS, processes of homeostatic adaptation began to develop (Martí and Armario, 1998; Girotti et al., 2006; Gądek-Michalska et al., 2011; Gądek-Michalska et al., 2012; Zelek-Molik et al., 2021), whereby the negative behavioral and physiological consequences of stress are not detected and possibly masked to ensure energy homeostasis (see Herman et al., 2016).

The aim of the current study was twofold. First, we aimed to check the impact of different durations of exposure to RS (3, 7, and 14 days) on the expression of glutamate signaling proteins, namely, p/t GluA1, p/t GluN2B, and mGluR1a/5, and vesicular glutamate transporters (VGLUT): VGLUT1 and VGLUT2 within the FC of rats. Second, after finding that 14 days is an efficient RS duration to develop hypofrontality, we investigated the impact of chronic RS on cognitive functions using novel object recognition (NOR) and elevated plus maze (EPM) behavioral tests. In biochemical studies, we tested the influence of chronic RS on the expression of p(Y530)Fyn, Fyn, p(S133)CREB, and CREB in the mPFC to identify which signaling pathway could be involved in RS-evoked maladaptation. Additionally, we assessed the therapeutic potential of β1AR blockade using betaxolol (BET, 5 mg/kg, p. o.) to alleviate behavioral and biochemical stress effects. The experimental design alongside the undertaken aims is depicted in Figure 1.

2 Materials and methods

2.1 Animals

Experiments were conducted on male Wistar rats purchased from Charles River, Germany. The animals were 6 weeks old, weighing approximately 164 g at the beginning of the experiments. They were housed in groups of 4–5 with unlimited access to commercial food and tap water in standard rat cages (UNO Housing, Zevenaar, Netherlands), except during the time of RS, which was administered during the light phase. The following standard laboratory conditions were maintained in the animal room: an artificial 12-h light/dark cycle (lights on from 7 a.m. to 7 p.m.) and a constant temperature of 22°C ± 2 °C. Before the onset of the experiments, the animals were allowed a 1-week habituation period. All procedures were approved by the Local Ethical Commission for Animal Experiments at the Maj Institute of Pharmacology, Polish Academy of Sciences, in Krakow (Permit No. 120/2018, dated 15/03/2018, 168/2018, and 10/05/2018) and fulfilled the requirements of EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.2 Experimental design

Two parts of experiments on two cohorts of animals were performed to obtain the data presented in this manuscript. The initial part served to evaluate the effects of different durations of RS exposure (3, 7, and 14 days) on the expression of selected glutamate signaling molecules (p/t GluA1, p/t GluN2B, mGluR1a/5, VGLUT1, and VGLUT2) in the FC (Figure 1A). In the second part of the experiment (Figure 1B), rats underwent 14 days of daily RS sessions, and half of them were treated with BET during the last 8 days of experiment to assess whether β1AR blockade could modulate RS effects on the measured behavioral and biochemical parameters. In the behavioral study, NOR and EPM tests were performed to assess recognition memory and anxiety, respectively. NOR was conducted 24 h after 7 days of RS exposure and a single BET application. EPM was performed 24 h after 14 days of RS exposure, with chronic BET application administered during the second week of treatment,

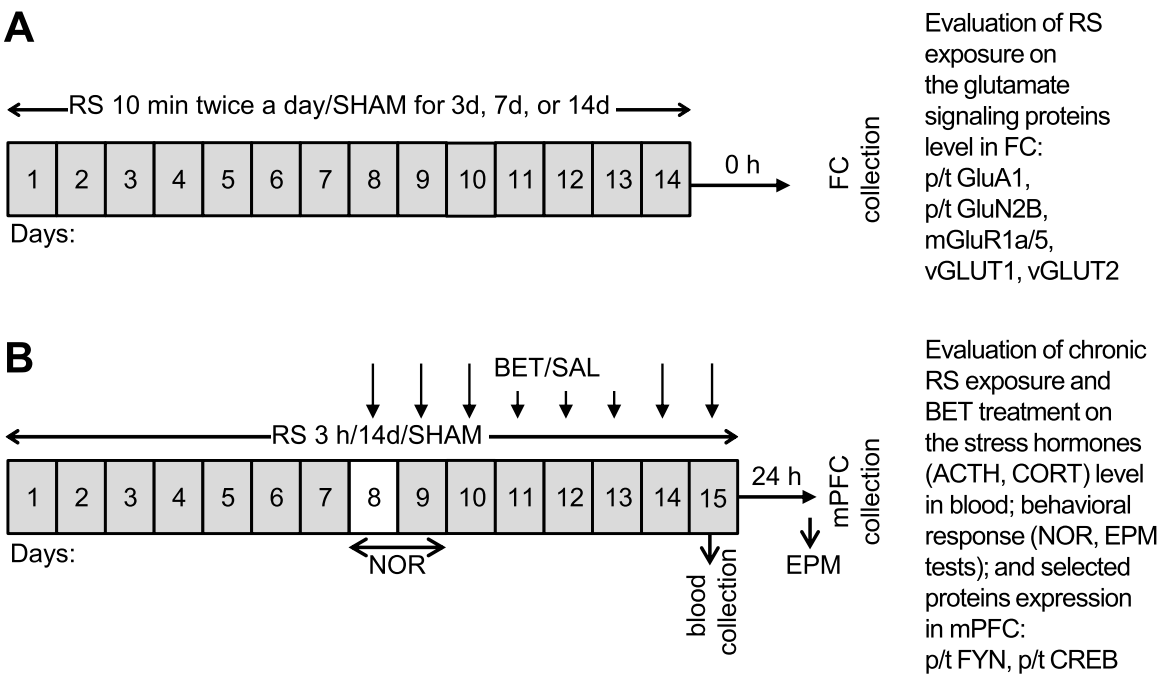

FIGURE 1
Schedule of experimental procedures undertaken in two parts of the study. **(A)** Mild RS applied 10 min twice a day for 3, 7, or 14 days, adopted to evaluate whether the mild RS exposure develops in FC of rats' bidirectional effect on the expression of glutamate signaling proteins and therefore can serve as a model of unadaptable stress. The following groups were generated in **(A)** control: RS 3d, RS 7d, and RS14d. **(B)** Regular RS procedure applied for 3 h daily for 14 days together with BET (5 mg/kg/po) treatment during the last 8 days of experiment to evaluate the body stress reaction, recognition memory, anxiety behavior, and indicated protein expression in medial FC (mPFC) after chronic RS and BET treatment. To avoid acute disturbances during familiarization and recognition, rats had 1 day break in RS/SHAM application on experimental day 8 (indicated by the white box), whereas on day 9, the NOR test was performed before the RS session. The following groups were generated in **(B)** SHAM/SAL, RS/SAL, SHAM/BET, and RS/BET.

immediately after RS. On the 8th experimental day, when RS was not applied to avoid disturbances in NOR training, BET was administered 2 h before the NOR test. In biochemical studies, we assessed the body's stress reaction by comparing adrenocorticotropin (ACTH) and corticosterone (CORT) levels among the experimental groups. Moreover, we measured the impact of RS and BET on the expression of p/t Fyn and p/t CREB proteins in the mPFC. Finally, we performed the qualitative immunofluorescence analysis of β1AR and GluA1, as well as colocalization analysis of β1AR–GluA1, to identify FC regions where these two receptors might interact.

2.3 Stress procedures

Two validated protocols for RS were adopted (Gadek-Michalska et al., 2015; Kusek et al., 2017; Rafa-Zabłocka et al., 2021). For the assessment of the timeline of glutamate signaling changes in FC rats, RS was conducted in metal tubes (diameter: 55 mm) for 10 min twice a day, for 3, 7, or 14 days. The control groups consisted of naive animals that remained in their home cages. Details of this procedure were described elsewhere (Gadek-Michalska et al., 2015). To assess the therapeutic impact of β1AR blockade on RS-evoked behavioral and biochemical parameters, the rats were placed in perforated plastic tubes (6.5 cm inner diameter) of adjustable length. The restraint allowed breathing and limited movements of the head and limbs. The RS procedure lasted 3 h daily for 14 days. After each stress session, the animals were removed from the restrainers and returned to their home cages. Control (SHAM) animals remained in their home cages during the stress sessions. Details of this procedure were described elsewhere (Rafa-Zabłocka et al., 2021). To monitor the rats' wellbeing and assess their stress response, body weight was recorded daily.

2.4 Betaxolol treatment protocol

To assess the role of β1AR in the studied mechanism and to analyze the therapeutic effect of its inhibition, rats were treated with BET, which is a specific antagonist of β1AR used clinically to treat hypertension and is shown to alleviate anxiety in stress models (Rudoy and Van Bockstaele, 2007). BET is a potentially effective tool for modulating brain functions impaired by stress due to its unique pharmacokinetic properties. It is a long-acting β1AR blocker with no intrinsic sympathomimetic activity, and it easily penetrates the blood–brain barrier (Ramos and Arnsten, 2007). In the second week of the RS procedure, treatment with BET (5 mg/kg, p. o.) (Alcon, Fort Worth, TX, United States) or 0.9% NaCl (0.5 mL/rat, p. o.) was introduced immediately after stress. Rats were treated for 8 consecutive days, from the 8th to the 15th experimental day. The dose and route of treatment were chosen based on the literature (Rudoy and Van Bockstaele, 2007) and our previous data (Rafa-Zabłocka et al., 2021).

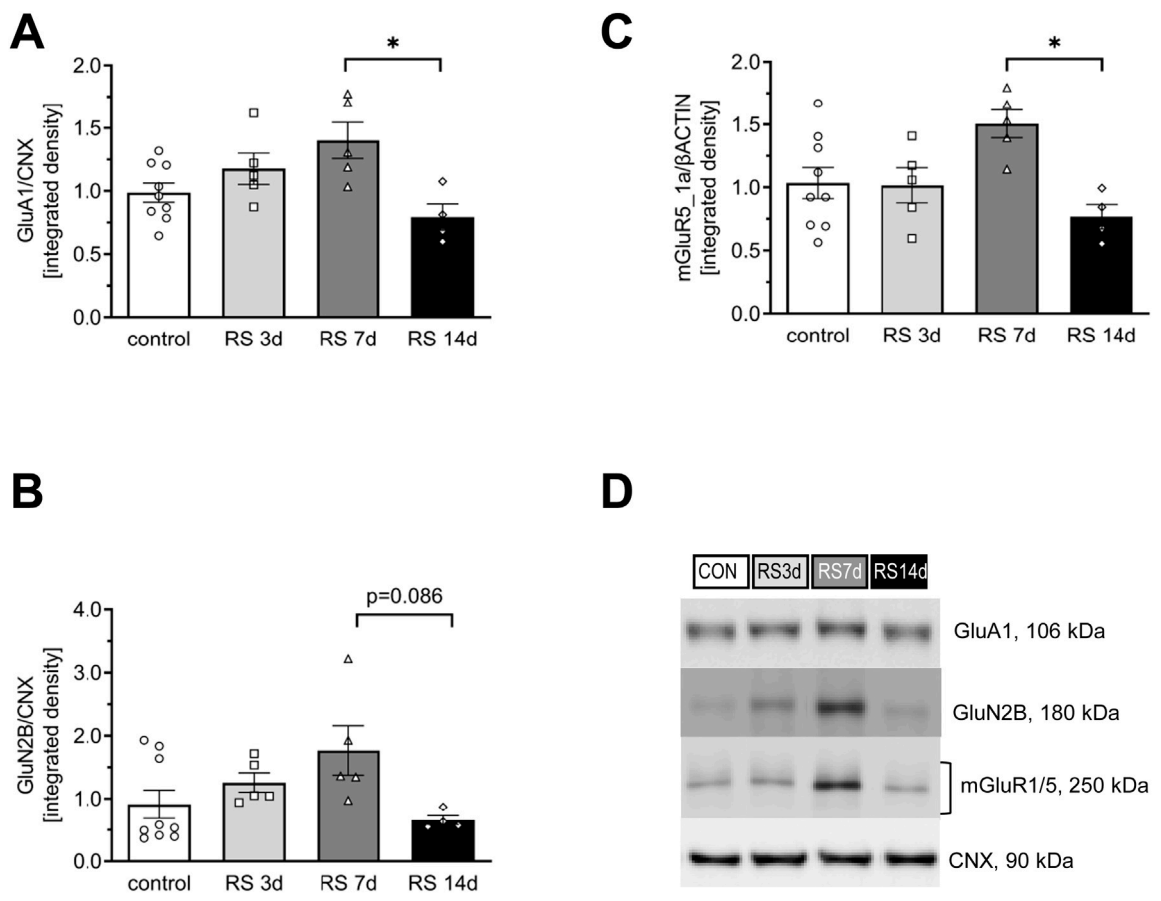

FIGURE 2
Influence of RS and its duration on the expression of selected glutamate receptors in the rat FC. **(A)** The expression of the GluA1 subunit of AMPA-R. **(B)** The expression of the GluN2B subunit of NMDA-R. **(C)** The expression of mGluR5/1a. **(D)** Representative immunoblots that illustrate GluA1, GluN2B, and mGluR5/1a expression in the FC of experimental groups. Data were calculated as control percentages and are expressed as mean ± SEM, N = 4–9/group. In **(A)** [F (3, 19) = 4,85; p = 0.011, in **(B)** [F (3, 19) = 2.99; p = 0.057], and in **(C)** [F (3, 19) = 4.57; p = 0.014]; *p < 0.05 (unequal N HSD).

2.5 Novel object recognition

The test consisted of two 5-min trials separated by a 1-day intertrial interval and was performed as previously described (Piotrowska et al., 2024). During the first trial (familiarization, T1), two identical objects were placed in the opposite corners, approximately 10 cm from the walls of the open field. In the second trial (T2, recognition), one of the objects was replaced with a novel object. Animals were returned to their home cage after T1 and T2. The height of the objects was comparable (~12 cm), and they were heavy enough to prevent displacement by the animals. The location of the novel object in the recognition trial was randomly assigned for each rat. Rats spending less than 5 s exploring the two objects during the trial were eliminated from the study. The exploration time of the objects was measured using the video tracking software EthoVision XT8 (Noldus, the Netherlands). Based on the exploration time of the two objects, a discrimination index was calculated as the time spent exploring the novel object minus the time spent exploring the familiar object.

2.6 Elevated plus maze

The apparatus for the elevated plus maze (EPM), made of Plexiglas and elevated to a height of 50 cm, consisted of two open arms (40 × 12 cm) and two closed arms (40 × 12 × 20 cm) arranged at 90° angles to each other, extending from a central platform (12 × 12 cm). The experiment was conducted under low-intensity light (30 Lux). Each rat was placed on the central platform of the maze facing an open arm. A single trial lasted for 5 min and was performed 23 h after the last BET or SAL treatment. Time spent in the open and closed arms, as well as the number of visits to the open and closed arms, was recorded using the video tracking software EthoVision XT8 (Noldus, the Netherlands). Time spent in the open arms and the number of open arm visits served as measures of anxiety.

2.7 Brain tissue sample and blood collection

Rats dedicated to checking the profile of changes in the expression of glutamate signaling elements in the FC were

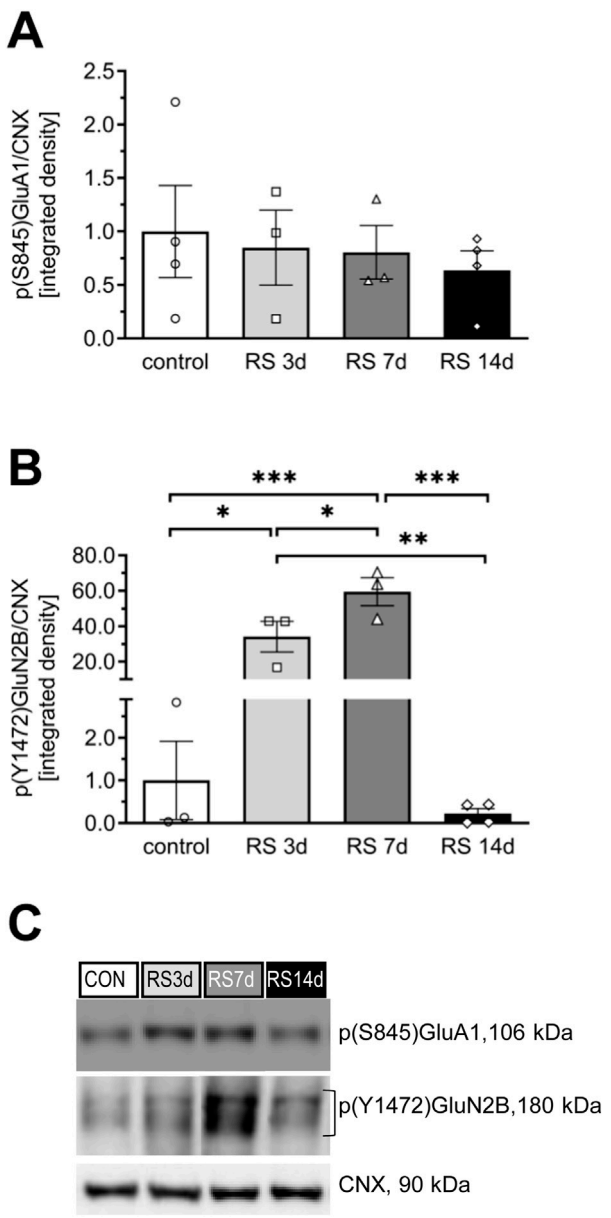

FIGURE 3
Influence of RS and its duration on the phosphorylation level of the GluA1 and GluN2B subunits in rat FC. **(A)** The expression of p(S845) GluA1. **(B)** The expression of p(Y1472)GluN2B. **(C)** Representative immunoblots that illustrate GluA1 and GluN2B phosphorylation levels in the FC of experimental groups. The phosphorylated GluA1 and GluN2B proteins were assessed on the same membrane as total proteins, and both were normalized to the same loading control. Therefore, the mutual representative blot for CNX is visualized in both Figures 2D, 3C. Results are expressed as a mean ± SEM, N = 3–4/group. In **(A)** [$F(3, 10) = 0.24$; $p = 0.868$] and in **(B)** [$F(3, 9) = 28.35$; $p < 0.0001$]. ***p< 0.001, **p< 0.01, and *p< 0.05 indicated in graph $p = 0.08$ (unequal N HSD).

decapitated immediately (0 h after the last stress session), and the brains were rapidly removed from the skulls. The FC (frontal part of the brain without the olfactory bulb, extending to 2.7 mm rostral to bregma) was excised on an ice-cold glass plate. During the last RS session, 0.5 mL of blood was collected from the tail veins of rats and placed in EDTA-coated tubes. These rats were decapitated the next day (24 h after the last RS and BET), and their brains were rapidly removed. The mPFC was dissected from 2 mm coronal slices (AP 4.7–2.7 mm rostral to bregma) using a rat brain matrix (Braintree Scientific, MA, United States), as described previously (Sun et al., 2014b). Isolated brain structures were immediately frozen on dry ice and stored at −80°C until assayed. For qualitative immunohistochemistry analysis, the whole brain from a decapitated rat in the SHAM/SAL group was immersed in 4% paraformaldehyde for further processing.

2.8 ELISA analysis of CORT and ACTH levels

Plasma from blood samples was isolated according to a previously described protocol (Zelek-Molik et al., 2021). In brief, blood samples were centrifuged at 3,000 × g for 15 min at 4 °C, and the plasma was then transferred to new 1.5-mL collection tubes and stored at −20 °C. CORT and ACTH concentrations were determined using an enzyme-linked immunosorbent assay (ELISA) method with commercially available Rat Corticosterone ELISA and Rat Adrenocorticotropic Hormone ELISA kits (Bioassay Technology Laboratory, Shanghai, China). The immunoenzymatic reaction was prepared and developed according to the manufacturer's instructions. Serum samples, tested in duplicates, were diluted fivefold before carrying out the assays. The absorbance was measured at 450 nm using a plate reader (Synergy MX, BioTek, Winooski, VT, United States). Hormone concentrations were calculated from standard curves fitted using four-parameter logistic equations in GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, United States). The sensitivity of ELISA assays was 0.24 ng/mL for CORT and 2.49 pg/mL for ACTH.

2.9 Immunoblotting

The procedure of immunoblotting was described previously (Zelek-Molik et al., 2021). In brief, total protein extraction was performed using radioimmunoprecipitation assay (RIPA) buffer (MilliporeSigma, Burlington, MA, United States). Equal amounts of protein extracts were diluted with a loading buffer containing an inclusion body solubilization buffer (G-Biosciences, Saint Louis, MO, United States) and a reducing agent, 1% 2-mercaptoethanol. The samples were then denatured at 45 °C for 30 min. Denatured samples were run on SDS-PAGE gels and transferred onto nitrocellulose membranes. The membranes were blocked using 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST; pH 7.6) for 1 h at room temperature and then incubated with specific primary antibodies. All studied glutamatergic receptors were assessed on the same membranes. After staining with Ponceau S, the membranes were horizontally cut at the 150 kDa level. Proteins—mGluR1/5 dimer (250 kDa) and p- and total GluN2B (180 kDa)—were assessed on the upper section, whereas GluA1 (106 kDa) was assessed on the bottom section. The expression of glutamate transporters, p/t Fyn, and p/t CREB was analyzed on separate gels. Following overnight incubation at 4 °C with primary antibodies and three washes using the blocking solution, the membranes were incubated with appropriate secondary antibodies for 1 h at room temperature, followed by

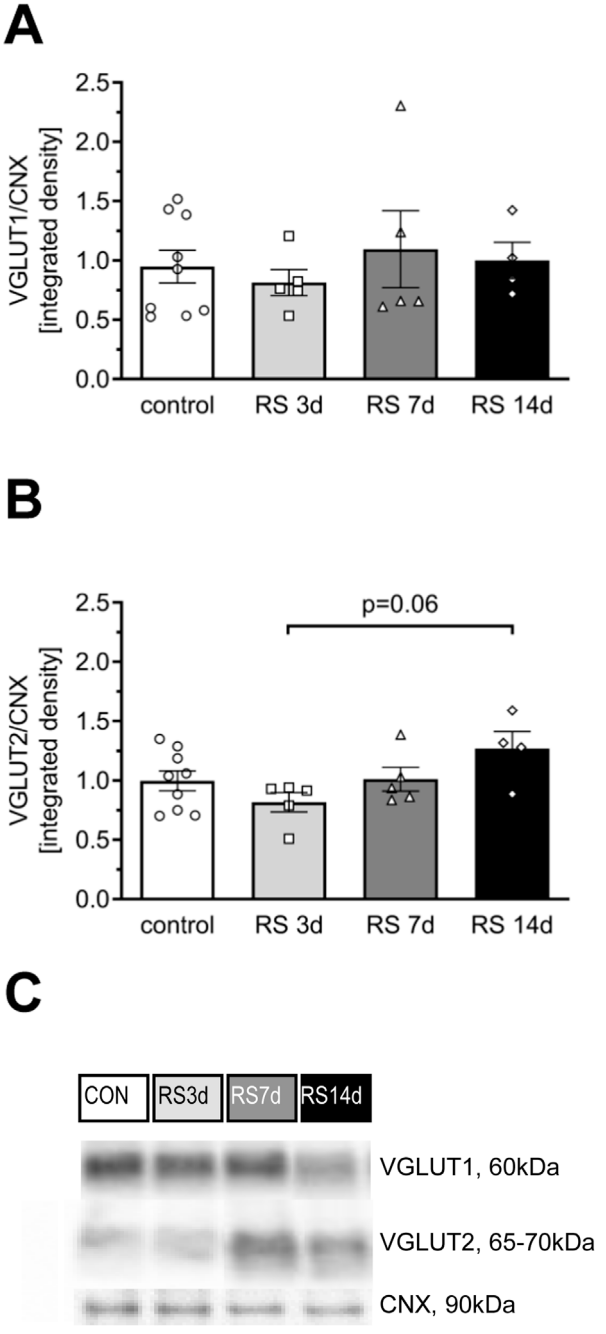

FIGURE 4
Influence of RS and its duration on the expression of selected glutamate transporters in the rat FC. **(A)** The expression of VGLUT1. **(B)** The expression of VGLUT2. **(C)** Representative immunoblots that illustrate VGLUT expression in the FC of experimental groups. Results are expressed as a mean ± SEM, N = 4–9/group. In **(A)** [F (3, 19) = 0.32; p = 0.807] and **(B)** [F (3, 19) = 2.66; p = 0.077] indicated in graph p = 0.06 (unequal N HSD).

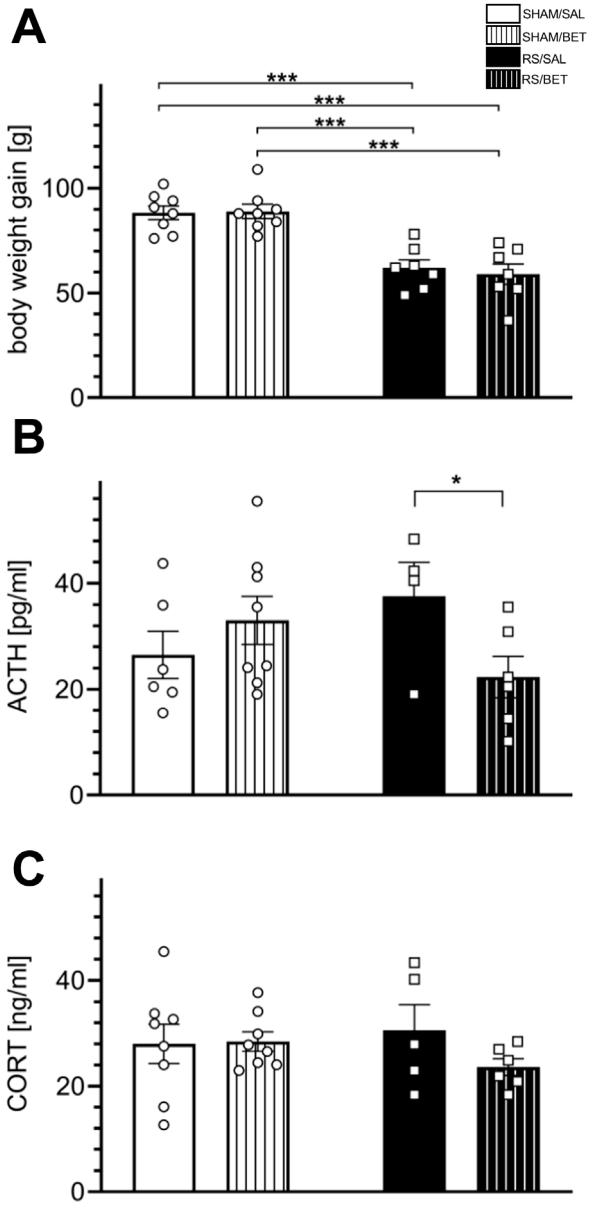

FIGURE 5
Body stress reaction to chronic RS and BET treatment. **(A)** Body weight gain during experimental procedures, **(B)** the concentration of ACTH, and **(C)** the concentration CORT. Stress hormones were assessed in plasma isolated from tail vein blood taken during the last RS session. White bars correspond to SHAM, black to RS pretreatment, and bars with patterns correspond to BET posttreatment. BET (5 mg/kg/po/14 days) applied immediately after RS. Results are expressed as a mean ± SEM; N = 4–8/group. In **(A)** RS [F (1,26) = 53.97; p< 0.001], BET [F (1,26) = 0.096; p = 0.76], and RS × BET interaction [F (1,26) = 0.22; p = 0.64]. In **(B)** RS effect [F (1,19) = 0.158; p = 0.69], BET effect [F (1,19) = 0.745; p = 0.20], and RS × BET interaction [F (1,20) = 4.238; p = 0.05]. In **(C)** RS effect [F (1,23) = 0.131; p = 0.72], BET [F (1,23) = 1.064; p = 0.31], and RS×BET effect [F (1,23) = 1.374; p = 0.25]. ***p< 0.001 (unequal N HSD).

three additional washes with TBST. Antibody binding was detected using an enhanced chemiluminescence kit (ECL Plus 32106, Thermo Fisher Scientific, Waltham, MA, United States). Equal protein loading was further confirmed by probing using anti-calnexin antiserum (1:5,000; ADI-SPA-865-F, Enzo Life Sciences, Farmingdale, NY, United States) or anti-β-actin antiserum (1:5,000; A5441, MilliporeSigma, Burlington, MA, United States). The following antibodies were used in the experiment: p(Y1472) GluN2B (1:1,000, M2442, MilliporeSigma, Burlington, MA, United States), GluN2B (1:1,000, 610416, BD Biosciences, San Jose, CA, United States), mGluR5/1a (1:2000, 2032-mGluR5/1a,

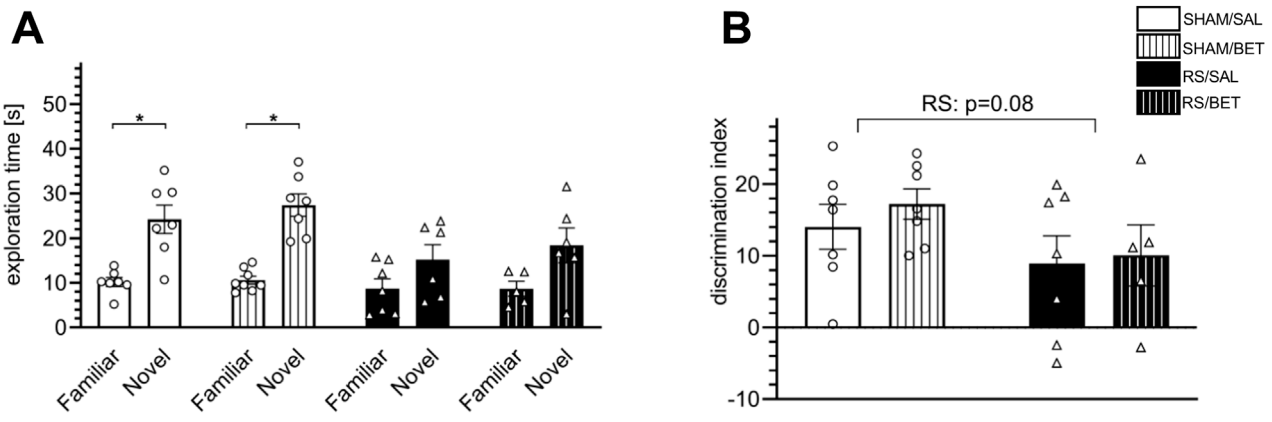

FIGURE 6
Effects of RS and single BET on the rats' behavior measured in the NOR test. **(A)** Exploration time of a novel and a familiar object by particular groups in the retention trial (T2). **(B)** Discrimination index (DI) measured as the time spent exploring the novel object minus time spent exploring the familiar object. White bars correspond to SHAM, black to RS pretreatment, and bars with patterns correspond to BET posttreatment; BET (5 mg/kg/po/1x) applied immediately after 7th RS session. Results are expressed as a mean ± SEM. N = 6–8/group. In **(B)** RS [F (1,22) = 3.3100; p = 0.08 (p-value indicated in the graph)], BET [F (1,22) = 0.40; p = 0.53], and RS × BET interaction [F (1,22) = 0.009; p = 0.76]. *p< 0.05 indicates significant difference in time spent exploring the novel object compared with the familiar object (t-test).

PhosphoSolution, Aurora, CO, United States), GluA1 (1:2000, ab31232, Abcam, United Kingdom), VGLUT1 (1:1,000, MAB5502, MilliporeSigma, Burlington, MA, United States), VGLUT2 (1:1,000, D7D2H, Cell Signaling Technology, Danvers, MA, United States), p (Y530)Fyn (1:1,000, ab182661, Abcam, United Kingdom), Fyn (1:1,000, ab125016, Abcam, United Kingdom), p (S133)CREB (1:1,000, 06–519, MilliporeSigma, Burlington, MA, United States), and CREB (1: 5,000, Cell Signaling Technology, Danvers, MA, United States). All Western blot analyses were performed at least twice to confirm the results. The chemiluminescence of specific signals was visualized using a multi-application gel imaging system, and the immunoreactive bands were quantified using an image analyzer (Multi Gauge V3.0, Fujifilm, Tokyo, Japan).

2.10 Immunohistochemistry

Rat brains were fixed in 4% paraformaldehyde overnight and processed as described previously (Bielawski et al., 2023), with further modifications. The fixed brains were embedded in paraffin and coronally sectioned at a thickness of 7 μm using a rotary microtome (Leica, Wetzlar, Germany). For GluA1 and β1AR staining, selected sections containing the mPFC and M1/M2 regions of the FC (bregma +3.0 mm) were subjected to deparaffinization and antigen retrieval using the microwave method with citrate buffer. Subsequently, these sections were immersed in a blocking solution consisting of 5% normal goat serum (S-1000–20; Vector Laboratories, CA, United States) dissolved in PBS. The localization of GluA1 and β1AR was confirmed by labeling with a mouse anti-GluA1 antibody (1:100, Sigma-Aldrich, St. Louis, Missouri, United States) and a rabbit anti-β1AR antibody (1:250, Abcam, United Kingdom). The primary antibodies bound to antigens were visualized using anti-mouse Alexa-488 and anti-rabbit Alexa-594 secondary antibodies (1:400; Molecular Probes, Eugene, OR, United States). Stained sections were examined and photographed in the widefield mode using a Leica TCS SP8 microscope.

2.11 Statistical analysis

All values are presented as percentages of controls and are expressed as the mean ± standard error of the mean (SEM), with group sizes ranging from N = 3 to 9 rats. The small sample size (N = 3–4) applied to the Western blot results shown in Figure 3, which were designed to verify whether the changes observed in Figure 2 of the manuscript are localized to the cell membrane. Thus, the small sample size should not interfere with the overall conclusions of the article. Statistical analyses were performed using Statistica 10 (Round Rock, TX, United States). Data were evaluated using one- or two-way analysis of variance (ANOVA), followed by a *post hoc* test (unequal N HSD or Fisher LSD) where appropriate. A significance level of $p < 0.05$ was considered indicative of a significant effect. Within-group comparisons of novel vs. familiar object recognition in the NOR test were conducted using a t-test for independent samples.

3 Results

3.1 Bidirectional profiles of changes evoked by RS on the level of glutamate receptor proteins in the rat FC

3.1.1 GluA1 protein

In the case of total GluA1, one-way ANOVA revealed a significant difference among the analyzed groups [F (3, 19) = 4.85; $p < 0.05$]. The *post hoc* analysis of the influence of different durations of restraint stress on the expression of GluA1 in the rat FC showed a gradual increase in protein levels in the RS3d and RS7d

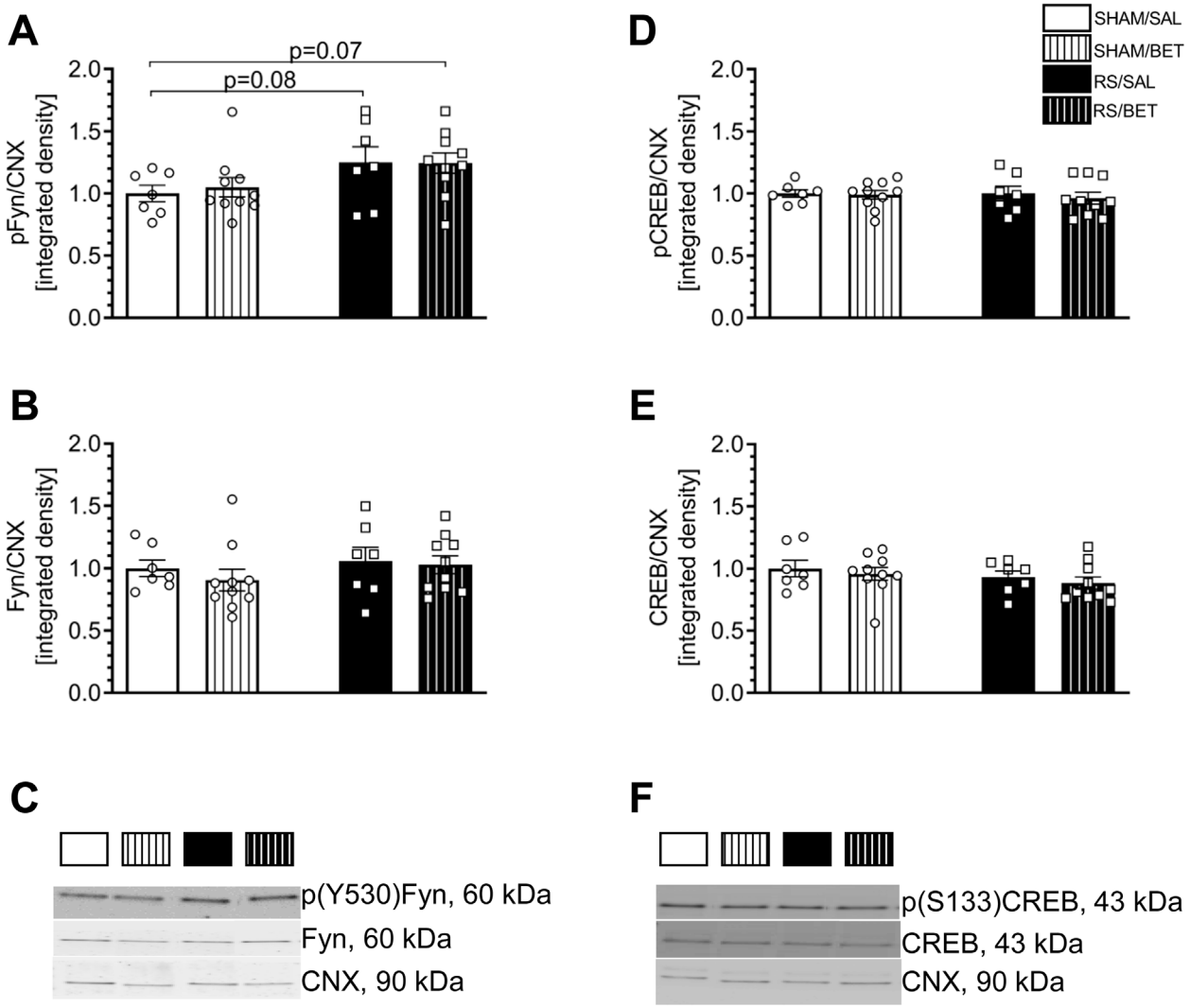

FIGURE 7
Effects of chronic RS and BET treatment on the phosphorylation and total level of Fyn and CREB proteins in the mPFC of rats measured by 24 h after the last treatment. **(A)** The expression of p (Y530)/Fyn. **(B)** The expression of Fyn. **(C)** Representative immunoblots for p(Y530)Fyn and Fyn. **(D)** The expression of p(S133)CREB. **(E)** The expression of CREB. **(F)** Representative immunoblots for p(S133)CREB and CREB. White bars correspond to SHAM, black to RS pretreatment, and bars with patterns correspond to BET post-treatment. BET (5 mg/kg/po/14 days) applied immediately after RS. Results are expressed as a mean ± SEM. N = 6–8/group. In **(A)** RS [F (1,30) = 6.0012; p = 0.02], BET [F (1,30) = 0.06; p = 0.81]; and RS × BET interaction [F (1,30) = 0,93; p = 0.76]. In **(B)** RS [F (1,30) = 1.07; p = 0.31], BET [F (1,30) = 0.05; p = 0.48], and RS × BET interaction [F (1,30) = 0.14; p = 0.70]. In **(D)** RS [F (1,30) = 0.09; p = 0.76], BET [F (1,30) = 0.29; p = 0.59], and RS × BET interaction [F (1,30) = 0.09; p = 0.80]. In **(E)** RS [F (1,30) = 1.67; p = 0.20], BET [F (1,30) = 0.72; p = 0.40], and RS × BET interaction [F (1,30) = 0.003; p = 0.95]. p = 0.08 and p = 0.07 values indicated in graph (Fisher LSD test).

groups compared to the control group (by 18% and 40%, respectively). The augmented GluA1 level in the RS7d group was on the borderline of statistical significance compared to the control (p = 0.08). Two weeks of RS application resulted in a 60% decrease in GluA1 levels compared to the RS7d group (and a 21% insignificant decrease compared to the control) (Figures 2A,D).

No significant changes were observed among groups in the phosphorylation level of p(S845)GluA1 [F (3, 10) = 0.24; p = 0.87] (Figures 3A,C).

3.1.2 GluN2B protein

In the case of total GluN2B, one-way ANOVA revealed an effect on the borderline of statistical significance [F (3, 19) = 2.99; p = 0.056]. Restraint stress initially evoked a gradual increase in GluN2B levels in the RS3d and RS7d groups compared to the control group, by 38% and 94%, respectively; however, this effect was statistically insignificant. Two weeks of RS resulted in a 63% decrease in GluN2B expression compared to the RS7d group (p = 0.086) and a 27% insignificant decrease compared to the control group (Figures 2B,D). A more pronounced effect was observed for the p(Y1472)GluN2B level [F (3, 9) = 28.35; p < 0.0001], which gradually increased in the RS3d and RS7d groups compared to the control group by approximately 30-fold (p < 0.05) and 60-fold (p < 0.01), respectively. After 14 days of RS, the p(Y1472)GluN2B level decreased below the control level (Figures 3B,C).

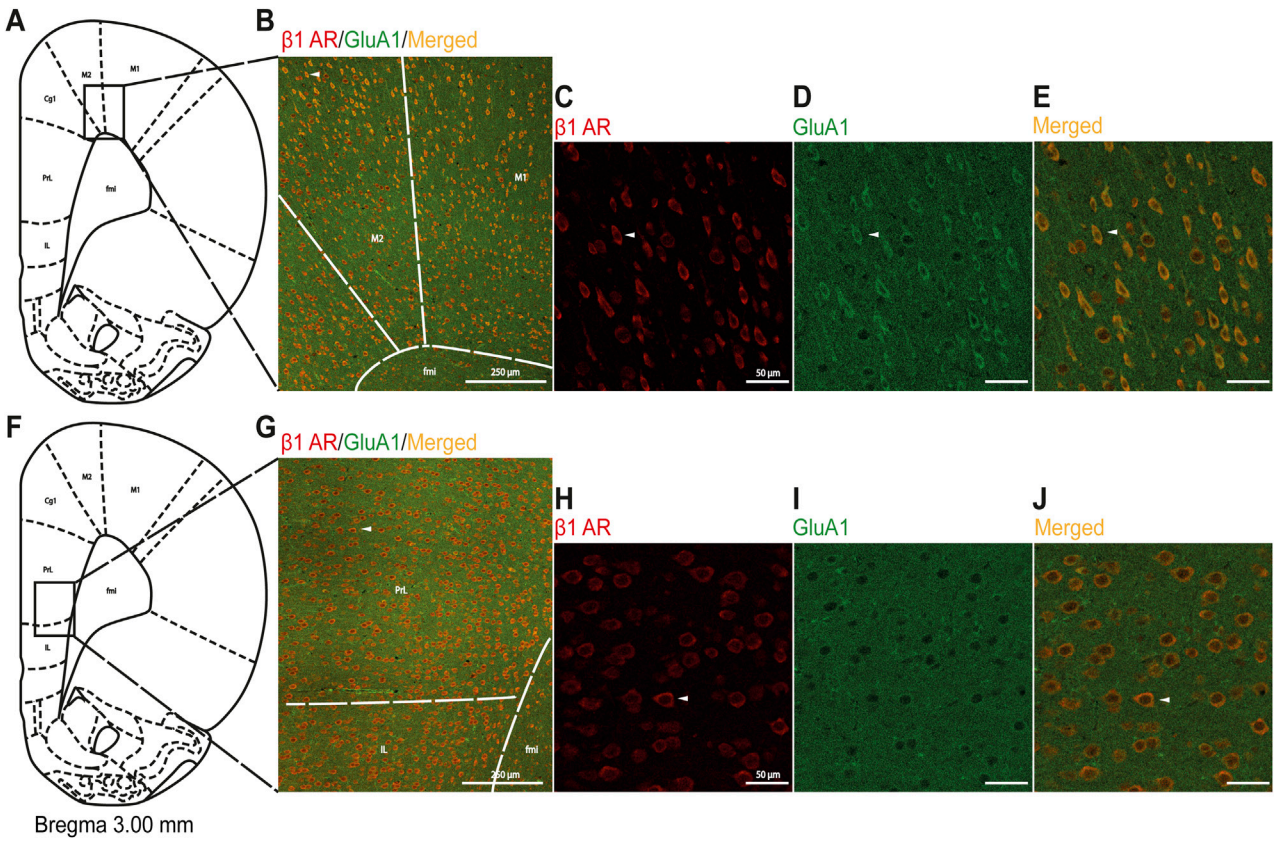

FIGURE 8
Immunofluorescent qualitative detection of colocalization of the β1AR and GluA1 subunits of AMPA-R in M1/M2 and mPFC regions. **(A)** Schematic illustration of the analyzed area for M1/M2, and **(B)** a representative image with drawn lines indicating borders among adjacent areas. In **(C-E)**, successively presented, representative magnified images with immunofluorescence staining for β1AR (red), GluA1 (green), and merged (orange) in M1/M2. **(F)** Schematic illustration of the analyzed area for mPFC, and **(G)** a representative image with drawn lines indicating borders among adjacent areas. In **(H-J)**, successively presented, representative magnified images with immunofluorescence staining for β1AR (red), GluA1 (green), and merged (orange) in mPFC. Scale bars in **(B)** and **(G)** 250 µm; in **(C-E)** and **(H-J)**: 50 µm. White arrows indicate the same cell at different magnifications in the upper and bottom panels. Abbreviations: Cg1, cingulate cortex; fmi, forceps minor of the corpus callosum; IL, infralimbic cortex; M1, primary motor cortex; M2, secondary motor cortex; PL, prelimbic cortex.

3.1.3 mGluR5/1a protein

One-way ANOVA revealed significant differences among the analyzed groups [F (3, 19) = 4.57; $p < 0.05$]. Restraint stress after 3 days of treatment did not alter the level of mGluR5/1a. An insignificant 51% increase was observed in the RS7d group compared to the control group. However, after 14 days of RS, mGluR5/1a expression was significantly decreased by 49% compared to the RS7d group and exhibited an insignificant 25% decrease compared to the control group (Figures 2C,D).

3.2 Profile of changes evoked by RS on the level of glutamate transporters in the rat FC

There were no significant differences among treatment groups in the level of VGLUT1 [F (3, 19) = 0.32; $p = 0.81$] (Figures 4A,C). One-way ANOVA for VGLUT2 expression revealed an effect on the borderline of statistical significance [F (3, 19) = 2.66; $p = 0.077$]. The *post hoc* analysis indicated that after a slight decrease in the RS3d group (by 19% compared to the control), VGLUT2 expression in the RS7d group returned to the control level, whereas in the RS14d group, it was insignificantly increased by 28% compared to the control. The increase in VGLUT2 expression in the RS14d group was on the borderline of statistical significance compared to the RS3d group (by 54%; $p = 0.06$) (Figures 4B,C).

3.3 Rat body response to prolonged RS exposure and BET treatment

3.3.1 Body weight

The initial body weight ±SEM of the animal groups analyzed in the study is as follows: SHAM/SAL – 167 ± 2.52 g, n = 8; SHAM/BET – 164 ± 3.31 g, n = 8; RS/SAL – 161 ± 3.36 g, n = 8; and RS/BET – 164 ± 3.31 g, n = 8. A two-way ANOVA revealed a significant effect of RS on body weight gain during the entire RS procedure [F (1, 26) = 53.97; $p < 0.001$]. There was no significant effect of BET [F (1, 26) = 0.096; $p = 0.76$] or the RS × BET interaction [F (1, 26) = 0.22; $p = 0.64$]. The *post hoc* analysis revealed that BET did not affect body weight gain, whereas the RS/SAL and RS/BET groups showed

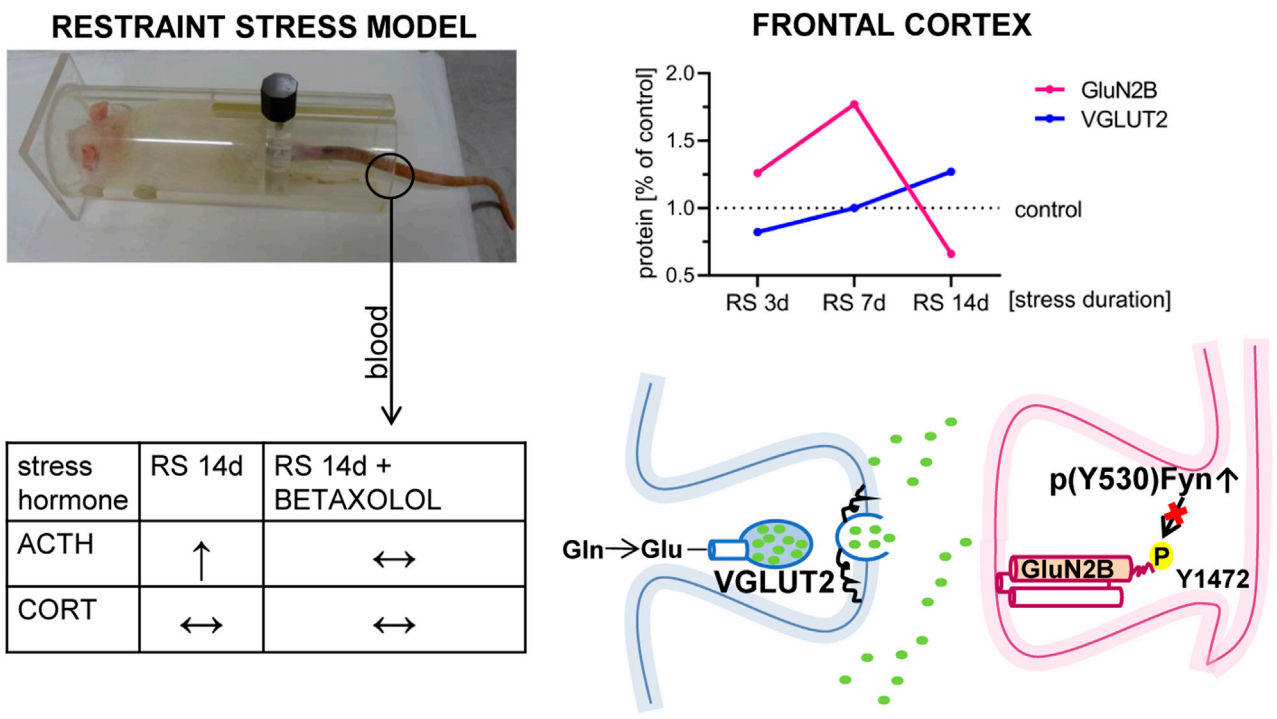

FIGURE 9
Hypothetical role of Fyn in the mechanism of GluN2B downregulation in the FC of stressed rats. Figure summarizes the results presented in the article. It depicted that RS applied for 14 days leads to a decrease in the GluN2B level in FC. GluN2B downregulation in FC, at least in the mPFC area, may be related to stress-altered Fyn activity. Although BET had no therapeutic effects on Fyn activity (see text for details), β1AR blockade normalized augmented by the stress blood level of ACTH.

reduced body weight gain compared to the SHAM/SAL group, by 30% and 33%, respectively (Figure 5A).

3.3.2 Plasma level of stress hormones

For ACTH concentration, two-way ANOVA revealed no significant effect of RS [$F(1, 19) = 0.158$; $p = 0.69$] or BET [$F(1, 19) = 0.745$; $p = 0.20$]. However, the RS × BET interaction effect was on the borderline of statistical significance [$F(1, 20) = 4.238$; $p = 0.05$]. A comparison of mean values among groups showed an insignificant increase in ACTH concentration in the RS/SAL group (by 42% compared to SHAM/SAL), which returned to the control level after BET treatment. The ACTH level in the RS/BET group was significantly reduced compared to that in the RS/SAL group ($p = 0.038$; LSD test) (Figure 5B). For CORT concentration, no significant main effects were observed: RS effect [$F(1, 23) = 0.131$; $p = 0.72$], BET effect [$F(1, 23) = 1.064$; $p = 0.31$], or RS × BET interaction [$F(1, 23) = 1.374$; $p = 0.25$] (Figure 5C).

3.4 The behavioral response of rats to prolonged RS exposure and BET treatment

3.4.1 Novel object recognition

To avoid acute disturbances during familiarization and recognition, the rats had a break from the stress session on the training day and until the test trial. Rats in the BET groups received the drug after familiarization. The test trial (T2) was performed only on rats that explored two novel objects in the training trial (T1) with no side preference. In T2, all tested rats in the SHAM groups spent significantly more time exploring the novel object, and a single BET treatment did not alter this preference (Figure 6A). Within-group comparisons showed significant results for SHAM/SAL [$t(12) = -4.24$; $p < 0.01$] and SHAM/BET [$t(13) = -6.62$; $p < 0.001$]. In contrast, rats in the RS/SAL group did not show a preference for exploring the novel object [$t(11) = -1.67$; $p = 0.13$], and the effect in the RS/BET group was also insignificant [$t(9) = -2.13$; $p = 0.06$]. The analysis of the discrimination index revealed a lower discrimination index in stressed animals. The RS effect was on the borderline of statistical significance [$F(1, 22) = 3.31$; $p = 0.08$]. No significant effects of BET [$F(1, 22) = 0.40$; $p = 0.53$] or RS × BET interaction [$F(1, 22) = 0.09$; $p = 0.76$] on the discrimination index in the NOR test were observed (Figure 6B).

3.4.2 Elevated plus maze

There were no significant effects of RS, BET, or RS × BET interaction on the time spent in the open arms (RS: [$F(1, 26) = 0.16$; $p = 0.69$], BET: [$F(1, 26) = 0.02$; $p = 0.89$], and RS × BET: [$F(1, 26) = 0.11$; $p = 0.75$]) or in the closed arms (RS: [$F(1, 26) = 0.01$; $p = 0.94$], BET: [$F(1, 26) = 0.46$; $p = 0.50$], and RS × BET: [$F(1, 26) = 0.02$; $p = 0.87$]) of the EPM apparatus. Similarly, there were no significant effects on the number of entries into the open arms (RS: [$F(1, 26) = 0.39$; $p = 0.84$], BET: [$F(1, 26) = 0.05$; $p = 0.82$], and RS × BET: [$F(1, 26) = 1.32$; $p = 0.26$]) or the closed arms (RS: [$F(1, 26) = 0.12$; $p = 0.73$], BET: [$F(1, 26) = 0.15$; $p = 0.70$], and RS × BET: [$F(1, 26) =$

0.0006; $p = 0.98$]). The mean time ±SEM spent by all tested animals in the open arms was 70.58 ± 7.40 s, which was more than twofold lower than the time spent in the closed arms (150.94 ± 9.90 s) (t = 6.50, df = 58, $p < 0.0001$, and unpaired t-test) (Supplementary Figure S1).

3.5 Effects of the prolonged restraint stress exposure and betaxolol treatment on Fyn and CREB expression and phosphorylation in the rat mPFC

3.5.1 Fyn protein

Stress augmented the level of p(Y530)Fyn independently of BET treatment. Two-way ANOVA revealed a significant RS effect [F (1, 30) = 6.0012; $p = 0.02$] on the level of p(Y530)Fyn. There were no significant effects of BET [F (1, 30) = 0.06; $p = 0.81$] or RS × BET interaction [F (1, 30) = 0.93; $p = 0.76$]. The *post hoc* Fisher LSD test showed a 25% increase in the phosphorylation level of p(Y530)Fyn in the RS/SAL group ($p = 0.082$) and the RS/BET group ($p = 0.066$) compared to the SHAM/SAL group (Figures 7A,C).

No significant effects of RS [F (1, 30) = 1.07; $p = 0.31$], BET [F (1, 30) = 0.05; $p = 0.48$], or RS × BET interaction [F (1, 30) = 0.14; $p = 0.70$] were observed on the expression of the total Fyn protein (Figures 7B,C).

3.5.2 CREB protein

Neither RS nor BET influenced p(Ser133)CREB or CREB expression in the mPFC. Two-way ANOVA results for p(Ser133) CREB are as follows: RS [F (1, 30) = 0.09; $p = 0.76$], BET [F (1, 30) = 0.29; $p = 0.59$], and RS × BET interaction [F (1, 30) = 0.09; $p = 0.80$] (Figures 7D,F). Similarly, two-way ANOVA results for CREB are as follows: RS [F (1, 30) = 1.67; $p = 0.20$], BET [F (1, 30) = 0.72; $p = 0.40$], and RS × BET interaction [F (1, 30) = 0.003; $p = 0.95$] (Figures 7E,F).

3.6 Qualitative comparison of β1AR–GluA1 colocalization in M1/M2 vs. mPFC

Based on the knowledge of the cooperation between βAR and GluA1, which is necessary to modulate intracellular stress signaling in the hippocampus (Joiner et al., 2010), we performed a colocalization analysis of β1AR with GluA1 to identify loci other than the mPFC within the FC where βAR could modulate glutamatergic signaling. The colocalization of β1AR and GluA1 proteins was more clearly visible in the M1/M2 regions than that in the mPFC (Figure 8).

4 Discussion

We demonstrated an increase in the level of glutamate receptors in the FC of rats after 7 days of RS exposure, followed by a decrease observed on the 14th day of RS. Furthermore, we showed that chronic RS impaired recognition memory and elevated the p(Y530) Fyn level in the mPFC in a manner independent of β1AR activity.

4.1 Bidirectional profile of RS evoked changes in the glutamate signaling proteins within FC

Preclinical studies utilizing genetic, biochemical, electrophysiological, pharmacological, and lesion techniques have revealed the involvement of glutamate receptors—particularly the GluA1 subunit of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA-R), the GluN2B subunit of N-methyl-d-aspartate receptors (NMDA-R), and the mGluR1a/5 dimer of group I metabotropic glutamate receptors (mGluRI)—in stress-affected synaptic functioning of the FC (Lu et al., 2009; Musazzi et al., 2015; Mikics et al., 2017; Zelek-Molik et al., 2021). In the present study, we observed a gradual increase in GluA1 and GluN2B levels in the FC of RS3d and RS7d groups compared to the control group, followed by a decrease in the RS14d group compared to the RS7d group. A similar bidirectional profile of changes was noted for mGluR1a/5. These results confirm the involvement of GluA1, GluN2B, and mGluR1a/5 in the FC as part of the mechanism underlying the stress pathology. The bidirectional profile of glutamate receptor level changes observed in this study aligns with findings from other stress models (for review, see Musazzi et al., 2015; Mayberg et al., 2005). However, the dynamics of stress-evoked changes in glutamate receptor levels observed in our study differed from those reported in the literature. We noted upregulation of GluA1, GluN2B, and mGluR1a/5 after 7 days of RS, whereas others detected augmented levels of glutamate receptors following acute RS (Yuen et al., 2009). A possible explanation for this discrepancy is the less severe RS protocol used in the first part (A) of our experiments (10 min twice daily) compared to the typically applied 2–3 h daily RS. This explanation aligns with the observations of Cristina Rabasa et al., who showed slower adaptation of HPA responses to homotypic stress with lighter intensity (Rabasa et al., 2015). The upregulation of glutamatergic receptor subunits in the FC after acute RS exposure has been associated with improved behavioral performance (Yuen et al., 2009). Assuming that an increased glutamate receptor level in the FC is a biomarker of improved working memory, our results suggest that this facilitation under mild RS conditions (10 min twice daily) persists longer, lasting at least 7 days. Notably, applying the same RS duration but with higher intensity (3 h daily) in the second part (B) of our experiments impaired recognition memory in rats. Following the increase in GluA1, GluN2B, and mGluR1a/5 levels observed in the FC of rats exposed to 7 days of RS, a downregulation was noted after 14 days of RS exposure. It should be noted that the expression of glutamate subunits of ionotropic receptors encompasses not only the membrane fraction but also other intracellular compartments, where GluA1 and GluN2B are synthesized and processed. However, in our study, the effects of RS appeared to be specific to membrane NMDA-Rs containing GluN2B subunits. This conclusion is supported by the observation that RS, in our study, had a similar effect on the phosphorylation level of these subunits, a process that occurs only at the plasma membrane (Goebel-Goody et al., 2012). Chronic RS and CORT administration have been associated with increased field potential amplitudes in the FC (Bobula et al., 2011) and enhanced presynaptic glutamate release (Liu et al., 2020; Modrak et al., 2023). In the present study, we demonstrated an

increased level of the presynaptic VGLUT2, likely reflecting the described glutamate release adaptation in the presynaptic elements of stressed synapses within the FC. VGLUT2 is considered a marker of thalamocortical and mesocortical projections (Liguz-Lecznar and Skangiel-Kramska, 2007; Gorelova et al., 2012). Therefore, changes in VGLUT2, but not in VGLUT1, suggest that RS affects glutamatergic inputs from the thalamus or ventral tegmental area. A similar pattern of changes in VGLUT levels was recently observed in a model of chronic psychosocial stress (Zelek-Molik et al., 2021), suggesting that both social and physical stressors affect similar cortical projections. The downregulation of glutamate receptor levels, together with the upregulation of VGLUT2 in the FC observed after 14 days of mild RS exposure, indicates that 14 days is an efficient duration for modeling stress-evoked hypofrontality, even under less severe stress conditions. Consequently, we selected 14 days of RS as an optimal period to further investigate the involvement of Fyn and CREB protein activity in stress pathology mechanisms, as well as to evaluate the therapeutic potential of systemic β1AR blockade applied after 1 week of RS.

4.2 Body stress response can be attenuated by β1AR blockade

To evaluate the applicability of the RS procedure as a model for stress-related hypofrontality in the context of potential homeostatic adaptation, we conducted a comprehensive analysis of the physiological and behavioral stress responses after chronic RS exposure. The slower body weight gain observed in stressed animals reflects profound physiological changes induced by prolonged stress exposure. This observation aligns with findings from other studies employing RS protocols (Magariños and McEwen, 1995; Harris et al., 2002; McLaughlin et al., 2007). The RS-induced reduction in body weight gain is unlikely to result from food restrictions during RS sessions, as rats were weighed both before the stress session and after a 21-h period of unrestricted food access. Furthermore, studies monitoring food intake in restrained animals have shown that RS can increase food consumption compared to control groups (Kleen et al., 2006). Thus, the slower weight gain in RS rats likely reflects the physiological effects of an uncontrollable stressor, which is known to influence the FC structure and function and contribute to the development of stress-related psychiatric disorders. In behavioral studies, rats exposed to RS (3 h daily) for 7 days spent a similar amount of time exploring familiar and novel objects in the NOR test, unlike non-stressed control rats. Consequently, the discrimination index was lower in RS rats than that in SHAM groups. This finding corroborates previous studies showing that rats exposed to daily RS for 1–3 weeks exhibit impaired object discrimination (Bowman et al., 2009; Bowman et al., 2022; Yuen et al., 2012; Luine et al., 2017), indicating deficits in visual (non-spatial) memory. However, chronic RS in our study did not induce anxiety in the EPM test, which differed from the results in male rats subjected to more severe RS protocols (6 h daily for 21 or 28 days) (Bowman et al., 2009; Chiba et al., 2012). Taken together, these findings suggest that the RS protocol used in our study (3 h daily for 14 days) is sufficient to impair recognition memory but lacks the intensity to induce anxiety. Analysis of stress hormone levels during the last RS session revealed no changes in CORT levels but an increase in ACTH levels, which was normalized by BET treatment. The absence of increased CORT levels during the 14th RS session aligns with the findings from other chronic RS models (Gądek-Michalska et al., 2013; Kusek et al., 2017) and likely reflects adaptive homeostasis (habituation) to a processive stressor, which is classified as "non-damaging" (Girotti et al., 2006; Davies, 2016). It is noteworthy, however, that the tail veins of RS rats appeared highly shrunken compared to non-stressed controls, making blood collection difficult (personal observation). This finding suggests stress anticipation by RS rats, despite unchanged CORT levels across experimental groups. The absence of elevated CORT levels may indicate a functional negative feedback mechanism that rapidly extinguishes HPA responses to predictable stressors, thereby preserving the capacity to respond appropriately to novel (heterotypic) stressors (Herman et al., 2016). Additionally, ACTH levels, rather than CORT, are considered markers of stressor intensity. Chronic exposure to a strong stressor has been shown to result in sustained ACTH elevation and reduced CORT levels compared to initial acute RS responses (Martí and Armario, 1998). The increased ACTH levels observed in our study suggest that the chronic RS protocol was sufficiently intense to induce prolonged stress anticipation and influence FC structure and function. The secretion of ACTH from the pituitary gland is primarily regulated by corticotropin-releasing hormone from hypothalamic neurons (Herman et al., 2016). However, pharmacological evidence indicates that the ACTH release is partially mediated by βAR stimulation in the anterior pituitary (Mezey et al., 1983). Our results suggest that endogenous β1AR stimulation is necessary for the stress-induced ACTH release. The therapeutic implications of ACTH reduction in stress-related disorders warrant further investigation. Nevertheless, our findings demonstrate that systemic β1AR blockade can alleviate the elevated ACTH levels. This observation aligns with evidence suggesting that pharmacological β1AR blockade mitigates stress-induced memory impairments and anxiety in animal models (Otis et al., 2015).

4.3 Fyn importance for signaling adaptations in mPFC after RS

Kinase Fyn, which is primarily associated with GluN2B phosphorylation (Goebel-Goody et al., 2012), shares structural similarities with other non-receptor tyrosine kinases. Functional analyses revealed that enzyme activation requires single phosphorylation at Y416 in the SH1 domain, whereas phosphorylation at Y527, located at the carboxyl (C-) terminus of the Fyn protein, leads to kinase downregulation (Sicheri and Kuriyan, 1997; Roskoski, 2005; Vatish et al., 2009). Notably, the entire C-terminus of Fyn is involved in the negative regulation of kinase activity (Sicheri and Kuriyan, 1997), and recent studies have identified Y530 as a critical regulatory site for the conformational changes that inactivate Fyn (Cuesta-Hernández et al., 2023). Given that phosphorylation at tyrosine 530 inactivates Fyn, our findings demonstrating that chronic RS upregulates p(Y530)Fyn levels in the mPFC suggest that chronic stress inhibits Fyn activity. Evidence from Fyn-deficient mice indicates that Fyn inactivity leads to impaired spatial learning, blunted LTP in the hippocampus (Grant et al., 1992), and heightened responsiveness to fear-

inducing stimuli (Miyakawa et al., 1994). In light of these findings, our data showing impaired object recognition along with altered Fyn phosphorylation in the mPFC of rats exposed to chronic RS suggest that the downregulation of Fyn activity plays a role in the mechanisms underlying stress pathology. This interpretation is supported by pharmacological studies indicating that Fyn activation is involved in the antidepressant effects of amitriptyline (Abe et al., 2019) and losartan (Diniz et al., 2018). Furthermore, decreased Src activity, a kinase closely related to Fyn, has been observed in the hippocampus in a mouse model of postpartum depression (Zhang et al., 2016). However, there is a conflicting source of evidence regarding Fyn's role in stress pathology. Some studies suggest a therapeutic benefit from the downregulation of Fyn activity in brain structures (Wang et al., 2022). Therefore, further research is required to elucidate the precise role of Fyn activity in the mechanisms of stress-related disorders.

4.4 β1AR pathway engagements in RS-evoked glutamate signal disturbances in mPFC

Our results suggest that Fyn may be directly involved in the mechanism of RS-evoked downregulation of GluN2B-containing NMDA receptors in the FC. However, the impact of stress on p(Y530)Fyn levels appears to be independent of β1AR activity, as BET treatment did not influence p(Y530)Fyn levels in the mPFC. Additionally, our study revealed that 24 h after chronic RS, the activity of the cAMP-responsive element-binding protein (CREB) remains unaltered in the mPFC of RS rats. CREB is a transcription factor that regulates numerous metabolic pathways, including β-AR signaling (e.g., Glebov-McCloud et al., 2024). This finding suggests that the β1AR–cAMP–CREB pathway is not involved in the cognitive impairments induced by exposure to homotypic stress. This result is consistent with the data from studies on stress-induced cocaine-seeking behavior (Briand and Blendy, 2013), but it contradicts with findings showing downregulation of CRTC1 (a CREB coactivator) in the mPFC of animals displaying depressive-like behavior following stress exposure (Wang et al., 2020). The discrepancy between these findings may be attributed to differences in the stress pathology models used. Based on our behavioral data, our model demonstrates spatial memory impairments, whereas CREB downregulation has been reported in mice exhibiting depressive-like behavior. Another potential explanation for the lack of detectable alterations in CREB activity in the mPFC of RS rats could be the time-limited role of CREB in stress-induced intracellular signaling. We measured p/t CREB levels 24 h after the final stress session, whereas CREB-dependent changes, such as those associated with fear learning in the hippocampus (Zelek-Molik et al., 2019) or glutamate signaling alterations in the mPFC during cocaine withdrawal (Sun et al., 2013; Sun et al., 2014a; Sun et al., 2014b), have been observed primarily within the first hours following stressor exposure. To identify potential targets within glutamate signaling for the therapeutic effects of β1AR blockade, we conducted immunohistochemical analysis in the FC region. This analysis revealed the colocalization of β1AR with AMPA-R containing GluA1, predominantly in the motor cortex (M1/M2). These findings suggest that investigating the regulatory effects of β1AR blockade on AMPA-R activity altered by stress, particularly in the M1/M2 region of the FC, would be a valuable avenue for future research.

4.5 Limitations

The present study has several limitations. The primary limitation is that the level of glutamate receptors was assessed in a broader area of the FC, whereas Fyn and CREB levels were measured specifically in the mPFC. The second limitation is the difference in timing, as glutamate receptors and transporters were evaluated immediately after RS, whereas Fyn and CREB levels were assessed 24 h post-RS. Last, only BET was used to evaluate the therapeutic effects of β1AR blockade on stress-induced alterations in glutamate signaling within the FC. Despite these limitations, the findings of this study provide an essential foundation for future research. Specifically, they highlight the potential involvement of the M1/M2 region in stress pathology and offer insights into the precise role of Fyn activity in the mechanisms underlying stress-induced disruptions in glutamate signaling within the FC.

4.6 Conclusions

Our results, summarized in Figure 9, confirm a bidirectional pattern of changes in glutamate receptor levels in the FC, which progressively develop during prolonged exposure to homotypic stress. These findings suggest that the dynamics of these changes correlate with the intensity of the applied daily stressors. The data indicate that chronic RS, accompanied by cognitive impairments, leads to a decrease in Fyn kinase activity in the mPFC, highlighting Fyn deactivation as a critical factor in the chronic stress-induced downregulation of GluN2B in the FC. Furthermore, the lack of BET impact on the assessed behavioral and biochemical parameters suggests that βAR activity is not involved in the mechanism underlying chronic RS-evoked GluN2B downregulation in the FC at least within the mPFC region.

Data availability statement

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

Ethics statement

The animal study was approved by the Local Ethics Committee for Animal Experiments at Maj Institute of Pharmacology at the Polish Academy of Sciences in Krakow. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AZ-M: conceptualization, formal analysis, investigation, methodology, project administration, supervision, validation,

visualization, writing–original draft, and writing–review and editing. AG-M: conceptualization, investigation, methodology, and writing–review and editing. MW: formal analysis, methodology, and writing–review and editing. AB: formal analysis, investigation, and writing–review and editing. KM: visualization and writing–review and editing. GK: methodology and writing–review and editing. IN: conceptualization, funding acquisition, supervision, and writing–review and editing.

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Conflict of interest

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

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Trends in research on novel antidepressant treatments

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Mood disorders, such as major depressive disorder and bipolar disorder, are among the most common mental illnesses and a leading cause of disability worldwide. Key symptoms of these conditions include a depressed mood or anhedonia, sleep and psychomotor disturbances, changes in appetite or weight, and fatigue or loss of energy. Prolonged cognitive disturbances further impair the ability to think or concentrate and are often accompanied by persistent feelings of worthlessness or excessive guilt. Collectively, these symptoms underscore depression as a serious, long-term global health issue. In addition, clinical studies indicate a growing number of patients experiencing difficulties in responding to treatment, even in the long term. This phenomenon poses significant challenges for healthcare professionals, families, and patients alike. As a result, there is an urgent need for therapies that are both rapid-acting and safe. This review aims to summarize the prevailing trends in research on novel antidepressants, emphasizing their diversity and multi-directional mechanisms of action. The development of rapid-acting drugs is increasingly focused on achieving high efficacy, particularly for treatment-resistant depression. Such advances offer the potential for rapid therapeutic effects without the prolonged and often tedious administration of older generation antidepressants. Findings from studies using animal models of depression continue to play a crucial role in predicting and designing new therapeutic strategies. These models remain indispensable for understanding the physiological effects of newly developed compounds, thereby guiding the creation of innovative treatments.

KEYWORDS

depression, animal models, RAAD, TRD, antidepressants, ketamine, psylocin

1 Introduction

Major depressive disorder (MDD) is a common and growing global health concern, affecting over 264 million people, with an estimated lifetime risk of 15%–18% (Murray et al., 2012; Bromet et al., 2011). Unlike short-lived, everyday mood fluctuations, depression represents a severe and persistent health condition. Patients report many comorbidities that reduce quality of life and life expectancy (Berk et al., 2023).

According to the DSM-5 (Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition), a diagnosis of a major depressive episode requires the presence of five or more symptoms for at least 2 weeks. Key symptoms include a depressed mood or anhedonia, sleep and psychomotor disturbances (agitation or retardation), changes in appetite or weight, and fatigue or loss of energy. Cognitive impairments, such as difficulties with concentration or decision-making, often accompany persistent feelings of worthlessness or excessive guilt.

The World Health Organization (WHO), in the 11th revision of the International Classification of Diseases and Related Health Problems (ICD-11), conceptualizes depression as a syndrome characterized by a clinically recognizable set of reported experiences (symptoms) and observed behaviors (signs) associated with distress and impaired functioning (Cuijpers et al., 2023; International Advisory Group for the Revision of ICD-10 Mental and Behavioural Disorders, 2011). At its most severe, depression can lead to suicide, which the WHO identifies as the second leading cause of death among individuals aged 15–29, accounting for approximately 800,000 deaths annually (Hawkins et al., 2018; Jensen et al., 2023; Murphy et al., 2021).

The discovery and widespread use of the first antidepressants revolutionized depression treatment. Grounded in the widely accepted monoamine theory of depression, first- and second-generation antidepressants, including serotonin and norepinephrine reuptake inhibitors, provided significant insights into mechanisms of action and treatment options. However, clinical trials have shown that these compounds are not equally effective in patients (Nunez et al., 2022; Salzman, 1996; Price et al., 1987). Furthermore, they often require weeks or months to achieve full effectiveness, underscoring the need for more effective and rapid-acting treatments for MDD (U.S. Food and Drug Administration (FDA) 2021; European Medicines Agency (EMA) 2013; Rush 2007; Mourilhe and Stokes, 1998).

In addition, one of the major challenges in treating depression is that many people who are prescribed antidepressants do not fully respond to pharmacotherapy. About 30% of patients treated for major depression do not respond satisfactorily to initial treatment. A significant number of patients have a poor prognosis at follow-up, with up to 20% still affected 2 years after onset (Fava et al., 2003). The STAR*D trial reported that one in three patients with unipolar depression did not achieve symptomatic remission after multiple antidepressant trials (Trivedi et al., 2006), and patients with bipolar depression did not benefit from antidepressant treatment combined with mood stabilizers (Bowden et al., 2012). These data indicate that a significant number of patients experience a phenomenon known as treatment-resistant depression (TRD) or refractory major depressive disorder (Berlim and Turecki, 2007; Tundo et al., 2023; Fu et al., 2024). Several authors have suggested distinguishing between difficult-to-treat and treatment-resistant depression on the basis of the longitudinal course of the illness. However, the common distinction indicates a clinical state resulting from a lack of response to treatment. As evidenced by the fact that several definitions have been proposed, the construct of TRD is very heterogeneous (Murphy et al., 2021).

2 Monoamine system

Current treatments for MDD are predominantly based on the monoaminergic system, as the monoamine hypothesis of depression suggests that a decrease in serotonin, norepinephrine, and dopamine levels in the central nervous system underlies the condition's pathophysiology. The effectiveness of antidepressants supports this hypothesis, as drugs that increase the levels of these neurotransmitters in the brain have consistently been shown to reduce symptoms of depression.

Four main classes of monoamine-targeting antidepressants have been developed: first-generation antidepressants (monoamine oxidase inhibitors [MAOIs] and tricyclic antidepressants [TCAs]), second-generation antidepressants (selective serotonin reuptake inhibitors [SSRIs] and serotonin-norepinephrine reuptake inhibitors [SNRIs]), and atypical antidepressants. Among these, SSRIs are the most commonly used today, as they primarily address the hypoactivity of monoamine neurotransmitter systems (Sharp and Collins, 2023). Guidelines from the National Institute for Health and Care Excellence (Leichsenring et al., 2023) and the American Psychiatric Association (APA, 2019) recommend selective serotonin reuptake inhibitors (SSRIs), among other options, as first-line treatments for moderate to severe major depression. They are generally better tolerated and safer in overdose situations compared to TCAs and other antidepressants like noradrenergic and specific serotonin antidepressants or MAOIs (Olfson and Marcus, 2009; NHS Information Centre, 2011). However, an important limitation of all these treatments is their delayed onset of action, often taking 3 weeks or more to produce noticeable effects. During this time, especially in younger populations, SSRIs may exacerbate pre-existing anxiety or suicidality (Walter et al., 2022; Jack et al., 2023; Lagerberg et al., 2023).

Antidepressants acting via the monoaminergic system include partial agonists targeting serotonin receptors such as 5-HT1A, 5-HT1B, 5-HT2A, 5-HT2C, 5-HT4, and 5-HT7. Other mechanisms involve serotonin transporter inhibitors, 5-HT1A receptor "biased" agonists, triple monoamine reuptake inhibitors, and MAO-A inhibitors. Psychedelics, such as psilocin, also act within this system (Lochmann and Richardson, 2018; Weilburg, 2004; Papp et al., 2024). Psilocin, the most extensively studied psychedelic, primarily targets serotonin receptors but ultimately enhances glutamate release and sustained excitatory neurotransmission in pyramidal neurons. This promotes a prolonged state of enhanced neural plasticity in corticolimbic circuits, a key factor in its antidepressant effects.

The antidepressant effects of psilocin are thought to be mediated through activation of the 5-HT2A-mGlu2 receptor complex, which is also crucial for the hallucinogenic behaviors induced by 5-HT2A receptor agonists (López-Giménez and González-Maeso, 2018; Erritzoe et al., 2024). Activation of 5-HT2A receptors promotes glutamate release in pyramidal neurons of the prefrontal cortex, leading to both antidepressant and anxiolytic effects. Research on mitigating the adverse acute effects of psilocybin suggests these effects are related to the occupancy of 5-HT2A receptors (Madsen et al., 2019; Lagerberg et al., 2023). Interestingly, chronic administration of SSRIs or SNRIs has been shown to lead to downregulation of 5-HT2A receptors in both animal and human studies (Kumar et al., 2019; Klimek et al., 1994; Meyer et al., 2001).

3 Glutamatergic system

Among newer, faster-acting pharmacological treatment strategies, those targeting the glutamatergic and GABAergic systems have shown considerable promise for the treatment of MDD and TRD. A particularly notable example is (R,S)-ketamine, whose rapid improvement in depressive symptoms

after administration (Swainson et al., 2019; Berman et al., 2000; Barbara et al., 2024) has significantly renewed interest in compounds that modulate glutamatergic receptors, sparking a wave of research and drug development in this area. (R,S)-Ketamine acts as an NMDA receptor antagonist. Its S (+) isomer, esketamine, has been approved in the European Union, United Kingdom, and United States for adults with TRD and for the rapid reduction of depressive symptoms in psychiatric emergencies related to MDD. Ketamine's effects, while mechanistically distinct from those of psilocin, share similarities in their activation of intracellular signaling pathways. These pathways produce long-lasting changes in synaptic function and morphology (neuroplasticity), proposed as a common mechanism underlying the therapeutic effects of ketamine and classical psychedelics (Aleksandrova and Phillips, 2021). While newly developed neuroplasticity-based strategies do not directly target glutamate modulation, they focus on activating the intracellular mammalian target of rapamycin complex 1 (mTORC1) signaling cascade. This pathway links glutamate modulation to the induction of neuroplastic changes.

Specific enantiomers, racemic mixtures or metabolites of ketamine have different affinities for NMDAR and have shown antidepressant activity in preclinical and clinical studies. Metabolites of ketamine such as S-norketamine, 2S,6S-hydroxynorketamine, 2S,6R-hydroxynorketamine and 2R,6S-hydroxynorketamine have shown antidepressant-like effects in animal models (Zanos et al., 2016; Yokoyama et al., 2020). Differences between (R)-ketamine and (S)-ketamine have been observed at behavioural and biochemical levels. Some preclinical studies also show that (R)-ketamine has stronger and longer-lasting effects and is safer than ketamine or (S)-ketamine. That suggest that (R)-ketamine could be a promising molecule and, like some metabolites, should be tested in clinical trials (Pochwat et al., 2022).

Among the many compounds under investigation, NMDA and metabotropic glutamate receptor antagonists and negative allosteric modulators appear particularly promising due to their ability to inhibit glutamatergic neurotransmission. These include broad-spectrum glutamatergic modulators, like some of the non-NMDA receptor agonists such as glycine site NMDAR partial agonists, NR2B antagonists, non-selective low-trapping NMDAR channel blockers or non-competitive NMDAR antagonists are active in models of depression (Machado-Vieira et al., 2017). NMDAR antagonists alone or as an adjunct to imipramine/SSRIs have produced antidepressant effects in animal models of depression (Poleszak et al., 2011).

Regarding metabotropic glutamate receptors, all three groups show antidepressant effects, especially under experimental conditions. For group I mGlu receptors, promising ligands include mGlu1 receptor antagonists, mGlu5 and partial mGlu5 receptor negative allosteric modulators, but also mGlu5 receptor inverse agonists. For group II mGlu receptors, antidepressant-like activity has been demonstrated by mGlu2/3 receptor agonists, mGlu2/3 receptor antagonists, and mGlu2/3 and selective mGlu2 and mGlu3 receptor negative allosteric modulators. To a lesser extent, group III mGlu receptors are being investigated. Group III mGlu receptor agonists, mGlu7 receptor negative allosteric modulators, mGlu7 receptor allosteric agonists and mGlu8 receptor agonists (Dogra and Conn, 2021). As shown, metabotropic glutamate receptor ligands excert antidepressant potential and, as some suggest, could be used as adjuncts to reduce the side effects of rapid-acting antidepressants (discussed in Pochwat et al., 2022).

Some experimental compounds exhibit intriguing multimodal mechanisms of action, including NMDA receptor antagonism combined with α-1 adrenergic receptor agonism. Additionally, a positive allosteric GABAA modulator is currently in phase III clinical trials (Clayton et al., 2023). It should be emphasised that a large number of NMDAR antagonists are effective in preclinical models of depression, but have been unsuccessful in clinical trials (Kishimoto et al., 2016). However novel glutamatergic modulators for the treatment of mood disorders encompass a diverse range of approaches. These advances are yielding new insights and opportunities for the development of rapid-acting treatments for MDD (for reviews, see Henter et al., 2021; Serretti, 2024).

4 Treatment-resistant depression

Treatment-resistant depression is a significant clinical challenge. While some studies report that over 35% of patients with depression are resistant to antidepressant treatment (Nemeroff, 2007; Thase, 2011; Thomas et al., 2013), more recent data highlight an even greater burden. According to Heerlein et al. (2021); Heerlein et al. (2022), approximately 69% of patients with difficult-to-treat depression in Europe did not respond to treatment within 1 year. Despite low remission rates, 60% of these patients remained on unchanged treatment regimens for extended periods. Some studies reported that in some populations even one-fourth of all depressive patients are affected by TRD (Gałecki et al., 2022). Mortality, particularly due to suicide and accidents, as well as comorbid conditions, is significantly higher in TRD patients (Döme et al., 2021; Reutfors et al., 2018; DiBernardo et al., 2018). Non-lethal self-harm is also more frequent in TRD patients compared to those without this condition (Parker and Graham, 2015). Furthermore, TRD is associated with worse disease outcomes and elevated rates of general and psychiatric comorbidities relative to other patients treated for depression.

Among recently introduced TRD treatments, esketamine has a special place. Recent studies confirm the efficacy, safety and safety and tolerability of esketamine in adults with TRD. In patients with comorbidities in the REAL-ESK study reported the later response, as well as the non-inferiority in effectiveness present novel and interesting findings (Martinotti et al., 2022). It should be noted that esketamine is only approved for TRD and emergency suicidality. It represents an important novelty in the pharmacological treatment of patients with depression, but its specific mechanism of action may be associated with craving behaviour and additional addictive potential (Leichsenring et al., 2023). A narrative review of the available literature on the most common clinical "misconceptions" and "stereotypes" about esketamine has recently been presented by Di Vincenzo et al. (2024).

Despite ongoing research, there is no universally effective treatment strategy for TRD. Current guidelines recommend several approaches, including dose optimization, switching antidepressants, or augmenting therapy with other pharmacological agents (Gabriel et al., 2023; Davies et al., 2019). The high heterogeneity in the treatment of patients with TRD and

the urgent need for standardised strategies and treatments specifically approved for TRD are highlighted. In addition to esketamine, some new studies describe the benefits of adding lithium or antipsychotics to adjuvant therapies for patients with TRD (Maina et al., 2023). Strong recommendations also exist for disorder-specific psychological therapies, such as cognitive analytic therapy, cognitive behavioral therapy (including internet-based CBT), and interpersonal therapy for depression (Ijaz et al., 2018). However, long-term treatment often proves discouraging for both patients and their families, emphasizing the urgent need for alternative therapeutic approaches.

5 Optimizing treatment strategies for TRD

The latest guidelines advocate for first addressing pseudo-resistance by ensuring adherence to treatment and optimizing antidepressant dose and duration. For combination therapies, the pairing of monoamine reuptake inhibitors with presynaptic α2-adrenoceptor antagonists has shown promise in improving efficacy in TRD patients. Additionally, targeting the opioid system with μ-receptor agonists, mixed (μ/κ) receptor agonists, or κ-receptor antagonists may provide novel avenues for treatment (Borbely et al., 2021). Agonism of the 5-HT1A receptor is another promising strategy for achieving rapid and sustained therapeutic effects in TRD (Smith et al., 2023; Papp et al., 2023).

Among augmentation strategies, second-generation antipsychotics and lithium remain the most evidence-supported options for managing TRD. Novel therapies, particularly those targeting the glutamatergic system, have garnered significant attention. Intravenous ketamine and intranasal S-ketamine are at the forefront of these developments. Since intranasal S-ketamine was approved by the U.S. FDA and the EMA in 2019, it has been introduced as a novel adjunctive therapy for TRD with specific indications and under laboratory control. These therapies offer hope for addressing the limitations of traditional treatment approaches. We identified three primary clinical approaches to optimize treatment for TRD: neurostimulation therapy, metabolic modulation, and ion level modulation.

5.1 Neuromodulations/neurostimulations treatments

Neuromodulation is a growing area of research interest based on clinical and preclinical reports (Hamani and Nóbrega, 2010; Figee et al., 2022; Papp et al., 2018). Non-invasive techniques include electroconvulsive therapy (ECT), magnetic seizure therapy (MST), transcranial direct current stimulation (tDCS), and repetitive transcranial magnetic stimulation (rTMS). Invasive procedures include neurosurgical vagus nerve stimulation (VNS) and deep brain stimulation (DBS), in which electrodes are implanted in discrete brain targets under MRI guidance. For affective disorders, as recommended by the Canadian Agency for Drugs and Technologies in Health, 2014, DBS for MDD is still under investigation. Acute and maintenance efficacy, as well as safety and tolerability, were classified at level 3 in 2016 (Milev et al., 2016). This classification means that recommendations are based primarily on epidemiological or risk factor studies, observational studies, randomized controlled trials with small samples, non-randomized controlled prospective studies or case series, or high-quality retrospective studies.

5.2 Metabolic modulation

The unadaptable stress response with the prolonged energy mobilization promotes dysglycaemia and insulin resistance that in turn alter mitochondrial structure and function generating oxidative stress, and inflammation leading to cellular damage (Picard et al., 2014). This mechanism likely underlies distinguished recently metabolic subtype of MDD (Krupa et al., 2023) and justifies the frequent co-occurrence of type 2 diabetes (TMD2) and depression (Guerrero Fernández de Alba et al., 2020). Insulin resistance has been considered as a factor responsible for more severe depressive symptoms and as a predictor of lack of response to antidepressant drugs. It should be noted that both in the clinics and preclinical studies the insulin-sensitizing interventions improved the effectiveness of antidepressive treatments. The antidepressive mechanism of insulin applied in combined MDD therapy seems to be related to the direct modulatory tone of insulin into serotoninergic and dopaminergic neurotransmission, which is known to be disrupted in patients with insulin resistance (see Krupa et al., 2023).

Cellular metabolic overactivity during exposure to stressor(s) augments the cellular (mainly mitochondrial) production and accumulation of oxygen reactive species (ROS) which in physiological state are neutralized by antioxidant defensive system primarily consisting of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), and glutathione S-transferase (GST) enzymes. Oxidative stress, defined as an imbalance between production and accumulation of ROS in cells and tissues is considered as an important factor implicated in the pathomechanism of MDD (Bell et al., 2024; Lievanos-Ruiz and Fenton-Navarro, 2024; Correia et al., 2023). Recent data, performed by proton magnetic resonance spectroscopy methodology revealed reduced levels of GSH in the occipital region of the cortex of patients with MDD (Bell et al., 2024). Although the precise pattern of depression evoked changes in the level and/or function of the antioxidant defensive system is not clear, strategies reducing the oxidative stress components seem to be effective in the reduction of depressive symptoms in the clinics (Bajpai, 2014; Zhang et al., 2022) and in the preclinical studies (see Correia et al., 2023).

5.3 Ion levels modulation

All biochemical processes necessary for the proper intracellular signal transduction, protein synthesis, activity, and other metabolic pathways require homeostasis of trace elements including zinc (Zn^{2+}), magnesium (Mg^{2+}), calcium (Ca^{2+}), copper (Cu^{2+}), iron (Fe^{2+}), and manganese (Mn^{2+}) ions (Meng et al., 2024). The monitoring of trace elements may serve as a diagnostic panel and provide information about the

effectiveness of antidepressive therapy applied to patients. Clinical observations regarding the serum levels of trace elements in depression patients pointed out decreased Zn2+, and Mg2+ serum levels as potential markers of depression and evidenced the beneficial effects of Zn2+, and Mg2+ supplementation in the treatment or prevention of depression (Szewczyk et al., 2018). Moreover, in the case of TRD, Zn2+ supplementation was shown to augment the efficacy of antidepressant pharmacotherapy (Siwek et al., 2010; Styczeń et al., 2016). Multiple preclinical studies supported clinical observation regarding the role of Zn2+, and Mg2+ deficiency in the expression of depressive-like behaviours (Szewczyk et al., 2018). Regarding the potential antidepressive mechanism of Zn2+, its role in direct activation of metabotropic GPR39 receptor is postulated, and modulation of GPR39 downstream CREB/BDNF/TrkB signalling and related neuroplasticity (Meng et al., 2024). In turn, antidepressive properties of Mg2+ seem to be related mainly to the blockade of the NMDA receptors overactivated in the forebrain in the presence of physical or anticipated stressors (Zelek-Molik et al., 2025). Additionally, Mg2+'s role in the regulation of the HPA axis, inflammation and oxidative stress, regulation of the glutamatergic, 5HT, dopamine and norepinephrine signalling and the sleep-wake cycle has been recognized. Available data suggest that neither depression nor antidepressant treatment affects the Fe2+ and Mn2+ levels. However, the concentration of Ca2+ and Cu2+ in the blood and brain of MDD patients was shown to be upregulated. Moreover, a positive correlation between the patient's severity of depression and Ca2+ and Cu2+ levels was detected (Meng et al., 2024). Typically, the antidepressive treatment with SSRIs was shown to alleviate the augmented level of Cu2+, however, treatment with lithium additionally increased Ca2+ concentration and may lead to hypercalcemia, recognized as a harmful side effect after lithium treatment.

6 Trends in the search for biomarkers to discover more efficient antidepressants

To assess the latest approaches in studying molecular targets and testing antidepressive strategies in preclinical models of depression, we performed a literature search using the PubMed database with keywords such as "depressive-like behavior" and "pharmacotherapy". This search yielded 191 review articles published within the last 5 years up to September 2024. After excluding irrelevant publications, non-English articles, and those restricted by open-access limitations, 64 scientific articles were selected, each describing currently tested targets and potential pharmacotherapies for depression.

Among the selected articles, a significant portion (43 articles) focused on the modulation of neuroinflammation, gut-brain axis activity, and BDNF related neuroplasticity processes in the forebrain. Additionally, ten articles explored the therapeutic potential of bioactive plant compounds, reflecting the growing interest in leveraging the therapeutic properties of plants in medicine.

Below, we summarize the most relevant findings from these reviews and provide references to the literature where readers can find detailed analyses and links to original studies.

6.1 Neuroinflammation

Impaired inflammatory responses have been identified as a significant factor in the pathomechanism of depression (e.g., Wang et al., 2024; Bielawski et al., 2023; Belzeaux et al., 2012). As mentioned above, depression-related neuroinflammation is connected to the excessive metabolic processes and oxidative stress occurrence (e.g., Correia et al., 2023). Treatment-resistant depression (TRD) and depressive-like behavior are associated with glial dysregulation, particularly in the frontal cortex and hippocampus (Sanadgol et al., 2023; Fang et al., 2023; Bansal et al., 2024; Furuyashiki et al., 2019). This dysregulation manifests as elevated levels of proinflammatory cytokines (Il1β, Il6, TNFα) in the blood and brain and overactivation of immune receptors (TNFR1 and TLR-4). These changes lead to the upregulation of the NF-κB pathway, heightened inflammatory responses, and depressive-like symptoms (Sanadgol et al., 2023; Sharma et al., 2024).

Additionally, the synthesis of proinflammatory lipid mediators such as prostaglandins and cysteinyl leukotrienes, linked to abnormal arachidonic acid turnover in the brain, has been observed in depressive-like behavior (Furuyashiki et al., 2019). Available antidepressants are largely ineffective in reducing neuroinflammation in TRD patients (Belzeaux et al., 2012) and in preclinical models (Duda et al., 2017; 2019), emphasizing the need to identify new therapeutic targets for depression-induced neuroinflammation.

One promising approach is targeting the NLRP3 inflammasome and its P2X7 receptor, which regulate the release of proinflammatory cytokines and are under investigation as potential antidepressive pharmacotherapy targets (Mokhtari and Uludag, 2023; Roy et al., 2023; Qi et al., 2023). Animal models have also shown that depressive-like behavior is accompanied by increased activity of fatty acid amide hydrolase (FAAH), an enzyme in the endocannabinoid system. FAAH inhibitors have demonstrated therapeutic potential without the side effects associated with direct CB1 agonists (Rafiei and Kolla, 2021). Furthermore, studies have revealed that increasing omega-3 fatty acid intake or its derivatives, such as resolvins, can attenuate neuroinflammation-related depression (Furuyashiki et al., 2019).

6.2 Gut-brain-axis

The gut microbiota is a key site of neurotransmitter synthesis, such as serotonin (5-HT), norepinephrine (NE), and dopamine (DA), which are impaired in depression (Bhatt et al., 2022). It is widely accepted that depression and depressive behaviours are associated with gut dysbiosis induced by excessive activation of the HPA axis and increased levels of cortisol/corticosterone in the blood. This results in increased intestinal permeability and increased translocation of gut bacteria and their metabolites (including short-chain fatty acids, neurotransmitters, and cytokines) into the circulation (a process also known as "leaky gut") (Halvorson et al., 2024). A leaky gut causes increased inflammation, changes in gut bacteria (including reduced levels of Bifidobacterium and *Lactobacillus*), and accompanying symptoms of depression, which can be alleviated by taking probiotics and prebiotics (Sharma et al., 2024; Sjöstedt et al., 2021). Depression often accompanies irritable

bowel syndrome (IBS), which is directly related to the occurrence of gut dysbiosis, effectively treated with SSRIs. The underlying mechanism of the therapeutic interaction between the microbiota and psychotic treatment is not clear, however. In contrast to the aforementioned therapeutic effects observed in SSRI-treated IBS, preclinical evidence shows that the metabolic disturbances observed with olanzapine are due to the drug-microbiota interaction, as the use of antibiotics that eliminate gut bacteria reversed the adverse side effects of olanzapine (Sjöstedt et al., 2021). Antidepressant therapy can also affect the composition of the microbiota. For example, MAO-A inhibitors (iproniazid) or SSRIs (sertraline, fluoxetine, paroxetine) have been shown to act like antibiotics, leading to microbial dysbiosis, which is associated with weight gain reported as a side effect after long-term SSRI use. In turn, vortioxetine, a 5-HT receptor modulator and SERT inhibitor (Sanchez et al., 2015) seems to exert its antidepressant effects by regulating the gut microbiota in patients with MDD (Ye et al., 2021). Understanding the precise mechanism linking dysbiosis with depression and dysbiosis induced by some antidepressants seems important for the development of improved antidepressants.

Currently, it is evidenced that gut microbiome communicates with the brain through the gut-brain axis, regulating stress responses, inflammation, and emotions (Guerrero-Hreins et al., 2021; Pinna, 2023). Glucagon-like peptide 1 (GLP-1), released from the colon, reaches the brain via the vagal afferent pathway and activates GLP-1 receptors (GLP-1R), which are widely expressed in limbic structures. This pathway is implicated in regulating feeding and emotional behaviors.

Clinical observations suggest that pharmacotherapy with GLP-1R agonists may induce demotivation and anhedonia with prolonged use (Dumiaty et al., 2024; Detka and Głombik, 2021). Preclinical studies have recently highlighted reelin, an extracellular matrix protein expressed in the brain and intestines, as a promising antidepressive compound (Halvorson et al., 2024). In the brain, reelin supports synaptic plasticity, while in the intestines, it interacts with receptors such as VLDLR, ApoER2, EphB2, and α3β1-integrins. Similarly to ketamine, reelin was shown to activate mTORC1, without NMDA receptors antagonism, suggesting that rescuing reelin level may offer rapid antidepressant effects avoiding the side (dissociative) effects related to therapy with ketamine (Johnston et al., 2023).

Epigenetic modifications of the gut microbiome and its metabolites, induced by diet, prebiotics, probiotics, specific antibiotics, fecal microbiota transplantation, or antidepressants, may have beneficial antidepressive effects (Nohesara et al., 2023). For example, augmentation of allopregnanolone (AP) signaling through PPAR-α (peroxisome proliferator-activated receptor α) in the colon has been shown to reduce gut dysbiosis and depressive symptoms (Pinna, 2023). Similarly, gastrointestinal peptides like ghrelin have emerged as potential targets for modulating dysbiosis-related depressive-like behavior (Gajewska et al., 2023).

6.3 Neuroplasticity

Dysregulation of neural plasticity is a well-established factor implicated in the pathophysiology of depression and is characterized by hypoactivation, synaptic loss and weakening, reduced connectivity, and decreased levels of neurotrophic factors (reviewed in Matar et al., 2024). Brain-derived neurotrophic factor (BDNF) is considered a critical mediator between antidepressant activity and the neuroplastic changes responsible for therapeutic effects. Impaired BDNF-TrkB signalling and its regulatory influence on NMDA and AMPA receptors in the forebrain leads to structural and functional changes in neurons and the development of psychiatric diseases, including depression (McGinty et al., 2015; Sun et al., 2014; Sharma et al., 2024; Zelek-Molik et al., 2021). Glutamatergic modulation of AMPA and NMDA receptors on cortical pyramidal neurons leads to upregulation of BDNF expression. Through this mechanism, glutamate release and AMPA receptor activation enhance BDNF binding to TrkB. This activates mTOR and ERK signalling, intensifying the expression of neuroplasticity-related genes, protein synthesis of synaptic components, and mechanisms that result in rapid and long-lasting local synaptogenesis (Pochwat et al., 2022).

Antidepressants enhance neuroplasticity through several pathways. By modulating the TrkB receptor and increasing the excitability of pyramidal neurons, these treatments amplify excitatory transmission. Receptor activity of antidepressants influences mTORC1 through serotonin transporters (as seen in classical antidepressants), NMDA, mGluR2, or AMPA receptors (in rapid-acting antidepressants like ketamine and its metabolites), 5HT2A/5HT1A receptors (as in psychedelics such as psilocin or lysergic acid diethylamide), or even through heterodimer formation, which potentiates neuroplasticity effects between receptor types (Brunello et al., 2024; Ilchibaeva et al., 2022). Further, downstream effectors including mTORC1, ERK, CREB, and elongation factor 2 (EF2) mediate intracellular effects of antidepressants by inducing plasticity-related genes and BDNF expression. These neuroplasticity-driven processes significantly reduce depressive symptoms by promoting synaptic remodelling and enhanced connectivity (Page et al., 2024; Moliner et al., 2023; Casarotto et al., 2021; Saarelainen et al., 2003). Although BDNF does not cross the blood-brain barrier (BBB), its levels in the brain have been shown to increase through physical exercise. This effect is mediated by the myokine irisin, a protein upregulated in muscle during exercise, which is cleaved from its precursor (FNDC5), secreted into circulation, and capable of crossing the BBB to stimulate BDNF synthesis in the hippocampus. This process supports neuroplasticity and related therapeutic outcomes (Colucci-D'Amato et al., 2020; Liu et al., 2024).

BDNF exerts its effects not only through high-affinity binding to TrkB receptors but also through low-affinity interactions with the neurotrophin receptor p75NTR and its co-receptor sortilin. These interactions activate a wide range of intracellular signalling pathways involved in cytoskeletal remodelling and plasticity. Recently identified downstream effectors of BDNF signalling that may serve as potential targets for new antidepressant therapies include non-receptor tyrosine kinases such as SFK/JAK/FAK (Wang et al., 2022) and FYN (Zelek-Molik et al., 2025); lysophosphatidic acid (LPA) and its receptors (Li and Li, 2024); the small GTPase RhoA (Rafa-Zabłocka et al., 2021); and adhesion G protein-coupled receptor GPR65 (Qi and Guan, 2024).

6.4 Pleiotropic antidepressive activity of plant compounds

Recent studies have highlighted the antidepressant potential of various phytochemicals, including gintonin (Kim H-J et al., 2017), ferulic acid (Dong and Zhao, 2023), ginsenoside Rg1 (Yang et al., 2022), flavonols (Jazvinšćak Jembrek et al., 2023), flavonoids (German-Ponciano et al., 2022; Ko et al., 2020), mitragynine (Johnson et al., 2020), and terpenoids such as linalool, linalyl acetate, geraniol, citronellol, and limonene (Muller et al., 2021; Agatonovic-Kustrin et al., 2020). While the precise mechanisms of antidepressant action and their clinical usefulness require further research, it has been observed that their effects often combine anti-inflammatory properties, improvements in brain-gut axis function, and positive effects on neuronal plasticity.

The anti-inflammatory effects of plant compounds have been linked to downregulation of the proinflammatory MAPK and NF-κB pathways (Wang et al., 2024; Dong and Zhao, 2023), upregulation of the heme oxygenase (HO) system via nuclear transcription factor erythroid 2-related factor 2 (Nrf2) (Wang et al., 2024), inhibition of MAO-A activity in the forebrain, and suppression of microglial activation and proinflammatory cytokine release (Dong and Zhao, 2023).

In addition to their anti-inflammatory properties, active plant compounds like ferulic acid, ginsenoside Rg1, and peptides from soybeans, leaves, and grains (e.g., pEL, rubimetide, and soy-deprestatin) have demonstrated antianxiety and antidepressant effects via gut-brain regulation (Dong and Zhao, 2023; Yang et al., 2022; Mizushige, 2021). These peptides are thought to activate serotonin, dopamine, and GABA systems in the brain through their interaction with the gastrointestinal tract and vagus nerves. Ferulic acid and ginsenoside Rg1 have also been shown to reduce blood corticosterone levels and brain glycogen, as well as alleviate dysbiosis in the colon by modulating microbiome composition and microbial metabolism.

Regarding their effects on impaired neuroplasticity in limbic structures, flavonoids have been found to exhibit antidepressant properties by increasing BDNF and 5-HT levels in these regions (German-Ponciano et al., 2022). Ginsenoside Rg1, ginkgolide A, curcumin, cannabidiol, lavender oil, extracts from Hericium erinaceus mushrooms, and other traditional African medicinal plants have been shown to regulate BDNF/cAMP/CREB signaling in the forebrain (Yang et al., 2022; Gutiérrez-Rodelo et al., 2023; Muller et al., 2021). Lavender oil, in particular, enhances dendritic complexity in primary neurons compared to corticosterone-treated neurons. Interestingly, lavender oil exerts its antidepressant effects without binding to monoamine transporters, neurotransmitter receptors, or GABAergic properties. Its mechanism includes moderate inhibition of voltage-dependent calcium channes (VDCC), which differentiates it from classical antidepressants (Muller et al., 2021).

7 Discussion

As discussed above, antidepressive pharmacotherapies are primarily focused on monoaminergic and glutamatergic neurotransmission, which have been shown to influence neuroplasticity in brain limbic structures and mitigate depressive symptoms. However, the clinical utility of currently available antidepressants is limited by insufficient effectiveness and numerous side effects in patients. This highlight the necessity to identify new molecular targets involved in the pathogenesis of depression and to discover compounds with greater therapeutic potential than those offered by current pharmacotherapy.

Animal models provide a unique opportunity to evaluate the effects of potential drugs in the context of a wide range of biological and behavioural actions and play a crucial role in drug development, as well as being used to determine drug efficacy and predict antidepressant efficacy. Rodent models of depression that satisfy critical criteria of face, predictive, and construct validity are most often based on acute, chronic, or social stress paradigms, or genetic predispositions (Duman, 2010; Willner et al., 2019). Traditionally, animal models have been employed to assess drug efficacy and predict antidepressant potential. However, the increasing global burden of TRD requires the development and validation of new preclinical models tailored to this disease.

The Lancet-World Psychiatric Association Commission on Depression (2022) has emphasised that the development and testing of therapies targeting early intervention and novel disease mechanisms is a critical priority. In this regard, variants of the diathesis-stress animal model, also referred to as the vulnerability-stress model, are consistent with these recommendations. The diathesis-stress model posits that an individual predisposed to vulnerability (biological or psychological) may develop depression following exposure to an acute stressor. This vulnerability may encompass biological factors (e.g., genetic predispositions, endocrine imbalances, inflammatory responses, or altered brain connectivity) or psychological traits (e.g., temperament, personality, past experiences, or beliefs) (Monroe and Simons, 1991). These elements are increasingly utilized in animal models with high translational potential to generate novel, rapid-acting antidepressant drug candidates (Papp and Willner, 2023; Papp et al., 2024).

Vulnerability factors stemming from early life stress, social stressors, genetics, or experimental manipulations contribute to the development of depression-like symptoms in animal models. These conditions are associated with alterations in energy metabolism, hypothalamic-pituitary-adrenal (HPA) axis dysfunction, genetic factors, and behavioral disturbances, all of which are relevant to the pathophysiology of depression in humans (Virijevic et al., 2024; Brivio et al., 2022; Willner and Belzung, 2015). While animal models cannot fully replicate the complexity of human depressive disorders, they successfully simulate core symptoms of depression, elucidate underlying mechanisms, and allow the testing of specific hypotheses regarding novel molecular targets. Moreover, animal models are critical for evaluating the potential of newly discovered mechanisms and for assessing the efficacy of drug candidates. Although limitations exist, these models remain invaluable for advancing our understanding of depression and the development of innovative therapies.

In their 2022 review, Borbély et al. highlighted that many phase II clinical trials focus on the effects of drug candidates in treatment-resistant depression (TRD), emphasizing the urgent need for therapies with efficacy in this challenging population. This trend

is mirrored in preclinical studies, where antidepressant candidates are often tested in TRD models. Demonstrating efficacy in such models significantly increases the likelihood of success in treating mood disorders. It appears that neuroplasticity-based mechanisms, such as TrkB dimerization and BDNF binding, likely represent a common pathway for many antidepressant therapies, underscoring the essential role of plasticity mechanisms in the long-term efficacy of pharmacotherapy. Understanding and enhancing these mechanisms may be key to improving antidepressant outcomes.

Interestingly, the mechanisms of action of many novel antidepressant drugs have shifted focus to the glutamatergic-GABAergic interplay rather than traditional monoaminergic neurotransmitter systems. New drug candidates include GABA-A potentiators, NMDA receptor antagonists or modulators, and mGlu2/3 receptor modulators. Psilocybin formulations and synthetic psychedelics targeting 5-HT2A receptor agonism are also being explored; however, concerns regarding their safety, including potential risks of addiction and other adverse effects, remain a critical area of debate.

Evidence supports the efficacy of rapid-acting therapies in providing a rapid reduction in depressive symptoms, which could improve patients' quality of life and reduce healthcare costs. However, no glutamatergic modulator tested to date has matched the rapid, robust and sustained antidepressant effects of (R,S)-ketamine and esketamine. In addition, the breadth of therapeutic effects of ketamine, such as its antisuicidal properties, anti-anhedonic effects or broader therapeutic potential, remains unmatched. The investigation of novel compounds for these specific indications remains a high priority.

7.1 Challenges and questions for future research

One of the significant challenges in developing new antidepressants is the heterogeneity of mood disorders. Despite significant progress, the pathophysiology of depression remains idiopathic and incompletely understood, complicating efforts to identify universally effective treatments.

Key questions for future research include:

Neuroplasticity and Treatment Response: How can neuroplasticity be effectively enhanced to improve treatment outcomes? What are the most promising molecular targets to stimulate plasticity mechanisms?

Mechanistic Contributions: How do intracellular signaling pathways, network activity, neuroinflammatory and neuroendocrine effects, early-life factors, and the microbiota system contribute to the clinical efficacy of antidepressants?

Combination Therapies: What are the additive or synergistic effects of pharmacotherapy when combined with psychotherapy or other interventions, and how can these combinations be optimized?

Addressing these questions will require an integrated approach, combining insights from neurobiology, pharmacology, genetics, and clinical practice. Continued exploration of novel compounds, combined therapies, and personalized treatment strategies, offers the best path forward to advancing the treatment of depression and TRD.

Author contributions

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Conflict of interest

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Ferroptosis, pathogenesis and therapy in AS co-depression disease

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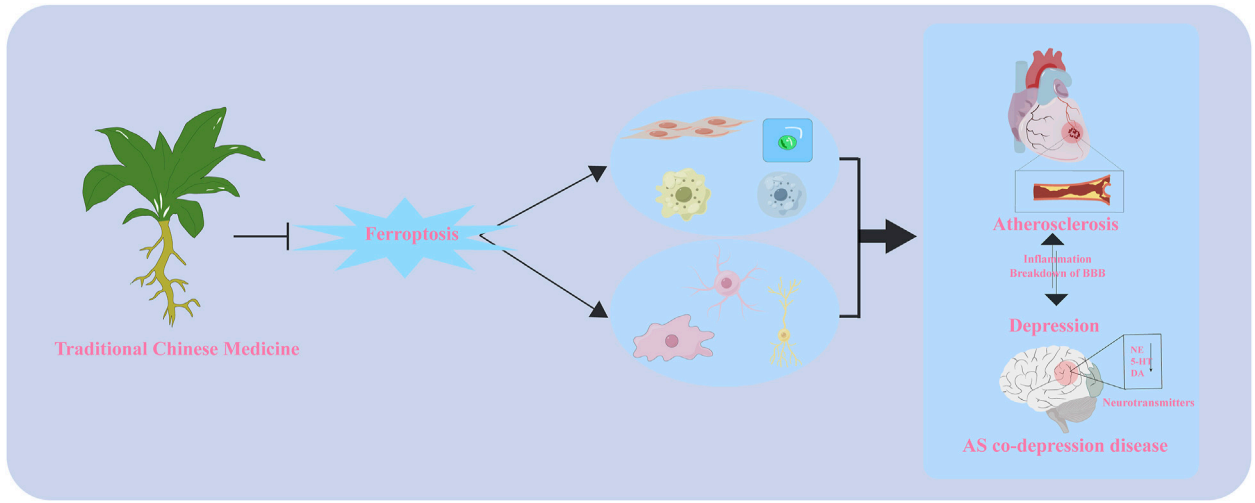
Atherosclerosis (AS)-related cardiovascular disease and depression are often comorbid, with patients with cardiovascular disease facing an increased risk of depression, which worsens AS. Both diseases are characterized by oxidative stress and lipid metabolism disorders. Ferroptosis, a form of cell death characterized by iron overload and harmful lipid peroxide accumulation, is found in various diseases, including AS and depression. Consistent with the iron deposition and lipid peroxidation (LPO) that characterize the ferroptosis mechanism, disturbances in iron and lipid metabolism are also crucial pathogenic mechanisms in AS and depression. The comorbid mechanisms are complex, posing challenges for clinical treatment. Chinese herbs hold significant potential owing to their multi-target pharmacological effects. Therefore, this review aims to investigate iron overload, LPO, and ferroptosis across various cell types, the shared pathogenesis of AS and depression with ferroptosis, and research on Chinese herbal medicine targeting ferroptosis in the treatment of anti-AS co-depression. This provides a comprehensive understanding of AS co-depression disease from the perspective of ferroptosis.

KEYWORDS

ferroptosis, atherosclerosis, depression, comorbid, pathogenesis, therapy

1 Introduction

Atherosclerosis (AS), the pathological basis of cardiovascular disease, is a threat to human life and health. According to the World Health Organization, cardiovascular disease accounts for up to 17.9 million deaths annually, representing approximately 30% of global mortality (Herrington et al., 2016; Tsao et al., 2023). AS is a chronic inflammatory condition characterized by endothelial cell damage, lipid accumulation within arterial walls, smooth muscle cell growth, and foam cell formation. Depression, a mood disorder characterized primarily by persistent feelings of sadness and anhedonia, arises from diverse causes. It has become a significant public health concern, with a global prevalence rate of 4.4% (Monroe and Harkness, 2022; WHO, 2017). Extensive research has established a strong relationship between depression and cardiovascular disease (Hare et al., 2014; Kawada, 2017; Li C. et al., 2020). Meta-analyses highlight the profound detrimental effect of depression on the development and outcomes of cardiovascular disease (Krittanawong et al., 2023). Similarly, with a co-prevalence of 17%–47%, depression is highly prevalent among individuals with cardiovascular disease (Mavrides and Nemeroff, 2013). Data from a



GRAPHICAL ABSTRACT

recent U.S. health survey reveals a significant positive connection between adult depression and plasma AS index (Ye et al., 2024). Furthermore, evidence from multi-omics studies (Huang et al., 2023), Mendelian randomization analyses (Lei et al., 2023; Shakt et al., 2024), and the vascular depression hypothesis (Alexopoulos et al., 1997) underscores the close relationship between AS and depression. Despite growing evidence that they occur simultaneously and affect each other, the mechanisms linking AS and depression are still poorly understood, which complicates effective treatment strategies.

The interaction mechanism between AS and depression is highly complex, involving multiple potential mechanisms, including inflammation (Chrysohoou et al., 2018; Halaris, 2017), hypothalamic-pituitary-adrenal axis dysfunction (Gu et al., 2012), endothelial dysfunction (Chrysohoou et al., 2018; Paranthaman et al., 2012), immune responses (Mousa et al., 2022), autonomic nervous system dysfunction (Pizzi et al., 2010), and intestinal flora and metabolite dysfunction (Liao et al., 2023). Lipid metabolism disorders play a crucial role, with numerous studies confirming alterations in lipid composition in the plasma and brain of patients with AS and depression. High-fat diets disrupt brain lipid metabolism and promote neuroinflammation, increasing depression risk (Wang A. et al., 2023). Mendelian randomization analysis reveals a causal connection between altered triglyceride, cholesterol levels, and depressive phenotypes (So et al., 2021). Elevated oxidized low-density lipoprotein (Ox-LDL) levels contribute to the development of major depression by inducing neuroinflammation (Almulla et al., 2023).

Additionally, a mouse model of AS-related depression was developed using APOE$^{-/-}$ mice to investigate the underlying pathogenesis. Differential phosphatidylethanolamine (PE) levels in the hippocampus and prefrontal cortex of the model mice are closely linked to ferroptosis (Hu et al., 2022). PE, the second most abundant phospholipid in living organisms, plays a crucial role in depression. Disruptions in PE metabolism can impair mitochondrial activity in the brain, contributing to depressive symptoms (Modica-Napolitano and Renshaw, 2004). Furthermore, individuals with seasonal depression exhibit altered plasma PE levels (Otoki et al., 2017). The role of ferroptosis in AS has also been extensively demonstrated (Fang et al., 2023). Stockwell further highlights its significance in cardiovascular and neurodegenerative diseases (Stockwell, 2022). Therefore, the study of AS co-depression diseases from the perspective of ferroptosis has a sufficient theoretical and experimental basis.

2 Method

This review aims to summarize the mechanism of ferroptosis in the pathogenesis of atherosclerotic comorbid depression and to highlight the research progress on the use of Chinese medicine in treating this condition by targeting ferroptosis. Keywords included the following: “ferroptosis,” “iron death,” “atherosclerosis,” “depression,” “cardiovascular and cerebrovascular disease,” “major depressive disorder,” and “Traditional Chinese medicine.” Searches were conducted using multiple online databases, including Google Scholar, PubMed, Scopus, Embase, Web of Science, and ScienceDirect, for English-language journal reports and articles published up to October 2024. References were manually screened from the extracted articles.

3 Overview of ferroptosis

Cell death is fundamental to organismal growth, development, senescence, and death, involving mechanisms such as apoptosis, necrotic apoptosis, autophagy, cellular pyroptosis, and necrosis, each with distinct characteristics (Moujalled et al., 2021). Ferroptosis, a regulated form of cell death distinct from apoptosis, was introduced by Dixon in 2012 (Dixon et al., 2012) and defined by the Cell Death Commission in 2018 (Galluzzi et al., 2018). The condition is characterized by redox imbalance and iron overload, with affected cells exhibiting smaller mitochondria, increased membrane density, and reduced cristae, while the nucleus shows minimal changes (Li J. et al., 2020). Cellular alterations include increased lipid peroxides, elevated reactive

oxygen species (ROS), and reduced Glutathione Peroxidase 4 (GPX4) levels (Chen et al., 2021b). Ferroptosis affects tumor development and various disorders, including neurological, ischemic, and cardiovascular conditions (Fang et al., 2023) and depression (Chen et al., 2023). Furthermore, ferroptosis is regulated by various processes and small molecules, including iron metabolism, lipid peroxidation (LPO), the XC-GSH-GPX4 axis, ferroptosis suppressor protein 1 (FSP1), coenzyme Q10 (CoQ10)-NAD(P)H pathway, guanosine triphosphate cycloheximide hydrolase 1, tetrahydrobiopterin (BH4)-dihydrofolate reductase pathway, autophagy, mitochondrial function, among others (Chen et al., 2021a; Song Y. H. et al., 2023).

4 Main mechanisms of ferroptosis

Ferroptosis primarily arises from the excessive accumulation of ROS on the cell membrane owing to metabolic dysfunction. Lipid peroxides and their metabolites, such as malondialdehyde (MDA) and 4-hydroxynonenal, damage proteins, membrane integrity, and DNA structure, leading to cell membrane hyperpermeability and ferroptosis (Lei et al., 2022). Lipid peroxide accumulation results from enzymatic and non-enzymatic processes (Rochette et al., 2022). Polyunsaturated fatty acids (PUFA) are converted into reactive lipid peroxides by fatty acid enzymes and through the Fenton reaction, which is driven by iron accumulation from disrupted iron metabolism.

4.1 Enzymatic pathway of ferroptosis

4.1.1 ACSL4/LPCAT3/LOX lipid metabolism pathway

Among the three families of lipid oxidases, lipoxygenase is crucial to the enzymatic processes driving ferroptosis. The other two families are cyclooxygenase and cytochrome P450. Lipoxygenase converts free PUFA into lipid oxides, which significantly affects iron redox reactions (Yang et al., 2016). PUFA oxidation-specific nonheme iron-containing enzymes are known as LOXs (Maheshwari, 2023). PE, derived from adrenoic acid (AdA) and arachidonic acid (AA), are key substrates for LPO. Metabolic abnormalities in PE directly influence lipid metabolic pathways involved in ferroptosis (Kagan et al., 2017).

The principal cause of ferroptosis is linked to the peroxidation of PUFAs (Gan, 2022) and PE (Amoscato et al., 2023), while monounsaturated fatty acids inhibit ferroptosis. Ferroptosis is caused by PE-AA/AdA-OOH, not by other phospholipid (PL)-OOH forms (Lei et al., 2019). The oxidation process of PE requires the involvement of ACSL4 (He et al., 2022) and LPCAT3 (Miura et al., 2021). PUFAs, particularly AA and adrenergic acid, are susceptible to oxidation owing to the weak C-H bonds in their diallyl group (Kagan et al., 2017). The LPO process proceeds as follows: Free AA/ADA is catalyzed by ACSL4 to form AA/AdA-CoA derivatives (Zhang H. L. et al., 2022). These AA/AdA-CoA derivatives are then synthesized with PE in the cell membrane by LPCAT3, producing AA/AdA-PE (Doll and Conrad, 2017). Finally, AA/AdA-PE undergoes oxidation through enzymatic and non-enzymatic pathways. The enzymatic pathway involves LOXs, while the non-enzymatic pathway is driven by hydroxyl radicals generated through the Fenton reaction. This ultimately leads to harmful lipid peroxide formation (Wang et al., 2021c).

The LPO process highlights ACSL4, LPCAT3, and LOXs as important targets for regulating ferroptosis. For instance, berberine inhibits endothelial ferroptosis and AS by suppressing ACSL4 expression (Hong et al., 2024). Xiaoyao San mitigates lipid metabolism disorder and depression in mice by inhibiting ACSL4 (Jiao et al., 2021). Myeloid LPCAT3 deficiency disrupts AA homeostasis in the liver, leading to metabolic imbalances and triglyceride accumulation (Bourgeois et al., 2021). Additionally, LOX inhibition reduces neuroinflammation and LPO by inhibiting inflammasome activation (Cakir-Aktas et al., 2023).

4.1.2 System Xc -/GSH/GPX4 pathway

Ferroptosis is mainly induced by inhibiting glutathione production, with the System Xc -/GSH/GPX4 pathway playing a key role in preventing LPO. The Xc-system facilitates intracellular cysteine (Cys) absorption, which is converted into glutathione. While the Xc-system is considered the primary source of Cys, its inhibition can also lead to Cys production through the sulfur transfer pathway. Both pathways help maintain intracellular Cys levels. However, Xc-system inhibition results in glutathione and Cys deficiencies, triggering ferroptosis (Li F. J. et al., 2022). This underscores the pivotal role of the Xc-system in preserving intracellular redox balance.

GPX4, also known as phospholipid hydroperoxide glutathione peroxidase, consists of approximately 170 amino acids and has a molecular weight of about 19 kDa. Numerous GPX family members, including GPX1–GPX8, are found in mammals (Nishida Xavier da Silva et al., 2022). However, only GPX4 scavenges membrane lipid hydroperoxides. This feature is attributed to its distinct amino acid composition and spatial arrangement. GPX4 utilizes reduced GSH as a critical substrate and oxidizes it to GSSH while converting PLOOH to fatty alcohol to suppress the LPO process (Liu Y. et al., 2023). Targeted metabolomics reveals that GPX4 overexpression and knockdown influenced the lethality of 12 ferroptosis inducers, highlighting the role of Gpx4 in ferroptosis regulation (Lou et al., 2024). RAS-selective lethal 3 (RSL3) inhibits GPX4 by covalently binding to it, leading to lipid peroxide accumulation and ferroptosis induction. Glutathione deprivation converts GPX4-catalyzed peroxides to alcohols, leading to Cys deficiency, further inactivating GPX4 and promoting ferroptosis (Ursini and Maiorino, 2020). Targeting GPX4 can improve AS (Yang et al., 2024b) and depression (Qian et al., 2023).

4.1.3 Ferroptosis suppressor protein 1(FSP1) -coenzyme Q10 (CoQ10)-NADPH pathway

The FSP1-CoQ10-NAD(P)H pathway is crucial in ferroptosis prevention, alongside the GPX4 pathway, the main defense mechanism. Apoptosis-inducing factor mitochondria-associated 2 (AIFM2), formerly FSP1, mitigates ferroptosis owing to GPX4 deletion. Ubiquinone (CoQ10) mediates this inhibition. FSP1 uses NAD(P)H, while the reduced form of ubiquinone captures lipid peroxyl radicals that cause LPO. Pharmacologically targeting FSP1 induces ferroptosis in various cancers, synergizing effectively with GPX4 inhibitors (Koppula et al., 2022). The FSP1-

CoQ10-NAD(P)H pathway, alongside glutathione and GPX4, functions as an independent parallel mechanism to mitigate ferroptosis and PL peroxidation. In the absence of GPX4, FSP1 effectively inhibits PL peroxidation and ferroptosis by using NAD(P)H to convert oxidized ubiquinone into ubiquinol (Doll et al., 2019).

In this pathway, CoQ10 is crucial, and the inhibition of its synthesis leads to increased LPO (Arslanbaeva et al., 2022). CoQ10 is primarily synthesized in the mitochondria, though its exact origin remains unclear. In the absence of GPX4 activity, dihydroacetate dehydrogenase inhibits ferroptosis in mitochondria by enhancing CoQH2 production (Mao et al., 2021). Moreover, BH4 prevents ferroptosis by producing CoQH2, inhibiting LPO, and scavenging free radicals (Zhang G. et al., 2023). Targeting FSP1 can improve AS (Xie et al., 2022) and depression (Wu X. et al., 2024).

4.1.4 Mevalonate (MVA) pathway

The MVA pathway is a key mechanism for inhibiting ferroptosis through interactions with the GSH-GPX4 and FSP1-CoQ10-NADPH pathways. It generates isoprenoid chemicals from acetyl coenzyme A. Acetyl coenzyme A condenses to form 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), which is then converted to MVA by HMG-CoA reductase. MVA is further metabolized into isoprenoid compounds such as gibberellin A, cholesterol, and isopentenyl pyrophosphate (IPP) (Lasunción et al., 2022). IPP is an intermediary molecule involved in ferroptosis regulation. Farnesyl phosphate synthetase converts IPP to farnesyl pyrophosphate (FPP), which is then converted into squalene by squalene synthase (SQS) and cyclized by squalene cyclase to produce cholesterol (Liang et al., 2023). IPP is crucial for synthesizing various biomolecules and, along with FPP, produces non-cholesterol compounds such as CoQ10 through the GGPS enzyme, which SQS inhibits (Chen et al., 2020). FIN56 induces ferroptosis by stimulating SQS and decreasing COQ10 production (Shimada et al., 2016).

Additionally, the MVA pathway is vital for GPX4 synthesis. Selenocysteine (Sec) must be incorporated into its catalytic center to enable its antioxidant function. This process requires IPP to activate isoprenyltransferase, which catalyzes the mutation of the Sec transporter RNA, facilitating GPX4 activity and maintenance (Jia et al., 2024). Consequently, FIN56 inhibits Sec integration into the catalytic subunit of GPX4, reducing its antioxidant capacity (Costa et al., 2023).

4.2 Non-enzymatic pathway of ferroptosis

4.2.1 Iron homeostasis

Iron is crucial for living organisms, and its deficiency can cause health issues. Intracellular iron is stored in two major forms—an unstable intracellular free ferrous iron pool and an inert form bound to ferritin (Fuhrmann and Brüne, 2022). Ferrous and ferric iron are forms of free iron, and excess Fe^{2+} can trigger the Fenton reaction, producing ROS (Bogdan et al., 2016) that induce ferroptosis and LPO. The importance of iron homeostasis is evident as the iron chelator deferoxamine effectively suppresses ferroptosis induced by intracellular iron overload (Costa et al., 2023). Additionally, iron is a cofactor for enzymes such as cytochrome P450 oxidoreductase and lipoxygenase, which synthesize lipid peroxides. Iron absorption, efflux, and cellular availability are crucial for ferroptosis.

4.2.2 Iron metabolism

Transferrin-bound trivalent iron binds to the transferrin receptor (TFR1), allowing direct cellular entry through endocytosis and converts it to ferrous iron through STEAP3. Ferrous iron is released by divalent metal transporter 1 (DMT1) and recycled into the cell membrane through TFR1 within the cellular labile iron pool. TFR1 expression is regulated by five iron-regulatory elements (IREs) in its 3′untranslated region. Under iron deficiency, iron-regulatory proteins (IRPs) bind to IREs to enhance TFR1 expression (Wang S. et al., 2024). Redox-active iron complexes within the unstable iron pool are stored in cells bound to ferritin, composed of light and heavy chain components. Ferritin stores free unstable iron. Nuclear receptor coactivator 4 (NCOA4) enhances free iron levels by binding to ferritin-heavy chains and aiding its transport through the lysosome. Moreover, iron is exported through PROM2-mediated exocytosis or the FPN1/SLC40A1 efflux pump, forming multivesicular aggregates.

4.2.3 Regulation of ferroptosis by iron metabolism

Ferroptosis can be inhibited by reducing iron uptake and using iron chelators. Conversely, transferrin removal inhibits ferroptosis, TFR1 upregulation increases susceptibility, and IRP2 inhibition confers cellular resistance (Jiang et al., 2021). Hepcidin, a liver-derived antimicrobial peptide, negatively regulates FPN1, leading to tissue iron overload and promoting ferroptosis, underscoring its crucial role in iron metabolism regulation. Typically, transferrin-bound iron predominates, but non-transferrin-bound iron (NTBI) can emerge in plasma during iron overload (Galy et al., 2024). NTBI comprises low molecular weight iron forms that can be absorbed and damage organs, unlike transferrin-bound iron. In summary, LPO pathways depend on intracellular iron metabolism, with disruptions leading to ferroptosis. The pathways and regulatory mechanisms of ferroptosis are outlined. (Figure 1). Specific types of cell ferroptosis play a crucial role in AS co-depression diseases.

5 Multiple cell ferroptosis promotes atherosclerosis

5.1 Ferroptosis of vascular endothelial cells promotes atherosclerosis

AS begins with vascular endothelial dysfunction, characterized by increased permeability, leukocyte adhesion, heightened inflammation, and accelerated plaque and thrombus formation. The negative effects of ferroptosis in endothelial cells during AS are well established. Bai et al. demonstrated that Ox-LDL, a potent pro-atherosclerotic factor, induces ferroptosis in mouse aortic endothelial cells by increasing iron deposition, lipid peroxides, and ROS while decreasing GPX4 levels. High-fat-fed $ApoE^{-/-}$ mice exhibit larger atherosclerotic plaques, severe ferroptosis, increased NOX production, and reduced GSH and xCT expression in the thoracic aorta compared to wild-type mice (Bai et al., 2020). Li et al. showed that injecting endothelial progenitor cell-derived extracellular vesicles containing miR-199a-3p into high-

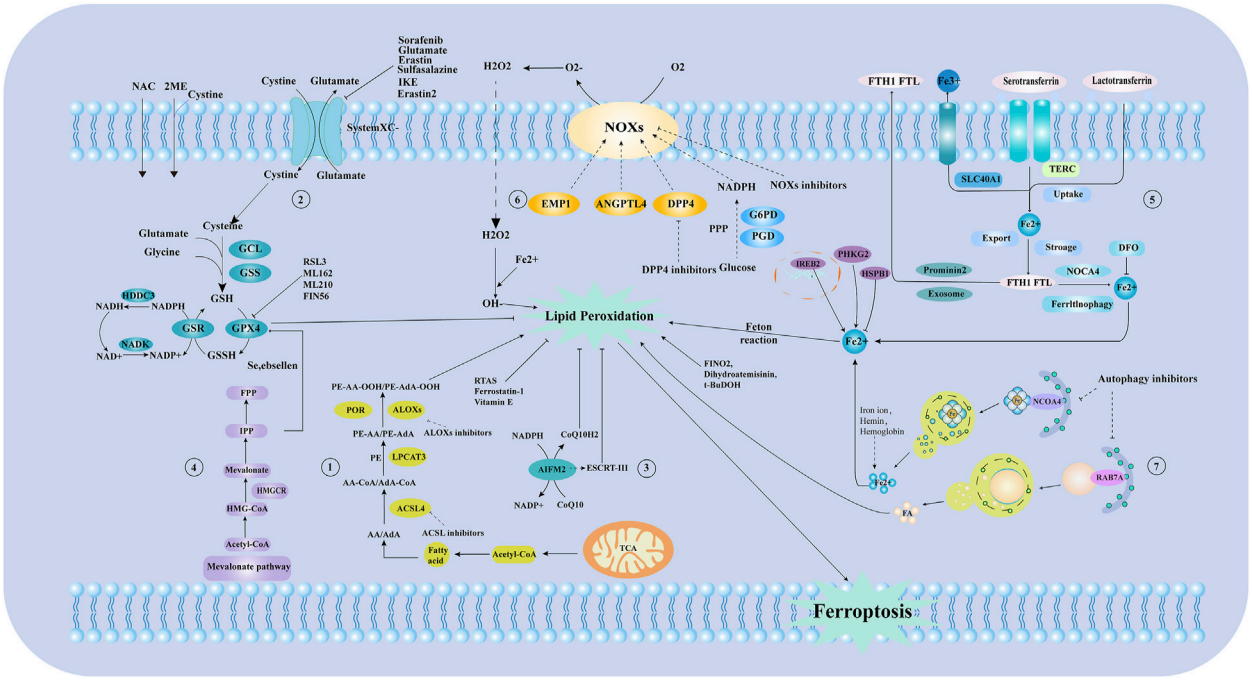


FIGURE 1
The mechanism of ferroptosis and iron metabolism. ① Lipid metabolic pathways. ② Xc-glutathione/glutathione peroxidase pathway. ③ Ferroptosis suppressor protein 1(FSP1) -Coenzyme Q10 (CoQ10)-NADPH pathway. ④ Mevalonate (MVA) pathway. ⑤ Process of iron metabolism. ⑥ Reactive oxygen species, free radical generation process. ⑦ The process of ferritin autophagy. NAC:N-Acetyl-L-cysteine; GCL: Glutamate-Cysteine Ligase; GSS: Glutathione Synthetase; GSH: Glutathione; GPX4: Glutathione Peroxidase 4; NADP: nicotinamide adenine dinucleotide phosphate; NAD: nicotinamide adenine dinucleotide; IPP: Isopentenyl pyrophosphate; FPP: Farnesyl pyrophosphate; HMG-COA:3-hydroxy-3-methyl glutaryl coenzyme A reductase; Acetl-CoA: Acetoacetyl-CoA; AA/AdA: Arachidonic acid/Adrenic acid; ACSL4:long-chain acyl-CoA synthetase 4; LPCAT3: Lysophosphatidylcholine acyltransferase 3; PE: Phosphatidyl ethanolamine; ALOXS: Arachidonate lipoxygenase; TCA: Tricarboxylic Acid Cycle; AIFM2/FSP1:Ferroptosis suppressor protein 1; EMP1: EPO mimetic peptide-1; ANGPTL4: Angiopoietin-Like Protein 4; DPP4: Dipeptidyl peptidase-4; G6PD: Glucose-6-phosphate dehydrogenase; PGD: Phosphogluconate Dehydrogenase; FTH1 FTL: Ferritin Heavy Chain 1, Ferritin Light Chain; TERC: Telomerase RNA Component; NOCA4: Nuclear Receptor Coactivator 4; DFO: Deferoxamine; RSL3: GSH peroxidase 4 inhibitors; HDDC3: HD Domain Containing 3; NADK: Nicotinamide adenine dinucleotide (NAD+) kinase; HMGCR: 3-hydroxy-3-methylglutaryl coA reductase; GSR: glutathione-disulfide reductase; 2 ME: 2-Methoxyestradiol; T-BuDOH: t-Butyl hydroperoxide; RAB7A:ras-related protein Rab-7a; ESCRT-III: Endosomal sorting complex required for transport III; CoQ10H2: Reduced coenzyme Q10; NOXs: NADPH Oxidases; SLC40A1: Solute carrier family 40 member 1; IREB2: Iron-Responsive Element Binding Protein 2; PHKG2: Phosphorylase Kinase Catalytic Subunit Gamma 2; HSPB1:Heat shock protein beta-1; PPP: pentose phosphate pathway; POR: cytochrome p450 oxidoreductase; CoQ10: Coenzyme Q10; System Xc -: Cystine/glutamate antiporter system; NCOA4: Nuclear receptor coactivator 4.

fat fed APOE mice reduced iron accumulation and LPO, increased xCT, GSH, and GPX4 levels in the aorta, and resulted in smaller atherosclerotic plaques (Li et al., 2021). Peng et al. identified a positive correlation between endothelial cell function and N-acetylneuraminic acid (Neu5Ac) from glucose metabolism. Neu5Ac facilitates SLC3A2 binding to ubiquitin, initiating P62-mediated degradation, which leads to LPO and ferroptosis in high-fat-fed ApoE$^{-/-}$ mice. This process is inhibited by Fer-1 (Xiang et al., 2023).

Iron accumulation from various sources significantly contributes to AS and vascular endothelial cell (VEC) ferroptosis. A key PL oxidation product present in atherosclerotic lesions is 1-palmitoyl-2-glutaryl-sn-glycero-3-phosphocholine (PGPC). Chen et al. show that PGPC treatment of human umbilical vein endothelial cells (HUVECs) increases fatty acid binding protein-3 (FABP3), ROS, LPO, and ferrous iron levels. It disrupts mitochondrial membrane potential and reduces glutathione and GPX4 expression. Ferroportin-1, a ferroptosis inhibitor, countered these effects. PGPC triggers ferroptosis through the CD36 receptor, impairing endothelial function (Chen et al., 2024). Iron accumulation from ferritin autophagy worsens AS, damaging endothelial cells. HUVEC exposed to high doses of ionizing radiation exhibits increased ferritin autophagy through the p38/NCOA4 pathway, leading to elevated iron levels, increased lipid peroxidation, and decreased antioxidant protein expression (GPX4, Nrf2, xCT, and SLC3A2). This cascade in significant VECs injury and ferroptosis (Wu Z. et al., 2023).

Additionally, enhancing VEC antioxidant activity is crucial for preventing ferroptosis and improving AS. Tumor necrosis factor-related protein 13 (CTRP13) is crucial for antioxidant defense and endothelial function. Du et al. developed a model of Ox-LDL-induced ferroptosis in HUVECs, showing that CTRP13 intervention increased antioxidant enzyme expression, preventing AS and protecting endothelial cells from ferroptosis (Du J. et al., 2024). Gualou-Xiebai (GLXB) inhibits ferroptosis in high-fat-fed ApoE$^{-/-}$ mice by reducing mitochondrial damage, boosting glutathione and superoxide dismutase levels, and decreasing LPO and MDA levels. *In vitro*, GLXB mitigated erastin-induced ferroptosis, alleviating Ox-LDL-induced damage in HUVEC (Zhu L. et al., 2024). Qixian granule

(QXG) is used to treat postmenopausal AS in women. Zhang et al. demonstrate that QXG inhibits GPX4 and FTH1-mediated ferroptosis by activating TRPML1 directly or through the GPER pathway, slowing AS progression. *In vitro*, QXG-treated serum prevents human aortic endothelial cells from proliferating, migrating, and inducing ROS and mitochondrial damage from Ox-LDL (Zhang et al., 2024).

5.2 Ferroptosis of macrophage promotes atherosclerosis

Macrophage death exhibits a dual role in atherogenesis. During the early stages of AS, moderate macrophage death reduces inflammation and metalloproteinase secretion. Conversely, in advanced stages, uncontrolled macrophage death exacerbates inflammation, promotes the formation of lipid-rich necrotic cores, and destabilizes plaques (Susser and Rayner, 2022; Wang L. et al., 2023). Numerous studies show the role of macrophage ferroptosis in promoting AS. Interleukin-23p19 (IL-23p19) is associated with cardiovascular conditions, including AS. Lu et al. created a mouse model of cardiac remodeling using transverse aortic constriction (TAC) and observed increased IL-23p19 expression levels in the heart after surgery, probably from cardiac macrophages. Silencing IL-23p19 reduces ferroptosis and M1 macrophage polarization in mice with TAC, improving cardiac remodeling and function recovery (Lu et al., 2024). Furthermore, Luo et al. report that activating Nrf2 and enhancing its nuclear translocation can inhibit Ox-LDL-induced ferroptosis in macrophages and resist ferroptosis in $ApoE^{-/-}$ animals on a high-fat diet (Luo et al., 2024).

Erythrocyte phagocytosis and macrophage ferroptosis are essential triggers of AS. Advanced atherosclerotic plaques exhibit intra-plaque (IP) angiogenesis. The fragile and leaky nature of IP arteries releases erythrocytes, which are subsequently phagocytosed by macrophages. This process leads to increased intracellular iron levels, LPO, and apoptosis. *In vitro* studies show that erythrophagocytosis by macrophages induces atypical ferroptosis, potentially causing plaque instability. Elevated heme oxygenase 1 and ferritin levels observed during this process were reduced by the ferroptosis inhibitor UAMC-3203. Research by Pauline Prall and colleagues using $ApoE^{-/-}$ mice reveals a significant decrease in carotid plaque thickness after 20 weeks, especially in plaques with hemorrhage or IP angiogenesis. This was accompanied by reduced expression levels of ferritin and IP heme oxygenase 1. Therefore, erythrophagocytosis-induced ferroptosis contributes to the formation of larger atherosclerotic plaques, but this effect can be reversed by UAMC-3203 (Puylaert et al., 2023). Liu et al. developed a red-line Jak2VF-expressing hyperlipidemic erythropoietin receptor Cre mice. They observed that these mice exhibit elevated iron and 4-HNE levels, increased erythrocyte phagocytosis by plaque macrophages, and enhanced macrophage ferroptosis, leading to greater necrosis in atherosclerotic plaques. These effects were amplified by selective erythropoiesis activation and inhibited by the ferroptosis inhibitor liproxstatin-1, indicating that Jak2VF signaling accelerates AS by promoting macrophage ferroptosis and erythrocyte phagocytosis (Liu W. et al., 2022).

5.3 Ferroptosis of vascular smooth muscle cells promotes atherosclerosis

Vascular smooth muscle cells (VSMCs) are essential in the early and advanced stages of AS. They infiltrate lesions, enlarge them and form a fibrous cap over the necrotic core. VSMC death causes vascular inflammation, loss of extracellular matrix (ECM) and collagen, weakening of the fibrous cap, and ultimately plaque rupture (Grootaert and Bennett, 2021; Xu X. D. et al., 2022). Ferroptosis of VSMCs is also a significant part of AS. Xie et al. observed that Fer-1 affects TFR1, FTH, and FTL expression in VSMC, reducing iron accumulation in atherosclerotic lesions *in vivo* and *in vitro*. Evidence suggests that VSMC ferroptosis may help enhance atherosclerotic lesions. Fer-1 fails to enhance the p53/SLC7A11/GPX4 pathway but improves the Nrf2/ferroptosis inhibitory protein 1 pathway, boosting resistance to LPO (You et al., 2023). Zhang et al. discovered that echinacea upregulates glutamate-cysteine ligase catalytic (GCLC) and modifier (GCLM) subunits in VSMC, maintaining the GSH balance essential for preventing AS. This regulation also reduces ferroptosis and matrix remodeling, both contributors to AS, by regulating GSH production (Zhang et al., 2023c). Mucosa-associated lymphoid tissue lymphoma translocation protein 1—a human cysteoaspartase paraprotein—induces ferroptosis in VSMCs through protein hydrolysis. A ferroptosis inhibitor reduces carotid artery neointimal plaques and AS in C57BL/6J and $ApoE^{-/-}$ mice, indicating that suppressing ferroptosis in VSMCs may alleviate proliferative vascular disease (Yan et al., 2023).

5.4 Ferroptosis of foam cells promotes atherosclerosis

Foam cells are essential in developing atherosclerotic lesions, from initial stages to advanced plaques. Macrophages—the primary source of foam cells—proliferate in the arterial intima after pro-inflammatory activation by endothelial cells, allowing them to migrate across the endothelial barrier. A small portion of VSMC and endothelial cells can differentiate into macrophages, which may then become foam cells by engulfing lipids. VSMC can also develop into foam cells (Chistiakov et al., 2017; Maguire et al., 2019). During plaque formation, foam cell death—primarily from ferroptosis and autophagy—accelerates atherosclerotic plaque development. Insufficient autophagy inhibits Nrf2-mediated antioxidant defenses, promoting iron deposition and LPO, leading to ferroptosis (Peng et al., 2022). Li et al. used bioinformatics to explore the roles of ferroptosis and isocitrate dehydrogenase 1 (IDH1) in foam cell formation. They observed that ferritin-1 can reverse the increase in macrophage ferroptosis and IDH1 levels caused by Ox-LDL. Inhibiting IDH1 reduces damage and apoptosis in Ox-LDL-treated macrophages while increasing Nrf2 levels, thereby decreasing foam cell formation (Li B. et al., 2022). Su et al. treated THP-1 macrophages with ferric ammonium citrate (FAC) and Ox-LDL, using SRT1 to activate SIRT1720 and rapamycin along with chloroquine to modulate autophagy. They observed that FAC exposure increases lipid ROS levels, decreases GPX4 and SIRT1 expression, and elevates IL-1β and IL-18 levels while reducing foam cell activity compared to that of macrophage

activity. These findings indicate that inhibiting ferroptosis in foam cells is essential for treating AS (Su et al., 2021).

In summary, ferroptosis in VECs, macrophages, VSMC, and foam cells contributes to AS. Treatment reduces ferroptosis levels in these cells and improves the condition, highlighting the importance of anti-ferroptosis strategies in the disease.

6 Multiple cells ferroptosis promotes depression

6.1 Ferroptosis of neurons promotes atherosclerosis

Brain cells are mainly involved in neurons, microglia, and astrocytes. Factors such as neuronal damage in the brain, inflammatory responses, oxidative stress, cell death, and altered neuroplasticity can influence depression (Malhi and Mann, 2018). Neurons are the fundamental structural and functional units of the nervous system. Studies show that iron-sag markers can be traced in cellular and animal models of neurodegenerative diseases (Cozzi et al., 2019). Fer-1 mitigates abnormal tsRNA expression profiles in the hippocampus tissues of the chronic unpredictable mild stress (CUMS) mouse model. Additionally, in an *in vitro* assay, ferroptosis is associated with reduced expression of SLC7A11 and GPX4 proteins, as well as ROS accumulation in corticosterone-constructed hippocampal progenitor neurons (Li E. et al., 2023). Xu et al. observed that alcohol consumption causes neuronal injury in the hippocampal and prefrontal cortex of mice. The alcohol-treated group shows reduced levels of synapse-associated proteins. Alcohol increases the number of iron-positively stained cells and the expression of the TFR1 protein. Following iron staining, GPX4 expression is minimized in the alcohol group. Conversely, the ferroptosis inhibitor ferritin-1 significantly prevents alcohol-induced damage to neurons *in vitro* and reverses the expression of the proteins mentioned above (Xu C. et al., 2022). In CUMS animals, decreased ferritin light chain 1 and Brain-derived neurotrophic factor (BDNF) levels are observed in the proteomics of hippocampal neurons, which are associated with hippocampal neuronal survival. Additionally, ferroptosis-associated proteins are differently expressed in these animals (Cao et al., 2021). Li et al. further demonstrated that enhancing hippocampal neuronal BDNF levels could prevent hippocampus neuronal ferroptosis and potentially enhance the antidepressant effects of electroconvulsive therapy (Li X. et al., 2023). The studies mentioned above highlight the significant roles that neuronal loss and ferroptosis play in depression.

6.2 Ferroptosis of microglia promotes atherosclerosis

Microglia make up approximately 0.5%–16.6% of the cells in the human central nervous system and are the sole remaining mononuclear phagocytes within the brain parenchyma (Askew et al., 2017). Microglia plays various roles in brain development, synaptic plasticity, neuronal synchronization, and neuroimmune homeostasis from embryo development to adulthood. They also interact with neurons, astrocytes, and oligodendrocytes via chemical signals or direct contact throughout the life cycle of an organism. Neuroinflammation progresses because of the combined effects of inflammation and microglial iron buildup, which can be inhibited to stop the progression of neuroinflammation (Liu S. et al., 2022). Depression is frequently accompanied by inflammation, and microglia are essential starting materials and controllers of the cynaroside (CNS) inflammatory cascade responses (Wang H. et al., 2022). Neuroinflammation is another significant mechanism in many neurodegenerative diseases and is often associated with persistent activation of microglia. Microglial energy metabolism is gaining prominence in neurodegenerative disorders, as numerous brain diseases are associated with changes in brain energy metabolism and constant inflammation. Moreover, energy metabolism strongly influences the inflammatory response of microglia (Aldana, 2019).

Inhibiting microglia ferroptosis to ameliorate CNS inflammation is a promising therapeutic tool for depression. Wang et al. utilized shRNA silencing of the microglial GPX4 gene to assess the anti-inflammatory effects of saikosaponin B2 (SSB2) in LPS-induced primary microglia and CUMS-induced mouse models of depression. Consequently, SSB2 exhibits anti-ferroptosis and anti-neuroinflammatory effects, attenuating the activation of the LPS-induced primary microglial TLR4/NF-κB signaling pathway in a GPX4-dependent manner (Wang et al., 2023c). Jiao et al. suggest that the antidepressant mechanism of Prowessan may involve the enhancement of the iron-death-associated PEBP1-GPX4 pathway. This pathway can control the expression of PEBP1, ERK1/2, GPX4, FTH1, ACSL4, and COX2, thereby enhancing the function of hippocampal astrocytes and microglial cells in CUMS model mice (Jiao et al., 2021). Yang et al. investigated the role of gallic acid in preventing ferroptosis in spinal microglial cells in rats with chronic pain and depression. They discovered that the anti-inflammatory and antioxidant properties of gallic acid decrease tissue iron concentration, improve mitochondrial damage, inhibit the P2X7-ROS signaling pathway, and reverse behavioral changes in these rats (Yang et al., 2023) (Table 1).

6.3 Ferroptosis of astrocytes promotes atherosclerosis

Astrocytes—the most abundant and largest class of glial cells in the mammalian brain—maintain and divide neuronal cells, aid in creating the blood-brain barrier, and regulate the onset of several disease processes (Khakh and Sofroniew, 2015). In culture, astrocytes are observed to shield neurons from oxidants and excitotoxins. This neuroprotective effect is thought to result from their ability to absorb glutamate and recycle free radicals. Astrocytes are involved in numerous neurodegenerative disorders and can significantly influence depression. In Orai1 knockout mice, the LPS-induced depression-like behavior, including the helplessness and pleasure deficit, is improved in astrocytes (Novakovic et al., 2023). Mice subjected to 6 weeks of prolonged stress followed by 6 weeks of social isolation exhibit symptoms of depression, accompanied by elevated levels of astrocyte activation in the dorsal and ventral dentate gyrus regions of the hippocampus (Du Preez et al., 2021). Prenatal exposure to air pollution in rodents

TABLE 1 The effects of microglia ferroptosis on depression.

Interventions	Subject	Pathway/ Protein	Mechanism	Results	Reference
Saikosaponin B2	CUMS mice	Inhibition of TLR4/NF-κB, GPX4↑	Anti-inflammatory, antioxidant, anti-ferroptosis	Depression↓	Wang et al. (2023c)
XiaoYao San	CUMS mice	Activation of PEBP1-GPX4	Improve microglia cell function	Depression↓	Jiao et al. (2021)
Gallic acid	CUMS rat	Adjustment P2X7-ROS	Inhibit ferroptosis in spinal microglia	Pain↓ and Depression↓	Yang et al. (2023)
Eicosapentaenoic acid	Mice	Nrf2↑ NLRP3↓	Inhibit microglia M1 polarisation and ferroptosis	Seizures↓ and Depression↓	Wang et al. (2022b)
Acupuncture	CUMS rat	SIRT1↑ Nrf2↑HO-1↑ GPX4↑	Anti-inflammatory and antioxidant, inhibits activation of microglia	Depression↓	Shen et al. (2024)
LPS	BV-2 cells	ALKBH5-PRMT2-β-catenin-GPX4	Promoting microglia ferroptosis and polarisation	Depression↑	Mao et al. (2024)
Cynaroside	CUMS mice	IRF1/SLC7A11/GPX4↑	Inhibition of microglia polarisation to M1 phenotype and reduction of inflammation and ferroptosis	Depression↓	Zhou et al. (2024)
Hydrogen sulfide	CUMS mice/BV-2 cells	SLC7A11/GPX4/CBS↑	Anti-inflammatory, Fe^{2+}↓ MDA↓ ROS↓ lipid peroxide↓	Depression↓	Wang et al. (2021b)

CUMS: Chronic unpredictable mild stress; TLR4:Toll-like receptor 4; NF-κB:Nuclear factor kappa-B; GPX4:Glutathione Peroxidase 4; PEBP1: Phosphatidylethanolamine Binding Protein 1; ROS:Reactive oxygen species; Nrf2:Nuclear factor ervthroid2-related factor 2; NLRP3:NOD-like receptor family pyrin domain containing 3; SIRT1:Sirtuin 1; HO-1:Heme oxygenase 1; ALKBH5:Alk B homolog 5; IRF1:Interferon Regulatory Factor 1; SLC7A11: Solute carrier family 7 member 11; MDA: malondialdehyde.

causes astrocyte degeneration, microglia activation, and disruption of the blood-brain barrier, leading to depressive tendencies in rats. These rats also show a twofold increase in hippocampus iron accumulation (Woodward et al., 2018).

Inhibition of astrocyte activation and ferroptosis ameliorates depression. CUMS depression model mice treated with the compound Chinese medicine XiaoYao San for 2 weeks showed a decrease in hippocampal body weight, total iron, and ferrous iron content. Changes were made in the concentrations of proteins associated with ferroptosis, including GPX4, FTH1, ACSL4, and COX2. The expression of glial fibrillary acidic protein—a marker of activated astrocytes—decreases, enhancing astrocyte glial cell activity (Jiao et al., 2021). Diffuse depression in neurons may be caused by a discrepancy in the antioxidant system, where the energy metabolic state of astrocytes plays a crucial role. ATP depletion in astrocytes leads to reduced glutathione levels and increased ferrous ions, reducing their antioxidant capacity. The iron chelating agent deferoxamine reduces astrocyte damage, suggesting that improvements in astrocyte functionality are partly attributed to the inhibition of their ferroptosis (Lian and Stringer, 2004).

As mentioned above, microglia, neurons, and astrocytes are involved in the pathological depression process. Changes in indicators of cellular ferroptosis are observed in all three types of cells, and effective improvements can be made using drugs or other intervening behaviors. This suggests that modulating ferroptosis in these 3 cell types plays an important role in depression (Figure 2).

7 Ferroptosis and cardiovascular disease co-depression

We have previously discussed the separate roles of iron death in AS and depression. Ferroptosis also plays a significant role in comorbidity. After a closed head injury, $ApoE^{-/-}$ mice exhibit elevated levels of iron in their brains, suggesting that $ApoE^{-/-}$ deficiency may increase the risk of oxidative damage to hippocampal tissue (Guaraldi and Shea, 2018). A randomized controlled clinical study of 26 patients with myocardial infarction, anxiety, and depression, compared to that of 26 healthy individuals, revealed that ferroptosis is involved in the pathogenesis of cardiovascular and cerebrovascular diseases associated with depression. *In vitro* studies show that upregulating sestrin2 decreases type I/II collagen and KEAP1 mRNA expression while increasing GPX4 and Nrf2 mRNA levels. Similar findings were observed with sestrin2 downregulation (Qian et al., 2023). Ferritin improves cognition by decreasing ROS and glutathione depletion in the MCAO model and downregulating elevated ferroptosis factors P11 and SLC53A7 (Ko et al., 2023).

8 Ferroptosis affects atherosclerosis and depression through different mechanisms

We have summarized the key mechanisms of cellular ferroptosis in AS and depression. However, disease development is often complex, involving multiple mechanisms and their interactions. Ferroptosis is also closely associated with inflammation, mitochondrial dysfunction, and the gut microbiota, which also play important roles in AS and depression.

8.1 Ferroptosis exacerbates peripheral inflammation

AS—a chronic inflammatory vascular disease—is closely associated with inflammation in its development (Kong et al., 2022). Macrophage-

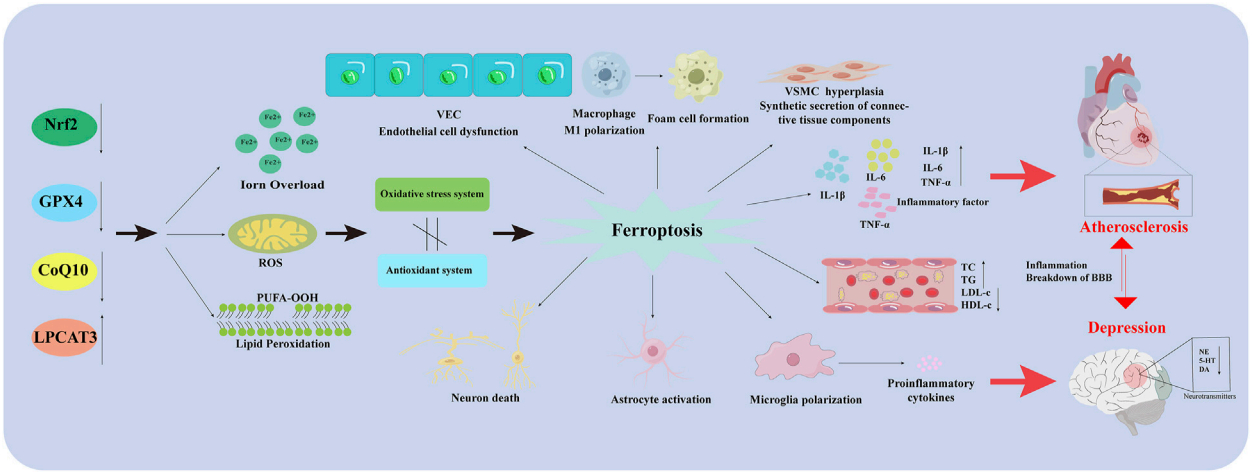


FIGURE 2
Ferroptosis in different types of cells in AS co-depression disease. Nrf2: Nuclear factor erythroid 2-related factor 2; GPX4: Glutathione peroxidase 4; CoQ10H2: Reduced coenzyme Q10; LPCAT3: Lysophosphatidylcholine acyltransferase 3; VEC: Vascular endothelial cell; PUFA-OOH: Polyunsaturated fatty acid-OOH; ROS: Reactive oxygen species; VSMC: Vascular Smooth Muscle Cell; IL-1β: Interleukin-1 beta; IL-6: Interleukin- 6; TNF-α: Tumor necrosis factor-α; TC: Total Cholesterol; TG: Triglyceride; LDL-C: Low-Density Lipoprotein Cholesterol; HDL-C: High density lipoprotein cholesterol; BBB: Blood Brain Barrier; NE: Norepinephrine; 5-HT: 5-hydroxy tryptamine; DA: Dopamine.

mediated inflammatory responses significantly contribute to AS progression, with various macrophage types observed in plaques. Excessive lipid uptake primarily drives polarization into M1-type macrophages. M2-type macrophages contain less intracytoplasmic iron and metabolize it more quickly than that of M1-type macrophages, which accumulate iron owing to their high ferritin content. Iron levels influence macrophage polarization: low levels inhibit anti-inflammatory responses, while high levels promote them (Cornelissen et al., 2019). Elevated iron levels increase macrophage secretion of matrix metalloproteinases, degrading the ECM and inducing AS plaque rupture. Iron overload also hinders lipoxygenase binding to the nuclear membrane, exacerbating inflammation and promoting AS formation (Ma et al., 2022). Ferroptosis—along with iron overload and imbalanced redox reactions—is closely associated with AS, contributing to macrophage foam cell formation. It increases ROS expression by promoting P53 acetylation, driving macrophage differentiation into the M1 phenotype (Zhou et al., 2018). Additionally, M2-type macrophages can be converted into M1-type macrophages when induced by iron nanoparticles (Laskar et al., 2013). Therefore, ferroptosis enhances M1-type macrophage polarization, worsens peripheral inflammation, and accelerates AS progression (Ma et al., 2022).

8.2 Ferroptosis exacerbates central inflammation

Neuroinflammation is primarily initiated by astrocytes and microglia in response to factors such as injury, infection, exposure to toxins, or autoimmune reactions. Microglia—the resident immune cells of the CNS—are characterized by elevated levels of iron (Liu S. et al., 2022). This is closely associated with iron overload-mediated depression and abnormal glial cell activation (Zeng et al., 2023). Research shows that synaptic plasticity theory is a widely accepted mechanism significant to the pathophysiology of depression, with BDNF signaling being one of these. BDNF downregulation, driven by various factors, causes neurotoxic effects and is crucial for synaptic plasticity in depression (Li Y. et al., 2024; Lomeli et al., 2024). Li et al. observed that iron overload through the ferricyanide/BDNF pathway may damage hippocampal neurons by reducing BDNF levels (Li J. et al., 2024). They also suggest that etomidate may have antidepressant effects by increasing BDNF, thereby protecting hippocampal neurons from ferroptosis (Li X. et al., 2023). Additionally, lactoferrin—an iron-binding glycoprotein—reduces NF-κB (p65) and TNF-α levels, lessening inflammation and depressive behaviors in rats subjected to chronic restraint stress. This is supported by findings in CUMS model mice, where iron buildup in hippocampal microglia influences neuronal degeneration and death (Ahmed et al., 2023; Gao et al., 2019). Microglia activation, increased pro-inflammatory cytokine expression, and ferroptosis in the hippocampus of CUMS mice are reversible with further erastin therapy (Ma et al., 2024). Zhang et al. observed that deferoxamine reverses neuropathological changes caused by inflammatory factor upregulation in a CUMS mouse model (Zhang W. et al., 2022). These findings indicate a clear link between depression onset and neurotoxicity from excess iron.

8.3 Ferroptosis affects atherosclerosis by modulating mitochondrial function

Iron is essential for lipid metabolism, protein synthesis, cellular respiration, and DNA synthesis, with mitochondria playing a key role in iron metabolism. Iron also contributes to adaptive mechanisms in cardiac control (Ravingerová et al., 2020), but its regulatory role in iron metabolism is often overlooked. The heart derives energy from iron-sulfur clusters (ISCs), produced by

mitochondria, the only sites of hemoglobin production. These ISCs contain hemoglobin and catalyze electron transfer through the bidirectional oxidation states of iron. Excessive iron accumulation in mitochondria causes oxidative stress, generating harmful free radicals that damage DNA, proteins, and lipids, ultimately impairing cardiac function (Kumfu et al., 2012). Altered mitochondrial morphology distinguishes ferroptosis from other cell death types. This alteration can lead to excessive ROS production, damaging VECs and causing mitochondrial dysfunction. Aortic endothelial cells (MAECs) from high-fat-fed ApoE$^{-/-}$ mice exhibited increased expression of mitoferrin 2 (Mfrn2), an iron transporter in the inner mitochondrial membrane. Silencing the Mfrn2 gene prevents mitochondrial iron overload in MAECs (Wang D. et al., 2021). Research on the role of ferroptosis in mitochondrial function and its effects on AS is limited. However, the studies mentioned above highlight its significance in AS and offer new insights for investigating the pathological mechanisms of AS through ferroptosis.

8.4 Ferroptosis affects depression by modulating mitochondrial function

Mitochondria are essential for cellular energy metabolism and play a key role in inducing ferroptosis through various metabolic processes. Intracellular ROS are primarily produced by mitochondrial metabolism. Superoxide is produced when electrons leak from, ETC., complexes I and III and they are converted into hydrogen peroxide by superoxide dismutase. This hydrogen peroxide reacts with ferrous ions to form hydroxyl radicals, which then transform the bis-allyl hydrogens in PUFAs into PUFA radicals. In the presence of oxygen, these unstable free radicals quickly form PUFA peroxyl radicals, which ultimately convert into PUFA hydroperoxides. This process promotes LPO, leading to ferroptosis owing to ROS produced by mitochondria. Elevated ferrous ions and iron homeostasis imbalances can further increase ROS through the Fenton reaction, also contributing to ferroptosis. Numerous researches show that oxidative stress and disruption of the electron transport chain in mitochondria are closely related to depression (Jiang et al., 2024; Khan et al., 2023; Scaini et al., 2022). Among them, mitochondria-mediated production is positively associated with depression incidence. Excess iron ions disrupt the electron transport chain and reduce ATP synthesis in the mitochondria. Research indicates that individuals with severe depression exhibit lower ATP and glucose metabolism in brain regions associated with mood regulation, including the bilateral insula, nucleus accumbens, and cingulate gyrus (Su et al., 2014). ATP levels in the hippocampus and prefrontal cortex of CUMS depression model mice were reduced, but injecting ATP into the lateral ventricle may significantly enhance their depressive behavior (Cao et al., 2013; Shen et al., 2020). Furthermore, issues with mitochondrial iron metabolism problems may lead to depression by disrupting mitochondrial structure and function, which are key sources of cellular ROS. This disruption may also relate to depression management via the ferroptosis signaling pathway (Song Y. et al., 2023).

8.5 Ferroptosis affects atherosclerosis by regulating gut flora

Gut microbes also play a key role in AS. Studies show significant changes in the gut microbiota of individuals with ASCVD. In a metagenomic analysis of the gut microbiome of 218 patients with ASCVD and 187 healthy patients, Jie et al. identified a strong association between gut microbiota and ASCVD (Jie et al., 2017). Animal models indicate that gut microbiota may contribute to ASCVD through mechanisms such as direct host invasion, immune system activation through lipopolysaccharide, altered metabolism, and the release of metabolites into the bloodstream. Trimethylamine N-oxide is the most established link to ASCVD, supported by human and animal studies (Barrington and Lusis, 2017). Recent studies show that iron storage in mice correlates positively with *Clostridium* spp. iron utilization, which is enhanced by dietary iron, intravenous iron, and chronic blood transfusions (La Carpia et al., 2019). An increase in *Clostridium* species has been observed in AS (Konturek et al., 2015). Additionally, a randomized controlled trial reveal that Qing-Xin-Jie-Yu Granule can modify gut flora, reduce AS, and prevent ferroptosis by modulating the GPX4/xCT pathway, thereby stabilizing AS plaques (Zhang et al., 2023b). These findings highlight the significant function of ferroptosis in AS and suggest that gut microbiota are a major contributor.

8.6 Ferroptosis affects depression by regulating gut flora

The brain-gut axis is a bidirectional communication network that connects cognitive and emotional functions of the brain to gut activity through neural pathways, neuroendocrine and neuroimmune systems, and gut microbiota (Mayer et al., 2022). Disturbances in gut microbiota and its metabolites have been observed in individuals with depression and animal models, suggesting a link between depression and disrupted gut microbiota, as well as impaired neural communication (Liu L. et al., 2023; Wang Y. et al., 2024). Imbalances in serum iron homeostasis can trigger inflammation and gut microbial diseases, leading to brain stress responses. Increased IL-1β levels elevate iron regulatory protein 1 and TFR1 in oligodendrocytes, while reduced iron transporter protein 1 expression leads to intracellular iron accumulation, contributing to neurodegenerative diseases (Yang J. et al., 2024). Studies indicate a strong connection between depression onset and IL-1β-mediated signaling pathways (García-García et al., 2022; Ng et al., 2022; Wang et al., 2018), with IL-6 playing a similar role (Zhang G. et al., 2023). Individuals with depression exhibit reduced BDNF levels, highlighting its potential as a diagnostic biomarker (Rana et al., 2021). Ferroptosis is linked to depression by suppressing BDNF expression, while gut microorganisms influence BDNF and modulate depression through the brain-gut axis (Matin and Dadkhah, 2024). Depression may be associated with gut flora dysbiosis, and probiotics could help alleviate its symptoms. Iron metabolism and ferroptosis may induce neurological damage through interactions with the central nervous system, gut microbiota, and brain-gut axis, positioning them as potential therapeutic targets for depression (Figure 3).

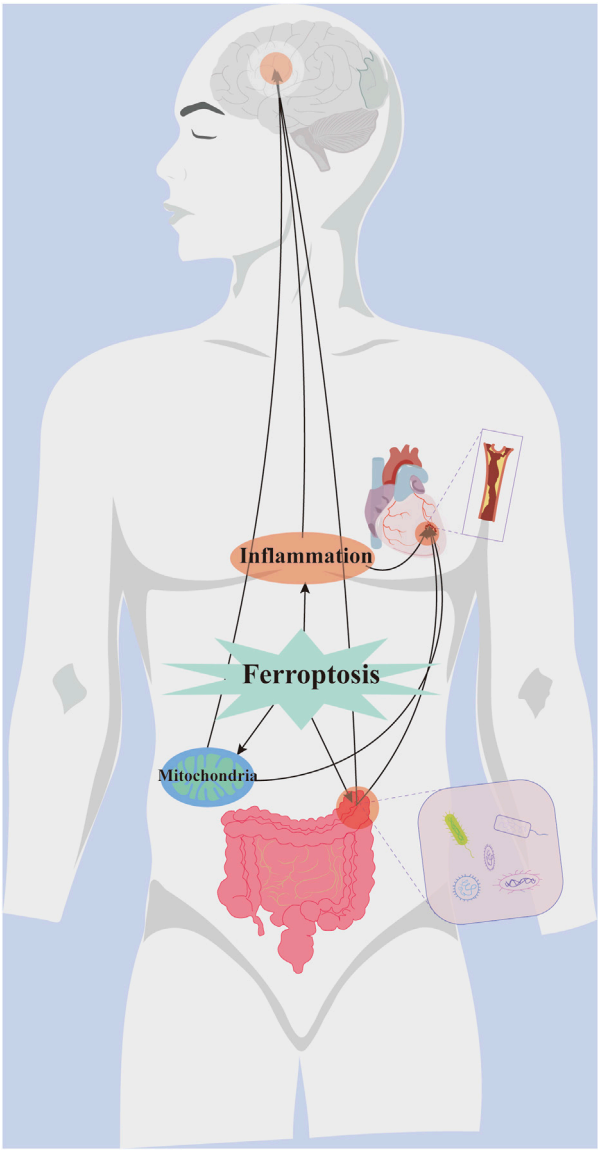


FIGURE 3
Crosstalk between ferroptosis and common pathogenesis of AS co-depression disease.

8.7 The role of ferroptosis in atherosclerosis with depression

Ferroptosis influences the onset of AS and depression through inflammation, mitochondrial dysfunction, and gut microbiota, respectively. It also plays an important role in the comorbidity of atherosclerotic cardiovascular disease and depression. In patients with chronic stable angina pectoris complicated by depression and anxiety, Melissa officinalis supplements have shown significant improvement in symptoms. Melissa officinalis exhibits strong antioxidant properties, regulates lipid metabolism (Petrisor et al., 2022), and can also inhibit ferroptosis (El Hajj et al., 2023). In patients with AS and depression, elevated levels of the inflammatory factors IL-6 and IL-10 are observed (Mousa et al., 2022), while ferroptosis exerts a significant pro-inflammatory effect. In ApoE$^{-/-}$ mice subjected to chronic mild stress, lipid metabolism disorders, increased adipocyte hypertrophy, and reduced PPARG gene expression promote AS, while PPARG exhibits an anti-ferroptosis effect (Mao et al., 2023). Therefore, the role of ferroptosis in AS and depression is bidirectional, involving interactions through various disease mechanisms.

In summary, the significant role of ferroptosis in AS and depression has been highlighted. Ferroptosis contributes to the onset and progression of AS co-depression directly or by influencing other mechanisms. Targeted regulation of ferroptosis through drugs could represent a novel approach for treating AS-depression comorbidities. However, commonly used ferroptosis modulators are often associated with significant side effects, including pulmonary toxicity (Parry et al., 2002), liver damage, and neurotoxic effects (Zaafar et al., 2024). Currently, Chinese medicine offers promise for treating AS and depression. Its compounds exhibit anti-ferroptosis effects with minimal side effects, enhancing patient acceptability. Therefore, we will review the anti-ferroptosis effects of Chinese medicine compounds and monomers in AS and depression.

9 The development of ferroptosis-focused Chinese medicine for the treatment of depression and atherosclerosis

9.1 Treatment of atherosclerosis with a Chinese medicine monomer targeting ferroptosis

Paeonia lactiflora, a medicinal herb native to China, is the source of Paeonol (Pae), a bioactive compound with potent antioxidant and lipid-lowering properties (Shi et al., 2020). Studies indicate that Pae reduces lipid accumulation and foam cell growth, thereby lowering atherosclerotic plaque formation (Shi et al., 2022). Additionally, studies reveal that Pae enhances Nrf2 protein expression, potentially preventing lipid accumulation in foam cells by reducing ferroptosis. Gao et al. reveal that Pae effectively reduced fat growth and aortic ferroptosis in ApoE$^{-/-}$ mice, outperforming ferritin-1 and simvastatin. *In vitro*, Pae inhibited lipid accumulation from Ox-LDL in foam cells undergoing ferroptosis. The SIRT1/Nrf2/GPX4 pathway was identified as a protective mechanism, with SIRT1 knockdown reversing this effect. Therefore, Pae may inhibit lipid accumulation and alleviate AS by targeting this pathway (Gao et al., 2024).

Hydroxysafflor yellow A (HSYA), a monochalcone glycoside derived from the Asteraceae family, is used to manage and prevent ischemic cerebrovascular diseases. Its anti-inflammatory, antioxidant, and anti-angiogenic properties help alleviate AS (Feng et al., 2023; Xue et al., 2021). HSYA reduces oxidative damage to HUVECs caused by H2O2 and inhibits ferroptosis during reperfusion, suggesting a protective role in heart tissues. Rong et al. administered HSYA to ApoE$^{-/-}$ mice and applied it in an Ox-LDL-induced hyperlipidemic model in HUVEC. HSYA suppressed ferroptosis in HUVEC, increased GSH, SLC7A11, and GPX4 levels, and significantly reduced atherosclerotic plaque formation. It also downregulated miR-429, which regulated

SLC7A11 expression. After SLC7A11 siRNA transfection or miR-429 mimicry, the antioxidative stress and anti-ferroptosis effects of HSYA were reduced. Therefore, HSYA holds promise as a therapeutic agent for AS (Rong et al., 2023).

Echinatin, a chalcone isolated from the Chinese herb licorice, possesses anti-inflammatory and antioxidant properties. Zhang established a mechanical property-based screening approach to identify compounds that alleviate arterial wall stiffness by affecting the interactions between VSMCs and the ECM. The study reveals that echinatin reduced ECM stiffness surrounding cultured VSMCs and upregulated glutamate cysteine ligases (GCLs) expression, including the catalytic (GCLC) and regulatory (GCLM) subunits. Further studies show that the upregulation of GCLC/GCLM in VSMC by echinatin helps maintain the homeostasis of GSH metabolism. Adequate glutathione is essential for counteracting AS (Zhang et al., 2023c). Icariin, the main active ingredient of epimedium, improves endothelial cell dysfunction. Wang et al. investigated the protective effect of icariin on Ox-LDL-treated VECs and ApoE$^{-/-}$ mice fed a high-fat diet. The findings reveal that icariin reduces the atherosclerotic plaque area and collagen fibers in aortic sinus tissue while enhancing mitochondrial activity and membrane potential. The levels of ROS in VECs were decreased. *In vivo* experiments, icariin reduced ferroptosis alleviated atherosclerotic lesions, and increased TFEB nucleation rate. These findings suggest that icariin could be a potential candidate for preventing VEC ferroptosis in cardiovascular disease treatment (Wang et al., 2023d). Tanshinone IIA (TSA) protects endothelial tissue from injury. We investigated its effect on ferroptosis in human coronary endothelial cells treated with TSA. TSA significantly reduces the excessive accumulation of total cell ROS and lipid ROS induced by ferroptosis inducers, restores glutathione content, and promotes nuclear translocation of Nrf2 (He et al., 2021).

9.2 Treatment of as with a Chinese medicine compound targeting ferroptosis

TCM compounds are widely used in AS treatment due to their multi-target effects, including the anti-ferroptosis mechanism. MaiJiTong Granules (MJT), a traditional Chinese medicine (TCM) formulation, contains Mucuna pruriens, Astragalus, Cinnamon, Danshen, Poria, and Paeonia lactiflora, which effectively treat cardiovascular diseases. Key ingredients, such as Astragalus saponin IV and ferulic acid, inhibit ferroptosis (Peng et al., 2021). Jia et al. developed a mouse model of AS through high-fat feeding to assess the efficacy of MJT in preventing and treating ferroptosis and AS. The study reveals that MJT exerts anti-inflammatory effects, lowers LDL levels, inhibits foam cell formation, and reduces plaque area and instability, thereby slowing AS progression. Additionally, MJT promotes ferroptosis and alleviates iron dysregulation and LPO during atherogenesis, reducing DMT1 and SOCS1/p53 expression through STAT1 phosphorylation (Shi et al., 2024).

DiDang decoction (DDD) is a TCM formula comprising rhubarb, peach kernel, leech, and gadfly, known for its ability to break up silt. Documented in the Typhoid Fever and Golden Chamber, it is used in China to treat AS and hyperlipidemia. DDD mitigates AS by preventing vascular fibrosis, preserving endothelial function, and reducing plaque rupture (Ren et al., 2017; Zhou et al., 2020). Wu et al. investigated the anti-ferroptosis effects of DDD through network pharmacology and *in vitro* experiments. They found that DDD therapeutically targets AS by enhancing mitochondrial function through the HIF-1 signaling pathway, reducing ROS levels, and modulating GPX4, Bcl2, and Bax protein levels, thereby benefiting AS and hyperlipidemia (Wu et al., 2023a).

The Qingxin Jieyu Granule (QXJYG), created by academician Chen Keji, is a key formula for ASCVD based on the "blood stasis and toxin" theory. It features ingredients such as Astragalus, Dangshen, and Huo Xiang. Previous studies show that QXJYG granules reduce inflammatory factors, modify intestinal flora to alleviate AS, and regulate iron metabolism in macrophages, stabilizing atherosclerotic plaques in individuals with stable coronary artery disease. Zhang et al. used RSL3 to induce macrophage ferroptosis *in vitro* and investigated the effects of Qing Xin Xie Du Granule on ferroptosis in ApoE$^{-/-}$ mice. QXJYG reduced inflammatory factors associated with ferroptosis, lowered lipid peroxides such as MDA, enhanced antioxidant capacity, and inhibited AS progression and plaque vulnerability. Additionally, QXJYG decreased total iron content compared to that of the model group and significantly increased GPX4/xCT expression in aortic tissues, reversing RSL3-induced ferroptosis in macrophages (Zhang et al., 2023b) (Table 2).

9.3 Treatment of depression with a Chinese medicine monomer targeting ferroptosis

Quercetin, a flavonoid found in many fruits and vegetables, exhibits anti-inflammatory, antioxidant, and antidepressant effects. It also exerts various pharmacological effects, including modulation of the intestinal microbiota (Li B. et al., 2024), anti-apoptosis (Ge et al., 2024), inhibition of inflammatory vesicle activation (Du L. et al., 2024), and modulation of glutamate receptors (Wang M. et al., 2024). Quercetin regulates micronutrients by chelating iron, scavenging ROS, and reducing ferroptosis induced by various pathological factors (Zoidis et al., 2021). Wang et al. used a rat model of perimenopausal depression (OVX-CUMS) to examine the effects of quercetin on serum elemental changes. The findings reveal that OVX-CUMS rats exhibit increased iron levels in the blood and prefrontal cortex, accompanied by reduced expression of ferroptosis-associated proteins SLC7A11 and GPX4, which improve following quercetin treatment (Wang D. et al., 2024). Furthermore, Zhu et al. investigated the effects of quercetin on the lipid metabolism gene PTGS2 in breast cancer-related depression. They found that quercetin improves lipid metabolism and intestinal flora while reducing ferroptosis markers (total iron, Fe2+, MDA, and ROS). These findings confirm the potential of quercetin to inhibit neuronal ferroptosis and enhance the immune response, offering relief from depression-associated breast cancer (Zhu Q. et al., 2024).

Gastrodin, the main active compound in the Aspalathus rhizome, exhibits significant neurobiological effects. It reduces β-amyloid deposition, inhibits glutamate production, prevents

TABLE 2 Traditional Chinese medicine targeting ferroptosis in the treatment of AS.

Interventions	Subject	Mechanism	Results	Reference
Paeonol	Foam cell	Upgrading of SIRT1/Nrf2/GPX4	Atherosclerosis↓	Gao et al. (2024)
Hydroxysafflor yellow A	HUVEC	Regulating miR-429/SLC7A11	Atherosclerosis ↓	Rong et al. (2023)
Echinatin	VSMC	Upgrading of GCLC/GCLM	Atherosclerosis ↓	Zhang et al. (2023c)
Icariin	VEC	Inhibits ROS and promotes autophagy	Atherosclerosis ↓	Wang et al. (2023d)
Tanshinone IIA	HCAEC	Inhibiting total ROS and lipid peroxidation, activation of Nrf2	Atherosclerosis ↓	He et al. (2021)
MaiJiTong granule	$LDLR^{-/-}$ mice	Activation of STAT6, DMT1 and SOCS1/P53 signalling pathways	Atherosclerosis ↓	Shi et al. (2024)
DiDang decoction	L-O2 cell	Activation of HIF-1 signalling pathway, reduces ROS levels and inhibits ferroptosis	Atherosclerosis ↓ Hyperlipidemia ↓	Wu et al. (2023a)
Qing-Xin-Jie-Yu Granule	J744A.1 cells	regulating the GPX4/xCT signaling pathway	Atherosclerosis ↓	Zhang et al. (2023b)

HUVEC:human umbilical vein endothelial cells; VSMC:vascular smooth muscle cell; GCLC: Glutamate-Cysteine Ligase; GCLM: Glutamate-cysteine ligase modifying subunit; VEC: vascular endothelial cell; $ApoE^{-/-}$:Apolipoprotein E Knockout; STAT6: Signal transducer and activator of transcription 6; DMT1:Divalent metal transporter 1; SOCS1: Suppressor of cytokine signaling 1; HIF-1: Hypoxia-inducible factor 1; $LDLR^{-/-}$:Low-Density Lipoprotein Receptor Knockout.

ferroptosis, and restores synaptic plasticity. Furthermore, it increases BDNF levels, promotes neurogenesis, inhibits microglial activation, and regulates dopamine concentrations. Clinical studies show the effectiveness of gastrodin in managing post-stroke depression (Berezutsky et al., 2022). Catalpol, derived from dihuang, a cyclic enol ether terpene in TCM, exhibits hypoglycemic, depressive, and neuroprotective effects (Wang Y. L. et al., 2021). Wu et al. used a network pharmacology approach to predict that Catalpol could inhibit ferroptosis and mitigate fluoxetine-induced liver injury. Subsequent molecular docking reveals that ATF3/FSP1-mediated ferroptosis plays a major role in fluoxetine-induced hepatic injury. Additionally, Catalpol can reverse and enhance the antidepressant effects of fluoxetine (Wu X. et al., 2024). CNS, an antioxidant flavonoid from Chinese honeysuckle, has recently garnered attention for its potential antidepressant benefits (Yang et al., 2021). Zhou et al. conducted transcriptome analysis and validation, showing that CNS inhibits LPO, ferroptosis, and inflammatory polarization through the IRF1/SLC7A11/GPX4 signaling pathway. Furthermore, *in vivo* experiments showed that CNS exhibited therapeutic effects comparable to those of fluoxetine, effectively ameliorating symptoms of anxiety, despair, and anhedonia while also inhibiting microglial activation in the hippocampus of mice subjected to CUMS. CNS was found to mitigate inflammation, lower ferroptosis levels, prevent microglial polarization to the M1 phenotype and promote overall mental health (Zhou et al., 2024). Additionally, herbal extracts such as silybin (Liu P. et al., 2023) and Lycium barbarum glycopeptide (Dai et al., 2023) have been shown to target ferroptosis with antidepressant effects.

9.4 Treatment of depression with a Chinese medicine compound targeting ferroptosis

Chinese herbal compounds are increasingly used to target ferroptosis in depression treatment. The antidepressant properties of Pure Essence, documented in the prescription compendium of the Taiping Welfare Pharmacy Bureau during the Song Dynasty, have been thoroughly validated through TCM theory, clinical applications, and pharmacological research. Furthermore, it is considered one of the classical formulas for antidepressant treatment (Ran et al., 2024). Animal studies show that it ameliorates depressive-like behavior in CUMS rats, enhances neurotransmitter transmission, and modulates gene expression in astrocytes (Wu Y. et al., 2024). Recent studies have increasingly focused on the role of ferroptosis in the pathophysiology of depression. Jiao et al. showed that free-powder administration could alleviate depressive behaviors in CUMS mice by modulating PEBP1-GPX4-mediated ferroptosis in the hippocampus. This effect was evidenced by alterations in total and ferrous iron levels in the hippocampus and an increase in the expression of PEBP1, ERK1/2, and key ferroptosis-related proteins, including GPX4, FTH1, ACSL4, and COX2 (Jiao et al., 2021). Similarly, Di Huang Yin Zi, another Chinese herbal compound, has shown potential antidepressant effects in the brains of post-stroke depressed rats. These effects are associated with increased ROS and MDA levels, as well as modifications in ferroptosis-related markers, such as Fe^{2+}, SLC7A11, and GPX4 (Yang et al., 2024c). In addition, a recent study suggests that Suanzaoren decoction modulates the DJ-1/Nrf2 signaling pathway, alleviating neuronal loss, synaptic damage, and ferroptosis associated with Alzheimer's disease. This modulation is evidenced by the upregulation of FPN1, DJ-1, Nrf2, GPX4, and SLC7A11 proteins in the hippocampus, coupled with the downregulation of TfR1, FTH1, FTL, and ACSL4 proteins (Long et al., 2024). Suanzaoren Decoction also exhibits multi-pathway and multi-targeted antidepressant effects, including anti-inflammatory actions and gut flora regulation (Du et al., 2023; Du Y. et al., 2024). While studies on antidepressants have not specifically focused on ferroptosis, it is likely that the actions of Suanzaoren Decoction contribute to the modulation of ferroptosis in depression (Table 3).

9.5 Chinese medicine targeting ferroptosis for treatment of atherosclerosis co-depression

The preceding discussion highlights various monomers and compounds that target ferroptosis for treating AS and depression.

TABLE 3 Traditional Chinese medicine targeting ferroptosis in the treatment of depression.

Interventions	Subject	Mechanism	Results	Reference
Quercetin	OVX-CUMS rat	Fe↓GPX4 and SLC7A11↑	Depression↓	Wang et al. (2024a)
Quercetin	BALB/c mice and primary hippocampal neurons	Fe, Fe^{2+},MDA and ROS↓	Depression↓	Zhu et al. (2024b)
Gastrodin	Mice	inhibit ferroptosis and restore synaptic plasticity	Depression↓	Berezutsky et al. (2022)
Catalpol	Mice	ATF3/FSP1 signalling mediates ferroptosis	Promoting antidepressant effect of fluoxetine↓	Wu et al. (2024a)
Cynaroside	CUMS mice	IRF1/SLC7A11/GPX4↑	Depression↓	Zhou et al. (2024)
Silybin	Mice	p53↓and SLC7A11↑GPX4↑STING↓	Neuroinflammation↓	Dai et al. (2023)
Lycium barbarum glycopeptide	Chronic restrain stress mice	IPLA2, GPX4↑,4-HNE and MDA↓	Anxiety and depressive behaviour↓	Ran et al. (2024)
XiaoYao San	CUMS mice	Activation of PEBP1-GPX4	Depression↓	Jiao et al. (2021)
Di-Huang-Yin-Zi	PSD rat	ROS, MDA, Fe^{2+}↓; SLC7A11 and GPX4↑ promotes P53	Depression↓	Long et al. (2024)
SuanZaoRen decoction	APP/PS1 mice	iron↓TfR1, FTH1, FTL and ACSL4 proteins↓ FPN1, DJ-1, Nrf2, GPX4 and SLC7A11↑	Inhibit neuron loss, synaptic damage	Du et al. (2024b)

OVX: ovariectomy; STING: stimulator of interferon gene; 4-HNE: 4-Hydroxynonenal; FTH1: Ferritin Heavy Chain 1; FTL:ferritin light chain; ACSL4:Long-chain acyl-CoA, synthetase 4; FPN1: Ferroportin 1.

Certain pharmacological agents, despite their differences, may share common mechanisms in targeting ferroptosis. For example, PAE not only protects against AS but also exerts neuroprotective effects by inhibiting neuronal ferroptosis in cerebral hemorrhage (Jin et al., 2021) and serves as an antidepressant (Wei et al., 2023). HSYA, known for its excellent blood-brain barrier permeability, improves depressive behavior by inhibiting hippocampal inflammation and oxidative stress (Liu Z. et al., 2022). Similarly, Icariin has shown promising antidepressant effects (Zeng et al., 2022). Quercetin also exhibits the potential to inhibit atherosclerotic ferroptosis while serving as an antidepressant by targeting ferroptosis-related mechanisms (Li H. et al., 2024). CNS inhibits the abnormal proliferation of aortic vascular smooth muscle cells (Kim et al., 2006). In summary, TCM effectively treats AS-related depression by targeting ferroptosis, which is also an important therapeutic target.

10 Conclusions and future perspectives

In conclusion, studying the mechanisms of comorbidity through the perspective of ferroptosis is essential, with GPX4 and Nrf2 being highlighted as critical targets. Cellular focus includes macrophages, endothelial cells, microglia, and neurons. Regarding treatment, Chinese medicine, known for its multi-target effects, holds significant potential. Ferroptosis not only directly contributes to AS but also interacts with inflammatory responses, energy metabolism, and the gut, accelerating disease progression. Targeting ferroptosis offers a promising therapeutic approach for AS and depression, providing a new strategy for addressing comorbidity. We highlighted the role of ferroptosis in comorbidities and emphasized its relevance in cardio-cerebrovascular diseases associated with AS and psychiatric disorders, such as depression. This underscores the broader significance of the ferroptosis mechanism across various diseases and provides insights into its involvement in other conditions.

Despite significant progress in understanding ferroptosis in AS and depression, its role in comorbid conditions remains unclear. In the clinical treatment of patients with atherosclerotic co-depressive disorders, combining lipid-lowering drugs and antidepressants often causes side effects such as nausea, dizziness, and vomiting, resulting in treatment failure. Therefore, identifying shared pathogenesis is crucial, and targeting ferroptosis represents a promising strategy. For example, deferoxamine, an iron-chelating agent, is an approved treatment for iron overload. It improves endothelium-dependent vasodilation in patients with coronary heart disease (Duffy et al., 2001), protects neurons from ferroptosis, and exhibits antidepressant effects (Trachtenberg et al., 2011). However, deferoxamine can cause growth delay, allergic reactions and bone abnormalities, and at high doses, it can cause nervous system damage (Entezari et al., 2022). Despite challenges such as poor solubility and instability, many active ingredients in Chinese medicine offer multi-target effects and lower toxicity, making them a preferable option.

TCM plays a significant role in treating atherosclerotic co-depression diseases through its anti-ferroptosis effects, but the specific molecular targets remain unclear. TCM consists of numerous components; however, only a few are absorbable, which complicates the understanding of their mechanisms and limits the scientific credibility of TCM. Anti-AS co-depression drugs targeting ferroptosis also have side effects. For example, thiazolidinediones, which inhibit ACSL4, can cause cardiac toxicity with prolonged use (Al Sultan et al., 2024), while zileuton, a lipoxygenase inhibitor, may lead to indigestion, nausea, and other issues. Although ferroptosis has been implicated in these diseases, its exact role in the occurrence and progression of comorbidities during specific pathogenesis remains

unclear. The mechanism of ferroptosis in comorbidities is not fully understood. These limitations highlight the direction of our efforts in studying comorbidity.

Author contributions

YZ: Conceptualization, Data curation, Formal Analysis, Writing–original draft. PR: Conceptualization, Software, Writing–review and editing. QL: Investigation, Writing–review and editing. XL: Software, Writing–review and editing. XC: Formal Analysis, Writing–review and editing. YW: Validation, Writing–review and editing. XW: Conceptualization, Funding acquisition, Writing–review and editing. JZ: Conceptualization, Formal Analysis, Funding acquisition, Visualization, Writing–review and editing.

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Conflict of interest

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

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Purinergic receptor antagonism reduces interictal discharges and rescues cognitive function in a mouse model of temporal lobe epilepsy

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Epilepsy is one of the most prevalent neurological disorders globally. Current treatments mainly target neuronal activity, often overlooking the involvement of astrocytes and microglia in epilepsy's pathophysiology. Here, we explored the impact of purinergic receptors, predominantly found in glial tissue, on epileptiform activity. We used TNP-ATP, a potent purinergic receptor antagonist, and conducted experiments using a mouse model of mesial temporal lobe epilepsy to examine behavioral performance and neural activity patterns. Our findings reveal that although TNP-ATP treatment did not significantly impact motor function or anxiety levels, it reduced both the amplitude and rate of hippocampal interictal discharges. Such reduction also affected the synchrony of associated neuronal spiking. Additionally, cognitive function, particularly hippocampus-dependent spatial memory and prefrontal cortex-dependent executive control, were partially restored. Moreover, neuronal recordings showed increased phase coherence between the hippocampus and prefrontal cortex for both slow (theta) and fast (gamma) oscillations in treated animals, indicating strengthened neural coordination between cortical regions upon purinergic receptor antagonism. These results underscore the potential role of purinergic receptor antagonists in improving behavioral and cognitive performance in epilepsy, providing novel insight into the use of these pharmacological agents as a therapeutic approach.

KEYWORDS

purinergic receptor, temporal lobe epilepsy, interictal discharges, cognitive dysfunction, cortical oscillation

1 Introduction

Epilepsy is a major neurological disorder affecting millions of people worldwide, with a noticeably higher prevalence in low- and middle-income countries (Thijs et al., 2019; Beghi, 2020). Characterized by its diverse seizure types, ranging from minor absences to severe generalized tonic–clonic seizures, epilepsy arises from imbalances in excitatory and inhibitory neurotransmitters in the brain, mainly glutamate and gamma-aminobutyric acid (GABA), respectively (Lévesque and Avoli, 2013; Akyuz et al., 2021). This imbalance leads to neuronal

hyperexcitability and hypersynchronized electrical brain activity, manifesting as electrographic seizures.

The primary treatment for epilepsy involves the use of antiseizure medications, yet their effectiveness is limited, highlighting the need for novel pharmacological strategies (Löscher and Schmidt, 2011). Recent advances in understanding epilepsy have drawn attention to the roles of astrocytes and microglia in its pathophysiology (Devinsky et al., 2013; Bedner and Steinhäuser, 2016). Indeed, during epileptic episodes, the release of glutamate and ATP activates purinergic receptors, which are prevalent on neurons, astrocytes, and especially microglia (Vezzani et al., 2019). Such activation triggers inflammatory responses via the release of pro-inflammatory cytokines (Avignone et al., 2008; Beamer et al., 2017).

P2X receptors (P2XRs), a subtype of purinergic receptors, are notably upregulated in microglia following seizures, suggesting a key role in epilepsy's progression (Beamer et al., 2017). For example, P2X7R antagonists have shown potential in modulating epileptiform activity, probably enhancing the effects of traditional antiepileptic drugs (Amhaoul et al., 2016; Beamer et al., 2017). The activation of ATP receptors, particularly the P2X7R, is increasingly recognized as a crucial factor in controlling brain hyperexcitability, inflammation, and potentially offering neuroprotection (Beamer et al., 2017; He et al., 2017).

Despite the expanding body of research, further study is necessary to establish the precise role of ATP signaling in epilepsy. ATP, often co-stored with classic neurotransmitters like GABA and glutamate, shows a significant increase in extracellular levels during pathological conditions (Illes et al., 2019). The rise in extracellular ATP levels is known to activate P2XRs on brain immune cells, exacerbating neuroinflammation, a key factor in the progression of epilepsy (Skaper et al., 2010). Given this, targeting ATP receptors, especially P2XRs, has been hypothesized to be instrumental in modulating neuronal excitability, neuroinflammation, and neuroprotection. Hence, we explored the therapeutic potential of targeting ATP receptors in epilepsy treatment, with a specific interest on TNP-ATP, a purinergic receptor antagonist, in a mouse model of mesial temporal lobe epilepsy. We concentrated on behavioral performance, cognitive function, and neural activity patterns, as these features remain little explored in preclinical models. We tested the hypothesis that purinergic receptor antagonism reduces interictal discharges and rescues cognitive function in a mouse model of low-level temporal lobe epilepsy. By examining both the behavioral and neural impacts of this treatment, our research contributes to substantiate the use of purinergic antagonists in epilepsy's pathophysiology and potential new treatment avenues.

2 Methods and methods

2.1 Animals

A total of 18 male mice were used in this study. Mice were housed in a temperature- and humidity-controlled room (22 ± 2°C) with food and water available ad libitum. A group of C57BL/6J mice (n = 10) were used for electrophysiological and behavioral experiments, whereas another group of Nestin-GFP mice (n = 8) were used for immunofluorescent experiments. Nestin-GFP mice, used for immunostaining experiments for astrogliosis, were kindly provided by Dr. Grigori Enikolopov at Cold Spring Harbor Laboratory (Cold Spring Harbor, NY, USA). These mice were crossbred with C57BL/6 mice for at least 10 generations (Mignone et al., 2004) and were kept at the animal house facilities at the UPV/EHU campus in Leioa, Spain in standard conditions of housing. All procedures were approved by the University of the Basque Country (UPV/EHU) Ethics Committees (Leioa, Spain) and Diputacio Foral de Bizkaia under protocol M20-2022-130 and M30-2022-129. All procedures followed the European directive 2010/63/UE and National Institutes of Health guidelines. Efforts were made to minimize the number of animals used and their suffering.

2.2 5-Bromo-2′-deoxyuridine (BrdU) administration

BrdU [5-Bromo-1-(2-deoxy-β-D-ribofuranosyluracil, 5-Bromouracil deoxyriboside); Sigma, St Louis, MO, USA] was diluted in sterile saline and administered through intraperitoneal injections at 150 mg/kg concentration (15 mg/mL of BrdU was diluted in sterile phosphate-buffered saline (PBS) with 0.01 N sodium hidroxyde (1% of the total solution). BrdU was administered intraperitoneally (3 injections, 24 h-apart) on the 3 last days of TNP-ATP (or vehicle) administration.

2.3 Immunohistochemistry

Mice were sacrificed 21 days after the KA injection. Experiments were performed essentially as described before following methods optimized for the use in transgenic mice (Encinas et al., 2006, 2011; Encinas and Enikolopov, 2008). Animals were deeply anesthetized and were subjected to transcardial perfusion with 25 mL of PBS followed by 30 mL of 4% (w/v) paraformaldehyde in PBS, pH 7.4. The brains were removed and post-fixed, with the same fixative solution, for 3 h at room temperature, then transferred to PBS and kept at 4°C. Quantitative analysis of cell populations in transgenic mice was performed by means of design-based (assumption free, unbiased) stereology using a modified optical fractionator sampling scheme as previously described (Encinas and Enikolopov, 2008). Slices were collected using systematic-random sampling. The right hemisphere was selected per animal. The hemisphere was sliced sagittally in a lateral-to-medial direction, from the beginning of the lateral ventricle to the middle line, thus including the entire dentate gyrus. We focused on the dentate gyrus because neurogenesis is well-documented in the subgranular zone, and this region is particularly vulnerable to epileptic insults. The 50 µm slices (cut using a Leica VT 1200S vibrating blade microtome, Leica Microsystems GmbH, Wetzlar, Germany) were collected in 6 parallel sets, each set consisting of 12 slices, each slice 300 µm apart from the next. The sections were incubated with blocking and permeabilization solution (PBS containing 0.25% Triton-100X and 3% BSA) for 3 h at room temperature, and then incubated overnight with the primary antibodies (diluted in the same solution) at 4°C. After thorough washing with PBS, the sections were incubated with fluorochrome-conjugated secondary antibodies diluted in the blocking and permeabilization solution for 3 h at room temperature. After washing with PBS, the sections were mounted on gelatin coated slides with DakoCytomation Fluorescent Mounting

Medium (DakoCytomation, Carpinteria, CA). Those sections destined to the analysis of BrdU incorporation were treated, before the immunostaining procedure, with 2 N HCl for 20 min at 37°C, rinsed with PBS, incubated with 0.1 M sodium tetraborate for 10 min at room temperature, and then rinsed with PBS. The GFP signal from the transgenic mice was detected with an antibody against GFP for enhancement and better visualization. The following antibodies were used: chicken anti-GFP (Aves Laboratories, Tigard, OR) at 1:1000 dilution; rabbit anti-S100β (Dako Cytomation) at 1:1000 and rat anti-BrdU (AbD Serotech, Kidlington, UK) at 1:1000. As secondary antibodies, AlexaFluor 488 goat anti-chicken (Molecular Probes, Willow Creek Road, Eugene, OR) at 1:500; AlexaFluor 647 goat anti-rabbit (Molecular Probes) at 1:500; AlexaFluor 568 goat anti-rat (Molecular Probes) at 1:500 were used. In randomly selected fields, at 63x, (by starting at the lateral tip of the upper blade of the GCL and then skipping 2 field and imaging the 3rd, the 6th etc.), following the GCL (upper and lower blade) along the dentate gyrus, 50–100 BrdU cells per animal were categorized as astrocytes, or reactive astrocytes following the criteria described previously (Encinas et al., 2006; Encinas and Enikolopov, 2008; Sierra et al., 2015). Astrocytes were identified as S100β-positive cells, negative for Nestin-GFP staining, with stellar morphology. Reactive astrocytes were defined as Nestin-GFP-positive cells immunopositive also S100β and with hypertrophic soma, thickened processes and star-like morphology. Those cells in the uppermost focal plane were not counted to avoid overestimation. The total number of astrocytes and of reactive astrocytes was estimated quantifying their number in the granule cell layer and hilus in one set of slices and then multiplying by 6.

2.4 Microdrive construction

Custom-made microdrives carrying 6 electrodes (Microprobes) were assembled for simultaneous bilateral electrophysiological recording of local field potentials in the prefrontal cortex and the dorsal CA1 area of the hippocampus. One electrode was inserted in the bundle targeting the prefrontal cortex, and two electrodes in the bundle targeting the hippocampus. Each electrode was cemented to a single fixed bundle and was connected to an 8-channel printed circuit board assembled to an Omnetics connector.

2.5 Stereotaxic surgery

Animals aged 60 days were used for implantation of microdrives. Mice were initially anesthetized with isoflurane (4% isoflurane with 100% O_2) before being placed in a stereotaxic frame, and anesthesia was maintained using isoflurane (1–2% isoflurane with 100% O_2) until the surgery was finished. Rectal temperature was monitored, and core temperature (37°C) was maintained with a homeothermic blanket. After incision in the scalp, two burr holes were drilled at stereotaxic coordinates targeting the prefrontal cortex (1.94 mm AP, 0.5 mm ML, from Bregma) and the CA1 region of the hippocampus (−1.54 mm AP, 1.5 mm ML from Bregma). We targeted the CA1 area because, as the main output region of the hippocampus, it establishes strong functional connectivity with the frontal cortex that is crucial for spatial navigation, memory formation and sleep coordination (Siapas and Wilson, 1998; Negrón-Oyarzo et al., 2018). The dura was removed, and a glass pipette was descended through the brain (2 mm DV) to inject KA directly on the hippocampus. After injection, the pipette was removed and the recording electrodes were lowered to the cortical surface. Two ground wires were attached to skull screws, and the microdrive was affixed to the skull with dental acrylic. After surgery, animals were maintained in individual cages in a room with controlled temperature (22 ± 2°C) and humidity, with food and water available ad libitum. Mice were allowed to recover for 1 week after surgery before beginning behavioral experiments and electrophysiological recordings. During recovery, weight and general health were monitored daily, and animals received an intraperitoneal dose of analgesic (Ketoprofen, 5 mg/kg/day) and antibiotic (Enrofloxacin, 5 mg/kg/day). One day before surgery, mice were I.P. injected with either saline (i.e., control) or TNP-ATP (i.e., treated, 10 mg/kg). The procedure was then repeated for 7 days after surgery.

2.6 Electrophysiological recordings

After a week of recovery from surgery, mice were placed in a custom-built booth, and microdrives were connected to a headstage (model RHD2116 Intan Tech, CA, USA). Neural signals were amplified (200 times), digitized (sampled at 20 kHz), filtered (0.5–5,000 Hz), and monitored through an amplifier board (RHD2000 evaluation system; Intan Tech, CA, USA).

2.7 Behavioral experiments

For the probabilistic feeding task, animals were exposed to a FED3 [feeding experiment device (Nguyen et al., 2016)] that dispensed a pellet (sweet, popped quinoa) from the central port every time the animal nose-poke on one of the lateral ports. The probability of receiving a pellet was larger in one lateral port (80 vs. 20%), and after a variable number of trails (15 ± 2), the rule was reversed. Both the operant behavior (video tracking) and the electrophysiological activity were simultaneously recorded for 10 min. For the metric spatial change task, animals underwent 4 sessions in a rectangular arena (50 cm × 30 cm × 30 cm high), each lasting 5 min. During the first 3 sessions, animals were allowed to explore two objects placed 20 cm apart and positioned 5 cm from the arena walls, with a 3-min intersession interval. In the last session (test), following a 10-min intersession interval, the objects were repositioned so that the separation between them was reduced to 10 cm (Goodrich-Hunsaker et al., 2008). Importantly, the exploration time of individual objects was not measured. Instead, the total exploration time for both objects combined was calculated and compared between the sample and test conditions.

2.8 Cross-frequency modulation

Phase-amplitude cross-frequency coupling modulation index for several frequency pairs of theta-band "phase-modulating" and 2–200 Hz "amplitude-modulated" components was evaluated. We first filtered spectral components of the LFPs in the theta frequency

(4–8 Hz) and amplitude bandwidth 4 Hz at 2-Hz-steps were used to obtain the amplitude between 2 and 200 Hz. We next calculated the Hilbert transform to obtain the instantaneous phase of theta oscillation and the instantaneous wideband amplitude oscillations. The modulation index was obtained by means a normalized entropy index described in Tort et al. (2008).

2.9 Cross-correlations

Interictal discharge onset was defined with point 0 to correlate both hippocampal neural activity and contralateral ID onset by applying the "sliding-sweeps" algorithm. The timestamps of contralateral interictal discharges and hippocampal spikes within a time window (0.5 and 1.5 s respectively) were considered as a template and were represented by a vector of spikes relative to $t = 0$ s, with a time bin (100 and 200 ms respectively). Next, the window was shifted to successive interictal discharges onset throughout the recording session, and an array of recurrences of templates was obtained. Cross-correlations were obtained by averaging the array and normalizing to the basal activity.

2.10 Granger causality

The multivariate Granger causality (MVGC) Matlab toolbox was used to assess pairwise causalities between LFP–LFP activity. This toolbox, available online,[1] allows a fast and accurate estimation of the Wiener–Granger causal inference in the frequency domain. Estimators were calculated with the standard ordinary least squares and with a model order of 50. Frequency resolution was set at 1000.

2.11 Spectrograms

Time-frequency spectra for LFP recordings were computed by a wavelet time frequency transformation (using Morlet wavelets) based on convolution in the time domain and using the FieldTrip function ft_freqanalysis.m.[2]

2.12 Histology

After completion of experiments, mice were anesthetized with isoflurane (3%), and then transcardially perfused with 20 mL of saline solution followed by 50 mL of 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4). The brain was removed, incubated overnight in 4% paraformaldehyde in PBS, and then stored in PBS containing 0.2% sodium azide. Coronal brain slices (60 µm) were prepared from paraformaldehyde-fixed brains with a vibratome (World Precision Instruments, Sarasota, USA) in ice-cold PBS. For visualization of the electrolytic lesions, slices were stained with Nissl staining, and images were acquired with a microscope (Nikon).

2.13 Locomotor patterns

DeepLabCut, an open-source video tracking system, was used to automatically collect the mouse's instantaneous position along the track. The animal trajectory was smoothed (mobile mean of 10 successive points) to avoid artifacts due to space discretization, and the animal's instantaneous speed was quantified as the mean displacement between adjacent frames divided by the frame time (camera acquisition at 30 fps).

2.14 LFP spectral power and coherence

For analysis of power of oscillatory activity, electrophysiological recordings were downsampled to 1,000 Hz, bandpass-filtered at 0.1–100 Hz, and power spectral density (PSD) and coherence were computed using multitaper Fourier analysis from the Chronux toolbox.[3] Interictal discharges were detected by high pass filtering (>40 Hz; Butterworth, order = 4) the CA1 LFP and the z-scored squared signal was calculated. Peaks above 5 standard deviations were identified as putative interictal discharges. For PSD analysis, field potentials were divided into 5,000 ms segments with 500 ms overlap, and a time-bandwidth product (TW) of 3 and 5 tapers. For spectral coherence, field potentials were divided into 2000 ms segments with 100 ms overlap, and a time-bandwidth product (TW) of 5 and 9 tapers. Mean spectral power and coherence measures were calculated for theta (6–10 Hz), slow-gamma (20–40 Hz), and fast-gamma (60–80 Hz) bands for the entire trial session, and separately for start, navigation, and escape phases of the task.

2.15 Spike sorting

Spike sorting was performed offline using MATLAB-based graphical cluster-cutting software, Mclust/Klustakwik toolbox (version 3.5). To detect spikes, broadband recordings sampled at 20 kHz were filtered at 600–5000 Hz, and events with amplitudes reaching 5–50 standard deviations over the mean were considered as candidate spikes. For each recording file, single channels from individual tetrodes were identified, and a single file was generated per channel. Then, generated files from the same electrode were clustered by Principal Components Analysis (PCA) using MClust 3.5 toolbox running on MATLAB. The spike features considered for clustering included peak amplitude (the maximum height of the waveform of each channel of each spike), timestamp (the time of occurrence of each spike), spike width (the duration in each channel of each spike), energy (the energy contained within the waveform of each channel of the spike), valley (the maximum depth of the waveform of each channel of the spike), and wave-PC (the contribution to the waveform due to the principal component). MClust automatically identified similar clusters by assigning values between 0.0–1.0 for each cluster, in which similar clusters displayed values close to 1.0. Similarity between clusters was manually confirmed through visual inspection of spike features. Final confirmation was done by examining the ISI

1 https://users.sussex.ac.uk/~lionelb/MVGC/html/mvgchelp.html#1

2 http://fieldtrip.fcdonders.nl/

3 http://www.chronux.org

for every single unit and establishing that no spikes were present at ISIs shorter than 2 ms. Finally, a file for every single unit including timestamps of spikes for every tetrode was generated and exported, and this file was used in the subsequent analysis on MATLAB.

2.16 Statistical analysis

Comparison between behavioral parameters (latency time, errors) and other normally distributed parameters were analyzed with parametric analysis (t-student test; one-way ANOVA followed by Bonferroni post-hoc test). Comparison between firing rates and other non-normally distributed parameters were analyzed with non-parametric tests (Mann–Whitney U-test; Kruskal-Wallis test followed by Dunn's multiple comparison post-hoc test). Linear correlations between parameters were analyzed by Spearman correlation test. Comparisons between the slopes of significant linear regressions were performed using a bootstrap resampling method with replacement and testing the differences between the distributions of the slopes over 1,000 iterations. Comparisons between PETHs were analyzed with the Wilcoxon test. Statistical analysis was performed with GraphPad Prism software or with MATLAB (The MathWorks Inc.). Significant differences were accepted at $p < 0.05$. Summary statistical results are presented in Supplementary Table 1.

3 Results

We injected a single dose of kainic acid (KA, 50 nL, 2.22 mM) in the right dorsal CA1 area of the hippocampus in anesthetized mice to model early stages of mesial temporal lobe epilepsy development (Bouilleret et al., 1999; Sheybani et al., 2018). This is considered an intermediate dose, with a single injection effectively inducing mild non-convulsive seizures (Bielefeld et al., 2017), making it suitable for studying interictal activity and behavioral performance (Figure S1). Concurrently, during stereotaxic surgery, we implanted an electrode array targeting both the medial prefrontal cortex and dorsal CA1 area (Figure S2). Furthermore, mice were randomly injected the day before surgery with either saline (i.e.; control) or TNP-ATP (i.e.; treated), a potent P2XR blocker (North and Surprenant, 2000), to evaluate its effect on epileptiform activity and cognitive performance. To assess the effect of TNP-ATP, we quantified astrogliogenesis and astrogliosis in the hippocampus (Figure S3). Treated mice exhibited a reduced number of astrocytes and reactive astrocytes compared to control mice. Since the expression of nestin is a hallmark of reactive astrocytes, we used nestin-GFP transgenic mice for this experiment (Mignone et al., 2004). Astrocytes expressed GFAP but not nestin-GFP, while reactive astrocytes expressed both biomarkers (Figure S3). Hence, TNP-ATP reduced both astrogliogenesis and astrogliosis.

Next, we evaluated epileptiform activity in control and treated animals. We identified interictal discharges by prominently large, rapid deflections in the local field potentials of the dorsal hippocampus (Figures 1A,B). The amplitude of interictal discharges was affected by TNP-ATP, as it was significantly decreased in treated animals when compared to control mice (two-sided Wilcoxon rank-sum test, $p = 0.015$, Figure 1C), consistently propagating from the injection site to the contralateral hemisphere (Figure 1D). Such propagation was slightly, though significantly, diminished by TNP-ATP (unpaired t-test, $p = 0.012$, Figure 1E). Importantly, high frequency activity (>40 Hz) was not different between treated and control animals (Figure S4), thus differences in baseline activity are unlikely to affect the proper determination and identification of interictal events. Furthermore, the incidence of interictal discharges was significantly diminished by TNP-ATP (unpaired t-test, $p = 0.019$, Figure 1F), particularly on the KA-injected hemisphere (unpaired t-test, $p = 0.007$). Conversely, TNP-ATP did not influence the duration of interictal discharges (Figure 1G), nor did it affect unit discharge (Figure 1H). However, we observed a significant decrease in the coupling of neuronal spiking to interictal discharges, as demonstrated by the crosscorrelogram between both activity patterns (unpaired t-test, $p = 0.012$, Figure 1I). During spontaneous behavior, animals in both control and treated groups explored and moved around the box at similar speeds (unpaired t-test, $p = 0.58$). However, the onset of interictal discharges triggered a consistent decrease in movement speed, thus altering movement patterns (Figure 1J). Interestingly, the speed decrease was slightly, though significantly, attenuated by TNP-ATP (one-way ANOVA, $p = 1.34\times10^{-7}$, Figure 1K). Collectively, these findings indicate that blocking P2XRs diminishes several key aspects of epileptiform activity in the temporal lobe.

We then assessed cognitive function in the epilepsy model. For this, we took advantage of the feeding experimentation device [FED (Nguyen et al., 2016)], a programmable pellet dispensing machine that releases food with varying probability (Figure 2A). A pellet was delivered upon nose-poking with larger probability in one spout of the FED (80%), and after a variable number of trails (15 ± 2), the rule was reversed. In order to maximize the amount of collected reward in this probabilistic feeding task, mice had to acquire the reversal rule. During early stages of training, both groups exhibited similar nose poking behavior, which significantly increased after several days of training (Pearson correlation, $p < 0.001$, Figure 2B). However, the rate of nose-poking was larger in treated animals when compared to control mice (Bootstrap test, $p < 3.1\times10^{-15}$, Figure 2B), thus suggesting increased motivation in the task. Similarly, both groups significantly increased the collected reward during training (Pearson correlation, $p < 0.01$, Figure 2C). Nevertheless, treated animals collected pellets at a higher rate compared to control mice (Bootstrap test, $p < 2\times10^{-18}$, Figure 2C). Further, latency for pellet collection quickly decreased over sessions for both treated and control animals (Pearson correlation, $p < 0.05$, Figure 2D), thus suggesting that treated animals were faster to acquire and reinforce nose poking behavior. Moreover, treated animals decreased their latency at a faster rate compared to control mice (Bootstrap test, $p < 10^{-20}$, Figure 2D). These findings suggest that treated mice displayed greater motivation and task engagement compared to control animals. However, the efficiency of pellet collection did not improve over time for either experimental group (Pearson correlation, $p > 0.05$, Figure 2E), suggesting that increased behavioral motivation did not reflect the acquisition of task-relevant rules. Indeed, alternance, the simplest behavioral strategy for pellet collection, did not change over time for either experimental group (Pearson correlation, $p > 0.05$, Figure 2F). Therefore, our results indicate that although cognitive flexibility remained unaffected, general task motivation and engagement improved in treated animals.

We then assessed neural activity in the hippocampus and prefrontal cortex during the feeding task. Theta oscillations (4–10 Hz) dominated hippocampal activity during task performance in both control and treated animals (Figure 3A), which were not affected by

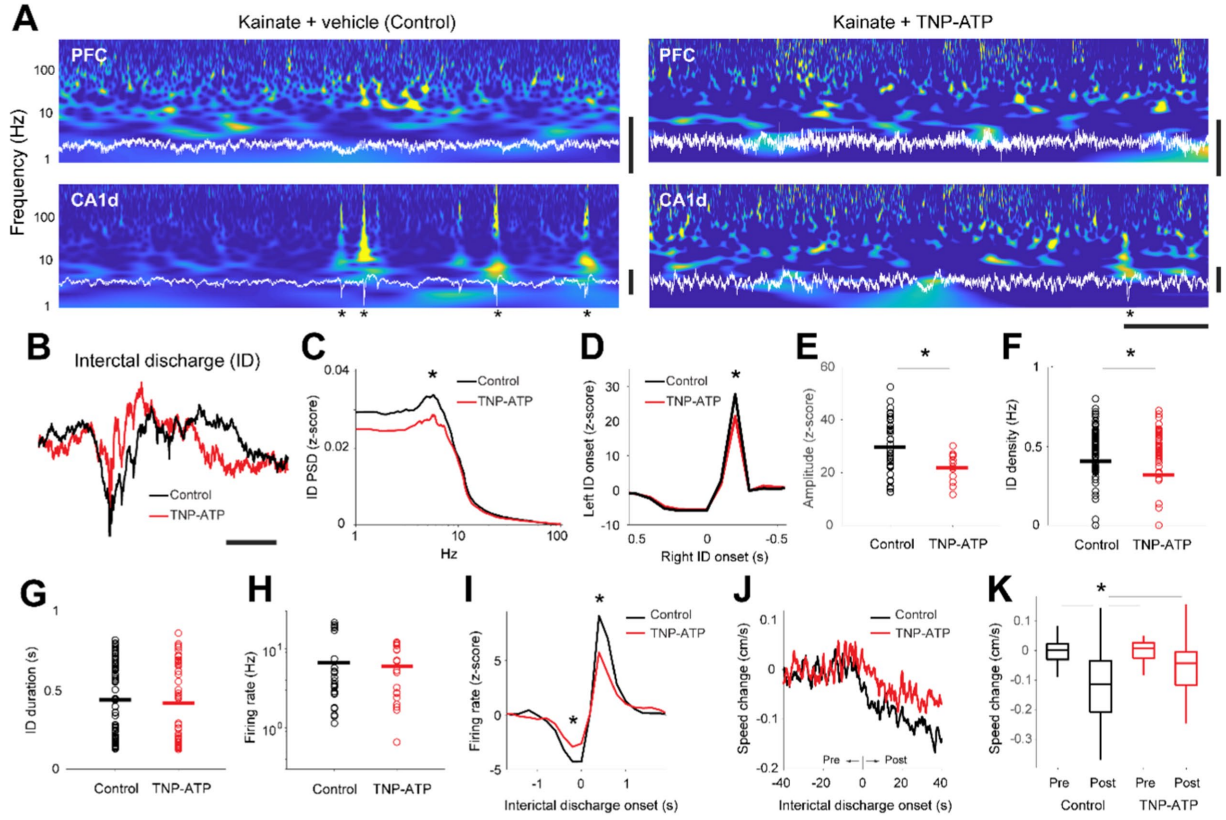

FIGURE 1
Interictal activity in the hippocampus of an epilepsy rodent model. **(A)** Spectrograms (blue) calculated from the local field potentials (white) recorded in the medial prefrontal cortex (PFC) and dorsal hippocampus (CA1), for animals injected with saline (left, kainite + vehicle) or a purinergic receptor antagonist (right, kainate + TNP-ATP). Asterisks depict interictal discharges in the dorsal hippocampus. Horizontal bar, 1 s; Vertical bars, 1 mV. **(B)** Overlay average interictal discharges (ID) recorded in the dorsal hippocampus from control (black) and TNP-ATP-treated animals (red). Scalebar, 200 ms. **(C)** Power spectral density of detected interictal discharges for control (black) and treated (red) animals. Power differed at peak frequencies (6 Hz) of interictal discharges (two-sided Wilcoxon rank-sum test, $z = 2.4$, $p = 0.015$). **(D)** Crosscorrelograms between interictal discharges detected on the right hemisphere (reference time) and left hemisphere. Note that interictal activity in the right hemisphere precedes left hemisphere discharges [unpaired t-test, $t(47) = 2.2$, $p = 0.031$, 27.7 ± 1.6 z-score, control; 21.4 ± 0.8 z-score, TNP-ATP]. **(E)** Amplitude of interictal discharges for control (black) and treated (red) animals (unpaired t-test, $p = 0.012$, 29.6 ± 1.6 z-score, control; 21.9 ± 0.9 z-score, TNP-ATP). **(F)** Density of interictal discharges for control (black) and treated (red) animals [unpaired t-test, $t(158) = 2.3$, $p = 0.019$, 0.41 ± 0.02 Hz, control; 0.32 ± 0.03 Hz, TNP-ATP]. **(G)** Duration of individual interictal discharges for control (black) and treated (red) animals [unpaired t-test, $p = 0.64$, $t(120) = 0.46$, 0.44 ± 0.03 vs. 0.42 ± 0.04 Hz]. **(H)** Average firing rates recorded from single units per session [unpaired t-test, $p = 0.69$, $t(40) = 0.39$, 6.7 ± 1.36 vs. 5.98 ± 0.96 Hz]. **(I)** Crosscorrelograms between interictal discharges and firing rates recorded from single units (before ID onset, unpaired t-test, $p = 0.012$, −4.3 ± 0.39 z-score, control; −2.9 ± 0.32 z-score, TNP-ATP; after ID onset, unpaired t-test, $p = 0.012$, 9.10 ± 0.95 z-score, control; 5.74 ± 0.80 z-score, TNP-ATP). **(J)** Average changes in locomotion speed during recordings of spontaneous behavior in reference to the onset of interictal discharges. **(K)** Box plots showing changes in locomotion speed preceding (pre) and following (post) the onset of interictal discharges. Note the 'control post' was larger than the other conditions. Asterisk depicts significant differences produced by TNP-ATP (one-way ANOVA, $p = 1.34 \times 10^{-7}$. *Post hoc* Tukey–Kramer's multiple comparison test; control post vs. control pre, $^*p = 1.1 \times 10^{-7}$; control post vs. TNP-ATP pre, $p = 3.3 \times 10^{-6}$; control post vs. TNP-ATP post, $p = 0.03$). Asterisks depict statistically significant differences.

TNP-ATP (two-sided Wilcoxon rank-sum test, $p = 0.25$, Figure 3B). In addition, the 4-Hz oscillation (Fujisawa and Buzsáki, 2011) was prominent in the prefrontal cortex, which was significantly decreased in treated animals (two-sided Wilcoxon rank-sum test, $p = 6.4\times10^{-9}$, Figure 3C). The power of faster activity patterns, like gamma oscillations (30–80 Hz), remained unaffected by purinergic blockade in both the hippocampus (two-sided Wilcoxon rank-sum test, $p = 0.48$) and prefrontal cortex (two-sided Wilcoxon rank-sum test, $p = 0.99$). No changes in cross frequency modulation were detected between the hippocampal theta rhythm and prefrontal cortex oscillations upon treatment (false discovery rate, $p > 0.05$, Figure 3D), yet the comodulation between slow 4-Hz cortical oscillations and gamma hippocampal oscillations was significantly enhanced by TNP-ATP (two-sided Wilcoxon rank-sum test, $p = 0.01$, Figure 3E). Moreover, we further investigated hippocampo-cortical interactions with Granger causality, which provides information about the directionality of connectivity between neural nodes (Friston et al., 2013). Hence, we found in control animals, and as previously reported (Broggini et al., 2016), a significant increase in ascending causality (i.e.; CA1 - > PFC) in both the theta and gamma bands during interictal intervals, whereas the descending drive (i.e.; PFC - > CA1) was not affected by TNP-ATP (false discovery rate, $p > 0.05$, Figure 3F). Moreover, the ascending drive was significantly reduced in treated animals, in both the theta range (two-sided Wilcoxon rank-sum test, $p = 3.3\times10^{-5}$, Figure 3G) and gamma band (two-sided Wilcoxon rank-sum test, $p = 8.2\times10^{-4}$, Figure 3H).

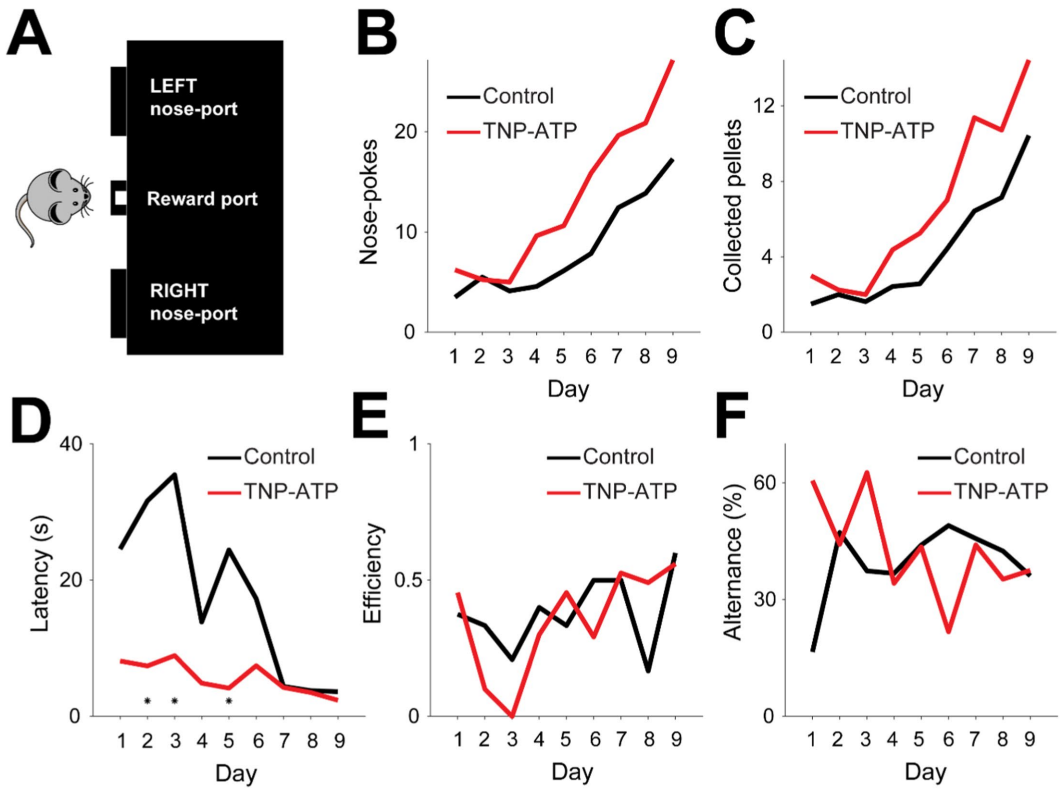

FIGURE 2
Behavioral performance during the probabilistic feeding task. **(A)** Diagram of the Feeding Experimentation Device (FED) used to implement the behavioral task. Mice were trained to nose poke on the lateral spouts to obtain reward (i.e., a small piece of food pellet) in the middle spout. Lateral spouts had fixed reward-delivering probabilities (80 and 20%), which were then reversed at the end of every training block (15 ± 2 trials). **(B)** Average number of nose pokes performed by mice in relation to training day. Both control and treated animals increased their number of nose pokes (Pearson correlations; control, $p = 3.5\times10^{-4}$; TNP-ATP, $p = 7.5\times10^{-4}$), yet the increase rate was significantly larger for TNP-ATP-injected animals (Bootstrap test for regression slopes, 100 iterations, $p = 5\times10^{-4}$; 1.67 ± 0.01 nose pokes/day, control; 2.77 ± 0.01 nose pokes/day, TNP-ATP). **(C)** Average number of collected reward by mice in relation to training day. Both control and treated animals increased the number of collected pellets, yet the increase rate was significantly larger for TNP-ATP-injected animals (Pearson correlation, control, $p = 4.9\times10^{-5}$; TNP-ATP, $p = 1.3\times10^{-3}$. Bootstrap test for comparing linear regression slopes, 100 iterations, $p = 0.02$, 1.05 ± 0.01 pellets/day, control; 1.55 ± 0.01 pellets/day, TNP-ATP). **(D)** Average latency to collect pellets from the middle nozzle by mice in relation to training day. Both control and treated animals progressively decreased their latency, yet it was significantly smaller for TNP-ATP-injected animals (Pearson correlation, control, $p = 3.1\times10^{-3}$; TNP-ATP, $p = 0.013$. Bootstrap test for comparing linear regression slopes, 100 iterations, $p = 10^{-4}$, −0.68 ± 0.01 s/day, control; −3.87 ± 0.03 s/day, TNP-ATP). **(E)** Task efficiency in relation to training day. Efficiency was calculated as the ratio between the total number of collected pellets and total nose pokes (Pearson correlation, control, $p = 0.35$; TNP-ATP, $p = 0.08$). **(F)** Alternance proportion in relation to training day (Pearson correlation, control, $p = 0.24$; TNP-ATP, $p = 0.09$).

Next, we assessed hippocampo-cortical coordination during performance of the probabilistic feeding task. For this, we behaviorally separated states of task engagement (i.e., nose poking or pellet collection) from non-engaged states (i.e., arena exploration or grooming) (Figure 4A). That is, animals were considered as task-engaged only during direct interaction with the FED. Typically, during task-engaged states mice exhibited little displacement, whereas during non-engaged states animals moved more around in the arena (Figure S5). Therefore, to control for variations in movement patterns, we analyzed only those task episodes where the movement speed fell within two standard deviations from the average speed at the FED (Figure S5). We then calculated and compared the phase coherence between field potentials recorded in the hippocampus and prefrontal cortex during task performance (one-sided Wilcoxon signed rank test, $p < 0.004$, Figure 4B) and found coupling in the theta band to be significantly larger during task engagement when compared to non-engaged states (one-sided Wilcoxon signed rank test, $p = 4.6\times10^{-4}$, Figure 4C). This result was specific to the theta band, as it was not observed in either the 4-Hz (one-sided Wilcoxon signed rank test, $p = 0.38$, Figure 4D) or during gamma oscillations (one-sided Wilcoxon signed rank test, $p = 0.53$, Figure 4E). Interestingly, theta phase coherence was larger upon the application of TNP-ATP when compared to control animals (two-sided Wilcoxon signed rank test, $p < 4.6\times10^{-3}$, Figures 4F,G), thus suggesting that the coordination between hippocampus and prefrontal cortex was stronger upon blocking purinergic transmission. We also recorded a group of animals with electrodes located in the posterior parietal cortex, which allowed us to compute the coordination between cortical regions. Neural coupling between prefrontal and posterior parietal cortex was not affected during task execution (Figure S6), thus enhanced inter-regional coordination seemed to be specific for the hippocampus and prefrontal cortex axis.

Finally, we further investigated behavioral performance in a spatial memory task. In this task, metric spatial change task (Kannangara et al., 2015), mice were trained to explore an arena with two objects. They then re-explored the same environment with

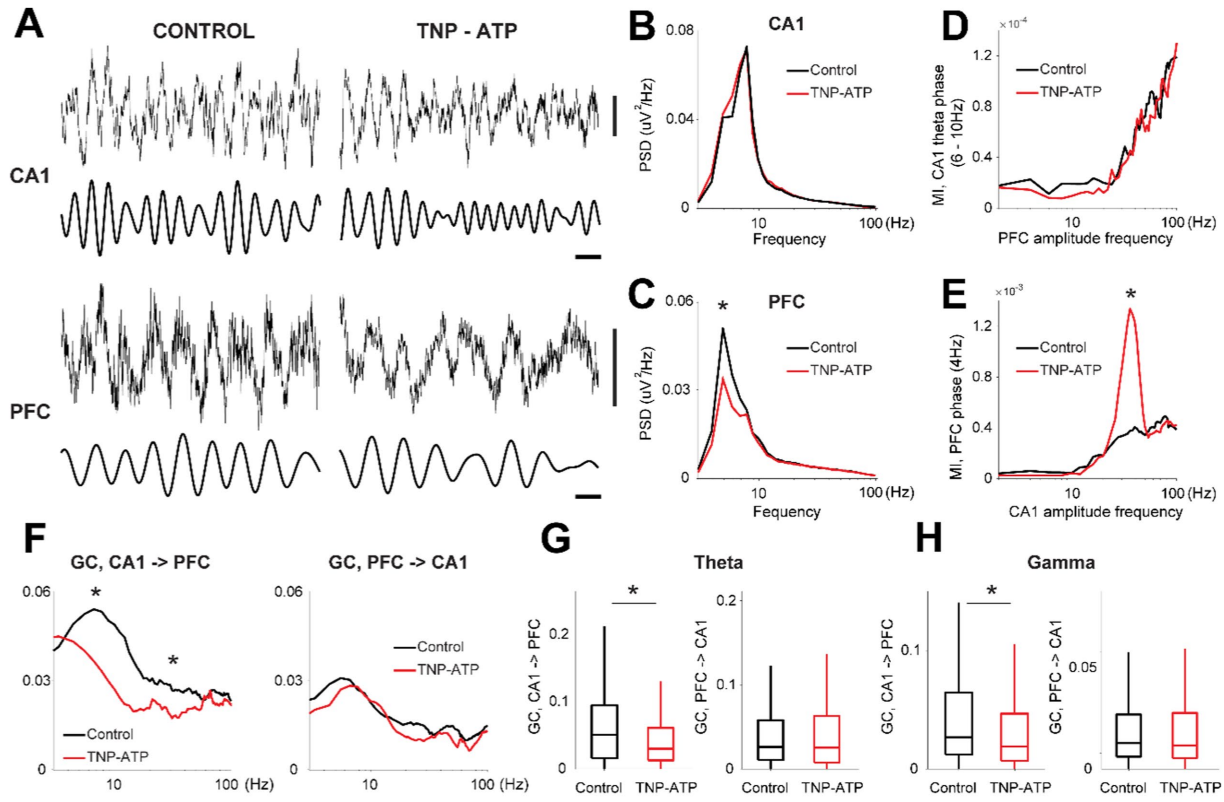

FIGURE 3
Oscillatory network activity during performance of a probabilistic feeding task. **(A)** Examples of cortical oscillations recorded during task performance. LFP (0.1–500 Hz), delta (3–4 Hz), theta (5–10 Hz). Horizontal bar, 200 ms; Vertical bars, 200 uV. Average power spectral distribution for LFP recording from hippocampus **(B)** and prefrontal cortex **(C)** for control and treated animals (two-sided Wilcoxon rank-sum test, $z = 3.5$, $p = 4.0\times10^{-4}$). Cross frequency modulation (MI, modulation index) between the phase of slow oscillations and the amplitude of fast oscillations for the hippocampus (**D**, theta) and prefrontal cortex (**E**, 4-Hz) for control and treated animals (two-sided Wilcoxon rank-sum test, $z = -2.57$, $p = 0.01$). **(F)** Granger causality for ascending (left, CA1 - > PFC) and descending (right, PFC - > CA1) drives for control and treated animals (two-sided Wilcoxon signed rank test with false discovery rate correction for multiple comparisons, $p < 0.001$). Box plot representation for the magnitude of Granger causality for theta (**G**, two-sided Wilcoxon rank-sum test, $p = 3.3\times10^{-5}$) and gamma (**H**, two-sided Wilcoxon rank-sum test, $p = 8.2\times10^{-4}$) frequency bands. Asterisks depicts significant differences.

the objects slightly displaced (Figure 5A). During the test trial, animals typically exhibit increased exploration of the objects if they detect changes in the environment, compared to the sample trial. This increase, measured by the discrimination index, serves as an indicator of hippocampus-dependent spatial memory. We observed that control animals did not recognize the displacement of objects in the test trial (Figure 5B). In contrast, treated mice showed a positive discrimination index, indicating that spatial memory was partially improved in relation to control mice (Figure 5C). These findings suggest that TNP-ATP may partially restore hippocampal function in a murine model of epilepsy, yet future studies (including control wild-type mice) are necessary to better understand the extent to which hippocampal function is normalized. At the end of experimental procedures, we exposed animals to an open field to assess their spontaneous behavior. General motor function seemed to be similar between control and treated animals, as their covered distance in the arena was not significantly different (Figure S7). Similarly, markers of anxiety, such as the time spent at the walls or number of fecal boli, were not significantly different between groups (Figure S7), thus suggesting that purinergic blockade does not affect motor function or anxiety levels in KA-injected animals.

4 Discussion

The pursuit of novel targetable pathological mechanisms underlying temporal lobe epilepsy is driven by the inadequacy of current antiepileptic drugs to control seizures in about one third of patients and their lack of impact on the underlying pathophysiology (Bialer and White, 2010; Pitkänen and Lukasiuk, 2011; Kobow et al., 2012). Among several proposals, altered purinergic signaling has accumulated evidence as a potential mechanism in epilepsy (Di Virgilio et al., 2023; Engel, 2023). Indeed, after injury to the central nervous system, ATP is massively released, enough as to activate the low-affinity P2XRs (Illes, 2020). The role of the P2XRs is versatile, as initially, their activation may contribute positively to neuroinflammation by triggering microglia activation and inflammasome-mediated IL-1 release, among other effects, as previously described (Roth et al., 2014; Sperlágh and Illes, 2014). However, this benefit is countered by the potential detrimental effects of aberrant P2XR expression, which can sustain neuroinflammation, induce neuronal death, and promote hyperexcitability (Murphy et al., 2012; Franceschini et al., 2015). Previous research has identified overexpression of P2X7Rs in epilepsy, but uncertainties persisted

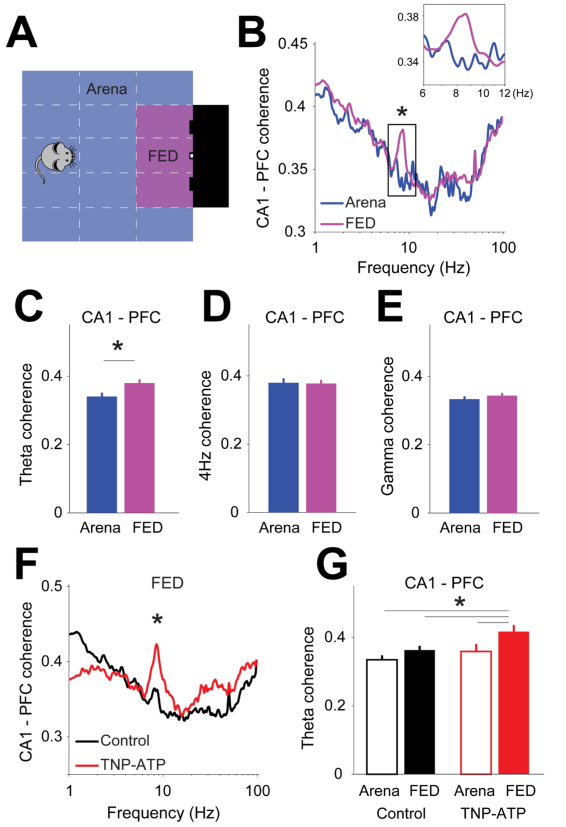

FIGURE 4
Cortical connectivity during performance of a probabilistic feeding task. **(A)** Definition of task-engaged (i.e., FED) and non-engaged (i.e., arena) zones during behavioral test. **(B)** Phase coherence between hippocampus and prefrontal cortex for task-engaged and non-engaged areas (one-sided Wilcoxon signed rank test with false discovery rate correction for multiple comparisons, $p < 0.004$). Inset depicts theta band phase coherence. Bar plots of phase coherence for theta (**C**, one-sided Wilcoxon signed rank test, $z = -3.5$, $p = 4.6\times10^{-4}$), 4-Hz (**D**, one-sided Wilcoxon signed rank test, $z = -0.87$, $p = 0.38$), and gamma (**E**, one-sided Wilcoxon signed rank test, $z = -0.63$, $p = 0.53$) frequency bands. **(F)** Phase coherence between hippocampus and prefrontal cortex for control and treated animals (two-sided Wilcoxon signed rank test with false discovery rate correction for multiple comparisons, $p < 4.6\times10^{-3}$). **(G)** Bar plots depicting theta oscillation phase coherence during task performance. Note blocking purinergic transmission increases coherence selectively during task-engaged states (two-way ANOVA: arena vs. FED, $p = 0.02$; control vs. TNP-ATP. Post-hoc Tukey–Kramer's test to correct for multiple comparisons, $p = 0.03$; control arena vs. TNP-ATP FED, $p = 8.9\times10^{-4}$; control FED vs. TNP-ATP FED, $p = 0.016$; TNP-ATP arena vs. TNP-ATP FED, $p = 0.002$). Asterisks depict significant differences.

regarding the specific cell types involved and the functional role of the receptor. The potential for targeting P2XR has been explored predominantly in models of acute status epilepticus (Doná et al., 2009; Kim and Kang, 2011; Jimenez-Pacheco et al., 2013). Nevertheless, the cognitive effects of purinergic signaling in epilepsy remain little studied. This gap in knowledge underscores the need for further investigation into the role of purinergic receptors in epilepsy and their potential as a therapeutic target.

Here, we studied the influence of purinergic receptor signaling on epileptiform activity and cognitive function in a murine model of mesial temporal lobe epilepsy, prompted by KA injection in the dorsal CA1 area. We targeted P2XRs using TNP-ATP, a selective purinergic receptor antagonist, and examined behavioral performance and neural activity patterns. Our results show that TNP-ATP treatment had little effect on motor function or anxiety levels, yet it reduced both the amplitude and incidence of hippocampal epileptiform activity. Neuronal spiking associated with interictal activity was also depressed. Furthermore, hippocampus-dependent spatial memory and prefrontal cortex-dependent executive function were partially restored when behaviorally assessed. These results support the potential role of purinergic receptor antagonists in improving behavioral and cognitive performance in epilepsy, suggesting novel insight into the use of these pharmacological agents as a therapeutic approach.

While our study demonstrates promising effects of TNP-ATP on seizure reduction and cognitive function, we acknowledge the complexities associated with the timing and duration of treatment. Indeed, the ED50 of TNP-ATP varies across P2X receptor subtypes, with high potency at P2X1 receptors (ED50 around 1 nM) but lower affinity for P2X7 receptors (ED50 in the micromolar range) (Bianchi et al., 1999). Based on these pharmacodynamic properties, the plasma concentration of TNP-ATP 24 h after administration is likely below the effective threshold for receptor blockade. This raises the possibility that the observed effects reflect a combination of direct receptor blockade and indirect compensatory mechanisms triggered by prolonged receptor inhibition. These compensatory effects likely develop from the first day of treatment, as TNP-ATP was administered daily beginning 1 day before the kainic acid lesion. To address this limitation, future studies should disentangle these direct and indirect effects, to establish the potential contribution of compensatory mechanisms in shaping the observed outcomes, recognizing their potential influence on epileptiform activity, neuroinflammation, and cognitive function.

Past advancements in understanding the role of purinergic signaling, particularly the involvement of P2X7Rs, have highlighted their significance in the inflammation and progression of epilepsy (Engel et al., 2016; Fischer et al., 2016; Beamer et al., 2017). Notably, P2X7R antagonists, while showing limited efficacy in acute anticonvulsant tests and in fully kindled rats as standalone treatments, demonstrated enhanced anticonvulsant effects when used in conjunction with antiepileptic drugs (Fischer et al., 2016). Our study did not focus explicitly on quantifying anticonvulsant effects, yet we observed a marked reduction in both the amplitude and frequency of interictal discharges. This suggests a significant anticonvulsant potential for purinergic receptor antagonists even when administered independently. The discrepancies between our findings and previous studies could be attributed, at least partly, to differences in the epilepsy models used and the timing of drug administration. Indeed, our research model is induced by a single intrahippocampal injection of kainic acid (Lévesque and Avoli, 2013), which gradually evolves into an epilepsy model, and immediately initiated treatment with TNP-ATP. Contrastingly, Fischer et al. (2016) utilized a combination of pentylenetetrazol and electrical kindling to induce epilepsy and applied purinergic blockers only post-epilepsy induction (Fischer et al., 2016). Hence, in our model, the epileptiform activity had not fully developed into status epilepticus at the time of applying the purinergic receptor antagonist. Furthermore, our results align with previous findings indicating seizure reduction and neuroprotection in a murine model of epilepsy induced by intra-amygdalar kainic acid injection (Engel et al., 2012; Jimenez-Pacheco et al., 2016). This consistency underscores the potential of purinergic receptor antagonists in epilepsy treatment, warranting further investigation.

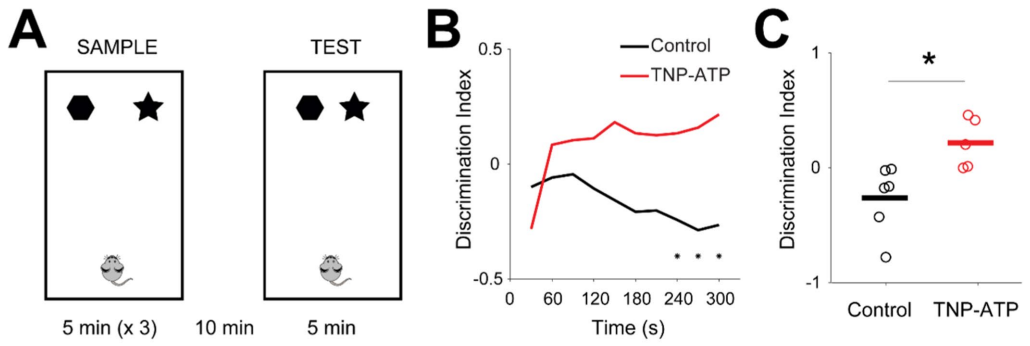

FIGURE 5
Spatial memory is rescued following blockade of purinergic receptors. **(A)** Diagram of the metric spatial change task. Mice were allowed to explore an arena containing two different objects, which were then positioned closer. **(B)** Discrimination index (ratio between the difference and the sum of exploration times of objects in the test trial and sample trial) in relation to time in the arena for control (black) and treated (red) animals (one-sided Wilcoxon signed rank test with false discovery rate correction for multiple comparisons, $p < 0.03$). **(C)** Average discrimination index for control (black) and treated (red) animals. Circles and lines represent individual mice and population averages, respectively [unpaired t-test, $t(9) = -3.03$, $p = 0.014$; -0.2 ± 0.11, control; 0.22 ± 0.09, TNP-ATP]. Asterisks point to statistically significant differences.

As explained, we also evaluated cognitive function. For this, we used the metric spatial change task (Kannangara et al., 2015) that assesses spatial memory. This test is particularly sensitive to spatial pattern separation, the brain's capacity to discriminate between similar, overlapping spatial representations and store them orthogonally, which largely resides in the dentate gyrus (Yassa and Stark, 2011). Adult neurogenesis, taking place in the dentate gyrus, is remarkably important for pattern separation (Clelland et al., 2009; Sahay et al., 2011) and we have previously shown that it is severely disrupted in epilepsy (Sierra et al., 2015; Muro-García et al., 2019). Hence, it was plausible to expect detrimental effects of epilepsy in both pattern separation and spatial memory formation. Indeed, our results are consistent with recent data from pilocarpine-induced status epilepticus showing pattern separation memory impairment (Choi et al., 2024). Moreover, our findings are consistent with the efficacy of pharmacological methods using valproate or endoneuraminidase to inhibit seizure-induced neurogenesis, which not only preserves hippocampal spatial memory but also rescues features of aberrant hippocampal neurogenesis (Jessberger et al., 2007; Pekcec et al., 2008).

The impact of epilepsy on temporal lobe function, particularly in the consolidation of episodic memories mediated by the hippocampus, has been well documented (Schwarcz and Witter, 2002; Helmstaedter and Kockelmann, 2006). Similarly, the integrity of the hippocampus is crucial for spatial memory, which aligns with our results in the metric test, corroborating previous studies (O'Keefe and Nadel, 1978; Moser et al., 2017). Additionally, recent studies are increasingly linking epilepsy with aspects of executive function, notably cognitive flexibility, which depends on the activity of the medial prefrontal cortex (Hermann et al., 2002; Helmstaedter and Witt, 2017). In our study, we observed that blocking purinergic transmission in a rodent model of epilepsy affected executive function. Although the treatment did not directly enhance task efficiency, measured by the ratio of rewards collected to trial attempts, nor did it alter the rate of alternation in nose poking, it significantly improved proactive nose poking behaviors and decreased the latency to collect rewards. These findings suggest enhanced task engagement upon purinergic receptor antagonism.

Importantly, the directionality and causality within the hippocampal-prefrontal cortex axis during task performance warrant further consideration. Enhanced theta- and gamma-band coordination between these regions, as observed in kainite-injected mice, may reflect a pathological enhancement of physiological directionality of information flow during specific phases of learning and memory retrieval (Broggini et al., 2016), which is decreased by our pharmacological treatment. This aligns with previous findings, which suggest that hippocampal ventral output to the prefrontal cortex is critical for retrieving stored information (de Mooij-van Malsen et al., 2023). Such restored coordination might underlie the observed improvements in behavioral performance and cognitive function in treated animals. While our study did not explicitly separate learning and retrieval phases, and our recordings were performed in the dorsal CA1 area, the data hints at the potential for TNP-ATP to facilitate these processes, a topic for further exploration in future studies.

Cortical regions are functionally connected during multiple behavioral states. The medial prefrontal cortex expresses dominant delta oscillations which are modulated by task demands and by hippocampal inputs. Indeed, there is abundant evidence of the modulation of prefrontal gamma activity by hippocampal oscillations (Siegel et al., 2012; Roux and Uhlhaas, 2014; Backus et al., 2016). In the reverse direction, the phase of cortical delta rhythms also modulates the amplitude of hippocampal gamma oscillations. Interestingly, our results show that blocking purinergic transmission was able to restore the coupling between slow cortical rhythms and fast hippocampal oscillations. Nonetheless, we found no effect in the opposite case. Moreover, we reproduced the previously described enhanced directionality in epilepsy of the theta-range activity from the dorsal hippocampus to the medial prefrontal cortex, as computed by Granger causality (Broggini et al., 2016). In our experiments, such causality was significantly decreased by purinergic receptor antagonism; yet, the opposite directionality, from the medial prefrontal cortex to the hippocampus was not modified by treatment. Importantly, our pharmacological treatment was able to restore the functional hippocampo-cortical connectivity selectively in the theta band during task execution, which has been previously demonstrated to be relevant for efficient task performance (Roux and Uhlhaas, 2014; Backus et al., 2016). These results suggest that output modulatory projections from the hippocampus are disrupted in the epilepsy model, but not input modulatory afferences, such as those arising from the medial prefrontal cortex. Additionally, blocking

purinergic receptors seems to partially restore the functional connectivity in the hippocampo-cortical axis. Overall, our results monitoring both the behavioral and functional connectivity impacts contributes to a substantiate the use of purinergic antagonists in epilepsy's pathophysiology and potential new treatment avenues.

Despite demonstrating that blocking purinergic signaling with TNP-ATP reduces seizure severity and improves behavioral outcomes, our findings must be interpreted in the context of several limitations. The overarching descriptor of purinergic receptor antagonism oversimplifies the complexity of purinergic signaling. Indeed, while we note that TNP-ATP inhibits P2X receptors, it is important to identify which of the seven P2X subtypes and potentially other purinergic receptors are involved. The purinergic system encompasses P2X, P2Y, and P1 receptors, each with distinct roles in neurotransmission and pathology; therefore, future studies will have to address a more precise identification of the receptor subtypes blocked for understanding the mechanisms behind our results.

Moreover, our study examined the acute effects of kainate, which induces seizures rather than chronic epilepsy, where spontaneous recurrent seizures are observed. Although we refer to epilepsy in our study, the present experiments primarily capture the acute seizure phase. In subsequent studies, it will be important to determine whether mice actually transition to a state of chronic epilepsy and to clarify when TNP-ATP is administered relative to both acute and potential chronic seizure episodes. Similarly, the extent to which TNP-ATP's effects on behavior are directly linked to its seizure-suppressing properties remains unresolved. While our data indicate that mice treated with TNP-ATP perform better in behavioral tests, correlating the degree of seizure reduction with behavioral outcomes would more definitively establish whether the improved behavior results primarily from decreased seizure severity or from additional neuroprotective or anti-inflammatory actions of TNP-ATP.

Finally, the pharmacokinetics of TNP-ATP in the brain, including its half-life and clearance, remains insufficiently characterized. Without confirming that TNP-ATP is still active at the time of kainate-induced seizures and behavioral testing, it is difficult to assert that all observed effects are indeed attributable to purinergic receptor blockade. Future studies employing more precise pharmacokinetic measurements, and possibly alternative, receptor-specific ligands, would clarify the duration and specificity of the action of TNP-ATP, thus strengthening our conclusions. By addressing these limitations, it will be possible to build a future stronger foundation for the therapeutic potential of purinergic antagonists in seizure-related disorders.

Data availability statement

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

Ethics statement

The animal study was approved by University of the Basque Country (UPV/EHU) Ethics Committees (Leioa, Spain) and Diputacio Foral de Bizkaia under protocol M20-2022-130 and M30-2022-129. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

NE: Conceptualization, Writing – review & editing, Data curation, Formal analysis, Investigation, Methodology. SM-S: Investigation, Methodology, Writing – review & editing. AL-V: Investigation, Writing – review & editing. TM: Investigation, Writing – review & editing. TM-G: Investigation, Writing – review & editing. GF: Investigation, Writing – review & editing. JE-P: Writing – review & editing, Project administration, Supervision. PF: Project administration, Supervision, Writing – review & editing, Conceptualization, Funding acquisition, Resources, Writing – original draft.

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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.

Generative AI statement

The authors declare that no Gen AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2025.1513135/full#supplementary-material

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