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New insights into intracellular pathways and therapeutic targets in CNS diseases

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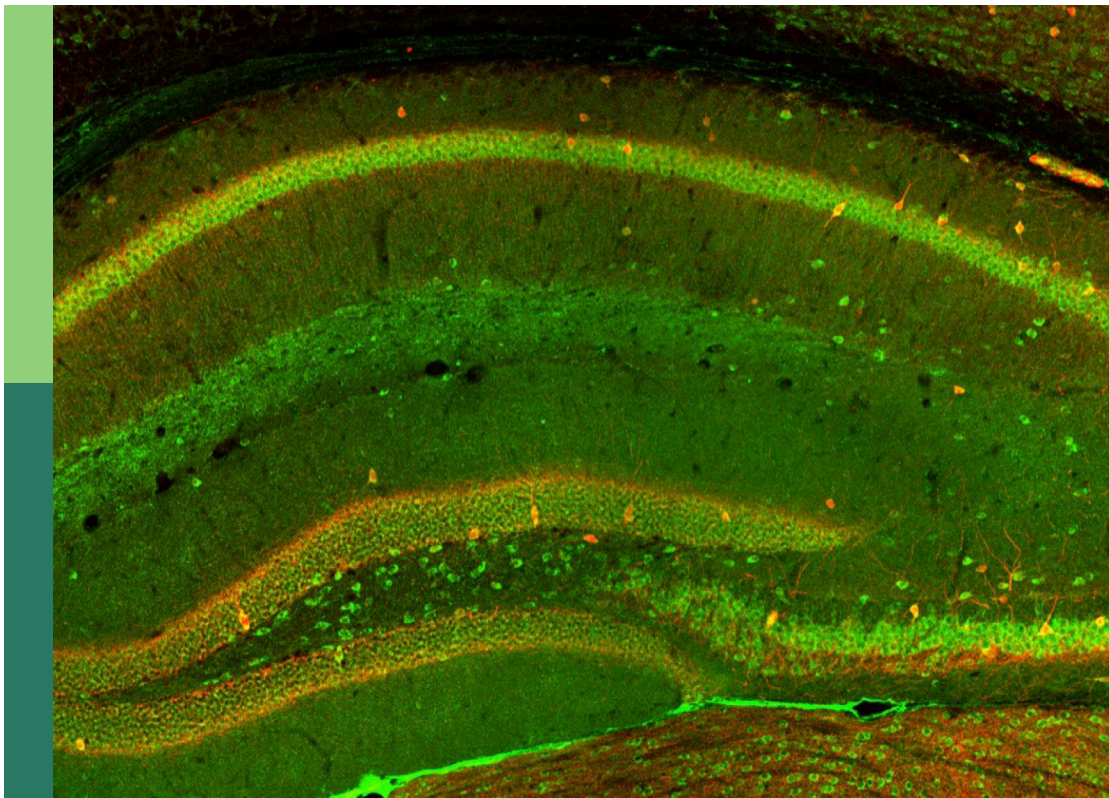

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New insights into intracellular pathways and therapeutic targets in CNS diseases

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Editorial: New insights into intracellular pathways and therapeutic targets in CNS diseases

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Editorial on the Research Topic
New insights into intracellular pathways and therapeutic targets in CNS diseases

The future of therapy in the realm of central nervous system (CNS) disorders unquestionably lies in the development of targeted, personalized treatments. The ever-expanding toolkit of cutting-edge technologies is enabling researchers to gain unprecedented insights into the complexities of the human brain, revealing intricate physiological pathways that govern neurological health. This editorial introduces a selection of studies featured in this Research Topic of *Frontiers in Cellular Neuroscience*, all of which focus on the dysregulation of intracellular signaling networks that play a critical role in neurological diseases. These studies offer not only fresh perspectives on the pathophysiology of these conditions but also new hope for developing effective treatments and, potentially, cures.

At the heart of these innovations is the recognition of the brain's unique and highly specialized nature. Every structure, every neuronal circuit, and each individual cell in the brain holds a specific role in regulating emotions, memory, cognition, and behavior. The fine-tuned balance of these elements must be preserved for healthy neurological functions. This delicate balance poses a critical question for the development of new therapies: what are the consequences of suboptimal treatment, particularly in diseases that affect the brain? How can the risks and benefits of experimental therapies be carefully weighed to ensure that patient dignity is respected, particularly when the stakes are so high? (Maidment et al., 2024).

In this Research Topic, we present a selection of studies that help to cope with these complex questions. One such contribution comes from Marino et al., who explore the phenomenon of "Brain Fog," a cognitive condition often experienced by patients undergoing radiotherapy for brain cancer. Though the term may initially seem metaphorical, Marino et al. argue that "Brain Fog" is, in fact, a distinct post-treatment condition that can have long-lasting effects on cognitive function. This phenomenon is particularly pronounced in glioblastoma patients, who are frequently subjected to radiotherapy. The study underscores the critical need to understand the biological mechanisms behind Brain Fog, particularly the role of protein misfolding and microglial

activation in neurodegeneration. Marino et al.'s work highlights the importance of studying the brain's microenvironment—a rapidly growing area in neuroscience that holds tremendous potential for the development of personalized, patient-centered treatments. Understanding how the brain's immune cells, such as microglia, interact with tumor cells and surrounding tissues could lead to more effective therapeutic strategies, reducing the cognitive side effects that often accompany cancer treatments.

However, treating glioblastoma remains one of the most formidable challenges in modern medicine. Glioblastoma is an aggressive form of brain cancer with a particularly poor prognosis and limited treatment options. Despite some advancements in surgical techniques and radiation therapy, survival rates for glioblastoma patients have remained low, with few meaningful breakthroughs since the approval of Temozolomide in the late 1990s (Jezierzański et al., 2024). The tumor's intracranial location, combined with the notorious very limited permeability of the blood-brain barrier, presents unique obstacles to drug delivery (Fukushima and de Groot, 2024). Moreover, the inherent heterogeneity of glioblastoma cells means that, even if one aspect of the tumor is targeted effectively, other subpopulations may remain unaffected, leading to tumor recurrence. Chiariello et al. delve into this problem, exploring novel approaches to intracranial drug delivery. They review a range of techniques that aim to bypass the blood-brain barrier and facilitate the targeted delivery of therapeutic agents directly to the tumor site. Despite early promise, many of these methods have encountered challenges, and the quest for more effective drug delivery platforms remains an ongoing endeavor. Chiariello et al.'s work emphasizes the need for continued innovation, particularly in the development of "smart materials" that can respond to the unique characteristics of the tumor microenvironment (Nehra et al., 2021). These advanced drug delivery systems may 1 day offer more precise, localized therapies that could significantly improve the prognosis for glioblastoma patients.

Another promising avenue of therapeutic development lies in drug repurposing—an approach that allows researchers to investigate existing, FDA-approved drugs for new therapeutic indications (Rani et al., 2024). This strategy offers several advantages, including shorter development timelines, lower costs, and reduced risks associated with safety and toxicity. One example of this approach is the work of Pallavicini et al., who investigate the potential of the citron kinase inhibitor Lestaurtinib in the treatment of medulloblastoma, a common and aggressive pediatric brain tumor. Lestaurtinib, which was originally developed for its effects on the Sonic Hedgehog (Shh) signaling pathway, exhibits a poly-pharmacological profile, including the ability to induce DNA damage and promote apoptosis. Pallavicini et al.'s study suggests that Lestaurtinib may hold promise as a repurposed treatment for medulloblastoma, highlighting the therapeutic potential of rethinking old drugs for new applications. This research exemplifies the power of drug repurposing in accelerating the availability of novel treatments for brain tumors and other CNS diseases.

Beyond traditional pharmacological approaches, this Research Topic also explores the role of endogenous molecules in CNS diseases. As the brain sciences continue to evolve, even well-known molecules are revealing new and unexpected functions. A fascinating example is the work by Cavalu et al., who investigate the role of orexin, a neuropeptide involved in regulating arousal, sleep-wake cycles, and appetite. Their research uncovers a dual role for orexin in both promoting neurodegeneration and potentially preventing cancer, depending on the context. While orexin may contribute to the survival of cancer cells, its involvement in apoptosis regulation in the brain suggests that it may also have a neuroprotective role under certain conditions. This discovery opens the door to further research into orexin-based therapies, potentially targeting orexin receptors to either alleviate neurodegenerative diseases or limit cancer cell proliferation.

Similarly, Shaikh et al. examine the therapeutic potential of modulating dopamine transporters in the context of Alzheimer's disease. Dopamine, a neurotransmitter essential for regulating mood, attention, and motor function, is often dysregulated in neurodegenerative diseases, including Alzheimer's (Saggu et al., 2024). By targeting dopamine transporters, Shaikh et al.'s work suggests that it may be possible to modulate dopamine signaling in a way that alleviates both motor and cognitive deficits seen in Alzheimer's patients. The ability to fine-tune dopamine signaling represents an exciting area of research with the potential to offer new treatment options for Alzheimer's and related disorders.

Microglia, the resident immune cells of the central nervous system, have also emerged as central players in the pathology of many neurological diseases (Abellanas et al., 2025). Once thought to be passive supporter of neuronal health, microglia is now recognized for its active role in regulating neuroinflammation and neuronal plasticity. Recent research has uncovered significant regional heterogeneity in microglial phenotypes, suggesting that microglia exhibit distinct behaviors depending on the brain region and disease context. In this Research Topic, several studies explore regional variations and the potential therapeutic implications of modulating microglial function. For example, research by Smith et al. reveals that microglia in neurogenic regions appear to be in a "primed" state, with heightened immune responses and antigen presentation capabilities, which could influence both their role in neurogenesis and their contribution to the pathogenesis of epilepsy.

One particularly exciting development is the discovery of post-translational modifications of microglial proteins, such as lactylation, which may influence microglial behavior and contribute to disease progression. By targeting specific post-translational pathways including histone acetyltransferases (HATs) and histone deacetylases (HDACs), researchers may be able to tune microglial hyperexcitability, either enhancing their protective functions or suppressing their harmful effects (Chen and Zhu, 2024). This underscores the importance of considering the precise role of microglia in different areas of the brain when designing therapies aimed at modulating glial functions that could offer new therapeutic opportunities for treating conditions such as Alzheimer's and CNS prion disease.

Therefore, the identification of activation markers and modulators that influence microglial function opens potential avenues for precision medicine. By targeting specific microglial subtypes or activation states, it may be possible to tailor treatments to individual patients, maximizing efficacy while minimizing side

effects. Personalized approaches therefore suggest a promising future for the treatment of CNS diseases, where therapies are not one-size-fits-all but instead are designed to address the unique characteristics of each patient's disease.

In conclusion, studies highlighted in this Research Topic offer a glimpse into the future of CNS disease treatment. Through the exploration of novel intracellular pathways, the reimagining of existing therapies, and the identification of new therapeutic targets, these studies pave the way for more effective, personalized interventions. As our understanding of the molecular and cellular underpinnings of neurological diseases continues to deepen, we are better equipped to develop treatments that not only address symptoms but also target the root causes of these conditions. The journey toward personalized and precision medicine offers hope for a future where CNS diseases are no longer an insurmountable challenge, but treatable conditions with well-defined and effective therapeutic solutions.

Author contributions

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Conflict of interest

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

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MINI REVIEW
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MicroRNA-124: A Key Player in Microglia-Mediated Inflammation in Neurological Diseases

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Neurological disorders are mainly characterized by progressive neuron loss and neurological deterioration, which cause human disability and death. However, many types of neurological disorders have similar pathological mechanisms, including the neuroinflammatory response. Various microRNAs (miRs), such as miR-21, miR-124, miR-146a, and miR-132 were recently shown to affect a broad spectrum of biological functions in the central nervous system (CNS). Microglia are innate immune cells with important roles in the physiological and pathological activities of the CNS. Recently, abnormal expression of miR-124 was shown to be associated with the occurrence and development of various diseases in CNS via regulating microglia function. In addition, miR-124 is a promising biomarker and therapeutic target. Studies on the role of miR-124 in regulating microglia function involved in pathogenesis of neurological disorders at different stages will provide new ideas for the use of miR-124 as a therapeutic target for different CNS diseases.

Keywords: microRNA-124, neurological disorders, microglia, biomarker, therapeutic target

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INTRODUCTION

Progressive loss of neurons and deterioration of neurological symptoms are the pathological basis and main features of neurological disorders. Neurological disorders have diverse etiologies and progress rapidly, which can be disabling and fatal (Ayaz et al., 2019). The main causes of neurological disorders include cerebrovascular diseases (CVDs), neurodegenerative diseases (NDDs), Alzheimer's disease (AD), Parkinson's disease (PD), motor neuron disease, Huntington's disease (HD), epileptic diseases, neurological tumors, traumatic diseases, and neuro autoimmune diseases (Santalucia, 2008; Song and Suk, 2017). Although the complex mechanisms of various neurological disorders have been widely studied, their precise etiologies remain largely unknown. However, most neurological disorders share common pathophysiological mechanisms and disease characteristics, particularly activated microglia (MG)-mediated neuroinflammatory responses.

MicroRNA (miR) is a multifunctional endogenous non-coding small molecule RNA, consisting of 18–25 nucleotide sequences and an incomplete 3′-untranslated region of the target mRNA molecule complementary combination. The miRs regulate gene expression and protein synthesis at the post-transcriptional level and participate in cell proliferation, differentiation, apoptosis, and other life activities (Yao et al., 2020). Emerging studies have shown that various miRs, such as miR-21, miR-124, miR-146a, and miR-132, play key roles in neurological disorders and may be feasible therapeutic targets (Gascon et al., 2014; Devaux et al., 2016). The miR-124 is among the most abundant miRs in the mammalian nervous system, showing a much higher level in the central nervous system (CNS) than in other organs and accounting for 5–48% of the total miRNA content

in the cerebral cortex (Landgraf et al., 2007; Treiber et al., 2012). miR-124 has three immature precursor sequences, miR-124-1, miR-124-2, and miR-124-3, which are on chromosomes 8p23.1, 8q12.3, and 20q13.33, respectively (Lagos-Quintana et al., 2002). miR-124 expression first occurs during neural differentiation and reaches a peak in mature neurons (Sonntag et al., 2012). During CNS development and mature nerve formation, miR-124 can inhibit cell proliferation and promote cell differentiation, thereby regulating nerve differentiation (Makeyev et al., 2007).

Microglia are innate immune cells of the CNS and play important roles as "executors" in neuroinflammation, functioning in immune monitoring and defense (Aguzzi et al., 2013). Previous studies suggested that miR-124 can regulate the polarization state of MG and that up-regulating the expression level of miR-124 can promote the transformation of MG from the pro-inflammatory M1 type to the anti-inflammatory M2 type (Yu et al., 2017; Periyasamy et al., 2018). miR-124 expression in MG has also been shown to reduce inflammation by downregulating tumor necrosis factor α (TNF-α) and major histocompatibility complex II, as well as by reducing reactive oxygen species (Louw et al., 2016). In addition, miR-124 acts as a key regulator of MG quiescence in the CNS and as a previously unknown modulator of monocyte and macrophage activation (Ponomarev et al., 2011). Herein, we review the role of miR-124 in regulating MG function in the pathogenesis of different neurological disorders, its target genes, changes in expression levels, and the pathogenic pathways that may be involved. We also describe the potential of miR-124 as a biomarker and therapeutic target (**Table 1**) for the diagnosis and prognosis of neurological disorders.

MicroRNA-124 ROLES IN NEUROLOGICAL DISORDERS

Although recent studies have revealed initial information on miR-124-related signaling pathway mechanisms, many potential mechanisms remain to be identified. Dysregulated miR-124 expression may be involved in the occurrence and development of various neurological disorders by regulating MG function (**Figure 1**). For example, decreased miR-124 expression in the nervous system is often an important link in neurodegenerative and glioma diseases (Kanagaraj et al., 2014; Wu et al., 2018; Serpe et al., 2021). A recent review on the role of miR-124 in the pathogenesis of neurological disorders described the pathophysiological processes of neurological disorders and novel therapeutic strategies for treating neurological disorders (Han et al., 2019).

MicroRNA-124 AND CEREBROVASCULAR DISEASES

Cerebrovascular diseases can lead to death or long-term disability and is a worldwide health concern. Plasma miR-124-3p levels were found to be elevated in patients with acute ischemic stroke (AIS) (Badacz et al., 2021), and the miR-124-3p level was positively correlated with the poor prognosis of these patients based on the modified Rankin score (Rainer et al., 2016). Higher plasma miR-124-3p levels were suggested to be associated with unfavorable outcomes in patients with AIS undergoing thrombolysis, and miR-124-3p is closely related with the severity of stroke (He et al., 2019). miR-124 levels dynamically changed in patients with AIS (Ji et al., 2016), rapidly decreasing within 24 h and gradually increasing at 48 h after ischemia (Sun et al., 2019). However, the opposite conclusion was reported in several other studies. The serum miR-124 level was decreased within 24 h after stroke onset and was negatively correlated with high-sensitivity C-reactive protein levels and the infarct volume; the authors hypothesized that serum miR-124 was suppressed in AIS, thus facilitating neuroinflammation and brain injury (Liu et al., 2015). Intracerebral hemorrhage (ICH) accounts for 15% of stroke cases, showing a mortality rate as high as 50% within the first month of ICH onset, with half of these deaths occurring within 48 h. Thus, rapid diagnosis and timely treatment are important for patients with ICH (Broderick, 1993; Burke and Venketasubramanian, 2006). In a previous study, the average plasma miR-124 concentrations increased by more than 100-fold in 24 h, and subsequently decreased on days 2, 7, and 14, reaching nearly normal levels on day 30 (Wang Z. et al., 2018). miR-124-3p levels were investigated as diagnostic biomarkers of acute stroke; plasma miR-124-3p levels were higher in patients with ICH than in those with AIS within 24 h of symptom onset. Therefore, the miR-124-3p level was suggested as a diagnostic biomarker for discriminating ICH from AIS (Leung et al., 2014). In other studies, miR-124 decreased immediately after carotid arterial injury but dramatically increased at days 7 and 14 after injury. Thus, miR-124 was proposed as a novel regulator of vascular smooth muscle cell proliferation and involved in the development of neointimal proliferation (Choe et al., 2017).

Additional studies in animal models have been performed to determine the role of miR-124 in the pathophysiological mechanism of CVD (Volný et al., 2015). The miR-124 expression level was significantly decreased in cerebral infarction mice group compared to that in the sham group; miR-124 exerted neuroprotective and anti-inflammatory effects in mice with cerebral infarction by shifting the polarization of MG/macrophages into the beneficial, anti-inflammatory M2 type MG (Hamzei Taj et al., 2016). Early miR-124 injection significantly increases neuronal survival and the number of M2-like polarized MG. Furthermore, the stroke lesion core was significantly reduced over time following early miR-124 injection. These neuroprotective and anti-inflammatory effects of early miR-124 treatment were pronounced during the first week with Arg-1 (a marker for M2 phenotype), indicating miR-124 as a novel therapeutic agent for neuroprotection after stroke (Hamzei Taj et al., 2016).

Glial scars present a major obstacle to neuronal regeneration after ischemic stroke. Knockdown of miR-124 in M2 type MG small extracellular vesicles inhibited glial scar formation and promoted stroke recovery (Li et al., 2021). MG contributed to the inflammatory response and exacerbated ICH-induced secondary injury in mice. miR-124 overexpression decreased pro-inflammatory cytokine levels, indicating that this miRNA ameliorates ICH-induced inflammatory injury by modulating

TABLE 1 | Circulating miR-124 expression as circulating biomarker in neurological disorders.

Neurological disorders	Disease model	Sample	miRNA expression change	Target genes/related pathway/function	References
Acute ischemic stroke, AIS	Patient	Plasma	miR-124-3p, increased	AIS diagnosis	Rainer et al., 2016
Acute ischemic stroke, AIS	Patient	Plasma	miR-124-3p, increased	AIS patient severity and prognosis	He et al., 2019
Acute ischemic stroke, AIS	Patient	Plasma	miR-124, decreased within 24 h	AIS diagnosis	Sun et al., 2019
Acute ischemic stroke, AIS	Patient	Serum	miR-124, decreased within 24 h	AIS diagnosis, predicting infarction volume	Liu et al., 2015
Intracerebral hemorrhage, ICH	Patient	Plasma	miR-124, increased within 24 h	ICH diagnosis	Wang Z. et al., 2018
Stroke	Patient	Plasma	miR-124-3p, increased	ICH and AIS discrimination	Leung et al., 2014
Parkinson's disease, PD	Patient	Plasma	miR-124, decreased	PD diagnosis	Li et al., 2017
Parkinson's disease, PD	Patient	Plasma	miR-124-3p, decreased	PD diagnosis	Ravanidis et al., 2020
Multiple sclerosis, MS	Patient	Monocytes	miR-124, decreased	Progressive MS diagnosis	Amoruso et al., 2020
Traumatic brain injury, TBI	Patient	Plasma	miR-124-3p, increased	TBI diagnosis	Vuokila et al., 2020
Traumatic brain injury, TBI	Patient	Plasma	miR-124-3p, increased	Evaluating TBI severity	Schindler et al., 2020
Traumatic brain injury, TBI	Patient	Serum	miR-124-3p, increased	TBI diagnosis	O'connell et al., 2020

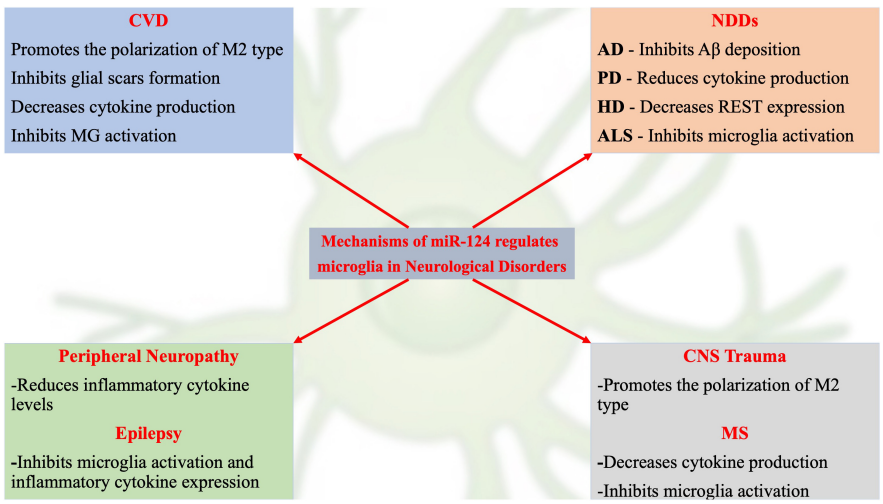

FIGURE 1 | Schematic representation of current hot topics and emerging mechanisms of miR-124 in regulating microglia in neurological disorders.

MG polarization toward the M2 phenotype (Yu et al., 2017). In an experimental rat subarachnoid hemorrhage model, Chen et al. demonstrated that delivery of exosomal miR-124 from neurons to MG was significantly reduced, accompanied by increased C/EBPα expression, which was inhibited by CX3CL1/CX3CR1 overexpression; thus, the CX3CL1/CX3CR1 axis may exert protective roles in subarachnoid hemorrhage by promoting the delivery of exosomal miR-124 to MG and inhibiting MG activation as well as the neuroinflammatory response (Chen et al., 2020).

Based on the above reports, although the circulating miR-124 expression level was not consistent among different CVD studies, in patients with AIS, the miR-124 level was elevated in most studies and associated with the poor prognosis of patients. miR-124 upregulation improved the prognosis of patients with CVD and reduced nerve cell injury through various mechanisms, such as the anti-inflammatory response, thereby playing a role in repairing the damaged CNS. However, the role of miR-124 as a protective agent for CVD by regulating miR-124 expression levels and the most effective method for miR-124 application requires further exploration.

MicroRNA-124 AND NEURODEGENERATIVE DISEASES

Alzheimer's Disease

Alzheimer's disease is a progressive NDD with an insidious onset. Clinically, AD is characterized by general dementia such as memory impairment, aphasia, apraxia, agnosia, visual space impairment, executive dysfunction, and personality and behavioral changes (Uddin et al., 2018). miR-124 is involved in the pathogenesis of AD through various mechanisms including MG activation and neuroinflammation (Bahlakeh et al., 2021).

Beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) is a critical enzyme that regulates the production of amyloid-β (Aβ), which is abnormally upregulated in AD (Peng et al., 2017). Upregulated MG exosomes miR-124-3p (exo-miR-124-3p) alleviated neurodegeneration in repetitive scratch-injured neurons *in vitro*. Furthermore, miR-124-3p targets Rela (an inhibitory transcription factor of ApoE), which may promote Aβ proteolytic breakdown and inhibit Aβ abnormalities (Ge et al., 2020). miR-124 is the critical regulatory factor in the hypoxia/Aβ-miR-124-BACE1-Aβ cycle. Additionally, activation of the EPAC-Rap1 pathway was shown to be involved in inhibiting miR-124 in the hippocampus under hypoxia or Aβ insult (Zhang et al., 2017). miR-124 expression was downregulated with aging and showed a decreased ability to regulate ApoE-dependent Aβ uptake by targeting regulatory factor X1 transcripts on BV2 MG.

Tau hyperphosphorylation forms neurofibrillary tangles, a crucial event in the pathogenesis of AD. miR-124-3p was shown to inhibit abnormal tau hyperphosphorylation by regulating the caveolin-1-PI3K/Akt/GSK3β pathway in AD (Kang et al., 2017). Abnormal activation of cyclin-dependent kinase-5 (CDK5) is involved in controlling hyperphosphorylation, which is mediated by calpain-induced cleavage of p35 to p25. miR-124-3p is a calpain-targeting miR which may inhibit the protein translation of calpain. An inverse correlation was found between miR-124-3p and calpain levels in AD specimens. Furthermore, miR-124-3p overexpression significantly reduced Aβ deposition and improved AD mouse behavior through posttranscriptional control of calpain (Zhou et al., 2019).

Synaptic loss is an early pathological event in AD. Although the underlying molecular mechanism is unclear, miRNAs have been suggested as important regulators of synaptic function and memory (Selkoe, 2002). Protein tyrosine phosphatase non-receptor type 1 (PTPN1) reduction dramatically induces synaptic impairments and memory decline. Wang et al. demonstrated the PTPN1 is a direct target of miR-124, and the miR-124/PTPN1 pathway is involved in synaptic dysfunction and memory loss in AD (Wang X. et al., 2018).

Based on these studies, miR-124 is involved in the pathogenesis of AD by promoting Aβ production, enhancing tau phosphorylation, impairing synaptic function, and influencing the MG activation state. Upregulated miR-124 protects patients with AD from pathological damage by inhibiting Aβ production and reducing tau phosphorylation and synaptic loss, providing a potential strategy for treating AD.

Parkinson's Disease

Parkinson's disease is a common NDD with a prevalence rate of approximately 1.7% among people over 65 years of age in China. Most PD cases are sporadic and less than 10% have a family history. The leading pathological change in PD is the degeneration of DA neurons in the midbrain, which leads to a significant reduction in the striatum DA. The exact etiology of this pathological change remains unclear. The miR-124 level was found to be decreased in the plasma of patients with PD, with an area under the receiver operating characteristic curve of miR-124 of 0.709 (95% CI 0.618–0.633, $p < 0.05$), indicating that miR-124 is associated with PD and a potential biomarker for PD diagnosis (Li et al., 2017). In addition, plasma miR-124-3p levels were decreased in patients with PD compared to those in healthy controls (Ravanidis et al., 2020).

miR-124 is involved in the pathogenesis of PD mainly through neuroinflammation and the regulation of autophagy (Angelopoulou et al., 2019). In a previous study, miR-124 was significantly downregulated in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced mouse model of PD and suggested to inhibit neuroinflammation during PD development (Slota and Booth, 2019). The expression of sequestosome-1 (p62) and phospho-p38 (p-p38) mitogen-activated protein kinase was significantly increased in lipopolysaccharide-treated immortalized murine BV2 cells in an MPTP-induced mouse model of PD. Furthermore, exogenous delivery of miR-124 suppressed p62 and p-p38 expression and attenuated the activation of MG in MPTP-treated mice, indicating that miR-124 inhibits neuroinflammation during PD development by targeting p62, p38, and autophagy (Yao et al., 2019). miR-124-3p expression was decreased in MPP^+-induced SH-SY5Y cells. miR-124-3p overexpression exerted protective effects by increasing cell viability and reducing cell apoptosis, caspase-3 activity, inflammatory factors TNF-α, and IL-1β levels as well as by attenuating MPP^+-induced neuronal injury. In addition, miR-124-3p targets STAT3, mediating the neuroprotective effect (Geng et al., 2017). Mitogen-activated protein kinase 3 (MEKK3) expression was increased in an MPTP-induced mouse model of PD, thus promoting the activation of MG by regulating nuclear factor (NF)-κB expression. Exogenous delivery of miR-124 inhibited MEKK3 and p-p65 expression and attenuated the activation of MG in the substantia nigra pars compacta of MPTP-treated mice, indicating miR-124 can inhibit neuroinflammation during the development of PD by regulating the MEKK3/NF-κB signaling pathways (Yao et al., 2018).

miR-124 may be involved in the pathogenesis of PD through other mechanisms. miR-124-3p expression was downregulated in 6-hydroxydopamine (6-OHDA)-treated PC12 and SH-SY5Y cells. Direct targeting of annexinA5 by miR-124-3p enhanced the viability of 6-OHDA-treated PC12 or SH-SY5Y cells, which was associated with stimulation of the extracellular signal-regulated kinase pathway (Dong et al., 2018). A calpain-p25-mediated increase in cdk5 expression leads to dopaminergic neuronal death in human PD and MPTP-PD models. Additionally, an interaction between miR-124 with calpain 1 was identified in experiments using miR-124 target protector sequences; miR-124 overexpression attenuated the expression of calpain 1/p25/cdk5 proteins and improved cell survival. Furthermore, miR-124 was suggested to regulate the expression of calpain 1/p25/cdk5/pathway proteins in dopaminergic neurons (Kanagaraj et al., 2014). miR-124 also affected dopamine receptor expression, neuronal proliferation, and apoptosis in MPTP-induced mouse models of PD. Endothelin 2 has been identified as a target of miR-124; miR-124 overexpression promoted dopamine receptor expression and neuronal proliferation, and suppressed neuronal apoptosis by downregulating endothelin 2 *via* activation of the Hedgehog signaling pathway (Wang et al., 2019). Bim is a

BH3-only protein shown to be involved in apoptosis of DA neurons in an MPTP model of PD, in addition to being a direct target of miR-124. Upregulation of miR-124 significantly reduced the loss of DA neurons in MPTP-treated mice by regulating Bim expression (Wang H. et al., 2016).

Furthermore, regulation of miR-124 expression is a promising strategy for PD treatment. In a previous study, miR-124 loaded nanoparticles (NPs) enhanced brain repair in PD. Intracerebral administration of miR-124 NPs increased the number of migrating neuroblasts and induced the migration of neurons into the lesioned striatum of 6-OHDA-treated mice. Thus, miR-124 NPs were considered as a potential new therapeutic approach for promoting endogenous brain repair mechanisms in NDDs (Saraiva et al., 2016b). In addition, miR-124 NPs were suggested to target both the MEKK3 and NF-κB pathways, and reduce inflammatory cytokine levels (Gan et al., 2019).

In general, the expression of miR-124 is down-regulated in various PD models, miR-124 serves as a protective factor in DA neurons in PD, and up-regulation of miR-124 can protect DA neurons in PD from injury via multiple mechanisms, including by inhibiting MG activation. Therefore, miR-125 may have a therapeutic role in PD.

Huntington's Disease

Huntington's disease is an autosomal dominant genetic disease characterized by degenerative changes to the nervous system. Clinically, HD mainly manifests as dance-like movement, progressive cognitive decline, and mental symptoms. Abnormal amplification of the CAG repeat sequence at codon 17 downstream of the IT15 gene initiation codon is the main cause of the disease. In this NDD, altered miR-124 expression can lead to abnormal gene regulation. In HD striatal mutant STHdh (Q111)/Hdh (Q111) cells, miR-124 expression was shown to be downregulated. Cyclin A2 (CCNA2) was identified a target gene of miR-124, and increased miR-124 expression in R6/2 mice altered CCNA2 expression and the proportion of cells in S phase in the HD cell model, indicating that downregulation of miR-124 expression increased CCNA2 expression in HD and was involved in deregulation of the cell cycle in STHdh (Q111)/Hdh (Q111) cells (Das et al., 2013). In contrast, miR-124 overexpression exhibited therapeutic effects in an HD model. Exosome-based delivery of miR-124 to the striatum of R6/2 transgenic HD mice reduced REST expression. Although the treatment did not produce significant behavioral improvement, it offered possible therapeutic strategies from a pathophysiological perspective (Lee et al., 2017). In summary, based on current limited evidence, miR-124 appears to play a protective role in the progression of HD.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS), the most common type of motor neuron disease, is a lethal NDD, the pathogenesis of which is not completely understood. The lesion involves both upper and lower motor neurons and is characterized by progressive limb weakness and muscle atrophy with pyramidal tract signs. Between 5% and 10% of patients with ALS have a family history; however, the number of discovered genes clearly associated with ALS remains very limited (Petrov et al., 2017). Protein misfolding and Cu/Zn superoxide dismutase 1 mutations are associated with inflammatory and neurotoxic pathways in ALS (Fan et al., 2020).

miR-124 expression was downregulated in the spinal cord and brainstem and upregulated in differentiated neural stem cells in G93A-superoxide dismutase 1 mice. Sox2 and Sox9 were identified as target genes of miR-124 and their protein levels showed opposite changes with miR-124 expression *in vivo* and *in vitro*. Thus, miR-124 was suggested to play an important role in astrocytic differentiation by targeting Sox2 and Sox9 in ALS transgenic mice (Zhou et al., 2018). Dysregulation of certain miRs can contribute to MG hyperactivation, persistent neuroinflammation, and abnormal macrophage polarization in the brain (Guo et al., 2019). Increased miR-124 expression caused persistent activation of NF-κB and matrix metalloproteinases 2 and 9, as well as upregulation of major histocompatibility complex-II, TNF-α, IL-1β, and inducible nitric oxide synthase gene expression, indicating induced M1 polarization. The data indicated that modulation of the inflammatory-associated miR-124 could determine early and late phenotypic alterations in the recipient N9-MG, providing a promising therapeutic approach for inhibiting MG activation and its associated effects in motor neuron degeneration (Pinto et al., 2017). In addition, MG-associated inflammatory biomarkers, such as NF-κB/Nlrp3-inflammasome and pro-inflammatory cytokines, were increased during the symptomatic stage of ALS, accompanied by upregulated miR-124 expression. These results highlight the role of miR-124 in activating MG and provide candidate miRs that may exert potential neuroprotective strategies in ALS therapy (Cunha et al., 2018).

MicroRNA-124 AND MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is an immune-mediated chronic inflammatory demyelinating disease of the CNS. Abnormal activation of cellular and humoral immunity eventually leads to loss of the myelin sheath in the CNS and damage to oligodendrocytes, some of which may involve axons and neurons (Musella et al., 2018). miR-124 was significantly increased in demyelinated hippocampi and cortices from postmortem MS brains and inversely correlated with memory performance (Dutta et al., 2013). However, miR-124 was significantly downregulated in monocytes from patients with progressive MS, indicating the complete loss of homeostatic monocyte function during the progressive phase of the disease (Amoruso et al., 2020).

Emerging evidence has indicated that neuroinflammation is an important contributor to MS and that miR-124 is involved in the pathogenesis of MS as a neuroinflammation regulator. Resveratrol shows potential as an effective therapeutic agent in experimental autoimmune encephalomyelitis, a murine model of MS. Resveratrol effectively decreased MS severity, including inflammation and CNS immune cell infiltration. Further investigations of the therapeutic mechanism showed that resveratrol could upregulate miR-124 expression and then suppress sphingosine kinase 1 expression (associated target gene of miR-124), indicating that upregulation of miR-124 can

suppress neuroinflammation and halt cell-cycle progression in activated encephalitogenic T cells (Gandy et al., 2019).

MicroRNA-124 AND PERIPHERAL NEUROPATHY

Neuropathic pain is caused by somatosensory dysfunction of the peripheral system and CNS and is associated with spontaneous pain, such as sensory disorders, paresthesia, hyperalgesia, and hypersensitivity. Although significant progress has been made in the pathogenesis and treatment of neuropathic pain, therapeutic results remain unsatisfactory (Scholz and Woolf, 2007; Van Hecke et al., 2014). Dysregulated expression of miRs plays key roles in neuropathic pain development. In a chemotherapy-induced peripheral neuropathic animal model, circulating miR-124 levels were increased, which correlated with axonal degeneration in both the dorsal root ganglion and sciatic nerve. Although these results cannot yet be applied in clinical practice, they provide positive evidence that circulating miR-124 can be used as a diagnostic biomarker for the early diagnosis of peripheral neuropathy (Peng et al., 2019). In the chronic constriction injury model of neuropathic pain in rats, miR-124 was upregulated; however, modulation of miRs did not appear to significantly contribute to changes in gene expression in the spinal cord in this chronic neuropathic pain model (Brandenburger et al., 2012).

miR-124-3p was dramatically downregulated in rats after chronic sciatic nerve injury; in contrast, miR-124-3p overexpression reduced the levels of inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and TNF-α, and inhibited mechanical allodynia and heat hyperalgesia. In addition, EZH2 was identified as a target of miR-124-3p. Therefore, miR-124-3p may promote neuroinflammation and neuropathic pain by targeting EZH2 as a promising therapeutic strategy for neuropathic pain (Zhang et al., 2019). Furthermore, miR-124 effectively reversed established hyperalgesia in morphine-induced persistent sensitization models. The mechanism may be associated with targeting of Toll-like receptor signals, highlighting the therapeutic potential of miR-124 in treating peripheral neuropathy (Grace et al., 2018).

MicroRNA-124 AND EPILEPSY

Epilepsy is a chronic neurological disorder characterized by recurrent seizures and caused by abnormal and synchronized firing of neurons in the brain (Alves et al., 2019). miR-124 expression was downregulated in epileptic patients and drug-induced epileptic rats. In two drug-induced rat epilepsy models, intra-hippocampal administration of miR-124 reduced the severity of epilepsy and prolonged the incubation period. cAMP-response element-binding protein1 (CREB1) was identified as a target gene of miR-124, and miR-124 overexpression repressed CREB1 expression, which is a key regulator in epileptogenesis, indicating miR-124 is involved in the pathogenesis of epilepsy by regulating CREB1 expression (Wang W. et al., 2016). miR-124 expression is dynamic during the three stages of mesial temporal lobe epilepsy (MTLE) development. miR-124 was significantly upregulated in hippocampal tissues in the acute and chronic stages of MTLE but nearly returned to normal in the latent stage, indicating that this miRNA can be used as a biomarker for MTLE diagnosis and therapeutic target for anticonvulsant drugs (Peng et al., 2013).

miR-124 expression was found to be low in refractory epilepsy rats, and upregulation of the miR-124 level increased the seizure interval, improved the cognitive function of rats, and promoted PI3K and AKT expression. Thus, miR-124 may play a protective role in temporal lobe epilepsy by promoting PI3K/Akt signaling pathway (Wang R. et al., 2020). miR-124 expression was also downregulated in status epilepticus rats. miR-124 upregulation reduced neuron-restrictive silencer factor expression and inhibited MG activation and inflammatory cytokine expression (Brennan et al., 2016).

Most studies support that miR-124 show low expression in epilepsy models, particularly in the acute phase of epilepsy or status epilepticus models. Upregulating the expression of miR-124 can protect against neuronal impairment in epilepsy, and studies have suggested that the protective effect of miR-124 can be achieved by inhibiting the inflammatory response in the CNS.

MicroRNA-124 AND CENTRAL NERVOUS SYSTEM TRAUMA

Traumatic Brain Injury

Traumatic brain injury (TBI) is defined as traumatic structural injury and/or brain dysfunction caused by external forces and is clinically characterized by a loss of consciousness, memory loss, altered mental state, neurological dysfunction, and intracranial damage (Ruff et al., 2009). miR-124-3p levels were elevated at 2 days post-TBI in both the blood and plasma of patients; an elevated plasma miR-124-3p level 2 days post-TBI was positively correlated with larger lesion area at the chronic time point in TBI models (Vuokila et al., 2020). Furthermore, miR-124-3p was detected only in patients with severe TBI (Schindler et al., 2020). The miR-124-3p level was elevated in the serum of patients with TBI (O'connell et al., 2020). However, miR-124-3p was downregulated in post-TBI hippocampal pathologies in experimental models and in humans (Vuokila et al., 2018).

Neuroinflammation is the characteristic pathological change occurring during acute nerve injury after TBI. Inhibiting the excessive inflammatory response is crucial for improving the prognosis of the nervous system. miR-124-3p was shown to promote anti-inflamed M2 polarization in MG, and an increase in the level of miR-124-3p in MG exosomes after TBI inhibited neuroinflammation and contributed to neurite outgrowth by facilitating their transfer into neurons. miR-124-3p exerted these effects by targeting PDE4B, thus inhibiting mTOR signaling activity (Huang et al., 2018). In addition, exo-miR-124 treatment was suggested to promote M2 polarization of MG and improve hippocampal neurogenesis and functional recovery after TBI (Yang et al., 2019). MG exo-miR-124-3p may also

inhibit neuronal autophagy and protect against nerve injury by facilitating their transfer into neurons for the treatment of nerve injury after TBI (Li et al., 2019).

Spinal Cord Injury

Spinal cord injury (SCI) is a highly disabling injury to the CNS caused by trauma. Destruction of the spinal cord (SC) structure can lead to an inflammatory response, immune injury, and other mechanisms in the damaged SC tissue, resulting in spinal cord dysfunction. miR-124 expression in neurons was shown to be significantly decreased within 7 days after SCI and may reflect the severity of SCI (Zhao et al., 2015).

miR-124 was decreased in a rat model of SCI. GTP-cyclohydrolase 1, a target gene of miR-124, plays an important role in SCI-induced neuronal apoptosis. miR-124 overexpression inhibited neuronal apoptosis in SCI by modulating GTP-cyclohydrolase 1 expression (Yuan et al., 2019). Tal1 is also a potential target gene of miR-124, and its downregulation promoted the proliferation of neuronal precursor cells and inhibited their differentiation, indicating miR-124 can mediate SCI repair by altering the expression of various mRNAs in rats (Wang J. et al., 2020). miR-124 was notably downregulated in SCI rats; miR-124 overexpression improved functional recovery and decreased the lesion size in SCI rats. BAX, an apoptosis regulator, is a target of miR-124; thus, miR-124 suppressed neuronal cell apoptosis in an SCI rat model by inhibiting BAX expression (Xu et al., 2019). miR-124 was shown to target pyridoxal kinase to accelerate the differentiation of bone marrow mesenchymal stem cells into neurocytes and promote SCI repair (Song et al., 2017). Exosomal miR-124-3p (exo-miR-124-3p) derived from bone marrow mesenchymal stem cells attenuated nerve injury induced by SC ischemia-reperfusion injury by regulating Ern1 and M2 macrophage polarization (Li et al., 2020). In addition, PI3K/AKT/NF-κB signaling cascades were involved in modulating MG via exo-miR-124-3p (Jiang et al., 2020).

Despite these different conclusions, the miR-124 expression level in the peripheral blood of patients with SCI showed an increasing trend and was positively correlated with the severity of SCI. In animal models, miR-124 expression was mainly downregulated, and miR-124 overexpression may play a protective role by inhibiting inflammation and neuronal apoptosis and by promoting nerve regeneration.

DISCUSSION AND PERSPECTIVES

Herein, we reviewed the role of miR-124 in neurological disorders and its mechanism in regulating pathophysiological processes. miR-124 is involved in the pathogenesis of neurological disorders through various mechanisms, mainly post-transcriptional regulation of gene expression, glial cell activation, and neuroinflammatory response. The feasibility of using miR-124 as a circulating biomarker and therapeutic target in the diagnosis of neurological disorders was also reviewed.

miR-124 is highly conserved and is among the most abundant miRs specifically expressed in the CNS. In addition, miR-124 is closely associated with nervous system development, injury, and repair (Han et al., 2019). Changes in miR-124 expression levels may play a key role in the occurrence of various neurological disorders. A review of previous studies revealed the extensive roles of miR-124 in the pathology of these disorders. Notably, miR-124 expression is downregulated during the acute phase of most neurological disorders, such as CVD, NDDs, neurological tumors, and TBI. However, this expression level changes dynamically. For example, the level is significantly downregulated in the early onset of AIS and, as the disease develops, it gradually returns to normal. Although the mechanism of changes in the miR-124 expression level is unclear, most studies revealed that a normal miR-124 level has a protective effect on the nervous system. Low miR-124 expression is correlated with the occurrence of neurological disorders, and miR-124 overexpression can protect neurons or have a therapeutic effect on diseases. miR-124 is abundantly expressed in the CNS and involved in the pathogenesis of various neurological disorders. The main function of miR-124 is regulating gene expression at the post-transcriptional level. miR-124 directly regulates many key pathogenic genes of the nervous system, such as the target gene BACE1, a critical enzyme that controls the production of Aβ. In addition, miR-124 is involved in activating MG in many neurological disorders. For example, miR-124 is significantly increased in M2-polarized MG in CVD and TBI and promotes the activation of MG by regulating NF-κB expression in PD. Furthermore, miR-124 participates in various neuroinflammation reactions; miR-124 inhibits neuroinflammation during the development of PD by regulating the MEKK3/NF-κB signaling pathways, suppressing neuroinflammation, and halting cell-cycle progression in activated encephalitogenic T cells in MS. Understanding the involvement of miR-124 in neurological disorders may facilitate the clinical application of miR-124 as a diagnostic biomarker and therapeutic target.

Changes in peripheral blood miR-124 levels may become a biomarker of neurological disorders. The miR-124-3p level was significantly increased in AIS patients and associated with poor prognosis. In addition, the miR-124-3p level in patients with ICH was higher than in patients with AIS, indicating that the level of this miRNA has diagnostic value for CVDs and can be used to distinguish between ischemic and hemorrhagic CVDs. Among NDDs, the miR-124 levels in the plasma of patients with PD were significantly lower than those in healthy controls. Furthermore, the miR-124 expression level in the monocytes of patients with MS was significantly decreased; this decline was also observed in patients with epilepsy. Although the expression level differs from that in some animal models, miR-124 expression in human patients may better reflect the actual clinical situation. Considering the wide and dynamic changes in miR-124 in neurological disorders, further clinical data are needed to determine its usefulness as a biomarker, and the specificity of miR-124 should be quantified in different neurological disorders. Furthermore, changes in the miR-124 expression level in the peripheral circulation differ from those in the brain tissue even in the same neurological disorders, as expected. The blood-brain barrier creates very different biochemical conditions between the brain and blood. Different tissue-specific miR-124 levels may be

caused by damage to the blood-brain barrier. Changes in miR-124 levels in the brain may be useful for targeted treatment.

The results of mechanistic studies showed that miR-124 exerted a protective effect on the nervous system *via* various mechanisms such as post-transcriptional regulation of gene expression, regulation of glial cell transformation to an anti-inflammatory phenotype, and inhibition of the neuroinflammatory response. However, miR-124 has several potential side effects. Since miR-124 has multiple target genes, further research is needed to confirm whether miR-124 interacts with other genes to produce unpredictable effects while exerting its therapeutic activities.

Virus-based delivery systems are highly efficient and can effectively deliver miRNAs to target cells. Intracranial injection of adeno-associated virus expressing miR-124-3p into AD mice significantly reduced Aβ deposition and improved the cognitive function of AD mice (Yao et al., 2019). Non-viral delivery systems are widely used in clinical studies because of their variety and relative safety (Yin et al., 2014). miR-124-loaded polymeric NPs were constructed to treat a 6-OHDA-challenged mouse model of PD, resulting in improved motor function (Saraiva et al., 2016a). Exosomes exhibit a high level of miR-124 expression (exosome-miR-124) which decreased REST target gene expression in R6/2 transgenic HD mice; this gene is involved in multiple links in the pathogenesis of HD (Lee et al., 2017). Although the *in vivo* delivery of miRs by adeno-associated virus, NPs, and exosomes has been successfully tested in various animal models, further *in vivo* experiments are needed before clinical application.

CONCLUSION

miR-124 plays an important role in the occurrence and development of various neurological disorders and may be useful as a biomarker for the diagnosis and prognosis of these disorders. However, additional human studies are needed to verify its clinical application prospects. Understanding the functional role of miR-124 in regulating pathological mechanisms and other regulatory pathways in different nerve injuries, cell types, and disease stages will facilitate the use of miR-124 as a therapeutic target for neurological disorders.

AUTHOR CONTRIBUTIONS

JZ and JW performed the literature search. JZ drafted the manuscript. JW critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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Innate Immune Tolerance in Microglia Does Not Impact on Central Nervous System Prion Disease

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Prion diseases such as Creutzfeldt-Jakob disease in humans, bovine spongiform encephalopathy in cattle, and scrapie in sheep, are infectious and chronic neurodegenerative diseases to which there are no cures. Infection with prions in the central nervous system (CNS) ultimately causes extensive neurodegeneration, and this is accompanied by prominent microglial and astrocytic activation in affected regions. The microglia are the CNS macrophages and help maintain neuronal homeostasis, clear dead or dying cells and provide defense against pathogens. The microglia also provide neuroprotection during CNS prion disease, but their pro-inflammatory activation may exacerbate the development of the neuropathology. Innate immune tolerance induced by consecutive systemic bacterial lipopolysaccharide (LPS) treatment can induce long-term epigenetic changes in the microglia in the brain that several months later can dampen their responsiveness to subsequent LPS treatment and impede the development of neuritic damage in a transgenic mouse model of Alzheimer's disease-like pathology. We therefore reasoned that innate immune tolerance in microglia might similarly impede the subsequent development of CNS prion disease. To test this hypothesis groups of mice were first infected with prions by intracerebral injection, and 35 days later given four consecutive systemic injections with LPS to induce innate immune tolerance. Our data show that consecutive systemic LPS treatment did not affect the subsequent development of CNS prion disease. Our data suggests innate immune tolerance in microglia does not influence the subsequent onset of prion disease-induced neuropathology in mice, despite previously published evidence of this effect in an Alzheimer's disease mouse model.

Keywords: microglia, prion disease, central nervous system, lipopolysaccharide, innate immune tolerance

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INTRODUCTION

Prion diseases (also known as transmissible spongiform encephalopathies) are sub-acute neurodegenerative diseases that affect humans and certain captive and free-ranging mammals including ovine, bovine, and cervid species. Infectious prions are considered to comprise of PrP^{Sc}, abnormally folded isoforms of the host-encoded cellular prion protein, PrP^{C} (Legname et al., 2004). High levels of PrP^{Sc} accumulate in affected tissues during prion disease. Once the prions infect the central nervous system (CNS), they ultimately cause extensive neurodegeneration and vacuolar

(spongiform) degeneration as well as microglial and astrocytic activation. Currently, no cures are available to treat prion-infected individuals.

The microglia are the parenchymal macrophages of the CNS and are considered to have essential roles in CNS development and homeostasis (reviewed in Prinz et al., 2019). During CNS prion disease the microglia appear to play a neuroprotective role and constrain the neurotoxic activation of reactive astrocytes (Zhu et al., 2016; Carroll et al., 2018; Bradford et al., 2021). However, the disease-associated activation of microglia can contribute to the development of neuropathology in some CNS disorders (Keren-Shaul et al., 2017), including prion diseases (Alibhai et al., 2016; Hu et al., 2021).

In the steady state, the continual production of factors derived from the commensal gut and lung microbiome plays an important role in maintaining microglial homeostasis and function (Erny et al., 2015; Hosang et al., 2022). However, ablation or modification of the commensal microbiomes at these sites can impede microglial responses to LPS treatment, infection with lymphocytic choriomeningitis virus (Erny et al., 2015), or autoimmunity in the CNS (experimental allergic encephalitis; Hosang et al., 2022). Studies have also shown how systemic inflammation or pathogen co-infection can modify the development of certain neurodegenerative disorders (Mabbott et al., 2020). In Alzheimer's disease patients, for example, systemic inflammation coincides with increased cognitive decline (Holmes et al., 2009) and alterations to the gut microbiome might enhance the progression of the neuropathology (Xie et al., 2022). Gastrointestinal infections might similarly increase the risk of Parkinson's disease (Nerius et al., 2020). Experimental studies have also shown how systemic LPS-mediated inflammation or gastrointestinal infections can exacerbate the progression of the clinical signs or the neuropathology in the brains of mice infected with prions (Combrinck et al., 2002; Cunningham et al., 2005; Donaldson et al., 2020) or ischemic brain damage (Denes et al., 2010).

In many of the above examples, the treatments or infections were initiated when detectable signs of neuropathology were already evident in the CNS. However, studies have shown how consecutive, systemic, LPS treatments can induce epigenetic changes in the microglia in the CNS that can persist in these cells for at least 6 months after their initial application (Wendeln et al., 2018). This epigenetic reprogramming induced innate immune tolerance in the microglia that rendered them hypo-responsive to subsequent stimulation. As a consequence, consecutive LPS treatment caused long-term changes in the microglia that months later were sufficient to alter the development of pathology in an experimental mouse model of stroke (focal cortical ischemia) and impede the subsequent development of neuropathology in a spontaneous transgenic mouse model of Alzheimer's disease-like pathology (Wendeln et al., 2018). Other studies have shown how similar systemic stimuli (Zhou et al., 2020), a high-fat diet (Vargas-Rodríguez et al., 2022), and early life thermal stress (Ben-Nun et al., 2022) can cause long-term changes in microglial responses that can impede their responses to subsequent pro-inflammatory stimuli. The immune tolerance induced in the microglia to these repeated stimuli may be a neuroprotective response to protect the CNS from repetitive or subsequent pro-inflammatory insults (Lajqi et al., 2020; Zhou et al., 2020). Conversely, sepsis was shown to induce innate immune training in microglia that increased susceptibility to amyloid-β induced neuropathology (De Sousa et al., 2021). Although effects on microglia were not determined, mild infection with the SARS-CoV-2 coronavirus has also been shown to cause the persistent pro-inflammatory activation of monocytes and macrophages that may have long-term effects on subsequent immune responses (Bohnacker et al., 2022).

The impact of innate immune tolerance in microglia on the subsequent development of CNS prion disease was not known. Therefore, in the current study, we used a mouse model of CNS prion disease to determine whether exposure to consecutive, systemic, LPS treatments soon after prion infection would cause similar long-term changes in the microglia that months later could modulate the development of the neuropathology. A thorough understanding of the factors that modulate microglial phenotype and function during CNS prion disease may identify novel targets for therapeutic intervention in these currently untreatable neurodegenerative disorders.

MATERIALS AND METHODS

Mice

Female C57BL/6J mice were purchased from Charles River (Margate, United Kingdom) and used throughout this study. The mice were maintained in-house under specific pathogen-free conditions and used in experiments at 6–10 weeks old. All *in vivo* mouse studies were performed under the authority of a UK Home Office Project License in accordance with the regulations of the UK Home Office "Animals (scientific procedures) Act 1986." Approval for the individual studies was obtained after review from the University of Edinburgh's ethical review committee.

In vivo Treatment With Bacterial Lipopolysaccharide

At the times indicated mice were given daily intraperitoneal (IP) injections with bacterial LPS derived from *Salmonella enterica* serotype Typhimurium (Sigma-Aldrich, Poole, Dorset, United Kingdom) at 500 μg/kg body weight in sterile PBS. Parallel groups of mice were injected IP with 50 μL sterile PBS as control.

Prion Infection and Clinical Disease Assessment

Groups of female C57BL/6J mice (5–6 mice/group) were injected intracerebrally (IC) into the right medial temporal lobe with 20 μL of a 1% (weight/volume) brain homogenate prepared from mice terminally infected with mouse-passaged ME7 scrapie prions. Following injection with prions, the mice were coded and assessed blindly at daily intervals for the clinical signs of prion disease. The mice were culled at a standard humane end-point upon the development of terminal clinical signs of prion disease, as described previously (Donaldson et al., 2020). Brains were

removed and cut in half sagittally across the midline to separate the 2 hemispheres. One brain half was immediately flash frozen at the temperature of liquid nitrogen for gene expression or protein analysis. The other brain half was fixed in 10% neutral buffered formalin for at least 48 h prior to histopathological processing.

Clinical prion disease was confirmed by histopathological assessment of the abundance and distribution of the spongiform vacuolar degeneration in paraffin-embedded, hematoxylin and eosin (H&E) stained coronal brain sections as described previously (Fraser and Dickinson, 1967). Vacuolar lesion profiles were prepared by scoring the presence and severity of the prion disease-specific vacuolation in nine gray matter and three white matter areas using a 0–5 scale: G1, dorsal medulla; G2, cerebellar cortex; G3, superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septum; G8, retrosplenial and adjacent motor cortex; G9, cingulate and adjacent motor cortex; W1, inferior and middle cerebellar peduncles; W2, decussation of superior cerebellar peduncles; and W3, cerebellar peduncles.

Real Time-Quantitative PCR (RT-qPCR) Analysis

Snap-frozen half brains were homogenized using Lysing Matrix D tubes (MP Biomedicals, Cambridge, United Kingdom) and a Ribolyser tissue homogenizer (Bio-Rad Laboratories, Watford, United Kingdom). Total RNA was extracted using RNABee (AmsBio, Abingdon, United Kingdom), purified using an RNeasy Mini kit (Qiagen, Manchester, United Kingdom), and treated with RNase-free DNase I (Promega, Southampton, United Kingdom) to remove genomic DNA. First-strand cDNA synthesis was performed using 1 μg total RNA and the SuperScript III Reverse Transcriptase (Life Technologies, Waltham, MA, United States), and mRNA amplified using Oligo DT (Promega). RT-qPCR was then performed using the primers listed in **Table 1** and FastStart Universal SYBR Green Master mix (Rox; Sigma-Aldrich) on an MX3005P RT-qPCR system (Agilent Technologies LDA UK Ltd., Stockport, Cheshire, United Kingdom). Cycle threshold values were analyzed using MxPro software (Agilent Technologies LDA UK Ltd.) and normalized relative to the reference gene *Rpl19* using the ΔΔCT method. Expression values were normalized so that the mean level in the 1xPBS-treated controls was 1.0.

TABLE 1 | RT-qPCR primers.

Gene	Forward primer	Reverse primer
Aif1	GGATCAACAAGCAATTCCTCGA	CTGAGAAAGTCAGAGT AGCTGA
Csf1r	AGGCAGGCTGGAATAATCTGACCT	CGTCACAGAACAGGACA TCAGAGC
Cx3cr1	CAGCATCGACCGGTACCTT	GCTGCACTGTCCGGTTGTT
Gbp2	GGGGTCACTGTCTGACCACT	GGGAAACCTGGGATGAGATT
Gfap	AGAAAGGTTGAATCGCTGGA	CGGCGATAGTCGTTAGCTTC
Iigp1	GGGGCAATAGCTCATTGGTA	ACCTCGAAGACATCCCCTTT
Il1β	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCT GCGAGATT
Il6	ACCAGAGGAAATTTTCAATAGGC	TGATGCACTTGCAGAAAACA
Il10	CCCTTTGCTATGGTGTCCTT	TGGTTTCTCTTCCCAAGACC
Itgam	TGGCCTATACAAGCTTGGCTTT	AAAGGCCGTTACTGAGGTGG
Psmb8	CAGTCCTGAAGAGGCCTACG	CACTTTCACCCAACCGTCTT
Prnp	TACCCTAACCAAGTGTAC TACAGGCC	TGGTACTGGGTGACGCA CATCTGCTC
Rpl19	GAAGGTCAAAGGGAATGTGTTCA	CCTTGTCTGCCTTCAGCTTGT
Srgn	GCAAGGTTATCCTGCTCGGA	TGGGAGGGCCGATGTTATTG
Tmem119	GTGTCTAACAGGCCCCAGAA	AGCCACGTGGTATCAAGGAG
Tnf	TGTGCTCAGAGCTTTCAACAA	CTTGATGGTGGTGCATGAGA

Serum Cytokine Quantitation by ELISA

Concentrations of interleukin-1β (IL-1β), IL-6, IL-10, and tumor necrosis factor-α (TNFα) in serum samples were measured using a mouse IL-1 beta, mouse IL-6, mouse IL-10, and mouse TNF alpha uncoated ELISA kits, respectively (Thermo Fisher Scientific), according to the manufacturers' instructions.

Immunohistochemistry and Image Analysis

Paraffin-embedded sections (thickness 6 μm) were deparaffinized and pre-treated by autoclaving in target retrieval solution (Dako) at 121°C for 15 min. Endogenous peroxidases were quenched by immersion in 4% hydrogen peroxide in methanol for 10 min. Microglia were detected by immunostaining with rabbit anti-allograft inflammatory factor-1 (AIF1; also known as ionized calcium binding adaptor molecule 1, Iba1) polyclonal antibody (Wako, Japan) and astrocytes were detected using rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody (Agilent Dako). Prior to immunostaining to detect PrP, sections were immersed in 98% formic acid for 10 min. PrP was then detected by immunostaining with mouse anti-PrP monoclonal antibody BH-1 (McCutcheon et al., 2014). Following addition of biotinylated secondary antibodies, immunostaining was revealed using the Vectastain avidin-biotin complex (ABC) kit (Vector Laboratories) with 3, 3′-diaminobenzidine tetrahydrochloride (DAB) as a substrate. Sections were counterstained with hematoxylin before mounting and imaging.

Stained sections were imaged using a Brightfield Eclipse Ni-E light microscope (Nikon Instruments Europe BV, Amsterdam, Netherlands), and images were captured using Zen 2 software (Carl Zeiss Ltd., Cambridge, United Kingdom). The abundance of the AIF1 +, GFAP +, and disease-specific PrP (PrP^d+) immunostaining on DAB stained sections was measured using Fiji/ImageJ software[1] using the analyze particle algorithm as described previously (Bradford et al., 2019). Briefly, DAB-stained images of the CA1 region of the hippocampus striatum radiatum were de-convoluted, intensity thresholds applied, and mean gray OD values measured from grayscale images using a scale of 0–255. Data for each image are expressed as a proportion of the total area of pixel area analyzed (% area coverage). The morphology of AIF1 + microglia (dendrite length, number of dendrite segments, number of dendrite branch points, and number of terminal points) in the CA1 region of the hippocampus striatum radiatum of each mouse was assessed on DAB stained sections using IMARIS (version 9.5.1) software (Bitplane, Zurich, Switzerland)

[1]https://imagej.nih.gov/ij/

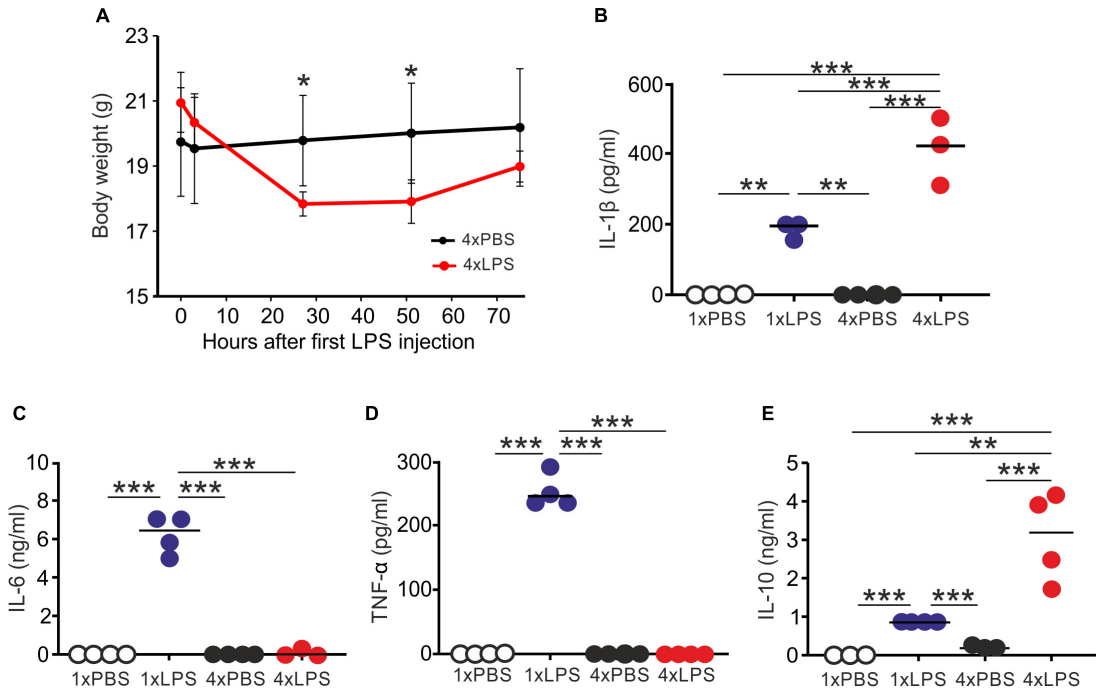

FIGURE 1 | Multiple LPS exposure induces innate immune tolerance. **(A)** Groups of mice (n = 4/group) were given four daily IP injections with either LPS or PBS (control) and body weight measured at intervals afterward. **(B,C)** Mice were given a single (1x) or four (4x) daily IP injections with either LPS or PBS (control) and serum collected 3 h after the final injection. **(B)** Concentration of IL-1β in the serum of mice given a single or four daily IP injections with either LPS or PBS. **(C)** Concentration of IL-6 in the serum of mice given a single or four daily IP injections with either LPS or PBS. **(D)** Concentration of TNF-α in the serum of mice given a single or four daily IP injections with either LPS or PBS. **(E)** Concentration of IL-10 in the serum of mice given a single or four daily IP injections with either LPS or PBS. N = 3–4 mice/group; horizontal bar, median. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

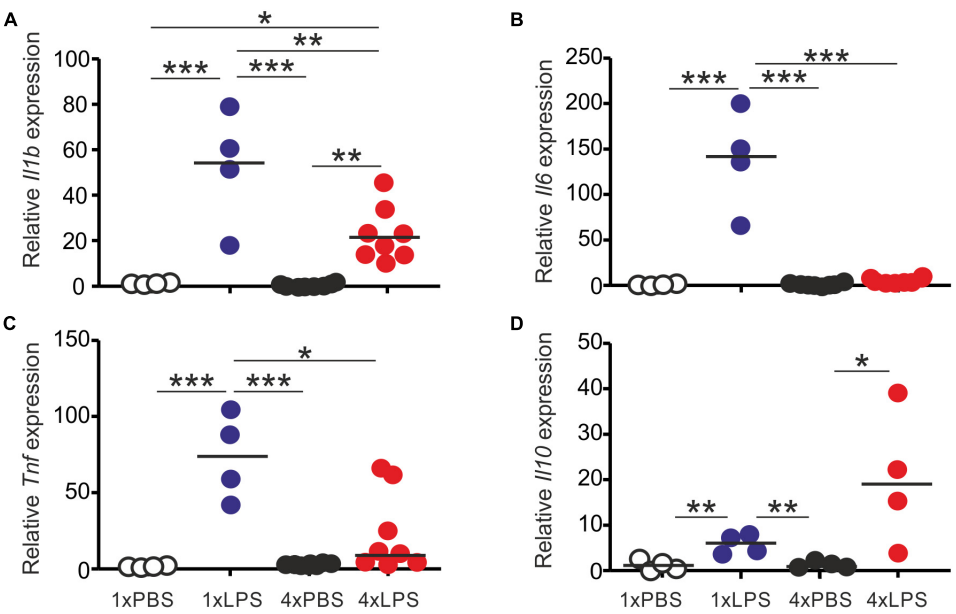

FIGURE 2 | Effect of systemic LPS treatment on expression of cytokine-encoding genes in the brain. Mice were given a single (1x) or four (4x) daily IP injections with either LPS or PBS (control). Brains were collected 3 h after the final injection and gene expression compared by RT-qPCR analysis. **(A)** Relative expression level of *Il1b* mRNA. **(B)** Relative expression level of *Il6* mRNA. **(C)** Relative expression level of *Tnf* mRNA. **(D)** Relative expression level of *Il10* mRNA. Gene expression data are normalized so that the mean level in the 1xPBS controls was 1.0. N = 4–8 mice/group; horizontal bar, median. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

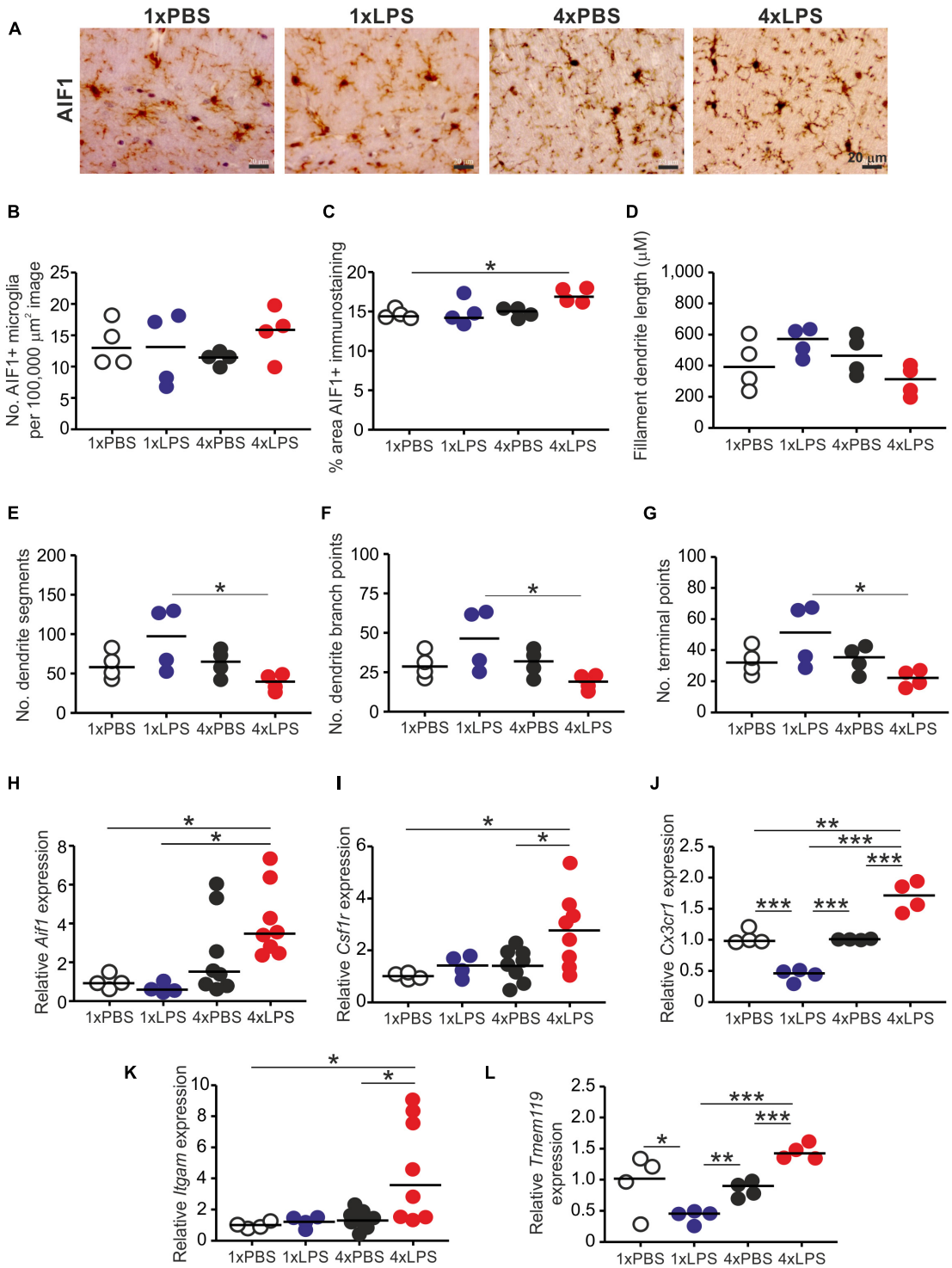

FIGURE 3 | Effect of systemic LPS treatment on microglia. Mice were given a single (1x) four (4x) daily IP injections with either LPS or PBS (control) and brains collected 3 h after the final injection. **(A)** Representative immunostaining of AIF1 + microglia (brown) in the CA1 region of the hippocampus striatum radiatum of each mouse. Scale bar 20 μm. **(B)** Relative abundance of AIF1+ microglia in 100,000 μm^2 images of the CA1 region of the hippocampus striatum radiatum of mice from each group. **(C)** Comparison of the magnitude of the AIF1+ immunostaining in the CA1 region of the hippocampus striatum radiatum of mice from each group. Assessment of AIF1+ microglia morphology in the CA1 region of the hippocampus striatum radiatum of mice from each group: **(D)**, mean filament dendrite length; **(E)**, mean number of dendrite segments; **(F)**, mean number of dendrite branch points; **(G)**, mean number of terminal points. **(H–L)** Relative expression level of *Aif1*, *Csf1r*, *Cx3cr1*, *Itgam,* and *Tmem119* mRNA in half brains from each mouse from each group. Gene expression data are normalized so that the mean level in the 1xPBS controls was 1.0. N = 4–8 mice/group; horizontal bar in histograms, median. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

as previously described (Bradford et al., 2017b). Data were collected from 28 to 32 microglia/group. Individual data points are presented as mean value/mouse.

Western Blotting

Prion-specific PrP^{Sc} was detected in flash-frozen brain samples by Western blotting as described previously (Bradford et al., 2017a). Briefly, brain homogenates (10% weight/volume) were prepared in NP40 lysis buffer [1% NP40, 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM TrisHCl (pH 7.5)]. To detect relatively proteinase-resistant PrP^{Sc} a sample of the homogenate was treated with 20 μg/ml proteinase K (PK) at 37°C for 1 h. The digestion was stopped by addition of 1 mM phenylmethylsulfonyl fluoride. Samples were then

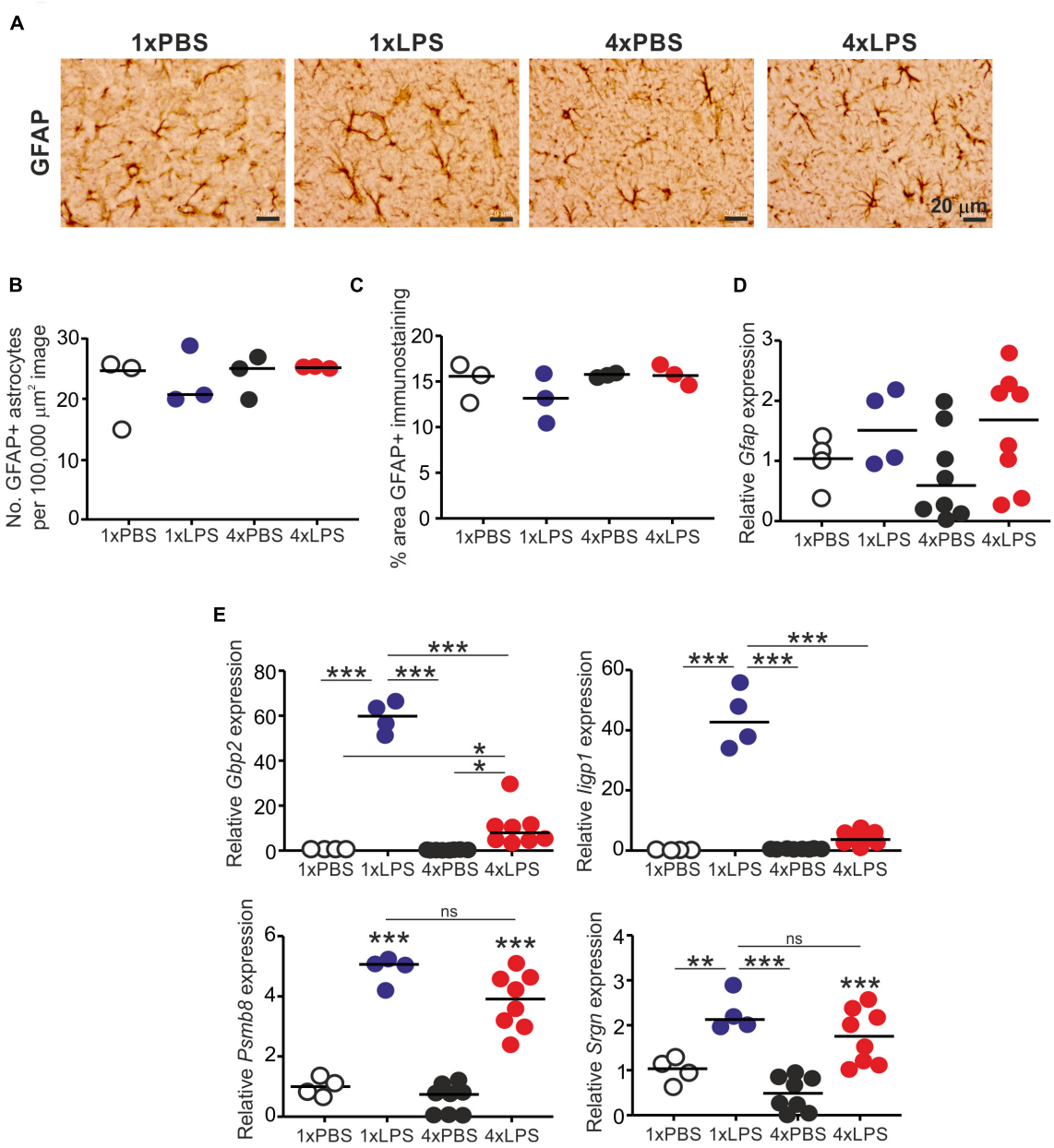

FIGURE 4 | Effect of systemic LPS treatment on astrocytes. Mice were given a single (1x) or four (4x) daily IP injections with either LPS or PBS (control) and brains collected 3 h after the final injection. **(A)** Representative immunostaining of GFAP + astrocytes (brown) in the CA1 region of the hippocampus striatum radiatum of each mouse. Scale bar 20 μm. **(B)** Relative abundance of GFAP+ astrocytes in 100,000 μm^2 images of the CA1 region of the hippocampus striatum radiatum of mice from each group. **(C)** Comparison of the magnitude of the GFAP+ immunostaining in the CA1 region of the hippocampus striatum radiatum of mice from each group. **(D)** Relative expression level of *Gfap* mRNA in half brains from each mouse from each group. **(E)** Relative expression level of *Gbp2*, *Iigp1*, *Psmb8,* and *Srgn* mRNA in half brains from each mouse from each group. Gene expression data are normalized so that the mean level in the 1xPBS controls was 1.0. $N = 3–8$ mice/group; horizontal bar in histograms, median. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significantly different.

denatured by incubation at 85°C for 15 min in 1X SDS sample buffer (Life Technologies) and separated via electrophoresis using 12% Tris-glycine polyacrylamide gels (Nupage, Life Technologies). Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes by semi-dry electroblotting and PrP detected using mouse monoclonal antibody BH1, and β-actin detected using mouse monoclonal antibody C4 (Santa Cruz Biotechnology). Membranes were subsequently stained with horseradish peroxidase-conjugated goat anti-species specific antibody (Jackson Immunoresearch) and visualized using chemiluminescence (BM Chemiluminescent substrate kit, Roche, Burgess Hill, United Kingdom). The relative abundance of PrP in each sample on the Western blots was compared by densitometric analysis using ImageJ software. The PrP abundance in each sample was normalized to β-actin and expressed as the percentage PrP in individual samples presented relative to the mean value in the PBS-treated controls which was set at 100%.

Statistics

Statistical significance between groups was tested using Prism 7.0 software (GraphPad, San Diego, United States). Datasets were first compared for normality using the Shapiro–Wilk normality test. Differences between groups were then compared using one-way ANOVA and *post hoc* Tukey multiple comparisons tests. Survival curve data for prion-infected mice in each treatment group were compared by Log-rank (Mantel-Cox) test. Data are expressed as dot plots of individual animal observations with median values indicated by a horizontal bar. Body weight and brain lesion profile data are presented as mean ± SD. Values of $P < 0.05$ were accepted as significant. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

RESULTS

Multiple Lipopolysaccharide Exposure Induces Innate Immune Tolerance

First, we compared the effects of single or multiple systemic LPS injections on cytokine production in the blood and brain. Groups of female C57BL/6J mice were treated daily with LPS by intraperitoneal (IP) injection for 1 or 4 days (1xLPS and 4xLPS, respectively) to induce innate immune training or tolerance, respectively (Wendeln et al., 2018). Parallel groups of mice were injected with PBS as a control. Brains and peripheral blood were collected 3 h after the final treatment. As anticipated, LPS treatment induced a transient period of mild sickness signs and weight loss which had begun to resolve by day 4 of treatment (**Figure 1A**). High levels of the pro-inflammatory cytokines interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNF-α) were detected in the serum 3 h after 1 dose of LPS (**Figures 1B–D**). However, the levels of IL-6 and TNF-α in the serum 4xLPS-treated mice were undetectable and consistent with the presence of high levels of the anti-inflammatory cytokine IL-10 in the serum at this time (**Figure 1E**). In the brain, genes encoding the cytokines IL-1β, IL-6, and TNF-α were similarly significantly induced within 3 h after a single dose of LPS (**Figure 2**). In contrast, expression of *Il1b*, *Il6,* and *Tnf* was significantly reduced

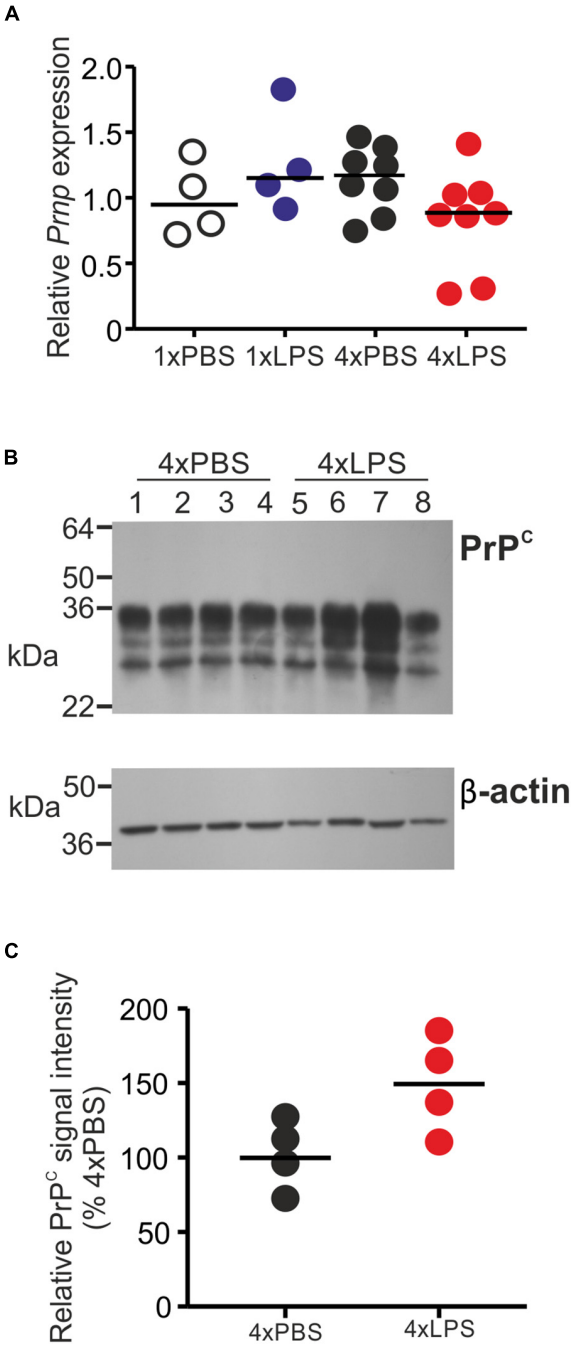

FIGURE 5 | Effect of systemic LPS treatment on PrP^{C} expression in the brain. Mice were given a single (1x) or four (4x) daily IP injections with either LPS or PBS (control) and brains collected 3 h after the final injection. **(A)** Relative expression level of *Prnp* mRNA in half brains from each mouse from each group. Gene expression data are normalized so that the mean level in the 1xPBS controls was 1.0. $N = 4–8$ mice/group. Not significantly different. **(B)** Western blot analysis of PrP^{C} protein (upper panel) and β-actin (lower panel) in half brains from mice given 4 daily IP injections with either LPS or PBS (4xPBS, 4xLPS, respectively). **(C)** Quantitation of the relative abundance of PrP^{C} protein in half brains from each group. The abundance of PrP^{C} in each sample was normalized to β-actin and expressed as the% relative to the mean value in the 4xPBS-treated controls. $N = 4$ mice/group; horizontal bar in histogram, median. Not significantly different, student's *t*-test.

after 4 consecutive daily doses of LPS when compared to mice given a single dose, and this was accompanied by an increase in the expression of *Il10* mRNA in the brains of 4xLPS-treated mice (**Figure 2**). Together, these data suggest that consecutive IP injections with LPS for 4 days induced innate immune tolerance in the periphery and brain, consistent with previous studies (Wendeln et al., 2018).

Impact of Systemic Lipopolysaccharide Treatment on Glial Cells in the Brain

Prion disease in the CNS is accompanied by extensive glial cell activation, and both the microglia and the astrocytes appear to play important roles in the development of the neuropathology (Zhu et al., 2016; Smith et al., 2020; Bradford et al., 2021). Since innate immune tolerance in microglia can influence the neuropathology in a mouse model of Alzheimer's disease (Wendeln et al., 2018), we next compared the effects of a single and four daily LPS treatments on microglia and astrocytes. Brains were collected 3 h after the last LPS or PBS treatment, and microglia were detected on histological sections by immunostaining for allograft inflammatory factor-1 (AIF1+ cells; **Figure 3A**). Although LPS treatment did not affect the abundance of microglia in the brain when compared to PBS-treated controls (**Figure 3B**), a significant increase in the abundance of AIF1+ immunostaining was detected in the brains of 4xLPS-treated mice compared to PBS-treated controls (**Figure 3C**). Morphometric analysis suggested this was accompanied by a significant reduction in microglia dendrite branching morphology (number of dendrite segments, dendrite branch points, and terminal points) in the brains of 4xLPS-treated mice compared to the microglia in the brains of

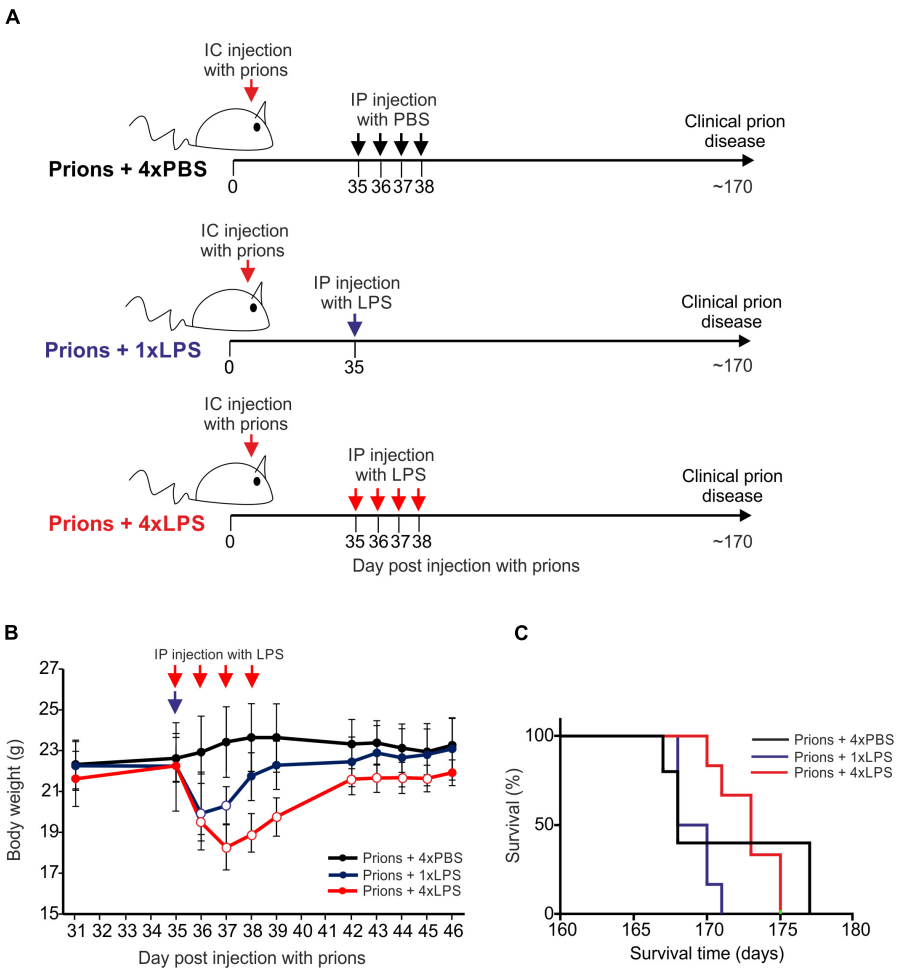

FIGURE 6 | Effect of systemic LPS treatment on CNS prion disease. **(A)** Cartoon describing the experimental design. Mice were first injected with ME7 scrapie prions directly into the brain by IC injection. Thirty-five days later, the mice were given either a single IP LPS injection (prions + 1xLPS) or four consecutive daily IP LPS injections (prions + 4xLPS) to induce innate immune training or tolerance, respectively. A parallel group of mice were given four consecutive daily IP PBS injections (prions + 4xLPS) as a control. **(B)** A single IP LPS injection (prions + 1xLPS) or four consecutive daily IP LPS injections (prions + 4xLPS) caused a transient reduction in body weight compared to prions + 4xPBS-treated controls. Open circles, $P < 0.05$ compared to prions + 4xPBS-treated controls. Closed circles, not significantly different compared to prions + 4xPBS-treated controls. **(C)** Survival curve for prion infected mice in each treatment group. $N = 5–6$ mice/group. Not significantly different, Log-rank (Mantel-Cox) test.

1xLPS-treated mice (**Figures 3D–G**). Consistent with these changes, the expression of the microglia-related genes *Aif1*, *Csf1r*, *Cx3cr1* (encoding C-X3-C motif chemokine receptor 1), *Itgam* (encoding CD11b), and *Tmem119* (encoding transmembrane protein 119) were also significantly increased in the brains of 4xLPS-treated mice (**Figures 3H–L**). In the brains of mice given consecutive daily systemic LPS treatment (4xLPS-treated mice) the increased expression of the homeostatic microglia-related genes *Csf1r*, *Cx3cr1,* and *Tmem119*, elevated expression of mRNA encoding the anti-inflammatory cytokine IL-10, coupled with the reduced expression of the pro-inflammatory cytokines *Il1b*, *Il6* and *Tnf* implied that their microglia had a homeostatic/anti-inflammatory phenotype.

Treatment with LPS did not affect the abundance of glial fibrillary acidic protein (GFAP)+ astrocytes (**Figures 4A,B**) or the abundance of GFAP protein or mRNA (**Figures 4C,D**). Factors including complement component C1q, IL-1α and TNF-α released from LPS-stimulated microglia can induce A1 neurotoxic astrocyte activation (Liddelow et al., 2017). Here, a single LPS treatment similarly induced the expression of the A1 neurotoxic reactive astrocyte-associated genes *Gbp2*, *Iigp1*, *Psmb8,* and *Srgn* in the brain (**Figure 4E**). Conversely, expression of *Gbp2* and *Iigp1* was significantly reduced in the brains of 4xLPS-treated mice (**Figure 4E**), implying that the innate immune tolerance induced by 4xLPS treatment had impeded the ability of the microglia to sustain A1 neurotoxic astrocyte activation. However, it was noticeable that expression of *Psmb8* and *Srgn* was not reduced in the brains of 4xLPS-treated mice when compared to 1xLPS-treated mice.

The level of cellular PrP^C in the brain can indirectly influence the rate of development of CNS prion disease in infected mice (Manson et al., 1994). However, the expression levels of PrP^C mRNA (*Prnp*) and protein were similar in the brains of LPS-treated mice compared to PBS-treated controls (**Figures 5A–C**).

Impact of Innate Immune Tolerance on Central Nervous System Prion Disease

Wendeln et al. (2018) showed how systemically induced innate immune tolerance causes epigenetic changes in the microglia that can influence the development of Alzheimer's disease-like neuropathology in the brain several months after LPS treatment. We therefore determined whether the innate immune tolerance induced in the brains after four daily LPS treatments could similarly months later modify CNS prion disease pathogenesis.

Groups of female C57BL/6J mice (n = 5–6/group) were first injected with ME7 scrapie prions directly into the brain by intracerebral (IC) injection. At 35 days post injection (dpi) with prions, and several weeks prior to the onset of detectable histopathological signs of microglia activation and prion-specific neuropathology in the brain (Donaldson et al., 2020; Bradford et al., 2021), the mice were then given a single IP LPS injection (prions + 1xLPS) or four consecutive daily IP LPS injections (prions + 4xLPS) to induce innate immune training or tolerance, respectively, as above (**Figure 6A**). A third group of mice were treated with PBS as a control (prions + 4xPBS). The mice were then monitored for the development of clinical signs of prion disease.

As above, peripheral LPS treatment caused a transient period of mild sickness behavior and weight loss when compared to the PBS-treated mice which had resolved by 7 days later (**Figure 6B**). All prion-infected PBS-treated control mice succumbed to clinical prion disease with a mean survival time of 172 ± 3 days (**Figure 6C** and **Table 1**). Treatment with a single or four consecutive daily doses of LPS did not significantly affect prion disease survival times. All the mice from the prions + 1xLPS and prions + 4xLPS treatment groups succumbed to clinical prion disease with similar survival times when compared to the prions + 4xPBS control mice (**Figure 6C** and **Table 2**). The timing of the duration to the initial onset of detection of the clinical signs of prion disease and the duration of the clinical phase were also similar in each group of mice irrespective of treatment (**Table 2**). These data clearly demonstrate that the innate immune tolerance induced after four daily LPS treatments did not affect the subsequent onset of the development of the clinical signs of prion disease.

Histopathological analysis of the brains from each group of clinically affected mice showed that the magnitude and distribution of the prion disease-specific vacuolation were similar in clinically affected prions + 1xLPS and prions + 4xLPS treated mice compared to prions + 4xPBS controls (**Figure 7A**). The magnitude of the microgliosis and reactive astrocytosis in response to CNS prion infection was also similar in the brains of mice from each group (**Figures 7B–F**). We also compared the levels of mRNA encoding the cytokines IL-1β, IL-6, TNF-α and IL-10 in the brains of mice from each

TABLE 2 | Effect of systemic LPS treatment on CNS prion disease.

Group	Incubation period[a]	Clinical phase[b]	Survival time[C]	Clinical disease incidence[d]
Prions + 4xPBS	159 ± 7 (160)	12 ± 5 (10)	171 ± 5 (168)	5/5
Prions + 1xLPS	164 ± 3 (167)ns	6 ± 5 (4)ns	169 ± 1 (169)ns	6/6
Prions + 4xLPS	165 ± 4 (167)ns	6 ± 4 (4)ns	173 ± 2 (173)ns	6/6

Mice were first injected with ME7 scrapie prions directly into the brain by IC injection. Thirty five days later the mice were given a either single IP LPS injection (Prions + 1xLPS) or four consecutive daily IP LPS injections (Prions + 4xLPS) to induce innate immune training or tolerance, respectively. A parallel group of mice were given four consecutive daily IP PBS injections (Prions + 4xLPS) as a control.

[a]Incubation period, mean duration (days ± SD) from time of IC injection with prions to time when first clinical signs of disease were detected. Median duration in brackets.

[b]Clinical phase, mean duration (days ± SD) from first detection of clinical signs of prion disease to development of terminal signs of clinical disease. Median duration in brackets.

[c]Survival time, mean duration (days ± SD) from time of IC injection with prions to development of terminal signs of clinical disease. Median duration in brackets.

[d]Clinical disease incidence, number of mice that developed terminal clinical signs of prion disease/number of mice injected IC with prions.

Statistical differences between groups were compared using One-Way ANOVA and post hoc Tukey multiple comparisons tests. Survival data compared by Log-rank (Mantel-Cox) test. ns, not significant.

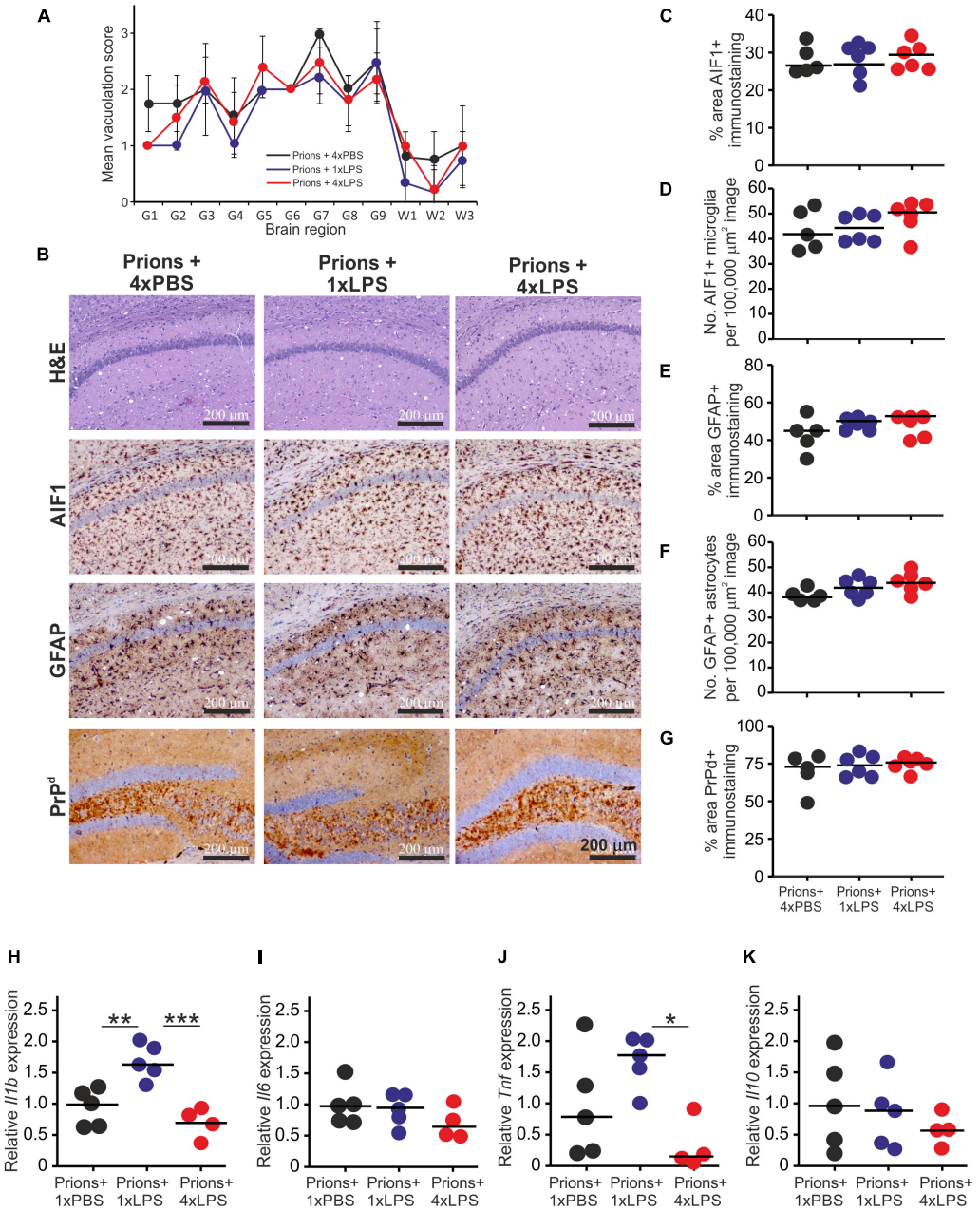

FIGURE 7 | Effect of systemic LPS treatment on the histopathological signs of prion disease in the brain. Mice were first injected with ME7 scrapie prions directly into the brain by IC injection. Thirty five days later the mice were given a either single IP LPS injection (prions + 1xLPS) or four consecutive daily IP LPS injections (prions + 4xLPS) to induce innate immune training or tolerance, respectively. A parallel group of mice were given four consecutive daily IP PBS injections (prions + 4xLPS) as a control. Brains were collected at the terminal stage. **(A)** The severity and distribution of the spongiform pathology (vacuolation) within each clinically affected brain from each treatment group was scored on H&E sections using a scale of 1–5 in nine gray matter regions and three white matter regions: G1, dorsal medulla; G2, cerebellar cortex; G3, superior colliculus; G4, hypothalamus; G5, thalamus; G6,hippocampus; G7, septum; G8, retrosplenial and adjacent motor cortex; G9, cingulate and adjacent motor cortex; W1, inferior and middle cerebellar peduncles; W2, decussation of superior cerebellar peduncles; and W3, cerebellar peduncles. **(B)** Representative images showing high levels of spongiform pathology (H&E, upper row), microgliosis (AIF1+ cells, brown, 2nd row), reactive astrocytes (GFAP+ cells, brown, 3rd row) and heavy prion disease-specific PrPd accumulation (brown, bottom row) in the hippocampus of all prion infected mice from each treatment group at the terminal clinical stage. Sections counterstained with hematoxylin to detect cell nuclei (blue). Scale bar, 200 µm. **(C,D)** Comparison of the magnitude of the AIF1+ immunostaining and abundance of AIF1 + microglia, respectively, in the CA1 region of the hippocampus of mice from each group. **(E,F)** Comparison of the magnitude of the GFAP+ immunostaining and abundance of GFAP+ reactive astrocytes, respectively, in the CA1 region of the hippocampus of mice from each group. **(G)** Comparison of the magnitude of the PrP^{d} + immunostaining in the CA1 region of the hippocampus of mice from each group. **(H)** Relative expression level of *Il1b* mRNA. **(I)** Relative expression level of *Il6* mRNA. **(J)** Relative expression level of *Tnf* mRNA. **(K)** Relative expression level of *Il10* mRNA. Gene expression data are normalized so that the mean level in the 1xPBS controls was 1.0. N = 4–6 mice/group; horizontal bar, median. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

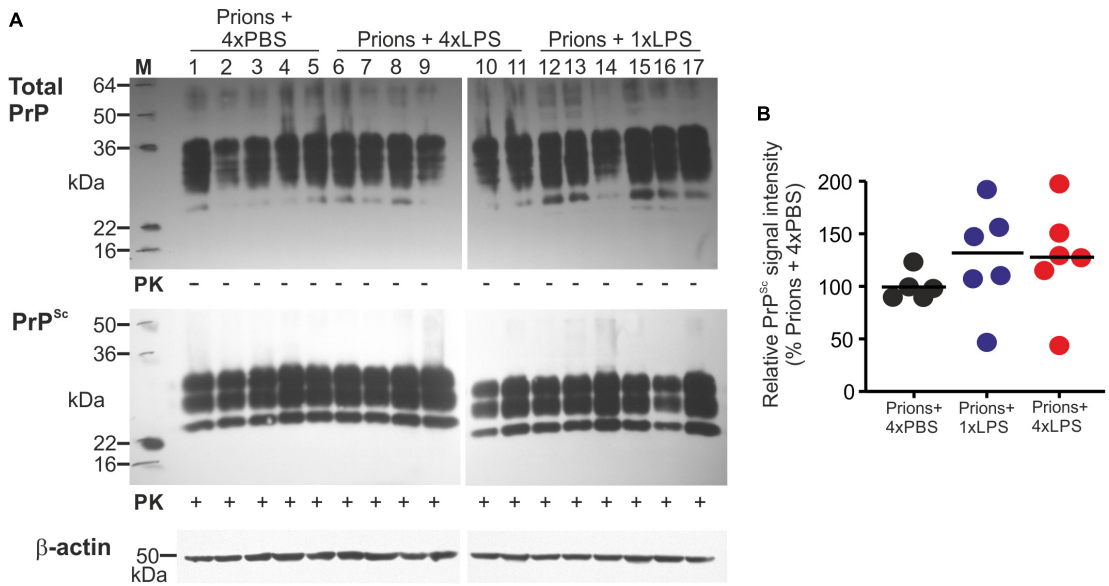

FIGURE 8 | Effect of systemic LPS treatment on prion disease-specific PrP^{Sc} accumulation in the brain. Mice were first injected with ME7 scrapie prions directly into the brain by IC injection. Thirty five days later the mice were given a either single IP LPS injection (prions + 1xLPS) or four consecutive daily IP LPS injections (prions + 4xLPS) to induce innate immune training or tolerance, respectively. A parallel group of mice were given four consecutive daily IP PBS injections (prions + 4xLPS) as a control. Brains were collected at the terminal stage. **(A)** Western blot analysis of prion disease specific PrP^{Sc} accumulation in half brains from each group. PK, proteinase-K. Upper panel, total PrP; middle panel, relatively PK-resistant prion disease-specific PrP^{Sc}; lower panel β-actin. **(B)** Quantitation of the relative abundance of PrP^{Sc} in half brains from each group. The abundance of PrP^{Sc} in each sample was expressed as the% relative to the mean value in the 1xPBS-treated controls. *N* = 5–6 mice/group; horizontal bar in histograms, median.

group. Innate immune tolerance induced after four daily LPS treatments did not affect the expression level of mRNA encoding these cytokines at the terminal stage of prion disease when compared to prions + 4xPBS controls (**Figures 7H–K**). However, microglia training in response to 1xLPS treatment coincided with a significant increase in *Il1b* expression at the terminal stage compared to prions + 4xPBS controls, and this increase was ablated in the brains of prions + 4xLPS treated mice (**Figure 7H**). The level of *Tnf* expression in the brains of prions + 4xLPS treated mice was also significantly reduced compared to prions + 1xLPS treated mice (**Figure 7J**).

A previous study demonstrated that 4 consecutive LPS treatments induced long-term modifications in the brain that months later were sufficient to decrease amyloid-β levels and plaque burdens in the APP23 mouse model of Alzheimer's disease pathology (Wendeln et al., 2018). However, LPS treatment did not subsequently affect the accumulation of prion disease-specific PrP^{Sc} accumulation in the brain at the terminal clinical stage (**Figures 7B,G**, **8A,B**). Together, these data clearly demonstrate that innate immune tolerance induced by consecutive peripheral LPS treatments does not induce long-term modifications that significantly affect the development of CNS prion disease pathogenesis.

DISCUSSION

The microglia are considered to provide neuroprotection during CNS prion disease (Zhu et al., 2016; Bradford et al., 2021), but their pro-inflammatory activation may exacerbate the development of the neuropathology in affected regions of the brain (Cunningham et al., 2005; Cunningham et al., 2009; Alibhai et al., 2016). Innate immune tolerance induced by consecutive systemic LPS treatment causes long-term epigenetic changes in the microglia in the brain that several months later can impede the accumulation of insoluble amyloid-β and plaque-associated neuritic damage in a transgenic mouse model of Alzheimer's disease-like pathology (Wendeln et al., 2018). We therefore reasoned that innate immune tolerance in microglia might similarly impede the subsequent development of CNS prion disease. However, in the current study, we clearly show that consecutive systemic LPS treatment did not affect the subsequent development of the neuropathology or survival times in a mouse model of CNS prion disease. Thus, whereas innate immune tolerance and the epigenetic modifications it causes in the microglia may influence the severity of the neuropathology of some neurological diseases including Alzheimer's disease and cerebral ischemia (Wendeln et al., 2018), our data suggest it is unlikely to impact on the development of CNS prion disease.

During the 1980s the UK human population is estimated to have been extensively exposed to bovine spongiform encephalopathy (BSE) prions via contaminated food (Wilesmith, 1993; Valleron et al., 2001). Despite this widespread risk of zoonotic disease transmission, the numbers of definite and probable human cases of variant Creutzfeldt-Jakob disease (vCJD) due to the consumption of BSE-contaminated food have remained low (*n* = 178, March 2022), with no new cases recorded since 2016 (Unit, 2018). However, a higher incidence

of prion disease-specific PrP accumulation has been detected in a retrospective screen of UK human appendix and tonsil samples (Hilton et al., 2004; Gill et al., 2013), raising the possibility that there may be many more asymptomatic individuals infected with vCJD. The reasons for the discrepancy between the reported number of probable and definite clinical vCJD cases and the estimated incidence of infected individuals in the UK are not known. Of course since these studies relied on the detection of disease-specific PrP by immunohistochemistry (IHC), it remains to be determined whether this reflects the presence of infectious vCJD prions (Gill et al., 2013). In the current study, we considered it plausible that systemic inflammation several months, or even years, before onset of the prion accumulation in the brain might cause innate immune tolerance that reduces the risk of subsequently developing CNS prion disease in sub-clinically infected individuals. However, using an experimental mouse model of CNS prion disease our data suggest that innate immune tolerance in the microglia is unlikely to have a significant impact on CNS prion disease susceptibility or the development of the neuropathology.

CNS prion infection has been suggested to induce a "primed state" in the microglia that exacerbates their subsequent responses to pro-inflammatory stimuli (Perry et al., 2003). As a consequence, a range of studies have shown how exposure to pro-inflammatory stimuli or a systemic infection during the CNS phase can switch the microglia from an anti-inflammatory phenotype to an activated disease-associated phenotype that can exacerbate the clinical signs and accelerate prion disease progression (Cunningham et al., 2005; Cunningham et al., 2009; Lunnon et al., 2011; Chouhan et al., 2022). In contrast, in the current study, the mice were treated with LPS several months before the induction of the primed state in the microglia and the onset of the neuropathology. Further studies are necessary to determine whether prolonged LPS exposure during the CNS phase would similarly impact on the development of the neuropathology, for example by impeding microglia polarization toward a disease-associated phenotype (Alibhai et al., 2016).

Whereas Wendeln et al. (2018) showed how the long-term effects of innate immune tolerance in the microglia impeded the development of amyloid-β-associated neuropathology in a transgenic mouse model of Alzheimer's disease-like pathology, our data show this did not affect the development of CNS prion disease pathology. These contrasting effects highlight important differences in the phenotypes of the microglia in the prion disease-affected brain when compared to other neurodegenerative disorders. For example, expression of triggering receptor expressed on myeloid cells-2 (TREM2) is important for the disease-associated activation of the microglia in a mouse model of Alzheimer's disease-like pathology (Keren-Shaul et al., 2017). In contrast, TREM2 deficiency has only a modest influence on CNS prion disease (Zhu et al., 2015). The absence of the commensal gut or lung microbiota can significantly affect microglial activation (Erny et al., 2015) and the development of autoimmunity in the CNS (Hosang et al., 2022), but we have shown that CNS prion disease was unaltered in the germ-free mice (Bradford et al., 2017b). Factors produced by the microglia can trigger neurotoxic A1 reactive astrocyte activation (Liddelow et al., 2017). However, the reactive astrocyte activation in the prion disease-affected brain occurs independently of stimulation from the microglia or microglia-derived factors (Hartmann et al., 2020; Bradford et al., 2021). Instead, during prion disease, the microglia appear to be acting to constrain the neurotoxic or dysregulated activation of reactive astrocytes (Bradford et al., 2021).

No therapies or cures are currently available to treat the prion diseases. The pharmacological modulation of microglia phenotype or activation status has been proposed a novel means to prevent or delay the development of the neuropathology (Zhu et al., 2016; Bradford et al., 2021; Hu et al., 2021). Our data clearly suggest that innate immune tolerance in the microglia is unlikely to have a significant impact on CNS prion disease. However, a thorough understanding of the impact of systemic inflammation on microglia during CNS prion disease could help identify important factors that influence the risk of developing prion disease and other important neurodegenerative conditions.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal studies were reviewed and approved by the University of Edinburgh's Ethical Review Committee.

AUTHOR CONTRIBUTIONS

NAM conceived the study and obtained funding. RP and BMB performed the experiments and analyzed the datasets. All authors designed the study, contributed to the writing of the manuscript, and approved the final draft.

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Distinct characteristics of microglia from neurogenic and non-neurogenic regions of the human brain in patients with Mesial Temporal Lobe Epilepsy

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The study of microglia isolated from adult human brain tissue provides unique insight into the physiology of these brain immune cells and their role in adult human brain disorders. Reports of microglia in post-mortem adult human brain tissue show regional differences in microglial populations, however, these differences have not been fully explored in living microglia. In this study biopsy tissue was obtained from epileptic patients undergoing surgery and consisted of both cortical areas and neurogenic ventricular and hippocampal (Hp) areas. Microglia were concurrently isolated from both regions and compared by immunochemistry. Our initial observation was that a greater number of microglia resulted from isolation and culture of ventricular/Hp tissue than cortical tissue. This was found to be due to a greater proliferative capacity of microglia from ventricular/Hp regions compared to the cortex. Additionally, ventricular/Hp microglia had a greater proliferative response to the microglial mitogen Macrophage Colony-Stimulating Factor (M-CSF). This enhanced response was found to be associated with higher M-CSF receptor expression and higher expression of proteins involved in M-CSF signalling DAP12 and C/EBPβ. Microglia from the ventricular/Hp region also displayed higher expression of the receptor for Insulin-like Growth Factor-1, a molecule with some functional similarity to M-CSF. Compared to microglia isolated from the cortex, ventricular/Hp microglia showed increased HLA-DP, DQ, DR antigen presentation protein expression and a rounded morphology. These findings show that microglia from adult human brain neurogenic

regions are more proliferative than cortical microglia and have a distinct protein expression profile. The data present a case for differential microglial phenotype and function in different regions of the adult human brain and suggest that microglia in adult neurogenic regions are "primed" to an activated state by their unique tissue environment.

KEYWORDS

microglia, primary human cell culture, M-CSF or CSF1, neurogenic niche, microglial heterogeneity

Introduction

Microglia are the brain's primary immune cells. They play important homeostatic roles and can modulate the functions of other brain cells. Microglia actively monitor neuronal synapses (Nimmerjahn et al., 2005; Wake et al., 2009), phagocytose debris (Chan et al., 2001; Simard et al., 2006), and can secrete both supportive and detrimental factors into the extracellular environment (Olah et al., 2011). Microglia communicate with other brain cells *via* cytokines and growth factors and are important cell types during development, in normal physiological states, and during injury and degenerative processes (de Haas et al., 2007). In these different situations, microglia can express specific cell surface receptors, have specific morphology, and produce different soluble molecules (Olah et al., 2011). Thus, microglia have marked phenotypic diversity which is influenced by their microenvironment including other cell types and soluble molecules in their surroundings. An important factor regulating microglial phenotype is the growth factor Macrophage Colony-Stimulating Factor (M-CSF). Development and differentiation of microglia is dependent on M-CSF signalling (Ginhoux et al., 2010) and M-CSF also increases microglial division (Lee et al., 1993; Vidyadaran et al., 2009; Yamamoto et al., 2010).

Normal rodent and human adult brains have regional differences in microglia density and immune protein expression (Mittelbronn et al., 2001; de Haas et al., 2008). For example, it has been demonstrated that white matter contains more microglia than grey matter (Mittelbronn et al., 2001). Furthermore, different brain regions show different microglial responses to ageing (Hart et al., 2012) and different phenotypes *in vitro* (Melief et al., 2012). The diverse nature of these brain immune cells has begun to be appreciated, but it is not fully known to what extent microglia vary in different brain regions and what factors/mechanisms produce these differences.

Two highly specialised areas of the adult brain are the neurogenic regions of the subventricular zone (SVZ) of the lateral ventricles and the dentate gyrus (DG) of the hippocampus. Here neural progenitor cells (NPCs) proliferate throughout life and give rise to new neurons (Eriksson et al., 1998; Curtis et al., 2007). Microglia are present in both the rodent and human SVZ and DG (Curtis et al., 2005; Goings et al., 2006; Hellstrom et al., 2011; Morrens et al., 2012) where they perform many important roles including cytokine production and phagocytosis (Walton et al., 2006; Sierra et al., 2010; Morrens et al., 2012). Investigation of the role of microglia in adult neurogenesis has led to findings of specific microglial characteristics within the SVZ and DG neurogenic regions. Microglia of the mouse SVZ were found to have a higher level of basal activation and proliferation, and greater expression of CD45 and CD11b, than non-neurogenic regions of striatum and corpus callosum (Goings et al., 2006). Following cortical injury, SVZ microglia did not, however, become more activated, despite being closer to the injury than the striatum and corpus callosum where microglia greatly increased activation compared to non-injured brains (Goings et al., 2006). A study of human microglial regional heterogeneity found that SVZ microglia clustered separately from microglia from the cortex, cerebellum or thalamus, and identified a phenotypic subset of microglia in the SVZ that was absent from the temporal and frontal cortex (Böttcher et al., 2019). Further evidence of a special phenotype of microglia in neurogenic regions is the unique proliferative capacity of microglia found in the SVZ of neonatal mice whereby SVZ cultures produced 20-fold greater yields of microglia than corresponding cortical cultures (Marshall et al., 2008; Marshall et al., 2014). However, adult brain cultures gave 10 times fewer microglia (Marshall et al., 2008) and it is unknown whether this unique proliferative capacity of subventricular microglia is true for the adult human brain as several other differences exist between the culture of rodent and human microglia.

The use of primary human microglia is an invaluable tool for neuroscience research (Dragunow, 2008a; Gibbons and Dragunow, 2010; Smith and Dragunow, 2014). Using human biopsy tissue from temporal lobe epilepsy surgeries, the microglia of the hippocampus and of the temporal horn of the lateral ventricle (herein referred to as "ventricular/Hp" microglia) were compared to microglia from the cortical

middle temporal gyrus. Major distinctions were found between microglia from these two separate human adult brain regions in terms of proliferation and immune protein expression.

Materials and methods

Tissue

Biopsy adult human brain tissue used for this study was obtained from patients undergoing surgery for drug-resistant temporal lobe epilepsy. This research was approved by the Northern Regional Ethics Committee and the University of Auckland Human Participants Ethics Committee and informed consent was obtained from all participants. All biopsy specimens were from temporal lobe epilepsy cases with a high degree of mesial temporal sclerosis (neuropathological grade 3–4, where grade 4 is maximal severity). Due to the nature of the surgery, all candidates had previously been on a range of medications, administered alone or in combination, including lamotrigine, phenytoin, sodium valproate, tegretol, and topiramate. All patients were male and in their third decade. Biopsy tissue with gross signs of sclerosis (i.e., the seizure focus) was removed by a pathologist for pathological examination and the remaining cortical and ventricular/Hp tissue was used for the culture and study of microglia.

Human glial cell isolation and culture

Microglia were isolated from two regions of adult human brain tissue using a method based on the cell isolation protocols previously optimised in our lab (Gibbons et al., 2011; Park et al., 2012, 2022; Smith et al., 2013a; Rustenhoven et al., 2016). The two brain regions compared were (1) cortical middle temporal gyrus and (2) the neurogenic regions of the hippocampal DG and the overlying SVZ of the lateral ventricle. Tissue of equivalent weights from both regions was processed concurrently by the same method. 1–2 g tissue was washed once in HBSS and visible blood vessels were removed with sterile forceps. The tissue was diced in a sterile petri dish into 1 mm^3 pieces. The tissue was placed in a 15 ml tube containing 10 ml enzymatic mix [100 U/ml DNase (Invitrogen, Carlsbad, CA, USA) and 2.5 U/ml papain (Worthington, Lakewood, NJ, USA) in Hibernate-A medium (Gibco, Waltham, MA, USA)] for chemical digestion. This was placed on a rotating device in a 37°C incubator for 10 min. The cell/enzyme mix was then removed from the incubator, triturated with a 10 ml pipette, and returned to the incubator for a further 10 min. This cell mix was then diluted with 10 ml DMEM/F12 (Gibco, CA, USA) with 1% B27 (Gibco, CA, USA) and passed through a 70 μm strainer (Bector Dickinson, Franklin Lakes, NJ, USA). The cells that passed through the strainer were centrifuged at 160 × *g* for 10 min and resuspended in 7 ml media [DMEM/F12 with 1% B27, 1% GlutaMAX (Gibco, CA, USA), 1% penicillin-streptomycin (Gibco, CA, USA), 40 ng/ml fibroblast growth factor (FGF; Peprotech, Cranbury, NJ, USA), 40 ng/ml epidermal growth factor (EGF; Peprotech, NJ, USA) and 2 μg/ml heparin (Sigma, St. Louis, MO, USA)] and seeded into a T25 flask. This media has previously been optimised for the survival of neural precursor cells which can also be obtained by this cell isolation method, but which are easily isolated and distinguishable from microglia (Park et al., 2012, 2022; Rustenhoven et al., 2016). On the following day (14–18 h later) the flasks were tapped and non-adherent cells and debris were removed and used for studies of neural precursor cells. The remaining adherent glia were maintained as previously described (Gibbons et al., 2011; Smith et al., 2013a) by adding 7 ml microglial media (DMEM/F12 with 10% FBS and 1% PSG) and culturing for 1–2 weeks. Cells cultured from both brain regions were plated simultaneously into adjacent wells of 96-well plates at 50,000 cells/ml for experimentation.

Cytokine treatment

Primary human glial cell cultures were treated in 96-well plates. 1 μl cytokine was added to 100 μl media. Cells were treated with 25 ng/ml recombinant human M-CSF (Sigma-Aldrich, MO, USA; in H_2O) or 1 ng/ml recombinant human IFNy (R&D Systems, Minneapolis, MN, USA; in PBS with 0.1% BSA) at 0 and 48 h. Total time of cytokine treatment was 96 h.

BrdU proliferation assay

Following 72 h exposure to 25 ng/ml M-CSF, 10 μM BrdU was added to the cells for 24 h. Cells were washed twice with PBS to remove excess BrdU and fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature and processed for immunocytochemistry as previously described (Smith et al., 2013b).

Immunocytochemistry

Cells were processed for immunocytochemistry as described previously using the specific antibodies detailed in **Table 1**, which we have previously characterised (Smith et al., 2013b). Controls omitting the primary antibodies gave no staining. Incubation of fixed cells with 0.5 μg Human Fc Block (BD

Biosciences, NJ, USA) for 30 min at room temperature prior to the addition of primary antibody produced no significant difference in staining (**Supplementary Figure 1**).

Quantitative image analysis of cell number, protein expression and microglial morphology

Immunocytochemical and morphological observations were quantified using a Discovery-1 automated fluorescence microscope (Molecular Devices, San Jose, CA, USA) and Metamorph (6.2.6 software, Molecular Devices) image analysis system as previously described (Dragunow, 2008b; Smith et al., 2010). Intensity thresholds were set based on the staining by each antibody and were adjusted to exclude false positives and false negatives. All images from one experiment were analysed with the same threshold. Results were logged automatically to Microsoft Excel spreadsheets.

For quantification of microglial morphology, the Journal "Microglial Shape" was written in Metamorph. The journal automatically thresholded each image to isolate CD45-positive microglia, then applied the *Integrated Morphometry Analysis* tool *Elliptical Form Factor* (length/breadth) to determine cell shape (Smith et al., 2013b).

Statistical analysis

Data from representative experiments are displayed, unless otherwise stated, with mean ± standard error of the mean (SEM). Experiments were replicated with cells from at least four different individuals. The F-test and Bartlett's test were used to check for equal variances. Statistical analysis was carried out using *t*-tests or ANOVA. When no significant interaction was found by two-way ANOVA, one-way ANOVA and Tukey's multiple comparison tests were used. In cases of unequal variance the equivalent non-parametric test was used (Mann Whitney test or Kruskal-Wallis test with Dunn's multiple comparison test). *P*-values of < 0.05 were considered statistically significant differences.

Results

Differential proliferation of microglia from ventricular/hippocampal and cortical regions

Microglia were cultured from two anatomical regions of biopsy adult human brain tissue from the same patients.

TABLE 1 Antibodies used for immunocytochemistry.

Antibody	Company	Catalogue #	Dilution
Rabbit anti-PU.1	Cell signalling	2258	1:500
Mouse anti-CD45	Abcam	ab8216	1:500
Rabbit anti-CSF-1R	Santa Cruz	Sc-692	1:50
Mouse anti-HLA-DP, DQ, DR	Dako	M0775	1:500
Rabbit anti-DAP12	Santa Cruz	Sc-20783	1:500
Mouse anti-C/EBPβ	Santa Cruz	Sc-7962	1:250
Mouse anti-IGF-1R	Millipore	MAB1120	1:50
Mouse anti-BrdU	Roche	11170376001	1:500
Rabbit anti-Ki67	Dako	A0047	1:500
Goat anti-rabbit IgG Alexa Fluor® 594	Invitrogen	A11012	1:500
Goat anti-mouse IgG Alexa Fluor® 488	Invitrogen	A11001	1:500
Goat anti-mouse IgG Alexa Fluor® 594	Invitrogen	A11005	1:500
Goat anti-rabbit IgG Alexa Fluor® 488	Invitrogen	A11008	1:500

Microglia isolated from the cortical middle temporal gyrus were compared to microglia isolated from the neurogenic regions of the hippocampal DG and the overlying SVZ of the lateral ventricle.

Microglia were visualised using antibodies to cell surface antigen CD45 and nuclear transcription factor PU.1 (Smith et al., 2013d; **Figures 1A,B**). It was found that the yields of microglia (as assessed by immunocytochemistry, **Figures 1A,B**) from the ventricular/Hp region (580,000 microglia per gram tissue, $n = 3$) were greater than those derived from the cortical middle temporal gyrus region (290,000 microglia per gram tissue, $n = 3$).

Adult human microglia cultured from middle temporal gyrus cortical regions have very low rates of proliferation *in vitro* (3.2 ± 0.7%; $n = 9$), confirming previous results (Gibbons et al., 2007; Smith et al., 2013b). In contrast, microglia derived from the lateral ventricle/Hp areas of the same patients had more variable levels of microglial proliferation, sometimes greatly exceeding that of microglia derived from the cortex. We observed greatly increased microglial proliferation in lateral ventricle/Hp cultures from three out of nine cases; small, marginal increases in another three cases; and no change in three other cases. The endogenous cell division marker Ki67 and the exogenous proliferation indicator BrdU were both used to confirm differences in proliferation and gave equivalent results (**Figures 1C–E**). Overall, the average basal percentage of dividing microglia in ventricular/Hp cultures was 9.1 ± 2.3% ($n = 9$). The increase in ventricular/Hp microglial division compared to cortical microglial division was variable between cases, but microglia from ventricular/Hp regions were found to

have on average a 3-fold (n = 9; p = 0.040; Mann Whitney test) higher proliferation rate than cortical microglia (Figure 1E).

Ventricular/hippocampal microglia have a greater proliferation response to Macrophage Colony-Stimulating Factor than cortical microglia

We have previously reported an increase in adult human microglial cell number with M-CSF treatment (Smith et al., 2013b). This increase in microglia cell number was due to increased microglial proliferation. Here we assessed whether microglia cultured from the ventricular/Hp region would also respond to M-CSF by increasing proliferation, given their higher level of basal proliferation.

Macrophage Colony-Stimulating Factor treatment increased proliferation of microglia cultured from the ventricular/Hp region (seven out of seven cases) as for microglia from the cortex. Furthermore, ventricular/Hp microglia had a larger proliferation response to M-CSF than cortical microglia (Figure 2A). At the individual case level, the percentage increase in proliferating microglia with M-CSF treatment was consistently greater for ventricular/Hp microglia than for cortical microglia (five out of seven cases). Although three out of nine cases did not have higher basal ventricular/Hp microglial proliferation compared to cortical microglia, these cases had a greater proliferation response to M-CSF in ventricular/Hp microglia compared to cortical microglia.

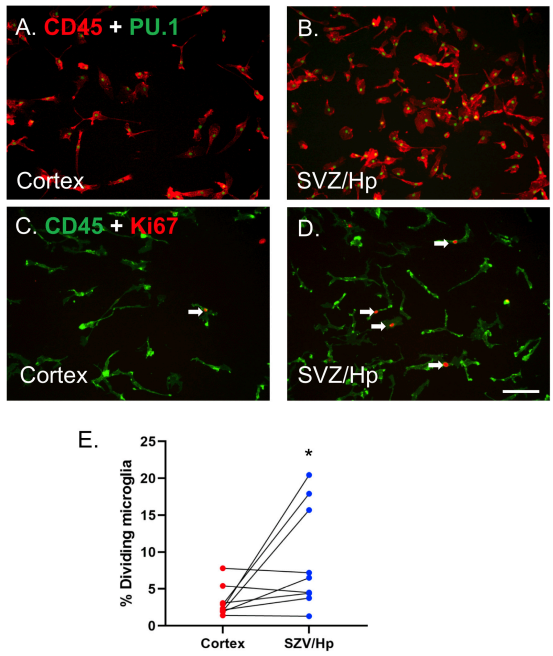

FIGURE 1
Microglia from neurogenic ventricular/Hp tissue proliferate more than microglia from non-neurogenic cortical tissue. **(A)** PU.1 (green nuclei) and CD45 (red) double-positive microglia are present in glial cultures from cortical human brain tissue. **(B)** A greater number of microglia are present in ventricular/Hp cultures. **(C,D)** Immunocytochemical images of CD45 microglial cell surface marker (green) and Ki67 cell division marker (red) showing minimal proliferation of cortical microglia **(C)** and greater basal proliferation of ventricular/Hp microglia **(D)**. Arrows indicate examples of Ki67-immunopositive microglia. Scale bar = 100 μm. **(E)** Quantification of the percentage of microglia cultured from cortical and ventricular/Hp regions that incorporate BrdU, showing a significantly greater percentage of dividing microglia in ventricular/Hp regions. Each data point indicates an individual case (n = 9). Cortex and SVZ/Hp samples from the same case are indicated by connecting lines. *indicates P value < 0.05.

Similar expression of PU.1 transcription factor and CD45 cell surface receptor in ventricular/hippocampal and cortical microglia

PU.1 is a microglial transcription factor which has been shown to be involved in microglial responses to M-CSF (Zhang et al., 1994; Celada et al., 1996; Gómez-Nicola et al., 2013; Smith et al., 2013b). Although there were significantly greater numbers of PU.1-positive microglia in ventricular/Hp cultures, no consistently significant difference was found between cortical and ventricular/Hp microglia in levels of PU.1 protein expression per microglial cell as quantified by the intensity of PU.1 staining (Figure 2B). However, as previously reported for cortical microglia, M-CSF increased the staining intensity of PU.1 indicating an increased amount of PU.1 protein expressed by microglia from both cortical and ventricular/Hp regions (Figure 2B).

It has previously been reported that adult human microglia constitutively express high levels of CD45 *in vitro* (Gibbons et al., 2007; Smith et al., 2013d) and here no difference in CD45 expression was found between ventricular/Hp and cortical microglia (data not shown).

Neurogenic region microglia express higher levels of proteins involved in Macrophage Colony-Stimulating Factor signalling

To further explore the increased ability of ventricular/Hp microglia to respond to M-CSF, M-CSF receptor (CSF-1R) expression was investigated. We have previously reported the expression of CSF-1R protein on cortical microglia cultured from adult human tissue (Smith et al., 2013b). As expected given their proliferation response to M-CSF, CSF-1R was expressed by microglia cultured from ventricular/Hp

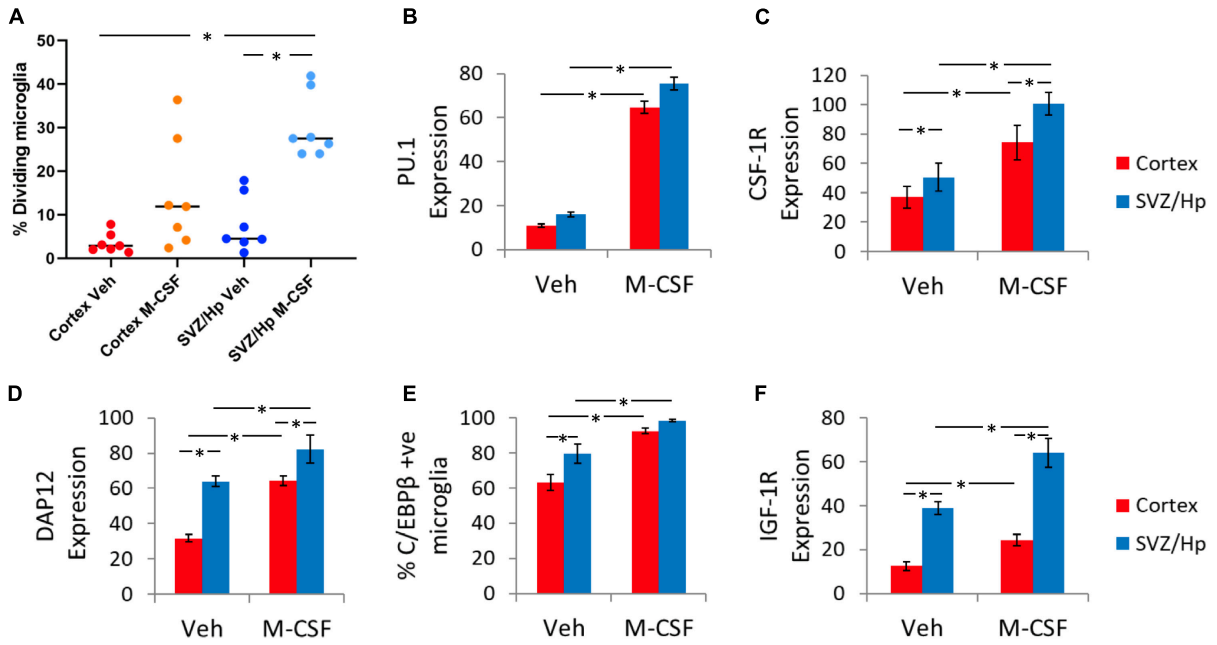

FIGURE 2
Basal and Macrophage Colony-Stimulating Factor (M-CSF)-induced microglial proliferation and expression of PU.1, CSF-1R, DAP12, CEBPβ, and IGF1 receptor. **(A)** Quantification of the percentage of microglia that incorporate BrdU under control conditions and with M-CSF treatment showing a significant increase in microglial division with M-CSF and an enhanced response in ventricular/Hp microglia. Each data point indicates an individual case (n = 7). **(B)** M-CSF significantly increases the intensity (arbitrary fluorescence units) of PU.1 expression (amount of PU.1 protein) in adult human microglia from neurogenic (ventricular/Hp) and non-neurogenic (cortical) regions of the adult human brain. **(C)** CSF-1R is more highly expressed in ventricular/Hp microglia than cortical microglia. A significant increase in intensity of receptor labelling is found for CSF-1R following M-CSF treatment. **(D)** Quantification of DAP12 staining intensity shows a significantly greater level of expression in ventricular/Hp microglia compared to cortical microglia, and a significant increase in DAP12 expression with M-CSF treatment. **(E)** Quantification of microglial C/EBPβ expression showing differential basal expression by cortical and ventricular/Hp microglia, and significant increases in the percentage of cortical and ventricular/Hp microglia which express C/EBPβ following M-CSF treatment. **(F)** Ventricular/Hp microglia express significantly more IGF-1R than cortical microglia, and a significant increase in intensity of IGF-1R is evident following M-CSF treatment. **(B–F)** Protein expression was quantified using an intensity threshold in Metamorph image analysis software, as described in the methods. *indicates P value < 0.05.

as well as cortical regions (Figure 2C). Basal levels of CSF-1R protein expression on cortical and ventricular/Hp microglia were compared and it was found that there was a higher amount of CSF-1R protein expressed by ventricular/Hp microglia than by cortical microglia (Figure 2C). We have previously found that treatment of adult human microglia with M-CSF increases their expression of CSF-1R (Smith et al., 2013b). Both ventricular/Hp and cortical microglia increased expression of CSF-1R following exposure to M-CSF (Figure 2C).

DAP12 is an adaptor protein found in microglia in the adult human brain (Satoh et al., 2011) and is involved in M-CSF signalling (Otero et al., 2009; Smith et al., 2013c). DAP12 expression was assessed in microglia from ventricular/Hp regions and from the cortex. Basal DAP12 expression levels were higher in ventricular/Hp microglia than in cortical microglia for most cases (six out of seven cases; Figure 2D). M-CSF produced an increase in DAP12 expression for cortical microglia (seven out of seven cases; Figure 2D) and this was also observed for ventricular/Hp microglia. Thus ventricular/Hp microglia have higher basal levels of DAP12 expression than cortical microglia and M-CSF treatment of cortical microglia increases their levels of DAP12 expression similar to basal levels in ventricular/Hp microglia (Figure 2D).

C/EBPβ is a transcription factor expressed by microglia which has been shown to be involved in M-CSF-mediated effects (Gómez-Nicola et al., 2013; Smith et al., 2013b). Total microglia were labelled for PU.1 and the percentage of microglia expressing C/EBPβ was quantified (Smith et al., 2013d). Higher C/EBPβ protein expression was found in ventricular/Hp microglia than in cortical microglia (Figure 2E). Furthermore, M-CSF produced an increase in C/EBPβ expression in microglia from both cortex and ventricular/Hp regions (Figure 2E).

Another growth factor that can act on microglia and have immunomodulatory effects is IGF-1. We have previously demonstrated IGF-1R protein expression in human adult microglia (Smith et al., 2013b) and here it was found that basal levels of IGF-1R protein expression are higher in ventricular/Hp microglia than cortical microglia (Figure 2F). We have also previously observed that IGF-1R expression increased on microglia from the cortex following M-CSF treatment. This

response was found to also be present in ventricular/Hp microglia (**Figure 2F**).

Neurogenic region microglia have a more "activated" phenotype

Two commonly assessed indicators of microglial phenotype are expression of HLA and microglial morphology. We were interested to know whether cortical and ventricular/Hp microglia differed in these measures. HLA is a widely used marker of microglial "activation" and the proportion of microglia expressing HLA basally *in vitro* is highly variable between cases and is further increased by exposure to the pro-inflammatory cytokine IFNy (Smith et al., 2013e). Ventricular/Hp and cortical microglia, from the same cases, were compared to see whether their basal HLA expression differed.

It was found that ventricular/Hp microglia had a greater propensity to express HLA than cortical microglia. In the majority of cases (six out of seven), more HLA was expressed by ventricular/Hp microglia than cortical microglia (**Figures 3A,B,E**). IFNy increases cortical adult human microglial expression of HLA (**Figure 3C**). This was also found to be true for the ventricular/Hp microglia (**Figure 3D**). However, due to higher basal HLA expression in ventricular/Hp microglia, this effect was not as pronounced as for cortical microglia (**Figure 3E**).

Human adult microglia are not uniformly shaped *in vitro*. Some are rounded and others have longer processes and extensions. In most of the cases (five out of seven cases) it was observed that microglia cultured from the ventricular/Hp region had rounder morphology than cortical microglia (**Figures 3F,G**). This difference in cell morphology was quantified and found to be significant (**Figure 3H**).

A morphological response to M-CSF was observed for both cortical and ventricular/Hp microglia populations. M-CSF caused microglia to become more elongated, confirming previous results (Smith et al., 2013b). The response was found to be quantitatively similar for ventricular/Hp and cortical microglia (data not shown).

Discussion

Here we report differential proliferation and protein expression of adult human microglia from two distinct brain regions from patients with Mesial Temporal Lobe Epilepsy—(1) the cortex and (2) the hippocampus and overlying ventricular lining.

The initial observation of spontaneously dividing ventricular/Hp microglia in normal culture conditions led to the further investigation of microglia from this region of the adult human brain in comparison to microglia from the cortical temporal lobe. Interestingly, microglia from the ventricular/Hp region were found to proliferate at a relatively high rate without growth factor stimulation, whereas cortical microglia have very low rates of basal proliferation. Using Ki67 immunocytochemistry and BrdU proliferation assays it was demonstrated that the increasing number of ventricular/Hp microglia in culture was due to cell division and not just a survival effect. This finding suggests that there are differences in intrinsic cell division mechanisms in these two microglial populations. Marshall et al. (2008) found that the huge expansive capacity of neonatal rodent SVZ microglia was diminished in the adult brain. The present study shows an increased proliferative capacity of human adult ventricular/Hp microglia, although it is relatively small compared to neonatal rodent microglia. CD45 expression has also previously been shown to be higher in rodent SVZ microglia than microglia in non-neurogenic regions (Goings et al., 2006), however, we did not find this to be the case in adult human microglia.

We investigated whether there was a correlation between microglial proliferation and disease severity, however, no correlation was found between the degree of epilepsy and ventricular/Hp microglial proliferation. From the clinical information available no common factor was evident among the three most highly proliferative cases. This may be due to the small sample size, similar age, and male sex of the patients, and the presence of severe epilepsy in all patients undergoing surgery to treat their symptoms. With larger sample sizes it may be possible to identify sub-populations of individuals with differential microglial proliferation in different brain regions. It is also a possibility that sub-sets of microglia with differential proliferative capacity exist *within* different brain regions, and that more highly proliferative microglia sub-sets were sampled from the three cases with the highest levels of proliferation.

Even more pronounced than the differences in basal proliferation was the difference in M-CSF-stimulated proliferation of ventricular/Hp microglia compared to cortical microglia. The majority of cases exhibited a significantly larger increase in microglial proliferation with M-CSF for the ventricular/Hp region compared to the cortex. M-CSF is present in the adult human brain and has been reported to be differentially expressed in varying disease states, thus this growth factor may stimulate microglial proliferation in the intact human brain.

Based on our previous findings with adult human microglia and characterisation of their responses to M-CSF (Smith et al., 2013b), we used a panel of microglial proteins to compare microglia isolated from the cortex and ventricular/Hp regions. To identify the mechanisms behind this differential proliferative response in microglia from two distinct brain regions, expression of the receptor for M-CSF (CSF-1R) was assessed. In concordance with their heightened response to M-CSF, higher expression of CSF-1R protein was found

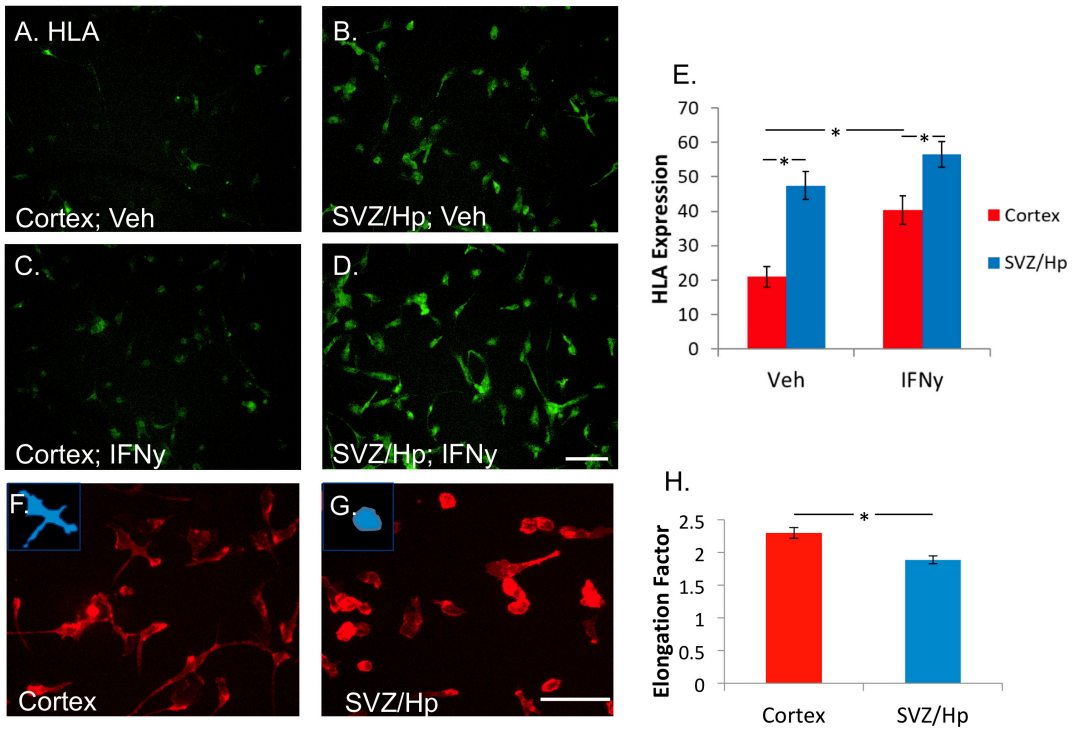

FIGURE 3
Microglia from the ventricular/Hp region express greater levels of HLA-DP, DQ, and DR are more rounded than cortical microglia. **(A)** In basal conditions without any treatment, a variable level of HLA-DP, DQ, DR is expressed by cortical adult human microglia. **(B)** Microglia from ventricular/Hp regions have a higher basal level of HLA-DP, DQ, DR expression. **(C)** IFNy (1 ng/ml, 96 h) increased cortical microglial expression of HLA-DP, DQ, DR, as well as that of ventricular/Hp microglia **(D)**. Scale bar = 100 μm. **(E)** Quantification of HLA expression (arbitrary fluorescence units) showing differential expression by cortical and ventricular/Hp microglia, and a significant increase in HLA expression with IFNy treatment. **(F)** Adult human microglia isolated from cortical tissue immunolabelled with the cell surface marker CD45 have a heterogeneous morphology with various extended processes. **(G)** Microglia isolated from ventricular/Hp regions have a rounder morphology with fewer processes. Insets, in panels **(F,G)** show representative morphology of cells. Scale bar = 100 μm. **(H)** Quantification of microglial morphology using Metamorph *Elliptical Form Factor* (a measure of elongation) image analysis demonstrates a significant difference in microglia shape between cortical and adjacent neurogenic regions. *indicates *P* value < 0.05.

on ventricular/Hp microglia. Higher CSF-1R expression by ventricular/Hp microglia was not paralleled by relatively high PU.1 expression, even though PU.1 has been shown to regulate CSF-1R gene expression (Zhang et al., 1994). However, greater expression of the C/EBPβ transcription factor in ventricular/Hp microglia could be mechanistically involved in their increased response to M-CSF. C/EBPβ has been shown to regulate CSF-1R gene expression (Zhang et al., 1996) and to be involved in M-CSF actions in disease states (Komuro et al., 2003; Marigo et al., 2010). Increased DAP12 expression in ventricular/Hp microglia could also be related to their increased response to M-CSF as we have previously shown that M-CSF treatment increases DAP12 expression in adult human microglia (Smith et al., 2013b), and M-CSF has been found to induce macrophage proliferation *via* DAP12 (Otero et al., 2009). Thus the machinery for M-CSF signalling—CSF-1R, DAP12 adaptor protein and C/EBPβ transcription factor—are more highly expressed in ventricular/Hp microglia, associated with a strong proliferative response to M-CSF.

The mitogenic growth factor IGF-1 may share some functional effects with M-CSF (Wessells et al., 2004; Gow et al., 2010) and has also been reported to be expressed in the adult brain (Connor et al., 1997). Here it was found that the receptor for IGF-1 (IGF-1R) was also expressed at higher basal levels in ventricular/Hp microglia compared to cortical microglia. The finding that protein levels of IGF-1R are increased upon M-CSF stimulation confirms our previous reports with adult human microglia (Smith et al., 2013b). IGF-1 has been reported within neurogenic regions (Anderson et al., 2002). Given the important role that IGF-1 is thought to play in adult neurogenesis, this intriguing finding of increased IGF-1R expression on ventricular/Hp microglia shows that IGF-1 may act through microglia, as well as other cell types including NPCs (Aberg et al., 2003; Monzo et al., 2013), in neurogenic regions to influence neurogenesis.

Regional differences in the response of rodent microglia to cytokine receptor-stimulation have previously been reported, and together these findings raise the question of whether there

are truly different "sub-populations" of microglia in different brain regions or whether all microglia will respond similarly if placed in the same environment (van Weering et al., 2011; Hanisch, 2013). The results from this study show that microglia from different brain regions retain differential phenotype and function *in vitro*, indicating some level of autonomous cell phenotype, but to what extent these microglial phenotypes are reversible *in vivo* is still unknown.

Functional microglial diversity in specific brain regions is likely necessary to accommodate the requirements of different brain regions, for example different energy requirements and cell types. The observation of a distinct profile of microglia from the ventricular lining and Hp, which are adult neurogenic regions, is intriguing as adult neurogenesis requires immune support (Martino et al., 2011; Morrens et al., 2012) but exactly how the finding of increased proliferation of microglia in stem cell niches has an effect on the NPCs residing there is unknown. NPCs and microglia both release trophic and immunomodulatory molecules (Pluchino et al., 2005; Martino and Pluchino, 2007). In fact NPCs are being discovered to have remarkable influence over immune activity and were found to have a distinct secretory protein profile (Mosher et al., 2012). Conditioned medium from primary mouse NPCs was found to induce microglial proliferation, chemotaxis and phagocytosis, while transplantation of NPCs or NPC conditioned medium significantly increased the numbers of dividing microglia *in vivo* (Mosher et al., 2012). Thus it seems that microglia and NPC have a two-way relationship, both contributing to maintenance of the neurogenic niche.

The rounder morphology and higher levels of HLA protein expression of ventricular/Hp microglia compared to their cortical counterparts are generally thought to be indicative of an "activated, pro-inflammatory" microglial phenotype (Graeber, 2010). With a concurrent increase in proliferation, this may identify an important microglia phenotype or sub-type with disease-relevant implications for neurogenic regions in Mesial Temporal Lobe Epilepsy. Furthermore, it is possible that an inflammatory environment resulting from seizure activity is responsible for the increased proliferation and activation of neurogenic region microglia in Mesial Temporal Lobe Epilepsy tissue. Further studies are required to address whether increased proliferation combined with high HLA expression results in a harmful or beneficial microglia phenotype in ventricular/Hp regions, both in patients with Mesial Temporal Lobe Epilepsy and in healthy controls. Specifically, assaying pro-inflammatory cytokine secretion from neurogenic region and cortical region microglia would advance our understanding of the functional implications of the current findings.

Conversely, adult human microglial responses to M-CSF have been shown to result in microglia with "surveying" characteristics. M-CSF induces a major morphological response in adult human microglia whereby they become elongated and bipolar (Smith et al., 2013b). Furthermore, M-CSF has been shown to reduce microglial HLA expression, including that of primary adult human microglia. However, microglia respond to many factors in their environment simultaneously and the phenotypic plasticity of microglia is highly evident here, where ventricular/Hp microglia are shown to be basally "activated" but have massive responses to M-CSF through proliferation, morphology change and multiple protein expression changes.

Our findings are in-line with a recent study of *post-mortem* human microglia which found that SVZ microglia had significantly higher expression of HLA-DR as well as CD45, CD68 (a lysosomal protein), and CD64 (Fc receptor), compared to microglia from the temporal and frontal cortex, by FACS and CyTOF (Böttcher et al., 2019). They also noted that these microglia expressed higher levels of proliferation markers (Böttcher et al., 2019). This suggests that our findings are indeed representative of region-specific differences in microglia and not an artefact of biopsy tissue.

A possible explanation for heightened ventricular/Hp microglial activation is their proximity to immune protein-containing cerebrospinal fluid in the lateral ventricles. It has been demonstrated that microglia in regions of the brain with a less defined blood-brain barrier, and thus increased exposure to plasma proteins, have a less ramified morphology than microglia from other regions (Cuadros and Navascues, 1998; Galea et al., 2007). However, we do not yet know whether the *in vitro* differences in morphology observed here are also present in human brains, or in 3D *in vitro* cultures.

This study utilises biopsy human brain tissues which are invaluable for understanding adult human brain disorders but which undoubtedly come with several limitations. As biopsy brain tissue cannot be taken from healthy individuals, this study is limited to tissue from chronically diseased individuals. It is unknown to what extent the disease process has led to the observations in this study, and whether the same holds true for healthy human brain tissue. Furthermore, although the seizure focus was removed by a pathologist before separation of cortical and ventricular/Hp tissue and not used for microglia isolation, an alternative explanation for the microglial differences observed in these two brain regions could be their relative distance from the seizure focal point, as even the temporal cortex microglia may be influenced by seizure activity in the neighbouring hippocampus, or a response to ongoing neurodegeneration in the hippocampal area as part of epilepsy pathology. Despite these limitations, use of this tissue forms an important part of our understanding of the role of microglia in the adult human brain.

In conclusion, we report fundamental differences in two regional populations of microglia in the adult human brain. Specifically, we observed higher rates of proliferation in neurogenic region microglia and a heightened proliferative response to the mitogen M-CSF. Furthermore, neurogenic region microglia display characteristics of activation. The functional differences among microglia based on regional variation adds to the emerging view of microglia as a highly heterogeneous and phenotypically diverse cell type and may

have relevance to the maintenance of the neurogenic niche in the adult human brain.

Data availability statement

The original contributions presented in this 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 University of Auckland Human Participants Ethics Committee and Northern Regional Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

Author contributions

AS and MD conceived and designed the experiments, interpreted the data, and wrote the manuscript. AS performed the cell isolation, cell culture, immunocytochemistry, image acquisition, and analysis. TP performed the isolation and culture of cells. MA performed the immunocytochemistry. RO, PB, EM, and RF contributed to materials and clinical information and revised the manuscript. All authors read 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/fncel.2022.1047928/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Blocking of non-specific antibody binding to Fc receptors did not alter staining patterns. Following fixation, cells were incubated with PBS or purified recombinant Fc protein to block non-specific binding of antibodies to the Fc receptor. Staining was then performed using the indicated primary and appropriate secondary antibodies at the concentrations used for all other experiments. Representative images are shown. Scale bar = 100 μm.

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Lestaurtinib inhibits Citron kinase activity and medulloblastoma growth through induction of DNA damage, apoptosis and cytokinesis failure

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Introduction: Medulloblastoma (MB), the most common malignant pediatric brain tumor, is currently treated with surgery followed by radiation and chemotherapy, which is accompanied by severe side effects, raising the need for innovative therapies. Disruption of the microcephaly-related gene Citron kinase (CITK) impairs the expansion of xenograft models as well as spontaneous MB arising in transgenic mice. No specific CITK inhibitors are available.

Methods: Lestaurtinib, a Staurosporine derivative also known as CEP-701, inhibits CITK with IC50 of 90 nM. We therefore tested the biological effects of this molecule on different MB cell lines, as well as in vivo, injecting the drug in MBs arising in SmoA1 transgenic mice.

Results: Similar to CITK knockdown, treatment of MB cells with 100 nM Lestaurtinib reduces phospho-INCENP levels at the midbody and leads to late cytokinesis failure. Moreover, Lestaurtinib impairs cell proliferation through CITK-sensitive mechanisms. These phenotypes are accompanied by accumulation of DNA double strand breaks, cell cycle block and TP53 superfamily activation in vitro and in vivo. Lestaurtinib treatment reduces tumor growth and increases mice survival.

Discussion: Our data indicate that Lestaurtinib produces in MB cells poly-pharmacological effects extending beyond the inhibition of its validated targets, supporting the possibility of repositioning this drug for MB treatment.

KEYWORDS

Lestaurtinib, CitK, drug repurposing, medulloblastoma, preclinical studies

1 Introduction

Medulloblastoma (MB) is the most common malignant pediatric brain tumor (1, 2) and has been traditionally classified into four biological subgroups: WNT, Sonic Hedgehog (SHH), Group 3 and Group 4 (3–6). Due to the overlap between Group 3 and Group 4, it has recently been re-classified in: WNT-activated, SHH-activated and TP53-wildtype, SHH-activated and TP53-mutant, non-WNT/non-SHH (7). In general, MB is treated with surgery, followed by radiation of the entire neuro-axis and high dose multi-agent chemotherapy (2). Despite long-term survival can be as high as 90% in the rare WNT subgroup, it is on average around 50% in the other subtypes, with worse prognosis in non-WNT/non-SHH patients (8). Moreover, radio-chemotherapy is frequently accompanied by severe neurological, cognitive, and endocrine side effects (8). For these reasons, effective therapies less disruptive of normal physiology are an unmet medical need.

MB cells share many molecular pathways with progenitor cells of the cerebellum (9–11). On this basis, genes that are selectively required during development for proliferation and genomic stability of normal neural progenitors may represent attractive targets for MB drug discovery. An interesting group of such genes is represented by primary hereditary microcephaly genes (MCPH), whose loss leads to significant reduction of head circumference and brain volume (12–14). Many MCPH genes have been proposed as specific targets for brain tumors therapy (15–17).

Citron Kinase protein (CITK) is the main product of the *CIT* gene, whose mutations are responsible for MCPH17 (18, 19). CITK is required in neural progenitors for cytokinesis (20, 21), mitotic spindle positioning (22) and chromosomal stability (23). Its loss induces DNA damage and apoptosis in SHH and non-WNT/non-SHH MB cells and reduces growth of both xenograft and transgenic MB (24, 25). CITK downregulation potentiates the effects of ionizing radiations (IR) and cisplatin treatment in reducing growth potential and colony forming activity of MB cells (24). Interestingly, these anti-proliferative effects of CITK loss may be engaged through TP53-dependent and TP53-independent mechanisms (24, 25). Genetic evidence (18), as well as rescue experiments in MB cells (25), indicate that kinase activity is essential for physiological and tumor-sustaining functions.

Although specific inhibitors of CITK activity are not yet available, assessment of 72 inhibitors against 456 human kinases (26) showed that Lestaurtinib has relatively high affinity for this protein.

2 Materials and methods

2.1 Kinase assays

For radioactive kinase assay, 150 ng of recombinant CITK (ab161903, Abcam, Cambridge, MA, USA) and 500 ng of MYPT1 (654-880) (#12-457, Merck, Sigma-Aldrich, Burlington, MA, USA) were incubated in kinase buffer (50mM Hepes pH7.4, 10mM MgCl2 5mM MnCl2, 0,5mM DTT) with 1μM of ATP and 5 mCi of [γ32P] ATP (6000 Ci mmol-1) (PerkinElmer), for 30 minutes at 30°C.

For ATP-consumption assays, ADP-Glo™ Kinase Assay (V6930, Promega Corporation, Madison, WI, USA) was used according to manufacturer protocol, with the indicated concentrations of Lestaurtinib (Tocris Biotechne group, Minneapolis, MN, USA).

2.2 Cell culture

ONS-76 cells (MB subtype: SHH) were kindly provided by Luigi Varesio (Gaslini Hospital, Genoa, Italy) and were cultured in RPMI medium (Euroclone, Milan, Italy) with 10% fetal bovine serum (FBS, Gibco, Gaithersburg, MD, USA). DAOY (MB subtype: SHH) and D283 (MB subtype: non-WNT/non-SHH) cells were obtained from ATCC and were cultured in MEM medium (Euroclone) and 10% FBS (Gibco). D341 cells (MB subtype: non-WNT/non-SHH) were obtained from ATCC and were cultured in MEM medium and 20% FBS (Gibco). Culture media were supplemented by nonessential amino acids, L-glutamine and sodium pyruvate (Gibco). All cell lines, were passed between 5 and 10 times from thawing of the original aliquots and routinely tested for Mycoplasma contamination. All cells were grown at 37°C, in a humidified incubator, with 5% CO_2.

2.3 Transfection of RNAi and constructs

Transfection for RNAi was performed as previously described (24) with published CITK double-stranded RNA (27). Non-targeting pool (Dharmacon, Lafayette, CO) was used as a negative control. Cells plated on six-well plates were transfected using 6.25 μl of the required siRNA (20 μM) together with 1.5 μL Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. To over express Cherry-tagged wild type CITK and kinase dead constructs, ONS-76 cells were transfected by Transit-it x1 (Mirus Bio LLC, Madison, WI, USA). The constructs used in this manuscript were previously published (27).

2.4 Analysis of cell proliferation

To analyze cell proliferation, 25000 ONS-76 and DAOY cells were seeded into 12-well plate, in medium containing DMSO or 100 nM Lestaurtinib and counted in triplicates after 24, 48 and 72 hours with Bio-Rad TC20 Automated Cell Counter. 20000 D283 cells and 30000 D341 cells were seeded into 12-well plate, in medium containing DMSO or 100 nM Lestaurtinib and counted in triplicates after 24, 48, 72 and 96 hours.

2.5 Colony forming assay

For colony forming assay, 300 cells were plated at day 1 for ONS-76 and DAOY, in medium containing DMSO or 100 nM Lestaurtinib. For D283 and D341, 6000 and 10000 cells were plated, respectively. Clonogenic assay were stopped after 7-10 days. After medium removal, colonies were incubated for 10 min with Nissl staining and then rinsed in water.

2.6 FACS analysis

For cell cycle analysis, cells were plated in 6-well plates and treated with DMSO or 100 nM Lestaurtinib for 24 hours. 50000 cells were collected, centrifuged at 200 g for 5 min, fixed in cold 70% ethanol overnight, washed with PBS two times and finally stained with 5 µL of propidium iodide (from 1 mg/mL of stock) in 500 µL of PBS and RNAse. For apoptosis detection, cells were plated in 6-well plates and treated with DMSO or 100 nM Lestaurtinib for 24 hours. 50000 cells were collected, centrifuged at 200 g for 5 minutes, and resuspended in 300 µL of binding buffer. Cells where then incubated with Annexin V FITC (3 µL) and propidium iodide staining solution (5 µL) for 15 to 30 minutes before flow cytometry.

2.7 CellTox™ green cytotoxicity assay

CellTox™ Green Reagent (Promega) was added to each well at the end point of treatment according to the manufacturer's instructions.

2.8 CellTiter-Glo® luminescent cell viability assay

CellTiter-Glo® Reagent (Promega) was added to each well at the end point of treatment according to the manufacturer's instructions.

2.9 Antibodies

The following antibodies were used: mouse monoclonal anti-citron (#611377; Transduction Laboratories, BD Biosciences, Franklin Lakes, NJ, USA), mouse monoclonal anti-vinculin (#V9131), mouse monoclonal anti-αTubulin (#T5168) from Sigma-Aldrich; rabbit polyclonal anti-cleaved Caspase 3 (#9661S), anti-TP53 phospho Ser15 (#9284S), anti-γH2AX (S139; 20E3; #2577), anti FLT3 (8F2 #3462) and anti-INCENP (P240, #2807) from Cell Signaling Technology (Danvers, DA, USA); rabbit polyclonal anti-TP73 (#ab14430) and rabbit monoclonal anti-53BP1 (#ab36823) from Abcam (Cambridge, UK); rabbit monoclonal anti-RAD51 (#sc-8349) and rabbit polyclonal anti p21 (#sc-756) from Santa Cruz (Dallas, TX, USA), rabbit polyclonal anti-pTSS INCENP (a kind gift of M.A. Lampson) (28).

2.10 Western blotting

Cells and tissues were lysed in RIPA buffer (1% NP40, 150 mmol/L of NaCl, 50 mmol/L of Tris-HCl pH 8, 5 mmol/L of EDTA, 0.01% SDS, 0.005% sodium deoxycholate, Roche protease inhibitors and PMSF) for 10 minutes, at 4 °C. Samples were clarified 10 min at 13,000 rpm at 4 °C. Mouse tissues were homogenized in the same buffer with pellet pestle (Z359971 Sigma-Aldrich). For immunoblots, equal amounts of proteins from both whole-cell lysates were resolved by SDS–PAGE and blotted to nitrocellulose membranes.

2.11 Immunofluorescence

Cultured cells were fixed for 10 min at RT with PFA 4%. D341 treated cells were first spun in Thermo Scientific Cytospin 4 (Thermo Fisher Scientific) at 500 rpm, for 10 min, and then processed for immunofluorescence (IF) as previously published (24). Primary antibodies were detected with anti-rabbit Alexa Fluor 488 or 555 and/or anti-mouse Alexa Fluor 488 or 555 (Molecular Probes, Invitrogen), used at 1:1000 dilution, for 30 min. Cells were counterstained with 0.5 µg/mL of DAPI for 10 min and washed with PBS. Finally, cell slides were mounted with Prolong (Thermo Fisher Scientific).

Tumors were fixed in 4% paraformaldehyde in PBS overnight at 4°C, slowly dehydrated with 30% sucrose, embedded with Tissue-TEK (O.C.T., Sakura Finetek, Alphen aan den Rijn, The Netherlands) and frozen by using isopentane. Twenty-micrometers of cryo-sections were rehydrated in PBS and processed for antigen retrieval 1h at 70°C in Antigen Retrieval Buffer (10 mM Na-Citrate pH 6.0, 10% Glycerol in PBS). Primary antibodies were diluted in Tx buffer (0,3% Triton X-100, 0,2% gelatin, 300 mM NaCl in PBS). Secondary antibodies were used at dilution 1:1000 in Tx buffer for 1 hour at RT. TUNEL assay was performed using the Click-iTTM Plus TUNEL Assay (Invitrogen) according to manufacturer protocol. To quantify midbody fluorescence signals, we used Integrated density from Fiji software, that is the sum of the values of the pixels in the image or selection, subtracting the cytoplasmic background; we then calculated the mean of control midbodies and used that value as reference for all midbodies of the same experiment (controls and treated).

2.12 Mouse strain

The mouse strain ND2:SmoA1 (expressing the constitutively active point mutant SmoA1 under the Neurod2 promoter in cerebellar granule cells), in congenic C57BL/6J background, was obtained from The Jackson Laboratory.

2.13 Experimental animal work

Experiments involving samples from mice treated with DMSO or Lestaurtinib have been performed conforming to the Italian laws on animal experimentation, under permission number 1128/2020-PR, released on 16th November 2020 from Italian Ministry of Health, Department of Public Veterinary Health.

2.14 Xenograft assays

Subcutaneous medulloblastoma xenografts were obtained by transplanting in eight-week-old male NOD-SCID mice (Jackson Laboratory) 1x10^6 ONS-76 cells in the flank. After 4 weeks, mice were treated with 10uL of DMSO or Lestaurtinib 100nM once a

week for 4 weeks. Xenograft tumors were measured every week and tumor volume was estimated as $4\pi r^3/3$.

2.15 Lestaurtinib *in vivo* treatment

Mice showing hunched posture, head tilt or weight loss were anesthetized by intraperitoneal administration of Ketamine (100 mg/ml; MSD Animal Health, Segrate, Italia) supplemented by Xylazine (20 mg/ml; Bayer; Leverkusen, Germany) and then placed in the stereotaxic apparatus. Mice were randomly divided into control and treatment groups. The treatment group received a stereotaxic microinjection of 1 µl of 100 nM Lestaurtinib in PBS in the tumor mass. The control group received a stereotaxic microinjection of 1 µl of DMSO in PBS.

2.16 MRI

MRIs were acquired at 7T with a Bruker Neo Avance (Bruker, Ettlingen, Germany) scanner with a 1H quadrature mouse brain volume coil. The animals were scanned every 7 days, starting from 10 weeks of age, to check the presence of tumors through T2-weighted (T2w) high-resolution images acquired with the following parameters: repetition time (TR) =4,000 milliseconds; echo time (TE) = 35.44 milliseconds; rare factor (RF) = 16; slice thickness= 0.5 mm; slice geometry = axial; number of slices = 20;field of view = 2.00 cm;matrix = 256 x 256;number of averages (NAV) = 3; total imaging time: 3 minutes 12 seconds. Animals bearing a tumor mass (size 1–15 mm3) were treated either with a stereotaxic microinjection of 100 nM Lestaurtinib or normal saline (control) starting from week 0 (first administration) to week 4, and imaged by MR with the sequence parameters reported above starting from week 0 to week 7, to monitor tumor progression. Before undergoing MRI, animals were anesthetized by intramuscular injection of 5 mg/kg of xylazine (Rompun; Bayer) and 20 mg/kg of tiletamine/zolazepam (Zoletil 100; Virbac). Tumor volume was assessed in each animal through MR data analysis, carried out with Fiji software. More in details, the tumor area was delineated in each slice of T2w high-resolution images by manually drawing regions of interest along tumor borders. The tumor volume in each slice was estimated multiplying each tumor area for the slice thickness (0.5 mm). Finally, the total tumor volume was estimated by adding up all the single slice volumes.

2.17 Survival analysis

Kaplan–Meier method was used to estimate survival. Ten-week-old littermates were divided in DMSO and Lestaurtinib cohorts and treated with once a week for 4 consecutive weeks with a stereotaxic microinjection of DMSO and Lestaurtinib 100 nM, respectively. All mice were euthanized at the onset of symptoms.

2.18 Statistical analysis

Statistical analyses were performed using Microsoft Office Excel (Version 16, Microsoft Corporation, Redmond, WA, USA) and GraphPad (Version 8, GraphPad Software, San Diego, CA, USA). Unpaired two-tails Student's *t*-test was used if not otherwise specified. Data are shown as the mean values of at least 3 independent experiments and standard error of the mean (mean ± SEM). Mann-Whitney test was used to analyze 53BP1 foci and Chi-Square test for percentage distribution using absolute frequency of experiments.

3 Results

3.1 Lestaurtinib inhibits citron kinase enzymatic functions

Since Lestaurtinib could bind CITK with high affinity (26), we evaluated if it could also inhibit CITK catalytic activity. We first resorted to a non-radioactive *in vitro* kinase assay (28) based on the recombinant catalytic domain of the human protein and on MYPT1, a known substrate of myotonic dystrophy kinase family members, which include CITK (29). Increasing concentrations of Lestaurtinib reduced the ATP consumption, with an IC50 of 90 nM (Figure 1A). We validated this data using the same enzyme and substrate to perform a radioactive kinase assay. Also in this case, we observed a consistent reduction in both CITK autophosphorylation and MYPT1 phosphorylation, at Lestaurtinib concentrations comparable to those effective in the non-radioactive assay (Figure 1B) (29).

We also tested whether, in intact cells, 100 nM Lestaurtinib could affect the phosphorylation of INCENP at residues 834-902, which has been reported as a CITK-dependent event (30). We tested this hypothesis on ONS-76, a MB SHH TP53 wildtype cell line known to be sensitive to CITK depletion. Lestaurtinib treatment significantly reduced phospho-INCENP at midbody, the narrow intracellular bridge that connects two daughter cells at the end of cytokinesis, without altering total protein levels in the same structure (Figures 1C-F).

It is well known that loss of CITK leads to cytokinesis failure in many cell types, including MB cells (24, 25). To test whether the same phenotype could be produced by Lestaurtinib, we used 4 different cell lines representing all aggressive MB subtype: ONS-76, DAOY for SHH MB TP53 mutated, D283 and D341 for non-WNT/non-SHH subtypes (7). In all the MB cells tested, 100 nM Lestaurtinib significantly increased the percentage of binucleated cells (Figures 1G, H). Time lapse microscopy analysis of Lestaurtinib-treated ONS-76 cells showed that the increase in binucleation is produced because of late cytokinesis failure, following relatively normal cleavage furrow ingression and initial midbody formation (Figure S1A, movie. S1 and S2). By western blot, we observed that Lestaurtinib treatment does not alter CITK protein levels in MB cell lines (Figure S1B, C). These data indicate that 100 nM Lestaurtinib may significantly inhibit CITK activity, as well as the late stages of cytokinesis which are affected by CITK loss (20, 27).

3.2 Lestaurtinib reduces proliferation of MB cells through CITK-sensitive mechanisms and impairs their clonogenic potential

We next tested whether Lestaurtinib affects the *in vitro* expansion of SHH and non-WNT/non-SHH cell lines. Similarly

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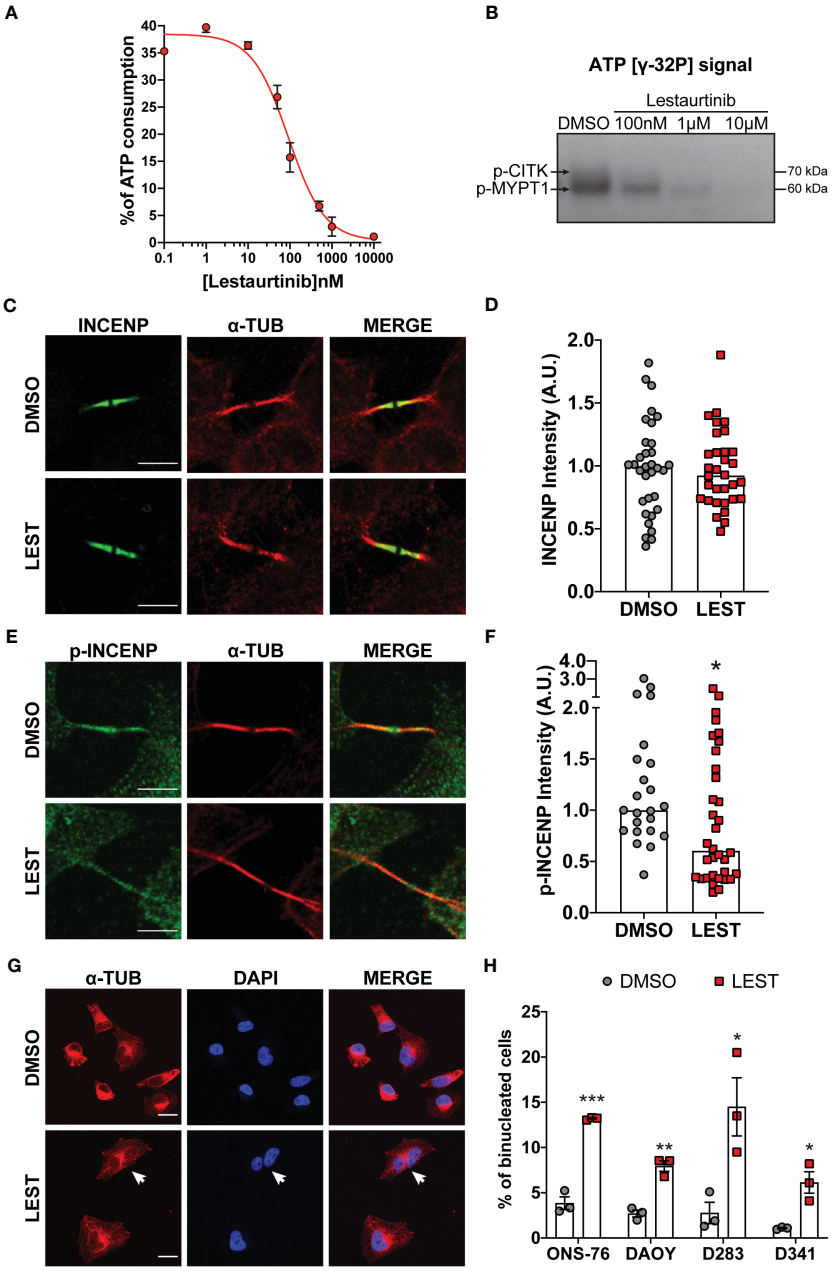

FIGURE 1
Lestaurtinib inhibits Citron Kinase catalytic activity and induces cytokinesis failure. **(A)** Quantification of ATP consumption was measured with ADP-Glo™ Kinase Assay, by incubating CIT kinase domain with recombinant human MYPT1 (see methods) and the indicated concentrations of Lestaurtinib. **(B)** CITK domain was incubated with MYPT1 in the presence of [γ-32P] ATP. The reactions were then analyzed by SDS-PAGE and autoradiography. p-CITK arrow indicates auto-phosphorylation, p-MYPT1 arrow the substrate phosphorylation. **(C-F)** Analysis of high magnification images of midbodies, in ONS-76 cells treated for 24 hours with DMSO or 100 nM Lestaurtinib (LEST) and immunostained for INCENP **(C)**, phospho (p)-INCENP **(E)**, as well as α-tubulin. Midbody intensity of INCENP and p-INCENP was quantified (**D** and **F**, respectively). Scale bars: 5 μm. **(G)** Representative IF image of ONS-76 cells analyzed 24 hours after treatment with DMSO or 100 nM Lestaurtinib and immunostained for α-tubulin. White arrow indicates a typical binucleated cells. Scale bars: 20 μm **(H)** Quantification of binucleated cells in ONS-76, DAOY, D283 and D341 treated as in **(G)** Each dot indicates an independent biological replicate. >200 cells were counted for each treatment conditions in each experiment. All quantifications were based on at least three independent biological replicates. *P<0.05, **P<0.01 ***P<0.01; unpaired two-tailed Student's t-test. A.U., arbitrary units.

to CITK downregulation (24, 25), 100 nM Lestaurtinib reduced the proliferation of all the tested MB cell lines (Figures 2A-D). Interestingly, the effect was particularly strong in the D283 cell line (Figure 2C), which are very sensitive to CITK loss (24). Moreover, Lestaurtinib reduced the clonogenic potential of all tested cell lines, as shown by colony forming assay (Figures 2E, F). Both the number and the size of the colonies were reduced.

To assess whether CITK is involved in the anti-proliferative effect produced by Lestaurtinib, we analyzed the growth potential of treated versus untreated ONS-76 cells, expressing control or CITK-specific

siRNAs. Compared to the untreated control, cell growth was reduced in all cases, but the effects of Lestaurtinib and CITK loss were not additive (Figure 2G). Next, we treated with 100 nM Lestaurtinib ONS-76 cells over expressing Cherry protein (control), as well as Cherry-tagged fusions of wild-type CITK and kinase-dead protein (CITK-D) (31). Interestingly, the active version of CITK rescued the Lestaurtinib anti-proliferative effect, while CITK-D had no effect (Figure 2H). Similar results were obtained for DAOY, D283 and D341 cell lines (Figures S2A, B). We next assessed the possible role in the MB context of known Lestaurtinib targets. FLT3, the target against which Lestaurtinib was first developed (32, 33), is not expressed at high levels in MB cell lines (Figure S2C). The other best known Lestaurtinib target is JAK2 (34). Accordingly, In MB cell lines, phosphorylation of the prominent JAK2 substrate STAT3 was inhibited as well as by the more selective JAK2 inhibitor Ruxolitinib (35) (Figures S2D, E). Nevertheless, Ruxolitinib did not impair proliferation of ONS-76 at any tested concentration and significantly reduced DAOY proliferation only at 10 µM concentration (Figure S2F). Taken together, these results are consistent with a relatively specific involvement of CITK activity in the growth-inhibitory effects produced by Lestaurtinib on MB cells.

3.3 Lestaurtinib induces cell cycle block, apoptosis and TP53 super family activation in MB cells

We next assessed whether Lestaurtinib treatment leads to the other prominent phenotypes observed in CITK-depleted MB cells: cell cycle arrest and apoptosis. Flow-cytometry analysis revealed that, in ONS-76 and DAOY, 24 hours of Lestaurtinib treatment produces a significant increase of cells in the G0/G1 phase, as well as a significant reduction of the proportion of tetraploid cells (Figures 3A, B). These results are consistent with a strong inhibition of cell-cycle progression, especially if considering the increase of binucleated cells detected by IF (Figures 1G, H). Moreover, flow cytometric determination of cells positive for the early apoptosis marker Annexin V revealed that the percentage of ONS-76 and DAOY cells undergoing programmed cell death is significantly increased (Figures 3C, D). Consistently, western blot and IF showed increased levels of cleaved caspase 3 (cCASP3) (Figures 3E-G). These phenotypes were associated with an induction of phosphorylated-TP53 protein in both ONS-76 and DAOY cells (Figure 3E). In addition, the latter cell line, which is known to express a mutated form of TP53 (36), showed increased levels of TP73 protein (Figure 3E). Accordingly, in both cell lines Lestaurtinib induced high levels of P21 (Figures 3E, F), a prominent downstream effector of TP53 family proteins (25). Similarly, we observed in Lestaurtinib treated D283 and D341 cells an increased percentage of cCASP3 positive cells by IF (Figures 3H, I). Finally, we observed in all Lestaurtinib treated cells an increase in cell death (Figure S3A), and a reduction of cell viability (FigureS3B). These data indicate that, as in the case of CITK knockdown, Lestaurtinib treatment for 24 hours induces cell cycle arrest and cell death in all MB cell lines, possibly involving activation of TP53 family downstream pathways (24, 25).

3.4 Lestaurtinib treatment increases DNA damage in MB cells

CITK loss leads to DNA double strand breaks accumulation (23–25), associated with reduced nuclear levels of the DNA repair protein RAD51 and impaired homologous recombination (24). Thereby, we wondered whether this also occurs in MB cells after Lestaurtinib treatment. In all cell lines, 24 hours of treatment with 100 nM Lestaurtinib induced significantly increased frequency of 53BP1 foci per nucleus (Figures 4A, B), which represent a consolidated marker for DNA double strand breaks. Moreover, compared to control conditions, we observed by western blot increased levels of γ H2AX (marker of any DNA damage), and reduced levels of total RAD51 (Figures 4C, D). These data indicate that Lestaurtinib induces DNA damage accumulation in MB cells, suggesting an impairment of homologous recombination.

3.5 Lestaurtinib reduces tumor growth in SmoA1 MB bearing mice and increases survival

We next evaluated whether the effects produced by Lestaurtinib *in vitro* may also occur *in vivo*. First, we performed a xenograft assay by subcutaneously injecting into immunodeficient mice ONS-76 cells. Palpable tumors developed within 4 weeks after injection and mice were treated with a weekly injection of 10 uL of DMSO or Lestaurtinib 100nM in the tumor mass. Compared to the rapid growth which we observed in DMSO- treated tumors, Lestaurtinib-injected tumors grew at significantly slower rate (Figures S4A-B). To analyze the effects of Lestaurtinib on a model that may more faithfully reproduce the conditions of human MB, we then moved to the ND2:SmoA1 mouse model, in which a constitutively active SmoA1 point mutant is expressed in cerebellar granule cells under the Neurod2 promoter, leading to a high incidence of MB (37). Tumors developing in this model closely mimic the human SHH MB type, since they are generated by accumulation of random mutations after the initial driver event, within immune competent animals (38). In particular we concentrated only on male mice, since the effects of estrogen on MB mouse model is debated (39, 40). To avoid the potential complication of the blood-brain barrier, we delivered Lestaurtinib directly to the tumor mass *via* stereotaxic injections. By histological analysis, we observed a strong increase in the frequency of pyknotic nuclei, throughout the tumor mass of Lestaurtinib treated tumors (Figures 5A, B). Accordingly, we detected significantly increased frequency of cells positive for cCASP3 and TUNEL (Figures 5C, D) (41–43). Interestingly, the non-affected cerebellar tissue, as well as the rest of CNS, did not show an increased number of apoptotic cells, in both histopathological and immunohistochemical analyses (data not shown). Finally, by western blot, we observed a significant increase of cCASP3 and γ H2AX in the tumor mass of Lestaurtinib treated mice, compared to DMSO treated mice (Figures 5E, F). These data indicate that Lestaurtinib injection is effective in inducing DNA damage and apoptosis in an orthotopic model of medulloblastoma. To assess the overall antitumor effect of Lestaurtinib treatment, we monitored by

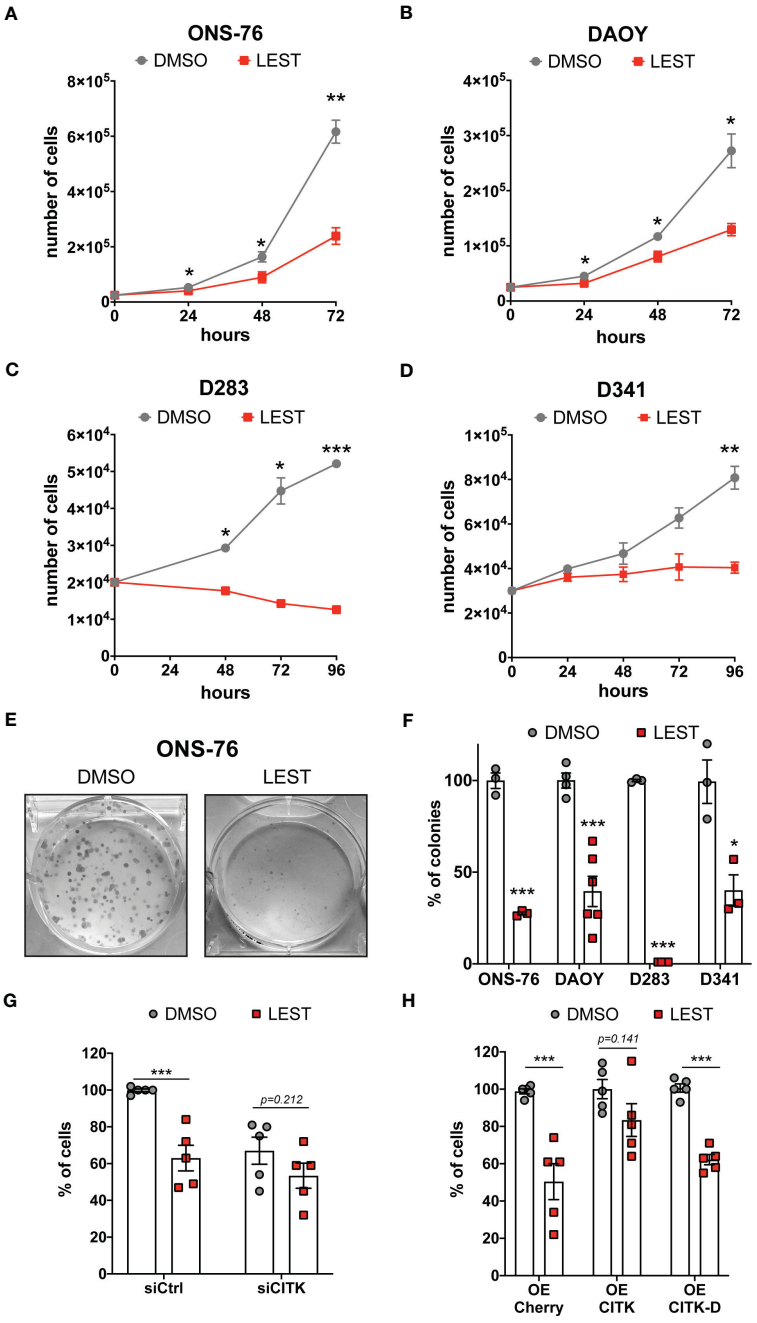

FIGURE 2
Lestaurtinib reduces MB cells proliferation and impairs the clonogenic potential **(A-D)** Cell proliferation assay: 25,000 ONS-76 **(A)** and DAOY cells **(B)** 20,000 D283 cells **(C)** and 30,000 D341 cells **(D)** were treated with DMSO or 100 nM Lestaurtinib. Growth curves were obtained by assessing cells' number in each well at the indicated times after treatment. **(E)** Images of ONS-76 colonies 10 days after treatment with DMSO or 100 nM Lestaurtinib and stained with crystal violet. **(F)** Quantification of the percentage of colonies formed after DMSO or 100 nM Lestaurtinib treatment; each dot indicates an independent biological replicate. **(G)** Quantification of ONS-76 cell number 48 hours after transfection of non-targeting (siCtrl) or CITK-specific siRNA (siCITK), treated with DMSO or 100nM of Lestaurtinib during the last 24 hours of the experiment. Each dot indicates the percentage of the corresponding negative control. **(H)** Quantification of ONS-76 cells number 48 hours after transfection of Cherry (control), wild type Cherry-tagged CITK (CITK) and K126A inactive mutant (CITK-D), treated with DMSO or 100nM of Lestaurtinib during the last 24 hours of the experiment. As above, numbers are expressed as percentage of control, in independent biological replicates. All quantifications were based on at least three independent biological replicates. Error bars, SEM. *P<0.05, **P<0.01 ***P<0.01; unpaired two-tailed Student's t-test.

MRI the growth of tumors that received four injections in consecutive weeks. This analysis showed that Lestaurtinib injection impairs medulloblastoma growth, from the beginning of treatment to three weeks after the last injection (Figures 6A, B). Finally, we evaluated whether Lestaurtinib significantly ameliorates the survival of SmoA1 mice. In particular, we treated transgenic mice with weekly intratecal Lestaurtinib or control injections, during four consecutive weeks, starting from 10 weeks, a time at which 90% of SmoA1 mice develop MB (25, 38). Long term follow up showed a significant increase in survival of the Lestaurtinib-treated mice (Figure 6C).

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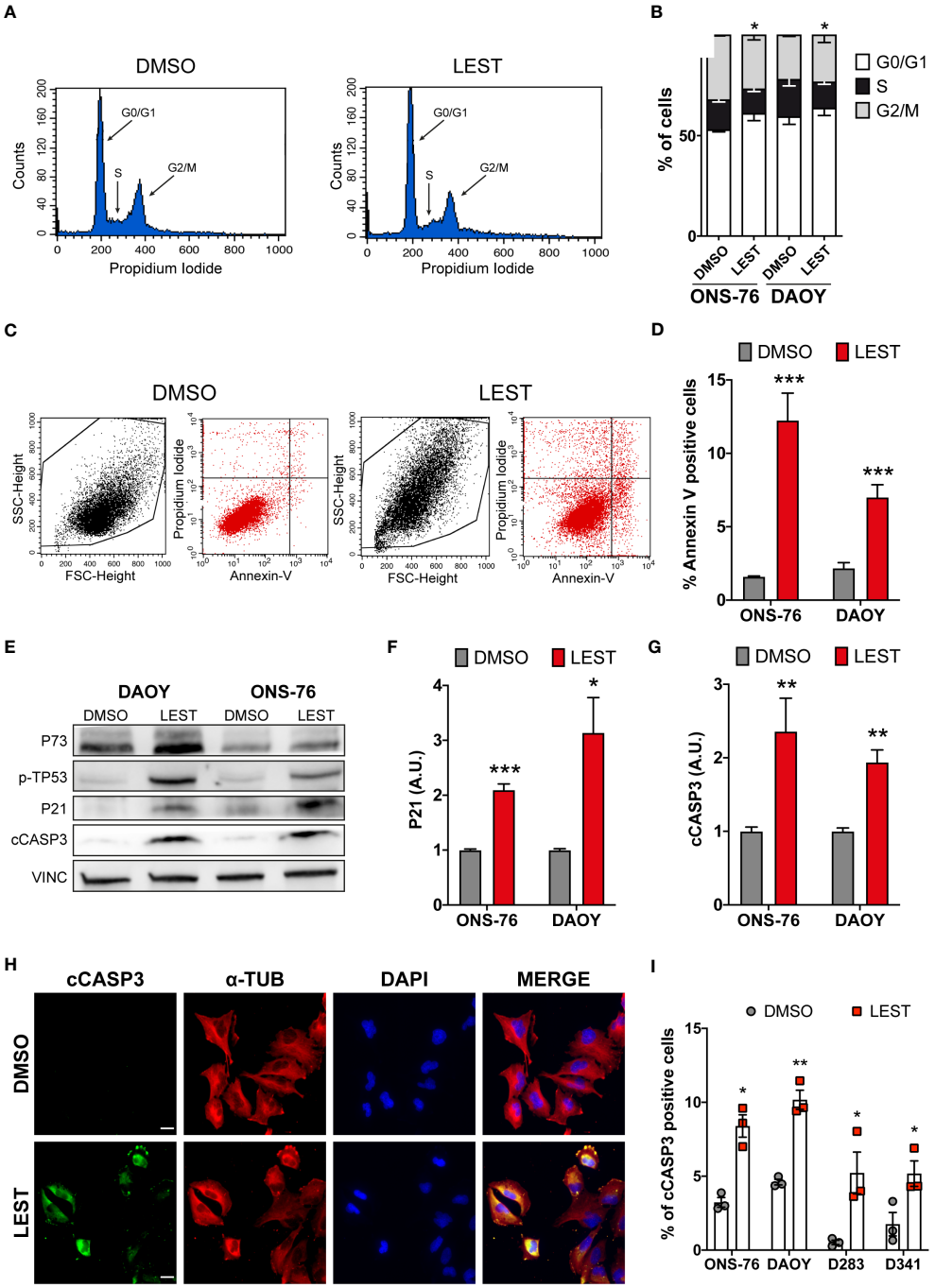

FIGURE 3
Lestaurtinib induces cell cycle block and apoptosis. **(A)** Exemplar cell-cycle profile of ONS-76 cells treated for 24 hours with DMSO or 100 nM Lestaurtinib. G0/G1, S and G2-M phases are shown. **(B)** Quantification of the percentage of ONS-76 and DAOY treated cells in the different cell-cycle phases; p=0,019 for ONS-76, p=0,04 for DAOY, Chi-Square test. **(C, D)** Quantification of Annexin V positive ONS-76 and DAOY cells, 24 hours after treatment with DMSO or 100 nM Lestaurtinib. **(E)** Western blot analysis of total lysate from ONS-76 cells and DAOY cells, 24 hours after treatment with DMSO or 100 nM Lestaurtinib. The levels of phospho-TP53, TP73, P21 as well as cleaved caspase 3 (cCASP3) were analyzed (loading control vinculin, VINC). **(F, G)** Quantification of the relative density of P21 **(F)**, and cCASP3 **(G)** in 4 replicates of the experiments shown in **(E)** Values are expressed as a ratio over the DMSO control average **(H)** Representative image of ONS-76 cells processed for immunofluorescence 24 hours after treatment with DMSO or 100 nM Lestaurtinib and immunostained for cleaved caspase 3 (cCASP3) and α-tubulin. Scale bars: 5 µm. **(I)** Quantification of the percentage of cCASP3 positive cells in the indicated cell lines, 24 hours after treatment with DMSO or 100 nM Lestaurtinib. All quantifications were based on at least three independent biological replicates. Error bars, SEM. *P<0.05, **P<0.01 ***P<0.01; unpaired two-tailed Student's t-test. Scale bars: 5 µm. A.U., arbitrary units.

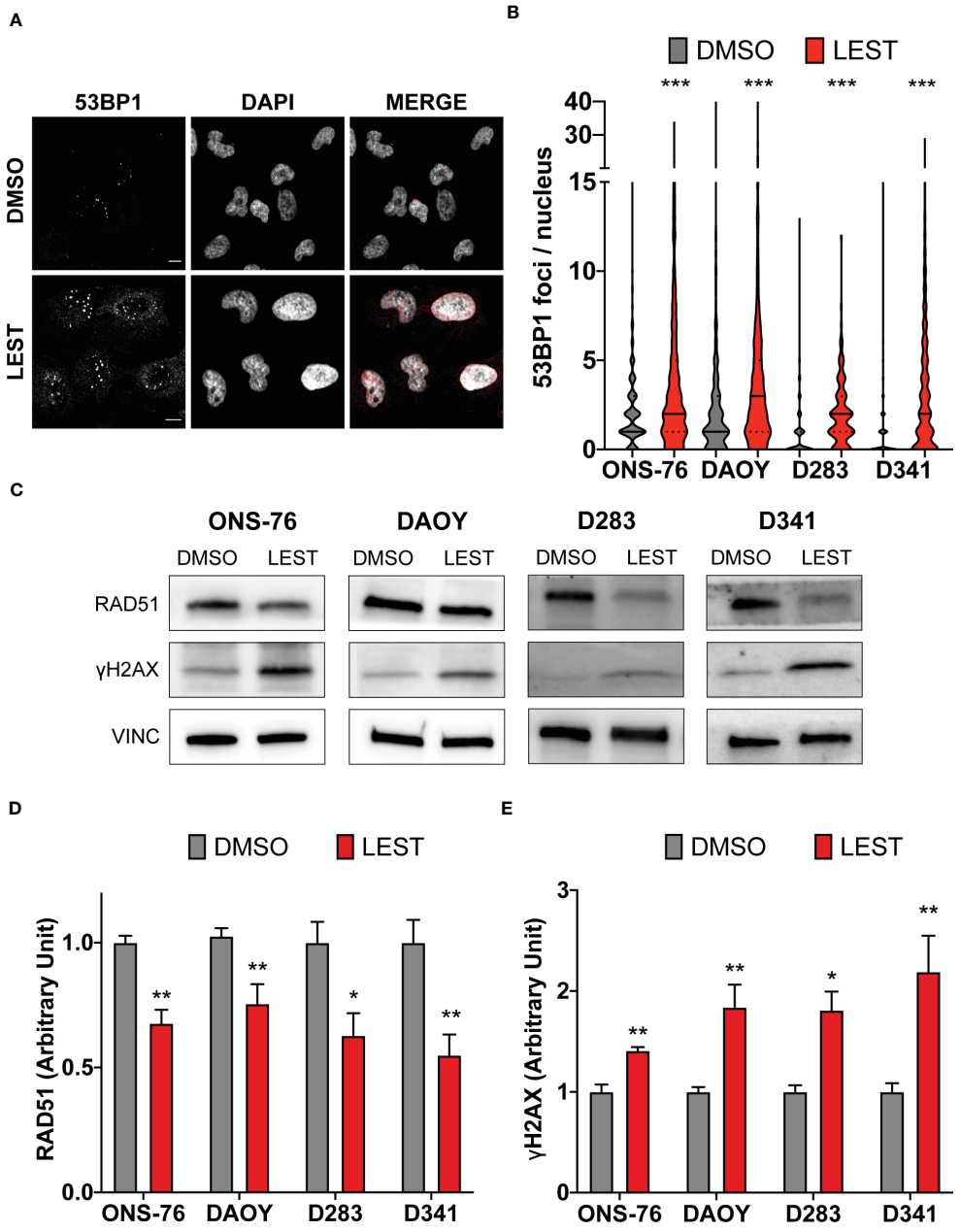

FIGURE 4
Lestaurtinib induces DNA damage accumulation in MB cells. **(A)** Representative image of ONS-76 cells immunostained for 53BP1 24 hours after treatment with DMSO or 100 nM Lestaurtinib. Scale bars, 10 µm. **(B)** Quantification and 53BP1 nuclear foci in the indicated cell lines, treated as in **(A)**; >250 cells were counted for each treatment conditions in each replicate. **(C)** Western blot analysis of total lysate from the indicated cell lines, 24 hours after treatment with DMSO or 100 nM Lestaurtinib. The levels of RAD51 and γH2AX were analyzed and the internal loading control was vinculin (VINC). **(D, E)** Quantification of the relative density of RAD51 **(D)** and γH2AX **(E)** in the indicated cell lines. All quantifications were based on at least three independent biological replicates. Error bars, SEM. *P<0.05, **P<0.01 ***P<0.01; unpaired two-tailed Student's t-test for blots, Mann–Whitney U test for 53BP1 foci.

Altogether, these results indicate that Lestaurtinib inhibits SmoA1 medulloblastoma progression.

4 Discussion

Protein kinases have emerged as one of the most prominent class of pharmacological targets for cancer treatment. Indeed, 89 kinase inhibitors have been so far approved worldwide (https://www.ppu.mrc.ac.uk/list-clinically-approved-kinase-inhibitors), with main applications in oncology. Although most of these molecules have been raised against specific targets, systematic studies of their kinome interactions revealed that many of them may hit multiple kinases (26, 44). This feature is traditionally considered not very desirable, for the obvious possibility of producing adverse side effects, but it has also been argued that a poly-pharmacological profile is not

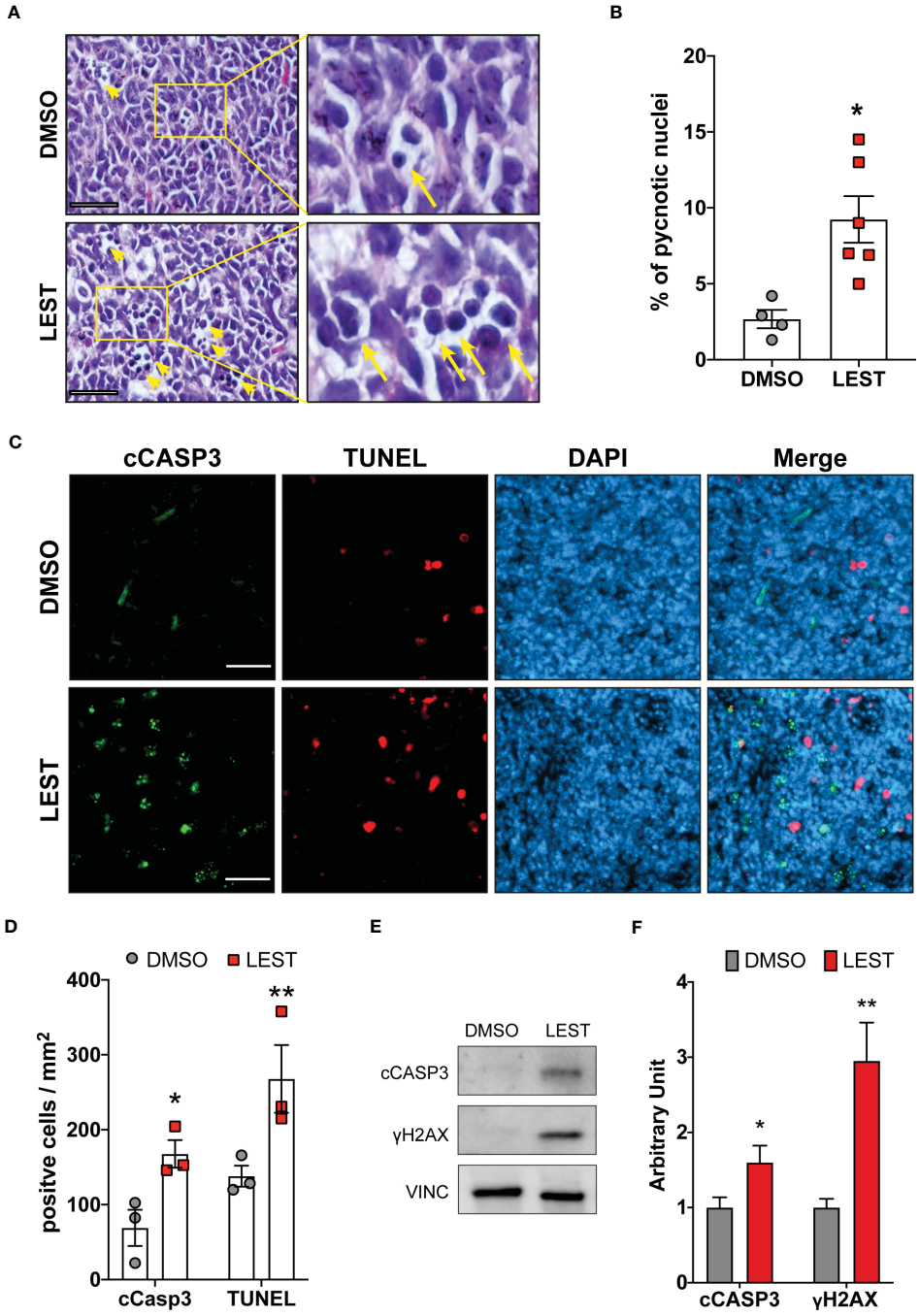

FIGURE 5
Lestaurtinib induces apoptosis and DNA damage accumulation in vivo. **(A)** Hematoxylin and eosin (H&E) staining of paraffin sections, obtained from MBs that developed in SmoA1 mice, treated with stereotaxic injection of 1µl DMSO or 1µl 100 nM Lestaurtinib (in PBS) for 24 hours. Yellow arrows indicate pyknotic nuclei. Scale bars: 20 µm. **(B)** Percentage of pyknotic nuclei in sections obtained as described in panel **(A)** Each dot indicates a treated animal. **(C)** Frozen sections from tumors obtained as in panel A were labeled by anti-caspase 3 (cCASP3) fluorescent immunostaining and TUNEL assay (red), counterstaining with DAPI. Scale bars: 50 µm. **(D)** Quantification of positive cells for mm^2 of cCASP3- and TUNEL-positive cells in frozen sections treated as described in panel **(C)** Each dot indicates a treated animal. **(E, F)** Western blot analysis of total cell lysates obtained from tumors obtained as described in **(A)** The levels of cleaved caspase 3 (cCASP3) and γH2AX were analyzed and the internal loading control was GAPDH. Average relative density of cCASP3 and γH2AX in DMSO or Lestaurtinib treated mice (N=6) is reported in panel **(E)** Error bars, SEM. *P<0.05, **P<0.01; unpaired two-tailed Student's t-test. A.U., arbitrary units.

necessarily a disadvantage for anti-cancer drugs (45–48). Indeed, not only single-target molecules may fail in the long run, due the emergence of drug resistance, but molecules inhibiting several targets may even result in safer and more effective action profiles.

Lestaurtinib (also known as CEP-701) is a Staurosporine derivative that inhibits the FLT3 tyrosine kinase at low nanomolar concentrations. For this reason, it was tested in clinical trials as targeted agent for treatment of acute myeloid

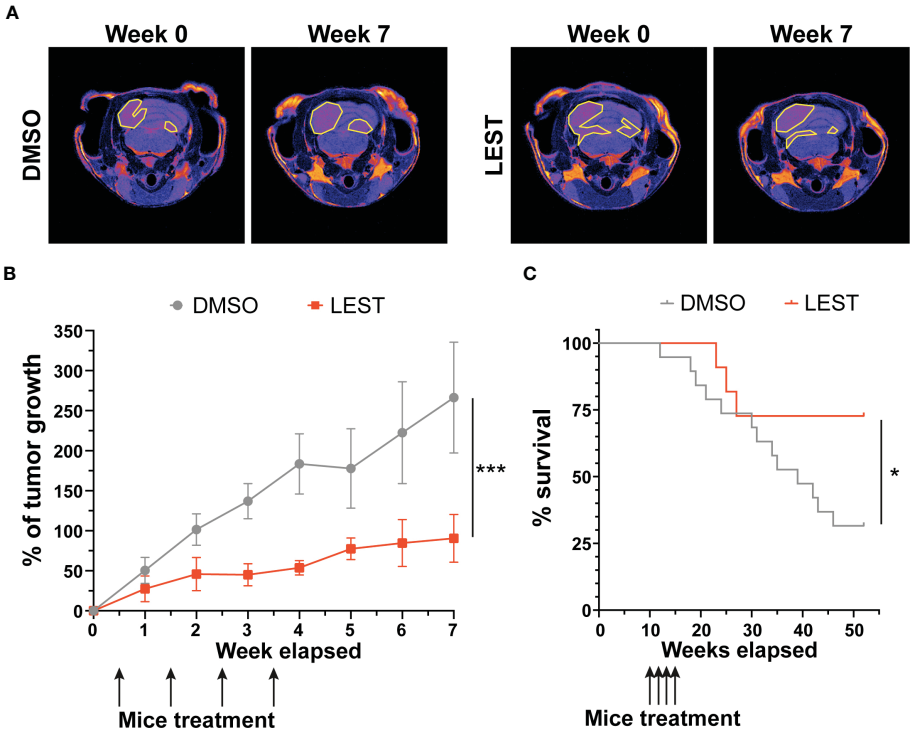

FIGURE 6
Lestaurtinib inhibits medulloblastoma progression in SmoA1 mice **(A)** Representative T2 weighted MRI-based follow up (from tumor detection, week 0, to last time point monitored, week 7) of mice treated for 4 consecutive weeks with intrathecal injection of DMSO or Lestaurtinib 100nM. Pseudocolorized images are displayed; yellow lines outline the tumor area in MRI sections. **(B)** Quantitative analysis of tumor growth in follow up experiment performed as described in **(A)** Tumor volumes were reconstructed, summing up the corresponding voxels in each section (N = 6). Error bars, SEM. ***P<0.01; different between linear regression of each growth curve. The black arrows indicate Lestaurtinib administration. **(C)**, Kaplan–Meier survival curves of control and mice treated for 4 weeks with Lestaurtinib at 10 weeks of age (N = 19 DMSO, 12 Lest). Log-rank (Mantel–Cox) test was used to compare survival between experimental groups (*P<0,05). The black arrows indicate Lestaurtinib administration.

leukemia (49) although it didn't produce significant clinical benefit, as compared to chemotherapy alone (32, 33). Lestaurtinib was also tested in patients with JAK2 mutations and myelofibrosis, who responded to treatment in some cases (34, 50). Considering its effectiveness as a TrkB inhibitor, it has been clinically tested in a Phase I trial in patients with refractory neuroblastoma, who showed sporadic responses (51). All clinical trials indicated that Lestaurtinib is well tolerated. On this ground, and considering that it is a promiscuous inhibitor of many other kinases (26), it is conceivable that Lestaurtinib could be repurposed for treating other tumor types.

In our previous works, we proposed CITK as a promising target for MB treatment, since its loss reduces growth and induces apoptosis of both xenograft and transgenic SHH MBs (24, 25). In the latter case, temporally-controlled genetic deletion of CITK in transgenic MB mice significantly improved survival, without evidence of adverse effects in other organs (25). No specific inhibitors for CITK have been developed so far. We found that Lestaurtinib inhibits CITK catalytic activity with an IC50 of 90 nM (Figure 1A, B), a concentration similar to the reported 85 nM K_d (26). Moreover, treatment of dividing MB cells at 100 nM significantly alters the midbody abundance of 834-902 phospho-INCENP, which is a known CITK substrate (30) (Figures 1C-F). The same concentration of Lestaurtinib produced most of the phenotypic effects obtained in MB cells by CITK depletion. It impairs cell proliferation and clonogenic potential in different types of MB cells (Figure 2). The growth inhibition is accompanied by late cytokinesis failure, accumulation of DNA-DSB and apoptosis. Importantly, these effects were also observed after *in vivo* injection, in MB spontaneously arising in the SmoA1 transgenic model. These data significantly extend the current knowledge about the possible biological effects produced by Lestaurtinib, which to our knowledge has not been linked to mitotic alterations or to DNA damage accumulation. Importantly, we obtained evidence that intratecal administration of Lestaurtinib affects the growth of SHH Medulloblastomas spontaneously arising in immunocompetent transgenic mice and increases their long-term survival (Figure 6), without producing severe side effects.

Considering the poly-pharmacological phenotype of Lestaurtinib, it is unlikely that all the effects described in this study are due to CITK inhibition. Indeed, the drug can bind with a K_d lower than 100 nM to many other kinases, including the well-known mitotic master players CDK2, AURKA and AURKB (26). The latter is a known interactor of CITK during cytokinesis and the cross regulation of the two proteins is crucial to correctly organize midbody proteins (30). Nevertheless, it is conceivable that CITK

may play a significant role in the complex networks engaged by Lestaurtinib in MB cells. Indeed, its effects were not additive with CITK depletion and the observed reduction of the proliferative potential was rescued by CITK overexpression (Figures 2G-H, Figure S3A, B). Under the therapeutic perspective Lestaurtinib could play a beneficial role in MB not only through the novel mechanisms which we have described in this study, but also through inhibition of other kinase-dependent pathways. Considering the critical relevance of mitotic kinases for the expansion of the normal neural progenitors (15)and the promising results obtained in a Phase I trial on refractory neuroblastoma patients (51), the poly-pharmacology profile of Lestaurtinib has the potential of being particularly effective in neuroectodermal tumors, such as MB. In conclusion, based on our results, we propose that Lestaurtinib is a promising lead for MB treatment.

Data availability statement

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

Ethics statement

The animal study was reviewed and approved by Italian Ministry of Health, Department of Public Veterinary Health, Permission number 1128/2020-PR, released on 16th November 2020.

Author contributions

Conception and design: GP, FD. Development of methodology: GP, RP, FD, ET. Acquisition of data: GI, GP, VB, MG, EF. Analysis and interpretation of data: GI, GP, MG. Writing of the manuscript: GI, GP, FD. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1202585/full#supplementary-material

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Targeting dopamine transporter to ameliorate cognitive deficits in Alzheimer's disease

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Alzheimer's disease (AD) is characterized by the pathologic deposition of amyloid and neurofibrillary tangles in the brain, leading to neuronal damage and defective synapses. These changes manifest as abnormalities in cognition and behavior. The functional deficits are also attributed to abnormalities in multiple neurotransmitter systems contributing to neuronal dysfunction. One such important system is the dopaminergic system. It plays a crucial role in modulating movement, cognition, and behavior while connecting various brain areas and influencing other neurotransmitter systems, making it relevant in neurodegenerative disorders like AD and Parkinson's disease (PD). Considering its significance, the dopaminergic system has emerged as a promising target for alleviating movement and cognitive deficits in PD and AD, respectively. Extensive research has been conducted on dopaminergic neurons, receptors, and dopamine levels as critical factors in cognition and memory in AD. However, the exact nature of movement abnormalities and other features of extrapyramidal symptoms are not fully understood yet in AD. Recently, a previously overlooked element of the dopaminergic system, the dopamine transporter, has shown significant promise as a more effective target for enhancing cognition while addressing dopaminergic system dysfunction in AD.

KEYWORDS

dopamine transporter, dopamine, Alzheimer's disease, mesocorticolimbic pathway, cognition

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by cognitive decline and behavioral abnormalities accompanied by impairments in various personality domains. AD has an intricate pathophysiology that is believed to be initiated by oxidative stress, leading to the deposition of amyloid-β (Aβ), tau hyperphosphorylation, neuroinflammation, and neurodegeneration (Tarkowski, 2003; Manczak et al., 2011; Leuner et al., 2012). The neuropathology in AD involves abnormal neuronal circuits (Busche et al., 2019) resulting from aberrant synaptic morphology and dysfunctional neurotransmitter systems (NTS) (Hsieh et al., 2006; Meyer-Luehmann et al., 2008; Kandimalla and Reddy, 2017). The synaptic dysfunction starts with impairment of long-term potentiation and eventually leads to synaptic depression (Selkoe, 2002; Palop and Mucke, 2010). Several NTS, such as acetylcholine, catecholamines (such as dopamine and norepinephrine), indoleamines (such as serotonin), and glutamate, have been associated with cognition, and their abnormal functioning is observed in AD.

The dopaminergic system, being a crucial NTS, exhibits decreased levels of dopamine receptors (Pan et al., 2019), dopamine neurotransmitter, dopaminergic neuronal count,

and connectivity in the ventral tegmental area (VTA)—hippocampus—nucleus accumbens (NAc) loop (Nobili et al., 2017; Cordella et al., 2018; Sala et al., 2021) in AD brains. While the administration of dopamine agonists has been found effective in restoring cortical plasticity in AD patients (Koch et al., 2014), it improves only frontal-lobe-related cognition without significantly impacting global cognition (Koch et al., 2020). Moreover, a newly studied component of the dopaminergic system, the dopamine transporter (DAT), has shown promise in increasing dopamine levels and attenuating disease progression when blocked. Based on these findings, we present evidence suggesting that targeting the DAT could be a potential strategy for alleviating cognitive dysfunction in mild to moderate AD.

2. Dopaminergic system—Role of dopamine transporter

The dopaminergic system primarily consists of dopaminergic neurons, receptors, the neurotransmitter dopamine, and dopamine transporter. The neurotransmitter released by dopaminergic neurons exerts excitatory and inhibitory effects by acting on presynaptic and postsynaptic receptors, known as dopamine receptors (Juárez Olguín et al., 2016). There are five dopamine receptors, namely D1, D2, D3, D4, and D5, which can be categorized into two groups: D1-like receptors (D1 and D5) coupled to G-stimulatory sites, and D2-like receptors (D2, D3, and D4) coupled to G-inhibitory sites (Bhatia et al., 2023). D1 receptors are the most abundant in the central nervous system, followed by D2, D3, D5, and D4 subtypes (Bhatia et al., 2023). These dopamine receptors are distributed in various brain regions with the possibility of co-existence of different dopamine receptors within the same neuron (Jaber et al., 1996; Perreault et al., 2011).

The D3, D4, and D5 receptors are primarily associated with cognition, while D1 and D2 receptors are linked to learning and memory (Gross and Drescher, 2012; Carr et al., 2017; Mishra et al., 2018). The final component of the dopaminergic system is the DAT, a transmembrane protein located in the presynaptic terminal of dopaminergic neurons responsible for dopamine reuptake. It plays an essential role in regulating synaptic dopamine levels, making it the key regulator of dopaminergic neuron connectivity (Miller et al., 2021). Multiple modulators, including D2 and D3 receptors, influence the dopamine transporter's function. Activation of D2 receptors increases dopamine transporter activity and dopaminergic reuptake (Ramamoorthy et al., 2011), whereas modulation by D3 receptors varies in a biphasic manner, with short-term activation increasing DAT surface expression and prolonged activation leading to inhibition (Zapata et al., 2007).

3. The anatomy and physiology of dopamine transporter

The DAT is a transmembrane protein belonging to the family of Na^+/Cl- -dependent neurotransmitter transporters and comprises 12 helices. These transmembrane helices (TMH) are interconnected through intracellular and extracellular loops (Bu et al., 2021). As a membrane-spanning protein belonging to solute carrier 6 transport family, the DAT undergoes conformational change in response to ligand binding (Shan et al., 2011). This change in its conformation is necessary for the translocation of dopamine into the neuron. Hence, blocking of this conformation results in dopamine efflux outside the neuron, as happens in response to cocaine binding (Huang et al., 2009). There are two main sites for ligand binding, a central or primary substrate binding site (S1) and a vestibular or secondary substrate binding site (S2), the latter of which greatly influences former's function (Shi et al., 2008; Shan et al., 2011). The antagonist attachment to the S1 competitively inhibits ligand binding, whereas the antagonist occupancy of S2 allosterically prevents ligand transport through DAT. Therefore, S1 and S2 along with other allosteric sites are the targets for the DAT modulators (Nepal et al., 2023).

Physiologically, the influx of dopamine requires its attachment with S1 in the presence of two Na+ and one Cl^- ion (Shan et al., 2011). The S1 binding of dopamine induces inward conformation of DAT that results in influx of released dopamine from the synaptic cleft back into the presynaptic neuron by coupling Na+ out of the cell and dopamine back into the axonal terminal (Shi et al., 2008). This process terminates dopamine neurotransmission and regulates the duration of dopamine's effect on its corresponding receptors. The TMH contain substrate binding sites for dopamine regulation extracellularly, with the larger Amino (N-) and Carboxy (C-) terminals extending to the cytoplasm to modulate the function of the DAT intracellularly (Vaughan and Foster, 2013).

The N-terminus contains residues for phosphorylation (Khoshbouei et al., 2004; German et al., 2015) and ubiquitination (German et al., 2015), while the C-terminus contains interaction domains for various proteins. These proteins include Parkin, which controls the cell surface expression of the DAT (Jiang et al., 2004), and α-synuclein, which regulates intracellular dopamine levels and modulates dopaminergic neuronal apoptosis (Dagra et al., 2021). Additionally, there are binding sites for lipid-raft protein-Flotillin and Ras-like protein Rin, which act as mediators of membrane mobility (Sorkina et al., 2013), and protein kinase C (PKC), which triggers endocytosis of the DAT (Navaroli et al., 2011).

Regarding post-translational modification, the DAT protein in dopaminergic pathways is continuously regulated by phosphorylation, followed by internalization, through three main kinase pathways: PKC, calcium-calmodulin dependent kinase II, and extracellular signal-regulated protein kinase (ERK) (Fog et al., 2006; German et al., 2015). After phosphorylation-mediated internalization, the DAT undergoes ubiquitination, a process crucial for protein homeostasis. The N-terminus determines whether the DAT will be recycled back onto the cell surface or completely degraded by the action of lysosomes (Boudanova et al., 2008; German et al., 2015), depending on the demands of the dopaminergic pathway (Figure 1).

On the other hand, dopamine synthesis depends on the activity of tyrosine hydroxylase (TH), which serves as the rate-limiting enzyme for converting the amino acid tyrosine into dopamine (Xiao et al., 2021). The DAT not only facilitates the degradation of dopamine, promoting its reuptake, but may also affect the levels of TH (Salvatore and Pruett, 2012; Salvatore et al., 2016). In this way, TH and the DAT work synchronously to maintain the desired levels of dopamine in the dopaminergic pathways of the brain. Moreover, the dopamine transport DAT is also responsible for modulating

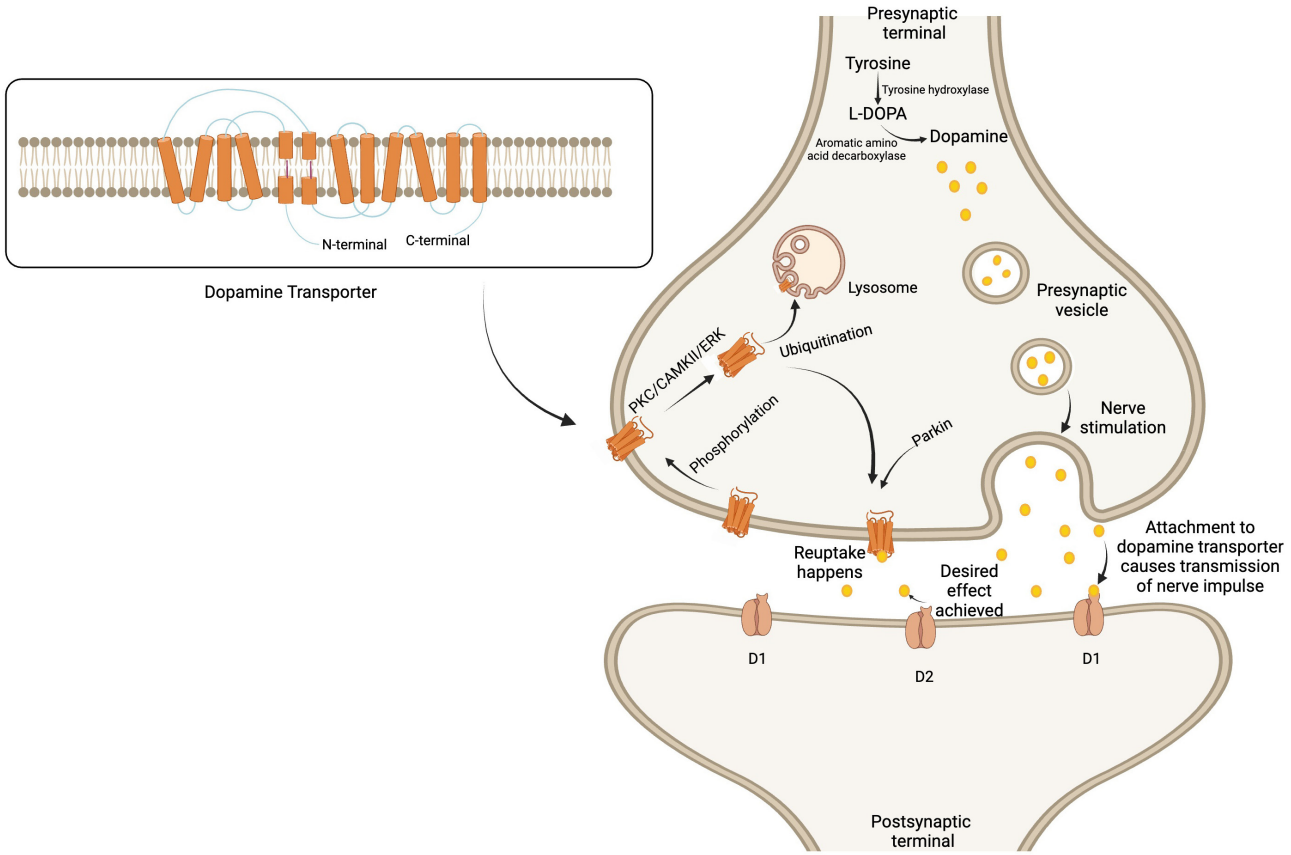

FIGURE 1
Regulation of dopamine metabolism and dopamine transporter surface expression in the synaptic cleft. Dopamine is formed from tyrosine and stored in the presynaptic vesicles to be released upon nerve stimulation. The secreted dopamine in the synaptic cleft is attached to the post-synaptic dopamine receptors to transmit the nerve impulse. Soon after achieving the desired effect, the dopamine transporter reuptake results in the termination of signals. The expression of the dopamine transporter is regulated by phosphorylation, followed by internalization through any of the three kinase pathways. The transporter then undergoes ubiquitination, which leads to either degradation by lysosomes or recycling back to the membrane. Created with BioRender.com.

membrane potential of the cell and hence the neuronal function in the dopaminergic pathways (Carvelli et al., 2004).

The DAT primarily regulates dopamine in most areas of the dopaminergic pathway. However, in specific regions like the hippocampus, it is mainly metabolized by norepinephrine transporter (NET) (Borgkvist et al., 2012), and in the prefrontal cortex (PFC), it is metabolized by Catechol-O-Methyltransferase (COMT) due to the scarcity of dopamine transporter in these areas (Lammel et al., 2008).

4. Mesocorticolimbic circuitry: the interplay of dopamine and the dopamine transporter in cognition

The brain has three main dopaminergic pathways: the nigrostriatal (NS), the mesocorticolimbic (MCL), and the tuberoinfundibular. While the role of dopaminergic modulation in cognition is not fully understood, dopamine in the striatum, midbrain, limbic system, and PFC is believed to be involved in memory and cognition through the NS and MCL pathways (Schott et al., 2006; McNamara et al., 2014; Herrera et al., 2020; Vassilev et al., 2021).

In the NS pathway, there is a dopamine connection between the substantia nigra and the striatum, while in the MCL pathway, the midbrain, limbic system, and PFC are interconnected through dopamine. The MCL circuit itself consists of two pathways: the mesocortical pathway, where dopamine neurons have their cell bodies in the VTA of the midbrain and extend nerve fibers to the PFC, and the mesolimbic pathway, where nerve fibers project to the NAc and other limbic structures (Krashia et al., 2022). Physiologically regulated dopamine innervation and neurotransmission are crucial for the proper functioning of these dopaminergic pathways. Reduction in dopaminergic neurons and/or dopamine neurotransmitter levels can lead to defective connectivity between linking areas.

Similar to NS pathway, the MCL pathway also plays a vital role in cognition, including learning, memory, and decision-making, modulated by dopamine activity in the frontal lobe, limbic system, and midbrain (McNamara et al., 2014; Engelhard et al., 2019; Coddington et al., 2023). Therefore, alterations in dopamine levels may likely contribute to cognitive deficits (Koch et al., 2014; Pan et al., 2019).

Dopamine signals are primarily terminated by either reuptake via transporters or enzymatic degradation by COMT (Caire et al., 2023). Altered levels of these dopamine-signal terminators can also impact cognition, as observed with the DAT inhibitors that improve memory and cognition in neurodegenerative diseases (as described below). Paradoxically, lower dopamine transporter levels have been observed in some psychiatric disorders, such as attention deficit hyperactivity disorder (Kurzina et al., 2020) and depression (Pizzagalli et al., 2019; Dubol et al., 2020). This paucity of dopamine in the absence of higher dopamine transporter in these disorders may be due to high extracellular levels of dopamine that eventually lead to an inability to replenish dopamine in synaptic vesicles, causing a lower amplitude of dopamine release per nerve impulse (Benoit-Marand et al., 2000). Additionally, other factors, such as interaction with α-synuclein in PD, can decrease dopamine transporter activity without affecting its concentration at the plasma membrane, resulting in reduced dopamine reuptake and subsequent lower extracellular dopamine (Swant et al., 2011; Pahrudin Arrozi et al., 2017). Furthermore, dopamine neuronal damage and degeneration may also be associated with decreased levels of the DAT, even without any defect in its activity (Cheng et al., 2010; Fazio et al., 2018).

Overall, both increased and decreased dopamine levels can lead to unwanted symptoms, highlighting the importance of maintaining a continuous check-and-balance of dopamine in synapses for the normal functioning of the dopaminergic pathways.

5. Modifications in mesocorticolimbic circuitry and dopamine transporter: implications for AD

Physiological aging leads to changes in the MCL circuitry, characterized by decreased dopamine levels, reduced expression of dopamine receptors, and synaptic dysfunction (Volkow et al., 1996; Kaasinen, 2000; Norrara et al., 2018). The normal aging process is also associated with a decline in the DAT level in certain brain regions, including the hippocampus, PFC, and putamen (Volkow et al., 1996). However, these alterations in the MCL loop are more pronounced in AD, mainly affecting the dopaminergic neuronal count and dopamine receptors' expression, except for D5, which is probably increased in the frontal lobe (Kumar and Patel, 2007).

Subsequently, the MCL loop neuropathology leads to decreased dopamine connectivity and impaired long-term potentiation in AD brain (Koch et al., 2014). Additionally, the damage to pyramidal neurons and synapses in the hippocampus and PFC, due to progressive neurodegeneration caused by amyloid plaque deposition and tau pathology, contribute to impaired cognition and memory (Kemppainen et al., 2003; Ambrée et al., 2009; Guzmán-Ramos et al., 2012).

Although various abnormalities were observed in the MCL pathway, no change in DAT activity was found in AD (Joyce et al., 1997). However, a newer molecular imaging study reported decreased DAT density and activity in both the MCL loop and caudate nucleus in the defective dopaminergic system in the AD brain (Sala et al., 2021). Despite the possibility of already reduced DAT levels, further blocking the DAT improved cognitive deficits, as recently observed in animal models of aging and AD (Xu et al., 2021; Yin et al., 2023). Even though several studies deduced temporary improvement in cognition due to increased synaptic dopamine, newer DAT blockers can repair cognitive deficits by reducing the disease's neuropathology. Although the exact mode of action is yet to be elucidated, the cognitive improvement is thought to be due to the inhibition of α-synuclein and $A\beta_{1-42}$ aggregation in the hippocampus and the promotion of lysosomal biogenesis and subsequent degradation of Aβ plaques (Xu et al., 2021; Yin et al., 2023).

Although the MCL pathway and the NS pathway may exhibit distinct roles, the DAT activity and the regulated level of synaptic dopamine are equally crucial for the normal functioning of both. The importance of DAT in MCL has yet to be explored so far, likely due to its negligible presence in some of the areas. However, its reduced levels in the NS loop are linked with cognitive impairment (Li et al., 2020; Fiorenzato et al., 2021). The DAT is found to be closely associated with cognition, as the uninhibited blockade or elimination of the DAT may worsen the disease pathology, as observed in DAT knock-out rodents showing severe cognitive deficits (Leo et al., 2018; Kurzina et al., 2020). Therefore, controlled inhibition of DAT function is crucial to avoid disturbing its physiological effects in the AD brain.

6. Effects of dopamine transporter modulators on cognition in AD

The DAT modulators with the potential to enhance cognition and memory can be classified into two main categories. The first category includes substrate-like competitive inhibitors that reduce dopamine reuptake and increase dopamine efflux. The second category comprises atypical or highly-specific DAT inhibitors that prevent dopamine reuptake, increasing dopamine concentration in synapses (Goodwin et al., 2009).

Surprisingly, none of these agents have been studied in neurodegenerative diseases despite their efficacy in improving cognition. Likewise, modafinil (diphenylmethyl-sulfinylacetamide), a prototype of a non-specific DAT inhibitor that also acts on NET and serotonin transporter in the striatum (Madras et al., 2006), has not garnered much interest from researchers in this field. In contrast, Yin et al. (2023) recently introduced a novel class of DAT modulators that exert their effects on DAT and lysosomal activity and, hence, can be termed "DAT-inhibitors-and-lysosomal-activity-promoters- (DILAP)". These drugs were tested on AD mice and effectively improved memory and cognitive deficits. Moreover, DILAP were also found to reduce intracerebral Aβ burden by promoting lysosomal synthesis and phagocytosis (Yin et al., 2023). Two examples of DILAP are the lysosome-enhancing compound LH2-051 and clomipramine hydrochloride (HCl), also known as Anafranil or S2541. LH2-051 inhibits the DAT, and its binding leads to translocation of the DAT from the plasma membrane to the lysosomal membrane via intracellular vesicles. The localization of DAT onto lysosome decreases the availability of the phosphorylating proteins and, therefore, promotes dephosphorylation of transcription factor EB (TFEB). The dephosphorylated TFEB then undergoes nuclear translocation, which enhances the expression of lysosomal

and autophagic genes, promoting lysosomal acidification and biogenesis (Yin et al., 2023). This increase in active lysosomes ultimately results in Aβ clearance and improved learning, memory, and cognition (Yin et al., 2023). The mentioned outcomes of dopamine transport inhibition and lysosomal activation are comparable with the effects of Clomipramine HCl that blocks the activity of the DAT along with the serotonin transporter and NET (Gillman, 2007; Yin et al., 2023). In this way, it may also be effective in attenuating AD neuropathology.

7. Implications of recent studies on alternative dopamine transporter inhibitors

The modulators acting as substrate-like agents for the DAT include amphetamine and methamphetamine, while DAT-specific agents comprise modafinil derivatives better known as (synthetic) modafinil analogs. Although both amphetamine and methamphetamine are non-specific, having more affinity NET receptors, they are potent inhibitors of DAT (Han and Gu, 2006; Docherty and Alsufyani, 2021). By acting as substrate-like competitive inhibitors, these agents can decrease dopamine reuptake and increase dopamine efflux (Goodwin et al., 2009) in brain regions such as the medial PFC (mPFC), dentate gyrus (DG) (Fog et al., 2006; Shyu et al., 2021), and NAc (Hedges et al., 2018). On the other hand, DAT-specific drugs selectively target DAT-mediated reuptake in the mPFC (Sagheddu et al., 2020; Kouhnavardi et al., 2022), NAc (Kouhnavardi et al., 2022), and hippocampus (Kristofova et al., 2018).

The mechanism of action of DAT inhibition is different among the three classes of drugs, i.e., atypical inhibitors, substrate-like competitive inhibitors and DILAP. The atypical DAT inhibitors increase the synaptic dopamine level by inhibiting DAT function (Loland et al., 2012). Whereas, the substrate-like inhibitors, like amphetamine increase synaptic dopamine levels by inhibiting DAT uptake, promoting DAT mediated reverse-transport of dopamine and facilitating exocytic dopamine release (Calipari and Ferris, 2013; Daberkow et al., 2013). Additionally, they may also stimulate internalization of the plasma membrane DAT, thereby further decreasing its availability and function (Wheeler et al., 2015). In comparison, the DILAP inhibit the DAT mediated dopamine reuptake, while promoting its translocation from the plasma membrane to the lysosomal membrane. This translocation increases expression of lysosomal and autophagic genes which promotes degradation of Aβ-plaques (Yin et al., 2023) (Figure 2).

Due to their high DAT specificity, synthetic modafinil analogs like R-modafinil, S-CE-123 (S-5-((benzhydrylsulfinyl)methyl) thiazole), S,S-CE158 (5-(((S)-((S)-(3-bromophenyl)(phenyl) methyl)sulfinyl)methyl)thiazole), and S-MK-26 ((S)-5-(((B(3-chlorophenyl)methyl)sulphinyl)methyl)thiazole) do not exert any effect on the reward pathway, making them less likely to cause addiction, abuse or withdrawal symptoms compared to the parent drug and other non-specific counterparts (Kristofova et al., 2018; Sagheddu et al., 2020; Hazani et al., 2022; Kouhnavardi et al., 2022). These modafinil analogs also have the potential to improve synaptic transmission and plasticity in the hippocampus (Kouhnavardi et al., 2022).

Considering that studies evaluating the effects of DAT inhibition showed improved cognition attributed to amyloid and α-synuclein in the MCL pathway, especially in the hippocampus and cortex (Xu et al., 2021; Yin et al., 2023), it is likely that other DAT inhibitors may exert a similar effect in the AD brain. As atypical DAT inhibitors also improve synaptic plasticity while having a minimal tendency for addiction, they could be prime candidates to be tested on AD rodent models for cognitive improvement.

8. How dopamine transporter can be a better target?

Previous studies on AD brains have shown normal and reduced levels of DAT (Joyce et al., 1997; Sala et al., 2021) and no change in DAT sites and TH levels in the VTA (Murray et al., 1995). Currently, there is no drug approved by the FDA for the treatment of the dopaminergic system in AD (Chopade et al., 2023), as both the L-Dopa and dopamine agonists failed to demonstrate compelling results to improve cognition in the elderly (Lebedev et al., 2020) and AD subjects (Koch et al., 2020), respectively. In comparison, the newer studies targeting the DAT in AD animal models have shown promise (Xu et al., 2021; Yin et al., 2023).

Since dopamine is involved in the NS pathway, which affects mood and motivation, the effects of DAT inhibitors should also be considered in that loop. A study by Udo et al. found an inverse correlation between the level of DAT in the caudate nucleus and the severity of apathy in AD (Udo et al., 2020). However, it is worth noting that the study mentioned the degeneration of dopaminergic neurons in the NS pathway as the cause of decreased DAT levels in presynaptic terminals. Therefore, apathy is likely due to the loss of these striatal dopaminergic neurons (Udo et al., 2020). In line with this notion, a newer study demonstrated the potential of specific DAT inhibitors to improve motivational impairments in rats (Kouhnavardi et al., 2022). Thus, DAT inhibitors may not worsen apathy, as the symptom is primarily a result of dopamine neuronal degeneration in the NS pathway.

Moreover, the development of novel heterocyclic compounds, such as CE-125 (4-((benzhydrylsulfinyl)methyl)-2-cyclopropylthiazole)) and CE-111 (4-(benzhydrylsulfinylmethyl)-2-methyl-thiazole), acting as DAT-specific inhibitors along with dopamine receptor modulators, have shown potential as better alternatives to modafinil-derived analogs by having multiple targets in the dysfunctional dopaminergic system (Saroja et al., 2016; Hussein et al., 2017). These atypical DAT inhibitors may effectively improve cognition in cases of defective dopaminergic pathways. Furthermore, the effects of DAT modulation for cognitive enhancement can be evaluated by targeting specific binding sites, which is now feasible after the identification of novel allosteric sites on the DAT protein (Cheng et al., 2017; Aggarwal et al., 2019).

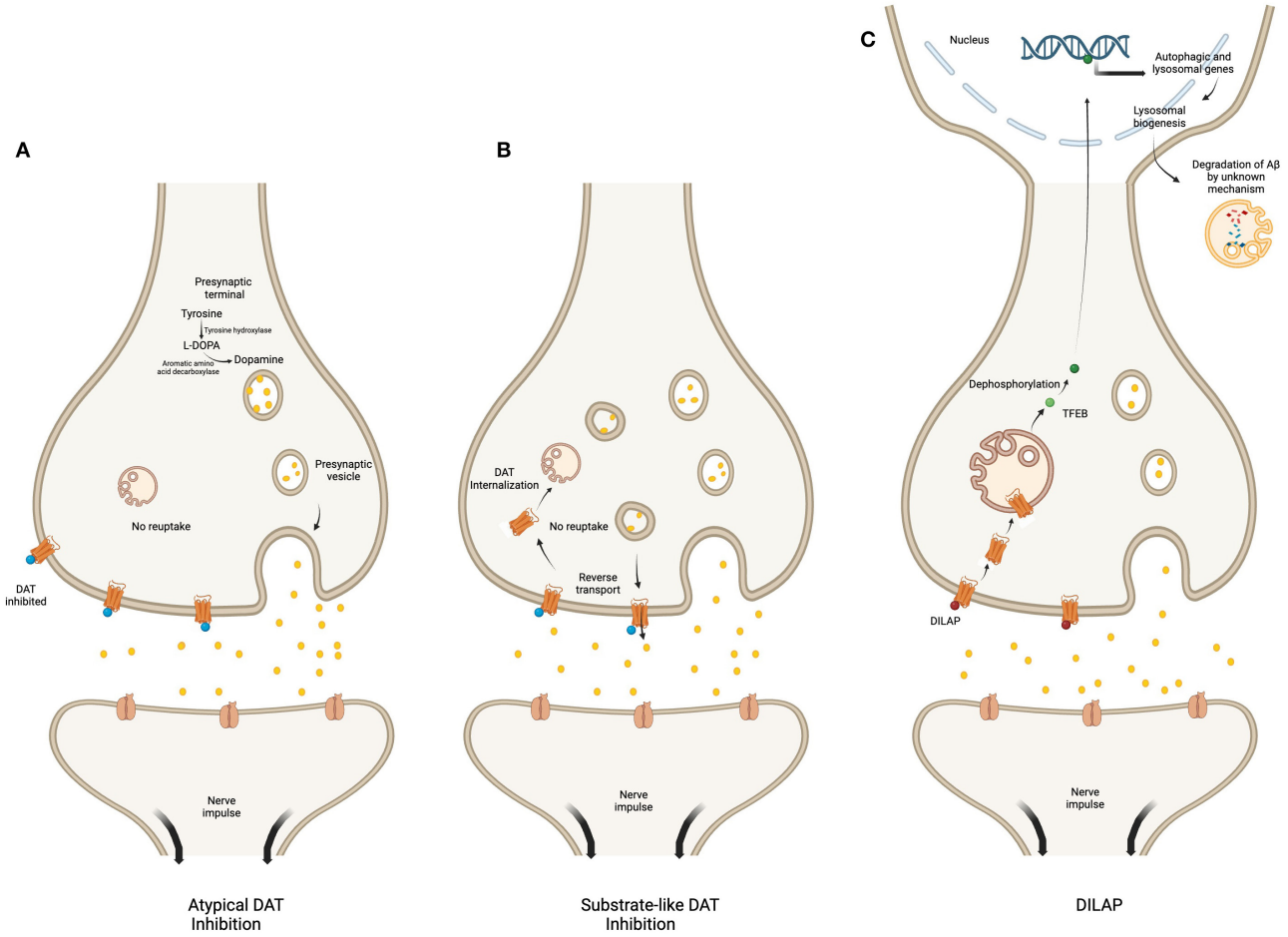

FIGURE 2
Regulatory mechanism of various DAT inhibitors. The figure compares the mechanism of action of atypical DAT inhibitor **(A)**, substrate-like DAT inhibitor **(B)**, and DILAP **(C)**. The atypical inhibitors bind DAT to block dopamine reuptake **(A)**. The substrate-like inhibitors block dopamine influx while stimulating the DAT-mediated reverse transport of DAT into the synaptic cleft; they also decrease DAT availability by promoting its internalization **(B)**. The DILAP inhibits DAT to increase synaptic dopamine, and their binding cause DAT translocation to the lysosomal membrane, which results in nuclear translocation of the dephosphorylated TFEB to increase expression of autophagic and lysosomal genes, ultimately leading to Aβ-plaques degradation by an unknown mechanism **(C)**. Created with BioRender.com.

9. Discussion and future directions

Studies found that the MCL pathway is affected more than the NS pathway in AD (Koeppe et al., 2008; Colloby et al., 2012; Sala et al., 2021). Therefore, targeting the abnormal MCL pathway can alleviate most dopamine-related cognitive deficits. For this purpose, DAT inhibitors can be considered as potential targets to improve cognition by reducing the dopamine reuptake. However, while inhibiting DAT, the precise regulation of dopamine in the MCL loop must be ensured, especially in the VTA-hippocampal circuit, to avoid dopamine disbalance and resulting adverse effects. If, for instance, DAT is excessively blocked, then the resultant hyperdopaminergic state can lead to dopamine dysregulation syndrome, promoting undesirable responses like gambling and drug addiction. Conversely, a hypodopaminergic state can lead to anxiety and apathy (Calabresi et al., 2013). Care must also be considered when generalizing DAT inhibition to the whole brain as certain chemical can downregulate DAT as well as TH gene expression (Mohamad Najib et al., 2023). However, considering that the dopamine is metabolized by COMT and NET in the PFC and hippocampus, respectively (Lammel et al., 2008; Borgkvist et al., 2012), the regulated substrate modification of the DAT would not severely alter the dopamine levels in these areas.

Presently, no drug is approved to treat the dopaminergic system-related cognitive deficits in AD. However, recent studies on DAT inhibitors demonstrate its potential to be an efficacious target, with the newly discovered DILAP possessing the ability to restore cognitive deficits by ameliorating AD neuropathology. The DILAP binds to DAT causing translocation of the latter to the lysosomal membrane in order to increase the lysosomal biogenesis and subsequent dissolution of amyloid plaque by a mechanism not understood yet. The exact mechanism of action of DILAP to improve cognition is yet to be elucidate, however, it is suggested to be linked with the attenuation of amyloid burden. Moreover, a study on an AD mouse model showed promising effects of Nilotinib, a tyrosine kinase inhibitor that reduces Aβ levels, prevents structural damage and degeneration

of dopaminergic neurons in the VTA, and restores VTA-hippocampal loop function, thereby reducing dopamine-related cognitive impairments (Barbera et al., 2021). This points toward the possibility of developing useful nootropics in combination with DAT inhibitors to improve MCL pathway functioning, ameliorate cognitive impairment, and mitigate AD neuropathology simultaneously. Furthermore, atypical DAT inhibitors can be tested with agents such as acetylcholinesterase inhibitors and N-methyl-D-aspartate receptor antagonists. Positive results from such combinations could help improve cognitive impairment and decrease the pathologic burden of the disease in the brain.

Author contributions

AS: Conceptualization, Methodology, Writing – original draft. MY: Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Writing – review & editing. FA: Writing – review & editing. JK: Writing – review & editing. ST: Writing – review & editing.

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Conflict of interest

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

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Overcoming challenges in glioblastoma treatment: targeting infiltrating cancer cells and harnessing the tumor microenvironment

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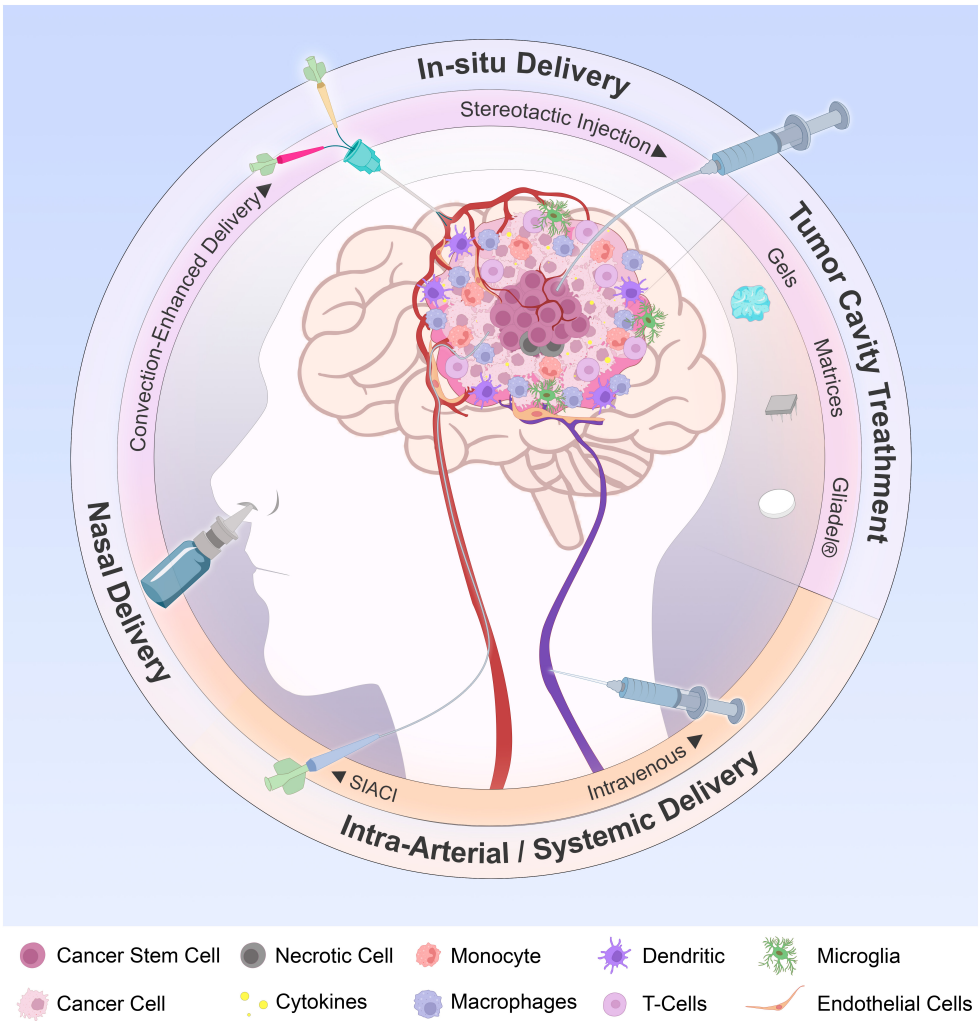
Glioblastoma (GB) is a highly malignant primary brain tumor with limited treatment options and poor prognosis. Despite current treatment approaches, including surgical resection, radiation therapy, and chemotherapy with temozolomide (TMZ), GB remains mostly incurable due to its invasive growth pattern, limited drug penetration beyond the blood-brain barrier (BBB), and resistance to conventional therapies. One of the main challenges in GB treatment is effectively eliminating infiltrating cancer cells that remain in the brain parenchyma after primary tumor resection. We've reviewed the most recent challenges and surveyed the potential strategies aimed at enhancing local treatment outcomes.

KEYWORDS

glioblastoma, local delivery, microenvironment, blood brain barrier, drug repurposing

Introduction

Glioblastoma is the most prevalent malignant primary brain tumor and recent scientific and technical advances have allowed for a deeper understanding of the etiologic relevance of the heterogeneity of GB. Based on the current classification of the tumors of the central nervous system (CNS), among adult-type diffuse gliomas, astrocytic tumors without mutations in the Isocitrate dehydrogenase (IDH) genes are termed glioblastomas IDH-wildtype (Torp et al., 2022). The presence of microvascular proliferation and/or necrosis and at least one molecular alteration identified as predictive of tumor aggressiveness, such as Epithelial Growth Factor Receptor (EGFR) amplification, Telomerase promoter (TERTp) mutations (Brás et al., 2023) up-regulation of chromosome 7 and loss of chromosome 10 (Zhao et al., 2023) allows for a diagnosis of glioblastoma IDH-wildtype CNS WHO grade 4 (since now, GB).



GRAPHICAL ABSTRACT
Current treatment approaches for glioblastoma and glioblastoma recurrences.

Despite all the innovation that generates the "holistic representation of the evolving disease" (Asleh et al., 2023), GB remains mostly incurable, with a median survival of 14 months and a 5-year survival rate of 5.7%, primarily due to its challenging characteristics: (i) invasive growth pattern, making complete surgical resection nearly impossible; (ii) localization beyond the blood-brain barrier (BBB), which severely limits drug penetration; (iii) resistance to conventional radiotherapy and chemotherapy (Grochans et al., 2022).

Indeed, GB is strongly refractory to most anti-tumor treatments, one of the main reasons lying in the difficulties of tackling its cellular heterogeneity. In the tumor, in fact, the cell population is represented by differentiated GB cells, stem-like cells (GSCs) as well as by some elements of the microenvironment milieu among which endothelial cells, vascular

Abbreviations: PLGA-PEG-PLGA, (D,L-lactic acid-co-glycolic acid)-b-poly(ethylene glycol)-b-poly(D, L-lactic acid-co-glycolic acid); ADSC, Adipose tissue-Derived mesenchymal Stem Cells; AMNPs, Allomelanin Nanoparticles; ABC, ATP-binding cassette; AF, Auranofin; BBB, Blood-Brain Barrier; CPT, Camptothecin; CCM, Cancer Cell Membrane; CBD, Cannabidiol; CNS, Centra Nervous System; CTX, Cetuximab; CLIC1, Chloride intracellular channel-1; CED, Convection Enhanced Delivery; Cy5, Cyanine5; DABG, Dialdehyde O^6 Benzylguanine; DTPA, Diethylenetriaminepentaacetic Dianhydride; DOX, Doxorubicin; EGFR, Epidermal Growth Factor Receptor; FDA, Food and Drugs Administration; FUS, Frequency Ultrasound; FUS-BBBO, FUS combined with circulating microbubbles can temporarily open the BBB; GA, Gambogic Acid; GB, Glioblastoma; GSCs, Glioma Stem Cells; AuNRs, Gold nanorods; HSV-TK, Herpes Simplex Virus-Thymidine Kinase; HIFU, High Intensity Focused Ultrasound; HA, Hyaluronic Acid; HQ, Hydroquinidine; ICAM-1, Intracellular Adesion Molecule 1; LNPs, Lipid Nanoparticles; LIPU, Low-Intensity Pulsed Ultrasound; M1NVs, Macrophage-Derived Nanovesicles; MRI, Magnetic Resonance Imaging; MPDA, Mesoporous Polydopamine; MB, Microbubbles; HePc, Miltefosine; anti-EphA3-TMZ@GNPsTMZ), Monoclonal antibody anti-EphA3 – TMZ-loaded gold nanoparticles; MDR, Multidrug resistance; OLA, Olaparib; p-gp, p-glycoprotein; PTX, Paclitaxel; PDX, Patient-Derived Xenograft; PARP, Poly (ADP-ribose) polymerase; PLGA, poly lactic-co-glycolic acid; PCL, Polycaprolactone; PGA, Polyglutamic Acid; PDEARGs, Prognostic differentially expressed angiogenesis-related genes; PD-1, Programmed Cell Death Protein 1; PD-L1, Programmed Death-Ligand 1; ROS, Reactive Oxygen Species; SiO_2, Silicate; SIACI, Superselective Intra-Arterial Cerebral Infusion; TMZ, Temozolomide; TrxR1, Thioredoxin Reductase 1; TRAC, Tissue-Reactive Anchoring of Click groups; TfR, Transferrin Receptor; TAM, Tumor-Associated Macrophages; TILs, Tumor-Infiltrating Lymphocytes; ZIP14, Zrt- and Irt-like (ZIP) proteins transporters.

pericytes, macrophages, and other types of immune cells (Zhao et al., 2023). Sequencing technology advances and transcriptional stratification has further corroborated the complexity of this picture contributing to further subdivide GB in proneural, classical and mesenchymal subtypes. In this scenario, the developmental dynamicity of GSCs add another layer of complexity to the heterogeneous nature of the tumors. GSCs are indeed considered at the top of hierarchic lineage of cells holding stem cell-like regeneration ability (Lathia et al., 2015) but share several common molecular markers with normal adult neural stem cells and progenitor cells, originating some ambiguity regarding their definition and identification (Zhang G. et al., 2023). Recently, single-cell RNA sequencing (scRNA-seq) on patient derived samples suggest that GBM cells do not exist as separate populations but rather evolve from stemness to differentiation (Yan et al., 2023). These findings are hardly good news, as GSCs have been found to be deeply implicated in tumor progression, drug resistance and tumor recurrence (Guo et al., 2023). The attention of researchers therefore turns to this class of cells and their plastic nature in search for new regulatory protein to be pharmacologically targeted as Achilles' heel of tumor cells survival. In this scenario, new homeostatic regulatory proteins were identified: for example, the ZIP14 (SLC39A14) protein, which mediates the cellular uptake of manganese, iron and zinc, has been indicated as a possible mediator of cellular ferroptosis-related cell death (Zhao et al., 2022; Zhang Y. et al., 2023). Similarly, the chloride intracellular channel-1 (CLIC1) is known to be instrumental for tumor proliferation in several solid tumor including GB and his overexpression on GSCs is inversely associated with patient survival (Setti et al., 2013), suggesting CLIC1 to be a potential target and prognostic biomarker (Randhawa and Jahani-Asl, 2023). Accordingly, a novel class of biguanide-based derivatives used as CLIC1-inhibitors has been recently developed and holds promises for the treatment of CLIC1-expressing glioblastomas (Barbieri et al., 2022).

Interestingly, the presence of stem cells in the bulk of glioblastomas has been also strongly associated to cancer multidrug resistance (MDR) (Mattei et al., 2021), which remains a serious challenge in GB therapy as it seriously limits the effects of different chemotherapeutic drugs (Tian et al., 2023). Indeed, as MDR has been often associated with the expression of p-glycoprotein (p-gp), an ATP-binding cassette (ABC) transporter that promotes efflux of chemotherapeutics from tumor cells, this protein has been recently suggested as a druggable therapeutic target (Hasan et al., 2023). Indeed, the p-gp inhibitor reversan, in association with magnetic nanoparticle-mediated hyperthermia, was able to increase the cytotoxic effect of Doxorubicine treatment to eliminate bulk tumors along with the GSC population (Hasan et al., 2023).

Mounting evidence has also been gathered to clarify the role of proteins of the tumor micro-environment (TME) mediating the regulation of cell adhesion and migration (Caverzán et al., 2023; Khan et al., 2023). In this view, TME becomes the scenario of the dynamic dialogue between tumor cells and tumor infiltrating immune system elements, mostly tumor-associated macrophages (TAMs), monocytes, T cells and resident microglia (Eisenbarth and Wang, 2023). TAMs indeed provide a major contribution to cancer growth and immunosuppression, causing resistance even to the most effective immunotherapies, ultimately paving the way to tumor recurrences (Zhang L. et al., 2023). Moreover, in aggressive brain tumors, TME host tumor associated hyper-vascularization. Indeed, the malfunctioning of the tight junctions in the endothelial cells of the tumor associated vascularization may favor the buildup of fluid in the tumor district leading to edema and increased intracranial pressure, partially restored with steroid anti-inflammatory therapy (Cenciarini et al., 2019).

Blood-Brain Barrier's pericyte's disruption and hypoxia sustain small vessel proliferation and, in the small vessels district, the dialogue between cancer cells proteins such Nestin and CD133 and endothelial biomarkers such as CD34 proteins facilitates brain metastasis, sustaining tumor cells circulation and subsequent tissue infiltration (Schiffer et al., 2018). More recently, the development of vascularization in GB was also described using genomic analytical tools, to create a risk prediction model for GB, where prognostic differentially expressed angiogenesis-related genes (PDEARGs) become potentially druggable prognostic biomarkers of regulatory networks and provide valuable insight to tackle the role of neovascularization in these tumors (Wan et al., 2023).

Recurrent tumors also exhibit changes in the microenvironment composition, characterized by higher levels of tumor-infiltrating lymphocytes (TILs), macrophages, and increased expression of Programmed Death-Ligand1 (PD-L1) and Programmed Cell Death Protein1 (PD-1) compared to primary tumors (Karschnia et al., 2023; White et al., 2023). This suggests a strong relationship between tumor heterogeneity, immune system involvement in recurrences (Hoogstrate et al., 2023; White et al., 2023) and supports the potential use of peritumoral microenvironmental markers for patient stratification (Riahi Samani et al., 2023). Furthermore, these findings may drive the development of novel precision immunotherapeutic tools (Pornnoppadol et al., 2023; White et al., 2023).

Importantly, a key challenge in GB treatment is effectively targeting and eliminating infiltrating cancer cells that persist in the brain parenchyma after primary tumor resection. Current treatment approaches involve a complex combination of surgical resection, radiation therapy, and concurrent chemotherapy using temozolomide (TMZ) (Fisher and Adamson, 2021). However, the anti-tumor efficacy of TMZ is significantly hindered by its limited ability to cross the BBB (reaching only 20% of blood concentration) (Grochans et al., 2022) and various cellular mechanisms conferring therapeutic resistance. Following the surgical removal of the primary tumor, a lower residual tumor volume is positively correlated with a more favorable prognosis. In this context, it is crucial to identify the microscopic tumor margins hidden within the brain parenchyma and prevent loco-regional recurrence (Bütof et al., 2022). Thus, the quest is to develop effective treatments capable of intercepting residual cells, possibly tumor stem cells, beyond the BBB while potentially countering the activity of favorable microenvironmental elements sustaining tumor invasiveness (Garcia-Diaz et al., 2023).

Here, we aimed at highlighting various strategies employed to overcome the challenges posed by the BBB. Nanomaterial-based delivery systems will be discussed, emphasizing their ability to enhance drug penetration into the brain and improve treatment efficacy and the use of nanomaterials loaded with single drugs or combination therapies. The focus will be centered on how these approaches can enhance the therapeutic effect by targeting multiple pathways or mechanisms involved in tumor growth and progression. Moreover, the review will delve into targeting druggable microenvironmental factors that contribute to the

development of recurrent tumors. Finally, we will explore the potential of local drug delivery strategies for drug repurposing will be examined. Understanding and targeting these factors may help preventing the growth of residual cancer cells and reduce the risk of recurrence.

Overcoming the BBB

The BBB poses a significant challenge for the systemic delivery of chemotherapeutic agents to treat intracranial diseases. It is composed of endothelial cells, pericytes, astrocytes, microglia, and neurons, forming a structural barrier that restricts the passage of molecules into the brain (Sprowls et al., 2019). Endothelial cells lining the brain's capillaries express tight junctions that seal the gaps between adjacent cells, preventing the free movement of molecules between the blood and the brain. Additionally, these cells lack fenestrations and pinocytic activity, further impeding the passage of substances. Pericytes, closely associated with endothelial cells, regulate capillary diameter and cerebral blood flow while supporting microvascular stability and the BBB's structural integrity. Astrocytes play a crucial role in maintaining the barrier function through their foot processes, which establish a direct interface between vascular and neuroglial compartments.

Under normal physiological conditions, the BBB's specific ion channels and transporters maintain an optimal microenvironment for brain function, regulating the levels of neurotransmitters, controlling ionic homeostasis, and protecting against neurotoxins. Water-soluble nutrients, metabolites and small lipid-soluble molecules can passively diffuse through the BBB and enter the brain (Kadry et al., 2020).

Blood-Brain Barrier's tight junctions and membrane transporters stringently control the exchange of molecules and ions between the blood and the brain. Limited penetration is observed for small lipophilic molecules or those agents that can actively enter by binding to carrier proteins such as transferrin receptor (TfR), epidermal growth factor receptor (EGFR), glucose, or immunoglobulins (Triguero et al., 1989; Arora et al., 2020; Cui et al., 2023; Pornnoppadol et al., 2023).

Concentrating systemic treatment at tumor site

Traditionally, the possibility of overcoming the BBB to concentrate systemically administered treatments (Thombre et al., 2023) and diagnostic tracers into the brain (Bae et al., 2023; **Figure 1A**) is achieved through temporary opening and partial disruption of tight junctions by physical interaction of focused high/low-frequency ultrasound (FUS) (Johansen et al., 2023; Mehta et al., 2023) as well as by using osmotic gradients (Cosolo et al., 1989; **Figure 1B**). Less conventional methods also include the use of isoflurane (Noorani et al., 2023) to increase the permeability of the brain to small hydrophilic molecules.

Extracranial magnetic resonance imaging (MRI) guided application of focused ultrasounds (FUS) in combination with circulating microbubbles (MB) has shown promise for the treatment of CNS diseases, including GB. High-intensity focused ultrasound (HIFU) can be used for cell and tissue-specific thermo-ablation, directly targeting GB cells (Johansen et al., 2023).

Low-intensity FUS has been FDA-approved for the treatment of untreatable tremors in Parkinson's disease (NCT03608553). This technique can ameliorate the symptoms in patients. On the other hand, the use of low-intensity FUS combined with circulating MB can temporarily open the BBB (FUS-BBBO) enabling the delivery of anticancer molecules into the brain that may have been ineffective when administered systemically. For example, the intracranial concentration of Panobinostat, administered intra-peritoneally in combination with ultrasound/Magnetic Resonance Imaging (FUS/MRI), was significantly increased compared to sole intraperitoneal injection (Martinez et al., 2023). This led to a significant reduction in tumor volume and extended survival in a patient-derived xenograft (PDX) orthotopic model.

FUS has also been beneficial in the delivery of newly designed conjugates, such as hyaluronic acid (HA) with camptothecin (CPT) and doxorubicin (DOX) (Sun et al., 2022). The flexibility of the polymer in these conjugates facilitates BBB crossing. Similarly, FUS application increased the tumor penetration of a new sensitizer system constituted using a polymeric block, the pH-sensitive polyglutamic acid (PGA) and the chemotherapeutic agent and sonosensitizer DOX, camouflaged with human U87-MG cell membranes administered systemically. This nano-system effectively delivered doxorubicin into U87-MG cells, resulting in tumor growth reduction and overcoming of drug resistance (Chen et al., 2023).

In recent developments, MB-FUS systems are being used actively in drug delivery for cancer immunotherapy. FUS-induced bursting of MB generates an acoustic emission-dependent expression of the proinflammatory marker ICAM-1. Simultaneously, MB-FUS enhances local delivery of anti-PD1 agents and promotes infiltration of T lymphocytes in the tumor microenvironment (Lee et al., 2022).

Other authors combined charged MB with the negatively charged Gambogic Acid (GA) (an active component of a traditional Chinese medicine effective as antiproliferative and tumor infiltration agent) loaded polymeric nanoparticle (GA/PLGA) to form a GA/PLGA-charged MB complex (Dong et al., 2022). Similarly, FUS application benefits the distribution and long-term accumulation of ^{89}Zr labeled/cetuximab, an anti EGFR antibody in a preclinical mouse model (Sacks et al., 2018).

In an innovative strategy, intracranial drug depots for small molecules were implanted in mice using bio-orthogonal click chemistry technology, which exploits extracellular matrix molecules to anchor tissue-reactive anchoring of click groups (TRAC). FUS and MB played a crucial role in enabling the non-invasive loading of these drug depots, allowing prolonged and spatially controlled treatment (Moody et al., 2023).

A phase 2 clinical trial (NCT03744026) has been designed for the use of a low intensity pulsed ultrasound (LIPU) device implanted into the skull of patients at the time of primary tumor surgical removal. A different trial (NCT04528680) combined LIPU with injected MB to efficiently increase the delivery of systemically infused albumin-linked paclitaxel in patients with recurrent glioblastoma. LIPU-MB is also being tested in combination with other therapies, such as albumin-bound paclitaxel and carboplatin (Sonabend et al., 2023).

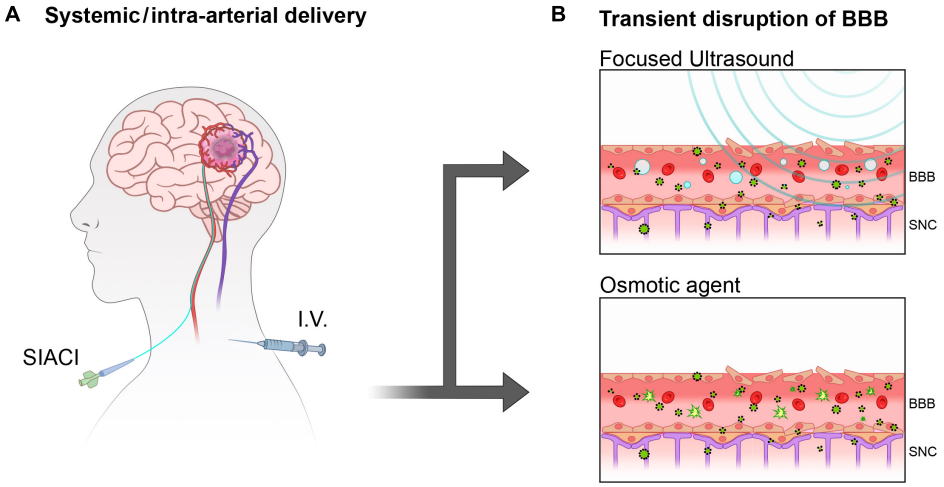


FIGURE 1
Overcoming BBB with systemically delivered therapies. **(A)** Superselective Intra-Arterial Cerebral Infusion (SIACI) and intravenous injection (I.V) are often administered in conjunction with by temporary opening of the BBB to facilitate delivery to the brain. **(B)** Focused ultrasound (FUS) and Osmotic forces are little invasive methods to temporary disrupt the tightly ordered pavement of the blood vessels constituting the BBB allowing blood-brain exchanges.

Overall, the application of FUS in combination with MB holds great potential for the treatment of CNS diseases, including glioblastoma. It allows for targeted treatment, enhanced drug delivery, and potential synergies with immunotherapy approaches.

In situ delivery of treatment

Maximizing the safe resection of tumor mass upon diagnosis is crucial for treatment, as the extent of residual volume directly correlates with patient survival. However, even after resection, there are often residual cells hidden within the uneven margins of the resected area, which can lead to tumor recurrence over time. *In situ* post peri-surgery applications aim to concentrate treatments to maximize the chances of killing these remaining cells. However, the presence of surrounding neurons necessitates minimizing functional deficits (**Figure 2**).

Currently, *in situ* delivery is obtained by convection-enhanced delivery (CED) (Sperring et al., 2023; Thompson et al., 2023) (Clinical Trial: NCT03043391) achieved by gently pushing drugs into the tumor (**Figure 2A**). Similarly, the intra-arterial delivery of chemicals, with or without the assistance of osmotic gradient (Chu et al., 2022; Wang et al., 2023) can result in effective intracranial treatment. However, the deposition of biocompatible drug-enriched injectable matrices (**Figure 2B**) that locally release active treatments seems to be the most promising strategy for intracranial controlled release. When resection is possible, patients are surgically implanted with a polymeric matrix for the semi-controlled release of carmustine, the FDA-approved Gliadel®. However, this implant has a degree of rigidity that limits its use. With the development of innovative biomaterials such as smart materials, polymeric matrices and hydrogels it will be possible to design more adaptable materials able to adhere to the surgical resection margins of the cavity (Gu et al., 2022; **Figure 2C**).

Many of these formulations can incorporate nanomaterials that are sensitive to environmental stimuli, including temperature (such as thermogels) (Gu et al., 2022), chemical stimuli such as acidity/CO_2 (Younis et al., 2023), reactive oxygen species (ROS) (Habra et al., 2022), magnetic force, or X-ray irradiation (Yun et al., 2022). Additionally, the customization of different nanoparticles (Idlas et al., 2023) and the loading of various molecules (Lin et al., 2023) allow for unlimited potential therapeutic combinations. In some cases, nanoparticles loaded with chemotherapeutics are dispersed into gel matrices (Gu et al., 2022), which function as a shelter to reduce molecule degradation and unwanted cytotoxicity (Erthal et al., 2023). New formulations for *in situ* delivery aim at optimizing the effects of first-generation anti-glioblastoma agents such as carmustine and temozolomide, either alone or in combination, to maximize their curative effects, for example overcoming the resistance to TMZ using the O_6 alkylguanine DNA alkyltransferase inhibitor, dialdehyde O_6 benzylguanine (DABG), tested in xenograft (Chu et al., 2023) or intracranially delivered, minimizing peripheral toxicity (Chen et al., 2022; Iturrioz-Rodríguez et al., 2023).

In situ delivery also presents an opportunity for targeting the tumor microenvironment and stimulating the tumor-suppressive immune system. Recently, hyaluronic acid (HA) has been utilized as an injectable delivery platform for combinatory treatments. HA-Doxorubicin and HA-CpG, an agonist of Toll-like receptor (Catania et al., 2023), were used to stimulate tumor-associated macrophages (TAM). Similarly, DOX-loaded mesoporous polydopamine (MPDA) nanoparticles were encapsulated in macrophage-derived nanovesicles (M1NVs) and used as immunostimulant effectors, while fibrin hydrogels serving as *in situ* delivery vehicles (Zhang R. et al., 2023).

GB recurrence has been recently modeled, in preclinical settings, by partially resecting the primary tumor. Bianco and coworkers have successfully demonstrated this approach (Bianco et al., 2017; Kubelt et al., 2023), and more recently, a minimally

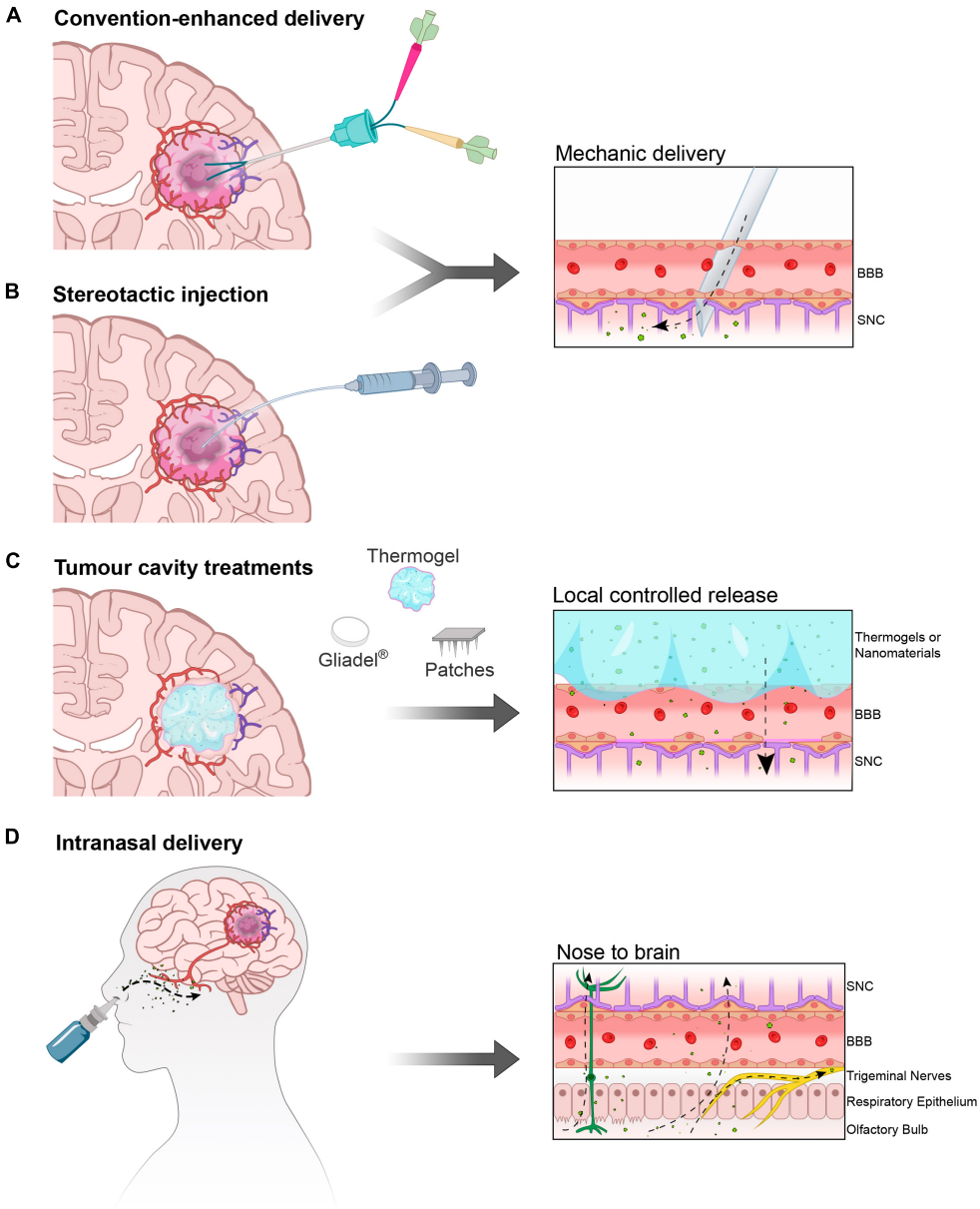


FIGURE 2
In situ and intranasal delivery. **(A)** Convection enhanced delivery (CED) and **(B)** injection of therapy solutions/gel directly into the parenchyma normally after primary tumor resection. **(C)** Deposition of matrices (solid polymers and gels) and mini-devices (micro-needles on patches) in direct contact with the after-resection cavity borders. **(D)** The intranasal route is a portal to the Central Nervous System as therapies inhaled or instilled on the nasal mucosa can bypass the BBB travelling through the olfactory neurons and trigeminal nerves, effectively reaching the Olfactory Bulb and from here different areas of the brain.

modified technique has been developed (Sun et al., 2023). Live imaging is frequently used to monitor the inevitable reappearance of the intracranial tumor due to residual cells and the presence of glioma stem cells (GSCs). Following resection, the cavity created in the brain tissue provides an opportunity for delivering curative treatments. One promising approach involves injecting a biodegradable thermosensitive triblock copolymer, poly (D,L-lactic acid-co-glycolic acid)-b-poly(ethylene glycol)-b-poly(D, L-lactic acid-co-glycolic acid) (PLGA-PEG-PLGA), that contain the anticancer therapy for sustained local release. These formulations mostly include TMZ (Zhang R. et al., 2023); however combinatorial treatment can be achieve also using less conventional anti-GB agents such as curcumin (Liang et al., 2023). Intra-tumor injection can also be performed with allosteric tissue elements (**Figure 2B**). In a phase I trial, a suicide gene therapy was attempted by injecting adipose tissue-derived mesenchymal stem cells (ADSCs) carrying the herpes simplex virus-thymidine kinase (HSV-TK) gene into patients with non-surgically removable recurrent GB (Oraee-Yazdani et al., 2023). A notable innovative therapeutic tool carrier has recently been developed (Sun et al., 2022). This carrier consists of allomelanin nanoparticles (AMNPs) loaded with a checkpoint inhibitor, CLP002, and camouflaged with cancer cell membrane coatings (CCM). The delivery system, named AMNP@CLP@CCM, can penetrate the BBB, interfering

with immune activity, and sustaining photo-thermal ablation with AMNPs. Ultimately, these combined effects will synergistically kill tumor cells.

The murine model of intracranial resection of primary tumor mass is frequently used for the preclinical investigation of treatment against recurrences, filling the resection cavity with the local therapy. "Smart" hydrogels, such as chitosan-based thermogels enriched with SiO_2-TMZ loaded nanoparticles, have proven effective in reducing recurrences in a U87-MG recurrence model. The use of silica particles as carriers represents an unconventional but equally effective choice compared to the more commonly used PCL-TMZ loaded nanoparticles (Gherardini et al., 2023). Similarly, photosensitive polymeric matrices have been previously employed to treat recurrences with paclitaxel (PTX) (Zhao et al., 2018). A novel approach to target residual cells in GB involved the use of polymeric microneedle patches designed to anchor within the irregular margin. These patches were loaded with polymer-coated nanoparticles containing either cannabidiol (CBD) or olaparib (OLA). The uniqueness of the study lies in both the choice of the carriers and the therapeutic agents (Muresan et al., 2023). OLA, a PARP inhibitor, has been utilized in combination with TMZ in both preclinical studies (Zampieri et al., 2021) and clinical trials (Lesueur et al., 2019). However, there are only a few encouraging reports on the use of CBD, the non-psychotropic component of cannabis, showing tumor growth reduction (Lah et al., 2022) and increased survival rates in glioblastoma patients (Likar et al., 2021).

The intranasal route

The intranasal route offers a direct pathway for active molecules to reach the CNS, making it an attractive option for drug delivery to the brain. This route serves two purposes: delivering drugs directly to the brain (nose-to-brain) and increasing peripheral vascular concentrations (nose-to-blood), similarly to pulmonary delivery methods. The olfactory nerve pathways serve as the gateway to the Olfactory Bulb, where drugs deposited on the nasal mucosa can travel through the olfactory neurons and trigeminal nerves, effectively reaching different areas of the brain (**Figure 2D**).

Through this way, active formulations can avoid first pass (enzymatic degradation and clearance) metabolism, increasing their bioavailability in the CNS, minimizing the occurrence of peripheral side effects. However, it is important to note that local damage of the nasal mucosa and brain tissue can limit the amount of active drugs that can be delivered.

In general, highly lipophilic drugs or nanoparticles with a low molecular weight are well-suited for intranasal delivery strategies. These characteristics enable better penetration and absorption through the nasal mucosa, facilitating efficient transport to the brain.

Recently, the utilization of intranasal routes for treating GB has been reviewed, focusing on the advantages and disadvantages for patients (Goel et al., 2022). Furthermore, in the past decade, the availability of tunable nanoparticles has stimulated multidisciplinary research to design and test various promising formulations (Montegiove et al., 2022). A recently completed clinical trial (NCT04091503) examined the safety and effectiveness of intranasal administration of TMZ in patients with GB and, recently, nanocarriers have been specifically designed for intranasal delivery. For instance, a monoclonal antibody anti-EphA3 was utilized to target TMZ-loaded gold nanoparticles (anti-EphA3-TMZ@GNPsTMZ) for photothermal therapy in nose-to-brain delivery (Yu et al., 2022). Chitosan-coated PLGA nanoparticles were optimized for nose-to-brain delivery of carmustine into the healthy Albino rat brain, resulting in significantly increased chemotherapeutic concentration compared to plasma levels. However, no indication of efficacy against tumor growth *in vivo* was reported (Ahmad et al., 2022).

Decorating nanoparticles with transferrin to achieve targeting of TfR appears to be an efficient approach for intranasal delivery, as already demonstrated (Sandbhor et al., 2023). Lipid nanoparticles (LNPs) were used for the co-delivery of paclitaxel (PTX) and miltefosine (HePc), a proapoptotic agent, resulting in significant tumor reduction and increased survival in mice after intranasal treatment compared to systemic Taxol® and nasal free drug administration. The role of meningeal pathways and of the lymphatic system in intranasal delivery has recently been studied revealing reduced liposomal transport in GB (Semyachkina-Glushkovskaya et al., 2022). The application of biocompatible infrared photo stimulation of meningeal lymphatic vessels in the cribriform plate could potentially enhance flux and delivery. Finally, we highlight few rare examples of nanoparticle tracing after intranasal delivery (Han et al., 2023) mapping their localization in the tumor site, brain parenchyma and more significantly intercepting accumulation in internal organs and overall body districts (NIR and SPECT/CT imaging), which is crucial for targeting and assessing off-target toxicity. Gold nanorods (AuNRs) tested as a platform for delivery in CNS were functionalized by adding the fluorescent dye Cyanine5 (Cy5) for optical imaging or a metal chelator, diethylenetriaminepentaacetic dianhydride (DTPA) hinged by PEG to ^{111}Indium for nuclear detection, allowing detection of the brain area distribution and the peripheral organ distribution of the NP after entering the CNS (Han et al., 2023).

In conclusion, the nose-to-brain route holds great potential for repurposing conventional drugs for novel therapeutic applications.

Drug repurposing

Drug repurposing is a cost-effective strategy for cancer treatment, serving as an alternative to *de novo* drug discovery. In the case of GB, candidate molecules for repurposing are often identified through biomedical and biogenetic profiling of patients and *in silico* docking simulations (McGowan et al., 2023; Roy et al., 2023).

Among these candidates, FDA-approved drugs that are currently used for treating other conditions have shown promise (Jones et al., 2023; Moretti et al., 2023; Murali and Karuppasamy, 2023; Vítovcová et al., 2023). Recent preclinical examples include Flubendazole, an inhibitor of microtubule growth that activates autophagy and STAT3-dependent apoptosis, and auranofin (AF), an inhibitor of TrxR1, used alone or in combination with the prooxidant menadione (Szeliga and Rola, 2022). Hydroquinidine (HQ) is another repurposing candidate (Yavuz and Demircan, 2023) to induce GBM cell death overcoming TMZ resistance that could be a good candidate for *in vivo* study.

Repurposing or repositioning anticancer agents that have demonstrated efficacy *in vitro* but failed to produce therapeutic effects *in vivo*, or those causing unacceptable off-target effects when administered systemically, could find a second life through local delivery. For example, intranasal administration of Temozolomide (TMZ) (Clinical Trial: NCT04091503) or systemic administration of Placlitaxel, in combination with albumin to induce osmotic opening of BBB (NCT04528680) is under investigation, as well as the intracranial deposition of Paclitaxel in OncoGel® NCT00479765 (Morales and Mousa, 2022) to elicit a stronger antitumor effect. A single dose of intra-arterial mannitol should facilitate the temporary opening of the BBB to allow superselective intra-arterial cerebral infusion (SIACI) (**Figure 1A**) of a high single dose of TMZ (up to 250mg/m2) to achieve a more efficient tumor site targeting (NCT01180816). Similarly, SIACI protocol with mannose osmotic pretreatment is attempted with Cetuximab (CTX), targeting the Epidermal Growth Factor Receptor (EGFR) is under study in a Phase I clinical trial to treat recurrent GB (Clinical Trial: NCT02861898).

Conclusion

Recent advances *in loco*-regional treatment in GB focused on overcoming the BBB and targeting microenvironmental proteins to enhance therapeutic efficacy. "The Holy Grail," "the Magic Bullet," "the Trojan Horse": these are all metaphors that can be used to define the goal to find a mythic nano-devise or a molecular tool that could be able to overcome the BBB and deliver its anticancer therapy for an effective personalized treatments to benefit GB patients. On the other hand, strategies such as focused ultrasounds with microbubbles, intracranial drug depots, and *in situ* delivery using nanomaterials and biomaterials show promise in improving drug delivery combating GB recurrence and improving patient's overall survival and quality of life.

Author contributions

MC: Conceptualization, Data curation, Funding acquisition, Project administration, Writing—original draft. GI: Conceptualization, Data curation, Methodology, Software, Writing—original draft. VB: Conceptualization, Data curation, Writing—original draft. LG: Conceptualization, Data curation, Methodology, Writing—original draft, Writing—review and editing.

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Conflict of interest

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

Publisher's note

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Glitches in the brain: the dangerous relationship between radiotherapy and brain fog

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Up to approximately 70% of cancer survivors report persistent deficits in memory, attention, speed of information processing, multi-tasking, and mental health functioning, a series of symptoms known as "brain fog." The severity and duration of such effects can vary depending on age, cancer type, and treatment regimens. In particular, every year, hundreds of thousands of patients worldwide undergo radiotherapy (RT) for primary brain tumors and brain metastases originating from extracranial tumors. Besides its potential benefits in the control of tumor progression, recent studies indicate that RT reprograms the brain tumor microenvironment inducing increased activation of microglia and astrocytes and a consequent general condition of neuroinflammation that in case it becomes chronic could lead to a cognitive decline. Furthermore, radiation can induce endothelium reticulum (ER) stress directly or indirectly by generating reactive oxygen species (ROS) activating compensatory survival signaling pathways in the RT-surviving fraction of healthy neuronal and glial cells. In particular, the anomalous accumulation of misfolding proteins in neuronal cells exposed to radiation as a consequence of excessive activation of unfolded protein response (UPR) could pave the way to neurodegenerative disorders. Moreover, exposure of cells to ionizing radiation was also shown to affect the normal proteasome activity, slowing the degradation rate of misfolded proteins, and further exacerbating ER-stress conditions. This compromises several neuronal functions, with neuronal accumulation of ubiquitinated proteins with a consequent switch from proteasome to immunoproteasome that increases neuroinflammation, a crucial risk factor for neurodegeneration. The etiology of brain fog remains elusive and can arise not only during treatment but can also persist for an extended period after the end of RT. In this review, we will focus on the molecular pathways triggered by radiation therapy affecting cognitive functions and potentially at the origin of so-called "brain fog" symptomatology, with the aim to define novel therapeutic strategies to preserve healthy brain tissue from cognitive decline.

KEYWORDS

brain fog, cognitive impairment, neuroinflammation, brain tumors, sigma receptors, P2X7 receptors, transcranial direct current stimulation, hyperbaric oxygen

1 Introduction

Cancer-related cognitive deficits (CRCDs), also known as "brain fog," are common among cancer patients (Hardy et al., 2023) and include difficulties in short-term and working memory, attention, processing speed, verbal fluency, and executive function (Jean-Pierre et al., 2012; McDougall et al., 2014; Prasad et al., 2015). Among the potential reasons for brain fog aside from psychological distress are chemotherapy-or radiation-related neurotoxicity (Hardy et al., 2023). However, the etiology of CRCDs remain still elusive as they arise during treatment but can also persist for an extended period after the end of chemo-or radio-treatment (Falleti et al., 2005; Eberhardt et al., 2006; Ahles et al., 2012).

Brain tumors are one of the most aggressive and detrimental forms of cancer. In particular, for brain tumors and for brain metastasis originating from extracranial tumors, the standard treatment includes surgery, chemotherapy and radiation. Glioblastoma multiforme (GBM) represents the most common and aggressive brain tumor in the aging population and accounts for 58% of all gliomas in the elderly. The prognosis for patients with GBM remains dismal, with overall survival of 12–18 months. Notwithstanding, though GBM is a rare tumor with a global incidence of less than 10 per 100,000 people, its poor prognosis makes it a crucial public health issue. Early diagnosis and the type of treatment chosen do not affect GBM patient survival rate, making screening programs unhelpful. Long-term survival in GBM, defined as survival beyond 3 years, remains scarce, with estimates ranging from 3 to 5%. While extensive research is underway to develop novel therapies for extending survival, the impact of tumor and treatment on the cognitive status of survivors remains relatively understudied.

The current standard of care for Glioblastoma multiforme is the Stupp's protocol, developed in 2005, which involves a two-stage approach: debulking surgery followed by a combination of radiotherapy (RT) and chemotherapy (Stupp et al., 2005). RT is the mainstay treatment (Marsh et al., 2010; McDuff et al., 2013; Owonikoko et al., 2014) due to its capability to uniformly penetrate both the brain and tumor parenchyma, overcoming resistant cells (McDuff et al., 2013). However, on the other side of the coin, cranial radiation may induce a cognitive decline, the most common radio-correlated neurotoxic effect at any patient's age observed also as a result of doses much lower than those that can cause radionecrosis (Makale et al., 2017).

Radiation therapy, an integral component of modern cancer treatment, holds particular significance for primary brain tumors. Often the sole modality that offers substantial survival and quality-of-life benefits, RT plays a crucial role in the management of these malignancies.

Over the past few decades, radiotherapy for brain tumors has undergone significant technological advancements across all aspects of treatment, including patient immobilization, imaging, treatment planning, and precise delivery. This includes better imaging, planning, and delivery methods. These advancements, especially in imaging and radiation technology, allow for more precise targeting of tumors and less damage to healthy brain tissue (Scaringi et al., 2018). In particular, the intensity-modulated radiation therapy (IMRT) and volumetric modulated arc therapy (VMAT) "shape" the radiation beams to closely fit the tumor's unique shape, minimizing harm to healthy tissue and reducing side effects while maximizing the treatment's effectiveness (Scaringi et al., 2018; Kotecha et al., 2021). Stereotactic irradiation represents an advanced iteration of conventional external beam radiation therapy (CRT). It uses special headgear to hold patients perfectly still, allowing incredibly precise targeting of the tumor with submillimeter accuracy. This reduces the amount of healthy brain tissue exposed to radiation, potentially lowering the risk of long-term side effects. The treatment can be given in one or multiple sessions, still delivering high doses to the tumor. Another relatively new technique is FLASH RT, defined as a single ultra-high dose-rate RT (higher than 40Gy/S), based on the proton's capacity of deliver little energy with the highest energy release in the target volume, leading no dose leakage and reducing damage on healthy tissue (Hughes and Parsons, 2020; Huang and Mendonca, 2021; Lin et al., 2022).

RT has established itself as one of the three mainstays of GBM treatment, alongside surgery and chemotherapy (Orth et al., 2014). Beyond its direct and indirect DNA damage-induced local control of target lesions in cancer cells, recent preclinical and clinical evidence suggests that RT may also modulate antitumor immune responses by inducing immunogenic cell death and reconfiguring the tumor microenvironment (TME). In particular, GBM is characterized by high inter-and intra-tumor heterogeneity and a very complex TME, composed not only of neoplastic cells but also of nervous cells (i.e., astrocytes and neurons), stem cells, fibroblasts, vascular as well as varieties of host and infiltrating immune cells. This has led many to evaluate RT as a partner therapy to immuno-oncology treatments, a research field very relevant in brain tumors, where the blood–brain barrier (BBB) significantly limits the penetration of antineoplastic drugs into the brain and consequently the achievement of therapeutic sufficiently high concentrations. While RT offers potential benefits in treating brain tumors, it is also associated with a common complication: cognitive decline. Despite the prevalence of this issue, the underlying mechanisms responsible for this dysfunction remain largely unclear. Consequently, there are currently no effective preventive measures or treatments available.

This review aims to shed light on this critical yet understudied issue.

2 The relationship between RT and brain fog

2.1 RT and neuroinflammation: mastering the duality of a double-edged blade

Our current understanding of the mechanisms underlying radiation-induced brain injury centers on the immediate depletion of neural stem cells and the subsequent disruption of hippocampus-mediated functions, including learning and memory. Indeed, different studies have documented that stress leads to a reduction of dendritic, spine, and synaptic material in the hippocampus and prefrontal cortex (Wager-Smith and Markou, 2011). Additionally, a single 10-min session of swim stress has been shown to cause dendritic length loss in the infralimbic cortex (Izquierdo et al., 2006). In the context of RT effects and cancer, it is well known radiation ability to induce an "immunogenic hub" of great relevance for the local (bystander effect) and remote (abscopal effect) antitumor effects, as described for several solid tumors by different groups (Formenti and Demaria, 2013; Baskar et al., 2014; Klammer et al., 2015; Marín et al., 2015). However, RT

could have a hidden side in the brain. Indeed, in addition to its direct cytotoxic effect on neuronal cells, RT may negatively impact on the cells of TME directly or by inducing the release of inflammatory mediators such as adenosine triphosphate (eATP), interferons, and chemokines to the extracellular space (Greene-Schloesser et al., 2012; Herrera et al., 2017) potentiating glioma cell growth and invasion or contributing to build up an immunosuppressive milieu (Wang and Haffty, 2018).

The same glioma cells are known to increase oxidative stress and stimulate the release of immunosuppressive molecules such as interleukin-6 (IL-6), IL-10 and tumor growth factor beta (TGF-β), which in turn reprogram the immune components of TME such as microglia to a pro-tumorigenic phenotype (Alghamri et al., 2021). This condition can lead to the loss of BBB integrity, exposing the brain to adverse substances from the periphery, and to host immune cells, that can disrupt the homeostasis of the CNS (Alghamri et al., 2021). In support of this, the impact of myeloid cells on TME is compared by Buonfiglioli and Hambardzumyan to "the mythological evil three-headed dog, Cerberus," that guards the underworld as well as microglia cells play a triple protecting and supporting role on tumor. In fact, these myeloid cells promote tumor growth, modulate immune suppression, and exacerbate cerebral edema (Buonfiglioli and Hambardzumyan, 2021).

Furthermore, several works showed that irradiated microglia may induce astrogliosis, release of neurotoxic factors, compromising the BBB integrity with consequent immune cells invasion and neuronal cell death (Hwang et al., 2006; Wilson et al., 2009; Liddelow et al., 2017). The induction of a reactive state in microglia following cranial irradiation treatment have been shown to be associated with deficits in neural precursor, neuronal cell population maintenance and neurogenesis, in synaptic structure and function, and myelin plasticity. During development and under normal physiological conditions, microglia play a crucial role in shaping neural circuit refinement by eliminating excess dendritic and synaptic connections (Stevens et al., 2007; Schafer et al., 2012). Moreover, these cells exhibit complex branching patterns and display remarkable mobility in response to injury or disease, rapidly migrating to the affected area to engulf cellular debris. Activated microglia are also observed in various neurodegenerative disorders, such as Alzheimer's disease (AD) (Hong et al., 2016) and Parkinson's disease (PD) (Lecours et al., 2018), where microglial activation contributed to aberrantly increased synaptic pruning. Notably, microglia and astrocytes are overexpressed in the brain until 140 days post-irradiation in rats (Desmarais et al., 2015). In addition, microglial cells were found in an activated status with a classic amoeboid phenotype, harboring few ramifications and increased body volume in the area near the irradiation focus, while showing a steady state morphology with extended processes in the distal area (Constanzo et al., 2020). It is also known that microglia exhibit remarkable plasticity, adopting a spectrum of activation states ranging from fully inflamed, characterized by the release of pro-inflammatory cytokines, to alternatively activated, distinguished by the secretion of anti-inflammatory cytokines or neurotrophins. Consequently, microglia can transit from a homeostatic, neurotrophic state to a neurotoxic state (Luo and Chen, 2012). *In vivo* experiments on rats and mice demonstrated a dose-dependent reduction in hippocampal neurogenesis following ionizing irradiation, with higher radiation doses resulting in more pronounced impairments in both proliferating precursor cells and newly formed neurons (Tada et al., 1999). The deficit in neurogenesis is due mainly to radiation-induced perturbations in the neurogenic niche, rather than cell-intrinsic effects on the precursor cells (Monje et al., 2002, 2003). The IL-6-mediated inhibition of neuronal differentiation caused by radiation-activated microglia was postulated to be the central element in this microenvironmental disruption (Monje et al., 2002). In preclinical models, the direct contribution of radiation-induced microglial inflammation to cranial irradiation-mediated memory impairments is strongly supported by the evidence that anti-inflammatory drugs targeting microglia or depletion of microglia (Monje et al., 2003) using CSF1R inhibitors (Acharya et al., 2016) restore hippocampal neurogenesis and enhance cognitive function following irradiation. Finally, a recent study employing a glioma mouse model underscores the significance of non-tumor factors in memory impairment following cranial irradiation. The study suggests that microglial activation triggered by radiation exposure plays a more prominent role in memory dysfunction than tumor growth itself (Feng et al., 2018). While the applicability of these findings to humans warrants further investigation, the specifics may differ across glioma subtypes.

Furthermore, irradiated brain tissues show pathologic features resembling aging-associated neurodegeneration, including reduced neurogenesis, chronic oxidative stress and inflammation (Mrak, 2009; Wang et al., 2014). In response to radiation exposure and subsequent DNA damage accumulation, cells can undergo various cell type-specific responses, one of which is cellular senescence (Eriksson and Stigbrand, 2010). Notably, senescent cells, despite their inability to replicate, may evade clearance and accumulate in tissues, persistently releasing inflammatory factors that contribute to tissue damage (Tripathi et al., 2021). Consequently, radiation-induced cellular senescence has emerged as a crucial mediator of tissue dysfunction, fueling chronic inflammation and exacerbating radiation-induced side effects. Moreover, a burgeoning body of research suggests that astrocyte senescence and astrocyte-derived neuroinflammation could be identified as potential contributors to radiation-induced brain injury. While astrocytes perform numerous neuroprotective functions, including the production of neurotrophic factors, they may also promote neurodegeneration in certain diseases, such as AD, which is thought to be associated with the induction of a senescence-associated secretory phenotype (SASP). In addition, animal models of radiation-induced brain injury have revealed the presence of hypertrophic astrocytes that persist for at least 12 months following radiation exposure (Suman et al., 2013; Turnquist et al., 2016). Notably, a significant proportion of these enlarged astrocytes exhibit senescence, a crucial pathological feature that likely extends to other brain disease processes. Following brain injury, astrocytes undergo proliferation as part of reactive astrogliosis, a process that can lead to replicative senescence (Pekny and Pekna, 2014; Herranz and Gil, 2018). Elevated secretion of the SASP cytokines, IL-6 and IL-1β has been observed in animal models of radiation-induced brain injury and is suspected to impede neurogenesis, thereby contributing to cognitive decline (Haveman et al., 1998; Monje et al., 2003; Rola et al., 2004; Lee et al., 2010; Yang et al., 2017). Therapeutic interventions that target and mitigate neuroinflammation using anti-inflammatory drugs have demonstrated enhanced neurogenesis in radiation-induced brain injury (Marmary et al., 2016). In fact, in animal models, IL-6 has been shown to exacerbate radiation-induced senescence, further emphasizing the crucial role of chronic neuroinflammation in promoting radiation-induced brain injury (Turnquist et al., 2019). It is also been reported that

the inhibition of full-length p53 regulates p21, RAD51, and IL-6, each of which has been shown to be important in radiation-induced injury and neurotoxicity. The same study also provided compelling evidence suggesting that the p53 isoform Δ133p53 holds therapeutic potential in preventing astrocyte senescence and mitigating astrocyte-mediated neuroinflammation. In addition, astrocyte dysfunction, even in the absence of neuronal or other cellular damage, can lead to memory loss. These abundant brain cells (not by chance, some gliomas more closely resemble cells of the astrocyte lineage) do play crucial roles, while their contribution to neurocognitive disorders such as dementia remains incompletely understood. A recent work by Licht-Murava et al. (2023) showed that abnormal immune activity in astrocytes is sufficient to cause cognitive deficits in dementia. In particular, the authors found that patients with AD or frontotemporal dementia have aberrant accumulation of TAR-DNA binding protein-43 (TDP-43) in hippocampal astrocytes. In Alzheimer's disease mouse models, inducing widespread or hippocampus-targeted TDP-43 accumulation in astrocytes resulted in progressive memory loss and localized alterations in antiviral gene expression. Furthermore, Disruptions in astrocytic TDP-43 function contribute to cognitive decline through abnormal chemokine-mediated signaling between astrocytes and neurons (Licht-Murava et al., 2023).

Finally, both *in vitro* and *in vivo* studies have demonstrated that the inflammatory response of microglia and astrocytes is mediated by PARP-1, with its activation triggering protein synthesis and proliferation (Gutierrez-Quintana et al., 2022). However, excessive PARP-1 activation can lead to detrimental consequences, including neuronal death, persistent microglial activation, and neuroinflammation. The most well-established mechanism by which PARP-1 contributes to neuroinflammation involves its regulation of pro-inflammatory transcription factors such as NF-κB, AP-1, and nuclear factor of activated T cells (Ullrich et al., 2001; Kauppinen and Swanson, 2005; Kauppinen et al., 2011; Martínez-Zamudio and Ha, 2014; Stoica et al., 2014; Raghunatha et al., 2020) (Figure 1). In particular, several studies have reported that nuclear translocation of NF-κB requires PARP-1 function. NF-κB is one of the best-characterized transcription factors, regulating the expression of multiple genes involved in immunity and inflammation. PARP-1 activity is strongly linked to BBB disruption observed in neuroinflammatory diseases. While the precise mechanisms remain to be fully elucidated, several studies suggest connections between PARP activation, edema formation, and heightened infiltration of peripheral immune cells into the brain parenchyma (Chiarugi and Moskowitz, 2003). These observations led to the evaluation of PARP-1 inhibitors as potential mitigators of neurotoxicity in animal models of CNS pathologies in which neuroinflammation plays a key role. Moreover, of particular significance for their potential applications in neuro-oncology, PARP inhibitors have demonstrated synergistic effects when combined with DNA-damaging agents like TMZ and RT, which together constitute the standard of care for GBM patients (Lescot et al., 2010; Wu et al., 2014; Rom et al., 2015).

2.2 RT induces ER stress-response leading to neurodegeneration

Although the correlation between RT and neuroinflammation is largely been discussed, the precise mechanisms of neurotoxicity and consequent neurodegeneration remain poorly understood at the molecular level (Friedl et al., 2022). We tried to address this issue investigating the interaction between RT and ER-stress response pathways, building upon existing evidence linking RT to ER stress and ER stress to neurodegeneration. This approach allowed us to formulate a hypothesis about the specific link between RT and neurodegeneration.

Approximately one-third of all proteins undergo post-translational modifications, folding, and trafficking within the endoplasmic reticulum (ER) (Black et al., 1981; Kim et al., 2008) and cells maintain a state of proteostasis through a complex network of signaling pathways that regulate protein synthesis, folding, trafficking, and degradation (Read and Schröder, 2021). In response to specific physiological or pathological conditions, the demand for protein synthesis can surge, overwhelming the protein-folding capacity of the ER lumen. This results in the accumulation of partially folded, misfolded, or unfolded proteins, a state known as ER stress (Gutierrez-Quintana et al., 2022). Mild ER stress is typically managed by the unfolded protein response (UPR). However, prolonged or persistent ER stress triggers constitutive UPR activation, which ultimately leads to the activation of cell death pathways. Disruptions in these processes can lead to the accumulation and aggregation of misfolded proteins within cells, triggering pathological consequences, just as neurodegeneration (Ren et al., 2021).

In particular, radiation can induce ER stress either directly or indirectly through the production of reactive oxygen species (ROS). In some cases, cancer cell clones that survive radiation therapy may do so by activating compensatory survival signaling pathways, such as the UPR. In particular, under radiation-induced ER stress, specific signaling by PERK, ATF6, and IRE1 may be activated, and augment the upregulations of UPR-related genes to recover and recycle misfolded proteins (Chatterjee et al., 2018). However, excessive activation of UPR in the surviving cell fraction resident in the irradiated field, was showed to cause either radioresistance in tumor cells (Urra et al., 2016) and induce an anomalous accumulation of misfolded protein in neuronal cells exposed to radiation, paving the way to the pathogenesis of neurodegenerative disorders (Wang et al., 2018). Indeed, neuronal cells are particularly susceptible to protein misfolding compared to non-neuronal cells. In non-neuronal cells, cell division helps to mitigate the effects of ER stress by repeatedly diluting unfolded peptides. In contrast, not-dividing post-mitotic neurons rely solely on the UPR for survival. Therefore, if the misfolding is not resolved and normal cellular functions are not restored, the UPR can trigger selective neuronal death or neurodegeneration due to the accumulation of aberrant proteins. This strongly supports the crucial role of ER stress in the pathogenic neuronal response (Hetz and Saxena, 2017; Wang et al., 2018). Thus, various neurodegenerative diseases display specific types of misfolded proteins (Lindholm et al., 2006; Remondelli and Renna, 2017). For example, AD, PD, Huntington's disease (HD), and ALS are characterized by a clinically silent period, during which aberrant proteins progressively aggregate and accumulate in the brain, leading to impaired synaptic function and ultimately neurodegeneration (Ciechanover and Kwon, 2015; Remondelli and Renna, 2017). These pathological conditions affecting the peripheral and CNS are also called "protein misfolding diseases."

In support of this, chronic ER dysfunction was showed to be highly associated with memory and cognitive impairment observed in different neurodegenerative diseases, like AD (Duran-Aniotz et al., 2014) and PD (Ryu et al., 2002; Colla et al., 2012). In addition, there is some evidence

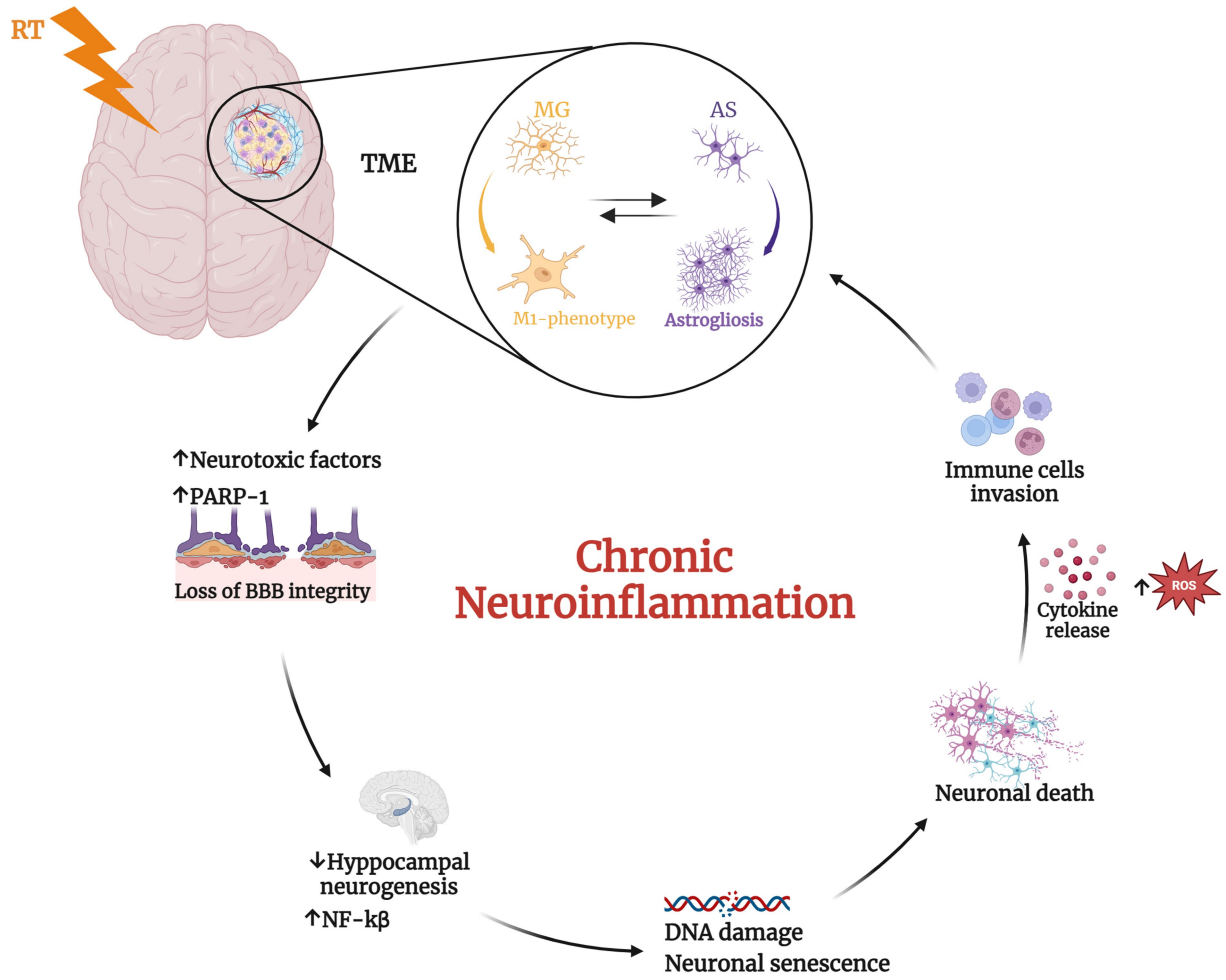

FIGURE 1
Radiotherapy (RT) induces significant changes in the tumor microenvironment. RT mediates the transition of microglial (MG) cells from a resting state to the activated pro-inflammatory M1-phenotype that induces prolonged activation of astrocytes (AS) leading to astrogliosis (Hwang et al., 2006; Wilson et al., 2009; Liddelow et al., 2017; Feng et al., 2018). This, in turn, promotes the phenotype switching of microglia in a vicious cycle. These phenomena determine neurotoxic factors release, the alteration of blood–brain barrier (BBB) integrity (Alghamri et al., 2021) and the upregulation of Poly ADP-ribose polymerase 1 (PARP-1), with consequent increase of nuclear factor kappa beta subunit (NF-kβ) and reduced neurogenesis (Ullrich et al., 2001; Chiarugi and Moskowitz, 2003; Kauppinen and Swanson, 2005; Kauppinen et al., 2011; Martínez-Zamudio and Ha, 2014; Stoica et al., 2014; Raghunatha et al., 2020; Gutierrez-Quintana et al., 2022). The consequent inflamed condition induces DNA damage, neuronal senescence, and ultimately neuronal death. These alterations further recall immune cells into the tumor microenvironment (TME) determining a chronic neuroinflammation status. Created with BioRender.com.

that PERK and IRE1, central in UPR signaling pathways, are important in neurodegenerative diseases due to their impact on synaptic functions and their capability to attenuate the effects of chronic ER stress. It has been shown that selectively lowering PERK expression in AD mice models prevents the aberrant phosphorylation of eIF2α and consequently improves synaptic plasticity and spatial memory consolidation (Costa-Mattioli et al., 2009) (Figure 2).

In addition, lowering of PERK expression in AD mice models prevent the aberrant phosphorylation of eIF2α and consequently improved synaptic plasticity and spatial memory consolidation (Costa-Mattioli et al., 2009). Moreover, suppression of eIF2α kinases alleviates AD-related plasticity and memory deficits (Ciechanover and Kwon, 2015). These findings, taken together, further support the hypothesis that UPR pathways are implicated in the disruption of cognitive and memory functionality and strategies aimed at restoring the proper proteostasis of neuronal cells could have important therapeutic effects (Hetz and Saxena, 2017). In fact, targeting pathways associated with abnormal ER stress with pharmacological treatment has been shown to rescue neuronal loss in PD *in vitro* models (Chung et al., 2013). There are also evidences that cells with a chronic and severe ER stress, for instance induced by RT, interfere with immunosuppressive environment of the CNS, supporting a link between neuronal cells under ER stress and glial cells leading to inflammation of brain microenvironment (Drake, 2015; Logsdon et al., 2016). As a case in point, ER-stress-induced astrocyte activation can induce a pro-inflammatory phenotype in microglial cells (Meares et al., 2014), which through their innate receptors, can recognize extracellular protein aggregates or oligomers as danger signals. This interaction triggers a neuroinflammatory response that initiates debris clearance via microglia-mediated phagocytosis (Sprenkle et al., 2017).

Notably, during the UPR the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and mitogen-activated protein

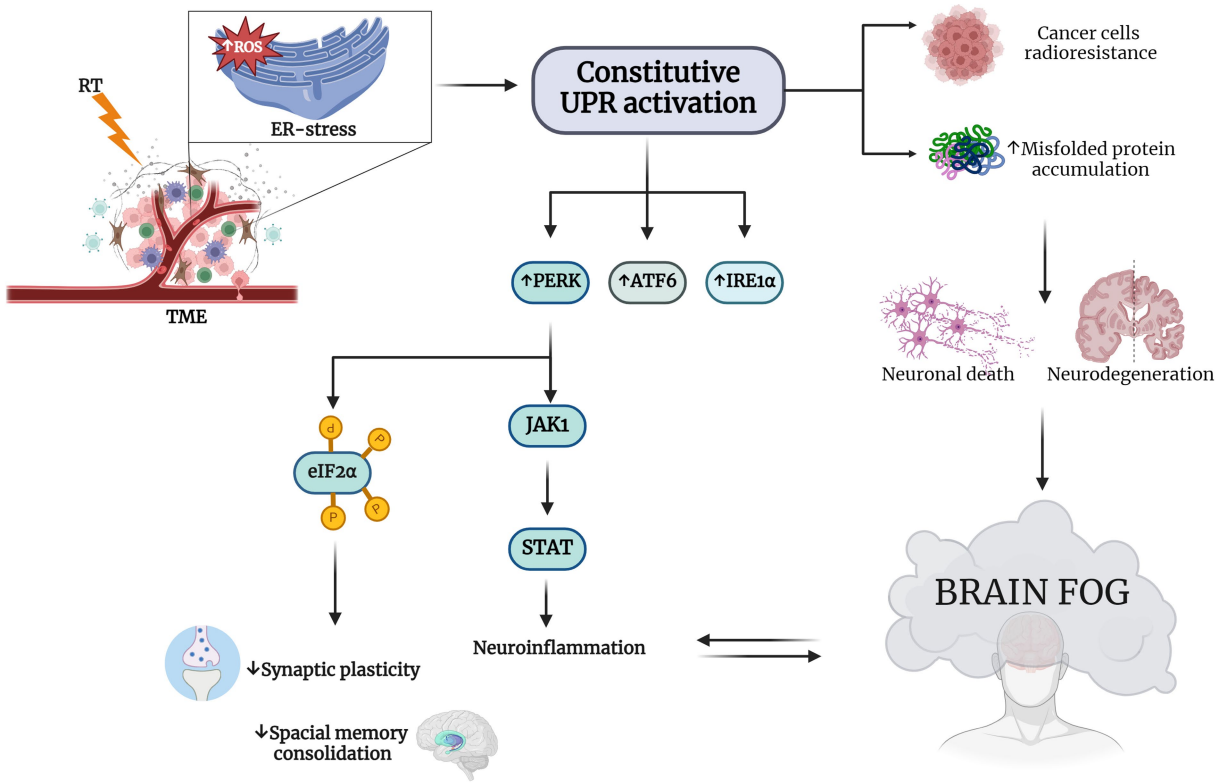

FIGURE 2
RT induces ER stress in TME and consequent brain fog. RT-induced endoplasmic reticulum (ER) stress-specific signaling that leads to a constitutive unfolded protein response (UPR) activation This increases the expression of eukaryotic initiation factor 2α (eIF2α) kinase (PERK), activating transcription factor 6 (ATF6) and type I transmembrane protein inositol requiring 1 α (IRE1α). Hyperactivation of UPR is a survival strategy from RT for cancer cells and results in misfolded protein accumulation in neuronal cells Chatterjee et al., 2018; Wang et al., 2018; Ren et al., 2021). The neurodegeneration induced by this accumulation may explain the cognitive impairment observed in patients treated with RT. PERK increasing determines on one hand the phosphorylation of eIF2α, decreasing synaptic plasticity and impairing spatial memory consolidation, and on the other hand, it causes neuroinflammation through Janus kinase 1 (JAK1) and the increasing of signal transducer and activator of transcription (STAT), with the consequent establishment of the so-called brain fog (Tang et al., 2001; van den Berg et al., 2001; Bellezza et al., 2014; Meares et al., 2014; Drake, 2015; Logsdon et al., 2016; Sprenkle et al., 2017). Created with BioRender.com.

kinase 1 (MAPK-1) were shown to play a pivotal role in mediating cell survival (Tang et al., 2001; van den Berg et al., 2001). Furthermore, in astrocytes it has been shown that UPR affects STAT pathways through the interaction of PERK and JACK1, further supporting the association between UPR and neuroinflammation (Meares et al., 2014), paving the way to our hypothesis that RT could lead to neurodegeneration through the exacerbation of ER-stress response pathways.

2.3 RT effects on proteasomal degradation system and neurocognitive disorders

To counteract against misfolded proteins, the ER system has in place quality control mechanisms, including the unfolded protein response (UPR), as well as ER-associated degradation (ERAD) (Taylor et al., 2002; Hartl, 2017), which interacts in a coordinated manner with the ubiquitin-proteasome system (UPS) (Araki and Nagata, 2011). This multicatalytic complex is also the main target of many cancer therapies, including radiation. It was reported that even subtle changes in cellular redox balance caused by irradiation (and other stress stimuli) profoundly impact proteasome function. This suggests that proteasomes act as sophisticated and highly sensitive stress sensors, rapidly and simultaneously orchestrating diverse cellular processes in response to radiation exposure (Pervan et al., 2005). In particular, ionizing radiation exposure has been shown to impair normal proteasome activity. This reduction in proteasome activity slows the degradation of proteins, leading to their further accumulation and exacerbating endoplasmic reticulum (ER) stress conditions. Studies utilizing proteasome inhibitors across various organisms have revealed its impact on memory processes, including consolidation, recollection, and extinction. In fact, within the nervous system, the proteasome plays a crucial role in protein degradation and maintaining cellular homeostasis in neurons, glial cells, thereby contributing to overall brain health (Davidson and Pickering, 2023). Moreover, because the proteasome degrades most short-lived cellular proteins, primarily the proteasome subtypes (26S), changes in its activity might significantly, and selectively, alter the life span of many signaling proteins and in particular, in brain cells, compromise several neuronal functions, such as gene transcription and neurotransmitter release. Emerging research has shed light on the neuron-specific functions of the proteasome, particularly its crucial role in facilitating long-term memory formation (Giulivi et al., 1994; Dantuma and Lindsten, 2010; Brodsky and Skach, 2011; Jung and Grune, 2013) and

potentiation (Pacifici et al., 1993), dendritic spine growth (Upadhya et al., 2004) and neurodevelopment (Dong et al., 2008; Hamilton et al., 2012), as well as synaptic plasticity (Hamilton and Zito, 2013). The proteasome also plays a regulatory role in clock proteins within the nervous system, influencing circadian rhythm (Ceriani et al., 1999).

Furthermore, the proteasome is linked to neuroinflammation and to some age-related neurodegenerative diseases (Pintado et al., 2017). In particular, during neuroinflammatory conditions the brain expresses cyclooxygenases-1 and 2 (COX-1 and COX-2), which release prostaglandins that induce proteasome inhibition that, in turn, hampers neuroinflammation. In particular, COX-2 is upregulated in both neurons and glial cells during neuronal injury (Consilvio et al., 2004; Ishii et al., 2005; Bi et al., 2012). Additionally, in a study of Pintado and colleagues, it was shown that proteasome inhibition in a rat model caused a worsening of neuroinflammation (Pintado et al., 2012). Neurological disorders have been also reported to be associated with the accumulation of ubiquitinated proteins in neuronal inclusion and also with signs of inflammation that may contribute to neurodegenerative processes (Lev et al., 2006; Ortega et al., 2007). Taken together, these considerations lead to the hypothesis that changes induced by radiotherapy in proteasome system trigger the neuroinflammation which in turn induce cognitive impairment and neurodegeneration (Figure 3).

Declines in the activity of the constitutive proteasome, observed with aging and neurodegenerative diseases, often coincide with an upregulation of the alternative proteasome form, known as the immunoproteasome. The immunoproteasome is a specialized variant that differs from the standard proteasome in three subunits, induced by inflammation and constitutively expressed in hematopoietic cells. Under non-inflammatory conditions, the immunoproteasome is only a small portion of the total cellular proteasome pool, as immunoproteasome expression is low in neurons and glia in absence of cytokine stimulation. In the CNS the immunoproteasome appears to be expressed both in immune and non-immune cells, including in astrocytes, bone marrow-derived immune cells, oligodendrocytes, and Purkinje cells (Speese et al., 2003; Eide et al., 2005; Hegde, 2010). Immunoproteasome expression is typically low in these regions but undergoes a significant increase in response to injury (Ferrington et al., 2008). For instance, after interferon-γ release and during neuroinflammation, cells are stimulated to produce ROS such as the H_2O_2 and the superoxide hydroxyl radicals, thus damaging the cellular proteome (Pearl-Yafe et al., 2003). Moreover, when neuronal accumulation of ubiquitinated proteins occurs, there is a switch from proteasome to immunoproteasome that increases the peptide reserve for antigen presentation. Indeed, a Pintado's *in vivo* study, showed that after the injection of lipopolisaccaride in rats with a higher proportion of immunoproteasome, proteasome inhibition induced the formation of neuronal aggresome-like structures. However, these modifications were not observed when proteasome inhibition was induced separately, suggesting that neuroinflammation is a crucial risk factor for intracellular protein accumulation and neurodegeneration (Pintado et al., 2012). Furthermore, the immunoproteasome plays a crucial role in glial cells, implying an interplay between the immunoproteasome and glia-mediated inflammatory responses, ultimately contributing to a pro-inflammatory environment (Orre et al., 2013; Jansen et al., 2014). Radiotherapy (RT) is known to trigger neuroinflammation, which in turn is associated with activation of the immunoproteasome. This activation has been linked to the formation of aggresome-like structures in neurons. Based on this chain of events, it's conceivable that RT contributes to neurodegeneration and cognitive impairment in GBM patients by triggering of inflammatory response pathways and accumulation of misfolded proteins potentially damaging neurons in healthy tissue surrounding tumor.

2.4 RT's CNS damage: a multifaceted attack

To recapitulate, RT triggers interconnected pathways, with neuroinflammation and glial activation as central players, ultimately leading to neuronal death, impaired communication, and cognitive decline known as brain fog. Understanding these pathways holds promise for developing neuroprotective strategies. The key pathways consist firstly in neuroinflammation: RT triggers inflammatory mediators (eATP, interferons, chemokines) & oxidative stress (IL-6, IL-10, TGF-β), leading to BBB disruption and further inflammation. This fuels neuronal death, memory issues, and glial activation (microgliosis & astrogliosis); secondly glial activation: microglial hyperactivity and astrogliosis release neurotoxic factors, damaging neurons and impairing communication; thirdly neurogenesis disruption: RT hinders new neuron formation in the hippocampus, impacting learning and adaptation; fourth DNA damage and senescence: DNA damage in astrocytes triggers a response leading to tissue damage and cognitive decline and fifth ER-stress: RT-induced the accumulation of misfolded proteins which activates UPR pathways, causing both radioresistance and neurodegeneration. Additionally, UPR interacts with JAK1, affecting STAT pathways and promoting neuroinflammation, that, in turn, stokes the vicious cycle that ends with brain fog (Table 1).

3 New horizons for clearing brain fog

Due to their highly invasive nature and extensive infiltration of brain tissue, GBM treatment often involves delivering high doses of RT (typically 60 Gy) to large brain volumes in an attempt to delay tumor recurrence and extend patient survival. However, this unavoidably exposes normal, functioning brain tissue to radiation, that causes devastating effects on brain function. Radiation-induced cognitive impairment manifests with acute (days to weeks after RT), early delayed (1–6 months after RT and often reversible), and late delayed effects (6 months or more after RT and usually irreversible and progressive). Late delayed effects include decreases in memory and executive functioning, among other deficits, further worsening the quality of life of GBM patients. The mechanisms underlying RT-induced neurotoxicity are still being studied and are known to be complex and multifaceted. This complexity makes it challenging to develop effective preventive measures to mitigate the adverse effects of RT on the brain. In the present review, we have focused on the molecular mechanisms that have been found to play a central role both in the etiology and pathogenesis of cognitive impairment due to RT and to important degenerative diseases, such as AD and PD.

About this, the sigma receptors (SRs), a class of ER transmembrane proteins, could represent an appealing target for the prevention of neurocognitive disorders. Sigma receptors (SRs) exist in two subtypes: sigma-1 receptor (S1R) and sigma-2 receptor (S2R). S1R resides on the mitochondria-associated endoplasmic reticulum (ER) membrane

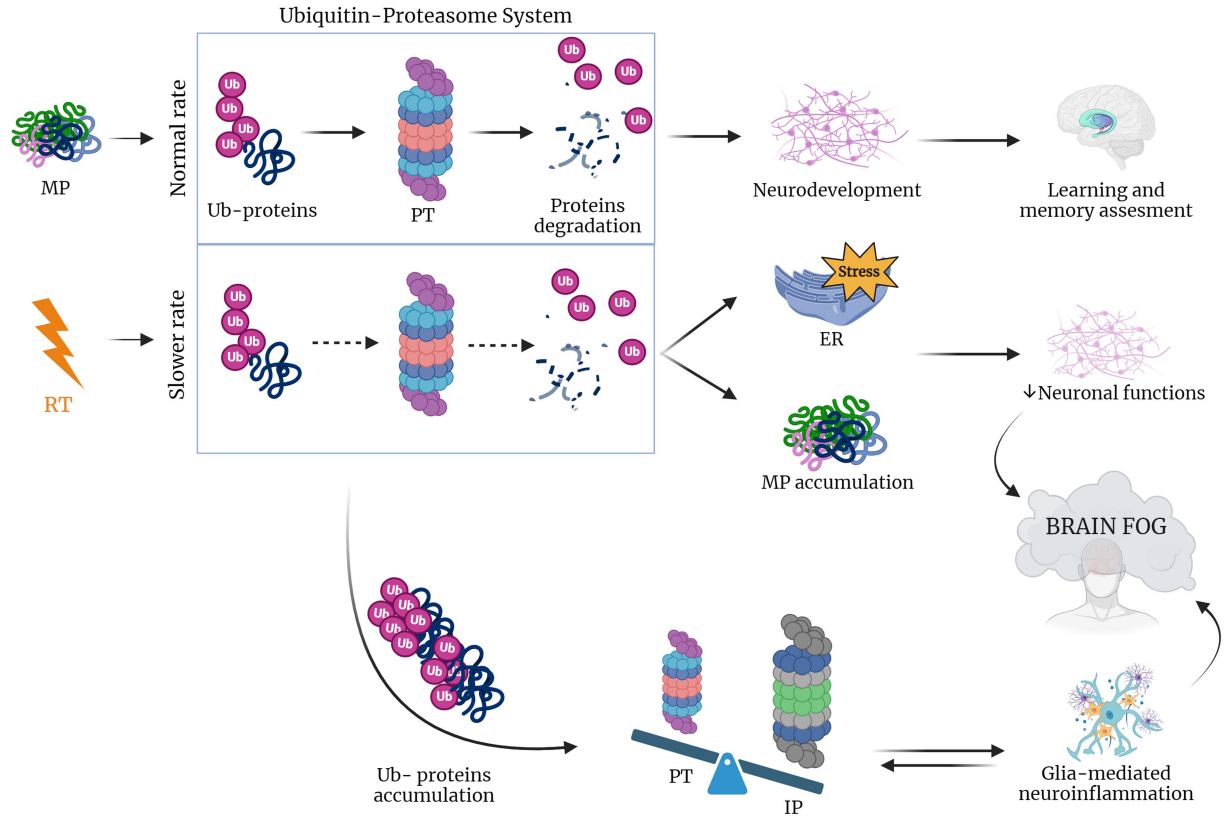

FIGURE 3
RT lowers UPR rate inducing brain fog. When protein misfolding occurs during physiological conditions proteins are ubiquitinated (Ub-proteins), activating the proteasome (PT) that in response induces protein degradation (Tang et al., 2001; van den Berg et al., 2001; Bellezza et al., 2014; Meares et al., 2014; Drake, 2015; Logsdon et al., 2016; Sprenkle et al., 2017). In the case of RT, this rate is lowered leading to the accumulation of Ub-proteins that shift the equilibrium to an increased impact of immunoproteasome, which leads to glia-mediated neuroinflammation. The latter, together with the accumulation of misfolded proteins and induced ER stress causes the compromise of neuronal function and the consequent brain fog establishment (Pearl-Yafe et al., 2003; Ferrington et al., 2008; Pintado et al., 2012; Orre et al., 2013; Jansen et al., 2014). Created with BioRender.com.

(MAM), while S2R is found in the ER-resident membrane. SRs exert chaperoning functions and modulate physio-pathological processes in the CNS. SRs are found abundantly in various brain cells, including neurons, astrocytes, microglia, and oligodendrocytes (Gundlach et al., 1986; Alonso et al., 2000; Hayashi and Su, 2004; Gekker et al., 2006; Zhao et al., 2014).

A multitude of studies have demonstrated that S1R plays a crucial role in promoting neuronal survival and restoring neuronal functions in neurodegenerative diseases. This neuroprotective effect is attributed to S1R's ability to modulate various cellular processes (including calcium homeostasis and glutamate activity), reducing the production of ROS, regulating ER and mitochondrial functions, and influencing reactive gliosis and neuronal plasticity (Nguyen et al., 2015; Ruscher and Wieloch, 2015). Emerging research has demonstrated the effectiveness of S1R-targeting drugs in alleviating symptoms associated with a wide range of neurodegenerative disorders, each with distinct underlying mechanisms. These disorders include learning and memory disorders, cognitive impairments, and neurodegenerative diseases such as AD, PD, ALS, MS, and HD (Maurice and Goguadze, 2017).

In addition to S1R, S2R has also been shown to play an important role in neurological diseases (Huang et al., 2014). S2R couples and interacts with surrounding proteins to actuate a wide variety of cellular processes being closely associated and interacting with key proteins including progesterone receptor membrane component 1 (PGRMC1). S2R and PGRMC1 are linked to learning and memory through mechanism of action studies and efficacy studies in *in vitro* and *in vivo* preclinical models. PGRMC1 is also a well-identified hormone receptor with multiple functions in AD (Xu et al., 2022), and α-synucleinopathies (Kline et al., 2017). Indeed, S2R modulators have been shown to ameliorate amyloid-β oligomer and α-synuclein oligomer-mediated deficits in neuronal trafficking (Izzo et al., 2014).

Based on the many pathways affected in neurodegenerative diseases, another possible good candidate could be the hyperbaric oxygen treatment (HBOT). This therapy has been used for over 50 years to treat various conditions, including decompression sickness and wound healing (Mensah-Kane and Sumien, 2023). Recent studies have shown promising results in using HBOT to treat conditions associated with neurodegeneration and functional impairments. In fact, HBOT has been shown to reduce neuroinflammation in severe brain disorders. It also has the ability to downregulate pro-inflammatory cytokines (IL-1β, IL-12, TNFα, and IFNγ) while upregulating an anti-inflammatory cytokine (IL-10), making it potentially cytoprotective (Kudchodkar et al., 2008). Moreover, combining hyperbaric oxygen (HBO) with RT was found to suppress inflammasome activation in an *in vitro* human microglia model

TABLE 1 RT's CNS damage: a multifaceted attack.

RT-induced pathways leading to CNS damage	References
Release of inflammatory mediators (eATP, interferons, chemokines)	Greene-Schloesser et al. (2012), Herrera et al. (2017)
Increase of oxidative stress through the release of immunosuppressive molecules (IL-6, IL-10, TGF-β) with loss of BBB integrity	Alghamri et al. (2021)
Induction of reactive state of microglia that induces astrogliosis, release of neurotoxic factors, neuronal cell death, memory dysfunctions	Hwang et al. (2006), Wilson et al. (2009), Liddelow et al. (2017), Feng et al. (2018)
Perturbation in neurogenic niche leading to deficits in neural precursors, dysfunction in hippocampal neurogenesis, synaptic structure	Tada et al. (1999), Monje et al. (2003), Rola et al. (2004), Stevens et al. (2007)
DNA damage accumulation leading to a senescence-associated secretory phenotype in astrocytes with tissue damage and cognitive decline	Monje et al. (2002), Eriksson and Stigbrand (2010), Turnquist et al. (2019), Tripathi et al. (2021)
higher expression level of NF-kB and PARP 1 and consequent neuronal death, and constitutive microglia activation	Stoica et al. (2014), Kauppinen and Swanson (2005), Kauppinen et al. (2011), Raghunatha et al. (2020), Martínez-Zamudio and Ha (2014), Ullrich et al. (2001), Gutierrez-Quintana et al. (2022), Chiarugi and Moskowitz (2003)
Induction of ER-stress through the production of ROS, upregulation of UPR pathways (PERK, ATF6, IRE1) causing radioresistance through MAPK-1 and NF-kB and accumulation of misfolded proteins in neuronal cells	Tang et al. (2001), van den Berg et al. (2001), Drake (2015), Logsdon et al. (2016), Pintado et al. (2017), Chatterjee et al. (2018), Wang et al. (2018), Ren et al. (2021)
interaction between PERK and JACK1 affecting STAT pathways leading to neuroinflammation	Bellezza et al. (2014), Meares et al. (2014), Sprenkle et al. (2017)
Changes in cellular redox balance which impact on proteasome that act as a stress signal causing degradation of proteins and exacerbating ER-stress	Pacifici et al. (1993), Giulivi et al. (1994), Dantuma and Lindsten (2010), Davidson and Pickering (2023)
Induction of COX-1 and COX-2 upregulation with the release of prostaglandins that induce proteasome inhibition that hampers neuroinflammation contributing to neurodegeneration	Consilvio et al. (2004), Ishii et al. (2005), Bi et al. (2012)
Induction of neuroinflammation with release of IFN-γ, production of ROS that damage constitutive proteasome with the consequent upregulation of the immunoproteasome, which in turn induces the formation of neuronal aggresome-like structures and neurodegeneration	Pearl-Yafe et al. (2003), Ferrington et al. (2008), Pintado et al. (2012), Orre et al. (2013), Jansen et al. (2014)

(Arienti et al., 2021). This effect was attributed to a reduction in the expression levels of pro-inflammatory cytokines IL-1β and IL-6. Similar results were obtained by Qian et al. who reported that, in animal models, HBO mitigates the inflammatory response associated with traumatic brain injury by modulating microglial inflammasome signaling (Qian et al., 2017). Recently, basic and clinical research has shown the potential of HBOT to treat neurodegenerative diseases (Huang and Obenaus, 2011; Huang et al., 2016; Shapira et al., 2018). The effectiveness of HBOT in improving age-related cognitive decline was evaluated in a study involving healthy elderly individuals (Jacobs et al., 1969). Male participants with an average age of 68 years, displaying clinical signs of intellectual deterioration, underwent cognitive assessments following 30 intermittent sessions of HBOT, which involved breathing pure oxygen at 2.5 times atmospheric pressure. HBOT enhanced cognitive function in these healthy older adults through mechanisms involving regional alterations in cerebral blood flow, as assessed by perfusion magnetic resonance imaging (Amir et al., 2020). Also, elderly patients with significant memory loss demonstrated enhanced cognition and increased cerebral blood flow following exposure to HBOT (Shapira et al., 2018). In the *in vivo* models of aging, HBOT effectively counteracted cognitive decline and hippocampal-dependent pathologies by enhancing cholinergic signaling pathways, protecting against apoptosis, and mitigating oxidative stress and inflammatory responses (Chen et al., 2016, 2017; Shwe et al., 2021).

Another common mental disease associated with brain tumors and RT is depression. The impact of stress on brain morphology has become increasingly evident through extensive research spanning several decades and depression is now clearly associated to chronic uncontrollable stress and the related neuroinflammation derived (Eyre and Baune, 2012; Iwata et al., 2013; Franco and Fernández-Suárez, 2015). Indeed, depressed patients usually exhibit increased inflammatory cytokines such as IL-1β, IL-6 and IFNγ, both in different brain regions and the periphery (Maes et al., 2009; Wager-Smith and Markou, 2011; Young et al., 2014). It is also known that enhanced levels of IL1β in the hippocampus lead to inflammation, that may contribute to depression (Kovacs et al., 2016). In this context, the role of ATP-gated transmembrane cation channel P2X7 receptor in the neuroinflammation is highlighted, due to its involvement in the IL-1β maturation (Potucek et al., 2006; Mingam et al., 2008; Piccini et al., 2008). These receptors are mainly located on microglia and activated in response to stress signals like increased level of ATP (Ferrari et al., 2006). Studies on peripheral immune cells demonstrated that activation of P2X7R induced oligomerization of NLR family pyrin domain containing 3 (NLRP3) with other proteins that in complex form the so-called inflammasome (Yue et al., 2017). The latter, as already discussed, is associated to neuroinflammation, leading to the neurodegeneration that can comprehend, in the light of the last discoveries, depression. Taken together, these findings may suggest NLRP3 inflammasome as a new therapeutic target for cognitive impairment related to radiation therapy (Alcocer-Gómez and Cordero, 2014).

Non-invasive brain stimulation (NIBS) may represent a new age of brain fog treatment. Neuromodulatory techniques stand as

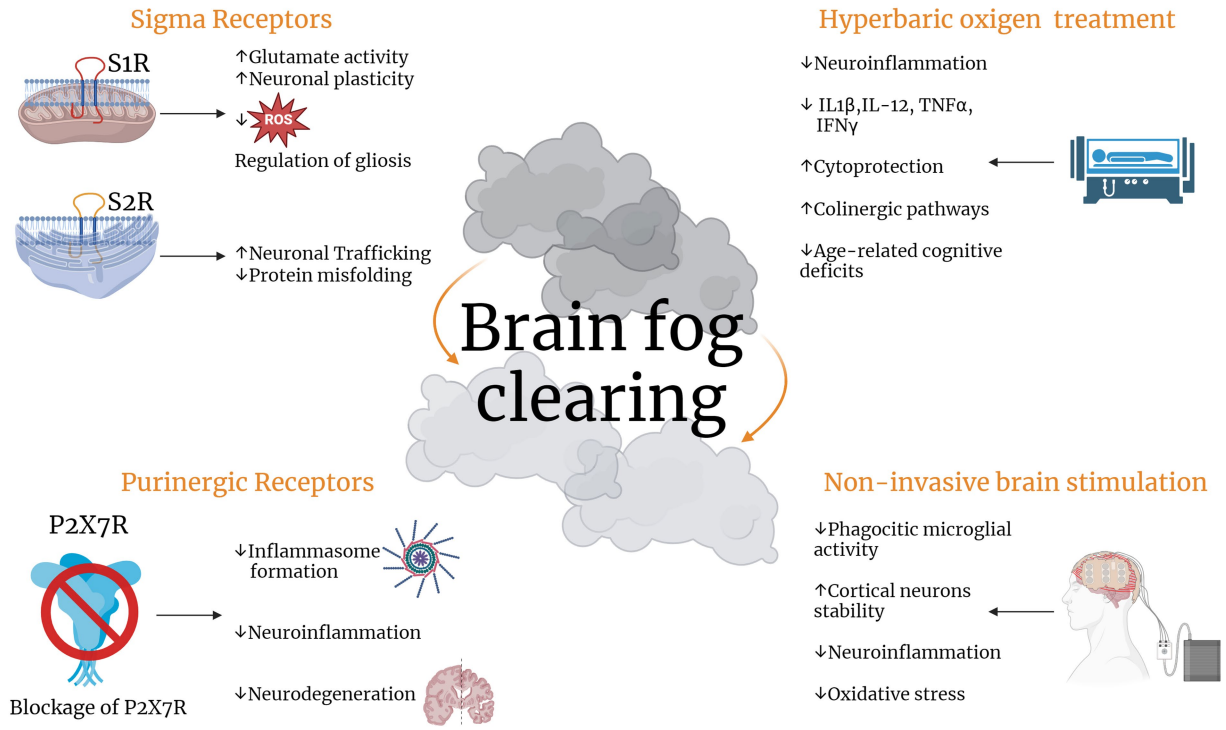

FIGURE 4
New therapeutic strategies to clear brain fog. Sigma receptors are ER transmembrane proteins represented by Sigma-1 receptor (S1R) and Sigma-2 receptor (S2R), that exert chaperoning functions and modulate physio-pathological processes like neuronal plasticity, glutamate activity as well as regulation glial cells reactivity. S1R receptor agonists preserve neurological functions while S2R antagonists ameliorate neuronal trafficking deficits derived by protein misfolding diseases. Hyperbaric oxygen treatment is involved in many neurodegenerative diseases. It reduces neuroinflammation through the inhibition of pro-inflammatory cytokines and mediate cytoprotection via the upregulation of anti-inflammatory cytokines. It also prevents cognitive impairments and hippocampal pathologies through the modulation of cholinergic pathways. ATP-gated transmembrane purinergic P2X7 receptor induces the formation of inflammasome by leading to oligomerization of NLRP3, posing the way to think at these receptors as a new therapeutic strategy to target cognitive impairment related to radiotherapy. Non-invasive brain stimulation techniques may add to the previously described agents and represent a new horizon on the brain fog treatment. They induce neuronal plasticity changes, reduce phagocytic microglial activity and neuroinflammation, and decrease oxidative stress while increasing cortical neurons stability. These could be innovative therapeutic target to clear brain fog. Created with BioRender.com.

robust alternatives to pharmacological interventions for neurological and neuropsychiatric disorders, primarily due to their numerous advantages, including non-invasiveness, enhanced safety, and minimal to negligible side effects (Peruzzotti-Jametti et al., 2013a). Though NIBS tools developed on magnetic and electric fields, and more recently on ultrasound, resulting in one of the fastest-growing fields in medicine, the concept of influencing the activity of the human brain by using external therapeutical strategies dates back to the 1st century AD (Cambiaghi and Sconocchia, 2018). The main non-invasive brain neuromodulatory approaches are repetitive transcranial magnetic stimulation (rTMS), transcranial direct current stimulation (tDCS), transcranial alternating current stimulation (tACS), random noise stimulation (RNS), transcranial ultrasound stimulation (TUS). Although the underlying mechanisms of action is slightly different among them, NIBS tools are known to induce long-lasting neuronal plasticity changes, associated to behavioral modifications in both humans (Zhao and Woodman, 2021; George et al., 2022) and animal models (Cambiaghi et al., 2020a; Cherchi et al., 2022). Interestingly, in addition to neuronal effects, both magnetic and electric stimulation after effects have been recently associated with different glial cell activity modulation. Of note, in ischemic mouse models rTMS promotes microglia anti-inflammatory cytokines production both *in-vitro* and *in-vivo* (Luo et al., 2022). In rodent models of brain ischemia and vascular dementia, tDCS lead to an attenuation of the inflammatory response in different brain regions. In particular, in the MCAO mouse model of brain ischemia, cathodal tDCS is able to preserve cortical neurons if applied in the acute phase (Peruzzotti-Jametti et al., 2013b), while it exerts positive effects on functional motor outcomes when delivered hours after the brain damage and inflammatory response, combined with a less phagocytic anti-inflammatory microglia activity (Cherchi et al., 2022). In the rat vascular dementia model, anodal tDCS reduces the levels of malondialdehyd and ROS, but enhances superoxide and glutathione, thus reducing the oxidative stress (Guo et al., 2020). Finally, mice exposed to a 14-days 5 Hz rTMS exhibit increased cell proliferation in the hippocampal dentate gyrus, in parallel with improved cognitive behavior (Ramírez-Rodríguez et al., 2022). In line with this, 5 days of high-frequency (15 Hz) rTMS showed an improved emotional behavior paralleled by enhanced prefrontal cortex morphological plasticity, both in terms of dendritic spine density and dendritic complexity of layers II/III and V (Cambiaghi et al., 2022). On the contrary, 1 Hz rTMS results in augmented mature granule cells and newly

generated neurons structural complexity, in association to antidepressant effects, though not affecting neurogenesis (Cambiaghi et al., 2020b). Especially, these latter observations on NIBS effects on glial cells, inflammation and neurogenesis well suits a strong interest for the treatment of brain fog associated to RT (Figure 4).

4 Conclusion

Radiotherapy (RT) is a common treatment for glioblastoma multiforme (GBM), but it can have adverse neurocognitive effects. The exact impact of RT on the quality of life in long-term GBM survivors is not fully understood. Predicting the clinical impact of RT is challenging because both the immediate and long-term effects of RT on quality of patient's life depend on various factors. These factors include radiobiological factors (RT dose, volume, timing, and duration), physiological factors (pre-existing brain function), and patient-related factors (age, sex, and comorbidities). While RT remains the most effective non-surgical treatment option for GBM, its effectiveness is limited by the inherent and adaptive radioresistance of these tumors, which contributes to their inevitable recurrence.

Radiation treatment planning should consider the brain's remarkable ability to adapt and recover by creating new neural connections, a crucial aspect of patient rehabilitation, as well as the sensitivity of the targeted brain regions. Even if the most severe effects occur months to years after radiation therapy, it is conceivable that decreasing the early impairment of brain parenchyma could likely prevent the propagation of the late-term effects of RT (Constanzo et al., 2020).

While our grasp of the underlying mechanisms of radiation-induced cognitive dysfunction remains incomplete, compelling evidence points to neuroinflammation as a significant contributor. Recent research has unveiled neuroinflammation as a pervasive feature in numerous CNS disorders, encompassing brain trauma, stroke, and various neurodegenerative processes. Bridging the gap between these preclinical findings and clinical practice holds the potential to enhance both survival rates and quality of life for brain tumor patients undergoing RT. Incorporating neuroinflammatory markers, cognitive function assessments, and quality of life measures into the design of future clinical trials based on RT treatment is crucial. Furthermore, literature data suggest a plausible link also between radiation neurotoxicity and UPR activation. However, targeting the UPR is still challenging, due to its role in physiological pathways that involve different organs, so it can have serious adverse effects if administered for a long time. Indeed, particular attention should be paid as the consequences on basal motor and cognitive functions could be severe and this aspect has to be taken into account (Hetz and Saxena, 2017).

Author contributions

NM: Conceptualization, Writing – original draft, Writing – review & editing. MB: Writing – review & editing. MEV: Writing – original draft, Writing – review & editing. MC: Writing – review & editing. AT: Conceptualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

Publisher's note

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Orexins in apoptosis: a dual regulatory role

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The orexins, also referred to as hypocretins, are neuropeptides that originate from the lateral hypothalamus (LH) region of the brain. They are composed of two small peptides, orexin-A, and orexin-B, which are broadly distributed throughout the central and peripheral nervous systems. Orexins are recognized to regulate diverse functions, involving energy homeostasis, the sleep-wake cycle, stress responses, and reward-seeking behaviors. Additionally, it is suggested that orexin-A deficiency is linked to sleepiness and narcolepsy. The orexins bind to their respective receptors, the orexin receptor type 1 (OX1R) and type 2 (OX2R), and activate different signaling pathways, which results in the mediation of various physiological functions. Orexin receptors are widely expressed in different parts of the body, including the skin, muscles, lungs, and bone marrow. The expression levels of orexins and their receptors play a crucial role in apoptosis, which makes them a potential target for clinical treatment of various disorders. This article delves into the significance of orexins and orexin receptors in the process of apoptosis, highlighting their expression levels and their potential contributions to different diseases. The article offers an overview of the existing understanding of the orexin/receptor system and how it influences the regulation of apoptosis.

KEYWORDS

orexins, apoptosis, CNS disorders, neuroprotection, cell signaling, new therapeutic target

1 Introduction

Orexins, alternatively referred to as hypocretins, are chemical messengers generated within limited clusters of neurons found in the lateral hypothalamic (LH) and perifornical hypothalamic regions (PFA). The term "orexin" is derived from the Greek word "orexis," which translates to hunger or appetite. The primary role of orexins lies in controlling wakefulness, alertness, food consumption, and behaviors associated with rewards by affecting specific clusters of brain nuclei (Sakurai, 2014).

There are two forms of orexin peptides, both derived from the cleavage of preproorexin (Karteris et al., 2004). These are orexin-A, which is made up of 33 amino acids, and orexin-B,

which is composed of 28 amino acids (Figure 1). Orexin-A can bind to both the orexin-1 receptor (OX1R) and, to a lesser extent, the orexin-2 receptor (OX2R), while orexin-B has a higher binding affinity for OX2R (Lang et al., 2004). Despite the widespread distribution of these receptors, research into orexin signaling has primarily focused on OX1R owing to the lack of an effective and available OX2R antagonist (Wang et al., 2018).

Neurons synthesizing orexins establish bidirectional connections with mediobasal hypothalamic structures responsible for managing food consumption (Sakurai, 1999). There is supporting evidence indicating that orexin neurons in the lateral hypothalamus (LH) can be considered "second-order" neurons, as they receive signals from neurons within the arcuate nucleus that express neuropeptide Y (NPY) and agouti-related peptide (AgRP) (Berthoud, 2002). This suggests that these neurons are involved in the integration processes that facilitate and promote food consumption (Reichelt et al., 2015). Nonetheless, there is also supporting evidence indicating that orexin neurons can function as "first-order" neurons, directly responding to metabolic cues such as leptin, glucose, and ghrelin. These neurons seem to exhibit characteristics of both first- and second-order neurons, participating in the intricate integration of signals from neuropeptides and adipostatic factors that govern feeding behavior (Adamantidis and De Lecea, 2009) (Figure 2).

Concerning feeding behavior, extensive documentation exists on the appetite-stimulating effects of orexin-A regarding food intake (Öztürk et al., 2022). Administering orexin-A directly into the cerebral ventricles has been demonstrated to boost food consumption in rodents. Moreover, in experiments where rats have options, they tend to preferentially increase their consumption of favored diets, particularly those rich in saturated fat, when exposed to orexin-A (Yamanaka et al., 1999). Blocking the orexin-A-induced increase in appetite and behavioral satisfaction has proven effective through the antagonism of the OX1R (orexin receptor 1) (White et al., 2005).

Orexin neurons form bidirectional connections with regions in the mediobasal hypothalamus that play a role in regulating food intake. New studies suggest that orexin neurons located in the lateral hypothalamus (LH) can function as "second-order" neurons, receiving input from neurons containing neuropeptide Y (NPY) and Agouti-related peptide (AgRP) situated within the arcuate nucleus. This suggests their involvement in the process of integrating signals related to food intake. Conversely, these orexin neurons can also function as "first-order" neurons, directly responding to metabolic signals such as leptin, glucose, and ghrelin. The intricacy of their functions likely encompasses both first- and second-order characteristics in the complex integration of signals from neuropeptides and signals related to adipostasis, which collectively govern feeding behavior.

2 Orexin receptors

In mammals, there exist two distinct types of orexin receptors, explicitly the OX1R and the OX2R (Nitkiewicz et al., 2010). Both of these receptors belong to class A of G-protein-coupled receptors (GPCRs) (Yang et al., 2021). Interestingly, OX1R is exclusive to

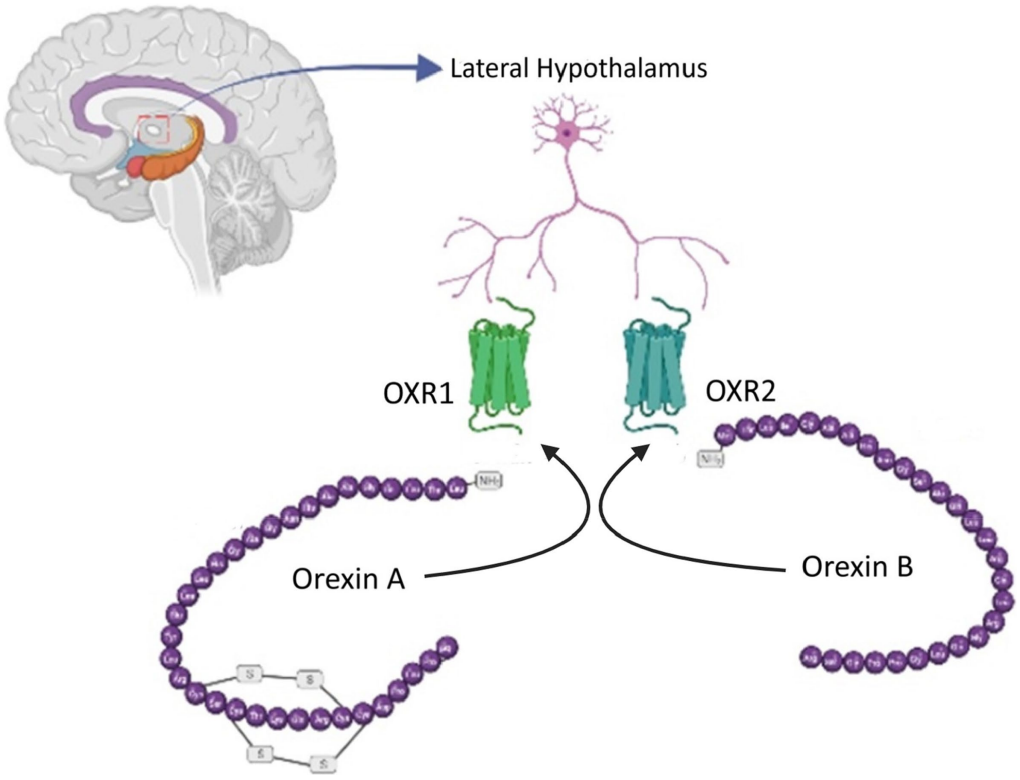

FIGURE 1
Orexin neurotransmitters. Two orexin peptides, orexin-A (33 amino acids) and orexin-B (28 amino acids), are generated from preproorexin cleavage. Orexin-A binds to both OX1R and, to a lesser extent, OX2R, while orexin-B exhibits a higher affinity for OX2R.

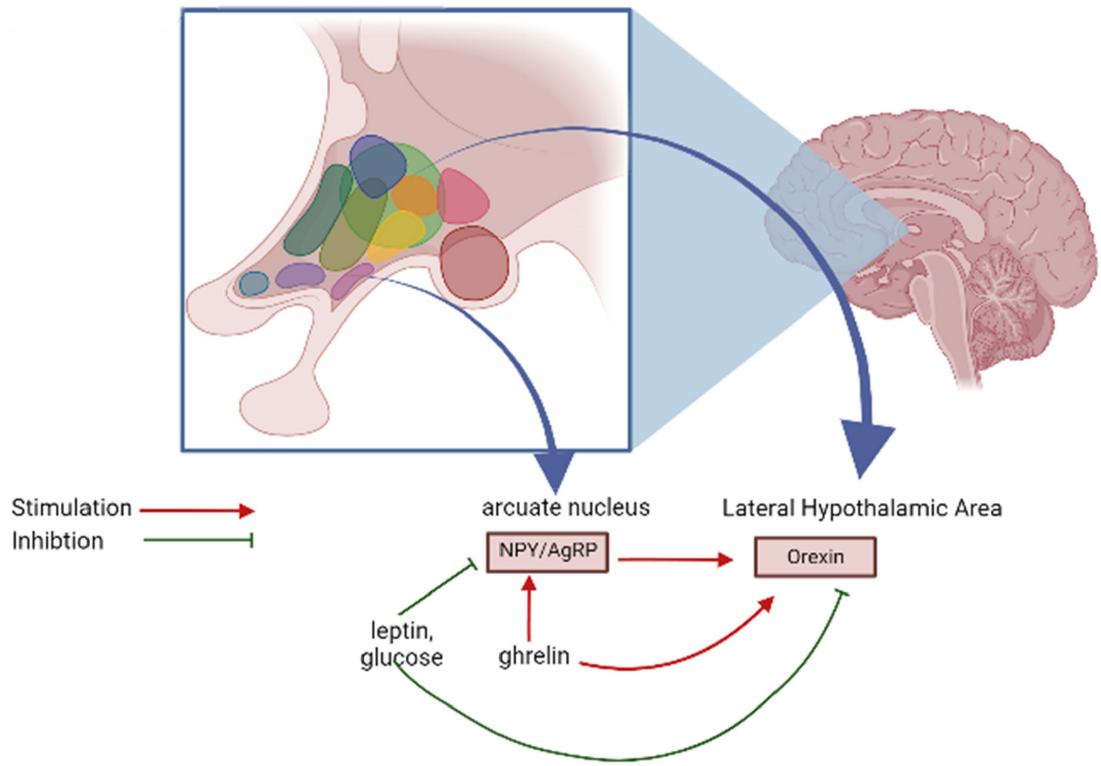

FIGURE 2
Regulation of orexin release.

mammals and is agreed to have developed from the original OX2R via gene duplication events that occurred throughout the early stages of mammalian evolution (Soya and Sakurai, 2020). Conversely, OX2R exists in all vertebrates, implying that it represents the ancestral form of orexin receptors (Tam et al., 2011). Due to its relatively recent phylogenetic origin compared to OX2R, OX1R appears to be involved in more intricate and complex physiological functions (Zhang et al., 2013). Research has indicated that mice lacking OX1R display behavior resembling anxiety (Demidova et al., 2022). Furthermore, it has been observed that OX1R within the locus coeruleus (LC) noradrenaline neurons contribute to the expression and consolidation of conditioned fear memory. This is achieved through the stimulation of LC neurons, which project to the lateral amygdala. This pathway also acts a role in the generalization of fear memory (Kaplan et al., 2022). OX1R has also been demonstrated to participate in heightened responses to conditioned cues, activating motivational responses in rats and influencing feeding behavior associated with reward (Choi et al., 2010). These results indicate that OX1R has a part to play in the emotional and motivational processes observed in mammals (Sakurai, 2014).

3 Molecular mechanisms of orexin/receptor interaction

Orexins are neuropeptides that play a key role in regulating various physiological functions, such as arousal, appetite, energy homeostasis, and stress response (Grafe and Bhatnagar, 2018). Orexins bind to two types of GPCRs, OX1R and OX2R, which have distinct but overlapping expression patterns and signaling pathways in the brain and peripheral tissues (Wang et al., 2018).

Understanding the molecular mechanisms of orexin interaction with receptors is essential for explaining the physiological and pathological roles of orexins and developing novel therapeutics for orexin-related disorders. Orexin A is composed of 33 amino acids, while orexin B contains 28 amino acids. They share a common C-terminal region that is crucial for receptor binding. Orexins adopt an alpha-helical conformation in solution and in complex with receptors, as revealed by nuclear magnetic resonance (NMR) and X-ray crystallography research (Yin et al., 2016). Orexins bind to the extracellular domains of OX1R and OX2R, which consist of seven transmembrane helices connected by three extracellular loops and an N-terminal tail (Vu et al., 2021).

The molecular interactions between orexins and receptors involve multiple contacts between the C-terminal region of orexins and the N-terminal tail, the first extracellular loop, and the second extracellular loop of receptors, The C-terminal region of orexins forms a hydrophobic core that interacts with a conserved pocket formed by the N-terminal tail and the first extracellular loop of receptors (Takai et al., 2006). The C-terminal region also contains a polar residue (Asn or Asp) that forms a hydrogen bond with a conserved residue (Tyr or His) in the second extracellular loop of receptors (Karhu et al., 2015). These interactions are essential for orexin binding and receptor activation. In addition, the N-terminal region of orexins contributes to receptor binding and selectivity by forming specific contacts with residues in the N-terminal tail and the first extracellular loop of

receptors (Karhu et al., 2019). The N-terminal region also modulates the conformational changes of receptors upon ligand binding, which affects the coupling efficiency of receptors to G proteins.

The binding of orexins to OX1R and OX2R induces conformational changes in the intracellular domains of receptors, which enable the interaction with various G proteins and downstream effectors (Couvineau et al., 2019). OX1R preferentially couples to Gq/11 proteins, which stimulate phospholipase C (PLC) and enhance intracellular calcium levels (Berrendero et al., 2018). OX2R couples to both Gq/11 and Gi/o proteins, which prevent adenylyl cyclase (AC) and decrease cyclic adenosine monophosphate (cAMP) (Urbańska et al., 2012). Both OX1R and OX2R can also couple to Gs proteins, which activate AC and increase cAMP levels. The differential coupling of OX1R and OX2R to G proteins results in distinct cellular responses to orexins in diverse tissues and cell types.

4 Physiological effects of orexins

The role of orexins extends beyond the central nervous system to the periphery. Orexin receptors are expressed not only in the brain, but also in various peripheral tissues, such as the gastrointestinal tract, kidneys, gonads, pancreas, and adrenal glands (Kirchgessner and Liu, 1999; Randeva et al., 2001; Nakabayashi et al., 2003; Liguori et al., 2018; Couvineau et al., 2019) (Table 1). These peripheral effects of orexins have been investigated in both animals and humans and have known to play important roles in regulating various physiological processes such as glucose homeostasis, energy balance, and cardiovascular function. Studies have shown that the orexin system plays a role in regulating glucose metabolism and insulin sensitivity in peripheral tissues, particularly in the liver (Greene et al., 2020). Orexin A has been shown to have insulin-sensitizing effects and to reduce liver glucose production. Moreover, the orexin system has been implicated in regulating body weight, body fat distribution, and metabolism. Orexin-A has been revealed to reduce body weight and improve insulin sensitivity in obese animals (Zarifkar et al., 2017). The orexin system has been shown to play a role in regulating inflammation, both in the central nervous system and in peripheral tissues (Couvineau et al., 2019).

In the gastrointestinal tract, orexins have been shown to increase gastric motility and promote the release of digestive enzymes, thereby increasing nutrient absorption (Baccari, 2010). In the pancreas, orexins have shown to stimulate the secretion of insulin and regulate glucose homeostasis (Nowak et al., 2005). Orexins also play a role in the regulation of energy balance by modulating the function of adipocytes and regulating the release of hunger-inducing hormones such as ghrelin (Suarez et al., 2020). In addition, orexins have shown to play a role in cardiovascular function by regulating blood pressure and heart rate (Carrive, 2013). Orexins have additionally been associated with the control of the stress response by influencing the functioning of the hypothalamic-pituitary-adrenal (HPA) axis and the release of CRH from the hypothalamus (Spinazzi et al., 2006).

Orexin is integral to the immune system, engaging with its receptors found on immune cells. Research indicates the presence of orexin receptors, specifically OX1R and OX2R, across a range of immune-related tissues and cells, such as T lymphocytes and myeloid cells, which points to a direct influence of orexins (Becquet et al., 2019). Treatment with Orexin A has been observed to alter the population of immune cells, potentially leading to immunosuppressive outcomes in specific cell types, including granulocytic and monocytic myeloid-derived suppressor cells. Furthermore, Orexin A has demonstrated sustained positive effects in managing autoimmune disorders, such as experimental autoimmune encephalomyelitis, showcasing its capacity to influence neuroinflammatory pathways (Becquet et al., 2019). In essence, the involvement of orexin within the immune system underscores its regulatory function and suggests significant implications for the management of inflammatory and autoimmune conditions.

TABLE 1 Physiological effects of orexin.

Organ	Effect of orexin	References
Brain	Regulates various physiological processes	Couvineau et al. (2019)
Gastrointestinal tract	Increases gastric motility, promotes enzyme release	Baccari (2010)
Kidneys	Not specified	
Gonads	Not specified	
Pancreas	Stimulates insulin secretion, regulates glucose homeostasis	Nowak et al. (2005)
Adrenal glands	Not specified	
Liver	Reduces liver glucose production, insulin sensitivity	Greene et al. (2020)
Peripheral tissues (general)	Regulates glucose metabolism, insulin sensitivity	Zarifkar et al. (2017)
Adipocytes	Modulates adipocyte function	Suarez et al. (2020)
Cardiovascular system	Regulates blood pressure, heart rate	Carrive (2013)
Stress response (hypothalamic–pituitary–adrenal axis)	Modulates stress response, regulates CRH release	Spinazzi et al. (2006)
Immune system	Implication for the management of inflammatory and autoimmune conditions	Becquet et al. (2019)

5 Apoptosis

Apoptosis is a programmed cell death that is believed to be vital in keeping the stability of cell populations within an organism. It is a well-controlled process that takes place due to various internal and external stimuli and is crucial for the normal functioning of cells and tissues. The term “apoptosis” was first used to describe this process due to the characteristic shrinkage and fragmentation of cells into smaller units known as apoptotic bodies (Elmore, 2007). Unlike apoptosis, necrosis is another type of cell death that occurs as a result of cellular injury from external factors such as toxins or trauma.

Necrosis is an uncontrolled process that can cause inflammation and harm to tissue, whereas apoptosis is a regulated process that does not result in inflammation or tissue damage (Fink and Cookson, 2005).

Apoptosis is triggered by signals received by the cell, including DNA damage, growth factor withdrawal, oxidative stress, and viral infections. These signals activate specific enzymes called caspases, which cause the degradation of cellular proteins, resulting in cell shrinkage, chromatin condensation, and fragmentation into apoptotic bodies (Jan and Chaudhry, 2019). This process of apoptosis is crucial for preserving the balance of cell populations in an organism. For instance, it plays a critical role in the development of the nervous system by selectively eliminating excess neurons to form functional neural circuits (Hollville et al., 2019). Additionally, it also helps in eliminating damaged or abnormal cells, thus avoiding the development of cancer and other diseases.

The molecular mechanisms that control apoptosis are complex and involve the triggering of specific signaling pathways and the interaction of many different proteins. The two main pathways that control apoptosis are the extrinsic pathway, which is activated by signals from outside the cell, and the intrinsic pathway, which is activated by signals within the cell (Elmore, 2007). Apoptosis is regulated by a delicate balance between pro-apoptotic and anti-apoptotic signals. Pro-apoptotic signals promote cell death, while anti-apoptotic signals inhibit cell death. Disruptions in this balance can result in a failure of apoptosis, which can contribute to the development of cancer and other diseases (Pistritto et al., 2016). Cancer cells often exhibit resistance to apoptosis, which allows them to proliferate uncontrollably. In addition, abnormal apoptosis has been implicated in numerous diseases, involving neurodegenerative disorders, autoimmune diseases, and cardiovascular disease (Ghavami et al., 2014). Because of its central role in regulating cell death, apoptosis is a critical target for the development of new therapies. For example, drugs that can enhance apoptosis in cancer cells have been developed and are being investigated in clinical trials. Conversely, drugs that can inhibit apoptosis in certain diseases, such as neurodegenerative disorders, are also being developed. The apoptotic process involves various cellular organelles like mitochondria, Golgi apparatus, lysosomes, endoplasmic reticulum, centrosomes, cell membrane, and nucleus.

6 Effect of orexins on caspases

The initiation of apoptosis and the triggering of proapoptotic proteins lead to the triggering of caspases. These proteases are accountable for the precise cleavage of particular protein substrates and hold considerable importance in controlling whether cells survive or undergo programmed cell death. Caspases also contribute significantly to various other cellular processes, embracing inflammation, cell proliferation, and differentiation (Shalini et al., 2015). Currently, 11 human caspases have been identified and can be separated into two groups: inflammatory caspases (caspase 1, 4, 5) and apoptotic caspases, which are further divided into initiator caspases (caspases 2, 8, 9, and 10) and executive caspases (caspases 3, 6, and 7) (Van Opdenbosch and Lamkanfi, 2019).

Cell demise initiated by Orexin A exhibited characteristics such as condensed chromatin structure, which is tightly packed, and it necessitated the activation of new gene transcription and protein synthesis, both of which are well-recognized indicators of programmed cell death. This phenomenon might also be linked to caspase activation alongside various other apoptotic pathways (Ammoun et al., 2006). In a similar vein, previous findings had proposed that the activation of orexin receptors had the potential to trigger apoptosis in rat C6 glioma cells by stimulating the caspase signaling pathways (Biegańska et al., 2012). In a separate investigation, Orexins were found to induce cell death through apoptosis, relying on caspase activity, and led to significant inhibition of cell growth within Chinese hamster ovary cells that had been transfected with OX2R cDNA. This effect was achieved by activating caspase 3 and caspase 9 (Voisin et al., 2006). The cell death induced by orexin-A exhibited features such as condensed chromatin, a tightly packed structure. Furthermore, it necessitated the initiation of new gene transcription and protein synthesis, which are well-established characteristics of programmed cell death. Additionally, this phenomenon might be connected to the activation of caspases, alongside other mechanisms associated with apoptosis (Ammoun et al., 2006).

Orexin receptor presence was detected in various cancer models. Orexins did not influence cell multiplication but instead facilitated cell death, as evidenced by observable alterations in cell structure, fragmentation of DNA, condensation of chromatin, release of cytochrome c into the cytosol, and activation of caspase-3 and caspase-7 enzymes. The occurrence of OX1R and the orexins' pro-cell death effects were evident in colon cancer cell lines such as Caco-2, SW480, and LoVo, but notably absent in typical colonic epithelial cells (Rouet-Benzineb et al., 2004). In colon cancer cell lines, Orexin acted as an intrinsic pro-apoptotic peptide by triggering the activation of OX1R (Rouet-Benzineb et al., 2004; Laburthe and Voisin, 2012). When treating chemoresistant pancreatic tumors, which had developed resistance to Nab-paclitaxel or gemcitabine through succeeding mice xenografts, it was discovered that orexin A exhibited a remarkable capability to significantly impede tumor growth (Voisin et al., 2022). This effect was achieved by boosting apoptosis mediated by caspase 3 and 7. Importantly, no resistance to orexin-A was observed in these tumors (Voisin et al., 2022). The caspase-mediated apoptosis process is generally governed by two primary pathways: the extrinsic pathway, regulated by caspase-8 and initiated through death receptors, and the intrinsic pathway, governed by caspase-9 and initiated within the mitochondria (Wen et al., 2012). Caspase-3, functioning as the downstream executor, facilitates the cleavage of cellular target proteins, ultimately leading to cell death. Research has provided evidence indicating that orexins can induce apoptosis and hinder cell proliferation in both human and rat pancreatic tumor cells and CHO cells. These effects are achieved via the stimulation of caspase-3 activity (Chen L. et al., 2013; Dayot et al., 2018) (Table 2).

Conversely, orexins have been observed to promote the proliferation of neuronal cells by inhibiting the activity of caspase-3. Apaf-1 (Apoptotic protease activating factor 1) acts as an activator of caspase-3 in the presence of cytochrome C. Interestingly, Apaf-1 expression was found to be absent in normal neuronal cells, potentially conferring resistance to apoptosis induced by cytochrome c in these cells (Shakeri et al., 2021). This lack of Apaf-1 expression in normal neural tissues renders them resistant to the apoptotic effects instigated by cytochrome c. This underscores the pivotal role of Apaf-1 in mediating apoptosis specifically in brain tumors, while sparing normal neuronal cells. Furthermore, research has found that treatment with

TABLE 2 The effects of orexin on different molecular pathways of apoptosis.

Target	Specific proteins targeted	Effects of orexin	References
Effect on caspases	Caspase-3, Caspase-7, Caspase-9	■ Promotes apoptosis by inducing Caspase-3, Caspase-7, and Caspase-9	Voisin et al. (2022)
	Cytochrome C	■ Suppresses cleaved caspase-9 and caspase-3	Suo et al. (2018)
		■ Cytochrome c release	Xu et al. (2021b)
		■ Inhibits cytochrome C expression	Xu et al. (2021b)
		■ Stimulation of the enzyme phosphotyrosine phosphatase SHP-2, followed by the initiation of mitochondrial apoptosis mediated by cytochrome c	Marcos and Coveñas (2023)
Mitochondrial mechanism of apoptosis	Bcl-2, Bax	■ Increases Bcl-2 expression ■ Decreases Bax expression ■ Raise Bcl-2/Bax ratio	Safdar et al. (2021)
Effect of orexins on receptor-related mechanism of apoptosis	TNF-α	■ Reduce TNF-α-mediated inflammation ■ Anti-apoptotic effect	Messal et al. (2018)
	Fas receptors	■ Reduces inflammation ■ Potentially exhibits antiapoptotic effects	Dondelinger et al. (2016)
		■ Provides insights into chemotherapeutic intervention challenges with Fas ligand-mediated apoptosis	Wolpin and Mayer (2008), Segal and Saltz (2009), Voisin et al. (2011)
Endoplasmic reticulum stress and apoptosis	GRp87 IRE1α XBP1 PERK Unfolded Proteins, mTOR-XBP1	■ Prevents ER stress and inflammation	Tsuneki et al. (2022)
	Ca2+	■ At higher concentrations, orexins increase Ca2+ release from the endoplasmic reticulum and increase apoptosis	Xia et al. (2009)
Modulation of p53 activity	p53	■ Modulates p53 activity, leading to inhibition of apoptosis	Sokołowska et al. (2014)
Regulation of Caspase-2	Caspase-2	■ Reduces caspase-2 activity	Sokołowska et al. (2014)

orexin A can significantly inhibit apoptosis induced by serum starvation, which was endorsed by reduced activity of caspase-9 (Liu et al., 2015). However, under the same conditions, orexin A had no impact on the activity of caspase-8, indicating that it can restrain the intrinsic apoptotic pathway and guard cells from undergoing apoptosis (Liu et al., 2015) (Table 2).

Other studies have supported these findings, indicating that orexin receptor activation reduces caspase activity and cellular apoptosis. In SGC-7901 gastric cancer cells, The expression of OX1R was observed and orexin A stimulated SGC-7901 cell proliferation and sustainability, diminished the pro-apoptotic activity of caspase-9, and guarded the cells from apoptosis in a dose-dependent manner (Liu et al., 2015). In a separate investigation, it was found that orexin A had an inhibitory effect on apoptosis in gastric cancer cells, and this inhibition was mediated through OX1R via the protein kinase B (Akt) pathway (Wen et al., 2015). Furthermore, it has been uncovered that the stimulation of OX1R may play a critical role in the development of pancreatic cancer and could serve as a promising therapeutic target for individuals with pancreatic cancer. This potential treatment avenue involves the modulation of the Akt/mTOR pathway alongside the stimulation of caspase 3 and 9 (Chen Y. et al., 2013; Suo et al., 2018).

7 Effect of orexins on mitochondrial mechanism of apoptosis

The significance of mitochondria in the process of apoptotic cell death is considerable and can be initiated by a range of different stimuli. A crucial stage within this series of events revolves around the release of cytochrome c and other proteins from the interior of mitochondria into the adjacent cytosol. This occurrence is a consequence of heightened permeability in the membranes of the mitochondria (Wang and Youle, 2009). Cytochrome c plays a pivotal role by serving as a foundation for the assembly of a structure known as the apoptosome. This apoptosome consists of both oligomeric proteins and procaspases-9 molecules. After activation, caspase-9 dissociates from the apoptosome and oversees the activation of other procaspase-9 molecules. This, in turn, triggers the activation of effector caspases, including caspase-3, -6, and -7 (Iordanov et al., 2005).

Mitochondria's role in controlling and enhancing the apoptotic pathway is facilitated by the Bcl-2 protein family. Back in 1988, Bcl-2 was primarily acknowledged as a gene product emerging from translocations, a prevalent genetic characteristic frequently observed in B-cell central follicular lymphoma (Reed et al., 1988). Elevated

quantities of Bcl-2, induced by the promoter of the light chain immunoglobulin, play a role in bestowing confrontation to apoptosis upon converted B cells. Bcl-2 is detectable in conjunction with several cellular components, encompassing the nuclear membrane, endoplasmic reticulum, and the outer mitochondrial membrane (Popgeorgiev et al., 2018). Moreover, within the extensive Bcl-2 protein family, there exist several other anti-apoptotic members, such as Bcl-xL, Bcl-W, Mcl-1, and A1. Additionally, there are pro-apoptotic proteins known as BH3-only proteins, which include Bax and Bak. These proapoptotic proteins have the ability to induce permeability transition in the mitochondrial membrane by forming pores. Collectively, all of these proteins constitute the broader Bcl-2 protein family (Willis et al., 2005; Lomonosova and Chinnadurai, 2008). Bax and Bak, and occasionally Bok, are effector molecules with the ability to change their conformation, associate with each other to form oligomers, and generate pores within the outer mitochondrial membrane. This sequence of events ultimately results in the permeability transition within the mitochondria (Cosentino and García-Sáez, 2017). As pores are formed in the outer mitochondrial membrane, simultaneous modifications occur in the structure of the inner membrane, along with a restructuring of cristae. These modifications facilitate the movement of proteins from the space between the mitochondrial membranes (intermembrane space) into the cytosol.

There is an alternative hypothesis proposing that the Bcl-2 protein family might play a role in the regulation of an existing multi-protein complex recognized as the permeability transition pore (PTP). This complex consists of a combination of soluble and integral proteins found within the mitochondrial membranes (Brenner et al., 2000; Azzolin et al., 2010). It's important to emphasize that almost all proteins comprising the mitochondrial permeability transition pore (MPTP) come in multiple forms, suggesting that their expression profiles could differ between normal and transformed cells. This pore complex is situated at specific points where the inner and outer mitochondrial membranes make contact. When the pore transitions into an "open" state, it leads to the expanding of the matrix and disruption of the membrane (Hurst et al., 2017). There is supporting evidence indicating that the formation of the mitochondrial permeability transition pore (MPTP) can play a role in post-apoptotic necrosis. Moreover, it can be initiated by the presence and actions of the p53 protein (Ying and Padanilam, 2016). When exposed to irradiation by UV and various stimuli, cytosolic p53 can relocate toward the outer membrane of mitochondria. There, it straightforwardly reacts with Bcl-2 proteins. This interaction can bind to both the antiapoptotic protein Bcl-xL and the proapoptotic proteins Bax and Bak. Consequently, this binding event triggers the generation of mitochondrial outer membrane permeabilization (MOMP), which subsequently leads to the activation of the caspase cascade (Qian et al., 2022).

Orexins induce the activation of certain tyrosine phosphorylation events in the OX1R receptor, targeting specific tyrosine-based patterns referred to as the immunoreceptor tyrosine-based inhibitory motif (ITIM) and the immunoreceptor tyrosine-based switch motif (ITSM) (El Firar et al., 2009). Due to these phosphorylation processes, it leads to the recruitment and activation of the phosphotyrosine phosphatase SHP-2 (Voisin et al., 2008). Consequently, this activation initiates mitochondrial apoptosis mediated by cytochrome c (Marcos and Coveñas, 2023).

In contrast, the administration of orexin A was observed to block the movement of cytochrome c from the mitochondria to the cytoplasm and its binding with Apaf-1 (Danial and Korsmeyer, 2004; Johnson et al., 2007; Xu et al., 2021b). Consequently, this interference would result in a decrease in the mobilization of procaspase 9, as shown in Table 2. Following treatment with orexin A, there was an observed increase in the expression of Bcl-2, accompanied by a decrease in the expression of Bax. Therefore, this led to an elevation in the Bcl-2/Bax ratio (Safdar et al., 2021). Furthermore, in the context of cerebral ischemia/reperfusion injury, orexin A demonstrated the inhibition of cytochrome C expression, as well as the cleaved forms of caspase-9 and caspase-3 (Xu et al., 2021b). As a result, it can be inferred that orexin A mitigates apoptosis by modulating the Bcl-2/Bax ratio and suppressing the expression of cytochrome C, cleaved caspase-9, and cleaved caspase-3. Orexin A also demonstrated a neuroprotective effect against cellular death induced by palmitic acid, as shown in Table 2. Specifically, orexin A attenuates palmitic acid-induced cell death in the hypothalamus through mechanisms that include a reduction in caspase-3/7-driven apoptosis, the Bcl-2 gene expression stabilization, and a reduction in the Bax/Bcl-2 expression ratio (Duffy et al., 2016). The intraperitoneal administration of orexin A resulted in the improvement of renal function and the alleviation of histological abnormalities in mice treated with cisplatin. Orexin A achieved this by curbing cisplatin-induced oxidative stress and decreasing apoptotic cell death through the inhibition of the p53-mediated pathway in these cisplatin-treated mice (Jo et al., 2022). Furthermore, when OXR1 was blocked in testicular cancer in mice, there was a notable increase in the expression levels of Bax, p53, and caspase 3. This observation implies that the activation of OXR1 potentially results in a reduction in apoptosis by inhibiting the p53/caspase 3 signaling pathway.

8 Effect of orexins on receptor-related mechanism of apoptosis

Substances that trigger apoptosis are categorized as ligands within the cytokine group known as the TNF superfamily. These ligands specifically engage with death receptors found within the TNF-R superfamily (Diaz Arguello and Haisma, 2021). These cytokines possess the ability to attach to a varied range of surface receptors, and as of now, more than 40 pairs of ligands and receptors have been recognized within this particular family (Aggarwal et al., 2012). The TNF superfamily consists of multiple members, among which are APRIL, TRAIL, TNF-α, FasL, RANKL, TWEAK, and lymphotoxinα (Devarapu et al., 2017). For instance, FasL demonstrates a unique binding preference for the Fas receptor, whereas TRAIL possesses the capability to engage with four separate receptors referred to as TRAIL-R1 through TRAIL-R4 (Diaz Arguello and Haisma, 2021). When the TNF-R superfamily is activated, its intracellular domain initiates the recruitment of adaptor proteins. For instance, in the case of Fas receptor activation by FasL, it associates with adaptor proteins such as FADD/MORT1. Subsequently, these adaptor proteins prompt the activation of procaspases 8 or 10 (Diaz Arguello and Haisma, 2021). Following this, these procaspases undergo maturation into their active states, and these active caspases subsequently trigger the activation of executive caspases. For instance, caspase 8 has the capacity to activate Bax, thus playing a role in apoptosis through the mitochondrial pathway (Diaz Arguello and Haisma, 2021).

The TNFR1 receptor can assemble into two distinct complexes that involve various adaptor proteins: complex I and complex II. Complex I comprises adaptor molecules such as TRADD, RIPK1, TRAFs, cIAP1 or 2, and cFLIP (Dondelinger et al., 2016). The activation of RIPK1 leads to the creation of a platform that initiates the activation of NF-κB and MAPK signaling pathways. This activation subsequently triggers the expression of prosurvival proteins and proinflammatory factors (Chen et al., 2019). Complex II, on the other hand, involves TRADD-adaptor proteins interacting with FADD-adaptor proteins, ultimately resulting in apoptosis. However, if the activation of TNF-R1 results in the degradation of cIAPs and inhibition of caspase activity via the RIPK1-mediated signaling, it prompts another cell death type called necroptosis (programmed necrosis) (Liu et al., 2016). Necroptosis is induced by various factors, including but not limited to TNFα, Fas, interferons, TRAIL/Apo2L, viral infections, and toll-like receptors (Jayaraman et al., 2021).

It is interesting to observe that besides OX1R, which initiates apoptosis through the intrinsic or mitochondrial pathway, it might modulate receptor-mediated mechanism of apoptosis (Voisin et al., 2011, 2022). Cancer cells are also known to express Fas receptors, which, acting as death receptors, initiate apoptosis through the extrinsic apoptosis pathway (Dondelinger et al., 2016). Regrettably, many cancer cell lines tend to exhibit some degree of resistance to Fas ligand-mediated apoptosis, even when they possess Fas receptors on their surface (Zhu et al., 2017). In contrast, it's worth noting that normal cells are highly sensitive to Fas-mediated apoptosis, responding with a high degree of susceptibility to this signaling pathway (Green and Ferguson, 2001). This significant difference in sensitivity between cancer cells and normal cells poses a substantial obstacle to the potential use of Fas receptor agonists as candidates for chemotherapy. If these agonists were employed, patients' cancer cells might remain relatively resistant to apoptosis, while their normal cells would be more susceptible to self-destruction. Similarly, resistance to apoptosis facilitated by TNF-related apoptosis ligand (TRAIL) has been observed at multiple points in the extrinsic apoptosis pathway in cancer, hindering the therapeutic utility of TRAIL as an apoptosis inducer in tumor cells. Efforts to utilize TNF and Fas ligand have also faced challenges due to the induction of NF-κB-mediated inflammation (Rex et al., 2019) and fulminant hepatic failure, respectively, (De Gregorio et al., 2020). Notably OX1R is evidently unaffected by the limitations typically associated with death receptors. Notably, OX1R induces apoptosis in colon cancer cells, even those resistant to 5-FU, as reported by Voisin and colleagues (Voisin et al., 2011, 2022). This unique characteristic positions OX1R as a promising candidate for therapeutic intervention, with OX1R agonists potentially enhancing the efficacy of traditional chemotherapy treatments in colon cancer (Wolpin and Mayer, 2008; Segal and Saltz, 2009).

Some studies stated that administration of orexin A reduced the formation of TNF-α and other cytokines and led to anti-inflammatory and antiapoptotic effects in colitis (Messal et al., 2018) (Table 2). This could be mediated by inhibition of NF-κB and MAPK/ERK signaling (Xu et al., 2021b). However, Excessive release of TNFα leads to a downregulation of OX2R as previously reported (Sun et al., 2018). The administration of TNFα significantly decreased (by 86%) OX2R protein levels through ubiquitination in B35 cells which was accompanied by a diminution in cIAP-1 and 2 levels (Zhan et al., 2011). These research studies revealed that TNF-α can impede the function of the orexin system by decreasing the levels of both Prepro-orexin and OX2R (Zhan et al., 2011). However, further studies are needed to investigate the effect of orexins on the TNF superfamily and their adaptor proteins.

9 Endoplasmic reticulum stress and apoptosis

The Endoplasmic Reticulum (ER) possesses a protein quality management system that depends on the assistance of protein chaperones and foldases. These components play a critical role in recognizing and facilitating the correct folding of misfolded proteins. If the process of folding is unsuccessful, it leads to the buildup of proteins that are misfolded within the lumen of ER, a condition referred to as ER stress (Haeri and Knox, 2012). These issues encompass problems like impaired glycosylation, reduced formation of disulfide bonds, a reduction in the concentration of calcium within the ER lumen, and disruptions in the transport of protein to the Golgi apparatus. The culmination of these events triggers the Unfolded Protein Response (UPR) signaling pathway, which is designed to either destroy or correct misfolded proteins and restore equilibrium within the Endoplasmic Reticulum (ER) (Amen et al., 2019). The UPR can initiate either an adaptive response or a reaction that results in apoptotic cell death. Following the ER stress onset, cells can activate several mechanisms, including: (1) reduced protein synthesis to prevent further accumulation of proteins (Almanza et al., 2019), (2) Enhanced gene transcription responsible for chaperone proteins encoding for instance GRP94 and BiP/GRP78 (Zhu and Lee, 2015), and (3) ER-accompanying degradation (ERAD) of proteins that fail to embrace their intrinsic conformation, thereby targeting them for disposal (Qi et al., 2017). Degradation can take place via autophagy or within the 26S proteasome. In mammals, 3 transmembrane proteins, specifically IRE-1α, PERK, and ATF-6α, typically associate with GRP78, a chaperone responsible for facilitating proper protein folding and preventing protein aggregation (Bravo et al., 2013). In cases of mild or transient ER stress, GRP78 separates from IRE-1, ATF-6α, and, PERK, triggering the unfolded protein response (UPR) to mitigate stress and uphold ER functionality (Wang et al., 2015). When the cell's adaptive reaction to ER stress falls short, programmed cell death is initiated through multiple mechanisms. These mechanisms encompass the IRE1-activated ASK1/JNK signaling, the PERK/eIF2-supported activation of the proapoptotic transcription factor CHOP, Bax/Bak-mediated calcium release into the cytosol, and the proteolytic stimulation of procaspase-12 (Merighi and Lossi, 2022). IRE1α stands out as one of the highly conserved sensors for ER stress. It's categorized as a type I transmembrane protein with dual functionalities, serving as both an endoribonuclease and a serine/threonine kinase. When GRP78 disengages, IRE1α experiences homo-oligomerization and trans-autophosphorylation events, which then activate its endoribonuclease and kinase domains (Ricci et al., 2021). As a result of this activation, IRE1α becomes engaged in facilitating the splicing of the mRNA intron responsible for encoding XBP1. This splicing process yields the XBP1 isoform (active), which subsequently initiates the gene expression responsible for ensuring the accurate folding of proteins within the lumen, the phospholipids biosynthesis, and the degradation of misfolded molecular structures (Yoshida et al., 2001). IRE1α also plays a role in engaging signaling pathways that lead to apoptotic cell death. For instance, it interacts with TRAF2 in the cytosol, leading to the activation of NF-κB, ASK, and JNK, ultimately

contributing to inflammation and apoptosis (Nishitoh et al., 2002; Tam et al., 2012; Junjappa et al., 2018). IRE1α has the capacity to interact with proapoptotic proteins like Bax and Bak. This interaction induces conformational changes and/or oligomerization within the ER membrane, resulting in the formation of a pore that permits the secretion of calcium into the cytosol (Kanekura et al., 2015). The heightened calcium concentration activates procaspase-12. Subsequently, procaspase-12 dissociates from the membrane, arrives the cytosol, and triggers the stimulation of procaspase-9, ultimately leading to the activation of caspase-3 (Moon, 2023). The calcium that entered the cytosol from the ER is promptly sequestered by the mitochondria. This event results in the depolarization of the inner mitochondrial membrane and initiates mitochondria-supported apoptosis.

PERK is a transmembrane sensor protein equipped with serine/threonine kinase activity within its cytoplasmic domain. When the endoplasmic reticulum (ER) experiences stress, PERK becomes active in a manner similar to IRE1. This activation process entails the separation of IRE1α from GRP78, its subsequent homooligomerization, and autophosphorylation (Rozpedek et al., 2016). After activation, active PERK phosphorylates the α-subunit of eIF2α (eukaryotic initiation factor 2α), which leads to a decrease in general protein synthesis. Paradoxically, this also results in an elevation in the translation of specific mRNAs, including those responsible for encoding the transcription factor ATF4 (Rozpedek et al., 2016). ATF4 plays a pivotal role in the cellular response to endoplasmic reticulum (ER) stress. It initiates the expression of other unfolded protein response (UPR) transcription factors, chaperones, proteins involved in autophagy regulation, and components of the oxidative stress response. One significant target of ATF4 is the gene responsible for producing the proapoptotic protein CHOP. CHOP, in turn, enhances the transcription of oxidase ERO1α, which results in the generation of reactive oxygen species (ROS) within the ER. Additionally, it triggers the release of calcium ions (Ca2+) into the cytosol by activating the inositol 1,4,5-trisphosphate receptor located on the ER membrane (Cao and Kaufman, 2014). The increased Ca2+ levels activate Ca2+/calmodulin-dependent protein kinase II (CaMKII), which, in turn, initiates apoptosis. CHOP further promotes apoptosis by inhibiting the antiapoptotic protein Bcl-2 and activating other proapoptotic factors. Additionally, it plays a role in the activation of caspase-8, further contributing to the apoptotic process (Hu et al., 2018).

Intervention with orexin A led to a substantial reduction in the expression of GRP78 and protected against cell damage induced by oxygen–glucose deprivation/reoxygenation (Kong et al., 2019). Orexin A exhibited an anti-apoptotic effect by diminishing the expression of components within the ER stress-related signaling pathways, including GRP78/IRE1α/JNK and GRP78/IRE1α/caspase-3/caspase-12 (Xu et al., 2021a), which provided new mechanistic evidence of the neuroprotective effect of orexin-A. Furthermore, treatment with orexin-A led to a reduction in the infarct volume in rats following cerebral ischemia–reperfusion injury, while also mitigating neuronal apoptosis (Xu et al., 2021a). Taken together, these discoveries imply that orexin-A has the potential to alleviate cerebral ischemia–reperfusion injury by inhibiting apoptosis mediated by ERS, thereby elucidating the mechanism underlying its neuroprotective properties (Table 2).

It has been observed that the administration of orexin-A can prevent the development of non-alcoholic steatohepatitis. Notably, orexin A promptly activates the mTOR-XBP1 pathway in the liver during periods of fasting (Tsuneki et al., 2022). The daily administration of orexin A was found to effectively reduce hepatic inflammation during the refeeding phase following fasting, and this effect was dependent on the presence of rapamycin. Consequently, it was proposed that orexin's action could potentially prepare the liver's adaptive response to anticipated endoplasmic reticulum (ER) stress through the mTOR/XBP1 pathway, thereby preventing an excessive occurrence of ER stress and inflammation in the liver (Tsuneki et al., 2022). Furthermore, a deficiency of orexin A in the hypothalamus could result in neuronal apoptosis, a process mediated by pathways that encompass IRE1α-XBP1, PERK, and unfolded proteins.

In response to stimulation by orexin-A, a common cellular reaction is the elevation of intracellular calcium ion (Ca2+) levels. Research has demonstrated that orexins induce intracellular Ca2+ transients in cells expressing the orexin receptors, whether they are receptor-transfected cells or cells naturally expressing these receptors. These Ca2+ transients serve as valuable indicators of orexin receptor activation (Kukkonen and Leonard, 2014). Typically, increases in intracellular Ca2+ concentration can result from various mechanisms. Studies conducted in Chinese hamster ovary (CHO) cells indicate that the origin of orexin-A-induced Ca2+ transients varies depending on the concentration of the ligand (Smart et al., 1999). At lower concentrations of orexin-A, the primary source of Ca2+ influx appears to be the opening of receptor-operated Ca2+ channels. However, at higher concentrations of orexin-A, it is suggested that the increases in Ca2+ are a consequence of both Ca2+ release from the endoplasmic reticulum and influx through store-operated Ca2+ channels. This concentration-dependent variation in the mechanism of Ca2+ entry points to a complex and regulated cellular response to orexin-A stimulation (Xia et al., 2009). Indeed, an excessive accumulation of Ca2+ or disruptions in the precise compartmentalization of intracellular calcium can contribute to the initiation of apoptosis. Proper regulation of intracellular calcium levels is essential for cell survival, and dysregulation can lead to cell death pathways, including apoptosis (Orrenius et al., 2003). Exactly, the apoptotic effect of orexin mediated by Ca2+ is contingent upon the intracellular concentration of calcium. The level of intracellular calcium plays a pivotal role in determining whether cells undergo apoptosis or not, and it can be influenced by various factors, including the actions of orexin (Table 2).

10 Effect of orexins on nuclear mechanisms of apoptosis

The process of nuclear-mediated apoptosis encompasses two primary pathways. The first pathway is associated with the activation of p53, which subsequently upholds the expression of proapoptotic proteins, including Noxa, Puma, and Bax (Oda et al., 2000; Nakano and Vousden, 2001; Chen L. et al., 2013). These proapoptotic proteins, once expressed, contribute to mitochondrial-driven apoptosis and initiate the caspase cascade. The activation of p53 can be triggered by various factors, including exposure to ionizing radiation and UV, heat shock, hypoxia, oxidative stress, and reduced temperatures (Renzing et al., 1996). In actively growing cells, p53 activation can result from DNA damage or errors in DNA replication. This leads to cell cycle arrest, a process mediated by the expression of the p21 gene product under the control of p53. If the cellular repair mechanisms are unable to effectively address the damage, the cells will then initiate their

apoptotic program as a safeguard against potential genetic instability (Williams and Schumacher, 2016).

The second pathway for inducing nuclear apoptosis involves the activation of caspase-2. Despite much research into its role in apoptosis, the function of caspase-2 remains unclear. Caspase-2 has a similar domain structure to initiator caspases-8 and -9 and becomes active through dimerization and autoprocessing (Bouchier-Hayes and Green, 2012). It comprises a higher MW protein platform featuring a CARD sequence that participates in protein–protein interactions. Specifically, the CARD in caspase-2 interacts with the CARD in RAIDD, functioning as an adaptor protein that facilitates the recruitment of caspase-2 into the activation complex (Vigneswara and Ahmed, 2020).

The protein known as "p53-induced protein with a death domain" (PIDD) is triggered by p53 and plays a role in p53-dependent apoptosis. The assembly of the PIDDosome, a complex comprising PIDD, RAIDD, and caspase-2, occurs when PIDD binds to RAIDD, which in turn recruits seven caspase-2 molecules. This recruitment leads to autoprocessing and activation of caspase-2 within the PIDDosome complex (Sladky and Villunger, 2020). PIDD also forms a separate complex with RIPK1 and NEMO in response to DNA damage (Weiler et al., 2022). The recruitment of RAIDD and RIPK1 into the PIDDosome occurs sequentially, with RIPK1 and NEMO first interacting with PIDD, followed by RAIDD and caspase-2 (Table 2).

There have been numerous studies investigating the effect of orexin on the p53 protein, a transcription factor that plays a crucial role in cell cycle regulation and DNA damage response. In *in vitro* studies, orexin has been shown to regulate the activity of p53, leading to changes in cell proliferation, apoptosis, and senescence. For example, orexin treatment has been reported to induce p53-mediated apoptosis in various cancer cell lines (Ammoun et al., 2006), including prostate (Malendowicz et al., 2011), and colon (Wen et al., 2016) cancer cells.

Conversely, orexin has also been shown to protect neurons against oxidative stress-induced apoptosis by stabilizing the p53 protein. *In vivo* research has further confirmed the role of orexin in p53 regulation. For example, a study in mice showed that orexin deficiency leads to increased p53 accumulation and apoptosis in the hypothalamus, suggesting a potential role for orexin in maintaining hypothalamic neuronal viability (Chen et al., 2015). Another study demonstrated that orexin receptor agonists can protect from oxidative stress-induced p53 activation and apoptosis in neuronal cell cultures (Sokołowska et al., 2014).

Recent research showed that orexin may also affect caspase-2. One study found that orexin reduced caspase-2 activity in neurons, suggesting a potential neuroprotective effect (Chung et al., 2007). This was further supported by a study, which showed that orexin treatment significantly reduced caspase-2-mediated cell death in a model of cerebral ischemia (Chen et al., 2011). A more recent study found that orexin-A could protect neurons against oxidative stress-induced cell death by regulating the balance between the pro-apoptotic protein, caspase-2, and the anti-apoptotic protein, Bcl-2. The study suggested that orexin-A may play a protective role in some neurodegenerative diseases by reducing oxidative stress and inhibiting the activation of caspase-2 (Naidoo, 2022).

11 Conclusion

In conclusion, the multifaceted effects of orexins on apoptosis highlight their complex and context-dependent role in cellular regulation. The evidence presented in this text demonstrates that orexins can modulate apoptosis through various mechanisms, including caspase activation, mitochondrial regulation, receptor-related pathways, endoplasmic reticulum stress, and nuclear mechanisms.

Orexins exhibit both pro-apoptotic and anti-apoptotic properties, depending on the cell type and conditions. In cancer models, orexins have been shown to promote apoptosis via the stimulation of caspases, particularly caspase-3 and caspase-9, in several cell lines. This pro-apoptotic effect has potential implications for cancer therapy, as it selectively targets cancer cells while sparing normal cells. Conversely, orexins also demonstrate anti-apoptotic effects by inhibiting caspase activity, particularly caspase-3, in neuronal cells. These effects contribute to neuroprotection against oxidative stress-induced apoptosis, which may have relevance in the context of neurodegenerative diseases. Furthermore, orexins influence apoptosis through mechanisms such as the regulation of Bcl-2/Bax ratio, cytochrome C expression, and modulation of p53 activity. These effects are intricate and contingent on the specific cellular and physiological contexts. The possible effects of orexin on different molecular mechanisms of apoptosis are summarized in Figure 3.

In summary, orexins play a pivotal role in the intricate regulation of apoptosis, with their actions varying depending on the cellular environment and signaling pathways involved. Understanding these diverse effects of orexins on apoptosis is crucial for elucidating their potential therapeutic applications in cancer treatment and neuroprotection.

12 Future perspectives

Further research is needed to fully understand the mechanisms by which orexins regulate apoptosis. The following areas could be explored: (1) Additional studies are needed to investigate the effects of orexins on apoptosis in different cell types and to determine whether the conflicting findings in the literature are due to differences in cell type; (2) More investigations are needed to understand the underlying mechanisms by which orexins regulate apoptosis, including the role of intracellular Ca2+ concentration, the p53 protein, caspases, and other signaling pathways; (3) The relationship between orexins and oxidative stress is complex and not fully understood. Supplementary research is required to clarify the role of orexins in reducing oxidative stress and its impact on apoptosis regulation; (4) Orexins have been shown to have neuroprotective effects in various contexts, but the underlying mechanisms are not well understood. Therefore, it is needed to determine the precise mechanisms by which orexins protect against neuronal injury and death; (5) The role of orexins in cancer is not well understood and requires further investigation to determine whether orexins can be used as therapeutic targets in cancer treatment and to clarify the factors that could determine their role as both pro- and anti-apoptotic agents in cancer cells.

In the domain of intrinsic apoptotic mechanisms, orexin is implicated in initiating a series of molecular events that culminate in apoptosis, commencing with the activation of p53. This crucial step leads to the mitochondrial release of cytochrome c, which subsequently activates caspase 9, an essential enzyme in the apoptotic process. Concurrently, orexin modulates the Akt pathway by upregulating key proteins involved in cellular stress responses. This modulation results in the activation of several critical factors that significantly influence apoptosis and cell survival. NF-κB plays a dual

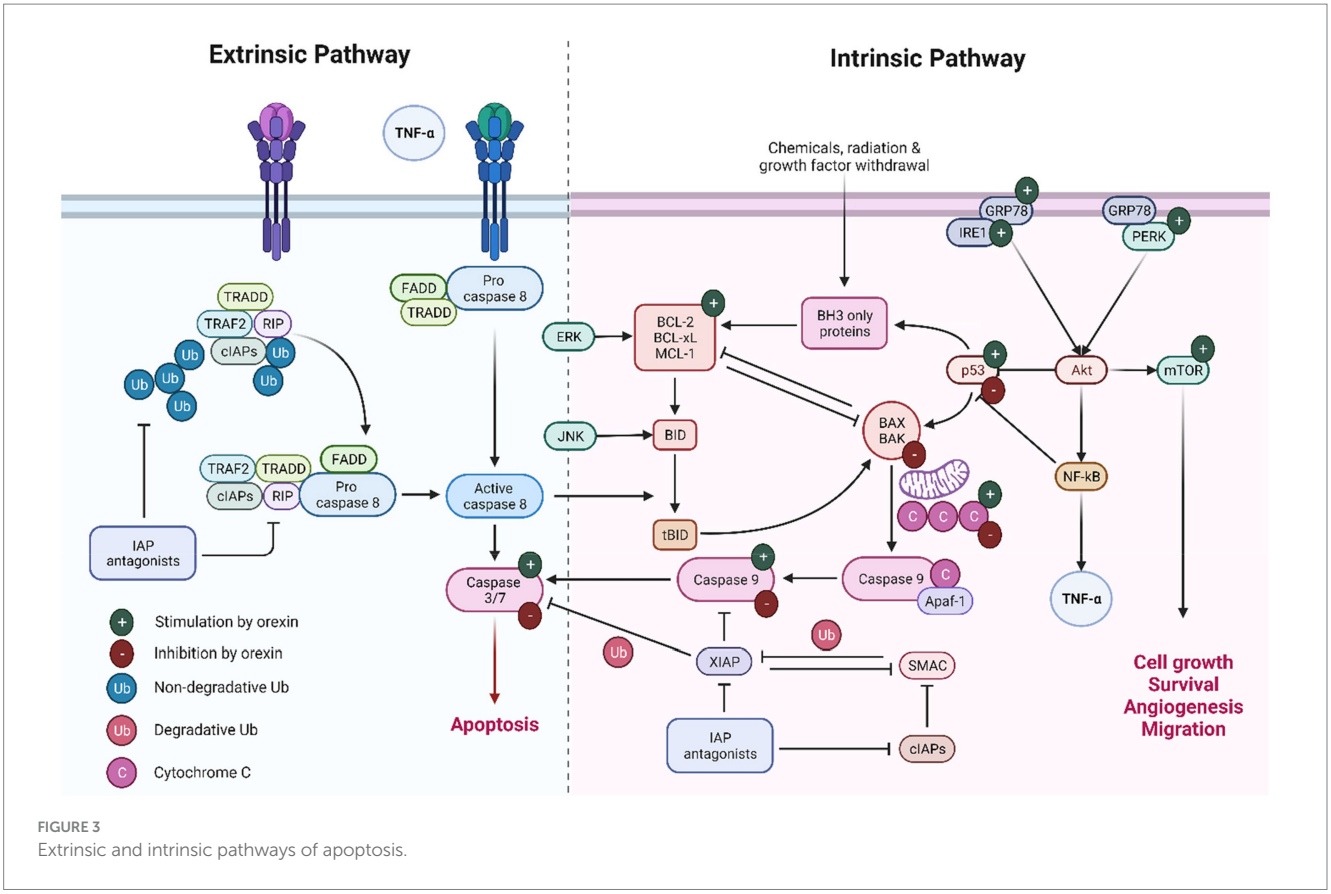

FIGURE 3
Extrinsic and intrinsic pathways of apoptosis.

role, predominantly acting as an anti-apoptotic agent by downregulating p53 expression, while mTOR promotes cell survival, migration, and angiogenesis. Interestingly, under certain conditions, orexin appears to downregulate the p53/cytochrome c/caspase 9 axis, thereby inhibiting apoptosis. In the context of extrinsic apoptotic mechanisms, orexin's influence on caspase 3 and caspase 7 is context-dependent, with the potential to either stimulate or inhibit these enzymes. In various cancer models, orexins increased apoptosis via induction of caspase 3 and 7. In chemoresistant pancreatic tumors, orexins effectively inhibit tumor development by enhancing caspase 3 and 7-mediated apoptosis. Additionally, orexins exhibit a contrasting role in neuronal cells by inhibiting caspase-3 activity and protecting against apoptosis.

Author contributions

SC: Conceptualization, Data curation, Investigation, Project administration, Software, Supervision, Validation, Writing – original draft, Writing – review & editing. SS: Conceptualization, Data curation, Investigation, Project administration, Software, Writing – original draft, Writing – review & editing. RH: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. MA-R: Conceptualization, Data curation, Investigation, Software, Validation, Writing – original draft, Writing – review & editing. MY: Conceptualization, Data curation, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. EE: Conceptualization, Data curation, Methodology, Formal analysis, Validation, Investigation, Writing – original draft, Writing – review & editing.

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Conflict of interest

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

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Glossary

AgRP	Agouti-related peptide
Aprf-1	Apoptotic protease activating factor 1
APRIL	Proliferation-inducing ligand
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
cAMP	Cyclic adenosine monophosphate
FADD	Fas-associated death domain protein
Fas	Cell surface death receptor
FasL	Fas ligand
FLICE	(FADD-like IL-1β-converting enzyme)-inhibitory protein
GPCRs	G-protein-coupled receptors
LC	Locus coeruleus
LH	Lateral hypothalamic
MAPK	Mitogen-activated protein kinase
MOMP	Mitochondrial outer membrane permeabilization
MORT1	Mediator of receptor-induced toxicity protein 1
MPTP	Mitochondrial PTP
NF-κB	Nuclear factor-kappa B
NMR	Nuclear magnetic resonance
NYP	Neuropeptide Y
OX1R	Orexin-1 receptor
OX2R	Orexin-2 receptor
PFA	Perifornical hypothalamic areas
PLC	Phospholipase C

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The lactate metabolism and protein lactylation in epilepsy

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Protein lactylation is a new form of post-translational modification that has recently been proposed. Lactoyl groups, derived mainly from the glycolytic product lactate, have been linked to protein lactylation in brain tissue, which has been shown to correlate with increased neuronal excitability. Ischemic stroke may promote neuronal glycolysis, leading to lactate accumulation in brain tissue. This accumulation of lactate accumulation may heighten neuronal excitability by upregulating protein lactylation levels, potentially triggering post-stroke epilepsy. Although current clinical treatments for seizures have advanced significantly, approximately 30% of patients with epilepsy remain unresponsive to medication, and the prevalence of epilepsy continues to rise. This study explores the mechanisms of epilepsy-associated neuronal death mediated by lactate metabolism and protein lactylation. This study also examines the potential for histone deacetylase inhibitors to alleviate seizures by modifying lactylation levels, thereby offering fresh perspectives for future research into the pathogenesis and clinical treatment of epilepsy.

KEYWORDS

protein lactylation, histone deacetylase, epilepsy, high-mobility group box 1, hypoxia-inducible factor-1α, lactate

Highlights

- The lactate metabolism and transport carried out in neurons and glial cells may influence the development of epilepsy by regulating protein lactylation levels.
- Lactate may mediate epilepsy-related neuronal loss by promoting HMGB1 lactylation.
- Ischemic stroke may promote HMGB1 lactylation by activating HIF-1, which can promote a shift in the mode of cellular energy acquisition from oxidative phosphorylation to glycolysis, thereby inducing post-stroke epilepsy.
- Histone deacetylases may affect protein lactylation by regulating the transcriptional activity of HIF-1.
- Histone deacetylase inhibitors may combat post-stroke epilepsy by modulating hypoxia-induced protein lactylation.

1 Introduction

Epilepsy, as a common chronic disease of the nervous system, has affected more than 70 million people of all ages worldwide, with the number continuing to rise annually (Eugen et al., 2019). The factors inducing seizures are diverse and multifaceted. Ischemic stroke is one of the common causes of epilepsy. Post-ischemic stroke epilepsy accounts for approximately 9% of all epilepsy cases, a figure slightly lower than that caused by cerebral hemorrhage. However, the growing number of individuals affected by ischemic stroke each year highlights its significant impact on public health (Lanqing et al., 2021; Carolina et al., 2021). The hypoxic

environment caused by ischemic stroke often forces brain tissue to obtain energy through glycolysis, thereby producing a large amount of lactate.

Lactate in the brain may affect the progression of various neuropsychiatric diseases through its roles in learning, memory, and emotional regulation. This process may be related to lactate-mediated post-translational modifications of proteins (Hagihara et al., 2021). Lactylation (La), a novel type of post-translational protein modification, was identified by Zhang et al. (2019). With the ongoing research into protein lactylation modification in recent years, HAGIHARA H et al. found that lysine lactylation (Kla) may be prevalent in neurons and glial cells in the brain.

Furthermore, the level of lysine lactylation is regulated by lactic acid levels, neural excitation, and social defeat stress (Hagihara et al., 2021). It suggests that increased neuronal excitability may be closely related to the level of protein lactylation in neurons.

Abnormal excitation of neurons is a key driver of epileptogenesis. Ischemia/reperfusion injury (I/R) can induce epileptogenesis by mediating abnormal excitation of neurons (Paudel et al., 2020; Esih et al., 2021; Endres et al., 2022). It can be speculated that ischemia-induced stroke may lead to increased glucose metabolism in the brain, resulting in the production of large quantities of lactate, which can be transformed into acetyl groups to participate in the acetylation of proteins related to neuronal excitation and thus cause post-stroke epilepsy (Hagihara et al., 2021; He et al., 2023). That is, protein lactylation may be a potential regulatory target for post-ischemic stroke epilepsy. While anti-epileptic drugs can help most patients control their seizures, approximately 30% of individuals with epilepsy do not respond to current clinical medications (Sun et al., 2021). This review mainly focuses on the impact of lactate metabolism and protein acetylation on epilepsy and changes in acetylation modifications controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Furthermore, based on the mechanisms of action of HDAC inhibitors (HDACIs) in regulating acetylation modifications, we discuss the possibility that they could provide a new strategy for treating post-stroke epilepsy.

2 The lactate mediates epilepsy by regulating the functions of neurons and glial cells

Seizures are usually localized in the hippocampus, with temporal lobe epilepsy (TLE) being the most common (Sun et al., 2021). Previous studies have suggested that neuronal hyperexcitability in epilepsy is primarily mediated by neuronal death and neuroinflammation, which is mediated by astrogliosis and microglial activation, and the relationship between neuronal death and neuroinflammation may be complex (Haenisch et al., 2015; Roh et al., 2023). Seizures are usually accompanied by significant glial cell proliferation and loss of neurons, and glial cell proliferation-mediated neuroinflammation often leads to neuronal death (Feng et al., 2019). Protein lactylation modifications have been found in the hippocampus and prefrontal cortex and are strongly associated with neuronal excitability (Hagihara et al., 2021).

Currently, there is insufficient evidence to confirm that protein lactylation directly induces epilepsy by regulating neuronal excitability. However, lactate, as the donor of lactide, can serve as an energy source for metabolic activities in neurons and glial cells during ischemia and hypoxia, potentially influencing the progression of post-stroke epilepsy.

2.1 Glial cells mediate epilepsy by regulating the release of neurotransmitters

Studies on status epilepticus suggest that astrocyte and microglial activity changes, which influence critical homeostatic processes—such as synaptogenesis, extracellular ion concentrations, and excitation-inhibition balance—may occur early in epilepsy development. Inhibiting glial cell activity has been shown to reduce susceptibility to epilepsy (Shen et al., 2023). Neuroglia, such as astrocytes, microglia, and oligodendrocytes, may influence neuronal excitability by regulating the production of glutamate, adenosine triphosphate (ATP), and γ-aminobutyric acid (GABA) in the central nervous system, thereby modulating the progression of epilepsy (Shen et al., 2023).

Glutamate is the primary excitatory neurotransmitter in the central nervous system and can induce seizures by increasing synaptic transmission in the hippocampal region. Although the death of neurons caused by cerebral infarction can reduce the synthesis of the neurotransmitter glutamate, previous studies have confirmed that the level of glutamate in the blood and cerebrospinal fluid of ischemic stroke patients is related to the severity of infarction and neurological dysfunction (Nicolo et al., 2019). Research has shown that stroke can mediate neuroinflammatory damage by activating astrocytes' TGF-β pathway, and the TGF-β signaling in astrocytes may have a pro-epileptic effect (Korotkov et al., 2020). Therefore, astrocytes may induce post-stroke epilepsy by mediating stroke-related glutamate excitotoxicity. Astrocytes primarily take up excessive glutamate from the synaptic cleft through two types of glutamate transporters, such as glutamate–aspartate transporter (GLAST) and glutamate transporter-1 (GLT1). The glutamate, which is transported into the cell by GLAST or GLT1, can be converted into glutamine by glutamine synthetase, which is a major precursor for the biosynthesis of the inhibitory neurotransmitter GABA (Sun et al., 2021; Peterson et al., 2021). Glutamate released by neurons is taken up by astrocytes and converted to glutamine to alleviate the excitotoxicity of glutamate on neurons. Glutamine serves as a source of energy for both glial cells and neurons (Nagy et al., 2018). Recent studies have shown that reducing the expression of nascent proteins and the activity of inward rectifying K+ channel subtype 4.1 (Kir4.1), respectively, leads to a decrease in the expression of GLAST and GLT1 in hippocampal astrocytes, which results in increased extracellular glutamate levels and decreased GABA release, leading to an increased risk of epileptogenesis (Sun et al., 2021; Centonze et al., 2023). Since knocking down nascent protein does not affect the expression of GABA transporter proteins in astrocytes, the reduction in GABA release may be attributed to reduced GABA synthesis (Sun et al., 2021). It has been shown that inhibiting the conversion of putrescine to spermine in astrocytes would lead to more remaining putrescine being available for the synthesis of GABA, thereby effectively reducing epileptic activity (Kovács et al., 2021).

Previous research suggests that compared to GLAST, GLT1 is the main transporter for the uptake of glutamate by astrocytes (Peterson

et al., 2021). In the intrahippocampal kainic acid model of TLE, GLT1 regulates the frequency of seizures and the total time spent in seizures by mediating the uptake of most glutamate in the dorsal forebrain (Peterson et al., 2021). GLT1 mitigates ischemic stroke-induced neuroexcitotoxicity by increasing glutamate reuptake in astrocytes, thereby reducing neuronal cell death, which is an important cause of post-stroke epilepsy (Wang et al., 2022). Post-translational modifications of GLT-1, including palmitoylation, ubiquitination, nitrosylation, and succinylation, can regulate the distribution of GLT-1 and the rate at which it transports glutamate (Peterson et al., 2021). Although it has not yet been confirmed that palmitoylation can alter the activity of GLT1, based on reports linking protein palmitoylation to neuronal excitability, GLT1 lactylation may affect the excitability of neurons by regulating the concentration of glutamate between synapses (Hagihara et al., 2021).

Previous studies have shown that calcium ions mediate the release of glutamate and ATP from neurotransmitter-activated astrocytes to promote neuronal firing patterns (Shen et al., 2023; Chen et al., 2023). However, other research suggests that the majority of the neurotransmitter glutamate in the brain is synthesized and released by neuronal cells, with astrocytes playing a role in regulating the concentration of glutamate between synapses through reuptake. Until the concept of "glutamatergic astrocytes" was proposed by DeCeglia et al., the ability of astrocytes to transmit information like neurons was unknown. Glutamatergic astrocytes are located mainly in the hippocampus and express the vesicular glutamate transporter protein 1 (vGLUT1), which specifically loads glutamate into synaptic vesicles and promotes its release into the synaptic gap (de Ceglia et al., 2023). Although vGLUT1-mediated glutamate release may promote the occurrence of epilepsy, it does not prove that the glutamate transported by vGLUT1 is synthesized by astrocytes rather than acquired by astrocytes from the synaptic cleft. Glutamate can not only directly induce epilepsy by mediating the excitotoxicity of neurons but also induce epilepsy related to brain tissue damage by altering the function of inhibitory interneurons. Previous studies have confirmed that brain tissue damage can lead to an increase in synaptic inputs generated by pyramidal neurons, thereby driving an increase in the excitability of surviving hilar interneurons, which in turn leads to the occurrence of post-traumatic epilepsy (Butler et al., 2017). This may be closely related to the glutamatergic synapses formed between pyramidal cells and interneurons in the hippocampus, as a previous study has shown that pyramidal cells may regulate the transition between inhibitory interneurons and disinhibitory interneurons through the glutamatergic synapses formed with interneurons (Tzilivaki et al., 2023).

During status epilepticus, neurons, astrocytes, and microglia release ATP, activating the purinergic receptor P2Y1 on astrocytes and further exacerbating neuronal excitotoxicity. Meanwhile, ATP released from damaged neurons can activate NLRP3 inflammatory vesicles by binding to the ATP-gated ion channel P2X7 in microglia, mediating inflammatory injury in brain tissue (Liu et al., 2022). Glial cells and neurons can sustainably increase neuronal excitotoxicity by releasing excitatory neurotransmitters. However, adenosine kinase synthesized by astrocytes converts ATP to adenosine, alleviating neuronal excitotoxicity by activating presynaptic A1 adenosine receptors, thereby blocking this self-sustaining cycle of neuronal activity (Shen et al., 2023; Boison and Steinhäuser, 2018). Meanwhile, as brain-resident immune cells, microglia catalyze the conversion of ATP to adenosine by expressing CD39, an extracellular ATP/ADP hydrolase encoded by Entpd1, which can reduce seizures (Hu et al., 2023). Although activated microglia may promote the occurrence of epilepsy by releasing glutamate and inflammatory cytokines (such as interleukin-1β, interleukin-6, and tumor necrosis factor-α), inhibiting glutamate uptake, and reducing GABAergic transmission (Shen et al., 2023), the above-mentioned astrocytes and microglia can play a dual role in promoting and inhibiting epilepsy, which may depend on different stages of epileptogenesis.

Previous studies have shown that oligodendrocytes, like astrocytes and microglia, regulate the release and reuptake of neurotransmitters between neuronal synapses, thereby mediating epileptogenesis and development. Oligodendrocytes have been shown to be involved in the regulation of glutamate levels in the brain (Shen et al., 2023).

2.2 Lactate interference with glial cell function

Glucose is the main energy source for the brain, and interfering with the glucose metabolic process may affect brain function. Lactate produced by glycolysis may mediate glial cell activation and neuronal functional impairment by promoting histone acetylation (Pan et al., 2022; Wei et al., 2023). The increase in histone H3K18 acetylation levels in the hippocampus can directly activate the nuclear factor kappa-B (NF-κB) signaling pathway by stimulating the promoters of Rat transcription factor (Rela) and NF-κB, which can enhance the production and release of pro-inflammatory factors and exacerbate the inflammatory damage associated with epilepsy (Wei et al., 2023; Cai and Lin, 2022). This pathological change may be amplified by a positive feedback loop between glycolysis/H4K12la/ pyruvate kinase isozyme type M2 (PKM2). As a key enzyme in the glycolytic pathway, PKM2 can ensure that the cell's energy metabolism switches from oxidative phosphorylation to glycolysis. The H3K18la mainly mediates the pro-inflammatory activation of microglia through the NF-κB signaling pathway. Microglia activation can exacerbate neuronal functional damage by promoting the formation of NLRP3 inflammasomes and Apoptosis-Associated Speck-Like Protein Containing a Caspase Recruitment Domain (ASC) specks (Pan et al., 2022).

Microglia cells have significantly higher levels of H4K12la at the promoters of hypoxia-inducible factor-1α (HIF-1α), PKM2, and lactate dehydrogenase (LDH), which leads to increased expression of these glycolysis-related proteins and further upregulates H3K18 and H4K12 acetylation (Pan et al., 2022; Wei et al., 2023). While there was no significant difference in H4K12la between the transgenic AD model mice and wild-type mice in the affected areas of their brains, the research suggests that the metabolism of astrocytes and neurons, like microglia, is regulated by lactate (Pan et al., 2022; Barros et al., 2023). Previous studies have suggested that cognitive impairment may be linked to the expression levels and activities of key enzymes in glycolysis, such as LDH, as well as glucose transporter protein 1 (GLUT1) and GLUT3, which are the primary cellular channels for glucose uptake in neurons and astrocytes, respectively (Yang et al., 2024; Wu et al., 2023). It is, therefore, possible that the glucose uptake of astrocytes and neurons may affect brain function by regulating lactate production. Based on the evidence of crosstalk between microglia,

astrocytes, and neurons, the functions of starry glial and neuronal cells may also be influenced by protein acetylation, as in microglia (Mayorga-Weber et al., 2022; Mason, 2017). The lactate/H4K12la/PKM2 feedback loop in microglia might continuously promote the acetylation of histones in neurons and astrocytes by providing lactate. Furthermore, as GLUT1 on astrocytes transports more glucose into the cell, aerobic glycolysis gradually replaces mitochondrial oxidative phosphorylation as these cells' main glucose metabolic pathway. The glucose taken up by neurons via GLUT3 not only participates in oxidative energy supply but also is metabolized by the pentose phosphate pathway to eventually produce glutathione to maintain redox balance (Mayorga-Weber et al., 2022) (Figure 1).

Cerebral ischemia can induce the proliferation of astrocytes and stimulate their glycolytic metabolism (Lv et al., 2015), leading to the release of lactate, which can then enter neuron cells through monocarboxylic acid transporter (MCT) to cause an increase in neuronal activity (Barros et al., 2023; Yang et al., 2024; Wu et al., 2023). With the release of lactate from astrocytes, the negative feedback on glycolysis gradually weakens, allowing astrocytes to continuously produce lactate for energy transport to the neuron cells (Barros et al., 2023). Additionally, it has been demonstrated that neurons can uptake lactate released by microglia via MCT2 (Mayorga-Weber et al., 2022). Consequently, microglia and astrocytes may maintain neuron energy metabolism by continuously supplying lactate. This lactate may affect the physiological functions of neurons by promoting histone lactylation in hippocampal tissue (Nagy et al., 2018; Wei et al., 2023) (Figure 1).

MCT1-4 show cell-type-specific distribution. MCT1/4 is primarily located on the membranes of astrocytes, with MCT4 being unique to astrocytes. MCT2 is expressed exclusively by neurons, and microglia mainly rely on MCT1 for lactate transport (Yang et al., 2024; Jády et al., 2016). Lactate/pyruvate is transported between cells via MCTs. The imported pyruvate may enter the mitochondria and preferentially serve as a substrate for the pyruvate dehydrogenase system (PDHc) to provide energy for the cells, while the imported lactate is first oxidized by LDH to produce NADH, which then follows a specific shuttle mechanism to enter the mitochondria. When the shuttling of NADH is impeded, it may prevent the oxidation of lactate (Nagy et al., 2018) as a product of glycolysis; lactate/pyruvate can be used by the mitochondria for oxidative phosphorylation. Astrocytes can release lactate through MCT1/4, which may then enter neuronal cells via MCT2. Finally, the lactate enters the mitochondria through the MCT1/2 channels in the neuron's cytoplasm (Yang et al., 2024; Wu et al., 2023; Yang et al., 2022), promoting mitochondrial energy metabolism and reactive oxygen species (ROS) production. The mitochondria ROS (mtROS) can induce dysfunction of neuronal mitochondria and damage the structure and function of synaptic elements (Jia et al., 2021). Moreover, the uptake of dysfunctional mitochondria released from glial cells by neurons can also lead to abnormalities in neural discharge (Zhang et al., 2023). Thus, lactate metabolism and mitochondrial function in glial cells can affect neuronal discharge frequency. It has been shown that an increase in the number of mitochondria and a disturbance in their dynamics in the hippocampus can lead to excessive excitation of hippocampal neurons and prolonged epileptic duration in mice (Bebensee et al., 2017; Lee et al., 2022) (Figure 1).

Activated microglia can cause neuronal damage by releasing dysfunctional mitochondria, whereas astrocytes can support neuronal survival by transferring functional mitochondria to them (Jia et al., 2023). Astrocytes might regulate neuronal activity by providing "powerhouses" (mitochondria) and the energy substrate lactate, thereby affecting anxiety-like behavior and cognitive deficits caused by seizures (Wu et al., 2023; Jia et al., 2023). Neurons can take up glucose through GLUT3 and break it down via glycolysis to generate energy, but their reliance on astrocytes for energy metabolism may help them avoid apoptosis induced by high glycolytic rates (Wu et al., 2023). Thus, the regulation of neuronal activity by astrocytes is closely related to mitochondrial function and lactate metabolism. Mitochondria have been shown to transfer between neurons and glial cells, and exogenous mitochondria can cross the blood–brain barrier to reduce ROS release induced by epilepsy, thereby reducing hippocampal neuron loss and glial activation (Jia et al., 2023). Numerous studies have confirmed that mitochondrial dysfunction can lead to increased lactate production through enhanced anaerobic glycolysis (Barros et al., 2023; Yang et al., 2024; Wu et al., 2023), and the lactate may mediate the occurrence and development of epilepsy by promoting protein lactylation modifications (Figure 1).

2.3 The impact of lactate metabolism on neuronal cell death

Previous studies have suggested that neuronal loss associated with programmed death plays an important role in the development of epilepsy (Liang et al., 2023).

2.3.1 NETosis

Neuroinflammation is one of the main causes of neuron loss (Li et al., 2021). Neuroinflammation is characterized not only by the proliferation of glial cells but also by disruption of the blood–brain barrier and migration of peripheral immune cells into the brain. Neutrophils infiltrate damaged brain tissue immediately after ischemic injury, and they can sustain neuronal damage around the infarct by releasing neutrophil extracellular trapping networks (NETs) (Li et al., 2021; Bernis et al., 2023; Byun et al., 2023). NETosis is a form of suicidal death for neutrophils, characterized by the formation and release of NETs, which primarily consist of double-stranded DNA, histones, and granule proteins (Li et al., 2021). During chronic inflammatory damage in the central nervous system, inhibiting the activity of key enzymes in the glycolytic pathway can block the activation of NETosis by reducing lactate levels (Awasthi et al., 2019; Ye et al., 2022; Wang et al., 2018). NETosis may exacerbate neuroinflammation by releasing inflammatory factors to induce neuronal loss in brain tissues after ischemic infarction. The hypoxic environment created by ischemic stroke can induce the occurrence of glycolysis, leading to the production of large amounts of lactate. The lactate may trigger NETosis and contribute to the development of post-stroke epilepsy by enhancing the lactylation of histones in neutrophils that infiltrate the ischemic focus.

2.3.2 Cuproptosis

Recent research has indicated that neuronal cuproptosis may be an important factor in the initiation and progression of TLE (Yang et al., 2023). Cuproptosis is a mitochondrial proteotoxic stress-dependent mode of regulated cell death (RCD), where the accumulation of copper in cells is crucial (Chen et al., 2023). Ferredoxin 1 (FDX1), a key protein in cuproptosis, can trigger

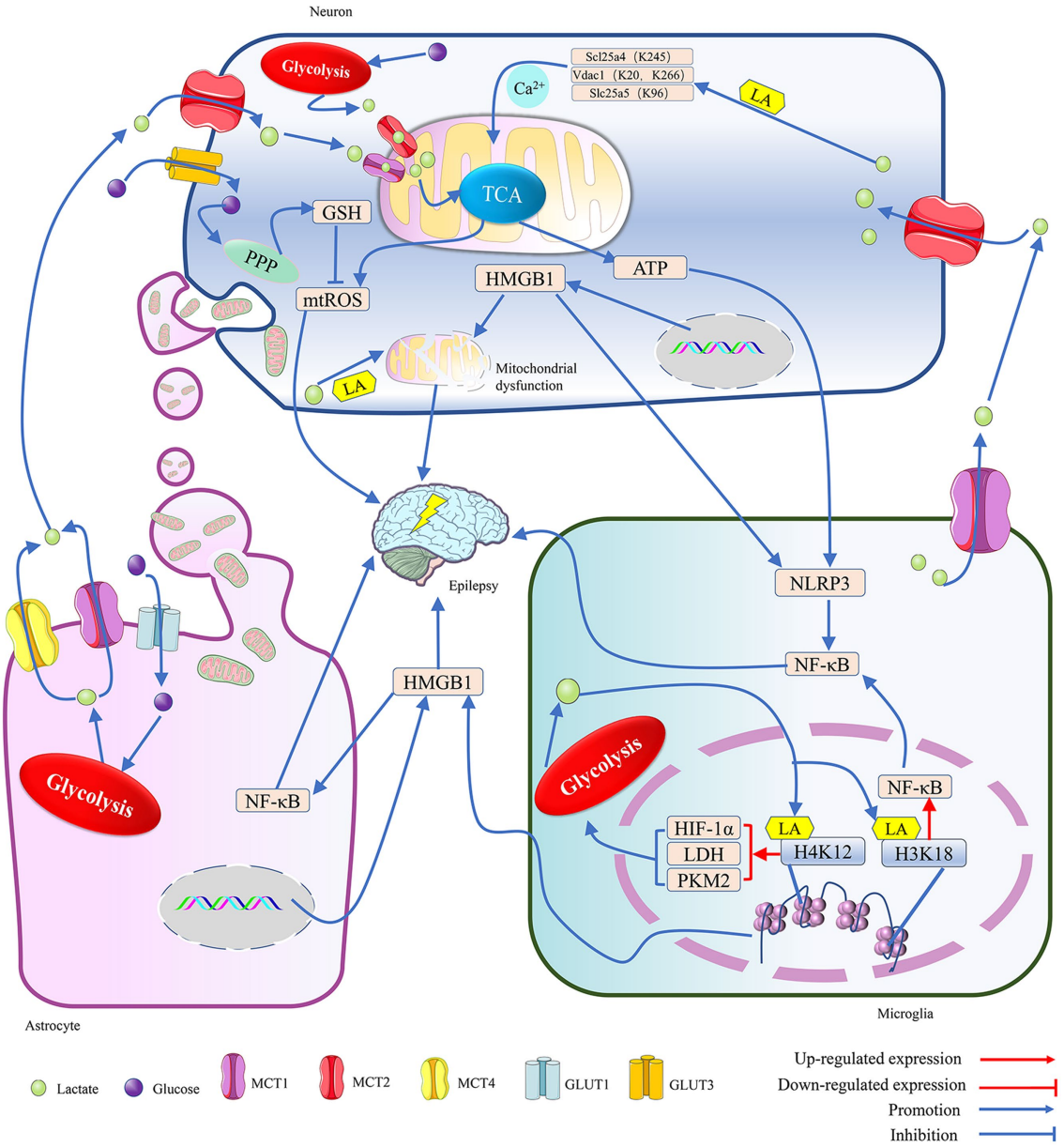

FIGURE 1
The metabolic crosstalk between neurons and glia involving lactate is implicated in epilepsy. 1. Both glucose and lactate can serve as energy substrates for neurons. Glucose is primarily taken up by neurons via GLUT3, while lactate, mainly provided by astrocytes and microglia, is mostly taken up by neurons via MCT2 during situations of intense stimulation of neural function. In a hypoxic environment, within neurons, the lactate generated from glucose through glycolysis and the lactate transferred into the cell by MCT2 are spread on the mitochondrial membrane by MCT1 and MCT2. There, they penetrate the TCA cycle, and the TCA contributes to the production of a significant amount of ATP and ROS through the promotion of oxidative phosphorylation. The ATP not only supports neuronal excitation but also activates the NLRP3/NF-κB pathway in microglia, leading to epilepsy. The mtROS may cause epilepsy by inducing oxidative stress in neurons, but PPP can reduce it by synthesizing glutathione, an antioxidant. Additionally, lactate can lead to epilepsy by enhancing the lactylation levels of proteins related to mitochondrial function, such as inducing $Ca2^{+}$ overload-mediated mitochondrial apoptosis by promoting lactylation of key proteins of the $Ca2^{+}$ signaling pathway, Scl25a4 (K245), Slc25a5 (K96) and Vdac1 (K20, K266). The transfer of HMGB1 from the nucleus to the cytoplasm can help coordinate homophilin lactylation, leading to epileptogenesis by mediating neuronal mitochondrial dysfunction. However, astrocytes maintain normal neuronal physiological activity and reduce epileptogenesis by providing healthy mitochondria to neurons. 2. In astrocytes, glucose taken up by the GLUT1 channel is converted into lactate, which is primarily exported by the MCT1/4 and used as an energy source by neurons. Both microglia and astrocytes can secrete HMGB1, which is a damage-associated molecular pattern and continues to induce the activation of glial cells and the inflammatory damage of neurons, leading to the occurrence of epilepsy. Meanwhile, HMGB1 secreted by microglia can mediate epileptogenesis through activation of the NF-κB pathway in astrocytes. 3. In a hypoxic environment, the lactate generated by glycolysis in microglia not only supports neuronal energy metabolism but also facilitates the lactylation of histones H3K18 and H4K12, which enhances the expression of NF-κB and glycolysis-related genes. NF-κB can trigger the onset of epilepsy, and the enzymes, such as LDH, HIF-1α, and PKM2, can further promote the production of lactate, which then helps in the lactylation of histones, ultimately intensifying the symptoms of epilepsy. Adenosine triphosphate (ATP); glucose transporter 1/3 (GLUT1/3); glutathione (GSH); high-mobility group box 1 (HMGB1); hypoxia-inducible factor 1 alpha (HIF-1α); K (Lysine); monocarboxylate transporter (MCT); mitochondria reactive oxygen species (mtROS); NLR family pyrin domain containing 3 (NLRP3); nuclear factor kappa-B (NF-κB); pentose phosphate pathway (PPP); pyruvate kinase isozymes M2 (PKM2); and tricarboxylic acid cycle (TCA).

mitochondrial protein lipidization, leading to mitochondrial protein toxicity stress. This form of stress is distinct from copper-induced mitochondrial oxidative stress and the iron-induced oxidative cell death associated with ferroptosis. As mitochondria are the primary targets in cuproptosis, the process includes the disintegration of the mitochondrial membrane and the functional loss of enzymes critical to the tricarboxylic acid cycle (Liu et al., 2022). When oxidative phosphorylation is inhibited, glycolysis is activated as the main energy source for cells (Barros et al., 2023). It seems that the occurrence of cuproptosis may help enhance the level of lactate. In turn, when aerobic glycolysis replaces the aerobic oxidation of sugars as the main energy source for cells, cuproptosis is likely to be suppressed (Xiong et al., 2023). It has been demonstrated that overexpression of FDX1 induces cuproptosis in tumor cells by catalyzing the lipoylation of PDH and α-ketoglutarate dehydrogenase (Schulz et al., 2023). However, HIF-1α can block FDX1-induced cuproptosis by indirectly inhibiting PDH (Chen et al., 2023). PDH promotes the entry of pyruvate into mitochondria and initiates the tricarboxylic acid cycle. Inhibition of PDH activity leads to mitochondrial energy depletion, which activates AMPK to promote cuproptosis (Xue et al., 2023). Copper overload may induce cuproptosis by mediating mitochondrial respiratory chain damage by over-activating the energy sensor AMPK. Cuproptosis can promote the release of the pro-inflammatory mediator High-Mobility Group Box 1 (HMGB1) (Liu et al., 2022; Xue et al., 2023) (Figure 2). It appears that cuproptosis-induced mitochondrial dysfunction may inhibit the exacerbation of cuproptosis by promoting the production of lactate, which has been shown to activate HIF-1α. HIF-1α may antagonize the effects of FDX1-mediated lipid acylation of PDH by inhibiting the activity of PDH to inhibit cuproptosis (Jiang et al., 2021).

2.3.3 Ferroptosis and disulfidptosis

Mitochondria, as the center of cellular energy metabolism, are not only the main target of cuproptosis but also the main site of iron utilization. The accumulation of excessive iron (Fe^{2+}) in mitochondria, which is a key factor in triggering ferroptosis, can lead to mitochondrial functional impairment, which exacerbates oxidative stress-induced cellular damage through the release of ROS, destructive lipids, mitochondrial DNA, and proteins (Moos et al., 2023). Ferroptosis is a form of programmed cell death caused by lipid peroxidation associated with energy metabolism (Zhao et al., 2020). The onset and progression of ferroptosis may involve disruption of mitochondrial function, which often induces the Warburg effect (Yang et al., 2023). Elevated lactate concentration in cells may regulate iron ion metabolism by promoting lactylation modification of proteins, thereby promoting cellular ferroptosis (Zhang et al., 2023). Nevertheless, exogenous lactate can assist cells in replenishing their energy resources by activating HCAR1 or MCT1, increasing ATP production. With the enhancement of ATP levels, the potency of AMPK is gradually reduced, resulting in the inhibition of ferroptosis or cuproptosis (Xue et al., 2023) (Figure 2). Mitochondrial dysfunction is not the sole factor inducing the Warburg effect in neurons. In HT22 cells, the activation of small-conductance calcium-activated K^+ (SK) channels may initiate aerobic glycolysis as the main energy source for the cell and reduce the generation of ROS during the process of mitochondrial energy metabolism to inhibit ROS-induced cellular oxidative stress, thereby preventing the cell from entering ferroptosis (Krabbendam et al., 2020).

With the continuous deepening of research on iron metabolism and ferroptosis in recent years, inhibiting ferroptosis in neuronal cells is considered a potential new strategy for treating epilepsy (Moos et al., 2023; Chen et al., 2020). It has been shown that the neuronal excitotoxicity inducer glutamate can promote ferroptosis by releasing mitochondrial ROS (Krabbendam et al., 2020). Cerebral infarction-induced cerebral ischemia and hypoxia promote the onset of glycolysis and lactate production in neuronal cells by upregulating the expression of LDHA (Yao and Li, 2023). Elevated lactate levels promote lactylation modification of lymphocyte cytoplasmic protein 1 in neuronal cells of Middle cerebral artery occlusion (MCAO) rats, thereby exacerbating cerebral infarction-induced neuronal loss, which is an important cause of post-stroke epilepsy (Zhang et al., 2023).

The neuronal solute carrier family 7 member 11 (SLC7A11)/GPX4 pathway may play a role in reducing the occurrence of epilepsy by inhibiting ferroptosis (Liang et al., 2023; Yuan et al., 2021). SLC7A11, a transport protein that facilitates the uptake of cystine and the efflux of glutamate, is instrumental in activating GPX4. This activation occurs through its role in cystine uptake, essential for the synthesis of glutathione, a key antioxidant that GPX4 uses to prevent lipid peroxidation and, consequently, ferroptosis in neuronal cells (Yang et al., 2023). However, the accumulation of disulfides, such as cystine, in the cell causes disulfide bond stress, which mediates the formation of disulfide bonds between actin cytoskeletal proteins and the breakdown of the actin filament (F-actin) network, ultimately leading to disulfidptosis (Liu et al., 2023). Therefore, the intake of cystine mediated by SLC7A11 can suppress neuronal ferroptosis while also triggering neuronal disulfidptosis. The efficiency of SLC7A11 in transporting cystine is affected by the level of glutamate in the cell. Although HIF-1α promotes the uptake of cysteine mediated by SLC7A11 by upregulating the expression of SLC1A1, lactate may not be involved in the resistance to ferroptosis driven by the glutamate metabolism of SLC7A11 (Yang et al., 2023).

2.3.4 Apoptosis

Recent research has shown that neuronal autophagy and apoptosis related to epilepsy are mediated by the mTOR signaling pathway (Liu et al., 2022), and these two forms of programmed cell death may exist in a complex interplay in the pathological changes leading to neuronal loss (Arab et al., 2023). Caspase-3 is one of the key effector molecules in executing cellular apoptosis. Activated caspase-3 and DNA-binding protein HMGB1 can rapidly transfer to mitochondria and degrade mitochondrial proteins, thereby mediating the loss of hippocampal CA1 and GABAergic interneurons to maintain sustained epilepsy (Kim et al., 2021). It is evident that HMGB1, like caspase-3, can mediate neuronal apoptosis-associated epilepsy by affecting mitochondrial structure and function. Recent studies have suggested that HMGB1 activation is regulated by lactylation modifications and that this process is closely linked to cellular lactate production (Yao and Li, 2023). Lactate promotes mitochondrial hyperfission by mediating an increase in lactylation of the mitochondrial Fission 1 protein (Fis1) lysine 20 (Fis1 K20la), which can contribute to mitochondrial hyperfission by inducing ATP depletion, mitochondrial ROS overproduction, and mitochondrial damage-mediated apoptosis. Conversely, activation of PDH downregulates lactylation of Fis1 K20 by decreasing the level of lactate produced by glycolysis, thereby alleviating the apoptosis-inducing effect of mitochondrial damage (An et al., 2023). Meanwhile, inhibiting H3 histone lactylation at H3K9

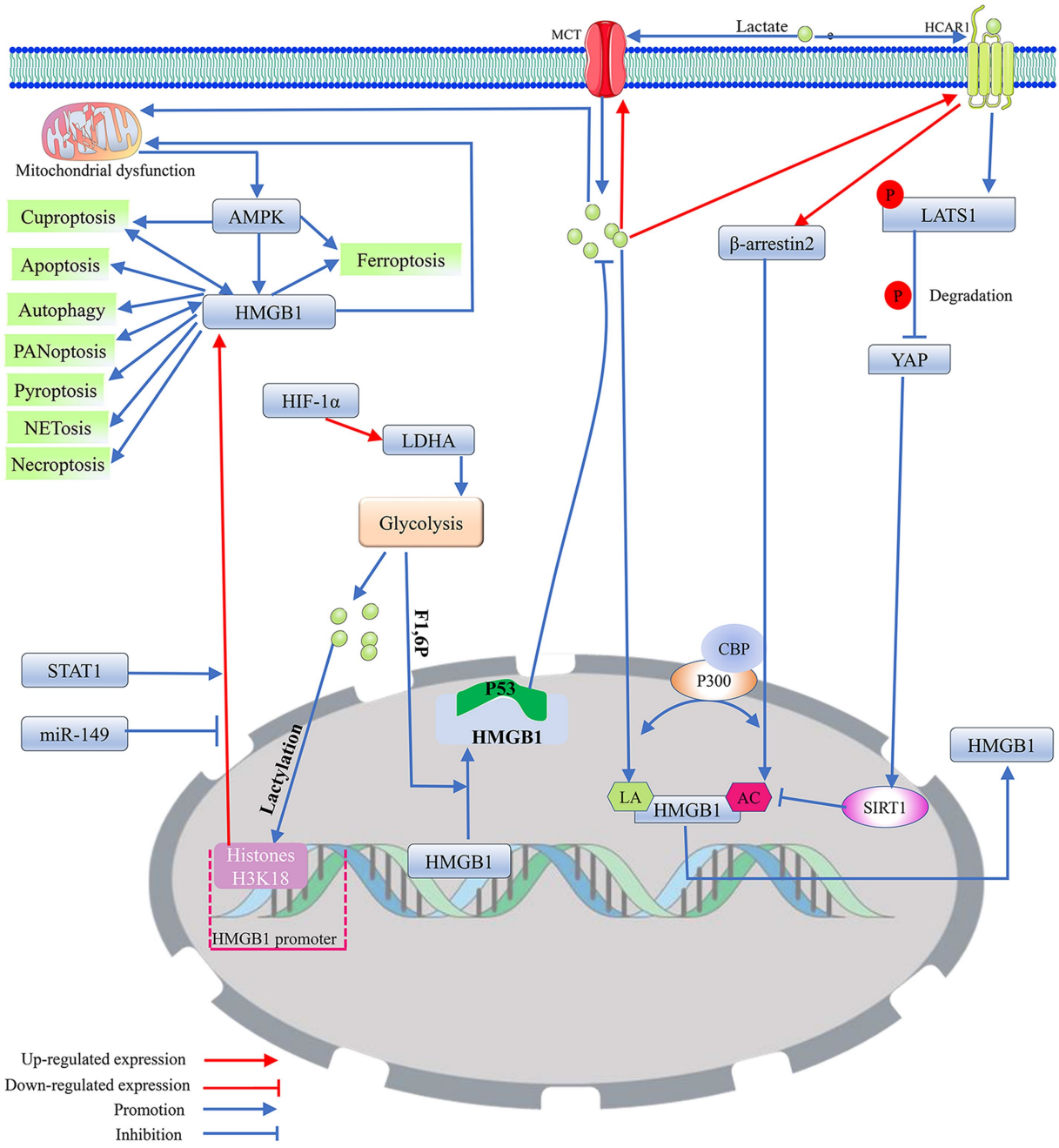

FIGURE 2
The potential regulatory mechanism of lactate on the biological activity of HMGB1. 1. As a DNA-binding protein, HMGB1 is usually located within the cell nucleus, but when it separates from the DNA strand and migrates out of the cell nucleus, its properties that enhance inflammation and immune responses are activated. Mitochondrial dysfunction promotes the nuclear translocation of HMGB1 through the activation of AMPK, thereby inducing HMGB1 mediated programmed cell death. Among them, cuproptosis and PANoptosis can further promote the nuclear translocation of HMGB1. In addition, miR-149 and STAT1 can affect HMGB1-mediated programmed cell death and mitochondrial dysfunction by regulating the expression level of HMGB1. 2. Exogenous lactate can upregulate β-arresin2 expression by binding to HCAR1, thereby promoting P300/CBP recruitment to the nucleus. P300/CBP promotes HMGB1 secretion and activation by catalyzing HMGB1 lactylation/acetylation. Meanwhile, the binding of exogenous lactate to HCAR1 can upregulate the level of LATS1 phosphorylation, which in turn promotes the phosphorylation and degradation of YAP. The degradation of YAP could promote the acetylation of HMGB1 by reducing SIRT1 activity. In addition, exogenous lactate can be taken up by cells via MCT, thereby mediating mitochondrial dysfunction by promoting protein lactylation, and mitochondrial dysfunction can promote HMGB1 activation. Elevated intracellular lactate levels upregulate the expression of MCT1 and HCAR1. 3. Transcription factor HIF-1α can promote glycolysis by enhancing the expression of LDHA. The lactate produced by glycolysis can not only directly promote HMGB1 lactylation but also enhance the expression of HMGB1 by upregulating the lactylation level of histone H3K18 in its promoter region. F1,6P, as an intermediate product of glycolysis, can bind to the K43/K44 sites of the HMGB1 protein to promote the separation of HMGB1 from DNA in the cell nucleus. The free form of HMGB1 can block P53 degradation by binding to the oncogenic factor P53, thereby reducing the production of lactate. Acetylation (AC); adenosine 5′-monophosphate-activated protein kinase (AMPK); CREB-binding protein (CBP); fructose-1,6-bisphosphate (F1,6P); hypoxia-inducible factor 1 (HIF-1); high-mobility group box 1 (HMGB1); hydroxy-carboxylic acid receptor 1 (HCAR1); lactylation (LA); large tumor suppressor kinase 1 (LATS1); lactate dehydrogenase A (LDHA); monocarboxylate transporter (MCT); signal transducer and activator of transcription 1 (STAT1); silencing regulatory protein 1 (SIRT1); and yes-associated protein (YAP).

and H3K14 sites can induce apoptosis (Xu et al., 2023). It follows that lactate may mediate mitochondrial function-related apoptosis by regulating lactylation modifications of histones and non-histone proteins.

2.3.5 Pyroptosis

Pyroptosis, as an inflammatory mode of programmed cell death, can mediate the expression and release of caspase/Gasdermin D (GSDMD)-associated inflammatory factors through the activation of inflammasomes (Xia et al., 2021). The Nucleotide-binding oligomerization domain-like receptor family card domain-containing protein (NLRP3) inflammasome is an important activator of pyroptosis related to epilepsy, and it has been demonstrated that STAT3 can promote the expression of NLRP3 by catalyzing the acetylation of histone H3K9 on the NLRP3 promoter, thereby activating the NLRP3/caspase-1 pathway and exacerbating the damage to neurons in epileptic mice (Jiang et al., 2021). Epilepsy-related neuronal pyroptosis is initiated by the accumulation of ROS caused by mitochondrial impairment in neurons (Xu et al., 2023). Mitochondrial dysfunction often forces cells to obtain energy through glycolysis. A study on brain ischemia found that lactate upregulates HMGB1 expression by promoting histone lactylation of the HMGB1 promoter, which may lead to neuronal pyroptosis (Yao and Li, 2023). It is closely related to the activation of the NLRP3 inflammasome by HMGB1 (Liu et al., 2022). Thus, lactate may affect the occurrence of epilepsy by regulating neuronal apoptosis.

2.3.6 Autophagy

Autophagy is a process by which cells degrade their own excess or aging organelles and misfolded proteins (Yang et al., 2020). Lactate and pyruvate can activate mitochondrial autophagy and autophagy in primary neurons and astrocytes by lowering intracellular hydrogen ion concentration at non-toxic concentrations, which facilitates the restoration of mitochondrial function for the protection of these cells from apoptosis and necrosis (Fedotova et al., 2021). It has been shown that a lack of neuronal autophagy contributes to epilepsy and that seizures may be further exacerbated by triggering autophagy dysfunction, thus creating a vicious cycle (Ali et al., 2023). Lactate not only protects neurons by regulating mitochondrial autophagy within the cells but also maintains neuronal activity by regulating mitochondrial autophagy in glial cells (Zhu et al., 2022). Moreover, lactate may further promote cytoplasmic acidification by inducing a shift in cellular energy metabolism from oxidative phosphorylation to glycolysis, thereby sustaining mitochondrial autophagy (Komilova et al., 2021). With the exacerbation of neuronal autophagy, the autophagy articulation protein Sequestosome 1 is heavily depleted, which can inhibit the activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mTOR pathway. Decreased mTOR activity impedes the downregulation of the transcription factor Hif-1α, resulting in increased expression of key glycolytic enzymes LDHA and hexokinase-2 (HK2) (Song et al., 2021). In addition, SIRT1, located in the nucleus and cytoplasm, directly or indirectly promotes the formation of autophagic vesicles and improves mitochondrial function, thus blocking epileptogenesis and development (Ali et al., 2023). Because both SIRT1 and lactate can mediate autophagy in neurons to control epileptogenesis, it is likely that there is an association between SIRT1 and lactate metabolism. A recent study has confirmed that lactate can improve long-term cognitive impairment in newborns repeatedly exposed to sevoflurane by activating SIRT1-mediated hippocampal neurogenesis and synaptic remodeling (Qiu et al., 2023).

2.3.7 Necroptosis

Necroptosis, which is a novel pattern of cell death associated with inflammation, is considered one of the complex mechanisms of neuronal death after status epilepticus (Roh et al., 2023). The accumulation of ROS due to mitochondrial damage can promote necroptosis of neurons in the hippocampus of acute epilepsy patients through activation of the RIPK1/RIPK3/MLKL pathway, and mitochondrial enzyme SIRT3 and the indole-derived small molecule NecroX-7 can alleviate seizures by suppressing the production of mitochondrial ROS (Roh et al., 2023; Song et al., 2021). This shows that mitochondria are important organelles that mediate epilepsy-related neuronal necroptosis. The glycolytic product lactate may regulate the activity of HIF-1α to block the release of mitochondrial ROS mediated by PDH, thereby regulating the necroptosis induced by RIPK (Chen et al., 2023; Jiang et al., 2021; Icard et al., 2023; Wong Fok Lung et al., 2020; Weindel et al., 2022; Luo et al., 2022). Furthermore, necroptosis is activated in hippocampal astrocytes and microglia to mediate neuroinflammation 4 h after status epilepticus induction (Wu et al., 2021).

2.3.8 PANoptosis

PANoptosis, which is an inflammatory programmed cell death driven by PANoptosome, has key features of pyroptosis, ferroptosis, apoptosis, and necroptosis (Lin et al., 2022; González-Rodríguez and Fernández-López, 2023). PANoptosis may be present in brain tissue I/R and is a new way of causing neuronal loss (González-Rodríguez and Fernández-López, 2023). Mitochondrial dysfunction likely affects PANoptosis of neurons, as it does most programmed forms of cell death (She et al., 2023). OGD/R induces PANoptosis of neurons by inducing mitochondrial fission and dysfunction (Zeng et al., 2023). It may be related to the hypoxia-induced enhancement of glycolytic metabolism, as several enzymes involved in glycolytic metabolism have been shown to directly regulate mitochondrial functions (Liu et al., 2023). Thus, mitochondrial oxidative stress and leakage of mitochondrial contents induced by multiple factors are important triggers of PANoptosis (Zeng et al., 2023; Liu et al., 2023; Bi et al., 2022). Numerous studies have demonstrated that the structural proteins of the PANoptosome are able to induce epilepsy-associated neuronal loss by mediating crosstalk in the neuronal programmed death pathway (Xia et al., 2023; Shi et al., 2023). PANoptosis may possess characteristics of various programmed cell deaths, including but not limited to pyroptosis, apoptosis, and necroptosis (Lin et al., 2022; González-Rodríguez and Fernández-López, 2023). Although it cannot currently be confirmed that PANoptosis includes cuproptosis, the evidence suggesting that (Fe-S) cluster damage can lead to cuproptosis, ferroptosis, and PANoptosis implies that these three programmed cell death pathways may be closely related to (Fe-S) cluster-mediated mitochondrial dysfunction (Chen et al., 2023; Lin et al., 2022). Therefore, PANoptosis, which possesses characteristics of all these programmed cell death types, may also be regulated by lactate metabolism, such as pyroptosis, apoptosis, and necroptosis.

2.4 Mitochondrial dysfunction intervention in the context of epilepsy-related neuronal programmed death

Accumulating evidence indicates that the occurrence and development of epilepsy are closely related to mitochondrial dysfunction (Moos et al., 2023). Damaged mitochondria can mediate many forms of neuronal programmed cell death by releasing mitochondrial contents, such as ROS, mitochondrial DNA, and mitochondria-associated proteins, leading to epilepsy-associated neuronal cell loss (Jia et al., 2023). Mitochondria can play an anticonvulsant and anti-neuroinflammatory role by synthesizing D-fructose 1,6-diphosphate, a glycolysis intermediate that inhibits sugar metabolism (Jia et al., 2023). It has been demonstrated that artificial mitochondrial transplantation may inhibit the loss of hippocampal neurons and the activation of glial cells by ameliorating the metabolic imbalance induced by mitochondrial dysfunction, thereby reducing hippocampal damage after seizures and ameliorating epilepsy-associated psychiatric and cognitive disorders (Jia et al., 2023). Therapeutic interventions aimed at improving mitochondrial function in the central nervous system may effectively alleviate neurological damage and cognitive impairments related to epilepsy. A recent study has demonstrated that honokiol attenuates mitochondrial dysfunction and its associated oxidative stress by regulating mitochondrial ROS homeostasis and that it may stabilize mitochondrial function through activation of mitochondrial DNA transcription mediated by the glutamate receptor N-methyl-D-aspartate receptor/AMPK/peroxisome proliferator-activated receptor γ-coactivator-1α/SIRT3 pathway (Wang et al., 2022). Overexpression of SIRT3 in neuronal cells is protective against ischemia–reperfusion injury in the mouse spinal cord (Gu et al., 2023). Meanwhile, the ketogenic diet-induced overexpression of SIRT1 can maintain the normal physiological function of neurons by promoting neuronal autophagy to eliminate protein aggregates and damaged mitochondria (Dyńka et al., 2022).

Brain injury and energy deficiency promote the transport of energy substrates, such as ketones, lactate, and acetoacetate, into cells by upregulating the number of MCT channels on the neuronal membrane, thereby ensuring normal neuronal function. Astrocytes may reduce glucose availability and frequency of epileptic seizures by producing ketones and shuttling them via MCT channels to neurons (Dyńka et al., 2022). However, some other studies suggest that transporting these energy substances and mitochondria may increase neuronal excitability, thereby triggering epilepsy. The energy substances and mitochondria transferred from glial cells to neurons may lead to neuronal excitation, an important factor in inducing epilepsy (Nagy et al., 2018; Wei et al., 2023; Jia et al., 2023). The increased synthesis of lactate caused by cerebral ischemia promotes mitochondrial apoptotic pathway-mediated neuronal death, which may induce the occurrence of post-stroke epilepsy by upregulating the lactylation levels of key proteins in the calcium signaling pathway, such as Scl25a4 (K245), Slc25a5 (K96), and Vdac1 (K20, K266) (Yao et al., 2023). However, when lactate and ketones replace glucose as the main energy source for neurons, glucose can enter the pentose phosphate pathway and produce a large amount of nicotinamide adenine dinucleotide phosphate, which can offset the oxidative effect of ROS released during the mitochondrial utilization of lactate for production (Mayorga-Weber et al., 2022). During status epilepticus, HMGB1, a non-histone DNA-binding protein, can be transferred from the cell nucleus to the mitochondria, thereby disrupting the energy metabolism balance of the mitochondria (Kim et al., 2021). HMGB1 in microglia can promote the release of inflammatory factors such as IL-1β and IL-18 by activating the NLRP3/NF-κB/MAPKs pathway, leading to the activation of the NLRP3 inflammasome in hippocampal neurons, which can subsequently lead to pyroptosis and apoptosis (Liu et al., 2022; Shen et al., 2018). The neuronal injury promotes the release of neurally mediated ATP, which reactivates the NLRP3/NF-κB/MAPKs pathway via binding to the purinergic receptor P2X ligand-gated ion channel 7 in microglia without HMGB1. HMGB1 may be a central element in the tandem of neuronal death and glial cell activation in the hippocampus, and HMGB1 can amplify the effects of neuroinflammatory injury by promoting the interaction between neuronal death and glial cell activation (Liu et al., 2022; Shen et al., 2018) (Figure 1).

2.5 Regulation of neuronal programmed death by HMGB1

2.5.1 HMGB1 and mitochondria

HMGB1 nucleus-to-cytoplasm translocation has been shown to maintain status epilepticus and drug-resistant temporal lobe epilepsy by mediating oxidative stress in brain tissue in neurons and glial cells (Pauletti et al., 2019). The function and activity of HMGB1 are determined not only by its subcellular localization but also by its oxidation–reduction status and post-translational modifications (Chen et al., 2023). Mitochondrial damage-mediated oxidative modification of HMGB1 promotes cytoplasmic accumulation and extracellular secretion of HMGB1. HMGB1 secreted into the extracellular compartment initiates the expression of NF-κB-mediated inflammatory factors (e.g., TNF-α and IL-1β) through activation of Toll-like receptor 4 (TLR4) on the cell surface, ultimately leading to inflammatory tissue damage (Zhao et al., 2023). Brain tissue ischemia induces HMGB1 translocation from the cell nucleus to the mitochondria and extracellular region in neurons and astrocytes, leading to neuronal apoptosis associated with epileptic status (Wang et al., 2020; Dai et al., 2023). During this process, the transposition of HMGB1 triggers the destruction of mitochondria, and the released contents of mitochondrial lysis further intensify HMGB1-mediated neuroinflammatory damage (Wang et al., 2020; Dai et al., 2023; Zhang et al., 2020) (Figure 2).

2.5.2 HMGB1 and various types of programmed cell death

Mitochondrial dysfunction can induce epileptogenesis by mediating multiple programmed cell deaths in neurons, and HMGB1 may intervene in epilepsy-associated multiple programmed cell deaths by modulating mitochondrial function. It has been demonstrated that HMGB1 released into the extracellular space can act as a damage-associated molecular pattern protein to mediate a variety of cellular programmed deaths (Chen et al., 2023; Tang et al., 2023). HMGB1, which is secreted extracellularly, may drive the disease process in epilepsy by inducing neuroinflammation and neuronal loss during the silent phase following the initial triggering event (Rosciszewski et al., 2019). HMGB1 transferred from the nucleus to the cytoplasm may activate the Myeloid differentiation factor 88 (MyD88)/NF-κB axis by

binding to TLR4. The MyD88/NF-κB axis can induce neurological deficits by promoting neuronal apoptosis, autophagy, and the release of inflammatory factors, such as IL-1β and TNF-α (Lei et al., 2022; Fu et al., 2023; Shang et al., 2020) (Figure 3). STAT1 may upregulate the expression level of HMGB1 by binding to the HMGB1 promoter, thereby affecting the biological effects mediated by the TLR4/MyD88/NF-κB pathway (Fu et al., 2023). In the MCAO model, impaired neurons induce NETosis by releasing large amounts of HMGB1, which induces NETosis and can even be extruded as part of the NET. The extruded HMGB1 may further induce NETosis and platelet activation, which is one of the important reasons for the elevation of HMGB1, thereby exacerbating neuronal inflammatory damage (Kim and Lee, 2020). miR-149 may reduce the occurrence of epilepsy by inhibiting HMGB1-mediated neuroinflammatory injury (Kwak et al., 2023; Parsons et al., 2022; Wang et al., 2022). Cuproptosis and PANoptosis may further induce the release of inflammatory factors and programmed cell death by promoting the release of HMGB1 (Liu et al., 2022; Kwak et al., 2023). In addition, ATP depletion and calcium ions can upregulate the level of HMGB1 phosphorylation through activation of AMPK, which can promote the translocation and activation of HMGB1 (Liu et al., 2022). It cannot be excluded that HMGB1 may induce various forms of programmed death in neurons by regulating mitochondrial function (Figure 2).

Neuroglia may be involved in HMGB1-mediated neuronal programmed cell death (Rosciszewski et al., 2019). HMGB1 induces NF-κB pathway activation in glial cells by activating signaling pathways involving TLR2, TLR4, and the receptor for advanced glycation end products (RAGE). Since activation of the NLRP3/NF-κB/MAPKs pathway in microglia is an important mechanism for HMGB1-induced hippocampal neuronal pyroptosis and apoptosis (Liu et al., 2022; Shen et al., 2018), the limited activation of NF-κB in astrocytes can lead to partial suppression of neuron loss in the absence of microglia, thereby producing a beneficial effect on controlling the occurrence of epileptic seizures (Cai and Lin, 2022; Rosciszewski et al., 2019). The damaged neurons can further promote the activation of microglia by secreting HMGB1 (Kitaoka et al., 2023). HMGB1 secreted by neurons can also directly act on astrocytes, thereby activating NF-κB p65-mediated inflammatory cascade effects (Kaya et al., 2023). It can be speculated that the intervention of glial cells may exacerbate the neuronal death effect induced by HMGB1.

2.5.3 The impact of lactate metabolism on the function of HMGB1

Research has shown that the increased protein lactylation level of hippocampal neurons is accompanied by upregulation of the HMGB1 expression level (Xie et al., 2023). Extracellular lactate can regulate cell death and immune cell polarization by binding to HCAR1 and transport protein MCT on the membrane (Zhai et al., 2022). Lactate primarily relies on the acetylase P300/CBP to introduce the lactide moiety into the lysine residue of HMGB1, thus directly participating in the lactylation of HMGB1. Meanwhile, exogenous lactate specifically increases the expression of β-arrestin2 by binding to HCAR1, which can catalyze the lactylation/acetylation of HMGB1 by facilitating the recruitment of P300/CBP to the nucleus, thereby mediating the secretion of HMGB1. Exogenous lactate promotes the phosphorylation and degradation of Yes-Associated Protein (YAP) by binding to HCAR. YAP can preserve SIRT1 expression and activation, and lactate-mediated degradation of YAP promotes HMGB1 acetylation by reducing SIRT1 activity. Lactate can induce the lactylation of HMGB1 in macrophages and promote its release from these cells (Yang et al., 2022). Fructose-1,6-bisphosphate, an intermediate product of glycolysis, serves as a novel HMGB1 ligand that promotes the segregation of HMGB1 from DNA in the nucleus by directly binding to K43/K44 on the HMGB1 protein. The free HMGB1 prefers to bind to P53 to form the HMGB1-P53 complex, which effectively prevents the degradation of P53, thus blocking glycolysis and removing damaged mitochondria (He et al., 2023; Li et al., 2023). Based on numerous reports that HMGB1 is an important gene in the regulation of glucose metabolism and mitochondria can regulate the activation of HMGB1, it is suggested that HMGB1 may be an important signaling factor related to mitochondrial dysfunction associated with protein lactylation modification-induced cell death (Li et al., 2023; Du et al., 2022; Liu et al., 2022) (Figure 2).

3 Regulating the level of protein lactylation may be a measure to intervene in the occurrence and development of epilepsy

Numerous studies have confirmed that histone acetylation is involved in regulating various cellular physiological activities (Table 1). Mitochondrial dysfunction often promotes the preferential catabolism of glucose by aerobic glycolysis to cause the accumulation of lactate, which can regulate cellular gene expression in the form of epigenetic modifications through regulating histone lactylation (He et al., 2023). Indeed, there exists a mutually promoting relationship between protein lactylation and mitochondrial dysfunction; mitochondrial dysfunction is a core element in the pathogenesis of epilepsy (Moos et al., 2023), and the disease process may be affected by protein lactylation.

3.1 Mechanisms involved in influencing the level of protein lactylation

Since lactate can be catalyzed to produce lactyl coenzyme A, which has been confirmed as a direct precursor of the lactyl group, factors that affect lactate synthesis and transport may be involved in the regulation of protein lactylation modification (Yang et al., 2022; Dong et al., 2022) (Figure 4).

3.1.1 The impact of proteins related to glucose metabolism on lactate production

Lactate is mainly derived from cellular glycolytic metabolism, and GLUT transfers glucose from the extracellular environment into the cell, thus providing the raw material for lactate production (Fan et al., 2023). HK2 catalyzes the conversion of glucose to glucose-6-phosphate, thereby driving the process of generating lactate (Rho et al., 2023). However, HK1 has been shown to be downregulated by lactate expression, thereby inhibiting the glycolytic pathway (Jiang et al., 2021). Phosphofructokinase-1 and phosphoglycerate kinase 1 (PGK1) progressively convert glucose-6-phosphoglycerate to 3-phosphoglycerate. Fructose-2,6-bisphosphatase 3 is a constitutive activator of phosphofructokinase-1, a key enzyme in glycolysis, and downregulation of fructose-2,6-bisphosphatase 3 and phosphofructokinase-1 expression in astrocytes reduces the production of lactate (Lv et al., 2015).

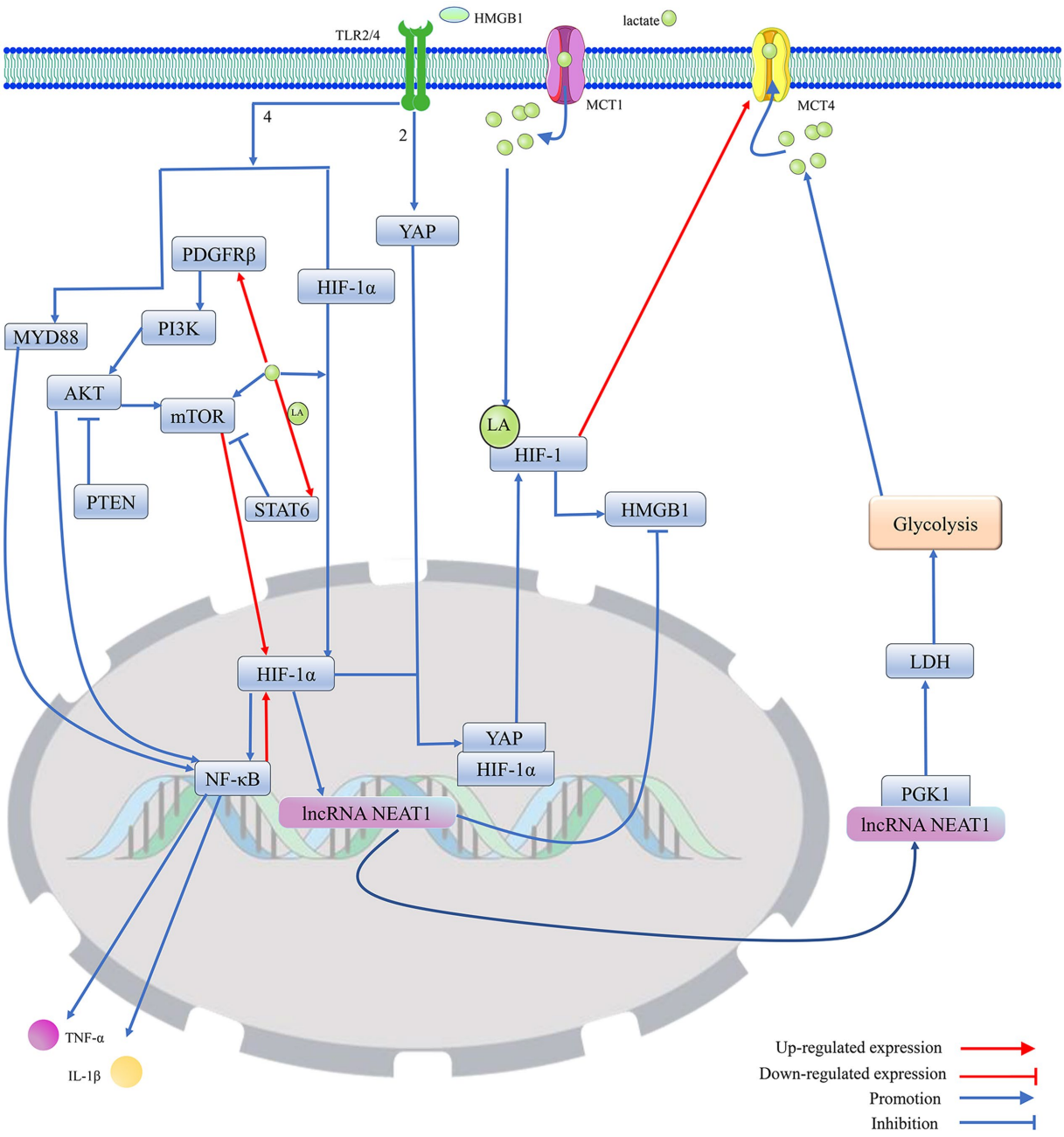

FIGURE 3
Protein lactonylation modification is an important influence in the process that is HIF-1-mediated HMGB1 activity. 1. Lactate, as a metabolite of cellular adaptation to hypoxic environments, is mainly produced by glycolysis. MCT1 and MCT4 are mainly responsible for the cellular uptake and release of lactate. Elevated intracellular lactate levels not only promote the translocation of HIF-1α from the cytoplasm to the nucleus to regulate gene expression but also stabilize the structure and enhance the biological activity of HIF-1 by promoting HIF-1 lactylation. Furthermore, lactate can promote the expression of HIF-1α by directly or indirectly enhancing the activation of mTOR. However, lactate can upregulate STAT6 expression by promoting histone lactylation, thereby blocking PDGFRβ/PI3K/AKT/mTOR-mediated HIF-1 expression. 2. Activated HIF-1 promotes the secretion and activation of HMGB1, which is secreted into the extracellular compartment and promotes the migration of YAP from the cytoplasm to the nucleus by binding to TLR2, thereby promoting the activation of HIF-1. Meanwhile, HMGB1, by binding to TLR4, not only directly promotes the migration of HIF-1 to the nucleus to exert transcriptional regulation but also activates the MYD88/NF-κB pathway to enhance the expression of HIF-1. However, HIF-1α can reduce the expression of HMGB1 by upregulating the transcript level of lncRNA NEAT1, thereby blocking the excessive activation of HIF-1 induced by HMGB1. The lncRNA NEAT1 can stabilize the structure of PGK1 by binding to PGK1, which mediates the glycolysis/lactate/HIF-1α pathway by increasing LDH activity, but HIF-1 can promote lactate efflux by upregulating the expression of MCT4, which blocks the excessive activation of HIF-1 induced by lactate. Furthermore, PTEN could block HIF-1 expression, which is mediated by the PDGFRβ/PI3K/AKT/NF-κB pathway, by inhibiting AKT, thereby reducing HMGB1-induced release of cellular inflammatory factors. Protein kinase B (AKT); hypoxia-inducible factor 1 (HIF-1); high-mobility group box. 1 (HMGB1); interleukin 1β (IL-1β); lactylation (LA); lactate dehydrogenase (LDH); monocarboxylate transporter (MCT); mechanistic target of rapamycin (mTOR); myeloid differentiation factor 88 (MYD88); nuclear enriched transcript 1 (NEAT1); platelet-derived growth factor receptor β (PDGFRβ); phosphoglycerate kinase 1 (PGK1); phosphatidylinositol 3-kinase (PI3K); phosphatase and tensin homolog (PTEN); signal transducer and activator of transcription 6 (STAT6); toll-like receptor (TLR); tumor necrosis factor-α (TNF-α); and yes-associated protein (YAP).

TABLE 1 The common sites of histone lactylation.

Associated diseases	Subjects	Sites	Lactylation-mediated biological effects	References
Prostate cancer	Prostate cancer cells	H3K18	It may trigger an adenocarcinoma-to-neuroendocrine transition.	He et al. (2023)
Ocular melanoma	Ocular melanoma cell lines (OCM1 and OM431)	H3K18	It can reduce the expression levels of Period Circadian Protein 1 and P53 in ocular melanoma cells by promoting the transcription of YTH Domain Family Protein 2, which can promote cell proliferation and migration.	Yu et al. (2021)
Colon cancer	Tumor-infiltrating myeloid cells	H3K18	It can activate the Janus Kinase 1-STAT3 signaling pathway by upregulating the expression of methyltransferase-like 3 (METTL3) in Tumor-infiltrating myeloid cells for promoting tumor growth.	Xiong et al. (2022)
Ulcerative Colitis	Bone marrow-derived macrophages (BMDMs)	H3K18	It can suppress M1 macrophage polarization and macrophage pyroptosis.	Sun et al. (2021)
Developmental abnormalities of the nervous system	Neural stem/progenitor cells (NSPCs)isolated from E13.5 mice forebrain 2. P19 embryonic carcinoma (EC) cells	H3K18	It can encourage P19 EC cell-derived NSPC to exit the cell cycle and differentiate into neurons.	Dai et al. (2022)
Malignant pleural effusion (MPE)	Human Natural killer T-like cells	H3K18	Upregulation of the H3K18la level in the promoter region of the FOXP3 gene can reduce the anti-tumor function of Natural killer T-like cells by promoting FOXP3 expression.	Wang et al. (2023)
Intestinal inflammation	Th17 cells	H3K18	It may promote Foxp3 expression through upregulation of ROS-driven IL-2 secretion, which stimulates the pro-inflammatory effects of macrophages.	Lopez Krol et al. (2022)
Sepsis	THP1 cells	H3K18	It can drive M2 polarization of Macrophages by increasing the expression of M2 markers (such as Arg1).	Ma et al. (2022)
pulmonary hypertension	Pulmonary artery smooth muscle cells	H3K18	It can promote cell proliferation and vascular remodeling.	Chen et al. (2023)
Uveal melanoma (UM)	Human uveal melanoma cells (92.1)	H3K18	It may inhibit tumor cell growth by promoting the expression of oxidative phosphorylation-related genes and cell growth quiescent markers.	Longhitano et al. (2022)
Clear cell renal cell carcinoma (ccRCC)	Immortalized renal epithelial cells, Von Hippel–Lindau inactive RCC cell lines, and RCC cell lines with endogenous Von Hippel–Lindau	H3K18	It can promote the proliferation and migration ability of ccRCC cells.	Yang et al. (2022)
Cerebral ischemia	N2a cells	H3K18	It can promote N2a cell scorching by upregulating the HMGB1 level.	Yao and Li (2023)
Alzheimer's disease	1. BV2 cells 2. Hippocampus tissue of AD model mice	H3K18	It can mediate neuroinflammation through direct stimulation of the NF-κB pathway to cause neurodegenerative aging-related disorder characterized by progressive cognitive impairment.	Wei et al. (2023)
Liver cancer	Liver cancer stem cells	H3K9 and H3K56	It can promote tumorigenesis.	Pan et al. (2022)
	The human HCC cells	H3K9 and H3K14	It can block apoptosis in tumor cells.	Xu et al. (2023)
Non-Small Cell Lung Cancer	NSCLC cell lines A549 and H1299	H4K8	It can inhibit the proliferation and migration of NSCLC cells by upregulating the expression of IDH3G and downregulating the expression of HK-1.	Jiang et al. (2021)
Alzheimer's disease (AD)	Microglia from 5XFAD mice	H4K12	It can stimulate microglial pro-inflammatory activation by promoting the expression of glycolytic genes.	Pan et al. (2022)

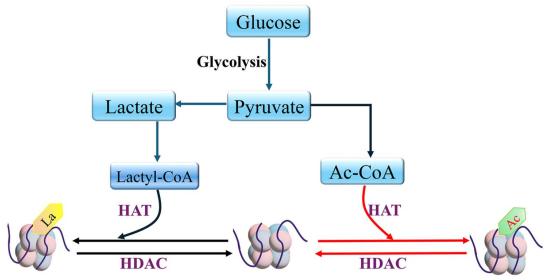

FIGURE 4
HDAC and HAT mediate histone lactylation and histone acetylation Lactyl coenzyme A (Lactyl-CoA); Acetyl-CoA (Ac-CoA).

Knockdown of PGK1 inhibits the M1 polarization of microglia, thereby preventing the alleviation of neuroinflammation by disrupting glycolytic metabolism in the MCAO rat model (Cao et al., 2023). The enzyme 3-phosphoglycerate is transformed into pyruvate by pyruvate kinase, which has two structural isoforms: PKM1 and PKM2. PKM1 consistently functions as a high-activity tetramer, while PKM2 can exist either as a low-activity dimer or a high-activity tetramer. The low-activity dimer form of PKM2 promotes the conversion of pyruvate to lactate, playing a significant role in driving glycolysis. In contrast, the high-activity forms of both PKM2 and PKM1 facilitate the tricarboxylic acid cycle. A recent study has shown that an increase in the expression of PKM2 and a higher PKM2/PKM1 ratio promote glycolysis (Zhang et al., 2017). In the nervous system, the knockout of PKM2 can result in the compensatory overexpression of PKM1, which may promote the transformation of the energy metabolism of microglial cells from glycolysis to oxidative phosphorylation (Pan et al., 2022). PDH and LDH are key enzymes in determining the fate of pyruvate, an intermediate product of glycolysis, and PDH promotes the conversion of pyruvate to acetyl-coenzyme A, while LDH can shift the metabolism of pyruvate toward the production of lactate (Yang et al., 2022; Jia et al., 2021; Icard et al., 2023). LDH contains two subtypes, LDHA and LDHB. LDHA prefers to convert pyruvate into lactate, while LDHB tends to convert lactate back into pyruvate. However, downregulation of both LDHA and LDHB inhibits glycolysis and reduces lactate production (Yang et al., 2022). Pyruvate Dehydrogenase Phosphatase 1 (PDP1) and PDK control the rate of pyruvate transformation into acetyl-CoA by regulating the activity of PDH. PDK inhibits PDH activity by promoting the phosphorylation of serine 293 on PDH, while PDP1 normally catalyzes PDH dephosphorylation to activate PDH (Chen et al., 2023; Karagiota et al., 2023; Kantarci et al., 2020). Pyruvate transport to the mitochondria is normally blocked by the upregulation of PDK, leading to the production of lactate (Kantarci et al., 2020). In addition, lncRNA Nuclear Enriched Transcript 1 (NEAT1) can increase the activity of PGK1 and inhibit the degradation of PGK1, thereby promoting glycolysis (Liang et al., 2022) (Figures 3, 5).

Previous studies have confirmed possible interactions between key enzymes of glycolytic metabolism. PGK1 accelerates lactate synthesis in microglia by upregulating the expression of PKM2 and LDHA (Cao et al., 2023). The dimer PKM2 can enter the nucleus to form a complex with prolyl hydroxylase 3, thereby promoting the expression of HIF-1α and its target genes (LDH, GLUT1, and PDK1) (Awasthi et al., 2019). Simultaneously, PKM2 may promote the generation of lactate by promoting glycolysis, which can increase the transcript levels of HIF-1α, PKM2, and LDHA through upregulation of H4K12la levels on the HIF-1α, PKM2, and LDHA promoter, thus promoting the further production of lactic acid (Pan et al., 2022). In addition, the knockdown of mitochondrial pyruvate carrier protein 1 promotes the conversion of pyruvate to lactate by blocking the entry of pyruvate into mitochondria (Gao et al., 2023). In turn, lactate reduces the transcription of HK1 by upregulating histone lactylation in the HK-1 promoter, which can inhibit glycolysis (Jiang et al., 2021).

Furthermore, lactate can indirectly regulate its own production by affecting the activity and transcriptional levels of key enzymes in the tricarboxylic acid cycle, such as ATP citrate lyase 2 and IDH3G (Jiang et al., 2021; Yang et al., 2023). Adenylate kinase 2, an ATP-metabolizing enzyme located in the mitochondria, also plays a role in this regulatory network. A specific variant, adenylate kinase 2 K28Ia, promotes glycolysis (Yang et al., 2023) (Figure 5).

3.1.2 Effect of HIF-1 on lactate metabolism

HIF-1, which contains both HIF-1α and HIF-1β subunits, is often activated under hypoxic conditions. HIF-1α, which is the main subunit responsible for functional regulation, often helps cells adapt to hypoxia by regulating the expression of some genes (Liang et al., 2023). HIF-1 affects lactate supply by regulating the expression of glycolysis-related proteins, such as GLUT1, HK2, PFK, PGK1, PKM, LDHA, and PDK (Chen et al., 2023; Jiang et al., 2021; Chen et al., 2023; Eschricht et al., 2015; Hu et al., 2019). There may be positive feedback between lactate and HIF-1 (Jiang et al., 2021; Hu et al., 2019). HIF-1 not only enhances cellular lactate production by promoting the expression of LDHA but also promotes lactate excretion through the upregulation of MCT4, which can maintain the continuity of glycolysis by avoiding the competitive inhibition of LDHA mediated by lactate excess (Huang et al., 2022). Additionally, HIF-1α induces the shift of energy metabolism from oxidative phosphorylation to glycolysis by downregulating the expression of PDH (Lu et al., 2022). HIF-1 can upregulate the expression of PDK and LDHA (Karagiota et al., 2023). PDK prevents pyruvate from being converted to acetyl-coenzyme A by catalyzing the phosphorylation of PDH, which may allow LDHA to convert pyruvate to lactate (Zhang et al., 2019; Chen et al., 2023; Huang et al., 2022). Knockdown of HCAR1 can block lactate-mediated cellular physiological activities by downregulating the transcriptional level of MCT (Wagner et al., 2015). HIF-1 and lactate have been shown to regulate lactate-mediated cellular physiological activities by upregulating MCT and HCAR1 expression (Longhitano et al., 2022; Huang et al., 2022). It is thus clear that HIF-1 can intervene in protein-lactylation-mediated biological effects by regulating the expression of functional proteins related to lactate metabolism and transport (Figure 5).

3.2 The impact of HIF-1 on epilepsy

Ischemia/hypoxia-induced elevation of HIF-1α expression in brain tissue can mediate cognitive deficits associated with hippocampal neuronal loss by inducing mitochondrial structural damage and upregulating pyroptosis-associated effectors, TNF-α and IL-1β (Zhao et al., 2022). HIF-1α/meme oxygenase 1 may promote the development of epilepsy by inducing ferroptosis of hippocampal neurons and reducing the activity of antioxidant enzymes in

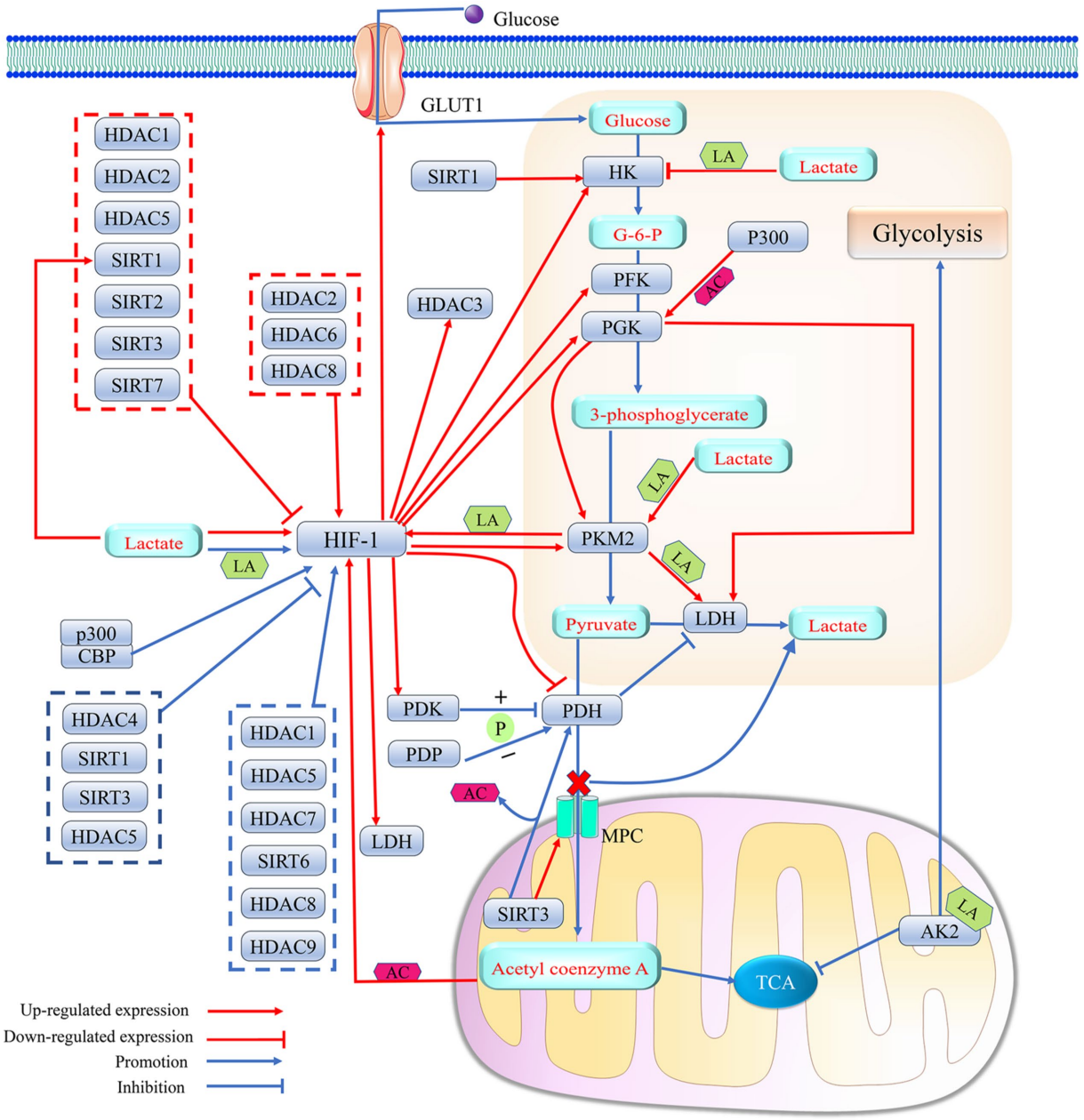

FIGURE 5
Histone deacetylase affects lactate metabolism by regulating HIF-1-mediated metabolic reprogramming effects. 1. HIF-1, as a transcription factor, enhances lactate production by regulating the expression levels of several key enzymes in glycolysis (HK, PGK, PFK, LDH, PDH and PKM2) and the glucose transport protein GLUT1. PDH promotes the entry of pyruvate into mitochondria via MPC and its conversion to acetyl coenzyme A, which initiates TCA by inhibiting the activity of LDH. HIF-1 catalyzes the phosphorylation of PDH by promoting the expression of PDK, thereby attenuating the activity of PDH. Elevated lactate levels in cells can promote lactylation modification of HIF-1α, which can elevate the transcription factor activity of HIF-1 to further enhance the efficiency of the glycolytic response. Histone acetylation or lactylation of the promoter of HIF-1 can promote HIF-1 expression. The level of promoter histone acetylation of HIF-1 is affected by the concentration of acetyl coenzyme A in cells, and the degree of HIF-1 lactylation can be regulated with PKM2. Meanwhile, the expression levels of both PKM2 and LDH can be upregulated by their promoter histone lactylation. A positive feedback loop consisting of lactate/PKM2/HIF-1/LDH further promotes protein lactylation. In addition, lactylation of the mitochondrial protein AK2 further promotes glycolysis and inhibits TCA. However, the positive feedback regulation of HIF-1α by lactate is not uncontrolled, and high lactate concentrations may block HK expression by upregulating histone lactylation levels in the HK promoter, thereby slowing down the onset of cellular glycolysis. Meanwhile, both PDP-mediated PDH dephosphorylation and SIRT3-catalyzed PDH deacetylation were able to produce less lactate by increasing PDH activity. 2. Histone acetylase P300 upregulates the expression level of PGK1, a key enzyme in glycolysis, by catalyzing its promoter histone acetylation. PGK1 promotes glycolysis by upregulating the expression of LDH and PKM2. Histone acetylase P300/CBP elevates the transcriptional activity of HIF-1 by promoting HIF-1 acetylation. 3. HDACs can intervene in lactate metabolism by regulating HIF-1 activity and/or expression levels. SIRT1 can even promote lactate production by directly upregulating the expression level of HK, while SIRT3 can help more pyruvate enter the mitochondria rather than converting it into lactate by promoting the expression of MPC. HIF-1 can act as a transcription factor to promote HDAC3 expression. Acetylation (AC); adenylate kinase 2 (AK2); glucose-6-phosphate (G-6-P); glucose transporter 1 (GLUT1); hexokinase (HK); histone acetylase P300; histone deacetylase (HDAC); hypoxia-inducible factor 1 (HIF-1); lactic dehydrogenase (LDH); lactylation (LA); mitochondrial pyruvate carrier (MPC); phosphorylation (P); pyruvate dehydrogenase kinase (PDK); pyruvate dehydrogenase (PDH); pyruvate dehydrogenase phosphatase (PDP); phosphoglycerate kinase (PGK); pyruvate kinase isozymes M2 (PKM2); phosphofructokinase-1 (PFK); silencing regulatory protein (SIRT); and tricarboxylic acid cycle (TCA).

hippocampal tissue (Liang et al., 2023). Numerous studies have suggested that cellular ferroptosis may be mediated by mitochondrial dysfunction and HMGB1 (Yang et al., 2023; Chen et al., 2023; Tang et al., 2023; Ge et al., 2023; Yuan et al., 2022). It can be inferred that HIF-1α may be involved in HMGB1-mediated mitochondrial dysfunction and programmed cell death associated with epilepsy.

3.2.1 HIF-1α and HMGB1

HIF-1α inhibits the expression of HMGB1 by upregulating the transcription level of lncRNA NEAT1, thereby reducing cell apoptosis and inflammatory damage (Luo et al., 2022). Indeed, HIF-1α, which may act as an upstream regulator of HMGB1, can affect the biological effects of HMGB1 by regulating its transcription level. Meanwhile, HIF-1α promotes the production of lactate from glycolysis by upregulating the expression level of LDHA, which exacerbates HMGB1-mediated ischemic neuronal pyroptosis by upregulating the histone H3K18la in the HMGB1 promoter (Yao and Li, 2023). Cell death usually favors the release of HMGB1 into the extracellular compartment. Extracellular HMGB1 can promote the expression of NF-κB and HIF-1α via binding to the TLR4 receptor (Peng et al., 2021). Lactate can enhance the translation level of NF-κB by promoting the translocation of the transcription factor HIF-1α into the nucleus (Peng et al., 2021). NF-κB located in the nucleus not only promotes the expression of inflammatory cytokines (IL-1β and TNF-α) but also enhances HMGB1 activity by elevating HIF-1α expression (Peng et al., 2021; Song et al., 2020). Thus, HIF-1α can regulate the function of HMGB1 through positive feedback. HMGB1 acts as a ligand for the cell surface receptor TLR2 to trigger YAP and HIF-1α nuclear translocation and activate the Hippo-YAP/HIF-1α pathway. Activated YAP not only promotes the expression of HIF-1α but also maintains HIF-1α stability by binding to HIF-1α in the nucleus, thereby promoting the expression of target genes of HIF-1α. Conversely, knockdown of HIF-1α may inhibit HMGB1 by reducing YAP nuclear translocation. It further confirms that HIF-1α can affect cell death mediated by HMGB1 (Zhang et al., 2019) (Figure 3).

3.2.2 HIF-1α and mitochondria

HIF-1α shifts energy production from oxidative phosphorylation to glycolysis by promoting the transcription of related genes, which reduces ROS production to reverse the mitochondrial dysfunction induced by oxidative stress (Li et al., 2019). Although HIF-1α does not stabilize mitochondrial function by promoting the transcription of mitochondrial DNA as SIRT3 does (Wang et al., 2022), HIF-1α, as a protein regulating iron homeostasis, decreases the partial pressure of oxygen, translocates to the mitochondria, and acts directly on the mitochondrial respiratory chain complex in hypoxia, thereby blocking the sustained effects of mitochondrial ROS on mitochondrial function and ultimately achieving multi-modal neuroprotection (Fouché et al., 2023). In addition, HIF-1α may exert cytoprotective effects by mediating mitochondrial autophagy (Lestón Pinilla et al., 2021; Fu et al., 2020). However, intermittent hypoxia induces cellular mitochondrial dysfunction via activation of HIF-1 (Moulin et al., 2022). In turn, ROS and lactate, which are generated with mitochondrial dysfunction, activate mTOR and HIF-1α-related signaling pathways (Tauffenberger et al., 2019; Ip et al., 2017), and the oxidative stress caused by mitochondrial dysfunction may protect the structure and function of HIF-1α by inhibiting its proteasomal degradation (Wu et al., 2023). This shows that there is an interactive relationship between HIF-1 and mitochondrial dysfunction and that the effect of HIF-1 on mitochondria is influenced by the extracellular environment and tissue variability (Figure 3). HIF-1, regulating glycolytic metabolism, can affect the level of protein lactylation by regulating lactate production. The protein lactylation may be one of the effective mechanisms by which HIF-1 regulates HMGBI, a core factor that mediates programmed death in multiple cells. Although the mechanisms of HIF-1-mediated neuronal loss, which is strongly associated with mitochondrial dysfunction-induced epilepsy, have not yet been clarified, it cannot be denied that HIF-1 may regulate the level of HMGBI lactylation to mediate programmed neuronal death or induce mitochondrial dysfunction to mediate the occurrence and development of epilepsy. It is speculated that HIF-1 may be an important target for regulating epilepsy-associated protein lactylation (Figure 6).

3.3 Regulation of protein lactylation by HATs and HDACs

3.3.1 Protein lactylation and delactylation catalyzed by HATs and HDACs

Previous studies have suggested that the acetyl group for protein acetylation is mainly supplied by acetyl coenzyme A and that the lactyl group for protein lactylation is mainly supplied by lactate (Dong et al., 2022). Acetyl coenzyme A and lactate in the body are mainly converted from pyruvate, an intermediate product of glycolytic metabolism, and both lactate modification and acetylation modification of proteins can be affected by glycolysis. Furthermore, it has been demonstrated that the mechanism of histone modification by lactate is similar to that of acetyl coenzyme A and that both protein lactylation and protein acetylation are regulated by acetylases and deacetylases (Moreno-Yruela et al., 2022) (Figure 4). Acetyltransferase P300, a newly identified lactonylated writer protein, was shown to promote gene expression by upregulating the levels of H3K9 acetylation, H4K12 acetylation, H4K16 acetylation, H4K12la, and H3K18la at the transcriptional start sites of pluripotency genes (Fan et al., 2023; Lin et al., 2022). P300 belongs to the nuclear A-type HAT. It has been demonstrated that the loss of P300 activity is associated with neuronal survival and long-term memory and that P300 may intervene in neuronal death by catalyzing histone acetylation or lactylation (Lu et al., 2015). P300 promotes PGK1 expression by catalyzing H3K27 acetylation in the PGK1 promoter region. PGK1 exacerbates ischemic stroke-induced brain injury by promoting microglia M1 polarization, inflammation, and glycolysis (Cao et al., 2023). Indeed, the regulation of protein lactylation by P300 can depend not only on the direct catalysis of lactide writing but also on the regulation of lactate production.

HDACs consist of HDAC1-11 and SIRT1-7 with deacetylase activity. A recent study revealed that HDACs, like P300, regulate protein lactylation and acetylation. Class I HDAC1, 2, and 3 are the most efficient lysine delactylases *in vitro*, mainly catalyzing the delactylation of H3K18 and H4K5. However, SIRT1-3, in vitro experiments, were shown to have a slight delactylation activity, and

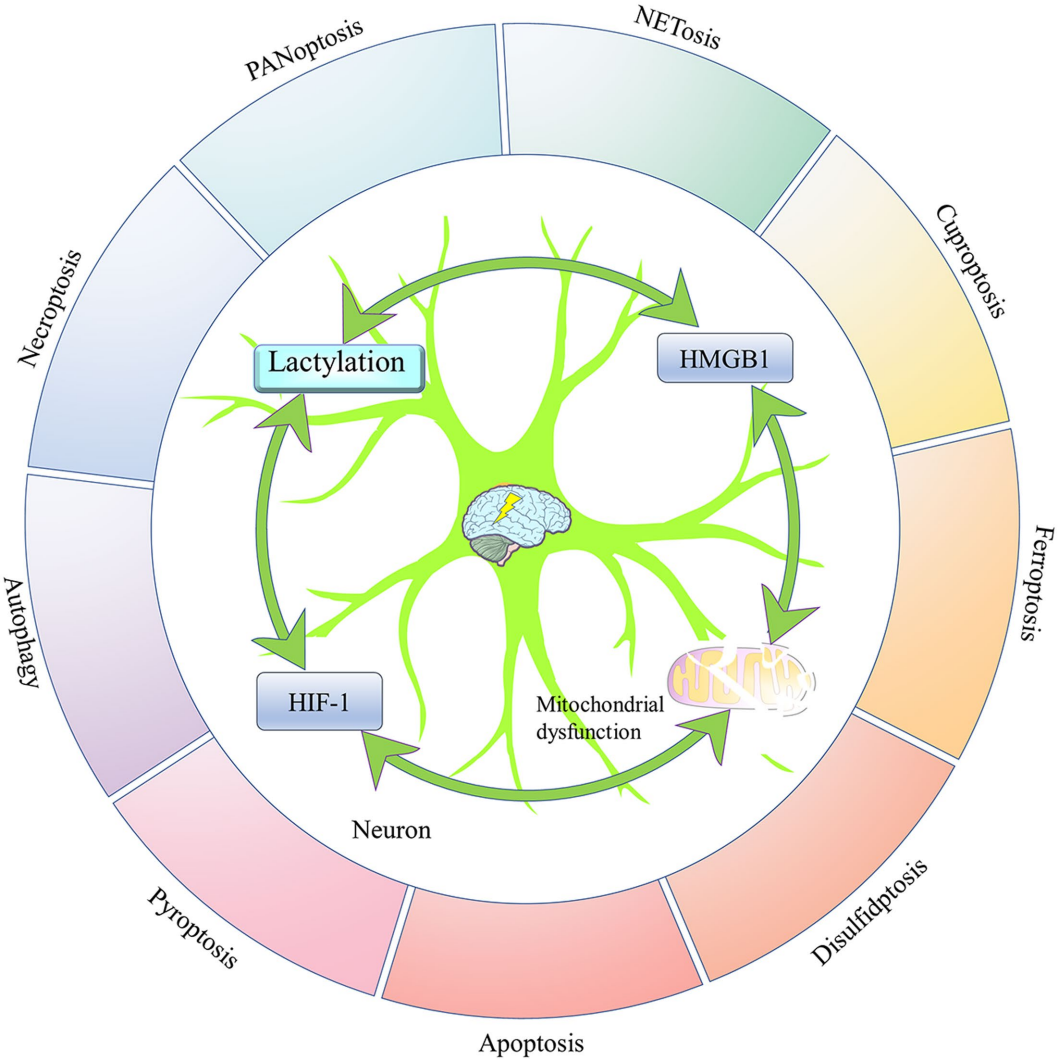

FIGURE 6
The four elements mediating epilepsy-related neuronal death. Neuronal death is one of the important pathological changes in epileptogenesis and development. Lactylation modification, HIF-1, and mitochondrial dysfunction all intervene in epilepsy-associated neuronal death by regulating the activity of HMGB1, which is a central factor in the regulation of multiple programmed cell deaths. In addition, there is an interactive relationship between lactylation modification, HIF-1, and mitochondrial dysfunction. Hypoxia-inducible factor 1 (HIF-1); and high-mobility group box 1 (HMGB1).

their main regulatory sites are in H3K18 and H3K5 (Fan et al., 2023; Moreno-Yruela et al., 2022). Furthermore, the knockdown of HDAC1-3 resulted in an increase in H4K5la but had little effect on pan-Kla and H3K18la. The differences in histone lactylation sites regulated by HDAC1-3 between in vitro and *in vivo* experiments suggest that the specificity of HDAC action sites may be affected by certain cofactors (Fan et al., 2023). However, some studies have suggested that HDAC1-3 predominantly regulates the level of H3K18la both in vitro and in vivo and that MS-275, a selective inhibitor of HDAC1-3, promotes gene expression by upregulating the levels of H3K14la and H3K18la (Dai et al., 2022). H3K18la can be widely distributed in the cells and tissues of humans and mice. Importantly, it has been discovered that H3K18la not only accumulates at promoters but also enriches in a tissue-specific manner at active enhancers, sharing a genomic distribution pattern with H3K27 acetylation (Galle et al., 2022). Mitochondrial dysfunction can promote pan-lysine lactylation and H3K18 lactylation without affecting pan-lysine acetylation and H3K27 acetylation (He et al., 2023). Lactylation and acetylation of histones may cooperate to execute the transcriptional regulation of genes. The acetylation of histones can competitively inhibit histone lactylation (Rho et al., 2023). However, HDAC inhibitors may activate neuronal transcriptional programs by mediating chemical modification of multiple forms of histone lysines, such as H3K9 acetylation, H3K9 crotonylation, H3K18 crotonylation, and H3K18la. There may be collaborative, rather than competitive, relationships between histone acetylation, butyrylation, and lactic acidification in neuronal cells (Dai et al., 2022). HDACs can not only regulate histone lactylation levels but also regulate lactylation modifications of non-histone proteins. SIRT1 is a potential non-histone delactylase in mammals (Sun et al., 2022). Meanwhile, HDACs affect the level of protein lactylation by regulating glucose metabolism. For example, SIRT1 promotes lactate production by positively regulating the expression of HK-2, a key enzyme in

glycolysis (Chen et al., 2022) (Figure 5), while SIRT6 has been shown to be an inhibitor of glycolysis (Sun et al., 2021) (Figure 1). SIRT3 promotes the conversion of pyruvate to acetyl coenzyme A by catalyzing the deacetylation of PDH, thereby reducing lactate production and downregulating the lactylation of mitochondrial proteins (An et al., 2023).

3.3.2 The role of HATs and HDACs in the regulation of HIF-1

HDACs regulate HIF-1α activity through various mechanisms. HDACs can regulate the activity of HIF-1α by affecting its expression level, degradation rate, and post-translational modifications (Table 2). Various factors may influence this process, such as tissue specificity and external environmental pressures (Geng et al., 2011).

It was shown that HDAC1 and HDAC2 downregulate the expression of fructose-1,6-bisphosphatase 1 by catalyzing the deacetylation of histone H3K27 in the enhancer of 1,6-bisphosphatase. Fructokinase 1,6-bisphosphate, a rate-limiting enzyme in gluconeogenesis, reduces the onset of glycolysis and decreases lactate production by inhibiting the expression of HIF-1α, thereby inhibiting histone lactylation (Yang et al., 2017; Fan et al., 2020). Meanwhile, HDAC2 promotes HIF-1α/6-phosphofructo-2-kinase (PFKFB3) axis-mediated neuroinflammatory injury by increasing the expression of HIF-1α (Liu et al., 2022). However, not all HDACs can act as upstream regulators of HIF-1, and there are currently few reports on the regulation of HIF-1 function by HDAC3, HDAC10, HDAC11, and SIRT5. It has even been suggested that HIF-1α may promote HDAC3 expression, serving as an upstream regulator of HDAC3 (Wang et al., 2021) (Figure 5). However, in epileptic hippocampal neurons, HDAC5 promotes HIF-1α enrichment at the PFKFB3 promoter by upregulating HIF-1α expression, thereby contributing to PFKFB3-induced neuronal apoptosis, oxidative stress, and inflammation (Pan et al., 2021).

Under hypoxic conditions, HDAC7-u enhances the transcriptional activity of HIF-1α, promoting the transcription of HIF-1α target genes. This effect is achieved by increasing HIF-1α's transcriptional activity rather than altering its transcriptional level (Shakespear et al., 2013). HDAC9 enhances the transcriptional activity of HIF-1 by catalyzing HIF-1 deacetylation, thereby mediating ischemic stroke-induced neuronal Ferroptosis in cortical layers (Sanguigno et al., 2023).

SIRT1 stabilizes Von Hippel-Lindau activity by deacetylating Von Hippel-Lindau, effectively reversing its acetylation. This modification enhances the stability and function of the Von Hippel-Lindau protein. Under normoxic conditions, hydroxylated HIF-1α accelerates HIF-1α degradation by binding to von Hippel–Lindau ubiquitin ligase complexes (Li et al., 2022; Sailhamer et al., 2010). Activation of HIF-1α under hypoxic conditions induces a shift in energy metabolism from oxidative phosphorylation to glycolysis, which facilitates cellular adaptation to the hypoxic environment to protect neuronal viability (Kaitsuka et al., 2020; Zhu et al., 2022). The knockdown or inhibition of the activities of SIRT2 and SIRT3 has been shown to increase the expression of HIF-1α, which in turn activates cellular glycolysis to ensure the energy supply for neurons in the ischemic core (Kaitsuka et al., 2020; Zhu et al., 2022). The glycolysis in neurons depletes NAD^+, which leads to the loss of biological activity of the NAD^+-dependent deacetylase SIRT1, thereby maintaining the stability of HIF-1α structure and function (Li et al., 2022). Blocking the negative regulatory effect of HDACs on HIF-1α activity may help brain tissue adapt to the hypoxic environment to a certain extent, but elevated HIF-1α activity may increase the risk of inflammatory injury (Li et al., 2022; Kaitsuka et al., 2020; Zhu et al., 2022). Furthermore, SIRT6 independently upregulated HIF-1α expression to promote glycolysis (You et al., 2022).

It was shown that P300 may intervene in the functioning of the central nervous system by regulating the activity of key enzymes of glycolysis (Cao et al., 2023; Lu et al., 2015) and that P300/CBP may enhance the expression of HIF-1α target genes by promoting lysine acetylation of the transcription factor HIF-1α (Wang et al., 2024). It can be speculated that P300 may indirectly regulate protein lactylation modification by affecting the lactate production of glycolysis by modulating the transcriptional activity of HIF-1α.

In summary, HAT and HDAC regulate protein lactylation *in vivo*. This regulation depends not only on the catalytic activities of these enzymes but also on the availability of the lactate donor, lactide. These enzymes also indirectly regulate the expression of proteins related to glycolytic metabolism, which are induced by the transcription factor HIF-1α. Additionally, HAT and HDAC directly influence the activity of key glycolytic enzymes, further impacting lactate production. These mechanisms highlight the complex interplay between histone modification and metabolic control in cellular processes.

3.4 Regulation of HIF-1 by other substances

Research indicates that the stability and activation of HIF-1 are not only regulated by HATs and HDACs but also closely related to oxygen levels, cellular iron content, and the activation of other signaling pathways (Liang et al., 2023).

The autophagy-related factor P62 may enhance the expression of glycolysis-related proteins by upregulating HIF-1α expression (Calvo-Garrido et al., 2019). In turn, the products and key enzymes of glucose metabolism can adjust the biological activity and expression level of HIF-1. In microglia, lactate not only upregulates the transcriptional level of HIF-1α by promoting the histone H4K12la of the HIF-1α promoter (Pan et al., 2022; Jiang et al., 2021) but also enhances the production of lactate by directly promoting the lactylation of HIF-1α (Luo et al., 2022).

Accumulating lactate may promote the expression of the transcription factor STAT6 by upregulating histone lactylation in the STAT6 promoter region (Lu et al., 2022). Furthermore, STAT6 may reduce HIF-1α expression by inhibiting the mTOR-mediated glycolipid metabolism regulation signaling pathway (Lu et al., 2022; Park et al., 2019) (Figure 3).

Meanwhile, PDP1 can drive the acetylation of histone H3 in the promoter of HIF-1 target genes and promote gene expression by promoting the production of the glucose metabolism intermediate product acetyl coenzyme A. The expression of the HIF-1 target gene PDK1 can counteract the effects of PDP1 and diminish the biological impact of HIF-1 (Karagiota et al., 2023) (Figure 5). The epigenetically important regulators HAT and HDAC have been shown to be major regulators of lactylation modification, and a large number of studies have demonstrated that HAT and HDAC are critical for the progression of epilepsy (Wang et al., 2022). The regulation of HIF-1

TABLE 2 The histone deacetylases regulating transcription factor HIF-1α.

Members	Subjects	Associated diseases	Mechanisms and effects	References
HDAC1	HEK293 T, SW48, and LOVO cell lines	Colorectal cancer	The HDAC1 may activate the HIF1α/vascular endothelial growth factor A signaling pathway by directly inhibiting the ubiquitination of HIF1α, which can promote tumor angiogenesis.	Chen et al. (2020)
	HAEC lines	Atherosclerosis	The HDAC1 reduces ROS accumulation and endothelial cell apoptosis in atheromatous plaques through downregulation of HIF1α expression	Wang et al. (2021)
	PC cell lines	Pancreatic Cancer (PC)	The HDAC1 can improve HIF-1α activity, which can promote aerobic glycolysis in tumor cells by inhibiting acetylation or degradation of HIF-1α.	Jin et al. (2021)
HDAC2	BV-2 microglial cells	Postoperative cognitive dysfunction	The HDAC2 may promote HIF-1α/PFKFB3 axis-mediated neuroinflammatory injury by increasing the expression of HIF-1α.	Liu et al. (2022)
	Human umbilical cord mesenchymal stem cell	Esophageal cancer	Blocking the binding of HDAC2 to the promoter of HIF-1α can activate the transcription of HIF-1α, which can promote tumor growth and metastasis.	Chen et al. (2023)
HDAC4	Osteoblast precursor cells	Diabetic osteoporosis	The HDAC4 can inhibit bone growth by blocking the HIF-1α/vascular endothelial growth factor A pathway.	Zhang et al. (2023)
HDAC5	Hippocampal neurons	Epilepsy	The HDAC5 may promote the synthesis of inflammatory factors IL-1β, TNF-α, and IL-6 by upregulating the expression of HIF-1α and PFKFB3, thereby inducing neuronal cell apoptosis, oxidative stress, and inflammation.	Pan et al. (2021)
	Rat Pheochromocytoma (PC)-12 Cells	Intermittent hypoxia	The HDAC5 can not only downregulate the transcription level of HIF-1 by reducing the acetylation of histone lysine residues but also inhibit the transcriptional activity of HIF-1 by lowering its acetylation levels.	Wang et al. (2021)
HDAC6	Th17 cell	Acute lung allograft rejection	Tubastatin A may downregulate HIF-1α expression levels and activity by specifically inhibiting HDAC6 activity, which can reduce acute allograft rejection.	Zhou et al. (2020)
HDAC7	Mouse macrophages	Inflammatory disease	The HDAC7-u, without regulating the transcriptional level of HIF-1α, may enhance HIF-1α transcriptional activity during hypoxia by binding to it, which can promote the transcription of HIF-1 α target genes.	Shakespear et al. (2013)
HDAC8	Human melanoma cell line A2058	Melanoma	The HDAC8 promotes the expression of HIF-1α target genes, such as hexokinase 2 (HK2) and glucose transporter protein 1 (GLUT1), by upregulating HIF-1α expression levels and elevating HIF-1α transcriptional activity, which can promote the proliferation and metastasis of melanoma cells.	Kim et al. (2023)
HDAC9	Primary cortical neurons	Ischemic stroke	The HDAC9 enhances the transcriptional activity of HIF-1 by catalyzing its deacetylation, thereby mediating ischemic stroke-induced neuronal iron death in the cortical layer.	Sanguigno et al. (2023)
SIRT1	bEnd.3 cells	Ischemic stroke	The Sirt1 may block HIF-1α/NOX2 signaling cascade-mediated microvascular endothelial cell destruction by promoting HIF-1α degradation.	Li et al. (2022)
	Stromal vascular fraction cells	Adipose fibrosis	The SIRT1 can inhibit the transcription of HIF-1 target genes by downregulating the expression level and acetylation level of HIF-1α.	Wu et al. (2023)
	Neuronal cells	Parkinson's Disease	The SIRT1 can suppress oxidative stress by maintaining HIF-1α in a deacetylated state to exert neuroprotective effects.	Ubaid et al. (2022)
SIRT2	Hippocampal neurons	Ischemic stroke	The SIRT2, in neurons, can downregulate the expression of HIF-1a under hypoxic conditions.	Kaitsuka et al. (2020)

(Continued)

TABLE 2 (Continued)

Members	Subjects	Associated diseases	Mechanisms and effects	References
SIRT3	A549 cells	Viral infections	Degradation of SIRT3 in damaged mitochondria increased cellular mROS levels to enhance HIF-1α stability and its target gene expression.	Gong et al. (2021)
	Primary murine juvenile chondrocytes	Osteoarthritis	The loss of Sirt3 may increase the expression of HIF-1a to induce a shift of chondrocyte metabolism from mitochondria to glycolysis.	Zhu et al. (2022)
	Bone marrow-derived macrophages (BMDMs)	Hepatic ischemic/reperfusion injury	The plasma membrane-bound G protein-coupled bile acid receptor knockdown promotes the acetylation, ubiquitination, and degradation of Forkhead Box Protein O3 by inhibiting SIRT3 expression, resulting in increased HIF-1α transcription.	Wang et al. (2020)
SIRT4	786-O cells and Caki-2 cells	Clear cell renal cell carcinomas	The SIRT4 binds to HIF-1α in a protein–protein interaction and directly inhibits HIF-1α expression, thereby blocking HIF-1α/HO-1-mediated tumor cell growth.	Tong et al. (2021)
SIRT6	PC9 cells and HCC827 cells	Non-small cell lung cancer	The SIRT6 may promote the growth of erlotinib-resistant non-small cell lung cancer cells by activating the HIF-1α/HK2 signaling axis to promote aerobic glycolysis in tumor cells.	You et al. (2022)
SIRT7	Hep3B cells	Unknown	Overexpression of Sirt7, without its deacetylation activity, can lead to a significant decrease in HIF-1α protein levels.	Hubbi et al. (2013)

by HAT and HDAC may be a potential pathway for their intervention in epilepsy-associated protein lactylation.

4 The impact of HDAC inhibitors on lactylation and epilepsy

Although the state of protein acetylation depends on the balance between HAT and HDAC, previous studies have shown that the imbalance between HAT and HDAC favors the latter, so the clinical development of HDAC inhibitors is focused on restoring this balance (Bezu et al., 2019). Currently, some HDAC inhibitors have been applied and studied as antiepileptic drugs (Wang et al., 2022). The protein lactylation induced by lactate is regulated by HAT and HDAC as protein acetylation (Yang et al., 2023). Suberoylanilide hydroxamic acid and trichostatin A, which are HDAC inhibitors, may reduce the rate of lactate production from glycolysis by promoting acetylation of key enzymes of glycolytic metabolism, thereby reducing protein lactylation modification (Wu et al., 2023; Zhang et al., 2022). It is undeniable that the antiepileptic effect of HDAC inhibitors may be partially mediated by the modulation of protein lactylation modifications.

4.1 The impact of HDAC inhibitors on neuronal loss and glial activation

The common HDAC inhibitors can be divided into four groups based on their structure: butyric acid derivatives (e.g., valproic acid), hydroxamic acid derivatives (e.g., suberoylanilide hydroxamic acid), benzamides (e.g., entestat) and cyclic tetrapeptides (e.g., romidepsin) (Bezu et al., 2019). Sodium butyrate can alleviate neurological damage caused by cerebral hypoxia-ischemia. The ability of trichostatin A to stimulate neurogenesis in the subgranular zone of the hippocampus may facilitate recovery from hypoxic–ischemic injury in the neonatal brain (Zalewska et al., 2020).

The conventional antiepileptic drug valproate may inhibit the activation of the NF-κb signaling pathway by promoting the acetylation of STAT1 and p65, thereby impeding microglia activation and attenuating I/R-induced neuroinflammation and brain damage (Dai et al., 2021). Suberoylanilide hydroxamic acid has been shown to prevent astrocyte and microglia activation, which can alleviate ischemia-induced neuroinflammation by inhibiting the deacetylation activity of HDAC1/2 (Dai et al., 2021), while the class IIa HDAC inhibitor MC1568 attenuates ischemic stroke-induced ferroptosis of cortical neurons by blocking HDAC9 transcription (Sanguigno et al., 2023). Furthermore, HDAC inhibitors significantly inhibit the immunological activation and aggregation induced by cerebral hemorrhage. Scriptaid has been proven to alleviate the inflammatory injury of cortical neurons caused by traumatic brain injury or cerebral hemorrhage by promoting the polarization of microglia/macrophages toward the protective M2 phenotype (Dai et al., 2021). Cerebral hemorrhage is highly susceptible to epilepsy, and the class I HDAC inhibitor entinostat protects neurons by inhibiting microglia activation, thereby ameliorating cerebral hemorrhage-induced neuroinflammatory damage (Bonsack and Sukumari-Ramesh, 2021). The class I HDAC inhibitor CI-994 and the HDAC1-specific inhibitor parthenolide may enhance synaptic plasticity in hippocampal neurons by inhibiting astrocyte and microglia immunoreactivity, thereby ameliorating epilepsy-induced cognitive impairment (Jia et al., 2023; Dai et al., 2021; Burns et al., 2022). RGFP966, the HDAC8-specific inhibitor WK2-16, and the HDAC3-specific inhibitor BG45 have been shown to reduce neuroinflammation-mediated hippocampal neuronal loss by

inhibiting the proliferative activation of glial cells, thereby maintaining the morphological and functional stability of the cerebral cortex (Dai et al., 2021; Wang et al., 2023). Additionally, FK228, also known as romidepsin, can exert a neuroprotective effect by inhibiting NETosis-mediated neural inflammation and promoting the regeneration of neurons (Thakur et al., 2024). HDAC inhibitors may reduce the occurrence of post-stroke epilepsy by alleviating neuronal loss and neuroinflammation induced by ischemic brain injury.

4.2 The biological activity of HIF-1 is regulated by HDAC inhibitors

HIF-1α not only affects ischemia-induced hippocampal neuronal loss by regulating lactate metabolism but is also closely associated with epilepsy-related hippocampal neuronal death and oxidative stress (Liang et al., 2023; Zhao et al., 2022). Valproate, which has been shown to inhibit class I and class II HDACs, may reduce HIF-1α-mediated hippocampal neuronal loss by lowering the protein level of HIF-1α, thereby achieving antiepileptic effects (Luo et al., 2013; Simeone et al., 2017). Sodium butyrate not only enhances ubiquitination of HIF-1α Lys532 by catalyzing its acetylation to target HIF-1α for proteasomal degradation (Naia et al., 2017), but also upregulates the expression level of HIF-1α by inhibiting HDAC2 (Bashir and Olaniyi, 2023). Moreover, butyrate sodium combined with curcumin, which is a less toxic natural pan-HDAC inhibitor derived from food, prevents PI3K/Akt axis-mediated inhibition of HIF-1α activity through inhibition of HDAC1 (Islam et al., 2023). The development of food-borne HDAC inhibitors may be beneficial for epilepsy control. The ketogenic diet can increase the production of β-hydroxybutyrate, which may alleviate seizures associated with neuroinflammation by promoting the acetylation of histone H3K9 and H3K14, similar to HDAC inhibitors (Simeone et al., 2017).

Suberoylanilide hydroxamic acid, vorinostat, which is the first acetylation-modifying drug approved by the US Food and Drug Administration, mainly inhibits the activity of HDAC1 and HDAC2. It has been demonstrated that vorinostat inhibits the activity of HDACs, including HDAC4, to promote HIF-1α acetylation, which can inhibit the biological activity of HIF-1α and promote HIF-1α degradation (Geng et al., 2011; Sailhamer et al., 2010). Meanwhile, vorinostat promotes intracellular lactate efflux by upregulating the expression of MCT1 and MCT4, and lactate released into the extracellular space enhances the inhibitory effect of vorinostat on HDAC activity (Radoul et al., 2019). In addition, N-hydroxy-7-(2-naphthylthio) heptanamide, a novel synthetic HDAC inhibitor, was shown to inhibit HIF-1α expression to a greater extent than Vorinostat by *in vitro* and in vivo experiments in breast cancer (Park et al., 2011). Panobinostat (LBH589), a novel pan-HDAC inhibitor, has been shown to potently inhibit the viability of HIF-1α (Ni and Ni, 2021). The pan-histone deacetylase inhibitor PCI-24781 induced cellular autophagy by upregulating the concentration of HIF-1α (Bhalla et al., 2013).

In summary, as HIF-1α can affect the level of protein lactylation by regulating the expression of key enzymes of glycolysis and lactate transporter proteins, HDAC inhibitors may control ischemic stroke-induced protein lactylation by regulating the activity of HIF-1α, thereby alleviating abnormal excitation of neurons (Hagihara et al., 2021; Wu et al., 2023; Zhang et al., 2022). It may be one of the potential mechanisms by which HDAC inhibitors could help prevent post-ischemic stroke epilepsy.

5 Discussion

Stroke is one of the leading causes of death worldwide. Ischemic strokes account for 87% of strokes in humans (González-Rodríguez and Fernández-López, 2023). The accumulation of lactate caused by ischemic stroke may mediate neuroexcitotoxicity and neuronal apoptosis through the upregulation of protein lactylation levels, which can lead to post-stroke epilepsy (Hagihara et al., 2021; Wang et al., 2022; Zhang et al., 2023). Reperfusion injury in ischemic foci of the brain is mainly mediated by mitochondrial dysfunction, which can be induced through over-activated glycolysis (She et al., 2023; Liu et al., 2023). Although reperfusion of blood improves the oxygen supply to the ischemic lesion, it does not ensure that the mode of energy metabolism in the ischemic lesion and its surrounding tissues is switched from glycolysis to oxidative phosphorylation, implying that reperfusion may not completely block lactate production. Although there is no evidence that neurons initiate the "Warburg effect," also known as "aerobic glycolysis," as tumor cells do (Lin et al., 2022), peripheral immune cells differentiated from bone marrow hematopoietic stem cells have been observed to display the "Warburg effect," such as macrophages and neutrophils (Chen et al., 2022). The "Warburg effect" of tumor cells is primarily for the rapid proliferation of tumor cells (Lin et al., 2022), while the "Warburg effect" of peripheral immune cells promotes an inflammatory cascade by supporting the expression of pro-inflammatory cytokines during the activation of immune cells (Zhang et al., 2019). Brain injury typically involves the disruption of the blood–brain barrier, and peripheral blood immune cells can easily migrate across the damaged barrier into the brain, where they work together with activated glial cells to exacerbate neuroinflammatory damage (Li et al., 2021; Bernis et al., 2023). After the restoration of blood supply to brain tissue, peripheral immune cells may continue to produce lactate and release it extracellularly, relying on the Warburg effect, and the lactate may be taken up by neurons via the MCT to promote neuronal protein lactylation, which may increase the risk of post-stroke epilepsy by mediating neuronal excitation (Hagihara et al., 2021; Zhang et al., 2019; Mayorga-Weber et al., 2022; Tröscher et al., 2021).

Furthermore, the enhancement of neuronal excitability further upregulates protein lactylation levels by promoting the conversion of glucose to lactate in the brain (Li and Freeman, 2015). However, as the lactate from aerobic glycolysis increases in peripheral immune cells infiltrating brain tissue, the level of protein lactylation is significantly upregulated, which can induce a shift of activated peripheral immune cells to an anti-inflammatory state for avoiding an unlimited extent of inflammatory damage to brain tissue (Zhang et al., 2019). It is evident that protein lactylation modifications mediate different biological effects in different cells.

Although protein lactylation alleviates ischemic stroke-induced neuroinflammation by affecting the activity of peripheral immune cells migrating to the ischemic lesion, the protein lactylation-mediated neuronal loss and glial cell activation should not be underestimated (Pan et al., 2022; Barros et al., 2023; Yang et al., 2024; Wu et al., 2023;

Yao et al., 2023). The modification of protein lactylation in neurons and glia is associated not only with the facilitation of glycolysis but also with the cellular transport of lactate. If both lactate production and intracellular transformation of lactate in neurons can be controlled, post-stroke epilepsy would be better prevented. Since HIF-1 can act as a transcription factor for key enzymes of the glycolytic pathway and lactate transporter proteins, inhibiting the transcriptional activity of HIF-1 may be a potential measure to combat post-stroke epilepsy (Longhitano et al., 2022; Karagiota et al., 2023; Huang et al., 2022). HDAC may affect the expression of HIF-1-related target genes by directly or indirectly regulating HIF-1 transcription, degradation, and bioactivity, thereby influencing cellular glucose uptake, mitochondrial function, and lactate metabolism (Table 2). However, research has confirmed that lactate increases the activity of SIRT1, which is a potential protein lactylation and acetylation modification regulator in mammals (Sun et al., 2022; He et al., 2023), and the ketogenic diet may exert an anti-epileptic effect by inhibiting hypoxia-enhanced glycolysis (Sun et al., 2021; Hou et al., 2017). Thus, inhibition of lactate production and lactate-mediated protein lactylation may be the key to reducing post-stroke epilepsy rather than inhibition of HDAC enzyme activity, and it has been demonstrated that the HDAC inhibitors sodium butyrate and trichostatin A control lactate production by modulating the activities of pyruvate kinase and LDH (Rodrigues et al., 2015).

HMGB1 is a core factor mediating programmed cell death in various cells, and the nucleus-to-cytoplasm translocation of HMGB1 in neurons and glial cells may be an important trigger for status epilepticus (Pauletti et al., 2019; Chen et al., 2023; Tang et al., 2023). It has been demonstrated that N-(2′-hydroxyphenyl)-2-propylvaleramide may promote HMGB1 acetylation through inhibition of HDAC1, thereby inducing translocation of HMGB1 from the nucleus to the cytoplasm. Meanwhile, N-(2′-hydroxyphenyl)-2-propylvaleramide also induced HMGB1 secretion by promoting ROS synthesis (Sixto-López et al., 2020). The HDAC8-specific inhibitors, such as PCI-34051 and PCI-48012, and Scriptaid, all promote HMGB1 acetylation, which triggers HMGB1-mediated biological effects by inhibiting HDAC activity (Lopez et al., 2015; Chi et al., 2017). However, the mechanism of HDAC inhibitors regulating the biological effects of HMGB1 includes promoting the HMGB1 acetylation and regulating HMGB1 expression. Chidamide, a novel oral selective HDAC inhibitor, can downregulate the expression of HMGB1 (Liu et al., 2021). The novel HDAC inhibitor HFY-4A induces apoptosis by upregulating HMGB1 expression (Yin et al., 2023). The HDAC inhibitor pemetrexed, combined with sildenafil, may upregulate the expression of HMGB1 and promote the extracellular release of HMGB1 by inhibiting HDAC6, HDAC2, HDAC4, and HDAC9 (Booth et al., 2017). In addition, HDAC inhibitors promote the formation of the HMGB1-P53 complex by inhibiting SIRT1 activity, thereby reducing lactate generated from glycolysis (He et al., 2023; Li et al., 2023; Ma et al., 2020; Wang et al., 2023). HDAC inhibitors may regulate the activity of HMGB1 in various ways, thereby indirectly modulating protein lactylation and cell death.

HDAC can affect lactate production by regulating the activity of the transcription factor HIF-1 or key enzymes of glycolysis. Conversely, lactate can inhibit HDAC activity like conventional HDAC inhibitors (Figure 5). Lactate has been shown to induce histone H3 and H4 hyperacetylation, which may promote the expression of some genes by inhibiting class I and II HDAC (Wagner et al., 2015). In $CD8^+$ T cells, lactate promotes protein acetylation by inhibiting HDAC activity, thereby inhibiting apoptosis (Feng et al., 2022). Lactate promotes the immunosuppressive effect of tumors by blocking the formation of a transcriptional repression complex between NF-κB and HDAC3 (Chang et al., 2021). Lactate can enhance HDAC6 activity by inhibiting HDAC11 activity, and HDAC6 upregulates IL-10 transcript levels and blocks the inflammatory effects of immune cells by promoting histone H3 acetylation in myeloid-derived suppressor cells and macrophages (Heim et al., 2020). Existing studies have confirmed that lactate can exert neuroprotective effects by regulating the expression levels of HDAC2/3/5 (Ding et al., 2020; Puri et al., 2019; Karnib et al., 2019; Genders et al., 2019). Lactate can alleviate inflammatory damage and protect neurological functions by modulating the function of HDACs.

There is a feedback regulation of HDAC expression by HIF-1α. HIF-1α in a hypoxic environment can enhance the deacetylation activity of HDAC2 (Wang et al., 2022). Meanwhile, HIF-1α can downregulate the transcript level of HDAC4 by binding to the promoter of HDAC4 (Pan and Zhao, 2021). The hypoxic environment drives HIF-1α to bind directly to the HDAC3 promoter, which reduces histone acetylation by upregulating HDAC3 expression (Wang et al., 2021; Yuan et al., 2022). However, the regulatory effects of HIF-1α on HDACs, like lactate, were not consistent. It has been shown that HIF-1α impairs the anti-inflammatory effect of Treg cells on pro-inflammatory macrophages by promoting the expression of SIRT2 in Treg cells, thereby contributing to ischemia- and hypoxia-induced neuroinflammatory injury (Shu et al., 2019). Both lactate and HIF-1α can act as HDAC inhibitors to regulate protein acetylation modifications. Based on the report of lactate mediating protein phosphorylation (Maschari et al., 2022), it is speculated that lactate not only induces protein lactylation but also alters protein function by regulating the levels of protein phosphorylation and acetylation.

Recent studies have suggested that histone acetylation and lactonylation may compete (Rho et al., 2023; Moreno-Yruela et al., 2022). The competition between the lactoyl and the acetyl groups for the epigenetic modifications of histone lysine is related not only to the activity of HDAC but also to the concentrations of lactic acid and acetyl coenzyme A. Pyruvate can be converted to lactate or acetyl coenzyme A by different enzymes, and the type of histone modification can be altered by regulating the activity of these enzymes (Dai et al., 2022). It has been shown that proteins tend to undergo acetylation rather than lactylation in the physiological state, while in hypoxia, histone lactylation can replace histone acetylation to regulate cellular function (Dai et al., 2022). However, it remains to be further explored whether ischemic stroke can mediate protein lactylation, replacing protein acetylation, which may alter cellular function by promoting glycolysis. Revealing this competitive relationship between lactylation and acetylation may facilitate the design of new therapeutic strategies for epilepsy after ischemic stroke. In addition, lactate and its mediated modification of protein lactylation can affect neuronal and glial cell function and the functional recovery of ischemic foci by modulating vascular endothelial cell function (Fan et al., 2023).

In summary, the modulation of protein lactylation levels by regulating lactate production and/or lactate membrane transport may be a potential strategy to combat epilepsy after ischemic stroke.

6 Conclusion

Ischemic brain injury results in lactate accumulation within the central nervous system, contributing to metabolic dysregulation. Lactate may induce post-stroke epilepsy by promoting protein lactylation in brain tissue. HAT and HDAC have been shown to modulate cellular function by catalyzing protein lactylation and delactylation, thereby mediating neuronal loss and glial cell activation, which are important factors contributing to epilepsy-associated neuronal hyperexcitability. Although the specific mechanism through which protein lactylation influences post-stroke epilepsy has not yet been validated, conducting research in this area may provide a new theoretical basis for using HDAC inhibitors in the clinical prevention and treatment of post-stroke epilepsy.

Author contributions

XK: Writing – original draft. SC: Writing – review & editing. QY: Conceptualization, Funding acquisition, Writing – review & editing.

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Conflict of interest

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

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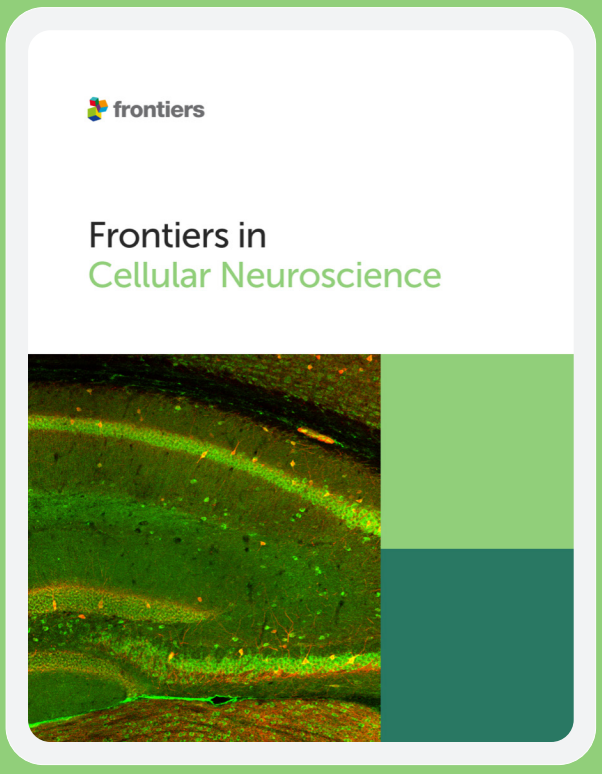

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