PLURIPOTENT CELLS FOR STROKE: FROM MECHANISM TO THERAPEUTIC STRATEGIES

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PUBLISHED IN: Frontiers in Cellular Neuroscience,
Frontiers in Molecular Neuroscience and Frontiers in Neuroscience

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88971-358-5
DOI 10.3389/978-2-88971-358-5

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PLURIPOTENT CELLS FOR STROKE: FROM MECHANISM TO THERAPEUTIC STRATEGIES

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Citation: Chen, Y., Yao, Y., Zhang, J., Hao, S., eds. (2021). Pluripotent Cells for Stroke: From Mechanism to Therapeutic Strategies. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-358-5

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frontiers in Cellular Neuroscience

EDITORIAL
published: 04 August 2021
doi: 10.3389/fncel.2021.738240

Editorial: Pluripotent Cells for Stroke: From Mechanism to Therapeutic Strategies

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Keywords: stroke, pluripotent cell, stem cell, pericyte, microenvironment, neural circuit

Editorial on the Research Topic

Pluripotent Cells for Stroke: From Mechanism to Therapeutic Strategies

Stroke, an acute central nervous system injury caused by cerebral ischemia or cerebral hemorrhage, is one of the major causes of death and the leading cause of long-term disability worldwide (Dalys and Collaborators, 2017; Wang et al., 2020). Thanks to early diagnosis, mini-invasive surgery, and advanced intensive care support, stroke survival rate has increased dramatically (Kim et al., 2020; Wang et al., 2020). Nevertheless, stroke survivors usually suffer from sequelae of neurological impairments and psychiatric disorders, which affect their daily functionality and working capacity. Although extensively studied, the molecular mechanisms underlying stroke pathophysiology remain not fully understood and innovative rehabilitation therapies for neural circuit remodeling after stroke are urgently needed.

Recently, pluripotent cell-based approaches have attracted more attention from scientists and physicians due to their possible neuroprotective and restorative effects on stroke. One major challenge, however, is that injury-induced microenvironments usually lead to obstruction in directional differentiation of pluripotent cells, and failure in reconstruction of neural circuits. Additionally, the conventional neurobehavioral evaluation and diffusion-weighted magnetic resonance imaging (MRI-DWI) cannot monitor the evolution of pluripotent cells differentiation. As a consequence, various pluripotent cell-based strategies exhibited uncertain neuroprotective efficacy in previous clinical trials.

This Research Topic contains 12 manuscripts, highlighting current understanding and future directions in pluripotent cell-based therapies in stroke. Due to their proliferation and differentiation potential, both endogenous and exogenous stem cells are candidates for neural regeneration and neural circuit remodeling. Liu et al. summarize the mechanisms, processes, and challenges of using stem cells in stroke treatment. They conclude that specific cell types, dosages and routes, and other issues for stem cell-based approaches still need to be optimized in the near future. Zhang et al. systemically review stem cell-based therapies for experimental ischemic stroke. They conclude that stem cell-based therapies are able to improve neurological function and reduce infarct volume, but further clinical studies are needed to verify these neuroprotective effects. Mesenchymal stem cells (MSCs) have been proven effective in ischemic stroke (Li et al.), intracerebral hemorrhage (Gong et al.), and neurodegeneration diseases, such as Alzheimer's Disease (Yan et al.). Islam et al. also report that human neuroepithelial precursor cells (cNEPs) grafted early after stroke are

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Edited and reviewed by:
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Specialty section:
This article was submitted to Cellular Neurophysiology, a section of the journal Frontiers in Cellular Neuroscience

Received: *08 July 2021*
Accepted: *13 July 2021*
Published: *04 August 2021*

Citation:
Chen Y, Yao Y, Zhang JH and Hao S (2021) Editorial: Pluripotent Cells for Stroke: From Mechanism to Therapeutic Strategies. Front. Cell. Neurosci. 15:738240. doi: 10.3389/fncel.2021.738240

able to improve functional recovery. Traditional Chinese medicine monomers, active components extracted from Chinese herbs, also exhibit the potential to activate proliferation and neurogenesis of neural stem cells after stroke (Wang et al.). Mechanistic studies show that canonical Wnt/β-catenin signaling and other molecules play a pivotal role in neural stem/progenitor cell differentiation (Kriska et al.).

Due to the limitations of neural stem cells, induced pluripotent stem cells (iPSCs) have been used as an alternative cell type in stroke treatment. iPSCs, produced by introducing specific transcription factors into somatic cells, exhibit similar differentiation potential as embryonic stem cells. Duan et al. summarize the current applications of iPSC therapy in ischemic stroke. In addition, inspired by iPSC and *in vivo* reprogramming technology, endogenous and reactive glial cells have been *in situ* converted to functional neurons for the treatments of stroke, neurodegenerative diseases, retinal diseases, and trauma in central nervous system (Wang et al., 2021).

In the concept of neuro-vascular network (Zhang et al., 2012), non-neuronal cells are equally important and play an essential role in neural circuit remodeling and neurofunctional recovery. Microglial cells, major immune cells in brain, are influenced by low dose of valproic acid after stroke, possibly via interleukin-6- and galectin-3-mediated extracellular matrix remodeling (Kuo et al.). Gan et al. elucidate the regulatory functions of lncRNAs on angiogenesis after stroke, which are important for vascular reconstruction and blood flow support of neural circuit remodeling. In addition, Cao et al. summarize the functions of pericytes as perivascular multi-potent cells and an important component of the blood-brain barrier in the central nervous system (Gautam and Yao, 2018). They discuss how pericyte dysfunction affects the pathophysiological processes of stroke. A subset of pericytes have been shown to trans-differentiate into neurons in the damaged tissue (Karow et al., 2018).

In summary, there are 12 outstanding manuscripts, including both original studies and comprehensive reviews, in this Research Topic. We summarize recent findings and challenges of pluripotent cell-based therapies in stroke, and propose to explore the mechanisms of directed differentiation and optimize pluripotent cells products & *in vivo* tracking techniques in further studies.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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

frontiers in Molecular Neuroscience

REVIEW
published: 04 February 2021
doi: 10.3389/fnmol.2020.613976

The Regulatory Functions of lncRNAs on Angiogenesis Following Ischemic Stroke

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Ischemic stroke is one of the leading causes of global mortality and disability. It is a multi-factorial disease involving multiple factors, and gene dysregulation is considered as the major molecular mechanisms underlying disease progression. Angiogenesis can promote collateral circulation, which helps the restoration of blood supply in the ischemic area and reduces ischemic necrosis following ischemic injury. Aberrant expression of long non-coding RNAs (lncRNAs) in ischemic stroke is associated with various biological functions of endothelial cells and serves essential roles on the angiogenesis of ischemic stroke. The key roles of lncRNAs on angiogenesis suggest their potential as novel therapeutic targets for future diagnosis and treatment. This review elucidates the detailed regulatory functions of lncRNAs on angiogenesis following ischemic stroke through numerous mechanisms, such as interaction with target microRNAs, downstream signaling pathways and target molecules.

Keywords: long non-coding RNAs, ischemic stroke, angiogenesis, endothelial cells, therapeutic targets

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Received: *04 October 2020*
Accepted: *28 December 2020*
Published: *04 February 2021*

Citation:
Gan L, Liao S, Xing Y and Deng S (2021) The Regulatory Functions of lncRNAs on Angiogenesis Following Ischemic Stroke. Front. Mol. Neurosci. 13:613976. doi: 10.3389/fnmol.2020.613976

INTRODUCTION

Stroke is a type of acute cerebral vascular disease with high disability and mortality rate. It is the second leading cause of death and the third leading cause of disability worldwide (Mozaffarian et al., 2016; Feigin et al., 2017). The age-adjusted stroke mortality rate was 35/100,000, and there were 16.9 million cases of incident first stroke in 2010. Recently, the incidence of stroke increases rapidly and it is the leading cause of death in China, as over two million new cases were diagnosed in 2017 (Feigin et al., 2014; Wu S. et al., 2019). Ischemic stroke accounts for ∼80–85% of all acute strokes, and its prevalence remarkably increases with advancing age (Randolph, 2016). Ischemic stroke is caused by cerebral artery occlusion, which reduces cerebral blood flow and causes neuronal cell death and brain dysfunction. The quality of life of patients can be severely affected by brain injury induced neurological deficits, leading to growing financial burden of health care.

It is well-established that early recanalization of blood vessels contributes to the restoration of blood supply in ischemic zone, and reduction of cerebral infarction area is the most effective treatment for acute ischemic brain injury. Angiogenesis following ischemic brain injury serves essential roles in clinical practice. Recent studies have indicated that enhancement of angiogenesis is a promising strategy for the treatment of ischemic stroke (Sun et al., 2020). Further understanding the detailed molecular mechanisms of post-ischemic stroke angiogenesis will facilitate the development of therapeutics.

Long non-coding RNAs (lncRNAs) are endogenous non-coding RNAs, which are >200 nucleotides in length but lack of open-reading frame. LncRNAs have been identified as key factors

in regulating the expression and function of protein-coding genes through numerous mechanisms. LncRNAs with complex secondary and tertiary structure serve essential roles on epigenetics, transcription, and post-transcriptional modification via binding with target DNA, RNA or protein (Mousavi et al., 2013; Toiyama et al., 2014). LncRNAs are key regulators in biological processes such as cell proliferation, differentiation, apoptosis, autophagy, and angiogenesis, and they are involved in the pathogenesis of various types of diseases including tumors (Takahashi et al., 2020; Zhang Y. et al., 2020), cardiovascular disorders (Mao et al., 2019; Bian et al., 2020), and nervous system diseases (Liang et al., 2020). LncRNAs are considered as novel regulators in the pathogenesis of ischemic stroke. A recent study has suggested that lncRNAs are involved in the regulation of post-ischemic stroke gene expression (Yan Y. et al., 2020). Clinical research has also revealed that dysregulation of lncRNAs could be an essential mechanism in ischemic stroke (Huang J. et al., 2019). Therefore, lncRNAs could be novel therapeutic candidates in future targeted therapy.

However, the detailed roles and underlying mechanisms of lncRNAs on the pathogenesis of ischemic stroke remain largely unknown. Therefore, in order to explore putative therapeutic targets, this review highlights the importance of specific lncRNAs in angiogenesis following ischemic stroke.

BIOLOGICAL FUNCTIONS OF LncRNAs

LncRNAs regulate gene expression through affecting epigenetics, transcription and translation. They serve essential physiological and pathological roles and are involved in numerous signaling pathways during the development of various diseases. LncRNAs are also considered as potential biomarkers. A previous study has indicated that H19 can target ACP5 protein directly and is a putative risk factor of ischemic stroke (Huang Y. et al., 2019). According to the subcellular distribution of lncRNAs, they are localized within the nucleus or cytoplasm, and previous studies have revealed their regulatory functions on gene expression at both transcriptional and post-transcriptional levels.

LncRNAs are involved in the regulation at the transcriptional level through acting as chromatin remodelers and transcriptional coactivators. Nuclear lncRNAs can regulate the functions of chromosomes by recruiting chromatin modifying complexes to specific sites on chromosomes, which consequently affects histone methylation and deacetylation; alternatively, lncRNAs can bind to regulatory transcription factors and modulate the transcription of target genes (Zhang C. L. et al., 2017; Lei et al., 2019; Zhang L. et al., 2019). At post-transcriptional level, cytoplasmic lncRNAs can regulate the expression of target gene indirectly through the downstream miRNAs (Zhuang et al., 2019). LncRNAs can also affect pre-mRNA splicing, mRNA translation and localization. For instance, some lncRNAs contain the complementary binding sites of target miRNAs and function as competing endogenous RNA (ceRNA) through binding certain miRNA competitively, which results in the upregulation of target mRNA (Cao et al., 2020). In addition, due to the existence of lncRNAs base-pairing with pre-mRNAs, the splicing of pre-mRNAs could be prevented, which subsequently affects the transcription of target genes (Szcześniak and Makałowska, 2016). Furthermore, certain lncRNAs are involved in the pathophysiology of diseases through binding to mRNA directly, which influences the degradation and translation of mRNAs (Wu H. et al., 2019).

REGULATORY MECHANISMS OF LncRNAs IN ISCHEMIC STROKE

LncRNAs are a group of non-coding RNAs involved in numerous biological processes and they are also considered as regulatory factors in ischemic stroke. In the human genome, ∼40% of differentially expressed lncRNAs were detected in the brain and localized in specific cell types and subcellular structures in neural regions, which serve essential roles during brain injury (Derrien et al., 2012; Wang et al., 2017b). A recent study revealed significant alterations of lncRNAs in the brain after cerebral ischemia, suggesting the important physiological and pathological roles of lncRNAs on initiating endothelial responses to ischemic stimuli at transcriptional and translational level. Differentially expressed lncRNAs were detected in blood samples from patients with acute ischemic stroke, rat models of middle cerebral artery occlusion (MCAO), and oxygen/glucose deprivation (OGD) cells (Guo et al., 2018; Barangi et al., 2019; Zhu et al., 2019). The abovementioned lncRNAs are involved in various biological processes of ischemic stroke including cell proliferation, apoptosis (Zhang L. et al., 2020), oxidative stress, inflammation (Zhang and Zhang, 2020) and autophagy (Luo et al., 2020). Previous studies suggested that lncRNAs exert various regulatory functions after stroke. For example, they act as miRNA sponges competing with mRNA for miRNA binding. Moreover, lncRNAs could also regulate the expression of target genes directly through numerous signaling pathways.

Regulation of Neurogenesis

Previous studies have revealed that altered levels of lncRNAs were associated with neurogenesis after stroke. A recent study indicated that N1LR inhibited neuronal apoptosis after ischemic stroke through inactivating p53 (Wu et al., 2017). Similarly, MEG3 interacted with p53 to mediate neuronal death (Yan et al., 2016). Furthermore, knockdown of MEG3 could suppress neuronal death by targeting the miR-21/PDCD4 signaling pathway (Yan et al., 2017). MEG3 induced autophagy and neuronal cell death via the miR-378/GRB2 axis, which subsequently suppressed the activation of Akt/mTOR signaling (Luo et al., 2020).

A previous study also indicated that MALAT1 promoted neuronal cell death and suppressed autophagy through targeting miR-30a in ischemic stroke (Guo et al., 2017). In consistence with these findings, H19 induced neuronal death by activating autophagy in ischemic stroke (Wang et al., 2017a). In addition, GAS5 inhibited cell death and suppressed neuronal survival through targeting the miR-137/Notch1 signaling pathway (Chen et al., 2018). Furthermore, SNHG14 promoted neuronal

apoptosis through microglia activation via regulating the miR-145-5p/PLA2G4A axis in cerebral infarction (Qi et al., 2017).

Regulation of Inflammation

Proinflammatory cytokines were released after ischemic brain injury, consequently activating NF-κB pathway that is involved in inflammatory responses. Nespas inhibited proinflammatory cytokine production and suppressed NF-κB signaling after ischemic stroke through TAK1 activation (Deng Y. et al., 2019). Gm4419 promoted neuroinflammation by inducing IκB phosphorylation and NF-κB signaling activation after ischemic stroke, and the levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 were increased (Wen et al., 2017). A previous study also indicated that H19 promoted neuroinflammation by M1 microglial polarization, which lead to increased production of TNF-α and IL-1β in ischemic stroke (Wang et al., 2017b).

Regulation of Angiogenesis

Previous studies have suggested that lncRNAs serve essential roles on the regulation of endothelial cell survival, vascular integrity and angiogenesis in stroke. In this review, the mechanisms underlying lncRNA-modulated angiogenesis are summerized.

Recent studies have revealed altered expression of lncRNAs in ischemic stroke (He et al., 2018; Ruan et al., 2020). Recently, lncRNA-mediated gene regulation has been intensively investigated. LncRNAs can regulate gene expression in ischemic stroke through affecting mRNA stability and epigenetic modifications. They function as decoys, scaffolds and enhancer RNAs of corresponding targets to regulate their functions. Furthermore, lncRNAs can interact with DNA and RNA by modulating pre-mRNA splicing or acting as miRNA sponge, resulting in impaired miRNA expression and functions. Numerous lncRNAs are associated with angiogenesis after ischemic stroke through affecting the transcription and translation (**Figure 1**, **Table 1**).

BRAIN MICROVASCULAR ENDOTHELIAL CELLS (BMECs)

BMECs are major structural and functional elements of brain microvasculature, which serve key roles on maintaining the integrity of the blood-brain barrier (BBB) and brain homeostasis by responding to the changes in cellular microenvironment dynamically. Ischemic stroke could cause structural and functional injuries to cerebrovascular endothelial cells, consequently increasing cerebrovascular permeability and damaging the BBB, which can result in neuroinflammation and neuronal damage. In addition, ischemic stroke results in BMECs injury and death. During cerebral ischemia, BMECs are stimulated by inflammatory mediators, which activates proinflammatory responses.

Angiogenesis after ischemic stroke promotes collateral circulation and restores blood supply in the ischemic area, subsequently reducing ischemic necrosis caused by ischemic injury and reversing the nerve damage (Li L. et al., 2017). LncRNAs were abundantly expressed in vascular endothelial cells and involved in endothelial biology and physiological functions.

Zhang et al. (2016) evaluated the expression levels of lncRNAs in primary BMECs after OGD using RNA-sequencing (RNA-seq) and differentially expressed lncRNAs were detected, including 147 up-regulated and 70 down-regulated lncRNAs. Among them, MALAT1 and SNHG12 are the most up-regulated lncRNAs. Highly conserved binding sites of transcription factors on the promoter region of lncRNAs reveals potential transcriptional regulation of lncRNAs, which could be associated with angiogenesis and serve essential roles on the response to ischemic stimuli; however, the detailed functions of lncRNAs in cerebral vascular endothelial biology is unclear.

A previous study indicated that cerebral vascular damage was aggravated in MALAT1 knock-out (KO) mice, where larger cerebral infarct volume and more severe neurological deficits were detected in response to ischemic insults (Zhang X. et al., 2017). Activation of MALAT1 was able to protect cerebral microvascular endothelial cells from ischemic injury (Yang et al., 2018). Additionally, SNHG12 was significantly up-regulated in microvascular endothelial cells under OGD/R conditions. Overexpression of SNHG12 inhibited brain microvascular endothelial cell death and inflammatory response, but promoted angiogenesis following OGD/R. Furthermore, SNHG12 suppressed brain microvascular endothelial cell injury by targeting miR-199a (Long et al., 2018). In addition, MACC1-AS1 was down-regulated in hypoxia-induced human brain microvascular endothelial cells (HBMECs). Overexpressed MACC1-AS1 reduced cell apoptosis and oxidative stress, while promoting cell proliferation, migration, and angiogenesis; meanwhile, cell permeability was decreased, which can affect cell barrier function. Moreover, MACC1-AS1 exert protective function of anti-apoptosis, pro-angiogenesis and anti-HBMECs injury via targeting the miR-6867-5p/TWIST1 signaling (Yan G. et al., 2020). Overexpression of FAL1 could reverse OGD/R-induced injury and cell death in brain microvascular endothelial cells through ameliorating oxidative stress, suppressing the secretion of pro-inflammatory cytokines and activating the PAK1/AKT signaling pathway (Gao M. et al., 2020).

BMECs AND INFLAMMATION

Accumulating evidence revealed the importance of inflammation in the pathogenesis of stroke, which involves the networking of numerous inflammatory cells and cytokines. Multiple inflammatory cells and cytokines participate in various stages of ischemic stroke. Aberrantly expressed lncRNAs in ischemic stroke are associated with the production of inflammatory factors and activation of various signaling pathways. Cerebral endothelial cells are stimulated by inflammatory mediators produced by neuronal cells in ischemic brains, which triggers proinflammatory activation. Activated cerebral endothelium could release inflammatory factors such as IL-1β, IL-6, MCP-1 and induce the activation of NF-κB pathway, consequently leading to cerebral ischemic injury (Shekhar et al., 2018). Moreover, OGD/R could aggravate inflammation by enhancing

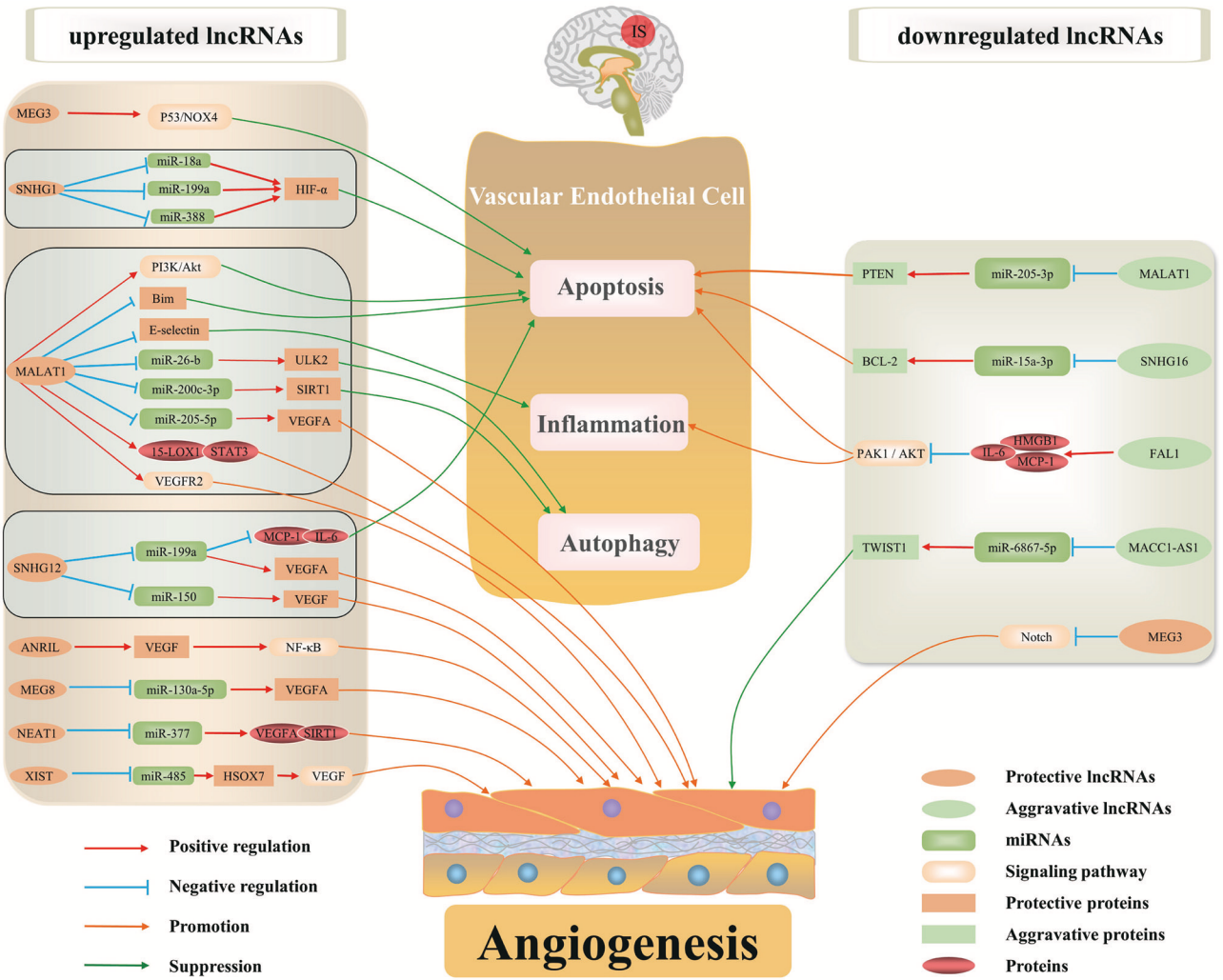

FIGURE 1 | The regulatory mechanisms of lncRNAs in ischemic stroke-induced angiogenesis. Some lncRNAs serve essential roles on angiogenesis after ischemic stroke by regulating inflammation, autophagy and apoptosis of BMECs, or directly regulating angiogenesis-associated proteins/signaling pathways to maintain vascular function and integrity. Certain lncRNA may regulate the expression of multiple target genes in various pathways involved in angiogenesis.

the production of proinflammatory cytokines. Additionally, lncRNAs could regulate cerebrovascular function in ischemic stroke through targeting apoptotic factors.

MALAT1 could bind to IL-6, E-selectin and MCP-1, which prevent OGD/R-induced inflammatory response in ischemic stroke by reducing the production of proinflammatory cytokines (Zhang X. et al., 2017; Yang et al., 2018). For instance, SNHG12 was up-regulated under OGD/R conditions in microvascular endothelial cells of mouse brain, which decreased the mRNA and protein levels of proinflammatory cytokines E-selectin, MCP-1 and IL-6, subsequently inhibiting the inflammatory response. Furthermore, SNHG12 exerts anti-inflammatory function in brain microvasculature cells after OGD/R by reducing ischemic cerebral vascular injury and parenchymal damage (Long et al., 2018). In addition, FAL1 was significantly down-regulated in OGD/R-stimulated HBMECs, and overexpression of FAL1 ameliorated OGD/R-induced oxidative stress by suppressing the production of IL-6, MCP-1 and HMGB-1. FAL1 might serve essential roles on regulating OGD/R-induced inflammation in HBMECs (Gao M. et al., 2020). LncRNAs were involved in the inflammatory response in BMECs, which might affect brain endothelial cell damage through modulating the release of inflammatory factors in BMECs.

BMECs AND AUTOPHAGY

Autophagy is a lysosome-dependent homeostatic process. As an endogenous defense mechanism, it might also inhibit cell death. It has been well-established that lncRNAs are involved in the pathogenesis of ischemic stroke through regulating cell autophagy, which protects BMECs from ischemic injury.

TABLE 1 | The roles of lncRNAs on angiogenesis in ischemic stroke.

lncRNA	Change in expression	Target miRNA	Target protein	Signaling pathway	Function	Reference(s)
MEG3	Decreased			Notch	Promote angiogenesis	Liu et al., 2017
MEG3	Increased			P53/NOX4	Promote apoptosis; inhibit angiogenesis	Zhan et al., 2017
MEG3	—	miR-9			Suppress proliferation and angiogenesis	He et al., 2017
MEG8	Increased	miR-130a-5p	VEGF-A		Promote cell viability, migration, angiogenesis	Sui et al., 2020
MALAT1	—			CREB/PGC-1α/PPARγ	Protect cerebrovascular endothelial cells and BBB integrity	Ruan et al., 2019
MALAT1	Increased/decreased			PI3K/Akt	Inhibit apoptosis	Xin and Jiang, 2017; Wang G. et al., 2018
MALAT1	Increased			VEGFR2	Promote angiogenesis	Zhang X. et al., 2018
MALAT1	Increased			15-LOX1/STAT3	Promote proliferation, migration, angiogenesis	Wang C. et al., 2019
MALAT1	Increased			MDM2/P53	Promote apoptosis	Zhang T. et al., 2018
MALAT1	Increased		E-selectin		Reduce inflammatory	Zhang X. et al., 2017; Yang et al., 2018
MALAT1	Increased		Bim		Reduce apoptosis	Zhang X. et al., 2017; Yang et al., 2018
MALAT1	Increased	miR-126		PI3K/Akt	Inhibit proliferation and induce apoptosis	Zhang L. et al., 2020
MALAT1	Increased	miR-26b	ULK2		Activate autophagy; suppress apoptosis	Li Z. et al., 2017
MALAT1	Increased	miR-200c-3p	SIRT1		Activate autophagy; suppress apoptosis	Wang S. et al., 2019
MALAT1	Increased	miR-205-5p	VEGFA		Promote proliferation, angiogenesis	Gao C. et al., 2020
MALAT1	Decreased	miR-205-3p	PTEN		Promote apoptosis	Gao and Wang, 2020
SNHG1	Increased	miR-18a	HIF-1α	HIF-1α/VEGF	Inhibit apoptosis, promote BMEC survival	Zhang L. et al., 2018
SNHG1	Increased	miR-199a	HIF-1α, VEGFA		Promote angiogenesis, promote BMEC survival	Wang Z. et al., 2018
SNHG1	Increased	miR-338	HIF-1α	HIF-1α/VEGF-A	Inhibit apoptosis	Yang and Zi, 2019
SNHG12	Increased	miR-199a	MCP1, IL-6, VEGFA		Inhibit inflammatory and cell death; promote angiogenesis	Long et al., 2018
SNHG12	Increased	miR-150	VEGF		Promote proliferation, migration, angiogenesis	Zhao et al., 2018
SNHG16	Decreased	miR-15a-5p	BCL-2		Promote cell apoptosis	Teng et al., 2020
ANRIL	Increased		VEGF	NF-κB	Promote angiogenesis	Zhang B. et al., 2017
FAL1	Decreased		MCP1, IL-6, HMGB1	PAK1/AKT	Promote apoptosis and inflammatory; inhibit proliferation	Gao M. et al., 2020
XIST	Increased	miR-485	SOX7	VEGF	Promote proliferation, migration, angiogenesis	Hu et al., 2019
MACC1-AS1	Decreased	miR-6867-5p	TWIST1		Promote apoptosis and oxidative stress, inhibit angiogenesis	Yan G. et al., 2020
NEAT1	Increased	miR-377	VEGFA, SIRT1		Promote angiogenesis	Zhou et al., 2019
Rmst	Increased	miR-150			Promote apoptosis; inhibit proliferation, migration	Qiao et al., 2020
MIAT	Increased	miR-204-5p	HMGB1		Inhibit angiogenesis	Deng W. et al., 2020

Autophagy can be activated by OGD/R as a defense response to protect BMECs against OGD-induced injury. Furthermore, lncRNAs are novel regulators involved in autophagy, and they can affect autophagy through downstream miRNAs; however, the underlying molecular mechanisms remain unclear.

Li Z. et al. (2017) revealed that MALAT1 was up-regulated in BMECs following OGD/R, which activated autophagy and promoted cell survival through down-regulating miR-26b and up-regulating ULK2. Wang S. et al. (2019) suggested that MALAT1 stimulated autophagy activation in BMECs via the miR-200c-3p/SIRT1 signaling, subsequently down-regulating p62 and inhibiting cell death. At present, the molecular mechanisms of autophagy in ischemic stroke are still unclear. Therefore, the detailed mechanisms of lncRNA-modulated regulatory functions on autophagy in BMECs under OGD/R conditions require further investigation.

BMECs AND APOPTOSIS

Dysfunction in the blood-brain barrier caused by the apoptosis of microvascular endothelial cells is one of the key pathological features of ischemic stroke, which contributes to secondary damage and poor prognosis in patients. LncRNAs might be involved in the apoptosis of BMECs through targeting apoptotic factors or various signaling pathways associated with the development of stroke. A recent study indicated that OGD/R down-regulated FAL1 and further reduced the phosphorylation of PAK1 and AKT, consequently promoting OGD/R-induced cell death. FAL1 could serve essential roles on regulating OGD/R-induced endothelial damage in HBMECs (Gao M. et al., 2020). Xin and Jiang (2017) revealed that the levels of MALAT1 in cells under OGD-R conditions were significantly increased, but down-regulation of MALAT1 was detected as oxygen levels increased. Additionally, MALAT1 suppressed OGD/R-induced apoptosis and the activity of caspase-3 in HBMECs by activating the PI3K/Akt pathway. These findings suggested that MALAT1 could protect HBMECs against OGD/R-induced endothelial damage. Furthermore, a previous study indicated that the expression of MALAT1 was down-regulated in OGD-induced apoptosis and negatively correlated with the expression of miR-205-3p. Subsequently, down-regulation of MALAT1 induced a reduction in PTEN levels, which further promoted OGD-induced apoptosis (Gao and Wang, 2020).

However, a recent study also revealed OGD-induced up-regulation of MALAT1 and down-regulation of miR-126 in HBMECs. In addition, knockdown of MALAT1 activated the PI3K/Akt pathway, increased the expression of phosphorylated PI3K/Akt and inhibited cell apoptosis. Moreover, MALAT1 down-regulated the expression of miR-126, which subsequently suppressed the proliferation and enhanced the apoptosis through inactivating the PI3K/AKT pathway. Therefore, up-regulation of MALAT1 could promote the development of ischemic stroke (Zhang L. et al., 2020). Another study indicated that MALAT1 could induce cell apoptosis. MALAT1 activated p53 signaling through targeting MDM2, consequently promoting the progression of ischemic stroke (Zhang T. et al., 2018). Furthermore, a previous study suggested that the expression of SNHG1 was remarkably increased in BMECs under OGD conditions and reversely correlated with the expression of miR-18a. Additionally, SNHG1 acted as miR-18a sponge and regulated the expression of its endogenous target HIF-1α, which inhibited cell apoptosis and promoted the survival of BMECs via the HIF-1α/VEGF signaling (Zhang L. et al., 2018). Another study also revealed that the levels of SNHG1 and miR-338 were up-regulated in OGD-induced BMECs. Knockdown of SNHG1 reduced the expression of HIF-1α/VEGF-A and promote the apoptosis of BMECs through targeting miR-338. As a result, OGD-induced injury in BMECs was aggravated. Therefore, SNHG1 exerted protective function against OGD-induced damage in BMECs via sponging miR-338 and up-regulate the HIF-1α/VEGF-A signaling (Yang and Zi, 2019). Additionally, the expression of SNHG12 was significantly increased in BMECs under OGD conditions, which inhibited cell death through targeting miR-199a (Long et al., 2018). Furthermore, SNHG16 suppressed OGD-induced apoptosis in HBMECs via the miR-15a-5p/Bcl-2 axis (Teng et al., 2020). In addition, up-regulation of Rmst inhibited cell proliferation/migration and promoted the apoptosis of OGD-induced cells through targeting miR-150 (Qiao et al., 2020). Moreover, MIAT was overexpressed in OGD-treated BMECs, and it could affect the injury of BMECs after cerebral ischemia through targeting the miR-204-5p/HMGB1 signaling (Deng W. et al., 2020). Taken all together, previous studies revealed the involvement of numerous lncRNAs in the apoptosis of BMECs after ischemic stroke.

BMECs AND ANGIOGENESIS

The key steps of angiogenesis include the proliferation and migration of blood vascular endothelial cells. The Notch and VEGF signaling pathways are involved in angiogenesis, which could regulate cell differentiation and migration, contributing to the formation of blood vessels. Ischemic stroke promoted the proliferation and migration of BMECs, subsequently activating angiogenesis. Angiogenesis is a complex process that involves the regulation of numerous genes. LncRNAs are novel key regulators associated with endothelial dysfunction after ischemic stroke. Thus, lncRNAs could be involved in angiogenesis of endothelial cells by maintaining vascular function and integrity through various pathways.

Michalik et al. (2014) revealed that silenced MALAT1 could suppress the proliferation of vascular endothelial cells, suggesting that MALAT1 was able to promote endothelial cell growth and might serve essential roles on protecting cerebral microvasculature against ischemic brain injury. Furthermore, lncRNAs regulated the expression of proximal protein, which are major angiogenic molecules, subsequently affecting the process of angiogenesis. In addition, MALAT1 could enhance angiogenesis by binding to VEGFR2 (Zhang X. et al., 2018). Wang C. et al. (2019) also indicated that OGD/R increased the expression levels of MALAT1, 15-LOX1, VEGF and pSTAT3. Knockdown of MALAT1 inhibited the proliferation and migration of BMECs, suggesting that MALAT1 could promote angiogenesis through activating the 15-LOX1/STAT3 axis.

Furthermore, lncRNAs might be involved in angiogenesis through numerous signaling pathways. A previous study indicated that HOTTIP promoted the growth of BMECs via

activating the Wnt/β-catenin axis (Liao et al., 2018). Zhang B. et al. (2017) also suggested that the expression of ANRIL was significantly up-regulated in the infarcted tissues of MCAO rat model. In addition, overexpression of ANRIL enhanced the expression of VEGF and promoted the angiogenesis by activating the NF-κB signaling.

LncRNAs could also function as ceRNAs of miRNAs to regulate the expression of target mRNAs, further affecting the process of angiogenesis. For instance, MEG3 is a key regulator in the proliferation and angiogenesis of BMECs by acting as miR-9 sponge (He et al., 2017). Moreover, MEG8 was up-regulated in OGD-treated BMECs. Silenced MEG8 inhibited the viability, migration and angiogenesis of BMECs via targeting miR-130a-5p. These findings suggested that MEG8 could protect BMECs from OGD-induced injury by promoting the angiogenesis following ischemic stroke through the miR-130a-5p/VEGFA axis (Sui et al., 2020). In addition, the expression of NEAT1 was elevated in BMECs under OGD conditions, which promoted the angiogenesis in BMECs by targeting the miR-377/SIRT1/VEGFA axis (Zhou et al., 2019). Both SNGH1 and SNGH12 were remarkably up-regulated in BMECs under OGD/R conditions, which promoted the survival and migration of BMECs. SNGH1 and SNGH12 exert their functions through targeting miR-199a and up-regulating their downstream molecules VEGFA and FGFb (Long et al., 2018; Wang Z. et al., 2018). Similarly, another study has suggested that up-regulation of SNHG12 could facilitate the restoration of neurological function in the infarct boundary zone of MCAO mice via the miR-150/VEGF signaling. Infarct volume was reduced, and cell growth/angiogenesis was enhanced by up-regulated SNHG12, suggesting that SNHG12 could promote angiogenesis following ischemic stroke (Zhao et al., 2018). Furthermore, XIST induced cell proliferation, migration and angiogenesis in BMECs via the miR-485/SOX7 axis (Hu et al., 2019). Another study also revealed that MALAT1 expression was increased in OGD/R-induced HBMECs. Up-regulated MALAT1 promoted cell proliferation and served protective roles on angiogenesis by targeting the miR-205-5p/VEGFA signaling in HBMECs under OGD/R conditions (Gao C. et al., 2020).

In summary, these findings indicated that lncRNAs including MALAT1, HOTTIP, ANRIL, MEG3, MEG8, NEAT1, SNHG1, SNHG12 and XIST were involved in post-ischemic stroke angiogenesis. Abovementioned lncRNAs are key regulators in angiogenesis by acting as ceRNA or targeting downstream molecules/pathways directly.

LncRNAs AS NOVEL DIAGNOSTIC AND PROGNOSTICS BIOMARKERS FOR ISCHEMIC STROKE

Recently, numerous lncRNAs have been identified using genome-wide analyses, and they are involved in various physiological and pathological processes in ischemic stroke through the regulation at both transcriptional and post-transcriptional levels. Previous findings revealed alteration of lncRNAs expression profiles in peripheral blood samples from patients with ischemic stroke, suggesting that lncRNAs could be novel diagnostic and prognostic biomarkers for this disease. A total of 206 lncRNAs were differentially expressed in the specimens from ischemic stroke patients, including 70 up-regulated and 128 down-regulated lncRNAs (Deng et al., 2018). Xu et al. (2020) revealed differential expression of 1,096 lncRNAs in the exosomes isolated from blood samples of patients with ischemic stroke, including 307 up-regulated and 789 down-regulated lncRNAs. Moreover, lnc-CRKL-2, lnc-NTRK3-4, RPS6KA2-AS1 and lnc-CALM1-7 could be potential biomarkers for the early diagnosis of ischemic stroke. Furthermore, Guo et al. (2018) suggested that 1,250 lncRNAs were differentially expressed in the peripheral blood samples following ischemic stroke. Additionally, ENST00000568297, ENST00000568243 and NR_046084 were identified as novel diagnostic biomarkers for ischemic stroke.

Moreover, the levels of lncRNAs might be associated with the severity of stroke. A previous study was performed on the plasma samples from patients with ischemic stroke, and up-regulated levels of lncRNAs including H19, MIAT, ANRIL, MEG3 and NEAT1 were detected. Up-regulation of abovementioned lncRNAs were positively correlated with the National Institutes of Health Stroke Scale (NIHSS) scores and infarct volume, which could affect the progression of ischemic stroke. Furthermore, the levels of H19 and NEAT1 were associated with the production of inflammatory factors including TNF-α, IL-6, IL-8, and IL-22. In addition, the expression levels of MIAT, ANRIL and NEAT1 were related to the up-regulation of high-sensitivity C-reactive protein. Abovementioned lncRNAs were considered as novel biomarker for the diagnosis of ischemic stroke (Wang et al., 2017b; Zhu et al., 2018; Zhang K. et al., 2019). In contrast, previous findings also revealed that ZFAS1 was significantly down-regulated in patients with large-artery atherosclerosis (LAA) stroke compared to non-LAA stroke group (Wang J. et al., 2019).

Some lncRNAs are associated with the prognosis of patients with ischemic stroke. A previous study indicated that the expression of MIAT was correlated with the therapeutic outcome. MIAT could be an unfavorable indicator of functional recovery, as reduced MIAT levels were detected in patients with favorable outcome, and vice versa. More importantly, the risk of death was higher in high MIAT expression group compared with the patients with low MIAT expression, in whom better prognosis and survival rate were observed (Zhu et al., 2018). In consistence with these findings, Wang et al. (2020) also suggested that MEG3 expression was associated with poor prognosis in patients with ischemic stroke. The risk of death was increased in high MEG3 expression group compared with the control. Furthermore, the expression levels of NEAT1 in patients with recurrence were elevated compared with non-recurrence group. In addition, patients with high NEAT1 expression exhibited shorter RFS compared with low NEAT1 expression group (Li et al., 2020). These studies revealed the expression profiles of lncRNAs in patients with cerebral ischemia, suggesting that lncRNAs could be potential diagnostic and prognostic biomarkers for ischemic stroke.

Previous studies have indicated that microvessel density in the peri-infarct region was increased in patients with ischemic stroke.

Angiogenesis was able to promote the recruitment of blood flow and metabolic nutrients into the infarct regions, subsequently facilitating functional recovery. Enhancement of angiogenesis is a promising therapeutic strategy for the treatment of ischemic stroke (Oshikawa et al., 2017; Zhang J. et al., 2019).

At present, the most effective treatment for ischemic stroke is vascular recanalization including interventional treatment and intravenous thrombolysis, which could restore the cerebral blood flow and reduce the infarction size. Until now, recombinant tissue plasminogen activator (rtPA) is the only FDA-approved drug for the treatment of ischemic stroke. However, the use of tPA is limited by its narrow therapeutic window and side effects including high risk of secondary bleeding. Patients with proximal occlusion in large arteries of the brain could be subjected to endovascular interventions. So far, no effective treatment is available for patients whose blood vessels cannot be recanalized at early stage. Therefore, new therapeutic strategies should be developed for patients with ischemic stroke, and enhancement of angiogenesis is a major target in the treatment of this disease.

RNA therapeutic strategies have been developed to intervene the angiogenesis after ischemic stroke. Therapeutic strategies by manipulation of lncRNAs have been evaluated in the treatment of cancer and ulcerative colitis (Velagapudi et al., 2017; Vautrin et al., 2019). RNA therapeutics mainly base on the inhibition or overexpression of certain RNAs. RNA knockdown technique was used to examine the functions of target RNAs. As lncRNAs are located in the nucleus and/or cytoplasm, antisense therapy and RNA interference (RNAi) are commonly used to induce the degradation of lncRNAs (Lennox and Behlke, 2016, 2020). MEG3 was upregulated in cerebral infarction area, and MEG3 knockdown vector was transfected into the striatum of ischemia/reperfusion rat, which reduced infarct size and promoted angiogenesis (Liu et al., 2017). Furthermore, treatment with nano polymer wrapped MEG3 shRNA conjugated with OX26 antibody was able to reduce the ischemic lesion volumes and enhance the angiogenesis in cerebral infarction area (Shen et al., 2018). MALAT1 was upregulated in mouse BMECs after OGD. Antisense LNA GapmeR was used to knockdown MALAT1, which increased cell death, aggravated inflammatory reaction and reduced vascular growth in BMECs (Michalik et al., 2014; Zhang X. et al., 2017). Chemical modifications of lncRNAs could also improve the therapeutic efficacy (Zhang H. et al., 2019). The effects of adeno associated virus (AAV), CRISPR/Cas9, gapmer antisense oligonucleotides (ASOs) remain to be further elucidated.

However, the translational value of most lncRNAs remains unclear. Firstly, in previous studies on the regulatory roles of lncRNAs in ischemic stroke, sample sizes are relatively small, and further large-scale cohort studies are required to confirm the existing findings. LncRNAs are not necessarily conserved in different species. Most studies on lncRNAs were performed on animals, but the effects of putative therapeutics on patients with ischemic stroke should be considered. Secondly, the detailed regulatory functions of lncRNAs should be identified. Better understanding of the mechanisms could improve drug development to reduce off-target effects. Furthermore, the complex nature of brain structure and function resulted in various regulatory mechanisms of lncRNAs in different types of brain cells involved in ischemic stroke, a better understanding of lncRNAs in specific cell types under normal and pathophysiological conditions could improve the specificity of lncRNA-targeted therapies. In addition, multiple lncRNAs are simultaneously involved in the regulation of angiogenesis after ischemic stroke, combinations of lncRNAs with different pathological targets could improve the therapeutic outcome of cerebral ischemia. The accuracy and stability of RNA-based therapeutics should be considered in clinical practice.

CONCLUSION

Emerging evidence has suggested that lncRNAs serve essential roles in the pathogenesis of ischemic stroke. LncRNAs could regulate the biological bahaviors of BMVECs through their target genes and serve essential roles on cell apoptosis, autophagy, inflammation, and angiogenesis. Furthermore, lncRNAs were closely associated with the prognosis of patients with ischemic stroke, and they could facilitate functional recovery by activating angiogenesis. In addition, lncRNAs are involved in angiogenesis by acting as miRNAs sponges or targeting the downstream signaling pathways/proteins directly. Therefore, lncRNAs might be potential diagnostic and prognostic biomarkers for ischemic stroke. However, only a few lncRNAs involved in angiogenesis have been studied, including MEG3, MEG8, MALAT1, SNHG1, SNHG12, SNHG16, ANRIL, FAL1, XIST, MACC1-AS1, NEAT1, Rmst, and MIAT. Therefore, further investigation is required to elucidate the detailed functions of lncRNAs in ischemic stroke. A better understanding of the underlying mechanisms could provide novel insights on the development of therapeutic targets and diagnostic biomarkers for patients with ischemic stroke.

AUTHOR CONTRIBUTIONS

LG and SL wrote the manuscript. YX and SD edited the manuscript. All authors contributed to the article and approved the submitted version.

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

frontiers in Cellular Neuroscience

REVIEW
published: 09 February 2021
doi: 10.3389/fncel.2021.628940

Mesenchymal Stem Cell-Based Therapy for Stroke: Current Understanding and Challenges

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Stroke, the most prevalent cerebrovascular disease, causes serious loss of neurological function and is the leading cause of morbidity and mortality worldwide. Despite advances in pharmacological and surgical therapy, treatment for functional rehabilitation following stroke is limited with a consequent serious impact on quality of life. Over the past decades, mesenchymal stem cell (MSCs)-based therapy has emerged as a novel strategy for various diseases including stroke due to their unique properties that include easy isolation, multipotent differentiation potential and strong paracrine capacity. Although MSCs have shown promising results in the treatment of stroke, there remain many challenges to overcome prior to their therapeutic application. In this review, we focus on the following issues: the scientific data from preclinical studies and clinical trials of MSCs in the treatment of stroke; the potential mechanisms underlying MSC-based therapy for stroke; the challenges related to the timing and delivery of MSCs and MSC senescence.

Keywords: mesenchymal stem cell, stroke, cell therapy, mechanisms, challenges

OPEN ACCESS

Edited by:
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Army Medical University, China

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

Received: *13 November 2020*
Accepted: *14 January 2021*
Published: *09 February 2021*

Citation:
Li W, Shi L, Hu B, Hong Y, Zhang H, Li X and Zhang Y (2021) Mesenchymal Stem Cell-Based Therapy for Stroke: Current Understanding and Challenges. Front. Cell. Neurosci. 15:628940. doi: 10.3389/fncel.2021.628940

INTRODUCTION

Stroke, one of the major diseases of the central nervous system, is a global health problem with limited treatment options. It is classified as hemorrhagic (13%), caused by rupture of blood vessels, or ischemic (87%), caused by disruption of blood supply (Kalladka and Muir, 2014). With an increasing elderly population, the mortality and morbidity of stroke are increasing. Approximately 15 million individuals worldwide are affected by stroke each year, of whom 5 million will ultimately die and 5 million will suffer long-term disability (Roy-O'Reilly and McCullough, 2014). Ischemic stroke is caused by occlusion of a supply artery due to embolus or thrombus. As a result of cerebral ischemia, excitatory amino acids react with tissues and generate a large number of calcium ions and free radicals. This produces carbon monoxide with consequent irreversible necrosis of brain cells (Rastogi et al., 2006). The necrotic portion, also known as the ischemic core, is surrounded by the peri-infarct region or penumbra that represents the functionally impaired but potentially salvageable tissue and is the primary target for the developing neuroprotective strategies (Candelario-Jalil and Paul, 2021). Rapid restoration of cerebral blood flow is the focus of the treatment for acute stroke. Currently, there are no proven options for stroke patients aside from dissolution of thrombus via tissue plasminogen activator (e.g., alteplase), or mechanical thrombectomy (Hacke et al., 2008; Powers et al., 2015; Saver et al., 2016). However, thrombolysis

has a narrow therapeutic window, being clinically effective only within 4.5 h after stroke and losing its effect when the thrombus is large or the stroke is extensive (Bhaskar et al., 2018). Fewer than 5% of ischemic stroke patients receive such treatment and still suffer post-treatment neurological deficits with no therapy available to promote recovery (Lyden et al., 2019). Mechanical thrombectomy exhibits the significant therapeutic efficacy in acute ischemic stroke caused by intracranial proximal artery occlusion. However, this technique is not yet fully developed, and the efficacy and safety of endovascular reperfusion beyond 6 h remains controversial (Berkhemer et al., 2015; Smith, 2019). Limited numbers of stroke patients can benefit from these approaches and achieve good outcomes (Detante et al., 2017).

Over the past decades, stem cell-based therapy has attracted great interest as an emerging treatment in stroke in the hope that it can repair the damaged central neural networks (Wan Safwani et al., 2017; Choi et al., 2018). Stem cell therapy displays significant effects of functional improvement for ischemic stroke, offering hope for the preservation of neural tissue in the acute phase of stroke and the replacement of lost tissue in the chronic stage (Wei et al., 2017).

MSCs are pluripotent, non-hematopoietic stem cells with the ability to differentiate into a diverse number of cell lineages, including chondrocytes, osteoblasts, and neuron-like cells (Uccelli et al., 2006; Williams and Keating, 2008). They can be isolated from almost all tissues in mammals including bone marrow (BM), adipose tissue or other tissues (Pinho et al., 2020) and are easy to culture and effectively expand. BM-MSCs are the most common, while in recent years adipose-derived MSCs have become increasingly popular due to their easy availability and high yield (Faghih et al., 2017; Perteghella et al., 2017). MSCs from bone marrow, adipose tissue have full trilineage (adipogenic, osteogenic, and chondrogenic) differentiation capacity and excellent immunomodulatory properties compared to other sources, and therefore represent the optimal stem cell sources for tissue engineering and regenerative medicine (Heo et al., 2016). Even in the acute stage of stroke, MSCs are suitable for transplantation and have substantial neurotrophic effects (Walczak et al., 2008). In addition, MSCs derived from adult tissues pose no risk of tumorigenesis and their low expression of major histocompatibility complex (MHC)-I and MHC-II antigens eliminates the need for immunosuppression following allogeneic administration (Bhatia and Hare, 2005; Williams and Hare, 2011). The therapeutic effects of MSCs are mediated by many mechanisms including anti-inflammation, anti-apoptosis, angiogenesis, and neurogenesis. They have become the focus of many preclinical and clinical studies (Li et al., 2016; Moniche et al., 2016). This review will focus on the application of MSCs in the treatment of stroke.

PRECLINICAL STUDIES

The application of MSCs in the treatment of stroke has been studied for nearly two decades. Several recent animal studies are summarized in **Table 1**. In most studies, Sprague Dawley (SD) rats or Wistar rats were used to establish a model of cerebral ischemia, induced by middle cerebral artery occlusion (MCAO). It has been shown that transplantation of MSCs following ischemic stroke promotes improvement of cerebral function (Toyoshima et al., 2015; Moisan et al., 2016; Hu et al., 2019) effectively protects ischemic neurons and restores brain damage (Son et al., 2019). However, several studies used young adult and healthy animals, without taking into account the fact that a large proportion of ischemic stroke patients are elderly and the presence of comorbidities such as hypertension and diabetes (Howells et al., 2010; Laso-Garcia et al., 2019). Herein, these animal models create the barriers to the translation of the findings to clinical trials. Therefore, we here focused on studies that incorporated comorbidities into animal models of stroke.

It has been estimated that about 75% of strokes occur in the elderly (Yousufuddin and Young, 2019). Shen et al. (2007a) selected 10–12 month-old female retired breeder rats to establish an ischemic stroke model, and confirmed the long-term neurological protective effects of MSC on ischemic stroke. In addition, Saraf et al. demonstrated that stroke induced CaN hyperactivation, triggering an apoptotic pathway in neurons that further led to neuronal death in middle-aged ovariectomized female rats. MSCs treatment rescued neurons and promoted neuronal survival via reducing CaN expression (Saraf et al., 2019). Hypertension is the major risk factor for all types of stroke (Hong, 2017; Cipolla et al., 2018), Hypertensive ischemic stroke models mostly use stroke-prone spontaneously hypertensive rats (SHRSP), an animal model that develops 100% hypertension without genetic modification, has cerebrovascular pathology and physiology very similar to that of human hypertension, and induces spontaneous strokes at a rate of more than 60% (Liao et al., 2013). Calio et al. (2014) demonstrated that MSCs induced an increase in the anti-apoptotic gene Bcl-2 and protected brain tissue through anti-apoptosis and antioxidation, suggesting that MSCs have a protective effect on neuronal cells in SHRSP rats. In another study, placental derived MSCs treatment greatly improved functional recovery and reduced infarct size in mice with hypertensive ischemic (Kranz et al., 2010). Diabetes is a definite risk factor for stroke. Patients with diabetes have an increased probability of developing ischemic stroke, and hyperglycemia exacerbates microvascular and macrovascular damage in ischemic strokes (Rehni et al., 2017; Lau et al., 2019). Therefore, studying diabetic stroke models is of great importance. It has been reported that 6 weeks after permanent MCAO, lesions were more severe in the hyperglycemic group than in the non-hyperglycemic group. Although human adipose tissue-derived MSCs treatment for hyperglycemic stroke rats did not reduce lesion size, it significantly improved neurological function (Gomez-de Frutos et al., 2019). Cui et al. (2016) demonstrated the beneficial effects of BM- MSCs in type 1 diabetic rats with stroke via mediating miR-145. In type 2 diabetic Wistar rats with stroke, treatment with exosomes harvested from BM-MSCs significantly improved blood-brain barrier (BBB) integrity, increased white matter remodeling, and promoted neural repair (Venkat et al., 2020).

TABLE 1 | Overview of animal studies of MSC-based therapy for stroke.

Animal species	Stroke type	Comorbidity	Cell source	Cell number	Delivery route	Timing	Results	References
SD	MCAO	–	BM	1×10^5	IA (carotid artery)	10 days	Neuronal regeneration	Hu et al., 2019
SD	MCAO	–	BM	3×10^6	IV (tail vein)	8 days	Angiogenesis	Moisan et al., 2016
Wistar	MCAO	–	BM	1×10^6	IA	1, 6, 24, and 48 h	Reduce infarction volume	Toyoshima et al., 2015
SD	MCAO	–	BM	2×10^5	IC (brain tissue)	1 days	Protect ischemic neurons	Son et al., 2019
Wistar	MCAO	Aging	BM	2×10^6	IA (carotid artery)	1 days	Long-term improvement in functional outcome	Shen et al., 2007a
SD	MCAO	Aging	BM	1×10^5	IA	6 h	Improve the functional outcome	Saraf et al., 2019
SHR	Stroke prone	Hypertension	BM	1×10^6	IC (atlanto-occipital membrane)	–	Neuroprotective and antioxidant potential	Calio et al., 2014
SHR	MCAO	Hypertension	Placenta	1×10^6	IV (tail vein)	8 and 24 h	Functional recovery	Kranz et al., 2010
SD	MCAO	Hyperglycemia	Adipose tissue	1×10^6	IV (tail vein)	48 h	Neurological recovery	Gomez-de Frutos et al., 2019
Wistar	MCAO	Diabetes	BM	5×10^6	IV (tail vein)	24 h	Neurorepair effects	Cui et al., 2016
Wistar	MCAO	Diabetes	BM	3×10^{11}	IV (tail vein)	3 days	Improve the functional outcome	Venkat et al., 2020

MCAO, middle cerebral artery occlusion; BM, bone marrow; IA, intra-arterial; IC, intracerebral; IV, intravenous.

Comorbidities in humans can profoundly affect stroke pathophysiology, lesion development, and recovery (Cho and Yang, 2018). Despite of the beneficial effects of MSCs, more preclinical studies are warranted to exploring MSC therapy for stroke due to the limited number of relevant studies in stroke comorbidity models. Therefore, the importance of using preclinical comorbidity model should be emphasized when establishing guidelines on how to improve the validity of animal models of stroke.

CLINICAL TRIALS

Although preclinical studies have shown that MSCs displays beneficial effects on stroke (Moisan et al., 2016; Cunningham et al., 2018), the safety problems inflammation, tumor development, metastasis in clinical trials have been reported (Gazdic et al., 2015; Dhere et al., 2016; Volarevic et al., 2018). Over the past few decades, the safety, feasibility and effectiveness of MSCs in the treatment of stroke have been widely studied in clinical trials (**Table 2**). Previous clinical trials have shown that MSCs isolated from different tissues have shown the high efficiency for stroke treatment (Detante et al., 2017; Wechsler et al., 2018; Chrostek et al., 2019; Cui et al., 2019; Suda et al., 2020). Several routes of delivery have been proposed including intracerebral (IC), intra-arterial (IA), and intravenous (IV) (Toyoshima et al., 2017). Among them, the intracerebral pathway is the most effective and invasive route. In contrast, the intravenous pathway is the least invasive, but the number of MSC cells reaching the injured brain is the most limited. The intra-arterial pathway is relatively neutral. In 2005, autologous BMSCs transplantation was performed intravenously for the first time in five patients with acute ischemic stroke and no adverse reactions were reported (Bang et al., 2005). In 2010, a large long-term study evaluated the safety and efficacy of autologous intravenous BMSCs transplantation and got similar results (Lee et al., 2010). In 2011, the reduction in infarct lesion volume and recovery of neurological function were obtained following administration of serum-expanded autologous BMSCs to chronic stroke patients (Honmou et al., 2011). Afterwards, a phase I/II study of intracerebral cell transplantation in patients with chronic stroke has reported that intracerebral transplantation of genetically modified MSCs significantly improved neurological function (Steinberg et al., 2016, 2018). A single-center, open-label Randomized Controlled Trial study showed that intravenous injection of autologous BMSCs also improved the motor function, suggesting that MSCs treatment is feasible therapeutic strategy for stroke (Jaillard et al., 2020).

Recently, Savitz et al. (2019) conducted a randomized, sham controlled, phase II trial in which autologous BM-derived aldehyde dehydrogenase (ALDH)-bright cells were transplanted intra-arterially to patients with disabling middle cerebral artery stroke and showed no adverse events in the treatment patients group, although there was a higher incidence of small lesions on MRI. In addition, superparamagnetic iron oxide-labeled BMSCs were also used to treat stroke patients and proved to be safe and tolerated (Shichinohe et al., 2017). Compared to BMSCs, adipose tissue-derived mesenchymal stem cells (AD-MSCs) are easier and safer to prepare without adverse side effects and ethical concerns (Ra et al., 2011; Gutierrez-Fernandez et al., 2013a,b). In 2014, allogeneic intravenous AD-MSCs transplantation was carried out in patients with subacute stroke. The results demonstrated that allogeneic AD-MSCs had no association with the development of tumors. The study concluded that AD-MSCs-based cell therapy was safe and could promote rehabilitation of stroke (Diez-Tejedor et al., 2014).

Human umbilical cord-derived MSCs (hUC-MSCs) have great advantages for stroke treatment due to low immunogenicity and no substantial ethical problems (Yin et al., 2019). A Phase

TABLE 2 | Clinical trials of MSC-based therapy for stroke.

Phase	Patients number	Delivery route	Cell source	Cell number	Timing	Results	References
I	5	IV	Autologous BM-MSCs	1×10^8	7 days	Improve in BI	Bang et al., 2005
II	16	IV	Autologous BM-MSCs	5×10^7	5–7 weeks	Improve in mRS	Lee et al., 2010
I	12	IV	Autologous BM-MSCs	1×10^8	36–133 days	Improve in NIHSS	Honmou et al., 2011
I	8	IV	Autologous BM-MSCs	$5–6 \times 10^7$	3 months–1 year	Improve in Fugle-Meyer and mRS, increase in number of cluster activation of Brodmann areas BA 4 and BA 6	Bhasin et al., 2011
II	20	IV	Allogeneic AD-MSCs	1×10^6 cells/kg	2 week	Safe and effective	Diez-Tejedor et al., 2014
I/IIa	18	IC	Modified MSCs (SB623)	dose-escalation: 2.5×10^6, 5.0×10^6, or 10×10^6	6–60 months	improve in ESS,NIHSS,Fugle-Meyer	Steinberg et al., 2016, 2018
II	48	IA	BM-ALDH^{br}cells	0.5×10^5–2.5×10^7	9–15 days	Safe	Savitz et al., 2019
I	10	IV	Allogeneic UC-MSCs	5×10^6–5×10^7/Kg	7–10 days	Safe and feasible	Laskowitz et al., 2018
II	16	IV	Autologous BM-MSCs	10×10^7–30×10^7	14 days	Improve in motor-NIHSS,Fugle-Meyer, task-related fMRI activity	Jaillard et al., 2020

BI, Barthel Index; mRS, modified Rankin Scale; NIHSS, National Institute of Health Stroke Scale; ESS, European Stroke Scale; BM, bone marrow; MSC, mesenchymal stem cell; UC-MSC, umbilical cord mesenchymal stem cell; ALDH^{br}, aldehyde dehydrogenase bright; IA, intra-arterial; IC, intracerebral; IV, intravenous.

I clinical trial using hUC-MSCs for acute stroke treatment demonstrated a significant functional recovery, indicating that hUC-MSCs treatment is safe and feasible option for acute stroke (Laskowitz et al., 2018).

Although MSC transplantation has been proven to be safe and feasible in small phase I/II trials, no significant improvement was observed in a randomized controlled intravenous phase II trial (Hess et al., 2017). Their efficacy in the treatment of ischemic stroke therefore remains controversial (Hess et al., 2017). Several reasons are proposed for the inconsistent results of MSC transplantation in ischemic stroke. First, trials differ in patient numbers, dose and type of cell delivery, timing of cell therapy, and treatment modalities. Second, the location/extent and severity of the lesions are different. The studies also applied different evaluation criteria when assessing neurological function and adverse reactions. More optimized and well-designed large sample multicenter studies are needed to explore the therapeutic efficacy of MSCs in ischemic stroke.

POTENTIAL MECHANISMS OF MSC THERAPY FOR STROKE

Currently, the underlying mechanisms of MSC-based therapy for stroke have not been fully elucidated. Many experimental studies have revealed that MSCs protect against stroke through multiple mechanisms including direct differentiation, paracrine effects and mitochondrial transfer (**Figure 1**).

MSC Differentiation

MSCs are pluripotent adult mesenchymal cells with the ability of self-renewal and multi-differentiation (Klimczak and Kozlowska, 2016). In the presence of injury and inflammation, MSCs are directly transplanted or homed to the damaged site (Shi et al., 2012). In a specific microenvironment of a tissue or organ, MSCs have the ability to divide and proliferate, differentiate and develop into the same cell type as the tissue or organ, including neuronal cells, and effect repair (Clevers et al., 2014; Shi et al., 2016). It has been reported that MSCs isolated from human umbilical cord can differentiate into neuron-like cells and maintain their immunomodulatory and antioxidant activities (Li et al., 2012).

Paracrine Effects

There is accumulating evidence that the ability of MSCs to differentiate into neuron cells cannot on its own explain the predominant benefits of MSC-based therapy (Ghittoni et al., 2005; Wang et al., 2012; Bang and Kim, 2019; Brown et al., 2020). Compared with BM-MSC transplantation, infusion of MSC-derived conditioned medium has been shown to equally improve injured brain function (Ghittoni et al., 2005; Gnecchi et al., 2006; Volarevic et al., 2011). A paracrine effect of MSCs is thus concluded to be a major mechanism underlying the benefits of MSC-based therapy for stroke (Liang et al., 2014). There is a close interaction between the soluble factors derived from MSCs and immune cells (such as dendritic cells, lymphocytes, natural killer cells, and macrophages) (Gao et al., 2014). A paracrine role is reflected in immune regulation: MSCs secrete soluble factors through direct cell-cell interaction, are involved in immune regulation and induce immune tolerance, and can improve and regulate the destructive inflammatory response (Wu et al., 2017; de Witte et al., 2018). Numerous paracrine components form a complex exocrine factor network to ensure the stability of cells and enhance the regeneration response. Many MSC-based tissue repair models are largely

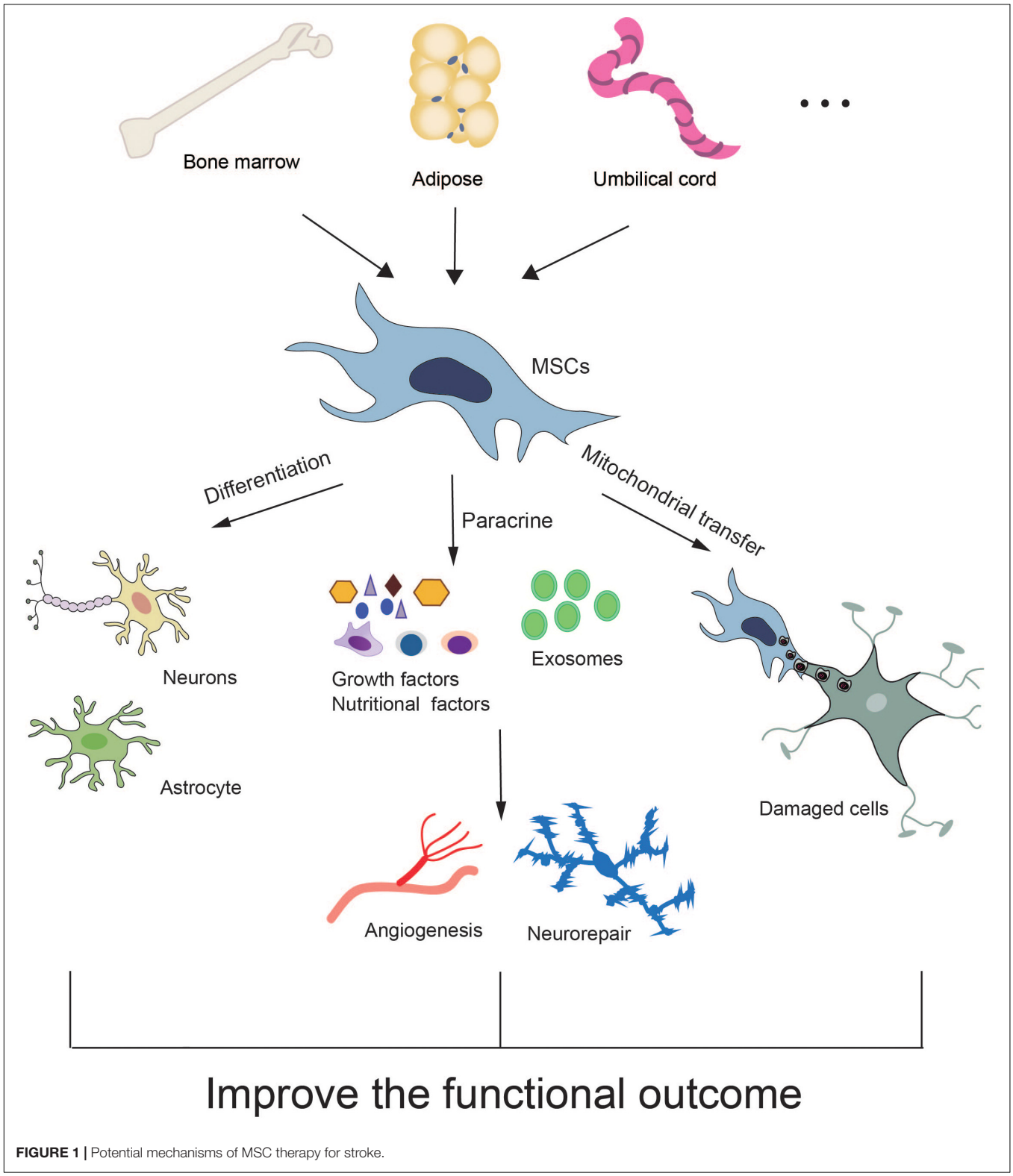

FIGURE 1 | Potential mechanisms of MSC therapy for stroke.

dependent on the paracrine action of MSCs (Weiss and Dahlke, 2019; Wu et al., 2020). Another manifestation of their paracrine effect is in the promotion of angiogenesis (De Luca et al., 2011; Gnecchi et al., 2016; Chen et al., 2020). Both basic fibroblast growth factor and vascular endothelial growth factor induce endothelial cell proliferation and migration to form new vascular branches from existing vascular branches (Gnecchi et al., 2006). The various nutritional factors secreted

by MSCs, including enzymes, growth factors, chemokines, matrix metalloproteinases, and adhesion molecules, all have an effect on several key steps of angiogenesis. They can induce the proliferation, migration and tubular formation of vascular endothelial cells, as well as inhibit apoptosis of endothelial cells (Kinnaird et al., 2004; Rehman et al., 2004). Transplantation of adipose tissue-derived MSCs has also been shown to promote angiogenesis and improve behavioral recovery in SD rats after MCAO operation (Mu et al., 2019). BM-MSCs can increase the expression of astrocyte-derived VEGF and BDNF in the ischemic boundary zone after stroke and promote angiogenesis, as well as the recruitment and proliferation of reactive astrocytes, leading to nerve injury repair (Guo et al., 2012; Zhang et al., 2017). Moreover, Human BM-MSCs have been shown to increase cerebral vascular generation in stroke lesions by releasing endogenous angiogenic factors that enhance the stability of new blood vessels (Moisan et al., 2016). Therefore, the paracrine effect of stem cells is likely to play an important role in increasing capillary density and angiogenesis in the damaged brain.

Notably, exosomes are the most important agents in the process of information transmission and inducing repair for many secreted cytokines (Elahi et al., 2020). The paracrine effect produced by their external secretion plays a critical role in stroke recovery (Xin et al., 2013a; Hao et al., 2014; Zhang Y. et al., 2015; Zhang et al., 2019). Exosomes from MSCs are 30–100 nm diameter lipid particles with a double membrane structure containing micro RNAs, mRNAs, DNAs, and bioactive substances such as protein and lipids. They display similar properties and functions to MSCs including low immunogenicity and the ability to stimulate nerve vascular repair with no risk of tumor formation (Yaghoubi et al., 2019). Increasing lines of evidence have confirmed that exosomes contribute significantly to the benefits of cell-based therapies, including the treatment of stroke, traumatic brain injury and other neurological diseases (Xin et al., 2013b; Zhang Y. et al., 2015; Stonesifer et al., 2017; Zhang et al., 2019). Dabrowska et al. (2019) demonstrated that intra-arterial delivery of exosomes derived from BM-MSCs reduced neuroinflammation induced by focal brain injury in ischemic stroke and this effect was comparable with that of transplanted BM-MSCs. In some mouse models of stroke, the long-term neuroprotection afforded by MSCs was closely associated with enhanced angiogenesis, and reduced post-ischemic immunosuppression (i.e., B cells, natural killer cells, and T-cell lymphocytosis), providing an appropriate external environment for successful brain remodeling (Doeppner et al., 2015; Hu et al., 2016). The secretion of a wide range of bioactive molecules is now considered the major mechanism of MSC-based therapy.

Mitochondrial Transfer

Mitochondrial transfer is a novel mechanism for stem cell therapy that has attracted wide attention. MSCs can transfer mitochondria to injured cells with mitochondrial dysfunction through a variety of ways to restore cell aerobic respiration and mitochondria function, leading to rescue of cell injury (Spees et al., 2006; Islam et al., 2012; Han et al., 2016). Mitochondrial dysfunction has been considered a sign of ischemia/reperfusion injury in the complex cell process, so mitochondrial transfer may be one of the mechanisms by which MSC treatment is of benefit for stroke (Han et al., 2020). Co-cultured BM-MSCs can transfer intact mitochondria through transient tunneling nanotubes (TNT) to damaged cells, restoring their mitochondrial function (Han et al., 2016). Yang et al. demonstrated that iPSC-MSCs could protect damaged PC12 cells by restoring mitochondrial function. This was not just due to the paracrine effect of MSCs, but also attributed to the mitochondria transferred from MSCs to the injured PC12 cells (Yang et al., 2020). There is accumulating evidence that mitochondrial transfer between MSCs and damaged cells is mainly mediated through tunneled nanotubes and microvesicles (Spees et al., 2006; Islam et al., 2012; Li et al., 2014; Murray and Krasnodembskaya, 2019). In addition, Babenko et al. (2015) reported that BM-MSCs can donate mitochondria to injured astrocytes and restore their mitochondrial function, demonstrating the protective function of MSCs on nerves. Tseng et al. (2020) demonstrated that transfer of mitochondria from MSCs to damaged neurons induced by oxidative stress *in vivo* and *in vitro* resulted in metabolic benefits. The researchers tagged BM-MSCs and tracked the transplanted mitochondria. They observed the mitochondria transfer and a protective effect on the damaged cerebral microvascular system in rats with cerebral ischemia (Liu et al., 2019; Yang et al., 2020). Thus, mitochondrial transfer from MSCs to damaged cells may offer a new avenue in the treatment of stroke.

CHALLENGES OF MSC THERAPY FOR STROKE

Although many animal studies and clinical trials of MSC-based therapy for stroke have obtained promising results,there remain many challenges to overcome before MSCs can be widely applied in clinical practice.

First, the optimum time for MSC administration remains controversial. Currently, most preclinical studies recommend transplanting MSCs during the acute stroke stage (<48 h). It has been reported that stroke can cause an increase in reactive oxygen species, activation of immune cells and production of pro-inflammatory cytokines in the acute phase, thus aggravating the secondary brain injury. MSC exosomes display immunomodulatory and neuroprotective effects, especially in the acute phase of stroke (Tobin et al., 2014; Vu et al., 2014). In contrast, some studies have shown that administration of MSCs in rats 1 month after stroke could also lead to neurological recovery (Shen et al., 2007b). MSCs can secrete many growth factors to activate the endogenous repair process, induce a decrease in glial scar and increase proliferation of cells in the subventricular area, promoting neurogenesis in the chronic phase of stroke (Shen et al., 2007b; Sinden et al., 2012). MSC transplantation has also been administered in the subacute or chronic stage of stroke in some clinical studies (Lalu et al., 2020). How to solve this knowledge gap and use available evidence to determine the optimal timing for cell therapy remains a major challenge.

Another challenge is to determine the best treatment. Although MSCs have shown general immune evasion and tolerance in clinical studies of stroke, an increasing number of preclinical studies have proved the therapeutic efficacy of conditioned medium (CM) and extracellular vesicles (EVs) derived from MSCs that reduces the dependence on and need for cells (Xin et al., 2013b; Cunningham et al., 2018). These cell-free substitutes can be cryopreserved with no concerns about cell survival. Cells can be preserved for a long time and transported around the world. Nevertheless, there is no clear consensus on the optimal culture conditions and pretreatment strategies to maximize the regenerative potential of MSC-derived CM or EVs (Reiner et al., 2017). Further clinical studies are needed to clarify their therapeutic value for stroke.

Third, the route of MSC administration is another major challenge. Previous studies have used both systemic and direct approaches such as IV, IA, and IC. Compared with more invasive routes (e.g., intrathecal and IC approaches), minimally invasive routes (e.g., IV and IA approaches) may cause less damage at the injection site although each route has its own advantages and disadvantages. How to choose a simpler and safer delivery route for MSCs is a major hurdle to their clinical application with much greater care required by the clinician.

Fourth, the best source of MSCs for stroke treatment has not been determined. Although most preclinical studies (>90%) use fresh MSCs from healthy, young donors, half of the clinical studies used autologous MSC products. Autologous MSCs may circumvent the logistical ethical problems and have been proven to be more effective than those obtained from healthy donors (Block et al., 2017; van de Vyver, 2017). Nonetheless, expanding enough stem cells for transplantation requires a long time, so it is impossible to use autologous MSC cells in the acute stage of stroke, especially from elderly patients or those with serious diseases. Genetic engineering or reprogramming to amplify MSCs can lead to uncontrolled proliferation and genetic abnormality, limiting their viability and therapeutic potential (Zhang J. et al., 2015). Furthermore, whether modified MSCs can successfully differentiate into fully functional neural cells in patients remains elusive (Strioga et al., 2012). One large randomized controlled clinical trial reported that cryopreserved allogeneic MSCs from healthy donors had poor viability and poor clinical efficacy (Matthay et al., 2019). Therefore, when using allogeneic cells from healthy donors care should be taken to assess their viability in order to match preclinical conditions.

Fifth, another challenge is the heterogeneity in study designs. The poor methodological rigorism of both preclinical and clinical studies may have contributed to the current conflicting results. Preclinical studies rarely adopted randomized or blinding designs, or carried out confirmatory studies, a pre-requisite of clinical trials (Macleod et al., 2008; Hirst et al., 2014). Similar problems also existed in clinical studies. Studies included RCT (Lee et al., 2010; Hess et al., 2017), single-arm trial (Steinberg et al., 2016), or case series (Honmou et al., 2011), and could not be compared. Previous clinical measures of efficacy reported included NIHSS, mRS, BI, Fugle-Meyer scale, and ESS. A unified method for evaluation of neurological function is lacking so it is difficult to reach a consistent conclusion about the safety and effectiveness of MSCs in clinical application. How to increase methodological rigorism is a great challenge for preclinical and clinical studies.

Sixth, senescence of MSCs has attracted extensive attention in recent years. The passage times of MSCs are limited. Extension of expansion time will inevitably lead to replicative senescence. Moreover, MSCs isolated from the elderly exhibit an aging phenotype with a decline in function, leading to decreased therapeutic efficacy (Wagner et al., 2008). Therefore, developing strategies to deal with MSC senescence is another future challenge (Kim and Park, 2017).

Finally,the comorbidities of patients are also the challenges for MSC therapy (Cui et al., 2019). Many stroke patients have comorbidities such as hypertension, diabetes and heart disease that may exert an impact on therapy efficacy (Chen et al., 2011). The medications such as antidiabetics and antiplatelet drugs often influence MSC function, limiting the therapeutic effects (Ortega et al., 2013). Unfortunately, most of preclinical studies haven't investigated influence factors, leading to a big knowledge gap in translate stroke research to clinic.

CONCLUSION

MSCs have many advantages: they are immune evasive, easy to harvest, expand and store for a long time, and convenient to manage in various ways. Additionally, their clinical use does not raise many ethical issues. Increasing evidence supports the potential of MSCs to treat stroke, but there are challenges to overcome. We have systematically reviewed the safety and efficacy of MSCs in the treatment of ischemic stroke and hemorrhagic stroke. In preclinical studies, MSC treatment has shown considerable efficacy in some neurological function tests, but there remains no large-scale randomized, double-blind, multicenter clinical study to prove their effectiveness. The heterogeneity of MSCs is the main barrier to their clinical application and therapeutic effect. Key parameters such as the source of MSCs, dosage, route of administration, administration time and other key parameters directly affect the application effect. More importantly, many clinical trials have similar limitations in detecting the role of MSCs, including small size, lack of a control arm, and inconsistent methods for use of MSCs. Homogenization and quality control are key issues in their clinical application. Future preclinical and clinical studies should consider adoption of a well-designed randomized controlled study design, method rigor and intervention measures, so as to determine the effect of MSC therapy in the treatment of stroke (Napoli and Borlongan, 2016; Squillaro et al., 2016). Nonetheless, despite these issues, MSCs have exciting potential as a means to protect neurons and improve the outcome for stroke patients.

AUTHOR CONTRIBUTIONS

WL and LS searched the literature and wrote the manuscript. BH, YH, and HZ searched the literature and provided comments. XL and YZ designed the study and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported in part by the High-level Hospital Construction Project of Guangdong Provincial People's Hospital (DFJH201918 to YZ, DFJH2020020 to WL), and a grant from the Science and Technology Planning Project of Guangzhou (201804010335 to BH) and Tibet Autonomous Region Research Projects (XZ2018-01-GB-09 to XL).

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

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
published: 10 February 2021
doi: 10.3389/fncel.2021.629356

Neurotrophin-3 Promotes the Neuronal Differentiation of BMSCs and Improves Cognitive Function in a Rat Model of Alzheimer's Disease

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Transplantation of bone marrow-derived mesenchymal stem cells (BMSCs) has the potential to be developed into an effective treatment for neurodegenerative diseases such as Alzheimer's disease (AD). However, the therapeutic effects of BMSCs are limited by their low neural differentiation rate. We transfected BMSCs with neurotrophin-3 (NT-3), a neurotrophic factor that promotes neuronal differentiation, and investigated the effects of NT-3 gene overexpression on the differentiation of BMSCs into neurons *in vitro* and *in vivo*. We further studied the possible molecular mechanisms. We found that overexpression of NT-3 promoted the differentiation of BMSCs into neurons *in vitro* and *in vivo* and improved cognitive function in rats with experimental AD. By contrast, silencing NT-3 inhibited the differentiation of BMSCs and decreased cognitive function in rats with AD. The Wnt/β-catenin signaling pathway was involved in the mechanism by which NT-3 gene modification influenced the neuronal differentiation of BMSCs *in vitro* and *in vivo*. Our findings support the prospect of using NT-3-transduced BMSCs for the development of novel therapies for AD.

Keywords: bone marrow-derived mesenchymal stem cells (BMSCs), neurotrophin-3 (NT-3), neuronal differentiation, Wnt/β-catenin, Alzheimer's disease 3

OPEN ACCESS

Edited by:
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Army Medical University, China

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

Received: *14 November 2020*
Accepted: *21 January 2021*
Published: *10 February 2021*

Citation:
Yan Z, Shi X, Wang H, Si C, Liu Q and Du Y (2021) Neurotrophin-3 Promotes the Neuronal Differentiation of BMSCs and Improves Cognitive Function in a Rat Model of Alzheimer's Disease. Front. Cell. Neurosci. 15:629356. doi: 10.3389/fncel.2021.629356

INTRODUCTION

Neurodegenerative diseases of the central nervous system, which cause nerve cell damage and neurologic impairment, are major and debilitating medical conditions that exert great burdens on patients, their families and healthcare services. Cognitive impairment, the dominant feature of Alzheimer's disease (AD), seriously affects the health and quality of life of many middle-aged and elderly people worldwide.

In recent years, stem cell transplantation has received increasing attention as a novel treatment for neurodegenerative diseases of the central nervous system. Bone marrow-derived mesenchymal stem cells (BMSCs) are multipotent stem cells derived from bone marrow that are potentially a good source of stem cells for transplantation. BMSCs have advantages that include availability in large numbers, straightforward harvesting via a simple procedure, immunotolerance and no restrictions with regard to morality and ethics (Xue et al., 2019; Ghahari et al., 2020).

Neurotrophin-3 (NT-3) is a multifunctional neurotrophic factor that plays important roles in the development and normal functioning of the nervous system (Chalazonitis, 2004; Cong et al., 2020). There is a growing body of evidence demonstrating that NT-3 promotes the survival of

neurons and repair of nerves (Mo et al., 2010; Rak et al., 2014; Wang Y. et al., 2018). Zhu et al. found that NT-3 induced the differentiation of progenitor BMSCs into neurons (Zhu et al., 2015). Moreover, previous research has suggested that the transplantation of BMSCs overexpressing NT-3 could promote recovery of locomotor function and nerve regeneration in a rat model of spinal cord injury (Wang et al., 2014). Recent study showed that NT-3 probably binds to the Brain Derived Neurotrophic Factor (BDNF) and jointly regulate neurogenesis and neural survival (Barh et al., 2017). Based on the above research, we speculated that the transplantation of BMSCs modified with the gene for NT-3 could have an impact on nerve regeneration and recovery of cognitive function that might be relevant to the treatment of AD.

In this study, we evaluated the effects of NT-3 on the differentiation of BMSCs into neurons *in vitro* and *in vivo* and on the recovery of cognitive function after BMSC transplantation in a rat model of AD. In addition, we investigated the mechanisms underlying any effects.

MATERIALS AND METHODS

Culture and Identification of BMSCs

BMSCs were isolated from Sprague-Dawley rats according to a previously described method, with some modifications (Jing et al., 2014). Briefly, the rat was killed by cervical dislocation, and the femurs and tibias were isolated under aseptic conditions and washed in pre-cooled phosphate-buffered saline (PBS). The two ends of each bone were snipped to expose the bone marrow cavity, which was washed several times with serum-free Dulbecco's Modified Eagle's Medium (DMEM). The bone marrow cells were collected, filtered and re-suspended in DMEM with 10% fetal bovine serum (FBS). The cells were seeded in T25 culture bottles for primary culture. Third-generation BMSCs were used for the subsequent experiments. The BMSCs were identified by flow cytometry using antibodies against CD90, CD44, CD45, and CD34. Adipogenic differentiation of BMSCs was induced by 5 mM glutamine, 1 mM dexamethasone, 100 nM insulin and 0.2 mM indomethacin and was assessed by staining with Oil RedO. Osteogenic differentiation of BMSCs was induced by 0.25 mM ascorbic acid, 10 mMβ-glycerophosphate and 1 mM dexamethasone and was assessed by staining with alizarin red.

Culture of Neuronal Cells

Primary neuronal cells were isolated from Sprague-Dawley rats within 24 h following their birth. The animal was killed by cervical dislocation, and the brain tissues were removed, minced and digested by 0.125% trypsin for 10 min at 37°Cunder sterile conditions. Then, the cells were washed in DMEM with 10% FBS to block trypsin activity. The cells were filtered using a 100 μm plastic mesh and re-suspended in Neurobasal-Amedium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with 2% B27 (Gibco) and 2 mM L-glutamine.

Lentivirus-Mediated Overexpression or Interference of NT-3 in BMSCs

The full-length rat NT-3 gene (NM_031073.2) was synthesized and cloned into a lentiviral expression vector. Short hairpin RNA (shRNA) was used to silence NT-3 expression. The sequences of the sh-NT-3 and negative control shRNA were 5'-GCAACAGAGACGCTACAAT-3' and 5'-CAGTACTTTTGTGT AGTAC-3', respectively. The sh-NT-3 and negative control shRNA were synthesized and constructed in an appropriate lentivirus vector. The above lentiviral vectors were packaged into lentiviruses in 293T cells by Wanleibio (Shenyang, China). For lentivirus-mediated transduction, the BMSCs were infected with concentrated virus and 6 μg/mL polybrene in serum-free medium. The medium was substituted with complete culture medium after 24 h. The NT-3 level was detected by western blot assay.

In vitro Induction of BMSC Differentiation

To induce the differentiation of BMSCs into neurons, two-compartment co-culture of BMSCs and neuronal cells was performed. Briefly, BMSCs overexpressing NT-3 or with silencing of NT-3 were seeded onto the lower layer of a transwell chamber at a density of 5×10^5/mL. After 2 days, the medium was substituted with Neurobasal-A medium, and neuronal cells were seeded onto the upper layer. On day 8 of co-culture, the expressions of neuron-specific enolase (NSE), class III β-tubulin and neurofilament (NF)-200 were detected by immunocytochemistry to identify differentiated neurons, as described below.

Immunocytochemistry Experiments

For the immunocytochemistry experiments, BMSCs were seeded onto slides in 6-well plates, fixed in 4% paraformaldehyde and blocked with 10% normal goat serum for 15 min at room temperature. The slides were incubated with primary antibodies against NSE (1:100, Abcam, Cambridge, UK), NF-200 (1:100, Abcam), class III β-tubulin (1:100, Abcam) or β-catenin (1:100, Proteintech, Wuhan, China) at 4°C overnight. Then, the slides were incubated with Cy3-labeled secondary antibodies (1:200, Beyotime, Haimen, China). The nuclei were stained with DAPI (4',6-diamidino-2-phenylindole). Images were acquired at a magnification of 400× from 5 random microscopic fields using a fluorescence microscope.

Quantitative Reverse Transcription Polymerase Chain Reaction (qPCR)

Total RNA was isolated from BMSCs and brain tissues using RNApure Total RNA Fast Isolation kit (BioTeke, Beijing, China) and reverse transcribed into complementary DNA using Super M-MLV Reverse Transcriptase (BioTeke). PCR was performed using SYBR Green PCR Master Mix (BioTeke). The gene primers were as follows; β-catenin: forward5'-AGCGACTAAGCAGGAAGGGAT-3', reverse5'-ACAGATGGCAGGCTCGGTAAT-3'; and β-actin: forward5'-GGAGATTACTGCCCTGGCTCCTAGC-3', reverse5'-GGCCGGACTCATCGTACTCCTGCTT-3. β-actin was used as an internal control. Relative quantification of gene expression was performed using the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis

Western blot assays were performed to evaluate protein levels in BMSCs and brain tissue. Briefly, total protein from BMSCs

or brain tissue was lysed in radioimmunoprecipitation assay (RIPA) buffer (Beyotime) and then denatured. The total and nuclear protein concentrations were detected using a bicinchoninic acid (BCA) protein estimation kit (Beyotime). Equal quantities of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5% fat-free milk for 1 h at room temperature, the membranes were incubated overnight at 4°C with the following primary antibodies: anti-NT-3(1:400, Boster, Wuhan, China), anti-β-catenin(1:500, Wanleibio), anti-β-actin(1:1000, Wanleibio) oranti-lamin B (1:1000, Wanleibio). Then, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000, Wanleibio) for 1 h at room temperature. The results were visualized using an electrochemiluminescence kit (Beyotime). The optical densities of the bands were quantified by Gel-Pro Analyzer software (Media Cybernetics, Rockville, MD, USA).

Animals

Adult (200–220 g) Sprague-Dawley rats were obtained from Beijing Vital River Laboratory Animal Co., Ltd. (Beijing, China). The rats were kept in a 12 h light/12 h dark cycle at room temperature (18–22°C) and a humidity of 40–60%. Rats were given free access to water and food during the experiments. All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee [2019 Ethics (005)]. The experiments were performed in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Generation of a Rat Model of AD Using Aβ1–42

Rats were injected intracerebroventricularly (ICV) with oligomeric Aβ1–42 to induce AD, as described in a previous study (Yang et al., 2017; Wang X. et al., 2018; Abshenas et al., 2020; Hour et al., 2020). Adult (200–220 g) Sprague-Dawley rats were deeply anesthetized with 10% chloral hydrate (0.35 mL/100 g) and immobilized to a stereotactic frame. An incision (1 cm) was made along the midline of the scalp to expose the bregma and coronal suture. A 5 μL volume of Aβ1–42 solution (Sigma, St. Louis, MO, USA) was injected into each side of the hippocampus for 5 min (stereotaxic coordinates: 3 mm posterior to the bregma, 2 mm bilateral from the midline,

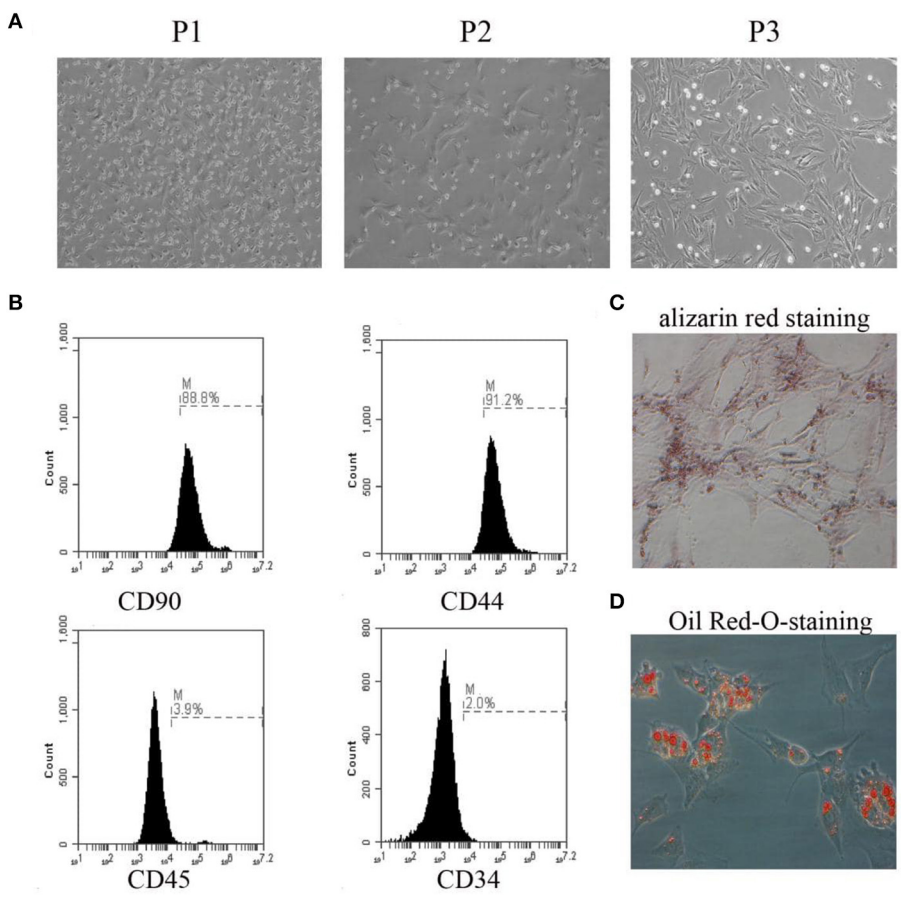

FIGURE 1 | Primary culture of BMSCs and identification. **(A)** The morphology of BMSCs from passage 1 to passage 3 was observed under the microscope. **(B)** The expression rates of CD90, CD44, CD45, and CD34 were detected by flow cytometry. **(C)** The osteogenic differentiation capability of BMSCs was assessed by alizarin red staining. **(D)** The adipogenic differentiation capability of BMSCs was assessed by Oil Red-O-staining. The results displayed were obtained from at least three independent experiments.

and 2.9 mm ventral to the skull surface). The needle was removed slowly 10 min after the injection. The rats were injected intraperitoneally with penicillin (4U/day) for 3 consecutive days after surgery to prevent infection.

Group Allocation and Cell Transplantation

The rats were divided randomly into the following four groups: PBS group, BMSC transplantation group, NT-3-BMSC transplantation group (overexpression of NT-3 in the BMSCs), and sh-NT-3-BMSC transplantation group (silencing of NT-3 in the BMSCs). Seven days after establishment of the AD model, control BMSCs, NT-3-BMSCs or sh-NT-3-BMSCs (1×10^5 cells in 10 μL PBS) were injected into each side of the hippocampus for 10 min at the same stereotaxic coordinates as described above. In the PBS control group, 10 μL PBS was injected into the hippocampus. One month after the injection of cells/PBS, the learning and memory ability of the rats was assessed using the Morris water maze (MWM) test. Following this, the rats were killed, and the brain tissues were collected for analysis as described above.

MWM

Hippocampal-dependent spatial memory was determined using the MWM test. The MWM consisted of a circular pool (diameter: 180 cm; height: 40 cm), an underwater platform and an automatic image acquisition and processing system. The pool was filled with water (22 ± 2°C) and divided into four quadrants: northeast (NE), northwest (NW), southwest (SW), and southeast (SE). A non-visible circular escape platform (diameter: 12 cm; height: 23 cm) was placed 1 cm below the water in the SW quadrant. In all trials, the rats were allowed up to 60 s to find the escape platform and were required to remain on it for 10 s. If a rat could not find the platform within 60 s, it was gently guided to the platform and maintained on it for 10 s, and the latency was recorded as 60 s. The rats were returned to their home cages after each test run. The training was carried out 4 times/day for 5 consecutive days and the escape latency was recorded.

Immunohistochemical Experiments

For immunohistochemical staining of brain tissues, frozen brain sections were deparaffinized and subjected to microwave heat-induced epitope retrieval. The sections were blocked with 10% normal goat serum for 30 min and incubated with primary antibodies against NSE (1:100, Abcam) or NF-200(1:100, Abcam) overnight at 4°C. The sections were then incubated with appropriate Cy3-conjugated secondary antibodies (1:200, Beyotime) for 60 min at room temperature. Images were obtained at a magnification of 400× from 5 random microscopic fields using a fluorescence microscope.

Statistical Analysis

Prism software (GraphPad, San Diego, CA, USA) was used for the analysis. All results are presented as the mean ± standard deviation (SD). Statistical comparisons were made using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test. Statistical significance was considered if the $P < 0.05$.

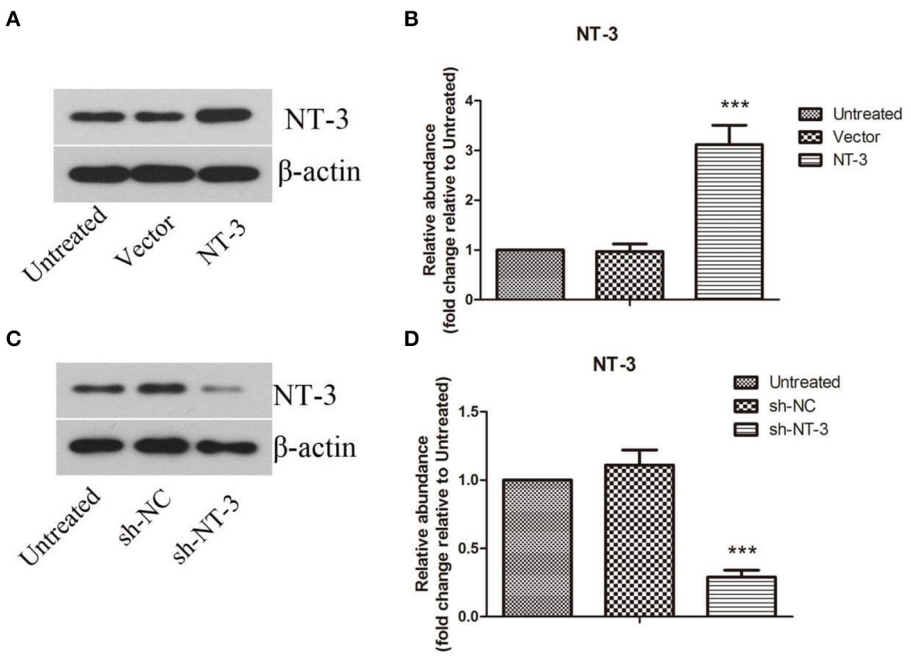

FIGURE 2 | Lentivirus-mediated overexpression or interference of NT-3 in BMSCs. **(A,C)** The protein level of NT-3 in BMSCs transfected by lentivirus was detected by western blot assay. β-actin was used as a loading control. **(B,D)** The densitometric analysis results were shown. The data were expressed as means ± SD ($n = 3$). The results displayed were obtained from at least three independent experiments. ***$P < 0.001$, versus the vector group or sh-NC group.

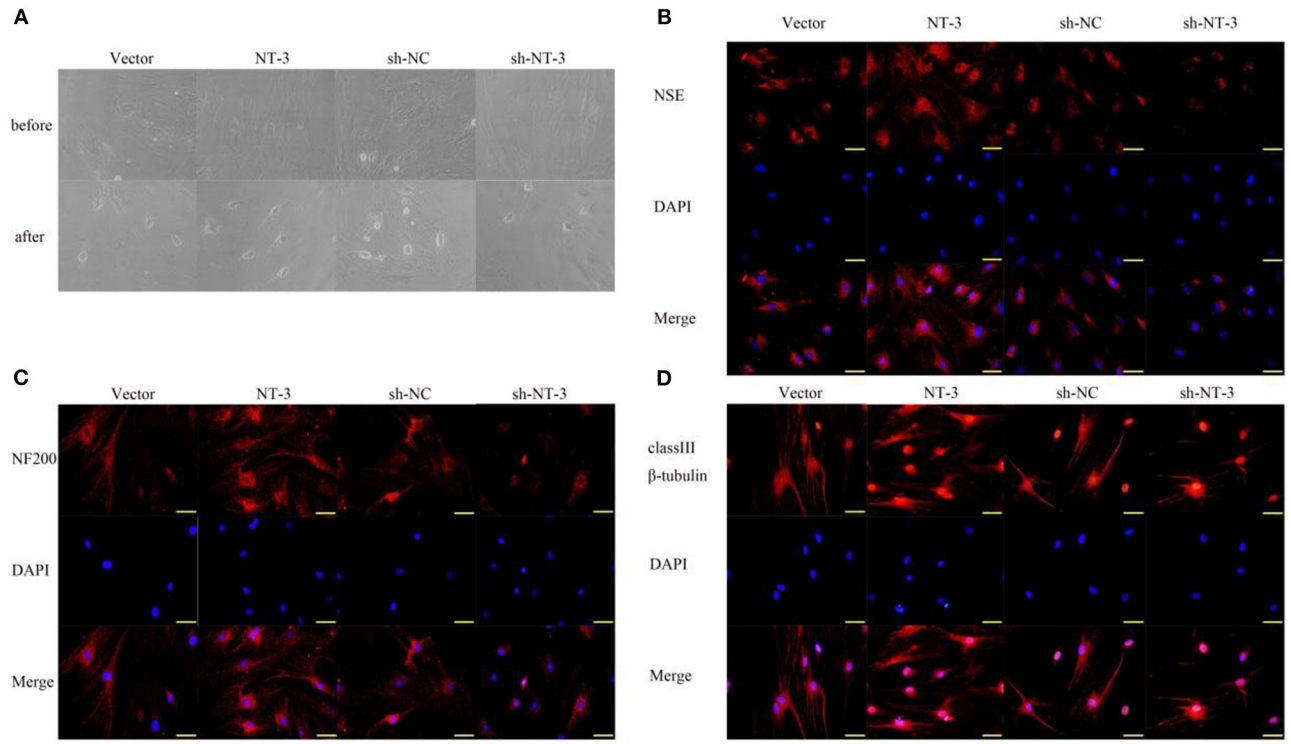

FIGURE 3 | Effect of NT-3 on the differentiation of BMSCs into neurons. **(A)** The morphology changes of BMSCs before and after neuronal differentiation induction were observed under the microscope. **(B–D)** The expressions of NSE, NF-200, and class III β-tubulin were assessed by immunofluorescence staining. Scale bars represent 50 μm. The results displayed were obtained from at least three independent experiments.

RESULTS

Characterization of BMSCs

Representative images of BMSCs from passage 1 to passage 3 are presented in **Figure 1A**. The identification of BMSCs was performed by flow cytometry using antibodies against CD90, CD44, CD45, and CD34. BMSCs were defined as CD90+/CD44+/CD45–/CD34– cells (**Figure 1B**). Osteogenic and adipogenic differentiation abilities were demonstrated by staining with alizarin red and Oil Red O, respectively (**Figures 1C,D**).

Expression of NT-3 in BMSCs

The protein levels of NT-3 in BMSCs after lentiviral-mediated transduction were detected by western blot assay. As shown in **Figures 2A,B**, the protein level of NT-3 in BMSCs was approximately doubled after gene transduction with lentivirus ($P < 0.001$). Successful interference of NT-3 expression in BMSCs was also confirmed (**Figures 2C,D**), and the efficiency of lentivirus-mediated interference was more than 70% ($P < 0.001$).

NT-3 Promotes Neuronal Differentiation of BMSCs *in vitro*

The differentiation of BMSCs into neurons was induced by two-compartment co-culture of BMSCs and neuronal cells. The morphologic characteristics of the BMSCs before and 8 days after co-culture are shown in **Figure 3A**. Some BMSCs exhibited a neuron-like morphology and became smaller and rounder. The neuron-like morphologic changes were more pronounced in the NT-3 overexpression group than in the vector control group, whereas silencing of NT-3 expression attenuated the morphologic changes. Immunostaining of NSE, NF-200, and neuronal class III β-tubulin in BMSCs was performed to further demonstrate differentiated neurons. As shown in **Figures 3B–D**, NT-3 overexpression enhanced the expressions of NSE, NF-200 and neuronal class III β-tubulin in BMSCs, whereas silencing of NT-3 resulted in weaker staining for NSE, NF-200, and neuronal class III β-tubulin.

NT-3 Promotes Neuronal Differentiation of BMSCs *in vitro* by Regulating the β-Catenin Expression

The mRNA expression of β-catenin in BMSCs was detected by qPCR (**Figure 4A**). Overexpression of NT-3 significantly increased the mRNA expression of β-catenin in BMSCs while interference of NT-3 suppressed β-catenin mRNA expression, as compared with the vector control group ($P < 0.001$). The protein levels of total and nuclear β-catenin were assessed by western blot assay. As shown in **Figures 4B–D**, the levels of total and nuclear β-catenin in BMSCs were enhanced significantly by NT-3 overexpression, compared with the vector group ($P < 0.001$). However, silencing of NT-3remarkably down-regulated the levels

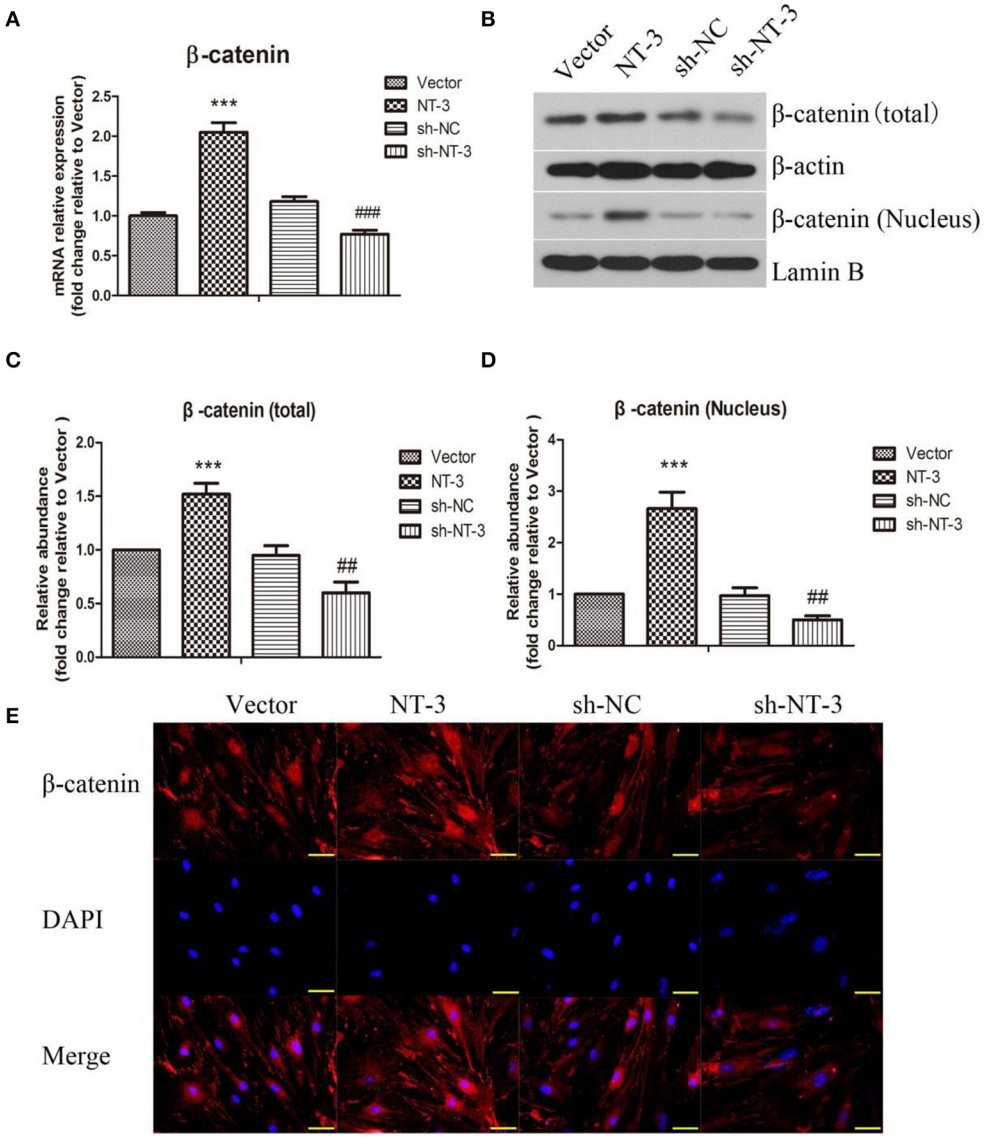

FIGURE 4 | Effect of NT-3 on Wnt/β-catenin signaling pathway in BMSCs. **(A)** The mRNA expression of NT-3 in BMSCs was assessed by qPCR. **(B)** The levels of total and nuclear β-catenin in BMSCs were determined by western blot assay. β-actin and Lamin B were used as loading controls. **(C,D)** The densitometric analysis results were shown. **(E)** The expression of β-catenin in BMSCs was detected by immunofluorescence staining. Scale bars represent 50 μm. The data were expressed as means ± SD ($n = 3$). The results displayed were obtained from at least three independent experiments. $^{***}P < 0.001$, vs. the vector group. $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$, vs. the sh-NC group.

of total and nuclear β-catenin ($P < 0.01$). Immunostaining of β-catenin in BMSCs further confirmed that the expression of β-catenin protein was increased by NT-3 overexpression but decreased by NT-3 silencing (**Figure 4E**).

Expression of NT-3 in Brain

The NT-3 level in brain tissue 1 month after BMSC transplantation was assessed by western blot assay (**Figures 5A,B**). Compared with the BMSC group, the NT-3 level in brain tissue was increased in the NT-3-BMSC group ($P<0.001$) but decreased in the sh-NT-3-BMSC group ($P < 0.01$).

Transplantation of BMSCs Modified With the NT-3 Gene Improves Cognitive Function in Rats With Experimental AD

The cognitive function of rats with experimental AD after transplantation of BMSCs was assessed using the MWM assay. On day 3 of training, the latencies were decreased in the BMSC and NT-3-BMSC groups compared with the

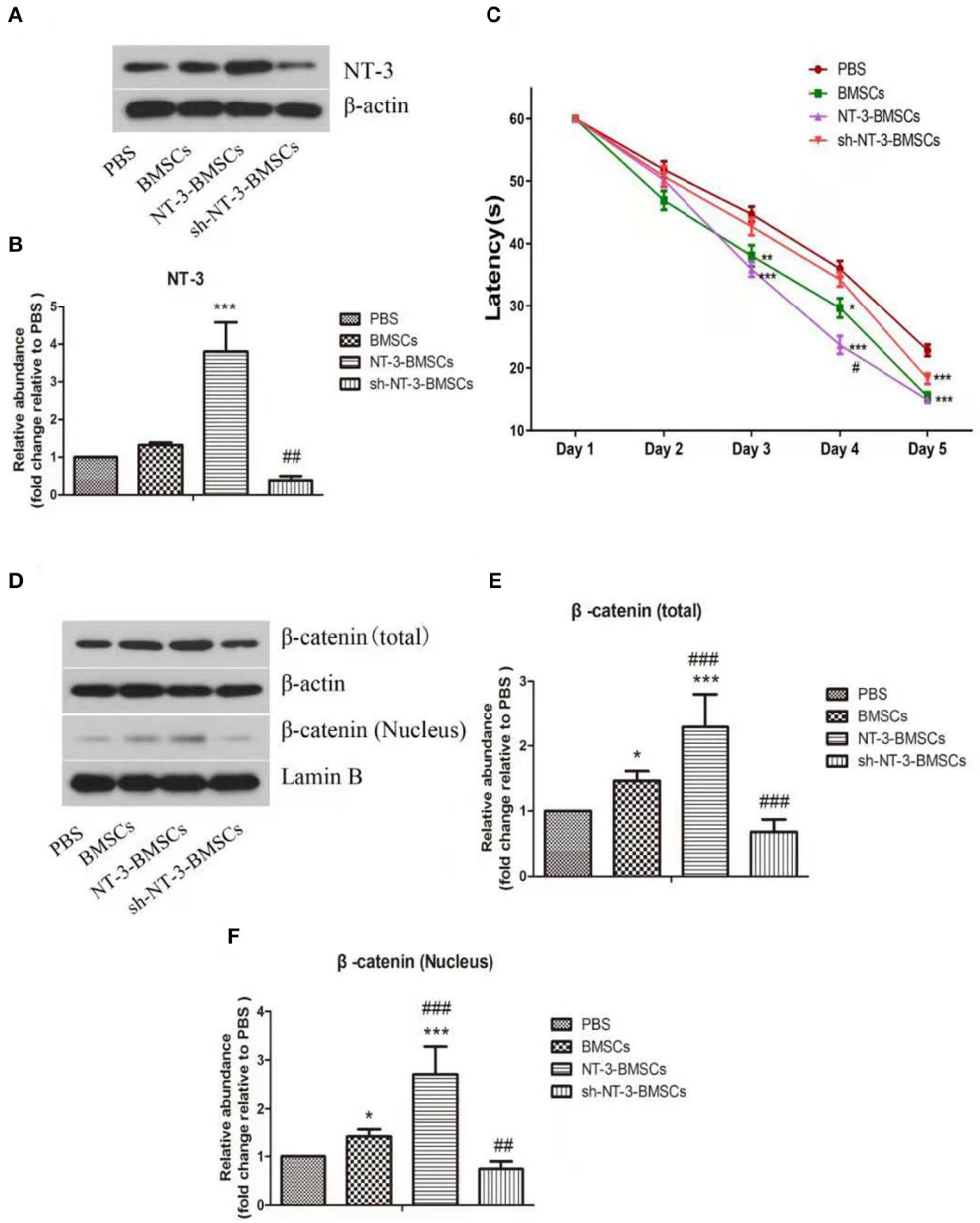

FIGURE 5 | Effect of NT-3 genetic modified BMSCs transplantation on the cognitive function and Wnt/β-catenin pathway in rats with AD. **(A)** The protein levels of NT-3 in brain tissues were assessed by western blot assay. β-actin was used as a loading control. **(B)** The densitometric analysis results were shown. **(C)** The cognitive function of the AD rats were determined by MWM. The mean escape latency of each group was shown. **(D)** The levels of total and nuclear β-catenin in brain tissues were determined by western blot assay. β-actin and Lamin B were used as loading controls. **(E,F)** The densitometric analysis results were shown. The data were expressed as means ± SD ($n = 4$). The results displayed were obtained from at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$; $^{***}P < 0.001$, vs. the PBS group. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$, vs. the BMSCs group.

PBS group (**Figure 5C**). However, there was no difference in latency between the sh-NT-3-BMSC group and PBS group. On day 4 of training, the decrease in latency was more obvious in the NT-3-BMSC group than in the BMSC group, whereas the sh-NT-3-BMSC group and PBS group showed little difference in latency. On day 5 of training, the latencies in the BMSC, NT-3-BMSC, and sh-NT-3-BMSC groups were decreased significantly compared with the PBS group, but there were no differences between the three BMSC groups.

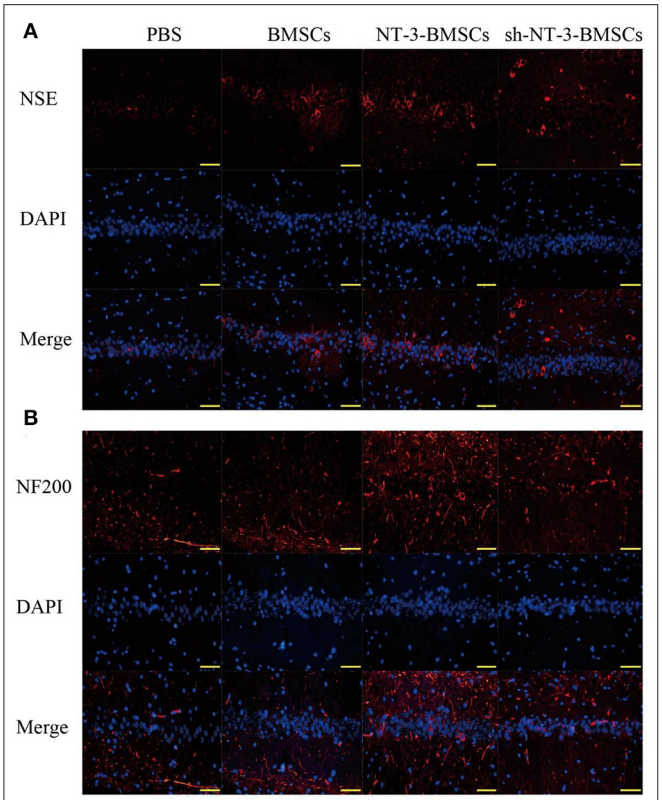

FIGURE 6 | Effect of NT-3 genetic modified BMSCs transplantation on neurogenesis in brain tissues. The expressions of NSE **(A)** and NF-200 **(B)** in the brain tissues were determined by immunofluorescence staining. Scale bars represent 50 μm. The results displayed were obtained from at least three independent experiments.

NT-3 Promotes the Neuronal Differentiation of BMSCs in Rats With Experimental AD by Regulating the β-Catenin Expression

The protein levels of total and nuclear β-catenin in brain tissue were assessed by western blot assay. As shown in **Figures 5D–F**, the levels of total and nuclear β-catenin in brain tissue were increased significantly in the BMSC group compared with the PBS group ($P < 0.05$), and the levels were further up-regulated in the NT-3-BMSC group ($P < 0.001$) but down-regulated in the sh-NT-3-BMSCgroup ($P < 0.01$).

NT-3 Promotes the Neuronal Differentiation of Transfected BMSCs in Rats With Experimental AD

Immunostaining of NSE and NF-200 was carried out to detect neurogenesis in brain tissue after transplantation of BMSCs. As shown in **Figures 6A,B**, the expressions of NSE and NF-200 in brain tissue were higher in the NT-3-BMSC group than in the BMSC group. However, the expressions of NSE and NF-200 were suppressed in the sh-NT-3-BMSC group.

DISCUSSION

AD, a neurodegenerative disease of the central nervous system, has become one of the major challenges facing medicine. In recent years, research into the transplantation of BMSCs has provided new ideas and prospects to meet this challenge. In 2000, Woodbury et al. reported that BMSCs could differentiate into neuron-like cells *in vitro* under suitable conditions. A growing body of evidence has demonstrated that genetic modification of BMSCs can promote the differentiation of BMSCs into neurons (Duan et al., 2014; Liu et al., 2015). In the present study, we have provided novel data showing that the transplantation of BMSCs modified with the NT-3 gene can affect neuroregeneration in rats with experimental AD and that the mechanisms involve regulation of the Wnt/β-catenin pathway.

We first evaluated the effect of NT-3 on the differentiation of BMSCs into neurons *in vitro*. Previous research had suggested that NT-3 could contribute to the neuronal differentiation of BMSCs (Moradian et al., 2017; Wu et al., 2018), but the relevant molecular mechanisms were not fully understood. Differentiated neurons were identified by detecting the expressions of NSE, NF-200, and class III β-tubulin. NSE is one of the enolases involved in the glycolytic pathway and is reported to be a highly specific marker for neurons (Isgro et al., 2015). NF-200 is a neuron-specific intermediate filament that plays a pivotal role in healthy neurons, while class III β-tubulin is expressed almost exclusively in neurons. According to our study, overexpression of NT-3 promoted the expressions of NSE, NF-200, and class III β-tubulin in BMSCs, while silencing of NT-3 suppressed their expressions. These results demonstrate that NT-3 facilitates the neuronal differentiation of BMSCs, consistent with previous studies.

β-catenin is an important member of the Wnt/β-catenin signaling pathway and is mainly expressed in the cytoplasm. When β-catenin translocate from the cytoplasm to accumulate in the nucleus, downstream targets are activated to induce a number of biologic effects such as cell proliferation and differentiation (Lai et al., 2009). The Wnt/β-catenin pathway has attracted more attention with advances in the study of the molecular mechanisms underlying neuronal differentiation of stem cells. Research has showed that the Wnt/β-catenin pathway participates in the formation of dopaminergic neurons and nerve regeneration (Tang et al., 2009). David et al. suggested that the Wnt/β-catenin pathway regulated the proliferation and neurogenesis of spinal cord neural precursors (David et al., 2010). Recently, the role of the Wnt/β-catenin pathway in the differentiation of BMSCs into neurons has been determined. It was demonstrated that the Wnt/β-catenin signaling pathway provided neuroprotection, promoted neurogenesis and improved neurocognitive function after transplantation of BMSCs into mice with traumatic brain injury (Zhao et al., 2016). Tsai et al. found that activation of the Wnt/β-catenin pathway promoted neurotrophic factors induced neuronal differentiation of BMSCs (Tsai et al., 2014). According to our results, activation of the Wnt/β-catenin pathway is involved in the mechanism by which NT-3 regulates the neuronal differentiation of BMSCs.

The transplantation of BMSCs has been reported to improve learning and memory ability in animal models of AD (Salem et al., 2014). Research has also suggested that the beneficial effect of BMSC transplantation in AD was through inhibition of neuronal cell death and recruitment of microglial immune responses (Ramezani et al., 2020). Moreover, Zhang et al. reported that the transplantation of BMSCs modified with the gene for brain-derived neurotrophic factor significantly improved the cognitive function of rats with experimental AD (Zhang et al., 2012). Based on this previous research, we further investigated the effects of transplantation of BMSCs modified with the NT-3 gene on neuroregeneration and cognitive function *in vivo* in a rat model of AD. Our results indicated that, compared with transplantation of unmodified BMSCs, the transplantation of BMSCs overexpressing NT-3 resulted in greater enhancements of NT-3 expression in brain tissue, neuroregeneration and cognitive function in rats with experimental AD. Furthermore, these effects resulted from activation of the Wnt/β-catenin pathway. By contrast, smaller effects were observed following the transplantation of BMSCs with NT-3 silencing. In our study, we found that NT-3 can affect the expression of β-catenin. But the mechanism is not clear. Previous study showed that NT-3 can link Trk receptor including Trk-A, Trk-B, and Trk-C, modulate intracellular signal transduction through PI3K-Akt and MAPK pathways, and regulate cell survival and differentiation (Wang et al., 2017).

In conclusion, our study suggests that overexpression of NT-3 promotes neuronal differentiation of BMSCs and that transplantation of NT-3-BMSCs accelerates neuroregeneration in rats with experimental AD via activation of the Wnt/β-catenin pathway. Silencing of NT-3 had the opposite effect. Our study may provide a theoretical basis for using transplantation of BMSCs modified with the NT-3 gene in the treatment of AD. Nevertheless, our study is not without limitations, first, we only found that NT-3 can promote the expression of β-catenin, and how NT-3 affects the Wnt/β-catenin pathway is not clear. Therefore, the probabilistic mechanism of NT-3 involvement in the treatment needs to be further studied. Second, previous studies have shown that NT-3 probably binds to the Brain Derived Neurotrophic Factor (BDNF) to regulate neurogenesis and nerve survival, and hence, the interaction of NT-3 and BDNF in neural differentiation of mesenchymal stem cells need to be further explored.

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/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of Jining First People's Hospital.

AUTHOR CONTRIBUTIONS

ZY and YD planned and conducted the experiments. XS, HW, CS, and QL contributed to the performing of the experiments. ZY and XS contributed to the performing of the data analysis and drafting the manuscript. YD revised the manuscript. All authors approved the final manuscript.

FUNDING

This research was conducted with funding by grants from Science and Technology Development Plan of Shandong province (2012GSF11838) to ZY.

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

frontiers in Cellular Neuroscience

REVIEW
published: 25 February 2021
doi: 10.3389/fncel.2021.637210

Insight Into the Mechanisms and the Challenges on Stem Cell-Based Therapies for Cerebral Ischemic Stroke

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Strokes are the most common types of cerebrovascular disease and remain a major cause of death and disability worldwide. Cerebral ischemic stroke is caused by a reduction in blood flow to the brain. In this disease, two major zones of injury are identified: the lesion core, in which cells rapidly progress toward death, and the ischemic penumbra (surrounding the lesion core), which is defined as hypoperfusion tissue where cells may remain viable and can be repaired. Two methods that are approved by the Food and Drug Administration (FDA) include intravenous thrombolytic therapy and endovascular thrombectomy, however, the narrow therapeutic window poses a limitation, and therefore a low percentage of stroke patients actually receive these treatments. Developments in stem cell therapy have introduced renewed hope to patients with ischemic stroke due to its potential effect for reversing the neurological sequelae. Over the last few decades, animal tests and clinical trials have been used to treat ischemic stroke experimentally with various types of stem cells. However, several technical and ethical challenges must be overcome before stem cells can become a choice for the treatment of stroke. In this review, we summarize the mechanisms, processes, and challenges of using stem cells in stroke treatment. We also discuss new developing trends in this field.

Keywords: stem cell, cell transplantation, ischemic stroke, brain regeneration, molecular mechanism

OPEN ACCESS

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

Received: *03 December 2020*
Accepted: *03 February 2021*
Published: *25 February 2021*

Citation:
Liu H, Reiter S, Zhou X, Chen H, Ou Y, Lenahan C and He Y (2021) Insight Into the Mechanisms and the Challenges on Stem Cell-Based Therapies for Cerebral Ischemic Stroke. Front. Cell. Neurosci. 15:637210. doi: 10.3389/fncel.2021.637210

INTRODUCTION

Stroke is a leading cause of functional impairment and death worldwide (Meschia et al., 2014). Approximately 795,000 people suffer from a stroke and more than 140,000 people die from it in the United States annually (Virani et al., 2020). There are three types of stroke: transient ischemic attack, ischemic stroke, and hemorrhagic stroke. Approximately 80% of strokes are ischemic (Thrift et al., 2001). Despite the high incidence of stroke, an effective therapy does not exist, particularly for chronic stroke. Currently, treatment for acute ischemic stroke is limited to efficient and fast removal of thrombus *via* intravenous use of tissue-type plasminogen activator (tPA) within 4.5 h, or endovascular mechanical thrombectomy within 6 h after symptom onset (Powers et al., 2018).

However, the narrow effective therapeutic window is the major limitation, and there is a high potential for hemorrhagic transformation. Consequently, few patients receive these therapies, which are compounded by the lack of existing rehabilitative therapy. Therefore, there is substantial interest in alleviating the post-stroke sequelae and improving restorative recovery.

Recent developments in stem cell biology have provided renewed hope for treating ischemic stroke. Stem cells are characterized by their potential to proliferate and differentiate, which makes stem cell transplantation the method of choice to facilitate neural regeneration, modulate microenvironments, and replace injured tissues. Nearly four decades of experimental evidence have proven the efficacy and safety of stem cell therapies in pre-clinical animal tests and clinical trials (Borlongan, 2019). In this review, we discuss the potential mechanisms, cell types, methods, and time for stem cell transplantation, current trends in stem cell-based therapy, and the challenges that need to be overcome.

POTENTIAL MECHANISMS OF STEM CELL THERAPY FOR ISCHEMIC STROKE

The etiology of ischemic stroke is due to a thrombotic or embolic blockage of an artery, resulting in acute loss of neurons, microglia, astrocytes, and oligodendroglia, as well as disruption of synapse structure. The pathophysiology of ischemic stroke remains unclear and involves a complex process. Increased apoptosis, inflammatory reaction, vascular remodeling, and neuronal injury are involved in ischemic stroke-induced neuronal death in the brain. Multiple potential mechanisms are involved in stem cell-based therapy for ischemic stroke (**Figure 1**), including cell migration and neurotrophic secretion, apoptosis and inflammation inhibition, angiogenesis, and neural circuit reconstruction. Therefore, stem cell therapy may be effective for stroke patients by replacing damaged neurons and promoting synaptic formation, as well as by stimulating angiogenesis, anti-apoptosis, and anti-inflammatory effects.

Cell Migration and Neurotrophic Secretion

It has been proven that the adult brain is capable of self-repair *via* endogenous generation of new neurons to replace neurons that have died (Arvidsson et al., 2002). However, the survival rate and the total number of new neurons are extremely low. Moreover, there is insufficient neurogenesis to replace the lost neurons. Providing enough exogenous stem cells may be more conducive for repairing the injured neurons. The blood-brain barrier (BBB) is disrupted after a stroke. The transplanted stem cells can easily cross the BBB to gather in the infarcted brain areas and reconstruct the BBB integrity (Bang et al., 2017; Sun et al., 2020). Those cells can differentiate into various types of cells forming nervous tissue (e.g., mature neurons, oligodendrocytes, and astrocytes) and release a host of neurotrophic factors and cytokines [e.g., vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF), nerve growth factor (NGF), insulin-like growth factor 1 (IGF-1)], which could promote neurogenesis to replace injured cells and improve neurological function (Ishibashi et al., 2004; Schinköthe et al., 2008; Kupcova Skalnikova, 2013).

Apoptosis and Inflammation Inhibition

Several studies have suggested that a reduction in apoptosis in the ischemic boundary area occurs following cellular therapy that is associated with improved neurological recovery in experimental models (Stonesifer et al., 2017; Sun et al., 2020). It was reported that the neuroprotective effects of human bone marrow mesenchymal stem cells (hMSCs) against cerebral ischemia could be antagonized by the apoptosis-related Bcl-2 antibody (Zhang et al., 2019). When hMSCs were co-cultured with oxygen-glucose deprived (OGD)-injured neurons, they triggered a series of events, including a reduction in rates of apoptosis and necroptosis, downregulation of the necroptosis-related receptor-interacting protein kinase 1 and 3, and deactivation of caspase-3, an enzyme involved in apoptosis (Kong et al., 2017). It was proven that stem cells could efficiently promote neurological functional recovery *in vivo* by preventing neuronal apoptosis (Sun et al., 2020). Also, cerebral ischemia was found to promote the release of damage-associated molecular patterns (DAMPs) and matrix metalloproteinases (MMPs), which led to a series of inflammatory responses such as astrocyte and microglia activation, proinflammatory cytokine and chemokine production, and infiltration of leukocytes and neutrophils (Jayaraj et al., 2019; Stanzione et al., 2020). Stem cells can orchestrate other cells to exert anti-inflammatory effects by decreasing their secretion of inflammatory markers such as interleukin 6 (IL-6), granulocyte colony-stimulating factor (G-CSF), interleukin 1 (IL-1), and tumor necrosis factor (TNF-α; Redondo-Castro et al., 2017). Stem cells can also secrete a wide range of other factors including anti-inflammatory cytokines, such as IL-10, IL-12, and TGF-β (Boshuizen and Steinberg, 2018).

Angiogenesis

Both *in vitro* and *in vivo* studies show that transplanted stem cells could promote neovascularization, stimulate angiogenesis and produce several angiogenic factors, which can be beneficial to functional recovery and neuronal regeneration (Zhang et al., 2011; Hicks et al., 2013; Zong et al., 2017). Transplanted stem cells can promote the secretion of VEGF and bFGF, which could enhance angiogenesis. Ryu et al. (2019) reported that *in vivo* treatment with MSCs significantly increased vessel length, vessel area, vessel volume, and the number of branching points. Kikuchi-Taura et al. (2020) demonstrated that gap junction-mediated cell-cell interaction was the prominent pathway for bone marrow mononuclear cells to activate angiogenesis after ischemia.

Neural Circuit Reconstruction

Axonal plasticity may be the basic mechanism of stem cell therapy (Boshuizen and Steinberg, 2018). After stem cell treatment, the numbers of axons and myelin sheaths increase in the rat hippocampus, corpus callosum, and corpus striatum (Li et al., 2016). Andres et al. (2011) proved that human neural precursor cell transplantation could promote dendritic plasticity

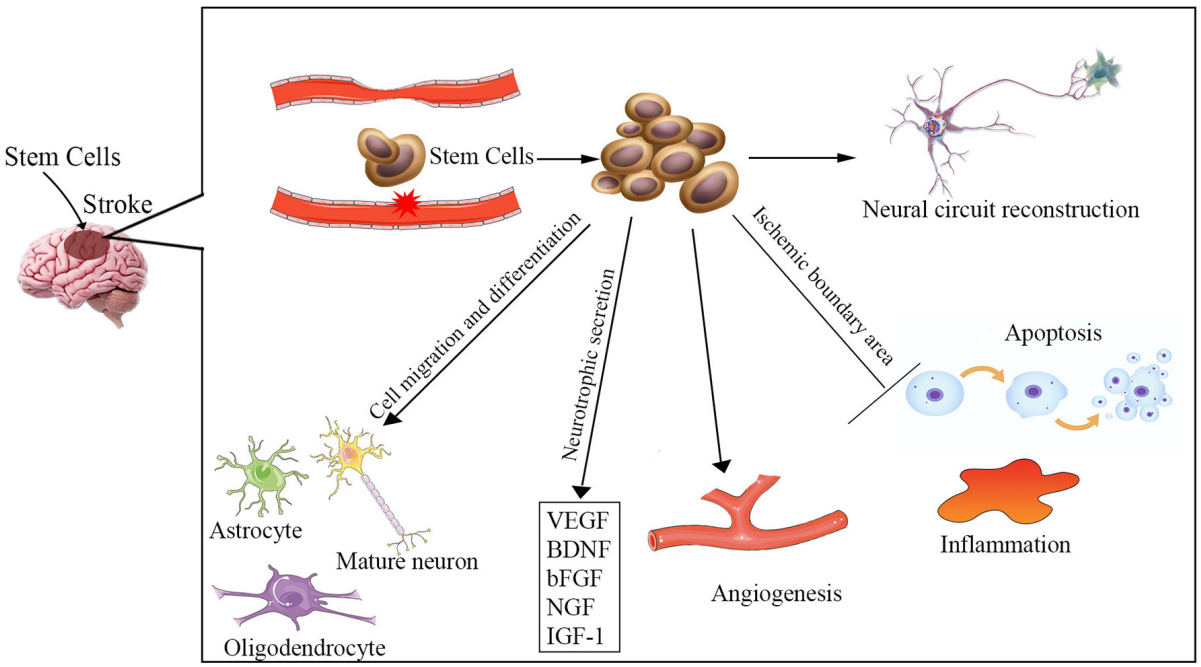

FIGURE 1 | Overview of the potential mechanisms of stem cell therapy for ischemic stroke. Neuronal injury, increased apoptosis, inflammatory reaction, and vascular remodeling are involved in the pathophysiology of ischemic stroke. The underlying mechanisms of stem cell therapy for ischemic stroke may be to reverse these processes, including cell migration and differentiation into various cells to replace damaged neurons, neurotrophic secretion, apoptosis and inflammation inhibition, angiogenesis, and enhancement of neural circuit reconstruction. VEGF, vascular endothelial growth factor; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; NGF, nerve growth factor; IGF-1, insulin-like growth factor 1.

and axonal rewiring, which are mediated by the secretion of VEGF. Stem cells can interact with the surrounding neural tissues and can enhance graft-host synaptic connectivity to form new neural circuity (Oki et al., 2012).

STEM CELL TYPES FOR TREATMENT OF ISCHEMIC STROKE

Stem cells are characterized by two unique properties: the capacity for self-renewal and differentiation into other cell types. In animal models, several types of stem cells were reported as efficient treatments for stroke (**Figure 2**), including neural stem cells (NSCs), neural progenitor cells (NPCs), embryonic stem cells (ESCs; Hicks et al., 2009), mesenchymal stem cells (MSCs; Steinberg et al., 2016), bone marrow mononuclear cells (BMMCs; Prasad et al., 2014), and induced pluripotent stem cells (iPSCs; Tornero et al., 2013). In clinical trials, the most commonly used stem cells are MSCs or NSCs, including wild-type and genetically modified cells.

Embryonic Stem Cells (ESCs)

ESCs are derived from the inner cell mass of blastocysts. They can differentiate into neurons, astrocytes, oligodendrocytes, and glial cells (Thomson et al., 1998; Wichterle et al., 2002). An advantage of using ESCs is their unlimited proliferative capacity *in vitro*. Several studies have demonstrated that the transplantation of differentiated ESCs can contribute to vascular regeneration and improved sensorimotor function after stroke (Oyamada et al., 2008; Hicks et al., 2009). However, ethical concerns, limited access to these cells, and the chances of forming tumors restrict widespread medical use of ESCs.

Endogenous Neural Stem/Precursor Cells (NSCs)

NSCs are found in the embryonic developing brain and the adult mammalian brain. They are present within specific regions, including the ventricular-subventricular zone (V-SVZ) of the lateral ventricles (Alvarez-Buylla and Garcia-Verdugo, 2002), the subgranular zone (SGZ) of the hippocampus (Djavadian, 2004), and the external germinal layer (EGL) of the cerebellum (Hatten and Heintz, 1995). In recent years, numerous studies have shown that NSCs within the V-SVZ/SGZ/EGL undergo continuous proliferation and differentiation in the middle cerebral artery occlusion (MCAO) animal model for at least 4 months following stroke ischemia (Thored et al., 2006). Several methods are available for the isolation and culture of NSCs *in vitro*. NSCs can be isolated directly from the SVZ/SGZ/EGL in adults or from the neuroectoderm of the developing embryonic brain (Martí-Fàbregas et al., 2010; Guo et al., 2012). Although this method is the most convenient, the number and survival rate of neurons from these proliferative cells were extremely low. This is likely to be because of an increased concentration of inflammatory

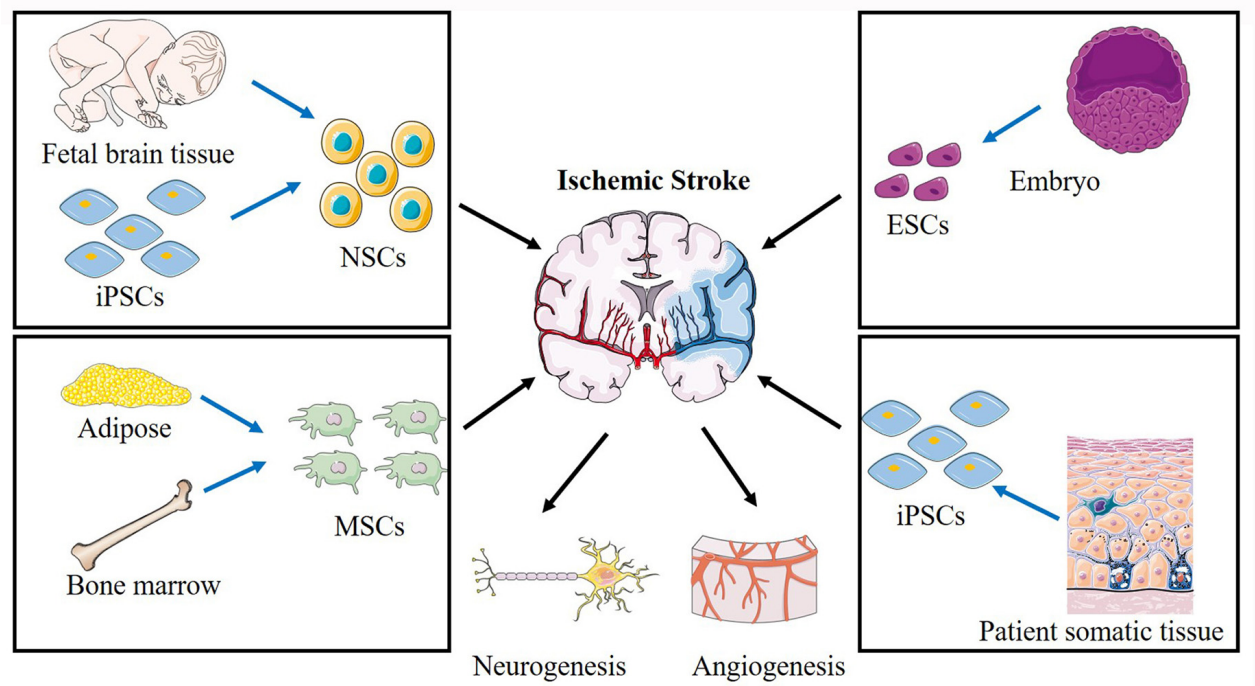

FIGURE 2 | Schematic of various stem cells applied in cerebral ischemic stroke and the proposed mechanisms. Different sources of NSCs, ESCs, MSCs, BMMCs, iPSCs are applied to treat ischemic stroke. NSCs, neural stem cells; ESCs, embryonic stem cells; MSCs, mesenchymal stem cells; iPSCs, induced pluripotent stem cells.

cytokines or inadequate nutritional support. Fortunately, various approaches can promote endogenous neurogenesis by promoting endogenous NSC survival, proliferation, and differentiation, which have provided a promising approach for treating stroke. For example, direct injection of certain growth factors, such as BDNF or VEGF, promotes the migration of endogenous NSCs to injured brain areas (Jin et al., 2002).

Mesenchymal Stem Cells (MSCs)

Numerous pre-clinical and clinical studies of stroke have been carried out using MSCs (Zheng et al., 2018). MSCs can be easily isolated from bone marrow, adipose tissue, peripheral blood, umbilical cord, dental pulp, and amniotic fluid (Yan et al., 2013). Bone marrow-derived MSCs (BMSCs) are the most frequently used MSCs in experimental studies exploring stroke treatments. BMSCs can be autologous and thereby avoid immune rejection and viral transmission (Bang et al., 2005). BMSCs have been considered the gold standard for cell therapy research. Several studies have proven that BMSCs have the potential to differentiate into neuronal cells *in vitro* upon treatment with various growth inducers (Ferroni et al., 2013; Yue et al., 2016). After MSCs transplantation, the microenvironment of damaged brain tissues can be modulated toward a more regenerative and anti-inflammatory *milieu* by decreasing the release of pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-6, or by secretion of anti-inflammatory factors. Also, the secretion of trophic factors and antiapoptotic molecules that promote neurogenesis and angiogenesis is increased. Some studies have also reported the use of human umbilical cord-derived MSCs for ischemic stroke treatment (Feng et al., 2020; Noh et al., 2020).

Bone Marrow Mononuclear Cells (BMMCs)

BMMCs are a heterogeneous population of cells that include monocytes, lymphocytes, hematopoietic stem cells, and mesenchymal, hematopoietic, and endothelial progenitor cells. BMMCs can be isolated autologously and conveniently cultured, which could be advantageous in acute ischemic stroke. In one clinical trial, it was reported that intravenous reinfusion of autologous BMMCs within 24–72 h of stroke onset might be feasible and effective (Savitz et al., 2011). Earlier transplantation of BMMCs may be more efficacious in enhancing recovery following stroke onset.

Induced Pluripotent Stem Cells (iPSCs)

iPSC are a type of pluripotent stem cell that can be generated directly from somatic cells. iPSC have been shown to differentiate into neuronal and glial phenotypes, and could potentially be used for the treatment of stroke. However, the process of obtaining iPSCs is expensive and time-consuming. Furthermore, the translational use of iPSCs has been limited due to the potential risk of oncogenesis and insertional mutagenesis, poor integration into host neuronal circuits, and production of immune-tolerable cells (Kooreman and Wu, 2010).

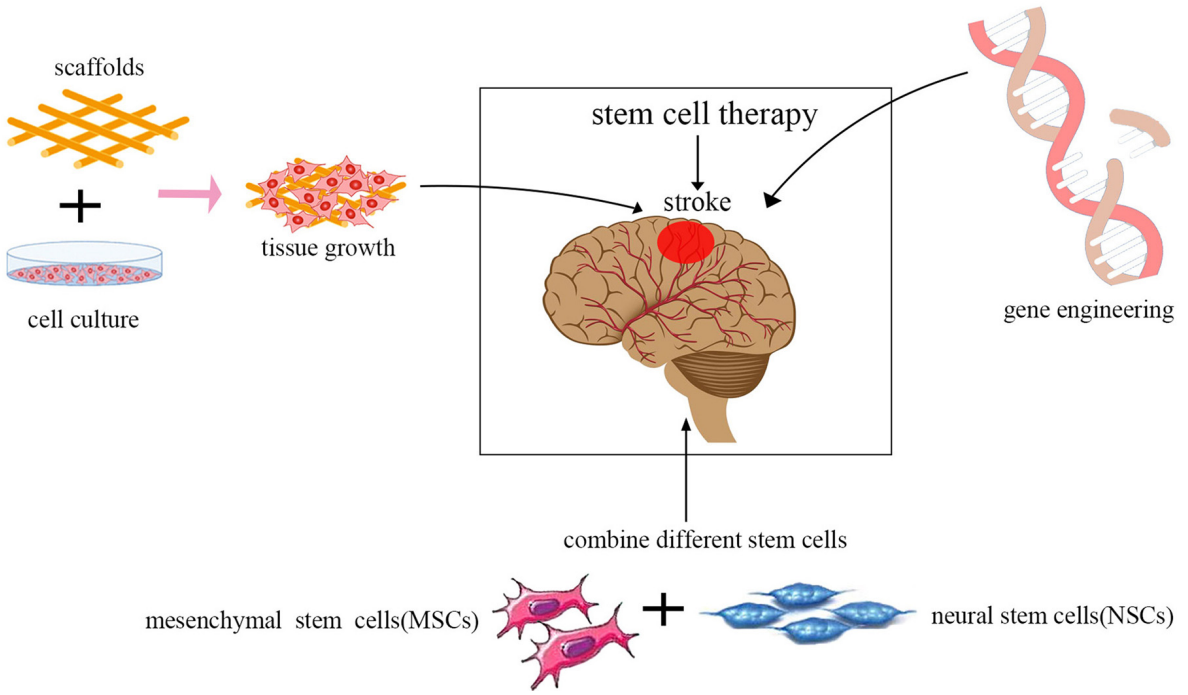

FIGURE 3 | Current trends in stem cell therapy for ischemic stroke. The integration of gene therapy, tissue engineering scaffolds, and the use of various stem cell types are the current trends in stem cell therapy.

STEM CELL TRANSPLANTATION

The most appropriate route for stem cell delivery remains unresolved. Cells can be transplanted using various delivery routes, including intracerebral, subarachnoid, and intranasal administration, as well as intravascular delivery *via* the tail vein (IV) or intra-artery (IA) injection. Among those methods, intracerebral administration is the most effective but most invasive method for exogenous stem cells to reach the injured region directly. Conversely, intravascular administration (IV or IA) is the least effective and least invasive method. Several concerns must be addressed in using the intracerebral or intraventricular method, including poor cell viability, invasiveness, immune rejection, and an uncertain prognosis, which present hurdles in the translational application of cell therapy (Wu et al., 2015). Regarding intravascular delivery methods, viable stem cells may not pass filtering organs, such as the lungs, liver, and spleen after IV administration. However, some studies promote the use of IA for stem cell administration as the shortest route toward the lesion, with improving cell engraftment and survival (Na Kim et al., 2017). The current animal studies or clinical trials did not systematically assess or report adverse events associated with the delivery route chosen. Only two animal studies explored the safety of the IA injection approach (Janowski et al., 2013; Yavagal et al., 2014). It has been reported that a higher incidence of strokes was observed due to microthrombus when injecting cells at a higher dose (2×10^6) but the same was not observed when a lower dose (1×10^6) was used to inject rats. However, this approach has not yet been tested in humans. Most of the current studies were conducted in animal models (**Table 1**; Brenneman et al., 2010; Chang et al., 2013; Kawabori et al., 2013; Cheng et al., 2015; Webb et al., 2018; Tian et al., 2019; Tobin et al., 2020; Asgari Taei et al., 2021). Further studies are needed to determine the best route for stem cell transplantation in treating stroke patients.

The appropriate timing of transplantation after ischemia is another critical factor affecting treatment outcomes and the survival of transplanted cells. In clinical trials, the time window of stem cell transplant administration ranged from 24 h to 2 years. Depending on the cell type or source, different time windows of administration possibly contribute to various levels of efficacy (Li et al., 2020). Several studies using the MCAO animal model have reported stem cell transplantations at times of 1 day (Chu et al., 2004; Zhang et al., 2009), 7 days (Kelly et al., 2004; Daadi et al., 2008), or 4 weeks (Jin et al., 2010) after the stroke. However, no studies have compared the effects of injection at different times. There are diverse opinions and a lack of consensus on the optimal timing of transplantation after stroke onset.

CURRENT TRENDS IN STEM CELL THERAPY

In recent years, stem cell therapeutics have become increasingly effective for ischemic stroke with the advancement of technology including the use of genetic and tissue engineering. There are some combination therapy strategies, such as the integration of

TABLE 1 | Experimental study for various stem cells transplantation into the ischemic brain.

References	Cell type	Animal model	Route of administration	Delivery timing (after ischemia)	Dose	Results
Asgari Taei et al. (2021)	Human ESC	Rat MCAO	left lateral ventricle	1, 24, and 48 h	1×10^6	Neurological deficits↓ Synaptic↑ Infarct volumes↓ Cerebral edema↓
Cheng et al. (2015)	NSC line C17.2	Rat MCAO	IV (tail vein)	24 h	5×10^6	Functional recovery↑ Neurogenesis↑ proliferation↑
Tian et al. (2019)	Leukemia inhibitory factor -transfected NSCs	Rat MCAO	IV (tail vein)	2 h	5×10^5	Lesion volume↓ Functional↑ Glial cell regeneration↑ Apoptosis↓
Webb et al. (2018)	NSCs-derived extracellular vesicle	porcine MCAO	IV	2, 14, and 24 h	2.7×10^6	Lesion volume↓ Cerebral diffusivity↑ Motor activity ↑ Exploratory behavior↑
Tobin et al. (2020)	interferon-γ–activated BMSCs	Rat MCAO	IV	4.5 h	5×10^6/kg	Functional recovery↑ Oligodendrogenesis↑ Microglia activation↓ Inflammation↓
Kawabori et al. (2013)	Rat BMSCs	Rat MCAO	ipsilateral striatum	1 or 4 weeks	1×10^5 or 1×10^6	Functional recovery↑ Differentiation ↑
Brenneman et al. (2010)	Autologous BMMCs	left MCA and left ICA occlusion	IA (carotid artery)	24 h	1×10^7 or 4×10^6	Neurological recovery↑ Infarct volume↓ Inflammation↓
Chang et al. (2013)	Human iPSCs	Rat MCAO	intracerebral	7 days	2×10^5	Behavioral↑ Neurogenesis↑ Inflammation↓ apoptosis↓

ESCs, embryonic stem cells; NSCs, neural stem cells; BMSCs, bone marrow-derived mesenchymal stem cells; BMMCs, bone marrow mononuclear cells; iPSCs, induced pluripotent stem cells; MCAO: middle cerebral artery occlusion.

gene therapy, tissue engineering scaffolds, and the use of various stem cell types (**Figure 3**).

Gene therapy is designed to introduce therapeutic genes into target cells. Transplantation of stem cells whose genes have been modified using viruses to express neurotrophic and growth factors, such as BDNF, VEGF, NGF, GDNF, Noggin, placental growth factor (PIGF), hepatocyte growth factor (HGF), erythropoietin (EPO), angiopoietin-1 (ANG-1), and IL-10 has been proven to be more effective in promoting neuronal tissue regeneration compared to the unmodified stem cells in experimental stroke animal models (Wang et al., 2004; Ding et al., 2011; Chen et al., 2013; Nakajima et al., 2017; Wang et al., 2018; Moxon et al., 2019). Bernstock et al. (2019) showed that NSCs modified by the small ubiquitin-like modifier (SUMO) could increase cell survival and enhance neuronal differentiation. Viral vectors are promising tools for the genetic modification of cells by integrating into the genome. However, the risk of oncogenesis can be increased greatly by altering the host gene expression patterns. Thus, more experimental animal studies are needed to evaluate systematically the safety of genetically modified stem cell therapy before this method is approved for wide use in clinical studies.

Stem cells combined with tissue engineering approaches are becoming increasingly popular in regenerative medicine, as well as for repairing the damaged central nervous system. Biological scaffolds can be modified and designed to meet most of the requisites of various tissues. They could promote the survival of cells. They could promote astrocyte infiltration into the stroke cavity rather than glial scar formation post-injury (Nih et al., 2017). An ideal tissue-engineered scaffold should have adequate histocompatibility and have a three-dimensional architecture that provides an ideal microenvironment for supporting cell adhesion, migration, proliferation, and differentiation without eliciting inflammatory responses *in vivo*. Furthermore, many biomaterials can reversibly bind to growth factors. Bible et al. (2009) reported that plasma polymerized allylamine (ppAAm)-treated poly-D, L-lactic-co-glycolic acid (PLGA) scaffold particles could attach NSCs *in vitro* at high density, and act as structural support for NSCs injected directly into the lesion cavity *in vivo*. Zhang et al. (2017) showed that transplantation of BMSCs in combination with a plasma scaffold into the cystic cavity after focal cerebral ischemia significantly reduced the infarct legion region, and motor function was dramatically improved. Moshayedi et al. (2016) reported that HA hydrogels could enhance the survival of transplanted cells and promote astrocytic differentiation *in vivo*.

Using a combination of multiple types of stem cells is another trend in cell therapy. Several studies have shown that various types of stem/progenitor cells and their derivatives can be more effective than a single type of stem cell for the reconstruction of damaged neural tissue after ischemic stroke, as different neurotrophic factors could be secreted by different cells. However, the detailed mechanism of this method remains unexplored, as well as the optimal composition of cell types, dose, and density to achieve the best neurofunctional recovery for ischemic stroke. In the study by Hosseini et al. (2015), MSCs from adult rat bone marrow were injected 1 day after middle cerebral artery occlusion (MCAO), and the NSCs from the ganglion eminence of a rat embryo at 14 days were transplanted 7 days after MCAO. The results showed that using a combination of MSCs and NSCs had a better neurological outcome, and fewer brain lesions were observed.

CHALLENGES TO OVERCOME IN THE CLINICAL APPLICATION OF STEM CELL THERAPY

Before stem cells can be successfully used in clinical translation, many setbacks must be overcome. First, tracking of transplanted cells and poor survival. Cells can be tracked *in vivo* with magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT/CT), positron emission tomography (PET), and bioluminescence imaging (BLI) using a dual green fluorescent protein-Luciferase (GFP-Luc) reporter system (Manley and Steinberg, 2012; Zheng et al., 2017). The work of Moshayedi et al. (2016) demonstrated that hyaluronic acid (HA) hydrogels can enhance the survival of transplanted cells for at least 6 weeks and can be tracked *in vivo* by MRI. Second, the clinical application of stem cells raises numerous ethical concerns, particularly for NSCs and ESCs. The application of iPSCs may avoid this problem. Additionally, the production of autologous iPSCs is feasible, but it is rather costly and would take several months for its production before the cells could be used for transplantation. Moreover, the clinical application of stem cells may raise numerous safety concerns. Allografts may cause immune rejection. Stem cells have the potential to differentiate into undesired tissues and can promote tumor growth and metastasis by enhancing the generation of new blood vessels and altering the tumor microenvironment (Lazennec and Jorgensen, 2008; Patel et al., 2010). Erdö et al. (2003) demonstrated that contamination of undifferentiated ES cells could promote tumorigenesis. Amariglio et al. (2009) reported that a patient with ataxia-telangiectasia who was treated with intracerebellar and intrathecal injection of donor-derived neural stem cell transplantation developed a brain tumor 4 years after the first treatment. More clinical research is warranted to explore the optimal transplantation plan (i.e., timing, route, and dose of administration).

CONCLUSION

Stem cells are very promising candidates for augmenting brain repair, to restore function following stroke treatment. During the last few years, various approaches to transplantation have been applied in ischemic stroke animal models and clinical trials. The outcomes of these studies have been encouraging, with the transplanted stem cells having various beneficial effects, including the reduced neurological deficit, reduced infarct area, reduced inflammation, and increased neurogenesis and angiogenesis. However, the clinical application of stem cell-based therapies remains in its infancy. Many issues still need to be resolved, including efficacy, safety, and feasibility. With the continuous exploration of regenerative medicine and the development of cell transplantation techniques, stem cell therapy

will help ischemic stroke patients achieve better neurofunctional recovery soon.

AUTHOR CONTRIBUTIONS

HL and YH conceived the main outline. HL wrote the manuscript. XZ and HC made the table and figure. SR, CL and YO took charge of manuscript revision in English. YH participated in the correction and final inspection of this review. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by a grant from the National Natural Science Foundation of China No. 81671154 to YH.

ACKNOWLEDGMENTS

We would like to acknowledge the help of all the staff involved in the manuscript drafting, article editing, and literature review.

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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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ORIGINAL RESEARCH
published: 25 February 2021
doi: 10.3389/fnins.2021.628983

Wnt/β-Catenin Signaling Promotes Differentiation of Ischemia-Activated Adult Neural Stem/Progenitor Cells to Neuronal Precursors

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

Received: 13 November 2020
Accepted: 18 January 2021
Published: 25 February 2021

Citation:
Kriska J, Janeckova L, Kirdajova D, Honsa P, Knotek T, Dzamba D, Kolenicova D, Butenko O, Vojtechova M, Capek M, Kozmik Z, Taketo MM, Korinek V and Anderova M (2021) Wnt/β-Catenin Signaling Promotes Differentiation of Ischemia-Activated Adult Neural Stem/Progenitor Cells to Neuronal Precursors. Front. Neurosci. 15:628983. doi: 10.3389/fnins.2021.628983

Modulating endogenous regenerative processes may represent a suitable treatment for central nervous system (CNS) injuries, such as stroke or trauma. Neural stem/progenitor cells (NS/PCs), which naturally reside in the subventricular zone (SVZ) of the adult brain, proliferate and differentiate to other cell types, and therefore may compensate the negative consequences of ischemic injury. The fate of NS/PCs in the developing brain is largely influenced by Wingless/Integrated (Wnt) signaling; however, its role in the differentiation of adult NS/PCs under ischemic conditions is still enigmatic. In our previous study, we identified the Wnt/β-catenin signaling pathway as a factor promoting neurogenesis at the expense of gliogenesis in neonatal mice. In this study, we used adult transgenic mice in order to assess the impact of the canonical Wnt pathway modulation (inhibition or hyper-activation) on NS/PCs derived from the SVZ, and combined it with the middle cerebral artery occlusion (MCAO) to disclose the effect of focal cerebral ischemia (FCI). Based on the electrophysiological properties of cultured cells, we first identified three cell types that represented *in vitro* differentiated NS/PCs – astrocytes, neuron-like cells, and precursor cells. Following FCI, we detected fewer neuron-like cells after Wnt signaling inhibition. Furthermore, the immunohistochemical analysis revealed an overall higher expression of cell-type-specific proteins after FCI, indicating increased proliferation and differentiation rates of NS/PCs in the SVZ. Remarkably, Wnt signaling hyper-activation increased the abundance of proliferating and neuron-like cells, while Wnt pathway inhibition had the opposite effect. Finally, the expression profiling at the single cell level revealed an increased proportion of neural stem cells and neuroblasts after FCI. These observations indicate that Wnt signaling enhances NS/PCs-based regeneration in the adult mouse brain following FCI, and supports neuronal differentiation in the SVZ.

Keywords: Wnt signaling, transgenic mouse, neural stem/progenitor cell, focal cerebral ischemia, adult neurogenesis, gliogenesis, patch-clamp technique, single-cell RNA sequencing

INTRODUCTION

Stroke is one of the leading causes of mortality worldwide, affecting a great number of people in developed countries and thus imposing a considerable economic burden on society (Woodruff et al., 2011; Rajsic et al., 2019). Aging, genetic predisposition, and unhealthy lifestyle, are all among the risk factors for stroke (Boehme et al., 2017). Ischemic stroke due to a blocked artery comprises more than 85% of all stroke cases (Woodruff et al., 2011); the most frequently affected vessel of the brain is the middle cerebral artery. Its occlusion causes poor blood flow followed by glucose and oxygen deprivation, resulting in cell death in a large cortical area of the brain (Rossi et al., 2007; Puig et al., 2018; Belov Kirdajova et al., 2020). Nevertheless, FCI also has a consistent impact on the physiological functions of cells residing in regions distant from the lesion (Ginsberg, 2003; Wang et al., 2017). Ischemic brain injury leads to motoric, sensoric, and cognitive dysfunctions, and it is accompanied by elevated neurogenesis in the neurogenic niches of the postnatal brain. Therefore, recent studies have attempted to treat or replace diminishing numbers of neural cells with the use of stem cells from various sources (Ruzicka et al., 2017), or by utilizing NS/PCs naturally residing in the adult brain (Groves et al., 2019) with the capability of differentiating to other cell types (Kriska et al., 2016; Butti et al., 2019; Vancamp et al., 2019).

Abbreviations: 4OHT, (Z)-4-hydroxytamoxifen; A488, Alexa Fluor hydrazide 488; A594/660, Alexa Fluor 594 or 660; aCSF, artificial cerebrospinal fluid; APC, adenomatous polyposis coli; B27, B27 supplement; bFGF, fibroblast growth factor-basic; BMP, bone morphogenetic protein; cDNA, complementary deoxyribonucleic acid; C_M, membrane capacitance; CNS, central nervous system; CO, corn oil; CO_2, carbon dioxide; COPs, committed oligodendrocyte precursors; Com, complex current pattern; C_t, cycle threshold; *Ctnnb1,* gene encoding β-catenin; CTRL, control, non-operated mice; Cy3, cyanine dye 3; DAPI, 4′,6-diamidino-2-phenylindole; DCX, doublecortin; Dkk1, Dickkopf 1; Dn, dominant negative; DNase, deoxyribonuclease; E2-3, exons 2–3; EGF, epidermal growth factor; EGFP, enhanced green fluorescent protein; EGFR, epidermal growth factor receptor; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; ERT2, mutant form of estrogen receptor; EtOH, ethanol; Ex3, exon 3; FACS, fluorescence-activated cell sorting; FCI, focal cerebral ischemia; FP, forward primer; *GAPDH, glyceraldehyde-3-phosphate dehydrogenase*; GFAP, glial fibrillary acidic protein; GSK-3β, glycogen synthase kinase 3β; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; i.p., intraperitoneal; IgG, immunoglobulin G; IR, input resistance; K^+, potassium; K_A, fast activating and inactivating outwardly rectifying K^+ current; K_A/C_M, current density of K_A; K_{DR}, delayed outwardly rectifying K^+ current; K_{DR}/C_M, current density of K_{DR}; K_{IR}, inwardly rectifying K^+ current; K_{IR}/C_M, current density of K_{IR}; LRP, low-density lipoprotein receptor-related protein; LV, lateral ventricle; MAP2, microtubule-associated protein 2; MCAO, middle cerebral artery occlusion; mRNA, messenger ribonucleic acid; n, number of specimens/cells; Na^+, sodium; Na^+/C_M, current density of Na^+ channels; NG2, neuron-glial antigen 2; *Nkd1, naked cuticle homolog 1*; NMDG, *N*-Methyl-D-glucamine; NS/PCs, neural stem/progenitor cells; OPCs, oligodendrocyte precursor cells; Out, outwardly rectifying current pattern; pA, polyadenylation site; pas, passive current pattern; PB, phosphate buffer; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; PDGFRα, platelet-derived growth factor receptor alpha; PFA, paraformaldehyde; PGK-Neo, neomycin resistance cassette; PLL, poly-L-lysine; PTB, pentobarbital; RP, reverse primer; RT, room temperature; RT-qPCR, reverse transcription quantitative polymerase chain reaction; S.D., standard deviation; S.E.M., standard error of the mean; SA, splice acceptor site; scRNA-seq, single-cell RNA sequencing; SGZ, subgranular zone; SVZ, subventricular zone; TAM, tamoxifen; TCF/LEF, T-cell factor/lymphoid enhancer factor; Td, tandem dimmer; *Tnfrsf19, tumor necrosis factor receptor superfamily, member 19*; *Troy, tumor necrosis factor receptor superfamily, member 19*; TTX, tetrodotoxin; V_M, membrane potential; WLS, Wntless; Wnt, Wingless/Integrated; WT, wild-type.

Adult neurogenesis in the CNS was first documented in the 1960s (Altman and Das, 1965). Under physiological conditions, it takes place in two particular regions of the mammalian brain – the SGZ of the dentate gyrus in the hippocampus and the SVZ of the lateral ventricles (LVs), which is adjacent to the striatum (Obernier and Alvarez-Buylla, 2019). These two regions are considered neurogenic zones of the adult brain, as they have been shown to comprise NS/PCs that are capable of generating distinct cell types *in vitro* as well as *in vivo* (Lie et al., 2005; Bizen et al., 2014). However, adult NS/PCs proliferate at a much slower pace than during embryogenesis (Furutachi et al., 2013). In addition to the two neurogenic regions of the adult CNS, there is good evidence that during ischemia, multiple neurogenic sites within the brain parenchyma are activated, lasting for more than 1 month after the induction of ischemic injury (Kokaia et al., 2006).

Neurogenesis, together with gliogenesis, largely depends on molecular and genetic inputs such as growth factors and cellular signaling pathways, creating a microenvironment, or niche, for NS/PCs. Moreover, these processes are also modulated during pathological states (O'Keeffe et al., 2009; Bowman et al., 2013; Lamus et al., 2020). The role of the canonical Wnt signaling pathway in the brain development has been well established (Chenn and Walsh, 2002; Machon et al., 2003, 2007; Kalani et al., 2008; Borday et al., 2018; Chodelkova et al., 2018). Nevertheless, recent research has directed its attention more on the function of this pathway in postnatal neurogenesis, and in the modulation of the properties of adult NS/PCs (Lie et al., 2005). The Wnt ligands belong to a group of secreted cysteine-rich glycosylated proteins that are involved in cellular processes, such as cell proliferation and differentiation, synaptic plasticity, or programmed cell death (Wiese et al., 2018; Palomer et al., 2019). In the absence of a Wnt signal, the multi-protein destruction complex is formed in the cytoplasm. Kinases of this complex put a molecular tag on β-catenin, the key factor of the whole cascade, and thus mark it for degradation in the proteasome. On the other hand, activation of the pathway stabilizes β-catenin. This stabilization is achieved via a negative regulation of glycogen synthase kinase 3β (GSK-3β), followed by the accumulation of β-catenin in the cytoplasm and its subsequent translocation to the nucleus, where it binds to the transcription factors T-TCF/LEF, and thus influences expression of Wnt target genes (Nusse and Clevers, 2017). Many of these genes are implicated in the proliferation and differentiation of neural precursors, or in self-regulation of the pathway, with its numerous negative feedback loops (Mikels and Nusse, 2006; ten Berge et al., 2008).

Wnt signaling influences the fate of postnatal NS/PCs. The activation of β-catenin-dependent transcription promotes the proliferation of precursor cells in the SVZ, while its inhibition reduces the number of newly generated cells (Adachi et al., 2007). Moreover, Wnt7a ligand increases the count of cells expressing neuronal markers, while suppressing gliogenesis (Prajerova et al., 2010). Similar effects of active Wnt signaling were also observed in our previous study on neonatal NS/PCs (Kriska et al., 2016). Additionally, it has been shown that hypoxia and FCI increase the number of NS/PCs in the

hippocampus and SVZ, promote the expansion of neuroblasts 1 month after MCAO, and that canonical Wnt signaling is involved in this process (Cui et al., 2011; Zhang et al., 2014; Knotek et al., 2020). The importance of Wnt signaling in the differentiation of NS/PCs to neurons under ischemic conditions was also documented by Zhang et al. (2016). Congruent with these findings, Zhang and Chopp (2016) highlighted the stroke-induced activation of NS/PCs, and their subsequent differentiation to neuroblasts, in their review. Moreover, Shruster et al. (2012) disclosed that Wnt signaling contributed to functional recovery following FCI in mice.

The aim of this study was to assess the effect of the canonical Wnt signaling pathway on the differentiation potential of NS/PCs under physiological conditions and following ischemia. Our data show that FCI increases the expression of Wnt target genes and cell-type-specific proteins, and concomitantly influences the electrophysiological properties of differentiated NS/PCs. Moreover, we found that the effect of active Wnt signaling on the differentiation of NS/PCs isolated from adult mice is greater after the induction of FCI, and that it promotes neurogenesis at the expense of gliogenesis. Therefore, we suggest that modulating Wnt signaling might ameliorate the negative effects associated with ischemic brain injury.

MATERIALS AND METHODS

Transgenic Animal Models

In our experiments, we used transgenic mouse strains that served as a source of NS/PCs, and as a tool for manipulating the canonical Wnt signaling pathway. The procedures involving the use of laboratory animals were carried out in accordance with the European Communities Council Directive from November 24, 1986 (86/609/EEC) and with the guidelines of the Institute of Experimental Medicine, Czech Academy of Sciences, which was approved by the Animal Care Committee (approval numbers 18/2011, 146/2013, and 2/2017). All efforts were made to minimize both the suffering and the numbers of mice assigned to the individual experiments. Young adult (postnatal day 50–56) males of three different transgenic mouse strains were utilized. These animals facilitated the manipulation of the canonical Wnt signaling pathway at different subcellular levels, specifically at the nuclear, membrane and cytoplasmic level (**Figure 1A**). First, we used the *Rosa26-tdTomato-EGFP/dnTCF4* mice (Janeckova et al., 2016) that produced dominant negative (dn)TCF4 protein from the *Rosa26* locus upon a Cre-mediated excision of a transcriptional blocker that was located upstream of *dnTCF4* cDNA. The dnTCF4 protein acted as a Wnt signaling inhibitor in the cell nucleus (**Figure 1B**). Moreover, this strain was designed to produce tandem dimeric (td) red fluorescent protein Tomato that was replaced by EGFP after Cre-mediated DNA excision. The *Rosa26-Dkk1* mice (Wu et al., 2008) were then used. After the Cre-mediated excision of a transcriptional blocker, the extracellular Wnt pathway inhibitor Dickkopf 1 (Dkk1) was produced from the ubiquitous *Rosa26* locus (**Figure 1C**), sequestering membrane receptors and thus inhibiting the pathway. Lastly, the $Catnb^{lox(ex3)}$ mice harbored the floxed allele of the *Ctnnb1* gene encoding protein β-catenin (Harada et al., 1999). In these mice the allele enabled conditional stabilization of β-catenin (**Figure 1D**). All these mouse strains were individually crossbred with the general Cre deletor mouse *Rosa26-CreERT2* that possessed tamoxifen (TAM)-inducible Cre recombinase fused with a ERT2 (Ventura et al., 2007; **Figure 1E**). According to the resulting genotype, the mice enabled the inhibition of Wnt signaling, either at the nuclear (genotype $Rosa26^{dnTCF4/CreERT2}$; further termed 'dnTCF4' mice/cells) or membrane level (genotype $Rosa26^{Dkk1/CreERT2}$; further termed 'Dkk1' mice/cells). Conversely, the $Ctnnb1^{del(ex3)/+}Rosa26^{+/CreERT2}$ mice (further termed 'Ex3' mice/cells) produced a stable form of β-catenin protein that hyper-activated the canonical Wnt signaling pathway upon the Cre-mediated excision of the DNA sequence encoding exon 3 of the *Ctnnb1* gene. The genetic background of all mouse strains was C57BL/6. We used wild-type (WT) C57BL/6 mice for single-cell RNA sequencing (scRNA-seq).

The Cre-recombination-mediated manipulation of Wnt signaling was achieved either *in vitro*, by the addition of 1 μM (Z)-4-hydroxytamoxifen (4OHT; Sigma-Aldrich, St. Louis, MO, United States) dissolved in ethanol (EtOH) into the differentiation medium or *in vivo*, by intraperitoneal (i.p.) injections of TAM (200 mg/kg of the animal's body weight; Toronto Research Chemicals, Toronto, ON, Canada) dissolved in corn oil (CO; Sigma-Aldrich, St. Louis, MO, United States). Tamoxifen injections were delivered in two doses, each once a day for two consecutive days, and experiments (induction of FCI or tissue collection) were performed on the third day after the last TAM injection. Cell cultures/mice treated only with the vehicle (EtOH or CO) were considered controls and labeled 'EtOH' and 'CO,' respectively, while cell cultures/mice with manipulated Wnt signaling were labeled '4OHT' or 'TAM.'

The Induction of Focal Cerebral Ischemia

Mice undergoing permanent MCAO, a procedure that has become a conventional model of FCI (Colak et al., 2011), were anesthetized with 2% isoflurane (Abbot, IL, United States) and maintained at 1% isoflurane using a vaporizer (Tec-3, Cyprane Ltd., Keighley, United Kingdom). An incision in the skin between the orbit and the external auditory meatus was made and the temporal muscle was retracted. A ∼1.5 mm hole was then drilled through the frontal bone, ∼1.0 mm rostrally to the fusion of the zygoma and the squamosal bone, and ∼3.5 mm ventrally to the dorsal surface of the brain. After the *dura mater* was opened and removed, the middle cerebral artery was exposed and cauterized with a pair of bipolar tweezers (SMT, Prague, Czechia) at a proximal location. The body temperature of the mouse was maintained at 37 ± 1°C using a heating pad throughout the surgery. After the operation, the mice were injected with 0.5 ml of saline solution subcutaneously. Analgesics were administered when necessary. The operated mice were labeled 'MCAO,' while the non-operated animals were considered controls and labeled 'CTRL'. Previously, we analyzed coronal brain slices using tetrazolium chloride staining and detected no ischemic lesions in either the non-operated mice or sham-operated animals, which were subjected to the same surgery

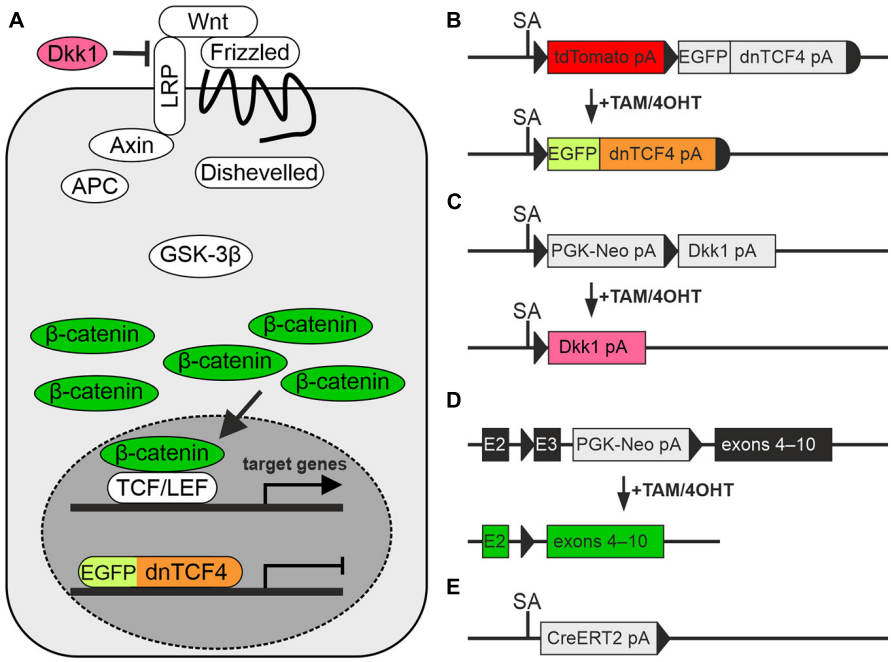

FIGURE 1 | Modulation of the Wnt signaling pathway in transgenic mice. **(A)** A simplified scheme of the canonical Wnt signaling pathway that shows by color-coding where the alterations in the cascade take place. **(B–E)** Schemes depicting genetic modifications present in the mouse strains used in the study. **(B)** The transcription of the Wnt-responsive genes in the nucleus is blocked by dominant negative T-Cell Factor 4 (dnTCF4; orange) fused to the enhanced green fluorescent protein (EGFP; light green). **(C)** Suppression of Wnt signaling at the membrane receptor level, due to the overexpression of a secreted Wnt inhibitor Dickkopf 1 (Dkk1; dark pink). **(D)** Activation of the pathway by the production of a stable variant of β-catenin. Cre-mediated excision of exon 3 (E3) removes the amino acid sequence involved in the degradation of the protein, and thus stable β-catenin aberrantly hyper-activates Wnt target genes (dark green). **(E)** Cre deletor mouse *Rosa26-CreERT2* carries the gene encoding tamoxifen/4-hydroxytamoxifen (TAM/4OHT)-inducible Cre recombinase, fused with a mutated form of the estrogen receptor (CreERT2). APC, adenomatous polyposis coli; E2-3, exons 2-3; GSK-3β, glycogen synthase kinase 3β; LRP, low-density lipoprotein receptor-related protein; pA, polyadenylation site; PGK-Neo, neomycin resistance cassette; SA, splice acceptor site; TCF/LEF, T-Cell Factor/Lymphoid Enhancer Factor; td, tandem dimer; Wnt, Wingless/Integrated.

procedure as the MCAO mice but without dura opening and vessel occlusion (Honsa et al., 2014). This distal model of MCAO has a high survival rate (>95%) and good reproducibility, as it typically yields an infarct lesion of a relatively small volume only in the cortical region (Honsa et al., 2013). To facilitate the orientation in the text, we provide a summarizing table of all combinations of the treatments used in our experiments in **Table 1**.

TABLE 1 | Treatments applied on neural stem/progenitor cells.

Experiment	Ischemia	Wnt signaling
In vitro	CTRL	EtOH
		4OHT
	MCAO	EtOH
		4OHT
In vivo	CTRL	CO
		TAM
	MCAO	CO
		TAM

Altogether eight different combinations of treatments were used in our analyses, depending on the type of an experiment, the (patho)physiological conditions of the brain, and the state of the canonical Wnt signaling pathway. 4OHT, (Z)-4-hydroxytamoxifen; CO, corn oil; CTRL, control; EtOH, ethanol; MCAO, middle cerebral artery occlusion; TAM, tamoxifen.

Tissue Isolation and Cell Culture Preparation

To prepare tissue or cell culture specimens for subsequent analyses, we prepared coronal sections of mouse brains and cut out lateral SVZs for further processing. Primary cultures were derived from NS/PCs isolated from the SVZs of the LVs; from both the right and left hemispheres from CTRL mice, while only from the SVZ ipsilateral to the site of injury in mice 3 days after MCAO (**Supplementary Figure 1**).

The animals were deeply anesthetized first with 4% isoflurane and subsequently with pentobarbital solution (PTB; 100 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO, United States), and perfused transcardially with an ice-cold isolation buffer containing (in mM): 110 *N*-Methyl-D-glucamine (NMDG)-Cl, 2.5 KCl, 24.5 $NaHCO_3$, 1.25 Na_2HPO_4, 0.5 $CaCl_2$, 7 $MgCl_2$, 20 glucose, osmolality 290 ± 3 mOsmol/kg. After subsequent decapitation, the brains were quickly removed from the skull and sliced into ∼500 μm coronal slices using vibratomes HM 650 V (MICROM International GmbH, Walldorf, Germany) or Leica VT 1200S (Baria, Prague, Czechia) and the SVZs were carefully

dissected out and cut into smaller pieces using a razor. To obtain a single-cell suspension, the tissue was first incubated with continuous shaking at 37°C for 45 min in 1 ml of papain solution (20 U/ml; Worthington, Lakewood, NJ, United States) with 0.2 ml of deoxyribonuclease (DNase; Worthington, Lakewood, NJ, United States). After papain treatment, the activity of the enzyme was inhibited with 1 ml of ovomucoid inhibitor solution (Worthington, Lakewood, NJ, United States). The tissue was then mechanically dissociated by gentle trituration using a 1 ml pipette and centrifuged at 1,020 × *g* for 3 min. After centrifugation, the supernatant was discarded and the cells were resuspended in 1 ml of proliferation medium containing Neurobasal-A medium (Life Technologies, Waltham, MA, United States), supplemented with the B27 supplement (B27; 2%; Life Technologies, Waltham, MA, United States), L-glutamine (2 mM; Sigma-Aldrich, St. Louis, MO, United States), antimicrobial reagent primocin (100 μg/ml; InvivoGen, Toulouse, France), and bFGF (10 ng/ml), and EGF (30 ng/ml); both were purchased from PeproTech, Rocky Hill, NJ, United States. The cells were subsequently filtered through a 70 μm cell strainer, into a 100 mm-diameter Petri dish containing an additional 9 ml of proliferation medium. The cells were cultured as neurospheres, at 37°C and 5% CO_2. After ∼12 days of *in vitro* proliferation, the formed neurospheres were collected and transferred into a 12 ml Falcon tube, and centrifuged at 1,020 × *g* for 3 min. The supernatant was discarded, and 1 ml of protease trypsin (Sigma-Aldrich, St. Louis, MO, United StatesA) was added to the pelleted neurospheres. After 3 min of trypsin incubation, 1 ml of trypsin inhibitor (Sigma-Aldrich, St. Louis, MO, United States) was added to the dissociated cells to block the proteolytic effect of trypsin. Subsequently, a negligible portion (∼100 μl) of the cell suspension was used to count cells in the hemocytometer. The rest of the suspension was centrifuged at 1,020 × *g* for 3 min and plated on poly-L-lysine (PLL)-coated (Sigma-Aldrich, St. Louis, MO, United States) coverslips placed in a 24-well plastic plate at the cell density of 6×10^4 cells/cm^2 in a differentiation medium. The differentiation medium had the same composition as the proliferation medium, but it was devoid of EGF and with a twofold (20 ng/ml) concentration of bFGF. The cells were cultured at 37°C and 5% CO_2, with a medium exchange on every third day. After 8–9 days of *in vitro* differentiation, the cells were used for electrophysiological measurements and immunocytochemical staining. To estimate the impact of Wnt signaling inhibition or activation on the differentiation of NS/PCs, 4OHT-treated cultures were compared with the corresponding EtOH-treated cells.

In order to prepare tissue slices for immunohistochemical analyses, the mice were deeply anesthetized with PTB (100 mg/kg, i.p.) and perfused transcardially using a saline solution (ArdeaPharma, a.s., Sevetin, Czechia) with 0.65% heparin (Zentiva Group, Prague, Czechia) at room temperature (RT), and subsequently with ice-cold 4% paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO, United States). The brain was then left in PFA for 3 more hours for thorough fixation, with subsequent transfer to the sucrose gradient for cryoprotection. The tissue was incubated in 10% sucrose for 12 h, then in 20% sucrose for 24 h, followed by 30% sucrose incubation for a further 72 h. Coronal sections of 30 μm were prepared using a cryostat (Leica CM1850, Leica Microsystems, Wetzlar, Germany) and subsequently stored at −20°C in a cryopreservation solution composed of 30% glycerol and 30% ethylene glycol in a PBS.

Tissue for RT-qPCR was isolated as described in **Supplementary Figure 1**. Briefly, the mice were anesthetized with 4% isoflurane and subsequently with 1% PTB and perfused transcardially with an ice-cold isolation buffer. After decapitation, the brains were quickly removed from the skull and cut into ∼500 μm coronal sections using a vibrating microtome. The SVZs were dissected out and cut into smaller pieces using a razor. Finally, the tissue was transferred into 2 ml Eppendorf tubes containing TRI Reagent (Sigma-Aldrich, St. Louis, MO, United States) for further RT-qPCR analysis.

To prepare a single-cell suspension for FACS and subsequent scRNA-seq, the tissue was isolated according to **Supplementary Figure 1**. The WT C57BL/6 mice were anesthetized with 1% PTB and perfused transcardially with an ice-cold isolation buffer containing (in mM): 136.0 NaCl, 5.4 KCl, 10.0 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5.5 glucose, with pH 7.4 and osmolality 290 ± 3 mOsmol/kg. After decapitation, the brains were quickly removed from the skull and cut into ∼750 μm coronal sections using a vibrating microtome. The SVZs with the adjacent striatum were dissected out and cut into smaller pieces using a razor. To obtain a single-cell suspension, the tissue was first incubated with continuous shaking at 37°C for 40 min in 1 ml of papain dissolved in the isolation buffer (20 U/ml) supplemented with 0.3 ml of DNase. After the papain treatment, the tissue was mechanically dissociated by gentle trituration using a 1 ml pipette and centrifuged at 300 × *g* for 3 min. After centrifugation, the supernatant was discarded and the cells were resuspended in 0.9 ml of the isolation buffer supplemented with 100 μl of an ovomucoid inhibitor solution and 50 μl of DNase. Afterward, the cells were centrifuged at 300 × *g* for 3 min, the supernatant was discarded, and the cell pellet was resuspended in Dulbecco's PBS (Sigma-Aldrich, St. Louis, MO, United States) and filtered through a 70 μm pre-separation filter (Miltenyi Biotec, Bergisch Gladbach, Germany). The single-cell suspension was then processed using a debris removal solution (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol for smaller amounts of tissue. The resulting cell suspension was resuspended in Neurobasal-A medium and kept at 4°C for further processing.

Electrophysiological Recordings

The electrophysiological properties of *in vitro* differentiated cells were recorded using the patch-clamp technique in the whole-cell configuration. Recording micropipettes with a tip resistance of ∼8–10 MΩ were made from borosilicate glass capillaries (Sutter Instruments, Novato, CA, United States) using a P-97 Brown-Flaming puller (Sutter Instruments, Novato, CA, United States) and subsequently filled with an artificial intracellular solution containing (in mM): 10 HEPES, 130 KCl, 0.5 $CaCl_2$, 2 $MgCl_2$, 5 ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), with pH 7.2, and in some cases also mixed with Alexa Fluor hydrazide 488 (A488; Molecular Probes, Carlsbad, CA, United States) for the visualization of recorded cells. The

measurements were made in an aCSF containing (in mM): 122 NaCl, 3 KCl, 1.5 $CaCl_2$, 1.3 $MgCl_2$, 1.25 Na_2HPO_4, 28 $NaHCO_3$, and 10 D-glucose (osmolality 300 ± 5 mmol/kg) and this solution was continuously gassed with 5% CO_2 to maintain a final pH of 7.4. All the recordings were made at RT on coverslips perfused with aCSF in the recording chamber of an upright Axioscop microscope (Zeiss, Gottingen, Germany), equipped with 2 electronic micromanipulators (Luigs & Neumann, Ratingen, Germany), and a high-resolution AxioCam HRc digital camera (Zeiss, Gottingen, Germany). Electrophysiological data were measured with a 10 kHz sample frequency using EPC9 or EPC10 amplifiers controlled by PatchMaster software (HEKA Elektronik, Lambrecht/Pfalz, Germany) and filtered using a Bessel filter.

The values of the V_M were measured by switching the EPC9/10 amplifiers to the current-clamp mode. Using the FitMaster software (HEKA Elektronik, Lambrecht/Pfalz, Germany), IR was calculated from the current value at 40 ms after the onset of the depolarizing 10 mV pulse from the holding potential of −70 mV. C_M was determined automatically from the Lock-in protocol by the software. Current patterns were obtained by hyperpolarizing and depolarizing the cell membrane from the holding potential of −70 mV to the values ranging from −160 mV to 40 mV in 10 mV steps, while the duration of each pulse was 50 ms. The inwardly rectifying potassium (K^+; K_{IR}), the K_A, and the K_{DR} current components were determined as follows. In order to isolate the K_{DR} current components, a voltage step from −70 to −60 mV was used to subtract the time- and voltage-independent currents. To activate the K_{DR} currents only, the cells were held at −50 mV, and the amplitude of the K_{DR} currents was measured at 40 mV, 40 ms after the onset of the pulse. The K_{IR} currents were determined analogously at −140 mV, also 40 ms after the onset of the pulse, while the cells were held at −70 mV. The K_A currents were measured at 40 mV and were isolated by subtracting the current traces, clamped at −110 mV from those clamped at −50 mV, and its amplitude was measured at the peak value. While measuring TTX-sensitive sodium (Na^+) currents, the cells were held at −70 mV, and the current amplitudes were isolated by subtracting the current traces measured in a solution containing 1 μM TTX (Alomone Labs, Jerusalem, Israel) from those measured in the absence of TTX in aCSF. The Na^+ current amplitudes were measured at the peak value. The current densities were calculated by dividing the maximum current amplitudes by the corresponding C_M values for each individual cell. The action potentials were obtained in the current-clamp mode and current values ranged from 50 to 1,000 pA in 50 pA steps, and the pulse duration was 300 ms.

After recording, the cells on the coverslips were fixed in 4% PFA dissolved in 0.2 M PB (pH 7.4) for 9 min, then transferred to 10 mM PBS (pH 7.2) and stored at 4°C for post-recording identification using immunofluorescence staining.

Immunochemistry and Confocal Microscopy

The coverslips with cells or 30-μm-thick coronal brain slices were washed 3 times for 10 min in PBS. They were then rinsed for 2 h at 4°C in blocking solution containing 5% Chemiblocker (Millipore, Billerica, MA, United States) and 0.5% Triton X-100 (Sigma-Aldrich, St. Louis, MO, United States) diluted in 10 mM PBS. The same blocking solution was used as the diluent for all primary and secondary antibodies. The specimens were incubated overnight at 4°C with primary antibodies. The following primary antibodies were used: rabbit polyclonal anti-GFAP (1:800; Sigma-Aldrich, St. Louis, MO, United States), mouse monoclonal anti-GFAP (1:800; Sigma-Aldrich, St. Louis, MO, United States) conjugated to cyanine dye 3 (Cy3), rabbit polyclonal anti-PDGFRα (1:200; Santa Cruz Biotechnology, Dallas, TX, United States), rabbit polyclonal anti-DCX (1:1000; Abcam, Cambridge, United Kingdom), mouse monoclonal anti-MAP2 (1:800; Merck Millipore, Billerica, MA, United States), mouse monoclonal anti-PCNA (1:1000; Abcam, Cambridge, United Kingdom). The next day, the specimens were washed 3 times for 10 min with PBS, which was followed by incubation with secondary antibodies for a further 2 h at 4°C. The secondary antibodies were goat polyclonal anti-rabbit/mouse IgG conjugated to A488, or Alexa Fluor 594 or 660 (A594/660; 1:200; Molecular Probes, Carlsbad, CA, United States). Next, the specimens were washed 3 times for 10 min in PBS and rinsed for 5 more minutes, at RT, in 300 nM 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes, Carlsbad, CA, United States) diluted in PBS for cell nuclei visualization. At the end of the procedure, the specimens were mounted onto microscope slides using Aqua Poly/Mount (Polysciences Inc., Eppelheim, Germany). Once the mounting medium solidified, the specimens were ready to be analyzed by confocal microscopy.

An LSM 5 DUO confocal fluorescence microscope (Zeiss, Gottingen, Germany), equipped with an Arg/HeNe laser and a 40× oil objective, was used for immunofluorescence analyses and, furthermore, the fluorescence signals were analyzed using the ImageJ software (NIH, Bethesda, MD, United States). Superimposed images of fluorescent stainings were obtained by overlaying several individual confocal planes. The images were subsequently digitally filtered and the immunopositive areas were used for quantification. The areas corresponding to the immunoreactivity of the cells in *in vitro* cultures were calculated in random regions of interest, and divided by the DAPI-positive area to normalize them to the cell number. Cells for immunocytochemical staining against β-catenin were fixed for 10 min in 4% PFA, permeabilized with 0.25% Triton X-100 for another 10 min, and washed in PBS. Overnight incubation with primary mouse monoclonal antibody (1:2000; BD Transduction Laboratories, San Jose, CA, United States) at 4°C was followed by incubation with goat anti-mouse secondary antibody conjugated to A488 dye for 1 h at RT. Finally, DAPI (Sigma-Aldrich, St. Louis, MO, United States) was used for counterstaining. Fluorescent microscopy images were taken from EtOH and 4OHT cultures. The A488 and DAPI fluorescence signals were analyzed with the ImageJ software, and the intensity of β-catenin staining was normalized to the cell number in every image. The obtained data were evaluated by Student's *t*-test.

The differentiation potential of progenitor cells of the SVZ *in situ* in coronal sections, was assessed by utilizing a spinning disk confocal fluorescent microscope Dragonfly

530 Andor (Oxford Instruments, Oxford, United Kingdom), which was equipped with Zyla 4.2 PLUS sCMOS camera and Fusion acquisition system. Superimposed images of GFAP, DCX, and PCNA stainings were obtained by overlaying 6–12 individual confocal images captured by a 20× objective. A series of the images was then digitally fused using the Fusion stitching tool. The obtained images were processed in the Imaris visualization software (Oxford Instruments, Oxford, United Kingdom). The immunopositive SVZ areas were dissected as shown in **Supplementary Figure 2** and quantified using Fiji ImageJ software (NIH, Bethesda, MD, United States). The areas corresponding to immunopositive cells of the SVZ were calculated and normalized to the DAPI-positive area using a macro written in the IJM scripting language for the Fiji software package (fiji.sc). First, the confocal data were split into individual channels. Next, segmentation of DAPI-stained nuclei was done by thresholding dialog and joined segmented nuclei were split by a watershed procedure. The number of the nuclei was then counted by the Particle Analyzer in Fiji. A similar segmentation approach of thresholding was also applied to the antibody channels. Finally, we identified and counted individual antibody-positive cells using the Image Calculator in Fiji that can create a picture with intersects of two images, and the Particle Analyzer that is capable of counting the numbers of the overlaps in the whole SVZ. Altogether six SVZ sections from two biological replicates were analyzed in each immunostaining data set.

Reverse Transcription Quantitative Polymerase Chain Reaction

From tissue samples, RNA was purified using a TRI Reagent (Sigma-Aldrich, St. Louis, MO, United States) or RNA Blue (Top-Bio, Prague, Czechia) according to the manufacturers' protocol. The reverse transcription of RNA and RT-qPCR were performed using LightCycler 480 SYBR Green I Master (Roche Diagnostics, Indianapolis, IN, United States). The primers for RT-qPCR are listed in **Supplementary Table 1**.

Single-Cell RNA Sequencing and Fluorescence-Activated Cell Sorting

The single-cell suspension for the scRNA-seq analysis was prepared as described above. Prior to sorting, the single-cell suspension derived from the WT C57BL/6 mice was stained for 15 min for lymphocytes and endothelial cells (CD45 and CD31; BioLegend Way, San Diego, CA, United States), and living cells (CellTrace calcein green; Thermo Fisher Scientific, Waltham, MA, United States). All antibodies were diluted in Neurobasal-A medium supplemented with 2% B27 and after staining, cells were washed in Neurobasal-A medium and kept at 4°C until sorting. Viable (Hoechst 33258-negative (Life Technologies, Carlsbad, CA, United States) and CellTrace-calcein green-positive) and CD45/CD31-negative cells were sorted using FACS (BD Influx, San Jose, CA, United States). The single cells were sorted into 1.5 ml Eppendorf tubes containing 200 µl of Neurobasal-A medium supplemented with 2% B27, and analyzed using a scRNA-seq approach.

The Chromium System (10× Genomics, Pleasanton, CA, United States) was used to generate barcoded single-cell cDNA libraries. The barcoded cDNA was then pooled and sequenced using the NextSeq 500 high-throughput sequencing system (Illumina, San Diego, CA, United States). The sequencing data were analyzed using a strategy for comprehensive integration of single-cell data developed by Stuart et al. (2019), and the identified cell clusters were matched to the molecular atlas of cells obtained from the adult SVZ published by Mizrak et al. (2019). Genes with significantly increased expression in the corresponding cluster, when compared to all other cells in the sample, are listed in **Supplementary Table 2**.

Data Analysis

The data are presented as means ± S.E.M. or as means ± standard deviation (S.D.) for a number (n) of specimens/cells. The Student's *t*-test was used to determine significant differences between the two experimental groups, and one-way or two-way ANOVA with Tukey's *post hoc* test, was performed to determine significant differences among more experimental groups. The significance was calculated in the GraphPad Prism software (San Diego, CA, United States), and the values of $p < 0.05$ were considered significant (*, one asterisk), $p < 0.01$ very significant (**, two asterisks), and $p < 0.001$ extremely significant (***, three or more asterisks).

RESULTS

This study is a logical continuation of our previous experiments performed on neonatal mice under physiological conditions, where we revealed that canonical Wnt signaling promotes neurogenesis at the expense of gliogenesis (Kriska et al., 2016). In order to gain a deeper insight into the differentiation of adult NS/PCs, we assessed here the impact of the Wnt signaling pathway under physiological conditions, as well as following the induction of FCI in the adult mouse brain. To elucidate the effect of the Wnt/β-catenin pathway on the differentiation potential of NS/PCs in non-operated (CTRL) mice and mice after the induction of permanent FCI (MCAO), we compared either *in vitro* cultures treated with 4OHT to their respective controls (EtOH), or tissue specimens from mice with manipulated Wnt signaling (TAM), to control mice with intact Wnt signaling (CO). We used a set of experimental approaches to disclose the effect of Wnt signaling manipulation on the mRNA, protein, and functional levels.

Cell Types Originated From Adult Neural Stem/Progenitor Cells Differentiated *in vitro*

Based on electrophysiological properties, together with previous post-recording immunocytochemical identification (Kriska et al., 2016), of EtOH-treated CTRL cells, we identified three cell types that represented *in vitro* differentiated adult NS/PCs. GFAP-positive astrocytes with a passive current pattern representing time- and voltage-independent K^+ currents possessed a relatively high average V_M (−85.26 ± 0.43 mV; n = 148) and low IR

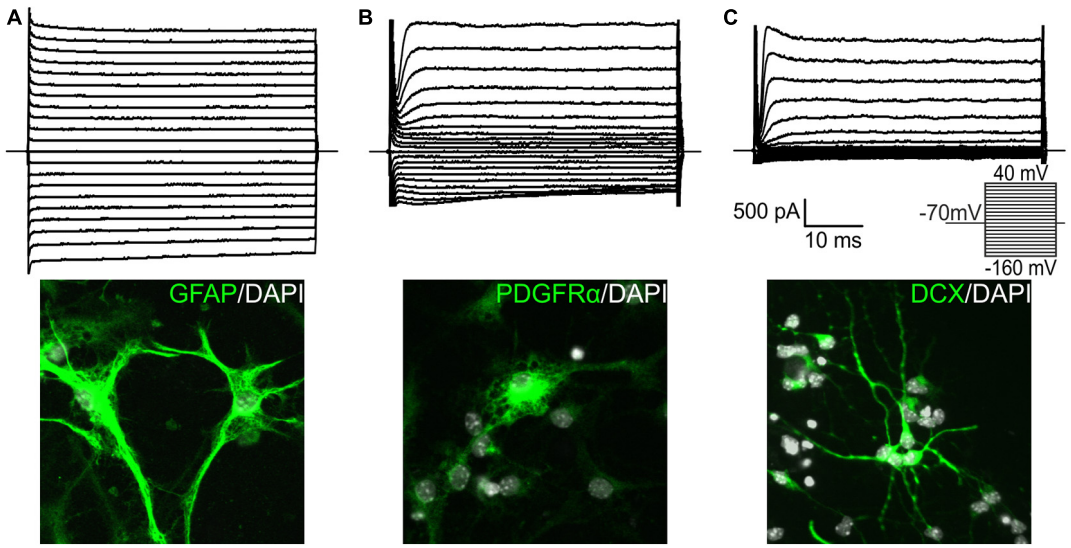

FIGURE 2 | Characterization of cell types identified among differentiated neural stem/progenitor cells. We identified three distinct cell types in the *in vitro* cultures, and supplemented them with illustrative images. Cells with a passive current pattern **(A)** were considered astrocytes as they were mostly GFAP-positive. They displayed predominantly time- and voltage-independent K^+ currents, together with small amplitudes of delayed outwardly rectifying K^+ currents (K_{DR}) and inwardly rectifying K^+ currents (K_{IR}). Cells displaying a complex current profile and expressing mainly PDGFRα **(B)** were considered precursor cells. They expressed fast activating and inactivating outwardly rectifying K^+ currents (K_A), K_{DR}, and K_{IR} currents. Doublecortin (DCX)-positive cells with an outwardly rectifying current pattern **(C)** were considered neuron-like cells, or neuroblasts, and expressed K_A and K_{DR} currents. Current patterns were obtained by hyper- and depolarizing the cell membrane from the holding potential of –70 mV to the values ranging from –160 to 40 mV in 10 mV increments (see the inset). DAPI, 4′,6-diamidino-2-phenylindole; GFAP, glial fibrillary acidic protein; PDGFRα, platelet-derived growth factor receptor alpha.

(73.45 ± 3.01 MΩ; n = 148; **Figure 2A**). PDGFRα-positive precursor cells with a complex current pattern expressed K_{IR} currents, together with K_{DR} and K_A currents (**Figure 2B**). They had V_M similar to astrocytes (−86.87 ± 0.58 mV; n = 127) and IR of medium values (150.92 ± 7.42 MΩ; n = 127). DCX- or MAP2-positive neuron-like cells (or neuroblasts) with an outwardly rectifying current profile composed of K_A currents and K_{DR} currents (**Figure 2C**) were characterized by V_M of −86.26 ± 0.86 mV (n = 174) and the highest IR values (1056.29 ± 66.21 MΩ; n = 174).

Analysis of the Effects of Wnt Pathway Modulation in Adult NS/PCs Differentiated *in vitro*

Next, we analyzed NS/PCs differentiated *in vitro* utilizing immunocytochemical staining against β-catenin, the principal element of the Wnt signaling; the NS/PCs were derived from "our" mouse models that enabled the Wnt signaling pathway manipulation. We did not detect any changes in the expression of this protein after Wnt signaling inhibition by dnTCF4 expression, as the manipulation of Wnt signaling took place in the nucleus; nevertheless, we identified its decreased amounts after Wnt signaling inhibition at the membrane receptor level. As expected, in cells with hyper-activated Wnt signaling, the expression of the protein was increased (**Figure 3**).

The incidence of the three cell types identified by the electrophysiological experiments was assessed by the patch-clamp technique in 413 EtOH- and 452 4OHT-treated cells from CTRL mice. Having analyzed the data from CTRL cells, we failed to identify any alterations in the cell incidence caused by the Wnt signaling pathway manipulation (**Figure 4A**). Nevertheless, 3 days after MCAO we found significant changes in the cultures with inhibited Wnt signaling at the membrane receptor level (**Figure 4B**). In these cultures, we observed decreased numbers of cells showing an outwardly rectifying current pattern [from 49.17 ± 3.61% in EtOH (n = 37) to 16.91 ± 5.11% in 4OHT (n = 13)] and an increased count of cells with a complex current profile [from 20.42 ± 3.25% in EtOH (n = 15) to 42.35 ± 4.93% in 4OHT (n = 34)].

The electrophysiological characterization of membrane properties was conducted utilizing the patch-clamp technique measurements on *in vitro* differentiated NS/PCs. Altogether 1,355 cells were analyzed, and the effect of Wnt signaling manipulation in CTRL and MCAO mice was investigated in cells with a passive, complex and outwardly rectifying current profile (**Tables 2**–**7**).

In cells with a passive current pattern, the changes in the membrane properties were sporadic (**Tables 2**, **3**). Wnt signaling manipulation only affected the passive electrophysiological properties. The strongest effect was identified in the V_M following the induction of FCI, where we discovered that following Wnt signaling inhibition, the cells became hyperpolarized. In dnTCF4 mice, the average V_M changed from −84.9 ± 0.7 mV (n = 25) to −87.1 ± 0.4 mV (n = 40), while in Dkk1 mice, its value changed from −86.0 ± 0.6 mV (n = 23) to −89.1 ± 0.3 mV (n = 32). Such hyperpolarization was observed in all analyzed cell types that were isolated from ischemic tissue. In CTRL

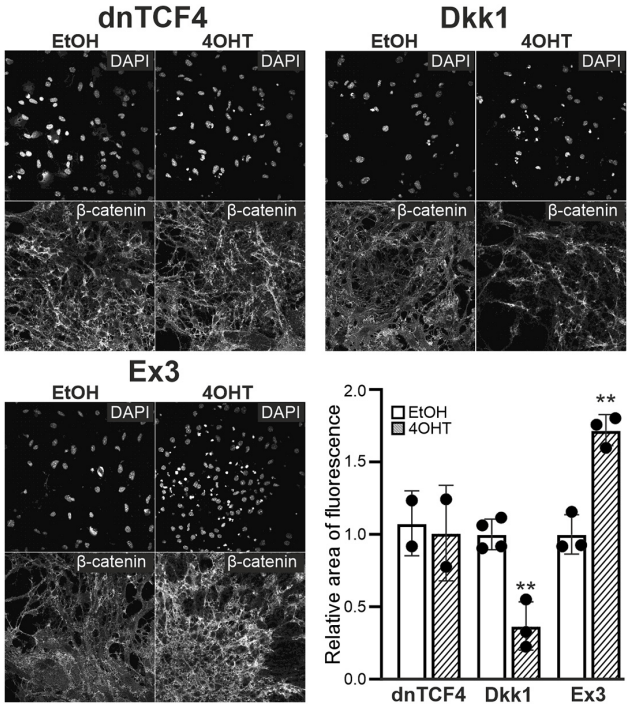

FIGURE 3 | Changes of β-catenin protein level upon Wnt pathway modulation. Representative fluorescence images show DAPI and β-catenin staining in differentiated adult neural stem/progenitor cells with inhibited (dnTCF4 and Dkk1), or activated (Ex3) Wnt signaling. Cells were only treated with either ethanol (EtOH) or with (Z)-4-hydroxytamoxifen (4OHT) dissolved in EtOH, and analyzed 8 days after the onset of *in vitro* differentiation. Diagram indicates quantification of β-catenin expression, showing the proportion of the area of positively-stained cells to the DAPI-positive area (n = 12). The values are represented as mean ± S.D. (standard deviation). Statistical significance was calculated using t-test; $^{**}p < 0.01$. DAPI, 4′,6-diamidino-2-phenylindole; Dkk1, Dickkopf 1; dnTCF4, dominant negative T-cell factor 4; Ex3, exon 3; n, number.

mice, we recorded higher IR after Wnt signaling inhibition at the membrane receptor level [from 49.1 ± 3.1 MΩ (n = 25) to 75.3 ± 4.5 MΩ (n = 25)], together with lowered C_M [from 31.4 ± 2.8 pF (n = 25) to 21.7 ± 1.7 pF (n = 25)]. Although we did not observe any differences in the current densities of K^+ currents, we detected a decreased incidence of cells showing K_{IR} currents following MCAO (data not shown). These results might correspond to astrocyte differentiation and their proliferative capacity following FCI.

In cells with a complex current pattern, we observed opposite effects of Wnt signaling inhibition and activation on the values of V_M (**Tables 4**, **5**). Inhibition of the pathway resulted in hyperpolarization [from −82.9 ± 1.0 mV (n = 43) to −88.6 ± 0.7 mV (n = 45) in CTRL dnTCF4 mice, and from −84.7 ± 1.3 mV (n = 15) to −88.2 ± 0.7 mV (n = 13) and from −87.2 ± 0.7 (n = 15) to −89.4 ± 0.6 mV (n = 34) in MCAO dnTCF4 and Dkk1 mice, respectively], while Wnt signaling activation led to depolarization of the membrane [from −88.8 ± 0.8 mV (n = 45) to −86.5 ± 0.7 mV (n = 58) in CTRL Ex3 mice]. Besides the passive membrane properties, Wnt signaling manipulation also influenced the expression of voltage-gated K^+ channels in this cell type. After Wnt signaling activation in CTRL mice, the densities of all examined K^+ currents decreased [significant decrease in K_{IR}, from 7.9 ± 0.7 pA/pF (n = 44) to 4.5 ± 0.5 pA/pF (n = 57), and in K_{DR}, from 42.1 ± 5.5 pA/pF (n = 44) to 25.6 ± 2.9 pA/pF (n = 58)]. This effect of Wnt signaling subsided after the induction of FCI, which is in accordance with our observation that under ischemic conditions, the majority of K^+ currents were upregulated, independently on the Wnt signaling inhibition/activation [significant increase in K_{IR} from 6.3 ± 1.4 pA/pF (n = 15) to 11.7 ± 1.6 pA/pF (n = 34) in Dkk1 mice and from 5.1 ± 0.9 pA/pF (n = 23) to 9.0 ± 1.4 pA/pF (n = 22) in Ex3 mice].

In cells displaying an outwardly rectifying current pattern, the effect of Wnt signaling inhibition on the V_M was reversed in CTRL and MCAO mice (**Tables 6**, **7**). In CTRL mice, the membrane was depolarized [from −85.5 ± 1.6 mV (n = 47) to −79.4 ± 1.8 mV (n = 47) in dnTCF4 mice and from −86.9 ± 1.3 mV (n = 65) to −81.8 ± 1.2 mV (n = 57) in Dkk1 mice]. Conversely, after the induction of FCI, the cells became hyperpolarized [significantly in dnTCF4 mice, from −77.4 ± 2.4 mV (n = 34) to −85.7 ± 1.4 mV (n = 19)], which was observed in all three cell types. The opposite impact of the Wnt signaling pathway on the V_M coincides with the expression of K^+ channels mediating outward currents. After Wnt signaling inhibition, the current densities of K_{DR} and K_A were decreased or increased only negligibly in CTRL mice [significant decrease in K_A, from 110.9 ± 9.8 pA/pF (n = 51) to 40.1 ± 7.2 pA/pF (n = 33), in Dkk1 mice] while in MCAO mice, their current densities increased [significant increase in K_{DR} in dnTCF4 mice, from 100.4 ± 5.3 pA/pF (n = 34) to 149.3 ± 19.5 pA/pF (n = 19), and significant increase in K_A in Dkk1 mice, from 103.7 ± 10.1 pA/pF (n = 35) to 180.3 ± 30.1 pA/pF (n = 10)]. A higher efflux of K^+ ions out of the cell could potentially explain the hyperpolarization observed after the induction of FCI in cells with an outwardly rectifying as well as a complex current profile.

Additionally, we identified changes in the incidence of neuron-like cells expressing voltage-dependent Na^+ channels (data not shown). Their counts increased both after Wnt signaling activation (from 3 to 11 cells; total numbers in both CTRL and MCAO cultures) and its inhibition in the cell nucleus (from 3 to 9 cells). Alternatively, the attenuation of the Wnt pathway at the membrane receptor level resulted in a smaller number of these cells (from 10 to 7 cells). Interestingly, the same trends were found in *in vitro* cultures derived from both CTRL as well as MCAO mice. Finally, the density of Na^+ currents was only significantly changed in non-operated Dkk1 mice. We identified its increase [from 9.8 ± 1.1 pA/pF (n = 8) in EtOH to 19.1 ± 4.3 pA/pF (n = 7) in 4OHT] in cells with an outwardly rectifying current pattern, which could add to the depolarization we observed in this cell type.

The electrophysiological analysis gave us an outline of the effect of Wnt signaling on the functional properties of differentiated NS/PCs. Wnt manipulation had a minimal impact on the electrophysiological properties of cells with

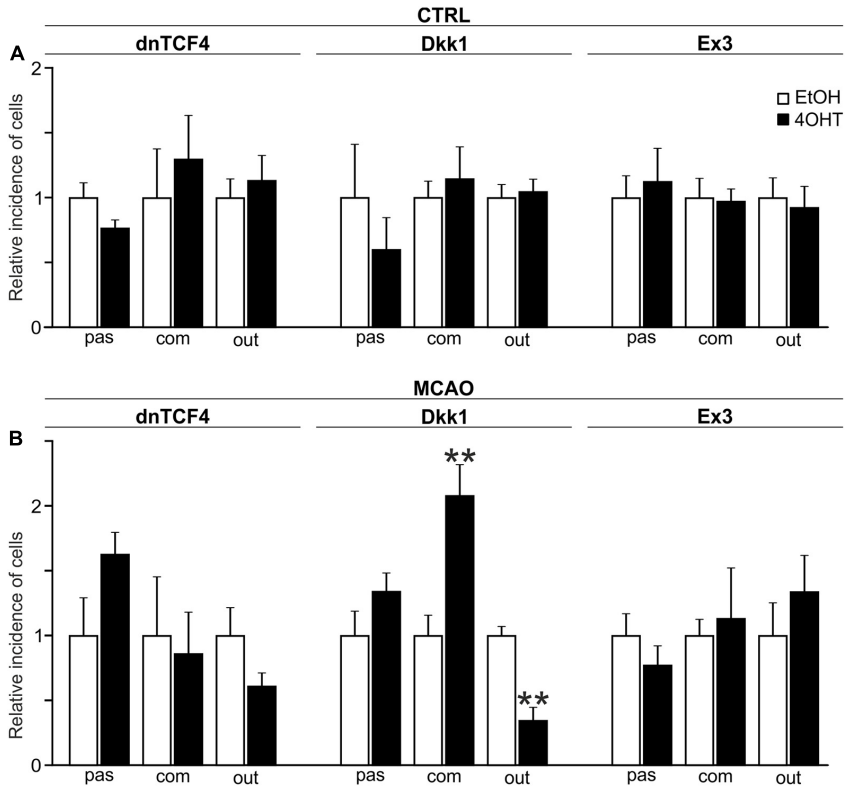

FIGURE 4 | Changes in the incidence of GFAP-, PDGFRα-, and DCX-positive cells after Wnt signaling manipulation in intact and ischemic mice. The incidence of cells showing passive (pas; GFAP$^+$ cells), complex (com; PDGFRα$^+$ cells) or outwardly rectifying (out; DCX$^+$ cells) current patterns was examined in neural stem/progenitor cell (NS/PC) cultures derived from adult control, non-operated (CTRL; **A**) as well as operated (MCAO; **B**) mice. The relative incidence of cells in controls was arbitrarily set to 1. Sixteen founder mice were used to derive NS/PCs from each mouse strain. The incidence was quantified from the following total number of cells (in brackets): CTRL-dnTCF4-EtOH (145), CTRL-dnTCF4-4OHT (141), CTRL-Dkk1-EtOH (116), CTRL-Dkk1-4OHT (114), CTRL-Ex3-EtOH (152), CTRL-Ex3-4OHT (197), MCAO-dnTCF4-EtOH (75), MCAO-dnTCF4-4OHT (72), MCAO-Dkk1-EtOH (75), MCAO-Dkk1-4OHT (79), MCAO-Ex3-EtOH (80), and MCAO-Ex3-4OHT (80). The incidence of cells in controls (EtOH) was compared to the incidence of the same cell types in cells with manipulated Wnt signaling (4OHT) with Student's *t*-test. $^{**}p < 0.01$. 4OHT, (Z)-4-hydroxytamoxifen; DCX, doublecortin; Dkk1, Dickkopf 1; dnTCF4, dominant negative T-cell factor 4; EtOH, ethanol; Ex3, exon 3; GFAP, glial fibrillary acidic protein; MCAO, middle cerebral artery occlusion; PDGFRα, platelet-derived growth factor receptor alpha.

TABLE 2 | Membrane properties of differentiated adult NS/PCs isolated from CTRL mice, and showing a passive current profile.

	dnTCF4		Dkk1		Ex3	
	EtOH	**4OHT**	**EtOH**	**4OHT**	**EtOH**	**4OHT**
V_M **[mV]**	−84.5 ± 0.7	−83.0 ± 1.0	−86.8 ± 0.9	−85.5 ± 1.0	**−85.4 ± 0.7**	**−87.6 ± 0.6***
IR [MΩ]	67.6 ± 4.2	66.7 ± 2.8	**49.1 ± 3.1**	**75.3 ± 4.5*****	89.4 ± 5.0	79.2 ± 4.0
C_M **[pF]**	27.1 ± 2.3	30.4 ± 2.9	**31.4 ± 2.8**	**21.7 ± 1.7****	22.6 ± 1.8	20.7 ± 1.4
K_{IR}/C_M **[pA/pF]**	2.9 ± 0.3	2.5 ± 0.4	2.4 ± 0.7	3.2 ± 0.4	3.5 ± 0.9	2.7 ± 0.3
K_{DR}/C_M **[pA/pF]**	4.0 ± 0.8	6.3 ± 1.5	7.4 ± 2.3	8.7 ± 3.0	5.8 ± 1.0	3.9 ± 0.6
n	62	55	25	25	61	63

4OHT, (Z)-4-hydroxytamoxifen; C_M, membrane capacitance; CTRL, control, non-operated mice; Dkk1, Dickkopf 1; dnTCF4, dominant negative T-cell factor 4; EtOH, ethanol; Ex3, exon 3; IR, input resistance; K_{DR}, delayed outwardly rectifying K^+ currents; K_{IR}, inwardly rectifying K^+ currents; K_{IR}/C_M, K_{DR}/C_M, current densities; n, number of cells; NS/PCs, neural stem/progenitor cells; V_M, membrane potential. Values in bold indicate significant differences between EtOH- and 4OHT-treated cultures; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

a passive current pattern. Conversely, the most significant changes were identified after FCI in cells with a complex and an outwardly rectifying current profile. We particularly observed hyperpolarized cells after Wnt signaling inhibition, which can be explained by higher current densities of K_{DR} and K_A. Furthermore, we only detected a few cells with voltage-dependent Na^+ channels. These results imply that Wnt signaling affects the distribution of K^+ and Na^+ channels, and thus influences the membrane properties of differentiated cells.

TABLE 3 | Membrane properties of differentiated adult NS/PCs isolated from MCAO mice, and showing a passive current profile.

	dnTCF4		Dkk1		Ex3	
	EtOH	**4OHT**	**EtOH**	**4OHT**	**EtOH**	**4OHT**
V_M **[mV]**	**−84.9 ± 0.7**	**−87.1 ± 0.4****	**−86.0 ± 0.6**	**−89.1 ± 0.3*****	−86.1 ± 0.3	−85.5 ± 0.6
IR [MΩ]	50.1 ± 3.6	47.2 ± 1.9	44.4 ± 3.5	46.3 ± 2.5	45.9 ± 2.1	48.3 ± 2.8
C_M **[pF]**	40.6 ± 5.0	36.3 ± 2.4	46.6 ± 4.7	36.5 ± 3.5	53.5 ± 9.5	51.4 ± 10.6
K_{IR}/C_M **[pA/pF]**	2.4 ± 0.4	1.9 ± 0.3	2.9 ± 0.6	4.2 ± 0.6	2.0 ± 0.3	2.1 ± 0.3
K_{DR}/C_M **[pA/pF]**	5.9 ± 1.1	4.2 ± 0.5	5.6 ± 1.0	4.6 ± 0.7	5.8 ± 0.7	4.7 ± 0.7
n	25	40	23	32	39	30

*4OHT, (Z)-4-hydroxytamoxifen; C_M, membrane capacitance; Dkk1, Dickkopf 1; dnTCF4, dominant negative T-cell factor 4; EtOH, ethanol; Ex3, exon 3; IR, input resistance; K_{DR}, delayed outwardly rectifying K^+ currents; K_{IR}, inwardly rectifying K^+ currents; K_{IR}/C_M, K_{DR}/C_M, current densities; MCAO, middle cerebral artery occlusion; n, number of cells; NS/PCs, neural stem/progenitor cells; V_M, membrane potential. Values in bold indicate significant differences between EtOH- and 4OHT-treated cultures; **$p < 0.01$, ***$p < 0.001$.*

TABLE 4 | Membrane properties of differentiated adult NS/PCs isolated from CTRL mice, and showing a complex current profile.

	dnTCF4		Dkk1		Ex3	
	EtOH	**4OHT**	**EtOH**	**4OHT**	**EtOH**	**4OHT**
V_M **[mV]**	**−82.9 ± 1.0**	**−88.6 ± 0.7*****	−89.0 ± 0.9	−86.9 ± 0.9	**−88.8 ± 0.8**	**−86.5 ± 0.7***
IR [MΩ]	138.2 ± 12.8	153.0 ± 13.8	136.4 ± 9.7	136.3 ± 9.0	**175.6 ± 14.0**	**138.6 ± 7.7***
C_M **[pF]**	**16.9 ± 1.6**	**12.7 ± 0.8***	14.5 ± 1.1	15.2 ± 1.3	15.7 ± 1.2	16.6 ± 1.1
K_{IR}/C_M **[pA/pF]**	6.1 ± 0.6	7.7 ± 0.9	8.3 ± 0.8	10.7 ± 1.2	**7.9 ± 0.7**	**4.5 ± 0.5*****
K_{DR}/C_M **[pA/pF]**	34.9 ± 5.1	52.8 ± 9.0	54.1 ± 6.3	76.0 ± 9.6	**42.1 ± 5.5**	**25.6 ± 2.9****
K_A/C_M **[pA/pF]**	22.3 ± 4.3	20.8 ± 3.7	**45.4 ± 6.3**	**25.1 ± 2.7***	19.2 ± 4.3	11.4 ± 2.4
Na^+/C_M **[pA/pF]**	28.2 ± 12.4	39.5 ± 5.4	11.8 ± 5.1	52.9 ± 0.0	19.7 ± 3.5	27.3 ± 5.7
n	43	45	39	38	45	58

*4OHT, (Z)-4-hydroxytamoxifen; C_M, membrane capacitance; CTRL, control, non-operated mice; Dkk1, Dickkopf 1; dnTCF4, dominant negative T-cell factor 4; EtOH, ethanol; Ex3, exon 3; IR, input resistance; K_A, fast activating and inactivating outwardly rectifying K^+ currents; K_{DR}, delayed outwardly rectifying K^+ currents; K_{IR}, inwardly rectifying K^+ currents; K_{IR}/C_M, K_{DR}/C_M, K_A/C_M, Na^+/C_M, current densities; n, number of cells; NS/PCs, neural stem/progenitor cells; V_M, membrane potential. Values in bold indicate significant differences between EtOH- and 4OHT-treated cultures; **$p < 0.01$, ***$p < 0.001$.*

TABLE 5 | Membrane properties of differentiated adult NS/PCs isolated from MCAO mice, and showing a complex current profile.

	dnTCF4		Dkk1		Ex3	
	EtOH	**4OHT**	**EtOH**	**4OHT**	**EtOH**	**4OHT**
V_M **[mV]**	**−84.7 ± 1.3**	**−88.2 ± 0.7***	**−87.2 ± 0.7**	**−89.4 ± 0.6***	−86.0 ± 0.5	−85.2 ± 1.3
IR [MΩ]	171.9 ± 33.5	120.7 ± 16.3	115.4 ± 26.3	111.8 ± 10.6	93.1 ± 11.4	110.6 ± 11.9
C_M **[pF]**	21.4 ± 3.5	20.3 ± 3.3	**23.3 ± 2.4**	**15.2 ± 1.2****	34.5 ± 13.8	18.9 ± 2.9
K_{IR}/C_M **[pA/pF]**	6.1 ± 1.0	6.3 ± 1.2	**6.3 ± 1.4**	**11.7 ± 1.6***	**5.1 ± 0.9**	**9.0 ± 1.4***
K_{DR}/C_M **[pA/pF]**	61.3 ± 9.4	52.4 ± 10.7	32.7 ± 8.0	79.6 ± 16.7	59.4 ± 15.1	54.0 ± 12.0
K_A/C_M **[pA/pF]**	15.9 ± 4.9	27.2 ± 8.7	26.5 ± 9.5	39.0 ± 7.9	38.7 ± 13.5	26.3 ± 11.7
Na^+/C_M **[pA/pF]**	– –	– –	– –	– –	– –	– –
n	15	13	15	34	23	23

*4OHT, (Z)-4-hydroxytamoxifen; C_M, membrane capacitance; Dkk1, Dickkopf 1; dnTCF4, dominant negative T-cell factor 4; EtOH, ethanol; Ex3, exon 3; IR, input resistance; K_A, fast activating and inactivating outwardly rectifying K^+ currents; K_{DR}, delayed outwardly rectifying K^+ currents; K_{IR}, inwardly rectifying K^+ currents; K_{IR}/C_M, K_{DR}/C_M, K_A/C_M, Na^+/C_M, current densities; MCAO, middle cerebral artery occlusion; n, number of cells; NS/PCs, neural stem/progenitor cells; V_M, membrane potential. Values in bold indicate significant differences between EtOH- and 4OHT-treated cultures; *$p < 0.05$, **$p < 0.01$.*

By comparing the current densities in control (CTRL-TAM) and post-ischemic (MCAO-TAM) animals, in which the Wnt signaling pathway was modified, we found that the blockage of Wnt signaling at the nuclear level (dnTCF4) had no effect on the current densities in any of the three cell types (**Tables 2**–**7**). On the other hand, Wnt signaling inhibition at the membrane receptor level (Dkk1) significantly increased the current densities of K_A channels in cells with a complex current pattern (from 25.1 ± 2.7 pA/pF to 39.0 ± 7.9 pA/pF; **Tables 4**, **5**) as well as in those displaying

TABLE 6 | Membrane properties of differentiated adult NS/PCs isolated from CTRL mice, and showing an outwardly rectifying current profile.

	dnTCF4		Dkk1		Ex3	
	EtOH	**4OHT**	**EtOH**	**4OHT**	**EtOH**	**4OHT**
V_M [mV]	**−85.5 ± 1.6**	**−79.4 ± 1.8***	**−86.9 ± 1.3**	**−81.8 ± 1.2****	**−86.2 ± 1.5**	**−77.8 ± 1.8*****
IR [MΩ]	997.1 ± 97.3	887.5 ± 96.0	**959.7 ± 97.2**	**352.4 ± 30.2*****	**1217.6 ± 134.3**	**877.8 ± 91.9***
C_M [pF]	8.4 ± 0.8	9.5 ± 0.6	**9.1 ± 0.5**	**16.1 ± 1.2*****	**9.1 ± 0.6**	**11.1 ± 0.8***
K_{DR}/C_M [pA/pF]	112.2 ± 7.1	118.3 ± 9.0	139.6 ± 8.9	150.5 ± 14.1	101.2 ± 4.7	88.5 ± 5.8
K_A/C_M [pA/pF]	84.5 ± 7.6	75.5 ± 12.3	**110.9 ± 9.8**	**40.1 ± 7.2*****	**80.6 ± 7.3**	**44.0 ± 7.1*****
Na^+/C_M [pA/pF]	– –	24.2 ± 12.8	**9.8 ± 1.1**	**19.1 ± 4.3***	12.4 ± 2.6	14.4 ± 4.3
n	47	47	65	57	62	61

*4OHT, (Z)-4-hydroxytamoxifen; C_M, membrane capacitance; CTRL, control, non-operated mice; Dkk1, Dickkopf 1; dnTCF4, dominant negative T-cell factor 4; EtOH, ethanol; Ex3, exon 3; IR, input resistance; K_A, fast activating and inactivating outwardly rectifying K^+ currents; K_{DR}, delayed outwardly rectifying K^+ currents; K_{DR}/C_M, K_A/C_M, Na^+/C_M, current densities; n, number of cells; NS/PCs, neural stem/progenitor cells; V_M, membrane potential. Values in bold indicate significant differences between EtOH- and 4OHT-treated cultures; *p < 0.05, **p < 0.01, ***p < 0.001.*

TABLE 7 | Membrane properties of differentiated adult NS/PCs isolated from MCAO mice, and showing an outwardly rectifying current profile.

	dnTCF4		Dkk1		Ex3	
	EtOH	**4OHT**	**EtOH**	**4OHT**	**EtOH**	**4OHT**
V_M [mV]	**−77.4 ± 2.4**	**−85.7 ± 1.4***	−78.1 ± 2.3	−84.3 ± 2.1	**−80.1 ± 2.5**	**−67.0 ± 3.4****
IR [MΩ]	1112.2 ± 132.8	1028.2 ± 123.1	1166.2 ± 157.2	741.8 ± 132.1	1230.8 ± 228.2	1018.7 ± 156.6
C_M [pF]	8.9 ± 0.4	8.8 ± 0.3	9.0 ± 0.4	7.9 ± 1.3	10.2 ± 1.1	9.1 ± 0.8
K_{DR}/C_M [pA/pF]	**100.4 ± 5.3**	**149.3 ± 19.5****	109.5 ± 6.7	137.1 ± 17.1	99.3 ± 6.6	114.6 ± 10.9
K_A/C_M [pA/pF]	80.8 ± 6.2	95.5 ± 12.1	**103.7 ± 10.1**	**180.3 ± 30.1****	87.5 ± 12.3	94.9 ± 19.4
Na^+/C_M [pA/pF]	11.6 ± 0.3	15.7 ± 2.3	12.3 ± 5.5	–	–	31.9 ± 10.4
n	34	19	37	13	18	24

*4OHT, (Z)-4-hydroxytamoxifen; C_M, membrane capacitance; Dkk1, Dickkopf 1; dnTCF4, dominant negative T-cell factor 4; EtOH, ethanol; Ex3, exon 3; IR, input resistance; K_A, fast activating and inactivating outwardly rectifying K^+ currents; K_{DR}, delayed outwardly rectifying K^+ currents; K_{DR}/C_M, K_A/C_M, Na^+/C_M, current densities; MCAO, middle cerebral artery occlusion; n, number of cells; NS/PCs, neural stem/progenitor cells; V_M, membrane potential. Values in bold indicate significant differences between EtOH- and 4OHT-treated cultures; *p < 0.05, **p < 0.01.*

an outwardly rectifying current profile (from 40.1 ± 7.2 pA/pF to 180.3 ± 30.1 pA/pF; **Tables 6**, **7**). We hypothesize that the diverse effect of Wnt signaling inhibition at different subcellular levels can be caused by the compensatory effect of endogenous Tcf4 produced in dnTCF4 mice (Janeckova et al., 2016). Interestingly, following FCI, Wnt signaling hyper-activation (Ex3) increased current densities of K_{IR} (from 4.5 ± 0.5 pA/pF to 9.0 ± 1.4 pA/pF), K_{DR} (from 25.6 ± 2.9 pA/pF to 54.0 ± 12.0 pA/pF), and K_A (from 11.4 ± 2.4 pA/pF to 26.3 ± 11.7 pA/pF) in cells with a complex current pattern (**Tables 4**, **5**), and K_{DR} (from 88.5 ± 5.8 pA/pF to 114.6 ± 10.9 pA/pF), K_A (from 44.0 ± 7.1 pA/pF to 94.9 ± 19.4 pA/pF) and Na^+ (from 14.4 ± 4.3 pA/pF to 31.9 ± 10.4 pA/pF) current densities in cells showing an outwardly rectifying current pattern (**Tables 6**, **7**). In cells with a passive current profile (**Tables 2**, **3**), Wnt signaling modulation following FCI had no impact on the current densities of voltage-dependent K^+ channels. This could be explained by the fact that the expression of voltage-dependent K^+ channels in cells showing a passive current pattern (**Tables 2**, **3**) is relatively lower than their expression in other cell types (**Tables 4**–**7**). Additionally, there was an IR decline in post-ischemic passive cells, presumably suggesting a reduced expression of two-pore domain K^+ channels (Zhou et al., 2009); however, this decline occurred regardless of Wnt signaling inhibition/activation (**Tables 2**, **3**).

Analysis of the Effects of Wnt Pathway Modulation in the Adult Brain

Changes in β-catenin protein levels upon Wnt signaling manipulation were assessed by immunoblotting of brain tissue lysates prepared from SVZs of adult mice of corresponding genotypes. However, the densitometric analysis revealed no consistent changes in the expression of total β-catenin or its non-phosphorylated, active form (data not shown). Additionally, we analyzed the expression of putative Wnt target genes, namely *Axin2*, naked cuticle homolog 1 (*Nkd1*), and *Troy* [alternative name tumor necrosis factor receptor superfamily, member 19 (*Tnfrsf19*)]. Overall, their expression was either downregulated or not significantly changed after Wnt signaling manipulation in CTRL mice of all strains (**Supplementary Figure 3**). The expression pattern was similar in MCAO mice; however, a significantly increased expression of the most Wnt target genes was observed. Moreover, hyper-activation of Wnt/β-catenin signaling induced by β-catenin stabilization was evident only in case of the *Axin2* gene (**Supplementary Figure 3**). These results indicate that brain tissue used for the analyses is more

heterogeneous than cell cultures as it contains, in addition to NS/PCs (and their descendants), also other cell types that might react to Wnt signaling manipulation differently.

Therefore, we performed immunohistochemical analysis in coronal brain sections where we analyzed the expression of DCX, GFAP, and a marker of dividing cells, PCNA, in the SVZ. In the vehicle-treated mice, we observed higher overall immunopositivity for all marker proteins after the induction of FCI (**Figures 5A–D**; due to the space constraints, the immunohistochemical staining is only shown for mice either non-operated or after FCI, indicated as 'CTRL CO' or 'MCAO CO,' respectively; for the immunohistochemical staining of all experimental groups, see **Supplementary Figure 4**). Furthermore, Wnt signaling inhibition caused higher expression of GFAP and lower expression of DCX in both CTRL and MCAO mice, while the immunopositivity of PCNA was decreased significantly only in MCAO mice (**Figures 5B,C**). These observations were more significant after Wnt signaling inhibition in the cell nucleus (**Figure 5B**). Conversely, Wnt signaling hyper-activation resulted in the overexpression of PCNA and DCX in CTRL mice, while this effect was diminished after FCI. Moreover, activation of the pathway led to the significantly decreased expression of GFAP only after the induction of FCI (**Figure 5D**).

To gain a deeper insight into the brain injury-induced cellular changes, we performed scRNA-seq of cells obtained from the brain of non-operated (CTRL) mice and animals 3 days after FCI. The SVZ (and adjacent striatum) was dissected, and the specimens were processed to obtain a single-cell suspension. The cells were subsequently FACS-sorted, and all living cells except leukocytes (CD45-positive cells) and vascular epithelium (CD31-positive) cells were used for the scRNA-seq analysis. The identified cell clusters were matched to the molecular atlas of cells obtained from the adult SVZ published by Mizrak et al. (2019) (**Supplementary Table 2**). Whereas the proportion of neural stem and transit amplifying cells, and two neuroblast subpopulations (neuroblasts 1 and 2), were after FCI increased, the number of matured neurons was reduced (**Figure 6A**). The latter observation could be attributed to the fact that the analyzed samples did not contain the site of injury in the cortex. Interestingly, the numbers of OPCs and COPs were reduced after FCI. Nevertheless, it should be noted that the OPCs and COPs counts were rather low in both situations. From four astrocyte subtypes, three of the subtypes (astrocytes 1 and 3, astrocytes 4_transit) were markedly decreased in brain tissue recovering from MCAO. Finally, a decrease in cells assigned to clusters representing mural cells (mainly pericytes) and fibroblasts was observed. After brain injury, pericytes contribute to the restoration of the blood-brain barrier. Immediately after brain damage, the number of pericytes decreases, but during the repair phase, pericytes undergo reactive pericytosis, i.e., the cells detach from cerebral blood vessels, change their shape and proliferate. Moreover, they form a cell mass demarcated by reactive astrocytes (Zehendner et al., 2015).

The *DCX* and *GFAP* transcripts were in CTRL mice and animals that underwent MCAO mainly distributed in the stem and progenitor cell clusters or in astrocytes, respectively. Additionally, *DCX* expression was emerging in COPs, supporting our previous observation that this subpopulation of neuron-glial antigen 2 (NG2)-positive glial cells might contribute to neurogenesis (Honsa et al., 2012). In contrast, FCI induced expression of proliferating cell marker *PCNA* in virtually all cell subpopulations (**Figure 6B**), which possibly relates to reactive gliosis after brain injury (Buffo et al., 2008).

The analysis of the expression of the putative Wnt target genes *Axin2*, *Nkd1*, and *Troy* brought less consistent results (**Figure 7**). Whereas *Axin2* was in CTRL tissue expressed mainly in the transit subpopulation of astrocytes, neural stem cells, transit amplifying cells, and COPs; *Nkd1* was mainly produced in oligodendrocytes and their precursors (OPCs and COPs). In contrast, *Troy* expression was predominantly detected in three astrocyte (astrocytes 2–4) subpopulations. Additionally, *Axin2* and *Troy* mRNA was detected in brain mural cells and fibroblasts, while all the three genes were expressed in endothelial cells. In the brain tissue after FCI, we observed *de novo* expression of *Axin2* and *Nkd1* in OPCs and transit astrocytes, respectively. Moreover, *Troy* expression was upregulated in virtually all astrocyte subpopulations (**Figure 7**). Next, we analyzed the expression profiles of all 19 mammalian Wnt ligands in brain cells of CTRL and MCAO mice, and identified a relatively high expression of *Wnt7b*, a ligand which activates canonical Wnt signaling (Daneman et al., 2009; Posokhova et al., 2015). Interestingly, in the healthy brain, *Wnt7b* transcripts were limited to two astrocyte subpopulations (astrocyte 1 and 3); however, after brain injury, the ligand was induced in the transit astrocyte subtype (astrocyte 4_transit) and in OPCs (**Figure 7**, right bottom diagram).

DISCUSSION

Transgenic Animal Models

Although the role of the canonical Wnt signaling pathway in neural development has been a main focus of countless studies, relatively little is known about its functions in adult neurogenesis and gliogenesis. This lack of information may stem from severe, constitutive dysregulations of this pathway that result in embryonic lethality (van Amerongen and Berns, 2006). To circumvent this issue, we used transgenic mouse strains that allowed TAM-induced, conditional manipulation of Wnt signaling. The same three mouse strains as in our previous study elucidating the effect of Wnt signaling on neonatal NS/PCs (Kriska et al., 2016), were used here; nevertheless, there were several differences in utilizing neonatal and adult mice in our experiments, as a variety of factors may influence the differentiation potential of adult NS/PCs. Among others, gender and age are the most important constituents in this process (Lembach et al., 2018; Vancamp et al., 2019). More specifically, clinical observations as well as experimental stroke confirmed that a high amount of estrogen in females acts as an endogenous neuroprotectant after the induction of cerebral ischemia (Hurn and Macrae, 2000), and that neurogenesis ceases with age

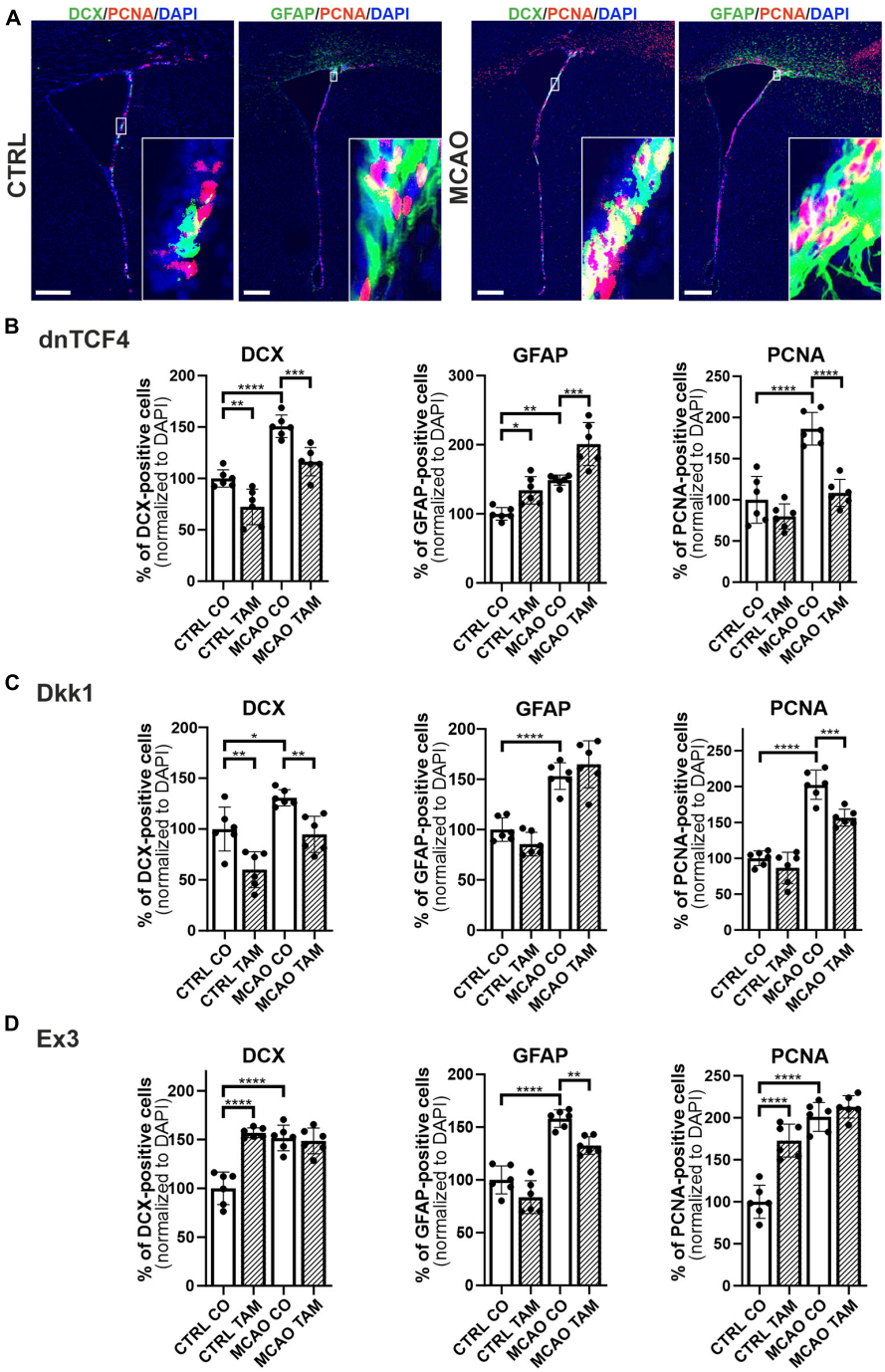

FIGURE 5 | Immunohistochemical analysis of changes in the production of cell-type-specific markers in the brain of transgenic mice after FCI. **(A)** Representative images of the SVZ isolated from CTRL and MCAO mice without Wnt pathway modulation. Increased stainings of neuroblasts marked by doublecortin (DCX), glial fibrillary acidic protein (GFAP)-positive astrocytes, and dividing cells harboring proliferating cell nuclear antigen (PCNA) were recorded 3 days after the induction of ischemia. Scale bar = 0.2 mm. Modulation of the canonical Wnt signaling pathway changed the incidence of cell types under control, physiological conditions (CTRL) as well as 3 days upon focal cerebral ischemia induced by middle cerebral artery occlusion (MCAO). Diagrams show quantification of immunohistochemical staining in the SVZ of CTRL and MCAO mice only treated with either tamoxifen (TAM) or vehicle, corn oil (CO). Tamoxifen-induced production of dominant negative human T-cell factor protein (dnTCF4; **B**) and Dickkopf 1 Wnt inhibitory protein (Dkk1; **C**) were used to achieve Wnt signaling inhibition and, conversely, production of constitutively active β-catenin (Ex3; **D**) was initiated in order to obtain Wnt pathway activation. Experiments were performed using two biological replicates and three technical replicates for each treatment ($n = 6$). Average values of the control mice (CTRL CO) were arbitrary set to 100% of immunogenic signal. Error bars represent standard deviation and one-way ANOVA was used to determine significant differences among the experimental groups; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Ex3, exon 3.

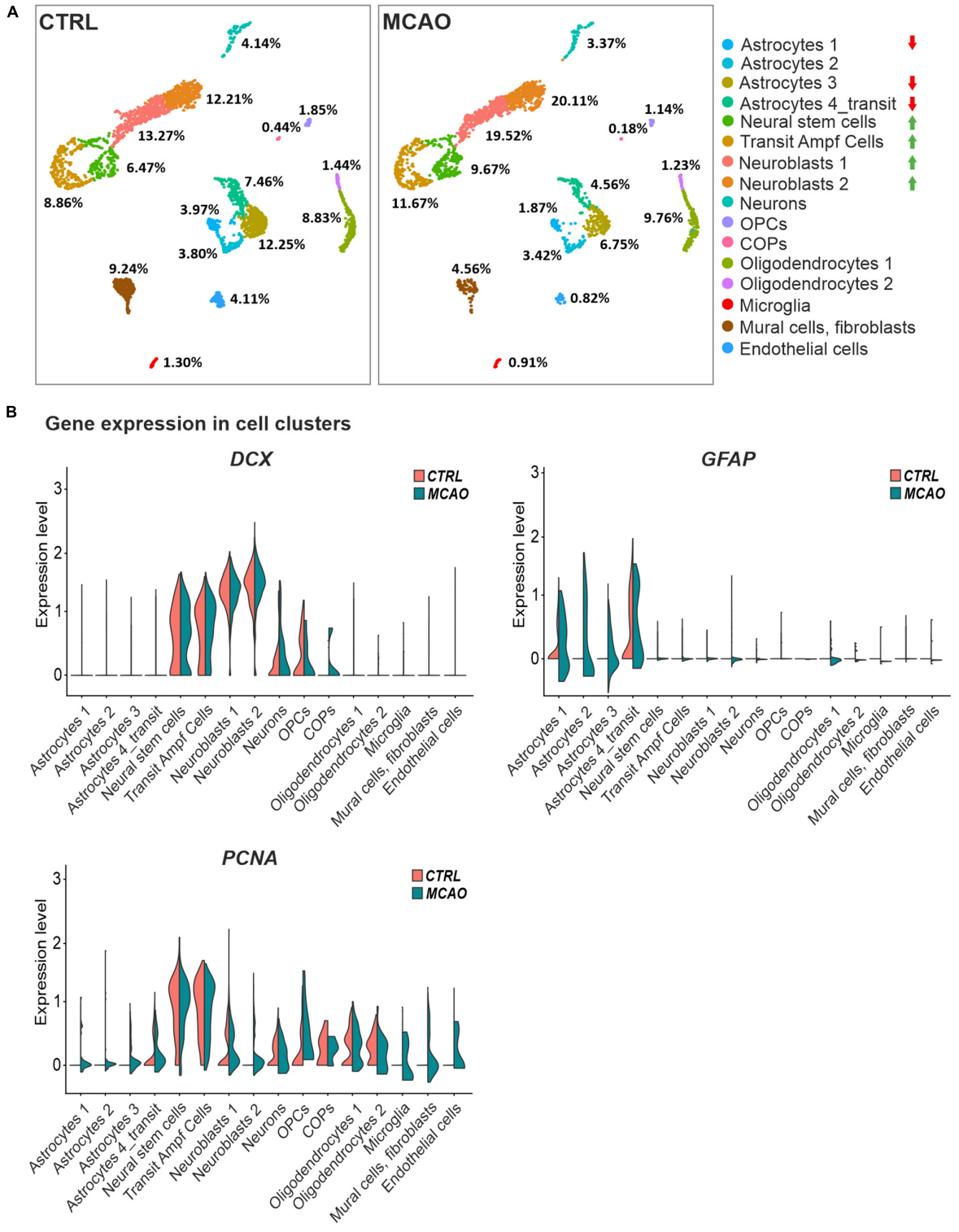

FIGURE 6 | Single-cell RNA sequencing analysis of cells obtained from the SVZ and striatum of the healthy (CTRL) and ischemic brain (MCAO). **(A)** Cell distribution in individual clusters (subpopulations); clusters with the most significant decrease or increase in cell counts after focal cerebral ischemia (FCI) are indicated by red or green arrows, respectively. The percentage of cells present in different cell clusters is indicated; RNA sequencing was performed on 3,694 and 2,728 cells obtained from the control or ischemic brain, respectively. After removing apoptotic cells, the remaining 2,923 (CTRL) and 2,193 (MCAO) cells were analyzed. More than 124,206 reads were obtained from one cell on average, representing 2,420 genes in the CTRL sample. In the brain after MCAO, the numbers were 175,361 reads per cell, representing 2,646 genes. **(B)** "Violin" plots showing the expression of *DCX*, *GFAP*, and *PCNA* in the indicated cell clusters. COPs, committed oligodendrocyte precursors; CTRL, control; *DCX*, doublecortin; *GFAP*, glial fibrillary acidic protein; MCAO, middle cerebral artery occlusion; OPCs, oligodendrocyte precursor cells; *PCNA*, proliferating cell nuclear antigen.

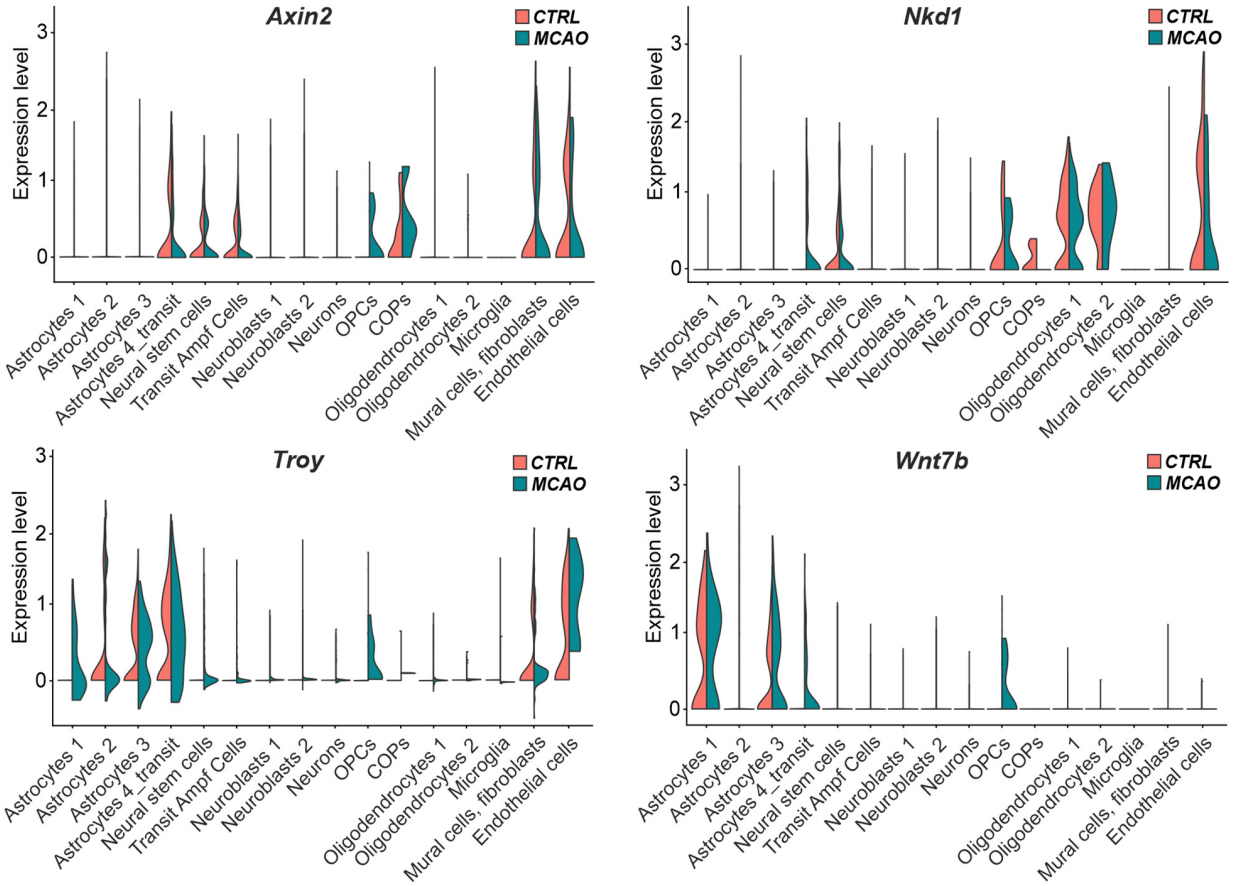

FIGURE 7 | "Violin" plots showing expression of Wnt-responsive genes and *Wnt7b* mRNA in the indicated cell clusters. COPs, committed oligodendrocyte precursors; CTRL, control; MCAO, middle cerebral artery occlusion; *Nkd1*, naked cuticle homolog 1; OPCs, oligodendrocyte precursor cells; *Troy*, tumor necrosis factor receptor superfamily, member 19; *Wnt7b*, Wingless/Integrated 7b.

(Lupo et al., 2019). For this reason, only male mice of a strictly defined age (postnatal day 50–56) were used in our experiments.

The Effect of the Wnt Pathway Modulation in Adult NS/PCs Differentiated *in vitro*

The Cre/loxP system, together with the well-defined *in vitro* conditions, guaranteed highly reproducible means of either Wnt/β-catenin pathway hyper-activation (Ex3), or its inhibition at two different subcellular levels – in the cell nucleus (dnTCF4) or at the membrane receptor level (Dkk1; **Figure 1**). Additionally, we assessed the impact of Wnt signaling in CTRL as well as MCAO mice, where the differentiated NS/PCs were evaluated 3 days after the induction of FCI. This time point represents an earlier phase of ischemia, and the differentiation of precursor cells has a different outcome in later phases (Li et al., 2010).

Our primary cultures consisted of three cell types that were previously identified in experiments using neonatal mice (Kriska et al., 2016). These cell types comprised of GFAP-positive astrocytes, DCX/MAP2-positive neuron-like cells, and PDGFRα-positive precursor cells (**Figure 2**). The results from immunocytochemical staining against β-catenin in CTRL cell cultures (**Figure 3**) also resembled those obtained in neonatal animals, showing an elevated expression of the protein after Wnt signaling activation (Ex3), and only a decreased expression of β-catenin after inhibition by secreted Wnt signaling antagonist Dkk1. Additionally, the inhibition of Wnt signaling in the cell nucleus (dnTCF4) did not affect the levels of the β-catenin protein, which is in accordance with the fact that the alteration in the pathway occurs downstream of the β-catenin destruction complex. Nevertheless, the results from neonatal mice were more pronounced, which could be partially attributed to the transition from the neonatal to adult NS/PCs niche, with possible changes in the responsiveness to intrinsic or extrinsic stimuli (Morrison and Spradling, 2008; Urbán and Guillemot, 2014). The exact moment when the switch from embryonic to adult neurogenesis occurs is elusive, but it has been suggested that it is between the first and the third postnatal week in the rodent dentate gyrus (Pleasure et al., 2000).

Wnt signaling manipulation altered the passive membrane properties, as well as the expression of K^+ channels in differentiated adult NS/PCs. We identified DCX/MAP2-positive neuron-like cells that showed the highest K_A and K_{DR} current

densities (**Tables 6**, **7**), which were comparable with previous studies from our laboratory (Prajerova et al., 2010). Moreover, we only characterized GFAP-positive astrocytes with very low densities of outwardly as well as inwardly rectifying K^+ currents (**Tables 2**, **3**). We observed decreased incidence of cells showing K_{IR} currents in NS/PCs-derived astrocytes following MCAO. Interestingly, a downregulation of K_{IR} channels was also identified in other types of neuropathologies (Bataveljić et al., 2012). K_{IR} expression is characteristic of mature astrocytes, and shifts these cells into the quiescent stages of the cell cycle (Higashimori and Sontheimer, 2007). These findings might signify that after the induction of FCI, astrocytes dedifferentiate and increase their proliferative activity. The other cell type represented PDGFRα-positive precursors that were defined by moderate values of K_A, K_{DR}, and K_{IR} (**Tables 4**, **5**), with a sporadic expression of voltage-gated Na^+ channels (data not shown). Similar membrane properties were also observed by Walz (2000). Additionally, we only identified significant changes in the density of Na^+ currents of neuron-like cells in CTRL mice. The inability to detect any significant changes in the cultures derived from MCAO mice could be caused by the overall decreased expression of voltage-dependent Na^+ channels after ischemia (Yao et al., 2002). However, another study identified exactly the opposite effect of ischemia on ion channels expression (Hernandez-Encarnacion et al., 2017). Regardless of the downregulation or upregulation, the effect of ischemic injury could overwhelm the effect of Wnt signaling manipulation in our model.

The incidence of the three cell types after Wnt signaling manipulation was not changed in the intact (CTRL) mice (**Figure 4A**). This could originate from the low responsiveness of these cells to Wnt signaling inhibition/activation, or in case of dnTCF4 and Dkk1 mice, by the relatively low level of the Wnt pathway inhibition caused by a compensatory effect of endogenous WT Tcf4 produced in dnTCF4 transgenic mice (Janeckova et al., 2016), or by the "dilution" of Dkk1 protein (Wu et al., 2008). Another reason for not detecting any changes in the cell incidence in CTRL cultures might also be the continuous, age-related depletion of the NS/PCs pool (Encinas et al., 2011) or the fact that NS/PCs prevail in a quiescent state (Codega et al., 2014). Thus, while Wnt inhibition adds to this BMP- and Notch-induced quiescence, the pathway activation in Ex3 mice might be ineffective in reverting the quiescent state (Urbán and Guillemot, 2014). Moreover, the majority of cells differentiated from NS/PCs undergo apoptosis and are rapidly phagocytized by microglia (Sierra et al., 2010). Interestingly, particular subpopulations of microglia have distinct effects on the differentiation potential of NS/PCs, as one subpopulation promotes astrogliogenesis and the other supports neurogenesis, while both inhibit NS/PCs proliferation (Vay et al., 2018). Nonetheless, the effect of Wnt signaling manipulation observed in neonatal cultures was (partially) reproduced with cells obtained from the adult brain after the induction of FCI. Specifically, Wnt signaling inhibition at the membrane receptor level suppressed neurogenesis (**Figure 4B**). The lower incidence of neuron-like cells corresponded with our observations from Western blotting experiments, where we identified a decrease in the expression of βIIItubulin, a neuronal marker, after Wnt signaling inhibition under ischemic conditions (data not shown). Why the changes in cell incidence were only observed in cell cultures originated from mice that underwent MCAO? The answer might be connected with the fact mentioned previously. Notch and BMP signaling maintain the quiescent (or dormant) state of adult NS/PCs. However, in response to brain ischemia, downregulation of Notch and BMP activity, and concomitant involvement of interferon gamma signaling, induce the activation/priming of NS/PCs (Llorens-Bobadilla et al., 2015). Another puzzling fact is the inconsistency between the effect of the two Wnt signaling inhibitors, dnTCF4 and Dkk1. An explanation could be the lower effectiveness of dnTCF4 in the Wnt pathway suppression and/or the contribution of the TCF/LEF-independent function(s) of β-catenin (Kelly et al., 2011; Valenta et al., 2012).

We obtained a relatively low yield of differentiated cells from adult mice, and this state did not improve even after a longer incubation of NS/PCs in proliferation medium. This phenomenon could be explained by the observation that adult NS/PCs are quiescent (Urbán and Guillemot, 2014). BMP signaling through the type IA receptor, which was identified in non-dividing cells of the adult hippocampus, is responsible for this behavior of precursor cells and active BMP signaling reversibly diminishes the proliferation of NS/PCs, while its inactivation only transiently enhances proliferation, with a subsequent reduction in the number of precursor cells (Mira et al., 2010). However, once young and old NS/PCs are activated, they exhibit similar proliferation and differentiation capacity (Kalamakis et al., 2019). Activated NS/PCs are characterized by the expression of EGFR (Codega et al., 2014). EGF is required for the increased proliferation of SVZ-derived cells (O'Keeffe et al., 2009). At the same time, NS/PCs might interconvert between the quiescent and activated state (Lugert et al., 2010; Codega et al., 2014). To enhance the cell yield, we increased the concentration of EGF in the proliferation medium by 50% (from 20 ng/ml in neonatal cultures to 30 ng/ml in adult cultures). However, to initiate the differentiation process of expanded NS/PCs, we had to omit this mitogenic agent in differentiation medium (Johe et al., 1996), which resulted in relatively low amounts of cells for electrophysiological measurements and other experiments.

The Effect of Wnt Pathway Modulation in Adult NS/PCs Differentiated *in vivo*

Changes in the expression levels of the putative Wnt target genes in response to Wnt pathway modulation did not completely meet our expectations; particularly the upregulation of the gene expression after β-catenin stabilization in the Ex3 mice treated with TAM (**Supplementary Figure 3**). Since a mixture of cells isolated from the adult brain was used for the analysis, our original assumption was that the presence of other cell types "contaminating" the isolates did respond to Wnt signaling modulation differently than NS/PCs (Wallmen et al., 2012). However, subsequent analysis of expression profiles at the single-cell level provided a slightly more "prosaic" explanation. Like other evolutionarily conserved signaling pathways, the Wnt

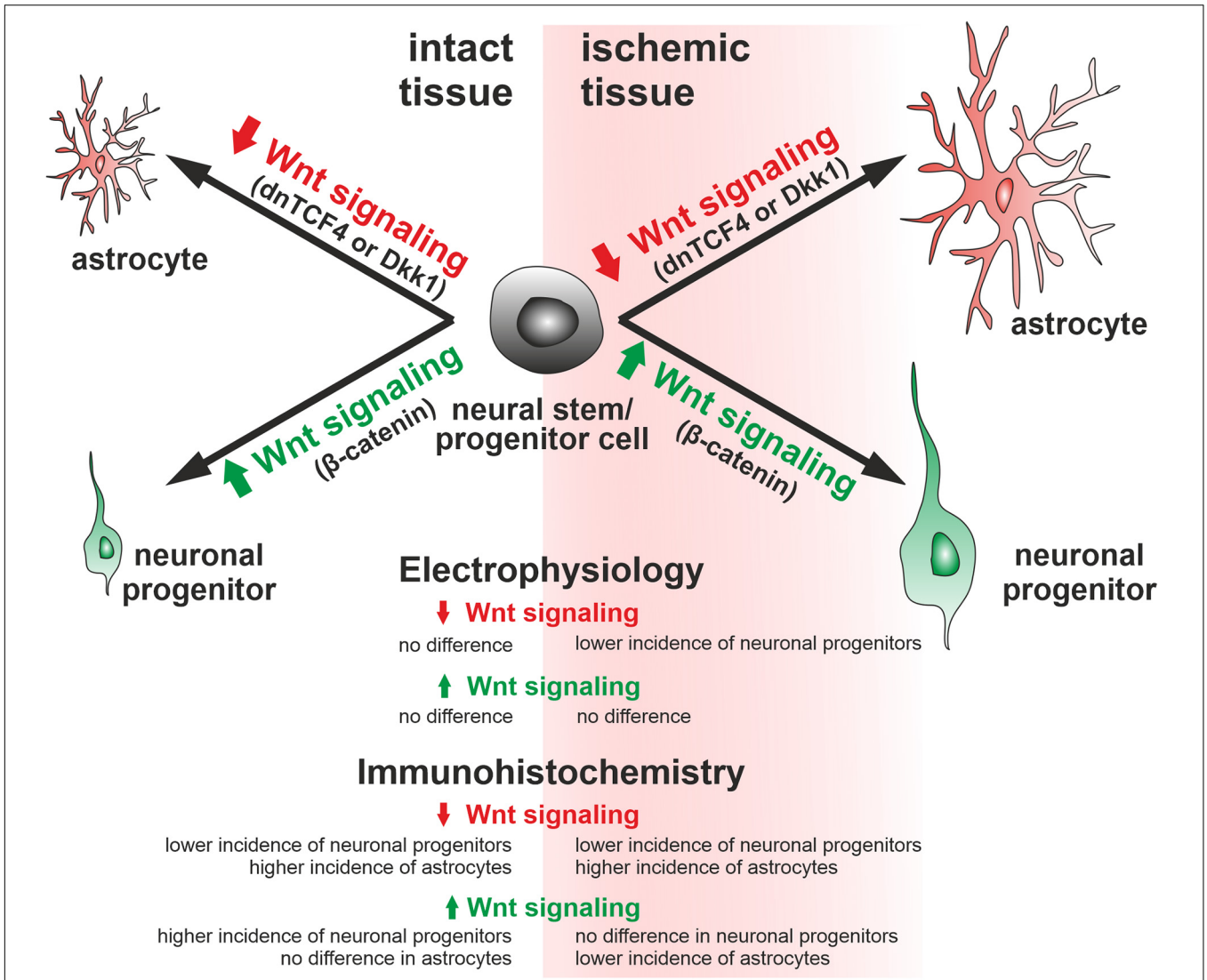

FIGURE 8 | Graphical representation of the changes observed in adult mice. According to our immunohistochemical analyses, Wnt signaling inhibition (dnTCF4 or Dkk1) led to the differentiation of neural stem/progenitor cells to astrocytes, while activation of the pathway (Ex3; constitutively active β-catenin) promoted neurogenesis. A similar impact of Wnt signaling manipulation after ischemia was also confirmed by the patch-clamp technique. Larger cells represent a greater effect of Wnt signaling after ischemia. Dkk1, Dickkopf 1; dnTCF4, dominant negative T-cell factor 4; Ex3, exon 3.

pathway does not always regulate the same group of genes and the transcriptional response to its activation (or inhibition) is thus tissue-specific; although there is a consensus that *Axin2* represents such a "universal" target gene (van de Moosdijk et al., 2020). The analysis of the expression levels of selected genes (*Axin2*, *Nkd1*, and *Troy*) in individual cell subpopulations clearly showed their different expression profile (**Figure 7**). Without experiments showing which target genes in the given brain region respond to Wnt pathway modulation, interpretation of the experiments described above will be complicated.

In order to disclose the differentiation potential of adult NS/PCs *in vivo*, we performed an exhaustive *in situ* immunohistochemical analysis of coronal slices with the SVZ region (**Figure 5**). This approach revealed an overall increased immunoreactivity of selected markers after FCI. Subsequent scRNA-seq showed that this increased positivity is mostly "due" to the cell subpopulations, which also produce the marker in healthy tissue (**Figure 6B**). Shruster et al. (2012) showed that stroke potently stimulates cell proliferation in the neurogenic niches, and that newly generated cells migrate to the injured striatum and cortex, while Wnt signaling promotes the survival of differentiated cells. Furthermore, we documented that Wnt signaling inhibition resulted in the abundance of GFAP in the SVZ, while, conversely, Wnt pathway hyper-activation leads to an overexpression of PCNA and DCX in the CTRL mice. This finding corresponds well with the data obtained from neonatal animals, where Wnt signaling activation, under physiological conditions, promoted neurogenesis at the expense of gliogenesis

(Kriska et al., 2016). Likewise, the neurogenic effect of the Wnt pathway was also observed by Mastrodonato et al. (2018) and Kase et al. (2019), which corroborates our findings from the immunohistochemical analysis.

As already mentioned, the histological analysis of the SVZ showed an increase in GFAP-positive cells after brain injury. However, in the expression profiling at the individual cell level, we observed a decrease in cells in most subpopulations of GFAP-positive astrocytes. How to interpret this discrepancy? One explanation may be the fact that in order to obtain sufficient amounts of viable cells for the scRNA-seq analysis, cells were isolated not only from the SVZ but also from the adjacent striatum. Another possibility is that a subpopulation of GFAP-expressing cells represents proliferative astrocytes that have the potential to dedifferentiate and act as adult stem cells in the neurogenic regions of the adult brain (Seri et al., 2001). We might speculate that these cells still contain detectable amounts of GFAP protein; however, the corresponding transcripts are not detectable at the given depth of sequencing.

Finally, we observed no significant effect of Wnt signaling hyper-activation of the PCNA positivity in the brain after FCI. This fact might be explained by an overall increase in the cell proliferation induced by ischemia (**Figure 6B**). Importantly, scRNA-seq provided a lead for a further analysis of the role of Wnt signaling in the healthy and diseased brain. Wnt ligands are secreted cysteine-rich glycosylated proteins. The posttranslational modifications also include acylation performed by endoplasmic-reticulum-resident acyltransferase porcupine. The modified Wnt molecules are released from the Wnt-producing cell by seven-pass membrane protein Wntless (WLS) (Bänziger et al., 2006). It would be very informative to use a conditional knockout allele of the *Wls* gene (Carpenter et al., 2010) in combination with a suitable Cre deletor strain to study the role of Wnt signaling in the brain tissue recovery.

CONCLUSION

In this study, we assessed the impact of the canonical Wnt pathway modulation on NS/PCs derived from the SVZ of adult mice. We observed that the Wnt pathway modulation had no effect on *in vitro* differentiated NS/PCs derived from the healthy brain. Nevertheless, when the cells were obtained from the ischemic brain, the Wnt pathway inhibition at the membrane level resulted in fewer neuron-like cells present in the cell cultures. Furthermore, the electrophysiological analysis indicated that blocking Wnt signaling affected the distribution of K^+ and Na^+ channels, and thus influenced the membrane properties of differentiated cells. The immunohistochemical analysis showed increased counts of DCX-, GFAP-, and PCNA-positive cells after the induction of FCI, while the effect of the Wnt pathway activation was more robust in the control, undamaged brain. More specifically, Wnt signaling hyper-activation increased the abundance of neuron-like DCX-positive precursors and proliferating cells, and Wnt pathway inhibition had the opposite effect (**Figure 8**). Our findings might help develop new strategies that ameliorate the negative effects of brain ischemia. Furthermore, it would be beneficial to determine whether active Wnt signaling might affect the course of other neurodegenerative diseases and neurological disorders, such as amyotrophic lateral sclerosis and schizophrenia.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-9935/.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care Committee of the Institute of Experimental Medicine, Czech Academy of Sciences (approval numbers 18/2011, 146/2013, and 2/2017).

AUTHOR CONTRIBUTIONS

MA and VK designed the experiments and supervised the project. MA, VK, JK, and LJ coordinated the experiments. JK was responsible for the tissue isolation and cell culture preparation. JK, PH, TK, DD, DKo, and OB contributed to the electrophysiological studies. JK, LJ, and OB acquired and analyzed the data from the immunochemical experiments. MC created and described the script for cell counting. DKi and PH performed the MCAO. MV carried out the Western blotting experiments. LJ performed and analyzed the scRNA-seq and the RT-qPCR experiments. LJ and VK supervised the breeding of the transgenic mouse strains. MT and ZK provided the $Catnb^{lox(ex3)}$ mice. JK and LJ wrote the manuscript. MA, VK, and TK revised the manuscript. All authors read and approved the final version of the manuscript.

FUNDING

This study was supported by the Czech Science Foundation (Grant Numbers 21-24674S, 19-02046S, and 19-03016S) and the Charles University Grant Agency (Grant Number 330119), and this publication is also a result of the "Advanced Bioimaging of Living Tissues" project (registration number CZ.2.16/3.1.00/21527) financed from the budget of the European Regional Development Fund and public budgets of Czechia through the Operational Programme Prague – Competitiveness. The Light Microscopy Core Facility of the Institute of Molecular Genetics of the CAS, v.v.i. was supported by MEYS (LM2015062) and OPPK (CZ.2.16/3.1.00/21547). None of these funding sources had any role in the design of the study, in the collection, analysis and interpretation of the data, in the writing of the report, or in the decision to submit this article for publication.

ACKNOWLEDGMENTS

We would like to express our gratitude to Marketa Hemerova and Helena Pavlikova for their technical assistance and to Frances Zatrepalkova for her helpful comments and suggestions. We also thank Sarka Kocourkova and Jan Kubovciak for scRNA-seq, Linda Berkova for bioinformatics analysis, and Matyas Sima and Zdenek Cimburek for cell sorting. The content of the manuscript has been used as part of a dissertation thesis, which can be found at the Thesis repository of Charles University (https://is.cuni.cz/webapps/zzp).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2021.628983/full#supplementary-material

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

frontiers in Cellular Neuroscience

REVIEW
published: 26 February 2021
doi: 10.3389/fncel.2021.628115

Traditional Chinese Medicine Monomers: Novel Strategy for Endogenous Neural Stem Cells Activation After Stroke

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Specialty section:
This article was submitted to Cellular Neurophysiology, a section of the journal Frontiers in Cellular Neuroscience

Received: *11 November 2020*
Accepted: *19 January 2021*
Published: *26 February 2021*

Citation:
Wang J, Hu J, Chen X, Lei X, Feng H, Wan F and Tan L (2021) Traditional Chinese Medicine Monomers: Novel Strategy for Endogenous Neural Stem Cells Activation After Stroke. Front. Cell. Neurosci. 15:628115. doi: 10.3389/fncel.2021.628115

Stem cell therapy, which has become a potential regenerative medical treatment and a promising approach for treating brain injuries induced by different types of cerebrovascular disease, has various application methods. Activation of endogenous neural stem cells (NSCs) can enable infarcted neuron replacement and promote neural networks' regeneration without the technical and ethical issues associated with the transplantation of exogenous stem cells. Thus, NSC activation can be a feasible strategy to treat central nervous system (CNS) injury. The potential molecular mechanisms of drug therapy for the activation of endogenous NSCs have gradually been revealed by researchers. Traditional Chinese medicine monomers (TCMs) are active components extracted from Chinese herbs, and some of them have demonstrated the potential to activate proliferation and neurogenesis of NSCs in CNS diseases. Ginsenoside Rg1, astragaloside IV (AST), icariin (ICA), salvianolic acid B (Sal B), resveratrol (RES), curcumin, artesunate (ART), and ginkgolide B (GB) have positive effects on NSCs *via* different signaling pathways and molecules, such as the Wingless/integrated/β-catenin (Wnt/β-catenin) signaling pathway, the sonic hedgehog (Shh) signaling pathway, brain-derived neurotrophic factor (BDNF), nuclear factor erythroid 2-related factor 2 (Nrf2), and heme oxygenase 1 (HO-1). This article may provide further motivation for researchers to take advantage of TCMs in studies on CNS injury and stem cell therapy.

Keywords: stroke, neural stem cells, traditional Chinese medicine monomers, neuroregeneration, central nervous system

Abbreviations: AD, Alzheimer's disease; ART, artesunate; AST, astragaloside IV; BBB, blood-brain barrier; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; ERK, extracellular regulated kinases; ESCs, embryonic stem cells; GB, ginkgolide B; I/R, ischemia/reperfusion; ICA, icariin; IL, interleukin; iPSCs, induced pluripotent stem cells; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MCAO, middle cerebral artery occlusion; MSCs, mesenchymal stem cells; NGF, nerve growth factor; NSCs, neural stem cells; NSPCs, neural stem/progenitor cells; OGD, oxygen-glucose deprivation; PI3K, phosphatidylinositide 3 kinase; PNS, panax notoginseng saponins; PSCs, pluripotent stem cells; RES, resveratrol; Sal B, salvianolic acid B; TCMs, traditional Chinese medicine monomers; Wnt/β-catenin, wingless/integrated/β-catenin.

INTRODUCTION

Almost 15 million people are affected by stroke every year, and because most are left disabled, stroke is a leading cause of disease burden worldwide (Johnston et al., 2009). Stroke, which is a sudden interruption of the blood supply to the brain, leads to neurological deficits, and the most effective treatments can only be applied in fewer than 20% of patients (Ojaghihaghighi et al., 2017). A series of pathophysiological changes appear after blood supply interruption, including local oxygen free radical and reactive oxygen species generation, cerebral edema, neuroinflammation, local inflammatory cell infiltration, and blood-brain barrier (BBB) destruction, resulting in irreparable neural disruption (Khoshnam et al., 2017; Datta et al., 2020). Neural stem cells (NSCs) can be activated, differentiate into different cell types, and migrate to ischemic lesions to promote postinjury repair through nutritional support, inflammatory response regulation, and functional restoration, indicating that NSCs are prime candidates for use in regenerative medicine (Grochowski et al., 2018). In the basic and clinical stroke research fields, stem cell therapy is considered to be a promising regenerative medical treatment and a promising approach for stroke (Sarmah et al., 2018). Among all the different methods of stem cell therapy, transplantation of exogenous stem cells and activation of endogenous or dormant stem cells are the primary safe and effective candidate strategies for stroke treatment (Yasuhara et al., 2020).

A variety of transplantable cell types, including embryonic stem cells (ESCs), adult tissue-derived stem cells, gene-edited stem cells, and induced pluripotent stem cells (iPSCs), have been used to generate both protective and regenerative effects in the stroke brain in laboratory studies. Tornero et al. (2017) transplanted iPSCs into stroke-injured adult rat cortices and observed improvements in neurological deficits and enhancement of the morphological development of both afferent and efferent nerves as well as their functional connections with host cortical neurons (Oki et al., 2012). Furthermore, grafted neurons can integrate into adult host neural networks and even into the brain's neural circuitry (Grønning Hansen et al., 2020; Palma-Tortosa et al., 2020). Researchers have adapted intravenous (IV) administration of autologous mesenchymal stem cells (MSCs), which has been studied extensively in animal models, for use in patients with ischemic stroke, and positive clinical outcomes have been achieved after stroke onset (Díez-Tejedor et al., 2014). However, successful transplantation of exogenous stem cells is still hindered by several technical and logistical problems. The specific cell type used is the principal concern due to ethical issues, and researchers must also consider the source as well as the location, dosage, route, and timing of administration (Fernández-Susavila et al., 2019; Kawabori et al., 2020). A series of conditions following a stroke, including neuroinflammation, and immune response activation not only seriously affect the survival of grafted NSCs but also facilitate their differentiation into glial cells; the potential tumorigenicity of transplanted cells is also a concern (Ideguchi et al., 2008; Datta et al., 2020). Microglia in the proinflammatory state may restrain phagocytes from clearing toxic debris and secreting tissue-repairing neurotrophic factors while contributing to neuronal cell death or further BBB disruption or impairing axonal regeneration (Lyu et al., 2020). Besides, it would be costly and labor-intensive to generate autologous iPSCs for personalized medicine in practice, and such a strategy would introduce the possibility of genetic defects that would reduce the therapeutic efficacy (Bang and Kim, 2019).

Therefore, activation of endogenous NSCs, with benefits including improvements in proliferation, directional migration, and differentiation, could be a more practical therapeutic approach to decrease stroke damage without the above disadvantages. The therapeutic effects of this approach can be achieved through nutritional support, regulation of the inflammatory response, directional replacement of neurons, reconstruction of neural circuits, restoration of neural function, and paracrine signaling involving nerve growth factor (NGF; Bain et al., 2013; Yu et al., 2016). Endogenous NSCs, which are abundant in the subgranular zone of the hippocampus and the subventricular zone of the germinal region of the brain, can differentiate into various types of cells to promote structural and functional repair of the brain and spinal cord tissue (Daynac et al., 2016; Song et al., 2018). However, the disastrous changes in the cellular microenvironment after stroke due to BBB damage, excitatory toxicity and neuroinflammation impact the survival, neurogenesis, and differentiation of NSCs (Zhong et al., 2021). The endogenous NSCs tend to differentiate into gliocytes rather than neurons, and the majority of them fail to reach the lesioned cortex. Therefore, developing an efficient and safe strategy to activate the transformation of endogenous NSCs into newborn neurons seems to be significant (Parent et al., 2002; Kreuzberg et al., 2010).

The use of traditional Chinese medicines, whether in the form of herbs, formulas, or monomers, to prevent and treat diseases of the central nervous system (CNS) has been a popular research topic for decades and has shown significant curative effects in many cases. A recent meta-analysis analyzed 67 randomized controlled trials and indicated that treatment with traditional Chinese medicines can enhance the clinical rehabilitation of stroke patients and significantly improve the recovery of neurological function to improve patient quality of life (Zhang et al., 2020). The relationship between traditional Chinese medicine treatments and stem cell therapy after stroke has also been investigated, and published studies have shown that these treatments benefit neural regeneration by promoting the proliferation and differentiation of NSCs (Li et al., 2015; Gao et al., 2017). Traditional Chinese medicine monomers (TCMs) are essential drugs that promote activation of endogenous NSCs after stroke. An increasing number of studies have explored the effects of TCMs on stroke. In this review article, we summarized the efficacy and mechanisms of TCMs that have been reported to activate NSCs in stroke (**Table 1**).

GLYCOSIDES

Ginsenoside Rg1

Ginsenoside Rg1 is the most abundant component isolated from Panax notoginseng saponins (PNS), the major bioactive

TABLE 1 | Effect of different traditional Chinese medicine monomers (TCM) on neural stem cells (NSCs) after stroke *in vitro* and *in vivo*.

Types	Sources	Components	Dose	Model	Objects	Pathways	Mechanisms	Stroke outcomes	References
Glycosides	*Panax notoginseng* (Burk) F. H. Chen	Ginsenoside Rg1	0.32 μg/ml	OGD	*In vitro*	None	Proliferation ↑ Differentiation ↑	NA	Gao et al. (2017)
			10, 20 μM/L	OGD	*In vitro*	Caspase 3 and Bax ↓ Bcl-2 ↑ P38 and JNK2 phosphorylation ↓	Apoptosis ↓ Oxidative stress ↓	NA	Li et al. (2017)
	Astragalus membranaceous (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao	Astragaloside IV	40 mg/4 ml once daily for 14 days	MCAO	Rat	BDNF-TrkB signaling pathway ↑	Proliferation ↑ Migration↑ Differentiation ↑ Maturation↑	Neurological behavioral deficits ↓ Infarction volume ↓	Ni et al. (2020)
			200 mg/kg *in vivo* 20 μM, *in vitro*	MCAO/Primary cell	*In vitro*/ Mouse	PI3K/Akt pathway↑ Wnt/β-catenin pathway ↑ IL-7 ↓	Apoptosis ↓ Proliferation ↑ Differentiation ↑	Anxiety-like behavior ↓	Sun et al. (2020)
Polyphenols	*Epimedium brevicornu* Maxim	Icariin	100 nM/L	None	*In vitro*	ERK	Proliferation ↑	NA	Huang et al. (2014)
			50, 100 μM/L	None	*In vitro*	Cyclin D1 and p21 ↑	Proliferation ↑	NA	Fu et al. (2018)
	Salvia miltiorrhiza Bunge (Lamiaceae)	Salvianolic acid B	50 μM/L once daily for 2 weeks	None	*In vitro*	PI3K/Akt signal pathway	Proliferation ↑	NA	Zhuang et al. (2012)
	Extract of *Veratrum grandiflorum*	Resveratrol	5 μmol/L	OGD	*In vitro*	SOD, Nrf2, HO-1, and NQO1↑	Apoptosis ↓ Oxidative stress ↓ Proliferation ↑	NA	Shen et al. (2016)
			5 μmol/L	OGD	*In vitro*	Shh signaling pathway ↑	Proliferation	NA	Cheng et al. (2015)
	Extract of *Curcuma longa* L	Curcumin	20 mg/kg, *in vivo* 0.5 μM, *in vitro*	None	*In vitro*/Rat	Wnt/β-Catenin Signaling pathway ↑	Proliferation ↑ Differentiation ↑	NA	Tiwari et al. (2014)
			1, 5 mg/ml	ICH	Rat	None	Oxidative stress ↓	Neurological behavioral deficits ↓ Hematoma size ↓	Marques et al. (2020)
Terpenoids	Extrcat of *Artemesia annua*	Artesunate	150 mg/kg, *in vivo* 0.8 μmol/L, *In vitro*	OGD/MCAO	*In vitro*/ Mouse	PI3K/Akt/FOXO-3a/p27Kip1 signaling pathway	Proliferation ↑	Neurological behavioral deficits ↓ Infarction volume ↓	Zhang et al. (2020)
	Ginkgo biloba L	Ginkgolide B	20 mg/kg, *in vivo* 40 and 60 mg/L, *in vitro*	MCAO	Rat	BDNF↑	Proliferation ↑ Differentiation ↑	Neurological behavioral deficits ↓	Zheng et al. (2018)

↑, *up-regulation;* ↓, *down-regulation.*

components extracted from the root of *Panax notoginseng* (Burk) F. H. Chen, can significantly reduce infarction volume and alleviate neurological deficits caused by cerebral ischemia/reperfusion (I/R; Zeng et al., 2014). Yang et al. (2020) speculated that the mechanisms of their neuroprotective effects could be related to anti-oxidative stress effects, anti-inflammatory and anti-apoptotic effects, promotion of BBB repair, and prevention of calcium overload. Furthermore, PNS are the primary ingredients of PNS tablet, Xuesaitong Soft Capsule, Xuesaitong Injection, and Xueshuantong Injection, all of which have been used in the treatment of acute ischemic stroke for many years in China (Yao et al., 2011; Yang et al., 2013; Li et al., 2018). Clinical research has suggested that Xuesaitong Soft Capsule relieves unstable angina with few side-effects (Yang et al., 2013). Li et al. (2012) included 30,884 cases in a new systematic post-marketing safety surveillance study to re-evaluate Xuesaitong Injection and found that it is safe for clinical use. Also, this medicine upregulates the expression of brain-derived neurotrophic factor (BDNF), a critical factor that promotes the repair and reconstruction of neurons associated with learning and memory and inhibits Tau protein phosphorylation in Alzheimer's disease (AD; Li et al., 2012). BDNF and its receptor tyrosine kinase receptor B (Trk B), have been proven to be closely related to the regulation of hippocampal neuron survival, nerve repair, and regeneration. Some studies have drawn attention to the association between serum BDNF and functional outcomes after stroke, but there seems to be little related evidence (Luo et al., 2019).

The effects of ginsenoside Rg1 in stem cells have been demonstrated. Ginsenoside Rg1 can dose-dependently promote the expression of neural cell adhesion molecule (NCAM) and synapsin-1 (SYN-1) to improve the cell proliferation and neural phenotype differentiation of both human and mouse adipose-derived stem cells (Xu et al., 2014; Dong et al., 2017). Rg1 has positive effects on the proliferation and differentiation of endogenous NSCs in AD rats and promotes the functional expression and maturation of human NSCs (Jiang et al., 2012; Zhao et al., 2018). Extracellular regulated kinases 1 and 2 (ERK1 and ERK2), which mediate neuroinflammation, may be vital molecular targets involved in Rg1-induced NSCs differentiation, and the effects of Rg1 on NSCs proliferation are most closely related to mechanisms involving the Hes1, cyclic adenosine monophosphate (CAMP)-protein kinase A (PKA) and phosphatidylinositol 3 kinase (PI3K)-AKT signal transduction pathways (Zhuang et al., 2009; Li et al., 2012; Zhao et al., 2012). As an effector of Notch signaling, which can improve the maintenance of NSCs during development, Hes 1 plays an important role in NSCs proliferation besides its role in astrogenesis (Kageyama et al., 2020). Metabolic abnormalities increase the risk of stroke, and reduce recanalization rates and increase the risk of hemorrhage, making tissue-type plasminogen activator (tPA) treatment potentially hazardous for stroke patients with diabetes mellitus or poststroke hyperglycemia (Jiang et al., 2020; Zhou et al., 2020). In mice subjected to middle cerebral artery occlusion (MCAO), administration of ginsenoside Rg1 was found to attenuate energy undersupply and amino acid and lipid metabolic disturbances and thereby to ameliorate neurological deficits and cerebral infarcts; however, slight differences were found between ginsenoside Rg1 treatment alone and combined NSCs transplantation and ginsenoside Rg1 treatment (Gao et al., 2020). Grafted ginsenoside Rg1-induced NSCs improved the learning and memory behavior in rat neonatal hypoxic-ischemic encephalopathy models. However, the isolated effects of ginsenoside Rg 1 on activating NSCs after stroke were unclear (Li et al., 2015). Increasing evidence has indicated the positive impact of Rg1 on NSC proliferation after oxygen-glucose deprivation (OGD), and glial-like directed differentiation is another effect of Rg1 on NSCs (Gao et al., 2017). Rg1 (10–20 μM) protects NSCs from OGD-induced cell death *via* apoptotic signaling and inhibits p38 and c-Jun N-terminal kinase2 (JNK2) phosphorylation to protect NSCs against oxidative stress (Li et al., 2017). The ginsenoside metabolite 20(S)-protopanaxadiol promotes the transition of NSCs from a state of proliferation to differentiation *via* induction of autophagy and cell cycle arrest, indicating that the process of ginsenoside metabolism may be directly involved in the activation of endogenous NSCs after stroke (Chen et al., 2020).

Astragaloside IV

Astragaloside IV (AST) is purified from the root of *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao and has been widely used in China for the treatment of hepatic, cardiovascular, and renal disorders (Fu et al., 2014). A systematic review performed to elucidate the relationships between AST and neurological disorders has revealed that the positive effects of AST on neurological disorders are due mainly to its anti-edema effect, which reduces lymphocyte infiltration and the numbers of dopaminergic neurons (Costa et al., 2019). Wang et al. (2017) searched eight databases to assess the function and possible mechanisms of AST in experimental stroke. Their investigation demonstrated that AST improves neurological deficits and reduces infarct volumes and BBB permeability and its antioxidant, anti-inflammatory and antiapoptotic properties provided neuroprotection during cerebral I/R injury (Wang et al., 2017).

The possible mechanisms of AST related to the proliferation of NSCs have been gradually revealed. Low-dose (10^{-6}/10^{-7} M) AST increases NSCs proliferation, high-dose (10^{-5} M) AST has no effects on cells, and low-dose of AST promotes NSCs differentiation partly through the Notch signaling pathway *in vitro* (Haiyan et al., 2016). Observed increases in the numbers of BrdU-positive and DCX-positive cells have proven that AST promotes adult neurogenesis in the mouse hippocampal dentate gyrus by regulating signal transduction through the chemokine (chemotactic cytokine)-family protein ligands CXCL1/CXCR *in vivo* (Huang et al., 2018). Chemokines and their receptors are widely expressed in the CNS, and CXCL 2 can enhance the viability of hippocampal neurons. Gao et al. (2018) confirmed that AST can induce the differentiation of NSCs into midbrain dopamine (DA) neurons, possibly through upregulation of sonic hedgehog (Shh), orphan nuclear hormone (Nurr1), and pituitary

homeobox 3 (Ptx3), in Parkinson's disease (PD). Wang et al. (2009) proposed that upregulation of the expression of NGF in the adult hippocampus, which is crucial for the survival and maintenance of neurons after stroke, is related to AST-induced proliferation and the neuronal differentiation of endogenous NSCs after transient forebrain ischemia. In a study conducted by Ni et al. (2020) after MCAO, Sprague–Dawley rats were intragastrically administered AST (40 mg/4 ml/kg, once daily) for 14 days to investigate the effect of AST after stroke. The results showed that AST treatment reduced the infarct volume, improved behavior, increased body weight, and promoted proliferation, migration, differentiation, and maturation of NSCs in the hippocampus by enhancing the BDNF-TrkB signaling pathway (Ni et al., 2020). Sun et al. (2020) discovered that interleukin (IL)-17, a proinflammatory cytokine, is a key effector of AST that regulates NSCs apoptosis and proliferation by upregulating the Wnt/β-catenin and Akt/GSK-3β pathways after the administration of 200 mg/kg AST *via* the tail vein for three consecutive days in MCAO model.

POLYPHENOLS

Flavonoids

Epimedium brevicornu Maxim, a traditional Chinese herb, is usually used to treat impotence, numbness, and osteoporosis (Pei et al., 2008). Icariin (ICA), the principal representative flavonoid of this herb, has shown potential preventive and therapeutic effects in nervous system diseases. ICA treatment accelerates locomotor function recovery, decreases the expression of inflammatory molecules, and enhances the expression of anti-inflammatory proteins after spinal cord injury (SCI), which proves that its protective effects may be induced by its antiapoptotic, antioxidant, and anti-inflammatory bioactivities (Jia et al., 2019). The results of a Morris water-maze test have indicated that ICA attenuates cognitive functional deficits in aging rats (Wu et al., 2012). Xiong et al. (2016) found that pretreatment with ICA reduces neurological deficit scores and infarct volumes through inhibition of inflammatory responses mediated by nuclear factor κB (NF-κB) and the peroxisome proliferator-activated receptor (PPAR) isotypes PPARα and PPARγ. Another PPAR isotype plays essential roles in cell proliferation and differentiation, lipid and glucose metabolism regulation, and inflammatory and oxidative responses. The anti-autophagic and anti-apoptotic properties of ICA protect mouse MSCs from OGD damage (Liu et al., 2020).

After the isolation and characterization of NSCs derived from 16 to 20-week-old human fetuses, Yang et al. (2016) found that low doses (0.1 and 1 μM) of ICA had a minimal effect on the proliferation of NSCs; conversely, a high dose (10 μM) markedly promoted proliferation and regulated related gene expression in human NSCs *in vitro*. Huang et al. (2014) found that treatment with 100 nmol/L ICA promoted the proliferation and neurosphere formation of mouse NSCs induced by ERK but had no effect on the differentiation of NSCs. Also, ICA can cause upregulation of the cell cycle genes cyclin D1 and p21 to affect the growth and proliferation of NSCs (Fu et al., 2018). ICA is also capable of activating quiescent NSCs in the hippocampi of aging (18- to 21-month-old) rats (Wu et al., 2012; Liu et al., 2020). The results of a study by Liu et al. (2018) show that after transient MCAO, combined ICA and MSCs treatment decreases the brain infarct volume, improved neurological motor deficits, and increases the expression of VEGF and BDNF to maximum levels in the hippocampus and frontal cortex through the phosphatidylinositide 3 kinase (PI3K)/ERK1/2 pathway. VEGF can promote endothelial cell regeneration and new blood vessel formation to protect learning and memory. ICA promotes the proliferation and differentiation of β-amyloid protein ($A\beta_{25-35}$)-treated hippocampal NSCs *via* the BDNF-TrkB-ERK/Akt signaling pathway, indicating that ICA has a positive effect on NSCs in CNS diseases (Quan et al., 2020). A growing number of researchers have paid attention to the applications of ICA in stem cell therapy because of the beneficial effects of this compound.

Phenolic Acids

Salvianolic acid B (Sal B) is a water-soluble product of *Salvia miltiorrhiza* Bunge (Lamiaceae) known as a treatment for cardiovascular and cerebrovascular diseases (Zhou et al., 2005). It is an ingredient in many formulas for stroke, such as Salvianolate Lyophilized Injection, Naoxintong, and *Salviae miltiorrliza*-borneol Jun-Shi coupled-herbs (Chen et al., 2016; Xue et al., 2016; Duan et al., 2019). Sal B reduces the memory impairments induced by cholinergic dysfunction and $A\beta_{25-35}$, and the protective mechanisms may be associated with its antioxidant and anti-inflammatory properties (Kim et al., 2011). In a microglia-neuron coculture system, Sal B was found to inhibit activation of microglia, and low production of nitric oxide (NO), tumor necrosis factor-α (TNF-α), IL-1β, and reactive oxygen species in hippocampal neurons indicated that it had a neuroprotective effect (Wang et al., 2010). Sal B treatment has been proven to be effective in stroke and to attenuate brain injury by reducing infarct volume and brain edema and increasing neurological scores; those effects are accompanied by inhibition of apoptosis and inflammation *via* activation of silent information regulator 1 (SIRT1) signaling, and has been proven to be effective in stroke (Lv et al., 2015). Delayed postischemic treatment with Sal B improves cognitive impairment after stroke in rats (Zhuang et al., 2012).

Multiple studies have focused on the effect of Sal B in stem cells. Sal B (1 μM) induces human ESCs to differentiate into hepatocytes, and the activation of the Wnt/β-catenin signaling pathway and the inhibition of the Notch pathway are involved in this process (Chen et al., 2018). Guo et al. (2010) found that Sal B treatment results in a substantial increase in stem cell proliferation, differentiation into neurons, and self-renewal *in vitro*. A study by Zhuang et al. (2012) reached the same conclusion and showed that the PI3K/Akt signaling pathway might be induced. A series of studies by Shu et al. (2015, 2018) have demonstrated that Sal B can potently not only potently promote iPSC proliferation and differentiation into neurons but also markedly attenuate the apoptotic ratio of iPSC-derived NSCs, possibly through the PI3K/AKT/glycogen synthase kinase 3β/β-catenin (PI3K/AKT/GSK3β/β-catenin) pathway. Thus, Sal B may be useful as an alternative treatment for the activation

of endogenous NSCs. Yan et al. (2020) found that combined application of Sal B and transplantation of MSCs is more effective in degenerated intervertebral disc repair than Sal B treatment or MSCs transplantation alone. However, as mentioned before, the effect is dose-dependent, and the cost is high.

Others

Resveratrol

Since it was first isolated from *Veratrum grandiflorum* in 1940, resveratrol (RES or 3,5,4′-trihydroxystilbene) has been reported to be an effective extract of many foods and Chinese herbs (Gambini et al., 2015). RES, a nonflavonoid polyphenol phytoalexin, has been used in medicinal preparations, such as *munakka* or *darakchasava*, for more than 2,000 years (Paul et al., 1999). It has been reported that RES reverses multidrug resistance in cancer cells and sensitizes cancer cells to standard chemotherapeutic agents when combined with clinically used drugs (Ko et al., 2017). A randomized, double-blind placebo-controlled phase II trial of RES in patients with mild to moderate AD including a total of 104 participants clearly showed that RES was detectable at a safe and well-tolerated concentration in the cerebrospinal fluid, indicating the feasibility of using RES to treat neurodegenerative diseases. Furthermore, RES modulates the CNS immune response by facilitating the activation of microglia/macrophages (Sawda et al., 2017). Besides, RES has been shown to have a positive effect against stroke by upregulating the antioxidative stress molecule Nrf2, proving the antioxidative activity of this compound (Shen et al., 2016).

Consistent with the multiple effects of RES, researchers have found that different RES working concentrations and treatment times can lead to the opposite effects in pluripotent stem cells (PSCs). Li et al. (2017) found that 50 or 500 nM RES maintained the pluripotent state of mouse ESCs after cell differentiation and that the kinase/signal transducers and activators of transcription 3 (JAK/STAT3) signaling pathway were activated, whereas the mammalian target of rapamycin (mTOR) signaling pathway was suppressed, to reinforce the self-renewal of mouse ESCs. However, Park et al. (2012) reported that when 20 and 50 μM RES were added to the stem cell culture medium, the proliferation of neural stem progenitor cells (NSPCs) was inhibited, and the proliferation of self-renewing cells in the hippocampus was reduced. Treatment with 10 μM RES delays entry into the S phase of the cell cycle and affects the mitochondrial metabolism of mouse ESCs, and adenosine monophosphate-activated protein kinase, and Unc-51-like autophagy activating kinase 1 (AMPK/ULk1)-dependent autophagy enhance the pluripotency of ESCs (Suvorova et al., 2019). RES (10 mM) maintains the stemness characteristics of human ESCs and promotes their proliferation through SIRT1/ERK signaling but did not influence the apoptotic rate of the cells (Safaeinejad et al., 2017). RES also promotes the long-term survival and neuronal differentiation of human NPCs in the rat brain (Yao et al., 2020). In one study, various concentrations of RES (1–100 μmol/L) were added to the culture medium, and the finding that there were more BrdU-positive cells in the 5 μmol/L group than in the OGD group demonstrated that RES increased the proliferation of NSCs after OGD.

Further research by Cheng et al. (2015) has suggested that the Shh signaling pathway mediates this renewal process. An experiment with a similar experimental design and dose was performed by Shen et al. (2016) but the researchers selected antioxidative stress-related signaling as an interventional target. The results proved that RES protected NSCs from apoptosis, increased the proportions of NSCs in the S and G2/M phases, and significantly upregulated the expression levels of Nrf2, heme oxygenase 1 (HO-1), and NAD(P)H: quinone oxidoreductase 1 (NQO1) after ODG, indicating that RES is capable of positively regulating NSCs in stroke (Shen et al., 2016).

Curcumin

Curcumin is the most abundant phytoconstituent among the curcuminoids and is extracted from the powdered rhizomes of *Curcuma longa* L (Priyadarsini, 2014). Curcumin is well known for its anti-inflammatory, anti-mutagenic, anti-carcinogenic, antimicrobial, antifibrotic, and antioxidant actions and has several effects on the CNS. The antitumor effect of curcumin is due to its inhibition of the growth, cell cycle progression, migration, and invasion of glioma cells, and most of these effects are remarkably dependent on mTOR-dependent ATG induction (Ryskalin et al., 2020). Curcumin has been reported to inhibit most activities of Aβ, including aggregation and Aβ-induced inflammation, *in vitro*, and oral administration of curcumin can improve behavioral impairments, inhibit Aβ oligomerization and deposition, and inhibit tau phosphorylation in the brains of AD animal models (Hamaguchi et al., 2010). Curcumin exerts protective effects against I/R injury by inducing the oxidative stress response, leukocyte infiltration, complement activation, and mitochondria-mediated mechanisms and protecting the BBB against disruption (Bavarsad et al., 2019).

It has been proven that neurogenesis in the context of stem cell therapy is another critical area in which curcumin functions. Kim et al. (2008) found that administration of 0.1 and 0.5 μM curcumin *via* direct dilution into the culture medium of hippocampal NSCs promoted NSC proliferation, whereas 10 μM curcumin had the opposite effect, indicating that the dose of curcumin is a crucial factor affecting the proliferation of NSCs. Also, Son et al. (2014) cultured spinal cord NSPCs with curcumin and reached the same conclusions regarding the dose of curcumin and the MAPK signaling pathway-mediated proliferation of NSCs. However, curcumin has some shortcomings, such as low absorption and metabolism, low water solubility, and rapid excretion from the blood circulation. Tiwari et al. (2014) applied curcumin-encapsulated PLGA nanoparticles (Cur-PLGA-NPs) to prove the positive influence of curcumin on adult neurogenesis. The results showed that even as little as 0.001 μM bulk curcumin promoted NSCs proliferation *in vitro* and that activation of the canonical Wnt/β-catenin pathway was involved in this process (Tiwari et al., 2014). Nanoemulsified curcumin could improve neurological behavioral deficits, decrease the size of hematoma and elicit antioxidant responses without exhibiting

cytotoxicity in the liver and kidney after stroke (Marques et al., 2020). The water-insolubility and low bioavailability of curcumin may impose restrictions on its passage through the BBB, but biocompatible formulations of organic substances and novel metal and oxide nanoparticles can be developed to increase its ability to pass through the BBB. Curcumin has the potential to play a more practical role in stem cell therapy after stroke due to its effects on other nervous system diseases.

TERPENOIDS

Artesunate

Artemisinin is a sesquiterpene lactone peroxide isolated from the leaves of the shrub *Artemesia annua* that is usually applied as an anti-malaria drug (Mohammadi et al., 2020). Artesunate (ART), also known as dihydroartemisinin-12-α-succinate, is a semisynthetic antimalarial compound derived from artemisinin that has high efficacy, solubility, and bioavailability and can be administered in many ways, including *via* the IV, intramuscular (IM), oral and rectal routes (Morris et al., 2011). Because of the superior qualities of ART, many of its effects have been investigated. ART can significantly induce cancer cell apoptosis, inhibit glioma cell growth and migration, and influence glioma cell metabolism (Wei et al., 2020). Gugliandolo et al. (2018) proved that ART can ameliorate traumatic brain injury (TBI)-induced lesions through its anti-inflammatory activity, and its protective effects are mediated through modulation of neurotrophic factors that play crucial roles in neuronal survival. Treatment with 200 mg/kg ART has been found to improve the modified Garcia score and decreased the brain water content in an animal model of subarachnoid hemorrhage (SAH). Moreover, ART treatment protects the BBB through activation of sphingosine-1-phosphate receptor-1 (S1P1), enhancement of PI3K activation, and stabilization of β-catenin in SAH mice (Zuo et al., 2017). *Via* immunofluorescence staining, Zhang et al. (2020) found remarkably enhanced numbers of cells in the G2/S phase and proliferation among NSPCs treated with 0.8 μmol/L ART. Also, PI3K/Akt/FOXO-3a/p27/Kip1 signaling is involved in the effects of ART after OGD *in vitro* and MCAO *in vivo*, and motor function recovery data indicate that ART may be a potential therapeutic agent for ischemic stroke treatment (Zhang et al., 2020). The inhibitory effect of ART on the proliferation of tumor cells is noticeable; however, a specific dose of ART is capable of promoting cell cycle progression in NSCs. Numerous studies have proven the protective effect of ART so that we can anticipate its therapeutic influence in the context of transplantation of NSCs after stroke. We will carry out further work in animal models and clinical trials to investigate and verify the direct effects of ART on NSCs after a stroke.

Ginkgolide B

Ginkgo biloba extracts (GBE), a classic popular herbal product isolated from *Ginkgo biloba* L., has been reported to have neuroprotective and antioxidant effects in cognitive disorders and AD (Singh et al., 2019). Ginkgolide B (GB) is a terpene lactone component of GBE that shares its characteristics. GB exhibits beneficial antiosteoporotic effects on osteoblasts throughout the differentiation process *via* activation of the Wnt/β-catenin signaling pathway (Zhu et al., 2019). In a focal I/R model, IV administration of GB (20 and 40 mg/kg) has been demonstrated to cause marked reductions in infarct volumes, brain edema, and neurological deficits; furthermore, it inhibits I/R-induced NF-κB and microglia activation and proinflammatory cytokine production, which demonstrates the anti-inflammatory and antiapoptotic activities of GB (Gu et al., 2012). Due to the brain-protective effect of GB, Fang et al. (2010) suggested that the most appropriate therapeutic window for GB was within 4 h in a rat MCAO model; also, their data suggested that the BBB permeability of GB was increased after I/R given to its elevated concentration. Intraperitoneal injection of GB (20 mg/kg) decreased the neurological deficit score after MCAO (Zheng et al., 2018).

In addition to the neuroprotective effects of GB against CNS damage, it is essential to reveal the underlying mechanisms of its effects related to stem cell therapy. Zhu et al. (2019) found that 20 μM GB did not activate the proliferation of rat bone MSCs, however, the promotion of differentiation was positively correlated with the dose of GB. Research has also demonstrated that culturing normal mouse ESCs with GB induces cell apoptosis by influencing reactive oxygen species generation, activating JNK and caspase-3, decreasing mitochondrial membrane potential (MMP), and inhibiting cell proliferation. Furthermore, treatment with 10 mM GB causes the resorption of blastocysts after implantation and leads to decreased fetal weight (Chan, 2006). On the other hand, GB (20 nM) inhibits the proliferation of mouse NSCs derived from the postnatal subventricular zone but promotes NSCs differentiation into neurons through the Wnt/β-catenin pathway (Li et al., 2018). In another study, an NSCs cell line was treated with 40 and 60 mg/L GB, and a shortened cell growth cycle was observed. Mice treated with GB (20 mg/kg) once daily after MCAO exhibited increased proportions of nestin-positive, NSE-positive, GFAP-positive, and BrdU-positive cells, and the mRNA expression of BDNF and epidermal growth factor indicated that GB promoted the proliferation and differentiation of NSCs after MCAO (Zheng et al., 2018).

SUMMARY

Currently, the TCMs research literature includes only a small number of high-quality studies, most of which are necessary studies, and a few clinical articles. However, TCMs could be effective tools to promote the neuroprotection, antiapoptotic differentiation, and proliferation of NSCs after stroke and their clinical safety has been validated by years of study. Different doses of TCMs can lead to opposing effects that may be caused by natural shortcomings such as water insolubility and low bioavailability. Nose-to-brain drug delivery (NBDD) vastly improves the efficacy of TCMs treatments by increasing their bioavailability and absorption rates and avoiding drug-induced gastrointestinal tract irritation. Additionally, biocompatible organic substance formulations and novel metal and oxide

nanoparticles can change medication profiles and reduce the required dose and side effects.

Classical pathways are involved in the effects of TCMs on the proliferation and differentiation of NSCs, including the Notch, PI3K/Akt, Wnt/β-catenin, and MAPK pathways. Molecules, including inflammatory factors, apoptosis factors, BDNF, and NGF are also involved. Studies have proven that almost all of these pathways/molecules mediate the process of CNS damage or support brain homeostasis maintenance. Ginsenoside Rg1 could promote the proliferation, differentiation, and maturity of NSCs through ERK1/2, Hes1 PI3K-AKT pathway, and p38 and JNK2 phosphorylation were under control after the admission of it in stroke models. Notch pathway was involved in AST induced differentiation of NSCs, NGF, IL-7, and BDNF-TrkB pathway took part in proliferation and the neuronal differentiation of NSCs after stroke *in vivo*. ICA promoted the proliferation and neurosphere formation of mouse NSCs induced by ERK but did not affect the differentiation of NSCs. The effect of AST and curcumin on NSCs after stroke was related to the Wnt/β-catenin pathway. PI3K/AKT/GSK3β/β-catenin was involved in the improvement of proliferation and differentiation of Sal B induced NSCs. PI3K/Akt/FOXO-3a/p27/Kip1 signaling was involved in the effects of ART after OGD *in vitro* and MCAO *in vivo*. The effects of TCMs on NSCs are regulated by multiple overlapping and interrelated signaling pathways that jointly constitute a regulatory network affecting neural regeneration. This kind of complex situation is probably related to drastic changes in physiology after lesion development. The interactions of TCMs and signaling pathways regulating the proliferation and differentiation of NSCs need to be further elucidated to clarify the multitarget and multi-pathway mechanisms and the comprehensive regulatory effects.

Large-scale clinical trials have led to a transformation in the clinical use of TCMs. However, issues remain. First, the ability of TCMs to cross the BBB and especially the enrichment rates in NSC pools in the subependymal region after infarction need to be determined quantitatively using high-performance liquid chromatography (HPLC), mass spectrometry, gas chromatography-mass spectrometry (GC-MS), or liquid chromatography-mass spectrometry (LC-MS). Second, specific molecular targets need to be identified. We need to return to the cellular level of stem research to explore the molecular mechanisms of TCMs because of the complexity of their systemic effects and because their neuroprotective effects may originate from sources other than the CNS. Also, studies on TCMs treatment combined with exogenous stem cell implantation are needed to determine whether TCMs can improve the microenvironment of stem cell proliferation after infarction. Such studies may provide new ideas for stem cell transformation therapies.

AUTHOR CONTRIBUTIONS

JW, XZC, and XJL reviewed the databases and analyzed information on subjects. HF, LT, and FW designed this review and worked on the manuscript revision. JW wrote the draft and modified this article. JH revised this article and replied to the reviewers in the modification phase. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by the Macau Young Scholars Program (AM201918), the Chongqing Natural Science Foundation (cstc2019jcyj-msxmX0458) of China, and the Science and Technology Development Fund, Macau SAR (Grant no. 0045/2019/AFJ). The funders had no role in the study design, data collection, or interpretation and had no role in the decision to submit the work for publication.

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

frontiers in Cellular Neuroscience

REVIEW
published: 24 March 2021
doi: 10.3389/fncel.2021.653367

Mesenchymal Stem Cells Transplantation in Intracerebral Hemorrhage: Application and Challenges

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Intracerebral hemorrhage (ICH) is one of the leading causes of death and long-term disability worldwide. Mesenchymal stem cell (MSC) therapies have demonstrated improved outcomes for treating ICH-induced neuronal defects, and the neural network reconstruction and neurological function recovery were enhanced in rodent ICH models through the mechanisms of neurogenesis, angiogenesis, anti-inflammation, and anti-apoptosis. However, many key issues associated with the survival, differentiation, and safety of grafted MSCs after ICH remain to be resolved, which hinder the clinical translation of MSC therapy. Herein, we reviewed an overview of the research status of MSC transplantation after ICH in different species including rodents, swine, monkey, and human, and the challenges for MSC-mediated ICH recovery from pathological microenvironment have been summarized. Furthermore, some efficient strategies for the outcome improvement of MSC transplantation were proposed.

Keywords: intracerebral hemorrhage, mesenchymal stem cells, different species, pathological microenvironment, optimizing strategy

OPEN ACCESS

Edited by:
Haruyuki Kamiya, Hokkaido University, Japan

Reviewed by:
Zhouping Tang, Huazhong University of Science and Technology, China
Haitao Wu, Institute of Basic Medical Sciences, China

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Specialty section:
This article was submitted to Cellular Neurophysiology, a section of the journal Frontiers in Cellular Neuroscience

Received: *14 January 2021*
Accepted: *01 March 2021*
Published: *24 March 2021*

Citation:
Gong Y-h, Hao S-l and Wang B-c (2021) Mesenchymal Stem Cells Transplantation in Intracerebral Hemorrhage: Application and Challenges. Front. Cell. Neurosci. 15:653367. doi: 10.3389/fncel.2021.653367

INTRODUCTION

Intracerebral hemorrhage (ICH) is one of the most devastating and life-threatening neurological diseases, which has led to high disability and mortality worldwide. Although ICH only accounts for ∼15% of all strokes, its mortality rate within 28 days is as high as ∼47%, and the surviving patients up to 25% are at risk of relapse ICH in the next 5 years (Chen Y. et al., 2020). Worse still, less than 12% of patients would have independent living ability at 1 year after ICH (van Asch et al., 2010). The incidence rate of ICH has not decreased with the development of medical and healthcare level, but shows an increasing trend in low- and middle-income countries (Wu and Anderson, 2020). This is probably due to the increased number of elderly people and the use of antiplatelet, anticoagulants, and thrombolytics (Carpenter et al., 2016; Wu et al., 2019). As of yet, considerable progress for ICH treatment has been made in preclinical research, however, there is still a lack of effective therapeutic strategies for the acute and long-term treatment of ICH in clinic, except for active rehabilitation.

Mesenchymal stem cell (MSC) therapy holds significant promise in regenerative medicine research and tissue engineering due to its unique properties, including extensive proliferation capacity, multiple differentiation potential, ease of isolation from various tissues, low

immunogenicity, paracrine activity, immunomodulatory function, and fewer ethical disputes (Zheng et al., 2018). The application of MSC therapies has represented an exciting treatment for stroke, especially for ICH patients suffering from neurological deficits and motor dysfunction. To date, ICH researchers have conducted much pre-clinical and clinical research based on MSC treatment with promising results (Bedini et al., 2018; Gao et al., 2018).

However, the prognostic differences between individuals, the influence of pathological environment on the characteristics of implanted MSCs, and the long-term safety based on MSC therapies are undefined, which is still a non-negligible obstacle to clinical translation. When emphasizing that MSC replacement therapy is a very promising treatment option for ICH, the interaction between the pathological microenvironment of the individual and the implanted MSCs should also be strictly considered. Therefore, this review summarized the application of MSCs in the treatment of ICH in different species (e.g., rodent, swine, monkey, and human) and their mechanism of promoting neurological recovery. Meanwhile, we emphasized the existing challenges faced by MSCs planted into the microenvironment of ICH injured brain tissues, including the impacts of mass effect, iron overload, and oxidative stress. Furthermore, we further discussed the MSC-based potential therapeutic methods that might have an optimized clinical transformation effect in the treatment of ICH.

INJURY MECHANISMS OF ICH

The cascade brain injury initiated by ICH is conventionally described as two consecutive pathological processes. In the first stage of primary brain injury, the resulting hematoma from the ruptured vessel could form a persistent mass effect, and mechanically stretch and compress the brain tissue to cause brain tissue damage. Subsequently, the secondary brain injury was mainly caused by the hematoma and its degradation products (i.e., hemoglobin, heme, and iron), which could induce microglia activation, inflammatory response, oxidative stress, brain edema, and blood-brain barrier (BBB) leakage. The secondary brain injury will further lead to permanent death of brain tissue and severe neurological deficits (Qureshi et al., 2009; Wilkinson et al., 2018).

BACKGROUND OF MESENCHYMAL STEM CELLS

Mesenchymal stem cells represent a versatile class of multipotent stem cells that hold promise to directly regenerate damaged tissues due to their potential of differentiation into almost any end-stage lineage cells (Han et al., 2019). Bone marrow-derived MSCs were the firstly-discovered and extensively studied MSCs, although they also had been isolated and characterized from tissues including adipose, dermis, cord blood, peripheral blood, synovial fluid, umbilical cord, placenta, amniotic fluid, fetal tissues, dental pulp, periosteum, and skeletal muscle (Murphy et al., 2013; Bedini et al., 2018). Various minimally invasive procedures had been used to isolate and expand MSCs *in vitro*, including abdominoplasty, bone marrow aspiration, and placental collection (Sherman et al., 2019). Therefore, MSCs could be obtained in quantity from the patients or third-party donors. These characteristics of easy availability and no ethical concerns make them widely used in regenerative medicine research (Wang et al., 2018). It should be noted that although these cells have many similarities, MSCs still exist with different gene expression patterns, differentiation profile, and clinical application potential according to their origin (Ghanta et al., 2018).

THE PROGRESS OF MSC-BASED THERAPIES IN DIFFERENT SPECIES AFTER ICH

Although the exact mechanism behind MSC-based therapies was still largely ambiguous, numerous studies had been conducted in animals and humans, and some therapeutic effects had been achieved with various therapy mechanisms. However, differences about the focus point and outcomes of MSC treatment in ICH still exist among species. Detailed research findings of MSC-based ICH intervention in different species will be introduced in subsequent sections, and a summary of the relevant studies is reported in **Table 1**.

MSC Transplantation in Rodents

At present, plenty of experimental research has been conducted on the treatment of rodent ICH with MSC transplantation, probably due to the versatility of rodent models. Large amounts of evidence confirmed that MSC transplantation is a promising treatment for neuronal recovery after ICH (Ma et al., 2015; Hu et al., 2016; Zheng et al., 2018). In view of the neurological function improvement effect of MSCs in ischemic rat brain, it was no doubt that the first MSC application in the rat ICH model also showed significant improvement in motor function (Zhang et al., 2006). To investigate the role of MSCs in ICH therapy, BrdU-labeled MSCs were delivered into the brain through carotid artery (CA), cervical vein (CV), or lateral ventricle (LV), respectively, at days 1, 3, 5, and 7 following ICH. Thereafter, BrdU-labeled MSCs were found in the brain of CA and LV delivery groups except CV injection. The majority of labeled MSCs could migrate into the ipsilateral cortex, bleeding area, and hippocampus. Additionally, double staining revealed that MSCs mainly differentiated into neurons in the hippocampus, while MSCs differentiated into neurons and astrocytes around the bleeding area. In the ipsilateral cortex, MSCs could differentiate into neurons, astrocytes, and oligodendrocytes. Taken together, these results demonstrated that the functional improvement of MSCs in ICH was probably related to their ability to differentiate into appropriate cell types. Furthermore, the nestin and doublecortin positive cells had been observed in the subventricular zone (SVZ) and nearby the lesion zone in the ICH animals received MSCs, indicating that the MSC transplantation could also enhance endogenous neurogenesis and differentiation (Otero et al., 2010). Therefore,

TABLE 1 | The application of MSC-based therapy in different species of hemorrhage stroke.

Species (model)	Modeling approach	Amount	Treatment times (postbleed)	Route of treatment	Results	References
Rat (ICH)	Collagenase VII	2×10^6 Cells	1, 3, 5 and 7 days	CA, CV, LV	Differentiated into neurons, astrocytes, and oligodendrocytes in the brain; The limb motor function and behavioral scores of the CA group and LV group were better than ICH-only and CV group	Zhang et al., 2006
Rat (ICH)	Collagenase IV	2×10^6 Cells	3 days	IC	Enhanced the endogenous neurogenesis and differentiation; Reduced the mNSS scores	Otero et al., 2010
Rat (ICH)	Autologous blood	3, 5, or 8×10^6 Cells	1 days	IV	Increased the immature neurons, neuronal migration, and synaptogenesis in the damaged brain region; Reduced tissue loss and NSS scores	Seyfried et al., 2006
Rat (ICH)	Collagenase VII	1×10^6 Cells	1 h	IV	Increased proliferation, neural regeneration, anti-apoptotic molecules, G-CSF and BDNF expression; Reduced apoptosis, hemorrhage volume and mNSS scores	Wang S.P. et al., 2012
Rat (ICH)	Collagenase VII	5×10^5 Cells	1 day	IC	Decreased apoptosis and inflammatory cell infiltration; Enhanced the endogenous neurogenesis, angiogenesis, BDFN and VEGF expression; Reduced the mNSS scores	Zhou et al., 2016
Rat (ICH)	Collagenase IV	5×10^6 Cells	2 h	IV	Decreased apoptosis, brain water content, inflammatory cell infiltration, microglia activation, inflammatory-associated cytokines, BBB permeability, mNSS scores; Upregulated anti-inflammatory cytokines	Chen et al., 2015
Rat (ICH)	Collagenase VII	5×10^5 Cells	2 days	IC	Reduced lesion volume, apoptosis, inflammatory factors Improved neurogenesis, angiogenesis, and behavioral recovery	Kim et al., 2015
Mice (ICH)	Collagenase A	1×10^6 Cells	1 day	IV	Suppressed the acute inflammation; Improved neurological deficits; Reduced the mNSS scores	Kuramoto et al., 2019
Rat (SAH)	Endovascular perforation	3×10^6 Cells	1 h	IV	Reduced brain water content, BBB disruption, neuronal injury, microglia activation, inflammatory cytokines expression Improved neurological function	Liu et al., 2019
Rat (ICH)	Collagenase VII	2×10^5 Flk-1^+ MSCs	1 day	IC	Reduced brain water content, hemorrhage volume, apoptosis, inflammatory cytokines, inflammatory cell infiltration, mNSS scores; Increased angiogenesis	Bao et al., 2013
Rat (ICH)	Collagenase VII	2×10^4 Cells	1 day	IC	Increased angiogenesis, anti-inflammation Reduced apoptosis, injury volume, mNSS scores	Liao et al., 2009
Mice (ICH)	Collagenase VII	3 to 4×10^5 Cells	2 days	IC	Reduced apoptosis, brain water content, AQP4 expression, inflammatory factors, mNSS scores	Zhang Y. et al., 2019
Rat (HICH)	Hemoglobin	1×10^5 Cells	6 h	IC	Increased neuron content; Reduced BBB permeability, microglia activation, apoptosis, pro-inflammatory factors, the edema and mNSS scores.	Ding et al., 2017
Rat (IVH)	Maternal blood	1×10^5 Cells	2 or 7 days	ICV	Decreased posthemorrhagic hydrocephalus, behavioral impairment, apoptosis, astrogliosis, inflammatory cytokines, and increased corpus callosum thickness, myelination at early stage of 2 days but not at 7 days	Park et al., 2016

(Continued)

TABLE 1 | Continued

Species (model)	Modeling approach	Amount	Treatment times (postbleed)	Route of treatment	Results	References
Swine (TBI)	Cortical impact	4×10^{13} particles	9 h and 1, 5, 9, and 13 days	IV	Shortened neurologic recovery time; Reduced the mNSS scores	Williams et al., 2019
Monkey (ICH)	Autologous blood	1 to 5×10^6 Cells	7 or 28 days	IC	Early treatment had more grafted cells uptake in the adjacent cortex and better results compared with later group MSCs treatment; Increased microvessel density and reduced neurologic deficit score	Feng et al., 2011
Human (ICH)	Spontaneous	20×10^6 cells	More than 6 months	ICV	Improved neurological status; Observed no adverse events	Al Fauzi et al., 2016
Human (ICH)	Spontaneous	Mean of 4.57×10^7 cells	1 year	IV	Improved neurological scores; Observed neither adverse event nor sign of de novo tumor development	Tsang et al., 2017
Human (SAH)	Aneurysmal	10×10^7 cells	3 days	IV	The patient achieved a rapid and favorable recovery	Brunet et al., 2019
Human (IVH)	Premature	5 or 10×10^6 cells	7 days	ICV	Decreased pro-inflammatory cytokines, VEGF in CSF; Observed no dose-limiting toxicities, serious adverse effects or mortality	Ahn et al., 2018
Human (ICH)	Spontaneous	1×10^6 cells	NA	CS	Decreased the brain edema, serum nerve injury marker molecules level; Increased the BDNF, NGF, and endothelial progenitor cells level	Zhang, 2016
Human (ICH)	Spontaneous	1.8×10^8 cells	14 days	IC	MSCs therapy had better functional outcomes in 5 years fellow-up Hu-MSCs graft had better outcomes than autologous BMSCs graft	Chang et al., 2016

ICH, intracerebral hemorrhage; SAH, subarachnoid hemorrhage; HICH, hypertensive intracerebral hemorrhage; IVH, intraventricular hemorrhage; TBI, traumatic brain injury; CSF, cerebrospinal fluid; CA, carotid artery; CV, cervical vein; LV, lateral ventricle; IC, intracerebral; IV, intravenous; ICV, intracerebroventricular; CS, cavum subarachnoidale; NSS, Neurological Severity Score; mNSS, modified NSS; NA, not available; G-CSF, granulocyte colony-stimulating factor; BDNF, brain-derived neurotrophic factor; VEGF, vascular endothelial growth factor; NGF, nerve growth factor; BBB, blood-brain barrier; AQP4, aquaporin-4; Hu-MSCs, human umbilical cord-derived mesenchymal stem cells; BMSCs, bone-marrow derived mesenchymal stem cells.

MSC therapy could mediate the transplanted and endogenous neurogenesis to reorganize the lost neuronal circuity after ICH. The observation of immature neurons and decreased tissue loss of striatum further confirmed the organizational restructuring role of MSCs in ICH (Seyfried et al., 2006, 2008).

The molecular mechanisms underlying MSC-mediated neurogenesis behavior was partly attributed to the trophic properties of MSCs, which could secrete neurotrophic and other chemokines to promote transplanted or endogenous stem cells proliferation and differentiation (Murphy et al., 2013; Baker et al., 2019). Previous studies demonstrated that tail vein delivered bone marrow-derived MSCs had increased the expression of brain-derived neurotrophic factor (BDNF), which was a crucial growth factor for the growth and differentiation of central nervous systems (Wang S.P. et al., 2012). Knockout of BDNF in MSCs had significant suppression in the effective treatment results of transplanted cells in intraventricular hemorrhage (IVH) (Ahn et al., 2017). Vascular endothelial growth factor (VEGF), which was closely related to angiogenesis, was also significantly upregulated in ICH brain tissues with transplanted MSCs (Zhou et al., 2016). In addition, glial cell-derived neurotrophic factor (GDNF) was also recognized as an important neurotrophic factor in the growth, differentiation, development, maintenance, and injury repair for several types of neurons. Not only transfection but also drug-induced increase of GDNF could significantly enhance the functional improvement of grafted MSCs on ICH (Yang et al., 2011; Lee et al., 2015; Deng et al., 2019). Furthermore, delivered hypoxia-preconditioned MSCs could reduce the tissue loss of the ipsilateral striatum, and enhance neuroregeneration and neurological functional recovery after ICH, which was attributed to the neuronal nourish and protect effect of MSC-mediated upregulation of GDNF, VEGF, and BDNF (Sun et al., 2015).

Neuronal cells insult caused by the primary brain injury and secondary brain injury after ICH was mainly located around the hematoma. Therefore, whether endogenous stem cells or exogenous stem cells could migrate to the perihematoma was a key factor for neuronal remodeling. To promote cells viability and migration, platelet-rich plasma (PRP)-derived scaffold was employed to deliver MSCs, and the contained fibronectin, fibrin, and adhesive proteins in the scaffold were expected to augment cell interaction and promote cell migration. Apparently, (PRP)-derived scaffold increased the biologic activity of donor cells and its integration in the injured tissue (Vaquero et al., 2013).

Although the potential neural recovery mechanism of MSCs in ICH is still largely undefined, apparently, it is not limited to the differentiation and remodeling of brain tissue. In fact, mechanisms involved in tissue repair, such as neurogenesis, angiogenesis, anti-inflammation, and anti-apoptosis, were all suggested to be associated with the functional recovery effect of transplanted MSCs in ICH (Chen et al., 2015; Kim et al., 2015; Xie et al., 2016; Kuramoto et al., 2019; Liu et al., 2019). It was well characterized that ICH-induced inflammation response was a key factor in secondary brain injury, which involved microglia activation, and infiltrating neutrophils release could induce BBB breakdown, vasogenic edema, and apoptosis of glia and neurons (Keep et al., 2012). Thus, a previous study had proposed a hypothesis that the behavioral recovery improved by MSC therapy might derive from the inhibition of inflammation cascade in ICH. In order to verify this hypothesis, Flk-1^+ MSCs was introduced for ICH (Bao et al., 2013). Results showed that Flk-1^+ MSCs-treatment dramatically decreased the neurons apoptosis, brain water content, inflammatory cells expression (e.g., microglia and neutrophils), and the mRNA levels of inflammatory mediators (e.g., IL-1β, IL-2, IL-4, IL-6, and TNF-α). Moreover, umbilical cord-derived MSCs administration had statistically increased vessel density, and reduced microglial activation and leukocytes infiltration at 3 days after ICH compared to the control group. These suggested that, except for neuronal regeneration, the underlying mechanisms of MSCs to accelerate neurological function recovery after ICH were also attributed to its effects of promoting angiogenesis and inhibiting inflammation (Liao et al., 2009). Other therapeutic mechanisms revealed that MSC transplantation-mediated inflammation suppression and down-regulation of aquaporin-4 (AQP4) protein expression could alleviate brain edema of ICH (Zhang Y. et al., 2019). The mechanism of transplanted MSCs inhibiting inflammation might be partly involved in the inhibition of iNOS expression (Ding et al., 2017). In addition, the microglial M2 polarization mediated by the secretory factors, insulin-like growth factor-1 (IGF-1) of stem cells, was considered to be a possible mechanism for stem cells improved hemorrhage stroke prognosis (Chen et al., 2019; Sun et al., 2020). Taken together, these studies demonstrated that anti-inflammation is a crucial mechanism involved in MSCs-mediated functional and tissue recovery after ICH.

Moreover, Adipose-derived stem cells administration rats of ICH found significantly decreased brain water content, brain atrophy, and apoptosis, indicating the multiple mechanisms of MSCs therapy in ICH recovery (Kim et al., 2007). Furthermore, other studies had demonstrated that MSCs transplantation improved BBB integrity after ICH, which was associated with the increased expression of TNF-α stimulated gene/protein 6 (TSG-6) and tight junction proteins (claudin-5 and zonula occludens-1) (Chen et al., 2015; Choi et al., 2018). As hypertension is the most common cause of cerebral hemorrhage clinically, a spontaneously hypertensive model was created to evaluate the long-term neuroprotective effects of MSCs in ICH, which would have great value for pre-clinical study. This study demonstrated that MSC treatment could reverse the BBB permeability and improve the neurological function after hypertensive ICH (Wang et al., 2015). For stem cell therapy, the conventional mode of administration is injection of cells into the brain directly or through blood vessels. However, the method of cells being injected into the brain is invasive, and regarding the method of vascular administration, it has been demonstrated that there were only a few cells homing to the brain or had embolization and possible tumorigenesis risk (Li P. et al., 2019; Mello et al., 2020). Thus, a novel way of intranasal route was applied for MSC transplantation therapy in collagenase IV injected mice (Sun et al., 2015). In addition, MSC-derived exosomes were also considered as a promising alternative strategy to solve the obstacle in MSC application (Bedini et al., 2018).

MSC Transplantation in Swine

Although different from the etiology of spontaneous ICH, the rehabilitation research of traumatic cerebral hemorrhage also had an important reference value for the clinical transformation of ICH intervention, especially in large animals. Exosomes were membrane-enclosed nanovesicles that contained numerous molecular constituents including cellular proteins, lipids, microRNAs and mRNAs, and had the properties of low immunogenicity, BBB penetrability, intercellular communication and neural regeneration mediation (Yang et al., 2017). Meanwhile, due to the neuroprotective effect of MSCs-generated exosomes in small animal traumatic brain injury (TBI) model, they were also highly expected in the treatment of traumatic cerebral hemorrhage in large animals (Zhang Y. et al., 2015). When Yorkshire swine were treated with MSCs-derived exosomes, they appeared to have faster neurologic recovery and obvious reduction of neurologic injury (Williams et al., 2019). The benefit outcomes in large animal models would provide a reliable evidence for the clinical application of preclinical research.

MSC Transplantation in Monkeys

The first study of MSC treatment for primates was conducted in an ICH model of intracranial autologous injected *macaca fascicularis monkey* (Feng et al., 2011). The MSC-treated group showed significantly improved neurologic deficit and microvessel density compared with the control group. Additionally, the serial ^{18}F-FDG PET scans found that the ^{18}F-FDG uptake in the adjacent cortex of the early treatment was increased significantly when compared with control group during recovery phase, and better treatment results were obtained in the MSC early treatment group (7 days) compared with late treatment group (28 days). However, the optimal timing of MSC transplantation for severe IVH in newborn rat pups was considered to be at early stage of 2 days instead of late at 7 days after induction (Park et al., 2016). Therefore, when extrapolating preclinical data into clinical trials, the narrow therapeutic time window of MSC transplantation for ICH intervention should be determined. Meanwhile, it is also worth noting that stereotaxical delivery of MSCs to the outside of the right putamen of *macaca fascicularis monkey* could induce the local tissue inflammation and necrosis, although this damage might

repair over time (Feng et al., 2014). Nevertheless, a total of four intravenous injections of MSCs (1×10^7 cells/kg) with 2 weeks interval did not affect the general health of monkeys (Wang Y. et al., 2012). These studies suggested that the delivery modes and therapeutic time window of MSCs applied for ICH were key factors associated to prognosis and required further elucidation.

MSC Transplantation in Humans

Mesenchymal stem cells have been extensively studied and confirmed as an effective treatment strategy for animal cerebral hemorrhage models, however, less research has been investigated in human cerebral hemorrhage. The first application of MSCs in humans was a case report about two persistent vegetative state patients of hemorrhagic stroke, and then they were followed-up with for a 1 year period (Al Fauzi et al., 2016). Patients were intraventricularly injected with MSCs (20×10^6 cells/2.5 mL) three times at intervals of 1 month using an Ommaya reservoir. The National Institute of Health Stroke Scale (NIHSS) scores of the two patients indicated that the functional status was obviously improved. A placebo-controlled study also found that two deliveries of MSCs (2×10^6 cells/3 mL) 1 month apart could significantly improve the neuro-restoration and clinical prognosis of ICH patients with severe disability (Tsang et al., 2017). In another case report of an 80-year-old SAH patient with a past medical history (i.e., diabetes, hypertension, and cardiac bypass) found that MSC transplantation gradually improved the consciousness of the patient at 3 weeks after SAH, and restored the ability of speaking and self-care (Brunet et al., 2019). Consistently, this procedure might provide a safe, feasible and effective treatment for patients with clinical cerebral hemorrhage in the future, and enhance the confidence in the treatment of patients with cerebral hemorrhage.

Based on previous experimental and clinical benefit, MSC transplantation was also carried out on premature infants to explore its safety and efficacy for severe IVH (Ahn et al., 2018). A total of nine premature infants were enrolled in this clinical trial, and different doses of MSCs were injected into the lateral ventricle (5×10^6 cells/kg; 1×10^7 cells/kg) on average at twelve postnatal days. All patients could tolerate the procedure well, and there were no immediate complications (i.e., allergies or death) which occurred within 6 h after transplantation. In these infant patients, four showed continual regression and improvement, and five received shunt placement due to the progressive ventriculomegaly before discharge. In most MSC transplanted patients, the inflammatory factors such as IL-6, TGF-β1/2, TNF-α, and IL-1β all exhibited a decreasing trend compared to before transplantation, that was consistent with that in animal experiments (Bao et al., 2013). Different from preclinical research, VEGF showed a reduced expression after MSC therapy, which was probably related to the up-regulation of baseline VEGF expression caused by hemorrhage insult (Tang et al., 2007). Meanwhile, Safety evaluation results showed both doses of MSCs did not induce the serious adverse events and dose-limiting toxicity, and the mortality was zero even in grade 4 IVH, which implied this strategy was safe when the dose was controlled within 1×10^7 cells/kg of MSCs. As severe IVH and subsequent posthemorrhagic hydrocephalus could cause long-term neurological disorders in surviving preterm infants, children with neurological sequelae underwent a noteworthy clinical trial in which they were treated with bone marrow-derived mononuclear cells (BMMNCs) that might have the synergic effect of hematopoietic stem cells (HSCs) and MSCs (Liem et al., 2019). Except for no observed any adverse side effect, BMMNC transplantation had made great progress in many aspects of neurodevelopment, such as motor function, fine motor skill, personal social, motor adaptive, and language skills, demonstrating the BMMNCs treatment effectiveness in promoting the social interaction and self-care ability of these children. This improvement in human-specific physiological functions was difficult to evaluate in preclinical animal studies, and the functional ameliorate illustrated that the transplanted stem cells had availably differentiated into region specific cell types and integrated into neural network appropriately.

Because of the mechanical damage of hematoma to brain tissue, clot evacuation was a commonly used intervention in the clinical treatment of ICH. Therefore, the combined therapy of MSC transplantation followed by minimally invasive hematoma removal was supposed as a promising treatment for ICH. Compared with surgical treatment, the combined group significantly decreased the brain edema, and increased the BDNF, NGF, and Endothelial progenitor cells level (Zhang, 2016). In a case report of a female patient with a past medical history of hypertension, the combined treatment also observed improved neurologic function consistent with preclinical studies (Zhang Q. et al., 2015; Zahra et al., 2020). However, there were discrepancies among combined therapy with respect to prognosis in different sources of transplanted MSCs, which might be due to differences in the viability, proliferation potential, and neurogenic efficacy of the tested cell line. A retrospective analysis of up to 5-years follow up had documented that the patients who received human umbilical cord-derived MSCs in the combined therapy group showed better outcome on functional recovery and complications than the bone-marrow MSCs combined therapy group and control group (Chang et al., 2016). Overall, the MSCs transplantation had promise to be safely and effectively applied in clinical ICH treatment, even so, the underlying regulatory mechanism, the optimal implantation time, and larger clinical trials still need to be further considered in future research.

DIFFERENCES BETWEEN PRECLINICAL AND CLINICAL STUDIES

Mesenchymal stem cell transplantation had demonstrated the positive therapeutic effects of facilitating tissue repair and prognosis of ICH in animal research and clinical trials. However, significant research differences still existed in the process of transforming preclinical research to clinical application. The uncertainty contained in the differences of this research might affect the therapeutic mechanisms of transplanted MSCs and the stability of clinical benefits from many aspects. Whether the therapeutic mechanisms of MSC therapy reported in animal

studies could effectively translate to the complex pathological environment of ICH patients was also unknown.

Research Subjects

Actually, the differences between experimental research and clinical trials had already appeared in the screening of research subjects. Healthy rodents were the most versatile research group in preclinical studies. However, patients with ICH usually had a variety of other diseases, because ICH often occurred in the antiplatelet, anticoagulants, and thrombolytics users or elder peoples (Brunet et al., 2019). In addition, the reported ICH patients also included a strict inclusion and exclusion criteria, which did not exist in the healthy animal research subjects (Ahn et al., 2018). Moreover, the small sample size and case report in clinical studies further increased the publication bias. Most importantly, none of the current animal ICH models could reproduce the actual pathological process of ICH. Taken together, in the process of extrapolating the trial results to clinical application, it was necessary to be further verified from the research objects, model establishment, sample size, and other aspects.

MSC Preparation

It is well known that the cell source, extraction, and expansion process of MSCs are direct factors associated with the biological characteristics and differentiation fate of implanted cells. In the rodent studies, MSCs derived from different sources (bone marrow, adipose, umbilical cord, placenta, dental pulp, and cord blood) had been used for ICH treatment and displayed an improved prognosis (Choi et al., 2018; Chen et al., 2019; Liu et al., 2019; Zhang Y. et al., 2019; Chen K.H. et al., 2020). Nevertheless, this trend of cell source balance had been tilted in human research. Except for a small amount of MSCs derived from umbilical cord or cord blood (Chang et al., 2016; Ahn et al., 2018), most of the transplanted MSCs were extracted through autologous bone marrow puncture in the clinical studies (Liem et al., 2019; Zahra et al., 2020). Different from this, the bone marrow-MSCs in the rodent studies were mainly derived from allogeneic femur and tibia. In addition, the MSCs transplantation in patients were all homogeneous, and MSCs implanted in rodents were also derived from heterogeneous (Chen K.H. et al., 2020). However, no research had focused on the influence of MSCs source or extraction differences on ICH treatment. Considering the risk and time consumption of autologous MSCs extraction, the immunogenicity of allogeneic MSCs, and the high cost of maintaining cells viability, optimized and practical MSCs product preparation still needed to be clarified in research to ensure the stability and predictability during transformation for clinical treatment.

Registration and Preliminary Results

Unlike animal research, registration was introduced to comprehensively and objectively record all clinical trials which had been conducted. However, many clinical studies did not report the registration information, and only a few studies had documented the registration number of ClinicalTrials.gov (Ahn et al., 2018; Zahra et al., 2020). Although the improved prognosis of MSC treatment had been widely reported, the results of animal studies were mainly histopathological examination and neurological evaluation (Liu et al., 2019), and the clinical studies were more about blood composition testing and neurological function scores (Zhang, 2016; Brunet et al., 2019). In fact, clinical results about MSCs treated ICH were more of a case report for the patients (Liem et al., 2019). The lack of control studies not only made the results occasional, but also greatly compromised the quality of clinical trials. Therefore, any conclusive reports about clinical therapeutic mechanisms or effects might be too early and not rigorous. The clinical application of MSCs for ICH treatment still needed more controlled and statistically significant clinical trials.

CHALLENGES OF ICH MICROENVIRONMENT ON MSCs THERAPY

The extracellular environment plays an important role in the proliferation, differentiation, and clinical prognosis of transplanted MSCs. In fact, the biomimetic microenvironment not only helps to maintain the advantages of MSCs (e.g., proliferation, differentiation, and immunoregulatory properties), but also helps to retain their phenotype, metabolism, adhesion, and response signal to surrounding factors (Kim H. et al., 2019). The central nervous system refers to a complicated system containing neurons, glial cells, blood vessels, and extracellular matrix, which closely interacts with the transplanted MSCs and determines its fate (Dooves et al., 2016). However, the composition of the brain tissue microenvironment with hemorrhage infiltration becomes more complex and changeable, including mass effect, erythrocyte lysate, thrombin, free radicals, cytotoxic, excitotoxic, inflammatory effects, and nitric oxide (Babu et al., 2012; Gao et al., 2018). Meanwhile, many different stimulations had been recognized as the initiators of the stress-induced premature senescence of MSCs, including mechanical stress, osmotic stress, ionizing radiation, reactive oxygen species, and hypoxia (Zhou et al., 2020). Therefore, the extracellular microenvironment of perihematoma should be strictly evaluated when exploring effective and translatable MSCs therapies for hemorrhage stroke (**Figure 1**).

Mass Effect

Mass effect is the main primary brain injury mechanism in ICH, which refers to the mechanical stretching and compression of the surrounding brain tissue by the hematoma (Wilkinson et al., 2018). Moreover, in the limited space of the skull, hematoma can lead to increased intracranial pressure and edema-derived mass effect (Guo et al., 2017). These would all result in a dynamic change and increase of mechanical stress in the intracranial microenvironment. So, the transplanted MSCs would sit in a microenvironment of mechanobiology with multiple complex mechanisms. Our studies had suggested that mechanical pressure enhanced the stretch-activated ion channel Piezo-2 expression *in vitro* and *vivo* as early as 8 h after ICH (Guo et al., 2018;

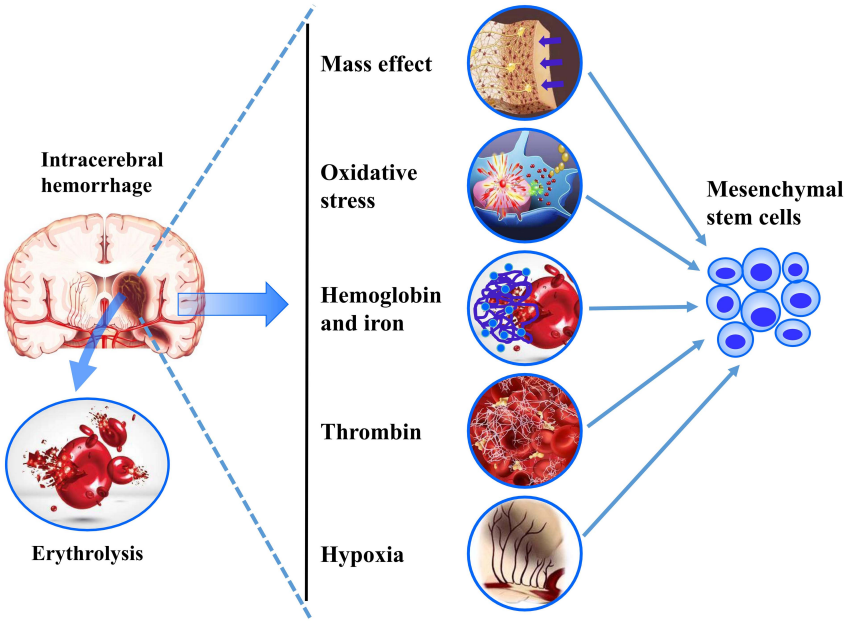

FIGURE 1 | A summarization of the challenges of ICH microenvironments on MSC transplantation.

Gong et al., 2019). *In vitro* data from adult *Drosophila* midgut feeding with indigestible food to generate mechanical loaded showed an increase enteroendocrine cells generation through Piezo protein upregulation, these suggested that the existence of stem cells in the fly midgut could, through mechanical signals, sense to modulate proliferation and differentiation (He et al., 2018). Mechanobiology, as the inductive niche of stem cells, could regulate their developmental processes and self-renewal (Vining and Mooney, 2017).

Thrombin

Thrombin, derived from blood after ICH, is an essential component of the coagulation cascade, which has been observed to induce the brain injury through activating the protease-activated receptors (PAR) (Gao et al., 2014; Mao et al., 2016). Although thrombin is a proven tissue damage mechanism in ICH, thrombin-activated platelet-rich plasma was reported to provide a higher proliferation rate and MSC marker expression in long-term cultured MSCs (Kocaoemer et al., 2007). Thrombin-preconditioned MSCs displayed a five-fold acceleration of MSC-derived extracellular vesicles biogenesis and a two-fold enrichment of their cargo contents, these were also regulated via PAR-mediated intracellular signaling pathways (i.e., ERK1/2, AKT, Rab5, and EEA-1) (Sung et al., 2019). Meanwhile, thrombin also could promote fibronectin secretion of MSCs via PAR-mediated ERK1/2 activation (Chen et al., 2014), and the fibronectin-formed adsorption force could regulate the transmission of the cell traction force and the lineage specifications of MSCs (Lin et al., 2018). Additionally, in the newborn rat model of unilateral carotid artery ligation, thrombin preconditioned human Whartong's jelly-derived MSC transplantation significantly enhanced the anti-inflammatory, anti-astroglial, anti-apoptotic effects, and neurological recovery (Kim Y.E. et al., 2019). Apparently, thrombin might have multiple undetermined influence mechanisms on the characteristics of transplanted MSCs during hemorrhage stroke.

Iron Overload

Intracerebral hemorrhage, except for the primary damage of mass effect, intraparenchymal blood, and its degradation products (hemoglobin, heme, and iron), could continue to insult the brain tissue through inducing the cytotoxic, excitotoxic, oxidative, and inflammatory effects (Babu et al., 2012). Among these, iron overload was considered as an essential factor to increase the lipid peroxidation, lethal reactive oxygen species (ROS) production and neuronal ferroptosis (Li et al., 2017; Wan et al., 2019). Worse still, the iron overload microenvironment also posed a fatal threat to the therapy of stem cells transplantation. It had been demonstrated that ferric ammonium citrate treatment was capable of markedly reducing the proliferation and pluripotency, and inducing apoptosis and senescence in MSCs (Yang et al., 2016). In addition, iron accumulation was found to elevate the ROS level and apoptosis of MSCs, suggesting that the damage mechanism of iron overload in MSCs transplantation may be related to oxidative stress (Lu et al., 2013; Yuan et al., 2019). Notably, interventions to reduce mitochondrial ROS accumulation could significantly reduce the apoptosis of MSCs (Yang et al., 2016; Yu et al., 2018). Besides, the *in vitro* and *in vivo* iron overload were found to have the ability to inhibit the osteogenic commitment and differentiation of MSCs in a dose-dependent manner (Balogh et al., 2016). Therefore, these results indicated that the secondary iron overload microenvironment

after ICH would affect not only the apoptosis but also the differentiation of transplanted MSCs.

Oxidative Stress

Although ROS is an inevitable product of the defense system and normal cellular metabolism, the imbalanced homeostasis of the oxidation-reduction system can significantly create disruption to the blood-brain barrier, cell death and structural damage. And that ROS aggregation could be induced by the metabolic products of hematoma, inflammatory cells, and excitatory amino acids after ICH (Qu et al., 2016). Meanwhile, ROS had been extensively considered as an important factor of senescence in MSCs (Ko et al., 2012; Zhou et al., 2020). The oxidative stress formed by sub-lethal doses of hydrogen peroxide could considerably reduce proliferation rate, and accelerate telomere attrition, and induce senescence-associated β-galactosidase expression and senescent morphological features (Brandl et al., 2011). The nuclear factor erythroid-2 related factor 2 (Nrf2) is a critical molecule to protect MSCs against oxidative stresses. It had been reported that the MSCs with transient expression of Nrf2 had the ability to resist the hypoxic and oxidative stress induced cell death and apoptosis (Mohammadzadeh et al., 2012). Oxidative stress preconditioning could protect the MSCs vitality through activating the Nrf2 pathway and upregulating its downstream target of the superoxide dismutase, catalase, and HO-1 (Zhang F. et al., 2019). Moreover, many strategies had been explored to avoid cell apoptosis and senescence initiated by oxidative stress, including pioglitazone, vaspin, ganoderic acid D, and vitamin E (Bhatti et al., 2018; Hu et al., 2019; Zhu et al., 2019; Xu et al., 2020). Taken together, before MSCs could be reliably and effectively used as hemorrhage stroke therapy, it is necessary to better understand the multiple microenvironmental regulatory mechanisms driving transplanted MSCs to form repair effect.

POTENTIAL STRATEGIES FOR MSCs TREATMENT ENHANCEMENT AFTER ICH

The above description highlights the critical function of ICH pathological microenvironment in determining the MSCs fate. The knowledge regarding mass effect and oxidative stress induced MSCs senescence provides a potential mechanism for the failure of MSCs-mediated clinical benefit in ICH. This indicated that effective therapeutic effect of MSCs on ICH required to strict regulation of the impacts of microenvironment on grafted MSCs. Facing the challenges of applying MSCs in the treatment of ICH, several optimizing strategies had been established and showed potential feasibility.

Preconditioning Treatments

Studies had shown that preconditioning of MSCs could effectively resist the impact of transplantation microenvironment and increase the regenerative potential of cells, although the underlying mechanism was unknown. Hypoxic preconditioning, pharmacological agents, and trophic factors exposure were commonly used to improve culture expansion conditions, enhance survival and proliferation, and avoid the possible changes of MSCs potency (Hu and Li, 2018; Nahhas and Hess, 2018). In the ICH mice model, the transplantation of hypoxic preconditioned MSCs or neural stem cell significantly increased the grafted-cells survival and the behavioral performance (Sun et al., 2015; Wakai et al., 2016). Additionally, apocynin preconditioned MSCs obviously improved the hematoma expansion, neuronal death, brain edema, and therapeutic efficacy in the acute stage of bacterial collagenase induced rat ICH model compared with native MSCs (Min et al., 2018).

Other than pharmacological and molecular preconditioning of the MSCs, the resistance of MSCs to the microenvironment could also be achieved using miRNA. MiRNA based treatments had the characteristics of delayed MSC senescence and multidimensional targets. Given the significant function of miRNA-126 in promoting angiogenesis, the miRNA-126 transfected MSCs were injected into the collagenase-induced ICH rats, leading to decreased brain water content and improved neurological score (Wang et al., 2020). In addition, the CX3CR1 overexpressed MSCs increased the viability and migration ability of transplanted MSCs, and improved the sensory and motor functions of the collagenase induced mice ICH model (Li G. et al., 2019). Moreover, GDNF-transfected MSCs could improve the neurological function of experimental ICH rats through enhancing neurotrophic factor secretion (Deng et al., 2019). Therefore, miRNA-based MSC therapy was expected to purposefully regulate the proliferation and differentiation ability of MSCs in the pathological microenvironment of ICH.

Optimizing ICH Microenvironment

Creating a standardized and locally beneficial microenvironment is another strategy to maintain the repair potential of transplanted MSCs. Iron overloaded and oxidative stress damage after ICH were recognized as not only critical damage factors for ICH, but also important causes of MSC senescence and apoptosis (Wan et al., 2019; Yuan et al., 2019). To improve the iron overload microenvironment after ICH, an injectable core-shell hydrogel was fabricated for ICH in situ therapy. The outer shell hydrogel with quick degradation property was loaded with iron chelator to eliminate iron overload, and the inner core hydrogel loaded with MSCs and growth factors displayed an improved MSCs survival and differentiation (Gong et al., 2020). In addition, surgical evacuation was a commonly used clinical approach to remove hematoma, and the benefits of combined treatment with MSCs transplantation might associate with the improvement of the transplanted cells microenvironment (Zhang Q. et al., 2015; Chang et al., 2016).

Exosomes Therapies

The focus of MSC therapy is cell replacement and neural reorganization, however, its embolization and possible tumorigenesis limitations make exosomes a potential alternative. Significantly, exosomes are even suggested to be the main therapeutic mechanism of MSCs treatment (Han et al., 2018). Exosomes are nano-sized extracellular vesicles, which can be isolated from the supernatant of cultured cells in exosome-free medium through centrifugation and other methods

(Bedini et al., 2018). Recently, treatment strategies based on exosomes of MSCs have been widely reported in many diseases. The potential mechanism of exosomes treatment in the ICH mainly comprises the anti-apoptotic, neurogenesis, angiogenesis, and anti-inflammation effects through its miRNAs (Cai et al., 2020; Duan et al., 2020).

The autologous blood ICH rats showed that the apoptotic and degenerative neurons in the miR-133b-modified MSC-derived exosomes treatment group was significantly reduced. It was believed that the neuroprotection effect of exosomes on ICH was mainly reflected in the anti-apoptotic effect of miR-133b through mediating RhoA and ERK1/2/CREB signaling pathway (Shen et al., 2018). The MSC-derived miR-206-knockdown exosomes were also confirmed to improve brain edema and neurological deficit in early brain injury of SAH rats, which was probably related to the suppression of neuronal apoptosis via BDNF/TrkB/CREB signaling (Zhao et al., 2019). Except for anti-apoptotic, the effects of neurogenesis and angiogenesis were also observed in the MSCs-derived exosomes treated ICH rats. The exosomes therapy remarkably increased the newly generated endothelial cells around the hematoma, mature neurons in the SVZ, and myelin in the striatum (Han et al., 2018). Moreover, the MSC-derived exosomes therapy was found to significantly alleviate early brain injury of SAH through restraining the HMGB1-TLR4 pathway activation and generating the anti-apoptosis and anti-inflammation effects (Xiong et al., 2020). Collectively, treatments based on exosomes are expected to provide a broader therapeutic strategy for ICH intervention.

CONCLUSION

Mesenchymal stem cell-based ICH treatments provide a unique opportunity to improve ICH outcome through multimodal therapeutic action. Multi-species preclinical studies have demonstrated promising evidence that MSCs can promote the neural network reconstruction and neurological function recovery through neurogenesis, angiogenesis, anti-inflammation, and anti-apoptosis mechanisms. Meanwhile, human clinical trials have further confirmed the safety and efficacy of MSCs-based therapy after ICH, and the potential to improve patient prognosis. However, additional studies aimed to better understand the pathological microenvironment related to the successful implantation of MSCs after ICH (e.g., mass effect, thrombin, oxidative stress, and hematoma degradation products) will ultimately augment the therapeutic effect and enhance the reliability and stability of clinical transformation. Furthermore, MSC-derived exosomes treatment might represent a promising way to deal with the complex pathological microenvironment of hemorrhagic stroke.

AUTHOR CONTRIBUTIONS

YG searched the literature and wrote the manuscript. BW and SH critically revised the manuscript. All authors have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This study was supported by the National Natural Science Foundation of China (Grant Nos. 11972099 and 31600770), the Chongqing Research Program of Basic Research and Frontier Technology (Grant Nos. cstc2018jcyjAX0836 and cstc2019jcyj-msxmX0753), and the Ph.D. Foundation of Southwest University of Science and Technology (No. 18zx7128).

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

frontiers in Cellular Neuroscience

SYSTEMATIC REVIEW
published: 14 April 2021
doi: 10.3389/fncel.2021.628908

Stem Cell-Based Therapy for Experimental Ischemic Stroke: A Preclinical Systematic Review

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Specialty section:
This article was submitted to Cellular Neurophysiology, a section of the journal Frontiers in Cellular Neuroscience

Received: *13 November 2020*
Accepted: *24 February 2021*
Published: *14 April 2021*

Citation:
Zhang X-L, Zhang X-G, Huang Y-R, Zheng Y-Y, Ying P-J, Zhang X-J, Lu X, Wang Y-J and Zheng G-Q (2021) Stem Cell-Based Therapy for Experimental Ischemic Stroke: A Preclinical Systematic Review. Front. Cell. Neurosci. 15:628908. doi: 10.3389/fncel.2021.628908

Stem cell transplantation offers promise in the treatment of ischemic stroke. Here we utilized systematic review, meta-analysis, and meta-regression to study the biological effect of stem cell treatments in animal models of ischemic stroke. A total of 98 eligible publications were included by searching PubMed, EMBASE, and Web of Science from inception to August 1, 2020. There are about 141 comparisons, involving 5,200 animals, that examined the effect of stem cell transplantation on neurological function and infarct volume as primary outcome measures in animal models for stroke. Stem cell-based therapy can improve both neurological function (effect size, −3.37; 95% confidence interval, −3.83 to −2.90) and infarct volume (effect size, −11.37; 95% confidence interval, −12.89 to −9.85) compared with controls. These results suggest that stem cell therapy could improve neurological function deficits and infarct volume, exerting potential neuroprotective effect for experimental ischemic stroke, but further clinical studies are still needed.

Keywords: stem cell-based therapy, ischemic stroke, middle cerebral artery occlusion, neuroprotection, preclinical systematic review

INTRODUCTION

Stroke is one of the leading causes of death and the first cause of acquired morbidity and mortality worldwide (Strong et al., 2007). Ischemic stroke is the most common type of stroke, defined as a syndrome characterized by a rapid onset of central nervous system function damage due to an interruption of the cerebral blood flow (Baker et al., 1998). However, recombinant tissue plasminogen activator (rtPA) remains the only pharmacological treatment approved by the Food and Drug Administration for thrombolysis in patients suffering from ischemic stroke (Wright et al., 2012; Jauch et al., 2013). Unfortunately, the relatively short therapeutic window of 3-4.5 h of rtPA and the risk of the devastating symptomatic intracranial hemorrhage limit its application (Chapman et al., 2014). Although the treatment protocols for acute ischemic stroke have been fundamentally altered because of the updated guidelines using endovascular techniques in Powers et al. (2015), this guideline concluded that certain endovascular procedures have been demonstrated to provide clinical benefit in selected acute ischemic stroke patients within a slightly prolonged therapeutic time window of 6 h after symptom onset. Thus, given the gap between the widespread occurrence of the disease and the limitations of conventional therapies available, novel intervention for ischemic stroke is urgently needed.

An interest in stem cell-based therapy was spawned by the limited eligibility for thrombolysis and failure of the neuroprotective paradigm (Jakala and Jolkkonen, 2012). Stem cells are undifferentiated biological cells that have the capacity to proliferate and differentiate into mature specialized cells and can divide to produce more stem cells (Mora-Lee et al., 2012). Various types of stem cells, including neural stem/progenitor cells (NSCs), embryonic stem cells, immortalized pluripotent stem cells (iPSCs), and tissue-derived stem cells such as mesenchymal stem cells (MSCs) and bone marrow mononuclear cells, have attained tremendous attention as a promising approach in nervous system diseases as disparate as motor neuron disease (Cabanes et al., 2007; Su et al., 2009), Parkinson's disease (Yasuhara et al., 2006), multiple sclerosis (Aharonowiz et al., 2008; Morando et al., 2012), and stroke (Jensen et al., 2013; Wang J. et al., 2013). There is now considerable preclinical literature on the possible benefits of stem cell transplantation following ischemic stroke. Stem cell may assist stroke recovery through cell replacement, neuroprotection, angiogenesis, endogenous neurogenesis, and modulation on inflammation and immune response (Hao et al., 2014).

To better foster the stem cell-based therapies that progressed into clinical trials, we need to further understand the optimal requirements for stem cell administration to improve the therapeutic effects on ischemic stroke. However, the ideal type of stem cell and from what donor species and tissue source, the appropriate time for injections, the number of cells needed, and the best administration route are still not clear. Furthermore, whether reports of efficacy in animal models are potentially biased in favor of positive results and whether the magnitude of integrative and protective effects is large enough to be potentially clinically meaningful are worth further investigation. Systematic review of preclinical data is a powerful analytical tool typically used to offer the most objective evidence for the efficacy of a treatment and improve the likelihood of success of future clinical trials (Murphy and Murphy, 2010). This systematic review included controlled studies of stem cell therapy in animals exposed to stroke compared with placebo control or no treatment in stroke animals, in which the outcome was measured with neurological function score and infarct size/infarct volume. The purpose is to evaluate the safety and efficacy of stem cell therapy for ischemic stroke and confirm the conditions of greatest efficacy.

METHODS

We strictly obeyed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement (Moher et al., 2010) to conduct this systematic review.

Eligibility Criteria

We included all controlled studies that compared stem cell therapy to placebo/vehicle or no-treatment *in vivo* models of ischemic stroke, in which the outcome was measured with neurological function score (NFS) and infarct size/infarct volume. To prevent bias, the inclusion criteria were prespecified as follows: (1) focal ischemic stroke, induced by transient middle cerebral artery occlusion (MCAO) or permanent MCAO, no restriction on animal species, as well as gender, age, weight, and sample size; (2) controlled studies with control group (receiving vehicle, saline, positive control drug, or no treatment) and experimental group (receiving allogeneic or autologous stem cell therapy), and there was no restriction on dosage, mode, and time of initial treatment; and (3) studies that have both the NFS and infarct size/infarct volume outcome measurement. The exclusion criteria were as follows: (1) other types of articles except animal experimental articles, including clinical articles, case reports, comments, reviews, abstracts, and *in vitro* studies; (2) non-focal cerebral ischemia model, such as global cerebral ischemia model, hypoxic–ischemic models or traumatic models; (3) non-single intervention, the administration for the experimental group was stem cell with another therapy or cell type; (4) non-controlled studies that lack a control group; (5) low-quality articles with quality scores lower than 5; and (6) published in other language except English.

Information Sources

Three databases (PubMed, EMBASE, and Web of Science) were searched for relevant published articles that assessed the effect of stem cells in animal models of cerebral ischemia and were reported in English in peer-reviewed journals up to August 1, 2020. The reference lists of all selected publications were also used to identify additional eligible studies.

Search Strategy

Studies of stem cells in animal models of cerebral ischemia were identified from three electronic databases (PubMed, EMBASE, and Web of Science). The keywords used in the search strategy were (stem cell OR stem OR multipotent OR mesenchymal OR cell therapy) AND (stroke OR cerebrovascular OR cerebral infarct OR cerebral ischemia/reperfusion OR middle cerebral artery OR middle cerebral artery occlusion). The Boolean (exact text) used in the search is in the **Supplementary Material**.

The publication time is from the inception of each database up to August 1, 2020. All searches were limited to studies on animals, published in English, and the stroke model was ischemic.

Study Selection

The first selection was made using the following additional filters: "stem cell" and "stroke." Then, a second selection was made from reading the titles and abstracts to assess if the content seemed to fit the inclusion criteria. Full text availability and criteria were verified before considering the inclusion of articles. The detailed research method was presented according to the PRISMA flow diagram.

Data Extraction

The following details were extracted by two investigators from the included studies: (1) publication year and the first author's name, type of ischemia (temporary or permanent); (2) characteristics of the animals used, including species, sex, and animal number per group; and (3) treatment information, including stem

cells (donor species and tissue source), intervention regime (anesthetic, time for injections, method of administration, and number of cells injected). When a publication reported more than one experiment or where an experiment contained more than one individual comparison, they were regarded as independent experiments, and data for every individual comparison from each experiment were extracted, respectively. If the experimental group of animals received different doses of stem cell administration with a single control group, we used the data for the highest dose. If neurobehavioral tests were performed at different times, we only extracted data for the final time point reported. If the published data were missing or only expressed graphically, we contacted the authors for further information, and when a response was not received, we measured the numerical values from the graphs by using digital ruler software or exclude them. For each comparison, we extracted the data of mean value and standard deviation from each experimental and control group of every study.

Risk of Bias Assessment

We assessed the methodological quality of the included studies by using the criteria according to a checklist as previously described (Macleod et al., 2004). These criteria were as follows: (1) peer-reviewed, which is an anonymized review process. The contribution will be initially assessed by the editor, and papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the quality of the paper; (2) statement of control of temperature; (3) random allocation to treatment or control; (4) blinded assessment of outcome; (5) use of anesthetic without significant intrinsic neuroprotective activity; (6) appropriate animal model which uses animals without relevant comorbidities (aged, diabetic, or hypertensive); (7) sample size calculation; (8) compliance with animal welfare regulations; and (9) statement of potential conflict of interests. Each item of the nine-item scale contributed one point, and each study was given a quality score. Two authors independently evaluated the methodological quality of the included articles. When one author thought that the quality score of one paper is higher than 5 and the other author thought that the score is lower than 5, we will discuss and consult with the corresponding author (G-QZ). The incidence of this situation is less than 1% because the two authors who evaluated the quality score have similar opinions basically.

Summary Measures

The main outcome measurements were the NFS and infarct size/infarct volume. Neurological function was mainly examined with modified neurological severity score, which is a composite of motor, sensory, reflex, and balance tests, and the higher score shows more severe injury. The measurement of infarct volume was mainly through immunohistochemistry and triphenyltetrazolium chloride staining. A total of 49 of the included studies conducted immunohistochemistry, and the specific markers used in this article to evaluate cellular ischemia were different. Most articles (75.5%) used one or two. Among them, glial fibrillary acidic protein and NeuN were the most common markers, with 37 and 26 articles using them, respectively. In addition, there are only nine, eight, seven, and five articles using dual adrenocortical hormone, microtubule-associated protein 2, tubulin III, and neuron-specific enolase. The lesion volume was determined as follows: corrected infarct area = [infarct - (ipsilateral hemisphere - contralateral hemisphere)] / contralateral hemisphere $\times$ 100, as described previously (Swanson et al., 1990), in order to eliminate the overestimation of infarct size and the measurement difference between different methods.

Synthesis of Results

Neurological function score and infarct volume were all considered as continuous data, and these outcome indicators conducted a global estimate of the combined effect sizes by calculating the standardized mean difference (SMD) or weighted mean difference (WMD) and 95% confidence intervals (CI) utilizing the random effects model. WMD is a standard statistic that measures the absolute difference between the mean values in two groups. It estimates the amount by which the experimental intervention changes the outcome on average compared with the control. It can be used as a summary statistic in meta-analysis when the outcome measurements in all studies are made on the same scale. SMD is used as a summary statistic in meta-analysis when the studies all assess the same outcome but measure the outcome in a variety of ways (Vesterinen et al., 2014).

Risk of Bias Across Studies

Publication bias was assessed by using funnel plot and Egger's test. The I^2 statistic was used for the assessment of heterogeneity.

Additional Analyses

To explore the impact of factors modifying the outcome measures, we conducted pre-stratified subgroup analyses according to the following variables: MCAO model induction, recipient species, recipient sex, donor species, anesthetic, number and type of cell used, time and route of administration, and manipulation of stem cells prior to implantation. Difference between groups was assessed by partitioning heterogeneity and using the χ^2 distribution with n-1 degrees of freedom (df), where n equals the number of groups. Meta-regression analyses were conducted to search for systematic differences among study design characteristics that potentially explained the sources and extent of heterogeneity between studies (Guyatt et al., 2012). The meta-regression was univariate rather than multivariate, and we calculated adjusted R^2 values to explain the proportion of the observed variability in the observed effect size for a group of experiments explained by variation in the independent variable in question (Antonic et al., 2013).

All statistical analyses were performed with RevMan version 5.3 and Stata version 15.0. Probability value $P < 0.05$ was considered significant.

RESULTS

Study Selection

Electronic searching identified 18,105 potentially relevant records, of which 13,876 articles remained after the removal of duplicates. After going through the titles and abstracts, 13,168 papers were excluded for clearly irrelevant content. By reading the full text of the remaining 708 articles which reported the efficacy of stem cell in experimental ischemic stroke, we obtained the full papers of 178 publications and assessed these for eligibility. Of these, 18 studies were excluded due to inadequate data for the outcome calculation, and 62 articles were excluded because their quality scores did not reach 5. Ultimately, 98 eligible studies remained for this meta-analysis, which included 141 comparisons of NFS and infarct volume (**Figure 1**).

Study Characteristics

The studies involved a total of 5,200 experimental subjects from two species: mouse ($n = 352$) and rats ($n = 4,848$). Eighty-four out of the 98 studies (85.7%) utilized temporary MCAO models, and 14 studies (14.3%) utilized permanent MCAO models. Isoflurane was used in 28 studies (28.6%), chloral hydrate in 19 studies (19.4%), and halothane in 15 studies (15.3%), which were the three most commonly used anesthetic. MSCs had been used in 57 studies (58.2%), followed by neural stem/progenitor cells (NSPCs) in 25 studies. The stem cells mostly used in these studies

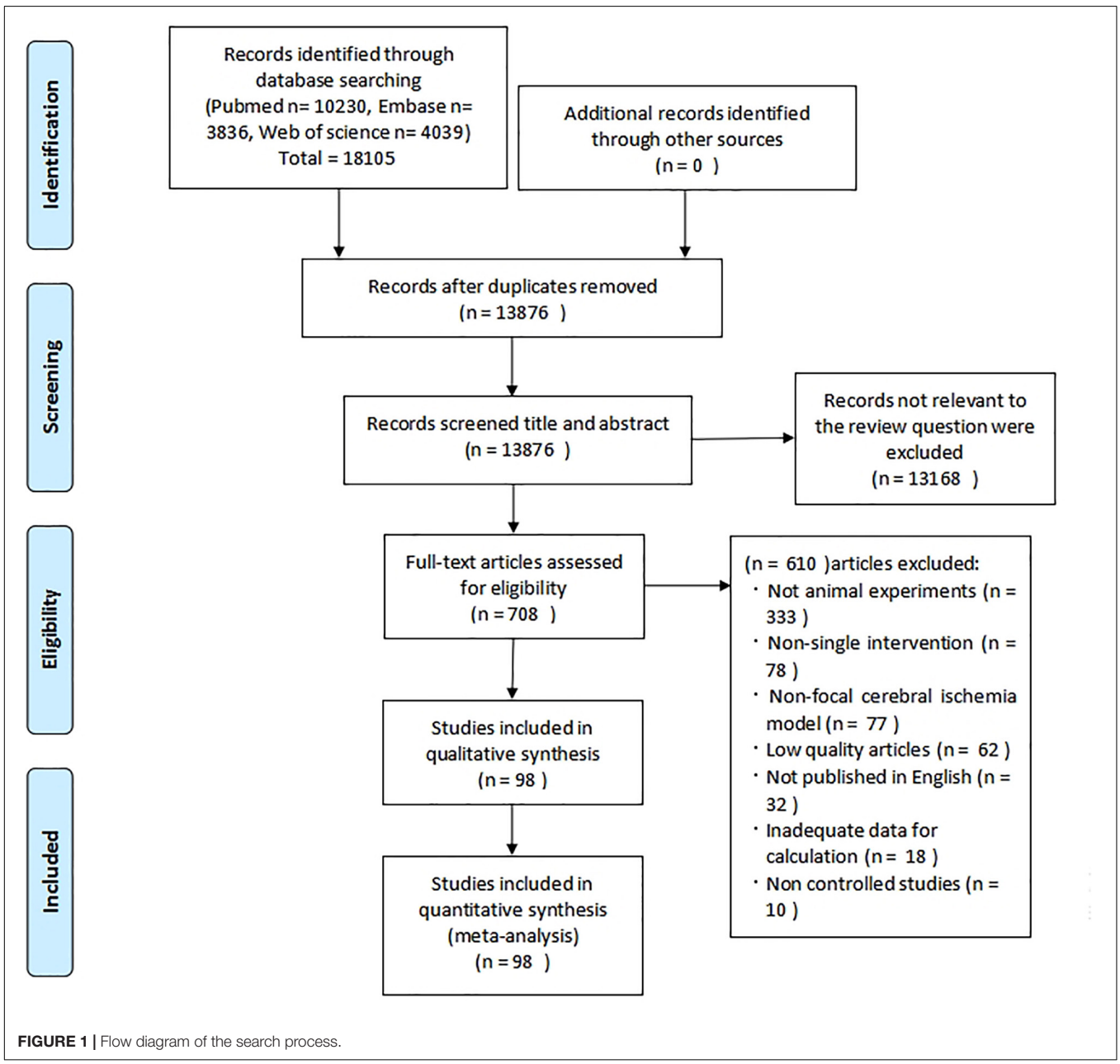

FIGURE 1 | Flow diagram of the search process.

were passage three or four cells (38.1%), and more than half of the studies (67%) used Dulbecco's modified Eagle's medium for the proliferation of stem cells. The dose of stem cells injected ranges from 5×10^4 to 1.2×10^7, and the time of administration was mainly focused on 1 to 7 days after operation. Stereotaxic administration in 46 studies was the most frequently reported method of administration. The study characteristics are shown in **Supplementary Table 1**, and information about the passage that was used for the stem cells is in **Supplementary Table 2**.

Risk of Bias Within Studies

The median quality score across the 98 studies was 5.8 (range, 5–8). Twenty-one studies failed to report whether there was temperature control. Random allocation to treatment group and blinded assessment of outcome were described in 72 and 58 studies, respectively. Nineteen studies had clearly said that there was no use of anesthetic with significant intrinsic neuroprotective activity. Only one study calculated the sample size necessary to achieve sufficient power. The declaration of compliance with animal welfare regulations and the potential conflict of interests were described in 85 and 54 studies, respectively. Other items, such as if it were peer-reviewed and the use of an appropriate animal model, which are animals without relevant comorbidities, were well reported in all these studies. The methodological quality of each study is summarized in **Supplementary Table 3**.

Meta-Analysis

One hundred forty-one comparisons of 98 included studies (Veizovic et al., 2001; Zhao et al., 2002, 2015, 2018; Kurozumi et al., 2004; Li et al., 2005, 2008, 2018, 2016; Boltze et al., 2006; Zhang et al., 2006, 2011; Shen et al., 2007, 2010; Andrews et al., 2008; Koh et al., 2008; Kamiya et al., 2008; Wu et al., 2008, 2015; Chen et al., 2009; Hicks et al., 2009; Liao et al., 2009; Stroemer et al., 2009; Cho et al., 2010; Jin et al., 2010; Leu et al., 2010; Bao et al., 2011; Lim et al., 2011; Gutiérrez-Fernández et al., 2011, 2013, 2015; Ikegame et al., 2011; Jiang et al., 2011, 2019; Liu et al., 2009, 2011; Song et al., 2011; Sugiyama et al., 2011; Inoue et al., 2013; Jensen et al., 2013; Wang H. et al., 2013; Du et al., 2014; Huang et al., 2014, 2018; Mitkari et al., 2014; Tang et al., 2014; Cheng et al., 2015, 2018; Hosseini et al., 2015a,c; Ma et al., 2015; Otero-Ortega et al., 2015; Eckert et al., 2015; Park et al., 2015, 2017; Yang et al., 2015, 2018, 2017; Wang et al., 2015; Bacigaluppi et al., 2016; Choi et al., 2016; Moisan et al., 2016; Ryu et al., 2016, 2019; Hou et al., 2017; Lin et al., 2017, 2020; Souza et al., 2017; Yamashita et al., 2017; Zhang J.J et al., 2017; Zhang T. et al., 2017; Zong et al., 2017; Zhu et al., 2017; Abd El Motteleb et al., 2018; Bi et al., 2018; Chi et al., 2018; Kong et al., 2018; Nito et al., 2018; Sowa et al., 2018; Yamaguchi et al., 2018; Yu et al., 2018; Zhang H.L. et al., 2018; Zhang G. et al., 2018; Cherkashova et al., 2019; He et al., 2019; Saraf et al., 2019; Sibov et al., 2019; Son et al., 2019; Tian et al., 2019; Vahidinia et al., 2019; Vats et al., 2019; Xie et al., 2019; Zuo et al., 2019; Lam et al., 2020; Salehi et al., 2020; Tobin et al., 2020; Paudyal et al., 2020; Oh et al., 2020) involving 5,200 animals examined the effect of stem cell transplantation on the neurological function and the infarct volume in animal models for stroke. The pooled analysis indicated that the animals in the treatment group significantly improved in neurological function more than the animals in the control group (SMD = −3.37, 95% CI −3.83 to −2.90, $P < 0.00001$); heterogeneity: $chi^2 = 802.16$, $df = 64$ ($P < 0.00001$), $I^2 = 92\%$ (**Figure 2**). The pooled analysis indicated that the animals in the treatment group significantly improved in infarct volume more than the animals in the control group (WMD = −11.37, 95% CI -12.89 to −9.85, $P < 0.00001$); heterogeneity: $chi^2 = 8898.96$, $df = 83$ ($P < 0.00001$), $I^2 = 99\%$ (**Figure 3**).

Stratified Meta-Analysis

In the stratified meta-analysis, the impact of study characteristics on the effect sizes was examined.

For neurological function, the stratified analysis showed that significant differences in effect sizes were observed relative to donor species ($P < 0.00001$) and time of administration ($P = 0.0003$). No significant differences in effect sizes were observed relative to the type of ischemia ($P = 0.13$), recipient species ($P = 0.22$), recipient sex ($P = 0.95$), anesthetic ($P = 0.23$), stem cell ($P = 0.59$), cell manipulations ($P = 0.39$), dose range ($P = 0.09$), and method of administration ($P = 0.16$) (**Supplementary Table 4**).

For infarct volume, the stratified analysis showed that significant differences in effect sizes were observed relative to the type of ischemia ($P = 0.001$), recipient sex ($P < 0.00001$), anesthetic ($P < 0.0001$), time of administration ($P = 0.03$), and method of administration ($P < 0.00001$). No significant differences in effect sizes were observed relative to recipient species ($P = 0.25$), donor species ($P = 0.36$), stem cell ($P = 0.47$), cell manipulations ($P = 0.55$), and dose range ($P = 0.54$) (**Supplementary Table 5**).

First of all, we do the subgroup analysis aimed at rat. The effect size of the transient MCAO model was larger than that of the permanent MCAO model both in NFS and infarct volume (**Figures 4A**, **5A**). It may be that persistent cerebral ischemia causes lasting damage to rat cerebral tissue. Similarly, sex with efficacy was higher in females, whether it is in NFS or infarct volume (**Figures 4B**, **5B**), which showed that female rats recovered better after stroke. Besides that, as for anesthetic, the effect size of halothane was larger, while the situation was different in the infarct volume (**Figures 4C**, **5C**). The result means that the use of halothane can better improve the neurological deficit, and the administration of isoflurane can do better in decreasing the infarct volume. This may be related to the intrinsic neuroprotective activity of these two anesthetics. Porcine as a source of stem cells was associated with substantial improvement in NFS, and human stem cells are better at decreasing the infarct volume (**Figures 4D**, **5D**). Meanwhile, higher estimates of effect size by NSPCs were observed in NFS, while efficacy was higher for other types of stem cell in infarct volume (**Figures 4E**, **5E**). A higher effect size of gene-modification cells in rat is shown, which is similar to the reported neuroprotective effects of gene-modification cells in the literatures (**Figures 4F**, **5F**), even though the degree of variance is not high. In addition, a moderate dose of stem cells ($1–5 \times 10^5$) can better improve the neurological deficit, but which certain dose is better for reducing infarct volume is unclear (**Figures 4G**, **5G**). It is better to give stem cell therapy as early as possible after stroke for efficacy was observed to be higher

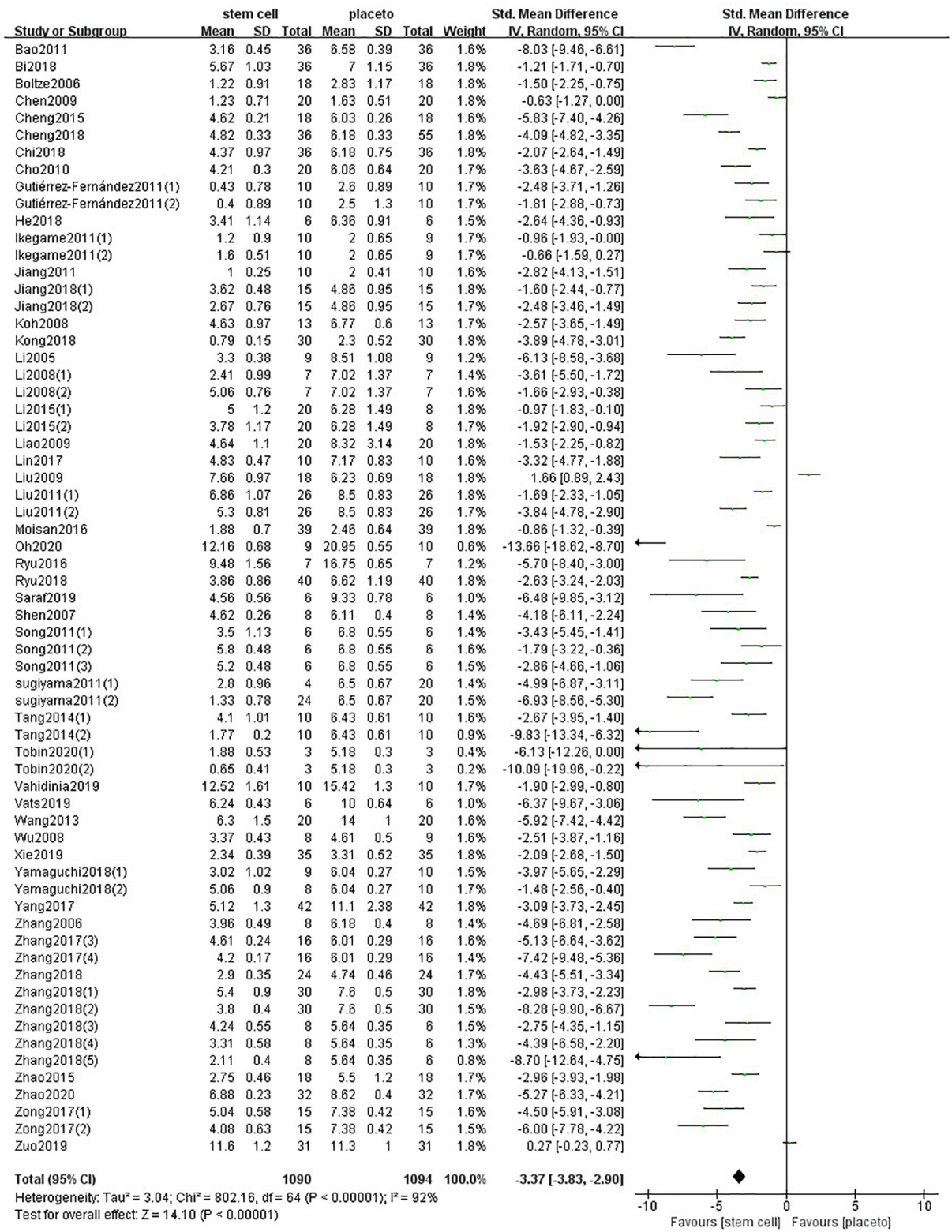

FIGURE 2 | Summary of the data included in the meta-analysis of the use of stem cells to treat ischemic stroke with individual comparisons ranked according to their effect on neurological function.

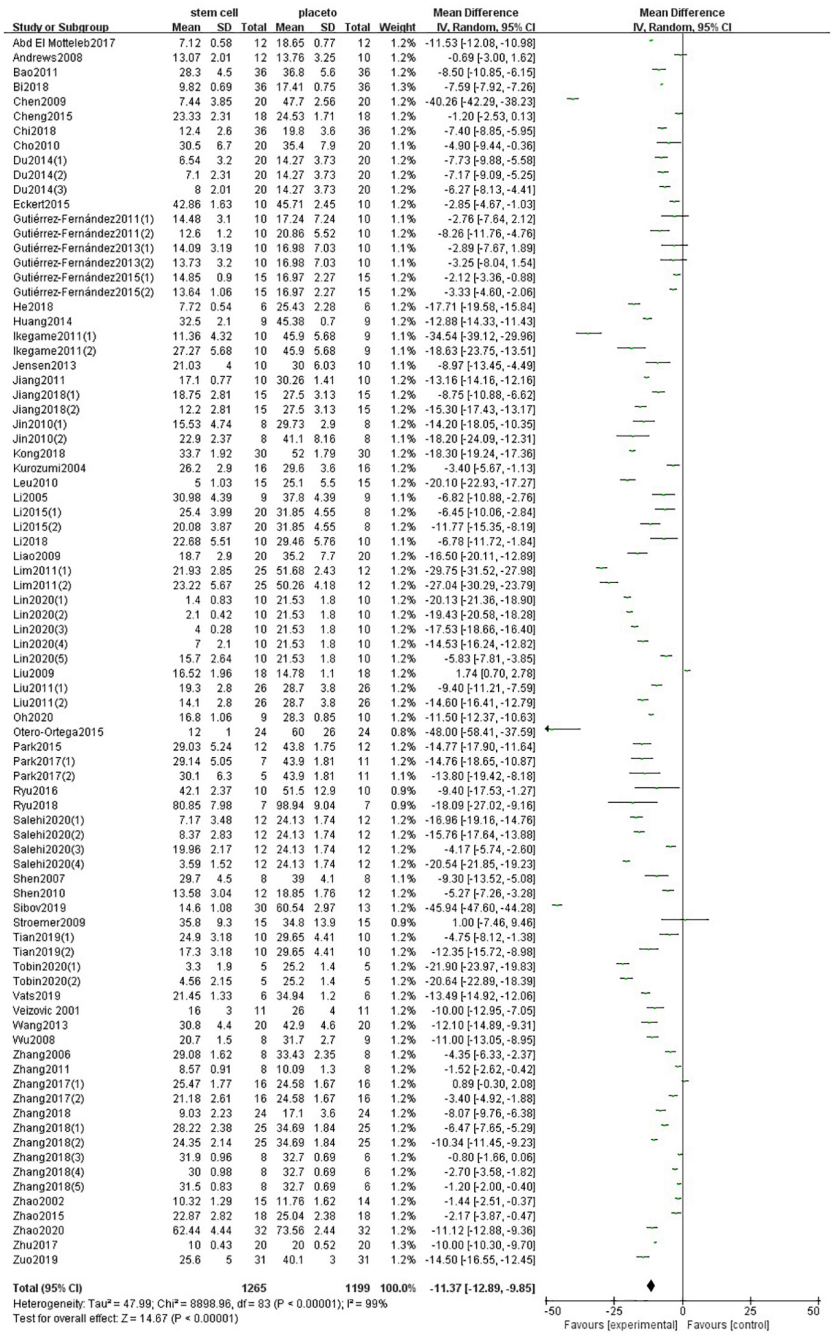

FIGURE 3 | Summary of the data included in the meta-analysis of the use of stem cells to treat ischemic stroke with individual comparisons ranked according to their effect on infarct volume.

within 1 day after stroke in infarct volume, and the effect size was similar to the group administrated at 7 days after stroke in NFS (**Figures 4H**, **5H**). Moreover, stereotaxic injection was more effective in NFS and got very close to systemic administration in improving infarct volume outcome (**Figures 4I**, **5I**).

Besides that, the subgroup analysis aimed at mice cannot be carried out because the date related to mice is not adequate enough.

Finally, we combined the data from the two species and conducted the subgroup analysis. In the analysis for the outcome measure according to NFS, the effect size of transient MCAO model was larger than that of the permanent MCAO model, and the situation was the same in infarct volume (**Figures 6A**, **7A**). The results illustrated that the transient MCAO model could improve the prognosis of stroke more effectively. Similarly, sex with efficacy was higher in males compared with females both in

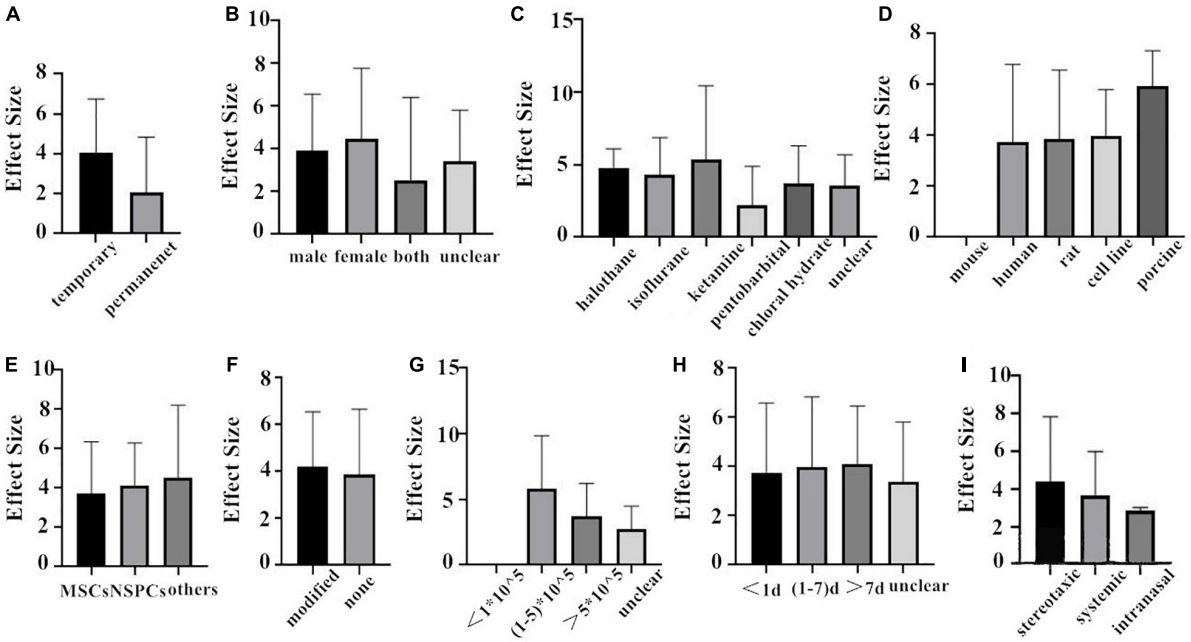

FIGURE 4 | The impact of study characteristics on the effect sizes aimed at rat. **(A)** Type of ischemia, **(B)** sex of animals, **(C)** anesthetic used, **(D)** donor species, **(E)** stem cell, **(F)** cell manipulations, **(G)** dose range, **(H)** time of administration, and **(I)** route of delivery.

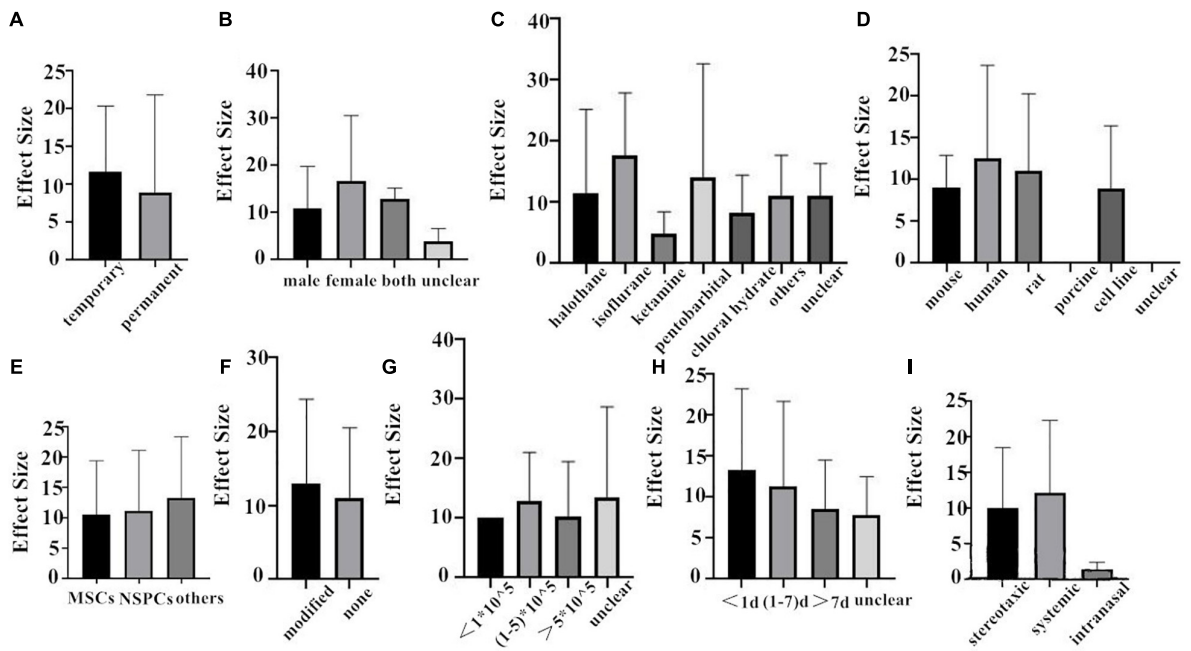

FIGURE 5 | The impact of study characteristics on the effect sizes aimed at rat. **(A)** Type of ischemia, **(B)** sex of animals, **(C)** anesthetic used, **(D)** donor species, **(E)** stem cell, **(F)** cell manipulations, **(G)** dose range, **(H)** time of administration, and **(I)** route of delivery.

NFS and infarct volume (**Figures 6C**, **7C**). It seems that males will recover better than females, but it seems different when concerning recipient species. Compared with mouse, rat was less effective at improving infarct volume but more effective in improving NFS (**Figures 6B**, **7B**). The use of halothane at MCAO induction was associated with a substantial improvement in NFS, while as for infarct volume, isoflurane seems better (**Figures 6D**, **7D**). This may be related to the intrinsic neuroprotective activity of these two anesthetics. Porcine, as a source of stem cells, was with substantial improvement in NFS, while mouse seems better

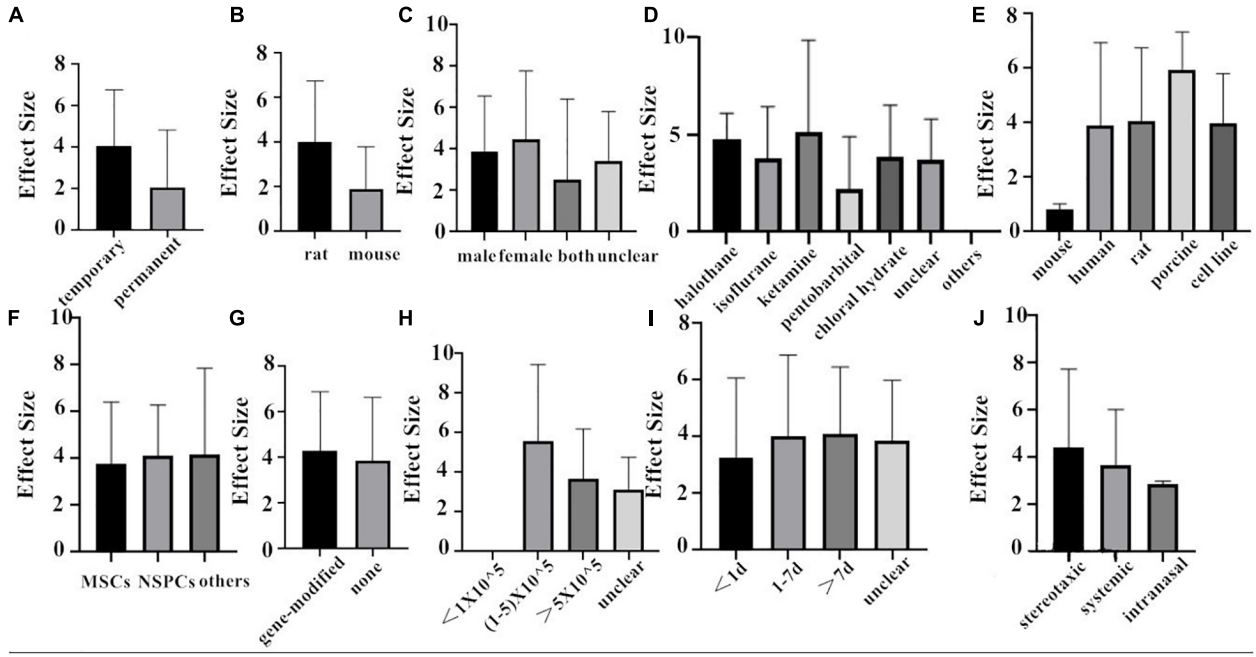

FIGURE 6 | The impact of study characteristics on the effect sizes of two species. **(A)** Type of ischemia, **(B)** species, **(C)** sex of animals, **(D)** anesthetic used, **(E)** donor species, **(F)** stem cell, **(G)** cell manipulations, **(H)** dose range, **(I)** time of administration, and **(J)** route of delivery.

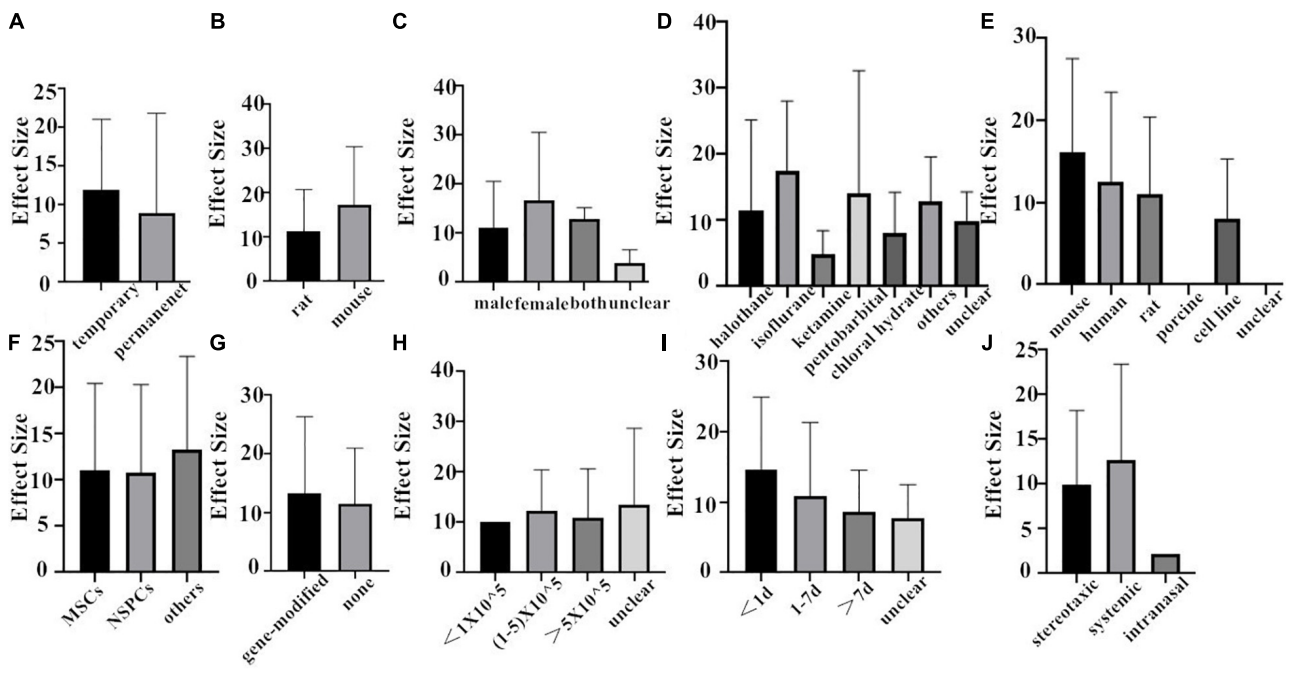

FIGURE 7 | The impact of study characteristics on the effect sizes of two species. **(A)** Type of ischemia, **(B)** species, **(C)** sex of animals, **(D)** anesthetic used, **(E)** donor species, **(F)** stem cell, **(G)** cell manipulations, **(H)** dose range, **(I)** time of administration, and **(J)** route of delivery.

in decreasing infarct volume (**Figures 6E**, **7E**). Meanwhile, in the subgroup analysis for the outcome of the type of stem cell, efficacy was higher for NSPCs in NFS, while the other types of stem cell such as MHP36 cells and SP cells had higher estimates of effect size in infarct volume (**Figures 6F**, **7F**). A higher effect size of gene-modification cells in rat is shown, which is similar to the reported neuroprotective effects of gene-modification cells in the literatures (**Figures 6G**, **7G**), even though the degree of variance

is not high. In addition, the effect size was observed to be larger with the dose of 1–5 × 10^5 in NFS and the unclear dose of stem cell in infarct volume (**Figures 6H**, **7H**). It illustrated that the injection of stem cells at a moderate dose could help reduce the neurological deficit effectively. As for the subgroup of the unclear dose, it is a group wherein the articles did not explicitly mention the dose of treatment, so we classified all of these articles into the unclear dose group. Improvement was seen in the stem cell therapy that was administrated at 7 days after cerebral ischemia in NFS, while in infarct volume, it was clear when the administration was done within 1 day after stroke (**Figures 6I**, **7I**). It means that stem cell therapy in the acute phase (24 h–7 days) can better rescue the neurons in the ischemic penumbra of the brain. Moreover, stereotaxic injection was more effective in NFS and got very close to systemic administration in improving infarct volume outcome (**Figures 6J**, **7J**).

Meta-Regression

To further explore heterogeneity among the studies, meta-regression was conducted to investigate the effect of characteristics on neurological function and infarct volume.

For neurological function, dose range (P = 0.045) was a significant source of heterogeneity, while type of ischemia (P = 0.057), recipient species (P = 0.108), recipient sex (P = 0.959), anesthetic (P = 0.154), donor species (P = 0.189), stem cell (P = 0.76), cell manipulations (P = 0.582), time of administration (P = 0.315), and method of administration (P = 0.83) had little effect on heterogeneity (**Supplementary Table 4**).

For infarct volume, we found that anesthetic (P = 0.028) and time of administration (P = 0.024) accounted for a significant proportion of the between-study heterogeneity in studies, while type of ischemia (P = 0.243), recipient species (P = 0.852), recipient sex (P = 0.44), donor species (P = 0.097), stem cell (P = 0.623), cell manipulations (P = 0.844), dose range (P = 0.884), and method of administration (P = 0.923) had little to do with heterogeneity (**Supplementary Table 5**).

Publication Bias

Funnel plot is a method to identify publication bias or other bias and judge whether there is bias in meta-analysis according to the degree of asymmetry. It (**Figure 8**) showed asymmetry, indicating a potential publication bias. Egger test is a statistical test of funnel plot asymmetry to determine whether publication bias or other bias has statistical significance. Through Egger's test, the publication not existed in neurological function data (**Figure 9**, $p < 0.163$), but in infarct volume data (**Figure 10**, $p = 0.001$).

DISCUSSION

Efficacy of Stem Cell-Based Therapy

The present study demonstrated that stem cell-based therapy improved neurological deficits and reduced infarct volume, reinforcing the evidence for a neuroprotective role of stem cell therapy in experimental ischemic stroke. However, our summary estimates should be considered as the average efficacy rather than the best estimate of a single "true" efficacy (Zhao et al., 2002) because of the existence of heterogeneity.

Methodological Considerations

In the present study, the overall quality of the included studies was relatively high. Thus, to some extent, the global estimated effect may be guaranteed because there were relatively few articles that did not use the blind method, did not randomly evaluate the result, or did not report the number of exits, which are key elements in the quality assessment of preclinical studies (Wardlaw et al., 2000). In addition, as a previous study once discussed (Macleod et al., 2004), the quality of study design is an important factor affecting the outcome. Especially when such trials are included in systematic reviews, the improvement of methodological quality will reduce bias (Xie et al., 2015).

Stratified Analysis

We found that the studies which used the transient MCAO model showed larger behavioral gains as well as infarct volume. The transient MCAO is also the most widely used animal models for experimental ischemic stroke and well simulates actual pathological conditions after thrombolysis in clinical practice. The recovery of cerebral blood flow plays a role on the improvement of neurological function, but the cerebral parenchyma was sensitive to reperfusion injury in the meantime, so there was no significant improvement in the infarct volume in a short experimental observation time.

Compared with mouse, rat was less effective at improving infarct volume but more effective in improving NFS. However, these accounted for a small proportion of the overall dataset, so these findings should be interpreted with caution. Meanwhile, the use of different types of anesthetic at MCAO induction was associated with recovery. In terms of neurological function score, the use of halothane showed a substantial improvement. For infarct volume, studies using isoflurane led to higher estimates of effect, possibly associated with its significant intrinsic neuroprotective activity (Macleod et al., 2004).

The most widely used stem cells in the studies were MSCs, but in the overall analysis, NSPCs were associated with a substantially larger effect in NFS, while other types of stem cells such as MHP36 cells and SP cells showed a higher improvement in infarct volume. These contrasts were less obvious in the effect size of manipulation of modified stem cell even though previous studies demonstrated that gene-modified stem cell can increase cell survival and enhance their function in cell therapy (Phillips and Tang, 2012). Studies that used these genetically modified stem cells do not show an obvious high estimate of effect in neurological function and infarct volume. Zhao et al. (2010) found increasing benefits from concentrations of as low as 10,000 implanted stem cells, while we see that the dose that significantly eased the neurological function recovery is 1–5 × 10^5. When concerning the decrease of infarct volume, it seems to be the abnormally high effect sizes reported in studies where the dose range was unclear. However, it should be interpreted with caution because these results are based on a small number of comparisons.

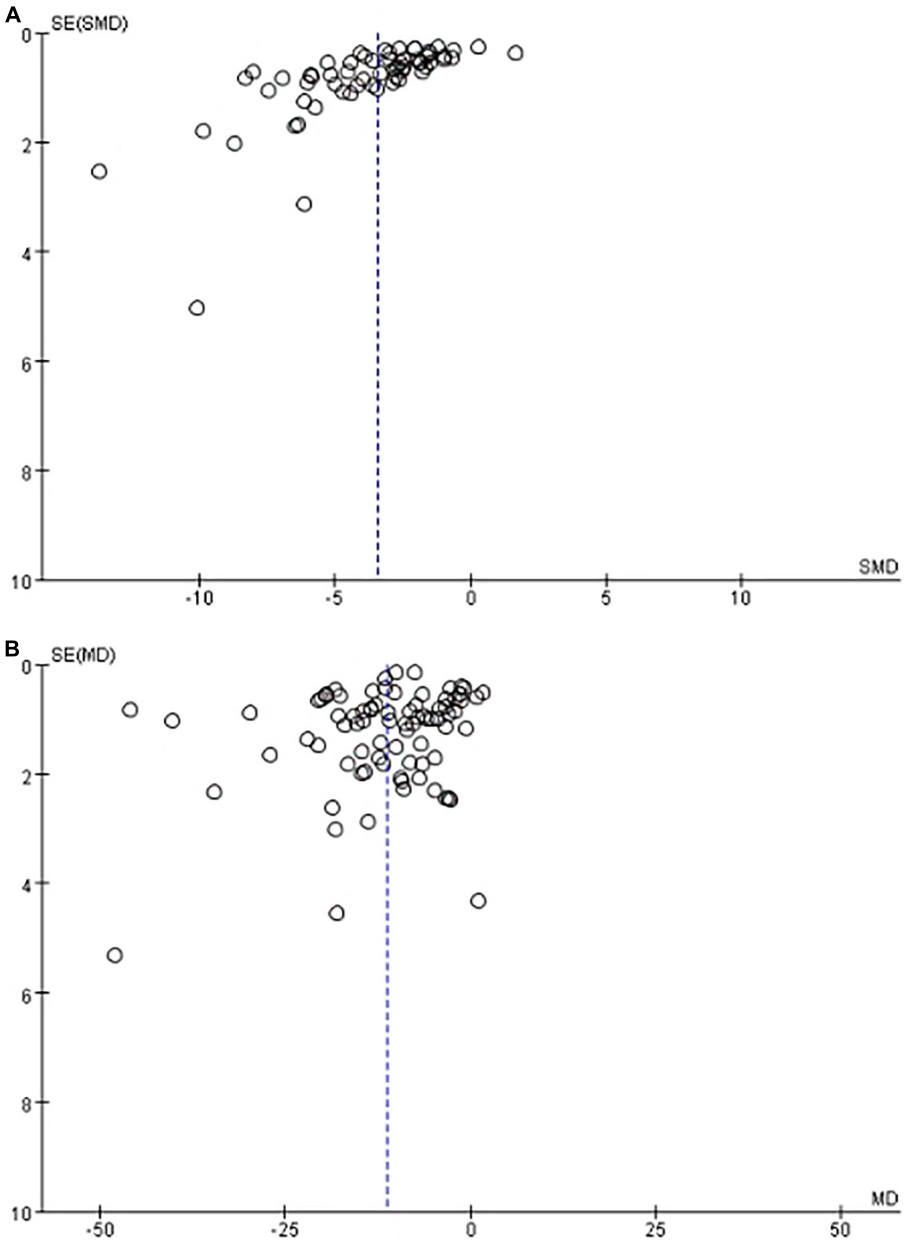

FIGURE 8 | The funnel plot of the effect of stem cell on neurological function **(A)** and infarct volume **(B)** after experimental ischemic stroke.

The optimal time and the best route for cell transplantation against ischemic stroke have always been the focus of controversy. Our results suggested that stem cell therapy within 24 h after cerebral ischemia can decrease the volume, and the administration that was employed at 7 days after ischemia can improve the function, which is only partly consistent with what Hosseini et al. (2015b) had reported. In addition, we found that the effect of three different methods of treatment was similar in promoting the recovery of neurological function, but in infarct volume, systemic delivery brought a higher efficacy than the way of stereotaxic and intranasal administration. Intranasal administration was actually *via* a specific site, the olfactory region, thus bypassing the blood–brain barrier and delivering therapeutic agents directly to the central nervous system (Illum, 2000; Thorne and Frey, 2001). Although it acts directly on brain tissue, its effect is not as good as expected. Given the size of the stem cell itself, the underlying feasibility and following therapeutic effect is worth investigating in greater depth in the future.

Limitations of Our Study

Firstly, our search strategy was only able to include the majority of published studies in three English databases; other languages and unpublished studies were not taken into account, which may lead

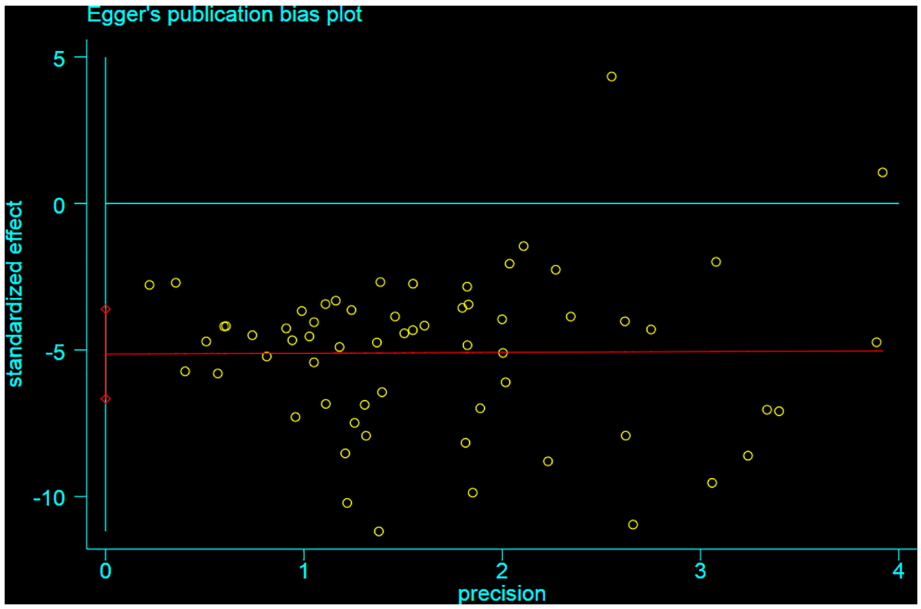

FIGURE 9 | Egger test for the assessment of the statistical significance of publication bias of neurological function.

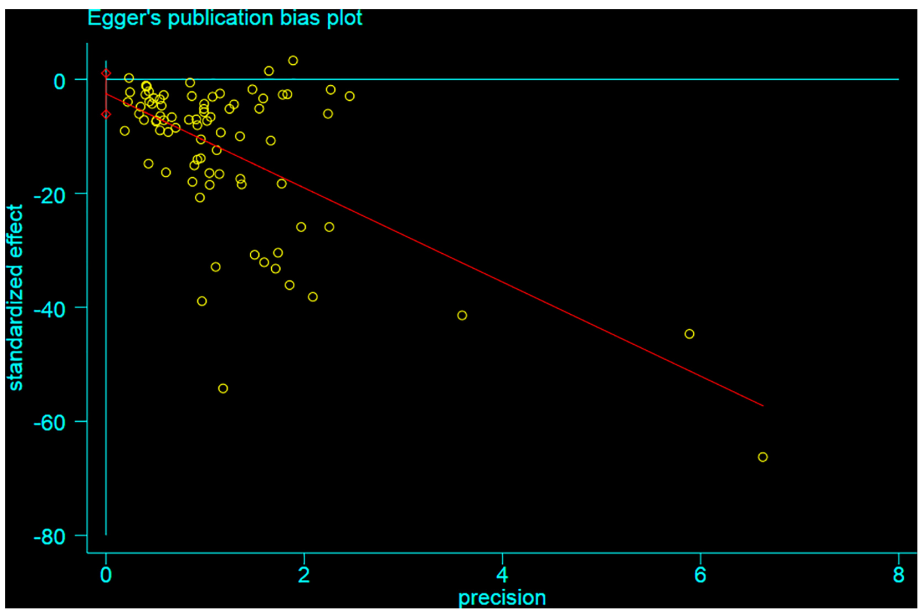

FIGURE 10 | Egger test for the assessment of the statistical significance of publication bias of infarct volume.

to a certain degree of selective bias (Guyatt et al., 2011). Secondly, there are many methods to build a stroke induction model, such as suture method, electric coagulation, photothrombosis, microembolism, thermocoagulation, and so on. Among the final studies included, there are more articles using rat to induce a stroke model, where suture method is more effective and convenient, so there are fewer articles using other methods. Therefore, our results may not be able to comprehensively assess the impact of various methods of stroke induction on the outcome measurements. Thirdly, analytic data in our meta-analysis were not available, such that of unpublished information of both study quality and study design features (the sex of a cohort of animals or the route of cell implantation); we have either analyzed that as unclear or inferred that these things that were not reported did not occur. Besides that, we present a series of univariate analyses; meta-regression might provide more robust insights, but these techniques are not well established (Zhao et al., 2002). Furthermore, our research is observational

rather than experimental, so we can only report associations rather than causation. Finally, we limited our analysis to studies that have both neurological function and infarct volume outcome measures. Thus, we will disregard other benefits seen in either of them as a main outcome indicator.

CONCLUSION

The present finding indicated that stem cell provided statistically significant benefits for stroke whether in neurological function or infarct volume. Therefore, the findings of the present systematic review, at least to a certain extent, provided supporting evidence for the future use of stem cell for stroke. However, the validity of these positive findings should be interpreted with caution due to some methodological limitations.

Besides that, even though the result we yielded is promising, certain safety concerns must also be properly addressed for its further use in the clinic in the future. First of all, tumor formation has been reported with the transplantation of stem cells. For example, one study declared that mice iPSCs expanded and formed much larger tumors in mice post-ischemic brain than in sham-operated brain until 28 days after transplantation (Kawai et al., 2010), but in a clinical trial conducted by Honmou et al. (2011) the safety of transplantation was shown. After 1 year of treatment, 12 patients who received stem cell therapy showed no adverse reactions such as central nervous system tumor, abnormal cell growth, venous thromboembolism, systemic malignant tumor, or systemic infection (Honmou et al., 2011). Therefore, before stem cells are allowed to be extensively used in the clinic, tumorigenesis is one of the next research points. In addition, whether the application of exogenous stem cell will cause immunological rejection and the ethical problem it brings also need to be paid attention to. Even though the current preclinical data is positive, studies of stem cell after stroke in humans thus far have focused on autologous cells, and so trials examining the safety of allogeneic stem cell in humans are needed.

On the other hand, there are NSPCs that existed in the subventricular and dentate gyrus zone of the brain. During ischemia, the proliferation and differentiation of endogenous neural stem cells is not sufficient enough to induce neural repair, which could contribute to the permanent disability of stroke patients (Bond et al., 2015). Thus, whether we can promote the proliferation and differentiation of stem cells in the brain through some exogenous interventions to improve the prognosis of stroke may be a novel therapeutic approach. Furthermore, whether we can promote the proliferation of endogenous stem cells through some exogenous interventions may also be a novel therapeutic approach. For example, Zhang et al. (2020) showed that treatment with artesunate promoted the proliferation of NSPCs *via* some molecular mechanisms, subsequently reducing the infracted brain volume and alleviating motor function impairment caused by MCAO. Zhuang et al. (2012) had undertaken a study to examine the promotive effect of salvianolic acid B (Sal B) on NSPC proliferation and neurogenesis. The result showed that Sal B was capable of promoting the proliferation of NSPC and neurogenesis *via* the PI3K/Akt signaling pathway. These findings suggested that it is possible that we could promote the recovery of stroke *via* endogenous stem cells. Besides that, the application of endogenous stem cells will avoid the possibility of tumorigenesis, immunological rejection, ethical problems, and other adverse reactions.

In addition, compared with some previous systematic reviews on stem cells for stroke, our review is more innovative. First of all, previous reviews on stem cell therapy for stroke are mostly about MSCs. We included studies of all kinds of stem cells and analyzed the different effects of different types of stem cell on neurological deficit and infarct volume. Secondly, through the assessment of the methodological quality of the literature, we only included studies whose quality score is higher than the average level in order to make the results more reliable and convincing. Finally, while other studies only discuss the effect of stem cell therapy on neurological deficit, our paper selected two outcome indicators—neurological function and infarct volume—and we are eager to make a more comprehensive assessment of stem cells on ischemic stroke.

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/s.

AUTHOR CONTRIBUTIONS

X-LZ, X-GZ, Y-RH, Y-YZ, P-JY, X-JZ, XL, and Y-JW analyzed the data and carried out the statistical analysis. G-QZ acted as an arbitrator in the review. G-QZ, X-LZ, and X-GZ conceived and designed the article, supervised the study, and contributed to finalize the manuscript. All authors participated in the study design, searched the databases, extracted and assessed the studies, and drafted the manuscript and reviewed the manuscript.

FUNDING

This project was supported by the grant of National Natural Science Foundation of China (H2902), the Young and Middle-Aged University Discipline Leaders of Zhejiang Province, China (2013277), and the Zhejiang Provincial Program for the Cultivation of High-Level Health Talents (2015).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2021.628908/full#supplementary-material

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

frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
published: 29 April 2021
doi: 10.3389/fncel.2021.654290

Transplantation of Human Cortically-Specified Neuroepithelial Progenitor Cells Leads to Improved Functional Outcomes in a Mouse Model of Stroke

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Edited by:
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University of Georgia, United States

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

Received: *15 January 2021*
Accepted: *08 April 2021*
Published: *29 April 2021*

Citation:
Islam R, Drecun S, Varga BV, Vonderwalde I, Siu R, Nagy A and Morshead CM (2021) Transplantation of Human Cortically-Specified Neuroepithelial Progenitor Cells Leads to Improved Functional Outcomes in a Mouse Model of Stroke. Front. Cell. Neurosci. 15:654290. doi: 10.3389/fncel.2021.654290

Stroke is a leading cause of death and long-term disability worldwide. Current therapeutic options are limited in terms of their time for implementation and efficacy in promoting recovery. Cell transplantation has been shown to have promise in several animal models however significant challenges remain, including the optimal source of cells to promote neural repair. Here, we report on the use of a population of human ESC derived, cortically specified, neuroepithelial precursor cells (cNEPs) that are neurally restricted in their lineage potential. CNEPs have the potential to give rise to mature neural cell types following transplantation, including neurons, astrocytes and oligodendrocytes. With a view towards translation, we sought to determine whether this human cell source was effective in promoting improved functional outcomes following stroke. Undifferentiated cNEPs were transplanted in a pre-clinical endothelin-1 (ET-1) model of ischemic motor cortical stroke in immunocompromised SCID-beige mice and cellular and functional outcomes were assessed. We demonstrate that cNEP transplantation in the acute phase (4 days post-stroke) improves motor function as early as 20 days post-stroke, compared to stroke-injured, non-transplanted mice. At the time of recovery, a small fraction (<6%) of the transplanted cNEPs are observed within the stroke injury site. The surviving cells expressed the immature neuronal marker, doublecortin, with no differentiation into mature neural phenotypes. At longer survival times (40 days), the majority of recovered, transplanted mice had a complete absence of surviving cNEPS. Hence, human cNEPs grafted at early times post-stroke support the observed functional recovery following ET-1 stroke but their persistence is not required, thereby supporting a by-stander effect rather than cell replacement.

Keywords: stroke, mouse behavior, human stem cell transplantation, stem cell survival and differentiation, human neuroepithelial cell, immunogenic response

INTRODUCTION

Stroke is a leading cause of death and disability in adults (Khoshnam et al., 2017). Current treatment options are limited and do not promote complete recovery after stroke, leaving patients with poor quality of life and socioeconomic hardship. One potential treatment to improve outcome following stroke involves transplantation of stem and progenitor cells (termed precursor cells). This approach has been shown to be effective in animal models of stroke (Mohamad et al., 2013; Xiong et al., 2017; Payne et al., 2018; Vonderwalde et al., 2019), however, several hurdles remain, including the identification of an optimal cell source to promote neural repair and functional recovery.

A variety of cell sources have been explored in transplant studies to treat stroke. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) can give rise to all cell types in the central nervous system (CNS), however they have reported tumorigenic capacity upon transplantation, limiting their efficacy (Thomson et al., 1998; Gao et al., 2016a). A recent approach to reducing the tumorigenic potential of transplanted cells involves the use of genome-editing strategies to insert a suicide gene into a cell division essential locus, allowing selective ablation of proliferating cells through administration of a pro-drug (Payne et al., 2019a). Other approaches to circumvent tumorigenicity include the transplantation of neurally committed cells with less tumorigenic potential (Gao et al., 2016b). For stroke application, previous studies have shown efficacy of both mesenchymal stem cells and neural precursors in improving motor recovery by enhancing host neuroplasticity as measured by increased synapse formation, without the need for integration and maturation (Wakabayashi et al., 2009; Oki et al., 2012; Vonderwalde et al., 2019). Additionally, studies suggest that neural stem cells mediate recovery through maturation and integration of transplanted cells (Zhou et al., 2015; Ficek et al., 2016). Herein, we have examined the potential of human ESC derived, cortical neuroepithelial cells (cNEPs). These neurally committed cells are present during brain development and have the capacity to generate cortical neural cell types *in vitro*. Hence, cNEPs provide regionally specific brain cells for transplantation in a cortical stroke injury model (Tornero et al., 2017; Payne et al., 2019b) and the opportunity to evaluate whether cNEPs promote improved functional outcomes by examining grafted cell survival, maturation and integration.

CNEPs are found in the developing neural tube, where they generate radial glia progenitors, neurons, astrocytes and oligodendrocytes (Martínez-Cerdeño and Noctor, 2018). *In vitro*, cNEPS can be derived from pluripotent stem cells, including iPSCs and ESCs (Guillaume et al., 2006; Payne et al., 2018). A previous study used iPSC derived cNEPS in a rat model of motor stroke that lesioned the cortex and striatum, and demonstrated minimal improvements in functional outcomes when transplanted in a hydrogel (Payne et al., 2019b). Whether ESC derived cNEPs can improve stroke outcomes in a mouse model of motor cortex stroke, has not been explored. Further, with a view towards translation and in accordance with stroke therapy academic industry roundtable (STAIRS) and the stem cell therapies as an emerging paradigm in stroke (STEPS) guidelines, it is important to demonstrate the efficacy of cNEPs in more than one model of stroke to advance therapeutics (Lapchak et al., 2013).

Here, we use an endothelin-1 (ET-1) model of focal ischemic stroke in the motor cortex that mimics the human condition. ET-1 is a potent vasoconstrictor that results in the formation of a lesion cavity, surrounding penumbra and formation of a glial scar—hallmarks of the cellular response to ischemic insult (Adkins et al., 2004; Horie et al., 2008; Erlandsson et al., 2011; Sachewsky et al., 2014). The model is reproducible and generates measurable functional impairments that permit the evaluation of the efficacy of transplanted cells in promoting neural repair. CNEPs were transplanted on post-stroke day 4 (PSD4; acute phase) and we observed a rapid and significant motor recovery by PSD20. Interestingly, at the time of recovery, the vast majority of cNEPS were no longer present in the injured tissue and surviving transplanted cells had immature neural phenotypes. Groups of mice that survived to PSD40 continued to display functional recovery in the absence of cell maturation and further, 67% of the recovered mice were devoid of surviving transplanted cells. Our study demonstrates that integration and maturation of cNEPs is not necessary for the recovery observed in this ET-1 model of stroke.

MATERIALS AND METHODS

H1 ESC Induction

Human H1 ESC (WiCell, Madison, WI, USA) were grown on Geltrex-coated (Thermo Fisher Scientific, Waltham, MA, USA) culture plates in mTESR1 medium (StemCell Technologies, Vancouver, BC, Canada) containing 1% PenStrep (Sigma–Aldrich, St. Louis, MO, USA). Cells were lifted with TrypLE (Thermo Fisher Scientific, Waltham, MA, USA) and passaged at 1.5×10^4 cells per cm^2, into induction and maintenance media containing a cocktail of small molecule inhibitors to generate cNEPs, as previously described (Payne et al., 2018; Varga et al., 2021). Briefly, H1 ESC were induced into cNEPs using media containing 50% DMEM-F12, 50% Neurobasal (Thermo Fisher Scientific), 0.5× N2 supplement (Gibco), 0.5× B27 supplement (Gibco), 1 mM Glutamax (Thermo Fisher Scientific), 25 mM 2-mercaptoethanol (Sigma). H1 ESC were passaged in media supplemented with 10 μM SB431542 and 100 nM LDN193189 and 10 μM Y27632. Media was changed every other day and kept for 8 days prior to passaging into maintenance media.

CNEP *In vitro* Culture and Differentiation

CNEPs were cultured on laminin-coated culture plates, lifted with TrypLE (Thermo Fisher Scientific, Waltham MA, USA) and passaged at 3×10^4 cells/cm^2 for 4–10 passages prior to transplantation. For transplant, cNEPs were pelleted and resuspended in regular artificial cerebrospinal fluid (aCSF) at 1×10^5 cells/μl and kept on ice for up to 2 h before intracranial injection. Media contained 50% DMEM-F12, 50% Neurobasal, 25 mM 2-mercaptoethanol, 1 mM Glutamax, 1× N2 supplement, 0.05× B27 minus vitamin A supplement (Gibco), further

supplemented with 2 μM CHIR99021 (Peprotech), 1 μM XAV939, 1 μM SB431542 (Peprotech), 10 ng/ml FGF2 (PeproTech), 50 nM LDN193189, 50 nM K02288, 50 nM AKTiVIII (Calbiochem) and 75 nM MK2206 (all other material from Selleck Chem). For transplant, cNEPs were pelleted and resuspended in regular artificial cerebrospinal fluid (aCSF) at 1×10^5 cells/μl and kept on ice for up to 2 h before intracranial injection.

For *in vitro* characterization, cNEPs were cultured in differentiation media containing 50% DMEM/F12 (Thermo Fisher Scientific, Waltham, MA, USA), 50% Neurobasal (Thermo Fisher Scientific, Waltham, MA, USA), 0.2× Insulin with Zinc (Thermo Fisher Scientific, Waltham, MA, USA), 0.2× N2 supplement (National Library of Medicine (2017), 0.1× B27 with Vitamin A (Thermo Fisher Scientific, Waltham, MA, USA), 25 mM 2-Mercaptoethanol (Sigma–Aldrich, St. Louis, MO, USA), 1 mM Glutamax (Thermo Fisher Scientific, Waltham, MA, USA) and 0.075× FBS (Wisent, Saint-Jean-Baptiste, QC, Canada). Media was changed every other day for up to 14 days. Cells were cultured in maintenance medium and fixed 48 h after plating (undifferentiated cells) or 14 days after plating (differentiated cells) with 4% paraformaldehyde, followed by immunocytochemistry. Cells were blocked with 5% normal goal serum (Sigma–Aldrich, St. Louis, MO, USA) in PBS and 0.5% TritonX-100 (Sigma–Aldrich, St. Louis, MO, USA) for 1 h at room temperature. Cells were incubated with primary antibodies diluted in blocking solution, overnight at 4°C. Immunocytochemistry was performed using DAPI (Invitrogen, Carlsbad, CA, USA), anti-OCT4 (1:200; BD Biosciences, 611202), anti-human Nestin (1:50; Millipore, Sigma, ABD69), anti-SOX2 (1:1,000; Abcam, AB97959), anti-BIII tubulin (1:1,000; Sigma, T8860), anti-DCX (1:250; AB18723), anti-Olig2 (1:200; AB9610) and anti-GFAP (1:1,000; Dako, Z0334). Secondary antibodies, Alexa Fluor488 (Invitrogen, Carlsbad, CA, USA) and Alexa Fluor568 (Invitrogen, Carlsbad, CA, USA) were diluted in 5% NGS. Cell markers colocalized with DAPI were counted from three images per well for differentiated cNEP cell counts.

Animals

Male Fox Chase SCID/Beige (Jackson Labs, Bar Harbor, ME, USA) mice, aged 10–15 weeks were used for all studies. Mice were housed with *ad libitum* food and water. Following stroke surgery, mice were housed individually. All experiments were conducted in accordance with the University of Toronto, Temerty Faculty of Medicine Animal Care Committee and with Canadian Council on Animal Care guidelines.

ET-1 Stroke

ET-1 stroke was performed as previously described (Vonderwalde et al., 2019). Briefly, the skull was exposed, a small burr hole was drilled at the site of the right sensorimotor cortex at AP: + 0.6 mm, ML: −2.2 mm lateral to bregma and DV: −1.0 mm. Mice received a 1 μl injection of 800 picomolar ET-1 in distilled H_2O (Millipore, Sigma, St. Louis, MO, USA) using a 2.5 μl Hamilton Syringe with a 26 gauge, 0.375″ long needle (Hamilton, Reno, NV, USA). ET-1 was injected at a rate of 0.1 μl/min. The needle was removed 10 min after the last injection. Mice were given saline, 5 mg/kg meloxicam as analgesic and allowed to recover. Mice were divided into two groups, Stroke alone and Stroke + cNEP transplant.

CNEP Transplant

On PSD4, mice in the Stroke + cNEP group received cNEP transplants. CNEPs were lifted with TrypLE (Thermo Fisher Scientific, Waltham, MA, USA) and added 1:1 with DMEM containing 10% FBS (Wisent, Saint-Jean-Baptiste, QC, Canada), counted, pelleted, and resuspended in aCSF at a concentration of 1×10^5 cells/μl, as previously described (Payne et al., 2019b). Cells were injected through the previously drilled burr hole at the same coordinates as the ET-1. One-hundred thousand cells in 1 μl of a CSF was injected using a Hamilton syringe. Control mice received 1 μl of a CSF.

Tissue Processing and Analysis

On PSD4, PSD8, PSD20 and PSD40, mice were deeply anaesthetized with 250 mg/kg Avertin (Sigma–Aldrich, St. Louis, MO, USA) and intracardially perfused with phosphate buffered saline (PBS) (10 ml/min for 3 min), followed by 4% paraformaldehyde at a rate of 6 ml/min for 5 min. Brains were removed and post-fixed at 4°C overnight in 4% paraformaldehyde and then stored in 30% sucrose until use. Brains were cryosectioned (−20°C) at 20 μm sections, placed on SuperFrost slides (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −20°C.

At the time of processing, sections were washed with PBS and blocked using 1% bovine serum albumin (Sigma–Aldrich, St. Louis, MO, USA), 4% normal goal serum (Sigma–Aldrich, St. Louis, MO, USA) and 0.5% TritonX-100 (Sigma–Aldrich, St. Louis, MO, USA) in PBS for 1 h at room temperature. Sections were incubated with primary antibodies: anti-human Nestin (1:50; Millipore, Sigma, ABD69), anti-HuNu (1:200; Millipore, Sigma, MAB1281), anti-SOX2 (1:1,000; Abcam, AB97959), anti-DCX (1:250; AB18723), anti-NeuN (1:100; Millipore, Sigma, ABN78), anti-GFAP (1:400, Sigma, G3893), anti-MBP (1:50; Abcam, AB7349), anti-Ki67 (1:200, AB15580) and anti-Iba1 (1:500, Wako 019–19741) in blocking solution overnight at 4°C. After 3 × 5 min PBS washes, tissue was incubated with secondary antibodies, Alexafluor488 and 568 (1:1,000; Invitrogen) at room temperature for 1 h. Another 3× PBS washes were performed and sections were mounted using Vectashield with DAPI (Vector, H-1,200). Images were acquired on an inverted Zeiss LSM880 laser scanning confocal and on an Axio Observer Zeiss microscope at 10× and 20× magnification for counting. Colocalized antibody staining with DAPI was counted manually. All brain sections through the stroke injury site were assessed and all sections (range 1–10 sections/brain) that contained transplanted cells were quantified. Cell counts were performed on sections from both treatment groups surrounding the stroke lesion and from sections containing HuNu+ cells from transplanted mice. Three 400 μm × 400 μm areas surrounding the stroke lesion (lateral, medial and inferior) were counted. For Iba1+ cells, the total numbers, as well as those ameboid in shape (with one or less processes), were counted in the same three areas surrounding the lesion

and in a minimum of three sections per brain from both treatment groups.

Histology

The sections were hydrated for 5 min each in 100% ethanol, 95% ethanol and 70% ethanol. Then, rinsed in distilled H_2O for 1 min and stained using 0.25% cresyl violet in 0.0025% glacial acetic acid and distilled H_2O for 15 min. Wash steps involved 2 min each of submersion in distilled H_2O and 95% ethanol. The sections were then placed in 0.25% glacial acetic acid in 95% ethanol for 1 min, dehydrated for 20 s in 100% ethanol, cleared using xylene and mounted using DPX mounting medium (Sigma–Aldrich, St. Louis, MO, USA). Images were taken at 5× magnification on an Axio Observer Zeiss microscope. AxioVision software and ImageJ were used to trace the stroke lesion as previously described (Vonderwalde et al., 2019) and volume was calculated using the average lesion area × the number of sections with lesions × the distance between the sections (160 μm).

Foot-Fault

The foot fault test was performed to assess motor function and coordination. Mice were placed on a metal grid (1 cm spaces) suspended 12 inches above a table surface and video recorded while mice traversed the grid for 3 min. Uninjured animals demonstrate good coordination and slip minimally during this task. The number of steps and the number of foot slips made with the forelimbs were counted and the difference in foot slip ratio was calculated as (number of contralateral paw slips − number of ipsilateral paw slips)/total number of steps × 100. Mice that did not exhibit a behavioral deficit in foot-fault greater than 1 standard deviation from their mean baseline values were removed from further analysis.

Catwalk

Catwalk was performed by placing mice at the entrance of a narrow glass walkway with a dark goal box on the opposite end of the walkway. As mice walked across the glass walkway to the goal box, paw prints as beam breaks were recorded on a video camera placed below the walkway. CatwalkXT software v10.6 (Noldus) was used to analyze print data and obtain quantitative parameters for gait analysis (Kappos et al., 2017). Terminal dual stance measured the duration of simultaneous contact of contralateral paws. Support Lateral measured the percent of the walk where simultaneous contact was made with lateral hindlimb and forelimb.

Statistical Analysis

Statistical analysis was conducted using V6 GraphPAD Prism and IBM SPSS v23. Foot fault and Catwalk were analyzed by repeated measures ANOVA, Tukey's *post hoc* test and across group comparisons at individual timepoints were analyzed with Student's *T*-test, Mann–Whitney *post hoc*. All data was represented as mean ± SEM. Significant differences are considered $p < 0.05$.

RESULTS

CNEPs Are Multipotent, Neurally Committed Cells *In vitro*

ES cells were plated and expanded in ES conditions prior to inducing and plating in cNEP conditions. To examine the neural differentiation potential of the cNEPs plated, cNEPs were collected and replated in maintenance media for 2 days (undifferentiated) or differentiation media for 14 days (differentiated) and immunocytochemistry was performed. Undifferentiated cNEPs expressed the NPC markers Sox2 and Nestin and did not express the pluripotency marker Oct4 expressed by ESCs (**Figure 1A**). Following 14 days of differentiation, the majority of cNEPs differentiated into DCX + neuroblasts (44.6 ± 5.2%) and BIII-tubulin + immature neurons (30.3 ± 5.5%), with fewer cNEPs expressing the astrocytic marker GFAP (15.9 ± 10.2%) and oligodendryocyte marker Olig2 (6.8 ± 1.7%; **Figures 1B,C**; Mujtaba et al., 1999; Payne et al., 2019b). Hence, similar to human iPSC derived cNEPs (Payne et al., 2019b), ESC derived cNEPs are multipotent, neurally committed cells *in vitro*.

Transplanted cNEPs Improve Motor Function Following Stroke

CNEPs were transplanted into the stroke lesion of immunocompromised SCID-Beige mice on PSD4 (acute phase) following an ET-1 induced stroke in the motor cortex (Vonderwalde et al., 2019). All mice were tested in the foot-fault task prior to stroke to establish baseline behavior, and again on PSD3 prior to cell transplantation (**Figures 2A,B**). Stroke injured mice displayed motor deficits in foot-fault at PSD3, relative to baseline performance (**Figure 2B**). On PSD4, one cohort of mice (Stroke + cNEP) received 1×10^5 cNEP cells into the stroke injury site. Stroke only mice received vehicle injections. Motor behavior was assessed in the Stroke + vehicle and Stroke + cNEP groups on PSD8, PSD20 and PSD40 (4, 16 and 36 days post-transplant). As shown in **Figure 2B**, mice that received cNEPs displayed significantly improved motor outcomes on PSD20 compared to non-transplanted mice and their motor performance was not significantly different from baseline (pre-stroke) values on PSD8 or PSD20. Mice that received cNEP transplant also had improved motor function compared to stroke untreated mice on PSD20 (**Figure 2B**). Mice that did not receive cNEP transplants continued to show significant motor deficits compared to their baseline on PSD20 (**Figure 2B**). Notably, the non-transplanted mice underwent spontaneous recovery by PSD40 and their motor function was not significantly different from the Stroke + cNEP group ($p = 0.93$). Hence, cNEP transplantation improved motor function following ET-1 induced motor cortical stroke as early as PSD20.

To assess motor coordination, we used the Catwalk for gait analysis on PSD20. A number of parameters have been shown to be affected following stroke, including terminal dual stance which measure the time that both hindlimbs remain on the platform (Liu et al., 2013; Caballero-Garrido et al., 2017).

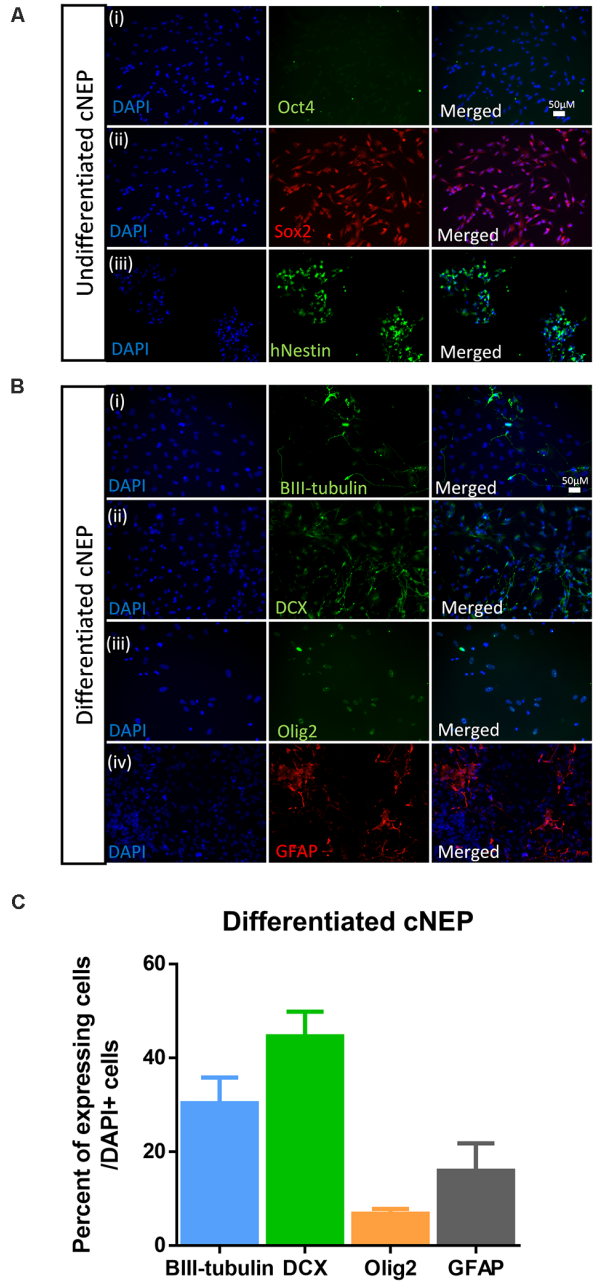

FIGURE 1 | Embryonic stem cell (ESC) derived cortical neuroepithelial cells (cNEPs) are neurally committed, multi-potent cells. **(A)** ESC were induced into cNEPs and then passaged or differentiated in media prior to immunocytochemistry. ESC derived cNEPs do not express the pluripotency marker OCT4 **(i)** and do express the undifferentiated neural precursor markers Sox2 **(ii)** and human Nestin (hNestin) **(iii)**. **(B)** cNEPs were plated on day 0 in differentiation media for 14 days. Subpopulations of cells express BIII-tubulin **(i)**, DCX **(ii)**, Olig2 **(iii)** and GFAP **(iv)**. Scale bars = 50 μm. **(C)** Quantification of differentiation markers. $n > 3$ independent experiments/condition.

Similar to previous reports using MCAO stroke, ET-1 stroked mice demonstrated a significant increase in left hindlimb stance on PSD20, that was reduced in mice that received cNEP transplantation (**Figure 2C**). Support lateral measures the percent of time during the walk that mice are simultaneously using ipsilateral paws (lateral forelimb and hindlimb). Again, similar to what is observed in MCAO stroked mice, ET-1 stroked mice demonstrated a decrease in the percent of the run that hindlimbs and forelimbs simultaneous make contact during locomotion. This parameter also improved with cNEP transplant on PSD20 (**Figure 2C**). These findings demonstrate that post-stroke impairments in motor coordination as measured by gait analysis are improved following cNEP transplantation. Immature cNEPs rescue motor deficit following a stroke.

Given the improved functional outcomes in transplanted mice, we next examined the cellular response in Stroke + cNEP treated mice. We assessed the differentiation profile of the surviving transplanted cells at the time of functional recovery by looking for co-labeled cells expressing human antigen markers, HuNu+ or hNestin+, with markers for mature neurons (NeuN), astrocytes (GFAP) and oligodendrocytes (myelin basic protein (MBP). As shown in **Figure 3A**, on PSD20, the vast majority of HuNu+ cNEPs expressed the neuroblast marker DCX (92 ± 11%; **Figures 3Ai–C**), with rare cells expressing the mature neuronal marker NeuN (2.6 ± 4.5%; **Figures 3Aii–C**). Virtually no HuNu+ cells expressed the astrocyte marker GFAP (**Figures 3Aiii–C**). We used Ki67 to look for proliferating cells and observed a small number of Ki67+/HuNu+ cNEPs (13 ± 13%) on PSD20 (**Figure 3Aiv**). As predicted, the contralateral hemisphere of Stroke + cNEP and Stroke only brains did not contain HuNU+ or Ki67+ cells (**Supplementary Figure 1**). Hence, on PSD20, surviving cNEP derived cells were DCX+, immature neurons.

Since transplanted human cells take longer to differentiate into mature phenotypes, we predicted that mature neurons may develop with longer survival times. We examined the brains of Stroke + cNEP mice on PSD40. Even at this longer survival time, transplanted cells continued to express DCX with rare HuNu+ or hNestin+ cells expressing the mature cell markers NeuN, GFAP or MBP (**Figures 3Bi–iii**). The contralateral hemisphere of Stroke + cNEP and Stroke only brains did not contain transplanted or proliferating cells (**Supplementary Figure 2**).

The number of surviving transplanted cells was quantified on PSD8 (a time when functional deficits were observed), PSD20 and PSD40 (when functional recovery is observed). The percentage of surviving cells (HuNu+ and DAPI+) relative to the numbers of transplanted cells (100,000 cells/brain) was not significantly different at any time examined (**Figure 3D**). Most striking, only three of the nine mice examined on PSD40 had surviving cNEPs (HuNu+ or hNestin+ cells). Hence, while Stroke + cNEP mice displayed improved motor function after PSD20, the recovery was not coincident with surviving cells on PSD40.

cNEP Transplantation Does Not Affect Stroke Lesion Volume or the Microglia/Macrophage Response

We next asked if the lesion volume was affected by the transplantation and associated with functional improvement.

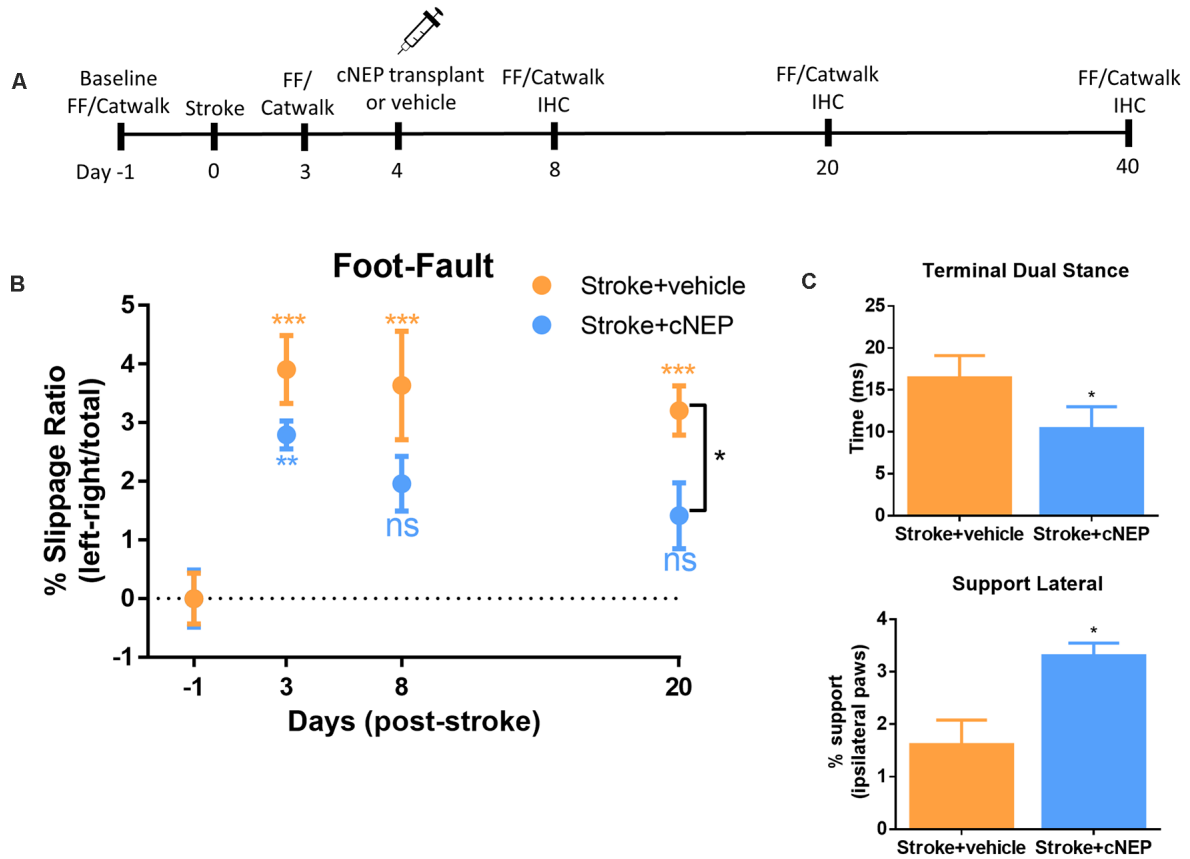

FIGURE 2 | cNEP transplantation improves motor outcomes of stroke by PSD20. **(A)** Schematic of experimental paradigm. FF, foot-fault and IHC, immunohistochemistry. **(B)** The percent slippage ratio was scored in Stroke + vehicle and Stroke + cNEP treated mice. Both groups display a motor deficit on PSD3 and PSD8 compared to baseline performance. Stroke + cNEP mice recover by PSD8 and PSD20 when compared to baseline and is significantly improved compared to Stroke + vehicle mice (Student's *T*-test, Mann–Whitney *post hoc*). Stroke + vehicle mice maintain the stroke motor deficit on PSD8 and PSD20, compared to baseline. Stroke + cNEP (n = 10 mice); Stroke + vehicle (n = 10 mice) Two-way ANOVA, Tukey's *post hoc*. **(C)** Catwalk was used to measure gait parameters after stroke with or without cNEP transplantation. Terminal dual stance measures the duration (ms) of simultaneous contact of both hindpaws. The duration of the left hindlimb terminal dual stance was significantly increased in Stroke + vehicle mice compared to Stroke + cNEP mice. Support lateral is a measurement of the time when ipsilateral paws (lateral forelimb and hindlimb) make simultaneous contact during the walking task Stroke + vehicle mice exhibited significantly impaired support lateral gait pattern compared to Stroke + cNEP treated mice (n=6 mice/group). All statistics were performed with Two-way ANOVA, Tukey's *post hoc*. Data shown as mean ± SEM. ns, not significant; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Cresyl violet stained images were quantified from cNEP transplanted mice sacrificed on PSD4 (prior to transplant) and PSD40. Cortical lesion volumes were not different between PSD4 and PSD40 in Stroke + cNEP transplanted mice (**Figure 4A**). Hence, cell transplantation does not lead to a change in the lesion volume at a time of functional recovery.

To determine whether cNEP transplantation affected the immunological response in stroke injured mice, the number of Iba1+ inflammatory cells (microglial and macrophages) were counted in the lesional and perilesional stroke injured cortex. SCID-beige mice lack the adaptive immune response but retain the innate immune response (**Figure 4B**). We observed no difference in the numbers of Iba1+ cells or the number of activated ameboid Iba1+ cells in Stroke only and Stroke + cNEP transplanted mice on PSD8, a time when immune cell activity is robust in response to stem cell transplantation (Walczak et al., 2007; **Figures 4B,C**). Hence, cNEP transplantation post-stroke does not elicit an enhanced neuroinflammatory response compared to Stroke alone.

DISCUSSION

Stem cell transplantation is a promising approach to treat the stroke injured brain. Despite the promise, a number of challenges still exist including the identification of an appropriate cell source, the location and timing of transplantation, and a clear understanding of the mechanisms that underlie the potential efficacy of the approach (Bliss et al., 2007). Here, we demonstrate that the acute transplantation of cNEPs in an ET-1 motor cortex stroke model can promote functional recovery as early as 16 days post-transplantation. We found that only a small fraction of the transplanted cells survive in the stroke injury site at the time of recovery, and despite their ability to differentiate into mature phenotypes *in vitro*,

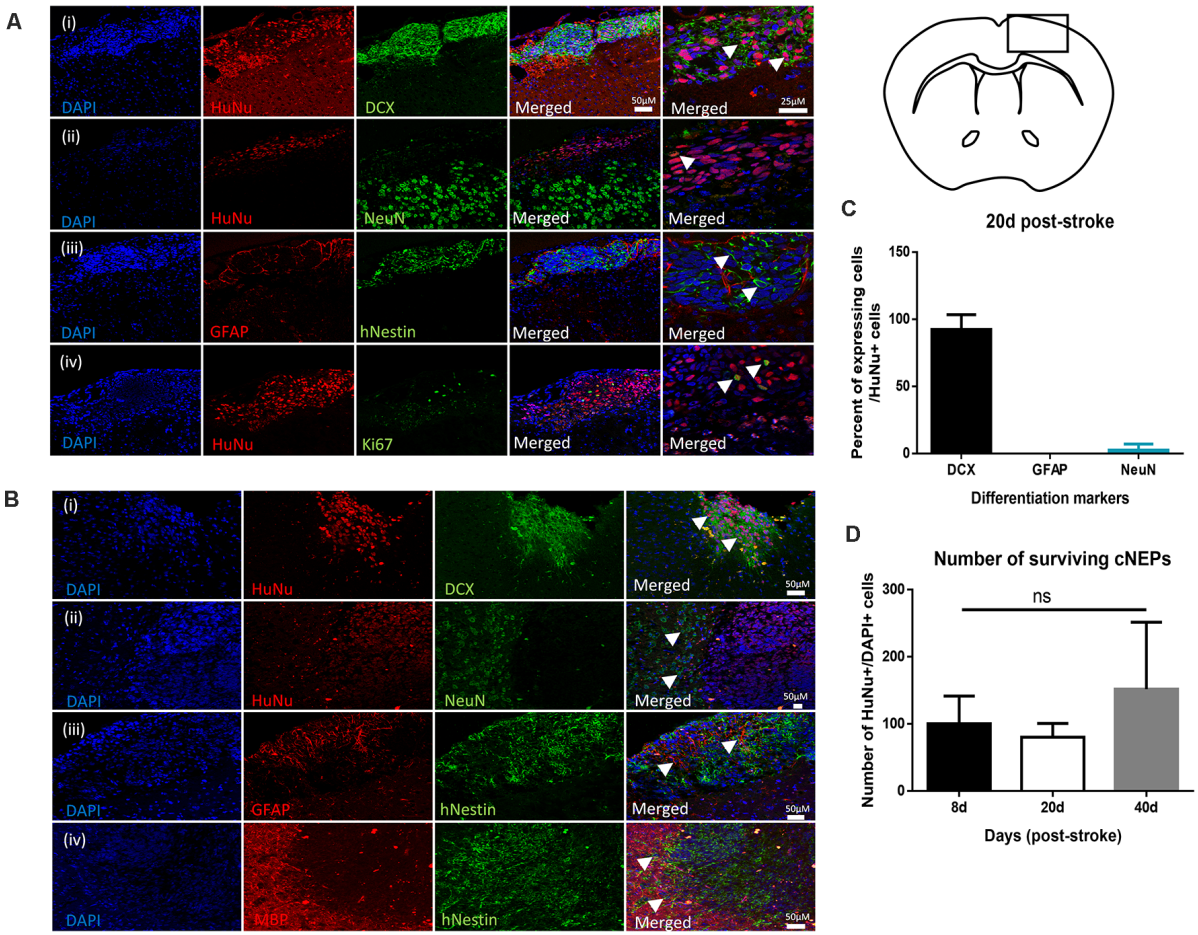

FIGURE 3 | Transplanted CNEPs express doublecortin, a marker of immature neurons. **(A)** Coronal schematic of forebrain at PSD20 HuNu+ or hNestin+ transplanted cNEPs express DCX **(i)**, low levels of NeuN **(ii)** and are GFAP negative **(iii)**. A small number of cells express Ki67 **(iv)**. Scale bar = 25 μm. **(B)** By PSD40, HuNu+ or hNestin+ transplanted cNEPs express DCX **(i)**, but do not colocalize with mature cell markers NeuN **(ii)**, GFAP **(iii)** or MBP **(iv)**. Scale bars = 50 μm. **(C)** Quantification of the differentiation phenotype of HuNu+ cNEP cells in the PSD20 brain showing majority of transplanted cells are DCX + immature neurons. (n = 3 mice). **(D)** The total numbers of HuNu+/DAPI+ cells showed no significant difference between the numbers of surviving cells on PSD8 (n = 3 mice), PSD20 (n = 3 mice) or PSD40 (n = 9 mice). Data represents mean ± SEM. One-way ANOVA, Tukey's *post hoc*; ns, not significant. Arrowheads indicate cells expressing mature or immature neuronal markers.

the surviving cNEPs remained immature *in vivo*, at the time of functional recovery. Further, we demonstrate that improved functional outcomes are not dependent on the persistence of the transplanted cells once improved functional outcomes have been achieved.

Cell integration and replacement are recognized as mechanisms for functional recovery (Englund et al., 2002; Falkner et al., 2016; Palma-Tortosa et al., 2020). This mechanism requires long term survival, maturation, and integration of transplanted cells to form synaptic connectivity with endogenous cells for functional benefits. Our study demonstrates that cNEP survival, maturation and integration are not required for the by-stander effect observed at early timepoints that give rise to the recovery we observed in this ET-1 stroke model. This is consistent with previous studies using human directly reprogrammed neural precursor cells in the same ET-1 mouse model of stroke (Vonderwalde et al., 2019). The complete absence of cells on PSD40 in 67% of the recovered mice, in combination with the lack of mature neurons suggests that cNEPs support recovery through "by-stander" effects such as secreting factors that enhance host neuroplasticity and cell maturation is not needed to maintain behavioral recovery (Xiong et al., 2017). The release of neurotrophic factors could significantly impact the stroke niche to induce angiogenesis, decrease immunogenic response and improve endogenous stem cell response (Horie et al., 2011; Oliveira et al., 2016). Indeed, the well documented ability of mesenchymal stem cell transplantation to support brain repair post-stroke is shown to be mediated by enhancing brain plasticity and/or reducing the inflammatory response (Kurozumi et al., 2005; Noh et al., 2016; Forsberg et al., 2020; Venkat et al., 2020). Identifying the factors that allow for recovery will be beneficial in determining future avenues of stroke treatment.

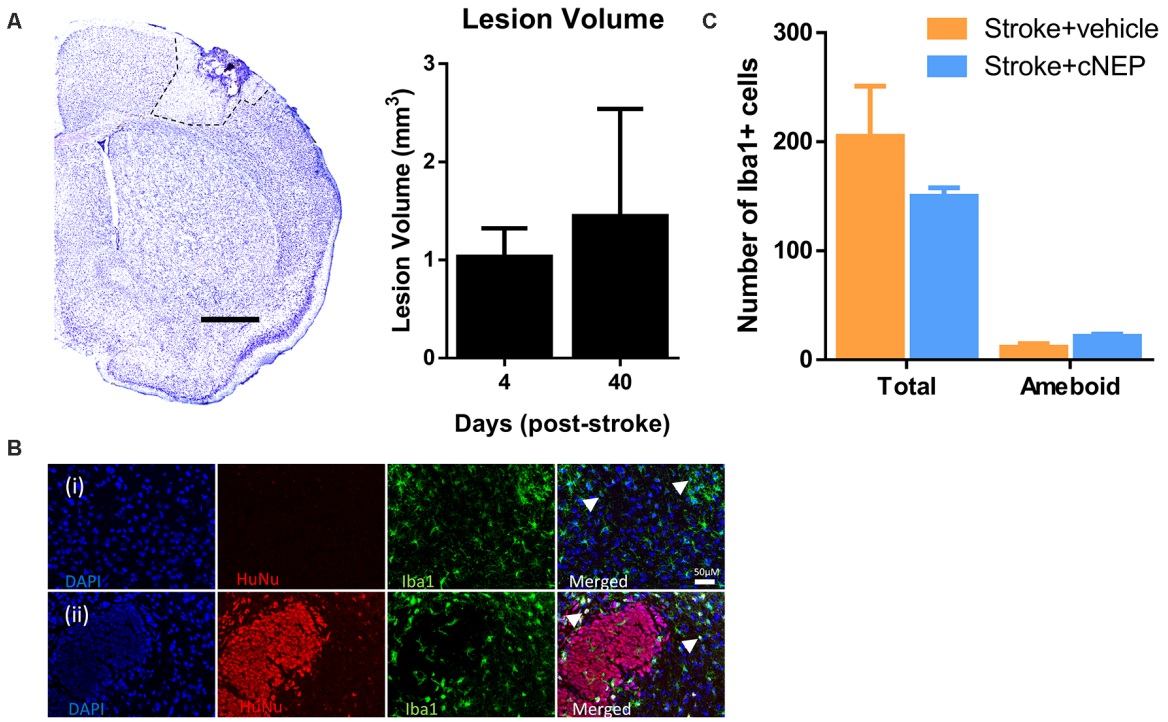

FIGURE 4 | Transplanted cells do not alter the lesion volume or the inflammatory cell response. **(A)** Cresyl violet cross section of the stroke injured cortex on post-stroke day 4 (PSD4). Dotted line delineates the stroke lesion. The lesion volume is not significantly different between PSD4 (pre-transplant) and PSD40 (Stroke + cNEPs). *n* = 3 mice/group; data represents mean ± SEM; Student's *t*-test. Scale bar = 500 μm. **(B)** Iba1+ inflammatory microglia were stained in Stroke+ vehicle **(i)** and Stroke + cNEP **(ii)** treated mice on PSD8. Scale bar = 50 μm. **(C)** Quantification of iba1+ cells showed no significant difference in the number of Iba1+ cells or activated Iba1+ cells between groups (*n* = 3 mice/group). Data represents mean ± SEM; Student's *t*-test. Arrowheads indicate iba1+ amoeboid shaped cells.

The differentiation profile of transplanted cNEPs suggests that unlike previous transplant studies, cNEPs do not contribute to the glial scar by turning into GFAP+ astrocytes (Payne et al., 2019b; Vonderwalde et al., 2019). Astrocytes have been shown to be beneficial following brain injury by limiting the extent of damage, releasing neurotrophic factors and clearing excess glutamate (Barreto et al., 2012; Liu and Chopp, 2016). However, astrocytes are also capable of releasing proinflammatory factors and inhibitory factors, such as chondroitin sulfates, that impair axonal growth (Barreto et al., 2012). Once activated, astrocytes exist within the brain in either anti-inflammatory or pro-inflammatory states (Giovannoni and Quintana, 2020) and reducing pro-inflammatory astrocyte activation has been shown to be beneficial for both behavioral and cellular outcomes after injury (Brambilla et al., 2005). Since the nature of the astrocytes derived from transplanted cells is not known, it is possible that the lack of CNEPs derived astrogliosis is advantageous.

Our study found that a small number of cNEPs expressed the proliferation marker Ki67, which is consistent with the previous study transplanting cNEPS into stroke injured rats (Payne et al., 2019b). Despite the positive functional outcomes and the fact that majority of mice had no surviving cells on PSD40, the presence of proliferating cells can be a concern for clinical application. Ablating proliferating cells using gene editing technologies that result in cell death upon expression of a cell division gene, CDK1, have been recently described (Liang et al., 2018; Payne et al., 2019a) and incorporating this Failsafe system into cNEPs would undoubtedly enhance their clinical application.

CNEP's can be derived from pluripotent stem cells, including iPSCs (Payne et al., 2018, 2019b) and ES cells (reported here). A previous report using iPSC derived cNEPs embedded in a hydrogel and transplanted in a subacute rat model of motor stroke, demonstrated limited improvements in functional recovery at >50 days post-stroke (Payne et al., 2019b). Payne et al. (2019b) found that most of the cNEPs survive following transplantation (on PSD50) and the majority differentiated into astrocytes. Several differences between the studies are noted, including the cell origin of cNEPs. IPSCs and ESCs have been shown to generate different progeny in the same culture conditions (Kim et al., 2010). Additionally, in the rat study cNEPs were delivered in a hydrogel which would modify the interaction of cNEPs with the host environment, potentially impacting the time of recovery and the differentiation profile of the transplanted cells. Further, the rat stroke model included cortical and subcortical ET-1 lesions, with the cell transplantation being limited to the cortex. Given that cNEPs are cortically specified, the limited recovery observed in their study may be related to the fact that striatal circuitry was disrupted and not impacted by the cNEP transplant. Together,

the outcomes of the *in vivo* studies comparing cells that behave similarly *in vitro*, highlight the importance of following the recommendations of STEPS and STAIRS that underscore the need to compare outcomes across different models, with a view towards translation (Lapchak et al., 2013).

When considering stem cell transplantation studies to treat stroke, there are a number of design parameters that can influence outcomes and make it challenging to compare across studies. For example; the cell source, the numbers of cells transplanted, the location and time of transplantation post-stroke and the vehicle used to transplant cells. Our study paradigm was based on the findings that stem cell transplantation into the stroke lesion provided greater survival and functional improvement following stroke, when compared to transplantation into surrounding parenchyma following ET-1 stroke (Ballios et al., 2015). In addition, we have found that the use of a hydrogel (HAMC) did not provide improved survival of human neural stem cells when transplanted into SCID-beige immunodeficient mice (Vonderwalde et al., 2019). Building on these studies we transplanted human cNEPs into the stroke lesion site suspended in regular cerebrospinal fluid in SCID-beige immunocompromised mice. Importantly, this method allowed us to avoid pharmacological immunosuppression, as these treatments have been shown to regulate endogenous neural precursor behavior which could influence the outcomes (Sachewsky et al., 2014). These experimental considerations are important for ensuring best approaches in pre-clinical stroke research.

Our study demonstrates that cortically specified, neurally committed neural precursor cells can promote rapid functional recovery when delivered in the acute phase post stroke without integration into the host circuitry. Future studies will determine the critical window of repair and identify the mechanism that serves to enhance neuroplasticity and functional recovery.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The animal study was reviewed and approved by University of Toronto, Temerty Faculty of Medicine Animal Care Committee.

AUTHOR CONTRIBUTIONS

RI designed and performed the experiments, data analysis, interpretation, wrote and edited the manuscript. BV and AN developed the cell culturing protocol. SD and IV performed experiments and data analysis. CM designed experiments and interpreted data, provided financial support, wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by Canada First Research Excellence Fund, Medicine by Design; and Heart and Stroke Foundation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2021.654290/full#supplementary-material.

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

frontiers in Neuroscience

REVIEW
published: 28 May 2021
doi: 10.3389/fnins.2021.628663

Induced Pluripotent Stem Cells for Ischemic Stroke Treatment

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Ischemic stroke is one of the main central nervous system diseases and is associated with high disability and mortality rates. Recombinant tissue plasminogen activator (rt-PA) and mechanical thrombectomy are the optimal therapies available currently to restore blood flow in patients with stroke; however, their limitations are well recognized. Therefore, new treatments are urgently required to overcome these shortcomings. Recently, stem cell transplantation technology, involving the transplantation of induced pluripotent stem cells (iPSCs), has drawn the interest of neuroscientists and is considered to be a promising alternative for ischemic stroke treatment. iPSCs are a class of cells produced by introducing specific transcription factors into somatic cells, and are similar to embryonic stem cells in biological function. Here, we have reviewed the current applications of stem cells with a focus on iPSC therapy in ischemic stroke, including the neuroprotective mechanisms, development constraints, major challenges to overcome, and clinical prospects. Based on the current state of research, we believe that stem cells, especially iPSCs, will pave the way for future stroke treatment.

Keywords: stem cells, ischemic stroke, cell therapy, treatment, mechanism

OPEN ACCESS

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

Received: *13 November 2020*
Accepted: *06 May 2021*
Published: *28 May 2021*

Citation:
Duan R, Gao Y, He R, Jing L, Li Y, Gong Z, Yao Y, Luan T, Zhang C, Li L and Jia Y (2021) Induced Pluripotent Stem Cells for Ischemic Stroke Treatment. Front. Neurosci. 15:628663. doi: 10.3389/fnins.2021.628663

INTRODUCTION

Stroke is one of the major central nervous system diseases associated with high disability and mortality rates, approximately 80% of the cases being ischemic stroke (Phipps and Cronin, 2020). Although there are many clinical treatment methods available currently for patients with ischemic stroke, including antiplatelet aggregation, anticoagulation, improving microcirculation, and improving brain metabolism, these do not have efficient regeneration and repair effects on the necrotic nerve cells in the ischemic area. The standard treatment method is to either use recombinant tissue plasminogen activator (rt-PA) or perform mechanical thrombectomy to restore blood flow. However, these treatments have certain limitations. The effective treatment time window of rt-PA is 4.5 h, and patients with large artery occlusion can be treated with thrombectomy within 6 h or even beyond 24 h of symptom onset. However, nearly 60% of patients did not achieve functional independence at 3 months after treatment in recent mechanical thrombectomy trials (Rabinstein, 2020; Shafie and Yu, 2021). When ischemic stroke occurs, apoptosis or necrosis of various cells is seen in the infarct areas. Therefore, the ideal treatment method would be to implant a certain type of cell that can replace the damaged cells. In recent years, stem cell transplantation technology has been successfully applied in heart disease modeling (Musunuru et al., 2018), diabetes mellitus (Kalra et al., 2018), macular degeneration (Bracha et al., 2017),

spinal cord injury (Csobonyeiova et al., 2019), cartilage regeneration (Castro-Viñuelas et al., 2018), and graft-vs.-host disease (Bloor et al., 2020). Stem cell transplantation is also being gradually considered for the treatment of some nervous system diseases, such as Alzheimer's disease (Robbins and Price, 2017), Parkinson's disease (Cobb et al., 2018), amyotrophic lateral sclerosis (Csobonyeiova et al., 2017), Huntington's disease (Tousley and Kegel-Gleason, 2016; Yoon et al., 2020), and ischemic stroke. In this review, we have summarized the progress that has been made in the development of cell therapy for ischemic stroke. We have particularly focused on the mechanism, current status, clinical application, development constraints, and future prospects of the application of induced pluripotent stem cells (iPSCs) in treating ischemic stroke.

CURRENT APPLICATIONS OF STEM CELLS

Stem cell transplantation technology is attractive as a novel option for the treatment of ischemic stroke, and utilizes different cell sources, including embryonic stem cells (ESCs), neural stem cells (NSCs), mesenchymal stem cells (MSCs), umbilical cord-derived blood cells, adipose-derived MSCs, dental stem cells, iPSCs, and some immortalized cell lines; the most recent studies in this field have primarily focused on the first three stem cell types. Here, we have discussed the current applications of different stem cell types in ischemic stroke, especially the iPSC therapy.

EMBRYONIC STEM CELLS (ESCS) IN ISCHEMIC STROKE

ESCs, a type of pluripotent cells, are derived from the inner mass of preimplantation embryo and have differentiation ability (Thomson et al., 1998). They are able to form specific nerve tissues, such as neurons, astrocytes, and oligodendrocytes (Wichterle et al., 2002). Therefore, ESCs have been regarded as a potential and ideal source of transplanted cells for neural disease therapy. Cell therapy based on ESCs has been shown to not only promote structural repair and functional recovery but also reduce the infarct size in a mouse model of ischemic stroke. After transplanting mouse ESCs into a severe focal ischemic rat cortex, ESC-derived cells expressing cell surface markers of neurons, astrocytes, oligodendrocytes, and endothelial cells could be found in the lesion area; moreover, the survival rate, neuronal differentiation, structural repair, and functional outcome were further improved by transplantation of ESCs overexpressing Bcl-2. Additionally, the intracerebral transplantation of mouse ESCs could have a positive effect on motor and sensory recovery and infarct size reduction in rats with focal ischemia (Wei et al., 2005; Nagai et al., 2010; Tae-Hoon and Yoon-Seok, 2012). Some studies have found nerve cells derived from human ESCs to be at risk of malignant transformation and teratoma formation, which is possibly caused by the stimulation of various local cytokines (Brederlau et al., 2006; Seminatore et al., 2010). From an ethical point of view, the limited sources and the associated high incidence of malignant transformation restrict the wide application of ESCs. Therefore, research on ESC application in stroke treatment is very limited.

NEURAL STEM CELLS (NSCS) IN ISCHEMIC STROKE

Neurogenesis was not possible until the discovery of NSCs, which have the ability to renew themselves and originate neurons, astrocytes, and oligodendrocytes (Reynolds and Weiss, 1992). Many studies have identified the existence of multipotent and self-renewing NSCs in different areas of the brain, including the subgranular zone in the dentate gyrus of the hippocampus and subventricular zone of the lateral ventricles, and the occurrence of endogenous neurogenesis in the adult brain (Gage, 2000; Koh and Park, 2017). Researchers have found that grafting of fetal neocortical tissue 1 week after focal brain ischemia improved the behavioral outcome and reduced secondary thalamic atrophy (Mattsson et al., 1997). Further research showed that transplanted human fetal NSCs were able to survive, migrate, and differentiate in ischemic stroke-damaged rats (Kelly et al., 2004; Darsalia et al., 2007). Human fetal NSCs were implanted into the ipsilateral striatum of a middle cerebral artery occlusion (MCAO) rat model 48 h after model establishment, and behavioral assessments were conducted at 6 and 14 weeks. Results showed that the grafted human NSCs survived in all rats and successfully differentiated to neuroblasts or mature neurons; functional recovery of the rats grafted with NSCs also improved at 6 and 14 weeks, including sensorimotor and spatial learning functions (Mine et al., 2013). Moreover, long-term survival and widespread distribution of NSCs were detected following intra-arterial delivery in an ischemic stroke rodent model, and cell differentiation was evident at 4 weeks (Zhang et al., 2020). In a cortical stroke model, stable graft survival and neuronal differentiation were successfully monitored, and human NSC transplantation was found to have a profound effect on network stability (Minassian et al., 2020). In addition, neural progenitor cells derived from human iPSCs were found to be more effective than mesenchymal stromal cells obtained from human placenta in suppressing the progression of experimental ischemic stroke by improving animal survival in the most acute period and accelerating the recovery of neurological deficit and body weight (Cherkashova et al., 2019). Rats that received pretreatment with human iPSC-NSCs and metformin recovered and had a faster decrease in infarct volume compared to the controls (Ould-Brahim et al., 2018). Furthermore, studies have shown that neuron stem cell extracellular vesicles (NSC EVs) could improve tissue and functional recovery in both rat and pig models of ischemic stroke, and possessed therapeutic potential (Webb et al., 2018a,b; Spellicy et al., 2020). However, NSC death or cell fate switch may happen in the case of hypoxia and ischemia, caused by a change in cell metabolism and irreversible switch from neurogenesis to gliosis via enhanced Notch signaling (Santopolo et al., 2020). Additionally, other obstacles also hinder NSC use in ischemic stroke treatment, including the limited resources

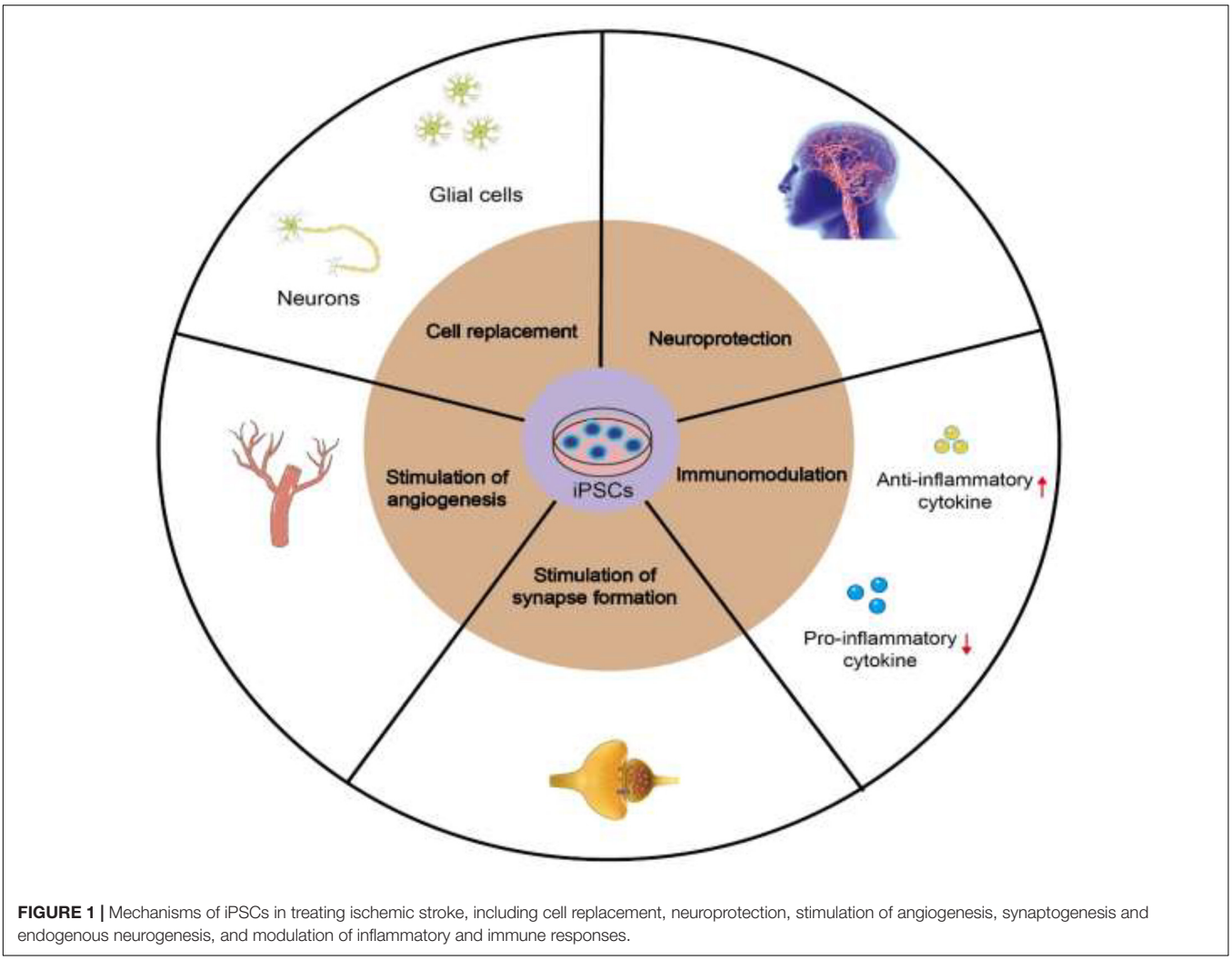

FIGURE 1 | Mechanisms of iPSCs in treating ischemic stroke, including cell replacement, neuroprotection, stimulation of angiogenesis, synaptogenesis and endogenous neurogenesis, and modulation of inflammatory and immune responses.

with ethical significance, insufficient biological understanding, minimal clinical exploration, and risk of tumorigenesis when derived from iPSCs (Alessandrini et al., 2019).

MESENCHYMAL STEM CELLS (MSCS) IN ISCHEMIC STROKE

MSCs are non-hematopoietic stem cells, which widely exist in various organs and tissues, and have a high degree of proliferation, along with self-renewal and multi-directional differentiation properties. MSCs can be obtained from several tissues, including the bone marrow, placenta, amniotic fluid, adipose tissue, umbilical cord, and dental pulp (Yalvac et al., 2009; Yang et al., 2011; Pittenger et al., 2019). At the preclinical level, transplantation of MSCs, especially BMSCs, improved functional recovery in MCAO model rats. MSC transplantation was shown to reduce the lesion volume (Koh et al., 2008; Li et al., 2010; Shen et al., 2011), enhance sensorimotor (Huang et al., 2013) and cognitive functions (Lowrance et al., 2015), promote angiogenesis and neurogenesis (Bao et al., 2011), modulate inflammatory and immune responses (Ohtaki et al., 2008), and improve synaptic recovery (Asgari Taei et al., 2021). Although some studies have shown no significant reduction of ischemic lesion volumes, the functional outcome nevertheless improved after the administration of MSCs; these discrepancies were suggested to be due to not recanalizing the middle cerebral artery (Zacharek et al., 2010; Gutiérrez-Fernández et al., 2013). Several clinical trials seem to have confirmed MSCs to be a potential option for the treatment of ischemic stroke; moreover, MSC therapy was demonstrated to be safe for subacute stroke patients in the long-term and may improve recovery after stroke according to a randomized controlled trial using an intravenously applied MSC culture expanded with fetal bovine serum (Lee et al., 2010; Díez-Tejedor et al., 2014; Steinberg et al., 2016; Levy et al., 2019; Lalu et al., 2020). Furthermore, MSC-derived extracellular vesicles (MSC EVs) played an important role in ischemic stroke as a treatment and potential biomarker (Otero-Ortega et al., 2019). However, no efficacy of MSC therapy was observed in the only randomized Phase II study until now (Hess et al., 2017). Hence, the efficacy of MSCs for stroke treatment remains to be determined.

INDUCED PLURIPOTENT STEM CELLS (IPSCS) IN ISCHEMIC STROKE

iPSCs form a class of cloned cells with characteristics similar to those of ESCs, and were first discovered by Takahashi and Yamanaka using retroviruses to integrate four factors (Sox2, Oct3/4, Klf4, and cMyc) into both mouse and adult human fibroblasts through corresponding vectors (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Till date, iPSCs have mainly been applied in three aspects, namely disease modeling, drug discovery, and regenerative therapy (Wilson and Wu, 2015). In recent years, iPSC-based cell therapy has developed rapidly and its potential has been studied for the treatment of many diseases. Retinal pigment epithelial cells differentiated from iPSCs have proven to be safe and effective in both preclinical models and clinical studies of macular degeneration (Li et al., 2012; Kamao et al., 2014; Yoshida et al., 2014; Sharma et al., 2019). As a novel alternative method, iPSC technology has attracted increasing attention in various other diseases, including ischemic stroke, heart failure, hematopoietic disorders, spinal cord injury, and liver disease (Lachmann et al., 2015; Hansel et al., 2016; Miyagawa and Sawa, 2018; Fernández-Susavila et al., 2019; Trawczynski et al., 2019). Since a detailed description of all these applications is beyond the scope of this review, we have focused specifically on the key issues related to ischemic stroke.

A variety of animal experiments have shown that the application of iPSCs plays an important role in ischemic stroke treatment, including improving sensorimotor function (Chen et al., 2010; Jiang et al., 2011; Gomi et al., 2012; Oki et al., 2012; Tatarishvili et al., 2014; Eckert et al., 2015; Lau et al., 2018; Oh et al., 2020), reducing lesion volume (Chen et al., 2010; Baker et al., 2017; Oh et al., 2020; Xia et al., 2020), promoting neurogenesis and angiogenesis (Oki et al., 2012; Oh et al., 2020), and exerting immunomodulatory and anti-inflammatory effects in the brains of rodents affected by ischemic stroke (Eckert et al., 2015). However, some animal experiments did not achieve the desired results, such as a significant reduction in infarct volume and improvement in behavior (Jensen et al., 2013; Kawai et al., 2010). Here, a summary of iPSC transplantation experiments in ischemic stroke models is shown in **Table 1**, and summarized the animal models of ischemic stroke, follow-up period and experimental results.

TABLE 1 | Summary of iPSC transplantation experiments in ischemic stroke models.

Model (reference)	Follow-up period	Results
MCAO rat (Xia et al., 2020)	2–28 days	iMSC-sEVs migrate to the brain, infarct size ↓, mNSS↓, error step number in the foot-fault test ↓, overall blood vessel density↑
MCAO rats (Jiang et al., 2011)	4–16 days	iPSCs migrate to the ischemic brain and differentiate into neural cells, no seizure or convulsive activity,%HLV↓, sensorimotor function↑
MCAO rats (Chen et al., 2010)	1–4 weeks	iPSCs-FG differentiate into astroglial-like and neuron-like cells, infarct size↓, motor function↑, anti-inflammatory cytokines ↑, pro-inflammatory cytokines ↓ but form teratoma in 4 weeks
Pig (Baker et al., 2017)	24 h to12 weeks	CBV, white matter integrity, neurometabolite abundance (NAA, Cr, Cho) ↑
Mouse (Gomi et al., 2012)	1–6 weeks	iPSCs differentiate into neuronal progenitors, axonal elongation, mNSS ↓ but immune rejection still exists after 6 weeks
MCAO pig (Lau et al., 2018)	1–12 weeks	Recovery of postural reactions, posture, mental status, and appetite
MCAO rats (Oki et al., 2012)	1–10 weeks	Extend axons to the GP, VEGF and recovery of fine forelimb movements ↑
MCAO rats (Tatarishvili et al., 2014)	1–8 weeks	Behavioral recovery ↑
MCAO rats (Eckert et al., 2015)	1–30 days	Behavioral recovery ↑, BBB leakage ↓, pro-inflammatory cytokine ↓, microglial activation ↓, adhesion molecules ↓, MCP-1 and MIP-1α ↓
MCAO rats (Oh et al., 2020)	1–12 weeks	Ep-iPSC-NPCs differentiated into neuronal and glial cells, sensorimotor and behavioral functional recovery ↑, MEP ↑, proliferating and migrating neural precursors ↑, astroglial scar formation ↓, microglial ↓, mNSS ↓, final infarct size ↓
MCAO rats (Jensen et al., 2013)	1–5 weeks	NSCs derived from human iPSCs survive and differentiate into neural cells, but without behavioral recovery or reduction of infarct size
MCAO rat (Kawai et al., 2010)	1–28 days	No significant difference in behavioral recovery and form larger tumors than the sham-operated group
MCAO rat (Tornero et al., 2017)	48 h to 6 months	iPSCs promoted synapse formation between neurons, and the grafted neurons received direct synaptic inputs from neurons

BBB, blood-brain barrier; CBV, cerebral blood volume; Cr, creatine; Cho, choline; GP, globus pallidus; iPSC, induced pluripotent stem cell; MSC, mesenchymal stem cell; MCAO, middle cerebral artery occlusion; iMSC-sEV, small extracellular vesicles secreted by MSCs derived from human iPSCs; mNSS, modified neurological severity score;%HLV, percentage hemisphere lesion volume; iPSC-FG, iPSCs with fibrin glue; MEP, motor-evoked potential; MCP-1, monocyte chemotactic protein 1; MIP-1α, macrophage inflammatory protein 1α; NAA, N-acetylaspartate; ep-iPSC-NPCs, neural precursor cells derived from human-iPSCs through episomal plasmid-based reprogramming; VEGF, vascular endothelial growth factor.

Mechanism of Action of iPSCs in Ischemic Stroke

Neurological deficit caused by ischemic stroke mainly occurs due to the loss of various nerve cells, including neurons and different types of glial cells. Implantation of iPSCs in animal models of ischemic stroke can effectively promote the recovery of nerve function. Multiple mechanisms have been proposed to account for these beneficial effects of iPSCs in treating ischemic stroke, including cell replacement, neuroprotection, modulation of inflammatory and immune responses, and stimulation of angiogenesis, synaptogenesis and endogenous neurogenesis (**Figure 1**).

Cell Replacement and Neuroprotection

iPSC-derived NSCs (iNSCs), including iPSCs generated by an episomal plasmid-based reprogramming technique, can differentiate into the three major neural lineages, namely neurons, astrocytes, and oligodendrocytes (Baker et al., 2017; Oh et al., 2020). The microenvironment may be a potential

factor contributing to the differentiation potential of iPSCs (Jiang et al., 2011). Baker et al. (2017) had found no significant difference in the number of neurons at the margin of the lesion between the iNSC-treated group and normal control animals 12 weeks after transplantation, whereas the number of neurons in the non-treated group was lower than that in normal control animals. Moreover, the expression levels of genes related to angiogenesis, neurotrophism, and inflammation were significantly altered in the brain tissues of the iNSC-treated group. Non-invasive longitudinal magnetic resonance imaging of stroke model animals provided evidence of brain metabolism recovery, white matter integrity, and cerebral blood perfusion after iNSC therapy at the tissue level. Another study found the transplantation of human iPSC-derived long-term expandable neuroepithelial-like stem cells (It-NESCs) into the striatum of stroke-injured rats to improve behavioral recovery, which could be observed in the early stages after transplantation (Oki et al., 2012). Importantly, hiPSC-lt-NESCs were shown to survive and differentiate to neurons in aged rats with ischemic stroke and further improve functional recovery (Tatarishvili et al., 2014). Together, these data suggested that iNSC treatment may have a neuroprotective effect, leading to reduced neuronal cell death in the cerebral cortex and inhibition of microglial activation, resulting in changes in gene expression that diminish inflammation and improve tissue recovery.

Modulation of Inflammatory and Immune Responses

Chen et al. (2010) had detected changes in inflammatory cytokine levels in the brain homogenate of MCAO model rats, 1 week after iPSC transplantation, using enzyme-linked immunosorbent assay. They found the levels of anti-inflammatory cytokines interleukin (IL)-4 and IL-10 in the brains of rats treated with subdural iPSCs cultured with fibrin glue (iPSC-FG) to be significantly higher than those of the simple cerebral ischemia control group, 1 week after subdural transplantation, whereas the levels of pro-inflammatory cytokines (IL-1β, TNF-α, IL-2, and IL-6) were significantly reduced. These results suggested that subdural-transplanted iPSCs-FG could have a possible paracrine effect, and may promote neuroprotection by increasing anti-inflammatory cytokines and decreasing pro-inflammatory cytokines. Eckert et al. (2015) had also supported the effect of early transplantation on host immune response, showing that transplantation of iPSC-derived NSCs into the hippocampus 24 h after stroke could attenuate the blood-brain barrier damage while reducing the expression levels of pro-inflammatory markers, microglial activation, and adhesion molecules, resulting in significant improvement of motor and sensorimotor functions in the first week after transplantation.

Stimulation of Angiogenesis

Vascular endothelial growth factor (VEGF) expression was detected in astrocytes and in the blood vessel wall of the damaged brain. The immune reactivity of VEGF was upregulated as early as 1 week after transplantation. Although the reactivity was relatively weak at 8 weeks after transplantation in aged rats, the result suggested that VEGF secretion has a certain effect on plasticity and inflammation in early recovery (Oki et al., 2012; Tatarishvili et al., 2014). Direct implantation of cells expressing the VEGF receptor fetal liver kinase-1, derived from iPSCs, into the ischemic area could effectively promote the formation of new blood vessels (Suzuki et al., 2010). In addition, the small extracellular vesicles secreted by MSCs derived from human iPSCs had the ability to promote angiogenesis and provide protection against brain injury after ischemic stroke by inhibiting signal transducer and activator of transcription 3 (STAT3)-dependent autophagy (Xia et al., 2020).

Stimulation of Synaptogenesis and Endogenous Neurogenesis

Further, the possibility of neural circuit reconstitution in the ischemic brain was investigated. Results indicated that iPSCs can differentiate into human telencephalic progenitors under serum-free culture of embryoid body-like aggregates, and these cells not only survived *in vivo* but also grew axons and extended to the cerebral ischemic area of the mice. iPSCs promoted synapse formation between host neurons, as demonstrated by fluorogold and synaptophysin staining of the host brain, and regulated the activity of transplanted neurons (Gomi et al., 2012; Tornero et al., 2017). In studies of human-to-human transplantation, neurons derived from PSCs were confirmed to have integrated into the adult host neural network (Grønning Hansen et al., 2020). Positive co-staining of presynaptic vesicle markers in some transplanted cells indicated these cells to participate in synaptic transmission. The transplanted cells also enhanced endogenous brain repair, including subventricular zone neurogenesis, reduction of inflammation, and formation of glial scars (Oh et al., 2020).

However, there was a lack of functional improvement and infarct area reduction, which were attributed to multiple potential factors, including the optional transplant timing after ischemic stroke, optional cell dose, and many variables of the cells themselves, such as the source, culture protocol, and differentiation stage, or other unknown reasons, suggesting that the transplanted cells need more time to mature and integrate into the neural network (Wechsler et al., 2009; Jensen et al., 2013).

Development Constraints and Possible Solutions

iPSCs are derived directly from the connective tissue of patients through a small biopsy and exhibit the same properties as ESCs, thereby overcoming the problems related to immune rejection and bypassing the need for embryos, to avoid ethical issues. Therefore, iPSCs can be generated in a patient-matched manner, each individual having his/her own PSC line. First, somatic cells, such as fibroblasts, are collected from the patient. Next, somatic cells are reprogrammed into iPSCs by the introduction of reprogramming factors. Thereafter, genetically corrected iPSCs are generated by genome editing. The corrected iPSCs differentiate into neurons or glial cells. Healthy iPSCs are obtained via quality assessments. Finally, cell therapy can be achieved by transplanting the cells into a patient with ischemic stroke (Alessandrini et al., 2019; Farkhondeh et al., 2019; Yasuhara et al., 2020; **Figure 2**). However, there are several

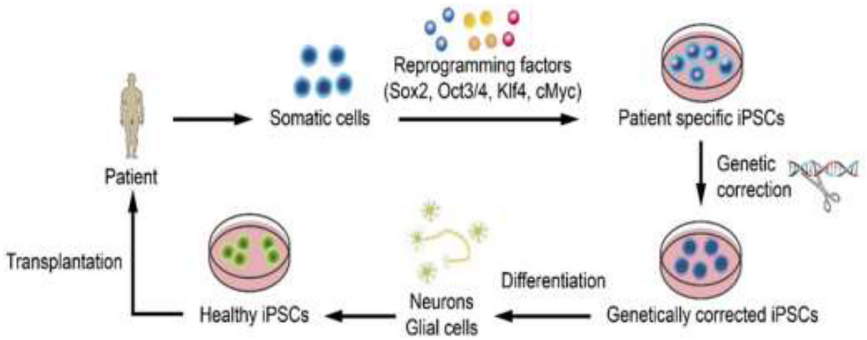

FIGURE 2 | Steps of iPSC-based therapy. First, somatic cells are collected from the patient. Next, somatic cells are reprogrammed into iPSCs by the introduction of reprogramming factors. Thereafter, genetically corrected iPSCs are generated by genome editing. The corrected iPSCs differentiate into neurons or glial cells. Healthy iPSCs are obtained via quality assessments. Finally, cell therapy can be achieved by transplanting the cells into a patient with ischemic stroke (Alessandrini et al., 2019; Farkhondeh et al., 2019; Yasuhara et al., 2020).

obstacles and limitations related to iPSC-based therapy that need to be overcome before clinical application.

Teratoma Formation

Although iPSC-NPCs could survive and migrate toward the lesion area, and were shown to alleviate the dysfunction induced by ischemic stroke, without tumor formation, during a 4-month period after transplantation (Jirak et al., 2019), the risk of tumorigenesis from iPSCs remains a major constraint for clinical application. A previous study had shown that direct injection of iPSCs into the ischemic brain caused teratoma formation in 100% of MCAO model rats 4 weeks after transplantation (Chen et al., 2010). In addition, the transplanted iPSCs expanded in the brain of post-ischemic mice and formed tumors that were larger than those formed in the sham-operated group (Kawai et al., 2010). The initial induction process of iPSCs required the use of a retrovirus carrying reprogramming factors, including Sox2, Oct3/4, Klf4, and cMyc, the latter two being oncogenes that may cause tumorigenicity in the host (Sun et al., 2010). Therefore, it is essential to strictly evaluate whether iPSCs and their derivatives could induce tumor growth in the host and to protect the same from happening.

Other studies have shown that, out of Oct4 and Klf4 as small-molecule compounds, Oct4 alone is sufficient to induce iPSCs (Shi et al., 2008; Kim et al., 2009). Some non-viral methods, such as plasmid vectors, minicircular DNA carriers, and proteins have been developed as a safer approach to generate iPSCs successfully (Cho et al., 2010; Okita et al., 2010; Rhee et al., 2011; Okano et al., 2013). Purification of cells before transplantation and some small-molecule inhibitors can effectively reduce the potential tumorigenicity of iPSCs. Quercetin/YM155-induced selective cell death (Lee et al., 2013) and pluripotent cell-specific inhibitors (PluriSIns) (Ben-David et al., 2013) were shown to be sufficient to completely inhibit teratoma formation by efficiently eliminating residual undifferentiated cells, which could increase the safety of iPSC-based treatments. The predifferentiation of iPSCs and establishment of long-term self-renewing neural cell lines are other effective strategies proposed to reduce the risk of tumor formation (Oki et al., 2012).

Low Induction Efficiency

Several solutions have also been put forward to overcome the problem of low iPSC induction rate. For example, stress-mediated p38 activation may enhance the low induction rate of iPSCs, and hyperosmosis has been reported to promote not only cell reprogramming but also iPSC generation (Xu et al., 2013). Expression of the mouse miR302/367 cluster could rapidly and successfully reprogram iPSCs, and the induction rate of this microRNA-based reprogramming method was shown to be 100 times greater than that of the original method (Anokye-Danso et al., 2011). As mentioned above, Sox2, Oct3/4, Klf4, and cMyc are of great importance in the generation of iPSCs. However, silencing of P53 with small interfering RNA and addition of undifferentiated embryonic cell transcription factor 1 (UTF1) could increase the production efficiency of iPSCs by 100 times, even if the oncogene c-Myc was removed from the combination (Nakagawa et al., 2008; Zhao et al., 2008). Another study confirmed that some small-molecule compounds can also effectively improve the induction efficiency. For instance, both DNA methyltransferase and histone deacetylase (HDAC) inhibitors play an important role in reprogramming efficiency. Valproic acid, an HDAC inhibitor, increased the reprogramming efficiency of iPSCs by more than 100 times, which could also be achieved without introduction of the oncogene c-Myc (Huangfu et al., 2008). Collectively, these findings highlighted the importance of achieving safety of the host after transplantation while ensuring a high induction rate.

Immune Rejection

Immune-like cells, with large nuclei, cluster around the transplanted cells, indicating immune rejection to continue after 6 weeks of transplantation (Gomi et al., 2012). Abnormal gene expression in some cells differentiated from iPSCs exhibited the potential to induce a T cell-dependent immune response in syngeneic recipients. This immunogenicity may be caused by the abnormal expression of antigens between ESCs and iPSCs due to epigenetic differences, leading to the destruction of peripheral immune tolerance (Zhao et al., 2011). The emergence of zinc finger nucleases, transcription activator–like effector

nucleases (TALENs), and the clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (Cas9) system, as powerful gene-editing tools, has now made it possible to modify genes efficiently and accurately. Genomic correction of mutant iPSCs may lead to healthy differentiated cells, thus avoiding the risk of immune rejection (Kim and Kim, 2014; Hockemeyer and Jaenisch, 2016; Ben Jehuda et al., 2018). In future, more attention should be paid to the occurrence of an immune response during iPSC transplantation, and additional research should be conducted to assess and avoid immune rejection.

Apart from the above limitations, overexpression of stromal cell-derived factor 1 alpha (SDF-1α) or optochemogenetic stimulation of iPSC-NPCs may be an effective strategy to enhance endogenous neurovascular regeneration and functional recovery after ischemic stroke (Chau et al., 2017; Yu et al., 2019). A study conducted in 2010 had found that subdural iPSCs-FG can significantly reduce the total infarct volume and improve the behavior of rats with MCAO, indicating subdural transplantation of iPSCs-FG to be a safer administration route (Chen et al., 2010). Transplantation of MSCs through the intra-arterial route increased the risk of cerebral lesions and did not improve functional recovery in a transient cerebral ischemia rat model (Argibay et al., 2017), whereas the intra-arterial transfer of MSCs/neurogenin-1 could block neuronal cell death and inflammation to ultimately improve functional recovery (Kim et al., 2020).

CONCLUSION AND FUTURE PROSPECTS

The explosion of research on stem cell therapy and its characteristics has ushered in a new era for the treatment of ischemic stroke. At the same time, iPSC technology has also become increasingly mature for the treatment of ischemic stroke, achieving considerable results in preclinical models, thereby bypassing the ethical and immune rejection concerns related to the use of ESCs. At present, iPSCs are considered a promising tool for clinical treatment, and are expected to be practically applicable in the near future. To realize this therapy, we should strive to overcome the remaining obstacles, and conduct wider and more in-depth clinical trials. Importantly, a consensus is required for optimizing the methods for cultivation and differentiation of iPSCs. In addition, different iPSC lines may vary in their ability to differentiate into different cell lines, a process that warrants further attention. The development and evaluation of various strategies may offer a promising solution to overcome the tumorigenicity and low efficiency of iPSC induction. Appropriate transplantation methods can improve the survival rate of transplanted cells and further strengthen the therapeutic effect. When choosing transplantation methods, in addition to determining the transplantation time and route, it would be necessary to determine the differentiation state, transplantation administration, dose, and auxiliary status before transplantation.

In summary, with rapid progress and improvement in the technology of iPSCs, and gaining better understanding of the reprogramming and therapeutic mechanisms, the ultimate goal of providing iPSCs safely and with relatively high efficiency to patients with ischemic stroke may become a reality in near future.

AUTHOR CONTRIBUTIONS

RD, YG, and RH searched for relevant literature and drafted the manuscript together. LJ and YL searched for relevant literature. YY and ZG revised the manuscript critically. TL and CZ were involved in preparing the figures and tables. LL and YJ provided professional guidance for this review and performed a final check of the manuscript. All authors contributed to the review of this manuscript and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation of China (grant nos. 81371385, U1604170, and 81870926).

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

frontiers in Molecular Neuroscience

ORIGINAL RESEARCH
published: 28 May 2021
doi: 10.3389/fnmol.2021.639145

Post-stroke Delivery of Valproic Acid Promotes Functional Recovery and Differentially Modifies Responses of Peri-Infarct Microglia

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Edited by:
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Chongqing University, China

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Specialty section:
This article was submitted to Molecular Signalling and Pathways, a section of the journal Frontiers in Molecular Neuroscience

Received: 08 December 2020
Accepted: 28 April 2021
Published: 28 May 2021

Citation:
Kuo T-T, Wang V, Wu J-S, Chen Y-H and Tseng K-Y (2021) Post-stroke Delivery of Valproic Acid Promotes Functional Recovery and Differentially Modifies Responses of Peri-Infarct Microglia. Front. Mol. Neurosci. 14:639145. doi: 10.3389/fnmol.2021.639145

The specific role of peri-infarct microglia and the timing of its morphological changes following ischemic stroke are not well understood. Valproic acid (VPA) can protect against ischemic damage and promote recovery. In this study, we first determined whether a single dose of VPA after stroke could decrease infarction area or improve functional recovery. Next, we investigated the number and morphological characteristic of peri-infarct microglia at different time points and elucidated the mechanism of microglial response by VPA treatment. Male Sprague-Dawley rats were subjected to distal middle cerebral artery occlusion (dMCAo) for 90 min, followed by reperfusion. Some received a single injection of VPA (200 mg/kg) 90 min after the induction of ischemia, while vehicle-treated animals underwent the same procedure with physiological saline. Infarction volume was calculated at 48 h after reperfusion, and neurological symptoms were evaluated. VPA didn't significantly reduce infarct volume but did ameliorate neurological deficit at least partially compared with vehicle. Meanwhile, VPA reduced dMCAo-induced elevation of IL-6 at 24 h post-stroke and significantly decreased the number of CD11b-positive microglia within peri-infarct cortex at 7 days. Morphological analysis revealed that VPA therapy leads to higher fractal dimensions, smaller soma size and lower circularity index of CD11b-positive cells within peri-infarct cortex at both 2 and 7 days, suggesting that VPA has core effects on microglial morphology. The modulation of microglia morphology caused by VPA might involve HDAC inhibition-mediated suppression of galectin-3 production. Furthermore, qPCR analysis of CD11b-positive cells at 3 days post-stroke suggested that VPA could partially enhance M2 subset polarization of microglia in peri-infarct cortex. Analysis of VPA-induced changes to gene expressions at 3 days post-stroke implies that these alternations of the biomarkers and microglial responses are implicated in the upregulation of wound healing, collagen trimmer, and extracellular matrix genes within peri-infarct cortex. Our results are the first to show that a low dose of VPA promotes

short-term functional recovery but does not alter infarct volume. The decreases in the expression of both IL-6 and galectin-3 might influence the morphological characteristics and transcriptional profiles of microglia and extracellular matrix remodeling, which could contribute to the improved recovery.

Keywords: distal middle cerebral artery occlusion (dMCAO), valproic acid (VPA), microglia activation, galectin-3 (Gal-3), ischemic stroke

INTRODUCTION

Stroke is one of the leading causes of death and adult disability worldwide (Feigin et al., 2015). Primary ischemic stroke causes the death of neurons, astrocytes, and oligodendrocytes, as well as blood capillary damage. This destruction results in a series of pathological reactions known as secondary injuries, including blood barrier disruption and inflammatory responses (Wang et al., 2011). Then, various types of inflammatory cells, such as microglia, T cells, and macrophages, are recruited to the lesion to remove cell debris (Wang et al., 2019). However, these inflammatory cells cause secondary cell death, expansion of the lesion region, and functional impairment of the body by elicit excessive production of cytokines and chemokines (Lambertsen et al., 2012; Lu et al., 2013). Most of the studies were concentrated on the neuroprotective property; thence, there is needed to identify drug targets or develop a new therapeutic way that would accelerate the recovery after ischemic brain injury by addressing postischemic pathologic mechanisms such as neuroinflammation (Anttila et al., 2018). A viable therapeutic strategy that modulates the brain inflammatory responses afterward stroke is recognized to boost functional recovery from ischemic stroke. However, few studies have taken this approach, and little is known about how recovery from stroke relates to neuroinflammation (Lakhan et al., 2009; Ahmad and Graham, 2010).

Valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, is widely used for the treatment of migraine and epileptic seizures and as a mood stabilizer in treating bipolar disorder (Chateauvieux et al., 2010). *In vitro*, treatment with VPA suppresses lipopolysaccharide-induced production of TNF-α and IL-6, attenuates glutamate-induced excitotoxicity, and inhibits ischemia-induced fast sodium and high-voltage-activated calcium currents (Suda et al., 2013; Mairuae and Cheepsunthorn, 2018). In rat models of transient ischemia, VPA exhibits anti-inflammatory and neuroprotective effects through the inhibition of HDAC activity and induction of HSP70, which attenuates ischemia-reperfusion injury. In addition, it ameliorates blood-brain barrier (BBB) disruption through inhibition of the nuclear translocation of nuclear factor-κB activation and matrix metalloproteinase 9 production (Ren et al., 2004; Wang et al., 2011; Suda et al., 2013). Furthermore, delayed VPA treatment could enhance white matter repair and neurogenesis in ischemic brain (Liu et al., 2012). In an animal model of spinal cord injury, VPA was further shown to decrease purinergic P2 × 4 receptor expression in activated microglia, as well as to ameliorate microgliosis (Masuch et al., 2016; Chen et al., 2018). Although the studies referenced above suggested that VPA therapy is involved in microglial activation, those investigations determined activated microglia based only on cellular density in brain sections immunolabeled to detect either ionized calcium binding adaptor molecule 1 (Iba1) or CD11b (Xuan et al., 2012). There have been no studies, however, aimed at better characterizing the morphological changes and polarized phenotype of peri-infarct microglia after VPA administration. Meanwhile, VPA therapy has been demonstrated to cause dose-dependent side effects, like depression, ataxia, arrhythmia, and rare but fatal hepatotoxicity (Anderson et al., 1992; Isoherranen et al., 2003). Relatively high doses of VPA of 300 mg/kg/day and above were shown to induce hepatotoxicity and neurological side effects in animal studies (Tai et al., 2014). Thus, we focused to study if the post-stroke injection of a lower dose of VPA, 200 mg/kg, in rats would promote recovery and if this recovery, if any, is implicated in altered biomarkers, cytokines, and transcriptional profiles within peri-infarct cortex. Furthermore, we quantitated the subtleties of microglial morphology with fractal analysis, soma size and circularity index following cortical ischemia-reperfusion insult because morphology has not yet been well studied. As microglia show obviously morphological changes on day 2 post-stroke in the peri-infarction cortex, we administered VPA after the onset of reperfusion. Here, we show how post-stroke of VPA administration, 200 mg/kg, promoted behavioral recovery during the short 2-day testing period, accompanied by modulating microglia/macrophage activation, and further explore the underlying molecular mechanisms.

Abbreviations: AAALAC International, Association for Assessment and Accreditation of Laboratory Animal Care International; APC, antigen-presenting cells; BBB, Blood-brain barrier; CCA, common carotid artery; cDNA, complementary DNA; CI, circularity index; DEGs, differentially expressed genes; dMCA, distal middle cerebral artery; dMCAo, distal middle cerebral artery occlusion; DMEM, Dulbecco's modified Eagle's medium; dscDNA, double-stranded cDNA; ELISAs, enzyme-linked immunosorbent assays; FBS, fetal bovine serum; GSEA, gene set enrichment analysis; HDAC, histone deacetylase; HSP70, heat shock protein 70; IACUC, Institutional Animal Care and Use Committee; Iba1, ionized calcium binding adaptor molecule 1; LPS, lipopolysaccharide; MACS, magnetic activated cell sorting; qPCR, quantitative PCR; RLE, Relative Log Expression; TMM, trimmed mean of *M*-values; TTC, 2,3,5-triphenyltetrazolium chloride; VPA, valproic acid; WGCNA, weighted gene co-expression network analysis.

MATERIALS AND METHODS

Animals

The total of 157 Male adult Sprague-Dawley rats (300–350 g; 36 Sham, 23 Sham + VPA, 50 dMCAo + vehicle, 48 dMCAo + VPA), based on previous experience involving similar experiment settings, were used for this study. Those animals were housed at the National Defense Medical Center's Animal

Center with a 12-h light/dark cycle, temperature of 25 ± 2°C, 55% humidity, 2–4 animals per cage, and *ad libitum* standard diet and water. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC; protocol number 16-258) of the National Defense Medical Center, Taiwan, R.O.C., which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). All experiments were performed in a blinded manner, and the experimental results are reported according to the ARRIVE guidelines.

Distal Middle Cerebral Artery Occlusion Model and VPA Treatment

A cortical stroke was induced in each rat by occluding the distal middle cerebral artery (dMCA) along with bilateral common carotid arteries (CCAs) (Chen et al., 1986). Each rat was anesthetized with 4% chloral hydrate (Sigma Aldrich, St. Louis, MO, United States) injected intraperitoneally (i.p.; 400 mg/kg), and lidocaine was used as a local anesthetic. The surgery was conducted as described earlier (Airavaara et al., 2010, 2009; Matlik et al., 2014). Briefly, the CCAs were separated through a cervical dissection. A small craniotomy was undertaken on the right side of the skull, and the right dMCA was ligated directly with a 10-0 suture. The CCAs were simultaneously occluded with non-traumatic arterial clips. After 90 min of ischemia, the suture around the MCA and the arterial clips were removed for restoration of blood flow (reperfusion). The body temperature of each rat was maintained at 37°C during the procedures until recovery from anesthesia, when the rat was returned to its home cage. A dose of 200 mg/kg of VPA or normal saline as a vehicle control was injected intraperitoneally into each rat, with the one injection made immediately after the onset of reperfusion. VPA was purchased from Sigma (MO, USA) and dissolved in physiologic saline (Xuan et al., 2012). 2,3,5-triphenyltetrazolium chloride (TTC) staining was used to quantified the infarction volume from 2-mm brain slices at day 2 post-stroke as described previously (Airavaara et al., 2010).

Behavioral Tests

A body asymmetry test was performed as described previously (Airavaara et al., 2010). First, lifting the rats above the testing table by the tails, and then to count the frequency of initial turnings of the head or upper body contralateral to the ischemic side. This trial was repeated 20 times in total. The modified Bederson's score was used to assessment the neurological deficits of all rats (Anttila et al., 2018). The cylinder test was carried out as described previously (Runeberg-Roos et al., 2016). The rats were placed inside a clear plastic cylinder (diameter: 35 cm) for 5 min. After raising back on the hind limbs, count the number of first front paw touches to the inner wall of the tube. Locomotor activity was measured using an infrared activity monitor for 1 h (Med Associates, St. Albans, VT, United States).

Assessment of Cytokines

Tissues from a total of 16 rats were used for the cytokine assay by enzyme-linked immunosorbent assay (ELISA). The ipsilateral peri-infarct cortical and hippocampal tissues were collected at 6, 24, and 48 h post-dMCAo. After homogenization in lysis buffer (PRO-PREP^{TM}, iNtRON Biotechnology, South Korea) and centrifugation at 15,000 *g* for 30 min, and then collected the supernatants and stored at −80°C. During quantification, the cytokines (TNFα, IL-1β, IL-6, and IL-10) were normalized to 150 μg of protein in the supernatant using a commercial ELISA kit (R&D Systems, Minneapolis, MN, United States) according to the manufacturer's instructions.

Histology, Immunostaining of Free-Floating Sections, and Image Acquisition

We utilized pentobarbital (90 mg/kg i.p.) to anesthetize the rats, which then were transcardially perfusion with 200 ml saline followed by 500 ml of 4% paraformaldehyde. Brains were dehydrated in 30% sucrose at 4°C and using a Leica CM3050 Cryostat to section coronally into 40-μm-thick slices. Sections were taken from 2.1 to −1.0 mm (striatum) relative to bregma, then stored in cryopreservant for storage (20% glycerol, 2% DMSO in 1XPBS). Sections were blocked with 4% BSA (Sigma-Aldrich) + 0.1% Triton X-100 (Sigma-Aldrich), then incubated with primary antibody (rabbit anti-CD11b 1:1000; Abcam) overnight at 4°C. The next day, sections were stained with secondary antibody, followed by incubation with secondary antibodies conjugated with Alexa Fluor® 488 or 568 (1:500). Digital imagining was carried out on an Olympus AX-80 microscope and attached DP-70 digital camera (Olympus America Inc., Center Valley, PA, United States) using a 40x objective. Three coronal brain sections per animal (between bregma −0.45 and −1.85) were imaged in the right hemisphere at each time point (sham, 1, 2, and 7 days post-dMCAo) (Morrison et al., 2017). The regions imaged were in the medial peri-infarct cortex extending 400 μm from the infarct border and in the dorsal striatum underlying the infarct. Therefore, the imaging yielded six digital photomicrographs per animal for analysis.

Unbiased Stereological Counting of CD11b-Positive Cells in the Peri-Infarct Cortex

CD11b-positive cells in the peri-infarct cortex were counted using unbiased stereology with a stereomicroscope (Olympus BX51) and the StereoInvestigator 6 program (MBF Bioscience) as previously described (Mijatovic et al., 2007). Selected four 40 μm thick coronal slices according to their location relative to the bregma (0.2, 0, −0.26, and −0.4 mm) to obtain results relatively free of bias in the distribution of the cells. This experiment only counted CD11b positive cells with clear microglia morphology. Approximately 80 randomly selected sites were analyzed for per area/slice to ensure accuracy and minimize error.

Fractal Analysis Using FracLac for ImageJ

For investigating the morphological changes of microglia following ischemic infarct, photomicrographs were acquired from the location (red square in **Figures 3A,C,E**), 300 μm

away from the infarct (peri-infarct cortex), where microglia activation was to be expected high. We utilized our computer-aided morphologic analysis to include fractal analysis (FracLac for ImageJ), which quantifies cell complexity (fractal dimension, fD) (Karperien et al., 2013). Five microglia randomly selected for this analyze within each photomicrograph (6 photomicrographs per animal). A total of 30 cells chosen for fractal analysis (using a grid and random number generator) per animal in each region. The additional structures that abut and surround each cell were eliminated from the analysis by manual deletion using a digitizing tablet and ImageJ. Binary images were then converted to outlines using ImageJ. Fractal dimension (fD) is the assessment of microglia complexity, which quantifies each cell's contour bounded by the endpoints and process lengths. FracLac for ImageJ calculates the microglia fD for each cell using a box plot protocol that determines the amount of pixel detail with increasing scale, where N = the number of pixels or "detail" at a particular scale (ε) (**Figure 5H** and **Table 1**; Morrison et al., 2017). These calculations and relationships were well described in the previous studies (Karperien et al., 2013; Morrison et al., 2017).

Manual Analysis of Microglia Morphology

FIJI software (version 2.0) was used for manual morphological analysis (Ferreira et al., 2014; Heindl et al., 2018). In order to prevent overlap with cells located in more superficial or deeper layers, slices containing an identified cells were processed as a maximum intensity projections. Maximum intensity projections of the cells were incepted to create a binary appearance. Quantification of microglia soma was completed using Fiji (Image J) Analyse Particles function with a particle size threshold of 10 pixels, to exclude small pixel nose and extract microglial soma size (green ellipse fit; **Figure 5J**). Through measurement of the area (white) and the perimeter (red) of a cell from the binary mask, the circularity index (CI) was determined (**Figure 5J** and **Table 1**).

Cell Culture and Treatment

Murine BV-2 microglial cells were gifted from Dr. Mei-Jen Wang (Huang et al., 2016) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin (all from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, United States) at 37°C under humidified 95% O2 and 5% CO2. The cells were then seeded into 24-well plates at a density of 1×10^5/well and maintained at 37°C under humidified 95% O2 and 5% CO2. To detect the expression of acetyl-histone H3, as well as the production of galectin-3 and heat shock protein 70 (HSP70), the medium was replaced with freshly prepared serum-free medium with or without lipopolysaccharide (LPS) (1 μg/ml) and/or a non-toxic dose of VPA (1.5 mM) for 6 h (Mairuae and Cheepsunthorn, 2018).

Western Blot Analysis

For western blotting, aliquots of the proteins were distributed by electrophoresis on sodium dodecyl sulfate–polyacrylamide gels (8% or 10%) and transferred to a polyvinylidene difluoride membrane. The membranes were rinsed in 0.01 M Tris-buffered saline (pH 7.4) containing 0.1% Triton X-100 for 10 min, blocked in 5% non-fat dry milk for 30 min, and then incubated overnight at 4°C in the presence of acetylated histone H3 antibody against acetylated histone H3 on lys9 (rabbit polyclonal; 1:1000; sigma-Aldrich), HSP70 (rabbit polyclonal; 1:1000; Santa Cruz), galectin-3 (rabbit polyclonal; 1:250; GeneTex), or actin (mouse polyclonal; 1:1000; Abcam). After washing three times with Tris-buffered saline, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody in Tris-buffered saline containing 5% non-fat dry milk for 2 h at room temperature. Immunoreactivity was detected by enhanced chemiluminescent autoradiography (ECL kit; Amersham Life Science, Arlington Heights, IL, United States). The western blots were captured with a digital camera, and the intensities were quantified with NIH Image J.

Protein Array Analysis

Brain protein levels were analyzed following dMCAo-induced cortical infarction. Rat brains were collected and homogenized 48 h after dMACo, and the expression levels of 67 proteins were measured using a RayBio® L-Series Rat 67 Antibody Array (RayBiotech, Norcross, GA, United States). Total protein was extracted from 250 mg of cortical tissue with 1 mL of ice-cold tissue protein extraction reagent containing protein degradation inhibitors (Kangcheng, Shanghai, China). Protein concentrations were determined by a BCA Protein Assay Kit (Kangcheng). The RayBio® L-Series Rat 67 Antibody Array membrane (RayBiotech) was blocked for 30 min by adding blocking buffer, and then incubated with the protein samples at room temperature for 2 h. The chip membrane was cleaned with buffer, and then incubated with biotin-labeled antibodies at room temperature for 2 h. After washing with buffer, the membrane was incubated with streptavidin (1:1000) coupled with horseradish peroxidase at room temperature for 2 h. Reacting with chemiluminescence reagent (RayBiotech, Norcross, GA, United States) in the dark and exposing to X-ray film, images were obtained using a film scanner (i3200, Kodak, Rochester, NY, United States). The original biomarker values were first centered and scaled by subtracting the mean of each biomarker from the data and then dividing it by the standard deviation, respectively (Zhou et al., 2017). Centering and scaling results in a uniform mean and scale across all the biomarkers, but leaves their distribution unchanged (Tusher et al., 2001; Yu et al., 2012).

Analysis of Differential Biomarker Expression

The biomarker values were summarized in terms of mean and standard deviation, or median with minimum and maximum responses across the groups. The fold change between groups was calculated as the ratio of the mean or median. If the biomarkers met or did not meet normality criteria across two groups, the significance of expression difference was evaluated by the paired *t*-test or signed-rank test, respectively. Biomarkers with a *P* adjusted value < 0.05 were considered to be differentially expressed (Tusher et al., 2001).

TABLE 1 | Summary of microglia/macrophage morphology measures.

	Measure	Unit	Range	Scale	Sampling	Interpretation
Fractal dimension	**Regression slope[*ln(N)/ln(?)*]**	**DB**	**1-2**	**Individual cell**	**24 cells/animal**	**Cell complexity**
Circularity Index	(4π[area]/[perimeter]2)	CI	0–1	Individual cell	24 cells/animal	Cell circularity

RNA Preparation and RNA-Sequencing

Rats were sacrificed on day 3 after MCAo surgery and perfused with 0.9% saline solution before the collection of tissue material (n = 4 for vehicle, n = 4 for VPA). RNA was extracted from tissues that were taken from the medial peri-infarct cortex on two 1 mm thick sections from positions A/P –0.9 to +0.1 and A/P +1.1 to +2.1. RNA was extracted with Trizol reagent and treated with DNase (#1906, Ambion). RNA purity and quantification were checked using SimpliNano™ - Biochrom Spectrophotometers (Biochrom, MA, United States). RNA degradation and integrity were monitored by Qsep 100 DNA/RNA Analyzer (BiOptic Inc., Taiwan). One μl RNA of each sample was utilized as input material for the RNA sample preparations. Sequencing libraries were set up by means of the KAPA mRNA HyperPrep Kit (KAPA Biosystems, Roche, Basel, Switzerland) following the manufacturer's recommendations, and index codes were come with attributing sequences to each sample. In a short, captured mRNA was disintegrated by incubating it at a high temperature in the presence of magnesium in KAPA Fragment, Prime and Elute Buffer (1x). First-strand complementary DNA (cDNA) was integrated using random hexamer priming. After converting the cDNA:RNA hybrid into double-stranded cDNA (dscDNA), dUTP was incorporated into the second cDNA strand accompanied by dAMP added to the 3′ ends of the resulting dscDNA. dsDNA adapters with 3′dTMP overhangs were ligated to library insert fragments to generate the library fragments carrying the adapters. For choosing cDNA fragments of preferentially 300∼400 bp in length, the library fragments were purified with the KAPA Pure Beads system (KAPA Biosystems, Roche, Basel, Switzerland). The library carrying appropriate adapter sequences at both ends was amplified using KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Roche, Basel, Switzerland) along with library amplification primers. Lastly, PCR products were purified using the KAPA Pure Beads system, and the library quality was assessed using the Qsep 100 DNA/RNA Analyzer (BiOptic Inc., Taiwan). The RNA-seq data have been deposited in the *RNA-Seq* database at Biotools-rat-RNA prelibrary under accession number BI-ANA-1416.

Bioinformatics

The original data from high-throughput sequencing (Illumina NovaSeq 6000 platform) was transformed into raw sequenced reads by CASAVA base celling and stored in FASTQ format (Love et al., 2014; Schurch et al., 2016). These data have been submitted to the NCBI BioProject database[1]. The NCBI SRA accession number for these data is PRJNA694497. After cleaning up low-quality reads and eliminating poor-quality bases (Bolger et al., 2014; Kim et al., 2015; Sahraeian et al., 2017), the obtained high-quality data were used for subsequent analysis. Read pairs from each sample were coordinated to the reference genome using the HISAT2 software (v2.1.0) (Kim et al., 2015; Sahraeian et al., 2017). For gene expression, the "Trimmed Mean of M-values" normalization (TMM) as well as "Relative Log Expression" normalization (RLE) was conducted using DEGseq (Wang et al., 2010; Love et al., 2014; Schurch et al., 2016). Differentially expressed genes (DEGs) analysis of two conditions was performed in R using DEGseq (without biological replicate) and DESeq2 (with biological replicate), which are based on the negative binomial distribution and Poisson distribution model, respectively (Anders et al., 2013; Li et al., 2016; Maza, 2016). The resulting p-values were adjusted using Benjamini and Hochberg's approach for controlling the FDR. GO (Kanehisa et al., 2008, 2019) enrichment analysis of DEGs was conducted using clusterProfiler (v3.10.1) (Yu et al., 2012). Gene set enrichment analysis (GSEA) (Subramanian et al., 2005) was performed with 1,000 permutations to identify enriched biological functions and activated pathways from the molecular signatures database (MSigDB). The MSigDB is a collection of annotated gene sets for use with GSEA software, including hallmark gene sets, positional gene sets, curated gene sets, motif gene sets, computational gene sets, GO gene sets, oncogenic gene sets, and immunologic gene sets (Liberzon et al., 2011, 2015). In addition, weighted gene co-expression network analysis (WGCNA) was used to construct the co-expression network based on the correlation coefficient of expression pattern using the WGCNA (v1.64) package in R (Zhang and Horvath, 2005; Langfelder and Horvath, 2008).

Real-Time Quantitative PCR From Microglia Isolated From the Sham or Stroke Cortex

The microglia harvest was performed as described previously (Anttila et al., 2018). Briefly, the CD11b-positive microglia within the cortex were isolated by magnetic activated cell sorting (MACS). On ice in HBSS without Ca^{2+} and Mg^{2+} dissected the ischemic cortex and the equivalent part of the brains from the sham animals. And then the Neural Tissue Dissociation kit (Miltenyi Biotec) and gentleMACS Dissociator (Miltenyi Biotec) was used to dissociate tissue. Followingly, the cells were suspended in 0.5% BSA in PBS and incubated with Myelin Removal Beads II (1:10, Miltenyi Biotec) for 15 min at 4°C and filtered through a LS column (Miltenyi Biotec) using a Quadro-MACS Separator (Miltenyi Biotec). Lastly, the cells were incubated at 4°C for 10 min with mouse anti-CD11b: FITC antibody (1:10, #MCA275FA, AbD Serotec) (Anttila et al., 2018). After washing along with resuspending in 0.5% BSA with 2 mM EDTA in PBS, cells were incubated with anti-FITC MicroBeads

[1]https://www.ncbi.nlm.nih.gov/sra

(1:10, Miltenyi Biotec) for 15 min at 4°C. Following resuspension in 0.5% BSA with 2 mM EDTA in PBS, the cell suspension was applied to a LS column placed on a QuadroMACS Separator. Total RNA was then extracted from the gentleMACS-isolated microglia using RNeasy Plus micro/mini kits (Qiagen, Hilden, Germany), according to the manufacturer's protocol.

RNA quality was assessed by means of an Agilent Bioanalyzer (Agilent, Santa Clara, CA, United States). For the rat samples, cDNA was synthesized by using an oligo-T18 primer and Maxima H minus reverse transcriptase (#EP0751; Thermo Fisher Scientific). Real-time quantitative PCR (qPCR) was performed with Lightcycler®480 SYBR Green I Master complemented with 2.5 pmol of primers (**Table 2**) following a Lightcycler®480 real-time PCR system (Roche Diagnostics). Reactions were performed in triplicate and analyzed with Lightcycler®480 Software. Using agarose gel electrophoresis to verify the length of the resulting PCR products. Gene expression was normalized to the geometric mean of ubiquitin-conjugating enzyme E2 I (Ube2i) expression levels.

Statistical Analysis

All graphs and statistics were performed in GraphPad Prism 6.0. The body asymmetry, Bederson's score, and locomotor activity data were analyzed using Bonferroni's *post hoc* test following two-way ANOVA. The infarction volume and qPCR results were analyzed using the two-tailed Student's *t*-test. Two-way ANOVA and Tukey's multiple comparisons *post hoc* test with the factors of "time" and "drug treatment" were used for the comparison of differences in ELISA results and parameters quantified from immunohistochemical staining. Values are presented as mean ± S.E.M. A statistically significant difference was defined as $p < 0.05$.

RESULTS

Low-Dose VPA Treatment Does Not Reduce Infarction Volume, but Promotes Short-Term Behavioral Recovery in Rat Cortical Stroke Model

In contrast with the results reported by prior studies, a single injection of the dose of VPA (200 mg/kg) had no effect on infarct volume measured at 48 h after stroke (**Figures 1A–C**). However, the treatment produced a rapid recovery of function, as seen from significantly reduced body asymmetry and Bederson's scores at 24 h post-stroke (**Figures 1D,E**). At 48 h, the rats injected with VPA also exhibited substantial reversal of injury-induced behavioral deficits in terms of body asymmetry, Bederson's neurological deficits, and the cylinder tests compared to the control group of vehicle-injected rats (**Figures 1D–F**). Spontaneous motor activity, as another indicator of hastened recovery, did not differ between the treatment groups (**Figures 1G,H**). These results clearly indicate that the low-dose VPA treatment significantly promoted functional recovery without reducing the size of the ischemic lesions.

TABLE 2 | Table of qPCR primers used in the study.

	Forward primer	Reverse primer
Clec10a	GAGAAAAACCAAGAGGCTGGT	CTAAGGCCCAGGGAGAACA
Tgfb1	CCTGGAAAGGGCTCAACAC	CAGTTCTTCTCTGTGGAGCTGA
CD163	CTCAGCGTCTCTGCTGTCAC	GGCCAGTCTCAGTTCCTTCTT
Arg1	TTTCCTTGCCTGCTTCTTC	TCCTGTCTCCGTATTCAGCC
iNOS	GCAGAA TGTGACCATCATGG	ACAACCTTGGTGTTGAAGGC
CD86	TCCTCCAGCAGTGGGAAACA	TTTGTAGGTTTCGGGTATCCTTGC
Ube2i	AGCTACGGATGCTTTTCAAAGA	CAGAAGGATACACGTTTGGATGA

VPA Decreases the Production of IL-6, but Not TNF-α or IL-1β in the Peri-Infarct Cortex

Since the levels of pro-inflammatory cytokines in experimental stroke have significant effects on infarction evolution (Lambertsen et al., 2012; Suda et al., 2013), we first characterized the cytokine responses in the proximal (peri-infarct cortex) and distal (ipsilateral hippocampus) region after dMCAo-induced cortical stroke, and then tested the efficacy of post-stroke subcutaneous VPA treatment in adult rats. Obvious increases in IL-1β, TNF-α, and IL-6 were observed as soon as 6 h after dMCAo within the peri-infarct cortex, where they remained elevated for 2 days after dMCAo (**Figures 2A,C,E**). Of additional interest, down-regulated IL-10 was observed 6 h after dMCAo in the peri-infarct cortex, where it remained decreased until 24 h (**Figure 2G**). In the area distal from infarct cortex (hippocampus), there were no differences in the levels of IL-1β, TNF-α, and IL-10 between the sham and dMCAo groups at different time points (**Figures 2B,F**). However, increased IL-6 was observed in hippocampal brain homogenates 6 h after dMCAo, where it remained elevated until 2 days (**Figure 2D**). These results suggest that dMCAo-induced up-regulation of IL-6 is not only involved in the region proximal to the infarct cortex, but also in the region distal to it (hippocampus).

To determine whether post-stroke VPA treatment is involved in inflammatory responses, we measured changes in the levels of pro- and anti-inflammatory cytokines in the peri-infarct cortex and hippocampal brain homogenates at 6, 24, and 48 h after dMCAo. The administration of VPA resulted in a significant decrease in IL-6 within the peri-infarct cortex at 24 h after dMCAo (**Figure 2C**), but not in the ipsilateral hippocampal area (**Figure 2D**). Meanwhile, the levels of IL-1, TNF-α, and IL-10 within the peri-infarct cortex and ipsilateral hippocampus exhibited no statistically significant differences between the two groups at different time points (**Figures 2B,F,H**), implying that post-stroke VPA treatment only suppresses the up-regulation of glial activation-induced IL-6 in the peri-infarct environment.

Post-Insult VPA Administration Reduces the Number of CD11b-Positive Cells on Day 7

Valproic acid has been demonstrated to exhibit a neuroprotective property in a rat model of dMCAo by promoting neuronal

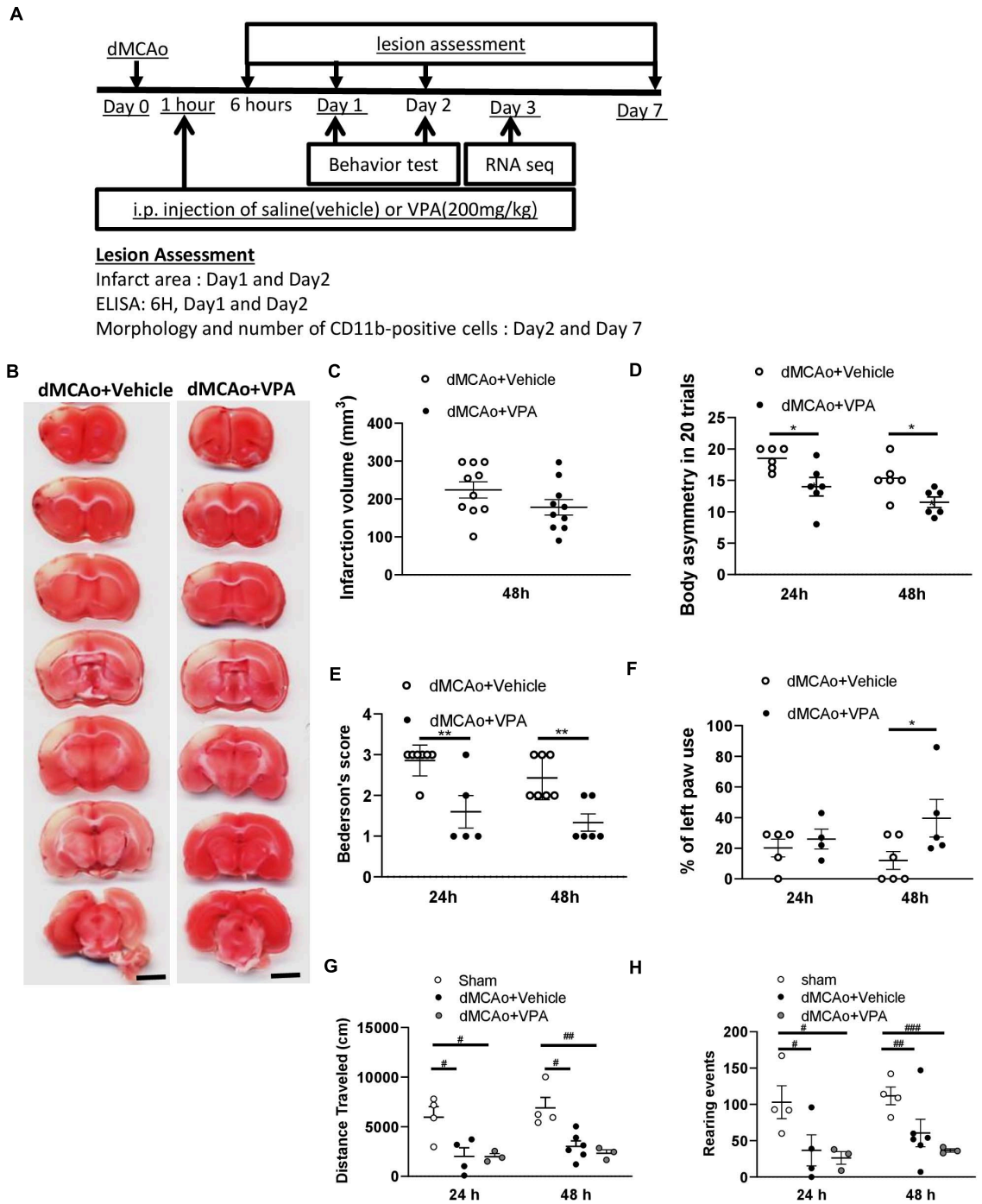

FIGURE 1 | Post-stroke single injection of VPA (200 mg/kg) does not reduce infarct area, but promotes functional recovery. **(A)** Experimental timeline. The rats underwent dMCAo surgery, and they were divided into two groups randomly. One hour after reperfusion, the animals received an intraperitoneal injection of either VPA or vehicle once, and their behavioral functions were evaluated on day 1 and 2. Rats were sacrificed for analysis at different time points. **(B)** A representative image showing infarct area stained with TTC in a series of coronal sections derived from these two groups of rats killed at 48 h after ischemia. **(C)** Quantified results of infarct volumes derived from animals killed at 48 h after the onset of dMCAo. **(D,E)** Effects of VPA (n = 7), vehicle (n = 8) on body asymmetry **(D)**, Bederson's neurologic test score **(E)**, $^{*}p < 0.05$, $^{**}p < 0.01$ indicate comparison with vehicle with Bonferroni's *post hoc* test following two-way ANOVA. **(F)** Forepaw use bias of the rats was assessed using the cylinder test on day 1 and 2 after dMCAo. $^{*}p = 0.039$ by Bonferroni's multiple comparisons test, following two-way ANOVA. **(G,H)** Effects of VPA (n = 5), vehicle (n = 6), and no treatment (n = 4) on vertical **(G)** and horizontal **(H)** activity measured for 30 min on day 1 and 2. $^{*}p < 0.05$, $^{**}p < 0.01$; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ by Bonferroni's multiple comparisons test, following one-way ANOVA. Scale bar: 5000 μm. The data represent mean ± SEM.

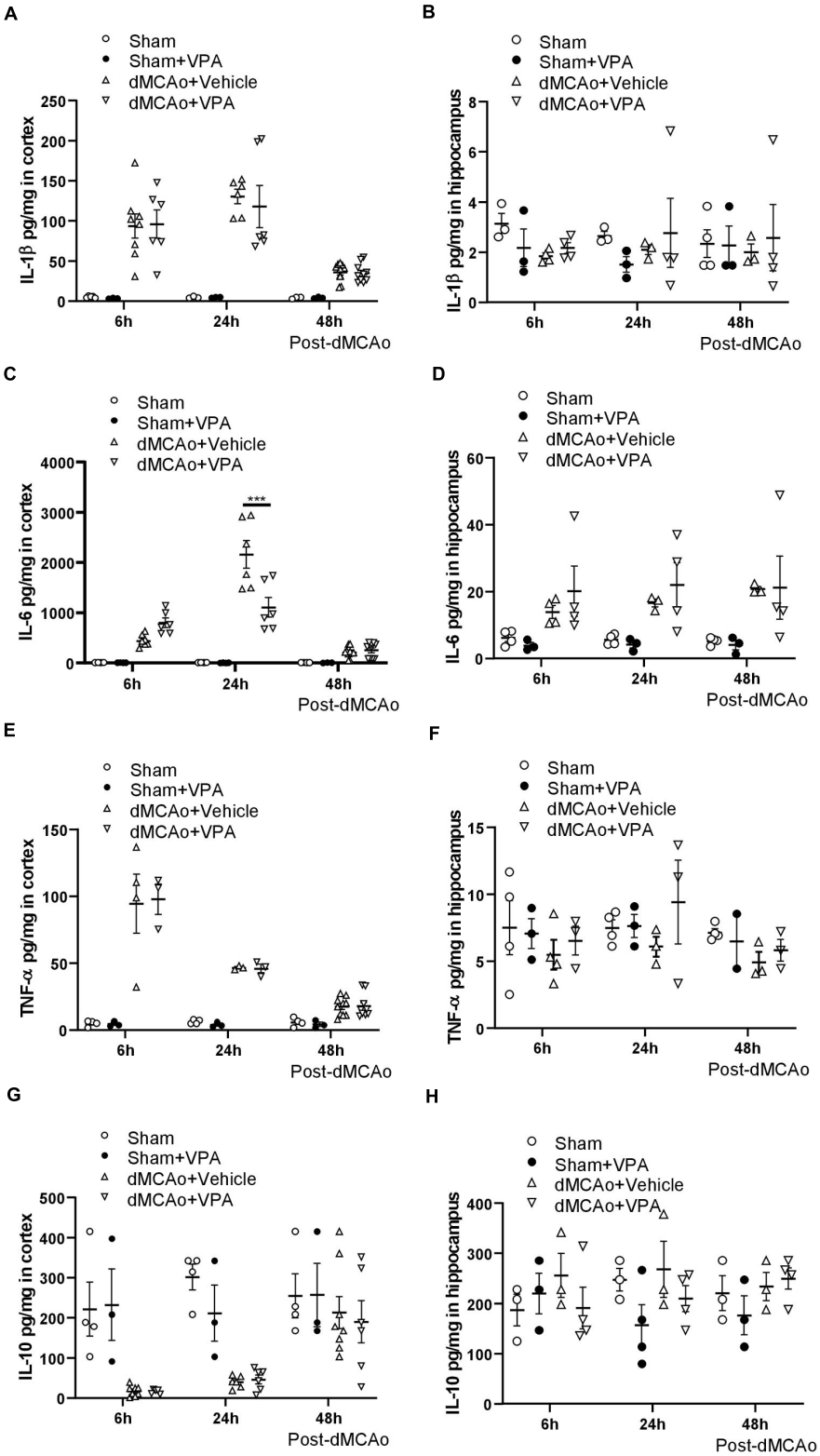

FIGURE 2 | Valproic acid suppresses IL-6 production in the peri-infarct cortex at 24 h after dMCAo surgery. **(A,B)** IL-1β concentrations in the peri-infarct cortex **(A)** and ipsilateral hippocampus **(B)** were measured by ELISA. **(C,D)** TNF-α concentrations in the peri-infarct cortex **(C)** and ipsilateral hippocampus **(D)** were measured by ELISA. **(E,F)** IL-6 concentrations in the peri-infarct cortex **(E)** and ipsilateral hippocampus **(F)** were measured by ELISA. **(G,H)** IL-10 concentrations in the peri-infarct cortex **(G)** and ipsilateral hippocampus **(H)** were measured by ELISA. ***$p < 0.001$ indicates comparison with the dMCAo + vehicle group with two-way ANOVA, Bonferroni's *post hoc* test. The data represent mean ± SEM.

survival as well as exerting anti-inflammatory effects, which would contribute to a reduction in microglial cell numbers (Suda et al., 2013). However, how post-stroke VPA treatment modulates the time course of microglia/macrophage activation in the peri-infarct area has not yet been investigated. The infarct was already well advanced at 6 h after dMCAo, reached a maximum volume by 48 h, and had contracted greatly by 7 days based on the volume of tissue exhibiting pale staining with TTC (**Figures 3A,C,E**). Thus, we first characterized the time course of CD11b-positive microglia/macrophages in the dMCAo-induced ischemic cortex. At 6 h post-stroke, few CD11b-positive microglia/macrophages with ramification were present in the peri-infarct region (**Figure 3B**). Microglia/macrophage activation peaked on day 2 post-stroke in the ischemic cortex, when the infarct core and peri-infarct zone were filled with $CD11b^+$ cells presenting with an amoeboid shape (**Figure 3D**). At 7 days post-stroke, CD11b-positive cells were obvious evident in the peri-infarct zone, but the morphology of microglia/macrophages with ramification of cell processes seemed to have recovered partially (**Figure 3F**). VPA treatment did not obviously reduce the number of CD11b-positive microglia/macrophages in sham group (**Figures 4A,B,G**) and on day 2 post-stroke (**Figures 4C,D,G**), but significantly decreased the CD11b-positive cell number in the peri-infarct zone at day 7 post-stroke (**Figures 4E–G**). These results suggested that the low-dose VPA treatment has delayed effects on the recruitment of microglia into ischemic cortex.

VPA Treatment Regulates Morphologic Responses of CD11b-Positive Cells After Ischemic Brain Injury

To further characterize the morphological responses of the microglia, the fractal dimensions and cell circularity of CD11b-immunolabeled cells were measured in separate sections from the same rats at 2 and 7 days post-stroke. Examples of CD11b-positive microglia (made binary and outlined) in the peri-infarct cortex with/without VPA administration are shown in **Figure 5**. The application of FracLac for Image J to microglia outlines resulted in fractal dimensions that ranged from 1.0029 to 1.4058 (available range is 1–2), with the lowest occurring in the peri-infarction region at day 2 post-stroke and the highest in the intact cortex with sham surgery (**Figures 5B,D,I**). There was no difference in the fractal dimensions of CD11b-immunolabeled cells in the intact cortex with or without VPA administration (**Figures 5C,I**). However, two-way ANOVA analysis showed that VPA treatment could increase the fractal dimensions of CD11b-labeled microglia/macrophages at 2 and 7 days post-stroke, compared with the saline group (**Figures 5D–G,I**). The above results suggest that microglial complexity is unchanged from sham in the impact region with or without VPA application, whereas, in the peri-infarct region, cell complexity was lower at 2 and 7 days post-stroke and was recovered close to the sham condition through VPA therapy. Using manual analysis, we investigated an additional measure of microglial morphology related to cell circularity: the circularity index. The soma size and circularity index results of the CD11b-labeled cells in the sham group were similar to those of the CD11b-labeled cells in the VPA-treated group (**Figures 5K,L**). However, the soma size as well as circularity index was maximally increased at 48 h post-stroke, and remained similarly elevated by 7 days (**Figures 5K,L**). The administration of VPA caused a significant reduction in the soma size and circularity index of CD11b-positive cells compared with the vehicle-treated group when assessed at 2 and 7 days after stroke (**Figures 5K,L**). Based on qualitative observations of the CD11b-labeled cell complexity, soma size and circularity, it was suggested that VPA administration could be implicated in microglia activation in the peri-infarct region.

VPA Suppresses the Level of Galectin-3 in the Peri-Infarct Cortex and LPS-Treated Microglia

It is widely accepted that epigenetic processes occur during ischemic stroke (Stanzione et al., 2020). HDAC inhibitors are known to promote a phenotypic shift in microglia and subsequently exert neuroprotective effects in animal models of neurodegenerative diseases (Li S. et al., 2019; Li et al., 2020). VPA has been shown to exhibit anti-inflammatory properties by suppressing the number of activated microglia in stroke rats (Suda et al., 2013). However, it is much less clear whether VPA ameliorates the activation of microglia/macrophages through the regulation of HDACs or the modulation of certain protein expression levels in these cells. Therefore, we adopted the antibody array analysis of 67 proteins in sham-operated, vehicle-treated, and VPA-treated ipsilateral cortex 2 days after dMCAo (**Table 3**). Only proteins with fold change > 2 or <0.25 and which were significantly different (adjusted $P < 0.05$) were included (**Figure 6A**). The level of galectin-3 was significantly higher in the vehicle-treated stroke rats compared with the sham-operated rats (**Figure 6B**). However, VPA treatment significantly suppressed the level of galetin-3 in the ischemic cortex compared with the vehicle-treated group cortex (**Figure 6B**). Western blotting showed that the galectin-3 protein level was remarkably reduced in the VPA-treated peri-infarct cortex (**Figures 6C,D**), consistent with the results obtained with the protein array analysis. In previous studies, the VPA dose used in animal studies to control seizures, 300 mg/kg, was demonstrated to induce an increase in acetylated histone H3 levels and to up-regulate HSP70 levels in the brain. In our study, the dosage of VPA, revised down to 200 mg/kg, also increased acetylated histone H3 levels in the peri-infarct cortex compared with vehicle-treated group cortex (**Figures 6C,D**). However, there was no difference in the levels of HSP70 between the two groups (**Figures 6C,D**). Since galectin-3 is expressed mainly in microglia/macrophages and up-regulated when activated, we next determined the epigenetic mechanism whereby VPA may regulate galectin-3 production in these cells. BV2 cells were challenged with 1 μg/ml LPS in the presence or absence of 1.6 Mm VPA for 6 h. Acetylated histone H3, galectin-3, and HSP70 expressions in the BV2 cells were subsequently assessed. As presented in **Figure 6E**, the addition of VPA resulted in an increase over the baseline acetylated histone H3 level but decreased galectin-3 expression compared with untreated control. In addition, when cells were

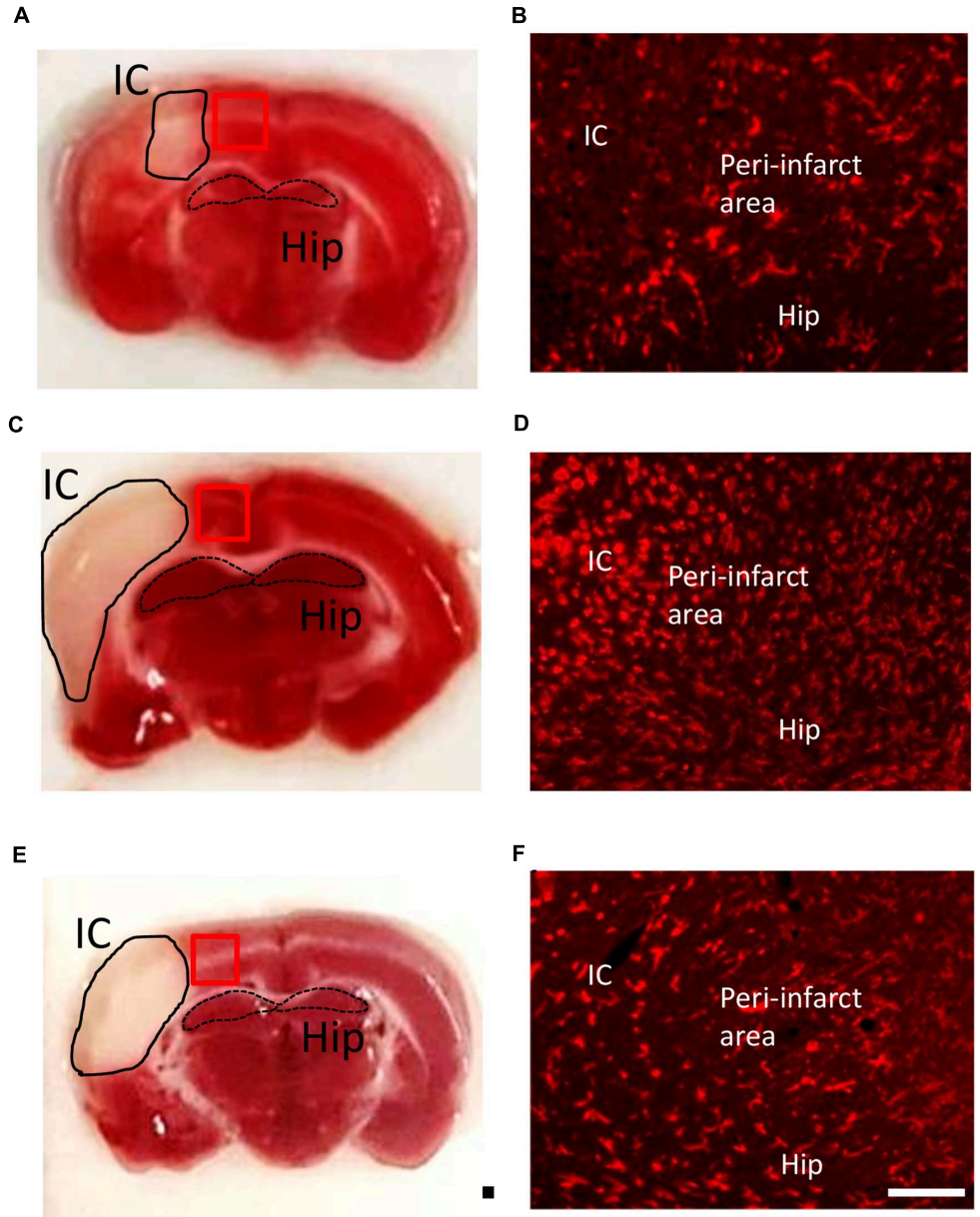

FIGURE 3 | Time course of microglia/macrophage activation after cortical stroke. Representative images of TTC staining and immunostaining of all microglia/macrophages (CD11b) from ischemic core (IC), peri-infarct area, and hippocampus (Hip) coronal sections at 1 **(A,B)**, 2 **(C,D)**, and 7 **(E,F)** days after 90-min dMCAo in rats. The morphological changes and increasing number of microglia/macrophages were obviously found at 2 days after ischemia. Scale bar is 50 μm (immunofluorescence of CD11b) and 5000 μm (TTC staining).

treated with LPS in the presence of VPA, acetylated histone H3 levels were significantly increased when compared with those receiving LPS treatment alone (**Figure 6F**). In contrast, administration with VPA was found to significantly decrease galectin-3 expression in LPS-treated cells as compared to LPS treatment alone (**Figure 6F**). However, the treatment of VPA had no effect on HSP70 expression under the condition of exposing BV-2 cells with or without LPS (**Figures 6E,F**). These results suggest that VPA, by promoting histone acetylation on H3, inhibits galectin-3 production in the microglia/macrophages under the baseline condition or in response to injurious stimuli.

Differential Gene Expression in Peri-Infarct Cortex With or Without VPA Treatment

Since the low dose of VPA was implicated as having an effect on the level of galectin-3 and in the morphological changes of microglia in the peri-infarct cortex, we naturally hypothesized

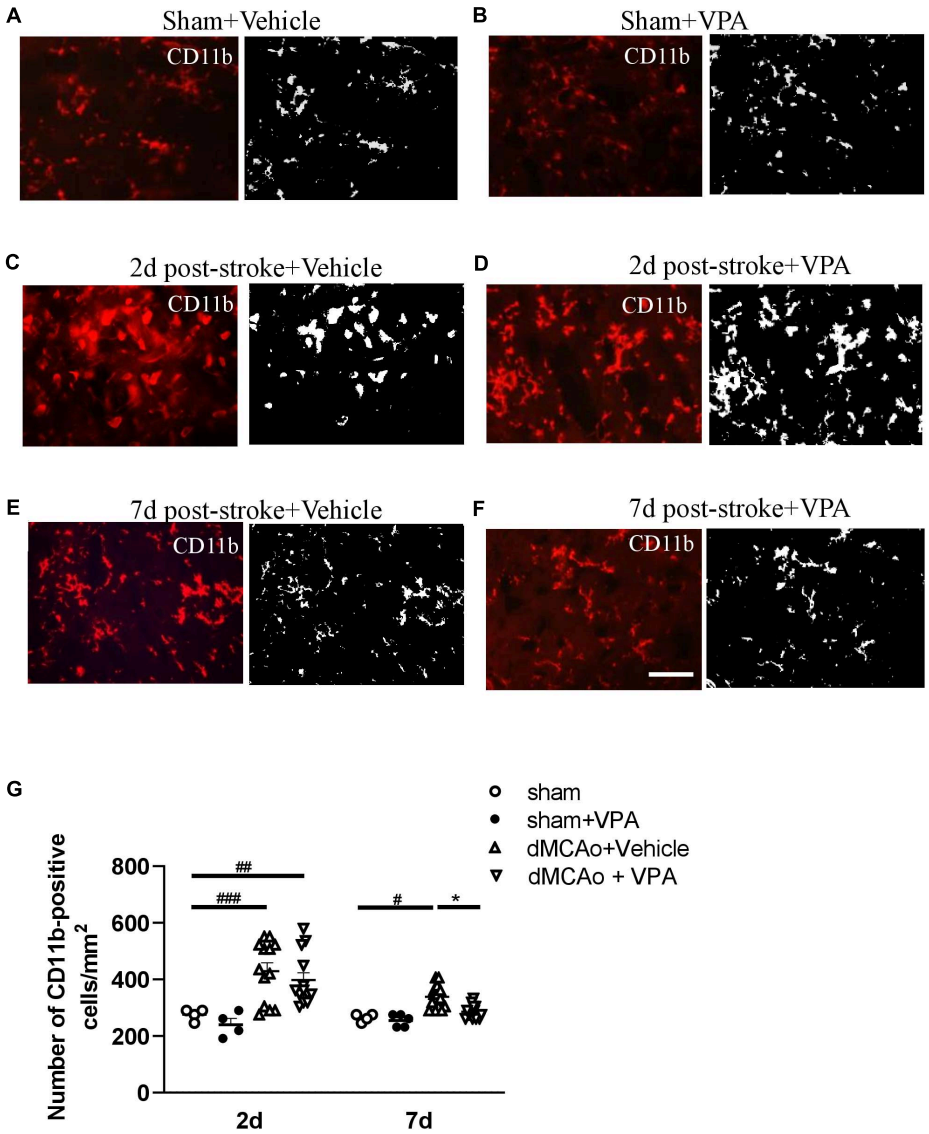

FIGURE 4 | Post-stroke intraperitoneal injection of VPA decreases the number of CD11b-positive cells in the peri-infarct cortex. **(A–F)** Representative photomicrographs of anti-CD11b immunofluorescent staining of the sham-operated cortex in vehicle-treated rats **(A)**, sham-operated cortex in VPA-treated rats **(B)**, peri-infarct cortex at 2 days after 90-min dMCAo in vehicle-treated rats **(C)**, peri-infarct cortex at 2 days after 90-min dMCAo in VPA-treated rats **(D)**, peri-infarct cortex at 7 days after 90-min dMCAo in vehicle-treated rats **(E)**, and peri-infarct cortex at 7 days after 90-min dMCAo in VPA-treated rats **(F)**. **(G)** Quantitation of CD11b-positive cells in the peri-infarct cortex at different time points showing accumulation of activated microglia/macrophages in the peri-infarct cortex at 2 and 7 days after ischemia. Original photomicrographs were subjected to a series of uniform Image J plugin protocols prior to conversion to binary images; binary images were then analyzed to calculate the number of CD11b-positive cells. #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$ indicate statistical difference between peri-infarct cortex and sham-operated cortex at each time point, Bonferroni's multiple comparisons test, following one-way ANOVA. *$p < 0.05$ indicates comparison with the dMCAo + vehicle group with two-way ANOVA, Bonferroni's *post hoc* test. Five tissue sections per rat were used for the analysis (n = 8–10). Scale bar: 20 μm. The data represent mean ± SEM.

that VPA treatment would regulate genes involved in tissue repair, as well as the phenotypes of immune cells. Comparison of gene profiles between vehicle-treated and VPA-treated groups was focused on the peri-infarct cortex at 3 days post-stroke (**Figure 7A**). Genes were identified as differentially expressed genes (DEGs) only when the fold difference between two groups was greater than 2 and the adjusted p-values were lower than or equal to 0.05. Volcano plots that graphically highlighted the DEGs that were significantly up- (red) or down- (blue) regulated in response to VPA treatment and vehicle treatment in the stroke rats were generated (**Figure 7B**). In the VPA-treated group, seven transcripts were differentially expressed compared to the vehicle-treated group, among which one gene was downregulated and six genes were upregulated (**Figure 7C**). The one down-regulated gene was a pseudogene. The up-regulated genes were related to components of the extracellular matrix (collagen

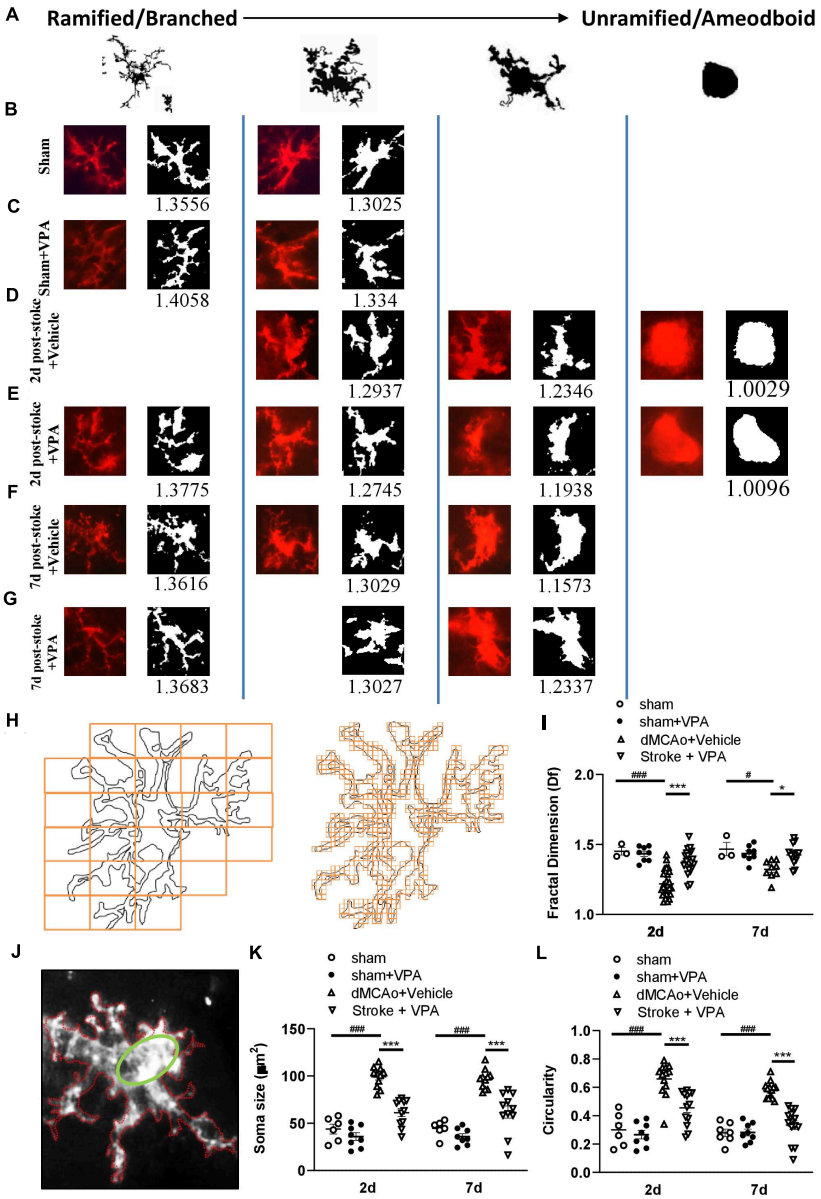

FIGURE 5 | Complexity analysis of CD11b-positive cell morphologies in the peri-infarct cortex with/without VPA administration. **(A)** Microglia/macrophages are morphologically dynamic cells able to change form from highly ramified to a completely amoeboid-like shape lacking processes. This transition can be very rapid under pathological conditions. The forms illustrated here represent snapshots of a transformation that is reversible at every time point, with variation with each form shown. **(B–G)** Complexity analysis of microglia/macrophages in CD11b-stained tissue. The process to prepare photomicrographs for complexity analysis: Original photomicrographs were subjected to a series of uniform ImageJ plugin protocols prior to conversion to binary images. Binary images were then analyzed by using FracLac for ImageJ, which quantifies single cell complexity (fractal dimension, fD). The calculated Df of the cell is shown below its binary image. Representative images of morphological changes in CD11b-staining cells within the sham-operated cortex in vehicle-treated rats **(B)**, sham-operated cortex in VPA-treated rats **(C)**, peri-infarct cortex at 2 days after 90-min dMCAo in vehicle-treated rats **(D)**, peri-infarct cortex at 2 days after 90-min dMCAo in VPA-treated rats **(E)**, peri-infarct cortex at 7 days after 90-min dMCAo in vehicle-treated rats **(F)**, and peri-infarct at 7 days after 90-min dMCAo in VPA-treated rats **(G)**. **(H)** Illustration of FracLac box counting method to derive fractal dimension calculations of a CD11b-positive cell outline. Shape detail is quantified as scale increase, represented by orange boxes. Box counting equation is summarized in **Table 1**. **(I)** Summary data and statistical analysis of fractal dimension at 2 and 7 days after ischemia. Fractal dimension was decreased in the peri-infarct cortex compared with the sham-operated cortex at 2 and 7 days post-stroke. However, VPA treatment restored the fractal dimension of CD11b-positive cells in the peri-infarct cortex at 2 days and 7 days post-stroke. All *post hoc* analyses are reported in the figure (2 days: ###$p < 0.001$ vs. sham and dMCAo + vehicle; ***$p < 0.001$ vs. dMCAo + vehicle and dMCAo + VPA; 7 days: #$p < 0.05$ vs. sham and dMCAo + vehicle; *$p < 0.05$ vs. dMCAo + vehicle and dMCAo + VPA). **(J)** The schemes illustrate graphically the calculation base for the circularity index for the manual analysis. Q22. **(K,L)** Soma size and circularity index were increased in the peri-infarct cortex compared with the sham-operated cortex at 2 and 7 days post-stroke. However, VPA treatment decreased the soma size and circularity index of CD11b-positive cells in the peri-infarct cortex at 2 and 7 days post-stroke. All *post hoc* analyses are reported in the figure (2 days: ###$p < 0.001$ vs. sham and dMCAo + vehicle; ***$p < 0.001$ vs. dMCAo + vehicle and dMCAo + VPA; 7 days: ###$p < 0.001$ vs. sham and dMCAo + vehicle; ***$p < 0.001$ vs. dMCAo + vehicle and dMCAo + VPA). Twenty-five to thirty cells per region of $n = 4$ rats in each group.

3a1, collagen 6a2, decorin, and cellular communication network factor 1) and included genes which code for coagulation-related surface proteins on the endothelial cells (thrombomodulin) and macrophage galactose-type lectin on M2 microglia (C-type lectin domain containing 10A) (**Figure 7C**). These data revealed a prominent upregulation of gene expression related to extracellular matrix remodeling in the stroke rats that received VPA treatment.

Gene Ontology (GO) Analysis From VPA- and Vehicle-Treated Ischemic Brains

To further reveal the functional categories of DEGs, gene ontology (GO) enrichment analysis was performed. GO enrichment analysis of DEGs (dMCAo + vehicle vs. dMCAo + VPA) identifies key biological processes, cellular components, and molecular functions. The top three significantly upregulated functional categories (adjusted P-value < 0.05) were enriched by DEGs in the VPA-treated group, with those DEGs being involved in wound healing, collagen trimmer and extracellular matrix functions (**Figures 7D,E**). Notably, the DEGs related to wound healing were also partially associated with collagen trimmer and extracellular matrix functions, suggesting that post-stroke VPA treatment might potentiate tissue remodeling in the peri-infarct cortex. Network-based GSEA (gene set enrichment analysis) was utilized to identify the functional associations amongst the genes affected by the VPA treatment (**Figure 7F**). Similar to the results obtained using enriched GO analysis, the top 10 functions of the up-regulated genes were associated with the extracellular space (adjusted P-value = 0.044), drug responses (adjusted P-value = 0.044), extracellular matrix (adjusted P-value = 0.044), positive regulation of gene expression (adjusted P-value = 0.044), basement membrane (adjusted P-value = 0.044), external side of the plasma membrane (adjusted P-value = 0.044), cell–cell adhesion (adjusted P-value = 0.046), wound healing (adjusted P-value = 0.046), heparin binding (adjusted P-value = 0.046), and angiogenesis (adjusted P-value = 0.046).

Post-stroke VPA Treatment Regulates Gene Expression Profile of Activated Microglia

Given that the C-type lectin domain containing 10A (*Clec10a*) was mainly expressed on the antigen-presenting cells (**APC**) and macrophages (Van Vliet et al., 2008), we wondered whether the upregulated *Clec10a* in VPA-treated peri-infarct cortex was also on the activated microglia. Therefore, we isolated CD11b-positive cells from the infarct area at day 3 post-stroke (**Figure 8A**), and then analyzed the mRNA level of *Clec10a* in these cells. Similar to the results obtained from immunofluorescence, VPA treatment did not obviously reduce the number of CD11b-positive microglia on day 3 post-stroke (**Figure 8B**). However, a significant increase in the mRNA level of *Clec10a* in the CD11b-positive microglia/macrophages was shown in the VPA-treated group (**Figure 8C**), suggesting that treatment with VPA increased *Clec10a* expression in the peri-infarct cortex and on activated microglia and macrophages. Since *Clec10a* has immunoregulatory properties and is induced on M2 microglia in neurological autoimmune disorders (Ilarregui et al., 2019), we next wanted to explore whether VPA could regulate the phenotypes or gene expression profiles of microglia. For additional evaluations of the status of microglia/macrophages, the mRNA levels of selected markers of the cytotoxic M1 phenotype (*iNOS* and *CD86*) and the cytoprotective M2 phenotype (*TGFβ1, CD163, Arginase-1*) were analyzed in these $CD11b^+$ cells. Our results indicated that VPA treatment does not change the expressions of *iNOS* or *CD86,* genes expressed in cytotoxic M1-type microglia (**Figure 8C**). In turn, the analysis of markers of M2 phenotype revealed a significant increase in the mRNA levels of *TGFβ1* and *CD163* but not *Arginase-1* in the $CD11b^+$ microglia in response to VPA exposure (**Figure 8C**), implying that VPA treatment did not cause collective up-regulation of M2-type marker genes in the peri-infarct cortex. Taken together, these findings indicate that post-stroke VPA treatment not only stimulates extracellular matrix remodeling, but also alters the morphological responses and gene expressions of activated microglia in the peri-infarct cortex.

DISCUSSION

This study provides evidence that a low dose of VPA post-stroke can improve the recovery of aspects of neurological function in rats, and that this effect of VPA is associated with the suppression of peri-infarct activity in microglia, including activity related to morphological changes and density. This attenuation of microglial activity may involve the suppression of galectin-3 expression and the upregulation of extracellular matrix remodeling in the peri-infarct cortex.

Accumulating evidence supports the notion that histone hypoacetylation and transcriptional dysfunction are involved in a large number of neurodegenerative conditions (Hu et al., 2017). Although postischemic treatment with a clinically relevant dose of VPA, specifically, 300 mg/kg, has been shown to reduce dMCAo-induced brain infraction, BBB disruption, and brain edema (Ren et al., 2004; Wang et al., 2011), this dose is too high for humans. As with many antiepileptic drugs, there are a number of adverse consequences associated with the use of VPA. Among these, valproate encephalopathy is a dose-related syndrome that can occur due to high VPA concentrations, particularly in new patients (Hamer et al., 2000). Moreover, VPA-associated pancytopenia and coagulopathy have been shown to be dose-dependent side effects (Attilakos et al., 2007). Furthermore, the teratogenicity of VPA limits its use in woman of childbearing age (Gotlib et al., 2017). In this study, we reduced the dose to 200 mg/kg of VPA and tested the therapeutic effects of this dose in rats. The results indicated that a single injection of VPA at a dose of 200 mg/kg in stroke rats did not reduce infarct volume but did significantly accelerate the reversal of behavioral deficits in rats with cerebral ischemic injury. The improvements of forepaw function and body symmetry occurred in the absence of changes in infarct volume and are broadly consistent with reports of improved outcomes without changes in infarct volume following VPA treatment in several studies (Lee et al., 2014).

TABLE 3 | Comparison on 67 biomarkers between dMCAo + vehicle and dMCAo + VPA groups.

Biomarker	dMCAo + VPA	dMCAo + vehicle	FoldChange	statistic	p.value	FDR	entrez_id
Galectin-3	4.01(3.2, 6.18)	15.43(5.14, 15.77)	3.850125136	Wilcoxon *W* = 2	0.003175	0.031042	83781
Adiponectin	2.21 ± 1.92	7.46 ± 3.27	3.368981525	*t* = –3.0969	0.019277	0.531042	246253
IL-1 ra	0(0, 0)	21.98(0, 75.63)	2198.554233	Wilcoxon *W* = 2.5	0.02537	0.531042	60582
IL-6	7.52(0, 22.56)	0(0, 1.73)	0.001327755	Wilcoxon *W* = 22	0.044909	0.531042	24498
FGF-BP	301.74 ± 135.3	460.75 ± 66.35	1.526962995	*t* = –2.3594	0.057642	0.531042	64535
IL-1 R6	225.24(0, 3432.54)	0(0, 0)	4.44E-05	Wilcoxon *W* = 20	0.072006	0.531042	171106
P-Cadherin	0(0, 0)	10.43(0, 22.32)	1044.471407	Wilcoxon *W* = 5	0.072006	0.531042	116777
TREM-1	0(0, 0)	4.49(0, 10.61)	449.8794155	Wilcoxon W = 5	0.072006	0.531042	301229
SCF	3.77 ± 2.71	11.03 ± 7.76	2.928412671	*t* = –1.976	0.105526	0.623708	60427
JAM-A	7.58 ± 7.42	16.97 ± 9.35	2.237719561	*t* = –1.7577	0.118792	0.623708	116479
IL-2 R alpha	0(0, 1.8)	1.21(0, 43.35)	121.5922312	Wilcoxon *W* = 5	0.118797	0.623708	25704
HGF	26.28(0, 48.32)	132.12(0, 180.73)	5.026618741	Wilcoxon *W* = 5	0.138792	0.623708	24446
Erythropoietin	0(0, 173.89)	67(0, 257.54)	6700.768421	Wilcoxon *W* = 5.5	0.161238	0.623708	24335
CD48	0(0, 0)	0(0, 13.61)	1	Wilcoxon *W* = 7.5	0.179712	0.623708	245962
CINC-2	0(0, 1.29)	0(0, 0)	1	Wilcoxon *W* = 17.5	0.179712	0.623708	171551
gp130	0(0, 0)	0(0, 204.37)	1	Wilcoxon *W* = 7.5	0.179712	0.623708	25205
RAGE	0(0, 93.74)	0(0, 0)	1	Wilcoxon *W* = 17.5	0.179712	0.623708	81722
Neuropilin-1	916.22 ± 718.22	1491.07 ± 633.66	1.627402803	*t* = –1.342	0.216978	0.690058	246331
Prolactin	49.11(2.2, 104.24)	67.37(45.9, 234.69)	1.371723009	Wilcoxon *W* = 6	0.222222	0.690058	24683
Prolactin R	0(0, 394.66)	199.46(0, 860.2)	19946.8758	Wilcoxon *W* = 6.5	0.235861	0.695791	24684
CTACK	42601.63 ± 25019.88	60571.41 ± 20800.69	1.421809662	*t* = –1.2349	0.253013	0.706855	362505
CNTF	5.56 ± 6.14	10.8 ± 7.51	1.940993959	*t* = –1.206	0.263573	0.706855	25707
TIMP-1	1297.87 ± 1033.13	2371.34 ± 1771.85	1.827096283	*t* = –1.1703	0.283371	0.726908	116510
Notch-2	571.4(523.82, 1436.6)	744.09(542.62, 1286.46)	1.30221903	Wilcoxon *W* = 7	0.309524	0.742871	29492
Activin A	0(0, 0)	0(0, 560.91)	1	Wilcoxon *W* = 10	0.423711	0.742871	29200
b-NGF	0(0, 0)	0(0, 11.38)	1	Wilcoxon *W* = 10	0.423711	0.742871	310738
CINC-3	0(0, 0)	0(0, 0.85)	1	Wilcoxon *W* = 10	0.423711	0.742871	114105
EphA5	0(0, 0)	0(0, 58.11)	1	Wilcoxon *W* = 10	0.423711	0.742871	79208
IFNg	0(0, 0)	0(0, 0.03)	1	Wilcoxon *W* = 10	0.423711	0.742871	25712
IL-13	0(0, 1.14)	0(0, 0)	1	Wilcoxon *W* = 15	0.423711	0.742871	116553
LIX	0(0, 0.28)	0(0, 0)	1	Wilcoxon *W* = 15	0.423711	0.742871	60665
RANTES	0(0, 0)	0(0, 0.13)	1	Wilcoxon W = 10	0.423711	0.742871	81780
TIMP-2	0(0, 0)	0(0, 2.41)	1	Wilcoxon *W* = 10	0.423711	0.742871	29543
CINC-1	0(0, 6.32)	0(0, 2.52)	1	Wilcoxon *W* = 16	0.440686	0.742871	81503
MIP-1 alpha	0(0, 368.16)	0(0, 62.21)	1	Wilcoxon *W* = 16	0.440686	0.742871	25542
Gas 1	26.56 ± 18.14	35.3 ± 22.7	1.32868067	*t* = –0.672	0.521405	0.848722	683470
Fractalkine	1130.84(826.69, 2588.39)	879.85(779.54, 1895.16)	0.778050805	Wilcoxon *W* = 16	0.547619	0.848722	89808
MCP-1	79.53(24.26, 161.42)	119.8(29.26, 372.45)	1.506219934	Wilcoxon *W* = 9	0.547619	0.848722	24770
IL-4	0.06 ± 0.08	0.09 ± 0.05	1.401720264	t = -0.6091	0.56102	0.848722	287287
Notch-1	271.33 ± 150.9	283.29 ± 147.24	1.044064267	*t* = –0.1268	0.902226	1	25496
B7-1	0(0, 0.37)	0(0, 0.33)	1	Wilcoxon *W* = 13	1	1	25408
B7-2	0(0, 6.95)	0(0, 16.11)	1	Wilcoxon *W* = 12	1	1	56822
Fit-3 Ligand	0.66(0, 2.1)	0.65(0, 2.36)	0.979628446	Wilcoxon *W* = 13	1	1	1.04E + 08
IL-10	0(0, 11.54)	0(0, 3.16)	1	Wilcoxon *W* = 13	1	1	25325
Neuropilin-2	329.77(166.34, 1581.33)	348.82(46.11, 1051.13)	1.057751003	Wilcoxon *W* = 13	1	1	81527
TCK-1	789.04(504.99, 6581.18)	1229.66(217.51, 3259.77)	1.558425886	Wilcoxon *W* = 13	1	1	246358
TWEAK R	0(0, 14.54)	0(0, 51.69)	1	Wilcoxon *W* = 12.5	1	1	302965
4-1BB	0(0, 0)	0(0, 0)	1	Wilcoxon *W* = 12.5			500590
IL-17F	0(0, 0)	0(0, 0)	1	Wilcoxon W = 12.5			301291
IL-1a	0(0, 0)	0(0, 0)	1	Wilcoxon *W* = 12.5			24493
IL-2	0(0, 0)	0(0, 0)	1	Wilcoxon *W* = 12.5			116562
IL-3	0(0, 0)	0(0, 0)	1	Wilcoxon *W* = 12.5			24495
TIM-1	0(0, 0)	0(0, 0)	1	Wilcoxon *W* = 12.5			140898
TNFa	0(0, 0)	0(0, 0)	1	Wilcoxon *W* = 12.5			24835
VEGF	0(0, 0)	0(0, 0)	1	Wilcoxon *W* = 12.5			83785

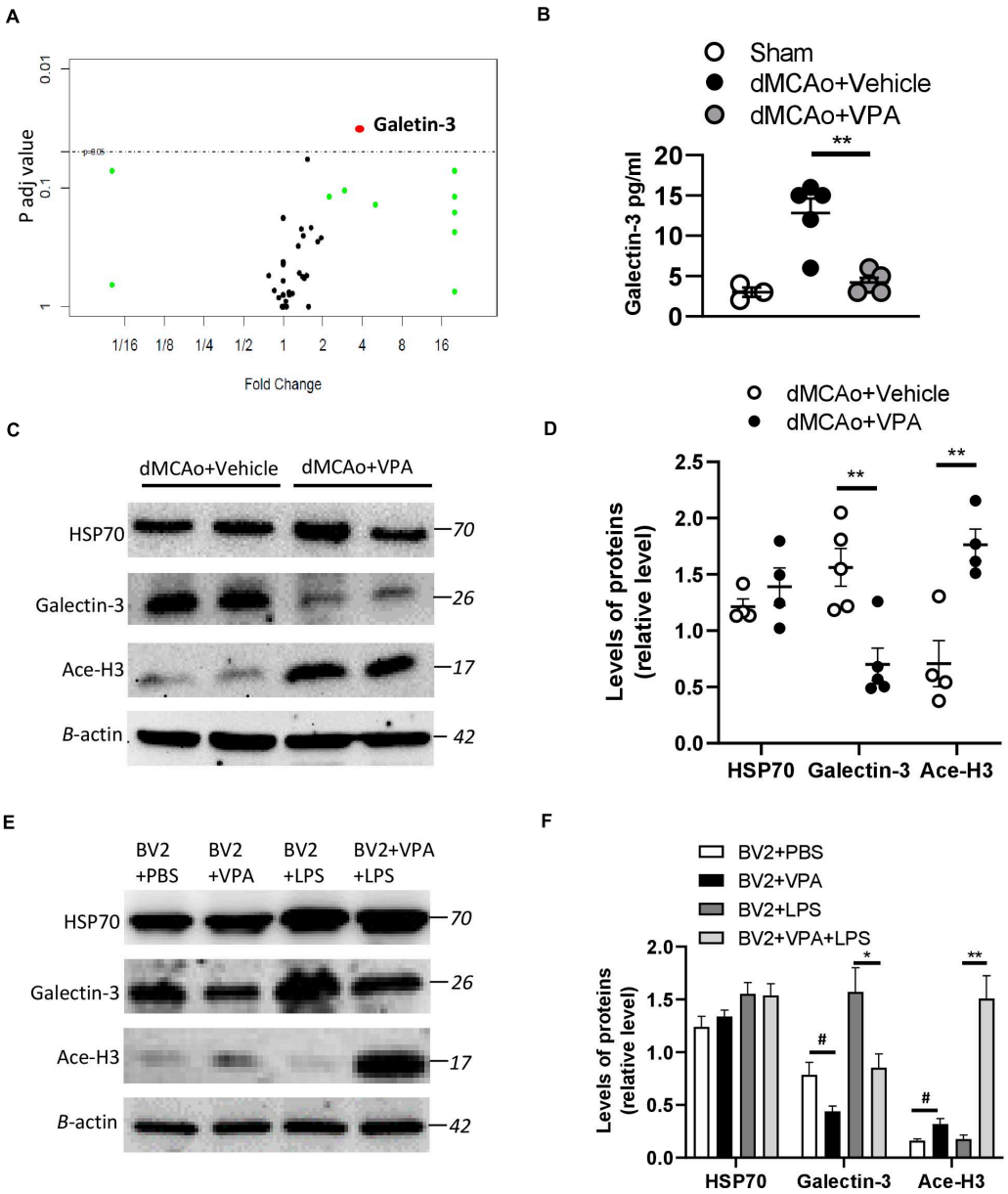

FIGURE 6 | Effects of VPA treatment on galectin-3 expression in the peri-infarct cortex and BV2 cells. **(A)** Volcano plot comparing the fold changes and adjusted p-values of 67 biomarkers in which each point represents a biomarker. **(B)** VPA suppresses galectin-3 production in the peri-infarct cortex of rats with ischemic brain injury. Galectin-3 concentrations in the peri-infarct cortex at 2 days after dMCAo surgery were measured by ELISA ($n = 5$). $^{**}p < 0.01$, compared with the vehicle-treated group. **(C)** Protein levels of HSP70, acetylated H3 (Ace-H3), galectin-3, and β-actin were determined by western blot analysis. **(D)** Quantified results of HSP70, Ace-H3, and galectin-3. Each column and vertical bar represents the mean ± SEM of 5 animals. $^{**}P < 0.01$, Student's *t*-test. **(E)** BV2 cells were activated using LPS (1 μg/ml) and, simultaneously, in the presence or absence of VPA as indicated. After 6 h of treatment, the protein levels of HSP70, acetylated H3 (Ace-H3), galectin-3, and β-actin were determined by western blot analysis. **(F)** VPA treatment enhances the levels of Ace-H3, but suppresses galectin-3 production in the BV2 cells with/without LPS exposure. $\#p < 0.05$ vs. control. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate comparison with LPS-treated BV2 cells with Student's *t*-test. The data represent mean ± SEM.

Thus, the findings of the present study suggest that cellular changes critical for recovery are initiated in the early stages after infract formation, and that they can be modulated by VPA treatment.

It has become increasingly that clear brain injury following ischemia is highly associated with the inflammatory response, which involves the infiltration of mononuclear phagocytes and activated microglia (Glass et al., 2010; Shi et al., 2011). Inflammatory cytokines (IL-1β, TNF-α, and IL-6) produced by peri-infarct microglia/macrophages would modulate tissue injury and have profound effects on infarct evolution (Lambertsen et al., 2012). In the past literature, VPA was demonstrated to inhibit

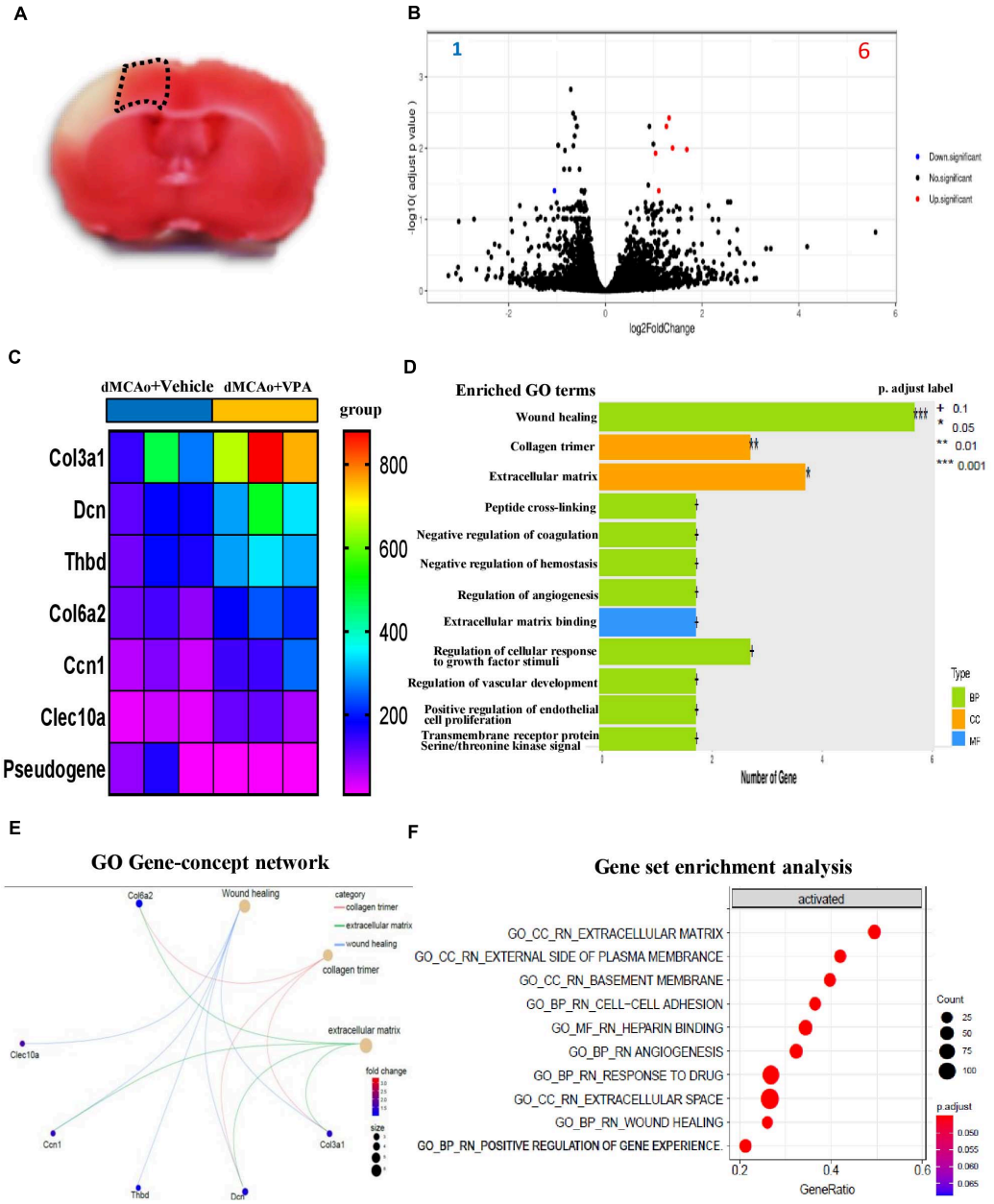

FIGURE 7 | Post-stroke VPA treatment regulates gene expression in the peri-infarct cortex at 3 days after ischemic brain injury. **(A)** A representative image of the brain sections used for the RNA-sequencing experiment. **(B)** Volcano plot comparing the log2fold changes and adjusted *p*-values of 15446 gene expressions. The red dots indicate genes upregulated (log2fold change > 1, adjusted *p*-value < 0.05), the blue dots indicate genes downregulated (log2fold change < –1, adjusted *p*-value < 0.05), and the black dots indicate genes with no significant change between dMCAo + vehicle and dMCAo + VPA. **(C)** Heatmap plot of DEGs in peri-infarct cortex between dMCAo + vehicle and dMCAo + VPA. **(D)** GO biological processes, cellular component, and molecular function over-representation analysis based on 7 DEGs. **(E)** Molecular network plot connected using GO over-representation analysis following VPA treatment in a rat model of dMCAo. **(F)** GO gene set enrichment analysis of genes upregulated by VPA.

TNF-α and IL-6 production induced by LPS in THP-1 cells, and this inhibition was linked to the suppression of NF-κB activation (Ichiyama et al., 2000; Mairuae and Cheepsunthorn, 2018). In animal studies of global brain ischemia, VPA further suppressed IL-1β production in the hippocampus (Xuan et al., 2012). Studies of cellular sources of TNF-α and IL-1β after MCAo have looked specifically at microglia and macrophages. However, IL-6 is upregulated in microglia and cortical neurons in stroke rats, and increases of IL-6 are more pronounced in the case of gray matter lesions (Eriksson et al., 1999; Suzuki et al., 1999). Based on our results, a single injection of VPA at a dose of 200 mg/kg was shown to suppress the up-regulation of IL-6 in cortex at 24 h

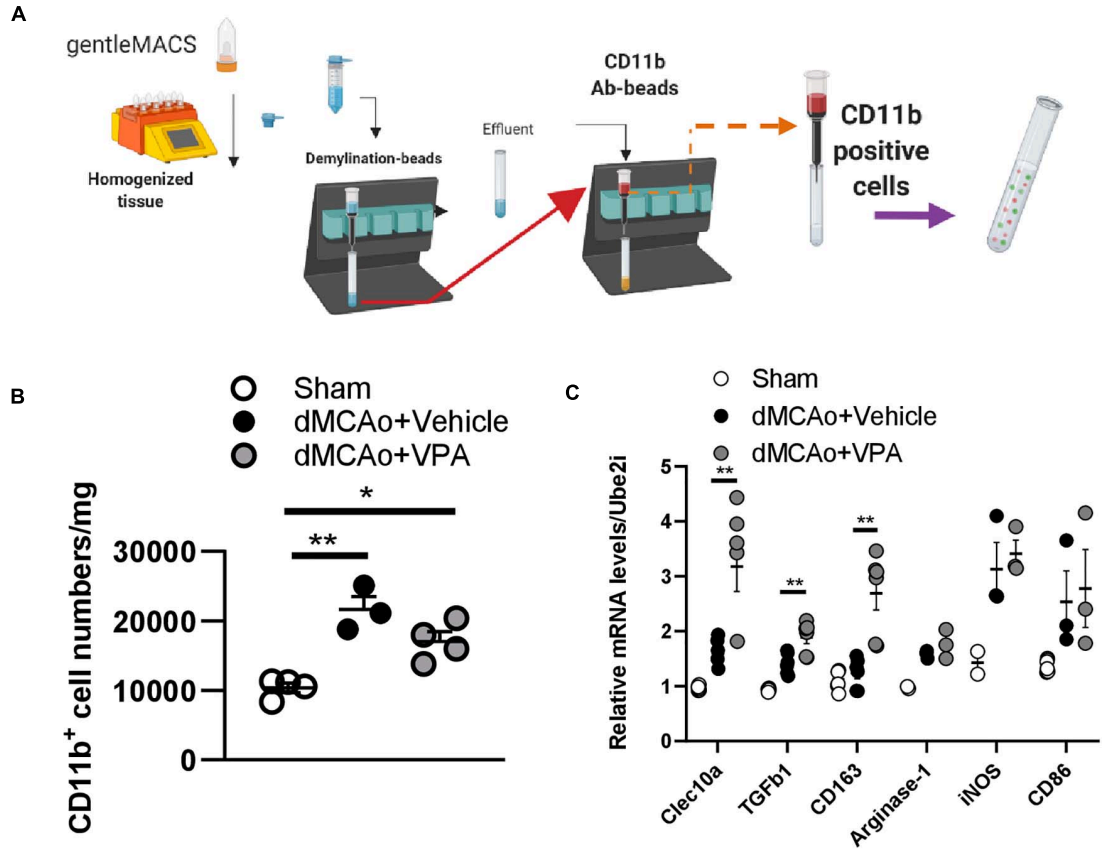

FIGURE 8 | Post-stroke VPA treatment enhances gene expressions of the alternative activated M2 phenotypes. **(A)** Male rats (12 weeks old) were subjected to sham operation or dMCAo surgery with/without VPA treatment. Microglia/macrophages were purified from sham cortex and peri-infarct cortex at day 3 post-dMCAo using gentleMACs Dissociator and CD11b antibody-beads. **(B)** There were significant differences in the number of CD11b-expressing microglia/macrophages between the sham group and dMCAo groups. **(C)** qPCR analysis of gene expression of alternative activated M2 phenotype (*Clec10a*, *TGFβ1, CD163,* and *Arginase-1*) and classical activated M1 phenotype (*iNOS* and *CD86*) in the CD11b-expressing microglia/macrophages of sham-operated, dMCAo + vehicle, and dMCAo + VPA groups. $^{*}P < 0.05$, $^{**}P < 0.01$, Student's *t*-test. $N = 3$–4 per group. The data represent mean ± SEM.

following dMCAo, suggesting that low-dose VPA therapy might both alleviate neuronal damage and dampen microglial activation in the early phase of cerebral ischemic injury.

The single injection of a low dose of VPA after stroke did not produce significant differences in the number of CD11b-positive microglia/macrophages within the peri-infarct cortex within the first 2 days. Thus, our findings suggest that the low dose of VPA did not produce gross alterations in CD11b-positive cell recruitment to the peri-infarct tissue in the early phase. However, in other studies regarding the effect of VPA on microglia/macrophage distribution, VPA was shown to greatly limit microglial density in the injured area (Xuan et al., 2012; Suda et al., 2013). This difference in response to focal ischemia compared with that in the present study is probably explained, at least in part, because the higher dose of VPA in those other studies produced decreases in cell loss and infarct size in those earlier investigations of stroke, and such protective effects can secondarily reduce microglial responses to ischemic damage.

Apart from the changes in microglial distribution in response to ischemic damage, ramified microglia would transform to amoeboid morphology and resume phagocytotic activity, which exacerbate neuroinflammatory responses (Sierra et al., 2016; Norris and Kipnis, 2019). It follows that microglia morphology, if quantified using sensitive methods, could provide insight into the neuropathology of distinct regions. Using two quantitative assessments of microglia complexity and circularity, we report that the microglia morphology was different from that in sham cortex in the peri-infarct cortex at 2 and 7 days post-stroke. In the peri-infarct region, most of the microglia became amoeboid or had increased circularity, while ramification in shape or higher complexity was less observed. One striking finding of our results is that the low-dose VPA treatment produced significant differences in the complexity and circularity of CD11b-positive microglia within 7 days compared with vehicle treatment, indicating that VPA greatly limits changes in microglial morphology after dMCAo. Simultaneously, the low-dose VPA treatment was shown to not only enhance the levels of acetylated histone H3 protein but also to suppress the levels of galectin-3 in the peri-infarct cortex. Since galectin-3 is predominantly expressed in the microglia of the adult rodent

brain (Lalancette-Hebert et al., 2012; Reichert and Rotshenker, 2019), we further argue that VPA treatment could suppress the LPS-upregulated galectin-3 levels in BV2 cells. It has been shown that galectin-3 could control microglia morphology by regulating the cytoskeleton (Hoyos et al., 2014; Reichert and Rotshenker, 2019). Thus, amoeboid microglia are rich in galectin-3 and display productive phagocytosis. Moreover, galectin-3 is required for microglia-mediated brain inflammation (Nomura et al., 2017; Siew et al., 2019), while the suppression of galectin-3 ameliorates microglia-mediated pathogenesis by decreasing NFκB activation (Siew et al., 2019). Interestingly, NFκB motifs have been identified in the promotor region of galectin-3 (Hsu et al., 1996), implying that NFκB and galectin-3 might be regulated in a positive feed-forward loop in microglia. In our study, VPA was shown for the first time to promote a morphological shift of peri-infarct microglia from the amoeboid to the ramified form, and that might be associated with galectin-3 suppression in the peri-infarct cortex or microglia. Although previous studies have found some epigenetic pathways wherein the chromatin remodeling proteins contribute to galectin-3 induction (Li Z. et al., 2019), we have not yet examined whether VPA regulating the level of galectin-3 is directly dependent on the epigenetic action or upregulation of the acetylation of NFκB p65 caused by the decrease of HDAC3 activity (Wang et al., 2011).

An increasing number of studies have demonstrated that VPA treatment could directly up-regulate genes associated with neuronal proliferation, differentiation, and neurotransmission, while down-regulating genes related to cell death and inflammation (Nikolian et al., 2018). Such changes may play roles in the benefits provided by VPA treatment following injury. In our study, RNA sequencing further revealed that VPA treatment exhibits a strong trend of upregulation of genes related to extracellular matrix remodeling in the peri-infarct cortex. Collagen 3a1, collagen 6a2, decorin, and cellular communication network factor 1, all of which are known to be part of the extracellular matrix, serve essential functions involved in the regulation of cellular processes and providing a permissive microenvironment to promote tissue repair (Nelimarkka et al., 2001; Davis and Senger, 2005; Kubota and Takigawa, 2007; Jarvelainen et al., 2015; Gregorio et al., 2018; Ucar and Humpel, 2018). Moreover, the provisional extracellular matrix serves as a pliable scaffold wherein mechanical guidance forces are established among endothelial cells, thereby providing critical support for vascular endothelium (Davis and Senger, 2005). Thrombomodulin is a membrane protein mainly expressed by endothelial cells. It is part of the anticoagulant protein C system, in which thrombomodulin binds with thrombin and promotes the cleavage of protein C and thrombin activatable fibrinolysis inhibitor, thereby inhibiting coagulation (Wenzel et al., 2014). Additionally, it interferes with inflammation, stabilizes barrier function, and promotes angiogenesis under pathological conditions (Watanabe-Kusunoki et al., 2020). *Clec10a* is a member of the C-type lectin receptor family and is expressed by myeloid APC, such as dendritic cells and macrophages (Ilarregui et al., 2019). Notably, *Clec10a* has also been shown to be upregulated on M2 microglia, which are involved in the processes of extracellular matrix reconstruction and tissue repair in the injured area. GSEA using GO also demonstrated a pattern of increased extracellular matrix, cell-cell adhesion, angiogenesis, and wound healing. Thus, the fact that these changes correlate with activated tissue repair and extracellular matrix suggests that VPA may help to restore normal functions.

In the present study, the analysis of VPA-induced changes to gene expression in the peri-infarct CD11b-positive cells at early time points revealed an up-regulation of the expression of *Clec10a, TGFβ1*, and *CD163*, but not *Arginase-1*. These genes are expressed in M2 phenotype microglia. The effect of VPA in terms of altering the expression of markers of alternative, anti-inflammatory microglia (M2 phenotype) is in agreement with what has been reported after spinal cord injury (Chen et al., 2018), but unlike what the authors of that report proposed, our data suggest that, during the early phase of cerebral ischemic injury, VPA did not decrease the expression of M1-associated markers (iNOS, CD86). This discrepancy implies that the single injection with a low dose of VPA only stimulated certain subtypes of alternatively activated microglia during pathology progression. Primarily M2 phenotype microglia with high expression of *Arginase-1* contribute to axon regeneration through anti-inflammatory effects in neurodegenerative diseases (Cherry et al., 2014). CD163 is a phagocytic marker of microglia/macrophages that functions as a membrane-bound scavenger receptor for cleaning extracellular haptoglobin-hemoglobin and has an immunoregulatory property associated with the resolution phase of inflammation (Etzerodt and Moestrup, 2013). *TGFβ1* has been recently shown to potentiate an adaptive activation of microglia to accelerate wound healing (Taylor et al., 2017). Collectively, these genes of the M2 phenotype are essential for repair processes, and their high expression in our study might reflect peri-infarct tissue repair in response to VPA treatment.

There were several limitations to the present study. First, although our data indicated that 200 mg/kg of VPA still exhibits a therapeutic effect in ischemic brain injury, we did not measure the kinetics of VPA in rats, and the dose we used on the rats in this study cannot be extrapolated to a human-equivalent dose. Second, our data demonstrated that post-stroke VPA treatment could suppress the upregulation of galectin-3, which is required for resident microglia activation in response to ischemic injury. However, galectin-3 knockout microglia failed to activate and proliferate, which was further associated with significant increases in the size of the ischemic lesions (Lalancette-Hebert et al., 2012). Thus, it is still necessary to elucidate the time courses of galectin-3 expression and function in microglia under pathological conditions. Finally, we did not have gene expression data from a sham group, so it is unclear if the effects of VPA on gene expression are restorative or *de novo*. Additional studies are already underway to fill in many of these gaps.

CONCLUSION

We found that a single injection of VPA following dMCAo was able to accelerate functional recovery in rats. Mitigating microglia activation through the suppression of upregulated

galectin-3 and altering the gene profiles of these cells could influence extracellular matrix reconstruction and contribute to the improved recovery.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI BioProject database, PRJNA694497.

ETHICS STATEMENT

The animal study was reviewed and approved by National Defense Medical Center's Animal Center. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

T-TK, Y-HC, and K-YT: conception and design. T-TK, VW, and K-YT: performing experiments, data analysis, and manuscript writing. T-TK, J-SW, Y-HC, K-YT: planning experiments, data analysis, and manuscript revision. All authors contributed to the article and approved the submitted version.

FUNDING

Supported by The Ministry of Science and Technology of Taiwan, ROC, MOST-2314-B-016-001-MY3, MOST-108-2314-B-016-058-MY2, and MOST-108-2314-B-016-027; Medical Research Project grants TSGH-C108-098, TSGH-C108-094, TSGH-D109-100, TSGH-D109-099, and TSGH-D109-097 from the Tri-Service General Hospital of Taiwan; and the National Defense Medical Center, ROC, MAB-108-025.

ACKNOWLEDGMENTS

The authors thank Mei-Jen Wang for the murine BV-2 microglial cell support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2021.639145/full#supplementary-material

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

frontiers in Neuroscience

REVIEW
published: 18 June 2021
doi: 10.3389/fnins.2021.629297

Pericytes for Therapeutic Approaches to Ischemic Stroke

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Pericytes are perivascular multipotent cells located on capillaries. Although pericytes are discovered in the nineteenth century, recent studies have found that pericytes play an important role in maintaining the blood—brain barrier (BBB) and regulating the neurovascular system. In the neurovascular unit, pericytes perform their functions by coordinating the crosstalk between endothelial, glial, and neuronal cells. Dysfunction of pericytes can lead to a variety of diseases, including stroke and other neurological disorders. Recent studies have suggested that pericytes can serve as a therapeutic target in ischemic stroke. In this review, we first summarize the biology and functions of pericytes in the central nervous system. Then, we focus on the role of dysfunctional pericytes in the pathogenesis of ischemic stroke. Finally, we discuss new therapies for ischemic stroke based on targeting pericytes.

Keywords: pericytes, ischemic stroke, cerebral blood flow, blood–brain barrier, therapeutic strategy

OPEN ACCESS

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

Received: *14 November 2020*
Accepted: *11 May 2021*
Published: *18 June 2021*

Citation:
Cao L, Zhou Y, Chen M, Li L and Zhang W (2021) Pericytes for Therapeutic Approaches to Ischemic Stroke. Front. Neurosci. 15:629297. doi: 10.3389/fnins.2021.629297

INTRODUCTION

Pericytes are perivascular multipotent cells located on capillaries. The perivascular cell was first discovered by Rouget in 1873, so it was named "Rouget's cell" (Rouget, 1873). Furthermore, according to the morphology and location of Rouget's cell, interacting with the underlying endothelial cells (ECs) that shared the basement membrane, Zimmermann renamed it "pericyte" in 1923 (Zimmermann, 1923). Subsequently, more studies discovered the origin and functions of pericytes. Pericytes in cerebral vessels develop from different layers of the germ. Faal et al. (2019) pointed out that pericytes of the central nervous system (CNS) were mainly derived from mesenchymal stem cells of the mesoderm. Some studies also discovered that pericytes of the forebrain originated from nerve crest cells, while pericytes of the midbrain, brain stem, and spinal cord originated from mesoderm stem cells (Etchevers et al., 2001; Korn et al., 2002; Kurz, 2009). There are pieces of evidence suggesting that pericytes in the brain can exert a large variety of functions (Bell et al., 2010; Armulik et al., 2011), including regulating cerebral blood flow (CBF), maintaining BBB integrity, regulating angiogenesis and inflammation, and acting as stem cells and progenitor cells (Gautam and Yao, 2018).

Ischemic stroke is a cerebrovascular disease due to the blockage of blood vessels and decreased blood supply in relevant brain regions (Li et al., 2014). When ischemic stroke occurs, a series of molecular and cellular events occur, ultimately leading to disruption of CBF, destruction of the blood—brain barrier (BBB), inflammation, glial cell activation, vascular malformation, and neuronal death (Moskowitz et al., 2010; Terasaki et al., 2014). Recent studies show that pericytes influence ischemic stroke pathology and contribute to progression and recovery.

In this review, we focus on recent studies on the biology and functions of pericytes during ischemic stroke. Specifically, we discuss the role of pericytes in CBF, BBB integrity, angiogenesis,

immune response, and scar formation and fibrosis (**Table 1**). Finally, we address recent findings on treatment strategies for ischemic stroke by targeting pericytes.

Pericytes and Cerebral Blood Flow in Ischemic Stroke

The question of whether pericytes can regulate CBF is still debatable. Pericytes have been demonstrated to regulate blood flow to the brain. For instance, actin and myosin-like filaments have been found in rat pericerebral cells (Le Beux and Willemot, 1978). Moreover, many kinds of contractile proteins, such as smooth muscle actin (SMA), have been identified in the culture of pericerebral cells, indicating that pericytes can synthesize the protein (Sieczkiewicz and Herman, 2003; Dore-Duffy and LaManna, 2007). Consistent with these findings, pericytes cultured *in vitro* have shown contraction due to intracellular Ca^{2+} (Kamouchi et al., 2004). Some studies reported that glutamate-induced capillary pericytes are mediated by prostaglandin E2 and nitric oxide, and vasodilation occurs before the arteriole dilation caused by electrical stimulation to increase blood flow (Hall et al., 2014; Kisler et al., 2017). Besides, Kisler et al. used pericyte-specific Cre mouse model with Cre-dependent human diphtheria toxin (DT) receptor (Kisler et al., 2020). DT led to rapid progressive loss of pericyte coverage of cortical capillaries; however, endothelial response, microvascular density, and neuron-evoked membrane potential responses remained. This study suggested that neurovascular uncoupling is driven by pericyte loss, not other vascular or neuronal dysfunction. The result supported the role of pericytes in CBF regulation (Kisler et al., 2020). Together, these results suggest that pericytes take an active part in regulating CBF under physiological conditions.

Pericyte contraction can be observed under pathological conditions as well, such as stroke. Research showed that in the process of stroke, pericytes could entrap red blood cells in the capillary contraction part, which obstruct the microcirculation (Yemisci et al., 2009). Similarly, Hall et al. (2014) reported that pericytes contracted capillaries and died quickly after ischemia, and pericyte death led to permanent capillary contraction; furthermore, the decreased time of CBF was prolonged even when arterial blood flow was restored. These pathological changes in pericytes after stroke were duplicated with the iCelligence electrical impedance system. Neuhaus et al. (2017) confirmed that chemical ischemia induced long-term and irreversible contraction of pericytes before death *in vitro*. These results suggest that the contraction and death of pericytes are involved in the pathogenesis of stroke by regulating CBF. Further studies have shown that the cause of pericyte death is partially mediated by glutamate, while free radical scavenging does not reduce pericyte death (Hall et al., 2014). Compared to this report, inhibition of nitrate-mediated oxidative stress has been demonstrated to reduce pericyte contraction induced by ischemia reperfusion and have a positive effect on tissue survival (Yemisci et al., 2009). In addition, in the rat model of focal cerebral ischemia, eddaravan, a free radical scavenger, can reduce infarct size by preventing pericyte contraction while promoting pericyte proliferation (Deguchi et al., 2014). Recently, Nelson et al. (2020) examined pericyte contractility using a new optogenetic model by inducing pericyte-specific CreER mouse line and ChR2 mouse and monitored and confirmed pericyte contractility *in vivo* and regulated capillary blood flow in the brain of aging mice under a two-photon microscope. Besides, Hartmann et al. (2021) also found that brain capillary pericyte optogenetic stimulation decreased lumen diameter and blood flow, but with slower kinetics than similar stimulation of mural cells on upstream pial and precapillary arterioles.

On the contrary, there are some researches indicating that pericytes cannot contract and regulate CBF as well. Using a variety of transgenic mice and two-photon microscopy, Hill et al. (2015) did not detect SMA expression in mice and human pericytes. They also showed that changes in blood vessel diameter and blood flow caused by optogenetic whisker stimulation and cortical spreading depolarization occurred in the microvessels covered by smooth muscle cell (SMC) but not in the capillaries covered by pericytes (Hill et al., 2015). In addition, by using a short-term middle cerebral artery occlusion (MCAO) model, it has been found that SMC contraction rather than pericyte contraction resulted in hypoperfusion and thus distal microvascular occlusion. Fernández-Klett et al. (2010) also found that the increase in CBF caused by neural activity can be explained by precapillary and penetrating arterioles, rather than pericyte in capillaries. These studies suggest that SMC, rather than pericyte, contributed to the regulation of blood flow under both physiological and pathological conditions. The difference is probably due to the similar structural and functional properties of pericytes and precapillary SMC (Hartmann et al., 2015), making it difficult to distinguish them completely. Some groups showed synaptic activity generated a synchronous $Ca2^{+}$ drop in pericytes and SMCs resulting in telangiectasia starting mainly from primary or secondary capillaries and then spreading along arterioles and downstream capillaries, which may be the location of pericytes in the proximal capillaries as the main regulator of CBF (Cai et al., 2018; Khennouf et al., 2018; Rungta et al., 2018). However, the main divergence of the two opinions is the definition of pericyte, since Hill et al. (2015) and Fernández-Klett et al. (2010) defined SMA-expression capillary cell as SMC, while Hartmann et al., 2015 defined it as pericyte. The findings of Hill et al. (2015) and Fernández-Klett et al. (2010) are identical to other findings once definition differences are taken into account. In summary, most of the studies show that pericytes with SMA expression can regulate CBF.

Pericytes and the Blood—Brain Barrier in Ischemic Stroke

The BBB is a dynamic barrier between blood and brain tissue that acts as a selective barrier to substances. Pericytes play an important role in the formation and maintenance of the BBB. In the CNS, a simplified neurovascular unit is composed of vascular cells (pericytes, vascular SMCs, ECs), glial cells (astrocytes, microglia, oligodendrocytes), and neurons (**Figure 1**). Pericytes are important components of the neurovascular unit and BBB. Daneman et al. (2010) have found that pericyte recruitment occurred 1 week before astrocyte recruitment during the

TABLE 1 | Functions of pericytes in ischemic stroke.

Functions	Roles	References
Regulating cerebral blood flow	Capillary contraction	Yemisci et al., 2009; Hall et al., 2014; Neuhaus et al., 2017; Kisler et al., 2020
Formation and maintenance of BBB	Regulation of vascular permeability Modulation of BBB integrity	Dohgu et al., 2005; Bai et al., 2015; Daneman and Prat, 2015
Angiogenic property	Blood vessel stabilization Revascularization	Dore-Duffy and LaManna, 2007; Stenzel et al., 2009
Immunological characteristics	Releasing anti-inflammatory cytokines/chemokines	Dohgu and Banks, 2013; Sakuma et al., 2016; Duan et al., 2018
Scar formation and fibrosis	Stem cell potential expressing PDGFRβ Neuroprotection	Makihara et al., 2015; Sakuma et al., 2016; Ozen et al., 2018

BBB, blood—brain barrier; PDGFRβ, platelet-derived growth factor receptor-beta.

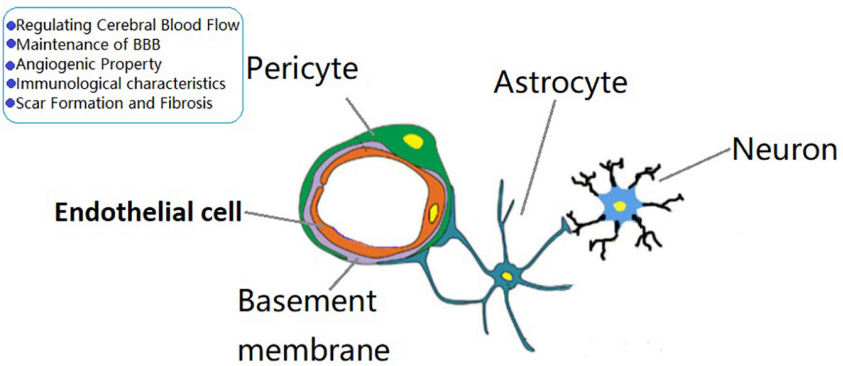

FIGURE 1 | The role of brain pericytes at the neurovascular unit (NVU). A simplified NVU is composed of vascular cells [pericytes, vascular smooth muscle cells, endothelial cells (ECs)], glial cells (astrocytes, microglia, oligodendrocytes), and neurons. Pericytes have the functions of regulating cerebral blood flow, maintenance of blood—brain barrier permeability, angiogenic characteristcs, immune responses, and scar formation and fibrosis.

formation of BBB. They also found that the coverage of pericyte played a crucial role in vascular permeability, and pericytes regulated BBB at the endothelial connectivity level (Daneman and Prat, 2015). Bell et al. (2012) found that apolipoprotein E maintained cerebrovascular integrity necessary for normal neuronal function by regulating the cyclophilin A–nuclear-factor-κB–matrix metalloproteinase pathway in pericytes in an isoform-specific manner. Al Ahmad et al. (2009) found that the increased integrity of endothelial barrier was related to pericytes and astrocytes, and pericytes protected the endothelial barrier better than astrocytes after thymic ischemia.

Several studies have shown that the loss of pericyte coverage led to destruction of BBB and endothelial barrier functions. Pericytes maintain BBB function by releasing angiopoetin-1 (Ang-1) and transforming growth factor-beta1 (TGF-β1). Increased occludin expression in ECs is associated with pericyte release of Ang-1. However, low levels of occludin cause the breakdown of tight junction (TJ) and blood vessel permeability (Hori et al., 2004). Dohgu et al. (2005) discovered that pericyte-derived TGF-β1 had the ability to directly influence BBB function. TGF-β1 activates mitogen-activated protein kinase (MAPK) signaling to increase the expression of TJ protein and P-glycoprotein (P-gp) expressed in EC to improve BBB function. However, Shen et al. (2011) showed that TGF-β1 promoted tyrosine phosphorylation of vascular endothelial-cadherin and claudin-5 in ECs and enhanced paracellular permeability. The role of pericyte-derived TGF-β1 in ischemic stroke needs further studies. Besides, Zlokovic (2013) showed that loss of pericytes disrupted cerebrovascular integrity and led to microvascular reductions amplifying vascular damage.

Although pericyte dysfunction is not directly associated with ischemic stroke, pericytes may be indirectly involved in ischemic pathogenesis by affecting vascular integrity. There is evidence that pericytes regulate BBB integrity through vascular endothelial growth factor (VEGF), thereby regulating ischemic injury. Sodium cyanide (NaCN) was used in an *in vitro* ischemia model, and it has been found to significantly increase the expression of VEGF in brain pericytes, while the conditioned medium from NaCN-treated pericytes destroyed vascular integrity in the *in vitro* BBB model (Bai et al., 2015). Meanwhile, it has been shown that VEGF mediated the increased permeability of BBB in ischemic brain (Zhang et al., 2000). In contrast, prolonged exposure to VEGF has been shown to improve the integrity of the BBB after ischemia (Zechariah et al., 2013). This difference may be due to differences in dose, treatment measures, and timing. Furthermore, pericytes influence BBB integrity through reactive oxygen species (ROS), thereby affecting the ischemic process. Studies have shown that nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 (NOX4) is highly upregulated in the peri-infarct area in the MCAO model. NOX4 has been shown to be primarily derived from pericytes, and the expression of NOX4 in pericyte was significantly increased in peri-infarct areas after MCAO. Further studies

showed that the overexpression of NOX4 in pericyte caused BBB destruction through upregulation of metalloproteinase 9, especially emphasizing the significant role of ROS in BBB integrity (Nishimura et al., 2016).

Angiogenic Property of Pericytes in Ischemic Stroke

Pericytes are closely related to the formation and stabilization of angiogenesis. The recruited pericytes enter new blood vessels to promote vascular maturation by releasing paracrine factors such as platelet-derived growth factor receptor-beta (PDGFRβ) (Dore-Duffy and LaManna, 2007). It has been reported that pericytes could stabilize the formation of capillary-like structures (Ramsauer et al., 2002). Persidsky et al. (2006) showed that pericytes were involved in the regulation of EC migration, proliferation, and differentiation. Furthermore, pericytes were reported to mediate the formation of new blood vessels through certain signaling molecules such as PDGFRβ, TGF-β, VEGF, and Ang-1 (Dore-Duffy and LaManna, 2007). Pericytes release VEGF to promote EC maturation (Hagedorn et al., 2004). Pericyte-derived TGF-β induces perivascular mesenchymal cells to differentiate into pericyte and SMCs (Sinha et al., 2004). Pericyte-released Ang-1 binds to Tie-2 in ECs, thereby promoting heparin binding to epidermal growth factor-like growth factor (HB-EGF) expression, which plays a key role in vascular stability (Stenzel et al., 2009).

Stroke is a cerebral blood circulation disease caused by stenosis, occlusion, or rupture of intracerebral arteries. Rebuilding blood flow to the brain in damaged areas aids in stroke recovery. Based on the important role of pericytes in angiogenesis, it is supposed that pericytes may contribute to stroke recovery by regulating angiogenesis. Recombinant human VEGF in the MCAO model has been shown to promote capillary growth and pericyte coverage, promote cerebral blood circulation, and thus reduce cerebral infarction area (Zechariah et al., 2013). In keeping with these observations, VEGF receptor inhibition accelerates cell death, inhibits EC proliferation, and exacerbates injury in neonatal stroke models (Shimotake et al., 2010). Based on these studies, it is speculated that pericytes have a positive effect in ischemic stroke by promoting angiogenesis.

It is important to point that angiogenesis may also have a negative effect on ischemic stroke. For example, elevated VEGF expression during ischemia was related to uncoupling of EC connectivity and increased vascular permeability and edema (Weis and Cheresh, 2005). In keeping with this finding, VEGF antagonism inhibited the formation of edema and tissue damage to some extent after MCAO in mouse brain (Nicholas van Bruggen et al., 1999). Furthermore, enhanced angiogenesis, including promoted EC activation and retinal hypervascularization, was observed in Notch1$^{\pm}$ Notch3$^{-/-}$ mice, a model of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) (Kofler et al., 2015). In addition, an increased incidence of ischemic stroke was related to type 1 and type 2 diabetes (Janghorbani et al., 2007). Angiogenesis in diabetes is persistent and uncontrolled (Cheng and Ma, 2015). In a word, pericyte angiogenic properties may play a dual role in ischemic stroke due to differences in the animal model, timing, and types of injury.

Immunological Characteristics of Pericytes in Ischemic Stroke

Pericytes take part in CNS defense by exhibiting non-specific and specific immune responses. A number of studies have reported that pericytes responded to pro-inflammatory signals. For example, chemokines and cytokines, including granulocyte colony-stimulating factor (G-CSF), interleukin (IL)-1 α, IL-6, and nitric oxide, are constitutively produced in brain pericytes of mice in normal conditions. In lipopolysaccharide (LPS)-induced inflammation, changes in expression levels of these chemokines and cytokines were accompanied by the induction of many new factors such as IL-5 and regulated upon activation normal T cell expressed and secreted factor (RANTES) (Kovac et al., 2011; Dohgu and Banks, 2013). With the increase of age and injury, pericytes with high expression of lysosomal acid phosphatase have phagocytosis. Pericytes may absorb substances in the blood or brain parenchyma through various endocytoses. When the BBB is broken down, pericytes can engulf the red blood cells (Castejon, 1984). Meanwhile, cultured rat pericytes expressed macrophage markers ED-2 and CD11b and were phagocytic with fluorescent dye-conjugated polystyrene beads and antibody-coated yeast polysaccharide, suggesting Fc receptor-independent and -dependent phagocytic activity (Balabanov et al., 1996). In summary, pericytes may have the immune cell-like characteristics and the ability to modulate immune responses.

Stroke is also a nervous system disease associated with the local inflammatory reactions and an immune response in the brain. It is supposed that pericytes may respond to CNS damage, including ischemic stroke, by transforming into microglia/macrophage-like cells (Boya, 1976). More recently, some studies have suggested that pericytes may acquire the microglia-like properties after ischemic stroke. First, it has been reported that the cells that express the regulator of G-protein signaling 5 (RGS5) after cerebral ischemia injury were mainly pericytes (Bondjers et al., 2003; Cho et al., 2003; Berger et al., 2005), which is associated with the proliferation and production of CD11b + and galactosin 3 + microglia cells (Ozen et al., 2018). Secondly, pericytes of the human brain acquire stemness characteristics and differentiate into a variety of lineages including microglia cells under oxygen and glucose deprivation simulating ischemic injury *in vitro* (Nakagomi et al., 2015). Meanwhile, pericyte stemness characteristics were detected in the ischemic regions of the mice, indicating that pericytes had multipotency in ischemic induction (Nakagomi et al., 2015; Gouveia et al., 2017). In addition, Iba1 + microglia cells express PDGFRβ in ischemic encephalopathy, showing that some microglia may come from post-ischemic pluripotent pericytes (Sakuma et al., 2016). Additionally, PDGFRβ + pericytes isolated from ischemic differentiated into microglia-like cells and acquired phagocytic activity (Sakuma et al., 2016). Recently, it has been reported that within 2 h of systemic inflammation, PDGFRβ mural cells of blood vessels rapidly secreted chemokine CC-chemokine ligand 2 (CCL2), which in turn increased

total neuronal excitability by promoting excitatory synaptic transmission in glutamatergic neurons of multiple brain regions (Duan et al., 2018). The study demonstrated *in vivo* that PDGFRβ cells functioned as initial sensors of external insults by secreting CCL2, which relayed the signal to the CNS. Through their gateway position in the brain, PDGFRβ cells are ideally positioned to respond rapidly to environmental changes and to coordinate responses. In summary, these studies show that pericytes can differentiate into microglia-like cells and perform related functions in ischemic conditions.

Pericytes and Scar Formation and Fibrosis in Ischemic Stroke

After injury of the CNS, glial cells are activated and form glial scar at the injury region by deposition of chondroitin sulfate proteoglycans, including neuroproteoglycans and phosphatase proteoglycans (Fawcett and Asher, 1999; McKeon et al., 1999; Silver and Miller, 2004; Li et al., 2013; Cregg et al., 2014). The function of scar tissue can keep toxic substances from spreading throughout the CNS (Fitch and Silver, 2008; Kawano et al., 2012). However, excessive or prolonged scar formation can inhibit axonal regeneration and hinder the recovery process, leading to fibrosis (Höke and Silver, 1996; Asher et al., 2001; Fitch and Silver, 2008). Besides, it is reported that astrocyte scar formation contributes to rather than prevents CNS axon regeneration (Anderson et al., 2016). The study showed that preventing or attenuating scar-forming astrocytes or ablating chronic astrocytic scars all failed to generate spontaneous regrowth of serotonergic axons in spinal cord injury (SCI) lesions. Sustained local delivery *via* hydrogel depots of required axon-specific growth factors not present in SCI lesions stimulated robust, laminin-dependent sensory axon regrowth past scar-forming astrocytes and inhibitory molecules in SCI lesions. By contrast, preventing astrocytic scar formation significantly reduced this stimulated axon regrowth.

It has been suggested that pericytes promoted scar formation and organ fibrosis. Pericytes are categorized into type I (Nestin-GFP^{-}/NG2-DsRed^{+}) and type II (Nestin-GFP^{+}/NG2-DsRed^{+}), producing adipocyte/fibroblast and nerve/myogen cells, respectively (Birbrair et al., 2013a,b). It has been reported that pericytes of type I gathered and contributed to scar formation in multiple organs (including brain, spinal cord, myocardium, and kidneys) after injury (Birbrair et al., 2014). It has been reported that in ischemic injury, PDGFRβ^{+} pericytes induced the fibrosis response in the kidney and CNS (Chen et al., 2011; Makihara et al., 2015). Compared to the control group, PDGFR$^{\pm}$ mice showed reduced fibrosis, reduced fibronectin deposition, and increased infarct size in the ischemic area, showing that PDGFRβ signaling-induced fibronectin production was essential for the repair process after ischemic stroke (Makihara et al., 2015). This discovery agrees with the neuroprotective effect of fibronectin in CNS injury (Tom et al., 2004; Yanqing et al., 2006).

Pericytes for Therapeutic Approaches to Ischemic Stroke

As discussed above, pericytes are involved in maintaining normal cerebrovascular function and play an important role in the pathological process of ischemic stroke. Thus, targeting pericytes may be an effective therapeutic method for ischemic stroke (Dalkara, 2019).

RGS5 protein that regulates vascular development was identified as a biomarker of pericytes (Bondjers et al., 2003). Ozen et al. (2018) found that the number of pericytes increased and the damage to the BBB decreased significantly after the RGS5 gene was knocked out in the model of permanent midbrain occlusion. In addition, loss of RGS5 in pericytes maintained aquaporin-4 (AQP4) expression in astrocytes and the integrity of TJs in ECs, reduced cerebral hypoxia, alleviated vascular leakage, and protected neurons in ischemic position (Ozen et al., 2018). AQP4 is a water channel protein expressed on astrocytic endfeet and plays an important role in BBB integrity. TJs have an important role in the maintenance of the BBB, and their unique expression in the brain correlates with BBB permeability. In acute stroke, there is degradation of TJs, resulting in loss of vascular integrity. Thus, targeting RGS5 might be a potential therapeutic strategy for ischemic stroke. Alarcon-Martinez et al. (2019) found that the α-SMA-mediated contractility in ischemic stroke and the effect of calcium in regulating contractile response could help in understanding the pericytes related to CBF at single-capillary level in ischemic stroke. Sun M. et al. (2020) found that sentrin/SUMO-specific protease 1 (SENP1) deletion in pericytes exacerbated infarct size and motor dysfunction after cerebral ischemia, though it had no effect on cognitive function. They also found that the deletion of pericyte-specific SENP1 significantly exaggerated neuronal damage after cerebral ischemia in mice. The knockdown of SENP1 in pericytes could activate apoptotic pathways and destroy the integrity of the cell barrier *in vitro*. These findings suggested that targeting SENP1 in pericytes might be a new therapeutic method for ischemic stroke (Sun M. et al., 2020). Besides, a study found that intracerebroventricular pleiotrophin (PTN) infusions prevented neuronal apoptosis in pericyte-ablated mice from persistent circulatory changes. PTN is a neurotrophic growth factor. The silencing of pericyte-derived PTN rendered neurons vulnerable to ischemic injury. This study demonstrated that pericyte loss was closely related to acute circulatory collapse and loss of PTN support. These findings suggested PTN support might be a new therapeutic method (Nikolakopoulou et al., 2019). Recently, Shibahara et al. (2020) demonstrated that pericyte-mediated fibrosis repair *via* PDGFR promotes functional recovery by enhancing peri-infarct oligodendrocyte formation and astrocyte proliferation following acute ischemic stroke. Besides, pericytes develop multipotency following experimental ischemia in mice, and these ischemia-induced multipotent stem cells (iSCs) can contribute to neurogenesis. This property of pericytes showed great potential in the treatment of neurovascular diseases (Geranmayeh et al., 2019). Pericytes from induced pluripotent stem cells (iPSC) are also expected to be used in autotransplantation therapy in ischemic stroke as acquiring BBB characteristics and binding to astrocytes, ECs, and neurons (Faal et al., 2019; Stebbins et al., 2019). Pericytes have been shown to acquire properties similar to stem cells and microglia after cerebral ischemia, providing another potential therapeutic strategy for recovery from ischemic stroke (Özen et al., 2014;

Nakagomi et al., 2015). Recently, Sun J. et al. (2020) generated pericyte-like cells (PCs) from human pluripotent stem cells (hPSCs). They found the cranial neural crest-derived pericyte-like cells (hPSC-CNC PCs) expressed typical pericyte markers and showed distinct pericyte properties. Moreover, when transplanted into a mouse model of MCAO with BBB breakdown, hPSC-CNC PCs efficiently improved neurological functional recovery in MCAO mouse model. The study indicated that hPSC-CNC PCs might represent an ideal cell source for the treatment of BBB dysfunction-related disorders and might be a new therapeutic method for ischemic stroke (Sun J. et al., 2020).

Although significant progress has been made in understanding the function of pericytes in the pathogenesis of ischemic stroke, some key issues remain to be studied further. First, although various markers have been used to identify pericytes, there are currently no pericyte-specific markers. It is important to note that the pericytes in most studies contained $PDGFR^{+}$ cells including both pericytes and SMCs. Secondly, pericytes are a diverse population of cells, and different types of pericytes may play different roles in ischemic stroke. The study in the future should focus on the identification of pericyte-specific and isotype-specific biomarkers, as well as drugs targeting pericytes or modulating their activities and autotransplantation of pericytes. Acquiring more information about pericytes will help us in studying pericytes in a novel way and facilitating the development of therapies for ischemic stroke.

AUTHOR CONTRIBUTIONS

LC searched for relevant literature and drafted the manuscript. YZ and MC searched for relevant literature and revised the manuscript critically. LL and WZ provided professional guidance for this review and performed a final check of the manuscript. All authors contributed to the review of this manuscript and approved the submitted version.

FUNDING

This study was supported by the National Natural Science Foundation of China 81870926 to LL.

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