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Novel therapeutic mechanisms targeting neuro-immune regulation of neurological disorders

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Novel therapeutic mechanisms targeting neuro-immune regulation of neurological disorders

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REVIEW
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The Role of NLRP3 Inflammasome in Alzheimer's Disease and Potential Therapeutic Targets

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Alzheimer's disease (AD) is a common age-related neurodegenerative disease characterized by progressive cognitive dysfunction and behavioral impairment. The typical pathological characteristics of AD are extracellular senile plaques composed of amyloid *ß* (Aβ) protein, intracellular neurofibrillary tangles formed by the hyperphosphorylation of the microtubule-associated protein tau, and neuron loss. In the past hundred years, although human beings have invested a lot of manpower, material and financial resources, there is no widely recognized drug for the effective prevention and clinical cure of AD in the world so far. Therefore, evaluating and exploring new drug targets for AD treatment is an important topic. At present, researchers have not stopped exploring the pathogenesis of AD, and the views on the pathogenic factors of AD are constantly changing. Multiple evidence have confirmed that chronic neuroinflammation plays a crucial role in the pathogenesis of AD. In the field of neuroinflammation, the nucleotide-binding oligomerization domain-like receptor pyrin domain-containing 3 (NLRP3) inflammasome is a key molecular link in the AD neuroinflammatory pathway. Under the stimulation of Aβ oligomers and tau aggregates, it can lead to the assembly and activation of NLRP3 inflammasome in microglia and astrocytes in the brain, thereby causing caspase-1 activation and the secretion of IL-1β and IL-18, which ultimately triggers the pathophysiological changes and cognitive decline of AD. In this review, we summarize current literatures on the activation of NLRP3 inflammasome and activation-related regulation mechanisms, and discuss its possible roles in the pathogenesis of AD. Moreover, focusing on the NLRP3 inflammasome and combining with the upstream and downstream signaling pathway-related molecules of NLRP3 inflammasome as targets, we review the pharmacologically related targets and various methods to alleviate neuroinflammation by regulating the activation of NLRP3 inflammasome, which provides new ideas for the treatment of AD.

Keywords: Alzheimer's disease, inflammation, neuroinflammation, NLRP3 inflammasome, mitochondrial dysfunction

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1 INTRODUCTION

Alzheimer's disease (AD) is a common neurodegenerative disease that occurs in the elderly, and is also called senile dementia. The main clinical manifestations of AD patients are the progressive decline of self-care ability, cognitive impairment, and neuropsychiatric abnormalities, which seriously affects the quality of life of AD patients. The typical pathological features of AD are senile plaques related to extracellular amyloid ß (Aβ) deposition and neurofibrillary tangles formed by hyperphosphorylation of intracellular microtubule-associated tau (d'Errico and Meyer-Luehmann 2020). With the aging of the global society becoming more and more prominent, the increasing number of AD patients has become a major public health problem, which has brought a heavy burden to individuals, the society and families.

AD is first described by the German physician Alois Alzheimer in 1906 and has a history of more than one hundred years (Sanabria-Castro et al., 2017). The pathogenesis of AD is complex and diverse, which mainly involves genetic and environmental factors (Dunn et al., 2019), Aβ toxicity (Benilova et al., 2012), tau hyperphosphorylation (Wang et al., 2014), central nervous system (CNS) inflammation (Kinney et al., 2018), synaptic dysfunction (Li et al., 2018a), cholinergic deficiency (Frost et al., 2017), oxidative stress (Islam et al., 2019), mitochondrial dysfunction (Cardoso et al., 2004), autophagy and mitophagy abnormalities (Reddy and Oliver 2019), lipid metabolism disorder (Zhu et al., 2019), imbalance of calcium homeostasis (Popugaeva et al., 2015), endoplasmic reticulum (ER) stress (Huang et al., 2015), etc. Although the amyloid cascade hypothesis and the Tau protein theory are currently accepted by most investigators, the continuous and excessive neuroinflammatory response also plays a central role in the pathogenesis of AD. The nucleotide-binding oligomerization domain-like receptor pyrin domain-containing 3 (NLRP3) inflammasome is crucial in the neuroinflammatory pathway and has recently been highlighted as a potential target for AD treatment.

Inflammasome is a type of cytosolic multiprotein complex and plays a crucial role in innate immunity. The concept of inflammasome is first proposed by Tschopp and his colleagues in 2002. It is mainly composed of three parts: intracytoplasmic pattern recognition receptors (PRRs), the adaptor protein domain and the effector domain cysteine protease pro-caspase-1 (Martinon et al., 2002). In the CNS, the inflammasome mainly presents in the cytoplasm of immune cells, neuronal cells, microglia and astrocytes (Minkiewicz et al., 2013; von et al., 2018; Hanslik and Ulland 2020), and can recognize pathogen-associated molecular patterns (PAMPs) or host-derived danger-associated molecular patterns (DAMPs). Among the many reported inflammasomes, the NLRP3 inflammasome is currently the most studied one. Just like the structure of the above-mentioned inflammasomes, the NLRP3 inflammasome includes the sensor protein NLRP3, the adaptor protein apoptosis-associated speck-like protein containing a CARD (caspase activation and recruitment domain) (ASC), and the effector protein (pro-caspase-1, a cysteine protease) (Schroder and Tschopp 2010). These three proteins can interact closely to regulate the function of NLRP3 inflammasome. Once NLRP3 recognizes the foreign pathogen molecules or internal danger signals, it will be activated and undergos self-oligomerization. Then NLRP3 binds to the pyrin domain (PYD) domain of the adaptor protein ASC, and recruits the protease pro-caspase-1 to form the NLRP3 inflammasome, which cleaves pro-caspase-1 into activated caspase-1 through autocatalysis. The activated caspase-1, as an inflammasome effector protein, is able to cleave the inactive pro-inflammatory cytokines pro-IL-1β and pro-IL-18 into mature forms of IL-1β and IL-18, respectively. Ultimately, IL-1β and IL-18 are released outside of the cell to play a variety of non-specific inflammatory roles (Martinon et al., 2002; Kelley et al., 2019). In addition, the activated caspase-1 can also mediate a type of inflammatory-related programmed cell death, which is called pyroptosis. A large amount of inflammatory substances released after cell pyroptosis will induce a strong inflammatory response (Fink and Cookson 2006; Shi et al., 2015).

More and more experimental evidence show that the activation of NLRP3 inflammasome is closely related to neurodegenerative diseases (Duan et al., 2020; Feng et al., 2021). Under the stimulation of Aβ plaques and tau aggregates, microglia and astrocytes mediate chronic neuroinflammatory response, neuronal death and pyroptosis through intracellular NLRP3 inflammasome, thereby driving the occurrence and progression of AD (Han et al., 2020b; Van Zeller et al., 2021). More importantly, pharmacological inhibition of NLRP3 inflammasome exhibits neuroprotective effects. The use of inhibitory treatment against NLRP3 inflammasome can reduce Aβ deposition and alleviate the cognitive impairment of AD mice (Yan et al., 2020b). In this review, we mainly summarize the mechanisms of NLRP3 inflammasome activation, and analyze its possible roles in the progression of AD. In addition, we also introduce the upstream and downstream signaling pathways of the NLRP3 inflammasome, as well as the latest developments regarding its potential targets and therapeutic strategies for AD treatment.

2 THE ACTIVATION AND REGULATION OF NLRP3 INFLAMMASOME

A certain number of exogenous or endogenous stimuli that induce the activation of NLRP3 inflammasome have been confirmed so far. The exogenous stimulating factors include lipopolysaccharide (LPS) (Ma et al., 2021), viral RNA (Allen et al., 2009), palmitate (Byeon et al., 2017), silica dioxide (Ko et al., 2020) and so on, while the damage-associated endogenous activators consist of ROS (Li et al., 2020a), cathepsin B (Bai et al., 2018), ATP (Amores-Iniesta et al., 2017), Aβ oligomers (Van Zeller et al., 2021), α-synuclein (α-syn) (Wang et al., 2020), etc. Although the process of NLRP3

inflammasome activation induced by the above factors has been extensively studied, the exact molecular mechanisms still need to be further explored. Current researches have shown that there are two main types of signaling pathways that are responsible for the activation of NLRP3 inflammasome. One is the canonical signaling pathway involving pro-caspase-1 recruitment and caspase-1 activation, and the other is the non-canonical signaling pathway, which is mainly related to the activation of mouse caspase-11 or human caspase-4 and caspase-5 induced by LPS.

2.1 Canonical NLRP3 Activation

As far as we know, the canonical NLRP3 inflammasome activation usually requires two steps: priming and activation (Yang et al., 2019). Generally speaking, in the resting state of cells, the basal levels of NLRP3 and IL-1β are considered to be insufficient to activate the inflammasome. Therefore, a priming step initiates the transcription of these targets. Priming signal (signal 1): NLRP3 is stimulated by danger signals (such as TLR4 agonists or endogenous molecules) to induce the expression of NF-κB, which up-regulates the transcription of NLRP3, IL-1β and IL-18 genes, resulting in the increased protein expression of NLRP3, pro-IL-1β and pro-IL-18 (Bauernfeind et al., 2009; Lamkanfi and Dixit 2014). Activation signal (signal 2): The second activation step is usually triggered by PAMPs or DAMPs (such as viral RNA, aluminum salt, ATP, Aβ, K^+ efflux, etc.), which allows the NLRP3 inflammasome to complete the assembly step. Then, the cysteine protease pro-caspase-1 is recruited through the adaptor protein ASC to form a large filamentous protein complex called ASC speck. Clustered pro-caspase-1 autocatalyzes and autocleaves to generate activated caspase-1, which cleaves the pro-IL-1β and pro-IL-18 to generate the activated forms IL-1β and IL-18. At the same time, activated caspase-1 can initiate pyroptosis through the lysis of gasdermin D (GSDMD) (Swanson et al., 2019).

Recently, many studies have provided convincing evidence that the priming step of NLRP3 inflammasome activation is not limited to the increase of transcription level. There is another way to affect the activity of inflammasome through ubiquitination and post-translational modification of NLRP3. A recent study described that the recruitment of NEK7 to NLRP3 is controlled by the phosphorylation status of NLRP3 S803 located within the interaction surface, in which NLRP3 S803 is phosphorylated upon priming and later dephosphorylated upon activation. Phosphomimetic substitutions of NLRP3 S803 abolish NEK7 recruitment and inflammasome activity in macrophages *in vitro* and *in vivo* (Niu et al., 2021). Furthermore, Tang et al. found that E3 ubiquitin ligase TRIM65 can bind to the nucleotide-binding and oligomerization domain (NACHT) domain of NLRP3, promote lys48-and lys63-linked NLRP3 ubiquitination and inhibit NEK7-NLRP3 interaction, thereby restraining NLRP3 inflammasome assembly and caspase- 1 activation (Tang et al., 2021). In contrast, deubiquitination of NLRP3 leads to its activation. Studies have reported that the E3 ubiquitin ligase TRIM31 can directly bind to NLRP3 to promote K48-linked polyubiquitination and proteasomal degradation of NLRP3, thereby inhibiting the activation of NLRP3 (Song et al., 2016).

2.2 Non-Canonical NLRP3 Activation

In the non-canonical activation pathway, the NLRP3 inflammasome mainly relies on caspase-11 in mice (the homologues caspase-4 and caspase-5 in humans). LPS generated by Gram-negative bacteria enters the cytosol and can bind to caspase-11 in mice, thereby triggering its oligomerization and activation. The activated caspase-11 can induce pyroptosis and produce pro-inflammatory cytokines (Downs et al., 2020).

2.3 The Regulation of NLRP3 Activation

As summarized in previously published reviews, the main mechanisms involved in the activation of NLRP3 inflammasome include K^+ efflux, cathepsin B released after lysosomal disruption, the change of extracellular Ca^{2+} homeostasis, and the production of reactive oxygen species (ROS) (Zhang et al., 2020), etc. We will not repeat any elaboration of the above activation mechanisms. However, in recent years, several direct or indirect ways have been reported to participate in the NLRP3 inflammasome activation. The latest studies have shown that mitochondrion is the central regulator of NLRP3 function. Mitochondrial reactive oxygen species (mtROS) production, mitochondrial DNA (mtDNA) release, mitochondrial-mediated apoptosis, mitochondrial calcium overload, and mitochondrial involvement in the localization of NLRP3 are all related to the regulation of NLRP3 activity (Zhou et al., 2011; Lawlor and Vince 2014; Rimessi et al., 2015). Therefore, we primarily discuss the roles of mitochondrial dysfunction, mitochondrial-associated endoplasmic reticulum membrane (MAM), autophagy and mitophagy in the activation and regulation of NLRP3 inflammasome in this review.

2.3.1 Mitochondrial Dysfunction and NLRP3 Inflammasome Activation

Mitochondrion is one of the organelles with a double-layer membrane structure in cells, and it is the metabolic center and energy factory of cells. It provides the substrate and energy required for the biosynthesis of the cell, and plays a decisive role in the fate of cells. Mitochondria produce mtROS during aerobic metabolism via respiratory chain. Various intracellular and extracellular damage factors, including ROS, misfolded protein aggregation (such as Aβ, Tau, α-syn, etc.), toxic drugs, etc., can damage the normal function of mitochondria (Cha et al., 2012; Szabo et al., 2020). When the function of mitochondria is impaired, the level of mtROS increases significantly. mtROS accumulates in the cytoplasm and interacts with the components of the NLRP3 inflammasome, thereby participating in the activation of the inflammasome. In an earlier study, Nakahira et al. found that mtROS produced by impaired mitochondria is necessary for macrophages to activate NLRP3 inflammasome in response to LPS and ATP (Nakahira et al., 2011). Moreover, there is accumulating evidence to demonstrate that the use of

chemical inhibitors to disrupt mitochondrial function can trigger the NLRP3 inflammasome activation. Mitochondrial dysfunction inducers such as rotenone (complex I inhibitor) can lead to increased levels of ROS, activation of NLRP3 inflammasome, and the expression of IL-1β in microglia (Sarkar et al., 2017). Furthermore, inhibitors or scavengers of mtROS can effectively restrain the activation of NLRP3 inflammasome. For example, the mtROS scavenger Mito-TEMPO inhibits the activation of NLRP3 inflammasome induced by injury factors and reduces the secretion of IL-1β (Ding et al., 2017). Consistent with these results, impaired clearance of damaged mitochondria will enhance the activation of NLRP3 inflammasome. However, there are also some inconsistent opinions about the relationship between mtROS and NLRP3 inflammasome activation. Some previous studies indicated that the activation of NLRP3 inflammasome may not depend on mtROS, but through other components of mitochondria (Iyer et al., 2013). Bauernfeind and his colleagues also showed that ROS inhibitors only blocked the priming step of NLRP3 inflammasome activation, while its direct activation step was not affected, which implied that the role of ROS was limited to the priming step of NLRP3 activation (Bauernfeind et al., 2011). Despite the existence of the above phenomenon, more and more evidence indicate that mtROS is located at upstream of NLRP3 inflammasome activation, and mtROS directly or indirectly participates in the process of NLRP3 inflammasome activation. Many drugs or chemical agents can alleviate the inflammatory effect of NLRP3 by reducing the level of mtROS.

Mitochondrial dysfunction causes increased mitochondrial breakage, which releases mtDNA, ATP, heat shock protein 60 (HSP60), mitochondrial transcription factor A (TFAM), cardiolipin, cytochrome c, etc. These substances can be considered as DAMPs to induce the activation of NLRP3 inflammasome (Dela and Kang 2018). Among them, mtDNA is the most extensively studied mitochondrial-derived activator. In an earlier study, Nakahira et al. showed that the release of mtDNA is crucial for the activation of NLRP3, which depends on the generation of ROS (Nakahira et al., 2011). Shimada et al. further used the 293 cells transfected with mtDNA to prove that mtDNA can directly bind to NLRP3 and mediate the activation of NLRP3 inflammasome. Conversely, macrophages lacking mtDNA severely reduce IL-1β production (Shimada et al., 2012). The increased levels of oxidized mtDNA (ox-mtDNA) in the cytoplasm can promote the binding with NLRP3 inflammasome, which leads to the co-localization of NLRP3 and ASC in the perinuclear space in endoplasmic reticulum-mitochondrial clusters (Zhong et al., 2018). Given that mtROS and ox-mtDNA are significantly related to the activation of NLRP3 inflammasome, a wide range of mitochondrial antioxidant drugs can attenuate the inflammasome activation. Epigallocatechin-3-gallate (EGCG) is a polyphenol with strong antioxidant properties. Luo et al. evaluated the protective effect of EGCG on acute pancreatitis (AP)-associated lung injury and found that EGCG could protect AP-associated lung injury by removing mtROS and its oxidation product ox-mtDNA. In addition, the antagonism of NLRP3 signaling by EGCG was affected in the presence of the mtROS stimulant rotenone or scavenger Mito-TEMPO (Luo et al., 2021). Idebenone is a highly acclaimed mitochondrial protective agent. In the oxygen glucose deprivation/reperfusion (OGD/R) injury model, Peng et al. found that mitochondrial dysfunction led to mtDNA translocation and mtROS production, as well as cytosolic accumulation of oxidized mtDNA, which promoted its binding to NLRP3. However, idebenone treatment effectively blocked this process, and alleviated NLRP3-mediated inflammatory damage after OGD/R (Peng et al., 2020). In short, increasing evidence show that mtDNA can be closely related to the expression of IL-1β through the NLRP3 inflammasome activation.

2.3.2 The Regulation of NLRP3 Inflammasome Activation by Mitochondrial-Associated Endoplasmic Reticulum Membrane

The morphological structure of mitochondria and ER in eukaryotic cells is highly dynamic, which provides opportunities for coupling between mitochondria and ER. It has been reported that the mitochondrial outer membrane and the ER membrane can form an interaction coupling site membrane structure with a stable interval, which is known as MAM (Hayashi et al., 2009). In some places, MAM is also called mitochondria-ER contact sites (MERCs). MAM plays an important role in material transfer and signal transduction. At present, MAM has become a well-known important way for the regulation of cholesterol, lipids, calcium metabolism, oxidative stress, inflammation and other functions (Yu et al., 2021). The relationship between MAM and inflammation is discovered as early as 2011. In unstimulated cells, NLRP3 is mainly located on the ER membrane and in the cytoplasm. However, upon activation, NLRP3 and ASC will redistribute and translocate to the MAM in the perinuclear region, which makes it easier to sense mitochondrial damage signals such as mtROS, cardiolipin, mtDNA, etc (Zhou et al., 2011). MAM can be regarded as a platform for inflammasome assembly and activation. During the formation of inflammasome, acetylated α-tubulin can migrate mitochondria to the perinuclear region and promote the assembly of ASC on mitochondria with NLRP3 on the ER (Misawa et al., 2013). Recent studies have shown that MAM participates in the regulation of DAMPs-mediated effects, antiviral responses, bacterial pathogen-mediated infections, and other inflammatory processes through direct or indirect action (Missiroli et al., 2018). Martinvalet also has introduced the important role of mitochondria and the ER contact sites in the development of immune response (Martinvalet 2018). The NLRP3 on the ER and the ASC on the mitochondrial combine with each other through CARD to form the NLRP3 inflammasome, and those mitochondrial outer membrane proteins involved in ER-mitochondrial binding, such as mitogen, can regulate the structural stability of MAM, thereby controlling the activation of

NLRP3 inflammasome. Mitochondrial antiviral signal protein (MAVS) is an adaptor molecule located on the outer mitochondrial membrane, which participates in the secretion of type I interferon. As an important component of MAM, it plays a pivotal role in regulating the host's natural immunity (Horner et al., 2015). Studies found that MAVS can recruit NLRP3 to mitochondria in response to viral infections. MAVS is linked to the N-terminal amino acid sequence of NLRP3, which is the basis of interaction between MAVS and NLRP3 (Subramanian et al., 2013). In addition, a study carried out by Guan et al. proved that MAVS is capable of stabilizing ASC and inducing the formation of cytosolic speck via recruiting the E3 ligase TRAF3 to ASC. Ubiquitination of ASC at Lys174 by TRAF3 is essential for speck formation and inflammasome activation. The deficiency of MAVS or TRAF3 will impair ASC ubiquitination and the formation of cytoplasmic speck, thereby reducing the NLRP3 inflammasome activation (Guan et al., 2015). Mitofusin 2 (MFN2) is a mitochondrial outer membrane GTPase, which plays an important role in the mitochondrial fusion process. Furthermore, MFN2 is also present on the ER membrane. MFN2 is enriched in MAM and enhances the structural stability of MAM. MFN2 on the ER bridges ER and mitochondria by engaging in homotypic and heterotypic complexes with mitofusin 1 or 2 on the surface of mitochondria (de Brito and Scorrano 2008). The stable MAM structure may provide a basis for the assembly of NLRP3 inflammasome. An earlier study showed that after infection with influenza virus or encephalomyocarditis virus (EMCV), MFN2 could interact with NLRP3 to promote the recruitment of NLRP3 to mitochondria, and subsequently induce IL-1β secretion. However, the secretion of IL-1β was significantly restored in MFN2 gene knockout cells (Ichinohe et al., 2013). Another study described that infection with *mycobacterium tuberculosis* up-regulated the expression of MFN2 and promoted the assembly and activation of the NLRP3 inflammasome (Xu et al., 2020). These researches imply that MFN2 may contribute to the stability of MAM structure, and promote the activation of NLRP3 inflammasome. However, the specific mechanism still needs further studies.

It is generally acknowledged that Ca^{2+} play an important role in NLRP3 inflammasome activation (Horng 2014). ER is the main Ca^{2+} reservoir in cells. The continuous transfer of Ca^{2+} from ER into the mitochondria will result in mitochondrial Ca^{2+} overload and dysfunction, which promotes the release of cardiolipin and mtDNA (Murakami et al., 2012). MAM is the main site that mediates the transportation of Ca^{2+} from ER to mitochondria, which is related to the distribution of Ca^{2+} transport channel proteins in the MAM region. The IP3R-GRP75-VDAC-MCU complex is a classic pathway that mediates the transport of ER Ca^{2+} to the mitochondria through the MAM region (Szabadkai et al., 2006). These proteins are also the constituent molecules of MAM. Inhibitors or gene knockouts against these molecules may attenuate NLRP3 inflammasome activation. We believe that the changes of MAM function will affect the activation of NLRP3 inflammasome. Therefore, drugs or compounds that cause changes in MAM function can regulate the NLRP3 inflammasome activation.

2.3.3 The Negative Regulation of NLRP3 Inflammasome via Autophagy and Mitophagy

Autophagy is a process of non-specific degradation of the cell's own components such as organelles and abnormal accumulation proteins through the lysosomal system. Hence, it is essential for maintaining cell homeostasis and survival (Mameli et al., 2021). Autophagy has been confirmed to be closely related to the NLRP3 inflammasome activation, as the response of eukaryotic cells to external stimuli. In an earlier study, Saitoh Tatsuya et al. reported that the important autophagy gene Atg16L1 regulated endotoxin-induced inflammasome activation. In LPS-stimulated macrophages, the deficiency of Atg16L1 could lead to activation of NLRP3 inflammasome and production of IL-1β (Saitoh et al., 2008). Furthermore, Atg5 is also an important autophagy-related gene. Atg5 acetylation can inhibit the maturation of autophagosomes and induce the activation of NLRP3 inflammasome. On the contrary, sirtuin 3 (SIRT3) can form a complex with Atg5 to block the acetylation of Atg5, which leads to impaired autophagy and accelerates the activation of NLRP3 inflammasome (Liu et al., 2018). As far as we know, there is mounting evidence show that autophagy is an important regulator of inflammasome, which negatively regulate the NLRP3 inflammasome activation. Autophagy can eliminate the endogenous activator DAMPs. In AD, autophagy alleviates the activation of NLRP3 inflammasome induced by Aβ oligomers via removing abnormally deposited and misfolded proteins (Wen et al., 2019). Mi-Hyang Cho et al. revealed that, in the microglia model, Aβ interacts with MAP1LC3B-II through OPTN/optineurin and is degraded by the autophagy process mediated by the PRKAA1 pathway (Cho et al., 2014). Deficiency or inhibition of autophagy can exacerbate the pathology of NLRP3 inflammasome-mediated neurodegenerative diseases (Qin et al., 2021). In contrast, autophagy inducers, such as rapamycin, AICAR, and metformin, can activate autophagy in microglia, which promotes the phagocytosis and degradation of misfolded protein aggregates in cells, thereby effectively inhibiting the excessive activation of NLRP3 inflammasome (Qiu et al., 2020).

Mitophagy is a process that selectively removes damaged mitochondria. Once mitochondrial dysfunction occurs, mitophagy can promote the renewal of mitochondria, thereby maintaining mitochondrial quality control. As mentioned above, there is growing evidence that damaged mitochondria activate the NLRP3 inflammasome through a variety of ways. Therefore, mitophagy can be considered as an important way to regulate the activation of NLRP3 inflammasome. Currently, multiple literatures demonstrate that mitophagy also negatively regulates the NLRP3 inflammasome activation. Mitophagy eliminates damaged mitochondria, avoids the release of endogenous molecules such as ATP, mtROS and mtDNA, thus reduces the activation of NLRP3 inflammasome (Mishra et al., 2021). Mitophagy inhibitors or gene knockouts can lead to mitophagy disorder, cause the accumulation of mtROS and

mtDNA in cells, and activate the NLRP3 inflammasome. Researching the role of Parkin, a central player in mitophagy, in host antiviral responses, Li et al. found that Parkin deficiency augments innate antiviral inflammation and promotes viral clearance by enhancing mtROS-mediated NLRP3 inflammasome activation (Li et al., 2019). On the contrary, mitophagy inducers can enhance the ability to clear dysfunctional mitochondria, thereby inhibiting NLRP3 inflammasome activation (Peng et al., 2021). Gao et al. reported in the nonalcoholic fatty liver disease (NAFLD) model that the expression levels of mitophagy markers PINK1 and Parkin was significantly diminished by deoxycholic acid (DCA) and the ability of mitophagy was impaired. However, after treatment with a specific mitophagy agonist carbonyl cyanide 3-chlorophenylhydrazone (CCCP), the ability of mitophagy was restored and the DCA-induced inflammasome response was prevented (Gao et al., 2021b). In conclusion, numerous current studies have shown that autophagy and mitophagy may be a self-limiting way to protect cells from excessive inflammation.

3 THE ROLE OF NLRP3 INFLAMMASOME IN ALZHEIMER'S DISEASE

Neuroinflammation is a double-edged sword. It is regarded as a defensive mechanism during the acute infection period and plays an anti-infection role. However, after its transfer to the chronic inflammation phase, excessive release of cytotoxic factors will cause inflammation activation. Increasing evidence from AD patients, *in vitro* cell models and *in vivo* animal models indicate that NLRP3 inflammasome plays an important role in AD. Saresella et al. showed that the expression level of NLRP3 inflammasome-related molecules was higher in severe AD patients than moderate ones via gene expression analysis of peripheral blood mononuclear cells (PBMCs) in AD patients. *In vitro* stimulation of PBMCs with LPS or Aβ42 could activate NLRP3 inflammasome. They believe that peripheral monocytes are likely to migrate across the blood-brain barrier (BBB) into the CNS and participate in the neuroinflammatory response of AD (Saresella et al., 2016). Mahmoudiasl et al. further detected increased expression levels of NLRP3, caspase-1, and inflammasome activation products IL-1β and IL-18 in the cerebral temporal cortex of AD patients (Ahmed et al., 2017). Aβ fibrils have unique structural characteristics and can be regarded as a kind of DAMPs, which are recognized by Toll-like receptors (TLRs) or nucleotide-binding oligomerization domain-like receptors (NLRs) and transmit pro-inflammatory signals. Early studies reported that the senile plaques are surrounded by activated microglia and astrocytes, and the glial cells around the Aβ plaques express higher levels of IL-1β (Apelt and Schliebs 2001). Subsequently, Halle et al. first described the role of NLRP3 inflammasome in the AD model. They found that Aβ activates the NLRP3 inflammasome in microglia, causing the maturation and secretion of IL-1β and IL-18. The increased amount of Aβ phagocytosed by microglia can cause lysosomal damage in the cytosol and the release of cathepsin B, and the latter can act as an endogenous danger signal to activate the NLRP3 inflammasome (Halle et al., 2008). Recent studies have shown that NLRP3 inflammasome is not only activated by fibrous Aβ aggregates, but also by lower molecular weight Aβ oligomers and fibrils. This suggests that the innate immune response of CNS triggered by Aβ activation may be before the onset of Aβ deposition (Luciunaite et al., 2020). The researchers further have explore the mechanisms by which Aβ activates the NLRP3 inflammasome and have found that this may involve two signals: the priming signal and the activation signa. When studying the inflammatory response of primary microglia to Aβ (1–42) protofibrils, Terrill-Usery et al. found that Aβ (1–42) protofibrils significantly upregulates the expression of IL-1β, TNFα mRNA and pro-IL-1β protein through the TLR/MyD88 pathway (Terrill-Usery et al., 2014). Similarly, the results of Liu et al. showed that Aβ(1–42) activates and up-regulates the expression of NLRP3 inflammasome-related molecules in BV-2 microglia via the TLR4/NLRP3 pathway and increases the secretion of IL-1β (Liu et al., 2020). These results indicate that Aβ fibrils can provide the priming signal for NLRP3 inflammasome activation. Another study revealed that Aβ induces the formation of NLRP3 inflammasome in a cathepsin-dependent manner. Under resting conditions, NLRP10 can bind to ASC and inhibit the assembly of NLRP3 inflammasome. However, after glial cells are treated by Aβ, cathepsin can be activated to promote the degradation of NLRP10, which makes it easier for NLRP3 and ASC to combine with each other to form inflammasomes (Murphy et al., 2014). This indicates that Aβ fibrils can also provide activation signals for NLRP3 inflammasome in an indirect way. In short, the above evidence mainly reflect that Aβ activates the NLRP3 inflammasome, and then participates in the pathogenesis of AD through IL-1β, IL-18 and other inflammatory cytokines. Moreover, it has been proposed that Aβ1-42 can also mediate GSDMD lysis through NLRP3-caspase-1 signal, and induce neuronal cell pyroptosis (Han et al., 2020).

In recent years, a large amount of data from cell experiments and animal models have confirmed that the activation of NLRP3 inflammasome can also affect the deposition and spread of Aβ. Heneka et al. found that, compared with APP/PS1 mice, NLRP3 and caspase-1 knockout AD model mice have a significantly enhanced ability of microglia to phagocytose Aβ and differentiate microglia into anti-inflammatory M2 type, which facilitates Aβ clearance (Heneka et al., 2013). In addition, the ability of microglia to clear Aβ can also be enhanced by inhibitors of NLRP3 or caspase-1, thereby reducing the accumulation of Aβ in the brains of APP/PS1 mice (Dempsey et al., 2017). These results confirm that the activation of NLRP3/caspase-1 inflammasome reduces the phagocytosis of Aβ by glial cells, which makes it easier for Aβ to accumulate in the cells. After comprehensive analysis of the related research results of Aβ and NLRP3 inflammasome, we speculate that when Aβ oligomers or fibrils activate NLRP3 inflammasome, it regulates the production of neurotoxic inflammatory cytokines such as IL-1β and IL-18. At the same time, it can induce pyroptosis of neurons by activating caspase-1 to mediate the lysis of GSDMD. On the other hand, the activation of NLRP3 inflammasome can conversely lead to increased Aβ

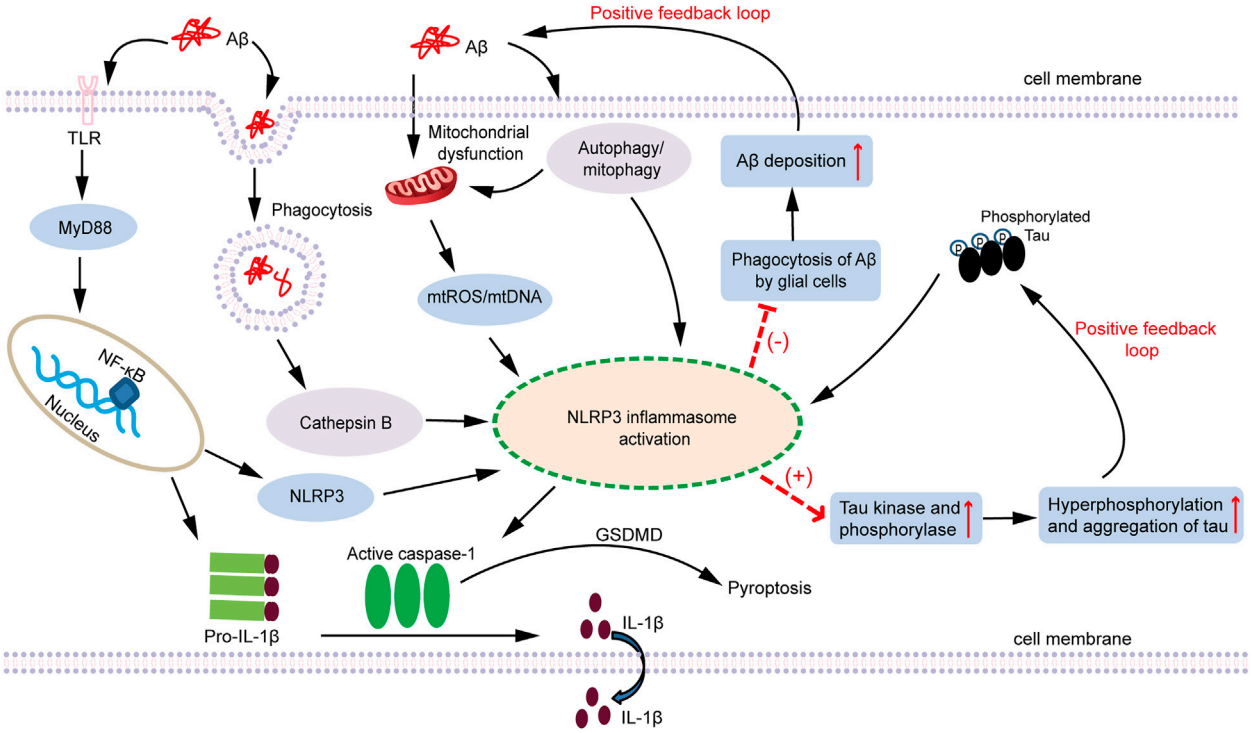

FIGURE 1 | A schematic diagram of the association between NLRP3 inflammasome activation and AD pathogenesis. Both Aβ oligomers and Tau aggregates are involved in the inflammatory response of AD. Fibrillar Aβ species are regarded as PAMPs that triggers NF-κB activation through pattern recognition receptors (such as TLRs) to elevate inflammasome components NLRP3 and pro-IL-1β. NLRP3, ASC, and pro-caspase-1 assemble together to form the NLRP3 inflammasome, which subsequently activates caspase-1, cleaves pro-IL-1β to produce the active form of IL-1β and secretes it extracellularly. In addition, phagocytosis of soluble Aβ also triggers lysosome leakage and consequently results in the emission of cathepsin B, which leads to NLRP3 inflammasome activation. Furthermore, Aβ oligomers can act as damaging stimulis to induce mitochondrial dysfunction, causing the production and accumulation of ROS, release of mtDNA, or cardiolipin externalization, which activates the NLRP3 inflammasome. Autophagy can not only clear Aβ, but also clear NLRP3, ASC and pro-caspase-1 inflammasome-related protein molecules. Mitophagy can selectively remove impaired mitochondria and relieve the release of damaging molecules within mitochondria. Therefore, autophagy and mitophagy can negatively regulate the activation of NLRP3. Aβ also indirectly regulate the activation of the NLRP3 inflammasome through the autophagy or mitophagy pathway. Moreover, the activation of NLRP3 inflammasome inhibits the phagocytosis of Aβ by glial cells, which contributes to the deposition of Aβ and facilitates the formation of Aβ plaques. In conclusion, Aβ can activate the NLRP3 inflammasome through different pathways. However, once the NLRP3 inflammasome is activated, it in turn increases the deposition of Aβ and the formation of Aβ plaque, which forms a positive feedback loop that amplifies Aβ pathogenic effect. Similar to the role of Aβ, Tau is regarded as an endogenous dangerous molecule that can activate the NLRP3 inflammasome. After the NLRP3 inflammasome is activated, it increases the activity of Tau kinase and phosphorylase, and facilitates the phosphorylation and aggregation of Tau, thereby also forming a positive feedback loop. Persistent activation of the NLRP3 inflammasome triggered by Aβ and Tau contributes to the development of chronic neuroinflammation, which ultimately leads to the neuronal loss and cognitive impairment. AD: Alzheimer's disease; Aβ: amyoid β; PAMPs: pathogen-associated molecular patterns; TLRs: Toll-like receptors; NF-κB: nuclear factor kappa B; NLRP3: nucleotide-binding oligomerization domain-like receptor pyrin domain-containing 3; ASC: apoptosis-associated speck-like protein containing a CARD; ROS: reactive oxygen species; mtROS: mitochondrial ROS; mtDNA: mitochondrial DNA; MyD88: myeloid differentiation factor 88; GSDMD: gasdermin D.

deposition and diffusion in glial cells, thereby inducing Aβ into the positive feedback loop, and ultimately contributing to the development of AD.

Although there are many researches to reveal the role of Aβ aggregates in the activation of NLRP3 inflammasome, there are limited studies on the relationship between Tau and NLRP3 inflammasome. In an earlier study, Kitazawa et al. used IL-1R blocking antibodies to inhibit IL-1β signaling in the 3xTg AD mouse model and found that they could significantly reduce the activity of tau kinase, such as cdk5/p25, GSK -3β, p38-MAPK, thereby reducing the level of tau phosphorylation (Kitazawa et al., 2011). This implies that the inflammatory effect after activation of NLPR3 inflammasome may have an impact on the pathogenic effect of Tau. In 2019, a major study revealed the influence of NLPR3 inflammasome on the pathology of tau. Ising et al. found that the loss of NLRP3 function could reduce the hyperphosphorylation and aggregation of tau by regulating tau kinase and phosphorylase. In addition, intracerebral injection of homogenate containing Aβ fibrils induced pathological changes of tau protein, which depends on the activation of NLRP3. Their study confirms that the activation of NLPR3 inflammasome in microglia plays an important role in the pathological changes of tau. Meanwhile, it also supports the Aβ cascade hypothesis in the pathogenesis of AD and the role of neurofibrillary tangles in the downstream development of Aβ-induced activation of microglia (Ising et al., 2019). In the same year, another highlighted study investigated whether Tau aggregates could activate NLRP3 inflammasome just like Aβ fibrils. They demonstrated that Tau activates NLRP3 inflammasome after being taken up by microglias, and its

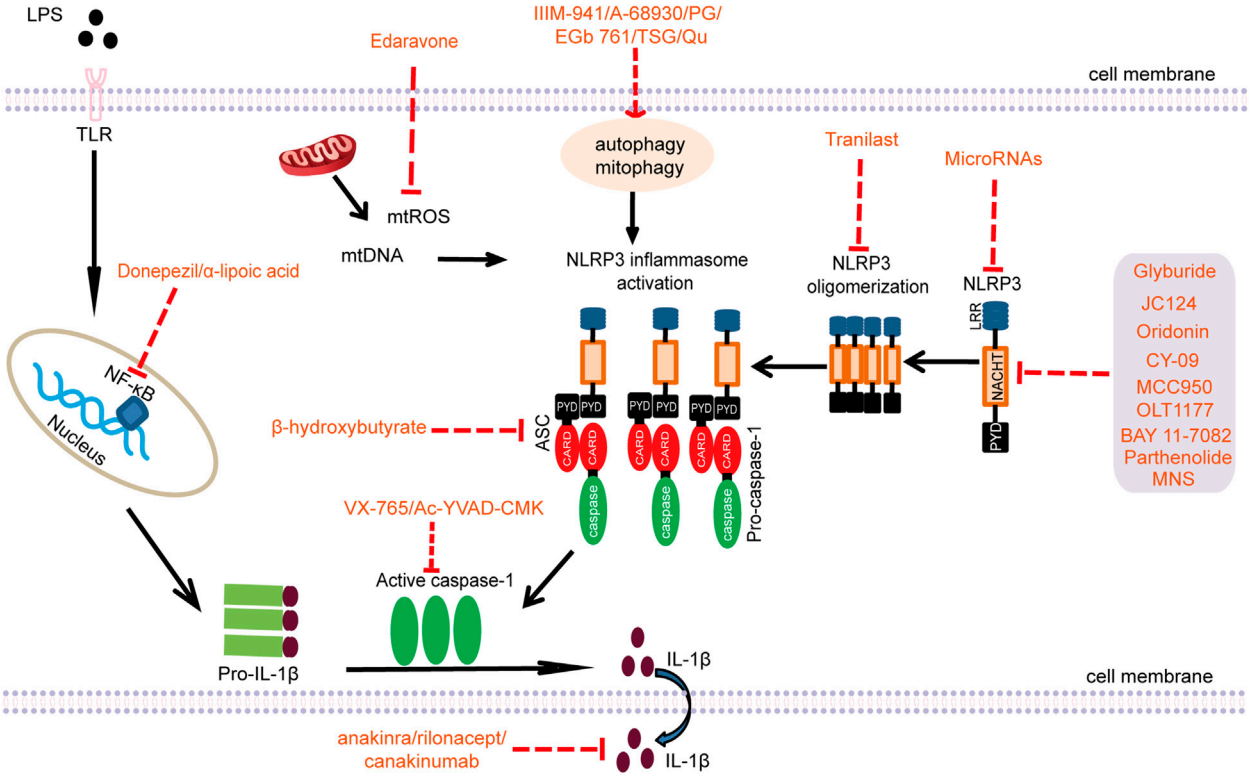

FIGURE 2 | Pharmacological targets of the NLRP3 inflammasome (LPS: lipopolysaccharide; TLR: Toll-like receptor; NF-κB: nuclear factor kappa B; mtROS: mitochondrial ROS; mtDNA: mitochondrial DNA; NLRP3: nucleotide-binding oligomerization domain-like receptor pyrin domain-containing 3; ASC: apoptosis-associated speck-like protein containing a CARD; PG: progesterone; TSG: 2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside; Qu: Quercetin; MNS: 3,4-methylenedioxy-β-nitrostyrene; CARD: caspase recruitment domain; NACHT: nucleotide-binding and oligomerization domain; PYD: pyrin domain; LRR: leucine-rich repeat).

activation mechanism is similar to that of Aβ. Moreover, Tau-induced pathology is alleviated in Tau transgenic mice with ASC gene deletion or NLRP3 targeting inhibitors (Stancu et al., 2019). Surprisingly, tau protein may also provide the priming signal for the activation of NLRP3 inflammasome. Panda et al. used tau-derived PHF6 peptide (VQIVYK) to stimulate microglia and found that VQIVYK in the form of fibrous aggregates upregulated the expression of NLRP3 at mRNA and protein levels in a dose- and time-dependent manner, ultimately leading to increased expression of IL-1β and IL-18 (Panda et al., 2021). Experimental results from *in vivo* also show that hyperphosphorylation of tau in the mouse brain significantly increases the activation of NLRP3 inflammasome and the up-regulation of IL-1β levels (Zhao et al., 2021). In summary, we speculate that the role of tau in NLRP3 inflammasome is similar to Aβ. On the one hand, tau aggregates activate the NLPR3 inflammasome to regulate the expression and secretion of IL-1β and IL-18 and participate in the pathological damage of tau. On the other hand, the activation of NLRP3 inflammasome can also increase the hyperphosphorylation and aggregation of tau through tau kinase and phosphorylase, thereby inducing tau to go through the positive feedback loop, and ultimately playing an important role in the pathogenesis of AD (**Figure 1**).

4 NLRP3 INFLAMMASOME INHIBITORS AS A POTENTIAL TARGET FOR THE TREATMENT OF ALZHEIMER'S DISEASE

With the comprehensive understanding of the molecular mechanism of NLRP3 inflammasome activation, since 2013, many published articles have paid more attention to the therapeutic value of targeted intervention of NLRP3 inflammasome in diseases. In view of the important role of NLRP3 inflammasome in the pathogenesis of AD, exploring its drug targets in the treatment of AD has also become a hot topic in its field. According to the characteristics of the formation and activation of NLRP3 inflammasome, some compounds that inhibit the activity of NLRP3 or interfere with its interaction with ASC are used to block the activation of NLRP3 inflammasome, which provides new ideas for the treatment of AD. Furthermore, considering the secretion of inflammatory factors downstream of the NLRP3 inflammasome and pyroptosis, the targeted intervention of caspase-1 activation and inhibition of downstream inflammatory factors of NLRP3 may also be a way to alleviate chronic inflammation in AD. The NLRP3 inflammasome and involvement of several upstream or downstream signaling pathways provide promising pharmacological targets for AD (**Figure 2**). At present, in the

TABLE 1 | The compounds or extractions targeting NLRP3 inflammasome pathways in AD.

Compounds or extractions	Mechanism	Cell or animal model	References
IL-1 inhibitors			
anakinra	IL-1 receptor antagonist	3xTg-AD transgenic mice	Kitazawa et al. (2011)
	IL-1 receptor antagonist	AD amyloidosis rat model	Qi et al. (2018), Batista et al. (2021)
rilonacept	IL-1inducible receptor	—	Giancane et al. (2016)
canakinumab	Antibody targeting IL-1β	—	Giancane et al. (2016)
NLRP3 inhibitors			
Glyburide	ATP sensible K^+ channels, downstream of the P2X7 receptor	—	Lamkanfi et al. (2009)
JC124	Inhibits the NLRP3 inflammasome and the activation of caspase-1	APP/PS1 or CRND8 APP transgenic mice	Fulp et al. (2018), Yin et al. (2018), Kuwar et al. (2021)
Oridonin	Covalent bond with NLRP3 in NACHT domain to block the interaction between NLRP3 and NEK7	$Aβ_{1-42}$ induced AD mice	Wang et al. (2014), Wang et al. (2016), He et al. (2018)
CY-09	Binds to the ATP binding motif of the NLRP3 NACHT domain to inhibit NLRP3 ATPase activity	—	Jiang et al. (2017)
MCC950	Walker B motif interaction and inhibition of ATP hydrolysis, selective inhibitor of NLRP3	APP/PS1 AD, Long evans rats, SAMP8 mouse	Qi et al. (2018), Coll et al. (2019), Fekete et al. (2019), Li et al., 2020b)
	Selective inhibitor of NLRP3	Microglia induced by Aβ aggregates	Luciunaite et al. (2020)
OLT1177	Binds to NLRP3 to inhibit its ATPase activity	APP/PS1 mice	Marchetti et al. (2018), Lonnemann et al. (2020)
Tranilast	Directly binds to the NACHT of NLRP3 and blocks NLRP3 oligomerization	—	Huang et al. (2018)
BAY 11–7082	Inhibits NLRP3 ATPase activity	APP 23 mice, BV2 cells	Ruan et al. (2019)
Parthenolide	Inhibits NLRP3 ATPase activity and caspase-1	Primary glial cells	Ou et al. (2020)
MNS	Inhibits the activity of NLRP3 ATPase through binding to the LRR and NACHT domains	—	He et al. (2014)
ASC inhibitors			
BHB	Prevents K^+ efflux and reduces ASC oligomerization and speck formation	5xFAD mouse	Youm et al. (2015), Shippy et al. (2020)
	Improves the cognitive function	AD patients	Ota et al. (2019)
Caspase-1 inhibitors			
VX-765	Inhibits caspase-1	AD J20 mouse	Flores et al. (2018), Flores et al. (2020)
Ac-YVAD-CMK	Inhibits caspase-1	APP/PS1 AD mice	Gu et al. (2021)
Plant-derived compounds			
Resveratrol	Inhibits TXNIP/TRX/NLRP3 signaling pathway	BV-2 cells	Feng and Zhang, (2019)
	Inhibits NF-κB/IL-1β/NLRP3 signaling pathway	AD mouse model induced by $Aβ_{1-42}$	Qi et al. (2019)
Pterostilbene	Inhibits the NLRP3/caspase-1 pathway	Microglia induced by $Aβ_{1-42}$	Li et al. (2018)
SFN	Inhibits the NLRP3 inflammasome	N9 microglial cells	Tufekci et al. (2021)
GB	Inhibits NLRP3 activation and promotes microglia M2 polarization	BV2 microglial cells induced by $Aβ_{1-42}$	Zhang et al. (2021b)
ABPPκ	Inhibits the expression of NLRP3, cleaved caspase-1, and ASC	BV2 microglia, Aβ oligomers-injected mice	Ge et al. (2021)
Chinese herbal medicines			
PK	Inhibits the NLRP3 inflammasome	5xFAD mouse	Kim et al. (2020)
DHM	Inhibits the NLRP3 inflammasome	APP/PS1 mice	Feng et al. (2018)
NSAIDs			
IND	Reduces the expression of IL-1β and caspase-1	AD rats induced by streptozotocin	Karkhah et al. (2021)
MicroRNAs	Directly or indirectly inhibits the expression of NLRP3	Glial cells, AD mice, and AD patients	Han et al. (2020), Feng et al. (2021), Wan et al. (2021)
Autophagy activators			
A-68930	Enhances the degradation of NLRP3 inflammasome by activating the AMPK/autophagy signaling pathway	BV2 cells, AD mice induced by $Aβ_{1-42}$	Cheng et al. (2020)
PG	Inhibits the activation of NLRP3-caspase-1 via enhancing the autophagy	Astrocytes	Hong et al. (2019)
EGb 761	Down-regulates the level of NLRP3 protein, reduces the activation of IL-1β and caspase-1 via autophagy	TgCRND8 AD model	Liu et al. (2015)
Mitophagy activators			
TSG	Prevents NLRP3 inflammation through mitophagy	APP/PS1 mice, BV2/N2a/SH-SY5Y cells	Gao et al. (2020)
Qu	Inhibits NLRP3 inflammation through mitophagy	Primary microglia, BV2 cells	Han et al. (2021)
ROS and NF-κB inhibitors			
α-lipoic acid	Inhibits NLRP3 via the NF-κB signaling pathway	BV-2 microglial cells	Kim et al. (2019)

(Continued on following page)

TABLE 1 | (*Continued*) The compounds or extractions targeting NLRP3 inflammasome pathways in AD.

Compounds or extractions	Mechanism	Cell or animal model	References
Edaravone	Reduces the production of mtROS, and inhibits the activation of NLRP3	Aβ-treated microglia	Wang et al. (2017)
Donepezil	Down-regulates NLRP3 and pro-IL-1β mRNA levels by inhibiting NF-κB/STAT3 phosphorylation	BV2 microglial cells, 5xFAD mice	Kim et al. (2021)

strategy of AD treatment, some compounds that directly inhibit the activity of NLRP3 ATPase include CY-09, MNS and OLT1177. There are some drugs interfering with ASC oligomerization, which is represented by *ß*-hydroxybutyric acid (BHB). The inhibitors of caspase-1 activation are VX-740 and VX-765. Biological agents targeting IL-1β mainly include IL-1β antibody canakinumab and recombinant IL-1β receptor antagonist anakinra. In the following section, we will review and summarize in detail the role and therapeutic value of the above interventions in AD (**Table 1**).

LRP3: nucleotide-binding oligomerization domain-like receptor pyrin domain-containing 3; AD: Alzheimer's disease; Aβ: amyoid β; ATP: Adenosine triphosphate; P2X7: P2X purinergic receptor 7; APP: amyloid precursor protein; APP/PS1: APPswe/PS1dE9; SAMP8: senescence-accelerated mouse prone 8; NACHT: nucleotide-binding and oligomerization domain; LRR: leucine-rich repeat; NEK7: NIMA-related kinase 7; MNS: 3,4-methylenedioxy-β-nitrostyrene; BHB: *ß*-hydroxybutyric acid; TXNIP: Thioredoxin interacting protein; TRX: Thioredoxin; SFN: Sulforaphane; GB: Ginkgolide; ABPPκ: *Achyranthes* bidentate polypeptide fraction *κ*; PK: Picrorhiza kurroa; DHM: Dihydromyricetin; NSAIDs: Nonsteroidal anti-inflammatory drugs; IND: Indomethacin; AMPK: Adenosine 5′-monophosphate (AMP)-activated protein kinase; PG: Progesterone; TSG: 2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside; Qu: Quercetin; ASC: apoptosis-associated speck-like protein containing a CARD; mtROS: mitochondrial ROS; NF-κB: nuclear factor kappa B; STAT3: Signal transducer and activator of transcription 3

4.1 IL-1β Antibodies and IL-1R Antagonists

IL-1β is usually present in cells as the precursor form pro-IL-1β. Pro-IL-1β has no biological activity. Activated caspase-1 can cleave pro-IL-1β into mature IL-1β through enzyme cleavage. At present, the strategy of targeting IL-1β has certain application prospects, but it also has some limitations. So far, there are mainly three biologics targeting IL-1β that have been used in the treatment of various inflammatory diseases. A recombinant IL-1 receptor antagonist anakinra, an inducible receptor rilonacept that binds to IL-1α and IL-1β, and the other one is IL-1β neutralizing antibody canakinumab (Giancane et al., 2016). These biological agents have been widely used to treat inflammation-related diseases, including autoimmune diseases (Sota et al., 2021), recurrent pericarditis (Fava et al., 2021), idiopathic arthritis (Autmizguine et al., 2015), gout (Perez-Ruiz et al., 2014). However, the clinical trials of these biologics in AD are rarely reported. Long-term injection of IL-1R blocking antibody to 3xTg-AD mice can significantly reduce brain inflammation, ameliorate cognitive impairment, relieve tau pathology, and partially reduce the level of Aβ oligomers (Kitazawa et al., 2011). In addition, Qi et al. found that anakinra can improve synaptic plasticity defects in a rat model of AD amyloidosis and eliminate the inhibitory effect on long-term potentiation (Qi et al., 2018). A recent study showed that anakinra can also alleviate synaptic loss and cognitive impairment in AD (Batista et al., 2021). For the application of inhibitors targeting IL-1β in AD, the ability of these biologics to cross the BBB, the ability to penetrate the brain tissue, and the side effects of drugs should be considered. Furthermore, upon NLRP3 inflammasome activation, in addition to the secretion of IL-1β, it also produces IL-18 and pyroptosis. Therefore, it is difficult to completely inhibit the pathogenic effect of NLRP3 inflammasome after blocking IL-1β. Recently, some new targets that participate in the regulation of NLRP3 inflammasome have been identified, which provides a new approach for AD therapy.

4.2 Specific Inhibitors of the NLRP3 Inflammasome

The activation of NLRP3 inflammasome depends on the integrity of the structure and function of NLRP3 and the assembly of NLRP3 inflammasome. Therefore, the potential therapeutic targets for NLRP3 inflammasome mainly include the NACHT domain of NLRP3, ASC and caspase-1, as well as other sites that affect its assembly. Targeting the pharmacological effects of NLRP3 inflammasome may be the best way to treat AD. Here, we mainly summarize several inhibitors for NLRP3 inflammasome activation and their therapeutic targets.

4.3 NLRP3 Activation Inhibitors

4.3.1 Glyburide

Glyburide is a sulphonylurea drug approved by the FDA, which treats type 2 diabetes by blocking the ATP-sensitive K^+ channel in *ß* pancreatic cells. Lamkanfi et al. found that glyburide has anti-inflammatory effects in an early study. It is the first identified compound that can inhibit the activation of NLRP3 inflammasome and the secretion of IL-1β induced by PAMPs, DAMPs and crystals. However, it has no effect on the activation of NLRC4 or NLRP1. The targets of glyburide still need to be further clarified. They found that glyburide targeted the signal components downstream of the P2X7 receptor and might act upstream of NLRP3 to inhibit the activation of caspase-1 (Lamkanfi et al., 2009). There are few studies on the application of glyburide in neurodegenerative diseases. Therefore, its therapeutic value in AD is still unclear.

4.3.2 JC124

JC124 is a specific small molecule inhibitor of NLRP3 inflammasome. In 2018, Fulp et al. developed the methylated analogue JC124 based on the sulfonamide analogue JC121 of glyburide. After oral administration, JC124 can penetrate through the BBB and enter the brain tissue. After JC124 treatment, APP/PS1 transgenic mice shows significant improvement in cognitive impairment (Fulp et al., 2018). In addition, Yin et al. demonstrated that JC-124 inhibits the lysis and activation of caspase-1 in CRND8 APP transgenic mice (TgCRND8) mice, and selectively restrains the formation of NLRP3 inflammasome, thereby effectively reducing Aβ deposition and microglia activation (Yin et al., 2018). After treatment with JC124 in the traumatic brain injury (TBI) model, it can significantly inhibit the activation of NLRP3 induced by injury, reduce the expression level of its downstream effector protein, and thus play a role in neuroprotection (Kuwar et al., 2019). A recent study has reported the preventive efficacy of JC124 in AD. They found that JC124 has the ability to inhibit neuronal inflammation, regulate the accumulation of Aβ and promote the alleviation of cognitive impairment. Moreover, improved synaptic plasticity and endogenous neurogenesis in the hippocampus are also observed (Kuwar et al., 2021). Therefore, JC124 is a new type of inhibitor targeting NLRP3 inflammasome, which can reduce the neuropathology of AD and improve cognitive function, thereby exhibiting neuroprotective effects.

4.3.3 Oridonin

Oridonin (Ori) is the main bioactive component of the natural anti-inflammatory Chinese medicinal herb Rabdosia rubescens, and has been proven to be as a specific covalent inhibitor of NLRP3 inflammasome. Under the stimulation of NLRP3 agonists such as monosodium urate crystals (MSU), ATP or cytosolic LPS (cLPS), Ori treatment inhibits NLRP3 inflammation and reduces IL-1β release (He et al., 2018). Regarding the target of Ori, studies have reported that Ori can directly bind to the NACHT domain of NLRP3. Ori forms a covalent bond with the cysteine 279 of NLRP3 in NACHT domain to block the interaction between NLRP3 and NEK7, thereby inhibiting NLRP3 inflammasome assembly and activation (He et al., 2018). In the AD mouse model, Ori inhibits the activation of microglia induced by Aβ1-42, reduces the release of inflammatory cytokines, prevents the loss of synapses, and improves the cognitive impairment of AD mice (Sulei Wang et al., 2014; Wang et al., 2016). In addition, it has also been observed in TBI that Ori treatment can significantly reduce the expression of NLRP3 inflammasome components (NLRP3, ASC and caspase-1), and restrict the secretion of IL-1β and IL-18 (Yan et al., 2020a). In addition to being widely used to treat inflammatory diseases, Ori also has potential neuroprotective effects. Therefore, Ori can be applied as a possible drug for long-term treatment of AD.

4.3.4 CY-09

CY-09 is a selective and direct NLRP3 inhibitor. In 2017, Jiang et al. confirmed the target of interaction between CY-09 and NLRP3. They found that CY-09 can directly bind to the Walker A motif of NLRP3, rather than NLRC4, NLRP1, NOD2, or RIG-1, which indicates the specificity of CY-09. CY-09 directly binds to the ATP binding motif of the NLRP3 NACHT domain to inhibit NLRP3 ATPase activity, thereby inhibiting the assembly and activation of NLRP3 inflammasome (Jiang et al., 2017). Based on the high specificity and good pharmacokinetic characteristics of CY-09 targeting NLRP3, it may become a new method for the treatment of diseases. Currently, CY-09 can be treated for inflammatory related diseases such as osteoarthritis (Zhang et al., 2021a), myocardial fibrosis (Gao et al., 2021a), hepatic steatosis (Wang et al., 2021), etc. However, the effect of CY-09 has not been reported in AD, and its application value should be explored as soon as possible.

4.3.5 MCC950

MCC950 is a small molecule compound of diarylsulfonylurea. It is a potent and selective small molecule inhibitor of NLRP3, which can block the activation of canonical and non-canonical NLRP3 at nanomolar concentrations. MCC950 specifically inhibits NLRP3 but not AIM2, NLRC4 or NLRP1 activation. MCC950 reduces IL-1β production *in vivo* and attenuates the severity of experimental autoimmune encephalomyelitis (EAE) (Coll et al., 2015). With the study of MCC950 target, the researchers have found that MCC950 can also specifically bind to NLRP3. It directly interacts with the walker B motif in the NACHT domain of NLRP3, which blocks the activity of NLRP3 ATPase and loses the ability to hydrolyze ATP, thereby blocking NLRP3 oligomerization and formation (Coll et al., 2019). This is further supported by another study. They have found that MCC950 can modify the active conformation of NLRP3 and prevent NLRP3 oligomerization (Tapia-Abellan et al., 2019). MCC950 is an effective and selective NLRP3 inhibitor, which has a wide range of applications in inflammatory diseases. However, here we mainly discuss the therapeutic effect of MCC950 in cognitive dysfunction diseases. Dempsey et al. found that, in the APP/PS1 AD mouse model, MCC950 can inhibit the activation of NLRP3 inflammasome in microglia, prevent the release of IL-1β, and promote the phagocytosis of Aβ by microglia, which reduces the accumulation of Aβ and improves the cognitive function (Qi et al., 2018). In addition, MCC950 can also completely inhibit the immune response after activation of NLRP3 inflammasomes induced by fibrils and low molecular weight Aβ aggregates (Luciunaite et al., 2020). MCC950 attenuates the reactivity of microglia induced by Aβ1-42 oligomers, blocks the activation of NLRP3 inflammasome, and eliminates memory impairment (Fekete et al., 2019). These results indicate that MCC950 can reduce Aβ-induced pathological events and enhance cognitive function. Some studies have also found that MCC950 improves the damage of synaptic plasticity (Qi et al., 2018), inhibits the activation of IL-1β induced by tau aggregates, and prevents tau-mediated pathological changes (Stancu et al., 2019). Li et al. reported that the administration of MCC950 improves the spatial memory and brain histology of senescence-accelerated mouse prone 8 (SAMP8), and reduces the deposition of Aβ in the mouse brain (Li et al., 2020). MCC950 may be a promising compound for AD treatment, but this also requires more animal experiments

and clinical drug observation trials for further evaluation. The improvement of drugs based on MCC950 can reduce its side effects and increase its neuroprotective efficacy and safety, which is also a potential strategy for the development of AD drugs.

4.3.6 OLT1177

OLT1177, also known as Dapansutrile, is an active *ß*-sulfonyl nitrile compound. OLT1177 is initially identified as a drug for the treatment of arthritis and is currently undergoing a phase II clinical trial for the treatment of acute gouty arthritis (Kluck et al., 2020). OLT1177 is a potent, selective and orally active inhibitor of NLRP3 inflammasome. The effect of OLT1177 is similar to that of MCC950. It blocks the canonical and non-canonical activation of NLRP3 inflammasome, and directly binds to NLRP3 to inhibit its ATPase activity (Marchetti et al., 2018). *In vitro* experiments have shown that nanomolar concentration of OLT1177 can specifically inhibit the activation of NLRP3 inflammasome and reduce the release of IL-1β and IL-18 (Marchetti et al., 2018). In a study, Lonnemann et al. provided some convincing evidence. Their results showed that OLT1177 inhibits the activation of NLRP3 inflammasome, thereby improving cognitive dysfunction and synaptic plasticity in AD mice, reducing the number of pathological plaque deposits in the cerebral cortex, and reducing the activity of microglia (Lonnemann et al., 2020). However, there are still few researches on the application of OLT1177 in neurodegenerative diseases. In short, considering that OLT1177 has good safety, pharmacokinetics and less side effects after oral administration, this makes OLT1177 to become an option for the treatment of AD in the future.

4.3.7 Tranilast

Tranilast is originally used as an anti-allergic drug, which has a good therapeutic effect on asthma, allergic rhinitis, idiopathic dermatitis and other allergic diseases. Now, other uses, such as myocardial fibrosis and anti-cancer treatment, are gradually being discovered (Chen et al., 2021; Osman et al., 2021). In 2018, Huang et al. first discovered that Tranilast is a direct NLRP3 inhibitor that can inhibit the NLRP3-NLRP3 interaction. Tranilast inhibits NLRP3 inflammasome activation in macrophages, but has no effects on AIM2 or NLRC4 inflammasome activation. Tranilast directly binds to the NACHT domain of NLRP3 and suppresses the assembly of NLRP3 inflammasome by blocking NLRP3 oligomerization (Huang et al., 2018). Moreover, the researchers have also reported that Tranilast increases the lysine 63 (K63)-linked ubiquitination of NLRP3, restricts NLRP3 oligomerization, blocks the assembly and activation of NLRP3 inflammasome, thereby improving vascular inflammation and atherosclerosis in $Ldlr^{-/-}$ and $ApoE^{-/-}$ mice (Chen et al., 2020). Tranilast can inhibit the formation of rat gliomas after oral administration, which indicates that Tranilast can cross the BBB (Platten et al., 2001). However, the therapeutic effect of Tranilast in AD is still unclear. Recent studies have reported that Tranilast can improve cognitive behavioral parameters and significantly increase memory-related proteins in Aβ-induced cognitive deficit model mice, thereby showing the potential for neuroprotection (Thapak et al., 2021). On the contrary, some researchers have put forward different views. Connors et al. found that Tranilast is likely to promote fibrillation by shifting Aβ monomer conformations to those capable of seed formation and fibril elongation, which indicates that elderly patients treated with Tranilast may increase the risk of AD (Connors et al., 2013). The role of Tranilast in AD still needs further research, and whether Tranilast plays a role in AD by inhibiting the activation of NLRP3 inflammasome is also unknown.

4.3.8 BAY 11–7082 and Parthenolide

BAY 11–7082 and Parthenolide are common NF-κB inhibitors. BAY 11–7082 can inhibit IκBα phosphorylation and prevent nuclear translocation of NF-κB. Parthenolide is a powerful natural anti-inflammatory drug derived from the medicinal plant Feverfew. As early as 2010, Juliana et al. found that BAY 11–7082 and Parthenolide can selectively inhibit the activity of NLRP3 inflammasome in macrophages, but this effect is not related to their inhibitory effect on NF-κB activity. They found that Bay 11–7082 and Parthenolide blocks ASC oligomerization via inhibiting NLRP3 ATPase activity. Surprisingly, in addition to directly inhibiting NLRP3, Parthenolide is also a direct inhibitor of caspase-1, while Bay 11–7082 has no such effect. Therefore, Bay 11–7082 selectively inhibits the NLRP3 inflammasome pathway, while Parthenolide inhibits the activity of multiple inflammasome pathways (Juliana et al., 2010). In the TBI model, Bay 11–7082 shows a similar effect to NLRP3 knockout, which significantly limits the NLRP3 inflammasome activation, reduces the levels of caspase-1 and IL-1β, and improves the cognitive function of model mice (Irrera et al., 2017). Additionally, the pretreatment of Bay 11–7082 can also block the activation of inflammasome through the pharmacological inhibition of NF-κB/NLRP3, thereby reducing neuronal damage and cognitive dysfunction in aged rats (Liu et al., 2021). In APP23 mice treated with kainic acid (KA), BAY 11–7082 attenuates KA-induced neuronal degeneration and Aβ deposition by inhibiting the activation of NLPR3 inflammasome, and ultimately improves the cognitive function (Ruan et al., 2019). These studies indicate that BAY 11–7082 has neuroprotective effects on AD. Parthenolide has been proven to have antioxidant and anti-inflammatory effects, but its role in the nervous system has not yet been elucidated. According to reports, Parthenolide can effectively reduce neuroinflammation and improve brain damage (Jun-An Wang et al., 2020). More importantly, the synthesis of Parthenolide derivatives with low toxicity, such as compound 8b (Ou et al., 2020), may bring hope for targeting NLRP3 inflammasome to treat AD.

4.3.9 3,4-Methylenedioxy-β-nitrostyrene

3,4-methylenedioxy-β-nitrostyrene (MNS) is a tyrosine kinase inhibitor. In 2014, He et al. first discovered the inhibitory effect of MNS on NLRP3 inflammasome activation. They found that MNS do not affect the activation of NLRC4 or AIM2 inflammasome, but specifically blocks NLRP3-mediated ASC speck formation and oligomerization. MNS directly binds to the nucleotide-binding and oligomerization domain (NOD) and leucine-rich repeat (LRR) domains of NLRP3 and inhibits the activity of NLRP3 ATPase, thereby blocking the assembly and activation of

inflammasome (He et al., 2014). It has previously been reported that MNS can inhibit platelet aggregation, tumor cell invasion and metastasis (Wang et al., 2007; Chen et al., 2015). At present, more attention should be paid to the application of MNS in inflammatory-related diseases by blocking the activation of NLRP3 inflammasome. The role of MNS in AD is still unknown, which requires more *in vivo* and *in vitro* experiments.

4.4 ASC Oligomerization Inhibitors

4.4.1 β-hydroxybutyrate

β-hydroxybutyric acid (BHB) is a ketone body produced by the oxidation of fatty acids in the liver under fasting conditions, which can provide alternative energy for the brain and heart. In 2015, Youm et al. first discovered that BHB can specifically inhibit the activation of NLRP3 inflammasome. They found that BHB inhibits NLRP3 inflammasome assembly and activation by preventing K^+ efflux and reducing ASC oligomerization and speck formation (Youm et al., 2015). Clinical evidence shows that long-term consumption of ketogenic formula can significantly improve the cognitive function of AD patients (Ota et al., 2019). In addition, BHB reduces the level of IL-1β by inhibiting NLRP3-mediated hippocampal neuroinflammation, thereby exerting an antidepressant effect (Yamanashi et al., 2017; Kajitani et al., 2020). Subsequently, BHB is also found to attenuate long-term stress-induced anxiety-related behaviors and plays an anti-anxiety effect (Yamanashi et al., 2020). Recently, in the 5xFAD mouse model, Shippy et al. revealed that the administration of BHB reduces Aβ plaque formation, microglial proliferation, ASC formation and caspase-1 activation, thereby alleviating AD pathology (Shippy et al., 2020). BHB can easily cross the BBB, which increases its therapeutic potential as a treatment strategy for AD.

4.5 Caspase-1 Activation Inhibitors

4.5.1 VX-765

Caspase-1 is an important component of NLRP3 inflammasome. Upon activation, caspase-1 promotes the production of IL-1β/IL-18, and at the same time mediates the pyroptosis through gasdermin D. VX-765 is a safe, effective, selective, and small molecule caspase-1 inhibitor. Early studies showed that VX-765 inhibits the production of IL-1β in forebrain astrocytes, thereby blocking epilepsy in rats (Ravizza et al., 2008). Currently, VX-765 has entered phase II clinical trials for patients with epilepsy. VX-765 is a non-toxic caspase-1 inhibitor that is permeable to the BBB. In the AD model, VX-765 prevents progressive Aβ deposition and reverses brain inflammation, synaptic loss, and memory impairment (Flores et al., 2018). In addition, VX-765 is promising as an effective drug to prevent the onset of cognitive deficits. Research by Flores et al. showed that treatment with VX-765 for 1 month before the onset of symptoms in AD J20 model mice could delay the cognitive impairment of mice by at least 5 months (Flores et al., 2020). Therefore, VX-765 represents a safe drug, which may have potential value in the early prevention of AD cognitive deficits and the improvement of cognitive dysfunction.

4.5.2 Ac-YVAD-CMK

Ac-YVAD-CMK is a selective and irreversible inhibitor of caspase-1, and prevents the expression of IL-1β. Ac-YVAD-CMK can inhibit the activation and infiltration of microglia around the hematoma in the rat model of cerebral hemorrhage, promote the transformation of microglia from M1 type to M2 type, and reduce the release of IL-1β/IL-18. At the same time, Ac-YVAD-CMK inhibits cell pyroptosis, improves nerve function, and exhibits neuroprotective effect (Lin et al., 2018; Liang et al., 2019). Infusion of Ac-YVAD-CMK into the lateral ventricle of aged rats can inhibit the production of hippocampal IL-1β, thereby improving the memory of aged rats and reversing the decrease of hippocampal neurons (Gemma et al., 2005, Gemma et al., 2007). In the AD model, AC-YVAD-CMK treatment improves spatial learning and memory impairment in APP/PS1 mice, reduces Aβ plaque deposition, and promotes membrane transport of GluA1 (Gu et al., 2021).

4.6 Plant-Derived Compounds and Chinese Herbal Medicines

Some plant-derived compounds and Chinese herbal medicines can inhibit the activation of NLRP3 inflammasome, and exhibit the effect of preventing and treating AD. Resveratrol is a natural polyphenol compound extracted from plants. Many studies have shown that resveratrol has anti-cancer, anti-oxidant, anti-inflammatory, anti-aging and other pharmacological effects (Kumar et al., 2021). In the nervous system, resveratrol can play a neuroprotective effect by inhibiting the activation of NLRP3 inflammasome. Feng et al. found that resveratrol significantly inhibits the proliferation and activation of BV-2 cells induced by Aβ through the TXNIP/TRX/NLRP3 signaling pathway, and reduces the expression levels of caspase-1 and IL-1β (Feng and Zhang 2019). Qi et al. also reported that resveratrol reduces Aβ-induced IL-1β production and mitochondrial dysfunction through the NF-κB/IL-1β/NLRP3 signaling pathway, improves learning and cognitive impairment, and plays an anti-dementia effect (Qi et al., 2019). Picrorhiza kurroa (PK) is a herbal medicine with antioxidant, anti-inflammatory, anti-allergic and anti-cancer effects. Kim et al. found that, in the hippocampus of 5xFAD mice, PK inhibits the activation of NLRP3 inflammasome, reduces the protein expression level of NLRP3 and the activity of caspase-1, thereby blocking the release of IL-1β (Kim et al., 2020). Dihydromyricetin (DHM) is a kind of plant flavonoids, which has many unique effects such as anti-oxidation, anti-thrombosis, anti-cancer, and anti-alcoholism. It is convinced that flavonoids can cross the BBB to regulate inflammation and exert neuroprotective effects (Youdim et al., 2003). In the AD model, DHM treatment can inhibit the activation of NLRP3 inflammasome in APP/PS1 mice and reduce the level of IL-1β. DHM, as a therapeutic drug that inhibits the activation of microglia by inhibiting NLRP3 inflammasome, contributes to prevent the progression of AD-like pathology and improve spatial memory (Feng et al., 2018). Pterostilbene is a natural compound with antioxidant, anti-inflammatory and neuroprotective activities. Li et al. reported that pterostilbene attenuates the neuroinflammatory response induced by Aβ1-42 in microglia via inhibiting the NLRP3/caspase-1 inflammasome pathway (Li et al., 2018b). Sulforaphane (SFN) is an isothiocyanate derivative

contained in cruciferous vegetables. SFN's anti-oxidation, anti-cancer, anti-inflammatory and other uses are being extensively studied. SFN also exhibits anti-inflammatory effects in the brain. Tufekci et al. found that SFN inhibits the secretion of IL-1β and IL-18 mediated by NLRP3 inflammasomes and the pyroptosis of microglia (Tufekci et al., 2021). Ginkgolide B (GB) is a plant ester compound extracted from Ginkgo biloba leaves. Through its anti-inflammatory, anti-oxidant and anti-apoptotic properties, GB exerts an effective neuroprotective effect on ischemic brain injury and neurodegenerative diseases. Zhang et al. found that GB treatment prevents the pathological process of AD and inhibits neuroinflammation by inhibiting NLRP3 inflammasome activation and promoting microglia M2 polarization (Zhang et al., 2021). *Achyranthes* bidentate has anti-inflammatory and antioxidant activities, and has been used in traditional Chinese medicine for the treatment of dementia and osteoporosis for a long time. Recent study has shown that *Achyranthes* bidentate polypeptide fraction κ (ABPPκ) can down-regulate Aβ oligomer-induced IκBα phosphorylation and NLRP3 expression *in vitro*. *In vivo*, pre-administration of ABPPk inhibits the activation of microglia in the CA3 region of the hippocampus, promotes the polarization of the microglia M2 phenotype, and reduces the expression of NLRP3, cleaved caspase-1 and ASC in the brain, thereby significantly improving the cognitive impairment of mice (Ge et al., 2021). In addition, there are other Chinese herbal medicines, including Dl-3-n-butylphthalide (Dl-NBP) (Wang et al., 2019), Shaoyao Gancao Tang (SG-Tang) (Chiu et al., 2021), and Liquiritigenin (LG) (Du et al., 2021), which can play a neuroprotective role in AD by inhibiting the NLRP3 inflammasome pathway. Therefore, these Chinese herbal medicines and extracts that can inhibit the activation of NLRP3 may be a promising and safe treatment for AD.

4.7 Nonsteroidal Anti-inflammatory Drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most widely used anti-inflammatory drugs in clinical practice. These drugs have a wide range of effects, including anti-inflammatory, anti-rheumatic, antipyretic, analgesic and so on, which are widely applied in rheumatic and painful diseases. NSAIDs mainly act by inhibiting the cyclooxygenase-1 (COX-1) and COX-2. Researches have shown that NSAIDs can delay the development or reduce the risk of AD by acting on the NLRP3 inflammasome pathway (Deardorff and Grossberg 2017). Early studies found that NSAIDs of the fenamate class (such as mefenamic acid) are effective and selective inhibitors of NLRP3 inflammasome, which can selectively inhibit the activation of NLRP3 inflammasome in macrophages and relieve the cognitive impairment of AD mice. The effect of NSAIDs on NLRP3 may be through the inhibition of the volume-regulated anion channel (VRAC), independently of COX enzymes (Daniels et al., 2016). Another study showed that Indomethacin (IND) reduces the expression of IL-1β and caspase-1 via inhibiting NLRC4 and NLRP3 inflammasomes, thereby improving neuroinflammation and memory impairment in AD (Karkhah et al., 2021). In a recent review, Hampel et al. have reported that, in transgenic AD mice, the researchers have found that NSAIDs not only exert neuroprotective effects by suppressing inflammatory effects, but also reduce early Aβ pathology through other mechanisms, thereby preventing memory decline. However, all controlled prospective trials from the clinic have not found positive therapeutic effects of NSAIDs in AD patients, or have limited their application due to the severe side effects (Hampel et al., 2020). So the researchers do not have positive data from patients supporting the hypothesis that NSAIDs are effective in AD. In humans, the occurrence of AD is related to many predisposing factors (such as age, genetics and environment, etc.), and its pathogenic mechanisms are also complex and diverse. This may lead to different mechanistic pathways for human AD and rodent AD disease models. Furthermore, human AD is a long-term asymptomatic chronic disease, and the relatively late treatment time point may also be a potential reason for the clinical inefficiency of NSAIDs. Because epidemiological data show that the incidence of AD decreases after long-term treatment with NSAIDs (Hampel et al., 2020). In the future, evaluation of the effect of NSAIDs in AD treatment requires more data from clinical trials.

4.8 MicroRNAs

MicroRNAs can directly target the NLRP3 inflammasome, and play an important role in the regulation of inflammation. MiR-138–5p can directly target the 3′-UTR of NLRP3 and inhibit the expression of NLRP3. Up-regulation of miR-138–5p inhibits the activation of NLRP3/caspase-1 axis and microglia, thereby attenuating hippocampal neuroinflammation and improving the cognitive function of the rat model (Feng et al., 2021a). MicroRNA-223 also directly targets and inhibits the expression of NLRP3, thereby reducing LPS-induced inflammation in microglia and improving neuronal function (Zhang et al., 2020). However, some studies have found that microRNAs also affect the expression of NLRP3 indirectly. MiR-194–5p can target TNF receptor associated factor 6 (TRAF6), which interacts with NLRP3 to promote the activation of NLRP3 inflammasome. Overexpression of miR-194–5p can reduce the interaction of TRAF6/NLRP3, thereby inhibiting the NLRP3 inflammasome activation and reducing neuroinflammation (Wan et al., 2021). A recent study found that the expression level of miR-22 in the peripheral blood of AD patients is lower than that of healthy people. MiR-22 regulates glial cell pyroptosis by targeting GSDMD, inhibits the activation of NLRP3 inflammasome, and reduces the release of inflammatory cytokines, thereby alleviating cognitive impairment in AD mice (Han et al., 2020a). In addition, miR-34c (Xu et al., 2019), miR-30e (Li et al., 2018c), and miR-7 (Zhou et al., 2016) can also directly target and inhibit NLRP3, regulate the activity of NLRP3 inflammasome, and improve the occurrence of neuroinflammation. Perhaps targeting microRNAs for regulating the activation of NLRP3 inflammasome may be a new direction for AD treatment.

4.9 Autophagy and Mitophagy Activators

As described in the previous regulation of NLRP3 inflammasome activation, autophagy and mitophagy have

been shown to regulate inflammasome activation. Therefore, any drugs that activate autophagy or mitophagy can negatively regulate NLRP3 inflammasome. IIIM-941 can induce autophagy through AMPK pathway to inhibit ATP-induced NLRP3 inflammasome activity (Ali et al., 2021). Dopamine D1 receptor agonist A-68930 enhances the degradation of NLRP3 inflammasome and reduces the secretion of IL-1β and IL-18 by activating the AMPK/autophagy signaling pathway, thereby improving the neuroinflammation and cognitive impairment of mice induced by Aβ1-42 (Cheng et al., 2020). Progesterone (PG) is a steroid with neuroprotective effects. Hong et al. found that PG inhibits the activation of NLRP3-caspase-1 inflammasome induced by Aβ via enhancing the autophagy of astrocytes, thereby exhibiting neuroprotective effects (Hong et al., 2019). In the TgCRND8 AD model, Ginkgo biloba extract EGb 761 can activate autophagy in microglia, down-regulate the level of NLRP3 protein, reduce the activation of IL-1β and caspase-1 induced by Aβ, and improve the cognitive ability of mice (Liu et al., 2015). Moreover, AICAR and metformin can activate PRKAA1 to enhance autophagy, which not only contributes to clear extracellular Aβ fibrils, but also inhibits Aβ-induced NLRP3 inflammasome activation and IL-1β release (Cho et al., 2014). 2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside (TSG) is one of the main active ingredients extracted from Polygonum multiflorum. It has been proven to be used in the treatment of AD. TSG can effectively alleviate the neuroinflammatory response induced by LPS via inhibiting the NLRP3 signaling pathway in microglia and neurons. In addition, TSG can also prevent LPS/ATP and Aβ-induced inflammation through AMPK/PINK1/Parkin-dependent enhancement of mitophagy, thereby exerting a neuroprotective effect (Gao et al., 2020). Quercetin (Qu) is a natural flavonoid compound with anti-inflammatory and antioxidant properties. Recent studies have found that Qu promotes mitophagy to enhance the clearance of damaged mitochondria, thereby inhibiting mtROS-mediated activation of NLRP3 inflammasome in microglia, and preventing neuronal damage (Han et al., 2021). We believe that the enhancement of autophagy and mitophagy in microglia may be a new strategy for the treatment of AD, but the safety of this treatment remains to be further observed.

5 ROS AND NF-κB INHIBITORS

α-lipoic acid (LA) is an antioxidant, and is frequently used in the treatment of diabetes. LA can easily pass through the BBB and play a protective role in the nervous system. Studies found that *α*-LA inhibits the activation of NLRP3 inflammasome in microglia, regulates the polarization of microglia from the M1 phenotype to the M2 phenotype, and reduces the neuroinflammatory response (Kim et al., 2019). Edaravone (EDA) is a free radical scavenger that has neuroprotective effects on cerebral infarction, amyotrophic lateral sclerosis and dementia. In the AD cell model, EDA significantly attenuates mitochondrial membrane potential (Δψm), reduces the production of mtROS, and inhibits the activation of NLRP3 inflammasome induced by Aβ (Wang et al., 2017). Donepezil is a reversible central acetylcholinesterase (AChE) inhibitor that can be used to improve the cognitive function of AD patients. Recent studies found that Donepezil can also inhibit LPS-induced AKT/MAPK signaling and NF-κB/STAT3 phosphorylation in BV2 microglia, and down-regulate NLRP3 and pro-IL-1β mRNA levels, thereby reducing neuroinflammation induced by NLRP3 inflammasome (Kim et al., 2021).

6 CONCLUSION AND FUTURE PERSPECTIVES

It has been nearly 2 decades since the NLRP3 inflammasome being discovered. With continuous studies, researchers have gained a certain understanding of the structure, composition, regulation and role of NLRP3, but its precise molecular mechanisms in diseases have not been fully elucidated. In recent years, the research of NLRP3 inflammasome in neurodegenerative diseases has attracted much attention. More and more evidences have confirmed that NLRP3 inflammasome activation plays an important role in the pathogenesis and progression of AD. More importantly, microglia and astrocytes play a crucial role in the chronic neuroinflammatory response of AD caused by NLRP3 inflammasome. In AD cells and animal models, the inhibitory measures against NLRP3 or its inflammasome constituent molecules can alleviate the inflammatory response, and reduce Aβ deposition, Tau phosphorylation and other pathological features, thereby improving AD-related behavioral abnormalities. Therefore, targeting NLRP3 inflammasome may be a new trend for AD treatment. The activation of NLRP3 inflammasome involves upstream signal related regulatory factors, priming signal, activation signal and downstream IL-1β and IL-18 effectors. In the early stage of drug development, researchers usually focus on strategies to block downstream inflammatory cytokines. Inhibitors targeting IL-1β as drugs for the treatment of neurological diseases have not achieved satisfactory clinical results. With the discovery of new drug targets, people gradually turn their attentions to NLRP3 and the constituent molecules ASC and caspase-1. This targeting effect is selective and efficient, which can ensure the specificity of the treatment to the greatest extent and reduce non-specific effects. In addition, the upstream-related regulatory factors of NLRP3 inflammasome activation can also become attractive pharmacological targets, but due to the complexity of the interaction of upstream signals, it may bring non-specific therapeutic roles. So far, although many compounds have successfully been identified to target NLRP3 inflammasome *in vitro* and *in vivo*, their therapeutic effects and safety in AD patients have yet to be verified by clinical trials. In the CNS diseases, the development of therapeutic drugs targeting the NLRP3 inflammasome needs to be evaluated by its permeability across the BBB. More importantly, under the premise of obtaining the desired therapeutic values, it will not cause toxic effects on the whole-body or CNS. In addition, AD is a

long-term chronic progressive disease, and usually requires intervention in the early stage of the disease. However, whether long-term use of targeted drugs for inflammasomes will affect the health of AD patients requires further evaluation. In view of the good safety and side effects of traditional Chinese herbal medicines and plant-derived compounds, they may provide new directions for the treatment of AD.

AUTHOR CONTRIBUTIONS

Author TL designed, collected literatures, and wrote the manuscript. Author SW and author QC collected some literatures. Author YZ and author LW revised the manuscript.

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Insight Into the Mechanism of Exercise Preconditioning in Ischemic Stroke

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Exercise preconditioning has attracted extensive attention to induce endogenous neuroprotection and has become the hotspot in neurotherapy. The training exercise is given multiple times before cerebral ischemia, effectively inducing ischemic tolerance and alleviating secondary brain damage post-stroke. Compared with other preconditioning methods, the main advantages of exercise include easy clinical operation and being readily accepted by patients. However, the specific mechanism behind exercise preconditioning to ameliorate brain injury is complex. It involves multi-pathway and multi-target regulation, including regulation of inflammatory response, oxidative stress, apoptosis inhibition, and neurogenesis promotion. The current review summarizes the recent studies on the mechanism of neuroprotection induced by exercise, providing the theoretical basis of applying exercise therapy to prevent and treat ischemic stroke. In addition, we highlight the various limitations and future challenges of translational medicine from fundamental study to clinical application.

Keywords: exercise preconditioning, ischemic stroke, neurprotection, apoptosis, neuroinflammation, oxidative stress

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INTRODUCTION

Stroke is primarily divided into hemorrhagic (intracranial hemorrhage and subarachnoid hemorrhage) and ischemic stroke. Ischemic stroke accounts for up to 80% of all strokes and is one of the most fatal global diseases with rapid onset, high mortality, and high disability [Amarenco et al., 2009; Hsieh et al., 2010 (accessed on 18 January 2022)]. The treatment principle behind ischemic stroke is to rapidly reconstruct blood reperfusion, restore oxygen supply to the brain, and remove harmful metabolites to reduce the cerebral infarction volume (Bhatia et al., 2010; Diprose et al., 2021). In recent years, neuroprotective agents have been studied based on anti-oxidation, anti-apoptosis, inhibition of excitatory amino acid release, anti-inflammation, vascular neuroprotection, and nanoparticles (Subedi and Gaire, 2021a; Chen et al., 2021; Zheng et al., 2021; Kaur and Sharma, 2022). However, most effective drugs in animal experiments often fail in clinical trials (Gladstone et al., 2002; Wahlgren and Ahmed, 2004). Therefore, finding other effective treatments besides drugs has been the emerging idea.

Ischemia tolerance has attracted wide attention as an effective protective strategy for cerebral ischemia. Ischemic preconditioning refers to tissue tolerance during long-term ischemic injury after one or more transient ischemia-reperfusion. It usually manifests as reduced cellular death, decreased cerebral infarct size, and improved organ dysfunction (Liu et al., 2021a; Correia et al., 2021; Ripley et al., 2021). Ischemic preconditioning is an effective neuroprotective method of endogenous cerebral ischemia, with exercise preconditioning being an essential type. Exercise preconditioning can effectively induce ischemia tolerance, exert neuroprotective effects, and alleviate brain damage

post-stroke by providing training multiple times before ictus. Compared with other preconditioning methods, its advantages are easy to master, operate clinically, and easily accepted by patients (Egan et al., 2014). Moreover, clinical and animal experiments have ascertained the neuroprotective effect of exercise preconditioning **Table 1**. The underlying mechanism involves regulating the inflammatory response, inhibiting oxidative stress and apoptosis, promoting neural regeneration, contributing to brain structure and function remodeling, and reducing tissue injury after cerebral ischemia (Sakakima, 2019; Hafez et al., 2020; Hafez et al., 2021).

MECHANISM OF EXERCISE PRECONDITIONING INDUCED CEREBRAL ISCHEMIA TOLERANCE

Attenuation of Neuronal Apoptosis

Apoptosis is programmed cell death, having the characteristics of selectivity, initiative, and reversibility. Cellular necrosis is characterized by cell swelling, membrane rupture, and random degradation of DNA. In contrast, cellular apoptosis involves dense chromatin, formation of DNA fragments, cytoplasmic foam, and apoptotic bodies (Park et al., 2021a; Moujalled et al., 2021; Saleem, 2021). Apoptosis is crucial in ischemic injury and is the primary form of delayed neuronal death after cerebral ischemia (Mitsios et al., 2007; Radak et al., 2017; Uzdensky, 2019). Therefore, brain damage will be alleviated if the occurrence and development of neuronal apoptosis are effectively prevented. Primarily, there are three apoptotic pathways: endoplasmic reticulum stress pathway, death receptor pathway, and mitochondrial pathway (Prentice et al., 2015; Redza-Dutordoir and Averill-Bates, 2016; Wei et al., 2018). In addition, many apoptosis-related genes and proteins are regulated and involved in apoptosis after cerebral ischemia (Ferrer et al., 2003; Uzdensky, 2019).

Previous studies have observed that exercise preconditioning can effectively alleviate cerebral ischemia associated tissue damage caused. One study revealed that preconditioned exercise retained more surviving neurons within the hippocampus of the ischemic brain tissue, effectively reducing neuronal death (Tahamtan et al., 2013). Another report depicted that exercise training could effectively induce autophagy and reduce neuronal apoptosis after stroke (Zhang et al., 2013). Exercise can induce the expression of the heat shock protein (HSP)-70, which attenuates apoptosis by inhibiting apoptosis-inducing factors and elevating anti-apoptotic proteins expression, such as Bcl-2, leading to the alleviation of cerebral ischemic injury (Zhang et al., 2011). Wang et al. (2019b) observed that preischemic treadmill exercise improves post ischemic brain injury outcomes by preserving both the old and newly formed HSP-72-containing neurons within rats. Similarly, Lin et al. (2015) proposed that preischemic treadmill exercise improves the outcome of ischemic stroke by elevating the numbers of neurons and glial cells containing HSP-20. In addition, several studies explored the potential mechanism underlying exercise-induced neuroprotection after ischemic stroke. Liebelt et al. (2010) suggested that exercise preconditioning can reduce neuronal apoptosis and cerebral infarction volume through upregulation of HSP-70 and ERK ½. Additionally, ERK and HSP-70 inhibitors could simultaneously eliminate the protective effects of exercise preconditioning on the brain. Other studies found that preischemic treadmill exercise reduced hippocampal microvascular injury after stroke, prevented zonula occludens-1 reduction in the hippocampus, and inhibited matrix metalloproteinase-9 (MMP-9) activation after stroke (Lee et al., 2019). Another team also revealed the changes of MMP-9 in stroke mice, and they observed that exercise preconditioning induced a better outcome than the control ischemic mice, manifested by reduced MMP-9, diminished infarct volume, and significantly improved neurological deficits (Naderi et al., 2018). Exercise preconditioning may inhibit MMP-9 activity by upregulating ERK1/2 expression and reducing neuronal apoptosis level after cerebral ischemia (Chaudhry et al., 2010). ERK-mediated signaling pathways are involved in ischemia-induced apoptosis and regulate Bax and Bcl-2 protein expression after stroke (Li et al., 2021b). The mechanism of exercise preconditioning affecting Bcl-2 and Bax proteins expression is similar to hypoxia preconditioning, among which caspase 3, Bcl-2, and Bax are the core members regulating neuronal apoptosis (Liu et al., 2021d). Choi et al. (2013) observed that short-term running exercises inhibited the division of DNA induced by hypoxic-ischemic injury. Thus, it effectively reduced the expression of caspase-3 and inhibited neuronal apoptosis (Choi et al., 2013). Zhang et al. (2019). showed that voluntary wheel running inhibits cellular apoptosis by downregulating the Bax/Bcl-2 ratio and caspase-3 protein expression. On further analysis, both mild exercise postconditioning and intense exercise postconditioning significantly decreased brain infarct volumes and apoptosis compared to the resting rats. Moreover, mild exercise postconditioning enhanced Bcl-2 expression and the Bcl-2/Bax ratio (Li et al., 2021a). Controversially, Li et al. (2017b) found that Bcl-2 expression was not affected by exercise after stroke, indicating the importance of the exercise time point. Terashi et al. (2019) investigated the neuroprotective effect of various frequency preconditioning exercises on neuronal apoptosis post cerebral ischemia in rats. They observed that high-intensity preconditioning exercise for three or more times per week exert neuroprotective effects by downregulating the Bax/Bcl-2 ratio and caspase-3 activation after stroke (Terashi et al., 2019). The above mentioned results indicate that both pre- or postconditioning exercise can potentially induce ischemic tolerance by regulating apoptosis and anti-apoptosis-related proteins. Therefore, exploring the most suitable time points, intensity and frequency of exercise should be incorporated in future studies.

Inhibition of Oxidative Stress

When the body is subjected to harmful stimulation, the oxidation-antioxidation balance system is broken, leading to oxidative tissue damage through the accumulation of reactive oxygen species (ROS) in cells (Lushchak et al., 2021). ROS mainly includes singlet oxygen, ozone, hydrogen peroxide, and oxygen-

free radicals. ROS can be produced through aerobic metabolism during normal physiological conditions, and the production and elimination of ROS maintain a dynamic balance in the body. Nitricoxidesynthas, cyclooxygenase, xanthine dehydrogenase/xanthine oxidase, reduced-type coenzyme II oxygenase, myeloperoxidase, and other enzymes promote ROS production. In contrast, superoxide dismutase, catalase, peroxidase, glutathione peroxidase, and other enzymes inhibit ROS production (Kalyanaraman, 2013; Griffiths et al., 2014; Moldogazieva et al., 2018). Increased oxygen free radical generation and/or decreased scavenging capacity of the anti-oxidation system in the injured area after cerebral ischemia contributes to ROS (Shao et al., 2020; Duan et al., 2021; Jelinek et al., 2021), leading to neuronal death (Li et al., 2018). Brain tissue is rich in lipids and is highly sensitive to oxidative damage caused by ROS, characterizing oxidative stress as an essential target in treating ischemic stroke (Liu et al., 2002; Liu, 2003; Schönfeld and Reiser, 2017).

Ostuka et al. (2021b) conducted an animal study investigating the role of exercise preconditioning in subarachnoid hemorrhage (SAH). It was found that preconditioning ameliorates early brain injury post SAH. Moreover, the expression of 4-hydroxynonenal and nitrotyrosine was reduced by Nrf2/HO-1 pathway activation, improving the oxidative stress indicators (Otsuka et al., 2021b). Another study from the same team revealed that exercise preconditioning could decrease ROS in focal brain ischemia (Otsuka et al., 2016). Leite et al. (2012) found that swim training could relieve oxidative damage under metabolic stress by inhibiting glutamic acid and promoting the release of nitric oxide. In addition, several animal studies have also established that exercise preconditioning can effectively reduce oxidative damage of brain tissue during cerebral ischemia-reperfusion. Long-term and short-term exercise preconditioning can elevate antioxidant enzyme levels in the hippocampus and cortex, reduce the malondialdehyde content, inhibit oxidative stress, thereby alleviating oxidative damage post cerebral ischemia-reperfusion. This effect was coupled with improved sensory-motor function and memory. Therefore, it suggests that reducing oxidative stress could be an essential mechanism of exercise preconditioning-induced cerebral ischemia tolerance (Radak et al., 2007; Schimidt et al., 2014; Sosa et al., 2015; Chrishtop et al., 2020). The combination therapy of exercise and scalp acupuncture counteracts ischemic brain injury through ROS downregulation, suggesting a potential therapeutic approach in stroke patients (Li et al., 2020b).

Hypoxia inducible factor-1α (HIF-1α) is a sensitive oxygen homeostasis regulator and can be rapidly induced by hypoxia/ischemia. It plays a vital role in ischemic stroke through various mechanisms, including oxidative stress regulation, apoptosis, inflammation, and angiogenesis (Guglielmotto et al., 2009; Miyata et al., 2011; Cheng et al., 2014; Jiang et al., 2020; Peng et al., 2020; Zhang et al., 2021a; He et al., 2021). Previous studies have also determined that HIF-1α is crucial in ischemic preconditioning, which reduces brain damage post cerebral ischemia (Liu et al., 2005). HIF-1α exhibits beneficial effects mediated by the Akt signaling pathway and neuroinflammatory response multi-modulation in remote ischemic preconditioning (Yang et al., 2018; Du et al., 2020). In addition, upregulation of HIF-1α expression by hypoxic preconditioning promotes angiogenesis and neurogenesis. It reduces neuronal death and improves neurological function post ischemic stroke (Chen et al., 2017). Moreover, HIF-1α is involved in attenuating hyperglycemia-enhanced hemorrhagic transformation through MMP-2 and MMP-9 inhibition post-stroke (Soejima et al., 2013). As one of the crucial ways of ischemic preconditioning, exercise-induced neuroprotection is significantly associated with HIF-1α. Exercise preconditioning enhanced HIF-1α expression, contributing to elevated glucose metabolism and ATP production rates after ischemic stroke (Dornbos et al., 2013). Furthermore, exercise preconditioning stimulates the release of HIF-1α. It enhances neurogenesis and angiogenesis (Li et al., 2017a), promoting synaptic plasticity (Li et al., 2020a), and reducing neuronal apoptosis (Otsuka et al., 2019). However, exercise preconditioning-induced neuroprotective effect could be quickly lost after exercise cessation. This outcome is a reminder that regulating HIF-1α expression in a time-dependent manner may potentially focus on the further treatment of ischemic stroke (Otsuka et al., 2021a).

Suppression of Inflammation

An inflammatory response is a pivotal part of the pathological process of ischemic brain injury. The inflammatory response involves a series of inflammatory cells and mediators, which have a dual effect of damage and repair in the occurrence and development of cerebral ischemia. Its effect is correlated with time, scope, and the severity of inflammation (Ceulemans et al., 2010; Wang et al., 2019a; Pluta et al., 2021). Studies have shown that inflammation factor expression in the ischemic region increased significantly within a few hours after cerebral ischemia, with tissue damage caused by various mechanisms, including microvascular occlusion, oxygen free radical generation cytotoxicity enzyme, and chemokine release (Zhang et al., 2021b; Ma et al., 2021).

Glial cells are a significant group of cells in the brain. The number of glial cells is 10–50 times that of neurons and has almost the same total volume as that of neurons. They are mainly categorized into astrocytes, oligodendrocytes, and microglias (Xu et al., 2020; Sancho et al., 2021). Microglia secretes inflammatory molecules at the injury site to protect healthy neurons and remove the dead ones. During cerebral ischemia, microglia are rapidly activated, presenting antigens, and releasing inflammatory factors like IL-1β, IL-6, and TNF-α. In contrast, during the recovery stage of the brain, microglia exhibits an anti-inflammatory role (Zhang, 2019; Berchtold et al., 2020; Kang et al., 2020; Subedi and Gaire, 2021b; Hou et al., 2021). Many scholars have explored the impact of microglia during exercise. High-intensity interval training elicited better responses at functional and cardiovascular levels than moderate-intensity continuous training after ischemic stroke. Thus, inflammasome-mediated pyroptosis could be suppressed by the anti-inflammatory effect of exercise due to the shifting of microglial polarization towards the neuroprotective M2 phenotype (Liu et al., 2021b). Moreover, treadmill exercises improved short-term memory, inhibited reactive astrogliosis

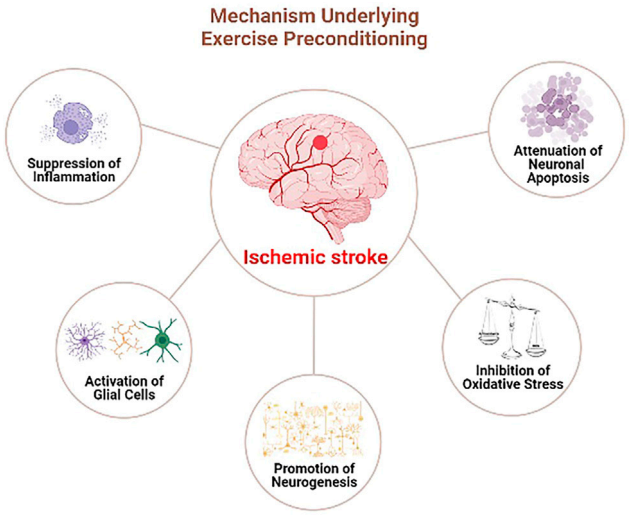

FIGURE 1 | The involved mechanisms underlying preconditioning exercise-induced neuroprotection in ischmeic stroke.

and microglial activation, and suppressed the expression of adhesion molecules and pro-inflammatory cytokines in hyperlipidemic rats (Park et al., 2021b). Casaletto et al. (2022) supported the conclusion that physical activity could be leveraged to reduce pro-inflammatory microglial states in humans through modifiable behavior. They monitored physical activities and cognitive performances in life and quantified the microglial activation and synaptic markers inside brain tissue at death (Casaletto et al., 2022). Treadmill exercise can significantly ameliorate cerebral ischemia-reperfusion injury through IL-4 expression elevation to promote M2 microglia polarization through the JAK1-STAT6 pathway (Lu et al., 2021).

Astrocytes are the most abundant cell type in the central nervous system responding to various disease states. They assist in clearing excessive potassium ions around neurons by regulating the osmotic balance of ions and water and maintaining the relative stability of the neuronal external environment (Jensen et al., 2013; Dinuzzo et al., 2017; Yang et al., 2021b). Astrocytes are also involved in the inflammatory response post cerebral ischemia (Gao et al., 2021; Mi et al., 2021; Kieran et al., 2022), although their roles are different in different stages of inflammation. In the initial phase of inflammation, astrocytes behave as antigen-presenting cells and secrete pro-inflammatory antigen-presenting cytokines to protect tissues from damage. During the inflammatory response and repair phase peak, astrocytes act as inflammatory regulatory cells, secreting anti-inflammatory cytokines and promoting tissue repair (Regunathan and Piletz, 2003). Jiang et al. (2021) investigated the physical exercise influence on activated astrocytes polarization. They observed that the impact of physical exercise on white matter repair and cognition improvement could be related to astrocytes polarization regulation, inducing myelin debris clearance and efficient remyelination (Jiang et al., 2021). He et al. (2017) revealed that voluntary wheel running accelerated glymphatic clearance, improved the expression and polarization of astrocytic aquaporin 4, attenuated neuroinflammation, and protected mice against synaptic dysfunction and decline in spatial cognition. In addition, Sun et al. (2018) observed that physical exercise released the immune response by decreasing cytokine levels and astrocytes population. Voluntary physical training could modulate the reactive astrocyte state, linked through astrocytic brain-derived neurotrophic factor (BDNF) to improve hippocampal cognition (Belaya et al., 2020).

Promotion of Neurogenesis

Traditionally, the non-regeneration of neurons is the main reason for the difficulty in neurological functional recovery (Caleo, 2015; Jones, 2017). Recently, researchers have identified that neurons have plasticity and the ability to repair post-injury, which can reshape nerve functions after ischemic stroke. Studies have found that ischemia-induced brain injury can be attenuated by regenerating neurons, synapses, and vessels, improving the defense capability of brain tissue. Moreover, the blood supply to the ischemic area can be restored, thereby promoting remodeling of neural function after ischemic injury (Yang et al., 2021a; Liu et al., 2021c; Zong et al., 2021; Puderbaugh and Emmady, 2022). The improved outcomes indicate that neural regeneration is an essential mechanism behind exercise preconditioning inducing ischemia tolerance (Shamsaei et al., 2015). Praag et al. observed that voluntary exercise ameliorates certain deleterious morphological and behavioral consequences of aging connected with neurogenesis regulation (van Praag et al., 2005). Another study found that treadmill exercise improved short-term and spatial memories by elevating neurogenesis and suppressing apoptosis within the hippocampal dentate gyrus of old-aged rats (Kim et al., 2010). Codd et al. revealed that elevated neurogenesis is sufficient to reverse hippocampal injury-induced deficits in either the damaged or intact hippocampus (Codd et al., 2020). Moreover, the improvement in hippocampal-based learning in aged mice after physical exercise is dependent on neurogenesis in the dentate gyrus and is regulated by growth hormone level changes. Specific changes in hippocampal circuitry underlying the cognitive improvements resulting from physical activity were also identified, suggesting dependency on neurogenesis activation in aged animals (Blackmore et al., 2021; Zhou et al., 2021). Cheng et al. (2020) observed that treadmill exercise promotes neurogenesis and myelin repair by upregulating the Wnt/β-catenin signaling pathway and improves the neurological deficit caused by focal cerebral ischemia/reperfusion. Similarly, Hong et al. (2020) showed that treadmill exercise enhanced motor function and short-term memory by elevating synaptic plasticity and neurogenesis in thrombotic stroke mice. Zhang et al. (2020) indicated that post-stroke exercise improved behavioral function recovery, where synaptogenesis was a beneficial factor.

BDNF plays a vital role in increasing synaptic plasticity and promoting neural regeneration. Xu et al. (2021) found an upregulation of BDNF and TrkB in the treadmill exercise group in rats. BDNF/TrkB signaling pathway could modulate the impact of exercise and the enriched environment by improving learning and memory in rats. BDNF expression

TABLE 1 | Summary of pre-clinical studies of exercise preconditioning in ischemic stroke.

Exercise type	Exercise manner	Species and model	Outcome	Involved signal	References
treadmill exercise	10 min/day (15–25 m/min), 5 days/week for 3 weeks	male Sprague–Dawley rats, 60 min of MCAO	reduced infarct volume and ameliorated sensorimotor function	upregulate BDNF, HIF-1α, and P2X7 receptor	Otsuka et al. (2021a)
treadmill exercise or swimming	Swim or run (15 m/min) 30 min/day, 5 days/week for 3 weeks	male Wistar rats, 30 min of MCAO	Increase brain trophic support and reduce brain damage	Increase the gene expressions of TrkB, TNF-α, and MMP2	Teymuri Kheravi et al. (2021)
treadmill exercise	4 weeks, the distance of exercise per week is about 5,000 m	male Sprague-Dawley rats, 90 min of MCAO	improve neurocognitive function	Increase the basal dopamine level	Fan et al. (2021)
treadmill exercise	25 min/day for 4 days, break for 2 days, and one acute bout for 30 min	male Wistar rats, embolic stroke model	reduce the neurovascular injury and improved functional outcomes	Increase the expression of peNOS and pAMPK	Hafez et al. (2020)
treadmill exercise	30 min/day (2 m/min for the first 5 min, 3 m/min for the next 5 min, 5 m/min for the last 20 min) for 4 weeks	male Wistar rats, bilateral common carotid arteries occlusion	ameliorate shot-term memory impairment and prevent microvascular injury in the hippocampus	prevente the reduction of ZO-1 in the hippocampus and inhibite the activation of MMP-9	Lee et al. (2019)
treadmill exercise	30 min (20 m/min), 30 min (30 m/min) and 60 min (30 m/min) for 1 week each	male Sprague-Dawley rats, MCAO	attenuate neurological injury	preserve old and newly formed HSP72-containing neurons	Wang et al. (2019b)
treadmill exercise	30 min/day (25 m/min) for 3 or 5 days/week for 3 weeks	male Sprague-Dawley rats, 60 min of MCAO	reduce infarct volumes, improve neurological scores and sensorimotor function	reduce the Bax/Bcl-2 ratio and caspase-3 activation	Terashi et al. (2019)
treadmill exercise	30 min/day (25 m/min) for 5 days/week for 3 weeks	male Sprague-Dawley rats, 60 min of MCAO	reduce ischemic neuronal cell death, induce neuron- and astrocyte-mediated brain ischemic tolerance	Increase expression of HIF-1α, and inhibit 14-3-3γ/p-β-catenin Ser37 anti-apoptotic pathway	Otsuka et al. (2019)
treadmill exercise	30 min/day for 5 days/week for 8 weeks	male Wistar rats, 60 min of MCAO	improve neurological function and BBB integrity	develop higher levels of cortical VEGF-A and striatal VEGF-R2	Rezaei et al. (2018)
treadmill exercise	40 min/day (18 m/min) for 5 days/week for 4 weeks	ovariectomized mice, permanent MCAO	diminish infarct volume, and improve neurological deficits	Decrease MMP-9, and increase IL-10	Naderi et al. (2018)
treadmill exercise	5 days/week for 4 weeks, time and intensity increase progressively	male wistar rats, 60 min of MCAO	reduce brain edema and decrease the neurological movement disorders	none	Shamsaei et al. (2015)
treadmill exercise	30 min/day (15 m/min) for 3 days/week for 4.5 weeks	male C57Bl/6 mice, 13 min of global cerebral ischemia	forced treadmill exercise induce a stress response, and lead to increased neuronal damage	Increase levels of NLRP3, galectin-3, IFNγ and IL-10	Svensson et al. (2016)
treadmill exercise	30 min/day (20 m/min) for 6 days/week	male Sprague Dawley rats, 90 min of MCAO	reduce brain infarct volume and neurological deficits	Increase SOD activity and decrease the concentration of MDA	Feng et al. (2014)
treadmill exercise	30 min/day (15 m/min) for 6 days/week for 3 weeks	male Sprague Dawley rats, 120 min of MCAO	improve neurological deficits, reduce infarct volume, mitigate pathological damage in the ischemic cortex	regulation of the TLR4/NF-κB signaling pathway and the inhibition of central and peripheral inflammatory cascades	Zhu et al. (2016)
treadmill exercise	30 min/day (25 m/min) for 5 days/week for 3 weeks	male Sprague Dawley rats, 60 min of MCAO	reduce neuronal apoptosis, oxidative stress, and infract volume, ameliorate motor function, increase astrocyte proliferation and angiogenesis	enhance expression of MK and BDNF	Otsuka et al. (2016)
treadmill exercise	30 min (20 m/min), 30 min (30 m/min) and 60 min (30 m/min) for 1 week each	male Sprague Dawley rats, 90 min of MCAO	attenuate brain infarct, glial apoptosis, and neurological deficits	Increase the numbers of both the HSP20-containing neurons and the HSP20-containing glia	Lin et al. (2015)
swimming	60 min/day for 6 days/week for 4 weeks	Sprague Dawley rats, 120 min of MCAO	reduce infarct volume	upregulate the expression of HIF-1α	Wang et al. (2015)
treadmill exercise	30 min/day (20 m/min) for 6 days/week for 3 weeks	male Sprague Dawley rats, 120 min of MCAO			Wang et al. (2014)

(Continued on following page)

TABLE 1 | (*Continued*) Summary of pre-clinical studies of exercise preconditioning in ischemic stroke.

Exercise type	Exercise manner	Species and model	Outcome	Involved signal	References
			reduce brain infarct volume, cerebral edema and neurological deficits	regulation of PKC-α-GLT-1-Glutamate and PI3K/Akt-GLT-1-Glutamate signal pathway	
treadmill exercise	30 min/day (20 m/min) for 5 days/week for 2 weeks	male Sprague Dawley rats, 120 min of MCAO	improve CBF and neurologic deficits, reduce infarct volume	Decrease ET-1 expression	Zhang et al. (2013)
treadmill exercise	30 min/day (18 m/min) for 5 days/week for 3 weeks	male wistar rats, 10 min of 4-vessel occlusion model	improve behavioral functions and maintain more viable cells in the dorsal hippocampus	none	Tahamtan et al. (2013)
treadmill exercise	30 min/day (30 m/min) for 5 days/week for 3 weeks	male Sprague Dawley rats, 120 min of MCAO	reduce neurological deficit and infarct volume, increase the rates of glucose metabolism	reduce ADP/ATP ratio, increase GLUT1, GLUT3, and PFK	Dornbos et al. (2013)
treadmill exercise	30 min/day (30 m/min) for 5 days/week for 3 weeks	Sprague Dawley rats, MCAO	reduce neuronal apoptosis	inhibit the expression of MMP-9 and ERK1/2 expression	Chaudhry et al. (2010)
treadmill exercise	30 min/day (30 m/min) for 5 days/week for 3 weeks	Sprague Dawley rats, MCAO	diminish neuronal injury, reduce infarct volume	upregulate HSP-70, ERK 1/2 and Bcl-x(L), downregulate Bax and AIF	Liebelt et al. (2010)
treadmill exercise	30 min/day (30 m/min) for 5 days/week for 3 weeks	male Sprague Dawley rats, 120 min of MCAO	Decrease neurological deficits, infarct volume and leukocyte infiltration	Reduce TNF-α, ERK 1/2, MMP-9 and ICDM-1 expression	Curry et al. (2010)

BBB, blood-brain barrier; BDNF, brain-derived neurotrophic factor; CBF, cerebral blood flow; ERK1/2, extracellular signal-regulated kinase one and 2; GLT-1, glutamate transporter-1; HIF-1α, hypoxia-inducible factor-1α; HSP, heat shock protein; ICDM-1, intercellular adhesion molecule-1; MCAO, middle cerebral artery occlusion; MDA, malondialdehyde; MK, midkine; MMP, matrix metalloproteinase-9; NF-κB, nuclear transcription factor-κB; NLRP3, nucleotide-binding oligomerization domain-like receptor containing pyrin domain 3; peNOS, phosphorylated endothelial nitric oxide synthase; SOD, superoxide dismutase; TLR4, toll-like receptor-4; TNF-α, tumour necrosis factor-α; TrkB, tropomyosin receptor kinase B; VEGF-A, vascular endothelial g PKC-α, protein kinase C-α; rowth factor A; VEGF-R2, vascular endothelial growth factor receptor 2; ZO-1, zonula occludens-1.

levels in the ischemic brain were significantly upregulated post exercise cessation in an animal study (Wang et al., 2020), consistent with another study (Xu et al., 2021). Interestingly, a meta-analysis summarized the effects of physical exercise with different intensities, duration, and frequency on peripheral BDNF levels among the sedentary elderly without any cognitive impairment. The results showed that physical exercise did not cause any significant difference in peripheral BDNF concentration (Fleitas et al., 2022), which indicates that BDNF expression in the brain and peripheral plasma are influenced differentially by exercises.

PROSPECTS

Therefore, exercise preconditioning could induce ischemia tolerance by inhibiting neural apoptosis and oxidative stress, regulating the inflammatory response, promoting neural regeneration, and exerting preventive and protective effects on the ischemic brain injury (**Figure 1**). Exercise preconditioning depicts a significant application prospect being a safe and slight side-effect strategy to prevent cerebral ischemia. Further studies on the neuroprotective mechanism of exercise preconditioning will identify new therapeutic targets for ischemic stroke. Moreover, supporting exercise training could provide a solid theoretical foundation as effective prevention and control measures of ischemic stroke patients.

However, many problems regarding exercise preconditioning require attention. First, the heterogeneity of population subgroups, including age, gender, dietary habits, etc., should be considered. Different hypoxic degrees, duration, and intensity will induce different effects. For example, how does exercise play a neuroprotective role in inducing cerebral ischemia tolerance among the elderly population with the most incidence of ischemic stroke? What type of exercise, frequency, intensity, and duration could harness the best results? Second, there is a lack of specific indicators to analyze the effect of exercise preconditioning. Applying mild stress may exacerbate the disease state rather than provide a cure in some disease cases. This outcome necessitates understanding the preconditioning and ischemic stroke mechanisms and the stress response of cells/tissues/organs at different stages of ischemic stroke. Moreover, it also requires searching for specific physiological biomarkers to improve the monitoring of disease progression or treatment effectiveness. In addition, the exercise preconditioning mechanism needs to be further explored. Does exercise directly affect the brain or protect brain function through peripheral effect? Which group of brain cells is more sensitive to exercise stimulation? Finally, combining exercise preconditioning with traditional medicine, nanomedicine, or other preconditioning methods needs to be studied, which could be a potential therapeutic approach for ischemic stroke.

AUTHOR CONTRIBUTIONS

YZ, YS and JH designed and drafted the manuscript. ZP revised the manuscript. All the authors finalized the paper and provided suggestions to improve it.

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Three-Day Continuous Oxytocin Infusion Attenuates Thermal and Mechanical Nociception by Rescuing Neuronal Chloride Homeostasis *via* Upregulation KCC2 Expression and Function

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Oxytocin (OT) and its receptor are promising targets for the treatment and prevention of the neuropathic pain. In the present study, we compared the effects of a single and continuous intrathecal infusion of OT on nerve injury-induced neuropathic pain behaviours in mice and further explore the mechanisms underlying their analgesic properties. We found that three days of continuous intrathecal OT infusion alleviated subsequent pain behaviours for 14 days, whereas a single OT injection induced a transient analgesia for 30 min, suggesting that only continuous intrathecal OT attenuated the establishment and development of neuropathic pain behaviours. Supporting this behavioural finding, continuous intrathecal infusion, but not short-term incubation of OT, reversed the nerve injury-induced depolarizing shift in Cl^- reversal potential *via* restoring the function and expression of spinal K^+-Cl^- cotransporter 2 (KCC2), which may be caused by OT-induced enhancement of GABA inhibitory transmission. This result suggests that only continuous use of OT may reverse the pathological changes caused by nerve injury, thereby mechanistically blocking the establishment and development of pain. These findings provide novel evidence relevant for advancing understanding of the effects of continuous OT administration on the pathophysiology of pain.

Keywords: neuropathic pain, oxytocin, chloride homeostasis, K+-Cl-cotransporter 2, continuous intrathecal drug delivery

INTRODUCTION

Neuropathic pain is a debilitating condition that affects 7–10% of the general population (Colloca et al., 2017). Unlike opioids and non-steroidal anti-inflammatory drugs for nociceptive pain, the medications used to treat neuropathic pain tend to only be modestly effective and can potentially cause multiple adverse reactions (Baron et al., 2010). Developing mechanism-based therapies for

neuropathic pain remains a major challenge. A growing body of literature has demonstrated the analgesic effects of the neuropeptide oxytocin (OT) in both humans and rodents (see reviews by Oxytocin and pain perception: from animal models to human research) (Gimpl and Fahrenholz, 2001; Honda and Takano, 2009; Koshimizu and Tsujimoto, 2009; Stoop, 2014; Boll et al., 2018; Herpertz et al., 2019). Electrical stimulation of the anterior part of the hypothalamic paraventricular nucleus increased OT concentration in the cerebrospinal fluid (CSF) and produced antinociception in rats (Martinez-Lorenzana et al., 2008), and intraperitoneal or intrathecal (i.t.) injection of OT was shown to block neuropathic pain in rats (Yang et al., 2007). Clinical data suggested that administration of OT in the cerebrospinal fluid (CSF) reduces surgical recovery time while decreasing pain and hypersensitivity in patients after injury (Wang et al., 2013). Considering it also plays a key modulatory role in emotions, stress and anxiety, which are well known to substantially influence pain perception (Apkarian et al., 2005; Apkarian, 2008; Baron et al., 2010; Peters, 2015; Tracy et al., 2015), OT has become a promising target for therapeutic interventions for pain.

Excitation/inhibition imbalance along the entire nociceptive pathway is considered a main driver in the development of neuropathic pain (Kahle et al., 2014). One of the mechanisms proposed for this imbalance involves compromised inhibition in the superficial dorsal horn of the spinal cord, leading to hyperactivity of spinal dorsal horn circuit, which is the main target for primary nociceptive afferents (Prescott, 2015). γ-aminobutyric acid (GABA) is the most critical inhibitory neurotransmitter in the central nervous system. The inhibitory efficiency of GABAergic transmission is determined primarily by the electrochemical gradient for Cl^-, which is depended by the intra and extracellular concentration of Cl^- (Ganguly et al., 2001). It has been demonstrated that Cl^- homeostasis is collapsed and Cl^- levels are elevated in spinal cord neurons under the pathophysiology of pain disorders (Coull et al., 2003). Recently, a body of evidence showed that compromised spinal inhibition resulted from downregulation of K^+-Cl^- cotransporter 2 (KCC2) and the subsequent disruption of intracellular chloride homeostasis (Coull et al., 2003; Price et al., 2009; Li et al., 2016; Mapplebeck et al., 2019). In mature central neurons, KCC2 is responsible for the low intracellular Cl^- concentration ($[Cl^-]_i$) that forms the basis for hyperpolarizing $GABA_A$ receptor-mediated responses. It regulates the formation (Li et al., 2007), functional maintenance and plasticity of glutamatergic synapses (Fiumelli et al., 2005; Gauvain et al., 2011; Chevy et al., 2015; Llano et al., 2015). Indeed, Modol's results indicate that nerve injury results in a reduction in the expression of KCC2 in the spinal dorsal horn that accompanies chronic pain, but prevention of the downregulation of KCC2 along the central sensory pathways relieves neuropathic pain after peripheral nerve injury (Modol et al., 2014). Loss of activity of this transporter is a key mechanism for chronic pain, and different groups demonstrated that renormalization of impaired KCC2 alleviated nerve injury-induced neuropathic pain (Gagnon et al., 2013; Kitayama, 2017). Leonzino et al. found that OT directly modulates the functional activity of KCC2 by promoting its phosphorylation and insertion/stabilization at the neuronal surface in an early developmental time window (Leonzino et al., 2016). However, little is known on how OT affects chloride homeostasis and the function of KCC2 in neuropathic pain.

In addition, the current understanding of mechanisms underlying OT analgesia is mainly based on studies using single or multiple injections of OT in animals. Little is known about the effects of continuous OT administration on pain processing. In this study, we adopted intrathecal drug delivery technique to administer OT centrally in nerve injured mice. Chronic intrathecal drug infusion through an implantable pump is a clinically available strategy to treat a number of neurological diseases (Ganguly et al., 2001; Kästner, 2010). Findings based on continuous intrathecal OT delivery in mice may provide more information on how OT targets the pathophysiology of pain and better implications for human therapy.

Thus, in the present study we adopted intrathecal drug delivery technique to compare the effects of a single or continuous intrathecal infusion of OT on pain behaviours in mice; we determined whether they block neuropathic pain by preventing the disruption of the intracellular Cl^- homeostasis in the spinal superficial dorsal horn, and whether it is mediated by restoring the KCC2 expression and function.

MATERIALS AND METHODS

Animals

All animal procedures were conducted in strict adherence to the guidelines of the International Association for the Study of Pain and were approved by the Animal Care and Use Committee of Health Science Center at Shenzhen University. 80 male C57BL/6 mice (5–8 weeks of age) were purchased from Guangdong Province Laboratory Animal Center (Guangzhou, China). 20 vGAT-ires-cre mice and 20 td-Tomato (Ai9) mice were purchased from Jackson Laboratory. The animals were housed in plastic cages (5 per cage) in a temperature-controlled environment on a 12 h/12 h light/dark cycle. Food and water were available *ad libitum*.

Reagents

Oxytocin (catalogue: H-2510) and [d(CH2)51,Tyr(Me)2, Thr4,Orn8,des–Gly–NH29]–vasotocin (dVOT, catalogue: H-2510) were purchased from Bachem AG (Bubendorf, Switzerland). TC OT39 (catalogue: 1078) was obtained from Tocris (Minnesota, United States).

Neuropathic Pain Model

The partial sciatic nerve ligation (pSNL) pain model was established according to previously described procedures (Seltzer et al., 1990). Briefly, the animals were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) and a tight ligation of approximately one-third to one-half the diameter of the right sciatic nerve (ipsilateral) was performed with 6–0 silk suture. In sham-operated mice, the nerve was exposed without ligation.

Behavioural Testing

Von Frey testing was performed to assess mechanical allodynia. The mice were habituated to the environment for 2 days before the testing began. All the behaviours were tested blindly. For testing mechanical allodynia, the mice were confined separately in boxes (14 × 18 × 12 cm) placed on an elevated metal mesh floor, and their hind paws were stimulated with a series of von Frey hairs with logarithmically increasing stiffness (0.16–2.00 g, Stoelting) situated perpendicularly to the central plantar surface. The 50% paw withdrawal threshold was determined by Dixon's up-down method. The hot plate test (Hot/Cold Plate, Cat. 35150, Ugo Basile, Italy) was used to examine thermal hyperalgesia. Each mouse was placed on the hot plate, and the latency of paw withdrawal from the heat stimulus was measured twice separated by a 5-min interval. The average value was used as the latency of response. All behavioural testing was done with the experimenters blinded to the treatment conditions.

Intrathecal Injection and Continuous Intrathecal Infusion of Drugs

OT (0.1 μg in 10 μL) or dVOT (0.1 μg/10 μL) was injected into the subarachnoid space through the intervertebral foramen between L4 and L6 (Hylden and Wilcox, 1980). For the intrathecal infusion of drugs, an osmotic minipump (model 1003D, ALZET, Cupertino, CA, United States) connected with a polyethylene catheter was deposited in a subcutaneous pocket following partial sciatic nerve ligation. The other end of the catheter was inserted from the atlanto-occipital membrane into the subarachnoid space until the tip of the catheter reached the lumbar spinal enlargement. OT and other reagents were then delivered continuously with a flow rate of 1 μL/h for 3 days from days 0 to 2 after pSNL surgery. The final dose of OT intrathecal infusion is 0.3 μg in 100 μL. (The volume delivery rate and the delivery duration of ALZET pumps are fixed at manufacture).

Quantitative RT-PCR

The animals were sacrificed and L4–6 spinal cord segments were collected in tubes with RNAlater (Qiagen Inc., Valencia, CA, United States) and stored at −80°C until RNA isolation. Total RNA was isolated from these tissues according to Chomczynski's method (Chomczynski and Sacchi, 1987) and reverse transcribed using Omniscript reverse transcriptase (Qiagen Inc., Valencia, CA, United States) at 37°C for 60 min. The reaction was performed in the presence of the RNase inhibitor rRNAsin (Promega, Madison, WI, United States) and an oligo (dT16) primer (Qiagen) to selectively amplify the mRNA. For quantitative PCR, 45 ng of cDNA was used as a template. Reactions were performed using Assay-On-Demand TaqMan probes and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster, CA, United States) according to the manufacturer's protocol. Reactions were run on a Real-Time PCR iCycler IQ (Bio-Rad, Hercules, CA, United States) with software version 3.0. The expression levels of *Kcc2* were normalized to *ß*-actin.

Western Blotting

The animals were sacrificed, and the L4-6 spinal cord segments were removed and stored at −80°C until assayed. The samples were homogenized and centrifuged to extract the protein, and the resulting preparations were saved. Equal amounts of protein were separated by 10% Tris-Tricine SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were then blocked in 5% non-fat milk for 1 h at room temperature, followed by overnight incubation with rabbit anti-KCC2 antibody (1:1000; ab49917, Abcam, United States) and *ß*-actin (1:2000; Sigma, United States) primary antibody. Immunoblots were then incubated for 1 h at room temperature with goat anti-rabbit polyclonal IgG (1:3000, ab205718, Abcam, MA, United States). Immunoblots were developed by chemiluminescent substrate and quantified using ImageJ software.

Immunohistochemistry

The mice were deeply anesthetized with isoflurane and transcardially perfused with PBS followed by 4% PFA. Lumbar L4-6 spinal cord segments sections were blocked and then incubated overnight at 4°C with rabbit antibodies against KCC2 (Abcam, ab49917, United States). The sections were then incubated for 30 min at 37°C with AF488-conjugated secondary antibodies (donkey, 1:500, Jackson Immuno-Research, West Grove, PA, United States), and the nuclei were stained with DAPI. The sections were viewed under Zeiss 880 inverted confocal microscopy, and images were collected using identical acquisition parameters and quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Silver spring, MD, United States) by experimenters blinded to treatment groups.

In Situ Hybridization

In situ hybridization was performed using the RNAscope system (Advanced Cell Diagnostics) following the manufacturer's protocol. Pre-treatment consisted of dehydration, followed by incubation with hydrogen peroxide and protease IV at room temperature. The Multiplex Fluorescent Kit v2 protocol was followed using commercial probes for the OT receptor (Oxtr, NM_001081147.1, #402658-C3). Images were captured by Zeiss 880 inverted confocal microscopy. Visualized cells with more than 5 puncta per cell were classified as positive neurons.

Electrophysiological Recordings

Adult (5–7 weeks) male mice were anaesthetized with urethane (1.5–2.0 g/kg, i.p.). The lumbosacral spinal cord was removed and submerged into ice-cold dissection solution saturated with 95% O_2 and 5% CO_2 at room temperature. Transverse slices (300–400 μm) were cut in a vibrating microslicer (VT1200s Leica). The slices were incubated at 32°C for at least 30 min in regular artificial cerebrospinal fluid (aCSF) equilibrated with 95% O_2 and 5% CO_2.

The following solutions were used: dissection solution containing (in mM) 240 sucrose, 25 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, and 3.5 $MgCl_2$ at pH 7.4; regular artificial CSF containing 135 NaCl, 2.5 KCl, 3 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 1 NaH_2PO_4, and 10 glucose at pH 7.4; and normal intrapipette solution for perforated recording containing 115 K-

methylsulfate, 25 KCl, 2 $MgCl_2$, 10 HEPES, 0.4 GTP-Na and 5 Mg-ATP at pH 7.2 and 310 mOsm.

To measure the reversal potential of GABA-evoked currents, a slice was placed in the recording chamber and completely submerged and superfused at a rate of 2–4 ml/min with aCSF. A perforated patch-clamp was applied to avoid changes in the $[Cl^-]_i$. To measure the chloride equilibrium potential (E_{Cl}), gramicidin D (80 μg/ml with an 0.8% DMSO final concentration from an 8 mg/ml stock in DMSO) was added to the intrapipette solution, and 6-cyano-7- nitroquinoxaline-2,3-dione (CNQX, 10 μM), DL-2-amino-5-phosphonovaleric acid (APV, 50 μM) and tetrodotoxin (TTX, 0.5 μM) were added to the aCSF solution. The tip of the patch pipette was filled with the normal intrapipette solution, while the rest of the pipette contained the gramicidin-containing solution. After forming a seal on the membrane, we waited 30 min for the gramicidin to effectively reduce the series resistance to below 100 MΩ. Membrane potential measurements were corrected for liquid junction potential, which was measured as in(Guo et al., 2014). GABA (1 mM) was puffed locally and instantaneously, and the puff pipette was aimed toward the recording pipette. Voltage ramps were applied from +8 to −92 mV over 200 ms at a holding potential of −42 mV. Since the voltage ramp might evoke a basal current, a control voltage ramp was first applied to record the basal current; 1 min later, GABA was puffed, followed by another voltage ramp, and then the GABA-evoked currents were recorded (Billups and Attwell, 2002). The reversal potential was analysed as in (Billups and Attwell, 2002).

Excitatory and inhibitory post-synaptic currents (EPSCs and IPSCs) recordings were made from lamina II inhibitory neurons. The patch-pipette solution contained (in mM) K-gluconate 135, KCl 5, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, HEPES 5, an Mg-ATP 5; or Cs_2SO_4 110, $CaCl_2$ 0.5, $MgCl_2$ 2, EGTA 5, HEPES 5, Mg-ATP5, tetraethylammonium (TEA)-Cl 5 (pH = 7.2) (Jiang et al., 2014). The former and latter solutions were used to record EPSCs and IPSCs, respectively. EPSC recordings were made at a holding potential (V_H) of −70 mV, where no IPSCs were observed, since the reversal potential for IPSCs was near −70 mV. IPSCs were recorded at a V_H of 0 mV, where EPSCs were invisible as reversal potential for EPSCs was close to 0 mV. Cs^+ and TEA were used to block K^+ channels expressed in the recorded neurons, and thus to easily shift V_H from −70 to 0 mV. GABAergic IPSCs were obtained in the presence of the glycine-receptor antagonist strychnine (1 mM). EPSC and IPSC events were detected and analysed using Mini Analysis Program 6.0. Signals were acquired using an Axopatch 700B amplifier and analysed with pCLAMP 10.3 software. Only neurons with resting membrane potential < −50 mV and stable access resistance were included.

Statistical Analysis

The data are expressed as means ± SEM and analysed with a *t*-test or variance (ANOVA) using one-way or mixed factorial designs as appropriate, followed by Bonferroni's *post hoc* test or simple-effects ANOVA. All statistical analyses were performed using GraphPad Prism 8.0. (GraphPad Inc., La Jolla, CA, United States). Significance was defined as $p < 0.05$.

RESULTS

Three-Day Continuous Intrathecal Infusion, but Not Short-Term Application of OT, Attenuated the Establishment and Development of Nerve Injury-Induced Nociceptive Behaviours in pSNL Mice

pSNL-induced nerve injury produced mechanical allodynia and thermal hyperalgesia in mice. This mechanical and thermal hypersensitivity started on day 1 and remained relatively stable from days 3 to 14 after nerve ligation (**Supplementary Figures S1A,B**).

An osmotic minipump was implanted immediately following partial sciatic nerve ligation. OT was then delivered with a flow rate of 1 μL/h for 3 days from days 0–2 after pSNL surgery. Mechanical allodynia and thermal hyperalgesia were tested at days 3, 5, 7 and 14 after pSNL surgery (**Figure 1A**). As shown in **Figures 1B,C**, infusion of OT (0.3 μg, 100 μL) for 3 days before the behavioural tests decreased nerve injury-induced nociceptive behaviours in mice. Compared with the vehicle, 3-days continuous infusion of OT increased the mechanical threshold in the von Frey test [$F(1,14) = 61.57$, $p < 0.001$; **Figure 1B**, $n = 8$] and paw withdrawal latency in the hot-plate test [$F(1,14) = 50.74$, $p < 0.001$; **Figure 1C**, $n = 8$] for 14 days, which was the longest period we tested, indicating that 3-days continuous intrathecal OT infusion may attenuate the establishment and development of nerve injury-induced neuropathic pain.

In comparison, the effect of a single injection of OT on pSNL-induced mechanical and thermal hypersensitivity was also tested on day 3 after nerve ligation, when the pain behaviours were well established (**Figure 1D**). Single intrathecal OT (0.1 μg/10 μL) significantly alleviated pSNL-induced mechanical allodynia [$F(1,14) = 42.59$, $p < 0.001$; **Figure 1E**] and thermal hyperalgesia [$F(1,14) = 29.66$, $p < 0.001$; **Figure 1F**] at 10 [$p < 0.001$] and 30 min [$p < 0.001$] after injection. This effect of OT was not observed at 60 min after the injection [$p > 0.05$; **Figures 1E,F**], indicating that the analgesic effect of a single intrathecal OT administration on nerve injury-induced pain behaviours is transient. OT at the doses used in the present study had no effect on the locomotor activity or motor coordination in mice (date not shown).

We found no significant differences between male and female mice in the analgesic effects of oxytocin [$p > 0.05$; **Supplementary Figure S4**].

The Effects of 3-days OT Infusion on Nerve Injury-Induced Nociceptive Behaviours Were Mediated by Oxtrs

To determine whether the effects of 3-days OT infusion on neuropathic pain were mediated by Oxtrs, its agonist or antagonist was administrated (**Figure 2A**). Co-intrathecal infusion (100 μL) of a selective Oxtr antagonist, dVOT (0.3 μg), with OT (0.3 μg) blocked the analgesic effect of OT on nerve injury-induced mechanical [$F(1,13) = 25.04$, $p =$

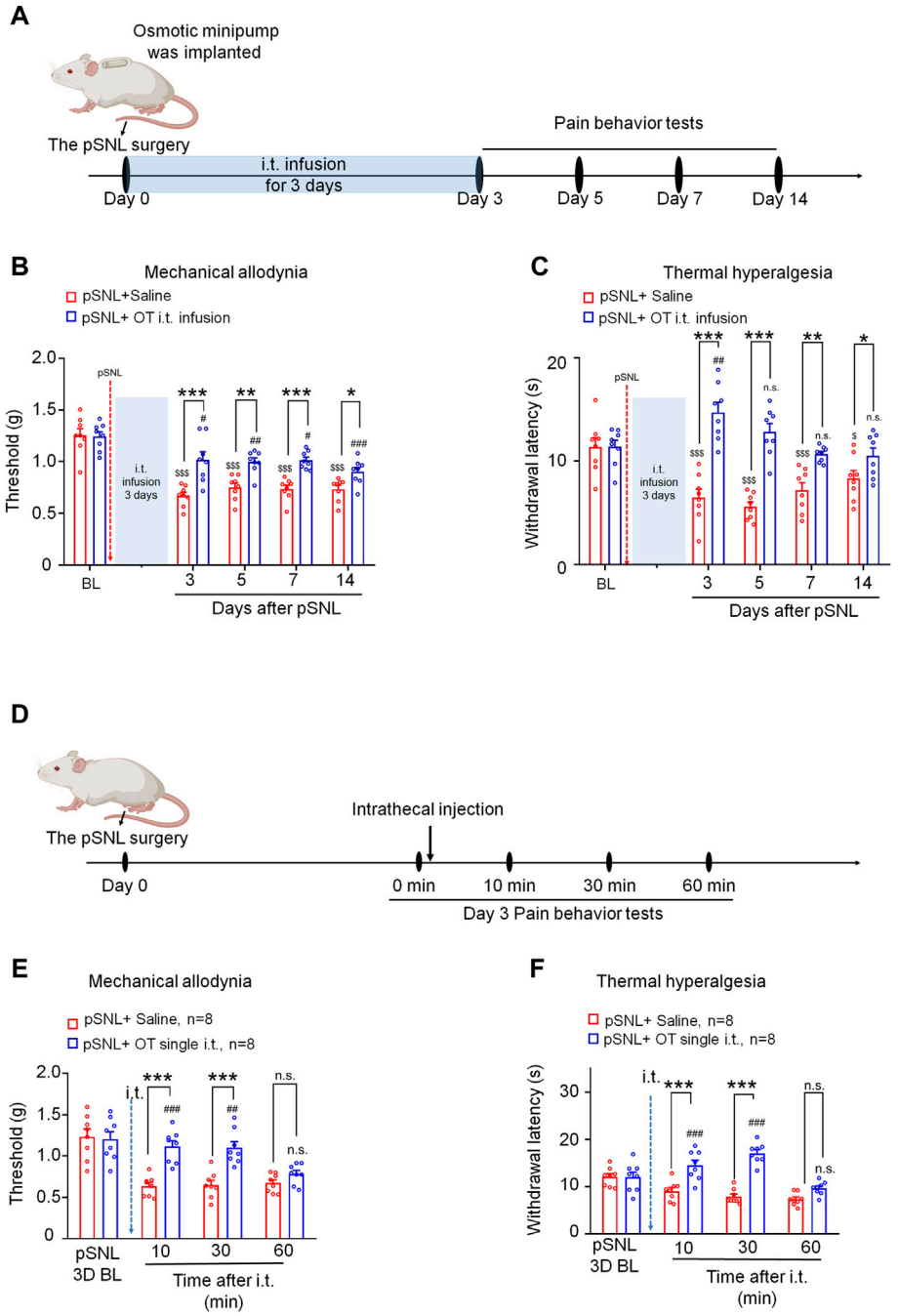

FIGURE 1 | Three-day continuous intrathecal infusion, but not short-term application of OT, attenuated the establishment and development of nerve injury-induced nociceptive behaviours in pSNL mice. **(A)** A schematic of the experimental design. **(B,C)** Continuous intrathecal OT infusion (0.3 μg/100 μL) for 3 days before behavioural tests decreased pSNL-induced mechanical allodynia **(A)** and thermal hyperalgesia **(B)** for 14 days. **(D)** A schematic of the experimental design. **(E,F)** A single intrathecal OT injection (0.1 μg/10 μL) relieved pSNL-induced mechanical allodynia **(E)** and thermal hyperalgesia **(F)** in mice. Two-way repeated-measures ANOVA with group as the between-subjects factor and day/time as the within-subjects factor. Data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ OT *vs.* saline; $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ *vs.* baseline; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#\#}p < 0.0001$ *vs.* baseline.

0.0002; **Figure 2B**, n = 7–8] and thermal hypersensitivity [F(1,12) = 28.92, $p < 0.001$; **Figure 2C**, n = 7]. The selective Oxtr agonists TC OT (0.3 μg/100 μL) produced significant analgesic effects which were equivalent to OT [von Frey test F(1,14) = 15.42, $p = 0.0015$; Hot-plat test F(1,14) = 29.80, $p < 0.0001$; **Figures 2D,E**; n = 8]. There results suggested that the 3-days intrathecal infusion of OT induced analgesic effect is mediated by the Oxtrs in the spinal cord.

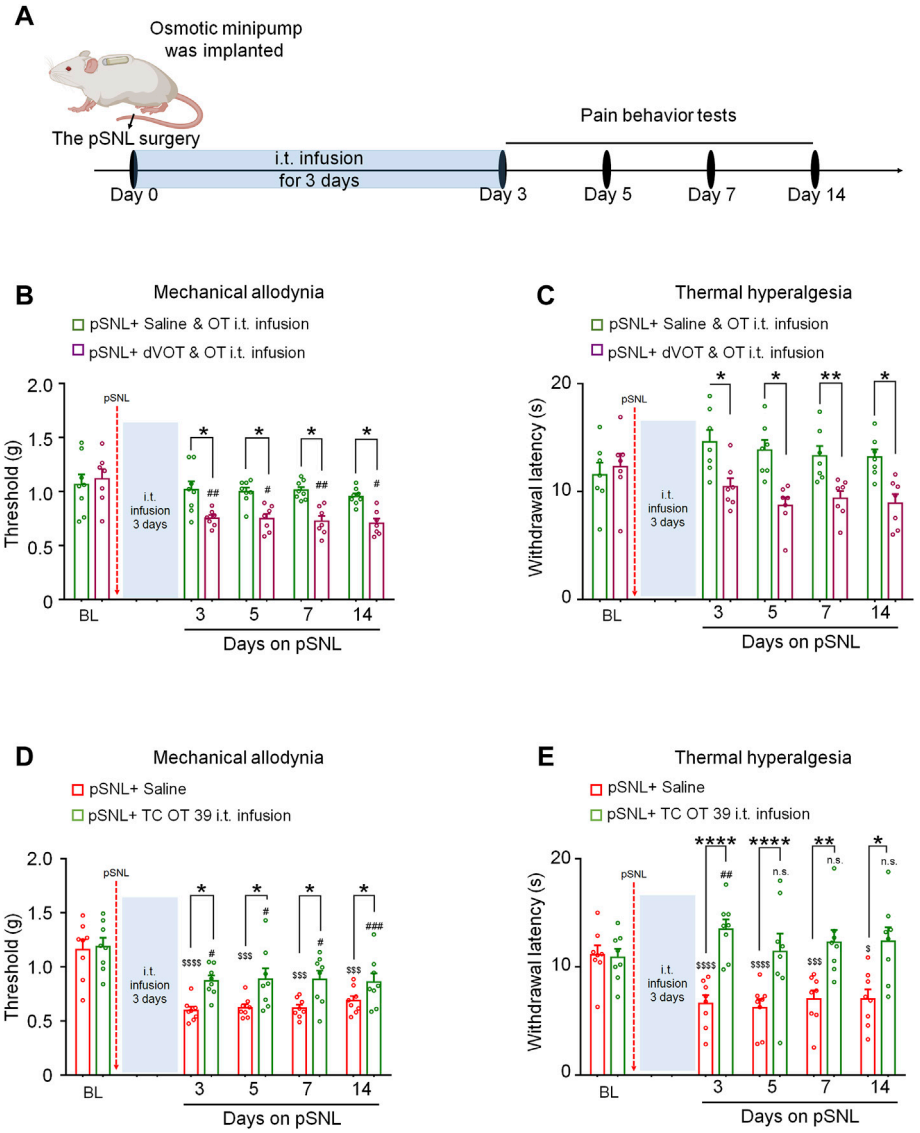

FIGURE 2 | The effects of 3-days OT infusion on nerve injury-induced nociceptive behaviours were mediated by OXTRs. **(A)** A schematic of the experimental design. **(B,C)** OT's effect on mechanical allodynia **(B)** and thermal hyperalgesia **(C)** was completely blocked by its selective antagonist, dVOT (0.3 μg/100 μL). **(D,E)** Selective OT receptor agonists, TC OT (0.3 μg/100 μL, intrathecal infusion) showed similar effects on mechanical allodynia **(D)** and thermal hyperalgesia **(E)** in pSNL mice. Two-way repeated-measures ANOVA with group as the between-subjects factor. Data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ TC OT *vs.* saline; OT *vs.* dVOT and OT. $^{\$\$\$}p < 0.001$, $^{\$\$\$\$}p < 0.0001$ *vs.* baseline; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* baseline.

Three-Day Continuous Intrathecal Infusion, but Not Short-Term Application of OT, Renormalized Neuronal Chloride Equilibrium Potential in Spinal Superficial Dorsal Horn

It was reported that neuronal intracellular chloride concentration was increased in the superficial dorsal horn after nerve injury (Yeo et al., 2021), we performed perforated patch-clamp recording in spinal cord slices derived from each group to investigate the effects of OT on chloride homeostasis (**Figure 3A**). Since $GABA_A$ receptor ($GABA_AR$) is the dominant chloride ion channel on the membrane of neurons in the superficial dorsal horn, GABA was puffed briefly to the recorded neuron to trigger transient chloride influx or efflux.

As voltage ramps were applied from +8 to −92 mV (**Figure 3C**), the GABA-evoked currents were recorded to evaluate chloride equilibrium potential (E_{Cl}^{-}). These currents were completely blocked by a selective $GABA_AR$ antagonist, bicuculline (10 μM), confirming that they were mediated by $GABA_AR$ (data not shown). The E_{Cl}^{-} in sham mice was −66.68 ± 1.22 mV (**Figures 3B–E**, n = 5–6, 3 mice per group),

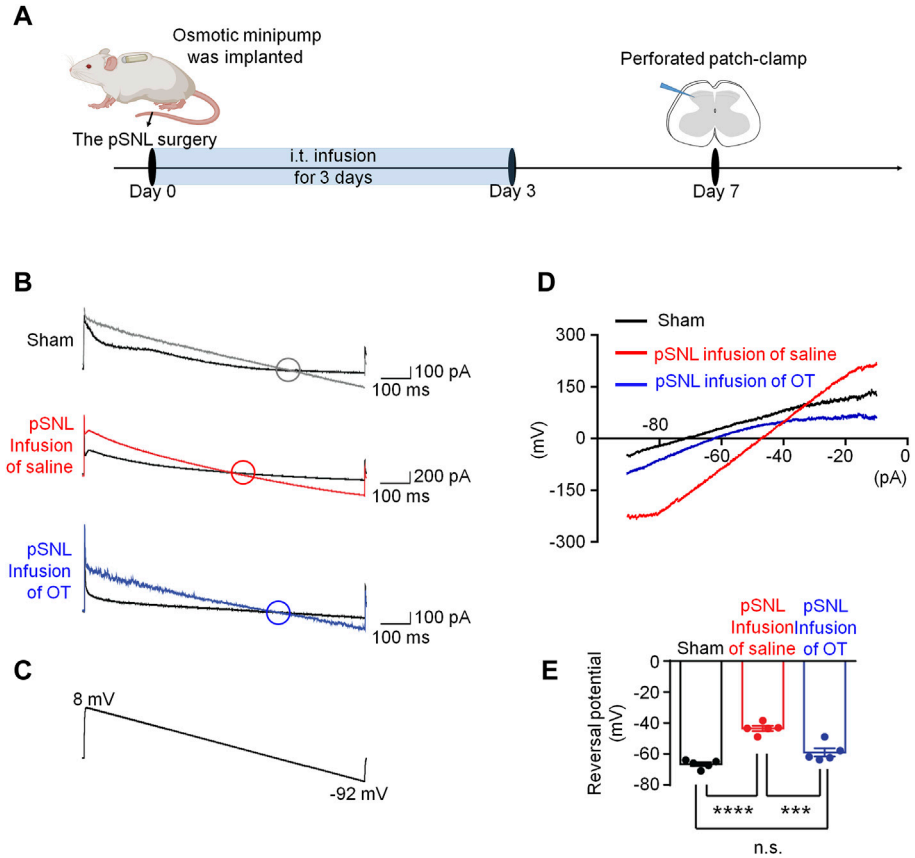

FIGURE 3 | Three-day continuous intrathecal OT infusion renormalized EGABA in spinal dorsal horn. **(A)** The schematics of the electrophysiological recording. **(B,C)** As voltage ramps applied from +8 to −92 Mv **(C)**, basal and GABA-evoked currents were recorded **(B)**. **(D,E)** Representative **(D)** and statistical **(E)** reversal potential of E_{GABA} recorded from slices of sham and pSNL mice treated with continuous OT or saline. One-way ANOVA followed by Bonferroni's post hoc test. Data are expressed as mean ± SEM. ***p < 0.001 sham *vs.* pSNL; ***p < 0.001 OT *vs.* saline infusion.

whereas that value in pSNL mice shifted to a more positive value of −43.54 ± 1.67 mV [p < 0.001 *vs.* sham group; F(2,12) = 36.26, p < 0.001; **Figures 3B–E**, n = 5 from 3-4 mice]. Continuous intrathecal infusion of OT reversed the value of E_{Cl}^- to −59.02 ± 2.69 mV, which was much closer to that of the sham mice [p > 0.05 *vs.* sham; **Figures 3B–E**, n = 5 from 3-4 mice], suggesting that 3-days infusion of OT was able to restore $[Cl^-]_i$ in pSNL mice.

In comparison, we also recorded the E_{Cl}^- using the spinal cord slices incubated with saline or OT for 30 min (short-term application, **Figure 4A**), and the reversal potentials were −44.34 ± 2.91 mV and −46.10 ± 3.10 mV, respectively [p > 0.05 *vs.* saline; F(2,12) = 31.71, p < 0.0001; **Figures 4B–E**]. Incubation of the spinal cord slices with OT for a relatively short time failed to restore the value of E_{Cl}^- in pSNL mice, suggesting that the effect of OT on E_{Cl}^- required relatively long-term application.

Three-Day Continuous Intrathecal OT Infusion Upregulated Spinal KCC2 Expression

Given that the shift of E_{Cl}^- in pSNL animals may be due to depressed function of KCC2, we analysed the transcriptional and expression levels of KCC2 in the spinal cord. Compared with the sham group, quantitative PCR data revealed a significant decrease in spinal *Kcc2* mRNA levels at both days 7 and 14 after pSNL surgery [p < 0.001 vs. sham; F(2,16) = 3.818, p = 0.0441; **Figure 5A**, n = 5 per group]. Intrathecal infusion of OT increased spinal *Kcc2* mRNA levels in pSNL mice compared with saline group [p < 0.01; F(2,16) = 3.818, p = 0.0441; **Figure 5A**, n = 5 per group].

Western blotting data also showed that nerve injury-induced a significant decrease in the protein levels of KCC2 in the spinal dorsal horn at days 7 and 14 after pSNL surgery [p < 0.0001 *vs.* sham; F(2,16) = 8.982, p = 0.0024; **Figures 5B,C**, n = 5 per group]. Intrathecal infusion of OT restored the protein levels of KCC2 but did not completely reverse this decrease [p < 0.01 *vs.* saline; F(2,16) = 8.982, p = 0.0024; **Figures 5B,C**, n = 5 per group]. Immunohistochemistry (IHC) of spinal slices from laminae II further supported the western blotting data, which showed that the KCC2 signal was widely expressed throughout the spinal dorsal horn in sham mice (**Figure 5D**). Nerve injury-induced a reduction in KCC2 expression at days 7 and 14 after pSNL surgery [p < 0.0001 *vs.* sham; F(2,12) = 8.119, p = 0.0059; **Figures 5D,E**]. Infusion of OT reversed this reduction [p <

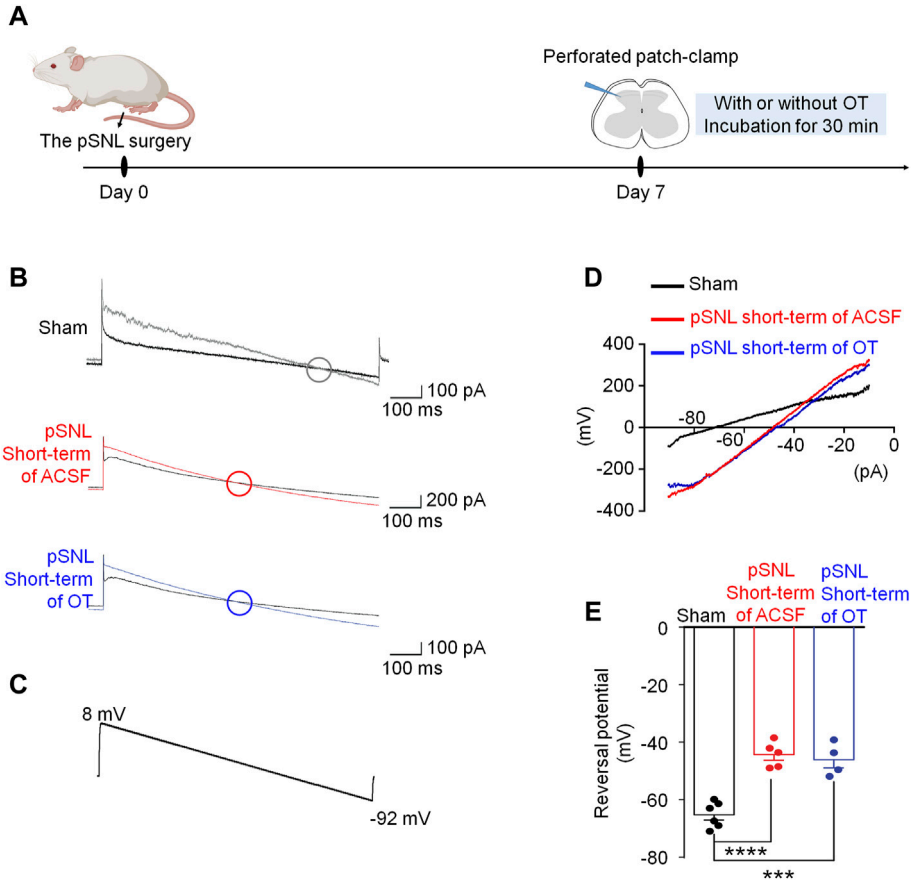

FIGURE 4 | Short-term OT incubation failed to renormalize EGABA in spinal dorsal horn. **(A)** The schematics of the electrophysiological recording. **(B,C)** As voltage ramps were applied from +8 to −92 Mv **(C)**, basal and GABA-evoked currents **(B)** were recorded. **(D,E)** The reversal potential of E_{GABA} recorded from slices of naïve and pSNL mice incubated with OT or saline. One-way ANOVA followed by Bonferroni's post hoc test. Data are expressed as mean ± SEM. ***$p < 0.001$, ****$p < 0.0001$ naïve *vs.* pSNL incubated with saline or OT.

0.01 *vs.* sham; F(2,12) = 8.119, $p = 0.0059$; **Figures 5D,E**, $n = 4$ per group] to some extent.

Oxtrs Are Functionally Expressed in Inhibitory Interneurons and OT Enhanced GABAergic Inhibitory Transmission Through Activation of Oxtrs in the Superficial Dorsal Horn

To further explore the underlying mechanism of OT on the regulation of E_{Cl}^-, we performed a novel *in situ* hybridization assay (RNAscope) to investigate the feature of Oxtr mRNA expression. Firstly, we used a novel *in situ* hybridization assay (RNAscope) to detect the properties of Otxr mRNA distributions in the superficial dorsal horn. As shown in **Supplementary Figure S2**, Oxtrs mRNA (white) were not expressed on microglia (green) and astrocytes (red), suggesting that majority of Oxtrs are located in the neurons. To test whether that Oxtrs were expressed on the inhibitory neurons in the spinal dorsal horn. Spinal cord slices derived from the vGAT-tdTomato mice were used, in which the inhibitory neurons were visualized by red fluorescence. As shown in **Figures 6A,B**, about 30% of vGAT + neurons (inhibitory neurons) expressed Oxtrs mRNA signalling in the in the spinal dorsal horn. Oxtr mRNAs were also found expressed in vGAT negative interneurons in the superficial dorsal horn.

We then performed whole-cell voltage clamp on the vGAT positive interneurons in the superficial dorsal horn. About 72% recorded $vGAT^+$ neurons ($n = 18$) produced an inward current when OT (0.5 μM) was perfused for 3 min at the V_H of −70 mV with an average of −10.40 ± 1.27 pA (upper trace in **Figures 6C–E**), but OT did not change the frequency and amplitude of spontaneous EPSCs in all of the examined $vGAT^+$ neurons [*t*-test, $p = 0.0663$, t (34) = 1.963 for frequency; $p = 0.6311$, t (34) = 0.4890 for amplitude; **Figure 6F**]. In the presence of the Oxtr antagonist dVOT (1 μM), OT failed to induce an inward current in all recorded vGAT positive interneurons in the superficial dorsal horn (**Figures 6G,H**, $n = 12$). In comparison, OT perfusion produced an inward current in 38% recorded vGAT negative neurons (**Supplementary Figures 3B,C**, $n = 13$).

Due to OT produced inward currents in some vGAT positive interneurons, we tested the effects of OT on GABAergic

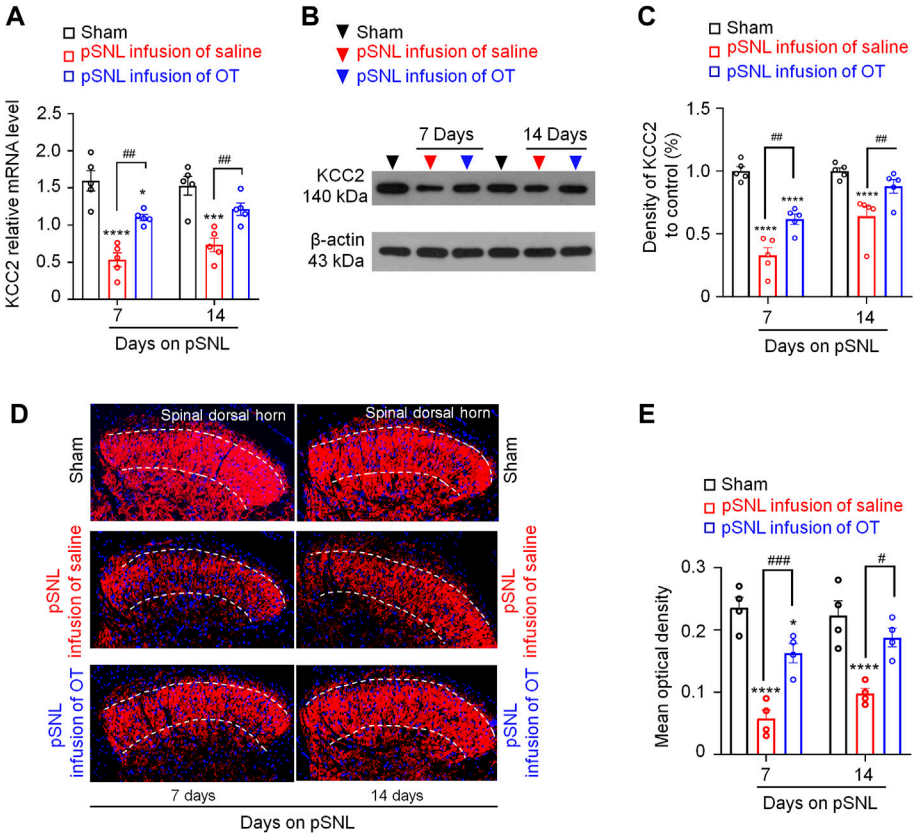

FIGURE 5 | Three-day continuous intrathecal OT infusion increased KCC2 expression in the spinal dorsal horn in pSNL mice. **(A)** Continuous intrathecal OT infusion increased spinal KCC2 mRNA on days 7 and 14 after pSNL. **(B,C)** Continuous intrathecal OT infusion upregulated spinal KCC2 protein levels on days 7 and 14 after pSNL. **(B)** Representative western blots of KCC2 and the loading control (β-actin) are presented for each group. **(D)** Representative image shows the staining of KCC2 (red) in naïve mice and in pSNL mice treated with saline or OT. DAPI was used to stain the cell nuclei (blue) **(E)** The intensity of KCC2 staining. One-way repeated measures ANOVA was used to analyse differences across days within each group. Simple effects ANOVA was used to confirm differences between groups at each time point. Data are expressed as mean ± SEM. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs.* saline; $^{***}p < 0.001$, $^{****}p < 0.0001$ *vs.* sham.

transmission in the spinal cord in the presence of a glycine-receptor antagonist, strychnine (1 μM). OT (0.5 μM) perfusion for 3 min increased the frequency and amplitude of spontaneous GABAergic IPSCs at the V_H of 0 mV from 5.02 ± 0.49 Hz to 13.61 ± 1.72 Hz and 9.40 ± 0.68 pA to 13.17 ± 1.30 pA, respectively (*t*-test, $p = 0.0009$, t (12) = 6.026 for frequency; $p = 0.0080$, t (12) = 3.899 for amplitude; $n = 7$; **Figures 7A,B**). Expectedly, OT enhanced GABAergic spontaneous transmission was total blocked by pre-treatment with a selective Oxtr antagonist, dVOT (1 μM, $p = 0.2498$, t (12) = 1.274 for frequency; $p = 0.2987$, t (12) = 1.138 for amplitude; $n = 7$; **Figures 7C,D**).

DISCUSSION

In this study, we demonstrated that three days of continuous intrathecal OT infusion alleviated subsequent pain behaviours for 14 days, whereas a single OT injection induced a transient analgesia for 30 min in mice. Supporting this behavioural finding, only continuous intrathecal infusion, but not short-term incubation of OT, reversed the nerve injury-induced depolarizing shift in Cl^- reversal potential, which was mediated by improving the function and expression of spinal K^+-Cl^- cotransporter 2 (KCC2). This result suggests that only continuous use of OT may reverse the pathological changes caused by nerve injury, thereby mechanistically blocking the establishment and development of pain.

Pain is a multidimensional experience that includes not only nociceptive and nocifensive components but also emotional-affective and cognitive components. As OT is involved in a wide range of behaviours, it is a promising target for the therapeutic pain intervention. The number of studies supporting that OT has antinociceptive effects grows steadily. Animal studies in particular have delivered robust evidence supporting this idea. Unfortunately, these findings have not been translated into therapeutics. We believe at least two issues have hampered the clinical use of OT. One is the poorly defined mechanisms of action of OT, and the other is difficulty with OT delivery to the central nervous system. Here, we adopted intrathecal drug delivery technique to administer OT centrally in nerve injured mice to understand how continuous use of OT acts on the pathological changes caused by nerve injury.

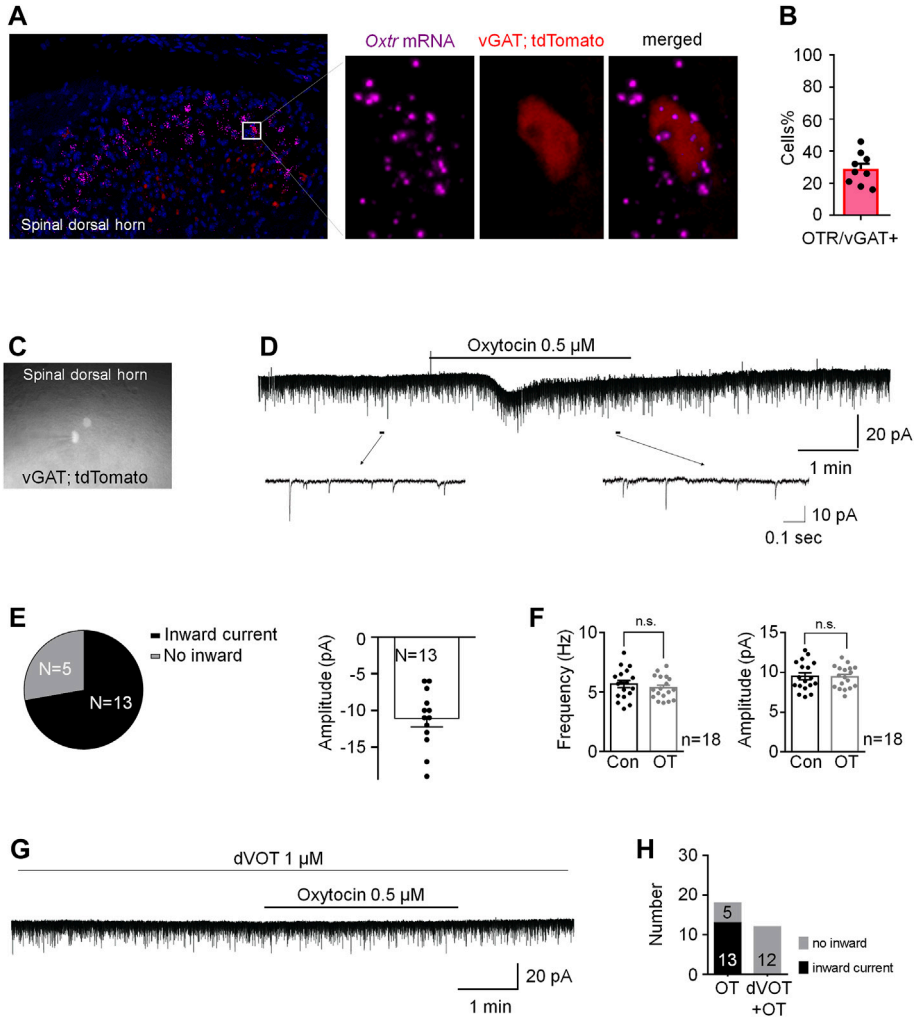

FIGURE 6 | OT produced an inward current in vGAT+ neurons through activation of Oxtrs in the superficial dorsal horn. **(A)** RNAscope showed that Oxtrs (pink) were expressed on the inhibitory neurons (red) in the spinal dorsal horn. Co-expression of a sample inhibitory neuron (red) and the puncta representing Oxtrs (pink) in the enlarged image. DAPI was used to stain the cell nuclei (blue). **(B)** percentage of Oxtrs expressed in the vGAT + neurons. **(C)** The vGAT^{+} interneurons in the superficial dorsal horn. **(D,E)** OT perfusion produced an inward current in 72% recorded vGAT + neurons (n = 18). **(F)** The frequency and amplitude of spontaneous EPSCs in all examined vGAT + neurons. Paired t-test. Data are expressed as mean ± SEM. **(G,H)** Selective Oxtr antagonist dVOT (1 μM) blocked OT induced inward currents in all recorded vGAT positive interneurons in the superficial dorsal horn (n = 12).

As the results showed in this study, continuous intrathecal OT infusion for three days alleviated subsequent pain behaviours induced by nerve injury. It is noteworthy that the pSNL mice that received the OT perfusion in advance showed continuous relief in pain behaviours for 14 days, which was as long as we tested, although the OT perfusion has stopped during behavioural tests. This result suggested that continuous intrathecal OT infusion may attenuate the establishment and development of nerve injury-induced neuropathic pain. In comparison, a single intrathecal injection of OT in intact or neuropathic pain model mice only induced a transient analgesia for 30 min. The short-term analgesic effect of a single administration of OT revealed in this study was compatible with the results derived from other pain models. For example, Yu found that the duration of analgesia of OT was within 1 hour in inflammatory pain (Yu et al., 2003), and Yang reported that the effects of intraventricular or intrathecal injection of OT lasted about 30 min in intact rats (Yang et al., 2007).

We also observed that intrathecal OT infusion not only reverse thermal hyperalgesia but induces analgesia one day after OT continuous infusion. A single injection of OT also showed an analgesia effect in the hotplate test 30 min after injection. This analgesic effect of OT may be related to presynaptic TRPV1 inhibition in the spinal cord (Sun et al., 2018). Since we found no significant differences between male and female mice in the analgesic effects of OT on day 3 after pSNL surgery (**Supplementary Figure S4**).We conducted the experiments using male mice in the present study. However, we cannot rule out sex differences in the effect of intrathecal OT infusion.

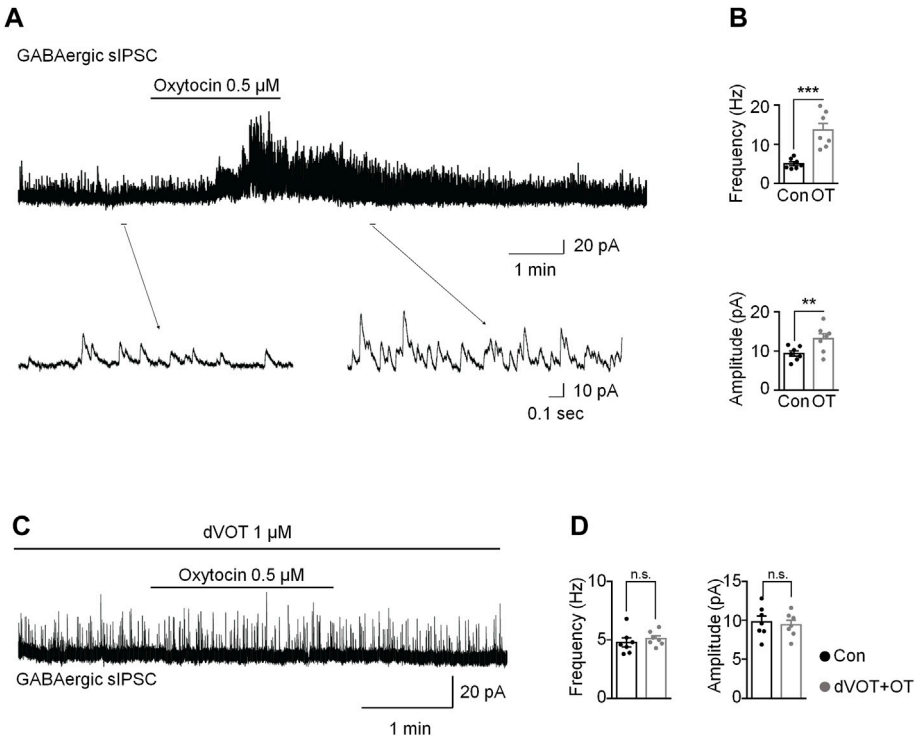

FIGURE 7 | OT enhanced GABAergic inhibitory transmission through activation of OXTRs in the superficial dorsal horn. **(A,B)** OT perfusion increased the frequency and amplitude of spontaneous GABAergic IPSCs. **(C,D)** The selective Oxtr antagonist dVOT blocked OT-enhanced GABAergic spontaneous transmission. Paired *t*-test. Data are expressed as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* control.

All the behavioural tested were conducted within 14 days after the pSNL surgery. Since inflammatory component existed post-surgery, the current results cannot rule out that anti-inflammatory mechanisms are involved in the analgesic effect of OT.

OT plays its effects by activating OT receptors, which belongs to the G protein-coupled receptor superfamily, together with the three structurally related arginine-vasopressin (AVP) receptors (V1aR, V1bR and V2R), forms a small receptor sub-family. All of these receptors bind to OT albeit with different affinities and eliciting different responses. Selective activating OXTRs by its agonist, TC OT produced significant analgesic effects which were equivalent to OT, whereas antagonizing OXTR by its antagonist, dVOT blocked the analgesic effect of OT in pSNL mice, indicating that intrathecal OT infusion induced analgesic effect is mediated by the OXTRs in the spinal cord.

The current understanding of mechanisms underlying OT analgesia is mainly based on studies using single or multiple injections of OT. The acute analgesic mechanisms of OT involve GABA, potassium channels, sodium channels and TRPV channels (Breton et al., 2008; Jiang et al., 2014). Little is known about the actions of continuous, relatively long-term OT administration on pain processing. It is proposed that nerve injury causes an imbalance between excitatory and inhibitory control in the nervous system, which is partially caused by a loss of inhibition in the dorsal horn of the spinal cord and which is in turn responsible for neuropathic pain (Kuner, 2010). The broken of neuronal intracellular Cl^- homeostasis is a major cause for the loss of inhibition in spinal dorsal horn. In order to investigate the underlying mechanisms of continuous intrathecal OT infusion on pain processing, we tested whether they block neuropathic pain by preventing the disruption of the intracellular Cl^- homeostasis in the spinal superficial dorsal horn, a key region in nociceptive information transmission; and whether it is mediated by restoring the KCC2 expression and function.

Firstly, we found that the chloride equilibrium potential (E_{Cl}^-) in pSNL mice was significantly shifted to a more positive value by using whole-cell patch-clamp technique, indicating an elevated level of $[Cl^-]_i$ in pSNL animals. The result was consistent with the previous finding that neuronal intracellular chloride concentration was increased in the superficial dorsal horn after nerve injury (Yeo et al., 2021). Only 3-days continuous intrathecal infusion, but not a short-term incubation of OT, restored the value of E_{Cl}^-, suggesting that only continuous intrathecal OT infusion was able to restore $[Cl^-]_i$. Considering neuronal chloride homeostasis plays important role in pain processing, this result indicated that continuous oxytocin infusion renormalized neuronal chloride homeostasis to attenuates neuropathic pain.

KCC2 (Cl^- extrusion) and NKCC1 (Cl^- uptake) are the most important chloride transporters in cortical neurons and therefore represent the main regulators of chloride homeostasis (Kaila, 1994; Delpire, 2000). The elevated level of $[Cl^-]_i$ in neurons

suggested a downregulation of KCC2 or an upregulation of NKCC1. Only continuous intrathecal infusion, but not a short-term incubation of OT, restored chloride homeostasis, and suggested the altered function of KCC2 or NKCC1 in pSNL animals.

Since it is reported that lack of Oxtr in neurons affects specifically KCC2 without impairing NKCC1 (Leonzino et al., 2016), we then used quantitative PCR, western blotting and immunohistochemistry to test whether the continuous intrathecal OT infusion upregulated spinal KCC2 expression and rescued the decrease in KCC2 expression by nerve injury. As the results showed, nerve injury induced a significant decrease in the expression levels of KCC2 after pSNL. Intrathecal infusion of OT restored the expression levels of KCC2 in the spinal dorsal horn.

Coull and his colleagues have shown that the inhibitory control in GABAergic neurons in the spinal dorsal horn can be lost when KCC2 activity is impaired, which can eventually lead to neuropathic pain (Coull et al., 2003). In mature central neurons, KCC2 is responsible for the low $[Cl^-]_i$ that forms the basis for hyperpolarizing $GABA_A$ receptor-mediated responses. Changes in KCC2 function and expression have been observed under various physiological and pathophysiological conditions. Nerve ligation often tends to decrease spinal KCC2 expression, which contributes to the development of neuropathic pain. Nerve injury-induced brain-derived neurotrophic factor (BDNF) release may account for the reduction in KCC2 (Kitayama, 2017). Therefore, it is indicated that spinal KCC2 expression is responsible for the development and maintenance of neuropathic pain. Continuous infusion of OT may attenuate the development and maintenance of neuropathic pain by restoring the alternations of KCC2.

As a small polypeptide, oxytocin is rapidly broken down in the gastrointestinal system. It has a very short half-life of 3–5 min in the blood. Although the half-life of OT is much longer in CSF (~28 min) than in the blood, it is known to penetrate the blood brain barrier only sparingly (Kang and Park, 2000), making oral or parenteral administration untenable. Thus, human OT effects on pain sensitivity have most frequently been investigated using the intranasal administration route. However, there are many constraints to the intranasal application of this neuropeptide that might contribute to the rather inconsistent findings in human studies. In one study, the elevation of OT levels in the CSF was observed only in one out of the six macaques that received intranasal OT (Lee et al., 2018). In 1984, Penn and Kroin introduced intrathecal administration of baclofen in humans to alleviate spasticity in severe cases (Penn and Kroin, 1984). Since then, intrathecal drug delivery has become an important treatment option for individuals with severe spasticity, dyskinetic cerebral palsy, stiff-man syndrome, and chronic pain (Penn and Mangieri, 1993; Saval and Chiodo, 2008; Eek et al., 2018). Drugs can be administered *via* an intrathecal route that allows for the placement of the medication in close proximity to the target receptors so that a much lower dose is needed. By using continuous intrathecal delivery, a steady drug concentration can be maintained within the central nervous system (Mathur et al., 2014). In a long-term (>10 years) clinical study where Baclofen was administrated intrathecally, patients reported a high level of treatment and life satisfaction (McCormick et al., 2016). These findings provide novel evidence relevant for advancing understanding of the effects of continuous OT administration on the pathophysiology of pain.

Many factors may mediate OT-induced KCC2 upregulation. It has been reported that BDNF may be the cause of the reduction in KCC2. As a neurotrophic factor, BDNF is produced and secreted mainly by microglia (Fujita et al., 2008). This study showed that Oxtrs were mainly expressed in the neurons, but not glia cells. So we speculate that OT did not upregulated of KCC2 through BDNF. In this study, we also found that OT enhanced GABAergic inhibitory transmission through activation of Oxtrs in the spinal dorsal horn, which may help us to understand the mechanisms underlying continuous OT's action on KCC2. We first confirmed by RNAscope that Oxtr mRNA was expressed on some of the inhibitory neurons in the spinal dorsal horn, although it was also observed in vGAT negative neurons. We then performed whole-cell voltage clamps to record the spontaneous EPSC in the inhibitory interneurons. OT perfusion produced an inward current without affecting the frequency and amplitude of spontaneous EPSCs in the inhibitory neurons. This result suggested that OT produced a depolarization in some inhibitory neurons without affecting glutamatergic transmission. As a result of the depolarization of inhibitory neurons, GABA may be released, which was further confirmed by the finding that OT enhanced GABAergic spontaneous transmission by increasing both the frequency and amplitude of spontaneous GABAergic IPSCs. These effects of OT on GABAergic inhibitory transmission were completely blocked by perfusion of a selective OTXR antagonist, dVOT. Ganguly et al. reported that GABAergic activity drove the increase in the level of KCC2 mRNA in mature neurons (Ganguly et al., 2001). Heubl et al. further demonstrated that enhancing $GABA_AR$-mediated inhibition confines KCC2 to the plasma membrane, while antagonizing inhibition reduces KCC2 surface expression by increasing the lateral diffusion and endocytosis of the transporter. This mechanism utilizes Cl^- as an intracellular secondary messenger and is dependent on the phosphorylation of KCC2 at threonines 906 and 1007 by the Cl^--sensing kinase WNK1. Taken together, we hypothesis that OT up-regulated KCC2 in neuropathic pain through the activation of GABAergic inhibitory transmission. However, this hypothesis is based on the transient actions of OT on the inhibitory neurons. Long-term application (3-days infusion) of OT may have many consequences on receptor binding, trafficking and expression. Therefore, we cannot rule out that the effect of OT on inhibitory neurons may be different when applied for a relatively long time, and that there are other mechanisms involved in OT-induced upregulation of KCC2.

CONCLUSION

To conclude, this study used an intrathecal delivery technique to demonstrate that continuous intrathecal OT infusion attenuated the subsequent establishment and development of nerve injury-induced neuropathic pain and renormalized neuronal chloride homeostasis *via* upregulation of KCC2 expression and function, which may be caused by OT-induced activation of GABA inhibitory transmission. These findings provide novel evidence relevant for advancing the understanding of the effects of continuous OT administration on the pathophysiology of pain.

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 animal study was reviewed and approved by the Animal Care and Use Committee of Health Science Center at Shenzhen University.

AUTHOR CONTRIBUTIONS

XB: Investigation, Methodology, Validation, Formal analysis. CR: Investigation, Methodology, Validation, Formal analysis. WG: Investigation, Methodology. JG: Investigation, Methodology. QZ: Investigation, Methodology. TL: Investigation, Methodology. WS: Investigation, Methodology. LX: Investigation, Methodology. DX: Investigation, Methodology. YeH: Investigation, Methodology. CJ: Conceptualization, Data curation, Funding acquisition, Resources, Writing—review and editing, Supervision. YuH: Conceptualization, Data curation, Writing—original draft, Writing—review and editing, Supervision, Project administration, Funding acquisition.

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SUPPLEMENTARY MATERIAL

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Mechanism Underlying Acupuncture Therapy in Spinal Cord Injury: A Narrative Overview of Preclinical Studies

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Spinal cord injury (SCI) results from various pathogenic factors that destroy the normal structure and function of the spinal cord, subsequently causing sensory, motor, and autonomic nerve dysfunction. SCI is one of the most common causes of disability and death globally. It leads to severe physical and mental injury to patients and causes a substantial economic burden on families and the society. The pathological changes and underlying mechanisms within SCI involve oxidative stress, apoptosis, inflammation, etc. As a traditional therapy, acupuncture has a positive effect promoting the recovery of SCI. Acupuncture-induced neuroprotection includes several mechanisms such as reducing oxidative stress, inhibiting the inflammatory response and neuronal apoptosis, alleviating glial scar formation, promoting neural stem cell differentiation, and improving microcirculation within the injured area. Therefore, the recent studies exploring the mechanism of acupuncture therapy in SCI will help provide a theoretical basis for applying acupuncture and seeking a better treatment target and acupuncture approach for SCI patients.

Keywords: acupuncture, spinal cord injury, therapy, mechanism, apoptosis, inflammation, oxidative stress, neuroprotection

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INTRODUCTION

Spinal cord injury (SCI) causes structural and functional damage through direct or indirect factors, leading to motor, sensory, and autonomic nerve dysfunction (McDonald and Sadowsky, 2002). The global incidence of SCI ranges from 3.6 to 195 per 1,000,000 (Jazayeri et al., 2015). In China, the incidence of traumatic SCI was standardized to 49.8 per 1,000,000 per year based on the 2010 census, and the mean age of patients at the time of injury was 43.7 ± 17.1 years (Jiang et al., 2021). SCI is a common cause of death and disability, with severe neurological dysfunction and complications, including neuropathic pain, pressure ulcers, and urinary tract infection. In addition, it causes a substantial psychological and social burden on patients, families, and the society (Wannapakhe et al., 2015; Gedde et al., 2019; Moshi et al., 2021). Pathophysiological changes after SCI include primary and secondary injuries. Compared with the unpredictability of primary injury, the underlying mechanism and effective treatment of secondary injury is the primary focus of the current SCI research (Belegu et al., 2007; Jeong et al., 2021). SCI is a dynamic pathological process causing nerve cell and nerve fiber edema at the initial stages, followed by microcirculation disorders due to damaged blood cells (Rivlin and Tator, 1978; Tator and Fehlings, 1991). Then, the nerve cell axons degenerate or die and are gradually replaced by glial cells (O'Shea et al., 2017; Lukacova et al., 2021).

TABLE 1 | Summary of preclinical studies of acupuncture therapy in spinal cord injury in recent 5 years.

Ref	Species	Acupuncture therapy	Outcome	Mechanism
Wang X et al. (2021)	Male SD rats	EA at *Dazhui* (GV14) and *Mingmen* (GV4) for 20 min daily until they were euthanized	Improve neurological function and promote the repair of the injured spinal cord tissue	Inhibit the Notch signaling pathway and regulate the downstream protein expressions (Delta1, Presenilin1, Hes1, and Hes5)
Dai et al. (2021)	Female C57BL/6 mice	EA at *Zusanli* (ST 36) and *Sanyinjiao* (SP 6) for 10 min daily for 6 days, followed by 1 day off and last for 4 weeks	Improve hindlimb motor function and protect neurons and myelinated axons	Inhibit inflammatory response and oxidative stress through activating the ApoE and Nrf2/HO-1 signaling pathway
Hu et al. (2021)	Female SD rats	EA at *Jiaji* (EX-B2) for 30 min daily for 2 weeks	Promote the recovery of spinal cord nerve function	Inhibit the expression of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α and the Nogo-NgR signaling pathway
Yang et al. (2021)	Female SD rats	EA at *Zhiyang* (GV9), *Jizhong* (GV6), *Yaoshu* (GV2), and *Changqiang* (GV1) twice a day for 8 weeks	Accelerate neural network reconstruction and restoration of spinal cord function	Increase the local production of NT-3, improve the hostile microenvironment of the injured spinal cord by dampening local inflammation, and foster the biological functions of MSC-derived neuron-like cells
Hongna et al. (2020)	Female SD rats	EA at *Jiaji* (EX-B2) for 30 min daily until they were euthanized	Improve locomotor function	Regulate autophagy flux and inhibit necroptosis
Lu et al. (2020)	Male SD rats	EA at *Ciliao* (BL32) and *Zhongliao* (BL33) for 20 min daily for 10 days	Improve neurogenic bladder (the *Ciliao* acupoint is superior to the *Guanyuan* point)	Reduce histomorphological abnormalities in interstitial cells of Cajal and inhibit the expression of hyperpolarization-activated cyclic nucleotide-gated channel proteins
Hu et al. (2020)	Male SD rats	EA at *Jiaji* (EX-B2) for 20 min daily for 7 or 14 days	Promote the recovery of the motor function	Affect the plasticity of peripheral nerve networks by regulating the Semaphorin 3A signal
Xu H et al. (2021)	Female SD rats	EA at *Zhiyang* (GV9), *Jizhong* (GV6), *Yaoshu* (GV2), and *Changqiang* (GV1) twice a day for 2 weeks	Promote the survival, axonal regrowth, and synaptic maintenance of spinal cord neurons	Trigger the synthesis and secretion of NT-3 by activating the CGRP/RAMP1/calcium/αCaMKII pathway
Cheng et al. (2020)	Male SD rats	EA at *Dazhui* (GV14) and *Mingmen* (GV4) for 30 min daily for a week	Improve functional recovery	Inhibit the phosphorylation of JNK/$p66^{Shc}$-mediated oxidative stress and reduce the p38MAPK-mediated microglial activation and inflammatory reaction
Zhou et al. (2020)	Male SD rats	EA at *Dazhui* (GV14), *Mingmen* (GV4), and *Jiaji* (EX-B2) for 20 min twice daily for 3 weeks	Improve hindlimb motor function	Twenty-nine upregulated and 139 downregulated miRNAs in the EA group. The MAPK, Wnt, and NF-κB signaling pathways are involved
Ding et al. (2020)	Male SD rats	Acupuncture combined with moxibustion at *Dazhui* (GV14), *Jiaji* (EX-B2), *Yaoyangguan* (GV3), *Zusanli* (ST36), and *Ciliao* (BL32) for 30 min daily for 7 or 14 days	Recover motor function, preserve the neuron cells, and alleviate the apoptosis of nerve cells	Improve the mRNA and protein levels of Shh and Gli-1
Li et al. (2020)	Male SD rats	EA at *Dazhui* (GV14) and *Mingmen* (GV4) for 20 min daily until they were euthanized	Improve locomotor function	Affect cell growth, apoptosis, and autophagy through the PI3K/AKT/mTOR signaling pathway
Song et al. (2022)	Male SD rats	EA at *Zusanli* (ST36) for 20 min daily until they were euthanized	Promote the recovery of neurological function	Stimulate ascending peripheral nerve conduction
Xiao et al. (2019)	Female SD rats	EA at *Yaoyangguan* (GV3), *Dazhui* (GV14), *Zusanli* (ST36), and *Ciliao* (BL32) for 20 min daily for 2 weeks	Promote axonal regeneration	Inhibit the Nogo/NgR and Rho/ROCK signaling pathway
Hong et al. (2021)	Male SD rats	EA at *Yaoyangguan* (GV3), *Dazhui* (GV14), *Zusanli* (ST36), and *Ciliao* (BL32) 20 min daily for 2 weeks	Improve lower limb movement function and spinal cord tissue morphology	Reduce mRNA and protein expression of RhoA and ROCKII, decrease p-MLC protein expression and p-MLC/MLC ratio, and suppress the cPLA2 activity and PGE_2 level

(Continued on following page)

TABLE 1 | (*Continued*) Summary of preclinical studies of acupuncture therapy in spinal cord injury in recent 5 years.

Ref	Species	Acupuncture therapy	Outcome	Mechanism
Xu et al. (2019)	Female SD rats	Fire needle at *Jiaji* (EX-B2) in 1/3 s daily	Improve lower limb locomotor function	Promote endogenous NSC proliferation differentiating into neurons by promoting the activation of Wnt/β-catenin and inhibiting the overexpression of ERK.
Prado et al. (2019)	Dog	EA at GV2, DU20, GV3a, and GV6; bilateral BL19, BL23, and BL24; unilateral KI3, ST36, LV3, and *Wei Jian* for 20 min three times a week for the initial 7 weeks and two times a week for 5 more weeks	Improve neurological function	None
Jin et al. (2019)	Female SD rats	EA at *Zhiyang* (GV9), *Jizhong* (GV6), *Yaoshu* (GV2), and *Changqiang* (GV1) daily for 8 weeks	Improve locomotor function	Enhance the survival and synaptic integration of grafted NT-3 and TRKC gene-overexpressing neural stem cell-derived neural network scaffold with the host spinal neural network by increasing the NT-3 level and activating the NT-3/TRKC/AKT signaling pathway
Alvarado-Sanchez et al. (2019)	Female Long–Evans rats	EA at *Mingmen* (GV4) per 30 min until they were euthanized	Improve motor function recovery and the amount of preserved spinal cord tissue	Decrease oxidative stress and lipid peroxidation
Zhang et al. (2019)	Female SD rats	Sacral EA intervention for 7 days	Inhibit apoptosis, protect nerve cells, promote the coordination of micturition reflex, and improve neurogenic bladder function	Improve the expressions of both NGF/TrkA signaling and Akt signaling
Wei et al. (2018)	Female C57BL/6 mice	EA at *Jiaji* (EX-B2) for 15 min for 5 days, followed by 1 day off and last for 4 weeks	Restore locomotor function	Inhibit the expression of PTEN and p53 and increase the levels of pmTOR/Akt/Erk and myelin basic protein
On-Ong-Arj et al. (2018)	Male Wistar rats	Yellow laser acupuncture at *Yaoshu* (GV2) for 10 min at 15 min, 6, 12, and 24 h after SCI on the first day, followed by 10 min daily for 7 days	Improve both motor deficit and neurodegeneration in the ventral horn of the spinal cord	Increase the expression of BDNF and inhibit inflammation, apoptosis, and oxidative stress
Wang et al. (2019)	Male Wistar rats	EA at *Neiguan* (PC6) and *Jianshi* (PC5)	Alleviate SCI-induced neuropathic pain	Inhibit the PI3K-mTOR signaling pathway
Wang et al. (2018)	Female Wistar rats	EA at *Dazhui* (GV 14) and *Baihui* (GV20) for 15 min daily for 2 weeks	Improve the recovery of nerve movement	Reduce the expression of platelet-activating factor and caspase-9 protein
Li et al. (2018)	Female Wistar rats	EA at *Jiaji* (EX-B2), *Mingmen* (GV4), and *Dazhui* (GV14) for 15 min daily for 6 days. After a 2-day interval, the second course started, with three courses in total.	Enhance the growth of nerve fibers and improve the hindlimb motor function recovery	None
Tu et al. (2018)	Male SD rats	EA at *Zusanli* (ST-36) and *Yanglingquan* (GB-34) performed between 09:00 and 11:00 daily for 7 days	Reduce mechanical allodynia and thermal hyperalgesia	Inhibit the activation of spinal microglia and block the BDNF-TrkB signaling pathway
Wang et al. (2017)	SD rats	EA at *Zusanli* (ST-36)-*Xuanzhong* (GB39) and *Futu* (ST32)-*Sanyinjiao* (SP6) for 30 min until they were euthanized	Improve hindlimb locomotor and sensory function	Systematic regulation of neurotrophic factors and their receptors
Tu et al. (2017b)	Male SD rats	EA at *Baihui* (GV20) and *Fengfu* (GV16) or *Dazhui* (GV14) and *Mingmen* (GV4)	EA stimulation at GV14 and GV4 promote the recovery of locomotor function	Improve mRNA and protein expression of BDNF and NT-3
Nascimento de Souza et al. (2017)	Male Wistar rats	Bee venom at a dose of 0.08 mg/kg injected subcutaneously at *Zusanli* (ST36) and *Yaoyangquan* (GV3) (20 μL at each point) once immediately after SCI and 24 h, 7, and 14 days after SCI.	Induce locomotor recovery	Reduce the expression of IL-6 and increase the expression of IL-10

(Continued on following page)

TABLE 1 | (*Continued*) Summary of preclinical studies of acupuncture therapy in spinal cord injury in recent 5 years.

Ref	Species	Acupuncture therapy	Outcome	Mechanism
Zhang J et al. (2017)	Male SD rats	EA at *Dazhui* (GV14) and *Mingmen* (GV4) for 20 min daily for 2 weeks	Promote spinal recovery	Promote the differentiation of neural stem cells into spinal neurons by enhancing Wnt1/β-catenin signaling
Zhu et al. (2017)	Male SD rats	EA at *Jizhong* (GV6) and *Zhiyang* (GV9) 30 min daily for 7 days	Promote the proliferation of neural stem cells and the survival of neurons	Promote the expression of neuronal markers Nestin, NeuN, and CGRP and inhibit cellular apoptosis and inflammation by downregulating miR-449a
Tu et al. (2017a)	Male SD rats	EA at *Dazhui* (GV14) and *Mingmen* (GV4) for 30 min at 30 min, 12, and 24 h after SCI.	Improve hindlimb locomotor function	Decrease the mRNA and protein expression of the subunits of NMDAR NR1 and NR2A
Liu and Wu (2017)	Female SD rats	EA at *Jizhong* (GV6) and *Zhiyang* (GV9) for 20 min daily for a week	Improve functional recovery and inhibit neuronal apoptosis	Reduce Bax and inhibit the sodium channel Nav1.3 expression by regulating miR-214
Zhao et al. (2017)	Male SD rats	EA at *Jizhong* (GV6) and *Zhiyang* (GV9) for 20 min every other day for 4 weeks	Improve motor function	Enhance the expression of IL-10, M2 marker CD206, NT-3, and the proportion of M2 macrophages
Escobar-Corona et al. (2017)	Male Wistar rats	EA at *Huantiao* (GB30), *Yinmen* (BL37), *Jizhong* (GV6), and *Zhiyang* (GV9) for 40 min every other day for 4 weeks	Improve gait locomotion, H-reflex, and ventral root potential	None

Abbreviations: αCaMKII: calmodulin-dependent protein kinase; BDNF: brain-derived neurotrophic factor; CGRP: calcitonin gene-related peptide; EA: electroacupuncture; MSC: mesenchymal stem cell; NMDARs: N-methyl-D-aspartate (NMDA) receptors; NSCs: neural stem cells; NT-3: neurotrophin-3; RAMP: receptor activity-modifying protein; SCI: spinal cord injury.

Aggravation of cellular, molecular, and other factors at different stages post SCI leads to a series of pathophysiological changes, reducing the spinal cord functional recovery (Tator, 1995). Timely and efficient intervention can partially stimulate potential nerve cells and axonal regeneration and resume the function of axons and neurons. The current treatments for SCI mainly include surgery, medication, and behavioral, physical, and supportive therapies (Becker et al., 2003; Boulenguez and Vinay, 2009; Ramer et al., 2014).

Acupuncture is a substantial alternative and adjunctive therapy for SCI and is a vital component of traditional Chinese medicine. Electroacupuncture, a method based on acupuncture combined with the micro-current wave of bioelectricity, was developed by combining traditional and modern medicines. In recent years, acupuncture–electroacupuncture has been widely used in clinical practices and exerts a significant neuroprotective effect against SCI and its complications (Paola and Arnold, 2003; Shin et al., 2009; Ma et al., 2015; Fan et al., 2018; Lu et al., 2020). Compared with other therapeutic methods, acupuncture is non-toxic and has a simple operation and low cost, but its mechanism remains unclear. This article summarizes the potential mechanism of acupuncture in SCI to provide the updated theoretical basis depicting various clinical applications of acupuncture in SCI patients (**Table 1**).

MECHANISM OF ACUPUNCTURE THERAPY IN SPINAL CORD INJURY

Reduction of Oxidative Stress

Free radicals can be generated and released after SCI. While the degree of oxidation exceeds the ability of the antioxidant system, excessive free radicals will initiate the oxidation chain reaction (Bringans et al., 2022). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) can efficiently react with intracellular macromolecules, causing cell death and tissue damage and subsequently aggravating SCI. The spinal cord contains many polyunsaturated fatty acids, thus making it sensitive to oxidative stress. The spinal cord neurons have active oxidative metabolism but low antioxidant capacity, making neurons and glial cells significantly vulnerable to oxidative stress. Hence, reactive oxygen metabolites accumulate, resulting in excessive consumption of antioxidants from tissues after SCI (Genovese and Cuzzocrea, 2008; Figueroa et al., 2013; Lim et al., 2013; Wojdasiewicz et al., 2020).

Superoxide dismutase (SOD) is an active protease scavenging free radicals and protecting cells from oxidative damage. It eliminates the oxidation products produced after SCI. The SOD level reflects the ability to clear free radicals and has a vital role in balancing oxidation and antioxidation. Malondialdehyde (MDA) is a lipid peroxidation metabolite, reflecting the degree of oxidative stress (Woźniak et al., 2016; Wu et al., 2017). The lipid peroxidation can interfere with Ca^{2+} transport from the cell membrane by inhibiting the Ca^{2+}-ATPase activity, causing intracellular Ca^{2+} overload and enhanced ion imbalance (Rohn et al., 1993; Rohn et al., 1996). In addition, oxidative stress post SCI destroys ion homeostasis both inside and outside the membrane. Moreover, abundant Ca^{2+} enters and accumulates within the mitochondria, leading to mitochondrial destruction, aerobic energy metabolism dysfunction, and inhibition of ATP synthesis (Brown et al., 2006; Visavadiya et al., 2013; Scholpa and Schnellmann, 2017). Studies have revealed that acupuncture, electroacupuncture, and laser acupuncture can reduce oxidative stress after SCI (Wu et al.,

2002; On-Ong-Arj et al., 2018; Alvarado-Sanchez et al., 2019). The results from a traumatic SCI model study showed that electroacupuncture at GV26 reduces radical hydroxyl concentration and increases lipid peroxidation. At the same time, stimulation of GV4 decreases oxidative stress and improves motor function recovery in the hind limbs of rats with paralysis, indicating electroacupuncture at GV4 could be a therapeutic alternative (Juarez Becerril et al., 2015). Jiang et al. found that electroacupuncture at *Shuigou* (DU26) and *Fengfu* (DU16) acupoints induce antioxidation effects by enhancing the SOD activity and decreasing the MDA level (Jiang et al., 2014). Similar to the effect of the reactive oxygen species (ROS) scavenger, acupuncture can inhibit superoxide anion production, decrease JNK/p66Shc-mediated ROS generation, upregulate the apolipoprotein E (ApoE) and nuclear factor E2-related factor 2 (Nrf2)/heme-oxygenase-1 (HO-1) signaling pathways, and reduce the ROS-induced p38MAPK and ERK activation in microglia after SCI (Choi et al., 2012; Dai et al., 2021). Notably, the inhibitory effect of electroacupuncture on p38MAPK is significantly enslaved to the acupuncture frequency (Cheng et al., 2020).

Inhibition of Neuronal Apoptosis

Apoptosis, predominantly neuronal apoptosis, is an essential pathological mechanism causing secondary spinal cord injury (Abbaszadeh et al., 2020; Shi et al., 2021). Axonal injury and neuronal apoptosis block nerve conduction pathways after SCI and aggravate secondary injuries. Therefore, inhibition of apoptosis can induce SCI recovery (Beattie, 2004). The anti-apoptotic mechanisms of acupuncture have been widely explored. Acupuncture protects the nerves and reduces apoptosis of neurons and oligodendrocytes, thus improving functional recovery after SCI (Cai and Shen, 2018). In addition, electroacupuncture can inhibit spinal cord neuronal apoptosis by increasing the Bcl-2 expression and inhibiting caspase-3 and Bax (Zhao et al., 2008; Shi et al., 2016; Liu and Wu, 2017; Zhu et al., 2017).

Poly-ADP ribose polymerase (PARP) is the most significant substrate of caspase-3, and activated PARP can cause apoptosis mediated by apoptosis-inducing factor (AIF) (Kang et al., 2004). Previous studies showed that electroacupuncture could ameliorate early brain injury after subarachnoid hemorrhage by inhibiting the PARP-1/AIF pathway (Lang et al., 2020). Moreover, electroacupuncture also reduces the PARP expression in cerebral ischemia/reperfusion and Parkinson's disease (Sun et al., 2003; Yu et al., 2020). Furthermore, Liu et al. found that apoptosis post SCI was accompanied by cleaved PARP upregulation and electroacupuncture treatment attenuation (Liu and Wu, 2017).

BNIP3 is a member of the Bcl-2 family that induces apoptosis by promoting mitochondrial permeability transport pore opening and mitochondrial damage (Yu et al., 2018). In addition, the BNIP3 expression is elevated in rats after SCI (Yu et al., 2018), and electroacupuncture at GV20-GB7 reduced BNIP3 after intracerebral hemorrhage (Guan et al., 2021).

Heat shock protein (HSP) is an endogenous stress protein with various biological protective effects. HSP family members such as HSP 70 and HSP 72 have a protective effect on neurons after SCI (Chang et al., 2014; Xu et al., 2021a; Kim et al., 2022). Acupuncture has been demonstrated to have a neuroprotective role in cerebral ischemia by regulating HSP 70 (Xu et al., 2014; Shi et al., 2017). Gao et al. reported that HSP 90 participates in electroacupuncture-induced analgesia in chronic neuropathic pain (Gao et al., 2021). Other signaling pathways, such as PI3K/Akt/Erk, Nogo/NgR, Rho/ROCK, and mTOR, may also include the acupuncture-related beneficial effects against SCI (Renfu et al., 2014; Wei et al., 2018; Xiao et al., 2019; Li et al., 2020).

The toxic effects of excitatory amino acids play an essential role in the pathogenesis of SCI. The glutamate ion receptor activated by the N-methyl-D-aspartate (NMDA) receptor induces excessive Ca^{2+} influx and destroys mitochondrial function, thus stimulating the death of neurons (Xie et al., 2014; Inquimbert et al., 2018). Studies found that electroacupuncture can protect the spinal cord after SCI by reducing the expression of the NMDA receptor subunit NR1 and NR2A in the injured area (Tu et al., 2017a). It can also alleviate mechanical allodynia by inhibiting the upregulation of NR2B after chronic constrictive injury (Zhao et al., 2019).

Recent studies have observed that electroacupuncture can improve the locomotor function by regulating autophagy flux and inhibiting necroptosis after SCI (Hongna et al., 2020). Furthermore, Fang et al. depicted that pre- and post-conditioning electroacupuncture alleviates spinal cord ischemia–reperfusion injury, partly through autophagy upregulation accompanied by apoptosis inhibition (Fang et al., 2017). Moreover, studies conducted in intracerebral hemorrhage rat models show the effect of ferroptosis inhibition by acupuncture (Kong et al., 2021; Li et al., 2022). Therefore, apoptosis, autophagy, necroptosis, and ferroptosis should be clarified in future acupuncture studies on SCI.

Restrain of Inflammatory Response

After SCI, infiltrating leukocytes attracted by the innate immune response leads to an inflammatory cascade in the area of injury, and an excessive inflammatory response damages the spinal cord tissue. In addition, leukocytes, microglia, astrocytes, and macrophages release many pro-inflammatory cytokines and chemokines, including interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (TNF-α), which aggravate local inflammation and damage axons and neurons (Zhou et al., 2014a; Tang et al., 2020a; Brockie et al., 2021; Hellenbrand et al., 2021). Therefore, regulating inflammatory factors and improving neuroinflammation is of great significance for the recovery of SCI.

Neuroprotection by acupuncture is partially mediated by inhibiting inflammation and microglial activation after SCI (Choi et al., 2010; Jiang et al., 2014). However, the inflammatory response in SCI has two sides; it exerts a positive reaction against injury and aggravates secondary injury post SCI. The pro-inflammatory macrophage/microglia (M1 subsets) and anti-inflammatory macrophage/microglia (M2 subsets) are significant. Therefore, regulating the polarization of M1 and M2 macrophages/microglia can affect the inflammatory response process after SCI (Buzoianu-Anguiano et al., 2021; Ding

et al., 2021; Hashemizadeh et al., 2022). Previous studies have shown that acupuncture can ameliorate SCI by regulating M1 and M2 macrophages (Zhao et al., 2017). It also reduces the release of pro-inflammatory cytokines such as IL-6, TNF-α, nitric oxide synthase, and cycloxygenase-2 (Choi et al., 2010).

The purinergic receptors P2X4 and P2X7 are overexpressed on the cell surface of spinal dorsal horn microglia involved in microglial activation, which significantly contributes to the inflammation after SCI (Deng et al., 2018; Du et al., 2019; Kobayakawa et al., 2019; Song et al., 2022). Electroacupuncture can inhibit P2X7 receptor-mediated microglial activation and attenuate neuropathic pain (Wu et al., 2021a). It can also relieve pain hypersensitivity by inhibiting P2X7 receptor-positive microglia after chronic constriction injury (Xu et al., 2016). In addition, acupuncture reduces diabetic peripheral neuralgia by downregulating the P2X4 expression in rat spinal microglia (Tang et al., 2020b).

The inflammasome is an essential component of host defense response, recognizing pathogen-associated molecular patterns and damage-associated molecular patterns. It mediates the release of pro-inflammatory factors after injury. The family of NOD-like receptors (NLRs) is a vital member of the inflammasome, with NLRP3 being the most studied inflammasome in central nervous system disorders. The ability of acupuncture to attenuate the inflammatory response through inflammasome regulation, especially NLRP3, has been explored in many neurological diseases, including autism (Zhao et al., 2022), postoperative cognitive dysfunction (Sun et al., 2021), depression (Li et al., 2021), Alzheimer's disease (Jiang et al., 2018; Zhang et al., 2021), cerebral ischemia (Jiang et al., 2019), and vascular dementia (Du et al., 2018). Further research is needed to explore the role of the inflammasome, including NLRs, in acupuncture-induced beneficial effects against SCI.

Choi et al. demonstrated that elevated p38MAPK accelerated the microglial secretion of inflammatory mediators after SCI. Electroacupuncture can effectively downregulate the p38MAPK phosphorylation level, inhibit microglial activation, and promote nerve regeneration (Choi et al., 2010). Hu et al. demonstrated that the combination of gangliosides with electroacupuncture at *Jiaojia* (EX-B2) has a more substantial effect in promoting the recovery of nerve function, which could be related to the inhibition of pro-inflammatory cytokines and the Nogo-NgR signaling pathway (Hu et al., 2021).

Improvement of Microcirculation Dysfunction

SCI can cause rupture, hemorrhage, and capillary embolism, leading to microcirculation dysfunction. Improved microcirculation can reduce cellular apoptosis and promote functional recovery (Tator and Koyanagi, 1997). Reduced blood flow and intramedullary vasospasm are seen after SCI. Vasoconstriction factors such as endothelin 1 (ET-1), prostaglandin E2 (PGE2), and thromboxane A2 (TXA2) cause vasospasm aggravation and blood flow reduction. As a result, the blood–spinal cord barrier gets disrupted, leading to inflammatory cell infiltration and spinal tissue edema (Tempel and Martin, 1992; Mitsuhashi et al., 1994; McKenzie et al., 1995; Wang et al., 2007; Sinescu et al., 2010).

Clinical studies conducted in healthy adults demonstrated that acupuncture influences the tortuousness of capillary loops, the diameter of the afferent loop, and capillary refill time, thereby regulating the microcirculation (Scardina et al., 2009; Yeh et al., 2021). In animal experiments, acupuncture can also improve the blood flow within the brain after hemorrhage or ischemia. It is primarily associated with the regulation of the vascular endothelial growth factor (VEGF), angiopoietin 1 (Ang-1), Ang-2, angiotensin II type I receptor, endothelin receptor, and EphB4/EphrinB2-mediated Src/PI3K signal pathways (Tian et al., 2013; Zhou et al., 2014b; Wu et al., 2021b). In addition, a study using the intervertebral disc extrusion model revealed that electroacupuncture improves microcirculation characterized by high blood flow, micro-vessel density, and reduced vacuolation within the white matter (Jiang et al., 2015). Acupuncture can also regulate microcirculation and attenuate neurological dysfunction by suppressing the cPLA2 activity and PGE2 level (Hong et al., 2021).

Attenuation of Glial Scar Formation

Glial cells play an essential role in the physiological function inside the spinal cord microenvironment and induce excessive hyperplasia of the glial scar under pathological conditions. On the one hand, a glial scar can limit the lesion expansion and protect the surrounding tissues from injury. On the other hand, it restricts neuronal regeneration (Faulkner et al., 2004; Pekny et al., 2014; Tran et al., 2018; Gu et al., 2019). During the spinal cord recovery, astrocytes proliferate and secrete a variety of extracellular matrices to form a glial scar, hindering the neural pathway recovery. The significant molecules participating in glial scar formation are chondroitin sulfate proteoglycans (CSPGs) and keratan sulfate proteoglycans produced by astrocytes (Zhang et al., 2006; Wang et al., 2021a; Tran et al., 2021). CSPG accumulation at the injured area inhibits the axonal growth, and reducing the CSPG expression can promote axonal regeneration and remyelination (Siebert and Osterhout, 2011). Electroacupuncture can downregulate the CSPG protein expression and stimulate axonal regeneration, leading to structural and functional recovery after SCI (Ding et al., 2011). It also stimulates the differentiation of transplanted bone marrow mesenchymal stem cells (MSCs) and promotes corticospinal tract regeneration across injured sites in the caudal cord, with CSPG protein involvement (Ding et al., 2013). Numerous studies have shown that acupuncture can restrict astrogliosis and alleviate neurological dysfunction caused by diseases such as hydrocephalus (Tida et al., 2018) and cerebral ischemia (Han et al., 2010; Tao et al., 2016; Young-Wook et al., 2019).

Glial fibrillary acidic protein (GFAP) is a crucial component of astrocytes. As an important marker of glial scar formation, GFAP depicts the proliferative state of astrocytes (Brenner, 2014; Yang and Wang, 2015). In addition, GFAP secreted by astrocytes forms a physical barrier to isolate damaged tissue, provides mechanical strength, and limits axonal growth due to the physical barrier (Pekny et al., 2014). Fire needle acupuncture and

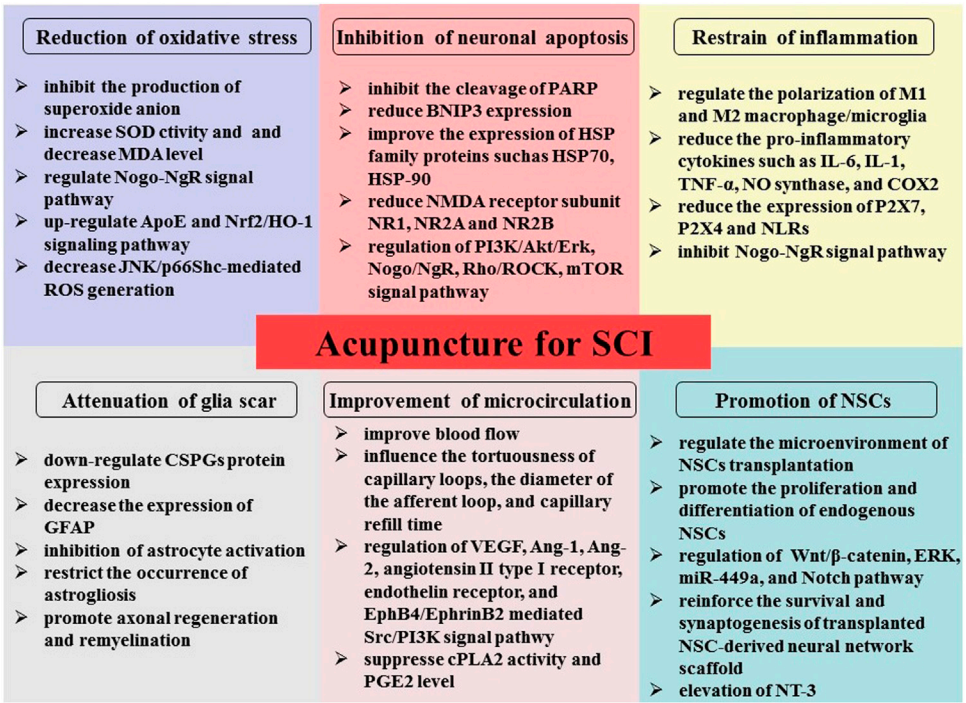

FIGURE 1 | Illustration of the possible mechanism underlying acupuncture therapy in SCI, including oxidative stress reduction, inflammation and apoptosis inhibition, microcirculation improvement, reduction of glial scar formation, and stimulation of NSC differentiation and proliferation.

electroacupuncture can decrease the GFAP expression, leading to the differentiation of neural stem cells (NSCs) and inhibition of astrocyte activation, respectively (Zhang et al., 2018; Xu et al., 2019). Liu et al. observed that electroacupuncture increases the gene and protein expression of GFAP and the platelet-derived growth factor (PDGF) after spinal cord transection, promoting locomotor function recovery (Liu et al., 2013). Interestingly, Wei et al. revealed that electroacupuncture elevates GFAP levels only at the early phase after SCI and reduces the GFAP expression later during recovery (Wei et al., 2017), indicating diverse functionalities of acupuncture in SCI. Choosing the time points and interval of acupuncture therapy exerting a better effect is an important issue that needs to be explored in future studies.

Promotion of Neural Stem Cell Proliferation and Differentiation

SCI induces damage to the segmentary neurons, axons, and glial cells at the injury site, forming a hole at the center of the spinal cord. The loss of neurons within the injured section and the disruption of the ascending sensory and descending motor tracts of axon conduction caused loss of the neurologic function. NSCs can differentiate into neurons, astrocytes, or oligodendrocytes, connect the spinal cord end, and rebuild neural pathways (Pereira et al., 2019; Vancamp et al., 2020; de Freria et al., 2021; Chen and Li, 2022). Several experimental studies have shown that acupuncture can induce the proliferation and differentiation of NSCs, thereby promoting the repair of injured nerves; however, the mechanism remains unclear (Tao et al., 2010; Zhang et al., 2013; Jiang et al., 2016; Dubrovsky et al., 2020).

Various hypotheses have been proposed to illustrate the acupuncture mechanism on NSCs. First, acupuncture could promote nerve regeneration and synaptogenesis by regulating the microenvironment of NSC transplantation and promoting SCI recovery (Tang et al., 2020c; Zhao et al., 2020; Yang et al., 2021). Second, electroacupuncture promotes the proliferation and differentiation of endogenous NSCs by regulating numerous endogenous signals. The upregulation of exosomal miR-146b, NeuroD1, the activation of the Notch pathway, and the downregulation of the PTEN expression are associated with acupuncture-induced improvement of neurological injury after ischemic stroke (Tao et al., 2014; Zhao et al., 2015; Sha et al., 2019; Zhang et al., 2020). In contrast, the potential signals of the acupuncture-induced NSC regulation in the SCI model include Wnt/β-catenin (Zhang et al., 2017a), ERK (Xu et al., 2019), miR-449a (Zhu et al., 2017), and Notch pathway (Wang et al., 2021b). Third, electroacupuncture reinforces the survival and synaptogenesis of transplanted NSC-derived neural network scaffolds as a neuronal relay bridging two severed ends of the injured spinal cord (Jin et al., 2019). Similarly, two other studies have shown that electroacupuncture facilitates the integration of the mesenchymal stem cell (MSC)–derived neural network into the transected spinal cord by elevating neurotrophin-3 (NT-3) (Ding et al., 2013; Yang et al., 2021). Moreover, pre-induction with NT-3 and retinoic acid after SCI before electroacupuncture could also promote the survival and differentiation of the grafted MSCs in gelatin sponge scaffolds (Zhang et al., 2014).

NT-3 is tightly associated with SCI recovery as the primary type of neurotrophic factor (Ding et al., 2009; Mo et al., 2016; Tu et al., 2017b). Electroacupuncture promotes the intrinsic growth ability of spinal neurons after SCI by activating the calcitonin gene-related peptide/α-calcium/calmodulin-dependent protein kinase/NT-3 pathway (Xu et al., 2021b). Additionally, electroacupuncture treatment can promote the differentiation and remyelination of MSCs and oligodendrocyte precursor cells, protect spinal motor neurons, and alleviate muscle atrophy after SCI, along with elevation of the NT-3 expression (Huang et al., 2011; Yan et al., 2011; Ding et al., 2015; Liu et al., 2015; Zhang et al., 2017b).

SUMMARY AND PROSPECTS

SCI is characterized by high mortality and disability, with complex regeneration and repair. We explained in detail the underlying mechanisms of acupuncture therapy for SCI, including oxidative stress reduction, inflammation and apoptosis inhibition, microcirculation improvement, glial scar formation reduction, and stimulation of NSC differentiation (**Figure 1**). This review could provide an experimental basis for better clinical application of acupuncture in SCI. However, SCI has complex pathophysiology. Therefore, significant research should be focused on the pathogenesis of acupuncture therapy to formulate mechanism-based specific intervention strategies and help SCI patients achieve better outcomes and recovery of impaired neurological function.

Although this review primarily summarizes recent preclinical studies, acupuncture clinical trials for SCI have shown positive results. Acupuncture alleviates the neurogenic bladder (Cheng et al., 1998; Honjo et al., 2000), chronic shoulder pain (Dyson-Hudson et al., 2001; Dyson-Hudson et al., 2007), neuropathic pain (Norrbrink and Lundeberg, 2011; Estores et al., 2017), and osteoporosis (Meng et al., 2014) and improves neurological (sensory and motor) functions (Wong et al., 2003). Interestingly, a study that enrolled seven healthy volunteers and three cervical SCI patients observed that the functional magnetic resonance imaging (fMRI) technique detected an activation centered at C6 and C2 cervical spinal cord levels by using acupuncture at L4 and L11, proving the existence of the meridians and points. An fMRI can be used as a harmless research and monitoring method to explore the effect of acupuncture therapy on SCI patients (Chen et al., 2007). However, most clinical trials are single-center trials with few subjects and are not conducted in a double-blinded manner.

Acupuncture can be an emerging therapy for the treatment of SCI as a simple, safe, and low-risk treatment. Although many basic studies and clinical trials have established the advantages of acupuncture in SCI, large-scale and multi-centric clinical trials are needed to authenticate the effect further. Moreover, the concept of precision medicine could further explore the best indicators in acupoint selection, stimulation frequency, starting time, and duration, for achieving individualized treatment. Thus, modern analytical techniques should be used to quantitatively analyze the variations in physiological and pathological indexes after acupuncture, which could popularize the global application of acupuncture.

AUTHOR CONTRIBUTIONS

KJ and XC contributed substantially to the conception and design of the work and drafting and revising the manuscript for important intellectual content. YS drafted parts of the manuscript. All authors approved the final version to be published and agreed to be accountable for all aspects of the work.

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Mechanisms Underlying Curcumin-Induced Neuroprotection in Cerebral Ischemia

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Ischemic stroke is the leading cause of death and disability worldwide, and restoring the blood flow to ischemic brain tissues is currently the main therapeutic strategy. However, reperfusion after brain ischemia leads to excessive reactive oxygen species production, inflammatory cell recruitment, the release of inflammatory mediators, cell death, mitochondrial dysfunction, endoplasmic reticulum stress, and blood–brain barrier damage; these pathological mechanisms will further aggravate brain tissue injury, ultimately affecting the recovery of neurological functions. It has attracted the attention of researchers to develop drugs with multitarget intervention effects for individuals with cerebral ischemia. A large number of studies have established that curcumin plays a significant neuroprotective role in cerebral ischemia via various mechanisms, including antioxidation, anti-inflammation, anti-apoptosis, protection of the blood–brain barrier, and restoration of mitochondrial function and structure, restoring cerebral circulation, reducing infarct volume, improving brain edema, promoting blood–brain barrier repair, and improving the neurological functions. Therefore, summarizing the results from the latest literature and identifying the potential mechanisms of action of curcumin in cerebral ischemia will serve as a basis and guidance for the clinical applications of curcumin in the future.

Keywords: cerebral ischemia, curcumin, neuroprotection, oxidative stress, inflammation, blood–brain barrier, apoptosis, mitochondrial dysfunction

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INTRODUCTION

Ischemic stroke is the most common type of stroke and is associated with high mortality and morbidity. Early restoration of blood supply to ischemic tissues is currently an effective treatment strategy that improves the energy metabolism, oxygen supply, and neurological outcomes. At present, recombinant tissue plasminogen activator (r-TPA) is used for thrombolytic therapy; however, with the limitation of usage within 4.5 h after the onset of stroke, only 3–5% of stroke patients meet the criteria and use r-TPA in a timely fashion (Wardlaw et al., 2014; Marlier et al., 2015; Moretti et al., 2015; Campbell et al., 2019; Campbell and Khatri, 2020). Therefore, current research focuses on exploring pathological mechanisms and discovering the novel potential therapeutic targets for cerebral ischemia. Cerebral ischemia causes acute brain injury, while reperfusion results in chronic brain injury. In the acute stage of ischemia, cellular homeostasis and microcirculation are impaired, cell energy metabolism is disrupted, and the structure of the blood–brain barrier (BBB) is destroyed. During the reperfusion period, these structures and functions are not restored; many

substances and cells that would not otherwise reach the brain, such as inflammatory cells and macromolecules of inflammatory factors, enter the brain through the damaged BBB. This leads to further aggravation of injury following cerebral ischemia (Pan et al., 2007; Jung et al., 2010; Badruddin et al., 2011). In short, the damage caused by cerebral ischemia and reperfusion involves oxidative stress, apoptosis, the inflammatory response, BBB destruction, and energy metabolism disorder, among other pathological mechanisms. Therefore, it is critical to developing drugs that can intervene with multiple targets.

Curcumin is the most important polyphenol active component of turmeric and is slightly soluble in water but soluble in ethanol and acetone. The ratio of compounds in turmeric is about 5% dimethoxylcurcumin, 15% demethoxylcurcumin, and 80% curcumin. It is challenging to dissolve, extract, and absorb curcumin, resulting in low bioavailability and limited clinical applications (Esatbeyoglu et al., 2012; Kotha and Luthria, 2019). In recent years, numerous drug delivery systems using liposomes, nanoparticles, and microemulsion as carriers have been successfully developed, which significantly increased the solubility, stability, and safety of curcumin, and greatly improved its biological activity in treating or preventing diseases, showing great promise for clinical application (Aggarwal and Sung, 2009; Mahmood et al., 2015; Abd El-Hack et al., 2021; Jabczyk et al., 2021; Feltrin et al., 2022).

As a natural medicine, curcumin has a wide range of beneficial pharmacological activities, including antitumor, anti-inflammatory, antioxidation, anti-apoptosis, etc. (Zhou et al., 2011; Mandal et al., 2020; Fu et al., 2021). Numerous studies have revealed the beneficial role of curcumin in cancer, diabetes, metabolic diseases, autoimmune diseases, atherosclerosis, arthritis, pulmonary diseases, etc (Aggarwal and Harikumar, 2009; Jabczyk et al., 2021; Mahjoob and Stochaj, 2021). Recently, researchers discovered that curcumin also has neuroprotective effects on various neurological diseases, including neuropsychiatric disorders, neurodegenerative diseases, traumatic brain injury, spinal cord injury, and epilepsy (Dhir, 2018; Bhat et al., 2019; Yavarpour-Bali et al., 2019; Yuan et al., 2019; Farkhondeh et al., 2020; Nebrisi, 2021; Lamanna-Rama et al., 2022). The involved mechanisms may include the mediation of neurotransmitters and the hypothalamus-pituitary-adrenal cortex axis, the release of neurotrophic factors, and the promotion of nerve regeneration, thereby influencing a variety of signaling cascades, enhancing vitality and differentiation of neurons, and ultimately enhancing neurological functions (Xu et al., 2006; Srivastava et al., 2018; Ramaholimihaso et al., 2020; Yang et al., 2020; Yang et al., 2021). Multiple *in vitro* and *in vivo* experiments have been carried out to investigate the role and mechanism of curcumin in cerebral ischemia and revealed that curcumin participates in the recovery of ischemic injury by inhibiting the oxidation, apoptosis and inflammation, protecting the BBB, and restoring mitochondrial functions (Ovbiagele, 2008; Bavarsad et al., 2019). A summary of recent studies on curcumin treatment for cerebral ischemia will assist in identifying its shortcomings and benefits, thereby guiding future research studies, clinical translational applications, and the exploration of novel therapeutic strategies for ischemic stroke.

Mechanisms of Curcumin Against Cerebral Ischemia

Recently, numerous studies have demonstrated the neuroprotective effect of curcumin in cerebral ischemia (Bavarsad et al., 2019; Ułamek-Kozioł et al., 2020; Subedi and Gaire, 2021). Curcumin can attenuate neurological dysfunction, and reduce infarct volume and brain edema, thereby improving the outcome of an ischemic stroke. Various mechanisms are involved, including the inhibition of oxidative stress, inflammation, apoptosis, calcium overload, and endoplasmic reticulum stress, as well as the restoration of BBB, and mitochondrial structural functions (**Supplementary Table S1**). The details are described below.

Curcumin Reduces Oxidative Stress

Brain tissues have a higher metabolic rate, demand for oxygen and polyunsaturated fatty acids, and lower levels of antioxidant enzymes compared with other organs, making the central nervous system more vulnerable to oxidative damage (Cenini et al., 2019; Torres-Cuevas et al., 2019; Bhatt et al., 2020). Oxidative stress caused by the disruption of homeostasis between oxidative and antioxidant systems are a key mechanism of cerebral ischemic injury (Li et al., 2018; Torres-Cuevas et al., 2019; Yang, 2019). As a vital signaling molecule in the brain, reactive oxygen species (ROS) directly or indirectly mediates several pathological processes after cerebral ischemia (Fraser, 2011; Olmez and Ozyurt, 2012; Orellana-Urzú a et al., 2020). It has been demonstrated that the activity of nitric oxide synthase (NOS), cyclooxygenase (COX), xanthine dehydrogenase/xanthine oxidase, myeloperoxidase, myeloperoxidase (MPO), and other enzymes promoting ROS production increase following stroke, whereas the activity of enzymes that prevent ROS production, such as superoxide dismutase (SOD), catalase, peroxidase, glutathione peroxidase (GSH-Px) decrease, consequently destroying the dynamic balance of ROS, and leading to its accumulation. Excessive ROS can trigger lipid peroxidation, DNA damage, and protein oxidation damage (Sorce et al., 2012; Bazmandegan et al., 2017; Shao et al., 2020; Su et al., 2020; Duan et al., 2021). Therefore, the use of free radical scavengers or other antioxidants is one of the primary therapeutic options for cerebral ischemia (Ahmadinejad et al., 2017; Davis and Pennypacker, 2017; Zhou et al., 2021).

Curcumin, as an antioxidant, accelerates the removal of ROS by activating the antioxidant enzymes and inhibiting the brain tissue damage induced by oxidative stress (Vajragupta et al., 2003; Namgyal et al., 2021). The antioxidative effect of curcumin in cerebral ischemia has been widely explored, and it has been noted that curcumin could partially exert neuroprotection by alleviating oxidative stress-induced injury post-stroke (Rathore et al., 2008; Mukherjee et al., 2019; Zhang et al., 2021). It was previously reported that pretreatment and posttreatment administration of curcumin both improved the antioxidative ability of the injured neurons (Wu et al., 2015), while immediate and delayed (24 h

after ischemia) treatments with curcumin both prevented ischemia-induced neuronal damage and oxidative insult, indicating the wide range time window of curcumin treatment in cerebral ischemia (Al-Omar et al., 2006).

Moreover, curcumin can lower the production and accumulation of ROS and oxidation products (MDA, lipid peroxidation, etc.) (Hosseinzadehdehkordi et al., 2015; Seo et al., 2017; Khan et al., 2019). Other formulations of curcumin with polyethylene glycol (PEG)-ylated polylactide-co-glycolide (PLGA) nanoparticles or solid lipid nanoparticles (C-SLNs) are also capable of reducing ROS levels (Mukherjee et al., 2019). Interestingly, a comparative study investigating the antioxidative effect of three curcuminoids (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) using a polymeric N-isopropyl acrylamide nanoparticle formulation determined that curcumin had the most potent antioxidant activity (Ahmad et al., 2013). In addition, curcumin elevates the activity and expression level of antioxidases (NADPH oxidase 2, SOD, CAT, GSH-Px, glutathione reductase, etc.) (Dohare et al., 2008; Kakkar et al., 2013; Wu et al., 2020). Awad *et al.* demonstrated that curcumin synergistically enhanced the inhibitory action of candesartan on brain ischemia through the suppression of oxidative stress, implying the beneficial combined effects and potential therapeutic strategy of curcumin and other drugs on cerebral ischemia in the future (Awad, 2011). Various signaling pathways are involved in curcumin-induced antioxidation. For example, curcumin could alleviate the oxidative damage by regulating the miR-1287-5p/LONP2 axis and miR-7/RelA p65 axis in an OGD/R model (Xu H. et al., 2019; Zhang et al., 2021). Another study described that dienone monocarbonyl curcumin analogs protected the cellular growth by eliminating ROS generation by activating the Nrf2/HO-1 signaling pathway (He et al., 2021). Similarly, curcumin and hexahydrocurcumin enhanced antioxidant defense partially through the Nrf2/HO-1 pathway in a rat stroke model (Wicha et al., 2017). In addition, other signaling pathways such as SP1/Prdx6 (Jia et al., 2017), AMPK/UCP2 (Pu et al., 2013), Golgi reassembly, and stacking protein 65 (GRASP65) (Lin et al., 2016) are also involved in the antioxidant properties of curcumin.

Curcumin Inhibits Cellular Apoptosis

Apoptosis is an autonomous and programmed process of cell death that is the predominant form of cell death in cerebral ischemia and is closely related to the prognosis of stroke patients (Ferrer and Planas, 2003; Mitsios et al., 2007; Uzdensky, 2019; Gao et al., 2020). Previous research has described that cell necrosis and apoptosis co-exist in the acute stage of cerebral ischemia, while apoptosis is the primary type of delayed cell death post-stroke. Indeed, following the stroke onset, necrosis mainly occurs in the ischemic central region, whereas apoptosis chiefly occurs in the ischemic penumbra (Ueda and Fujita, 2004; Radak et al., 2017). The mechanism of apoptosis induced by cerebral ischemia is intricate and involves not only alterations in the expression of apoptosis-related genes but is also regulated by myriad internal and external factors. The mechanisms that mediate ischemic stroke-induced apoptosis mainly include the mitochondrial and endoplasmic reticulum stress and death receptor pathways (Cao et al., 2001; Zheng et al., 2003; Broughton et al., 2009; Iurlaro and Muñoz-Pinedo, 2016).

The use of anti-apoptotic agents or therapeutic strategies can protect against cell injury after cerebral ischemia (Rami et al., 2008; Luo et al., 2019; Youssef et al., 2021). A large number of studies have reported that various traditional Chinese medicines, including curcumin, can effectively alleviate cellular apoptosis after cerebral ischemia and improve neurologic dysfunction (Dong et al., 2016; Yu et al., 2020; Zhu et al., 2021). Curcumin can upregulate the expression of anti-apoptotic proteins such as Bcl-2 and downregulate the expression of apoptosis-related proteins such as Bax and caspase-3, thus effectively inhibiting cellular apoptosis and attenuating cerebral ischemia-induced injury (Xie et al., 2018; Xu L. et al., 2019). The specific mechanism of curcumin alleviating apoptosis after cerebral ischemia is well-documented. Curcumin-laden exosomes target ischemic brain tissues and alleviate ROS-mediated mitochondrial apoptosis (He et al., 2020). Additionally, curcumin can alleviate ischemia-induced brain injury and cell apoptosis via repressing CCL3, elevating glucose transporter (GLUT)1 and GLUT3, inactivating the TLR4/MyD88/MAPK/NF-κB and Wnt/JNK1 pathways, and promoting MEK/ERK/CREB, and PI3K/Akt pathway activation (Xia et al., 2018; Xu L. et al., 2019; Wang C. et al., 2020; Wu et al., 2020; Zhou et al., 2020). Xu et al. (2018) showed that a combination of curcumin and vagus nerve stimulation restored behavioral deficits by inhibiting apoptosis after cerebral ischemia, with the involvement of the Akt/ERK2 pathway. Notably, curcumin inhibits cellular damage and apoptosis by diminishing the endoplasmic reticulum stress (ERS) (Cheng et al., 2020; Keshk et al., 2020; Zhou et al., 2022). Chhunchha et al. (2013) reported that curcumin abated hypoxia-induced ERS-mediated cell death in mouse hippocampal cells by enhancing peroxiredoxin 6 (Prdx6) expressions and inhibiting NF-κB activation. Another *in vitro* research using the neuroblastoma cells exposed that curcumin relieved neurotoxicity via regulating the PERK-eIF2α pathway (Yan et al., 2022). Last, curcumin mitigated axonal injury and neuronal cellular apoptosis through the PERK/Nrf2 signaling pathway in a rat diffuse axonal injury model (Huang T. et al., 2018).

However, it is worthwhile noting that curcumin could play an antitumor role by promoting the apoptosis of tumor cells (Notarbartolo et al., 2005; Giordano and Tommonaro, 2019; Walker and Mittal, 2020). Furthermore, exploration of the mechanism of curcumin in diverse diseases and its effect on apoptosis under contrasting conditions will assist in evaluating the safety and effectiveness of curcumin treatment in cerebral ischemia in the future.

Curcumin Diminishes the Inflammatory Cascade

Neuroinflammation plays a key role in the progression of cerebral ischemia. Following cerebral ischemia, microglia, astrocytes, and neutrophils, as the main effector cells, release a large number of inflammatory cytokines, such as interleukins, chemokines, and tumor necrosis factor (TNF), induce neuronal apoptosis, and

contribute to microvascular dysfunction, secondary cerebral hemorrhage, and cerebral edema (Wang et al., 2019b; Shi et al., 2019; Jurcau and Simion, 2021). The activation and infiltration of inflammatory cells, as well as the synthesis and secretion of adhesive molecules and inflammatory mediators, promote the inflammatory cascade (Barrington et al., 2017; Hendriksen et al., 2017; Živančević et al., 2021).

Curcumin has been shown to possess anti-inflammatory properties in various neurological disorders, including acute brain injuries (spinal cord injury (Zhang N. et al., 2017), traumatic brain injury (Sun et al., 2020), stroke (Miao et al., 2016), and subarachnoid hemorrhage (Wakade et al., 2009)), and neurodegenerative diseases (Alzheimer's disease (Hamaguchi et al., 2010), Parkinson's disease (Ojha et al., 2012), Huntington's disease (Ullah et al., 2017), and multiple sclerosis (Mohajeri et al., 2015)). It attenuates the inflammatory response after cerebral ischemia through multiple mechanisms. For instance, curcumin can reduce the induction and release inflammatory cytokines such as IL-6, IL-1β, TNF-α, and COX-2 (Zhang Y. et al., 2017; Wicha et al., 2017). In addition, curcuminoids decrease neutrophil rolling and adhesion to the cerebrovascular endothelium, lower neutrophil numbers, and inhibit neutrophil activation, thereby ameliorating ischemic brain injury (Funk et al., 2013). NF-κB is a regulatory factor with diverse transcriptional effects, which are activated after cerebral ischemia and participates in the transcription of relevant target genes contributing to the inflammatory response. Numerous researchers have demonstrated that the anti-inflammatory effect of curcumin in cerebral ischemia is tightly associated with the modulation of NF-κB (Li et al., 2016, 2017; Li et al., 2021). Ran et al. (2021) observed that curcumin ameliorated white matter injury after ischemic stroke via NF-κB suppression and NLRP3 inflammasome inhibition in a rat stroke model. Triblock copolymer nanomicelles loaded with curcumin also exert an anti-inflammatory effect by inhibiting the NF-κB pathway after cerebral ischemia (Li et al., 2021). Other studies assessing the link between NF-κB and curcumin established that the anti-inflammatory impact of curcumin in cerebral ischemia is mediated by the inhibition of the TLR4/MyD88/MAPK/NF-κB, TLR2/NF-κB, and PPAR γ/NF-κB pathways (Liu et al., 2013; Tu et al., 2014; Wang C. et al., 2020). Likewise, the modulation of the TLR4/p38/MAPK, SIRT1 and JAK2/STAT3 pathways (Li L. et al., 2015; Miao et al., 2016; Huang L. et al., 2018) are involved in curcumin-induced inhibition of inflammation in cerebral ischemia. As a recent hotspot area in stroke, ERS also contributes to inflammation and apoptosis in cerebral ischemia. Zhu et al. (2017) described the inhibitory effect of curcumin on ERS by downregulating the expression of GADD153 and caspase-12 in a rat stroke model. Meanwhile, an *in vitro* study exposed that curcumin attenuated neurotoxicity in the hippocampus by suppressing the ERS-associated TXNIP/NLRP3 inflammasome activation in an AMPK-dependent manner (Li Y. et al., 2015).

Microglia are in a resting state under physiological conditions and play the role of "immune monitoring and defense" in the microenvironment of brain cells. Conversely, they are rapidly activated and polarized in the pathological state (Hu et al., 2015; Ma et al., 2017). After the onset of cerebral ischemia, microglia play a contrasting role in brain injury or neuroprotection through M1 or M2 polarization (Xiong et al., 2016; Zhao et al., 2017; Xue et al., 2021). M1 microglia have cytotoxic effects and cause inflammatory tissue damage, whereas M2 microglia have a neuroprotective effect and promote tissue repair and regeneration. The latter congregate in the ischemic area during cerebral ischemia and release inflammatory factors to enhance the inflammatory response. Interestingly, curcumin has a profound regulatory influence on microglial responses, shifting the microglial phenotype from the pro-inflammatory M1 state toward the anti-inflammatory and tissue-reparative M2 phenotype, and inhibiting microglia-mediated pro-inflammatory responses (Hu et al., 2012). The results from both *in vivo* MCAO and *in vitro* OGD models have corroborated that curcumin reduces inflammation through the inhibition of M1 microglial activation and by weakening the increase in TNF-α and IL-1β (Liu et al., 2017; Wang et al., 2019a).

Curcumin Has a Protective Effect on the Integrity of the BBB

The BBB is predominantly composed of cerebral microvascular endothelial cells, astrocytes, basal lamina, and pericytes. The primary function of BBB is to prevent the diffusion of macromolecules into the brain parenchyma and maintain the stability of the internal environment of the nervous system (Huber et al., 2001; Obermeier et al., 2013; Langen et al., 2019; Alahmari, 2021). After the occurrence of cerebral ischemia, several mediators cause direct damage to the BBB components, which are exacerbated by apoptosis, oxidative stress, and inflammatory reaction, thus increasing the permeability of the BBB and aggravating brain edema and neurologic injury (Jin et al., 2010; Jiang et al., 2018; Kunze and Marti, 2019). Numerous studies have explored the protective role and mechanism of action of curcumin on BBB after ischemic stroke. Curcumin can protect the integrity of BBB and reduce brain edema by the upregulation of aquaporin 4 and tight junction proteins such as zonula occluden 1 (ZO-1), occludin, and claudin-5, and the downregulation of matrix metalloproteinase 9 (MMP-9), intercellular adhesion molecule-l (ICAM-1), and vascular cell adhesion molecule-l (VCAM-1) (Li et al., 2017; Wang et al., 2019a; Wicha et al., 2020; Wu et al., 2021). Furthermore, curcumin attenuates cerebral capillary endothelial cell damage by inhibiting the expression of inducible nitric oxide synthase (iNOS) and the generation of NO(x) (nitrites/nitrates contents), thereby preventing BBB damage (Jiang et al., 2007). The protection of shear rate can also prevent neutrophil adhesion to the cerebrovascular microcirculation and block early microvascular inflammation (Funk et al., 2013). Mo et al. (2021) found that curcumin exhibited a protective effect against cerebral ischemia by reducing the BBB dysfunction through protein kinase C-θ (PKC-θ) signaling. In addition, it was previously reported that curcumin ameliorates the permeability of the BBB during hypoxia by upregulating the expression of HO-1 in brain microvascular endothelial cells (Wang et al., 2013).

Despite many studies demonstrating the protective effect of curcumin on BBB, there are still unanswered questions such as which curcumin formulations and routes of administration can penetrate the BBB more rapidly. What is the main mechanism through which curcumin prevents BBB injury and how to determine the optimal dose and administration interval with favorable safety and efficacy profiles. Further studies are warranted to develop and identify potential treatment strategies for cerebral ischemia.

Curcumin Improves Mitochondrial Dysfunction and Calcium Overload

The mitochondrion is the main structure for regulating cellular calcium homeostasis. Cellular calcium overload can lead to ROS generation, mainly released from mitochondria, and induce oxidative stress (Kirkinezos and Moraes, 2001; Brookes et al., 2004; Peng and Jou, 2010). Mitochondrial permeability transition pore (mPTP) is a ROS-dependent protein complex between the mitochondrial inner and outer membrane. Calcium overload and oxidative stress in mitochondria can induce the opening of mPTP through lipid peroxidation and mitochondrial respiratory chain damage, thus reducing the mitochondrial membrane potential and releasing cytochrome C (Armstrong, 2006; Rottenberg and Hoek, 2017). The latter is a small molecule protein located in the inner membrane of mitochondria, which serves as an electron carrier between the mitochondrial respiratory chain complex III and complex IV. Its release activates caspase-9, which in turn activates the executor of apoptosis protein caspase-3, and ultimately leads to neuronal apoptosis (Kadenbach et al., 2004; Choi et al., 2007). Therefore, the destruction of mitochondrial structural integrity and functional homeostasis is a significant pathological change in cerebral ischemia injury. Protecting the mitochondrial structure and function is the focus of neuroprotection after cerebral ischemia.

Curcumin can alleviate cerebral ischemic injury by preserving the mitochondrial function and minimizing mitochondrial injury, elevating mitochondrial membrane potential, mitochondrial complex I activity, mitochondrial cytochrome c levels, and maintaining the mitochondrial membrane integrity (Rathore et al., 2008; Kakkar et al., 2013; Miao et al., 2016; Zhang Y. et al., 2017; Wang et al., 2019c). Moreover, curcumin may exert neuroprotective effects by increasing mitochondrial biogenesis, including nuclear respiratory factor-1, mitochondrial transcription factor A, and mitochondrial number (Wang et al., 2005; Liu et al., 2014). He et al. (2020) uncovered that curcumin-laden exosomes alleviated cerebral ischemia-reperfusion injury by inhibiting the ROS-mediated mitochondrial apoptosis. In another study, Mondal et al. (2019) discovered that tetrahydrocurcumin epigenetically mitigated mitochondrial dysfunctions by regulating the mitochondrial tissue inhibitor of metalloproteinase 2 (TIMP-2) through hypermethylation of the CpG islands of TIMP-2 promoter. Furthermore, curcumin can relieve Ca^{2+} dysregulation (Shukla et al., 2008), which may be associated with the inactivation of the P2X7 receptor (Wang Z. et al., 2020). However, the crosstalk and interactions of mitochondrial dysfunction, oxidative stress, calcium overload, and apoptosis in cerebral ischemia are complex. Further research is necessary to reveal the specific neuroprotective mechanism of curcumin in this complicated pathological process.

Curcumin Regulates Autophagy

Autophagy is a ubiquitous occurrence in eukaryotic animals in which cells phagocytose their own cellular components into vesicles and subsequently fuse with lysosomes to form autophagolysosomes, which breakdown to maintain the cell metabolism and organelle renewal (Mizushima et al., 2008; Mizushima and Komatsu, 2011). It is instrumental in maintaining cell survival and intracellular homeostasis under stressful conditions such as ischemia and hypoxia; however, immoderate autophagy may promote cell death (Smith et al., 2011; Kubisch et al., 2013; Choi et al., 2018). So far, the researchers have detected more than 30 autophagy-related genes involved in regulating autophagy. Cerebral ischemia is known to activate autophagy. However, the role and mechanism of autophagy in cerebral ischemia remain elusive (Wang et al., 2021). The influence and effect of autophagy may be dependent on the degree of ischemic injury and duration of ischemia (Sun et al., 2018; Wang et al., 2018; Wolf et al., 2019; Hou et al., 2022).

Curcumin can exert a beneficial impact by mediating autophagy, thereby inducing antitumor (Masuelli et al., 2017), anti-fibrotic (Kong et al., 2020), anti-apoptotic (Chen et al., 2021), and neuroprotective effects (Forouzanfar et al., 2020). Many studies have illustrated that curcumin attenuates cerebral ischemic injury with the involvement of autophagy. Curcumin can exert neuroprotective effects by suppressing the overactivated autophagy, with a diminished LC3-II/LC3-I ratio (Tyagi et al., 2012; Huang L. et al., 2018; Zhang et al., 2018). Conversely, other researchers hypothesize that curcumin attenuates cerebral ischemia-reperfusion injury by improving mitophagy, with an elevated LC3-II/LC3-I ratio (Wang and Xu, 2020). The difference between curcumin on autophagy may be correlated with the administration time point and dosage of curcumin, the stage of ischemic injury, and other factors. The dynamic alterations in autophagy regulated by curcumin in cerebral ischemia need to be explored in further research. Interestingly, Hou et al. (2019) identified that inhibition of autophagy caused a decrease in HIF-1α and an attenuation in HIF-1α induced autophagy suppression under OGD/R conditions, indicating the importance of the interaction of autophagy and HIF-1α underlying curcumin-induced neuroprotection in brain ischemia.

SUMMARY

Turmeric is a traditional Chinese medicine widely used in food and medicine and has been used to treat various diseases for millennia. Akin to many natural products, turmeric has a variety of biological activities with low toxicity. As a critical active component of turmeric, curcumin has been found to play a neuroprotective role in the treatment of cerebral ischemia through various mechanisms, such as antioxidant activity, anti-apoptosis, anti-inflammatory

activity, and BBB protection. However, there are unresolved questions. First, the clinical application of curcumin is challenging. At present, most of the studies are experimental by nature, and related clinical trials are limited. Although basic research has achieved favorable results, it should be noted that animals and humans have significant differences in terms of drug applications, such as drug dosage and frequency, administration route, and treatment time points. In addition, it has a strong desire to further illustrate the effectiveness, safety, and stability of curcumin in the body through clinical trials, and choose the optimal treatment strategy. Second, the effect of curcumin combined with other drugs and treatment methods should be explored to determine the potential mechanism of their synergistic effects in promoting the therapeutic effect of curcumin. Furthermore, curcumin has a wide range of therapeutic targets, making it challenging to focus on just one. Therefore, an effective strategy to maximize the efficacy of curcumin is by accelerating the development of drug delivery systems based on nanoparticles and other carriers and to carry out targeted modification in the new forms of curcumin. Last but not least, it is imperative to further deepen our understanding of the biological and pharmacological activities of curcumin. Considering that curcumin is almost insoluble in water and has a short half-life and low bioavailability, further studies are warranted to determine its application in cerebral ischemic therapy.

AUTHOR CONTRIBUTIONS

FF and ML contributed to the design of the review and revised the manuscript. FF drafted the manuscript. ML revised the manuscript. All the authors read and approved the final version of the manuscript.

SUPPLEMENTARY MATERIAL

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The Involvement of Caspases in Neuroinflammation and Neuronal Apoptosis in Chronic Pain and Potential Therapeutic Targets

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Chronic pain is a common, complex and unpleasant sensation following nerve injury, tissue trauma, inflammatory diseases, infection and cancer. It affects up to 25% of adults and is increasingly recognized as the leading cause of distress, disability and disease burden globally. Chronic pain is often refractory to most current analgesics, thus emphasizing the requirement for improved therapeutic medications. It is of great importance to elucidate the specific pathogenesis of chronic pain with different etiologies. Recent progress has advanced our understanding in the contribution of neuroinflammation and glial cells (microglia and astrocyte) activation in the plasticity of excitatory nociceptive synapses and the development of chronic pain phenotypes. Oxidative stress-associated neuronal apoptosis is also identified to be a pivotal step for central pain sensitization. The family of cysteine aspartate specific proteases (Caspases) has been well known to be key signaling molecules for inflammation and apoptosis in several neurological conditions. Recent studies have highlighted the unconventional and emerging role of caspases in microgliosis, astrocytes morphogenesis, chemokines release, cytokines secretion and neuronal apoptosis in initiating and maintaining synaptogenesis, synaptic strength and signal transduction in persistent pain hypersensitivity, suggesting the possibility of targeting caspases pathway for prevention and treatment of chronic pain. In this review, we will discuss and summarize the advances in the distinctive properties of caspases family in the pathophysiology of chronic pain, especially in neuropathic pain, inflammatory pain, cancer pain and musculoskeletal pain, with the aim to find the promising therapeutic candidates for the resolution of chronic pain to better manage patients undergoing chronic pain in clinics.

Keywords: caspase, chronic pain, neuroinflammation, neuronal apoptosis, synaptic plasticity, spinal cord

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INTRODUCTION

Pain is officially declared as "The Fifth Vital Sign" (Walid et al., 2008). Chronic pain is characterized by pain that sustains or recurs for longer than 3 months (Klein, 2015; Treede et al., 2019). Chronic pain remains to become a major medical issue which affects at least 25% of the general population and imposes a heavy financial burden to patients and healthcare systems worldwide (Gereau et al., 2014; Mills et al., 2019). It frequently presents spontaneous pain, allodynia and hyperalgesia, as a

result of nerve injury, cancer, chemotherapy, tissue trauma and inflammation (Ji et al., 2014; Ji et al., 2016; Baral et al., 2019). Patients with chronic pain also often experience insomnia, depression, anxiety and cognitive impairments, which is known to be associated with worsening pain and a serious threat to their quality of life (Moriarty et al., 2011; Tajerian et al., 2014; Tracey et al., 2019). The etiopathogenesis of chronic pain is still debated and, consequently, are the strategies for treating this condition (Ji et al., 2018; Tracey et al., 2019). Chronic pain is identified to be refractory to most analgesics currently (opioids, non-steroidal anti-inflammatory drugs and anticonvulsants) in use (Chou and Huffman, 2007; Tracey et al., 2019), thus emphasizing the urgent need for investigating the specific molecular mechanism that underlies the generation and persistence of chronic pain with different etiology.

Peripheral nociceptive sensitization (trigeminal ganglion and dorsal root ganglion, DRG) and central nociceptive sensitization (spinal cord and brain) mediated changes of neural plasticity in pain neurocircuits contributes to chronic pain phenotypes (Latremoliere and Woolf, 2009; Luo et al., 2014; Bliss et al., 2016; Han et al., 2016; Ji et al., 2018). While acute pain is an essential defensive response involving inflammation, chronic pain that is critically initiated by continuous neuroinflammation can be pathologic and maladaptive (Ji et al., 2016). Neuroinflammation involves glial cells (microglia and astrocyte) activation, chemokines (CCL1, CCL2, CCL7, CXCL1) release and pro-inflammatory mediators (TNF-α, IL-1β, IL-18, BDNF, PGE2) secretion in pain neural circuitry that, subsequently, mediates excitatory neuronal plasticity and synaptic transmission for producing and sustaining chronic inflammatory pain, chronic neuropathic pain, chronic fracture-associated pain, as well as chronic cancer pain (Zhang et al., 2013; Ji et al., 2014; Ni et al., 2019; Qiang and Yu, 2019; Wang et al., 2020b). Accumulating evidence emphasizes that oxidative stress drive neuronal apoptosis and sensitize nociceptors in the pathogenesis of chronic pain, such as chemotherapy-induced peripheral neuropathy (CIPN) and opioid-induced hyperalgesia (OIH) (Zhang et al., 2014; Shu et al., 2015; Grace et al., 2016a; Yousuf et al., 2020; Squillace and Salvemini, 2022). Nevertheless, the involvement of specific molecular signaling in neuroinflammation and neuronal apoptosis remains controversial.

Caspases are a family of conserved aspartate-specific cysteine proteases, which generally exhibits similar structures and presents in the cytoplasm in an inactive form (pro-caspases) (Graham et al., 2011). When an appropriate stimulus is given, caspases will be activated, dimerized and cleaved to form a heterotetramer, the active form of the enzyme (Van Opdenbosch and Lamkanfi, 2019). Activated caspases represent unique catalytic properties and can specifically recognize certain tetrapeptide motifs and cleave an aspartate residue in their substrates, executing programmed cell death (apoptosis) induced by a variety of injuries, including cytokines, chemokines, inflammatory damage and excitotoxicity (Van Opdenbosch and Lamkanfi, 2019). The human caspases family can be subdivided into three functional groups: apoptosis initiator caspases (Caspase-2, 8, 9, and 10), apoptosis effector caspases (Caspase-3, 6, and 7), and inflammatory caspases (Caspase-1, 4, 5, 11, and 12). Initiator caspases elicit the apoptosis signal while the effector caspases carry out the mass proteolysis that leads to apoptosis. Inflammatory caspases do not function in apoptosis but are rather involved in inflammatory signaling and other types of cell death such as pyroptosis (Julien and Wells, 2017).

The caspases have been gradually recognized as a cardinal contributor in neuroinflammatory responses and neuronal apoptosis in a wide variety of neurological and neuropsychiatric disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease, multiple sclerosis, amyotrophic lateral sclerosis, tauopathies and age-related macular degeneration (Flores et al., 2018; Kirby et al., 2019). Intriguingly, recent progress has advanced our understanding in the unconventional properties of the caspases in mediating the perception of pain. Of note, caspase-1, caspase-3 and caspase-6 are identified as key signaling molecules for nociception induction and persistence by regulating neuroinflammation, neural apoptosis and synaptic plasticity (Berta et al., 2014; Berta et al., 2017a; Gao et al., 2018) in the spinal dorsal horn (Chen et al., 2018; Ji et al., 2018). In this review, we provide a more comprehensive view on the emerging role of caspases in the mechanisms responsible for various pain states. Apart from nerve injury-induced neuropathic pain, we discuss caspases cascades in chronic inflammatory pain, cancer pain, chemotherapy-induced peripheral neuropathy and opioid-induced hyperalgesia. In particular, we summarize the latest basic and clinical advance in this field, and propose the potential therapeutic targets for the resolution of chronic pain in the clinical setting.

CASPASES AND NEUROPATHIC PAIN

Caspase-1

Neuropathic pain is primarily triggered by direct nerve trauma in the neurocircuits of peripheral and central somatosensory nervous system. Caspase-1 is the prototypical member of inflammatory caspases involved in cytokine maturation. Dysregulation of inflammasome is strongly associated with the human inflammatory diseases by the enhancement of Caspase-1 activity (Venero et al., 2013). The NOD-like receptor protein 3 (NLRP3) inflammasome are cytosolic multiprotein complexes, which consists of inactive pro-caspase-1 (Liang et al., 2022). When the recruitment of pro-caspase-1 into NLRP3 inflammasome occurs following the exposure to noxious stimuli, pro-caspase-1 will be auto-cleaved to become mature caspase-1 with high bioactivity (Liang et al., 2022). Then, caspase-1 can cleave the pro-IL-1β and pro-IL-18 to generate the activated forms IL-1β and IL-18, further mediating the extracellular secretion of IL-1β and IL-18, which facilitates the transmission of painful information (Rocha et al., 2020; Chen R. et al., 2021). Xu and his colleagues reported that mice with a chronic constriction injury (CCI) of sciatic nerve experience pain-like behaviors followed by the increase of NLRP3 and activated caspase-1 expression in neurons and astrocytes in the

TABLE 1 | Caspase-1 and its associated signaling molecules in rodent models of pain.

Pain conditions	Rodent models	Up-Regulation of signaling molecules	References
Neuropathic pain	CCI, C57BL/6 mice	Caspase-1 and NLRP3 in the spinal cord	Xu et al. (2019)
	CCI, C57BL/6 mice	Caspase-1, ASC and NLRP3 in the spinal cord	Tonkin et al. (2018)
	CCI, F344 rats	Caspase-1, DAMP, P2X7R and TLR4 in the spinal cord	Grace et al. (2018)
	CCI, F344 and SD rats	Caspase-1, DAMP, IL-1β, NLRP3, P2X7R and TLR4 in the spinal cord	Grace et al. (2016a)
	CCI, SD rats	Caspase-1, ASC, IL-1β and NALP1 in the spinal cord	Li et al. (2013)
	CCI, SD rats	Caspase-1, ASC, IL-1β, IL-18 and NLRP3 in the spinal cord	Xie et al. (2017)
	CCI, Wistar rats	Caspase-1, MMP-9, IL-1β, IL-6 and IL-18 in the spinal dorsal horn and DRG	Jurga et al. (2017)
	SCI, C57BL/6J mice	Caspase-1, IL-1β and IL-18 in the spinal cord	Qian et al. (2017)
	SNL, C57BL/6J mice	Caspase-1 and NLRP3 in spinal glial cells	Pan et al. (2018)
	SNL, SD rats	Caspase-1 in DRG	Zhang et al. (2015b)
	SNL, SD rats	Cleaved caspase-1, ASC, IL-1β, IL-18, NF-κB, NLRP3 and TNF-α in the spinal cord horn	Wang et al. (2021a)
	CIPN: oxaliplatin, SD rats, C57BL/6 mice	Caspase-1, IL-1β and NLRP3 in the spinal dorsal horn	Wahlman et al. (2018)
	CIPN: oxaliplatin and paclitaxel, C57BL/6 mice	Caspase-1, ASC and NLRP3 in the spinal cord	Tonkin et al. (2018)
	CIPN: oxaliplatin, Swiss mice and C67BL/6 mice	Caspase-1, IL-1β, *GFAP* mRNA and TNF-α in the spinal cord	Agnes et al. (2021)
	CIPN: paclitaxel, SD rats	Caspase-1, IL-1β and NLRP3 in DRG and sciatic nerve	Jia et al. (2017)
Inflammatory pain	Carrageenin injection, C57BL/6 mice	Caspase-1, IL-1β maturation, COX-2 and PGE2 in paw skins	Cunha et al. (2010)
	CFA or ceramide injection, C67BL/6 mice	Caspase-1, IL-1β and NLRP2 in DRG; caspase-1 and NLRP3 in spinal dorsal horn neurons	Matsuoka et al. (2019)
	CFA, SD rats and CB2 receptors KO mice	Caspase-1, ACS, IL-1β and NLRP3 in the skin tissue	Gao et al. (2018)
	Hindpaw incision, C57BL/6 mice	Caspase-1 near the wounds	Liang et al. (2010)
	LPS, Balb/c mice	Caspase-1, ASC, IL-1β and NLRC4 in the brain and spinal cord	Cagli et al. (2021)
	LPS, Wistar rats	Caspase-1, ASC, IL-1β and p-P38 in the spinal cord	Clark et al. (2006)
	MIA knee injection, SD rats	Caspase-1, ASC, IL-1β, IL-18 and NLRP3 in fibroblast-like synoviocytes	Ma et al. (2020)
Cancer pain	Walker 256 cells injection in tibial cavity, SD rats	Caspase-1, ASC and NLRP3 in the spinal cord	Chen et al. (2019)
Postoperative pain	Thoracotomy, SD rats	Caspase-1, IL-1β, IL-6, TLR4, TNF-α and in the spinal dorsal horn	Hu et al. (2020)
	Laparotomy, SD rats	Caspase-1, IL-1β, NF-κB, NLRP3, TLR4 and TNF in the spinal dorsal horn	Grace et al. (2019)

Abbreviations: ASC, apoptosis-associated speck-like protein containing a Caspase activation and recruitment domain; CB2, cannabinoid receptor type 2; CCI, chronic constriction injury; CFA, complete Freund's adjuvant; CIPN, chemotherapy induced neuropathic pain; CPTP, chronic post-thoracotomy pain; COX-2, cyclooxygenase-2; DAMP, damage associated molecular patterns; DRG, dorsal root ganglion; GFAP, Glial fibrillary acidic protein; IL-1β, interleukin-1β; KO, knockout; LPS, lipopolysaccharide; MIA, monosodium iodoacetate; MMP-9, matrix metalloproteinase-9; NALP1, NACHT leucine-rich-repeat protein 1; NF-κB, nuclear factor-kappa-B; NLRC4, NOD-like receptor C4; NLRP3, NOD-like receptor protein 3; P2X7R, P2X7 receptor; PGE2, Prostaglandin E2; SCI, spinal cord injury; SD rats, Sprague Dawley rat; SNL, sciatic nerve ligation; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-alpha.

superficial dorsal horn of spinal cord, and that pharmacological inhibition of caspase-1 activation attenuates CCI-induced mechanical allodynia and thermal hyperalgesia (Xu et al., 2019). Moreover, Grace et al. (2018) demonstrated that morphine treatment after CCI-induced peripheral nerve injury results in persistent damage associated molecular patterns (DAMP) release, which is critical for formation/activation of spinal caspase-1-dependent NLRP3 inflammasomes, thus causing the extension of CCI induced neuropathic allodynia, whereas inhibition of caspase-1, or IL-1β in the spinal dorsal horn reversed prolonged allodynia. Additionally, morphine induces the phosphorylation of p38, and up-modulates the expressions of Nuclear Factor-κB (NF-κB) subunit P65, toll-like receptor 4 (TLR4) and ionotropic P2X receptors (P2X4 and P2X7) in spinal microglia in CCI rats (Grace et al., 2016b). These alternations are involved in the activation of caspase-1 and the secretion of downstream cytokine IL-1β, which mediates the long-term increase of activity and excitability in nociceptive sensory neurons, and results in the production and persistence of chronic neuropathic pain. In addition to the CCI model, NLRP3 and activated caspase-1 expression were also significantly elevated in spinal glial cells of mice with partial sciatic nerve ligation (pSNL)-induced neuropathic pain (Pan et al., 2018). Meanwhile, the synthesis of caspase-1 in DRG of SNL rats is also increased (Zhang Y. et al., 2015), suggesting that activation of caspase-1 in peripheral nervous system is the pathophysiological basis for pronociceptive hypersensitivity in chronic neuropathic pain. Furthermore, Qian and his colleagues manifest that spinal suppression of caspase-1 activation can effectively reduce the synthesis of cytokines IL-1β and IL-18 and ameliorate mechanical allodynia phenomena in a model of neuropathic pain with spinal cord injury (SCI) (Qian et al., 2017).

In addition to neuropathic pain induced by nerve injury, many antineoplastic agents are capable of eliciting CIPN, which is characterized by mechanical allodynia, a pain evoked by non-nociceptive stimuli such as light touch (Shim et al., 2019). Repetitive injections of intraperitoneal paclitaxel generate and sustain long-lasting CIPN in rats, which is accompanied by NLRP3 over-expression, caspase-1 activation and IL-1β

TABLE 2 | Caspase-3 and its associated signaling molecules in rodent models of pain.

Pain conditions	Rodent models	Up-Regulation of signaling molecules	References
Neuropathic pain	CCI, Kunming mice	Caspase-3, calpain-1 in the neurons of spinal cord	Yang et al. (2018)
	CCI, SD rats	Caspase-3 and GAP-43 in the spinal cord	Wu et al. (2012)
	CCI, SD rats	Caspase-3 and TNF-α in the spinal cord	Hu et al. (2015)
	CCI, SD rats	Cytochrome-C-positive neurons and cleaved caspase-3-positive neurons in the spinal cord	Fu et al. (2017)
	CCI, SD rats	Caspase 3 and HIF-1α in the spinal cord	Mo et al. (2018)
	CPN, C57BL/6 mice	Caspase 3 in the ACC	Wang et al. (2020c)
	SCI, SD rats	Caspase 3, caspase-8, IL-1β and IL-18 in the spinal cord	Turtle et al. (2017)
	SCI, SD rats	Caspase 3, CD68(+), GFAP, iNOS, MDA, NMDAR1, 3-NT, TNF-α in the spinal cord	Lv et al. (2017)
	SCI, SD rats	Caspase-3 mRNA, Bcl-2-associated X protein, COX-2, interleukins, iNOS and TNF-α in the spinal cord	Cui et al. (2021a)
	SCI, Wistar rats	Caspase 3 in the spinal cord	Hajimashhadi et al. (2017)
	SNL, Wistar rats	Caspase-3, ATF-3 and anoctamin-1in DRG	García et al. (2018)
	PSNL, albino mice	Caspase-3, COX-2, IL-1β, IL-6, iNOS, TNF-α in the spinal cord	Khan et al. (2021)
	CIPN: paclitaxel, SD rats	Caspase-3 in DRG	Choi et al. (2013)
	CIPN: paclitaxel, C57BL/6 rats	Caspase 3 and RhoA in DRG	Chine et al. (2019)
	CIPN: paclitaxel, Wistar rats	Caspase 3, NF-kB p65, TNF-α in the sciatic nerve	Al-Massri et al. (2018)
	STZ-induced diabetes, SD rats	Caspase-3, hydroperoxides, lipid peroxides, NOX2 and NOX4 in the sciatic nerve	Ji et al. (2017)
	STZ-induced diabetes, SD rats	Caspase 3, AGE and BAX in the sciatic nerve tissue	Yu et al. (2021)
	STZ-induced diabetes, SD rats	Caspase 3, CX3CL1 in DRG, p38 MAPK in macrophage	Huang et al. (2014)
	STZ-induced diabetes, Wistar rats	Caspase-3 and the Bax/Bcl-2 ratio in the spinal cord	Rasoulian et al. (2019)
inflammatory pain	CFA, C57BL/6 mice	Caspase 3, BAX, NF-KB, NMDAR, TNF-α, P38 phosphorylation in the anterior cingulate cortex	Fan et al. (2018)
Cancer pain	Walker 256 cell intraperitoneal injection, SD rats	Cleaved caspase-3, ATF6, GRP78, p-IRE1 and p-PERK in the spinal cord	He et al. (2019)
	Walker 256 cell injection in tibia cavity, SD rats	Caspase-3, Iba-1, and the mRNA levels of IL-1β, TNF-α and IL-6 in CSF-CN	Chen et al. (2021a)
	MRMT-1 cell injection in tibia cavity, SD rats	Cleaved caspase-3, Bcl-2/BAX ratio and Drp1 in the spinal cord	Li et al. (2019a)
Musculoskeletal pain	Tibial fractures	Caspase-3 and LRRTM1 in the spinal dorsal horn	Zhang et al. (2020)

Abbreviations: ACC, anterior cingulate cortex; BAX, B-cell lymphoma 2-associated X apoptosis regulator; Bcl-2, B-cell lymphoma-2; CCI, chronic constriction injury; CD68(+), CD68-positive cells; CIPN, chemotherapy induced neuropathic pain; COX-2, cyclooxygenases-2; CPN, common peroneal nerve ligation; CSF-CN, cerebrospinal fluid-contacting neurons; CX3CL1, chemokine (C-X3-C motif) ligand 1; DRG, dorsal root ganglion; GAP-43, growth associated protein-43; GFAP, glial fibrillary acidic protein; GRP78, glucose regulatory protein 78; HIF-1α, hypoxia inducible factor-1α; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; LRRTM, leucine-rich repeat transmembrane neuronal protein; MDA, malondialdehyde; NF-κB, nuclear factor-kappa-B; NMDAR, N-methyl-d-aspartate receptor; 3-NT, 3-nitrotyrosine; pSNL, partial sciatic nerve ligation; p-IRE1, phosphorylated inositol-requiring enzyme-1; p38 MAPK, p38 mitogen-activated protein kinase; NOX2, NADPH oxidases 2; p-PERK, phosphorylated protein kinase RNA-like endoplasmic reticulum kinase; SCI, spinal cord injury; SNL, sciatic nerve ligation; STZ, streptozotocin; TNF-α, tumor necrosis factor-α.

secretion in DRG and sciatic nerve at 3 weeks after paclitaxel application. Furthermore, inhibition of caspase-1 activity reduces the occurrence of CIPN and alleviates the severity of CIPN (Jia et al., 2017). Simultaneously, up-regulations of caspase-1 and NLRP3 in the spinal dorsal horn were verified in oxaliplatin-associated CIPN in rats (Wahlman et al., 2018). The aforementioned findings identified that inhibition of caspase-1-dependent neuroinflammation may offer a novel therapeutic strategy for neuropathic pain control (**Table 1**).

Caspase-3

Caspase-3 is known as an executioner caspase in apoptosis because of its potent role in coordinating the destruction of cellular structures such as DNA fragmentation or degradation of cytoskeletal proteins (McIlwain et al., 2015). Caspase-3 is one of the key indicators of apoptosis, and studies in the field of chronic pain with caspase-3 are still in its exploratory stage. Wu et al. (2012) elucidated that sciatic nerve injury induced by the CCI model not only initiates chronic neuropathic pain, but also increases the expression of caspase-3 in the spinal cord and caspase-3-dependent apoptosis of dorsal horn neurons, which is associated with up-regulation of growth associated protein 43 (GAP-43) expression. Behavioral results also indicate that inhibition of caspase-3 activity by both pharmacological therapy with caspase-3 inhibitor Z-DEVD-FMK and caspase-3 knockdown attenuates the thermal hyperalgesia in CCI rats (Wu et al., 2012). Li et al. also found that microRNA-212-3p controls peripheral neuropathic allodynia and sodium voltage-gated channel alpha subunit 3 (Nav 1.3) through inhibiting caspase-3 cleavage and B-cell lymphoma 2-associated X apoptosis regulator (BAX) expression in rats with CCI surgery (Li Y. et al., 2019). EphrinB/EphB signaling, the most important

TABLE 3 | Caspase-6 and its associated signaling molecules in rodent models of pain.

Pain conditions	Rodent models	Up-Regulation of signaling molecules	References
Neuropathic pain	SNI, SD rats	Caspase-6 in DRG	Berta et al. (2016)
	CIPN: paclitaxel, C57BL/6 mice, *Casp6* $^{-/-}$mice	Caspase-6 in DRG	Berta et al. (2016)
Inflammatory pain	Formalin, CFA, C57BL/6 mice, *Casp6* $^{-/-}$mice	Caspase-6, TNF-α in the spinal cord	Berta et al. (2014)
Musculoskeletal pain	Tibial fracture C57BL/6 mice	Caspase-6, AMPAR-induced current in dorsal horn neurons, GluA1-containing AMPAR trafficking, netrin-1 release, spine density in spinal cord	Cui et al. (2021b)
Opioid-induced hyperalgesia	Remifentanil SD rats	Caspase-6, CCL21, CXCR3 in spinal cord	Wang et al. (2020a)

Abbreviations: AMPAR, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; CCL21=C-C Motif Chemokine Ligand 21; CIPN, chemotherapy induced neuropathic pain; CXCR3, C-X-C Motif Chemokine Receptor 3; DRG, dorsal root ganglion; SNI, spared nerve injury; TNF-α, tumor necrosis factor-α

subfamily of receptor tyrosine kinases (RTKs) in humans, is one of pivotal cascades in the spinal pain processing and nociceptive synaptic plasticity (Deng et al., 2017; Peng et al., 2019). Recent evidence discloses that caspase-3 activation in the spinal dorsal horn neurons but not microglia and astrocyte is implicated in EphrinB/EphB signaling dependent neuropathic pain formation in a mouse model of CCI (Yang et al., 2018) (**Table 2**).

Furthermore, spinal caspase-3 cleavage is required for axonal degeneration, mitochondrial dysfunction, oxidative stress and apoptosis in the pathogenesis of CIPN after intraperitoneal vincristine stimulation in mice (Chen et al., 2020). The over-expression of apoptosis-related proteins of BAX, BCL2, and caspase-3 in the sciatic nerve is reported in streptozotocin (STZ)-induced diabetic peripheral neuropathy in rats (Yu et al., 2021). Blocking caspase-3 signaling cascades can reduce spinal neuronal apoptosis and down-regulate nociceptor hyper-responsiveness, which may emerge as a promising strategy for the treatment of neuropathic pain. Conversely, peripheral nerve injury induced by common peroneal nerve (CPN) ligation blocks long-term depression (LTD) induction and caspase-3 expression in the anterior cingulate cortex (ACC). Electrophysiological and behavioral tests found that disrupting the link between caspase-3 and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor in the ACC inhibits LTD expression and induces peripheral pro-nociception phenotypes (Wang YJ. et al., 2020). Restoration of LTD through caspase-3 accumulation in the ACC relieves persistent mechanical allodynia (Wang YJ. et al., 2020), which may be used as a promising therapeutic approach for the management of chronic pain (**Table 2**). Acetyl-l-carnitine (ALCAR) is a short chain ester of carnitine L-isomer, which is the predominant acyl-carnitine and is involved in the redox reactions to eliminate reactive oxygen species, and finally, can increase acetylcholine levels, thus having neuroprotective action. Di Cesare Mannelli et al. (2007) found the decreased effect of ALCAR on the induction of apoptosis, the release of cytosolic cytochrome C, the activation of caspase-3, and the fragmentation of the genome in CCI rats. And that means ALCAR may be an agent suitable for clinical use in the prevention of nervous tissue cell death after peripheral nerve trauma *via* the inhibition by caspase-3. Di Cesare Mannelli et al. (2013) also found that antioxidants (such as silibinin and α-tocopherol) can ameliorate the symptoms of neuropathy and protect astrocytes from caspase-3-dependent apoptotic signaling activation in oxaliplatin-treated rats.

Caspase-6

Caspase-6 is also tightly associated with the pathophysiology of neuropathic pain. Peripheral nerve injury after CCI intervention is demonstrated to cause the release of activating transcription factor-3 (ATF3) and caspase-6 from axonal terminal, which then acts on microglia to trigger their activation. After microglial activation, p38 will be phosphorylated to further induce TNF-α release, and brain-derived neuro-trophic factor (BDNF) expression, which inducing central sensitization and supporting the transition from acute pain to chronic pain (Berta et al., 2016). Caspase-6 inhibition significantly reverse the development of mechanical allodynia in a rat model of neuropathic pain following spared nerve injury (SNI) of sciatic nerve. Moreover, caspase-6 deletion attenuated behavioral mechanical allodynia in the paclitaxel-induced CIPN, although the mechanisms that produce neuropathic pain after exposure to chemotherapeutics may be fundamentally different from those operating after nerve injury (Berta et al., 2017b). (**Table 3**)

Other Caspases

In recent years, in addition to caspase-1, caspase-3 and caspase-6, other caspases have gained great emphasis on neuropathic pain syndromes. Specifically, increase of caspase-9, apoptosis, mitochondrial reactive free oxygen species (fROS), lipid peroxidation, glutathione (GSH), transient receptor potential vanilloid-1 (TRPV1) current density, and calcium concentrations in the DRG and hippocampus was detected in STZ-induced diabetic peripheral mechanical allodynia and thermal hyperalgesia (Düzova et al., 2021). Application of metabotropic glutamate receptor 1 (mGluR1) antagonist can prevent CCI-induced neuropathic pain by reducing the synthesis of caspase-7 in the spinal dorsal horn and inhibiting the process of caspase-7 dependent neuronal apoptosis

(Siniscalco et al., 2008). However, whether caspase-9 and caspase-7 inhibitors can provide definitive relief of chronic pain is a scientific question that requires to be addressed.

CASPASES AND INFLAMMATORY PAIN

Caspase-1

Inflammatory pain is evoked by inflammation-associated stimuli and is often established in animal models using wound incision or injection of inflammatory chemical irritants, such as complete Freund's adjuvant (CFA), carrageenan or lipopolysaccharides (LPS) (Pan et al., 2021). Recent literatures have demonstrated that intraplantar administration of complete Freund's adjuvant (CFA) or ceramide not only down-regulates paw withdrawal mechanical threshold and paw withdrawal thermal latency, but also up-regulates the expression of the NOD-like receptor protein 2 (NLRP2)/caspase-1/IL-1β in small-sized DRG primary sensory neurons and the generation of NLRP3/caspase-1 in spinal dorsal horn neurons (Matsuoka et al., 2019; Hua et al., 2022). Intrathecal injection of a caspase-1 inhibitor Z-YVAD-FMK impairs CFA-induced inflammatory pain hypersensitivity through inhibiting IL-1β secretion in DRG (Matsuoka et al., 2019). Liang et al. also found, in the mouse model of hind paw incision, that caspase-1 activity was significantly increased in peri-incisional tissues. Caspase-1 inhibitor VRTXSD727 significantly reverses mechanical allodynia and thermal hyperalgesia, and reduces the synthesis and release of macrophage inflammatory protein-1α (MIP-1α), granulocyte-colony stimulating factor (G-CSF), Prostaglandin E2 (PGE2), as well as IL-1β around the wound incision (Liang et al., 2010). Additionally, caspase-1 knockout mice exhibit the impairment in the mechanical allodynia induced by intraplantar exposure to carrageenin, TNF-a and exogenous CXCL1, respectively. Meanwhile, caspase-1 deficiency suppresses carrageenin-induced PGE2 production, IL-1β maturation and cyclooxygenase-2 (COX-2) accumulation (Cunha et al., 2010). These detailed evidences strongly suggest the importance of caspase-1-dependent inflammatory cascades in the pathophysiology of inflammatory hyper-nociception (**Table 1**).

Caspase-3

Caspase-3 plays an important role in inflammatory pain. N-methyl-d-aspartate (NMDA) receptor, an ionotropic glutaminergic receptor, consists of the primary GluN1 subunit and one or more GluN2A-D modulatory subunits (Zhang et al., 2014). Activation of NMDA receptor is a leading determinant in the hyper-excitability of nociceptive neurons and central synaptic plasticity underlying pain-associated syndromes (Chen et al., 2016; Xu et al., 2020). The excitotoxicity of NMDA receptor is GluN2B dependent (Zhang et al., 2021). Notably, CFA injection into hind paw can not only aggravate neuronal apoptosis but also increase the expressions of Bax, caspase-3 and GluN2B-containing NMDA receptors in the ACC. Inhibiting caspase-3-dependent cascades protects neuronal survival, reduces GluN2B-containing NMDA receptor electrophysiological function and attenuates chronic inflammation-induced mechanical allodynia and thermal hyperalgesia (Fan et al., 2018) (**Table 2**).

Caspase-6

In 2014, a preclinical study by Berta et al. showed that caspase-6 is specifically expressed in C-fiber axonal terminals in the superficial spinal cord dorsal horn, and co-localizes with calcitonin-gene-related peptide (CGRP), suggesting the transportation of caspase-6 in peptidergic primary afferents to spinal central terminals, which sustaining nociception-related synaptic potency. Moreover, injections of formalin or bradykinin into the plantar induce the cleavage and activation of caspase-6 in nociceptive neurons of spinal dorsal horn. Spinal application of specific caspase-6 inhibitor Z-VEID-FMK or caspase-6 neutralizing antibody or delivery of caspase-6 siRNA around the sciatic nerve can effectively relieve the inflammatory pain induced by formalin intervention. Similarly, caspase-6 gene knockout reduces bradykinin-induced spontaneous pain, CFA-induced mechanical allodynia, and carrageenan-induced heat hyperalgesia, respectively. In addition, spinal exposure to recombinant caspase-6 not only facilitates microgliosis and microglial activation to result in TNF-α secretion, but also increases glutamate release from primary afferent terminals to enhance excitatory postsynaptic currents (Berta et al., 2014). These detailed results identify that caspase-6 activation may be a predominant feature of neuroinflammation and neuron-microglia interaction, as well as a key driver of synaptic plasticity and central sensitization, thereby mediating persistent inflammatory pain. Thus, targeting the caspase-6/TNF-α cascades may offer a novel choice for treating inflammatory pain states by microglial and synaptic modulation.

Other Caspases

In addition, recent report recapitulates the elevated concentration of caspase-11, NOD-like receptor C4 (NLRC4), ASC, and IL-1β in the brain and spinal cord of mice with lipopolysaccharide (LPS)-induced inflammatory heat hyperalgesia (Cagli et al., 2021), suggesting the implication of caspase-11-dependent NLRC4 inflammasome in pain perception.

CASPASES AND CANCER PAIN

Caspase-1

Cancer pain is also an important category of chronic pain and have the distinctive characteristic of both neuropathic pain and inflammatory pain processing (Wang K. et al., 2020; Wang K. et al., 2021). Mounting evidence reveal that the expression of NLRP3 inflammasome, including NLRP3, apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), and caspase-1, were significantly increased in a time-dependent manner in bone cancer pain (Chen et al., 2019). Behavioral tests confirmed that both single and repetitive treatment with NLRP3 inhibitor MCC950 markedly attenuated cancer pain behaviors (Chen et al., 2019), suggesting that the activation of NLRP3/ASC/Caspase-1 signaling cascades is an essential step for the initiation and maintenance of central pain sensitization following bone cancer (**Table 1**).

Caspase-3

Bioactivity of endoplasmic reticulum (ER) is vital for life yet toxic if dysfunction of ER occurs. Especially, ER stress plays a positive role in acute pain perception and chronic pain sensitization (Zhang E. et al., 2015; Inceoglu et al., 2015). Bone cancer pain not only induces bone destruction and unbearable mechanical allodynia, but also up-regulates the spinal expression of ER stress markers, including glucose-regulated protein 78 (GRP78), activating transcription factor-6 (ATF6), phosphorylated protein kinase RNA-like endoplasmic reticulum kinase (p-PERK), phosphorylated inositol-requiring enzyme-1 (p-IRE1) and cleaved caspase-3. Intrathecal blockage of ER stress impairs caspase-3 cleavage-dependent apoptosis in dorsal horn neurons and relieves bone cancer pain. More importantly, spinal therapy with a specific caspase-3 inhibitor Z-DEVD-FMK is enough and effective against bone cancer pain (He et al., 2019). Also, the modulation of Bax overload and caspase-3 cleavage in mitochondrial fission and apoptosis in bone cancer pain is confirmed by other research teams (Li MY. et al., 2019; Chen P. et al., 2021). Overall, this suggests that preventing ER stress-induced cellular dysfunction and caspase-3-dependent neuronal apoptosis may be a new approach for treating cancer pain (**Table 2**).

CASPASES AND MUSCULOSKELETAL PAIN

Caspase-3

Musculoskeletal pain refers to pain in the muscles, bones, ligaments, tendons, and nerves. Chronic musculoskeletal pain patients in general show signs of peripheral/central sensitization. Dynamic recruitment of GluA1-containing AMPAR at spinal synapses contributes to central sensitization underlying pain-associated syndromes (Luo et al., 2014; Zhang et al., 2018; Wang Z. et al., 2020; Liu et al., 2020). Leucine-rich repeat transmembrane protein 1 (LRRTM1) is demonstrated to mediate post-synaptic translocation of AMPA receptor and synaptogenesis (Bhouri et al., 2018; Schroeder et al., 2018). But the regulation of LRRTM1 in pain development remains underestimated. We recently revealed that tibial fracture with intramedullary pinning causes long-lasting mechanical allodynia and cold allodynia after orthopedic surgery in mice, along with the upregulated caspase-3 activity (but not caspase-3 protein expression) and LRRTM1 expression in spinal dorsal horn (Zhang et al., 2020). Pharmacological intervention with caspase-3 specific inhibitor Z-DEVD-FMK reduces fracture-associated behavioral pain and LRRTM1-mediated alterations in excitatory synaptic plasticity. Spinal exposure to recombinant caspase-3 evoked acute pain phenotypes and spinal LRRTM1 over-expression is reversed by LRRTM1 deficiency (Zhang et al., 2020). Collectively, this demonstrates the tight interaction between caspase-3 and LRRTM1 in inducing AMPA receptor trafficking and chronic central sensitization, ultimately regulating the formation and maintenance of fracture-associated pain. Sure, it will be of great interest to investigate how fracture trauma regulates caspase-3 activation and thus mediates the onset of chronic pain (**Table 2**).

Caspase-6

Our latest study provides several lines of evidence to confirm the requirement of caspase-6 in musculoskeletal pain induced by tibial fracture with intramedullary pinning (Cui W. et al., 2021). First, behavioral tests showed that tibial fractures after orthopedic operation initiate and persist postsurgical mechanical allodynia and cold allodynia, which is detectable on 3 days, peaks on 7–14 days, and sustains for at least 21 days. Second, biochemical tests found that tibial fracture up-regulates spinal active caspase-6 activity, GluA1-containing AMPA receptor trafficking, spine density in dorsal horn neurons. Third, spinal delivery of specific caspase-6 inhibitor Z-VEID-FMK and caspase-6 neutralizing antibody is sufficient to reduce the recruitment of GluA1-containing AMPA receptor at synapses and the amounts of mushroom spines, thereby attenuating fracture-associated chronic pain. Fourth, electrophysiological tests manifested that pharmacological inhibition of caspase-6 blocks AMPA receptor-mediated excitatory post-synaptic currents in the dorsal horn neurons in fracture animals (Cui W. et al., 2021). These above-mentioned results demonstrate that caspase-6-mediated changes in excitatory synaptic structure and functional plasticity is an important mechanism for the formation and maintenance of spinal nociception sensitization after fracture and orthopedic surgery, which provides a promising approach for chronic fracture pain therapy. However, there are several outstanding questions regarding how caspase-6 mediates AMPA receptor post-synaptic trafficking and ultimately triggers musculoskeletal pain. Simultaneously, in addition to the mechanism of affecting receptor transport, future researches are warranted to explore whether there are epigenetic regulations that interfere with the expression of glutamate receptors.

CASPASES AND POSTOPERATIVE PAIN

The requirement of caspase-1 for postoperative pain development has also been clarified. Extension of laparotomy-associated postoperative pain after morphine treatment is attributed to up-regulation of inflammatory genes, including those encoding caspase-1, NLRP3, TLR4, NF-κB, IL-1β, and TNF-α (Grace et al., 2019). Thoracotomy induces persistent postoperative behavioral pain, along with the spinal up-modulation of caspase-1 and TLR4 co-localization in dorsal horn neurons (Hu et al., 2020). The decrease of mechanical withdrawal threshold is attributed to caspase-1-dependent microglial activation and the overload of inflammatory mediators (TNF-α, IL-6, and IL-1β) in spinal dorsal horn. Additionally, the alleviation of postoperative mechanical allodynia by intrathecal therapy of caspase-1 inhibitor (Ac-YVAD-CMK) is reversed after TLR4 agonist treatment (Hu et al., 2020), further indicating the tight interaction between caspase-1 and TLR4 in spinal nociception transduction and central sensitization (**Table 1**).

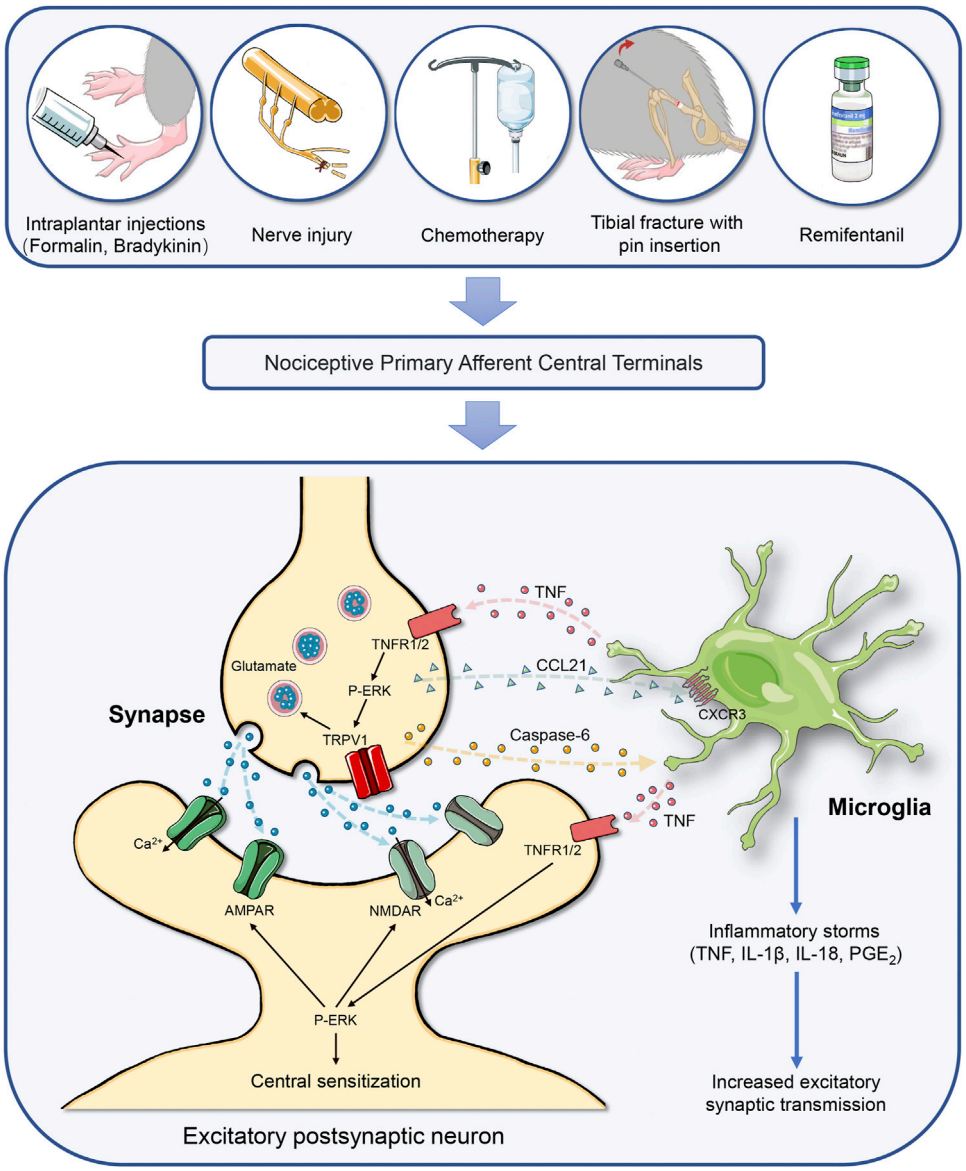

FIGURE 1 | Caspase-6 cascades-mediated neuron-microglia interaction in the spinal cord initiates chronic pain through neuroinflammation and central nociception sensitization. Chronic pain is categorized as inflammatory pain, neuropathic pain, cancer pain, musculoskeletal pain, and drug treatment-induced pain, which is driven by neuroinflammation in the spinal cord dorsal horn. Painful insults, which includes peripheral tissue inflammation, nerve trauma, fracture with orthopedic surgery, chemotherapy and opioids treatment, result in the hyperexcitability of primary sensory neurons and trigger the release of caspase-6 from the central terminals of primary nociceptive afferents, which causing microgliosis and microglia activation, and subsequent microglial TNF-α secretion. Then, the interaction between TNF-α and TNFR at presynaptic sites causes the release of glutamate *via* ERK and TRPV1 pathway. Activation of TNFR at postsynaptic neurons also facilitates the phosphorylation of ERK, which drives central sensitization via positive modulations of NMDAR and AMPAR and subsequent intracellular calcium influx. Simultaneously, caspase-6 cleavage promotes chemokine CCL21 release from presynaptic neurons, which elicits microglia activation via acting on its specific receptor CXCR3. Microglia activation further increases the secretion of the pro-inflammatory mediators (IL-1β, IL-18, and PGE2). These regulations of excitatory synaptic transmission by microglial mediators at pre-synaptic, post-synaptic, and extra-synaptic sites drive central sensitization in the nociception circuits, leading to the development of chronic pain. Abbreviations: AMPAR, a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; CCL21, chemokine (C-C motif) ligand 21; CXCR3, chemokine (C-X-C motif) receptor 3; ERK, extracellular signal-regulated kinase; IL-1β, interleukin-1β; IL-18, interleukin-18; NMDAR, N-methyl-D-aspartate receptor; p-ERK, ERK phosphorylation; PGE2, prostaglandin E2; TNF-α, tumor necrosis factor-α; TNFR, tumor necrosis factor receptor; TRPV1: transient receptor potential vanilloid-1.

CASPASES AND OPIOID-INDUCED HYPERALGESIA

Remifentanil is a potent short-acting μ-opioid agonist, regarded as an important component of balanced anesthesia in the clinical setting. Unfortunately, the intraoperative exposure to remifentanil elicits behavioral OIH phenotype in animals and patients (Zhang et al., 2017; Zhang et al., 2018; Li et al., 2021). Population-based studies also found that remifentanil can elevate peripheral mechanical nociceptive sensitivity, elicit

TABLE 4 | The inhibitors targeting caspases cascades in chronic pain treatment in rodents.

Target	Inhibitors	Administration route	Rodent models of pain	References
Caspase-1	Z-YVAD-FMK	Intrathecal injection	CFA, C67BL/6 mice	Matsuoka et al. (2019)
		Intrathecal injection	LPS, Wistar rats	Clark et al. (2006)
	Ac-YVAD-CMK	Intrathecal injection	Thoracotomy, SD rats	Hu et al. (2020)
		Intraplantar injection	Hind-paw incision, C57BL/6 mice	Liang et al. (2010)
	VRTXSD727	Oral gavage	Hind-paw incision, C57BL/6 mice	Liang et al. (2010)
	VX-765	Intraperitoneal injection	SNL, SD rats	Wang et al. (2021b)
Caspase-3	Z-DEVD-FMK	Intrathecal injection	CCI, SD rats	Wu et al. (2012)
		Intrathecal injection	Walker 256 cell intraperitoneal injection, SD rats	He et al. (2019)
		Intrathecal injection	Tibial fracture, C57BL/6 mice	Zhang et al. (2020)
Caspase-6	Z-VEID-FMK	Intrathecal injection	Formalin, C57BL/6 mice	Berta et al. (2014)
		Intrathecal injection	Formalin, CD1 mice	Chen et al. (2018a)
		Intrathecal injection	SNI, SD rats	Berta et al. (2017b)
		Intrathecal injection	Tibial fracture, C57BL/6 mice	Cui et al. (2021b)
		Intrathecal injection	Remifentanil infusion, SD rats	Wang et al. (2020a)

Abbreviations: CCI, chronic constriction injury; CFA, complete Freund's adjuvant; CPN common peroneal nerve; LPS, lipopolysaccharide; SD rats, Sprague Dawley rat; SNI, spared nerve injury; SNL, sciatic nerve ligation.

hyperalgesia area around the wound incision, and trigger OIH, even leading to chronicity of postoperative pain (Fletcher and Martinez, 2014; Zhang et al., 2016). Chemokines and their receptors-associated neuroinflammation is essential for OIH generation (Yang et al., 2016; Zhu et al., 2017). Wang et al. (2020a) confirmed that remifentanil infusion causes mechanical allodynia and thermal hyperalgesia, along with the spinal increase in the cleavage of caspase-6, the release of CCL21 in neurons and the expression of CXCR3 in microglia. Spinal inhibition of caspase-6 activation ameliorates OIH behavior and spinal CCL21/CXCR3 accumulation. Exogenous caspase-6 also evokes acute mechanical pain and represents microglial activation, which is impaired after spinal CCL21 neutralization. This suggests the contribution of caspase-6 in CCL21 signaling in chronic pain perception. However, the downstream target molecules through which caspase-6 upregulates the synthesis of CCL21 need to be further investigated. As a result, interactions of microglia-neurons are triggered by caspase-6 activation in synaptic plasticity and the formation and maintenance of chronic pain, as the potential pain circuits in the spinal cord dorsal horn is shown in **Figure 1**.

SPECIFIC CASPASES INHIBITORS AS A POTENTIAL CANDIDATE FOR CHRONIC PAIN TREATMENT

Despite decades of clinical investigation and medical advancement, current approaches for chronic pain-relief are still limited (Tracey et al., 2019). Non-steroidal anti-inflammatory drugs and acetaminophen must be cautiously administered in patients with gastrointestinal diseases, renal dysfunction and hepatic insufficiency (Bindu et al., 2020; Ishitsuka et al., 2020). Tricyclic antidepressants, norepinephrine reuptake inhibitors, NMDA receptor antagonists and α_2-δ anticonvulsants are only partially beneficial to neuropathic pain and several dose-limiting adverse-effects including sedation, somnolence and dizziness may block their practical utilization (Staahl et al., 2009; Thompson et al., 2019). Opioids, as 1st-line analgesics, frequently cause constipation, nausea, addiction, tolerance and hyperalgesia (Zhang et al., 2016; Imam et al., 2018; Colvin et al., 2019). Thus, alternative agents for pain control are urgently required. In view of the pivotal role of caspases in the pathogenesis of chronic pain, their targeting agents in the management of chronic pain have been identified as above-mentioned. The summarization and detail on therapeutic value of these drugs are shown in **Table 4**.

SUMMARY

We have summarized the role of the caspases in the development of chronic pain with different etiologies, with a view to providing new ideas for the management of chronic pain. Although significant progress has been made in preclinical study, most of the studies have only been conducted at the behavioral level, and the upstream and downstream molecular mechanisms have been poorly investigated, so there are plenty of issues that still need to be addressed. Future research should include the following directions: 1) high selective inhibitors developed for caspase-1, caspase-3 or caspase-6 can release chronic pain by inhibiting neuroinflammation, altered excitatory synaptic plasticity or neuronal apoptosis in animal models; however, whether these inhibitors can alleviate chronic pain in patients and be safely utilized in clinics remains to be explored. 2) In addition to caspases-1, 3, 6, 7, 9, and 11, it has not been conclusively established whether other members of the caspase family are also involved in the regulation of chronic pain. 3) most studies on the role of caspases in the occurrence and development of chronic pain were conducted in male animals. Considering the sex differences in the formation mechanism of chronic pain (Chen et al., 2018; Luo et al., 2018; Luo et al., 2019a; Luo et al., 2019b; Luo et al., 2021), future research should emphasize whether the caspase signaling is involved in the

formation of chronic pain in female animals. 4) Despite recent advances in pain therapy, visceral pain remains poorly understood. Recent study confirms that antibiotic-induced microbial changes resulted in neuro-immune responses and visceral pain attenuation in wild type but not in caspase-1/11 knockout mice (Aguilera et al., 2021), supported the notion of the inflammasome as a promising therapeutic target in the visceral pain. Therefore, further study of caspase family and visceral pain will be a promising field. 5) It is unclear whether there is any interaction between different caspases in specific pain conditions, which needs further investigations. Anyway, the findings of the above will further provide new interventional targets for the management of chronic pain and promote the development of novel drugs related to the caspase cascades.

AUTHOR CONTRIBUTIONS

LZ and HZ designed, collected literatures, and wrote the manuscript; NL, ZL, and YL collected some literatures and created figures and tables; LZ and YY revised the manuscript.

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GLOSSARY

ALCAR Acetyl-l-carnitine

AMPAR alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

ACC anterior cingulate cortex

AD Alzheimer's disease

ASC apoptosis-associated speck-like protein containing a Caspase activation and recruitment domain

ATF3 activating transcription factor-3

BAX B-cell lymphoma 2-associated X apoptosis regulator

BDNF brain-derived neuro-trophic factor

CCI chronic constriction injury

CCL21 C-C Motif Chemokine Ligand 21

CFA complete Freund's adjuvant

CGRP calcitonin-gene-related peptide

CIBP cancer-induced bone pain

CIPN chemotherapy induced neuropathic pain

COX-2 cyclooxygenase-2

CPN common peroneal nerve

CPTP chronic post-thoracotomy pain

CSF-CN Cerebrospinal fluid-contacting nucleus

CXCL1 Chemokine (C-X-C motif) ligand 1

CXCR3 C-X-C Motif Chemokine Receptor 3

DAMP damage associated molecular patterns

DRG dorsal root ganglion

ER endoplasmic reticulum

ERK extracellular signal-regulated kinase

GAP-43 growth associated protein 43

GFAP Glial fibrillary acidic protein

GSH glutathione

G-CSF granulocyte-colony stimulating factor

IL-1β interleukin-1beta

LPS lipopolysaccharide

LRRTM1 leucine-rich repeat transmembrane protein 1

LTD long-term depression

MDA malondialdehyde

MIP-1α macrophage inflammatory protein-1α

mGluR1 metabotropic glutamate receptor 1

NF-κB Nuclear Factor-κB

NLRP3 NOD-like receptor protein 3

NMDA N-methyl-d-aspartate

NLRC4 NOD-like receptor C4

NP neuropathic pain

3-NT 3-nitrotyrosine

OIH opioid-induced hyperalgesia

PD Parkinson's disease

p-ERK ERK phosphorylation

PGE2 Prostaglandin E2

p-IRE1 phosphorylated inositol-requiring enzyme-1

p-PERK phosphorylated protein kinase RNA-like endoplasmic reticulum kinase

pSNL partial sciatic nerve ligation

RTKs receptor tyrosine kinases

SCI spinal cord injury model

SNI spared nerve injury

SNL sciatic nerve ligation

STZ streptozotocin

TLR4 toll-like receptor 4

TNF-α Tumor necrosis factor-α

TRPV1 transient receptor potential vanilloid-1.

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SYSTEMATIC REVIEW
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Effects of Noninvasive Brain Stimulation Combined With Antidepressants in Patients With Poststroke Depression: A Systematic Review and Meta-Analysis

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Objective: To evaluated the efficacy and safety of noninvasive brain stimulation (NIBS) combined with antidepressants in patients with poststroke depression (PSD).

Methods: Seven databases were searched to identify randomized controlled trials of NIBS combined with antidepressants in the treatment of PSD based on the international classification of diseases (ICD-10) criteria and exclusion criteria. The retrieval time was from the database establishment to 31 October 2021. Two researchers independently screened the identified studies through the search strategy, extracted their characteristics, and evaluated the quality of the included literature. Cochrane Collaboration's tool was used to assess risk of bias. RevMan 5.3 software was applied for meta-analysis.

Results: A total of 34 randomized controlled trials were included, involving 2,711 patients with PSD. Meta-analysis showed that the total effective rate was higher in the combined therapy than the antidepressant alone [odds ratio (OR): 4.33; 95% confidence interval (CI): 3.07 to 6.11; $p < 0.00001$]. The Hamilton depressive scale (HAMD) score was significantly lower in repeated transcranial magnetic stimulation (rTMS) (≤10 Hz) combined with antidepressant than in antidepressant alone [standard mean difference (SMD): −1.44; 95% CI: −1.86 to −1.03; $p < 0.00001$]. No significant difference was seen in rTMS (>10 Hz) combined with antidepressant versus antidepressant alone (SMD: −4.02; 95% CI: −10.43 to 2.39; $p = 0.22$). In addition, combination therapy more strongly improved the modified Barthel index (MBI) scale than antidepressants [mean difference (MD): 8.29; 95% CI: 5.23–11.35; $p < 0.00001$]. Adverse effects were not significantly different between two therapies (OR: 1.33; 95% CI: 0.87 to 2.04; $p = 0.18$).

Conclusion: Low-frequency rTMS (≤10 Hz) combined with antidepressants tends to be more effective than antidepressants alone in patients with PSD, and there are no significant adverse effects. In addition, combined therapy may enhance quality of life after stroke. Combination therapy with high-frequency rTMS (>10 Hz) showed no advantage in treating PSD. The transcranial electrical stimulation (TES) combined with antidepressants might be

more effective than antidepressants alone, which are needed to confirm by more clinical trials since the.

Keywords: noninvasive brain stimulation, poststroke depression, repeated transcranial magnetic stimulation, antidepressant, depression

INTRODUCTION

Stroke is now the third leading cause of death worldwide (Benjamin et al., 2017). About 795,000 people in the United States experience new or recurrent stroke every year and, on average, a person has a stroke every 40 s (Benjamin et al., 2019). In addition to dyskinesia, patients with stroke often have psychological and emotional problems. One of the most common psychiatric complications of stroke is poststroke depression (PSD), which has an incidence in the first year after stroke as high as 33% (Hackett and Pickles, 2014). It severely affects the rehabilitation process after stroke and also exerts a heavy burden on patients' family and on society. Despite their prevalence, depression and other mood-related deficits generally get the least attention. Accordingly, mood disorders need to be addressed during the rehabilitation process of stroke to improve quality of life.

Antidepressants are currently the mainstay of treatment for PSD, but certain adverse reactions are inevitable (Hackett et al., 2008; Coupland et al., 2011). For example, tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs) increase the risk of cardiovascular and anticholinergic adverse effects. Fluoxetine has also been reported to be unable to improve PSD symptoms (Robinson et al., 2000; Rice et al., 2021), and some patients with stroke do not respond to antidepressants (Anderson et al., 2004; Hackett et al., 2008). Thus, an effective combination therapy for PSD is urgently required.

Repeated transcranial magnetic stimulation (rTMS) and transcranial electrical stimulation (TES) have been proven to be effective in boosting upper limb rehabilitation and improving aphasia after stroke (Vines et al., 2008; Szaflarski et al., 2011; Hu et al., 2018; Lefaucheur et al., 2020; Kuzu et al., 2021). More and more clinical trials have recently focused on noninvasive brain stimulation (NIBS) treatment of PSD (George et al., 1995; Jorge et al., 2008; Tang et al., 2018), and most have identified positive effects. We have found that the clinical effect of NIBS combined with antidepressants may be better than that of antidepressants alone, and many studies have also mentioned this possibility (Slotema et al., 2010; Brunoni et al., 2013). The current meta-analysis evaluated the efficacy and safety of NIBS combined with antidepressants in the treatment of PSD to provide evidence-based information for clinical decision-making and guideline recommendations.

MATERIALS AND METHODS

Search Strategy

Relevant randomized controlled trials (RCTs) of NIBS combined with antidepressants in the treatment of PSD were retrieved from the following databases: PubMed, EMBASE, Web of Science, CNKI, Cochrane Library, Biology Medicine Disc (CBM), and the Wanfang database. The retrieval time was from database establishment to October 2021. Search criteria were formulated according to different databases. The keywords included "noninvasive brain stimulation," "repeated transcranial magnetic stimulation," "transcranial direct current stimulation," "transcranial magnetic stimulation," "antidepressant," "antidepressant drugs," "western medicine," "after stroke," "poststroke," and "depression". Only English and Chinese articles were considered.

Inclusion Criteria

The literature included conformed to the following inclusion criteria (I) participants: patients were diagnosed with PSD and included those with ischemic stroke and hemorrhagic stroke, with no limit on the degree of depression. The diagnosis of PSD met the international classification of diseases (ICD-10) criteria for organic mental disorder (Brämer, 1988), and the score of Hamilton rating scale for depression (HAMD) exceeds 7 (Hamilton, 1960), the first onset, and the diagnosis of stroke was confirmed by magnetic resonance imaging (MRI) or computed tomography (CT), along with being down in spirits, fatigue and lack of interest. (II) study type: RCT; (III) interventions and comparisons: studies comparing the combination of noninvasive brain stimulation and antidepressants with antidepressants alone, such as fluoxetine, paroxetine, sertraline, fluvoxamine, citalopram, maprotiline, imipramine, amitriptyline, doxepin, and chlorimipramine, with only rTMS and TES chosen as noninvasive brain stimulation in this analysis and no frequency limit in the rTMS; (IV) primary outcomes: total effective rate and Hamilton depressive scale (HAMD) score; and (V) secondary outcomes: adverse effect rate and modified Barthel index (MBI) scale score.

Exclusion Criteria

Exclusion criteria of this study were as follows (I) language: non-English or non-Chinese studies; (II) study type: not RCTs, such as animal experiments, reviews, retrospective studies, case reports, conference, and comments; (III) duplicate records, those with incomplete, unclear or inconsistent outcomes, or those with missing information that could not be obtained from the authors; and (IV) studies without a control group or with placebo stimulation or NIBS at a different frequency to the control group.

Data Extraction and Management

Two researchers independently searched and browsed the databases according to the retrieval strategy and then carefully read the full article and extracted the characteristics of the

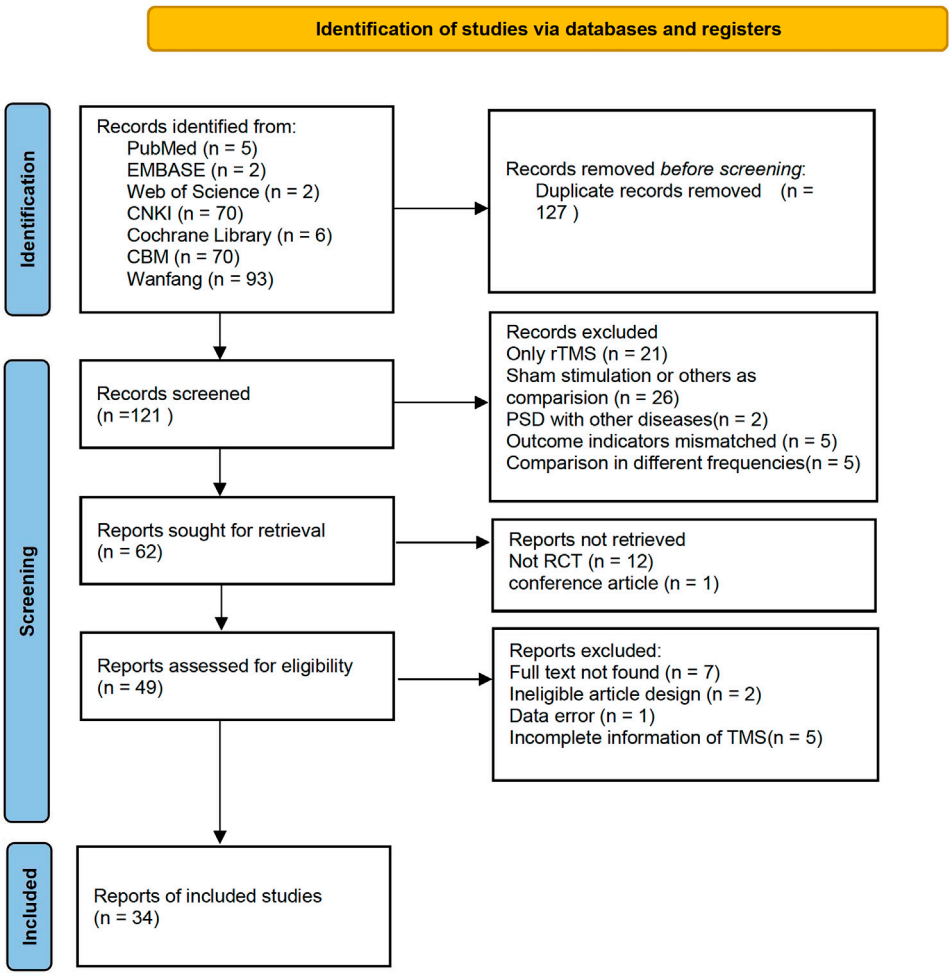

FIGURE 1 | Flow diagram of article selection.

included literature. The following information on the included literature was recorded: authors' names, publication year, sample size, participant age, intervention, control, outcome indicators, and stimulation frequency, intensity, orientation, control, and duration. Any disagreements were negotiated and discussed with a third researcher.

Quality Assessment

Cochrane Collaboration's risk of bias tool was used to assess the quality of the included studies. The tool considers six items: selection bias, performance bias, detection bias, attrition bias, reporting bias, and other biases. Each item was judged as one of three levels: low risk, unclear risk, or high risk.

Statistical Analysis

We used RevMan 5.3 software to perform this meta-analysis. The weighted mean difference was used for continuous variables, whereas the odds ratio (OR) was used for dichotomous variables. All data were calculated with 95% confidence intervals (95% CIs). Heterogeneity analysis and sensitivity analysis were also performed using RevMan 5.3. The random-effects model was selected if significant heterogeneity was identified ($p < 0.05$ or I^2>50%). Subgroup analysis and investigation of heterogeneity in subgroups were conducted when necessary. The fixed-effects model was selected if the heterogeneity was low ($p \geq 0.05$ or $I^2 \leq 50\%$). Reporting bias was assessed by funnel plot, with dissymmetry indicating significant reporting bias in the analysis.

RESULTS

Selection of Results

A total of 555 records were identified in the electronic databases. Of these, 248 records remained after the two researchers read the titles. After the deletion of duplicates and exclusion of 71 studies due to inconsistent primary standards after abstract screening, the full text of 50 articles were read for further assessment. Finally, 34 studies were selected for analysis. **Figure 1** shows the flow diagram of the article selection.

The 34 selected studies (Sun and Song, 2013; Hm, 2014; Ma and Ma, 2015; Wang and Ding, 2015; Xing and Wang, 2016; Tan

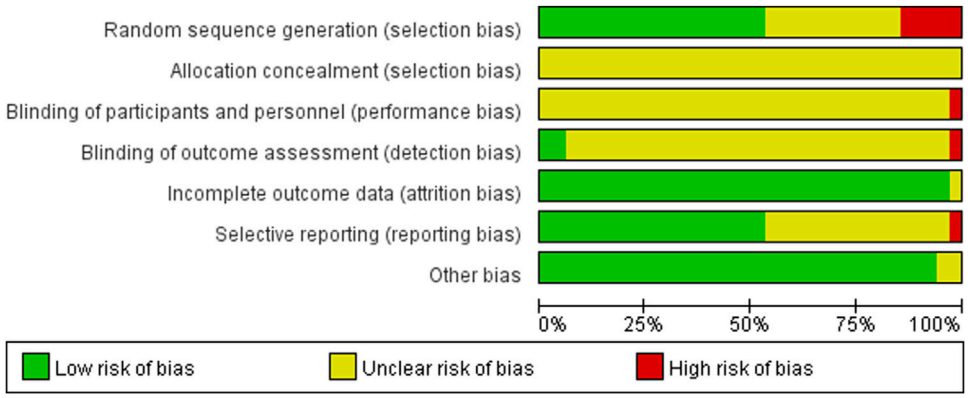

FIGURE 2 | Risk of bias.

and Zhou, 2017; Wang and Wen, 2017; Yang and Shi, 2018; Zhu, 2018; Wang and Li, 2019; Zhang, 2019; Wang and Qin, 2020; Wang and Wu, 2020; Yang and Hu, 2020; Wei, 2021) included a total of 2,784 patients, with 1,391 patients in the NIBS combined with antidepressant group and 1,393 patients in the antidepressant alone group. The basic characteristics of the included studies are summarized in **Supplementary Table S1**, including the authors' names, publication year, sample size, participant age, type of stroke, intervention, control, outcome indicators, and stimulation frequency, intensity, orientation, control, and duration. There was no significant difference in the baseline data between the two groups. The quality assessment of the included studies is shown in **Figures 2**, **3**.

Meta-Analysis Results

The main indicators were the HAMD score and the total effective rate after treatment. The secondary outcome indicators were the MBI score and adverse effects after treatment.

HAMD Score

Thirty-four of the selected studies (Wei, 2021; Zhu, 2018; Zhang, 2019; Yang and Hu, 2020; Yang and Shi, 2018; Xing and Wang, 2016; Wang and Wen, 2017; Wang and Qin, 2020; Wang and Wu, 2020; Wang and Ding, 2015; Wang and Li, 2019; Hm, 2014; Tan and Zhou, 2017; Sun and Song, 2013; Ma and Ma, 2015; Xj, 2018; Lu and Yang, 2016; Liu, 2015; Liu and Wang, 2020; Li and Chen, 2019; Li and Pan, 2013; L, 2014; Li, 2017; Li and Liang, 2016; Hu and Chen, 2020; Hl, 2021; Gan and Wang, 2015; Xy, 2014; Du, 2005; Xr, 2017; Cheng, 2011; Tian, 2018; Che and Chang, 2018), which involved 2,711 patients, reported the HAMD score as an outcome indicator. Because heterogeneity test analysis showed that there was significant heterogeneity among the included articles (I2 = 96%, $p < 0.00001$), a random-effects model was used to combine results. Subgroup analysis was performed according to intervention frequency and antidepressant category (Zhu, 2018; Zhang, 2019; Yang and Hu, 2020; Yang and Shi, 2018; Xj, 2018; Wei, 2021; Wang and Wen, 2017; Ma and Ma, 2015; Liu and Wang, 2020; Li, 2017; Li and Liang, 2016; L, 2014; Hu and Chen, 2020; Hm, 2014; Gan and Wang, 2015; Du, 2005; Cheng, 2011; Che and Chang, 2018; Fj, 2020). The meta-analysis showed that the difference was significant (SMD: −1.44; 95% CI: −1.86 to −1.03; $p < 0.00001$) (**Figure 4**). However, studies using rTMS combined with fluoxetine with a frequency exceeding 10 Hz showed no significant effect after treatment (SMD: −4.02; 95% CI: −10.43 to −2.39; $p = 0.22$).

Total Effect Rate

Seventeen of the included studies (Zhu, 2018; Zhang, 2019; Xy, 2014; Xing and Wang, 2016; Wei, 2021; Hm, 2014; Wang and Qin, 2020; Wang and Li, 2019; Tian, 2018; Lu and Yang, 2016; Liu and Wang, 2020; Li and Chen, 2019; Hl, 2021; Li and Liang, 2016; Cheng, 2011; L, 2014; Fj, 2020), which involved 1,406 patients, reported the total effect rate as an outcome indicator. There was significant heterogeneity among the included articles (I2 = 0%, $p < 0.00001$). Accordingly, the fixed-effects model was used to combine results. The meta-analysis showed that the difference was significant (OR: 4.33; 95% CI: 3.07 to 6.11; $p < 0.00001$) (**Figure 5**).

MBI Score

Seven of the selected studies (Xy, 2014; Xj, 2018; Tan and Zhou, 2017; Li and Pan, 2013; L, 2014; Hl, 2021; Cheng, 2011), involving 572 patients, reported the MBI score as an outcome indicator. The included articles showed significant heterogeneity (I2 = 86%, $p < 0.00001$) and the random-effects model was therefore used to combine results. The meta-analysis showed that the difference was significant (MD: 8.29; 95% CI: 5.23 to 11.35; $p < 0.00001$) (**Figure 6**).

Adverse Effect Rate

The adverse effect rate was reported as an outcome indicator in 12 of the included studies (Zhu, 2018; Zhang, 2019; Yang and Hu, 2020; Wang and Wu, 2020; Tian, 2018; Sun and Song, 2013; Liu, 2015; Liu and Wang, 2020; Li and Liang, 2016; L, 2014; Hl, 2021; Fj, 2020), which involved 981 patients. Because heterogeneity test analysis showed that there was significant heterogeneity among the included articles (I2 = 47%, $p = 0.04$), the fixed-effects model was used to combine results. The meta-analysis showed that there was no significant difference in the adverse effect rate between the two groups (OR = 1.33; 95% CI: 0.87–2.04, $p = 0.18$) (**Figure 7**).

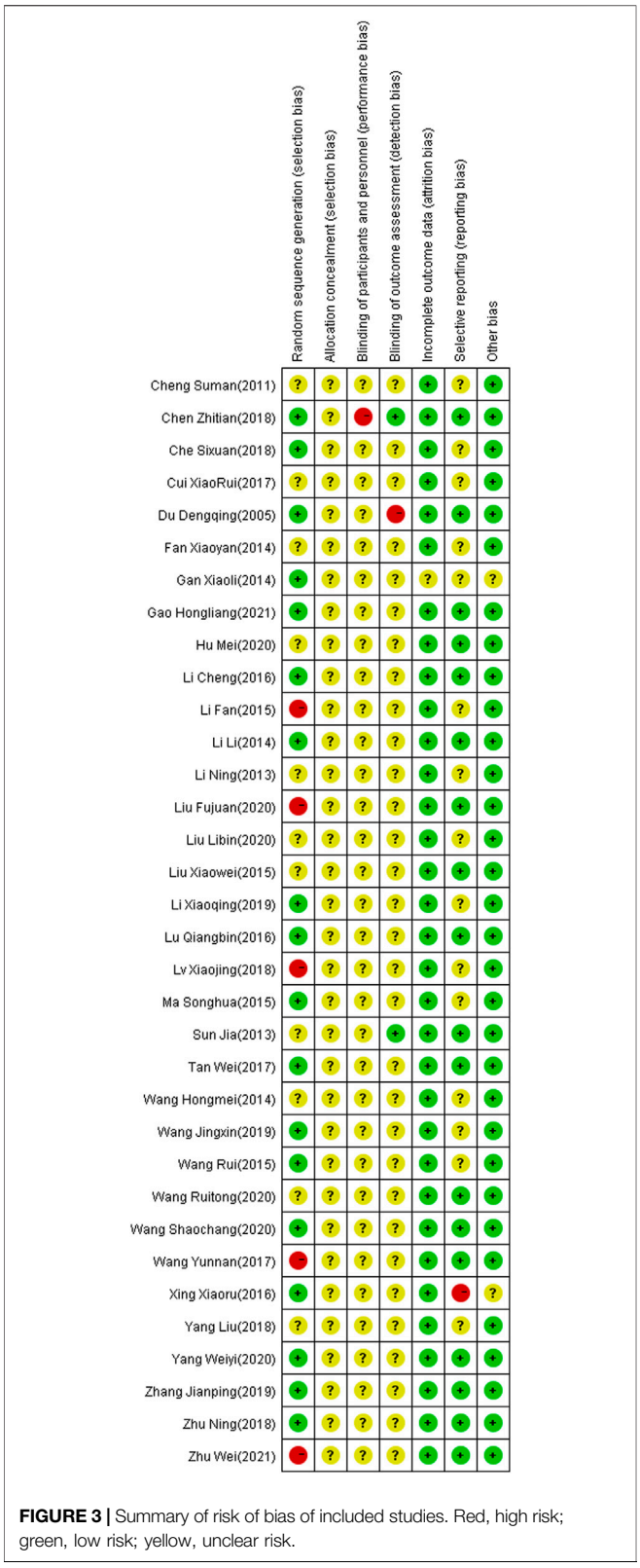

FIGURE 3 | Summary of risk of bias of included studies. Red, high risk; green, low risk; yellow, unclear risk.

Adverse reactions mainly included behavioral toxicity, nervous system abnormalities, and cardiovascular system abnormalities. Behavioral toxicity included somnolence and epilepsy. Nervous system abnormalities commonly included headache. Digestive system abnormalities included nausea, vomiting, and indigestion. In the NIBS combined with antidepressant group, 36 patients had headache, three had insomnia, three had thirst, eight had nausea, 12 had vomiting, and two had cardiovascular system abnormalities. In the antidepressant group, four patients had headaches, three had insomnia, four had thirst, five had nausea, 13 had vomiting, one had fatigue, and one had cardiovascular system abnormalities.

Sensitivity Analysis

Sensitivity analyses of each outcome indicator were performed by excluding single articles one-by-one to test the effect of each study on the pooled effect size. In the meta-analysis of the HAMD score, the heterogeneity decreased from 92% to 34% after deleting the study by Liu FJ from 2020 (Fj, 2020). The results showed that this heterogeneity was mainly due to this study. There was no qualitative change in the combined effect for all outcome indicators. Thus, the pooled results of the included studies were steady.

Publication Bias

Funnel plot analysis was used to analyze the publication bias of the HAMD score, total effect rate, and adverse effects. There was no obvious publication bias in the studies of the total effect rate and adverse effects. The poor symmetry of the funnel plot indicated the existence of a publication bias due to the study by Liu LB from 2020 (Liu and Wang, 2020). After deleting this study, the combined effect was not changed but the total heterogeneity decreased to 79%. The publication bias results for the HAMD score analysis are shown in **Figure 8**.

DISCUSSION

Our meta-analysis included 34 studies of the effects of NIBS combined with antidepressants for patients with PSD. The results showed that the combination of NIBS and antidepressants might have a better effect on PSD and could improve the depression scale score and quality of life compared with antidepressants alone. It is well known that guidelines recommend rTMS for the treatment of major depression, and many meta-analyses have shown that TMS intervention with PSD was positive (Shen et al., 2017; Liu et al., 2019; Shao et al., 2021), but a growing number of studies have recommended multi-module combination therapy and population-specific personalized treatment (Wang et al., 2019; Nestor and Blumberger, 2020), which warrants further research on the frequency and site of TMS intervention. The use of low-frequency TMS by Daniel R Schaffer significantly improved depression with cognitive impairment, suggesting that low-frequency TMS is more effective in specific populations (Schaffer et al., 2021). Compared with previous reviews (Bucur and Papagno, 2018; Liu et al., 2019), our analysis had the following advantages (I) combination NIBS and antidepressant therapy; (II) internationally recognized depression assessment scales; (III) inconsistent results with previous studies due to negative outcomes of high-frequency

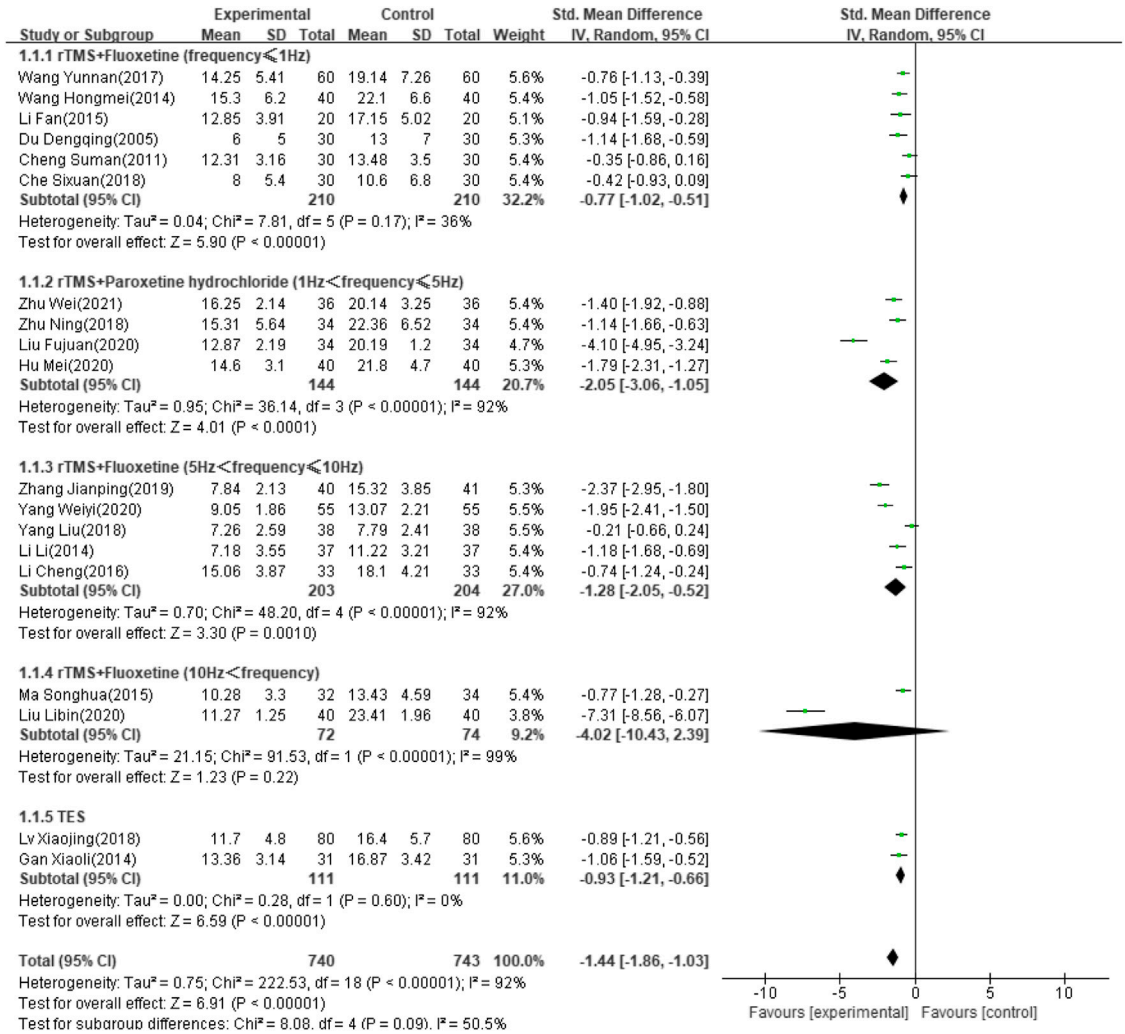

FIGURE 4 | Forest plot of HAMD score.

TMS combined with antidepressants; and (IV) the inclusion of more than 30 studies. There have been no studies evaluating NIBS in combination with antidepressants for PSD, although combination therapy is more clinically appropriate. Therefore, this meta-analysis may have a greater reference value than previous reviews.

According to the results of this analysis, combined NIBS and antidepressant therapy reduced the HAMD score of PSD more than antidepressants alone. However, this result was highly heterogeneous. We grouped the studies by a variety of clinically relevant factors, including age, intervention frequency and intensity, drug type, type of stroke, and stimulation orientation and duration. In the final analysis, rTMS combined with fluoxetine (less than 1 Hz and between 5 and 10 Hz) was more effective than fluoxetine alone, but the effect was not better with a frequency exceeding 10 Hz. TES combined with antidepressants improved the HAMD score more than antidepressants alone, although only two included articles examined this combination. After deleting the study by Liu FJ from 2020 (Fj, 2020) due to its high heterogeneity, rTMS combined with paroxetine was also more effective in reducing the HAMD score. This previous study by Liu FJ (Fj, 2020) was probably a retrospective study due to its vague description and was excluded from the pooled effect. Martijn (Arns et al., 2010) also commented that there were possibly differential effects of different rTMS stimulation frequencies, although many searches concluded that high-frequency rTMS has the same effect as low-frequency rTMS or antidepressants (Berlim et al., 2013).

The results of 17 studies (Zhu, 2018; Zhang, 2019; Xy, 2014; Xing and Wang, 2016; Wei, 2021; Wang and Qin, 2020; Hm, 2014; Hl, 2021; Wang and Li, 2019; Tian, 2018; Lu and Yang, 2016; Liu and Wang, 2020; Li and Chen, 2019; Li and Liang, 2016; Cheng, 2011; L, 2014; Fj, 2020) also indicated that NIBS combined with antidepressants was better than antidepressants alone regarding the total effect rate. Moreover, for the MBI score, seven studies (Xy, 2014; Xj,

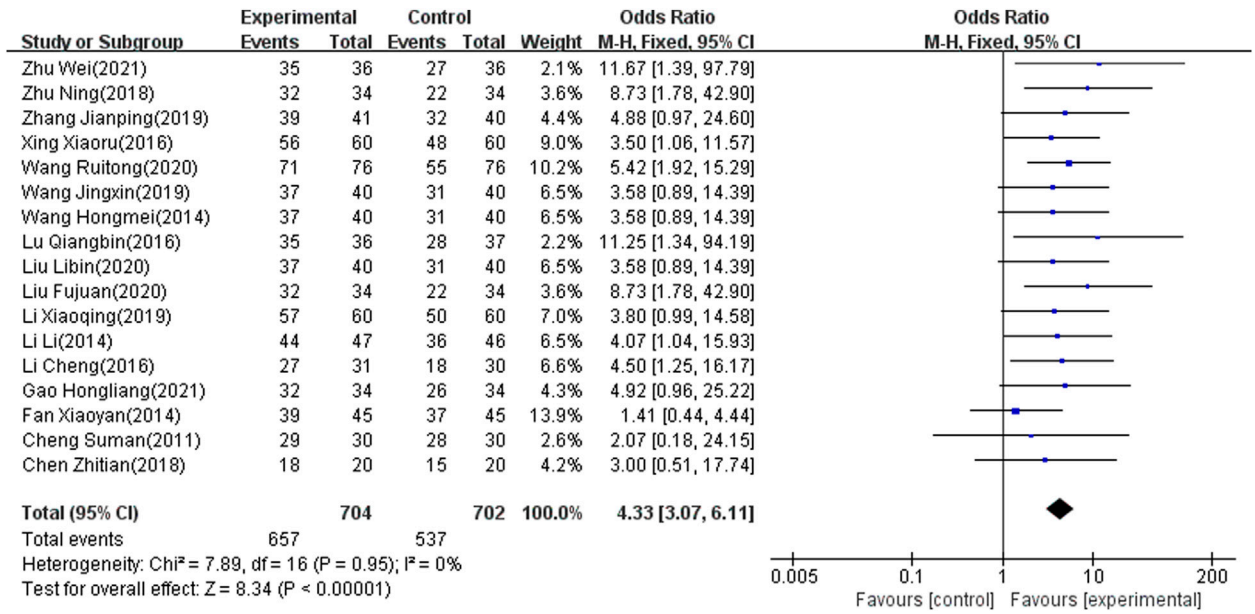

FIGURE 5 | Forest plot of total effect rate.

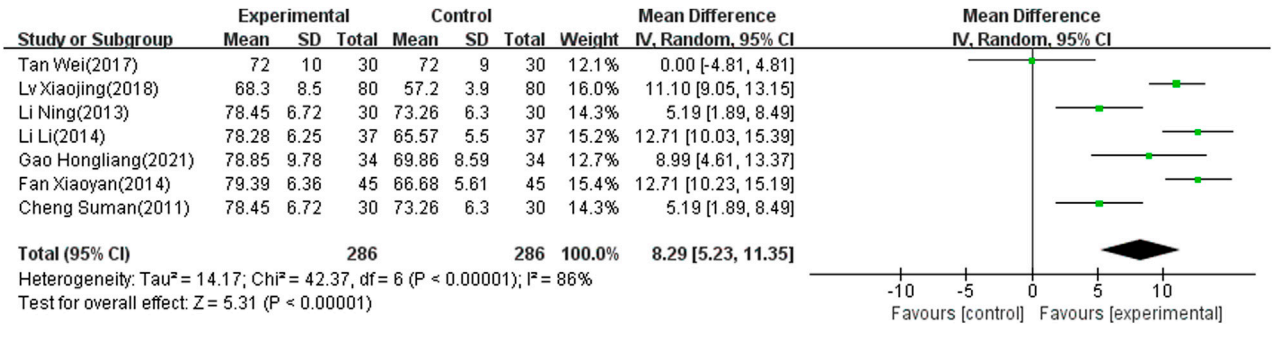

FIGURE 6 | Forest plot of MBI score.

2018; Tan and Zhou, 2017; Li and Pan, 2013; L, 2014; Hl, 2021; Cheng, 2011) showed that the combination therapy has potential benefits in patients with PSD. Combination therapy may be able to improve quality of life after stroke. Since a few included articles reported MBI scores, the meta-regression did not be conducted. Subgroup analyses were added based on clinical characteristics, including frequency, intensity and location of intervention, degree of depression, and course of disease. Heterogeneity still could not decrease to a reasonable range. We used sensitivity analysis to find no articles causing high heterogeneity, and adopted a random effect model. This result is stable and conservative. Some studies (Zhu, 2018; Zhang, 2019; Yang and Hu, 2020; Ma and Ma, 2015; L, 2014; Fj, 2020) have reported headache, nausea, vomiting, insomnia, thirst, and fatigue in both control and experimental groups. The adverse reactions may be caused by antidepressants. Twelve studies (Zhu, 2018; Zhang, 2019; Yang and Hu, 2020; Wang and Wu, 2020; Tian, 2018; Sun and Song, 2013; Liu, 2015; Liu and Wang, 2020; Li and Liang, 2016; L, 2014; Hl, 2021; Fj, 2020) demonstrated consistent and stable results in adverse reaction rates. This suggests that combined NIBS and antidepressant therapy is safe.

There is still a contradiction between the advantages and disadvantages of the different frequencies of NIBS, and the effects of different frequencies of NIBS are still disputed. Different frequencies of rTMS have been shown to reduce fluorodeoxy glucose F18 (^{18}F-FDG) uptake in the dorsal cortical region while simultaneously increasing ^{18}F-FDG uptake in the ventral region (Parthoens et al., 2016). The rTMS decreased glucose metabolism in the stimulated temporal region, with increases in the bilateral precentral, ipsilateral superior and midfrontal, prefrontal, and cingulate gyri. This suggests that 1 Hz rTMS could induce cortical

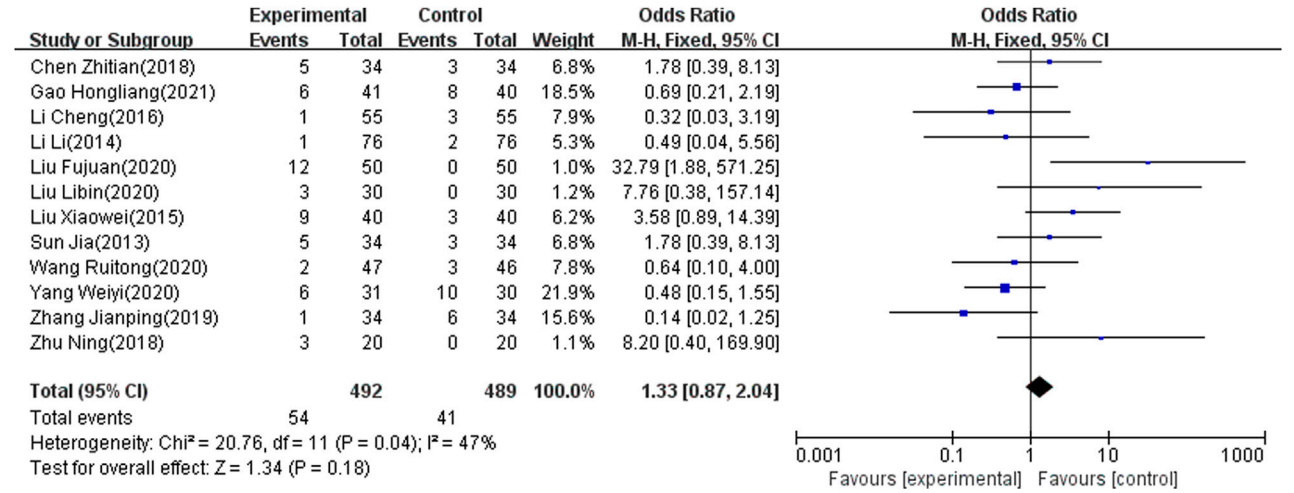

FIGURE 7 | Forest plot of side effect rate.

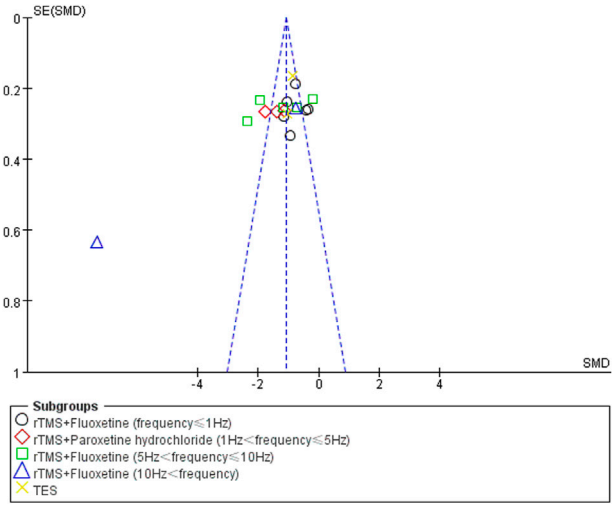

FIGURE 8 | Funnel plot of publication bias. SE, standard error; SMD, standard mean difference.

regulation and extensive changes in the neural network through long-range neuronal connectivity (Lee et al., 2013). Studies have also shown that low-intensity TMS mainly stimulates low-threshold inhibitory neurons (Duan et al., 2018). High-frequency TMS caused greater activation than low-frequency TMS in normal humans. However, oxidative stress, lipid peroxidation, and protein oxidation were found in the neural tissue of stroke patients. Any of these pathophysiological processes may be related to PSD (Nabavi et al., 2015). Kimbrell et al. (1999) also reported that the antidepressant response to rTMS might depend on the pretreatment cerebral metabolism and the stimulation frequency. Thus, it is possible that patients with PSD are more sensitive to low-frequency TMS. Due to abnormal expression of amine neurotransmitters and cytokine expression after stroke, the combination of antidepressants with rTMS may be more effective with the mild stimulation of low-frequency TMS.

The mechanism of PSD is still unclear, which may involve neurobiological pathways, inflammation and apoptosis mechanisms (Robinson and Jorge, 2016; Medeiros et al., 2020). Robinson (Robinson et al., 1984; Narushima et al., 2003) suggested that lesions in the left frontal lobe or left basal ganglia were associated with PSD. And focal brain stimulation using rTMS was only effective when administered to the left dorsolateral prefrontal cortex in patients with vascular depression (Jorge et al., 2008). A meta-analysis (Carson et al., 2000) showed that stroke site was not associated with depression, and a study (Wei et al., 2015) suggested a significant association between stroke in the right hemisphere and the incidence of depression. Some studies have hypothesized that stroke lesion area is related to depression degree, which could be explained by some pro-inflammatory factors (Spalletta et al., 2006). For example, the increase of IFN-γleads to the cascade reaction of other pro-inflammatory cytokines IL-6, IL-1βand TNF-α, which aggravates depression. Secondly, IFN-γcan affect the HPA axis (Capuron et al., 2003), leading to increased adrenocortical hormone and cortisol levels, resulting in increased reactive oxides (Altieri et al., 2012; Ferrari and Villa, 2017), which further cause cell death and damage. Proinflammatory factors also stimulate the activity of indoleamine 2, 3-dioxygenase, which degrades tryptophan, the biological precursor of serotonin, into a toxic metabolite (Bansal and Kuhad, 2016). Compared with common depression, PSD is associated with focal ischemia, which leads to programmed cell death, cell swelling, or cell necrosis and a series of complex events related to cellular and molecular mechanisms (Brouns and De Deyn, 2009). Whether the neuroanatomical location of stroke affects depression remains controversial. It remains unknown whether the severity of stroke is positively correlated with the severity of depression, or whether there

are differences in depression at different times after stroke. It is hoped that more RCTs will be designed in this direction in the future.

LIMITATIONS AND PROSPECTS

This meta-analysis emphasized the clinical efficacy and depression improvement of combination therapy in PSD patients but also examined quality of life and safety. However, all included RCTs were from China, which may indicate publication bias. Funnel plot analysis revealed that a study by Liu LB (Liu and Wang, 2020) had significant publication bias due to selective reporting of outcomes. Accordingly, the result should be treated with caution. This meta-analysis was not registered and there may be a small deviation, but we still strictly followed the procedures of systematic evaluation. In addition, some indicators were significantly heterogeneous. Therefore, caution is required for these findings. More basic studies are needed to determine the mechanism underlying the effect of low-frequency TMS combined with antidepressants on depression after stroke. Moreover, large multicenter studies are needed to assess the best frequency and type of depression drugs to promote the final translation of combination treatment into daily clinical practice and guidelines.

CONCLUSION

Our analysis demonstrate that low-frequency rTMS(10 ≤ Hz) combined with antidepressants tends to be more effective than antidepressants alone in patients with PSD and there are no significant adverse effects. In addition, combined therapy may boost quality of life after stroke. Combination therapy with high-frequency rTMS (>10 Hz) showed no advantage in treating PSD. The transcranial electrical stimulation (TES) combined with antidepressants may be more effective than antidepressants alone. More randomized controlled studies with detailed design for different stroke periods, depression levels and stroke location are needed to verify this conclusion.

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.

AUTHOR CONTRIBUTIONS

HC conceived the conception and design of the study. JL did the search of studies, performed the meta-analytic statistics. JL, JF, and YJ did the search of studies, performed the data extraction and prepared the tables and figures. JH was involved in the study selection and did the data extraction. HZ prepared the references and provided the detailed critical comments. JL and JF wrote the manuscript. All authors approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Artesunate Therapy Alleviates Fracture-Associated Chronic Pain After Orthopedic Surgery by Suppressing CCL21-Dependent TREM2/DAP12 Inflammatory Signaling in Mice

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Chronic pain after bone fracture and orthopedic surgery is often refractory to most analgesics currently in use, thus emphasizing the urgent need for improved therapeutic medications. Chemokine-dependent neuroinflammation is critical for excitatory synaptic plasticity and central nociception sensitization. Recent studies have focused on the inhibition of inflammatory responses by artesunate, the first anti-malaria drug extracted from artemisinin. The present study investigated the analgesic effects and potential targets of artesunate in a mouse model of chronic pain induced by tibial fracture and orthopedic surgery. Three injections of artesunate were intrathecally administered on a daily basis from days 4 to 6 after fracture. We reported that repetitive exposure to artesunate (10 and 100 μg but not 1 μg) dose-dependently prevented fracture-induced mechanical and cold allodynia. Moreover, single intrathecal injection of artesunate (100 μg) alleviated the established chronic pain on day 14 after fracture surgery. Intraperitoneal artesunate (10 and 50 mg kg^{-1}) therapy was effective against chronic fracture pain. Intriguingly, artesunate inhibited the upregulation of spinal chemokine CCL21, triggering receptor expressed on myeloid cells 2 (TREM2) and DNAX-activating protein of 12 kDa (DAP12) expressions and microglia activation in fracture mice. Furthermore, spinal CCL21 neutralization attenuated the severity of fracture-associated post-surgical pain. Exogenous CCL21-induced acute inflammatory pain was impaired by artesunate therapy. Additionally, the pharmacological blockage of TREM2 reduced recombinant CCL21-elicited behavioral hypernociception. The present findings demonstrate that artesunate therapy reduces the initiation and maintenance of fracture-associated chronic postoperative pain by inhibiting CCL21-dependent TREM2/DAP12

Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA, analysis of variance; CFA, complete Freund's adjuvant; DAP12, DNAX-activating protein of 12 kDa; DMSO, dimethyl sulfoxide; H_2O_2, hydrogen peroxide; i.p., intraperitoneal; i.t., intrathecal; LPS, lipopolysaccharide; SEM, standard error of mean; TNF-α, tumor necrosis factor-α; and TREM2, triggering receptor expressed on myeloid cells 2.

inflammatory signaling and microglia activation, thus suggesting that artesunate could emerge as a therapeutic strategy for fracture pain management.

Keywords: artesunate, bone fracture, CCL21, chronic pain, DAP12, TREM2, spinal cord

INTRODUCTION

With the rapid development of industry, construction, and transportation and the aggravation of population aging, the occurrence of fracture following industrial accidents, construction injuries, traffic injuries, and osteoporosis in the elderly also increases (Chen et al., 2017). Chronic pain after bone fracture and orthopedic surgery is clinically common and imposes a heavy financial burden to patients worldwide. It has been reported that the incidence of chronic pain after ankle and wrist fractures is 61.7% and that the incidence of chronic pain after tibial fracture is 55.1% (Friesgaard et al., 2016; Khan et al., 2016). Treatment of fracture pain remains a dramatic challenge to pain physicians. Recent reports have recapitulated the requirement of neuroinflammation for spinal pain sensitization, which is critical for multiple nociceptive perceptions after peripheral nerve injury, cancer, and bone fracture (Chen et al., 2018; Baral et al., 2019; Li et al., 2021). Yet, the specific molecular pathogenesis underlying fracture pain continues to be elusive.

Chemokine-mediated neuroinflammation involves microglia activation and neuronal plasticity in pain neurocircuits that, subsequently, maintain the pain phenotype (Ji et al., 2014; Ji et al., 2016). Chemokine CCL21, as a cardinal microglia-activating factor localized in dorsal horn neurons, is indicated to mediate excitatory synaptic transmission via microgliosis in pain states, ranging from chronic neuropathic pain to persistent cancer pain (Biber et al., 2011; Zheng et al., 2019; Hirth et al., 2020). Triggering receptors expressed on myeloid cells 2 (TREM2) and DNAX-activating protein of 12 kDa (DAP12) in microglia are gradually recognized as the downstream of microglial inflammatory signaling in pronociceptive facilitation during chemotherapy-induced peripheral neuropathy and nerve trauma-induced neuropathic allodynia (Hu et al., 2018; Wang Y. et al., 2020). Nevertheless, whether spinal CCL21 contributes to fracture-associated chronic pain via TREM2/DAP12 pathway remains largely unknown.

Given that current analgesics including opioid agents and non-steroidal anti-inflammatory drugs have several side effects and may interfere with bone healing (Kidner et al., 2009; Lisowska et al., 2018; Tracey et al., 2019), alternative agents for fracture-associated chronic pain control are urgently required. Artesunate, as an active derivative of artemisinin (Qinghaosu) with little toxicity, has been generally utilized to treat malaria for recent decades (Zou et al., 2020). Remarkably, artemisinin and its derivatives provide multi-therapeutic protections of anti-inflammation, anti-oxidation, anti-cancer, and anti-viral infection in a wide variety of pathophysiological disorders, such as sepsis, neurodegeneration, ischemia-reperfusion injury, tumors, and severe coronavirus disease (COVID-19) caused by SARS-CoV-2 (Duan et al., 2019; Gendrot et al., 2020; Kasaragod et al., 2020; Bang et al., 2021). Furthermore, artesunate is recently identified as an effective analgesic prescription for remifentanil-induced hyperalgesia and complete Freund's adjuvant (CFA)-induced acute inflammatory pain via the maintenance of oxidative homeostasis in rodents (Guruprasad et al., 2015; Zhang et al., 2022). However, whether artesunate ameliorates fracture-associated chronic pain by modulating spinal inflammatory responses requires to be further investigated.

In this study, we characterized the potential role of intrathecal (i.t.) artesunate in chronic postoperative pain using a mouse model of tibial fracture with intramedullary pinning. Spinal expressions of CCL21, TREM2, and DAP12 were measured to verify the nociceptive pathogenesis and anti-nociceptive targets of artesunate in our orthopedic model. Our findings identified that the inhibition of neuroinflammation by artesunate may offer a novel therapeutic strategy for pain control after orthopedic surgery.

MATERIALS AND METHODS

Animals

Adult male C57BL/6J mice, 8–10 weeks old, were raised in an artificially regulated 12-h light–dark environment with food and water *ad libitum*. All animals were purchased from the experimental animal center of the Chinese Academy of Military Medical Science. All experimental studies and protocols were conducted in strict accordance with the International Association for the Study of Pain directives and approved by the Animal Ethical and Welfare Committee of Tianjin Medical University (Tianjin, China).

Drugs and Administration

Artesunate (MedChemExpress, HY-N0193, China) was dissolved in 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, D2650, United States) for i.t. injection. Recombinant CCL21 (Abcam, ab201361, United Kingdom), a neutralizing antibody against CCL21 (anti-CCL21, R&D Systems, AF457, United States) and a neutralizing antibody against TREM2 (anti-TREM2, R&D Systems, 1729-T2, United States), was dissolved in normal saline or 10% DMSO for i.t.; delivery and the doses of these reagents were chosen based on previous reports (Hu et al., 2018; Wang et al., 2020a; Zhang et al., 2022). The intrathecal injection was performed under brief anesthesia of sevoflurane (induction, 3.0%; surgery, 1.5%; Maruishi Pharmaceutical Co., Ltd., Japan) and made between the levels of L_5 and L_6 using a 30-G needle (Donnelly et al., 2021); 5 μl of the reagent was given when the reflexive tail flick was observed.

Surgery

The tibial fracture-associated postoperative pain model was established according to the previously described procedures (Zhang et al., 2018; Cui et al., 2021). Briefly, animals were anesthetized with sevoflurane (induction, 3.0%; surgery, 1.5%) by a nose mask under sterile conditions. Muscles were disassociated following an incision from the knee to the

midshaft of the left tibia. After osteotomy, a 0.38-mm stainless steel pin was inserted into the tibia intramedullary canal, and the incision was sutured with 6–0 prolene. For sham operation, the incision and muscle disassociation were made identically without tibial fracture with pinning.

Behavioral Testing

All tests were conducted between 10:00 a.m. and 3:00 p.m. on that day in a temperature-controlled room at 24°C. The baseline threshold was tested 1 day before the treatment, and the mice were habituated 2 h per day in the testing apparatus for 3 days prior to the baseline threshold test.

In the von Frey test, the mice were placed on a test platform with a grid spacing of 1.5 mm, covered with a plexiglass box of 49 cm × 33 cm × 40 cm, and allowed to acclimatize for 2 h. The paw withdrawal threshold (PWT) of the mice was measured with the von Frey filaments (Stoelting, United States) between 0.16 and 2 g using the up-and-down method. Starting with 0.16 g to stimulate the left hind paw, licking or withdrawal during the 5 s stimulus was considered as a positive response. The force was reduced in the case of a positive reaction; otherwise, the force was increased, and finally the PWT was calculated (Zhang et al., 2018; Cui et al., 2021). With reference to the frequency behaviors, a 0.16 g von Frey filament was used to stimulate left hind paws for 10 times with a 30 s interval, and the percentage of withdrawal responses was calculated as frequency (Zhang et al., 2018; Zhang et al., 2021).

For measurement of cold allodynia, two acetone applications (20 μl each) were gently applied to the left hind paw bottom using a pipette and the responses to acetone were scored: 0, no response; 1, quick withdrawal, paw stamping, or flicking; 2, prolonged withdrawal or repeated flicking of the paw; and 3, repeated paw flicking and licking (Cui et al., 2021; Zhang et al., 2021).

The same investigator blinded to the treatments collected the behavioral data.

ELISA Analysis

An enzyme-linked immunosorbent assay (ELISA) was used to measure the concentrations of CCL21 (ab208985, Abcam), TREM2 (SAB2501170, Sigma), and DAP12 (EM8531, Wuhan Fine Biotech Co., China) in the $L_{4\text{-}5}$ segments of spinal cord. The spinal cord tissues were collected before and after tibial fracture, as well as at hour 12 after exogenous CCL21 administration. The spinal cord tissues were homogenized in a lysis buffer containing protease and phosphatase inhibitors. The tissue samples were centrifuged at 12,500 ×g for 10 min, and the supernatant was collected. The BCA protein assay (Pierce) was employed to determine protein concentrations. For each reaction in a 96-well plate, 100 μg of proteins of samples were used. All ELISA experiments followed the manufacturer's protocol. The optical densities of samples were measured using an ELISA plate reader (Bio-Rad) at a wavelength of 450 nm, and the levels of CCL21, TREM2, and DAP12 were calculated using the standard curves and normalized to the total protein levels.

Immunofluorescence

The mice were deeply anesthetized and transcardially perfused with pre-cooled PBS following 4% paraformaldehyde. The whole spinal cord was blown out using the hydraulic pressure method. The L_4–L_5 spinal cord was dissected and dehydrated in 30% sucrose for 2 days. The tissues were then frozen in OCT and cut into 5-μm frozen sections using a cryostat (Leica Biosystems, Germany). The sections were blocked with 0.3% Triton X-100 for 10 min and 5% goat serum for 1 h. They were then incubated with primary antibodies overnight at 4°C. The following primary antibody was used: anti-Iba-1 (1:200, Abcam, ab178847, United Kingdom). After rinsing three times with PBS, the sections were incubated with a fluorescence-labeled secondary antibody for 1 h. Images were collected using a fluorescence microscope (Olympus, Japan), and the analysis was performed using Image J software.

Statistical Analysis

All statistical analyses were performed with SPSS 18.0 software (SPSS, United States). All animals were randomly assigned to experimental conditions. All data were expressed as mean ± standard error of mean (SEM). The sample size was calculated as previously described (Zhang et al., 2018; Cui et al., 2021; Zhang et al., 2021). The Shapiro–Wilk test was used for determining the normality of data distribution, and parametric statistics were applied. The homogeneity of variance was validated using the Levene test. The statistical analyses of behavioral data were performed by two-way analysis of variance (ANOVA) with Bonferroni *post hoc* comparisons. The results of biochemical experiments were analyzed using one-way ANOVA with Bonferroni *post hoc* comparisons. A p value <0.05 was considered statistically significant.

RESULTS

Tibial Fracture Generates Chronic Postoperative Pain and Increases Spinal Expressions of CCL21, TREM2, and DAP12 After Orthopedic Surgery

First, we did not observe any significant differences in basal mechanical and cold sensitivities between two groups ($p > 0.05$, $n = 8$; **Figures 1A–C**). The von Frey test revealed that sham surgery did not change the postoperative paw withdrawal threshold and frequency in comparison with baseline ($p > 0.05$, $n = 8$; **Figures 1A,B**). The acetone test did not detect any marked alternation in cold response scores after sham operation ($p > 0.05$, $n = 8$; **Figure 1C**). Interestingly, as compared to sham animals, tibial fracture caused persistent (>21 days) postoperative pain (mechanical allodynia), as indicated by significantly decreased paw withdrawal thresholds [$F(1, 70) = 332.5$, $p < 0.001$, $n = 8$, two-way ANOVA; **Figure 1A**] and increased paw withdrawal frequency [$F(1, 70) = 471.6$, $p < 0.001$, $n = 8$, two-way ANOVA; **Figure 1B**] after orthopedic treatment. As parallel, the postoperative pain represented prolonged cold allodynia by a long-lasting elevation of cold response following fracture with pin insertion, as compared to sham intervention [$F(1, 70) = 166.7$, $p < 0.001$, $n = 8$, two-way ANOVA; **Figure 1C**].

Neuroinflammation-driven synaptic plasticity in the spinal cord dorsal horn is a central feature of pathological pain with

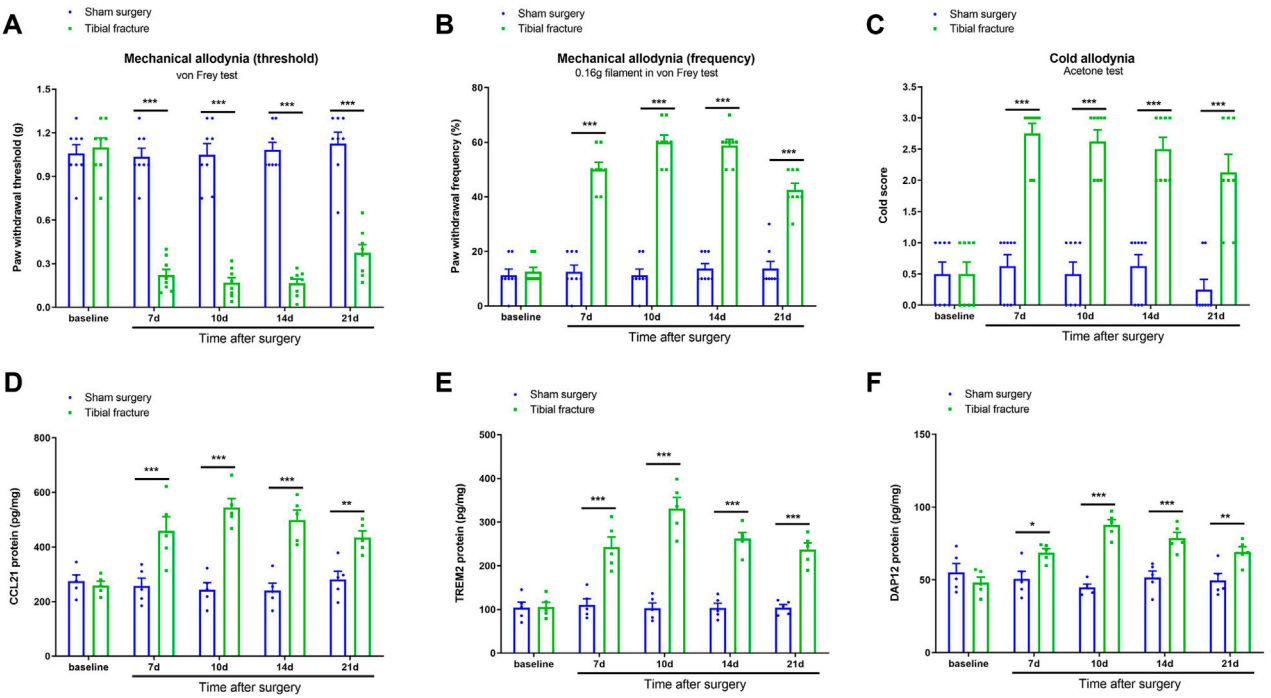

FIGURE 1 | Fracture-associated behavioral allodynia and spinal over-expressions of CCL21, TREM2, and DAP12 after orthopedic surgery in mice. The development of mechanical allodynia was assessed by paw withdrawal mechanical threshold **(A)** and paw withdrawal mechanical frequency to 0.16 g filament **(B)** in the von Frey test after sham surgery and tibial fracture ($n = 8$). The development of cold allodynia was assessed by cold response scoring **(C)** in acetone test after sham surgery and tibial fracture ($n = 8$). The spinal dorsal horn $L_{4\text{-}5}$ segments were collected for biochemical experiments. **(D–F)** ELISA identified the increased levels of spinal CCL21, TREM2, and DAP12 proteins after tibial fracture ($n = 5$). All the data are expressed as mean ± SEM and analyzed by two-way ANOVA with Bonferroni *post hoc* comparisons. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. group sham surgery.

different etiologies (Ji et al., 2014; Ji et al., 2018; Qiang and Yu, 2019; Wang et al., 2020b). Thus, the present study emphasized the specific molecular signaling of spinal nociceptive information transmission following fracture with pin insertion. ELISA exhibited that sham operation failed to affect the expression of CCL21, TREM2, and DAP12 in the spinal dorsal horn ($p > 0.05$, $n = 5$; **Figures 1D–F**). Noteworthy, our biochemical results showed that spinal levels of CCL21, TREM2, and DAP12 were considerably upregulated within 7 days, peaked at 14 days, and continued for at least 21 days in animals undergoing tibial fracture and orthopedic surgery ($p < 0.05$, $n = 5$; **Figures 1D–F**), which was consistent with the time course of chronic postoperative pain phenotypes. All these data suggest that tibial fracture with pin insertion initiates persistent spinal over-expression of CCL21, TREM2, and DAP12, which may be essential for the pathogenesis of chronic fracture pain.

Intrathecal Pretreatment With Artesunate Prevents Persistent Postoperative Pain Following Tibial Fracture and Orthopedic Surgery

To examine the effect of artesunate on basal nociceptive sensitivity, i.t., artesunate (1, 10, and 100 μg) was injected in naïve animals. We found that as compared to baseline, artesunate treatment did not impair peripheral mechanical and cold sensitivity in naïve mice ($p > 0.05$, $n = 6$; **Figures 2A–C**), suggesting that DHA therapy at 1, 10, and 100 μg was safe for our model. Then, to explore the potential role of artesunate in chronic fracture pain, i.t., artesunate (1, 10, and 100 μg) was administered daily for three consecutive days on days 4–6 (in the early phase) after tibial fracture with orthopedic surgery. Also, artesunate (i.t., 100 μg) was injected on days 4–6 in animals after sham surgery. Intriguingly, the i.t. therapy of artesunate at 10 and 100 μg but not 1 μg reduced fracture-associated postoperative pain, as characterized by the abrupt increase in paw withdrawal mechanical threshold [$F(5, 210) = 173.8$, $p < 0.001$, $n = 8$, two-way ANOVA; **Figure 2D**], the significant decrease in paw withdrawal mechanical frequency [$F(5, 210) = 127.0$, $p < 0.001$, $n = 8$, two-way ANOVA; **Figure 2E**], and the considerable reduction in cold scores [$F(5, 210) = 64.93$, $p < 0.001$, $n = 8$, two-way ANOVA; **Figure 2F**]. Such analgesia of artesunate strongly lasted for 1–7 days after the termination of the third treatment in a dose-dependent manner.

We further detected the levels of CCL21 and TREM2/DAP12 to verify whether these inflammatory mediators were involved in artesunate analgesia in fracture mice. Notably, artesunate pretreatment (100 μg) reduced the spinal up-modulation of CCL21, TREM2, and DAP12 expressions after tibial fracture with pin insertion ($p < 0.05$, $n = 5$; **Figures 3A–C**). Microglia

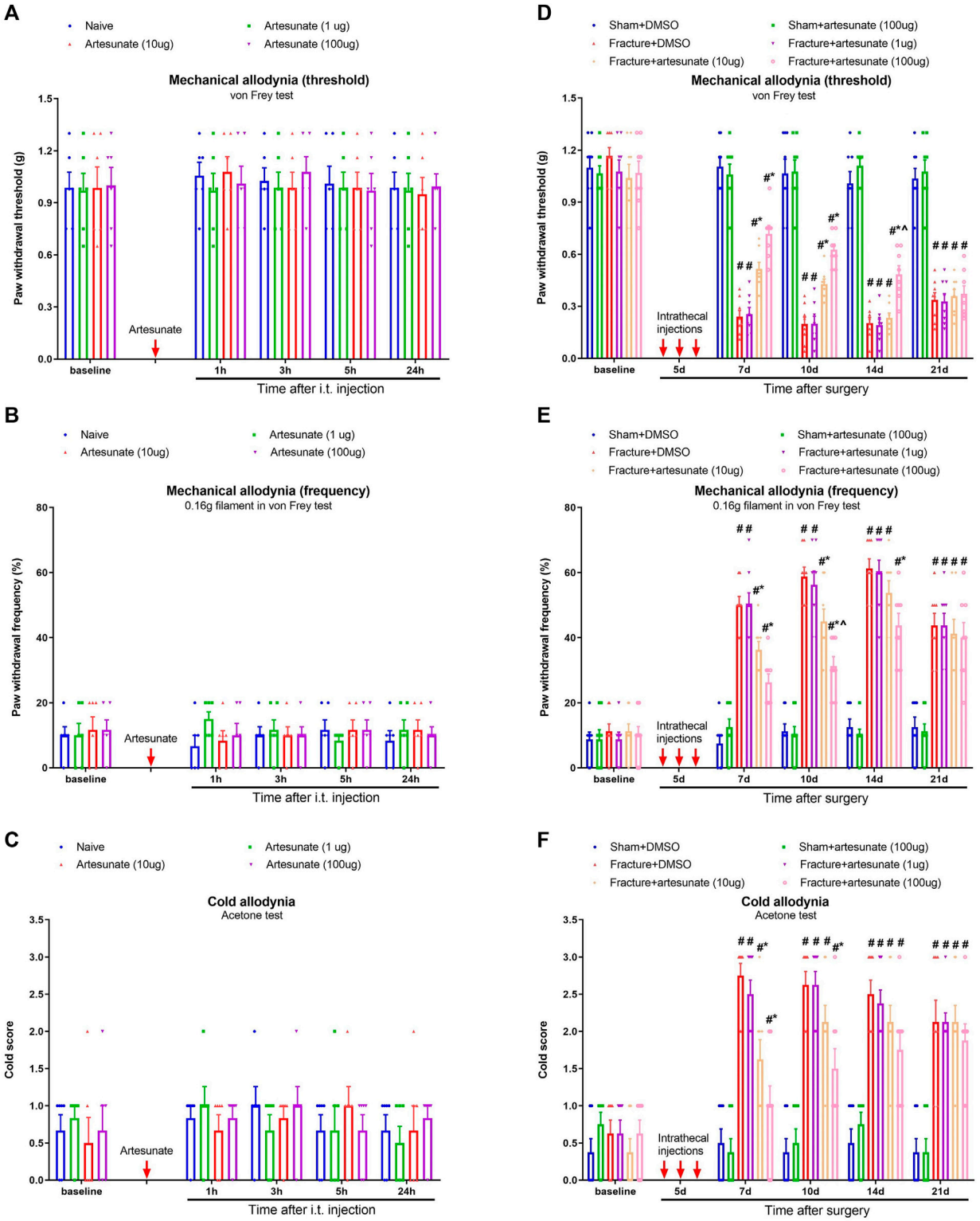

FIGURE 2 | Intrathecal pre-administration of artesunate reduces fracture-associated postoperative pain. **(A–C)** Single injection of artesunate (1, 10, and 100 μg) was intrathecally administered in naïve mice. **(D–F)** Intrathecal artesunate (1, 10, and 100 μg) was administered daily for three consecutive days on days 4, 5, and 6 (indicated by red arrows) after tibial fracture with orthopedic surgery. Also, artesunate (i.t., 100 μg) was injected on days 4, 5, and 6 after sham surgery. All behavioral results are mean ± SEM ($n = 6–8$) and analyzed by two-way ANOVA with Bonferroni *post hoc* comparisons. $^{\#}p < 0.05$ vs. group sham + DMSO, $^{*}p < 0.05$ vs. group fracture + DMSO, $^{\wedge}p < 0.05$ vs. group fracture + artesunate (10 μg).

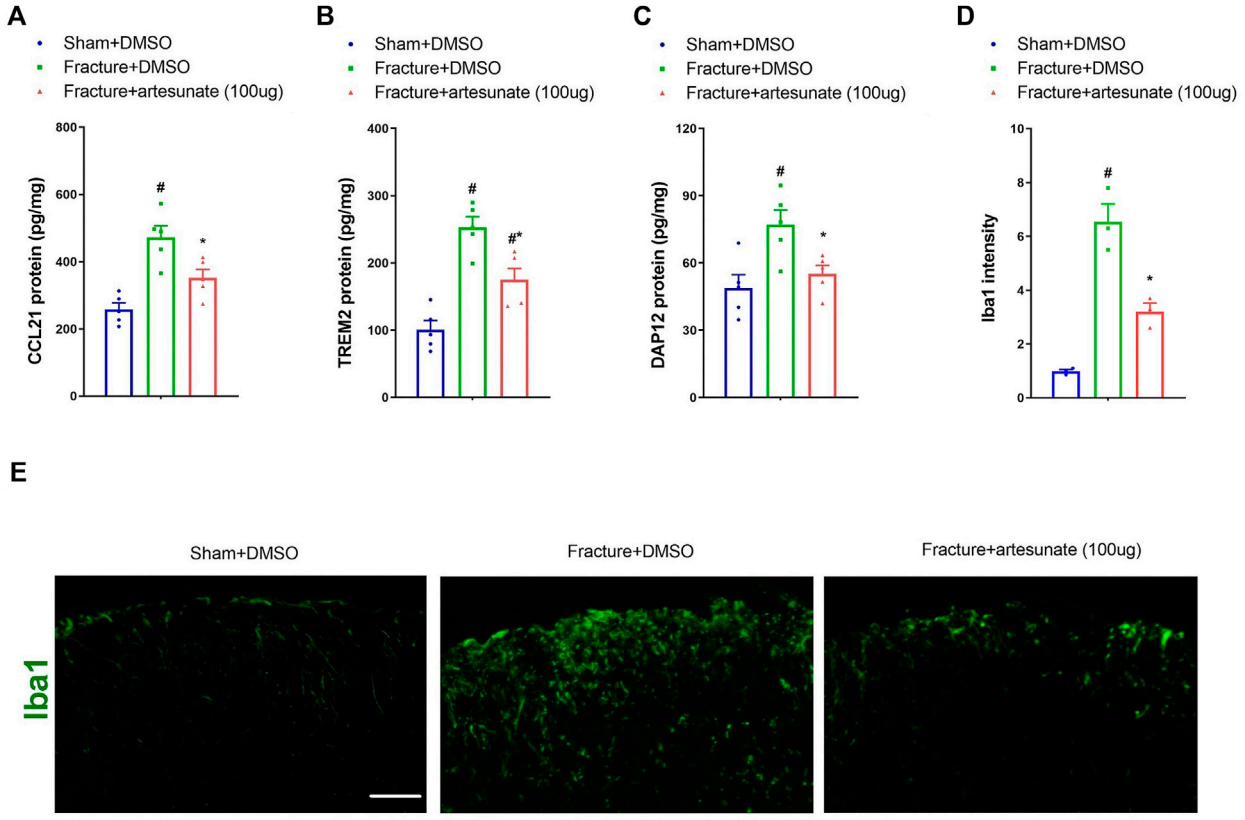

FIGURE 3 | Intrathecal pre-administration of artesunate reduces fracture-associated spinal over-expressions of CCL21, TREM2, and DAP12 and microglia activation. Intrathecal artesunate (100 μg) was administered daily for three consecutive days on days 4, 5, and 6 after tibial fracture with orthopedic surgery. All biochemical data were collected on day 7 after sham and fracture surgeries. **(A–C)** ELISA identified that pretreatment with artesunate downregulated the increased levels of spinal CCL21, TREM2, and DAP12 proteins after tibial fracture. **(D,E)** Immunohistochemistry staining showed representative photomicrographs of the marker of microglia activation (Iba1) in the spinal dorsal horn after fracture intervention and artesunate treatment (scale bar, 50 μm). All biochemical results are expressed as mean ± SEM (n = 3–5) and analyzed by one-way ANOVA with Bonferroni *post hoc* comparisons. $^{\#}p < 0.05$ vs. group sham + DMSO, $^{*}p < 0.05$ vs. group fracture + DMSO.

activation in the spinal dorsal horn of mice with fracture pain has been revealed in previous reports (Li et al., 2015; Shi et al., 2015; Zhang et al., 2016). Herein, we also found the suppression of fracture-related spinal microglia activation by artesunate treatment ($p < 0.05$, $n = 3$; **Figures 3D,E**). All these data suggest that artesunate therapy prevents chronic fracture pain *via* spinal inhibition of neuroinflammation.

Postoperative Treatment With Intrathecal Artesunate Impaired the Established Persistent Pain After Tibial Fracture and Orthopedic Surgery

After the prevention of fracture pain by artesunate was conformed, we further assessed the efficiency of postoperative i.t., artesunate therapy at improving chronic pain. The von Frey tests detected that the single administration of artesunate (i.t., 100 μg) on 14 days after fracture (in the late phase) produced a rapid and transient attenuation of the established mechanical allodynia for 5 h, as reflected by the increase in paw withdrawal threshold [F (1, 70) = 86.87, $p < 0.001$, $n = 8$, two-way ANOVA; **Figure 4A**] and the decrease in paw withdrawal frequency [F (1, 70) = 45.34, $p < 0.001$, $n = 8$, two-way ANOVA; **Figure 4B**] in fracture animals. As parallel, this therapy of artesunate also suppressed the established cold allodynia for 3 h [F (1, 70) = 12.06, $p < 0.001$, $n = 8$, two-way ANOVA; **Figure 4C**].

Spinal Inhibition of CCL21 Attenuates Tibial Fracture-Associated Postoperative Pain

To further examine whether the CCL21 pathway is central for the development of chronic fracture pain, repetitive anti-CCL21 (i.t., 0.1, 1, and 10 μg) was delivered on a daily basis from days 4 to 6 (in the early phase) after tibial fracture with pin insertion. Strikingly, pretreatment with anti-CCL21 at 10 μg but not 0.1 and 1 μg generated a significant alleviation of mechanical and cold allodynia, which sustained for more than 4 days after three injections, as demonstrated by the increase of paw withdrawal threshold [F (5, 210) = 192.9, $p < 0.001$; $n = 8$, two-way ANOVA; **Figure 5A**] and the decrease of cold scores [F (5, 210) = 57.76, $p < 0.001$, $n = 8$, two-way ANOVA; **Figure 5B**] in fracture-treated animals. Moreover, the single injection of anti-CCL21 (10 μg) on

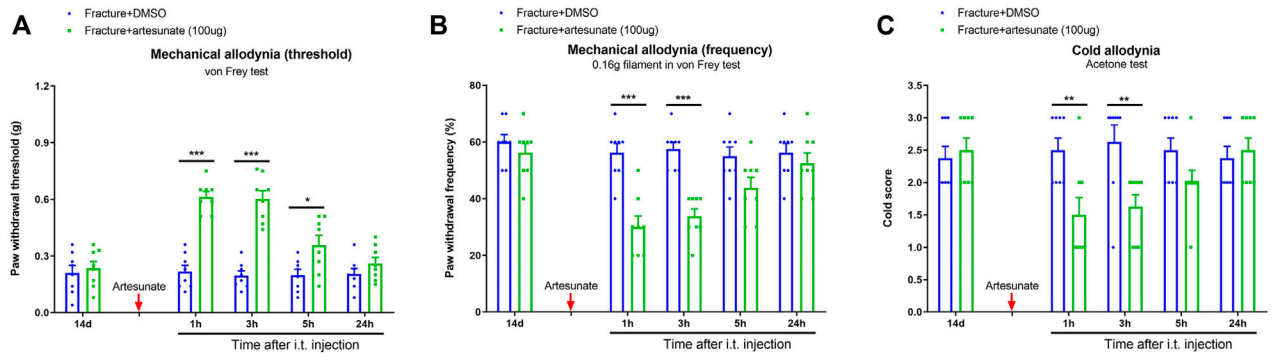

FIGURE 4 | Intrathecal post-treatment of artesunate impairs the established chronic pain after tibial fracture and orthopedic surgery. Artesunate (i.t., 100 μg, indicated by a red arrow) was injected on day 14 (in the late phase) after tibial fracture with pin insertion. The development of mechanical allodynia was assessed by paw withdrawal mechanical threshold **(A)** and paw withdrawal mechanical frequency to 0.16 g filament **(B)** in the von Frey test after tibial fracture. The development of cold allodynia was assessed by cold response scoring **(C)** in acetone test after tibial fracture. Results are mean ± SEM (n = 8) and analyzed by two-way ANOVA with Bonferroni *post hoc* comparisons. *p < 0.05, **p < 0.01, ***p < 0.001 vs. group fracture + DMSO.

day 14 after fracture intervention attenuated the established mechanical allodynia for 3 h [F (1, 70) = 36.96, p < 0.001, n = 8, two-way ANOVA; **Figure 5C**] and cold allodynia for 1 h [F (1, 70) = 7.39, p = 0.008, n = 8, two-way ANOVA; **Figure 5D**]. Thus, these behavioral data suggested that the spinal CCL21 pathway contributes to the production and persistence of chronic postoperative pain following tibial fracture procedures.

Intrathecal Artesunate Therapy Protects Against CCL21-Induced Acute Pain Phenotype and Spinal Increases of TREM2 and DAP12

Then, we further tested the hypothesis that artesunate would control the spinal CCL21-dependent nociception sensitization. We previously reported that acute exposure to recombinant CCL21 (i.t., 0.1 μg) directly initiated an acute allodynia (Wang et al., 2020a). Herein, artesunate (i.t., 100 μg) was injected 60 min prior to CCL21 administration. Interestingly, we found that artesunate intervention ameliorated CCL21-associated mechanical allodynia [F (2, 84) = 142.1, p < 0.001, n = 8, two-way ANOVA; **Figure 6A**] and cold allodynia [F (2, 84) = 26.39, p < 0.001, n = 8, two-way ANOVA; **Figure 6B**]. Furthermore, intrathecal exposure to CCL21 elevated the spinal concentrations of TREM2 and DAP12, which was compromised by artesunate pretreatment (p < 0.05, n = 5; **Figure 6C**). Collectively, these detailed data further illustrated that CCL21-dependent inflammatory signaling (TREM2/DAP12) might be a therapeutic target of artesunate analgesia in pain conditions.

Pharmacological Inhibition of TREM2 Reduces Fracture-Associated Chronic Pain and CCL21-Induced Acute Pain

Next, we evaluated the effect of TREM2 signaling on pathological pain development. Intriguingly, a neutralizing antibody against TREM2 (anti-TREM2, i.t., 2 μg) ameliorated the established mechanical allodynia [F (1, 70) = 37.75, p < 0.001, n = 8, two-way ANOVA; **Figure 7A**] and cold allodynia [F (1, 70) = 16.14, p < 0.001, n = 8, two-way ANOVA; **Figure 7B** following tibial fracture with pin insertion. As parallel, the pre-administration of anti-TREM2 (i.t., 2 μg) reduced the exogenous CCL21-induced upregulation of peripheral mechanical sensitivity [F (1, 56) = 25.2, p < 0.001, n = 8, two-way ANOVA; **Figure 7C**] and cold sensitivity [F (1, 56) = 15.32, p < 0.001, n = 8, two-way ANOVA; **Figure 7D**]. These results indicate a substantial interaction between CCL21 and TREM2/DAP12 in spinal neuroinflammation and pain transduction.

Systemic Therapy of Artesunate Alleviates Fracture-Associated Behavioral Pain After Orthopedic Surgery

Given that artesunate is often administered *via* intraperitoneal injection for the treatment of several diseases in rodents (Cheong et al., 2020; Bang et al., 2021), we finally investigated whether intraperitoneal artesunate therapy was also beneficial to chronic fracture pain. Thus, we administered artesunate (10 and 50 mg kg^{-1}) following intraperitoneal injection on 14 days after orthopedic surgery. Interestingly, the systemic delivery of artesunate at 10 and 50 mg kg^{-1} relieved the fracture-induced mechanical allodynia and cold allodynia, as manifested by the elevation in paw withdrawal mechanical threshold [F (2, 105) = 31.23, p < 0.001, n = 8, two-way ANOVA; **Figure 8A**] and the decrease in cold response scores [F (2, 105) = 8.59, p < 0.001, n = 8, two-way ANOVA; **Figure 8B**]. Such analgesia of intraperitoneal artesunate sustained for 1–3 h.

DISCUSSION

In this current study, the major findings are as follows: first, tibial fracture originates from the persistent mechanical allodynia and cold allodynia with the spinal up-modulation of CCL21 and TREM2/DAP12 expressions following orthopedic surgery. Second, the intrathecal delivery of artesunate prevents fracture-associated

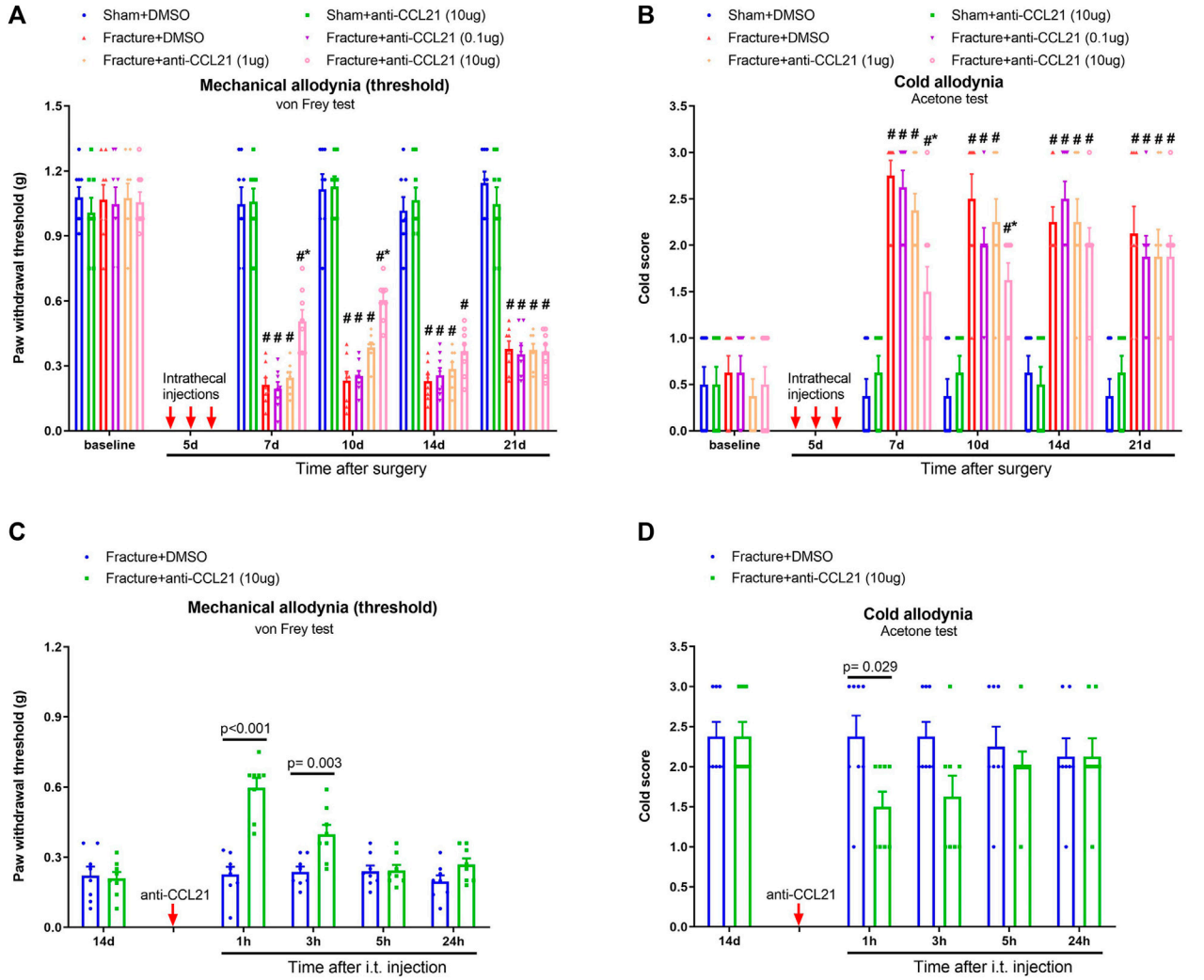

FIGURE 5 | Spinal neutralization of CCL21 reduces fracture-associated postoperative pain. **(A,B)** A neutralizing antibody against CCL21 (anti-CCL21) was intrathecally injected (0.1, 1 and 10 μg) on days 4, 5, and 6 (indicated by red arrows) after fracture surgery. Mechanical allodynia and cold allodynia were measured by the von Frey test and acetone test, respectively. **(C,D)** Intrathecal injection of anti-CCL21 (10 μg) on day 14 (indicated by a red arrow) after fracture surgery significantly inhibited the established mechanical and cold allodynia. All behavioral results are mean ± SEM ($n = 8$) and analyzed by two-way ANOVA with Bonferroni *post hoc* comparisons. $^{\#}p < 0.05$ vs. group sham + DMSO, $^{*}p < 0.05$ vs. group fracture + DMSO.

allodynia in a dose-dependent manner and reduces fracture-caused CCL21 over-expression, as well as the TREM2/DAP12 accumulation in the spinal dorsal horn. Third, the postoperative therapy of both intrathecal and intraperitoneal artesunate attenuates the established fracture allodynia. Fourth, spinal CCL21 neutralization is sufficient to impair the generation and maintenance of fracture-associated pain. Fifth, the pre-administration of artesunate impairs exogenous CCL21-evoked acute inflammatory pain by reducing the spinal TREM2/DAP12 overload. Sixth, the pharmacologic inhibition of TREM2/DAP12 also ameliorates fracture-associated chronic pain and CCL21-elicited acute pain. These results therefore elucidate a previously undescribed role of artesunate as an alleviator of chronic pain following tibial fracture and orthopedic surgery by the inhibition of CCL21-dependent TREM2/DAP12 neuroinflammatory signaling.

Neuroinflammation driven by chemokines is a cardinal feature of chronic pain following peripheral tissue damage, nerve trauma, chemotherapy, and cancer (Ji et al., 2014; Ji et al., 2018; Baral et al., 2019). We previously identified the requirement for chemokine CCL1 and its receptor CCR8 in neuroinflammation and neuronal excitability in fracture-associated pain generation and chronification (Wang et al., 2020b). The contribution of caspase-6 to the upregulation of chemokine CCL21 in the development of opioid-induced hyperalgesia has been recently revealed (Wang et al., 2020a). Moreover, the spinal inhibition of caspase-6-dependent neuroinflammation is effective against functional potentiation in excitatory nociceptive synapses and pain chronification after tibial fracture (Cui et al., 2021). Given that caspase-6 regulates fracture-associated pain and CCL21

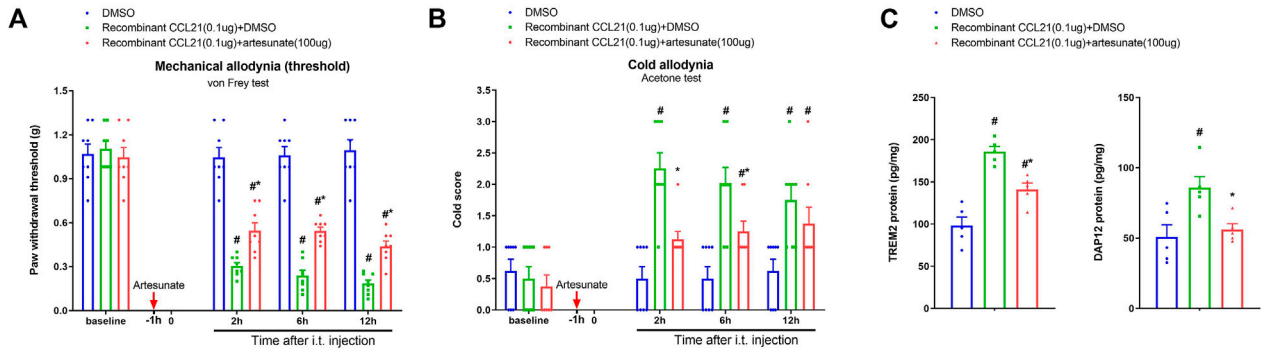

FIGURE 6 | Induction of acute pain by exogenous CCL21 and attenuation of allodynia by artesunate. Artesunate (i.t., 100 μg, indicated by a red arrow) was injected 60 min prior to recombinant CCL21 (i.t., 0.1 μg) application. **(A,B)** Exogenous CCL21-induced acute inflammatory pain was alleviated by the pre-application of artesunate. All behavioral results are mean ± SEM ($n = 8$) and analyzed by two-way ANOVA with Bonferroni *post hoc* comparisons. All biochemical data were collected at hour 12 after intrathecal injection. **(C)** ELISA identified that pretreatment with artesunate downregulated the increased levels of spinal TREM2 and DAP12 proteins after exogenous CCL21 exposure. All biochemical results are expressed as mean ± SEM ($n = 5$) and analyzed by one-way ANOVA with Bonferroni *post hoc* comparisons. $^{\#}p < 0.05$ vs. group DMSO, $^{*}p < 0.05$ vs. group recombinant CCL21 (0.1 μg) + DMSO.

pathway is an important downstream effector of caspase-6 in spinal pain transmission (Wang et al., 2020a; Cui et al., 2021), we examined whether CCL21 contributes to persistent fracture pain. Herein, we initially reported the spinal long-lasting increase of CCL21 expression in mice with tibial fracture and orthopedic surgery, consistent with the time course of chronic mechanical allodynia and cold allodynia. This is the first study demonstrating that spinal CCL21 neutralization not only prevents but also reduces fracture-associated postoperative allodynia. Additionally, we revealed that exogeneous CCL21 intervention following spinal application elicits acute pain behaviors in animals. This evidence strongly suggests the identification of spinal CCL21 pathway in the development of persistent pain after fracture. Still, how spinal CCL21 mediates neuroinflammatory process in chronic fracture pain remains to be investigated.

It is noteworthy that the involvement of TREM2/DAP12 in neuron–microglia interactions is indispensable for neuroinflammation-associated pain perception in several rodent models (Guan et al., 2016; Kobayashi et al., 2016; Wang Y. et al., 2020). Especially, TREM2, as a pivotal factor for microgliosis, is upregulated and promotes the recruitment of cytokines in the pathogenesis of cisplatin-induced peripheral neuropathic pain (Ma et al., 2022). However, the requirement of TREM2 and DAP12 for the pathophysiology of chronic fracture pain is virtually unknown. Interestingly, this is the first study wherein spinal concentrations of TREM2 and DAP12 represent a robust elevation after tibial fracture with pin insertion and TREM2 neutralization impairs chronic fracture pain. Simultaneously, it is clarified, for the first time, that spinal exposure to CCL21 upregulates TREM2 and DAP12 levels and the pharmacological blockage of TREM2 reduces CCL21-caused acute pain. These detailed data point to the possibility that spinal CCL21 over-expression facilitates TREM2 and DAP12 accumulation to further cause nociception phenotypes and that inhibiting this may provide a novel therapeutic target for pain conditions.

Artemisinin and its derivatives perform a potent anti-neuroinflammatory protection on Alzheimer's disease (Qiang et al., 2018), traumatic brain injuries (Zhou et al., 2020), and lipopolysaccharide (LPS)-induced cognitive dysfunction (Lin et al., 2021). Recent investigation has highlighted that artesunate therapy downregulates the severity of bacterial infection, the release of inflammatory mediators, nociception-like phenomena, and septic death (Bang et al., 2021). Artesunate is also effective against opioid-induced acute hyperalgesia and chemical irritant-induced acute inflammatory pain (Guruprasad et al., 2015; Zhang et al., 2022). However, no literature has mentioned the therapeutic role of artesunate in fracture-associated chronic pain. To the best of our knowledge, the present study is the first to uncover that repetitive injections of i.t. artesunate (10 and 100 μg but not 1 μg) prevent long-lasting mechanical and cold allodynia after tibial fracture and orthopedic surgery in a dose-dependent manner. We then discovered the mitigation of the established chronic fracture pain and the prevention of exogenous CCL21-induced acute inflammatory pain by the single application of artesunate (100 μg). Furthermore, this is the first study in which artesunate treatment disrupts spinal CCL21 over-expression and TREM2/DAP12 accumulation in fracture animals. Artesunate also reverses the exogenous CCL21-caused spinal overload of TREM2/DAP12. Strikingly, the intraperitoneal delivery of artesunate (10 and 50 mg kg^{-1}) is sufficient and effective in abrogating fracture-associated chronic pain. Taken together, these results emphasize that artesunate therapy protects against the generation and maintenance of fracture-associated chronic pain through inhibiting spinal CCL21-dependent TREM2/DAP12 inflammatory signaling and microglia activation. Indeed, microgliosis is capable of raising excitatory neuronal responsiveness by promoting the secretion of tumor necrosis factor-α (TNF-α) (Berta et al., 2014). Thus, it will be of great interest to explore whether TNF-α is a

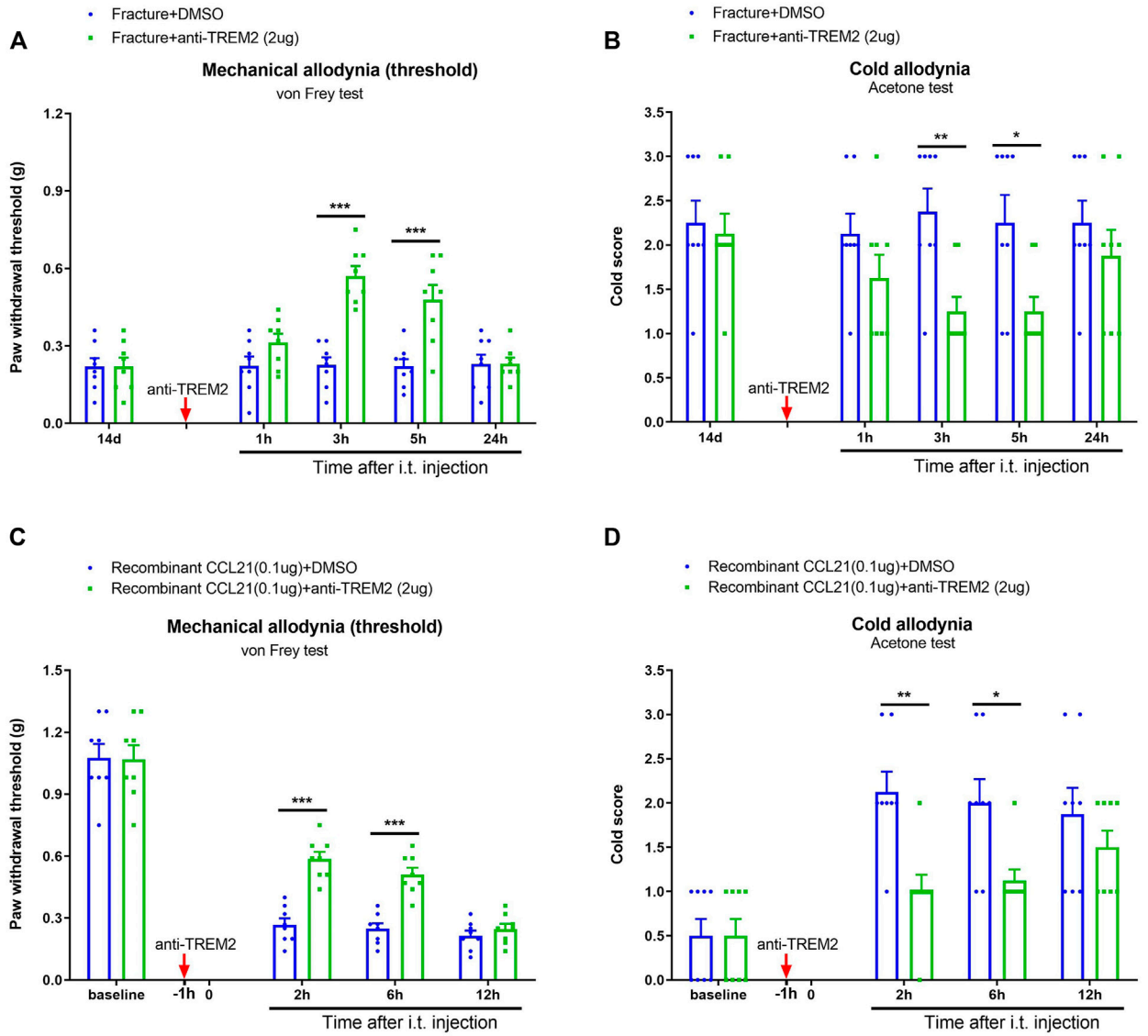

FIGURE 7 | Spinal neutralization of TREM2 reduces fracture-associated chronic pain and CCL21-induced acute pain. **(A,B)** A neutralizing antibody against TREM2 (anti-TREM2, i.t., 2 μg, indicated by a red arrow) was injected on day 14 after tibial fracture. Behavioral test showed the attenuation of the established fracture-associated mechanical allodynia and cold allodynia by anti-TREM2. **(C,D)** Anti-TREM2 (i.t., 2 μg, indicated by a red arrow) was injected 60 min prior to recombinant CCL21 (i.t., 0.1 μg). Exogenous CCL21-induced acute inflammatory pain was alleviated by the pre-application of anti-TREM2. All results are mean ± SEM ($n = 8$) and analyzed by two-way ANOVA with Bonferroni *post hoc* comparisons. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

potential downstream effector in the anti-nociceptive mechanisms of artesunate therapy.

Apart from its anti-inflammatory properties, artemisinin and its derivatives can activate the antioxidant system to exert potent neuroprotective effects on hydrogen peroxide (H_2O_2)-induced retinal neuronal dysfunction, sodium nitroprusside-induced cortical neurotoxicity, and neurodegenerative disease (Zheng et al., 2016; Yan et al., 2017; Li et al., 2019). Oxidative insult-related spinal nociception sensitization has been identified to be a cardinal step for the generation of fracture-induced pain (Guo et al., 2018; Guo et al., 2021). Accordingly, further investigations are warranted to study whether oxidative molecular signaling is involved in artesunate analgesia for fracture intervention. Previous reports disclosed that neuroinflammation underlies the central pain sensitization and allodynia initiation *via* α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor activation in dorsal horn neurons of fractured animals (Wang et al., 2020b; Cui et al., 2021). However, the connection between artesunate and AMPA receptor in our model remains largely undefined. There are several limitations to be considered. First, we did not evaluate the anti-nociceptive potency of artesunate treatment for females, which should be addressed by further studies.

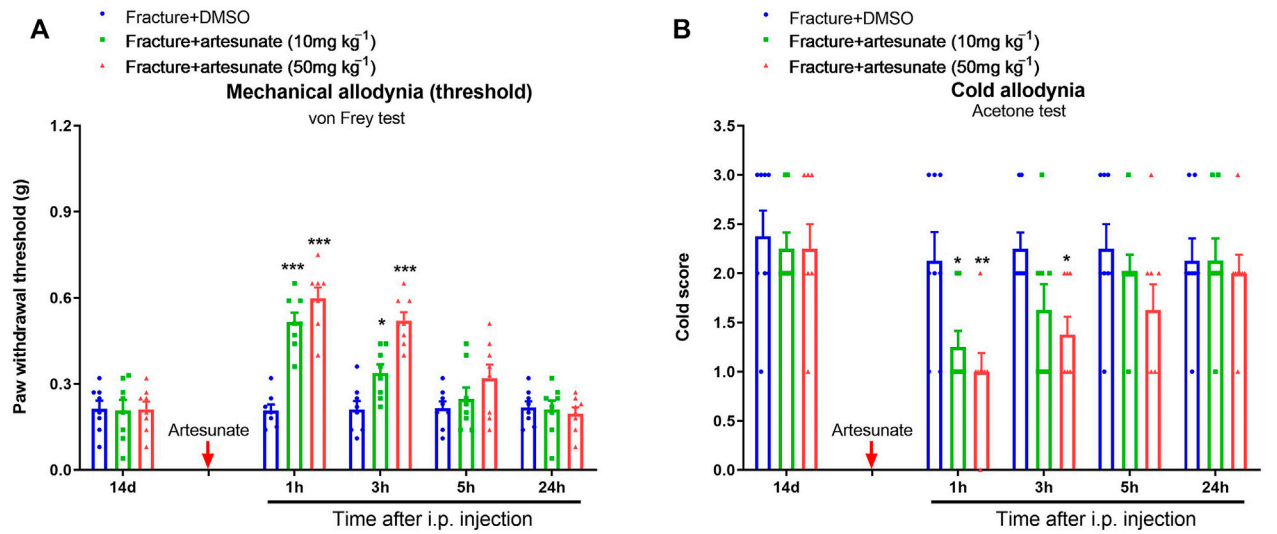

FIGURE 8 | Systemic post-treatment of artesunate impairs the established chronic pain after tibial fracture and orthopedic surgery. Artesunate (10 and 50 mg kg^{-1}, indicated by a red arrow) was intraperitoneally (i.p.) injected on day 14 (in the late phase) after tibial fracture with pin insertion. The development of mechanical allodynia was assessed by paw withdrawal mechanical threshold **(A)** in the von Frey test after tibial fracture. The development of cold allodynia was assessed by cold response scoring **(B)** in acetone test after tibial fracture. Results are mean ± SEM ($n = 8$) and analyzed by two-way ANOVA with Bonferroni *post hoc* comparisons. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. group fracture + DMSO.

Second, given that all experiments in our study were performed using adult male C57BL/6J mice (8–10 weeks old) without significant different body weights, we selected the same dose of artesunate through intrathecal injection, but it is unclear whether this dose of artesunate is sufficient to animals with larger body weights. Third, the intrathecal injection of drugs or antibodies can both affect spinal cord and DRG, so it will be important to explore whether the CCL21-dependent TREM2/DAP12 pathway in DRG is involved in artesunate analgesia. Fourth, although we administered the spinal application of exogenous CCL21 protein and neutralizing antibody against CCL21 to elucidate the mechanism of artesunate analgesia, it will be of great importance to further utilize spinal knockdown and over-expression of CCL21 gene in future. Another limitation is the failure to investigate whether other artemisinin derivatives (such as dihydroartemisinin and artemether) are also effective in controlling chronic fracture pain.

In summary, the current findings recapitulate an unconventional pharmacological role of artesunate in the prevention and alleviation of fracture-associated postoperative pain by inhibiting spinal CCL21-dependent TREM2/DAP12 inflammatory processes. These results suggest that artesunate administration and CCL21 neutralization may generate innovative therapeutic concepts for a targeted neurotherapeutic strategy for fracture pain in clinics.

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 animal study was reviewed and approved by the Animal Ethical and Welfare Committee of Tianjin Medical University (Tianjin, China).

AUTHOR CONTRIBUTIONS

LZ and YL conceived the experiment; NL, CW, HZ, YW, and TG collected the data; YZ, GW, and YY analyzed the data; and LZ, NL, and YL wrote the article.

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Phosphatase and Tensin Homology Deleted on Chromosome 10 Inhibitors Promote Neural Stem Cell Proliferation and Differentiation

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Phosphatase and tensin homology deleted on chromosome 10 (PTEN) is a tumor suppressor gene. Its encoded protein has phosphatase and lipid phosphatase activities, which regulate the growth, differentiation, migration, and apoptosis of cells. The catalytic activity of PTEN is crucial for controlling cell growth under physiological and pathological conditions. It not only affects the survival and proliferation of tumor cells, but also inhibits a variety of cell regeneration processes. The use of PTEN inhibitors is being explored as a potentially beneficial therapeutic intervention for the repair of injuries to the central nervous system. PTEN influences the proliferation and differentiation of NSCs by regulating the expression and phosphorylation of downstream molecular protein kinase B (Akt) and the mammalian target of rapamycin (mTOR). However, the role of PTEN inhibitors in the Akt/mTOR signaling pathway in NSC proliferation and differentiation is unclear. Dipotassium bisperoxo (picolinoto) oxovanadate (V) [bpv(pic)] is a biologically active vanadium compound that blocks PTEN dephosphorylation and suppresses its activity, and has been used as a PTEN lipid phosphatase inhibitor. Here, bpv(pic) intervention was found to significantly increase the number of rat NSCs, as determined by bromodeoxyuridine staining and the cell counting kit-8, and to increase the percentage of neurons undergoing differentiation, as shown by immunofluorescence staining. Bpv(pic) intervention also significantly increased PTEN and mTOR expression, as shown by real-time PCR analysis and western blotting. In conclusion, PTEN inhibitor bpv(pic) promotes the proliferation and differentiation of NSCs into neurons.

Keywords: Pten, mTOR, neural stem cells, proliferation, differentiation

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INTRODUCTION

As an anti-oncogene with dual specific phosphatase activity, phosphatase and tensin homology deleted on chromosome 10 (PTEN) has become a research hotspot in recent years. It plays an important role in a variety of diseases, including cancer, liver disease (Ikeda et al., 2020; Chen et al., 2021), and diabetes (Lu et al., 2021), where it is involved in cell migration, proliferation,

differentiation, apoptosis, and metabolism (Yamada and Araki, 2001; Hamada et al., 2005; Salmena et al., 2008; Chow and Salmena, 2020).

PTEN mainly catalyzes the conversion of phosphatidylinositol trisphosphate (PIP3) to phosphatidylinositol biphosphate (PIP2) by inhibiting the classical phosphatidylinositol 3 kinase (PI3K)-serine/threonine kinase (Akt) signaling pathway (Song et al., 2005). When PI3K receives signals from tyrosine kinase and G protein-coupled receptors, activated PI3K converts PIP2 to PIP3, and reduces PIP3 to PIP2. PIP3 then binds to the N-PI3KPH domain of downstream Akt, which is transferred from the cytoplasm to the cell membrane (Park et al., 2010).

With the assistance of 3-phosphoinositol-dependent protein kinase 1, PIP3 activates Akt by phosphorylating its threonine phosphorylation site (Thr308) or serine phosphorylation site (Ser473). Activated Akt then activates mammalian target of rapamycin (mTOR). The PI3K/Akt/mTOR signaling pathway activates and regulates cell proliferation, differentiation, and migration (Jung et al., 2021). The pathway is also involved in the repair and regeneration of central nerve injuries, as shown by PTEN gene knockout using a PTEN inhibitor or small interfering RNA which accelerated the growth of axons at the injured site (Lu et al., 2020). Although PTEN is not required to determine cell fate in the central nervous system (CNS), it was shown to function in NSC differentiation, where its expression changes dynamically. PTEN expression begins in the late stages of mouse CNS development and peaks in adulthood. It is widely expressed in the brain of adult mice, especially in neurons (Li et al., 2020; Yu et al., 2020).

mTOR is an important signaling molecule in the PTEN signaling pathway, which regulates pentameric neuronal ASH2-like, histone lysine methyltransferase complex subunit at the transcriptional level (Nguyen and Anderson, 2018). Consequently, it affects neuronal differentiation and directional axonal outgrowth (Jia et al., 2021). PI3K/AKT/mTOR signaling was shown to regulate neuronal cell maturation and differentiation, while Park (Park et al., 2008) reported regeneration of the optic nerve after PTEN knockdown following the reactivation of PI3K/Akt/mTOR signaling. PTEN also regulates neuronal apoptosis, proliferation, renewal, and differentiation, and inhibits neuronal regeneration by inhibiting transduction of the PI3K/AKT signaling pathway. Thus, inhibiting PTEN promotes the survival and differentiation of NSCs.

Vanadium and vanadium peroxide compounds are widely used as general inhibitors of protein tyrosine phosphatase, especially bisperoxovanadium compounds which include dipotassium bisperoxo (picolinoto) oxovanadate (V) [bpV(pic)] (vanadium diperoxys 5-hydroxypyridine). Bpv(pic) is a specific inhibitor of PTEN that promotes neural stem cell (NSC) proliferation and differentiation *in vitro* and *in vivo*, with no significant effect on cell survival (Guan et al., 2021). Together, these findings suggest that PTEN plays an important role not only in peripheral nerve damage but also in the repair and regeneration of central nerve injury.

In this study, we examined the role of a PTEN inhibitor in NSC proliferation and differentiation. We found that inhibiting PTEN expression decreased neuronal proliferation and differentiation through the activation of PI3K/Akt/mTOR signaling. Our findings enhance our understanding of the mechanism of NSC differentiation during neurogenesis.

MATERIALS AND METHODS

Cell Lines and Reagents

Sixteen-day-old pregnant SD rats (Guan et al., 2015) were provided by the Laboratory Animal Center of Nantong University. This study was conducted in accordance with the recommendations of the National Institutes of Health Laboratory Animal Care and Use Guidelines. The isolated fetal rat cerebral cortex was removed under aseptic conditions, meninges were stripped in Dulbecco's modified Eagle medium (DMEM) containing 0.25% trypsin for 10 min, and the cell suspension was obtained in DMEM containing 5% horse serum and 10% fetal bovine serum (Gibco, Grand Island, NY, United States) at a density of 1×10^6 cells/ml. Cells were then cultured at 37°C with 5% CO_2 in DMEM supplemented with neurobasal neuron-specific medium (Gibco) containing 1% B-27 supplement and 0.25% L-Glutamine.

Proliferation of NSCs After bpv(pic) Intervention

After harvesting, the second generation of NSCs was seeded into 24-well plates at a density of 5×10^4 cells/mL. Bpv(pic) (ATCC, Manassas, VA, United States) was added to the intervention group at a final concentration of 200 nmol/L (Thellung et al., 2019). NSCs were cultured for 5–7 days at 37°C with 5% CO_2, then the number of cells was determined using the cell counting kit-8 (CCK-8; Abcam) and compared between the two groups. Briefly, cell proliferation was measured by adding 100 μL DMEM/F-12 and 10 μL CCK-8 reagent to each plate, and incubating for 8 h at 37°C with 5% CO_2. The absorbance at 425 nm was then measured using the Multiskan MK33 microplate reader (Thermo Electron Corporation, Shanghai, China). Bromodeoxyuridine (BrdU) solution was also added to the intervention group at a final concentration of 5 μmol/L to stain proliferating neonatal neurons which were observed using an Olympus IX71 microscope.

NSC Differentiation

NSCs were inoculated at a density of 5×10^4 cells/ml into 24-well plates with polylysine-coated glass slides in differentiation solution (DMEM/F-12 supplemented with 1% fetal bovine serum) which was changed after 2 h (Guan et al., 2015). Bpv(pic) was added to the intervention group at a final concentration of 200 nmol/L, and all cells were cultured for a further 7 days. Cells were then incubated with the following primary antibodies at 4°C for 16 h: rabbit anti-rat βIII tubulin antibody (diluted 1:1000; Abcam), mouse anti-rat glial fibrillary acidic protein (GFAP) antibody (diluted 1:1000; Abcam), and rabbit anti-rat receptor interacting protein (RIP) antibody (diluted 1:1000; Abcam). They were then incubated with goat

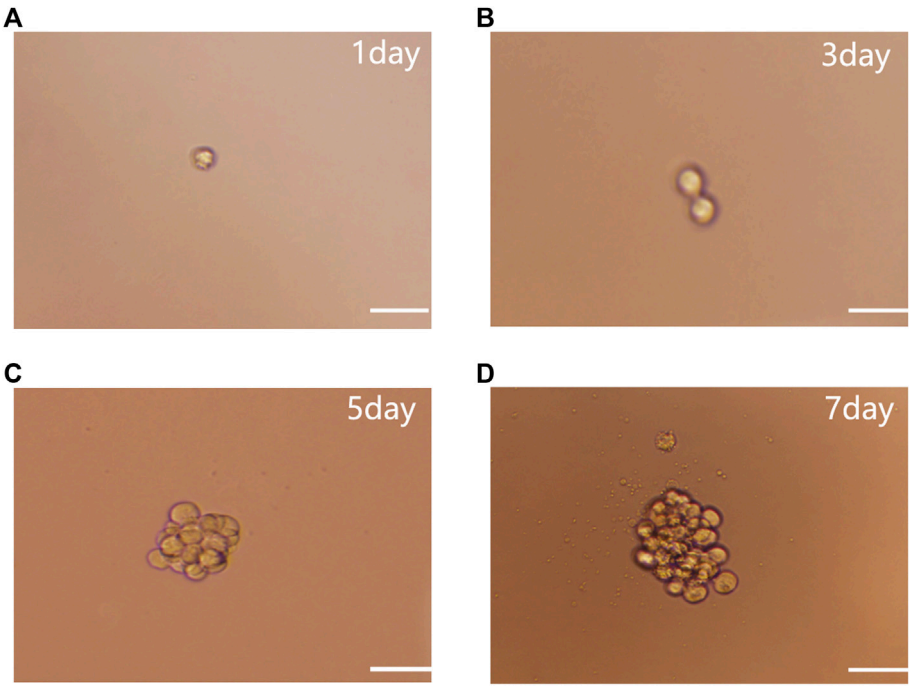

FIGURE 1 | NSC single cell cloning. **(A)** Single cell culture. **(B)** After 3 days culture. **(C)** After 5 days culture. **(D)** Proliferation to form NSCs after 7 days subculture. Scale bar: 100 μm.

anti-rabbit IgG H&L (Alexa Fluor® 594) (diluted 1:1000; Abcam) secondary antibodies at 20°C for 2 h. DNA was stained by immediately incubating the slides in 4′,6-diamidino-2-phenylindole (0.2 mg/ml) for 2 min. Slides were stored in the dark at 4°C, then six fields of view per slide were randomly selected. The percentage of positively staining cells in each field was calculated under an Olympus IX71 microscope, and the average value was compared between control and intervention groups.

Real-Time PCR Analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, United States), then reverse-transcribed into cDNA using the Omniscript RT Kit (Qiagen) according to the manufacturer's instructions. PCR was carried out using the following conditions: 95°C for 2 min, then 30 cycles of 95°C for 15 s, 54°C for 30 s, and 72°C for 1 min (Guan et al., 2015). Primer sequences were: mTOR-F: 5′-AGGAGGGACGTTTGC TCAGA-3′ and mTOR-R: 5′-TCCCTCACTGAACACAGCAG-3′; PTEN-F: 5′-ACCAGGACCAGAGGAAACCT-3′ and PTEN-R: 5′-TTTGTCAGGGTGAGCACAAG-3′; and β-actin-F: 5′-AGGCATCCTGACCCTGAAGTAC-3′ and β-actin-R: 5′-TCT TCATGAGGTAGTCTGTCAG-3′.

Western Blotting

Membranes were incubated with primary antibodies against β-actin (diluted 1:3000; Abcam), PTEN (diluted 1:1000; Abcam), and mTOR (diluted 1:1000; Abcam).

Statistical Analysis

All assays were performed in duplicate a total of three times. Data are expressed as the mean ± SEM, and were analyzed by the Student's t-test and one-way analysis of variance. SPSS v. 17.0 statistical software was used for analysis, and p values ≤ 0.05 were considered statistically significant.

RESULTS

NSCs Self-Renewed and Proliferated

Single-cell cloning experiments showed that individual NSCs (**Figure 1A**) divided after 3 days (d) of culture (**Figure 1B**), exhibited colonies of 15–28 cells after 5 days (**Figure 1C**), and proliferated to form a colony of around 50 cells after 7 days (**Figure 1D**). This suggests that colony formation occurred through the self-renewal and proliferation of NSCs rather than the aggregation of individual NSCs.

Bpv(pic) Promoted NSC Proliferation

BrdU staining showed that the number of NSCs in the intervention group (65 ± 6 cells) was significantly higher than in the control group (42 ± 5 cells) ($p < 0.05$) (**Figure 2A**). Absorbance values were 0.997 ± 0.085 and 0.788 ± 0.083 for the intervention and control groups, respectively. The CCK-8 assay found that bpv(pic) significantly inhibited the proliferation of the intervention group compared with the control ($p < 0.05$). These data together suggest that bpv(pic) promoted the proliferation of NSCs (**Figure 2B**).

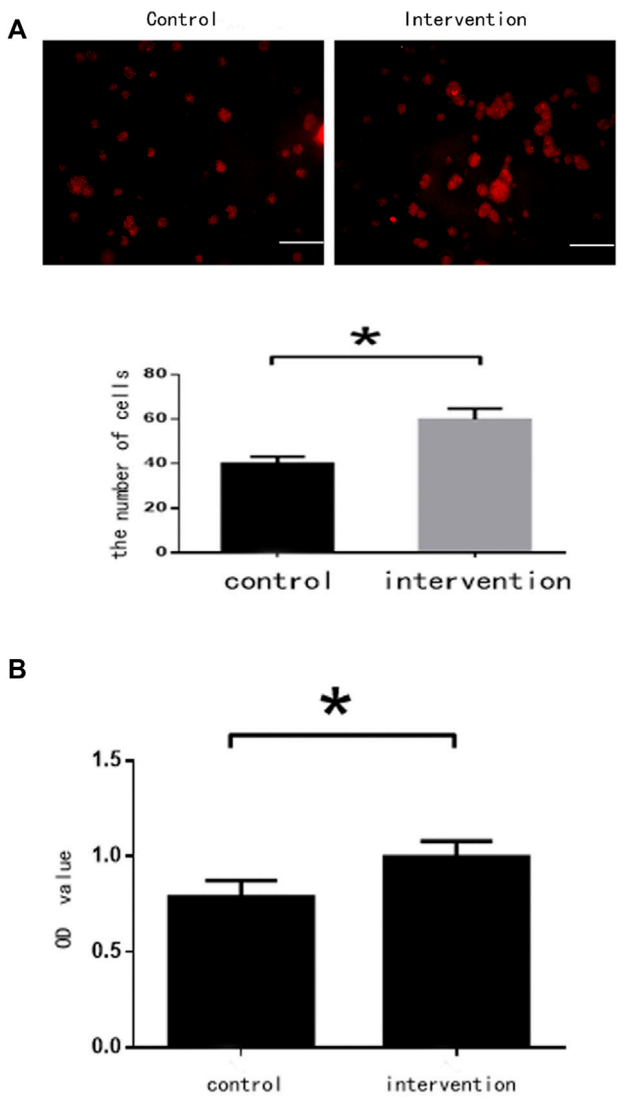

FIGURE 2 | Cell assessments 5 days after bpv(pic) intervention. **(A)** Cells after staining with BrdU. Scale bar: 100 μm. **(B)** Cell proliferation as detected by the CCK-8 assay.*$p < 0.05$.

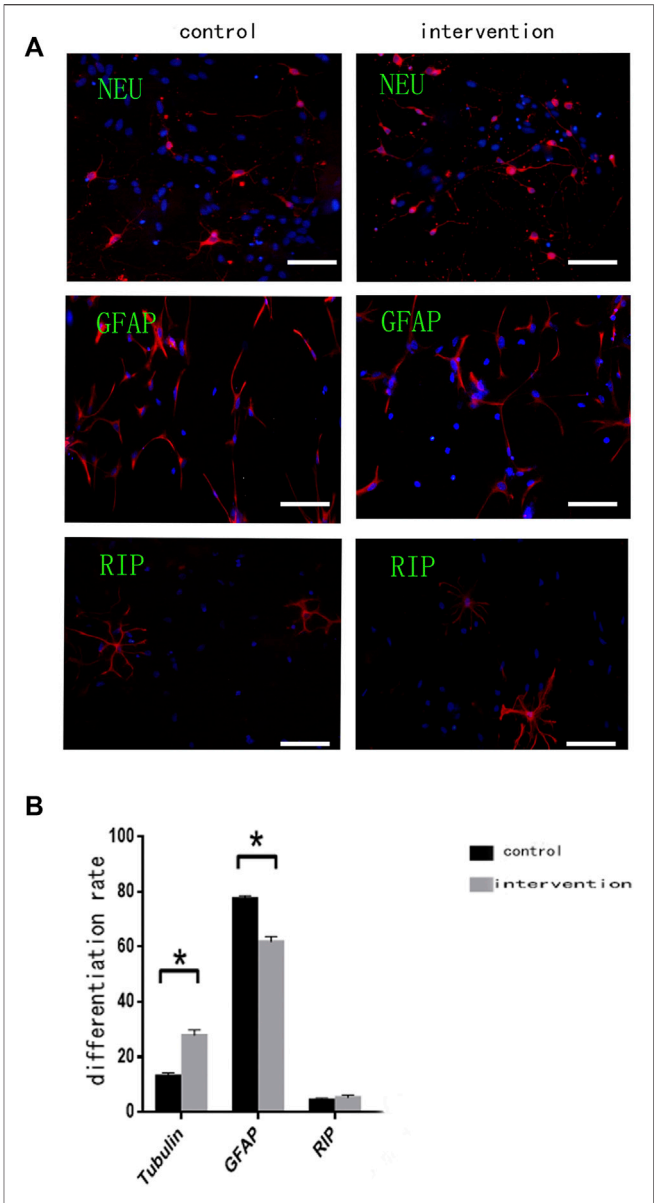

FIGURE 3 | Bpv(pic) modulates the differentiation of NSCs. **(A)** Immunofluorescence using anti-β-Tubulin III, anti-GFAP, and anti-RIP antibodies after bpv(pic) intervention. Scale bar: 100 μm. **(B)** Statistical analysis of immunofluorescence. *$p < 0.05$.

Bpv(pic) Promoted the Differentiation of NSCs Into Neurons and Inhibited Their Differentiation Into Glial Cells

Immunofluorescence staining (**Figure 3A**) with anti-βIII tubulin, anti-GFAP, and anti-RIP antibodies showed that the percentage of NSCs differentiating (**Figure 3B**) into neurons was significantly higher in the intervention group (27.860 ± 1.927%) than in the control group (13.120 ± 1.130%) ($p < 0.05$). Moreover, the percentage of differentiated glial cells was significantly lower in the intervention group (61.900 ± 1.840%) than in the control group (77.520 ± 1.035%) ($p < 0.05$). Some NSCs differentiated into oligodendrocytes, but there was no significant difference in the percentage of these between the two groups ($p > 0.05$).

Bpv(pic) Enhanced the Expression of mTOR and PTEN in NSCs

RT-PCR (**Figure 4A**) and western blotting (**Figure 4B**) were used to detect the expression of mTOR and PTEN at mRNA and protein levels, respectively. We observed significantly increased expression of mTOR and PTEN in the intervention group compared with the control group ($p < 0.05$), with a greater increase seen in mTOR expression.

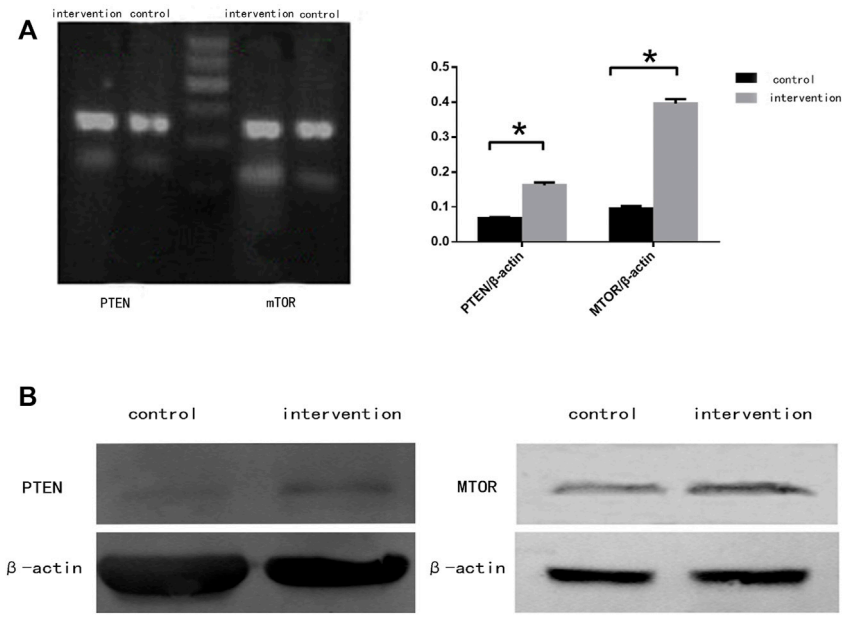

FIGURE 4 | mTOR and PTEN expression in NSCs 5 days after bpv (pic) intervention. **(A)** mRNA expression of mTOR and PTEN in rat NSCs. **(B)** Protein expression of mTOR and PTEN in rat NSCs. β-actin was used as a loading control. *$p < 0.05$.

DISCUSSION

Nerve regeneration and repair play important roles in nerve function recovery. NSCs are key cells in these processes because of their potential for self-renewal and multidirectional differentiation (Rueger and Androutsellis-Theotokis, 2013), although further research is needed to fully understand their involvement (Saha et al., 2012). Inhibiting PTEN expression was shown to increase the survival and proliferation of mesenchymal stem cells in myocardial infarction (Feng et al., 2020), while the proliferation of NSCs and neural progenitor cells is significantly increased following PTEN deletion. Thus, the study of molecular mechanisms that affect NSC proliferation and differentiation is crucial to promoting the repair of neural function.

PTEN is the first tumor suppressor gene known to encode a protein with phosphatase activity. It plays an important role in a variety of diseases by affecting cell proliferation, differentiation, apoptosis, and metabolism, and achieves its physiological effects by interacting with a series of downstream effector molecules (Kuchay et al., 2017). mTOR is one such signal molecule in the PTEN signaling pathway, which is activated through phosphorylation and mediates a series of downstream molecules to promote the synthesis of cellular proteins and cell growth (Yoon and Chen, 2008).

Bpv(pic) is a compound that changes the structure and inactivates the cysteine residues within the catalytic region of protein tyrosine phosphatases, including PTEN. Therefore, we used bpv(pic) as a PTEN inhibitor to investigate its effects on NSC proliferation and differentiation (Mak et al., 2010; Zhang et al., 2017). Bpv(pic) was previously shown to significantly enhance NSC proliferation using a mechanism involving activation of the Akt/mTOR signaling pathway (Zeng and Zhou, 2008; Li et al., 2009). In the present study, we observed a significantly higher number of NSCs after bpv(pic) treatment compared with the control. Additionally, we detected significantly increased expression of PTEN and mTOR in NSCs treated with bpv(pic). This increase in mTOR reflects inhibition of the action of PTEN and an increase, rather than a corresponding decrease, in PTEN expression itself. Because bpv(pic) did not interfere with PTEN expression at the molecular level, bpv(pic) combined with downstream molecules of PTEN, leading to positive feedback that increased PTEN expression (Que et al., 2007; Winbanks et al., 2007). After bpv(pic) treatment, downstream pathways were activated to increase the expression level of mTOR and affect cell proliferation and differentiation. In nerve cells, the function of mTOR must be maintained within a certain range to promote cell differentiation. However, there is currently no consensus on whether inhibiting PTEN to increase mTOR expression Chappell et al., 2011 promotes or inhibits cell differentiation.

Our findings suggest that bpv(pic) inhibits the expression of PTEN and promotes the migration and differentiation of NSC into neurons, thus enhancing the repair of central nervous system injuries. This should be explored in future work to investigate potential treatments of central nerve injury.

CONCLUSION

In summary, the PTEN inhibitor bpv(pic) promoted the proliferation of NSCs and their differentiation into neurons to some extent. This demonstrates the potential of bpv(pic) to be used in the recovery and treatment of CNS injuries.

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

Animal experiments were approved by the Experimental Animal Ethics Committee of the Affiliated Haian Hospital of Nantong University. All animal experiments were performed in accordance with the recommendations of the National Institutes of Health Laboratory Animal Care and Use Guidelines. Appropriate measures were taken to minimize the use of animals as well as their suffering.

AUTHOR CONTRIBUTIONS

XJL and YXG wrote the paper and conceived of and designed the experiments. YQC; JL and JHS analyzed the data. CG; SC and JRD collected and provided the samples for this study. All authors have read and agreed to the published version of the manuscript.

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REVIEW
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Neuroinflammation Involved in Diabetes-Related Pain and Itch

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Diabetes mellitus (DM) is a global epidemic with increasing incidence, which results in diverse complications, seriously affects the patient quality of life, and brings huge economic burdens to society. Diabetic neuropathy is the most common chronic complication of DM, resulting in neuropathic pain and chronic itch. The precise mechanisms of diabetic neuropathy have not been fully clarified, hindering the exploration of novel therapies for diabetic neuropathy and its terrible symptoms such as diabetic pain and itch. Accumulating evidence suggests that neuroinflammation plays a critical role in the pathophysiologic process of neuropathic pain and chronic itch. Indeed, researchers have currently made significant progress in knowing the role of glial cells and the pro-inflammatory mediators produced from glial cells in the modulation of chronic pain and itch signal processing. Here, we provide an overview of the current understanding of neuroinflammation in contributing to the sensitization of the peripheral nervous system (PNS) and central nervous system (CNS). In addition, we also summarize the inflammation mechanisms that contribute to the pathogenesis of diabetic itch, including activation of glial cells, oxidative stress, and pro-inflammatory factors. Targeting excessive neuroinflammation may provide potential and effective therapies for the treatment of chronic neuropathic pain and itch in DM.

Keywords: neuroinflammation, diabetes mellitus, diabetic pain, diabetic itch, sensitization

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INTRODUCTION

Diabetes mellitus (DM), one of the most serious metabolic diseases, is becoming the largest global epidemic of the 21st century, which causes multiple serious complications, such as neuropathic pain and diabetic itch. DM seriously affects the lives and economics of individuals, families, and societies (Stratton et al., 2000; Madsen et al., 2019; Calcutt, 2020; Rayego-Mateos et al., 2020; Schmitz et al., 2021). Diabetic neuropathy is one of the most prevalent comorbidities in patients with type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM), which results in chronic pain and itching (Dewanjee et al., 2018; Zakin et al., 2019). More than 50% of diabetic patients develop diabetic neuropathy (Papanas and Ziegler, 2015; Feldman et al., 2019). Diabetic peripheral neuropathy (DPN), as the common form of diabetic neuropathy, leads to neuropathic pain with a characteristic

Abbreviations: AGEs, advanced glycation end-products; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNS, central nervous system; DM, diabetes mellitus; DPN, diabetic peripheral neuropathy; IL, interleukin; MGO, methylglyoxal; Nav, voltage-gated sodium; NMDA, N-methyl-D-aspartic acid; pDN, painful diabetic neuropathy; PNS, peripheral nervous system; SCs, schwann cells; SGCs, satellite glial cells; STZ, streptozotocin; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; TNF-α, tumor necrosis factor-alpha; TRPA1, transient receptor potential channel ankyrin 1.

TABLE 1 | Terms and related definitions or description.

Terms	Definitions or description	References
Neuropathic pain	The pain caused by a somatosensory nerve lesion or disease	Haanpää et al. (2011) Loeser and Treede (2008)
Diabetic neuropathy	People with diabetes usually develop this neurodegenerative disorder that affects the sensory axons, autonomic axons, and some motor axons	Calcutt (2020)
Diabetic peripheral neuropathy (DPN)	The most common form of diabetic neuropathy is featured by injury to neurons, SCs, and blood vessels within the nerve. The consequence is distressing and costly clinical sequelae, such as leg amputations, foot ulcerations, and neuropathic pain with a characteristic "stocking-glove" pattern	Feldman et al. (2019)
Painful diabetic neuropathy (pDN)	Diabetics experience pain directly as a result of abnormalities in the somatosensory system	Tesfaye et al. (2013) Jensen et al. (2021)
Central sensitization	Increase in the sensitivity of neurons in the central pain or itch pathway to normal or subthreshold afferent input. When peripheral injury or inflammation occurs, persistent stimulation of nociceptors or pruriceptors leads to an increase in excitability of central pathways or a decrease in the activity of inhibitory pathways	Loeser and Treede (2008) Cevikbas and Lerner (2020)
Peripheral sensitization	The nociceptors and pruriceptors in the PNS have an increase in responsiveness or a decrease in threshold to the stimulation in their receptive fields	Loeser and Treede (2008) Gao and Ji (2010) Lavery et al. (2016)
Itch (pruritus)	An uncomfortable cutaneous sensation that initiates the desire to scratch	Ikoma et al. (2006) Lee et al. (2016)
Chronic itch	An unpleasant sensation that leads to intensive scratching lasting 6 weeks or longer	Cevikbas and Lerner (2020)

"stocking-glove" pattern. Neuropathic pain, one type of chronic pain, is caused by a lesion or dysfunction of the peripheral or central somatosensory nervous system (Calcutt, 2020). Over one-third of patients with diabetic neuropathy develop neuropathic pain (Veves et al., 2008; Bansal et al., 2014). In recent years, diabetic neuropathic pain (DNP) is getting more and more attention, numerous studies have been conducted to identify the underlying pathological mechanisms in the hope of developing related therapeutic targets, even if the procedure is full of challenges and failures.

Itching (also termed pruritus) is the irritating sensation in the skin that initiates a desire for scratching (Ikoma et al., 2006; Lee et al., 2016; Dong and Dong, 2018). Patients with systemic diseases such as skin, kidney, or liver diseases suffer from chronic itching that is debilitating and has a serious impact on their quality of life (Yosipovitch and Bernhard, 2013). Chronic itching is also a common symptom of diabetic neuropathy. Unfortunately, the etiology of itching involved in diabetic neuropathy remains poorly understood and therapeutic strategy is inadequate.

Hallmarks of neuroinflammation includes the infiltration of immune cells, as well as the activation of glial cells [e.g., Schwann cells (SCs), satellite glial cells (SGCs), microglia and astrocytes], and increased of inflammatory mediators (e.g., pro-inflammatory cytokines, chemokines) in the peripheral nervous system (PNS) and central nervous system (CNS). Accumulating evidence suggests that neuroinflammation plays a significant role in the pathogenesis and progression of chronic pain and itching (Ellis and Bennett, 2013; Ji et al., 2014; Perera et al., 2015; Borghi et al., 2019; Cevikbas and Lerner, 2020), particularly neuroinflammation-driven sensitization contributes to the development and maintenance of DNP and chronic itching.

Here we review the current progress of neuroinflammation in PNS and CNS that contributes to the induction and maintenance of DNP, as well as existing treatment therapies for this pain. We highlight the important roles of neuroinflammation-driven sensitization involved in DNP. In addition, the neuroinflammation mechanisms contributing to the pathogenesis of diabetic itching are also summarized. An expanding understanding of the contribution of neuroinflammation-driven neuropathic pain and chronic itching in diabetes is helping to identify new therapeutic targets for the treatment of neuropathic pain and chronic itch in diabetes.

DEFINITIONS AND TERMS ASSOCIATED WITH DIABETES-INDUCED PAIN AND ITCH

To better understand this review, some definitions, and terms associated with diabetes-induced pain and itching are listed in **Table 1**.

EPIDEMIOLOGY

Approximately 6.9%–10% of the general population is affected by neuropathic pain (Bouhassira et al., 2008; van Hecke et al., 2014; St John Smith, 2018). The increasing incidence is probably due to the aging population, high incidence of diabetes, and improved survival of cancer patients with subsequent chemotherapy (St John Smith, 2018). There is a higher incidence of chronic neuropathic pain in female patients than in male patients (8% vs. 5.7%), and in adults over 50 years of age than in those under 49 years of age (8.9% vs. 5.6%) (Bouhassira et al., 2008). Diabetes Atlas (9th edition, United Nations, 2019) edited by the international diabetes federation (IDF) describes 460 million (prevalence is ~9.3%) diabetic patients in the general population in 2019 (Saeedi et al., 2019), and more than half of these patients suffered from neuropathy (Dyck and Giannini, 1996; Pop-Busui et al., 2009; Callaghan et al., 2015), of whom ~1/3 develop neuropathic pain (Daousi et al., 2004; Abbott et al., 2011; Bouhassira et al., 2013). The prevalence of painful diabetic neuropathy (pDN) is ranging from 10 to 50% of all DM patients, (Abbott et al., 2011; Bouhassira et al., 2013; Alleman et al., 2015;

TABLE 2 | Prevalence of pain and itch in diabetes in different areas, assessment methods in different studies.

Patients and area	Number of diabetic patients	Prevalence (%)	Methods	Reference
Pain				
Patients with diabetes in northwest England	n = 15,692	21	Questionnaire (NSS and NDS)	Abbott et al. (2011)
Patients with diabetes in France nationwide	n = 766	20.3	Questionnaire (DN4 and MNSI), monofilament test	Bouhassira et al. (2013)
Patients with diabetes in United Kingdom	n = 350	16.2	Questionnaire (VAS and McGill Pain) and examination	Daousi et al. (2004)
Patients with diabetes in Italy	n = 816	13	Clinical examination and diagnostic tests	Truini et al. (2018)
Patients with T2DM in Denmark	n = 5,114	10	Questionnaire (DN4 and MNSIq)	Gylfadottir et al. (2020)
Itch				
Patients with diabetes in the United Kingdom	n = 300	18.4	Interviewed and clinical examination	Neilly et al. (1986)
Diabetic outpatients in Japan	n = 2,656	26.3	Questionnaire	Yamaoka et al. (2010)
Patients with T2DM in Taiwan, China	n = 385	27.5	Questionnaire	Ko et al. (2013)
Children with T1DM in Poland	n = 100	22	NRS and Questionnaire (4IIQ)	Stefaniak et al. (2020)
Patients with T2DM in Poland	n = 109	35.8	NRS and Questionnaire (4IIQ)	Stefaniak et al. (2021b)

DN4, Diabetic Neuropathy 4; NDS, neuropathy disability score; NSS, neuropathy symptom score; MNSI, Michigan Neuropathy Screening Instrument; VAS, visual analog scale; 4IIQ, Four-item Itch Questionnaire; NRS, numerical rating scale.

Truini et al., 2018), as shown in **Table 2**. The prevalence of pDN varies among different studies, many reasons are due to the differences, containing case definition criteria used, participants selected, sample size, and types of diabetes (Ziegler et al., 2014). Amazingly, a recent study has shown a higher prevalence of neuropathic pain in patients with pDN (73.11% of 1,547) in mainland China (Zhang et al., 2021), all these data demonstrate the seriousness of pDN in diabetic patients. Itching is also a relatively frequent symptom in patients with diabetes. Although itching has been first investigated in DM in the late 1920s, until now, literatures about diabetic chronic itching are still limited. An intense scratching habit lasting more than 6 weeks is classified as chronic itching (Cevikbas and Lerner, 2020). The prevalence of chronic itching in the general population is ~22% (Weisshaar, 2016), and the prevalence in DM is quite various, ranging from 18.4 to 35.8% (Neilly et al., 1986; Ko et al., 2013; Stefaniak et al., 2021a; Stefaniak et al., 2021b). This huge difference can be attributable to inconsistent definitions, varied tools for itch evaluation, age, gender, and diabetic populations with different diabetes types.

NEUROINFLAMMATORY MECHANISMS UNDERLYING DIABETES-RELATED NEUROPATHIC PAIN

Accumulating evidence suggests that neuroinflammation is closely related to chronic pain responding to stimuli (Perera et al., 2015; Ji et al., 2016; Ji et al., 2018; Borghi et al., 2019). The inflammation in PNS and CNS is characterized by the following: 1) an increase in the permeability of the blood-spinal cord barrier and blood-brain barrier (BBB), 2) infiltration of leukocytes, as the outcome of increased vascular permeability, 3) secretion and production of pro-inflammatory mediators (e.g., pro-inflammatory cytokines or chemokines), and 4) activation of glial cells causing the production of glial mediators that can regulate pain sensitivity (Ellis and Bennett, 2013; Ji et al., 2013; Ji et al., 2014).

It is well known that chronic pain results from neuronal plasticity in pain processing pathways. Neuronal plasticity involved in pain signal transmission consists of peripheral sensitization and central sensitization (Hucho and Levine, 2007; Basbaum et al., 2009; Gold and Gebhart, 2010; Woolf, 2011; Luo et al., 2014). Next, we highlight the important roles of neuroinflammation in promoting peripheral sensitization and central sensitization and involvement in DNP.

Neuroinflammation and Peripheral Sensitization in DNP

As a result of inflammation and tissue injury, the critical characteristic of peripheral sensitization of nociceptors is presented by a decrease in threshold and an increase in response to noxious stimuli and spontaneous activity (Rosenberger et al., 2020). The hyperexcitability of sensory neurons in both patients and rodent models with diabetes presents as spontaneous activity and an altered stimulus-response function (Thrainsdottir et al., 2003; Kim et al., 2012; Nowicki et al., 2012). The presence of this aberrant activity is essential to the development and maintenance of DNP.

Increasing reports suggest that SCs which ensheath the nerve fibers in the PNS are vital victims in the state of chronic hyperglycemia, causing demyelination in patients with diabetic neuropathy (Gumy et al., 2008; Dunnigan et al., 2013). SCs express both neurotrophins and their receptors. However, in diabetic patients or rodent models of diabetes, the robust decrease of neurotrophins in SCs results in unable to guide and support the regeneration of nerve fibers (Leinninger et al., 2004; Richner et al., 2014). In one previous study, streptozotocin (STZ)-induced diabetes reduces the level of ciliary neurotrophic factor (CNTF), an important neurotrophic factor from SCs (Calcutt et al., 1992). Some other studies have suggested that SCs and T cells interact with each other in diabetes. *Tang* et al.,

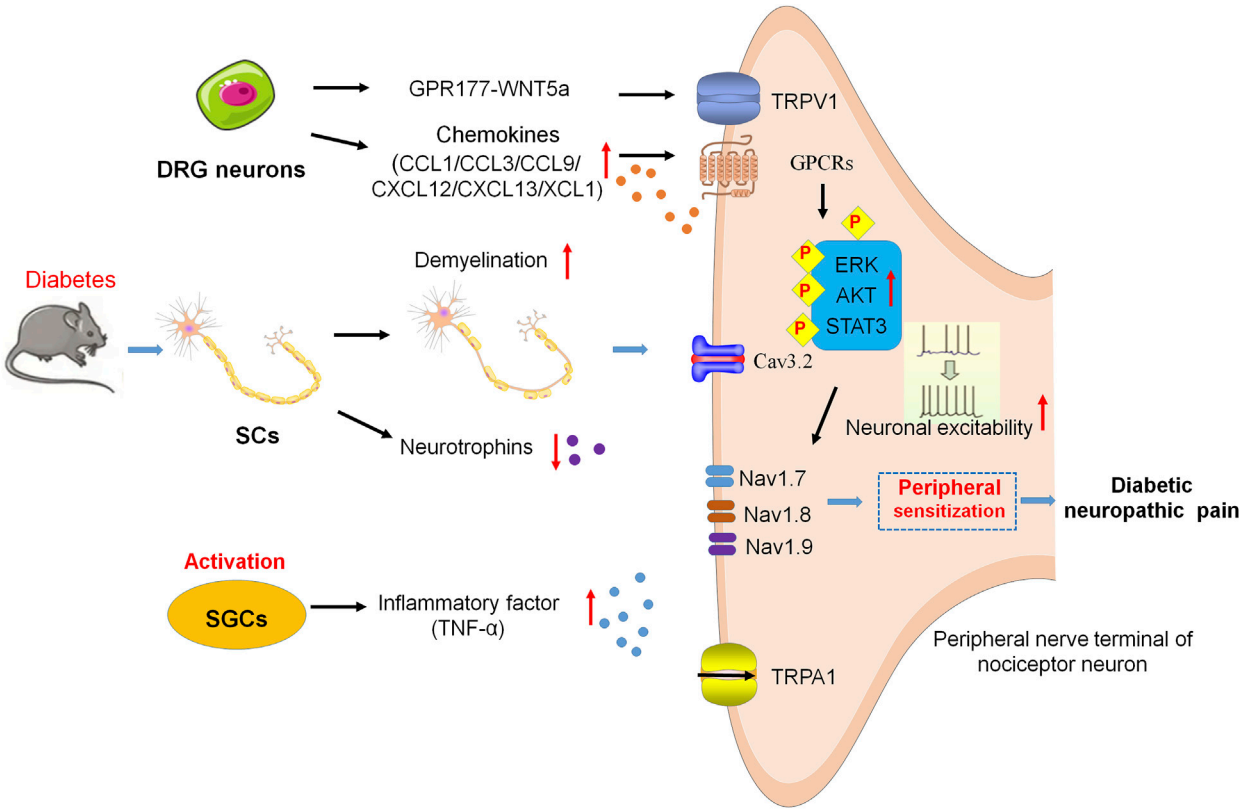

FIGURE 1 | Neuroinflammation and peripheral sensitization in DNP. SCs are damaged and decrease neurotrophin expression, resulting in demyelination of axons and failure of nerve regeneration. SGCs release TNF-α and then enhance the excitability of peripheral nociceptive neurons. Cav3.2 activity results in hyperexcitability of DRG neurons via the glycosylation of extracellular arginine residues in diabetes. Chemokines activate the signaling cascades, such as ERK, AKT, and STAT3, to sensitize the Nav1.7, Nav1.8, Nav1.9, and TRPA1, leading to hypersensitivity and hyperexcitability of peripheral nociceptive neurons. In addition, GPR177 derives DNP via WNT5a/TRPV1 interaction.

reported that levels of CXCR3 and phosphalated-p38 (p-p38) in the peripheral blood mononuclear cell (PBMC) of DPN patients are significantly increased. CXCR3 is elevated in CD8 (+) T cells via the p-p38 under high glucose conditions, and then promotes CD8 (+) T-cell recruitment into the diabetic nerves by CXCL9, CXCL10, and CXCL11 produced from glucose-stimulated SCs. Furthermore, results demonstrated that the upregulation of TNF-α, FasL, and PD-L1 in CD8 (+) T cells stimulating with SCs, which, in return, induce significant apoptosis of SCs, indicating the interaction of $CD8^+$ T cells and SCs plays a key role in the development of DPN (Tang et al., 2013) (**Figure 1**).

Several previous studies have revealed that SGCs in ganglia are important for PNS functionality and glia activation. SGCs contact with each other and enwrap neuronal soma in ganglia. Deterioration of this communication among SGCs under pathological conditions leads to abnormal pain signal transmission (Dublin and Hanani, 2007; Huang et al., 2013). In T1DM and T2DM mice, the increased levels of glial fibrillary acidic protein (GFAP) are considered as the activation of SGCs, which have been shown to be associated with the induction of neuropathic pain (Hanani et al., 2014; Liu et al., 2016). In T2DM rats, the upregulation of purinergic signaling promotes the activation of SGCs, increases tumor necrosis factor-alpha (TNF-α) release from SGCs, and enhances the excitability of dorsal root ganglion (DRG) neurons, which brings about the pain sensitivity (Liu et al., 2016; Gonçalves et al., 2018) (**Figure 1**).

The activity and status of ion channels within sensory neurons largely determine the transmission and processing of pain signals (Bennett and Woods, 2014; Waxman and Zamponi, 2014). Ion channels [e.g., voltage-gated sodium channels (Nav), potassium channels, calcium channels (Cav), and transient receptor potential channels (TRP)] are participated in resting and action potentials (Waxman and Zamponi, 2014). In peripheral sensory neurons, three particularly prevalent Nav-isoforms are identified and named as Nav1.7, Nav1.8, and Nav1.9 (Dubin and Patapoutian, 2010; Hameed, 2019). In addition to setting the excitability of the terminal, Nav1.7 and Nav1.9 also function as threshold channels for amplifying the sensory signal, while Nav1.8 plays the role in the upstroke of action potentials in nociceptors (Blair and Bean, 2002). Potassium channels act as important breaks in the excitability of sensory neurons. T-type Ca2+ channels have also been found to play an important role in pDN by regulating the excitability of nociceptors in the subthreshold range. The activity of Cav3.2 is increased in diabetes via the glycosylation of arginine residues within

extracellular membranes, which causes DRG neurons to be hyperexcitable. (Orestes et al., 2013). The changes in ion channels such as genetic variants, epigenetic modification, and abnormal expression, have all been implicated in the pathogenesis of neuropathic pain. *Sun et al.* reported that the increased expression of Nav1.8 is implicated in pDN, and such an increase reduces the failure probability of conduction in unmyelinated C fiber nociceptors, and then promotes more impulse conduction to the CNS, which results in neuropathic pain (Sun et al., 2012). Transient receptor potential vanilloid receptor-1 (TRPV1) ion channels are the important molecules involved in peripheral sensitization and pain modulation of chronic pain, which are widely expressed in nociceptive DRG neurons (Moore et al., 2018). A recent study reported that the orphan G protein-coupled receptor 177 (GPR177)-mediated wingless-related mammary tumor virus integration site 5a (WNT5a) secretion from A-fiber DRG neurons drives DNP by directly activating the TRPV1 channel and resulting in rapid currents and calcium elevations in DRG neurons (Xie et al., 2022). GPR177 and WNT5a are also found co-expressed in human DRG neurons, and pain intensity is positively related to WNT5a secretion in cerebrospinal fluid (CSF) among DNP patients (Xie et al., 2022). DNP is alleviated by interfering with WNT5a/TRPV1 interaction, thus providing a potential therapeutic target and intervention strategy for the clinical treatment of DNP (Xie et al., 2022) (**Figure 1**).

In addition, patients with diabetes have higher levels of reactive metabolites such as methylglyoxal (MGO), which post-translationally modify Nav1.8, then result in sensory neuron hyperexcitability, and finally lead to the development of diabetic pain (Bierhaus et al., 2012; Hansen et al., 2015). Rodent models of pDN showed signs of hypersensitivity in response to MGO via the activation of the sodium channel Nav1.8 and the transient receptor potential channel ankyrin 1 (TRPA1) (Bierhaus et al., 2012; Huang et al., 2016). It has been reported that MGO regulates the BBB permeability by producing the redistribution of junctional proteins, containing claudin-5 and β-catenin (Tóth et al., 2014), resulting in an increase in brain vessel permeability to MGO (Li et al., 2013). One previous research demonstrated that MGO specifically affects the integrated stress response (ISR) in IB4 positive DRG neurons *in vitro* and vivo diabetic models. The mechanical hypersensitivity of diabetic mice induced by MGO is attenuated by blocking the ISR (Barragán-Iglesias et al., 2019) (**Figure 1**).

Neuroinflammation and Central Sensitization in DNP

Increasing studies also suggest that neuroinflammation-drives central sensitization play a crucial role in the neuropathic pain via acting on both PNS and CNS of diabetics (Loeser and Treede, 2008; Ji et al., 2018). The key features of neuroinflammation in CNS are the activation of glial cells (e.g., astrocytes and microglia), resulting in the upregulation of inflammatory mediators such as pro-inflammatory cytokines and chemokines. These chemokines and cytokines work as potent

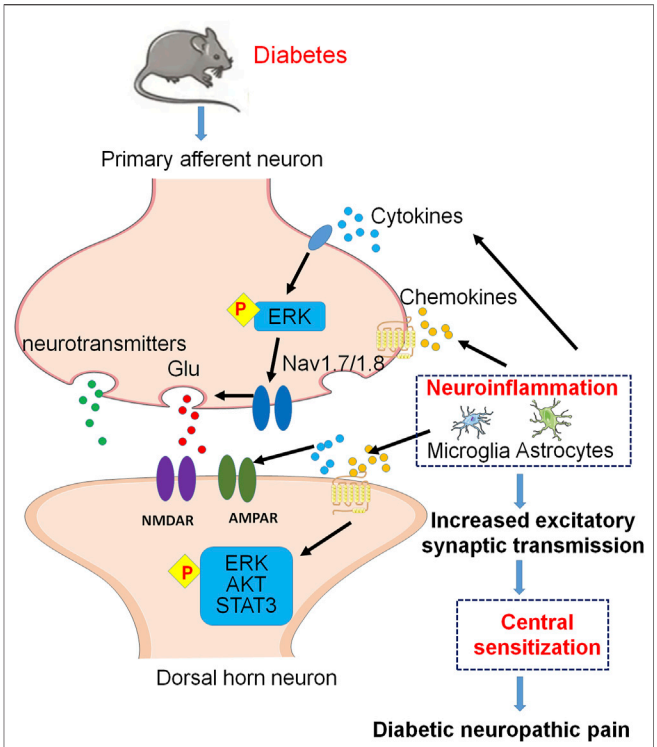

FIGURE 2 | Molecular mechanisms of neuroinflammation and central sensitization in excitatory synapses of the spinal dorsal horn under DNP. Cytokines and chemokines from spinal glial cells activate pERK in primary afferent terminals and finally enhance glutamate (Glu) release via activation of Nav1.7 and Nav1.8. At postsynaptic membrane, activation of postsynaptic Glu receptors contributes to central sensitization. In addition, cytokines and chemokines activate postsynaptic pERK, pAKT, and pSTAT3 signaling pathways, which contribute to central sensitization of DNP.

neuromodulators in the CNS that play a key role in triggering and maintaining the hyperalgesia and allodynia under chronic pain conditions (Samad et al., 2001; Kawasaki et al., 2008; Gao et al., 2009).

In diabetic neuropathy, synaptic transmission within the spinal cord is increased by enhancing the input from spontaneously active nociceptors, which further amplifies nociceptive signaling (Woolf, 2011). It is also believed that this occurs because of a temporal and spatial accumulation of nociceptive signal inputs, causing the neurons in the spinal dorsal horn to have a heightened response to their inputs. Under diabetic neuropathy conditions, microglial cells transform to a pro-inflammatory phenotype, which releases pro-inflammatory factors [e.g., TNF-α, interleukin (IL)-6, IL-1β] and brain-derived neurotrophic factor (BDNF), further amplify nociceptive signal transmission in the spinal dorsal horn, and promote mechanical hypersensitivity in pDN (Tsuda et al., 2008; Salter and Beggs, 2014; Sun et al., 2015; Liu M et al., 2019). Consistent with the microglia activation, the activation of astrocytes is also enhanced in diabetic mice (Liu M et al., 2019). In the T2DM animal model, there is a correlation between ERK activation [phosphorylated ERK (pERK)] in spinal superficial neurons and astrocytes and hypersensitivity to pain, and pERK inhibition may provide a new treatment

for diabetes-related pain (Xu et al., 2014). In addition, peripheral inflammation accompanied by prolonged nociceptive stimulation also increases the release of neurotransmitters [e.g., glutamate, BDNF, calcitonin gene-related peptide, and substance P] from the peripheral sensory fibers into the spinal dorsal horn and trigeminal nucleus. The increase of these neurotransmitters leads to the hyperexcitability of neurons in the spinal cord and supraspinal centers commonly referred to as central sensitization (Woolf, 1983; Woolf and Salter, 2000) (**Figure 2**).

An essential step for central sensitization is the activation of NMDARs and AMPARs at postsynaptic membrane surfaces (Ji et al., 2003; Latremoliere and Woolf, 2009). The previous study has shown that spinal activated astrocytes dramatically increase expression of IL-1β which may induce NMDAR phosphorylation in spinal dorsal horn neurons to enhance pain signal conduction in *db/db* mouse used widely as an animal model of T2DM. Therefore, the Astrocyte-IL-1β-NMDAR-Neuron axis unveils a novel mechanism underlying astrocyte-induced allodynia (Liao et al., 2011).

Chemokines and Chemokine Receptors Involved in Diabetic Neuropathic Pain

Under both normal and pathological conditions, chemokines contribute to cell survival, proliferation, and inflammation via activating intracellular signaling pathways (Jiang et al., 2020). Accumulating evidence suggests that chemokines and their receptors also contribute to chronic pain via enhancing neuroinflammation in the PNS and CNS (Van Steenwinckel et al., 2011; Zhang et al., 2012; Zhang et al., 2013; Zhang et al., 2017; Fyfe, 2018; Lin King et al., 2019; Lu et al., 2021). Studies in the past decade have shown that several chemokines and their receptors are implicated in the pathogenesis of DPN, and associated signaling pathways of the chemokine pairs are involved in the mechanisms of diabetic neuropathy pain (Menichella et al., 2014; Jiang et al., 2016; Zychowska et al., 2017; Jayaraj et al., 2018; Rojewska et al., 2018; Liu S et al., 2019) (**Figure 2**).

Previous studies have demonstrated the crucial role of CCL1 in the pathogenesis of diabetic neuropathy caused by STZ. As a mediator of neuroimmune interactions, CCL1 plays an important role in the DNP through CCL1/CCR8 cross-talk (Zychowska et al., 2017). In a study of STZ-induced diabetes mice, CCL3 and CCL9 levels are increased in the lumbar spinal cord, while neutralizing antibodies against CCL3 or CCL9 delay neuropathic pain symptoms following STZ administration, and the application of CCR1 antagonist also alleviates pain-related behavior in diabetic neuropathy (Rojewska et al., 2018).

In the high-fat diet (HFD)-induced mouse model of T2DM, the increase of CXCL12 expression is detected in DRG neurons, and CXCL12/CXCR4 signaling contributes to the development of pain in diabetes through enhancing calcium influx and excitability of Nav1.8 positive DRG neurons, as well as promoting inflammatory cell infiltration (Menichella et al., 2014). Reducing CXCR4-mediated nociceptor hyperexcitability can reverse pDN in HFD mice, suggesting that CXCR4 in Nav1.8 positive DRG neurons is involved in the development of mechanical allodynia in HFD-induced diabetes (Jayaraj et al., 2018).

Our data have shown the spinal CXCL13/CXCR5 axis participates in neuropathic pain. Through neuron-to-astrocyte cross-talk, CXCL13 is upregulated in spinal neurons after spinal nerve ligation and activates spinal astrocytes by interacting with its receptor CXCR5 (Jiang et al., 2016). In the spinal dorsal horn of *db/db* mice with thermal hyperalgesia and mechanical allodynia, the CXCL13 and CXCR5 are also significantly increased, and the phosphorylation of cell signaling kinases, including pERK, phosphorylated AKT (pAKT) and phosphorylated signal transducer and activator of transcription proteins 3 (pSTAT3) are upregulated. Further evidence showed that CXCL13/CXCR5 signaling contributes to diabetic pain *via* activating pERK, pAKT, and pSTAT3 cell signaling pathways and promoting the production of TNF-α and IL-6 in the spinal cord of diabetic mice (Liu S et al., 2019).

The expression of XCL1 and XCR1 in the lumbar spinal segments (L4 to L6) of the STZ-induced DPN mice is increased. More evidence suggested that XCR1 is expressed mainly on neurons in the pathology of DN. XCL1 intrathecal injection enhances nociceptive transmission in naive mice, and XCL1 neutralizing antibody administration diminishes allodynia/hyperalgesia in STZ-induced diabetic mice (Zychowska et al., 2016).

Advanced Glycation End-Products Involved in DNP

High levels of glucose lead to the glycation of several functional and structural proteins, resulting in producing advanced glycation end-products (AGEs). AGEs change gene expression and activation of nuclear factor-κB (NF-κB) via interacting with AGE-specific receptor (RAGE), thus inducing pro-inflammatory cytokines (e.g., IL-1α, IL-6, and TNF-α) (Neumann et al., 1999; Singh et al., 2014). In the spinal dorsal horn, TNF-α and IL-1β can act as neuromodulators to induce spinal synaptic plasticity such as long-term potentiation, and further promote neuropathic pain (Sorge et al., 2015; Taves et al., 2016).

NEUROINFLAMMATORY MECHANISMS UNDERLYING DIABETES-RELATED CHRONIC ITCH

Itch is an unpleasant cutaneous sensation that is accompanied by scratching or the desire to scratch (Ikoma et al., 2006; Lee et al., 2016; Dong and Dong, 2018). However, many similarities have been found between chronic pain and chronic itch (Ji, 2015; Moore et al., 2018; Ji et al., 2019). The cell bodies of itch sensory neurons are also located in the DRGs and trigeminal ganglia, and most itch neurons belong to C-type neurons (Ringkamp et al., 2011; LaMotte et al., 2014). The itch signals are generated in the primary afferent sensory fibers in the skin and then transmitted through the DRG neurons to the spinal dorsal horn neurons, and finally to the brain neurons (Ikoma et al., 2006; Han and Dong, 2014). Over the past decade, extensive research has been conducted on the mechanisms of itch, including peripheral and central neural mechanisms such as receptors and pathways involved in itch perception (Dong and Dong, 2018). According to the researchers, there are two main causes of the itch

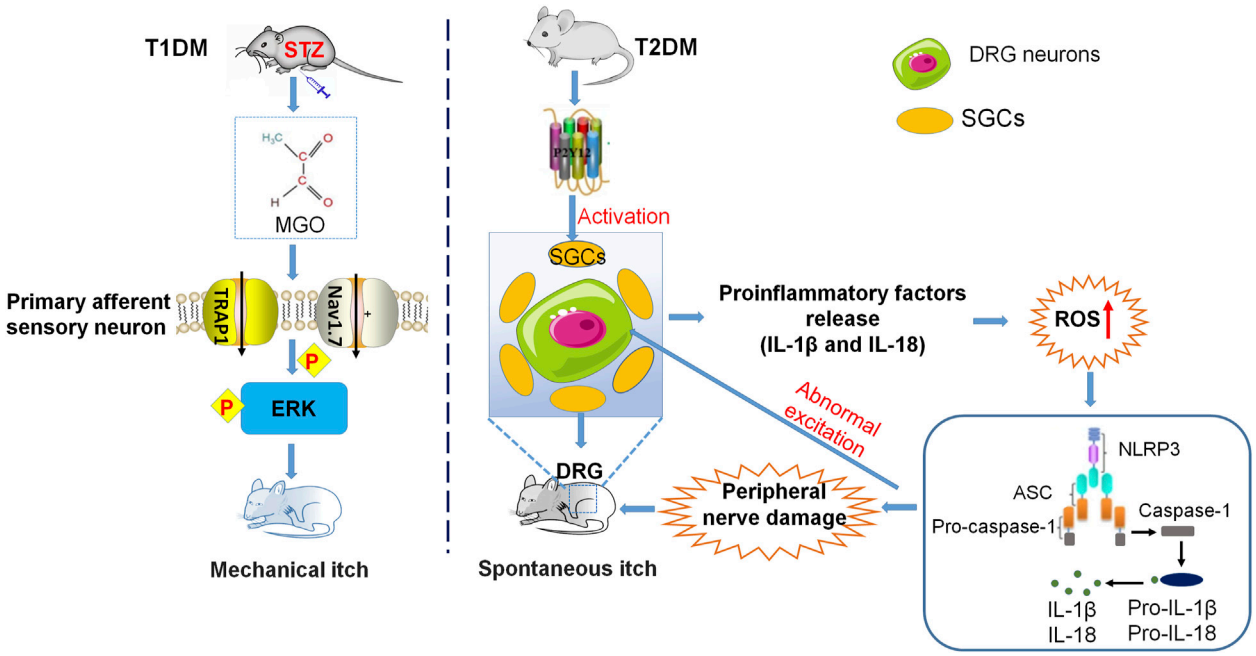

FIGURE 3 | Neuroinflammatory mechanisms underlying diabetes-related chronic itch. The mechanical itch induced by MGO or in the STZ-induced mouse model of T1DM is mediated by activation of TRPA1, Nav1.7, and ERK in the DRG neurons. In the T2DM mouse model, the upregulation of P2Y12 expression in SGCs contributes to the increase of ROS, followed by the activation of NLRP3 inflammasome, the upregulation of inflammatory cytokines, and the damage to peripheral nerves. These changes finally result in DRG neuron hyperexcitability and sensitization.

in diabetics, containing skin xerosis and diabetic polyneuropathy, suggesting that itch originates from dermatology or neurology (Stefaniak et al., 2021b). Additionally, oxidative stress and nerve inflammation contribute to diabetic polyneuropathy (Hagen and Ousman, 2021).

Previous data have shown that sensitization is also a common mechanism in itch processing. Peripheral sensitization caused by the C fibers in the epidermis plays important role in pruritus sensitization (Ikoma et al., 2006; Tominaga and Takamori, 2014). Several other results indicated that spinal sensitization occurs frequently in atopic dermatitis (AD) model mice, but may not in psoriasis model mice (Shiratori-Hayashi and Tsuda, 2021). Mechanical itch (also known as touch-evoked itch) is a notable feature of chronic itch, and also a prominent mark in diabetic neuropathy (Bourane et al., 2015). Other evidence suggests that mechanical itch is related to central sensitization (Pan et al., 2019; Sakai and Akiyama, 2020). However, mechanisms of chronic itch in diabetes are not fully understood owing to inadequate related studies. Here, we summarize the inflammation mechanisms that participated in diabetic itch, including activity and status of ion channels, oxidative stress, and pro-inflammatory factors (**Figure 3**).

Ion Channels Mediate Mechanical Itch in Diabetic Itch

Increasing evidence has suggested that TRPV1 and TRPA1 are the downstream effectors of itch-related inflammatory factors and are involved in itch signals on the nerve fibers. During a pathological state, pruritus-related inflammatory factors such as IL-31, IL-4, and NGF, stimulate TRPV1 and TRPA1 repeatedly, resulting in a decrease in the threshold of itch sensation and causing chronic itch (Moore et al., 2018; Xie and Li, 2019). Both pain and itch are direct effects of immune dysfunction, since the release of pro-inflammatory mediators by immune cells and epithelial cells after tissue injury can directly activate or sensitize pain and pruritus neurons, causing hypersensitivity to pain and pruritus (Ji, 2015). Chronic itch and chronic pain caused by peripheral sensitization have been reported to be induced by inflammatory mediators, which require the activation of TRPA1 and Nav1.7 (Basbaum et al., 2009). It is widely recognized that MGO is a potential mediator of itch in diabetes. Incubation of MGO induces inward currents and calcium influx in TRPA1-expressing HEK293 cells or DRG neurons. (Cheng et al., 2019). Mechanical itch evoked by MGO or in STZ-induced T1DM mice is dependent on the activation of TRPA1, Nav1.7, and the pERK signaling pathway in DRGs and spinal cord (Cheng et al., 2019).

Oxidative Stress Contributes to Diabetic Itch

Oxidative stress is an important factor in the pathogenesis of DM, especially in T2DM, which activates JNK, NF-κB, and p38 MAPK pathways to cause inflammation (Lamb and Goldstein, 2008; Agrawal and Kant, 2014). Previous studies have also shown that chronic and acute itching is related to oxidative stress (Liu and Ji, 2013; Zhou et al., 2019). ND7-23 cells (a cell line derived from the

dorsal root ganglia) exhibit a significant increase in intracellular reactive oxygen species (ROS) after MGO treatment. MGO or STZ-induced mechanical itching is significantly reduced by intraperitoneal injection of antioxidant α-lipoic acid (ALA), indicating that oxidative stress contributes to diabetic itch (Cheng et al., 2019). Moreover, T2DM mice with chronic itch exhibit significantly higher levels of ROS in the DRG cells, suggesting that these compounds play an important role in diabetic itch (Xu et al., 2022).

Pro-Inflammatory Factors in Diabetic Itch

Just like in chronic pain, pro-inflammatory factors such as cytokines and chemokines are also crucial in the pathogenesis of chronic itch (Liu et al., 2012; Storan et al., 2015). Diabetes was complicated by peripheral nerve injury results in an increase in the secretion of neuroinflammatory factors that can activate sensory C fibers and is accompanied by paraesthesia, suggesting that diabetic itch is due to abnormal discharges from damaged peripheral C fibers (Yamaoka et al., 2010; Yosipovitch and Bernhard, 2013). Spontaneous itching is an important indicator for evaluating itch behaviors. Recent studies have indicated that the number of spontaneous scratches in T2DM model mice is significantly increased. The increase of P2Y12 expression and SGC activity in these diabetic mice promotes the upregulation of ROS content, further activates the NLRP3 inflammatory body, and then produces inflammatory cytokines such as IL-18 and IL-1β. These inflammatory cytokines, in turn, cause peripheral nerve injury, abnormally excite DRG neurons, and result in spontaneous scratching. Treatment of P2Y12 shRNA or antagonist ticagrelor inhibits the spontaneous itch behaviors in the mouse model of T2DM (Xu et al., 2022).

STRATEGIES OF TREATMENT

Approaches to Treatment of Diabetic Pain

In recent years, targeted treatment of neuropathic pain is disappointing for a series of reasons as follows: 1) the underlying pathogenic mechanisms involved in neuropathic pain in diabetes are complex and not fully clarified, resulting in inadequate engagement of the claimed drug targets (Ji et al., 2014), 2) a translational gap from animal models of diabetes to patients with diabetes (King et al., 2009; Mogil, 2009), and 3) the serious side effects of existing analgesic drugs such as sedation, respiratory inhibition, tolerance, addiction and hyperalgesia following acute or chronic treatment.

Up to now, only glycemic control can prevent or slow down diabetic neuropathy progression in T1DM, but not in T2DM (Callaghan et al., 2012). Current evidence shows an association between diabetes and secondary complications with chronic inflammation. In addition to anti-inflammatory drugs, a multitude of hypoglycemic drugs such as thiazolidinediones, dipeptidyl peptidase-4 inhibitors, and metformin, have been found to reduce inflammation and improve outcomes. However, for all these hypoglycemic agents, it is necessary to distinguish between the anti-inflammatory effects produced by better glucose control and those related to the intrinsic anti-inflammatory effects of pharmacological compounds (Kothari et al., 2016).

According to the consensus from multiple guidelines and systematic reviews (Attal et al., 2010; Bril et al., 2011; Griebeler et al., 2014; Finnerup et al., 2015; Waldfogel et al., 2017), several drugs are supported to apply in the treatment of DNP, including calcium channel a2δ ligands (e.g., gabapentin and pregabalin) (Freeman et al., 2008; Moore et al., 2009; Griebeler et al., 2014; Finnerup et al., 2015; Pop-Busui et al., 2017), serotonin and noradrenaline reuptake inhibitors (SNRIs, e.g., duloxetine, venlafaxine) (Rowbotham et al., 2004; Wernicke et al., 2006; Zilliox and Russell, 2010; Tesfaye et al., 2013; Pop-Busui et al., 2017), and tricyclic antidepressants (TCAs, e.g., amitriptyline, nortriptyline, and desipramine) (Max et al., 1987; Max et al., 1991; Max et al., 1992; Boyle et al., 2012). However, these drugs do not clarify the potential pathogenesis for DNP.

Given the important roles of neuroinflammation such as cytokines and chemokines in the pathogenesis of DNP, targeting the pro-inflammatory mediators may provide a novel approach to treating DNP. There are three possible approaches for developing drugs that target chemokines and their receptors, including 1) blocking or neutralizing antibodies, 2) small-molecule inhibitors, and 3) small interfering RNA (siRNA). For example, antibodies that neutralize CCL3, CCL9, or XCL1 delay diabetic neuropathic pain symptoms. Similarly, CCR1 antagonist J113863 also attenuates pain-related behaviors in the diabetic pain model (Zychowska et al., 2016; Rojewska et al., 2018). Mechanical allodynia is alleviated in db/db mice following the injection of CXCR5 shRNA (Liu S et al., 2019).

More and more evidence has suggested that there is an inflammatory environment in the islets of patients with T2DM, including high levels of cytokines and chemokines, and immune cell infiltration. Therefore, many drugs targeting inflammatory cytokines such as TNF-α, IL-6, and IL-1β, are used to reduce insulin resistance and improve insulin secretion, further alleviating the complications of diabetes (Agrawal and Kant, 2014; Esser et al., 2015). For example, both troglitazone and gliclazide can reduce the TNF-α level in rodent models of diabetes. N-acetylcysteine (an anti-oxidant) attenuates the TNF-α levels in a dose-dependent manner, contributing to a decrease in the incidence and severity of diabetic neuropathy (Sagara et al., 1996). Tocilizumab (a monoclonal antibody targeting IL-6), drugs targeting IL-1β (e.g., anakinra, canakinumab, and other monoclonal antibodies), appear to reduce insulin resistance by reducing their pro-inflammatory effects in adipose tissue and muscle (Goldfine and Shoelson, 2017). Piroxicam statistically decreases the action potential amplitude of sensory neurons enhanced by STZ (Parry and Kozu, 1990). Nonsteroidal anti-inflammatory drugs (NSAIDs) reduce inflammation by inhibiting cyclooxygenase

(COX) enzymes and are widely used in the prevention and treatment of T2DM (Bellucci et al., 2017). Moreover, drugs that target vascular endothelial growth factors (such as Pegaptanib and Avastin) and chemokines are used for the treatment of diabetic retinopathy (Kastelan et al., 2013). The current research studies shown that these drugs against pro-inflammatory mediators have certain therapeutic effects on diabetes, but cannot reverse the development of diabetes, overall, more studies are needed to validate these results. In addition, the selective blocking of Nav1.7 function has been successfully applied to trigeminal neuralgia, but the expected effect in diabetic neuropathy needs further to explore (Zakrzewska et al., 2017). The decrease of calcium influx via interfering Cav3.2 expression can also reduce pain hypersensitivity in diabetic mice (Messinger et al., 2009).

Approaches to Treatment of Diabetic Itch

Currently, the mechanism involved in chronic itching, especially diabetic itching are poorly understood, resulting in limited effective therapies for chronic itching. Generally, treatment should be based on the therapeutic principle: finding out the cause, treating the primary diseases, avoiding the inducing factors, and moisturizing the skin (Greaves, 2005; Song et al., 2018). For diabetic itch, the optimal strategy is the treatment or prevention of causal diseases, that is, the maintenance of normal blood glucose (Steinhoff et al., 2018). In addition, some anti-inflammatory drugs targeting cytokines and chemokines (described in **6.1**) to treat the primary disease of diabetes probably also be beneficial to the treatment of itching induced by diabetes. Furthermore, experiments are needed in the future to confirm the anti-pruritic effect of these drugs on diabetes. In animal models of diabetes, knocking out of *Trpa1*, the blocker of Nav1.7, and TRPA1, antioxidants, and ERK inhibitor U0126 alleviate itching in mice evoked by STZ or MGO (Cheng et al., 2019). In addition, P2Y12 may be a promising target for the treatment of itching in T2DM (Xu et al., 2022).

Overall, drugs targeting diabetic itch patients are still inadequate, and further studies are needed to provide more information on the treatment efficacy.

PERSPECTIVE

As the most common chronic complication of DM, diabetic neuropathy results in chronic pain and itching. Our understanding of diabetic neuropathy continues to advance, especially neuroinflammation and sensitization-driven pain in diabetic neuropathy. However, the mechanism underlying pDN and chronic itching is still not fully revealed, hindering the development of therapies to treat diabetic pain and itch. Notably, chronic pain and itching are typically accompanied by anxiety, depression, and sleep disturbances, therefore, the development of drugs targeting inflammation not only helps treat diabetic pain and itching but also helps alleviate the development of mental illness in diabetic patients. Currently, many promising drugs in animal models or preclinical studies are aborted in clinical trials, which may be related to the insufficient representativeness of animal models, poor drug design, and design defects of clinical trials (Malik, 2016). Although regulatory agencies have approved a number of drugs and therapies to relieve the chronic pain and itch, it is worth noting that none of them are designed to target diabetes-specific mechanisms, while their efficacy varies from patient to patient and is confined to small subgroups of patients (Finnerup et al., 2010). Therefore, it is urgent and necessary to develop targeted drugs for diabetic pain and itching in the future.

AUTHOR CONTRIBUTIONS

X-XF designed and wrote the manuscript. HW, H-LS, and JW drew the schematic diagrams. Z-JZ initiated, supervised, and revised the manuscript.

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The beneficial roles of apelin-13/APJ system in cerebral ischemia: Pathogenesis and therapeutic strategies

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The incidence of cerebral ischemia has increased in the past decades, and the high fatality and disability rates seriously affect human health. Apelin is a bioactive peptide and the ligand of the G protein-coupled receptor APJ. Both are ubiquitously expressed in the peripheral and central nervous systems, and regulate various physiological and pathological process in the cardiovascular, nervous and endocrine systems. Apelin-13 is one of the subtypes of apelin, and the apelin-13/APJ signaling pathway protects against cerebral ischemia by promoting angiogenesis, inhibiting excitotoxicity and stabilizing atherosclerotic plaques. In this review, we have discussed the role of apelin-13 in the regulation of cerebral ischemia and the underlying mechanisms, along with the therapeutic potential of the apelin-13/APJ signaling pathway in cerebral ischemia.

KEYWORDS

apelin-13, APJ, cerebral ischemia, pathway, angiogenesis, atherosclerotic plaque, excitotoxicity

1 Introduction

Cerebral ischemia is a serious threat to human health, and is associated with high morbidity, disability and mortality. The rapidly aging population, as well as significant changes in lifestyle and diet brought about by the socio-economic development in China in recent years, has significantly increased the risk of stroke. It is currently the primary cause of death and disability among adults in China (Wang et al., 2015; Wang et al., 2017a; Guan et al., 2017). Therefore, it is crucial to devise suitable intervention methods in order to improve the prognosis of patients with cerebral ischemia and reduce the burden of disease.

Studies increasingly show that the apelin/apelin receptor (APJ) signaling pathway is involved in the occurrence and development of cerebral ischemia (Tables 1, 2). APJ is an orphan G protein-coupled receptor that was discovered by O'Dowd et al. (1993), and apelin is its endogenous ligand. The apelin/APJ system is ubiquitous in the peripheral and

TABLE 1 The evidence from clinical trials demonstrating the role of apelin in stroke.

Subject	Main findings	Citation
68 MMD patients, 25 MCAO patients, 29 healthy controls	Apelin-13 is significantly increased in MMD patients than MCAO patients independent of NO and VEGF.	Wu et al. (2022)
60 patients with high risk of stroke (AF and non-AF group), 34 healthy controls	Apelin might be used to rule out AF in patients with high risk of stroke	Bohm et al. (2021)
109 AIS patients treated with intravenous thrombolysis	Apelin can help effectively forecast the occurrence of HT in AIS patients after intravenous thrombolysis, as an independent protective factor of HT.	Zhu et al. (2021)
156 ischemic stroke patients, 79 hemorrhagic stroke patients, 235 healthy controls	Higher vaspin, apelin, and visfatin levels might be associated with increased stroke risk	Yu et al. (2021)
244 AIS patients, 167 healthy controls	Serum apelin-13 may be a potential prognostic biomarker for AIS. Serum apelin-13 levels is lower in the patients than healthy controls, patients with a NIHSS score ≤3 had higher apelin-13 levels. There is an association between apelin-13 and death or major disability at the 3-months follow-up, the patients with high apelin-13 levels show a lower incidence of stroke and combined events at the 1-year follow-up	Wang et al. (2020)
168 AIS patients, 58 healthy controls	No difference of apelin between AIS patients and control group, and no difference of apelin between stroke subgroups with and without significant ipsilateral carotid stenosis	Kadoglou et al. (2014)

central nervous systems, and regulates blood pressure, myocardial contraction, immune response, angiogenesis, cancer development and other biological processes (Hosoya et al., 2000; Kawamata et al., 2001; Li et al., 2008; Barnes et al., 2010; Yang et al., 2016a). The currently known subtypes of apelin include apelin-12, apelin-13, apelin-17, apelin-28, and apelin-36, of which apelin-13 is the predominant subtype found in the heart, brain and hypothalamus. Previous studies have shown that apelin-13 plays an important role in cerebral ischemia (Duan et al., 2019; Wang et al., 2020) and ischemic stroke, and changes in the expression level of endogenous apelin-13 following ischemia has diagnostic and therapeutic relevance. Exogenous apelin-13 supplementation in ischemic stroke patients can play a neuroprotective role by regulating multiple signaling pathways. In this review, we have summarized the role and mechanism of apelin-13 in cerebral ischemia, in order to offer new insights into its diagnosis and treatment.

2 Apelin-13

2.1 Biological characteristics of apelin-13

The gene encoding the arginine and lysin-rich apelin precursor peptide is located on chromosome Xg 25–26.1, and consists of three exons and two introns. The precursor peptide contains multiple potential sites of post-translational enzymatic processing, and can therefore generate multiple active apelin peptide fragments. For instance, cleavage of the apelin precursor peptide by angiotensin converting enzyme 2 (ACE2) generates isoforms of varying lengths such as aplein-12, apelin-13, apelin-17, and apelin-36. These isoforms differ in terms of tissue distribution, physiological and pharmacological effects, and binding strength with APJ (Lee et al., 2000; Reaux et al., 2001; De Mota et al., 2004). Furthermore, the biological activity of apelin, especially involving receptor binding and intracellular receptor transport, is greatly influenced by the size of the molecular fragment. Smaller apelin isoforms typically display stronger binding to APJ (Kleinz and Davenport, 2005; Carpéné et al., 2007).

Apelin-13 is a short peptide consisting of 13 amino acids. The N-terminal of apelin-13 binds to the APJ receptor, while the C-terminal is mainly involved in regulating its biological activity (Kawamata et al., 2001; Medhurst et al., 2003). It is degraded into an inactive form in the presence of ACE2 (Vickers et al., 2002), and is also modified into the more stable and active pyroglutamyl apelin-13. Studies show that pyroglutamyl apelin-13 is the most biological relevant subtype of apelin present in healthy human plasma (Mesmin et al., 2010; Zhen et al., 2013). Nevertheless, apelin-13 is ubiquitously expressed in the digestive system, cardiovascular system, central nervous system (CNS), kidneys, adipose tissue and retina, whereas apelin pro-peptide is predominantly present in the heart, lungs, kidneys and endothelial cells of large blood vessels (O'Carroll et al., 2000). Furthermore, apelin mRNA has been detected in the mammary glands, heart, lungs, brain, kidneys and other tissues of rats. Based on these findings, we can surmise that apelin has wide-ranging functions in humans as well as rodents. In particular, the presence of apelinergic neurons in the brain suggests that apelin may regulate food intake and digestion, pituitary hormone release and circadian rhythms (Reaux et al., 2002).

2.2 The APJ receptor

The apelin receptor APJ, also known as angiotensin II (Ang II) receptor-like 1, is a G protein-coupled receptor consisting of 380 amino acids with seven transmembrane structures. It is

TABLE 2 The evidence from experimental trials demonstrating the role and mechanism of apelin in stroke.

Subject	Apelin treatment	Main findings	Citation
MCAO/R rats	Apelin-13 is injected into the tail vein 5 min before reperfusion	Apelin-13 attenuates injury following ischemic stroke by targeting MMP, endothelin-B receptor, occludin/claudin-5 and oxidative stress	Gholamzadeh et al. (2021a)
HT22 cells (OGD/R)	The cells are treated with 0.1 μM Apelin-36	Apelin-36 protects against OGD/R-induced oxidative stress and mitochondrial dysfunction by promoting SIRT1-mediated PINK1/Parkin-dependent mitophagy	Shao et al. (2021a)
Spragu-Dawley rats (MCAO/R), SH-SY5Y cells (OGD/R)	Apelin-13 (50 μg/kg) is injected into the right ventricle of rats at the onset of reperfusion; SH-SY5Y cell is treated with 10–7 M apelin-13 for 5 h	Apelin-13 inhibits apoptosis and excessive autophagy by upregulating Bcl-2 and activating mTOR signaling pathway after cerebral ischemia/reperfusion injury	Shao et al. (2021b)
Wistar rats (MCAO/R)	Intravenous injection of apelin-13 (10, 20, and 40 μg/kg) *via* tail vein 5 min before reperfusion	Apelin-13 improve sensory-motor balance defects by reducing neural death and infarct volume, and restoration of serum NO levels after cerebral ischemia	Gholamzadeh et al. (2021b)
Sprague-Dawley rats (SAH)	Apelin-13 (10 mg/kg) is injected into the lateral cerebral ventricle at 0.5 h after SAH.	Apelin-13 attenuates early brain injury following subarachnoid hemorrhage *via* suppressing neuronal apoptosis through the GLP-1R/PI3K/Akt signaling	Liu et al. (2019)
Sprague-Dawley rats (SAH)	Apelin-13 (25 μg/kg, 50 μg/kg, and 100 μg/kg) is injected intracerebroventricularly immediately after SAH induction	Apelin-13 attenuates early brain injury through inhibiting inflammation and apoptosis in rats after SAH.	Shen et al. (2022)
Sprague-Dawley rats (MCAO/R), PC12 cells (I/R)	Apelin-13 (30 μg/kg, 60 μg/kg, and 120 μg/kg) is injected intracerebroventricularly 15 min before reperfusion in rats; PC12 cells are pretreated with apelin-13 (0.5, 1, and 1.5 μM) for 6 h	Apelin 13 protects against I/R-induced ROS-mediated inflammation and oxidative stress through activating the AMPK/GSK-3β pathway *via* AR/Gα/PLC/IP3/CaMKK signaling, and further upregulates the expression of Nrf2-regulated antioxidant enzymes	Duan et al. (2019)
CD-1 mice (MCAO/R)	15 μl Apelin-12 is intracerebroventricularly injected 15 min before reperfusion	Apelin-12 inhibits the JNK and p38MAPK signaling pathway of the apoptosis-related MAPKs family, thus offering protection to neurons from ischemia-reperfusion injury	Liu et al. (2018)
118 MCAO patients and 22 controls patients; Sprague-Dawley rats (MCAO)	Pretreatment of apelin-17 (1 μmol/L) in rats	Plasma apelin-17 levels in ischemic stroke patients are positively associated with enhanced collateral circulation, which may have resulted from an apelin-17-induced cerebral artery dilation mediated through the NO-cGMP pathway	Jiang et al. (2019)
Wistar rats (MCAO/R)	Apelin-13 (10 μl) is injected intracerebroventricularly 30 min before MCAO in rats	Apelin-13 can attenuate activate neuronal apoptosis by inhibiting eIF2-ATF4-CHOP-mediated ER stress, involvement of Gαi/Gαq- CK2 signaling	Wu et al. (2018)
Wistar rats (MCAO/R)	10 μl apelin-13 (0.03 μg/μl) or10 μl apelin-36 (0.05 μg/μl) is injected into the right lateral ventricle at 2 h after MCAO.	Post-stroke administration of low-dose apelin-36 could attenuate infarct volume and apoptosis, which is associated with the inhibition of ERS/UPR activation. Low dose of apelin-13 had no protective effect in rats with ischemic stroke	Qiu et al. (2017) Chu et al. (2017)

(Continued on following page)

TABLE 2 (*Continued*) The evidence from experimental trials demonstrating the role and mechanism of apelin in stroke.

Subject	Apelin treatment	Main findings	Citation
AQP4 +/+ and AQP4 −/− mice (MCAO/R)	Apelin-13 (50 μg/kg) is injected intracerebroventricularly 15 min before reperfusion	Apelin-13 protects BBB from disruption after cerebral ischemia both morphologically and functionally, which is highly associated with the increased levels of AQP4, possibly through the activation of ERK and PI3K/Akt pathways	
Spragu-Dawley rats (MCAO/R); priamary neurons, astrocytes, and endothelial cells (OGD/R)	Apelin-13 (50 μg/kg) is injected intracerebroventricularly 15 min before or immediately after reperfusion in rats; the cells treat with apelin-13 (100 μmol/L)	Protective effects of apelin-13 on ischemic neurovascular unit injuries are highly associated with the increase of VEGF binding to VEGFR-2, possibly acting through activation of ERK and PI3K/Akt pathways	Huang et al. (2016)
Mice (MCAO/R)	Apelin-13 (100 μg/kg) is injected intracerebroventricularly 15 min before reperfusion	Apelin-13 protects against apoptosis by activating AMP-activated protein kinase pathway in ischemia stroke	Yang et al. (2016b)
C57/BL6 mice (BOCCA)	Intranasal administration of apelin-13 (4 mg/kg) is given 30 min after the onset of stroke and repeat once daily	Apelin-13 exert neuroprotective effect after ischemic stroke, through reducing inflammatory activities, decreasing cell death, and increasing angiogenesis	Chen et al. (2015)
Wistar rats (MCAO/R)	Apelin-13 (0.1 μg/g) diluted in 10 μl physiological saline is injected into the lateral ventricle	Apelin-13 is neuroprotective against cerebral ischemia/reperfusion injury through inhibition of neuronal apoptosis	Yan et al. (2015)
Wistar rats (MCAO/R)	Apelin-13 (50 ng/kg, 10 μl) is injected intracerebroventricularly at the onset of reperfusion	Apelin-13 is neuroprotective for neurons against I/R through inhibiting the neuroinflammation	Xin et al. (2015)
ICR mice (MCAO/R)	Apelin-13 (10 μg/kg, 50 μg/kg, 100 μg/kg, 5 μl) is injected intracerebroventricularly 15 min before reperfusion	Apelin-13 protects the brain against ischemia/reperfusion injury through activating PI3K/Akt and ERK1/2 signaling pathways	Yang et al. (2014b)
ICR mice (MCAO/R, H/I)	Apelin-36 (0.1 μg in 10 μl saline) is injected into the left lateral ventricle at 30 min before MCAO; apelin-36 (1 μg in 100 μl saline) is administrated intraperitoneally at the beginning of recovery (H/I)	Apelin-36 protects against ischemic brain injury by reducing apoptosis *via* activating the PI3K/Akt pathway	Gu et al. (2013)
Wistar rats (MCAO/R)	Apelin-13 (25, 50, and 100 μg in 5 μl saline) is injected intracerebroventricular at the beginning of ischemia	Apelin-13 improves infarct volume, brain edema, and apoptosis, but not change neurological dysfunction after cerebral ischemia	Khaksari et al. (2012)
Primary mouse cortical neurons	Cortical neurons are incubated with different concentrations of apelin-13 (10 p.m. - 5 nM)	Apelin may block apoptosis and excitotoxic death *via* regulating Akt/ERK pathway and attenuating intracellular Ca^{2+} accumulation	Zeng et al. (2010)
Sprague-Dawley rats (SAH)	Apelin-13 (15 μg/kg, 50 μg/kg, and 150 μg/kg in 10 μl sterile saline) is injected intracerebroventricularly at 30 min after SAH induction	Exogenous apelin-13 binding to APJ attenuates early brain injury after SAH by reducing ERS-mediated oxidative stress and neuroinflammation, which is at least partly mediated by the AMPK/TXNIP/NLRP3 signaling pathway	Xu et al. (2019)
Sprague-Dawley rats (SAH)	Apelin-13 (15 μg/kg, 50 μg/kg, and 150 μg/kg in 10 μl sterile saline) is injected intracerebroventricularly at 30 min after SAH induction	Apelin-13 could exert its neuroprotective effects *via* suppression of ATF6/CHOP arm of ERS-response pathway in the early brain injury after SAH.	Xu et al. (2018)

currently the only known apelin-13 receptor so far, and is highly expressed in neurons and glial cytoplasm in caudate nucleus, corpus callosum and hippocampus (Hosoya et al., 2000; Medhurst et al., 2003). APJ relays the signals through Gα subunit (Gαi or Gαq) of G protein. The structure of APJ is similar to that of the Ang II type I (AT1) receptor, although it cannot bind to Ang II (O'Dowd et al., 1993). In addition, G protein-independent signaling pathways are also involved in the activation of the apelin/APJ system. Upon binding to apelin, APJ is activated and recruits G protein-coupled receptor kinases (GRKs), resulting in APJ phosphorylation. The inhibitor protein (β-arrestin) then rapidly binds to APJ, resulting in receptor desensitization, and activation of the G protein-independent signaling pathways (Chen et al., 2014; Chen et al., 2020).

2.3 The tissue distribution pattern of apelin-13 and APJ

Apelin 13 is widely distributed in the CNS, with high expression levels in neurons and oligodendrocytes, and relatively lower expression in the astrocytes. Apelin 13 mRNA has been detected in the spinal cord, brain stem, cerebral cortex, hypothalamus, cerebellum, striatum, and hippocampus (O'Carroll et al., 2000). The differential expression pattern of apelin 13 and APJ in the CNS is indicative of multiple physiological or pathological functions. Both APJ and apelin are highly expressed in the hypothalamus, the master regulator of the neuroendocrine and humoral balance. The co-localization of apelin and hypothalamic arginine vasopressin (AVP) neurons suggests that apelin may regulate body fluid balance, feeding and drinking behavior and the HPA axis by interacting with AVP (De Mota et al., 2000; Reaux-Le Goazigo et al., 2004). In addition, the distribution of apelin in hypothalamus and pituitary region also indicates that apelin may be involved in the regulation of neurological and adenohypophysial hormones (Brailoiu et al., 2002; Yang et al., 2019).

Several studies have shown that apelin and APJ are highly expressed in the cardiovascular system, and can enhance myocardial contraction, reduce cardiac load, dilate blood vessels, promote angiogenesis, and regulate cardiac electrical conduction (Maguire et al., 2009; Aydin et al., 2014; Yu et al., 2014). Interestingly, the apelin/APJ system is also expressed in the cerebral blood vessels, and regulates vascular function. For instance, some studies have demonstrated that apelin can promote vasodilation in cerebral vessels (Nagano et al., 2019; Mughal et al., 2020). Mughal et al. (2018) found that apelin inhibits nitric oxide (NO)-dependent relaxation of cerebral arteries by activating APJ and inhibiting large-conductance, calcium-activated K channel in cerebral arterial smooth muscle cells, partially *via* a PI3K-dependent mechanism (Modgil et al., 2013). In addition, apelin promotes development of new blood branches from preexisting cerebral vessels following ischemic stroke (Han et al., 2015; Hiramatsu et al., 2017; Wu et al., 2017). Jiang et al. (2019) correlated the increased levels of plasma apelin-17 in ischemic stroke patients with enhanced collateral circulation, which can be attributed to cerebral artery dilation induced by apelin-17 *via* regulating the NO-cGMP pathway.

2.4 The neuroprotective effects of apelin 13

There is ample evidence demonstrating the neuroprotective effects of apelin-13. It can protect neuronal cells against apoptosis and excitotoxic injury by inhibiting NMDA-induced intracellular Ca^{2+} accumulation, oxidative stress, mitochondrial damage, cytochrome C release and caspase-3 activation *via* the ERK1/2 signaling pathway (Zeng et al., 2010). In addition, one study showed that supraspinal administration of apelin-13 in mice induced antinociception *via* the opioid receptor (Xu et al., 2009). The same group reported that apelin-13 relieved acetic acid-induced visceral pain in mice when injected into the subarachnoid space, and this analgesic effect was blocked by opioid receptor antagonists (Lv et al., 2012). Similarly, Hajimashhadi et al. (2017) demonstrated that intrathecal injection of apelin-13 increased the autonomic activity and relieved signs of pain in rats with spinal cord injury. However, one study showed that peripheral administration of apelin-13 reduced the latency of painful stimuli and enhanced pain sensitivity in a dose- and time-dependent manner (Canpolat et al., 2016), and intrathecal administration of ML221, an APJ antagonist, transiently reduced chronic constriction injury-induced pain hypersensitivity (Xiong et al., 2017). These findings suggest that the spinal apelin/APJ system may drive neuropathic pain. Thus, the regulatory effects of apelin-13 on pain may depend on the route of administration, as well as the type and degree of pain, and needs further clarification.

Previous studies have shown that apelin-13 can enhance the consolidation of passive avoidance learning and memory in mice, and these protective effects are neutralized by antagonists of α-adrenergic, cholinergic, dopamine, 5-hydroxytryptophan and γ-aminobutyric acid receptors, as well as inhibitors of nitric oxide synthesis (Telegdy et al., 2013). In a mouse model of chronic stress-induced memory deficit, apelin-13 significantly improved the cognition of new objects and memory deficit of Y maze, likely through to the upregulation of BDNF (Shen et al., 2019). In addition, exogenous apelin-13 attenuated cisplatin-induced cognitive dysfunction by activating the BDNF/TrkB signaling pathway and suppressing neuroinflammation. Apelin-13 is also known to relieve the symptoms of anxiety in mice, and these anti-anxiety effects may be related to α, *ß* adrenergic, dopamine

and 5-HT receptors since they were blocked by the administration of phenbenzamine, haloperidol, propranolol, and dimethylergometrine (Telegdy and Jászberényi, 2014). Apelin-13 also reversed depression-like behavior in rats subjected to chronic social defeat stress and chronic water immersion restraint stress by regulating microglial polarization, and ameliorating a dysfunctional HPA axis and hippocampal glucocorticoid receptor (Dai et al., 2018; Tian et al., 2018; Zhou et al., 2020).

A clinical study on 126 patients with severe TBI and 126 healthy controls found that lower serum level of apelin-13 in the patients correlated significantly with increased severity of TBI, and was an independent predictor of short-term mortality, indicating that serum apelin-13 is a promising prognostic biomarker for severe TBI (Zhuang et al., 2021). The protective effects of apelin-13 in TBI are associated with inhibition of autophagy (Bao et al., 2015), suppression of neuronal apoptosis through the GLP-1R/PI3K/Akt signaling (Liu et al., 2019), and mitigation of blood-brain barrier (BBB) destruction and brain edema (Bao et al., 2016a). Early brain injury (EBI) is at present considered to the key determinant of the neurological function and clinical outcomes of subarachnoid hemorrhage (SAH) (Sehba et al., 2012; Fujii et al., 2013). Apelin-13 can attenuate EBI by inhibiting neuronal apoptosis and degeneration, and reducing the release of inflammatory cytokines such as TNF-α and IL-1β in the CSF. These protective effects were neutralized upon administration of the APJ inhibitor ML221 (Shen et al., 2022). The anti-apoptosis effect of apelin-13 in SAH may be related to the activation of the GLP-1R/PI3K/Akt signaling pathway (Liu et al., 2019). Xu et al. (2019) found that exogenous apelin-13 can alleviate EBI by suppressing endoplasmic reticulum (ER) stress-induced NLRP3 inflammasome activation and oxidative stress after SAH. Furthermore, the APJ inhibitor dorsomorphine reversed the neuroprotective effects of apelin-13 in SAH. Another study by Xu et al. (2018) confirmed that apelin-13 reduced neuronal apoptosis and prevented BBB disruption after SAH, and eventually improved EBI by alleviating ER stress partly *via* the ATF6/CHOP pathway. Intracerebral hemorrhage (ICH) shares certain pathological characteristics with SAH. Intracerebroventricular administration of apelin-13 improved motor function and brain edema after ICH by reducing neuronal death, which demonstrates its therapeutic potential (Bao et al., 2016b).

3 Apelin-13 and cerebral ischemia

The apelin/APJ system is closely associated with the pathogenesis of ischemic stroke, which is currently the most common cerebrovascular disease. Clinical studies suggest that apelin is related to the diagnosis and prognosis of cerebral ischemia, while studies in animal and cellular models indicate that exogenous apelin-13 can effectively reduce infarct volume and cerebral edema, and improve neurological function after cerebral ischemia.

3.1 Clinical studies

In a follow-up cohort study, Wang et al. (2017b) found that the variant rs9943582 of APJ gene was not significantly associated with ischemic stroke in the Chinese Han population. Consistent with this finding, another clinical study reported that the variant rs9943582 was not associated with the age at onset and clinical outcomes of ischemic stroke (Zhang et al., 2017). However, other clinical studies have reported contradictory findings. One study conducted in China on 244 AIS patients recruited within 24 h of stroke onset and 167 healthy controls showed that serum apelin-13 levels were lower in the patients compared to the healthy controls. In addition, patients with NIHSS score ≤3 had higher apelin-13 levels than those with NIHSS score >3. Low apelin-13 level in the patients was associated with death or major disability within 3-months, whereas patients with high apelin-13 levels showed a lower incidence of stroke and combined events after 1-year. These findings indicated that serum apelin-13 is a potential prognostic biomarker for acute ischemic stroke (Wang et al., 2020). Another clinical study demonstrated that higher apelin levels were associated with increased risk of stroke (including ischemic and hemorrhagic stroke) (Yu et al., 2021). Intravenous thrombolytic therapy (ITT) is commonly used to treat acute ischemic stroke, although it can enhance the risk of hemorrhagic transformation (HT). To analyze the predictive significance of apelin on HT in acute ischemic stroke patients after ITT, Zhu et al. (2021) analyzed the data of 109 acute ischemic stroke patients that received ITT, and found that a higher HT grade was associated with lower apelin level and increased levels of interleukin-1β (IL-1β) and IL-6. Moreover, lower apelin was also related with a higher risk of death of patients with both ischemic stroke and HT, indicating that apelin is an independent protective factor in stroke patients. Atrial fibrillation (AF) is associated with a high risk of stroke, and should therefore be detected in a timely manner. Bohm et al. (2021) showed that apelin levels were significantly lower in stroke patients with AF compared to the non-AF group in a multicenter, matched-cohort, and only apelin was identified as an independent predictor of AF. Thus, apelin administration should be considered in patients with high risk of stroke to exclude the possibility of AF. However, another clinical trial shown that apelin level did not differ between stroke patients and healthy individuals, and was not associated with cardiovascular mortality and morbidity during follow-up. This discrepancy can be

attributed to differences in sample size and patients selection, and measurement assays for apelin. Further large-scale multicenter clinical trials are needed, along with detailed subgroup analysis, to clarify the therapeutic value of apelin in stroke.

3.2 Mechanistic investigation

3.2.1 Apelin-13 protects against blood-brain barrier disruption after cerebral ischemia

The BBB controls the exchange of substances between blood and brain tissue, allows nutrients to pass and prevents harmful substances from entering, thereby protecting the CNS. Given that the secondary injuries after cerebral ischemia is closely related to the morphological and structural destruction of BBB, protecting the integrity of the latter and alleviating cerebral edema are increasingly being considered as treatment options for ischemic stroke (Huang et al., 2020; Parvez et al., 2022). Apelin-13 can reduce BBB permeability and brain vasogenic edema after ischemia by mitigating oxidative stress, and inhibiting the expression of matrix metalloproteinases (MMP) and endothelin-B receptor (Gholamzadeh et al., 2021a). Furthermore, the protective effect of apelin-13 on BBB post-stroke is significantly associated with the elevated expression of aquaporin-4 (AQP4), which is partly achieved by activating the extracellular signal-regulated kinase (ERK) and PI3K/Akt pathways (Chu et al., 2017). Several factors are involved in the destruction of BBB after ischemic stroke, such as inflammatory cytokines, microvessel and endothelial cell injury, and the degradation of extracellular matrix. It remains to be explored how apeline-13 affects these pathological pathways.

3.2.2 Apelin-13 promotes angiogenesis after cerebral ischemia

The apelin/APJ system plays an important role in embryonic vascular development and adult angiogenesis (Cox et al., 2006). Both APJ and apelin are expressed in retinal vascular endothelial cells, and apelin promotes the proliferation and chemotaxis of these cells, as well as formation of capillary tubes. In addition, the apelin/APJ system may also be involved in endothelial cell proliferation and neovascularization (Tao et al., 2010; Zhang et al., 2013). Apelin-13 can promote proliferation, migration and tube formation in myocardial microvascular endothelial cells, as well as angiogenesis *via* modulation of AMPK and Akt signaling (Yang et al., 2014a). Knocking out APJ in glioblastoma cells reduced tumor growth and angiogenesis, suggesting that targeting the apelin/APJ system is a promising strategy for preventing angiogenesis in glioblastoma (Amoozgar et al., 2019; Frisch et al., 2020).

Apelin-13 plays an important role in the formation of collateral circulation. A clinical trial demonstrated that apelin-13 was significantly increased in patients with moyamoya disease compared to those with middle cerebral artery occlusion independent of NO and VEGF. Given that moyamoya disease has better collateral circulation compared to ischemic stroke, high plasma levels of apelin may be indicative of good collateral circulation (Wu et al., 2022). Furthermore, intranasal administration of apelin-13 increased the number of new vessels in the area surrounding infarction, restored the local cerebral blood flow, and promoted long-term functional recovery by upregulating vascular endothelial growth factor (VEGF) and MMP-9 (Chen et al., 2015). Apelin-13 can protect neurovascular units from ischemic injury by increasing the expression of VEGF and VEGFR2, and promoting VEGF binding to VEGFR-2 by activating the ERK and PI3K/Akt pathways (Huang et al., 2016). Cerebral blood flow blockade is often accompanied by hypoxia, which activates the apelin/APJ system, and consequently promotes endothelial cell proliferation *via* the PI3K/Akt and MAPK signaling pathways (Zhang et al., 2015; Zhang et al., 2016).

3.2.3 Apelin-13 inhibits excitotoxicity after cerebral ischemia

Aspartic acid, glutamate and glycine are excitatory neurotransmitters that are mainly distributed in the synaptic terminals of neurons in the CNS. The main excitatory amino acid released after cerebral ischemia is glutamate, which binds to the excitatory amino acid receptors on the postsynaptic membrane, resulting in neurotoxicity and neuronal damage. Nerve cells are rich in NMDA, which mediates the excitotoxicity of glutamate. Glutamate activates NMDA receptors and triggers a massive Ca^{2+} influx through the specific ion channel, resulting in intracellular calcium overload in the early stage of ischemia, and eventually cell death (Hossmann, 1994; Lai et al., 2014). Apelin-13 can reduce NMDA activity by directly reducing the ion flow potential of the NMDA receptor membrane. In addition, apelin-13 also inhibits NMDA in a dose-dependent manner by activating the pro-survival Ca^{2+}, IP3, PKC, MEK-1/2, Akt, and Raf/ERK-1/2 signaling pathways, thereby antagonizing the excitotoxicity effects of glutamate and alleviating neuron injury (Cook et al., 2011; O'Donnell et al., 2007). Another experimental study established that apelin protects against NMDA-induced retinal neuronal death *via* APJ receptor by activating Akt and ERK1/2, and downregulating TNF-α (Ishimaru et al., 2017). Zeng et al. (2010) showed that apelin-13 can prevent serum deprivation-induced changes

in Akt and ERK1/2 phosphorylation, and attenuate NMDA-induced intracellular Ca^{2+} accumulation, which in turn inhibits apoptosis and excitotoxic death.

3.2.4 Apelin-13 promotes the stability of atherosclerotic plaques

Apelin-13 has been implicated in atherosclerosis in several studies on account of its immunoreactivity in human aortas and coronary arteries. Furthermore, apelin/APJ expression patterns are inversely correlated to human aortic and coronary atherosclerosis (Kostopoulos et al., 2014). In addition, serum apelin levels are negatively correlated with the severity of arterial stenosis, and positively correlated with the stability of atherosclerotic plaques, indicating its value as a potential biomarker of atherosclerotic plaque stability (Zhou et al., 2014). Consistent with this, a clinical trial conducted on 235 (114 black, 121 white) rheumatoid arthritis patients showed that apelin concentration in the serum was associated with altered levels of plaque stability mediators (MMP-2, MMP-9) and atherosclerosis, in a manner partly dependent on population origin and systemic inflammatory status (Gunter et al., 2017). A recent study showed that the apelin/APJ system is involved in the development of atherosclerosis by influencing vascular smooth muscle cells (Luo et al., 2018). Moreover, apelin is up-regulated in human atherosclerotic coronary artery and localized to the plaque along with macrophages and smooth muscle cells (Pitkin et al., 2010). Another study confirmed that apelin-13 significantly improves plaque stability by increasing collagen content and decreasing MMP-9 expression, reducing inflammatory cell infiltration (neutrophils and macrophages) and intracellular reactive oxygen species (ROS) content (Fraga-Silva et al., 2018). Furthermore, PINK1/Parkin-mediated mitophagy promotes apelin-13-induced vascular smooth muscle cell proliferation by AMPKα and exacerbates atherosclerotic lesions (He et al., 2019).

4 Summary and prospects

The apelin-13/APJ signaling axis is ubiquitous in the peripheral and central nervous systems. Apelin-13 is an endogenous neuroprotective molecule that regulates various physiological and pathological processes in the brain. Following cerebral ischemia, apelin-13 promotes angiogenesis, increases the stability of atherosclerotic plaques and reduces excitatory toxicity, thereby improving prognosis. At present, little is known regarding the function of the apelin-13/APJ pathway, and its mechanisms have not been clarified. To this end, we first need to clarify the biological functions and mechanism of apelin-13/APJ signaling in cerebral ischemia, and the long-term effects of activating this pathway. Secondly, novel APJ receptor agonists or antagonists have to be developed to verify the feasibility and efficacy of the apelin-13/APJ system as an intervention target in ischemic stroke. In addition, the variation loci related to the apelin/APJ system, their relationship with brain structure and function, and their impact on the prognosis of cerebral ischemia also need to be elucidated. Finally, the injection route, injection time and treatment frequency of apelin-13 in pre-clinical studies need to be optimized before clinical studies on ischemic stroke patients.

Author contributions

All the authors participated in discussing the literature. JC and YY supervised the research, JL and ZC wrote and revised the manuscript. All authors read and approved the final manuscript.

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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Morinda officinalis oligosaccharides mitigate chronic mild stress-induced inflammation and depression-like behaviour by deactivating the MyD88/PI3K pathway *via* E2F2

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Morinda officinalis oligosaccharides (MOs) are natural herbal extracts that have been shown to exert antidepressant effects. However, the mechanism of this effect remains unclear. Here, we explored the mechanism by which MOs improved experimental depression. Using a chronic mild stress (CMS) murine model, we examined whether MOs could protect against depressive-like behaviour. Lipopolysaccharide (LPS)- and ATP-treated BV2 cells were used to examine the potential mechanism by which MOs mediate the inflammatory response. We found that MOs prevented the CMS-induced reduction in the sucrose preference ratio in the sucrose preference test (SPT) and shortened the immobility durations in both the tail suspension test (TST) and forced swim test (FST). We also noticed that MOs suppressed inflammatory effects by deactivating the MyD88/PI3K pathway *via* E2F2 in CMS mice or LPS- and ATP-stimulated BV2 cells. Furthermore, overexpression of E2F2 blunted the beneficial effects of MOs *in vitro*. Collectively, these data showed that MOs exerted antidepressant effects in CMS mice by targeting E2F2-mediated MyD88/PI3K signalling pathway.

KEYWORDS

depression, inflammation, MyD88/PI3K, E2F2, morinda officinalis oligosaccharides

1 Introduction

Major depressive disorder (MDD), which is also known as depression, is a mental disease characterized by a wide range of symptoms, including depressed mood, cognition dysfunction, and abnormalities in appetite and sleep (Fava and Kendler, 2000). MDD is a seriously disabling public health problem with a very high prevalence. Approximately 350 million people suffer from depression, leading to great societal and economic burdens worldwide (Athira et al., 2020). At present, mainstream antidepressants to treat depression act through monoaminergic mechanisms (Belujon and Grace, 2017). Unfortunately, 2/3 of patients do not achieve remission after one course of treatment, and 1/3 fail to remit after four treatments (Rush et al., 2006). Therefore, it is urgent to develop new effective drugs for the treatment of depression.

Inflammation is the result of immune system activation, which often manifests as a localized reaction resulting from irritation, injury, or infections (Beurel et al., 2020). The brain possesses specialized immune cells called microglia that are activated by various stimuli. Cytokines participate in the induction and effector phases of inflammatory responses and are predominantly produced by immune cells, including microglia (Beurel et al., 2020). Previous evidence has shown that inflammation plays a vital role in depression. Patients with depression show an increased inflammatory response (Howren et al., 2009; Ye et al., 2018). Higher levels of inflammation increase the risk of the development of depression (Nowak et al., 2019). Furthermore, antidepressant therapies have been reported to inhibit inflammatory effects (Kalkman and Feuerbach, 2016).

The PI3K/AKT pathway belongs to the family of serine/threonine protein kinases and is implicated in the regulation of several downstream target proteins, including NF-κB, through the phosphorylation of IκBα and p65 (Ju et al., 2020). This pathway is involved in inflammation and depression. Abnormalities in this pathway have been found in inflammation-mediated depression induced by lipopolysaccharide (LPS) (Guo et al., 2019; Sun et al., 2021). Vilazodone, a novel antidepressant, improves the depressed mood of MDD patients, which is associated with reduced NF-κB activity (Eyre et al., 2017). Increased phosphorylation levels of PI3K, AKT, and p65 induced by notoginsenoside R1 exert significant antidepressant efficacy in CUMS rats by alleviating the inflammatory response in the hippocampus (Zhan et al., 2022). MyD88 is an essential component of TLR signalling that binds to PI3K and influences the PI3K/AKT/NF-κB pathway (Gelman et al., 2006; Laird et al., 2009; Wang S. et al., 2018). The interactions between MyD88 and PI3K have been observed by using an immunoprecipitation assay (Laird et al., 2009). Gelman et al. found that the MyD88 death domain is required for NF-κB activation, and MyD88 mutations abolish the association of MyD88 and PI3K, as well as the phosphorylation of AKT, in T cells (Gelman et al., 2006). Another study reported that E2F2 controls the PI3K/AKT/NF-κB axis by binding to MyD88 (Wang S. et al., 2018).

Morinda officinalis oligosaccharides (MOs) are bioactive compounds extracted from the roots of this plant. MOs have been approved by the China Food and Drug Administration (CFDA) for use as a prescribed traditional herbal medicine to treat depression (Li et al., 2021). Significant attenuation of behavioural deficits was found in rodents exposed to chronic unpredictable stress after treatment with MOs (Xu et al., 2017). However, the mechanism underlying MO-related antidepressant effects has not been fully elucidated. Recently, MOs have been shown to suppress hippocampal inflammation in poststroke rats (Li et al., 2021). Therefore, in the current study, we investigated whether MOs mitigated depression by targeting hippocampal inflammation by using a chronic mild stress (CMS) mouse model of depression and a lipopolysaccharide (LPS)- and adenosine triphosphate (ATP)-induced cellular model of inflammation.

2 Materials and methods

2.1 Animals and treatment

Male C57BL/6 mice (10–12 weeks of age) were used in this study. The mice were housed under a constant temperature of 23 ± 1°C with a 12-h light and dark cycle and free access to food and water. All animal experiments were approved by the Institutional Review Board at the Affiliated Guangji Hospital of Soochow University.

The mice were randomly assigned to five groups (12 per group): 1) control group; 2) CMS + saline group (model); 3) CMS + 25 mg/kg MO group (MO-L); 4) CMS + 50 mg/kg MO group (MO-H); and 5) CMS +20 mg/kg fluoxetine group (Flu). Mice in the CMS groups were housed individually and exposed to two random stressors per day for 7 weeks. The stressors included food deprivation (24 h), water deprivation (24 h), cage tilting (24 h), damp bedding (24 h), inversion of the day/night light cycle, restraint in a tube (2 h), and tail clipping (1 min). MOs (Tongrentang) and fluoxetine (Sigma–Aldrich) were administered (i.g.,) once daily for three consecutive weeks starting in week 5.

2.2 Cell culture and treatment

Murine BV2 microglial cells were obtained from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS) and 1% penicillin–streptomycin solution in a humidified 5% CO_2 atmosphere at 37°C. BV2 cells were pretreated with MOs (2.5, 5, 10 mg/ml) for 24 h and then treated with LPS (10 μg/ml) and ATP (5 mM) for another 12 h (Supplementary Figure S1).

For the E2F2 overexpression experiment, BV2 cells were transfected with vectors overexpressing E2F2 (Supplementary

Figure S2) or empty vectors using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's protocol.

2.3 Luciferase reporter assay

The wild-type (WT) or mutant (MUT) MyD88 promoter was cloned into a pGL3-basic vector (Promega). Then, HEK239T cells were cotransfected with either pcDNA3.1 or pcDNA3.1-E2F2 using Lipofectamine 2000 according to the manufacturer's instructions. Luciferase activity was determined using a Dual Luciferase Assay (Promega).

2.4 Sucrose preference test

The SPT was performed to assess anhedonia in mice according to a published procedure (Shen et al., 2020). Briefly, all mice were exposed to two bottles of 1% sucrose for 48 h, followed by 24 h of water deprivation. On the testing day, the animals were provided a pre-weighed bottle of 1% sucrose and a pre-weighed bottle of drinking water for 2 h (Shen et al., 2020). The positions of the two bottles were switched at the midway point of the test to avoid side preferences. At the end of the test, the bottles were weighed, and sucrose preference was calculated as the percentage of sucrose solution intake divided by the total fluid intake for each mouse.

2.5 Tail suspension test

The TST was performed according as previously described (Meng et al., 2020). Each mouse was suspended by the tail from a vertical bar for 6 min, and the total immobility time of the final 4 min of the 6-min testing period was analysed by ANY-MAZE software. The animals were judged to be immobile when they hung passively and ceased moving their limbs and body.

2.6 Forced swim test

The FST was conducted as previously described (Zhu et al., 2020). Each mouse was individually placed in a plastic cylinder (diameter: 30 cm; height: 40 cm) containing 25 cm of water at 24 ± 1°C and allowed to swim for 6 min. The immobility time was recorded during the final 4 min of the 6-min test. Mice were judged to be immobile when they made only minimal movements to keep their head above water.

2.7 Open field test

The OFT was performed as reported previously (Sevastre-Berghian et al., 2020). The mice were placed in the centre of the open field arena and allowed to freely explore the area for 5 min. Their movements were recorded, and the total distance travelled and time spent in the central area were analysed by ANY-MAZE software.

2.8 Quantitative real-time PCR (qRT–PCR)

Total RNA was extracted from hippocampi or cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocols. Quantitative RNA analysis was performed using Nanodrop spectrophotometry. After reverse transcription of total RNA into cDNA, SYBR Green qPCR Master Mix was used for real-time PCR detection according to the manufacturer's instructions. The expression of target genes was normalized to GAPDH mRNA levels. The primers used in this study are listed below.

2.9 ELISA

The levels of TNF-α (ab208348, Abcam), IL-1α (SEKM-0001, Solarbio) and IL-1β (ab197742, Abcam) in serum and cells were measured by ELISA using commercially available kits according to the manufacturer's protocol.

2.10 Immunofluorescence

Immunofluorescence staining was performed as previously reported (Lyu et al., 2018). Sections or cells were fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.5% Triton X-100 for 20 min, and blocked with 3% BSA for 1 h. Then, the samples were incubated with primary antibodies against Iba1 (ab178847, Abcam, 1:100), E2F2 (AF4100, Affinity, 1:100) and p-NF-κB p65 (AF 2006, Affinity, 1:200) overnight at 4°C, followed by incubation with goat anti-rabbit IgG Alexa Fluor® 488 (ab150077, Abcam, 1:200) for 1 h at room temperature. Cell nuclei were labelled with 4′-6-diamidino-2-phenylindole (DAPI), and images were obtained with a confocal microscope (Olympus Fluoview TM1000).

2.11 Western blotting

Western blotting was performed as previously described (Ning et al., 2014). Total protein was extracted from hippocampi or cells using RIPA lysis buffer and quantified by a bicinchoninic acid protein assay kit. Equal amounts of protein were then subjected to SDS-PAGE and transferred onto PVDF membranes. After being blocked with 5% nonfat milk for 1 h, the blots were probed with primary antibodies against E2F2 (AF4100, Affinity, 1:1,000), MyD88 (DF6162, Affinity, 1:1,000), p-PI3K (ab182651, 1:1,000,

Primers	Forward	Reverse
E2F2	TCGCAGAGACCATAGAGCCT	GGATTGGGGACAGGAACTGG
MyD88	AGGCATCACCACCCTTGAT	ATTAGCTCGCTGGCAATGGA
TNF-α	AGGCACTCCCCCAAAAGATG	CCACTTGGTGGTTTGTGAGTG
IL-1α	CAACGTCAAGCAACGGGAAG	CAAACTTCTGCCTGACGAGC
IL-1β	GAAATGCCACCTTTTGACAGTGA	GTCCTCATCCTGGAAGGTCC
GAPDH	GGGTCCCAGCTTAGGTTCATC	TACGGCCAAATCCGTTCACA

Abcam), PI3K (ab191606, Abcam, 1:1,000), p-AKT (ab38449, Abcam, 1:1,000), AKT (ab8805, Abcam, 1:500), p-NF-κB p65 (AF 2006, Affinity, 1:1,000), NF-κB p65 (AF5006, Affinity, 1:1,000) and GAPDH (ab22555, Abcam, 1:3,000) overnight at 4°C. The corresponding secondary antibodies (ab6728, Abcam, 1:4,000) were added and incubated for 1.5 h at room temperature. Immunoreactive bands were visualized by an enhanced chemiluminescence reagent and quantified by NIH ImageJ software.

2.12 MTT assay

Cell viability was determined by the MTT assay (Cui et al., 2020). Cells were plated into 96-well plates and treated with different concentrations of MOs for 24 h. Subsequently, 20 μL of MTT (5 mg/ml) was added to the wells. After incubation for 4 h at 37°C, the formazan was dissolved in dimethyl sulfoxide (DMSO). Optical density (OD) values were measured at a wavelength of 570 nm by a microplate reader.

2.13 Statistical analysis

The data are presented as the means ± SD. The results were analysed using GraphPad Prism 6.0 software. Significant differences were determined by Student's t test or analysis of variance (ANOVA). Differences of $p < 0.05$ were considered statistically significant.

3 Results

3.1 Activation of MyD88/PI3K signaling in chronic mild stress-exposed mice

First, we examined the changes of the MyD88/PI3K pathway in the hippocampus of CMS-exposed mice. The sucrose pereference ratio was significantly decreased in stressed mice over the control group, suggestive of successful CMS modeling (Figure 1A). It is noteworthy that CMS mice exhibited higher MyD88 expression and phosphorylation levels of PI3K, AKT and NF-κB p65 than nonstressed controls ($p < 0.01$), which suggested the activation of MyD88/PI3K signalling in depression (Figure 1B).

3.2 *Morinda officinalis* oligosaccharides alleviate depressive behaviour and inflammation by suppressing the E2F2-mediated MyD88/PI3K pathway in chronic mild stress-exposed mice

To investigate whether MOs alter depressive behaviour in CMS-exposed mice, we performed the SPT, TST and FST (Figures 2A–C). The mice that underwent the CMS challenge exhibited decreases in sucrose preference in the SPT and increases in immobility durations in the TST and FST ($p < 0.01$), and these effects were attenuated by MOs or Flu administration ($p < 0.05$, $p < 0.01$), indicating the antidepressant efficacy of MOs and Flu in CMS-induced depression. However, no differences were detected in the OFT test ($p > 0.05$, Figure 2D).

Next, we evaluated inflammation in the mice. Since abnormal activation of microglia, the immunologic guardian cells of the brain, is a major contributor to depression-related inflammation, we measured Iba1 (a key marker of microglia) fluorescence intensity to assess microglial activation (Brites and Fernandes, 2015; Wang Y.-L. et al., 2018). The stressed mice exhibited significant increases in Iba1 immunoreactivity (Control: 0.401 ± 0.0594, Mod: 0.754 ± 0.0293, MOs-L: 0.604 ± 0.0451, MOs-H: 0.471 ± 0.0751), concomitantly with enhanced levels of proinflammatory cytokines, including TNF-α (PCR: Control: 1.02 ± 0.053, Mod: 1.83 ± 0.186, MOs-L: 1.39 ± 0.154, MOs-H: 1.24 ± 0.144; ELISA: Control: 132 ± 22.0 pg/ml, Mod: 294 ± 26.8 pg/ml, MOs-L: 256 ± 20.4 pg/ml, MOs-H: 175 ± 25.2 pg/ml), IL-1α (PCR: Control: 1.02 ± 0.103, Mod: 2.36 ± 0.168, MOs-L: 1.72 ± 0.199, MOs-H: 1.57 ± 0.117; ELISA: Control: 7.90 ± 1.00 pg/ml, Mod: 16.8 ± 1.38 pg/ml, MOs-L: 14.9 ± 1.58 pg/ml, MOs-H: 11.3 ± 0.880 pg/ml) and IL-1β (PCR: Control: 0.994 ± 0.0635, Mod: 2.58 ± 0.384, MOs-L: 1.93 ± 0.139, MOs-H: 1.53 ± 0.118; ELISA: Control: 21.7 ± 4.64 pg/ml, Mod: 49.9 ± 4.69 pg/ml, MOs-L: 43.5 ± 2.95 pg/ml, MOs-H: 33.5 ± 3.56 pg/ml),

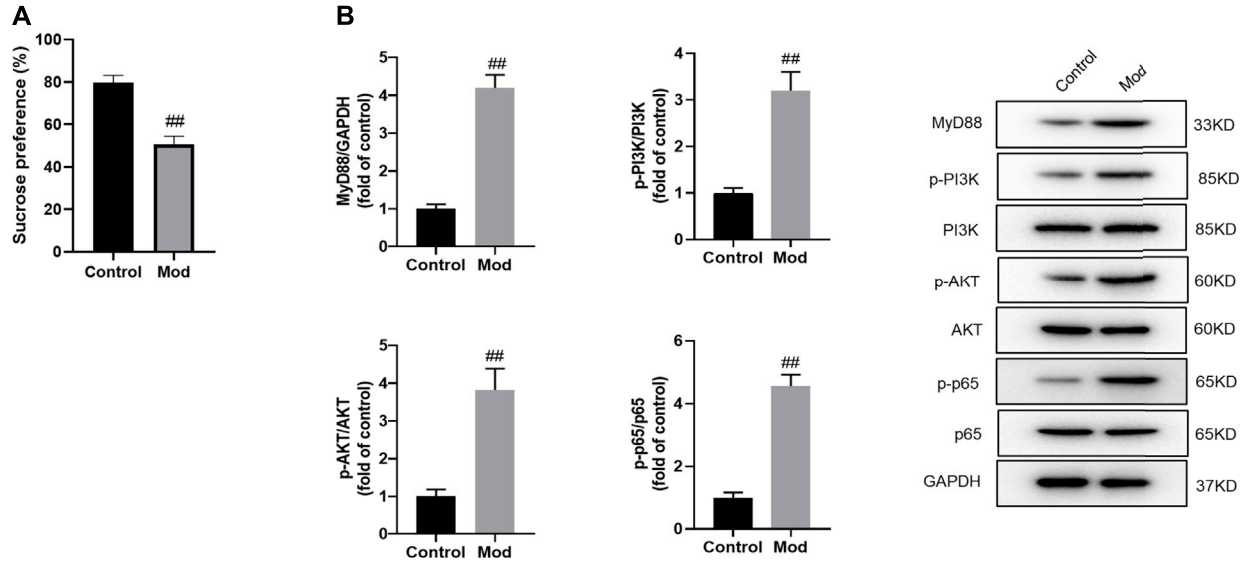

FIGURE 1
Protein expression of MyD88, p-PI3K, p-AKT and p-NF-κB p65 in the hippocampus of CMS-exposed mice. **(A)** Mice were subjected to the CMS protocol for 4 weeks, then sucrose preference test was performed. **(B)** Densitometric analysis and representative western blots of MyD88, p-PI3K, p-AKT and p-NF-κB p65 expressed in the hippocampus. Data are expressed as means ± SD (n = 3). #p < 0.05; ##p < 0.01.

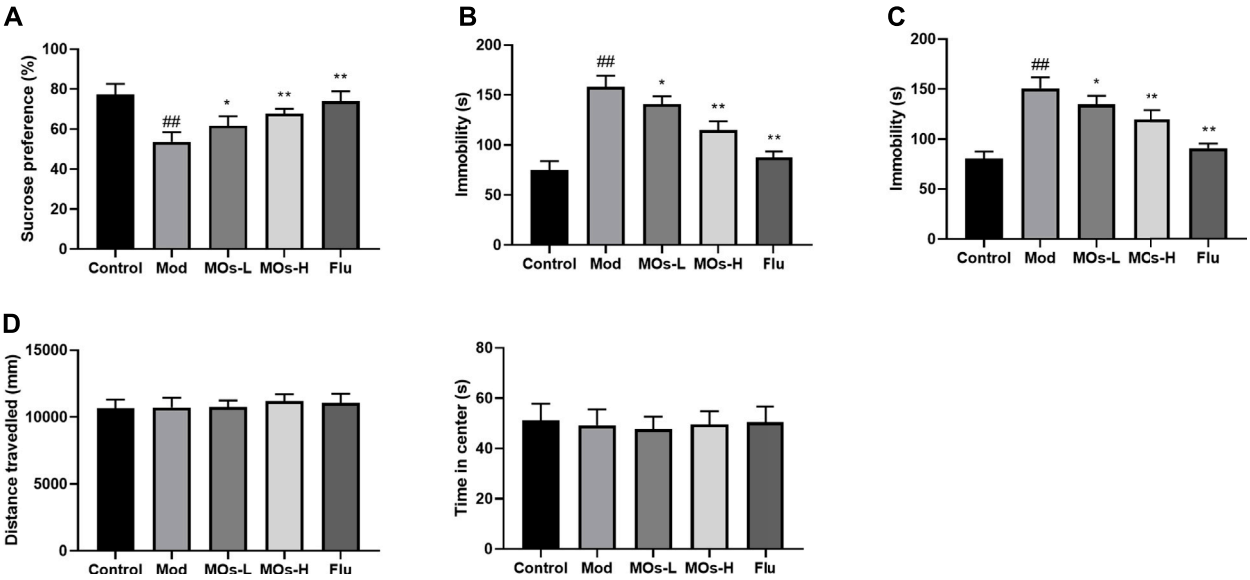

FIGURE 2
MOs block CMS-induced depressive-like behavior. **(A)** Mean sucrose preference (%) in the sucrose preference test. **(B)** The duration of immobility during the tail suspension test. **(C)** The duration of immobility during the forced swim test. **(D)** Total distance travelled and time spent in the center zone in the open field test. Data are expressed as means ± SD (n = 10). ##p < 0.01 vs. control; *p < 0.05; **p < 0.01 vs. Mod; ns, not significant.

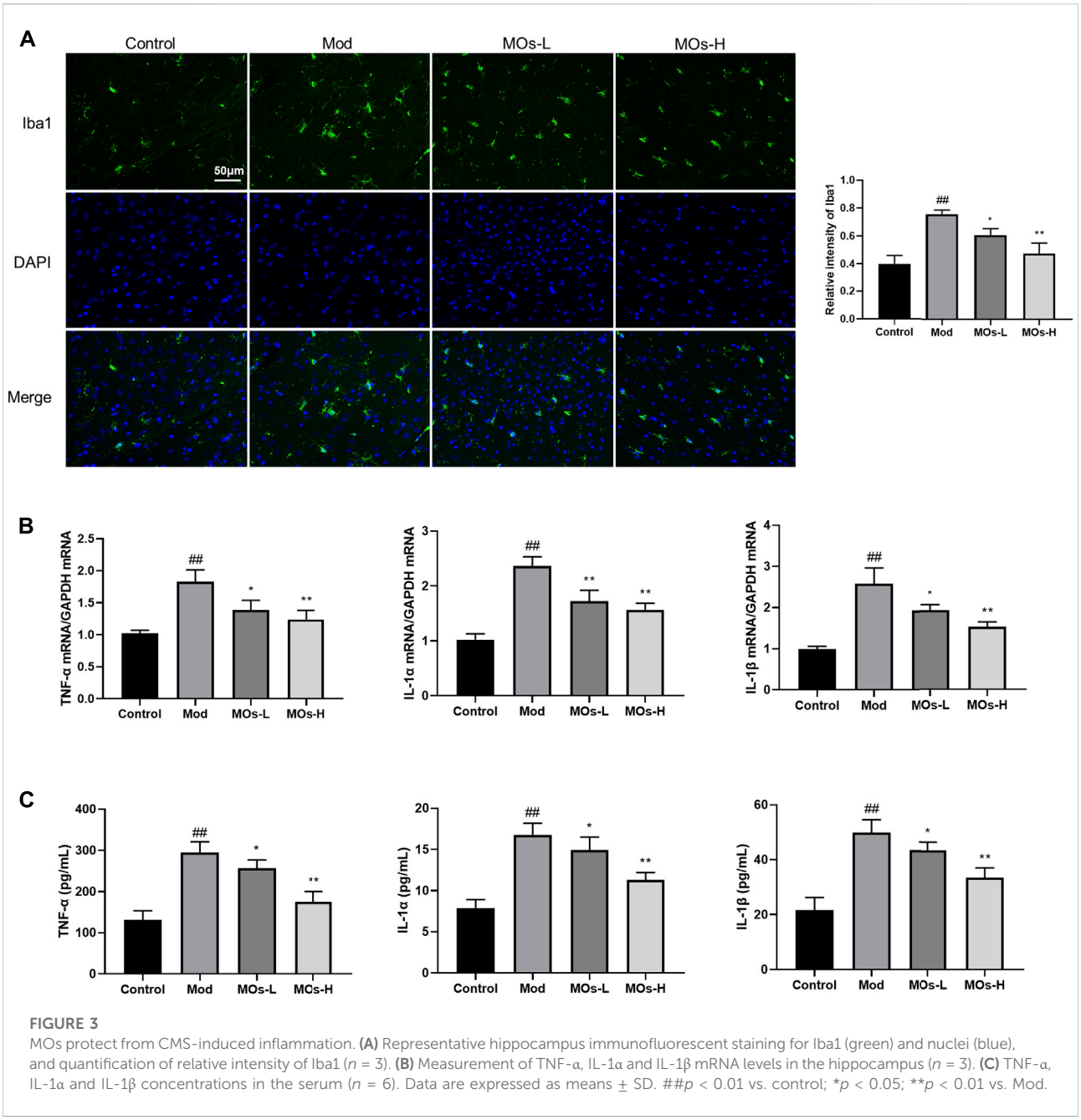

FIGURE 3
MOs protect from CMS-induced inflammation. **(A)** Representative hippocampus immunofluorescent staining for Iba1 (green) and nuclei (blue), and quantification of relative intensity of Iba1 (n = 3). **(B)** Measurement of TNF-α, IL-1α and IL-1β mRNA levels in the hippocampus (n = 3). **(C)** TNF-α, IL-1α and IL-1β concentrations in the serum (n = 6). Data are expressed as means ± SD. ##p < 0.01 vs. control; *p < 0.05; **p < 0.01 vs. Mod.

suggesting the activation of microglia after CMS modelling ($p < 0.01$, Figures 3A–C). However, these effects were suppressed by MO treatment ($p < 0.05$, $p < 0.01$). Furthermore, PCR and western blotting revealed that the mRNA expression of E2F2 and MyD88 and the protein levels of E2F2, MyD88, p-AKT, p-NF-κB p65 and p-PI3K were upregulated by CMS exposure ($p < 0.05$) and downregulated by MO intervention ($p < 0.05$, $p < 0.01$, Figures 4A–H). Together, these findings showed that MOs mitigated CMS-induced depressive behaviour and inflammation by deactivating E2F2-controlled MyD88/PI3K signalling in mice.

3.3 *Morinda officinalis* oligosaccharides inhibit E2F2 binding to MyD88

To validate the interaction between MyD88 and E2F2, luciferase reporter assays were performed in HEK293T cells (Figures 5A–C). Cotransfection of the MyD88-promoter-WT plasmid and E2F2 vector markedly increased luciferase expression compared with the effect of the MyD88-promoter-WT plasmid lacking the E2F2 vector ($p < 0.01$). However, cotransfection of the MyD88 promoter-MUT plasmid and E2F2 vector did not affect

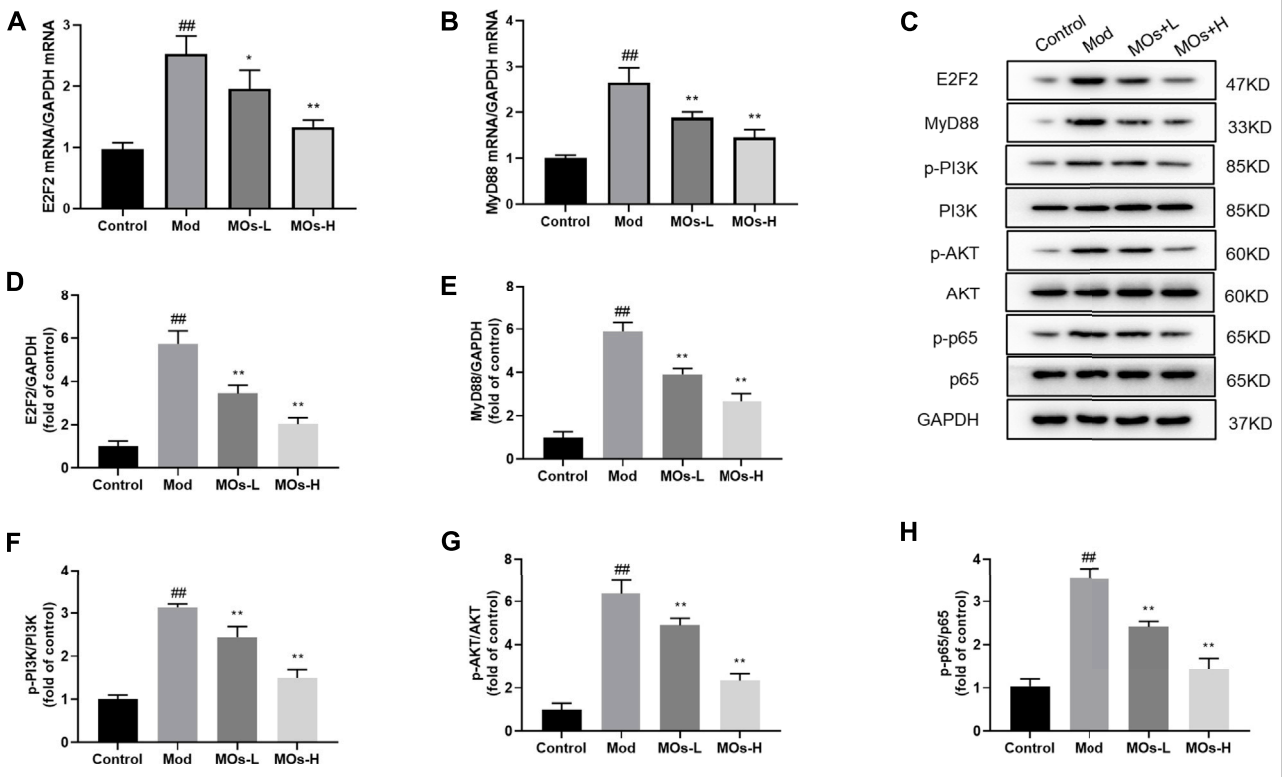

FIGURE 4
Effects of MOs on E2F2-regulated MyD88/PI3K pathway in the hippocampus. **(A–B)** qRT-PCR analysis of gene expression of E2F2 and MyD88 in hippocampus tissues. **(C–H)** Immunoblot analysis of E2F2, MyD88, p-PI3K, p-AKT and p-NF-κB p65 in hippocampus tissues. Data are expressed as means ± SD (n = 3). ##p < 0.01 vs. control; *p < 0.05; **p < 0.01 vs. Mod.

luciferase activity (p > 0.05). These findings showed that E2F2 could directly bind to the promoter of the MyD88 gene. We then assessed the effect of MOs on this binding process. Surprisingly, MO treatment inhibited the binding of E2F2 and the MyD88 promoter, as demonstrated by lower luciferase expression in the MyD88-promoter-WT + MO-H-E2F2 group than in the MyD88-promoter-WT + NC-E2F2 group (p < 0.01).

3.4 *Morinda officinalis* oligosaccharides protect against lipopolysaccharide- and adenosine triphosphate-induced inflammation *via* E2F2-mediated MyD88/PI3K signalling in BV2 cells

We also examined the protective effect of MOs in LPS- and ATP-induced cellular models of inflammation. Given that the maximum concentration of MOs that exerted no cytotoxicity in BV2 cells was 10 mg/ml, this dose was selected as the high dose for use in subsequent experiments (Figure 6A). As expected, MOs prevented LPS- and ATP-induced reductions in cell viability and the promotion of TNF-α, IL-1α and IL-1β levels (p < 0.05, p < 0.01, Figures 6B,C). Subsequently, we examined the effect of MOs on the E2F2-mediated MyD88/PI3K signalling pathway (Figures 7A,B). The results showed that MOs decreased E2F2 immunoreactivity and the protein expression of E2F2, MyD88, p-PI3K, p-AKT and p-NF-κB p65 in LPS- and ATP-exposed BV2 cells (p < 0.05, p < 0.01). Thus, MOs exerted anti-inflammatory effects *via* the E2F2-regulated MyD88/PI3K pathway *in vitro*.

To further elucidate the role of E2F2 in the anti-inflammatory effect of MOs, BV2 cells were transfected with E2F2 overexpression vectors (Figures 8A–H). We found that E2F2 overexpression reversed MO-induced decreases in MyD88 mRNA expression; MyD88, p-PI3K, p-AKT and p-NF-κB p65 protein expression; and p-NF-κB p65 immunoreactivity (p < 0.01), resulting in increased mRNA levels of proinflammatory factors (TNF-α, IL-1α and IL-1β, p < 0.01). These findings suggested that E2F2 inhibition was required for MO-induced anti-inflammatory effects *in vitro*.

4 Discussion

In the current study, we investigated the inflammatory mechanism related to the antidepressant activity of MOs. We

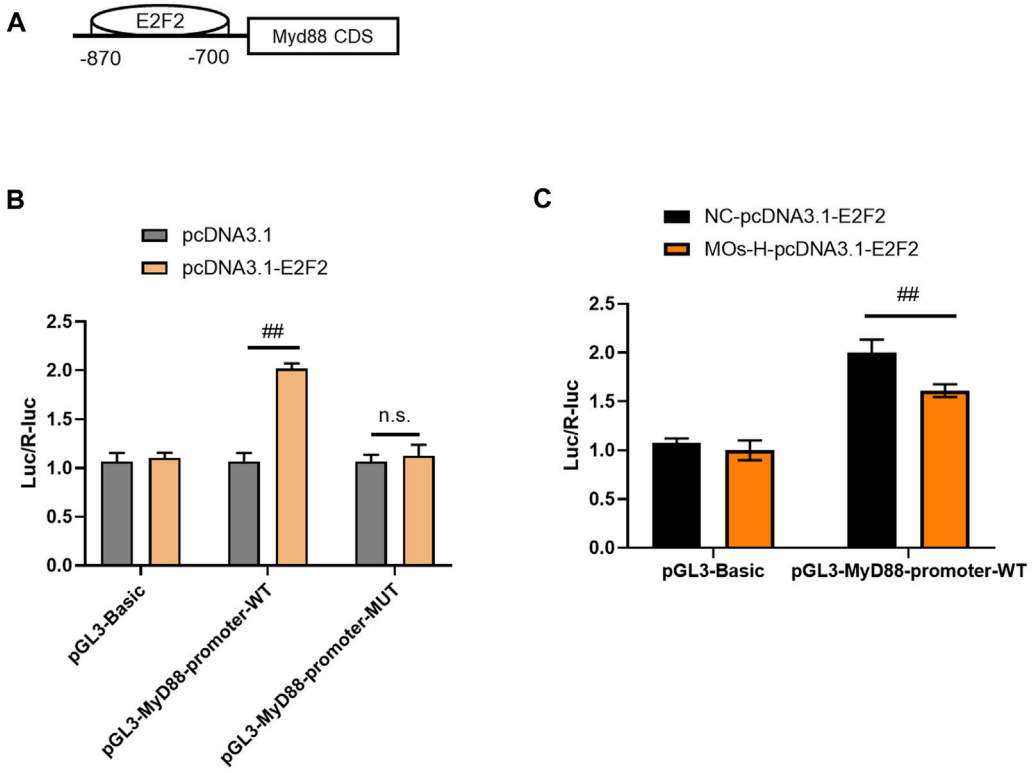

FIGURE 5
MOs inhibit E2F2 binding to MyD88 in HEK239T cells. **(A)** Schematic representation of the E2F2 binding motif in the MyD88 promoter. **(B)** Luciferase reporter gene assay of E2F2 and MyD88. **(C)** Effects of MOs (50 mg/kg) on E2F2 binding to MyD88 in the HEK239T cells. Data are expressed as means ± SD (n = 3). ##p < 0.01; n. s. not significant.

found that MOs ameliorated the depressive-like symptoms in mice underwent CMS protocol. MOs also inhibited CMS- or LPS + ATP-induced high levels of inflammation by targeting the MyD88/PI3K signaling pathway *via* E2F2. Additionally, overexpression of E2F2 reversed MO-produced anti-inflammatory effect *in vitro*.

Depression is a common psychiatric disorder associated with marked suffering. Since synthetic antidepressants have obvious disadvantages, such as limited efficacy, side effects and high prices, the antidepressant properties of natural medicines are attracting increasing attention (Liu et al., 2015). Feng et al. reported that Bupleuri Radix attenuated depression-like behaviour in rats by regulating metabolic profiles and the gut microbiota (Feng et al., 2020). A double-blind, randomized clinical trial revealed that crocin extracted from saffron (*Crocus sativus* L.) mitigated depressive symptoms in patients with breast cancer during chemotherapy (Salek et al., 2021). In the FST, the antidepressant efficacy of silexan, an essential oil from the flowering tops of *Lavandula angustifolia*, was comparable to the tricyclic antidepressant imipramine after 9 days of treatment (Friedland et al., 2021). In the current study, we investigated the antidepressant activity of the herbal medicine MOs by using a chronic mild stress (CMS) mouse model. CMS is one of the most widely used rodent models of depression. The primary variable measured in CMS is sucrose preference; stressed mice show reductions in sucrose consumption, which is interpreted as anhedonia, a core symptom of depression (Ramaker and Dulawa, 2017). Here, we found that MOs inhibited the reduction in sucrose consumption in the sucrose preference test and increased immobility durations in the tail suspension and forced swim tests, indicating the antidepressant activities of MOs in depressive rodents and further supporting the potential use of natural medicines in treating depression. It is noteworthy that although CMS rodents have been reported to have abnormalities such as decreased duration in the central zone in the OFT test, we did not observe any changes in the OFT like some other studies, which might be associated with differences in CMS protocols (Zhou et al., 2019; Shan et al., 2020; Xia et al., 2020).

Inflammation is an essential immune response that enables survival during infection or injury and maintains tissue homeostasis under a variety of noxious conditions (Medzhitov, 2010). Studies have indicated that inflammation regulates a wide variety of diseases, including depression.

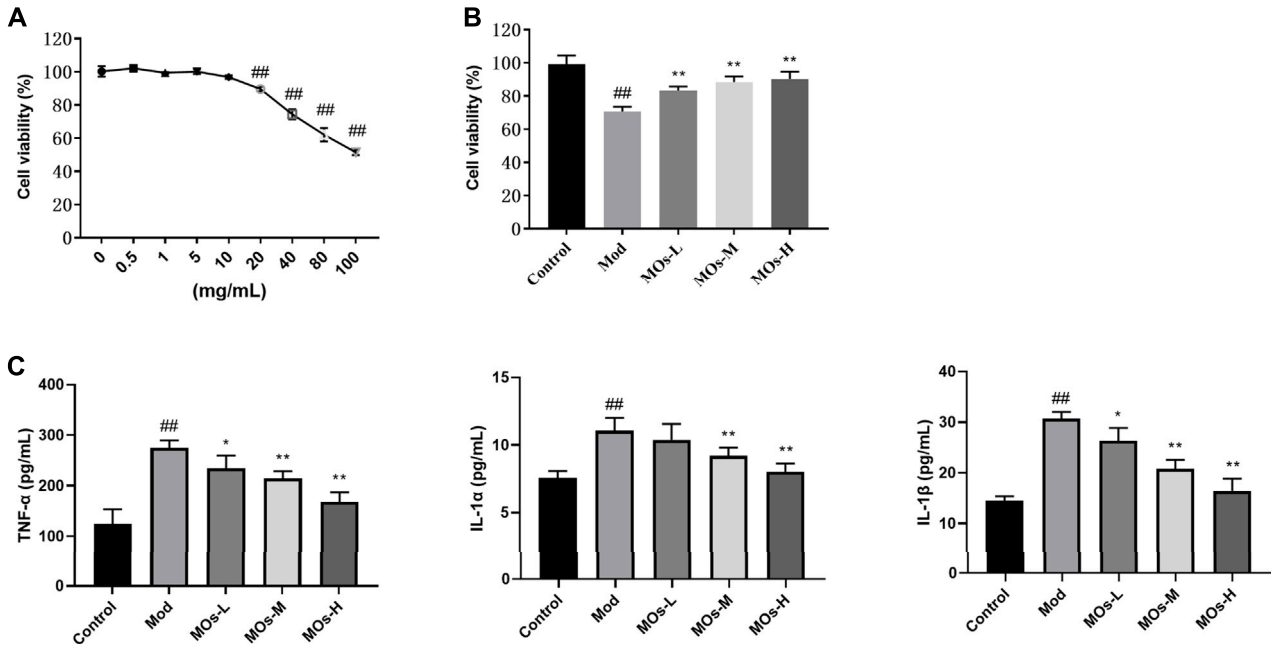

FIGURE 6
Protective effects of MOs against LPS- and ATP-induced inflammation in BV2 cells. **(A)** BV2 cells were administrated with different concentrations of MOs for 24 h, and the cell viability was detected using the MTT assay. **(B)** Cell viability assay of BV2 cells after 24 h of MOs treatment followed by LPS and ATP stimulation. **(C)** ELISA assays to determine the production of TNF-α, IL-1α and IL-1β in BV2 cells. Data are expressed as means ± SD (n = 6). ##p < 0.01 vs. control; *p < 0.05; **p < 0.01 vs. Mod.

Compared with healthy controls, patients with major depression have exhibit increased TNF-α and IL-1β levels both in the cerebrospinal fluid and in the peripheral blood circulation (Raison et al., 2006). The administration of interferon (IFN)-α (a potent inducer of proinflammatory cytokines) to treat cancer results in the development of depressive symptoms in a high percentage of patients (Capuron et al., 2002). In addition, antidepressants, such as selective serotonin reuptake inhibitors (SSRIs), exert negative immunoregulatory effects, suppressing the release of proinflammatory cytokines, such as IFN-γ, TNF-α, IL-1β and IL-6, and stimulating the generation of anti-inflammatory cytokines, such as IL-10 (Xia et al., 1996; Maes et al., 1999). In this study, we found that the CMS protocol increased Iba1 expression as well as TNF-α, IL-1α, and IL-1β levels in the hippocampus or serum, while MOs successfully inhibited this effect in CMS-exposed mice, leading to the attenuation of depression-like symptoms. These findings confirmed the involvement of inflammation in the aetiology of depression and MO-induced antidepressant efficacy.

The MyD88/PI3K pathway is an important pathway for regulating inflammation and depression. MyD88 is an adapter protein that mediates signal transduction for most TLRs. Previous evidence suggests that MyD88 can bind to the lipid kinase enzyme PI3K, which phosphorylates downstream target AKT and enhances inflammatory response *via* the NF-κB signal (Laird et al., 2009; Shorning et al., 2020). Patients with major depression have higher levels of IL-6 than healthy controls (Wang et al., 2019). Aerobic exercise inhibits MyD88/NF-κB signalling and hippocampal inflammation, which contributes to improvements in desperate behaviour and hippocampal function (Qu et al., 2020). LPS enhances the levels of IL-1β, IL-6, and TNF-α in the hippocampus and evokes depressive-like behaviours in mice, and these effects are alleviated by baicalin through the PI3K/AKT pathway (Guo et al., 2019). Astrocyte-specific reductions in Men1 levels enhance NF-κB activation and IL-1β production, leading to the development of depression in mice, and these effects can be rescued by an NF-κB inhibitor (Leng et al., 2018). In this work, upregulated MyD88 mRNA expression and enhanced phosphorylation of PI3K, AKT and NF-κB p65 were observed following CMS modelling. However, MO intervention greatly normalized the mRNA expression and protein levels of the MyD88/PI3K axis, reflecting the importance of this signalling pathway in MO-induced anti-inflammatory effects on depression. Furthermore, E2F2 overexpression blocked the MO-induced anti-inflammatory effect on LPS- and ATP-induced BV2 cells, confirming the necessary role of E2F2 in MO-mediated antidepressant activities. Previously, MOs have been demonstrated to generate antidepressant activities in rodents by suppressing hippocampal inflammation through the

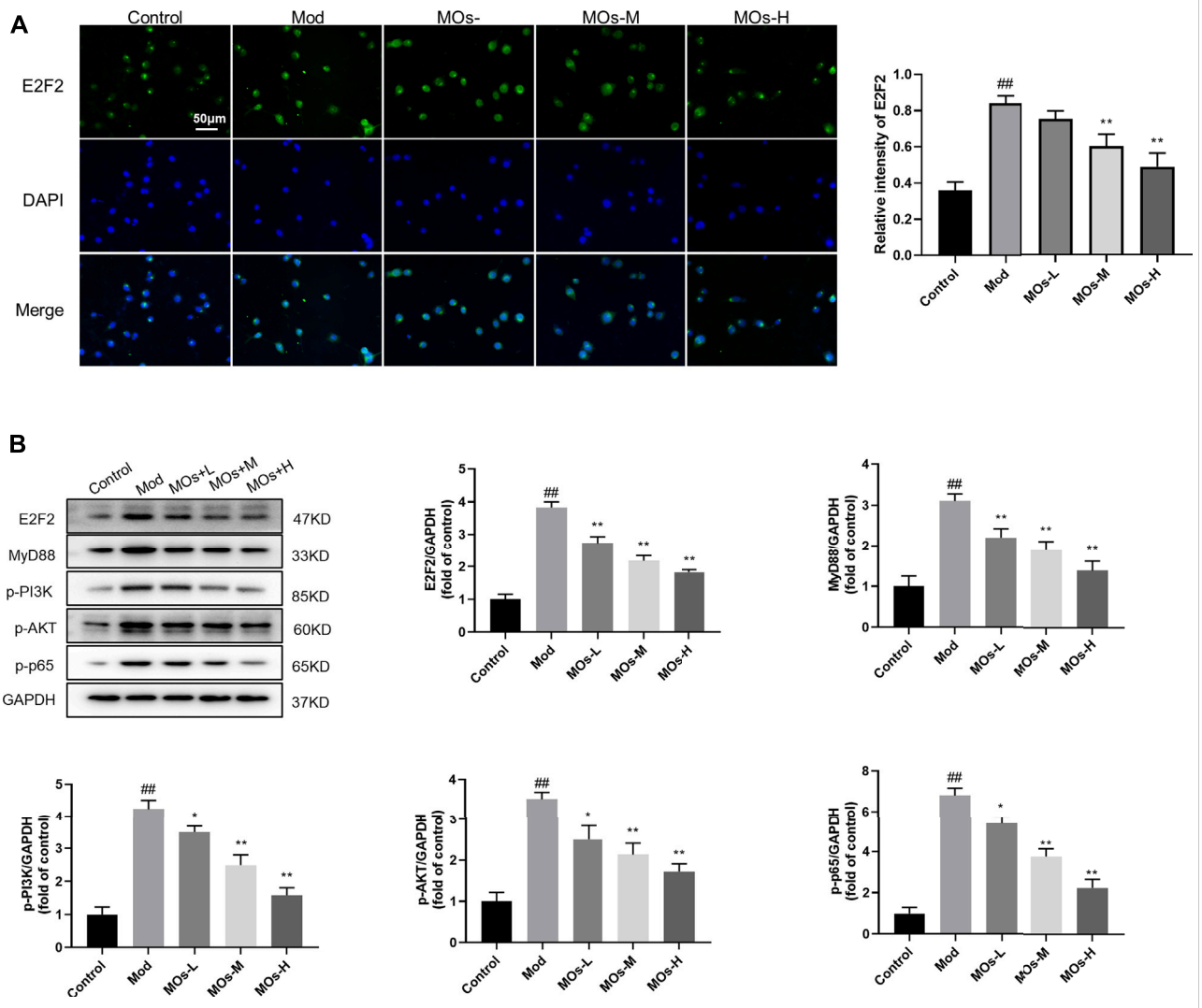

FIGURE 7
Effects of MOs on E2F2-controlled MyD88/PI3K pathway in LPS- and ATP-stimulated BV2 cells. **(A)** Representative hippocampus immunofluorescent staining for E2F2 (green) and nuclei (blue), and quantification of relative intensity of E2F2. **(B)** Protein expression levels of E2F2, MyD88, p-PI3K, p-AKT and p-NF-κB p65 in BV2 cells. Data are expressed as means ± SD ($n = 3$). ##$p < 0.01$ vs. control; *$p < 0.05$; **$p < 0.01$ vs. Mod.

microglial NLRP3 inflammasome (Li et al., 2021). Here, we observed that hippocampal MyD88/PI3K signalling was also an important pathway by which MOs attenuated hippocampal inflammation and ultimately induced antidepressant effects. In addition, the PI3K signalling was activated by E2F2 binding to the MyD88 promoter in the present study, which was consistent with the results observed by Wang et al. in rheumatoid arthritis (Wang S. et al., 2018).

There are certain limitations to the study. First, we only assessed the antidepressant effect of MOs in the CMS model, and other models, such as the LPS-induced depression model, should be used in future studies to fully clarify the antidepressant efficacy of MOs. Second, although the *in vitro* results indicated the important role of E2F2 in MO-induced antidepressant effects, animal studies with E2F2-overexpression vectors might be needed to elucidate the role of E2F2 in MO-mediated antidepressant effects. Third, conventional antidepressants have a major disadvantage in their several-week-long lag period of therapeutic efficacy, and whether MOs can exert a rapid onset antidepressant effect is worth further exploration.

In summary, our data demonstrated that MOs alleviate experimental depression and inflammation *via* the E2F2-mediated MyD88/PI3K signalling pathway. This work provides a promising molecular agent for the treatment of depression.

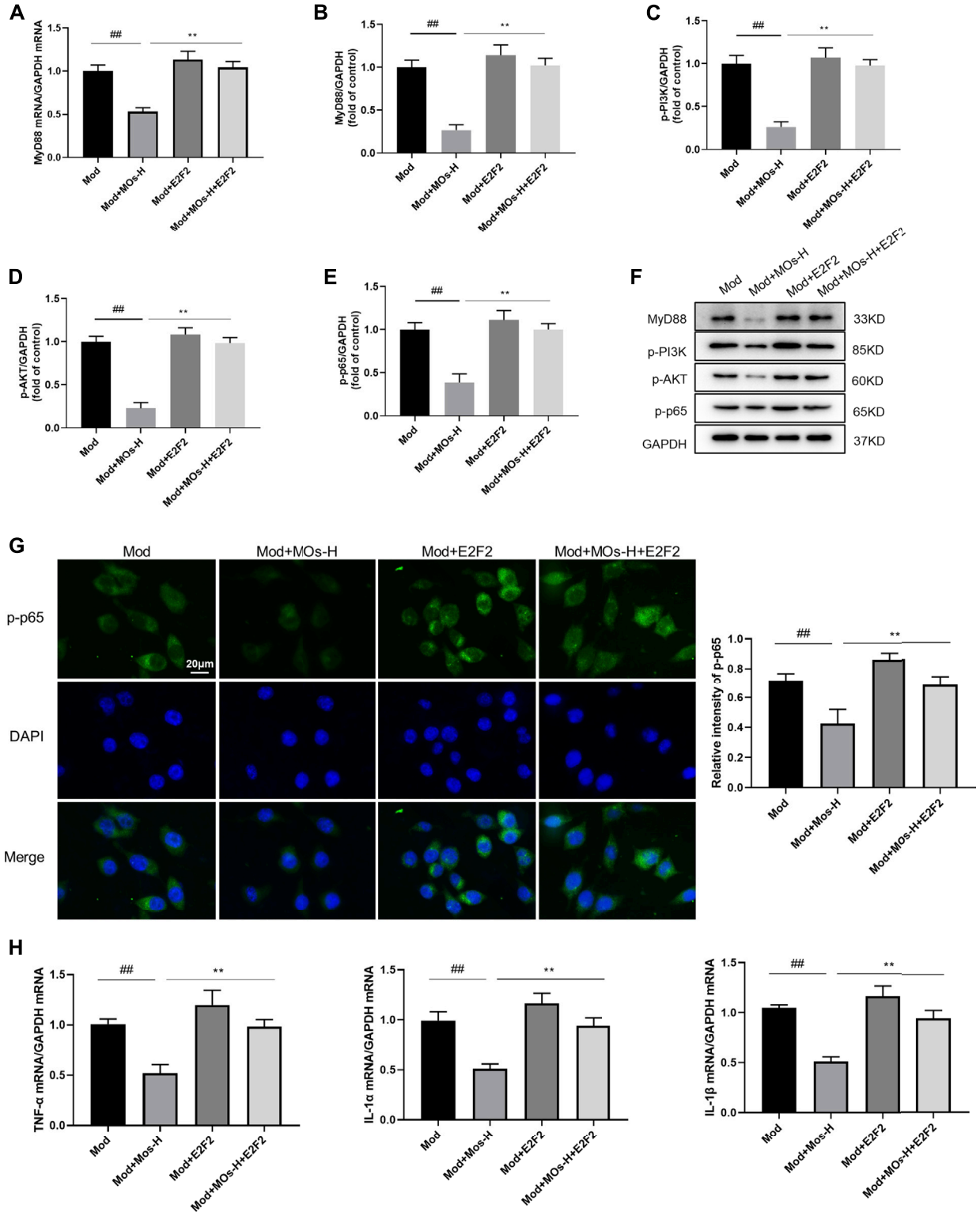

FIGURE 8
Effects of MOs in LPS- and ATP-stimulated BV2 cells were reversed by overexpression of E2F2. **(A)** qRT-PCR analysis of MyD88 mRNA expression in BV2 cells. **(B–F)** Immunoblot analysis of MyD88, p-PI3K, p-AKT and p-NF-κB p65 in BV2 cells. **(G)** Representative hippocampus immunofluorescent staining for p-NF-κB p65 (green) and nuclei (blue), and quantification of relative expression of p-NF-κB p65. **(H)** TNF-α, IL-1α and IL-1β mRNA levels in BV2 cells. Data are expressed as means ± SD (n = 3). ##p < 0.01; **p < 0.01.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by All animal experiments were approved by the Institutional Review Board at the Affiliated Guangji Hospital of Soochow University.

Author contributions

YZ, LH, and ZZ designed the experiments; XY, TX, GY, PW, and QQ performed the experiments; WT and ZZ analyzed the data; ZZ and WT wrote the paper. QJ, JW, LH, and YZ edited the manuscript. All authors participated in the preparation of the manuscript and approved the final manuscript.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.855964/full#supplementary-material

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Single-cell sequencing of brain tissues reveal the central nervous system's susceptibility to SARS-CoV-2 and the drug

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Background: The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) caused the current COVID-19 pandemic, resulting in a public health crisis that required immediate action. The SARS-CoV-2 virus enters human cells via three receptors, namely cathepsin, angiotensin-converting enzyme 2 (ACE2) and SARS-CoV receptors. Cathepsin destroys the spike protein (S protein), thereby allowing the entry of viral nucleic acid into human host cells.

Methods: Utilizing single-cell transcriptome analysis of brain tissues, the vulnerability of the central nervous system to infection with SARS-CoV-2 in humans was investigated.

Results: ACE2 is mainly expressed in endothelial cells, with the highest levels found in ageing endothelial cells. Drug prediction suggests that (-)-catechin reduces the effects of COVID-19 on the nervous system. Immunohistochemistry analysis showed that ACE2 was mainly expressed in cerebral vessels. Immunofluroscenceresults showed the co-expression of CD31 and ACE2 in human tissues. Western blot further showed that ACE2 expression was higher in old rats than in young rats.

Conclusion: This study provides insight into the mechanism of SARS-CoV-2 brain invasion. Accordingly, patients with neurological symptoms who are infected with SARS-CoV-2 should be given individualised care.

KEYWORDS

SARS-CoV-2, single-cell, epigallocatechin, CNS, ACE2, catechins

Introduction

During the early days of December 2019, a novel transmittable infection of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spread rapidly across China (Ladner et al., 2020). The World Health Organization (WHO) classified SARS-CoV-2 as a worldwide viral pandemic on 11 March 2020 (Lambertini et al., 2021; Omolaoye et al., 2021). Millions of individuals globally have been impacted by the SARS-CoV-2 virus. SARS-CoV-2 infection is a heterogeneous illness (Bhattacharyya and Thelma, 2020; Mavian et al., 2020), with extensive clinical characteristics, such as asymptomatic infection, septic shock, acute respiratory distress syndrome (ARDS), mild upper respiratory tract infection, multi-organ failure and mortality (Boras et al., 2021).

Respiratory viral infections, like other forms of viral infections, invade the central nervous system (CNS) via the hematoma or various neural retrograde routes. In terms of the CNS, it is infiltrated by a viral agent via the circulatory system. Moreover, certain viruses can invade neurons in the peripheral nervous system and then utilizes the axonal transport mechanism to obtain entry into the CNS (Schwerk et al., 2015; Dahm et al., 2016a). In the hematoma pathway, a virus can successfully invade the endothelium of the blood-brain barrier (BBB) or the epithelium of the blood-cerebrospinal fluid barrier (BCSFB) in the choroid plexus (CP), which is located in the ventricular system of the brain, or leukocytes, which can serve as a vector for dispersion towards the CNS (Argyris et al., 2007; Atluri et al., 2015). Furthermore, numerous SARS-COV-2-infected individuals experienced signs of neurological symptoms such as vomiting, nausea and headache. A clear association between these symptoms and unfavourable outcomes has been widely reported. Additionally, Moriguchi et al. presented the very first incidence of encephalitis/meningitis correlated with SARS-CoV-2 infection in the cerebrospinal fluid (CSF) that did not result in a positive nasal polyp test (Moriguchi et al., 2020). Furthermore, it is unclear if SARS-CoV-2 can infiltrate the CSF or the CNS of asymptomatic individuals. Nonetheless, the vulnerability of human CNS cells to SARS-CoV-2 and its specific pathogenic processes remain largely unknown.

Catechins, a category of phenolics that are predominantly found in foodstuffs, including cocoa, tea leaves, vegetables, fruits and wine, have been well recognized for their intriguing health-promoting functions, such as antioxidative, antibiotic, neuroprotective, anti-inflammatory, and anticarcinogenic functions. As a result, green tea is one of the richest and most available catechin sources, containing (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG) and (−)-epicatechin (EC) predominantly (Wang et al., 2021). These compounds have the capacity to destroy and also inhibit the spread of pathogenic pathogens. Numerous investigations have shown that EGCG suppresses influenza virus multiplication in cell cultures and that catechin has viable viricidal actions against a wide range of viruses, including those of the Flaviviridae, Orthomyxoviridae and Retroviridae families. Furthermore, EGCG acts against the human immunodeficiency virus (HIV) by inhibiting the enzymatic activities of the herpes simplex virus 1 and 2 (HSV-1 and HSV-2), hepatitis C virus (HCV) and HIV-1 reverse transcriptase (Liu et al., 2021). Based on the principle of reverse expression, catechin has the potential to prevent SARS-COV-2 from entering the CNS.

Therefore, understanding the expression patterns of ACE2 in the nervous system is crucial in determining the neural system's vulnerability to SARS-CoV-2 infection. This study examines the expression of ACE2 and associated genes in brain tissues, aiming to elucidate the susceptibility of the CNS to SARS-CoV-2 infection.

Materials and methods

Datasets

A single-cell RNA-seq expression profile of the mouse brain vascular system was obtained from the Gene Expression Omnibus (GEO) repository (GSE60361). The gene expression levels in each cell were analysed. Genes with an expression level of less than 0.1% of the total number of cells in the study were excluded. Eventually, 3005 cell samples from the dataset were selected for analysis, and the samples satisfied the quality control standards.

Clustering and dimensionality reduction

The Seurat package (version: 3.2.2) in the R software (version: 4.0.2) was utilized to conduct principal component analysis (PCA) using the PCElbowPlot and JackStraw functions to identify key principal components (PCs). To determine gene heterogeneity in each cell group, the FindAllMarkers utility in Seurat was utilized. Following this, cell clustering and visual analysis of UMAP were performed utilizing the RunUMAP platform. The singleR package was subsequently utilized to annotate the marker genes, and CellMarker was thereafter employed to correct them based on their features.

Pathway and process enrichment analysis

Pathway and process enrichment analysis was performed using Metascape (https://metascape.org/gp/index.html). Pathways and processes enrichment studies were performed using the following ontology resources for the ACE2-related gene list: PANTHER Pathway, WikiPathways,

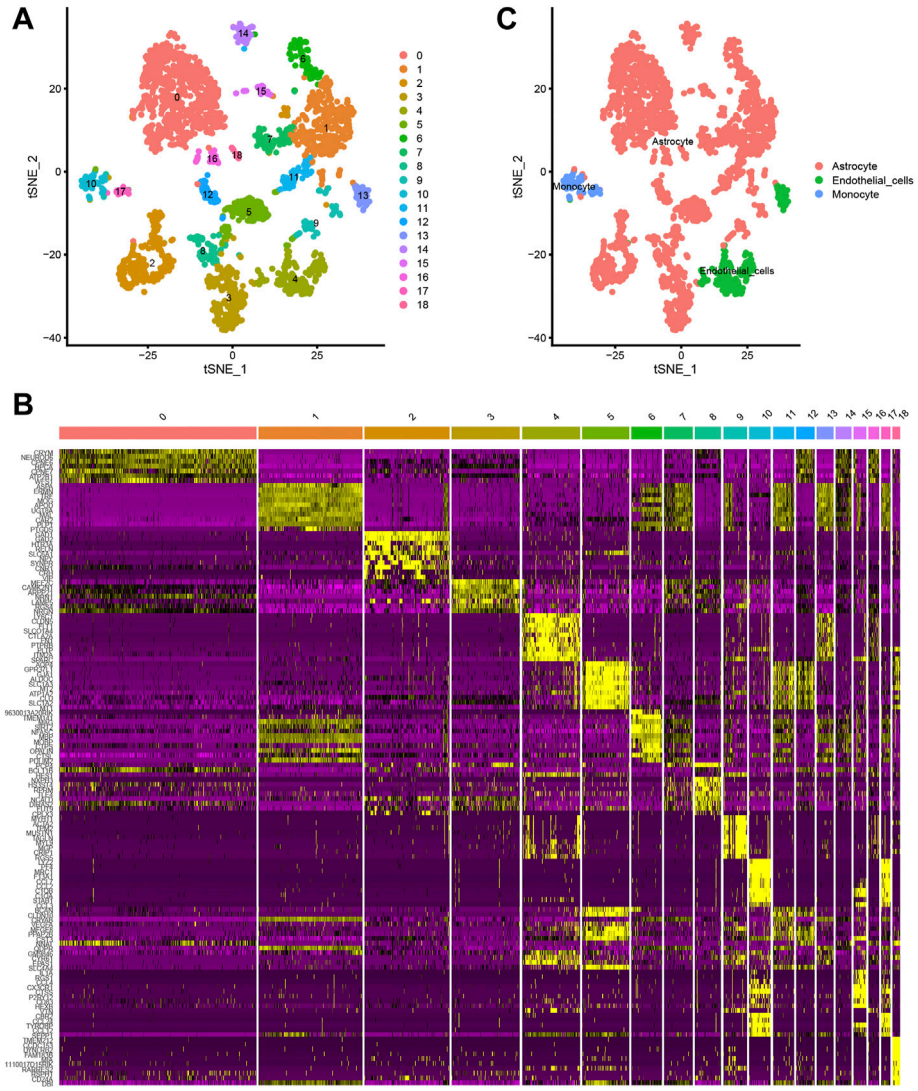

FIGURE 1
(A, B) The t-distributed stochastic neighbour embedding (t-SNE) technique classifies 18 cell clusters using the relevant PCs identified via principal component analysis. **(C)** A total of 18 clusters were identified using differential analysis. The top 10 marker genes in each cell cluster are shown in the heatmap.

Transcription Factor Targets, PaGenBase, DisGeNET, TRRUST, CORUM, Canonical Pathways, Reactome Gene Sets, GO Biological Processes, KEGG Pathway and COVID. Moreover, all genes in the genome served as the enrichment background. To collect and classify terms based on their affiliation commonalities, a *p*-value less than 0.01, the least count of three and an enrichment factor of more than 1.5 (the enrichment factor denotes the ratio of the recorded counts and anticipated counts) were used. Furthermore, *p*-values were determined utilizing the accumulative hypergeometric distribution, whereas q-values were derived utilizing the Benjamini–Hochberg technique, which involves multiple tests. Kappa score was used for the hierarchical clustering of the enriched terms, wherein sub-trees with a similarity degree of more than 0. 3 were deemed to be a cluster. The most significant statistical term inside a cluster was selected to serve as the cluster's representative term (Hochberg and Benjamini, 1990).

Protein-protein interaction enrichment analysis

To obtain the gene list of ACE2-related proteins, PPI enrichment analysis was performed utilizing different databases, includingInWeb_IM9, OmniPath8, BioGrid7, and

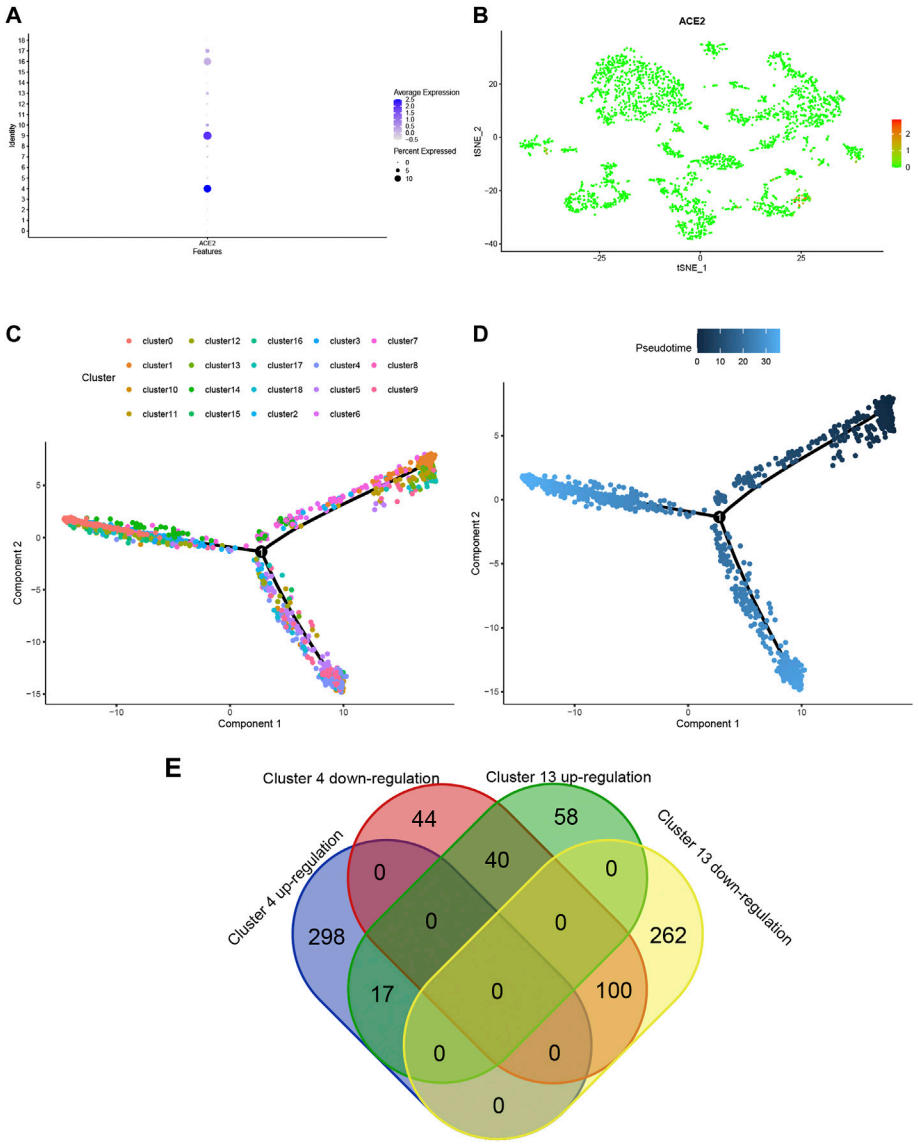

FIGURE 2
(A) Bubble plot of ACE2 expression in different cell clusters. **(B)** Dot plot shows ACE2 expression in each cell cluster. **(C,D)** The trajectory plot in pseudotime of each cell cluster using Monocle analysis. Different colours represent different cell states. **(E)** Venn plot of Cluster 4 and Cluster 13 in up- and down-regulated states.

STRING6 (Li et al., 2017; Oughtred et al., 2019; Szklarczyk et al., 2019). Furthermore, only the physical interaction function was used in STRING (with a physical score greater than 0.132) and BioGrid databases. The resulting network comprised the selection of proteins that have established physical interplays with a minimum of one other component on the list. When the networks consisted of approximately 3–500 proteins, the Molecular Complex Detection (MCODE) method was employed to determine the components of the network that significantly correlated with each other (Bader and Hogue, 2003).

Small molecules identification

To predict relatively small active molecules that might attenuate the existing biological state of ACE2-related endothelial cells, an evaluation of the ACE2-related endothelial cells was performed *via* the comparison of the differentially expressed genes (DEGs) between clusters 4 and 13 against those found in the Connectivity Map database (CMap, http://www.broadinstitute.org/cmap/). Initially, the DEGs were classified into two groups, namely downmodulated and upmodulated groups. Subsequently, for gene set enrichment

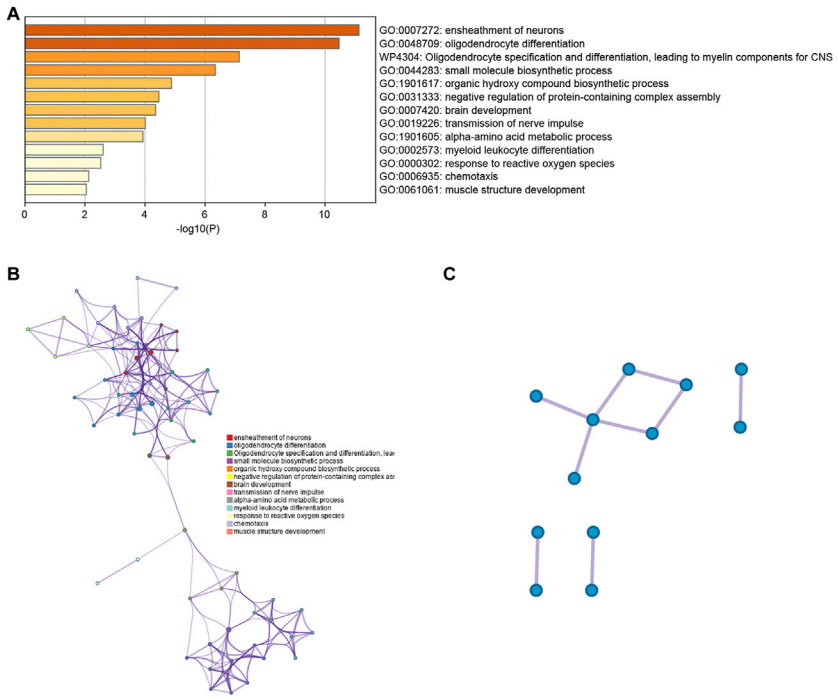

FIGURE 3
(A) Bar graph representing the enriched terms in the gene list, which are shaded according to their *p*-values. **(B)** A network of enriched terms, shaded according to cluster-ID, with nodes sharing a common cluster-ID often situated adjacent to each other. **(C)** The gene list reveals a network of protein-protein interactions and MCODE components.

analysis (GSEA), different expression significant probesets were selected from each group and evaluated, resulting in enrichment scores that ranged from −1 to +1. Furthermore, small molecules with positive connectivity values close to +1 were found to drive gene expression in cluster 13, while those with negative connectivity values close to −1 showed increased similarities between genes and small molecules, which might attenuate cluster 4's status.

Human protein atlas database analysis

HPA (https://www.proteinatlas.org/) is a database containing details on cell and tissue distributions among the 24,000 proteins found in the human body. It employs specialised antibodies and immunohistochemical technologies to examine the dispersion and expression of every protein in 48 different types of normal human tissues, 12 different types of blood cells, 47 different types of cell lines and 20 different types of tumour tissues. These tissues are collected from 144 distinct normal and 216 distinct tumour tissues, thus guaranteeing that the immunohistochemical findings are representative of the population. Thus, using this database, both the prognostic value and protein expression levels of the most possible hub genes in brain tissues were validated.

Immunofluorescence

Juvenile Sprague–Dawley (SD) rats aged 4 weeks and old SD rats aged 12 months were selected and subsequently treated with intracardiac perfusion of 0.1 mmol phosphate-buffered saline (PBS) and 4% paraformaldehyde. Eight-micrometre coronal cryosections were incubated and blocked with 5% bovine serum albumin (BSA) for 2 h. Frozen sections were then incubated overnight at 4°C with primary antibodies. The primary antibodies used were anti-CD31 antibody (1:2000, Abcam, ab9498) and anti-ACE2 antibody (1:1000, Proteintech, 21115-1-AP). After overnight incubation, frozen sections were incubated with fluorescent secondary antibody (1:2000, Abcam) at room temperature for 2 h. Then, the sections were washed thrice with PBS and covered with fluorescent fixation medium containing 4′,6-diamidino-2-phenylindole (DAPI) (1:1000, Solarbio). Image acquisition was performed using an Olympus fluorescence microscope with an eyepiece magnification of ×10 and an objective lens magnification of ×20. The exposure time of each section was 20 ms. Particle fluorescence intensity of ACE2 was calculated using ImageJ software (National Institutes of Health, United States) after filming, and each group included six animals.

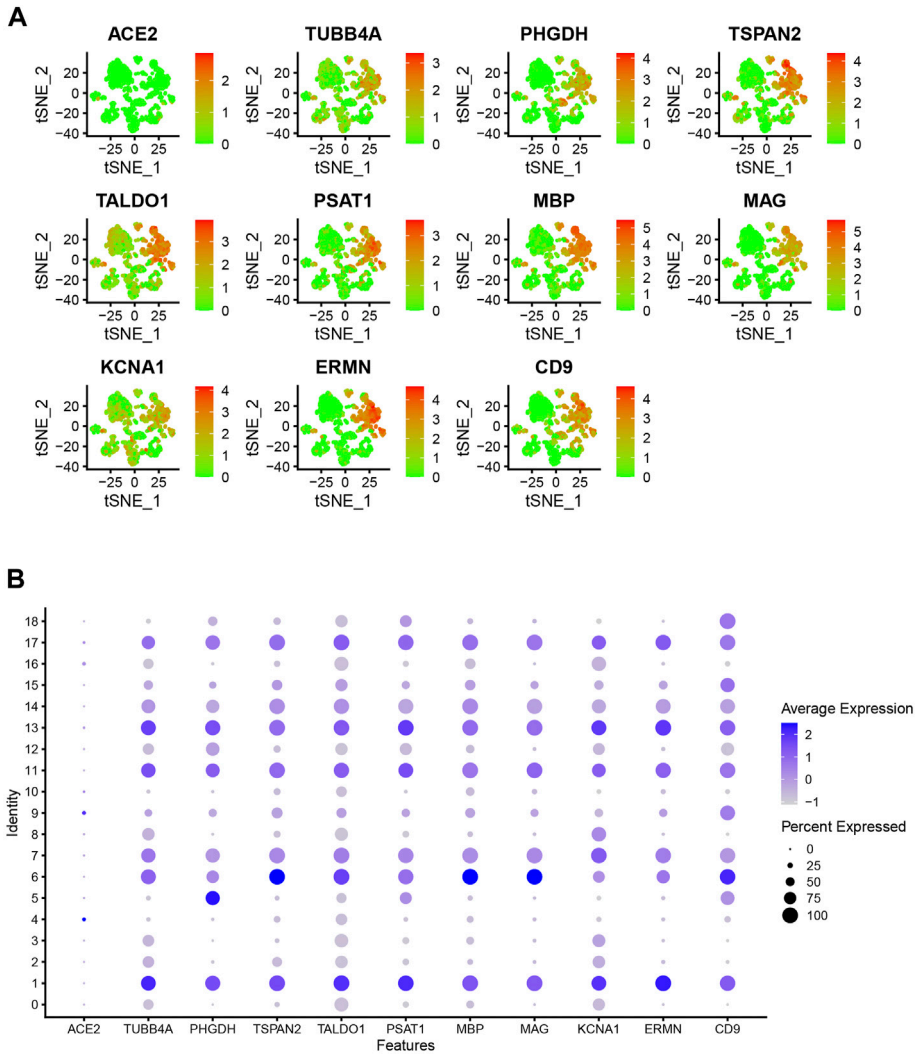

FIGURE 4
(A) Dot plot shows hub gene expressions in each cell cluster. **(B)** Bubble plot shows hub gene expressions in each cell cluster.

Western blot

Brain tissues from juvenile and aged SD rats were lysed in RIPA buffer (Solarbio, Beijing, China), following this protease and phosphatase inhibitors were added and then the sample was denatured at 100°C for 15 min. The protein samples were then separated using 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. Next, PVDF membranes were blocked with 5% skim milk powder solution for 1 h, incubated with primary antibodies, including anti-ACE2 antibody (1:1000, Proteintech, 21115-1-AP) and anti-Tubulin antibody (1:10000, Abmart, M20005) overnight, followed by secondary antibodies (1:5000) for 2 h at room temperature. The bands were visualised using an ECL kit chemiluminescence reagent (Billerica Millipore, MA, United States). Protein band signals were detected using the Chemidoc detection system (Bio-Rad, Hercules, CA, United States) and quantified by the ImageJ software (National Institutes of Health, United States).

Results

Utilization of scRNA-seq data to analyze and identify 15 cell clusters in brain tissues

A total of 3,005 cells from 67 mice were acquired in this study. t-distributed stochastic neighbour embedding (t-SNE) technique was then used to divide the cells into 18 distinct clusters (Figure 1A). Differential expression analysis facilitated the identification of

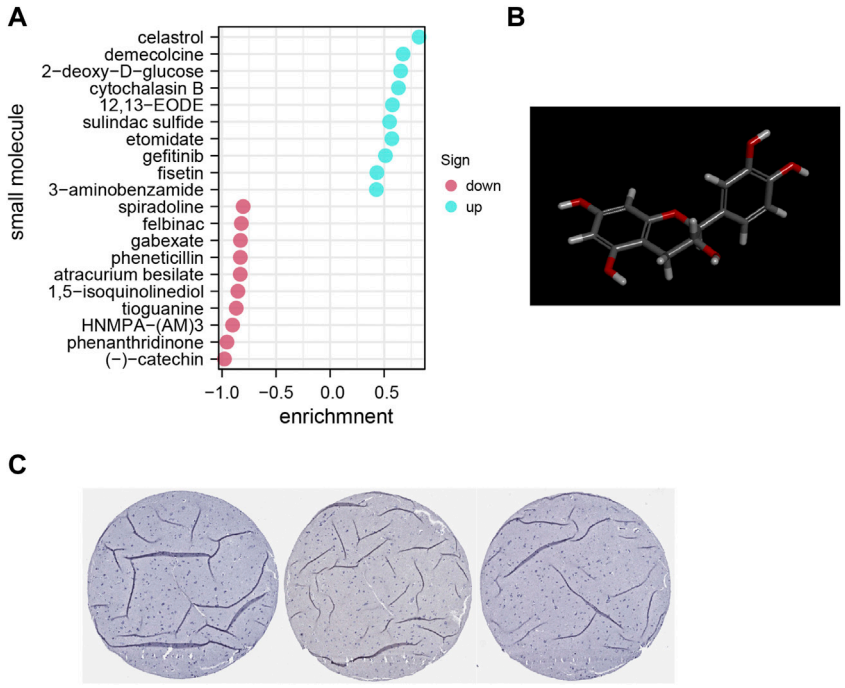

FIGURE 5
(A) Pop plot showing the top 20 small compounds capable of reversing gene expression. **(B)** Structure of top molecule. **(C)** Representative immunohistochemistry staining results reveal the protein level expression of ACE2 in brain tissues.

marker genes of the 18 different cell clusters (|logFC| >1 and adjusted *p*-value < 0.05) (Figures 1B,C). Annotations of the types of cells in the 18 cell clusters were performed utilizing singleR and the CellMarker database (Figure 1D).

Identification of two separate types of endothelial cells with unique biological functions and differentiation states

Using ACE2 as the marker, individual brain cells were successfully identified. Cluster 4 (endothelial cells) showed the highest average expression of ACE2 (Figure 2A). The endothelial cells were classified into two groups, namely cluster 4 and cluster 13 (Figure 2B). Furthermore, a considerable differentiation propensity was observed between cluster 13 with low-ACE2-expression in the former branch and cluster 4 within the latter branch on performing pseudotime trajectory analysis. This suggests that ageing endothelial cells are more susceptible to the SARS-CoV-2 virus (Figures 2C,D).

DEGs of cluster 4 and cluster 13

To explore the expression model of cluster 4 and cluster 13, a Venn qplot was drawn to show the up-regulated genes and downregulated genes in each cluster (Figure 2G). Genes with opposite expressions were considered to be DEGs in these two classifications.

Enrichment analyses

For a thorough understanding of the biological mechanisms of DEGs between clusters 4 and 13, Metascape was used to conduct GO and KEGG pathway enrichment analyses. The DEGs were found to be mostly enriched in the ensheathment of neurons, oligodendrocyte differentiation and oligodendrocyte specification, leading to myelin components for CNS, small molecule biosynthetic process, and organic hydroxy compound biosynthetic process (Figures 3A,B).

Establishment of the PPI network, module analysis and localization

The PPI network for the DEGs and MCODE showed that TUBB4A, TSPAN2, TALDO1, SCD5, PSAT1, PHGDH, MSMO1, MBP, MAG, KCNA1, ERMN and CD9 play a key role in SARS-CoV-2 infection (Figure 3C). Moreover, the hub genes are highly expressed in some astrocytes. The interaction of endothelial cells and

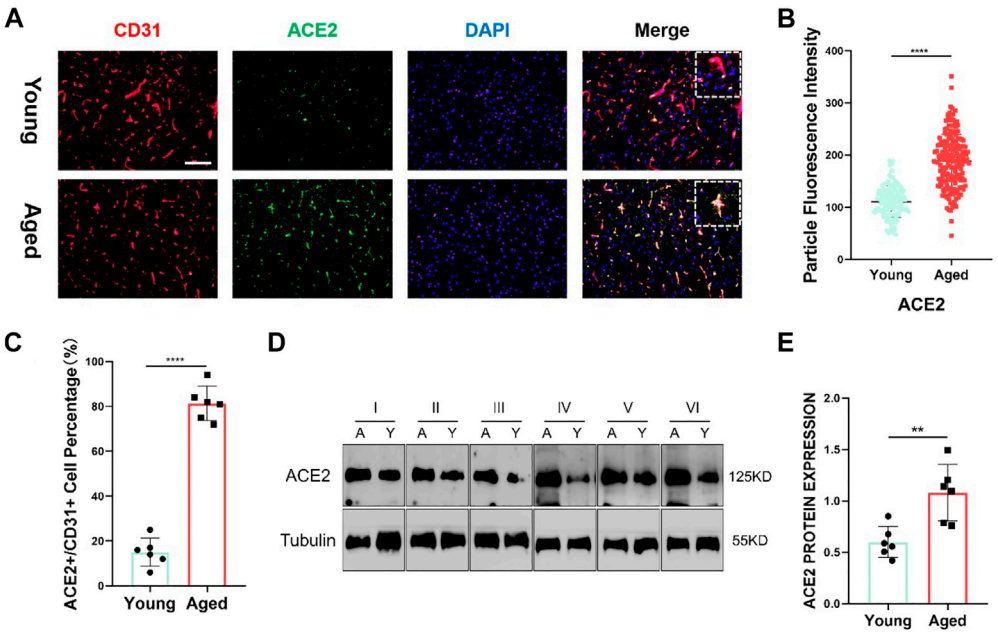

FIGURE 6
(A) Representative images of immunofluorescence staining for CD31 (red), ACE2 (green) and DAPI (blue) in the juvenile and aged Sprague–Dawley (SD) rats. Scale bar = 100μm; *n* = 6. **(B,C)** ACE2 shows a significant difference between the two groups. Data are presented as mean ± standard deviation (SD); **** *p*-value < 0.0001; *n* = 151 and 183 **(D)** Representative western blot images of ACE2 and tubulin from the two groups. *n* = 6. **(E)** Tubulin was used as a protein loading control; mean ± SD of eight independent experiments. ***p*-value < 0.01.

astrocytes can cause SARS-COV-2 to enter the CNS. The expression of these hub genes among cell subsets is shown in Figures 4A,B.

Identification of related active small molecules

The DEG data that had been classified into the upmodulated and downmodulated groups were entered into the CMap database, where it was subjected to further integration with small molecule treatments to evaluate and identify potential therapeutic medicines for ACE2-related endothelial cells. Figure 5A illustrate the top 20 relevant small molecules and their enrichment scores, respectively. A significant negative score was found to be associated with the small molecules of phenanthridinone (enrichment score = −0.954) and (−)catechin (enrichment score = −0.977), suggesting that these molecules characterize cluster 13. These prospective small molecule medications have the capacity of attenuating gene expression, thereby identifying potential novel pathways and molecular processes for innovative targeted treatments focusing on the CNS. However, further research is required to determine the specific significance of these potential small compounds.

ACE2 protein expression in the brain

Furthermore, utilizing clinical samples from the HPA repository, the ACE2 protein expression level was determined. Immunohistochemical results showed that the gene was mainly expressed in cerebral vessels (Figure 5B). Representative images of immunofluorescence staining for CD31 (red), ACE2 (green) and DAPI (blue) in the young and aged SD rats are shown in Figure 6A. Additionally, immunofluorescence showed that ACE2 was mainly expressed in endothelial cells and was significantly highly expressed in the brain endothelium of aged rats compared to that of young rats ($p < 0.0001$; Figures 6B,C). Furthermore, the western blot results showed that ACE2 expression in the brain in aged rats was significantly higher than that in young rats (Figures 6C,D).

Discussion

Respiratory viruses are capable of infecting the upper respiratory system in humans, resulting in mild illnesses in most cases (Gunathilake et al., 2021). However, in susceptible groups, such as neonates, infants, older adults and immunocompromised individuals, these pathogens may also impact the lower respiratory tract, resulting in more serious

infections such as pneumonia (Desforges et al., 2019). Furthermore, due to the virus's ability to adapt quickly and transcend the species barrier, most of these infections, including SARS-CoV and influenza A, have sometimes caused epidemics or pandemics. They have also been correlated with more significant clinical illnesses and even death (Berth et al., 2009). Additionally, various studies over decades have reported that certain respiratory viruses have neural-invasive abilities, indicating that they may migrate from the respiratory system into the CNS (Dahm et al., 2016b). Viruses that infect human CNS cells can subsequently induce various forms of encephalopathy, such as encephalitis and long-term neurologic illnesses. Although various therapeutic compounds are currently being investigated, there remains a scarcity of effective and reliable therapeutic regimens to treat SARS-CoV-2. Moreover, studies regarding SARS-CoV-2 in the CNS remain scarce.

Generally, an infection stimulates the endothelial cells to release chemokines, which improves vascular permeability and allows viruses to get through the first layer of the BBB (Mladinich et al., 2021). Furthermore, viruses commonly employ proteins produced by the endothelium and enter these cells. While SARS-Cov-2 infections are commonly limited to the airways, it has been reported to cross the epithelial barrier and infiltrate the CNS. This is consistent with the mechanism of other respiratory viral pathogens, such as influenza virus, Nipah virus and respiratory syncytial virus (RSV). In this study, brain endothelial cells showed significant expression levels of the enzyme ACE2. The SARS-CoV-2 virus enters the host cell via the SARS-CoV receptor ACE2. Hence, it was speculated that SARS-CoV-2 employs the ACE2 receptor for intracellular penetration into the CNS by infecting endothelial cells. The time analysis of cells showed that endothelial cells in the advanced stage had higher expressions of ACE2. This suggests that elderly patients are more likely to be infected by SARS-CoV-2 via the endothelial cells of the CNS.

Several patients with SARS-CoV-2 (i.e., who had a positive RT-PCR test) also experienced the loss of smell, despite not experiencing nasal obstruction dysgeusia, albeit exhibiting swelling in the olfactory cleft, which was validated using magnetic resonance imaging. The olfactory cleft is responsible for the flow of odours to the olfactory epithelium and then to the olfactory bulb. The olfactory epithelium (commonly referred to as the olfactory mucosa) consists of olfactory receptor neurons, basal cells and epithelial cells, all of which function together to create 'smell'. When TNF-alpha (TNF-α) and interleukin-1 beta (IL1β) are released, the above cells react to create a "smell". Notably, SARS-CoV-2 infection has been demonstrated to contribute to a higher production of TNF-α and IL1β. Consequently, the pathogenesis of SARS-CoV-2 could impact the lower respiratory tract while simultaneously impacting surrounding cells (such as those found in the respiratory tract), resulting in affecting the CNS.

Increasing evidence has identified the SARS-Cov-2 virus as the source of EGCG's antiviral effects (Upadhyay et al., 2020) Furthermore, it has been shown that EGCG attenuated the enzymatic activities of the coronavirus 3CL protease, preventing the virus from replicating. Moreover, EGCG has the ability to control particular targets such as the RdRp and viral S protein. It has also been shown to be effective in preventing the reproduction of SARS-CoV-2 in cell incubation experiments. Molecular docking studies also show that EGCG inhibits SARS-CoV-2 entry into the target cell by interfering with the RBD in the viral membrane that binds to ACE2. This study suggests that EGCG could prevent SARS-CoV-2 from entering the CNS through endothelial cells by inhibiting its expression. In order to verify the utility of EGCG in anti-SARS-CoV-2 treatments, more pre-clinical investigations, clinical trials and epidemiological analyses are necessary.

The findings in the present research are restricted as only mouse tissue samples were used. Nonetheless, this study provides proof that SARS-CoV-2 could infiltrate the CNS through a large number of susceptible cells. Moreover, endothelial cells in elderly patients have a greater susceptibility to infection by SARS-CoV-2. Furthermore, the influence of SARS-COV-2 on the CNS requires more attention.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ Supplementary Material.

Ethics statement

The animal study was reviewed and approved by The animal study was reviewed and approved by the Nantong University Animal Ethics Committee. (S20180220-002).

Author contributions

The lead authors, including ZW and ZL, took charge of the analysis and drafting of the manuscript. ZS performed the preliminary statistical analysis, drafted the first and final drafts, and responded to the reviewer comments. HM and CC were in charge of data collecting and organizing, and they all had a role in writing the final revisions of the text. YX and PG were responsible for organizing the funding, designing the initial concept, and contributing to the writing of final drafts.

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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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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.971017/full#supplementary-material

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Effects of different anesthetic depth during propofol anesthesia on postoperative recovery 24 h after arthroscopic day surgery: A randomized clinical trial

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Background: This study aimed to compare the effects of different depths of sedation during propofol anesthesia on postoperative recovery 24 h after knee arthroscopy day surgery in adult patients.

Methods: This prospective randomized controlled trial involved 126 patients (ASA physical status 1–2) who were scheduled to undergo arthroscopic day surgery. Patients were randomly divided into two groups: the light-sedation (L-Group) or deep-sedation (D-Group). In the L-group, the bispectral index values were kept in the range of 50–59; in the D-group, the bispectral index values were maintained in the range of 40–49. The Quality of Recovery-15 (QoR-15) score assessed 24 h postoperatively using a 15-item questionnaire was the primary outcome. Secondary outcomes included Athens Insomnia Scale scores, postoperative pain scores, nausea or vomiting.

Results: The total QoR-15 score 24 h postoperatively was similar in the two groups (L-group median:130, IQR [127–132] vs. D-group median:131, IQR [126–135], $p = 0.089$). But among the five dimensions of the QoR-15, physiological comfort was significantly better in the D-group than L-group ($p < 0.001$). The time to open eyes ($p < 0.001$), follow the command ($p < 0.001$) and to extubation ($p < 0.001$) after surgery in the L-group were shorter than the D-group. The Athens Insomnia Scale scores ($p < 0.001$) and incidence of dreaming ($p = 0.041$) at the first postoperative night in the L-group was significantly higher than those in the D-group. Propofol consumption in the L-group was less than D-group ($p < 0.001$).

Conclusion: For patients undergoing arthroscopic day surgery, general anesthesia with high-bispectral-index (50–59) cannot improve the total QoR-15 score 24 h postoperatively after surgery, but can lessen propofol consumption, reduce the time of extubation and anesthesia recovery period, compared with low-bispectral-index (40–49). Patients exposed to general anesthesia with low-bispectral-index values (40–49) may have better quality sleep and physical comfort than those with high-bispectral-index values (50–59).

Clinical Trial Registration: http://www.chictr.org.cn/showproj.aspx?proj=126526, identifier ChiCTR2100046340

KEYWORDS

bispectral index, anesthesia, arthroscopic, ambulatory, quality of recovery

Introduction

Early recovery after surgery under general anesthesia predicts early discharge. Recovery from general anesthesia is a critical perioperative period, and plays an important role in the promotion of the effect of clinical surgical treatment from the perspective of both physiological stability and patient satisfaction (Kehlet and Dahl, 2003). Intraoperative depth monitoring of anesthesia is crucial to ensure a rapid revive and functional recovery of patients postoperatively. The bispectral index has been recognized as one of the most commonly used indicators to judge the level of sedation and depth of anesthesia, and enable the doctors to properly adjust the anesthetic dose and avoid intraoperative awareness (Gan et al., 1997; Myles et al., 2004). Studies have revealed that deep anesthesia can increase the long-term postoperative mortality of patients who undergo major surgery (Liu et al., 2019; Short et al., 2019). However, there are few studies on the effects of the depth of anesthesia on short-term postoperative functional recovery during day surgery ambulatory. Bispectral index values between 40 and 60 are optimal for depth of sedation, which can avoid intraoperative awareness and delay of wake up (Avidan et al., 2008; Chiang et al., 2018). However, the range of best depth sedation is relatively wide.

Therefore, we conducted a randomized controlled trial to compare the effects of different depths of anesthesia on postoperative recovery of patients who underwent daytime knee arthroscopy. We hypothesized that the quality of recovery scores 24 h postoperatively of light-sedation (bispectral index: 50–59) was superior to deep-sedation (bispectral index: 40–49) after knee arthroscopy day surgery. Assessing the improvement of interventions on patient experience after anesthesia and surgery requires an emphasis on patient-centered outcome measures. The quality of recovery-15 scale was selected in this study to assess recovery in five dimensions 24 h after surgery (emotional state, physical comfort, psychological support, physical independence, and pain) (Bowyer and Royse, 2016).

Materials and methods

Study design and study population

The trial was approval from the Ethics Committee of the First Affiliated Hospital of Anhui Medical University (Ethical Application Reference: PJ 2021-06-09 Anhui, China) and was registered at the Chinese Clinical Trial Registry (ChiCTR2100046340) on 14 May 2021, http://www.chictr.org.cn/showproj.aspx?proj=126526. In this trial, patients aged 18–65 years with ASA I–II, who were scheduled to undergo arthroscopic day surgery under general anesthesia (GA) from June 2021 to September 2021, were enrolled. The exclusion criteria were severe cardiopulmonary system diseases, endocrine system diseases: pituitary tumors, severe diabetes, pheochromocytoma, and other mental diseases, including schizophrenia, depression, alcoholism, opioid dependence; Parkinson's disease, Alzheimer's disease, severe insomnia, and inability to understand visual analog scale and quality of recovery-15, cases in which patients were unable to take care of themselves in their preoperative lives, hemorrhagic disease history, or abnormal coagulation function.

Randomization

Before surgery, the researchers recruited the patients and obtained written informed consent. All the included patients were randomly divided in two groups at a 1:1 proportion using computer-generated randomization: L-group (bispectral index: 50–59) and D-group (bispectral index: 40–49). The numbers for allocation were packaged in opaque envelopes, which could only be observed by the anesthesia providers. Randomization was done on the morning of surgery using a computer-generated randomization table (simple randomization without restrictions). During a preanesthetic visit to the inpatient ward before surgery, the patients were asked to familiarize with the quality of recovery-15 questionnaire. The patients, outcome evaluators, and data information analysts were blinded to the trial intervention.

Anesthetic procedure and intervention

Standardized monitoring processes were conducted during anesthesia and operation. Before anesthesia induction, the patients were assessed by the quality of recovery-15 questionnaire (Stark et al., 2013; Bu et al., 2016). GA was then induced by intravenous injection of sufentanil (0.4 $\mu g\,kg^{-1}$), propofol (2.0 $mg\,kg^{-1}$), and cisatracurium (0.2 $mg\,kg^{-1}$). After attaining a sufficient depth of anesthesia, an I-gel laryngeal mask was utilized according to the patient's body weight (size 3 for

weights <50 kg, size 4 for 50–70 kg, or size 5 for weights >70 kg). An anesthesiologist with more than 5 years of experience was arranged to intubate the patients. All operations were performed by one surgical team.

Anesthesia was maintained using remifentanil (0.02–0.5 μg kg^{-1} min^{-1}), propofol (4–8 mg kg^{-1} h^{-1}), and cisatracurium (0.02–0.05 mg kg^{-1} h^{-1}). The bispectral index was monitored in two groups. In the L-group, the bispectral index values were kept in the range of 50–59; in the D-group, the bispectral index values were maintained in the range of 40–49. The criteria to trigger intervention to adjust the dosage of propofol to bring back the BIS into the target range was set as the BIS index being out of the targeted range for 30 s. And, the maintenance time of BIS index of targeted range was recorded. The end-tidal CO_2 ($EtCO_2$) was kept between 35 and 45 mmHg. Patients were given 6–8 ml/kg of Ringer's lactate solution as early as the induction period, followed by continuous infusion of Ringer's lactate solution at a rate of 5–7 ml kg^{-1} h^{-1} until the end of surgery. Intraoperative heart rate (HR) was maintained at 50–90 beats per min; if HR < 50 beats/min, atropine (0.3–0.5 mg) was administered; if HR > 90 beats/min, esmolol was administered (0.3–0.6 mg kg^{-1}. If the systolic blood pressure increased or dropped by 20% more than the baseline, nicardipine (5–10 μg kg^{-1}) and ephedrine (3 mg) was given. The infusion of anesthetic drugs did not stop until the end of surgery. Approximately 15 min before the end of subcuticular closure, the anesthesiologist intravenously injected 5 μg of sufentanil for the postoperative analgesia. Ondansetron, 0.1 mg/kg, was used for antiemetic prophylaxis. At the end of the surgery, the surgeon injected 10 ml of 0.5% ropivacaine into the joint cavity for postoperative analgesia. After the operation, all patients were transported to a postanesthesia care unit. An I-gel laryngeal mask was removed by the anesthesiologists and nurse who were blinded, when the $EtCO_2$ was below 45 mmHg on spontaneous respiration, and when the patient was able to follow voice commands. Flurbiprofen (50 mg) was given intravenously when the VAS score was above 3 during the postoperative period.

Outcome measures

In this study, the primary outcome was the global quality of recovery-15 score assessed 24 h postoperatively in five dimensions: emotional state (4 items), physical comfort (5 items), psychological support (2 items), physical independence (2 items), and pain (2 items) (Bowyer and Royse, 2016). The total score on the QoR-15 ranges from 0 (the poorest quality of recovery) to 150 (the best quality of recovery). By contrast, the secondary outcome was the time to open eyes, follow voice command and extubation, hospital stays, hospitalization costs (cost from discharge to admission), and postoperative pain scores. We defined the time to open the eye as the time from the end of surgery to the opening of the eyes. Time to follow the voice commands was defined as the time from the end of surgery to the time patients responded as instructed. Additionally, the time of extubation was defined as the time from the end of surgery to removal of I-gel laryngeal masks. After surgery, the patients were asked by investigators to rate the pain of incision at 1, 6, and 24 h postoperatively using the visual analog scale (VAS) (0 = none, 10 = most severe), the Ramsay Sedation Scale (RSS) scores, the condition of sleep on the first night and postoperative nausea and vomiting (PONV) were also recorded. The incidences of awareness and dreaming was followed up on the first postoperative day. The aforementioned parameters were evaluated by the same doctor who was blinded to the different patient groups. In addition, mean arterial pressure (MAP) and HR were noted down at different time points: baseline, 5 min after intubation, 5 min after tourniquet start and release, end of surgery and extubation.

Sample size estimation and statistical analysis

The primary outcome measure was the global quality of recover-15 score. We selected this score as the scale of sample size evaluation. According to our preliminary study conducted under GA with bispectral index values 40–49, the quality of recovery-15 scores postoperatively (at 24 h) were equivalent to 128 (12.5). In the published data, a change of 8 for the quality of recovery-15 scores was identified as clinically significant (Myles et al., 2016). We hypothesized that this trial would have 90% power to detect an increment of 8 in the quality of recovery-15 scores at a significance threshold of 0.05. Furthermore, the Power Analysis and Sample Size software (version 15.0, NCSS, LLC, United States) calculated that 53 patients per group were required. Considering a 20% withdrawal rate, we included 63 patients in each group.

Data were collected and recorded and analyzed using the Statistical Package for Social Sciences software (version 22.0, IBM Corporation, United States). The normality of quantitative variables was assessed with the Shapiro–Wilk test. Categorical variables were expressed as a number (n) and percentage (%). The quantitative variables were expressed as mean (SD), median [IQR], median (range). The mean values of age, weight, height, BMI, duration of surgery and anesthesia times were analyzed using the independent-samples *t*-test. The QoR-15 score, perioperative cumulative anesthetic dosage, time to open eyes, follow voice command and extubation, AIS scores, hospital stays, hospitalization costs were analyzed by the Mann–Whitney U-tests. The effects of intervention over time for the outcomes of interest (postoperative pain scores and hemodynamic values) were assessed using the repeated-measures analysis of variance (ANOVA) model group by time interaction. For measures that indicated significant group by time interaction effects, post hoc-

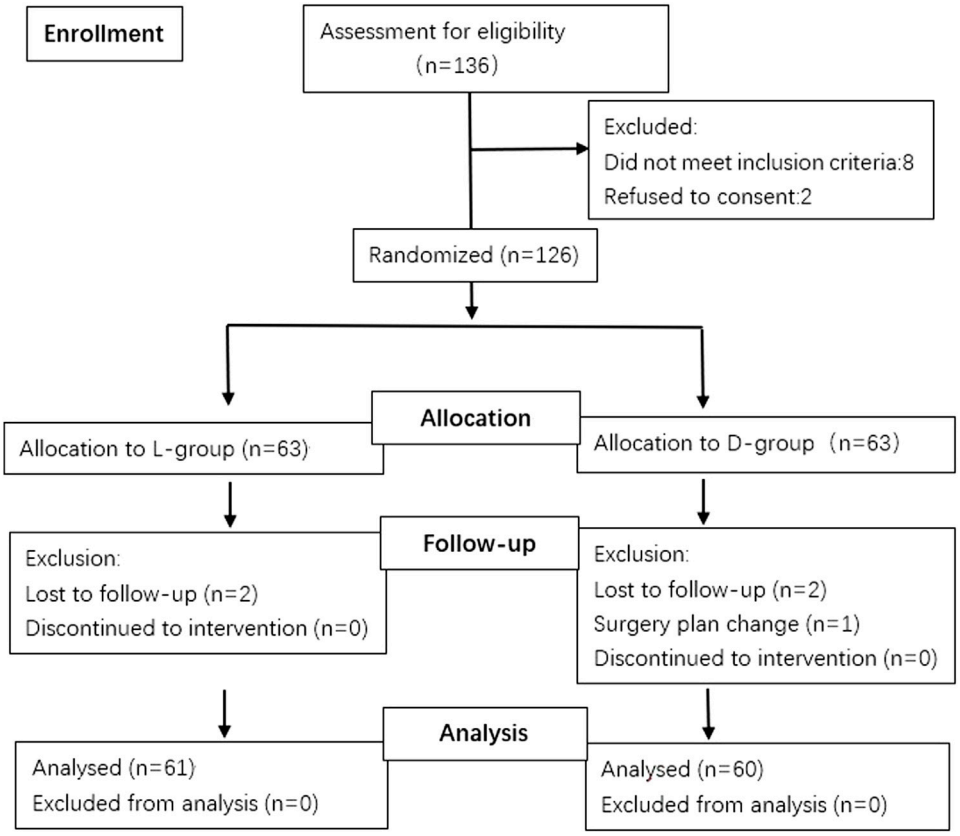

FIGURE 1
Consort flow chart that outlines patients' assignment and treatment protocols. Patients were allocated into two groups (L-group, D-group) to receive different depths of sedation with bispectral index value maintaining in the range of 50–59 or 40–49 respectively, following a computer-generated randomization code.

analysis on differences between the two groups were assessed by the independent sample *t*-test with Bonferroni correction. The Chi-squared test or Fisher's exact test was used to compare the number of patients based on the dream, PONV, and the ASA classification rates. Two-sided *p*-values of less than 0.05 were utilized to denote statistical significance.

Results

A total of 136 patients were screened for this study from 1 June 2021, to 1 September 2021. In addition, two patients refused to consent and eight did not meet the inclusion criteria, leaving 126 for primary randomized: 63 patients in the L and 63 in the D groups. Among the randomized patients, 4 were lost to follow-up because of study withdrawal after surgery, and 1 had changed surgery plan. Thus, 121 patients were remained for the final analysis: 61 patients in the L-group, 60 in the D-group (Figure 1). The patients' demographic profiles were comparable between the two groups (Table 1). No differences in age, gender, body mass index (BMI), ASA classification and basic bispectral index value were observed between two groups. The perioperative profiles of the patients, such as operative, anesthetic time, time of maintenance with target bispectral index values range, vasoactive drug consumption (ephedrine, atropine), the preoperative quality of recover-15 scores and RSS scores, had no significant differences between both groups (Tables 1, 2; Figure 2). However, significant differences in time to eye opening ($p < 0.001$), follow the voice command ($p < 0.001$) and extubation time ($p < 0.001$) were observed between the L and D groups. There were no patients who reported intraoperative awareness.

No differences in the total QoR-15 scores 24 h postoperatively were observed in the L-group ($p = 0.089$, Table 3). But among the five dimensions of the QoR-15, physiological comfort was significantly better in the D-group than L-group ($p < 0.001$, 48 [46–40] vs. 46 [45–47.5]). The time to eye opening, follow voice command, and extubation in the group were shorter than the D-group (6 [5 to 8] vs. 9 [8 to 11] min, $p < 0.001$; 7 [6 to 9] vs. 11 [9 to 13] min, $p < 0.001$; 9 [8 to 10]

TABLE.1 Baseline characteristics of included patients in the study.

	Group L (n = 61)	Group D (n = 60)	*p*-value
Age (yr)			
Mean ± SD	45 ± 11	42 ± 13	0.188
Range	18–63	18–59	
Sex, n (%)			0.524
Female	32 (52.5%)	28 (46.7%)	
Male	29 (47.5%)	32 (53.3%)	
BMI (kg/m^2)	24.1 ± 2.6	23.9 ± 2.6	0.751
ASA classification, n (%)			0.792
I	14 (23.0%)	15 (25.0%)	
II	47 (77.0%)	45 (75.0%)	
Basic BIS value	96 ± 1.5	96 ± 1.9	0.702
Operative time (min)	43.7 ± 12.8	46.2 ± 15.3	0.338
Anesthetic time (min)	68.9 ± 12.4	72.8 ± 16.0	0.186
Remifentanil consumption (ug)	500 [385–675]	573 [429–676]	0.147
Sufentanil consumption (ug)	30 [30–35)	32 [30–35]	0.815
Propofol consumption (mg)	346 [250–429]	412 [359–600]	<**0.001**$^{\#}$

AbbreviationsBMI, body mass index; ASA, american society of anesthesiologists; BIS, Bispectral index. The values are expressed as means ± SD, median [interquartile range] or number of patients (percentage). #$p < 0.05$.

TABLE.2 Perioperative profiles of the patients.

	Group L (n = 61)	Group D (n = 60)	*p*-value
Maintenance time of target BIS range (min)	62 ± 17	66 ± 17	0.163
Time to open eyes (min)	6 [5–8]	9 [8–11]	<**0.001**$^{\#}$
Time to follow the command after surgery (min)	7 [6–9]	11 [9–13]	<**0.001**$^{\#}$
Time to extubation (min)	9 [8–10]	12 [10–14]	<**0.001**$^{\#}$
Atropine (mg)	0 (0–0.5)	0 (0–0.5)	0.388
Ephedrine consumption (mg)	6 [0–12]	8 [0–12]	0.563
RSS	2 [1–3]	2 [2–4]	0.085
Intraoperative awareness	0	0	NA
Hospital Stay (h)	23 (17–48)	23 (21–48)	0.609
Hospitalization costs (¥)	12,263 [12,016–12502]	12,355 [11,999–12850]	0.332
AIS scores at the first postoperative night	4 [3–6]	2 [1–3]	<**0.001**$^{\#}$
Patients having dream, n (%)			**0.041**$^{\#}$
Yes	16 (26%)	7 (12%)	
No	45 (74%)	53 (88%)	
Postoperative VAS score			0.127
1 h	0 (0–4)	1 (0–4)	
6 h	1 (0–4)	1 (0–6)	
24 h	1 (0–4)	1 (0–4)	
PONV, n (%)			0.131
Yes	9 (15%)	4 (7%)	
No	52 (85%)	56 (93%)	

Abbreviations BIS, bispectral index; RSS, ramsay sedation scale; VAS, visual analogue scale; AIS, athens insomnia scale; PONV, postoperative nausea and/or vomiting. The values are expressed as means ± SD, median (interquartile range[range]), median (range) or number of patients (percentage). #$p < 0.05$.

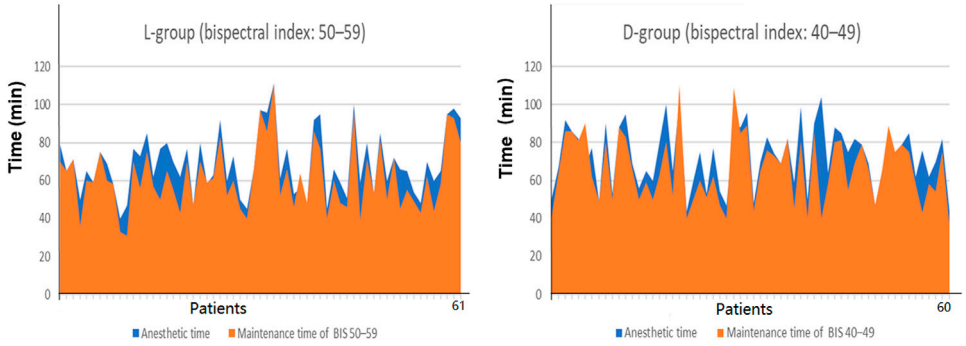

FIGURE 2
Percent of the time maintained in the target BIS values range and anesthetic time. Intraoperative maintenance time of low-bispectral-index values (40–49) and high-bispectral-index value (50–59) was insignificant ($p = 0.163$). Notes: *X*-axis in stands for patients in each group. The area of the orange range represents the time of target bispectral-index values range. The area of the blue range represents the anesthetic time.

TABLE 3 The QoR-15 scores (121 patients) before surgery and 24 h after surgery between two groups.

	Group L (n = 61)	Group D (n = 60)	*p*-value
Preoperative score			
Physical comfort	49 [46–49]	48.5 [47–49]	0.870
Physical independence	20 [20–20]	20 [20–20]	0.193
Pain	15 [14.5–16]	16 [15–17]	0.313
Psychological support	20 [20–20]	20 [20–20]	0.516
Emotional state	39 [37.5–40]	39 [38–40]	0.858
Total QoR-15 score	142 [139–144]	142.5 [140–145]	0.279
Postoperative score			
Physical comfort	46 [45–47.5]	48 [46–49]	**<0.001**#
Physical independence	7 [7–7]	7 [7–7]	0.344
Pain	18 [17–18.5]	18 [17–19]	0.958
Psychological support	20 [20–20]	20 [20–20]	0.135
Emotional state	39 [38–39.5]	39 [38–40]	0.155
Total QoR-15 score	130 [127–132]	131 [126–135]	0.089

AbbreviationsQoR-15, quality of recovery-15; The values are expressed as means ± SD, or median [interquartile range]. #$p < 0.05$.

vs. 12 [10 to 14] min, $p < 0.001$, respectively, Table 2). The Athens Insomnia Scale scores ($p < 0.001$) and incidence of dreaming ($p = 0.041$) at the first postoperative night in the L-group was significantly higher than the D-group (4 [3 to 6] vs. 2 [1 to 3], $p < 0.001$; 26 vs 12%, $p = 0.041$, Table 2). Propofol consumption in the L-group was less than the D-group ($p < 0.001$, Table 1).

Hemodynamic profiles, such as HR and MAP, were compared between the two groups. No significant differences were observed in MAP at baseline, 5 min after intubation, 5 min after tourniquet onset and release, end of surgery and extubation between both groups (Figure 3). Furthermore, perioperative opioid consumption (sufentanil, remifentanil), postoperative visual analog scale for incision site pain between the two groups were not significantly different and the difference in the incidence of PONV between the both groups was also insignificant (Tables 1, 2).

Discussion

The main findings of this study indicated that compared with the D-group (bispectral index: 40–49), GA for patients undergoing knee arthroscopy day surgery (with bispectral index values in the range of 50–59) did not improve the total QoR-15 score 24 h postoperatively after surgery but was able to lessen

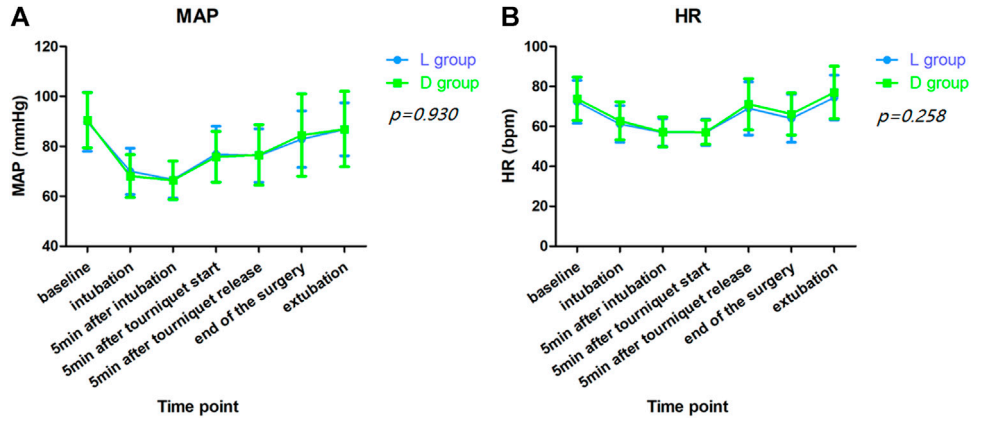

FIGURE 3
Hemodynamic values. **(A)**. Mean artery pressure (MAP); **(B)**. Heart rate (HR). No significant differences were observed in MAP (p = 0.930) and HR (p = 0.258) at baseline, 5 min after intubation, 5 min after tourniquet onset and release, end of surgery and extubation between both groups by repeated measures analysis of variance.

propofol consumption, the time of recovery from the anesthesia, and extubation. Furthermore, patients who were exposed to GA (with bispectral index values in the range of 40–49) have better quality sleep and physical comfort than light sedation (bispectral index: 50–59) at the first night after surgery.

Knee arthroscopy is a common clinical day surgery (Romina et al., 2017). Promoting day surgery can reduce the length of stay and enhance recovery after surgery, which could reduce the risk of venous thromboembolism and hospital-acquired infections (Bailey et al., 2019). The quality of recovery from GA can impact patient safety, patient satisfaction and medical costs (Fritz et al., 2013). Several studies had reported that bispectral index monitoring for general anesthesia may result in lower anesthetic doses, a lower incidence of anesthesia awareness, and faster patient recovery (Liu, 2004; Fritz et al., 2013; Lewis et al., 2019). In this study, propofol consumption in the L-group was less compared with the D-group, and no patients were reported intraoperative awareness. However, the total QoR-15 scores 24 h after knee arthroscopy day surgery in the L-group (bispectral index: 50–59) was not higher than the D-group (bispectral index: 49–49) (p > 0.05). This means that light sedation (bispectral index: 50–59) cannot improve the quality of recovery from GA 24 h postoperatively compared with deep sedation (bispectral index: 40–49). Therefore, it may not require maintaining bispectral index values in the range of 40–49 and consume more anesthetics for knee arthroscopy. McCormick et al. suggested that prolonged cumulative double-low conditions (low MAP (<75 mmHg) and low-bispectral-index values (<45)) were associated with mortality (McCormick et al., 2016). Furthermore, Yoon et al. reported that the cumulative duration of double-low conditions [low MAP (<45 mmHg) and low-bispectral-index values (<40)] were associated with 90-days postoperative mortality, and not with a 180-days postoperative mortality (Yoon et al., 2020). The appropriate dose for a given patient may contribute to the faster recovery and lower medical costs by reducing the time during the operating room and PACU (Dexter et al., 1999; Myles et al., 2004; Clark et al., 2009; Bosslet et al., 2010). In this study, light sedation (bispectral index: 50–59) reduced the time to eye opening, follow to voice command, and extubation and enhancement of the recovery from anesthesia compared with the D-group (bispectral index: 40–49), which was consistent with previous studies (Leslie et al., 2005; Mashour et al., 2012). However, no significant difference in medical costs and hospital stays was observed between the L-group (bispectral index: 50–59) and D-group (bispectral index:40–49), which means that light sedation (bispectral index: 50–59) cannot save hospitalization spending and reduce the length of stay. Additionally, there were no significance differences in hemodynamic profiles, vasoactive drug consumption (ephedrine, atropine), opioid consumption (sufentanil, remifentanil), and postoperative visual analog scale (VAS). Compared with the D-group (bispectral index: 40–49), maintaining bispectral index values at 50 to 59 may not increase opioid and vasoactive drug consumption, and may not affect the postoperative VAS and the occurrence of PONV.

High-sleep quality after surgery is one of the important guarantees for postoperative rehabilitation of patients (Rosenberg-Adamsen et al., 1996; Chen et al., 2017). Studies showed that sleep disturbance are more likely to occur after surgery owing to postoperative pain, environmental changes, trauma and other factors, and may contribute to neurological, cardiovascular complications, and may lead to increased morbidity (Redwine et al., 2000; Leung and Bradley, 2001; Alhola and Polo-Kantola, 2007; Krenk et al., 2012). Therefore, improving the sleep quality after surgery probably has a positive effect on the recovery of surgical patients. In this study, the Athens Insomnia Scale scores associated with the sleeping period

of the first night after knee arthroscopic surgery in the L-group (Bis 50–59) was higher than the D-group (bispectral index: 40–49). Thus, low-bispectral index values (40–49) can improve insomnia conditioned and the quality of sleep during the first night after surgery. This phenomenon may be one reason for the better physical comfort score in the D-group (bispectral index: 40–49). Different propofol consumptions may contribute to the above phenomenon. Dinesh Pal's findings showed that propofol could modulate sleep homeostasis by compensating for sleep debt in sleep-deprived rats, thus satisfying the need for both rapid and nonrapid eye movement sleep patterns (Pal et al., 2011). Evidence suggested that sufentanil may impair sleep and sleep architecture and insomnia may increase anesthetic consumption, but there was no difference in opioid consumption between the two groups (Erden et al., 2016; Tripathi et al., 2020; Yang et al., 2021). Increased propofol consumption in the D-group (bispectral index: 40–49) may be the possible reason for the improvement of the quality of sleep during the first night after surgery. Another positive result is that the numbers of patients reported of dreaming at first night sleep postoperatively in the D-group was less than the L-group. Combination of Athens Insomnia Scale scores, the occurrence of dreaming, and low-bispectral-index values (40–49) improved the first sleep quality by reduction in AIS scores and incidence of dreaming.

Before the study, we hypothesized that high-bispectral-index values (50–59) improves the quality of recovery scores 24 h postoperatively after knee arthroscopy day surgery, when compared to low-bispectral-index values (40–49). However, the results were contrary to our expectations. During the operation, intraoperative maintenance time of low-bispectral-index values (40–49) and high-bispectral-index value (50–59) was insignificant ([66 ± 17 vs. 62 ± 17] min, $p = 0.163$, table 2 and Figure 2). Propofol consumption in the L-group (bispectral index 50–59) was less and high-bispectral-index values (50–59) can shorten anesthesia recovery period. In addition, for patients with insomnia, low-bispectral-index values (40–49) may be more suitable. This may contribute to patients' physical comfort score. Several studies suggested that patients with sleep disorders may benefit from operations performed in the morning and GA under a median bispectral index level of 39 may contribute to better recovery of cognitive function 4–6 weeks postoperatively compared with a median bispectral index level of 51, particularly with respect to the ability to process information (Farag et al., 2006; Song et al., 2020). The understanding of the influences of different depths of anesthesia on postoperative cognitive function requires additional research. The aforementioned facts are the reasons for the results of this trial.

This trial is associated with several limitations. First, this is only a single-center study. Thus, a multicenter study would be better for testing our hypothesis. Second, as no "gold standard" exists for the assessment of the quality of recovery after surgery and anesthesia, the quality of recovery-15, was commonly used recently for validations. More measures should be developed to assess the quality of recovery. Third, the duration of surgery and hospitalization of patients were short, and the time to observe was limited; it is difficult to compare the long-term effects on the patients. Finally, the effects of different bispectral index values on the older patients or the children are unknown.

In conclusion, this study demonstrated that in patients who undergo arthroscopic day surgery, GA with high-bispectral index values (50–59) cannot improve the total QoR-15 score 24 h postoperatively but can lessen propofol consumption, accelerate the time of anesthetic recovery compared with low-bispectral-index values (40–49). Patients exposed to GA with low-bispectral-index values (40–49) have better quality sleep and physical comfort than those with high-bispectral-index values (50–59).

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 human participants were reviewed and approved by the First Affiliated Hospital of Anhui Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

Study design: MN, YS, HZ, CC, ZX, LC, and YL Ethics approval and registration: MN, YS, and LC Patient recruitment: MN, YS, HZ, and LS Data collection: MN, YS, HZ, and LS Data analysis: MN, YS, LC, and YL Drafting: MN, CC, ZX, and YL Final approval of the manuscript: all authors.

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Conflict of interest

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

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COMMD4 is a novel prognostic biomarker and relates to potential drug resistance mechanism in glioma

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Background: Glioma as the most frequently discovered tumor affecting the brain shows significant morbidity and fatality rates with unfavorable prognosis. There is an urgent need to find novel therapeutic targets to overcome the low chemotherapeutic efficacy of glioma. This research examined whether the copper-metabolism-domain protein, COMMD4, had predictive and therapeutic significance in glioma.

Methods: Using the freely accessible CGGA (The Chinese Glioma Atlas) and TCGA (The Cancer Genome Atlas) databases, we examined the function of COMMD4 in GBM and LGG. CIBERSORT and TIMER were utilized to assess the associations between COMMD4 and immune cells. The Gene Set Enrichment Analysis (GSEA) was employed to examine the functional data. Furthermore, the link between COMMD4 expression and predicted treatment response was evaluated via CellMiner Cross-Database. Meanwhile, qRT-PCR was conducted to examine COMMD4 expression in human glioma. Finally, Migration and invasion of glioma cells (U-87, U-251) were assessed using transwell assays. R was used to analyze the statistical data.

Results: According to our findings, COMMD4 expression level was higher in patients having grade-dependent glioma who also showed an unfavorable prognosis. Furthermore, qRT-PCR confirmed the high expression of COMMD4 in glioma tissues and cells. Additionally, using integrated correlation analysis, we acquired significant prognostic findings between isocitrate dehydrogenase 1(IDH1) and COMMD4. Meanwhile, a link between COMMD4 and many tumor-infiltrating immune cells was observed. GSEA and drug response analysis revealed the potential mechanism of COMMD4 in drug resistance of glioma.

Conclusion: The current findings validated COMMD4 as a novel biological marker, which might offer insights into the possible drug resistance mechanisms and the impact of the immune microenvironment on glioma. COMMD4 might be used to predict glioma prognosis.

KEYWORDS
CGGA, TCGA, COMMD4, glioma, drug resistance, mast cells

1 Introduction

Glioma is the most prevalent malignancy affecting the central nervous system (CNS), with roughly 4.7 cases per 100,000 persons being diagnosed each year (Larjavaara et al., 2007; Ostrom et al., 2013). Currently, the standard treatment plan for glioma is the combination of chemotherapy, radiotherapy, and surgical intervention. However, the prognosis remains unfavorable owing to a low sensitivity of glioma to radiotherapy and chemotherapy (Tonn et al., 2012; Jiang et al., 2016; Peng et al., 2018). Thus, a breakthrough in the treatment of glioma is critical. Nonetheless, the molecular mechanism in glioma is incompletely understood, hindering the development of novel treatment methods for glioma diagnosis and management (Lin et al., 2017).

In 2016, the World Health Organization (WHO) revised its categorization of CNS malignancies. According to the WHO classifications, adult diffuse gliomas are commonly identified and categorized by the nuclear retention, identification of the 1p/19q chromosomal co-deletion, and mutations in isocitrate dehydrogenase 1 (IDH1) or isocitrate dehydrogenase 2 (IDH2) genes. (Louis et al., 2014; Louis et al., 2016) In the 2021 revised version, novel molecular indicators, including telomerase reverse transcriptase (TERT) promoter alterations and epidermal growth factor receptor (EGFR) gene amplification, are required to classify adult patients with gliomas. Additional molecular indicators in gliomas include tumor protein p53 (TP53) mutation, which is related to poor prognosis and response to treatment (Louis et al., 2020; Louis et al., 2021).

The diffuse glioma encompasses both lower-grade gliomas (LGG) and glioblastomas (GBM), but a subgroup of tumors within each grade responds significantly differently to treatment. (Phillips et al., 2006) Even with such a heterogeneity, almost all glioma patients receive alkylating chemotherapy. The use of alkylators such as temozolomide (TMZ) could improve overall patient survival, but many patients experience only limited benefits. DNA repair enzyme O6-methylguanine DNA methyltransferase (MGMT) is thought to be the most effective mechanism of glioma resistance to TMZ. (Stupp et al., 2005; Hegi et al., 2008) Therefore, for the development of novel molecular targeting therapeutics, it is essential to identify tumor-specific pathways underlying DNA damage repair response initiation and hyperactivation.

The copper metabolism MURR1 domain (COMMD) protein family has ten members. COMMD proteins exert key roles in carcinogenesis, progression, invasion, and metastasis. (Green, 2003; Maine and Burstein, 2007; Wang et al., 2021) COMMD4 is a protein-coding gene belonging to the COMMD family, and is expressed at a high level in non-small cell lung cancers (NSCLC) and hepatocellular carcinoma (HCC). (Mao et al., 2011; Suraweera et al., 2020; Wang et al., 2021) Previous reports showed that in NSCLC cells, COMMD4 depletion results in apoptosis mediated by mitotic catastrophe, indicating that COMMD4 might serve as a therapeutic target. Nonetheless, it is unknown if COMMD4 could be employed as a biological marker for glioma and its involvement in gliomas is also unclear.

The data used in this research were obtained from the CGGA (Chinese Glioma Genome Atlas) and TCGA (The Cancer Genome Atlas) databases. Potential association between immune infiltration levels and COMMD4 in LGG and GBM was examined utilizing CIBERSORT. In addition, the Tumor Immune Estimation Resource (TIMER) was applied to evaluate the density of distinct Tumor-Infiltrating Immune Cells (TIICs). The link between COMMD4 expression and drug response was analyzed by CellMiner. This research improves the current understanding of the mechanisms and functions of COMMD4 in glioma.

2 Materials and methods

2.1 Retrieval and pre-processing of data from the cancer genome atlas

The LCG and GBM gene expression data and clinical data were extracted from the TCGA database (http://tcga-data. nci.nih.gov). The whole dataset had 698 tumors and 5 normal samples. (Neftel et al., 2019; Huang et al., 2021) Glioma sequencing data were generated utilizing the RNAseq - HTSeq platform and Strawberry Perl software (version 5.32.1). R (version 4.1.1) were used to conduct all the processing operations.

2.2 Clinical data and the CGGA mRNA matrix

The CGGA database (http://www.cgga.org.cn) is China's most comprehensive glioma genome repository, which provided this study with 1319 glioma samples. Informed consent was obtained before the acquisition of all these samples. Premised on this information, we determined the variations and the survival values in COMMD4 expression. In addition, we obtained additional datasets including the mRNAseq_325 (Illumina HiSeq 2000 or 2500), mRNAseq_693 (Platform: Illumina HiSeq) and mRNA_array_301 (Agilent Whole Human Genome (array)) datasets. The mRNAseq_693 dataset contained 693 glioma samples, and the mRNAseq_325 dataset contained 325 glioma samples. After

TABLE 1 Baseline of CGGA patients' information.

		Total	Low expression	High expression	χ2	*p*
PRS_type	primary	502	253	249	1.0919	0.5793
	Recurrenrt	222	113	109		
	Secondary	25	10	15		
Grade	WHO II	218	143	75	36.6424	0
	WHO III	240	121	119		
	WHO IV	291	112	179		
Gender	Male	442	224	218	2.6105	0.1062
	Female	267	152	115		
Age	< =41	342	188	154	5.7294	0.017
	>41	407	188	219		
Radio_status	No	124	62	62	0.0024	0.961
	Yes	625	314	311		
Chemo_status	No	229	128	101	4.2792	0.0386
	Yes	520	248	272		
IDH_mutation_status	Wildtype	339	151	188	7.9289	0.004
	Mutant	410	225	185		
1p19q_codeletion_status	Non-codel	594	290	304	2.1825	0.1396
	Codel	155	86	69		

TABLE 2 Cox analysis of the CGGA database.

Id	HR	HR.95L	HR.95H	*p* Value
COMMD4	1.276706	1.144555	1.424117	<0.001
Histology	4.486991	3.695058	5.448654	<0.001
Grade	2.883411	2.526415	3.290853	<0.001
Gender	1.04351	0.865536	1.258081	0.655
Age	1.623833	1.345161	1.960236	<0.001
Radio	0.928909	0.719933	1.198546	0.571
Chemo	1.647389	1.327807	2.043888	<0.001
IDH_mutation	0.317158	0.262089	0.383798	<0.001
1p19q_codeletion	0.230575	0.169012	0.314561	<0.001

TABLE 3 Cox analysis of the TCGA database.

Characteristics	Total(N)	HR (95% CI)	*p* Value
COMMD4	695	2.238 (1.750–2.861)	<0.001
Histological type	695		
Astrocytoma	195	Reference	
Glioblastoma	168	6.791 (4.932–9.352)	<0.001
Oligoastrocytoma	134	0.657 (0.419–1.031)	0.068
Oligodendroglioma	198	0.580 (0.395–0.853)	0.006
WHO grade	634		
G2	223	Reference	
G3	243	2.999 (2.007–4.480)	<0.001
G4	168	18.615 (12.460–27.812)	<0.001
Gender	695	1.262 (0.988–1.610)	0.062
Age	695	4.668 (3.598–6.056)	<0.001
IDH status	685	0.117 (0.090–0.152)	<0.001
1p/19q codeletion	688	4.428 (2.885–6.799)	<0.001

that, we employed the limma packages to normalize and batch the two mRNAseq matrices. Table 1 shows the clinicopathological parameters of patients whose clinical data from the CGGA database were complete. The survival and gene expression of COMMMD4 were listed in Tables 2, 3 using R software.

2.3 Interaction analysis of gene expression profiles

GEPIA (http://gepia.cancer-pku.cn/) is an online interactive server that comprises 8587 normal clinical specimens and the RNA seq data of 9736 tumors acquired from TCGA and The Genotype-Tissue Expression (GTEx) datasets. GEPIA was utilized here to investigate the clinical functions of COMMD4. (Tang et al., 2017) The bipartite method was applied to classify the COMMD4 expression into high- and low-expression groups. In addition, the "survival" modules were utilized to examine the links between COMMD4 expression and glioma patients' prognosis. Furthermore, the variation in the expression levels of COMMD4 between the tumor and normal samples was evaluated by the boxplot modules with the disease status as variables (normal or tumor). We employed the Wilcoxon rank-sum test to examine the

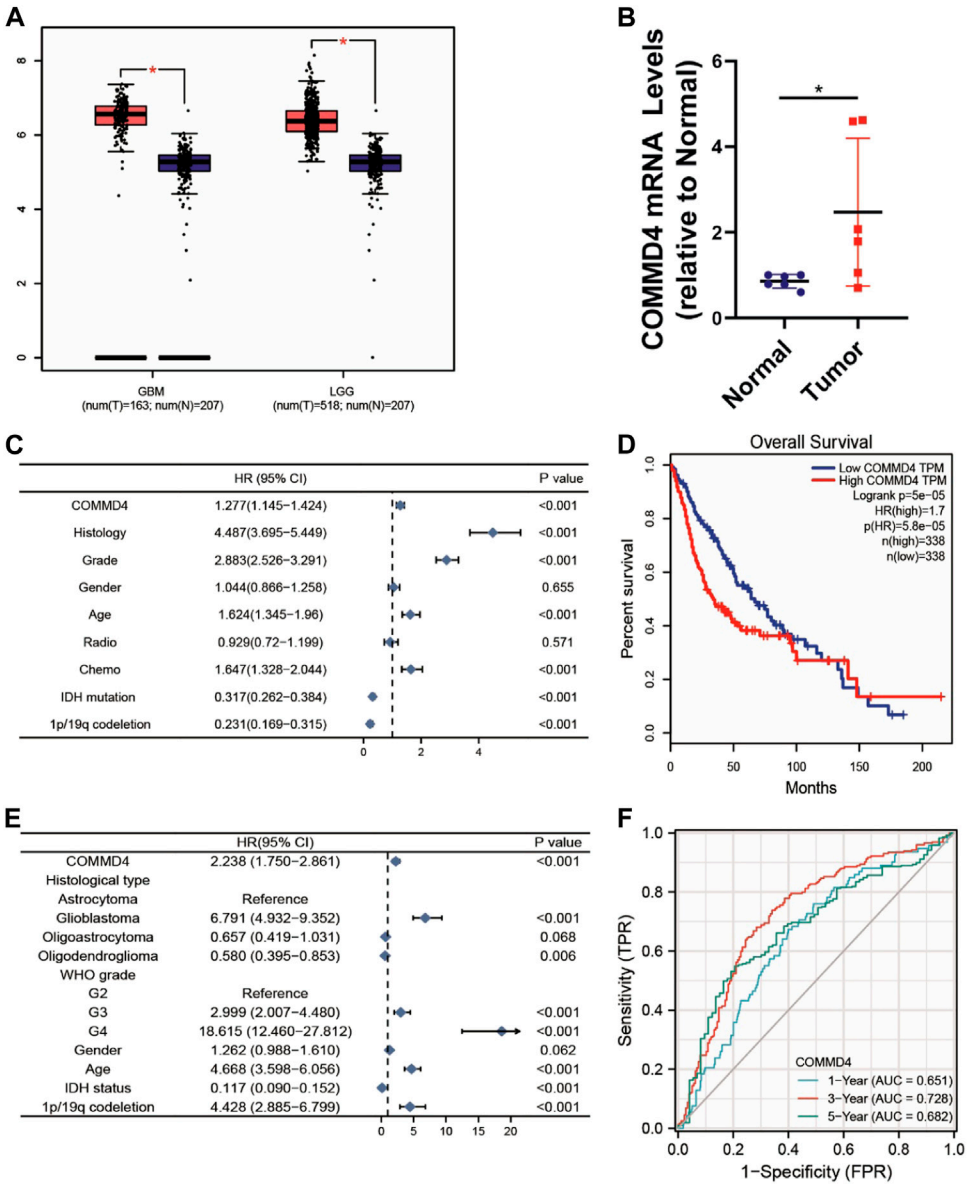

FIGURE 1
(A) COMMD4 expression differs significantly between GBM and LGG. **(B)** qRT-PCR assays to measure the mRNA expression level of COMMD4 in paraneoplastic tissue and tumor tissue from glioma patients. (*$p < 0.05$, with student's t-test). COMMD4 expression and other clinicopathological parameters derived from **(C)** CGGA dataset and **(E)** TCGA datasets were subjected to a univariate Cox analysis. **(D)** GEPIA was used to assess the survival curves of various COMMD4 expression levels. **(F)** The time-dependent receiver operating characteristic (ROC) curves for survival rates over one, three, and 5 years.

links between COMMD4 expression and grade, 1p/19q codeletion status, and IDH mutation status. The R software was used with the tools such as survminer, survival, and ggplot.

2.4 Univariate cox analysis

The links between histology, grade, 1p/19q-codeletion status, IDH mutations, and COMMMD4 expression were analyzed by the Univariate Cox analysis. We performed a statistical study using data from the CGGA and TCGA databases with the survival function in R (version 4.1.1).

2.5 Gene set enrichment analysis analysis

GSEA including KEGG and GO analyses was employed to examine the functional enrichment of COMMMD4 expression.

The biological coherence and correlations among each predicted module were investigated using GO analysis with differentially expressed mRNAs in the GO categories. To explore key pathways linked with COMMMD4 expression, KEGG analysis was carried out.

2.6 Immune cell infiltration assessment

Associations of TIICs with gene expression profiles in tumor tissues were assessed with the ssGSEA and CIBERSORT algorithms. The ssGSEA technique was used to calculate the relative infiltration levels of 24 distinct immune cells in the TCGA dataset. The "ggplot2" software was used to visualize the calculated Spearman correlations of 24 distinct immune cell infiltrations with hub genes. In cell type development, the CIBERSORT method employs a vector regression model. The consistent performance of CIBERSORT could be used to evaluate cellular heterogeneity on gene expression profiles of complex tissues. The algorism was then introduced to transfer the standard-annotated gene expression data to the CIBERSORT website after being applied to the LM22-signed matrix (Lin et al., 2021; Sun et al., 2022; Zhang et al., 2022). The data obtained were classified into low- and high-COMMMD4 expression subgroups in order to examine the variations in the percentage of immune cells, including macrophages, T cells, monocytes, NK cells, dendritic cells, and B cells.

2.7 Tumor immune estimation resource database analysis

Tumor Immune Estimation Resource (TIMER) (https://cistrome.sh.inyapps.io/timer/) was utilized to visualize the correlations between the series of variables in 32 kinds of cancers and over 1000 TCGA samples and immune infiltration levels. (Li et al., 2017) TIMER uses a deconvolutional statistical approach to produce an inference on multiple TIICs. Gene modules were employed to examine the connection between COMMD4 expression levels and TIICs, which included $CD8^+$ T cells, B cells, macrophages, neutrophils, dendritic cells, and $CD4^+$ T cells. The log2 TPM was applied to show the level of gene expression.

2.8 Single-cell analysis

Tabula Muris (https://tabula-muris.ds.czbiohub.org/) is a single-cell transcriptome tool containing over 100,000 cells from 20 different tissues and organs. (Tabula Muris Consortium et al., 2018) Using this database, we examined the associations of COMMD4 expression levels with various types of cells and tissues, including endothelial cells and T lymphocytes. Fluorescence-activated cell sorting (FACS) was also employed here to analyze the connections between COMMMD4 expression and distinct types of cells with great sensitivity and coverage.

2.9 COMMD4 and drug response

A link between COMMD4 expression and drug responsiveness was established by CellMiner (http://discover.nci.nih.gov/cellminer/). CellMiner, which was created by the Genomic and NCI, CCR, DTB, Pharmacology Facilit, NIH, is a query tool and database created for cancer researchers to facilitate the incorporation and evaluation of molecular as well as pharmacologic data for the NCI-60 tumor cell lines. The NCI-60 is a panel comprising 60 distinct human tumor cell lines, and is utilized by the National Cancer Institute's Developmental Therapeutics Program to identify more than 100,000 chemical compounds and natural products (Shankavaram et al., 2009).

2.10 Quantitative RT-PCR

Total RNA was extracted from paraneoplastic tissue and tumor tissue from glioma patients of different grades using the TRIzol reagent (Sigma-Aldrich, United States). Cell line samples were processed in the same way. Then, RNA from each sample (2 μg) was reverse-transcribed into cDNA, after which reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed using the FastStart universal SYBR ®Green Master (Roche, United States) in an ABI QuantStudio5 Q5 real-time PCR System (Thermo Fisher Scientific, United States). The template for the reaction was selected as cDNA at a reaction volume of 20 μl (10 μl of PCR mixture, 0.5 μl reverse and forward primers, 2 μl of cDNA template, and an appropriate volume of water). For the PCR reactions, the cycling conditions began with DNA denaturation at 95°C for 30 s (s), followed by 45 cycles for 15 s at 94°C, 30 s at 56°C, and 20 s at 72°C. Each sample was performed in triplicates. The $2^{-\Delta\Delta CT}$ method was adopted to obtain threshold cycle (CT) measurements, which were standardized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels in all samples. The mRNA expression levels were compared to paracancerous tissue controls. The following are the sequences of primer pairs for the target genes:

Gene	Forward primer sequence (5–3)	Reverse primer sequence (5–3)
COMMD4	TTCTTGGCGCGATGAGGTTC	TCAGAGGGCGTGACTCCATA
GAPDH	AATGGGCAGCCGTTAGGAAA	GCCCAATACGACCAAATCAGAG

2.11 Cell culture and drug

Human glioma cell lines U-87 and U-251 were obtained from ATCC (Beijing Beina Chuanglian Biotechnology Institute) and cultured in F12 and DMEM containing 10% fetal bovine serum (Gibco, Carlsbad, CA, United States), respectively. Both cell lines were stored in a humidified incubator at 37°C with 5% CO2. Temozolomide was procured from MCE (CAT# HY-17364). Dissolution of temozolomide was carried out in dimethyl sulfoxide

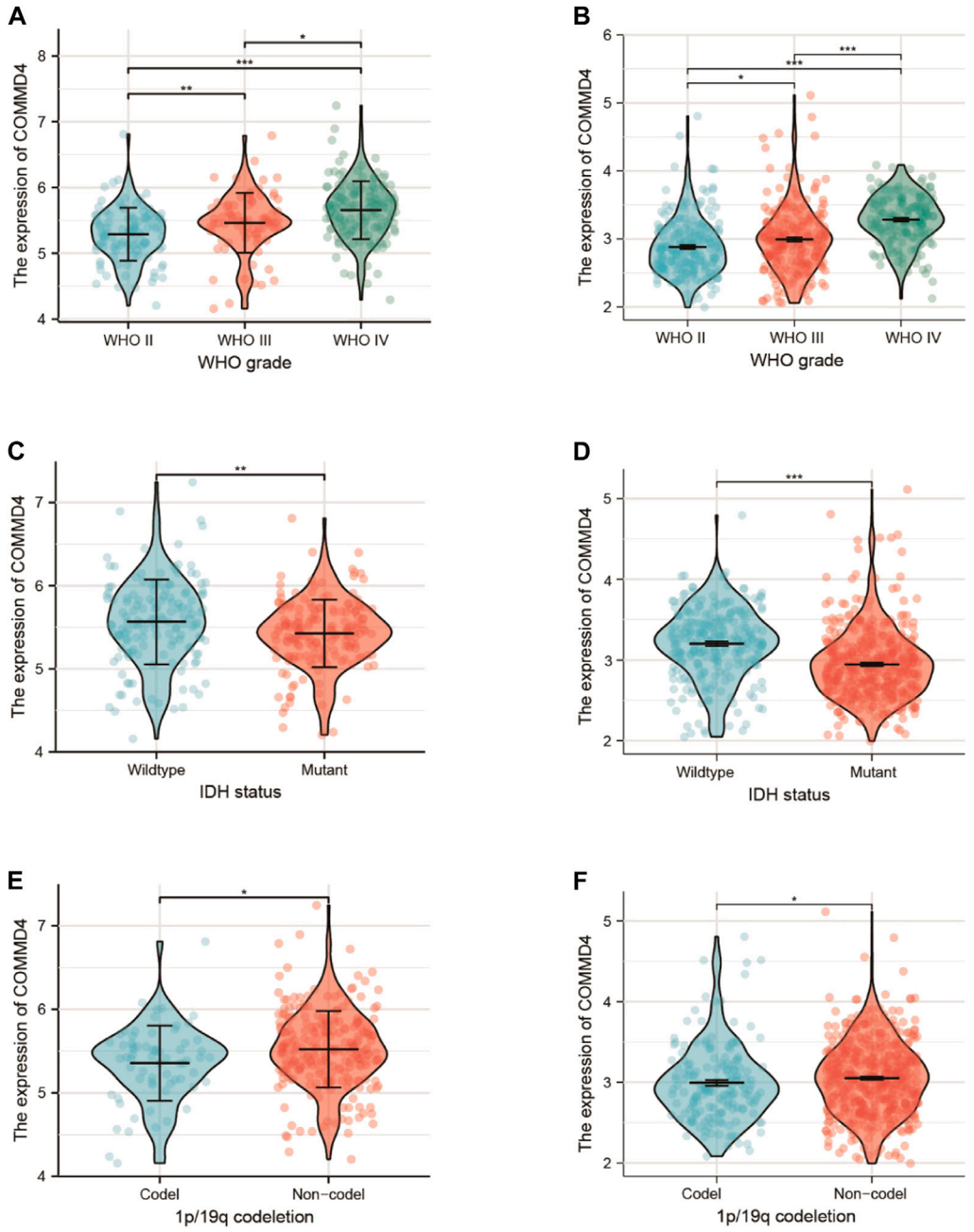

FIGURE 2
Expression of COMMD4 in CGGA **(A)** WHO grades. **(C)** IDH status-stratified distribution. **(E)** 1p/19q-codeletion status distribution. COMMD4 expression in TCGA **(B)** WHO grades. **(D)** IDH status-stratified distribution. **(F)** 1p/19q-codeletion status distribution.

(DMSO, Beyotime). Finally, it was co-cultured with cells at a concentration of 20 μM/ml.

2.12 Transwell assay

Transwell assays for migration and invasion of glioma cells (U-87, U-251) were performed. Briefly, cells (5 × 104) were inoculated into chambers coated (for invasion) or uncoated with Matrigel (BD Biosciences, San Jose, CA) (for migration). Serum-free medium was added to the upper layer and a complete DMEM medium was added to the lower layer. After 24 h of incubation, migrating or invading cells were fixed with 4% paraformaldehyde and stained with 0.1% crystalline violet. Counting under a light microscope.

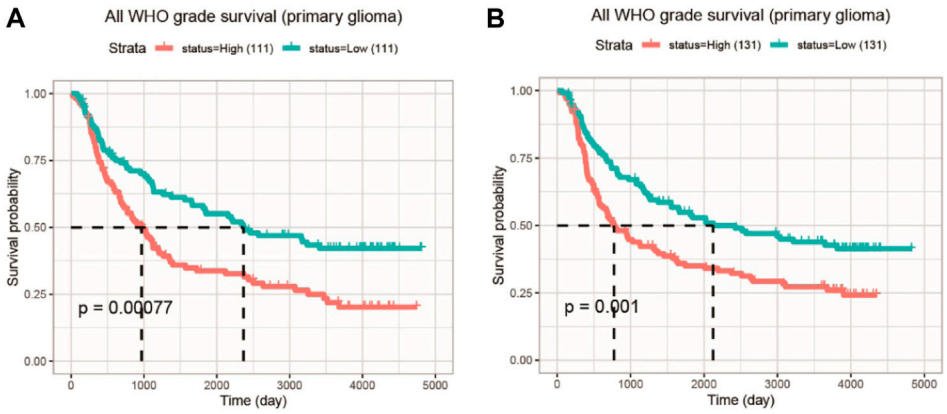

FIGURE 3
The KM survival curve illustrating the expression of COMMD4 in GBM and LGG patients **(A)** Dataset ID: mRNAseq_325-Primary Glioma **(B)** Dataset ID: mRNA_array_301-Primary Glioma.

3 Results

3.1 Relationship between COMMD4 expression and glioma survival status

COMMD4 expression level was elevated in both GBM (num (N) = 207, num (T) = 163) and in LGG (num (N) = 207 num, (T) = 518; Figure 1A). Furthermore, the COMMD4 overexpression was indicative of a more unfavorable overall survival (OS) (num (high) = 338, num (low) = 338, $p < 0.001$; Figure 1D). By using the bipartite technique, the expression level of COMMD4 in normal and malignant tissues was classified into 2 groups (low- and high-expression groups). These findings demonstrated that COMMD4 expression levels were greater in tumor tissues and were linked to a worse OS.

3.2 COMMD4 as an independent predictor for glioma patients

Based on the CGGA and TCGA databases, univariate Cox analysis was conducted to assess the utility or practicality of COMMD4 expression. Factors such as COMMD4 expression ($p < 0.001$), histology (astrocytoma, oligodendroglioma, Glioblastoma) ($p < 0.05$), grade (WHO grade) ($p < 0.001$), chemotherapy ($p < 0.001$), IDH mutation ($p < 0.001$) and 1p19qcodeletion ($p < 0.001$) (Figures 1C,E) were determined premised on the univariate analysis. According to the receiver operating characteristic (ROC) analysis, the area under the curve (AUC) of COMMD4 was found to be 0.651, 0.728, and 0.682 for one-, three-, and 5-year survival, respectively (Figure 1F).

3.3 The relationships between COMMD4 expression and world health organization grade, isocitrate dehydrogenase 1 phenotype in the chinese glioma atlas and the cancer genome atlas

The relationships between COMMD4 expression, WHO grade, and IDH1 state were analyzed in the two different datasets. In both datasets, comparable associations between COMMD4 expression levels and WHO glioma grades could be found (Figures 2A,B). The elevated COMMD4 expression level was linked to greater glioma malignancy, according to the findings. Furthermore, the IDH-wildtype group showed substantially elevated COMMD4 expression level compared with that in the IDH-mutant subgroup (Figures 2C,D). The 1p/19q-non-codeletion (non-codel) group had a considerably elevated COMMD4 expression level compared with that of the 1p/19q-codeletion group (Figures 2E,F), which was calculated using the Wilcoxon rank-sum test. These findings illustrated that COMMD4 was expressed at a high level in the 1p19q-non-codeletion and IDH-wildtype groups.

3.4 Survival analysis and expression of COMMD4 in primary gliomas derived from the chinese glioma atlas database

With the two CGGA datasets, an integrative survival analysis was performed to examine the association of COMMD4 expression with survival of glioma patients. In Dataset 1 (ID: mRNAseq 325), patients in the high-COMMD4 expression group with primary glioma demonstrated an unfavorable prognosis ($p < 0.001$; Figure 3A). Furthermore,

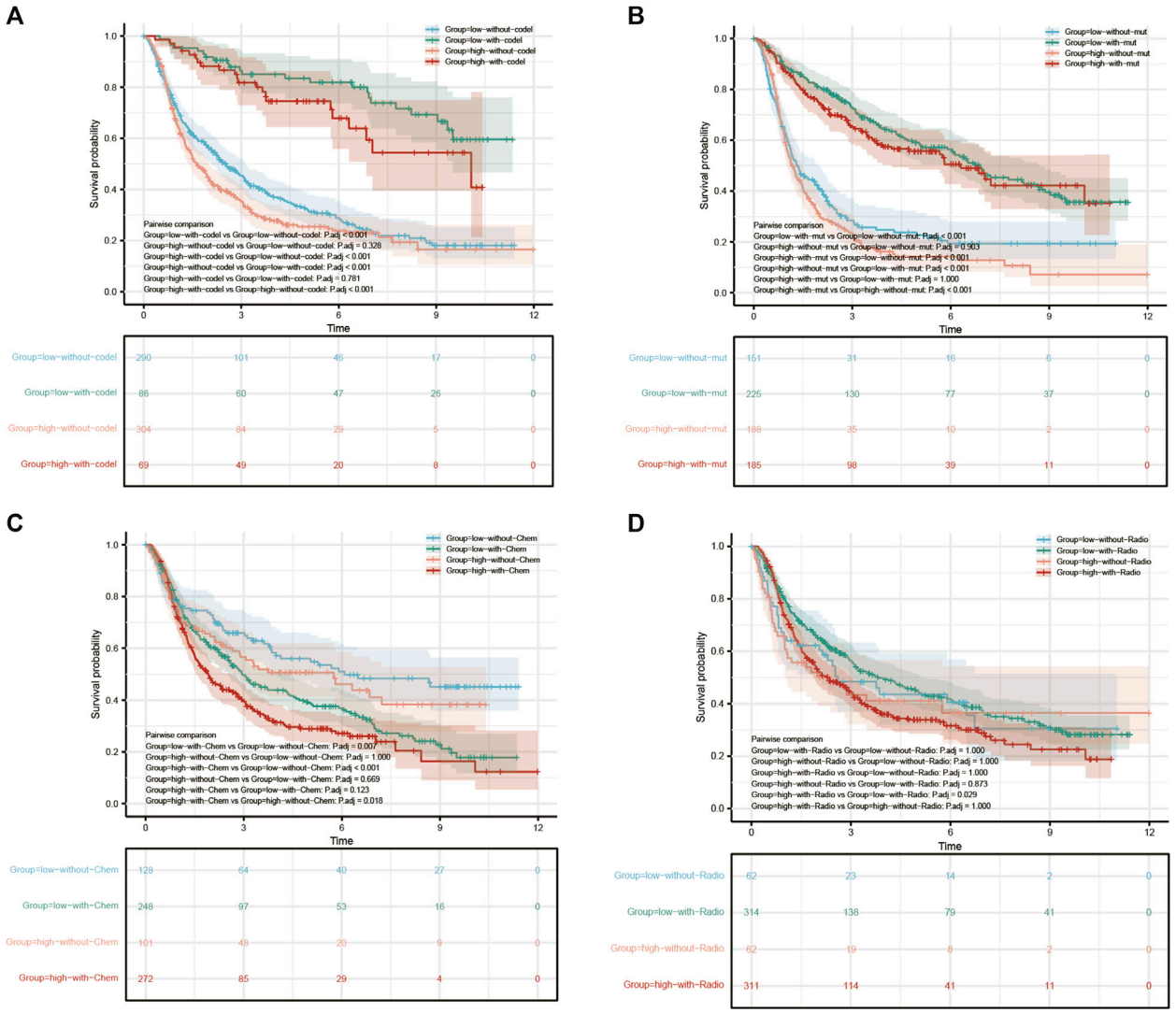

FIGURE 4
Survival analysis of GBM and LGG patients with varying levels of COMMD4 expression in comparison with **(A)** 1p/19q status **(B)** IDH mutation **(C)** chemotherapy **(D)** radiotherapy.

the high-expression group in Dataset 2 (ID: mRNA array 301) had a significantly unfavorable prognosis in primary glioma (p = 0.001; Figure 3B).

3.5 Multifactorial integrated survival analysis in the chinese glioma atlas database

To further analyze the clinical relevance of COMMD4, 1p19q status (Figure 4A), IDH1 genotypes (Figure 4B), chemotherapy (Figure 4C), radiotherapy (Figure 4D) were incorporated as parameters in a multivariate analysis. As demonstrated by the 1p19q status, COMMD4 overexpression and 1p19q non-codeletion (orange in Figure 4A) were associated with the poorest prognosis. Notwithstanding a high expression level of COMMD4, the survival rate remained high in the IDH1-R132-mutant groups (red Figure 4B). Thus, COMMD4 could be seen as a viable marker in the corresponding IDH1 genotypes ($p < 0.0001$). Following that, we examined the link between COMMD4 expression and the survival of patients receiving chemotherapy, and the worst prognosis was found in the high-COMMD4 expression group after chemotherapy (red in Figure 4C). However, favorable prognoses were reported in patients in the low-COMMD4 expression group who did not receive chemotherapy (blue in Figure 4C). As a result, patients

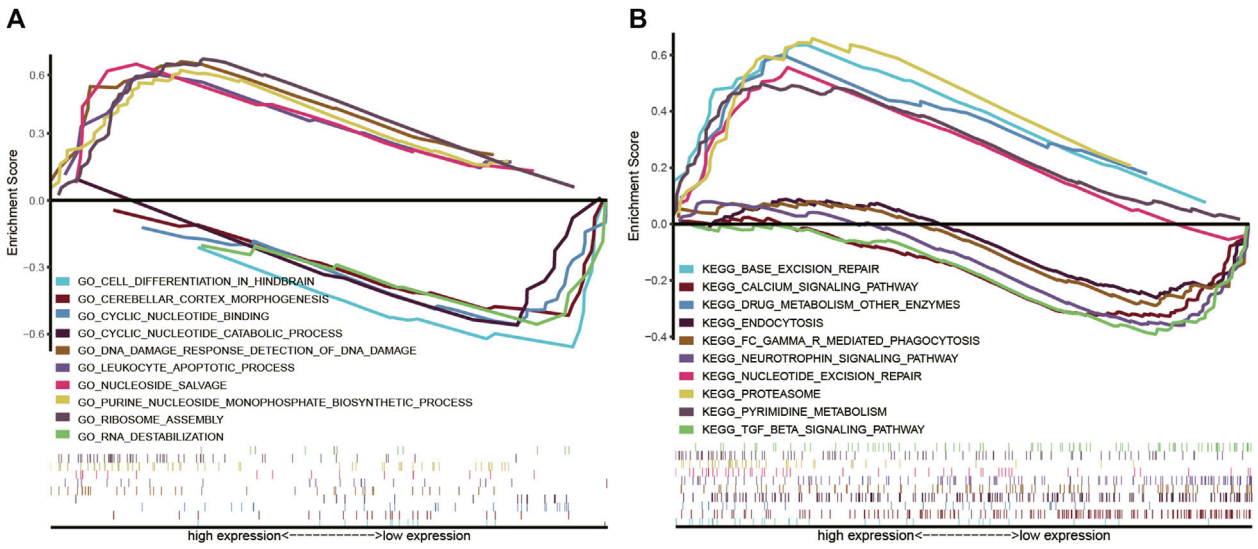

FIGURE 5
(A) GO findings show the pathways that were associated with COMMD4. **(B)** KEGG analyses showed the pathways that were linked to COMMD4.

receiving chemotherapy with a low COMMD4 expression level may benefit more. Similarly, patients in the high-COMMD4 expression group receiving radiotherapy (red in Figure 4D) were found to have unfavorable prognosis in comparison to those in the low-expression group with radiotherapy (green in Figure 4D).

3.6 Gene set enrichment analysis investigation of COMMD4-related pathways

We performed GO and KEGG analysis to examine the potential biological role of COMMD4. We identified five gene pathways strongly linked to COMMD4 expression, and discovered that COMMD4 was remarkably related to repair-related and immune-related gene pathways. According to the findings of GO analysis, the five pathways closely associated with the elevated level of COMMD4 overexpression included DNA damage response detection, leukocyte apoptotic process, nucleoside salvage, purine nucleoside monophosphate biosynthetic process, and ribosome assembly. Additionally, five inversely correlated categories were found, including cell differentiation in the hindbrain, cyclic nucleotide-binding, RNA destabilization, cerebellar cortex morphogenesis, and cyclic nucleotide catabolic process (Figure 5A). The findings of KEGG analysis indicated that the five pathways were positively linked to upregulation of COMMD4 expression, including base excision repair, drug metabolism of other enzymes, nucleotide excision repair, proteasome, and pyrimidine metabolism. Similarly, the five categories inversely linked to COMMD4 expression upregulation were FC gamma r mediated phagocytosis, calcium signaling pathway, endocytosis, TGF beta signaling pathway, and neurotrophin signaling pathway (Figure 5B).

3.7 Associations between COMMD4 expression and tumor-infiltrating immune cells

The relationship between TIICs in glioma and COMM4 expression levels was investigated. According to our results, the COMMD4 expression had a negative correlation with Tgd, TFH, Tem, Tcm, Th1 cells, Th2 cells, and Mast cells. (Figure 6A). To further verify the relationship between TIICs in glioma and COMMD4, the 703 TCGA samples and the 1018 CGGA samples were separated into low- and high- COMMD4 expression groups. According to the samples from the CGGA database, immune cell infiltration level (activated Mast cells, resting memory $CD4^+$ T cells, activated memory $CD4^+$ T cells, Neutrophils) was shown to be considerably lower in the high-risk group than the low-risk group. Furthermore, the infiltration levels of immune cells (activated Mast cells, naive CD4 T cells, activated NK cells, Monocytes, and naïve CD4 T cells) were considerably lowered in the high-risk group than the low-risk group. In both the CGGA (Figure 6B) and the TCGA (Figure 6C) databases, Mast cell activation ($p < 0.05$) was greatly attenuated in the high-COMMD4 expression subgroup.

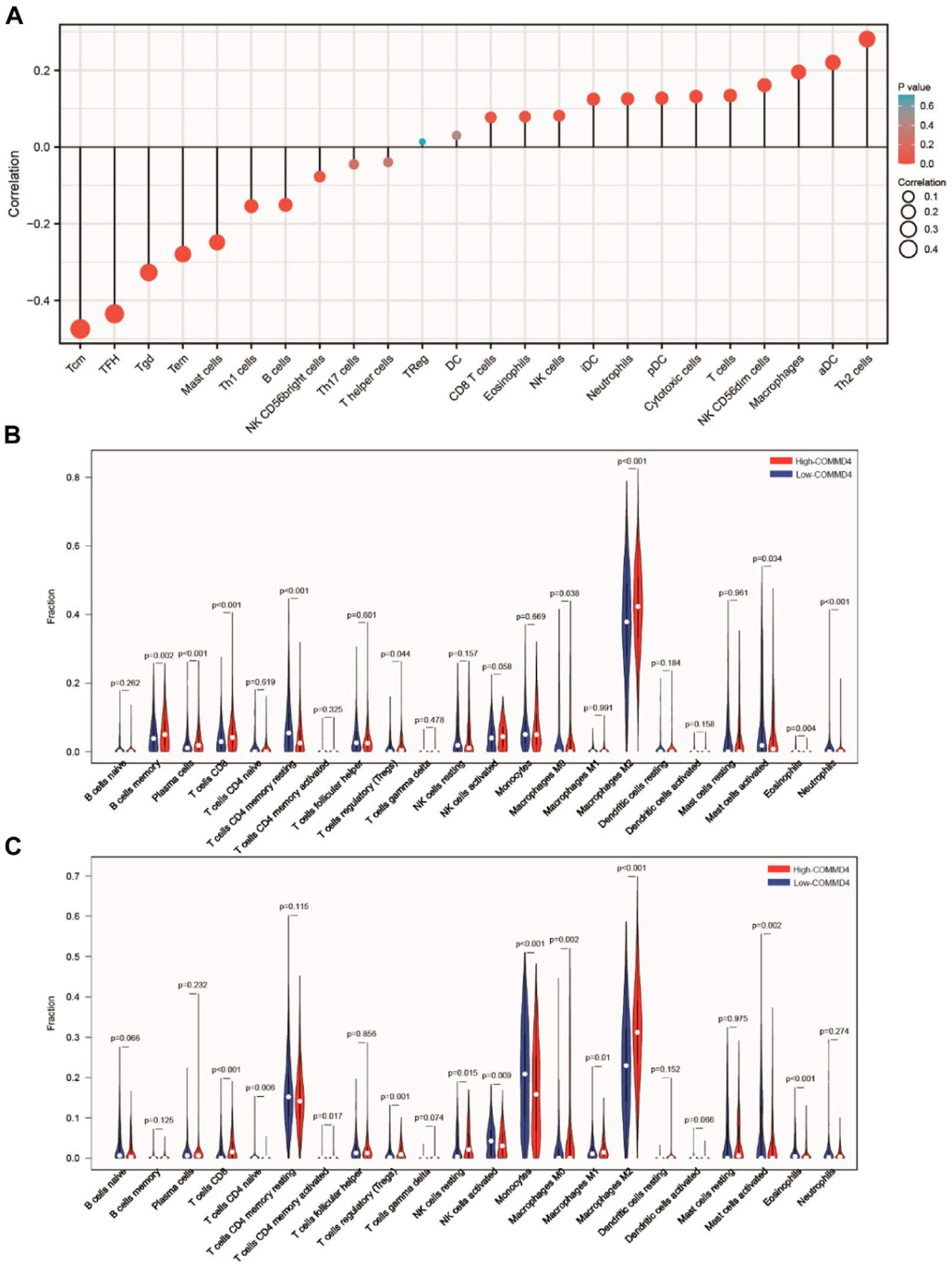

FIGURE 6
(A) The findings of the relative ratios of TIIC computed utilizing the ssGSEA method premised on the TCGA dataset. The relative ratios of TIIC derived utilizing the CIBERSORT method premised on the **(B)** CGGA and **(C)** TCGA datasets.

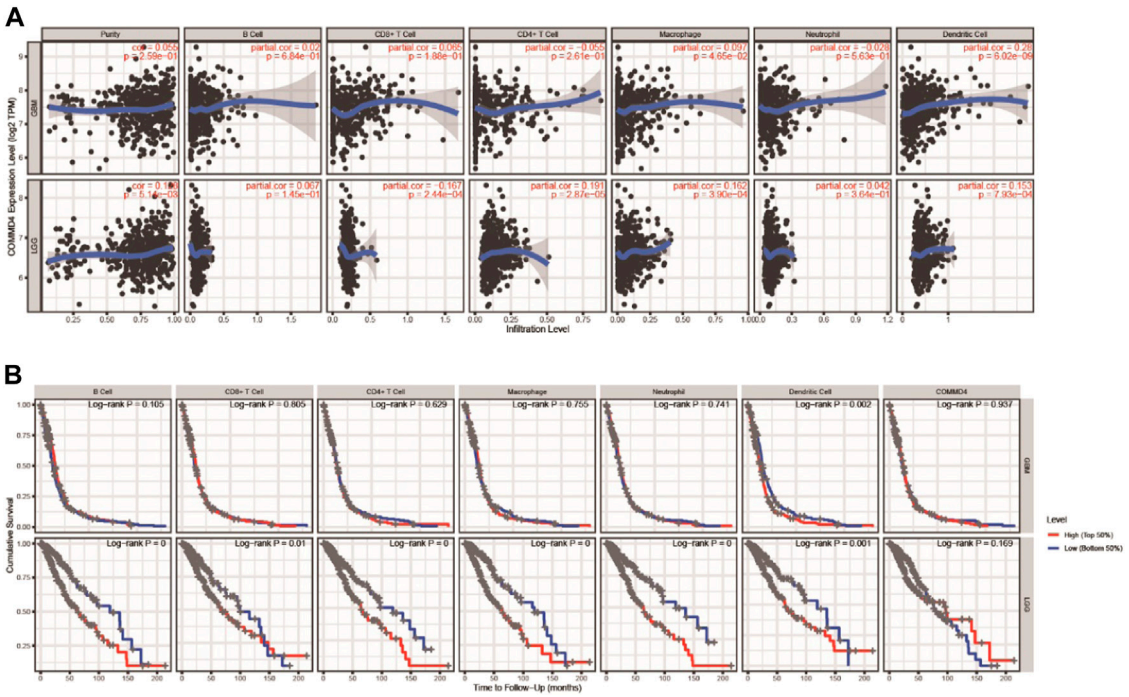

FIGURE 7
(A) In GBM and LGG, COMMD4 expression levels have substantial associations with infiltration levels of B cells, T cells, Macrophages, Neutrophils, and DCs. **(B)** B cells, T cells, Macrophages, Neutrophils, and DCs are all associated with overall survival in patients with GBM and LGG.

3.8 COMMD4 expression was related to the infiltration levels of immune cells and overall survival in glioblastomas and lower-grade gliomas from tumor immune estimation resource

The TIMER database was used to investigate whether the immune infiltration levels in glioma were linked to the COMMD4 expression levels. The infiltration levels of $CD8^+$ T lymphocytes was inversely linked to the expression of COMMD4 (r = −0.167, p = 2.44e-04) (Figure 7A) in LGG. Furthermore, the factors of neutrophils, DCs, macrophages, T and B cells were related to the OS rate in LGG and GBM (Figure 7B).

3.9 COMMD4 expression and cells from various organs were examined by single-cell analysis

The Tabula Muris database was used to examine the associations between COMMD4 expression and cells. Glioma were closely associated with astrocytes of the brain pericyte, neuron, oligodendrocyte precursor cell, oligodendrocyte, endothelial cell, and Bergmann glial cell, as shown in Figure 8A, and were displayed using t-SNE from FACS cells. As depicted in Figure 8B, COMMD4 was primarily associated with oligodendrocytes.

3.10 COMMD4 and drug responsiveness

COMMD4 expression was inversely related to drug responsiveness among patients treated with 5–Fluoro deoxy uridine, Amonafide, Vorinostat, Cladribine, Triethylenemelar, Hydroxyurea, Thiotepa, SNS−314, Methylprednisolone, Karenitecin, Pracinostat, and Gemcitabine. Figure 9 depicts the association between COMMD4 expression and predicted drug responsiveness.

3.11 The COMMD4 expression in human glioma

The level of COMMD4 expression in paraneoplastic tissue and tumor tissue from glioma patients was initially examined in this research. According to the RT-qPCR data, the expression of COMMD4 was up-regulated in glioma tissues relative to adjoining tissues (Figure 1B). Furthermore, glioma cells invasion and migratory abilities were evaluated by using transwell assay results in Figures 10A–D exhibited that the abilities of invasion and migration

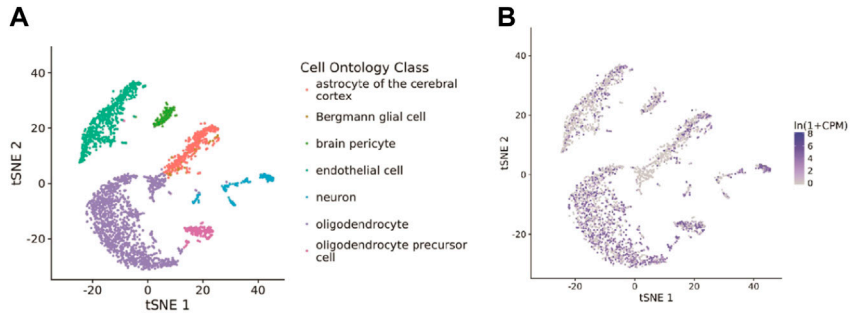

FIGURE 8
Single-cell analysis of COMMD4 expression **(A)** The cells that were linked to the tissues extracted from the brain. **(B)** The COMMD4 expression in tissues extracted from the brain.

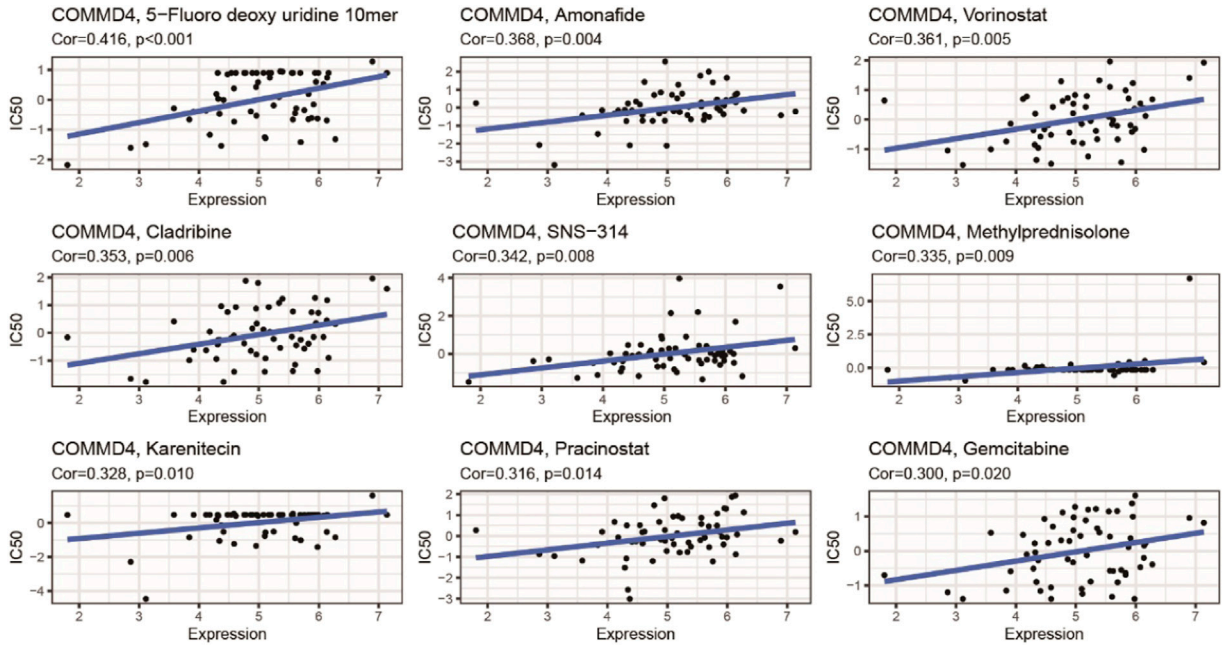

FIGURE 9
An illustration of the relationship between COMMD4 expression and expected medication response.

of U87 and U125 cells were conspicuously reduced by TMZ as comparison to the control group. In addition, results from qRT-PCR indicated the expression of COMMD4 was significantly upregulated after the induction of TMZ in these two cell lines (Figure 10E).

4 Discussion

Glioma is a type of brain tumor that originates from glial cells in the CNS and accounts for over 80% of all malignancies occurring in the brain. (Chen et al., 2017; Zhong et al., 2019)Surgical intervention and postoperative enhanced radiotherapy and chemotherapy are widely implemented in glioma treatment. (Bush et al., 2017; Malta et al., 2018) Glioblastoma, on the other hand, has an unfavorable prognosis, with a median survival period of shorter than 2 years. Therefore, viable biomarkers for early glioma detection are beneficial to patient management and prognosis.

This investigation proved the significance of COMMD4 in the pathogenesis of glioma, identified a new possible therapeutic target for glioma treatment and a prognostic indicator. In LGGs and GBMs, patients diagnosed with

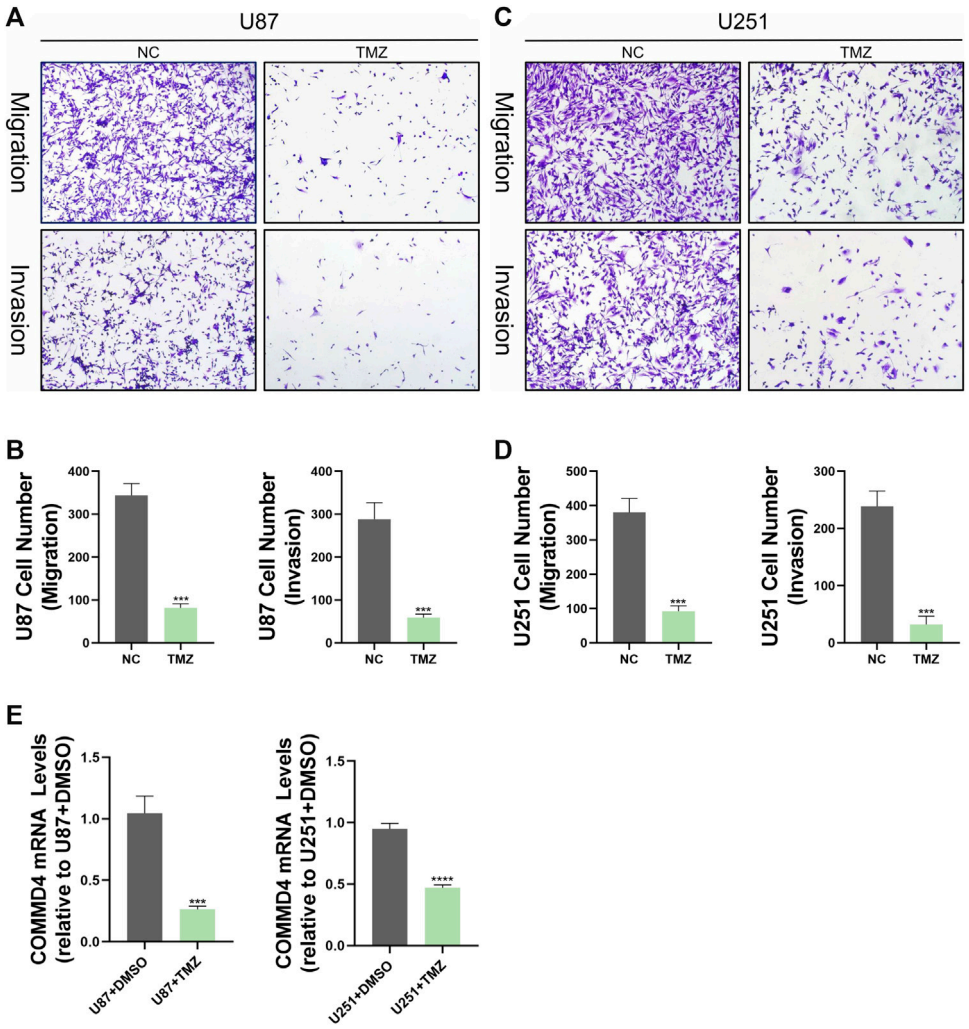

FIGURE 10
TMZ inhibits migration and invasion of glioma cells *in vitro* and reduces COMMD4 expression. **(A–D)** Transwell assay images of migration and invasion in the NC and TMZ groups, and quantitative counts of cell numbers **(E)** Relative quantitative analysis of COMMD4 expression in the NC and TMZ groups.

glioma exhibited low survival rate and elevated COMMD4 expression level. This study also examined the relationships between COMMD4 expression and IDH1 status. IDH1 phenotypes, according to the WHO, are an innovative diagnostic technique employed in clinical settings, and IDH1 mutation status is utilized to classify diffuse glioma in adults. The elevated expression levels of COMMD4 accelerated the malignant progression of glioma, as evidenced by the IDH1-wildtype patients' unfavorable survival. Furthermore, we compared chemotherapy and radiotherapy to highlight the role of COMMD4 and identified COMMD4 as a molecular marker for glioma patients' prognosis.

Although the mechanisms of COMMD4 in glioma cells are unknown, various research reports have shown that it is intimately linked to tumor genomic stability and apoptosis. Suraweera et al. found that COMMD4 was subjected to overexpression in NSCLC cells, and that siRNA knockdown of COMMD4 attenuated cell proliferation and viability. After being exposed to DNA-damaging agents, cell death was more accelerated. Following COMMD4 knockdown, non-small cell lung cancer (NSCLC) cells experienced mitotic catastrophe and apoptosis. Meanwhile, higher expression of COMMD4 has been found in NSCLC and was linked to unfavorable prognosis in adenocarcinoma (ADC). In addition, a previous report illustrated that

COMMD4 maintains genomic integrity through regulating chromatin structure at DSB sites. Moreover, the researchers also discovered that cells lacking COMMD4 are more susceptible to multiple DNA-damaging agents that induce DSBs and are less effective in repairing DSBs. Though the association between COMMD4 and glioma was not yet completely clarified, it could be speculated that COMMD4 influenced the development of pathophysiological pathways of glioma based on our findings and previous research on COMMD4 (Suraweera et al., 2020; Suraweera et al., 2021).

GSEA was used to conduct GO terms and KEGG pathway analyses to further examine the possible biological roles of COMMD4 in glioma. In samples exhibiting low and high levels of COMMD4, GSEA indicated substantial differences in GO term and KEGG pathway enrichment. In particular, GESA analysis illustrated an enrichment of several immune-related and repair-related gene sets in the high-COMMD4 group, including leukocyte apoptotic process, DNA damage response detection of DNA damage, nucleoside salvage, and nucleotide excision repair. Notably, according to a growing body of research, DNA damage repair and immunological infiltration are both implicated in cancer advancement and drug resistance. These data indicated that COMMD4 was implicated in the progression of glioma. In glioma development, high COMMD4 expression level might affect mechanisms of treatment resistance and tumor immunology. Our findings suggested that the upregulation of COMMD4 expression was linked to a poor prognosis. We hypothesized that elevated COMMD4 expression level had a pivotal regulatory function in these oncogenic pathways, and this resulted in a poorer prognosis for glioma patients.

From CellMiner, we discovered that COMMD4 expression was adversely related to drug responsiveness in patients treated with Amonafide and Cladribinethe. The drug resistance of Amonafide and Cladribinethe may be related to the DNA damage repair function of COMMD4 (De Isabella et al., 1995; Liu et al., 2011). Furthermore, GSEA confirmed the substantial enrichment of immune-related gene sets in the high-COMMD4 expression group. We then examined the relationship between infiltration levels of immune cells in glioma and COMMD4 expression. COMMD4 expression demonstrated a strong negative association with the infiltration level of mast cells (MC), according to CIBERSORT analysis. Mast cells are specific immune system cells that release a wide range of physiologically active chemicals, which can activate, regulate, or decrease the immune response. (Gordon and Galli, 1990; Falduto et al., 2022; Fereydouni et al., 2022) When exposed to FceRI, Human MCs produce substantial levels of granulocyte-macrophage colony-stimulating factor (GM-CSF), according to Fereydouni et al. This is significant because both GM-CSF and TNF-α have been shown to attenuate tumor cell proliferation, promote tumor regression, and improve anti-tumor co-therapies. (Yan et al., 2017; Josephs et al., 2018; Plotkin et al., 2019) Our findings suggested that the negative impact of COMMD4 on glioma could be resulted from the reduced density of mast cells. We speculated that COMMD4 may have certain effects on tumor immunity.

In summary, this is the first research exploring the function of COMMD4 in glioma. COMMD4 level was elevated in gliomas and COMMD4 was associated with tumor grade. In addition, qRT-PCR verified the high expression of COMMD4 in glioma tissues and cells. Furthermore, a high level of COMMD4 overexpression was related to an unfavorable prognosis and impaired infiltration of immune cells in glioma. Finally, the primary glioma pathway mediated by COMMD4 may be connected to genomic stability, which may be associated with glioma treatment resistance. The study also had certain limitations, for instance, there was an absence of *in vitro* and *in vivo* trials. Thus, additional research was encouraged to identify COMMD4 as a viable prognostic marker in glioma treatment resistance.

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 human participants were reviewed and approved by Ethics Committee of the Affiliated Hospital of Nantong University (Approval No: 2018-K020). The patients/participants provided their written informed consent to participate in this study.

Author contributions

ZHL, ZCL, PPG, and HYH carried out experiments and analysis. ZHL, HYH, PPG, ZCL, YDS, BW, LP, and WCW wrote the manuscript. ZHL, PPG, and LP conceived the study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.974107/full#supplementary-material

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